Pumilio 2 controls translation by competing with eIF4E for 7-methyl guanosine cap recognition

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ABSTRACT

Pumilio 2 (Pum2) interacts with the 3' UTR-containing pumilio binding element (PBE) of RINGO/SPY mRNA to repress translation in *Xenopus* oocytes. Here, we show that Pum2 also binds directly to the 5' 7mG cap structure; in so doing, it precludes eIF4E from binding the cap. Using deletion analysis, we have mapped the cap interaction domain of Pum2 to the amino terminus of the protein and identified a conserved tryptophan residue that mediates this specific interaction. Reporter mRNA-based assays demonstrate that Pum2 requires the conserved tryptophan to repress translation in injected *Xenopus* oocytes. Thus, in addition to its suggested role in regulating poly(A) tail length and mRNA stability, our results suggest that vertebrate Pumilio can repress translation by blocking the assembly of the essential initiation complex on the cap.

Keywords: pumilio; cap; translation

INTRODUCTION

The meiotic divisions in *Xenopus* oocytes require a translational cascade that culminates in “mature” germ cells that are competent for fertilization. One translational control mechanism that induces this oocyte maturation transition is cytoplasmic polyadenylation (Richter 2006). One factor that is critical for this process is CPEB, an RNA binding protein that associates with the cytoplasmic polyadenylation element (CPE), a 3'UTR sequence that targets specific mRNAs for polyadenylation, during maturation. Polyadenylation, in turn, is regulated by several CPEB-associated factors that assemble on the 3' end of the mRNA. These include (1) the cleavage and polyadenylation specificity factor (CPSF), a tetrameric complex that binds the polyadenylation hexanucleotide AAUAAA; (2) PARN, a deadenylase; (3) Gld2, a poly(A) polymerase; and (4) ePAB, a poly(A) binding protein (Barnard et al. 2004; Kim and Richter 2006, 2007). The activity of the complex is mediated by multiple, temporally regulated CPEB phosphorylation events during maturation. Despite the presence of an active Gld2 in the complex, CPE-containing mRNAs have short

poly(A) tails in the immature oocyte cytoplasm due to a dominant counteracting effect of the deadenylase PARN. As a result, pre-mRNAs that are polyadenylated in the nucleus rapidly undergo deadenylation following export of the mRNA to the cytoplasm. During maturation, phosphorylation of CPEB serine 174, which is catalyzed by Aurora A (Mendez et al. 2000) or MAP kinase (Keady et al. 2007), causes PARN to be expelled from the RNP complex; this process results in Gld2-catalyzed default polyadenylation (Kim and Richter 2006). ePAB, which is initially bound to CPEB, dissociates from it when CPEB undergoes a second round of phosphorylation events catalyzed by cdk1 (Mendez et al. 2002; Kim and Richter 2007). Once liberated from CPEB, ePAB then binds to the newly elongated poly(A) tail and protects it from subsequent degradation. ePAB also interacts with the initiation factor eIF4G, which helps stimulate translation (Kim and Richter 2007).

Another CPEB-interacting factor that regulates translation of mRNAs during oocyte maturation is Maskin. Despite being tethered to the 3' end of mRNA, Maskin exerts a silencing influence on translation initiation by binding the cap-binding factor eIF4E and preventing it from interacting with eIF4G. Because an eIF4E-eIF4G association is required for the recruitment of the 40S ribosomal subunit to the 5' end of the mRNA, translation is inhibited (Cao and Richter 2002; Cao et al. 2006). Following polyadenylation, Maskin dissociates from eIF4E, thereby allowing eIF4G to bind eIF4E and initiate translation.

Cyclin B1 is often the cofactor that binds to and activates cdk1. During the very early phase of oocyte maturation,

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however, this task is at least partly assumed by the RINGO/SPY protein (Ferby et al. 1999; Padmanabhan and Richter 2006). Although oocytes have little RINGO/SPY protein, they do contain moderate levels of dormant RINGO/SPY mRNA (Ferby et al. 1999). The translation of RINGO/SPY mRNA in oocytes is repressed by Pumilio 2 (Pum2), a sequence-specific RNA binding protein that interacts with the pumilio binding element (PBE) present in the 3' UTR of RINGO/SPY mRNA. This Pum2-directed repression probably occurs in coordination with DAZL and ePAB, two other RNA binding proteins (Collier et al. 2005). Upon the induction of oocyte maturation, Pum2, but not DAZL or ePAB, dissociates from RINGO/SPY mRNA, which is then translated (Padmanabhan and Richter 2006). Newly synthesized RINGO/SPY binds to and activates cdk1, which in turn phosphorylates CPEB on six sites. These events induce ePAB to dissociate from CPEB and bind the newly elongated poly(A) tail, as well as the initiation factor eIF4G. ePAB may help eIF4G displace Maskin from eIF4E, leading to 40S ribosomal subunit recruitment to the mRNA.

In yeast and metazoans, Pumilio or pumilio-like proteins (Pumilio-FBF or PUF proteins) repress translation of specific mRNAs that harbor a 3'UTR cis element, the PBE or Nanos response element (NRE) (Wharton et al. 1998; Gu et al. 2004; Hook et al. 2007; Kaye et al. 2009). These sequences are thought to function primarily by recruiting factors that control RNA stability and cytoplasmic 3' end formation (Goldstrohm et al. 2006). While investigating aspects of Maskin association with eIF4E by affinity chromatography with immobilized cap analog (m^7G -Sepharose), we noticed that Pum2, like Maskin, was retained on the affinity matrix and that it was competed off by excess cap analog. This result prompted us to investigate whether Pum2 was an eIF4E binding protein that could function like Maskin or other eIF4E binding proteins such as *Drosophila* Cup (Nakamura et al. 2004). To our surprise, Pum2 did not bind eIF4E, but instead bound directly to the cap analog via a conserved tryptophan residue. The interaction of Pum2 with the cap structure presumably precludes eIF4E from accessing the cap, since a Pum2 protein variant that harbored a mutation at the tryptophan residue was ineffective in repressing translation of a PBE containing reporter. From these results, we infer that this member of the PUF family of proteins represses translation by a novel mechanism.

RESULTS

Maskin is a CPEB-associated factor that is retained on m^7G -Sepharose resin (i.e., cap analog composed of $m^7G(5')ppp(5')G$ affixed to Sepharose) through an interaction with eIF4E, the cap binding protein. We noted previously that the association of Maskin with eIF4E was controlled in part by phosphorylation changes of Maskin

catalyzed by cdk1 and calcineurin during the embryonic cell cycle in *Xenopus* (Cao et al. 2006). An M-phase arrested cytostatic factor (CSF) extract derived from *Xenopus* eggs, when supplemented with calcium, progresses through the cell cycle with successive rounds of metaphase occurring approximately every 30 min (e.g., Rauh et al. 2005; Cao et al. 2006; Mochida and Hunt 2007). Aliquots of a CSF extract progressing through the cell cycle were applied to m^7G -Sepharose and the material retained on the matrix was then eluted in SDS sample buffer and analyzed by Western blots. Figure 1A shows that as the extract progressed through the cycle (i.e., 0–20 min in the presence of calcium), increasingly greater amounts of Maskin were retained on the m^7G -Sepharose resin (GTP was added to the extract prior to chromatography to reduce nonspecific adsorption) (Stebbins-Boaz et al. 1999; Cao et al. 2006). When the extract was supplemented with free cap analog (i.e., to compete for protein binding with the immobilized analog) in addition to GTP, very little Maskin was retained on the matrix. eIF4E association with m^7G -Sepharose was unchanged during calcium-induced entry into the cell cycle, while the addition of excess free analog resulted in reduced eIF4E binding to the matrix. Because Pum2 contains a YXXX Φ motif (where Φ is any hydrophobic amino acid, often a leucine), which is common among eIF4E binding proteins (Richter and Sonenberg 2005; Padmanabhan and Richter 2006), we suspected that it might also be retained on the m^7G -Sepharose via binding to eIF4E. Indeed, similar to Maskin, progressively more Pum2 was retained on the cap analog matrix as the cell cycle progressed (Fig. 1A). These results suggest that Pum2 interacts with the cap or a cap-binding factor like eIF4E to control translation.

To further confirm that Pum2 binds the cap, directly or indirectly, mRNA encoding epitope-tagged Pum2 was injected into oocytes. Following an incubation period, a homogenate was prepared and passed over a m^7G -Sepharose matrix or, as a control, GDP-Sepharose column. Pum2, as well as eIF4E, were both retained on the m^7G -Sepharose matrix, but not on the GDP matrix (Fig. 1B). The same extracts were supplemented with GTP or GTP plus cap analog and applied to m^7G -Sepharose. In the presence of excess free cap analog, but not free GTP, both Pum2 and eIF4E failed to be retained on the m^7G -Sepharose matrix (data not shown). These data further suggest that Pum2 is either a cap or eIF4E-binding protein.

mRNAs encoding Pum2 and eIF4E were translated in separate reticulocyte lysates, which were then combined and applied to m^7G -Sepharose in the presence of GTP or GTP plus cap analog. As shown in Figure 1C, Pum2 bound the m^7G -Sepharose in the presence of GTP, but not the free cap analog. eIF4E also bound to the m^7G -Sepharose resin when GTP was present, and was reduced, but not eliminated, when free cap analog was present. These data also indicate that Pum2 directly or indirectly interacts with the cap structure.

FIGURE 1. Pum2 binds the cap. (A) Cytostatic Factor (CSF) extracts were prepared from *Xenopus* eggs and subsequently supplemented with calcium to induce entry into the cell cycle. At 0, 10, and 20 min after calcium addition, the extracts were supplemented with GTP and applied to m⁷G-Sepharose resin (lanes 1–3). In addition, some extract (no calcium) was supplemented with cap analog and also applied to the m⁷G-Sepharose resin were probed on Western blots for Maskin, Pum2, and eIF4E. The load fractions (i.e., 10% of total initial extract) were also probed for the same proteins (lanes 4–6). (B) *Xenopus* oocyte extracts were applied to GDP-Sepharose or m⁷G-Sepharose; the bound material was probed for Pum2 and eIF4E on a Western blot. The load fraction (10% of total) was also probed for the same proteins. (C) Reticulocyte lysates were primed with mRNA encoding Pum2 or eIF4E in the presence of ³⁵S-methionine (lanes 3,4). Equal volumes of the lysates were mixed, supplemented with GTP or cap analog, and applied to m⁷G-Sepharose. Pum2 and eIF4E that were retained on the resin were detected by SDS-PAGE and phosphorimaging. (D) *E. coli*-expressed Pum2 and eIF4E were applied to m⁷G-Sepharose or GDP-Sepharose columns; the bound material was examined by Western blotting (lanes 1–4). Ten percent of the load fractions were also probed by Western blotting (lanes 5,6). (E) Varying amounts of reticulocyte-synthesized Pum2 and eIF4E (i.e., μL of lysate) were applied to m⁷G-Sepharose resin in the presence of GTP or GTP plus cap analog and the amount retained was analyzed by Western blotting (top, bottom). In some cases, the lysates were mixed in the amounts indicated prior to being applied to m⁷G-Sepharose (bottom panel, lanes 1–5). In these cases, the lysates contained GTP but no free cap analog.

The experiments noted above do not address whether Pum2 bound the m⁷G-Sepharose directly or via eIF4E. To distinguish between these possibilities, we used yeast two-hybrid analysis, protein coimmunoprecipitation assays, and in vitro pull-down experiments; in no case could we detect an interaction between Pum2 and eIF4E (data not shown). This prompted us to determine whether Pum2, in the absence of eIF4E, would be retained on m⁷G-Sepharose.

Previous attempts at expressing recombinant Pum2 protein in *E. coli* have been limited to its RNA binding PUF domain (Wang et al. 2001). To obtain a very limited amount of soluble full-length recombinant Pum2, we used NDSB 201, a nondetergent sulfobetaine that helps solubilize proteins from inclusion bodies (Vuillard et al. 1995). Soluble *E. coli* expressed histidine-tagged Pum 2 was applied to m⁷G-Sepharose or GDP-Sepharose matrices. As a control, recombinant eIF4E was applied to separate matrices in parallel. Figure 1D shows that in separate experiments both recombinant Pum2 and eIF4E were retained on cap analog, but not GDP Sepharose. Relative to eIF4E, the Pum2 binding was low, which could be due to either lower affinity or insolubility of some protein and/or improper folding upon re-solubilization.

To investigate whether Pum2 and eIF4E compete for binding to m⁷G-Sepharose, mRNAs encoding these proteins were translated in separate reticulocyte lysates, which were then applied to m⁷G-Sepharose in the presence of GTP or free cap analog. As noted above, Pum2 and eIF4E bound to the m⁷G-Sepharose resin in the presence of GTP, but not the free analog (Fig. 1E, top). A constant amount of Pum2-containing lysate (25 μL) and increasing amounts of eIF4E-containing lysate (1–10 μL) were then applied to m⁷G-Sepharose in the presence of GTP. As more eIF4E was retained on the matrix, progressively less Pum2 was retained. However, increased Pum2 retention caused no detectable decrease in eIF4E retention (Fig. 1E, bottom). These results suggest that Pum2 and eIF4E compete for binding to the cap analog.

Tryptophan 344 is necessary for Pum2 interaction with the cap analog

To determine the regions and residues necessary for Pum2 binding to cap analog, several deletion mutants in Pum2 were generated. mRNAs encoding these proteins were translated in reticulocyte lysates in the presence of ³⁵S-methionine; the lysates were then applied to m⁷G-Sepharose in the absence or presence of free analog. The binding of Pum2 and the various mutant proteins was expressed as a percent of the total protein (i.e., % input) that bound the m⁷G-Sepharose resin. Figure 2A shows that the preponderance of Pum2 binding activity resided in residues 334–689. Because other cap binding proteins form a “pocket” of aromatic residues into which the cap is inserted (Fechter and Brownlee 2005), we sought to identify aromatic residues of Pum2 within amino acid region 334–689 that could serve as a pocket for cap binding. Several of these residues were individually changed to glycine; one change in particular, the substitution of W344, which is conserved among Pum2 proteins from several vertebrate species (Fig. 2B), consistently abrogated cap binding when the source of the Pum2 was mRNA-primed reticulocyte lysates (Fig. 2C), or recombinant protein from *E. coli* (Fig. 2D). For

FIGURE 2. Pum2 W344 mediates cap binding and translational repression. (A) Pum2 deletion mutants and a W344G point mutant were expressed in reticulocyte lysates and applied to m⁷G-Sepharose; the percent bound was determined by Western blotting. (B) Sequence alignment of a selected region of Pum2 protein among animal species. The underlined W (corresponding to W344 in *Xenopus laevis*) is conserved among these and probably most other vertebrates as well. (C) Pum2 WT, Pum2 W344G, eIF4E, and ePAB were expressed in reticulocyte lysates, applied to GDP-Sepharose or m⁷G-Sepharose, and proteins that were retained were analyzed by Western blotting. (D) Recombinant Pum2 WT and W344G were applied to m⁷G-Sepharose and the bound material analyzed by Western blotting.

comparison, the strong binding of eIF4E and the lack of significant binding of ePAB to m⁷G-Sepharose are also shown (Fig. 2C).

To investigate whether Pum2 represses translation in a W344-dependent manner, we performed an RNA-protein tethering assay. That is, by anchoring an RNA binding protein (Pum2) directly to the RNA (via the λ N-B box system, see below), a functionally isolated translation system can be obtained. Thus, the effects of a mutant Pum2 on translation can be examined with minimal interference from endogenous Pum2 or other RNA binding proteins, since they do not interact with the B box sequence. Oocytes were injected with RNA encoding WT or W344G Pum2 that were fused to the phage λ N protein (Baron-Benhamou et al. 2004). After overnight incubation, the oocytes were injected a second time with luciferase RNA whose 3' UTR contained five phage λ B box stem-loop structures. While WT Pum2 reduced translation of the reporter by ~58%, the W344G mutant protein had little effect even though similar amounts of the proteins were synthesized (Fig. 3). In addition, similar amounts of the luciferase reporter RNAs synthesized in vitro with trace amounts of ³²P-UTP remained stable after the injected oocytes were incubated overnight (Fig. 3, top). In similar experiments, oocytes were first injected with luciferase RNA whose 3' UTR contained or lacked the RINGO/SPY pumilio binding elements (PBEs), and then injected with mRNAs encoding WT or W344G Pum2 proteins. Pum2 repressed luciferase RNA translation by ~65%, while W344G Pum2 had little effect. Moreover, WT Pum2 repression required the presence of the PBE in the luciferase 3' UTR. Similar amounts of WT and W344G Pum2 proteins were synthesized in the oocytes and all of the reporter RNAs were equally stable (Fig. 3). These data,

together with those in Figure 2, indicate that Pum2 represses translation by competing with eIF4E to bind the cap, and that the Pum2–cap interaction requires tryptophan 344.

DISCUSSION

Translational control by 3' UTR binding proteins is widespread in eukaryotes, yet there are few examples where the molecular mechanisms by which this regulation occurs are known in detail. Some cases where the mechanisms have been defined include lipoxigenase mRNA, where the 3' UTR binding proteins hnRNP K and E1 regulate 40S–60S subunit joining at the initiation AUG codon (Ostareck et al. 2001), and actin mRNA, where ZBP1 also binds the 3' UTR to mediate 40S–60S subunit joining (Hüttelmaier et al. 2005). Another example includes several maternal mRNAs, whose translation is controlled by the CPEB–Maskin–eIF4E complex

FIGURE 3. Pum2 represses translation in a W344-dependent manner. RNAs encoding Pum2 WT and W344G fused to λ N protein were injected into 10 oocytes. Following an overnight incubation, the oocytes were then injected with luciferase RNA containing 5 B boxes in the 3' UTR. The oocytes were then homogenized and luciferase activity was determined (histogram) as was expression of the fusion proteins by Western blotting (bottom). The top portion shows an autoradiogram indicating that the relative levels of ³²P-UTP trace-labeled luciferase RNAs at the end of the incubation period were similar. Other RNAs encoding Pum2 WT and W344G (no fusion) were injected into oocytes with luciferase RNA, whose 3' UTR contained or lacked a PBE. Luciferase activity, expression of the heterologous proteins, and relative amount of the luciferase reporter RNAs that remained after the incubation period were determined as above. The amount of luciferase activity in the absence of heterologous Pum2 was used as the standard against which the other values were normalized. Each experiment was performed three times; the bars on the histograms refer to SEM. The data are statistically significant ($P < 0.05$, Student's t -test).

noted in the Introduction. The CPEB–Maskin–eIF4E paradigm is mimicked in large part by Bruno and Cup (Nakamura et al. 2004) and FMRP and CYFIP (Napoli et al. 2008), where an eIF4E-interacting protein (Cup, CYFIP) is linked to a 3' UTR binding protein (Bruno, FMRP) (see Fig. 4 for a comparison of the Pum2–cap and CPEB–Maskin–eIF4E interactions). Another interesting example is Bicoid, which not only binds to mRNA 3' UTRs, but also to 4E-HP, a protein that binds the cap directly. In this case, 4E-HP blocks the association of eIF4E with the cap on specific mRNAs (Cho et al. 2005). Here, we show that translational control by Pum2 appears to be a variation on the Bicoid–4E-HP paradigm; it combines the activities of Bicoid–4E-HP into one protein. By binding to both a 3' UTR PBE and the cap structure of the same mRNA, Pum2 may preclude initiation by eIF4E on specific mRNAs.

Pum2 is one member of a large family of proteins designated PUF (acronym for pumilio and fem-3-binding factor [pumilio-FBF]), which are characterized by a similar RNA binding domain, the PUF domain composed of eight α helical repeats (Lu et al. 2009). PUF proteins repress translation in a manner that can involve deadenylation (Goldstrohm et al. 2006; Kimble and Crittenden 2007); presumably, a deadenylated RNA loses the poly(A) binding protein (PABP)–eIF4G interaction, which normally stimulates cap-dependent translation (Derry et al. 2006). In another instance, the yeast PUF6 protein represses translation by inhibiting recruitment of the 48S preinitiation complex into 80S ribosomes (Deng et al. 2008). These results, together with those presented here, indicate that Pumilio-PUF proteins can repress translation in multiple ways.

The equilibrium dissociation constants (K_d) of several cap binding proteins for the cap range $\sim$ 100-fold, from 10 nM for the nuclear cap binding complex CBC20/CBC80 (Wilson et al. 1999), to micromolar amounts for vaccinia virus VP 39 (Hu et al. 1999). Although we have been unable to determine a K_d of Pum2 for the cap because of its relative insolubility, based on the data presented in Figure 1E, we suspect that it is low, and may be about one-tenth that of eIF4E (the K_d of eIF4E for the cap is 260–280 nM) (Niedzwiecka et al. 2002; Scheper et al. 2002). However, Pum2 also binds to the PBE located in the 3' UTR with nanomolar affinity, which could influence, perhaps even increasing, its affinity for the cap. Moreover, a weak affinity for the cap may be necessary to alleviate Pum2-mediated repression (Padmanabhan and Richter 2006). We also

noted a cell cycle stage specific association of Pum2 with the cap, suggesting a role for post-translational modifications in ovo that may be required for more avid binding or, on the other hand, its release from the cap structure.

Cap binding proteins are generally thought to have pockets formed by aromatic residues into which the cap is inserted (Evdokimova et al. 2001; Cho et al. 2005; Fechter and Brownlee 2005; Kiriakidou et al. 2007;). In Pum2, W344 is probably one component of this pocket; our efforts to identify additional residues in the putative pocket were not successful. Nonetheless, Pum2 combines two binding specificities, with the cap and the 3' UTR PBE, to repress translation. Argonaute has been similarly shown to bind the cap through conserved hydrophobic residues (Kiriakidou et al. 2007), although the importance of this interaction for translational repression is controversial (Eulalio et al. 2008). The list of cap binding proteins is an ever-expanding one and includes multiple eIF4E proteins from several species (Rhoads 2009). Some of these eIF4E-like proteins either have a low binding affinity for eIF4G or do not bind it at all. It is therefore possible that some of these proteins might act like 4E-HP or Pum2 to repress translation by competing with bona fide eIF4E.

MATERIALS AND METHODS

Cap analog binding

Cap analog (m^7 GpppG) Sepharose was purchased from Amersham-Pharmacia and equilibrated in binding buffer (50 mM Tris-HCl, pH 7.5, 30 mM NaCl, 1 mM dithiothreitol, 2.5 mM MgCl₂, 0.5 mM 3-(1-pyridino)-1-propane sulfonate (NDSB-201, Calbiochem), 5% glycerol, and complete protease inhibitor (Roche). In some experiments, 0.1% 2-mercaptoethanol was substituted for the dithiothreitol and 1 M urea was substituted for the NDSB-201. NDSB 201 is a nondenaturing zwitterionic compound that is used to promote proper protein folding. GDP Sepharose was generated and was equilibrated in the same buffer (Sonenberg et al. 1979). Twenty-five microliters of bead slurry were supplemented with BSA (20 μ g/mL), preincubated with 250 μ M GTP or cap analog, and then incubated with varying amounts (although usually $\sim$ 20 μ L) of reticulocyte lysate primed with in vitro synthesized mRNA, recombinant Pum2 or eIF4E expressed in *E. coli* ($\sim$ 0.5 μ g), or extract from oocytes injected with mRNA encoding myc-tagged Pum2 for 1 h at 4 C. The beads were then washed five times with 0.25 mL of binding buffer before being heated in SDS sample buffer. This material was then either examined by Western blotting or SDS-PAGE and phosphorimaging. Chromatography on the cap analog or GDP Sepharose took place either in batch or in a column (i.e., a plastic pipet tip stoppered with glass wool). Additional details on cap analog affinity chromatography may be found elsewhere (Stebbins-Boaz et al. 1999; Cao et al. 2006).

Immunoprecipitations

Myc 9E10 monoclonal antibody was covalently conjugated to protein A Sepharose. Oocytes injected with myc-Pum2 RNA

FIGURE 4. Pum2 and CPEB-mediated translational control. Comparison of the Pum2–cap interaction with the CPEB–Maskin–eIF4E interaction. The PBE refers to the pumilio binding element and the CPE to the cytoplasmic polyadenylation element.

(1 $\mu\text{g}/\mu\text{L}$) were homogenized in IPHB buffer containing 0.1% NP-40 (10 oocytes in 0.25 mL). The insoluble material was removed by centrifugation and the supernatant was incubated with 25 μL myc antibody or nonspecific IgG conjugated beads for 1 h at 4 C. The beads were then washed 5 times with 0.25 mL RIPA buffer before elution in SDS sample buffer.

Luciferase assays

Reporter RNAs were generated by fusing the RINGO/SPY 3' UTR, containing or lacking the PBE (i.e., mutant $\Delta 6$) (Padmanabhan and Richter 2006) to the Renilla luciferase open reading frame (clone pRLTK8myc) (Nottrott et al. 2006). Clones containing the phage λ N and 5 B box sequences were a gift from Dr. D. Moazed (Harvard Medical School). The λ N sequence was PCR amplified and inserted into the NcoI/EcoRI site of pET30a that was in-frame with Pum2 WT and 344G. The B box sequence was inserted into the NotI/HpaI of pRLTK8myc.

RNAs were prepared using T7 mMessage machine (Ambion) and were subsequently polyadenylated with *E. coli* poly(A) polymerase. Twenty-five nanograms of RNA (encoding Pum2, λ N-Pum2 fusion proteins) were injected per oocyte; ~ 20 – 25 oocytes were injected, which were then incubated overnight before extracts were prepared for immunoprecipitation or a second injection of RNA. In these cases, about 0.5 fmol of reporter RNA was injected per oocyte. After 2 h of incubation, 5–20 oocytes were homogenized and prepared for luciferase assays with the Promega Dual-Luciferase Reporter Assay kit. Insoluble material was removed by centrifugation and 10 μL of extract was assayed according to the manufacturer's instructions.

Mutant proteins

Histidine-tagged Pum2 deletion proteins were expressed in and isolated from Tuner DE3 cells (Novagen). For some experiments, Pum2 protein that was enriched in inclusion bodies was solubilized in HEPES-NaOH containing 6 M guanidine HCl for 1 h before rapid dilution (1:10) in HEPES buffer that contained 1 M NDSB 201. Deletions and point mutations in Pum2 were generated by PCR of template DNA.

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