Two distinct mechanisms underlie dosage sensitivity in Pumilio1-associated diseases

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Abstract

The RNA-binding protein Pumilio1 (PUM1) is associated with distinct diseases: a mild, late-onset ataxia and a severe, wide-ranging neurodevelopmental syndrome. The ataxia patients retain 75% of normal PUM1 levels, the syndromic patients ~50%, but why this should cause such different phenotypes was unclear. Target dysregulation did not explain the difference, so we developed a PUM1 interactome for the mouse brain. This revealed RNA-binding proteins involved in aspects of RNA metabolism not previously associated with PUM1 (e.g., alternative splicing), as well as proteins in unexpected pathways (e.g., cyclosome). We focused on a subset of RNA-binding proteins and found that the effects of PUM1 loss on these proteins and their targets varied with brain region and sex. In patient cells, mice, and *in vitro*, mild PUM1 loss affected only PUM1-specific targets, whereas PUM1 haploinsufficiency destabilized these native complexes and altered expression of shared targets. We propose that dosage sensitivity can involve distinct mechanisms, and that there may be more intimate coordination among RNA-binding proteins than is readily evinced by more traditional *in vitro* approaches.

Impact statement

Most studies of RNA-binding proteins focus on identifying their targets, but identifying their protein

partners *in vivo* provides important insights into their functions and loss of function.

Introduction

To respond with agility to a specific perturbation, cells must modify their protein repertoire, much of which is controlled at the post-transcriptional level by RNA-binding proteins (RBPs). RBPs regulate RNA localization, transport, translation, splicing, and decay, and they orchestrate hundreds of pathways that are responsible for proper biological functions (Hentze *et al*, 2018; Keene, 2007b). RBPs can coordinate or compete with each other or exert mutual influence (Dassi, 2017), and they also interact with microRNAs (miRNAs), which are small non-coding RNAs that suppress the expression of their target genes by binding to a short complementary seed region in the 3' UTRs of mRNAs. The RBP and microRNA machinery are particularly important in neurons, whose plasticity demands a rapid local (axonal) response to stimuli despite distance from the nucleus (Mauger *et al*, 2016). Disruptions in RBP function underlie several complex neurological disorders: for example, large CAG expansions in FMRP cause Fragile X syndrome, but milder “premutations” in FMRP cause adult-onset Fragile X-associated tremor and ataxia syndrome (FXTAS) (Lukong *et al*, 2008). The RBPs TDP43 and FUS are both involved in amyotrophic lateral sclerosis, with TDP43 mutations also linked to frontotemporal dementia (Humphrey *et al*, 2020; Ravanidis *et al*, 2018). Despite recent interest in these and other RBPs implicated in neuronal function, we still know relatively little about RBP interactions and regulation (Darnell & Richter, 2012; Khalil *et al*, 2018; Ravanidis *et al.*, 2018).

We recently discovered that the RBP Pumilio1 (PUM1) is involved in two distinct human diseases (Gennarino *et al*, 2018; Gennarino *et al*, 2015). PUM1-associated developmental delay and seizures (PADDAS) is a wide-ranging neurodevelopmental disorder, while PUM1-related cerebellar ataxia (PRCA) is a slowly progressive, pure ataxia with onset in mid-life. Although the severity of the PRCA and PADDAS phenotypes tracks with the levels of functional PUM1 (75% of wild-type levels in PRCA, 40-60% in PADDAS), precisely what is happening at the molecular level was unclear. PUM1 comprises a highly conserved RNA-binding domain composed of eight tandem repeats known as Puf homology domains (HDs), and the mutation that produces mild disease (PRCA), T1035S, lies within this domain and impairs RNA binding—but the most severe PADDAS mutation (R1147W) is outside this domain and does not seem to impair RNA binding (Gennarino *et al.*, 2018; Gennarino *et al.*, 2015). Moreover, the PADDAS phenotype is wide-ranging and variable. The most severe mutation, R1147W, causes small stature, physical dysmorphologies, cognitive and language delays, motor impairments, and seizures starting in infancy. The enormous difference in phenotypic outcomes between mutations in the RNA-binding domain and outside of it, plus the fact that the six then-known PUM1 targets were

upregulated to similar extents in the PRCA and PADDAS patient cell lines (Gennarino *et al.*, 2018), led us to hypothesize that PRCA is caused by deregulation of PUM1 targets, but that PADDAS results from disruption of PUM1's interactions with its protein partners along with de-repression of the targets of these complexes.

To test this hypothesis would require identifying PUM1 interactors in the mouse brain. Although a great deal is known about the PUMILIO/FBF (PUF) family of RBPs, of which PUM1 and its homolog PUM2 are members, surprisingly little is known about PUM1 function in the postnatal mammalian brain. PUM1 was initially discovered in *Drosophila* for its contributions to embryonic patterning (Lin & Spradling, 1997) and stem cell maintenance (Leeb *et al.*, 2014). Studies in fly, worm, and germ cells show that Pumilio proteins regulate mRNA targets through at least three distinct mechanisms: they can collaborate with the miRNA machinery to repress targets (Friend *et al.*, 2012; Kedde *et al.*, 2010; Miles *et al.*, 2012); they can recruit the CCR4-NOT deadenylase complex to shorten poly(A) tails and promote mRNA degradation (Temme *et al.*, 2014; Van Etten *et al.*, 2012; Weidmann *et al.*, 2014); and they can antagonize poly(A)-binding proteins to repress translation (Goldstrohm *et al.*, 2018). PUM1/2 can also activate certain mRNAs (Bohn *et al.*, 2018; Uyhazi *et al.*, 2020).

Protein interactions in general, and those of PUF family members specifically, can be organism-, transcript-, and even condition-specific (Miller & Olivas, 2011). We therefore took an unbiased approach by using *in vivo* proteomics to identify PUM1's native partners. Because *Drosophila* studies show that Pumilio can change its protein partners in different neuronal types (Muraro *et al.*, 2008), we first examined the brain as a whole and then repeated our analyses in three brain regions that most highly express PUM1. We then determined the effect of PUM1 loss on a focused subset of RBP interactors that were the most highly connected within the interactome, using *PUM1* heterozygous and knockout mice and patient-derived cell lines. We also identified targets shared among these interactors and examined their responses to PUM1 insufficiency in mice and cell lines from patients bearing the T1035S and R1147W mutations. Our results support our hypothesis and underscore the need to examine all the interactions an RBP engages in, not just its targets. (Note that, because we deal with both mouse and human proteins in this paper, we will refer to the mouse proteins with lower case (e.g., Pum1) and the human proteins in all caps (PUM1).)

Results

Establishing the Pumilio1 interactome across the adult mouse brain

To identify Pum1 protein partners, we performed co-immunoprecipitation (IP) for Pum1 on whole brains from 10-week-old wild-type mice, followed by liquid chromatography with tandem mass

spectrometry (LC-MS/MS, or simply mass spec); we used IP against IgG (IP-IgG) as a negative control (see Materials and Methods). Because of the intrinsic variability of the mass spec technique, we combined and homogenized whole brains from two 10-week-old wild-type mice per sample (1 female and 1 male), aliquoting half of each sample for IP against either Pum1 or IgG, then performed six biological replicates (six samples, 12 mice total) for each mass spec experiment against IP-Pum1 and IP-IgG. Post-IP western blot detected no residual Pum1 from brain tissue, confirming that our protocol recovers virtually all of Pum1 (**Figure 1–figure supplement 1A**).

Principal component analysis (PCA) readily separated the IP-Pum1 interactors from the control IP-IgG (**Figure 1–figure supplement 1B**). To increase the likelihood that our candidates would prove to be genuine interactors, the proteins had to have at least two unique peptides present in at least five out of six IP-Pum1 samples and also show at least an average >1.5-fold difference between IP-Pum1 and IP-IgG in label-free quantification intensity (LFQ-intensity, see Materials and Methods). Our analysis yielded 412 putative Pum1 interactors (**Figure 1A** and **Supplementary Table 1**). We incorporated mammalian protein-protein interaction data from CORUM and the Human Protein Atlas by using g:GOST (g:Profiler; see Materials and Methods) (Raudvere *et al*, 2019) to reveal 22 clusters of functionally related proteins (**Figure 1A**). Among the putative interactors were those that have been previously identified, supporting the validity of our approach. For example, our mass spec identified Nanos1, the mammalian homologue of *Drosophila* Nanos, which was one of the first proteins found to interact with Pumilio during fly development (Wang *et al*, 2001); it also identified Pum2 and Fmrp, which associate with Pum1 in neural progenitor cells (Zhang *et al*, 2017). Some of the identified proteins belong to families previously found to interact with PUM1 *in vitro*, such as Cnot1, which is the central scaffold of the CCR4-NOT complex (Enwerem *et al*, 2021; Van Etten *et al.*, 2012). Translation initiation factors (cluster 3) have been found to cooperate with Puf proteins in other species (Blewett & Goldstrohm, 2012), and human PUM2-mediated repression was found to require PABPC1, while our mass spec yielded Pabpc4 (Weidmann *et al.*, 2014). Nanos1 and Pabpc4 have numerous connections among the clusters. Cluster 8, which includes proteins that have been associated with RNA fate regulation, was the most strongly interconnected with other clusters.

Most of the proteins, of course, have never been associated with PUM1. Among the candidate interactors with the greatest fold-change were many members of the anaphase-promoting complex (cluster 21). This E3 ubiquitin ligase complex targets substrates for degradation and is essential for neuronal survival (Almeida *et al*, 2005; Gieffers *et al*, 1999; Sivakumar & Gorbsky, 2015). We performed preliminary validation by reciprocal co-IP between Pum1 and Anapc1, which scaffolds the APC/C complex, and between Pum1 and Fzr1, which forms its catalytic pocket (Sivakumar & Gorbsky, 2015)

(**Figure 1–figure supplement 1C, D**). Cluster 11 was also unexpected, as it contains members of both mTORC1 (Rptor, Cad, Tsc1, Tsc2) and mTORC2 (Rictor) pathways, which are involved in a variety of cellular functions from promoting cell growth to autophagy (Liu & Sabatini, 2020). Reciprocal co-IP with Rptor and Cad confirmed interactions with Pum1 (**Figure 1–figure supplement 1C, D**).

Given the plethora of putative interactors to investigate, we repeated the mass spec experiments on the cerebellum, hippocampus, and cortex, where Pum1 is most highly expressed (Gennarino *et al.*, 2015), to see which pathways emerged as predominant. Because our mass spec protocol requires at least 5 mg of protein, we combined cerebellar tissue from eight 10-week-old wild-type mice (4 female and 4 male), performing the experiment in triplicate (for a total of 24 mice); we did the same for cortical tissue. For the hippocampus, we combined tissues from ten wild-type mice (5 of each sex) for three experiments (30 mice; see Materials and Methods). As before, we selected proteins with at least two unique peptides and an average of >1.5 fold-change between IP-Pum1 and the IP-IgG. PCA again showed a clear separation of Pum1 samples from the IgG and indicated that the Pum1 hippocampal and cortical interactomes are more similar to each other than to that of the cerebellum (**Figure 1–figure supplement 2A**). We identified 854 putative Pum1 interactors in the cerebellum, 423 in the hippocampus, and 598 in the cortex (**Supplementary Table 1**). 467 were unique to the cerebellum, 140 to the hippocampus, 229 to the cortex, and 154 unique to the rest of the brain (i.e., excluding these three regions). Only 88 candidates were shared among these three brain regions and the whole brain (**Figure 1–figure supplement 2B**, yellow dots; **Figure 1–figure supplement 2C**). To our surprise, the only brain region to show interaction between Pum1 and Pum2 was the cortex (**Figure 1–figure supplement 2C**).

The region-specific proteins expanded the list of Pum1 interactors in the pathways described initially (**Figure 1–figure supplement 2D-F**). For example, Cnot1 and Cnot2 turned up in all three brain regions, but Cnot10 appears to be cortex-specific (**Figure 1–figure supplement 2F**). There were many proteins involved in translation initiation, with Eif3b being specific to the hippocampus (**Figure 1–figure supplement 2B**). Rbfox1 was specific to the cortex, and Rbfox2 to the cerebellum (**Figure 1–figure supplement 2B**), consistent with previous work showing that Rbfox1 mediates cell-type-specific splicing in cortical interneurons (Wamsley *et al.*, 2018) and that Rbfox2 is needed for cerebellar development (Gehman *et al.*, 2012). The brain-region-specific analysis did not yield many additional interactors in the APC/C (**Figure 1–figure supplement 2D**) or mTOR pathways (**Figure 1–figure supplement 2E**). It did, however, reveal many pathways that have never been associated with Pum1 (**Supplementary Table 1**). Taken as a whole, these data suggest that the RNA-silencing, CCR4-NOT, translational initiation, polyA regulation, and splicing machineries are fine-tuned according to brain region or neuronal cell types, whereas the APC/C and mTOR machineries remain fairly consistent, at least across these three brain

regions.

This is not to say that certain interactions are necessarily limited to these brain regions. They may occur in regions not tested; they may occur across the whole brain, but at levels that did not reach the threshold we set. It could also be that some interactors are expressed more highly in specific brain regions, though this is not the determining factor for the interaction with Pum2, which is expressed to an equivalent degree in all three brain regions (**Figure 1–figure supplement 2G**).

We then performed DAVID Gene Ontology analysis for the hits from both the whole brain and each brain region (**Figure 1B** and **Supplementary Tables 2**). The dominant categories were ubiquitin ligases (anaphase-promoting complex [APC/C], E2/E3 and K11-linked ubiquitin) and RBPs involved in various aspects of RNA metabolism (RNA silencing, 3'UTR binding, mRNA stability, transport, and splicing). We decided to prioritize the RBPs in cluster 8 (**Figure 1A**)—Fmrp and Ago2 (involved in RNA silencing), Pum2, Cnot1, and Rbfox3 (an alternative splicing factor)—for three reasons. First, this cluster was the most highly interconnected with other clusters; second, RNA-related categories were prominent in the gene ontology analyses for both whole brain and all three brain regions; and third, these RBPs have been well studied and would allow us to more readily test the consequences of PUM1 loss.

Pum1 associates with Pum2, Fmrp, Ago2, and Cnot1 in the absence of mRNA

We first performed co-IP followed by western blot to confirm Pum1 association with Pum2, Fmrp, Ago2, Rbfox3 and Cnot1 (**Figure 2A, left panel**). Because Fmrp and Ago2 both bind Mov10 *in vitro* (Kenny *et al*, 2020; Kenny *et al*, 2014), we also blotted for murine Mov10, even though it did not appear among our mass spec results. Mov10 was pulled down with Pum1, likely in concert with Fmrp (**Figure 2A**). Reciprocal co-IP using antibodies against Pum2, Ago2, Fmrp, Rbfox3, Cnot1, and Mov10 followed by western blot confirmed these associations (**Figure 2B**). To verify the specificity of the antibodies, we co-IPed Pum1 and blotted for all six RBPs in *Pum1*^{-/-} mouse brain and detected none of them (**Figure 2–figure supplement 1A**). As negative controls, we tested other proteins associated with the RISC complex (Ago1 and Ago3) that did not appear in our mass spec data, and our co-IP experiments found no interactions (data not shown).

To exclude the possibility that the co-IPs simply recovered other proteins that are co-bound to target RNAs but are not part of the same complex as the protein of interest, we treated the mouse brain samples with RNase and verified that no detectable RNA remained (see Materials and Methods). Pum1 still associated with Pum2, Fmrp, Ago2, and Cnot1 in the absence of mRNA, but not Rbfox3 or Mov10 (**Figure 2A, right panel**). Interestingly, a previous *in vitro* study observed PUM1 interaction with AGO2 only in the presence of RNA in T-REx 293 cells (Sternburg *et al.*, 2018). We repeated the RNase

experiments in HEK293T cells, which confirmed our results (except for RBFOX3, which was not detectable in these cells) (**Figure 2C**).

Some interactions among these RBPs require PUM1

We next performed reciprocal co-IP in wild-type and *Pum1*^{-/-} 10-week-old mouse brains for Pum2, Ago2, Fmrp, Cnot1, Rbfox3, and Mov10. Pum2 interacted with Cnot1 only in the presence of Pum1 (**Figure 3A**). Unexpectedly, Fmrp associated with Mov10 and Rbfox3 only in the presence of Pum1 (**Figure 3B-D**). Fmrp and Cnot1 did not associate in the wild-type brain (**Figure 3B, left**) but they did so in the absence of Pum1 (**Figure 3B, right**). Ago2 associated with Pum1, Fmrp, Cnot1 and Mov10 in wild-type brain, but in the absence of Pum1 it no longer interacted with Fmrp or Mov10 (**Figure 3E**). Mov10 associated with Fmrp but not with Ago2 (**Figure 3D and E**). Rbfox3 associated with Cnot1 (**Figure 3C**) but not *vice versa* (**Figure 3F**). IPs against Pum2, Ago2, Fmrp, Cnot1, Rbfox3, and Mov10 with and without RNase treatment in wild-type and *Pum1*^{-/-} mouse brains (**Figure 3–figure supplement 1A-F**) confirmed that, in the absence of Pum1, these interactions require RNA.

These results underscore the importance of examining RBP interactions *in vivo*, in specific contexts, with and without RNase treatment. For example, previous work showed that AGO2, PABPC1, GW182 and CNOT1 interact with nuclear EGFR to regulate VEGF signaling in tumor cell lines (Dittmann *et al*, 2017), but neither GW182 nor EGFR turned up in either our whole-brain or regional interactomes. In fact, none of GW182's three mammalian paralogues (Tnrc6A, Tnrc6B, or Tnrc6C), which interact with all four human AGO proteins, was pulled down in our mass spec against Pum1 (**Supplementary Table 1**).

In murine neurons, therefore, Pum1 associates with Pum2, Fmrp, Ago2, and Cnot1, with or without RNA (**Figure 3A-F and Figure 3–figure supplement 1A-F**). Pum1 seems to be required for association between Fmrp, Mov10 and Rbfox3, and between Ago2 and Fmrp, Mov10, and Cnot1. In the absence of Pum1, the associations between Pum2, Fmrp, Ago2 and Cnot1 require RNA (**Figure 3–figure supplement 1A-F**).

Pum1 loss alters RBP levels by brain region and sex

If these are *bona fide* interactors of Pum1, then we would expect loss of Pum1 to alter their abundance. *Pum1* heterozygous and knockout mice showed changes in the quantities of Pum2, Ago2, and Mov10 proteins across the brain (**Figure 4–figure supplement 1A**), but only *Pum2* showed changes in mRNA levels (**Figure 4–figure supplement 1B**). Interestingly, Ago2 and Mov10 levels fell only in male mice (**Figure 4–figure supplement 1A**), so we decided to pursue this sex difference by brain region. We quantified Pum2, Fmrp, Ago2, Rbfox3, Cnot1 and Mov10 in the cerebellum, cortex, and hippocampus.

We first measured Pum1 mRNA and protein levels to confirm the reduction of Pum1 in our *Pum1*^{+/-} or *Pum1*^{-/-} mice in each brain region (**Figure 4A-C**, and **Figure 4—figure supplement 2A**). Pum2 protein levels rose in all three brain regions, as did its mRNA (**Figure 4A-C**, and **Figure 4—figure supplement 2A**).

Fmrp protein expression was upregulated in all three brain regions in male KO mice, but in female KO cerebella Fmrp was reduced by almost 70% (**Figure 4A**). (We should note that sex differences in Fmrp expression have been previously reported (Singh *et al*, 2007; Singh & Prasad, 2008).) Three other proteins also showed divergent responses to Pum1 loss according to sex and brain region: Ago2 (up in female but down in male cerebella, with the opposite pattern in hippocampus and cortex), Rbfox3 (in the hippocampus), and Cnot1 (in the cerebellum) (**Figure 4A-C**). None of them showed any changes in mRNA levels (**Figure 4—figure supplement 2A**), despite the fact that *Fmr1* and *Cnot1*, like *Pum2*, have a Pumilio Response Element (PRE) (Zamore *et al*, 1997)—sometimes known as a Pumilio Recognition Element (PRE) or Pumilio Binding Element (PBE)—in their 3'UTR. It may be that the loss of Pum1 causes sex- and region-specific reorganization of these complexes, or that there are additional sex-specific Pum1 interactors.

Pum1 loss dysregulates Ago2 and the microRNA machinery in mouse cerebella by sex

Since loss of Pum1 function has deleterious effects on the cerebellum in both mice (Gennarino *et al*, 2015) and humans (Gennarino *et al*, 2018), we were curious to know whether the divergence of Ago2 protein levels in males and females extended to cerebellar miRNAs. We performed miRNAseq. Hierarchical heatmap clustering of miRNA expression in *Pum1*^{-/-} and WT male and female cerebella at 10 weeks of age revealed that the expression of 166 miRNAs (**Supplementary Table 3**) diverged between the two sexes in parallel with Ago2 expression (**Figure 4D**).

To examine the functional consequences of this Ago2/miRNA dysregulation, we looked at the expression of downstream targets that are co-bound by those miRNAs in 10-week-old wild-type and *Pum1*^{-/-} male and female cerebella. We first selected all the miRNAs with at least a 25% change in expression in either direction, for a total of 19 miRNAs. Using TargetScan and CoMeTa (Agarwal *et al*, 2015; Gennarino *et al*, 2012) we identified 6832 putative targets that are co-bound by at least 2 out of 19 possible miRNAs. To reduce this list to a more manageable size for testing expression changes by qPCR, we prioritized targets that are co-bound by at least 8 miRNAs, for a total of 49 putative targets. *Pum1*^{-/-} male and female cerebella showed gene expression changes for 44 out of these 49 targets, which correlated with the sex-dependent differences in Ago2 levels (**Figure 4E**, **Figure 4—figure supplement 2B** and **Supplementary Table 4**).

The top targets of Pum1, Pum2, Fmrp, Ago2, and Rbfox3 are shared

So far our *in vivo* proteomics results seem to be borne out by changes in the levels of protein interactors upon loss of Pum1. Ideally, we would purify recombinant proteins to perform gel filtration assays to get a better handle on the nature of the interactions, but Pum1 is difficult to purify because it is prone to aggregation (Miller & Olivas, 2011). If the complexes Pum1 forms with these RBPs are physiologically relevant, however, then they should co-regulate at least some of the same mRNA targets. Indeed, one corollary of the "regulon theory," which posits that mRNA targets in the same pathway are co-regulated (Blackinton & Keene, 2014; Keene, 2007a, b; Keene & Lager, 2005), is that there should be a discernible set of RBPs that do the co-regulating.

To test this hypothesis, we downloaded and reanalyzed all the high-throughput sequencing UV-crosslinking and immunoprecipitation (HITS-CLIP) data that is available from the murine brain. Fortunately, this data exist for Fmrp (Maurin *et al*, 2018), Ago2 (Chi *et al*, 2009), Rbfox3 (Weyn-Vanhentenryck *et al*, 2014), Pum1, and Pum2 (Zhang *et al*, 2017). Then, to determine whether and how much the targets overlap, we performed gene set enrichment analysis (GSEA) (Subramanian *et al*, 2005). GSEA is a method for determining whether members of a particular class of genes from set *S*, such as the targets of a particular RBP like Pum1, are randomly distributed throughout another gene set list *L*, which is ranked (in this case) according to reads per kilobase of transcript, per million mapped reads (RPKM). Genes from *S* that are enriched (over-represented) at the top of *L* are expressed more than would be expected; the converse is true for genes over-represented at the bottom of *L*. Here, we performed pairwise GSEA analysis using Fmrp as the basis for comparison (because it has the largest dataset) and Pum1, Pum2, Ago2 and Rbfox3. As negative controls, we used HITS-CLIP data from mouse brain for four RBPs that did not show up as Pum1 interactors in our mass spec: Mbnl2 (Charizanis *et al*, 2012), Nova (Zhang *et al*, 2010), Apc (Preitner *et al*, 2014), and Ptpb2 (Licatalosi *et al*, 2012).

Strikingly, Pum1 HITS-CLIP targets were preferentially distributed in the top 5th percentile of all Fmrp targets, with an enrichment score (ES) of 0.93 (the maximum is 1) and a FDR of 0 (**Figure 5A**, *blue line represents ES*). Pum2, Ago2, and Rbfox3 showed nearly identical patterns (**Figure 5A**). There was no significant overlap between the targets of Fmrp and those of any negative control (Nova had the highest ES, but this was only 0.36 with a rank max of 45th percentile and FDR=1; **Figure 5B**).

To ascertain whether the highest-ranking Fmrp targets correspond to the genes with the highest probability of being Pum1 targets, we performed a more detailed GSEA. We first divided the Fmrp ranked target list into 10 equal bins according to percentile. We then repeated GSEA of Pum1 HITS-CLIP data for each bin and found that 648 of the 1194 identified Pum1 targets (54%) are in the top 10th

percentile of Fmrp targets, with an ES of 0.8; moreover, this was the only statistically significant bin (**Figure 5C**). This was also true for Pum2, Ago2, and Rbfox3 (**Figure 5C**). Neither Pum1, Pum2, Rbfox3, Fmrp, nor Ago2 targets were enriched among any of the target lists of the negative controls ranked by RPKM (**Figure 5—figure supplement 1A**, and data not shown).

We then performed the corresponding analysis using the Pum1 target list as the basis for comparison instead of the Fmrp list. We ran GSEA on each of the four Pum1 partners against the list of Pum1 target genes (again ranked by RPKM), and each partner's targets are within the top 20% of the Pum1 list (**Figure 5D**). Specifically, Fmrp's targets reside in the top 10th percentile (with an ES of 0.81), Pum2's targets within the 16th percentile (ES=0.9), Ago2's targets within the 18th percentile (ES=0.76), and Rbfox3's targets within the 19th percentile (ES=0.67). The four RBPs used here as negative controls have a minimum rank at the 37th percentile, and the best ES was 0.26 for Apc; none of the five reached statistical significance (**Figure 5D**). These analyses demonstrate that there is substantial overlap among the highest-ranked targets of Pum1, Pum2, Fmrp, Ago2, and Rbfox3.

We then decided to look at the targets shared by Pum1, Pum2, Ago2, and Rbfox3 and see how they distribute within Fmrp. The result was striking, with an ES of 0.93 falling within the top 5th percentile (**Figure 5E**); 141 out of 175 common targets were within the top 10th percentile (bin 1) of Fmrp targets, with 99 within the top 5th (**Figure 5F and Supplementary Table 5**). This contrasts with the negative controls, for which the best ES was 0.41 within the top 40th-60th percentile (**Figure 5G**). DAVID gene ontology analysis of those 175 common targets between Ago2, Pum1, Pum2, Fmrp, and Rbfox3 revealed pathways enriched in neurons and axonal projections (**Figure 5—figure supplement 1B and C**). Previous studies have shown that Pum1 and Pum2 cooperate with the microRNA machinery to suppress certain targets (Gennarino *et al.*, 2015; Kedde *et al.*, 2010). Among Fmrp HITS-CLIP targets, there were almost 300 microRNAs. Pum1 HITS-CLIP has 60 miRNAs, only four of which are not shared with Fmrp; Pum2 HITS-CLIP has no miRNAs that are not shared with either Pum1 or Fmrp (**Figure 5—figure supplement 1D and Supplementary Table 6**).

Because neuronal genes tend to be long (Zylka *et al.*, 2015) and RPKM can introduce bias toward longer genes (Mandelbourn *et al.*, 2019), we wanted to ensure that the targets were actually more highly expressed. Since the published HITS-CLIPs data from other studies were acquired from mice at different ages (but most at 3 weeks of age) and these studies did not typically perform RNAseq in parallel with HITS-CLIP from the same tissue and age, we decided to perform RNAseq from WT mice at 3 weeks of age in triplicate to determine a baseline level of gene expression in mouse brain expressed as DESeq2-normalized counts (Love *et al.*, 2014) (see Materials and Methods and **Supplementary Table 7**). We then normalized RPKM values and repeated the GSEA enrichment analysis. The enrichment between the

targets of Pum1 and those of Pum2, Ago2, Fmrp, and Rbfox3 was retained even after gene expression normalization (**Figure 5—figure supplement 1E-G**), with a minimum ES of 0.78, while the maximum score of the negative controls was 0.38 (**Figure 5—figure supplement 1E, F**). The shared targets of Pum1, Pum2, Rbfox3, and Ago2 were even more enriched, with an ES of 0.84 (**Figure 5—figure supplement 1H**).

PUM1 interactors are destabilized in cell lines from PADDAS, but not PRCA, patients

Having identified PUM1 interactors and shared targets, we were now in a position to test our two-pronged hypothesis about how *PUM1* mutations can lead to different diseases. To determine whether either mutation destabilizes PUM1 interactors, we compared their levels in patient-derived cell lines with those in controls. For PADDAS, we compared fibroblasts from one 9-year-old female patient (R1147W; subject 1, or S1) with fibroblasts from three different 9-year-old female healthy controls. For PRCA, we compared lymphoblastoid cells from two female PRCA patients (both with the T1035S mutation; subjects 2 and 3, or S2 and S3, 59 and 58 years old, respectively) with lymphoblastoid cells from three different 58-year-old female healthy controls (Gennarino *et al.*, 2018). We first measured how much PUM1 we could pull down from the patient-derived cell lines compared to their respective controls. Post-IP did not detect any residual PUM1 from PADDAS or PRCA cell lines, or their controls, confirming that our protocol was able to pull down virtually all of the PUM1 protein from patient-derived cell lines (**Figure 6—figure supplement 1A, B**). IP against PUM1 followed by western blot showed that PADDAS cells had 49%, and PRCA cells 76%, of the amount of PUM1 found in healthy controls (**Figure 6A and B**), consistent with our previous report (Gennarino *et al.*, 2018).

Co-IP confirmed that PUM1 associates with PUM2, FMRP, AGO2, CNOT1, and MOV10 in patient cell lines (**Figure 6A and B**). The mild T1035S variant reduced PUM1 binding to AGO2 by ~21% (**Figure 6B**). The more severe R1147W variant, however, reduced PUM1 association with AGO2, CNOT1, and MOV10 by ~84%, ~59%, and ~90%, respectively (**Figure 6A**). Interaction with FMRP did not appear to be affected. (We could not examine the effect of PUM1 mutations on RBFOX3, which is not expressed in fibroblasts or lymphoblastoid cells.)

Western blot analysis revealed that the proteins that lose their association with the R1147W variant undergo reduction in their expression levels (**Figure 6C**). Note that MOV10's association with R1147W was greatly reduced (**Figure 6A**) even though its protein levels were unchanged (**Figure 6C**). AGO2 and CNOT1 levels were unchanged in the PRCA cell line but were ~50% lower in the PADDAS cell line (**Figure 6C**). The mRNA levels of *PUM1*, *AGO2*, *CNOT1*, and *MOV10* did not change (**Figure 6—figure supplement 1C**), confirming that the reductions in their respective protein levels were due to the loss of

interaction with PUM1-R1147W.

One limitation of these studies is that we are implicitly comparing different cell types. Since we haven't been able to procure lymphoblasts from PADDAS patients or fibroblasts from PRCA patients, we sought to confirm our findings *in vitro*. We inserted a GST-tag at the N-terminal of *AGO2*, *CNOT1*, and *FMRP* (see Materials and Methods). (*AGO2* and *CNOT1* were the only proteins whose levels changed in fibroblasts, so *FMRP* is used here as a control.) We also cloned a Myc-tag polypeptide at the N-terminal of *PUM1*-WT, *PUM1*-R1147W (PADDAS) and *PUM1*-T1035S (PRCA) (see Materials and Methods). We then co-transfected the same amount of Myc-tagged PUM1 proteins with the GST-tagged interactors, followed by western blot to quantify the amount of GST-tagged protein retrieved relative to the quantity of PUM1 proteins pulled down in the IP. GST-*AGO2* associated with Myc-PUM1-R1147W 76% less than it did with Myc-PUM1-WT (**Figure 7A**), in alignment with our observations in the PADDAS cell lines (**Figure 6A, B**). We also found ~35% less interaction with *CNOT1* (**Figure 7B**) but no decrease in PUM1 association with *FMRP* (**Figure 7C**), again in accord with our findings in patient-derived cells (**Figure 6A, B**).

We also examined the effects of the PUM1 mutant on PABPC1, PABPC4, EIF4A3, and EIF4G3 (**Figure 1A**), which various studies have indicated interact with each other (Cheng & Gallie, 2007; Gallie, 1998; Le *et al*, 1997) and seems to be important for Pumilio activities but not necessarily as its interactors (Goldstrohm *et al*, 2018; Menon *et al*, 2004). We cloned a GST-tag at the respective N-terminals and performed the experiment as described above. While the R1147W mutation reduced interaction with PABPC1 by roughly 40%, it actually increased interaction with PABPC4 by 350% (**Figure 7-figure supplement 1A and B**). Strikingly, PUM1-R1147W increased interaction with EIF4A3 and EIF4G3 by 1600% to 2760% (**Figure 7-figure supplement 1C and D**).

These data suggest that the effects of the R1147W mutation, which does not impair PUM1 binding to mRNA (Gennarino *et al*, 2018), are due to the loss of interactions with RBPs that repress mRNA with a concomitant gain of function with translational initiation proteins.

R1147W mutation dramatically destabilizes PUM1

We next tested the PUM1 variants in a protein stability assay. We measured the half-life of the three Myc-tagged PUM1 proteins by separately transfecting 125ng of each. After cycloheximide treatment (T0) to block translation, we quantified the amount of protein at different time points: 30min, 1h, 2h, 4h, and 5h, compared to T0 (**Figure 7-figure supplement 1E**). The half-life of PUM1-WT was roughly 5.5 hours, the half-life of PUM1-T1035S was ~2 hours, and that of PUM1-R1147W slightly less than 1 hour (**Figure 7-figure**

supplement 1E).

We also wondered whether the R1147W mutation might be impaired in binding with WT PUM1. It has been thought that Puf proteins do not dimerize (Edwards *et al*, 2001; Zamore *et al*, 1999), but recent work supports this possibility (Qiu *et al*, 2019). We therefore co-transfected each of the Myc-tagged PUM1 variants with the same amount of GST-tagged *PUM1*-WT and used an empty vector as a negative control. First, we confirmed that IP against Myc-PUM1-WT pulled down GST-PUM1-WT. IP against Myc-PUM1-R1147W pulled down 51% of the total GST-PUM1-WT, while the interaction between Myc-PUM1-T1035S and GST-PUM1-WT remained unchanged (**Figure 7D**). The same interaction was observed after RNase treatment, suggesting that mammalian PUM1 dimerizes without RNA (**Figure 7-figure supplement 1F**). These data suggest that the R1147W mutation might exert a dominant-negative effect on wild-type PUM1.

The combination of lower protein levels and marked protein instability helps explain why the R1147W phenotype is better represented by the *Pum1* null mice than by the heterozygotes (Gennarino *et al.*, 2018).

Shared targets are upregulated in PADDAS but not PRCA

We had hypothesized that PRCA would involve dysregulation of PUM1 targets, whereas PADDAS would involve both destabilization of PUM1 interactors and dysregulation of downstream targets of those interactors. We therefore tested the effects of the T1035S and R1147W mutations on validated PUM1-specific targets (Chen *et al*, 2012; Gennarino *et al.*, 2015; Zhang *et al.*, 2017) that are not in the HITS-CLIP data for the other RBPs but are expressed in both fibroblasts and lymphoblasts. PUM1-specific mRNA were dysregulated to very similar extents in PRCA and PADDAS patient cells, with only a few targets being up to 20% more upregulated in PADDAS (**Figure 7E**).

We then examined the mRNA levels of all 175 targets shared between PUM1, PUM2, AGO2, FMRP, and RBFOX3 (**Figure 5E** and **Supplementary Table 5**) in the same PRCA and PADDAS patient cell lines. Of these shared targets, 55 were expressed in both PADDAS fibroblasts and PRCA lymphoblastoid cells. Of these 55, 52 shared targets were upregulated in PADDAS but not in PRCA (**Figure 7F**), by an average of two-fold (ranging from a low of 121% for IDS to 347% for TLK1). There was little or no change in most of these targets in PRCA cells, though levels of CALM1, ATP2A2, CREB1, and GNAQ fell by ~40%, and CALM2, TAOK1, and UBE2A by ~20% (**Figure 7F**).

These results support our hypothesis that the symptoms observed in PRCA are attributable to the dysregulation of PUM1-specific target genes, while PADDAS involves both protein partner destabilization and dysregulation of the partner proteins' targets.

Discussion

Since our initial study describing PUM1-related diseases (Gennarino *et al.*, 2018), we and others have identified additional PADDAS and PRCA patients (Bonnemason-Carrere *et al.*, 2019; Gennarino *et al.*, 2018; Imaizumi *et al.*, 2019; Lai *et al.*, 2019). In our sample, R1147W accounts for the majority of PADDAS patients, and T1035S for the majority of PRCA, which supports the value of studying these particular two mutations as causing the most severe and the most mild forms of PUM1-related disease. The question that drove the present study is: why should the 25% drop in PUM1 levels from PRCA to PADDAS produce such different phenotypes, especially when R1147W is not impaired in binding to mRNA? Our data strongly support our hypothesis that the difference is not due to a linear increase in the de-repression of mRNA targets but is rather a function of an additional mechanism coming into play: the destabilization of numerous interactions and the de-repression of their downstream targets. We draw this conclusion from several lines of evidence. First, when Pum1 was reduced by 50% (in heterozygous mice) or 100% (in knockout mice), there were changes in the levels of associated proteins that depended on brain region and sex. Second, we found changes in the levels of these proteins in patient cell lines, despite unchanged mRNA levels. Third, our *in vitro* studies using Myc- and GST-tagged proteins showed that AGO2 and CNOT1 lose their interaction with PUM1-R1147W, which itself binds less to WT PUM1. Fourth, not all interactions were diminished: the responses to R1147W ranged from a loss of 40-70% to a gain of 2760%. Together these data persuade us that a 50% loss of PUM1, whether through heterozygosity or the R1147W mutation, disrupts interactions with native partners and differentiates PADDAS from PRCA.

Diseases involving dosage-sensitive proteins

There are other dosage-sensitive proteins that produce different phenotypes depending on their expression level (Tan & Zoghbi, 2019), and our results raise the possibility that interacting complexes may be disrupted once expression falls below a certain threshold. What that threshold might be, exactly, is not clear, but for PUM1 it seems to be somewhere between the 75% of wild-type levels of PUM1 seen in PRCA and the 60% level estimated for the R1139W mutation that produced a milder form of PADDAS (Gennarino *et al.*, 2018). In this context it is worth noting that a recent study found that, below a threshold of ~70% of normal levels of FMRP, there were steep decreases in IQ for each further decrement in FMRP levels, even as small as 5-10% (Kim *et al.*, 2019). The amount of loss that can be sustained for a given protein would likely depend on its usual quantities, and it is possible that for some proteins, the difference in phenotype between greater and lesser abundance may indeed reflect a linear progression from mild to

severe. For example, a mutation in *MECP2* that reduces the protein levels by only 16% causes a mild form of Rett syndrome: the child developed normally until the age of two years, then regressed, lost acquired language and manual skills, and developed hand stereotypies (Takeguchi *et al*, 2020). She eventually regained the ability to repeat words in an echolalic fashion and never developed seizures, but the phenotype was clearly Rett syndrome. The relationship between protein levels and disease features in *MeCP2*-related disorders may be more linear because this protein is so extraordinarily abundant (Sandweiss *et al*, 2020). Similarly, in proteopathies such as Alzheimer's or Parkinson's disease, genetic duplications of *APP* or *SNCA* cause earlier onset of what is recognizably the same disease (Chartier-Harlin *et al*, 2004; Rovelet-Lecrux *et al*, 2006).

In contrast, there are diseases in which the phenotypes do not simply range from mild to severe versions of the same symptoms, but seem to take on a different character. The polyglutamine diseases, such as Huntington's disease and the spinocerebellar ataxias, come to mind, perhaps because it was through studying spinocerebellar ataxia type 1 (SCA1), that PUM1's role in neurological function was discovered (Gennarino *et al.*, 2015). In the polyglutaminopathies, the disease-causing protein bears an abnormally expanded CAG tract that tends to expand upon intergenerational transmission. Although the range of normal and pathogenic repeat tract lengths differs from one polyglutamine disease to another, larger expansions are more unstable, cause earlier onset, and affect far more tissues than smaller expansions (Orr & Zoghbi, 2007). For example, adult-onset SCA7 presents as ataxia, but infantile SCA7 affects the entire nervous system, the heart, and the kidneys, and leads to death by two years of age (Bah *et al*, 2020). Another example is Huntington's disease, where the juvenile form frequently lacks the classic chorea yet produces seizures, which are not a feature of the adult-onset disease; brain morphometry is also quite different in adult- and juvenile-onset cases (Tereshchenko *et al*, 2019). In this family of diseases, therefore, the mechanism is technically the same—repeat expansion—but different tissues have different thresholds for the CAG repeats. In the case of PUM1-related disease, it seems that an additional mechanism comes into play for the more severe phenotypes, beyond upregulation of mRNA targets. Interestingly, FMRP, which harbors a dynamic CGG repeat, is also associated with very different diseases, through two different mechanisms. Very large expansions silence the gene and produce Fragile X syndrome, whereas premutations are thought to cause the adult-onset Fragile-X-associated tremor/ataxia syndrome through RNA toxicity (Salcedo-Arellano *et al*, 2020).

Implications of the interactome

It has never been clear how the various modes of action attributed to PUM1 or other RBPs relate to one another, in large part because studies typically focus on one protein or pathway at a time. Our data

suggest that the three mechanisms of repression that have been proposed for PUM1 may be coordinated in neurons, insofar as PUM1, PUM2, FMRP, AGO2, MOV10, CNOT1 and RBFOX3 (and related proteins in specific brain regions) either interact or are so close to each other within the ribonucleosome that the loss of Pum1 or RNA can change the composition of the complexes that are identified by co-IP, in ways that are specific to brain region and sex. In this context it is worth noting that a very recent study found alternative splicing is altered in hippocampal slices from *Fmrp*-deficient mice; this observation was attributed to changes in H3K36me3 levels (Shah *et al*, 2020). Our data suggest that FMRP has an even closer relationship with the RBFOX protein family and alternative splicing machinery than suspected. Indeed, recent work has provided tantalizing glimpses of close interactions among various kinds of RNA metabolism. For example, members of the RBFOX family of proteins may, depending on their interactors (and perhaps cell type, sex, age, and species), be involved in microRNA processing in the nucleus and translation in the cytoplasm (Conboy, 2017). The FMRP/MOV10 complex appears to be involved in regulating translation through miRNA, with evidence that this role may change according to cell type (Kenny *et al*, 2020). Another study used quantitative mass spec to examine how *Fmrp* expression levels change with age in the wild-type rat dentate gyrus, and found differences in the levels of myriad proteins; among the 153 proteins with the most significant changes in levels were Pum1, Pum2 and Papbc1 (Smidak *et al*, 2017). The region-, sex-, and age-specificity of certain interactions indicates that unraveling RBP interactomes *in vivo* will require considerable finesse.

Although we did not pursue the entire interactome here, future studies investigating interactions between PUM1 and members of the mTOR pathway and anaphase-promoting complex should be very fruitful. As we were preparing this work, a new study demonstrated that mTORC1 is involved in postsynaptic responses in hippocampal glutamatergic neurons, whereas mTORC2 modulates presynaptic release (McCabe *et al*, 2020). Given the importance of proteostasis at the synapse—and its failure in many neurological diseases (Zoghbi & Bear, 2012)—the notion that different protein-regulating activities might be coordinated is intuitively appealing.

There is still much to be understood about how PUM1 works, and where and how the various RNA metabolic machineries come into contact. The fact that several associations we tested are RNA-independent suggests that certain complexes may form prior to binding mRNA. Could different pools of PUM1 form different complexes that determine whether and where a target is bound? Do the subtle differences between brain regions in the choice of interacting partners from the same pathways influence target choice, activity, or both? Given that mRNA is much larger than proteins, is the quantity of these RBPs so great that they coat the RNA? What other proteins may be involved in ‘handing off’ an mRNA from one pathway to another? Although we began this study to better understand Pum1 functions in

neurons, and thus have been rather Pum1-centric, it is by no means certain that Pum1 is the only protein that links these different molecular machineries. It may be simply the first of several missing links that we didn't realize were missing. Creating RBP interactomes should lead to a more complex yet realistic picture of RBP roles in neuronal function and in neurological disease.

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Author Contributions

S.B. designed and performed molecular experiments, analyzed and interpreted the data, and drafted the manuscript. N.D.P. performed the qPCR experiments, *in vitro* immunoprecipitation assays, analyzed the data, and edited the manuscript. A.C. performed all the brain region dissections in mice, performed the qPCR experiments and maintained the *Pum1* mutant mouse colony. M.C. performed the cloning experiments. V.L.B. interpreted data and wrote the manuscript. P.P and R.K.S. performed mass spectrometry and helped analyze the data. V.A.G. conceived the study, analyzed and interpreted the data, performed molecular experiments, and wrote the manuscript.

Declaration of Interests

The authors declare not competing interests

Materials and Methods

Ethical statement and mouse strains. All animal procedures were approved by the Institutional Animal Care and Use Committee at Columbia University, New York under the protocol AC-AAAU8490. Mice

were maintained on a 12-hr light, 12-hr dark cycle with regular chow and water ad libitum. *Pum1* knock-out mice were generated as previously described (Chen *et al.*, 2012). C57BL/6J wild-type mice were purchased from Jackson Laboratory and maintained as described above. For brain dissection, mice were anesthetized with isoflurane, and the brain rapidly removed from the skull and lysed in the appropriate buffer according to the experiment (see Materials and Methods Details).

HEK293T cell culture and maintenance. Human embryonic kidney immortalized 293T (HEK293T) cells were grown in DMEM (GenDepot, #CM002-320) supplemented with 10% of heat-inactivated fetal bovine serum (FBS [GenDepot, #F0901-050) and 1% penicillin/streptomycin (GenDepot, #CA005-010). All cells were incubated at 37 °C in a humidified chamber supplemented with 5% CO₂. HEK293T cells were later processed according to the needs of specific experiments (described below).

Patient-derived cell lines: fibroblasts and lymphoblasts. PADDAS and PRCA patient cell lines are the same as those reported previously (Gennarino *et al.*, 2018). The consent form for each subject specifically allows for sharing of medical information and physical exam findings; the sharing of cell lines from the PADDAS and PRCA subjects and the controls was approved under the Baylor College of Medicine IRB H-34578 and the Columbia University Medical Center IRB-AAAS7401 (Y01M00).

Primary fibroblasts from the *PUM1* PADDAS patient and the age- and sex-matched controls were generated as previously described (Gennarino *et al.*, 2018). Briefly, cells were isolated from skin biopsies taken from the patient or age-matched controls using standard methodology (Barch and Association of Cytogenetic Technology, 1991) and placed in a transport medium (Ham's F10, Thermo Scientific, #11550043). The skin specimen was later removed from the transport medium using a sterile technique (in a Class II biohazard cabinet) and transferred to a sterile Petri dish where it was cut into small pieces (< 0.5 mm) using sterile scalpel blades. These pieces were transferred to the lower surface of a 25 cm² culture flask (6-8 pieces per flask) which had been pre-moistened with 1-2 mL of AmnioMAX Complete Medium (Thermo Scientific, #11269016) supplemented with 1% penicillin/streptomycin (GenDepot, #CA005-010). Cell cultures were maintained at 37 °C in a humidified incubator supplemented with 5% CO₂. When cell growth was observed around the edges of the tissue, usually 3 to 5 days later, 2 to 3 mL of AmnioMAX Complete Medium were added. Once growth was established and the tissue was anchored to the flask, another 8 mL of AmnioMAX Complete Medium was added. Thereafter, the medium was renewed every 3 to 4 days until ready for sub-culturing.

Lymphoblastoid cells from *PUM1* PRCA patients and the age- and sex-matched controls were generated as previously described (Gennarino *et al.*, 2018). Briefly, lymphoblastoid suspension cell

cultures were grown in RPMI 1640 medium (Invitrogen, #11875093) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biological, Flowery Branch, #S11195H) and 1% penicillin/streptomycin (GenDepot, #CA005-010). Cell cultures were maintained at 37°C in a humidified incubator supplemented with 5% CO₂. Medium was renewed every 2 to 3 days.

Immunoprecipitation experiments using mouse brain tissue. Mouse brain tissues were gathered from an equal number of 10-week-old male and female mice as follows: whole brains from two mice; hippocampi from ten mice; cerebral cortices from eight mice; and cerebella from eight mice. Each grouping is considered one sample for one experiment. Samples were processed with a dounce homogenizer using a lysis buffer consisting of 200mM NaCl₂, 100mM NaPO₄, 20mM Hepes pH 7.4, 1% Triton X (which should disrupt all but the strongest protein-protein interactions) and complemented by 1X of Xpert Protease and 1X of Phosphatase Inhibitor Cocktail Solutions (GenDepot, #P3100-100, #P3200-020). Following homogenization, the samples were placed on ice for 15 minutes then centrifuged at 14,800 rpm at 4°C for 25 minutes to remove the debris from the supernatant. The supernatant was then moved to 1.5 ml tubes (Beckman microfuge tube #357448) and spun down in a Beckman ultra-centrifuge (Optima Max XP) at 4°C for 25 minutes at 44,000 rpm. 10% of the protein lysate was stored as input and only 1% was loaded for western blot. The protein extract was later divided into two aliquots, one for IP against the protein of interest (antibodies listed below) and the other for IP against IgG, and was then incubated with 30 µL of Dynabeads™ Protein G (Invitrogen, #10004D) and 5 µg of antibody overnight at 4°C on a rotisserie tube rotator. The next day, the beads were washed four times with the same lysis buffer used for IP and resuspended in 40µL of elution buffer (consisting of lysis buffer, NuPAGE 10X Reducing Agent [Invitrogen, #NP0009], NuPAGE LDS sample buffer at 1X final concentration [Invitrogen, #NP0007]) and boiled at 95°C for 10 minutes before the samples were loaded in the NuPAGE 4%–12% Bis-Tris Gels (Invitrogen, #NP0335BOX & #NP0336BOX) for further resolution and western blot analysis.

For the IP with RNase treatment, the beads were resuspended in 400 µL of lysis buffer after the three final washes and divided into two separate 1.5 ml tubes of 200 µL each. To establish the dose required to remove all RNA, we tested different amounts of RNase I (Invitrogen, #EN0602) and found that 4 µL was enough to render RNA undetectable both by denaturing gel and cDNA amplification. The negative control was to go without RNase treatment; both were incubated at 37°C for 15 min on a rotisserie tube rotator. After incubation, all the samples were washed one last time with 500 µL of lysis buffer and then eluted in 20 µL of elution buffer. We used the same protocol for all the IP processed by mass spectrometry.

The antibodies used for IP were: goat α -PUM1 (Bethyl Laboratories, #A300-201A), rabbit α -PUM2 (Bethyl Laboratories, #A300-202A), rabbit α -FMRP (Abcam Cambridge, #ab17722), rabbit α -AGO2 (Abcam Cambridge, #ab32381), rabbit α -NeuN (Thermo Fisher Scientific, #PA5-37407), rabbit α -CNOT1 (Cell Signaling Technology, #44613), rabbit α -MOV10 (Bethyl Laboratories, #A301-571A), rabbit α -RAPTOR (Cell Signaling Technology, #2280), rabbit α -CAD (Bethyl Laboratories, #A301-374A), rabbit α -ANAPC1 (Bethyl Laboratories, #A301-653A), and rabbit α -FZR1 (Thermo Fisher Scientific, #PA5-85303).

N.B.: The reason the inputs are separated from the IPs and then collaged is because *in vivo* we load only 1% of the lysate, which we later run off the same blot of the IPs and expose the membrane to see either the input (which usually appears first) or the IPs (which for some of them appear with a longer exposure). This is common practice when working with *in vivo* tissues (De Maio *et al*, 2018) (Lee *et al*, 2008) (Rousseaux *et al*, 2018) (Di Grazia *et al*, 2021) (Jin *et al*, 2020) (Lee *et al*, 2020).

Immunoprecipitation experiments from HEK293T and patient-derived cell lines. HEK293T cells and patient-derived fibroblasts or lymphoblastoid cells were lysed by pipetting up and down with a 1000 μ l tip in a lysis buffer consisting of 200mM NaCl₂, 100mM NaPO₄, 20mM Hepes pH 7.4, 1% Triton X and complemented by 1X of Xpert Protease and 1X of Phosphatase Inhibitor Cocktail (GenDepot, #P3100-100, #P3200-020). The rest of the protocol is the same as described above for mouse brain tissue, except that we used 2.5 μ g of primary antibody for IP.

Co-Immunoprecipitation in-gel digestion for mass spectrometry. Immunoprecipitated samples were separated on NuPAGE 4-12% gradient SDS-PAGE (Invitrogen, #NP0335BOX & #NP0336BOX) and stained with SimplyBlue (Invitrogen, #LC6060). Protein gel slices were excised and *in-gel* digestion performed as previously described (Shevchenko *et al*, 2006), with minor modifications. Gel slices were washed with 1:1 Acetonitrile and 100mM ammonium bicarbonate for 30 min then dehydrated with 100% acetonitrile for 10 min until shrunk. The excess acetonitrile was then removed and the slices dried in a speed-vacuum at room temperature for 10 minutes. Gel slices were reduced with 5 mM DTT for 30 min at 56°C in an air thermostat, cooled down to room temperature, and alkylated with 11 mM IAA for 30 min with no light. Gel slices were then washed with 100 mM of ammonium bicarbonate and 100% acetonitrile for 10 min each. Excess acetonitrile was removed and dried in a speed-vacuum for 10 min at room temperature and the gel slices were re-hydrated in a solution of 25 ng/ μ l trypsin in 50 mM ammonium bicarbonate for 30 min on ice and digested overnight at 37 °C in an air thermostat. Digested peptides were collected and further extracted from gel slices in extraction buffer (1:2 ratio by volume of

5% formic acid: acetonitrile) at high speed, shaking in an air thermostat. The supernatants from both extractions were combined and dried in a speed-vacuum. Peptides were dissolved in 3% acetonitrile/0.1% formic acid.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS). The Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer was used for peptide tandem mass spectroscopy (MS/MS). Desalted peptides were injected in an EASY-Spray™ PepMap™ RSLC C18 50cm X 75cm ID column (Thermo Scientific) connected to the Orbitrap Fusion™ Tribrid™. Peptide elution and separation were achieved at a non-linear flow rate of 250 nl/min using a gradient of 5%-30% of buffer B (0.1% (v/v) formic acid, 100% acetonitrile) for 110 minutes, maintaining the temperature of the column at 50 °C during the entire experiment. Survey scans of peptide precursors are performed from 400 to 1500 *m/z* at 120K full width at half maximum (FWHM) resolution (at 200 *m/z*) with a 2×10^5 ion count target and a maximum injection time of 50 ms. The instrument was set to run in top speed mode with 3-second cycles for the survey and the MS/MS scans. After a survey scan, MS/MS was performed on the most abundant precursors, i.e., those exhibiting a charge state from 2 to 6 of greater than 5×10^3 intensity, by isolating them in the quadrupole at 1.6 Th. We used collision-induced dissociation (CID) with 35% collision energy and detected the resulting fragments with the rapid scan rate in the ion trap. The automatic gain control (AGC) target for MS/MS was set to 1×10^4 and the maximum injection time was limited to 35ms. The dynamic exclusion was set to 45s with a 10ppm mass tolerance around the precursor and its isotopes. Monoisotopic precursor selection was enabled.

LC-MS/MS data analysis. Raw mass spectrometric data were analyzed using the MaxQuant environment v.1.6.1.0 (Cox & Mann, 2008) and Andromeda for database searches (Cox *et al*, 2011) at default settings with a few modifications. The default was used for first search tolerance and main search tolerance (20 ppm and 6 ppm, respectively). MaxQuant was set up to search with the reference mouse proteome database downloaded from Uniprot (<https://www.uniprot.org/proteomes/UP000000589>). MaxQuant performed the search for trypsin digestion with up to 2 missed cleavages. Peptide, site and protein false discovery rates (FDR) were all set to 1% with a minimum of 1 peptide needed for identification; label-free quantitation (LFQ) was performed with a minimum ratio count of 1. The following modifications were used for protein quantification: oxidation of methionine (M), acetylation of the protein N-terminus, and deamination for asparagine or glutamine (NQ). Results obtained from MaxQuant were further analyzed using the Perseus statistical package (Tyanova *et al*, 2016) that is part of the MaxQuant distribution. Protein identifications were filtered for common contaminants. Proteins were

considered for quantification only if they were found in at least two replicate groups. Significant alterations in protein abundance were determined by ANOVA with a threshold for significance of $P < 0.05$ (permutation-based FDR correction). Pum1 protein interactors were later considered if they were found in at least five out of six mass spec experiments for whole brain and in at least two out of three experiments for each respective brain region with a fold-change of >1.5 between LFQ-PUM1-WT and LFQ-IgG-WT (see **Table S1**).

Protein-protein interaction map. The protein-protein interaction map for the whole brain (**Figure 1A**) was generated by Cytoscape (<https://cytoscape.org/>) (Otasek *et al*, 2019) and the interactions among the Pum1 interactors were inferred from Corum (Giurgiu *et al*, 2019) and the Human Protein Atlas (Thul *et al*, 2017) by g:GOST which is a package of g:Profiler (<https://biit.cs.ut.ee/gprofiler/gost>) (Raudvere *et al*, 2019). The protein-protein interaction to generate the brain region-specific map (**Figure 1-figure supplement 2B**) was generated by Cytoscape.

Protein quantification and western blot analysis. Patient-derived lymphoblastoid, fibroblast cell lines, and control cell lines were collected at 6×10^6 cell confluence and processed for protein extraction. For mouse tissues, we processed either half brain (the other half was processed for RNA extraction, see below) or the entire hippocampus, cortex, or cerebellum for protein extraction. Mouse tissues or cell pellets were subsequently lysed with modified RIPA buffer consisting of 25 mM Tris-HCL, pH 7.6, 150 mM NaCl, 1.0% Tween 20, 1.0% sodium deoxycholate, 0.1% SDS, completed with 1X Xpert Protease and 1X Phosphatase Inhibitor Cocktail Solutions (GenDepot, #P3100-100 & #P3200-020). Cells were lysed by pipetting them up and down with a p1000 tip and then placed on ice for 20 min followed by centrifugation at 14,800 rpm at 4°C for 25 minutes. Mouse brain tissues were lysed starting from a 18G 1 ½” (Becton Dickson, #305196) and move down to 21G 1 ½” (Becton Dickson, #305167) and finally to 26G 1 ½” (Becton Dickson, #305111) syringe needles by pipetting them up and down until the lysate passed through the needle smoothly. Proteins were quantified by Pierce BCA Protein Assay Kit (Thermo Scientific, # PI23225) and the absorbance was measured by NanoDrop OneC (Thermo Scientific). Consequently, proteins were resolved by high resolution NuPAGE 4%–12% Bis-Tris Gel (Invitrogen, #NP0335BOX & #NP0336BOX) according to the manufacturer’s instructions. All the blots were acquired on the G:BOX Chemi XX9 machine (Syngene; Frederick, MD) using GeneSys software 1.6.5.0. Gel exposures were determine by the software.

Antibodies used for all the western blot experiments were: goat α -PUM1 [1:2500, (Bethyl Laboratories, #A300-201A)], rabbit α -PUM1 [1:2000, (Abcam Cambridge, #ab92545)], rabbit α -PUM2

[1:2000, (Bethyl Laboratories, # A300-202A)], rabbit α -FMRP [1:1000, (Abcam Cambridge, #ab17722)], rabbit α -AGO2 [1:1000, (Abcam Cambridge, #ab32381)], rabbit α -NeuN (Rbfox3) [1:1000, (Thermo Scientific, #PA5-37407)], rabbit α -CNOT1 [1:1000, (Cell Signaling Technology, #44613)], rabbit α -MOV10 [1:2000, (Bethyl Laboratories, #A301-571A)], and mouse α -GAPDH [1:10000, (Millipore, #CB1001)].

RNA extraction and quantitative real-time PCR (qPCR). Human fibroblast, lymphoblastoid, and respective control cell lines were harvested at 6×10^6 confluence prior to RNA extraction. For mouse tissues, half of the whole brain (the other half was processed for protein extraction, see above) or the entire hippocampus, cortex, or cerebellum were processed for RNA extraction. The RNA was collected for both human cells, mouse brain and brain region tissues using the miRNeasy kit (QIAGEN, # 217004) according to the manufacturer's instructions. RNA was quantified using NanoDrop OneC (Thermo Fisher Scientific). cDNA was synthesized using Quantitect Reverse Transcription kit (QIAGEN, # 205313) starting from 1 μ g of RNA. Quantitative RT-polymerase chain reaction (qRT-PCR) experiments were performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules) with PowerUP SYBR Green Master Mix (Applied Biosystems, #A25743). Real-time PCR runs were analyzed using the comparative C_T method normalized against the housekeeping human gene *GAPDH* or mouse *Gapdh*, depending on the experiment (Vandesompele *et al*, 2002).

Protein stability assay. HEK293T cells were seeded in 12-well plates for 24h and then transfected with 125 ng of either *PUM1* WT or mutant cDNA and one of the interactors using the jetPRIME Transfection Reagent (Polyplus transfection, #55-132) according to the manufacturer's protocol. After 24 h cells were treated with 0.1 mg/ml of cycloheximide (Fisher Scientific, #AAJ66004XF), which we considered time zero. Cells were later collected after 30min, 1h, 2h, 4h and 5h of treatment and processed for protein extraction and western blot analysis. Antibody: mouse α -Myc antibody [1:2000, (Cell Signaling Technologies, #2276)].

MicroRNA library construction and sequencing. Library preparation and microRNA sequencing was performed by LC Sciences according to the following criteria. Total RNA was extracted from cerebellum of WT and *Pum1*^{-/-} male and female at 10 weeks of age in triplicate, for a total of 12 samples using the miRNeasy kit (QIAGEN, # 217004) according to the manufacturer's instructions. The total RNA quality and quantity were assessed with Bioanalyzer 2100 (Agilent Technologies, Santa Clara) with RIN number > 7.0. Approximately 1 μ g of total RNA were used to prepare the small RNA library according to the

protocol of TruSeq Small RNA Sample Prep Kits (Illumina, San Diego). Then the single-end sequencing 50bp was performed on an Illumina Hiseq 2500 at LC Sciences (Hangzhou, China) following the vendor's recommended protocol.

MicroRNA sequencing bioinformatic analysis. Raw reads were subjected to an in-house program, ACGT101-miR (LC Sciences, Houston) to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, unique sequences of 18–26 nucleotides in length were mapped to specific species precursors in miRBase 22.0 (<http://www.mirbase.org/>) by BLAST search to identify known miRNAs and novel 3p- and 5p-derived miRNAs. Length variation at both 3' and 5' ends and one mismatch inside of the sequence were allowed in the alignment. The unique sequences mapping to specific species of mature miRNAs in hairpin arms were identified as known miRNAs. The unique sequences mapping to the other arm of known specific species precursor hairpins opposite the annotated mature miRNA-containing arm were considered to be novel 5p- or 3p-derived miRNA candidates. The remaining sequences were mapped to other selected species precursors (with the exclusion of specific species) in miRBase 22.0 by BLAST search, and the mapped pre-miRNAs were further BLASTed against the specific species genomes to determine their genomic locations. The last two were also defined as known miRNAs. The unmapped sequences were BLASTed against the specific genomes, and the hairpin RNA structures containing sequences were predicted from the flank 80 nt sequences using RNAfold software (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>). The criteria for secondary structure prediction were: (1) number of nucleotides in one bulge in stem (≤ 12), (2) number of base pairs in the stem region of the predicted hairpin (≥ 16), (3) cutoff of free energy (kCal/mol ≤ -15), (4) length of hairpin (up and down stems + terminal loop ≥ 50), (5) length of hairpin loop (≤ 20), (6) number of nucleotides in one bulge in mature region (≤ 8), (7) number of biased errors in one bulge in mature region (≤ 4), (8) number of biased bulges in mature region (≤ 2), (9) number of errors in mature region (≤ 7), (10) number of base pairs in the mature region of the predicted hairpin (≥ 12), (11) percent of mature region in stem (≥ 80).

RNA sequencing. The Genomic and RNA Profiling Core at Baylor College of Medicine first conducted Sample Quality checks using the NanoDrop spectrophotometer and Agilent Bioanalyzer 2100. We then used Illumina TruSeq Stranded Total RNA library preparation protocol (p/n 15031048, revE, document #RS-122-9007) along with ThermoFisher's ERCC RNA Spike-In Control Mixes Protocol (publication number 4455352, rev. D). Our process is as follows:

Illumina TruSeq stranded total RNA sample preparation protocol: Total RNA samples were

normalized to 250ng each, based on picogreen quantitation, and ERCC RNA Spike-In Controls (p/n 4456739) were incorporated into each sample. Using the TruSeq Stranded Total RNA Library Prep Gold Kit (p/n RS-122-2301), samples then underwent ribosomal RNA reduction and subsequently fragmented and primed with random hexamers to produce first strand cDNA. During second strand cDNA synthesis, RNA templates were removed and replaced with cDNA strands containing dUTP. The ds-cDNA was then purified using Beckman Coulter AMPure XP beads (p/n A63881).

Library preparation: Libraries were created from the cDNA by attaching an adenosine to the 3' end and ligating unique adapters to the ends (for more information on this process, see below). The ligated products underwent 15 cycles of amplification and a final bead purification. The resulting libraries were quantitated using the NanoDrop spectrophotometer and fragment size assessed using the Agilent 2100 Bioanalyzer. A qPCR assay was performed on the libraries to determine the concentration of adapter ligated fragments using the Applied Biosystems ViiA 7 Quantitative PCR instrument and a KAPA Library Quant Kit (p/n KK4824). All samples were pooled equimolarly and re-quantitated by qPCR, and also re-assessed on the Bioanalyzer.

Cluster generation by bridge amplification: HiSeq 2500 HOM. Using the concentration from the ViiA7 qPCR machine above, 26pM of equimolarly pooled library was loaded onto three lanes of a high output v4 flowcell (Illumina p/n PE-401-4001) and amplified by bridge amplification using the Illumina cBot machine (cBot protocol: PE_HiSeq_Cluster_Kit_v4_cBot_recipe_v9.0). PhiX Control v3 adapter-ligated library (Illumina p/n FC-110-3001) is spiked-in at 2% by weight to ensure balanced diversity and to monitor clustering and sequencing performance. A paired-end 100 cycle run was used to sequence the flowcell on a HiSeq 2500 Sequencing System (Illumina p/n FC-401-4003). An average of 129 million read pairs per sample was sequenced.

End repair converts the overhangs caused by fragmentation into blunt or polished ends. The 3' to 5' exonuclease activity of the End Repair mix removes the 3' overhangs, and polymerase activity fills in the 5' overhangs.

Adenylation: a single 'A' nucleotide is added to the 3' ends of the blunt fragments.

Adapter ligation: an adapter is ligated to the 3' end of the DNA fragment with a corresponding 'T' nucleotide.

Absolute expression: The absolute gene expression was calculated as DESeq2-normalized counts as previously described (Love *et al.*, 2014) (**Table S7**).

Samples: RNAseq was performed in triplicate from whole C57BL/6J mouse brains at 3 weeks of age.

Gene Set Enrichment Analysis (GSEA). GSEA was performed as previously described (Subramanian *et*

al., 2005). The cumulative distribution function was conducted by performing 1000 random gene-set membership assignments. A nominal p-value < 0.01 and an FDR < 0.25 were used to assess the significance of the enrichment score (ES). HITS-CLIP data were obtained from the literature and were initially acquired as follows: *Pum1* and *Pum2* from neonatal murine brains (Zhang *et al.*, 2017), *Fmrp* from cerebellum, cortex, and hippocampus together (Maurin *et al.*, 2018), *Ago2* from neocortex at embryonic day 13 (Chi *et al.*, 2009), *Rbfox3* from mouse brain (age not specified) (Weyn-Vanhentenryck *et al.*, 2014), *Nova* from mouse brain (age not specified) (Zhang *et al.*, 2010), *Ptpb2* from neocortex at embryonic day 18.5 (Licatalosi *et al.*, 2012), *Mbnl2* from hippocampus at 8-12 weeks of age (Charizanis *et al.*, 2012), and *Apc* from mouse brain at embryonic day 14 (Preitner *et al.*, 2014). For consistency all the HITS-CLIP data were ranked according to their RPKM.

For the normalized RPKM-GSEA analysis we performed RNAseq experiments (as described above), in triplicate, from WT C57BL/6 mouse brain at 3 weeks of age (we chose this age to be comparable to the other HITS-CLIP datasets) to infer the basal level of gene expression by DESeq2-normalized counts (see above). We then normalized the RPKM values by the DESeq2-normalized counts and repeated the GSEA enrichment analysis.

Gene ontology analyses. All gene ontology analyses were performed with David Gene Ontology (GO) and only categories with FDR<0.05 were considered. David GO for the *Pum1* interactome considered the entire interactome as background. For GSEA HITS-CLIP targets shared among *Pum1*, *Pum2*, *Fmrp*, *Ago2*, and *Rbfox3*, we considered the entire set of all targetomes together as background.

Myc and GST cloning procedure with in vitro immunoprecipitation (IP) assays. Human *PUM1* full-length cDNA was amplified by PCR and subcloned in a pRK5 plasmid containing the Myc tag sequence (Addgene, pRK5-Myc-Parkin #17612) at the N-terminal by using *SalI* (New England Biolabs, # R3138S) and *NotI* (New England Biolabs, #R0189S) restriction enzymes to replace *Parkin* with *PUM1*. For GST, the human full-length *PUM1* cDNA was, again, subcloned first in the pRK5 plasmid containing the GST tag sequence (Addgene, pRK5-HA GST RagC wt, #19304) at the N-terminal by using *SalI* and *NotI* restriction enzymes to replace *RagC* with *PUM1*. Human *FMRP*, *AGO2* and *CNOT1*, full-length cDNA were cloned and contain the GST tag sequence at the N-terminal as described for GST-*PUM1*.

To introduce the T1035S or R1147W mutations we used the QuikChange II XL Multi Site-Directed Mutagenesis kit (Agilent Technologies, #200521). The primers for the single mutagenesis experiments were designed by QuikChange software (Stratagene, San Diego, <https://www.genomics.agilent.com/primerDesignProgram.jsp>).

For IP, HEK293T cells were seeded in 6-well plates for 24 h and then transfected with 250 ng of either WT or mutant PUM1 plasmid with one of the interactors using the jetPRIME Transfection Reagent (Polyplus transfection, #55-132) as per the manufacturer's protocol. pRK5-Myc empty plasmid (no cDNA) was used as a negative control. After 48 h, the cells were collected and processed for immunoprecipitation. Protein lysates were incubated overnight at 4°C with mouse α -Myc antibody (1:400, [Cell Signaling Technologies, #2276]) on a rotisserie tube rotator. The next day, the beads were washed four times with an IP lysis buffer and resuspended in 40 μ L elution buffer (lysis buffer, NuPAGE 10X Reducing Agent [Invitrogen, #NP0009], NuPAGE LDS sample buffer 4X [Invitrogen, #NP0007]) and boiled at 95°C for 10 minutes before loading the samples in NuPAGE 4%–12% Bis-Tris Gels (Invitrogen, #NP0335BOX & #NP0336BOX) for further resolution and western blot analysis. Antibodies: mouse α -Myc antibody [1:2000, (Cell Signaling Technologies, #2276)], rabbit α -GST antibody [1:1000, (Cell Signaling Technologies, #2625)], mouse α -GAPDH [1:10000, (Millipore, #CB1001)].

Primers. For the qPCR analysis to unambiguously distinguish spliced cDNA from genomic DNA contamination, specific exon primers were designed to amplify across introns of the gene tested. The primers for all genes tested were designed with Primer3 (Koressaar & Remm, 2007; Untergasser *et al*, 2012). Cloning primers were manually designed to amplify the longest spliced gene isoform tested; if there was more than one isoform according to the UCSC Genome Browser (<https://genome.ucsc.edu/>), we chose the longest. See **Supplementary Table 8** for primer sequences.

Quantification and Statistical Analysis

Experimental design

For protein and RNA quantification from patient-derived cell lines, we used values from at least six independent experiments with three biological replicates for each experiment. At every stage of the study, the experimenter was blinded to the identity of control and patient-derived cell lines. For example, for the data regarding both human patient-derived cell lines and mice, Experimenter #1 made a list of samples and controls to be tested, and Experimenter #2 randomized this list and re-labeled the tubes; Experimenter #2 was the only person with the key to identify the samples. These samples were then distributed to Experimenter #3 to culture the cells, then to Experimenter #1 to perform western blots and qRT-PCR, and lastly Experimenters #1 and #4 analyzed the data. Only then was the key applied to identify the samples. For mouse experiments, the experimenters were randomized and blinded as described above. The number of animals used and sex, and the specific statistical tests used, are indicated for each experiment in the figure legends. Sample size was based on previous experience using the same mice (Gennarino *et al.*,

2015).

Software and statistical analysis. Statistical significance was analyzed using GraphPad Prism 8 (<https://www.graphpad.com/scientific-software/prism/>) and Excel Software (Microsoft). All data are presented as mean $\pm$ SEM. Statistical details for each experiment can be found in the figures and the legends. The range of expression levels in qPCR was determined from at least six independent experiments with three biological replicates by calculating the standard deviation of the Δ Ct (Pfaffl, 2001). The range of expression levels in western blots was determined from at least six independent experiments with at least six biological replicates. P values were calculated by Student's T-test or analysis of variance with Tukey's post hoc analysis. For the IP and protein quantification in patient cell lines in **Figure 7**, we had only one PADDAS patient, so the six repeated experiments were technical replicates rather than biological replicates. We therefore calculated the statistical significance based on these technical replicates in comparison to the three biological replicates (i.e., healthy controls).

Data Availability

Materials and reagents

Further information and requests for resources, reagents, and mouse models used in this manuscript should be directed to and will be fulfilled by Vincenzo A. Gennarino (vag2138@cumc.columbia.edu).

Code and raw data

No software was generated for this project. All software used in this study is publicly available and links are provided as appropriate in different sections of the Materials and Methods. Mass spectrometry, RNA sequencing and microRNA sequencing raw data generated during this study are available at PRIDE Archive at <https://www.ebi.ac.uk/pride/archive>, and Gene Expression Omnibus (GEO) at <https://www.ncbi.nlm.nih.gov/geo/> with the accession numbers pending.

Main figure titles and legends

Figure 1. A brain-specific Pum1 interactome. (A) Network of putative Pum1 interactors in 10-week-old mouse brain (circles connected to Pum1 by gray lines). Interactions between interactors (purple lines) inferred by g:GOST from Corum and the Human Protein Atlas (see Materials and Methods). The proteins in each of the 22 clusters are listed to the right. All putative Pum1 interactors are available in **Supplementary Table 1**. (B) Bubble plots show the top categories from gene ontology analyses of Pum1 interactors from whole brain, hippocampus, cortex, and cerebellum. Only the categories with fold enrichment >1.5 and FDR<0.05 are shown; not all are labeled because of space limitations. The full list of gene ontology categories are available in **Supplementary Table 2**.

Figure 2. Validation of Pum1 associations with post-transcriptional RNA-binding proteins in mouse brain. (A) Representative western blot of proteins pulled down by IP against Pum1 compared to IgG from wild-type mice brain without (*left*) and with (*right*) RNase treatment. In this panel, after IP-Pum1, we immunoblotted for Pum1 (positive control), Pum2, Fmrp, Ago2, Rbfox3, Cnot1, and Mov10. (See Materials and Methods.) (B) Representative western blot of the reciprocal IPs against Pum2, Fmrp, Ago2, Rbfox3, Cnot1 or Mov10. Each IP was immunoblotted against Pum1 and the putative interactor. (C) Representative western blots of the same proteins validated in panels A and B after IP against PUM1 with or without RNase treatment from HEK293T cell lines. In panels A through C, IP against IgG was used as a negative control and Input (1% from the initial protein lysate) as a loading control. The numbers on the right are the respective molecular weights expressed in kilodaltons (kDa). All the experiments were repeated at least six times. All mice were sacrificed at 10 weeks of age. See **Figure 2–source data 1** for the original blots.

Figure 3. Effects of Pum1 loss on interactions among the six RNA-binding proteins. Representative western blot of the proteins pulled down by (A) Pum2, (B) Fmrp, (C) Rbfox3, (D) Mov10, (E) Ago2, and (F) Cnot1 from WT and *Pum1*^{-/-} mouse brain at 10 weeks of age. IP against IgG was used as a negative control, and Input (1% from the initial protein lysate) as a loading control. Molecular weights to the right are expressed in kilodaltons (kDa). All the experiments were repeated at least four times. Since Rbfox3 and Mov10 interactions with Pum1 are RNA-dependent we did not perform IP from *Pum1*^{-/-} mouse brain. See **Figure 3–source data 1** for the original blots.

Figure 4. Pum1 interactors and the microRNA machinery show brain region- and sex-specific responses to Pum1 loss. Representative western blots of Pum1, Pum2, Fmrp, Ago2, Rbfox3, Cnot1, and

Mov10 in (A) cerebellum, (B) hippocampus, and (C) cortex in both male (left panel) and female (right panel) WT, *Pum1*^{+/-}, and *Pum1*^{-/-} mice. The same number of males and females at 10 weeks of age was used for a total of at least 18 mice per genotype. Graphs below show quantification for each protein by brain region, sex, and genotype. All data were normalized to Gapdh protein levels. All experiments were performed at least 9 times per genotype from mice at 10 weeks of age (data represent mean $\pm$ SEM). *P* values were calculated by two-tailed Student's *t* test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. See **Figure 4—figure supplement 2A** for mRNA quantification for each interactor, brain region, and sex. P1 indicates Pumilio1. See **Figure 4—Source data 1** for all western blot quantifications. (D) Heatmap showing 166 microRNAs from mouse cerebellum in *Pum1*^{-/-} male and female mice at 10 weeks of age that were dysregulated (fold change -3 to +3) relative to wild-type cerebellum. Three cerebella per genotype and sex were analyzed. The full list of miRNA names and fold changes are available in **Supplementary Table 3**. (E) Heatmap showing mRNA quantification by qPCR for 49 targets co-bound by a minimum of eight dysregulated miRNAs (>20% change) from panel D. Three cerebella per genotype and sex were analyzed. Statistical significance and magnitude of dysregulation are illustrated for both male and female in **Figure 4—figure supplement 2B**. The entire list of targets predicted to be co-bound by at least two miRNAs are reported in **Supplementary Table 4**. See **Figure 4—source data 1** and **2** for the original blots and quantifications.

Figure 5. Pum1 and its RNA-binding protein interactors share many neuronal mRNA targets. (A) Enrichment plots generated by Gene Set Enrichment Analysis (GSEA) of Pum1, Pum2, Rbfox3, and Ago2 HITS-CLIP targets plotted against Fmrp HITS-CLIP data ranked by RPKM. Pum1, Pum2, Rbfox3, and Ago2 targets are enriched at the top 10th percentile of the Fmrp targets with FDR=0. (B) GSEA analysis scores of HITS-CLIP data from each negative control (Apc, Nova, Ptpb2, and Mbnl2) plotted against Fmrp HITS-CLIP data ranked by RPKM. The negative controls have a maximum enrichment score of 0.36 for Apc ranking at the top 44.7% with FDR=1. (C) GSEA analysis scores of Pum1, Pum2, Rbfox3, and Ago2 HITS-CLIP data plotted against Fmrp HITS-CLIP data divided into 10-percentile bins and ranked by RPKM shows the shared targets are among the top percentiles of targets for each protein. (D) GSEA analysis scores of the HITS-CLIP data for Fmrp, Pum2, Ago2, Rbfox3 and four negative controls (Apc, Nova, Ptpb2, and Mbnl2) against Pum1 HITS-CLIP data ranked by RPKM. The targets of Fmrp, Ago2, Pum2, and Rbfox3 are enriched at the top 5th to 18th percentile of Pum1 targets. (E) GSEA analysis of the shared targets between Pum1, Pum2, Ago2, and Rbfox3 against Fmrp showing that they are enriched in the top 5th percentile of Fmrp targets ranked by RPKM. (F) Pum1, Pum2, Ago2, and Rbfox3 shared targets plotted against Fmrp HITS-CLIP targets ranked by RPKM and divided into 10-

percentile bins shows that all of their respective targets are enriched at the top 10th percentile of the Fmrp targets ranked by RPKM. (G) GSEA analysis scores of the targets shared by Pum1, Pum2, Ago2, and Rbfox3 and the four negative controls (Apc, Nova, Ptpb2, and Mbnl2) plotted against Fmrp. At best the negative controls are enriched at the top 40% with a maximum ES of 0.41. For all the GSEA analyses, the False Discovery Rate (FDR) was provided by GSEA: **FDR<0.05 and ***FDR<0.01. ES=Enrichment score (blue line). Note that lowest rank at max percentage indicates stronger targets in the rank (see Materials and Methods). The full list of shared targets is reported in **Supplementary Table 5**.

Figure 6. The R1147W mutation, but not T1035S, destabilizes PUM1 interactors. (A) IP against PUM1 from PADDAS (R1147W) patient (Subject 1 or S1)-derived fibroblasts confirms the interactions between PUM1 (used here as a positive control), and PUM2, FMRP, AGO2, CNOT1, and MOV10. *Bottom panel*: protein quantification shows loss of interaction between PUM1-R1147W and AGO2, CNOT1 and MOV10 compared to three age- and sex- matched control fibroblasts. Input (1%) was used as a loading control and IP against IgG was used as a negative control. (B) IP against PUM1 from two PRCA (T1035S) patient (S2 and S3)-derived lymphoblastoid cell lines confirm the interaction between PUM1 (used here as positive control), and PUM2, FMRP, AGO2, CNOT1, and MOV10. *Bottom panel*: protein quantification shows a slight decrease in interactions with FMRP and AGO2, and a slight increase with CNOT1 and MOV10, compared to age- and sex- matched lymphoblastoid controls. Input (1%) was used as a loading control and IP against IgG was used as a negative control. In **A** and **B**, the amount of protein pulled down compared to IP-PUM1 was quantified as follows: $[IP^X / (Input^X / GAPDH^X)] / [IP^{PUM1} / (Input^{PUM1} / GAPDH^{PUM1})]$, where X is the protein of interest. (C) Representative western blots (*left panels*) and relative quantification (*bar graphs to the right*) of protein levels for PUM1, PUM2, FMRP, AGO2, CNOT1, and MOV10 in PADDAS patient-derived fibroblasts and PRCA patient-derived lymphoblastoid cells compared to their respective age- and sex- matched fibroblast (for PADDAS patients) and lymphoblastoid (for PRCA patients) controls. Data were normalized to Gapdh protein levels. From **A** to **C**, all the experiments were performed at least six times. Data represent mean $\pm$ SEM. P values were calculated by two-tailed Student's t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See **Figure 6–source data 1** for the original blots.

Figure 7. RBP complexes with PUM1 R1147W are destabilized *in vitro* and shared neuronal targets

are upregulated only in PADDAS. (A-D) Representative western blots and relative IP quantification (*bar graphs*) of IP against Myc-PUM1-WT, Myc-PUM1-T1035S (PRCA), or Myc-PUM1-R1147W (PADDAS) followed by immunoblotting for: **(A)** GST-AGO2, **(B)** GST-CNOT1, **(C)** GST-FMRP, and **(D)** GST-PUM1-WT. Myc- and GST-tagged proteins were co-transfected in HEK293T cells in equal quantities (250ng each). The molecular weights were expressed in kilodaltons (kDa). The amount of protein pulled down compared to IP-PUM1 was quantified as $[IP^X / (Input^X / GAPDH^X)] / [IP^{PUM1} / (Input^{PUM1} / GAPDH^{PUM1})]$, where X is the protein of interest. **(E)** qPCR analysis of validated PUM1-specific targets from PADDAS patient-derived fibroblasts (S1, blue bars) compared to three age- and sex-matched fibroblast control cell lines, and PRCA patient-derived lymphoblastoid cell lines (S2 and S3, orange bars) compared to the three age- and sex-matched lymphoblastoid control cell lines. **(F)** mRNA level quantification of PUM1 neuronal targets in common with FMRP, PUM2, AGO2, and RBFOX3 (**Figure 5E** and **Supplementary Table 5**) in fibroblasts from subject 1 (PADDAS patient, R1147W) compared to the three age- and sex-matched control fibroblast lines (blue bars), and in lymphoblastoid cells from subjects 2 and 3 (PRCA patients, T1035S) compared to the three age- and sex-matched control lymphoblastoid cell lines (orange bars). For **E** and **F**, all data were normalized to *GAPDH* mRNA levels and experiments performed at least three times. Only genes expressed in both fibroblasts and lymphoblasts are represented here for a total of 10 for panel **E** and 55 for panel **F**. Data represent mean $\pm$ SEM. P values were calculated by two-tailed Student's t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The full list of shared targets expressed in fibroblast and lymphoblast cell lines is reported in **Supplementary Table 5**. See **Figure 7—source data 1** for the original blots.

Supplemental figure titles and legends

Figure 1—figure supplement 1. Immunoprecipitation (IP) antibody specificity against Pum1 from mouse brain and Pum1 interactor validation. **(A)** Pre-IP, IP, and post-IP against Pum1 and IgG from wild-type mouse brain show the specificity of the antibody used against Pum1. Pre-IP represents 1% from the initial protein lysate as a loading control while 10% of the protein lysate after IP-Pum1 was loaded as post-IP. The numbers on the right show molecular weight in kilodaltons (kDa). **(B)** Principal component analysis (PCA) of IP-Pum1 followed by mass spectrometry (MS) in WT adult mouse brains compared with IP against IgG; each dot represents two combined samples for a total of 6 samples processed by mass spectrometry. **(C)** Representative western blot of Rptor, Cad, Anapc1 and Fzr1 proteins pulled down by IP against Pum1 (used here as positive control) from WT mice. **(D)** Representative western blot of the reciprocal co-Ips against Anapc1, Fzr1, Rptor, and Cad. Each co-IP was immunoblotted against Pum1 and the pulled down protein used here as a reference protein to confirm the respective protein-protein

interaction. For **C** and **D**, IP against IgG was used as a negative control, and Input (1% from the initial protein lysate) as a loading control. Molecular weights are expressed in kilodaltons (kDa) to the right. All the experiments were repeated at least four times. All mice were sacrificed at 10 weeks of age with an equal number of male and female. See **Figure 1–figure supplement 1–source data 1** for the original blots.

Figure 1–figure supplement 2. Pum1 interactors differ by brain region. (A) Principal component analysis (PCA) of IP-Pum1 followed by mass spectrometry (MS) in cortex, hippocampus, and cerebellum from WT mice. IP against IgG was used as a negative control. Each dot represents a total of 3 samples processed by MS for each brain region. (B) Pum1 interactome from cerebellum, hippocampus, cortex and the rest of the brain (i.e., excluding those three regions) showing all Pum1 putative interactors with a fold change > 1.5 (see also **Supplementary Table 1**), for a total of 1,500 proteins. Node colors represent different brain regions or the overlap between two or more brain regions as noted. (C) After IP against Pum1 we immunoblotted for Pum1 (positive control), Anapc1, Cnot1 and Pum2. While Cnot1 and Anapc1 can be pulled down from all three brain regions, Pum2 can only be pulled down from cortex. For **A–C** we combined cerebellar and cortical tissues from eight wild-type mice (4 male and 4 female) for a total of 24 mice in triplicate for cerebellum and cortex, while for hippocampus we combined tissues from ten wild-type mice (5 female and 5 male) for a total of 30 mice in triplicate. All mice were 10 weeks of age. IP against IgG was used as a negative control. Molecular protein weights are expressed in kilodaltons (kDa). (D–F) The three most enriched protein complexes among the Pum1 interactors for each brain region are shown in (D) for the anaphase promoting complex/cyclosome (APC/C); (E) for the mTOR pathway; (F) for RNA-silencing (RISC), CCR4-NOT, translation initiation and polyA binding. Edge colors (D, E, and F) represent a specific brain region: red for cortex, green for cerebellum, blue for hippocampus, and gray for proteins in common between two or more brain regions. (G) Western blot analysis at 10 weeks of age to evaluate Pum2 expression levels in eight different brain regions as well as whole brain. Pum2 is highly expressed in olfactory bulbs and amygdala, and expressed at similar levels in hippocampus, cerebellum, and cortex. HP: hippocampus; CB: cerebellum; CX: cortex; MB: midbrain; OB: olfactory bulbs; AM: amygdala; SN: substantia nigra pars compacta; BS: brain stem; WB: whole brain. See **Figure 1–figure supplement 2–source data 1** for the original blots.

Figure 2–figure supplement 1. Immunoprecipitation (IP) against Pum1 from whole brain in *Pum1*^{-/-} mice. IP against Pum1 in *Pum1*^{-/-} mouse demonstrates the complete absence of Pum1 and thus the specificity of the anti-Pum1 antibody. IP against IgG was used as a negative control, and Input (1% from the initial protein lysate) as a loading control. Molecular weights at the right are in kilodaltons (kDa). All

the experiments were repeated at least four times. IP against IgG was used as a negative control, and Input (1% from the initial protein lysate) as a loading control. Equal numbers of male and female mice were sacrificed at 10 weeks of age. See **Figure 2–figure supplement 1–source data 1** for the original blots.

Figure 3–figure supplement 1. Immunoprecipitation (IP) against Pum1 interactors from whole brain in wild-type and *Pum1*^{-/-} mice with and without RNase treatment. (A-F) Representative western blot of the proteins pulled down by (A) Pum2, (B) Fmrp, (C) Ago2, (D) Cnot1, (E) Mov10, and (F) Rbfox3 from wild-type (WT, *left panel*) and *Pum1*^{-/-} (*right panel*) mouse brain without (*left panel*) and with (*right panel*) RNase treatment. IP against IgG was used as a negative control, and Input (1% from the initial protein lysate) as a loading control. Molecular weights at the right are in kilodaltons (kDa). All the experiments were repeated at least four times. IP against IgG was used as a negative control, and Input (1% from the initial protein lysate) as a loading control. Equal numbers of male and female mice were sacrificed at 10 weeks of age. See **Figure 3–figure supplement 1–source data 1** for the original blots.

Figure 4–figure supplement 1. Protein and mRNA quantification from whole brain in WT, *Pum1*^{+/-} and *Pum1*^{-/-} mice. (A) Representative western blot with relative quantifications of Pum1, Pum2, Fmrp, Ago2, Rbfox3, Cnot1, and Mov10 from the whole brain in WT, *Pum1*^{+/-} and *Pum1*^{-/-} mice. All data were normalized to Gapdh protein levels. The numbers on the right are the respective molecular weights expressed in kilodaltons (kDa). (B) mRNA level quantification by qPCR of *Pum1*, *Pum2*, *Fmrp*, *Ago2*, *Rbfox3*, *Cnot1*, and *Mov10* from whole brain in WT, *Pum1*^{+/-} and *Pum1*^{-/-} mice. Again, all data were normalized to *Gapdh* mRNA levels. All the experiments were conducted with equal number of male (at least 6 per genotype) and female (at least 6 per genotype) mice at 10 weeks of age, for a total of at least 12 mice per genotype, performed at least six times per genotype (data represent mean ± SEM). The *p* values were calculated by the Student's *t* test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. See **Figure 4–figure supplement 1–source data 1** for the original blots.

Figure 4–figure supplement 2. mRNA quantification of Pum1 interactors by brain region and sex and the 49 targets co-bound by at least eight dysregulated miRNAs in mouse cerebellum. (A) mRNA levels in cerebellum, hippocampus, and cortex in male and female for all the validated Pum1 interactors. The same number of mice were used here as in **Figure 4A-C** for a total of at least 9 mice per genotype and sex at 10 weeks of age. All data were normalized to *Gapdh* mRNA levels. All the experiments were performed at least six times (data represent mean ± SEM). (B) qPCR in cerebellum of male (*left*) and female (*right*) mice at 10 weeks of age for the 49 targets co-bound by at least eight dysregulated miRNAs (with minimum 20% change in expression) from **Figure 4E** and **Supplementary**

Table 4. All the experiments were performed in triplicate for three different biological replicates for both male and female (data represent mean $\pm$ SEM). The p values were calculated by two-tailed Student's t test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 5–figure supplement 1. Pum1, Pum2, Ago2, Fmrp, and Rbfox3 share a number of targets in the mouse brain. (A) Gene Set Enrichment Analysis (GSEA) of Pum1 HITS-CLIP data plotted against HITS-CLIP data from the negative controls (RNA binding proteins that did not show up in the Pum1 interactome: Ptpb2, Nova, Apc, and Mbnl2) reveals no significant enrichment. (B-C) Gene ontology analysis of the HITS-CLIP targets shared between Pum1, Pum2, Fmrp, Ago2, and Rbfox3 reveals enrichment for certain (B) biological functions and (C) cellular localization. Only categories with $FDR < 0.05$ and fold enrichment > 5 were plotted in B and C. (D) Venn diagram of miRNAs identified by Pum1, and Pum2 shows almost 100% overlap with the miRNAs pulled down by Fmrp HITS-CLIP. The full list of shared miRNAs is reported in **Supplementary Table 6**. (E) GSEA of HITS-CLIP data normalized against gene expression in mouse brain at 3 weeks of age (see Materials and Methods) of Pum1, Pum2, Rbfox3, and Ago2 against Fmrp HITS-CLIP data ranked by normalized RPKM. (F) GSEA of HITS-CLIP data normalized against gene expression in mouse brain at 3 weeks of age (see Materials and Methods) of Mbnl2, Apc, Nova, and Ptpb2 plotted against Fmrp HITS-CLIP data ranked by normalized RPKM. Mbnl2, Ptpb2, Apc, and Nova were used as negative controls. (G) Fmrp, Pum2, Ago2, Rbfox3 shared targets, and targets of the four negative controls, plotted against Pum1 HITS-CLIP data ranked by normalized RPKM. (H) Pum1, Pum2, Ago2, Rbfox3 shared targets plotted against Fmrp HITS-CLIP data ranked by normalized RPKM. The RNAseq gene expression changes are reported in **Supplementary Table 7**. For all GSEA analyses the False Discovery Rate (FDR) was provided by GSEA, $***FDR < 0.01$. ES=Enrichment score (blue line). Note that lowest rank at max percentage ES indicates stronger targets in the rank.

Figure 6–figure supplement 1. PUM1 validation experiments in patient-derived cells. (A-B) Pre-IP, IP, and post-IP against PUM1 and IgG from (A) PADDAS fibroblasts and (B) PRCA lymphoblastoid cells. In both cell lines we were able to pull down 100% of PUM1. Pre-IP represents 1% from the initial protein lysate as a loading control, while 10% of the protein lysate was loaded as post-IP. Molecular weights provided at right in kilodaltons (kDa). (C) mRNA level quantification for all of the immunoblotted proteins in **Figure 6C** in PADDAS and PRCA patient-derived cell lines compared to their respective age-, sex-, and cell-type-matched controls. All data were normalized to *GAPDH* mRNA levels, and experiments performed at least six times. Data represent mean $\pm$ SEM. See **Figure 6–figure supplement 1–source data 1** for the original blots.

Figure 7–figure supplement 1. PUM1 validation experiments *in vitro*. (A–D) Representative western blots (upper panel) and relative IP quantification (bottom panel) of IP against Myc-PUM1-WT, Myc-PUM1-T1035S (PRCA), or Myc-PUM1-R1147W (PADDAS) followed by immunoblotting for: (A) GST-PABPC1, (B) GST-PABPC4, (C) GST-EIF4A3, and (D) GST-EIF4G3. Myc- and GST-tagged proteins were co-transfected in HEK293T cells in equal quantities (250ng each). The molecular weights were expressed in kilodaltons (kDa). The amount of protein pulled down compared to IP-PUM1 was quantified as $[IP^X / (Input^X / GAPDH^X)] / [IP^{PUM1} / (Input^{PUM1} / GAPDH^{PUM1})]$, where X is the protein of interest. (E) PUM1 half-life in HEK293T at different time points, from zero (cycloheximide treatment) to 5h total upon transfection of Myc-PUM1-WT, Myc-PUM1-T1035S, or Myc-PUM1-R1147W. The half-life of the R1147W mutant is significantly shorter than that of WT and T1035S. All data were normalized to GAPDH and compared to time zero. The experiments were performed at least four times and the data represent mean $\pm$ SEM. The *p* values were calculated by two-tailed Student's *t* test. ***p* < 0.01, ****p* < 0.001. (F) Representative western blots of IP with RNase treatment against Myc-PUM1-WT, Myc-PUM1-T1035S (PRCA), or Myc-PUM1-R1147W (PADDAS) followed by immunoblotting to test binding between PUM1 proteins without the RNA. The numbers on the right are the respective molecular weights expressed in kilodaltons (kDa). See **Figure 7–figure supplement 1–source data 1** for the original blots.

Supplementary table titles and legends

Supplementary Table 1. Pum1 mass spectrometry results from whole brain and three separate brain regions in 10-week-old mice. All the proteins have an FDR < 0.05, and only proteins with a Ratio LFQ-Pum1-WT/LFQ-IgG-WT > 1.5 are reported here.

Supplementary Table 2. Gene Ontology analysis of Pum1 mass spectrometry from whole brain and the three brain regions. Only categories with fold enrichment of >1.5 and FDR<0.05 are shown.

Supplementary Table 3. MicroRNA names and sequences with log2 fold change as represented in Figure 4D. PC = Predicted Candidate.

Supplementary Table 4. Predicted target genes for all the differentially expressed miRNA (with at least 25% change) in cerebellum of male and female in wild-type and *Pum1*^{-/-} mice at 10 weeks of age. The target genes tested to generated Figure 4E and Figure 4–figure supplement 2B have at least 8 co-bound miRNAs.

Supplementary Table 5. HITS-CLIP targets common to Pum1, Pum2, Ago2, Rbfox3, and Fmrp.

1174 **Supplementary Table 6. microRNAs that overlap between Pum1, Pum2, and Fmrp HITS-CLIP**
 1175 **data.**

1176 **Supplementary Table 7. RNAseq in mouse brain at 3 weeks of age.**

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1178 **Supplementary Table 8. All primer sequences used in this study.**

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Figure 5. Botta et al.

Figure 6. Botta et al.

Figure 7. Botta et al.

Figure 1—figure supplement 1. Botta et al.

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