RNA-binding protein GLD-1/quaking genetically interacts with the mir-35 and the let-7 miRNA pathways in Caenorhabditis elegans

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1. Summary

Messenger RNA translation is regulated by RNA-binding proteins and small non-coding RNAs called microRNAs. Even though we know the majority of RNA-binding proteins and microRNAs that regulate messenger RNA expression, evidence of interactions between the two remain elusive. The role of the RNA-binding protein GLD-1 as a translational repressor is well studied during Caenorhabditis elegans germline development and maintenance. Possible functions of GLD-1 during somatic development and the mechanism of how GLD-1 acts as a translational repressor are not known. Its human homologue, quaking (QKI), is essential for embryonic development. Here, we report that the RNA-binding protein GLD-1 in C. elegans affects multiple microRNA pathways and interacts with proteins required for microRNA function. Using genome-wide RNAi screening, we found that nhl-2 and vig-1, two known modulators of miRNA function, genetically interact with GLD-1. gld-1 mutations enhance multiple phenotypes conferred by mir-35 and let-7 family mutants during somatic development. We used stable isotope labelling with amino acids in cell culture to globally analyse the changes in the proteome conferred by let-7 and gld-1 during animal development. We identified the histone mRNA-binding protein CDL-1 to be, in part, responsible for the phenotypes observed in let-7 and gld-1 mutants. The link between GLD-1 and miRNA-mediated gene regulation is further supported by its biochemical interaction with ALG-1, CGH-1 and PAB-1, proteins implicated in miRNA regulation. Overall, we have uncovered genetic and biochemical interactions between GLD-1 and miRNA pathways.

2. Introduction

MicroRNAs (miRNAs) are approximately 21 nucleotide-long endogenous regulatory RNAs that mediate translational regulation through binding to
the 3'UTR of target mRNAs. Over the past decade, they have been implicated in many biological processes and are now considered to be major modulators of gene expression [1,2]. After biogenesis, miRNAs and Argonauta proteins (AGO; ALG-1/ALG-2 in <C. elegans>) form the miRNA-induced silencing complex (miRISC) together with GW182 proteins (AIN-1/AIN-2 in <C. elegans>) [3]. VIG-1 is thought to function in the miRNA pathway and was identified as an interactor of miRNA/Argonauta complexes by co-immunoprecipitation [4]. Mammalian TRIM32 and <C. elegans> NGL-2 also interact with AGO and promote miRNA activity [5,6].

GLD-1 is a member of a highly conserved RNA-binding protein family, characterized by the signal transduction and activation of RNA (STAR) domain [7]. GLD-1 affects <C. elegans> germline development and maintenance by translational repression of a variety of target proteins [8–14]. A key role for GLD-1 in modulating DNA damage-induced germline apoptosis was uncovered via the hypomorphomic <gld-1(op236)> allele [13]. In <gld-1(op236)> mutants, GLD-1 is unable to bind the 3'UTR of cep-1/p53, whereas translational repression of GLD-1 targets mediating developmental regulation is unaffected. <gld-1(op236)> is unique among <gld-1> alleles in showing no overt defect in germ cell development at the permissive temperature. However, at the restrictive temperature, <gld-1(op236)> animals are sterile, and undifferentiated germ cells accumulate [13]. The mammalian orthologue of GLD-1 is quaking/QKI, which functions in translational regulation during neuronal development [15,16]. GLD-1 and QKI are functionally conserved, and ectopically expressed QKI in worms recognizes GLD-1 target sequences [17]. Although GLD-1 biochemically interacts with AIN-2, the functional consequences of this interaction have not yet been determined [18]. Lastly, miRNA-related functions of gld-1 have not been documented by mutational analysis, and a gld-1 phenotype affecting somatic development of animals has not been reported.

Deleting the vast majority of known <C. elegans> miRNAs individually does not result in obvious overt phenotypes [19]. Phenotypes tend to arise when several members of a miRNA family are deleted [20]. Alternatively, mutating miRNA pathway genes also generate sensitized system that helps us to unravel miRNA function [21]. Such synthetic phenotypes point towards the existence of extensive redundancy in miRNA-mediated gene regulation. Caenorhabditis elegans genetics allows for using ‘sensitized’ genetic backgrounds to study subtle phenotypes associated with redundant mechanisms of miRNA-mediated gene regulation.

Initially aiming to identify genes required for GLD-1-mediated translational regulation, we performed a genome-wide RNAi screen for enhancers of the gld-1(op236)/hypomorphomic allele. This screen identified vlg-1 and nhl-2, both of which are modulators of miRNA function, thus suggesting that GLD-1 might affect general miRNA-mediated gene regulation. We indeed found that gld-1 enhances multiple let-7 and mir-35 family miRNA phenotypes affecting somatic development. Using stable isotope labelling with amino acids in cell culture (SILAC)-based proteomics, we show that the upregulation of the histone miRNA-binding protein CDL-1 is partially responsible for the genetic interactions between GLD-1 and let-7 miRNA. A role for GLD-1 in miRNA-mediated gene regulation is further supported by the interaction of GLD-1 with ALG-1, CGH-1 and PAB-1, proteins previously implicated in miRNA-mediated gene regulation.

### 3. Material and methods

#### 3.1. Strains and animal handling

Strains used in this paper were TG34 <gld-1(op236)>I, TG2209 <vig-1(kx2536)>Ib, TG2129 gld-1(op236)I; vig-1(kx2536)>I, TG1725 nhbl-2(ok818>III, TG2130 gld-1(op236)>I; nhbl-2(ok818)III, MT14119 nD50II, TG2133 gld-1(op236)>I; nD50II, GR1432 let-7(mg279)X, TG1684 gld-1(op236)>I; let-7(mg279)X, VT1142 nD51V; mir-84 (n5037)X; cts139, TG2134 gld-1(op236)>I; nD51V; mir-84 (n5037)X; cts139, SU93 jcs1IV, TG2017 gld-1(op236)>I; jcs1IV, TG2018 jcs1IV; let-7(mg279)I, TG2019 gld-1(op236)>I; jcs1IV; let-7(mg279)X, MT2124 let-60(n1046)IV, TG2135 gld-1(op236)>I; let-60(n1046)IV, SD551 let-60(ga89)IV, TG2136 gld-1(op236)>I; let-60(ga89)IV, TG1828 mals105V, TG1825 gld-1(op236)>I; mals105V, TG1826 mals105V; let-7(mg279)X, TG1827 gld-1(op236)>I; mals105V; let-7(mg279)X, TG2137 gld-1(op236)>I; HT2 I; mals105V; let-7(mg279)X, TG2138 gld-1(op236)/glnd-1(q485)I; mals105V; let-7(mg279)X, TG2131 gld-1(op236)>I; vig-1(kx2536)I; mals105V, SX493 Pcol-10::GFP::lin-41–3'UTR(mj32)II, TG2039 Pcol-10::Gld-1::mCherry::gld-1–3'UTR(qtEx2039), TG2139 Pcol-10::GFP::lin-41–3'UTR(qtEx2039), TG2041 Pcol-10::mCherry::H2B::gld-1–3'UTR(qtEx2041), SX1257 Pcol-10::GFP::lin-41–3'UTR(mj32)II; Pcol-10::mCherry::unc-54–3'UTR(mj117) IV, TG2212 Pcol-10::GFP::lin-41–3'UTR(mj32)II; Pcol-10::mCherry::unc-54–3'UTR(mj117) IV; Pcol-10::let-7(mg279)X, TG2213 gld-1(op236)>I; Pcol-10::GFP::lin-41–3'UTR(mj32)II; Pcol-10::mCherry::unc-54–3'UTR(mj117) IV; Pcol-10::let-7(mg279)X, TG1769 gld-1(op236)>I; Pcol-10::GFP::lin-41–3'UTR(mj32)II; Pcol-10::mCherry::unc-54–3'UTR(mj117) IV, OZ152 gld-1::GFP::FLAG; glnd-1(q485)I (ozs12), SX2695 gld-1(op236)>I; col-10::GFP::lin-41 deletion (mjs35)II; unc-119(ed3) III; let-7(mg279)X, SX2696 gld-1(op236)>I; col-10::GFP::lin-41 deletion (mjs35)II; unc-119(ed3) III; let-7(mg279)X, SX2297 col-10::GFP::lin-41 deletion (mjs35)II; unc-119(ed3) III.

Caenorhabditis elegans larvae were grown on Escherichia coli strain OP50 at 20°C unless otherwise stated. let-60(ga89) mutants were grown at 20°C and their progeny was shifted to 25°C at the L1 larval stage and scored at adult stage for the presence of multi-vulva phenotype. Microscopic analysis of the animals was carried out by anaesthetizing with levamisole and observed using a Zeiss axio_scope.

#### 3.2. RNAi in Caenorhabditis elegans

We used the whole-genome RNAi library generated by Ahringer Laboratory [22,23], and a modified version of the RNAi feeding protocol described in [24]. RNAi-expressing bacteria were grown from frozen stocks overnight at 37°C in LB medium containing 50 μg ml⁻¹ ampicillin and 10 μg ml⁻¹ tetracycline in 96-well plates. A fresh culture seeded from the overnight culture was incubated at 37°C in 96-deep-well plates until OD₆₀₀ was 0.6–1 and then induced with 1 mM IPTG for 2 h at 20°C. L1 larvae were dispensed into 96-well plates (10 worms well⁻¹) in 100 μl M9 medium supplemented with 50 μg ml⁻¹ ampicillin, 10 μg ml⁻¹ tetracycline, 10 μg ml⁻¹ cholesterol, 0.1 μg ml⁻¹ fungizone and 1 mM IPTG. Induced bacteria (50 μl) were also dispensed into each well, and worms were grown at 20°C with constant shaking at
3.3. Generation of transgenic lines

The pgld-1::mCherryHis::glg-1–3’UTR (GA_AA006, gEx2041) construct was generated by cloning the gld-1 promoter (amplified using primers 5’-atatatatatgcccgcGCTGATGTTAACCTGTAAG-3’ from genomic DNA) using Ascl and NotI enzymes. mCherryHis was amplified using primers 5’-atatatatatatgcccgcGGTCGTGAATTCCCTTACGA-3’ and 3’-atatatatatatgcccgcTTCGATCACTC-3’ and digested with NotI and FseI. The glg-1 3’UTR was amplified using primers 5’-atatatatatatgcccgcGACTGAAAGCCAGGTACCTTATTC-5’ and 3’-atatatatatatgcccgcTTACTTGCTGGAAGTGTACTTG-5’ and digested with NotI and FseI. The glg-1 coding region was amplified from genomic DNA using primers 5’-atatatatatatgcccgcGTGGTCTCAAAGGGTGAAG-3’ and 3’-atatatatatatgcccgcATGCCGTCGTGCACCACTC-3’ and digested with ApgI and HindIII. mCherry was amplified using primers 5’-atatatatatatgcccgcGAATTTTGGAATTCccccagtgtt-5’ and 3’-GAATTTTGGAATTCcaaaattcgtt-3’ and digested with NotI and HindIII. The glg-1 promoter using primers 5’-atatatatatatgcccgcGCTGATGTTAACCTGTAAG-3’ and 3’-atatatatatatgcccgcGACTGAAAGCCAGGTACCTTATTC-3’ and digested with NotI and HindIII. The glg-1 3’UTR was amplified using primers 5’-atatatatatatgcccgcGAATTTTGGAATTCccccagtgtt-5’ and 3’-GAATTTTGGAATTCcaaaattcgtt-3’ and digested with NotI and HindIII. The glg-1 promoter using primers 5’-atatatatatatgcccgcGCTGATGTTAACCTGTAAG-3’ and 3’-atatatatatatgcccgcGACTGAAAGCCAGGTACCTTATTC-3’ and digested with NotI and HindIII.

3.5. Northern blotting

Total RNA was extracted using Qiazol (Qiagen) with 300 μl of 0.7 mm zirconia beads at 4°C from synchronized adult-stage worms and separated on 15% polyacrylamide gels (SequagelFlowGel). Gels were blotted onto Hybond-N (Amersham) membrane using a semi-dry electro blotted, and chemical cross-linking was carried out using l-ethyl-3-3-dimethylaminopropyl carbodiimide (EDC) as previously described [32]. Blots were probed with labelled let-7 RNA and U6 snRNA DNA oligonucleotides as previously described [33].

3.6. SILAC in nematodes

SILAC growth conditions, subcellular fractionations, peptide preparations and mass spectrometry were carried out as described in [34–36]. The number of proteins detectable by mass spectrometry was increased by subcellular fractionation [34]. For determining B to A and C to A ratios, we considered proteins that were only detected in one experiment if a minimum of two peptides were detected (the detection of two or more independent peptides all passing though MAXQUANT adding to the statistical significance [37]). We also considered proteins detected in two independent experiments but excluded those where the standard error of the mean based on multiple detected peptides from the same protein was bigger than the mean. In the B to A comparison, 2708 proteins passed those criteria, whereas 2927 passed in the C to A comparison. We focused on the 2179 proteins that were reliably detected in both datasets (see electronic supplementary material, table S1).

4. Results

4.1. A genetic screen identifies vig-1 and nhl-2 as enhancers of gld-1(op236)

We performed a genetic screen to search for genes whose inactivation enhances the glg-1(op236) defect. glg-1(op236) animals have wild-type germlines at 20°C but are sterile at 25°C [13]. In a whole-genome RNAi feeding screen, we looked for genes whose depletion by RNAi causes sterility at 20°C specifically in glg-1(op236) mutants but not in wild-type animals (figure 1a). Out of over a hundred initial candidates (data not shown), 20 reproducibly showed reduced progeny in the glg-1(op236) background (figure 1b). Of these, deletions were available for seven candidates, and double mutants of five of these with glg-1 confirmed the reduced progeny or sterility phenotype observed in the RNAi screen. A detailed analysis of synthetic phenotypes with hecd-1, cel-1 and lar-1 will be described elsewhere.
Interestingly, vig-1 and nhl-2 were previously identified as modulators of the miRNA pathway [4,5]. We observed that a significant portion of gld-1(op236); nhl-2(ok818) animals are sterile and have stacked oocytes in their germlines and no fertilized embryos in the uterus (electronic supplementary material, figure S1a,b). Analysis of gld-1(op236); vig-1(ok2536) animals revealed that the entire germline fills with undifferentiated cells and animals become sterile within 12 h of reaching adult stage (electronic supplementary material, figure S1c,d). In addition, embryos laid from these animals show a highly penetrant embryonic lethality phenotype (electronic supplementary material, figure S1e). The sterility phenotypes of the gld-1(op236); nhl-2(ok818) and gld-1(op236); vig-1(ok2536) double mutants confirm the results of our RNAi screen. Unexpectedly, 29% of the surviving gld-1(op236); vig-1(ok2536) larvae have arrested development and abnormal somatic morphology (figure 1c,d). ‘Extruding bumps’ are readily observed at various positions along the body axis of these animals. Using the col-19::GFP hypodermal cell marker, we found that hypodermal cells accumulate in these protrusions rather than being regularly spaced along the body axis (figure 1c). In addition, double-mutant animals appear shorter and sick. Sterility and embryonic lethality phenotypes have already been described for dcr-1 (dicer endonuclease) mutants as well as for alg-1/alg-2 Argonaute double mutants [38–40]. Given this similarity, and as both VIG-1 and NHL-2 modulate miRNA function, we decided to investigate possible genetic interactions between gld-1 and miRNAs.

4.2. gld-1 genetically interacts with mir-35 family miRNAs

To investigate an interaction between gld-1 and miRNAs, we started by making double mutants of gld-1(op236) and mir-35 family miRNAs. The mir-35 family of miRNAs comprises eight members (mir-R3–35–42), which are highly enriched in oocytes [20] and are required for embryonic development. While mir-35 family mutants do not individually exhibit an observable phenotype, combined mutation of either all or most mir-35 family members causes severe embryonic and larval lethal phenotypes [20], similar to those we observed in the gld-1(op236); vig-1(ok2536) double mutant. The expression pattern and phenotypes of mir-35 family miRNAs make them suitable to investigate possible genetic interactions with gld-1.

A deletion mutant, nDf50, which removes all mir-35 family miRNAs except for mir-42, causes a temperaturesensitive embryonic and early larval lethality [20]. At 20°C, we observed that 33% of mir-35–41(nDf50) animals die either during embryogenesis or at the L1 larval stage. In gld-1(op236); mir-35–41(nDf50) double-mutant embryonic and larval lethality increases to 67%, indicating a strong genetic interaction between gld-1 and the mir-35 miRNA family (figure 2a, left). Given that both mir-35–41(nDf50) and gld-1(op236); mir-35–41(nDf50) mutants lay similar numbers of eggs (figure 2a, right), the synthetic interaction between gld-1 and mir-35 family miRNAs must specifically affect early embryonic development.
indicates cuticle that failed to shed.

during L4 to young adult transition. (\(\frac{1}{4}\) viable. Number of eggs laid per worm is shown in the right graph. Each experiment was carried out in quadruplicate (m, maternal genotype; z, zygotic genotype. Synchronized L1 stage animals were grown to adult stage and assayed for lethality owing to internal hatching of embryonic and larval lethality is depicted for mir-35 – 41(nD50) and gld-1(op236); mir-35 – 41(nD50) mutants at 20°C (left graph), gld-1(op236) animals are 100% viable. Number of eggs laid per worm is shown in the right graph. Each experiment was carried out in quadruplicate (m, maternal genotype; z, zygotic genotype. Synchronized L1 stage animals were grown to adult stage and assayed for lethality owing to internal hatching of embryos. Owing to the sterility of gld-1(null) animals, gld-1(null); let-7(mg279) phenotype is determined by slow movement and lack of pharyngeal activity during L4 to young adult transition. (c) Representative picture of a gld-1(op236); let-7(mg279) worm. The accumulation of late stage embryos is evident. Arrowhead indicates cuticle that failed to shed.

4.3. gld-1 genetically interacts with let-7 family miRNAs

Next, we decided to investigate whether gld-1 can genetically interact with other miRNA families. The let-7 family (let-7, mir-48, mir-84, mir-241 and mir-795) miRNAs are much more studied compared with mir-35 family miRNAs during C. elegans development. One of the phenotypes in let-7 mutants relates to molting [41]. Caenorhabditis elegans has four larval stages, and each larval stage ends with a molt. Moulting starts with a sleep-like stage called lethargus during which worms slow down feeding and movement. During lethargus, a new cuticle is synthesized, and molting ends with the removal of the old cuticle [42]. A supernumerary fifth molt has been described in let-7(mg279); mir-84(tm1304) double mutants [41], during which adult animals cease to move and stop pharyngeal activity. Subsequently, affected animals fail to lay eggs and die owing to the internal hatching of embryos.

We did not observe such a phenotype in gld-1(op236) and in the hypomorphic let-7(mg279) single mutant, but to our surprise, this phenotype occurred in 84% of gld-1(op236); let-7(mg279) double mutants (figure 2b and electronic supplementary material, movie S1). The mg279 allele has a promoter mutation that reduces let-7 expression [43]. As previously observed for let-7(mg279); mir-84(tm1304) double mutants [41], gld-1(op236); let-7(mg279) animals with only partially shed cuticles can be observed (figure 2c). gld-1(q485) null; gld-1(op236); let-7(mg279) and gld-1(q485) null; let-7(mg279) double-mutant worms show supernumerary moulting phenotypes confirming that the synthetic phenotypes are really caused by mutations of the gld-1 gene. Heterozygous gld-1(op236)+; let-7(mg279) animals have wild-type appearance (figure 2b) consistent with gld-1(op236) behaving as a recessive allele. gld-1(op236) m+ z; let-7(mg279) (m, maternal genotype; z, zygotic genotype) animals have a comparable phenotype with gld-1(op236) m– z; let-7(mg279) animals (figure 2b), showing that maternal contribution of gld-1 does not affect the supernumerary moulting phenotype. To extend our analysis, we investigated possible genetic interactions of gld-1(op236) with the remaining let-7 family members mir-48, mir-84 and mir-241. Forty-two per cent (n = 43) of mir-48 mir-241; mir-84 triple mutants die owing to a burst vulva during the L4 to adult transition reminiscent to the let-7(null) phenotype [44]. The penetrance of this phenotype is enhanced by gld-1(op236), with 64% (n = 81, p = 0.013 Fisher’s exact test) lethality observed in the gld-1(op236); mir-48 mir-241; mir-84 quadruple mutant. gld-1(op236) did not affect levels of mature let-7 miRNA, thereby ruling out the possibility that GLD-1 has an essential, non-redundant role in miRNA processing (see electronic supplementary material, figure S2). Our results show that gld-1 can genetically interact with the let-7 miRNA family during somatic development when the let-7 miRNA pathway is sensitized through mutations of the let-7 family miRNAs.

4.4. gld-1(op236) affects let-7 regulation of hypodermal development

In order to better understand the extent of genetic interactions between gld-1 and the let-7 miRNA, we focused on the role of let-7 miRNA in hypodermal development. During the L4 to adult transition, let-7 downregulates lin-41, a TRM-NHL domain protein that keeps the transcription factor LIN-29 in an inactive state possibly through miRNA regulation as described for mammalian systems [45]. LIN-29 transcriptionally activates adult-stage-specific genes such as collagen col-19 [46]. Either lack of let-7 or disrupted let-7 function, causes loss of col-19 expression owing to increased LIN-41 expression that leads to reduced LIN-29 activity (figure 3a).
ectopic alae are indicated by small T-bars. (Figure 3a) Simplified diagram of the let-7 pathway leading to col-19 expression. (b) col-19:GFP expression in hypodermal hyp7 cells (error bars = s.e.m. of triplicate results). (c) Seam cell fusion defects assayed by the ajm-1::GFP junction marker upon either control RNAi or glp-1 RNAi (error bars = s.e.m. of quadruplicate results, n = 20 for each replicate). In glp-1 RNAi, only the animals without a germline were assayed. (d) Representative pictures of col-19::GFP expressing worms. Numbers indicate worms. Note the complete absence of signal in worm number 2 in the right panel. (e) Representative images of animals showing adult-stage alae and seam cell fusions. In wild-type worms, complete alae and complete seam cell fusion can be seen. Strong ectopic junctions (arrow heads), weak ectopic junctions (small, thin arrows) and lack of junctions (not shown) are observed in glp-1 RNAi, only the animals without a germline were assayed. Schematic drawing of the seam cell fusion defects observable by AJM-1::GFP. (f) Schematic drawing of the seam cell fusion defects observable by AJM-1::GFP.

[41] A transcriptional reporter expressing GFP under the control of the col-19 promoter reveals that both gld-1(op236) and let-7(mg279) single mutants have unaltered col-19::GFP expression (figure 3b). Interestingly, only 28% of gld-1(op236); let-7(mg279) double-mutant animals have wild-type levels of transgene expression (figure 3b) and 47% of double-mutant animals do not express col-19:GFP in the hypodermal hyp7 cells (figure 3d). col-19:GFP expression is not affected in gld-1(op236); let-7(mg279) (1.5%; n = 66) again indicating recessiveness of gld-1(op236). Conversely, reduced expression (60.5%; n = 114) in gld-1(op236);gld-1(q485); let-7(mg279) strains indicates that the phenotype is due to a mutation of gld-1.

At the end of the L4 larval moult, lateral seam cells fuse and form extracellular structures called alae [47]. The timing of seam cell fusion and alae formation is controlled by let-7 family miRNAs [43]. Similarly, mutations in C. elegans genes encoding AGO (alg-1, alg-2) and GW182 proteins (ain-1, ain-2) also have seam cell fusion and alae formation defects [18,38]. We assayed seam cell fusion using the AJM-1::GFP junction marker as indicated (figure 3f). Analysis of differential interference contrast (DIC) images and the AJM-1::GFP junction marker indicate defects in alae formation and seam cell fusions in gld-1(op236), let-7(mg279) and gld-1(op236); let-7(mg279) worms (right hand panel). In the left-hand panels, partial alae or lack of alae are indicated by dashed lines and ectopic alae are indicated by small T-bars. (f) Schematic drawing of the seam cell fusion defects observable by AJM-1::GFP.

4.5. GLD-1 affects let-60 signalling

To check whether the genetic interactions of gld-1 with the let-7 miRNA family are restricted to the hypodermal development, we looked into the let-60/RAS pathway that functions during vulva formation [48]. mir-84 and let-7 antagonize let-60/RAS signalling in vulval precursor cells that are not destined to form the vulva. Such regulation can be assessed in vivo using let-60/RAS gain-of-function alleles that induce
ectopic vulva formation by triggering excessive MAP kinase signalling. The system is sensitized by maintaining the let-60(n1046) gain-of-function allele in a heterozygous state or switching let-60(ga89) temperature-sensitive gain-of-function allele from 20 to 25°C. gld-1(op236); let-60(n1046)/+ (figure 4a) and gld-1(op236); let-60(ga89) (figure 4b) double mutants have an increased incidence of forming multiple vulvae, indicating that GLD-1 also affects vulva induction.

4.6. Germline expression of gld-1 is not necessary for somatic phenotypes

GLD-1 protein expression has previously been reported in the germline [7]. We therefore wished to determine whether gld-1 expression also occurs in somatic tissues, and whether the genetic interactions we observe during somatic development depend on a germline. To determine whether a germline is required for seam cell fusion defects in gld-1(op236) and gld-1(op236); let-7(mg279) animals, we used RNAi to inactivate glp-1, which is essential for germline development [49]. We observed that the defective seam cell fusion phenotype occurs to a similar extent in animals lacking a germline as in those with a germline (figure 3c). Furthermore, somatic expression of an mCherry::H2B transcriptional reporter (the histone fusion used to focus diffuse, low level cytoplasmic GFP expression to the nucleus) under the control of the gld-1 promoter and the gld-1 3’UTR was mainly localized to the head, tail and ventral side of the animals (see electronic supplementary material, figure S3). DIC microscopy analysis indicates that most of the positive cells are neuronal cells in the head and tail ganglia and the ventral nerve cord. Interestingly, let-7 and at least one let-7 target, hbl-1, is also reported to have a similar expression pattern [50], although it is not known to what extent let-7 miRNA phenotypes require hypodermal or neuronal expression. It is likely that high levels of background fluorescence masked low levels of gld-1 reporter expression in other cell types. We could not confirm this localization pattern by antibody staining owing to background problems. However, we generated transgenic animals expressing full-length gld-1 fused to histone::GFP by an operon linker, generated by single copy insertion using the MosSCI technique [29,51,52]. This transgene shows the same pattern of somatic expression, shows expression in embryos and rescues gld-1(null) in the germline (electronic supplementary material, figure S4). Even though these reporter constructs might not exactly represent endogenous GLD-1 expression, together with our genetic results, they suggest somatic roles for gld-1.

4.7. Overexpressing a lin-41 3’UTR construct acts as a ‘sponge’ to sequester let-7 miRNA and provides a sensitized system to assay GLD-1 activity

One of the targets of let-7 miRNA during larval development is the lin-41 mRNA [45]. Hypodermal defects in gld-1(op236); let-7(mg279) could be the result of mis-regulation of lin-41 mRNA. To investigate this possibility, we generated a single copy MosSCI insertion of GFP::lin-41–3’UTR fusion construct under the control of the col-10 promoter that ensures expression in hypodermal tissues. We did not observe any phenotype (figure 5a) in wild-type or in gld-1(op236) animals. However, let-7(mg279) mutants showed a low penetrance bursting through the vulva phenotype reminiscent to let-7(null) phenotype (figure 5a). We likened this observation to a sponge-like effect of the GFP::lin-41–3’UTR towards let-7 miRNA. miRNA sponges are complementary target sequences that can sequester the miRNA from its endogenous target [53]. In C. elegans, such a sponge was used for the lin-4 miRNA [54]. The penetrance of the vulva-bursting phenotype is dramatically enhanced in gld-1(op236); let-7(mg279) double mutants
expressing the GFP::lin-41–3'UTR (let-7 sponge) (figure 5a). Thus, gld-1(op236) specifically enhances the let-7-dependent phenotypes, and the extent of genetic interactions between gld-1 and the let-7 miRNA pathway becomes more evident when the let-7 miRNA pathway is further compromised. Expression of a let-7 sponge with a deletion of the 3 let-7 binding sites or expression of the unrelated unc-54 3'UTR did not cause any bursting phenotype in let-7(mg279) and in gld-1(op236); let-7(mg279) animals supporting the specificity of the let-7 sponge and the interactions between gld-1 and let-7 miRNA (figure 5a).

We next expressed gld-1 under the control of the col-10 promoter in the hypodermis to investigate whether such expression of gld-1 might cause any phenotype associated with the loss of let-7 targets. col-10::GLD-1 expression in the hypodermis caused a loss of adult-stage alae (figure 5b) and a dumpy phenotype (shortened size; figure 5c,d). A dumpy phenotype also occurs following mutation of let-7 targets such as lin-41 [45]. Co-expression of the let-7 sponge partially rescues the dumpy and loss of alae phenotypes (figure 5b–d). Based on these results, we cannot exclude the possibility that gld-1 and let-7 miRNA function in parallel pathways during the hypodermal development. Equally, GLD-1 expression in the hypodermis might cause unrelated phenotypes. However, another likely interpretation of these experiments is that GLD-1 and let-7 act in conjunction to excessively repress target mRNAs possibly in the same pathway, and that reducing the 'dose' of let-7 using the sponge alleviates target gene repression.

4.8. SILAC in nematodes identifies proteome wide changes in gld-1 and let-7 mutants

Our results suggest that GLD-1 and let-7 synergistically affect animal development. We wanted to determine whether the
phenotypes we observed are due to the mis-regulation of either a single or small number of genes, as opposed to affecting multiple target genes. In addition, we wanted to determine whether GLD-1 and the let-7 miRNA regulate distinct or same targets. To address this question, we used nematode SILAC to systematically quantify protein levels [34,36]. The strongest genetic interaction between gld-1 and let-7 occurs in the let-7 sponge system (figure 5a). Thus, to assess changes in the proteome of the respective strains, we used the following experimental set-up. For the SILAC experiment synchronized L1 larvae of three strains, namely (A) [let-7 sponge], (B) let-7(mg279); [let-7 sponge] and (C) gld-1(op236); let-7(mg279); [let-7 sponge] were grown up to the young-adult stage until the bursting phenotype just becomes visible and subjected to quantitative mass spectrometry (figure 6a). As the animals expressing let-7 sponge alone do not display any phenotype, we considered them as the baseline similar to using wild-type. Thus, by comparing the animals with a weak phenotype (B) to animals with a strong phenotype (C), we aimed to identify proteins whose expression change might be responsible for the bursting through the vulva phenotype and help explain the interaction between gld-1 and the let-7 miRNA. In a SILAC experiment, the relative ratios of two sets of proteins can be measured by differential isotope labelling. We then determined proteins differentially expressed when B was compared with A. At the same time, we also determined differentially expressed proteins comparing C with A. As a control, we analysed the levels of GFP expression under the control of lin-41 3’UTR sponge by Western blotting. GFP levels are higher in the let-7(mg279); let-7 sponge animals, compared with the let-7 sponge only (B to A; electronic supplementary material, figure S5a). We identified a similar rise in GFP levels in SILAC experiments, and the level of GFP was further increased in gld-1(op236); let-7(mg279); let-7 sponge animals (C to A; electronic supplementary material, figure S5b). This result confirms the sensitivity of our SILAC-based approach. In the B/A comparison, 2708 proteins passed our specificity criteria, whereas 2292 passed in the C/A comparison (see Materials and methods). We focused on comparing the abundance of the 2179 proteins that were reliably detected in both datasets. The ratios of (C) gld-1(op236); let-7(mg279); [let-7 sponge] protein to (A) [let-7 sponge] protein shown on the y-axis are compared with the (B) let-7(mg279); [let-7 sponge], with (A) [let-7 sponge] ratios depicted on the x-axis (figure 6a).

Among these 2179 proteins, 252 overlap with the 1084 previously described GLD-1 targets (figure 6a, coloured in blue) [55,56]. Proteins (239) overlap with 1322 predicted let-7 targets (figure 6a, coloured in red, mirWIP database [57]). Fifty-four proteins are predicted to be both GLD-1 and let-7 targets (figure 6a, coloured in purple). The relative abundance of the majority of suspected GLD-1 and let-7 co-targets do not change when the C/A ratios are compared with the B/A ratios. However, three such candidate proteins, namely cdl-1, dnj-2 and B0303.3 were more prominently upregulated in C/A.

We tested whether the depletion of these three proteins suppresses the vulva-bursting phenotype of the gld-1(op236); let-7(mg279); [let-7 sponge] strain (figure 6b). Targeting the [let-7 sponge] which is ac10-10; GFP::lin-41 3’UTRconstruct by GFP RNAi lead to a complete suppression of the vulva-bursting phenotype and thus served as a positive control. The depletion of one of the candidates, namely cdl-1 lead to a reduced vulva-bursting phenotype consistent with the notion that the upregulation of CDL-1 in the gld-1(op236); let-7(mg279) background might contribute to the vulva-bursting phenotype. Indeed, cdl-1 3’UTR harboura a GLD-1 and a let-7 binding site (electronic supplementary material, figure S7). CDL-1 is a histone mRNA hairpin binding protein required for expression of histones [58–61]. It is expressed in all somatic cells with strong expression in proliferating cells such as hypodermal cells, intestal cells and the germ cells [59]. CDL-1 is required for larval development and affects vulva morphology [59,61]. However, the suppression of the vulva-bursting phenotype by CDL-1 RNAi in gld-1(op236); let-7(mg279); [let-7 sponge] animals is not complete. This indicates that other factors also contribute to the vulva-bursting phenotype.
4.9. Identification of GLD-1-containing complexes

Our data are consistent with GLD-1 either directly or indirectly interacting with the miRNA pathway. To investigate this genetic interaction at biochemical level, we next aimed to purify proteins that interact with GLD-1. We used GLD-1 antibodies to immunoprecipitate GLD-1 complexes in wild-type animals and used mass spectrometry to identify GLD-1 interactors. To verify the specificity of GLD-1 interactions, we also pulled down GLD-1 using a GFP-binder from animals expressing a GLD-1::GFP fusion protein and focused our subsequent analysis on proteins specifically pulled down in both purification approaches (figure 7a). Among the GLD-1 interactors identified, many are associated with RNA and some are involved in RNA-mediated gene regulation (figure 7a). We identified the CGH-1 helicase, CAR-1 and the Y-box domain proteins CEY-2, CEY-3 and CEY-4, all previously reported to be components of a complex localized to RNP granules in the C. elegans germline, and that are likely to function in translational repression [62]. Importantly, CGH-1 is proposed to act in mRNA-mediated gene expression. Depletion of cgh-1 enhances the defects of let-7 family mutants and CGH-1 biochemically interacts with ALG-1, AIN-1 and NHL-2 [5]. Indeed, ALG-1 also co-purified with GLD-1 in our experiments (figure 7a). Other proteins identified as GLD-1 interactors include PAB-1 and SQD-1. PAB-1 is a C. elegans poly(A) binding protein and also a component of AIN-1, AIN-2 and CGH-1 complexes, suggesting a role in translational regulation and mRNA-mediated repression [18,63,64]. SQD-1 is the C. elegans orthologue of the Drosophila squid hnRNP protein, previously identified as an AIN-2 and mir-35 miRNA-associated protein [18,65]. PAB-1, CGH-1, CAR-1, CEY-1-4 and SQD-1 were also identified as GLD-1 protein interactors in a recent study [66]. In the same study, interaction of CGH-1 with GLD-1 was shown to depend on the presence of RNA. Considering all the GLD-1 interactors have RNA-binding domains, the presence of RNA might be essential in all these interactions. In summary, GLD-1 interactors identified in this and previous studies associate with proteins known or suspected to be involved in mRNA-mediated gene repression.

We next investigated whether GLD-1 interactors affect let-7 miRNA function similar to GLD-1. We therefore assessed the extent of vulva-bursting upon RNAi of those interactors in let-7 sponge and let-7(mg279); let-7 sponge animals. RNAi against alg-1, ain-1 and ain-2, core miRNA components, were used as positive controls. As expected, alg-1 RNAi induces a strong vulva-bursting phenotype in both let-7 sponge and let-7(mg279); let-7 sponge animals (figure 7b). ain-1 and ain-2 RNAi induced the vulva-bursting phenotype only in the sensitive let-7(mg279); let-7 sponge animals. Among the GLD-1 interactors besides alg-1 RNAi, cgh-1 and pab-1 RNAi also induced a strong vulva-bursting phenotype in the let-7(mg279); let-7 sponge animals, supporting their role in miRNA function.

5. Discussion

The starting point of our work was the identification of two miRNA effector genes, vig-1 and nhl-2 as genetic enhancers of gld-1(op236) (figure 1). Both genes had previously been shown to affect miRNA function [4,5], although not in the germline. It is possible that the germline phenotypes we observed are related to a miRNA-associated function, although it is equally likely that VIG-1 and NHL-2 may, either directly or indirectly, affect GLD-1 function, or else may act in conjunction with GLD-1 to mediate translational repression of GLD-1 targets in the germline. Different phenotypes observed in the gld-1(op236); vig-1(ok2536) and the gld-1(op236); nhl-2(ok818) double mutants suggest that vig-1 and nhl-2 function in different pathways in the germline. We will investigate these possibilities in future studies.

Intrigued by the genetic interactions with those miRNA effector genes, we investigated possible roles of gld-1 in multiple miRNA-mediated pathways and uncovered novel roles for gld-1 during the somatic development of C. elegans. Given that gld-1 phenotypes and expression were previously only described for the germline, we were surprised to find GLD-1 phenotypes associated with somatic development. Enhancement of mir-35 family embryonic and larval lethal phenotypes may be explained by perturbation of maternal miRNA pools derived from gld-1(op236) germlines that may enhance mir-35 phenotypes. However, it is unlikely that such a model can explain the genetic interactions we observed between gld-1 and let-7 family miRNAs. let-7-related

| protein names | total peptide (background subtracted) | % mean coverage | protein name (49) |
|---------------|--------------------------------------|----------------|------------------|
| CGH-1         | 20 (1)                               | 19.4           | 48.7             |
| CAR-1         | 13 (2)                               | 11.9           | 37.6             |
| CEY-4         | 7 (1)                                | 18.4           | 32.4             |
| CEY-3         | 6 (1)                                | 15.7           | 29.2             |
| CEY-2         | 13 (5)                               | 34.8           | 29.4             |
| ALG-1         | 12 (3)                               | 4.5            | 113.1            |
| PAB-1         | 90 (49)                              | 22.4           | 71.6             |
| RPL-23        | 6 (0)                                | 27.2           | 15.0             |
| SQD-1         | 5 (0)                                | 10.7           | 33.7             |
| WOSGI11.6     | 13 (5)                               | 13.0           | 73.2             |
| T22F3.3       | 18 (6)                               | 9.2            | 101.6            |
| C14F11.1      | 6 (1)                                | 10.2           | 45.6             |
phenotypes arise much later during development, making a mechanism involving the maternal contribution of miRNAs unlikely. Indeed, we show that glld-1(op236) m- z- animals have a comparable phenotype with glld-1(op236) m- z- animals (figure 2). Furthermore, we observed that let-7 phenotypes are enhanced by glld-1 even when glp-1 RNAi animals lacking a germline were analysed. Finally, recent data published by the modENCODE consortium indicate that glld-1 is transcribed in glp-1 mutants that lack a germline [67]. Consistent with this, using two independently generated reporters we observed glld-1 reporter expression in somatic tissues. Unfortunately, GLD-1 antibody staining was not successful owing to unspecific background in somatic tissues.

We focused on the let-7 miRNA pathway, whose components have been much more characterized in C. elegans. By using already established tools, we could show that glld-1 affects multiple let-7 miRNA regulated pathways (figures 2–4). Importantly, we generated a sensitized system using a let-7 sponge and showed that glld-1(op236) specifically enhances let-7 loss-of-function phenotypes (figure 5). Our let-7 sponge system confirms the notion that the miRNA pathways are highly redundant. let-7 miRNA levels in wild-type animals are sufficient to regulate both the endogenous targets and also an additional transgene target (let-7 sponge). However, when the let-7 miRNA levels are limiting, such as in the hypomorphic let-7(mg279) mutants, endogenous targets are not efficiently dealt with when the let-7 sponge is present (figure 5a). Only in this ‘very sensitive’ situation, a role for glld-1 in the let-7 miRNA pathway becomes apparent. This finding can be explained by the robustness and redundancy of the let-7 miRNAs and also by the target genes whose mis-regulation is well tolerated. When we expressed GLD-1 specifically in the hypodermis, we observed defects in alae formation and dumpy animals. Even though these phenotypes might be unspecific, as we do not know whether GLD-1 is expressed in this tissue, co-expressing the let-7 sponge partially suppresses these phenotypes. This further supports the involvement of glld-1 either in the let-7 pathway or in a parallel pathway. This hypothesis is further supported by the functions of the GLD-1 interactors we identified (figure 7): ALG-1 is a core component of the miRNA machinery [38], and CGH-1 can interact with ALG-1 to modulate miRNA-mediated gene regulation [5]. PAB-1 interacts with AIN-1/2, core miRISC components [18,63], and PAB-1 homologues are required for miRNA-mediated gene regulation in mammalian cells [68]. We have shown that the GLD-1 interactors CGH-1 and PAB-1 affect let-7 miRNA function (figure 7).

Hundreds of genes are regulated by GLD-1, and it is likely that the expression of an even higher number of genes is modulated by miRNAs. In this context, how can we explain the genetic and biochemical interactions we observe? To address this question, we took a state of the art proteomics approach based on SILAC in nematodes. With this approach, we were able to detect the differential expression of more than 2000 proteins, and we were able to compare the relative abundance of these proteins in animals with weak and strong phenotypes. When we tested some of the upregulated proteins for suppression of the vulva-bursting phenotype associated with the glld-1(op236); let-7(mg279); [let-7 sponge] strain, we detected a strong suppression with the RNAi-mediated knockdown of cdll-1 gene. cdll-1 is a predicted let-7 miRNA target and it was identified as a GLD-1 target [55–57]. Thus, cdll-1 is a strong candidate to be co-regulated by both let-7 and GLD-1. However, we cannot rule out the possibility that the cdll-1 upregulation is not directly controlled by let-7 or GLD-1 and it may arise owing to secondary effects. Either way, we can conclude that CDL-1 upregulation in a let-7 and GLD-1-dependent manner is in part responsible for the vulva-bursting phenotype.

Overall, we suggest two possible mechanisms for GLD-1 and miRNA interactions that are not mutually exclusive and may act in parallel. First, mutation of both GLD-1 and miRNAs could mis-regulate several targets within a single pathway, leading to the observed phenotypes. For instance, moulting defects in let-7 mutants are partly due to mis-regulation of the nuclear hormone receptors nhr-23 and nhr-25 [41], and nhr-23 is a predicted GLD-1 target [55,56]. We could not detect NHR-23 in our SILAC experiments. Similarly, some of the phenotypes we observed (figure 5b) might be explained by negative regulation of LIN-28 by GLD-1 as it was reported for the germline [55]. However, we did not detect changes in LIN-28 protein levels, which is why we did not follow up on this lead.

A second possibility is that GLD-1 and miRNAs may co-regulate a subset of miRNAs. The interactions we observed between GLD-1, CGH-1, PAB-1 and ALG-1 in GLD-1 complexes support such a mechanism. Consistent with this model, GLD-1 appears to associate with several 3’UTRs that are known or predicted miRNA targets in C. elegans. For example, the potential mir-35 targets, lin-23 and glld-1 [69], have also been identified as GLD-1 targets [55]. Similarly, lin-28 and ztf-7 are let-7 miRNA targets [70,71] and these genes have also been identified as GLD-1 targets [55].

We are aware that biochemical interactions reflect total animal extracts and that most GLD-1 protein expression is likely to occur in the germline. Thus, the association of GLD-1 with 3’UTRs in somatic cells may be underrepresented in biochemical experiments aimed to determine GLD-1 targets [55]. GLD-1 has been extensively studied in the germline and consensus-binding motifs have been defined [55,72]. We also know that GLD-1 appears to bind RNA as a dimer and that GLD-1 RNA binding is enhanced when multiple binding sites are available [72]. Still, we know next to nothing regarding how GLD-1 actually confers translational repression. It is intriguing that several proteins previously associated with miRNA regulation biochemically interact with GLD-1. CGH-1 interacts with the miRISC complex and GLD-1 in an RNA-dependent manner [5,66], and therefore raises the possibility of being the mediator between the two translational repression mechanisms. In a recent study, one of the C. elegans poly(A) binding proteins, PABP-2, was shown to antagonize let-7 miRNA function [73]. In our study, we show that PAB-1 is required for proper let-7 function and this is in line with the interactions between PAB-1 and AIN-1 [63]. These results indicate that different poly(A) binding proteins may affect miRNA function in opposite ways and perhaps these effects depend on other RNA-binding proteins like GLD-1. We therefore speculate that the mechanisms of GLD-1-mediated translational repression and miRNA-mediated translational repression may overlap. It will be interesting to investigate this potentially-shared mechanism in the future.

By taking advantage of multiple genetically sensitized miRNA pathways that act during C. elegans development to reliably assess subtle changes in gene expression, we could implicate GLD-1 in miRNA-mediated gene regulation. The mammalian GLD-1 homologue QKI has been reported to co-localize and interact with ALG2 [74], and recently QKI was shown to directly interact with and stabilize miR-20a, revealing...
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