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ORIGINAL ARTICLE

mir-35 is involved in intestine cell G1/S transition and germ cell proliferation in *C. elegans*

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MicroRNA (miRNA) regulates gene expression in many cellular events, yet functions of only a few miRNAs are known in *C. elegans*. We analyzed the function of *mir-35*-41 unique to the worm, and show here that *mir-35* regulates the G1/S transition of intestinal cells and germ cell proliferation. Loss of *mir-35* leads to a decrease of nuclei numbers in intestine and distal mitotic gonad, while re-introduction of *mir-35* rescues the mutant phenotypes. Genetic analysis indicates that *mir-35* may act through Rb/E2F and SCF pathways. Further bioinformatic and functional analyses demonstrate that *mir-35* targets evolutionally conserved *lin-23* and *gld-1*. Together, our study reveals a novel function of *mir-35* family in cell division regulation.

***Keywords*:** miRNA; *C. elegans*; *mir-35*; G1/S transition; germ cell proliferation
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Introduction

miRNAs are non-coding transcripts of 19-25 nucleotides in eukaryotes. Since *lin-4* was first identified in *C. elegans* [1], hundreds of miRNA species, with important functions in a variety of biological processes, have been identified in various multi-cell organisms. These small RNAs regulate gene expression mainly through degradation of target mRNAs [2], termination of translation [3], and chromatin modification [4]. In *C. elegans*, there are currently about 170 miRNA genes listed in the miRBase sequence database (http://www.mirbase.org); however, only a few have been studied in detail [5-14]. Because deleting most miRNAs individually does not lead to any overt phenotypes/abnormalities in development and differentiation [15], it has been a popular accord that functional redundancy of different miRNAs (miRNAs share the same or have diverse and overlapping target mRNAs) may contribute to the limited progress of miRNA study in the worm. The complexity of functional redundancy of miRNAs may further be contributed by tandem-arrayed miRNA gene clusters in the worm genome.

Because miRNAs normally function in a subtle way to safeguard or buffer developmental or physiological processes, and/or to coordinate multiple gene regulatory pathways, functions of individual miRNAs could hardly be distinguishable in normal conditions. Recent miRNA studies suggest that previously unknown miRNA functions may be revealed under sensitized or stressed conditions [16]. For instance, *mir-1* has been indicated in modulating synaptic function when neuro-musculature signaling is compromised [17]. Recently, Brenner *et al*. reported that in sensitized situations, either genetically or environmentally, critical roles of several known miRNAs could be revealed, indicating that a group of seemingly unrelated miRNAs works redundantly on distinct target

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Abbreviations: CDC (Cell Division Cycle related), CKI (Cyclin-dependent Kinase Inhibitor), CUL (CULin), CYE (CYclin E), DPL (DPLike protein), EFL (E2F-like protein), GLP (abnormal Germ Line Proliferation), GLD (defective in Germ Line Development), LIN (abnormal cell LINeage), *mir-35* (microRNA-35), RB (Retinoblastoma protein), SCF (Skp1-Cul1-F box)
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mRNAs of parallel regulatory genetic pathways, and/or on the very same targets that integrate distinct genetic pathways in a group of specialized cells [16]. Therefore, against genetic and/or environmental changes, micro effects of individual miRNAs would be amplified.

The *mir-35* family (*mir-35-41* cluster and *mir-42*) and its expression pattern were first described in 2001 [14]. Later in a genome-wide analysis of miRNA promoter activities, the *pri-mir-35-41* was found ubiquitously expressed in embryo and all larva stages [18]. Early embryonic death was evident when the entire cluster, including *mir-42*, was deleted [14]; however, the genetic basis of how the *mir-35* family affects post-embryonic worm development was not explored. We sought to study the function of *mir-35* family in a temperature sensitive mutant, *gk262*, in which only the *mir-35-41* cluster was deleted (Figure 1A). Combining genetics and other approaches, we discovered that *mir-35* functions in G1/

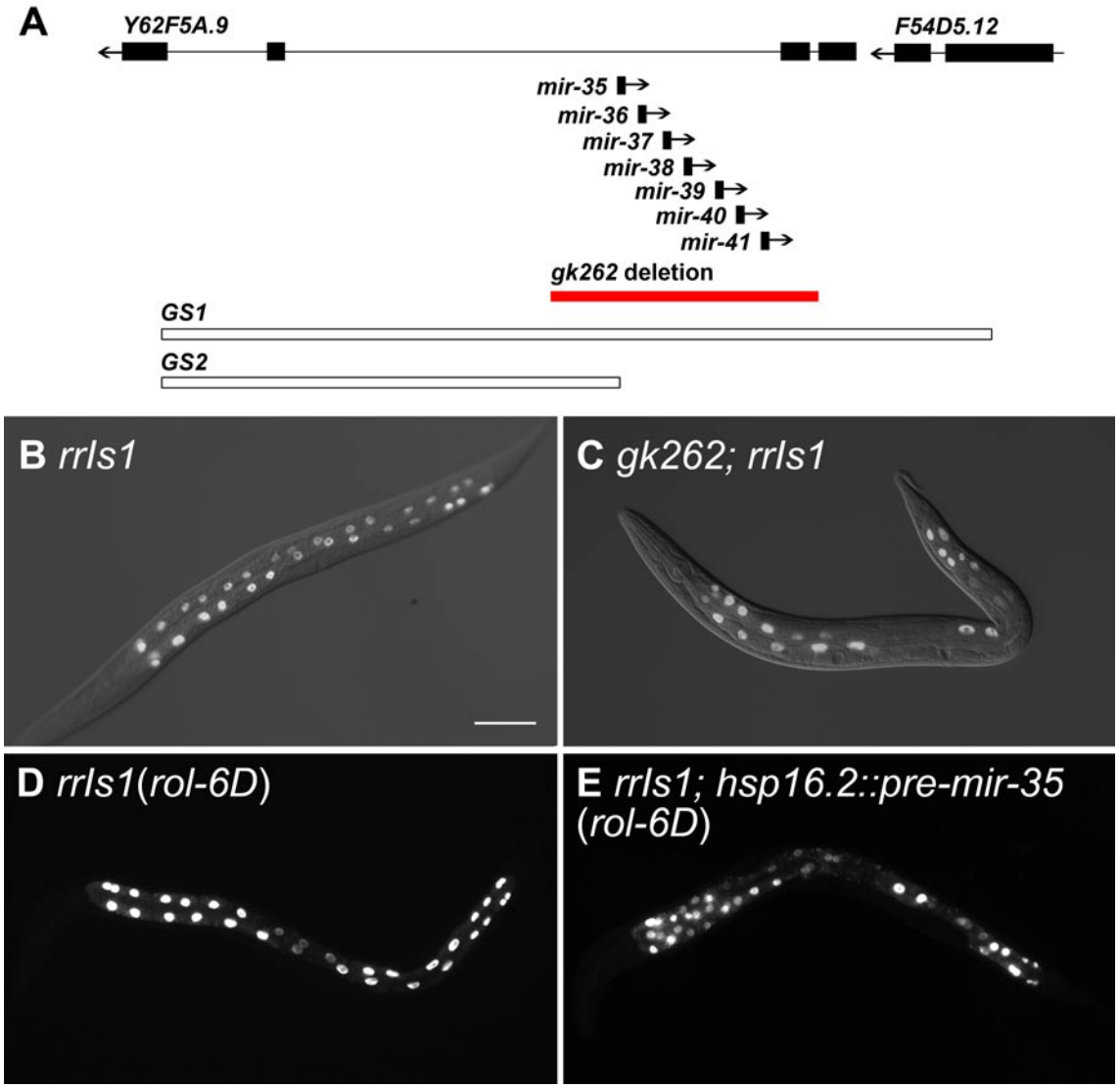


Figure 1 *mir-35* affects the cell cycle control in intestinal cells. **(A)** The schematic genomic location of *mir-35-41* cluster. The mutant *gk262* has a 1 268 bp deletion at *Y62F5A.9* locus, removing the whole *mir-35-41* cluster. GS1 is a 4.06 kb-fragment that covers the entire *mir-35-41* locus. GS2 is a 2.16-kb fragment upstream of *mir-35-41* cluster locus, which includes the promoter of *mir-35*. **(B)** The *rrls1*[*elt-2::GFP*] marker visualizes the nuclei of the intestine cells. **(C)** *gk262* animals exhibit a decrease in the intestine nuclei number, **(D**, **E)** *mir-35* over-expression increases the intestine nuclei number. **(D)** *rrls1* carrying a *rol-6D* co-microinjection marker as a control. **(F)** The transgenic *hsp-16.2::pre-mir-35*(*rol-6D*) animals show a increase in the intestine nuclei number. The scale bar represents 100 μm.

Table 1 Number of intestinal nuclei in *gk262* mutants

	20 °C		25 °C (temperature shift)	
	rrIs1	*gk262; rrIs1*	*rrIs1*	*gk262; rrIs1*
3-fold stage	19.0 ± 0.6 (49)*, 18-20**	17.0 ± 2.3 (45), 11-20	18.8 ± 1.0 (56), 17-20	16.0 ± 2.3 (48), 11-20
L3/L4 stage	31.0 ± 1.4 (83), 27-34	26.3 ± 3.2 (141), 19-34	30.7 ± 1.6 (93), 26-34	22.5 ± 3.4 (102), 16-30

*The number in the bracket is the total animal number examined.
**The dash indicates the range of the number.

Table 2 Number of intestinal nuclei in transgenic lines expressing *mir-35-41* cluster members at 25 °C

Strains		Intestinal nuclei
rol-6D		32.0 ± 1.1 (91)*, 28-34**
gk262; GS1(*rol-6D*)		32.0 ± 2.0 (46), 29-34
gk262; GS2::*pre-mir-35*(*rol-6D*)	line1	31.6 ± 2.1 (51), 30-35
	line2	31.5 ± 2.9 (47), 29-34
gk262; GS2::*pre-mir-36*(*rol-6D*)	line1	32.1 ± 1.8 (43), 29-35
	line2	31.2 ± 1.3 (39), 29-34
gk262; GS2::*pre-mir-37*(*rol-6D*)	line1	32.0 ± 2.0 (50), 28-38
	line2	31.5 ± 1.7 (33), 28-35
gk262; GS2::*pre-mir-38*(*rol-6D*)	line1	32.6 ± 1.5 (38), 29-35
	line2	31.7 ± 1.7 (31), 28-35
gk262; GS2::*pre-mir-39*(*rol-6D*)	line1	31.5 ± 1.6 (48), 27-35
	line2	30.8 ± 1.4 (34), 28-34
gk262; GS2::*pre-mir-40*(*rol-6D*)	line1	31.6 ± 1.7 (37), 27-35
	line2	31.0 ± 1.5 (42), 28-35
gk262; GS2::*pre-mir-41*(*rol-6D*)	line1	31.1 ± 1.6 (55), 27-34
	line2	30.7 ± 1.5 (34), 27-34
gk262; ges-1::*pre-mir-35*(*rol-6D*)	line1	33.5 ± 1.6 (49), 30-38
	line2	33.3 ± 1.7 (50), 29-38
hsp16−2::*pre-mir-35*(*rol-6D*) (20 °C)	line1	35.6 ± 2.0 (55), 32-41
	line2	35.3 ± 1.0 (67), 33-37
	line3	35.4 ± 1.4 (81), 32-39

*The number in the bracket is the total animal number examined.
**The dash indicates the range of the number.

S transition to regulate intestinal cell cycle and germ cell proliferation.

G1/S transition is governed by three identified pathways, CKI (Cyclin-dependent Kinase Inhibitor), SCF (Skp1-Cul1-F box) and Rb (Retinoblastoma protein)/E2F. We find that *mir-35* participates in G1/S transition mainly through inhibiting Rb/E2F and SCF pathways during intestine development. In the gonad, GLP-1 (abnormal Germ Line Proliferation)/Notch signaling is essential to promote germ line divisions in distal mitotic region [19-22]. GLD (defective in Germ Line Development) family plays a key role in determining meiosis entry and indirectly regulates mitotic proliferation of the germline [23-27]. We found that *mir-35* may down-regulate *gld-1* to ensure a normal germline proliferation in distal mitotic region. Together, our study reveals that *mir-35* coordinates distinct signaling/regulatory pathways in cell cycle and proliferation.

Results

Lacking mir-35 causes decrease of intestinal nuclei numbers

In *C. elegans*, wild-type embryo has 20 intestine cells, each of which has a diploid nucleus at 3-fold stage [28]. After hatching, some intestinal nuclei replicate without cell division, thus 30 to 34 diploid nuclei are present in the 20 intestine cells since late L1 stage [28]. Transgenic GFP line *rrIs1*[*elt-2::GFP*] specifically labels intestine nuclei (Figure 1B). When *rrIs1* was introduced into

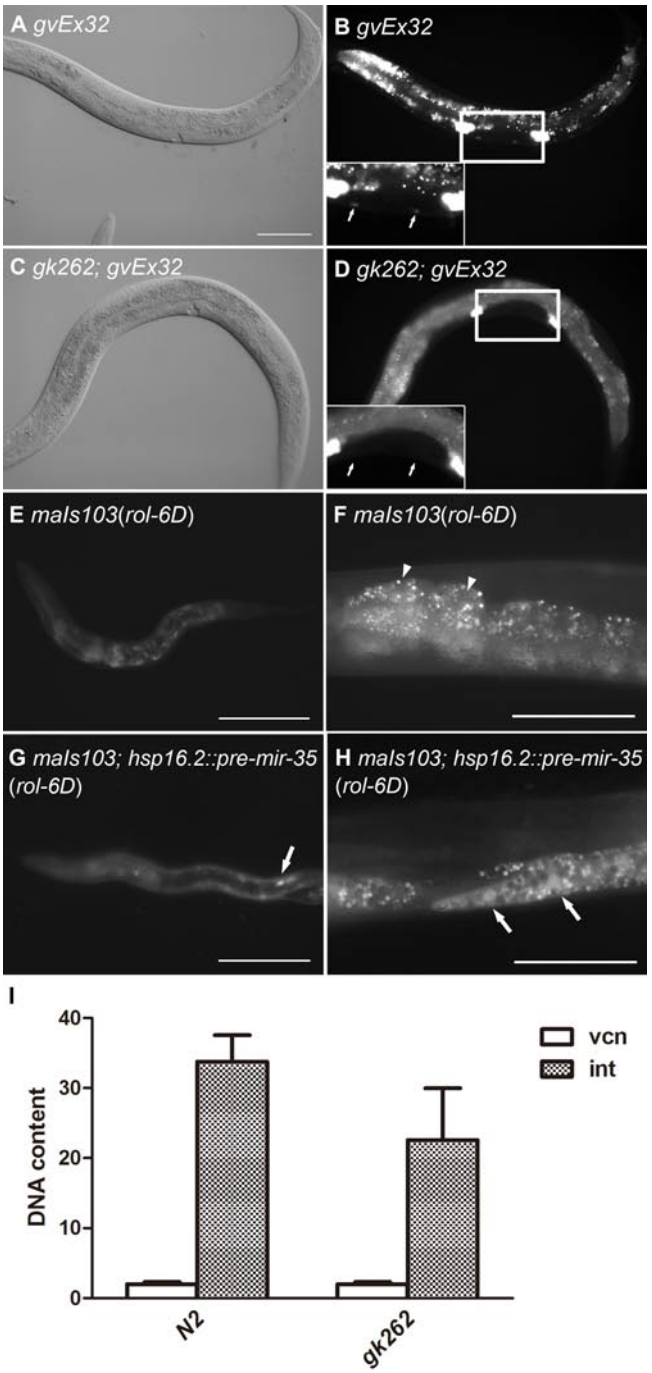


Figure 2 *mir-35* affects DNA replication. **(A**, **B)** The *gvEx32* [*cye-1::GFP*; *rol-6D*] marker visualizes the nuclei in the process of DNA replication. The DIC **(A)** and the *cye-1::GFP* **(B)** picture of the same animal at early L4 stage. In the enlarged region, the arrows indicate two hypodermal cell nuclei that are replicating DNA. **(C**, **D)** *gk262*; *gvEx32* shows less GFP-expressing cells after the temperature shift. The DIC **(C)** and the *cye-1::GFP* **(D)** picture of the same animal at the same stage as shown in **A** and **B**. In the enlarged region, the arrows indicate two hypodermal cell nuclei that lose DNA replication. **(E**, **F)** The *maIs103*[*rnr::GFP*] marker also visualizes the nuclei in the process of DNA replication. While at the arrest L1 **(E)** and adult **(F)** stage, all the somatic cell nuclei stop DNA replication. The arrowheads in F indicate intestinal nuclei, which stop DNA replication. **(G**, **H)** *mals103* carrying a *hsp-16.2::pre-mir-35* transgene could induce DNA replication in the intestinal nuclei at the arrest L1 **(G)** and adult **(H)** stage after heat-shock. The arrows in **G** and **H** indicate intestinal nuclei in which DNA is synthesized. The scale bars represent 100 μm. **(I)** Intestinal ploidy measurement in N2 and *gk262*. Ventral cord neuron nuclei were used as an internal 2n standard. White bar indicates the average DNA content of over 50 ventral cord nuclei in 10 independent animals. Black bar indicates the average DNA content of over 50 intestinal nuclei in 10 independent animals. Error bars represent standard deviation (SD).

the *mir-35* mutant *gk262*, less intestinal nuclei were observed than the wide type, at 3-fold and L3/L4 stages, respectively (Table 1, $P < 0.001$, Student *t*-test). As a temperature sensitive deficiency, *gk262* is embryonic lethal at 25 °C. Using temperature shift assay (i.e., synchronized young adults were allowed to lay eggs for 1 h at 20 °C, and the eggs were shifted to and maintained at 25 °C), we found *gk262* survivors had only an average of 16.0 ($n = 48$) and 22.5 ($n = 102$) intestinal nuclei at 3-fold and L3/L4 stages, respectively, significantly less than the wide type (Figure 1C, Table 1, $P < 0.001$, Student *t*-test). The DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) nuclear staining also indicated such a decrease (data not shown). Therefore, intestinal E lineage is abnormal in *gk262*.

gk262 has a 1 268 bp deletion at the *Y62F5A.9* locus, removing the gene's entire 2nd exon and portions of 1st and 2nd introns. *mir-35-41* cluster resides in an opposite orientation of *Y62F5A.9* and is completely missing in *gk262* (Figure 1A). A 4.06 kb-PCR product (GS1) that covers the entire *mir-35-41* locus and partial *Y62F5A.9* (Figure 1A), could fully rescue embryonic lethality and intestinal defect of *gk262* at 25 °C, i.e., there were 32.0 ± 2.0 °C ($n = 46$) intestinal nuclei in transgenic *gk262; GS1*. We also used a 2.16-kb fragment (GS2, Figure 1A) to drive the expression of each of the seven members of the *mir-35* cluster, and every resulted construct completely rescued *gk262* intestine defects at 25 °C (Table 2). When *pre-mir-35* was driven by an intestine specific *ges-1* promoter and expressed in *gk262*, the intestinal-defects could be fully rescued (Table 2). In fact, excess intestinal nuclei could also be observed in the transgenic *gk262* (Table 2). When we mis-expressed either *pre-mir-35* or *pre-mir-35-41* with a heat-shock promoter, we found that the intestinal nuclei number was further

Table 3 Number of intestinal nuclei in the double mutants of *gk262* and G1/S related genes at L3/L4 stage

		20 °C		25 °C	
		Control	*gk262*	Control	*gk262*
SCF	*cdc-25.1* (*rr31*)	61.3 ± 2.8 (30)*, 55-65**	44.2 ± 7.8 (26), 32-55		
Rb/E2F	*lin-35* (*n745*)	31.3 ± 2.2 (43), 28-35	31.0 ± 2.4 (45), 27-39	31.4 ± 2.0 (50), 28-35	31.6 ± 1.7 (49), 29-36
	lin-36 (*n766*)	33.5 ± 2.1 (48), 29-38	30.6 ± 2.0 (47), 26-35	30.7 ± 2.4 (47), 25-35	30.4 ± 2.1 (50), 25-34
	efl-1 (*se1*)	30.9 ± 1.9 (50), 27-35	30.4 ± 2.7 (40), 24-36	30.0 ± 1.6 (46), 28-34	29.7 ± 1.9 (48), 25-34

*The number in the bracket is the total animal number examined.
**The dash indicates the range of the number.

Table 4 Number of intestinal nuclei in the double mutants of *gk262* and G1/S related genes at 3-fold stage

		20 °C		25 °C	
		Control	*gk262*	Control	*gk262*
Inhibitor	*cki-1* (*RNAi*)	28.8 ± 1.7 (31)*, 26-32**	20.9 ± 2.0 (18), 16-24	29.4 ± 1.4 (54), 25-32	21.5 ± 2.4 (48), 15-28
SCF	*lin-23* (*RNAi*)	42.8 ± 3.6 (17), 35-50	37.2 ± 5.7 (23), 30-50	43.4 ± 4.6 (92), 35-57	40.2 ± 3.3 (42), 29-46
Rb/E2F	*dpl-1* (*RNAi*)	22.9 ± 2.3 (55), 19-28	16.7 ± 2.0 (43), 14-20	22.6 ± 2.7 (72), 19-41	15.7 ± 2.1 (57), 11-20
	lin-9 (*RNAi*)	20.9 ± 1.9 (44), 19-23	17.9 ± 1.8 (71), 14-22	20.4 ± 1.1 (56), 18-22	17.4 ± 1.4 (81), 14-22
	lin-15B (*RNAi*)	21.0 ± 1.7 (39), 18-23	19.1 ± 1.7 (31), 13-21	20.2 ± 1.2 (58), 17-23	18.1 ± 1.7 (54), 12-21

*The number in the bracket is the total animal number examined.
**The dash indicates the range of the number.

increased (Figure 1D-1E; Table 2, $P < 0.001$, Student *t*-test). These results indicate that the loss of the *mir-35-41* cluster is responsible for *gk262* intestine defects.

mir-35 affects DNA replication of intestine nuclei

In *C. elegans*, all intestinal nuclei endoreduplicate their DNA prior to each of four molts, thereby producing 32n nuclei in adult intestine cells [28]. DNA content of intestinal nuclei in adult *gk262* was measured. Using normally divided ventral cord neuron as 2n control, partially visualized by transgenic GFP lines *juIs14*[*acr-2::GFP*] and *juIs76*[*unc-25::GFP*] (data not shown) , we found that DNA content of intestinal nuclei was 22.6 ± 7.4 ($n = 68$) in *gk262*, much less than that of 33.3 ± 3.8 ($n = 52$) in wild type (Figure 2I, $P < 0.01$ Student *t*-test). Moreover, we followed nuclear DNA synthesis in *gk262* using two transgenic GFP lines, *maIs103*[*rnr::GFP*] and *gvEx32*[*cye-1::GFP; rol-6D*], both of which specifically marked nuclei entering S phase (Figure 2A, 2B, 2E and 2F). We observed less GFP-expressing nuclei in *gk262; gvEx32* (Figure 2C and 2D). Furthermore, heat-induced ectopic expression of *pre-mir-35* in L1 arrest and adult *maIs103* worms resulted in *rnr::GFP* expression in intestinal nuclei (Figure 2E-2H). These results indicate that *mir-35* could trigger DNA synthesis in quiescent cells (i.e. cells in terminally differentiated state), thus regulating G1/S transition of the cell cycle in intestine cells.

mir-35 engages in the Rb/E2F and SCF pathways in regulating G1/S transition

To determine how *mir-35* affects G1/S transition, we analyzed genetic interactions of *mir-35* and pivotal genes in the three main regulatory pathways. CKI-1 was reported to negatively regulate G1/S transition [29-31] as one of the most important CKIs. In *cki-1*(*RNAi*)*, gk262*, the number of intestinal nuclei was between those of *cki-1*(*RNAi*) and *gk262* at 3-fold stage (Tables 1 and 4), showing a partial suppression effect of *cki-1* on *gk262*. Meanwhile, *cki-1*(*RNAi*); *gk262* led to an additive effect on embryonic lethality. These data indicated that *cki-1* function may be mediated partially through *mir-35*.

The SCF complex functions as an E3 ubiquitin ligase and degrades candidate target proteins, including CYE-1 (cyclin E-1) [32, 33] and CDC-25.1 (Cell Division Cycle related) [34], to negatively regulate G1/S transition. In *C. elegans*, *cul-1* (CULlin) and *lin-23* (abnormal cell LINeage) have been identified to encode main components of this complex [35, 36], and depletion of *lin-23* leads to intestinal hyperplasia [34]. We found *lin-23*(*RNAi*); *gk262* showed a high rate of embryonic lethality and *lin-23*(*RNAi*)-induced hyperplasia could hardly be restored in *gk262* (Tables 3 and 4), suggesting that *lin-23* may function downstream of *mir-35*. We also examined the genetic interaction between *cdc-25.1* and *mir-35*. Because known loss-of-function alleles of *cdc-25.1* do not show any intestine defect [37, 38], and *cdc-*

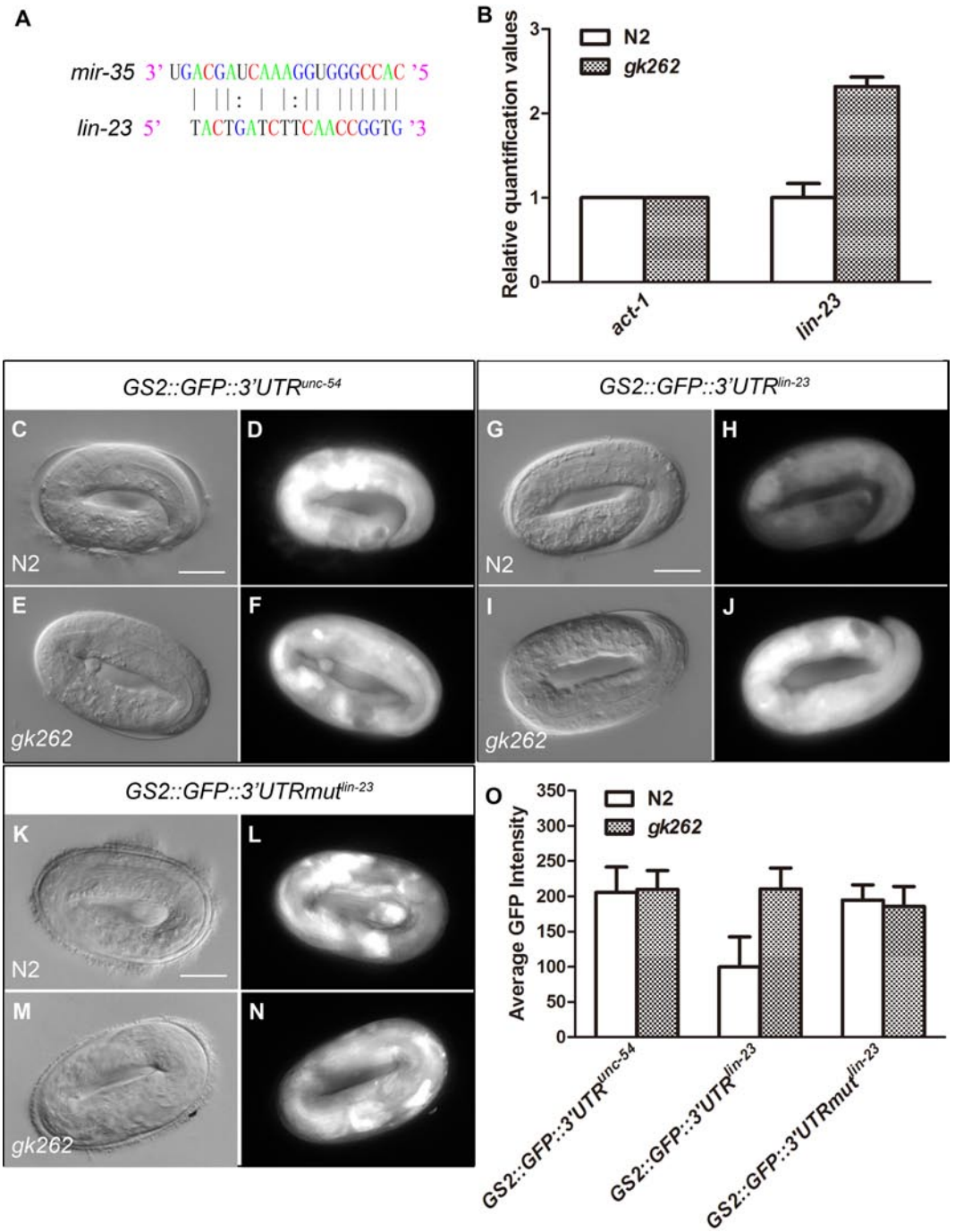
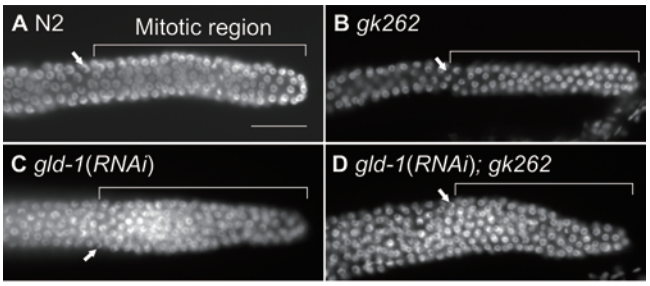
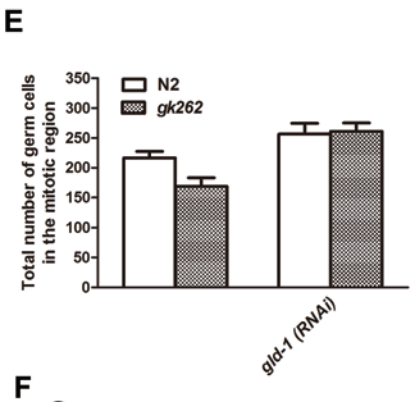


Figure 3 *lin-23* may be a direct target of *mir-35*. **(A)** *lin-23* is predicted as a target of *mir-35* by the bioinformatics program miRanda. Solid lines indicate perfect match, while dash lines indicate non-perfect match. **(B)** The mRNA level of *lin-23* is up regulated in the *gk262* at young adult stage revealed by qRT-PCR. **(C**-**N)** *in vivo* GPF reporter experiment shows that *lin-23* is a direct target of *mir-35*. **(C**-**F)** The 3′ UTR of *unc-54* has no binding site for *mir-35*, which is used as a control, no obvious difference of GFP level could be observed between wild type and *gk262*. The DIC **(C**, **E)** and the GFP **(D**, **F)** picture of the same embryo at 2-3 fold stage. **(G**-**J)** A much higher GFP level in *gk262* was observed compared to that of the wild type. The DIC **(C**, **E)** and the GFP **(D**, **F)** pictures are of the same embryos at 2-3 fold stage. **(K**-**N)** The *3'UTRmut*$^{lin-23}$ has a mutated binding site for *mir-35*. No obvious difference of GFP level could be observed between wild type and *gk262*. The DIC **(K**, **M)** and the GFP **(L**, **N)** pictures are of the same embryos at 2-3 fold stage. The scale bar represents 25 μm. **(O)** Quantification of average GFP intensity of the embryos harboring the *GS2::GFP::3'UTR*$^{unc-54}$, *GS2::GFP::3'UTR*$^{lin-23}$ and *GS2::GFP::3'UTRmut*$^{lin-23}$. Error bars represent SD.





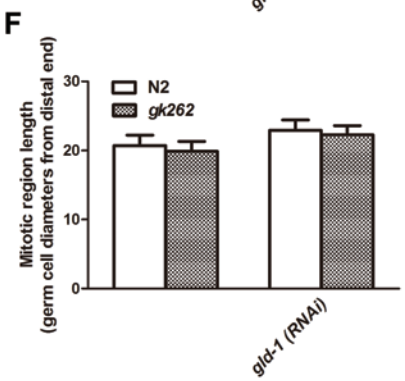


Figure 4 *mir-35* affects adult germ line mitosis. **(A-D)** DAPI staining of distal ends of gonads, dissected from adult hermaphrodites. **(B)** *gk262* mutant shows a thinner mitotic region than N2 **(A)**, but the lengths of both are nearly the same. In **(C)** *gld-1(RNAi)* and **(D)** *gld-1(RNAi)*; *gk262*, the mitotic regions overgrow, while the lengths of the mitotic regions are not changed. The arrows indicate the crest-like germ cells in the transition zones. The scale bar represents 50 μm. **(E)** Total number of germ cells in the mitotic region of N2 and gk262, and the progeny of animals performed *gld-1(RNAi)*. Error bars represent SD. **(F)** Mitotic region length (germ cell diameters from distal end) of N2 and *gk262*, and the progeny of animals performed *gld-1(RNAi)*. Error bars represent SD.

*25.1*RNAi-treated *gk262* and *lin-23(e1883)* lines are early embryonic lethal (data not shown), we took advantage of a dominant gain-of-function *cdc-25.1* mutant *rr31*, which induces hyperplasia in the intestine [39]. Double mutant *rr31*; *gk262* is early embryonic lethal at 25 °C. However, the *rr31*; *gk262* line could develop into fertile adulthood at 20 °C, while the hyperplasia in the intestine is suppressed (Tables 3 and 4), consistent with the fact that over-expression of Lin-23 reduced *cdc-25.1*(*rr31*)-dependent intestine hyperplasia [34].

The Rb/E2F pathway include *lin-35* Rb [40], *lin-36* [41], *dpl-1* (DP-Like) and *efl-1* (E2F-Like) [42]. *lin-35, lin-36* and *efl-1* negatively regulate S phase entry [43, 44], while *dpl-1* acts as both a positive and negative regulator [44, 45]. We found that *lin-35*(*n745*), *lin-36*(*n766*) and *efl-1*(*se1*) could fully suppress intestinal nuclei phenotype of *gk262*, at both 20 °C and 25 °C (Tables 1 and 3, $P > 0.05$, Student *t*-test), indicating that *mir-35* regulates G1/S transition in an Rb/E2F-dependent manner.

lin-9 Mip130/TWIT and *lin-15B* were also identified as negative G1 regulatory proteins [44, 46], however, *gk262; lin-9*(*n112*) and *gk262; lin-15B*(*n309*) were embryonic lethals. *lin-9*(*RNAi*)- and *lin-15B*(*RNAi*)-injected *gk262* showed limited restoration of intestine nuclei loss (Tables 3 and 4) at 25 °C, suggesting that *lin-9* and *lin-15B* may have no direct genetic interaction with *mir-35*. In summary, *mir-35* inhibits the retinoblastoma protein/E2F and SCF pathways in regulating G1/S transition.

mir-35 targets lin-23 to regulate G1/S transition

lin-23 is a potential target of *mir-35* (Figure 3A). Using qRT-PCR, we found that *lin-23* gene expression level was up-regulated in *gk262* at 25 °C (Figure 3B). To further examine whether *lin-23* is the target gene of *mir-35*, the 3′ UTR of *lin-23* and GS2 (Figure 1A) were used to yield the *GS2::GFP::3′UTR*$^{lin\text{-}23}$ reporter DNA construct. A much higher GFP level in *gk262*, compared to that of the wild type (Figure 3G-3J and 3O, $n > 50$, $P < 0.001$, Student *t*-test) was observed. In contrast, the *unc-54* 3′ UTR, which is lacking of *mir-35*-binding site, is not responsive to *mir-35* (Figure 3C-3F and 3O, $n > 50$). When the binding site of *mir-35* on *lin-23* 3′ UTR was mutated, we found no obvious difference of GFP level between wild type and *gk262* harboring the transgenic *GS2::GFP::3′UTRmut*$^{lin\text{-}23}$ (Figure 3K-3N and 3O, $n > 50$). Therefore, *lin-23* is most likely a direct target of *mir-35* in G1/S transition regulation.

mir-35 affects germ line proliferation

Because *gk262* has a smaller brood size compared to the wild type, we suspected that *mir-35* might play a role in germ line proliferation. Indeed, adult *gk262* mitotic region and transition zone were significantly smaller than those of wild type animals (Figure 4A and 4B), while the length of mitotic region remained normal (Figure 4F). There are less mitotic germ cells (about 22% less nuclei,

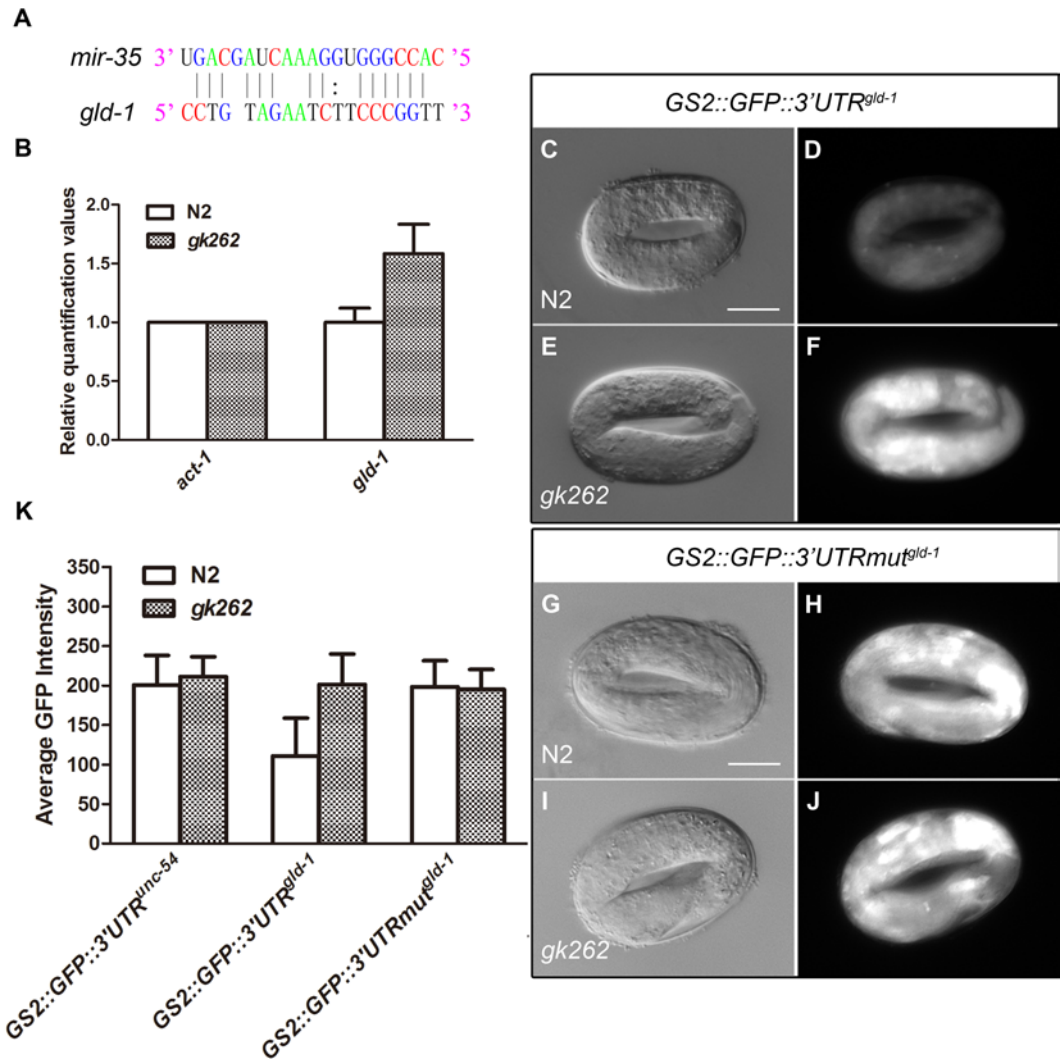


Figure 5 *mir-35* may target *gld-1*. **(A)** *gld-1* is predicted as a target of *mir-35* by the bioinformatics program miRanda. Solid lines indicate perfect match, while dash lines indicate non-perfect match. **(B)** The mRNA level of *gld-1* is up regulated in the *gk262* at young adult stage revealed by qRT-PCR. **(C-J)** *in vivo* GPF reporter experiment shows that *gld-1* is a direct target of *mir-35*. **(C-F)** A much higher GFP level in *gk262* was observed compared to that of the wild type. The DIC **(C**, **E)** and the GFP **(D**, **F)** pictures are of the same embryos at 2-3 fold stage. **(G-J)** The *3'UTRmut*$^{gld-1}$ has a mutated binding site for *mir-35*. No obvious difference of GFP level could be observed between wild type and *gk262*. The DIC **(G**, **I)** and the GFP **(H**, **J)** pictures are of the same embryos at 2-3 fold stage. The scale bar represents 25 μm. **(K)** Quantification of average GFP intensity of the embryos harboring the *GS2::GFP::3'UTR*$^{unc-54}$, *GS2::GFP::3'UTR*$^{gld-1}$ and *GS2::GFP::3'UTRmut*$^{gld-1}$. Error bars represent SD.

Figure 4F) in the entire mitotic region (Figure 4E).

It appeared that *mir-35* might restrict a gonad-specific factor(s) in the mitotic region at the end of the distal gonad arm to regulate a critical germ line proliferation signal. GLD-1 is such a candidate because it is expressed mainly outside of the tip portion of the mitotic region of gonad and inhibits germ line mitosis [22, 24]. Bioinformatics prediction also revealed that *gld-1* may be a direct target of *mir-35* (Figure 5A). *gld-1*(*RNAi*) led to a larger mitotic region and transition zone (Figure 4A and 4C). Compared to untreated wild type, there were about 19% more germ cells in the mitotic region of *gld-1*(*RNAi*) animals, while the length of mitotic region increased insignificantly (Figure 4E and 4F). Furthermore, *gld-1*(*RNAi*) suppressed *gk262*'s defects in gonad germ cell number and size of mitotic region (Figure 4B-4F). Noticeably, major changes of the mitotic region normally occurred outside the tip portion of the distal end of gonad arm in *gk262* and *gld-1*(*RNAi*) animals (Figure 4A-4D), indicating that *mir-35* and *gld-1* function mainly

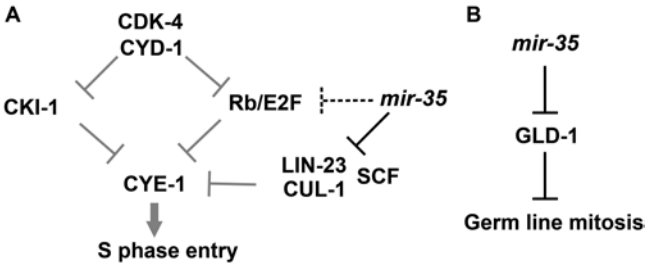


Figure 6 Hypothetical model of *mir-35* family function during cell cycle. **(A)** In the intestinal cells, the *mir-35* family suppresses Rb/E2F and SCF pathways to promote the entry of S-phase. *lin-23* may be the direct target of *mir-35* in the SCF pathway, while in the Rb/E2F pathway, the direct target is unknown. **(B)** In the distal gonad, the *mir-35* family targets *gld-1* to repress the germ line mitosis.

in germ line proliferation, rather than initial germ cell fate determination and division.

mir-35-41 cluster targets gld-1

To further test whether *gld-1* is a *mir-35* target gene, we performed quantitative PCR, and found a significantly higher level of *gld-1* in *gk262* (Figure 5B). We also conducted an *in vivo* GFP reporter experiment, using the transgenic cassette *GS2::GFP::3′UTR*$^{gld\text{-}1}$. We found that the transgene showed stronger GFP expression in *gk262* than in wide type (Figure 5C-5F and 5K, $n > 50$, $P < 0.001$, Student *t*-test). When we mutated the *mir-35*-binding site of *gld-1* 3′ UTR, we observed no obvious difference of GFP level between wild type and *gk262* harboring the transgenic *GS2::GFP::3′UTRmut*$^{gld\text{-}1}$ (Figure 5G-5K, $n > 50$). Taken together, *mir-35* targets *gld-1* in regulating germ cell proliferation.

Discussion

We have discovered that the *mir-35* family of *C. elegans* plays an important role in regulating G1/S transition of the cell cycle in intestine and cell proliferation in germ line. In intestinal cells, this regulation is mediated by inhibiting the ubiquitous Rb/E2F pathway and the SCF pathway, in which *lin-23* is a direct target of *mir-35*. In the distal mitotic arm of germ line of hermaphrodites, *mir-35* targets *gld-1* directly to ensure proper mitotic proliferation of germ cells (Figure 6). Thus, our study reveals how *mir-35* participates in the global tune-up of cell cycle/proliferation in the intestine and germ line of *C. elegans*, and indicates that distinct regulatory pathways of cell division/proliferation may be joined by connected miRNAs, in addition to components shared by these pathways.

Alvarez-Saavedra and Horvitz previously showed that without the *mir-35* family , most animals undergo developmental arrest at the early embryonic stage and a few show delayed development at normal temperature [14]. During the initial phase of the present study, we suspected that cell cycle defect might be one of the reasons that cause the developmental abnormality, and indeed, we found genetic interactions between *mir-35* and Rb/E2F-related genes. Furthermore, we found a few fertile *gk262; lin-36*(*n766*) worms at 25 °C. Additionally, in a pilot EMS-induced mutagenesis suppressor screen, three recovered lines which suppress the intestinal cell cycle defects barely survived at 25 °C (data not shown). Therefore, the fact that depletion of the *mir-35* family leads to cell cycle defect, may at least partially explain the cause(s) of early developmental abnormality of *gk262*.

In mammalian and cancer cells, several miRNAs have been reported to regulate the G1/S transition of the cell cycle. For instance, *miR-17* and *miR-20a* temper an E2F1-induced G1 checkpoint [47], and *miR-195* regulates G1/S transition [48]. Additionally, *miR-18* mediates the degradation of *βTrCP1* (mammalian *lin-23* homolog) mRNA, which encodes the main component of the SCF complex [49]. Together with our findings in *C. elegans* intestine, it appears that posttranscriptional regulation of G1/S transition by miRNAs is well conserved across species. However, other than *lin-23* of the SCF pathway, we did not find any target genes of *mir-35* in the Rb/E2F pathway, either by bioinformatics prediction or microarray analysis. There are two possible explanations. First, there are target genes of *mir-35* yet to be identified in the Rb/E2F pathway. Indeed, by bioinformatics data, over 300 candidate target genes of the *mir-35* family have not been studied, or even annotated. Among these genes, some encode proteins that share similar functional domain with that of EFL-1, such as F49E12.6, thus potentially are targets of *mir-35-41*. The second explanation is that Rb/E2F and SCF pathways might cross-talk so that Rb/E2F could bypass or partially substitute SCF function in the G1/S transition. This explanation is supported by the experiments showing a synthetic interaction between *lin-35* and *lin-23* in Rb-regulated cell proliferation [50]. Our experiments presented here indicate that *mir-35* participates in adjusting components of both Rb/E2F and SCF pathways, suggesting a cross-talk between the two pathways is necessary for proper cell proliferation. The reduction of proliferating germ cells in *gk262* initially suggested to us that both Rb/E2F and SCF pathways may be also negatively regulated by *mir-35*. However, Rb is known to be ubiquitously expressed, although the *lin-35* mutant does not show abnormal gonad development [51,

52]. Similarly, *lin-23* is expressed in the gonad, but loss of LIN-23 function does not lead to germ cell proliferation defect [36]. Thus, we currently could not exclude that a cross-talk of Rb/E2F and SCF also happens in the gonad.

Because major cell loss was observed in the mitotic region close to the transition zone in *gk262* (Figure 4A and 4B), it is highly possible that *mir-35* may inhibit a gonad-specific factor(s), which is mainly expressed in the mitotic region and restricts proliferation. It is known that GLP-1/Notch signaling is both necessary and sufficient for germline proliferation at the expense of differentiation, while Gld-1 inhibits germline mitosis and negative feedback regulates GLP-1/Notch signaling [53-55]. In the mitotic region, GLP-1 proteins are present close to where DTC is [56, 57], while GLD-1 is present close to the transition zone [24, 26], making *gld-1* a top candidate gene responsible for the loss of germ cells in *gk262*. Indeed, our experiments indicate that *mir-35* directly targets *gld-1* and a higher than normal level of GLD-1 inhibits germ cell mitosis (Figures 4 and 5). Knock down of GLD-1, in both N2 and *gk262*, leads to more germ line proliferation in the entire mitotic region, except in the most distal portion. This phenotype is consistent with another aspect of GLD-1 function, i.e., a lower than normal level of GLD-1 promotes germ cell proliferation [58]. The bipartite distribution of GLP-1 and GLD-1 in gonad may also explain why abnormal GLP-1 or GLD-1 level could lead to gonad tumor, a sign of out-of-control mitotic proliferation in hermaphrodites. Interestingly, the mammalian GLD-1 homolog, quaking RNA-binding protein I, is also involved in glioblastoma [59]. We suggest that through modifying GLD-1 expression level and subsequent changes of GLP-1/Notch signaling, *mir-35* regulates germline proliferation in the gonad, although the potential involvement of a cross-talk between Rb/E2F, SCF and GLP-1/Notch pathways is not excluded.

The expression of *mir-35-41* is ubiquitous and starts at the onset of gastrulation (our unpublished observation also supports this view). It is therefore not surprising that development of more than one organ is affected when the *mir-35-41* cluster is deleted. In addition to intestine and gonad defects, we did observe somatic sex transformation in *gk262* hermaphrodites, i.e., increased incidence of ectopic growth of the ray structure (2-fold increase vs wild type) and male-specific neurons (8-fold increase). The fact that the *mir-35* family has multiple functions strengthens a notion that miRNAs participate in a wide range of biological processes, likely due to their "one to more" working mode [5, 6, 60, 61]. How do *mir-35-41* achieve organ-specific function? Our experiments presented here show that *mir-35-41* target multiple components of cell proliferation control pathways, to ensure correct cell numbers in the intestine and gonad. By qRT-PCR, we also found that *mir-35-41* directly or indirectly regulate genes such as *fem-2*, *fem-3* and *xol-1*, which all encode proteins required in the somatic sex determination pathway [62-65] (our unpublished data). It remains to be seen elusive if such a regulatory scheme of a miRNA family applies to all multi-cell organisms. At least in *D. melanogaster*, a miRNA cluster is found to impair hedgehog signaling [66].

Precise cell cycle control is essential for genetic stability and accurate cell division of any multi-cell species, employing many if not all conserved regulatory pathways during animal development and differentiation. Although the *mir-35-41* family is unique to *C. elegans*, perhaps representing a newly evolved miRNA species in *Caenorhabditis* lineage, the target genes, i.e., *lin-23* and *gld-1*, identified in the present study are highly conserved across species. The fact that mammalian cell cycle is regulated by a variety of miRNA species and our present study suggest that targets of miRNAs in cell cycle and proliferation pathways are well conserved in evolution, yet many factors required for the fine regulations remain to be identified. Thus, a systematic search for and research on novel targets of the *mir-35* family in *C. elegans*, as exemplified by our study, will further our understanding of cell cycle and proliferation regulation.

Materials and Methods

Culture conditions and strains

C. elegans strains were maintained at 20 °C as described by Brenner [67]. The temperature-sensitive strains were maintained at 20 °C and in temperature shift assay, synchronized young adults were allowed to lay eggs for 1 h at 20 °C, and the eggs were shifted to and maintained at 25 °C. For RNAi experiments in the temperature-sensitive strains, we placed dsRNAs injected worms at 20 °C for 8 h for RNAi to take effect, shifted injected worms to 25 °C and obtained 3-fold embryos (RNAi) developed from laid eggs of RNAi animals. Mutations used in this study were as follows: *cdc-25.1*(*rr31*)*I; rrIs1*, *lin-35*(*n745*)*I*, *mir-35*(*gk262*)*II*, *lin-36*(*n766*)*III*, *lin-9*(*n112*)*III*, *efl-1*(*se1*)*V*, *lin-15AB*(*n309*)*X.* Transgenic markers: *rrIs1*[*elt-2::GFP*] [39], *maIs103*[*rnr::GFP*] [29], *gvEx32*[*cye-1::GFP; rol-6D*] were gifts from M Krause.

Nomarski fluorescent microscope examination

Live animals were mounted to M9 solution in 3% agar pads, viewed under Zeiss microscopes and imaged by a Zeiss AxioCam.

Rescue gk262 phenotype by microinjection

To rescue *mir-35-41*(*gk262*), a region (GS1) from 2.16-kb upstream of *mir-35* to 1.09-kb downstream of the *mir-41* locus, was amplified from genomic DNA with PCR primers (5′ ATTCCTCAACCATCGCCT 3′ and 5′ ATTATCGACGGAGTGCTCG 3′). A region (GS2) was amplified with PCR primers (5′ AATAAGCT-

TATTCCTCAACCATCGCCT 3′ and 5′ CGGGATCCTAGTTCTAATGGAAAGTACCA 3′) and cloned into the plasmid pPD49.78 to replace the *hsp16.2* promoter. Individual members of the *mir-35-41* cluster were cloned into the modified pPD49.78 with primers listed below:

pre-mir-35 5′ CGGGATCCCCCAACTATTATTCTCGGAT 3′; and 5′ CGGGTACCTGGAGCAAGTGGAAAAGAT 3′,

pre-mir-36 5′ ACTGGATCCTCTTTTCCACTTGCTCCAC 3′ and 5′ TATGGTACCATTTTAAGATATTTTATCTTTCCG 3′,

pre-mir-37 5′ ACTGGATCCCCCCGACGCGGAAAGATAA 3′ and 5′ TATGGTACCCTCACAGAGAAACCACGAGGACC 3′,

pre-mir-38 5′ ACTGGATCCTCGTGGTTTCTCTGTGAGCC 3′ and 5′ TATGGTACCGCTGAATGGTAGAATGTAGGGG 3′,

pre-mir-39 5′ ACTGGATCCTTCTGGAGTTTTCCCCTACATTC 3′ and 5′ TATGGTACCTGAGGTGCGGACAGGAGG 3′,

pre-mir-40 5′ ACTGGATCCGTGTCTCCGAACCTCCTGTCC 3′ and 5′ CATGGTACCCCCACTTAAAAATAACTCACCTTGA 3′,

pre-mir-41 5′ ACTGGATCCCAGCTAAGGTGCGGGTACA 3′ and 5′ TATGGTACCAGTGCAACGAGTTTTGGACA 3′.

The promoter of *ges-1* was amplified with the primers 5′ GCGCATGCTCACCAATACCTTTAGTGAC 3′ and 5′ GGGGATCCCTGAATTCAAAGATAAGATATGT 3′.

All constructs were injected (co-injected with pRF-4[*rol-6*(*su1006*)] at 80 ng/μl) to the *gk262* at 20 ng/μl.

Over-expression of mir-35 and mir-35-41

To over express *mir-35* and *mir-35-41*, we amplified the *pre-mir-35* (primer information is identical to last section) and *pre-mir-35-41* (5′ CGGGATCCCCCAACTATTATTCTCGGAT 3′ and 5′ CGGGTACCAACAGCAAAAAAAGAGCAGG 3′). The DNA fragments of *pre-mir-35* and *pre-mir-35-41* were cloned into the plasmid pPD49.78 containing *hsp16.2* promoter. Plasmids were injected (co-injected with pRF-4[*rol-6*(*su1006*)] at 80 ng/μl) to the N2 at 20ng/μl. Transgenic embryos between 0 and 3 h post egg lying, and transgenic animals at arrest L1 and adult stages were heat shocked for 1 h at 33 °C to induce over-expression of *mir-35* or *mir-35-41*.

RNAi by microinjection

Single-strand RNA was transcribed using Promega *in vitro* transcription kit. The PCR primers are listed below and were amplified from wild-type N2 cDNA:

cki-1 5′ GTCGTTGCCTTTTCGGTCGT 3′ and 5′ CGAGTTCTGATCGTTGGACG 3′,

lin-23 5′ GTCGTTGCCTTTTCGGTCGT 3′ and 5′ CGAGTTCTGATCGTTGGACG 3′,

dpl-1 5′ AGTTGGAGGATCTAGTGGAG 3′ and 5′ GACATAGTGCTGGTGTAAGG 3′,

cdc-25.1 5′ ATCTGGTCGTGTAGCCCTCA 3′ and 5′ ATGCTGCTTGTCTTGCTTCC 3′,

lin-9 5′ TCTTGCTCTGTCTCCGCCT 3′ and 5′ CTTTCGAGCATCTACGTGGC 3′,

lin-15B 5′ CTTGGCGGTGATTGCTCG 3′ and 5′ GCACATTGGCTGGTCTCG 3′,

gld-1 5′CTCTGAATCGCCATCTCGTT 3′ and 5′ TGTTGTTGACTGAAGAAGCC 3′.

The single-stranded RNAs of each gene were annealed to form dsRNA for injection. Embryos from injected animals (24 and 48 h post microinjection) were collected for further analysis.

DAPI staining

Approximately 50 mutant animals were sampled on a microscope slide, covered and quickly frozen in liquid nitrogen. After removing cover slip, each slide was placed in methanol, then acetone for 10 minutes each at –20 °C. After air drying, specimens were treated with DAPI [68] and were ready to be observed.

Gonad dissection

We performed gonadal dissection essentially as Francis *et al*. described [53]. Briefly, adult worms were picked onto an unseeded NGM plate, immersed in 2 ml M9 buffer containing 0.2 mM of levamisole, suspended and transferred to a clean dish. Paralyzed worm was be-headed at level of pharynx with miniature surgical blades, let out the entire gonad arms and transferred with exposed gonads into a 0.5 ml Eppendorf tube for later DAPI staining.

DNA quantification

To quantify DNA content, nuclei images of DAPI-stained animals were taken with a Zeiss AxioCam and images were analyzed with NIH ImageJ 1.40 g software. Using ventral nerve cord nuclei as a 2n DNA standard, C values of intestinal nuclei were estimated by their DAPI-based densitometric quantifications [69, 70].

RNA isolation and quantitative real time PCR

Synchronized wild-type and mutant animals were kept at 20 °C until the worms reached L4 larval stage and the synchronized L4 animals were shifted to 25 °C for 24 h. Total RNA of young adults was extracted using Trizol reagent (Invitrogen). Residual genomic DNA was removed by RNase-free DNase (QIAEN). cDNA was synthesized using random hexamers and SuperScriptII Reverse Transcriptase (Invitrogen). Real-time PCR was performed using SyBR Green PCR Master Mix (Applied Biosystems) on a 7500 Real Time PCR system (Applied Biosystems). Using *act-1* as an internal control, relative fold change for transcripts was calculated using comparative C_T ($2^{-\Delta\Delta CT}$) method. Initial data analysis was carried out using the Applied Biosystems real-time PCR software. Three independent samples were prepared and run in triplicate. Three pairs of primers are listed below:

act-1 5′ CCGTGAAAAGATGACCCAAATC 3′ and 5′ GGTACGTCCGGAAGCGTAGA 3′,

lin-23 5′ TGTTCCGCCTGCAGTTTGA 3′ and 5′ GTCCAAAAAGTCCCAAATGAGAA 3′,

gld-1 5′ GGAAAGTGCTCACCGTGGAA 3′ and 5′ TGTTCGAGTGCTGCTTGCA 3′

GFP reporter assay

GFP reporters *GS2*::*GFP*::*3′UTR*$^{unc-54}$, *GS2*::*GFP*::*3′UTR*$^{lin-23}$, *GS2*::*GFP*::*3′UTRmut*$^{lin-23}$ *GS2*::*GFP*::*3′UTR*$^{gld-1}$ and *GS2*::*GFP*::*3′UTRmut*$^{gld-1}$ were based on plasmid pPD95.75. GS2 fragment was amplified and cloned into the plasmid pPD95.75, whose original *3′UTR*$^{unc-54}$ was replaced with *3′UTR*$^{lin-23}$ (5′ CGCGAATTCATGGCAGTACATTTGATAGT 3′ and 5′ ATTGGGCCCATGGCAGATTGTGGAGTA 3′), or *3′ UTR*$^{gld-1}$ (5′ GGAGAATTCAAAGTGCTCACCGTGGAA 3′ and 5′ ATAGGGCCCATGGACAGATTGGGAAGG 3′). Using overlap PCR, the mutated 3′ UTR of *lin-23* was amplified with two pairs of primers: 5′ CCAGAATTCTAGTCAGTTTCTTTTCCG 3′ and 5′ GGTTTGGTTGATTTCTCGTGGGGTTGAAGATCAGTAAA 3′, 5′ CACGAGAAATCAACCAAACCCCTTCCCAACTTTTC-

CTCT 3′ and 5′ TAAGGGCCCATGGCAGATTGTGGAGTAGC 3′. The mutated 3′ UTR of *gld-1* was amplified with two pairs of primers: 5′ GCGGAATTCTCAGGTCCAGTTTTGATGTC 3′ and 5′ TTTTTGATGACTACATCTTTGGCGGAAGATTCTACAGG G 3′, 5′ CAAAGATGTAGTCATCAAAAACCCGCTAGTTAGAT ATGTTC 3′ and 5′ TTAGGGCCCACAGATTGGGAAGGGG 3′. Reporters were injected (co-injected with pRF-4[*rol-6*(*su1006*)] at 80 ng/μl) to the N2 at 20 ng/μl.

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