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Preparation of cooperative RNA recognition complexes for crystallographic structural studies

Chen Qiu¹, Aaron C. Goldstrohm², Traci M. Tanaka Hall¹

¹Epigenetics and Stem Cell Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709

²Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN 55455

Abstract

It is essential that mRNA-binding proteins recognize specific motifs in target mRNAs to control their processing, localization, and expression. Although mRNAs are typically targets of many different regulatory factors, our understanding of how they work together is limited. In some cases, RNA-binding proteins work cooperatively to regulate an mRNA target. A classic example is *Drosophila melanogaster* Pumilio (Pum) and Nanos (Nos). Pum is a sequence-specific RNA-binding protein. Nos also binds RNA, but interaction with some targets requires Pum to bind first. We recently determined crystal structures of complexes of Pum and Nos with two different target RNA sequences. A crystal structure in complex with the *hunchback* mRNA element showed how Pum and Nos together can recognize an extended RNA sequence with Nos binding to an A/U-rich sequence 5' of the Pum sequence element. Nos also enables recognition of elements that contain an A/U-rich 5' sequence, but imperfectly match the Pum sequence element. We determined a crystal structure of Pum and Nos in complex with the *Cyclin B* mRNA element, which demonstrated how Nos clamps the Pum-RNA complex and enables recognition of the imperfect element. Here we describe methods for expression and purification of stable Pum-Nos-RNA complexes for crystallization, details of the crystallization and structure determination, and guidance on how to analyze protein-RNA structures and evaluate structure-driven hypotheses. We aim to provide tips and guidance that can be applied to other protein-RNA complexes. With hundreds of mRNA-binding proteins identified, combinatorial control is likely to be common, and much work remains to understand them structurally.

Keywords

Pumilio; Nanos; X-ray crystallography; cooperative RNA binding; PUF protein

1. INTRODUCTION

Post-transcriptional regulation, mediated by hundreds of mRNA-binding proteins (mRBPs), plays an essential role in controlling gene expression. mRBPs recognize their target mRNAs

through specific sequence or structural motifs often in the 5' or 3' untranslated regions (UTRs), acting as an initial step leading to regulation of RNA processing, localization, translation and degradation (Mitchell & Parker, 2014; Singh, Pratt, Yeo, & Moore, 2015). An individual mRBP is capable of recognizing hundreds of mRNAs. A second RBP can add an additional layer of selectivity when its binding site is in the vicinity of the first binding site. Cooperative binding of the two RBPs yields a more stable complex while each individual protein might bind only weakly or transiently. X-ray crystallography is a valuable technique to visualize the protein-RNA complexes and provide insights into combinatorial target RNA recognition and regulation by multiple RBPs. Hundreds of structures of individual RBPs in complex with their cognate RNA are available. However, structural knowledge of cooperative binding by multiple RBPs to RNA is limited. It is more challenging to obtain a stable protein-RNA complex consisting of multiple proteins for crystallographic studies. Here we describe methods for preparing a ternary complex of *Drosophila* Pumilio (Pum) and Nanos (Nos) proteins and their target RNA for biochemical and structural studies. We also discuss some techniques and strategies that might be helpful for crystallizing protein-RNA complexes in general.

Drosophila Pum is a founding member of the eukaryotic PUF (Pum and *fem-3* mRNA-binding factor, FBF) protein family (Zamore, Williamson, & Lehmann, 1997). PUF proteins are characterized by a sequence-specific RNA-binding domain known as the PUM-homology domain (PUM-HD), and the domain is evolutionarily conserved across eukaryotes (Fig. 1A) (Wickens, Bernstein, Kimble, & Parker, 2002; Zhang et al., 1997). The prototypical PUF recognition element (PRE) contains 5'-UGUAnAUA-3' (n = any base) (X. Wang, McLachlan, Zamore, & Hall, 2002). Yeast and worm encode multiple PUF proteins exhibiting different sequence specificities with varying length of n from 1 to 5 in the middle region (Gerber, Herschlag, & Brown, 2004; Koh et al., 2009). Crystal structures of PUM-HDs in complex with RNA reveal modular RNA recognition by eight PUM repeats arranged in a crescent shape (Lu & Hall, 2011; M. T. Miller, Higgin, & Hall, 2008; X. Wang et al., 2002; Y. Wang, Opperman, Wickens, & Hall, 2009; Wilinski et al., 2015; Zhu, Stumpf, Krahn, Wickens, & Hall, 2009). Typically, one PUM repeat comprising three α helices uses three amino acid residues in the second α helix to contact one RNA base in a sequence-specific manner. PUF proteins bind to PREs usually found in the 3'-UTR of target mRNAs and recruit partner proteins leading to repression of protein expression and mRNA degradation (M. A. Miller & Olivas, 2011). PUF proteins function as broad-spectrum regulators in higher eukaryotes for mRNAs involved in embryonic, germline and neuronal development (Gerber, Luschnig, Krasnow, Brown, & Herschlag, 2006; Goldstrohm, Hall, & McKenney, 2018; Kershner & Kimble, 2010).

A classic example of PUF-mediated regulation is demonstrated by the essential role of *Drosophila* Pum in establishing the embryonic body pattern through repression of maternal *hunchback* (*hb*) mRNA (Forbes & Lehmann, 1998; Lehmann & Nusslein-Volhard, 1987). Pum protein and *hb* mRNA are distributed throughout the *Drosophila* embryo (Macdonald, 1992). Nos protein forms a gradient, emanating from the posterior to the anterior end (Barker, Wang, Moore, Dickinson, & Lehmann, 1992; C. Wang & Lehmann, 1991). At the posterior, *hb* mRNA is repressed by Pum in concert with Nos, whereas at the anterior, Hb protein is produced due to the absence of Nos (Lehmann & Nusslein-Volhard, 1991; Murata

& Wharton, 1995; Sonoda & Wharton, 1999). The proper spatial distribution of Hb protein is critical for proper body pattern formation: no abdominal segments are formed in embryos lacking *pum* or *nos*. Extensive genetic and biochemical studies have revealed the importance of two copies of Nos Response Elements (NREs) in the 3'-UTR of *hb* mRNA (Wharton & Struhl, 1991). Both NRE1 and NRE2 contain two conserved elements termed Box A and Box B. The Box B sequence corresponds to a PRE (5'-UGUAHAUA, H=C/U), which is bound by Pum. RNA-bound Pum recruits Nos, which binds RNA upstream of the PRE via its C-terminal zinc-finger (ZF) domain (Fig. 1A) (Curtis et al., 1997). Nos subsequently recruits the CNOT deadenylase complex through its N-terminal Nos effector domain, resulting in deadenylation and decapping of the mRNA (Raisch et al., 2016). Box A has been shown recently to be a binding site for a third mRBP called Brain Tumor (Brat), which also synergistically inhibits *hb* mRNA translation (Arvola, Weidmann, Tanaka Hall, & Goldstrohm, 2017; Loedige et al., 2014; Sonoda & Wharton, 2001).

Our crystal structures of Pum-Nos-NRE ternary complexes illuminated the mechanisms of Pum-Nos cooperativity at the atomic level (Fig. 1B) (Weidmann et al., 2016). Pum recognizes the PRE sequence within the ternary complex and also as observed in our Pum-PRE binary complex. Additionally, Nos embraces both Pum and RNA, effectively clamping them together. The Nos tandem ZFs bind 3 nucleotides immediately upstream of the PRE, and the Nos C-terminal tail after the ZF domain interacts with a loop region between repeats 7 and 8 of the PUM-HD. Nos alone does not bind the NRE in the absence of Pum. Nevertheless, Nos enhances Pum binding to RNA. Another known Pum-Nos target *Cyclin B* (*CycB*) mRNA contains an imperfect PRE: 5'-UGUAAUUU (Asaoka-Taguchi, Yamada, Nakamura, Hanyu, & Kobayashi, 1999) (Fig. 1A). Pum alone does not form a stable complex with the *CycB* mRNA element, but addition of Nos results in a stable ternary complex. A crystal structure of a ternary complex of Pum and Nos with the *CycB* PRE showed how the imperfect PRE sequence is tolerated to allow Pum binding (Fig. 1B). We also used SEQRS (*in vitro* selection, high-throughput sequencing of RNA, and sequence specificity landscapes) analysis, which revealed that Nos alters the binding specificity of Pum. A 4-nt Nos-binding site (NBS) with A/U rich sequences was identified upstream of the 5'-UGUA of the PRE, while sequence selection at the 3'-end of the PRE became relaxed.

2. EXPRESSION AND PURIFICATION OF PUM-NOS-RNA TERNARY COMPLEXES

2.1. Expression system

cDNAs encoding *Drosophila* Pum (residues 1091–1426) and Nos (289–401) were subcloned into the pSMT3 vector, and plasmids were transformed into *E. coli* strain BL21-CodonPlus (DE3)-RIL (Agilent). The pSMT3 vector (kindly provided by Christopher Lima, Sloan Kettering Institute) has become our first choice when working with a new protein (Mossessova & Lima, 2000). The N-terminal fused His₆-SUMO tag improves protein solubility and is cleavable by the SUMO protease, Ulp1. Ulp1 recognizes the tertiary structure of the ubiquitin-like protein SUMO rather than an amino acid sequence so the cleavage is highly specific. When the BamHI restriction site is used for the 5' end cloning, only a serine residue is left at the N-terminus of the expressed protein after Ulp1 cleavage.

The cleaved His₆-SUMO tag runs at ~17 kDa on SDS-PAGE with a calculated MW of 13.7 kDa. The Pum and Nos proteins were purified using the protocol below (Fig. 2). Steps 1 to 11 are the same for Pum and Nos and can be performed in parallel.

For this work, we focused on the structures of truncated Pum and Nos because the full length proteins expressed poorly. The optimal truncated proteins were determined in part by analysis of sequence homology, specifically the well-conserved PUM-HD of Pum and the tandem ZFs of Nos. In addition, functional assays for RNA-binding and translational repression were employed to delineate the regions of Pum and Nos necessary for RNA recognition and synergistic mRNA regulation.

2.2. Protein expression

1. On day 1, inoculate a small volume (10–50 mL) culture of Luria-Bertani (LB) medium containing 50 µg/mL of chloramphenicol and 50 µg/mL kanamycin with *E. coli* from freshly transformed colonies or frozen glycerol stock. Inclusion of chloramphenicol maintains the pACYC plasmid containing extra copies of tRNA genes in the CodonPlus strain. The culture should contain at least 5 mL for each liter of expression culture. Grow overnight at 37 °C with shaking at 220–250 rpm.
2. The next morning, day 2, add 5 mL overnight culture to inoculate a 1 L culture of LB medium containing 50 µg/mL kanamycin. Include 0.5 mM Zn(OAc)₂ in the media for Nos expression since Nos contains tandem ZFs. Incubate cultures at 37 °C with shaking at 220–250 rpm until the OD₆₀₀ reaches ~0.6. Lower the incubator temperature to 16 °C. Induce protein expression with 0.2 mM IPTG and incubate the culture overnight at 16 °C with shaking.
3. On day 3, spin down the cultures. Wash the cell pellets with PBS and store the pellets at –20 °C for later use or continue the purification.

2.3. Protein purification

4. Resuspend the cell pellet from 1 l culture in 40 mL pre-chilled lysis buffer containing 20 mM Tris (pH 8.0), 0.5 M NaCl, 20 mM imidazole, 5% (v/v) glycerol, and 0.1% (v/v) β-mercaptoethanol. If the cell pellet is freshly prepared, do a freeze-thaw to improve cell lysis efficiency by sonication.
5. Sonicate to disrupt the cells for 30 sec at 60% amplitude, cycling 1 sec on and 2 sec off. Repeat 3 times. Spin the lysate at 28,000 x g for 35 min to pellet and remove the insoluble material. Save a small aliquot (~50 µL) of the total cell lysate and supernatant fraction for SDS-PAGE analysis (Fig. 2).
6. Mix the supernatant with 5 mL Ni-NTA resin (Qiagen) pre-equilibrated with lysis buffer in a 50-mL Falcon tube and rotate for 1 hr at 4 °C to bind His-tagged protein. To prepare the resin, we transfer 10 mL of the 50% slurry to a Bio-Rad Econo-Pac column, elute the storage solution, wash the resin with lysis buffer, and transfer the resuspended resin to the Falcon tube.

7. After protein binding, spin the lysate and resin mixture at 500 x g for 5 min at 4 °C. Remove the supernatant containing unbound proteins. Save a small aliquot (~50 µL) of the supernatant solution for SDS-PAGE analysis (Fig. 2). Add 50 mL lysis buffer to the tube and transfer the resuspended resin to a gravity column. We use Bio-Rad Econo-Pac columns.
8. Wash the resin with lysis buffer until no protein is detected in the washes. Usually 200–300 mL wash is sufficient. We monitor this by performing a rapid protein detection assay. We mix 9 µL of wash elution with 50 µL diluted Bio-Rad Protein Assay Dye Reagent (1 part dye concentrate to 4 parts distilled water). The yellow dye solution turns blue in the presence of protein. The high NaCl concentration in the lysis and wash buffer helps to reduce nucleic acid contamination.
9. Elute protein with a buffer containing 20 mM Tris (pH 8.0), 50 mM NaCl, 0.2 M imidazole (a 2 M imidazole stock solution is pH adjusted to 8.0), and 1 mM DTT. Monitor by rapid protein detection assay and stop eluting when the dye solution no longer changes color. Usually 50 mL eluent is collected. Note the NaCl concentration is reduced from 500 mM in the lysis and wash buffer to 50 mM in the elution buffer. Using the low-salt elution buffer avoids dialysis or dilution to prepare the sample for subsequent ion-exchange or heparin-affinity chromatography. Imidazole has no effect on protein binding to a heparin or Q column. It is typically good practice to check the purity of the eluted protein and the progression of the purification by SDS-PAGE (Fig. 2).
10. To cleave the fusion protein, add 100 µL Ulp1 (1 mg/mL) to the eluent. Incubate at 4 °C overnight. We obtained the Ulp1 expression plasmid from Dr. Christopher Lima at Sloan Kettering Institute. The His-tagged Ulp1 was purified by one-step Ni-chelating column. One liter of *E. coli* culture yields more than 20 mg Ulp1. The Ulp1 protease is aliquoted and stored at –80 °C. The incubation time for Ulp1 cleavage varies for different proteins. Some fusion proteins can be completely cleaved within 1–2 hr at 4 °C. For those proteins it is better to do 2-hr incubation and then move to the next purification step on the same day. Some proteins require more cleavage time, such as overnight incubation; however, we have had proteins precipitate during overnight Ulp1 cleavage reactions. This step should be assessed by SDS-PAGE the first time protein is prepared and cleaved.
11. Filter the protein solution using Millex-GP syringe filters with 0.22 µm pore size. Apply the protein solution to a 5-mL Hi-Trap Heparin column (GE Healthcare) equilibrated in Buffer A containing 20 mM Tris (pH 8.0) and 1 mM DTT. Elute the protein with a linear NaCl gradient from 5% to 100% Buffer B containing 20 mM Tris (pH 8.0), 1 mM DTT, and 1 M NaCl. Pum co-elutes with His-SUMO in a single peak at 260 mM NaCl from the Heparin column (Fig. 2). Nos separates from the majority of His-SUMO. However, an SDS-PAGE gel of the Nos preparation shows some faster migrating fragments that are likely to be degradation products (Fig. 2).

12. To remove contaminating His-SUMO from Pum, pooled peak fractions are applied to 1 mL Ni-NTA resin. His-SUMO binds to the Ni-NTA resin and Pum flows through.
13. Concentrate purified Pum (after removal of His-SUMO) and Nos (eluted from the Heparin column) separately using Amicon Ultra Centrifugal Filter Units (EMD Millipore), 30,000 and 10,000 NMWL (nominal molecular weight limit), respectively. Measure the OD₂₈₀ with a NanoDrop or other spectrophotometer. Stop concentrating when the OD₂₈₀ is in the range of 1 to 2. Determine the protein concentration using a calculated protein extinction coefficient based on the numbers of tryptophan and tyrosine residues. The online tool ProtParam in ExPASy can be used to calculate the extinction coefficient (Gasteiger E., 2005).
14. One liter of bacterial culture yields about 30 mg of Pum or 1 mg of Nos. The yield of Nos is lower, and we do not typically have excess protein to store. The concentrated Pum protein that is not used immediately can be flash frozen in liquid nitrogen and stored at −80 °C. After thawing the protein, a gel filtration column will prepare the protein for use.

2.4. Formation and purification of a Pum-Nos-RNA complex

15. A 16-nt *hb* NRE2 RNA (RNA_{*hb*}, 5′-AAAUUGUACAUAAGCC) and a 14-nt *CycB* NRE RNA (RNA_{*CycB*}, 5′-UAUUUGUAAUUUAU) were custom synthesized by Dharmacon. Deprotect the RNAs as directed using the supplied buffer and dry using a SpeedVac. Resuspend the pellet in a buffer of 10 mM Tris, pH 7.5 and 1 mM EDTA. The RNA concentration is determined by OD₂₆₀ using a NanoDrop or other spectrophotometer (for a 1 cm pathlength, the OD₂₆₀=1 for a solution of 40 µg/mL RNA). RNA is stored at −20 °C.
16. Mix Pum, Nos, and RNA in a molar ratio of approximately 1:1.1:1.1 and incubate at 4 °C overnight. Since a minor amount of the Nos protein was partially degraded, the calculated protein concentration based on UV₂₈₀ may not be accurate. To compensate, we included excess Nos in the binding reaction. Any unbound Nos protein will be separated, along with excess RNA, in the subsequent gel filtration step. With OD₂₈₀ values of 1–2 for the two proteins, the concentration of the complex is ~100 µM.
17. Run the assembled complex mixture on a Superdex 200 10/300 GL column (GE Healthcare) equilibrated in 20 mM Hepes (pH 7.4), 0.15 M NaCl, and 2 mM DTT. The maximum sample loading volume for the 10/300 GL column is 500 µL. If the sample volume is >500 µL, concentrate using an Amicon Ultra Centrifugal Filter Unit with a 30,000 NMWL. In this case, the molecular weight of the ternary complex of Pum-Nos-RNA is about 54 kDa. The extra Nos protein with a MW of 12 kDa, including the degraded products that lack the C-terminus and fail to interact with Pum, can pass through the 30,000 NMWL filter membrane. Thus the concentration step can contribute to purification of the ternary complex. Always centrifuge the sample in a microcentrifuge at

maximum speed (~16,000 x g) for 10 min to remove possible precipitation before loading onto the column.

18. The Pum:Nos:RNA complex elutes from the Superdex 200 10/300 GL column at 14.7 mL. Pool the peak fractions and concentrate to OD₂₈₀ ~7. The ratio of OD₂₆₀/OD₂₈₀ is about 1.7, indicating the presence of RNA. Spin down the sample in a microcentrifuge at maximum speed (~16,000 x g) for 10 min. Set up the crystallization experiment immediately.

3. CRYSTALLIZATION AND STRUCTURE DETERMINATION OF A PUM-NOS-RNA COMPLEX

3.1. Crystallization

3.1.1 Crystallization screening and optimization—Initial crystallization screening was carried out using commercial sparse-matrix screens. 250 nL sample was mixed with 250 nL reservoir solution in a 96-well sitting drop plate using a Mosquito® robot (TTP Labtech). Crystals were observed in multiple conditions with (NH₄)₂SO₄ for the sample of Pum-Nos-RNA_{hb}. A grid screen of varying (NH₄)₂SO₄ concentration and pH was performed in a 24-well hanging-drop vapor diffusion format. Single crystals were grown in the condition of 1.1 M (NH₄)₂SO₄, 0.1 M MES, pH 5.6.

During co-crystallization of a complex of multiple macromolecules (with more than one protein and/or with RNA), it is always unknown whether the crystals contain all of the components. Verification that crystals have the desired content at an early stage can avoid the waste of time and effort in optimizing hits with some missing components. One way to determine the content of crystals is to analyze them on a polyacrylamide gel. Crystals are retrieved and washed carefully before dissolving in water or directly in gel-loading buffer. The presence of RNA can be checked by TBE-urea gel, staining with an RNA dye such as methylene blue. Alternatively, diffraction data can be collected if the initial crystals are of diffraction quality. The unit cell content of the crystal can be analyzed for probable protein residue number in terms of the Matthews coefficient. With the structures of one or two components available, one can perform molecular replacement to see whether there is extra density for the third component even with low resolution data. After the protein-RNA complex composition is confirmed, further optimization can be carried out to obtain crystals diffracting to higher resolution.

3.1.2 Crystallization of the Pum-Nos-RNA_{CycB} complex—Crystals of Pum-Nos-RNA_{CycB} were obtained in the condition of 0.9 M (NH₄)₂SO₄, 0.1 M MES, pH 5.6 by microseeding with the Pum-Nos-RNA_{hb} complex crystals.

1. Transfer a few *hb* crystals to a 1.5-mL microcentrifuge tube with a seed bead (Hampton Research) and 50 µL of 1.1 M (NH₄)₂SO₄, 0.1 M MES, pH 5.6. Vortex the tube for 2 min.
2. Make 10-fold serial dilutions of the seed stock with 1.1 M (NH₄)₂SO₄, 0.1 M MES, pH 5.6; (1, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴).

3. Set up a 24-well plate with varying concentrations of $(\text{NH}_4)_2\text{SO}_4$ from 0.8 M to 1.2 M in the reservoir. Pipet 2 μL *CycB* sample and 2 μL diluted seed stock on a cover slide for hanging drop vapor diffusion crystallization.

3.1.3 Crystallization of the Pum-RNA complex—To address the question whether Nos induces any structural change in Pum, we also grew crystals of the Pum-RNA binary complex.

1. Thaw a tube of frozen Pum protein from step 13 in section 2.4 on ice. Centrifuge the protein at 16,000 $\times$ g for 10 min to remove any precipitation.
2. Run a Superdex 75 10/300 GL column (GE Healthcare) with buffer of 20 mM Hepes (pH 7.4), 0.15 M NaCl, and 2 mM DTT. Pool the peak fractions and concentrate to OD_{280} around 3 to 4.
3. Mix Pum protein with RNA at a molar ratio of 1:1.2. Keep on ice for 2 hr. We usually make the RNA stock concentration 0.5–1 mM, so the protein will not be too dilute after mixing with RNA.
4. Centrifuge the sample at 16,000 $\times$ g for 10 min. Set up crystallization screens.

PUF proteins bind to short single-stranded RNAs. Different from the ternary complex preparation, purification of the PUF-RNA binary complex by a gel filtration column is not necessary. One advantage is that a variety of RNA oligonucleotides can be screened simply by mixing purified protein with different RNA. Indeed the sequence and length of RNA affect the outcome of crystallization. In this case, two RNAs were used for screening: an 8-nt PRE sequence (5'-UGUACAUA) and a 12-nt sequence including 4 nt upstream of the PRE (5'-AAAUUGUACAUA). Only the 8-nt RNA produced crystals, while the 12-nt did not crystallize. The initial crystals of the Pum-RNA complex appeared in clusters of rods. To grow single crystals, the microseeding method was used as described above. The optimized condition contained 15% (w/v) PEG 3350, 0.1 M bis-tris, pH 6.5, and 0.2 M NH_4OAc .

3.2 Structure determination

Crystals were transferred to a cryoprotective-solution containing the crystallization solution with an additional 20% v/v glycerol and flash frozen in liquid nitrogen. X-ray diffraction data were collected at the SER-CAT Beamline 22-ID at the Advanced Photon Source, Argonne National Laboratories.

3.2.1 The Pum-Nos-RNA ternary complex—Crystals of the Pum-Nos-RNA_{hb} complex belong to the P6₅22 space group with unit cell dimensions: $a=b=137.0$ Å, $c=221.4$ Å, $\alpha=\beta=90^\circ$, $\gamma=120^\circ$. There is one complex in an asymmetric unit, corresponding to 78% solvent and a Matthews coefficient of 5.7. The high solvent content correlates with the low-resolution diffraction limit of the crystals. The best crystal diffracted to 3.7 Å Bragg spacings, although with a relatively large crystal size of $0.2 \times 0.2 \times 0.4$ mm. Crystal dehydration has been used as a post-crystallization treatment to remove excess solvent, tighten crystal packing, and thus improve diffraction. We tried dehydration by increasing the precipitant concentration in either the drop or the reservoir from 1.1 M $(\text{NH}_4)_2\text{SO}_4$ to 2.4 M and equilibrating overnight or for four days. However, crystal diffraction did not improve.

The dataset was processed and scaled using HKL2000 (Otwinowski & Minor, 1997). The structures of *Drosophila* Pum (PDB: 3H3D) (Edwards, Pyle, Wharton, & Aggarwal, 2001) and zebrafish Nos ZF domain (PDB: 3ALR) (Hashimoto et al., 2010) were used as the search model for molecular replacement with Phenix Phaser (Adams et al., 2010). One round of Phenix.refine revealed the density of the bound RNA. Iterative rounds of manual model building with COOT (Emsley & Cowtan, 2004) and refinement with Phenix led to the final structure of Pum-Nos-RNA_{hb} with $R_{\text{work}} = 0.264$ and $R_{\text{free}} = 0.30$. In the structure only residues 316–385 of Nos were modeled with visible density, while the Nos construct contained residues 289–401. More than 20 residues at both the N- and C- termini were disordered. Removing disordered regions from protein constructs often increases the likelihood of crystallization and improves crystal quality. The Nos protein sequence contains poly-serine and poly-asparagine in the region preceding the first ZF: $^{292}\text{SNSSNNNNNNN}_{302}$. We truncated the Nos construct to 307–401. However, the shortened Nos protein aggregated. Taken together, the 3.7 Å structure of the Pum-Nos-RNA_{hb} ternary complex was the best we could achieve. Despite the modest resolution, the structure provides molecular insights into the Pum-Nos cooperative RNA recognition.

Crystals of the Pum-Nos-RNA_{CycB} complex diffracted even more weakly than the *hb* crystals, with the resolution of 4.0 Å. The structure was refined with the high-resolution Pum protein structure as the reference model.

3.2.2 The Pum-RNA binary complex—Crystals of the Pum-RNA binary complex belong to the C2 space group with unit cell dimensions: $a=194.9$ Å, $b=29.5$ Å, $c=62.0$ Å, $\alpha=\gamma=90^\circ$, $\beta=101.2^\circ$. There is one complex in an asymmetric unit, corresponding to 44% solvent and a Matthews coefficient of 2.2. The crystal diffracted to 1.14 Å resolution. The structure was determined by molecular replacement using the *Drosophila* Pum structure (PDB: 3H3D) as the search model.

4. ANALYSIS OF PROTEIN:RNA CRYSTAL STRUCTURES

With a beautiful crystal structure of a protein:RNA complex in hand, it is satisfying to admire the outcome of weeks, months, or perhaps years of work. Yet harvesting the fruit of that labor lies in the in-depth analyses of the crystal structure. It is helpful to make a list of all the questions that you have and what to look for in the structures. Some common questions for all structures:

1. What is the overall fold of the protein? Is it similar to other proteins? What features are conserved? What are unique features?
2. Using the structure to make a structure-based sequence alignment, are the typical functional residues conserved? What are the roles of well-conserved amino acid residues?
3. Does the structure of the protein change upon ligand binding? What might be the importance of conformational changes for function?
4. Do observed protein-RNA contacts provide information about RNA-binding sequence or structure specificity?

4.1. Characterizing Protein:RNA Interactions

Of course, one of the first things we do when we have a new protein:RNA structure is to look at all of the protein:RNA contacts. One can generate a list of sets of protein atoms and solvent molecules that are within ~ 3.5 Å of the RNA. However, for structures like our PUF protein:RNA complexes, where the RNA is of a manageable length, it is best to look at the protein:RNA interactions manually. We typically follow along the RNA and look at the protein and solvent atoms that are within ~ 3.5 Å of the RNA and determine whether the interaction is chemically reasonable. This practice is also good for quality control of the structure. We use MolProbity (Davis et al., 2007) to assess our structure, and sometimes we find an Arg/Gln/His side chain flip that produces better contacts with the RNA. We usually draw a diagram that illustrates the contacts. We classify interactions into those that appear to be sequence specific because they interact with the RNA bases and those that interact with the RNA backbone. We examine which bases are recognized specifically and which are not. We also determine whether RNA structures are recognized, such as a set of RNA backbone interactions that can only occur if the RNA folds in a particular way. MolProbity's characterization of the RNA structure is also helpful for identifying RNA structural elements.

In the specific case of the Pum:Nos:RNA and Pum:RNA complexes, we examined interactions of both Pum and Nos with the *hb* RNA and noted differences in interactions with the *CycB* RNA. Our ternary complex structures were of modest resolution, so we were limited in our ability to identify water-mediated interactions. However, our Pum:*hb* NRE RNA binary complex structure was of very high resolution, so we used this structure to provide details.

4.1.1. Pum-*hb* RNA interactions.—We found that Pum recognizes the PRE within the *hb* NRE RNA similar to what had been observed in previous Pumilio:RNA complexes.

- a. Pum uses characteristic tripartite recognition motifs (TRMs) to bind to specific RNA bases using the PUF RNA recognition code: NQ/Y for uracil, SQ/R or CQ/R for adenine, and SE/N for guanine.
- b. Pum did not specifically recognize the nucleotide at position 5 (+5C), instead this base directly stacked with the 4th base. We had previously identified this type of RNA recognition, the 'base omission' recognition mode, for some human Pum protein and RNA combinations (Lu & Hall, 2011).
- c. Recognition of the *hb* RNA PRE by Pum was the same in the binary and ternary complexes.

4.1.2. Pum-*CycB* RNA interactions.—We found that the interactions of Pum with the *CycB* RNA included 5' interactions that were similar to those with the *hb* RNA and 3' interactions that were distinct (Fig. 1B).

- a. In our structures of ternary complexes of Pum and Nos with *hb* or *CycB* RNAs, the structure and recognition of the common 5'-UGUA sequence was the same.

- b. In contrast, the 3' divergent sequence of the *CycB* NRE RNA required adjustments in the RNA structure for Pum to bind to 'mismatched' +5A and +6U bases. The +8U base of *CycB* RNA was not resolved, but interaction with +7U was the same for *hb* and *CycB* RNAs.

4.1.3. Nos-RNA interactions.—We found that Nos interacted with three nucleotides immediately upstream of the PRE in the *hb* and *CycB* RNAs (Fig. 3A).

- a. Nos forms a binding pocket for the −1U nucleotide.
- b. Nos binds to the −2A and −3A nucleotides on the surface of the two zinc fingers.

4.1.4. Pum protein conformational changes.—We also discovered changes in the Pum structure with formation of the ternary complex that resulted in additional RNA interaction. If multiple structures of a protein are available, aligning the structures and assessing structural changes may turn up interesting differences. A previous structure of Pum alone required truncation of the C terminus for crystallization, and in the crystal lattice, the C-terminal regions of two independent Pum molecules interacted (Edwards et al., 2001). Because of this truncation, the Pum protein in our binary complex contained 22 additional residues that formed two α helices at the C-terminus. In the presence of Nos, the final α helix unfolded and the peptide formed part of the RNA interaction surface in the ternary complex.

4.2. Characterizing Protein:Protein Interactions

The crystal structures of the ternary complexes revealed crucial interactions between the C-terminal tail of Nos and the non-RNA-binding surface of Pum (Fig. 3). Just as we examined the structures for all contacts between the protein and RNA, we also looked at interactions between the two proteins. We looked specifically at the roles of residues that had previously been shown to be important for Pum and Nos function. A *nos* mutant with a strong phenotype resulted from a deletion of seven residues in the C-terminal region of Nos (Arrizabalaga & Lehmann, 1999), and our structures showed that the deleted region includes residues that interact with Pum. In addition, our structures revealed that Nos interacts with Pum between PUM repeats 7 and 8. This finding indicates why an insertion in this loop disrupted Pum function and why Nos could only interact with human Pum1 when three residues in the equivalent loop are deleted (Sonoda & Wharton, 1999). Other mutations that disrupt complex formation were also found at this interface (Wharton, Sonoda, Lee, Patterson, & Murata, 1998).

4.3. Methods to Evaluate Structure-Driven Hypotheses

We took advantage of several different methods to evaluate complex formation, translational repression, and RNA sequence specificity (Weidmann et al., 2016).

- a. Electrophoretic mobility shift assays (EMSAs) were used to assess complex formation (Black D.L., 1998; Ryder, Recht, & Williamson, 2008). Although EMSAs have their limitations as a non-equilibrium method, they have the advantage of visualizing unbound RNAs and distinct complexes. The ability to observe a supershifted RNA band when both Pum and Nos bound to the PRE

RNA was helpful. We could manipulate the two proteins or RNA using this *in vitro* method. This approach revealed critical elements for complex formation and clearly demonstrated that Nos enables binding of Pum to the *CycB* RNA.

- b. Cell-based reporter assays to assess translational repression were used to determine the effects of Nos variations (Van Etten, Schagat, & Goldstrohm, 2013). We took advantage of the fact that *Drosophila* D.mel-2 cells do not express Nos and the level of Pum expression is not sufficient to repress a reporter mRNA in the absence of exogenous Nos. Since we relied upon the endogenous Pum, we could alter the reporter RNA sequence or Nos protein.
- c. An *in vitro* selection technique called SEQRS (Campbell et al., 2012) was used to identify the consensus RNA motif for Pum alone or in the presence of Nos. This method is thoroughly described in a recent review (Lou et al., 2017). The strength of this method is in the ability to analyze the sequences as enrichment advances through rounds of selection.

5. CONCLUDING REMARKS

The breadth of information about RNA-binding proteins and their motifs continues to expand with the development of novel genomics methods. These data provide the opportunity to explore the range of RNA recognition strategies by mRBPs. X-ray crystallography remains an effective method to determine the molecular mechanisms of protein-RNA interaction. It provides high-resolution information and is particularly well suited for medium-to-large sized complexes (50–200 kDa). Collaborative mRBP complexes have been identified more slowly. Often they begin with genetic studies that indicate regulation of a common pathway followed by careful biochemical studies to firm up the relationships. The story of Pum and Nos cooperative regulation arose from *Drosophila* genetics and developed through a series of studies by multiple groups. The crystal structures of Pum-Nos-RNA complexes have added a satisfying picture to illustrate new principles of RNA recognition by mRBPs working together to dynamically control gene expression.

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Figure 1.

Crystal structures of Pum-Nos-RNA ternary complexes. A. Schematic drawings of Pum and Nos structural domains and RNA sequences. The eight repeats forming the PUM-HD RNA-binding domain are indicated by yellow rectangles, and the two ZF domains of Nos are represented by blue rectangles. The C-terminal tail of Nos is colored red. NRE RNA sequences bound by Pum and Nos are underlined orange and blue, respectively. The PRE consensus sequences are shown in black uppercase letters at highly conserved positions and in the *CycB* sequence, nucleotides that do not match the consensus are red. The Nos binding sequence is A/U-rich and shown in lowercase. B. Superposition of crystal structures of Pum

and Nos in complex with *hb* (orange, PDB ID 5KL1) and *CycB* (light blue, PDB ID 5KL8) RNAs. The two RNAs are shown with the protein structures from the Pum-Nos-RNA_{*hb*} complex. Pum is shown as a ribbon diagram, and Nos is shown as a surface representation. The PUM-HD and ZF domains are colored as in the schematic drawings.

Figure 2.

Flowchart of protein purification and Pum-Nos-RNA ternary complex formation. Coomassie-stained SDS-PAGE gels showing samples at different stages of complex preparation are shown. In the top gel, the lanes are aliquots of total cell lysate (Total), soluble supernatant fraction (Soluble), remaining proteins flowing through the Ni column (Flow Thru), and imidazole eluate from the Ni column (Eluate). A chromatogram of the Superdex 200 gel filtration purification of the Pum-Nos-RNA ternary complex is shown, and the pooled peak fractions are indicated by the red box. Fractions from this peak are shown in the bottom SDS-PAGE gel.

Figure 3.

Nos clasps the RNA nucleotides 5' of the Pum binding site with its ZF domains and wraps around Pum with its C-terminal tail. Two orientations of the Pum-Nos-RNA_{hb} ternary complex are shown. Pum (yellow) is shown as a surface representation, and Nos is shown as a ribbon diagram. The ZFs and C-terminal tail are colored as in Fig. 1.