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

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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.

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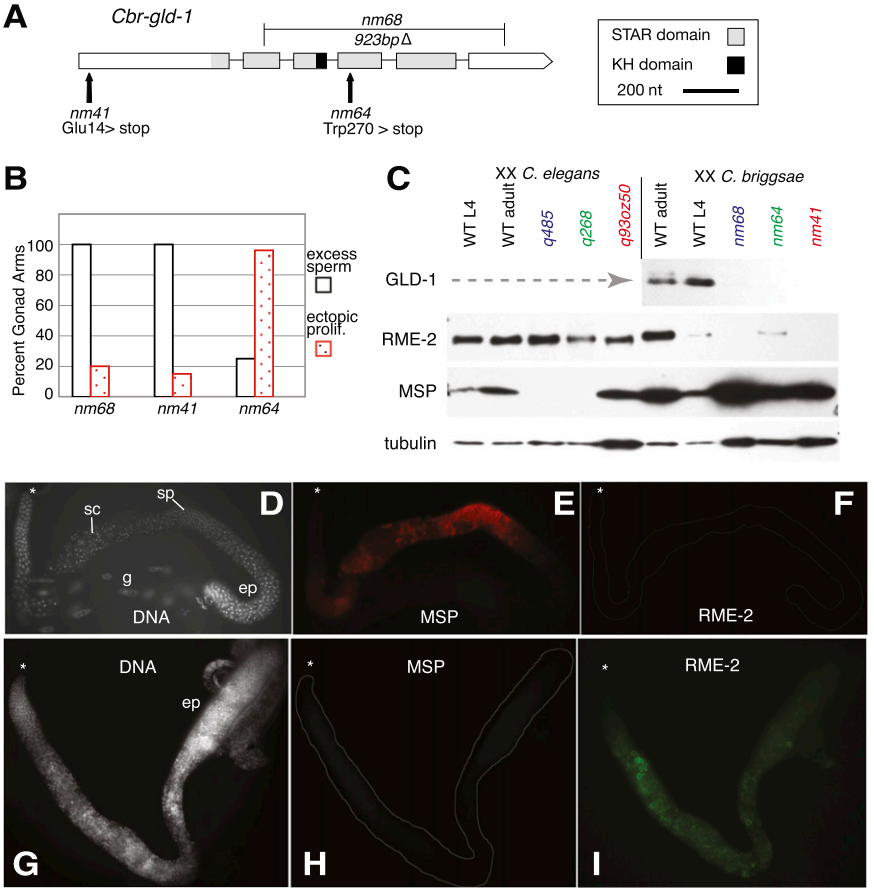

Fig. 1. *C. briggsae* and *C. elegans gld-1* mutations produce opposite sexual transformations of the hermaphrodite germ line. (*A*) Structure of *Cbr-gld-1*, with exon-intron boundaries, conserved coding domains, and mutant lesions indicated. (*B*) Phenotypic distributions of *Cbr-gld-1* alleles measured 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*

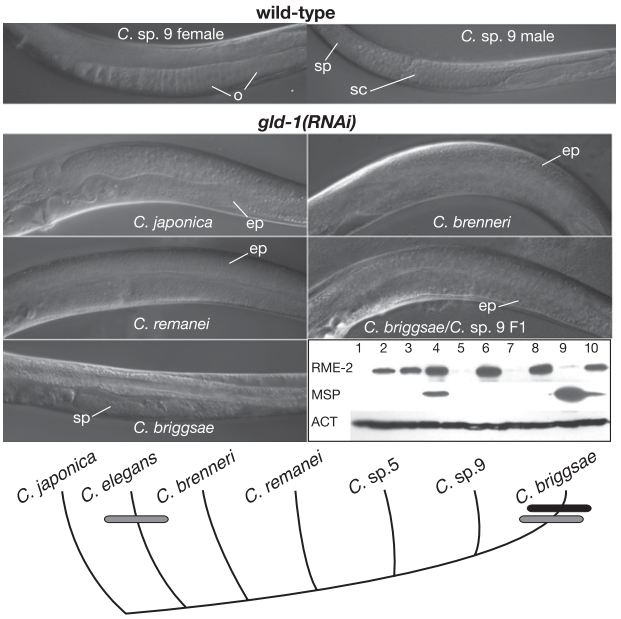

Fig. 2. *Cbr-gld-1* sperm repression is of recent origin, but its role in meiotic progression is ancient. (*Top*) Gonads of wild-type *C.* sp. 9 (representative of gonochoristic species). (*Left*) Unmated XX female, showing oocytes (o) of one ovarian arm. (*Right*) XO male with spermatids (sp) and primary 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.

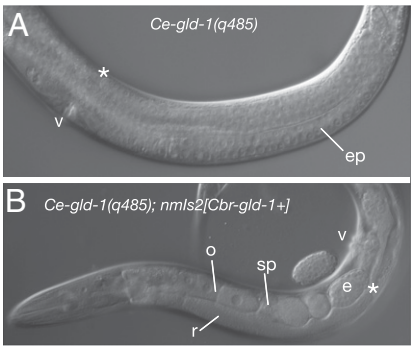

Fig. 3. *Cbr-gld-1* can fully substitute for *C. elegans gld-1*. (*A*) Germ-line phenotype of adult XX *C. elegans gld-1(q485)* mutants showing extensive ectopic proliferation of oocyte-fated cells that have exited meiosis and reentered mitosis. *ep*, Ectopic proliferation. The distal tip of the anterior 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).

EVOLUTION

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.

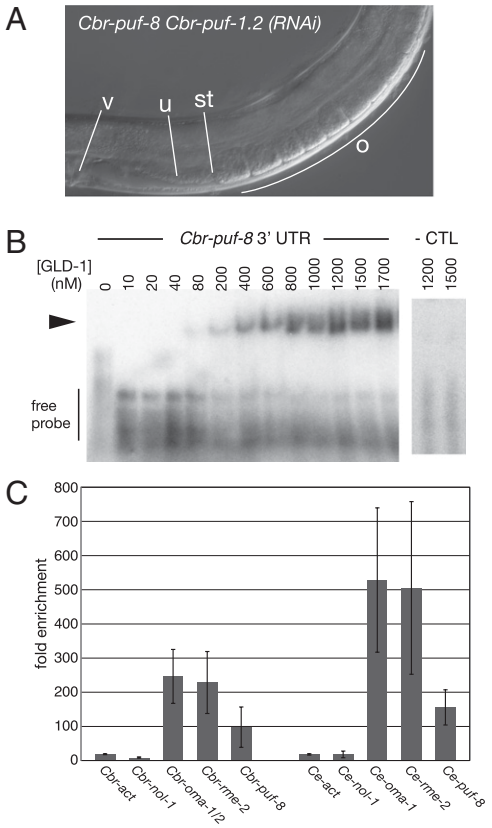

Fig. 4. *Cbr-puf-8* is a sperm-promoting target of *Cbr*-GLD-1. (*A*) *Cbr-puf-8 (RNAi);Cbr-puf-1.2(RNAi)* feminizes the hermaphrodite germ line. Of the animals that produce gametes, XX adults have only oocytes (o) and have an empty spermatheca (st) and uterus (u). v, vulva. (*B*) GLD-1 binds *Cbr-puf-8* 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-

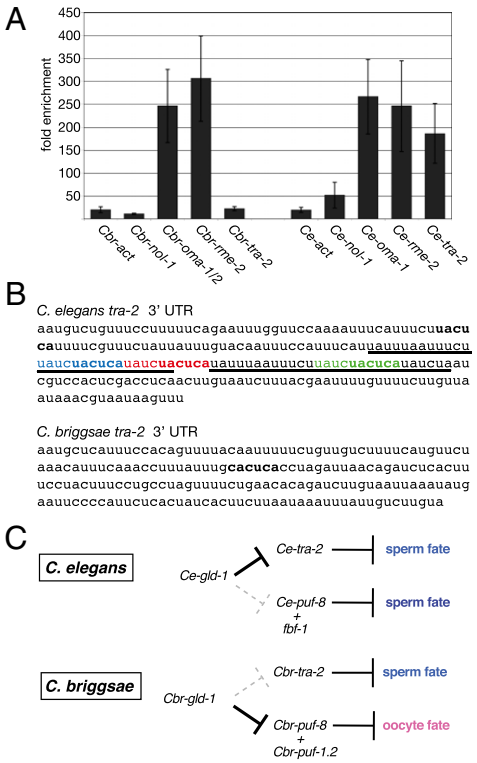

Fig. 5. *tra-2* mRNA associates with GLD-1 in *C. elegans* but not *C. briggsae*. (*A*) Comparison of *tra-2* association with GLD-1 in *C. briggsae* and *C. elegans*. qRT-PCR enrichments for GLD-1 IP vs. mock IP RNA preparations are given for negative controls, positive controls, and *tra-2*; each is 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* germ-line 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*).

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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.