An Interaction Network of RNA-Binding Proteins Involved in *Drosophila* Oogenesis

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**In Brief**
The interaction network of six RBPs involved in *Drosophila* oogenesis has been determined using both label-free and dimethyl labeling MS-based proteomics. These RBPs have overlapping functions in *Drosophila* development. The results reported 26 novel associations validated *in vitro*, including interactions with splicing factors and translational regulators, suggesting a mechanism for how RBPs may regulate maternal transcripts during oogenesis. Given the evolutionary conservation of the selected RBPs, the study provides the foundation for future functional and structural studies across systems.

**Highlights**
- Label-free and dimethyl labeling MS analysis of 6 RBPs from *Drosophila* ovaries.
- Functionally related RBPs show overlapping proteomes.
- Selective co-purification of splicing factors and translational regulators.
- Validation of 26 novel interactions by co-immunoprecipitation.

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An Interaction Network of RNA-Binding Proteins Involved in Drosophila Oogenesis

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During Drosophila oogenesis, the localization and translational regulation of maternal transcripts relies on RNA-binding proteins (RBPs). Many of these RBPs localize several mRNAs and may have additional direct interaction partners to regulate their functions. Using immunoprecipitation from whole Drosophila ovaries coupled to mass spectrometry, we examined protein-protein associations of 6 GFP-tagged RBPs expressed at physiological levels. Analysis of the interaction network and further validation in human cells allowed us to identify 26 previously unknown associations, besides recovering several well characterized interactions. We identified interactions between RBPs and several splicing factors, providing links between nuclear and cytoplasmic events of mRNA regulation. Additionally, components of the translational and RNA decay machineries were selectively co-purified with some baits, suggesting a mechanism for how RBPs may regulate maternal transcripts. Given the evolutionary conservation of the studied RBPs, the interaction network presented here provides the foundation for future functional and structural studies of mRNA localization across metazoans.

The post-transcriptional regulation of gene expression requires several trans-acting factors that regulate the life cycle of an mRNA (1). Many of these factors are RNA-binding proteins (RBPs) that interact with the maturing mRNAs to form functional messenger ribonucleoprotein complexes (mRNPs), interconnecting various steps of RNA metabolism, thereby controlling gene expression (1–3). In Drosophila oogenesis, mRNPs are first assembled in the nurse cell nucleus, providing a platform for the formation of larger dynamic assemblies in the cytoplasm, regulating mRNA transport, silencing and localized translation. Several RBPs have been identified and extensively studied in Drosophila development. Some of the well characterized and evolutionary conserved examples include the double-stranded-RNA-binding protein (dsRBP) Staufen (Stau) (4–11), the DEAD-box helicases Vasa (Vas) (12–20) and elf4AIII (21–25), the CCHC-type zinc finger protein Nanos (Nos) (26–30) and the heterogeneous nuclear ribonucleoproteins (hnRNPs) Hrp48 and Glorund (Glo) (31–33). Binding of these proteins is essential for the proper expression of four key maternal transcripts: bicoid (bcd), oskar (osk), gurken (grk), and nanos (nos) that are critical to define the future embryonic axes.

During Drosophila oogenesis, the anterior-posterior axis is established through the localization of bcd to the anterior pole and localization of osk and nos to the posterior pole of the oocyte. Accumulation of grk at the antero-dorsal corner determines the dorso-ventral axis of the embryo. The posterior targeting of osk requires several RBPs including Stau, elf4AIII, Hrp48, Glo and Vas (4, 25, 34–38). Once localized, translation of osk initiates the assembly of the pole plasm by anchoring Vas to the posterior of the oocyte, a critical step in the formation of germ cells (39–41). This also results in posterior localization and activation of nos, essential for the embryonic abdominal patterning (42). Remarkably, many components of the osk mRNP regulate multiple transcripts. For example, Stau is also essential for the anterior accumulation of bcd mRNA in the eggs (43, 44). Hrp48, Glo and Vas regulate the localization and translation of both osk and grk transcripts (35–38, 40, 45–47). Glo also represses nonlocalized nos in the oocytes (48), whereas Vas promotes nos translation in the embryos (49).

In addition to their functions in establishing oocyte polarity, these RBPs have various other roles during Drosophila oogenesis. For example, Hrp48 and Glo are required in nurse cells for the regulation of chromosome organization (38, 47). They have also been implicated as regulators of alternative splicing, like their mammalian homologs (38, 50–52). During early oogenesis, both Vas and Nos are involved in the maintenance of germline stem cells, in oocyte differentiation, and other aspects of oocyte development (26, 35, 53–56). In embryos, Nos functions in germline development (53, 57–61) and further promotes the inclusion of germline cells in the developing ovary (53, 62). In addition to oogenic processes, Nos and Stau are also involved in the development of the Drosophila nervous system (63, 64).

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Many RBPs in Drosophila oogenesis have overlapping functions that are likely differentially regulated. Little is known about this regulation and it may involve several as yet unidentified mRNP components. To comprehensively identify RBP interactors, we carried out a systematic in vivo purification screen of GFP-tagged RBPs coupled with MS. We employed both labeled and label-free MS methods and identified several proteins significantly enriched with the purified RBPs. The interactomes of the individual RBPs were largely independent with some overlap. Our screen identified several previously unknown interactions, many of which we validated in vitro. This work presents an extended interaction network of RBPs in Drosophila, offering a new reference point for future functional and structural studies of mRNA localization.

**EXPERIMENTAL PROCEDURES**

**Cloning and DNA Constructs**—For cloning purposes, total RNA was extracted from WT ovaries using the TRI-Reagent (Sigma), according to the manufacturer’s instructions. RNA was reverse transcribed using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (Thermo Fisher Scientific), in the presence of oligo(dT)15 primers. To express proteins in human HEK293 cells, genes of interest were amplified from Drosophila cDNA, or in some cases from the Drosophila Genomics Resource Center (DGRC) clones, using standard PCR conditions. Accession numbers of all the genes cloned are provided in supplemental Table S1. Fragments were cloned into mammalian expression vectors based on pEGFP-C1 (CLONTECH), bearing an N-terminal EGFP tag or modified to contain either HA or HA-Flag tags (provided by Elisa Izaurralde, MPI Tübingen). Full-length cDNAs were cloned, except for the protein Nucampolin (Ncm), where a sequence encoding amino acids 359-664 was amplified. The boundaries were designed based on the MIF4G domain of the human ortholog CWC22, which has been shown to bind elf4AIII (65). To serve as a control, either MBP or EGFP alone was used.

**Drosophila Stocks**—All flies were kept at room temperature on standard Drosophila medium. Oregon R flies were used as WT. Fosmid lines expressing GFP-tagged proteins (66) were purchased from the Vienna Drosophila Resource Center (VDRC) 318283, 318719, 318195, 318157, 318898, 318766). To generate the control fly-line expressing the tag only, the tag sequence was cloned in a modified pUAST-attB vector (67) (without UAS sites or SV40 poly(A) signal), downstream of a moderately expressing exu promoter using KpnI and BamHI sites. The purified vector was injected into embryos from a recombinant stock with a genotype y[1] M{vas-int.Dm} ZH-2A w[*]; and BamHI sites. The purification of RBPs in Drosophila of previously unknown interactions, many of which we validated.

**Immunoprecipitation from Drosophila Ovaries**—Ovaries from well-fed flies were dissected in PBS and stored at –80 °C. For immunoprecipitation, frozen ovaries were thawed on ice in lysis buffer (50 mM Tris-HCl pH 7.5 at 4 °C, 100 mM NaCl, 250 mM Sucrose, 0.1% Nonidet P-40 and 1 mM DTT) and pooled together in required numbers (see supplemental Table S2). Ovaries were homogenized with a glass pestle in a tissue homogenizer in lysis buffer (320 μl/40 flies) supplemented with protease inhibitors (CompleteTM, EDTA-free Protease inhibitor mixture, Roche). Lysates were cleared by centrifugation at 21,000 × g for 20 min at 4 °C and 5 μl of RNAse A/T1 (Thermo Fisher Scientific) was added to the supernatants. After incubation at 4 °C for 30 min, lysates were cleared again and 30-60 μl of GFP-TRAP MA beads (Chromotek, Planegg-Martinsried, Germany) were added. The mixtures were incubated for 1 h at 4 °C in rotation. Beads were washed with lysis buffer and proteins were eluted as described above.

**Western Blotting and Detection**—Eluates were separated on 10% polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were blocked in PBS containing 5% milk powder and 0.1% Tween-20. HA-tagged, HA-Flag-tagged and GFP-tagged proteins were detected using HRP-conjugated monoclonal anti-HA (1:5000, BioLegend #901501) or polyclonal anti-GFP antibodies (1:2000, Thermo Fisher Scientific #A11222) respectively. Blots were developed with ECL (GE Healthcare) reagents, as recommended by the manufacturer, and imaged using an Amersham Pharmacia Bio-Tech Imager 600 (GE Healthcare). The raw immunoblots are shown in supplemental data S6.

**Experimental Design and Statistical Rationale**—For analysis of proteins interacting with each tagged RBP, both label-free and dimethyl labeling MS experiments were performed and raw data were processed by the MaxQuant software as described below. Proteome data comprised a total of 21 raw files (3 biological replicates from each sample) for label-free MS and 2 raw files (2 biological replicates from each sample) for dimethyl labeling MS. Tag alone was used as a negative control for both analyses.

**Mass Spectrometry Measurements**—For proteome measurements, eluates were separated on a NuPAGE Bis-Tris precast 4-12% gradient gel (Invitrogen). Samples were run ~2 cm into the gel and bands were visualized with a 0.1% Colloidal Coomassie Blue stain (Sera, Heidelberg, Germany). Proteins were digested in-gel using trypsin. Peptides were desalted and purified on C18 StageTips (68). LC–MS analysis was carried out on a nanoLC (Easy-nLC 1200, Thermo Fisher Scientific) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) through a nanoelectrospray ion source (Thermo Fisher Scientific), as described previously (69). In brief, peptides were eluted using a segmented gradient of 10%–50% HPLC solvent B (80% ACN in 0.1% formic acid) at a flow rate of 200
For identification of protein interactions, the data were analyzed in a pairwise fashion i.e. each individual bait group against the control group. Proteins were filtered based on the identification of minimum three valid values in at least one replicate group. As the data followed a normal distribution, the missing values were imputed (using 0.3 standard deviations width reduction and 1.8 standard deviations downshift) enabling statistical analysis. Both-sided Welch’s t test was used with an α0 value of 2, to control the artificial within-group variance. For each test, to filter the rows, a requirement of at least 2 valid values in the bait group was set, further controlling the effects of imputation. A 5-10% FDR cutoff (permutation-based; number of randomizations: 250 without preserving groupings) was set to determine significantly enriched proteins. The same pipeline was employed for all the pairwise analyses.

For analyzing dimethyl labeling MS data, the ratio for each sample was normalized to the median of the distribution through MaxQuant (74), to correct for mixing errors. Normalized ratios were Log2-transformed and ratios from duplicates were plotted against each other. Statistically significant differences in abundance were determined by applying an arbitrary ratio threshold of 1 in Log2 scale (2-fold).

All the scatter plots and the volcano plots were generated using GraphPad Prism v. 7.0.0. For creating networks or subnetworks, Cytoscape v. 3.7.1 (75) was used. To integrate IP-MS data with literature, information from databases like String v. 11.0 (76) and FlyBase (77) were used. From String, only experimental data with medium confidence range was considered. Physical interaction data from FlyBase (77) were extracted for each protein individually. Node size calculation: for dimethyl labeling data, average of the enrichment ratios of duplicates was calculated for each protein. If a protein was found to be associated with more than one bait or identified in both label-free and labeled MS data, the highest fold change value was considered, irrespective of the experiment type. In cases where several nodes were combined into one, the highest value among the respective individual components was considered. Fig. 4 was created using Gephi v. 0.9.2 (78). Modules were detected with an algorithm described in (79), with randomization on, using edge weights and a resolution of 0.5. Force-field-based clustering was performed using the Force Atlas 2 Plugin. Baits were re-positioned manually for clarity.

For GO term analysis, the DAVID (Database for Annotation, Visualization and Integrated Discovery) v. 6.8 functional annotation tool was used (80, 81), which adopts Fisher’s Exact test to measure the gene-enrichment in annotation terms. The following parameters were used: background: Drosophila genome; count threshold (minimum number of genes for that term) of 2; maximum ease score (modified Fisher’s Exact P-value) of 0.01. To reduce redundancy in the GO terms, the DAVID output was fed into REVIGO (Reduce + Visualize Gene Ontology) (82) and p-values were used to select and cluster GO terms with a similarity score of 0.7 (medium). The Drosophila database was used to find the GO term sizes (supplemental data S1).

RESULTS

Tagged Proteins Recapitulate the Endogenous Localization Patterns—To purify RBP complexes from fly ovaries under native conditions, we used transgenic fly lines generated by recombineering. We used the “Tagged FlyFos Transgene-Ome” resource (ITRG) (66) expressing C-terminally-tagged proteins under the regulation of their endogenous promoters. These lines carry a 40 kDa tagging cassette consisting of “2XTY1-sGFP-V5-preTEV-BLRP-3XFLAG” that can be used for both in vivo visualization and affinity purification. From the ITRG library, we selected six RBPs for IP-MS, eIF4AII, Glo, Hrp48, Nos, Stau and Vas. To serve as a control, we
generated a transgenic line expressing the tag alone (hereafter referred to as GFP), under the promoter of a moderately expressing gene (exu). To ensure that the RBP fusions are functional in vivo, we checked their localization patterns at different stages of oogenesis. All the proteins were found to be localized as expected (supplemental Fig. S1A–S1f). In agreement with previous work, we observed eIF4AIII-GFP in the nucleus of the nurse cells and localized to the posterior of the oocyte at stage 5 of oogenesis (supplemental Fig. S1C; 25). eIF4AIII was also reported to enrich weakly at the posterior of the oocyte at stage 9 (25), which we could not detect in our samples. We could detect Glo-GFP in the nucleus of the nurse cells, the oocyte and follicle cells, consistent with the antibody-based localization of endogenous Glo (supplemental Fig. S1D; 48). Hrp48-GFP was present in the cytoplasm of the nurse cells and localized to the posterior of the oocytes at stage 9, similar to Vas-GFP (supplemental Fig. S1E, S1G; 46, 83). We also observed the nuage localization of GFP-tagged Vas at early stages, consistent with previous studies (supplemental Fig. S1G; 83). At stage 8, Stau-GFP was concentrated at the anterior of the oocyte, whereas stage-9 egg chambers showed a strong posterior enrichment (supplemental Fig. S1F; 4). In the nurse cells of stage-10 egg chambers, Nos-GFP showed a strong uniform GFP expression, as also observed by Wang and colleagues (62). In addition to the localization patterns of the tagged RBPs, we also checked their ability to rescue the effects of mutations that cause either lethality or sterility, as summarized in supplemental Fig. S1J. Although only 3 out of 6 transgenes assayed were able to fully substitute for the endogenous copy, their localization in the endogenous patterns suggests that their interactions driving localization during oogenesis have been maintained.

**Label-Free MS Combined with Statistical Analysis Recovers Known Associations**—For IP, we lysed whole ovaries in mild conditions of salt and detergent, and purified the complexes using the GFP-TRAP system (Chromotek) (Fig. 1A). An IP from flies expressing GFP alone was used as a negative control to identify proteins binding nonspecifically to the tag. Because we were interested in identifying RNA-independent protein-protein interactions, the experiments were carried out in the presence of RNases. As the transgenes are regulated by their endogenous promoters, they had varying levels of expression, as observed by Western blotting (Fig. 1B). To compensate for this variability in the IP-MS analysis, we adjusted the number of flies dissected for each transgene (supplemental Table S2).

All the samples were prepared in biological triplicates and the resulting spectra were searched against the *Drosophila melanogaster* proteome database (Fig. 1A, supplemental Fig. S2A). For confident identification of proteins and accurate intensity-based Label-Free Quantification (LFQ), we processed the raw data using the MaxLFQ module of the MaxQuant software (74, 84). Additionally, we activated the “matching between runs” algorithm to quantify unidentified or unsequenced peptides in the samples, by transferring peptide identifications among replicates. The global analysis of the proteomes resulted in the identification of 15,005 peptides mapping to 1878 protein groups, at a FDR of 1% at the peptide and protein level. Of these, 1841 unique protein groups were quantified in at least one of the 21 samples, which account for 87.5% of the total ovary proteome of *Drosophila* (85). The average correlation within replicates ranged from 0.71 (Glo) to 0.92 (Hrp48), suggesting overall good reproducibility of the data (Fig. 1C). In addition, the visualization of LFQ intensities of all the samples as a heat map demonstrates that all the baits were consistently enriched (supplemental Fig. S2C). The replicate profiles looked largely similar, with only minor differences. However, the number of proteins quantified with each bait varied highly, as marked by the absence of information in the heat map (supplemental Fig. S2C).

For statistical analysis, we considered only those proteins that were quantified in all three replicates of a given sample. To identify significantly enriched proteins, we employed the Welch’s t test (with 5–10% FDR cutoff), post-imation, on each bait-control matrix. The results are presented as volcano plots in Fig. 2A. All the baits were highly enriched and we observed a minimal background, indicating the high specificity of the purifications. For the baits eIF4AIII-, Stau- and Glo-GFP, where fewer proteins were reproducibly quantified, we considered statistical significance up to 10% FDR. The low number of detections for these proteins may be because of the loss of interactions on RNase treatment.

For all the baits, we found several known interactants to be reproducibly enriched over control, mostly with statistical significance (Fig. 2A, supplemental Data S5). For example, we co-purified all the other core components of the Exon Junction Complex (EJC) with eIF4AIII-GFP (25) and the NOT proteins with Nos-GFP (86, 87). We also detected known partners of Vas, involved in both pole plasm assembly in the oocyte (Oskar (Osk); Gustavus (Gus); Fat facets (Faf); F-box synaptic protein (Fsn); Fmr1) and production of germline piRNAs in the nuage (Tejas (Tej); Spindle-E (SpnE); Kumo (Qin); Tapas), with high confidence (88). Previously identified Hrp48-interacting partners such as Ovarian Tumor (Otu), Cup, PABP, Squid (Sqd) and Syncrip (Syp), involved in maternal mRNA regulation were also co-purified with the Hrp48-GFP bait (47, 89, 90). This indicates that our experimental conditions and analysis pipeline can preserve and identify true interactions. In addition, we also identified proteins that are known to be indirectly associated with Vas, such as the Cullin proteins (91) and Tudor-domain proteins Tudor (Tud) (92) and Krimper (Krimp) (93), suggesting that we not only recovered direct interactants, but whole complexes functional in distinct pathways (supplemental Fig. S3). With the Glo-GFP bait, we reproducibly identified other hnRNPs, including Hrp48 and the splicing factor Half pint.
(Hfp), consistent with previous reports (38, 94). Similarly, we could also recover an interaction of Stau-GFP with the spliceosomal protein SmB (95). However, the enrichment was not significant for these proteins, suggesting weak or transient interactions.

Quantitative Analysis Complements the Label-Free MS Data—To detect fold changes in protein abundances with high precision, we used dimethyl labeling MS. This approach is advantageous for Drosophila, where metabolic isotopic labeling is still challenging. To confirm the results of our label-free analysis, we carried out dimethyl labeling MS for the Hrp48- and Vas-GFP samples. We chose Hrp48 and Vas as these proteins express well and they are involved in the localization and translational regulation of both osk and grk during Drosophila oogenesis (35–37, 40, 45–47). We carried out the experiments in duplicates and purified the samples the same way as for label-free MS (supplemental Fig. S2B). After in-gel digestion, we labeled the peptides with heavy, medium, or
Fig. 2. Interactants, including known and new partners, were significantly enriched. A. Volcano plots of proteins identified to be associated with each bait in the label-free MS analysis, after filtering and data imputation. The significance of enrichment was calculated using the two-tailed Welch’s t-test, with FDR < 0.05, s0 = 2 for Nos, Hrp48 and Vas, and FDR < 0.1, s0 = 2 for elf4AIII, Glo and Stau. For each bait-control pair, the resulting differences between the logarithmized means of the two groups “Log2(bait/control)” and the negative logarithmized p values were plotted against each other. B. Scatter plots of the proteins identified to be associated with Hrp48-GFP and Vas-GFP in the dimethyl labeling MS analysis. Normalized ratios (Log2) of both the replicates were plotted against each other. Dotted lines mark the proteins with more than 2-fold change over control, in each replicate. IP from GFP sample served as a control. “N” denotes the number of protein groups plotted and “r” denotes the Pearson correlation coefficient. Each identified protein is represented as a dot in light gray; each bait is highlighted in green; significantly enriched proteins are highlighted in pink; known interactants are highlighted in blue; background binders are highlighted in dark gray; empty circle represents control.
light isotopes and inverted the labels in the replicate, to mini-
mimize the variability because of the labeling procedures. As
before, the raw data were processed with the MaxQuant
software (74), providing confident identification of proteins
(1% FDR) and normalized protein-abundance ratios. The
analysis of the Vas- and Hrp48-associated proteomes
resulted in the identification of 4027 peptides, mapping to
615 protein groups. The replicates showed high correlation
and the abundance ratios calculated could be well dupli-
cated. We considered as a hit those proteins that we identi-
fied with an abundance ratio of 2 in both replicates. Con-
sistent with the label-free analysis, we found several known
interactors, most of them reproducibly enriched (Fig. 2B). To
check how the two analyses relate to each other, we mapped
the proteins identified in labeled MS onto the label-free MS
data. As shown in Fig. 3, the proteins that were significantly
enriched in the labeled MS followed the same distribution
profile and showed up to 47% overlap (for Vas) with those
enriched in the label-free MS analysis. Background proteins
identified in labeled MS (<2 fold in both replicates) showed a
similar profile when graded on the corresponding label-free
MS data (Fig. 3). To get a comprehensive view of the pro-
teomes associated with Hrp48 and Vas, we combined the
enriched proteins from both analyses.

**Global Analysis of RBP Interactomes Reveals Novel Protein Interactions**—To understand how the proteomes identified with each bait interact with each other, we built a composite network of all statistically signifi-
cant interactants. Although each bait has interaction partners that do not interact with any of the other baits (for example, Stau interaction with RNA silencing proteins Dicer and its co-factor Loquacious (Loqs)), the network is also highly connected. In addition to the con-
siderable overlap in the proteomes of the functionally rela-
ted hnRNPs Hrp48 and Glo, we observed that Hrp48 and
Vas also shared a significant number of interacting proteins
(supplemental Fig. S4, supplemental Data S3). This overlap

![Quantitative label-based MS data support the label-free MS data](image-url)
suggests the interplay of these protein modules in the localization of transcripts during *Drosophila* development (96). To gain a systemic understanding of the network, we also added the known protein-protein associations from the String v. 11.0 (76) and FlyBase (77) databases. Next, we carried out a modularity analysis to identify highly connected communities of proteins (Fig. 4A, supplemental Data S4). As expected, many proteins involved in oogenesis, mRNA localization, translational regulation and germ cell formation were selectively enriched with all the baits. Additionally, proteins involved in neurogenesis and splicing were also overrepresented, consistent with the well-studied function of selected RBPs in these processes (Fig. 4B).

Interestingly, we observed ribosomal proteins (components of both large and small subunits) to be significantly enriched with Vas-GFP and Hrp48-GFP, but not with the other RBPs analyzed (Fig. 5, supplemental Fig. S4). Previous studies have shown the requirement of Vas in translational activation of osk, nos and grk mRNAs (35, 36, 40, 49). However, the molecular mechanism by which Vas activates translation is unclear. Studies in *Drosophila* have shown that Vas directly binds the translation initiation factor elf5B (dIF2) to positively regulate grk and mei-P26 translation and possibly other germline-specific transcripts (97–99). In addition, Vas also interacts genetically with the translation initiation factor elf4A for efficient germ cell formation (100). However, neither elf5B nor elf4A were detected or enriched in our data set. Instead, other translation initiation factors involved in the formation of the pre-initiation complex, such as elf2, elf3 and the cap-binding complex of elf4E-4G were selectively co-purified (Fig. 5). This is consistent with the recently reported interaction of elf3 subunits with Vas in the *Drosophila* oocytes (56).

Hrp48 is required for the translational repression of osk and grk mRNAs (45–47). Consistent with this, we co-purified with Hrp48-GFP (in both label-free and labeled MS experiments) several P-body-components, associated with the RNA repression/decay machinery, most notably the deadenylase and decapping complexes (Fig. 6). Ribosomal proteins and translation initiation factors were also significantly enriched with the Hrp48-GFP bait, similar to Vas-GFP (Fig. 5). This includes elf3d, which has recently been reported to interact with Hrp48 to translationally repress the msl-2 mRNA (101).

**In Vitro Validation of Protein-Protein Interactions**—To validate the results of our IP-MS analysis, we co-expressed bait-candidate pairs in cultured mammalian HEK293 cells (Fig. 7). This system can be effectively used to study direct interactions of *Drosophila* proteins because it reduces the likelihood of endogenous proteins mediating indirect associations. For validation, we selected significantly enriched candidates identified in the label-free MS analysis. Additionally, we also considered functionally relevant partners, enriched with a >2 times fold change but excluded by statistical filtering. For Hrp48 and Vas, where information from differential labeling was also available, we selected candidates among the interacting proteins identified in both data sets.

Typically, we co-expressed an EGFP (referred to as GFP)-tagged bait with an HA-tagged candidate protein and used the GFP-TRAP system (Chromotek) to carry out IPs in the presence of RNases. We observed that small proteins (<25kDa) expressed poorly as fusions with HA or HA-Flag. In these cases, we switched the tags. Substitution with the GFP tag improved the expression of the candidate proteins in all the cases tested. To be able to validate the interactions of such small proteins, we co-expressed a GFP-tagged candidate with an HA-Flag-tagged bait and performed the IP with anti-Flag (Fig. 7B, 7D). Because Vas does not express well as an HA- or HA-Flag fusion, we could not test the interactions of small protein candidates with Vas. As negative controls, we used MBP or GFP. We also included known interactions as positive controls, wherever possible. All the tested candidates are indicated in supplemental Fig. S5.

Out of 90 protein-protein interactions assayed, we could confirm 32 interactions (35%), of which 26 were found to be novel (summarized in Fig. 7J). Similar studies from *Drosophila* oocytes or embryos have shown a validation rate of <25% (56, 102). All positive interactions were confirmed at least 3 times, in independent experiments. In addition, we were also able to validate some of the interactions by reciprocal IP, as shown in Fig. 7C. To further confirm that our MS analysis pipeline effectively separated background binders from true interactants, we also tested Nucleophosmin (Nph), which was depleted in all IP-MS data sets. We could not detect interactions of Nph with any of the 4 baits tested in vitro (data not shown), in line with the MS data. Additionally, we also tested Sdq that has been reported to interact with Hrp48 in an RNA-dependent manner (47). The negative results further confirm that our experimental conditions effectively disrupted RNA-mediated associations. However, we cannot exclude the possibility that some interactions could be indirect and mediated by human factors or residual RNA impurities. Also, many interactions that were determined to be significant in the MS data analysis could not be validated in our in vitro assay. This could be because of the lack of post-translational modifications, protein misfolding, low affinity or indirect interactions. To visualize the co-IP results, we integrated the validated interactions with the IP-MS data (both labeled and label-free) and information from the literature to create a subnetwork (supplemental Fig. S6). As the majority of the validated interactants are known regulators of maternal mRNAs, this subnetwork highlights an extended interaction network potentially involved in the regulation of mRNAs during *Drosophila* oocyte development.

**DISCUSSION**

This study presents a proteome interaction network of six RBPs (elf4AIII, Hrp48, Glo, Nos, Stau and Vas) required for the localization of maternal mRNAs in *Drosophila*. To
Interactome of RNA Binding Proteins from Drosophila Ovaries

A

B

GO:0002181 Cytoplasmic translation
GO:0051298 Centrosome duplication
GO:0032543 Mitochondrial translation
GO:0068413 Translational initiation
GO:0000027 Ribosomal large subunit assembly
GO:0001731 Formation of translation preinitiation complex
GO:0002208 Neurogenesis
GO:0000398 mRNA splicing, via spliceosome
GO:0017148 Negative regulation of translation
GO:0007095 Mitotic G2 DNA damage checkpoint
GO:0005840 Ribosome
GO:00022625 Cytosolic large ribosomal subunit
GO:0000932 P-body
GO:00033296 Eukaryotic 48S preinitiation complex
GO:00005737 Cytoplasm
GO:0005852 Eukaryotic translation initiation factor 3 complex
GO:0005875 Microtubule associated complex
GO:0005730 Nucleolus
GO:0071013 Catalytic step 2 spliceosome
GO:0000228 Nuclear chromosome
GO:00003735 Structural constituent of ribosome
GO:0003723 RNA binding
GO:0003729 mRNA binding
GO:0003743 Translation initiation factor activity
GO:0005515 Protein binding
GO:0019643 RNA binding
GO:0005524 ATP binding
GO:0004535 poly(A)-specific ribonuclease activity
GO:004404 ATP-dependent RNA helicase activity
GO:0070180 Large ribosomal subunit mRNA binding
construct this network, we purified complexes associated with bait proteins, in an RNA-independent manner and used the MaxLFQ algorithm (84) for label-free relative protein quantification. The accuracy of this approach is comparable to labeled MS techniques such as SILAC (103). However, despite our stringent RNase treatment, it is possible that some of the recovered interactions are mediated by stretches of RNA protected from RNase cleavage by interacting proteins. By statistical filtering, we could separate background and specific binders for each bait. Several well characterized interactions were significantly enriched with most baits, indicating the efficacy of our workflow. To complement these data, we also obtained MS data from dimethyl labeling for a subset of the baits. We were able to validate 32 of the interactions assayed, including several novel associations.

In addition to the known regulators of mRNA localization and oocyte patterning, we co-purified nuclear and cytoplasmic complexes involved in different aspects of RNA metabolism. Our results highlight the diverse functions of these complexes in the post-transcriptional regulation of maternal mRNAs. The purification with the Stau-GFP bait of Loqs, a component of the RNA interference (RNAi) machinery is one such example. Loqs is a conserved cytoplasmic dsRBP. The protein participates in the biogenesis and processing of small noncoding RNAs that operate within the RNAi pathway (104). RNAi plays an important role in Drosophila germline development and the early phase of osk repression (96). Drosophila Loqs mutant females are sterile and their ovaries fail to sustain germ line stem cells (105). We found that both Dicer and Loqs are highly enriched in the Stau IP. We could confirm the Stau-Loqs interaction in vitro. This suggests a potential role for Stau in translational repression of osk by associating with the RNAi machinery. Although no such evidence has been presented in Drosophila, recent reports in other insects (D. citri and L. decemlineata) (106, 107) and the nematode C. elegans (11) have shown a requirement for Stau in RNAi responses. These results suggest a conserved role for Stau in RNAi-mediated gene silencing.

Nuclear Processing is Intrinsically Linked to Cytoplasmic Targeting of Maternal mRNAs—In addition to their crucial role in pre-mRNA processing, splicing factors also affect the cytoplasmic fates of mRNAs. SmB, a spliceosomal Sm protein is a known osk mRNP component. SmB contributes to germ-cell specification, at least in part by facilitating osk mRNA localization (95) and fails to localize to the posterior of the oocyte in the absence of Vas (108). Several Sm proteins have also been detected to be associated with Vas in the oocytes (56). Consistent with this, we co-purified SmB with Stau, Glo, Hrp48 and Vas. We also purified other splicing regulators with several of our baits, including Hfp (109) and the SR family proteins SC35 and SF2 (110, 111). Both SC35 and SF2 could be validated for their interaction with Glo and Stau, whereas Hfp bound elF4AIII and Stau in vitro. Hfp was previously shown to interact with Hrp48 and Glo (38) and SF2 co-purifies with the short isoform of Osk (112). These results together with the well-studied role of SR proteins in cytoplasmic regulation of gene expression including mRNA export, decay, and translation in mammalian systems (113, 114) suggest that splicing factors are bona fide components of the mRNA localization machinery.

Translational Regulation of Maternal mRNAs by Hrp48 and Vas—In agreement with the function of Vas in enhancing the translation of maternal mRNAs (35, 36, 40, 49), we co-purified several ribosomal proteins and translation initiation factors with Vas-GFP. Our results suggest that Vas may recruit factors involved in translation initiation. This is also supported by the interaction of Vas with elF5B and elF3 subunits, as previously reported (56, 97–99).

In contrast to Vas, Hrp48 is a known translational repressor (45–47). In line with the localization of Hrp48 to P-bodies (115), we co-purified several components of the mRNA decay machinery, including the CCR4-NOT deadenylase complex with Hrp48-GFP. It is possible that these interactions are indirect and mediated by BicudalC (BicC) and Belle (Bel). These proteins negatively regulate target mRNAs together with the CCR4-NOT complex (116, 117). We demonstrated in vitro binding of Hrp48 with both BicC and Bel. This suggests that by recruiting these proteins, Hrp48 may regulate the nos and osk mRNAs, possibly via CCR4-NOT mediated deadenylation (Fig. 8; ref. (116–119)). The function of Hrp48 in nos regulation remains to be investigated (Fig. 8).

However, the parallel enrichment of ribosomal proteins and P-body-components with Hrp48-GFP indicates its bifunctional role in modulating translation. Several lines of evidence from Drosophila, including binding of Hrp48 to a derepressor element in the osk 5’UTR (45), identification of Hrp48 as a part of a protein complex functioning in translational enhancement of Hsp83 mRNA (120) and interaction of Hrp48

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**Fig. 4.** The global interactome reveals a connected network. A, Interaction network of significantly enriched proteins, identified to be associated with each bait in the MS analysis. For Hrp48 and Vas, proteins from both labeled and label-free analyses were considered. Proteins are represented as nodes whereas edges represent the interactions. Green nodes represent the baits and the interactants are colored differently, based on their modularity class. The layout is based on a force-field analysis with the baits re-positioned for clarity. Edges representing interactions are colored differently for each bait: Vas in cyan; Hrp48 in green; elF4AIII in dark green; Glo in blue; Stau in red; Nos in orange and databases in gray. The edges have a unit weight (representing a known interaction). Node size (except for baits) represents the fold change (Log2) over control. For simplicity, respective subunits of the elF3 complex, cytoplasmic, and mitochondrial ribosomal proteins (separately for small and large subunits) were combined into a single node. B, Proteins were functionally annotated for their roles in biological processes, molecular functions and cellular components using GO term analysis. Only top ten terms in each category, with $p < 0.01$ are shown. The numbers represent the gene count involved in the respective GO term.
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with CPEB (cytoplasmic polyadenylation element binding) protein Orb (this study) support the dual nature of Hrp48. hnRNP A2, the mammalian homolog of Hrp48, also exhibits the ability to mediate both translational stimulation (121) as well as repression (122), further strengthening the argument.

Identification of Novel Genes with a Potential Role in Drosophila Oogenesis—Along with several known regulators of maternal mRNAs, we identified the protein products of many previously uncharacterized genes. One such example is CG5726, which encodes for a protein with a MIF4G-like domain. This domain is found in many proteins involved in RNA metabolism including translation initiation factors, NMD factors and nuclear cap-binding proteins (65, 123–126). With no identifiable orthologs in humans, CG5726 protein shows up to 50%
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A. Glo

B. Glo

C. Glo

D. Stau

E. Hrp48

F. eIF4AII

G. Vas

H. Stau

I. Nos

J. Tested Positive Novel

| Protein | Tested | Positive | Novel |
|---------|--------|----------|-------|
| Glo     | 8      | 3        | 1     |
| Stau    | 14     | 7        | 7     |
| Hrp48   | 28     | 10       | 8     |
| Nos     | 6      | 3        | 3     |
| Vas     | 11     | 4        | 4     |
| Total   | 90     | 32       | 26    |
In vitro validation of interactions with bait proteins.

A, E–I, Lysates from human HEK293 cells expressing GFP-tagged baits and HA-tagged (or HA-Flag- tagged) candidates were immunoprecipitated using an anti-GFP nanobody coupled to magnetic beads. HA- tagged MBP served as a negative control. Inputs and eluates were analyzed by Western blotting using anti-GFP and anti-HA antibodies. For GFP-tagged proteins, 1–3% of the input and 10% of the eluates were loaded, whereas for HA-tagged (or HA-Flag- tagged) proteins, 1–3% of the input and 90% of the eluates were analyzed. 

B, D, HEK293 cells were co-transfected with HA-Flag-tagged baits and GFP-tagged candidates. GFP tag served as a control. Cell lysates were immunoprecipitated with anti-Flag antibody and analyzed by Western blotting. For HA-Flag-tagged proteins, 3–4% of the input and 10% of the eluates were loaded, whereas for GFP-tagged proteins, 3–4% of the input and 90% of the eluates were analyzed. C, Cells were transfected the same way as in panel B, and the lysate was immunoprecipitated with an anti-GFP nanobody coupled to magnetic beads. For GFP-tagged proteins, 3.4% of the input and 10% of the eluates were loaded, whereas for HA-Flag-tagged protein, 3.4% of the input and 90% of the eluates were analyzed. In each panel, cell lysates were treated with RNases before immunoprecipitation. Novel interactions are highlighted in red. J, Summary of interactions assayed by co-IPs in human HEK293 cells.

Model of Hrp48-mediated protein associations in the regulation of maternal mRNAs. A subset of protein complexes interacting with Hrp48, possibly involved in the regulation of maternal mRNAs is shown. Many of these proteins were also found to be enriched in Vas-associated complexes. Note that only in vitro validated interactions of Hrp48 and Vas are shown here, except for their association with the translation machinery which was identified in the IP-MS data. Proteins are represented as circles and the interactions are represented as edges. Dotted gray lines represent the known interactions from databases; blue lines represent the interaction identified in the IP-MS data; green lines represent the in vitro validated novel interactions. Black arrows represent positive regulation whereas the red arrows represent negative regulation of mRNAs.
sequence identity among Drosophilids. In early embryos of *Drosophila melanogaster*, CG5726 interacts with short Osk (112). In this study, we found CG5726 to be interacting with multiple RBPs: Glo, Hrp48 and Vas, suggesting its possible role in translational regulation.

Consistent with the well-documented role of RNA helicases in oogenesis and fertility (88, 127–130), we identified two putative DEAD-box helicases, CG10077 and Mahe (Maheshvara). Although CG10077 is an ortholog of human DXD17, Mahe is an evolutionary conserved regulator of Notch signaling (131). We could recapitulate the interaction of Mahe with both Hrp48 and Glo, and CG10077 with Glo in our co-IP assay. Additionally, we found an interaction of CG13090 (Ubiquitin activating enzyme 4) with Nos. Mutations in this gene have been shown to impair ovarian stem cell functioning (132), which fits well with the role of Nos in maintenance of germ line stem cells during oogenesis (53, 55). The functional characterization of the protein interactions uncovered in this work will require further in vivo studies.

With certain limitations such as partial functionality of bait proteins or loss of transient or weak interactions, our ability to recover many known associations and validation of several newly identified interactions indicates the general reliability of our data. Although we performed the experiments in a transcript-independent manner, cluster-based analysis of the complexes can be used to identify functional units potentially regulating different mRNAs. Integrating isolated complexes into interaction networks will further enable functional insights into poorly characterized proteins. Given the evolutionary conservation of several RBPs, our study provides a framework to transfer information to other systems, enhancing our understanding of regulation of RBPs and their diverse roles in developmental processes.

**DATA AVAILABILITY**

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD016680. The annotation spectra for the label-free MS have been documented on MS-Viewer (http://msviewer.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msviewer) and can be accessed by the search key “ydwohmubp3”. The annotation data for dimethyl labeling MS are provided in supplemental Data S7.

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**Author contributions**—P.B., B.M., and F.B. designed research; P.B., J.M., and K.S. performed research; P.B., B.M., and F.B. analyzed data; P.B., K.S., and F.B. wrote the paper; J.M. and K.S. contributed new reagents/analytic tools.

**Conflict of interest**—Authors declare no competing interests.

**Abbreviations**—The abbreviations used are: EGFP, enhanced green fluorescent protein; elF, eukaryotic initiation factor; EJC, exon junction complex; FDR, false discovery rate; GO, gene ontology; HA, Hemagglutinin; hnRNP, heterogeneous nuclear ribonucleoproteins; IP, immunoprecipitation; Lfq, label-free quantification; MBP, maltose-binding protein; mRNP, messenger ribonucleoprotein; NMD, nonsense-mediated decay; PFA, paraformaldehyde; piRNA, piwi-interacting RNA; RBP, RNA-binding protein; SILAC, stable isotope labeling with amino acids; UAS, upstream activating sequence.

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