RNA regulatory networks diversified through curvature of the PUF protein scaffold

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Proteins bind and control mRNAs, directing their localization, translation and stability. Members of the PUF family of RNA-binding proteins control multiple mRNAs in a single cell, and play key roles in development, stem cell maintenance and memory formation. Here we identified the mRNA targets of a \textit{S. cerevisiae} PUF protein, Puf5p, by ultraviolet-crosslinking-affinity purification and high-throughput sequencing (HITS-CLIP). The binding sites recognized by Puf5p are diverse, with variable spacer lengths between two specific sequences. Each length of site correlates with a distinct biological function. Crystal structures of Puf5p–RNA complexes reveal that the protein scaffold presents an exceptionally flat and extended interaction surface relative to other PUF proteins. In complexes with RNAs of different lengths, the protein is unchanged. A single PUF protein repeat is sufficient to induce broadening of specificity. Changes in protein architecture, such as alterations in curvature, may lead to evolution of mRNA regulatory networks.
RNA-binding proteins control an messenger RNA (mRNA)’s life, including its translation, movement and destruction. These events underlie diverse biological processes, ranging from early development to memory formation. Regulatory proteins bind simultaneously to short RNA sequences, typically in 3’-untranslated regions (3’UTRs), and to protein effectors that determine the RNA’s fate. The RNA-binding specificities of the proteins determine which mRNAs are controlled, while effectors determine the outcomes.

RNA regulatory networks, in which a single RNA-binding protein controls multiple mRNAs, are widespread. For example, cytoplasmic polyadenylation element-binding protein, an RNA recognition motif-containing protein, binds to and regulates many mRNAs that participate in the regulation of embryonic cell cycles, while Nova co-regulates multiple mRNAs with roles in alternative polyadenylation and splicing. As a result, RNA-binding proteins integrate post-transcriptional controls, as DNA-binding proteins coordinate transcriptional regulation.

To understand RNA regulatory circuits in molecular terms, we need to know which mRNAs are controlled, how they are recognized, and how the networks change during evolution. PUF proteins are exemplary mRNA regulators. They bind to the 3’UTRs of many mRNAs and do so through single-stranded RNA-binding elements. For example, Puf3p of yeast binds nuclear-encoded mRNAs with roles in the mitochondria. Similarly, PUF proteins in Caenorhabditis elegans, Drosophila and humans control an overlapping battery of mRNAs with established roles in stem cells. The RNA-binding specificities of most PUF proteins are defined in part by three amino acids (tripartite recognition motifs, TRMs) in each of eight tandemly reiterated PUF repeats. TRMs, annotated as XY–Z, recognize specific bases through edge-on (residues X and Y) and stacking interactions (residue Z). Specificity also can be achieved through the requirement for a base that does not contact the protein, but is solvent-exposed. RNA immunoprecipitation and microarray (RIP-chip) studies suggested that Saccharomyces cerevisiae Puf5p binds ~200 mRNAs that contain 10–nt-long binding elements. Genetic analysis has implicated Puf5p in multiple cellular functions, including lifespan, cell wall integrity, and chromatin structure and mating type switching, consistent with the view that it participates in the control of diverse groups of mRNAs.

In this work, we used ultraviolet-crosslinking and high-throughput sequencing to define ~1,000 high-confidence RNA targets of Puf5p in vivo. These targets possess unexpected diversity of binding element lengths, with the same RNA sequence features at the two ends but varying numbers of nucleotides in between. The lengths of sites correlate with the biological functions of the targets. The crystal structures of Puf5p–RNA complexes revealed that the RNAs assume altered conformations to accommodate a fixed protein architecture. The plasticity in binding element length is driven by the flattened curvature of the PUF protein scaffold. The findings suggest ways in which alterations in protein curvature result in new specificities and enable the evolution of new RNA networks.

Results

Identification of Puf5p RNA targets. Using in vivo ultraviolet-crosslinking and high-throughput sequencing (HITS-CLIP), we identified >1,000 mRNAs to which Puf5p binds in S. cerevisiae, representing 16% of the yeast transcriptome. The strain analysed contained a PUF5 gene fused to a tandem affinity purification (TAP) tag. Cells were irradiated during mid-log phase. After lysis and mild RNase treatment, Puf5p was stringently purified through tandem affinity steps and SDS–polyacrylamide gel electrophoresis. The purification of crosslinked complexes was effective, as evidenced by western blotting. Complexes whose RNA components had been 32P-end labelled exhibited heterogenous, slower mobilities than Puf5p alone. To identify RNAs bound to Puf5p, adapters were ligated, the protein digested, and the RNAs converted to complementary DNAs that were analysed by high-throughput sequencing. The adapters contained random bar codes, so that PCR duplication events could be discarded. Upon aligning the sequence reads to the yeast genome, we found that the majority of peaks were within 3’UTRs of mRNAs, and
that the set of target mRNAs were distinct from, but overlapped, those of other yeast PUF proteins. We obtained 16,300,145 and 11,100,468 reads from two biological replicates. Of these, 616,401 and 491,532 (6 and 7%) mapped to unique locations in the *S. cerevisiae* genome, after filtering by quality score and removing PCR duplicates (Fig. 1a; Supplementary Fig. 1a). The functional enrichment of targets detected was only minimally affected by changing the filtering methods we used (Supplementary Fig. 1b). A total of 1,439 peaks were identified, representing a total of 1,190 RNAs (Supplementary Data 1). Of these, 1,043 (88%) mapped to mRNAs. The remaining 12% mapped to non-coding RNAs, including small nucleolar RNA, small nuclear RNA, non-coding RNA and transfer RNAs (Supplementary Fig. 2). Of the peaks in mRNAs, most resided in 3′UTRs (68%), but a fraction mapped to open reading frames (ORFs; 28%) or 5′UTRs (4%; Fig. 1d; see Supplementary Data 1 for complete gene list). Approximately 7% of Puf5p targets defined by RIP-chip overlap with those of Puf3p (Supplementary Fig. 3b). The overlap with Puf3p targets is 30% (Supplementary Fig. 3a; see the Discussion section). We conclude that Puf5p is a broad regulator of a distinct set of mRNAs in *S. cerevisiae*.

The breadth of binding element lengths associated with Puf5p is unusual among PUF proteins (Fig. 2c). For example, three other PUF proteins—human PUM2 (ref. 14), *S. cerevisiae* Puf4p and *C. elegans* FBF-2 (ref. 7)—show a single dominant length of 9 nt in vivo, measured either by CLIP methods22 or inferred from RIP-chip5 (Fig. 2c). Essentially the same behaviour was observed for each protein in vitro24. To examine the sequence preferences of Puf5p *in vitro*, the purified protein was incubated with an RNA library in which 20 consecutive nucleotides had been randomized, generating a theoretical complexity of four20 RNA sequences24. Bound RNAs were eluted, and the process repeated five times. The RNAs were analysed by high-throughput sequencing. In this method, termed SEQRS, the number of reads obtained is a proxy

PuF5p-binding elements range in length from 8 to 12 nt. We developed stringent criteria to select a set of 1,043 high-confidence targets that we used to identify RNA sequence elements bound by PuF5p. We first defined significant peaks as an enrichment of independent reads in a specific genic region (modified false discovery rate (modFDR)<0.01)15 (Supplementary Fig. 1a). To identify high-confidence targets, we required that a peak contain 1-nt or 2-nt deletions in multiple reads (a strong indicator that these RNAs had been crosslinked to Puf5p)6 and a minimum of 10 reads per peak. In addition, these criteria had to be satisfied in at least two biological replicates (Supplementary Fig. 1a). Normalized peak heights at specific loci were reproducible between the biological replicates (Pearson correlation coefficient 0.90; Fig. 1e). Previous studies validated several putative targets of Puf5p by showing they were regulated by that protein *in vivo*17,18. Among the best characterized are SMX2 and *HO* mRNA19,20, which are used here as examples (Fig. 2a). With both mRNAs, peaks lay over the previously characterized binding elements in their 3′UTRs (Fig. 2a).

We identified five classes of PuF5p-binding elements ranging from 8 to 12 nt, each comprising a 5′-UGUA tetranucleotide sequence and a 3′-UA with a variable length spacer region in between (Fig. 2b). We performed an unbiased search of the complete set of high-confidence targets for over-represented sequences in peaks using multiple em for motif elicitation (MEME)21. The position weight matrix we obtained consists of a sequence and a 3′-terminal UA with a variable length spacer region in between (Fig. 2b). Seventy-one per cent of the 1,043 targets, and 66% of the total number of peaks (1,439), contained at least one PuF5p-binding element. Peaks without enriched sequences may reflect contacts that were less sequence-specific or mediated by interactions between PuF5p and RNA-bound factors. The sequences between UGUA and UA display little difference compared with the background nucleotide frequencies surrounding the sites (Supplementary Fig. 4), though adenosine was modestly enriched 1- or 2-nt upstream of the 3′-terminal UA in 9- and 10-nt elements (Supplementary Fig. 4a), and guanosines were uncommon (Supplementary Fig. 4c).

The breadth of binding element lengths associated with Puf5p *in vivo* is unusual among PUF proteins (Fig. 2c). For example, three other PUF proteins—human PUM2 (ref. 14), *S. cerevisiae* Puf4p and *C. elegans* FBF-2 (ref. 7)—show a single dominant length of 9 nt in vivo, measured either by CLIP methods22 or inferred from RIP-chip5 (Fig. 2c). Essentially the same behaviour was observed for each protein *in vitro*24. To examine the sequence preferences of Puf5p *in vitro*, the purified protein was incubated with an RNA library in which 20 consecutive nucleotides had been randomized, generating a theoretical complexity of four20 RNA sequences24. Bound RNAs were eluted, and the process repeated five times. The RNAs were analysed by high-throughput sequencing. In this method, termed SEQRS, the number of reads obtained is a proxy

**Figure 2 | PuF5p binding element exhibits flexibility.** (a) PuF5p interaction peaks map to previously characterized PuF5p binding sites. Orange and green lines represent reads mapped for each replicate. SMX2 has a peak over the 9-nt-binding element. *HO* has a broad peak over two binding elements: a 9 nt lower affinity site and a 8/10 nt higher affinity site. (b) Binding elements identified in high-confidence PuF5p target mRNAs. The MEME-derived logo is shown on the left, which was deconvoluted into five binding elements of 8-12 nt in length. (c) Distribution of binding element lengths for four PUF proteins representing three species. Results from CLIP (red), SEQRS5,6 (light blue), RIP-chip5,7 (green) and PAR-CLIP24 (dark blue) experiments are compared, where available, and shown as enrichment relative to the predominant length for each protein, which is set to 1. The consensus RNA sequence element for each protein is shown, where N is A, C, G or U.
for affinities measured in vitro\textsuperscript{24}. Re-analysis of data obtained with PuF5p\textsuperscript{24} revealed that the number of reads for each site length yielded a pattern similar to that seen in HITS-CLIP, in that 9- and 10-nt sites were the most abundant (Fig. 2c). Eight, 11 and 12-nt sites were less prevalent in SEQR8 than in vivo, but above background in vitro. Indeed, for each protein analysed, we observed greater binding for sub-optimal lengths in the cell than in vitro (Fig. 2c). Many factors affect binding in vivo, including protein–protein interactions and RNA accessibility.

Site length correlates with biological function. The majority of the sites bound by PuF5p consist of individual elements, in which only a single site of unambiguous length is present in the CLIP peak (Fig. 3a; Supplementary Table 1; Supplementary Data 2). Gene ontology (GO) analysis of the PuF5p targets suggests that each binding element length found in mRNAs correlates with a distinct biological role (Fig. 3b; Supplementary Table 2). Surprisingly, when we analysed mRNAs with different binding site lengths separately, RNAs with 8-nt binding elements were over-represented for mitochondrial organization (hypergeometric distribution test—correction Holm–Bonferroni, \( P = 9.5\times 10^{-4} \)), and 10-nt for ribosome biogenesis (hypergeometric distribution test—correction Holm–Bonferroni, \( P = 3.6\times 10^{-4} \)), and 10-nt sites for regulation of gene expression (hypergeometric distribution test—correction Holm–Bonferroni, \( P = 2.9 \times 10^{-4} \)), 11-nt sites for translation (hypergeometric distribution test—correction Holm–Bonferroni, \( P = 3.6\times 10^{-4} \)) (Fig. 3b). A total of 12-nt sites did not correlate with a specific GO term. GO analysis of the mRNAs with binding elements in ORFs or 5′-UTRs, revealed that only 10-nt-binding elements in ORFs were associated with a GO term, positive regulation of pseudohyphal growth (hypergeometric distribution test—correction Holm–Bonferroni, \( P = 8.1\times 10^{-4} \)).

The 8-nt PuF5p elements lie in a subset of mRNAs that also were bound to PuF3p in PAR-CLIP experiments\textsuperscript{25}. PuF5p associated with \( \sim 1,000 \) nuclear-encoded mRNAs with mitochondrial functions\textsuperscript{25}. Even if the criteria selecting high-confidence PuF5p targets are relaxed—not filtering for gapped reads—a very similar enrichment emerges (Supplementary Fig. 1b). Similarly, 22% of the 9-nt PuF5p elements lie in mRNAs that bind PuF4p and are enriched for genes with ribosome assembly and nucleolar functions. Of the PuF5p targets with GO annotations mitochondrial organization or ribosome biogenesis, 46 and 31% are PuF5p or PuF4p targets, respectively\textsuperscript{9}. We suggest that the restricted specificities of PuF5p and PuF4p for 8- and 9-nt sites, respectively, underlie the correlations between PuF5p length of binding sites and their biological functions. In particular, we suggest that the broadened specificity of PuF5p enabled its recruitment to pre-existing RNA regulatory circuits in the S. cerevisiae lineage (see the Discussion section).

Alternate RNA conformations adapt to fixed protein scaffold. To understand how PuF5p accommodates a wide range of target site lengths, we determined crystal structures of the PuF5p RNA-binding domain bound to RNAs of 9–12 nt (Fig. 4; Table 1), including the recognition sequences in SMX2 (9 nt, 2.35-Å resolution), MFA2 (10 nt, 2.15-Å resolution), AA72 (11 nt, 2.5-Å resolution) and AMN1 (12 nt, 2.8-Å resolution) mRNAs (sites of 8 nt did not crystallize). Each RNA corresponded to a high-confidence 3′UTR-binding element in HITS-CLIP (Fig. 2a; Supplementary Figs 5,6). The RNAs bound with affinities that corresponded to our structural observations, in that higher affinity RNA-binding sites correlated with larger numbers of protein–RNA contacts (Fig. 4; Supplementary Fig. 7; Supplementary Table 3).

Despite the varying lengths and sequences of the RNA-binding sites, the overall conformation of PuF5p was unchanged in the four crystal structures (root mean square deviation \( < 0.7 \) Å over all Cx atoms or \( < 1.1 \) Å over all protein atoms). The protein scaffold comprises eight \( \alpha \)-helical repeats flanked by a short N-terminal sequence and a C-terminal helix (R8) (Fig. 4a). The C-terminal repeats 5–8 bound the 5′-UGUA RNA sequence, while repeats 1 and 2 bound the UA-3′ element (Fig. 4a,b). Repeats 3 and 4 lie opposite the variable central regions of the RNAs (Fig. 4c–f). While the overall architecture of PuF5p resembles that of other PUF proteins, PuF5p’s repeats are more irregular in length and structure than seen in human PUM1 and S. cerevisiae PuF5p and PuF4p (Supplementary Fig. 8a). For example, repeats 7 and 8 in PuF5p are unusually long (64 and 72 residues versus 36 in a typical repeat) with extended \( \alpha \)2 and \( \alpha \)3 helices and inter-helix loops. The positions of the \( \alpha \)3 helices relative to the \( \alpha \)1 and \( \alpha \)2 helices are also more varied in PuF5p than PuF3p or PuF4p (Supplementary Fig. 8a).

Since the curvature of the PuF5p scaffold is fixed, RNAs of different lengths adopt different conformations, as described below. Recognition of the 5′-UGUA and 3′-UA elements by repeats 5–8 and 1–2, respectively, are identical in all structures. Differences in RNA conformation and recognition are found opposite the central repeats 3 and 4.

PuF5p binds to the 9-nt SMX2 RNA site by recognizing all but the central fifth base. (Fig. 4c). Bases 1–4 and 6–9 are each recognized by a PUF repeat (Supplementary Fig. 5a). However, the fifth base, C5, lies in an atypical conformation, in which the plane of the base is parallel to the axis of the protein, within van der Waals bonding distance of the side chain of Cys381 in repeat 5 (Fig. 4c). The ribose rings of C5 and U6 adopt C2′-endo conformations to accommodate positioning base C5. We refer to this conformation as ‘5-stacked’.

PuF5p binds the 10-nt MFA2 site similarly to the 9-nt SMX2 site, but an additional base is accommodated by turning the eighth base away from the RNA-binding surface opposite repeat 3, which we refer to as ‘8-flipped’ (Fig. 4d). The positions for all but the eighth base overlap with the 9-nt SMX2 RNA, and the protein–RNA recognition pattern is similar, though the seventh base is a uracil in MFA2 and an adenine in SMX2 (Fig. 4b; Supplementary Fig. 5a).

PuF5p appears to recognize only the 5′-UGUA conserved element and two additional 3′ bases of the 11-nt AA72 site (Fig. 4e; Supplementary Fig. 5b), consistent with weaker binding of PuF5p to this site than 9- or 10-nt sites (12- or 2-fold weaker binding, respectively, Supplementary Table 3). A 2.5-Å crystal structure of PuF5p:AA72 reveals electron density for bases 1–5 and for two 3′ bases bound to PuF5p repeats 1 and 2. In contrast to the parallel orientation of base 5 in 9 and 10-nt sites, base A5 of AA72 stacks directly with base A4 and forms a van der Waals contact with the side chain of Cys381 in PuF5p repeat 5 (Fig. 4f). We refer to this conformation as ‘5-stacked’. Using the consensus sequences as a guide, we modelled the 3′ bases as the conserved U10 and A11 bases and did not model bases 6–9. However, alternate conformations of the RNA are possible, including a conformation similar to that of the 12-nt AMN1 site.

PuF5p binds to the longer 12-nt AMN1 site with a distinct RNA conformation. Unlike the conformations of the shorter length binding sites, bases A4, A5 and C6 stack directly with each other opposite repeat 5. We refer to this conformation as ‘Triple-stacked’. Residues in repeat 5 (Cys381 and Lys385) contact bases A5 and C6 (Fig. 4f). PuF5p repeat 4 does not interact with an RNA base using its edge-interacting residues, but base U7 is bound to repeat 3 (Fig. 4f). Electron density was observed for bases 1–7 and two 3′ bases bound to PuF5p repeats 1 and 2. We modelled the 3′ bases as the conserved U11 and A12.
bases, as we did for the 11-nt AAT2 site, and bases 8–10 were not included in the model.

**Curvature as a determinant of specificity.** The flatter RNA-binding surface of PuF5p contributes to its specificity by creating a more extended RNA-binding surface. PuF5p possesses the least curved RNA-binding surface observed among PUF proteins to date (Supplementary Fig. 8b) and binds to the longest RNA target sequences identified thus far. PuF3p preferentially binds 8-nt sites and exhibits the greatest curvature among the yeast PUFs (Supplementary Fig. 8b); this reflects the regular spacing of RNA-binding helices, which matches the spacing of bases in an extended RNA chain. PuF4p, which binds 9-nt-binding sites, is intermediate in curvature, between PuF3p and PuF5p (Supplementary Fig. 8b).

**Figure 3 | Binding element lengths correlate with biological functions.** (a) Network representation of PuF5p mRNA targets visualized in Cytoscape 3.0.1 (ref. 47). Binding elements were defined as UGUN(x)UA within 25 bp of the peak summit. Large red hubs indicate the length of the binding element. Each node (small circle or square) represents one HITS-CLIP peak in an mRNA. Green square nodes represent mRNAs containing an individual binding element of either 8, 9, 10, 11 or 12 nt; nodes with only one binding element (edge) are placed at the outer periphery of the diagram. Green square nodes with two lines indicate that the HITS-CLIP peak contained two non-overlapping binding elements. Most binding elements were unambiguously of a single length. In a minority of elements, two different lengths of binding elements co-reside in a single sequence. Circles represent these mRNAs with ‘overlapping’ binding elements: for example, ‘8–10’ means a single site of the sequence UGUNNNNNUAUA, which possesses both 8- and 10-nt elements depending on the 3'-UA used, and either sequence may be used in vivo, and ‘8–10, 9–11’ means that two distinct overlapping sites are present under the peak. The key to the right is a colour code for each combination of overlapping binding element lengths (nt). The numbers of mRNAs containing overlapping binding elements are provided in Supplementary Table 1. (b) Gene ontology term enrichment for mRNAs belonging to each length of binding element using SGD YeastMine. GO terms that are significantly over-represented in the gene list are bolded for each binding element length. Numbers of genes in the most enriched GO terms are in parentheses.
Discontinuous AMN1 and 9-nt with atoms coloured by element (carbon, grey; nitrogen, blue; oxygen, red; lines. RNA-interacting residues and MFA2 RNA. Puf5p is shown as a ribbon diagram with PUM repeats coloured between Puf5p repeats (rectangles) and SMX2 RNA (Fig. 5a). These proteins were identified as orthologues using SYNERGY, which relies on the species tree, sequence similarity and synteny. Their binding preferences versus length of site were evaluated using a set of RNAs 8–12 nt in length, conforming to the sequence UGU(A)₃-UA using the yeast three-hybrid system. All the RNAs thus maintained the 5'-UGUA and 3'-UA critical for S. cerevisiae Puf5p interaction and contained a single 3'-UA element to define the target length unambiguously. In the three-hybrid assay, the level of expression of a reporter gene (LacZ) is a proxy for the affinity of the interaction.

Puf5p proteins across Ascomycota exhibited broad binding specificities. Pu5 proteins bound similarly to sites of 9, 10, 11 and 12 nt (Fig. 5b). The more restricted specificities of Pu5 for 9-nt sites (Fig. 5c), and Pu5 for 8-nt sites (Fig. 5d), also were conserved across the entire phylum, with the exception of S. pombe Pu3. This protein bound a broad range of site lengths, unlike its orthologues in other species that showed preference for 8-nt sites.

The broadened specificity of S. pombe Pu3 appears to have arisen exclusively in the fission yeast lineage, which enabled us to probe how that broadening arose during evolution. We reasoned that the broadening was not due to the identity of the RNA-interacting TRMs, as the residues are identical among all the Pu3p orthologues (with the exception of repeat 3 in N. crassa, with a Gln to Arg substitution). To identify the key regions of the proteins that confer specificity, we prepared chimeras in which segments of the S. cerevisiae and S. pombe proteins were exchanged (Fig. 6a). The specificity profile—broad or narrow—was conferred by PuF repeats 6–8. A chimeric protein possessing repeats 6–8 from S. pombe exhibited broad specificity, while a chimera with repeats 6–8 of the S. cerevisiae protein had narrow specificity (Fig. 6a,b). The protein sequences in repeat 6 contain a divergent region among Pu3p orthologues (Supplementary Fig. 9). Indeed, substitution of S. pombe repeat 6 alone into an S. cerevisiae scaffold was sufficient to confer the broad specificity profile (Fig. 6b).

Discussion
Pu5p is a broad regulator of RNAs in S. cerevisiae, binding to > 1,000 RNA targets, constituting ~16% of the transcriptome. A total of 71% of these targets possess recognizable binding elements beginning with a 5'-UGU sequence, which range in

![Image](https://example.com/image.png)

Figure 4 | Crystal structures of Pu5p in complex with representative 9-12 nt target mRNAs. (a) Crystal structure of Pu5p in complex with 10-nt MFA2 RNA. Pu5p is shown as a ribbon diagram with PUM repeats coloured alternately blue and red. Two disordered loops are indicated with dotted lines. RNA-interacting residues and MFA2 RNA are shown as stick models with atoms coloured by element (carbon, grey; nitrogen, blue; oxygen, red; sulfur, yellow; phosphorus, orange). (b) Schematic diagram of interactions between Pu5 repeats (rectangles) and MFA2 RNA bases (ovals). Hydrogen bonds are indicated by dotted lines. (c) Interactions between repeats 3–5 of Pu5p and 9-nt SMX2 RNA. (e) 10-nt MFA2 RNA, 11-nt AAT2 RNA or 12-nt AMN1 RNA (f). Hydrogen bonds are indicated by dotted lines. Discontinuous F₀ – F₁ electron density (contoured at 3σ) following base A5 in the 11-nt AAT2 RNA is shown in e.
length from 8 to 12 nt. The variations in length are accommodated by conformational adaptations of the RNA onto a fixed protein scaffold. The wide range of mRNA target site lengths is consistent with prior studies that linked Puf5p to a spectrum of functions, including cell wall integrity \(^{11}\) and chromatin structure\(^{10}\).

The biological functions of target mRNAs are correlated with the length of binding elements they possess. How does this correlation arise? We propose that the correlation is imposed by the length of binding elements they possess. How does this impose the requirement for these RNA conformational changes? We consider the implications of the different lengths of binding sites in specific subcellular compartments (such as P-bodies), in which proteins and RNAs are present at high concentrations, and low complexity, Q/N-rich regions present in Puf3p, Puf4p, and Puf5p proteins that could facilitate aggregation\(^{32}\).

RNAs of different lengths adopt a broad range of conformations when bound to Puf5p. The flatter, extended scaffold of Puf5p, combined with its specificity for 5' and 3' sequences, imposes the requirement for these RNA conformational variations and permits recognition of 8–12-nt length RNAs. The elegance of this arrangement is that very similar sets of atomic contacts between amino acids and RNA bases are maintained in the different complexes, despite the range of RNA lengths they possess. For example, 18 of the 21 edge-on contacts made between Puf3p and its RNA target are also made in mRNPs with mitochondria-related functions, while Puf4p binds 9-nt-binding elements in mRNPs with roles in ribosomal biogenesis and assembly\(^{6}\).

Two PUF proteins that bind the same site could do so sequentially, competitively or cooperatively. Genetic studies demonstrate that Puf4p and Puf5p redundantly control the decay rate of common targets\(^{30}\). In the absence of one of the proteins, the other is sufficient. However, for other common targets, the actions of two PUF proteins may be sequential. For example, MRPL8 mRNA is a target of both Puf5p and Puf3p, possesses a single binding element, and is localized to the mitochondrial periphery in a Puf3p-dependent manner\(^{31}\). Puf5p could exchange with Puf3p, facilitating repression (Puf5p) \textit{en route} to localization to the mitochondria (Puf3p).

While 71% of Puf5p targets possess discernible binding elements, 29% do not. RNAs without binding elements may associate with Puf5p indirectly, perhaps through a protein to which it and Puf5p are bound. Crosslinking to RNAs without sites could also be driven by their high concentrations in specific subcellular compartments (such as P-bodies), in which proteins and RNAs are present at high concentrations, and low complexity, Q/N-rich regions present in Puf3p, Puf4p, and Puf5p proteins that could facilitate aggregation\(^{32}\).

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whether that protein binds 8-nt sites (S. cerevisiae) or accommodates 8, 9 or 10-nt sites (S. pombe). S. cerevisiae repeat 6, which induces narrow specificity, contains additional residues relative to the same region of the S. pombe protein, which, although not near the RNA-binding residues, may alter the structure with corresponding effects on specificity (Supplementary Figs 8a and 9).

From an evolutionary perspective, the broadening of Puf5p's specificity may have enabled new regulatory inputs into existing RNA circuits. Perhaps, the ability of Puf5p to recognize a wide range of target lengths arose after ancestral proteins (for example, Puf3p) already regulated batteries of RNAs with related functions (Supplementary Figs 8a and 9). From an evolutionary perspective, the broadening of Puf5p's specificity may have enabled new regulatory inputs into existing RNA circuits. Perhaps, the ability of Puf5p to recognize a wide range of target lengths arose after ancestral proteins (for example, Puf3p) already regulated batteries of RNAs with related functions (Supplementary Figs 8a and 9).

Figure 5 | Binding element preference across phylum Ascomycota. (a) Phylogeny of Ascomycota fungi. Each subphylum is indicated: budding yeast—yellow; filamentous fungi—green; and the fission yeast—blue. Species used for RNA-binding studies below are highlighted. (b-d) RNA-binding element length preferences for Puf5p, Puf4p and Puf3p orthologues. RNA binding was assayed using the yeast three-hybrid system48. RNAs tested were 8 (5'-UGUAUAUA-3'), 9 (5'-UGUAAAAUA-3'), 10 (5'-UGUAAAAAUA-3'), 11 (5'-UGUAAAAAAUA-3') or 12 (5'-UGUAAAAAAAAUA-3') nucleotides in length. Raw luminescence values per cell for each biological replicate (n = 3) were averaged then normalized to controls where the 5'-U sequence was mutated to ACA. Error bars represent s.d. The full set of six species could not be tested for all five binding element lengths. N. crassa does not have a Puf4 ortholog; C. albicans and S. pombe Puf4p failed to specifically bind any RNA; E. gossypii Puf4p clones could not be obtained.

Methods
HITS-CLIP. HITS-CLIP was performed on 81 of S. cerevisiae harbouring an integrated TAP tag sequence at the C terminus of the endogenous puf5 locus (GE YSC1178-202231131) per biological replicate. Cells were pelleted, washed with water and distributed into Petri dishes. The cells were ultraviolet irradiated with 1,000-mJ wavelength 254 light. Pelleted cells were snap frozen in liquid nitrogen for 1 h. Beads were washed with 10 ml CBB (25 mM Tris (pH 7.5), 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 10 mM β-mercaptoethanol) at 25 °C. A measure of 30 μl of RQ1 DNASE (Promega, M6101) and 10 μl 1:10,000 dilution of RNase A (Promega A7973) were added and incubated at 37 °C for 10 min. The lysate was cleared by centrifugation then incubated with 400 μl pre-washed calmodulin Sepharose beads (GE 17-0529-01) rotating at 4 °C for 1 h. Beads were washed with 10 ml CBB and then incubated in 3 ml CEB (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM EDTA, 0.1% IGEPAL, 10 mM β-mercaptoethanol) at 4 °C, followed by washing with 2 ml CEB. The supernatant was incubated with 400 μl pre-washed IgG beads (Life Technologies 11202D) rotating at 4 °C for 1 h. Following incubation, beads were washed 1 × with PNK (50 mM
Figure 6 | Evolution of Puf3p broadened RNA specificity. (a) Schematic representations of chimeras tested in b. PUM repeats are represented by circles; S. cerevisiae, blue; S. pombe, green. (b) Broadened S. pombe Puf3 specificity is linked to repeat 6. RNA-binding element length preferences for the chimeric proteins were assayed using the yeast three-hybrid system with 8, 9 or 10 nt RNAs as in Fig. 5. Raw luminescence values per cell for each biological replicate (n = 3) were averaged then normalized to an acaAAAUA mutant negative control, which depresses binding >100-fold49. Error bars represent s.d.
Protein–RNA crystallization. All experiments were conducted in the 15–20% (v/v) glycerol and flash frozen in liquid nitrogen. For phasing, a Puf5p:SMX complex crystal was soaked in 17.5% (w/v) PEG 3350, 0.5 M KI, 0.1 M CBTP and 15% (v/v) glycerol for 5 min and then flash frozen.

X-ray data collection. X-ray data for structures of the 9-, 10- and 12 nt RNA complexes were collected at the SER-CAT beamline at the Advanced Photon Source, Argonne National Laboratory. Data for the 11 nt RNA complex and the isodeoxosaccharide crystal were grown in the same conditions as the P21212 structure as the search model. Data and refinement statistics are shown in Table 1. All models show good geometry according to MolProbity: 95–98% of the residues are in favoured regions of the Ramachandran plot, and there are no outliers.

Electrophoretic mobility shift assays. RNA oligonucleotides were radio labelled using 32P-γ-ATP and T4 polynucleotide kinase (New England Biolabs) following the manufacturer’s instructions. Serially diluted PuF5p was mixed with 100 pmol labelled RNA in buffer containing 10 mM HEPES, pH 7.4; 50 mM NaCl, 1 mM EDTA; 0.1 mg ml⁻¹ bovine serum albumin; 0.01% (v/v) Tween 20 and 0.1 mg ml⁻¹ yeast tRNA. After overnight, incubation at 4 °C, 4-μl loading dye (15% v/v Ficoll 400 and 0.01% bromophenol blue) was added to each 20-μl reaction before gel loading. Novex TBE gels (10%; Invitrogen) were run at 100 V at 4 °C for 30 min to resolve the samples. The gels were dried and exposed to storage phosphor screens. The screens were scanned using a Molecular Dynamics Typhoon phosphorimaging system (GE Healthcare). The band intensities were analysed using the ImageQuant. K values were calculated using GraphPad Prism by fitting the data to a hyperbolic function of binding and a Hill coefficient of 0.93 of PuF5p was active, as determined using the method described in reference 46. The reported Kd values were not adjusted.

Yeast three-hybrid assays. Each orthologous PuF protein-binding domain was cloned into activation domain–protein fusion plasmid, pGADT7 (ref. 28). Oligonucleotides representing each RNA sequence were ordered from Integrated DNA Technologies (IDT) and cloned into the hybrid RNA plasmid, p3HR2 (ref. 28). All experiments were conducted in the S. cerevisiae strain YBR1-1 (MATa, ura3-52, leu2-3, -112, his3-200, trp1-1, ade2, lys2:[LexAop]-HIS3, URA3:[LexAop]-lacZ and LexA-MS2 M52 coat (N55K)). Strains were lithium acetate transformed with appropriate combinations of plasmids and plated on synthetic dextrose media lacking uracil and leucine. Single colonies were selected and allowed to grow to stationary phase, then density and grown for about 4 h. OD₅₃₀ for each culture was measured, then 50 μl of culture was added to 50 μl of Beta-gal (Promega E4720) and incubated for 1 h in the dark. Luminescence was measured by microplate reader (Bio-Tech Synergy 4). Raw luminescence was normalized to OD₅₃₀ and each biological replicate (n = 3) was then averaged and s.d. was calculated.

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Author contributions

D.W., C.Q., Z.T.C. and M.N. performed the experiments. All authors analyzed the data and prepared the manuscript.

Additional information

Accession codes: The Illumina FASTQ sequence files have been deposited in the Sequence Read Archive (accession numbers SRR2085177 and SRR2084791). The atomic coordinates and structure factors for the crystal structures of Puf5p in complex with SMX2 (PDB IDs: 5BYM and 5BZV), MFA2 (PDB ID: 5BZ1), AA72 (PDB ID: 5BZU) and AMN1 (PDB ID: 5BZ5) RNAs have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank.

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