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Translational repression of a *C. elegans* Notch mRNA by the STAR/KH domain protein GLD-1

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SUMMARY

In *C. elegans*, the Notch receptor GLP-1 is localized within the germline and early embryo by translational control of *glp-1* mRNA. RNA elements in the *glp-1* 3′ untranslated region (3′ UTR) are necessary for repression of *glp-1* translation in germ cells, and for localization of translation to anterior cells of the early embryo. The direct regulators of *glp-1* mRNA are not known. Here, we show that a 34 nucleotide region of the *glp-1* 3′ UTR contains two regulatory elements, an element that represses translation in germ cells and posterior cells of the early embryo, and an element that inhibits repressor activity to promote translation in the embryo. Furthermore, we show that the STAR/KH domain protein GLD-1 binds directly and specifically to the repressor element. Depletion of GLD-1 activity by RNA interference causes loss of endogenous *glp-1* mRNA repression in early meiotic germ cells, and in posterior cells of the early embryo. Therefore, GLD-1 is a direct repressor of *glp-1* translation at two developmental stages. These results suggest a new function for GLD-1 in regulating early embryonic asymmetry. Furthermore, these observations indicate that precise control of GLD-1 activity by other regulatory factors is important to localize this Notch receptor, and contributes to the spatial organization of Notch signaling.

Key words: Translation, Notch, STAR, GLD-1, *Caenorhabdtis elegans*

INTRODUCTION

The regulation of cytoplasmic mRNAs is critical to growth, development and cell function (Gray and Wickens, 1998; Proud, 2001). For many genes, mRNA translation and localization is precisely controlled to specify the timing, levels, and intracellular location of protein activity. In early embryogenesis of several metazoans, embryonic polarity and cell division depend on cascades of mRNA regulation within the cytoplasm of oocytes and embryonic cells (de Moor and Richter, 2001; Wickens, 2000). One common theme among these systems is the control of mRNA translation by elements in the 3′ untranslated regions (3′ UTRs) of specific mRNAs. The mechanisms that mediate 3′ UTR-dependent translational control and that link translational regulation to cell signaling, division and polarity are not well understood.

In the *C. elegans* embryo, post-transcriptional regulation is crucial for the specification of early cell fates (Goodwin and Evans, 1997; Rose and Kemphues, 1998). During the first cell division, polarity within the zygote leads to the localization of cell fate regulators to specific embryonic cells (Fig. 1). For example, the Notch membrane receptor, GLP-1, is localized to anterior cells, while one of its ligands, the Delta-like APX-1, is localized to a single posterior cell (Evans et al., 1994; Mickey et al., 1996). The localization of GLP-1 and APX-1 is probably crucial for spatially constraining cell signaling that regulates anterior cell fates (Mello et al., 1994; Priess et al., 1987). In addition, several transcription factors, including the caudal homolog PAL-1, are localized to posterior cells where they control posterior cell development (Bowerman et al., 1993; Hunter and Kenyon, 1996; Lin et al., 1998). The mRNAs of most of these factors are made in the germline during oogenesis and delivered to all cells of the embryo after fertilization. Thus, translational and/or post-translational controls are critical for distinct patterns of protein localization in the *C. elegans* embryo.

For GLP-1 and PAL-1, localized expression in the embryo is achieved by translational regulation through 3′ UTR elements in their mRNAs (Evans et al., 1994; Hunter and Kenyon, 1996). Both the timing and location of translation is precisely controlled during germ cell development and early embryogenesis (Fig. 1A). After its transcription in the germline, *glp-1* mRNA is distributed to mitotic and meiotic germ cells, oocytes and to all cells of the early embryo (Crittenden et al., 1994; Evans et al., 1994). However, GLP-1 protein is restricted to mitotic germ cells of the distal end of the germline, where it promotes mitotic renewal of germline stem cells (Austin and Kimble, 1987; Crittenden et al., 1994). GLP-1 expression disappears as germ cells enter prophase of meiosis, and continues to be repressed in oocytes and one-cell zygotes. After fertilization, *glp-1* translation begins in the 2-cell embryo but is then restricted to anterior cells (Evans et al.,

1994). The *glp-1* 3′ UTR is sufficient both to repress mRNA translation in germ cells and to localize translation to anterior cells of the embryo (Evans et al., 1994). Different regulatory regions within the *glp-1* 3′ UTR mediate different aspects of *glp-1* translational control (Fig. 1B). A 61 nucleotide (nt) region, called the SCR (for Spatial Control Region), is necessary for repression of translation in posterior cells of the embryo and contains sequences conserved among three nematode species (Evans et al., 1994; Rudel and Kimble, 2001). A separate 129 nt region is necessary for full translational repression in oocytes (Evans et al., 1994). These studies suggest that a complex regulatory system functions through *glp-1* 3′ UTR elements to spatially restrict the GLP-1 receptor to mitotic germ cells and to anterior cells of the early embryo.

The factors that directly control *glp-1* translation are not known. Several genes required for different aspects of *glp-1* regulation have been identified in mutant screens. Genes that control early cell polarity are necessary for GLP-1 localization in the embryo, presumably because they regulate the activities or localization of *glp-1* regulators (Crittenden et al., 1997; Rose and Kemphues, 1998). In addition, two functionally redundant proteins, MEX-5 and MEX-6, act downstream of polarity genes and promote GLP-1 expression in anterior embryonic cells (Schubert et al., 2000). MEX-5 and 6 contain Zn-finger like domains and could be RNA-binding proteins, but it is not known how these factors affect *glp-1* mRNA regulation.

In the germline, the KH-domain protein GLD-1 is required to restrict germ cell mitosis and GLP-1 expression to the distal tip region of the gonad (Crittenden et al., 1994; Francis et al., 1995a). GLD-1 is a member of the STAR family of RNA-binding proteins, several members of which have been linked to regulation of various signaling pathways (Vernet and Artzt, 1997). Germ cells in *gld-1* null mutants fail to progress from early meiotic prophase to oogenesis, and instead they proliferate inappropriately, forming germline tumors (Francis et al., 1995a). These tumorous germlines express GLP-1 throughout the gonad (Crittenden et al., 1994). Because mutations in other genes that cause excessive proliferation also cause ectopic GLP-1 expression (Berry et al., 1997; Crittenden et al., 1994), the increased GLP-1 expression in *gld-1* mutants could be an indirect result of excessive germ cell mitosis. Alternatively, it could be due to a more direct loss of *glp-1* mRNA repression. GLD-1 has been implicated as a direct RNA-binding regulator of translation of other *C. elegans* mRNAs (Jan et al., 1999; Lee and Schedl, 2001; Xu et al., 2001). Moreover, GLD-1 has multiple functions in germ cell development, and has multiple mRNA targets (Francis et al., 1995a; Francis et al., 1995b; Lee and Schedl, 2001). Interestingly, GLD-1 is also expressed in the early embryo and is localized to posterior blastomeres of the embryo (Jones et al., 1996). The embryonic functions of GLD-1 are not known. Thus GLD-1 may control a variety of germ cell and embryonic functions by specific interactions with numerous mRNAs.

In this paper, we show that two distinct types of translational control elements reside within the *glp-1* SCR. One element is required for repression of translation in posterior cells of the embryo, and also contributes to translational repression in the germline. A second element is required for derepression of translation in anterior cells of the early embryo. Furthermore, we show that GLD-1 binds directly and specifically to the repressor element of the SCR, and is required in vivo for repressing GLP-1 expression at two distinct developmental stages. The results suggest a new function for GLD-1 in regulating early embryonic asymmetry, and that functional interactions between GLD-1 and other factors contribute to the localization of this Notch receptor. These interactions are probably important for spatial organization of Notch signaling in both the gonad and early *C. elegans* embryo.

MATERIALS AND METHODS

Strains and plasmids

The *C. elegans* N2 (Bristol) strain was used for all experiments. All plasmids used for making reporter mRNAs derive from pJK370, which is similar to pJK350 (Evans et al., 1994), but has a backbone derived from pPD16.43 (Fire et al., 1990). pTE4.0 contains the full-length wild-type *glp-1* 3′ UTR (Evans et al., 1994). All base substitutions in the full-length *glp-1* 3′ UTR were made in pTE4.0. To make lacunc mRNAs, a *Sal*I to *Bgl*II PCR fragment of the *unc-54* 3′ UTR (Evans et al., 1994) was cloned into pJK370, and synthetic *Xba*I and *Hin*dIII sites were inserted 130 nt upstream of the poly(A) tract to make pTE3.0. The 34 nt wild-type and mutant SCR fragments with *Xba*I and *Hin*dIII ends were cloned into pTE3.0. For synthesis of in vitro RNA probes, tagged RNAs and competitors, a 71nt *Xba*I to *Hin*dIII *glp-1* 3′ UTR fragment, which includes the 61 nt SCR, and the 34 nt SCR sub-fragments were cloned into pBluescript (KSII+). All RNAs made from these vectors also contain a 5′ end of 34 nt vector sequence. A partial *gld-1* cDNA cloned into pBluescript, and a GST-*gld-1* fusion construct were kindly provided by E. B. Goodwin. cDNAs from the *C. elegans gld-1*-like genes T21G5.5 and K07H8.9 were generated by RT-PCR. All DNAs were sequenced by the University of Colorado Cancer Center DNA Sequencing and Analysis Core Facility, which is supported by the NIH/NCI Cancer Core Support Grant (CA46934).

RNA precipitations

All steps were performed at 4°C unless otherwise noted. Synchronized adult hermaphrodites were grown as described previously (Barbee et al., 2002), and were homogenized using a French Press in homogenization buffer (10 mM Hepes pH 7.2, 75 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM EGTA, 50 mM sucrose, 5% glycerol, 1 mM DTT, EDTA-free protease inhibitors; Roche). Homogenates containing 50-100 mg of protein were brought to 600 mM KCl, and then spun at 10,000 ***g*** for 10 minutes. Samples were diluted to 150 mM KCl, with buffer containing 10 mM Hepes pH 7.2, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT. Micrococcal nuclease (15 U/ml) (Amersham Pharmacia) was added and the extracts were incubated at room temperature for 20 minutes. EGTA was added to 2 mM to inhibit the nuclease, and the extract was spun at 100,000 ***g*** for 1 hour. Digoxigenin-RNAs (20 nM) and heparin (1 mg/ml) were incubated with the supernatants for 1 hour. Anti-digoxigenin magnetic particles (1 mg magnetic particles per 35 pmol of RNA; Roche), were pre-washed twice in wash buffer (20 mM HEPES pH 7, 75 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.05% Nonidet P-40), and were then incubated with extracts for 1 hour. The magnetic particles were separated for 5 minutes using a magnetic particle separator (Roche) and the unbound extract was removed. The magnetic beads were washed three times with wash buffer for 10 minutes. Finally, the proteins were eluted twice for 10 minutes using wash buffer with 1 M KCl. The proteins eluted from the RNA precipitations were concentrated and dialyzed with wash buffer using centricon filters (Amicon). Digoxigenin RNA (dig-RNA) was made using T7 Megascript transcription reagents (Ambion) with ATP, GTP and CTP at 6.7 mM, UTP at 5.2 mM, and digoxigenin-11-UTP (Roche) at 1.7 mM. After 4-6 hours of

transcription, DNase I was then added and unincorporated nucleotides were removed by gel filtration.

RNA-binding assays

UV cross-linking

Radiolabeled probes were transcribed with T7 polymerase (Gibco) using α-^{32}P-labeled UTP (2.6 μM), with unlabeled UTP (10 μM), and GTP, ATP and CTP at 0.5 mM. Binding reactions (20 μl) contained 2 μl concentrated eluted proteins with wild-type SCR probe (0.5 nM to 2 nM) in buffer consisting of 10 mM Hepes pH 7, 2 mM $MgCl_2$, 80 mM KCl, 2 mM EGTA, and 1 mM DTT. Binding reactions were incubated on ice for 45 minutes, and then were UV irradiated in 0.5 ml Eppendorf tubes for 5 minutes using 254 nm bulbs in a Stratalinker (Stratagene). RNAse A and RNAse T1 were used to digest the RNA, and the proteins were separated by SDS-PAGE and visualized by phosphoimager.

GST GLD-1 pulldown

GST-GLD-1 fusion protein used for some experiments was a generous gift of E. B. Goodwin, and for other experiments was made and purified as described (Jan et al., 1999). Similar results were obtained with both preparations. ^{32}P-labeled probes were incubated with 200 nM GST-GLD-1 fusion protein in 10 mM Hepes pH 7, 2 mM $MgCl_2$, 80 mM KCl, 2 mM EGTA, 0.57 mg/ml heparin, 1 mM DTT, at 4°C for 1 hour. Equal amounts of 50% slurry of glutathione beads were added to each binding reaction. The beads were allowed to bind for 30 minutes, then they were spun at 500 ***g*** for 5 minutes. The unbound fraction was removed and the beads washed five times for 10 minutes with wash buffer. The labeled RNA remaining with the beads was then counted using a scintillation counter.

Filter binding assay

^{32}P-labeled RNA probe was made as described above except with a 10:1 ratio of UTP to α-^{32}P-labeled UTP. Binding reactions containing buffer (10 mM Hepes pH 7, 2 mM $MgCl_2$, 80 mM KCl, 2 mM EGTA, 1 mM DTT), recombinant GST-GLD-1, 60 nM labeled RNA with or without excess amounts of unlabeled RNA (made using a Megascript transcription kit; Ambion) were incubated for 1 hour at 4°C. The reaction mixtures were then filtered through nitrocellulose and DE81 membranes to collect protein/RNA complexes and unbound RNA, respectively (Wong and Lohman, 1993). The RNA bound to each membrane was determined by phosphoimaging.

For western blots, total extracts or concentrated eluates from digoxigenin-RNA pull downs were separated on 10% SDS-PAGE, transferred to nitrocellulose and detected with rabbit antibodies against GLD-1 (generously provided by Elizabeth Goodwin) as described previously (Jan et al., 1999). To compare immunoblots of eluates pulled down by different RNAs, an equal percentage of total eluates were loaded on SDS gels.

Reporter mRNA assays and in situ hybridization

Reporter mRNAs were made and injected as described previously (Evans et al., 1994). All mRNAs were tested for integrity by gel electrophoresis, and by injection into adult somatic cells. Only mRNAs that ran as a tight single band and that produced strong β-gal expression in somatic cells were assayed, and 3-5 separate mRNA preparations were used for each mRNA. Reporter mRNAs were injected at 50 nM unless otherwise specified. In situ hybridizations were done as described using an antisense *lacZ* probe (Evans et al., 1994).

RNAi and immunofluorescence

RNAi was performed by microinjection of dsRNA essentially as described (Montgomery and Fire, 1998). PCR products that contained T7 and T3 promoters were amplified from a *gld-1* cDNA plasmid (generously provided by E. B. Goodwin), or from cDNA encoding the GLD-1-related genes T21G5.5 or K07H8.9, and were gel purified, extracted with phenol/chloroform, and ethanol precipitated. Sense and anti-sense RNA strands were made separately using Megascript (Ambion), and were annealed by heating and slow cooling. One-day old N2 adult hermaphrodites were injected into one gonad with dsRNA (0.5-1 mg/ml), and then incubated at 20°C, or 25°C for various times. Essentially the same *gld-1(RNAi)* phenotypes were seen at all temperatures, and included the following classes in temporal sequence: F_1 sterile animals with tumorous gonads, F_1 embryonic arrest (up to 25%), and then Po sterility with oogenesis and germ cell hyper-proliferation defects. For the experiments shown in Fig. 5, *gld-1(RNAi)* and control animals were incubated at 25°C for 15 hours, but the same effects on GLP-1 expression were detected at 20°C after 24 hours of incubation (data not shown). For T21G5.5 or K07H8.9, no RNAi phenotypes were detected through 48 hours of incubation at 20°C (data not shown). For GLP-1 staining, gonads were dissected from injected animals, fixed, and stained with antibodies and DAPI as described previously (Barbee et al., 2002; Evans et al., 1994). GLP-1 was detected using rabbit antibodies against the LNG region (a gift from Judith Kimble), and P-granules using the K76 monoclonal (a gift from Susan Strome, through the Developmental Studies Hybridoma Bank, the NICHD, and the University of Iowa, Dept. of Biological Sciences, Iowa City, IA 52242).

RESULTS

A 34 nucleotide sub-region of the *glp-1* SCR is sufficient for translational control.

Previous work suggested that the spatial control region (SCR) of the *glp-1* 3′ UTR is necessary for translational repression in posterior cells of the embryo (Evans et al., 1994). To determine if the SCR is sufficient for embryonic regulation, we inserted SCR fragments into the *unc-54* 3′ UTR in a *lacZ* reporter mRNA vector. The *unc-54* 3′ UTR is derived from a muscle myosin gene and supports unregulated translation in both germ cells and embryos (Evans et al., 1994). Capped and polyadenylated mRNAs encoding β-galactosidase (β-gal) and carrying modified *unc-54* 3′ UTRs were directly injected into hermaphrodite gonads, and injected animals were stained for β-gal. Reporter mRNA containing the *unc-54* 3′ UTR alone (lacunc mRNA) expressed β-gal in all regions of the gonads and weakly in all cells of early embryos (lacunc in Fig. 1C, data not shown). In contrast, expression from lacunc mRNAs with the entire *glp*-1 SCR or a 34 nt SCR fragment was mostly restricted to anterior cells of early embryos (lacunc(34WT) in Fig. 1D and 1E, and data not shown). Of the stained 4- to 16-cell embryos with lacunc(34WT) mRNA, 96% showed anterior localized β-gal expression (n=24). This localized translation pattern is similar to *lacZ* mRNA containing the full-length *glp-1* 3′ UTR (Fig. 2B, Table 1). Therefore, we conclude that these 34 nucleotides are sufficient for repression in posterior cells of the embryo. The strong translation of *lacZ* mRNA with the 34 nt *glp-1* region in embryos compared to *unc-54* alone suggests that these *glp-1* elements may also stimulate translation in the embryo.

The *glp-1* SCR contains both repression and derepression elements

To further define the *glp-1* translational control elements, we made base substitution mutations within the 34 nt sub-region of the SCR, and assayed them using reporter mRNAs (Fig. 2A-D, Table 1). These mutations were assayed in the context of the full-length *glp-1* 3′ UTR (lacglp mRNAs). Substitution of

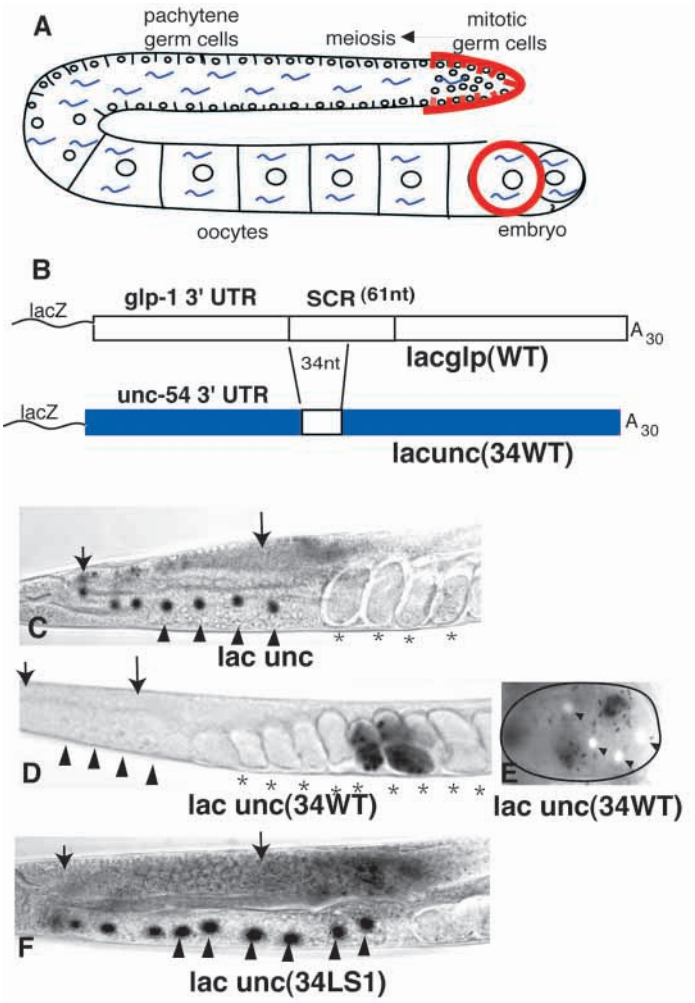

Fig. 1. The Spatial Control Region of the *glp-1* 3′ UTR is sufficient for translational control in the embryo. (A) A diagram of a single hermaphrodite gonad arm. Mitotic germ cells proliferate near the distal end, and as they move from this region they enter meiosis, differentiate into oocytes, and are then fertilized. GLP-1 protein (in red) is expressed in mitotic germ cells and in anterior cells of the early embryo, but *glp-1* mRNA is found throughout (blue lines). (B) Schematic of the *glp-1* 3′ UTR and a chimeric *unc-54* 3′ UTR used in *lacZ* reporter mRNAs. *lacZ* coding sequences include a nuclear localization signal. (C) Whole mount of a hermaphrodite injected with lacunc mRNA (no *glp-1* sequences) and stained with X-gal. β-gal activity (dark nuclear stain) was detected in the distal arm (arrows) and in oocyte nuclei (arrowheads), and weakly in embryos (asterisks). The site of injection is indicated by the large arrow. Injected *lacZ* mRNAs are typically excluded from the distal tip region for unknown reasons (data not shown). (D) A whole mount of a hermaphrodite injected with lacunc(34WT) mRNA. β-gal activity was not detected in the distal arm (arrows) or in oocytes (arrowheads), but was strongly detected in embryos older than the 4-cell stage (asterisks). (E) An 8-cell embryo from an animal injected with lacunc(34WT) mRNA stained with X-gal and DAPI. Dark β-gal staining was seen in the four anterior (AB) nuclei; one AB nucleus is not in focus. Staining was not detected or was very weak in four posterior cells (small arrowheads). (F) Whole mount hermaphrodite injected with lacunc(34LS1) mRNA which contains a mutation in the GRE of the SCR (see Fig. 2). Staining was detected in the distal arm (large arrow), near the gonad bend (small arrow), in oocytes (arrowheads), and in some older embryos (not shown).

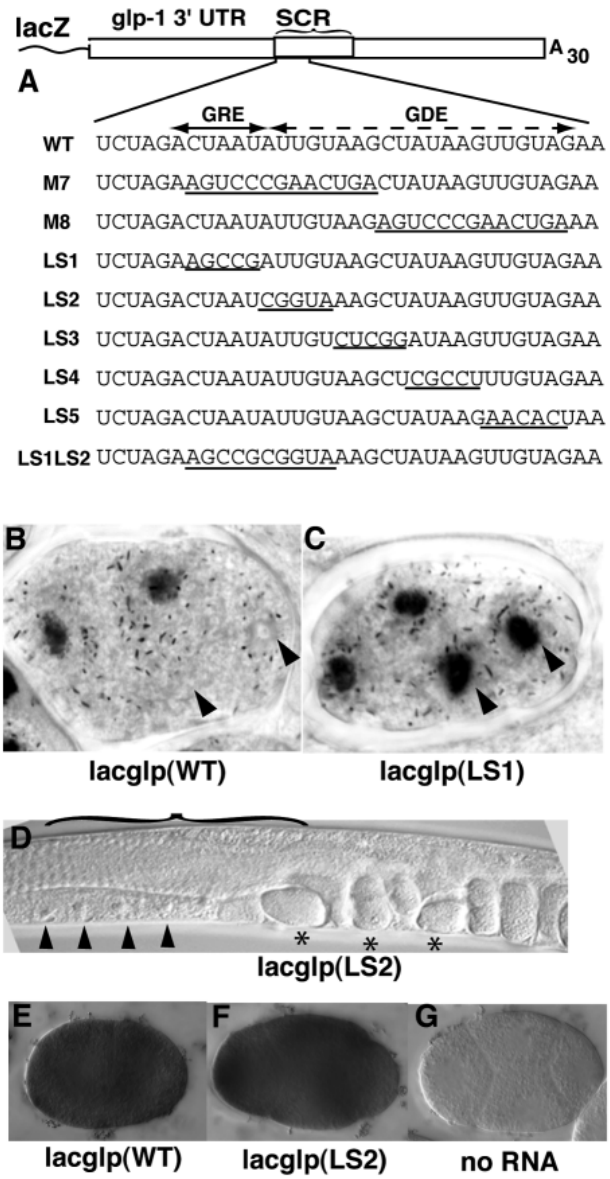

Fig. 2. Mutational analysis of the *glp-1* SCR. (A) Base substitution mutations were made within the full-length *glp-1* 3′ UTR in *lacZ* reporter mRNAs. The 34 nt sub-region of the SCR is shown, and mutations are underlined. The location of the GRE and GDE elements suggested by the data in (B-F) and in Table 1 are shown at the top; arrowheads indicate that these elements may overlap and/or contain additional untested nucleotides. (B) A 4-cell embryo from an animal injected with *lacZ* mRNA carrying the wild-type *glp-1* 3′ UTR [lacglp(WT)] and stained for β-gal. Dark staining can be seen in the anterior cells ABa and ABp, but not in posterior cells (arrowheads). (C) A 4-cell embryo from an animal injected with lacglp(LS1) mRNA. Dark staining can be seen in all four cells. (D) A whole mount of a hermaphrodite injected with lacglp(LS2) mRNA; no staining was detected in the distal arm (bracket), oocytes (arrowheads), or embryos (asterisks). (E-G) In situ hybridization to detect injected *lacZ* reporter mRNA in embryos with a *lacZ* probe. (E) A 2-cell embryo carrying lacglp(WT) mRNA. (F) A 4-cell embryo carrying lacglp(LS2) mRNA. (G) A 4-cell embryo from a non-injected animal.

Table 1. Effects of SCR base substitutions on reporter mRNA translation

Reporter mRNA	Total gonads injected	% gonads with strong β-gal expression in embryos*	% gonads with weak β-gal expression in embryos*	% embryos with anterior localization† (*n*)
lacglp(wt)	89	26	9	93 (27)
lacglp(M7)	48	35	27	0 (20)
lacglp(M8)	29	0	41	–
lacglp(LS1)	110	20	17	0 (24)
lacglp(LS2)	93	0	0	–
lacglp(LS3)	76	4	13	–
lacglp(LS4)	48	0	13	–
lacglp(LS5)	39	8	21	–
lacglp(LS1LS2)	69	41	16	3 (30)

*Staining in embryos was scored as 'strong' if β-gal was easily detected at 50× magnification, and 'weak' if staining was detected only at 400×. All mRNAs produced strong expression when injected into somatic cells.

†Four- to 16-cell embryos in which staining and cell identity could be unambiguously determined were scored as 'anterior localized' if staining was strong in AB-derived cells and undetectable in at least two (4- to 6-cell embryos) or four (8- to 16-cell embryos) P1-derived cells. – indicates that localization β-gal could not be scored because of low or absent staining.

13 nucleotides near the 5′ end caused un-localized β-gal expression in embryos (M7 in Fig. 2A, lacglp(M7) in Table 1). This loss of repression in posterior cells was similar to deletion of the SCR; repression in oocytes was mostly maintained while repression in posterior cells of the embryo was lost. Substitution of 5 nucleotides within this region also resulted in un-localized expression in most embryos (lacglp(LS1) in Fig. 2C, Table 1), although partial asymmetry could be detected in a few embryos (data not shown). Therefore, these mutations disrupt an element that represses translation in posterior cells of the embryo. We call this element the *glp-1* Repression Element (GRE). At a minimum, the GRE is defined by the five nucleotides disrupted in the LS1 mutant, but may include additional bases (Fig. 2A).

Surprisingly, base substitutions adjacent to the GRE strongly or partially inhibited β-gal expression in the embryo (lacglp(LS2) in Fig. 2D, lacglp(M8) and lacglp(LS2-LS5) in Table 1). In contrast, all of these mutant mRNAs produced strong β-gal expression when injected into intestinal cells (data not shown). The loss of embryonic expression from these mutant mRNAs could be due to defective activation of translation or by decreased mRNA stability in the embryo. When injected animals were examined by in situ hybridization with a *lacZ* RNA probe, lacglp mRNAs with the LS2 or LS4 mutations were detected at similar intensities to wild-type or LS1 mRNAs in both gonads and all cells of early embryos (Fig. 2E,F, data not shown). These results suggest that the LS2 and LS4 mutations do not dramatically reduce mRNA stability. Furthermore, when both the GRE and these neighboring sequences were simultaneously disrupted by base substitution or deletion, strong but un-localized translation was detected in early embryos (lacglp(LS1LS2) and lacglp(M7) in Table 1) (Evans et al., 1994). Therefore, an RNA element defined by mutants LS2-LS5 promotes translation in the embryo by inhibiting GRE-mediated repression. We call this element the GDE, for *glp-1* Derepressor Element (Fig. 2A).

We noticed that GRE mutation or SCR deletion in lacglp mRNAs also caused a small but reproducible increase in the number of gonads with β-gal expression (data not shown) (Evans et al., 1994). However, full repression of germline translation requires RNA elements outside of the SCR (Evans et al., 1994). To examine GRE function in the germline independently of other *glp-1* elements, we compared the translation of lacunc mRNA carrying a wild-type 34 nt SCR fragment [lacunc(34WT)] to one with the LS1 mutation in the GRE [lacunc(34LS1)]. Lacunc(34WT) mRNA translation was strongly inhibited in the germline; β-gal expression was restricted to embryos in 75% of the injected gonads that stained (*n*=20) (Fig. 1D). None of these gonads expressed β-gal in early meiotic germ cells of the distal arm or near the gonad bend (Fig. 1D), while 25% expressed β-gal within later stage oocytes that were closest to the proximal end of the gonad (not shown). In contrast, lacunc(34LS1) mRNA was strongly translated in germ cells (compare Fig. 1D to 1F). Of the gonads injected with lacunc(34LS1) that produced β-gal, 100% had staining within the distal arm and 96% in the oocytes (*n*=23). These results suggest that the GRE not only functions in embryos, but also contributes to repression of *glp-1* translation in the gonad. GRE activity was strongest within more distal regions of the gonad that contain germ cells in early stages of oogenesis. Because the GRE is not essential for germline repression within the intact *glp-1* 3′ UTR, it functions redundantly with other *glp-1* RNA elements (see Discussion) (Evans et al., 1994).

GLD-1 directly binds the *glp-1* GRE

To identify proteins that bind to *glp-1* RNA elements of the SCR, RNA-binding factors were affinity precipitated from crude adult extracts using tagged RNAs (see Materials and Methods). RNA-binding proteins in enriched fractions were detected by UV cross-linking to ^{32}P-labeled RNA probes. Wild-type tagged SCR RNA pulled out polypeptides of 58 kDa (p58) and 30 kDa (p30) that could be cross-linked to an RNA probe containing both the GRE and GDE (Fig. 3A, lane 1). In contrast, tagged RNA that contained the M7 mutation failed to pull out either p58 or p30 (Fig. 3A, lane 2). To examine the specificity of p58 and p30 further, RNAs containing GRE or GDE mutations were tested for their ability to bind these proteins. UV cross-linking of p58 and p30 was strongly disrupted by the LS1 mutation in the GRE, but not by the LS2 or LS5 mutations within the GDE (Fig. 3B). The LS3 and LS4 mutations partially attenuated cross-linking to both p58 and p30. Excess amounts of unlabeled RNA containing the LS1 mutation was a poor competitor of ^{32}P-labeled wild-type probes for cross-linking to p58 and p30 (Fig. 3C). In contrast, excess wild-type RNA, or RNAs with any of the GDE mutations, displaced the labeled wild-type probe (Fig. 3C, data not shown). Together, these experiments demonstrate that both p58 and p30 specifically require the GRE for binding to the *glp-1* SCR. Sequences that overlap with the GDE also appear to promote but are not essential for their binding to *glp-1* RNA (Fig. 3).

Several previous observations suggest that p58 could be the KH-domain protein, GLD-1. First, GLD-1 is expressed where the *glp-1* GRE functions (in meiotic germ cells of the distal arm and posterior blastomeres of the embryo) (Jones et al., 1996). Second, GLD-1 runs at 58 kDa on SDS gels (Jones et al., 1996). Third, there is increased expression of GLP-1 in the sterile tumorous gonads of *gld-1* mutants (Crittenden et al.,

1994). Therefore, we tested whether p58 could be GLD-1 by western blot of *glp-1* RNA binding proteins with GLD-1 antibodies. Strikingly, GLD-1 antibodies labeled both 58 kDa and 30 kDa polypeptides in the fraction pulled out by the dig-tagged wild-type SCR RNA (Fig. 3D, lane 1). In contrast, no GLD-1 was detected in fractions pulled out by RNA with the M7 mutation in the GRE and GDE (Fig. 3D, lane 2). Because western blots of total worm lysate revealed only a single 58 kDa band (data not shown), the 30 kDa species are likely to be proteolytic fragments of GLD-1. These results suggest that both p58 and p30 labeled by UV cross-linking are GLD-1, although either (or both) may also include other proteins. Nonetheless, taken together, these observations suggest that GLD-1 is a specific RNA-binding protein for the GRE in the *glp-1* 3′ UTR.

To determine if GLD-1 can bind the GRE directly, a recombinant purified GST-GLD-1 protein fusion was tested for its ability to bind *glp-1* RNAs in vitro. Using a GST pull-down assay, we found that ^{32}P-labeled RNA containing the GRE and the GDE was pulled down by GST-GLD-1, but RNA with the LS1 mutation in the GRE was not (Fig. 4A). Neither wild-type nor mutant RNAs were pulled down using GST alone (data not shown). To more carefully examine the specificity of GLD-1 binding, we used a filter binding assay to test the abilities of

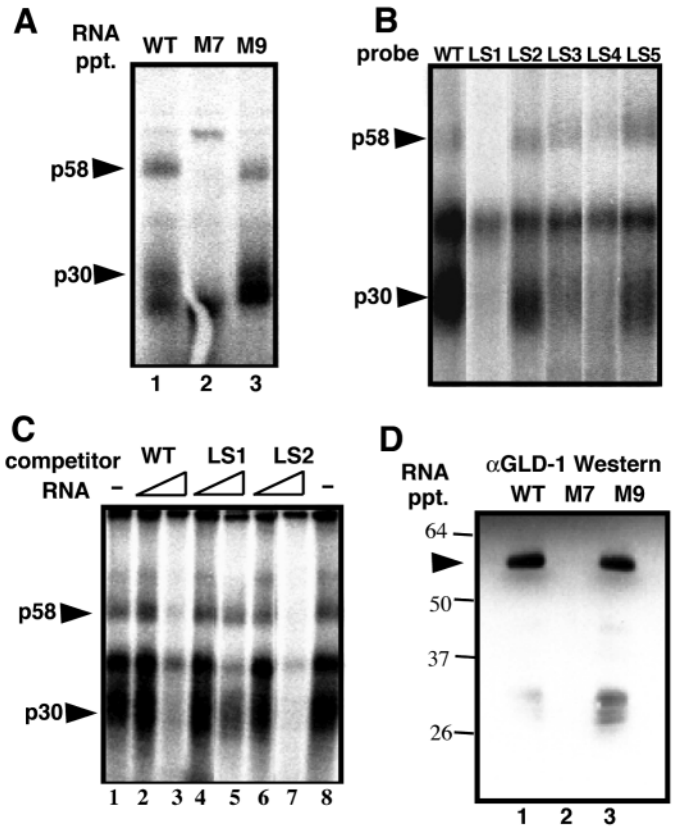

Fig. 3. GLD-1 is pulled out of crude extracts by tagged SCR RNA. (A) Proteins were pulled out of crude extracts using digoxigenin (dig)-labeled RNAs that included the entire 61 nt *glp-1* SCR (total RNA size, including vector sequence, was 104 nt). Precipitated proteins were subjected to UV cross-linking to a ^{32}P-labeled RNA containing 34 nt of the wild-type *glp-1* SCR (the GRE/GDE region in Fig. 2; total RNA probe was 68 nt). 58 and 30 kDa (p58 and p30) proteins labeled by the probe are indicated. Dig-labeled RNAs were either wild type, had the M7 mutation in the GRE and GDE (see Fig. 2), or a 13 nt mutation (M9) at the 3′ end of the SCR, downstream of the 34 nt GRE/GDE region (not shown). (B) Proteins pulled out by wild-type dig-SCR RNA were UV cross-linked to ^{32}P-labeled probes that had 34 nt of wild-type or mutant SCR sequences (refer to Fig. 2). The ratios of p58 to p30 varied from prep to prep, possibly due to proteolysis (data not shown). (C) Competition of wild-type ^{32}P-labeled probe by unlabeled wild-type or mutant RNAs, as assayed by UV cross-linking. Unlabeled RNAs were added at 10- and 500-fold molar excess of labeled probe. Lanes 1 and 8 have no competitor RNA added. (D) A western blot of proteins pulled out by dig-tagged wild-type (WT), M7 or M9 SCR RNAs (71 nt) probed with GLD-1 antibodies. Protein samples used for this blot were from the same samples as used for UV cross-linking in A. The arrow marks the position of the single band detected in total worm homogenate (data not shown).

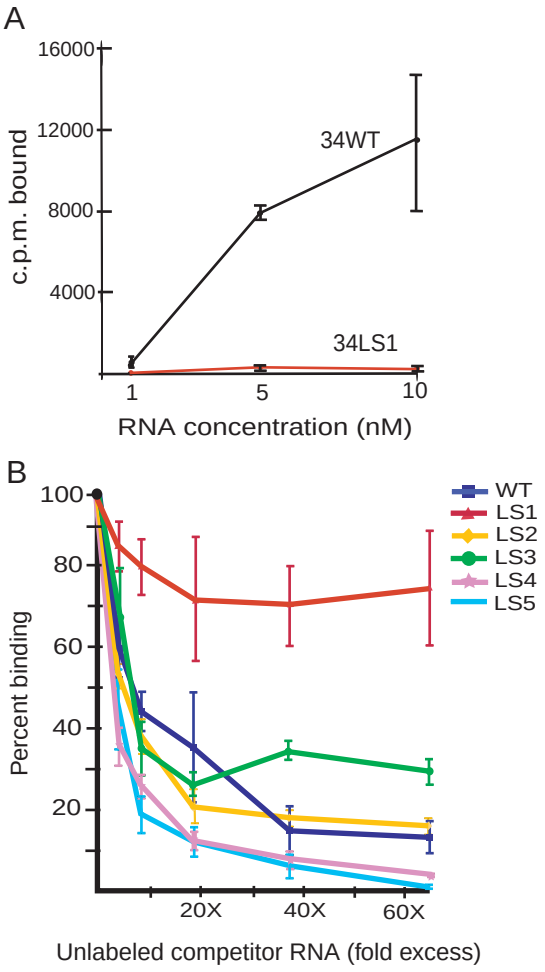

Fig. 4. Recombinant GLD-1 binds directly and specifically to the GRE of *glp-1* RNA. (A) GST pull down of radiolabeled *glp-1* 34 nt RNAs by GST-GLD-1. Shown are means of two binding reactions±the variance from a single experiment; similar results were seen in three separate experiments. (B) Competitive displacement of GST GLD-1 from ^{32}P-labeled SCR (34 nt) with unlabeled wild-type or mutant RNAs. A filter-binding assay was used to detect GLD-1 binding to wild-type ^{32}P-labeled 34 nt RNA that contains both the GRE and GDE. Increasing concentrations of unlabeled 34 nt RNAs were added to the binding reactions. 100% binding is the amount bound in absence of any competitor. Shown is the mean±s.d. of three to four separate binding reactions.

unlabeled mutant RNAs to compete with labeled wild-type probe for binding to GLD-1 (Fig. 4B). Unlabeled wild-type RNA and RNAs with mutations in the GDE were effective competitors for binding to GLD-1. In contrast, the LS1 mutation within the GRE greatly inhibited competition for GLD-1 binding. We conclude that GLD-1 binds directly to the *glp-1* 3′ UTR and binding depends specifically on the GRE, suggesting that GLD-1 could be a repressor of *glp-1* mRNA translation.

GLD-1 is required for GLP-1 repression in germ cells and embryos

To determine if GLD-1 is required for translational control of endogenous *glp-1* mRNA, we disrupted GLD-1 function and stained for endogenous GLP-1 protein by immunofluorescence. Because most loss-of-function *gld-1* mutants fail to make oocytes and embryos (Francis et al., 1995a), these mutants cannot be used to examine *gld-1* function in embryos or in adult gonads undergoing oogenesis. Therefore, we reduced *gld-1* function in adult hermaphrodites by RNA interference (RNAi). Injection of *gld-1* double-stranded RNA (dsRNA) caused a progression of phenotypes with time after injection that are consistent with the known effects of *gld-1* mutations (Francis et al., 1995a) (see Materials and Methods). RNAi of two *gld-1*-related genes (T21G5.5 and K07H8.9) had no detectable phenotypes, supporting the specificity of *gld-1(RNAi)* for GLD-1 depletion (data not shown). Strikingly, *gld-1* RNAi caused a dramatic increase in GLP-1 expression in the gonads of injected animals (Fig. 5B,C). In wild-type control animals, GLP-1 was expressed in the mitotic region of the distal gonad tip but was not detected where germ cell nuclei enter meiosis (Fig. 5A, see Fig. 1 for orientation). However, in *gld-1*(RNAi) animals, GLP-1 expression extended well into the distal arm and occasionally into parts of the proximal arm (Fig. 5B,C). In some *gld-1(RNAi*) gonads with high GLP-1 expression, most germ cell nuclei appeared to have normally entered meiotic pachytene and were still making functional oocytes (Fig. 5B, and data not shown). This result suggests that ectopic expression of GLP-1 does not require the formation of germline tumors, although it does not distinguish between direct and indirect effects. Regardless, we conclude that GLD-1 is required for repression of GLP-1 expression in the distal arm of the adult hermaphrodite germline.

RNAi of *gld-1* also affected the regulation of GLP-1 expression in early embryos. At the 4 to 8-cell stages, 36% of *gld-1*(RNAi) embryos (n=36) had GLP-1 staining in posterior blastomeres that was equivalent or nearly equivalent to staining in anterior blastomeres (Fig. 5E). In contrast, none of 4- to 8-cell embryos (n=62) from non-injected mothers had high GLP-1 staining in posterior cells, and all showed enrichment of GLP-1 in anterior cells (Fig. 5D). The posterior expression of GLP-1 seen in *gld-1*(RNAi) embryos was higher than the very low levels detected in all 1-cell zygotes examined, suggesting that posterior expression of GLP-1 was not due to perdurance of protein translated in the germline (data not shown). Therefore, these data suggest that GLD-1 is required for normal translational repression of *glp-1* mRNA in posterior cells of the embryo. The incomplete penetrance of GLP-1 mis-localization could indicate that GLD-1 only partially contributes to *glp-1* mRNA regulation in the embryo.

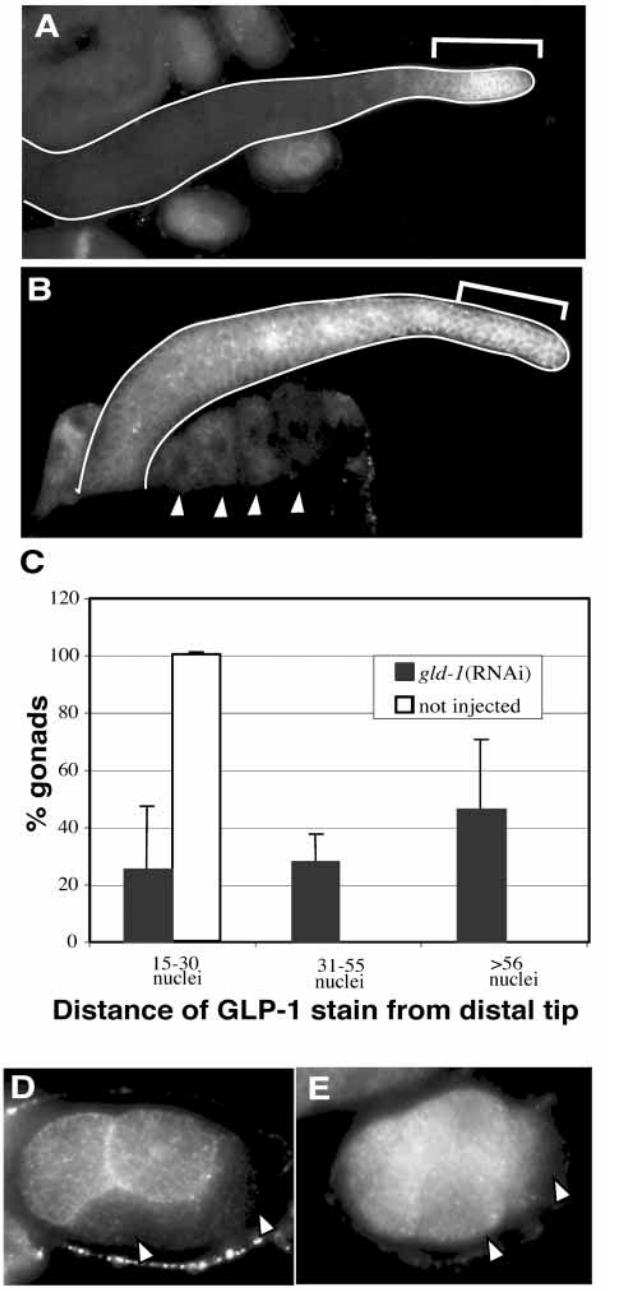

Fig. 5. GLD-1 depletion disrupts regulation of endogenous GLP-1 expression. Animals were injected with *gld-1* dsRNA and then stained for GLP-1 protein by immunofluorescence after incubation for 15 hours at 25°C. Gonads are outlined in A and B. (A) A control gonad dissected from a non-injected animal. GLP-1 staining was restricted to the mitotic proliferation zone of the germline (white bracket). (B) A gonad dissected from a *gld-1(RNAi)* animal. GLP-1 staining extends beyond the mitotic region (bracket) of the distal arm. This gonad also contained oocytes (arrowheads) with diakinesis nuclei, as judged by DAPI staining (not shown). (C) Quantitation of the expansion of GLP-1 staining from the distal tip, following *gld-1(RNAi).* The number of nuclei were counted from the distal tip to where GLP-1 staining was lost from peripheral membranes in individual gonads. Numbers represent mean±s.d. from three experiments (n=8-17 gonads for each experiment). (D) A 4-cell embryo from a non-injected control animal. (E) A 4-cell embryo from a *gld-1(RNAi)* animal. The two posterior blastomeres are indicated (arrowheads) in D and E.

Alternatively, GLD-1 may be only partially depleted in these RNAi experiments. Using immunofluorescence, we found that GLD-1 staining in *gld-1(RNAi)* animals was reduced but not eliminated in both gonads and embryos under the conditions used for these assays (data not shown). By the time GLD-1 staining was severely reduced, gonads from injected animals produced no embryo progeny. Thus, GLD-1 could be an essential repressor of *glp-1* translation in the early embryo.

DISCUSSION

Several of the observations above suggest that the KH-domain protein, GLD-1, functions as a direct repressor of *glp-1* translation. First, endogenous GLD-1 can associate specifically with *glp-1* RNA containing the GRE repression element. Second, recombinant GLD-1 binds specifically and directly to a 34 nucleotide fragment of the *glp-1* SCR in vitro. Third, binding of GLD-1 to *glp-1* RNA is specifically disrupted by mutations in the GRE that also disrupt repression of translation in meiotic germ cells and early embryos. Finally, depletion of endogenous GLD-1 causes inappropriate expression of endogenous GLP-1 at similar times and places of development where the *glp-1* GRE functions. These results strongly suggest that GLD-1 is a repressor of *glp-1* translation at two developmental stages, early prophase of germ cell meiosis and early in embryogenesis.

GLD-1 is a conserved member of the STAR/Quaking/GSG family of proteins that have been linked to various signal transduction and developmental events. In *C. elegans*, GLD-1 has been shown to control sex determination and oogenesis in the germline by regulation of several germline mRNAs (Francis et al., 1995a; Jan et al., 1999; Lee and Schedl, 2001; Xu et al., 2001). Our studies suggest a new function for GLD-1 in the control of early embryonic asymmetry. This function contributes to the localization of a Notch receptor to anterior cells of the embryo. In other organisms, STAR family members have been connected to various protein kinase signaling cascades (Vernet and Artzt, 1997). In *C. elegans*, GLD-1 regulates the translation of *tra-2*, which encodes a key membrane receptor for sex determination (Jan et al., 1999; Kuwabara et al., 1992). GLD-1 also associates with mRNA encoding a Raf protein kinase in the germline (Lee and Schedl, 2001). Our results link GLD-1 function to the control of another signaling system, the Notch pathway. Therefore, this family of RNA binding proteins may be generally important for regulation of a variety of different signaling events that control important developmental decisions in metazoans.

These results suggest that GLD-1 contributes to the spatial organization of Notch signaling in both the embryo and germline. In the embryo, localization of GLP-1/Notch ligands and the receptor is probably important for the spatial patterning of anterior cell fates (Mello et al., 1994; Priess and Thomson, 1987). We suggest that GLD-1 participates in this process by repression of *glp-1* translation in posterior cells of the embryo. In the germline, GLD-1 repression of *glp-1* could be part of a negative feedback system of RNA regulation that controls spatial organization of Notch signaling and germ cell development. GLP-1 is expressed in mitotic germ cells of the distal tip, where it maintains the mitotic stem cell population (Austin and Kimble, 1987; Crittenden et al., 1994). Interestingly, GLD-1 expression is inhibited in the mitotic distal tip region by a process dependent on the RNA-binding proteins FBF-1 and FBF-2, which can directly bind the *gld-1* 3′ UTR (Crittenden et al., 2002; Jones et al., 1996). This may be important to permit full expression of the GLP-1 receptor in these cells. As germ cells move away from the distal tip and the source of the ligand for GLP-1 signaling, they enter meiosis and begin to express GLD-1 and probably other factors (see below), which then repress the translation of *glp-1* mRNA. GLD-1 is essential to promote exit from mitosis and female gamete differentiation (Francis et al., 1995a; Francis et al., 1995b). However, genetic studies suggest that GLP-1 down regulation by GLD-1 only weakly contributes to inhibition of germ cell mitosis (Francis et al., 1995b). It could be that GLD-1 repression of *glp-1* mRNA and other mRNAs, coupled with localization of the GLP-1/Notch ligand to the somatic distal tip cell (Henderson et al., 1994), provide redundant systems to spatially constrain GLP-1 signaling and organize mitotic proliferation of germline stem cells in the gonad.

Our studies and previous observations suggest that the precise localization of *glp-1* translation in the embryo requires regulation of GLD-1 by other factors. In the early embryo, localized translation requires not only the GRE but also the GDE that promotes translation. Because the GDE is only needed to regulate the GRE, we suggest it functions as a binding site for a derepression factor that inhibits GLD-1 binding to the GRE or its activity (Fig. 6). Localization of translation could be most simply achieved by localization of the derepressor to anterior cells. However, GLD-1 is greatly enriched in posterior cells (Jones et al., 1996). It could be that a derepressor is necessary to inhibit low residual levels of GLD-1 that persist in anterior cells. Perhaps, localization of both GLD-1 and derepressor factors ensures tight localization of GLP-1 and other cell fate regulators. Alternatively, another un-localized repressor may also contribute to *glp-1* mRNA repression through the GRE.

In the germline, the regulation of *glp-1* translation is surprisingly complex. Our results suggest GLD-1 is required to repress *glp-1* translation in early meiotic germ cells, and part

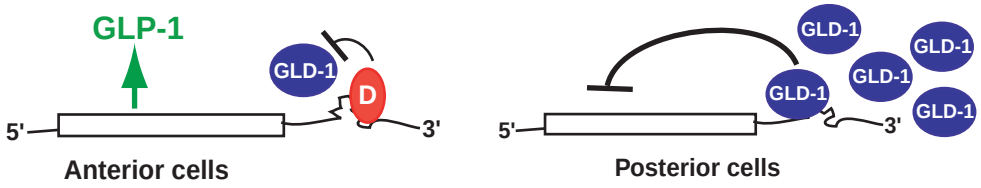

Fig. 6. A model for GLD-1 and *glp-1* mRNA regulation in the early embryo. In posterior cells, GLD-1 binds to the GRE, probably with other factors (not shown), and represses *glp-1* mRNA translation. In anterior cells, a derepressor (D) binds to the GDE to inhibit GLD-1 binding or activity, leading to *glp-1* translation. Localization of *glp-1* translation is further ensured by enrichment of GLD-1 in posterior cells.

of this control is likely to result from direct binding of GLD-1 to the GRE. However, several observations suggest that other factors are also involved in germline repression of *glp-1*. First, a distinct region of the *glp-1* 3′ UTR is required for repression of translation in the germline (Evans et al., 1994). Second, GLD-1 disappears in differentiating oocytes of the proximal arm, whereas *glp-1* mRNA continues to be repressed (Crittenden et al., 1994; Jones et al., 1996). These two observations suggest that other regulatory factors repress *glp-1* mRNA in oocytes, and indicate distinct regulatory systems that function in different spatial domains in the gonad. Third, mutations in the GRE only weakly disrupted repression by the full-length *glp-1* 3′ UTR in the gonad, whereas GLD-1 depletion caused a robust increase in *glp-1* translation in early meiotic germ cells. These results may indicate that GLD-1 repression of germline *glp-1* may be partly indirect by affecting unknown *glp-1* RNA-binding factors, or there may be other binding sites for GLD-1 elsewhere within the *glp-1* 3′ UTR.

GLD-1 regulates a variety of different mRNAs, but how it specifically controls these mRNAs is not well understood. Although some sequence similarity exists among the GLD-1 binding regions of other mRNAs, the GRE region of *glp-1* has only limited similarity to these other elements in primary sequence (Goodwin et al., 1993; Jan et al., 1999; Lee and Schedl, 2001; Xu et al., 2001). In contrast, the nucleotides within and surrounding the GRE are mostly conserved in *glp-1* homologs of different nematode species (Rudel and Kimble, 2001). The binding site in *glp-1* may fold into a structure related to these other RNA targets, or *glp-1* RNA may bind a distinct binding site in GLD-1. Alternatively, distinct binding partners for GLD-1 may promote recognition, and specific regulation, of different mRNAs. The F-box protein FOG-2 is a GLD-1 binding protein that is specifically required for a sex determination function of GLD-1 (Clifford et al., 2000). Perhaps other specific factors may interact with GLD-1 to control *glp-1* RNA, and distinct complexes may function in the germline and early embryo. It will be important to identify these interactions, and the interactions with more general translation factors, to understand the molecular mechanisms that lead to distinct but precise patterns of mRNA regulation.

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