A starvation-induced noncoding RNA modulates expression of Dicer-regulated genes

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Although much has been learned about short noncoding RNAs, long noncoding transcripts are largely uncharacterized. Here, we describe Caenorhabditis elegans rncs-1, a highly base-paired, 800-nucleotide noncoding RNA expressed in hypodermis and intestine. Transcription of rncs-1 is modulated in response to food supply. Although highly double-stranded, we show that rncs-1 RNA is not a substrate for Dicer because of branched structures at its termini. However, rncs-1 RNA inhibits Dicer cleavage of a second dsRNA in vitro, presumably by competition. We validate this observation in vivo by demonstrating that mRNA levels of several Dicer-regulated genes vary with changes in rncs-1 expression. Certain viruses express dsRNA to compete with cellular dsRNA-mediated pathways, and our data suggest that rncs-1 provides a cellular correlate of this phenomenon.

With a growing number of genomes sequenced, it is clear that an increase in organismal complexity is not explained by an increase in protein-coding genes. The genome of the ~1,000-cell nematode Caenorhabditis elegans contains ~20,000 protein-coding genes, an only slightly smaller number than the 22,000 genes predicted for humans (1). Certainly, posttranscriptional processes such as RNA splicing and editing account for 22,000 genes predicted for humans (1). Certainly, posttranscriptional processes such as RNA splicing and editing account for 22,000 genes predicted for humans (1). Obviously, our data suggest that the double-stranded structure of rncs-1 allows it to modulate expression of Dicer-regulated genes.

Results

rncs-1 Is Expressed in Intestine and Hypodermis and Enriched in Males. C. elegans develop from embryo through four larval stages (L1–L4) into adults. Postembryonic development depends on the presence of food, and worms arrest at the beginning of L1 under starved conditions (L1 arrest). Further, lack of food and high culture density in early development prompt entry into an alternative larval form called dauer. To study rncs-1 expression, we isolated total RNA from synchronized cultures at each developmental stage and from arrested L1s and dauer larvae from starved and crowded liquid culture. Northern blot analysis revealed an RNA species of ~800 nt (Fig. 1C). The transcript was present at constant levels in embryos, larvae, and adults but increased in arrested L1 worms and dauers.

To investigate the spatial expression of rncs-1, we injected Prncs-1::GFP, a reporter with ~2 kb of putative rncs-1 regulatory and promoter sequences driving expression of green fluorescent protein (Fig. 1A), into wild-type (N2) worms. GFP expression initiated in the early gastrula (data not shown). We observed robust expression of Prncs-1::GFP in the midgut (E cell lineage) starting at the 28-cell stage and continuing into adulthood (Fig. 1 D–F). By the comma stage (Fig. 1D), fluorescence was also visible in the embryo periphery in cells that give rise to hypodermis. In L1 larva and subsequent stages, we observed strong expression of GFP in hypodermal cells, including Hyp 7 syncytium and head and tail hypodermis. The expression pattern was identical in hermaphrodites and males, but adult hermaphrodites displayed fluorescence in vulval epithelium. Expression was absent in seam cells, nervous system, and pharynx. As described below, like rncs-1, the Prncs-1::GFP reporter showed increased expression during starvation. Although fluorescence intensity was enhanced under starved conditions, the spatial expression pattern was unchanged.

Expression of the Prncs-1::GFP transgene was also enhanced in males. We observed an ~2.5-fold increase in rncs-1 expression in total RNA prepared from wild-type, well fed males, compared with hermaphrodites (Fig. 1 G and H). In contrast, differences in mRNA levels were not observed for mRNAs of let-413 and...
eps-8, intestinal genes involved in midgut formation and maintenance (8, 9) (Fig. 1H).

**rncs-1 Transcription Is Regulated by Food Supply.** To determine whether the increase in *rncs-1* levels in arrested L1 and dauer larvae was a response to lack of food, we removed food from cultures of L4 worms and harvested worms for RNA isolation over 2 days. We observed an ~3-fold induction of *rncs-1* levels over unstarved controls in as little as 1 h of starvation [Fig. 2A and B and supporting information (SI) Fig. S1]. After 10 h, *rncs-1* RNA reached a maximum of ~6-fold enrichment, and this level was maintained for the 48-h starvation period. After 48 h of starvation, food was reintroduced, and within 6 h *rncs-1* levels decreased to baseline. Unlike *rncs-1*, the general population of polyadenylated RNA did not increase in the absence of food (Fig. 2B, dashed line).

The *C. elegans* dauer is a nonfeeding stage, and entry into dauer is mediated by sensory integration of two environmental cues: lack of food and abundance of dauer pheromone (daumone). High levels of daumone in the medium, indicative of high population density, prompt entry into dauer despite sufficient food (10). We took advantage of the different pathways to dauer to investigate whether *rncs-1* was elevated because of absence of food in the medium or lack of food in the digestive tract. When RNA was isolated from dauer larvae purified from exhausted liquid culture, where starvation conditions and high pheromone levels existed, we observed increased levels of *rncs-1*. Similarly, in RNA from wild-type L4 larvae starved for indicated times and then reintroduced to food for several hours (48-h starved, 1- to 6-h fed); 18S RNA, loading control (B). Quantified Northern blot data for two independent cohorts of worms. Black diamonds/solid line, *rncs-1* RNA; white squares/dashed line, total polyadenylated RNA detected by an oligodT probe; black arrowhead, time of food addition; error bars, SEM. Transcript levels were normalized to 18S RNA and are shown relative to unstarved 0-h sample. The possibility that the increase in *rncs-1* levels compared with RNA was caused by reduction of total RNA during starvation was excluded by measuring total RNA yield per worm in a starvation time course (Fig. S1). (C) Comparison of *rncs-1* levels in well-fed L3 larvae (L3), dauer larvae isolated from starved and overcrowded liquid culture (daus), and dauer larvae grown with food on dauer pheromone-rich plates (dauph). (D) Northern blot of GFP mRNA and *rncs-1* in RNA from wild-type worms carrying the *P*rncs-1::GFP transgene that were subjected to a starvation/feeding time course; 18S RNA, loading control.
Although these experiments do not rule out a component of regulation at the level of RNA stability, they indicate that upstream regulatory and promoter sequences likely contribute to regulation of \textit{rncs-1} in response to food supply. In fact, the upstream regulatory sequence of \textit{rncs-1} contains seven motifs with the (A/T)GATA(A/G) consensus recognized by GATA transcription factors, including three extended GATA consensus sites often found in \textit{C. elegans} intestinal genes (Fig. S2A) (11). Indeed, starved animals in which the mRNA coding for the intestinal GATA factor ELT-2 was targeted by RNAi showed a significantly weaker induction of \textit{rncs-1} (∼2.5-fold compared with ∼7-fold) (Fig. S2B and C).

**Terminal Branched Structures Protect rncs-1 RNA from Processing by Dicer.** dsRNA, when introduced into \textit{C. elegans}, is cleaved by the RNase III enzyme Dicer into ∼23-nt small interfering RNAs (siRNAs). The \textit{rncs-1} secondary structure contains an almost perfectly double-stranded helix of ∼300 bp (Fig. 1B), suggesting that \textit{rncs-1} RNA may be a substrate for Dicer. Although \textit{C. elegans} Dicer has not been purified in an active form, Dicer activity can be assayed by incubating \textsuperscript{32}P-labeled dsRNA in embryo extracts (12). We used this assay to test whether siRNAs are generated from \textit{rncs-1} RNA in \textit{vitro} (Fig. 3). When \textsuperscript{32}P-labeled \textit{rncs-1} RNA was incubated in extract, no small RNAs were observed (Fig. 3B, lane 1); the absence of detectable siRNAs persisted over multiple reaction times and RNA and extract concentrations (Fig. S3A; data not shown).

Human Dicer cleaves dsRNA more efficiently from its termini (13), and we speculated that the branched structures flanking the central RNA helix of \textit{rncs-1} hindered processing. We synthesized \textit{rncs-1} derivatives lacking one or both terminal structures (Fig. 3A, substrates 2–4) and a substrate consisting of the \textit{rncs-1} helix with all mismatches repaired, which served as a positive control (Fig. 3A, substrate 5). Incubation of \textit{rncs-1} derivatives in extract produced siRNAs when at least one helix terminus was blunt-ended (Fig. 3B, lanes 2–5). This indicated that the terminal branched structures of \textit{rncs-1} protect it from cleavage by Dicer \textit{in vitro}; because both termini provided protection, yet different sequences, protection was not caused by a specific sequence. Northern blot analyses of RNA isolated from adult \textit{C. elegans} showed similar levels of \textit{rncs-1} in wild-type, \textit{dcr-1(ok247)}, and \textit{rde-4(ne299)} animals (Fig. S3B). These mutant strains are incapable of siRNA production (14, 15), and thus, consistent with our \textit{in vitro} studies, under these conditions \textit{rncs-1} is not a substrate for Dicer \textit{in vivo}.

**Analysis of rncs-1 Deletion and Overexpressing Lines.** In hopes of understanding the \textit{in vivo} role of \textit{rncs-1} we analyzed a deletion strain \textit{rncs-1(tm1632)} (Fig. 1B), and transgenic lines overexpressing \textit{rncs-1} in a \textit{rncs-1(tm1632)} or wild-type background. All animals appeared healthy. Because \textit{rncs-1} is induced during starvation, we assayed survival during starvation and dauer formation (data not shown), \textit{rncs-1(tm1632)} and overexpressing lines were indistinguishable from wild-type animals in these assays.

We noticed that overexpressing lines had an increased frequency of males among hermaphroditic self-progeny. To quantify this defect, for each strain tested, 500–1,000 L4 hermaphrodites were transferred to a separate plate and grown to day 1 of adulthood. Progeny were isolated by hypochlorite treatment and synchrony was assayed during starvation and dauer formation (data not shown), \textit{rncs-1(tm1632)} and overexpressing lines were indistinguishable from wild-type animals in these assays. Occurrence of males (XO genotype) in offspring of \textit{rncs-1} overexpressing lines to test this idea. We first searched for Dicer-regulated genes with altered expression in \textit{rncs-1(tm1632)}
animals. Our laboratory recently used microarray analyses to identify genes whose mRNA levels are Dicer-dependent (18). We chose 18 genes from these datasets with a predicted or confirmed expression pattern similar to that of rncs-1 and used quantitative RT-PCR (qRT-PCR) to compare their expression in wild-type and rncs-1(tm1632) animals. If rncs-1 antagonizes Dicer activity in vivo, genes normally silenced by Dicer would show a further decrease in expression in an rncs-1-deficient background, and we identified five genes with this property (Fig. 4A). Reduction in mRNA levels varied from \(\sim 50\%\) (F53A9.2), to \(\sim 10\%\) (T07C5.1). Dicer-dependent silencing of F35D11.3 was not further enhanced in the rncs-1(tm1632) mutant, and this gene was included as a negative control.

There were many possible explanations for the decreased expression of Dicer-regulated genes in rncs-1(tm1632) animals. However, if rncs-1 was truly competing with endogenous dsRNA (i.e., precursors of siRNA or microRNA [miRNA]) for binding to Dicer, animals overexpressing rncs-1 should show increased expression of Dicer-regulated genes. Indeed, genes with reduced mRNA levels in the rncs-1(tm1632) mutant had increased mRNA levels when rncs-1 was overexpressed (+, Fig. 4B).

### Table 1. Frequency of spontaneous male offspring

<table>
<thead>
<tr>
<th>Strain*</th>
<th>H/M</th>
<th>% Males</th>
<th>P value, (\chi^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>11,802/5</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td>N2 + rncs-1 + GFP</td>
<td>6,846/43</td>
<td>0.624</td>
<td>(&lt;0.0001^*)</td>
</tr>
<tr>
<td>N2 + GFP</td>
<td>8,312/11</td>
<td>0.132</td>
<td>(&lt;0.0001^*)</td>
</tr>
<tr>
<td>rncs-1(tm1632)</td>
<td>9,654/4</td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td>rncs-1(tm1632) + rncs-1 + GFP</td>
<td>9,646/33</td>
<td>0.341</td>
<td>(&lt;0.0001^*)</td>
</tr>
<tr>
<td>rncs-1(tm1632) + GFP</td>
<td>4,370/5</td>
<td>0.114</td>
<td>0.017, 0.114^#</td>
</tr>
<tr>
<td>rncs-1(tm1632) + rncs-1 + RFP</td>
<td>3,101/6</td>
<td>0.193</td>
<td>0.009^#</td>
</tr>
</tbody>
</table>

*GFP under rncs-1 promoter, RFP under myo-2 promoter.
†Compared with N2.
‡Compared with N2 + rncs-1 + GFP.
§Compared with rncs-1(tm1632).
¶Compared with N2 + rncs-1 + RFP.
\(\sim\) Compared with rncs-1(tm1632) + rncs-1 + RFP.

qRT-PCR analyses verified that Dicer mRNA levels were unchanged in these strains (data not shown).

For each gene, we also quantified mRNA levels in dcr-1(ok247) mutant animals. We then compared how much wild-type mRNA levels were altered in dcr-1(ok247) animals with how much they were altered in rncs-1(tm1632) and overexpressing strains (Fig. 4C). For the five Dicer-regulated genes we observed a broad range in regard to how much the wild-type expression level was altered in dcr-1(ok247) knockout animals (circles), in rncs-1(tm1632) knockout animals (squares), and in rncs-1-overexpressing lines (triangles). Interestingly, the magnitude of response for a given gene in one strain correlated with its response in other strains (Fig. 4C). For example, in each strain, F35A9.2 showed the greatest change, C30F12.6 showed an intermediate response, and T07C5.1 was least affected. The characteristic way in which each gene responded in the various strains suggested that the same property of the gene was being interrogated in each case. It seems likely that this property is the relative amount of mRNA associated with each gene, compared with the dsRNA involved in its silencing (see Discussion).

F53A9.2 expression was most sensitive to changes in Dicer and rncs-1 (Fig. 4C), and for this gene, but not others, mRNA levels were also increased by the \(P_{\text{rncs-1}:GFP}\) transgene. We found that this transgene, as often observed in repetitive, transgenic arrays (19), produces both sense and antisense transcripts (Fig. S4). Analysis of additional transgenic strains showed a correlation with F53A9.2 expression and the amount of dsRNA synthesized by the transgenic array and verified that the effect of dsRNA on F53A9.2 was independent of sequence (Fig. S5).

Northern blot analyses revealed a 16- to 19-fold increase in rncs-1 in overexpressing lines compared with wild type (Fig. S4), the same order of magnitude as the 6-fold increase in rncs-1 that occurs during starvation (Fig. 2B). Further, from qRT-PCR assays of mRNA from the Dicer-regulated genes we estimate rncs-1 is 14- to 125-fold more abundant in unstarved animals (SI Materials and Methods). If dsRNA involved in silencing these genes was equimolar to the mRNA, rncs-1 would be present at an excess similar to that which showed competition in vitro (Fig. 3D). However, in repeated assays, we did not observe a simple up-regulation of Dicer-regulated genes under starved conditions. Instead, expression of rncs-1-sensitive genes showed a large degree of fluctuation during starved conditions.
Starvation initiates many events, in addition to up-regulation of rncs-1, that presumably are responding to subtle differences between experiments, despite our careful attempts to maintain constancy.

siRNAs Are Reduced by rncs-1 Overexpression. If the change in expression of Dicer-regulated genes in response to changes in rncs-1 levels was caused by inhibition of Dicer by rncs-1, we should also see changes in levels of small RNAs that were Dicer products. Indeed, using sense-oriented DNA oligonucleotide probes (to detect antisense RNA), we detected small RNAs complementary to F53A9.2 and C30F12.6 (black arrowheads, Fig. 5 A and B). dcr-1(ok247) animals are sterile, and because homozgyous animals are isolated from F1 progeny of heterozygous mothers, they contain some maternal Dicer. Thus, as expected, small RNAs were reduced but not eliminated in this strain (Fig. 5 A). Previous assays of miRNAs in dcr-1(ok247) show that reduction in the ~23-nt miRNA correlates with an accumulation of the pre-miRNA (20). C30F12.6 reduction of small RNAs in dcr-1(ok247) coincided with appearance of a ~65-nt band reminiscent of a pre-miRNA (white arrowheads, Fig. 5 A and B). Although future studies will be necessary to delineate the role of Dicer in regulating the C30F12.6 and F53A9.2 genes, possibly the former is regulated by a miRNA and the latter an siRNA.

Consistent with our competition model, F53A9.2 and C30F12.6 small RNAs were reduced in strains overexpressing rncs-1 (Fig. 5 B and C). Reduction in small RNAs correlated with the increase in mRNA (Fig. 4 B and Fig. S6 A and B). Previous studies suggest that small RNAs act catalytically, and this finding agrees with our data. In the N2 (+) strain, F53A9.2 mRNA levels increased ~12-fold in response to a 2-fold decrease in small RNA levels (Figs. 4 B and 5 C; Fig. S6 A). Thus, it is not surprising that small RNAs of C30F12.6 changed only slightly upon rncs-1 overexpression (~20%), Fig. 5 B and C) because a very moderate up-regulation of its mRNA occurred (1.5- to 3.5-fold; Fig. 4 B). We did not detect changes in small RNAs in the rncs-1(tm1632) mutant (data not shown), likely because of limited sensitivity of the Northern blot analysis.

Discussion

rncs-1 is a ncRNA whose double-stranded structure is unprecedented among characterized ncRNAs. Changes in rncs-1 expression lead to changes in expression of several Dicer-regulated genes, suggesting that rncs-1 binds to Dicer or accessory dsRBPs to compete with endogenous dsRNA involved in silencing. Although rncs-1 now provides an example of a cellular RNA that modulates activity of a dsRBP, viruses have long been known to employ such strategies (21-23).

What Is Important for Modulating Expression of Dicer-Regulated Genes? Both C. elegans and Homo sapiens encode only a single Dicer, but the enzyme is responsible for regulating expression of numerous genes. How Dicer coordinates precise expression of many genes is entirely unexplored, and our data provide clues in this regard. The Dicer-regulated genes we analyzed showed a broad range in sensitivity to changes in rncs-1 expression or the deletion of the dcr-1 gene [dcr-1(ok247); Fig. 4]. Yet, the magnitude of response observed for a given gene under one condition correlated with the magnitude of its response in other conditions (Fig. 4 C). Genes showing a large change in expression in dcr-1(ok247) animals showed large changes when the levels of rncs-1 were altered. Our data suggest that Dicer is limiting compared with its dsRNA substrates (siRNA or miRNA precursors), and for a given gene, the amount of dsRNA substrate relative to the cognate mRNA differs. High mRNA levels that correlate with equally high levels of dsRNA would be very sensitive to the loss of DCR-1 but relatively insensitive to rncs-1 levels because it would be hard to compete with the high levels of dsRNA substrate. However, high mRNA levels associated with much less than equimolar dsRNA would show only a small increase in dcr-1(ok247) animals, but the low level of silencing would be very sensitive to rncs-1 levels. A gene that gives rise to low levels of mRNA but equimolar amounts of dsRNA would be very sensitive to the loss of Dicer, and sensitive to changes in rncs-1. This latter scenario correlates with the F53A9.2 gene we analyzed, and in fact, this gene is associated with very low levels of mRNA (SI Materials and Methods). These considerations may explain why only a subset of the Dicer-regulated genes we assayed were affected by rncs-1 levels and why processing of the abundant lin-4 mRNA is not altered by rncs-1 overexpression (Fig. S6 C).

What Is the Biologic Role of rncs-1? We observed a decrease in mRNA levels of several Dicer-regulated genes in the rncs-1(tm1632) mutant compared with wild type. Thus, rncs-1 modulates expression of these genes in vivo. However, as yet, we have not observed a physiological phenotype in rncs-1(tm1632) animals that correlates with this molecular phenotype. We did, however, observe an increased frequency of males in rncs-1-overexpressing lines. The low numbers of progeny in starved animals precluded our efforts to determine whether increased levels of rncs-1 during starvation also leads to increased male progeny. However, initiation of genetic exchange by switching from asexual to sexual reproduction is a common strategy for adaptation to challenging environments (24), and it remains possible that rncs-1 is involved in such a strategy. In this regard, rncs-1 may reiterate a theme of ncRNAs, many of which relate to stress (3).

rncs-1 functions are mediated by its structure rather than its sequence, so it is not surprising that sequence searches have not revealed homologs. Here, rncs-1 is similar to other long ncRNAs, including essential transcripts like mammalian Xist, that for
unknown reasons are poorly conserved in sequence, even in closely related species (25). Although the sequence of rncs-1 is unique and not repeat-derived, inverted DNA repeats with extensive base-paired stems are recombinogenic and cause genome instability (26). This could explain the absence of an identifiable rncs-1 homolog in Caenorhabditis briggsae and Caenorhabditis remanei, species closely related to C. elegans. Indeed, in C. briggsae and C. remanei the region of the X chromosome syntenic with the rncs-1 neighborhood underwent an inversion, with a putative breakpoint close to rncs-1. The fact that C. elegans has retained an unstable element in its genome hints at a selective advantage provided by rncs-1.

Materials and Methods

Strain designation, cloning of rncs-1, and construction of transcription templates are described in Si Materials and Methods.

Strains and Culture. C. elegans strains were grown under standard conditions (27): rncs-1(tm1632) was backcrossed six times to generate BBS15 (Si Materials and Methods). Arrested L1 were harvested after hatching embryos in M9 for 16–24 h. Males were picked to ~99% purity from progeny of male-fertilized hermaphrodites.

For starvation time courses, L4 from well fed liquid culture (Fig. 2 A) or plates (Fig. 2 D) were starved in M9. Dauer larvae were harvested from liquid culture grown to high density/food exhaustion, by treatment with 1% SDS (28). Phenomenic plates were seeded with Escherichia coli OP50 and contained sufficient partially purified daumone to induce ~100% dauer formation in N2 at 20°C. Adults placed on each plate laid eggs for 8 h before removal. After 3 days, progeny that entered dauer were harvested.

Transgenics. For the Ptm1632::GFP reporter, ~2,000 bp of upstream sequences of genomic rncs-1 (Fig. 1 A) were introduced into the multiple cloning site of pPD49.26. Plasmid was injected into N2 worms. Lines overexpressing rncs-1 were produced by injecting a tm1632::GFP reporter to generate BB17 or with prf4 into N2 to generate BB18 (Figs. 4 and 5). Excess nonspecific DNA (1-kb ladder; Invitrogen) was cojected.

Northern Blot Analysis and RT-PCR. For nonintegrated lines, ~99% pure transgenic populations were handpicked. For integrated lines, transgenic populations were handpicked.

In Vitro Transcription. Internally 32P-labeled RNA was transcribed with SuperScript II reverse transcriptase and oligo(dT) primers (Invitrogen). Samples were treated with RNase H (New England Biolabs) and diluted 4-fold with ddH2O. Five microliters of cDNA was used per PCR in a LightCycler 2.0 instrument with the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche). Primer pairs spanned at least one exon–exon junction and produced products of 150–300 bp.

In Vitro Dicer Activity Assay. Extract preparation and Dicer activity assays were as described in ref. 17, but for competition assays glycerol was 20%, and extract was cleared by centrifugation (100,000 × g; 60 min). Dicer assays (20 μl) contained 30 μg of total protein and 10 nM labeled RNA. Reactions with rncs-1 as inhibitor were preincubated (5 min; 20°C) with unlabeled rncs-1 before adding labeled 300-bp unc-22 dsRNA.

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Supporting Information

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SI Materials and Methods

Strains. The following strains were used in our studies: N2, BB15 rncs-1(tm1632) X, BB16 uuEx6 [pPrncs-1::GFP], BB17 rncs-1(tm1632) uuEx7 [rncs-1(+) pPrncs-1::GFP], BB32 uuEx7 [rncs-1(+) pPrncs-1::GFP], BB18 uuEx8 [rncs-1(+) pRF4], BB1 dcr-1(ok2477); unc-32(e189III) (1), BB13 rde-4(ne299) III; BB65 rncs-1(tm1632) uuEx12 [rncs-1(+) pPhyto-3:RFP].

Cloning of rncs-1. rncs-1 was amplified from N2 genomic DNA by a PCR (primers SH21/SH22, Table S1) and cloned into pGEM3zf(+). To eliminate the intron, exons 1 and 2 were amplified individually from the genomic clone. Exon 1 primers SH45 and SH46 (Table S1) linked an EcoRI site to the 5’ end and the most 5’ nucleotides of exon 2, including an XmnI site, to the 3′ end of the product. Exon 2 was amplified with primers SH47 and SH48 (Table S1), adding a 3′ HindIII site. Exon 1 product was digested with EcoRI and XmnI, exon 2 product with XmnI and HindIII. The rncs-1 cDNA clone pGEM52G was made by three-way ligation into the EcoRI and HindIII sites of pGEM3zf(+).

Transcription Templates. rncs-1 was transcribed from HindIII-linearized pGEM52G using T7 RNA polymerase. Templates for rncs-1 (derivatives) were generated by a PCR of cDNA segments with T7 promoter sequences linked to 5′ primers: nt 1–342 (derivative 2, 5′ strand), nt 438–800 (derivative 2, 3′ strand), nt 71–393 (derivative 3, 5′ strand), nt 394–709 (derivative 3, 3′ strand), nt 71–342 (derivative 4, 5′ strand), nt 438–709 (derivative 4, 3′ strand). Template for the 3′ strand of derivative 5 was made by amplifying nt 71–342 with a T7 promoter on the 3′ primer. Derivative 4 is an intermolecular duplex; primers were modified to replace the tetraloop (nt 392–395) with two GC base pairs. Templates for the 300-bp uEx6 5′ dsRNA were made by a PCR of part of exon 6 with T7 promoter sites linked to the 5′ primer for sense or the 3′ primer for antisense.


Calculation of Relative RNA Abundance. To determine the approximate relative abundance of rncs-1 and mRNAs targeted by Dicer, RNA levels were quantified from Northern blotting (rncs-1) and qRT-PCR (F53A9.2, B0222.3, C30F12.6) by using the act-1 mRNA as a reference point. qRT-PCR data could not be reliably obtained for rncs-1 because of its highly double-stranded secondary structure.

Relative act-1 and rncs-1 levels were determined from Northern blotting (Fig. S4A), by using PhosphorImager analyses with ImageQuant software (Molecular Dynamics) taking Northern blot probe length (i.e., number of labeled nt) and exposure time into account. We found that act-1 mRNA is 100- to 200-fold more abundant than rncs-1 in unstarved N2 adults.

Relative act-1 and Dicer target mRNA levels were estimated from crossing point data obtained during qRT-PCR. Although this method assumes nearly equal efficiency of the qRT-PCR primer pairs, this is a reasonable assumption as efficiency coefficients for all primers pairs ranged from 1.9 to 2. Average crossing points were as follows: act-1, 8.9; F53A9.2, 23.6; B0222.3, 21.5; C30F12.6: 21.3.

By using a standard curve of crossing point plotted against the log of mRNA concentration, the following relative abundance was determined: act-1, 1; F53A9.2, 1/12,500; B0222.3, 1/3,300; C30F12.6, 1/2,800.

By using act-1 mRNA as a reference point, the estimated relative abundance of rncs-1 compared with Dicer target mRNAs in unstarved N2 calculates as: rncs-1, 1; F53A9.2, 1/63–1/125; B0222.3, 1/16–1/33; C30F12.6, 1/14–1/28.

Note that these estimates only reflect the relative abundance of RNAs on a whole-worm basis. Further, this is not a true estimate of the relative abundance of rncs-1 to the dsRNA involved in silencing the mRNA because the nature of this dsRNA is unknown. If the dsRNA arose from antisense transcription, the amount of Dicer target mRNA (sense strand) would define the maximum dsRNA possible.

Fig. S1. Total RNA yield per worm during starvation. From a starved culture of L4 larvae an equal number of worms was removed at each of the time points indicated. Total RNA was extracted (see Materials and Methods), and RNA yield was measured by spectrophotometry. RNA yield relative to unstarved control was calculated [(RNA per starved worm)/(RNA per unstarved worm)] - 100%. The average of four experiments is plotted; error bars, SEM. The data indicate that the observed increase in rncs-1 levels relative to rRNA upon starvation (Fig. 2 A and B) cannot be accounted for by an overall loss of total RNA, which is largely rRNA. For example, after 3 h of starvation, when an ~5-fold increase of rncs-1 is observed, total RNA per worm levels remain at ~90% of unstarved control (compare with Fig. 2B).
Fig. S2. rncs-1 induction during starvation depends on the GATA transcription factor ELT-2. (A) Diagram of nucleotides 1009000 to 1014000 on the X chromosome, including the 847-nt rncs-1 gene and part of its immediate neighbors. Gray boxes, coding exons; white boxes and arrows, noncoding exons; connecting lines, introns. Diamonds represent locations of the (A/T)GATA(A/G) motifs recognized by GATA-type transcription factors, with white diamonds indicating the extended TGATAAGA consensus frequently found in Caenorhabditis elegans intestinal genes. (B) Northern blot of rncs-1 in young adult wild-type worms fed with Escherichia coli expressing dsRNA against GFP (dsGFP) or elt-2 (dsELT-2). f, worms harvested well fed; st, worms harvested after 3 h of starvation; blot was reprobed for 18S rRNA. (C) Bar height represents mean rncs-1 levels as quantified by Northern blot analyses in three independent experiments (error bars, SEM). To verify successful targeting by RNAi, elt-2 mRNA levels were quantified by real-time quantitative RT-PCR (tabulation below graph; numbers indicate mean mRNA levels relative to the dsGFP fed control). The results indicate that ELT-2 is involved in starvation up-regulation of rncs-1, although at present we do not know whether this involves direct binding of ELT-2 to promoter elements. Starvation induction of rncs-1 was unchanged in deletion strains lacking ELT-4 or ELT-7, two additional GATA transcription factors in the C. elegans intestine (data not shown).
Resistance of rncs-1 to cleavage by Dicer. (A) Eight hundred-base pair CAT duplex dsRNA (cat d) and full-length rncs-1 RNA were transcribed in vitro and incubated in wild-type C. elegans embryo extract for the times indicated. CAT duplex RNA is cleaved into siRNAs, whereas rncs-1 is resistant to cleavage. Note that the highly stable intramolecular duplex of rncs-1 (~590 kcal/mol, as calculated by mfold) prevents full denaturation of this RNA in formamide/urea. This results in the migration of full-length rncs-1 as two separate bands (asterisks), the upper of which represents nondenatured rncs-1 whose migration is retarded by the branched terminal structures. (B) Northern blot of rncs-1 in RNA samples from young adult wild-type (N2), dcr-1(ok247), and rde-4(ne299) worms. Numbers below lanes indicate relative average (rel. ave.) and SEM, calculated by Northern blot analyses of three independent samples of each strain. (C) Gel shift assay of rncs-1 (50 pM) with recombinant RDE-4 protein. As little as 50 nM RDE-4 protein is required for complete loss of free rncs-1. (D) Inhibition of Dicer activity by rncs-1 derivatives. Full-length rncs-1, single-stranded RNA of rncs-1 sequence (ssRNA, nt 71–393), and derivative substrate 4 were used as competitor RNA in a competition experiment (see Fig. 3 C and D and Materials and Methods). Substrate 5 was used as the labeled substrate. Full-length rncs-1 and substrate 4, but not ssRNA, inhibit Dicer cleavage, as indicated by the disappearance of siRNA signal with increasing competitor RNA concentration. This result demonstrates that the ability to inhibit Dicer activity in vitro depends on the presence of dsRNA but is independent of the terminal structure of the duplex.
Fig. S4. Characterization of RNA levels in transgenic lines. (A) Northern blot of six samples each of unstarved N2, the rncs-1(tm1632) rescue line carrying Prcns-1::rncs-1, and wild-type worms carrying Prcns-1::rncs-1. Average rncs-1 levels in the overexpressing lines relative to the N2 control were quantified from this blot and are shown below the autoradiogram (±SEM). (B) rncs-1 levels in well fed wild-type (N2), rncs-1(tm1632) mutants (tm1632), a rncs-1 rescue line carrying Prcns-1::rncs-1 and Prncs-1::GFP [tm1632/H11001] as well as in wild-type worms carrying Prncs-1::GFP [N2(gfp)] were analyzed by Northern blotting. The tm1632 deletion strain produces no detectable rncs-1, whereas rncs-1 levels are increased over wild-type levels in the tm1632(+1) rescue line. The N2(gfp) strain, which introduces copies of GFP driven by the rncs-1 promoter, shows wild-type expression of the endogenous rncs-1 locus. Representative results from Northern blot analyses of several experiments (n = 2–6, depending on strain) are shown. (C) Possible scenario for dsRNA generation from transgenic arrays and RT-PCR strategy for detection of antisense RNA (asRNA). As shown, arrays containing copies of promoter::GFP transgenes in tandem and opposing orientation give rise to stretches of asRNA (orange) by read-through transcription. Hybridization of antisense transcripts to sense RNA (green) produces dsRNA. asGFP RNA can be detected by RT-PCR with an asGFP-specific RT primer followed by PCR amplification of DNA. (D) Detection of asGFP transcripts in wild-type worms carrying the Prcns-1::GFP transgene. We previously reported the occurrence of asGFP and dsGFP in worms carrying a sur-5::GFP, Phsp16–2::GFP (IR) transgene (strain 3hsp) (2). Using the illustrated RT-PCR approach (antisense primer, AAAGATCCCAACGAAAAGAGAGACCACAT; sense primer, GTATAGTTCATCCATGCCATGTGTAATCCC; see Materials and Methods for RT conditions), we detected asGFP sequence in the 3hsp strain, the 514d strain (in which the 3hsp array was integrated into the genome), and in wild-type worms carrying the Prcns-1::GFP transgene [N2(gfp)]; wild-type worms lacking a transgenic array (N2) showed no antisense-specific RT-PCR product. (E) Starvation-induced increase of read-through GFP asRNA in worms carrying the Prcns-1::GFP transgene. RNA isolated in the starvation time course experiment shown in Fig. 2D was analyzed by GFP antisense-specific RT-PCR. Samples were removed for gel electrophoresis after the indicated number of PCR cycles within the linear amplification range. Detectable antisense RNA levels increase upon prolonged starvation and fall to unstarved levels after 6 h of refeeding. This behavior parallels that of rncs-1 RNA, consistent with the idea that the asRNA is a product of read-through transcription from the rncs-1 promoter within the transgenic array.
Fig. S5. Increase in F53A9.2 mRNA levels in response to transgenic arrays that produce sense and antisense transcripts. (A) mRNA levels of F53A9.2 were analyzed by real-time quantitative PCR (see Materials and Methods) in various strains grown at 20°C (strains defined in legend for Fig. S4D). We observed up-regulation of F53A9.2 in 514d, hsp3, and N2(gfp) strains, but not wild-type animals lacking a transgene (N2). This result is consistent with our hypothesis that the F53A9.2 mRNA increase observed upon expression of the Prncs-1::GFP transgene is caused by low levels of GFP dsRNA produced from the transgene. The average of two independent experiments is shown relative to wild type (N2); error bars, SEM. (B) Overexpression of F53A9.2 in response to heat shock-induced expression of hairpin GFP. The 514d strain, carrying a sur-5::GFP, Phsp16–2::GFP(IR) transgene, overexpresses a long-GFP inverted repeat hairpin when subjected to heatshock (2). We tested whether overexpression of GFP hairpin dsRNA, like overexpression of rncs-1, results in increase of F53A9.2 expression. The average of two independent experiments is shown; error bars, SEM. Under heat shock conditions (white bars), 514d samples show greatly increased levels of F53A9.2 mRNA compared with samples grown at 20°C (gray bars). In comparison, a lesser increase was observed in wild-type worms upon heat shock compared with normal growth temperature. We thank Jeff Habig and Joe Aruscavage for heatshock samples.
Fig. S6. Correlation of decrease in siRNA and increase in mRNA levels in response to rncs-1 overexpression. (A) Juxtaposition of the increase in F53A9.2 mRNA (Left) and the decrease in F53A9.2 siRNA (Right) observed in dcr-1 mutant and rncs-1-overexpressing lines. The data are plotted relative to wild-type RNA levels (N2 – 1) and are reiterations of data presented in Figs. 4 and 5. (B) The average magnitude of increase in mRNA was plotted against the average magnitude of decrease in siRNA observed for different strains [N2, dcr-1(ok247), tm1632(+), and N2(+)]. Black diamonds represent data points for F53A9.2 (see A), and white diamonds are data points for C30F12.6 (compare Figs. 4 and 5). A linear trend is observed with greater decreases in siRNAs corresponding to greater increases in mRNA. (C) Northern analyses of lin-4 miRNA and pre-miRNA (pre) in N2-, dcr-1-, and rncs-1-overexpressing lines from 1 μg (lanes 1 and 2) or 3 μg (lanes 3–5) size-selected RNA. lin-4 miRNA processing is not affected upon overexpression of rncs-1.
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