The STAR/Maxi-KH domain protein GLD-1 mediates a developmental switch in the translational control of *C. elegans* PAL-1

Darcy Mootz, Diana M. Ho* and Craig P. Hunter†

Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA
*Present address: Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
†Author for correspondence (e-mail: hunter@mcb.harvard.edu)

Accepted 30 March 2004

Development 131, 3263-3272
Published by The Company of Biologists 2004
doi:10.1242/dev.01196

Summary

Translational control is an essential mechanism of gene control utilized throughout development, yet the molecular mechanisms underlying translational activation and repression are poorly understood. We have investigated the translational control of the *C. elegans caudal* homolog, *pal-1*, and found that GLD-1, a member of the evolutionarily conserved STAR/Maxi-KH domain family, acts through a minimal *pal-1* 3′ UTR element to repress *pal-1* translation in the distal germline. We also provide data suggesting that GLD-1 may repress *pal-1* translation after initiation. Finally, we show that GLD-1 represses the distal germline expression of the KH domain protein MEX-3, which was previously shown to repress PAL-1 expression in the proximal germline and which appears specialized to control PAL-1 expression patterns in the embryo. Hence, GLD-1 mediates a developmental switch in the control of PAL-1 repression, allowing MEX-3 to accumulate and take over the task of PAL-1 repression in the proximal germline, where GLD-1 protein levels decline.

Key words: GLD-1, PAL-1, Translational control, *C. elegans*, STAR/Maxi-KH Domain, Post-initiation repression

Introduction

The regulation of mRNA translation is an essential mechanism of gene control utilized in diverse processes ranging from metabolism to cell growth and differentiation. Translational control offers clear advantages in enucleate and quiescent cells, but it is equally valuable in transcriptionally active cells, where it affords precise spatial and temporal control of protein expression. Some of the best-characterized examples of translational regulation come from studies on *Drosophila* anterior-posterior axis formation, which is controlled by morphogen gradients established through spatially restricted translational activation and repression. For example, *bicoid* mRNA is localized to the anterior pole of the oocyte and is translated only after fertilization to yield an anterior-to-posterior protein gradient (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988). In contrast, *caudal* mRNA is uniformly localized, and Caudal protein is present in a reciprocal posterior-to-anterior gradient as a result of translational repression by Bicoid (Mlodzik and Gehring, 1987; Dubnau and Struhl, 1996; Rivera Pomar et al., 1996).

Studies on translational control mechanisms have repeatedly converged on regulatory elements in the 5′ and 3′ untranslated regions (UTRs). Through both genetic and biochemical studies, numerous examples of UTR regulatory elements and their associated trans-acting factors have been discovered (reviewed by Wickens et al., 1996). One of the best-understood examples of 5′ UTR-mediated repression is iron response element (IRE)-mediated repression of ferritin synthesis. Under low cellular iron levels, the iron regulatory protein binds the IRE in the ferritin mRNA 5′ UTR and is thought to sterically inhibit recruitment of the small ribosomal subunit (Gray and Hentze, 1994; Muckenthaler et al., 1998). 3′ UTR-mediated control is more prevalent than 5′ UTR-mediated control in developmentally regulated genes (Wickens et al., 1996). The key role of the 3′ UTR is not surprising, given that the 3′ UTR is less evolutionarily constrained than both the coding sequence and the 5′ UTR; the coding sequence must code for a functional polypeptide, while the 5′ UTR must have a secondary structure that is amenable to ribosome scanning. Recent data suggest factors bound to 3′ UTRs may control translation in part, by regulating the interaction of eIF4E, the 5′ cap binding protein, with eIF4G, the adaptor protein that binds eIF4E and recruits the small ribosomal subunit via its interaction with eIF3. For example, in *Xenopus*, many maternal mRNAs are regulated by a 3′ UTR element called the CPE (cytoplasmic polyadenylation element) and the CPE binding protein, CPEB (Mendez and Richter, 2001). A ternary complex of CPEB, Maskin and eIF4E is hypothesized to circularize mRNAs and repress translation initiation by masking the eIF4G binding site on eIF4E (Stebbins-Boaz et al., 1999). Similarly, in *Drosophila*, Bicoid is hypothesized to simultaneously bind eIF4E and the *caudal* 3′ UTR, thereby preventing recruitment of eIF4G to *caudal* mRNA (Niessing et al., 2002).

Patterning of the *C. elegans* embryo relies upon the asymmetric distribution of maternal regulatory proteins among early blastomeres. Translational control is implicated in asymmetric expression of maternal PAL-1, the Caudal

homolog. Like Caudal, PAL-1 is expressed in the posterior of the embryo where it functions in posterior patterning (Hunter and Kenyon, 1996; Edgar et al., 2001). PAL-1 protein is first detected in posterior blastomeres beginning at the four-cell stage, even though *pal-1* mRNA is present in all cells of the early embryo (Hunter and Kenyon, 1996). Translational control of PAL-1 expression is suggested by the ability of the *pal-1* 3′ UTR to restrict the expression of a *lacZ* reporter RNA to posterior blastomeres; *lacZ* RNA injected into hermaphrodite gonads is expressed in all blastomeres, whereas *lacZ::pal-1* 3′ UTR RNA is expressed primarily in posterior blastomeres (Hunter and Kenyon, 1996).

Control of maternal PAL-1 expression must begin in the germline to ensure that embryos do not inherit PAL-1 protein. The gonad consists of two U-shaped arms in which presumptive oocyte nuclei progressively mature while moving from the distal to the proximal region of each arm (Fig. 1A). In the distal region, syncytial nuclei progress from mitosis to meiotic pachytene, while in the proximal region, nuclei complete meiosis I and become cellularized as they mature into oocytes. *pal-1* mRNA is present throughout the germline (Nematode Expression Pattern DataBase (NEXTDB), http://nematode.lab.nig.ac.jp), yet PAL-1 protein is observed only transiently; low levels are observed in immature oocytes found in the bend of the gonad arm (Fig. 1A) (D.M. and C.P.H., unpublished). *mex-3* is hypothesized to repress *pal-1* translation in mature oocytes and early embryos. PAL-1 is present at high levels in oocytes and all cells of early embryos that lack maternal *mex-3*, and expression of *lacZ::pal-1* 3′ UTR RNA is not restricted to posterior blastomeres in *mex-3* mutant embryos (Hunter and Kenyon, 1996). Interestingly, *mex-3* encodes a putative RNA binding protein, containing two KH (K homology) domains, that is expressed cytoplasmically in a pattern complementary to that of PAL-1 (Draper et al., 1996). Hence, MEX-3 may directly mediate the translational repression of *pal-1* in oocytes and early embryos.

It is unclear how PAL-1 expression is repressed in the hermaphrodite gonad prior to the appearance of MEX-3. MEX-3 is detected only in the proximal gonad arm (Draper et al., 1996), and no ectopic PAL-1 is detected in the distal gonad arm of *mex-3* mutant hermaphrodites (D.M. and C.P.H., unpublished). We have investigated PAL-1 repression in the distal gonad arm and here we provide evidence that a member of the conserved STAR/Maxi-KH domain family, GLD-1, binds a minimal element in the *pal-1* 3′ UTR and represses *pal-1* translation. We also show that GLD-1 represses the distal germline expression of MEX-3, thus allowing MEX-3 to accumulate and take over the task of PAL-1 repression in the proximal gonad arm where GLD-1 is absent. Finally, we provide data suggesting that GLD-1 may repress translation after initiation.

Materials and methods

Nematode strains and culture

Standard culture techniques were used (Brenner, 1974). The following strains were used: Bristol N2 (wild-type), JK1466 [*gld-1(q485)/dpy-5(e61) unc-13(e51)*], BS1077 [*ozEx54* [GLD-1::GFP-FLAG, unc-119(+)]; *unc-13(e51) gld-1(q485)*], AZ212 [*unc-119(ed3)* ruIs32[*unc-119(+) pie-1::GFP::H2B*] and HC93 [*unc-119 (ed3) qtIS5*[*unc-119(+) pie-1::GFP::H2B::2X GRE*]. Worms for sedimentation and RNase protection experiments were synchronized as L1 larvae by allowing embryos to hatch in the absence of food. Worms of the appropriate developmental stages (verified at 250× magnification) were cleaned by sucrose flotation, washed 3× with M9 Buffer and 1× with water, and flash-frozen.

Reporter RNA experiments

All *pal-1* 3′ UTR sequence was cloned into pJK370 which bears an NLS::*lacZ* coding sequence and a poly(A) tract of 30 residues. Residue 1 in Fig. 2 corresponds to the third residue following the *pal-1* stop codon. Capped RNAs were synthesized essentially as described previously (Evans et al., 1994) and poly(A)+ purified using Oligotex kits (Qiagen) according to manufacturer's instructions, with the exception that RNA was not denatured before incubation with Oligotex resin. RNA concentration was determined spectrophotometrically, and RNA integrity was verified by electrophoresis. Two or more preparations of RNA were injected for each construct. RNAs (50 nM) were injected into the distal gonad arm, and worms were stained with X-gal (Fire et al., 1990) after a 6-hour recovery at 25°C. In situ hybridizations were performed 75-90 minutes following injections using a *lacZ* antisense probe as described previously (Seydoux and Fire, 1995). Strong signal in the in situ hybridization experiments was defined as being visible through a Nomarski filter at 100×.

Transformation and RNAi

The GH::2X GRE construct was created by inserting two copies of the GRE and the *pal-1* cleavage and polyadenylation signal after the stop codon of pKS114 (kindly provided by K. Subramanian and G. Seydoux), which is derived from pJH4.52 (G. Seydoux, personal communication), yet lacks the *pie-1* 3′ UTR. The extra-chromosomal array line was generated using the complex array method (Kelly et al., 1997) and the integrated line used in RNase protection assays was created using microparticle bombardment (Praitis et al., 2001). RNAi was performed using bacteria expressing RNA hairpins (Winston et al., 2002). Worms were placed on RNAi bacteria as L4 larvae and the F_1 adults exhibited highly penetrant *gld(–)* and *mex-3(–)* phenotypes. For the double RNAi, bacteria were mixed together in equal volumes.

Gradients

Sucrose gradient buffer contained 50 mM Tris-HCl (pH 8.5), 25 mM KCl, 10 mM $MgCl_2$, and 1% protease inhibitor cocktail (Sigma, P8215) (EDTA was added to 0.1 M to some gradients). Linear gradients were made by overlaying 5.5 mls 0% sucrose gradient buffer on 5.5 mls 56% sucrose gradient buffer in a 14×89 mm tube. Tubes were first placed horizontally for 2 hours at room temperature, and then placed vertically for 30-45 minutes at 4°C (the linearity of the gradients made by this method was confirmed by measuring the refractive index of multiple fractions). 0.5 ml was removed from the gradient tops before sample loading. Polysome extracts were prepared by grinding frozen worms to a fine powder using a mortar and pestle on dry ice. The resulting worm powder was added to 3-4 volumes of ice-cold buffer consisting of 0.2 M Tris-HCl (pH 8.5), 50 mM KCl, 25 mM $MgCl_2$, 1 mM DTT, 2 mM EGTA, 0.2 mg/ml heparin, 500 U/ml RNasin, 2% PTE, and 1% protease inhibitor cocktail (EDTA was added to 0.1 M in some samples). Samples were centrifuged for 10 minutes at 16,000 ***g*** and supernatant was layered on a sucrose gradient. Gradients were centrifuged at 4°C for 1.5 hours at 40,000 rpm in a SW-41 rotor. 0.2 or 0.5 ml fractions were collected manually from the top of the gradients and RNA was extracted using Trizol (Gibco). Absorbance (260 nm) measurements were made on an aliquot of each purified RNA fraction. Pooled fractions were analyzed for *pal-1* or *mex-3* mRNA using Ambion's RPAIII kit. Metrizamide gradient analysis was performed as described by Olsen and Ambros (Olsen and Ambros, 1999). Ribosomal fractions were identified by the index of refraction as described by Olsen and Ambros (Olsen and Ambros, 1999). Only the top 90% of the gradient was analyzed

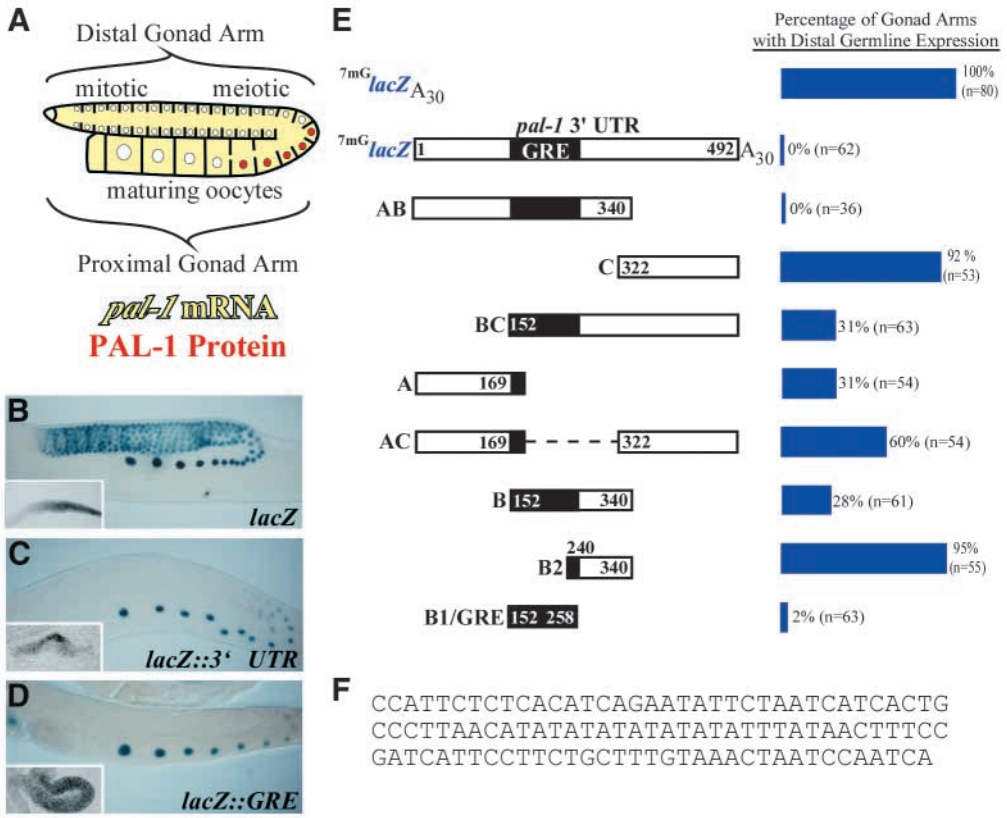

Fig. 1. Multiple regions of the *pal-1* 3′ UTR repress translation in the distal gonad arm. (A) Schematic of an adult gonad arm illustrating the progressive maturation of germ cells. *pal-1* mRNA (yellow) is present throughout the germline, yet PAL-1 protein (red) is detectable only in immature oocytes (47% of gonad arms (n=49) exhibit no PAL-1 expression). (B-D) Gonad arms injected with *lacZ*, *lacZ*::3′ UTR and *lacZ*::GRE RNA, respectively, and stained with X-gal. Insets show dissected distal gonad arms that were injected with the same RNAs (except in D, where *lacZ*::2X GRE RNA was used) and hybridized with *lacZ* antisense probe. (E) Schematics of *lacZ* reporter constructs with blue bars denoting the percentage of injected gonad arms with distal germline expression. Regions A, B, and the GRE mediate repression. (F) Sequence of the GRE.

because of the high metrizamide viscosity at the bottom. Fractions analyzed by western blot were concentrated by TCA precipitation.

Other methods

Antibody staining was performed as described in Huang et al. (Huang et al., 2002). The biotin pull-down assays were performed essentially as described by Lee and Schedl (Lee and Schedl, 2001), except that 4 picomoles of each RNA were used and worms were fragmented using a mortar and pestle as described above.

Results

The *pal-1* 3′ UTR is sufficient to confer translational repression in the distal gonad arm

To determine which regions of *pal-1* mRNA are required for germline repression, we used a reporter RNA assay in which *lacZ* RNAs containing *pal-1* sequence were injected into the distal gonad arm. The *pal-1* 3′ UTR was sufficient to repress distal germline expression, since *lacZ*::3′ UTR RNA was never expressed in the distal germline (n=62), while control *lacZ* RNA was always expressed throughout the distal germline (n=80) (Fig. 1B,C,E). However, unlike endogenous PAL-1, *lacZ*::3′ UTR RNA was expressed throughout the proximal germline, perhaps reflecting perdurance of β-galactosidase transiently translated in immature oocytes and/or the need for additional *pal-1* sequence to promote subsequent repression.

Several lines of evidence suggest that the absence of distal germline expression observed following injection of *lacZ*::3′ UTR RNA is due to translational repression, rather than RNA degradation or transport into the proximal germline. First, similarly strong β-galactosidase signal was detected in oocytes and embryos following injection of *lacZ*::3′ UTR RNA and *lacZ* RNA, suggesting that the *lacZ*::3′ UTR RNA was not subject to significant degradation. Second, β-galactosidase was detected in oocytes as late as 8 hours post-injection of *lacZ*::3′ UTR RNA (signal in oocytes is first detected 70 minutes post-injection). Since germline nuclei are continuously passing from the distal to the proximal gonad arm, this result suggests that the RNA translated in the proximal germline at the end of the 8-hour period was present in the distal germline throughout much of the incubation period. Finally, *lacZ* and *lacZ*::3′ UTR RNA were detected in the distal germline by in situ hybridization in a comparable percentage of gonad arms (90%, n=31 and 79%, n=63, respectively) (Fig. 1B,C, insets).

It is possible that the detected *lacZ*::3′ UTR RNA was degraded and not competent for translation, therefore we determined the efficiency with which unstable RNA can be detected. Since uncapped RNAs are translated inefficiently and degraded rapidly in multiple systems (reviewed by Mitchell and Tollervy, 2000), we injected uncapped *lacZ* RNA and found that it was not expressed in the germline (n=16) and was inefficiently detected by in situ hybridization. Only 33% (n=27) of injected gonad arms exhibited signal, and of these, only one (4% of total) exhibited strong signal. This is in contrast to the high percentage of gonad arms that exhibited a strong in situ signal following injection of *lacZ* or *lacZ*::3′ UTR RNA (89% and 62%, respectively). These data indicate that degraded RNA yields a minimal in situ signal, and suggest that the *lacZ*::3′ UTR RNA is intact, but translationally repressed.

Multiple *pal-1* 3′ UTR elements inhibit germline expression

To identify regions of the *pal-1* 3′ UTR responsible for this

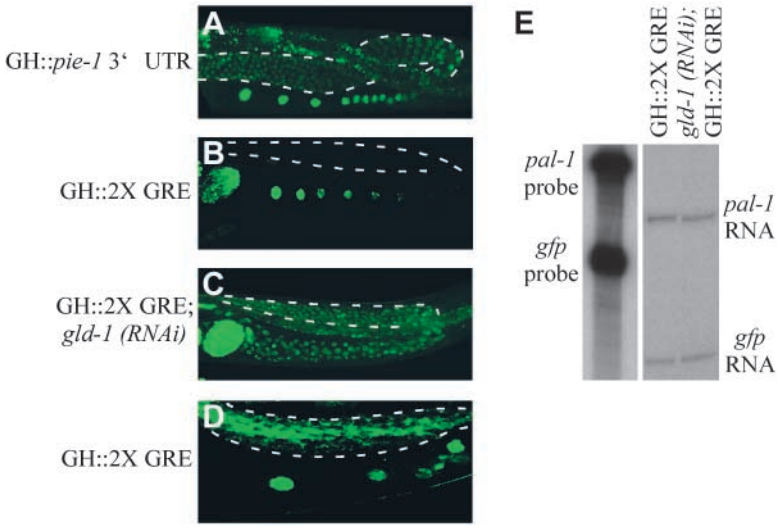

Fig. 2. *gld-1* mediates the translational repression activity of the GRE. The distal gonad arms are outlined and the autofluorescent intestine is visible in A and C. (A) GH::*pie-1* 3′ UTR is expressed in nuclei throughout the proximal and distal germline. (B) GH::2X GRE is expressed in proximal germline nuclei, but not distal germline nuclei. (C) GH::2X GRE is ectopically expressed in distal germline nuclei following *gld-1* RNAi. The characteristic *gld-1*(–) proximal germline tumor is evident. (D) Multi-second exposure of a dissected GH::2X GRE gonad arm. Weak expression is observed in the distal germline cytoplasm. (E) RNase protection assays with *pal-1* and *gfp* probes and 60 μg of total RNA from wild-type or *gld-1 (RNAi)* GH::2X GRE L4 larvae (integrated transgene). *gld-1* does not destabilize *pal-1* or GH::2X GRE mRNA.

translational repression activity, we performed deletion analysis of the *lacZ*::3′ UTR RNA. For ease of discussion, the first, middle, and last thirds of the 3′ UTR have been designated A, B and C, respectively. These thirds average 175 nucleotides in length and they overlap by approximately 18 nucleotides (Fig. 1E). Region C has minimal germline repression activity, as full germline repression is maintained when region C is deleted (construct AB, Fig. 1E), and *lacZ* RNA bearing region C alone is expressed in the distal germline of 92% (n=53) of injected gonad arms.

In contrast, both region A and region B are necessary for complete distal germline repression. *lacZ*::3′ UTR RNA is never expressed in the distal germline, whereas *lacZ*::3′ UTR RNAs lacking region A or B (constructs BC and AC) are expressed in the distal germline in 31% and 60% of gonad arms, respectively. Importantly, like the *lacZ*::3′ UTR RNA, these two constructs (and all other deletion constructs) were expressed in the proximal germline, thus showing that functional reporter RNAs were injected. The germline repression activities of region A and B are further supported by the observation that constructs that contain either region A or B in isolation were sufficient to repress distal germline expression of *lacZ* reporter RNA in approximately two-thirds of gonad arms.

A minimal germline repression element (GRE) represses translation

Deletion of region B from the *lacZ*::3′ UTR construct impaired repression more significantly than deletion of region A, therefore we further mapped repression activity within region B. We found that the second half of B (B2) lacks repression activity, while the first half (B1) robustly inhibits germline expression; *lacZ*::B1 RNA was expressed in the distal germline in only 2% (n=63) of gonad arms (Fig. 1D,E). Hence, B1, a 107-nucleotide element (Fig. 1F), is both necessary and sufficient for robust germline repression and hereafter B1 will be referred to as the germline repression element, or GRE. Importantly, *lacZ* RNA bearing two copies of the GRE is efficiently detected in the distal germline by in situ hybridization, suggesting that the GRE represses translation, as opposed to regulating RNA stability or localization (Fig. 1D, inset).

GLD-1 mediates the translational repression activity of the GRE

To understand how the GRE promotes translational repression, we sought to identify trans-acting factors that mediate its activity. A clear candidate is GLD-1, a cytoplasmic RNA binding protein (STAR/Maxi-KH domain protein) that represses the expression of at least four genes (Jones and Schedl, 1995; Jones et al., 1996; Jan et al., 1999; Lee and Schedl, 2001; Xu et al., 2001; Marin and Evans, 2003). GLD-1 is expressed in the distal, but not the proximal germline, and thus is expressed at the right time and place to be a *pal-1* repressor (Jones et al., 1996). To assess the genetic interaction between GLD-1 and the GRE, we constructed a transgenic line in which expression of a GFP::histone H2B fusion protein is driven by a germline promoter (*pie-1*) and under the regulation of two tandem copies of the GRE (GH::2X GRE). The expression pattern of this reporter recapitulated that of the *lacZ*::GRE reporter, as nuclear GFP was observed throughout the proximal germline, but not the distal germline (Fig. 2B). Importantly, a similar transgene that differs only by the presence of the *pie-1* 3′ UTR instead of the *pal-1* GREs is expressed in nuclei throughout both the distal and proximal germline (Fig. 2A), indicating that the lack of distal germline expression of the GFP::2X GRE reporter is due to the repression activity of the GRE. In addition, upon long exposures (several seconds) of dissected GH::2X GRE distal gonad arms using a CCD camera, we noted weak cytoplasmic GFP expression (Fig. 2D), which is discussed below.

Following bacterially mediated RNA interference (RNAi) of *gld-1* in GH::2X GRE worms, GFP was detected in virtually all nuclei of the distal germline (Fig. 2C), indicating that GLD-1 acts through the GRE to inhibit distal germline expression. Because *gld-1* null adults have a proximal germline tumor which is formed by nuclei that exit meiotic pachytene and proliferate mitotically (Francis et al., 1995a), we considered the possibility that the ectopic GH::2X GRE expression in the distal germline was a secondary consequence of the aberrant proximal germline development. However, we eliminated this explanation because we observed ectopic reporter expression at the extreme distal ends of the adult gonad, where germline development appears normal, and because we observed ectopic

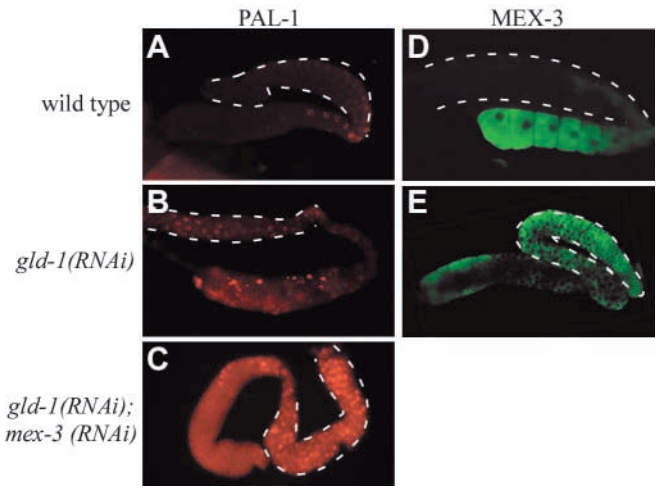

Fig. 3. *gld-1* represses the distal germline expression of both PAL-1 and MEX-3. The distal gonad arms are outlined. (A,B,D,E) Anti-PAL-1 (A,B) and anti-MEX-3 (D,E) staining in wild-type (A,D) and *gld-1(RNAi)* (B,E) gonad arms. (A,D) No expression is detected in the distal germline. PAL-1 is detectable in immature oocytes in 53% of gonad arms (*n*=49) and the staining shown here is an extreme example illustrating the maximal PAL-1 expression observed. (B,E) Ectopic expression is observed in the distal germline. (C) Anti-PAL-1 staining in a *gld-1 (RNAi)*; *mex-3 (RNAi)* gonad arm. Compared to *gld-1* RNAi worms, significantly more ectopic PAL-1 is observed in the distal germline (see text for quantitation).

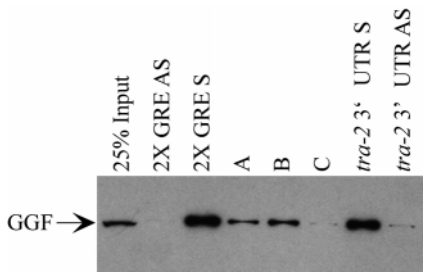

Fig. 4. GLD-1 selectively binds *pal-1* 3′ UTR elements that mediate germline repression in vivo. Biotinylated 3′ UTR RNA fragments were incubated with extracts prepared from adults expressing a rescuing GLD-1::GFP::FLAG fusion protein (GGF). Biotinylated RNA was isolated using streptavidin magnetic beads and bound proteins were subjected to SDS-PAGE and western analysis with anti-FLAG antibody. S, sense RNA; AS, antisense RNA. This assay shows the expected specificity, as GGF is captured by *tra-2* 3′ UTR RNA that bears GLD-1 binding sites, but not by a *tra-2* antisense RNA. 2X GRE, A, and B RNAs capture GGF, while antisense 2X GRE and C RNAs do not, showing that binding activity in extracts correlates with in vivo repression activity.

expression in *gld-1* L4 larvae, which do not yet have a tumorous germline (Francis et al., 1995a) (data not shown). To determine whether *gld-1* controls the stability or the translation of GH::2X GRE RNA, we quantified the amount of GH::2X GRE in wild type and *gld-1* RNAi worms. Duplicate RNase protection assays indicated that the amount of reporter RNA and endogenous RNA was not significantly changed following *gld-1* RNAi, suggesting that GLD-1 represses translation as opposed to destabilizing the RNA (Fig. 2E).

GLD-1 also represses the expression of endogenous PAL-1 in the distal germline, as ectopic PAL-1 immunofluorescence was detected in the distal germline of adult *gld-1* null mutants (*q485*) and RNAi worms (Fig. 3B). This ectopic PAL-1 signal was restricted to small clusters of nuclei instead of being distributed throughout the distal germline, like the ectopic GFP expression of GH::2X GRE reporter following *gld-1* RNAi. We hypothesized that derepression of endogenous *pal-1* translation was incomplete in *gld-1* mutants because of additional repression elements, and asked whether *mex-3*, which functions to repress PAL-1 expression in the proximal germline, is ectopically active in the distal germline of *gld-1* mutants. Indeed, we observed ectopic MEX-3 throughout the distal germline of *gld-1* null mutants (*q485*) and RNAi worms (*n*=59), and we observed a clear increase in ectopic PAL-1 in the distal germline of *mex-3(RNAi); gld-1(RNAi)* worms (Fig. 3C,E). In 100% of *gld-1 (q485)* and RNAi worms, between 0-50 distal germline nuclei express PAL-1 (*n*=63), whereas in 71% of *gld-1(RNAi)*; *mex-3(RNAi)* worms, between 50-200+ distal germline nuclei express PAL-1 (*n*=41). Hence, in wild-type worms, *gld-1* inhibits both PAL-1 and MEX-3 expression in the distal germline, while MEX-3 inhibits PAL-1 expression in the proximal germline. Interestingly, a leaky switch from GLD-1 to MEX-3-mediated repression may account for the transient PAL-1 expression in the bend of the gonad arm (Fig. 1A), which is precisely where GLD-1 levels decline and MEX-3 levels rise (Draper et al., 1996; Jones et al., 1996).

GLD-1 physically interacts with germline repression elements

GLD-1 could repress *pal-1* translation directly by binding the GRE or indirectly by controlling the expression of a *pal-1* regulator that acts through the GRE. To distinguish between these possibilities, we tested the ability of biotinylated GRE RNA to capture a rescuing GLD-1::GFP::FLAG fusion protein (GGF) from adult extracts. Biotinylated RNA was isolated using streptavidin magnetic beads, and bound proteins were subjected to SDS-PAGE and western analysis with anti-FLAG antibody. As shown in Fig. 4 (lanes 7 and 8) and by Lee and Schedl (Lee and Schedl, 2001), this assay shows the expected specificity as GGF is pulled-down by a *tra-2* 3′ UTR RNA that bears GLD-1 binding sites, but not by antisense *tra-2* 3′ UTR RNA (Jan et al., 1999). We found that sense, but not antisense 2× GRE RNA captured GGF (Fig. 4, lanes 2 and 3). Taken with the genetic interactions between GLD-1 and the GRE, this result strongly suggests that GLD-1 binds the GRE in vivo, either by physically contacting the GRE, or by contacting protein(s) that physically contact the GRE. We next tested the GLD-1 binding activity of other 3′ UTR elements and found that binding activity in extracts correlates with in vivo repression activity; regions A and B both efficiently captured GGF, while region C did not (Fig. 4, lanes 4, 5 and 6), suggesting that GLD-1 is a major regulator of *pal-1* translation.

Repressed *pal-1* mRNA co-fractionates with ribosomes

How does GLD-1 repress *pal-1* translation? GLD-1 may interfere with translation initiation or it may interact with the translation machinery after initiation to impair elongation or termination. The weak cytoplasmic expression of the GH::2X GRE reporter in the distal germline (Fig. 2D) is consistent with

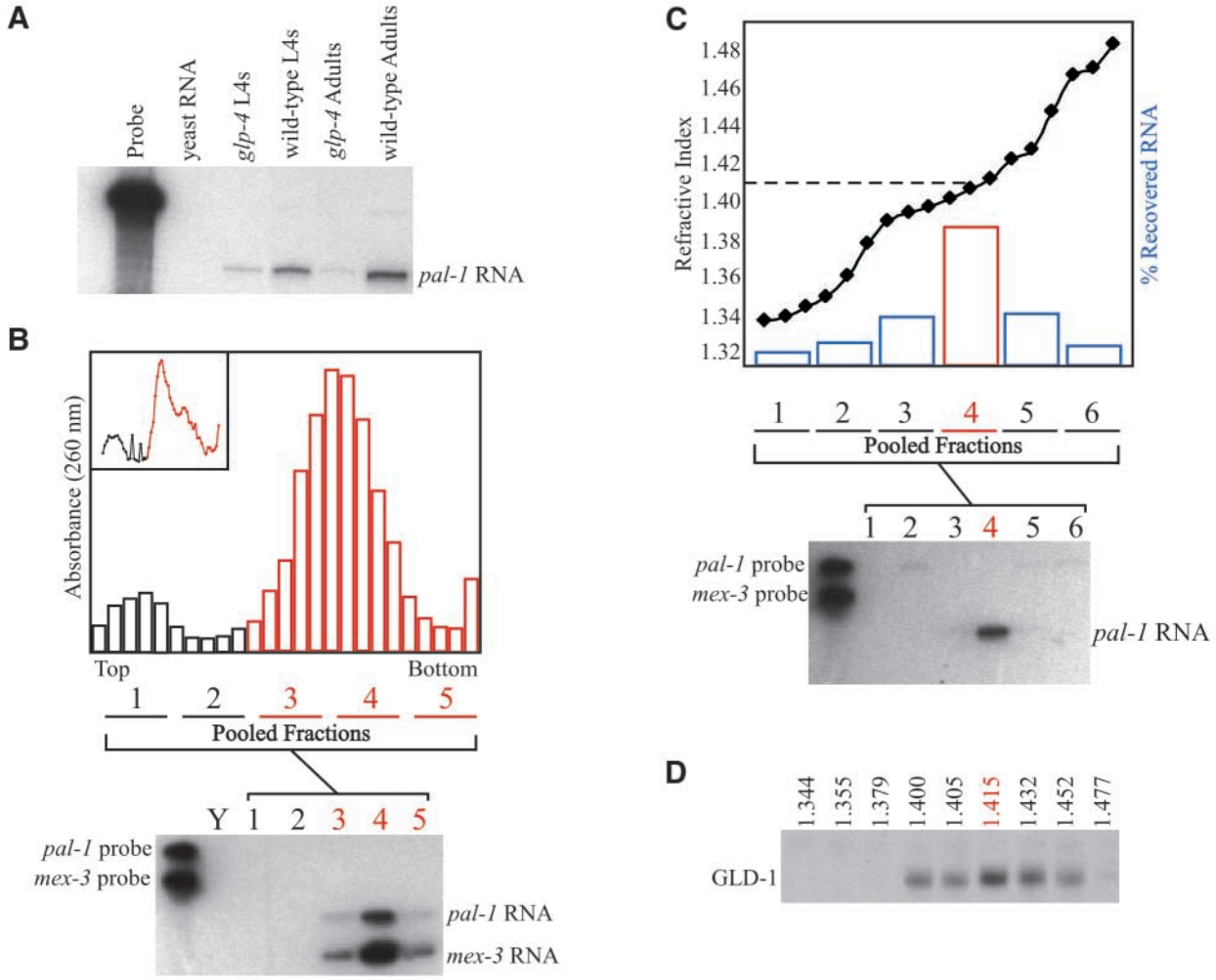

Fig. 5. *pal-1* mRNA and GLD-1 protein co-fractionate with ribosomes. (A) RNase protection assays with *pal-1* probe and 60 μg of yeast RNA or RNA from wild-type and *glp-4 (bn2ts)* L4 larvae and adults. Densitometry of two replicates indicates that approximately 75% of *pal-1* mRNA from wild-type L4s and 95% of *pal-1* mRNA from adults is associated with the germline. (B) A representative histogram illustrating the total RNA in 25 0.5 ml fractions taken manually after worm extracts were fractionated by sucrose density gradient sedimentation. Inset shows the absorbance profile when 56 0.2 ml fractions were taken from a gradient run under the same conditions. The fractions containing ribosomes are indicated in red and bars below the graph indicate the fractions pooled for RNase protection assays. Both *pal-1* and *mex-3* mRNA co-fractionate with ribosomes. Y, control containing only yeast RNA. (C) Metrizamide equilibrium sedimentation of L4 larvae. Curve denotes the refractive index of the gradient fractions, while the bars denote the percentage of total RNA recovered in the pooled fractions. RNase protection assays on pooled fractions indicate that *pal-1* mRNA is found at the predicted refractive index for *C. elegans* ribosomes under these conditions (1.412, indicated by dotted line and red bar). The *mex-3* probe shown in the input lane was not included in the RNase protection assays. (D) Distribution of GLD-1 protein from adult hermaphrodites following metrizamide sedimentation. The index of refraction is indicated above each lane. Western analysis with anti-GLD-1 antibody indicates that GLD-1 is enriched in the predicted ribosome fraction (red).

GLD-1 repressing translation after initiation. We hypothesize that this cytoplasmic GFP signal may represent mature GFP tethered to ribosomes by the incompletely translated histone H2B (C-terminal domain of the fusion protein), as there is precedence for tethered proteins exhibiting activity when their C-termini are extended to allow protrusion from the ribosome exit tunnel (reviewed by Kramer et al., 2001). To test the idea that PAL-1 expression may be inhibited after translational initiation, we asked whether *pal-1* mRNA co-fractionates with polysomes using the following two independent methods of sedimentation: (1) sucrose density sedimentation, which separates cellular components based on size, weight and shape (sedimentation coefficient), and therefore resolves free mRNA, ribosomal subunits, and monosomes, disomes, etc.; and (2) metrizamide equilibrium sedimentation, which separates complexes based on buoyant density and therefore causes monosomes and polysomes of all sizes to co-fractionate. The distribution of ribosomes (monosomes and polysomes) was determined by measuring the amount of total RNA (primarily ribosomal RNA) in fractions taken manually from the gradient tops. The fractions were then pooled and RNase protection assays were performed to determine the distribution of *pal-1* mRNA relative to the ribosomes.

For these experiments, we used worm samples that contained abundant germline *pal-1* mRNA, but no PAL-1 protein – hermaphrodites of the last larval stage (L4), which

synthesize maternal mRNAs but do not yet produce oocytes, and young adult hermaphrodites, which have just completed the L4 molt and do not yet produce oocytes. Germline enrichment of *pal-1* mRNA in L4s and adults is supported by RNase protection experiments indicating that very little *pal-1* mRNA is present in *glp-4 (bn2ts)* L4s and adults, which have a greatly reduced germline (Beanan and Strome, 1992) (Fig. 5A). Duplicate comparisons of wild-type and *glp-4* worms indicate that approximately 75% of *pal-1* mRNA from wild-type L4s and 95% of *pal-1* mRNA from adults is associated with the germline.

We found that virtually all *pal-1* mRNA from L4 larvae and young adults co-fractionated with ribosomes in sucrose gradients. In two independent L4 gradients, 91% and 97% of *pal-1* mRNA was recovered in the ribosome fractions, while in the young adult gradient, 93% of *pal-1* mRNA was recovered in the ribosome fractions (Fig. 5B and data not shown). Because the absorbance of only 25 gradient fractions was measured, our analysis did not resolve the ribosomal subunits or resolve monosomes from disomes, etc., so we refer to monosomes and polysomes collectively as ribosomes. When we manually took 56 fractions, a more typical pattern of RNA absorbance was observed (Fig. 5B, inset). Importantly, *pal-1* mRNA was not restricted to the first ribosome fraction, but was instead distributed throughout the ribosome fractions. To test whether the co-sedimentation of *pal-1* mRNA with ribosomes is consistent with *pal-1* mRNA physically associating with ribosomes, we fractionated extracts in the presence of EDTA, which causes ribosomal subunits to dissociate and release mRNAs. Virtually all *pal-1* mRNA (over 90%) was shifted to slower-sedimenting fractions after EDTA treatment (data not shown), suggesting that repressed *pal-1* mRNA may be physically associated with ribosomes in L4s and young adults. However, it is also possible that *pal-1* mRNA is associated with other heterogeneous, fast-sedimenting mRNP complexes that are EDTA sensitive.

Similar results were obtained from L4 larval extracts fractionated on a metrizamide gradient (Fig. 5C). *pal-1* mRNA was found in the same fraction as monosomes and polysomes of all sizes (fraction four), whose position is inferred both from the total RNA peak (ribosomal RNAs) and from the index of refraction of *C. elegans* ribosomes under these conditions (Olsen and Ambros, 1999). In addition, to determine whether GLD-1 may mediate post-initiation repression, we assayed metrizamide gradient fractions by immunoblot and found that GLD-1 protein from adults was enriched in the ribosome containing fraction (Fig. 5D).

gld-1 as a general repressor in the distal gonad arm

Because *gld-1* inhibits the distal germline expression of the *pal-1* regulator MEX-3, we asked whether *gld-1* also inhibits the distal germline expression of proteins that repress PAL-1 expression in the early embryo: SPN-4, MEX-5 and MEX-6 (Huang et al., 2002). SPN-4 is an RRM protein (Gomes et al., 2001), while MEX-5 and MEX-6 are 70% identical CCCH zinc finger proteins with overlapping functions (Schubert et al., 2000). Because the individual functions of MEX-5 and MEX-6 have not been defined with respect to PAL-1 regulation, we refer to these proteins collectively as MEX-5/6. SPN-4 and MEX-5/6 are expressed in the proximal, but not distal, gonad arms of wild-type adults (N. N. Huang and C.P.H., unpublished)

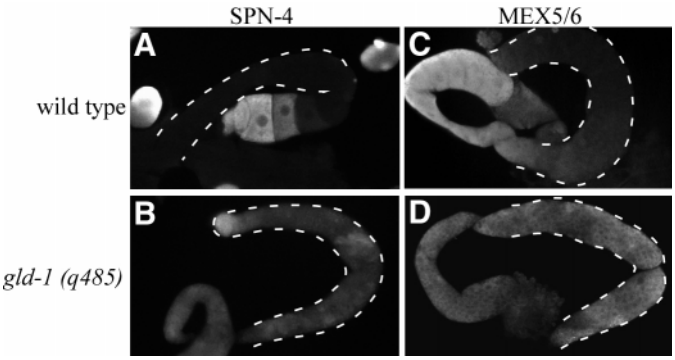

Fig. 6. *gld-1* represses the distal germline expression of SPN-4 and MEX5/6. The distal gonad arms are outlined. (A,B) Anti-SPN-4 and (C,D) anti-MEX5/6 staining in wild-type (A,C) and *gld-1(q485)* (B,D) gonad arms. Expression is not detected in the distal germline of wild type (A,C) but ectopic expression is observed in *gld-1(q485)* (B,D).

(Schubert et al., 2000) (Fig. 6A,C). In *gld-1* null mutants (*q485*), we observed that 80% (*n*=25) and 100% (*n*=28) of gonad arms exhibited ectopic SPN-4 and MEX5/6 expression, respectively (Fig. 6B,D). Hence, *gld-1* directly or indirectly represses the distal germline expression of all three proteins known to repress PAL-1 expression at later developmental stages.

To begin to understand how *gld-1* represses the expression of proteins other than PAL-1, we asked whether repressed *mex-3* mRNA, like *pal-1* mRNA, co-purifies with ribosomes. Indeed, when extracts made from young adults (lacking oocytes) were fractionated via sucrose density gradient sedimentation, virtually all *mex-3* mRNA co-fractionated with ribosomes (Fig. 5B). This sedimentation profile of *mex-3* mRNA was abolished when ribosomes were disrupted by EDTA treatment (data not shown), consistent with the idea that like *pal-1*, the translation of *mex-3* may be inhibited after translation initiation.

Discussion

A developmental switch in PAL-1 repression

Our data suggest a developmental switch in which *pal-1* translation is blocked in the distal germline by a broad spectrum translation repressor, the STAR/Maxi-KH domain protein GLD-1, and subsequently blocked in the proximal germline by a more specific repressor, the KH domain protein MEX-3 (Fig. 7). Our classification of GLD-1 as a broad spectrum repressor derives from our current finding that GLD-1 repress the distal germline expression of four proteins – PAL-1, MEX-3, SPN-4 and MEX-5/6 – combined with previous work showing: (1) that GLD-1 represses the expression of four other proteins in the distal germline (Jan et al., 1999; Lee and Schedl, 2001; Xu et al., 2001; Marin and Evans, 2003) and (2) that 13 additional mRNAs are enriched following GLD-1 immunoprecipitation (Lee and Schedl, 2001). Hence, GLD-1 appears to function as a general repressor in the distal germline, providing a common mechanism for repressing the expression of maternal mRNAs whose products are not needed until oocyte maturation or embryogenesis. Such a broad spectrum repressor conserves limited maternal resources and may have

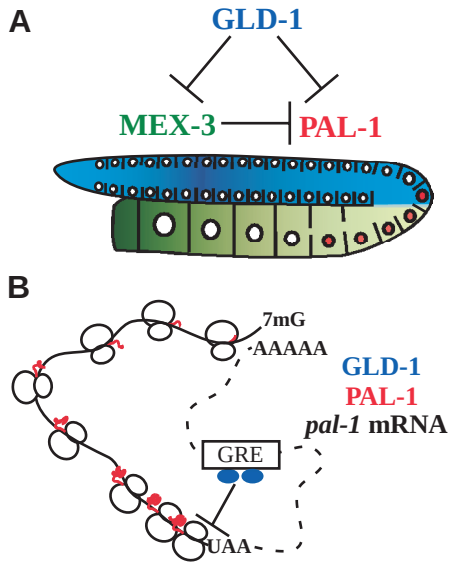

Fig. 7. Proposed model for PAL-1 repression in the germline. (A) GLD-1 represses the translation of both *pal-1* and *mex-3* in the distal gonad arm, while MEX-3 represses *pal-1* translation in the proximal gonad arm. GLD-1 and MEX-3, shown in blue and green respectively, have graded expression patterns in the gonad, with lowest levels found in the bend. A leaky switch from GLD-1- to MEX-3-mediated repression may account for the restricted PAL-1 expression (red) in the bend of the gonad arm. (B) GLD-1 binds the GRE and may repress *pal-1* translation after ribosome loading.

evolutionary advantages. Interestingly, GLD-1 represses the expression of MEX-3, thus enabling a switch to MEX-3-mediated repression in the proximal gonad arm, where GLD-1 protein is absent. To our knowledge, this is the first description of a 'hand-off' in which a translational regulator represses a secondary repressor of one of its targets. The switch from GLD-1 to MEX-3-mediated repression appears to be leaky, as PAL-1 expression is often transiently observed in the gonad precisely where GLD-1 levels decline and MEX-3 levels rise. Unlike GLD-1, which has multiple mRNA targets and multiple functions during development, MEX-3's primary role appears to be PAL-1 repression, since the *mex-3(–)* phenotype can largely be suppressed by removing *pal-1* activity (Hunter and Kenyon, 1996).

Translational repression of *pal-1* by GLD-1

lacZ reporter RNA experiments suggest that the *pal-1* 3′ UTR is sufficient to confer translational repression in the distal germline. Specifically, *lacZ*::3′ UTR RNA is detected in the distal germline when no β-galactosidase protein is detected, and both the intensity and the time-course of β-galactosidase expression in the proximal germline suggest translational repression. Through deletion analysis of the *lacZ*::3′ UTR RNA, we identified two regions, A and B, that possess germline repression activity. Within region B, we identified the GRE, a minimal element of 107 nucleotides that robustly inhibits distal germline expression. Similar to the *lacZ*::3′ UTR RNA, *lacZ* RNA under the regulation of two copies of the GRE is stable in the distal germline and is expressed strongly in the proximal germline, suggesting that the GRE is a translational repression element.

The STAR/Maxi-KH domain protein GLD-1 mediates the translational repression activity of the GRE. GLD-1 is specifically precipitated from worm extracts by GRE RNA, and GLD-1 represses the distal germline expression of both PAL-1 and a GH::2X GRE reporter, without destabilizing the respective mRNAs. GLD-1 could be the only regulator of the GRE, since the GH::2X GRE reporter is ectopically expressed in all distal germline nuclei following *gld-1* RNAi, and removal of *mex-3* activity in addition to *gld-1* activity does not noticeably increase the level of ectopic expression (data not shown). In contrast, repression of full-length *pal-1* mRNA may require regulators in addition to GLD-1, as ectopic PAL-1 is detected only in a subset of distal germline nuclei in *gld-1* mutants. Moreover, even following reduction of ectopic *mex-3* activity through *gld-1; mex-3* double RNAi, PAL-1 is still not detected in all nuclei. This apparently incomplete derepression of PAL-1 expression could be due to the failure to eliminate all GLD-1 and MEX-3 protein via double RNAi, or due to the up-regulation of another PAL-1 repressor in the germline of *gld-1* (RNAi) worms. Indeed, we found that SPN-4 and MEX-5/6, which repress PAL-1 expression in embryos, are ectopically expressed in the distal germline following *gld-1* RNAi, and they may contribute to PAL-1 repression. Alternatively, there may be additional protein(s) that normally contributes to PAL-1 repression in the distal gonad arm.

GLD-1 is homologous to a sub-family of KH domain proteins known as the GSG or STAR domain family (Jones and Schedl, 1995), whose members include the evolutionarily conserved Quaking protein, mammalian Sam68 and SF1 and *Drosophila* How (Vernet and Artzt, 1997). The ~200 amino acid STAR domain consists of an enlarged KH RNA-binding domain (maxi-KH domain) flanked by conserved residues on both sides (Vernet and Artzt, 1997). While the functions of these family members are not well understood, they have been implicated in various aspects of RNA metabolism, including mRNA splicing, nuclear export and translation (Arning et al., 1996; Larocque et al., 2002; Saccomanno et al., 1999). Other than GLD-1, only one family member, the mouse Quaking I isoform 6, has thus far been implicated as a translational regulator, and this is based on its ability to repress *tra-2* expression when expressed in *C. elegans* (Saccomanno et al., 1999).

Specificity of GLD-1 regulation

GLD-1 was previously shown to repress the expression of *tra-2*, *rme-2*, *mes-3* and *glp-1* mRNAs (Jan et al., 1999; Lee and Schedl, 2001; Xu et al., 2001; Marin and Evans, 2003). GLD-1 can directly bind the 3′ UTRs of all these mRNAs, yet minimal binding sites with translational repression activity in vivo have been described only for *tra-2* and *glp-1* (Goodwin et al., 1993; Marin and Evans, 2003). The GRE is the third element that mediates *gld-1* repression in vivo, yet it does not share obvious primary or secondary structures with the previously identified elements, and a comparison of all known and putative binding sites reveals only limited homology between two targets, raising the question of how GLD-1 regulates many disparate mRNAs. GLD-1 may specifically bind mRNAs in vivo in cooperation with co-factor(s). For example, the novel F-box-containing protein, FOG-2, is implicated as a co-factor specifically in the regulation of *tra-2* translation. FOG-2 forms a ternary complex with GLD-1 and

the *tra-2* 3′ UTR (Clifford et al., 2000). *fog-2* is not required for *pal-1* repression, as no ectopic PAL-1 was observed in the germline of *fog-2* mutants (data not shown). Alternatively, GLD-1 may be guided to its targets by small non-coding RNAs or microRNAs (miRNAs). The *C. elegans* miRNA *lin-4* is hypothesized to repress *lin-14 and lin-28* expression after translation initiation by binding imperfect complementary elements in 3′ UTRs of these mRNAs (Olsen and Ambros, 1999; Seggerson et al., 2002). In addition, the *Drosophila* and mouse homologs of the Fragile X translational repressor associate with the miRNA *mir2b* and the non-coding RNA *BC1*, respectively (Caudy et al., 2002; Ishizuka et al., 2002; Zalfa et al., 2003). In support of the hypothesis that miRNAs may participate in GLD-1-mediated repression, we have observed ectopic PAL-1 expression in the distal germline of worms with impaired miRNA processing (data not shown).

Post-initiation translational repression

There are four explanations for our observation that translationally repressed *pal-1* mRNA co-fractionates with ribosomes: (1) *pal-1* mRNA could be a component of large heterogeneous ribonucleoprotein particles (mRNPs) that have the density of ribosomes as well as the sedimentation coefficients of polysomes of various sizes; (2) *pal-1* mRNA could be in a mRNP associated with ribosomes actively translating other messages; (3) PAL-1 protein could be degraded co-translationally in a manner dependent on the *pal-1* 3′ UTR but independent of PAL-1 protein sequence (since *lacZ* and *gfp* reporters are accurately regulated); or (4) *pal-1* mRNA may be associated with ribosomes that have slowed or stalled while translating it. The post-initiation repression mechanism is the simplest explanation, and it is the only mechanism that also explains the cytoplasmic GFP signal we observed in the distal germline of animals expressing the GH::2X GRE transgene (Fig. 2D). Reversing the order of the two protein coding regions in this transgene never resulted in GFP signal in the distal germline, but these constructs were poorly expressed in the proximal germline (data not shown). Like *pal-1* mRNA, GLD-1 also co-fractionates with ribosomes, and our working hypothesis is that GLD-1 is a component of a post-initiation repression complex in the distal germline. This derives from the fact that GLD-1 directly or indirectly represses the distal germline expression of at least eight proteins (four described in this paper) and that for at least two of these, PAL-1 and MEX-3, post-initiation repression is implicated.

Although polysome analysis has been performed on one other GLD-1 target, *tra-2* (Goodwin et al., 1993), it is unclear as to whether GLD-1 represses TRA-2 expression before or after translation initiation. For example, the polysome analysis was performed on adults, yet GLD-1 represses *tra-2* translation in larvae (Jan et al., 1999). Also, the interpretation of the data is hindered by the fact that *tra-2* activity is regulated both in the soma and germline of males and hermaphrodites (reviewed by Kuwabara and Perry, 2001). All these populations of mRNA were analyzed together in the polysome analysis, but GLD-1 is required only for the repression of *tra-2* mRNA in the hermaphrodite germline (Francis et al., 1995a; Francis et al., 1995b; Jan et al., 1999). The trans-acting factor(s) required for repression in the male soma and germline are unknown and the mechanism of repression may be different.

There is a small but growing list of mRNAs subject to post-initiation repression, yet GLD-1 is the one of the first trans-acting proteins to be implicated in this process. For example, the mRNAs encoding *C. elegans* LIN-14 and LIN-28 co-fractionate with polysomes when the proteins are not detectable, but no trans-acting proteins involved in repression have been identified (Olsen and Ambros, 1999; Seggerson et al., 2002). Similarly, roughly half of *Drososphila nanos* mRNA is found associated with polysomes in embryos with no detectable protein, and although the RNA-binding protein Smaug can repress Nanos expression, it is not known whether it represses the polysomal or sub-polysomal population of *nanos* mRNA (Simbert et al., 1996; Dahanukar et al., 1999; Clark et al., 2000). Despite the growing number of examples, the mechanisms of post-initiation repression remain unknown. The identification of GLD-1 as a putative regulator of post-initiation repression may provide an inroad for the molecular dissection of this process.

We thank T. Evans and J. Kimble for pJK370, T. Schedl for the BS1077 strain, N. Huang for SPN-4 and MEX5/6 antibodies, K. Subramanian and G. Seydoux for pKS114, B. Goodwin for the *tra-2* 3′ UTR plasmid and anti-GLD-1 antibody, and the *Caenorhabditis* Genetics Center for strains. We also thank V. Ambros for helpful discussions and K. Carniol and C. Molodowitch for critical comments on the manuscript. This work was supported by an NIH grant to C.P.H. (GM616677).

References

Arning, S., Gruter, P., Bilbe, G. and Kramer, A. (1996). Mammalian splicing factor SF1 is encoded by variant cDNAs and binds to RNA. *RNA* **2**, 794-810.

Beanan, M. J. and Strome, S. (1992). Characterization of a germ-line proliferation mutation in C. *elegans*. *Development* **116**, 755-766.

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nusslein-Volhard, C. (1988). The role of localization of bicoid RNA in organizing the anterior pattern of the Drosophila embryo. *EMBO J.* **7**, 1749-1756.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Caudy, A. A., Myers, M., Hannon, G. J. and Hammond, S. M. (2002). Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev.* **16**, 2491-2496.

Clark, I. E., Wyckoff, D. and Gavis, E. R. (2000). Synthesis of the posterior determinant Nanos is spatially restricted by a novel cotranslational regulatory mechanism. *Curr. Biol.* **10**, 1311-1314.

Clifford, R., Lee, M. H., Nayak, S., Ohmachi, M., Giorgini, F. and Schedl, T. (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.

Dahanukar, A., Walker, J. A. and Wharton, R. P. (1999). Smaug, a novel RNA-binding protein that operates a translational switch in *Drosophila*. *Mol. Cell* **4**, 209-218.

Draper, B. W., Mello, C. C., Bowerman, B., Hardin, J. and Priess, J. R. (1996). MEX-3 is a KH domain protein that regulates blastomere identity in early *C. elegans* embryos. *Cell* **87**, 205-216.

Driever, W. and Nusslein-Volhard, C. (1988). A gradient of bicoid protein in Drosophila embryos. *Cell* **54**, 83-93.

Dubnau, J. and Struhl, G. (1996). RNA recognition and translational regulation by a homeodomain protein. *Nature* **379**, 694-699.

Edgar, L. G., Carr, S., Wang, H. and Wood, W. B. (2001). Zygotic expression of the caudal homolog *pal-1* is required for posterior patterning in Caenorhabditis elegans embryogenesis. *Dev. Biol.* **229**, 71-88.

Evans, T. C., Crittenden, S. L., Kodoyianni, V. and Kimble, J. (1994). Translational control of maternal *glp-1* mRNA establishes an asymmetry in the *C. elegans* embryo. *Cell* **77**, 183-194.

Fire, A., Harrison, S. W. and Dixon, D. (1990). A modular set of lacZ fusion vectors for studying gene expression in Caenorhabditis elegans. *Gene* **93**, 189-198.

Francis, R., Barton, M. K., Kimble, J. and Schedl, T. (1995a). *gld-1*, a tumor suppressor gene required for oocyte development in *Caenorhabditis elegans*. *Genetics* **139**, 579-606.

Francis, R., Maine, E. and Schedl, T. (1995b). 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.

Gomes, J. E., Encalada, S. E., Swan, K. A., Shelton, C. A., Carter, J. C. and Bowerman, B. (2001). The maternal gene *spn-4* encodes a predicted RRM protein required for mitotic spindle orientation and cell fate patterning in early *C. elegans* embryos. *Development*. **128**, 4301-4314.

Goodwin, E. B., Okkema, P. G., Evans, T. C. and Kimble, J. (1993). Translational regulation of *tra-2* by its 3′ untranslated region controls sexual identity in *C. elegans*. *Cell* **75**, 329-339.

Gray, N. K. and Hentze, M. W. (1994). Iron regulatory protein prevents binding of the 43S translation pre-initiation complex to ferritin and eALAS mRNAs. *EMBO J.* **13**, 3882-3891.

Huang, N. N., Mootz, D. E., Walhout, A. J., Vidal, M. and Hunter, C. P. (2002). MEX-3 interacting proteins link cell polarity to asymmetric gene expression in *Caenorhabditis elegans*. *Development* **129**, 747-759.

Hunter, C. P. and Kenyon, C. (1996). Spatial and temporal controls target *pal-1* blastomere-specification activity to a single blastomere lineage in *C. elegans* embryos. *Cell* **87**, 217-226.

Ishizuka, A., Siomi, M. C. and Siomi, H. (2002). A *Drosophila* fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev.* **16**, 2497-2508.

Jan, E., Motzny, C. K., Graves, L. E. and Goodwin, E. B. (1999). The STAR protein, GLD-1, is a translational regulator of sexual identity in *Caenorhabditis elegans*. *EMBO J.* **18**, 258-269.

Jones, A. R. and Schedl, T. (1995). Mutations in *gld-1*, a female germ cell-specific tumor suppressor gene in *Caenorhabditis elegans*, affect a conserved domain also found in Src-associated protein Sam68. *Genes Dev.* **9**, 1491-1504.

Jones, A. R., Francis, R. and 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.

Kelly, W. G., Xu, S., Montgomery, M. K. and Fire, A. (1997). Distinct requirements for somatic and germline expression of a generally expressed *Caenorhabditis elegans* gene. *Genetics* **146**, 227-238.

Kramer, G., Ramachandiran, V. and Hardesty, B. (2001). Cotranslational folding – omnia mea mecum porto? *Int. J. Biochem. Cell. Biol.* **33**, 541-553.

Kuwabara, P. E. and Perry, M. D. (2001). It ain't over till it's ova: germline sex determination in *C. elegans*. *BioEssays* **23**, 596-604.

Larocque, D., Pilotte, J., Chen, T., Cloutier, F., Massie, B., Pedraza, L., Couture, R., Lasko, P., Almazan, G. and Richard, S. (2002). Nuclear retention of MBP mRNAs in the quaking viable mice. *Neuron* **36**, 815-829.

Lee, M. H. and 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.

Marin, V. A. and Evans, T. C. (2003). Translational repression of a *C. elegans* Notch mRNA by the STAR/KH domain protein GLD-1. *Development* **130**, 26323-26332.

Mendez, R. and Richter, J. D. (2001). Translational control by CPEB: a means to the end. *Nat. Rev. Mol. Cell. Biol.* **7**, 521-529.

Mitchell, P. and Tollervey, D. (2000). mRNA stability in eukaryotes. *Curr. Opin. Genet. Dev.* **10**, 193-198.

Mlodzik, M. and Gehring, W. J. (1987). Expression of the caudal gene in the germ line of Drosophila: formation of an RNA and protein gradient during early embryogenesis. *Cell* **48**, 465-478.

Muckenthaler, M., Gray, N. K. and Hentze, M. W. (1998). IRP-1 binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the cap-binding complex eIF4F. *Mol. Cell* **2**, 383-388.

Niessing, D., Blanke, S. and Jackle, H. (2002). Bicoid associates with the 5′-cap-bound complex of caudal mRNA and represses translation. *Genes Dev.* **16**, 2576-2582.

Olsen, P. H. and Ambros, V. (1999). The *lin-4* regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol.* **216**, 671-680.

Praitis, V., Casey, E., Collar, D. and Austin, J. (2001). Creation of low-copy integrated transgenic lines in *Caenorhabditis elegans*. *Genetics* **157**, 1217-1226.

Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W. J. and Jackle, H. (1996). RNA binding and translational suppression by bicoid. *Nature* **379**, 746-749.

Saccomanno, L., Loushin, C., Jan, E., Punkay, E., Artzt, K. and Goodwin, E. B. (1999). The STAR protein QKI-6 is a translational repressor. *Proc. Natl. Acad. Sci. USA* **96**, 12605-12610.

Schubert, C. M., Lin, R., de Vries, C. J., Plasterk, R. H. and Priess, J. R. (2000). MEX-5 and MEX-6 function to establish soma/germline asymmetry in early *C. elegans* embryos. *Mol. Cell* **5**, 671-682.

Seggerson, K., Tang, L. and Moss, E. G. (2002). Two genetic circuits repress the *Caenorhabditis elegans* heterochronic gene *lin-28* after translation initiation. *Dev. Biol.* **243**, 215-225.

Seydoux, G. and Fire, A. (1995). Whole-mount *in situ* hybridization for the detection of RNA in *Caenorhabditis elegans* embryos. *Methods Cell Biol.* **48**, 323-337.

Simbert, C. A., Wilson, J. E., Kerr, K. and Macdonald, P. M. (1996). smaug protein represses translation of unlocalized *nanos* mRNA in the *Drosophila* embryo. *Genes Dev.* **10**, 2600-2609.

Stebbins-Boaz, B., Cao, Q., de Moor, C. H., Mendez, R. and Richter, J. D. (1999). Maskin is a CPEB-associated factor that transiently interacts with elF-4E. *Mol. Cell* **4**, 1017-1027.

Vernet, C. and Artzt, K. (1997). STAR, a gene family involved in signal transduction and activation of RNA. *Trends Genet.* **13**, 479-484.

Wickens, M., Kimble, J. and Strickland, S. (1996). Translational control of developmental decisions. In *Translational Contro*l (ed. J. W. B. Hershey, M. B. Mathew and N. Sonenberg), pp. 411-50. Plainview, New York: Cold Spring Harbor Laboratory Press.

Winston, W. M., Molodowitch, C. and Hunter, C. P. (2002). Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science* **295**, 2456-2459.

Xu, L., Paulsen, J., Yoo, Y., Goodwin, E. B. and Strome, S. (2001). *Caenorhabditis elegans* MES-3 is a target of GLD-1 and functions epigenetically in germline development. *Genetics* **159**, 1007-1017.

Zalfa, F., Giorgi, M., Primerano, B., Moro, A., Di Penta, A., Reis, S., Oostra, B. and Bagni, C. (2003). The Fragile X syndrome protein FMRP associates with *BC1* RNA and regulates the translation of specific mRNAs at synapses. *Cell* **112**, 317-327.