Independent recruitments of a translational regulator in the evolution of self-fertile nematodes

Alana V. Beadell[a,b,1], Qinwen Liu[a], Dorothy M. Johnson[a,2], and Eric S. Haag[a,b,3]

[a]Department of Biology, and [b]Program in Behavior, Evolution, Ecology, and Systematics, University of Maryland, College Park, MD 20742

Edited by Iva Greenwald, Columbia University, New York, NY, and approved October 28, 2011 (received for review May 20, 2011)

Pleiotropic developmental regulators have been repeatedly linked to the evolution of anatomical novelties. Known mechanisms include *cis*-regulatory DNA changes that alter regulator transcription patterns or modify target-gene linkages. Here, we examine the role of another form of regulation, translational control, in the repeated evolution of self-fertile hermaphroditism in *Caenorhabditis* nematodes. *Caenorhabditis elegans* hermaphrodites initiate spermatogenesis in an otherwise female body through translational repression of the gene *tra-2*. This repression is mediated by GLD-1, an RNA-binding protein also required for oocyte meiosis and differentiation. By contrast, we show that in the convergently hermaphroditic *Caenorhabditis briggsae*, GLD-1 acts to promote oogenesis. The opposite functions of *gld-1* in these species are not gene-intrinsic, but instead result from the unique contexts for its action that evolved in each. In *C. elegans*, GLD-1 became essential for promoting XX spermatogenesis via changes in the *tra-2* mRNA and evolution of the species-specific protein FOG-2. *C. briggsae* GLD-1 became an essential repressor of sperm-promoting genes, including *Cbr-puf-8*, and did not evolve a strong association with *tra-2*. Despite its variable roles in sex determination, the function of *gld-1* in female meiotic progression is ancient and conserved. This conserved role may explain why *gld-1* is repeatedly recruited to regulate hermaphroditism. We conclude that, as with transcription factors, spatially localized translational regulators play important roles in the evolution of anatomical novelties.

germ cells | translation | breeding systems | mutant

Many important adaptations involve localized modifications of development. Because the genes that regulate development often function in multiple times and places, mutations in *cis*-regulatory elements that locally alter their expression are expected to offer a simple route to tissue-specific changes in function (1, 2). This circumvention of pleiotropy by changes in gene regulation, recently dubbed the Stern-Carroll Rule (3), has been borne out in both animals and plants (e.g., refs. 4–10). In each case, transcriptional enhancers appear to have been the target of selection.

In *Caenorhabditis* nematodes, self-fertile hermaphrodites evolved independently from females in *Caenorhabditis elegans* and *Caenorhabditis briggsae* (11–14). Selfing is an important reproductive adaptation that profoundly affects the efficacy of natural selection (15), population genetic variation (16–18), and genome content (19). However, the limited XX spermatogenesis that underlies hermaphroditism represents a developmental novelty worthy of study in its own right. In particular, the prominence of posttranscriptional gene regulation in the germ line (20, 21) suggests self-fertility may evolve by mechanisms that are distinct from those described in the soma.

Here we compare the role of GLD-1, a regulator of translation (22), in *C. elegans* and *C. briggsae* germ-line sex determination. GLD-1 is an RNA-binding protein of the STAR (for signal transduction and activation of RNA metabolism) family. STAR proteins are implicated in diverse cellular processes, including cell division, gametogenesis, apoptosis, and embryonic and larval development, and are found across the Metazoa (e.g., refs. 23–27). *C. elegans* GLD-1 is a germ-line–specific, pleiotropic translational repressor (22, 28, 29) required for the mitosis/meiosis decision of germ-line stem cells, meiotic progression of oocyte-fated cells, and specification of *C. elegans* hermaphrodite sperm in an otherwise female body (30, 31).

In this study we show that *gld-1* has been recruited to regulate hermaphrodite development in *C. briggsae*. However, it acts to promote oogenesis, rather than spermatogenesis as in *C. elegans*. These alternative roles are the result of differences in the *cis*-regulatory RNA of a conserved sex-determination gene, *tra-2*, and in the downstream function of a conserved target, *puf-8*. Our results provide insights into how pleiotropic translational regulators, as with transcription factors, are redeployed during phenotypic evolution.

Results

Characterization of *Cbr-gld-1* Mutations. In a screen for recessive mutations that cause germ-line–specific sexual transformation in *C. briggsae* hermaphrodites, the alleles *nm41* and *nm64* manifested excess sperm and ectopic proliferation of germ cells (Fig. 1 and Fig. S1). The overproduction of sperm resembled the phenotype reported for RNA interference (RNAi) knockdown of *Cbr-gld-1* (14). Mutants differed, however, in that simultaneous feminization was not required for the frequent formation of tumors. Both *nm41* and *nm64* fail to complement, genetically map to *Cbr-gld-1*, and are associated with a premature stop codon in its ORF (Fig. 1*A*). Neither allele produced detectable GLD-1 protein (Fig. 1*C*). However, they differed in expression of the oocyte marker RME-2 (Fig. 1*C*) (32) and in the frequencies of their mutant phenotypes (Fig. 1*B*), and thus one or both alleles might retain residual function.

To confirm the *Cbr-gld-1* loss-of-function phenotype, we isolated the deletion mutant *nm68*, which eliminates conserved sequences important for RNA binding and homodimerization (Fig. 1*A*) (33). As with *nm41* and *nm64*, no GLD-1 protein is detectable in *nm68* homozygotes. We infer that *nm68* likely represents a null allele. Like *nm41*, *nm68* causes some ectopic germ-cell proliferation and a high frequency of excess spermatogenesis, with no evidence of oocyte-fated cells. XO *Cbr-gld-1* mutants are normal, as judged by differential interference microscopy (DIC) microscopy and mating assays, and Cbr-GLD-1 expression in males is low relative to hermaphrodites (Fig. S2). This finding indicates that, as in *C. elegans* (30), *Cbr-gld-1* has at most a nonessential role in the *C. briggsae* XO male germ line.

Author contributions: A.V.B., Q.L., and E.S.H. designed research; A.V.B., Q.L., D.M.J., and E.S.H. performed research; A.V.B. contributed new reagents/analytic tools; A.V.B., Q.L., D.M.J., and E.S.H. analyzed data; and A.V.B. and E.S.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]Present address: Department of Biochemistry, University of Wisconsin, Madison, WI 53706.

[2]Present address: Biochemistry, Cellular, and Molecular Biology Graduate Program, The Johns Hopkins School of Medicine, Baltimore, MD 21205.

[3]To whom correspondence should be addressed. E-mail: ehaag@umd.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1108068108/-/DCSupplemental.

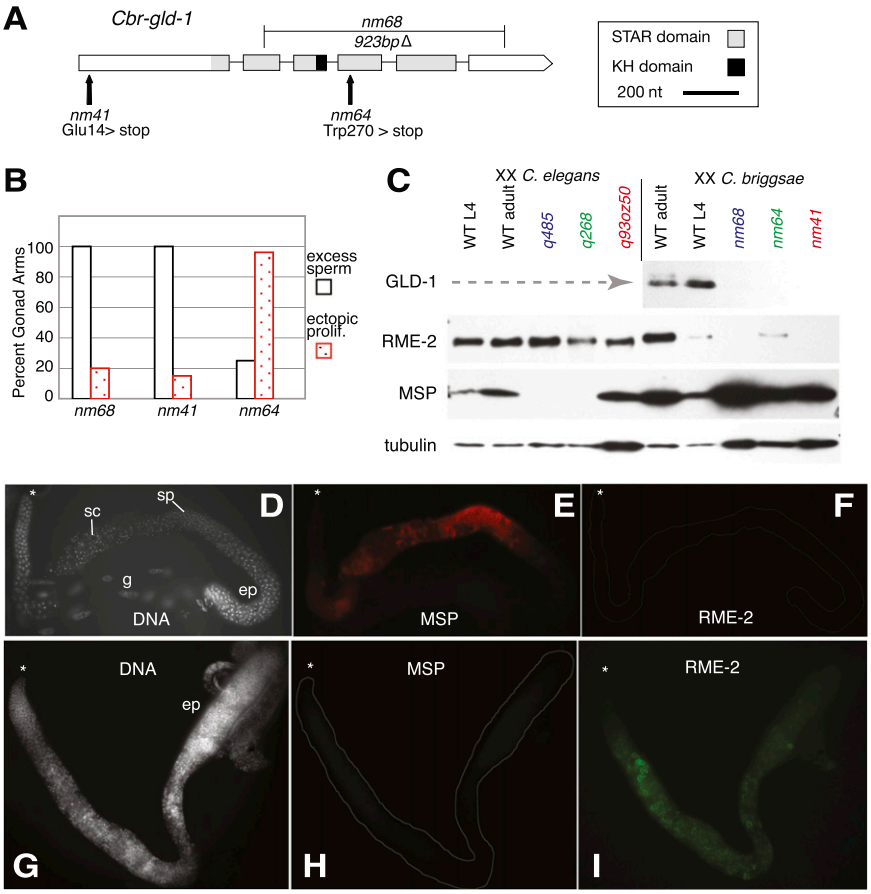

Fig. 1. *C. briggsae* and *C. elegans gld-1* mutations produce opposite sexual transformations of the hermaphrodite germ line. (*A*) Structure of *Cbr-gld-1*, with exon-intron boundaries, conserved coding domains, and mutant lesions indicated. (*B*) Phenotypic distributions of *Cbr-gld-1* alleles measured in XX animals on day 1 of adulthood by DIC microscopy of Hoechst-33258–stained gonads. n = 200 gonad arms examined for each genotype. *ectopic prolif.*, Mitotic proliferation of germ cells proximal to the distal stem-cell niche (Fig. S1). (*C*) Immunoblots of *C. elegans* and *C. briggsae* wild-type L4 and adult hermaphrodites and mutant adults of indicated genotype. *q485* is a null allele of *Ce-gld-1* (30); *q268* and *q93oz50* have premature stop lesions affecting the equivalent codons altered in *nm64* and *nm41*, respectively [but note that *q93oz50* also harbors a downstream mutation (30)]. RME-2 and MSP antibodies mark oocytes and spermatocytes, respectively; tubulin is a loading control. (*D–F*) *C. briggsae gld-1(nm68)* XX extruded gonad, stained with Hoechst-33258 (*D*), anti-MSP (*E*), and anti–RME-2 (F). (*G–I*) *C. elegans gld-1(q485)* XX extruded gonad, stained as in *D* to *F*. spermatocytes (sc), sperm (sp), ectopic proliferation (ep) Experimental manipulations in the two species were performed simultaneously and identically; gonads are representative of each. (Magnification, 140×.)

Chromosome staining and immunohistochemistry with an anti-phosphohistone H3 antibody (a mitotic marker) in XX *Cbr-gld-1* mutant germ lines indicates that, as for *C. elegans gld-1* (30), ectopic germ-cell proliferation results from a failure of germ cells to complete the meiotic program (Fig. 1 *B* and *D–F*, and Fig. S1). Unlike *C. elegans* mutants, however, isogenic *Cbr-gld-1* mutant gonads vary substantially in the extent and location of ectopic proliferation and gametogenesis along the proximal-distal axis (Fig. 1*D* and Fig. S1). *Cbr-gld-1* germ cells that ectopically proliferate fail to express detectable amounts of the sperm marker major sperm protein (MSP) or RME-2 (Fig. 1 *D–F*), consistent with an un- or de-differentiated cell state. Because XX *nm68* mutants develop tumors, but *Cbr-gld-1(RNAi)*, with moderate concentrations of dsRNA, can fully masculinize without tumors (14) (Table S1), a low level of *Cbr-gld-1* activity may be required for reliable XX sperm development. Other interpretations are possible, however, including a cryptic female or intersexual origin for *Cbr-gld-1* mutant germ-line tumors.

To further investigate *Cbr-gld-1* germ-line tumor formation, we examined interactions with other sex-determination genes. Mutations in *Cbr-tra-2* and *Cbr-tra-1* that masculinize the XX germ line and soma (34) suppress *Cbr-gld-1(nm68)* tumors, and the germ line remains masculinized (Table S1). When only the hermaphrodite germ line is feminized with *Cbr-fog-3(RNAi)* (35), *Cbr-gld-1(RNAi)* produces germ-line tumors (14) (Table S1). Furthermore, *Cbr-gld-1(RNAi) Cbr- fog-3(RNAi)* double-knockdown in *C. briggsae* wild-type XO males also results in completely penetrant tumor formation in a male somatic gonad (Table S1). Thus, *Cbr-gld-1(lf)*-mediated tumors form in oocyte-fated germ lines, regardless of somatic sex or karyotype, as in *C. elegans* (31). *Cbr-tra-1* mutants also produce robust oocytes as they age (34, 36). *Cbr-gld-1; Cbr-tra-1* mutants produce neither tumors nor differentiated oocytes, but do often produce sperm normally (Table S1).

Phylogenetic Survey of *gld-1* Function. Orthologs of *gld-1* exist across *Caenorhabditis* (Fig. S3), and their highly XX-biased expression is conserved (Fig. S2) (29). We knocked down *gld-1* in females of *Caenorhabditis japonica*, *Caenorhabditis brenneri*, *Caenorhabditis remanei*, and *C. briggsae*/*C.* sp. 9 F1 hybrids (37), and in *C. briggsae* hermaphrodites (Fig. 2). This process eliminated differentiated oocytes in all cases, but only caused germline masculinization in *C. briggsae*. In gonochoristic *gld-1(RNAi)*, the female germ line largely fills with ectopically proliferating germ cells that fail to express detectable amounts of RME-2 or MSP (Fig. 2), but males suffer no observable defects and are fertile. We conclude that the XX female ancestors of *C. briggsae* relied on *gld-1* for oocyte meiosis and differentiation, but not for repression of the sperm fate. Thus, *Cbr-gld-1* was recruited into germ-line sex determination during (or possibly subsequent to) the evolution of self-fertility.

Context-Dependent Role of *gld-1* in Hermaphrodite Sex Determination. *gld-1* is pleiotropic (31), has hundreds of target mRNAs (38), and is itself both positively and negatively regulated at the mRNA (39–41) and protein (42, 43) levels. The opposite sex-determination phenotypes for *gld-1* in *C. briggsae* and *C. elegans* could be intrinsic to *gld-1* itself. Alternatively, the strong conservation of GLD-1 sequence (Fig. S4) and expression pattern (14) suggested that factors with which GLD-1 interacts may be responsible. To test these alternatives, we introduced a *Cbr-gld-1* transgene into *C. elegans gld-1(q485)*-null mutants. Two transgenic lines expressing *Cbr*-GLD-1 (Fig. S5) restored both robust XX spermatogenesis and normal oogenesis to *gld-1*

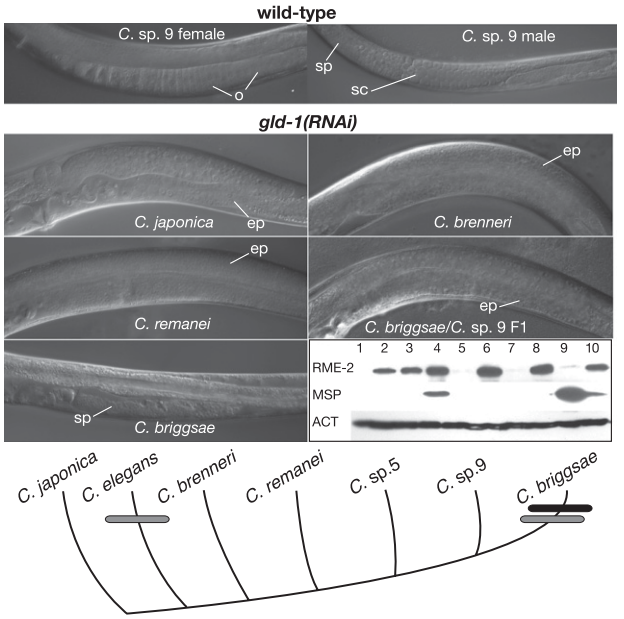

Fig. 2. *Cbr-gld-1* sperm repression is of recent origin, but its role in meiotic progression is ancient. (*Top*) Gonads of wild-type *C.* sp. 9 (representative of gonochoristic species). (*Left*) Unmated XX female, showing oocytes (o) of one ovarian arm. (*Right*) XO male with spermatids (sp) and primary spermatocytes (sc) in the single-armed testis. (*Middle*) *gld-1(RNAi)* phenotypes in gonochoristic *Caenorhabditis*. *ep*, ectopic proliferation; *sp*, sperm. DIC micrographs of gonads of adult XX progeny of injected mothers are shown (absence of sperm in gonochoristic species was confirmed by staining with Hoechst-33258 dye). (*Lower Right*) Immunoblots of proteins from untreated (lanes 2, 4, 6, 8, 10) or *gld-1* loss-of-function (lanes 1, 3, 5, 7, 9) XX adults of *C. japonica* (lanes 1 and 2), *C. elegans* (lanes 3 and 4), *C. remanei* (lanes 5 and 6), *C. briggsae*-*C.* sp. 9 F1 hybrids (lanes 7 and 8), and *C. briggsae* (lanes 9 and 10). Loss-of-function for *C. elegans* was via *gld-1(q485),* for *C. briggsae* was via *Cbr-gld-1(nm68)*, and via species-specific *gld-1(RNAi)* treatments for all others. For *C. briggsae*-*C.* sp. 9 hybrids, the *C. briggsae* sequence was used. (*Bottom*) Phylogenetic interpretation (tree compiled from refs. 37 and 62), with inferred origins of self-fertility (gray) and of *Cbr-gld-1*'s role as a sperm repressor (black) indicated.

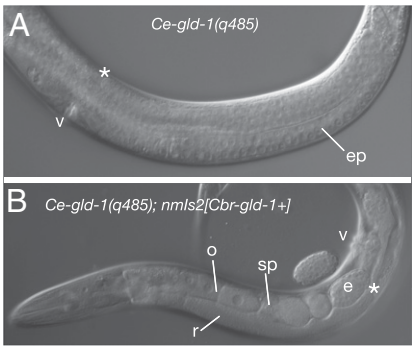

Fig. 3. *Cbr-gld-1* can fully substitute for *C. elegans gld-1*. (*A*) Germ-line phenotype of adult XX *C. elegans gld-1(q485)* mutants showing extensive ectopic proliferation of oocyte-fated cells that have exited meiosis and reentered mitosis. *ep*, Ectopic proliferation. The distal tip of the anterior gonad is marked with an asterisk. *v*, vulva. (*B*) *Ce-gld-1(q485)* homozygote bearing an HA epitope-tagged wild-type *Cbr-gld-1* transgene with a normal rachis (r), oocytes (o), sperm (sp), and abundant selfed embryos (e). The distal tip of the anterior gonad is marked with an asterisk. *v*, vulva. Micrograph is representative of the animals with this genotype. (Magnification, 100×.)

(q485) homozygotes (Fig. 3). Thus, the opposite roles of *gld-1* in *C. elegans* and *C. briggsae* germ-line sex determination are because of species-specific context.

***Cbr-puf-8* Is an Oogenesis-Promoting Target of Cbr-GLD-1.** As GLD-1 represses mRNA translation (22, 25), we asked whether the *Cbr-gld-1* excess sperm phenotype could be explained by an association between Cbr-GLD-1 and sperm-promoting mRNAs. To address this question, GLD-1-associated mRNA was immunoprecipitated from wild-type worms (Fig. S6*A*). An initial survey using quantitative RT-PCR (qRT-PCR) failed to implicate any known *C. briggsae* sperm-promoting mRNAs (Fig. S6*B*). To identify new sperm-promoting targets, we queried a whole-genome microarray with Cbr-GLD-1–associated mRNA and performed RNAi knockdown of 125 putative target genes. Only knockdown of the Puf family RNA-binding protein gene, *Cbr-puf-8*, produced the expected feminized mutant phenotype (Table 1), but the effect was weak.

C. elegans puf-8 also acts in germ-line sex determination, doing so redundantly with another Puf gene, *fbf-1* (44) to inhibit the sperm fate. Although opposite in phenotype, we surmised that, as in *C. elegans*, other Puf family members might act redundantly with *Cbr-puf-8* to regulate germ-line sex. Knockdown of *Cbr-puf-8* in combination with each of the other Puf genes positively enriched in the microarray analysis (CBG09894, CBG13175, and CBG01774) produced no sex-determination disruptions, nor did selected double and triple knockdowns. We next examined three other *C. briggsae* Puf genes related to *fbf-1/2* (45), *Cbr-puf-1.1* (CBG02701), *Cbr-puf-1.2* (CBG13460), and *Cbr-puf-2* (CBG02702). Knockdown of *Cbr-puf-1.2* is weakly feminized on its own, but strongly enhanced the feminization of *Cbr-puf-8(RNAi)* (Fig. 4*A* and Table 1). This feminization is not observed in XO male siblings (Table 1).

Consistent with *Cbr-puf-8*'s role as a major sex-determining target of Cbr-GLD-1, we find that *Cbr-puf-8(RNAi); Cbr-puf-1.2 (RNAi)* fully suppresses the sperm production of *Cbr-gld-1 (RNAi)* (Table 1). This triple knockdown also produces surprisingly normal oocytes in a minority of animals, although most had no overtly differentiated gametes. We also observed a reduced germ-line phenotype with *Cbr-puf-8(RNAi)*, especially in combination with *Cbr-gld-1(RNAi)* (Table 1), which is also seen in a minority of *C. elegans puf-8* mutants (46). In addition to suppressing *Cbr-gld-1(lf)* germ-line masculinization, the triple knockdown of *Cbr-gld-1; Cbr-puf-8; puf-1.2* partially rescues this reduction in germ-cell number. *Cbr-puf-8* and *Cbr-puf-1.2* may therefore have antagonistic roles in germ-cell proliferation, similar to *C. elegans fbf-1* and *fbf-2* (40, 45).

A likely GLD-1 binding site (33, 38) is present at nucleotides 25–31 3′ of the *Cbr-puf-8* stop codon. The purified STAR domain of Cbr-GLD-1 and a synthetic *puf-8* 3′ UTR fragment containing this region interact in vitro without other factors (Fig. 4*B*). Taken together, the genetic and molecular evidence are consistent with the *Cbr-puf-8* mRNA being a direct target of Cbr-GLD-1, and suggest that de-repression of *Cbr-puf-8* is a major contributor to the germ-line masculinization of *Cbr-gld-1* mutants.

The 3′UTR of *C. elegans puf-8* also contains a potential GLD-1 binding site (33), and Ce-GLD-1 and *Ce-puf-8* mRNA interact in vivo (Fig. 4*C*). Because *Ce-puf-8* promotes the oocyte fate with *fbf-1* (44), this finding raised the possibility that *Ce-puf-8* hyperactivity contributes to the feminization of *C. elegans gld-1* mutants. However, in an epistasis test, XX *puf-8 fbf-1; gld-1* triple homozygotes fail to produce differentiated gametes, and germ cells resemble those of *gld-1* single mutants (Fig. S7).

Differential *tra-2*–GLD-1 Association in *C. elegans* and *C. briggsae*. Germ-line hyperactivity of *tra-2* is a cause of germ-line feminization in XX *Ce-gld-1* mutants (22, 28). *C. briggsae tra-2* plays a conserved female-promoting role in both the soma and germ line (34, 47). *Cbr-tra-2* mRNA can also be repressed via its 3′ UTR in the soma (48), which in *C. elegans* is mediated by SUP-26 (49), and potentially the GLD-1 paralog ASD-2 (50) (Fig. S3).

Table 1. Genetic interactions between *Cbr-puf-8* and other Puf family genes

Targets of RNAi	Germ-line phenotype*,†			
	Sperm only	Sperm + oocytes	Oocytes only	Reduced germ line, no gametes
Cbr-gld-1‡	240	0	0	0
Cbr-puf-8	0	172	9	4
Cbr-puf-1.2	0	294	6	0
Cbr-gld-1 + *Cbr-puf-8*	0	0	4	122
Cbr-puf-8 + *Cbr-puf-1.1*	0	77	0	0
Cbr-puf-8 + *Cbr-puf-1.2*	0	8	144	0
Cbr-puf-8 + *Cbr-puf-1.2* (XO progeny)	22	0	0	0
Cbr-puf-8 + *Cbr-puf-2*	0	128	6	1
Cbr-gld-1 + *Cbr-puf-8* + *Cbr-puf-1.2*	0	3	43	109

*All phenotypes were scored by DIC microscopy in the progeny of injected wild-type hermaphrodites within the first two days of adulthood. All progeny XX unless otherwise noted.

†Only sex determination or germ cell proliferation phenotypes are given. Other phenotypes include aberrant or delayed gametogenesis, (largely proximal) ectopic germ-cell proliferation, and degenerate proximal-most oocytes.

‡*Cbr-gld-1(RNAi)* was used here because it rarely induces ectopic proliferation and because there is no convenient phenotypic marker for *Cbr-gld-1*.

However, it is unclear whether *Cbr-tra-2* is regulated by *Cbr*-GLD-1 in the germ line. We therefore asked whether reduced association of *tra-2* mRNA and GLD-1 in *C. briggsae* vs. *C. elegans* might contribute to their different *gld-1* phenotypes. To address this question, we immunoprecipitated GLD-associated mRNA from both species, and compared the extent of *tra-2* mRNA enrichment. Although positive and negative controls were enriched to a similar extent in both species, only in *C. elegans* is there strong association between GLD-1 and *tra-2* mRNA (Fig. 5*A*). Stronger association between GLD-1 and *tra-2* mRNA in *C. elegans* may equate with stronger germ-line *tra-2* repression in that species compared with *C. briggsae*.

To explain this difference in GLD-1–*tra-2* interaction, we compared the *tra-2* 3′UTR sequences of *C. elegans* and *C. briggsae.* GLD-1 binds a short motif in its mRNA targets, the STAR protein-binding element (SBE) (33) or GLD-1-binding motif (GBM) (38). In *Ce-tra-2*, GLD-1 binding sites are found within larger direct repeat elements (DREs) (28), containing three SBEs/GBMs, plus a fourth candidate site more 5′ in the 3′ UTR (Fig. 5*B*). In contrast, the *Cbr-tra-2* 3′ UTR lacks DREs and possesses only a single GBM variant. As GLD-1 association with mRNA is determined by the strength and number of GBMs within UTRs (38), this suggests the differential association of *tra-2* and GLD-1 in *C. elegans* and *C. briggsae* is because of differences in *cis*-regulatory RNA sequences in the *tra-2* 3′ UTR.

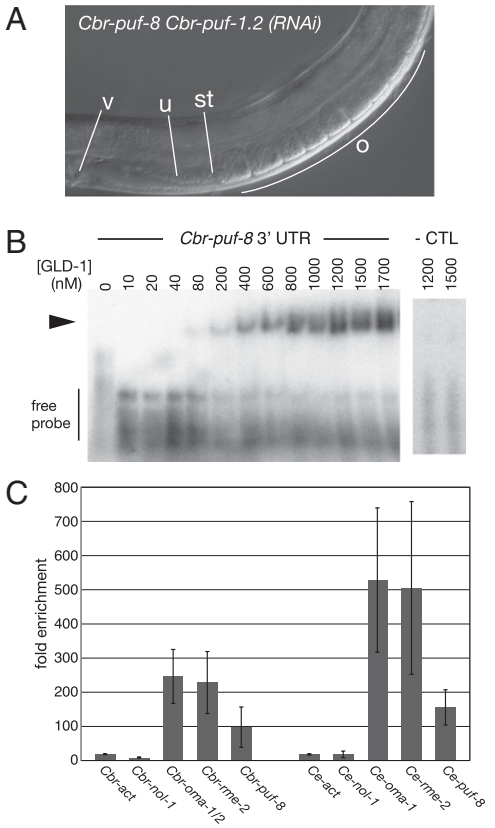

Fig. 4. *Cbr-puf-8* is a sperm-promoting target of *Cbr*-GLD-1. (*A*) *Cbr-puf-8 (RNAi);Cbr-puf-1.2(RNAi)* feminizes the hermaphrodite germ line. Of the animals that produce gametes, XX adults have only oocytes (o) and have an empty spermatheca (st) and uterus (u). v, vulva. (*B*) GLD-1 binds *Cbr-puf-8* 3′ UTR directly in vitro. Wedge, Cbr-GLD-1–dependent complex in gel-shift assay. –CTL RNA is derived from the *Cbr-tra-2* 5′ UTR. (*C*) Conserved association of *puf-8* with GLD-1 in *C. briggsae* and *C. elegans*. qRT-PCR enrichments for GLD-1 immunoprecipitated (IP) vs. mock IP RNA preparations are given for negative control (pan-*actin* and *nol-1*), positive control (*oma-1/2* and *rme-2*), and *puf-8*. Each is expressed as the average of at least three biological replicates (± SEM). (Magnification, 140×.)

Discussion

The above data demonstrate that GLD-1 had an ancestral function in the regulation of female meiotic progression, and that it has been independently recruited to promote or limit hermaphrodite spermatogenesis in *C. elegans* and *C. briggsae*, respectively. We provide evidence that these alternative roles are because of both *cis*-regulatory changes in a key GLD-1 target mRNA (*tra-2*) and downstream changes that alter the output of a conserved interaction (GLD-1/*puf-8*). A model summarizing our interpretation of these results is presented in Fig. 5*C*.

Evolution of *cis*-Regulatory Elements in the tra-2 mRNA. The *C. elegans tra-2* DRE is not found in other sequenced *Caenorhabditis* genomes. Multimerized GLD-1 binding sites in the context of species-specific perfect repeats strongly indicates a recent evolutionary event. Further supporting the functional significance of increased SBE/GBM number, *C. elegans tra-2* mutants possessing only one DRE exhibit dominant, hermaphrodite-specific germ-line feminization (28, 51). GLD-1 binds RNA as a dimer, but each protomer can potentially bind separate sequence elements (33). Thus, perhaps four GLD-1 dimers could be recruited to the *C. elegans tra-2* 3′ UTR, but the single SBE of *Cbr-tra-2* suggests a maximum of one. Different stoichi-

EVOLUTION

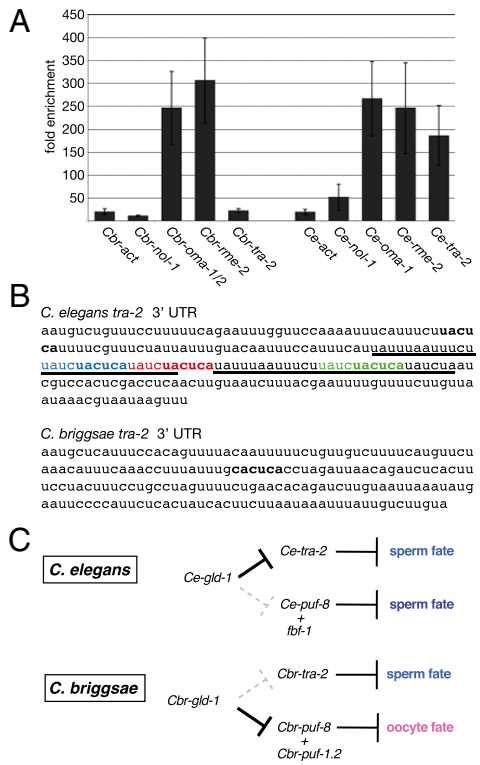

Fig. 5. *tra-2* mRNA associates with GLD-1 in *C. elegans* but not *C. briggsae*. (*A*) Comparison of *tra-2* association with GLD-1 in *C. briggsae* and *C. elegans*. qRT-PCR enrichments for GLD-1 IP vs. mock IP RNA preparations are given for negative controls, positive controls, and *tra-2*; each is expressed as the average of at least three biological replicates (± SEM). (*B*) Recent multimerization of the GLD-1–binding site in the *C. elegans tra-2* 3′ UTR. The experimentally determined *tra-2* 3′ UTR sequences for *C. elegans* (12) and *C. briggsae* (13) are shown. For *C. elegans tra-2*, the boldface hexanucleotides indicate exact matches to the SBE "conservative consensus" (UACU[C/A]A) that binds GLD-1 (14); for *C. briggsae tra-2* they indicate the single variant motif (CACUCA). The 28-nt direct repeat element (DRE) in the *Ce-tra-2* UTR is underlined, and the three perfect repeats of the SBE-bearing 10-mer are shown in blue, red, and green type. (*C*) Model for different contexts of GLD-1 sex-determination function in *C. elegans* and *C. briggsae*. Heavy solid bars indicate strong or genetically significant repression; dashed gray lines signify weaker or genetically insignificant repression.

ometry of the GLD-1/*tra-2* mRNA complex may explain some or all of the differential affinity and different *gld-1* mutant phenotypes we find in vivo.

Another *C. elegans*-specific factor that may contribute to the *Ce*-GLD-1/*tra-2* association is FOG-2, an F-box protein that binds GLD-1 and is specifically required for XX spermatogenesis (14). The exact role of FOG-2 in *C. elegans tra-2* regulation is not known, although it is not required for GLD-1 binding of *tra-2* mRNA in vitro (42). Interestingly, *C. briggsae* hermaphrodite spermatogenesis also requires its own species-specific F-box protein, SHE-1 (48), at elevated temperatures, but SHE-1 does not physically associate with Cbr-GLD-1.

Alternative Roles for *puf-8* Orthologs in Hermaphrodite Sex Determination. *C. briggsae* GLD-1's novel role in sperm repression is likely achieved, at least in part, through direct translational repression of *Cbr-puf-8*. Cbr-PUF-8 is itself a translational regulator, and its *C. elegans* ortholog is also involved in sex determination. However, as with GLD-1, its sexual roles differ, as it promotes oocyte production (with *fbf-1*) in *C. elegans* (44) but sperm production (with *Cbr-puf-1.2*) in *C. briggsae*. Because *Cbr-puf-8* (alone or with *Cbr-puf-1.2*) is not required for male spermatogenesis (Table 1), and the female ancestors of *C. briggsae* did not produce sperm (by definition), its role in *C. briggsae* germline sex determination likely arose during the evolution of selfing in that lineage.

C. elegans GLD-1 and *puf-8* mRNA also associate in vivo (Fig. 4*C*). However, *puf-8 fbf-1* fails to restore spermatogenesis to mutants lacking GLD-1 (Fig. S7) or its cofactor, FOG-2 (44). This finding suggests that the Ce-GLD-1–*puf-8* interaction is a minor factor regulating germ-line sex compared with Ce-GLD-1–*tra-2*, but pleiotropy of all three genes complicates interpretation. The roles of *gld-1* and *puf-8* in *C. elegans* germ-line stem-cell proliferation (52, 53) also suggest their interaction might be conserved primarily for this. The mRNA targets of PUF-8 and its ancestral functions in *Caenorhabditis* are unknown. Thus, *puf-8* represents an important subject for future comparative studies.

Translational Control and the Evolution of Development. Recent studies have stressed the importance of *cis*-regulatory DNA and transcription factors in the evolution of novel phenotypes (1, 54, 55). This work highlights the role of another level of regulation, translational control, in the convergent evolution of a significant adaptation, self-fertile hermaphroditism. Analogous to transcriptional evolution, evolutionarily dynamic *cis*-acting sequences in mRNAs (e.g., *tra-2*) can interact with conserved, pleiotropic translational control factors (e.g., GLD-1). In addition, the activity of an RNA-binding protein may be modulated by interactions with protein cofactors (e.g., FOG-2), which may have more recent evolutionary origins and narrower functions. As a result, target mRNAs may gain or lose regulation by a given RNA binding protein, or may have the functional significance of existing regulation (e.g., Cbr-GLD-1–*puf-8*) changed. Tissues where precise control of mRNA translation or localization is especially important to development or physiology would be expected to make particular use of this mechanism for adaptation. This study has highlighted the nematode germ line as one such tissue, but early embryos (56–58) and nervous systems (59) may show similar dynamics.

Materials and Methods

Cbr-gld-1 alleles *nm41* and *nm64* were recognized by sterile F_2 progeny of ethyl methanesulfonate -mutagenized AF16 animals, as previously described (27). *Cbr-gld-1(nm68)* was identified in PCR-based deletion screens (12). Microscopy and immunohistochemistry were performed according to standard methods (*SI Materials and Methods*). Immunoblots used a custom chicken anti–GLD-1 antibody (see below and *SI Materials and Methods*) or an anti–GLD-1 polyclonal antibody, generously provided by T. Schedl (Washington University, St. Louis, MO). Anti-MSP and anti–RME-2 antibodies were gifts of D. Greenstein (University of Minnesota, Minneapolis, MN) and B. Grant (Rutgers University, Piscataway, NJ), respectively. RNA interference was assessed by scoring progeny of mothers microinjected with dsRNA at concentrations of ~2–3 μg/μL (*SI Materials and Methods*). *C. elegans gld-1* mutant strains (see *SI Materials and Methods* for details) were obtained from the Caenorhabditis Genetics Center, T. Schedl, and J. Kimble (University of Wisconsin, Madison, WI). Rescue of *q485* homozygotes by *Cbr-gld-1* was mediated by plasmid pAD-g6, a Gateway-based (Invitrogen) derivative of pCR50 [gift of C. Richie and A. Golden (National Institutes of Health, Bethesda, MD)], (*SI Materials and Methods*). Biolistic transformation of *C. elegans* was performed by standard methods (60) (see *SI Materials and Methods*).

RNA immunoprecipitations (RIP) used an affinity-purified polyclonal chicken antibody developed against a peptide from near the N terminus of *Cbr*-GLD-1 (Open Biosystems) and goat anti-IgY agarose beads (Aves Labs) using a modified version of a published protocol (61) (*SI Materials and Methods*). For microarray analysis, five replicates of mock (IgY) vs. anti–GLD-1 and three of total mRNA vs. anti–GLD-1 RIPs were performed. cDNA was hybridized to Agilent 44 K dual-color arrays containing at least two probes for all predicted *C. briggsae* protein-coding genes. Data analysis is described in *SI Materials and Methods*. qRT-PCR assays on RIP RNA were performed with gene-specific primer pairs (*SI Materials and Methods*). Gel-shift mobility assays were performed using the purified STAR domain of Cbr-

GLD-1 and in vitro transcribed, 5′ radiolabeled *Cbr*-puf-8 3′UTR (*SI Materials and Methods*).

ACKNOWLEDGMENTS. We thank D. Greenstein, B. Grant, T. Schedl, J. Kimble, and J. Mann for antibodies; C. Richie and A. Golden for pCR50; T. Schedl, J. Kimble, and the Caenorhabditis Genetics Center for worm strains; I. Hamza, G. Seydoux, J. Williamson, S. Kwan, S. Sukarev, and I. Yanai for technical assistance; and T. Schedl, G. Seydoux, D. Rasoloson, V. Reinke, R. Ellis, and J. Kimble for helpful discussions. This work was supported by National Institutes of Health Award 5R01GM079414 (to E.S.H.), a University of Maryland W. T. and M. T. Hockmeyer Fellowship (to A.V.B.), and a Howard-Hughes Medical Institute Undergraduate Research Fellowship (to D.M.J.).

1. Carroll SB (2008) Evo-devo and an expanding evolutionary synthesis: A genetic theory of morphological evolution. *Cell* 134:25–36.
2. Stern DL (2000) Evolutionary developmental biology and the problem of variation. *Evolution* 54:1079–1091.
3. Haag E, Lenski R (2011) L'enfant terrible at 30: The maturation of evolutionary developmental biology. *Development* 138:2633–2637.
4. Chan YF, et al. (2010) Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a Pitx1 enhancer. *Science* 327:302–305.
5. Sucena E, Delon I, Jones I, Payre F, Stern DL (2003) Regulatory evolution of shavenbaby/ovo underlies multiple cases of morphological parallelism. *Nature* 424: 935–938.
6. Gompel N, Prud'homme B, Wittkopp PJ, Kassner VA, Carroll SB (2005) Chance caught on the wing: *cis*-Regulatory evolution and the origin of pigment patterns in Drosophila. *Nature* 433:481–487.
7. Manceau M, Domingues VS, Mallarino R, Hoekstra HE (2011) The developmental role of Agouti in color pattern evolution. *Science* 331:1062–1065.
8. Williams TM, et al. (2008) The regulation and evolution of a genetic switch controlling sexually dimorphic traits in *Drosophila*. *Cell* 134:610–623.
9. Piazza P, et al. (2010) *Arabidopsis thaliana* leaf form evolved via loss of KNOX expression in leaves in association with a selective sweep. *Curr Biol* 20:2223–2228.
10. Clark RM, Wagler TN, Quijada P, Doebley J (2006) A distant upstream enhancer at the maize domestication gene tb1 has pleiotropic effects on plant and inflorescent architecture. *Nat Genet* 38:594–597.
11. Cho S, Jin SW, Cohen A, Ellis RE (2004) A phylogeny of *Caenorhabditis* reveals frequent loss of introns during nematode evolution. *Genome Res* 14:1207–1220.
12. Hill RC, et al. (2006) Genetic flexibility in the convergent evolution of hermaphroditism in *Caenorhabditis* nematodes. *Dev Cell* 10:531–538.
13. Kiontke K, et al. (2004) *Caenorhabditis* phylogeny predicts convergence of hermaphroditism and extensive intron loss. *Proc Natl Acad Sci USA* 101:9003–9008.
14. Nayak S, Goree J, Schedl T (2005) *fog-2* and the evolution of self-fertile hermaphroditism in *Caenorhabditis*. *PLoS Biol* 3:e6.
15. Morran LT, Parmenter MD, Phillips PC (2009) Mutation load and rapid adaptation favour outcrossing over self-fertilization. *Nature* 462:350–352.
16. Barriére A, Félix MA (2005) High local genetic diversity and low outcrossing rate in *Caenorhabditis elegans* natural populations. *Curr Biol* 15:1176–1184.
17. Cutter AD, Baird SE, Charlesworth D (2006) High nucleotide polymorphism and rapid decay of linkage disequilibrium in wild populations of *Caenorhabditis remanei*. *Genetics* 174:901–913.
18. Graustein A, Gaspar JM, Walters JR, Palopoli MF (2002) Levels of DNA polymorphism vary with mating system in the nematode genus *Caenorhabditis*. *Genetics* 161: 99–107.
19. Wang J, Chen PJ, Wang GJ, Keller L (2010) Chromosome size differences may affect meiosis and genome size. *Science* 329:293.
20. Merritt C, Rasoloson D, Ko D, Seydoux G (2008) 3′ UTRs are the primary regulators of gene expression in the *C. elegans* germline. *Curr Biol* 18:1476–1482.
21. Puoti A, Gallegos M, Zhang B, Wickens MP, Kimble J (1997) Controls of cell fate and pattern by 3′UTRs: The *C. elegans* sperm/oocyte decision. *Cold Spring Harb Symp Quant Biol* 62:19–24.
22. Jan E, Motzny CK, Graves LE, Goodwin EB (1999) The STAR protein, GLD-1, is a translational regulator of sexual identity in *Caenorhabditis elegans*. *EMBO J* 18: 258–269.
23. Arning S, Gruter P, Bilbe G, Kramer A (1996) Mammalian splicing factor SF1 is encoded by variant cDNAs and binds to RNA. *RNA* 2:794–810.
24. Ebersole T, Chen Q, Justice M, Artzt K (1996) The quaking gene product necessary in embryogenesis and myelination and combines features of RNA binding and signal transduction proteins. *Nat Genet* 12:260–265.
25. Lee MH, Schedl T (2001) Identification of in vivo mRNA targets of GLD-1, a maxi-KH motif containing protein required for *C. elegans* germ cell development. *Genes Dev* 15:2408–2420.
26. Lukong K, Richard S (2003) Sam68, the KH domain-containing superSTAR. *Biochim Biophys Acta* 1653:73–86.
27. Volk T, Israeli D, Nir R, Toledano-Katchalski H (2008) Tissue development and RNA control: "HOW" is it coordinated? *Trends Genet* 24:94–101.
28. Goodwin EB, Okkema PG, Evans TC, Kimble J (1993) Translational regulation of *tra-2* by its 3′ untranslated region controls sexual identity in *C. elegans*. *Cell* 75:329–339.
29. Jones AR, Francis R, Schedl T (1996) GLD-1, a cytoplasmic protein essential for oocyte differentiation, shows stage- and sex-specific expression during *Caenorhabditis elegans* germline development. *Dev Biol* 180:165–183.
30. Francis R, Barton MK, Kimble J, Schedl T (1995) *gld-1*, a tumor suppressor gene required for oocyte development in *Caenorhabditis elegans*. *Genetics* 139:579–606.
31. Francis R, Maine E, Schedl T (1995) Analysis of the multiple roles of *gld-1* in germline development: Interactions with the sex determination cascade and the *glp-1* signaling pathway. *Genetics* 139:607–630.
32. Grant B, Hirsh D (1999) Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. *Mol Biol Cell* 10:4311–4326.
33. Ryder SP, Frater L, Abramovitz D, Goodwin E, Williamson J (2004) RNA target specificity of the STAR/GSG domain posttranscriptional regulatory protein GLD-1. *Nat Struct Mol Biol* 11:20–28.
34. Kelleher DF, et al. (2008) Comparative genetics of sex determination: Masculinizing mutations in *Caenorhabditis briggsae*. *Genetics* 178:1415–1429.
35. Chen P, Cho S, Jin S, Ellis R (2001) Specification of germ cell fates by FOG-3 has been conserved during nematode evolution. *Genetics* 158:1513–1525.
36. Hill R, Haag E (2009) A sensitized genetic background reveals evolution near the terminus of the *Caenorhabditis* germline sex determination pathway. *Evol Dev* 4: 333–341.
37. Woodruff G, Eke O, Baird SE, Félix M, Haag E (2010) Insights into species divergence and the evolution of hermaphroditism from fertile interspecies hybrids of *Caenorhabditis* nematodes. *Genetics* 186:997–1012.
38. Wright JE, et al. (2010) A quantitative RNA code for mRNA target selection by the germline fate determinant GLD-1. *EMBO J* 30:533–545.
39. Crittenden SL, et al. (2002) A conserved RNA-binding protein controls germline stem cells in *Caenorhabditis elegans*. *Nature* 417:660–663.
40. Suh N, et al. (2009) FBF and its dual control of *gld-1* expression in the *Caenorhabditis elegans* germline. *Genetics* 181:1249–1260.
41. Suh N, Jedamzik B, Eckmann CR, Wickens M, Kimble J (2006) The GLD-2 poly(A) polymerase activates *gld-1* mRNA in the *Caenorhabditis elegans* germ line. *Proc Natl Acad Sci USA* 103:15108–15112.
42. Clifford R, et al. (2000) FOG-2, a novel F-box-containing protein, associates with the GLD-1 RNA-binding protein and directs male sex determination in the *C. elegans* hermaphrodite germline. *Development* 127:5265–5276.
43. Jeong J, Verheyden JM, Kimble J (2010) Cyclin E and Cdk2 Control GLD-1, the mitosis/meiosis decision, and germline stem cells in *Caenorhabditis elegans*. *PLoS Genet* 7: e1001348.
44. Bachorik JL, Kimble J (2005) Redundant control of the *Caenorhabditis elegans* sperm/oocyte switch by PUF-8 and FBF-1, two distinct PUF RNA-binding proteins. *Proc Natl Acad Sci USA* 102:10893–10897.
45. Lamont LB, Crittenden SL, Bernstein D, Wickens M, Kimble J (2004) FBF-1 and FBF-2 regulate the size of the mitotic region in the *C. elegans* germline. *Dev Cell* 7:697–707.
46. Subramaniam K, Seydoux G (2003) Dedifferentiation of primary spermatocytes into germ cell tumors in *C. elegans* lacking the pumilio-like protein PUF-8. *Curr Biol* 13: 134–139.
47. Kuwabara PE (1996) Interspecies comparison reveals evolution of control regions in the nematode sex-determining gene *tra-2*. *Genetics* 144:597–607.
48. Jan E, Yoon JW, Walterhouse D, Iannaccone P, Goodwin EB (1997) Conservation of the *C. elegans tra-2* 3′UTR translational control. *EMBO J* 16:6301–6313.
49. Mapes J, Chen JT, Yu JS, Xue D (2010) Somatic sex determination in *Caenorhabditis elegans* is modulated by SUP-26 repression of *tra-2* translation. *Proc Natl Acad Sci USA* 107:18022–18027.
50. Carmel AB, Wu J, Lehmann-Blount KA, Williamson JR (2010) High-affinity consensus binding of target RNAs by the STAR/GSG proteins GLD-1, STAR-2 and Quaking. *BMC Mol Biol* 11:48.
51. Doniach T (1986) Activity of the sex-determining gene *tra-2* is modulated to allow spermatogenesis in the *C. elegans* hermaphrodite. *Genetics* 114:53–76.
52. Ariz M, Mainpal R, Subramaniam K (2009) *C. elegans* RNA-binding proteins PUF-8 and MEX-3 function redundantly to promote germline stem cell mitosis. *Dev Biol* 326: 295–304.
53. Hansen D, Wilson-Berry L, Dang T, Schedl T (2004) Control of the proliferation versus meiotic development decision in the *C. elegans* germline through regulation of GLD-1 protein accumulation. *Development* 131:93–104.
54. Shapiro MD, et al. (2004) Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature* 428:717–723.
55. Doebley J, Stec A, Hubbard L (1997) The evolution of apical dominance in maize. *Nature* 386:485–488.
56. Marin VA, Evans TC (2003) Translational repression of a *C. elegans* Notch mRNA by the STAR/KH domain protein GLD-1. *Development* 130:2623–2632.
57. Kingsley E, Chan X, Duan Y, Lambert J (2007) Widespread RNA segregation in a spiralian embryo. *Evol Dev* 9:527–539.
58. Johnstone O, Lasko P (2001) Translational regulation and RNA localization in Drosophila oocytes and embryos. *Annu Rev Genet* 35:365–406.
59. Wang D, Martin K, Zukin R (2010) Spatially restricting gene expression by local translation at synapses. *Trends Neurosci* 33:173–182.
60. Praitis V, Casey E, Collar D, Austin J (2001) Creation of low-copy integrated transgenic lines in *Caenorhabditis elegans*. *Genetics* 157:1217–1226.
61. Kershner AM, Kimble J (2010) Genome-wide analysis of mRNA targets for *Caenorhabditis elegans* FBF, a conserved stem cell regulator. *Proc Natl Acad Sci USA* 107: 3936–3941.
62. Kiontke K, Sudhaus W (2006) Ecology of Caenorhabditis species (Jan. 2006). *Wormbook*, ed The *C. elegans* Research Community. Available at http://www.wormbook.org.

Supporting Information

Beadell et al. 10.1073/pnas.1108068108

SI Materials and Methods

Nematode Genetics. *Cbr-gld-1* alleles *nm41* and *nm64* were recognized by sterile F_2 progeny of ethyl-methanesulfonate-mutagenized AF16 animals and isolated by sibling selection, as previously described (1). After backcrosses to unmutagenized AF16, tight linkage to *Cbr-gld-1* was demonstrated via a polymorphism adjacent to *Cbr-gld-1* that distinguishes AF16 and HK104 strains. Mutations were identified by resequencing the entire GLD-1 coding regions. *Cbr-gld-1(nm68)* was identified by PCR-based deletion screens, as previously described (2); *nm41* and *nm64*, and *nm64* and *nm68* fail to complement each other. *Caenorhabditis elegans* strains BS263 [*gld-1(q93oz50)*] and BS2 [*gld-1(q268)*] were the gift of T. Schedl (Washington University, St. Louis, MO). *C. elegans* strains JK1466 [*gld-1(q485)/dpy-5 (e61)unc-13(e51) I*] and BS3156 [*unc-13(e51) gld-1(q485)/hT2 [dpy-18(h662)] I; +/hT2[bli-4(e937)] III*] were obtained from the Caenorhabditis Genetics Center. *C. elegans gld-1; puf-8 fbf-1* triple mutants were derived from strain JK3752 [*gld-1(q485)/ccIs4251 unc-15(e73); fbf-1(ok91) puf-8(q725)/mIn1[mIs14 dpy10(e128)]*], kindly provided by J. Kimble (University of Wisconsin, Madison, WI).

Rescue of BS3156 *q485* homozygotes by *Cbr-gld-1* was mediated by plasmid pAD-g6 (see below for details). Biolistic transformation of *C. elegans* via *unc-119* rescue was performed by standard methods (3, 4). Specifically, we grew *Ce-unc-119(ed3)* worms at 20 °C on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain HB101. We harvested 1 mL of gravid animals, bombarded with 10 μg of plasmid, and recovered animals to 80 10-cm NGM plates seeded with HB101 at 25 °C. After 10–12 d, we screened worms for both wild-type moving and GFP-pharynx expressing animals. We identified 19 plates with more than three such animals and singled four animals per plate to fresh standard NGM plates to maintain lines and check for continued transgene expression.

Construct for Cross-Species Rescue of *gld-1(q485)*. The construct used for bombardment transformation, pAD-g6, was derived from two distinct tripartite Multisite Gateway (Invitrogen) plasmid constructs, pCR50 and pAD-g1. pCR50 (a gift of C. Richie and A. Golden, National Institutes of Health, Bethesda, MD) combines the pharyngeal reporter *myo-2::*GFP with an unmanipulated rescuing genomic fragment of *Caenorhabditis briggsae unc-119*, which was inserted into a unique restriction site. pAD-g1 combines 923 nucleotides upstream of the AF16 *Cbr-gld-1* start codon (cloned into pDONR P4-P1R), the entire *Cbr-gld-1* coding region C-terminally tagged by HA and FLAG epitopes via fusion PCR (cloned into pDONR 221), and 1,354 nt downstream of the *Cbr-gld-1* stop codon (cloned into pDONR P2R-P3). These three plasmids were combined in the Multisite LR reaction to create pAD-g1. The entire tagged *Cbr-gld-1* locus of pAD-g1 was then excised and cloned by blunt ligation into a unique BamHI site in pCR50, creating pAD-g6.

Immunoblots and Immunohistochemistry. Immunoblots and gonad immunohistochemistry used a custom GLD-1 chicken polyclonal antibody (see below) or an anti–GLD-1 polyclonal antibody, generously provided by T. Schedl (Washington University, St. Louis, MO), made against the C-terminal 82% of *C. elegans* GLD-1 that also recognizes *C. briggsae* GLD-1. Anti-major sperm protein (MSP) and anti–RME-2 (an oocyte marker) antibodies were the gifts of D. Greenstein (University of Minnesota, Minneapolis, MN) and B. Grant (Rutgers University, Piscataway, NJ), respectively, and antitubulin, antiactin, and anti-HA antibodies were purchased from Sigma. Immunohistochemistry protocols were slightly modified from those of T. Schedl, using a methanol/formaldehyde fix for 10–15 min at room temperature. Fluorescently conjugated secondary antibodies were Alexa 488 and Alexa 555 (Molecular Probes-Invitrogen), used at 1:1,000 to 1:2,000 dilutions. All gonads were dissected in the same conditions and were blocked, incubated in antibodies, and washed simultaneously in the same conditions.

rme-2 is a conserved in vivo target mRNA of *Ce*-GLD-1 and Cbr-GLD-1 (5) (Figs. 4 and 5). It is thus surprising that Cbr-RME-2 expression is not detectable in *Cbr-gld-1* loss-of-function treatments (e.g., Fig. 1). However, RME-2's expression appears to be subordinate to overall germ-line sex, and we use RME-2 in this limited capacity to show the lack of female character of *Cbr-gld-1* mutant germ cells.

RNA Interference. *C. briggsae* AF16 adult virgin hermaphrodites were reared at 20 °C, injected in the gut with dsRNA at concentrations of ∼2–3 μg/μL, and recovered to seeded plates at 20 °C. Phenotypes were assessed by scoring progeny 3–4 d later (young-adult stage) under the stereomicroscope and, if found to be abnormal, with differential interference microscopy (DIC) microscopy. The progeny of at least four injected mothers were scored for each experiment. Oligonucleotide primers were designed to amplify the same ∼750-bp region for all *gld-1* orthologs (corresponding to nucleotides 753–1,626 of *C. elegans gld-1*), obtained from WormBase (WS210). For *Cbr-puf-1b* and *Cbr-puf-8* knockdown, gene-specific primers were used to amplify regions with high divergence between Puf family members. Primer sequences are available upon request.

Immunoprecipitation of GLD-1 and Analysis of Associated RNA. GLD-1 RNA immunoprecipitations (RIP) used a custom, affinity-purified polyclonal chicken antibody (produced by Open Biosystems, Huntsville, AL). A peptide antigen representing residues 16–36 of *Cbr*-GLD-1, which is conserved in *C. elegans* and *Caenorhabditis remanei* orthologs (Fig. S1*A*), was used to immunize two female chickens (operating under NIH OLAW assurance number A-373801). We obtained one affinity-purified polyclonal chicken antibody specific to GLD-1.

Messenger RNA complexed with *Cbr*-GLD-1 were immunoprecipitated from young AF16 wild-type XX adults and recovered using a modified version of a protocol kindly provided by A. Kershner and J. Kimble (University of Wisconsin, Madison, WI) (6), adjusted for our chicken antibody and modified as described below.

Synchronous, mostly hermaphroditic populations of *C. briggsae* AF16 were grown on 15-cm NGM plates at 20 °C and seeded thickly with OP50. Separate populations of worms were grown in parallel and each kept separate through growth and processing to become biological replicates. When their average age was young gravid adult, animals were rinsed off plates and washed in M9 buffer for more than 30 min to clear gut bacteria. We recovered ∼1-mL pellets of settled worms (by gravity settling or brief, low-speed centrifugation) per replicate. Each pellet was washed once in buffer A (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA), twice in lysis buffer [20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 0.2 mg/mL heparin, plus 1× EDTA-free Mini Complete Protease Inhibitor Mixture (Roche), 2 mM DTT, 200 U/mL recombinant RNasin (Promega)], and finally resuspended 1:1 in lysis buffer.

Worms were poured into a cold mortar and ground in liquid N_2 into a fine powder. This powder was thawed on ice, passed ~15 times through a dounce homogenizer while still on ice, and resuspended to a 2-mL final volume with lysis buffer. Lysates were spun at 10,000 × *g* for 10 min at 4 °C to pellet insoluble debris, and supernatants were precleared with goat anti-IgY agarose-coupled beads (PrecipHen; Aves Labs) by rotation for 30 min at 4 °C. Half of the precleared lysate of each replicate was added to 15 μg chicken anti–GLD-1–bound PrecipHen and half to 15 μg total IgY-bound PrecipHen (using unconjugated total IgY; Jackson Immunoresearch), and rotated for ~8 h at 4 °C.

To harvest the RNA bound to GLD-1, beads were pelleted by mild centrifugation and washed 10 min with tumbling at 4 °C in lysis buffer, followed by four 10-min tumbling washes at 4 °C in wash buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT, 10 U/mL RNasin). Bound RNA was released from GLD-1 with 1 mL TRI Reagent (Ambion), followed by rocking/shaking for 30 min. Next, 200 μL chloroform was added to the TRI Reagent-supernatant and a modified phenol:chloroform extraction and ethanol precipitation was performed using Qiagen RNeasy columns. RNA was eluted in RNase-free water. During the experiment, samples were removed after preclearing and after the immunoprecipitation (IP) to check for both the quality of RNA recovered by agarose gel electrophoresis and the effectiveness of IP by Western blot.

RNA from anti–GLD-1 and anti-IgY immunoprecipitations and from total input for each biological replicate was reverse transcribed using SuperScript III (Invitrogen) according to the manufacturer's instructions. Next, 0.5 μL cDNA was used as template with the Light Cycler 480 SYBR Green I kit (Roche) for quantitative real-time PCR, according to manufacturer's instructions. After scrutinizing melting curve analyses to ensure amplification of a single appropriate product, data were analyzed with LinRegPCR (7). For microarray analysis, a modified version of a *C. briggsae* long-oligonucleotide Agilent 44-K probe set (8) was developed and used for two-color hybridizations. Three replicates of RIP RNA vs. total mRNA and five replicates of RIP RNA vs. mock (IgY) IP RNA were performed, and only probes showing significant enrichment in both control classes were considered for further analysis.

Gel Mobility-Shift Assay. A maltose-binding protein fusion protein with the STAR (signal transduction and activation of RNA metabolism) domain of Cbr-GLD-1 (amino acids 135–329) was produced from a derivative of a plasmid encoding the analogous *C. elegans* construct (9) (a gift of S. Kwan and J. Williamson, Scripps Research Institute, La Jolla, CA). The fusion protein was expressed and purified by affinity and ion-exchange chromatography steps, as described in Ryder et al (9) using HPLC (Pharmacia; courtesy of S. Sukharev, University of Maryland, College Park, MD). Next, a 300 nt 3′ fragment of Cbr-puf-8 (whose 5′ end is the stop codon) was amplified from genomic DNA via semi-nested PCR. An oligo-dT reverse primer and two gene-specific forward primers were used, the inner of which was tailed with the phage T7 promoter sequence. Gel-purified template DNA was transcribed in vitro using the T7 Megascript kit (Ambion). Transcripts were end-labeled with [γ^{32}P] ATP and polynucleotide kinase (New England Biolabs) according to the manufacturer's instructions. For mobility-shift experiments, 20 fmoles of labeled RNA was incubated for 2 h with varying concentrations of MBP-Cbr-GLD-1 STAR and resolved on native polyacrylamide gels, as previously described (10).

1. Kelleher DF, et al. (2008) Comparative genetics of sex determination: masculinizing mutations in *Caenorhabditis briggsae*. *Genetics* 178:1415–1429.
2. Hill RC, et al. (2006) Genetic flexibility in the convergent evolution of hermaphroditism in *Caenorhabditis* nematodes. *Dev Cell* 10:531–538.
3. Merritt C, Gallo C, Rasoloson D, Seydoux G (2009) Transgenic solutions for the germline. *Wormbook*, ed The *C. elegans* Research Community. Available at http://www.wormbase.org.
4. Praitis V, Casey E, Collar D, Austin J (2001) Creation of low-copy integrated transgenic lines in *Caenorhabditis elegans*. *Genetics* 157:1217–1226.
5. Nayak S, Goree J, Schedl T (2005) *fog-2* and the evolution of self-fertile hermaphroditism in *Caenorhabditis*. *PLoS Biol* 3:e6.
6. Kershner AM, Kimble J (2010) Genome-wide analysis of mRNA targets for *Caenorhabditis elegans* FBF, a conserved stem cell regulator. *Proc Natl Acad Sci USA* 107:3936–3941.
7. Ramakers C, Ruijter J, Deprez R, Moorman A (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 13:62–66.
8. Yanai I, Hunter C (2009) Comparison of diverse developmental transcriptomes reveals that coexpression of gene neighbors is not evolutionarily conserved. *Genome Res* 19:2214–2220.
9. Ryder SP, Frater L, Abramovitz D, Goodwin E, Williamson J (2004) RNA target specificity of the STAR/GSG domain posttranscriptional regulatory protein GLD-1. *Nat Struct Mol Biol* 11:20–28.
10. Chritton JJ, Wickens M (2010) Translational repression by PUF proteins in vitro. *RNA* 16:1217–1225.

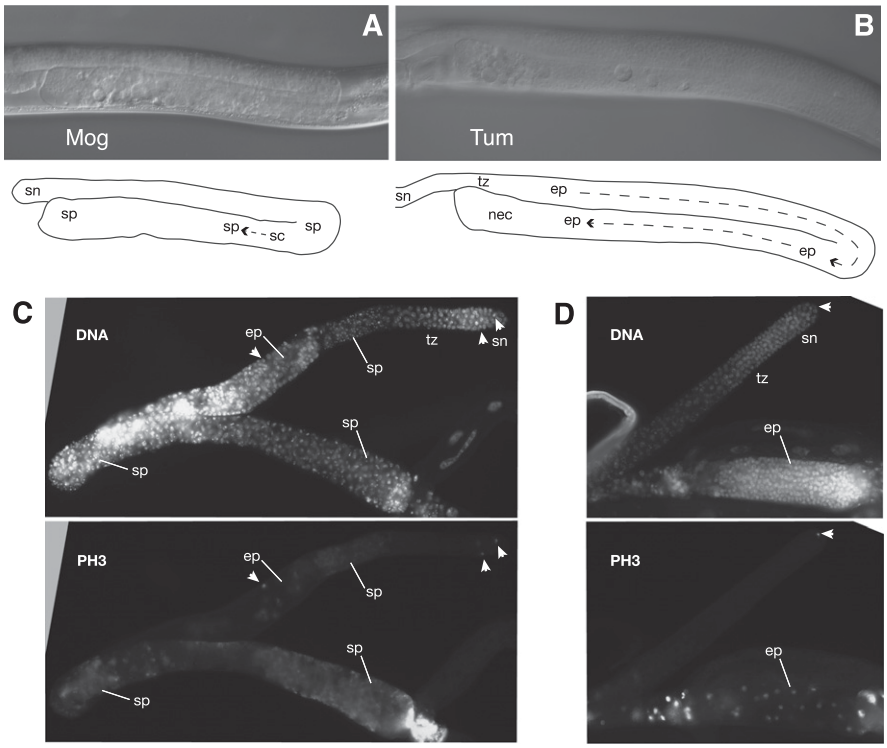

Fig. S1. Variable masculinization and ectopic proliferation in *Cbr-gld-1* mutants. (*A* and *B*) One gonad arm of a whole-mounted XX *Cbr-gld-1(nm41)* mutant adult with extensive masculinization of the germ line (Mog; *A*) or extensive ectopic proliferation and no differentiated gametes (Tum; *B*). (*Upper*) A DIC micrograph; (*Lower*) a tracing of the germ line with cell types indicated (ep, ectopic proliferation; nec, necrosis; sc, spermatocyte; sn, stem cell niche; tz, transition zone from mitotic to meiotic domains). In *A*, sperm (with highly compact nuclei) exist in multiple locations, in at least one case proximal to a population of spermatocytes (sc) that will presumably produce more sperm with time. (*C* and *D*) Fluorescence micrographs of extruded, single gonad arms of XX *Cbr-gld-1(nm41)* mutant adults. (*Upper*) Hoechst staining of DNA; (*Lower*) staining of the same gonad with anti-phosphohistone H3 (PH3) antibody, which identifies nuclei of cells in mitosis (arrowheads). In both cases, nuclei in the stem cell niche stain with PH3, as expected, but so do those in proximal to the niche. In *C*, these nuclei can be seen between two of three distinct subpopulations of differentiated spermatids, but in *D* they are found exclusively in the proximal half of the germ line. Cytoplasmic staining observed in differentiated spermatocytes is likely to be nonspecific. (Magnification, 150×.)

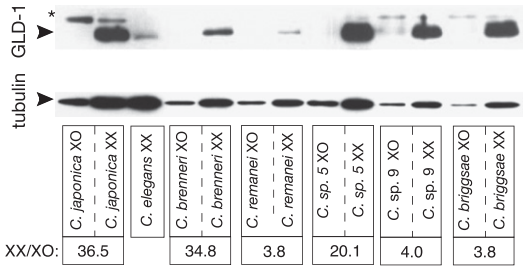

Fig. S2. Conservation of XX-biased GLD-1 expression. GLD-1 immunoblot (*Upper*) of male and XX (either female or hermaphrodite) protein samples from various *Caenorhabditis* species (40 animals per lane). The XX/XO ratio of tubulin-normalized GLD-1 expression is given below the name of each non-*elegans* species.

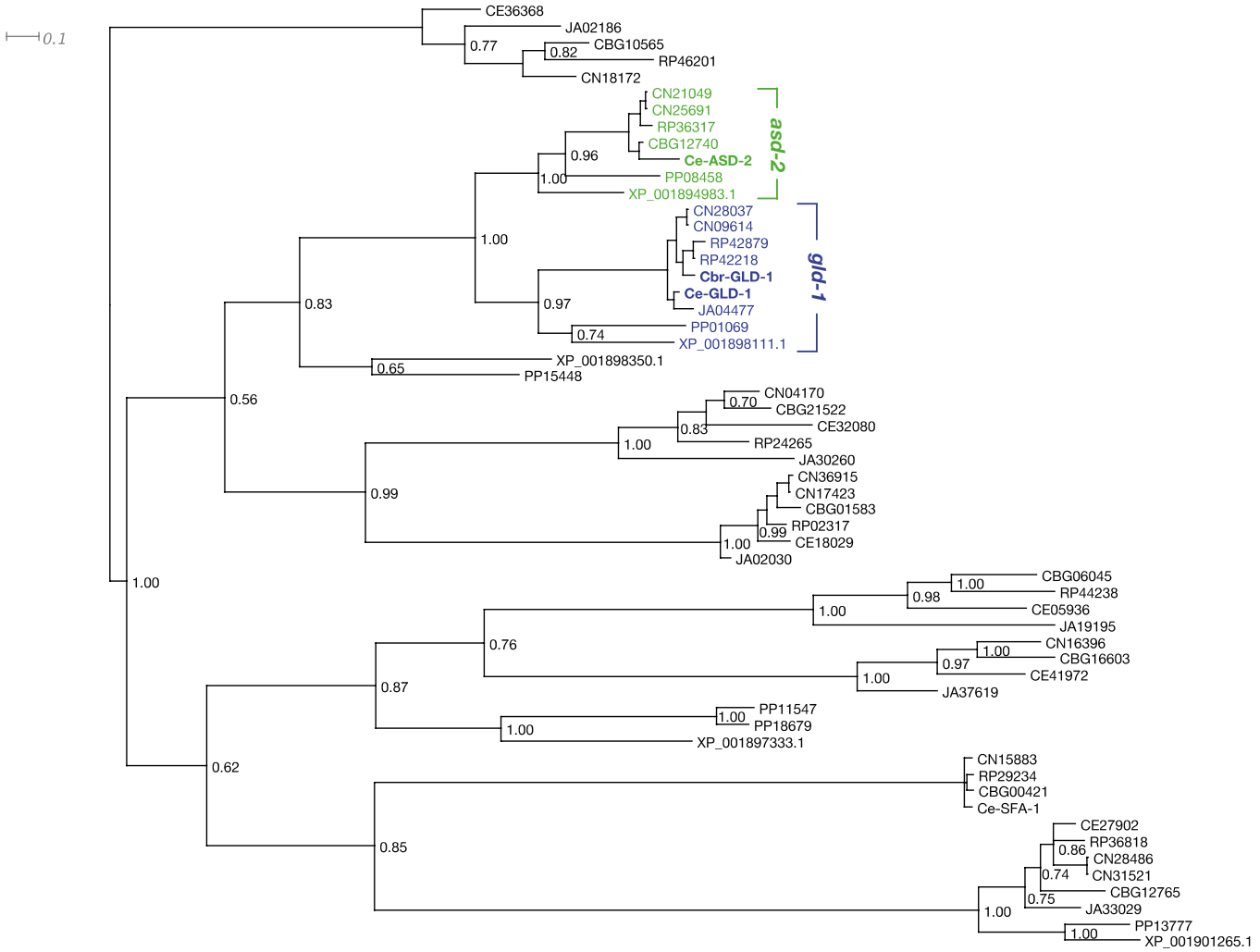

Fig. S3. Nematode STAR family phylogeny. Amino acid-based Bayesian phylogeny of STAR family proteins from seven sequenced nematode genomes. The GLD-1 clade is highlighted in blue, and Ce-GLD-1 and Cbr-GLD-1 are further emphasized with bold type. Green shading indicates the ASD-2 (also known as STAR-2) clade sister to GLD-1. Taxon prefixes for gene predictions are: Ce or CE, *C. elegans;* Cbr or CBG, *C. briggsae;* RP, *C. remanei;* CN, *C. brenneri,* JA, *Caenorhabditis japonica;* PP, *Pristionchus pacificus;* XP, *Brugia malayi*. Numbers at nodes are posterior probabilities; some are omitted for visual clarity, but their values are similarly high. The phylogeny was inferred using the STAR domain and flanking sequences (446 characters total) in Mr. Bayes (1), as implemented in the CIPRES Science Gateway with a mixed amino acid model and invgamma rate model (2). Standard diagnostics confirmed stationarity.

1. Ronquist F, Huelsenbeck J (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572−1574.
2. Miller MA, et al. (2009) The CIPRES Portals. in *CIPRES* (http://www.phylo.org/). Accessed May 1, 2009.

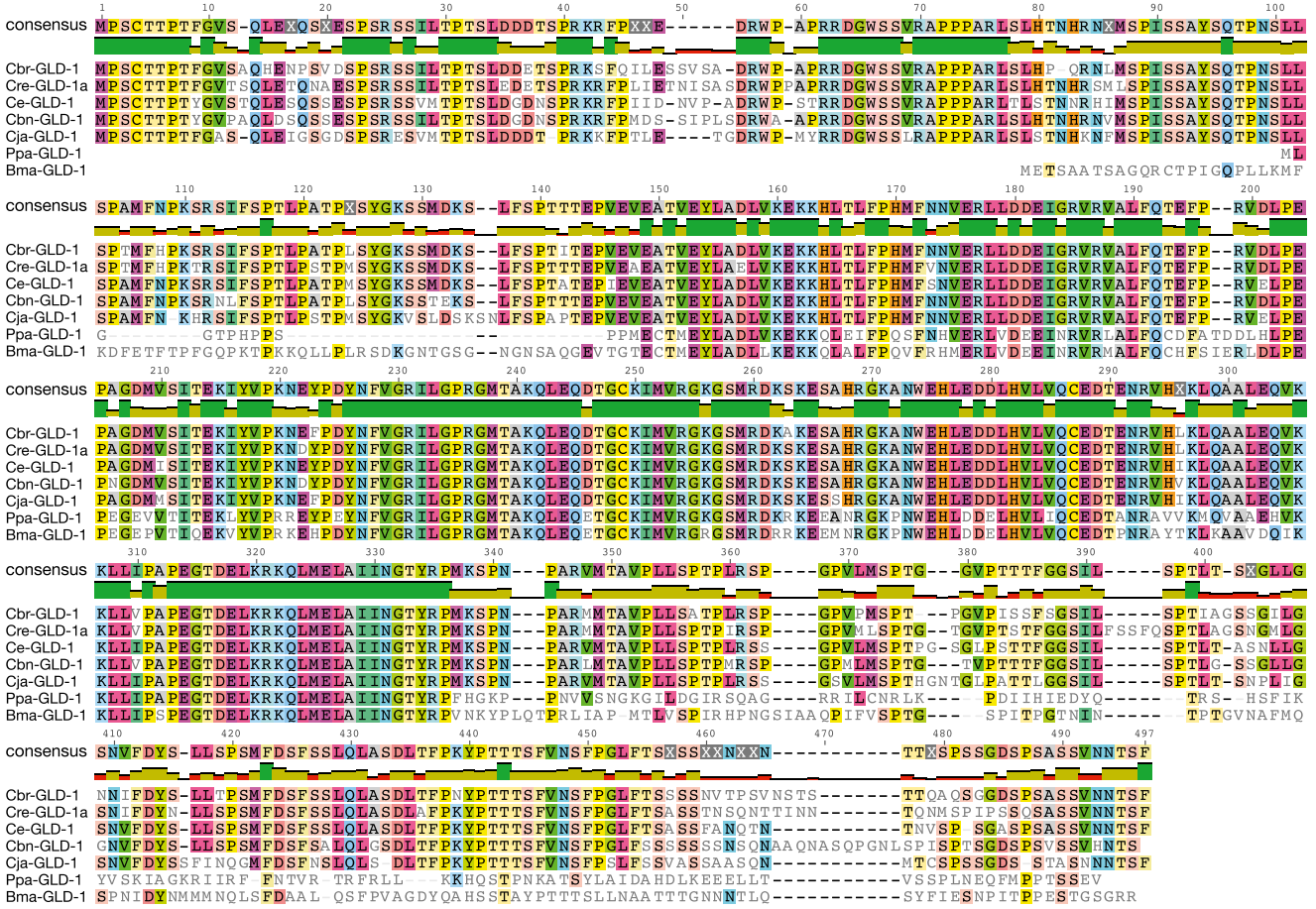

Fig. S4. Alignment of complete GLD-1 orthologs from seven nematode species. Sequence prefixes are as above. Alignment was produced with ClustalW (1) using default parameters implemented in the Geneious software package. The STAR/GSG domain spans from near amino acids 150–336. For *C. remanei* and *C. brenneri*, only one of two GLD-1 predictions are shown; for *C. remanei* these are likely to be recently duplicated paralogs, and for *C. brenneri* they likely represent allelic variants. Strength of conservation at each position is indicated by extent of green beneath the consensus.

1. Larkin MA, et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947–2948.

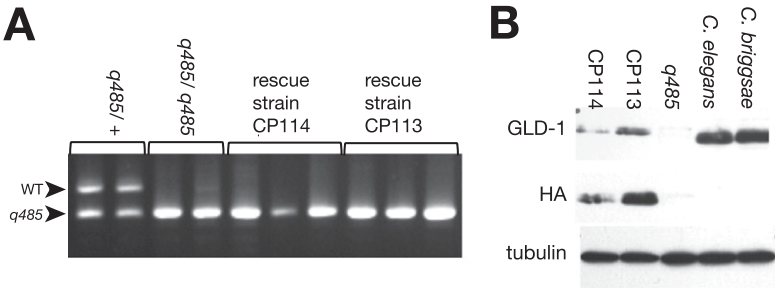

Fig. S5. Rescue of *C. elegans gld-1(q485)* mutants by a *Cbr-gld-1* transgene. (*A*) Agarose gel of PCR products amplified with primers flanking the *Ce-gld-1 (q485)* deletion, demonstrating that rescued strains CP113 and CP114 lack the wild-type allele. (*B*) GLD-1 and HA immunoblots in control and rescued *gld-1 (q485)* homozygous lines. Each lane has protein extract from 50 adults. Blot was first probed with anti–GLD-1 antibody, stripped, and reprobed with anti-HA antibody, then stripped a second time and reprobed with antitubulin antibody.

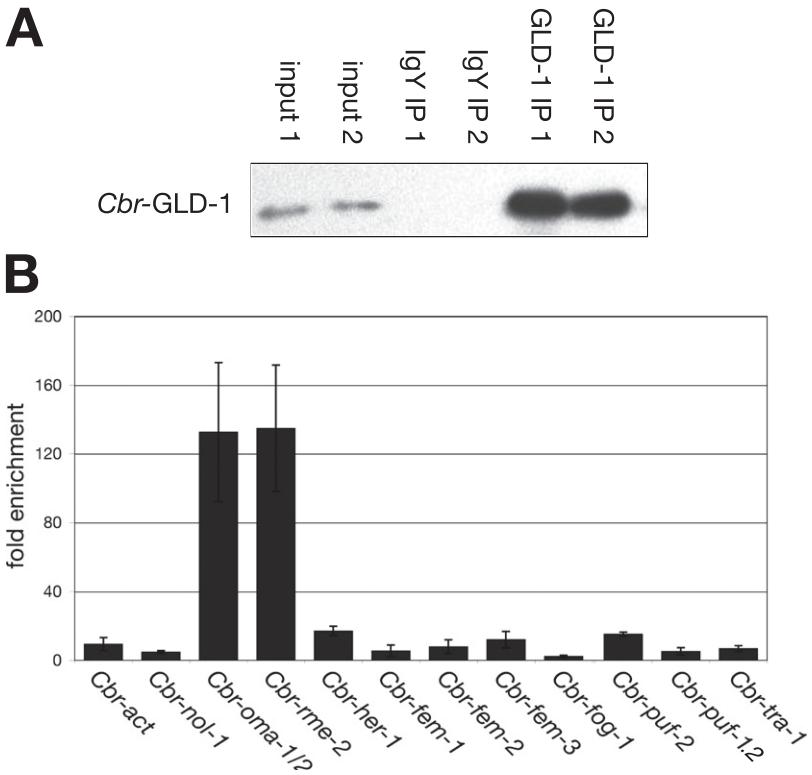

Fig. S6. GLD-1 RIP and RIP-qRT-PCR analysis of candidate Cbr-GLD-1 sperm-promoting target mRNAs. (*A*) IP of *Cbr*-GLD-1 from whole-worm lysates. Chicken anti–GLD-1 or total IgY was used for IP and mock IP, respectively, and rabbit anti–GLD-1 (1) was used for immunoblotting; 1% of input lysates and 20% of IP or mock IP beads were loaded, and 1 and 2 refer to biological replicates. (*B*) qRT-PCR of negative control (*Cbr-act* and *Cbr-nol-1*), positive control (*Cbr-oma-1/2* and *Cbr-rme-2*), and candidate sperm-promoting mRNAs associated with IP GLD-1, expressed as the average ratio of anti–GLD-1 IP to mock IgY IP (see text for details) of at least three biological replicates (error bars = SEM). *Cbr-fog*-3 and *she-1* were also assayed in this experiment, but produced IP and mock IP signals too low to quantify, consistent with nonenrichment.

1. Jones AR, Francis R, Schedl T (1996) GLD-1, a cytoplasmic protein essential for oocyte differentiation, shows stage- and sex-specific expression during *Caenorhabditis elegans* germline development. *Dev Biol* 180:165–183.

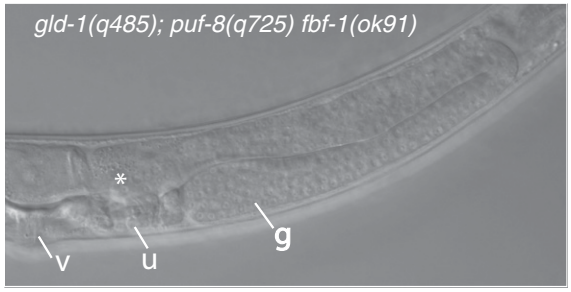

Fig. S7. Triple mutant analysis of *C. elegans gld-1, puf-8, and fbf-1*. The strain JK3752 was used to identify triple homozygotes. These animals produce no obvious gametes, and resemble those of *gld-1(q485)* single mutants. g, germ line; u, uterus; v, vulva.

Table S1. Suppression of *Cbr-gld-1* ectopic proliferation by masculinizing mutations

Germ-line phenotype	Source and observation		
	XX Animals		
	Cbr-gld-1(nm68) (from Fig. 1*B*)	*Cbr-tra-1(nm2)* (from ref. 1)	1/4 *Cbr-gld1(nm68); Cbr-tra-1(nm2)* n = 300*
Sperm only	80%	55%	50%
Sperm + oocytes	0%	35%	43%
Ectopic proliferation + sperm	20%	0%	0%
Single-worm genotyping†	*Cbr-tra-1(nm2); Cbr-gld-1(+/+)*	*Cbr-tra-1(nm2); Cbr-gld-1(nm41/+)*	*Cbr-tra-1(nm2); Cbr-gld-1(nm41/)*
Sperm only	0	0	5
Sperm + oocytes	6	19	0
Ectopic proliferation + sperm	Not observed	Not observed	Not observed
Ectopic proliferation	0	0	1
	Cbr-gld-1(nm68) (from Fig. 1*B*)	*Cbr-tra-2(nm1)* (from ref. 2)	1/4 *Cbr-gld1(nm68); Cbr-tra-2(nm1)* n = 120*
Sperm only	80%	100%	100%
Sperm + oocytes	0%	0%	0%
Ectopic proliferation + sperm	20%	0%	0%
	Cbr-gld-1(RNAi) (n = 230)‡	*Cbr-fog-3 (RNAi)* n = 40	*Cbr-gld-1(RNAi);*‡ *Cbr-fog-3(RNAi)* n = 32
Sperm only	94%	0%	3%
Oocytes only	0%	60%	0%
Sperm + oocytes	5%	40%	0%
Ectopic proliferation	0%	0%	97%
	XO Animals		
	1/4 *Cbr-gld1(nm68)* n = 200*	*Cbr-fog-3* RNAi n = 40	*Cbr-gld-1(RNAi);*‡ *Cbr-fog-3(RNAi)* n = 15
Sperm only	100%	0%	0%
Oocytes only	0%	60%	0%
Sperm + oocytes	0%	40%	0%
Ectopic proliferation	0%	0%	100%

n = Number of germ lines scored; hermaphrodites possess two independent gonad arms. Percentages may not sum to 100 for each genotype because phenotypic classes without sex determination or tumor formation consequence (such as "germ-cell death" and "aberrant somatic gonad") have been omitted.

*Because no convenient phenotypic marker currently exists for *Cbr-gld-1*, all *Tra* offspring (i.e., those with a male soma) of a doubly heterozygous selfing mother were scored with DIC microscopy for the germ-line phenotype. One quarter of the *Tra* F1 animals examined this way should be *Cbr-gld-1(nm68)* homozygotes, and we verified that there was no bias against *Cbr-gld-1* mutant animals by randomly genotyping 48 *Tra* F1 progeny for the *nm68* deletion mutation and checking for statistically significant deviations from the expected frequency. A second experiment in which animals of known phenotype were genotyped is described in the main text.

†In this experiment, animals chosen to be scored by DIC microscopy and then genotyped were not sampled randomly, but rather chosen to emphasize oogenic *Tras* and apparently normal males.

‡We used *Cbr-gld-1* RNAi in this experiment for two reasons. First, double RNAi allows the genotype of all F1 progeny to be known with certainty. Second, as *Cbr-gld-1(nm68)* produces ectopic germ-line proliferation in XX animals, but *Cbr-gld-1(RNAi)* does so rarely, an increase in the proportion of animals with ectopic proliferation in *Cbr-gld-1; Cbr-fog-3* double loss-of-function animals is most easily assessed with RNAi.

1. Hill RC, Haag ES (2009) A sensitized genetic background reveals evolution near the terminus of the *Caenorhabditis* germline sex determination pathway. *Evol Dev* 11:333–342.
2. Kelleher DF, et al. (2008) Comparative genetics of sex determination: masculinizing mutations in *Caenorhabditis briggsae*. *Genetics* 178:1415–1429.