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

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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 pachytylene, 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 [glp-1(q485)/dpy-5(e61) unc-13(e3)]; BS1077 [ceEx34 [GLD-1::GFP-FLAG, unc-119(+)]; unc-13(e3) glp-1(q485)], AZ212 [unc-119(ed3) rul-32[unc-119(+)] pce-1::GFP::H2B] and HC935 [unc-119 (ed3) qtd55[unc-119(+)] pce-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 cleared 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 pH4.52 (G. Seydoux, personal communication), yet lacks the pce-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 RNAi protection assays was created using microparticle bombardment (Prairits 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 F1 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 MgCl2, 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 MgCl2, 1 mM DTT, 2 mM EDTA, 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.
GLD-1 represses pal-1 translation

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
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
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).

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 germine nuclei express PAL-1 (n=63), whereas in 71% of gld-1(RNAi); mex-3(RNAi) worms, between 50-200 distal germine 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 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.

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...
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 ribosomes 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 ribosomes. We fractionated extracts in the presence of EDTA, shown), suggesting that repressed slower-sedimenting fractions after EDTA treatment (data not shown), consistent with the idea that pal-1 mRNA was shifted to EDTA sensitive. Virtually all which causes ribosomal subunits to dissociate and release 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) (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 represses 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
GLD-1 represses the translation of both \( \text{pal-1} \) and \( \text{mex-3} \) in the distal gonad arm, while MEX-3 represses \( \text{pal-1} \) 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 in the bend of the gonad arm. (B) GLD-1 binds the GRE and may repress \( \text{pal-1} \) translation after ribosome loading.

### Translational repression of \( \text{pal-1} \) by GLD-1

\( \text{lacZ} \) reporter RNA experiments suggest that the \( \text{pal-1} \) 3’ UTR is sufficient to confer translational repression in the distal germline. Specifically, \( \text{lacZ}::3’ \) 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 \( \text{lacZ}::3’ \) 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 \( \text{lacZ}::3’ \) UTR RNA, \( \text{lacZ} \) 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 \( \text{gld-1} \) RNAi, and removal of \( \text{mex-3} \) activity in addition to \( \text{gld-1} \) activity does not noticeably increase the level of ectopic expression (data not shown). In contrast, repression of full-length \( \text{pal-1} \) mRNA may require regulators in addition to GLD-1, as ectopic \( \text{PAL-1} \) is detected only in a subset of distal germline nuclei in \( \text{gld-1} \) mutants. Moreover, even following reduction of ectopic \( \text{mex-3} \) activity through \( \text{gld-1} \); \( \text{mex-3} \) double RNAi, \( \text{PAL-1} \) is still not detected in all nuclei. This apparently incomplete derepression of \( \text{PAL-1} \) 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 \( \text{gld-1} \) (RNAi) worms. Indeed, we found that \( \text{SPN-4} \) and MEX-5/6, which repress \( \text{PAL-1} \) expression in embryos, are ectopically expressed in the distal germline following \( \text{gld-1} \) RNAi, and they may contribute to \( \text{PAL-1} \) repression. Alternatively, there may be additional protein(s) that normally contributes to \( \text{PAL-1} \) 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 \( \text{tra-2} \), \( \text{rme-2} \), \( \text{mes-3} \) and \( \text{glp-1} \) 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 \( \text{tra-2} \) and \( \text{glp-1} \) (Goodwin et al., 1993; Marin and Evans, 2003). The GRE is the third element that mediates \( \text{gld-1} \) 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 \( \text{tra-2} \) 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 
unhypthesized to repress *lin-14* and *lin-28* expression after 
translation initiation by binding imperfect complementary 
elements in 3' UTRs of these miRNAs (Olsen and Ambros, 
1999; Seggerson et al., 2002). In addition, the *Drosophila*
and mouse homologs of the Fragile X translation repressor 
asociate with the miRNA *mir-2* and the non-coding RNA 
*BCL*, 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 
eterogeneous 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 
translating other messages; (3) PAL-1 protein could be 
is detected by the FRAGILE X repressor 
associate with the miRNA *mir-2* and the non-coding RNA 
*BCL*, 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).

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 *Drosophila 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.

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expression of the caudal homolog *pal-1* is required for posterior patterning 

Translational control of maternal *gfp-1* mRNA establishes an asymmetry in the 


