**File S1 for Rounds *et al.*, 2021, *Genetics*.**

**Supplemental Materials and Methods**

**Bulk *Drosophila* Head Isolation**

Thousands of isolated *Drosophila* heads were required to complete the immunoprecipitation experiments detailed in this study. To ensure feasibility, reproducibility, and efficiency, heads were isolated essentially as described in (Tian *et al.* 2013; Chow 2015). Briefly, whole adults were transferred from −80°C storage to a 15 ml conical tube and submerged in liquid nitrogen. Using a vortex mixer (02215365, Fisher Scientific), tubes were vortexed (speed 10, contact-activated) for six 3-second intervals, returning to liquid nitrogen between each. Resulting slurries were separated by a stack of stainless-steel sieves on dry ice. In this stack, a 710 µm U.S.A. standard No. 25 test sieve rested atop a 425 µm U.S.A. standard No. 40 test sieve (EW-59987-12 and EW-59987-16, Cole-Parmer). After brief, vigorous shaking on dry ice, only *Drosophila* heads remained on the smaller sieve, enabling exact counting and isolation on dry ice. Heads were returned to −80°C storage until further use.

**Immunoblotting**

Samples previously diluted in modified 2X Laemmli sample buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.2 M dithiothreitol (DTT), 0.02% bromophenol blue; final pH of 6.8) (Laemmli 1970) were again centrifuged at 16,100×g for 5 minutes at room temperature to clear insoluble material, magnetized as appropriate, and collected as supernatants. If the color of any Input sample solutions yellowed, indicating acidification by leftover acetone, all input samples were neutralized by an equal volume of 1 M Tris, pH 8.0. Samples were resolved on a 7.5% precast, “stain-free” polyacrylamide gel supplemented with UV-reactive trihalo compounds (456-8023, Bio-Rad). Then, these trihalo compounds were activated by 45-second UV exposure, covalently adding small, persistent fluorophores to sample proteins and allowing total protein visualization in all subsequent steps. Next, samples were transferred to a polyvinylidene difluoride (PVDF) membrane (0.2 µm; 162-0177, Bio-Rad) and blocked at room temperature in 4% non-fat milk in TBS-T (0.1% Tween). Membranes were sequentially incubated in primary antibodies, secondary antibodies, and Clarity ECL (1705061, Bio-Rad) at room temperature, with each incubation separated by washes in TBS-T (0.1% Tween). Immune-reactive species, total protein loading, and Kaleidoscope protein ladder (161-0375, Bio-Rad) were then visualized with a ChemiDoc MP digital gel imager (Bio-Rad). Primary antibodies and dilutions used are as follows: rabbit α-Atx2 at 1:1,000 (gift of Dr. Chunghun Lim, (Lee *et al.* 2017)), rabbit α-alpha tubulin at 1:1,000 (ab52866, Abcam), rabbit α-Nab2 (1:4,000) (first described in (Pak *et al.* 2011)), mouse α-Fmr1 at 1:200 (sc-57005, Santa Cruz Biotechnology), and mouse α-FLAG at 1:500 (F1804, Sigma-Aldrich). Secondary antibodies and dilutions used are as follows: goat α-rabbit HRP at 1:4,000 (Jackson) and goat α-mouse HRP at 1:1,000.

**Independent *DESeq2* normalization of IP and Input sample read counts**

Importantly, we chose to perform two independent *DESeq2* analyses, once on the 12 IP samples and once on the 12 Input samples, to normalize sample read counts for inter-library comparisons, rather than performing a single *DESeq2* analysis on all 24 samples at once. In our view, this sample separation method produces the most properly normalized read counts for inter-sample comparison because, when considered as a whole group, samples from RNA IP experiments violate some of the assumptions underlying the *DESeq* median-of-ratios read normalization strategy (Anders and Huber 2010; Anders *et al.* 2012). Specifically, the *DESeq* software within *DESeq2* normalizes for library size and composition under the assumption that most genes are expressed similarly across samples. This is not expected to be true between IP and Input samples, but it is a reasonable assumption within these groups. Thus, *DESeq* represents a valid count normalization strategy within, but not across, each group. Further discussion on this subject may be found in the *DESeq2* vignette (Love *et al.* 2020) available from Bioconductor (Gentleman *et al.* 2004; Huber *et al.* 2015). Notably, either normalization strategy produces broadly similar results (data not shown)—we argue those produced by independent normalization are only moderately more stringently, accurately normalized.

**RNA Sequencing Analysis—Gene Ontology**

The *Statistical overrepresentation test* tool in the PANTHER software web interface (Mi *et al.* 2019) was employed for Gene Ontology (GO) analysis (Ashburner *et al.* 2000; The Gene Ontology Consortium 2019). Exact parameters, GO database version information, and access dates used for this analysis are detailed in Supplemental Table 1. For PANTHER compatibility, prior to upload gene symbols were converted to FlyBase IDs (FBgnxxxxxxx) using the Flybase ID Validator at http://flybase.org/convert/id (database release FB2020\_04). Critically, GO term enrichment in given gene lists was evaluated in reference to only the 5,760 genes in the testable set (see*Materials and Methods* and *Results*), not to the total 17,753 genes annotated in the BGDP6.22 release of the *Drosophila* genome described above. This restriction controls for the effect of sample type on GO term overrepresentation testing and prevents, for example,the mis-identification of GO terms enriched in all female *Drosophila* heads as being enriched specifically in Nab2-associated RNAs. Three gene lists were analyzed for GO term overrepresentation by PANTHER: transcripts significantly associated with *Both Nab2 and Atx2* (28), *Only Nab2* (113), and *Only Atx2* (75). On a technical note, 134 genes in the testable set, along with 5, 7, and 3 genes included in the *Both Nab2 and Atx2*, *Only Nab2*, and *Only Atx2* sets, respectively, are not protein-coding genes or are otherwise unannotated in the PANTHER database and were thus automatically excluded by PANTHER from these GO analyses and related enrichment calculations.

For each transcript set, analyses were conducted separately for each of the three top-level GO domains—molecular function, biological process, and cellular component—using the PANTHER “complete” GO term sets. For each gene list, overrepresented GO terms (nominal *p*-value<0.05) were identified and, to avoid redundancy and increase explanatory power, were filtered through a process of “Hierarchical Selection” to identify the top 3 or 6 “independent” GO terms. First, overrepresented terms were sorted hierarchically by PANTHER. That is, less specific parent terms in the GO hierarchy (e.g. “regulation of nervous system process”) were grouped with their more specific child terms (e.g. “negative regulation of neuronal action potential”), and the resulting term families were rank-ordered by the fold enrichment of their most specific child term. Then, for each term family, the most specific term with at least two members in the given RBP-associated transcript list was kept; the remaining terms in each family were discarded. Within-family ties for term specificity in the GO hierarchy were resolved with fold enrichment, keeping only the most enriched term of each tie. The remaining GO terms were re-ordered by fold enrichment, and the top 3 or 6 were reported in the bar graphs presented here. GO term accession numbers and additional term information were obtained as necessary from the AmiGO 2 GO database web tool (Carbon *et al.* 2009).

**Literature Cited in Supplemental Materials and Methods**

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