**Mechanisms of mRNA regulation by Pumilio and Nanos**

by

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# DEDICATION

This work is dedicated to my wife, Brittany Bowman, whose partnership permits me to be the person I am meant to be.

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I cannot thank my mentor, Aaron Goldstrohm, enough. However, I will try to succinctly state why I am immensely grateful for his guidance. Aaron has transformed my curiosity into capability. The thoughtfulness and patience he exercised while training me was integral to my success. He is straightforward and transparent with his management. His interest in the science is contagious. He has been supportive of all of my endeavors to enhance my development, even those that kept me from the bench.

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Lastly, I must mention how much I love and appreciate my family, who imparted in me the sense and gifted me the opportunity to pursue my passions.

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# ABBREVIATIONS

| | |
|---|---|
| 4EHP | →4E Homologous Protein |
| ARE | →adenosine and uridine rich element |
| ARE-BP | →adenosine and uridine rich element binding protein |
| 4EHP | →4E Homologous Protein |
| Bcd | →Bicoid (*Drosophila*) |
| Brat | →Brain Tumor |
| CBC | →Cap-binding Complex |
| CNOT4 | →CCR4-NOT transcription complex subunit 4 |
| CDS | →Coding sequence |
| CNBP | →Cellular Nucleic Acid Binding Protein |
| CPE | →Cytoplasmic Polyadenylation Element |
| CPEB | →Cytoplasmic Polyadenylation Element Binding protein |
| CPSF | →Cleavage and polyadenylation specificity factor |
| CycB | →Cyclin B (*Drosophila*) |
| CycB1 | →Cyclin B1 (*Homo Sapiens*) |
| DM1 | →Myotonic Dystrophy Type 1 |
| DM2 | →Myotonic Dystrophy Type 2 |
| DMPK | →Myotonic Dystrophy Protein Kinase |
| DNA | →Deoxyribonucleic acid |
| eEF1A | →eukaryotic Elongation Factor 1A |
| eIF2 | →eukaryotic Initiation Factor 2 |
| eIF3 | →eukaryotic Initiation Factor 3 |
| eIF5 | →eukaryotic Initiation Factor 5 |
| eIF4E | →eukaryotic Initiation Factor 4E |
| eIF4F | →eukaryotic Initiation Factor 4F |
| eRF1 | →eukaryotic Release Factor 1 |
| eRF3 | →eukaryotic Release Factor 3 |
| FBF | →Fem-3 Binding Factor |
| GLD-3 | →germline development defective 3 |
| Hb | →Hunchback |
| IRE | →Iron Response Element |
| IRP1 | →Iron Response Element Binding Protein 1 |
| MBNL1 | →muscleblind like 1 |
| miRNA | →micro ribonucleic acid |
| mRNA | →messenger ribonucleic acid |
| MSI1 | →Musashi 1 |
| NIM | →NOT1 Interacting Motif |
| Nos | →Nanos |

| | |
|---|---|
| NPC | →Nuclear Pore Complex |
| NRD | →Nanos repression domain |
| NRE | →Nanos Response Element |
| PABP | →Poly-Adenosine Binding Protein (all species) |
| pAbp | →poly-Adenosine binding protein (*Drosophila*) |
| PABPC1 | →Poly-Adenosine Binding Protein Cytoplasmic 1 (*Homo sapiens*) |
| PAP | →Poly-Adenosine polymerase |
| Para | →Paralytic |
| Poly(A) | →Poly-Adenosine |
| PRE | →Pumilio Response Element |
| PUF | →Pumilio and Fem-3 Binding Factor |
| Pum | →Pumilio |
| RD | →repression domain |
| RISC | →RNA induced silencing complex |
| RNA | →Ribonucleic acid |
| RNAP II | →RNA Polymerase II |
| rRNA | →ribosomal ribonucleic acid |
| ZnF | →Zinc Finger |

# CHAPTER 1

# INTRODUCTION

## 1.1 The central dogma

The proper flow of genetic information is crucial to all life. Every living thing contains an initial set of instructions for its assembly in its genome. Long deoxyribonucleic acid (DNA) polymers encode functional components that work together to guide the construction of macromolecules, organelles, cells, tissues, and ultimately whole organisms. Distinct integral processes are responsible for decoding the information within DNA and creating usable materials. DNA contains four monomeric nucleotide constituents that make up its code: Adenosine (A), Thymine (T), Guanosine (G), and Cytosine (C). A single strand of DNA contains many nucleotides linked together via phosphodiester bonds. The order in which nucleotides are linked together is referred to as a DNA sequence. DNA is typically double stranded; a single strand is bound to another opposite strand with what is termed a "complementary" sequence. Adenosines in one DNA strand bind to Thymines in the opposite strand, and Guanines pair with Cytosines

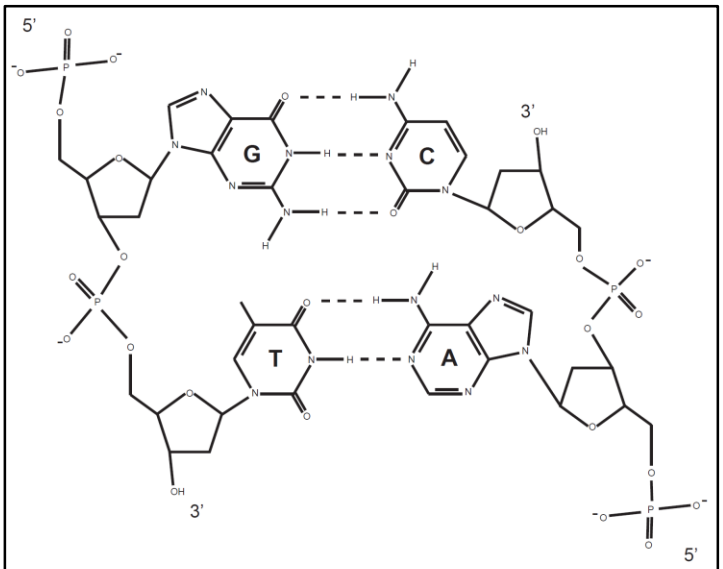


**Figure 1.1- Representation of double stranded DNA base pairing.** The molecular organization of Guanine-Cytosine and Thymine-Adenosine base pairs between complementary DNA strands are depicted. Phosphodiester linkages, 5' phosphates, and 3' hydroxyls are also shown. Dotted lines represent hydrogen bonds made between complementary bases

(Fig. 1.1). Ribonucleic acid (RNA) polymerases read the information contained in one strand of double stranded DNA, termed the coding strand, and generate a single stranded RNA copy of the DNA in a process known as transcription. The resulting RNA transcript is nearly identical to the DNA coding strand with a few exceptions: RNA is single stranded, a hydroxyl group replaces a hydrogen at the 2' position of the sugar ring within each nucleotide, and the Thymine nucleotide in DNA is replaced by Uracil (U) in RNA (Fig. 1.2). Many RNA molecules become functional themselves and serve necessary structural and enzymatic roles. One major class of transcripts is messenger RNAs (mRNAs), which code for proteins. The code within mRNAs exists as sequences of trinucleotide units (codons) that specify a particular polypeptide sequence. Beginning with an AUG start codon, every succeeding three nucleotides "in frame" determine one of twenty amino acids to incorporate. Corresponding amino acids are added sequentially until a stop codon (UGA, UAA, and UAG) signifies the end of the code. The ribosome, a complex composed of many RNAs and proteins itself, interprets the message in mRNA and physically links each amino acid together in the polypeptide chain.

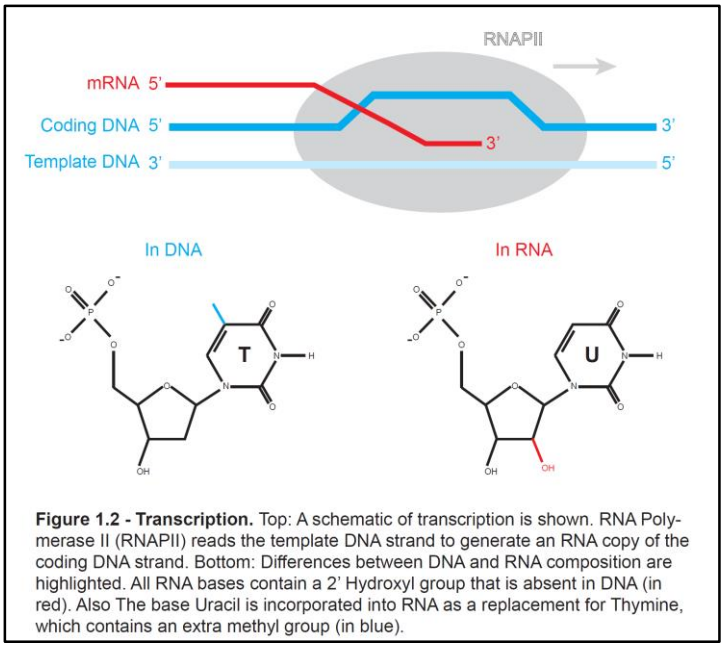


**Figure 1.2 - Transcription.** Top: A schematic of transcription is shown. RNA Polymerase II (RNAPII) reads the template DNA strand to generate an RNA copy of the coding DNA strand. Bottom: Differences between DNA and RNA composition are highlighted. All RNA bases contain a 2' Hydroxyl group that is absent in DNA (in red). Also The base Uracil is incorporated into RNA as a replacement for Thymine, which contains an extra methyl group (in blue).

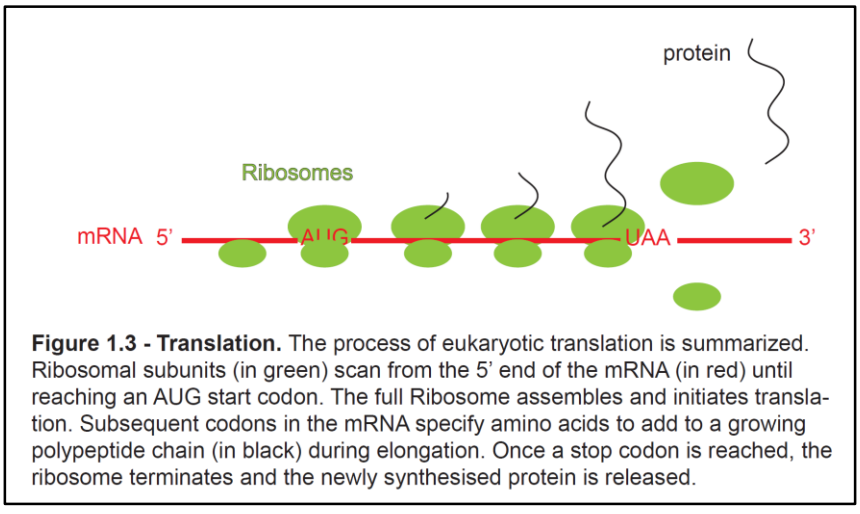


**Figure 1.3 - Translation.** The process of eukaryotic translation is summarized. Ribosomal subunits (in green) scan from the 5' end of the mRNA (in red) until reaching an AUG start codon. The full Ribosome assembles and initiates translation. Subsequent codons in the mRNA specify amino acids to add to a growing polypeptide chain (in black) during elongation. Once a stop codon is reached, the ribosome terminates and the newly synthesised protein is released.

Protein synthesis in this manner is known as translation (Fig. 1.3). The myriad proteins produced by the ribosome go on to serve many cellular functions.

Certainly, different organisms contain varying

sets of protein-coding DNA genes that underlie biodiversity. However, not all genes within an organism are expressed (turned on) at all times and in all tissues. Changes in the level, timing, and site of gene expression results in major differences both within species (e.g. “identical” twins) and between closely related species (e.g. Chimpanzees and humans). Thus, tight control of the processes of transcription (DNA → RNA) and translation (RNA → protein) is essential to the development and homeostasis of an organism. This dissertation will focus on the regulation of gene expression at the post-transcriptional level (RNA → protein). Hallmarks of such regulation include modifying the rates of mRNA destruction, which removes mRNA to prevent its expression, and the inhibition of translation, which blocks protein synthesis but may leave an mRNA message intact for future use. Relevant to the work presented here, strategies employed in eukaryotic cells will be discussed.

## 1.2 Eukaryotic mRNA regulation

### 1.2.1 mRNA transcription and processing

The life cycle of a eukaryotic mRNA begins as it is transcribed from DNA by RNA polymerase II (RNAP II) (Wade and Struhl 2008). Nucleotide polymers like DNA and RNA are synthesized in a 5’ to 3’ manner, corresponding to the numbered nomenclature of the carbon atoms at each terminus of the nucleotide chain (Fig 1.4). Since polymerases use nucleotide triphosphates as substrates for chain elongation, the first nucleotide in a growing mRNA chain will contain an exposed 5’ triphosphate. While the mRNA is being transcribed, this 5’ end is protected through a process known as capping (Perales and Bentley 2009) (Fig 1.5). The capping process is exclusive to eukaryotes. During capping, the triphosphate is trimmed to a diphosphate, and a guanosine triphosphate nucleotide is added via a unique 5’ to 5’ linkage. Additionally, this newly formed Guanosine cap is methylated, further distinguishing the mRNA 5’ end. This distinct structure protects the mRNA from enzymes

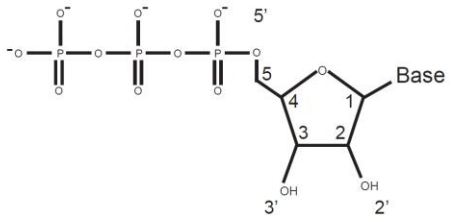


**Figure 1.4 - Ribonucleotide Triphosphate.** The numbering of atoms on an rNTP is pictured. The 3’ hydroxyl of a growing RNA chain reacts with the 5’ phosphate of an rNTP to form a new phosphodi-ester bond.

that would otherwise recognize and destroy the free 5' phosphates. The cap also promotes later events in the maturation of the mRNA.

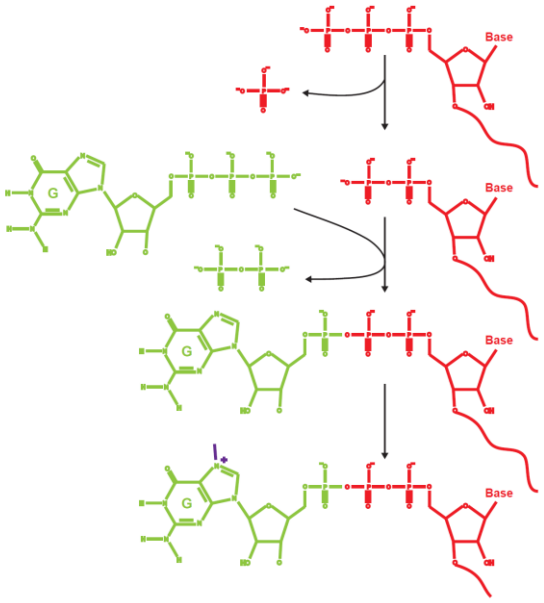


**Figure 1.5 - mRNA 5' capping.** The steps of co-transcriptional capping are depicted. 1) The 5' triphosphate is trimmed to a diphosphate. 2) GTP is added to the mRNA through a 5'-5' linkage. 3) The G base is methylated (in purple) at the 7 position.

Mature eukaryotic mRNAs contain a code for a single protein that will be translated by the ribosome, the coding sequence (CDS), and 5' and 3' flanking sequences which are not translated (untranslated regions - UTRs). Many premature mRNAs are made up of both exons, sequence retained in the final mRNA, and introns, sequence that is removed. The process of intron elimination is known as splicing and occurs co-transcriptionally (Perales and Bentley 2009) (Fig 1.6). The 3' termini of the mRNA is determined by a cleavage event. An mRNA signal sequence (AAUAAA) is encoded at the intended end of a transcript. Upon being transcribed, the signal is recognized by the cleavage and polyadenylation specificity factor (CPSF). This leads to an endonucleolytic (i.e. cuts between nucleotides) cleavage of the growing mRNA about 25-30 nucleotides downstream of the signal (Mandel et al. 2008; Perales and Bentley 2009) (Fig 1.7). Coupled to cleavage, a poly-Adenosine polymerase (PAP) then adds numerous Adenosine nucleotides onto to the 3' end. The binding of nuclear poly-Adenosine binding proteins (PABP) helps to ensure proper poly-adenylation (Kuhn et al. 2009). Like the 5' cap, this 3' poly-Adenosine (poly(A)) tail is also protective and is important in the mRNA's fate.

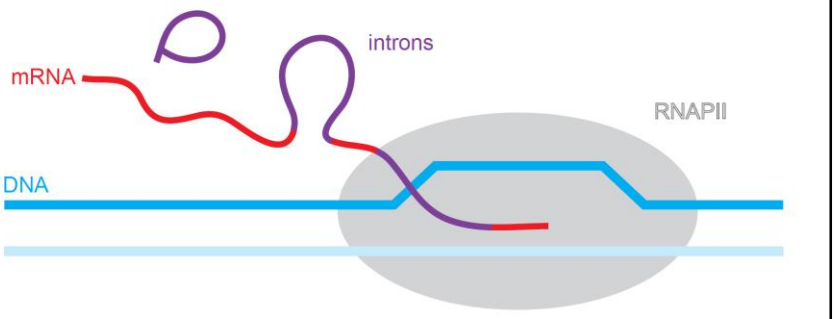


**Figure 1.6 - Splicing of mRNA introns.** The process of splicing is depicted. A newly synthesized mRNA contains exons (in red) and introns (in purple). Eukaryotic mRNAs are capped (in green) and introns are spliced out co-transcriptionally.

### 1.2.2 mRNA export and translation initiation

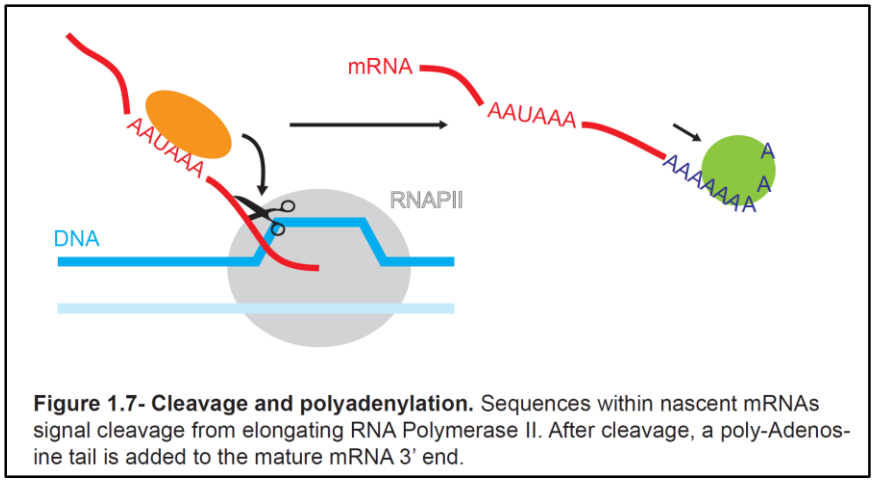


**Figure 1.7- Cleavage and polyadenylation.** Sequences within nascent mRNAs signal cleavage from elongating RNA Polymerase II. After cleavage, a poly-Adenosine tail is added to the mature mRNA 3' end.

A mature capped and poly-adenylated mRNA molecule must be transported from the eukaryotic nucleus into the cytoplasm before translation can occur. The nuclear Cap-binding Complex (CBC) recognizes the 5' cap and directs mRNA to the nuclear pore complex (NPC) where it is exported into the cytoplasm (Lewis and Izaurralde 1997; Stutz and Izaurralde 2003). After clearing the NPC, nuclear factors which recognize the 5' cap and 3' poly(A) tail are replaced by cytoplasmic protein complexes that serve to promote mRNA translation. The cap is bound by the eukaryotic Initiation Factor 4E (eIF4E), while the poly(A) tail is coated with cytoplasmic poly(A) binding proteins (PABP) (Gebauer and Hentze 2004). Another protein, eukaryotic Initiation Factor 4G (eIF4G), bridges eIF4E and pAbp through direct interactions with each. Together, this links the 5' and 3' ends of an mRNA and forms the eukaryotic Initiation Factor 4F (eIF4F) complex (Fig 1.8). Formation of eIF4F promotes the assembly of more initiation components at the 5' end, including an initiator methionine transfer RNA (tRNA), eukaryotic Initiation Factors 2, 3, and 5 (eIF2, eIF3, and eIF5), and the 40S ribosomal subunit (Aitken and Lorsch 2012). This complex scans from the 5' end until it reaches an AUG start codon within the mRNA, signifying the beginning of a CDS. In the correct context, this AUG will promote the incorporation of the 60S ribosomal subunit and signal the transition from initiation into elongation.

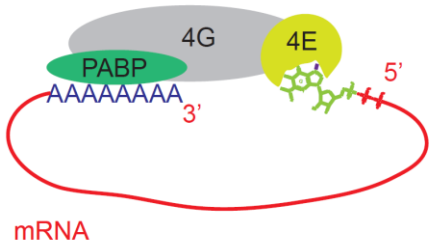


**Figure 1.8- The closed loop.** The mRNA 5' cap and 3' poly(A) tail are bound by eIF4E and PABP. The bound ends are joined by interactions eIF4G to form a closed loop.

### 1.2.3 Translation elongation and termination

Elongation of translation proceeds within the A, P, and E sites of the 80S (60S + 40S) ribosome. The A site acts as in entry for aminoacylated tRNAs, tRNAs “charged” with a single amino acid. The anticodon stem of a tRNA molecule specifies which mRNA codon it will recognize in the A site through base-pairing interactions. Along with other structural features, the anticodon stem may also determine what amino acid a charged tRNA will contain (Pang et al. 2014). In this way, each mRNA codon determines each amino acid in the polypeptide chain assembled during translation. The AUG start codon is recognized by an initiator tRNA charged with the amino acid methionine, but this tRNA is unique as it is present in the 40S ribosome scanning complex and begins occupying the P site (the location of Peptidyl transfer). After the start, each subsequent tRNA must be recruited to the ribosome according to the mRNA codon residing in the A site. Eukaryotic Elongation Factor 1 A (eEF1A) is responsible for bringing charged tRNAs to the A site. The protein eEF1A exists in a guanosine triphosphate (GTP) bound or guanosine diphosphate (GDP) bound state. The GTP-bound form readily associates with charged tRNAs and thus is important for A site recruitment. However, the charged tRNA cannot donate its amino acid to an elongating polypeptide while it remains associated with eEF1A. When a correct codon-anticodon pair assembles in the A site, the GTP bound to eEF1A is hydrolyzed to GDP. This causes dissociation of eEF1A, allowing the charged tRNA in the A site to participate in peptide elongation. Ribosomal RNA (rRNA) catalyzes the peptidyl transfer reaction. The amino acid in the P site links with the amino acid in the A site

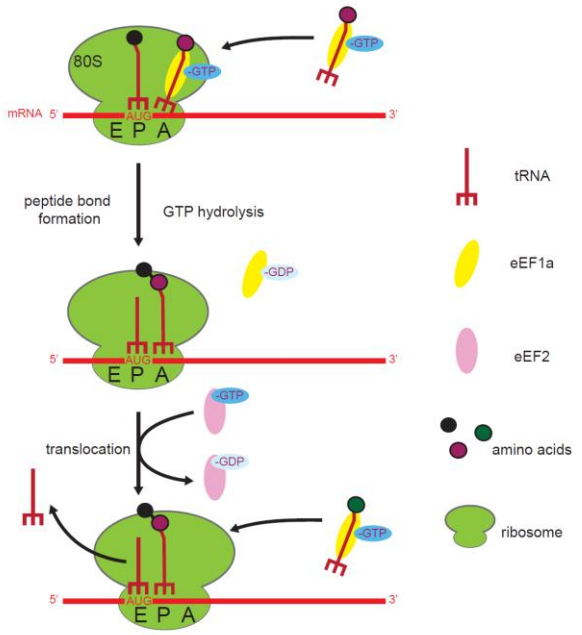


**Figure 1.9 - Translation elongation.** The major steps of translation elongation are depicted. Translation initiates at an AUG start codon. The eEF1a protein recruits charged tRNAs to the 80S ribosome A site. Correct pairing induces hydrolysis of GTP and loss of eEF1a, allowing peptide bond formation. GTP hydrolysis in eEF2 translocates the amino-acyl tRNA from the A site to the P site. The uncharged tRNA leaves from the E site and the cycle continues until termination.

via peptide bond formation and leaves the tRNA in the P site uncharged, depositing the elongating polypeptide chain onto the A site tRNA. Using the energy from hydrolysis of a bound GTP to GDP, the eukaryotic Elongation Factor 2 (eEF2) translocates the polypeptide bound tRNA from the A site to the P site and the uncharged tRNA from the P site to the E site (Dever and Green 2012). The uncharged tRNA then Exits the E site and the P site peptidyl-tRNA is ready for another round of addition (Fig 1.9).

The termination of translation also involves manipulation of the ribosome A and P sites (Dever and Green 2012). Elongation proceeds until an mRNA stop codon occupies the A site. No normal tRNA can decode the stop codon. Instead, eukaryotic Release Factor 1 (eRF1) binds to the stop codon in the A site. In another GTP hydrolysis dependent interaction, eRF1 and eukaryotic Release Factor 3 (eRF3) promote polypeptide release from the P site tRNA, liberating the nascent protein from the ribosome (Fig 1.10). PABP proteins can contribute to translation termination through direct interactions with eRF3 (Hoshino et al. 1999; Osawa et al. 2012). After termination, the ATP-Binding Cassette subfamily E member 1 (ABCE1) protein couples ATP hydrolysis to the dissociation of the remaining 80S ribosome into its 40S and 60S constituents, which can then be recycled into initiation complexes.

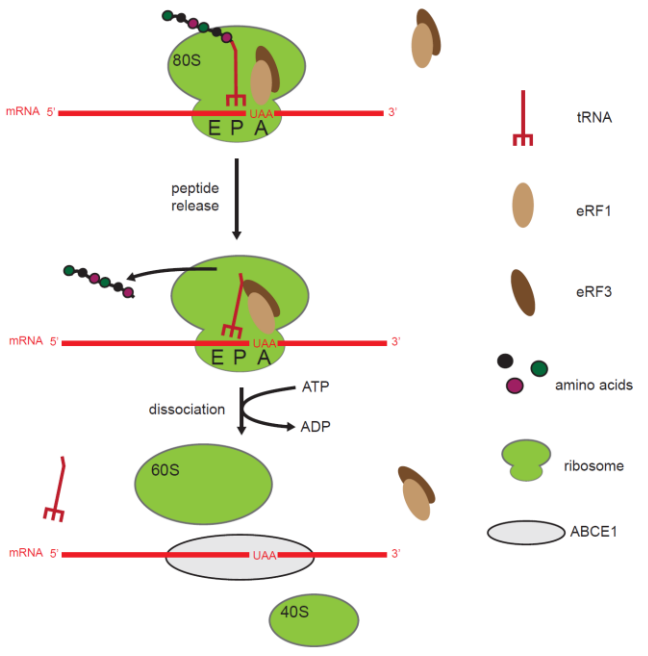


**Figure 1.10 - Translation termination.** The major steps of translation termination are highlighted. The eRF1 protein recognizes a stop codon in the A site. eRF3 triggers peptide release. The ABCE1 protein couples ATP hydrolysis to complex dissociation. The free components go on to initiate further translation.

### 1.2.4 mRNA turnover

Gene expression can be modulated by controlling the rate of translation or modulating the level of mRNA. The level of mRNA can be controlled through its synthesis during transcription and also by managing the rate of mRNA destruction. Decay of mRNA proceeds in a highly regulated stepwise fashion. The 5' cap and 3' poly(A) tail serve as protection

from decay factors, thus the first steps in mRNA destruction involves removal of these modifications. Proteins known as deadenylases catalyze enzymatic removal of the poly(A) tail; deadenylases remove adenosines from the 3' end, eventually leaving an mRNA with a short oligo(A) 3' end. Two major eukaryotic deadenylase complexes exist, the CCR4-NOT complex and the PAN complex (Wahle and Winkler 2013). Some reports propose that the PAN complex is responsible for initial deadenylation, while CCR4-NOT is necessary for complete deadenylation (Yamashita et al. 2005). Other data suggests that each complex may target separate groups of mRNAs (Sun et al. 2013). Both complexes can be recruited directly or indirectly through association with PABP (Ezzeddine et al. 2007; Siddiqui et al. 2007). Regardless of the mechanism of deadenylation, an mRNA lacking a tail becomes a better substrate for both the exosome, a complex which degrades RNA nonspecifically from the 3' end, and the decapping complex (Tharun and Parker 2001). The Like Sm (Lsm) 1-7 complex recognizes short tailed mRNAs and interacts with the decapping complex proteins 1 and 2 (Dcp1 and Dcp2) to facilitate their activity. The enzyme Dcp2 cleaves the 5' cap from the mRNA, exposing the 5' end of the transcript. Once an mRNA is decapped, it is susceptible to rapid 5'-3' decay by the exoribonuclease 1 (XRN1) protein (Garneau et al. 2007) (Fig 1.11). Beyond the ultimate degradation of an mRNA, loss of these modifications is immediately coupled to translational output. Loss of the poly(A) tail excludes PABP from an mRNA, precluding the formation of the closed loop and its stimulation of translation. Loss of the cap prevents recognition by eIF4E and subsequent assembly of the 40S ribosomal scanning

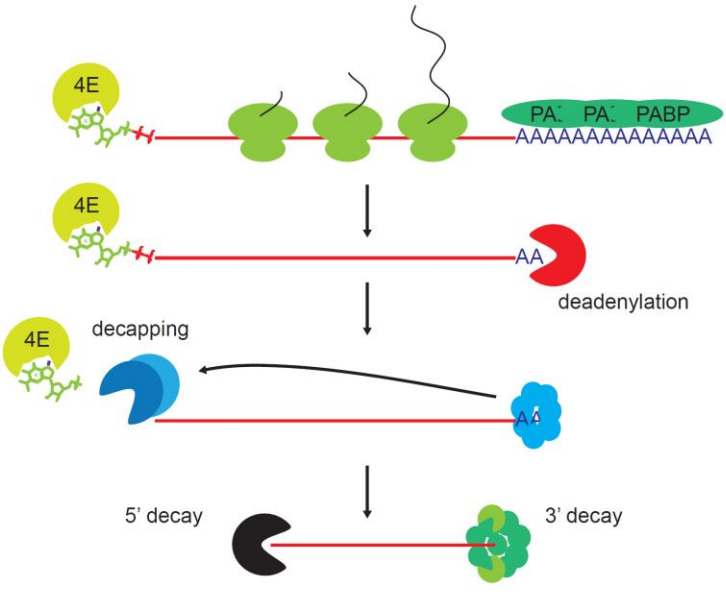


**Figure 1.11- mRNA turnover.** Steps involved in mRNA turnover are illustrated. Deadenylation removes the 3' poly(A) tail and removes PABP, limiting translation. The Lsm1-7 complex helps to recruit the decapping complex. An uncapped mRNA is degraded from its 5' end by XRN1 and from its 3' end by the exosome.

complex. Consequently, in addition to reorganizing initiation, elongation, and termination factors, many examples of mRNA regulation rely on directly modifying or interfering with recognition of the cap and poly(A) tail.

### 1.2.5 *Cis* and *trans* mRNA regulatory elements

Beyond the CDS that determines the identity of synthesized protein, an mRNA molecule also contains sequences which determine its relative processing, its localization in space, the efficiency of its translation, and its ultimate lifespan. Nucleotide sequences within an mRNA that guide these outcomes are considered to be *cis* regulatory elements. Some *cis* elements form secondary and tertiary structures, giving form to the mRNA for specific purpose. Other *cis* sequences or structures serve to recruit so-called *trans* factors, which are accessory proteins or other molecules that mediate an mRNA regulatory outcome. Many *trans* factors are specific to a particular *cis* element, while others recognize features common to all mRNAs (e.g. the cap, poly(A) tail). Introns and UTRs are common sites of *cis* regulatory elements. RNA binding proteins (RBPs) are a large class of proteins which exert regulatory activity on *cis* elements within mRNAs.

### 1.2.6 The importance of RNA binding proteins

RBPs make up a large portion of translated proteins. To date, greater than 1500 RBPs have been identified in mammalian cells and many are important to critical processes (Gerstberger et al. 2014). During splicing, RBP recognition of *cis* elements in both introns and exons can serve to inhibit or promote inclusion or exclusion of specific exons (Chen and Manley 2009). This alternative splicing contributes to the protein diversity within an organism by generating multiple proteins with potentially different function from the same gene. A stem loop structural element in the mRNAs coding for histones, the proteins responsible for the organization of DNA into chromosomes, serves as an alternative processing element. These histone stem loops are bound by the Stem Loop Binding Protein (SLBP) instead of CPSF, leading to cleavage at the 3'UTR that does not result in polyadenylation (Dominski and Marzluff 2007). The specific processing of histone mRNAs ensures their expression is properly timed and controlled. The Iron Response Element (IRE) is a *cis* regulatory element that acts in opposite fashion

depending on its location within an mRNA (Anderson et al. 2012). IRE-binding protein 1 (IRP1) is an RBP that recognizes the IRE sequence. When cellular iron is high, IRP1 dissociates from the 5'UTR of ferritin mRNA, allowing ribosome scanning and increased translation of ferritin protein, which in turn stores the excess iron. If iron is low, IRP1 bound to the 3'UTR of transferrin receptor mRNA blocks 3' degradation, stabilizing this message and increasing the level of transferrin receptor. In this way, IRP mediates the response to iron levels in the cell.

Dysregulation of RBPs is directly linked to human disease. For example, in patients presenting symptoms of myotonic dystrophy types 1 and 2 (DM1 and DM2), a trinucleotide and tetranucleotide repeat expansion (CTG and CCTG) occurs in the DNA sequences of the mytonic dystrophy protein kinase (DMPK) and the cellular nucleic acid-binding protein (CNBP), respectively (Ranum and Cooper 2006). Due to these expansions, the DMPK mRNA 3'UTR and the first intron of the CNBP mRNA contain long CUG containing structures which form foci within nuclei. The CUG elements serve as a sponge for the muscleblind like 1 (MBNL1) RBP that normally acts in splicing. The MBNL1 aggregation on the CUG elements leads to a loss of function and ultimately contributes to the disease. RBPs may contribute to the proliferation of cancer as well. In human cancer cell lines, alternative cleavage and polyadenylation results in many mRNAs bearing significantly shortened 3'UTRs (Mayr and Bartel 2009). Shortened mRNAs coding for oncogenes (genes that contribute to cancer progression) were seen to be highly expressed, as RBPs and other *trans* factors that target the 3'UTR for regulation could no longer bind.

### 1.2.7 The 3'UTR: a hotspot for post-transcriptional regulation

The 3'UTR is a common target for the regulation of mRNA stability and translation. A well-studied example is that of adenosine and uridine rich elements (AREs) present in the mRNA 3'UTR of many inflammation genes (Beisang and Bohjanen 2012). These elements are recognized by ARE binding proteins (ARE-BPs). Multiple ARE-BPs exist, and their effects on bound mRNAs can differ. Some ARE-BPs promote mRNA instability, accelerating decay, while other ARE-BPs are protective. While ARE-BPs engage 3'UTR sites with minor sequence specificity, other factors have evolved to bind more distinct

mRNA targets. The large family of small RNAs known as microRNAs (miRNAs) use base complementarity with mRNA 3'UTRs to recruit RNA induced silencing complexes (RISCs) (Huntzinger and Izaurralde 2011). A miRNA RISC engaged with an mRNA target evicts PABP from the poly(A) tail to inhibit translation and recruits both PAN and CCR4-NOT deadenylase complexes to initiate its destruction (Fabian et al. 2009; Zekri et al. 2013) (Fig 1.12). There are many instances where an RBP structure can specify recognition of a unique 3'UTR sequence. For instance, the RBP Musashi 1 (MSI1) binds to the sequence (G/A)U1-3AGU in mRNA 3'UTRs and results in repression of protein expression (Kawahara et al. 2008). An MSI1 interaction with PABP interferes with eIF4F assembly and thus inhibits translation. The work presented in this dissertation will focus on the protein Pumilio (Pum), a member of the larger family of RBPs known as PUFs (Pumilio and Fem-3 binding factor), and its partner Nanos (Nos), a zinc finger protein implicated in Pum function. PUFs bind sequence specific cis elements, mainly in 3'UTRs, to coordinate localization, translation, and destruction of mRNAs (Miller and Olivas 2011). The function of Pum and Nos is not unlike that of ARE-BPs, miRNAs, or MSI1; however, the mechanistic details of regulation remain to be elucidated.

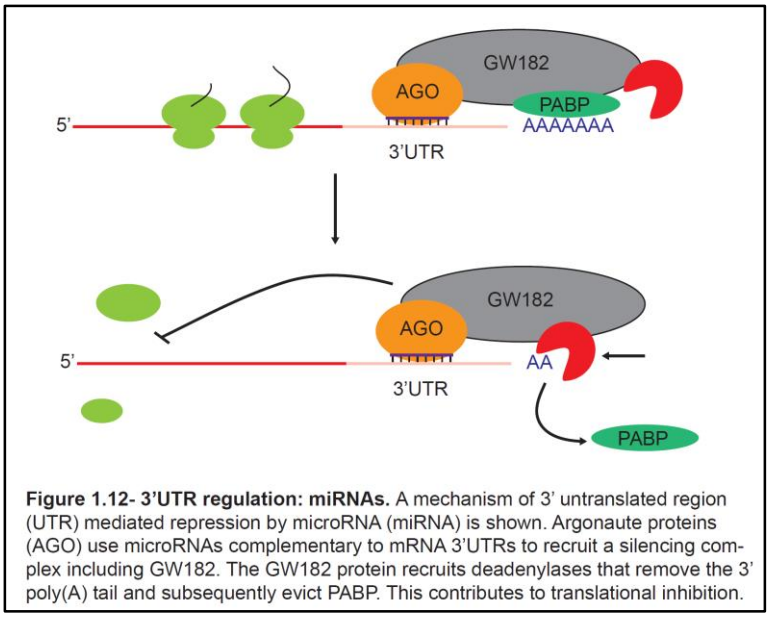


**Figure 1.12- 3'UTR regulation: miRNAs.** A mechanism of 3' untranslated region (UTR) mediated repression by microRNA (miRNA) is shown. Argonaute proteins (AGO) use microRNAs complementary to mRNA 3'UTRs to recruit a silencing complex including GW182. The GW182 protein recruits deadenylases that remove the 3' poly(A) tail and subsequently evict PABP. This contributes to translational inhibition.

## 1.3 The PUF family of proteins

### 1.3.1 *Drosophila* Pumilio and *Caenorhabditis elegans* Fem-3 binding factor

The founding member of the PUF family of RBPs, Pum, was identified in *Drosophila melanogaster* (fruit fly) in 1987 (Nusslein-Volhard et al. 1987). The fly embryo is unique; early stages of development occur in a shared bulk cytoplasm known as a syncytium where the placement of mRNAs and proteins determine what and where structures

ultimately form. A number of genes are expressed from the female parent fly genome and are transmitted into the egg cell, so-called maternal effect genes. Maternal effect genes control multiple events in the early development of the fly embryo, including maturation of the germline stem cells and anterior-posterior axis determination. Female flies that do not express wild type Pum produce embryos lacking abdominal segments, a result of impaired posterior definition. This effect was linked to Pum-mediated regulation of an anterior determinant gene Hunchback (Hb) (Tautz 1988). The function of Pum in the posterior was also dependent on the protein Nos (Irish et al. 1989). This Nos and Pum regulation is dependent on regions of the Hb mRNA 3'UTR, initially termed the Nos Response Elements (NRE) (Wharton and Struhl 1991) (Fig 1.13). It was later clarified that Pum was essential for Hb regulation; Pum is an RBP that binds directly to the Hb NREs (Barker et al. 1992; Murata and Wharton 1995). The model that emerged involved Pum binding to the NRE and recruitment of Nos to inhibit Hb translation and trigger deadenylation (Wreden et al. 1997; Sonoda and Wharton 1999). The Hb RNA is certainly not the sole target of Pum. Based on RNA immunoprecipitation experiments from fly embryos and adult flies, Pum associates with nearly 1000 mRNAs in the transcriptome (Gerber et al. 2006). These observations of Pum were the basis for proceeding studies of PUF proteins, including this dissertation.

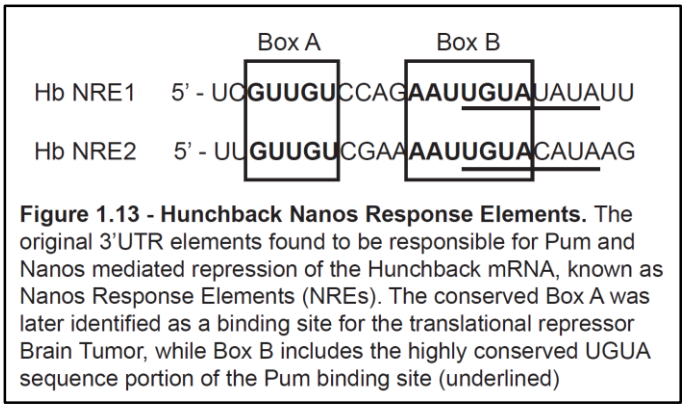


**Figure 1.13 - Hunchback Nanos Response Elements.** The original 3'UTR elements found to be responsible for Pum and Nanos mediated repression of the Hunchback mRNA, known as Nanos Response Elements (NREs). The conserved Box A was later identified as a binding site for the translational repressor Brain Tumor, while Box B includes the highly conserved UGUA sequence portion of the Pum binding site (underlined)

In 1997, a protein similar to Pum was discovered in *Caenorhabditis elegans* (nematode) (Zhang et al. 1997). *C.elegans* are hermaphrodite worms that produce both sperm and oocytes from their germ cells (Zanetti and Puoti 2013). Repression of the *fem-3* gene is necessary for germ cells to switch from spermatogenesis to oogenesis. A *fem-3* mutant with persistent expression of the FEM-3 protein produces only sperm, while a loss of FEM-3 function results in worms that only produce oocytes. The protein responsible for this regulation was named *fem-3* Binding Factor (FBF) (Zhang et al. 1997). Like Pum, Fem-3 Binding Factor (FBF) is an RBP that recognizes a *cis* element in a 3'UTR. FBF binds to an element similar to an NRE within the *fem-3* mRNA and also acts

to inhibit translation. It was found that a specific region of the FBF protein bearing 8 repeated amino acid sequence elements contained the RNA binding activity. Coincidentally, a similar repeated sequence was identified in the C-terminal region of Pum that also contained RNA binding activity (Zamore et al. 1997). A subsequent search for homologous proteins in other organisms found other instances of the 8 repeat structure throughout eukaryotes including two proteins in humans (Zamore et al. 1997; Zhang et al. 1997). These proteins became known collectively as PUF proteins for their founding members Pum and FBF.

### 1.3.2 PUFs across eukaryotes

The PUF RNA binding domain is present in many integral proteins throughout eukaryotes. The first protein identified bearing a Pum like region was YGL023, now known as Puf4p (Barker et al. 1992). The budding yeast *Saccharomyces cerevisiae* is a eukaryotic single celled organism that does not maintain two sexes for reproduction. However, yeast can exchange genetic information with cells of the opposite “mating type”. Each cell can switch between the “a” or “α” mating type through a genetic rearrangement catalyzed by the HO endonuclease protein (Haber 2012). Expression of *HO* is tightly controlled at the transcriptional and post-transcriptional level. Puf4p binds the *HO* mRNA 3’UTR and recruits the CCR4-NOT deadenylase complex to negatively regulate expression (Hook et al. 2007). Through an adjacent binding site, another yeast PUF protein, Puf5p, recruits eIF4E interacting protein 1 (EAP1) to promote 5’ decapping and accelerate mRNA decay (Blewett and Goldstrohm 2012).

In *Xenopus laevis* (frog) a pumilio homolog (XPum) was found to regulate an important mediator of oocyte maturation, Cyclin B1 (CycB1) (Nakahata et al. 2001). Before maturation, many mRNAs including CycB1 exist in a “masked” state of limited expression, lacking a long poly(A) tail. When ready, cytoplasmic polyadenylation elements (CPE) in the 3’UTR are bound by the CPE binding protein (CPEB), signaling polyadenylation of the transcript and subsequent translation. XPum binds an NRE like sequence in the CycB 3’UTR and interacts with CPEB to further repress CycB. In fact, XPum can counteract CPEB mediated polyadenylation, instead retaining a short tail on CycB (Nakahata et al. 2003). Conserved PUFs in other organisms also mediate germline

regulation akin to XPum: FBF in *C. elegans,* Pumilio in flies (discussed in detail in 1.4.2), and PUM1 and PUM2 in mice and humans (Jaruzelska et al. 2003; Chen et al. 2012).

PUF proteins are also necessary for neuronal regulation from flies to mammals. The Pumilio2 protein localizes to stress granules in rat hippocampal neurons and is thought to regulate local translation through repression of eEF1A and eIF4E mRNAs (Vessey et al. 2006). Human PUM2 is responsible for the formation of similar granules in human HeLa cells. The presence of Pumilio2 in rat hippocampal neurons has large effects on neuronal morphology. Depletion of Pumilio2 protein results in an increase in dendritic extensions and excitatory synapses, while over-expression of Pumilio2 significantly reduces dendritic development, dendritic spine number, and excitatory synapses (Vessey et al. 2010). A similar relationship is observed between the expression of Pumilio2 and visual cortical pyramidal neuron excitability in mice. Depletion of Pumilio2 relieves repression of mRNA encoding sodium channel voltage-gated type VIII alpha (Scn8a), a channel responsible for maintaining membrane potential (Driscoll et al. 2013). Again, the loss of Pumilio2 leads to hyper-excitable synapses while its overexpression results in a loss of synaptic firing. Beyond the nervous system, > 2000 mRNA transcripts associate with human PUM1 and PUM2 in HeLa cells, underscoring the myriad potential pathways in which PUFs could participate (Galgano et al. 2008). PUFs across eukaryotes control many diverse important functions, yet they do so through a conserved mechanism of sequence recognition made clear by the elucidation of the PUF RNA binding domain's structure.

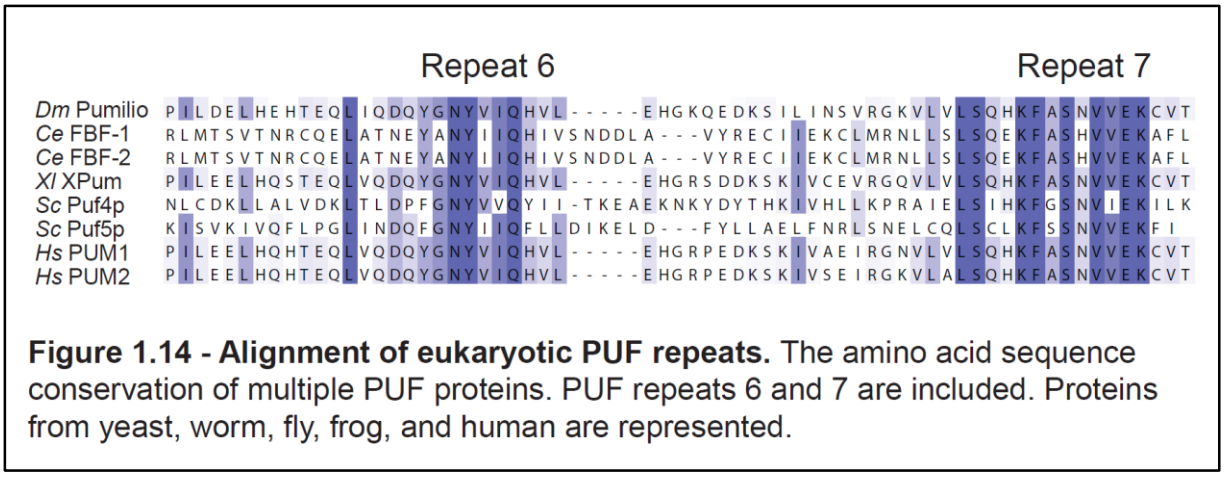


**Figure 1.14 - Alignment of eukaryotic PUF repeats.** The amino acid sequence conservation of multiple PUF proteins. PUF repeats 6 and 7 are included. Proteins from yeast, worm, fly, frog, and human are represented.

### 1.3.3 PUF structure

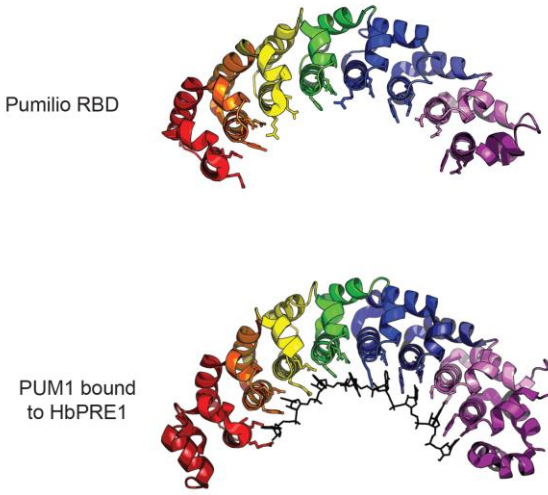


**Figure 1.15 - Structures of the PUF RNA binding domain.** Top: X-ray crystal structure of *Drosophila* Pumilio RNA binding domain from Edwards et. al 2001. Bottom: Structure of human PUM1 bound to the Hb Pum Response Element 1 (HbPRE1 UGUAUAUA) from Wang et. al 2002. Each of eight three-helical repeats is highlighted.

The definition of the PUF family of proteins relied on the homology across eukaryotes in the eight repeated elements in the Pum and FBF RNA binding domains (Fig 1.14) (Barker et al. 1992; Zamore et al. 1997; Zhang et al. 1997). The first X-ray diffraction mediated crystal structure of the conserved Pum RNA binding domain revealed the coordination of these three helical repeats as a crescent shaped molecule with a concave RNA-binding surface and an opposite surface for binding to protein partners (Edwards et al. 2001). The crystal structure of human PUM1 was solved at the same time and revealed that the same structure is conserved (Wang et al. 2001). The first RNA elements *Drosophila* Pum was thought to bind was the two Hb NREs, which each contain a so-called Box A and Box B element thought to be necessary for Pum binding (Wharton and Struhl 1991) (Fig. 1.13). It had been unknown what specific nucleotides were engaged by the RNA binding face of PUF proteins, but this was elucidated when the structure of human PUM1 bound to RNA was solved (Wang et al. 2002). Surprisingly, PUM1 bound directly to an eight nucleotide sequence including only a portion of Box B. PUM1 was crystallized bound to UGUAUAUA from NRE1 and UGUACAUA from NRE2 (Fig 1.15). Each PUF repeat of the PUM1 RNA binding domain binds a single nucleotide in the RNA. Three amino acids within each repeat confer nucleotide specificity; two amino acids form hydrogen bonds with the Watson-Crick face of the base, while a third amino acid contributes stacking interactions between each base (Fig 1.16). PUF proteins rarely deviate from this mode of binding. Some PUFs can recognize G in the fourth position (FBF-like) or accommodate extra nucleotides within the binding site through base flipping

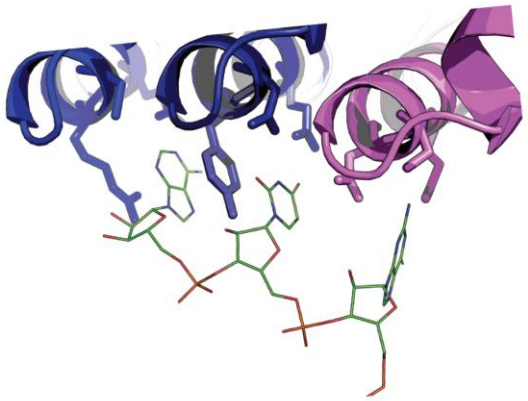

**Figure 1.16 - PUF nucleotide recognition.** Zoomed in view of PUM1 bound to HbPRE1. Repeats 5, 6, and 7 recognize $G_2$, $U_3$, and $A_4$, respectively. Side chains involved in recognition are shown.

(FBF2, Puf4p, Puf3p), but each of eight repeats will always bind one nucleotide in a binding site specified by their amino acid triad (Miller et al. 2008; Wang et al. 2009; Zhu et al. 2009). When fly Pum is immunoprecipitated from fly embryos or adult flies, the most enriched RNA motif associated with Pum is UGUAHAUA, where H is any nucleotide except G (Gerber et al. 2006). This minimal 8 nucleotide binding site will be referred to as the Pum response element (PRE).

## 1.4 Targets and mechanisms of Pumilio

### 1.4.1 Hunchback and embryonic body patterning

The first PUF target identified was Drosophila Hunchback (Hb), a transcription factor whose activity is necessary for proper anterior segmentation of the fly embryo (Lehmann and Nusslein-Volhard 1987). Transcriptional activation of zygotic Hb is dependent on the anterior localized Bicoid (Bcd) protein. However, the zygotic genome is silenced in the early stages of embryogenesis; the zygote does not produce new mRNAs. The mRNAs that exist during this time are deposited into the embryo by the mother fly, and all gene expression must be regulated at the post-transcriptional level. Hb protein presents as a gradient emanating from the anterior pole of the embryo even at these early stages of development, underlying a maternal source of Hb mRNA (Tautz and Pfeifle 1989). The maternal Hb mRNA exists throughout the embryo, thus its translation must be specifically inhibited in the posterior. The Hb mRNA contains two PREs in its 3'UTR that result in Pum dependent regulation. Pum expression is not limited to the posterior; however, Pum repression of Hb persists only in this region. Instead, the Nos protein is the spatial determinant of Hb regulation. Nos is expressed as a gradient beginning at the posterior pole and extending into the embryo (Barker et al. 1992). The combination of Pum and Nos in the posterior of the embryo results in translational

silencing of Hb mRNA and loss of the Hb poly(A) tail (Tautz 1988; Irish et al. 1989; Murata and Wharton 1995; Wreden et al. 1997; Sonoda and Wharton 1999). Pum and Nos could also effectively regulate mRNAs lacking a poly(A) tail, but could not regulate mRNAs lacking the normal 5' cap (Chagnovich and Lehmann 2001). Another protein partner, Brain Tumor (Brat), was also recruited to Hb through an RNA dependent interaction with Pum and Nos and contributed to Hb repression (Sonoda and Wharton 2001). Brat interacts with the 4E homologous protein (4EHP) to compete for recognition of the cap by eIF4E, blocking translation (Cho et al. 2006). This accessory repression is important for abdominal segmentation as it refines the Hb protein gradient; brat mutant embryos have similar segmentation defects to pum and nos embryos (Sonoda and Wharton 2001). These findings contributed to a model of Pum repression wherein Pum binds the PRE through its RNA binding domain and recruits Nos and Brat for deadenylation and translational inhibition (Fig 1.17). More recently, Brat has been identified as maintaining its own RNA binding activity which is enhanced by Pum (Loedige et al. 2014). The Brat binding site resides in Box A of the NRE sequence, explaining the necessity for both Box A and Box B in Pum dependent regulation of Hb mRNA.

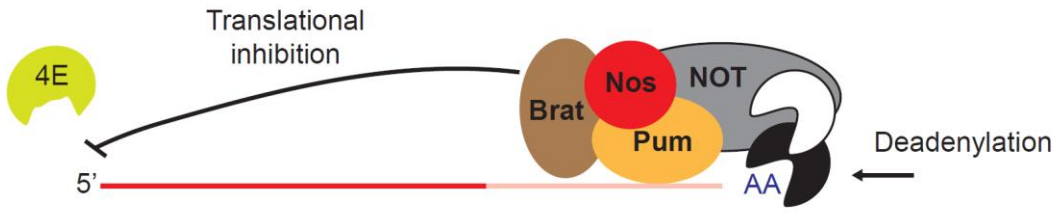


**Figure 1.17- Original model of Pumilio repression.** The first proposed mechanism of Pumilio mRNA repression. Pum binds PRE elements in 3'UTRs and recruits Nanos. Nos then recruits Brat to inhibit cap recognition and the CCR4-NOT complex to cause target deadenylation.

### 1.4.2 Cyclin B and germline stem cell maintenance

In addition to controlling abdominal segmentation in the early developing embryo, Pum and Nos are also important for maintenance of the developing *Drosophila* germline through regulation of another PRE containing mRNA of the Cyclin B (CycB) gene. CycB regulates progression of the cell cycle into mitosis and its destruction is necessary for entry into anaphase (Fung and Poon 2005). Thus, blocking the expression of CycB

prevents cell proliferation. In the developing fly embryo, CycB mRNA becomes localized at high levels in the posterior pole, eventually becoming incorporated into the germline precursor pole cells. The pole cells form before the rest of the syncytial embryo cellularizes (Asaoka-Taguchi et al. 1999). Translation of CycB protein is limited until gastrulation, after the pole cells physically migrate to what will become the fly ovaries. The localization and translational regulation of the CycB mRNA can be attributed to elements in its 3'UTR that resemble the Box A and Box B of the Hb NRE (Dalby and Glover 1993).

Pum protein is present throughout the embryo during development, but high levels of Nos protein are specifically retained within the pole cells (Wang et al. 1994). Pole cells without maternal Nos expression fail to migrate to the midgut region during development, resulting in flies with underpopulated and inviable germlines (Forbes and Lehmann 1998). Both *pum* and *nos* mutants are impaired for pole cell migration and experience premature expression of CycB, resulting in precocious entry into mitosis (Asaoka-Taguchi et al. 1999). Because Nos and Pum are also necessary for maintaining asymmetric cell division in these primordial germ cells as well as mature germline stem cells, this early proliferation event results in terminal differentiation (Gilboa and Lehmann 2004; Wang and Lin 2004). The regulation of CycB translation is normally dependent on a weak PRE (UGUAAUUUAUA), but Pum's requirement for binding can be precluded by tethering Nos directly to the CycB mRNA (Kadyrova et al. 2007). From yeast 2-hybrid data, Nos was found to interact with the protein CCR4-NOT transcription complex subunit 4 (CNOT4) homolog, Cnot4. From this evidence, it was proposed that Pum binds the PRE of CycB solely to recruit Nos and the CCR4-NOT deadenylase complex (through Cnot4) to promote translational inhibition (Kadyrova et al. 2007). However, Cnot4 is not a constitutive member of the CCR4-NOT complex in *Drosophila* (Temme et al. 2010). Instead, CNOT4 may play a role in ubiquitin mediated protein decay (Sun et al. 2015).

### 1.4.3 Paralytic and neurological function

Pum protein also controls important neurological processes in the fly including motor function, neuronal morphology, and long-term memory (Schweers et al. 2002; Dubnau et al. 2003; Ye et al. 2004). At the neuromuscular junction (nmj), where a synapse

separates a motor neuron from muscle fiber, proper ion concentrations are necessary for signal transmission. Ion currents control how voltage-gated Calcium ($Ca^{2+}$) channels respond to nerve impulses, the amount of $Ca^{2+}$ taken up by the motor neuron, and ultimately the level of neuro-transmitter released into the synapse (Fig 1.18) (Frank 2014). The combined length and amplitude of these effects make up neuronal "excitability". Repeated stimulation of a motor neuron results in long-term facilitation (LTF): extended release of neurotransmitters that promotes an equivalent response in the muscle (Jan and Jan 1978). The time it takes the NMJ to achieve a state of LTF is proportional to neuronal excitability. The motor neurons of mutant *pum* larva reach LTF faster than their wild type counterparts and adult *pum* flies suffer from motor defects (Schweers et al. 2002). Conversely, larva that over-express Pum cannot reach LTF. This dependence on Pum is directly correlated with changing levels of the PRE containing mRNA for Paralytic (Para), a sodium ($Na^{+}$) channel protein (Mee et al. 2004). Interestingly, the functional PRE (UGUAAAUA) necessary for Pum regulation resides in the Para CDS (Muraro et al. 2008). While Nos is necessary for all Pum regulation of Para, Brat is only required in certain motor neurons.

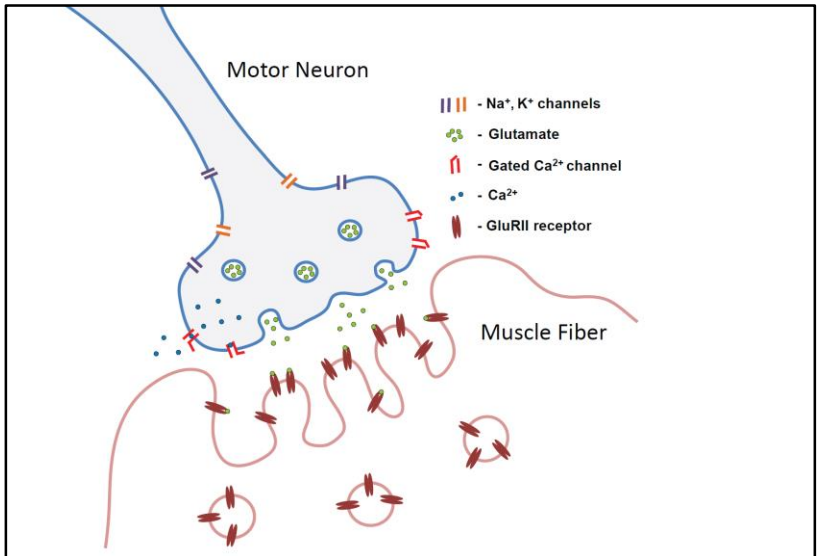


**Figure 1.18 - The *Drosophila* neuromuscular junction.** A schematic of the fly neuromuscular junction (nmj). Levels of Sodium and Potassium channels control the relative response of voltage-gated Calcium channels to nerve stimulation. Presynaptic Calcium uptake triggers neurotransmitter release (Glutamate), and the amount of receptor at the post-synaptic muscle fiber helps determine signal intensity.

## 1.5 Nanos and its homologs

### 1.5.1 *Drosophila* Nanos

In each identified case of Pum regulation in *Drosophila* (Hb, CycB, and Para), Nos is necessary for the PRE dependent activity. Nos is proposed to recruit translational inhibitors (Brat and 4EHP) as well as decay factors (CCR4-NOT), but how it determines

which strategy to utilize or what other factors may be necessary remain unknown. Nos is the defining member of a family of Zinc Finger (ZnF) proteins, containing conserved

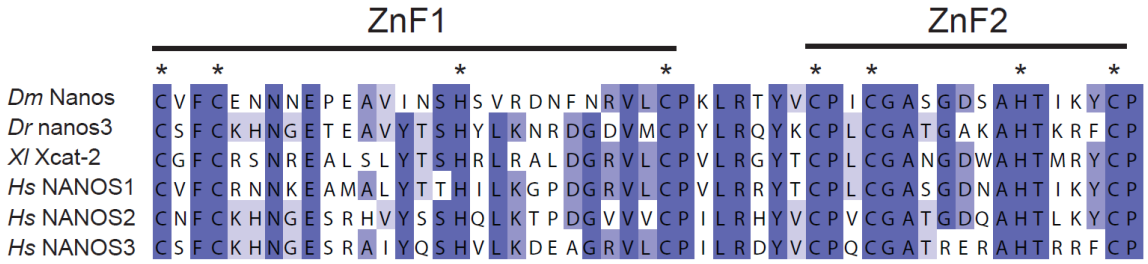


**Figure 1.19 - Alignment of Nanos family Zinc Fingers.** The amino acid sequence conservation of multiple Nanos homologues. The conserved tandem CCHC Zinc Fingers (ZnF) are inculded. Proteins from fly, fish, frog, and human are represented. Asterisks indicate Cysteines and Histidines responsible for metal coordination.

tandem ZnF domains at its C-terminus (Fig 1.19). Each ZnF coordinates one Zinc ion using a combination of three cysteines and one histidine (Curtis et al. 1997). A portion of Nos containing the ZnF region and a C-terminal extension is suggested to bind RNA non-specifically *in vitro* (Curtis et al. 1997). However, expression of Nos proteins with mutations that abrogate zinc binding by either ZnF (C315Y, C354Y) or truncations of the N-terminus or C-terminus cannot rescue *nos* defects of abdominal segmentation in the embryo (Curtis et al. 1997). Mutations in Pum that block Nos recruitment have been identified, but how Nos interacts with Pum is unclear (Edwards et al. 2001).

### 1.5.2 The conservation of Nanos proteins

Nanos homologues exist in many eukaryotes, but are absent in yeast. The only feature conserved throughout NANOS proteins is the tandem ZnF regions. A structure of *Danio rerio* (zebrafish) Nanos3 tandem ZnF illustrates coordination of Zinc by key conserved residues and this construct can recapitulate nonspecific RNA binding, albeit at high µM concentrations (Fig. 1.20) (Hashimoto et al. 2010). Outside of

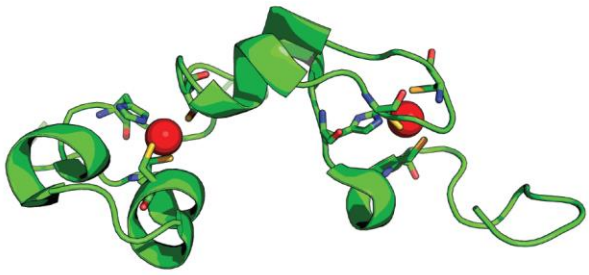

**Figure 1.20 - Structure of zebrafish nanos3 ZnFs.** X-ray crystal structure of *Danio rerio* nanos3 Zinc Fingers (ZnF) from Hashimoto et. al 2010. Cysteine and Histidine residues that coordinate Zinc (in red) are shown.

the ZnF, a conserved element in the N-terminus of *X. laevis* Nanos1 was identified which could repress mRNAs independently of a poly(A) tail or a 5' cap (Lai et al. 2011). Curiously, this region is absent from *Drosophila* Nos. Structural evidence suggests that this conserved region acts as a NOT1 interacting motif (NIM) in human NANOS1, NANOS2, and NANOS3 (Bhandari et al. 2014). While this may support a CCR4-NOT mechanism of Nos mediated repression, it was also observed that this region mediated deadenylation independent translational repression. Because the NIM is absent in *Drosophila* Nos, it is not clear whether the recruitment of NOT1 is integral to Pum and Nos cooperative repression.

### 1.5.3 Interactions between NANOS and PUF proteins

The direct association of Nos and Pum has been observed in an RNA dependent manner through the yeast 3-hybrid system and at high µM concentrations using pull-down assays *in vitro* (Sonoda and Wharton 1999; Edwards et al. 2001). These interactions depended on a region between repeats 7 and 8 of the Pum RNA binding domain. Additionally, one piece of evidence suggests that a C-terminal motif (ITMEDAI) in Nos, which is not conserved in other NANOS proteins, is necessary for this interaction with Pum (Sonoda and Wharton 1999). In contrast, the ZnF region of human NANOS1 is reported to interact with the human PUM2 RNA binding domain between repeats 7 and 8 (Jaruzelska et al. 2003). *Xenopus* pumilio1 and nanos1 also interact, however the details of their binding are unknown (Nakahata et al. 2001). Co-expression of human PUM2 and NANOS3 results in repression of the E2F transcription factor 3 mRNA, but their individual contributions were not investigated (Miles et al. 2012). It is unclear whether the PUM2-NANOS3 interaction is direct or indirect. In short, the understanding of how PUF and NANOS homologues interact, if their interaction is truly conserved, remains incomplete.

## 1.6 Conclusions

The activity of PUF proteins represents a microcosm of important post-transcriptional regulation. The control of mRNA decay and translation are integral to proper gene expression. Pum, the founding PUF, mediates multiple important biological processes (embryogenesis, germline stem cell maintenance, neurological function) and does so via multiple mechanisms (CCR4-NOT mediated deadenylation, Brat mediated

translational inhibition). When research for this dissertation began, there was no direct functional evaluation of these models outside of the whole fly. Some direct interactions with suggested partners had been demonstrated, but the details of interaction with even the necessary partner Nos were not robustly verified. I set out to critically investigate the models and mechanisms of Pum regulation using a *Drosophila* cell based model. Cell lines provide a living system in which expression of every gene can be measured via multiple means, increased through transfection, and depleted by double stranded RNA interference (dsRNAi). The question this research addresses can be simplified to, “What is necessary for Pumilio repression?”

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# CHAPTER 2

# REPRESSION AND REGULATORY DOMAINS OF PUMILIO

Portions of the work presented in this chapter were originally published as:

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*Molecular and Cellular Biology* January 2012. Vol. 32 No. 2**:** 527-540
Authors: Chase A. Weidmann and Aaron C. Goldstrohm

## 2.1 Abstract

*Drosophila* Pumilio (Pum) is an RNA binding protein (RBP) that potently represses specific mRNAs. In developing embryos, Pum regulates a key morphogen, Hunchback (Hb), in collaboration with the cofactor Nanos (Nos). To investigate repression by Pum and Nos, we created cell-based assays and find that Pum inhibits translation and enhances mRNA decay independent of Nos. Nos robustly stimulates repression through interactions with the Pum RNA binding domain (RBD). We programmed Pum to recognize a new binding site, which garners repression of new target mRNAs. We show that cofactors Brain Tumor (Brat) and eIF4E Homologous Protein (4E-HP) are not obligatory for Pum and Nos activity. The conserved RBD of Pum was thought to be sufficient for its function. Instead, we demonstrate that three unique domains in the amino-terminus (N-terminus) of Pum possess the major repressive activity and can function autonomously. The N-termini of insect and vertebrate PUFs are related and we show that corresponding regions of human PUM1 and PUM2 have repressive activity. Other PUF proteins lack

these repression domains. Our findings suggest that PUF proteins have evolved new regulatory functions through protein sequences appended to their conserved PUF repeat RBDs.

## 2.2 Introduction

Precise regulation is required for expression of the appropriate quantity of proteins at the proper time and location. Post-transcriptional regulation of messenger RNAs (mRNAs) is an integral control point mediated by cis-acting sequences and trans-acting regulators (Gebauer and Hentze 2004). PUF (Pumilio and Fem-3 binding factor) proteins are a family of mRNA regulators defined by a conserved RBD (Wickens et al. 2002). PUFs exert their function by selectively binding to single-stranded RNA sequences with high affinity and specificity (Zamore et al. 1997; Zhang et al. 1997; Wang et al. 2002; Lu et al. 2009). *Drosophila* Pum, the focus of this study, binds the consensus sequence 5'-UGUANAUA (Murata and Wharton 1995; Zamore et al. 1997; Wharton et al. 1998; Gerber et al. 2006).

Pum controls diverse processes including stem cell proliferation (Lin and Spradling 1997; Forbes and Lehmann 1998; Asaoka-Taguchi et al. 1999; Parisi and Lin 2000), motor neuron function, and memory formation (Schweers et al. 2002; Dubnau et al. 2003; Mee et al. 2004; Menon et al. 2004). Pum was initially identified by its requirement for embryonic development. Mutations in Pum disrupt abdominal segmentation (Lehmann and Nusslein-Volhard 1987b). Early embryonic development is regulated through intricate expression patterns of maternally derived mRNA transcripts while the genome is transcriptionally silent (Wickens et al. 2000). During this stage, Pum regulates the mRNA encoding Hb (Lehmann and Nusslein-Volhard 1987a; Tautz 1988; Barker et al. 1992). For proper development, Hb protein must be restricted to the embryonic anterior, yet the mRNA is distributed throughout the embryo (Tautz 1988; Tautz and Pfeifle 1989). The zinc finger (ZnF) protein Nos spatially restricts Hb expression (Nusslein-Volhard et al. 1987; Lehmann and Nusslein-Volhard 1991; Wharton and Struhl 1991). A gradient of Nos emanates from the posterior pole, opposing the Hb gradient (Wang and Lehmann 1991). Two RNA sequences located in the 3' UTR of Hb mRNA, Nos Response Elements (NREs), are necessary for repression of Hb (Wharton and Struhl 1991). The NREs are,

in fact, binding sites for Pum, which is evenly dispersed throughout the embryo (Barker et al. 1992; Macdonald 1992; Murata and Wharton 1995). In the posterior, Nos partners with Pum on the NREs to form a ternary Nos-Pum-NRE complex that represses Hb (Sonoda and Wharton 1999).

Pum repression correlates with shortening of the 3' poly-Adenosine (poly(A)) tail of target mRNAs (*i.e.* deadenylation) (Wharton and Struhl 1991; Wreden et al. 1997; Chagnovich and Lehmann 2001; Gamberi et al. 2002). Yeast and *C. elegans* PUFs also enhance deadenylation (Olivas and Parker 2000; Jackson et al. 2004; Goldstrohm et al. 2006; Goldstrohm et al. 2007; Hook et al. 2007; Suh et al. 2009). However, multiple lines of evidence indicate that additional repression mechanisms exist. Pum inhibits mRNAs that lack a poly(A) tail, implicating a poly(A) independent mechanism (Chagnovich and Lehmann 2001). Pum repression was shown to be dependent on the 5' 7-methyl guanosine cap (Chagnovich and Lehmann 2001); however, in the *Drosophila* eye, Pum inhibited a reporter whose translation was driven by an internal ribosome entry site, suggesting a cap independent mechanism (Wharton et al. 1998). Therefore, Pum likely uses multiple means to repress mRNAs, though the precise mechanism(s) remain unknown.

To inhibit Hb, Pum is thought to recruit co-repressors. Assembly of the Pum-Nos complex on the Hb NRE recruits the Brat protein (Sonoda and Wharton 2001). Like Pum and Nos, Brat promotes formation of the correct Hb gradient and abdominal segments (Sonoda and Wharton 2001). A lack of Brat shifts the Hb gradient and limits segmentation. A similar phenotype is produced by mutations in 4EHP, which partners with Brat (Cho et al. 2006). 4EHP competes with eIF4E for binding to the 5' cap structure and inhibits translation (Cho et al. 2005). Inactivation of 4EHP, or mutations that abrogate its recruitment, shifts the Hb gradient towards the posterior and reduce abdominal segmentation, though the effect is not fully penetrant (Cho et al. 2006). Therefore, recruitment of 4EHP by Brat is proposed to interfere with cap-dependent translation to refine the Hb protein gradient (Cho et al. 2006).

Nos may be an obligatory cofactor for Pum repression. Supporting this notion, Nos is necessary for Pum repression of Hb, Cyclin B (CycB), and Paralytic in the embryonic

posterior, primordial germ cells, and larval neurons, respectively (Wharton and Struhl 1991; Kadyrova et al. 2007; Muraro et al. 2008). One study suggested that the main function of Pum is to recruit Nos (Kadyrova et al. 2007). Orthologs of Nos serve as cofactors for PUFs in *C. elegans* and *Xenopus* (Kraemer et al. 1999; Nakahata et al. 2001). However, PUFs in yeast repress though no Nos ortholog is present. Evidence in *Drosophila* also hints that Nos might not be essential in all contexts. For instance, Pum regulates Bicoid (Bcd) and CycB in the anterior of the embryo, where Nos is not detected (Gamberi et al. 2002; Vardy and Orr-Weaver 2007). Therefore, the universal necessity of Nos in Pum repression remains questionable.

The carboxy-terminal (C-terminal) RBD of Pum binds Nos and Brat, which in turn recruits 4EHP (Sonoda and Wharton 1999; Sonoda and Wharton 2001; Edwards et al. 2003; Cho et al. 2006). Because over-expression of the Pum RBD partially rescued embryonic segmentation defects in *Pum* mutant embryos, this region was thought to be sufficient for function (Wharton et al. 1998). Biochemical studies focused on the 336 amino acid RBD because it is amenable to purification, whereas full length Pum (1533 amino acid) has been recalcitrant (Zamore et al. 1997; Zamore et al. 1999). The functions of regions outside the RBD are obscure, though some evidence hints at their importance (Barker et al. 1992; Wharton et al. 1998), (Menon et al. 2004; Muraro et al. 2008). Analysis of the molecular functions of these sequences has awaited development of new approaches to measure their activities.

In the research presented here, we develop assays that measure repression by Pum and Nos. We uncover two modes of Pum-mediated repression: one that is dependent on Nos and another that is not. We examine the roles of co-repressors Brat and 4EHP and find that they are dispensable for either mode. Furthermore, we engineered a new Pum protein with altered RNA binding specificity to direct repression of a new target mRNA. A key discovery was that full length Pum mediates robust repression, whereas the RBD displays weak activity. The major repressive activity of Pum resides within three unique repression domains in the protein's N-terminus. We show that equivalent regions of human PUFs also exhibit repressive activity.

## 2.3 Results

### 2.3.1 A cell-based reporter assay recapitulates canonical Pum repression

To dissect repression by Pum and Nos, we used the D.mel-2 cell line (ATCC® #CRL-1963™), derived from *Drosophila* embryo Schneider 2 (S2) cells (Schneider 1972). We created a plasmid that expresses a reporter mRNA encoding *Renilla* luciferase, a bioluminescent enzyme derived from sea pansy, and containing efficient cleavage and poly-adenylation signals within a minimal 3'UTR. To study regulation by Pum and Nos, one or three NRE sequences (Wharton and Struhl 1991) were inserted into the 3'UTR to create Rn1xNRE and Rn3xNRE, respectively (Fig. 2.1). As a control, the NRE sequences were mutated by changing the $U_1G_2U_3$ trinucleotide, crucial for Pum binding, to ACA in Rn3xNREmut (Fig. 2.1). Reporter plasmids were individually transfected into D.mel-2 cells. As an internal control, a plasmid expressing a luciferase protein derived from fireflies was co-transfected (FF control, Fig. 2.1). To measure protein expression, the enzymatic activities of *Renilla* and firefly luciferases were assayed. Transfection efficiency of each sample was normalized by calculating a relative response ratio of *Renilla* activity divided by Firefly activity. Relative response ratios and standard errors for all experiments are reported in Tables 2.1 – 2.17. We observed that *Renilla* expression from reporters with three NREs was slightly reduced when compared to those lacking NREs, suggesting putative Pum repression (Fig. 2.2). However, the 3xNRE reporter was slightly elevated

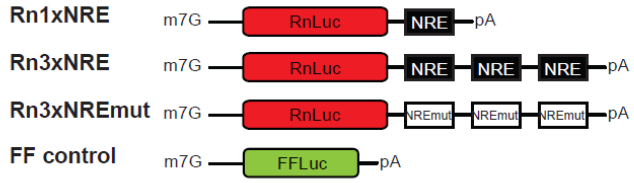



**Figure 2.1 - Luciferase reporters.** Diagrams of Renilla (Rn) and Firefly (FF) luciferase mRNA reporters with wild type (NRE) or mutant (NREmut) Nanos Response Elements are depicted.

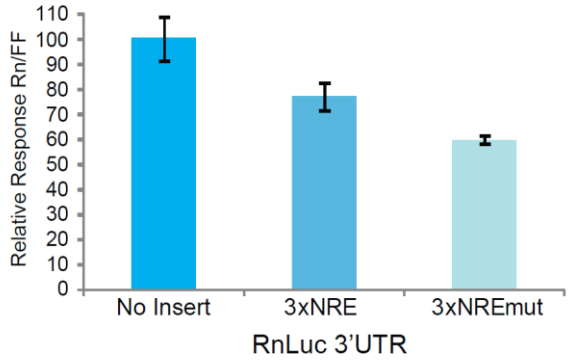



**Figure 2.2 - Reporter expression in D.mel-2 cells.** Relative response ratios of Renilla reporters: a no 3'UTR insert control, the 3xNRE reporter, and the 3xNREmut reporter.

when compared to reporters with mutant NREs, complicating interpretation (Fig. 2.2). RNAi mediated depletion of endogenous Pum caused a 1.8 fold increase in Rn3xNRE expression, but did not affect Rn3xNREmut (Fig. 2.3), indicating that endogenous Pum inhibits the mRNA with Pum binding sites. In the same experiment, attempts to deplete Nos through double stranded RNA (dsRNA) mediated interference had no effect (Fig. 2.3).

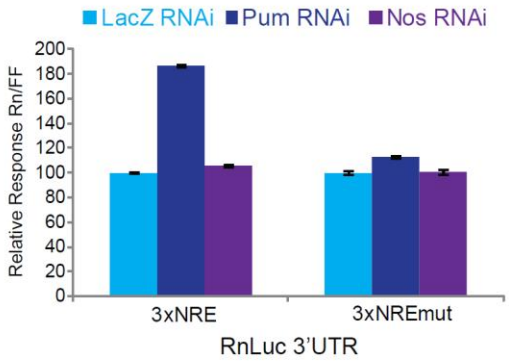



**Figure 2.3 - Reporter Response to RNAi.** Expression of 3xNRE and 3xNREmut Renilla reporters upon treatment with LacZ, Pumilio, or Nanos RNAi. Expression is normalized to LacZ treated samples.

A possible explanation for the minimal difference between 3xNRE and 3xNREmut expression is that a key regulator - Pum or Nos - may be limiting. Therefore, we measured expression of Pum and Nos in D.mel-2 cells. Reverse transcription followed by quantitative polymerase chain reaction (qRT-PCR) revealed that D.mel-2 cells express Pum mRNA (cycle threshold ($C_t$) of 27.0); however, Nos mRNA was not detected. A constitutively expressed ribosomal subunit Rpl32 was detected at $C_t$ of 24.2 (Fig. 2.4). Nos mRNA was detectable when cells were transfected with a Nos expression plasmid, demonstrating that the qRT-PCR assay is valid (Fig. 2.4). Nos, Pum, and Rpl32 were not detected in control reactions lacking template (No Template Control, NTC, Fig. 2.4) or reverse transcriptase (data not shown). These results demonstrate that D.mel-2 cells express Pum but not detectable amounts of Nos, supported by microarray analysis of mRNA expression in S2 cells (Cherbas et al. 2011).

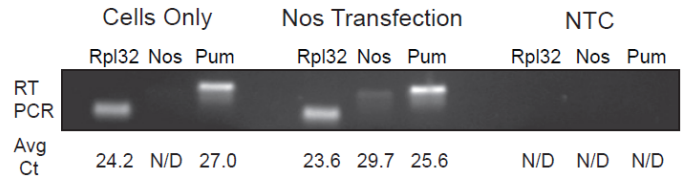



**Figure 2.4 - Nanos Expression in D.mel-2 cells.** qRT-PCR analysis of Nanos (Nos), Pumilio (Pum) or positive control Rpl32 mRNAs from D.mel-2 cells (cells only) or cells transfected with Nos expression plasmid. Average cycle threshold (Avg Ct) values are indicated below corresponding samples. NTC: No Template Control reactions; N/D: Not Detected.

The effect of Nos was tested by transfecting increasing amounts of a Nos

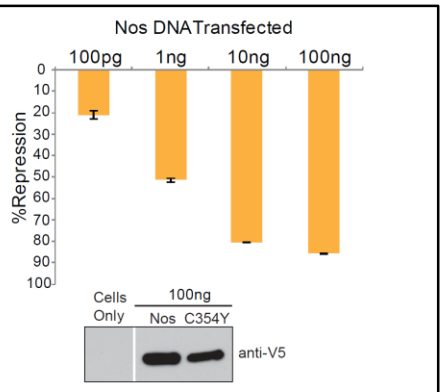
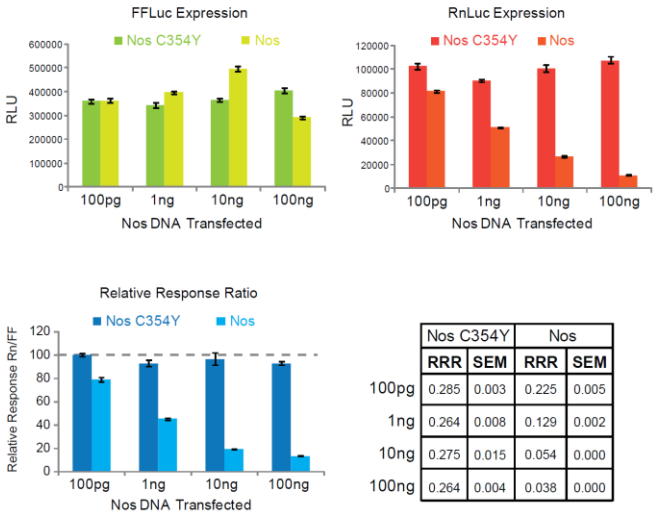



**Figure 2.5 - Nanos Repression of RnLuc 3xNRE.** Percent repression by Nos, reported for the indicated mass of transfected plasmid, was calculated relative to mutant Nanos C354Y.




| | Nos C354Y | | Nos | |
|---|---|---|---|---|
| | RRR | SEM | RRR | SEM |
| 100pg | 0.285 | 0.003 | 0.225 | 0.005 |
| 1ng | 0.264 | 0.008 | 0.129 | 0.002 |
| 10ng | 0.275 | 0.015 | 0.054 | 0.000 |
| 100ng | 0.264 | 0.004 | 0.038 | 0.000 |

**Figure 2.6 - Raw Nanos repression data.** Raw luminometer data for Firefly and Renilla expression from the Nanos transfection gradient (Figure 2.5). The Relative Luminescence Unit (RLU) value for Renilla luciferase expression is divided by firefly RLU values to create relative response ratios (RRR) for each sample. Control RRRs within a single experiment are normalized to 100. The original numeric RRRs and standard error of the mean (SEM) are listed.

expression plasmid into D.mel-2 cells along with the Rn3xNRE reporter (Fig. 2.5, raw data are reported in Fig. 2.6). As a control, equivalent samples were prepared using an inactive Nos mutant wherein the cysteine at position 354 of the ZnF was changed to a tyrosine (C354Y). Loss of zinc binding at this site is reported to impede nonspecific RNA binding by Nos (Curtis et al. 1997). Western blotting confirmed expression of both wild type and mutant Nos proteins (Fig. 2.5). To measure Nos mediated repression, luciferase activities were measured. Next, a normalized response ratio was determined for each amount of transfected Nos, relative to the equivalent amount of Nos C354Y. Nos inhibited expression of the Rn3xNRE reporter mRNA in a dose dependent manner (Fig. 2.5). Transfection of 100ng of Nos expression plasmid caused 85.6% repression of Renilla expression (Fig. 2.5). Titration of Nos expression plasmid over a 2000-fold range demonstrated that Nos repression plateaus at 10 ng and continues to repress greater than 80% up to 200ng with no observed squelching

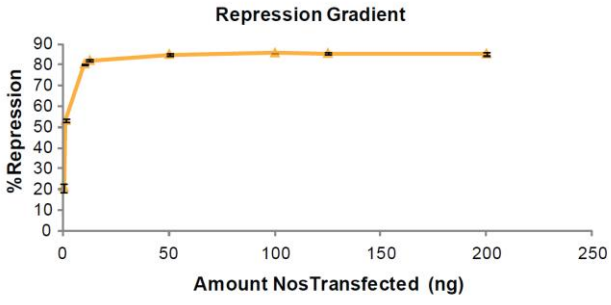



**Figure 2.7 - Nanos repression gradient.** Percent repression of RnLuc 3xPRE versus mass of transfected Nanos expression plasmid, per 6-well. Percent repression values were derived from dual luciferase assays. Error bars represent SEM values.

effect (Fig. 2.7). Therefore, this assay recapitulates the ability of Nos to cause repression of NRE-bearing target mRNA.

### 2.3.2 Nos dependent and independent Pum repression

#### 2.3.2.1 Nos inhibits protein expression and reduces levels of target mRNAs

Nos targets mRNAs through direct interactions with its partner, Pum, and the NRE (Sonoda and Wharton 1999). To address the necessity of the NRE for Nos-directed regulation, the effect of Nos on varying *Renilla* reporters was tested (Fig. 2.1). Reporters were transfected into cells with plasmid expressing either wild type or mutant Nos C354Y. Each sample was split into three portions: luciferase activity assays were performed on one, qRT-PCR was performed on RNA from another, and western blots were performed on the final portion (Fig. 2.8). Wild type but not mutant Nos repressed luciferase expression from both Rn1xNRE (76% repression) and Rn3xNRE (85% repression) reporters, whereas the mutant Rn3xNREmut reporter was not repressed (Fig. 2.8). Repression of mRNA level corresponded to the observed change in luciferase protein. Rn1xNRE and Rn3xNRE mRNAs were reduced by 72% and 79%, respectively, whereas the Rn3xNREmut mRNA was not affected (Fig. 2.8). Western blot analysis confirmed expression of wild type and mutant Nos proteins (Fig. 2.8). These data show that the NRE is necessary for Nos-directed regulation, in agreement with data from embryos (Wharton and Struhl 1991), and that Nos potently decreases reporter protein and mRNA levels.

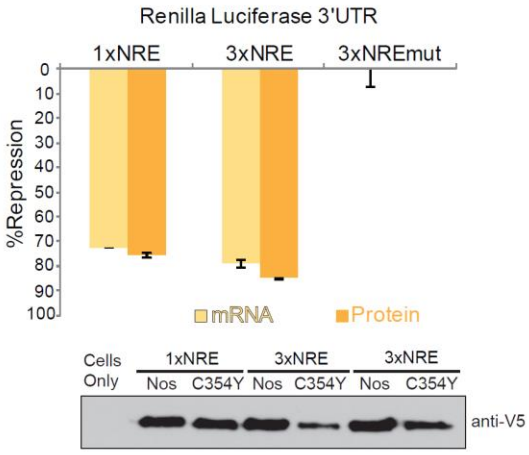



**Figure 2.8 - Nanos repression of varying 3'UTRs.** Percent repression of luciferase protein (dual luciferase assay) and mRNA (qRT-PCR). See Table 2.1 for corresponding data. Western blotting confirms protein expression in each sample.

### 2.3.2.2 Depletion of Pum and Nos, but not Brat or 4EHP, abrogates Nos-dependent repression

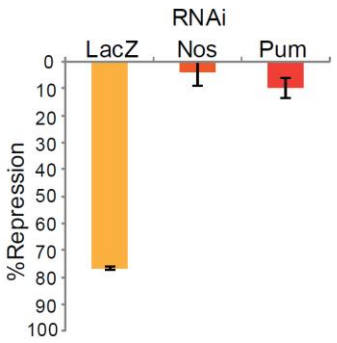



**Figure 2.9 - Repression is Pum dependent.** Percent repression by Nanos in cells treated with dsRNA for: LacZ (control), Nos, or Pum

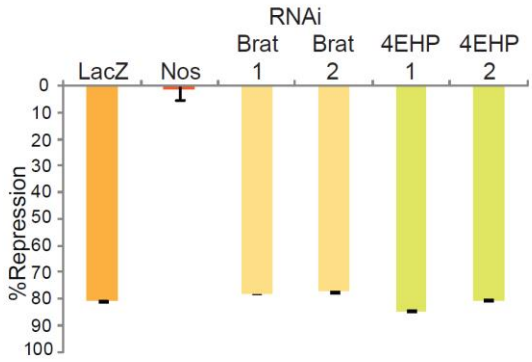



**Figure 2.10 - Depletion of Brat and 4EHP has no effect on Nanos repression.** Percent repression by Nanos in cells treated with dsRNA for: LacZ (negative control), Nos, Brain Tumor (Brat) or 4EHP.

Pum and Nos assemble on the NRE and are thought to recruit Brat and 4EHP to inhibit translation (Sonoda and Wharton 2001; Cho et al. 2006). We sought to examine the role of each co-repressor in Nos mediated repression. Using qRT-PCR, we confirmed expression of endogenous Brat and 4EHP mRNAs in D.mel-2 cells with specific $C_t$ values of 23.3 and 24.2, respectively. We then depleted each protein by RNA interference using specific double-stranded RNAs (dsRNAs). As a negative control, cells were treated with dsRNA corresponding to the bacterial LacZ gene. Rn3xNRE reporter and Nos expression plasmids were subsequently transfected into these dsRNA-treated cells. As before, Nos dependent repression was calculated relative to mutant Nos C354Y. Depletion of exogenously expressed Nos or endogenously expressed Pum almost completely abolished repression, whereas non-targeting LacZ dsRNA had no effect (Fig. 2.9). These results demonstrate that Pum is essential for Nos dependent repression of the NRE containing mRNA and validate the RNAi efficacy. Surprisingly, depletion of Brat or 4EHP had no effect on Nos-directed repression (Fig. 2.10). This result was corroborated using two different dsRNAs that targeted separate regions of the Brat or 4EHP coding sequences (Fig. 2.10). We confirmed depletion of each mRNA by qRT-PCR. Exogenously expressed Nos mRNA was depleted by up to 95% by treatment with Nos dsRNA (data not shown). Across multiple experiments, specific dsRNAs depleted

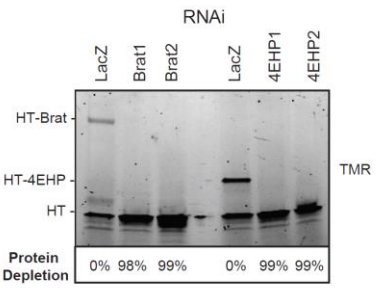



**Figure 2.11 - Efficacy of RNAi.** Fluorescent imaging of SDS Polyacrylamide (4-20%) separated TMR-labeled HaloTag (HT) Brat or 4EHP proteins. Protein depletion by dsRNA to Brat or 4EHP was calculated relative to control LacZ RNAi. HT alone served as a transfection control.

Pum mRNA by up to 67%, Brat mRNA by up to 80%, and 4EHP mRNA by up to 84% (data not shown). To further verify the RNAi efficacy, we tested the ability to deplete Brat or 4EHP proteins by over-expressing fluorescently labeled HaloTag (HT) fusions of Brat or 4EHP (Fig. 2.11). Both dsRNAs for Brat or 4EHP completely ablated expression (98-99% depletion) of HT-Brat and HT-4EHP, respectively, as measured by fluorescence detection (Fig. 2.11). As an internal control, HaloTag was also over-expressed and was not depleted by the dsRNAs (Fig. 2.11). We conclude that Nos and Pum collaborate to repress. Moreover, Nos dependent repression remains effective when Brat and 4EHP are significantly depleted.

### 2.3.2.3 Pum represses mRNAs independent of Nos

The observation that Pum represses mRNAs in the anterior of the developing embryo, where Nos is not detected, suggests that Nos may not be absolutely required (Gamberi et al. 2002; Vardy and Orr-Weaver 2007). While depletion of endogenous Pum in D.mel-2 cells increased RnLuc3xNRE expression, RNAi of Nos had no effect (Fig. 2.3). Since Nos is undetectable in D.mel-2 cells, this indicates that Pum can repress independently of Nos (Fig. 2.4).

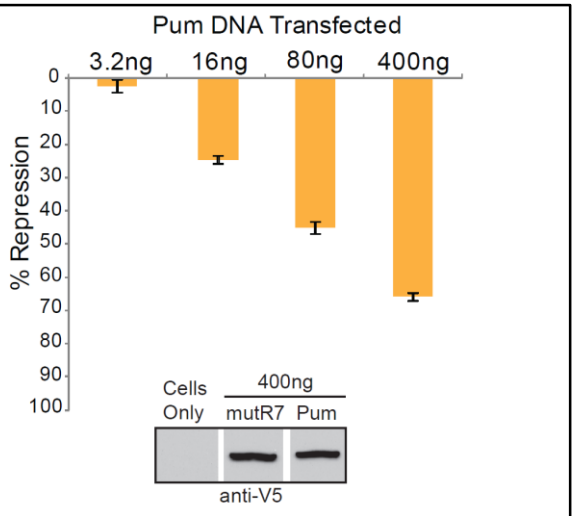
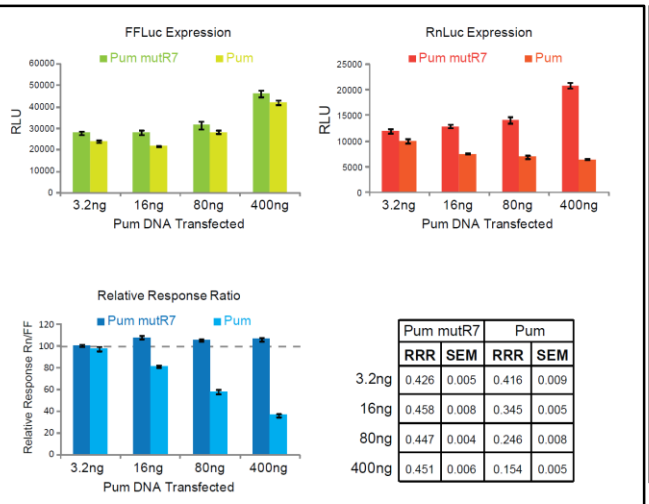



**Figure 2.12 - Pum Repression of RnLuc 3xNRE.** Percent repression of Rn3xNRE reporter by the indicated mass of transfected Pum plasmid, relative to mutant Pum mutR7.

We tested the ability of Pum to repress NRE containing reporters by transfecting a Pum expression plasmid, which caused dose-dependent repression of Rn3xNRE (Fig. 2.12, raw data are reported in Fig. 2.13). At the highest expression level, Pum repressed expression by 66% (Fig. 2.12). A broader titration range of Pum is shown in Figure 2.14.




| | Pum mutR7 | | Pum | |
|---|---|---|---|---|
| | RRR | SEM | RRR | SEM |
| 3.2ng | 0.426 | 0.005 | 0.416 | 0.009 |
| 16ng | 0.458 | 0.008 | 0.345 | 0.005 |
| 80ng | 0.447 | 0.004 | 0.246 | 0.008 |
| 400ng | 0.451 | 0.006 | 0.154 | 0.005 |

**Figure 2.13 - Raw Pumilio repression data.** Raw luminometer data for Firefly and Renilla expression from the Pumilio transfection gradient (Figure 2.12). The Relative Luminescence Unit (RLU) value for Renilla luciferase expression is divided by firefly RLU values to create relative response ratios (RRR) for each sample. Control RRRs within a single experiment are normalized to 100. The original numeric RRRs and standard error of the mean (SEM) are listed.

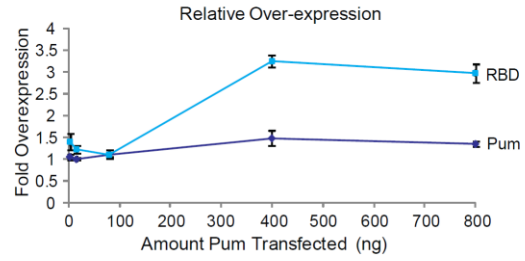



**Figure 2.15 - Pum relative over-expression.** Samples in Figure 2.14 were also subjected to qRT-PCR measurements of Pumilio mRNA, and fold over-expression was calculated relative to endogenous Pumilio levels in D.mel-2 cells alone. These values are plotted versus the amount of DNA transfected in each sample.

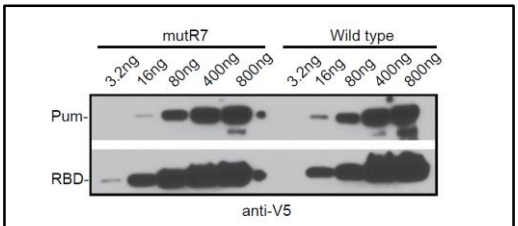



**Figure 2.16 - Pum protein expression.** Western blotting of samples from Figures 2.14 and 2.15 using anti-V5 antibody to detect over-expressed protein.

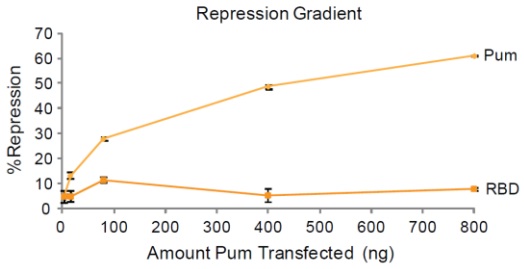



**Figure 2.14 - Pum Repression gradient.** Percent repression of RnLuc 3xPRE versus mass of transfected Pumilio (Pum) or Pumilio RNA binding domain (RBD) expression plasmid, per 6-well. Percent repression values were derived from dual luciferase assays. Error bars represent SEM values.

Repression increased as the dosage of Pum increased, with no indication of squelching (Fig. 2.14). The assay is very responsive to modest increases in Pum. Over-expression at maximum repression, measured by qRT-PCR, was 1.34 fold above endogenous Pum (Fig. 2.15). Pum repression values were calculated relative to equivalent amounts of an inactive mutant Pum (Pum mutR7) that is incapable of binding to RNA by way of alanine substitutions in the RNA recognition amino acids in the seventh PUF repeat (Fig. 2.12). Both wild type and mutant Pum proteins were expressed (Fig. 2.12) and increased in response to the mass of transfected expression plasmid (Fig. 2.16). The data indicate that Pum can indeed repress in a Nos independent fashion.

### 2.3.2.4 Pum reduces protein and mRNA levels in NRE-dependent manner

To verify that Nos independent Pum repression is mediated by the NRE, we examined the effect of Pum on reporter protein and mRNA levels using Rn1xNRE,

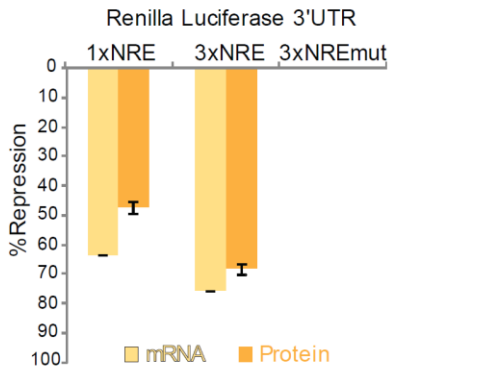



**Figure 2.17 - Pumilio repression of varying 3'UTRs.** Percent repression by Pum of the Renilla luciferase protein and mRNA expression for the indicated reporters.

Rn3xNRE, and control Rn3xNREmut reporters. Pum potently decreased NRE-containing reporter mRNA levels and caused a corresponding reduction in *Renilla* luciferase protein activity (Fig. 2.17). Rn1xNRE and Rn3xNRE mRNAs were reduced 63% and 76%, respectively (Fig. 2.17). Luciferase expression was repressed by 47% for Rn1xNRE and 68% for Rn3xNRE (Fig. 2.17). Mutation of the NRE alleviates repression entirely (3xNREmut, Fig. 2.17). These results demonstrate that Pum over-expression elicits Nos independent repression of NRE containing reporters by reducing protein and mRNA expression.

### 2.3.2.5 Nos and Brat are not necessary for Pum mediated repression

We further interrogated the potential involvement of Nos and Brat in Pum repression. Although Nos expression was undetectable in D.mel-2 cells, we wished to use multiple strategies to eliminate the possibility that trace amounts of Nos, below the limit of detection, might be sufficient to aid Pum repression. First, we utilized Pum mutants that are inactive for interaction with either Nos or Brat. Mutation of F1367S in Pum blocks interaction with Nos (Edwards et al. 2001; Edwards et al. 2003). The G1330D mutation in Pum, originally identified as the *pum*$^{680}$ allele that eliminates abdominal segmentation (Lehmann and Nusslein-Volhard 1987b; Wharton et al. 1998), binds the NRE but is unable to recruit Brat into the Pum-Nos-NRE complex (Edwards et al. 2001; Sonoda and Wharton 2001). We tested the ability of these mutants to repress Rn3xNRE reporter relative to wild type Pum and mutant Pum mutR7. Neither F1367S nor G1330D affected Pum repression (Fig. 2.18).

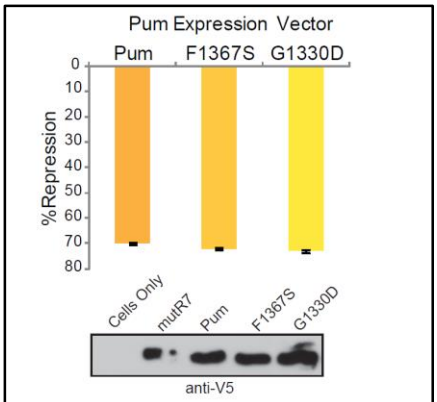



**Figure 2.18 - Binding site mutations do not affect Pumilio.** Percent repression of the Rn3xNRE reporter by wild type, mutant F1367S or mutant G1330D Pumilio. Repression was calculated relative to Pum mutR7. Western blotting confirms expression of each Pum variant.

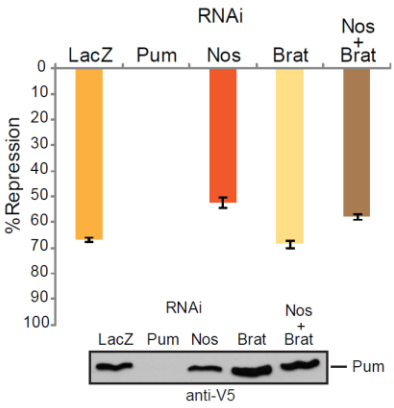



**Figure 2.19 - Depletion of Nos and Brat does not block Pum repression.** Percent repression by wild type Pum (400ng) in cells treated with dsRNA to LacZ control, Pum, Nos, Brain Tumor (Brat), or both Nos and Brat.

As an additional means of assessing participation of Nos and Brat in Pum repression, cells were treated with corresponding dsRNAs to induce RNA interference, and the resulting impact on repression by over-expressed Pum was measured. As a positive control, RNAi depletion of Pum completely alleviated repression (Fig. 2.19). Treatment with LacZ dsRNA had no effect on Pum repression (Fig. 2.19). RNAi of Nos, Brat, or simultaneous knockdown of Nos and Brat had negligible effects on Pum repression (Fig. 2.19). Therefore, using three approaches, we have shown that Pum can repress by a mechanism that is independent of Nos and Brat: 1) Pum represses in a Nos-deficient cells, 2) Mutations in Pum that inhibit Nos and Brat ternary complex formation do not affect repression, and 3) Depletion of Nos, Brat, or both does not alleviate Pum repression. These findings provide strong evidence indicative of a previously uncharacterized regulatory function of Pum, which we now explore.

### 2.3.3 Pum contains multiple repression and regulatory domains

#### 2.3.3.1 The N-terminus of Pum is necessary for optimal repression

To characterize the domains of Pum that are necessary for repression, we examined the activities of the RBD and full length Pum. The RBD is composed of eight PUF repeats located at the C-terminus of Pum protein (amino acids 1091-1426) (Fig. 2.20) and is necessary and sufficient for high affinity binding to the NRE RNA and interaction with co-repressors Nos and Brat (Zamore et al. 1997; Wharton et al. 1998; Sonoda and Wharton 1999; Zamore et al. 1999; Edwards et al. 2001; Sonoda and Wharton 2001; Edwards et al. 2003). Outside of the RBD, no domains or motifs have been documented. However, within the large N-terminal region of Pum (aa1-1090), we


**Figure 2.20 - Diagram of Pumilio domains.** Pumilio Conserved Motifs, PCMa and PCMb, are indicated in green and red. The RNA binding domain (RBD) is also labeled with PUF repeats indicated by yellow boxes.

identified two regions conserved in PUF proteins from insects to vertebrates, designated Pum Conserved Motifs “a” and “b”, (PCMa and PCMb, Fig. 2.20).

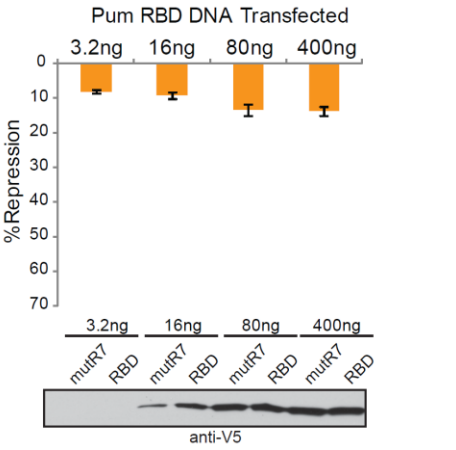



**Figure 2.21 - RBD repression gradient.** Percent repression of Rn3xNRE by Pum RBD, calculated relative to mutant Pum RBD mutR7. Expression of RBD across the gradient was observed with a western blot.

Repression by the Pum RBD was measured relative to an RNA binding defective mutant RBD mutR7. The RBD repressed the Rn3xNRE reporter by 8-15% (Fig. 2.21). While repression increased slightly over a gradient of transfected RBD, the maximum level of repression did not exceed 15% (Fig. 2.21). Under identical conditions, the full length Pum protein repressed by 57% while the RBD repressed by 11% (Fig. 2.22). This difference cannot be attributed to poor protein expression of the RBD; western blotting revealed that the RBD expressed to a higher level than full length Pum (Fig. 2.22). We scrutinized repression by Pum and the RBD over a 250 fold range of transfected plasmids (Fig. 2.14). At each transfected amount, full length Pum repressed greater than the RBD. Repression by the RBD never exceeded 15%; whereas repression by full length Pum continued increasing up to 61% at the maximum amount of transfected plasmid (Fig. 2.14). The RBD mRNA was maximally over-expressed by 3.31 fold and full length Pum by 1.34 fold, relative to endogenous Pum mRNA (Fig. 2.15). Comparison of

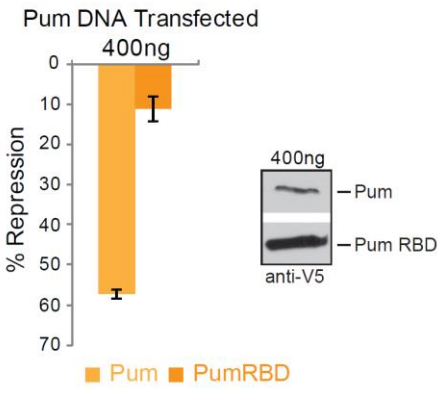



**Figure 2.22 - Comparison of full length Pum and the Pum RNA binding domain.** Percent repression of Rn3xNRE by full length Pum or RBD.

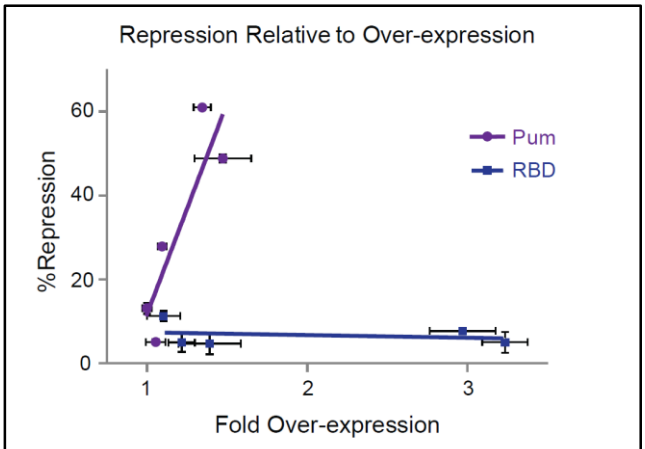



**Figure 2.23 - Pumilio versus RBD repression.** Percent repression versus fold over-expression from Figure 2.14 and 2.15 with best-fit lines plotted for each data set.

conditions where mRNAs and proteins were over-expressed at similar levels (*e.g.* 400ng Pum and 80ng RBD), shows that Pum repressed 49% while the RBD only repressed 11% (Fig. 2.16 and 2.23). Therefore, differential repression does not result from disparate expression levels. These results indicate that the N-terminal 1090 amino acids of Pum contain the major repressive activity, illuminating a previously unknown function.

### 2.3.3.2 Programming Pum RNA binding specificity confers repression of a new target mRNA

We engineered a Pum protein with altered RNA binding specificity that recognizes a new binding site, allowing examination of exogenously introduced Pum mutants without potential interference by the endogenous protein. Previous studies deciphered an RNA binding code for PUF repeats (Wang et al. 2002; Cheong and Hall 2006; Lu et al. 2009). Three amino acids of each PUF repeat recognize one nucleotide (Wang et al. 2002). The third base of the Pum binding site is a uracil ($U_1G_2\mathbf{U_3}$), which interacts with amino acids N1306, Y1307, and Q1310 of the sixth PUF repeat. The two flanking amino acids, N1306 and Q1310, make hydrogen bonds with $U_3$ while Y1307 mediates base stacking interactions between $U_3$ and the following nucleotide base, $A_4$ (Wang et al. 2002). By changing the RNA recognition amino acids of repeat 6 (N1306S and Q1310E), we programmed the mutant Pum R6SYE to bind an NRE sequence with $U_1G_2\mathbf{G_3}$ trinucleotide, instead of $U_1G_2U_3$, in the Rn3xNRE UGG reporter.

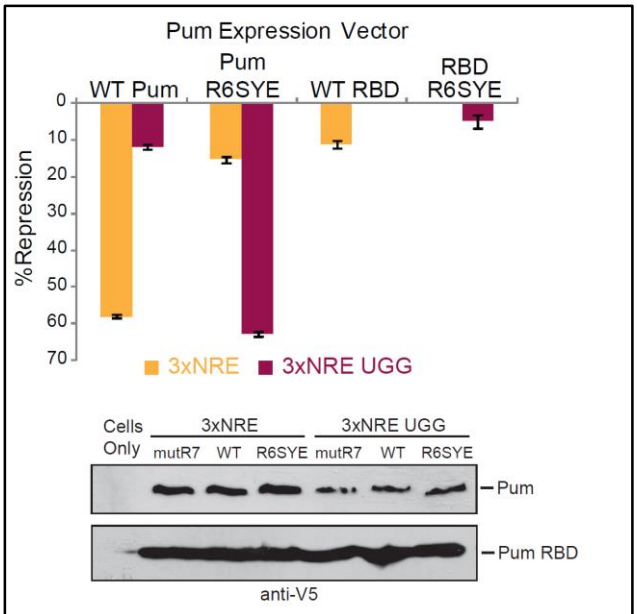



**Figure 2.24 - Engineering Pumilio specificity.** Percent repression of the Rn3xNRE with wild type or UGG NREs by full length Pum, wild type (WT Pum) and programmed mutant (Pum R6SYE), or by Pum RBD, wild type (WT RBD) or programmed mutant (RBD R6SYE).

We tested the ability of wild type Pum to repress the Rn3xNRE and Rn3xNRE UGG reporter mRNAs. As expected, Pum repressed the wild type NRE reporter by 58% but only slightly affected the Rn3xNRE UGG reporter by 12% (Fig. 2.24). We then measured the activity of Pum R6SYE, which minimally

repressed the wild type NRE by 15% (Rn3xNRE, Fig. 2.24). In contrast, Pum R6SYE dramatically repressed the Rn3xNRE UGG reporter by 63%. This result demonstrates that PUF proteins can be programmed to repress new target mRNAs.

We then used Pum R6SYE to examine the activity of the RBD relative to full length protein. We considered that repression by the exogenous Pum RBD tested in Figure 2.21 and 2.22 might be antagonized by endogenous Pum. This concern could be eliminated by the altered specificity approach. First, we confirmed that the wild type Pum RBD repressed the Rn3xNRE reporter by 11% but was incapable of repressing the Rn3xNRE UGG reporter (Fig. 2.24). Next, repression by RBD R6SYE was examined. RBD R6SYE repressed the UGG reporter weakly (5%) but had no effect on the reporter bearing wild type NREs (Fig. 2.24). Expression of Pum RBD and derivatives was confirmed by western blot (Fig. 2.24). These results reaffirm that repression by the RBD is substantially deficient relative to full length Pum.

### 2.3.3.3 The RBD is sufficient for Nos stimulation of repression

The effectiveness of Pum R6SYE created the opportunity to further examine Nos dependent repression. We hypothesized that inefficient repression by the RBD might be caused by the lack of Nos in D.mel-2 cells. Because Nos enhances repression by endogenous Pum of the wild type NRE reporter (Fig. 2.5), we circumvented this issue using the altered specificity approach. Using the Rn3xNRE UGG reporter, the ability of Pum RBD R6SYE to respond to Nos was tested. While Nos and the RBD R6SYE mutant have low activity when tested individually (Fig. 2.25, 8% and 11% repression, respectively), when expressed together, they synergize to repress by 34% (Fig. 2.25). To confirm that Nos dependent

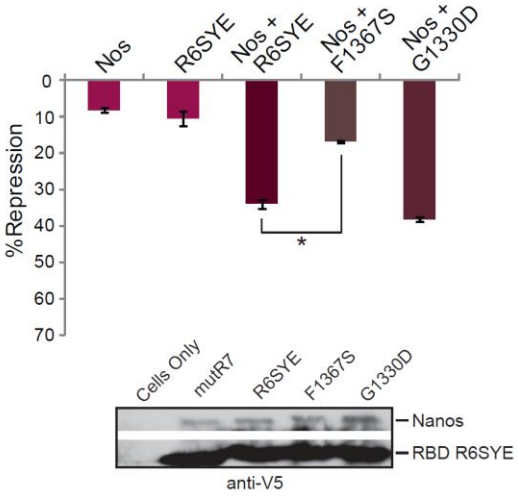



**Figure 2.25 - Nanos enhances the RBD.** Percent repression Nos and Pum RBD R6SYE using the Rn3xNRE UGG reporter. Mutations of F1367S or G1330D in Pum RBD R6SYE block binding to Nanos or Brain Tumor, respectively. Mean values with standard error are indicated in

repression is mediated by interaction with the RBD, we tested the ability of Nos to affect the RBD R6SYE mutant, F1367S, which disrupts the Pum-Nos interaction (Edwards et al. 2001). RBD R6SYE F1367S with Nos repressed by 17% — less than the additive repression of the Nos and RBD R6SYE controls combined (Fig. 2.25); therefore, Nos cannot synergize with the Pum RBD in the absence of a direct protein interaction. Using a RBD R6SYE with the G1330D mutation, we tested the requirement of interaction with Brat (Sonoda and Wharton 2001). Nos stimulated repression by RBD R6SYE G1330D to 38% (Fig. 2.25). We confirmed expression of Nos and RBD R6SYE proteins by western blot (Fig. 2.25). We conclude that repression by the RBD is enhanced by interaction with Nos, but enhancement does not require binding to Brat.

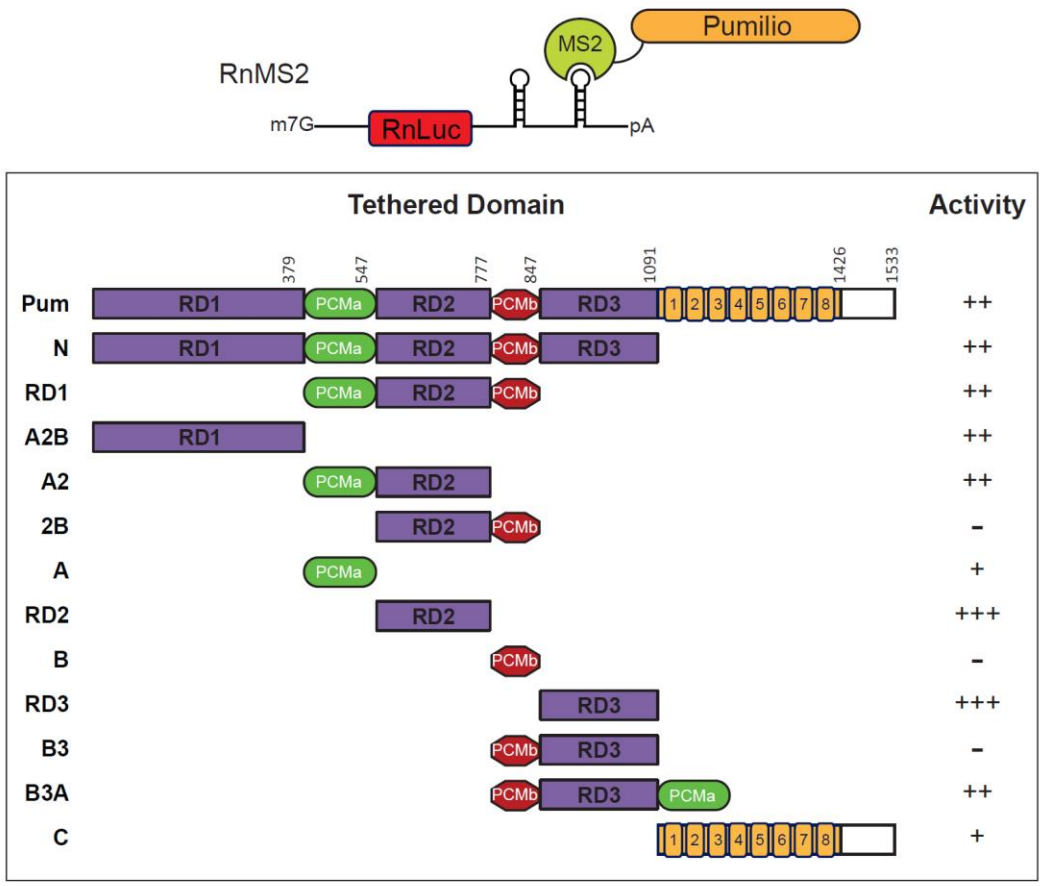



**Figure 2.26 - The tethered function assay.** Diagram of tethered-function Renilla luciferase reporter with tandem stem-loop binding sites for MS2 coat protein in the 3'UTR (RnMS2). Schematic of test proteins fused to MS2 coat protein (MS2) including full length Pum and segments with indicated amino acid positions. Relative repression by Pum segments is based on the scale: (-) <10% repression, (+) 10-30%, (++) 31-60%, and (+++) >60% repression.

### 2.3.3.4 The N-terminal portion of Pum contains the major repression activity

Full length Pum exhibits greater repression than the RBD, indicating that the major repression domain resides outside of the PUF repeats. To separate Pum repression and RNA binding activities, we utilized a tethered-function approach. Pum, or portions thereof, were fused to the MS2 coat protein (MS2), which binds a specific RNA stem-loop. A *Renilla* luciferase reporter was constructed with two MS2 binding sites in a minimal 3'UTR (Fig. 2.26, RnMS2). If the test protein represses when tethered by MS2, then reporter expression will be reduced. As a control, Firefly luciferase was co-expressed. Luciferase activities were normalized by dividing *Renilla* signals by those of Firefly to calculate relative response ratios. Values generated using test proteins were compared to control MS2 protein.

When tethered, full length Pum repressed by 60% (Fig. 2.27), a magnitude similar to that observed for repression of Rn3xNRE (Fig. 2.12). The N-terminal two-thirds of Pum (N, amino acids 1-1090) repressed by 50% whereas the C-terminal RBD (C, aa1091-1533) repressed by 22% (Fig. 2.27). Repression is dependent on tethering because, when not fused to MS2, full length Pum and RBD had no effect on RnMS2 (data not shown). Consistent with results in Figure 3, the C-terminal RBD represses inefficiently. Therefore, the N-terminal 1090 amino acids contain the major repressive activity of Pum.

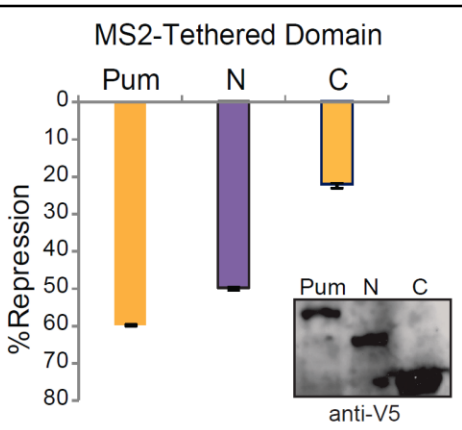



**Figure 2.27 - Tethered repression.** Percent repression of RnMS2 by tethered Pum, the N-terminus (N) and C-terminus (C), calculated relative to MS2 control. Western blot confirms expression of tagged proteins

### 2.3.3.5 Multiple domains within the Pum N-terminus have autonomous repression activity

We further dissected repression by the Pum N-terminus using the tethered-function assay. Internal deletions did not cause loss of repression, thus we reasoned that the N-terminus harbors multiple repression domains (Fig. 2.28). Six segments of Pum

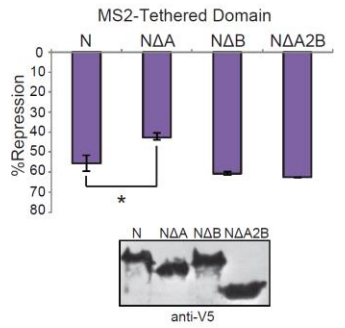



**Figure 2.28 - PCMa and PCMb deletions.** Percent repression of RnMS2 reporter by tethered Pumilio N-terminus containing internal deletions of single domains. Deletions of PCMa, PCMb, and aa379-847 are included. Statistical significance ($p < 0.05$) from a two-tailed t-test is indicated.

were then separately fused to MS2 including amino acids 1-378 (Region 1), 379-547 (PCMa, Region A), 548-776 (Region 2), 777-847 (PCMb, Region B), 848-1090 (Region 3), and 1091-1533 (Region C) (Fig. 2.26). When tethered, three segments repressed more efficiently than the N-terminus: region 1, 48% repression; region 2, 76% repression; and region 3, 68% repression (Fig. 2.29). PCMb did not repress while PCMa and region C repressed to lesser degree (24% and 26% repression) (Fig. 2.29). Therefore, multiple domains of Pum can independently repress an mRNA. We termed Regions 1, 2, and 3 Repression Domains (RDs) for their ability to stimulate robust repression when tethered.

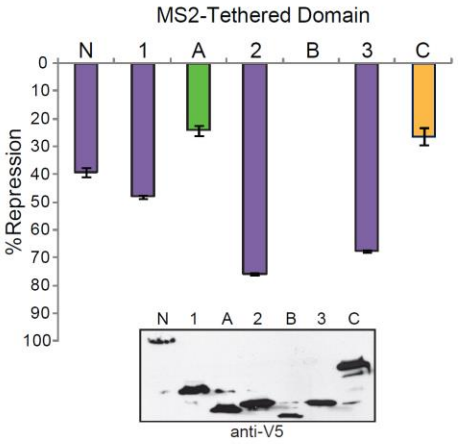



**Figure 2.29 - Multiple Pum repression domains.** Percent repression of RnMS2 reporter by tethered Pum segments, named corresponding to landmarks as in 2.26. Western blot confirms expression.

### 2.3.3.6 Conserved motifs modify the activity of an autonomous Pum repression domain

The role of PCMa and PCMb remained unclear. We reasoned that these domains may regulate Pum function. To investigate this idea, we created tethered constructs with one or both conserved motifs connected to a Pum repression domain (RD2, aa548-776) (Fig. 2.30).

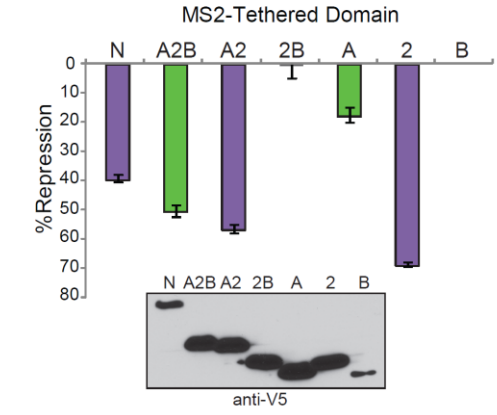



**Figure 2.30 - PCMa and PCMb are Auto-regulatory.** Percent repression of RnMS2 by the indicated MS2-tethered Pum segments.

By itself, RD2 had maximal activity (Fig. 2.30, 69% repression). When PCMa was fused to RD2 (A2, aa379-776), the activity remained robust (Fig. 2.30, 56% repression). Strikingly, when PCMb was fused to RD2 (2B, aa548-847), activity was completely lost (Fig. 2.30, 1% repression). In agreement with Fig. 2.29, PCMa and PCMb exhibited weak or no repression on their own (Fig. 2.30). When PCMb was

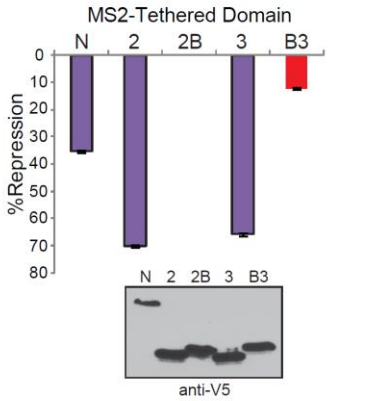



**Figure 2.31 - PCMb inhibits RD2 and RD3.** Percent repression by Pum repression domains 2 and 3 alone (RD2 and RD3) or fused to PCMb (2B or B3). Western blotting confirmed expression of tagged proteins.

fused to RD3 (B3, aa777-1090), activity was also severely reduced (Fig. 2.31, 12% repression). Western blots confirmed protein expression (Fig. 2.30, 2.31). The data show that PCMb inhibits repression domains in Region 2 and Region 3.

We measured the activity of a segment containing PCMa, RD2, and PCMb; this construct (A2B, aa379-847) repressed the mRNA by 50% (Fig. 2.30). A similar restoration of repression is observed when PCMa is fused to a construct containing PCMb and RD3 (2B3, aa777-1090 + aa379-547) (Fig. 2.32). Deletion of PCMa from the tethered N-terminus also leads to a small but significant decrease in repression (Fig. 2.29). We speculate that PCMa may antagonize the negative regulatory function of PCMb, perhaps via auto-inhibitory interactions.

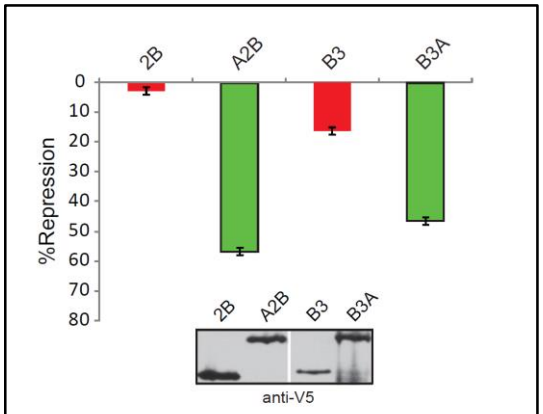



**Figure 2.32 - PCMa activity is modular.** Percent repression of RnMS2 by the indicated MS2-tethered Pum segments. PCMa has been fused to the C-termionus of B3 to generatee B3A.

### 2.3.3.7 The N-terminal Pum repression domains reduce protein and mRNA levels

Full length Pum reduces protein expression and mRNA levels with comparable efficiency (Fig. 2.8). We next tested the ability of individual Pum RDs to do the same. Using the tethered-function approach, we measured the effect of the N-terminus and C-terminus on luciferase protein and mRNA expression. Reduction of protein levels correlated with reduction of mRNA levels for both the full length protein (Fig. 2.33, Pum, 45% protein and 32% mRNA) and the C-terminus (Fig. 2.33, Region C, 10% protein and 6% mRNA). The N-terminus caused a substantially greater effect on protein expression than

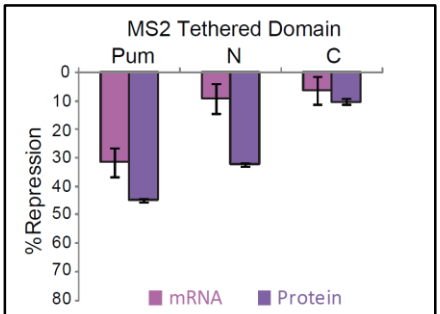



**Figure 2.33 - N-terminus represses translation.** Percent repression of RnMS2 mRNA and protein by tethered full length Pum, Pum N-terminus or Pum C-terminus.

mRNA (Region N, 32% protein and 9% mRNA, Fig. 2.33). When each N-terminal repression domain was tested, similar differences were observed for RD1 (42% vs 18%), RD2 (72% vs 42%), and RD3 (62% vs 45%) (Fig. 2.34). This suggests that the N-terminal repression domains inhibit translation to a greater extent than they enhance mRNA degradation.

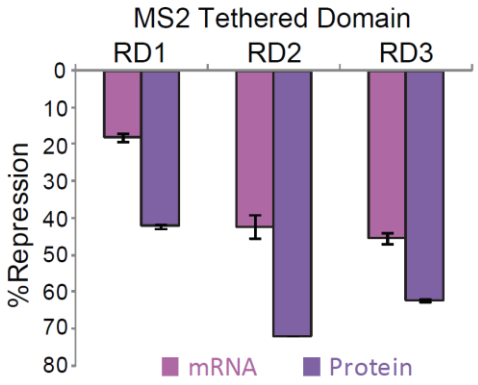



**Figure 2.34 - RDs repress translation.** Percent repression of RnMS2 mRNA and protein by tethered Pum N-terminal repression domains.

### 2.3.3.8 N-terminal repression domains are conserved in human PUF proteins

We hypothesized that the N-terminal repression domains of Pum may be conserved by other PUF proteins. Insect and vertebrate PUF proteins share a similar architecture including a highly conserved C-terminal RBD (>80% identical, Fig. 2.35) and N-terminal domains (Fig. 2.35) including PCMa and PCMb motifs (Fig. 2.35). We compared repression by the N-termini of human PUM1 (Hs PUM1-N; aa1-827) and PUM2 (Hs PUM2-N; aa1-704) to that of *Drosophila* Pum (Dm Pum-N) using the tethered-function assay (Fig. 2.36). All three proteins were expressed, as confirmed by western blotting (Fig. 2.36). Human PUM1-N and PUM2-N repressed RnMS2 reporter by 47% and 36%, respectively, comparable to the 35% repression caused by the N-terminus of Pum (Fig. 2.36). Next, we tested whether the regions of human PUM1 corresponding to Pum repression domains possessed autonomous repressive activity. When tethered, region 1 of human PUM1 (Hs PUM1-1; aa1-150) lacked repressive function (Fig. 2.37). However, PUM1 region 2 (HsPUM1-2, aa309-459)

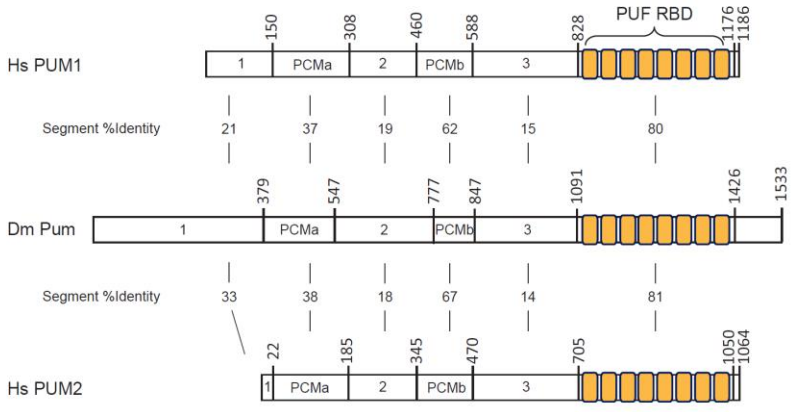



**Figure 2.35 - PUF domain conservation.** Comparison of human PUM1 and PUM2 to Drosophila Pumilio. Percentage of identical amino acids between segments is indicated.

and region 3 (HsPUM1-3, aa589-827) repressed 48% and 82%, respectively (Fig. 2.37). These results show that the N-termini of human PUFs contain potent repressive domains, indicating a conserved regulatory function.

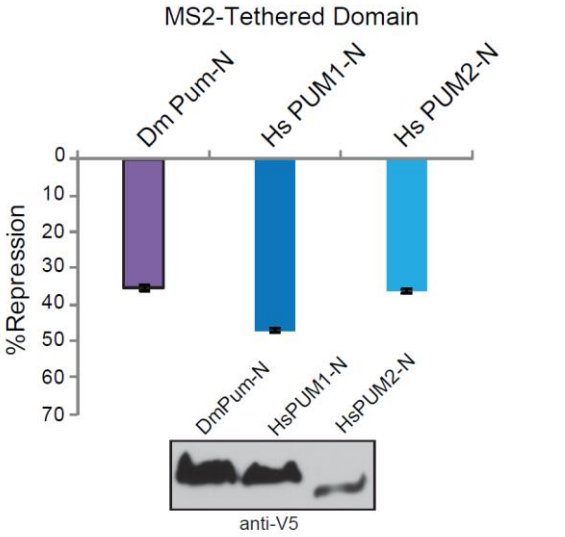



**Figure 2.36 - Human PUF N-termini repress.** Percent repression of RnMS2 by the tethered N-terminus of Pumilio (Dm Pum-N) and human PUM1 (Hs PUM1-N) and PUM2 (Hs PUM2-N).

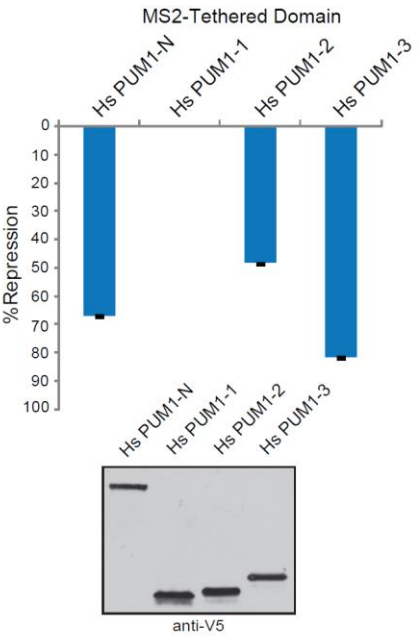



**Figure 2.37 - Human PUF repression domains.** Percent repression by MS2-tethered regions of human PUM1 corresponding to Pumilio repression domains (Hs PUM1-1, Hs PUM1-2, Hs PUM1-3).

Table 2.1: Data From Figure 2.8

| | Nos C354Y | | Nos | |
|---|---|---|---|---|
| | **RRR** | **SEM** | **RRR** | **SEM** |
| 1xNRE | 0.270 | 0.005 | 0.066 | 0.002 |
| 3xNRE | 0.295 | 0.005 | 0.045 | 0.001 |
| 3xNREmut | 0.164 | 0.004 | 0.183 | 0.003 |

Table 2.2: Data From Figure 2.9, 2.10

| | Nos C354Y | | Nos | |
|---|---|---|---|---|
| **RNAi** | **RRR** | **SEM** | **RRR** | **SEM** |
| LacZ | 0.341 | 0.010 | 0.080 | 0.002 |
| Nos | | | 0.328 | 0.017 |
| Pum | | | 0.309 | 0.012 |
| LacZ | 0.413 | 0.006 | 0.080 | 0.001 |
| Nos | | | 0.407 | 0.015 |
| Brat 1 | | | 0.090 | 0.001 |
| Brat 2 | | | 0.094 | 0.001 |
| 4EHP 1 | | | 0.064 | 0.002 |
| 4EHP 2 | | | 0.081 | 0.001 |

Table 2.3: Data From Figure 2.17

| | Pum mutR7 | | Pum | |
|---|---|---|---|---|
| | **RRR** | **SEM** | **RRR** | **SEM** |
| 1xNRE | 0.409 | 0.016 | 0.216 | 0.008 |
| 3xNRE | 0.459 | 0.032 | 0.147 | 0.008 |
| 3xNREmut | 0.194 | 0.001 | 0.204 | 0.007 |

Table 2.4: Data From Figure 2.18

| Pum mutR7 | | Pum | | F1367S | | G1330D | |
|---|---|---|---|---|---|---|---|
| **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** |
| 0.333 | 0.008 | 0.099 | 0.001 | 0.092 | 0.001 | 0.090 | 0.003 |

Table 2.5: Data From Figure 2.19

| | Pum mutR7 | | Pum | |
|---|---|---|---|---|
| **RNAi** | **RRR** | **SEM** | **RRR** | **SEM** |
| LacZ | 0.368 | 0.014 | 0.123 | 0.003 |
| Pum | | | 0.791 | 0.032 |
| Nos | | | 0.175 | 0.007 |
| Brat | | | 0.117 | 0.005 |
| Nos + Brat | | | 0.156 | 0.004 |

Table 2.6: Data From Figure 2.21

| | RBD mutR7 | | RBD | |
|---|---|---|---|---|
| | **RRR** | **SEM** | **RRR** | **SEM** |
| 3.2ng | 0.470 | 0.005 | 0.467 | 0.002 |
| 16ng | 0.488 | 0.078 | 0.460 | 0.005 |
| 80ng | 0.531 | 0.012 | 0.439 | 0.008 |
| 400ng | 0.508 | 0.007 | 0.438 | 0.006 |

Table 2.7: Data From Figure 2.22

| | mutR7 | | Wild Type | |
|---|---|---|---|---|
| | **RRR** | **SEM** | **RRR** | **SEM** |
| Pum | 0.429 | 0.005 | 0.183 | 0.004 |
| RBD | 0.415 | 0.003 | 0.370 | 0.013 |

Table 2.8: Data From Figure 2.24

| | mutR7 | | Wild Type | | R6SYE | | |
|---|---|---|---|---|---|---|---|
| | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | |
| Pum | 0.603 | 0.019 | 0.253 | 0.003 | 0.511 | 0.006 | 3xNRE |
| RBD | 0.667 | 0.008 | 0.592 | 0.006 | 0.740 | 0.008 | |
| Pum | 0.459 | 0.020 | 0.405 | 0.003 | 0.171 | 0.003 | 3xNRE UGG |
| RBD | 0.514 | 0.006 | 0.525 | 0.002 | 0.489 | 0.009 | |

Table 2.9: Data From Figure 2.25

| RBD mutR7 | | Nos | | RBD R6SYE | | Nos+R6SYE | | Nos+F1367S | | Nos+G1330D | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** |
| 0.518 | 0.009 | 0.474 | 0.003 | 0.462 | 0.010 | 0.341 | 0.007 | 0.430 | 0.002 | 0.319 | 0.003 |

Table 2.10: Data From Figure 2.27

| MS2 | | Pum | | N | | C | |
|---|---|---|---|---|---|---|---|
| **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** |
| 0.234 | 0.002 | 0.094 | 0.001 | 0.117 | 0.001 | 0.182 | 0.001 |

Table 2.11: Data From Figure 2.29

| MS2 | | N | | 1 | | A | | 2 | | B | | 3 | | C | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** |
| 0.145 | 0.002 | 0.089 | 0.002 | 0.076 | 0.001 | 0.111 | 0.003 | 0.035 | 0.000 | 0.162 | 0.002 | 0.047 | 0.001 | 0.107 | 0.005 |

Table 2.12: Data From Figure 2.30

| MS2 | | N | | A2B | | A2 | | 2B | | A | | 2 | | B | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** |
| 0.197 | 0.010 | 0.120 | 0.003 | 0.098 | 0.004 | 0.086 | 0.003 | 0.195 | 0.008 | 0.162 | 0.005 | 0.062 | 0.002 | 0.212 | 0.006 |

Table 2.13: Data From Figure 2.31

| MS2 | | N | | 2 | | 2B | | 3 | | B3 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** |
| 0.219 | 0.001 | 0.142 | 0.001 | 0.065 | 0.001 | 0.242 | 0.003 | 0.075 | 0.001 | 0.192 | 0.001 |

Table 2.14: Data From Figure 2.33

| MS2 | | Pum | | N | | C | |
|---|---|---|---|---|---|---|---|
| **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** |
| 0.167 | 0.001 | 0.092 | 0.001 | 0.113 | 0.001 | 0.150 | 0.002 |

Table 2.15: Data From Figure 2.34

| MS2 | | 1 | | 2 | | 3 | |
|---|---|---|---|---|---|---|---|
| **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** |
| 0.106 | 0.001 | 0.061 | 0.001 | 0.030 | 0.000 | 0.040 | 0.001 |

Table 2.16: Data From Figure 2.36

| MS2 | | Dm Pum-N | | Hs PUM1-N | | Hs PUM2-N | |
|---|---|---|---|---|---|---|---|
| **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** |
| 0.165 | 0.001 | 0.107 | 0.001 | 0.087 | 0.001 | 0.106 | 0.001 |

Table 2.17: Data From Figure 2.37

| MS2 | | Hs PUM1-N | | Hs PUM1-1 | | Hs PUM1-3 | |
|---|---|---|---|---|---|---|---|
| **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** | **RRR** | **SEM** |
| 0.213 | 0.007 | 0.123 | 0.004 | 0.202 | 0.007 | 0.072 | 0.003 |

## 2.4 Discussion

Pum and Nos control important functions including development (Lehmann and Nusslein-Volhard 1987b; Lehmann and Nusslein-Volhard 1991; Wang and Lehmann 1991), stem cell proliferation (Lin and Spradling 1997; Forbes and Lehmann 1998; Asaoka-Taguchi et al. 1999; Parisi and Lin 2000) and learning (Dubnau et al. 2003). Previous analyses of Pum and Nos function were restricted to mutant or transgenic *Drosophila*. The experiments presented in this work build upon these studies to elucidate the mechanism of regulation. We developed a reporter assay that recapitulates Nos dependent repression. Nos mRNA is not detectable in D.mel-2 cells (Fig. 2.4) (Cherbas et al. 2011), but exogenous expression of Nos confers potent repression of an mRNA bearing Hb NREs (Fig. 2.5 and 2.8). Pum is essential for Nos repression (Fig. 2.9) (Lehmann and Nusslein-Volhard 1991; Barker et al. 1992). Therefore, Nos activates Pum. Acting together, Nos and Pum inhibit protein expression and cause a corresponding decrease in mRNA level (Fig. 2.8). The data are consistent with Nos and Pum collaborating to repress Hb mRNA in the *Drosophila* embryo (Lehmann and Nusslein-Volhard 1987b; Tautz 1988; Lehmann and Nusslein-Volhard 1991; Wharton and Struhl 1991; Barker et al. 1992; Murata and Wharton 1995; Sonoda and Wharton 1999).

Pum also represses independently of Nos (Fig. 2.12). Without Nos, endogenous Pum in D.mel-2 cells minimally represses the NRE-bearing reporter (Fig. 2.3); however, efficient repression was elicited by increasing the concentration of Pum (Fig. 2.12, Fig. 2.14). A likely explanation is that the amount of endogenous Pum is insufficient to efficiently repress. Like Nos dependent repression, Pum potently decreased reporter protein and mRNA levels (Fig. 2.17). Several facts support the conclusion that Nos was not necessary for repression by Pum. First, Nos is not detectable in D.mel-2 cells. Also, RNAi of Nos did not affect Pum repression (Fig. 2.19). Furthermore, a mutation that blocks Nos binding to Pum (F1367S) did not alleviate repression (Fig. 2.18). Nos independent Pum repression is supported by observations that Pum regulates Bcd and CycB mRNAs in the anterior of *Drosophila* embryos, where Nos is below the limit of detection (Gamberi et al. 2002; Vardy and Orr-Weaver 2007).

The finding that Pum represses independently of Nos raises the question: what is the function of Nos? One logical answer is that Nos strengthens Pum repression. The observation that Nos activates endogenous Pum supports a model wherein Nos enhances the RNA binding activity of Pum. Indeed, the association of Nos with the RBD of Pum is sufficient for enhancement (Fig. 2.25). Previous work strengthens this hypothesis: Nos and Pum interact with each other and both associate with the NRE RNA through a network of protein-protein and protein-RNA interactions that may cooperate to enhance binding (Sonoda and Wharton 1999; Sonoda and Wharton 2001). The necessity of Nos could be obviated by increasing the level of Pum (Fig. 2.12), likely resulting from increased occupancy of the NRE reporter. Another hypothesis is that binding of Nos to Pum might displace a negative regulatory factor, resulting in activation of endogenous Pum. Nos may also collaborate with Pum to recruit co-repressors. The Nos-Pum-NRE complex is thought to recruit Brat and 4EHP to refine regulation of the Hb gradient (Sonoda and Wharton 2001; Cho et al. 2006). However, RNAi depletion of Brat and 4EHP did not abrogate Nos dependent repression (Fig. 2.10). We interpret this as evidence that Nos and Pum repress mRNAs through additional mechanisms (see below), with the caveat that residual Brat and 4EHP might be sufficient to support Nos dependent repression. As an alternative model, Nos and Pum may collaborate to recruit the Ccr4-Not deadenylase complex through interactions with Not4 and Pop2 subunits, respectively (Kadyrova et al. 2007; Goldstrohm and Wickens 2008). These models are further addressed in Chapters 3 and 4.

Potent Pum repression in the absence of Nos indicates that Pum independently inhibits protein expression and/or enhances mRNA decay. Involvement of known co-repressors, Brat and 4EHP, is improbable because recruitment of these proteins depends on Nos (Sonoda and Wharton 2001; Cho et al. 2006). Furthermore, a mutant Pum (G1330D) that cannot bind Brat is fully active for Nos independent repression (Fig. 2.18). In addition, depletion of Brat by RNAi did not affect Pum repression (Fig. 2.19). These findings reveal that mechanisms other than Brat-4EHP mediated inhibition of 5’ cap dependent translation are utilized by Pum. Previous studies concluded that Brat, and therefore 4EHP, are dispensable for Pum repression in certain contexts. For instance, Pum repression of CycB in embryonic pole cells does not require Brat (Sonoda and

Wharton 2001; Kadyrova et al. 2007). Furthermore, while Brat is necessary for Pum repression in motor neurons, it is not essential in other neurons (Muraro et al. 2008). Finally, the G1330D mutant Pum, which is deficient for recruitment of Brat, is functional for regulation of dendritic morphology in sensory neurons (Ye et al. 2004). We do not dismiss the importance of Brat and 4EHP in embryonic development. Instead, these findings illustrate that while Brat and 4EHP facilitate repression of Hb in the embryo, in other contexts, Pum represses by other means.

We identified Pum domains that mediate Nos independent repression. The Pum RBD has modest repressive activity compared to the full length protein (Fig. 2.14, 2.22, 2.24), indicating that regions outside of the RBD must confer repressive activity. Previous analysis of the ability of Pum transgenes to rescue abdominal segmentation defects in a *pum* mutant embryo support this conclusion. Whereas over-expression of the Pum RBD partially rescued segmentation defects, the full length Pum fully restored proper embryonic development (Barker et al. 1992; Wharton et al. 1998). Indeed, we discovered three repression domains within the N-terminal two-thirds of Pum that provide the major repressive activity (Fig. 2.29). These unique RDs (i.e. aa1-378, 548-776, and 848-1090) do not share sequence homology. Each functions autonomously when tethered to mRNA (Fig. 2.29). Because all known Pum cofactors (i.e. Nos, Brat, 4EHP, and Pop2) interact with the RBD (Sonoda and Wharton 1999; Sonoda and Wharton 2001; Cho et al. 2006; Kadyrova et al. 2007), the N-terminal repression domains likely function through novel mechanism(s). While full length Pum affects both mRNA and protein levels almost equally (Fig. 2.8, 2.17), the individual repression domains affect protein expression more than mRNA levels (Fig. 2.33 and 2.34). This suggests translational inhibition may be their predominant function.

The repressive function of the Pum N-terminus may be evolutionarily conserved. Sequence alignments indicated that the N-terminus of vertebrate PUMs, including human PUM1 and PUM2, are related to the Pum N-terminus (Fig. 2.35). When tethered, the N-terminal portions of human PUM1 and PUM2 repressed, providing evidence that human PUFs are repressors (Fig. 2.36). Two regions in human PUM1 are autonomous repression domains (Fig. 2.37). These regions are small (Region 2, 152 amino acids and

Region 3, 240 amino acids), share 19% and 15% identity with Pum, and do not contain previously identified motifs. We propose that they may contact novel co-repressors, which remain to be identified.

We compared the Pum N-terminus to other PUF proteins; no detectable relationship could be found with six *S. cerevisiae* PUFs or twelve *C. elegans* PUFs. Instead, these PUFs have evolved unique sequences, appended to their RBDs, whose function remains unknown. We also searched the non-redundant protein sequence database (www.ncbi.nlm.nih.gov) using the BLAST algorithm (Altschul et al. 1990) to identify protein sequences similar to the Pum N-terminus: no proteins, other than PUF family members, share homology. The broad implication is that members of the PUF family have evolved unique domains, appended to the evolutionarily conserved PUF repeat RNA binding module, which may confer unique regulatory activities to individual PUFs. Consistent with this idea, specific PUFs have been shown to affect translation, mRNA degradation, mRNA localization, and for one PUF, activation of target mRNAs (Olivas and Parker 2000; Tadauchi et al. 2001; Gu et al. 2004; Goldstrohm et al. 2006; Garcia-Rodriguez et al. 2007; Saint-Georges et al. 2008; Kaye et al. 2009; Chritton and Wickens 2010; Quenault et al. 2011).

We identified two sequence motifs in the N-terminus of Pum, designated PCMa and PCMb, which are conserved between insect (*e.g. Drosophila*) and vertebrates (*e.g.* humans) (Fig. 2.35). PCMb encompasses a motif in Xenopus PUM2 proposed to interfere with cap-dependent translation (Cao et al. 2010). However, when tethered, PCMb does not repress (Fig. 2.29), nor does deletion of PCMb diminish Pum repression (Fig. 2.28). In addition, mutation of a PCMb tryptophan residue (W783) proposed to contact the 5' cap (Cao et al. 2010) had no effect (data not shown). Therefore, we find no evidence that the putative cap binding motif of PCMb is important for Pum repression. Instead, PCMb negatively affects RD2 (aa548-776) and RD3 (aa848-1090) (Fig. 2.31). While PCMa had weak repressive activity on its own, it could counteract the inhibitory effect of PCMb (Fig. 2.30) and deletion of PCMa caused a minor but significant drop in repression (Fig. 2.28). The precise roles of PCMa and PCMb remain to be determined; we speculate that they may have auto-regulatory functions.

We successfully programmed Pum to repress a new target mRNA. By changing the RNA recognition amino acids in the sixth PUF repeat of Pum from NYQ to SYE, the specificity was altered from uridine to guanosine, thereby conferring repression to an mRNA with an altered binding site (3xNRE UGG, Fig. 2.24). This experiment provides the proof-of-principle that PUF proteins with programmed RNA binding specificity can be engineered to repress new mRNAs. While programmed Pum fully represses its new target, a similarly programmed RBD lacks substantial activity (Fig. 2.24), further emphasizing the importance of the N-terminal repression domains. This finding has important implications for future engineering of PUFs. The Pum RBD provides a protein module with low intrinsic regulatory activity that can be programmed to bind new RNA sequences. Functional domains – either repression or activation domains - can be attached to this module to create novel RNA regulators. Consistent with this idea, a recent study reported that addition of splicing effector domains and a nuclear localization signal transformed a PUF RBD into a splicing regulator (Wang et al. 2009).

An important question for future research is: how do the Pum repression domains function? A probable hypothesis is that the repression domains inhibit the translation machinery. Alternatively, the repression domains may activate enzymes that degrade mRNAs. In future experiments, we seek to identify co-repressor(s) that interact with these domains. Also worth consideration is why Pum possesses multiple repression domains. These domains may recruit the same co-repressor, either acting redundantly or collaboratively. Alternatively, each repression domain could bind to a different co-repressor, perhaps affecting different steps in the gene expression pathway (e.g. translation initiation or mRNA degradation). In this case, their individual repressive activities would collaborate to increase the efficiency of repression. Addressing these crucial questions will help reveal how Pum regulates mRNAs to control diverse biological functions.

## 2.5 Materials and Methods

*Plasmids.* To create pAc5.1 FF control, Firefly luciferase was PCR amplified from pGL4.13 (Promega) and inserted into plasmid pAc5.1/V5-His A (Invitrogen). Reporter plasmids were created by inserting the *Renilla* luciferase open reading frame with a

minimal 3'UTR into pAc5.1. This 3'UTR contains a multiple cloning site and cleavage and poly-adenylation signal from psiCHECK1 (Promega). To create the reporters, oligonucleotides encoding wild type NREs (Rn1xNRE and Rn3xNRE) or mutant NREs (Rn3xNREmut) were inserted into Xho1 and Not1 sites in multiple cloning site of pAc5.1 *Renilla* luciferase. The NRE sequences, derived from *Drosophila* hunchback (NM_169234), are as follows (Pum site underlined, mutations bold):

NRE (5'-UUGUUGUCGAAAAUUGUACAUAAGCCAA)

NRE mutant (5'-UU**CA**U**CA**CGAAAAU**ACA**ACAUAAGCCAA)

NRE UGG (5'-UUG**G**UG**G**CGAAAAUUG**G**ACAUAAGCCAA).

The RnMS2 reporter plasmid for the tethered-function assays was created by inserting oligonucleotides containing two MS2 binding sites into the Xho1 and Not1 sites in the 3'UTR of pAc5.1 *Renilla* luciferase. The sequence of the tandem MS2 binding sites is: 5'AAAACATGAGGATCACCCATGTCTGCAGGTCGACTCTAGAAAACATGAG GATCACCCATGTC (stem-loops are underlined). *Drosophila* Pumilio (NP_731315.1) and the Pumilio RBD (aa 1091-1426) were amplified by RT-PCR from oligo-dT primed cDNA from S2 cells and inserted into pIZ/V5-His vector (Invitrogen). Nanos (NP_476658.1) was cloned from whole fly cDNA and also inserted into the pIZ/V5-His vector. Mutations in Pumilio and the RBD were created by Quickchange site-directed mutagenesis (Stratagene). RNA binding defective Pumilio and RBD were created by mutating amino acids S1342A, N1343A, E1346A of the seventh PUF repeat. Pumilio R6SYE mutants were created by mutating N1306S and Q1310E of the sixth PUF repeat. The Pumilio and RBD mutants F1367S or G1330D were also created by site-directed mutagenesis. To create defective Nanos mutant, amino acid C354Y was mutated by site-directed mutagenesis. For the tethered-function expression vectors, DNA encoding MS2 coat protein was amplified from the pLexA N55K three-hybrid vector and fused in-frame to the N-terminus of Pumilio. Control plasmid pIZ MS2 was created by inserting MS2 coding sequence into the pIZ plasmid. The control pIZ-HT plasmid was created by inserting the HaloTag (Promega) ORF into pIZ plasmid. Brat (NP_476945.1) or 4EHP (NP_788729.1)

coding sequences were amplified from S2 cell cDNA and inserted into pIZ-HT to create HT-Brat and HT-4EHP.

*Cell culture.* D.mel-2 cells (Invitrogen) were cultured in Sf-900™ III serum-free media (Invitrogen) with 5mL/L Penicillin-Streptomycin using standard cell culture techniques. Cells were grown at 28°C.

*Transfections.* D.mel-2 cells were transfected with plasmid DNA using Effectene (Qiagen) according to manufacturer's specifications. Unless otherwise noted, standard transfection conditions of 2.2mL per well of a 6-well plate are as follows: 1.6mL D.mel-2 cells ($1.5x10^6$cells/mL), 600µl Sf900™ III media, 10ng of *Renilla* reporter plasmid DNA, 5ng firefly control plasmid DNA, and Effectene. For transfection of Pumilio expression vectors, 400ng of DNA was used unless otherwise noted in the figures. For Nanos expression, 10ng was the standard amount, unless otherwise noted. Cells were harvested for dual luciferase assay, western blotting, TMR labeling and fluorescence detection, and qRT-PCR after two days of growth following transfection. Where necessary, total DNA transfected in each sample was held constant by balancing transfection with empty pIZ vector.

*RNA interference.* Double stranded RNAs corresponding to each target gene were generated by in vitro transcription from DNA templates. The templates were created by PCR amplification of regions of 250-600bp of open reading frame from either plasmid vectors or cDNA from D.mel-2 cells. Both forward and reverse PCR primers had T7 promoters appended. Oligos used are listed below with T7 sequence underlined and gene specific sequence in bold:

**LacZ control**

Forward Primer:

5'-dGGATCCTAATACGACTCACTATAGGG**TGACGTCTCGTTGCTGCATAAAC**

Reverse Primer:

5'-dGGATCCTAATACGACTCACTATAGGG**GGCGTTAAAGTTGTTCTGCTTCATC**

**Pumilio**

Forward Primer:

5’-dGGATCCTAATACGACTCACTATAGGG**GTCAAGGATCAGAATGGCAATCATGT**

Reverse Primer:

5’-dGGATCCTAATACGACTCACTATAGGG**CTTCTCCAACTTGGCATTGATGTGC**

**Nanos**

Forward Primer:

5’-dGGATCCTAATACGACTCACTATAGGG**CATTCCACTCGCCACCCACTGG**

Reverse Primer:

5’-
dGGATCCTAATACGACTCACTATAGGG**CTAAACCTTCATCTGTTGCTTGTAGTAAC**

**Brain Tumor - 1**

Forward Primer:

5’-dGGATCCTAATACGACTCACTATAGGG**CAGATCTTCGACAAGGAGGGACG**

Reverse Primer:

5’-dGGATCCTAATACGACTCACTATAGGG**CATACCCACTGGCGCCAGTTGG**

**Brain Tumor - 2**

Forward Primer:

5’-dGGATCCTAATACGACTCACTATAGGG**CAACGAGCTGAACGAGACGCACC**

Reverse Primer:

5’-dGGATCCTAATACGACTCACTATAGGG**GGTGTGACTGTTGGTGGTGGCC**

**4EHP - 1**

Forward Primer:

5’-dGGATCCTAATACGACTCACTATAGGG**CGATGCTCGGGGAGCAGTTCC**

Reverse Primer:

5’-dGGATCCTAATACGACTCACTATAGGG**CAATGGGCCTTTATTAATTGAAACATA**

**4EHP - 2**

Forward Primer:

5’-dGGATCCTAATACGACTCACTATAGGG**GCAGTACGAGACGAAAAACTGGCC**

Reverse Primer:

5’-dGGATCCTAATACGACTCACTATAGGG**CGACCATGTGCAGCGACTTGC**

From each PCR template, dsRNA was transcribed *in vitro* with T7 RiboMAX Large Scale RNA Production System (Promega), treated with TURBO DNase (Ambion) for 3 hours, and purified using the SV Total RNA Isolation System (Promega). For knockdown of each gene’s expression, 6µg of dsRNA per well of a 6-well plate was added to cells 10 minutes before transfection of reporters and expression vectors.

*Luciferase Assays.* Luciferase assays were performed two days post-transfection. To do so, 100µl of transfected D.mel-2 cells were plated into three or four wells of a 96-well plate. Firefly and *Renilla* luciferase expression was measured using the Dual-Glo Luciferase Assay (Promega) according to the manufacturer’s specifications and the GloMax Multi+ Detection System (Promega) Luminometer. The measured relative light units (RLU) were used to calculate a relative response ratio (RRR) using the equation RRR = *Renilla* RLU/Firefly RLU. Response ratios are displayed normalized to mutant controls (set to 100). A percent repression value was then calculated as Percent Repression = $100x(1-RRR_{variable}/RRR_{mutant})$. For Nanos-stimulated repression, $RRR_{mutant}$ corresponds to the Nanos C354Y mutant. For Pumilio repression, Pumilio mutR7 (S1342A, N1343A, E1346A) was used as the mutant control. For RNAi treated samples,

percent repression was calculated for each sample relative to the negative control Pum mutR7 treated with LacZ control dsRNA using the equation Percent Repression = 100x(1-$RRR_{variable}/RRR_{control}$). To measure activation of the Pumilio RBD R6SYE by Nanos, the $RRR_{mutant}$ control was measured from cells expressing RBD mutR7. Displayed response ratios and percent repression in the tethered-function assay was determined relative to the control samples expressing MS2CP from the pIZ MS2CP plasmid, using the equation Percent Repression = 100x(1-$RRR_{variable}/RRR_{MS2CP}$). To measure experimental error, we calculated standard error of the mean (SEM) from triplicate or quadruplicate samples in each experiment. The reported SEMs are from technical replicates and are representative of multiple biological replicates performed at different times from different cell populations. Data were analyzed using Microsoft Excel. A graph of Pumilio repression relative to fold over-expression was created using the GraphPad Prism software.

*Western blotting.* For western blotting analysis, 1mL aliquots were taken from the same transfected D.mel-2 samples used for dual luciferase expression analysis. Two days post-transfection, cells were centrifuged at 1000xg for 3 minutes and pellets were lysed for one hour on ice in lysis buffer (0.5% Igepal CA-630 (USB), 50mM Tris-HCl pH 8.0, 0.5mM EDTA, 2mM $MgCl_2$, 150mM NaCl, 20nM PMSF, 1µg/mL Aprotinin, 1µg/mL Pepstatin, 1µg/mL Leupeptin). Lysates were cleared by centrifugation at 16,000xg for two minutes, and supernatants were saved as whole cell protein extracts. Extracts were separated via SDS polyacrylamide (12%) gel electrophoresis (Tris-Glycine running buffer) and proteins were transferred onto Immobilon-FL PVDF membranes (Millipore). Membranes were blocked in blocking buffer (PBS, 5% milk, 0.01% Tween 20), probed with V5-antibody (Invitrogen), washed in buffer, probed with HRP-conjugated goat anti-mouse IgG (Thermo Scientific), washed again, covered in ECL Western Blotting Reagent (Pierce), and imaged (luminescence) on autoradiography film.

*Fluorescent labeling and visualization of HaloTag protein constructs.* Protein extracts from HaloTag expression cells were harvested as above and mixed with HaloTag TMR Ligand (900nM final) for 30 minutes on ice in the dark. After labeling, extracts were separated via SDS polyacrylamide (4-20%) gel electrophoresis (Tris-Glycine running buffer) and protein fluorescence (532 Ex/580 Em) was measured with a Typhoon Trio+

Imager (GE Healthcare). Relative fluorescence was quantified using ImageQuant TL software (GE Healthcare).

*RNA isolation and cDNA preparation.* For isolation of RNA, 1mL of transfected D.mel-2 cells was centrifuged at 1000xg for 3 minutes, washed twice in PBS, and lysed with QIAzol reagent (Qiagen) according to the manufacturer's specifications. Upon ethanol precipitation and resuspension, whole cell RNA was treated with TURBO DNase (Ambion) for 3 hours. For isolates prepared from the tethered-function assay, RNA was purified from cell pellets using Maxwell LEV simplyRNA Cells (Promega). RNAs were primed with random hexamers (IDT) for synthesis of cDNAs using the GoScript Reverse Transcriptase (Promega). The final concentration of RNA in RT reactions was 60ng/µl.

*Quantitative PCR.* To measure endogenous mRNA levels, quantitative PCR was performed on 5µl of cDNA product in a 50µl reaction using 100nM of specific primers and GoTaq qPCR Master Mix (Promega). To measure Firefly and *Renilla* luciferase mRNAs, multiplexed qPCR was performed in 25µl reactions with 200nM fluorescent primers (Biosearch Technologies) and Plexor® Master Mix (Promega). Reactions were performed with a C1000 thermal cycler equipped with the CFX96 Real-Time System (Bio-Rad). Standard control reactions were performed without reverse transcriptase or without RNA template. For GoTaq reactions, cycling conditions were performed using the following sequence of steps: 1) 95°C for 3 min, 2) 95°C for 10 sec, 3) 65°C for 30 sec, 4) 72°C for 40 sec, with steps 2–4 repeated for 40 cycles. For Plexor reactions: 1) 95°C for 2 min, 2) 95°C for 5 sec, 3) 60°C for 35 sec, with steps 2–3 repeated for 40 cycles. In the case of Figure 2.4, GoTaq qRT-PCR was performed for 30 cycles and products were visualized by 0.8% agarose gel electrophoresis. Each qPCR reaction was analyzed via a thermal melting curve and gave a single peak with the expected melting temperature. Amplification efficiencies of each primer set were optimized. Plexor primers were optimized at 100% for the Plexor qPCR protocol, while all other primers had efficiencies between 90-110% with 65°C elongation steps.

Cycle thresholds ($C_t$) were measured using CFX Manager software (Bio-Rad) for GoTaq reactions, while the raw data were imported into Plexor Analysis Desktop (Promega) for Plexor® reactions. Differences in mRNA levels were calculated using the

$\Delta\Delta C_t$ method. For analysis of RNAi depletion of endogenous mRNAs (i.e. Pumilio, Nanos, Brain Tumor, and 4EHP), $C_t$ values were measured and normalized to the internal control Rpl32 mRNA for each sample using the equation: $\Delta C_{t\ target\ RNAi} = C_{t\ target} - C_{t\ control}$. A normalized $\Delta C_{t\ control\ RNAi}$ was also calculated for each mRNA in the LacZ dsRNA treated samples. To measure relative changes in each mRNA level, $\Delta\Delta C_t$ was calculated for each gene as $\Delta\Delta C_t = \Delta C_{t\ target\ RNAi} - \Delta C_{t\ control\ RNAi}$. The fold change in mRNA level was then calculated as $2^{-\Delta\Delta Ct}$. For the measurement of reporter mRNA levels, the same method was used but the normalizations were calculated relative to the internal control Firefly mRNA (FF control). The $\Delta C_t$ for each sample was calculated as $\Delta C_t = C_{t\ \textit{Renilla}} - C_{t\ firefly}$. To measure changes in reporter mRNA levels induced by Pumilio or Nanos, $\Delta\Delta C_t$ was calculated as $\Delta C_{t\ WT} - \Delta C_{t\ mutant}$ where "mutant" refers to samples expressing RNA binding defective Pumilio (mutR7) or Nanos (C354Y), as indicated in the figure legends. The fold change was then calculated from $2^{-\Delta\Delta Ct}$. Relative changes in reporter ratios were represented as normalized to the mutant controls (set to 100). Percent repression values were derived using the equation: Percent Repression = 100x(1-fold change).

*qPCR primer sequences.*

**Firefly Luciferase Reporter**

Forward Primer: 5'-dGATCCTCAACGTGCAAAAGAAGC

Reverse Primer: 5'-d FAM-isoC-TCACGAAGGTGTACATGCTTTGG

***Renilla* Luciferase Reporter**

Forward Primer: 5'-d CAL Fluor Orange 560-isoC-CGCAACTACAACGCCTACCTTC

Reverse Primer: 5'-dCCCTCGACAATAGCGTTGGAAAA

**Rpl32**

Forward Primer: 5'-dGCCCAAGGGTATCGACAACAG

Reverse Primer: 5'-dGCACGTTGTGCACCAGGAAC

**Pumilio**

Forward Primer: 5’-dGCCTGATGACCGATGTCTTTGG

Reverse Primer: 5’-dCGATTTCCTGCTGCTGCTCC

**Nanos**

Forward Primer: 5’-dCTGGCTCGATGCAGGATGTG

Reverse Primer: 5’-dGTCTGCAGCTGGGCAGGATT

**Brain Tumor**

Forward Primer: 5’-dCAACTACAGACGGGCATTCAGG

Reverse Primer: 5’-dGCCCGAATGTAACCAAAGGTG

**4EHP**

Forward Primer – 5’-dCCAGCGTGCAGCAGTGGTGG

Reverse Primer – 5’-dCAAACGTTCTCCCAGGCCCG

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# CHAPTER 3

# MECHANISMS OF mRNA REPRESSION BY THE PUF RNA BINDING DOMAIN

Portions of the work presented in this chapter were originally published as:

"The RNA binding domain of Pum antagonizes poly-adenosine binding protein and accelerates deadenylation"

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## 3.1 Abstract

Pumilio and Fem-3 Binding Factor (PUF) proteins are potent repressors that serve important roles in stem cell maintenance, neurological processes, and embryonic development. These functions are driven by PUF protein recognition of specific binding sites within the 3' untranslated regions (UTRs) of target mRNAs. In this study, we investigated mechanisms of repression by the founding PUF, *Drosophila* Pumilio (Pum), and its human orthologs. Here we evaluated a previously proposed model wherein the Pum RNA Binding Domain (RBD) binds Argonaute which in turn blocks the translational activity of the eukaryotic elongation factor 1A (eEF1A). Surprisingly, we found that Argonautes are not necessary for repression elicited by *Drosophila* and human PUFs in cells. A second model proposed that the RBD of Pum represses by recruiting deadenylases to shorten the mRNA's poly-adenosine (poly(A)) tail. Indeed, the RBD

binds to the Pop2 deadenylase and accelerates deadenylation; however, this activity is not crucial for regulation. Rather, we determined that the poly(A) is necessary for repression by the RBD. Our results reveal that poly(A) dependent repression by the RBD requires the poly(A) binding protein, pAbp. Furthermore, we show that repression by the human PUM2 RBD requires the pAbp ortholog, PABPC1. Pum associates with pAbp but does not disrupt association of pAbp with the mRNA. Taken together, our data support a model wherein the Pum RBD antagonizes the ability of pAbp to promote translation. Thus the conserved function of the PUF RBD is to bind specific mRNAs, antagonize pAbp function and promote deadenylation.

## 3.2 Introduction

Protein expression is controlled at multiple levels including translation and mRNA stability (Garneau et al. 2007; Jackson et al. 2010; Schwanhausser et al. 2011). For example, the efficiency of mRNA translation is promoted by the 5' 7-methyl guanosine cap and the 3' poly(A) tail, which are respectively recognized by the eukaryotic initiation factor 4F complex, eIF4F, and the poly(A) binding protein, pAbp (Jackson et al. 2010). Enzymatic removal of the poly(A) tail (i.e. deadenylation) and 5' cap (i.e. decapping) can antagonize translation and initiate mRNA degradation (Goldstrohm and Wickens 2008; Li and Kiledjian 2010).

Translation and mRNA stability are controlled by interaction of trans-acting regulators with cis-acting RNA elements. A prototypical example of these regulators is the PUF family of proteins, named after founding members *Drosophila melanogaster* Pum and *Caenorhabditis elegans* Fem-3 Binding Factor (FBF) (Wickens et al. 2002). PUFs are present in all eukaryotes and share a conserved RNA binding domain (RBD) composed of eight repeated motifs. The RBD binds with high affinity and specificity to 8-10 nucleotide regulatory sequences that are predominantly found in 3'UTRs of mRNAs (Zamore et al. 1997; Zamore et al. 1999; Wang et al. 2002; Lu et al. 2009). PUF binding sites are prevalent in the transcriptome and hundreds of mRNAs copurify with individual PUFs (Gerber et al. 2004; Gerber et al. 2006; Galgano et al. 2008; Morris et al. 2008; Hafner et al. 2010). As a consequence, the impact of PUFs on gene expression is likely substantial. Analysis of the biological functions of PUFs supports this idea: they control

diverse functions including development, fertility, cell proliferation, and neurological processes (Lehmann and Nusslein-Volhard 1987; Lin and Spradling 1997; Zhang et al. 1997; Forbes and Lehmann 1998; Asaoka-Taguchi et al. 1999; Crittenden et al. 2002; Dubnau et al. 2003; Mee et al. 2004; Ye et al. 2004).

PUF proteins repress target mRNA expression by inhibiting translation and/or inducing mRNA degradation (Miller and Olivas 2011), but the mechanisms and cofactors involved remain to be fully elucidated. Results discussed in Chapter 2 revealed that human and *Drosophila* PUFs possess multiple domains that contribute to repression (Weidmann and Goldstrohm 2012). For all PUFs studied to date, the conserved RBD contributes to repression; therefore, we focused on dissecting the mechanism of repression by the RBDs of *Drosophila* Pum and human PUFs, PUM1 and PUM2. To do so, we used recently developed assays that specifically measure their ability to repress target mRNAs (Van Etten et al. 2012; Weidmann and Goldstrohm 2012).

Multiple mechanisms have been proposed to account for repression by the RBD. Initially, the repressive activity of the Pum RBD was thought to depend on two partners, Nanos and Brain Tumor; however, our results revealed that they are not essential for Pum mediated repression (Weidmann and Goldstrohm 2012). Early research in multiple organisms found that PUF repression correlated with shortening of the poly(A) tail of target mRNAs (Ahringer et al. 1992; Wreden et al. 1997; Olivas and Parker 2000; Chagnovich and Lehmann 2001). Subsequently, the RBD of PUFs from *S. cerevisiae, Drosophila, C. elegans*, and human were shown to bind orthologs of Pop2, a deadenylase enzyme that shortens the poly(A) tail of mRNAs in a 3' to 5' direction, indicating a conserved role for the RBD in deadenylase recruitment to target mRNAs (Goldstrohm et al. 2006; Kadyrova et al. 2007; Suh et al. 2009; Van Etten et al. 2012). In addition, Pop2 forms a heterodimer with another deadenylase, Ccr4, as a part of the Ccr4-Not deadenylase complex (Goldstrohm and Wickens 2008). These facts support a model in which the PUF RBD represses mRNAs by recruiting deadenylases to enhance the rate of poly(A) tail shortening. Consistent with this model, functional data from yeast and humans demonstrate that deadenylases contribute to the efficiency of PUF repression (Goldstrohm et al. 2006; Goldstrohm et al. 2007; Hook et al. 2007; Van Etten et al. 2012).

Because the poly(A) tail plays a pivotal role in translation through the action of pAbp (Kuhn and Wahle 2004; Jackson et al. 2010), PUF enhanced shortening of the poly(A) tail could reduce synthesis of the encoded protein and/or promote mRNA decay. In the research presented here, we measured the impact of deadenylases, the poly(A) tail, and pAbp in the mechanism of repression by Pum.

A recent study proposed, based on biochemical data, that PUF RBDs can inhibit translation by blocking polypeptide elongation (Friend et al. 2012). The RBD of the *C. elegans* PUF, FBF, was found to bind the CSR-1 protein, one of 27 nematode Argonaute orthologs (Wedeles et al. 2013). Together, FBF and CSR-1 were reported to interact with the translation elongation factor, eEF1A, and inhibit its GTPase activity, which is essential for translation. This mechanism may apply to the RBD of human PUMs as well (Friend et al. 2012). Like FBF, PUM2 bound to Argonaute orthologs and eEF1A and specific mutations of conserved phenylalanine and threonine residues were reported to disrupt PUM2 binding to eEF1A and Argonautes, respectively. *In vitro* translation assays using a rabbit reticulocyte extract provided functional evidence that the PUM2 RBD inhibits translation. Wild-type PUM2 RBD impeded translation whereas PUM2 RBD mutants, defective for binding to eEF1A or Argonautes, had no repressive effect. Given that the amino acids that mediate interaction with eEF1A and Argonautes are conserved in the RBDs of PUFs, this could be a conserved mechanism (Friend et al. 2012). In this report, we examine the role of Argonaute proteins in PUF repression *in vivo*.

In the present study, we scrutinized the mechanisms that underlie repression mediated by the RBDs of *Drosophila* and human PUFs. Our results indicate that the PUF-Argonaute interactions are not required for PUF mediated repression of protein expression. Instead, we find repression by the Pum RBD is completely dependent on the poly(A) tail. The RBD promotes deadenylation, dependent on the Pop2 and Ccr4 deadenylase enzymes, and interacts with Pop2. However, while blocking deadenylation stabilizes the mRNA, it does not prevent repression of protein synthesis. We find that the crucial mechanism of RBD mediated repression depends on the poly(A) binding protein, pAbp. Consistent with these observations, the Pum RBD associates with pAbp. Together our data support a mechanism wherein the Pum RBD targets pAbp to interfere with its

ability to promote translation. The RBD does not displace pAbp from the mRNA, but instead antagonizes its ability to promote translation. Upon triggering repression of protein synthesis, deadenylation occurs as a subsequent effect. Finally, our data reveal that the additional Pum repression domains inhibit protein expression by a pAbp and poly(A) independent mechanism.

## 3.3 Results

### 3.3.1 PUFs and Argonautes

#### 3.3.1.1 Mutations in the Argonaute and eEF1A binding motifs do not alter PUF Repression

We first tested the requirement of a PUF-Argonaute-eEF1A interaction using a mutational approach and cell-based reporter assays that we previously developed to measure PUF repression by *Drosophila* and human PUFs (Van Etten et al. 2012; Weidmann and Goldstrohm 2012). A PUF responsive reporter gene was created by inserting three copies of the Pum Response Element (PRE) into the 3'UTR of a *Renilla* luciferase gene to create RnLuc 3xPRE. Mutations within the RBD of *C. elegans* FBF and human PUM2 have been identified that abolish binding to Argonaute and eEF1A but do not affect RNA binding, as measured by *in vitro* binding assays (Friend et al. 2012). Alignment of *C. elegans*, human, *Drosophila*, and *S. cerevisiae* PUFs revealed that a threonine residue required for Argonaute-binding and the phenylalanine residue required for eEF1A-binding are conserved throughout PUFs (Fig. 3.1) (Friend et al. 2012). Interestingly, the Argonaute binding threonine is conserved in S*. cerevisiae* PUFs, though Argonautes are not present in this species (Meister 2013). Conservation of

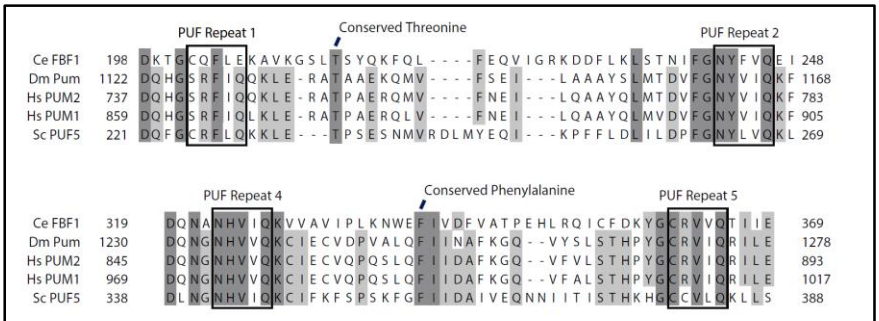


**Figure 3.1 - Conserved motifs within the PUF RBD.** Alignment of Argonaute and eEF1A binding regions of PUFs from *Caenorhabditis elegans* (Ce FBF1), *Drosophila melanogaster* (Dm Pum), *Homo sapiens* (Hs PUM1 and PUM2), and *Saccharomyces cerevisiae* (Sc PUF5), reported by Friend et al, 2012 (Friend et al., 2012). The conserved Argonaute and eEF1A binding amino acids are indicated at the top, and regions of complete and near complete conservation are highlighted in dark and light gray, respectively. RNA recognition motifs of each PUF repeat are also labeled.

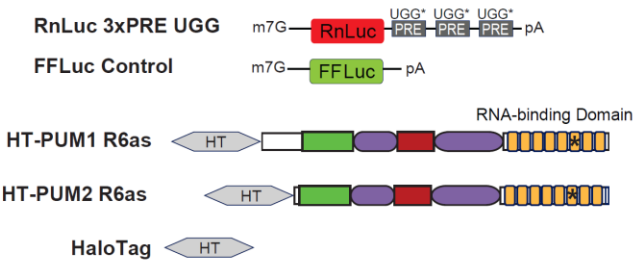


**Figure 3.2 - Human cell expression vectors.** Schematic of reporters and PUM constructs used in HEK293 cell experiments. UGG* denotes altered Pum Response elements (PREs) in the 3' untranslated region (UTR). FFLuc served as a control. Human PUM1 and PUM2 were expressed as Halotag (HT) fusions. The * in the RBDs signifies altered specificity in PUF repeat 6 (R6as) that confers binding to the UGG PRE. Colored boxes indicate conserved repression domains and regulatory motifs.

these residues in *Drosophila* and human PUFs indicated that our functional assay could be used to assess their roles in repression. We created mutant versions of human PUM1 and PUM2 that correspond to the previously identified mutations that disrupt binding to Argonautes (T874E of PUM1; T752E of PUM2) or eEF1A (F990R of PUM1; F866R of PUM2) and tested their ability to repress the *Renilla* luciferase reporter in human cells (Friend et al. 2012). Because endogenous PUM1 and PUM2 in HEK293 cells repress the RnLuc 3xPRE reporter, we modified the PRE sequences (Fig. 3.2, RnLuc 3xPRE UGG) so that only exogenously introduced PUF proteins with altered RNA binding specificity (Fig. 3.2, PUM1 R6as or PUM2 R6as) can regulate the reporter, as previously described (Van Etten et al. 2012). When expressed in human cells, the altered specificity PUM1 and PUM2 repressed the RnLuc 3xPRE UGG reporter by 62% and 64%, respectively, relative to the negative control, Halotag (Fig. 3.3). When mutations in the Argonaute or eEF1A binding sites were introduced into the altered specificity PUFs (PUM1 T874E or F990R; PUM2 T752E or F866R), their capacity for repression was not compromised (Fig. 3.3), indicating that these binding interfaces are not required for repression by human PUMs in living cells.

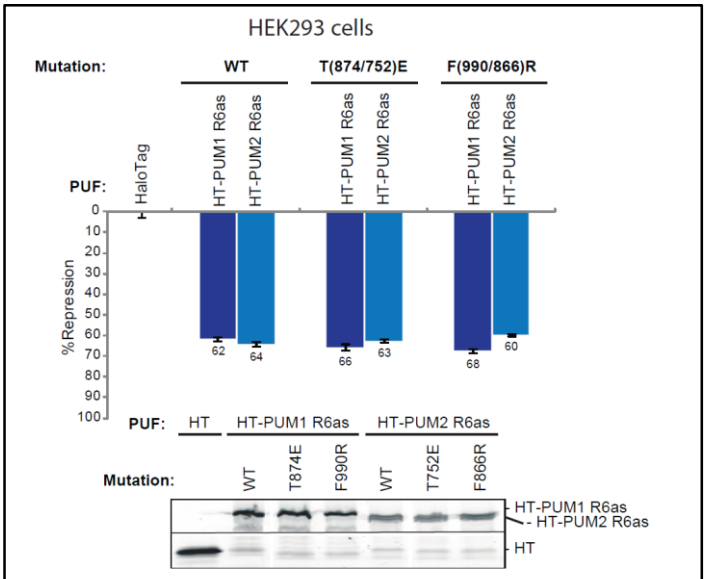


**Figure 3.3 - Repression by AGO and eEF1A binding mutants.** Percent repression in human HEK293 cells of the RnLuc 3xPRE UGG reporter by human PUM1 R6as and PUM2 R6as test proteins. Expression of each test protein was verified by fluorescent detection of TMR-labeled Halotag fusions.

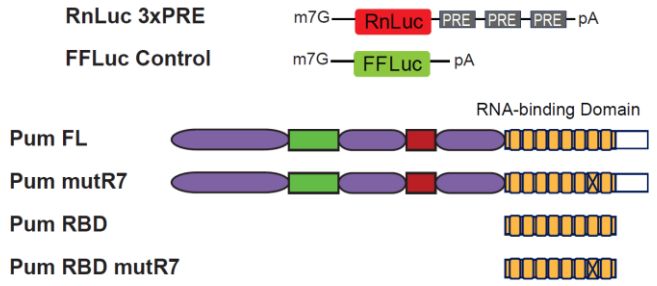


**Figure 3.4 - *Drosophila* cell expression vectors.** Representation of constructs used in D.mel-2 cell experiments. The Renilla reporter 3'UTR contains three wild-type PREs. Pumilio (Pum) constructs include full length (FL) and the RNA binding domain (RBD). Colored boxes indicate conserved repression domains and regulatory motifs. The X in negative control Pum mutR7 constructs represents mutations that block RNA binding and repression.

We next assessed the role of Argonaute and eEF1A binding residues in repression by *Drosophila* Pum. The level of endogenous Pum in *Drosophila* D.mel-2 cells is insufficient to efficiently repress RnLuc 3xPRE (Fig. 3.4); yet, moderate over-expression of Pum causes repression (Van Etten et al. 2012; Weidmann and Goldstrohm 2012). This system provides an excellent means of studying Pum structure and function. Full-length *Drosophila* Pum (Fig. 3.4, Pum FL) potently repressed the PRE-bearing reporter, whereas the Pum RBD repressed to a lesser degree (Weidmann and Goldstrohm 2012). Mutations that inactivate Pum RNA binding activity (Fig. 3.4, Pum mutR7), or change the PRE, completely blocked repression (Weidmann and Goldstrohm 2012). Using this approach, we tested the activity of Pum with mutations in the predicted Argonaute (T1137E) and eEF1A (F1251R) binding residues by measuring repression of RnLuc 3xPRE (Fig. 3.4). Full-length Pum T1137E retained the repression activity of wild-type Pum (Fig. 3.5, Pum FL WT at 73% repression vs. Pum FL T1137E at 74% repression) and Pum F1251R repressed at slightly below wild-type level (Fig. 3.5, Pum FL F1251R, 61% repression). We considered that the repressive activity of Pum's RBD might be obscured by additional repression domains that we previously identified in the amino-terminus of the protein (Weidmann and

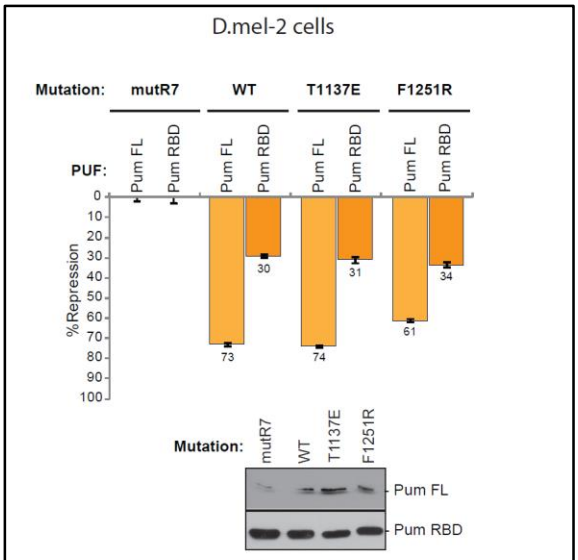


**Figure 3.5 - Pumilio AGO and eEF1A binding mutants.** Percent repression of RnLuc 3xPRE reporter by wild-type (WT), Argonaute-binding defective (T1137E), and eEF1A binding-defective (F1251R) Pum FL and Pum RBD test constructs in D.mel-2 cells. Western blot confirms expression of test constructs.

Goldstrohm 2012). Therefore, we tested the effect of these mutations on the activity of the RBD (Fig. 3.4). Wild-type Pum RBD, RBD T1137E, and RBD F1251R all repressed between 30% and 34% (Fig. 3.5). We conclude that these conserved residues are not necessary for repression by full length Pum or the conserved RBD.

### 3.3.1.2 Argonaute associates with human and *Drosophila* PUFs

The lack of detectable functional impact of mutation of the Argonaute binding residues of *Drosophila* and human PUFs compelled us to assess whether the mutations did indeed prevent this association. HEK293 cells were transfected with FLAG-tagged AGO1 and Halotag fusions of either wild- type or the T752E mutant PUM2. AGO1 was selected because it was reported to bind strongly (Friend et al., 2012). Halotag alone was used as a negative control and a Halotag fusion of CNOT6L, a deadenylase known to associate with Argonaute, served as a positive control (Fabian et al. 2009). Complexes were purified using a FLAG antibody from RNase-treated cell extracts. PUM2 and CNOT6L co-immunoprecipitated with FLAG-AGO1 but were not detected in the mock eluates (Fig. 3.6, compare lanes 14 and 16 to lanes 10 and 12). These findings are consistent with biochemical data of Friend et al (2012) but, surprisingly, we detect robust association between the PUM2 T752E mutant and AGO1 (Fig. 3.6, lane 15).

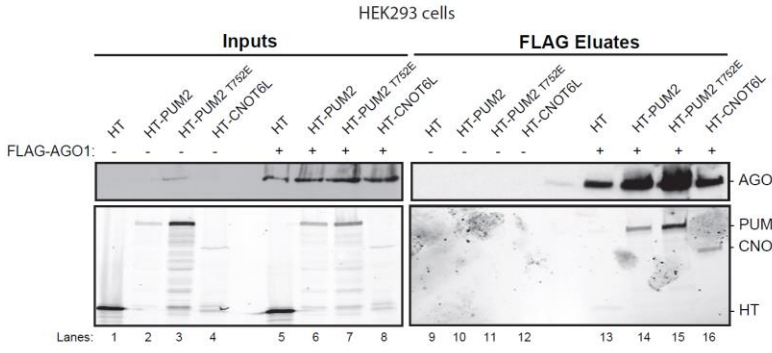


**Figure 3.6 - Co-IP of PUM2 and AGO1.** Co-immunoprecipitation of PUM2 with Argonaute 1 (AGO1) from RNase treated HEK293 cell extracts. Western blotting detected FLAG-tagged AGO1 in the inputs and eluates from anti-FLAG immunoprecipitations. Halotag (HT) or Halotag fused to wild-type or mutant (T752E) PUM2 or CNOT6L were detected by covalent labelling of HT with fluorescent TMR ligand. As a negative control, FLAG immunoprecipations were performed from extracts that expressed Halotag prey proteins but not FLAG-AGO1.

We also assessed binding of *Drosophila* Pum to Argonaute and the effect of the equivalent T1137E mutation. V5 epitope-tagged Pum RBD, fused to Halotag, was purified from RNase-treated D.mel-2 cell extracts that co-expressed FLAG-tagged Ago2 and the negative control protein, myc-tagged Lsm11. After washing, bound

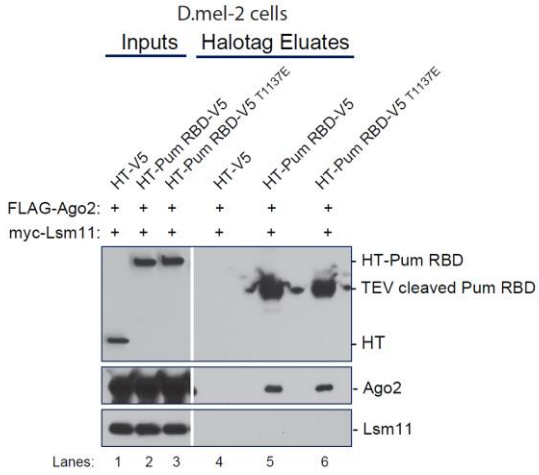


**Figure 3.7 - Pulldown of Pum RBD and Ago2.** *Drosophila* Argonaute 2 (Ago2) associates with the RNA binding domain (RBD) of Pumilio. HT alone or HT-Pum RBD fusions were co-expressed in D.mel-2 cells with FLAG-tagged Ago2 or, as a negative control, myc-tagged Lsm11. Halotag pulldowns were performed from cell extracts and bound proteins were eluted via TEV protease cleavage. Western blots of input cell extracts and Halotag eluates were probed to detect wild-type or mutant (T1137E) Pum RBD, Ago2 and Lsm11.

complexes were eluted by cleavage of the Halotag-Pum RBD fusion with TEV protease. Ago2 co-eluted with Pum RBD whereas Lsm11 did not (Fig. 3.7, lane 5). This was true for *Drosophila* Ago2, but no interaction between Pum and Ago1 was detected (data not shown). This result provides evidence that Drosophila Pum, like *C. elegans* FBF and human PUM2, associates with Argonaute. However, the Pum T1137E mutation did not disrupt binding of Pum to Ago2 (Fig. 3.7, lane 6). Further, an alanine substitution, T1137A, also possessed wild-type repression activity and bound Ago2 (data not shown). In summary, our data show that mutations reported to abrogate PUF interaction with Argonaute do not effect repression or Argonaute association.

### 3.3.1.3 The repression and Argonaute binding activities of the Pumilio RNA binding domain can be separated

Because mutation of the residues purported to mediate PUF binding to Argonaute did not in fact prevent the interaction, we sought an alternative way to assess the functional relevance to Pum repression. The tethered function assay provided an ideal means to dissect the regions of the RBD necessary for Argonaute binding and repression (Coller and Wickens 2002). In this assay, regions of Pum were fused to the phage protein MS2 which binds specific RNA stem-loop structures in the 3'UTR of a *Renilla* reporter gene (Fig. 3.8, RnLuc MS2). The regions of

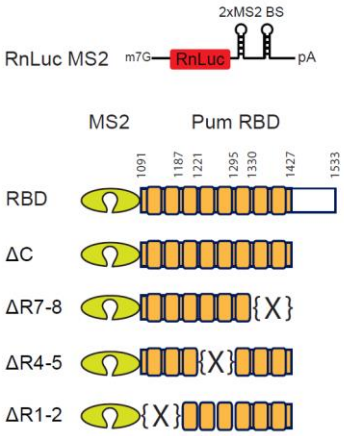


**Figure 3.8 - Tethered function constructs.** Diagrams of the tethered function reporter and Pum RBD constructs. The RBD and truncations were fused to the MS2 RNA binding protein. Amino acid numbers where deletions were made are indicated.

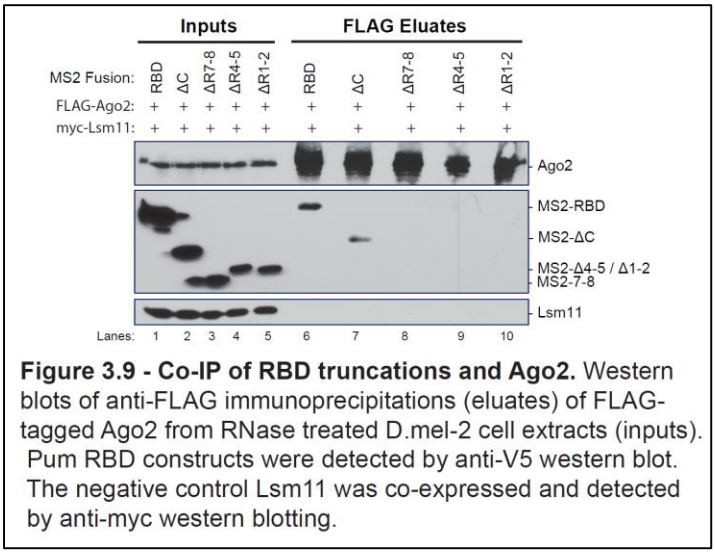


**Figure 3.9 - Co-IP of RBD truncations and Ago2.** Western blots of anti-FLAG immunoprecipitations (eluates) of FLAG-tagged Ago2 from RNase treated D.mel-2 cell extracts (inputs). Pum RBD constructs were detected by anti-V5 western blot. The negative control Lsm11 was co-expressed and detected by anti-myc western blotting.

the RBD necessary for association with Ago2 and for repression of RnLuc MS2 were mapped via a series of truncations with C-terminal V5 epitope tags (Fig. 3.8). These proteins were expressed in D.mel-2 cells with FLAG-tagged Ago2. Anti-FLAG immunoprecipitations were then performed from RNase-treated cell extracts. The RBD and a C-terminal truncation (ΔC) both copurified with FLAG-Ago2 (Fig. 3.9, lanes 6 and 7), but the ΔR1-2, ΔR4-5, ΔR7-8 deletions of the PUF repeats prevented the RBD-Ago2 interaction (Fig. 3.9, lanes 8-10), indicating that multiple PUF repeats are necessary to contact Ago2.

Having identified Pum truncations that no longer bind Ago2, we next tested their repressive activity in the tethered function assay (Fig. 3.10). We found that the RBD, ΔC and ΔR7-8 each repressed the RnLuc MS2 reporter; however, neither ΔR4-5 nor the ΔR1-2 were active (Fig. 3.10). Expression of each protein was verified by western blotting (Fig. 3.9, lanes 1-5). Most significantly, the tethered region lacking PUF repeats 7 and 8 (ΔR7-8) was active for repression though it did not bind Ago2 (Fig. 3.9 and 3.10). Based on this analysis, we can conclude that the interaction between Pum and Ago2 is dispensable for repression by the RBD.

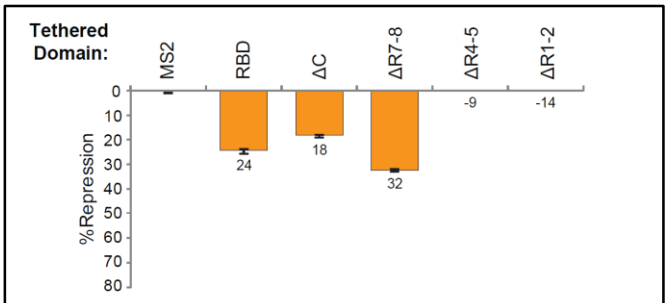


**Figure 3.10 - Tethered repression of RBD truncations.** Tethered function reporter assays measured repression by Pum RBD constructs in D.mel-2 cells. Percent repression, calculated relative to MS2 negative control, is graphed. Mean values derived from four replicates are plotted along with standard error.

### 3.3.1.4 Depletion of Argonaute proteins does not hinder PUF repression in cells

We further tested the requirement for Argonautes in repression by Pum by depleting Argonautes using RNA interference (RNAi). Efficient RNAi was demonstrated

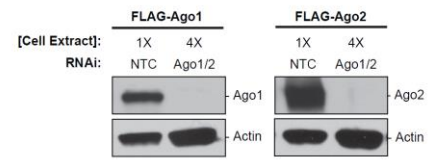


**Figure 3.11 - Argonaute RNAi.** Efficient depletion of FLAG-tagged Ago1 and Ago2 in D.mel-2 cells. Western blots were performed using anti-FLAG antibody and, as a loading control, anti-Actin. Non-targeting control (NTC) treated extracts served as a control. Four times the amount of Ago-depleted extracts were loaded to highlight knockdown efficiency.

by the depletion of over-expressed Ago1 and Ago2 proteins (Fig. 3.11). We also confirmed RNAi depletion of endogenous Argonautes by quantitative Reverse Transcriptase coupled with Polymerase Chain Reaction (qRT-PCR); Ago1 and Ago2 mRNA levels were depleted by up to 70% and 86%, respectively (Fig. 3.12). In cells treated with non-targeting control dsRNA (NTC), Pum FL repressed RnLuc 3xPRE mRNA by 70% and the Pum RBD repressed by 24% (Fig. 3.12, NTC), consistent with our previous findings (Weidmann and Goldstrohm 2012). Depletion of either Argonaute, Ago1 or Ago2, did not affect repression by Pum FL or the Pum RBD (Fig. 3.12, Ago 1, Ago2) nor did simultaneous knockdown of both Ago1 and Ago2 (Fig. 3.12, Ago1/Ago2). Therefore, depletion of Argonautes does not affect Pum repression in cells.

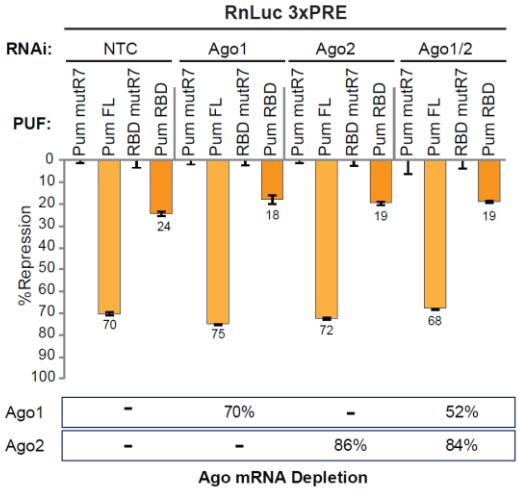


**Figure 3.12 - Pum repression with Ago RNAi.** Percent repression of RnLuc 3xPRE by Dm Pum FL or Dm Pum RBD was measured in D.mel-2 cells. RNAi was performed using dsRNAs to Ago1, Ago2, or both. NTC dsRNA served as a control. Percent repression by Pum FL or RBD was calculated relative to Pum FL mutR7 or RBD mutR7, respectively. RNAi depletion of endogenous Argonaute mRNAs were verified via qRT-PCR, and the percent depletions are indicated.

We also measured the effect of Argonaute depletion on the repressive activity of the human PUM1 in D.mel-2 cells. PUM1 achieved 53% repression in cells treated with the non-targeting control dsRNA (Fig. 3.13), consistent with our previous observation (Van Etten et al. 2012). Efficient depletion of Ago1, Ago2, or both did not alleviate PUM1 mediated repression (Fig. 3.13). Thus, like *Drosophila* Pum, human PUM1 is able to efficiently repress a PRE-containing mRNA when Argonautes are depleted. It is notable that, because eEF1A is an essential translation factor, it was not feasible to test its role in PUF repression using RNAi; however, because the eEF1A interaction with the RBD was Argonaute-dependent (Friend et al. 2012) and Argonaute is not necessary, this point is likely inconsequential.

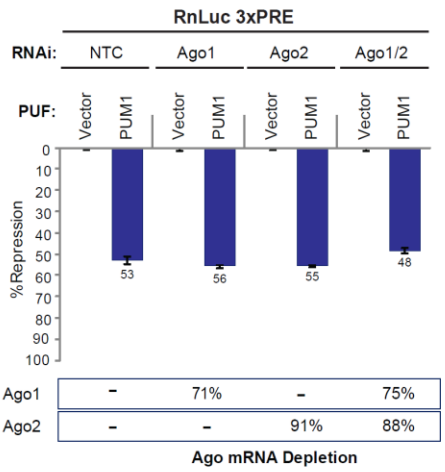


**Figure 3.13 - PUM1 repression with Ago RNAi.** Percent repression of RnLuc 3xPRE by human PUM1 was measured in D.mel-2 cells depleted of Ago1, Ago2, or both by RNAi. Repression by PUM1 was calculated relative to the negative control, empty expression vector. RNAi depletion of endogenous Argonaute mRNAs was verified by qRT-PCR.

Finally, we measured the effect of Argonaute depletion on the regulation of an mRNA bearing the 3'UTR from a natural Pum target. Well-characterized targets of Pum, such as the mRNA encoding the morphogen Hunchback, are not expressed in D.mel-2 cells; therefore, we appended the 3'UTR of the *Hunchback* mRNA to a *Renilla* luciferase reporter (Fig. 3.14, RnLuc Hb 3'UTR). To assess the effect of endogenous Pum and Argonaute on RnLuc Hb 3'UTR expression, we performed RNAi with non-targeting control dsRNA, or dsRNA targeting Pum, or dsRNA targeting both Ago1 and Ago2. When Pum was depleted, the RnLuc Hb 3'UTR reporter expression increased by about 2-fold (Fig. 3.14). In contrast, no effect on RnLuc Hb 3'UTR expression was detected when Argonautes were depleted (Fig. 3.14). We conclude that endogenous Pum represses the Hb 3'UTR reporter, but Argonautes are not required for this effect.

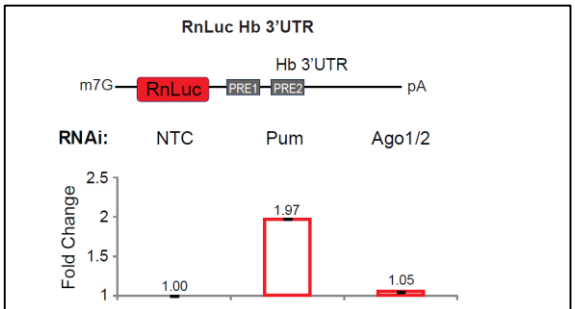


**Figure 3.14 - Hunchback reporter expression with Pum and Ago RNAi.** Fold change in expression of the RnLuc reporter with the Hb 3'UTR reporter was measured in D.mel-2 cells treated with NTC dsRNA, Pum dsRNA, or Ago1 and Ago2 dsRNAs. Pum Response Elements 1 and 2 (PRE1 and PRE2) within the Hb 3'UTR are binding sites for Pumilio. Reporter expression was normalized to a Firefly luciferase control and fold change was calculated relative to the NTC sample.

### 3.3.2 Mechanisms of repression by the PUF RNA binding domain

#### 3.3.2.1 The RBD of Pum enhances deadenylation dependent on the Pop2 and Ccr4 deadenylases

The data reported above demonstrate that interaction with Argonautes is not necessary for RBD mediated repression. Consequently, alternative mechanisms(s) must account for the observed PUF mediated repression *in vivo*. In several model systems

PUF repression correlates with shortening of the poly(A) tail of target mRNAs; therefore, we next measured the effect of the Pum RBD on deadenylation (Ahringer et al. 1992; Wreden et al. 1997; Olivas and Parker 2000; Chagnovich and Lehmann 2001; Gamberi et al. 2002; Goldstrohm et al. 2006). We investigated the ability of the wild-type and mutR7 Pum RBD to affect poly(A) tail length of the RnLuc 3xPRE reporter using a transcriptional shutoff strategy (Fig. 3.15). RNA samples were collected at specific time points following inhibition of synthesis with Actinomycin D. To measure poly(A) tail length, the 3' end of the RnLuc 3xPRE mRNA was liberated by RNase H cleavage with a specific antisense DNA oligonucleotide and detected by northern blotting. Inclusion of a poly-thymidine oligonucleotide in a control reaction removed the poly(A) tail, serving as a marker (Fig. 3.15, dT).

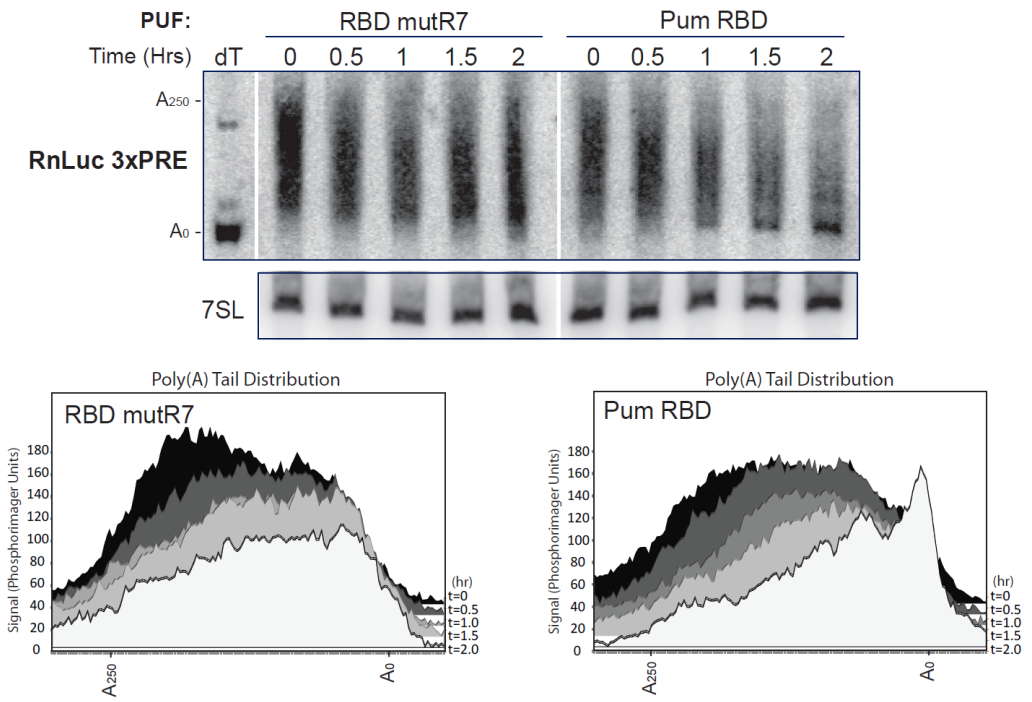


**Figure 3.15 - The Pum RBD accelerates deadenylation.** Above: Denaturing poly-acrylamide northern blotting of RNA prepared from D.mel-2 cells over a time course following addition of Actinomycin D. To resolve the poly(A) tail, the RnLuc 3xPRE mRNA was liberated with RNase H. A deoxy-Thymidine oligo (dT) was added to one reaction to remove the poly(A) tail (A0). Northern blot was probed to detect the RnLuc 3xPRE 3' cleavage product and the internal control 7SL, a stable non-coding RNA. Length of poly(A) tails are marked on the left. Below: Phosphorimager quantification of poly(A) length distribution for each lane from the Northern blot.

At the initial time point, a heterogeneous population of poly(A) tails were present, including new mRNAs with long poly(A) tails of up to 250 nucleotides in addition to intermediates at various stages of deadenylation (Fig. 3.15, time = 0). Over the two hour time course, we observed that deadenylation of RnLuc 3xPRE progressed relatively slowly in the presence of RBD mutR7 (Fig. 3.15). In contrast, when the wild-type Pum RBD was present, the poly(A) tail of RnLuc 3xPRE was more rapidly shortened, with the majority of RnLuc 3xPRE mRNA having almost no poly(A) tail after two hours (Fig. 3.15). In the last three time points (1, 1.5, 2 hours), deadenylated RnLuc 3xPRE intermediate accumulated (Fig. 3.15, Pum RBD). As an internal control, the levels of non-adenylated 7SL RNA were unchanged over the time course that samples were collected (Fig. 3.15, 7SL). These results demonstrate that the RBD of Pum is sufficient to accelerate deadenylation of the reporter mRNA.

In yeast, PUF mediated deadenylation depends on Pop2-Ccr4 deadenylase heterodimer, wherein the PUF directly interacts with the Pop2 subunit (Goldstrohm et al. 2006; Goldstrohm et al. 2007; Hook et al. 2007). This interaction is thought to be a conserved feature of PUF repression, because orthologs of PUFs and Pop2 from *C. elegans* and humans have been reported to bind each other (Goldstrohm et al. 2006; Suh et al. 2009; Van Etten et al. 2012). Furthermore, *in vitro* evidence indicates that *Drosophila* Pum binds to Pop2 (Kadyrova et al. 2007). We first asked whether RNAi depletion of Pop2 and Ccr4 (*Drosophila* Twin) would affect Pum RBD promoted deadenylation of the RnLuc 3xPRE mRNA. We confirmed depletion of epitope-tagged Pop2 and Ccr4 by western blotting (Fig. 3.16). Next, northern blotting was performed to measure the effect of deadenylase depletion on the poly(A) tail of RnLuc 3xPRE mRNA. In cells treated with non-targeting control RNAi, the reporter was deadenylated (Fig. 3.17, NTC); however, depletion of Pop2 and Ccr4 prevented the ability of Pum RBD to accelerate deadenylation (Fig. 3.17, Pop2 + Ccr4). In fact,

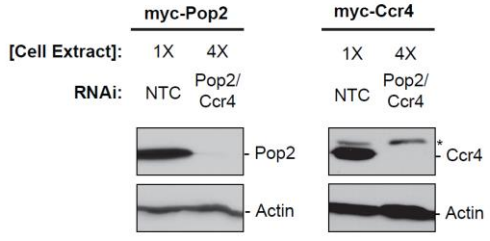


**Figure 3.16 - Deadenylase RNAi.** Western blotting of myc-tagged Pop2 and Ccr4 depletion by RNAi in D.mel-2 cells. Four times the amount of Pop2 and Ccr4 depleted extract was loaded relative to the NTC extracts to highlight knockdown efficiency. Myc antibody was used for Pop2 and Ccr4 detection. Western blot of Actin served as a loading standard. A nonspecific band was also detected by the myc antibody in all cell extracts (*).

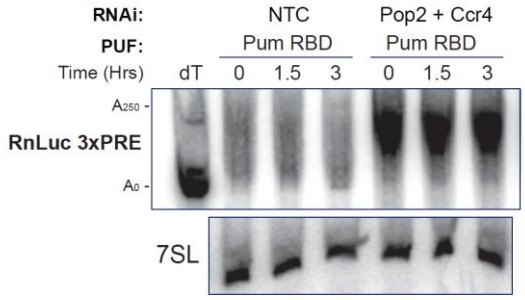


**Figure 3.17 - Reporter deadenylation is CCR4-NOT dependent.** Denaturing poly-acrylamide Northern blotting of RnLuc 3xPRE from D.mel-2 cells expressing Pum RBD and treated with either non-targeting control (NTC) dsRNA or both Pop2 and Ccr4 dsRNAs.

deadenylation was completely blocked: the RnLuc 3xPRE mRNA was stabilized with a long poly(A) tail throughout the three hour time course. These data indicate that the Pop2-Ccr4 deadenylase complex is necessary for Pum RBD mediated deadenylation of RnLuc 3xPRE.

Given the observations that deadenylation of the Pum reporter mRNA depends on Pop2-Ccr4 deadenylase, we wished to confirm that Pum binds to the Pop2 subunit of the deadenylase complex. To do so, the Pum RBD was fused to Halotag and the V5 epitope (HT-RBD-V5) and co-expressed in D.mel-2 cells with myc-tagged Pop2. As a negative control, Pop2 was also co-expressed with Halotag-V5 alone (HT-V5). Expression of HT-V5, HT- RBD-V5 and the myc-Pop2 protein was confirmed in the cell extracts (Fig. 3.18, Inputs). Halotag proteins were affinity purified from RNase treated extracts and the bound proteins were eluted with TEV protease and detected by western blotting. Pop2 substantially co-eluted with the Pum RBD (Fig. 3.18, HT-Pum RBD-V5), but was not detected in the Halotag control eluate (Fig. 3.18, HT-V5). As a negative control, we probed for Actin protein and found no enrichment by HT-RBD (Fig. 3.18). These results demonstrate that the Pum RBD associates with Pop2. The fact that the association was maintained in the presence of RNases indicates that RNA does not

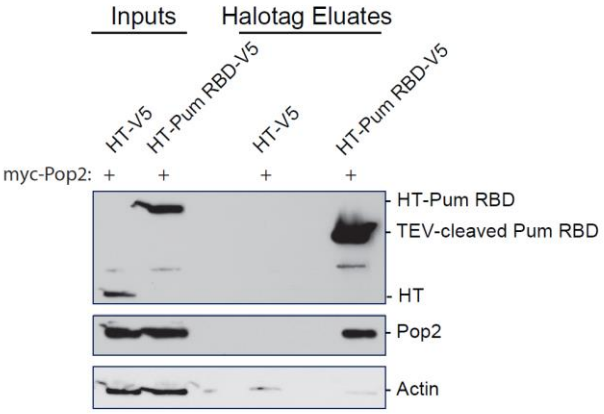


**Figure 3.18 - Pulldown of Pum RBD and Pop2.** Western blotting of Halotag pull-down assay demonstrating association of Pop2 with Pumilio RBD. V5-tagged Halotag (HT-V5) alone or Halotag-Pum RBD (HT-Pum RBD-V5) fusions were co-expressed with myc-tagged Pop2. Cell extracts (Inputs) were treated with RNases. HaloLink-bound complexes were eluted with TEV protease (Eluates). Blots were probed with V5 antibody to detect Pum RBD or with myc antibody to detect Pop2. Actin western blot served as a negative control.

mediate the interaction. Together with the data demonstrating that the RBD promotes deadenylation, the interaction with Pop2 suggests that the Pum RBD may recruit the deadenylase to the target mRNA to enhance deadenylation.

### 3.3.2.2 A poly(A) tail is necessary for repression by the Pum RNA binding domain

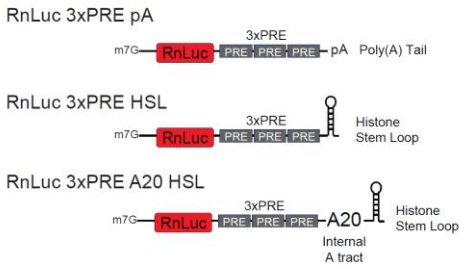


**Figure 3.19 - Histone stem loop reporters.** Diagrams represent Renilla luciferase (RnLuc) reporters with poly-adenylated or non-adenylated 3' ends. Reporters contain three PUF response elements (PREs). The RnLuc 3xPRE HSL reporter has a non-adenylated 3' end generated by Histone Stem Loop (HSL) processing elements. RnLuc 3xPRE A20 HSL has an internal poly-adenosine tract of 20 nucleotides (A20) between the PREs and HSL.

The observation that the RBD of Pum accelerated deadenylation indicated that poly(A) dependent regulation may be the mechanism by which it represses protein expression. If so, then repression should depend on the presence of a poly(A) tail. To test this idea, we compared Pum mediated repression of a reporter bearing a poly(A) tail to one that lacks poly(A). To create a non-adenylated 3' end, the cleavage/polyadenylation elements of the RnLuc 3xPRE reporter were replaced by a Histone Stem Loop (HSL), which is processed by a unique 3' end formation pathway (Marzluff et al. 2008) (Fig. 3.19). Consistent with earlier observations, full-length Pum repressed the RnLuc 3xPRE pA reporter by 79% and the Pum RBD repressed by 19% (Fig. 3.20, 3xPRE pA: Pum FL and Pum RBD). In contrast, repression of the non-adenylated RnLuc 3xPRE HSL reporter by Pum FL was diminished to 36% (Fig. 3.20, 3xPRE HSL). Strikingly, the RBD was unable to repress an HSL reporter (Fig. 3.20, 3xPRE HSL: Pum RBD) and instead promoted expression – the basis of this effect is currently unknown. We conclude that the 3' poly(A) tail is necessary for repression by the Pum RBD. Consistent with this result,

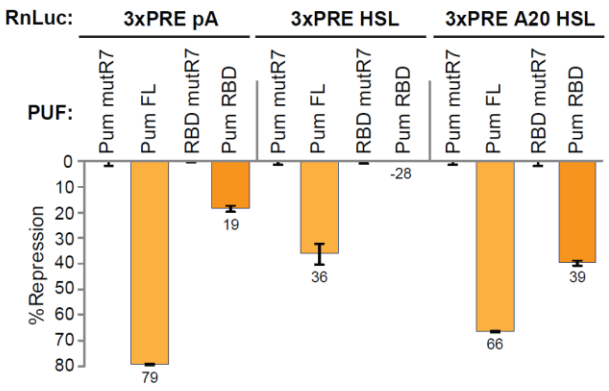


**Figure 3.20 - Pum repression of HSL reporters.** Percent repression of the RnLuc 3xPRE reporters by full length Pum FL and Pum RBD in D.mel-2 cells. Percent repression by Pum FL and RBD was calculated relative to Pum FL mutR7 and RBD mutR7, respectively.

repression by full-length Pum is reduced in the absence of a poly(A) tail, reflecting the loss of repression by its RBD. The remaining poly(A) independent repressive activity of full-length Pum likely emanates from the N-terminal repression domains (Weidmann and Goldstrohm 2012).

The requirement of the poly(A) tail for repression can be interpreted in several ways. Pum RBD could repress by promoting shortening of the poly(A) tail, thereby reducing translation and/or mRNA stability. Alternatively, Pum RBD could interfere with the function of poly(A) binding protein (pAbp), which coats the poly(A) tail and promotes translation initiation. To distinguish between these models, we created a new reporter mRNA with a non-adenylated HSL 3' end and an internal poly(A) tract (Fig. 3.19, RnLuc 3xPRE A20 HSL). The internal poly(A) tract is 20 nucleotides long, which is sufficient to bind at least one molecule of pAbp (Kuhn and Wahle 2004). Importantly, because deadenylases are 3' exoribonucleases that do not degrade internal poly(A) tracts (Goldstrohm and Wickens 2008), the RnLuc 3xPRE A20 HSL mRNA is not subject to deadenylation. Pum FL repressed the RnLuc 3xPRE A20 HSL mRNA by 66%, which is similar in magnitude to RnLuc 3xPRE pA with a normal 3' poly(A) tail (Fig. 3.20). Pum RBD repressed RnLuc 3xPRE A20 HSL by 39%, demonstrating that the internal poly(A) tract restored and strengthened repression (Fig. 3.20). We conclude that Pum RBD repression depends on the presence of poly(A). These results suggest that RBD mediated repression may require co-occupancy of the mRNA by Pum RBD and pAbp.

We also considered the possibility that poly(A) could promote repression by facilitating RNA-binding by Pum, perhaps mediated by pAbp. If so, then strengthening binding of Pum to the RNA should overcome the poly(A) requirement. Pum binds to the PRE with a dissociation constant in the low nanomolar range (Zamore et al. 1997; Zamore et al. 1999). To strengthen this interaction, we utilized a modified MS2 coat protein and binding site interaction with an order of magnitude stronger binding (Lim et al. 1994;

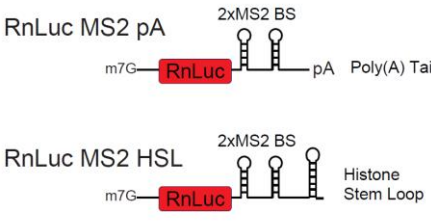


**Figure 3.21- MS2 HSL reporters.** RnLuc reporters with two MS2 protein binding sites were used for tethered function assays, including poly-adenylated RnLuc MS2 pA and non-adenylated RnLuc MS2 HSL.

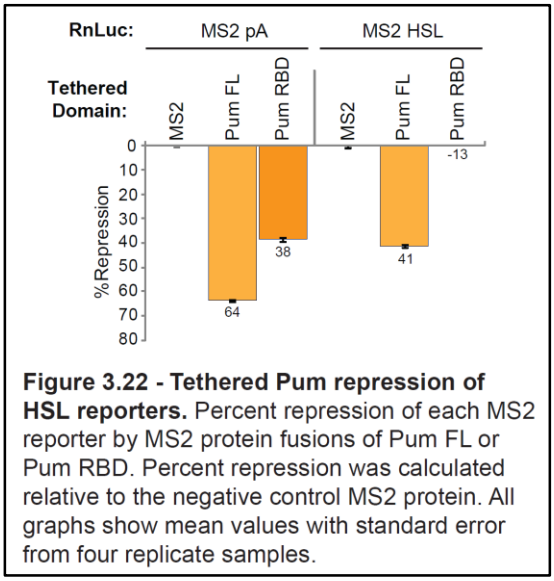


**Figure 3.22 - Tethered Pum repression of HSL reporters.** Percent repression of each MS2 reporter by MS2 protein fusions of Pum FL or Pum RBD. Percent repression was calculated relative to the negative control MS2 protein. All graphs show mean values with standard error from four replicate samples.

Johansson et al. 1998). Two reporters were used in this analysis: RnLuc MS2 pA, with a 3' poly(A) tail generated using efficient cleavage/poly-adenylation elements, and the RnLuc MS2 HSL reporter, with a non-adenylated 3' end generated by the HSL (Fig. 3.21). The results corroborate those described for the 3xPRE reporter. When tethered, Pum FL repressed the RnLuc MS2 HSL reporter less efficiently (41%) than the RnLuc MS2 pA reporter (64%) (Fig. 3.21). Importantly, tethered Pum RBD repressed the poly-adenylated reporter by 38% whereas repression of the HSL reporter was completely alleviated (Fig. 3.22) and, as observed for the 3xPRE HSL reporter, Pum RBD slightly enhanced expression of RnLuc MS2 HSL. We conclude that poly(A), in the form of a 3' tail or an internal poly(A) tract, is necessary for repression by the Pum RBD and contributes to the full magnitude of repression by the full-length Pum protein. Moreover, strengthening the association of Pum RBD with the mRNA did not lessen the poly(A) dependence, suggesting that poly(A) functions beyond facilitating the RBD-mRNA interaction.

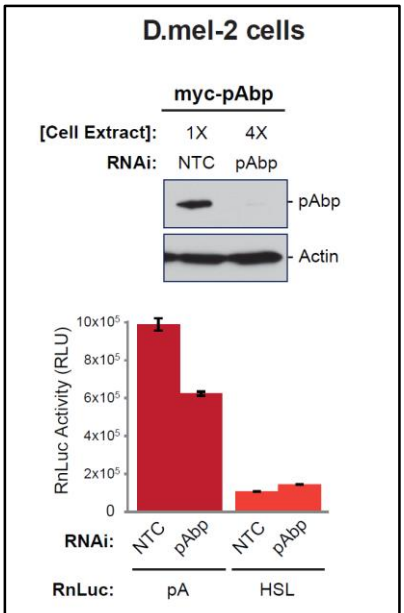


**Figure 3.23 - pAbp RNAi.** Top: western blot demonstrates efficient RNAi depletion of myc-tagged pAbp from D.mel-2 cells. Bottom: Relative Light Units (RLU) measured in non-targeting control or pAbp dsRNA treated cells. Reporters included RnLuc pA and RnLuc HSL.

### 3.3.2.3 Poly(A) binding protein is necessary for repression by *Drosophila* and human PUF RNA binding domains

Having found that poly(A) is necessary for repression by the Pum RBD, we wished to determine if this property is mediated by pAbp. To do so, we depleted pAbp using RNAi. Depletion of epitope-tagged pAbp protein was confirmed by western blotting (Fig. 3.23). In addition, using qRT-PCR, we measured an 84% decrease of endogenous pAbp mRNA relative to the negative control RNAi. Next, we

confirmed that depletion of pAbp reduced poly(A) stimulated translation. Indeed, poly-adenylated Renilla luciferase reporter expression was reduced by 40% relative to non-targeting control RNAi (Fig. 3.23, RnLuc pA), consistent with pAbp's general role in promoting translation. Importantly, reporter luciferase activity remained more than three orders of magnitude above background, permitting measurement of Pum activity in subsequent experiments. As an additional control, we measured the effect of pAbp depletion on a non-adenylated RnLuc HSL reporter. Knockdown of pAbp did not reduce luciferase activity (Fig. 3.23, RnLuc HSL), consistent with the fact that HSL translation does not utilize pAbp (Marzluff et al. 2008).

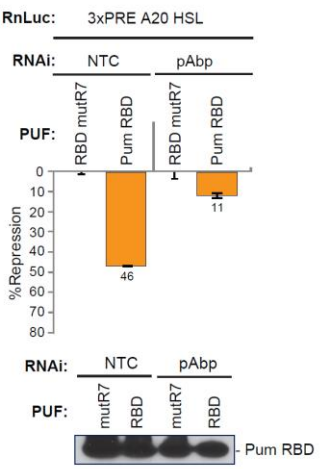


**Figure 3.24 - pAbp RNAi blocks RnLuc A20 HSL repression.** Percent repression of RnLuc 3xPRE A20 HSL reporter by the Pum RBD in D.mel-2 cells treated with dsRNA to pAbp or NTC dsRNA. Percent repression was calculated relative to RBD mutR7 negative control.

To evaluate the role of pAbp in Pum repression, we first took advantage of the ability of the Pum RBD to repress the RnLuc 3xPRE A20 HSL mRNA, which bears an internal A20 tract and a non-adenylated 3' end (Fig. 3.19). This reporter permits analysis of the effect of pAbp independent of potential interplay with deadenylation. As shown in Fig. 3.24, the Pum RBD repressed the RnLuc 3xPRE A20 HSL by 46% in the control sample. When pAbp was depleted, repression was substantially diminished to 11% (Fig. 3.24). This residual repression may result from incomplete pAbp depletion. We conclude that poly(A) tract dependent repression by Pum RBD requires pAbp.

We next evaluated the roles of Pop2 and Ccr4 deadenylases and pAbp by depleting each protein using RNAi and measuring the effect on repression of the RnLuc 3xPRE pA reporter. Simultaneous knockdown of Pop2 and Ccr4 did not prevent repression by full-length Pum, but the activity was reduced from 74% to 64% (Fig. 3.25). In contrast, depletion of Pop2 and Ccr4 did not alleviate repression by the RBD, indeed it was slightly enhanced (Fig. 3.25). Note that we confirmed efficient depletion of Pop2 and Ccr4 proteins by these dsRNAs, which blocked deadenylation (Fig. 3.16 and 3.17). We conclude that deadenylation is not required for Pum RBD mediated repression; despite

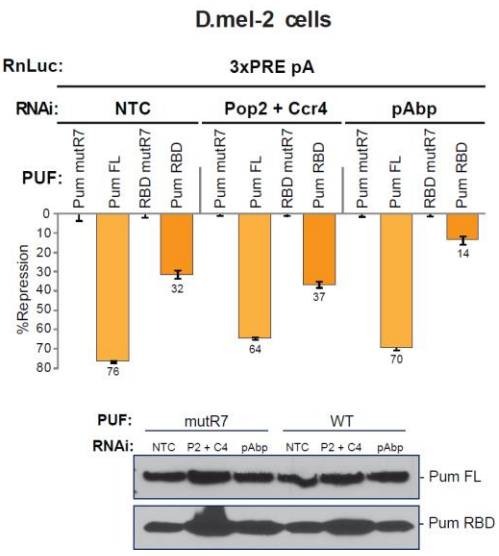


**Figure 3.25 - Pum RBD repression is pAbp dependent.** Percent repression of RnLuc 3xPRE pA reporter by Pum FL and Pum RBD in D.mel-2 cells treated with NTC, Pop2 and Ccr4, or pAbp dsRNAs. Percent repression was calculated relative to negative control Pum FL mutR7 and RBD mutR7.

the fact the RBD accelerates deadenylation dependent on Pop2 and Ccr4 deadenylases (Fig. 3.15 - 3.18).

When pAbp was knocked down, Pum RBD repression of RnLuc 3xPRE pA decreased from 32% to 14% (Fig. 3.25); therefore, pAbp plays an important role in RBD mediated repression of poly-adenylated mRNA, in agreement with the internal poly(A) tract data (Fig. 3.24). In contrast, Pum FL repression was largely unaffected (Fig. 3.25), highlighting a pAbp-independent function of N-terminal repression domains of Pum that we previously characterized (Weidmann and Goldstrohm 2012). It is important to note that the general effect of pAbp depletion (Fig. 3.23) did not prevent measurement of Pum mediated repression because the experimental design measures repression within each RNAi condition (e.g. repression by wild-type Pum RBD measured relative to the mutR7 negative control within pAbp depleted cells).

To further analyze the role of pAbp in Pum repression, we used the tethered function approach. When fused to MS2, Pum FL and Pum RBD repress the RnLuc MS2 pA reporter to a degree comparable to their effect on the RnLuc 3xPRE, 67% and 32%, respectively (Fig. 3.26), consistent with our previous analysis

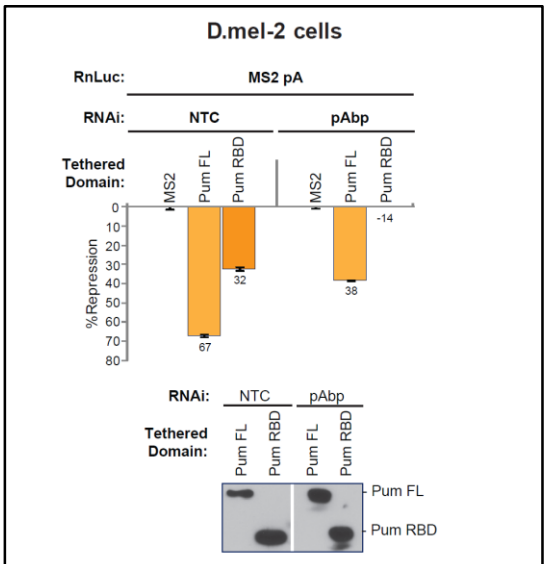


**Figure 3.26 - Tethered RBD repression is pAbp dependent.** Percent repression of RnLuc MS2 pA reporter by MS2 alone, a MS2-Pum FL fusion, and a MS2-Pum RBD fusion in D.mel-2 cells treated with NTC or pAbp dsRNAs. Percent repression by tethered constructs was calculated relative to MS2 alone. Western confirms expression.

(Weidmann and Goldstrohm 2012). Knockdown of pAbp reduced Pum FL repression from 67% to 38%, whereas repression by the Pum RBD was entirely lost (Fig. 3.26). In fact, without pAbp, the RBD slightly stimulated reporter protein expression (Fig. 3.26) to a degree similar to the RBD's effect on HSL reporters (Fig. 3.20 and 3.22). Western blotting confirmed that expression of tethered constructs was unaffected by pAbp knockdown (Fig. 3.26). Interestingly, repression by tethered Pum FL and RBD is more sensitive to pAbp depletion. The results confirm that repression by the Pum RBD depends on pAbp, whereas the N-terminal repression domains in full length Pum can repress independent of this cofactor.

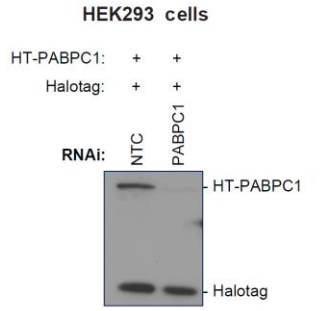


**Figure 3.27 - PABPC1 RNAi in human cells.** Western blotting, using anti-Halotag antibody, demonstrating efficient RNAi depletion of human PABPC1, fused to Halotag (HT-PABPC1), in HEK293 cells that co-expressed an internal control Halotag protein.

The finding that pAbp was necessary for repression by the RBD of *Drosophila* Pum suggested that pAbp may also be involved in repression by the conserved RBD of human PUFs. To test this idea, we performed RNAi to deplete the human pAbp ortholog, PABPC1, from HEK293 cells. Efficient knockdown of epitope-tagged PABPC1 was confirmed by western blotting (Fig. 3.27). A co-expressed Halotag protein, included as an internal control, was not affected by RNAi depletion of PABPC1 (Fig. 3.27). To specifically detect RBD mediated repression, we utilized the 3xPRE UGG reporter (Fig. 3.1) to measure repression activity of PUM2 RBD with altered RNA binding specificity, fused to Halotag (Fig. 3.28, HT-PUM2 RBD-R6as). A mutant version of the reporter, RnLuc 3xPREmt, wherein the PREs were mutated to eliminate PUM2 binding and repression (Van Etten et al. 2012), was included as a negative control. We observed that PUM2

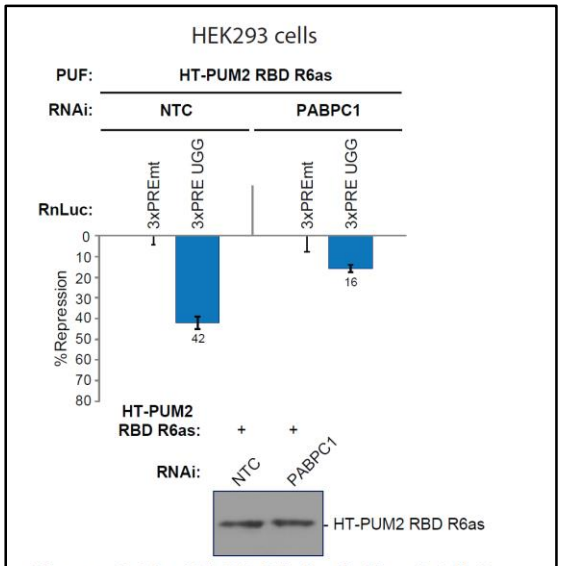


**Figure 3.28 - PABPC1 depletion inhibits PUM2 repression.** Percent repression of RnLuc 3xPRE UGG reporter by human PUM2 RBD R6as, with altered RNA binding specificity, in HEK293 cells treated with either non-targeting control (NTC) or PABPC1 siRNAs. Percent repression of the RnLuc 3xPRE UGG reporter was calculated relative to the negative control reporter, RnLuc 3xPREmt, wherein the PREs are inactivated by mutations.

RBD R6as repressed RnLuc 3xPRE UGG by 42% in cells transfected with non-targeting control siRNA (Fig. 3.28, NTC). In contrast, PABPC1 depletion substantially reduced PUM2 RBD R6as repression to 16% (Fig. 3.28). Importantly, PABPC1 depletion did not affect expression of HT-PUM2 RBD R6as (Fig. 3.28). We conclude that PABPC1 is required for repression by human PUM2 RBD. Taken together, our data demonstrate that poly(A) binding protein plays a conserved role in repression by the RBD of *Drosophila* and human PUFs.

### 3.3.2.4 The Pum RNA binding domain associates with pAbp

The functional connection between the RBD and pAbp prompted us to search for a physical association between these two proteins. To test this idea, we fused the Pum RBD to Halotag and a V5 epitope (HT-RBD-V5) and co-expressed this protein in D.mel-2 cells with either myc-tagged pAbp or, as a negative control, myc-tagged Lsm11. As a negative control, Lsm11 and pAbp were also co-expressed in cells with V5-tagged Halotag. Cell extracts were treated with RNases to degrade RNA that might bridge the two proteins. Halotag proteins were captured and bound complexes were eluted using TEV protease. Western blotting with anti-V5 antibody shows that HT-V5 and HT-RBD-V5 expressed efficiently in D.mel-2 cells, and an equivalent amount of RBD was cleaved off of the resin in both Lsm11 and pAbp samples (Fig. 3.29, lanes 6 and 8). pAbp copurified with HT-RBD-V5, but not HT control (Fig. 3.29, compare lane 8 to lane 7). The Lsm11 control protein did not associate with HT or HT-RBD-V5, demonstrating specificity (Fig. 3.29, lanes 5 and 6). These results

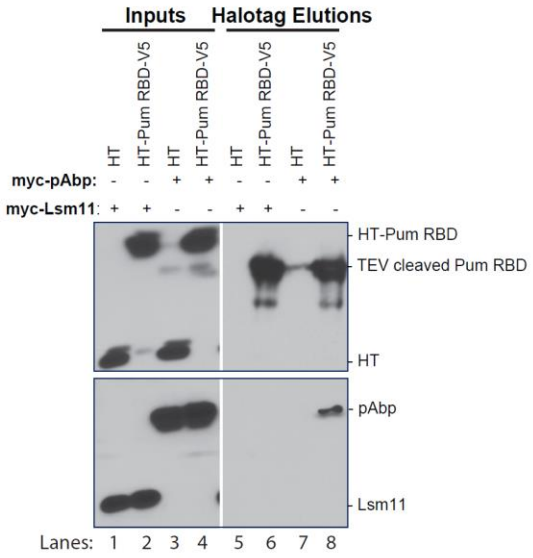


**Figure 3.29 - The Pum RBD associates with pAbp.** V5-tagged HT alone or HT-Pum RBD fusions (HT-Pum RBD-V5) were co-expressed in D.mel-2 cells with myc-pAbp or, as a negative control, myc-Lsm11. Cell extracts were treated with RNases and HT fusions were purified over a HaloLink resin. Bound proteins were eluted with TEV protease. Western blots were probed with V5 antibody for detection of Pum RBD and myc antibody for detection of prey proteins, Lsm11 or pAbp

reveal that the Pum RBD associates with pAbp independent of RNA, providing a physical link between the Pum RBD and its cofactor.

### 3.3.2.5 The Pum RNA binding domain does not displace pAbp from a target mRNA

Based on our results showing that pAbp is necessary for poly(A) dependent repression by the Pum RBD and that Pum interacts with pAbp, we hypothesized that Pum may promote repression by interfering with pAbp's ability to promote translation. One potential mechanism could be that Pum displaces pAbp from the poly(A) tail of a target mRNA. To test the hypothesis, we measured the effect of wild-type and mutant Pum RBD on the association of pAbp with the RnLuc 3xPRE mRNA. To do so, FLAG-tagged pAbp was immunoprecipitated, RNA was purified from the eluate, and pAbp-associated mRNA was then detected by northern blotting. We first validated this RNA co-immunoprecipitation assay by co-expressing FLAG-tagged pAbp with RnLuc reporters bearing different 3' ends including a normal poly(A) tail, an HSL, or an internal 20 Adenosine tract terminating in a HSL (A20 HSL). As negative controls, mock anti-FLAG immunoprecipitations were also performed from cells that expressed each reporter but not FLAG-pAbp (Fig. 3.30). Immunoprecipitation of FLAG-pAbp was confirmed by western blot (Fig. 3.30). As expected, poly-adenylated RnLuc mRNA was substantially enriched by pAbp, with 44-fold enrichment relative to the mock eluate (Fig. 3.30). Importantly, the HSL reporter was not enriched; however, the introduction of the internal Adenosine tract (A20 HSL) conferred pAbp enrichment (Fig. 3.30). This control

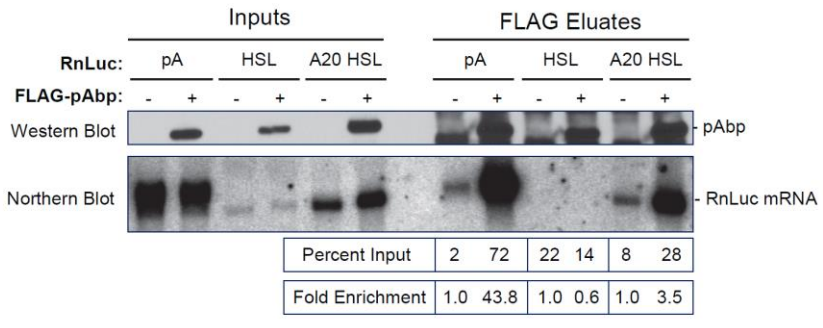


**Figure 3.30 - pAbp RNA immunoprecipitation.** Co-immunoprecipitation of poly-adenylated mRNA with pAbp from D.mel-2 cells. Western blotting comfirmed FLAG-tagged pAbp expression in inputs and in purified eluates. Northern blotting detected Renilla luciferase (RnLuc) reporter mRNAs in input and eluates, including RnLuc pA, RnLuc HSL, or RnLuc A20 HSL. Mock transfected cells served as controls. Renilla mRNA measured in each eluate was quantified as a percentage of the total RNA present in the input cell extracts. From these values, fold enrichment of each RNA in pAbp eluates was calculated relative to the corresponding mock transfection.

validates our ability to specifically enrich for pAbp associated mRNAs.

Using this RNA immunoprecipitation assay, we then tested whether the Pum RBD affects the association of pAbp with the PRE-containing reporter mRNA. Three replicate FLAG-pAbp immunoprecipitations were performed from cells that co-expressed either the RnLuc 3xPRE pA (Fig. 3.31) or the RnLuc 3xPRE A20 HSL (Fig. 3.32) mRNAs along with

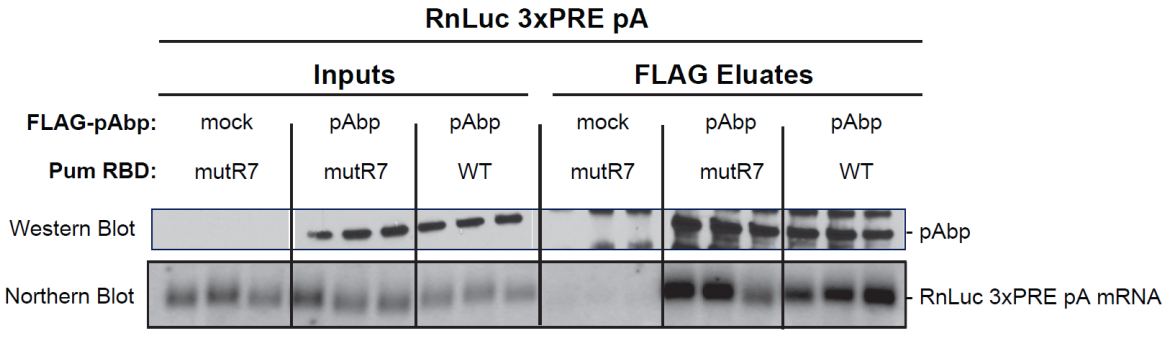


**Figure 3.31 - pAbp RNA IP with Pum RBD expression and RnLuc pA.** FLAG-pAbp immunoprecipitations performed from cells expressing wild-type (WT) or mutR7 Pum RBD with RnLuc 3xPRE pA reporter, as in Figure 3.30. Three replicates were performed. Expression and enrichment of pAbp in eluates was verified with western blotting using a FLAG antibody. Northern blotting was performed to detect RnLuc 3xPRE pA RNAs in the input cell extracts and eluates.

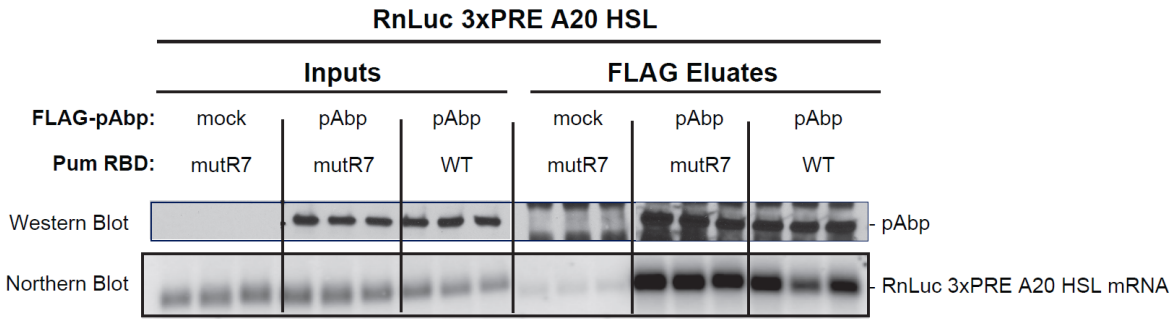


**Figure 3.32 - pAbp RNA IP with Pum RBD and RnLuc A20 HSL.** The same experiment as in Figure 3.31 except RnLuc 3xPRE A20 HSL reporter was used.

either wild-type or mutant Pum RBD. Inputs and FLAG eluates were assayed by northern blotting and FLAG-pAbp enrichment was confirmed via western blotting (Fig. 3.31 and 3.32). RnLuc 3xPRE pA and RnLuc 3xPRE A20 HSL mRNAs were enriched by 25-30 fold in the FLAG-pAbp eluates but not in mock eluates. We also performed luciferase assays on these samples to verify that RBD mediated repression is effective under these

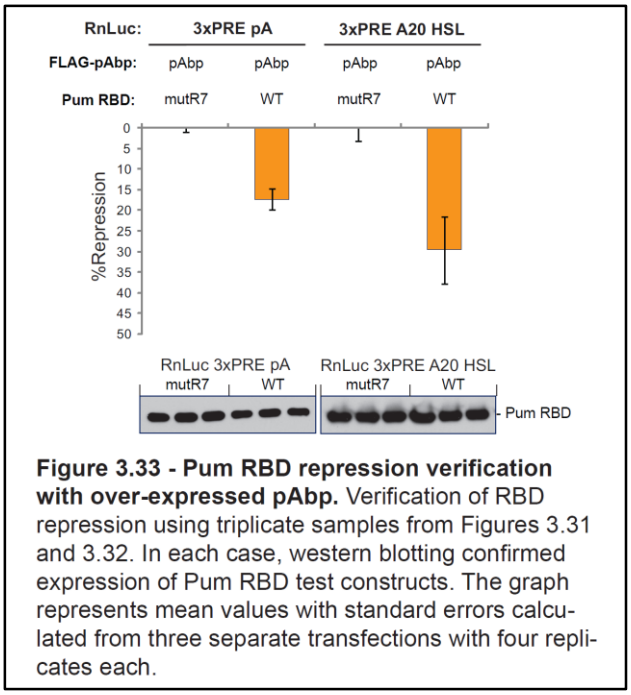


**Figure 3.33 - Pum RBD repression verification with over-expressed pAbp.** Verification of RBD repression using triplicate samples from Figures 3.31 and 3.32. In each case, western blotting confirmed expression of Pum RBD test constructs. The graph represents mean values with standard errors calculated from three separate transfections with four replicates each.

conditions (i.e. that expression of FLAG-pAbp does not alter repression) (Fig. 3.33). Consistent with the results in Figure 3.20, the RBD repressed the 3xPRE pA reporter by 17% and repression of the 3xPRE A20 HSL reporter was slightly enhanced at 29% (Fig. 3.33). Importantly, wild-type Pum RBD did not significantly change the level of enrichment of the RnLuc 3xPRE pA and RnLuc 3xPRE A20 HSL mRNAs in FLAG-pAbp eluates (Fig. 3.34). We conclude that the RBD does not displace pAbp from an mRNA to elicit repression. Instead, the data suggest that the RBD-pAbp interaction antagonizes the ability of the poly(A)-bound pAbp to enhance translation.

## 3.4 Discussion

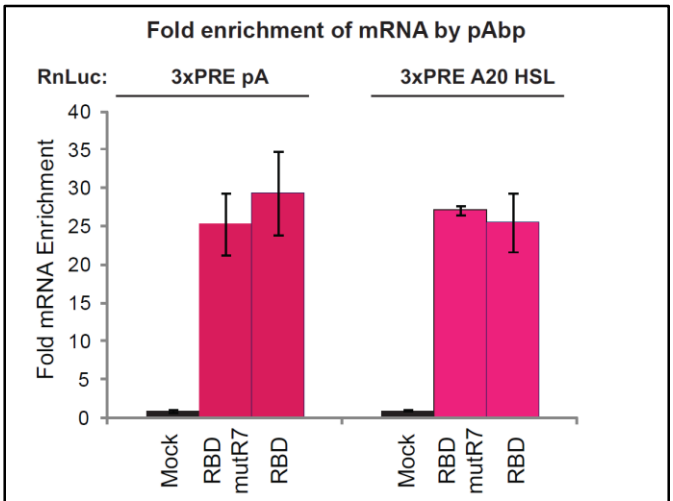


**Figure 3.34 - Pum RBD does not affect reporter enrichment by pAbp .** Fold enrichment of RnLuc 3xPRE pA or RnLuc 3xPRE A20 HSL by pAbp with co-expressed wild-type or mutR7 Pum RBD as quantified from the experiments in Figures 3.31 and 3.32. The graph represents mean values with standard errors calculated from three replicate samples.

*Drosophila* and human PUF proteins possess multiple domains that contribute to repression of protein expression from target mRNAs (Weidmann and Goldstrohm 2012). For example, *Drosophila* Pum has four repression domains that can function autonomously including the RBD and three repression domains located in the amino-terminus (Weidmann and Goldstrohm 2012). The current challenge is to dissect how each repression domain acts to inhibit translation and/or promote mRNA degradation. In this study, we focused on the

mechanism of repression elicited by the evolutionarily conserved RBD. First, we evaluated two mechanisms proposed to account for RBD mediated repression including inhibition of translation elongation by a PUF-Argonaute-eEF1A ternary complex and acceleration of deadenylation achieved via recruitment of Pop2-Ccr4 deadenylases.

Biochemical data led to a model wherein the PUF RBD forms a complex with Argonaute that in turn binds eEF1A (Friend et al. 2012). Our analysis represents the first evaluation of this model in *Drosophila* and human cells. Our results confirm that human PUMs associate with Argonaute and reveal that the PUF-Argonaute interaction is conserved by *Drosophila* Pum. However, we found that mutations in conserved sites reported to inactivate PUF-Argonaute binding *in vitro* did not eliminate the PUF-Argonaute association of *Drosophila* and human PUFs. Moreover, these mutations had no effect on PUF repression in cells. In these contexts, multiple PUF-Argonaute contacts, or other protein partners, may compliment the binding sites that were identified *in vitro.*

Our functional data indicate that the interaction of *Drosophila* and human PUFs with Argonautes is not essential for repression in cells. First, we identified truncations of the RBD that eliminated the association with Argonaute but retained repressive activity. Multiple regions outside of the reported Argonaute binding site were found to be necessary for the PUF-Argonaute interaction. However, when tethered to mRNA, an RBD truncation (deletion of PUF repeats 7 and 8) that did not associate with Argonaute was still fully active for repression. Second, RNAi depletion of Argonautes did not alleviate repression by *Drosophila* Pum or human PUMs, nor did mutations reported to prevent binding to eEF1A. These observations held true for target mRNAs with a minimal 3'UTR with PRE elements and for a target mRNA that contains the 3'UTR of the natural Pum target mRNA, *Hunchback*. Taken together, this evidence indicates that the PUF-Argonaute-eEF1A complex does not play an essential role in the mechanism of repression by *Drosophila* Pum or human PUFs. Notwithstanding, it remains possible that the PUF-Argonaute-eEF1A complex could contribute to regulation of particular target mRNAs in specific contexts. An intriguing possibility is that a PUF-Argonaute interaction could participate in combinatorial control of target mRNAs regulated by both PUFs and microRNAs. Interestingly, human and *Drosophila* PUMs were recently reported to

collaborate with microRNA mediated repression (Kedde et al. 2010; Miles et al. 2012). If true, we would anticipate that collaborative repression would be dependent on the major effector protein of microRNA mediated repression, GW182 (Tritschler et al. 2010). Notably, we depleted GW182 from *Drosophila* cells and saw no effect on repression by the Pum RBD (data not shown). Our interpretation of this result is qualified by the fact that the reporters used in the present study are not predicted to be regulated by microRNAs, and the observations by Friend et al. were reported to be microRNA independent (Friend et al. 2012). Thus, future studies are necessary to address whether the PUF-Argonaute interaction might participate in combinatorial control.

Multiple studies indicate that deadenylation plays a role in PUF RBD mediated repression. For example, PUF repression correlates with deadenylation of target mRNAs (Ahringer et al. 1992; Wreden et al. 1997; Olivas and Parker 2000; Goldstrohm et al. 2006). Our data and previous work demonstrate that the conserved RBD of yeast, *C. elegans*, *Drosophila*, and human PUFs interact with the Pop2 subunit of the Pop2-Ccr4 deadenylase complex (Goldstrohm et al. 2006; Hook et al. 2007; Kadyrova et al. 2007; Suh et al. 2009; Van Etten et al. 2012). In this study, we showed that Pop2 copurifies with the RBD of Pum. Genetic analysis in yeast demonstrated that PUF mediated deadenylation depends on Pop2 and Ccr4, and the yeast Puf4 protein requires Pop2 and Ccr4 to repress protein expression (Goldstrohm et al. 2006; Goldstrohm et al. 2007; Hook et al. 2007). PUF regulated deadenylation could be reconstituted with purified PUF RBD and Pop2-Ccr4 deadenylase complex (Goldstrohm et al. 2006; Goldstrohm et al. 2007; Hook et al. 2007). Furthermore, human PUFs interact with multiple isoforms of Pop2 and Ccr4 deadenylases and their ability to repress is diminished when deadenylation is blocked (Van Etten et al. 2012). These findings all supported a model in which the RBD of PUF proteins recruits the deadenylases to target mRNAs , thereby promoting poly(A) tail shortening. This effect results in diminished translational output and subsequently can lead to mRNA decay. Indeed, we show here that the Pum RBD promotes deadenylation and requires the poly(A) tail to repress. Consistent with this model, we showed that deadenylation of the Pum target mRNA was fully dependent on Pop2 and Ccr4 deadenylases.

Surprisingly, we found that repression by *Drosophila* Pum RBD persists when Pop2 and Ccr4 are depleted by RNAi. Depletion of the deadenylases caused only a minor reduction in repression by full-length Pum. Further, while poly(A) is necessary for Pum repression, this requirement can be fulfilled by an internal poly(A) tract that is not susceptible to deadenylation. Therefore, deadenylation is not a primary mechanism of repression by *Drosophila* Pum RBD. We hypothesize that deadenylation may be a secondary effect and may serve to increase efficiency or reinforce the regulatory switch by diverting the repressed mRNA to the decay pathway.

If deadenylation is not the primary mechanism, then what is the trigger of Pum RBD mediated repression? Our analysis revealed that repressive activity of the RBD is fully dependent on the presence of poly(A), whether at the 3' end or as an internal poly(A) tract, in the PRE containing target mRNA. This led us to test the role of the poly(A) binding protein, pAbp. Indeed, we found that pAbp was necessary for RBD mediated repression *in vivo*. We also identified a physical link between Pum and pAbp. Biochemical evidence further supports the importance of pAbp in PUF repression. Using a translationally active yeast extract, the pAbp ortholog, Pab1p, was shown to participate in translational inhibition by the RBD of yeast Puf5 (Chritton and Wickens 2011). In this same yeast extract, the RBD of *C. elegans* FBF also inhibited translation in a Pab1p dependent manner (Chritton and Wickens 2011). In addition, pAbp co-localizes with PUFs in ribonucleoprotein granules in rat neurons (Vessey et al. 2010). Together with our *in vivo* evidence, these findings suggest that the involvement of pAbp in repression by PUFs may be an evolutionarily conserved feature. In support of this, we found that RNAi depletion of PABPC1 reduced repression by human PUM2 RBD in HEK293 cells. Future *in vivo* analysis of the importance of pAbp orthologs in repression by other PUF proteins will be necessary to confirm this prediction.

Poly(A) binding protein enhances translation, making it an opportune target for negative regulators of protein expression (Tritschler et al. 2010; Brook et al. 2012). For instance, the translational inhibitor PAIP2 (poly-adenosine binding protein interaction protein 2), the sequence specific repressors Musashi, and the micro-RNA Induced Silencing Complex (miRISC) component GW182 have all been shown to bind pAbp

orthologs (Khaleghpour et al. 2001; Karim et al. 2006; Kawahara et al. 2008; Fabian et al. 2009). Binding of PAIP2, Musashi, or GW182 to pAbp disrupts its interaction with the 5’ cap-bound eIF4F complex, resulting in reduced translation efficiency (Khaleghpour et al. 2001; Karim et al. 2006; Duncan et al. 2009; Moretti et al. 2012; Zekri et al. 2013). Together with SXL, the RNA binding protein UNR also targets pAbp but reduces translation by an alternate mechanism; UNR interferes with ribosome recruitment by the assembled initiation factors (Duncan et al. 2009). PAIP2 and miRISC also repress by displacing PABPC1 from the poly(A) tail (Karim et al. 2006; Duncan et al. 2009; Moretti et al. 2012; Zekri et al. 2013). In the case of miRISC, displacement of PABPC1 is thought to lead to subsequent deadenylation, mediated by miRISC recruitment of the Ccr4-Pop2 deadenylase complex (Moretti et al. 2012; Zekri et al. 2013). Drawing on these examples, we consider potential mechanisms for pAbp dependent repression by Pum. Pum did not displace pAbp from the target mRNA, distinguishing the mechanism of repression from that of miRISC or PAIP2. Alternatively, Pum interaction with pAbp may interfere with pAbp binding to eIF4G. We note that our attempts to detect association of eIF4G with Pum RBD have been unsuccessful. Supporting this model, Chritton and Wickens showed that the Pab1-eIF4G interaction is required for repression by yeast Puf5 *in vitro* (Chritton and Wickens 2011). As a result, Pum would disrupt the “closed loop” contacts between 5’ cap bound eIF4F and poly(A) bound pAbp, resulting in diminished translation initiation. It is also possible that the pAbp-eIF4G interaction remains unaffected by the PUF-pAbp complex. In this scenario, Pum RBD would act like SXL-UNR, interacting with pAbp to block ribosome recruitment. Future detailed mechanistic analysis of Pum regulated translation will be necessary to distinguish these models.

While we focused on poly(A) dependent repression by the RBD in the present study, our data emphasize that additional mechanisms of PUF repression exist. Full-length *Drosophila* and human PUFs retain repressive activity, albeit reduced in magnitude, which is independent of poly(A), pAbp, and Pop2-Ccr4 (Chagnovich and Lehmann 2001; Van Etten et al. 2012). Our previous work showed that the Pum RBD is one of four repression domains in Pum and that the amino-terminus of fruit fly and human PUFs exhibit robust repressive activity (Weidmann and Goldstrohm 2012). These

observations argue that additional mechanisms of repression, elicited by the amino-terminal repression domains of Pum, PUM1, and PUM2, remain to be identified.

Based on conservation of the RBD throughout eukaryotes, it was originally suspected that members of the PUF family may repress by the same means (Wickens et al. 2002; Spassov and Jurecic 2003). Indeed, enhancement of mRNA decay by the RBD via recruitment of deadenylases is a conserved feature, though the contribution to the magnitude of repression appears to differ between PUFs in different organisms (Goldstrohm et al. 2006; Hook et al. 2007; Blewett and Goldstrohm 2012; Van Etten et al. 2012). While repression by *Drosophila* Pum is largely unaffected by depletion of deadenylases, repression by human PUM1 was substantially reduced by deadenylase depletion and by over-expression of a dominant negative deadenylase (Van Etten et al. 2012). Additional evidence comes from analysis of yeast PUFs. Both Puf4 and Puf5 accelerate deadenylation, and repression by Puf4 depends on Pop2 and Ccr4 (Goldstrohm et al. 2006; Hook et al. 2007; Blewett and Goldstrohm 2012). In contrast, while Puf5 does promote poly(A) shortening, it can circumvent deadenylation by recruiting the Eap1 protein to enhance decapping of the target mRNA (Blewett and Goldstrohm 2012). Thus, an emerging principle is that individual PUFs can have different corepressor requirements and dominant repressive mechanisms. Analysis of yeast Puf6 lends additional support as Puf6 was shown to uniquely target eIF5B to inhibit translation (Deng et al. 2008).

Beyond the RBD, PUF proteins from organisms such as yeast and *C. elegans* differ substantially at the amino acid level from those found in insects and vertebrates (Spassov and Jurecic 2003; Weidmann and Goldstrohm 2012). The divergent polypeptide sequences of different PUF proteins may confer unique regulatory functions. Further adding to the regulatory potential, PUFs from insects and vertebrates have evolved multiple repressive domains, each of which can act independently (Weidmann and Goldstrohm 2012). Thus, individual PUFs may assemble distinct regulatory complexes depending on the context *in vivo.* We anticipate that members of this ancient protein family have evolved strategies of regulation that remain to be revealed, with the pAbp and

poly(A) dependent mechanism of the RBD contributing to the maximal efficiency of repression.

## 3.5 Materials and Methods

**Plasmids.** Plasmids used in this study included pAc5.1 FFluc, pAc5.1 RnLuc, pAc5.1 RnLuc 3xPRE, pIZ Pum FL, pIZ Pum FL mutR7, pIZ Pum RBD, and pIZ Pum RBD mutR7, all of which were previously described in Chapter 2 (Weidmann and Goldstrohm 2012). The PRE sequences were derived from the natural Pum target mRNA, *Hunchback* (Murata and Wharton, 1995; Zamore et al., 1999; Zamore et al., 1997). The pAc5.1 RnLuc Hb 3'UTR reporter was created by inserting the 3' untranslated region of the *Drosophila Hunchback* mRNA (NM_169233.2 ) into the XhoI and NotI restriction sites downstream of Renilla luciferase coding sequence in pAc5.1 RnLuc vector. The Hb 3'UTR was amplified from *Drosophila* genomic DNA using the following primers (Hb sequence underlined, restriction sites in bold):

Hb 3'UTR Forward:
5'-GCAG**CTCGAG**GTTCCCCATCACCATCACCTTG

Hb 3'UTR Reverse:
5'-CACC**GCGGCCGC**AATTTGACTTTGGACTGTTGGTATTGTTTG

The pIZ PUM1 plasmid, expressing human PUM1, was previously described (Van Etten et al. 2012). Mutant versions of *Drosophila* Pum (NP_001262403.1), reported to inhibit binding to Argonaute or eEF1A (Friend et al. 2012) were created by site-directed mutagenesis with the following primers (mutations in bold):

Pum T1137E Forward:
5'-CCAACAGAAGTTGGAGCGGGCC**GAG**GCCGCCGAGAAGCAAATGG

Pum T1137E Reverse:
5'-CCATTTGCTTCTCGGCGGC**CTC**GGCCCGCTCCAACTTCTGTTGG

Pum F1251R Forward:
5’-GGACCCCGTGGCGCTGCAG**CG**CATCATCAATGCTTTCAAGGGTCAGG

Pum F1251R Reverse:
5’-CCTGACCCTTGAAAGCATTGATGATG**CG**CTGCAGCGCCACGGGGTCC

The psiCheck1-based RnLuc 3xPRE, RnLuc 3xPRE UGG, and RnLuc 3xPREmt reporter plasmids; the pGL4.13 FFLuc internal control; and the pFN21A-based expression vectors for Halotag, Halotag human CNOT6L, or Halotag versions of human PUM1 and PUM2 R6SYE derivatives were previously described (Van Etten et al. 2012). For pFN21A PUM2 RBD R6as, aa705-1050 of human PUM2 (NP_056132.1) was cloned into the flexi sites of pFN21A (Promega) and R6as (N921S, Q925E) was generated via site directed mutagenesis as in Van Etten et al., 2012. Human PUF mutants were created based on the mutations reported to abrogate binding to Argonautes or eEF1A (Friend et al. 2012), by site directed mutagenesis of PUM1 (NP_001018494.1) and PUM2 (NP_056132.1) using the following primers:

PUM1 T874E Forward:
5’-GCTCAAACTGGAGCGTGCC**GA**ACCAGCTGAGCGCCAGC

PUM1 T874E Reverse:
5’-GCTGGCGCTCAGCTGGT**TC**GGCACGCTCCAGTTTCAGC

PUM1 F990R Forward:
5’-GTGTACAGCCCCAGTCTTTGCAA**CG**TATCATCGATGCGTTTAAGGGACAGG

PUM1 F990R Reverse:
5’-CCTGTCCCTTAAACGCATCGATGATA**CG**TTGCAAAGACTGGGGCTGTACAC

PUM2 T752E Forward:

5’-CATACAGCAAAAACTAGAGAGAGCT**GAA**CCAGCTGAGCGACAGATGG

PUM2 T752E Reverse:
5’-CCATCTGTCGCTCAGCTGG**TTC**AGCTCTCTCTAGTTTTTGCTGTATG

PUM2 F866R Forward:
5’-GTGTTCAGCCACAGTCACTACAG**CG**CATCATTGATGCTTTCAAGGGACAAG

PUM2 F866R Reverse:
5’-CTTGTCCCTTGAAAGCATCAATGATG**CG**CTGTAGTGACTGTGGCTGAACAC

For FLAG immunoprecipitation in HEK293 cells, the coding sequence for human AGO1 (NP_036331.1) was inserted with an N-terminal 3xFLAG tag into the pF5A vector (Promega) to create pF5A N3xFLAG AGO1. pFN21A PABPC1 was generated by inserting the coding sequence of human pAbp, PABPC1 (NP_002559.2) into the flexi sites of pFN21A (Promega). For Halotag pulldown assays, the Halotag coding sequence from pFN18A (Promega), a C-terminal TEV cleavage site, and the Sgf1 restriction site were inserted into the pIZ V5-His A vector (Invitrogen) to create the HT control. *Drosophila* Pum was then inserted in frame and C-terminal to Halotag and a TEV protease cleavage site. For HT-RBD, containing amino acids 1091-1533, the N-terminus of Pum (aa1-1090) was deleted via inverse PCR. The pIZ myc-Lsm11 (NP_610522.1) vector and the empty pUB myc and pUB FLAG vectors (Ubiquitin 63E promoter, SV40 poly(A) site, and pUC19 backbone) were provided by Dr. Eric Wagner. pUB myc-pAbp was generated by inserting the coding sequence of *Drosophila* pAbp (NP_725750.1) into the pUB vector downstream of the myc tag, and pIZ myc-Pop2 and pIZ myc-Ccr4 were generated by inserting the coding sequences of *Drosophila* Pop2 (NP_648538.1) or *Drosophila* twin (NP_732966.1) with an N-terminal myc tag into pIZ. pUB FLAG-Ago1 and pUB FLAG-Ago2 were created by inserting the coding sequence of *Drosophila* Ago1 (NP_001246314.1) and *Drosophila* Ago2 (NP_730054.1) into the pUB FLAG vector downstream and in frame with the FLAG tag. For the tethered function assays, the pAc5.1 RnLuc 2xMS2 reporter, pIZ MS2-Pum FL, and pIZ MS2-RBD (aa1091-1533) was previously described in Chapter 2 (Weidmann

and Goldstrohm 2012). To create truncations of the Pum RBD, fused to MS2, inverse PCR was used with pIZ MS2-RBD to delete aa1427-1533 (pIZ MS2-RBDΔC) and subsequently aa1330-1426 (pIZ MS2-RBDΔR7-8), aa1222-1294 (pIZ MS2-RBDΔR4-5), or aa1091-1186 (pIZ MS2-RBDΔR1-2). For the HSL reporters, a histone stem loop (HSL) and a histone downstream element (HDE) were inserted in place of the SV40 cleavage/poly-adenylation element to create pAc5.1 RnLuc HSL, pAc5.1 RnLuc 3xPRE HSL, and pAc5.1 RnLuc MS2 HSL (Weidmann and Goldstrohm 2012). The sequence added is as follows, with HSL and Histone downstream element underlined: 5'-**GGTCCTTTTCAGGACC**ACAAACCAGATT**CAATGAGATAAAATTTTCTGTT**. Inverse PCR was performed to insert 20 Adenosines upstream of the HSL to create the pAc5.1 RnLuc A20 HSL and pAc5.1 RnLuc 3xPRE A20 HSL reporters.

**Cell Culture.** D.mel-2 cells (Invitrogen) were cultured in Sf-900 III serum-free medium (Invitrogen) with 50 Units/mL penicillin and 50 µg/mL streptomycin using standard cell culture techniques. Cells were grown at 28°C. HEK293 cells were cultured as previously described (Van Etten et al. 2012).

**Transfections.** Transfections were performed as previously described for D.mel-2 cells (Chapter 2, Weidmann and Goldstrohm 2012) and HEK293 cells (Van Etten et al. 2012). When transfections were performed for transcription shutoff experiments, Effectene (QIAGEN) reagents were scaled up to 20 mL total volume in a T-150 flask: 4.55 ng FFLuc, 9.1 ng RnLuc, 3636 ng pIZ vector, 818 µl EC buffer, 29.1 µl Enhancer, 36.36 µl Effectene, 14.6 mL D.mel-2 cells ($1.5x10^6$ cells/mL), and 5.4 mL Sf900III media. To inhibit transcription, Actinomycin D (Sigma) was added at 5 µg/mL final concentration, 48 hours post-transfection. Aliquots of cells at each indicated time point were removed, pelleted at 1000 x g for 3 minutes, and frozen at -80°C until RNA isolation. For experiments with RnLuc 3xPRE HSL and RnLuc 3xPRE A20 HSL reporters, 50 ng and 20 ng of the indicated reporter, respectively, was transfected per well of a 6-well plate, to ensure comparable levels of expression to pA reporters. To compare the effect of pAbp depletion on RnLuc pA and RnLuc HSL, 50ng of the indicated RnLuc plasmid and 400ng

of empty pIZ vector was transfected into 6-wells treated with either control or pAbp dsRNA.

In the siRNA experiment assessing PABPC1 knockdown efficiency, FuGENE HD (Promega) was used to transfect 80 ng pFN21A PABPC1 and 20 ng pFN21A Halotag control into 96-wells treated with siRNAs. In experiments testing the effect of siRNA-mediated depletion of PABPC1 on repression activity of HT-PUM2 RBD R6as, FuGENE HD was used to transfect 5 ng pGL4.13 FFLuc, 10 ng psiCheck1 RnLuc 3xPRE UGG or RnLuc 3xPRE ACA, and 85 ng pFN21A PUM2 RBD R6as into 20,000 cells in each well of a 96-well plate.

**RNA Interference.** As previously described, double-stranded RNAs (dsRNAs) were in vitro transcribed for RNAi including: non-targeting control (NTC) LacZ, PumN, Pop2, and Ccr4 (Van Etten et al. 2012; Weidmann and Goldstrohm 2012). The following primers were used to generate templates for production of Argonaute and pAbp dsRNAs, with T7 promoter sequence underlined and gene specific regions bolded:

AGO1 Forward:
5’-GGATCCTAATACGACTCACTATAGG**CCAATCACTTCCAGGTGACAATGC**

AGO1 Reverse:
5’-GGATCCTAATACGACTCACTATAGG**CCACTGCGAGGGCCTTACG**

AGO2 Forward:
5’-GGATCCTAATACGACTCACTATAGG**GGATGGAGCAACTCAGGTGGC**

AGO2 Reverse:
5’-GGATCCTAATACGACTCACTATAGG**GAGGAATAATCACAATTGCCAGATCG**

pAbp Forward:
5’-GGATCCTAATACGACTCACTATAGG**GCGTATGCAGCAGCTGGGACAG**

pAbp Reverse:
5'-GGATCCTAATACGACTCACTATAGG**CCTTGCAATTGCTGTGGAATTGGC**.

Corresponding regions were amplified via PCR from D.mel-2 cDNA and dsRNA was transcribed *in vitro* and purified as previously described (Van Etten et al. 2012; Weidmann and Goldstrohm 2012). For knockdown, cells in one well of a 6-well plate, with total volume 1.6 mL, was treated with 6 µg of each dsRNA for 5 minutes before transfection. For knockdown during transcription shutoff assays, 20 mL total volume was treated with 60 µg of each dsRNA for 5 minutes before transfection.

For RNAi in HEK293 cells, depletion of PABPC1 was performed through the use of On-target Plus Smartpool siRNA (L-019598-00) or a Non Targeting Control siRNA (Dharmacon). Twenty thousand HEK293 cells were plated per well of a 96 well plate in antibiotic free medium. 24 hours later the cells were transfected with 10 fmol of siRNA using Dharmafect 1 (Dharmacon) according to the manufacturer's protocol. Forty-eight hours after siRNA treatment, cells were transfected with reporters and expression vectors. Forty-eight hours post-transfection, luciferase assays to measure RnLuc and FFLuc activities were performed and cell lysates were prepared for western blot analysis.

**Alignments.** Alignments of the PUF RNA binding domain were performed using the open source bioinformatics software Jalview 2.8 (www.jalview.org) using the MafftWS alignment (Waterhouse et al. 2009).

**Luciferase Assays.** Luciferase assays were performed as previously described using dual glo assay (Promega) (Van Etten et al. 2012; Weidmann and Goldstrohm 2012). A relative response ratio (RRR), from RnLuc signal/FFLuc signal, is calculated for each sample. The ratio is normalized to the control (set to 100). Percent repression is derived from the equation $100*(1-(RRR_{WT}/RRR_{Negative\ Control}))$, where $RRR_{WT}$ is from a sample transfected with an active regulator and $RRR_{Negative\ Control}$ comes from a sample transfected with an equivalent amount of an inactive negative control. Inactive controls for Pum FL and Pum RBD were created by mutating the RNA recognition amino acids in the 7$^{th}$ PUF repeat, which prevents RNA binding and repression, to create pIZ Pum FL mutR7 and pIZ Pum RBD mutR7 plasmids (Weidmann and Goldstrohm 2012). Empty pIZ

vector was used as the inactive control for human PUM1 in *Drosophila* cells. The control for tethered function experiments was the MS2 expression vector, pIZ MS2 (Weidmann and Goldstrohm 2012). For the RnLuc Hb 3'UTR reporter, fold change reporter expression was calculated as $RRR_{RNAi}/RRR_{NTC}$, where $RRR_{RNAi}$ is from sample treated with targeting dsRNAs and $RRR_{NTC}$ is from sample treated with non-targeting control dsRNAs.

To measure repression by altered specificity human PUMs in HEK293 cells, the pFN21A Halotag expression vector served as a negative control and percent repression was calculated as previously described (Van Etten et al. 2012). For RNAi depletion of PABPC1 in HEK293, percent repression of RnLuc 3xPRE UGG reporter was calculated relative to the negative control reporter, RnLuc 3xPREmt, as previously described (Van Etten et al. 2012).

**Immunoprecipitation.** For FLAG immunoprecipitations from HEK293 cell, 3 mLs of 200,000 cells/mL were transfected. Mock samples were transfected using Fugene HD with 3 µg of Halotag prey plasmids (HT, HT-PUM2, HT-PUM2 T752E, and HT-CNOT6L), while FLAG-AGO1 samples were transfected with 750 ng pF5A FLAG-HsAGO1 bait and 2.25 µg of Halotag prey plasmids. Forty-eight hours post-transfection, cell pellets were resuspended in 500 µl lysis buffer containing 0.5% Igepal CA-630 (USB), 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 2mM $MgCl_2$, and 150 mM NaCl. Protease inhibitors were also added to final concentrations of 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 µg/ml aprotinin, 10 µg/ml pepstatin, and 10 µg/ml leupeptin. Lysates were passed through a 25 gauge needle 5 times and were then cleared at 16,000 x g for ten minutes at 4°C. Cleared lysates were treated with final concentrations of 20 U/mL RNase ONE (Promega), 8 µg/mL RNase A (Fermentas), and 500 nM HaloTag TMR ligand (Promega). A portion was kept as the input. The extract was then bound to 10 µl bed volume of EZview Red Anti-FLAG M2 affinity resin (Sigma) (equilibrated with lysis buffer and blocked for 30 minutes at 4°C with 500 µg/ml BSA). Binding proceeded for 12 hours at 4°C. Beads were washed 1 time in 1 ml lysis buffer and three times in lysis buffer lacking detergent with 500 mM NaCl. Beads were resuspended in 30 µl elution buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA, and 2 mM $MgCl_2$. To elute bound complexes, 3xFLAG peptide (Sigma) was added to 150 ng/µl. Elution proceeded with end-over-end rotation

for 30 minutes at 4°C. Eluates were separated from the resin using a Micro Bio-spin column (Bio-Rad).

For FLAG immunoprecipitations from D.mel-2 cells, 2 mLs of 1.5 million cells/mL were transfected (Effectene) with 300 ng of MS2-V5 prey plasmids (RBD, RBDΔC, RBDΔR7-8, RBDΔR4-5, RBDΔR1-2), 50 ng FLAG-Dm Ago2 bait and 50 ng myc-Lsm11 negative control. Immunoprecipitation proceeded similar to the above protocol with minor differences. Each cell pellet was lysed in 300 µL lysis buffer containing 0.5% Igepal CA-630 (USB), 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 150 mM NaCl. Protease inhibitors were also added to final concentrations of 2 mM phenylmethylsulfonyl fluoride [PMSF], 20 µg/ml aprotinin, 20 µg/ml pepstatin, and 20 µg/ml leupeptin. Lysates were treated with 50 U/mL RNase ONE and 10 µg/mL RNase A. After binding, the resin was washed 3 times in lysis buffer and 3 times in lysis buffer lacking detergent. Beads were eluted for 24 hours at 4°C in 50 µl elution buffer containing 50 mM Tris pH 8.0, 300 nM NaCl, 2 mM EDTA, and 300 ng/µl of 3xFLAG peptide.

**FLAG-pAbp RNA Immunoprecipitation.** The FLAG-pAbp RNA immunoprecipitations (RNA-IP) in D.mel-2 cells followed the FLAG immunoprecipitation described above with the following key differences. For control in Figure 9A, 2 mLs of D.mel-2 cells at 1.5 million cells/mL were transfected with 100 ng of RnLuc reporter plasmids (pA, HSL, A20 HSL) and 50 ng of either empty pUB vector or FLAG-pAbp. In the RNA-IPs with the Pum RBD, Reporter plasmids (20 ng RnLuc 3xPRE pA or 30 ng RnLuc 3xPRE A20 HSL) were co-transfected with 50 ng of either pUB vector or FLAG-pAbp and 350 ng of either RBD mutR7 or RBD plasmids. The RNA-IP lysis buffer consisted of 50 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, protease inhibitors, 0.2% Igepal CA-630 (USB), and 200 U/mL RNasin. In addition, beads were washed 1 time in lysis buffer and 3 times in lysis buffer with reduced detergent (0.01%). No elution with 3xFLAG peptide was performed; input lysates and IP pellets were directly subjected to Trizol RNA purification or SDS elution.

**Halotag Pull-down Assays.** For pull-down assays, 300 ng bait plasmid (pIZ Halotag alone or pIZ Halotag-Pum RBD aa1091-1533 was cotransfected with 100 ng prey

plasmid (pIZ myc-Lsm11, pIZ myc-Pop2, or pUB myc-pAbp) into 1 well of a 6-well plate for each bait-prey combination. For pull-down of Ago2 by HT-RBD, 300 ng of pIZ Halotag-PumRBD or pIZ Halotag-PumRBD T1137E bait, 50 ng of pUB FLAG-Ago2 prey, and 50 ng of pUB myc-Lsm11 control were cotransfected into one 6-well. Two milliliters of transfected cells were pelleted at 1000 x g for 3 minutes and washed twice in phosphate buffered saline (PBS). Pellets were resuspended in 300 µl lysis buffer containing 0.5% Igepal CA-630 (USB), 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 150 mM NaCl. Protease inhibitors were also added to final concentrations of 2 mM phenylmethylsulfonyl fluoride [PMSF], 20 µg/ml aprotinin, 20 µg/ml pepstatin, and 20 µg/ml leupeptin. Lysates were passed through a 25 gauge needle 5 times and incubated at 4°C for one hour. Lysates were then cleared at 16,000 x g for fifteen minutes at 4°C. A portion of the resulting supernatant was kept as the input. The extract was then bound to 20 µl bed volume of Halolink beads (Promega) (equilibrated with lysis buffer and blocked for 30 minutes at 4°C with 500 µg/ml BSA). During the binding, 100 units/ml RNase ONE (Promega) and 10 µg/ml RNase A was added to degrade RNA. Binding proceeded for 24 hours at 4°C. Beads were washed three times in 1 ml lysis buffer and three times in lysis buffer lacking detergent with increased NaCl (300 nM NaCl for Ago2, 750 nM NaCl for pAbp). Beads were resuspended in 30 µl elution buffer containing 50 mM Tris-HCl pH 8.0 and 300 mM NaCl. To cleave Halotag fusions, thereby eluting bound complexes, 5 units of AcTEV protease (Invitrogen) was added and incubated for 12 hours at 4°C. Eluates were separated from the Halolink beads using a Micro Bio-spin column (Bio-Rad).

**Western blotting.** Western blotting from luciferase assay samples was performed as previously described in Chapter 2 (Weidmann and Goldstrohm 2012). For Western blotting of Halotag pull-downs, input samples were diluted ten-fold in Elution Buffer and were separated along with TEV elutions via SDS-polyacrylamide (12%) gel electrophoresis and proteins were transferred onto Immobilon-P membranes (Millipore). All membranes were probed with either V5 monoclonal antibody (Invitrogen), c-myc (9E10) antibody (provided by Dr. Eric Wagner), anti-HaloTag monoclonal antibody (Promega), or monoclonal anti-FLAG M2 antibody (Sigma). Secondary detection was performed using horseradish peroxidase conjugated goat anti-mouse IgG (Thermo

Scientific). Signals were detected using Immobilon western chemiluminescent substrate (Millipore) and autoradiography film.

**Fluorescent labeling and visualization of Halotag protein constructs.** Protein extracts from HEK293 cells expressing Halotag fusions were harvested from each well of a 96-well plate in 20 µl of lysis buffer (0.5% Igepal CA-630 [USB], 50 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, 2 mM MgCl2, 150 mM NaCl) with 1 x Protease Inhibitor cocktail (Promega) and mixed with 900 nM Halotag TMR Ligand (Promega) for 30 min on ice, protected from light. For labeling of FLAG IPs, refer to FLAG IP methods. After labeling, extracts were separated via SDS polyacrylamide (12%) gel electrophoresis and detected by fluorescence imaging with a Typhoon Trio imager (GE Healthcare).

**RNA isolation.** For isolation of RNA, 4 ml of transfected D.mel-2 cells were centrifuged at 1,000 x g for 3 minutes, washed twice in PBS, and RNA was purified from cell pellets using Maxwell 16 LEV SimplyRNA tissue kit and the Maxwell 16 instrument (Promega). Total RNA preparations were utilized for Northern blotting or cDNA preparation. For pAbp RNA-IP experiments, Trizol reagent (Ambion) was used for RNA purification according to manufacturer's protocols.

**Northern analysis.** Northern blotting was performed as previously described (Blewett and Goldstrohm 2012). RNA was separated in a denaturing 0.85% agarose MOPS/formaldehyde gel. RNAs were transferred to Immobilon NY+ membrane (Millipore). Membranes were then UV-crosslinked and probed for the RNAs indicated. For RnLuc reporter, a $^{32}$P body-labeled, antisense RNA probe was created by in vitro transcription. The following primers were used to amplify templates for creation of RnLuc RNA probes. The T7 promoter sequence is underlined and gene specific regions are bolded.

RnLuc forward primer: 5'-**GCCCGTGGCTAGATGCATCATCC**

RnLuc reverse primer:

5’-GGATCCTAATACGACTCACTATAGG**GCGGACAATCTGGACGACGTCGG**.

For detection of RnLuc reporter for poly(A) tail analysis, primers included the

RnLuc 3’ forward primer: 5’-**GGGCGAGGTTAGACGGCCTACCCT**

RnLuc 3’ reverse primer:
5’-GGATCCTAATACGACTCACTATAGG**GCGGCCAGCGGCCTTGG**.

The 7SL RNA was detected on northern blots using a $^{32}$P 5’ end-labeled DNA oligo with the following sequence.
7SL Probe:
5’-CACCCCTGGCCCGGTTCATCCCTCCTTAGCCAACCTGAATGCCACGG.

Northern blots of poly(A) tail length, including RNase H cleavage, were performed as previously described (Blewett and Goldstrohm 2012). Total RNA, 20 µg, was annealed to a cleavage oligo specific to the RnLuc reporter. The oligo used for RNase H cleavage bore the sequence 5’-CCTTGAATGGCTCCAGGTA. Oligo dT cleaved control reactions contained 5 µg of oligo $dT_{15}$ (IDT). Two units of RNase H (NEB) were added to each reaction. RNAs were cleaved 1 hour at 37 °C, then precipitated with 0.3 M Sodium Acetate, and 3 volumes 100% ethanol for 1 hour at -20°C. RNAs were pelleted, washed with 70% ethanol, and resuspended in denaturing RNA loading buffer. RNAs were separated in 5% polyacrylamide, 7 M urea gels with 0.5 X Tris-Borate EDTA running buffer. Electrophoretic transfer to Immobilon NY+ was performed with TransBlot (Bio-Rad) for 1 hour at 60 Volts. Membranes were then probed as described above. Blots were visualized using a Typhoon Trio (GE) phosphorimager and quantitated using ImageQuant software (GE).

**cDNA preparation and qPCR.** For measurement of endogenous mRNA knockdown, RNAs were primed with random hexamers (IDT) for synthesis of cDNAs using GoScript reverse transcriptase (Promega). The final concentration of RNA in these reactions was 500 µg/mL. To measure endogenous mRNA levels, quantitative PCR

(qPCR) was performed on 5 µl of cDNA product in a 50 µl reaction using 100 nM of gene specific primers and GoTaq qPCR master mix (Promega) as described previously (Weidmann and Goldstrohm 2012). Standard negative control reactions were performed without reverse transcriptase. Differences in mRNA levels were calculated using the $\Delta\Delta C_T$ method. $C_T$ values were measured and normalized to the internal control Rpl32 mRNA to generate $\Delta C_T$. $\Delta\Delta C_T$ was derived relative to the non-targeting control $\Delta C_T$ (Livak and Schmittgen 2001; Schmittgen and Livak 2008). qPCR primers for Pop2 and Ccr4 were previously published (Van Etten et al. 2012). The qPCR primer sequences for additional *Drosophila* genes are as follows:

Rpl32 Forward: 5'-GCCCAAGGGTATCGACAACAG
Rpl32 Reverse: 5'-GCACGTTGTGCACCAGGAAC
Ago1 Forward: 5'-CCAGATGCGTCGCAAGTATCG
Ago1 Reverse: 5'-CGGGTAGCGCAATTTCATGC
Ago2 Forward: 5'-GTGAGCGACGGCCAGTTTCC
Ago2 Reverse: 5'-GAACTTGTTCGATGTCGTTACGTCG
pAbp Forward: 5'-CGTCGCTCGTTGGGCTATGC
pAbp Reverse: 5'-GCGACGAAGAGAAGGATCACGC

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# CHAPTER 4

# PUMILIO ENHANCEMENT BY NANOS

## 4.1 Abstract

*Drosophila* Pumilio and Nanos proteins function together to control diverse developmental processes, germline stem cell maintenance, and neurological functions including memory formation. Pumilio belongs to a conserved eukaryotic family of RNA binding proteins that bind with high affinity and specificity to Pumilio Response Elements (PREs), consequently inhibiting protein synthesis. Nanos belongs to a conserved family of tandem zinc finger proteins. Together they are implicated in mRNA localization, translational inhibition, and decay. We found that Nanos robustly stimulated Pumilio-mediated repression by two mechanisms. First, Nanos directly stimulates the RNA binding activity of Pumilio. Nanos binds to Pumilio and increases the affinity of Pumilio for PRE containing RNA. In fact, Nanos stimulates Pumilio binding to RNAs that are not normally bound by Pumilio. This effect is mirrored in cells; Nanos stimulates Pumilio-dependent repression of mRNAs bearing weak/degenerate PREs. Therefore, Nanos expands the repertoire of Pumilio target mRNAs. Second, we discovered that Nanos possesses a repression domain that synergistically promotes repression with Pumilio. The Nanos repression domain (NRD) can function independently of Pumilio when tethered to a reporter mRNA. Depletion of previously proposed corepressors Brain Tumor, Ccr4-Not deadenylase, or Cup do not affect NRD function. Together, these data reveal a new mechanism of Nanos repression that contributes to combinatorial control by Pumilio. We propose that Nanos augments the spatiotemporal control of mRNAs by

Pumilio; Nanos specifies how tightly Pumilio binds mRNA and tunes the level of repression.

## 4.2 Introduction

*Drosophila melanogaster* Pumilio (Pum) and *Caenorhabditis elegans* Fem-3 Binding Factor (FBF) are the founding members of the family of RNA binding proteins (RBPs) known as PUFs (Wickens et al. 2002). PUFs throughout eukaryotes each contain a series of eight repeated helical motifs that make up the conserved PUF RNA binding domain (RBD). Unique among RBPs, each PUF repeat specifies recognition of a single nucleotide and can act as part of a modular array (Wang et al. 2002; Miller et al. 2008; Wang et al. 2009). Working together, repeats of PUF RBDs bind tightly to specific 8-10 nucleotide regulatory sequences within mRNAs to promote post-transcriptional regulation of expression (Zamore et al. 1997; Wang et al. 2002; Miller and Olivas 2011). In this way, PUFs control integral processes like embryonic development, germline maintenance, and neurological function (Nusslein-Volhard et al. 1987; Zhang et al. 1997; Forbes and Lehmann 1998; Asaoka-Taguchi et al. 1999; Dubnau et al. 2003; Mee et al. 2004; Ye et al. 2004).

The sequence of PUF binding sites is largely conserved among eukaryotes. PUFs most similar to *Drosophila* Pum, like the human PUF proteins PUM1 and PUM2, associate strongly with the RNA sequence UGUAHAUA (Gerber et al. 2004; Gerber et al. 2006; Galgano et al. 2008; Morris et al. 2008; Hafner et al. 2010). Though Pum binds this sequence independently, regulation of targets *in vivo* requires the partner protein Nanos (Nos) (Barker et al. 1992; Forbes and Lehmann 1998; Muraro et al. 2008). This is true for multiple targets identified *in vivo*: including the regulation of the sodium channel Paralytic (Para) in the nervous system, the cell cycle regulator Cyclin B (CycB) in the germline, and the anterior morphogen Hunchback (Hb) in the early embryo. Nos was proposed to be necessary to recruit both the Brain Tumor (Brat) protein and the Ccr4-Not deadenylase complex to cause translational inhibition (Cho et al. 2006; Kadyrova et al. 2007). However, our group has demonstrated Nos independent repression in cells by multiple autonomous repression domains within Pum (Chapters 2 and 3) (Weidmann and

Goldstrohm 2012; Weidmann et al. 2014). While the addition of Nos could enhance Pum repression, the mechanism by which Nos does so is unclear.

Nos proteins contain tandem Zinc fingers (ZnF) that coordinate metal ions using a combination of cysteine and histidine amino acids (CCHC) (Curtis et al. 1997). Nos proteins are reported to bind RNA with low affinity and no specificity *in vitro* (Curtis et al. 1997; Hashimoto et al. 2010). Mutant *nos* embryos resemble *pum* mutants, lacking abdominal segmentation due to loss of Hb regulation**.** Expression of Nos proteins with mutations that abrogate zinc binding by either ZnF (C315Y, C354Y) or truncations missing portions of the Nos N-terminus or C-terminus cannot rescue mutant *nos* embryos (Curtis et al. 1997). Pum and Nos have been demonstrated to interact in an RNA dependent manner through co-immunoprecipitation experiments and yeast three-hybrid assays (Sonoda and Wharton 1999; Edwards et al. 2001). The *Xenopus* Nanos homolog, Xcat2, can pull down *Xenopus* Pumilio from embryo extracts, and Human PUM2 and NOS1 are also purported to interact through the PUF RBD (Nakahata et al. 2001; Jaruzelska et al. 2003). Mutations in Pum that block Nos recruitment have been identified (Edwards et al. 2001). One such mutation, F1367S, prevents Nos dependent enhancement of Pum repression in cells (Chapter 2) (Weidmann and Goldstrohm 2012). The details of this Pum-Nos interaction are unknown. The evidence of Nos RNA binding and the conserved interaction of Nos homologues with PUF RBDs might suggest a role for Nos in stimulating Pum binding to RNA.

Features of Pum regulation *in vivo* hint at a role for Nos in Pum PRE complex formation. The presence of Nos exclusively in the developing embryo posterior restricts Pum repression of the Hunchback mRNA to this location. This results in localized expression of Hb protein in the anterior and subsequently proper abdominal segmentation (Lehmann and Nusslein-Volhard 1987; Irish et al. 1989; Wharton and Struhl 1991; Murata and Wharton 1995; Wreden et al. 1997; Chagnovich and Lehmann 2001; Cho et al. 2006). The Hb mRNA contains two perfect Pum response elements (PREs) of the forms UGUAUAUA and UGUACAUA. In cells, mRNA reporters bearing these elements can be repressed simply by expressing more Pum (Weidmann and Goldstrohm 2012). For some reason, the level of Pum in embryos is insufficient without Nos. Ectopic expression of Nos

in the embryo anterior extends the repression of Hb and also permits Pum regulation of the Bicoid (Bcd) mRNA (Gamberi et al. 2002)**.** The Bcd mRNA contains a near perfect PRE with a centrally located Guanine (UGUAGAUA) not normally favored in Pum binding sites. More concentrated amounts of Nos are necessary for Pum regulation of the CycB mRNA in primordial germ cells and this necessity persists into germline stem cells (Forbes and Lehmann 1998; Gilboa and Lehmann 2004)**.** Low amounts of Nos are unable to stimulate Pum repression of CycB mRNA in somatic cells (Kadyrova et al. 2007). The PRE element within CycB is particularly degenerate, containing extra nucleotides within the center (UGUAauuUAUA). How these varying PRE elements augment Nos and Pum regulation is unknown. The varying requirements of Nos for each target may underlie differences in the affinity of Pum for each PRE. Nos might enhance Pum repression by stimulating binding. This could explain why over-expression of Pum can overcome the necessity for Nos (Chapter 2, Weidmann and Goldstrohm 2012).

Multiple models implicate Nos in the recruitment of repression activity to Pum complexes. For example, the Brat protein can assemble into a quaternary structure with Nos, Pum, and RNA (Edwards et al. 2001; Sonoda and Wharton 2001). Brat's recruitment of 4EHP competes for recognition of the 5' cap, inhibiting translation. A NOT interacting motif (NIM) exits within the N-termini of Nos homologs, but this motif is absent in *Drosophila* Nos (Lai et al. 2011; Bhandari et al. 2014). An interaction between the Not4 protein and the Nos N-terminus (NosN), identified through a yeast two-hybrid approach, is purported to be a link between Pum and NOT in flies (Kadyrova et al. 2007). Binding of NosN to the 4E binding protein, Cup, has also been described (Verrotti and Wharton 2000). Recruitment of any such element to PRE bound Pum would elicit repression, and could be responsible for the ability of Nos to enhance Pum.

We set out to test each model of Nos enhanced Pum repression. First, we created a cell based reporter assay that measures the ability of Nos to regulate the Hb 3'UTR. We dissected the regions of Nos necessary for this regulation. As with rescue of the *nos* embryo (Curtis et al. 1997), truncation or mutation of the N-terminus, ZnFs, or C-terminus of Nos protein diminished repression. However, a minimal construct of the Nos ZnFs and the C-terminal extension was sufficient for significant activity. We purified a recombinant

form of this truncated protein and tested its ability to form a ternary complex with the Pum RBD and RNA *in vitro*. Through electrophoretic mobility shift assays, we discovered that the presence of Nos increased the affinity of Pum RBD for PRE RNA significantly. Moreover, Nos enabled RBD binding to RNA it could not bind alone, including RNAs with mutations in the conserved PRE. Using the cell based assay, we confirmed that expression of Nos conferred repression on mRNAs containing these same PREs. We also uncovered a new RNA independent interaction between the Nos N terminus and Pum. Finally, using a tethered function approach, we identified a novel repression domain within the Nos N-terminus that appears to operate via a mechanism independent of previously identified Nos binding partners.

## 4.3 Results

### 4.3.1 Nanos enhancement of Pumilio Repression

#### 4.3.1.1 Pumilio dependent Nanos regulation of the Hunchback 3'UTR

To recapitulate Nos and Pum mediated repression, we designed mRNA reporters using the open reading frame of *Renilla* luciferase fused to the 3'UTR of *Drosophila melanogaster* Hunchback mRNA (Weidmann et al. 2014) (Fig. 4.1, RnLuc Hb 3'UTR WT). To ensure the reporting of Pumilio dependent repression, mRNAs with mutations in one or both PRE sites were also generated (Fig. 4.1; mt1, mt2, and mt1-2). As described in Chapters 2 and 3, reporters were transfected into D.mel-2 cells and Firefly luciferase (FFLuc) was co-expressed as a control (Weidmann and Goldstrohm 2012; Weidmann et al. 2014). Reporter expression is measured as a relative response ratio (RRR) of RnLuc to FFLuc luminescence. *Renilla* luciferase activity was 1.5 higher in cells expressing the Hb 3'UTR mt1-2 reporter than cells expressing the Hb 3'UTR WT reporter, likely resulting from endogenous Pumilio repression (data not shown). D.mel-2 cells do not express detectable levels of Nos mRNA (Weidmann and Goldstrohm 2012). To measure the effect of Nos on RnLuc

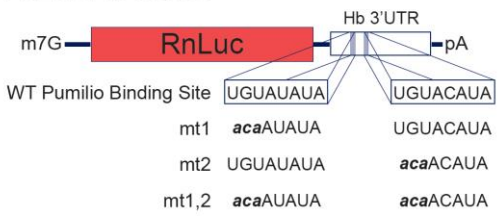


**Figure 4.1 - A Hunchback 3'UTR reporter.** Renilla luciferase reporters bearing the wild type or mutant Hunchback 3' untranslated region are diagrammed. The wild type sequence of both Pumilio binding sites is outlined. Sequences below each site illustrate mutations present in the mt1, mt2, and mt1-2 RnLuc reporters.

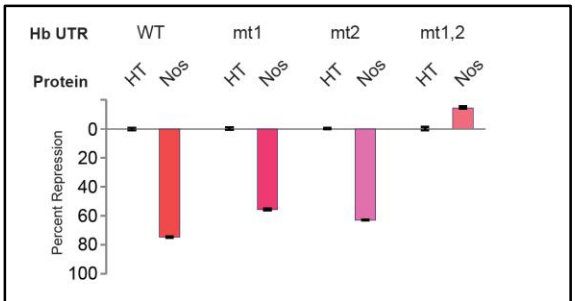


**Figure 4.2 - Nanos repression of Hb 3'UTR.** Percent repression of RnLuc Hb 3'UTR reporters when co-expressed with Halotag (HT) or Halotag fused to full length Nanos (Nos) in D.mel-2 cells. WT, mt1, mt2, and mt1-2 reporters are described in figure 4.1.

Hb 3'UTR reporters, we expressed Nos fused to the HaloTag (HT, Promega) protein and measured reporter luminescence (Fig. 4.2). Cells expressing the HT protein alone were used as a normalization for full reporter expression (Fig. 4.2). A percent repression value can then be calculated with the equation $100*(1-(RRR_{Repressor}/RRR_{Control}))$, wherein the repressor is HT-Nos and the control is HT alone. By this metric, Nos repressed the RnLuc Hb 3'UTR WT reporter by 75% (Fig. 4.2, WT). This repression is similar in magnitude to the Nos enhanced Pum repression observed on 3xNRE containing reporters (Chapter 2) (Weidmann and Goldstrohm 2012). Mutation of either PRE site resulted in slightly diminished repression (56% and 63%, Fig. 4.2, mt1 and mt2). A loss of both PRE sites eliminates repression, with expression of Nos resulting in a 15% *activation* of RnLuc Hb 3'UTR mt1-2 expression (Fig. 4.2, mt1-2). Expression of HT and HT-Nos was confirmed through fluorescence of labeled HT protein (Fig. 4.3). Thus, this assay faithfully captures Nos-enhanced Pumilio repression of the Hb 3'UTR.

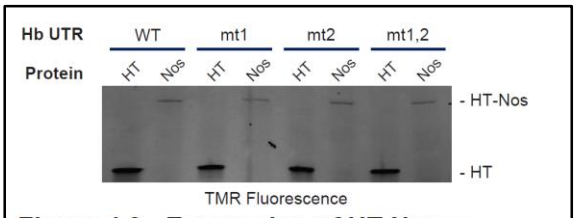


**Figure 4.3 - Expression of HT-Nanos.** Extracts from cells expressing Halotag (HT) alone or fused to Nanos (Nos) were labeled with HaloTag TMR fluorescent ligand. Fluorescent detection enabled visualization of expressed proteins.

Nos is a 401 amino acid (aa) protein that contains a conserved set of tandem Cysteine$_2$-Histidine-Cysteine (CCHC) zinc fingers (ZnF) (Fig. 4.4, region Z). Outside of this ZnF containing region, Nos has a 295aa N-terminus and a short 26aa C-terminal extension (Fig. 4.4, regions N and C). We designed a set of mutations and truncations of Nos to identify regions of the protein

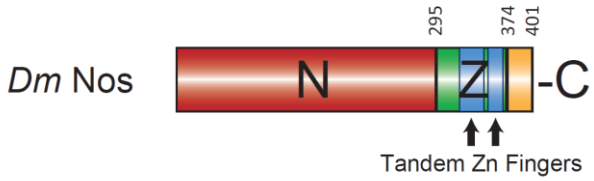


**Figure 4.4 - *Drosophila* Nanos domains.** A diagram of the Nanos protein is shown. Tandem Zinc (Zn) Fingers (in blue) lie within a region of Nanos important for its function *in vivo* (region Z). The relative lengths of th Nanos N-terminus (in red) and short C-terminal extension (in yellow) are depicted.

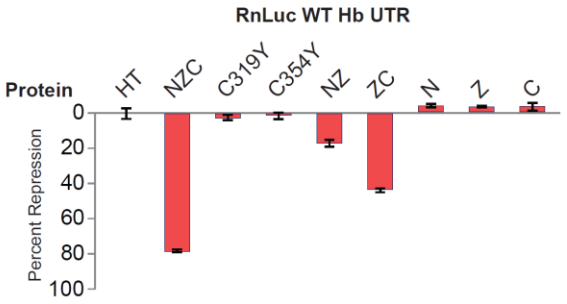


**Figure 4.5 - Repression by Nanos mutants.** Percent repression of RnLuc Hb 3'UTR WT reporter by co-expressed Halotag (HT) or Halotag fused to Nanos mu-utants and truncations in D.mel-2 cells. Mutations in Nanos Zn fingers (C319Y and C354Y) are included. The NZC nomenclature is described in Figure 4.4

necessary for the enhancement of Pum repression. To assess the necessity of the ZnFs, we compared wild-type Nos repression to that of two mutant variants known to abrogate metal binding (Curtis et al. 1997) (Fig. 4.5, C319Y and C354Y). These variants have the first critical Cysteine in one of the two ZnFs mutated to Tyrosine. Both the C319Y and C354Y mutations completely blocked repression caused by Nos, as reporter expression levels mirrored that of the HT control (Fig. 4.5). Metal binding to both of the ZnF regions is therefore required for Nos to stimulate Pum repression. To explore whether other regions of Nos are important for Pum enhancement, we also made truncations of Nos lacking the C or N-terminal domains (Fig. 4.5, NZ and ZC). Removal of the 26aa C-terminal region nearly eliminated repression; full length Nos repressed by 79% while the NZ truncation repressed by only 12% (Fig. 4.5, NZC and NZ). Removal of the 295aa N-terminus had a more modest effect, retaining 43% repression activity (Fig. 4.5, ZC). No separate Nos region could support repression, including the ZnF-containing Z region (Fig. 4.5, N, Z, and C). Differences in expression could not explain loss of activity, as each Nos variant was expressed at similar levels to the wild-type (Fig. 4.6). Taken together, these findings suggest a role for the N and C regions of Nos in Pum enhancement. The ZnFs are absolutely critical for function, and the C region is necessary for significant enhancement. The N region has only a partial role in Pum enhancement. With a region of Nos sufficient for induction of Pum repression (ZC), we began investigating possible mechanisms of Nos-mediated enhancement. One hypothesis is that Nos affects Pum's ability to bind to RNA.

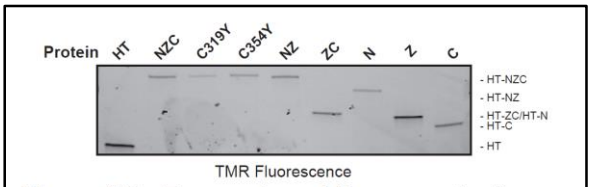


**Figure 4.6 - Expression of Nanos mutants.** Protein expression analysis from Figure 4.5. Extracts from cells expressing Halotag (HT) alone or fused to Nanos mutants and truncations were labeled with HaloTag TMR fluorescent ligand and visualized on an SDS-PAGE gel.

### 4.3.1.2 Formation of a stable ternary Pumilio-Nanos-PRE complex *in vitro*

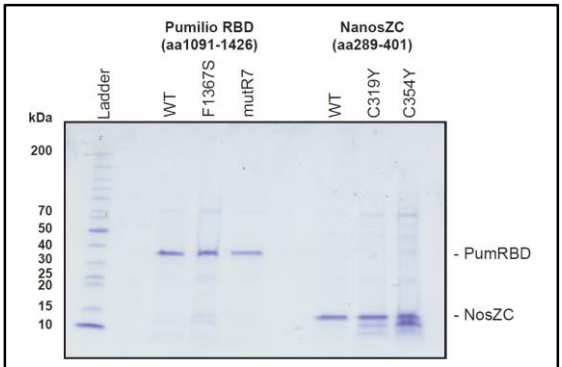


**Figure 4.7 - Purification of recombinant Pum and Nos.** Coomassie stained SDS-PAGE of Pumilio RNA binding domain (RBD) and Nanos ZC expressed and purified from bacteria. Expected sizes of cleaved RBD and NosZC are 39 kDa and 14kDa, respectively. Constructs included wild type (WT) Pum RBD, a Nanos binding-deficient RBD (F1367S), and an RNA binding-deficient RBD (mutR7). WT NanosZC and Zn finger mutants of NanosZC (C319Y and C354Y) are also included.

We aimed to reconstitute the Pum-Nos-NRE complex using an electrophoretic mobility shift assay (EMSA) in order to evaluate whether Nos can contribute to Pum RNA binding. To do so, we expressed and purified recombinant versions of Pum and Nanos. The Pum constructs contained only the region necessary for Nos enhancement, the RBD. Recombinant Nos consisted exclusively of the minimal region sufficient for stimulation of repression, ZC (Fig. 4.7). As controls, we also purified RBD mutants which could not associate with Nos (F1367S) or could not associate with RNA (mutR7) (Fig. 4.7). In addition, Nos ZC constructs with the ZnF mutations C319Y or C354Y were also purified (Fig. 4.7). To track complex formation, we mixed a 5' Cy5 labeled PRE containing RNA with combinations of our purified proteins (Fig. 4.8). The labeled RNA contained 26 nucleotides of the original Hb Nanos Response Element 2, which contains a perfect PRE sequence (UGUACAUA). The addition of 40 nM wild-type Pum RBD resulted in a near complete shift of the PRE RNA (Fig. 4.8, lanes 1-3). Addition of Nos ZC at a concentration equimolar to RBD resulted in a consequent super-shift, suggesting ternary complex formation (Fig. 4.8, lane 4). We then tested the EMSA properties of Nos-binding deficient F1367S RBD (Fig. 4.8, lanes 5-8). The RBD-PRE complex formed readily but no super-shift was observed when Nos ZC was added (Fig. 4.8,

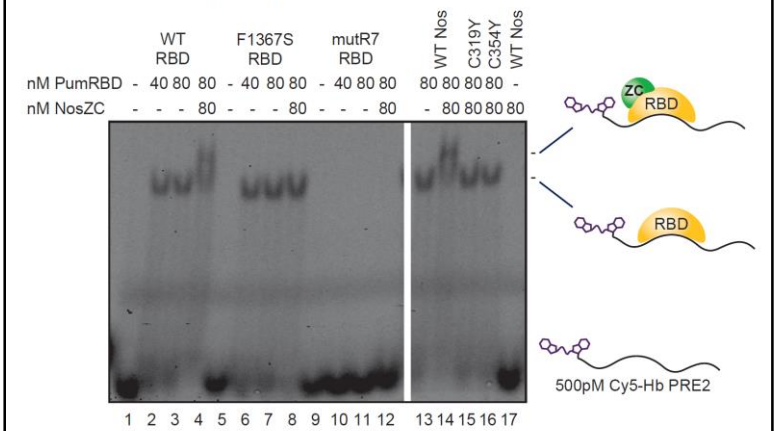


**Figure 4.8 - Formation of a Pum Nos RNA ternary complex.** Fluorescent Eletrophoretic Mobility Shift Assay (EMSA). Indicated concentrations of protein and RNA were combined. Bound and unbound complexes were separated on a native PAGE gel. RNA was visualized by fluorescence detection. Illustrations of binary and ternary complexes are provided.

lanes 6-8). This interaction site on the RBD is thus necessary for ternary complex formation *in vitro* (Fig. 4.8, lane 8) and for Nos enhanced repression in cells (Chapter 2) (Weidmann and Goldstrohm 2012). As expected, the mutR7 RNA-binding deficient RBD could not shift RNA (Fig. 4.8, lanes 9-12). While Nos can form ternary complexes with wild-type RBD and PRE at this concentration, it cannot shift RNA on its own (Fig. 4.8, compare lanes 4 and 14 to 12 and 17). Mutations in either Nos ZnF eliminates ternary complex formation as well (Fig. 4.8, lanes 15-16). The F1367 interaction with Nos on the RBD is necessary for ternary complex formation *in vitro* (Fig. 4.8, lane 8) and for Nos enhanced repression in cells (Chapter 2) (Weidmann and Goldstrohm 2012). The ZnF are absolutely required for the assembly of Nos into Pum-PRE (Fig. 4.8, lanes 15-16) and for PRE dependent repression in cells (Fig. 4.5). Thus, the conditions in the EMSA are consistent with Nos enhanced repression in cells being a result of Pum-Nos complex formation. We then asked whether this complex formation affected the Pum RBD's ability to bind RNA.

#### 4.3.1.3 Nanos increases the affinity of Pumilio for PRE and non-PREs *in vitro*

In order to quantitate the effect of Nos on the association of Pum RBD with the PRE, we designed a minimal 16nt RNA based on the PRE within the Hb NRE2 (Fig. 4.9, HbPRE2). To observe HbPRE2 in our EMSA assay, we radiolabeled the 5' nucleotide with $^{32}$P. While keeping the concentration of RNA constant, we titrated the amount of Pum RBD (0-200 nM) over multiple equilibrium binding reactions and observed changes in native gel mobility of the RNA (Fig. 4.9). Through quantitation of the fraction of RNA bound (shifted RNA/total RNA) at each Pum RBD concentration, we were able to measure an observed dissociation constant ($K_{d\ obs}$) (Fig. 4.9). Gathering data from replicate EMSAs,

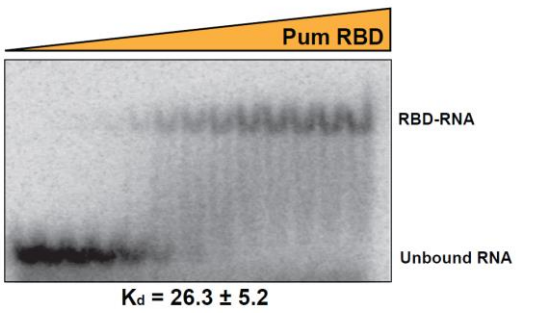


**Figure 4.9 - RBD binding to HbPRE2.** Radioactive Eletrophoretic Mobility Shift Assay (EMSA). Increasing concentrations of Pumilio RNA Binding domain (Pum RBD, 0-200nM) was added to 300pM radiolabeled RNA. The RNA sequence is based on the second Hunchback Pum response element (HbPRE2). Bound and unbound complexes were separated on a native PAGE gel. RNA was visualized by radiography. A dissociation constant $K_d$ (and associated S.E.M.) was quantified from quadruplicate gels.

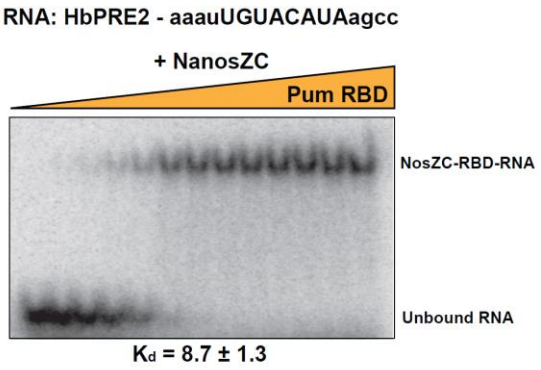


**Figure 4.10- NosZC-RBD binding to HbPRE2.** The same experiment as in Figure 4.9, but with 400nM of NosZC added to each sample.

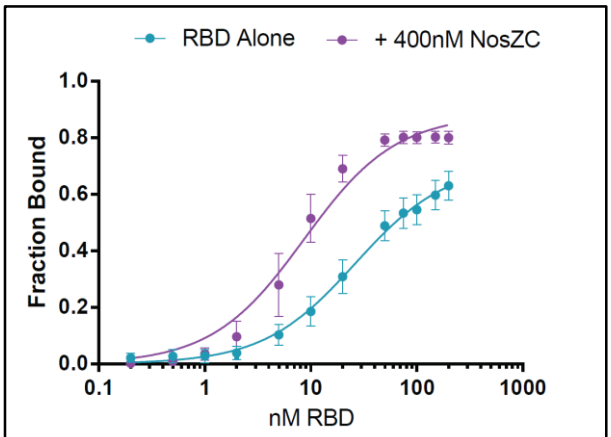


**Figure 4.11 - NosZC enhances RBD RNA binding.** Quantification of fraction bound data from Figures 4.9 and 4.10. Each data set is an average of four replicate gels. Bars represent S.E.M.

the $K_{d\ obs}$ was calculated to be 26nM for Pum RBD binding to HbPRE2 (Fig. 4.9). We then tested whether the presence of Nos might change the $K_{d\ obs}$. Nos has no measurable ability to shift RNAs at low concentration, but it can bind to an RBD-RNA complex (Fig. 4.8). We again performed the RBD titration under identical conditions, except NosZC was included at a constant concentration above that of the highest RBD concentration (400nM, Fig. 4.10). Only two species of RNA were observed, unbound and ternary complex (Fig. 4.10). Importantly, no shift was observed without Pum RBD, even though NosZC was present, simplifying interpretation of the EMSA (Fig. 4.10, first lane). With NosZC present, the $K_{d\ obs}$ was measured at 8.7nM (Fig 4.10). This is a 3-fold increase in the affinity of the Pum RBD for HbPRE2 (Fig. 4.11). An increase in Pum's affinity for RNA could facilitate the enhanced repression observed in cells.

Because Nos had a significant effect on Pum binding to the HbPRE2, we questioned whether a similar effect could be observed on PRE sites derived from other mRNA targets. To this end, we generated 16-19nt RNAs based on other PREs from the first Hb NRE, the Bcd 3'UTR, and CycB 3'UTR (Fig. 4.12, HbPRE1, BcdPRE, and CycBPRE). Surprisingly, none of these PREs were stably bound by the Pum RBD in our EMSA conditions and $K_{d\ obs}$ values could not be reliably measured (Fig. 4.12, left panels). This held true with concentrations of RBD up to 1µM. Remarkably, the addition of NosZC resulted in RNA binding close to HbPRE2 affinity (Fig. 4.12, right panels). With NosZC included, the $K_{d\ obs}$ for HbPRE1, BcdPRE, and CycBPRE were measured at 59.6nM,

46.1nM, and 12.0nM, respectively (Fig. 4.12). This enhancement constitutes a range of greater than 16-80 fold increases in affinity (Fig. 4.13). Moreover, the observed modified affinities match closely with that of HbPRE2, a site that is regulated independently by Pum in cells and together with Nos *in vivo*.

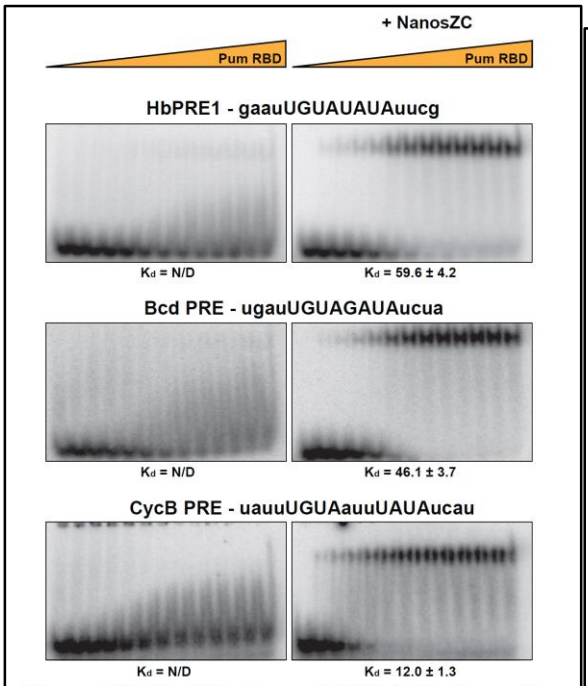


**Figure 4.12 - NosZC enhances RBD binding to varying PREs.** Radioactive EMSA as in Figures 4.9 and 4.10., but with varying PRE RNAs. RNA sequences derived from the first Hunchback PRE (HbPRE1), the Bicoid PRE (Bcd PRE), and the CyclinB PRE (CycB PRE) were used. The Pum binding sites are capitalized. Gels on the left represent reactions with only RNA and RBD, while those on the right include NosZC. The concentration range of the RBD is now 0-1000nM, and the concentration of NosZC was increased to 1000nM.

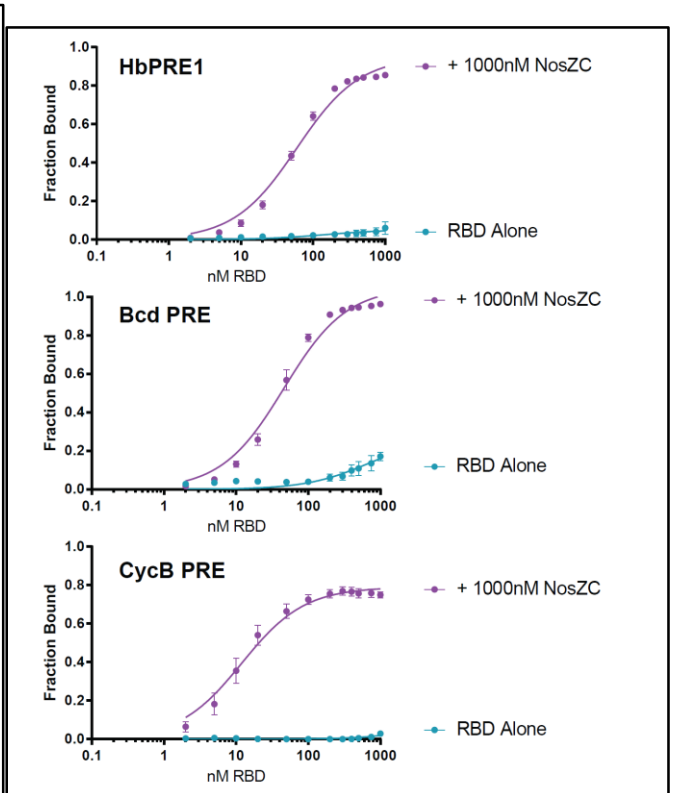


**Figure 4.13 - NosZC significantly enhances RBD binding.** Quantification of fraction bound data from Figure 4.12. Each data set is an average of three replicate gels. Bars represent S.E.M.

After witnessing such drastic changes in RNA binding, we were curious whether Nos could confer Pum binding to non-PRE RNAs. Mutant versions of the HbPRE2 RNAs were created harboring moderate to strong changes in the conserved $U_1G_2U_3$ trinucleotide: including a $U_3$ to G mutation or $U_1G_2U_3$ to ACA (Fig. 4.14, HbPRE2 G3 and HbPRE2 ACA). These PRE elements do not confer regulation of a reporter by Pum in cells (Chapter 2) (Weidmann and Goldstrohm 2012). The HbPRE2 G3 RNA behaved similarly to the weak endogenous PREs from Hb NRE1, Bcd, and CycB (Fig. 4.14, top panels). Pum RBD alone could not stably bind HbPRE2 G3, but the inclusion of NosZC resulted in a $K_{d\ obs}$ of 59.9nM (Fig. 4.14). This represents at least a 16-fold change in

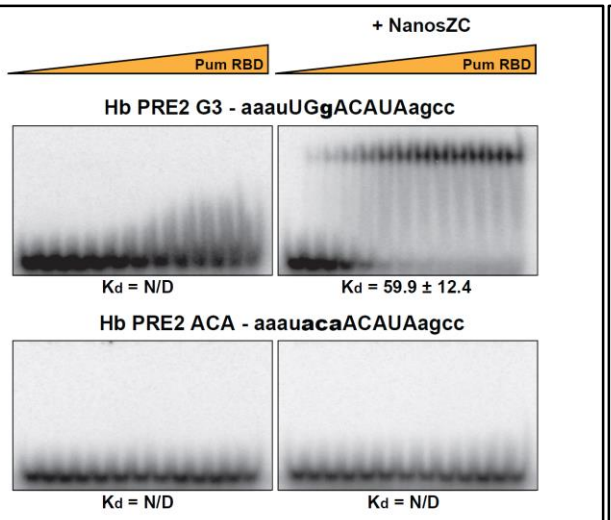


**Figure 4.14 - Ternary complex tolerates some mutation of the PRE.** Radioactive EMSA as in Figure 4.12, but with mutant HbPRE2 RNAs. Pum binding sites are capitalized while mutations are lowercase and bold. Gels on the left represent reactions with only RNA and RBD, while those on the right include NosZC. The concentration range of the RBD is 0-1000nM, and the concentration of NosZC is 1000nM.

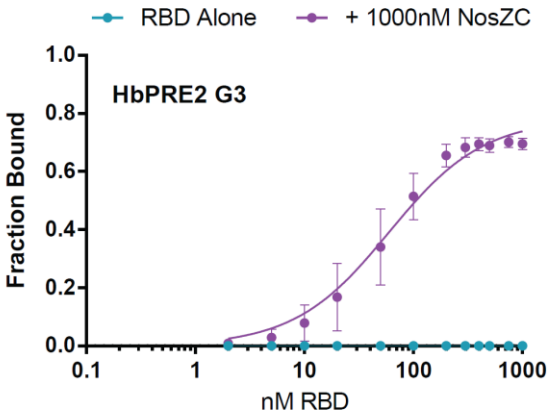


**Figure 4.15 - NosZC enhances RBD RNA binding to a PRE mutant.** Quantification of fraction bound data from Figure 4.14. The data set is an average of three replicate gels. Bars represent S.E.M.

binding affinity and results in an interaction that rivals a real Pum target RNA (Fig. 4.15). However, NosZC does not confer non-specific binding activity to the Pum RBD, as no binding of HbPRE2 ACA was observed with or without Nos (Fig. 4.14, bottom panels). The EMSAs collectively show that Nos can act as a potent enhancer of Pum RNA binding to both strong and weak PREs, relaxing specificity and improving affinity. It became important to test whether Nos can confer Pum binding and repression onto mRNAs that Pum does not normally target in cells.

### 4.3.1.4 Nanos triggers Pumilio repression of weak PREs in cells

To assess the ability of Nos to stimulate Pum repression of mRNAs containing weak PREs, we turned to our cell based reporter assay. To compare the effects of Nos on different sequence elements, we generated *Renilla* reporters bearing minimal 3'UTRs that contain each PRE tested in our EMSA binding assays. Outside of the 19-20nt region of insertion, each reporter was identical. Reporters were generated with sequences including an empty multiple cloning site (RnLuc MCS), HbPRE2, HbPRE1, BcdPRE, CycBPRE, HbPRE2 G3, and HbPRE ACA. Each reporter was co-expressed in cells with the FFLuc control and a gradient of HT-Nos (Fig. 4.16). The percent repression of each *Renilla* reporter was measured comparing each sample with increasing HT-Nos to a reporter alone sample in the same experiment. As an added negative control, the

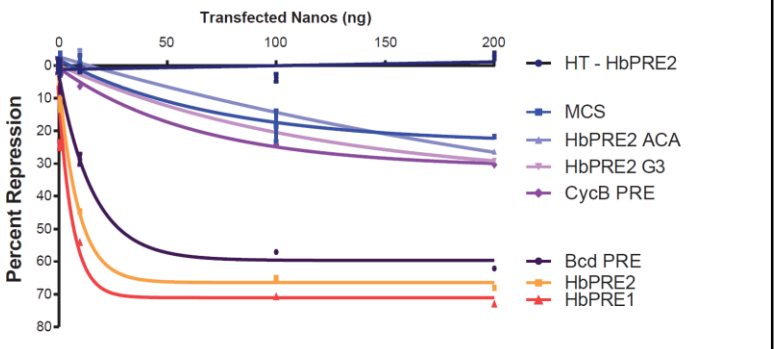


**Figure 4.16 - Nanos enhances Pum repression of varying PREs in cells.** Percent repression of varying RnLuc PRE reporters when co-expressed with increasing amounts of HT-Nanos in D.mel-2 cells. PRE elements are described in the text. The negative control (HT - HbPRE2) was measured from cells co-expressing the HbPRE2 reporter and increasing amounts of HT alone.

HbPRE2 was also co-expressed with a gradient of HT alone (Fig. 4.16). Across the entire expression gradient, HT alone had no effect on the HbPRE2 reporter (Fig. 4.16). High amounts of transfected HT-Nos resulted in some repression of the MCS reporter, however (21% at 200ng, Fig. 4.16). Repression of the HbPRE2 reporter was robust, increasing from 12% repression with 1ng of transfected Nos to 68% repression with 200ng of transfected Nos (Fig. 4.16). A similar effect was seen with the HbPRE1 reporter (24% to 73%, Fig. 4.16). The BcdPRE responded more moderately: repressed by 7% at 1ng, 29% at 10ng, 57% at 100ng, and 62% at 200ng (Fig. 4.16). Repression of the CycBPRE was limited with low transfected Nos (only 6% at 10ng) and only reached 30% repression at the highest amount (Fig. 4.16). The HbPRE2 G3 reporter behaved similarly to CycBPRE, achieving 30% repression at high amounts of transfected Nos (Fig. 4.16). Finally, repression of the HbPRE2 ACA reporter was also nonexistent at low Nos transfection amounts (Fig. 4.16, 1 and 10ng), but was measurable at high transfection amounts (Fig. 4.16, 100 and 200ng, 15% and 26%). While the repression of CycBPRE, HbPRE2 G3, and HbPRE2 ACA is only slightly enhanced (30%, 29%, and 26%) when compared to MCS (22%), these differences are statistically significant ($p < 0.001$). We conclude that Nos can confer Pum repression to reporters with PRE elements proportional to Pum-RNA binding affinity.

#### 4.3.1.5 Identification of a novel Nanos-Pumilio interaction

The reason why deletion of the Nos N-terminus could moderately impede the enhancement of Pum repression in cells (Fig. 4.5) while the ZC was entirely sufficient for the enhancement of Pum RNA binding (Fig. 4.9 - 4.15) was unclear. The N-terminus of Nos could have varying roles: it could assist in Pum-Nos complex formation, it may

augment and improve the enhancement of RNA binding, or it may assist in recruitment of factors necessary for translational repression. To assess the role of the N-terminus in Pum complex assembly, we employed a co-immunoprecipitation approach from cells. As bait, we created a vector for expression of 3xFLAG-tagged Pum (FLAG-Pum). As prey, vectors expressing an HT control, HT-Nos, HT-NosN, or HT-NosZC were used. Each prey was co-transfected into cells with either FLAG-Pum or a mock empty expression vector. After allowing time to express each protein, cells were lysed and treated with RNases 1 and A. Pum protein complexes were purified with anti-FLAG beads (Fig. 4.17). Bait and prey proteins were expressed at similar levels in each sample (Fig. 4.17, Input). HT-Nos and HT-NosN were both enriched by the FLAG IP exclusively in samples expressing FLAG-Pum (Fig. 4.17, FLAG IP). Importantly, the HT negative control was not enriched by FLAG IP in either mock or FLAG-Pum expressing samples (Fig. 4.17, FLAG IP HT). The HT-NosZC construct was also not enriched by FLAG IP in either sample, supporting the idea that the interaction between NosZC and Pum may be RNA dependent. Together, these results suggest that the Nos N-terminus is responsible for maintaining an RNA independent association with Pum in cells. This novel interaction may be partially responsible for the loss of repression observed when the Nos N-terminus is deleted. Additionally, the Nos N-terminus may also contribute repression activity to the complex, as has been reported previously (Kadyrova et al. 2007). Nos homologs also have been reported to maintain N-terminal derived repression activity (Lai et al. 2011; Bhandari et al. 2014).

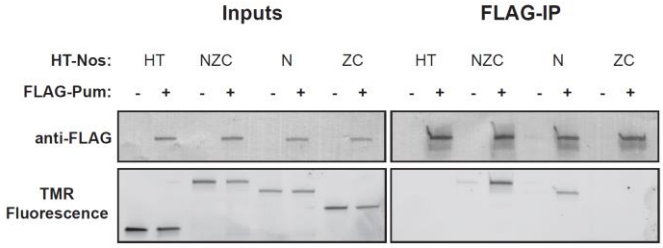


**Figure 4.17 - Pumilio interacts with the Nanos N-terminus independent of RNA.** Co-immunoprecipitation of FLAG-Pum with HT-Nanos truncations from RNase treated D.mel-2 cell extracts. Western blotting detected FLAG-tagged Pumilio (FLAG-Pum) in the inputs and eluates from anti-FLAG immunoprecipitations. Halotag (HT) or Halotag fused to Nanos variants were detected by covalent labelling of HT with fluorescent TMR ligand. As a negative control, FLAG immunoprecipations were performed from extracts that expressed Halotag prey proteins but not FLAG-RBD..

### 4.3.2 A Pumilio independent Nanos repression

#### 4.3.2.1 Tethered function identifies a repression domain in Nanos

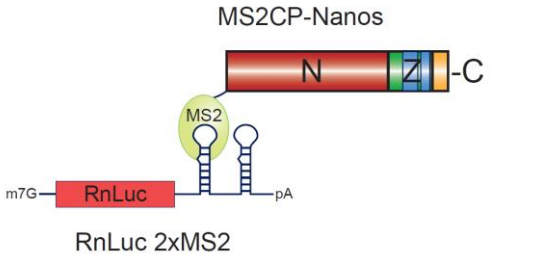


**Figure 4.18 - Nanos tethered function assay.** Diagram of the tethered function reporter and Nanos constructs. Nanos and truncations were fused to the MS2 RNA binding protein. The N, Z, and C regions are as described in Figure 4.4.

To confirm whether Nos possesses intrinsic repression capacity independent of Pum, we turned to the tethered function assay (Chapter 2 and 3) (Coller and Wickens 2002). We created plasmids expressing fusions of Nos regions to the phage MS2 coat protein, which binds a specific RNA stem loop structure (Fig. 4.18). We co-expressed these fusions with a *Renilla* luciferase reporter bearing two MS2 binding sites in its mRNA 3'UTR (Fig. 4.18, RnLuc 2xMS2). Using FFLuc as the transfection control, we used luminescence output to quantify RRR for a series of tethered Nos mutants and truncations much like in Fig. 4.5: constructs included tethered NZC, C354Y, NZ, ZC, N, and Z. Expression ratios were normalized to samples expressing the MS2 coat protein alone (set to 100). Compared to the MS2 control, tethered full length Nos repressed 69% (Fig. 4.19, NZC). Tethered Nos repression does not require both ZnFs, as the tethered C354Y mutant was able to repress by a similar amount (60%, Fig. 4.19, C354Y). The C-terminal extension was also dispensable; tethered NZ repressed 67% (Fig. 4.19, NZ). Conversely, deletion of the Nos N-terminus abrogated tethered repression (12%, Fig. 4.19, ZC). Moreover, tethering the Nos N-terminus alone was sufficient for near full repression activity (63%, Fig. 4.19, N). Very minimal activity was observed when Z alone was tethered (7%, Fig. 4.19, Z). Together, these results indicate a role for the Nos N-terminus in inhibiting mRNA expression.

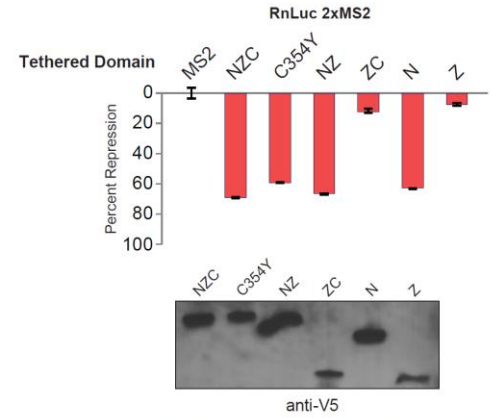


**Figure 4.19 - The Nanos N-terminus has repression activity.** Percent repression of RnLuc 2xMS2 reporter when co-expressed with MS2 coat protein or MS2 fused to Nanos variants and truncations in D.mel-2 cells. Mutation of one Nanos Zn finger (C354Y) is included. The NZC nomenclature is described in Figure 4.4. Western blotting detects expression of V5-tagged protein constructs.

### 4.3.2.2 RNAi depletion of existing partners do not affect Nanos repression

Pum dependent and independent mechanisms of Nos have been proposed previously through interactions with Pum and other partners (Verrotti and Wharton 2000; Sonoda and Wharton 2001; Cho et al. 2006; Kadyrova et al. 2007; Lai et al. 2011; Bhandari et al. 2014). The tethered Nos activity we observed thus could be derived from one of these sources: Pum, Brat, Ccr4-Not, or the 4E binding protein Cup. To evaluate the necessity of these proteins in tethered Nos repression, we applied dsRNA interference of each gene to the tethered function assay (Fig. 4.20). Before transfection of reporters and tethered NosN, cells were incubated with dsRNA complementary to endogenous mRNAs. As a control, dsRNA targeting the absent LacZ gene was used. With control dsRNAi, tethered NosN repressed by 64%, mirroring the normal level of activity seen in Fig. 4.19 (Fig. 4.20, Control). Depletion of either Pum or Brat did not impede repression (70% and 74%, Fig. 4.20, Pum and Brat). Depletion of either the Pop2 and Ccr4 deadenylases or the Not1 deadenylase complex scaffolding protein led to a slight impairment of repression (60% and 53%, Fig. 4.20, Pop2 + Ccr4 and Not1). Lastly, knockdown of Cup had no effect on NosN activity (67%, Fig. 4.20, Cup). Depletion of the Pum, Brat, Pop2, and Ccr4 proteins utilizing these dsRNAs has been verified previously (Chapter 2 and 3) (Weidmann and Goldstrohm 2012; Weidmann et al. 2014). Knockdown of each endogenous mRNA was validated by qRTPCR from each experiment. The mRNAs encoding Pum, Brat, Pop2, Ccr4, Not1, and Cup were depleted by 75%, 78%, 39%, 93%, 84%, and 88%, respectively. The data suggest that the tethered Nos N-terminus may operate via a novel mechanism, as depletion of factors necessary for other Nos mediated deadenylation and translational repression does not relieve activity. This repression may contribute to the enhancement of Pum repression by Nos.

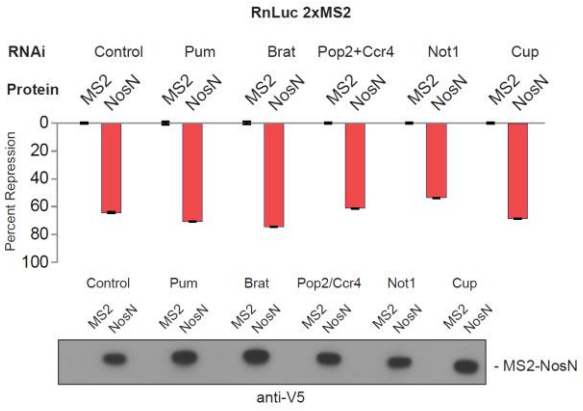


**Figure 4.20 - The Nanos N-terminus has novel activity.** Percent repression of RnLuc 2xMS2 reporter when co-expressed with MS2 coat protein or MS2 fused to the Nanos N-terminus in D.mel-2 cells. Cells were treated with a NTC dsRNA or dsRNAs corresponding to each indicated gene. Western blotting detects expression of V5-tagged protein constructs.

## 4.4 Discussion

### 4.4.1 Multiple domains of Nanos are necessary for enhanced Pum repression

*Drosophila* Nanos is required for Pum-mediated repression of multiple mRNAs *in vivo* (Barker et al. 1992; Wreden et al. 1997; Forbes and Lehmann 1998; Asaoka-Taguchi et al. 1999; Sonoda and Wharton 1999; Gilboa and Lehmann 2004; Ye et al. 2004; Kadyrova et al. 2007; Muraro et al. 2008). Pum binding and Nos recruitment to the targets Hunchback, Paralytic, and Cyclin B are crucial for development, motor function, and fertility, respectively**.** However, Pum causes translational repression, accelerates deadenylation, and limits target mRNA levels all in the absence of Nos (Chapters 2 and 3, Weidmann and Goldstrohm 2012; Weidmann et al. 2014). If Nos is not necessary for Pum activity, why is Nos required *in vivo*? We evaluated two hypotheses: 1) Nos could enhance Pum RNA binding, restricting where Pum could engage its targets and 2) Nos may recruit additional activity that permits full repression by Pum.

D.mel-2 cells, which do not express detectable levels of Nos, were a perfect system to test what effect Nos had on Pum-dependent repression. We created a reporter mRNA bearing the 3'UTR of the Pum target Hunchback, whose regulation depends on Nos *in vivo*. The number of Pum binding sites in the Hb 3'UTR and the amount of Nos protein confers a precise level of regulation upon the Hunchback transcript during embryo development (Wharton and Struhl 1991). Our results in D.mel-2 cells fully recapitulated Hb regulation; Nos expression blocked translation of our reporter in a manner dependent on Pum binding sites. One Pum binding site was sufficient for repression, but the presence of both was slightly more inhibitory than either alone. With Nos-Pum regulation accurately reconstituted in cells, we sought to dissect what regions of Nos were important for activity.

Nos has a 295 amino acid (aa) N-terminus and a short 26aa C-terminal extension that flank an 80aa region including tandem zinc fingers (ZnFs). One of the first *nos* mutant alleles identified was $nos^{L7}$, which expresses a protein with an in-frame deletion of 7 amino acids (aa376-382, ITMEDAI) in the Nos C-terminal extension (Lehmann 1988). The $nos^{L7}$ allele results in a loss of function; embryos generated from $nos^{L7}$ flies are small,

lack abdominal segments, and are ultimately inviable (Lehmann 1988). This deletion in Nos precludes formation of a Pum-Nos-RNA complex as measured by multiple binding assays, including yeast three-hybrid and *in vitro* pull-down (Wharton et al. 1998, Sonoda and Wharton 1999). We observed that deletion of the Nos C-terminal extension, which includes the ITMEDAI sequence, significantly impairs Nos repression of an Hb 3'UTR reporter in *Drosophila* cells. We speculate that this region of Nos serves as a binding site for Pum; without this interaction, Nos cannot enhance Pum repression.

The defining characteristic of the Nanos family of proteins is their tandem CCHC type ZnFs (Curtis et al. 1997). These ZnFs are conserved from flies to humans and are purported to confer non-specific RNA binding to Nos and its homologs (Curtis et al. 1997; Hashimoto et al. 2010). Interruption of metal coordination by either Nos ZnF results in abdominal segmentation and fertility defects in flies similar to other *pum* and *nos* mutants (Curtis et al. 1997). We observed that mutations in these ZnFs (C319Y and C354Y) also completely blocked Nos stimulated repression of the Hb 3'UTR reporter. Thus, the ZnFs and C-terminal extension of the Nos protein are critical to Pum enhancement in cells and likely underlie effects observed *in vivo*. If the C-terminal extension is necessary for Pum interaction, perhaps the RNA binding capacity of the ZnFs is important for enhancement.

The N-terminus of Nos is not highly conserved and its role in translational repression is unknown. Injection of RNAs expressing Nos proteins lacking the N-terminus cannot rescue the *nos* phenotype, suggesting that this region plays a role in Nos dependent regulation (Curtis et al. 1997). In D.mel-2 cells, we observed that truncation of the Nos N-terminus cuts repression of the Hb 3'UTR reporter in half. The Nos N-terminus, therefore, contributes activity to the more crucial ZnFs and C-terminal extension. It is important to understand how each region contributes to Pum repression.

### 4.4.2 Nanos enhances Pum repression by increasing affinity for RNA

Reports of Nos RNA binding dependent on Zinc coordination and the observation that Nos stimulates Pum repression through the RBD led us to question whether Nos might affect Pum's affinity for RNA. The observed Nos RNA binding was very weak and nonspecific, but it might contribute in a combinatorial fashion to Pum's specific sequence

recognition. To test this, we expressed and purified recombinant variants of the Pum RBD and the Nos ZC region and directly assessed their ability to bind RNA through electrophoretic mobility shift assays. As expected, the Pum RBD bound the HbPRE2 RNA readily; yet we did not observe RNA binding by NosZC even at μM concentrations. However, when Nos was added together with RBD and PRE, a ternary complex of Pum, Nos, and RNA was observed. Mutation of either Nos ZnF (C315Y or C354Y) prevented ternary complex formation. The F1367S mutation in the Pum RBD also obviated complex formation, consistent with past reports of impaired Nos association and synergistic repression (Edwards et al. 2001, Weidmann and Goldstrohm 2012). These results favor a model of Pum dependent recruitment of Nos to RNA. Does the ternary complex mediate recruitment of factors that inhibit protein synthesis or does the association with Nos change Pum's affinity for RNA? Either mechanism could be responsible for Pum enhancement.

The *C. elegans* PUF protein FBF-2 is an example where association with other partners into a ternary complex affects RNA binding and protein recruitment. Germline development defective 3 (GLD-3) and the cytoplasmic polyadenylation element binding protein (CPEB), CPB-1, compete for interaction with FBF-2 (Menichelli et al. 2013). GLD-3 behaves like Nos; GLD-3 cannot associate with RNA alone, but will assemble in a ternary complex with RNA and FBF-2 (Wu et al. 2013). GLD-3's association with FBF-2 did not affect RNA affinity, it was only recruited to the RNA by FBF-2. CPB-1 can bind RNA by itself, but in complex with FBF-2 it alters PUF specificity *in vitro* for the nucleotides flanking the PUF binding site (Campbell et al. 2012a). Additionally, like Nos, CPB-1 increases the affinity of FBF-2 for certain mRNA sequences, including *C. elegans* Cyclin B (CYB-1) (Menichelli et al. 2013). An interaction between a human PUF and a human CPEB, PUM2 and CPEB3, is also conserved (Campbell et al. 2012b). The interactions of GLD-3 and CPB-1 are maintained through a binding site on FBF-2 analogous to the Nos interaction region on the Pum RBD: both sites exist between the seventh and eight repeat of the PUF RBD. Competing interactions of CPB-1 and GLD-3 could determine whether FBF-2 requires specific flanking nucleotides in the RNA binding site to interact and repress. Nos may operate through a similar mechanism. Mutations in the four nucleotides upstream of the Hb PRE result in a loss of proper regulation *in vivo* (Wharton et al. 1998).

Nanos may confer added specificity through its own direct interaction with these nucleotides. We measured the Pum RBD's affinity for the PRE in the presence or absence of Nos. By itself, the RBD's affinity for PRE RNA was strong; we observed a $K_d$ of 26 nM. When we measured the assembly of the same amount of RBD and RNA into ternary complex with Nos, the association was even stronger (8 nM). GLD-3 does not affect RNA binding by FBF-2 (Wu et al. 2013). CPB-1 can increase FBF-2 affinity for a weak CYB-1 PRE about 5-fold and slightly alters specificity (Menichelli et al. 2013). Nos can actually increase the Pum RBD's affinity for even a strong PRE site.

The ability of Nos to significantly enhance Pum binding to a perfect PRE RNA was striking. We wondered whether Nos could assist retention of Pum on sites which are more weakly associated, perhaps shifting the specificity even more so than CPB-1 does to FBF-2. We sampled RNAs of varying PRE "strength" derived from multiple Pum targets or created through mutation of the HbPRE2 RNA. Of the RNAs tested, only the HbPRE2 RNA could stably associate with the Pum RBD alone. To our surprise, even the PRE sequence derived from the first Hb mRNA NRE (HbPRE1) was not bound with high affinity. Upon closer inspection, we attribute this to an ability of the HbPRE1 16mer to form stem loops or self-dimers under our binding conditions; 8 of 16 nucleotides in HbPRE1 can base pair with a ΔG of -6.3 kcal/mol. Nonetheless, this RNA was readily incorporated into the Pum-Nos-PRE ternary complex, highlighting the improvement of Pum-RNA affinity. The BcdPRE, CycBPRE, and an HbPRE2 mutant ($U_3$ to $G_3$) behaved similarly. None were bound by the Pum RBD alone, but the presence of Nos mediated strong incorporation into the ternary complex. In fact, only the HbPRE2 triple mutant (HbPRE ACA) did not respond to the addition of Nos. These results show that Nos significantly improves Pum RNA binding and can cause greater than 80-fold increases in affinity for weak PRE sites. This will substantially expand the number of Pum targets *in vivo* in addition to greatly enhancing Pum repression activity.

We then asked whether each PRE element tested *in vitro* could confer Nos enhanced Pum repression on a reporter mRNA. The results corroborated our own *in vitro* analyses and validate observations *in vivo*. The perfect PRE sites, HbPRE1 and HbPRE2, were significantly repressed even with small amounts of Nos expression, much like Hb

regulation in the embryo posterior. The BcdPRE, which contains a central G residue normally absent in PREs (UGUAGAUA), conferred a moderate amount of repression. In the embryo, Bcd mRNA repression is observed in the anterior upon ectopic expression of Nos (Gamberi et al. 2002). Repression of the CycBPRE reporter required larger amounts of transfected Nos, mirroring the requirement for concentrated Nos protein in pole cells where CycB translation is repressed (Kadyrova et al. 2007). Mutation of $U_3$ to G in the HbPRE2 reporter resulted in behavior resembling the CycB PRE. Surprisingly, even the strong mutant HbPRE2 ACA, where the conserved $U_1G_2U_3$ triplet has been changed to ACA, was slightly repressed by high amounts of transfected Nos. High amounts of Nos also resulted in repression of a reporter without any PREs in the 3'UTR, but this repression was significantly reduced compared to those containing PREs. We attribute this background to degenerate PRE-like sites intrinsic to the reporter coding sequence (CDS). The Paralytic mRNA is regulated through such a CDS intrinsic site (Muraro et al. 2008).

As measured via EMSAs, weak PREs could be bound with similar affinity to strong PREs when Nos was present. In cells, the level of repression is proportional to the Nos-Pum-RNA affinity. The HbPRE2 is bound most strongly by Pum and Nanos and can be repressed by adding small amounts of Nos in cells. The CycBPRE or $G_3$ PRE were only bound by Pum when Nos was present, and are only repressed when large amounts of Nanos are expressed. The ACA PRE mutant is not bound *in vitro* by Pum even when Nos is present, and it is repressed more weakly than other PRE reporters at high amounts of Nos expression. Thus, the level of Nos determines what affinity is necessary for a PRE to be bound and, to a certain extent, the magnitude of repression.

### 4.4.3 The Nanos N-terminus contains a novel Pum interaction domain

If RNA binding enhancement, which occurs through the Nos ZC regions, was sufficient for Pum enhancement, why does removal of the Nos N-terminus affect repression? The Nos N-terminus could be performing multiple roles, including reinforcing the Pum-Nos interaction or recruiting other factors which might aid in repression. To directly test the former hypothesis, we performed Co-IPs of Pum and Nanos. We were

surprised to find an RNA independent association of the two proteins, as this has not been observed previously. Using truncations of the Nos protein, we further dissected the region of Nos necessary for this interaction. The Nos N-terminus was necessary and sufficient for RNA independent association with Pum. Interactions between the PUF RBD and Nanos homologues have been observed by our group *in vitro* and by other groups using yeast hybrid assays and pulldown experiments with proteins from flies, frogs, and humans (Sonoda and Wharton 1999; Edwards et al. 2001; Nakahata et al. 2001; Jaruzelska et al. 2003). These interactions all depend on regions of Nos encompassing regions Z and C. We speculate that the novel association between Pum and the Nos-N terminus, coupled to the interaction of NosZC and the Pum RBD, might assist in Pum-Nos-RNA complex formation. This model might explain the partial loss of Nos mediated repression when the N-terminus is excluded. Further experiments are necessary to determine whether this interaction is direct and to identify what regions of Pumilio are required.

### 4.4.4 The Nanos N-terminus contains a Pum independent repression domain

The Nos N-terminus may serve another role by recruiting factors that enhance repression function rather than RNA binding. To evaluate the ability of each Nos domain to repress protein expression, we used the MS2 tethering assay to link Nos directly to an mRNA reporter. This method of Nos recruitment resulted in robust repression of the 2xMS2 reporter, much like the effect of untethered Nos expression on RnLuc Hb 3'UTR. However, similar mutations and truncations of tethered Nos had vastly different outcomes on reporter expression. Mutation or deletion of Nos Z and C had no effect on repression by tethered Nos. In fact, the Nos N-terminus was sufficient for near complete activity. There are many candidates for factors that might underlie the activity of this newly identified Nos repression domain (NRD); Pum, Brat, the NOT complex, and Cup have all been reported to bind Nos and function in translational regulation or mRNA decay. To assess which factors might contribute to NRD activity, we performed RNAi depletion of each. Only depletion of NOT complex components caused a minor reduction of repression. This suggests that the Nos N-terminus may be able to recruit an as yet unidentified translational regulator. This model could explain why the N terminus of Nos

is necessary for full repression of the Hb 3'UTR reporter in cells. Recruitment of the unknown factor by the NRD may contribute to the enhancement of Pum repression *in vivo*.

### 4.4.5 Implications and future directions

In summary, we have characterized two new mechanisms of Pum repression enhancement by Nos. Nos improves the binding affinity of the Pum RBD for RNA targets through assembly of a ternary complex. This strengthens binding to the PRE consensus and can also confer binding on non-canonical Pum targets. This ability of Nos extends to the enhancement of Pum repression of PRE and non-PRE containing reporter mRNAs in cells. A newly identified Pum interaction with the Nos N terminus may aid in ternary complex formation. Further, the Nos N-terminus contains an effective repression domain that remains active when other known Nos partners are depleted. Together, enhanced RNA binding and the recruitment of new repression activity could increase the magnitude of Pum repression and expand the breadth of Pum mRNA targets (Fig 4.21).

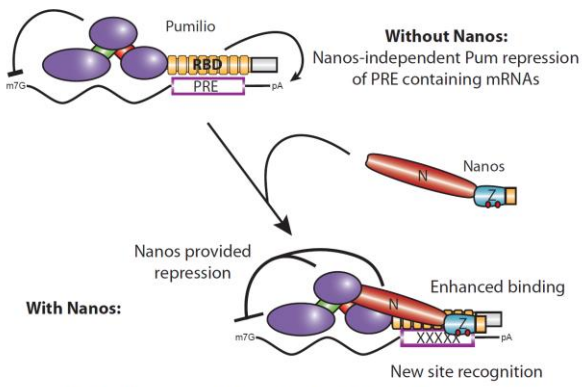


**Figure 4.21 - Nanos enhances Pumilio repression.** A model of Nanos-enhanced Pum repression. By itself, Pumilio can bind and repress mRNAs with high affinity PRE elements. The RBD (yellow) and N-terminal repression domains (purple) contribute activity. Assembled with Nanos, Pumilio can recognize new targets with degenerate PRE-like elements. The Nanos ZC region binds to the Pum RBD. The Nanos N-terminus also associates with Pumilio through an uncharacterized interaction. Recruitment of the Nanos N-terminus contributes repression to enhance Pum regulation.

How do these findings inform the biology of Pum and Nos proteins? Nos, through N-terminal and C-terminal interactions, shifts Pum from a partially inactive state to a fully active state. Nos does so through enhancement of RNA binding and the addition of repression. For the Hunchback RNA, this results in posterior specific Pum binding and repression, resulting in proper embryonic development. In the developing pole cells and germline stem cells, high concentrations of Nos confer Pum regulation on the weak PREs of Cyclin B mRNA. This prevents premature differentiation of the germline and ultimately maintains asymmetric stem cell division. In the nervous system, Nos could be localized in specific synaptic regions to control plasticity at the neuromuscular junction through

regulation of the sodium channel, Paralytic. The Paralytic mRNA has a PRE within its CDS, and Nos enhanced RNA binding may be required for Pum to access this site.

It is unclear whether Nos and Pum homologs interact synergistically like the fly proteins. PUFs are present throughout eukaryotes, while NOS proteins are conserved among metazoans. Germline regulation by PUFs and NOS proteins is a common feature (Jaruzelska et al. 2003, Lai et al. 2011, Nakahata et al. 2001), while only PUFs are implicated in the regulation of the mammalian nervous system (Driscoll et al. 2013, Vessey et al. 2010). Loss of the retinoblastoma tumor suppressor results in increased expression of Nos in flies and NOS in human cells, and consequently repression of mRNA targets containing PREs (Miles et al. 2014). Only the ZnFs of Nos and NOS proteins are highly conserved, yet the Nos N and C-terminal regions are both important for optimal enhancement of Pum repression. Additionally, the sequence between repeats 7 and 8 of the Pum RBD thought to interact with Nanos only exists in flying insects. Because mammalian PUFs and NOS proteins are reported to interact, the nature of their association must be distinct from Nos and Pum. The N-termini of NOS proteins from fish, amphibian, and mammals contain a motif that interacts with the NOT1 subunit of the CCR4-NOT deadenylase complex and tethering this domain to mRNA is sufficient to cause translational repression (Bhandari et al. 2014, Lai et al. 2011). The *Drosophila* Nos N-terminus does not contain this motif yet is capable of translational repression independent of the NOT complex. The interaction and enhancement mechanism of PUFs and NOS proteins might have changed, but the functional outcomes may remain. Whether or not mammalian NOS proteins are capable of enhancing PUF RNA binding is unknown.

The remaining questions are mechanistic in nature. How is the Pum-Nos complex assembled and how does this impart strengthened RNA binding? More quantitative analyses are required to assess the contributions of Nos to $k_{on}$ and $k_{off}$ rates of Pum RNA binding. It remains unclear whether Nos facilitates complex formation through a transient interaction with RNA or by changing the Pum RBD's ability to engage RNA, putatively through binding induced structural changes. Looking at the big picture, can Nos or proteins like Nos confer changes in the identities of Pum targeted RNAs transcriptome-

wide? Are the mRNAs targeted by Pum alone distinct from those bound when Nos is present? New approaches leveraging next generation sequencing technologies would inform what these changes might be. Lastly, what role does the Nos N-terminus play in the enhancement of RNA binding, if any? Importantly, what is/are the mechanism(s) of the NRD? Understanding the effects of the NRD on mRNA deadenylation, decay, and translation would inform what components are necessary for repression. Does the availability of each repression mechanism control the ultimate fate of an mRNA target? Depending on the collection of co-repressors available, Pum repression might lead to transcript silencing and storage, deadenylation and decay, or even activation of expression. Understanding how Nos modulates Pum repression remains integral to revealing how all PUF proteins might be regulated.

## 4.5 Materials and Methods

**Plasmids.** Plasmids used in this study included pAc5.1 FFluc, pAc5.1 RnLuc Hb 3'UTR, pAc5.1 RnLuc 2xMS2, pIZ HT, and the empty pUB 3xFLAG vector (mock transfections of Fig. 4.17), all of which were previously described in Chapters 2 and 3 (Weidmann and Goldstrohm 2012; Weidmann et al. 2014). The described changes to the RnLuc Hb 3'UTR reporter plasmid (UGU – ACA for each PRE) to create pAc5.1 RnLuc Hb 3'UTR m1, mt2, and mut1-2 were generated using inverse PCR. The pIZ HT-Nanos expression vector was created by inserting the coding DNA sequence of *Drosophila* Nanos protein (NP_001262723.1) into the XbaI restriction site of pIZ HT in frame with an upstream HaloTag coding sequence and TEV cleavage site and downstream V5 epitope and His6 elements. Importantly, we found that the Nanos sequence amplified from whole fly cDNA was that of Nanos isoform B, which lacks the 19 amino acid sequence aa14-VGVANPPSLAQSGKIFQLQ-32 present in the N-terminus of full length Nos isoform A (NP_476658.1). This sequence was not present in any constructs presented here and is thus unnecessary for all activities mentioned. However, the amino acid positions noted in the text correspond to Nos isoform A. This was meant to prevent confusion in reporting of the originally identified Cysteine mutations (C319Y and C354Y, which are C300Y and C335Y in isoform B) and domain separations. The C319Y and C354Y mutations were generated via Quickchange site directed mutagenesis, while the following truncations

were generated with inverse PCR: NZ includes aa1-373, ZC includes aa289-401, N includes aa1-294, Z includes aa289-373, and C includes aa374-401.

Bacterial expression vectors for expression of recombinant Pum and Nos were created with the pFN18K vector (Promega), which allows fusion of an N-terminal HaloTag to a protein of interest for purification. Sequence encoding the RNA binding domain of Pum (aa 1091-1426 of NP_001262403.1) was inserted into pFN18K via the Flexi® Cloning System (Promega) to create the Pumilio RBD WT vector. Quickchange site directed mutagenesis was used to generate the F1367S RBD mutant and the RBD mutR7 (S1342A N1343A E1346A) vectors. An identical approach was applied to create pFN18K NosZC and the C319Y and C354Y mutant vectors. A triple FLAG-tag was added with inverse PCR into the pFN18K Pumilio RBD such that the epitope would be incorporated at the N-terminus of the purified and eluted protein. Similarly, a V5 sequence was added to pFN18K NosZC such that the final protein included a C-terminal epitope.

Reporters used for Nos enhancement of Pum repression with varying PRE elements were all made based on the pAc5.1 RnLuc plasmid. Two changes were made to generate the pAc5.4 RnLuc base vector (RnLuc MCS in Fig. 4.16). First, a second cleavage and polyadenylation element intrinsic to the pAc5.1 vector was removed via inverse PCR, leaving only the strong SV40 element included in the original *Renilla* vector sequence. Second, a sequence within the RnLuc ORF that produced a near PUF site in the mRNA was synonymously mutated to eliminate possible background. The pAc5.4 RnLuc MCS vector contains a 3'UTR sequence between the stop codon and the SV40 cleavage and polyadenylation signal of the form:

(XhoI and NotI restriction sites in bold, insertion site is underlined)
5'TTCTAGGCGATCG**CTCGAG**CCCGGGAATTCGTTTAAACCTAGA**GC**GGCCGC**T**GGCCGC

To generate each pAc5.4 reporter, complementary DNA oligos (IDT) bearing wild type and mutant PRE elements from endogenous Pum targets were inserted into the XhoI and Not1 restriction sites of the pAc5.4 RnLuc MCS reporter. The oligos used are as follows:

(Restriction site overhangs in bold, PRE sequences underlined, mutations lowercase)

HbPRE2 Forward:

5’-**TCGA**CGAAAATTGTACATAAGCC

HbPRE2 Reverse:

5’-**GGCC**GGCTTATGTACAATTTTCG

HbPRE2 G3 Forward:

5’-**TCGA**CGAAAATTG**g**ACATAAGCC

HbPRE2 G3 Reverse:

5’-**GGCC**GGCTTATGT**c**CAATTTTCG

HbPRE2 ACA Forward:

5’-**TCGA**CGAAAAT**aca**ACATAAGCC

HbPRE2 ACA Reverse:

5’-**GGCC**GGCTTATGT**tgt**ATTTTCG

HbPRE1 Forward:

5’-**TCGA**CCAGAATTGTATATATTCG

HbPRE1 Reverse:

5’-**GGCC**CGAATATATACAATTCTGG

BcdPRE Forward:

5’-**TCGA**AAGTGATTGTAGATATCTA

BcdPRE Reverse:
5’-**GGCC**TAGATATCTACAATCACTT

CycBPRE Forward:
5’-**TCGA**GACTATTTGTAATTTATATC

CycBPRE Reverse:
5’-**GGCC**GATATAAATTACAAATAGTC

The FLAG-Pum vector used for co-immunoprecipitation of Nos and Nos truncations was generated by PCR amplification of full length Pumilio sequence including V5 and His6 tags from the pIZ Pumilio vector (Chapter 2) (Weidmann and Goldstrohm 2012) and insertion into the pUB FLAG vector. The pIZ MS2-Nanos plasmid was achieved through insertion of Nanos sequence in frame between the upstream MS2 coat protein and the downstream V5 His6 using KpnI and XbaI restriction sites. As with pIZ HT-Nos vectors, a combination of Quickchange site directed mutagenesis and inverse PCR was employed to generate MS2-Nos C354Y, MS2-NZ, MS2-ZC, MS2-NosN, and MS2-NosZ.

**Cell Culture.** D.mel-2 cells (Invitrogen) were cultured in Sf-900 III serum-free medium (Invitrogen) with 50 Units/mL penicillin and 50 µg/mL streptomycin using standard cell culture techniques. Cells were grown at 28°C.

**Transfections.** Transfections were performed very closely to previously described for D.mel-2 cells (Chapters 2 and 3) (Weidmann and Goldstrohm 2012; Weidmann et al. 2014). Each Effectene (QIAGEN) 6-well transfection was composed of 5ng FFLuc control plasmid, 10ng of RnLuc plasmid, 200ng total of expression vector, 43-44µl of EC buffer, 1.6µl of enhancer, 2µl of Effectene, 300µl of new sf900 III media, and 1.6mL of D.mel-2 cells (1M/mL). For untethered Nos experiments where mass was not indicated, 10ng of Nos expression vector was balanced to 200ng total with empty pIZ vector. For every Nos transfection gradient, pIZ was also used to balance total expression vector to 200ng. When using MS2 tethered Nos, 50ng was the standard amount. For transfections used

in Co-Immunoprecipitation of FLAG-Pum and HT-Nos, the transfections included 100ng of pUB plasmid bait, 300ng of pIZ-HT prey, 92µl of EC buffer, 3.2µl of enhancer, 4µl of Effectene, 300µl of new sf900 III media, and 1.6mL of D.mel-2 cells (1.5M/mL).

**RNA Interference.** As previously described, double-stranded RNAs (dsRNAs) were in vitro transcribed for RNAi including: non-targeting control (NTC) LacZ, Pum, Brat, Pop2, and Ccr4 (Van Etten et al. 2012; Weidmann and Goldstrohm 2012; Weidmann et al. 2014). The following primers were used to generate templates for production of Not1 and Cup dsRNAs, with T7 promoter sequence underlined and gene specific regions bolded:

Not1 Forward:
5'-GGATCCTAATACGACTCACTATAGG**CTTTACGCTCAGTTGCTGCAGGACC**

Not1 Reverse:
5'-GGATCCTAATACGACTCACTATAGG**CAAAGCAATCGCCTGAGTTCCCAC**

Cup Forward:
5'-GGATCCTAATACGACTCACTATAGG**GTACCACAATGGCAAGTCGCAGC**

Cup Reverse:
5'-GGATCCTAATACGACTCACTATAGG**ACGTTTCTCGCTCTGTTTCGCC**

Corresponding regions were amplified via PCR from D.mel-2 cDNA and dsRNA was transcribed *in vitro* and purified as previously described (Van Etten et al. 2012; Weidmann and Goldstrohm 2012; Weidmann et al. 2014). For knockdown, cells in one well of a 6-well plate, with total volume 1.6 mL, was treated with 6 µg of each dsRNA for 5 minutes before transfection.

**Luciferase Assays.** Luciferase assays were performed as previously described using dual glo assay (Promega) (Chapters 2 and 3) (Weidmann and Goldstrohm 2012;

Weidmann et al. 2014). A relative response ratio (RRR), from RnLuc signal/FFLuc signal, is calculated for each sample. The ratio is normalized to the control (set to 100). Percent repression is derived from the equation 100*(1-($RRR_{WT}$/$RRR_{Negative\ Control}$)), where $RRR_{WT}$ is from a sample transfected with an active regulator and $RRR_{Negative\ Control}$ comes from a sample transfected with an equivalent amount of an inactive negative control. The pIZ-HT vector was used as the inactive control for HT-Nos constructs. The control for tethered function experiments was the MS2 expression vector, pIZ MS2.

**Immunoprecipitation.** For FLAG immunoprecipitations from D.mel-2 cells, two duplicate 6-wells of 2 mLs of 1.5 million cells/mL were transfected (Effectene) with 300 ng of pIZ HT prey plasmids (HT control, HT-Nanos, HT-N, or HT-ZC) and 100 ng bait (empty pUB or FLAG-Pum). Forty-eight hours post-transfection, the 4mL of transfections were pelleted for 3 minutes at 1000xg and lysed in 250 µL lysis buffer containing 0.2% Igepal CA-630 (USB), 50 mM Tris-HCl (pH 8.0), 5mM $MgCl_2$, and 150 mM NaCl. Protease inhibitors were added to final concentrations of 2 mM phenylmethylsulfonyl fluoride [PMSF], 20 µg/ml aprotinin, 20 µg/ml pepstatin, and 20 µg/ml leupeptin. RNase ONE and RNase A were also added to 50 U/mL and 10 µg/mL, respectively. Lysates were passed through a 25 gauge needle 5x before incubation on ice for 1 hour. Lysates were cleared by centrifugation at 16,000xg for 2 minutes. Lysates were then diluted 4-fold into buffer containing 50 mM Tris-HCl (pH 8.0), 5mM $MgCl_2$, and 150 mM NaCl. A portion of this diluted lysate (40µl) was saved as input. FLAG containing complexes were precipitated from remaining volume using 20µl M2 Affinity Resin (Sigma) that had been washed three times in lysis dilution buffer. Complexes were bound four 24 hours at 4°C. After binding, the resin was washed a total of 9 times in 1mL wash buffer (50 mM Tris-HCl (pH 8.0), 5mM $MgCl_2$, and 300 mM NaCl) with a decreasing amount of detergent (3x with 0.5% Igepal CA-630, 3x with 0.2%, and 3x with 0.05%). Beads were eluted for 24 hours at 4°C in 50 µl elution buffer containing 50 mM Tris pH 8.0, 300 nM NaCl, 5 mM MgCl, 0.05% Igepal CA-630 and 300 ng/µl of 3xFLAG peptide.

**Bacterial Expression and Purification of Recombinant Protein**. KRX bacterial cells (Promega) were transformed with pFN18K plasmids, plated on LB media with kanamycin, and grown at 37°C. A single colony was moved into 25mL of liquid culture with agitation at 37°C for 16 hours. 10mL of this culture was diluted into 750mL of 2xYT media with 25µg/mL Kanamycin and 2mM $MgSO_4$ and incubated at 37°C with shaking. When the culture reached $OD_{600}$ of 0.7-0.9, cells were induced by adding 5mL of 20% Rhamnose (0.1% final) and grown at 37°C for 3 hours. Cells were pelleted at 7000xg for 10 minutes, washed in 30mL of Bug Wash (50 mM Tris-HCl, pH 8.0, 10% Sucrose) and pelleted again.

Pellets were resuspended in 25mL binding buffer including 50mM Tris-HCl pH 8.0, 0.5mM EDTA, 2mM $MgCl_2$, 150mM NaCl, 1mM DTT, 0.05% Igepal CA-630, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml pepstatin, and 10 µg/ml leupeptin. To lyse cells, lysozyme was added to a final concentration of 0.5mg/mL and cells were incubated at 4°C for 30 minutes with gentle rocking. $MgCl_2$ was then increased to 7mM concentration and DNase I (Roche) was added to 10µg/mL before continuing incubation for another 20 minutes. Lysates were cleared at 50,000xg for 30 min at 4°C. HaloTag containing proteins were bound with 500µl of pre-washed HaloLink Resin (Promega) for 24 hours at 4°C. Beads were washed 3 times with Wash Buffer (50mM Tris-HCl pH 8.0, 0.5mM EDTA, 2mM $MgCl_2$, 1M NaCl, 1mM DTT, 0.5% Igepal CA-630) and 3 times with Elution Buffer (50mM Tris-HCl, pH 7.6, 150mM NaCl, 1mM DTT, 20% Glycerol). After washing, beads were resuspended in 400µl of Elution Buffer with 30U of AcTEV protease (Invitrogen) and cleavage proceeded for 24 hours at 4°C. After removal of beads, the concentration of cleaved protein was measured compared to BSA standards through SDS-polyacrylamide (4-20%) gel electrophoresis and subsequent Coomassie staining.

**Fluorescent and Radioactive Electrophoretic Mobility Shift Assays.** All RNA binding reactions were performed in RNA Binding Buffer (50mM Tris-HCl pH 7.6, 150mM NaCl, 2mM DTT, 2µg/mL BSA, 0.01% Igepal CA-630, 0.02% Bromophenol Blue, 20% Glycerol). Reactions were left to equilibrate for 3 hours at 4°C. A 5% native polyacrylamide TBE mini-PROTEAN gel (BIO-RAD) was pre-run for 3 hours at 50V before loading 5µl of each sample. Gels were run at 50V for 2-2.25 hours at 4°C. For fluorescent EMSAs, RNA was

imaged immediately via fluorescence imaging with a Typhoon Trio imager (GE Healthcare). For the fluorescent EMSAs, the concentration of target RNA was held constant at 1nM, while the concentrations of Pum RBD and Nos ZC are as noted in Figure 4.8. The fluorescent RNA target used contained the following sequence (the PRE element is underlined):

Cy5 Nanos Response Element RNA

5’-Cy5-rUUGUUGUCGAAAAUUGUACAUAAGCC

For the $K_{d\ obs}$ measurements, radioactive RNA oligos were used at a constant concentration of 300pM. RNAs were labeled with $^{32}P$ at their 5’ ends using T4 DNA Ligase (NEB) with ATP [λ-$^{32}P$] (Perkin-Elmer). RNA oligos (IDT) used included the following (with PRE elements underlined and mutations in lowercase bold):

HbPRE2 RNA:
5’-rAAAUUGUACAUAAGCC

HbPRE2 G3 RNA:
5’-rAAAUUG**g**ACAUAAGCC

HbPRE2 ACA RNA:
5’-rAAAU**aca**ACAUAAGCC
HbPRE1 Forward:
5’-rGAAUUGUAUAUAUUCG

BcdPRE Forward:
5’-rUGAUUGUAGAUAUCUA

CycBPRE Forward:
5’-rUAUUUGUAAUUUAUAUC

Replicate EMSAs (N = 3) were performed for each RNA to calculate $K_{d\ obs}$. For HbPRE2 RNA, the concentration of NosZC in the +NosZC EMSAs was held constant at 400nM while the gradient of Pum RBD concentrations included 0nM, 0.2nM, 0.5nM, 1nM, 2nM, 5nM, 10nM, 20nM, 50nM, 75nM, 100nM, 150nM, and 200nM. For all other RNAs tested, the +NosZC condition held NosZC concentration at 1µM while Pum RBD concentration gradient included 0nM, 2nM, 5nM, 10nM, 20nM, 50nM, 100nM, 200nM, 300nM, 400nM, 500nM, 750nM, and 1000nM. Gels containing radioactive RNAs were dried at 70°C for 30 minutes and vacuum affixed to Whatman paper. The radioactive gels were then exposed to a storage phosphor screen for 16 hours. The signal on the screen was captured with a Typhoon Trio imager (GE Healthcare) and subsequently quantified using ImageQuant TL Software (GE Healthcare). Fraction bound values were plotted against RBD concentration for multiple EMSAs and $K_{d\ obs}$ was calculated via nonlinear regression analysis within the GraphPad Prism software (GraphPad Software, Inc.).

**Western blotting.** Western blotting from luciferase assay samples was performed as previously described in Chapters 2 and 3 (Weidmann and Goldstrohm 2012; Weidmann et al. 2014). Proteins were separated via SDS-polyacrylamide (4-20%) gel electrophoresis and transferred onto Immobilon-P membranes (Millipore). All membranes were probed with either V5 monoclonal antibody (Invitrogen) or monoclonal anti-FLAG M2 antibody (Sigma). Secondary detection was performed using horseradish peroxidase conjugated goat anti-mouse IgG (Thermo Scientific). Signals were detected using Immobilon western chemiluminescent substrate (Millipore) and autoradiography film.

**Fluorescent labeling and visualization of Halotag protein constructs.** Protein extracts from D.mel-2 cells expressing HaloTag fusions were incubated with 100nM HaloTag TMR Ligand (Promega) for 30 min on ice, protected from light. After labeling, extracts were separated via SDS polyacrylamide (4-20%) gel electrophoresis and detected by fluorescence imaging with a Typhoon Trio imager (GE Healthcare).

**cDNA preparation and qPCR.** For measurement of endogenous mRNA knockdown, RNAs were primed with random hexamers (IDT) for synthesis of cDNAs

using GoScript reverse transcriptase (Promega). The final concentration of RNA in these reactions was 200 µg/mL. To measure endogenous mRNA levels, quantitative PCR (qPCR) was performed on 5 µl of cDNA product in a 50 µl reaction using 100 nM of gene specific primers and GoTaq qPCR master mix (Promega) as described previously in Chapters 2 and 3 (Weidmann and Goldstrohm 2012; Weidmann et al. 2014). Standard negative control reactions were performed without reverse transcriptase. Differences in mRNA levels were calculated using the $\Delta\Delta C_t$ method. $C_t$ values were measured and normalized to the internal control Rpl32 mRNA to generate $\Delta C_t$. $\Delta\Delta C_t$ was derived relative to the non-targeting control $\Delta C_T$ (Livak and Schmittgen 2001; Schmittgen and Livak 2008). qPCR primers for Rpl32, Pum, Brat, Pop2, and Ccr4 were previously published (Van Etten et al. 2012; Weidmann and Goldstrohm 2012; Weidmann et al. 2014) The qPCR primer sequences for additional *Drosophila* genes are as follows:

Not1 Forward: 5'- CTTTAACTCGAGCAGCGACTACAGC

Not1 Reverse: 5'- CTGGTTCTGTTGCGTGTACAGTGC

Cup Forward: 5'- CTGAAGGCGATCCTCGGCC

Cup Reverse: 5'- CGATCCATGTCCGTCAAGCG

## 4.6 References


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# CHAPTER 5

# CONCLUSIONS

## 5.1 Summary of Thesis

### 5.1.1 Repression and regulatory domains of Pumilio

This dissertation expands the understanding of how PUF proteins work to control the flow of genetic information. My findings elucidate combinatorial control of mRNA regulation by the multifunctional RNA binding proteins Nanos and Pumilio. This work has focused on molecular mechanisms, but the results apply to the important biological processes directed by PUF post-transcriptional regulation. Contributions to the field include elucidation of regions of PUFs necessary for activity, a novel means of regulating PUF activity, new factors involved in PUF mediated repression, and, in the case of Nanos, how partner proteins can influence the outcome of PUF control through RNA binding enhancement and accessory repression recruitment.

My first major finding addressed the existing model of *Drosophila* Pumilio (Pum) repression. Nanos (Nos), and sometimes Brain Tumor, are necessary for Pum mediated repression of important mRNAs throughout the fly life cycle. The existing model suggested that Pum was only necessary for RNA binding, and the partners Nos and Brain Tumor recruited factors necessary for translational repression. I developed and optimized a cell-based luciferase reporter assay that measures Pum repression. I observed that while Nos could stimulate Pum repression, both Nos and Brain tumor were dispensable for Pum's activity (Chapter 2). Additionally, the RNA binding domain (RBD) of Pum was

not sufficient for full repression. Instead, a combination of four repression domains, including three N-terminal regions and the RBD, were necessary for complete activity.

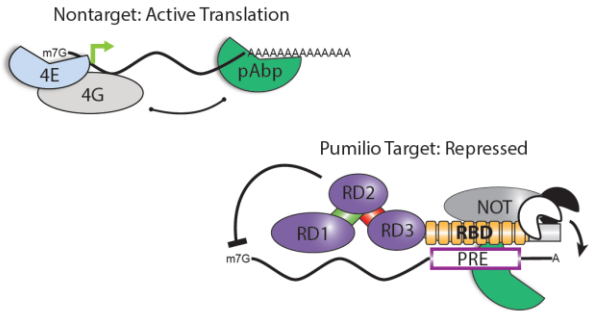


**Figure 5.1 - Multifunctional Pumilio repression.** Model of Nanos independent Pumilio repression based on data from Chapters 2 and 3. mRNAs not bound by Pumilio are actively translated. mRNAs with Pum response elements (PREs) are bound by Pumilio and undergo pAbp interference and accelerated deadenylation caused by the RNA binding domain (RBD). Additionally, three repression domains (RDs) in the Pumilio N-terminus mediate poly(A) independent repression

Each domain could repress independently when tethered directly to an mRNA. The ability of PUF N-terminal domains to repress was conserved in human PUM1 and PUM2 as well. My work discovered these multiple sources of repression activity and revealed how Pum can use multiple mechanisms to achieve target inhibition (Fig. 5.1).

Another important discovery was made during the analysis of the Pum N-terminus: two conserved regulatory motifs that differentially modulate repression domain function. One domain, PCMb (Pumilio conserved motif B), could completely block the activity of two Pum repression domains. Another region, PCMa (Pumilio conserved motif A), counteracts inhibition by PCMb. The PCMa region was also somewhat modular, as it could be fused in a non-native conformation to restore repression to the inhibited PCMb-RD3 construct. This was the first demonstration of auto-regulatory domains within the Pum N-terminus (Fig. 5.2).

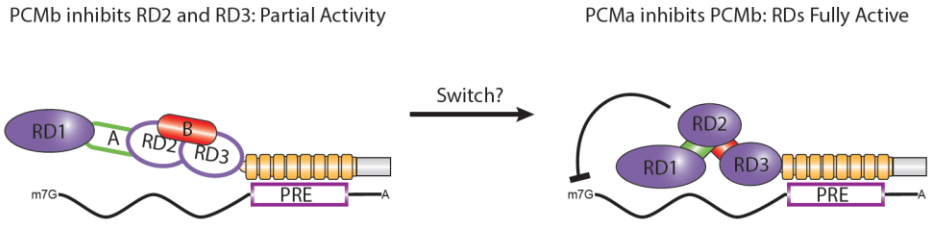


**Figure 5.2 - Autoregulatory regions mediate repression domain activity.** Model of Pumilio autoregulatory switch. The Pumilio conserved motif B (PCMb) inhibits repression domain (RD) 2 and 3 activity. The presence of Pumilio conserved motif A (PCMa) counteracts PCMb, restoring repression activity. Factors that control the interaction between these motifs, and thus determine Pumilio activity, are unknown.

### 5.1.2 Mechanisms of mRNA repression by the PUF RNA binding domain

My work has also clarified a portion of Pum's Nanos independent repression mechanism (Chapter 3). I first evaluated a model of repression wherein the PUF RBD bound to Argonaute proteins to interfere with translation elongation through eEF1A. While I confirmed the association of PUF RBDs (fly and human) with Argonautes, I showed that the interaction was not integral to PUF function. Depletion of Argonaute proteins or elimination of Argonaute-RBD binding did not affect repression by the PUF RBD. Instead, I confirmed the conserved role of deadenylation in RBD activity. I showed that the Pum RBD could accelerate deadenylation of an mRNA target. This deadenylation was dependent upon the Ccr4-Not deadenylase complex. Homologs of the NOT complex subunit, Pop2, interact directly with PUF proteins in eukaryotes ranging from yeast to humans. RBD mediated deadenylation acceleration is likely a conserved mechanism of all PUF proteins.

However, further experiments revealed that deadenylation was a consequence of Pum RBD repression, not a prerequisite. RBD activity did depend on the presence of a poly(A) tail, however, as it was unable to repress an mRNA that terminated with a Histone stem loop. This could be overcome by insertion of a poly(A) tract within the 3'UTR. I found that this was due to the RBD's reliance on the poly(A) binding protein, pAbp, for translational inhibition. The RBD binds pAbp and prevents its ability to stimulate translation. Unlike other regulators, the RBD does not exclude pAbp from poly(A) RNA, it merely blocks pAbp's ability to promote initiation. I saw that repression by a human PUF protein was also impaired by depletion of the human pAbp homolog, PABPC1. This is evidence of a conserved mechanism of translational inhibition by PUF RBDs. One important caveat: the N-terminal Pum repression domains do not require pAbp or poly(A) and operate through an as yet unknown mechanism. This allows Pum to control gene expression in different contexts where one pathway may or may not be available.

### 5.1.3 Pumilio enhancement by Nanos

My work has also led to a new interpretation of the role that Nos plays in Pum repression. Nos is certainly necessary for regulation of specific Pum mRNA targets *in*

*vivo*, but my experiments offer a new explanation for this requirement beyond a simple co-recruitment model (Chapter 4). Pum can repress without Nos, but the addition of Nanos stimulates Pum activity. Using my cell based assay, I was able to identify regions of Nanos sufficient for Pum enhancement. The Nos zinc fingers and C-terminal extension (ZC) were most important, but the Nos N-terminus also contributed. I tested whether Nos had any effect on the binding of RNA by the Pum RBD. The Nos ZC region improved the affinity of the RBD for an RNA containing the Pum Response Element (PRE) *in vitro*. In fact, Nos ZC permitted the Pum RBD to bind to RNAs with weak PRE sites that the Pum RBD could not bind to alone. Thus, Nos enhances Pum RNA binding. The expression of Nos in cells could also confer Pum repression on mRNA reporters bearing degenerate or mutant PRE sites. This enhancement of RNA binding is likely why Nos is required *in vivo* for Pum repression; the localization of Nos determines where Pum will bind RNAs. For example, this explains why Pum repression of the Hunchback mRNA is restricted to the posterior where Nanos is expressed. It also may underlie why high concentrations of Nanos are necessary in the pole cells to regulate through the Cyclin B PRE, a highly degenerate binding site. These observations also suggest that Pum, with the help of Nos, could bind an entirely new set of mRNA targets without canonical PREs (Fig. 5.3).

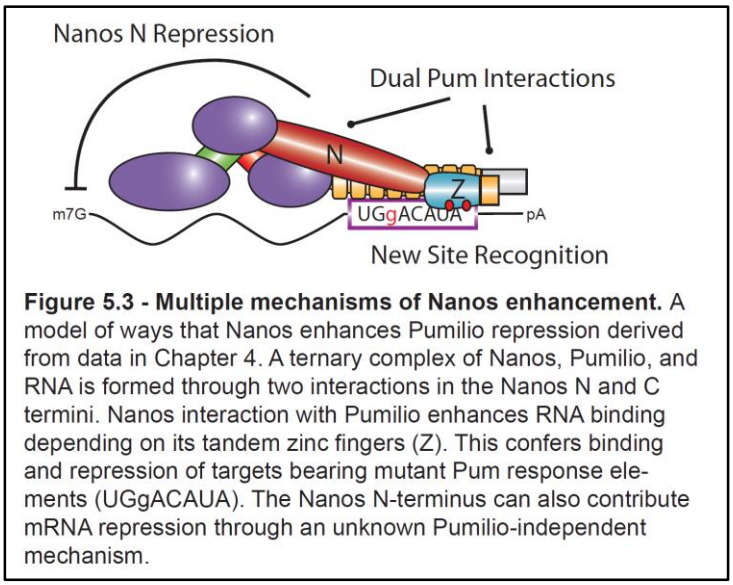


**Figure 5.3 - Multiple mechanisms of Nanos enhancement.** A model of ways that Nanos enhances Pumilio repression derived from data in Chapter 4. A ternary complex of Nanos, Pumilio, and RNA is formed through two interactions in the Nanos N and C termini. Nanos interaction with Pumilio enhances RNA binding depending on its tandem zinc fingers (Z). This confers binding and repression of targets bearing mutant Pum response elements (UGgACAUA). The Nanos N-terminus can also contribute mRNA repression through an unknown Pumilio-independent mechanism.

Lastly, I uncovered new roles for the Nos N-terminus that could contribute to Pum enhancement. First, the Nanos N-terminus is responsible for an RNA independent association with Pum. This could reinforce formation of a Pum-Nos-RNA complex or assist in the recruitment of Pum co-repressors. Second, I found that tethering the Nos N-terminus can lead to Pum-independent repression. Depletion of other proposed Nanos partners (Brat, Not, or Cup) did not impede activity, suggesting a novel mechanism of repression. Nos might recruit additional repression activities to the complex to ensure

target inhibition. The mechanism of repression utilized by Pum and Nanos N-termini remain to be elucidated in detail.

## 5.2 Future Directions

### 5.2.1 How do the N-terminal repression domains work?

While my work has made significant strides in understanding PUF activity, it has also generated new lines of inquiry. Importantly, through what mechanisms do the N-terminal Pum repression domains (RDs) exert their function? Their sequence is not very conserved, although both *Drosophila* and human PUFs maintain repression domains in their N-termini. A conserved structure may be important for function. However, based on sequence analysis and structure prediction, the RD sequences appear to be highly disordered and of low complexity. For instance, the Pum RDs contain multiple regions of poly-glutamine thought to confer prion-like aggregation (Salazar et al. 2010). These domains also mediate assembly into distinct aggregate foci. Mammalian Pumilio 2 (Pum2) exists in Ribonucleoprotein (RNP) particles localized in dendrites and also assembles into larger aggregate stress granules (Vessey et al. 2006). Stress granules and another type of RNP granule, processing bodies, are thought to be sites of mRNA storage and perhaps direct mRNA turnover through coordination of decay factors (Decker and Parker 2012). Preliminary experiments with further truncations of each RD have not clarified what is necessary. Multiple motifs within each repression domain, including those without poly-glutamine, seem to contribute to full activity.

Our lab has pursued multiple approaches to identify partners involved in activity: including a limited RNAi screen, a yeast 2-hybrid approach, and mass spectrometry. We have identified putative interactors, but no factor necessary for repression was identified. A genome-wide RNAi screen could identify factors involved in function, but the reporter assay must be adapted for this procedure. Likely candidates might include enhancers of decapping, translation initiation factors, or ribosomal components. Refinements in the mass spectrometry approach might also be helpful. This could include purification of RNA bound complexes rather than immunoprecipitation of the protein domains alone. Such an approach might enrich for active Pum complexes rather than all factors which can bind or regulate. Microscopy would be useful to test whether each RD changes the localization

of mRNA targets. If the RDs truly aggregate in granules, this should be easy to observe. Alternatively, the Pum RDs may have some amount of activity towards RNA on their own, possibly nucleolytic or RNA binding. *In vitro* assays could address whether the RDs bind and or degrade RNA molecules. A necessity for this approach would require new methods of purifying these domains. Similar approaches will be useful in describing the mechanism(s) of the Nanos repression domain as well.

### 5.2.2 What is the purpose and mechanism of autoregulation?

Analysis of the Pum N-terminus revealed the existence of auto-regulatory domains PCMa and PCMb. These regions are more conserved than the repression domains themselves and are arrayed in a similar fashion in fly and human PUFs. How do these auto-regulatory domains operate? Work describing the fly phosphoproteome revealed that region PCMa is highly phosphorylated (Zhai et al. 2008). This post-translational modification may act as a switch between active and inactive RDs. The modification state of PCMa might determine whether PCMb plays an inhibitory role. Upon kinase/phosphatase activity on PCMa, PCMb could shift its regulatory state. Mutational analysis of each phosphorylated site in PCMa would be necessary to confirm their role in regulation. How PCMa and PCMb modulate the RDs is also unclear. There is no current evidence that PCMb can act to inhibit RD2 or RD3 in cells when it is not physically fused, however PCMa added *in trans* has a limited ability to block PCMb inhibition. PCMb may inhibit recruitment of co-repressors through a change in localization or through modifications of local structure. PCMa might counteract this activity through association with PCMb. We have initiated *in vitro* binding assays meant to identify direct interactions between PCMa and PCMb. Microscopy approaches could delineate a mechanism of differential localization from one of dynamic structure. Tethering PCMb to each RD might drastically change its position in the cell. Structural characterization of the RDs alone and fused to each regulatory motif could highlight how a switch might occur. It is also possible that Nos might interact with these motifs, although new *in vitro* interaction and functional assays would need to be developed to separate other mechanisms of Nos enhancement from PCMa/b modulation.

### 5.2.3 Towards a complete mechanism of PUF RBD mediated repression

My work contributes mechanistic insight to conserved mechanisms of PUF RBD repression. While Argonautes were not necessary for RBD activity, poly(A) and pAbp were required. Multiple reports suggest a link between PUFs and small RNA mediated repression (Kedde et al. 2010; Friend et al. 2012; Miles et al. 2012). These events may be mRNA specific and underlie co-regulation by PUFs and RISCs in particular contexts. The use of poly(A) binding proteins to inhibit translation appears to be a mechanism conserved from yeast PUFs to human PUFs (Chritton and Wickens 2011; Weidmann et al. 2014). Since the RBD does not evict pAbp from mRNA, it is unclear how exactly translation inhibition is achieved. The RBD and pAbp interact in an RNA independent manner, but the nature of this interaction has not been fully described. Is the interaction direct? Does pAbp binding to the RBD exclude eIF4G from binding to pAbp? A combination of recombinant pull-downs and RNA-immunoprecipitation experiments might address these questions.

A surprising feature of RBD regulation was the lack of a requirement for the Ccr4-Not deadenylase complex. PUF RBDs throughout eukaryotes maintain direct interactions with the Pop2 deadenylase subunit. Indeed, my work confirmed that the RBD accelerates Ccr4-Not dependent deadenylation. However, this activity was not required. Instead, it appears that deadenylation is a consequence of pAbp directed translational inhibition. Depletion of pAbp eliminated reporter deadenylation. This was an unexpected result, as the presence of pAbp is thought to prevent deadenylation by Ccr4-Not. However, the Ccr4-Not complex can be recruited by pAbp through accessory proteins (Ezzeddine et al. 2007). It is possible that RBD binding initiates a conformational change in pAbp that results in a shift from being protective to promoting degradation. What allows deadenylation to proceed? Pop2 and the RBD also interact independent of RNA, yet deadenylation is not accelerated unless pAbp dependent translational inhibition has occurred. Perhaps the RBD-pAbp interaction occludes the pAbp-poly(A) interface without fully evicting pAbp from the RBD bound complex. A better understanding of the RBD-pAbp-Pop2 interaction is necessary to test this hypothesis.

### 5.2.4 What is the nature of RNA binding and repression enhancement by Nanos?

The most intriguing finding was that of the role of Nanos in enhancing Pum RNA binding. The interaction between Pum, Nos, and RNA has been known for many years. However, the formation of the complex could only be inferred indirectly from pull-downs and yeast hybrid assays (Sonoda and Wharton 1999; Edwards et al. 2001). I was excited to demonstrate the ternary complex with purified proteins and native EMSA. It was proposed that Pum had to bind Nos to confer repression onto its targets through recruitment of the Ccr4-Not complex (Kadyrova et al. 2007). Instead, I found that Nos has a large effect on the RBD's ability to bind to RNA. Strikingly, Nos conferred Pum binding *in vitro* and repression in cells to RNAs with degenerate Pum response elements. How can Nanos do this? Three hypotheses remain to be tested: 1) Nanos could be transiently interacting with RNA and orienting the PRE for binding by the RBD, 2) Nanos might bind the RBD and encourage a conformation with higher RNA affinity, or 3) RBD binding to a PRE provides a platform for Nos binding, which subsequently locks the RBD in place. While EMSA has proven useful for generating relative $K_d$ measurements, it has been difficult to infer actual rates. More precise approaches would assist in the determination of $k_{on}$ and $k_{off}$ values. A stopped-flow approach might be able to capture a relatively fast $k_{on}$. Single molecule approaches with fluorescently labeled proteins and immobilized RNA could also be appropriate for monitoring the kinetics of complex formation. Alternatively, structural information on this complex could deliver insight into how the association of Nanos changes Pum's engagement with mRNA.

Lastly, because the effect of Nanos is so drastic *in vitro* and in cells, it is important to evaluate our assumptions about what a Pum target really is. If proteins like Nanos can significantly change the affinity of PUFs and putatively relax their specificity, there may be a novel subset of mRNAs that PUFs regulate. One might ask whether the presence of Nanos can significantly change what targets PUM binds transcriptome-wide. Advanced sequencing technology will allow measurement of changes in PUM binding and regulation globally. Are targets of Pum alone distinct from targets of the Pum-Nos complex? High throughput sequencing coupled to cross-linking and immunoprecipitation (HITS-CLIP) or Photoactivatable ribonucleoside cross-linking and immunoprecipitation (PAR-CLIP) are

both techniques that might assess whether Nos simply expands the list of Pum targets by relaxing specificity or actually changes Pum targets by altering specificity. Experiments *in vivo* reveal that mutations in nucleotides upstream of the Hunchback PRE impair Nos-Pum-RNA complex formation and Hunchback regulation (Wharton et al. 1998). This site might be bound by Nos and thus determine the altered specificity of the Nos-Pum complex compared to Pum alone. This could be quickly tested *in vitro* using the established EMSA protocol for ternary complex formation. Additionally, a systematic evolution of ligands by exponential enrichment (SELEX) approach might also be appropriate to screen for changes in the RNAs that Pum-Nos might prefer.

In conclusion, my work has moved the field of PUF regulation forward on many fronts. This dissertation has answered many questions and raised many more. It lays the foundation for future studies of the mechanisms of PUF regulation, and it informs how PUFs might be controlled within the biological processes they operate.

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