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Drosophila Pur-α binds to trinucleotide-repeat containing cellular RNAs and translocates to the early oocyte

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Pur-α was identified as a DNA-binding protein with high affinity for the single-stranded PUR-motif $(GGN)_n$. Bound to DNA, Pur-α can both activate and repress transcription. In addition, Pur-α binds to RNA and may participate in nuclear RNA export as well as transport of cytoplasmic neuronal mRNP granules. The heritable trinucleotide-repeat expansion disease Fragile X associated Tremor and Ataxia Syndrome (FXTAS) leads to interaction of Pur-α with mutant, abnormally long $r(CGG)_n$ stretches, which appears to titrate the protein away from its physiologic mRNA targets into nuclear RNA-protein aggregates. We examined the function of Drosophila Pur-α and demonstrate that the protein accumulates in the growing oocyte early in oogenesis. Co-purifying proteins reveal that Pur-α is part of transported mRNP complexes, analogous to its reported role in nerve cells. We analyzed the subcellular localization of mutant GFP-Pur-α fusion proteins where either nucleic acid binding or dimerization, or both, were prevented. We propose that association with mRNAs occurs in the nucleus and is required for nuclear export of the complex. Furthermore, efficient translocation into the oocyte also requires RNA binding as well as dimerization. RNA binding assays demonstrate that recombinant Drosophila Pur-α can bind $r(CGG)_4$ with higher affinity than previously thought. Related sequences, such as $r(CAG)_4$ and the consensus sequence of the *opa*-repeat $r(CAG)_3CAA$, can also associate with Pur-α in vitro and in vivo. The mRNA target spectrum of Pur-α may therefore be larger than previously anticipated.

Introduction

Regulation of gene expression takes place both at the level of transcription and beyond. The post-transcriptional steps, such as processing, nuclear export and—if applicable—cytoplasmic transport, depend on specific protein factors that interact with the mRNA. The protein Pur-α was identified due to its binding to single-stranded, purine-rich DNA such as the Pur-motif $(GGN)_n$.[1,2] Vertebrate Pur-α is a transcription factor and—depending on the context—can either activate or repress gene expression. Furthermore, it associates with certain viral proteins and is an important player in oncogenic transformation (reviewed in ref. 3). Biochemical analysis of neuronal RNA-transport granules detected the presence of Pur-α as a tightly associated protein that is required for mRNA transport and the progression of transport granules, revealing that Pur-α also functions in the cytoplasm and binds RNA.[4] Deletion of *pur*-α in mice results in perinatal lethality and abnormal brain morphology,[5,6] consistent with a neuronal requirement of Pur-α. RNase treatment disrupted the transport granules and liberated Pur-α together with its paralog Pur-β,[4] indicating that the mRNP transport complex must be stabilized by RNA.

Trinucleotide repeat expansion diseases, such as Huntington's disease or Fragile-X associated Tremor and Ataxia Syndrome (FXTAS) are caused by extended stretches of tandemly repeated nucleotide triplets.[7] The mutations are believed to arise due to polymerase slippage during replication and the affected patients suffer from progressive neurodegeneration. In many cases, this expansion occurs within the coding sequence of the affected gene leading to long stretches of a single amino acid (e.g., poly-Q stretches caused by the $(CAG)_n$ repeats in Huntingtin). This may lead to abnormal expression levels and/or impair protein function.[8] In other cases, however, the expanded repeat is found in non-coding regions of the mRNA, suggesting that translation into homopolymeric stretches of amino acids may not be required for the pathology. Furthermore, the expanded repeat sequence can be inserted into the non-coding portion of a heterologous transcript and still induce corresponding pathological changes in

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animal models of FXTAS[9] and spinocerebellar ataxia type 3.[10] As a consequence, at least part of the pathology of the trinucleotide repeat expansion diseases may be due to RNA toxicity that operates in trans rather than a deregulation of the trinucleotide-repeat carrying transcript itself. In animal models of trinucleotide-repeat disorders, the severity of the toxic effect can be influenced by modulating the expression of RNA-binding proteins such as Drosophila Muscleblind (*mbl*) or Pur-α (*pur*-α), leading to the hypothesis that the toxicity of abnormally expanded trinucleotide repeats may at least in part be caused by titrating away these proteins from their physiologic mRNA targets.[9,10] Although the genes harboring expanded trinucleotide-repeats are often broadly expressed, the nervous system appears to be most sensitive and patients suffer predominantly from neurodegenerative processes. Not surprisingly, animal models of these diseases have therefore focused on the analysis of effects in the nervous system, and neuropathological changes are discernible in *pur*-α knockout mice.[5,11]

Analysis of the amino acid sequence revealed that Pur-α contains three occurrences of a characteristic sequence stretch called PUR-repeat; the first two repeats fold together into one nucleic acid binding domain, while the third repeat mediates protein dimerization by forming an intermolecular nucleic acid binding domain with the C-terminal PUR-repeat of a second Pur-α molecule. Crystallographic analysis of Drosophila Pur-α has revealed the three dimensional structure of its nucleic acid binding domain, leading to the identification of amino acids that are essential for nucleic acid binding.[12] In the fruitfly *Drosophila melanogaster*, a number of genes that are important for the development of the nervous system also take part in key signaling events during oogenesis.[13] The extensive transport and localization of mRNAs prior to translation that characterizes Drosophila oogenesis is also reminiscent of the dendritic transport of mRNAs in neurons.[14-17] In the early Drosophila embryo, an estimated 70% of all mRNAs show a specific subcellular localization pattern and most of these are positioned already during oogenesis.[18]

We therefore analyzed the localization and co-purifying proteins of Drosophila Pur-α during oogenesis with a combination of fluorescence microscopy and biochemical tools, revealing that Pur-α protein is part of mRNA transport complexes in the ovary. We transgenically expressed point mutant and truncated Pur-α proteins fused to GFP and conclude that RNA binding as well as protein dimerization are required for proper localization. The protein levels are particularly high in polar cells, a pair of specialized somatic cells in each egg chamber that are important to coordinate migration of the follicle cells over the egg chamber.[19] In vitro and in vivo binding studies demonstrate that Pur-α has moderate sequence specificity, showing preferential binding to purine-rich trinucleotide repeats that are much shorter than the ones created by disease-causing mutations. We noticed that many developmental genes, e.g., Notch (*N*) share a purine rich trinucleotide repeat motif previously referred to as the *opa*-repeat.[20] A 12-mer consensus derived from this repeat is bound by Pur-α in vitro and mRNAs containing such a sequence motif have a higher propensity to associate with GFP-Pur-α in vivo. Thus, the *opa*-repeat containing genes may be among the physiological targets of Pur-α.

Results

Localization and transport of Pur-α during oogenesis. We developed monoclonal antibodies to examine the localization of Pur-α protein during oogenesis. In the germarium, Pur-α was detected in somatic follicle cells as well as the descendants of the germ line. Staining within the cytoplasm was not uniform; rather, the protein was distributed over many small particles or aggregates. In early egg chambers, Pur-α was equally abundant in follicle cells and nurse cells, but accumulated in the developing oocyte. During the early stages in oogenesis the oocyte nucleus is transcriptionally silent and pumping of the nurse cell content into the oocyte has not been initiated.[21,22] The most straightforward interpretation is therefore that Pur-α is actively transported into the oocyte. Pur-α was a predominantly cytoplasmic protein in all cell types and developmental stages of the Drosophila ovary, but weak nuclear staining can be observed (**Figs. 1A and S1** for high-resolution). The first two PUR-repeats of Pur-α fold together to generate a nucleic acid binding domain that prefers purine-rich nucleic acids (both DNA and RNA[12]). The third PUR-repeat can mediate protein dimerization by folding to a similar domain as repeats one and two in an intermolecular interaction. We dissected the influence of domain architecture and RNA binding on Pur-α localization and transport during oogenesis with the help of transgenic GFP-fusion constructs.

The expression of a cDNA-based, full-length GFP-Pur-α protein faithfully recapitulated the localization of endogenous Pur-α (**Fig. 1B**). A structure-guided truncation of the C-terminal PUR-repeat, leading to monomeric Pur-α that retains the ability to bind nucleic acids,[12] was less efficiently transported into the oocyte (**Fig. 1C**). A similar reduction of transport was detected upon introduction of two point mutations (R80A, R158A) in PUR-repeats I and II, leading to strongly reduced nucleic acid binding while retaining correct folding,[12] in the context of full-length Pur-α (**Fig. 1D**). A combination of both, i.e., monomeric Pur-α with impaired nucleic acid binding, was no longer transported into the oocyte. Furthermore, the accumulation in granules and the predominantly cytoplasmic localization of Pur-α were lost. This indicates that Pur-α shuttles between nucleus and cytoplasm and that nucleic acid binding of Pur-α is required for efficient nuclear export (**Fig. 1E**). The third PUR-repeat has not been biochemically characterized. Upon dimerization, it may be capable of binding nucleic acids; we therefore created transgenes in which corresponding point mutations were present in all three PUR-repeats in the context of a full-length GFP-Pur-α protein (R80A, R180A, R226A). This protein retained the ability to dimerize with wild-type Pur-α (**Fig. S2A**). The localization of the GFP-fusion protein was similar to the one of truncated Pur-α, i.e., reduced transport into the oocyte and an increased proportion of protein that can be found in the nucleus (**Fig. 1F**). In summary, our results are consistent with nucleo-cytoplasmic shuttling and oocyte directed transport of Pur-α.

Pur-α is enriched in the polar cells of the follicular epithelium. A detailed examination revealed that the cells at the anterior and posterior tip of each egg chamber show particularly strong Pur-α expression (see **Fig. 1A**, red arrowheads). This is also recapitulated by our GFP-Pur-α fusion construct driven by the ubiquitin promotor but not a control construct expressing only GFP (**Fig. 1B**), arguing that post-transcriptional regulation, such as protein or mRNA stability, is responsible for the difference in protein level. Based on their position, their negative staining for the transcription factor *eya* (**Fig. 2A**) and their positive staining for the cell-adhesion molecule *fas-III* (**Fig. 2B and C**), we identified the Pur-α enriched cells at the extremities of the egg chamber as the polar cells. These cells have a common ancestor with the stalk cells and play a pivotal role in separation of subsequent egg chambers, migration and organization of the follicular epithelium as well as establishment of the A/P-axis within the oocyte. GFP-Pur-α expression remains high in the polar cells after their migration together with the border cells (**Fig. 2D**). Specification of these polar cells depends on a relay of Notch and JAK/STAT signaling between subsequent germ line cysts as they bud out of the germarium.[23,24]

Drosophila Pur-α is part of mRNP transport complexes. Our results indicate that Pur-α may participate in the transport of mRNAs into the growing oocyte. To gain further insight into the molecular basis for this, we analyzed the proteins that co-purify with Pur-α. We prepared protein extracts from transgenic ovaries expressing either full-length GFP-Pur-α or, as a control, GFP only. With the help of GFP-trap® affinity-beads, we then purified GFP-containing protein complexes, separated the associated proteins by SDS-PAGE and performed mass spectrometry to identify these proteins. With a cutoff of 20% sequence coverage, we identified several known components of mRNP transport complexes in oocytes, notably Tral, Exu, Yps and Me31B, as the major proteins that were specifically associated with Pur-α (**Table S1**, only proteins that were not detected in the GFP control immunoprecipitate are shown). The well-known RNA binding protein Staufen was not detected in the GFP-Pur-α immunoprecipitate, indicating that Pur-α associates only with a subset of all mRNPs present in the ovary.

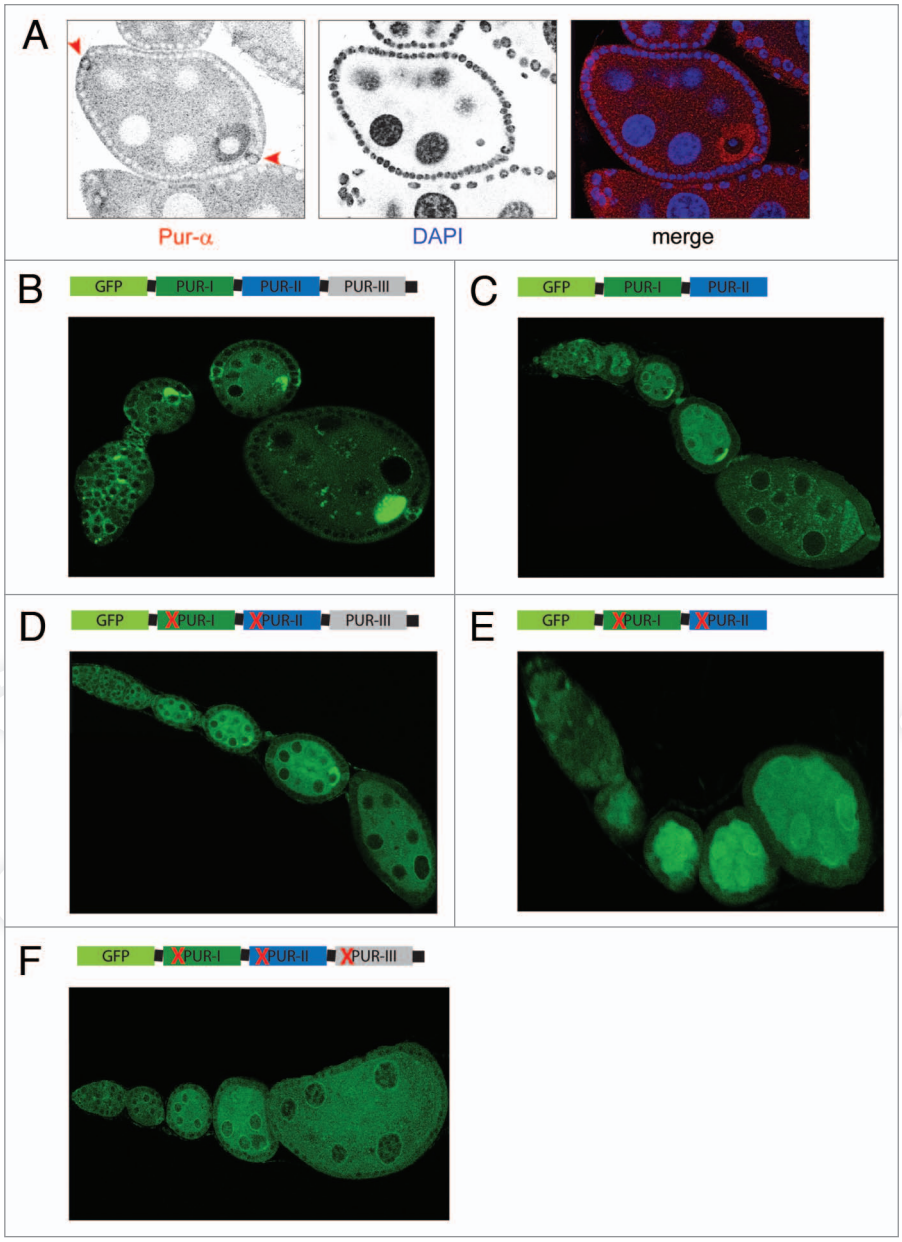

Figure 1. Localization of Pur-α during Drosophila oogenesis. (A) Immunofluorescence detection of Pur-α protein in a stage 8–9 oocyte using the rat monoclonal antibodies 7H8 and 1E10 developed in this study; DNA was stained with DAPI. Arrowheads indicate the position of cells with high expression levels of Pur-α at the anterior and posterior tip of the oocyte. See Figure S1 for a larger image showing more detail of Pur-α localization. (B) Fluorescence image of transgenically expressed, full-length GFP-Pur-α during early oogenesis. (C) Localization of truncated GFP-Pur-$\alpha_{40\text{-}185}$, a fusion protein that has lost the potential to dimerize; (D) Localization of full-length GFP-Pur-α containing point mutations in the first and second PUR-repeat (R80A, R158A) that prevent nucleic acid binding; this protein retains the ability to dimerize (also with endogenous wild-type Pur-α). (E) Localization of mutant, truncated GFP-Pur-α protein; this protein can neither bind to RNA nor dimerize. Note that in comparison with (C), the fusion protein is no longer excluded from the nucleus. (F) Localization of full-length GFP-Pur-α with point mutations in all three PUR-repeats (R80A, R158A, R226A); this protein can no longer bind nucleic acids; while dimer-formation is still possible, this will not reconstitute a functional RNA-binding domain due to the point mutation. Note that in comparison with (B) the fusion protein is also no longer excluded from the nucleus.

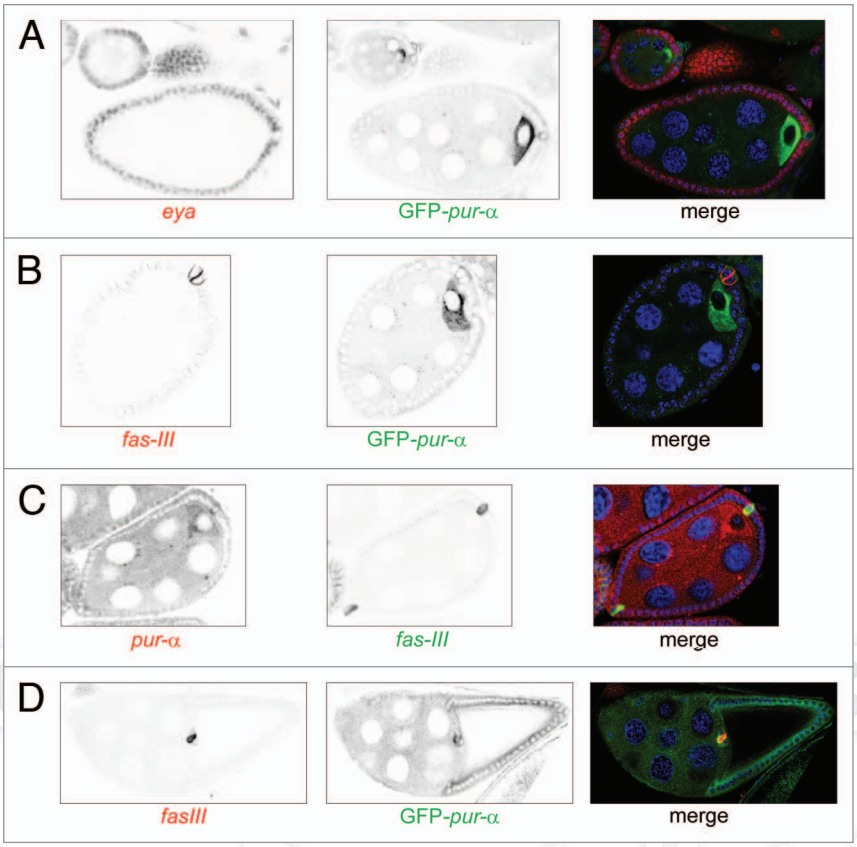

Figure 2. Polar cells express high levels of Pur-α. (A) Immunofluorescence staining of the transcription factor *eya*, which is not expressed in the polar cells, and transgenic GFP-Pur-α. (B) Immunofluorescence staining of the cell adhesion molecule *fas-III*, present only in the polar cells of the developing cyst, and transgenic GFP-Pur-α. (C) Co-staining of endogenous Pur-α and *fas-III*. (D) Immunofluorescence of *fas-III* and GFP-Pur-α in a stage 10 oocyte; Pur-α levels are maintained high during migration of the polar cells through the nurse cells.

To validate the mass spectrometry results we repeated the GFP affinity purification from ovary extracts, followed by western blotting. To address the functional and structural requirements for co-purification, we also included GFP-fusions of several mutations and truncations of Pur-α. The association of Cup, Tral and Me31B with full-length GFP-Pur-α could be validated by western blotting with available antibodies (**Fig. 3**). A fusion protein containing only the PUR-repeats I and II showed the same extent of interaction as full-length protein, indicating that dimerization is not required for these associations. On the other hand, the introduction of point mutations leading to deficient RNA binding in both PUR-repeats (R80A, R158A) strongly reduced the association with the three factors. This was also true—though to a lesser extent—in the context of full-length Pur-α. A second set of point mutations (R65A, R142A) diminishes RNA binding less than the previous one[12] and impaired association of Cup, Tral and Me31B to a lesser extent (**Fig. 3**). Taken together, our results suggest that the association of Pur-α with mRNP transport components is likely mediated by mRNAs bound to Pur-α. We observed a similar RNA-dependent co-purification of dFmr1, Ago1 and Ago2 with Pur-α expressed in cultured S2-cells (**Fig. S2B**).

RNA binding characteristics of Pur-α. The capacity of Pur-α to bind nucleic acids is crucial for its incorporation into the mRNP transport particle (**Fig. 3**) and competition of expanded CGG repeats with normal mRNAs may underlie some of the pathologic changes associated with the trinucleotide repeat disorder FXTAS.[9] If the extended repeats represent overly efficient binders for Pur-α, then similar but shorter repeats should be bound by Pur-α under normal conditions. We therefore tested whether short trinucleotide repeats are also bound by Pur-α. Previous in vitro studies using electrophoretic mobility shift assays (EMSA) demonstrated binding of full-length Pur-α as well as a truncated protein containing only PUR-repeats I and II to $r(CGG)_4$ with a moderate affinity of 1–2 μM.[12] We repeated the affinity measurements comparing EMSA and fluorescence anisotropy, a solution based assay that does not require separation of bound and free probe (**Fig. 4** and inserts; quantification and curve-fitting is shown in **Figs. S3–S8**). The affinity of Pur-α to $r(CGG)_4$ was 176 ± 60 nM, significantly higher than the affinity we obtained with EMSA (2,331 ± 346 nM). To rule out that this difference was due to a measurement artifact we repeated the experiment with a different solution-based technique, microscale temperature gradient thermophoresis (MST[25]) and obtained an affinity of 365 ± 34 nM. It appears thus that the Pur-α RNA affinity in solution is considerably higher than previously thought. We tested several other trinucleotide repeat sequences for in vitro binding: While the sequence $r(GGA)_4$ bound with high affinity to Pur-α (149 ± 9 nM), $r(CAG)_4$ showed a lower affinity. The sequences $r(CUG)_4$ and $r(CAA)_4$ showed only low-affinity binding. We noticed that many developmental genes in Drosophila—e.g., *N*, *dsh* or *Dl*—share a CAG-rich RNA sequence motif previously referred to as the *opa*-repeat.[20] We could demonstrate binding of an oligonucleotide with the sequence of the *opa*-repeat, $(CAG)_3CAA$, to Pur-α in vitro, albeit with a lower affinity than $r(CAG)_4$.

Are similar sequences also associated with Pur-α in vivo? To answer this question we prepared ovary extracts from transgenic flies expressing either a GFP-Pur-α fusion or GFP alone and immunoprecipitated the protein-RNA complexes with GFP-trap beads as described above for the proteomic analysis. We then isolated RNA from input and bound fractions and performed microarray analysis. We observed that overall, RNA-binding of Pur-α is pervasive: About 6,000 genes were detectable in all conditions, but no group of mRNAs that clearly associated more than the general average with GFP-Pur-α was discernible. Since recovery values cannot be directly calculated from the microarray data, we calculated relative changes in the input and bound fraction between the GFP-Pur-α and GFP samples, then ranked the genes

according to this "fold change." If an mRNA has a propensity to associate with Pur-α, then it should rank higher in the "bound" list than in the "input" list. Conversely, if a gene is not associated with GFP-Pur-α, then its relative rank should be higher in the input list than in the bound list. While the pervasive binding did not allow firm conclusions for any individual mRNA, we could use these lists (**Table S1**) to search for trinucleotide repeat enrichment in the bound list relative to the input. To this end, we determined the number of N_3 occurrences for each gene (using only the coding sequence of the longest isoform) as well as the occurrence of each trinucleotide twice, three and four times in a row within the same coding sequence (yielding total occurrences, doublets, triplets and quadruplets, resp.). For a transcriptome-wide description we then constructed cumulative sum curves for the ranked input and bound lists for each of the 64 trinucelotides and the *opa*-repeat. If the cumulative sum curve for the bound list lies above the curve for the input list for a given trinucleotide, this sequence has a propensity to associate with GFP-Pur-α, whereas a cumulative sum curve that falls below the one for the input list indicates that the corresponding trinucleotide is depleted in the GFP-Pur-α immunoprecipitate.

As shown in **Figure 4**, the graphs derived from the RNA co-immunoprecipitation are consistent with our in vitro binding data. This fact was observable with all three permutations of the trinucleotides examined and the determined association with GFP-Pur-α became more pronounced for doublet and triplet occurrences compared with total occurrences. This indicates that direct trinucleotide repeats are preferentially bound by GFP-Pur-α (**Figs. S10–S14**). We noticed that—consistent with the moderate sequence specificity we found in vitro—the longer mRNAs associated slightly more efficiently with GFP-Pur-α independent of their sequence (**Fig. S15** and cumulative sum curves for CDS length). We therefore calculated the area between the two curves for each trinucleotide and subtracted the length-dependent contribution. In a list ranked according to the remaining difference between the curves, a preference for purine-rich trinucleotides is visible for both total and triplet occurrences. The *opa*-repeat $(CAG)_3CAA$—of which 787 instances were detected—also showed a clear propensity to associate with GFP-Pur-α in vivo, ranking on par with the $(CGG)_3$ permutations (**Fig. 5**). To quantify the recovery rates we analyzed individual mRNAs in a GFP-Pur-α vs. a GFP-only immunoprecipitation from ovary extracts by quantitative RT-PCR (**Fig. S16**). Consistent with the pervasive RNA binding observed in our arrays, we found that all the mRNAs we analyzed had a higher recovery rate with GFP-Pur-α. There was a trend for higher recovery for the trinucleotide-repeat containing mRNAs (e.g., *N*, *ago2*, *dsh*, see also **Fig. S9**) than for the four control mRNAs (*act5C,* α*-tub, gapdh, rp49*).

Is the trinucleotide-repeat character of the sequence a required feature, or is a clustering of purine-bases sufficient for preferential association? To distinguish these possibilities, we repeated our bioinformatics analysis by calculating the cumulative sum curves for dinucleotide repeats. Similar to the situation with trinucleotides, we found that the purine-rich dinucleotides were preferentially recovered. This recovery also increased with the number of repeats, indicating that most likely a high local density of purine bases is sufficient for binding of Pur-α (**Figs. S17 and S18**). However, we observed that while a trinucleotide-repeat character of the sequence may not be required for binding to Pur-α, the occurrence of two consecutive, identical trinucleotides is more frequent than three consecutive and identical dinucleotides in the coding region of the roughly 6,000 ovary-expressed Drosophila mRNAs (**Fig. S19**). Thus, while Pur-α probably also binds purine-rich regions with complex sequences, the trinucleotide repeats are likely physiological targets.

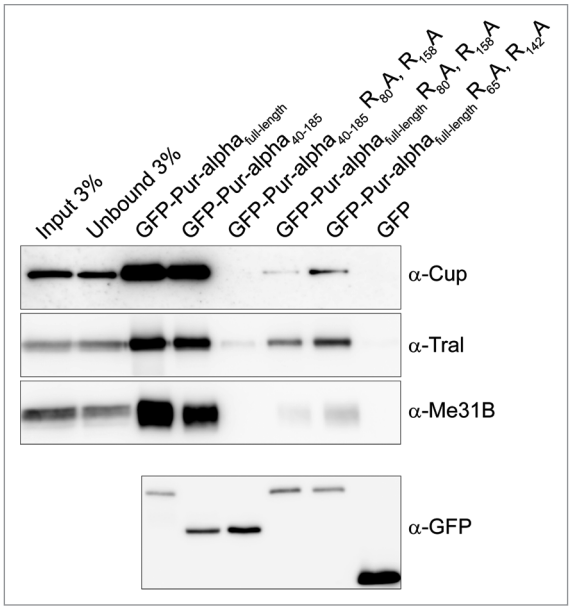

Figure 3. Western-Blot validation of Pur-α interactors. Co-immunoprecipitation of Cup, Tral and Me31B with full-length GFP-Pur-α, truncated GFP-Pur-$\alpha_{40\text{-}185}$, point mutant and truncated GFP-Pur-$\alpha_{40\text{-}185}$ (R80A, R158A), full-length GFP-Pur-α with point mutations in PUR-repeats I and II (R80A, R158A) and full-length GFP-Pur-α with a weaker point-mutation in PUR-repeats I and II (R65A, R142A). The capacity of Pur-α to bind RNA correlates with the extent of co-immunoprecipitation. Note that mutations which abolish RNA binding also lead to loss of the co-purifying proteins.

In conclusion, our binding studies demonstrate that Drosophila Pur-α can associate both in vitro and in vivo with various purine-rich RNA sequences, in particular repeats of GGA, AAG, CGG, CAG. These sequences are apparently preferentially bound when they occur in direct repeats of two or more trinucleotides, considerably fewer than the extended, disease-causing repeats found in e.g., FXTAS. Potential physiologic mRNA targets of Drosophila Pur-α are the *opa*-repeat containing mRNAs, many of which have known functions in development and/or neurogenesis.

Discussion

Overexpression of Pur-α can reduce the toxicity of trinucleotide repeat RNA in a model for the neurodegenerative disease Fragile X Tremor and Ataxia Syndrome (FXTAS), a fact that may be explained by the sequestration of endogenous Pur-α protein onto the toxic RNA and away from its physiologic targets.[9] Mammalian Pur-α is present in neuronal mRNP transport

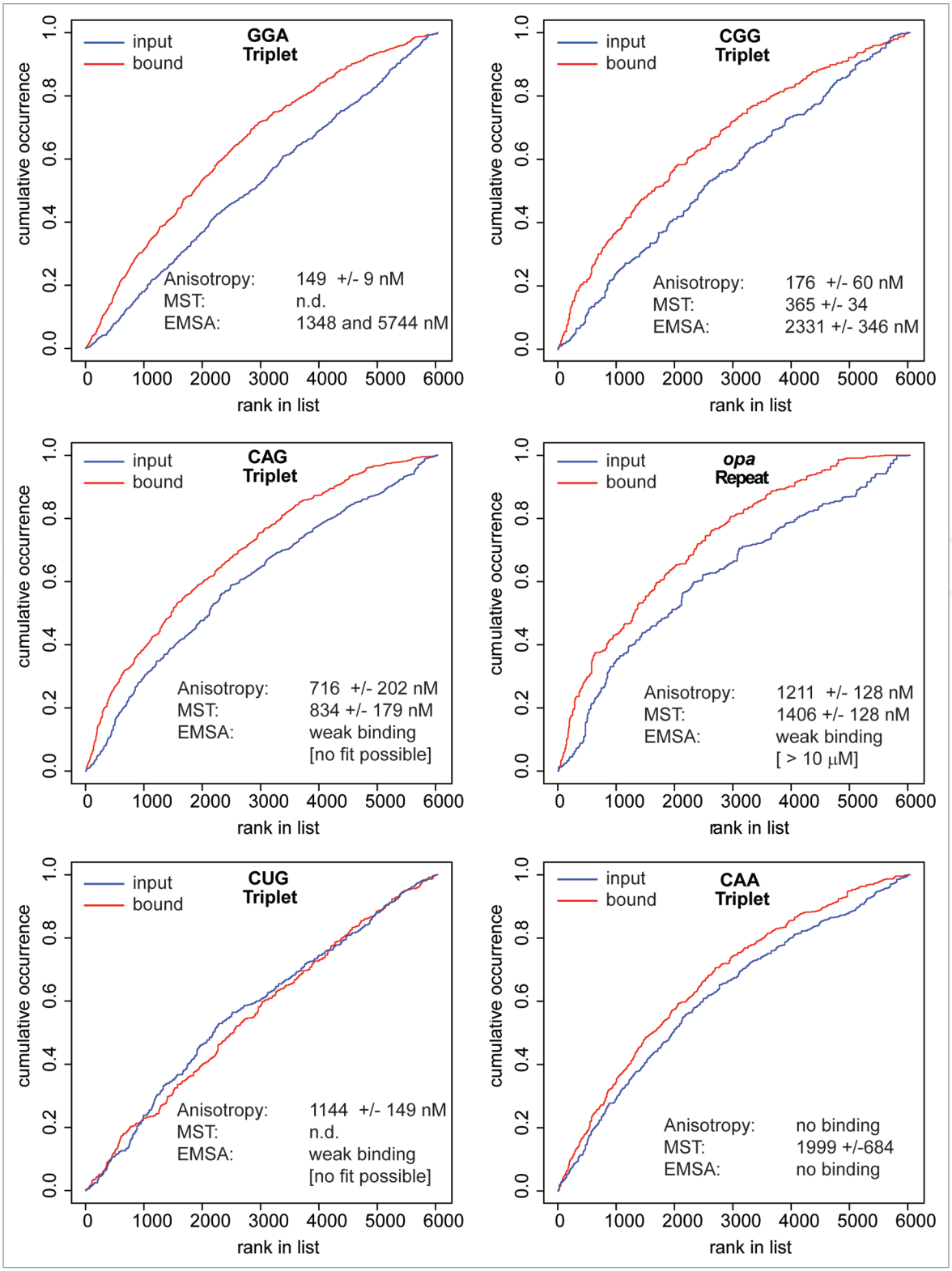

Figure 4. Characterization of RNA-binding by Drosophila Pur-α. The graphs represent the cumulative distribution of the indicated sequences as a direct triplet repeat (e.g., GGA GGA GGA) in the gene lists ranked according to fold change between ovaries of GFP only and GFP-Pur-α expressing flies. The curves were calculated for RNA extracted from the input material (blue curves) and the RNA obtained after α-GFP-Pur-α immunoprecipitation (red curves). If a sequence motif has a propensity to associate with GFP-Pur-α, the red curve should fall above the blue curve. The insets show the binding constants obtained with recombinantly expressed GST-Pur-$\alpha_{40\text{-}185}$ using fluorescence anisotropy, microscale temperature gradient thermophoresis (MST) and electrophoretic mobility shift assays (EMSA). The solution-based assays (anisotropy and MST) show substantially higher binding affinities than the electrophoretic assay.

particles, but beyond this fact little is known about the normal function of Pur-α protein.[4] We demonstrate that Drosophila Pur-α forms complexes with the oocyte mRNA transport system, and appears to shuttle between nucleus and cytoplasm. Binding studies with purified recombinant protein as well as RNA co-immunoprecipitation revealed that Pur-α has the potential to bind to a large number of mRNAs but prefers sequences with a high local density of purines such as certain physiologically occurring trinucleotide repeats.

Functional architecture of drosophila Pur α. A previous study identified three so-called PUR-repeats in the Drosophila Pur-α sequence; crystallographic analysis revealed that two repeats form a nucleic acid-binding domain with a whirly-like fold.[12] Dimerization of Pur-α, which forms a third, intermolecular RNA binding domain, is necessary to achieve optimal transport into the oocyte. Assembly of multiple RNA binding sites is a common strategy among RNA transport factors that likely increases affinity, specificity or half-life of the mRNP complex, i.e., the chance for the cargo to remain bound throughout the entire transport time.[26] The binding affinities we determined in vitro are significantly higher than previously thought but still in a moderate range (150 nM and higher). Considering the solution structure model calculated from SAXS-data, a 12-mer RNA binding substrate may be too short to make contacts with more than one pur-domain.[12] Furthermore, while the GST-Pur-$\alpha_{40\text{-}185}$ truncation we employed dimerizes via the GST-tag, it lacks the third RNA binding domain that is normally formed between both subunits. It is therefore possible that longer stretches of CAG/CGG repeats, as found e.g., in the *Notch opa*-repeat,[20] are bound with higher affinity by wild-type Pur-α. While in vitro assays demonstrate that Pur-α is capable of binding RNA autonomously, its affinity may be increased by interacting cofactors. For example, binding of murine Pur-α to single-stranded DNA is substantially enhanced in the presence of calmodulin.[27]

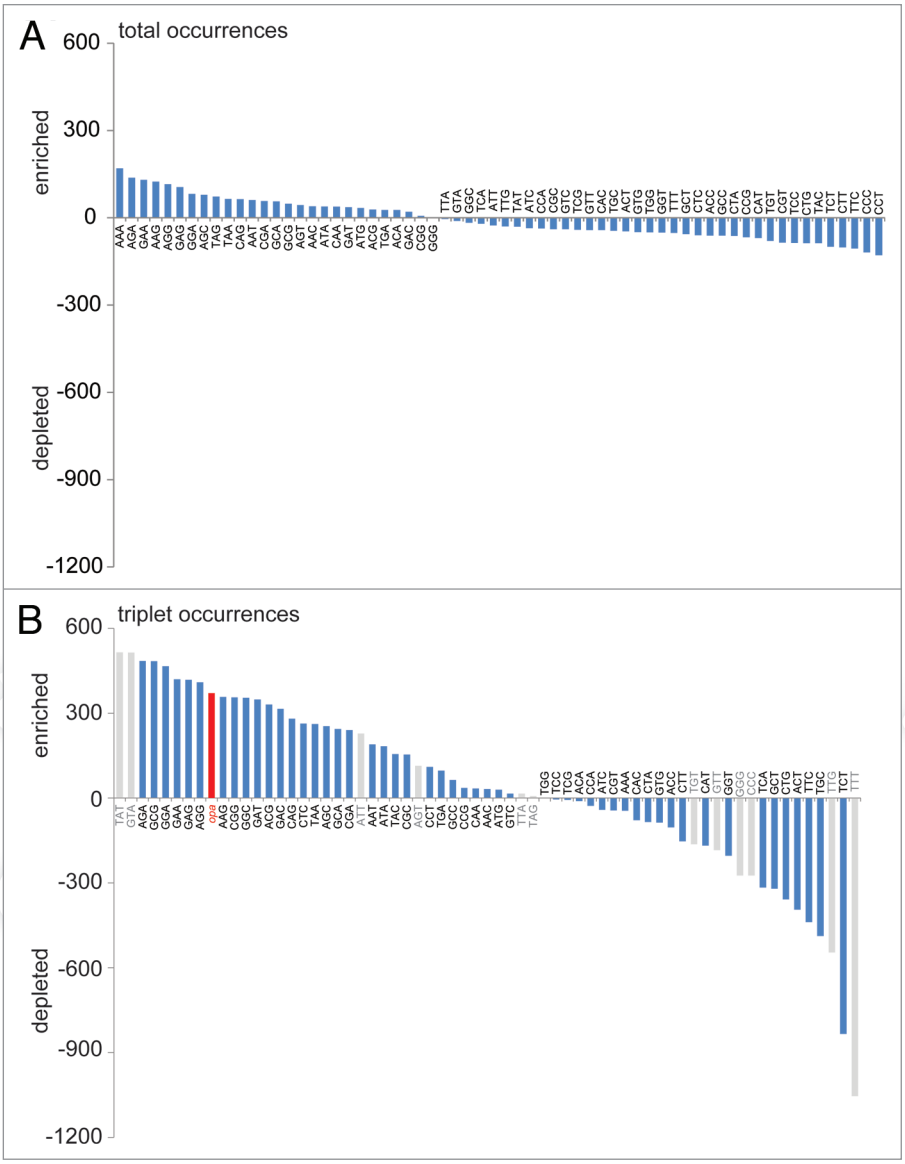

Figure 5. Enrichment of trinucleotide sequences in GFP-Pur-α immunoprecipitates. The relative enrichment of each trinucleotide sequence was calculated according to the area between the curves for the input and bound samples. For this calculation we corrected for a sequence-independent preferential recovery of longer mRNAs (see Fig. S16). Both parts are depicted using the same scale. (A) Enrichment/depletion calculated based on the cumulative distribution of total occurrences of each trinucleotide sequence within the coding sequences of all detected transcripts. This graph indicates that a pruine-rich character of the sequence is sufficient to induce moderate preferential association with Pur-α. (B) Enrichment/depletion calculated based on the cumulative distribution of triplet occurrences of each trinucleotide sequence within the coding sequence of all detected transcripts (see curves in Fig. 4 for sample curves). The gray bars represent trinucleotides for which less than 50 triplet occurrences were found; the coarse nature of the resulting cumulative curves render the calculation of the area and correction for length-preference imprecise; these values should therefore be interpreted with caution. Analogous calculations for the *opa*-repeat (CAG CAG CAG CAA) were also included (red color). When comparing the two graphs one can appreciate that a direct repeat of purine-rich sequences, leading to a high local density of purines, substantially increases the affinity for Pur-α.

Full-length Pur-α protein carrying mutations that render it incapable of nucleic acid binding becomes detectable in the nucleus, indicating that under normal circumstances Pur-α may shuttle between nucleus and cytoplasm. Analogous nucleo-cytoplasmic shuttling has been described for Cup, one of the Pur-α binding partners we identified,[28] indicating that Cup may already associate with the Pur-α mRNP in the nucleus. The identification of Pur-α as part of nuclear aggregates formed during neurodegeneration[9] is also consistent with

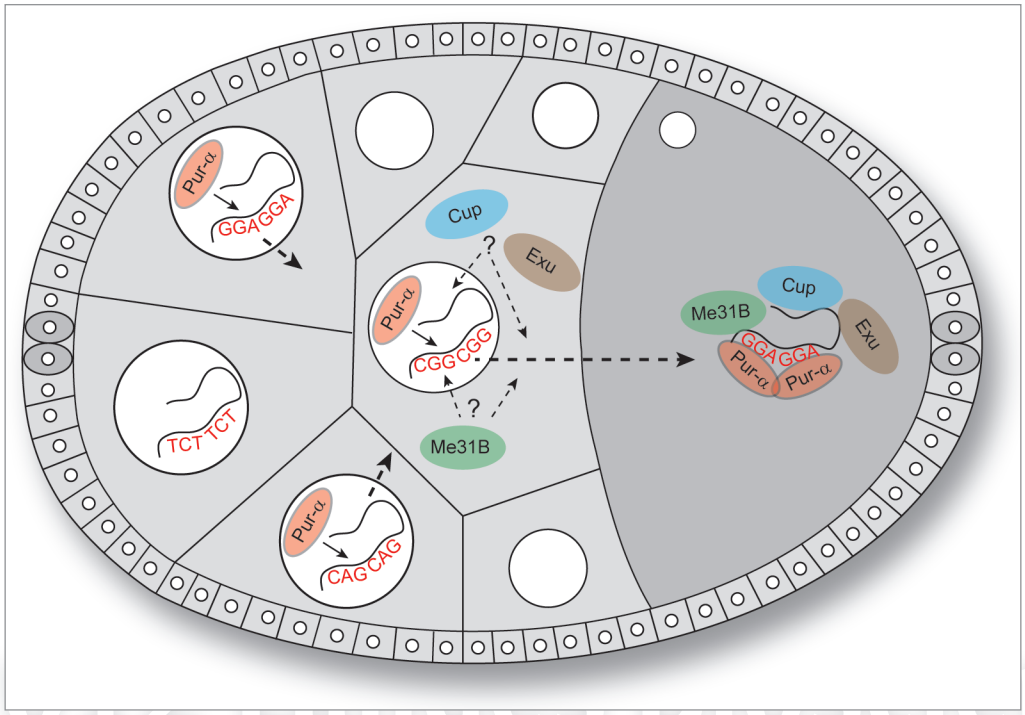

Figure 6. Localization and RNA-binding of Pur-α during Drosophila oogenesis. Pur-α protein is expressed in both somatic and germ line cells but enriched in the growing oocyte and the polar cells of the follicular epithelium (indicated by the gray shading). While oocyte enrichment is the result of active transport (the oocyte nucleus is transcriptionally inactive), high levels in the polar cells must be cell-type dependent. Since the transgenically expressed GFP-Pur-α protein also shows this enrichment, whereas the control construct expressing only GFP does not, the difference must be due either to stabilization of Pur-α protein in the polar cells or de-stabilization of Pur-α in the other follicle cells. The RNA-binding specificity of Pur-α is moderate, but purine-rich trinucleotide sequences, in particular those occurring as direct repeats, have a higher propensity to associate with Pur-α. Since point mutant proteins with an RNA binding defect accumulate in the nucleus, association with mRNA precedes nuclear export of Pur-α. Furthermore, export is less efficient without bound mRNA. It is unclear whether the other mRNP components we identified as co-purifying with Pur-α associate in the nucleus or in the cytoplasm to form the mature, transported mRNP.

the notion that Pur-α shuttles between nucleus and cytoplasm. Finally, an analysis of proteins that interact with Pur-α in vitro identified many candidates, including nuclear factors such as histones. Genetic analysis demonstrated that the RNA helicase RM62/Lip and the actin-binding protein Hts function as modulators of rCGG repeat induced toxicity.[29] We also identified three of these proteins in our analysis GFP-Pur-α interactors in ovaries (Ypsilon Schachtel and Hsc4) or somatic S2-cells (dFmr1), despite the very different technical approaches and tissues (ovaries or cells vs. brain extract). A common conclusion of both studies is that Pur-α likely associates with mRNPs before their export from the nucleus, then travels together with the mRNP to the cytoplasm. We propose that during oogenesis RNA-binding enables Pur-α to interact with the transport machinery, resulting in oocyte accumulation (summarized in **Fig. 6**). Whether association of other factors with the mRNP occurs in a hierarchical order, and whether this happens in the nucleus or only after export, is unclear. However, our in vitro studies demonstrate that Pur-α can bind to physiologic mRNA targets without the need for prior binding of other proteins. There are several striking examples of maternal mRNAs that localize to specific domains in the oocyte (e.g., posterior pole, anterior-dorsal region). We did not consistently observe enrichment of Pur-α in a particular region of the oocyte. This may indicate that Pur-α interacts with mRNAs predominantly during the transport phase and not during anchoring at their destination. Alternatively, the combination of a large number of specific localization patterns could also be perceived as a roughly uniform distribution.

What is the physiologic role of RNA-bound Pur-α? In the Drosophila ovary, Pur-α is expressed in both the follicle cells as well as in the germ line, and it is reasonable to assume that the composition of Pur-α containing complexes may differ between those two lineages (with respect to associated proteins and/or mRNAs). The ovary extracts we prepared are most likely predominated by the germ line form of Pur-α complexes, simply because the mature eggs contribute the greatest share. Our identification of Cup and Me31B as prominent interactors underscores this fact because both of them are expressed only in the germ line.[30,31] In mouse brain extracts, Pur-α associates with the Fragile X mental retardation protein (FMRP).[4,9,32,33] While dFmr1 was not identified in our proteomic analysis from ovaries, we could confirm complex formation between Drosophila Pur-α and dFmr1, the dFmr1-interacting protein Ago2 and the miRNA-effector Ago1 in the somatic S2 cell culture line (**Fig. S2B**). These associations depend on the ability of Pur-α to bind to RNA, though overexpressed Ago2 still interacts to some extent with mutant Pur-α. Again, the most likely scenario is that many of these interactions are mediated by RNA. It thus appears that it is the mRNA that

recruits Pur-α to the transport and/or RNAi complexes, rather than the inverse. In this scenario, Pur-α's role is not to determine substrate specificity with respect to the mRNA; rather, it may have a regulatory role such as modulating translation activity or serve as a RNA chaperone to assist in the formation of specific secondary structures. Indeed, inhibition of translation by Pur-α has been observed in vitro[34] and Pur-α was found associated with translational repressors in cells.[35] The absence of obvious dominant-negative effects for our GFP-Pur-α mutants with reduced RNA binding suggests that no essential factors are titrated away from the transport complexes, consistent with the notion that the interactions with Pur-α are mediated by RNA.

Our analysis of RNA-binding demonstrates a preference for repetitive, purine-rich short sequence motifs. These can be found in many mRNAs, and accordingly we detected that most mRNAs can associate to some extent with overexpressed GFP-Pur-α in the ovary. However, RNA binding may be more specific at physiologic levels, where potentially modulating co-factors are present in corresponding amounts. New approaches to study protein-RNA interactions such as PAR-clip may reveal more specific interactions and greatly improve resolution of the bound sequence motif. However, in the case of Pur-α this technique may be misleading since the most widely employed strategy depends on crosslinks to a modified uridine residue. Yet, the pyrimidines and in particular uridines are clearly underrepresented in the mRNAs associated with Pur-α (**Fig. 5**). It appears that preferential association of as little as two trinucleotide repeats with Pur-α can occur, demonstrating that the physiologic mRNA binding mode may be similar to the one occurring on toxic RNA. One can therefore speculate that mammalian Pur-α also associates with normal-length trinucleotide repeat containing mRNAs.

Pur-α localizes to the dendritic part of vertebrate and Drosophila neurons.[9,33] When the expressed rCGG-repeat RNA reaches a critical threshold, Pur-α and another rCGG-binding protein, hnRNP A2/B1, are presumably sequestered from their endogenous targets and this may contribute to the neurodegenerative disease fragile X associated tremor and ataxia syndrome (FXTAS[9]). Nuclear retention of these proteins in the aggregates nucleated by toxic mRNAs may be a cause of the neuropathology, but it is also possible that specific mRNAs may not be able to fulfill their normal function when Pur-α association is not properly maintained. An unusual mode of non-AUG mediated translation initiation has recently been described for extended polyglutamine stretches, challenging the notion of RNA toxicity[36,37] and adding control of non-AUG mediated translation initiation as a potential function for trinucleotide-repeat binding factors. A knockout of Pur-α in mice is viable until shortly after birth, where the mutant animals die from neurological abnormalities such as seizures and tremor.[5] Under certain circumstances, mutant animals can survive for several months and develop consistent tremor and histological changes in the brain.[11] The murine phenotype thus resembles certain aspects of human trinucleotide repeat expansion diseases; in particular, the brain appears to be the organ most sensitive to loss of Pur-α. Hypomorphic *pur*-α alleles as well as transgenic RNAi in Drosophila results in no obvious phenotype (data not shown), but residual activity can be assumed in both cases.

Recently, formation of double-stranded RNA via bidirectional transcription of expanded trinucleotide repeats and processing by the RNAi machinery has been proposed as a novel mechanism that contributes to RNA toxicity.[38] While Pur-α associates with RNAi factors (**Fig. S1B**), it is dispensable for RNAi and miRNA-mediated silencing (data not shown). We would therefore not expect Pur-α to be part of the dsRNA-mediated pathogenic mechanism.

Mammalian Pur-α also functions as a DNA-binding protein and can function as a transcriptional activator as well as a repressor.[3] Whether this is also true for the Drosophila protein is currently unclear. An exciting new development is the observation that transgenic expression of an $r(CGG)_{60}$ repeat in Drosophila leads to elevated steady-state transcript levels of the transposons *gypsy*, *copia* and *I-element*. Overexpression of Pur-α as well as hnRNP A2/B1 can remedy the toxicity,[9] in the case of hnRNP A2/B1 via its interaction with heterochromatin protein 1 (HP1) and the restoration of transposon silencing.[39] Whether similar effects can be achieved by overexpression of Pur-α or whether suppression of toxicity occurs through a different mechanism remains to be shown. Clearly, further work is required to investigate the functions of Pur-α beyond nucleic acid binding.

Materials and Methods

Fly stocks. Fly stocks were maintained on standard agar food at 25°C. The Pur-α sequence was PCR amplified from fly cDNA and N-terminal GFP-fusions of the described Pur-α-protein variants were cloned *Bam*HI/*Not*I into the backbone of pKF63, resulting in expression under control of the ubiquitin promotor.[40] Point mutations were generated with overlap extension PCR as described in reference 12. The plasmids were injected into w^{1118} embryos (Rainbow Transgenic Flies, Newbury Park/Ca, USA) and transgenic lines were established after backcrossing to w^{1118}.

Protein extraction, co-immunoprecipitation, western blotting and mass spectrometry. Ovaries were dissected, washed once in PBS (Invitrogen) and the cells lysed with a microcentrifuge-tube fitting pestle in lysis-buffer.[41] To reduce the lipid content, extracts were passed through a Sephadex G-10 spin-column placed in a microcentrifuge tube. For immunoprecipitation 0.5–2 μg total protein were incubated with 30 μl of GFP-trap A beads (Chromotek, Martinsried, Germany) for 60 min at 4°C, then washed with lysis buffer containing 1% Triton as described in reference 41. The bound fractions were analyzed either by western blotting or separated on a 4–20% polyacrylamide gradient gel (Pierce Thermo Fisher, Rockford, IL USA) and slices corresponding to a size range of ~30 kDa were cut out. Protein identification was performed by LC-MS/MS (ZfP München core facility). The used antibodies were: anti-Cup, anti-Me31B, anti-Tral[30,42] and anti-GFP (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA USA). Secondary antibodies were HRP-coupled donkey anti-mouse IgG H + L (Dianova, Hamburg/Germany)

and images were acquired on a Fuji LAS-3000 mini system (Fujifilm, Düsseldorf, Germany).

Generation of monoclonal anti-Pur-α (Drosophila) antibodies. Monoclonal rat anti-Drosophila-Pur-α antibodies were prepared as described in reference 43, using GST-Pur-$\alpha_{40\text{-}255}$ as antigen.

Fluorescence microscopy. Ovaries were dissected in FACS-Flow (Becton Dickinson, Franklin Lakes, NJ USA), fixed for 15 min with 4% formaldehyde and washed three times for 10 min in PBS + 0.2% Triton X-100 (PBT), then blocked for 1–4 h with PBT containing 5% normal goat serum (NGS). Primary antibodies were added in blocking solution and incubated overnight at 4°C. Ovaries were washed 3 times with PBT, secondary antibodies were diluted in PBT and incubated 2–24 h at 4°C, followed by three 10 min washing steps with PBT. DNA was stained with DAPI (final concentration 0.5 μg/ml) for 5 min at RT. Ovaries were washed with PBT three times, mounted with DABCO glycerol mounting medium[44] and examined with a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

The following antibodies were used: monoclonal mouse anti-FasIII 7G10 and anti-eya 10H6 (both 1:200, DSHB); and monoclonal rat anti Pur-α 7H8 and 1E10 (this study).

Secondary antibodies: AlexaFluor488-conjugated anti-mouse, Cy3-conjugated anti-mouse and anti-rat (all 1:200, Molecular Probes Invitrogen).

Electrophoretic mobility shift assays. Recombinant GST-Pur-$\alpha_{40\text{-}185}$ was prepared as described in reference 12, except that the GST tag was not removed to induce dimerization, then diluted serially in binding buffer (20 mM Tris pH 7.4, 100 mM KAc, 3.5 mM $MgCl_2$, 0.1% BSA, 0.01% Tween). After incubation for 30 min at room temperature with 100 nM fluorescein-conjugated RNA-oligonucleotides in the dark, the reaction mixtures were loaded onto 4% 0.5x TBE polyacrylamide gels in 1x native loading dye (2x loading dye: 10% Glycerin, 2 μg/ml BSA, 2 mM DTT, 0.2 μg/ml Salmon Sperm DNA (SSD)) and analyzed after electrophoresis (180 V, 20 min) with a Typhoon 9400 Variable Mode Imager (GE Healthcare; Freiburg, Germany) (excitation 488 nm, emission 520 nm). The bound to total ratio was determined using ImageJ (rsbweb.nih.gov/iJ/).

Fluorescence anisotropy and microscale temperature gradient thermophoresis measurements. The binding reactions were performed under identical conditions, in many cases the same reaction was analyzed in parallel by EMSA and fluorescence anisotropy and/or microscale temperature gradient thermophoresis. For fluorescence anisotropy, 40 μl of the reactions were transferred into a dark, low-binding surface 96-well plate and measured in a Tecan Infinite M1000 plate-reading fluorescence spectrometer. Thermophoresis measurements were conducted by loading standard capillaries with binding reaction mix and then analyzed as described in reference 45, on a Nanotemper Monolith 1 instrument.

Microarray and data analysis. RNA was extracted with Trizol according to the manufacturer's instructions, then analyzed on Affymetrix Drosophila 2.0 microarrays. Expression values were calculated with R!/Bioconductor and the ranked lists were constructed in Excel. The array data has been submitted to the ArrayExpress database under the accession number E-MEXP-3546. Trinucleotide as well as dinucleotide frequencies and cumulative sum curves were constructed with ad hoc PERL and R! scripts (available upon request).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at: www.landesbioscience.com/journals/rnabiology/article/19760

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