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Antagonism between GLD-2 Binding Partners Controls Gamete Sex

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SUMMARY

Cytoplasmic polyadenylation is a key mechanism of gene control. In *Caenorhabditis elegans,* GLD-2 and GLD-3 provide the catalytic and RNA-binding subunits, respectively, of a major cytoplasmic poly(A) polymerase (PAP). Here, we identify RNP-8 as a second GLD-2 partner. RNP-8 binds GLD-2 and stimulates GLD-2 activity to form a functional PAP, much like GLD-3. Moreover, GLD-2/RNP-8 and GLD-2/GLD-3 exist as separate complexes that form selectively during development, and RNP-8 and GLD-3 appear to have distinct RNA-binding specificities. Therefore, GLD-2 can form either of two discrete PAPs. In *C. elegans* hermaphrodites, gamete production begins with spermatogenesis and transitions later to oogenesis. We suggest that the combinatorial use of GLD-2 contributes to this transition, as GLD-2/GLD-3 promotes spermatogenesis, whereas GLD-2/RNP-8 specifies oogenesis. Indeed, RNP-8 and GLD-3 antagonize each other, as evidenced by genetic cosuppression and molecular competition for GLD-2 binding. We conclude that GLD-2 and its binding partners control gamete identity.

INTRODUCTION

Cytoplasmic polyadenylation controls mRNA stability and translation, and, hence, it is a key mechanism of gene control (Richter, 2000; Wickens et al., 2000). Indeed, translational control, rather than transcriptional regulation, appears to be the prevailing mechanism for gene control in germ cells and early embryos (Wickens et al., 2000). Regulated polyadenylation is also important at the synapse for long-term memory and learning (Huang et al., 2002; Keleman et al., 2007; Kwak et al., 2008; Rouhana et al., 2005; Si et al., 2003). Despite its importance to early animal development and learning, the molecular mechanisms that regulate cytoplasmic polyadenylation are just emerging.

One major cytoplasmic poly(A) polymerase (PAP) consists of a catalytic subunit and an RNA-binding moiety. The catalytic subunit was discovered in fission yeast and *Caenorhabditis elegans* (Read et al., 2002; Saitoh et al., 2002; Wang et al., 2002) and exists in virtually all eukaryotes, including flies, frogs, mice, and humans (Barnard et al., 2004; Benoit et al., 2008; Kwak et al., 2004; Nakanishi et al., 2006; Rouhana et al., 2005). This catalytic subunit, called GLD-2 in metazoans, belongs to the nucleotidyl transferase superfamily (Aravind and Koonin, 1999; Wang et al., 2002), as does canonical nuclear PAP (Bard et al., 2000). An RNA-binding domain is present in canonical PAP (Bard et al., 2000), but apparently not in GLD-2 (Wang et al., 2002).

GLD-2 appears to be recruited to select RNAs by an RNA-binding partner in worms, flies, and vertebrates. In *C. elegans*, GLD-2 associates with GLD-3, which harbors five KH domains and belongs to the Bicaudal-C family of RNA-binding proteins (Eckmann et al., 2002; Wang et al., 2002). Importantly, GLD-3 stimulates GLD-2 PAP activity in vitro (Wang et al., 2002), and GLD-3 and GLD-2 colocalize in cytoplasmic germ granules (Eckmann et al., 2002; Wang et al., 2002), which have been implicated in mRNA regulation (Seydoux and Braun, 2006). Therefore, GLD-2 and GLD-3 together form an active cytoplasmic PAP in the *C. elegans* germline. In *Xenopus*, GLD-2 exists in a complex with cytoplasmic polyadenylation element binding protein (CPEB); cleavage and polyadenylation specificity factor (CPSF); and RBM9, an RRM protein (Barnard et al., 2004; Papin et al., 2008; Rouhana et al., 2005). However, a direct interaction between RNA-binding proteins and GLD-2 has not been demonstrated in vertebrates or flies.

In budding yeast, the closest GLD-2 homolog is Trf4. Similar to *C. elegans* GLD-2, yeast Trf4 has little PAP activity on its own, but it is associated with Air1 and Air2, which are closely related putative RNA-binding proteins that stimulate Trf4 PAP activity (LaCava et al., 2005; Vaňáčová et al., 2005; Wyers et al., 2005). Trf4, Air1 or Air2, plus the Mtr4 helicase form the TRAMP complex, which acts in the nucleus to polyadenylate and thereby degrade selected RNAs. By contrast, GLD-2 and GLD-3 act in the cytoplasm (Eckmann et al., 2002; Wang et al., 2002) to polyadenylate and thereby activate target mRNAs (Suh et al., 2006). Therefore, GLD-2/GLD-3 and the TRAMP complex appear to be functionally divergent.

C. elegans GLD-2 controls multiple aspects of germline development (Kadyk and Kimble, 1998; Wang et al., 2002). To accomplish its varied roles, we proposed that GLD-2 might function combinatorially, interacting with distinct RNA-binding proteins to control specific functions. Consistent with that idea, GLD-2 and GLD-3 have similar, but not identical, biological roles. Most relevant to this work are the roles that GLD-2 and GLD-3 play in the sperm/oocyte decision. Normally, XO male germlines produce sperm continuously, whereas XX hermaphrodite germlines make sperm transiently in larvae and oocytes in adults.

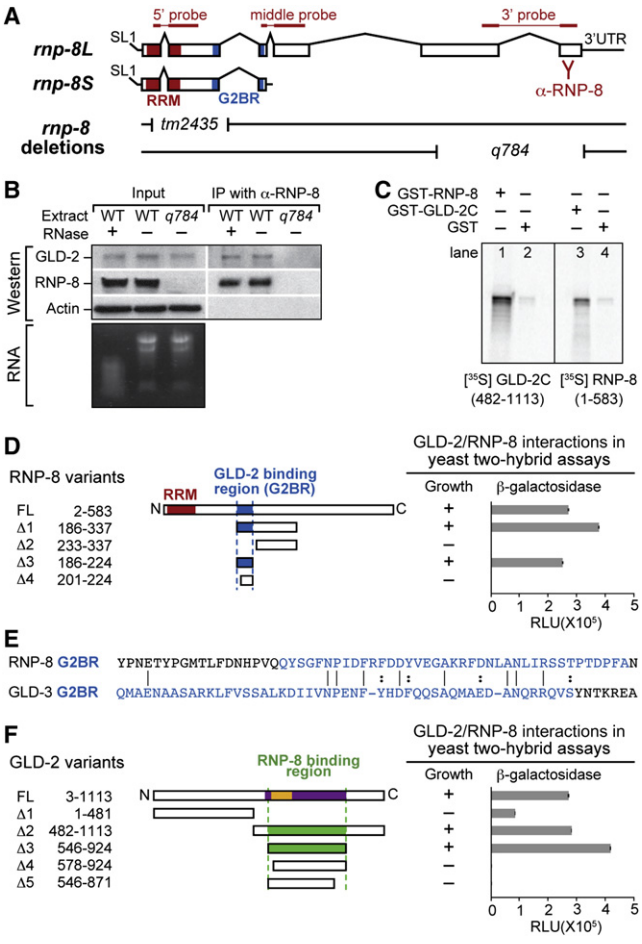

Figure 1. RNP-8 Interacts with GLD-2 in an RNA-Independent Manner

(A) The *rnp-8* locus. Upper, *rnp-8* transcripts are predicted to encode proteins with an N-terminal RRM (red) and a GLD-2 binding region (G2BR) (blue). Boxes, exons; connecting lines, introns; SL1, *trans*-spliced leader; 3′UTR, 3′ untranslated region; α-RNP-8, polyclonal antibody. Lower, *rnp-8* deletions are shown by gaps.

(B) Coimmunoprecipitation (Co-IP) of RNP-8 and GLD-2 from either wild-type (wt) or *rnp-8(q784)* adult extracts. Western blots of RNP-8, GLD-2, and actin as a control. Immunoprecipitations (IP) were done with α-RNP-8, either in the presence or absence of RNase A. For the input lanes, 1% worm extracts was loaded, but input and IP panels were exposed for different times. The efficiency of IP was ~1%–10% in the repeated experiments.

(C) In vitro binding of RNP-8 and GLD-2. Left, an in vitro-translated and ^{35}S-labeled GLD-2 fragment (GLD-2C) was incubated with purified GST-RNP-8 or GST alone (lanes 1 and 2); right, in vitro-translated and ^{35}S-labeled RNP-8 was incubated with purified GST-GLD-2C or GST alone (lanes 3 and 4).

(D) GLD-2 binding region (G2BR) in RNP-8. RNP-8 variants were fused to the Gal4 activation domain and tested in yeast two-hybrid assays for interaction with full-length GLD-2 fused to the LexA DNA binding domain. FL, full length. Results were scored by growth and β-galactosidase assays. For the growth assay, "–" refers to no growth and "+" refers to growth in the absence of histidine and the presence of 100 mM 3-aminotriazole. β-galactosidase was measured in Relative Light Units (RLU), and values are represented as mean ± SEM of three replicates. A small RNP-8 fragment (Δ3, amino acids 186–224) composed of 39 amino acids was sufficient for the GLD-2 interaction.

(E) Comparison of GLD-2 binding regions (G2BRs) in RNP-8 and GLD-3, aligned with the EBLOSUM62 program (European Bioinformatics Institute). Blue letters, amino acids in each G2BR; black letters, amino acids outside the G2BRs. Lines connect identical amino acids; two dots mark similar amino acids. The two G2BRs overlap for 33 amino acids and are 24% identical and 36% similar.

(F) RNP-8 binding region in GLD-2. GLD-2 variants were fused to the LexA DNA binding domain and tested for their interaction with full-length RNP-8 fused to the Gal4 activation domain. A large GLD-2 fragment (Δ3, amino acids 546–924) was sufficient for the RNP-8 interaction. Abbreviations are as in (D). Orange, catalytic domain; purple, central domain.

GLD-3 is essential for continued spermatogenesis at the expense of oogenesis (Eckmann et al., 2002), but no role in the sperm/oocyte decision had been observed previously for GLD-2 (Kadyk and Kimble, 1998).

In this paper, we report the identification of a second GLD-2 partner, RNP-8, and we investigate its relationship to GLD-2 and GLD-3. RNP-8 interacts with GLD-2 in yeast, in vitro and by coimmunoprecipitation from worm extracts; it possesses an RNA recognition motif (RRM), binds RNA, and enhances GLD-2 PAP activity; and, finally, it is enriched in oogenic germlines, is barely detectable in spermatogenic germlines, and localizes to germ granules. We find that GLD-2 is a gender-neutral enzyme that can either masculinize or feminize the germline. By contrast, GLD-3 promotes the sperm fate (Eckmann et al., 2002), whereas RNP-8 promotes the oocyte fate. GLD-3 and RNP-8 are genetically antagonistic with respect to gamete identity and compete with each other for binding GLD-2. Moreover, they exist in distinct complexes with GLD-2 and appear to have distinct RNA-binding specificities. We propose that GLD-2 governs gamete sex in a combinatorial fashion, driving the sperm fate with GLD-3 and the oocyte fate with RNP-8.

RESULTS

Identification of RNP-8, a GLD-2 Interacting Protein

R119.7 was identified in a yeast two-hybrid screen for proteins that interact with GLD-2, a screen that also identified GLD-3 (Wang et al., 2002). Briefly, from a screen of 2,000,000 transformants, 28/118 positives corresponded to the R119.7 predicted open reading frame. The R119.7 amino acid sequence contains a predicted RRM in its N-terminal region (amino acids 11–79) (Figure 1A), which gives the gene its name *rnp-8* (*rib*o*nu*cleo*p*rotein). To analyze *rnp-8*, we characterized its transcripts and generated key reagents, including affinity-purified anti-RNP-8 antibody (α-RNP-8), a polyclonal antibody that specifically recognizes the RNP-8 C terminus, and *rnp-8(q784)*, a deletion mutant that removes that C-terminal region (Figure 1A; see subsequent Results and Experimental Procedures).

To confirm the interaction between RNP-8 and GLD-2, we first asked whether RNP-8 and GLD-2 coimmunoprecipitate from worm extracts. To this end, we incubated extracts prepared from either wild-type or *rnp-8(q784)* adult hermaphrodites with α-RNP-8 that had been coupled to protein A beads. GLD-2 was coimmunoprecipitated from wild-type extracts, but not from *rnp-8(q784)* extracts (Figure 1B). RNase addition did not abrogate the association (Figure 1B); thus, the GLD-2/RNP-8 interaction is RNA independent. In addition, RNP-8 was coimmunoprecipitated with α-GLD-2 antibody (Figure 5G). Therefore, RNP-8 and GLD-2 associate with each other in extracts and are likely complexed in living worms.

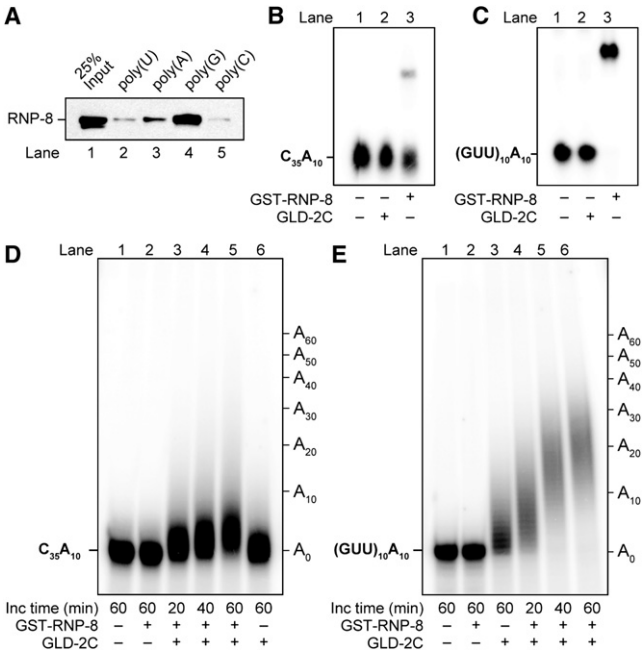

Figure 2. RNP-8 Binds RNA and Stimulates GLD-2 PAP Activity

(A) RNA homopolymer-binding assays with purified recombinant RNP-8. Proteins were incubated with poly(U), poly(A), poly(G), or poly(C) that had been coupled to beads, and their retention was analyzed by western blot (lanes 2–5).

(B and C) Electrophoretic mobility shift assays of RNAs bound to RNP-8 or GLD-2. The ^{32}P-labeled RNAs were incubated with no protein (lane 1), purified recombinant GLD-2C (lane 2), or GST-RNP-8 (lane 3); they were then analyzed on a 5% native polyacrylamide gel by autoradiography. (B) $C_{35}A_{10}$ RNA. (C) $(GUU)_{10}A_{10}$ RNA.

(D and E) Polyadenylation assays. A ^{32}P-labeled RNA substrate was incubated in the presence of ATP with purified recombinant proteins (GLD-2, RNP-8, or both) for 20–60 min, as noted, and was analyzed on a 10% polyacrylamide gel by autoradiography. (D) $C_{35}A_{10}$ RNA. (E) $(GUU)_{10}A_{10}$ RNA.

To ask whether the GLD-2/RNP-8 interaction is direct, we generated glutathione S-transferase (GST) fusions and performed pull-down assays in vitro. A large GLD-2 fragment (GLD-2C: amino acids 482–1113), which spans its conserved and catalytically active domains (Wang et al., 2002), was ^{35}S-labeled by in vitro translation and incubated with beads carrying GST-RNP-8. GLD-2C was retained by GST-RNP-8, but not by GST on its own (Figure 1C, lanes 1 and 2). In the converse experiment, RNP-8 was retained by GST-GLD-2C, but not by GST alone (Figure 1C, lanes 3 and 4). Therefore, the GLD-2/RNP-8 interaction appears to be direct, a conclusion confirmed by using recombinant purified proteins (Figure 5E). We conclude that RNP-8 and GLD-2 associate with each other both in vitro and in worm extracts, and that their interaction is direct and RNA independent.

RNP-8 is the second protein identified as a GLD-2 partner; the first was GLD-3 (see Introduction). To be able to compare the GLD-2 binding regions in the two GLD-2 partners, we used the yeast two-hybrid assay and found a stretch of 39 amino acids (amino acids 186–224) that was both necessary and sufficient for GLD-2 binding (Figure 1D). Previously, the GLD-2 binding region in GLD-3 was narrowed to 49 amino acids (Eckmann et al., 2004). Alignment of the two GLD-2 binding regions showed limited sequence similarity, but both regions are predicted to form an α helix (Figure 1E). In the GLD-2 protein, we identified a relatively large fragment (amino acids 544–924) that is required for the RNP-8 interaction (Figure 1F). This region of GLD-2 comprises both catalytic and central domains and is essentially the same as that found for binding to GLD-3 (Eckmann et al., 2004).

RNP-8 Stimulates GLD-2 PAP Activity In Vitro

The discovery of RNP-8 as a GLD-2 partner raised the possibility that this RRM-containing protein might bind RNA and tether GLD-2 PAP activity to specific mRNAs. We first tested the idea that RNP-8 binds RNA by using RNA homopolymers. RNP-8 was strongly retained by poly(G), but poorly retained by poly(U), poly(A), or poly(C) (Figure 2A). Based on that sequence preference, we designed two RNA oligomers to test RNP-8 binding in vitro: $C_{35}A_{10}$ was predicted to bind RNP-8 poorly or not at all, and $(GUU)_{10}A_{10}$ was predicted to bind RNP-8 well. Indeed, when equimolar concentrations of purified recombinant RNP-8 and the RNA oligos were used, RNP-8 bound $(GUU)_{10}A_{10}$ better than $C_{35}A_{10}$ (Figures 2B and 2C, lane 3). In contrast, GLD-2C was not capable of binding either of these RNAs on its own (Figures 2B and 2C, lane 2).

We next asked if RNP-8 can recruit GLD-2 PAP activity to RNA in vitro. To this end, purified recombinant GLD-2C and GST-RNP-8 were incubated with a labeled RNA substrate (either $C_{35}A_{10}$ or $(GUU)_{10}A_{10}$) and ATP. After incubation, the reaction mixture was separated on a denaturing gel. RNP-8 alone did not stimulate incorporation with either substrate (Figures 2D and 2E, lane 2). GLD-2 on its own was capable of minor ATP incorporation (Figure 2D, lane 6; Figure 2E, lane 3), but the combination of GLD-2 and RNP-8 together greatly enhanced incorporation (Figure 2D, lanes 3–5; Figure 2E, lanes 4–6). Importantly, the length of the poly(A) tail increased with incubation time and was stimulated more with $(GUU)_{10}A_{10}$ than with $C_{35}A_{10}$ as the RNA substrate. We conclude that GLD-2 and RNP-8 work together in a manner that is directly analogous to the GLD-2/GLD-3 heterodimer (Wang et al., 2002), and we suggest that RNP-8 provides RNA sequence specificity to GLD-2-mediated polyadenylation.

The *rnp-8* Locus Is Expressed in the Germline

A biological function of RNP-8 was not apparent from genomic-level RNAi studies (i.e., no defects were seen) (Piano et al., 2002). To more rigorously investigate its biological role, we began by analyzing *rnp-8* gene products in wild-type animals and two mutants. The *rnp-8(q784)* deletion removes 1223 bp from the 3′ end of the locus and is predicted to delete 233 and insert 7 novel amino acids to the C terminus; the predicted RNP-8(q784) mutant protein leaves intact both the RRM and GLD-2 binding region (G2BR) (Figure 1A). The *rnp-8(tm2435)* deletion, a kind gift from the National Bioresource Project of Japan, deletes 626 bp and inserts 9 bp at the 5′ end of the locus; it removes both the RRM and G2BR and shifts the reading frame (Figure 1A). Both *rnp-8(q784)* and *rnp-8*(*tm2435*) homozygotes are viable and largely self-fertile (see below).

Two *rnp-8* transcripts were detected on northern blots (Figures 1A and 3A) and were confirmed by cDNA analysis (data not shown). A 2.2 kb mRNA, dubbed *rnp-8L*, contains 6

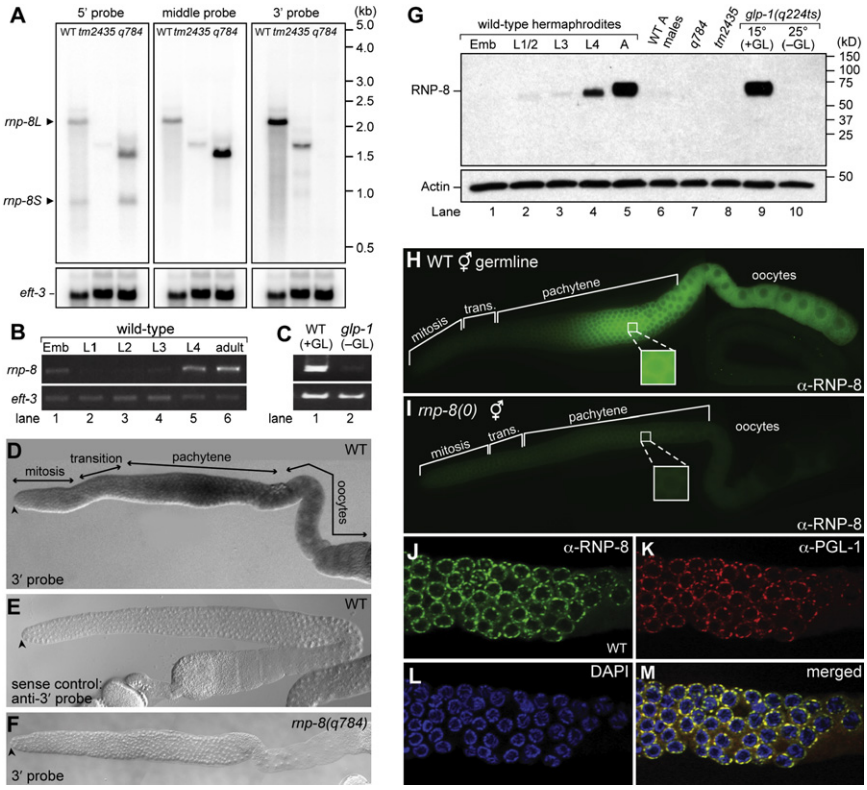

Figure 3. *rnp-8* Transcripts and RNP-8L Protein

(A) Two *rnp-8* transcripts are detected in northern blots of mRNAs prepared from adults with wild-type, *rnp-8(tm2435)* homozygous, and *rnp-8(q784)* homozygous genotypes. cDNA probes are shown in Figure 1A. *eft-3* mRNA was the loading control; right, molecular weight markers (kb).

(B) Staged expression of *rnp-8* mRNA. RT-PCR analysis of *rnp-8* mRNAs, with primers recognizing both *rnp-8L* and *rnp-8S*. *eft-3* mRNA was the control. Emb, embryo; L1–L4, first–fourth larval stage.

(C) Germline expression of *rnp-8* mRNA. RT-PCR analysis of *rnp-8* mRNAs, with primers that recognize *rnp-8L*; a similar experiment was performed with primers for the *rnp-8S* transcript with equivalent results. RNA was prepared from wild-type adults, which possess normal germline tissue (+GL), or from *glp-1(q175)* mutant adults, which have essentially no germline (−GL) (Austin and Kimble, 1987).

(D–F) Germline distribution of *rnp-8* mRNA in dissected adult hermaphrodite germlines. Arrowhead, distal end. All hybridizations and images were treated identically. (D) Wild-type (wt) germline probed with an antisense strand corresponding to exons 5 and 6 (3′ probe; Figure 1A). (E) Wild-type (wt) germline probed with a sense strand of the same fragment as in (D) (anti-3′ probe). (F) *rnp-8(q784)* mutant germline probed with an antisense strand of the same fragment as in (D) (3′ probe).

(G) Western blot probed with rabbit α-RNP-8 (top) or α-actin (bottom). α-RNP-8 recognizes the RNP-8L protein as a major band at ~65 kD. On a longer gel, this single band resolves into several bands, which likely represent posttranslational modifications. α-RNP-8 cannot detect RNP-8S. Emb, embryos; L1–L4, first–fourth larval stages; A, adult; *q784*, *rnp-8(q784)* homozygotes; *tm2435*, *rnp-8(tm2435)* homozygotes. *glp-1(q224ts)* hermaphrodites raised at the permissive temperature (15°C) have a nearly normal germline (+GL), but those raised at the restrictive temperature (25°C) have essentially no germline (−GL).

(H–M) Immunocytochemistry of extruded adult hermaphrodite germlines. (H and I) (H) Wild-type and (I) *rnp-8(tm2435)* germlines were stained with rabbit α-RNP-8 (green), and images were obtained on a fluorescence microscope. RNP-8 is abundant in meiotic pachytene germ cells and oocytes. Insets, RNP-8 is cytoplasmic and enriched in granules. RNP-8 is also detected, at a much lower level, in cytoplasmic granules in the distal germline. (J–M) Wild-type germline was double stained with (J) rat α-RNP-8 (green) and (K) α-PGL-1 (red), and images were obtained on a confocal microscope. (M) RNP-8 and PGL-1 overlap in all granules (yellow). Photos were taken in the pachytene region.

exons; a 0.75 kb mRNA, dubbed *rnp-8S*, contains exons 1–3. Both are transpliced to SL1 and polyadenylated in cDNAs. *rnp-8L* and *rnp-8S* are predicted to encode proteins of 583 and 230 amino acids, respectively. Both proteins include the RRM and G2BR (Figure 1A). The *rnp-8(tm2435)* deletion generates a shorter mRNA that is vastly reduced on northern blots (Figure 3A); the *rnp-8(q784)* deletion generates truncated transcripts that have a poly(A) tail.

We used both RT-PCR and in situ hybridization to analyze *rnp-8* mRNAs during development. The *rnp-8L* and/or *rnp-8S* mRNAs were present in embryos, were rare in first- and second-stage larvae (L1 and L2), and were abundant in later-stage larvae and in adults (Figure 3B); moreover, both transcripts were greatly diminished in mutants lacking a germline (Figure 3C; data not shown). This profile suggests that *rnp-8* is expressed in the germline, which we confirmed by in situ hybridization. The adult wild-type germline contains mitotically dividing germ cells at the distal end and maturing meiotic germ cells in more proximal regions. *rnp-8L* was detectable, but low, in the mitotic region and transition zone, which contains early meiotic prophase germ cells, and it was abundant in the pachytene region and developing oocytes, as assessed with an antisense-strand probe (Figure 3D), but not with a sense-strand control probe (Figure 3E). The antisense-strand probe was directed against sequence within the *q784* deletion and, as a result, did not hybridize to RNA in *rnp-8(q784)* germlines (Figure 3F).

To visualize the RNP-8L protein, we used α-RNP-8, an affinity-purified rabbit polyclonal antibody raised against the C-terminal 19 amino acids (524–542, Figure 1A). In wild-type protein extracts, α-RNP-8 recognized a major protein, which corresponded in size to predicted RNP-8L (Figure 3G). In extracts, prepared from *rnp-8(tm2435)* or *rnp-8(q784)* mutants, RNP-8L was no longer detectable (Figure 3G, lanes 7 and 8). Similarly, as assessed with immunocytochemistry, α-RNP-8 recognized protein in wild-type, but not in mutant germlines (Figures 3H and 3I; data not shown). The absence of a signal in *rnp-8(q784)* confirms the specificity of α-RNP-8, but it does not address whether RNP-8 is absent from this strain because the deletion removes the sequence used to raise α-RNP-8; however, the absence of a signal in *rnp-8(tm2435)* confirms that this mutant eliminates most or all RNP-8L protein. We also suggest that

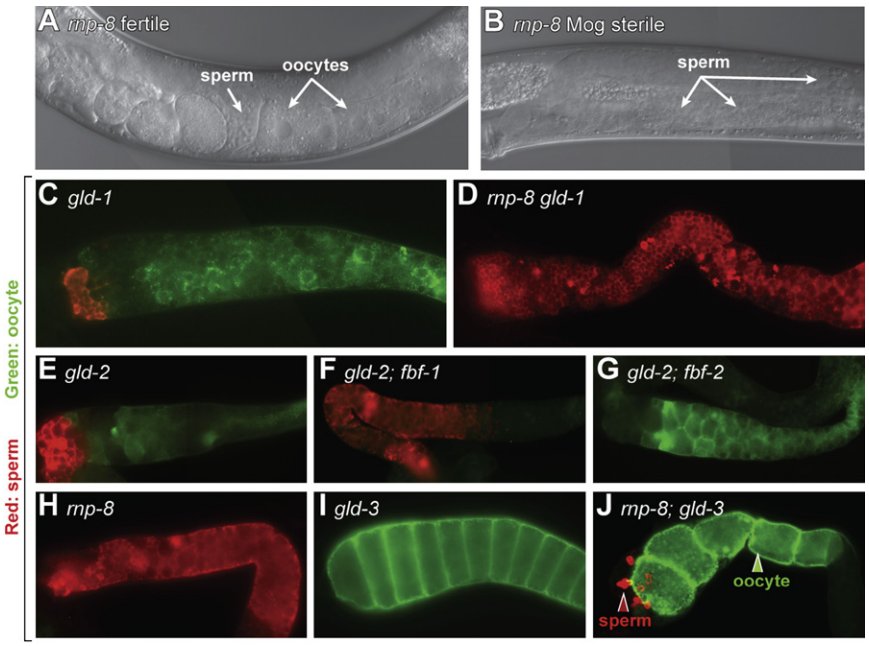

Figure 4. *rnp-8* Promotes the Oocyte Fate and Is Reciprocally Antagonistic with *gld-3* in the Sperm/Oocyte Switch

(A and B) DIC microscopy of *rnp-8(tm2435)* hermaphrodite adults.

(C–J) Adult hermaphrodite germlines were extruded and double stained with SP56 (sperm-specific marker; red) and α-RME-2 (oocyte-specific marker; green). Photos show the proximal germline. (C) *gld-1(q485)*. (D) *rnp-8(tm2435) gld-1(q485)*. (E) *gld-2(q497)*. (F) *gld-2(q497); fbf-1(ok91)*. (G) *gld-2(q497); fbf-2(q738)*. (H) *rnp-8(tm2435)*. (I) *gld-3(q730)*. (J) *rnp-8(tm2435); gld-3(q730)*.

RNP-8S is not made in *rnp-8(tm2435)* because this deletion removes most of the *rnp-8S* coding region and shifts the reading frame. Therefore, *rnp-8(tm2435)* is likely to be a strong loss-of-function or null mutant; henceforth, *rnp-8(tm2435)* is referred to as *rnp-8(0)*.

RNP-8L Is Enriched in Oogenic Germlines and Colocalizes with Germ Granules

Attempts to obtain an RNP-8 antibody that recognizes the common region present in both RNP-8L and RNP-8S have not yet been successful. We therefore used α-RNP-8 to assay RNP-8L developmental regulation and subcellular localization. On western blots, RNP-8L was barely detectable in embryos, increasingly detectable in larvae, and abundant in adult hermaphrodites (Figure 3G, lanes 1–5). Moreover, RNP-8L protein was absent from mutants with no germline (Figure 3G, lane 10). These results suggest that RNP-8L is abundant in the adult hermaphrodite germline, a conclusion supported by immunocytochemistry. Immunostaining also revealed that RNP-8L protein is predominantly cytoplasmic (Figures 3H–3M), colocalizes with the PGL-1 germ granule marker (Figures 3J–3M) (Kawasaki et al., 1998), and is abundant in the proximal pachytene region and developing oocytes (Figure 3H). GLD-2 shows a similar distribution and also colocalizes with PGL-1 (Wang et al., 2002); thus, RNP-8 and GLD-2 are likely to colocalize in germ granules.

RNP-8L was detectable in males (Figure 3G, lane 6), but much less abundant than in hermaphrodites, suggesting a sex-specific role. As assessed by immunocytochemistry of male germlines, RNP-8L was limited to the mitotic region, transition zone, and distal pachytene region; it was absent from proximal pachytene cells, spermatocytes, and mature sperm (data not shown). Indeed, RNP-8L was similar in both hermaphrodite and male distal germlines, both with respect to its low level and its distribution in cytoplasmic granules (Figure 3H; data not shown). As assessed by western blot, RNP-8L was also low in mutant adult hermaphrodites that made only sperm and no oocytes (data not shown). We conclude that abundant RNP-8L is associated with oogenesis.

rnp-8 Promotes the Oocyte Fate

Most *rnp-8(0)* hermaphrodites were self-fertile with a superficially normal germline (Figure 4A) and no obvious somatic defect. However, some *rnp-8(0)* hermaphrodites were sterile and made only sperm, the Mog (for *M*asculinization *o*f *g*ermline) phenotype (Figures 4B and 4H); Mog germlines were also seen after *rnp-8* RNAi (data not shown). The *rnp-8(0)* Mog germlines displayed abundant SP56 (sperm-specific marker) (Ward et al., 1986), but no RME-2 (oocyte-specific marker) (Grant and Hirsh, 1999) (Figure 4H).

The *rnp-8* Mog phenotype shows that RNP-8 promotes the oocyte fate, but its low penetrance suggests that RNP-8 acts with other regulators to specify the oocyte fate. To test this idea, we asked if *rnp-8(0)* enhanced mutations with a low-penetrance Mog phenotype, such as *fbf-1(0)* (Crittenden et al., 2002) and *nos-3(0)* (Kraemer et al., 1999). Indeed, both were enhanced (Table 1), suggesting that RNP-8 promotes the oocyte fate as part of a regulatory network.

We also examined the effect of *rnp-8(0)* on *gld-1(0)* germlines, which are tumorous (Francis et al., 1995a). Normally, the GLD-1

Table 1. *rnp-8* Promotes the Oocyte Fate

	Germline Defects[b] (Percentage of Animals)			
		Sterile		
Genotype[a]	Fertile	Sperm Only	Other[c]	n
Wild-type	100	0	0	>1000
rnp-8	90	9	1	534
fbf-1[d]	99	1	0	1792
rnp-8; fbf-1	70	29	1	227
nos-3[e]	99.7	0.2	0.1	2000
rnp-8; nos-3	55	40.5	4.5	198

The *gld-3 nos-3* germline is tumorous (Eckmann et al., 2004).

[a] All animals were XX hermaphrodites; all alleles were strong loss-of-function putative nulls.

[b] Animals were first scored for fertility or sterility; then, sterile germlines were scored for gametes by DIC.

[c] Germlines had a variable and ambiguous morphology.

[d] Crittenden et al. (2002).

[e] Kraemer et al. (1999).

Table 2. Genetic Analysis of Sperm/Oocyte Regulators

	Genotype[a]	Germline Defects[b] (Percentage of Animals) Sperm + Oocyte	Sperm Only	Oocyte Only	n
A	Wild-type	100	0	0	>1000
	rnp-8[c]	76	24	0	268
	gld-1	60	0	40	57
	rnp-8; gld-1 (1 da)	0	100	0	78
	rnp-8; gld-1 (2 da)	0	100	0	46
	rnp-8; gld-1 (3 da)	0	100	0	45
B	*gld-2*	100	0	0	200
	fbf-1	100	0	0	45
	rnp-8; fbf-1[c]	29	71	0	31
	gld-2; fbf-1	3	97	0	150
	gld-3 fbf-1	100	0	0	36
	fbf-2	94	0	6	36
	rnp-8; fbf-2	97	3	0	34
	gld-2; fbf-2	0	0	100	49
	gld-3 fbf-2	0	0	100	38
C	*gld-3*	68	0	32	82
	rnp-8; gld-3	99	1	1	150

The *gld-1; gld-3* germline is tumorous (Eckmann et al., 2004).

[a] All animals were XX hermaphrodites; all alleles were strong loss-of-function putative nulls.

[b] Gamete sex was scored as the percentage of germlines with SP56 and α-RME-2 ("Sperm + Oocyte"); SP56, but not α-RME-2 ("Sperm Only"); or α-RME-2, but not SP56 ("Oocyte Only"), staining. All germlines were dissected from adults 1 day past L4, except as noted; 2 da and 3 da indicate 2 days and 3 days past L4, respectively.

[c] Using this scoring method, the percentage of germlines with sperm only is higher than the percentage of sperm-only sterile animals (Table 1), because each animal contains two germlines. If one germline makes oocytes, the animal is fertile.

protein promotes meiotic maturation (Francis et al., 1995a) and also promotes meiotic entry in parallel with GLD-2 (Kadyk and Kimble, 1998). The result was dramatic: *gld-1(0)* germlines were 0% Mog, as expected, but *rnp-8 gld-1* double mutant germlines were 100% Mog (Figures 4C and 4D; Table 2A). The XX *rnp-8 gld-1* double mutants lost their germline tumors and made excess sperm and no oocytes (Figure 4D). This unexpected germline masculinization was seen in *rnp-8 gld-1* adults at 1 day, 2 days, and 3 days past L4, scored either by Nomarski to visualize the diagnostic size and shape of each gamete or with sperm- and oocyte-specific antibodies (Figure 4D; Table 2A; data not shown). A germline tumor typical of *gld-1(0)* single mutants could be restored to the *rnp-8 gld-1* double mutant by using *fog-1* RNAi to feminize its germline, as expected because only oogenic *gld-1* germlines are tumorous (Francis et al., 1995b; data not shown). We conclude that RNP-8 functions redundantly with GLD-1 to promote the oocyte fate.

GLD-2 Can Promote Either Sperm or Oocyte Fate

Previous work found no role for GLD-2 in gamete sex: XX *gld-2(0)* hermaphrodites made both sperm and oocytes, and XO *gld-2(0)* males made sperm only, although both gametes were defective (Figure 4E; Table 2B) (Kadyk and Kimble, 1998). We reasoned that GLD-2 might affect gamete sex in a sensitized mutant background, and we asked if *gld-2(0)* might enhance mutants with low-penetrance Mog or Fog (*F*eminization *o*f *g*ermline) phenotypes. In these experiments, young adult germlines were scored with sperm- and oocyte-specific markers. We found that *gld-2* enhanced *fbf-1(0)* germline masculinization, and that it also enhanced *fbf-2(0)* germline feminization (Figures 4F and 4G; Table 2B). Importantly, *gld-3(0)* also enhanced *fbf-2(0)*, but did not affect *fbf-1(0)* (Table 2B). We conclude that GLD-2 does in fact influence gamete sex, and that GLD-2 can promote either the sperm or oocyte fate, depending on its interactions with other regulators. Importantly, both *gld-2* and *rnp-8* enhance the *fbf-1* Mog phenotype, and both *gld-2* and *gld-3* enhance the *fbf-2* Fog phenotype (Table 2B).

RNP-8 and GLD-3 Antagonize Each Other in the Sperm/Oocyte Decision

Both RNP-8 and GLD-3 partner with GLD-2 and stimulate GLD-2-mediated polyadenylation in vitro (this work; Wang et al., 2002). However, RNP-8 governs oocyte specification (this work), and GLD-3 promotes the sperm fate (Eckmann et al., 2002). Given those opposite roles, we reasoned that RNP-8 and GLD-3 might antagonize each other. To explore this idea, we compared gamete sex in *rnp-8(0)* and *gld-3(0)* single mutants as well as in *rnp-8; gld-3* double mutants. In each mutant, we focused on young adult hermaphrodite germlines, 1 day past L4, and scored the production of sperm or oocytes by staining with sperm- and oocyte-specific markers. For *rnp-8(0)*, 24% of the germlines made sperm but not oocytes, and the rest made both gametes (Figure 4H; Table 2A); for *gld-3(0)*, 32% made oocytes but not sperm, and the rest made both gametes (Figure 4I; Table 2C). By contrast, nearly all (99%) *rnp-8 gld-3* double mutants made both sperm and oocytes (Figure 4J; Table 2C). Therefore, removal of both RNP-8 and GLD-3 restores the germline to a quasi-normal state, in which both sperm and oocytes are made. This result underscores the idea that RNP-8 and GLD-3 are part of a well-buffered regulatory network. We conclude that *rnp-8* and *gld-3* are antagonistic in their effect on the sperm/oocyte decision.

RNP-8 and GLD-3 Can Compete with Each Other for GLD-2 Binding

RNP-8 and GLD-3 both possess a small G2BR, and both interact with the same GLD-2 region (Eckmann et al., 2004). Therefore, we postulated that the functional GLD-3/RNP-8 antagonism might rely on competition between RNP-8 and GLD-3 for GLD-2 binding. To test this idea, we first used a modified yeast two-hybrid assay (Figure 5A). Specifically, we coexpressed three proteins: RNP-8 fused to the GAL4 activation domain (AD-RNP-8), GLD-2 fused to the LexA DNA-binding domain (BD-GLD-2), and a test protein (e.g., GLD-3). To monitor interactions, we assayed expression from a *lacZ* reporter gene and also growth on plates lacking histidine (Figure 5B, left). Western blots were used to ensure that proteins were expressed (Figure 5B, right). By both assays, AD-RNP-8 and BD-GLD-2 interacted strongly in the absence of test protein, but they interacted poorly when GLD-3 was introduced as the test protein (Figure 5B, lanes 1 and 2). GLD-3 inhibition was specific because two other test proteins, FOG-1 RNA-binding protein and chicken pyruvate kinase, had virtually no effect (Figure 5B, lanes 3

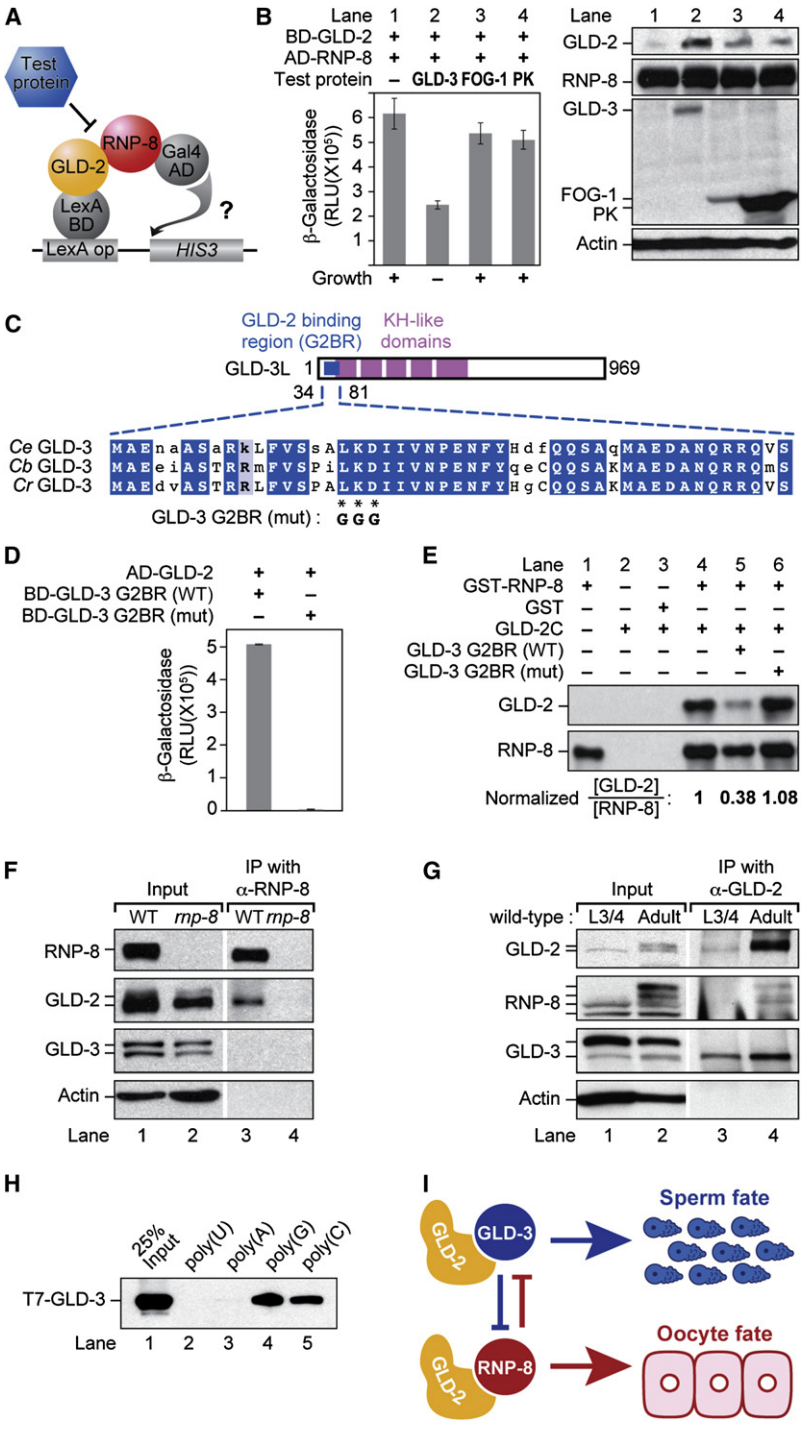

Figure 5. RNP-8 and GLD-3 Proteins Compete with Each Other for GLD-2 Binding

(A) Schematic of the variant yeast two-hybrid assay used to test the effect of a test protein (e.g., GLD-3) on the GLD-2/RNP-8 interaction.

(B) Full-length GLD-3 interferes with the GLD-2/RNP-8 interaction in yeast. Left, β-galactosidase reporter assay (upper) and growth assay (lower); right, western blots showing that test proteins are expressed. Lane numbers in the left and right panels mark the same experiments. LexA binding domain fusion protein, BD-GLD-2; Gal4 activation domain fusion protein, AD-RNP-8; test proteins, GLD-3 (lane 2), FOG-1 (lane 3), and chicken pyruvate kinase, PK (lane 4). β-galactosidase was measured in Relative Light Units (RLU), and values are represented as mean ± SEM of three replicates.

(C) Identification of conserved residues in the GLD-3 G2BR to design control peptide. Upper, schematic of GLD-3 with KH domains (purple) and N-terminal G2BR (blue) (Eckmann et al., 2004). Lower, sequence alignment of G2BRs of three GLD-3 homologs: *Ce*, *C. elegans*; *Cb*, *C. briggsae*; *Cr*, *C. remanei*. Identical amino acids, dark blue; similar amino acids, light blue. Asterisks mark three amino acids changed to glycine in the GLD-3 G2BR (mut) control peptide.

(D) Using a yeast two-hybrid assay. Wild-type (wt) GLD-3 G2BR interacts with GLD-2, but GLD-3 G2BR (mut) does not. β-galactosidase was measured in Relative Light Units (RLU), and values are represented as mean ± SEM of three replicates.

(E) Competition between RNP-8 and GLD-3 G2BR for GLD-2 binding. In vitro assay with recombinant purified GLD-2C and RNP-8 proteins plus GLD-3 G2BR synthetic peptides. RNP-8 specifically interacts with GLD-2 (compare lanes 3 and 4). GLD-3 G2BR (wt) peptide interferes with this interaction (lane 5), whereas GLD-3 G2BR (mut) peptide does not (lane 6). Lower, quantitation of GLD-2 protein abundance relative to RNP-8, obtained by measuring band intensity with ImageJ software (rsbweb.nih.gov/ij).

(F) Co-IP with α-RNP-8 and either wild-type (wt) or *rnp-8(q784)* mixed-stage extracts. Western blots of RNP-8, GLD-2, GLD-3, and actin as a control. For the input, 1% worm extracts was loaded. For GLD-2, lanes 1 and 2 are not comparable to lanes 3 and 4, because the bands in lanes 1 and 2 were so heavily loaded that the film had to be exposed for a shorter time.

(G) Co-IP with α-GLD-2 and either larval (L3/4) or adult wild-type extracts. Western blots of GLD-2, RNP-8, GLD-3, and actin as a control. This blot was prepared from a longer gel than that used in other figures. As a result, GLD-2, RNP-8, and GLD-3 were detected as multiple bands, which likely represent posttranslational modifications. The smudges in lanes 3 and 4 of the RNP-8 blot were unavoidable; the antibody heavy chain ran close to RNP-8, and both antibodies (for IP and blotting) were generated from rabbit. For the input, 1% worm extracts was loaded. For GLD-2, lanes 1 and 2 are not comparable to lanes 3 and 4, because the bands in lane 4 were so heavily loaded that the film had to be exposed for a shorter time.

(H) RNA homopolymer-binding assays with purified recombinant GLD-3. Proteins were incubated with poly(U), poly(A), poly(G), or poly(C) that had been coupled to beads, and their retention was analyzed by western blot (lanes 2–5).

(I) Model for combinatorial control of sperm and oocyte specification by GLD-2/RNP-8 and GLD-2/GLD-3. Activation may be direct or indirect, as discussed in the text; also, the GLD-2 complexes must be part of a well-buffered regulatory network that controls gamete choice, as discussed in the text.

and 4). We also tested the GLD-3/RNP-8 competition in reverse, using BD-GLD-3 and AD-GLD-2 to monitor the interaction and RNP-8 as the test protein, with similar results (data not shown).

To test competition in vitro, we first developed a binding assay with purified recombinant GST-RNP-8 and GLD-2C proteins. When assayed in vitro, GST-RNP-8 retained GLD-2C (Figure 5E, lane 4), but GST alone did not (Figure 5E, lane 3), which confirms the direct interaction between RNP-8 and GLD-2. As an antagonist, we prepared a synthetic 49 amino acid GLD-3 peptide that spans the minimal G2BR (Eckmann et al., 2004); the synthetic

wild-type peptide is dubbed GLD-3 G2BR (wt). As a control, we mutated three conserved amino acids to generate a different synthetic peptide, GLD-3 G2BR (mut) (Figure 5C). GLD-3 G2BR (wt) bound to GLD-2 in a yeast two-hybrid assay, but GLD-3 G2BR (mut) did not (Figure 5D). Finally, we asked if GLD-3 G2BR interferes with the binding between recombinant RNP-8 and GLD-2, by using the in vitro binding assay. Indeed, GST-RNP-8's retention of GLD-2 was severely compromised by the GLD-3 G2BR (wt) peptide, but not by the control GLD-3 G2BR (mut) peptide (Figure 5E, lanes 5 and 6). We also attempted to test if GLD-3 G2BR could antagonize the RNP-8 stimulation of GLD-2 PAP activity in vitro, but GLD-3 G2BR abolished GLD-2 catalytic activity even in the absence of RNP-8; thus, this experiment was not possible.

GLD-2 Complexes with RNP-8 and GLD-3 In Vivo

To ask if GLD-2/GLD-3 and GLD-2/RNP-8 form distinct complexes in vivo, we immunoprecipitated RNP-8 or GLD-2 from wild-type worm extracts and examined their associations, both with each other and with GLD-3. Immunoprecipitation with α-RNP-8 brought down GLD-2, but not GLD-3, from extracts made from mixed-stage worms (Figure 5F). Therefore, the GLD-2/RNP-8 complex is likely to be separate from the GLD-2/GLD-3 complex.

We next immunoprecipitated GLD-2 from spermatogenic L3/L4 extracts and from oogenic adult extracts. In larvae, GLD-2 binds almost exclusively to GLD-3, even though RNP-8 is present at the same stage (Figure 5G). This result suggests that GLD-3 can compete successfully with RNP-8 in spermatogenic germlines. In adults, GLD-2 brings down both GLD-3 and RNP-8 (Figure 5G). The GLD-3 association in oogenic germlines was not unexpected, because GLD-2 and GLD-3 promote meiotic entry and meiotic maturation in oogenic germlines (Eckmann et al., 2002, 2004). We conclude that GLD-2/RNP-8 and GLD-2/GLD-3 complexes form selectively during development, and do so in a way that is consistent with GLD-2/GLD-3 promoting the sperm fate and GLD-2/RNP-8 promoting the oocyte fate.

RNP-8 and GLD-3 Appear to Have Distinct RNA-Binding Specificities

To explore the mechanism by which RNP-8 and GLD-3 exert opposite effects on gamete sex, we investigated their RNA-binding specificities, by using a homopolymer assay and purified recombinant RNP-8 and GLD-3 proteins. We found that RNP-8 was retained strongly with poly(G) and weakly with poly(U), poly(A), or poly(C) (Figure 2A), as noted above. However, GLD-3 was retained strongly with both poly(G) and poly(C), but it was barely retained with poly(U) or poly(A) (Figure 5H). Therefore, RNP-8 and GLD-3 appear to have distinct RNA-binding specificities in vitro, which suggests that they are likely to have distinct RNA-binding specificities in vivo.

DISCUSSION

GLD-2 Provides the Catalytic Subunit for Two Distinct Poly(A) Polymerases

Previous work identified the GLD-2/GLD-3 PAP (Wang et al., 2002). Here, we identify a second distinct GLD-2-dependent enzyme: the RNP-8 protein binds GLD-2 and stimulates its PAP activity in vitro; RNP-8 and GLD-2 coimmunoprecipitate from worm extracts; they colocalize in cytoplasmic germ granules; and they share a common biological role (see below). Discovery of GLD-2/RNP-8 shows that GLD-2 can interact with distinct RNA-binding proteins. Whereas GLD-3 is a Bicaudal-C homolog bearing five KH motifs (Eckmann et al., 2002), RNP-8 harbors a single RRM domain. Thus, the two proteins are likely to have distinct binding specificities. Indeed, using purified recombinant proteins, we show that RNP-8 and GLD-3 both bind RNA, but appear to do so with different specificities. Therefore, GLD-2/GLD-3 and GLD-2/RNP-8 form molecularly distinct enzymes.

RNP-8 Governs Oocyte Fate Specification

The biological role of RNP-8 was determined by using an *rnp-8* null mutant and several double null mutants. As a single mutant, *rnp-8* displays only a low-penetrance germline sexual transformation, but even this infrequent defect reveals that RNP-8 can be essential for oocyte fate specification, at least in some germlines. More compelling is the finding that an *rnp-8* mutation dramatically increases germline masculinization by mutations in any of three other genes. Most important is *gld-1*. As a single mutant, *gld-1* null mutants are not masculinized, but removal of both GLD-1 and RNP-8 fully transforms germlines from oogenic to spermatogenic. Therefore, GLD-1 and RNP-8 stand out as key regulators of oocyte specification.

A likely hypothesis is that GLD-1 together with the GLD-2/RNP-8 PAP promotes the oocyte fate, although we are unable to test this idea genetically because *gld-1 gld-2* germlines are tumorous and fail to make gametes (Hansen et al., 2004; Kadyk and Kimble, 1998). The proposed redundancy of GLD-1 and GLD-2/RNP-8 parallels the known redundancy of GLD-1 and GLD-2/GLD-3 for control of meiotic entry (Eckmann et al., 2004; Kadyk and Kimble, 1998). An attractive idea, albeit speculative, is that the controls of meiotic entry and oocyte fate specification rely on similar regulatory circuits. The mechanism by which GLD-1 and GLD-2 specify oocytes likely relies on GLD-1 translational repression and GLD-2 translational activation (Jan et al., 1999; Lee and Schedl, 2001; Suh et al., 2006). An important challenge for the future is to identify the target mRNAs of both GLD-1 and GLD-2/RNP-8 that are critical for the oocyte fate.

RNP-8 and GLD-3 Antagonism

The GLD-2 partners, RNP-8 and GLD-3, have opposite effects on gamete identity: RNP-8 promotes the oocyte fate (this work), whereas GLD-3 promotes the sperm fate (Eckmann et al., 2002). Previous studies suggested that GLD-3 directs spermatogenesis by inhibiting FBF (Eckmann et al., 2002). Here, we propose that GLD-3 also promotes the sperm fate by an additional mechanism, competition with RNP-8 for GLD-2 activity. In support of this idea, *rnp-8* and *gld-3* mutations suppress each other's gamete identity defects, and RNP-8 and GLD-3 proteins compete with each other for GLD-2 binding. Importantly, GLD-2/RNP-8 and GLD-2/GLD-3 exist as separate complexes that form selectively during development. We do not yet understand how GLD-2 associates with the correct partner during development. One possibility is that the ratio of RNP-8 and GLD-3 abundance controls the ratio of GLD-2/RNP-8 and GLD-2/GLD-3 complexes. Alternatively, posttranslational

modifications of RNP-8, GLD-3, or GLD-2 might control their association. Regardless, RNP-8 and GLD-3 are antagonists by both genetic and molecular criteria.

Models for GLD-2 Combinatorial Control

GLD-2 forms two discrete PAPs that have distinct functions. We envision two simple models to explain how these two discrete enzymes may control gamete sex. Both models rely on competition for GLD-2 binding, and both invoke combinatorial control (Figure 5I). One idea is that GLD-2/GLD-3 and GLD-2/RNP-8 activate sperm-specifying and oocyte-specifying mRNAs, respectively. By this scenario, GLD-2/GLD-3 would directly promote the sperm fate, in addition to its many other roles. Alternatively, GLD-2/GLD-3 might promote the sperm fate indirectly, by precluding formation of GLD-2/RNP-8. By this model, GLD-2/GLD-3 would drive gender-neutral events (e.g., meiotic entry, meiotic progression), whereas GLD-2/RNP-8 would be specialized for activating oocyte-specific mRNAs. Other models are, of course, possible. Regardless of the actual mechanism, we emphasize that GLD-2 and its partners are likely to control gamete sex in a combinatorial fashion.

Combinatorial control is a major mechanism of developmental regulation. Many examples exist for transcription factors (e.g., bHLH proteins) (Molkentin and Olson, 1996; Remenyi et al., 2004), and a few exist for RNA regulators (e.g., PUF proteins and CPEB) (Pique et al., 2008; Wickens et al., 2002). This work demonstrates that GLD-2-related enzymes can also act with distinct partners to achieve specific biological outcomes. Indeed, GLD-2 enzymes control development throughout the animal kingdom, and they also influence memory in *Drosophila* (Kwak et al., 2008) and perhaps in mice (Rouhana et al., 2005). Therefore, the discovery of GLD-2 partners with antagonistic effects in the nematode may be of broad-ranging significance.

EXPERIMENTAL PROCEDURES

Nematode Strains and Methods

Strains were maintained at 20°C, as described (Brenner, 1974). Wild-type was the N2 Bristol strain. Mutations and balancers were as follows: LGI: *rnp-8(tm2435* and *q784)*, *gld-1(q485)*, *gld-2(q497)*, *hT2[qIs48]*; LGII: *gld-3(q730)*, *nos-3(q650)*, *fbf-1(ok91)*, *fbf-2(q738)*, *mnIn1[mIs14 dpy-10(e128)]*; LGIII: *glp-1(q175* and *q224ts)*, *hT2[qIs48]*. For *rnp-8* RNAi, a double-stranded RNA corresponding to *rnp-8(242-982)* (numbering begins at AUG of the spliced transcript) was injected into wild-type adult hermaphrodite germlines at a concentration of 1 mg/ml. *fog-1* RNAi was carried out by feeding L4s bacteria expressing double-stranded RNAs and scoring F_1 progeny (Kamath and Ahringer, 2003).

Production of α-RNP-8 and α-GLD-2 Antibodies

To generate α-RNP-8 polyclonal antibodies (Cocalico Biologicals), rats and rabbits were injected with a Keyhole-limpet-hemocyanin-conjugated peptide (Genemed Synthesis) corresponding to amino acids 524–542 of RNP-8. Antibodies from antisera of each species were purified on an affinity column with SulfoLink Coupling Gel (Pierce) coupled to the unconjugated peptide as recommended by the manufacturer. α-GLD-2 rabbit polyclonal antibody was generated by Strategic Diagnostics Inc. (SDI) by using antigen corresponding to amino acids 171–270 of GLD-2 (ZC308.1a), and α-GLD-2 rat antiserum was generated by Cocalico Biologicals by injecting purified GST-GLD-2C (aa 482–1113) proteins into rats.

Coimmunoprecipitation

For RNP-8 coimmunoprecipitation (co-IP), wild-type and *rnp-8(q784)* hermaphrodites were grown on standard NGM agar plates, collected as adults (Figure 1B) or mixed stage (Figure 5F), and washed multiple times in M9 buffer. For each strain, the equivalent of 1 ml packed animals was resuspended in lysis buffer (50 mM HEPES [pH 7.5], 10 mM KCl, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, EDTA-free protease inhibitor cocktail [Roche]). Worms were frozen immediately in liquid nitrogen and pulverized with a cooled mortar and pestle. Frozen extracts were thawed on ice, homogenized in a glass homogenizer (Pyrex), and cleared by centrifugation at 10,000 × g for 10 min at 4°C. For each immunoprecipitation, 1–3 mg of the precleared extract was incubated overnight at 4°C with 5 μg purified rabbit α-RNP-8 that was coupled to protein A beads (Pierce). The beads were subsequently washed six times with wash buffer (50 mM Tris-HCl [pH 7.5], 10 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, EDTA-free protease inhibitor cocktail).

GLD-2 co-IP followed essentially the same protocol as described above, except that 20 μg purified rabbit α-GLD-2 was incubated with worm extract, coupled to protein A beads, and washed with wash buffer containing 100 mM NaCl. Bound proteins were analyzed by western blots. Rat α-GLD-2 antiserum (1:500 dilution), rabbit α-GLD-2 (1:2000 dilution), rabbit α-RNP-8 (1:1000 dilution), rabbit α-GLD-3L antiserum (1:500 dilution) (Eckmann et al., 2002), and α-actin C4 (1:40,000 dilution; MP Biomedicals) were used as primary antibodies.

GST Pull-Downs

GLD-2C (482–1113) and RNP-8 (1–583) were cloned into pGEX-6P-1 and a modified pGEX-4T-1 (C-terminal His_6 tag) (Amersham), respectively. Protein purification was performed essentially as described (Bernstein et al., 2005). Purified GST, GST-GLD-2C, and GST-RNP-8 were immobilized on glutathione agarose beads (GE Healthcare), and ^{35}S-radiolabeled GLD-2C and RNP-8 were translated by using a rabbit reticulocyte translation system (TnT Quick Coupled; Promega). For pull-down assays, GST fusion proteins and labeled proteins were incubated in 1× binding buffer (40 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM $MgCl_2$, 10 mM DTT, 0.1% NP-40, 10% glycerol, 50 μg/ml BSA, EDTA-free protease inhibitor cocktail) for 2 hr at 4°C. Beads were washed twice with 1× binding buffer and twice with 1× high-salt (0.3 M NaCl) PBS and resuspended in SDS loading buffer. Eluted proteins were analyzed by SDS-PAGE and exposed to a Phosphor Imager (Molecular Dynamics).

For the modified binding assays, the GST moiety was first cleaved from GST-GLD-2C by PreScission protease (GE Healthcare) prior to its incubation with purified GST-RNP-8 and GLD-3 G2BR peptide (either wt or mut; Genemed Synthesis) in 1× PAP buffer (25 mM Tris-Cl [pH 8], 40 mM KCl, 1 mM $MgCl_2$, 0.05 mM EDTA, 0.5 mM DTT, 0.1% NP-40, 10% glycerol, 100 μg/ml BSA) for 1.5 hr at 4°C, which was immediately followed by a 1 hr incubation with glutathione agarose beads. Beads were washed three times with 1× high-salt (0.3 M NaCl) PBS. Proteins were analyzed by western blots.

In Vitro RNA Binding and Polyadenylation Assays

A purified recombinant protein (either 100 nM GST-RNP-8 or 300 nM GLD-2) was mixed with 200 fMole ^{32}P-labeled RNA oligo (either $C_{35}A_{10}$ or $(GUU)_{10}A_{10}$) incubated in 1× PAP buffer supplemented with 1 μg yeast tRNA (Ambion) at 25°C for 30 min, and the product was analyzed on a 5% native polyacrylamide gel and exposed to a Phosphor Imager.

To assess polyadenylation, purified recombinant proteins, ^{32}P-labeled RNA oligo, and 0.5 mM ATP were mixed in 1× PAP buffer and set on ice; the reaction was started by transfer to 25°C for a set time and was stopped by formamide addition. RNAs were analyzed on a 10% polyacrylamide gel and exposed to a Phosphor Imager.

Northern Blots and In Situ Hybridization

Northern blots were performed with reagents and conditions as specified in the NorthernMax-Gly kit (Ambion). A total of 0.6 μg poly(A)+ RNA was loaded on each lane and separated in a 1% LE-agarose gel. RNAs were transferred to an Ambion BrightStar-Plus membrane and probed with RNA probes made by using the Strip-EZ RNA Kit (Ambion). 5′ probe: 136–489 bp; middle probe: 618–982 bp; 3′ probe: 1443–1744 bp of *rnp-8*, where numbering begins at the AUG in the spliced transcript.

In situ hybridization on dissected gonads was performed essentially as described (Jones et al., 1996), by using a sense or an antisense single-stranded digoxigenin-labeled *rnp-8(1443-1744)* 3′ probe. Probes were applied to

gonads and hybridized at 48°C for 48 hr. After hybridization and washing, the gonads were incubated with alkaline-phosphatase-conjugated α-digoxigenin (Fab2 fragment) (Roche) at 4°C overnight. Gonads were stained with BCIP/NBT substrate (Sigma), mounted, and viewed with a Zeiss Axioplan 2 microscope.

Immunocytochemistry

Germlines were fixed and incubated with antibodies as described (Lee et al., 2007). Images were obtained either on a Zeiss LSM510 confocal microscope or on a Zeiss Axioplan 2 microscope. RNP-8 was detected with either rabbit α-RNP-8 (1:1000 dilution) or rat α-RNP-8 (1:50 dilution), and PGL-1 was detected with rabbit α-PGL-1 (1:100 dilution; gift from S. Strome). The sperm-specific SP56 (1:300 dilution; gift from S. Ward) and the oocyte-specific α-RME-2 (1:500 dilution; gift from B. Grant) antibodies were used as primary antibodies.

Modified Yeast Two-Hybrid Assay

Modified yeast two-hybrid assays were performed as described (Bernstein et al., 2002), with minor modification. Plasmids encoding chimeric proteins were cotransformed into strain L40–ura (Invitrogen). Reporter (lacZ) expression was assayed by using the Beta-Glo Assay system (Promega).

GLD-3 Protein Purification and RNA Homopolymer-Binding Assay

GLD-3 (1–960) was cloned into pET-21b(+) (Novagen), and cells were extracted by using B-PER Bacterial Protein Extraction Reagent (Pierce). Inclusion bodies were solubilized by using Inclusion Body Solubilization Reagent (Pierce). Protein purification was performed under denaturing conditions, as specified in the Ni-NTA purification system (Invitrogen). Purified protein was refolded by dialysis with a Slide-A-Lyzer Dialysis Cassette (Pierce) as described by the manufacturer.

The RNA homopolymer-binding assay was performed essentially as described (Nykamp et al., 2008). A purified recombinant protein (either GST-RNP-8 or T7•tag-GLD-3-His_6) was mixed and incubated for 30 min at 4°C in binding buffer (containing 100 mM NaCl) with 50 μg of the indicated homopolymer immobilized on Sepharose or agarose. Beads were washed three times, and eluted proteins were analyzed by western blots with rabbit α-RNP-8 (1:1000 dilution) or α-T7•tag monoclonal antibody (Novagen; 1:5000 dilution).

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