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Post-transcriptional Regulatory Functions of Mammalian Pumilio Proteins

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Abstract

Mammalian Pumilio proteins, PUM1 and PUM2, are members of the PUF family of sequence specific RNA-binding proteins. This review explores their mechanisms, regulatory networks, biological functions, and relevance to diseases. Pumilio proteins bind an extensive network of mRNAs and repress protein expression by inhibiting translation and promoting mRNA decay. Oppositely, in certain contexts they can activate protein expression. Pumilio proteins also regulate non-coding RNAs. The non-coding RNA, *NORAD*, can in turn modulate Pumilio activity. Genetic analysis provides new insights into Pumilio protein function. They are essential for growth and development. They control diverse processes including stem cell fate and neurological functions such as behavior and memory formation. Novel findings show that their dysfunction contributes to neurodegeneration, epilepsy, movement disorders, intellectual disability, infertility, and cancer.

Keywords

Pumilio; PUM1; PUM2; RNA-binding protein; Post-transcriptional gene regulation

RNA decay

Pumilio proteins are conserved regulators of mRNA fate

Post-transcriptional mechanisms dynamically control the magnitude and spatio-temporal pattern of protein expression, orchestrated by cis-acting RNA features and trans-acting RNA-binding factors [1, 2]. This review focuses on mammalian Pumilio proteins, which belong to the eukaryotic PUF family of sequence-specific, RNA-binding proteins that control mRNA fate [3]. Pumilio was discovered in *Drosophila* by virtue of its essential role in embryonic development [4–6]. Thereafter, Pumilio proteins have emerged as archetypal

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post-transcriptional regulators, and much is known about invertebrate orthologs [3, 4, 7, 8]. This review describes progress in understanding the regulatory roles of mammalian Pumilio proteins, including new insights into their functions in stem cells, fertility, development, and the nervous system and how their dysfunction contributes to neurological diseases and cancer.

Defining characteristics of Pumilio proteins

Mammals possess two cytoplasmic ‘classical’ Pumilio proteins, PUM1 and PUM2, which are closely related to each other (76% identical) and to *Drosophila* Pumilio (30% identical) (Figure 1). The classical Pumilio proteins are characterized by exquisite sequence specificity dictated by a unique, highly conserved RNA-binding domain, the Pumilio Homology Domain (Pum-HD) (80% identical between human and *Drosophila*). The Pum-HD comprises eight structural repeats of a ~36 amino acid motif (Figure 1A). These motifs were recognized in founding members *Drosophila* Pumilio (Figure 1B) and *Caenorhabditis elegans* *fem-3* binding factor (FBF) and are referred to as Pum/PUF repeats [9–12].

Classical PUMs ranging from insects to humans also share a unique N-terminal region that lacks homology to other proteins and is predicted to be intrinsically disordered overall (Figure 1A and B)[13]. The N-terminal regions of PUMs show higher rates of evolution, including variable low complexity regions, insertions, and deletions, with the exception of two segments designated Pumilio Conserved Motifs (PCMa and PCMb)(~37% and ~62% identity between *Drosophila* and human PUMs, respectively)(Figure 1C)[13]. It is noteworthy that multiple isoforms of PUM1 and PUM2 encoding mRNAs have been cataloged (for example, see NCBI Gene ID: 9698 for human *PUM1* and Gene ID: 23369 for human *PUM2*), including alternative transcription initiation sites, 5′ exons, internal exons, and 3′ processing events. These mRNA isoforms have the potential to impact the function and expression of the PUMs, an area of inquiry that remains unexplored.

Mammals also have two ‘divergent’ PUMs, PUM3/Puf-A and NOP9 (Figure 1D) that are conserved in eukaryotes [14]. They have Pum-HD regions; however, their RNA-binding domains are extended with 11 Pum repeats [15, 16]. In contrast to classical PUMs, divergent PUMs have predicted nuclear localization signals (NLS), are predominantly nucleolar, and function in ribosome biogenesis [17, 18]. The PUM3 family appears to bind to structured RNA or DNA and lacks sequence specificity, making these proteins quite distinct from classical PUMs [15]. NOP9 proteins are a hybrid of classical PUM and PUM3 traits; they bind to both specific sequences or structured elements in pre-ribosomal RNA [16, 19–21]. As less is known about their functions in mammals, this review does not address divergent PUMs further.

Structure and specificity of Pumilio proteins

Epiphanies in Pumilio function came when *Drosophila* Pumilio was shown to bind short RNA motifs in the 3′ UTR of the *hunchback* (*hb*) mRNA to control embryogenesis [11, 22, 23] and *C. elegans* FBF was shown to bind a related motif in the *fem-3* mRNA to control gametogenesis [12]. In both instances, the Pum-HD regions conferred specific RNA interaction and resulted in repression of their respective target mRNA. Although the

functions of mammalian PUM1/2 were not known at that time, their Pum-HDs bound to the same RNA motifs in the *hb* mRNA [11, 24]. The binding specificities for Pumilio proteins from a variety of species, including *Drosophila* to humans, are now well-characterized [11, 22, 24–35]. The consensus binding site for proteins closely related to Pumilio, 5'UGUAHAUA, herein referred to as the Pumilio Recognition/Response Element (PRE), is well conserved.

Structural studies illuminated how Pumilio proteins specifically recognize RNA sequences. Crystal structures of the Pum-HDs of *Drosophila* Pumilio and human PUM1 revealed α -helical Pum repeats that formed a curved structure (Figure 2A)[36, 37]. The most highly conserved amino acid residues line the inner concave surface of the Pum-HD. Crystal structures of PUM1 in complex with the PRE from *hb* mRNA illuminated the elegant mechanism of RNA sequence recognition by Pum repeats [31]. Each repeat employs three side chains, referred to as a tripartite recognition motif (TRM)[38], to recognize an RNA base. Two side chains form hydrogen bond or van der Waals contacts with an edge of the base and a third side chain forms a stacking interaction. Remarkably, the particular sets of side chains specify the RNA base recognized [39]. For example, the TRM motif NYxxQ (x is a hydrophobic residue) uses edge-interacting Asn and Gln and base-stacking Tyr to specify uracil (Figure 2B). This TRM can be abbreviated NQ/Y. The PUM1-RNA structure also revealed TRMs that specify adenine (CQ/R or SQ/R) and guanine (SE/N). Selection experiments later identified TRMs that specify cytosine (SR/Y)[40, 41].

Once mRNA targets of mammalian PUMs were identified, crystal structures of human PUM1 and PUM2 in complex with their PREs showed that PUM1 and PUM2 bind similarly to these sequences as to *hb* RNA (Figure 2C)[32]. These structures also showed that sequence variations at the 5th nucleotide position result in different modes of RNA recognition in that region. The Hoogsteen edge of an A5 base may be recognized instead of the Watson-Crick edge, and a C5 base may stack directly with the A4 base and not contact the protein (highlighted in Figure 2C). The fact that *Drosophila*, mouse, and human PUMs have identical TRMs embedded within highly similar Pum-HDs of nearly identical structure (1.2–1.6 Å rmsd between *Drosophila* and human, 1.4 Å rmsd between human PUM1 and PUM2) can explain their conserved PRE specificity ([25, 36 37, 42]. The modular recognition of RNA sequence has enabled prediction of PUM binding sites in target RNAs and rational design of Pumilio proteins with engineered RNA specificity and novel functions [31, 38, 43–49].

Mechanisms of PUM-mediated repression (PMR)

Discovery of the mechanisms of mRNA regulation by Pumilio proteins is crucial for understanding their functions. Analyses of invertebrate orthologs established that they repress protein and mRNA levels of target mRNAs to which they bind [3, 4, 7, 8]. This inhibitory function carries over to mammals; PUM1 and PUM2 repress PRE-containing target mRNAs, manifested by reduced levels of that mRNA and the encoded protein [50], hereon referred to as PUM-mediated repression (PMR).

Stability and translation of mRNAs are intermingled. Translation is promoted by two key mRNA modifications, the 5' 7-methyl guanosine cap (5' cap) and the 3' poly-adenosine tail,

which are bound by eIF4E and poly(A) binding proteins (PABP), respectively [51]. The cap and poly(A) tail also protect the RNA from spurious degradation, and their removal typically initiates mRNA decay [52]. Given their pivotal roles in translation and stability, the 5' cap and 3' poly(A) tail are nodes for regulatory processes. Indeed, invertebrate Pumilio proteins repress by promoting deadenylation and, in several instances, decapping [53–56].

In mammals, PMR results in degradation of target mRNAs [29, 50, 57]. Biochemical analysis showed that components of the major deadenylation machine, the Ccr4-Not complex (CNOT), copurify with human PUM1 and PUM2 [50, 54, 58]. Recruitment of the CNOT complex is a conserved mechanism of PMR, as the Pum-HD of Pumilio orthologs from yeast to humans directly interact with the Pop2/Caf1/CNOT7/CNOT8 deadenylase enzyme subunit (Figure 3)[54, 59–62]. This conclusion is supported by functional analyses, which showed that blocking CNOT activity by deletion, RNAi depletion, or dominant negative approaches reduced PMR in multiple species including humans [50, 54, 55, 60–63].

Analysis of *Drosophila* Pumilio identified three additional repression domains (RDs) in the N terminus (Figure 1C) that reduce protein and mRNA expression levels [13]. The PUM RDs can function autonomously when artificially directed to an mRNA. These novel RDs do not share sequence homology with each other or known domains. While the RDs are among the least conserved parts of PUMs (Figure 1C), experimental evidence indicates that analogous regions of human PUM1/2 possess repression activity (Figure 3)[13]. An important future goal will be to determine the regulatory mechanisms of the PUM N-terminal RDs.

The generality of PUM-mediated RNA decay has been borne out by transcriptome-wide analyses which found that the PRE is among the most strongly correlated features with mRNA instability [64–66]. Massive parallel reporter assays showed that PREs destabilize mRNAs during vertebrate embryogenesis [67]. Analysis with reporter mRNAs indicated that the major effect of PREs is to reduce mRNA levels [33, 50, 68]. Moreover, depletion of PUM1/2 in human cells stabilized hundreds of PRE-containing transcripts, whereas over-expression of PUM1/2 destabilized their target mRNAs [33, 69].

Inhibition of translation is also involved in PMR. Compelling evidence supports that antagonism of the translational activity of poly(A) and PABP is a second conserved repression mechanism (Figure 3) [50, 61]. Biochemical and cell-based data from invertebrates and humans indicate that PABP and poly(A) are required for efficient repression by the Pum-HD of multiple orthologs [61, 70]. Mechanistically, the Pum-HD is thought to associate with PABP and to disrupt its ability to promote translation, apparently without dislodging it from the mRNA [61, 70]. At this time it is unclear if mammalian PUMs interact with PABP and how this mechanism interfaces with deadenylation.

Additional mechanisms of PUM-mediated translational inhibition have been proposed. First, *Xenopus* PUM2 was shown to bind to the 5' cap of mRNAs, and mutation of the cap-binding motif alleviated translational repression in oocytes [71]. This cap-binding motif is in PCMb, and does not coincide with the Pumilio N-terminal RDs (Figure 1C). The potential for this mechanism to contribute to PMR by mammalian PUMs has not been addressed.

Second, PUM orthologs were reported to inhibit translation elongation in vitro by inhibiting translation elongation factor eEF1a through an Argonaute-mediated mechanism [72]; however, disrupting this potential mechanism did not affect PMR in cells [61].

Do PUMs act on mRNAs before or after they engage the translation apparatus? Conflicting observations have been reported; in one case PUM2 did not associate with poly-ribosome bound mRNAs [73], whereas another study observed that a minor population of PUM1/2 cofractionates with poly-ribosomes [34]. A recent study showed that PMR resulted in diminished poly-ribosome association of mRNAs with two or more PREs, but the magnitude of the effect was modest relative to the effect on mRNA abundance [68]. The effect of PUMs on translation may be dependent on the context of specific mRNA or biological conditions. For instance, protein expression of several mRNAs is affected by PMR to a larger extent than changes in the level of that mRNA [33, 34]. Future analyses of the transcriptome-wide impact of PUMs on translation may help address these issues.

The PUM-mediated activation (PMA) paradox

New data reveal that mammalian PUM1/2 can activate certain mRNAs. For example, PUM1/2 bind PREs in the 3'UTR of the mRNA encoding the transcription factor FOXP1 and enhance its expression [74]. Another study identified more than 100 PUM-activated mRNAs that contain a PRE, half of which are known to be bound by PUMs [33]. While seemingly paradoxical, such bifunctional control of mRNAs is not without precedent; for example, the Iron Response Element Protein has positive or negative effects, depending on the context of its binding site [75]. Also, the cytoplasmic poly-adenylation element binding protein (CPEB) can switch from repressor to activator in response to developmental cues [76]. In fact, activation of mRNAs has been observed for PUM orthologs in multiple species [60, 77–80]. While the mechanism of PMA in mammals is unknown, the phenomenon is transcript-specific, suggesting that mRNA features may be key determinants. Evidence in non-mammalian model organisms indicates that activation by PUM orthologs involves mRNA stabilization, cytoplasmic poly-adenylation, and enhancement of translation [60, 78–80]. Perhaps PUM1/2 collaborates with other regulatory factors, such as poly-adenylation factors, to promote mRNA stability and/or translation or, alternatively, displaces a dominant regulator from the target mRNA.

Overlapping functions of PUMs

The presence of two classical mammalian PUMs raises the question: does each PUM have a specialized, unique function or are they redundant? Their Pum-HDs are 90% identical, have identical TRMs that confer indistinguishable RNA specificities, and associate with overlapping sets of mRNAs [29, 30, 32, 34, 35]. Both PUMs repress target mRNAs with comparable magnitude [50]. Moreover, the two PUMs are functionally redundant in cells; both must be simultaneously depleted to fully alleviate PRE-mediated repression [33, 50]. In the case of PUM-activated mRNAs, again both PUMs appear to participate [33, 74]. Based on these observations, it is likely that PUMs are redundant with overlapping functions, consequently both PUMs should be investigated when analyzing mRNA regulation.

PUM1 and PUM2 proteins and mRNAs are coincidentally expressed in a wide range of tissues and cell types throughout development (Figure 4)[27, 81, 82](see also Illumina Body Map 2 Project and the Human Protein Atlas [83]). Based on these observations, it is reasonable to predict that their regulatory functions broadly overlap. In certain tissues, enrichment of either *PUM1* or *PUM2* mRNAs are observed (Figure 4). For instance, *PUM2* mRNA is more abundant in blood and the cerebellum of the brain than *PUM1*, whereas *PUM1* is more prevalent in skeletal muscle. Still, our knowledge of differential expression of the two PUM proteins in diverse cell types and stages of development remains incomplete, and deeper understanding could reveal unique, context- specific roles.

Insights into the PUM regulatory networks

To understand the impact of PUMs on gene expression, it is necessary to define the repertoire of transcripts that they regulate. Based on current knowledge, three criteria should be applied to define PUM target RNAs, including (1) the presence of a PRE site that is (2) bound by PUM1/2 resulting in (3) a functional consequence on the fate of that target RNA, including PMR or PMA. Numerous studies support these conclusions for individual genes (cataloged in Table S1, Bohn et al, 2018)[33]. Here, we summarize transcriptome-wide analyses of PUM1/2 regulatory networks based on one or more of these criteria.

High resolution definition of the PRE [11, 28–32, 35, 84] enabled prediction of thousands of potential PUM targets across the transcriptome. In humans, mRNAs and non-coding RNAs from 7822 genes contain PREs, ranging from 1–19 sites (cataloged in Table S6 of Bohn et al, 2018)[33]. Interestingly, several ncRNAs contain the highest density of PREs [33, 69, 85]. These observations suggest that PUMs may broadly impact gene expression.

An extensive collection of PUM1/2-bound RNAs from more than 4000 unique genes have been identified from human or mouse cells and several tissues [27, 29, 30, 34, 35, 85]. PRE sequences are enriched in more than half of these mRNAs, consistent with direct PUM targeting[33]. The remainder may be indirectly bound (i.e. PUM1/2 bind another RBP that is in direct contact with that RNA), could be recognized via combinatorial mechanisms that alter PUM specificity, or in some instances might be false positives.

PUM-mediated regulation of the transcriptome has been investigated by either deleting, depleting, or over-expressing PUM1 and/or PUM2, thereby identifying many hundreds of affected RNAs [33, 34, 69, 85]. Of those, hundreds of RNAs are direct targets, based on their inclusion in PREcontaining and PUM-bound datasets [33]. These analyses were restricted to a few cell/tissue types; thus, to capture the full impact of PUMs on gene expression, future analyses should pursue PUM targets in multiple contexts. Moreover, previous global analyses focused on RNA levels. To fully measure the influence of PUMs, ribosome profiling and quantitative proteomic approaches should be applied to measure changes in the translated proteome.

What can we learn from these PUM regulatory networks? First, the location of PREs is a determinant of regulation. PREs are most prevalent in 3'UTRs in the predicted, bound, and regulated target mRNAs [27, 29, 30, 33] and are less prevalent in 5'UTR and coding sequences. PREs in 3'UTRs correlate most strongly with PMR, as do PREs in ncRNAs,

whereas PREs in coding regions show a weaker correlation [33]. In contrast, 5'UTR PREs do not contribute to PUM-induced changes in RNA levels. A single PRE is necessary and sufficient for regulation, and more PREs correlates with increased regulation, an effect that saturates at 3–4 PREs [29, 33, 68]. Still, computational modeling indicates that we have more to learn, because parameters of PRE number, location, and PUM occupancy do not fully account for the observed regulation. Given that each PUM target RNA likely has a unique constellation of cis-elements and trans-acting RNA-binding factors that control its fate, it is necessary to determine how PUM1/2-mediated regulation integrates with those factors in the context of individual transcripts.

Characterization of PUM1/2 regulatory networks provides insights into the diverse cellular processes and molecular functions that they can affect. Here we summarize the significantly enriched gene ontology categories among the predicted, bound, and regulated PUM targets (Table 1) [27, 29, 30, 33–35, 85–90]. Future research should focus on investigating the direct impact of PUMs on these pathways and processes in normal and disease states. Non-coding RNAs were also present in the PUM regulatory network, but as few have documented functions, interpreting these connections awaits characterization of the ncRNAs themselves.

Localization of PUM1/2 to intracellular granules

Specific RNAs and proteins can condense into membrane-less ribonucleoprotein (RNP) granules. Classical PUMs operate in the cytoplasm and several studies report that PUM1/2 can be enriched in three types of cytoplasmic RNP granules: stress granules, processing bodies (P-bodies), and neuronal transport granules [91–94]. Stress granules form in response to environmental stresses and are defined by their content of translationally inactive RNPs containing certain translation factors, RNA-binding proteins, and mRNAs [95]. Both PUM1 and PUM2 were observed in stress granules induced by oxidative stress and viral infection [29, 93, 96]. Moreover, over-expression of PUM2 can induce stress granule formation in lieu of stress [91]. PUM1/2 are enriched in purified P-bodies [94], which are RNP granules that are enriched with mRNA decay factors and translationally inactive mRNAs. In neurons, RNP granules are actively transported along dendrites to deliver mRNAs to synapses, where they can be locally translated in response to synaptic activity. PUM2 is present in RNP granules that traffic in dendrites, in addition to being present throughout the neuronal cell body [73, 91].

The presence of PUM1/2 in RNP granules raises important questions. Foremost, does localization have a functional consequence? Could RNP granules act to sequester or store PUMs and their target mRNAs for subsequent re-animation? Alternatively, does granule localization contribute to PUM-mediated repression? Because PUM1/2 repress mRNAs under conditions where RNP granules are not present, localization to granules is unlikely to be obligatory. In the case of neuronal RNP granules, PUM2 may bind and translationally repress specific transcripts whilst they are being localized to the synapse; those mRNAs could then be de-repressed in response to synaptic activation. The determinants of PUM1/2 incorporation into granules remains unknown. Low complexity, intrinsically disordered regions of proteins facilitate RNP granule formation [95] and properties of RNAs can also drive formation [93, 94, 97]. In the case of PUMs, both concepts may be relevant; PRE-

containing mRNAs and ncRNAs are enriched in stress granules [93], and the Pum-HD and disordered N terminus are necessary for PUM2 association with stress granules [91].

Insights into regulation of PUM1/2 activity

The activities of PUMs must be modulated to avoid deleterious effects on gene expression. One homeostatic mechanism is auto- and cross-regulation through negative feedback. *PUM1* and *PUM2* mRNAs have 8 and 13 PREs, respectively [33], and multiple studies reported PUM1/2 binding to their own and each other's mRNAs in cultured cells, stem cells, and brain [29, 30, 34, 88], and experimental evidence supports that depletion of one PUM can lead to increased expression of the other PUM [34, 86].

The activities of PUM1 and PUM2 may be modulated by post-translational modifications. Multiple sites of phosphorylation, ubiquitylation, and methylation have been mapped on each PUM (Figure 1), though the modifying enzymes and consequences of these modifications remain unknown. Thus far, only one site has been shown to affect PUM activity; phosphorylation of S714 of PUM1 is stimulated by growth factors and promotes binding and repression of the CDKN1B/p27 mRNA [86]. Surprisingly, S714 is outside of the RNA-binding domain and it is unclear how this modification affects the PUM-RNA interaction.

PUM activity can be modulated by a cytoplasmic, long ncRNA *NORAD* (NcRNA Activated by DNA damage) [69, 85]. *NORAD* contains 17 PREs, is avidly bound by PUMs, and therefore was proposed to act as a competitive inhibitor. Indeed, depletion of *NORAD* enhanced PMR of PUM-bound, PRE-containing mRNAs, whereas its over-expression reduced PMR. *NORAD* is induced by DNA damage, and depletion of *NORAD* leads to genome instability [85]. Likewise, PUMs affect genome stability, and this effect partly depends on *NORAD*. Transcriptome-wide analysis indicates that PUM1/2 and *NORAD* have opposing effects on an overlapping set of 193 genes, including replication, repair, and mitotic factors [85].

Even with its high PRE content and abundant expression, *NORAD* contains a minor fraction of all PREs in expressed transcripts [33, 98]. How can *NORAD* competitively inhibit PUM? A new study reports that *NORAD* binding to PUM1/2 may be facilitated by the RNA-binding protein SAM68 [98]. Like *NORAD*, SAM68 counteracts PUM2 repression and is necessary for *NORAD* to inhibit PMR. Mechanistically, PUM2 and SAM68 interact in an RNase-resistant manner. Based on these observations, the *NORAD*-SAM68 RNP is proposed to inhibit PUMs by binding them more avidly than other PRE-containing target RNAs. Interestingly, *NORAD* is itself subject to PMR, indicative of a PUM-*NORAD* feedback loop [33]. Thus PUM and *NORAD* functions are intertwined, but much remains to be learned with regard to when, where, and how the PUM-*NORAD* regulatory mechanism controls gene expression.

Combinatorial control can alter the regulatory activity and target specificity of invertebrate Pumilio orthologs [4, 25, 79, 99]. In mammals, a collection of RNA-binding proteins have been reported to associate with PUMs, including orthologs of Nanos (i.e. NANOS1, 2, 3) [100–103], Brain Tumor (i.e. TRIM71) [104], and CPEB [99]. These observations suggest

combinatorial control of PUM1/2 RNA-binding and regulatory activities; however, this remains to be proven. The FMRP protein is an intriguing example; it associates with PUM1 and PUM2, and they bind partially overlapping sets of nearly 200 mRNAs, including each other's mRNAs [29, 30, 34]. While PUMs and FMRP share some neurodevelopmental phenotypes [34, 57, 105, 106], their functional relationship has not been investigated. Additionally, members of the DAZ/Boule family of RNA-binding proteins, which have important roles in the germline, were reported to associate with human PUM2, though their influence on its activity remains unknown [107].

PUM1/2 may interface with microRNA (miRNA)-mediated regulation. Binding sites of miRNAs are enriched near PREs in a subset of PUM target mRNAs [30, 33]. Further, PUM1/2 associate with Argonaute proteins, the core component of the miRNA-induced silencing complex (RISC) [61, 72]. While Argonautes are not required for PMR *per se* [57, 61], they could act in a combinatorial manner. Two studies reported functional interactions between PUMs and miRNAs. In the first case, miR-221 repression of the *CDKN1B/p27* mRNA was reported to be PUM dependent [86]. In this mRNA, the miR-221 binding sites can base pair with the PREs, and PUM was proposed to disrupt that interaction, thereby enabling repression by miR-221. But *CDKN1B* mRNA is likely the exception, because complementarity of a PRE with a miRNA site is rare. In the second example, PUMs and PREs in the *E2F3* mRNA were shown to potentiate repression by miRNAs that target *E2F3*, though the mechanism remains unknown [87]. A germane study systematically assessed the functional interplay of PRE and let-7 miRNA sites and found that they operate independently, with additive effects on mRNA regulation [68].

Further research is necessary to determine the ability of PUM1/2 partners to modulate RNA binding, PMR, or PMA. We suggest the following criteria for interrogating combinatorial control: Both RBPs should bind to the same mRNA; they may do so cooperatively and/or with altered specificity. Both RBPs should make significant contributions to controlling the fate of that mRNA. Their combined activities may be additive, synergistic or, alternatively, their partnership may cause a new regulatory outcome.

Biological functions of mammalian Pumilio proteins

New studies illuminate the biological functions of mammalian PUM1/2 and the disease phenotypes resulting from their dysfunction, as summarized in Table 2. Here we explore their functions in stem cell biology, growth and development, in the germline and nervous system. We also discuss discoveries that link PUMs to cancer, infertility, and neurological disorders.

Control of stem cell fate—A prescient review suggested that the “primordial function of PUF proteins is to sustain mitotic proliferation of stem cells” [3]. Since then, accumulating data reveal that mammalian PUMs regulate stem cell fate in diverse contexts. In cultured embryonic stem cells (ESCs), PUM1 is required to exit self renewal, wherein it promotes differentiation by repressing pluripotency transcription factors [88]. In effect, PUM1 rewires the gene expression circuitry from ESC towards differentiation. While depletion of PUM1 reduced differentiation of ESCs, depletion of PUM2 had no effect, distinguishing its

function from PUM1. As discussed below, PUMs also regulate the fate of neuronal, germline, and hematopoietic stem cells.

Regulation of growth and development—Genetic analysis in mice revealed that PUMs are necessary for viability, growth and development. Combined knockout of *PUM1* and *PUM2* (*PUM1&2*) resulted in lethality during embryogenesis [34], whereas individual *PUM* knockouts cause diminished growth and size throughout lifespan [27, 34, 108–110]. This function appears to be relevant to humans, as *PUM1* mutations are linked to reduced growth and development [105]. Interestingly, neural-specific *PUM1&2* knockout also resulted in reduced growth and body weight, indicating an important role of brain function in this phenotype [34].

Regulation of gametogenesis—Pumilio orthologs in *Drosophila* and *C. elegans* have roles in germline stem cells wherein they regulate gametogenesis, and their disruption negatively impacts fertility [12, 111–113]. This role is conserved in mammals, as knockout of *PUM1* or *PUM2* reduces germline function. PUM1 is important for male fertility, wherein it suppresses activation of the p53 pathway within germ cells [27]. In the absence of PUM1, the testes are smaller and the germ cells undergo premature apoptosis as spermatocytes. Male mice with a *PUM2* gene trap mutation have smaller testes and degenerated seminiferous tubules [109]. While mice lacking either *PUM* gene exhibit reduced gametogenesis, they can still reproduce, perhaps sustained by the remaining PUM.

In the female germline, PUM1 is important for fertility and production of viable oocytes [110]. *PUM1* knockout mice are subfertile, produce smaller litters, and exhibit reduced ovarian follicle formation and oocyte development. Meiosis is delayed with defective chromosome synapsis. The target mRNAs underlying these phenotypes remain unknown. PUM1 does affect expression of the synaptonemal complex component, *Sycp1*, though this mRNA does not possess a PRE, suggesting that PUM1 may regulate synaptonemal complex disassembly or stability, rather than *Sycp1* mRNA directly. In contrast, females with *PUM2* knockout have normal fertility [109, 110], providing an example of PUM1 specific function. While both PUMs are present in ovaries, PUM1 protein is enriched in oocytes relative to PUM2; thus, PUM2 levels in the female germline may be insufficient to compensate.

Roles in neurogenesis, neuronal function, behavior, learning and memory formation—*Drosophila* and *C. elegans* PUM orthologs function in the nervous system to control memory formation and neural morphology and electrophysiology [4, 77]. Astonishingly, mammalian PUMs also regulate these and other neurological processes. PUM1 and PUM2 are expressed in neural stem cells, progenitors, and mature neurons throughout the developing brain [34, 57, 89, 91, 108]. PUM2 was shown to control morphology and electrophysiology of cultured hippocampal neurons wherein it directly regulates mRNAs encoding voltage-gated sodium channel Nav1.1 (SCN1A) and Nav1.6 (SCN8A gene) [73, 114]. Transcripts from ten voltage-gated sodium ion channels have PREs and are potential PUM targets [33], suggesting a general role for PUMs in control of electrophysiology. Inactivation of *PUM2* by gene trap caused hyperactivity and behavioral deficiencies in spatial and memory tests and nest building [90, 108]. This latter phenotype is shared by *PUM1* knockout mice [57].

Loss of PUM function contributes to seizures. In humans, *PUM1* mutations are linked to epilepsy [105]. The relationship of PUM1 to seizures has not been reported in mouse models; however, inactivation of *PUM2* increased the propensity for seizures [90, 108]. *PUM2* inactivation did not affect overall hippocampal neuronal activity, but decreased paired-pulse inhibition, which can contribute to spontaneous seizures. Hundreds of mRNAs were upregulated in response to *PUM2* inactivation in the brain, including those involved in synaptic transmission and structure [90]. Moreover, PUM2 dynamically affected expression of Nav1.1 (*SCN1A*), Nav1.2 (*SCN2A*), and Nav1.6 (*SCN8A*) encoding mRNAs, which are involved in epilepsy [114, 115]. Perturbation of gamma-aminobutyric acid (GABA) activity, an inhibitory neurotransmitter, may also contribute to propensity of seizures [116]. PUM1/2 repress the *ABAT* mRNA, which encodes the enzyme that catabolizes GABA [33]; and therefore may stabilize GABA. PUM2 also represses *GABRA2*, a subunit of the GABA type A ligand-gated chloride channel, which is important for GABA mediated inhibitory activity [90]. Thus, deficiency of PUM1/2 activity may dysregulate GABA and its receptor, which theoretically could contribute to increased susceptibility to seizures.

New studies establish key roles of mammalian PUMs in brain development, neurogenesis and neurological functions [34, 57, 73, 89]. Simultaneous neural-specific *PUM1&2* knockout caused deficiency in spatial learning and memory [34]. The brains of these *PUM1&2* knockout mice exhibited reduction of the dentate gyrus region of the hippocampus, which is important for learning and memory [117]. The dentate gyrus contains the neural stem cells that produce progenitors which subsequently differentiate into hippocampal neurons. The neural stem cell population in the brains of the *PUM1&2* knockout mice did not appear to change, but the population of neural progenitors increased whereas the number of immature neurons decreased, indicating that PUMs promote proliferation and differentiation [34]. PUMs are necessary for survival of immature neurons, as apoptosis increased in the dentate gyrus of the double knockout mice. These observations were corroborated by neurosphere assays, wherein neural stem cells from *PUM1&2* knockout showed reduced proliferation, self-renewal and differentiation, along with increased apoptosis. Another study analyzed the role of PUM2 in brain development and provides evidence for its importance in cortical neurogenesis and differentiation [89].

An extensive set of mRNAs are likely controlled by PUMs in the brain. Hundreds of PREcontaining mRNAs bound by PUM1 and PUM2 in mouse brain were identified and are enriched in neuro-developmental functions [34, 89]. Widespread changes in gene expression were observed in the *PUM1&2* knockout brains, with more than 7000 differentially expressed genes, whereas single PUM knockouts had a small number of affected mRNAs [34]. More than 300 of the differentially expressed mRNAs were bound by PUMs, with evidence of both PMR and PMA.

In several cases, the levels of PUM-bound target mRNAs did not change in the *PUM1&2* knockout brain, but the level of encoded protein was altered, indicative of translational control [34]. A new study suggests a mechanism where PUM2 may collaborate with eIF4E-Transporter (4E-T) to inhibit expression of proteins during neuronal differentiation [89]. 4E-T inhibits translation by binding to the translation initiation factor eIF4E [118]. In the brain, PUM2 and 4E-T target an overlapping set of nearly 300 mRNAs, including those of

neuronal specification transcription factors Brn1 and Tle4 [89]. During embryonic development, *Brn1* and *Tle4* mRNAs are coexpressed in progenitor cells whereas in postnatal stages they are expressed in mutually exclusive sets of neurons. Depletion of PUM2 or 4E-T caused aberrant co-expression of Tle4 and Brn1 in neuronal precursors and differentiating neurons. PUM2 and 4E-T co-immunoprecipitate, but whether this results from direct protein contacts or co-occupancy of target mRNAs remains unknown. Overall, this work suggests that PUM2 and 4E-T collaborate to repress expression of specification proteins during neurogenesis [89]. It is noteworthy that PUM2 and 4E-T also independently target many mRNAs, indicating that their regulatory function is not likely fully interdependent.

Roles in neurodegeneration—Recent evidence links PUM1 to a neurodegenerative disease [57]. In mice, *PUM1* knockout or haploinsufficiency caused neurodegeneration. The mechanism involves PUM1 repression of *Ataxin1* mRNA (*ATXN1*). In humans, poly-glutamine expansion in Ataxin1 leads to spinocerebellar ataxia type 1 (SCA1), a neurodegenerative disease characterized by loss of coordination and balance, muscle stiffness, weakness, cognitive and sensory impairment, and death. In mice, over-expression of Ataxin1 causes SCA1-related phenotypes. Knockout or depletion of PUM1 results in increased Ataxin1 expression, motor dysfunction, incoordination and loss of Purkinje neurons and reduced neuronal arborization in the cerebellum, resembling SCA1 [57]. Consistent with importance of PUM1 repression of Ataxin1 protein expression, haploinsufficiency of *Ataxin1* alleviated the effect. Moreover, over-expression of PUM1 suppressed neurodegeneration caused by Ataxin1 with poly-Q expansion, whereas *PUM1* haploinsufficiency exacerbated the disease phenotype.

Genomics provided the first evidence linking human *PUM1* to neurological disorders and emphasizes its neurodevelopmental roles [105]. Two classes of PUM1-associated neurological disorders were characterized by developmental delay, intellectual disability, seizures, and ataxia. PADDAS is an infantile onset PUM1-associated disorder with developmental disability, ataxia, and seizures. PRCA is an adult onset PUM1-related cerebellar ataxia disorder. These syndromes were discovered in a cohort of 15 patients, are likely quite rare, and it remains possible that other loci may contribute. Most PADDAS patients had heterozygous deletions encompassing the *PUM1* gene. Two PADDAS patients had either of two *PUM1* missense mutations. A patient with PUM1 R1139W had ataxia, incoordination, chorea, spasticity, and reduced growth. Another patient with PUM1 R1147W had epilepsy, ataxia, hypotonicity, developmental delay, visual impairment, scoliosis, facial dysmorphism, low bone density, and brain morphology abnormality. In the case of PRCA, an afflicted family was identified with a *PUM1* missense mutation, T1035S, which is associated with a partially penetrant autosomal dominant syndrome with cerebellar degeneration, ataxia, incoordination, and speech disorder. It remains unclear how these mutations affect PUM1 protein function. The PADDAS mutations R1139W and R1147W reside in the eighth repeat of the Pum-HD and are surface-exposed, whereas the PRCA PUM1 T1035S mutation is within the sixth repeat of the Pum-HD and is partially exposed (Figure 2A). None of these changes are on the RNA-binding surface. All three mutants exhibited reduced PUM1 protein levels, which may manifest in the disease state. In cultured

cells from PADDAS and PRCA patients, expression of several PUM targets was affected, including *ATXN1*, supporting a loss of function. Further investigation by over-expression of PUM1 mutants showed that R1147W repressed several target mRNAs, whereas R1139W or T1035S did not. In hippocampal neurons, wild type PUM1 and T1035S reduced arborization when over-expressed, whereas R1139W and R1147W had less of an effect. Additional research is necessary to determine the impact of PADDAS and PRCA mutations on PUM1 protein turnover, RNA binding, repression, protein interactions with corepressors, and intracellular localization.

Roles in hematopoiesis and leukemia—Mammalian PUM1/2 have been implicated in hematopoiesis and leukemia. Normally, hematopoietic stem cells (HSCs) in bone marrow differentiate to produce the different types of blood cells. Leukemia occurs when cancer-driving mutations give rise to leukemia stem cells (LSCs) that proliferate and disrupt blood and tissue function. New evidence indicates that PUM1/2 play a critical regulatory role in the maintenance and proliferation of normal HSCs and impact acute myeloid leukemia (AML)[74]. PUM1/2 are highly expressed in human and mouse HSCs and are over-expressed in primary AML samples and cell lines [74, 82]. Moreover, upon individual depletion of PUM1/2, the cell cycle was disrupted, proliferation decreased and apoptosis increased in both normal human and mouse HSCs as well as AML cells [74]. *FOXP1* mRNA, encoding a transcription factor involved in cell proliferation and differentiation, was identified as a direct target of PUM1/2. As noted earlier, PUM1/2 directly bind and activate the *FOXP1* mRNA, which in turn represses expression of cell cycle inhibitors such as CDKN1B/p27, thereby promoting proliferation. Whether PMA is widespread in hematopoiesis or leukemogenesis remains unknown. Notably, PUM1 can also directly repress expression of CDKN1B/p27 tumor suppressor [33, 86], potentially reinforcing the FOXP1 pathway in HSCs and AML cells. Numerous other mRNAs are likely also affected by PUM1/2 in HSCs and LSCs and remain to be discovered.

Concluding Remarks

Tremendous progress has been made in elucidating the regulatory roles and biological functions of mammalian Pumilio proteins. Still, many important questions remain regarding how they regulate target mRNAs in diverse biological contexts. PUMs are broadly expressed in the human body and are predicted to bind a wide array of the transcriptome, thus they are likely to affect many additional biological processes. It is our hope that this review will serve to focus and accelerate new discoveries. This important endeavor will promote our understanding of diseases that result from dysfunction of Pumilio proteins and will enable therapeutic strategies to correct those defects.

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Highlights:

- Mammalian Pumilio proteins recognize specific RNA sequences via a highly conserved Pum-HD domain.
- Pumilio proteins bind and regulate a large number of RNAs.
- Pumilio proteins repress target mRNAs by antagonizing translation and promoting RNA degradation.
- In certain contexts, Pumilio proteins may activate gene expression.
- Pumilio proteins regulate stem cell fate, development, and neurological functions.
- Dysfunction of Pumilio proteins contributes to neurodegeneration, epilepsy, ataxia, infertility, and cancer.

Outstanding Questions:

1. What are the biological roles of PUMs?
2. What diseases are caused by dysfunction of PUMs?
3. Which mRNAs are regulated by PUMs in relevant cell, tissue types, and developmental contexts?
4. What are the precise mechanisms of PUM-mediated regulation?
5. How is PUM activity regulated?

Figure 1. Features of classical cytoplasmic PUMs and divergent nucleolar PUMs. (A) Diagrams of human PUM1 and PUM2 proteins showing length in amino acid (aa) residues, sequence motifs, secondary structure, disordered versus ordered regions (computed by JRONN) [119] and hydrophobic versus hydrophilic amino acid content (adapted from Protein Data Bank: <https://www.rcsb.org>). Post-translational modifications (PTM) including methylation, phosphorylation, and ubiquitylation from Uniprot (<https://www.uniprot.org>) and Phosphosite (<https://www.phosphosite.org>) are shown at the top. Motifs designated by a single letter represent low complexity regions

enriched for that amino acid residue (A = alanine rich, Q = glutamine rich, S = serine rich, G = glycine rich). The TRMs within each Pum repeat (R1-R8) are also shown. (B) Diagram for founding member, *Drosophila* Pumilio, is shown for comparison. (C) Plot of relative sequence conservation versus amino acid residue position of 82 Pumilio protein orthologs including insects, fish, reptiles, birds, marsupials, mammals, primates and humans, generated using Clustal Omega [120], Consurf server [121], and Emboss Plotcon (<http://www.bioinformatics.nl/cgi-bin/emboss/plotcon>). For reference, conservation is plotted relative to functional domains defined for *Drosophila* Pumilio including three repression domains (RD1–3), Pumilio Conserved Motifs (PCMa and PCMb), and the Pum repeats (R1-R8) of the Pum- HD. Troughs represent sites of insertion. Peak height is proportional to conservation of sequence identity. (D) Diagrams of the divergent Pumilio orthologs PUM3 and NOP9. Motifs include predicted nuclear localization signals (NLS) and C-terminal Penguin Like (CPL) motif (PFam PF08144).

Figure 2. Structure and RNA recognition by PUM1 and PUM2.

(A) High conservation of the RNA-binding surface of PUM proteins. Ribbon diagram of a crystal structure of human PUM1 in complex with *hb* NRE RNA is shown colored according to the degree of amino acid sequence conservation calculated using the Consurf server [117]. The most highly conserved positions are colored maroon, and the least conserved are colored cyan. The specific PUM1 amino acid residues that are mutated in PADDAS (R1139W and R1147W) and the PRCA (T1035S) are indicated by space-filling spheres. (B) PUM1 RNA recognition code. Specific interaction of Pum repeats with U, A, G, and C

nucleotides is shown. (C) Ribbon diagram of a crystal structure of human PUM2 in complex with *erk2* PRE RNA. The RNA-binding helices are colored maroon. PUM2 binds to the *erk2* PRE RNA using the alternative 'base-omission mode,' where bases A4 and C5 (green) are directly stacked and R888 in repeat 5 (green) does not stack between bases A4 and C5.

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Figure 3. Model of PUM1/2 repression mechanisms.

PUM proteins bind the PRE, typically located in the 3'UTR of target mRNAs. Repression of the target mRNA is mediated in at least three ways: (A) The Pum-HDs of PUMs antagonize the translational activity of poly(A) binding protein (PABPC1). (B) PUMs promote translational repression and mRNA decay by recruiting the CCR4-NOT deadenylase complex (CNOT). (C) The N-terminal regions of PUM orthologs confer repression, but the mechanism remains to be determined.

Figure 4. Gene expression of human *PUM1* and *PUM2* mRNAs across 53 tissue types. Data were obtained from the GTEx consortium database. Violin plots showing median, interquartile range, and density for expression values of mRNAs for each PUM, in normalized, logio Transcripts per Million units (TPM), were generated for the indicated tissues using the GTEx portal website (<http://www.gtexportal.org/>) on 9/5/2018 using data from 10,294 samples.

Table 1:

Gene ontology categories enriched in PUM regulatory networks.

Gene Ontology Category	Examples	References
Signaling pathways	RAS, GTPase, WNT, Hedgehog, Notch, PDGF, EGF receptor, MAP kinase, serine/threonine phosphatase, TGF beta, and insulin receptor binding	[27, 29, 30, 33, 34, 89, 122]
Control of cell death	Negative regulation of apoptosis	[27, 30, 33, 34]
Cell migration and adhesion	Extracellular matrix glycoproteins, intracellular adhesion molecules, integrins, and cadherins	[29, 33, 34, 89]
Core cellular functions	Genome replication and stability, DNA repair, mitosis, spindle assembly and chromosome segregation	[29, 30, 33, 85]
Cell cycle and proliferation regulators	CDK/Cyclins, CDK inhibitors, and G1/S transition factors	[27, 29, 30, 33, 34, 86, 89]
Metabolic pathways	Oxidative phosphorylation, ubiquinone metabolism, nucleotide metabolism, fatty acid metabolism, pantothenate kinase activity	[27, 29, 30, 33, 34]
Transcription regulators	DNA-binding transcription factors including those involved in cancer and stem cell fate	[29, 30, 33, 88, 89]
Neurological functions	Nervous system development, differentiation, morphology, axon guidance, nerve signaling and impulse transmission, neurotransmitter transport, secretion, binding, and metabolism, and voltage-gated ion channels	[30, 33, 34, 57, 89, 90]
Neurodegenerative disease genes	Parkinson's, cerebellar ataxia, fragile X syndrome, and Alzheimer's disease	[30, 33, 34, 57, 105]
Cancer genes	330 cancer genes have PREs, 209 were bound by PUM1/2, and at least 45 have been shown to be regulated by PUMs, including tumor suppressors and oncogenes	[29, 30, 33, 86, 87]

Table 2:

Major functions of PUM1 and PUM2 and consequences of their dysfunction.

PUM Function	PUM Dysfunction	Approaches	References
Growth	Reduced body size	Mouse knockout, naturally occurring human mutations	[27, 34, 105, 108–110]
Development	Embryonic lethality, developmental disability	Mouse knockout, naturally occurring human mutations	[34, 105]
Stem cell fate	Defects in embryonic, germline, hematopoietic, and neural stem cell proliferation and differentiation	Mouse knockout, RNAi in human and mouse cells	[27, 34, 74, 82, 88]
Gametogenesis	Reduced fertility	Mouse knockout	[27, 109, 110]
Hematopoiesis	Promote acute myeloid leukemia	RNAi in human and mouse cells, analysis of mouse	[74, 82]
Neurogenesis	Neurodegeneration	Mouse knockout, RNAi in mouse brain	[34, 57, 89]
Neural electrophysiology	Epilepsy	Mouse knockout, RNAi in rat neurons, naturally occurring human mutations	[90, 105, 108, 114]
Behavior	Hyperactivity, deficient nesting behavior	Mouse knockout	[57, 90, 108]
Learning and memory formation	Diminished spatial memory, intellectual disability	Mouse knockout, naturally occurring human mutations	[34, 57, 105]
Motor function	Cerebellar ataxia	Mouse knockout, naturally occurring human mutations	[57, 105]