microRNAs play critical roles in the survival and recovery of *Caenorhabditis elegans* from starvation-induced L1 diapause

Xiaochang Zhang, Rebecca Zabinsky, Yudong Teng, Mingxue Cui, and Min Han[1]

Howard Hughes Medical Institute and Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309

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Environmental stresses and nutrition availability critically affect animal development. Numerous animal species across multiple phyla enter developmental arrest for long-term survival in unfavorable environments and resume development upon stress removal. Here we show that compromising overall microRNA (miRNA) functions or mutating certain individual miRNAs impairs the long-term survival of nematodes during starvation-induced L1 diapause. We provide evidence that miRNA miR-71 is not required for the animals' entry into L1 diapause, but plays a critical role in long-term survival by repressing the expression of insulin receptor/PI3K pathway genes and genes acting downstream or in parallel to the pathway. Furthermore, miR-71 plays a prominent role in developmental recovery from L1 diapause partly through repressing the expression of certain heterochronic genes. The presented results indicate that interactions between multiple miRNAs and likely a large number of their mRNA targets in multiple pathways regulate the response to starvation-induced L1 diapause.

age-1 | developmental timing | GW182 | unc-31 | ain-1

Food deprivation is a life-threatening challenge that animals frequently face as individuals and as species. Different organisms have developed versatile growth arrest strategies to overcome starvation-induced metabolic and developmental problems. The coordinated entrance into developmental arrest, long-term survival, and the reinitiation of development upon food availability are important biological processes to investigate.

The nematode *Caenorhabditis elegans* responds to starvation by entering developmental arrest at multiple stages of its life cycle (1). When late, first larval stage (L1) worms sense unfavorable conditions, they enter an alternative and long-lived larval stage called dauer larvae (or dauer diapause). However, when newly hatched L1 worms encounter an environment with no food, developmental programs arrest and the worm enters L1 diapause. Unlike dauer diapause, L1 diapause is not accompanied by life cycle changes and has not been shown to require certain signaling pathways that control the formation of dauer diapause [such as TGF-β signaling (*daf-1*, *daf-7*) and nuclear hormone receptor (*daf-12*)] (2, 3). Furthermore, worms that are long-lived due to dietary restriction or decreased mitochondrial respiratory rates are short-lived during L1 diapause, suggesting that the mechanisms controlling L1 starvation survival are different at least in some aspects from those controlling aging (3).

Previous studies showed that the release of postdocking calcium-regulated dense-core vesicles, the insulin receptor (InsR) pathway, the AMPK pathway, and protein chaperones are required for the long-term survival of starved L1 worms (2–4). The roles of InsRs have also been implicated in arresting the cell cycle in germ cells and a portion of somatic cells during L1 diapause (2, 4). Upon entering L1 diapause, RNA polymerase II quickly accumulates and pauses at promoter regions, and this accumulation was speculated to stop transcription and facilitate the immediate reinitiation of gene expression when food becomes available (2). However, the mechanisms that coordinate the long-term survival, overall developmental arrest, and reinitiation remain to be investigated.

microRNAs (miRNAs) are well known for their functions in controlling developmental timing in the nematode (5, 6). Surprisingly, the majority of the *C. elegans* miRNAs and miRNA families are not essential for development or viability under normal culture conditions (7, 8). The key properties of miRNA-mediated gene silencing, such as one miRNA targeting multiple mRNAs and one target gene being regulated by multiple miRNAs, suggest that (*i*) miRNAs are excellent candidate regulators for coordinating multiple aspects of molecular and cellular activities for specific physiological functions and (*ii*) miRNAs likely function in a synergistic or additive manner to regulate sets of genes under specific physiological conditions that are not limited to those related to development (9). Consistent with these ideas, several recent lines of evidence suggest that miRNA *let-7* and the heterochronic genes *lin-42* and *hbl-1* are required to regulate the starvation-induced dauer diapause (10–12) and that a number of miRNAs including *lin-4* and *mir-71* are involved in regulating life span (13, 14). Furthermore, a recent study suggests that the expression of certain miRNAs is differentially regulated by starvation-induced dauer diapause (15). However, it remains unclear how, and to what extent, miRNAs coordinate animal survival and development in response to stresses. In this study, we addressed the questions of whether and how miRNAs impact developmental arrest and the long-term survival of early L1 stage worms in response to food starvation.

Results

Intestinal miRNAs Play Critical Roles in L1 Starvation Survival. AIN-1 and AIN-2 are GW182 family proteins that are essential and partially redundant components of miRNA-induced silencing complexes (miRISCs) in *C. elegans* (16, 17). To investigate the roles of miRNAs in animal survival during starvation-induced L1 diapause, we impaired the overall miRISC function with loss-of-function (*lf*) mutants of *ain-1* (*ku322, ku425*, *and tm3681*) and *ain-2(tm2432)* and examined their L1 starvation survival rate (*Materials and Methods*). We found that *ain-1* but not *ain-2* mutants displayed a significant reduction in L1 starvation survival rate compared with that of wild type (Fig. 1 *A* and *D*). We further found that this survival rate reduction of *ain-1* mutants was overcome by ectopic expression of the AIN-2 protein in the intestine but not in the muscle (Fig. 1*A* and Fig. S1*A*). This is consistent with the previous reports that AIN-1 and AIN-2 are functional homologs with overlapping biochemical roles (16, 17). These results suggest that miRNAs act in the intestine, and possibly in other tissues, to promote L1 starvation survival. The overall effect of miRNAs on L1 starvation survival is expected to

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[1]To whom correspondence should be addressed. E-mail: mhan@colorado.edu.

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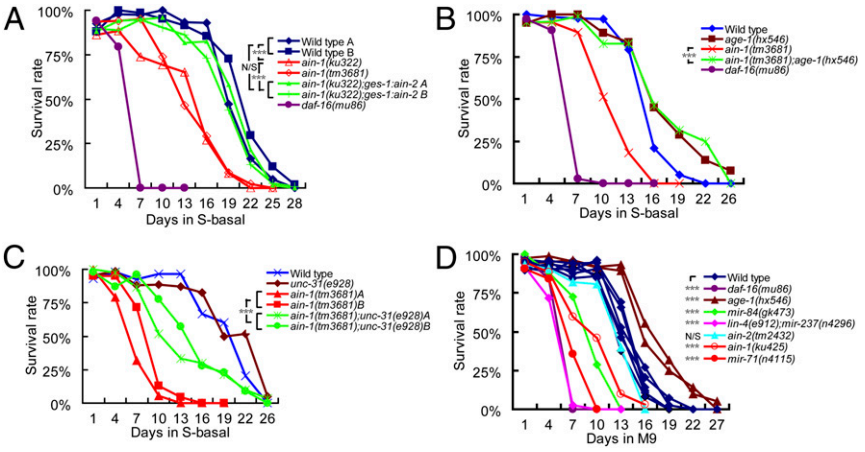

Fig. 1. Compromising overall miRNA function dramatically reduces the survival rate of L1 worms in starvation-induced diapause, and the effect can be significantly suppressed by an *age-1*/PI3K mutation. (*A*) Survival rate curves of wild-type and mutant strains, as indicated. The two *ain-1* loss-of-function alleles displayed significant reductions in L1 starvation survival rate. The reduced survival of *ain-1 (ku322, lf)* worms was suppressed by an intestine-expressed *ges-1promoter:ain-2::GFP* transgene (A and B represent two replicates). The result of a negative control using a *myo-3promoter:ain-2::GFP* transgene is shown in Fig. S1. Wild-type strains A and B are an N2 strain recently obtained from the *C. elegans* Genetic Center (reference 257) and an N2 strain from the laboratory stock, respectively. (*B*) Survival rate of single and double mutants to indicate the functional relationship between *ain-1* and *age-1*. The low survival rate associated with *ain-1 (tm3681, lf)* was largely overcome by the addition of the *age-1(rf)* allele. (*C*) The reduced L1 starvation survival rate of *ain-1(lf)* mutants was significantly suppressed by a null allele of *unc-31*. *A* and *B* represent replicated experiments of the indicated genotypes. (*D*) A representative chart of the L1 starvation survival rates of different miRNA mutants.

be significantly stronger than that reflected by the data in Fig. 1*A* because the *ain-1* mutations reduce, but do not eliminate, miRISC functions.

Previous studies indicate that the InsR pathway plays a dominant role in regulating L1 starvation survival and that reducing the activity of the insulin receptor *daf-2*, the PI3Kinase *age-1*, or the upstream regulator *unc-31* results in increased L1 starvation survival rate (2, 3). We found that the reduced survival rate of *ain-1* was suppressed by either reduction of *age-1* function or loss of *unc-31* function (Fig. 1 *B* and *C*), suggesting that a significant portion of the overall miRNA functions in L1 diapause is upstream of, or in parallel to, the InsR pathway.

To identify individual miRNAs that play prominent roles in L1 diapause, we screened 72 available mutant strains of individual miRNAs and miRNA families (87 miRNAs in total) using the L1 starvation assay. We identified 10 miRNA mutants that showed reduced survival rates with a stringent standard, as well as a few miRNA mutants with slightly increased survival rates (Table S1, Fig. 1*D*, and Fig. S1*B*). The effect observed in *ain-1(lf)* mutants is likely the consequence of the combined effects of attenuating functions of these individual miRNAs.

***mir-71* Deletion Drastically Reduces L1 Starvation Survival and the Defect Is Partially Suppressed by Mutations in *age-1*/PI3K and *unc-31*.** Among short-lived miRNA mutants, a *mir-71* deletion mutant, *mir-71(n4115)* (referred to as *mir-71(lf)* hereafter), displayed a severe reduction in L1 starvation survival rate (Table S1 and Fig. 2*A*). Because the InsR pathway was previously shown to play a prominent role in L1 diapause (2, 3), we examined genetic interactions between miR-71 and different components of the InsR pathway. Reduction-of-function mutation (*rf*) in the *age-1*/PI3 kinase gene, *age-1(hx546)*, made worms long-lived in the L1 starvation assay and was able to suppress the reduced L1 survival rate of *mir-71(lf)*; the rate of the double mutants was comparable to that of wild type (Fig. 2*A*). We next examined the relationship between miR-71 and UNC-31, which functions upstream of AGE-1 during L1 diapause by regulating calcium-regulated dense-core vesicle fusion and the release of an insulin-like ligand (3). We found that the *unc-31(e928 null)* mutation was also able to partially, but significantly, suppress the reduced survival rate associated with *mir-71(lf)* (Fig. 2*B*). These results suggest that a significant portion of the miR-71 activities in L1 diapause survival may be devoted to regulating the activities of UNC-31–mediated InsR/PI3K signaling and that the rest of miR-71 activity may regulate UNC-31–independent pathways.

We further examined the functional relationship between miR-71 and DAF-16, a FOXO transcription factor acting critically and negatively downstream of AGE-1/PI3K in the InsR pathway. We found that the poor survival rate of *daf-16(mu86) (lf)* was further decreased by *mir-71(lf)* (Fig. 2*C*), consistent with the notion that a portion of miR-71 activities regulate genes that act in parallel to UNC-31–mediated InsR/PI3K signaling for long-term survival during L1 diapause.

miR-71 Likely Directly Represses the Expression of *age-1* and *unc-31* by Acting on Their 3′ Untranslated Regions. To test whether the activity of the InsR pathway was down-regulated by miR-71, we

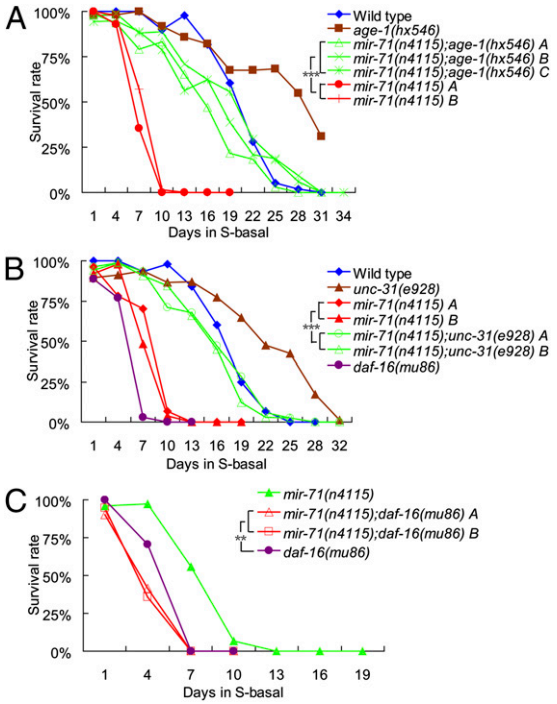

Fig. 2. Mutating miR-71 drastically reduces the survival rate of animals in L1 diapause, and the effect can be suppressed by mutations of insulin receptor pathway genes *age-1* and *unc-31*. (*A*) The *mir-71(n4115, lf)* mutant displayed severe reduction in L1 starvation survival rate, and the reduced survival rate of *mir-71 (lf)* was suppressed by a reduction-of-function allele of *age-1(hx546)*. (*B*) The severely reduced survival rate of the *mir-71(lf)* mutant was suppressed by a null allele of *unc-31(e928)*. (C) The poor survival rate of *daf-16(mu86, null)* was enhanced by *mir-71(lf)*. A, B, and C represent replicated experiments of the indicated genotypes.

first examined the endogenous expression of components of the InsR pathway in *mir-71(lf)*. We found that the mRNA level of UNC-31 was up-regulated by about 20% in *mir-71(lf)* (Fig. 3*A*). Because miRNA-mediated gene silencing may cause translational inhibition or mRNA degradation or both (19), the relatively small increase of UNC-31 in *mir-71(lf)* animals was still consistent with *unc-31* being a target of miR-71.

We found that the 3′UTRs of several genes of the InsR pathway, including *unc-31*, *age-1*, *pdk-1*, *akt-2*, and *sgk-1*, contain predicted miR-71 targeting sites (as predicted by TargetScan and mirWIP). The computation-based prediction that *age-1* and *pdk-1* are potential targets of miR-71 was also reported in a recent study focusing on miRNA functions in aging where the mRNA level of *pdk-1* was shown to be up-regulated in *mir-71* worms (14). Among these potential miRNA targets, the predicted miR-71–targeting sites in the 3′UTRs of *age-1* and *unc-31* are conserved between *C. elegans* and *Caenorhabditis briggsae*, leading us to focus further analyses on these two genes.

We used a dual-color 3′UTR reporter system (18) to test the computational, prediction-based hypothesis that the 3′UTRs of *age-1* and *unc-31* are directly regulated by miR-71 (Fig. 3*B* and *Materials and Methods*). Specifically, an *rpl-28:histone-24::mCherry:let-858 3′UTR* construct that drives constitutive and ubiquitous mCherry expression was used as an internal control, and a *4×NLS::GFP* construct driven by the same *rpl-28* promoter and containing the 3′UTR of *age-1* or *unc-31* was used as the reporter (Fig. 3*B*). The reporter construct, the control plasmid, and a transformation marker plasmid were coinjected into worms to generate the extrachromosomal arrays for analysis. If the 3′UTR of *age-1* or *unc-31* is repressed by miR-71, the GFP expression will be repressed in tissues where miR-71 is expressed in wild-type worms, but derepressed in the same tissues of *mir-71 (lf)* worms. We focused our analyses on intestine cells because (*i*) *mir-71promoter:GFP* was ubiquitously expressed in the pharynx, neurons, intestine cells, and other tissues throughout development (20) (Fig. S2*A*); (*ii*) we found strong expression of *rpl-28* promoter-driven reporters in the intestine nuclei; and (*iii*) more importantly, our result presented in Fig. 1*A* and Fig. S1*A* indicated a dominant role of intestinal miRNAs in regulating L1 starvation survival. We observed constitutive expression of *histone-24::mCherry* in both the control and *mir-71(lf)* worms (Fig. 3 *C* and *E*). In contrast, the nuclear-localized GFP expression under the control of the 3′UTR of *age-1*(Fig. 3 *C* and *D*) or *unc-31* (Fig. 3 *E* and *F*) was strongly repressed in the control worms, but prominently derepressed in *mir-71(lf)* mutant worms. These results suggest that miR-71 regulates the expression of *unc-31* and *age-1* through their 3′UTRs.

miR-71 Is Not Required for Arresting Seam Cell or M-Cell Divisions During L1 Diapause. DAF-16 (the FOXO homolog in *C. elegans*) has been shown to play an important role in cell cycle arrest and developmental progression partly by promoting *cki-1* expression in some somatic cells during L1 arrest (2). To determine whether miR-71 also plays a prominent developmental role in L1 diapause, we first examined the expression of a *cki-1promoter:GFP* (21) in V cells (epithelial seam cells dividing in early and mid-L1 stages). Interestingly, we found that the *cki-1promoter:GFP* was highly expressed in *mir-71(lf)* worms, and there were even additional cells that expressed the GFP in 7-d–starved *mir-71(lf)* L1 worms (Fig. 3*G* and Fig. S3*A*). Furthermore, the divisions of M cells and seam cells, as well as the expression of *lin-4promoter: YFP*, were suppressed in starved *mir-71(lf)* L1 worms similar to starved wild-type L1 worms (Fig. 3 *H* and *I* and Fig. S3 *B* and *C*). These results indicate that miR-71 is not essential for arresting seam cell or M-cell divisions during L1 diapause, suggesting that miR-71 function is distinct from DAF-16 function.

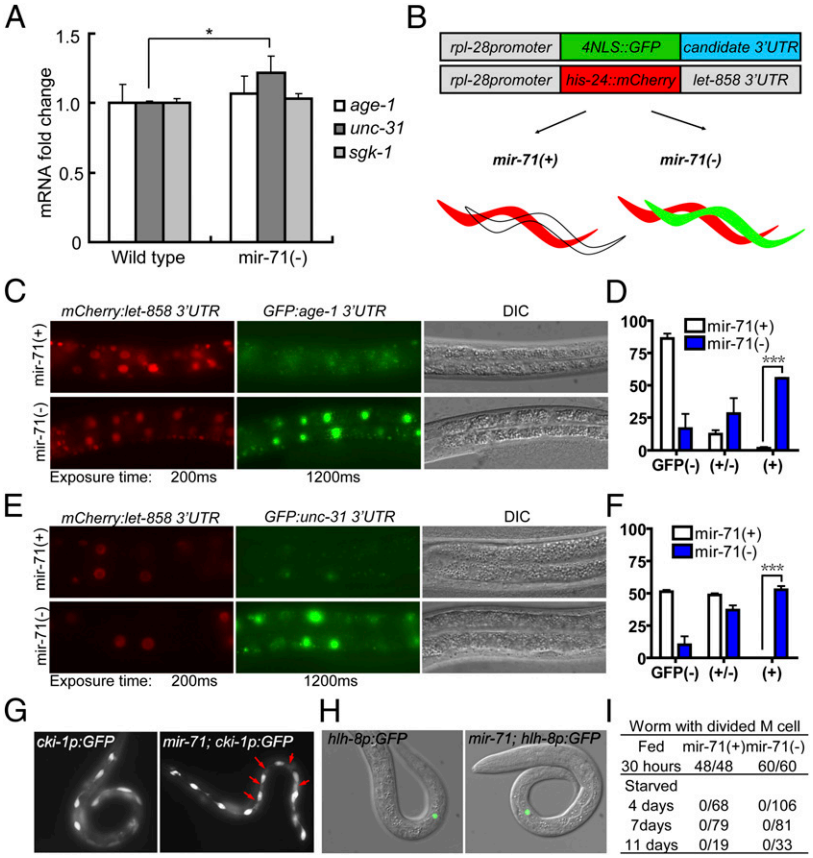

Fig. 3. miR-71 represses the expression of *age-1* and *unc-31* through the actions on their 3′UTR, but miR-71 is not required for arresting M cell division during L1 diapause. (*A*) Results of quantitative RT-PCR analysis of mRNA levels of indicated genes in 1-d–starved wild type and *mir-71(lf)* [labeled in Figs. 3 and 4 as *mir-71(–)*] L1 worms. The transcript level of *unc-31* was increased in *mir-71(lf)* worms, compared with that of wild-type controls that were normalized to the value of 1. **P* < 0.05. (*B*) A cartoon drawing illustrating the design of the dual-color 3′UTR reporter system (details in *Materials and Methods*) (18). (*C*) Fluorescence and differential interference contrast (DIC) images showing that the *age-1* 3′UTR reporter was repressed in *mir-71(+)* worms (3/4 transgenic lines) but not in *mir-71 (lf)* worms (4/4 transgenic lines). (*D*) Fractions of worms that carry 3′UTR reporter transgene and show no GFP expression [GFP(–)], weak GFP expression [GFP(+/–)], and comparable GFP expression to mCherry [GFP(+)]. The proportions of GFP(+) worms were compared between *mir-71(+)* and *mir-71(–)* mutants; *n* = 128 and 71, respectively. (*E*) Fluorescence and DIC images showing that the *unc-31* 3′UTR reporter was repressed in *mir-71(+)*worms (2/2 transgenic lines) but not in *mir-71(lf)* worms (4/4 transgenic lines). (*F*) Two transgenic lines from each genotype in *E* were analyzed in the same ways as those in *D*. *n* = 26 for *mir-71(+)* and *n* = 33 for *mir-71(–)*. (*G*) Fluorescence images showing the lateral views of 7-d–starved L1 worms expressing a *cki-1promoter:GFP(cki-1p:GFP)*. The reporter is strongly expressed in H and V cells in both wild-type and *mir-71(lf)* worms. Note that there are extra GFP-positive cells (red arrows) in *mir-71(lf)* mutants. See Fig. S3 for the top view images. (*H* and *I*) Fluorescence images (*H*) and statistical data (*I*) showing that the M cell diveded in fed animals but remained undivided in 4-, 7-, or 11-d–starved L1 wild-type and *mir-71(lf)* worms.

miR-71 Regulates the Timing of Vulval Cell Division in Animals Recovering from L1 Diapause. Consistent with reported analyses (7, 14), we did not observe any obvious developmental defects associated with well-fed *mir-71(lf)* mutant animals. We thus asked whether miR-71 was required for the reinitiation of developmental programs during the recovery phase after L1 starvation. We noted that even though >90% of the *mir-71(lf)* worms were able to recover from 4 d of L1 starvation, most of them displayed defects in vulval development (protruding vulva or vulvaless) and a severe reduction in brood size (Fig. S4*A*).

We further examined worms recovering from 4 d of L1 starvation and found that around 90% of the *mir-71(lf)* mutants displayed retarded vulval precursor cell (VPC) division, compared with less than 5% in wild type (Fig. 4*A*). Furthermore, *mir-71promoter:GFP* is highly expressed in the VPCs at L3 stage (Fig. S2*B*), and the severity of the VPC timing defect of the *mir-71(lf)* mutants depended on the length of time that *mir-71(lf)* L1 mutants were starved (Fig. 4*B*). In worms that recovered from 4 d of L1 starvation, we also found that a significant portion of the *mir-71(lf)* mutants displayed egg-laying defects and overproliferating or precociously reflexed gonads.

miR-71 May Regulate Developmental Timing During Recovery from L1 Diapause by Repressing the Expression of Genes in both UNC-31/AGE-1/DAF-16–Dependent and –Independent Pathways. We found that the vulval defects of recovering *mir-71(lf)* worms were strongly suppressed by an *unc-31(null)* mutation and partially suppressed by the *age-1(rf)* mutation (Fig. 4*C* and Fig. S4*B*), indicating that UNC-31 and AGE-1 have negative effects on proper vulval development during L1 diapause recovery and that miR-71 may antagonize such effects by repressing their expression.

Because *daf-16(mu86 lf)* worms displayed slow growth after 3 d of L1 starvation (delayed on average ~12 h, n = 23), and because the insulin receptor pathway negatively regulates the activity of DAF-16, we speculated that the VPC timing defect of starved *mir-71(lf)* worms may be due mainly to the reduced activity of *daf-16*. If this were true, the starved *mir-71(lf); daf-16(lf)* double-mutant worms should show a slow growth phenotype similar to that of *daf-16(lf)* worms, but no specific VPC timing defect. However, we found that the VPC timing defect of *mir-71 (lf)* was dramatically enhanced in *mir-71(lf); daf-16(lf)* double mutants recovered from either 1 or 3 d of L1 starvation and that the *mir-71(lf); daf-16(lf)* double mutants displayed an even slower rate (~24 h delay) of overall growth than that of *daf-16(lf)* worms (~12 h delay) when recovered from 3 d of L1 starvation (Fig. 4*D*). These results indicate that miR-71 plays a significant role in larval development of animals recovering from L1 diapause and likely does so by regulating the expression of components of the insulin receptor/DAF-16 pathway, as well as factors acting downstream, or in parallel to, DAF-16.

***hbl-1* and *lin-42* Are Likely Two Additional miR-71 Targets with Roles in Timing Regulation of VPC Division in Animals Recovering from L1 Diapause.** To understand how miR-71 affects VPC division, we searched its predicted targets for potential genes involved in regulating developmental timing. We found that the known developmental timing genes, *hbl-1*, *lin-42*, and *lit-1*, were at the top of the list (TargetScan). All three genes play roles in specifying the second larval stage (L2) developmental programs; loss-of-

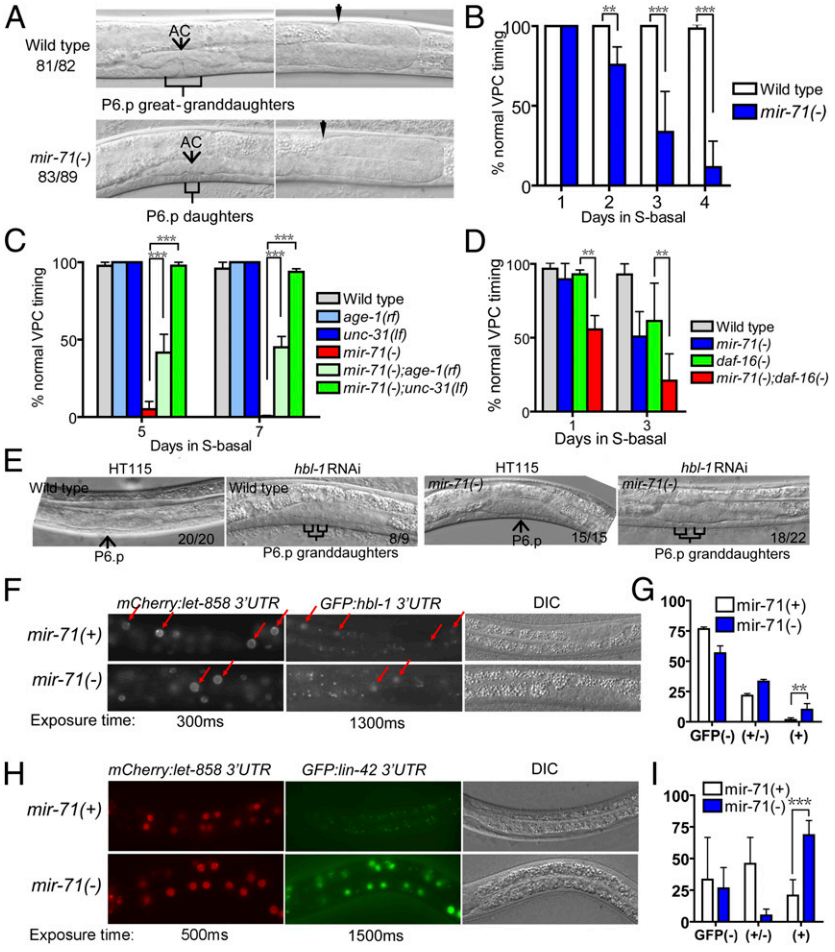

Fig. 4. miR-71 regulates vulval cell division during recovery of starved L1 worms. (*A*) Differential interference contrast (DIC) images showing L4 worms recovered from 4-d–starved L1 worms. Whereas the vulva of wild-type worms developed into the pyramidal stage (81 of 82 worms), the P6.p of *mir-71(n4115, lf)* mutant worms divided only once (83 of 89 worms). (*Right* panels) The gonad of the same animals in the *Left* panels to indicate the similar developmental stage. AC, anchor cell. (*B*) Bar graph showing the correlation between the severity of the retarded vulval precursor cell (VPC) timing defect of *mir-71(lf)* mutants and the duration of L1 starvation. n > 40 for each genotype at every time point. (*C*) Bar graph showing that the delayed VPC timing defects of *mir-71 (lf)* worms was suppressed by an *unc-31(lf)* mutation and partially suppressed by an *age-1(rf)* mutation. n > 40 for every genotype at each time point. (*D*) Bar graph showing that the delayed VPC timing defect of *mir-71 (lf)* worms was enhanced by *daf-16(lf)* after 1 or 3 d of L1 starvation. Note that the *daf-16(lf)* worms recovering from 3 d of L1 starvation displayed a ~12-h delay in overall development and that the *mir-71(lf); daf-16(lf)* double mutants displayed an ~24-h delay. n > 30 for every genotype at each time point. (*E*) DIC images showing that *hbl-1(RNAi)* caused precocious VPC divisions in late L2/early L3 in both wild-type and *mir-71(lf)* worms recovered from 4 d of L1 starvation. The numbers on each image indicate how many worms of the examined ones displayed the indicated phenotype. (*F*) Fluorescence and DIC images showing that an *hbl-1* 3′ UTR reporter was repressed in *mir-71(+)* worms and slightly derepressed in *mir-71(lf)* mutants. Red arrows point to representative intestine nuclei. (*G*) Statistical analysis of data in *F*; n = 60 for each. (*H*) Fluorescence and DIC images showing that a *lin-42* 3′UTR reporter was repressed in *mir-71(+)* worms (2/2 transgenic lines) and prominently derepressed in *mir-71(–)* worms (2/2 transgenic lines). (*I*) Statistical analysis of data in *H*; n = 30 for each.

function mutations in these genes are associated with precocious expression of the L3 cell fates in hypodermal cell lineage (skipping the L2 program) (22–25). Moreover, the expression of *hbl-1* is repressed by *let-7* family miRNAs at L3 during normal development, and the hyperactivity of *hbl-1* caused by failure of miRNA regulation leads to retarded development (26). Therefore, the retarded vulval development phenotype associated with *mir-71(lf)* mutants could potentially be caused by a collective effect of hyperactivities in these known timing regulators.

To test the hypothesis that these developmental timing genes mediate the regulatory role of miR-71 in larval development during recovery from starvation-induced L1 diapause, we examined whether knocking down HBL-1 function can suppress the retarded VPC timing defect of *mir-71(lf)*. Consistent with the observation described above, the 4-d–starved *mir-71(lf)* mutants recovering on the RNAi control plates displayed the highly penetrant retarded defect in VPC division. In contrast, the *mir-71(lf)* mutant worms recovering on *hbl-1(RNAi)* displayed precocious VPC divisions similar to that seen in wild type (Fig. 4*E*). This result suggests that miR-71 likely functions upstream of, or in parallel to, HBL-1 in regulating VPC timing. In starved L1 worms, we detected only a slight increase in the mRNA level of *hbl-1* in *mir-71* mutants compared with that in wild type (~10%), which may not be biologically significant. We then compared the expression of a *hbl-1 3′UTR* reporter (18) in the *mir-71(lf)* mutants with that in wild type and found that the expression of this reporter was slightly derepressed at L3 in the *mir-71* mutant (Fig. 4 *F* and *G*). This is consistent with *hbl-1* being one of the downstream targets of miR-71, although this modest effect alone is not expected to account for the vulval developmental phenotype in *mir-71* mutant. This result is also consistent with the prediction from a miRISC immunoprecipitation analysis that *hbl-1* is likely a target of one or more miRNAs, in addition to the *let-7* family miRNAs, during early development (18). The strong suppression of the *mir-71(lf)* defect by *hbl-1(RNAi)*, and the relatively weak effect of miR-71 on *hbl-1* expression, are consistent with the idea that miR-71 exerts its role by modulating activities of multiple genes related to *hbl-1* function in developmental timing.

To determine the functional relationship of miR-71 with LIN-42 and LIT-1, *mir-71(lf); lin-42(lf)* L1 worms were starved for 4 d and recovered on *lit-1(RNAi)* plates. Knocking down *lit-1* by RNAi in *mir-71(lf); lin-42(lf)* double mutants caused no significant suppression of the VPC timing defects of *mir-71(lf)* worms. However, we found that the reporter transgene with the *lin-42* 3′ UTR was significantly repressed in wild-type worms, but derepressed in the *mir-71(lf)* worms (Fig. 4 *H* and *I*). These results indicate that *lin-42* is likely one of the targets regulated by miR-71 during the L1 starvation recovery phase, but the major developmental defects of *mir-71(lf)* are due to the collective effect of changes in expression of many target genes, including those acting downstream of, or in parallel to, *lin-42* to regulate VPC divisions.

Discussion

Although the complete removal of miRNA functions causes embryonic lethality or infertility in worms, a partial disruption of overall miRNA functions by mutating either *ain-1* or *ain-2* provides an effective way to investigate miRNA functions (16, 17). Our analysis of L1 diapause using the *ain-1(lf)* mutants has generated valuable information regarding miRNA functions for long-term L1 survival, suggesting that miRNAs expressed in the intestine play critical roles during L1 diapause and that a significant portion of these miRNA activities are involved in modulating the UNC-31–InsR pathway. These results compelled us to examine specific interactions between individual miRNAs and their targets to gain mechanistic insights. Although the L1 diapause defect associated with *ain-1(lf)* mutations was quite robust, it reflected only a part of miRNA functions because the *ain-1(lf)* alleles themselves reduce but do not eliminate miRISC functions. The effects of eliminating all miRNA functions are expected to be much stronger, which is perhaps consistent with the additive effect of the defects associated with mutations of many individual miRNAs shown in Fig. 1*D*.

Following the studies by Horvitz and colleagues (7, 8) that showed that single mutations of the vast majority of miRNAs did not reveal obvious developmental or growth defects, our study reinforces the idea that most miRNAs do not regulate specific physiological functions through a robust regulatory interaction between one miRNA and one target. Instead, many specific physiological functions, such as the starvation-induced stress response, are regulated by a miRNA-target network, often involving multiple miRNAs and a large number of their targets. On one hand, we showed that deletions of a good number of miRNAs have varying impacts on the L1 diapause survival rate, although they may effect the rate through different mechanisms. On the other hand, the role of a particular miRNA (miR-71) is executed by repressing the expression of many genes in multiple pathways. Our genetic analysis indicated that for both L1 diapause survival and developmental recovery functions, miR-71 regulates expressions of genes in both the insulin receptor-dependent and -independent pathways. Furthermore, the observed derepression of individual genes by *mir-71(lf)* seemed too weak to account for the phenotype, consistent with the idea that a prominent phenotype of an miRNA mutation is caused by the collective effect of changing expression in many genes, an important property of miRNA-mediated gene regulation. To gain a thorough understanding of the miRNA–target interactions for starvation-induced L1 diapuase, biochemical and genomic approaches (17, 18, 27) may be helpful in identifying other targets of miR-71 and targets of other miRNAs involved in the process, given the limitations of the existing target prediction programs (28).

The InsR pathway has been repeatedly shown to be critical in stress responses (2, 3). Components of the InsR pathway, including *age-1*, have recently been predicted to be targets of miR-71 in its role in aging (14). Our data provide the experimental evidence that two components of the InsR pathway are likely direct targets of miR-71 in its role in a specific physiological process, L1 diapause (see a model in Fig. S5). It is also worth mentioning that multiple components of the InsR pathway, including *age-1*, *pdk-1*, *akt-2*, and *daf-16*, are predicted to be targets of the *let-7* family miRNAs. It seems plausible that miRNAs that control developmental timing are also involved in regulating the metabolic rate through repressing the InsR pathway activity. As pointed out above, multiple miRNAs in addition to *miR-71* and the *let-7* family miRNAs have roles in L1 diapause, and they may regulate the expression of many diverse targets that may include, but are not limited to, factors involved in UNC-31–InsR-signaling activities.

Unlike classical heterochronic miRNAs such as *lin-4* and *let-7*, the role of miR-71 in vulval cell division is essential in animals recovering from starvation-induced L1 diapause, but not in animals hatched on plates with food. We speculate that the expression of heterochronic genes controlling the L2/L3 programs, including that of *hbl-1* and *lin-42*, are increased during L1 diapause to arrest the developmental progression, and miR-71 is probably required to suppress these "excess" signals during the recovery phase (Fig. S5). However, miR-71 does not appear to regulate all post-embryonic development during L1 diapause recovery. For example, we observed a robust retarded mutant phenotype in the vulval lineage but did not see obvious defects in seam cell differentiation or alae formation. The latter results are consistent with the observation that miR-71 is not expressed in the seam cells. It is possible that other miRNAs, including those in the *let-7* family, control developmental timing in other tissues during the recovery phase after L1 starvation. We would also like to point out that, similar to its role in starvation survival, miR-71 likely regulates the expression of multiple targets in addition to the tested genes (*unc-31*, *hbl-1*, and *lin-42*) to influence developmental recovery; the

developmental defects may not necessarily be caused mainly by changed expressions of the genes tested in our study.

A recent study showed that the expression of miR-71 was significantly increased relative to other miRNAs in starved L1 worms (15). This result is consistent with the observation that miR-71 is specifically required for the starvation-induced stress response (Fig. S5). Using an *mir-71promoter:GFP* reporter (20), we did not observe obvious difference between the levels of GFP expression in 1-d–starved and nonstarved L1 animals, raising a possibility that miR-71 is maintained at a high level during L1 diapause when the expression of many other genes is reduced. We asked whether the expression of miR-71 was regulated by DAF-16, which is required during L1 diapause for long-term survival (2). However, we found that the expression of *mir-71promoter:GFP* was not decreased in *daf-16(lf)* L1 mutants that were starved for 24 h (Fig. S6). This result suggests that the high expression of miR-71 during L1 diapause is induced or maintained by other signaling pathways.

Materials and Methods

Nematode Strains and Methods. Worms strains were grown and maintained at 20 °C as described (29). The following strains were used: N2 (wild type), *age-1(hx546)*, *daf-16(mu86)*, MH2385 *ain-1(ku322)*, *ain-1(ku425)*, *ain-1(tm3681)*, *ain-2(tm2432)*, DA509 *unc-31(e928)*, MT2257 *lin-42(n1089)*, VT2084 *unc-119 (ed3) III; maIs352[unc-119(+) + Pmir-71:GFP]*, RG733 *wIs78 IV*, *JR667 unc-119 (e2498::Tc1) III; wIs51*, NH263 *hlh-8::GFP*, VT825 *dpy-20(e1282) IV*; *maIs113*, *PS4997 unc-119(e2498) III; syIs179*, MH3489 *hbl-1 3′UTR* reporter. MT12993 *mir-71(n4115)* worms were outcrossed with N2 for four generations before any test except the initial screen. *hbl-1*– and *lit-1*–feeding RNAi experiments were carried out following standard protocols (30, 31).

L1 Starvation Survival Assay and Statistical Analysis. L1 starvation assay was adapted from a previously described protocol (3). Briefly, worms were well fed for at least two generations, and gravid adults were bleached with hypochlorite and sodium hydroxide. The eggs were transferred to plates seeded with HB101 and bleached again 3 d later. The resulting eggs were hatched in 4–6 mL S-basal without cholesterol in 15-mL tubes (Greiner), which were placed on an end-over-end rocker (VWR) at 20 °C. A total of 16–24 h later, the density of newly hatched L1 worms was adjusted to three to five worms per microliter S-basal. To determine viability, 20-μL aliquots (60–100 worms) were placed every 3 d onto two 6-cm nematode growth medium (NGM) plates seeded with OP50, and the numbers of L1 worms were recorded as number of plated worms (Np). Three days later, the number of worms that were L2 or older was recorded as number of survived worms (Ns), and the survival rate was calculated as Ns/Np, which is an estimation of survived worms in the whole population. Survival curves were drawn in Excel. To compare the survival rates between strains, we simulated the survival rate of each genotype to 100 arbitrary "individual worms" and performed the log-rank test in Graphpad Prism 4. Also with Prism 4, the median life span was calculated with a nonlinear regression analysis using the following equation: Y = bottom + (top − bottom)/{1+10^[(LogEC50-X) × HillSlope]}. In this equation, bottom and top were set as 0 and 100, respectively. The median life span is equal to EC_{50}. The data for 3′UTR expression and for VPC timing were analyzed using χ^2 test. In Figs. 1–4, "*", "**" and "***"represent a P value equal or less than 0.05, 0.005, and 0.001, respectively. Bar diagrams represent mean and SD in Figs. 1–4.

3′UTR Reporters and Microscopy. PCR and molecular cloning were carried out following standard protocols. 3′UTRs of genes of interest were cloned into the modified pPD129.57 vector as described previously (18). The primers that were used to amplify the 3′UTR of candidate genes are available upon request. Individual GFP reporter constructs for candidate genes (4 ng/μL) and the mCherry internal control plasmid (4 ng/μL) were mixed with *unc-119* rescuing plasmid (20 ng/μL) and pBluescript KS+ (72 ng/μL) and coinjected into *unc-119(ed3)* and *mir-71(n4115); unc-119(ed3)* worms following standard protocols (32). Non-Unc stable transgenic lines were maintained, and the expression of GFP and mCherry were observed under a Zeiss Axiovision II microscope. For examining the *age-1 3′UTR* reporter, the *rol-6(d)* marker (100 ng/μl pRF4) was used instead of the *unc-119(+)* plasmid. Images were pseudocolored in Photoshop CS3 (Adobe) and assembled in Illustrator CS3 (Adobe).

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