Intermittent fasting (IF) is a dietary restriction regimen that extends the lifespans of Caenorhabditis elegans and mammals by inducing changes in gene expression. However, how IF induces these changes and promotes longevity remains unclear. One proposed mechanism involves gene regulation by microRNAs (miRNAs), small non-coding RNAs (~22 nucleotides) that repress gene expression and whose expression can be altered by fasting. To test this proposition, we examined the role of the miRNA machinery in fasting-induced transcriptional changes and longevity in C. elegans. We revealed that fasting up-regulated the expression of the miRNA-induced silencing complex (miRISC) components, including Argonaute and GW182, and the miRNA-processing enzyme DRSH-1 (the ortholog of the Drosophila Drosha enzyme). Our lifespan measurements demonstrated that IF-induced longevity was suppressed by knock-out or knockdown of miRISC components and was completely inhibited by drsh-1 ablation. Remarkably, drsh-1 ablation inhibited the fasting-induced changes in the expression of the target genes of DAF-16, the insulin/IGF-1 signaling effector in C. elegans. Fasting-induced transcriptome alterations were substantially and modestly suppressed in the drsh-1 null mutant and the null mutant of ain-1, a gene encoding GW182, respectively. Moreover, miRNA array analyses revealed that the expression levels of numerous miRNAs changed after 2 days of fasting. These results indicate that components of the miRNA machinery, especially the miRNA-processing enzyme DRSH-1, play an important role in mediating IF-induced longevity via the regulation of fasting-induced changes in gene expression.

Dietary restriction (DR) increases lifespan and prevents age-related diseases in many organisms, ranging from yeast to mice (1–4). Many forms of DR, including caloric restriction (CR), intermittent fasting (IF), and protein restriction (1, 2, 5), exist. An increasing number of studies indicate that fasting stimuli induce health benefits, such as lifespan extension and the prevention of diabetes and cardiovascular disease, and are considered to be a plausible intervention for slowing the rate of aging in humans (1, 5–8). Our previous studies have shown that IF significantly extends the lifespan of Caenorhabditis elegans, and IF-induced longevity is mediated by the fasting-induced transcriptional alterations by two transcription factors: DAF-16, the insulin/IGF-1 signaling (IIS) pathway effector (9), and AP-1, the stress-activated MAP kinase JNK pathway effector (10). Thus, the importance of transcriptional changes in IF-induced longevity has been well documented. However, roles of post-transcriptional regulation in fasting-induced signaling remain ambiguous.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that post-transcriptionally regulate gene expression (11–13). In the miRNA pathway, primary miRNA transcripts are cleaved by the microprocessor complex, which is composed of the ribonuclease (RNase) III enzyme Drosha/DRSH-1 and its cofactor DGCR8 (DiGeorge syndrome critical region gene 8)/PASH-1 (11–13). The processed products, termed precursor miRNAs, are exported to the cytoplasm, where the precursor miRNA stem-loop is processed by another RNase III, Dicer, thus generating mature miRNAs (11–13). Mature miRNAs form the miRNA-induced silencing complex (miRISC) with Argonaute protein (ALG-1 or ALG-2) and GW182 protein (AIN-1 or AIN-2), and the miRISC recognizes and represses target gene expression (12, 14, 15). Multiple roles of miRNAs in animals and plants have been reported in many biological processes, including aging (16–23). Recently, mir-71 and mir-228 have been shown to regulate CR-induced longevity through transcription factors PHA-4 and SKN-1 (21). In addition, mir-80 null mutants show age-related phenotypes that are similar to those of eat-2 mutants (the model of CR) (22). The expression of Dicer decreases with aging; this decrease is suppressed by CR in mouse adipose tissues and C. elegans (23). The expression levels of several miRNAs are altered after 12 h of fasting in C. elegans at the L4 stage (24). However, the involvement of miRNA in IF-induced longevity remains unaddressed.

In this study, we examined the role of the miRNA machinery in fasting-induced transcriptional changes and longevity in C. elegans. Our results indicated that the miRISC components and the miRNA-processing enzyme Drosha/drsh-1 were up-regulated by fasting and that knockdown or knock-out of
miRISC components suppressed IF-induced longevity. Remarkably, drsh-1 null mutation completely suppressed IF-induced longevity and inhibited the fasting-induced up-regulation of the target genes of DAF-16, the IIS pathway effector. Also, the majority of the fasting-induced transcriptome alterations were suppressed in drsh-1 null mutants. Additionally, our miRNA array analysis indicated that the expression levels of a number of miRNAs changed after 48 h of fasting starting at day 2 of the adult stage. These results reveal involvement of the miRNA machinery, particularly the miRNA-processing enzyme DRSH-1, in fasting-induced changes in gene expression and IF-induced longevity.

Results

Fasting induces changes in the expression of miRISC components

We examined the expression levels of miRISC components in worms under fed and fasting conditions. Remarkably, the genes encoding Argonaute proteins, alg-1 and alg-2, and the genes encoding GW182 proteins, ain-1 and ain-2, were up-regulated 2–3-fold after 48 h of fasting (Fig. 1A). Our immunoblot analysis revealed that the ALG-1 protein (GFP::ALG-1, left) and ALG-2 protein (ALG-2::HA, l) under fed and fasting conditions are shown. Protein extracts were from zals5 (alg-1:p::GFP::alg-1 + rol-6(su1006)), a strain overexpressing alg-1, and alg-2(ok304); pkl2256 (alg-2::HA + rol-6(su1006)), a strain overexpression alg-2. Histone H3 was used as the loading control. The relative intensity of GFP::ALG-1 in fasting conditions was 2.48 ± 0.51 compared with that in fed conditions (p = 0.007, unpaired Student’s t test, n = 3). The relative intensity of ALG-2::HA in fasting conditions was 1.20 ± 0.35 compared with that in fed conditions (p = 0.300, unpaired Student’s t test, n = 4).

miRISC is involved in IF-induced longevity

Because fasting-induced gene expression alterations underlie IF-induced longevity (9, 10), we considered the possibility that fasting might induce gene expression changes and longevity, at least partly through the miRNA machinery. To test this possibility, we measured the lifespan of the null mutants of miRISC components, alg-1(gk214), alg-2(ok304), ain-1(tm3681), and ain-2(tm1863), and found that the IF-induced longevity was significantly suppressed in the null mutants of alg-1, ain-1, and ain-2 (Fig. 2, A and B, Table 1). Because we observed developmental defects in alg-1 null mutants, we knocked down miRISC components (alg-1, alg-2, ain-1, and ain-2) by using RNAi. Because the Argonaute proteins are required for normal development (25, 26), we performed alg-1 or alg-2 RNAi after completion of development to prevent developmental defects. In the case of ain-1 and ain-2, we performed RNAi starting from the egg stage. The lifespan measurements revealed that the RNAi of alg-1, alg-2, or ain-1, compared with the control RNAi, caused a significant decrease in the IF-induced longevity (Fig. 3, A and B, Table 1). These results demonstrate that miRISC components are involved in regulation of the IF-induced longevity.

Expression of the miRNA-processing enzyme Drosha/DRSH-1 is enhanced by fasting, and drsh-1 ablation suppresses IF-induced longevity

To further examine the involvement of miRNA machinery in the fasting response, we focused on the miRNA-processing enzyme Drosha/DRSH-1 and its partner Pasha/PASH-1. Our quantitative RT-PCR (qRT-PCR) analysis revealed that the expression levels of drsh-1 and pash-1 were significantly up-regulated after fasting, similarly to the miRISC components (Fig. 4A). To test whether DRSH-1 is involved in IF-induced longevity, we used the drsh-1 null mutants. Our lifespan measurements indicated that the null mutation of drsh-1 completely suppressed IF-induced longevity but did not affect the lifespan in ad libitum feeding conditions (Fig. 4B and Table 1). However, it has previously been shown that a loss-of-function mutation of pash-1 resulted in shortened lifespans under ad libitum conditions in the absence of fluorodeoxyuridine (FUDR) (27). As the difference in the use of FUDR might affect the result, we also measured the lifespan of drsh-1 mutants in the absence of FUDR. The obtained results showed that the lifespan of drsh-1 mutants was shorter than that of wild type in the absence of FUDR (Fig. 4C and Table 1), consistent with a previous report (27). In any case, our results suggest that the miRNA-processing pathway is activated by fasting and is required for IF-induced longevity.

DRSH-1 is required for fasting-induced changes in the IIS target gene expression

The IIS pathway plays an important role in IF-induced longevity (9, 10). Therefore, we investigated the effect of drsh-1 ablation on fasting-enhanced DAF-16 target genes (aqp-1, dod-6, mtl-1, hil-1, hsp-12.6, and sod-3). qRT-PCR measurements indicated that the fasting-induced up-regulation of these genes was significantly suppressed in the drsh-1 null mutant, whereas the expression of some genes was up-regulated in ad
Figure 2. IF-induced longevity is suppressed in null mutants of miRISC components. A, survival curves of WT worms (upper left), alg-1 (gk214) (upper right), and alg-2 (ok304) mutants (lower left) in ad libitum (AL) and IF are shown. The mean lifespan of 3 independent experiments are shown (lower right). The error bars represent S.D. *, p < 0.05; #, p < 0.05; †, p < 0.05, one-way ANOVA followed by Tukey’s test. *, #, and † represent significant differences between strains in ad libitum conditions, between strains in IF conditions, and between ad libitum and IF in each genotype, respectively. B, survival curves of WT worms (upper left), ain-1 (tm3681) (upper right), and ain-2 (tm1863) mutants (lower left) in ad libitum and IF are shown. Mean lifespan of 4 independent experiments are shown (lower right). The error bars represent S.D. *, p < 0.05; #, p < 0.05; †, p < 0.05, one-way ANOVA followed by Tukey’s test. *, #, and † represent significant differences between strains in ad libitum conditions, between strains in IF conditions, and between ad libitum and IF in each genotype, respectively.

Table 1
IF-induced longevity in various mutants and RNAi-treated worms

|                | Mean lifespan (days) | Extension by IF | n* | No. of trials |
|----------------|----------------------|-----------------|----|--------------|
| WT            | AL† 18.9 ± 3.3       | 58.0            | 216 3 |
|               | IF 29.5 ± 2.2        | −4.4            | 225 3 |
| alg-1(gk214)  | AL 12.3 ± 1.5        | 53.8            | 205 3 |
|               | IF 11.5 ± 0.5        | 174 3           |
|               | alg-2(ok304)         | IF 28.2 ± 0.9   | 234 3 |
|               | AL 18.6 ± 2.6        | 70.4            | 156 4 |
|               | IF 26.3 ± 3.9        | 205 4           |
| ain-1(tm3681) | AL 15.0 ± 1.4        | 34.3            | 195 4 |
|               | IF 19.9 ± 2.3        | 220 4           |
| ain-2(tm1863) | AL 17.8 ± 1.4        | 30.7            | 263 4 |
|               | IF 23.1 ± 2.8        | 224 4           |
| ctrl RNAi     | AL 19.8 ± 1.8        | 57.8            | 262 3 |
|               | IF 31.2 ± 2.4        | 253 3           |
| alg-1 RNAi    | AL 17.4 ± 0.7        | 38.3            | 264 3 |
|               | IF 24.1 ± 2.2        | 233 3           |
| alg-2 RNAi    | AL 17.6 ± 0.9        | 48.0            | 265 3 |
|               | IF 26.1 ± 2.4        | 273 3           |
| ctrl RNAi     | AL 14.7 ± 2.5        | 60.3            | 321 5 |
|               | IF 23.3 ± 2.2        | 305 5           |
| ain-1 RNAi    | AL 13.5 ± 2.7        | 44.4            | 325 5 |
|               | IF 19.2 ± 1.8        | 317 5           |
| ain-2 RNAi    | AL 16.3 ± 3.0        | 57.5            | 273 4 |
|               | IF 25.3 ± 2.1        | 268 4           |
| WT            | AL 17.4 ± 0.6        | 49.1            | 165 3 |
|               | IF 25.9 ± 1.4        | 183 3           |
| drsh-1(ok369) | AL 17.9 ± 1.3        | −2.2            | 197 3 |
|               | IF 17.6 ± 2.0        | 183 3           |
| WT FUDR(−)    | AL 17.4 ± 0.8        | 135 3           |
| drsh-1(ok369) | FUDR(−)              | AL 12.8 ± 0.4   | 130 3 |
| WT ctrl RNAi  | AL 14.9 ± 1.5        | 56.8            | 213 4 |
|               | IF 23.3 ± 2.4        | 306 4           |
| WT daf-16 RNAi| AL 13.5 ± 1.6        | 50.3            | 205 4 |
|               | IF 20.1 ± 0.5        | 301 4           |
| drsh-1(ok369)| ctrl RNAi            | AL 16.5 ± 1.0   | −4.5 | 279 4 |
|               | daf-16 RNAi          | AL 15.8 ± 1.6   | −3.3 | 279 4 |

* n, the total number of worms.
† AL, ad libitum.
† p < 0.05, one-way ANOVA followed by Tukey’s test between ad libitum (AL) and IF in each genotype.

libitum conditions in the mutant compared with the WT worms (Fig. 5). To further investigate the relationship between DRSH-1 and the IIS pathway, we examined expression of the fasting-repressed DAF-16 target gene ins-7 (9). The results indicated that ins-7 expression was not down-regulated by fasting in drsh-1 null mutants, whereas the expression was substantially decreased by fasting in WT worms (Fig. 5). These results indicate that DRSH-1 is involved in the regulation of the IIS pathway.

We then examined the effect of simultaneous depletion of miRNA synthesis and insulin signaling on the lifespan and expression of the DAF-16 target genes. daf-16 RNAi shortened the lifespan of drsh-1 mutants (Fig. 6A and Table 1), indicating that DAF-16 regulates the lifespan in a DRSH-1-independent manner under ad libitum conditions. daf-16 RNAi partially suppressed IF-induced longevity in wild type and did not fur-
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The expression levels of several miRNAs change in response to fasting at the L4 stage in C. elegans (24). To examine the fasting-induced changes in miRNA expression in adult worms, we performed miRNA array experiments. The results indicated that the expression levels of numerous miRNAs underwent substantial changes in response to fasting (Fig. 8, left). The top 10 miRNAs whose expression was up-regulated or down-regulated by more than 1.5-fold after fasting are listed (Fig. 8, right). Previous reports have identified “age-related miRNAs” as miRNAs whose expression levels change during aging (19, 20). Our analysis indicated that this group of age-related miRNAs (Fig. 8, colored miRNAs, supplemental Tables S1 and S2) is significantly enriched in fasting-induced up-regulated and down-regulated miRNAs (p < 0.0002, Fisher's exact test).

Discussion

In this study, we demonstrate that miRNA machinery, particularly the miRNA-processing enzyme Drosha/DRSH-1, is involved in fasting-induced changes in gene expression and IF-induced longevity in C. elegans. Our analysis revealed that miRISC components (alg-1, alg-2, ain-1, and ain-2) and the miRNA-processing enzyme drsh-1 are up-regulated by fasting, thus suggesting that the miRNA machinery is activated in response to fasting. The expression of miRNA machinery proteins (Argonaute, Dicer, and Drosha) in mouse adipose tissues.

Figure 3. IF-induced longevity is suppressed by knockdown of miRISC components. A, survival curves of control RNAi- (upper left), alg-1 RNAi- (upper right), and alg-2 RNAi-treated (lower left) worms in ad libitum (AL) and IF are shown. Mean lifespan of 3 independent experiments are shown (lower right). The error bars represent S.D. *, p < 0.05; #, p < 0.05; t, p < 0.05, one-way ANOVA followed by Tukey’s test. *, #, and t represent significant differences between strains in ad libitum conditions, between strains in IF conditions, and between ad libitum and IF in each genotype, respectively. B, survival curves of control RNAi- (upper left), ain-1 RNAi- (upper right), and ain-2 RNAi-treated (lower left) worms in ad libitum and IF are shown. Mean lifespan of 4 to 5 independent experiments are shown (lower right). The error bars represent S.D. *, p < 0.05; #, p < 0.05; t, p < 0.05, one-way ANOVA followed by Tukey’s test. *, #, and t represent significant differences between strains in ad libitum conditions, between strains in IF conditions, and between ad libitum and IF in each genotype, respectively.

drsh-1 depletion did not suppress expression of DAF-16 target genes under fed conditions, when daf-16 RNAi suppressed it (Fig. 6B). daf-16 RNAi partially suppressed fasting-induced changes in expression of DAF-16 target genes, drsh-1 depletion, and simultaneous depletion of daf-16 and drsh-1 completely suppressed them. These results suggest that DRSH-1 and DAF-16 act in parallel pathways in lifespan regulation under ad libitum conditions, and that DRSH-1 is involved in DAF-16 regulation under fasting conditions, which could contribute to IF-induced longevity.

Fasting-induced transcriptome alterations are suppressed by the ablation of miRNA machinery components

These results suggest that DRSH-1 plays an essential role in IF-induced longevity and is involved in regulation of the IIS pathway. It has previously been shown that the IIS pathway also plays a partial but important role in IF-induced longevity (9, 10). Thus, we considered the possibility that DRSH-1 might also be involved in other pathways in the fasting response in addition to the IIS pathway. We performed microarray analysis using drsh-1, ain-1, and daf-16 null mutants under both fed conditions and fasting conditions. To validate the involvement of drsh-1 and ain-1 in fasting-induced gene expression changes, we compared the induction rates of all genes in the mutants with the induction rates of all genes in WT worms (Fig. 7). The correlation between WT worms and drsh-1 null mutants (r = 0.601) was low compared with the correlation between WT worms and ain-1 null mutants (r = 0.907) or daf-16 null mutants (r = 0.808), thus suggesting a greater role of DRSH-1 in the fasting response.

Fasting induces substantial changes in the expression of miRNAs

The expression levels of several miRNAs change in response to fasting at the L4 stage in C. elegans (24). To examine the fasting-induced changes in miRNA expression in adult worms, we performed miRNA array experiments. The results indicated that the expression levels of numerous miRNAs underwent substantial changes in response to fasting (Fig. 8, left). The top 10 miRNAs whose expression was up-regulated or down-regulated by more than 1.5-fold after fasting are listed (Fig. 8, right). Previous reports have identified “age-related miRNAs” as miRNAs whose expression levels change during aging (19, 20, 28). Our analysis indicated that this group of age-related miRNAs (Fig. 8, colored miRNAs, supplemental Tables S1 and S2) is significantly enriched in fasting-induced up-regulated and down-regulated miRNAs (p < 0.0002).
has been reported to decrease with aging, and these decreases are suppressed by CR (23). The age-dependent decrease of Dicer in C. elegans is also suppressed by CR (23). This previous report has indicated that activity of the miRNA machinery is altered during aging and is regulated by food availability in mice and C. elegans. However, the involvement of the miRNA machinery in DR-induced longevity has remained unaddressed. In the present study, our results indicated that IF-induced longevity is suppressed by knock-out or knockdown of miRISC components and is completely inhibited by drsh-1 null mutations. Because miRISC and DRSH-1 are required for miRNA synthesis and function (11–15), miRNAs appear to play an important role in IF-induced longevity. Our analyses indicated that fasting-induced transcriptome alterations are significantly and modestly suppressed by the abrasion of drsh-1 and ain-1, respectively. This result correlates with the complete and partial suppression of IF-induced longevity observed after the ablation of drsh-1 and ain-1, respectively, and suggests that the fasting-induced gene expression alteration underlies the IF-induced longevity. Collectively, our results suggest that miRNAs play an important role in the IF-induced longevity by mediating the fasting-induced gene expression alterations.

Figure 4. Expression of the miRNA-processing enzyme Drosha/DRSH-1 is enhanced by fasting, and drsh-1 ablation suppresses IF-induced longevity. A, relative mRNA expression levels of drsh-1 and pash-1 in fed and fasting conditions in WT worms are shown. The mean value from 3 to 4 independent experiments in the WT worms in fed conditions was set to 1. The error bars represent S.D. *, p < 0.05, unpaired Student’s t test. B, survival curves of WT worms (left) and drsh-1(ok369) mutants (middle) in ad libitum (AL) and IF are shown. The mean lifespan of 3 independent experiments are shown (right). The error bars represent S.D. *, p < 0.05; †, p < 0.05; †, p < 0.05; †, p < 0.05, one-way ANOVA followed by Tukey’s test. *, †, and † represent significant differences between strains in ad libitum conditions, between strains in IF conditions, and between ad libitum and IF in each genotype, respectively. C, survival curves of WT worms (solid line) and drsh-1(ok369) mutants (dotted line) under ad libitum conditions in the absence of FUDR (left). The mean lifespan of 3 independent experiments are shown (right). The error bars represent S.D. *, p < 0.05, unpaired Student’s t test.
result from the difference in the stage (L4 stage in the previous study versus day 2 adult in our study) and the duration of fasting stimulus (12 h in the previous study versus 48 h in our study). Two days of fasting decrease the expression of mir-80, which is shown to be an anti-longevity miRNA (22), and increases the expression of mir-34, a pro-longevity miRNA (29). These fasting-induced changes in miRNAs expression may contribute to IF-induced longevity.

The IIS pathway plays an important role in IF-induced longevity (9, 10). Our qRT-PCR measurements indicated that fasting-induced changes in expression of the DAF-16 target genes are completely suppressed by drsh-1 null mutation, thus suggesting that DRSH-1 may regulate IF-induced longevity at least partly through the IIS pathway. Our microarray analyses showed that not only DAF-16 target genes but also the majority of the fasting-induced genes are regulated by DRSH-1, thus suggesting that other pathways are also regulated by the miRNA machinery. The pathways that are related to miRNA machinery in fasting conditions remain to be determined. Because the miRNA machinery is conserved between nematodes and mammals (30–32), our findings may provide a new approach to the prevention of age-related diseases in humans.

Additional studies are needed to better understand the mechanisms of miRNA-mediated IF-induced longevity.

**Experimental procedures**

**Strains and growth conditions**

All strains were maintained at 20 °C on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50, as previously described (33). The following strains were used in this study: wild type (WT) N2, alg-1(gk214), alg-2(ok304), ain-1(tm3681), drsh-1(ok369), H127;[bli-4(e937) let-7[q782]] qsl48], zits5[alg-1::FEP::alg-1 + rol-6(su1006)], and alg-2(ok304); pkls2256[alg-2::HA + rol-6(su1006)]. These strains were provided by the Caenorhabditis Genetics Center (CGC), and ain-2(tm1863) which was provided by the National BioResource Project. All mutants were outcrossed with our laboratory WT at least twice before use.

**MicroRNA microarray analysis**

Synchronized WT eggs were obtained by the bleaching method (33). Worms from these synchronous eggs were raised in normal conditions, and young adult animals were transferred to NGM plates that contained 200 μg/ml of FUDR. The day on which the animals were transferred to FUDR-containing NGM plates was defined as t = 0 day. On day 2, the animals were transferred to FUDR-containing NGM plates that were seeded with or without UV-kill OP50. The animals were collected at day 2 in fed conditions and at day 4 in fed or fasting conditions, and total RNA was extracted with TRIzol reagent (Invitrogen) from frozen animals. The miRNA content in total RNA was analyzed using an Agilent 2100 Bioanalyzer. Then, 1 μg of total RNA was labeled with a FlashTag Biotin HSR RNA Labeling Kit (Affymetrix, Santa Clara, CA) for Affymetrix GeneChip® miRNA arrays (Affymetrix) according to the manufacturer’s recommendations. A simple colorimetric enzymelinked oligosorbent assay was used to confirm successful biotin labeling. After labeling, the samples were hybridized on Affymetrix GeneChip® microarrays, washed, stained, and scanned according to the manufacturer’s instructions (Affymetrix). The array data were normalized by global normalization with Robust Multichip Average and Detection Above Background by using the Expression Console software (Affymetrix).

**Quantitative RT-PCR**

Total RNA was reverse transcribed into single-stranded cDNA using ReverTra Ace qPCR RT master Mix with gDNA remover (TOYOBO) according to the manufacturer’s protocol. Quantitative RT-PCR was performed with an ABI 7300 Real-Time PCR system (Applied Biosystems) using SYBR Premix Ex Taq™ II (TAKARA). The relative mRNA levels were determined using the ΔΔCt method. All values were normalized to the expression of *rol-6*, a *C. elegans* housekeeping gene. The mean values of 3 to 6 experiments in wild type under fed conditions were set to 1. The primers used for DAF-16 target genes have been described previously (9). The primers in this study were as follows: *alg-1* forward: 5'-CAGCATGTCGAAGGAGACGA-3'; *alg-1* reverse: 5'-CGTACTTGAGAGATGTGA-3'; *alg-2* forward: 5'-GGTGACCTGGAATGTGA-3'; *alg-2* reverse: 5'-CGTGAGCGTGAGATTGTA-3'.

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**Figure 5. DRSH-1 plays an important role in fasting-induced alterations in DAF-16 target genes.** Relative miRNA expression levels of seven DAF-16 target genes (*aqp-1, dod-6, mtl-1, hil-1, hsp-12.6, sod-3, and ins-7*) in fed and fasting conditions in WT worms and *drsh-1(ok369)* mutants are shown. The mean value from 3 to 4 independent experiments in WT worms in fed conditions was set to 1. The error bars represent S.D. *p < 0.05; #, p < 0.01; †, p < 0.005, one-way ANOVA followed by Tukey’s test. * represents significance, † represents significance in fed conditions, # represents significance in fasting conditions, and † represents significance between strains in each genotype, respectively.
Figure 6. Effects of simultaneous depletion of DRSH-1 and DAF-16 on the lifespan and the expression of DAF-16 target genes. A, survival curves of control RNAi- or daf-16 RNAi-treated WT worms (left upper) and drsh-1(ok369) mutants (left lower) under ad libitum (AL) and IF conditions are shown. The mean lifespan of 4 independent experiments are shown (right). The error bars represent S.D. *, p < 0.05; #, p < 0.05; †, p < 0.05, one-way ANOVA followed by Tukey’s test. *, #, and † represent significant differences between strains in AL conditions, between strains in IF conditions, and between AL and IF in each genotype, respectively. B, relative mRNA expression levels of seven DAF-16 target genes (aqp-1, dod-6, mtl-1, hil-1, hsp-12.6, sod-3, and ins-7) in control RNAi- or daf-16 RNAi-treated WT worms and drsh-1(ok369) mutants in fed and fasting conditions are shown. The mean value from 3 to 4 independent experiments in WT worms under fed conditions was set to 1. The error bars represent S.D. *, p < 0.05; #, p < 0.05; †, p < 0.05, one-way ANOVA followed by Tukey’s test. *, #, and † represent significant differences between strains in fed conditions, between strains in fasting conditions, and between fed and fasting in each genotype, respectively.

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reverse: 5’-AGAGCACACGTCCTTTGACT-3’; ain-1 forward: 5’-GAGAAGCAAACGTACCT-3’; ain-1 reverse: 5’-TGGTACCTGGACATCGTT-3’; ain-2 reverse: 5’-CCATCGTGGAATGAATAC-3’; ain-2 forward: 5’-GGTGGTGGGATTATTGGAC-3’; drsh-1 forward: 5’-GATGTGTCTGATGACCTCAG-3’; drsh-1 reverse:
MicroRNA machinery regulates fasting-induced longevity

Figure 7. Fasting-induced transcriptome alterations are suppressed by the ablation of miRNA machinery components. Correlation of gene expression profiles in WT worms and drsh-1(ok369) (upper), ain-1(tm3681) (middle), or daf-16(mu86) mutants (lower). The log2 FC for day 4 adult worms under fed and fasting conditions are plotted for each gene.

5'-CTTTTACTTCTCCGGTCTTTGTCC-3', pash-1 forward: 5'-TCGTCCTCTCACTCGGAACCG-3', pash-1 reverse: 5'-CCGGTTCTGGTCAACGATCT-3', sgo-1 forward: 5'-GGCATTTGGTTTCATGTG-3', sgo-1 reverse: 5'-GGCAGCTCGGCAATACCG-3'.

RNA interference

RNA interference was performed by the feeding method as previously described (34). An empty vector (pPD129.36) was used as the control. Some of the RNAi clones (alg-1, ain-1, and daf-16) were constructed by PCR amplification with the follow-
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Figure 8. Fasting induces changes in the expression of miRNAs. Scatter plots comparing the log2-fold change (FC) of miRNA expression levels in total RNA from day 4 adult worms under fed conditions or 2 days (48 h) of fasting conditions. The red dots represent the genes whose expression changes were more than 1.5 or less than 0.67 in response to fasting. The expression levels of day 4 adult worms were normalized to the expression levels of day 2 adult worms. The solid lines indicate FC = 1.5 and = 0.67. The tables list the top 10 miRNAs whose expression level was changed after 48 h of fasting. The up-regulated (middle) and down-regulated (right) genes are shown. The colored miRNAs represent age-related miRNAs (refer to text).

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