RACK-1 regulates let-7 microRNA expression and terminal cell differentiation in Caenorhabditis elegans

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Introduction

microRNAs (miRNAs) are approximately 22-nt long, small non-coding RNAs that regulate gene expression at the post-transcriptional level. Most animal miRNA genes are transcribed by RNA polymerase II to generate the primary miRNA transcript (pri-miRNAs) and are subsequently processed by the nuclear RNase III Drosha and its partner DGCR8/Pasha. The processed products, ~70-nt long hairpin precursor miRNAs (pre-miRNAs) and are subsequently processed by the nuclear RNase III Drosha and its partner DGCR8/Pasha. The processed products, ~70-nt long hairpin precursor miRNAs (pre-miRNAs), are transported to the cytoplasm and cleaved by the cytoplasmic RNase III Dicer to produce short mature miRNAs. Mature miRNAs are assembled into miRNA-induced silencing complexes (miRISCs) with the core Argonautes and multiple cofactors. miRISCs bind to miRNA target sites with imperfectly complementary sequences typically in the 3′ untranslated region (3′ UTR) and trigger translational repression and/or mRNA degradation (for a review, see refs. 1–4).

miRNAs were initially discovered in C. elegans. The founding miRNAs lin-4 and let-7 were identified as heterochronic genes that regulate stage-specific developmental events such as cell proliferation and differentiation.5–10 In the early larval stages, the lin-4 miRNA represses LIN-14 and LIN-28 to program the L1-to-L2 and L2-to-L3 transitions, respectively.5–8,11 The progression from L2 to L3 is also controlled by the let-7 family miRNAs, miR-48, miR-84, and miR-241, that repress HBL-1.12–15 In the L3 and L4 stages, the let-7 miRNA and its paralogs (miR-48, miR-84, and miR-241) repress HBL-1 and LIN-41, leading to activation of LIN-29 expression.9,16 The adult transcription factor LIN-29 promotes the L4-to-adult switch (the L/A switch),17,18 in which the lateral hypodermal seam cells exit the cell cycle and terminally differentiate (depicted in Fig. 1A) (for a review, see refs. 20–25). Gene regulation by the lin-4 and let-7 miRNAs is conserved in vertebrates.26,27 The vertebrate miR-125 (a lin-4 ortholog) and let-7 miRNAs are expressed in the late stages of embryonic development but are largely absent in the early embryos, embryonic stem (ES) cells, and embryonal carcinoma (EC) cells,20,21 mirroring the timing of expression of lin-4 and let-7 during C. elegans larval development. In addition, the miR-125 and let-7 miRNAs regulate LIN28 and TRIM71, the mammalian ortholog of C. elegans LIN-41, via the complementary sites in the 3′ UTR.27,29,30 In humans, more than a thousand miRNAs have been found and predicted to regulate greater than...
60% of protein-encoding genes. miRNA dysfunction is often associated with developmental defects, diseases, and cancers (for a review, see refs. 22 and 34–38). miR-125 has been shown to be involved in several kinds of leukemia and let-7 in many types of cancers, including those of lung, liver, and breast.39-43

The receptor for activated C kinase 1 (RACK1) is a versatile scaffold protein, belonging to the family of WD40 repeat proteins and sharing significant homology to the β subunit of G-proteins (Gβ).44,45 Although RACK1 was originally identified as a receptor for protein kinase C, it is now known to be physically or functionally associated with numerous other proteins. RACK1 has also been suggested to be a key mediator of various pathways in the cell, since it shuttles proteins to distinct cellular compartments and plays an important role in many biological processes. In addition, many reports have indicated that a change in RACK1 expression is involved in a variety of cancers (for a review, see ref. 46). RACK1 is also a core component of the eukaryotic 40S ribosomal subunit and recently has been shown to interact with Argonaute proteins and miRNAs.47-53 Jannot et al. have proposed that the ribosome-bound RACK1 is required for

Figure 1. For figure legend, see page 1997.
proper miRNA functions in *C. elegans* and humans by playing a crucial role in recruitment of miRISCs to the ribosome. In their study, reduction of RACK-1 in *C. elegans* by RNAi increased the levels of *lin-4* and *let-7* miRNAs, implying that RACK-1 is not required for loading and stabilization of miRNAs. Although the miRNA levels were increased, the downregulation of targets was impaired. The accumulation of miRNAs was proposed to result from a slower turnover of the miRISCs that are unable to reach mRNA targets due to the lack of RACK-1. In a study using the Huh7 human hepatoma cell line, Otsuka et al. observed that knockdown of RACK1 did not change the levels of endogenous mature miR-122, miR-22, miR-140, and miR-185, but instead reduced the amount of miRNAs in Ago2-containing complexes. They have proposed that RACK1 functions after miRNA maturation and is required to load mature miRNAs into miRISCs. Given these contradictory findings, the distinct functions of RACK1 in the miRNA pathway remain obscure (for a review, see ref. 56).

Here we show evidence that RACK-1 plays a predominantly negative regulatory role in the let-7 miRNA pathway in *C. elegans*. We found that reduction of RACK-1 in *C. elegans* increased the *let-7* miRNA and, more importantly, that it significantly suppressed the retarded terminal differentiation of seam cells in animals carrying the hypomorphic *let*-7(n2853) allele or lacking the *let-7* family miRNA genes *mir-48* and *mir-241*. The increase in mature *let-7* may result from an increase in the levels of pre-*let-7*, which acts upstream of Dicer-mediated pre-miRNA processing. Interestingly, we found no increase in the transcription of *let-7* or the levels of primary *let-7*. Moreover, reduction of RACK-1 increased several, but not all, precursor miRNAs tested in this study. Our results suggest that, in addition to the functions previously proposed in distinct steps of the miRNA pathway, RACK1 may negatively impact the biogenesis of a subset of miRNAs at the post-transcriptional level.

**Results**

**RNAi-mediated knockdown of rack-1 suppresses the lethality of the hypomorphic let-7(n2853) allele but not the null let-7(mn112) allele**

We previously identified the *C. elegans* RACK-1 as an Argonaute ALG-1-associated protein by mass spectrometry, and the RACK-1-ALG-1 interaction has also been demonstrated by Jannot et al. 51,57 ALG-1 is one of the 2 Argonaute proteins associated with the miRNA pathway in *C. elegans*. To determine whether RACK-1 may play a role in miRNA function, we investigated the effect of *rack-1* knockdown on *let-7*-related heterochronic phenotypes. First, we knocked down *rack-1* expression in *let-7*(n2853) or *let-7*(mn112) mutant animals and scored the suppression of lethality, which has previously been used to identify *let-7* functional partners and/or downstream targets.59,61 The hypomorphic *let-7*(n2853) temperature-sensitive allele carries a G-to-A substitution in the *let-7* “seed region” that may reduce target silencing and lead to a decrease in the levels of mature *let-7* miRNA. The *let-7*(mn112) allele harbors a deletion in the *let-7* locus that completely prevents the production of the mature *let-7* miRNA. Let-7 loss of function results in heterochronic phenotypes such as retarded hypodermal cell differentiation and vulval abnormalities, and the mutant animals die by bursting through the vulva at the L/A switch.59,10 Knocking down negative functional partners of *let-7* has been shown to suppress the lethality of the *let-7*(n2853) allele by elevating the levels of the partially functional miRNA or modulating target silencing.60,62 Knocking down *let-7* downstream targets usually suppresses both the *let-7*(n2853) and *let-7*(mn112) alleles.59 In our hands, knocking down the 2 well-studied *let-7* targets bhl-1 or *daf-12* efficiently suppressed the lethality of both alleles.

**Figure 1** (See opposite page). RNAi-mediated knockdown of *rack-1* suppresses the retarded terminal differentiation of seam cells in *let-7*(n2853) animals via the heterochronic gene pathway. (A) Upper panel: schematic depiction of the *C. elegans* heterochronic gene pathway, which temporally regulates the developmental fates of seam cells. For simplicity, heterochronic genes *lin-4*, *lin-14*, and *lin-28* are not depicted. The 3 *let-7* family members *mir-48*, *mir-84*, and *mir-241* repress the transcription factor *HBL-1* and promote the L2-to-L3 transition. *let-7* represses *HBL-1* and *LIN-41* in the late larval stage (hashed line). Reduction in *HBL-1* and *LIN-41* levels derepresses *LIN-29* that promotes the L/A switch. ALG-1 and ALG-2 are essential for miRNA-mediated gene regulation (arrows). Lower panel: reciprocal temporal expression of the *let-7* family of miRNAs (in green) and their targets, *HBL-1* and *LIN-41* (in red). The black curve represents the derepression of *LIN-29* at the L/A switch. (B) Lineage of the seam cells V1-V4 and V6 in wild-type, *let-7*(n2853), *lin-29(n333)*, and *mir-48 mir-241(n1251)* animals investigated in this study. In wild-type animals, the seam cells V1-V4 and V6 divide asymmetrically at each larval stage. A symmetrical proliferative division (shown in bold) occurs early in the L2 stage and expands the seam cell number from 10 to 16. At the L/A switch, the seam cells terminally differentiate, exit the cell cycle, fuse with neighboring seam cells and secrete a cuticular collagen structure known as “alae” (depicted by 3 short solid lines). *let-7*(n2853) animals show a retarded phenotype in which the L4 fate of seam cells is reiterated and the terminal differentiation is delayed. In *lin-29(n333)* animals, seam cell lines continue to divide after the L4 molt and maintain larval characteristics. In *mir-48 mir-241(n1251)* animals, seam cell undergo a repetition of L2 fate divisions in the L3 stage, resulting in extra seam cells and delayed terminal differentiation. (C-E) Merged images of fluorescence and Nomarski micrographs from the same focal plane of animals carrying the *wls51[scm:gfp]* transgene, which serves as a nuclear seam cell marker, in the wild-type genetic background, *let-7*(n2853) and *lin-29(n333)*, respectively. The numbers of seam cells are shown in parentheses (mean ± SD). Synchronized L1 animals were placed onto bacteria expressing mock or *rack-1*(RNAi) feeding vectors and seam cell numbers were scored during the stages indicated. *rack-1*(RNAi) suppressed the reiterated divisions of seam cells at the L/A switch in *let-7*(n2853) but not in *lin-29(n333)* mutant animals. yAd, young adult. Scale bars represent 50 μm. Corresponding box-and-whisker plots of seam cell counts are shown on the bottom. n, the numbers of animals assayed. In these and all subsequent box-and-whisker plots, the top and bottom ends of each box represent the 75th and 25th percentiles, respectively. The line in the middle of each box shows the median value and the ends of the whiskers represent the data minimums and maximums. ***p < 0.001 by an unpaired two-tailed Student t test. (F) Rack-1 protein levels in mock RNAi and *rack-1*(RNAi) animals were analyzed by western blot analysis using a rabbit polyclonal antibody against RACK-1. GAPDH protein was used as the loading control.
Interestingly, knockdown of rack-1 showed no suppression of let-7(mn112) but strongly suppressed the hypomorphic let-7(n2853) allele at the non-permissive temperature (Table 1). This result implies that rack-1(RNAi) affects let-7-mediated gene regulation, with RACK-1 acting as a negative functional partner of let-7 but perhaps not as one of the let-7 downstream targets. Since RACK-1 functions pleiotropically, it is possible that rack-1(RNAi) suppresses vulva bursting of let-7(n2853) animals through parallel pathways. However, this possibility is less likely, because rack-1(RNAi) did not suppress let-7(mn112)-mediated lethality.

Depletion of RACK-1 suppresses the retarded terminal differentiation of seam cells in let-7(n2853) mutant animals
To determine whether the suppression of let-7(n2853) lethality by rack-1(RNAi) reflected a role for rack-1 in the heterochronic gene pathway, we examined how rack-1 knockdown affected the differentiation patterns of lateral hypodermal seam cells during the late larval stages and at the L/A switch, which are regulated by let-7 and its downstream targets. The let-7 miRNA accumulates in the L3 and L4 stages, repressing HBL-1 and LIN-41 and indirectly activating the most downstream heterochronic gene lin-29.13 lin-29 encodes an adult-specific transcription

Figure 2. Depletion of RACK-1 suppresses the retarded terminal differentiation of seam cells in mir-48 mir-241(nDf51) animals. mir-48 mir-241(nDf51) animals carrying the wls51[scm:gfp] transgene were monitored for the seam cell proliferation patterns from late L3 to young adulthood following depletion of RACK-1. (A) Mock RNAi; (B) rack-1(RNAi). Feeding RNAi was initiated in L4-stage parental (P) animals to ensure sufficient exposure to RNAi before counting seam cell numbers (mean ± SD in parentheses) from the L3 stage of progeny. mir-48 mir-241(nDf51) animals have ~25 seam cells by the L3/L4 transition, reflecting the repetition of L2-stage proliferation pattern in V1-V4 and V6 seam cells during the L3 stage. In the mock RNAi control, these seam cells underwent further wild-type-like proliferation patterns, and their number temporarily increased to ~47 by the early L4. It then decreased to ~26 in the late L4 stage when one of each pair of daughter cells differentiated and fused with hyp7. At the L/A switch, some but not all V1–V4 and V6 seam cells underwent a supernumerary division, resulting in the presence of ~35 seam cells. Animals under rack-1(RNAi) showed no significant difference in the seam cell proliferation patterns from the control during the L3 and L4 stages but failed to display a reiterated division at the L/A switch. yAd, young adult. Scale bars represent 50 µm. (C) mir-48 mir-241(nDf51) animals that were homologous for the rack-1(ok3676) deletion allele showed seam cell proliferation patterns similar to those seen in rack-1(RNAi) animals. (D) Grouped box-and-whisker plots of seam cell numbers in (A–C), n, the number of animals assayed. ***P < 0.001 by unpaired two-tailed Student’s t test.
factor that promotes adult cell fates (depicted in Fig. 1A).\textsuperscript{7,19,63} In wild-type animals, seam cells proceed with stem cell-like divisions during each larval stage, but at the L/A switch, they exit the cell cycle, fuse with neighboring seam cells, and produce the cuticular collagen structure known as alae (depicted in Fig. 1B).\textsuperscript{44} We scored seam cell nuclei using a nuclear seam cell marker sem::gfp encoded by wls51 as previously described.\textsuperscript{38,65} We found that knockdown of rack-1 in wild-type animals did not change the number of seam cells at the L/A switch (Fig. 1C). In hypomorphic let-7(n2853) animals grown at the non-permissive temperature, LIN-41 is derepressed due to lower let-7 function, leading to attenuated LIN-29 expression.\textsuperscript{9,10} Consequently, a subset of seam cells (V1–V4 and V6) reiterate the L4-stage cell proliferation pattern at the L/A switch, leading to an increase in seam cell number. let-7(n2853) animals also fail to generate complete adult alae at the L/A switch (depicted in Fig. 1B).\textsuperscript{9} Interestingly, rack-1(RNAi) suppressed the reiterated seam cell divisions (average 17 ± 1.7 [± SD] vs. 25 ± 2.9 in the control) (Fig. 1D) and the defective alae formation in let-7(n2853) mutants (Table S1). By contrast, in lin-29(n333)-null mutants, rack-1(RNAi) did not suppress this reiteration after L4 (depicted in Fig. 1B).\textsuperscript{17-19} When rack-1(RNAi) was performed in lin-29(n333) animals, the seam cell number increased from 15 ± 0.9 in the late L4 stage to 26 ± 2.2 after the L4 molt, the same pattern seen in the mock RNAi control (average 15 ± 0.7 in late L4 and 26 ± 2.7 in adults) (Fig. 1E). In addition, rack-1(RNAi) did not suppress the alae defect of lin-29(n333) (Table S1). This indicates that the effect of rack-1(RNAi) on hypodermal cell development may require lin-29. Taken together, the genetic interaction data suggest that depletion of RACK-1 suppresses the retarded phenotypes in hypomorphic let-7(n2853) mutants at the L/A switch, and this effect may take place in the heterochronic gene pathway at a position upstream of lin-29.

To directly verify depletion of RACK-1 by RNAi, we generated a rabbit polyclonal antibody against a 19-mer amino acid sequence of RACK-1. When animals were exposed to rack-1(RNAi) from the L1 stage, almost no RACK-1 was detected in the lysates obtained from animals at the L/A switch (Fig. 1F).

Depletion of RACK-1 suppresses the retarded terminal differentiation of seam cells in mir-48 mir-241(nDf51) double mutants

The let-7 paralogs mir-48, mir-84, and mir-241 contain the same seed region as let-7 and function redundantly with each other.\textsuperscript{14} They begin to be expressed during the L2 stage, repressing HBL-1 and mediating the L2-to-L3 transition (depicted in Fig. 1A).\textsuperscript{12-15} mir-48 mir-241 double- and mir-48 mir-241; mir-84 triple-null mutants display a repetition of L2-specific seam cell proliferation in the L3 stage, including one round of symmetric divisions and one round of asymmetric divisions, that results in an increase of seam cell number in the later stages (depicted in Fig. 1B).\textsuperscript{14} The double or triple mutants fail to generate complete alae at the L4 molt. This alae defect is strongly suppressed by depletion of HBL-1 and/or LIN-41, suggesting that miR-48, miR-84, and miR-241 may also contribute to repression of HBL-1 and LIN-41 in the late larval stages.\textsuperscript{14} We sought to determine whether depletion of RACK-1, which suppresses the let-7(n2853) phenotypes, also suppresses the retarded phenotypes at the L/A switch seen in the let-7 paralog mutants. We detected an increase in the seam cell numbers in mir-48 mir-241(nDf51) double mutants at the late L3 (average 25 ± 1.4) and late L4 (average 26 ± 1.6) stages, vs. the seam cell number ( = 16) seen in wild-type animals, reflecting the repetition of L2-stage proliferation events during the L3 stage (Fig. 2A). At the L/A switch, some but not all V1–V4 and V6 seam cells underwent a supernumerary division, and the seam cell number increased to 35 ± 3.9 on average (Fig. 2A), mirroring the retarded alae phenotype that has been reported previously as well as the results we obtained (Table S1).\textsuperscript{14} Depletion of RACK-1 by RNAi or a rack-1(ok3676)-null allele significantly repressed the supernumerary seam cell division at the L/A switch (Fig. 2B and C, young adult; 25 ± 2.0 seam cell nuclei in rack-1(RNAi), 28 ± 4.1 in rack-1(ok3676); Fig. 2D, box-and-whisker plots). In addition, the defective alae formation in mir-48 mir-241(nDf51) double mutants was significantly suppressed by rack-1(RNAi) (Table S1). With these multiple lines of evidence, we propose that depletion of RACK-1 may enhance let-7-mediated gene regulation, which suppresses the hypomorphic let-7(n2853) allele and also compensates for the developmental defects at the L/A switch in mir-48 mir-241(nDf51) mutants.

Depletion of RACK-1 enhances the precocious heterochronic phenotypes caused by a hunchback-like 1 (hbl-1) loss-of-function mutation

We wanted to further investigate whether removal of RACK-1 not only suppresses retarded heterochronic phenotypes, but also causes precocious seam cell development. In wild-type animals, seam cells exhibit terminal differentiation at the L/A switch and fuse into a syncytium.\textsuperscript{66} Overexpression of let-7, or depletion of let-7 targets such as LIN-41 or HBL-1, have been shown to result in precocious fusion of seam cells.\textsuperscript{9,10,12,13} We monitored seam cell fusion using the ajm-1::gfp reporter of wls79 as previously described.\textsuperscript{66} Although we did not observe significant precocious seam cell fusion in rack-1(RNAi) wild-type animals (data not

| Genotype     | RNAi\textsuperscript{a} | Suppression (% viable animals\textsuperscript{b}) | n |
|--------------|---------------------------|-----------------------------------------------|---|
| let-7(n2853) | Mock                      | 5                                             | 668|
|              | rack-1                    | 87                                            | 779|
| let-7(mn112) | Mock                      | 2                                             | 54 |
|              | rack-1                    | 4                                             | 64 |
|              | daf-12                    | 52                                            | 71 |
|              | hbl-1                     | 64                                            | 78 |

\textsuperscript{a}Synchronized L1 animals were subjected to feeding RNAi at 20 °C. The RNAi vector L4440 was used for mock experiments. \textsuperscript{b}RNAi suppression of the lethality associated with the let-7(n2853) temperature-sensitive and let-7(mn112) null alleles at 20 °C. The percentages of animals escaping vulva bursting after the L4-to-adult molt were scored. daf-12(RNAi) and hbl-1(RNAi) completely suppressed let-7(n2853) lethality. n > 100. For let-7(mn112), SP231 mndp1(XV)/+; unc-3(e151); let-7(mn112) X was used and only Unc animals homologous for the let-7(mn112) allele were scored.

Table 1. Suppression of let-7 mutation lethality

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shown), we found that depletion of RACK-1 enhanced the pre-cocious fusion of seam cells in the hbl-1(mg285) mutant background (Fig. 3A and B).13

In addition to precocious fusion, hbl-1(mg285) seam cells also exhibit premature cell cycle exit.12,13 However, while this phenotype is completely penetrant in lin-41 loss-of-function mutations,9,10 in hbl-1(mg285) animals a portion of seam cell nuclei fail to exit the division cycle permanently at the L3 molt, but instead undergo a further division during the late L4 stage.12,13 We sought to determine whether rack-1(RNAi) affects this process in hbl-1(mg285) mutants. In hbl-1(mg285) animals undergoing mock RNAi, we detected 16 seam cell nuclei at the L3 molt (data not shown) and higher numbers of seam cell nuclei in the late L4 (average 22 ± 2.4) and young adult stages (average 21 ± 1.9) (Fig. 3C and D).12,13 rack-1(RNAi) completely prevented additional divisions after L3, with the number of seam cell nuclei remaining at 16 from the L3 molt to the adult stage (average 16 ± 0.8 in late L4 and 16 ± 0.5 in young adults) (Fig. 3C and D). These results show that rack-1(RNAi) enhances the precocious heterochronic phenotypes of hbl-1(mg285) and may support the hypothesis that depletion of RACK-1 enhances let-7-mediated gene regulation.

Depletion of RACK-1 suppresses retarded male tail morphogenesis resulting from lin-41 gain-of-function mutations

To determine whether rack-1(RNAi) affects let-7-mediated gene regulation in other cell types, we investigated male tail tip morphogenesis that has been reported to be regulated by the let-7-lin-41 circuit.67 The C. elegans male tail tip undergoes a dramatic morphologic change during the L4 stage, in which 4 epidermal tail tip cells fuse with each other and retract anteriorly under the larval cuticle, resulting in a rounded or “peloderan” tail.68,69 The hypomorphic let-7(n2853) allele and 2 lin-41 gain-of-function (gf) alleles, lin-41(bx37) and lin-41(bx42), cause a delay in retraction and produce pointed or

Figure 3. Depletion of RACK-1 enhances the precocious heterochronic phenotypes in hbl-1(mg285) animals. rack-1(RNAi) increased the percentage of (A) late-L3 and (B) mid-L4 hbl-1(mg285) animals exhibiting precocious seam cell fusion. Upper panel: fluorescence images of hbl-1(mg285) wIs79[ajm-1::gfp; scm-1::gfp] animals grown under RNAi. Arrowheads point to the adherent junctions of unfused seam cells. Animals containing any unfused seam cells were scored and shown as “w/ unfused s.c.”. Only animals displaying fusion in all seam cells were considered completely precocious, shown as “w/ fused s.c.” in the figure. s.c., seam cells. Lower panel: Nomarski images showing developmental stages. Arrows point to the distal tip of the gonad. Asterisks indicate the prospective vulva position. V, the developing vulva. Scale bars represent 20 µm. Brackets indicate a statistically significant difference calculated with a chi-square test. (C) Merged fluorescence and Nomarski images taken at the same focal plane of late-L4 wIs51[scm::gfp]; hbl-1(mg285) animals grown under RNAi. The seam cell numbers are shown in parentheses (mean ± SD). Scale bars reflect 50 µm. (D) Young adult hbl-1(mg285) animals were assayed as stated in (C). yAd, young adult. For (C and D), box-and-whisker plots of seam cell numbers are shown on the right. ***P < 0.001 by unpaired 2-tailed Student t test.
“leptodran” (Lep) tails. The Lep phenotype can be categorized into 3 classes (“outside”, “inside”, and “knob”) depending on whether the tail tip extends beyond the posterior edge of the fan or has the shape of a small knob (Fig. 4A–C). In previous studies, knocking down lin-41 by RNAi has been shown to rescue the Lep phenotype in lin-41 gain-of-function mutants, and also cause precocious male tail morphogenesis or an “over-retracted” (Ore) tail in a small proportion of animals. In this work, under mock RNAi, most lin-41 (bx37) and lin-41 (bx42) males displayed a leptodran (Lep) tail tip (Fig. 4A–C), and only a very low percentage of animals exhibited a wild-type-like tail (bx37: 6%; bx42: 2%). lin-41 (RNAi) caused a high percentage of lin-41 gain-of-function males to exhibit a wild-type-like tail (bx37: 71%; bx42: 51%) and a small fraction to display an Ore tail. Interestingly, we found that male tail morphogenesis was also sensitive to depletion of RACK-1. When fed with rack-1 (RNAi) bacteria, the percentage of males with a wild-type-like tail (bx37: 42%; bx42: 20%) was significantly higher than that seen in the control (Fig. 4D–G).

The 3 classes of the Lep phenotypes (“outside”, “inside”, and “knob”) represent different strengths of the mutation, and thus a simple way to assess the Lep phenotype is to calculate the Lep severity index (LSI = [%knob + {2 × %inside} + {3 × %outside}] / 3) as previously described. As shown in Figure 4G, the LSI in the control RNAi (bx37: 89; bx42: 94) reflected the very high penetrance of the Lep phenotype. rack-1 (RNAi) reduced the LSI significantly (bx37: 50; bx42: 68), but lin-41 (RNAi) reduced the LSI still further (bx37: 9; bx42: 14). It has been shown that male tail morphogenesis is very sensitive to LIN-41 activity in a dosage-dependent manner. Since we have found that depletion of RACK-1 suppressed let-7-related retarded phenotypes in hypodermal cells, it is possible that rack-1 (RNAi) also partially reduces LIN-41 activity in male tail morphogenesis through let-7-mediated repression. However, we cannot exclude the possibility that

![Figure 4](https://www.landesbioscience.com(Cell Cycle)
rack-1(RNAi) affects male tail morphogenesis through other parallel pathways.

Depletion of RACK-1 enhances let-7-mediated gene regulation in an Argonaute ALG-1-dependent manner

In addition to morphological phenotypes, we monitored hypodermal expression of the mali105[col-19::gfp] reporter,8,14 which has been used to determine developmental timing in numerous studies, to more directly assess the effect of rack-1(RNAi). The adult-specific collagen gene col-19 is activated by LIN-29,18,19 and in a mutant lacking 2 let-7 family members, miR-48 and miR-84, col-19::gfp expression is retarded in the main body hypodermal syncytial hyp7 cell but is normal in the seam cells following the L/A switch.14 Overall, the hyp7 cell appears to display a more penetrant retarded phenotype than seam cells with respect to LIN-29 activation. Only a low percentage of let-7(n2853) young adult animals displayed col-19::gfp expression in hyp7 (Fig. 5A), while

Figure 5. Depletion of RACK-1 enhances col-19 expression in let-7(n2853) adult animals, and this effect may require the Argonaute protein ALG-1. (A–D) mali105[col-19::gfp]; let-7(n2853) animals were synchronized at L1 and subjected to RNAi. col-19::gfp expression was scored in young adults. (A) Upper panel, a representative Nomarski image showing developmental stage, V, vulva. Lower panel, a merged image of fluorescence and Nomarski micrographs from the same focal plane showing col-19::gfp expression in seam cells. A representative seam cell is indicated. Scale bars represent 50 µm. (B–D) Merged fluorescence and Nomarski micrographs showing col-19::gfp expression in seam cells and/or hyp7 in each RNAi experiment. Representative seam cells and hyp7 nuclei are indicated. The percentages of animals grown under RNAi exhibiting col-19::gfp expression in hyp7 are shown below. n, the number of animals assayed. Empty vector, bacteria expressing the L4440 vector used to dilute RNAi (at a 1:1 ratio) in single knock-down for a fair comparison of results from single and double RNAi experiments. (E–H) mali105[col-19::gfp] animals in the wild-type genetic background were subjected to the same set of RNAi experiments as in (A–D).
GFP was detected in some seam cells at the same stage. For this reason, we chose to employ col-19::gfp expression in hyp7 as a heterochronic marker. We knocked down rack-1 in let-7(n2853) animals and detected high expression of col-19::gfp in hyp7 (Fig. 5B). A similar pattern was also seen in the experiments using mir-48 mir-241(nDf51) animals (Fig. S1). These results agree with our earlier finding that depletion of RACK-1 suppressed the reiterated seam cell divisions at the L/A switch in let-7(n2853) and mir-48 mir-241(nDf51) animals (Figs. 1 and 2). If this effect acts through miRNA function, it should be attenuated or partially blocked by...

**Figure 6.** Depletion of RACK-1 increases the levels of let-7 and pre-let-7 miRNAs. (A) The levels of let-7 and pre-let-7 miRNAs were analyzed by northern blot analysis of RNA samples purified from synchronized rack-1(RNAi) and mock RNAi animals at the L/A switch. (B) Oscillating levels of let-7 were detected by northern blot analysis of RNA samples from synchronized rack-1(RNAi) and mock RNAi animals collected at the indicated time points. Asterisks indicate the time of the L/A switch. (C) The levels of pre-miRNAs (pre-let-7, pre-miR-58, pre-lin-4, pre-miR-44, pre-miR-48, pre-miR-70, and pre-miR-241) in dcr-1(RNAi) and dcr-1(RNAi); rack-1(RNAi) animals were detected by northern blot analysis. For (A–C), U6 snRNA was used as a loading control. (D) The levels of indicated pre-miRNAs in dcr-1(RNAi); rack-1(RNAi) animals were quantitated by the Phosphorimager and compared with the levels found in dcr-1(RNAi) animals (the horizontal dash line, set as 1-fold). Values were obtained from at least 3 independent biological duplicates (represented in C). Empty vector, bacteria expressing the L4440 vector used to dilute dcr-1(RNAi) (at a 1:1 ratio) in single knockdown for a fair comparison of results from single and double RNAi experiments. Error bars represent SD ***P < 0.001; *P < 0.05 by paired one-tailed Student t test. (E) Late L4-stage animals expressing the plk-7B:gfp transgene under the indicated RNAi were observed by fluorescence (upper) and Nomarski (lower) microscopy. In the upper panel, representative seam cells and hyp7 nuclei are indicated. In the lower panel, arrows point to the distal tip of the gonad. V, vulva. Scale bars represent 20 μm. (F) Quantification of GFP in (E) was performed by measuring the mean pixel intensity of GFP detected in 3 seam cell nuclei posterior to the developing vulva and 3 neighboring hyp7 nuclei for all animals, respectively. Six animals were assayed for each RNAi experiment. The relative GFP expression for each animal was calculated by normalization to the average expression in mock RNAi animals. Error bars represent SD ***P < 0.001; n.s., not significant by unpaired 2-tailed Student t test.
a loss of active miRISCs. Thus, we knocked down alg-1 along with rake-1(RNAi) and examined the results. Although bacteria expressing each RNAi vector were diluted at a 1:1 ratio in these double knockdown experiments, our western blot analysis and real-time quantitative RT-PCR assays detected no significantly lower efficiencies of rake-1(RNAi) or alg-1(RNAi) (Fig. S2). Moreover, let-7(n2853) animals displayed col-19::gfp expression in hyp7 under the diluted rake-1(RNAi) as when the RNAi was undiluted (Fig. 5B). Interestingly, alg-1(RNAi) predominantly reduced the net effect of rake-1(RNAi) on col-19::gfp expression in let-7(n2853) animals (Fig. 5C). Depletion of LIN-41 restored col-19::gfp expression in the presence of alg-1(RNAi) and, thus, served as a control, indicating that alg-1(RNAi) may prevent the GFP expression through the let-7-lin-41-lin-29 circuit (Fig. 5D).

We found that alg-1(RNAi) also inhibited col-19::gfp expression in hyp7 in wild-type animals, which is consistent with previous studies that observed let-7 retarded phenotypes in wild-type animals under alg-1/alg-2(RNAi). The GFP expression could be restored by simultaneous depletion of LIN-41 but not by depletion of RACK-1 (Fig. 5E–H). alg-1(RNAi) also caused defective alae formation, which was not suppressed by rake-1(RNAi) (Table S1). Taken together, these results agree with our hypothesis that depletion of RACK-1 enhances let-7-mediated gene regulation, and suggest that this effect may, in part at least, act through ALG-1. However, it is unlikely that rake-1(RNAi) enhances let-7 function by increasing the number of miRISCs, since the protein levels of ALG-1 were unchanged in rake-1(RNAi) animals (Fig. S2A).

Depletion of RACK-1 increases mature let-7 levels by acting upstream of Dicer-mediated pre-let-7 processing

To determine whether depletion of RACK-1 alters let-7 biogenesis, we analyzed synchronized late L4-stage wild-type animals by northern blot analysis when rake-1 was knocked down. We detected an increase in let-7 in rake-1(RNAi) animals (Fig. 6A; Fig. S2; 1.5-fold). Previous studies have shown an oscillating expression pattern of let-7 during development.71 To avoid misinterpreting let-7 levels due to analysis of only a single time point, we analyzed animals collected every 2 h from mid-L4 to early adulthood. We found that the levels of mature let-7 varied in a limit range through time and oscillated roughly in parallel with pre-let-7 levels, often peaking slightly after pre-let-7, and the overall expression of let-7 over time was increased by depletion of RACK-1 (Fig. 6B). Interestingly, this increase in let-7 was no longer observed following alg-1 knockdown (Fig. S2B), which is consistent with a previous finding by Jannot et al. that RACK-1 affects miRNA levels in an ALG-1-dependent manner.72 As a consequence, our earlier observation that rake-1(RNAi) failed to restore col-19::gfp expression in let-7(n2853); alg-1(RNAi) mutants (Fig. 5) not only can be explained by a requirement for ALG-1, but also by a decline in let-7 levels.

In addition to increased let-7, we have also observed a rise in pre-let-7 in rake-1(RNAi) animals (Fig. 6A and B), suggesting that the accumulation of let-7 may stem from miRNA biogenesis but not a slow turnover of mature let-7 as Jannot et al. have proposed.71 To better detect pre-let-7, we performed a double knockdown to reduce DCR-1 and RACK-1 simultaneously and then examined pre-miRNAs from animals at the L/A switch. Knockdown of dcr-1 impaired pre-miRNA processing and yielded a readily detectable accumulation of pre-let-7 (Fig. S3).87,82 We clearly detected a 2-fold increase in pre-let-7 levels with rake-1(RNAi) when dcr-1 was knocked down (Fig. 6C and D; Fig. S3), suggesting that rake-1(RNAi) affects let-7 biogenesis upstream of Dicer. Interestingly, we also detected increased levels of several pre-miRNAs, including pre-lin-4 and pre-miR-48 (Fig. 6C and D), showing that RACK-1 may be involved in the biogenesis of a set of, but not all, miRNAs. However, statistically significant changes in the levels of mature lin-4 and miR-48 in these samples were not observed (data not shown). We have not examined how depletion of RACK-1 affects the levels of lin-4 and miR-48 from the L1 to L3 stages, and thus the role of RACK-1 in the early development of C. elegans remains unclear.

To investigate whether depletion of RACK-1 increases the transcription of pri-let-7 in the nucleus, we monitored the expression of the plet-7B::gfp transgene that carries a let-7 promoter fused to a gfp reporter.73 For a positive control, we knocked down lin-42, which encodes the Period protein homolog,74,75 by the Arrhinger F47F6.1 RNAi construct,76 and detected significantly increased expression of plet-7B::gfp in seam cells and hyp7 (Fig. 6E and F). lin-42 mutations or depletion of LIN-42 by RNAi have been found to cause precocious heterochronic phenotypes,77,78 as well as accumulation of mature let-7 and increased pri-let-7 transcription.77 In contrast to the results for lin-42(RNAi), there was no change in plet-7B::gfp expression when rake-1 was knocked down. In addition, we did not detect significant changes in the amount or timing of expression of pri-let-7 from the L4 stage to adulthood in rake-1(RNAi) animals by real-time quantitative RT-PCR (data not shown). These results suggest that RACK-1 may function post-transcriptionally in miRNA biogenesis. Taken together, we show that RACK-1 controls the levels of a set of pre-miRNAs through an as yet unelucidated mechanism. In this way, RACK-1 may orchestrate the biogenesis and regulatory potential of miRNAs.

**Discussion**

RACK1 acts pleiotropically in many biological processes, complicating our ability to determine its distinct functions in the miRNA pathway. Previous studies have proposed different roles of RACK1 in miRNA gene transcription, primary miRNA processing/stability, the loading of miRNAs into miRISCs, and the recruitment of miRISCs to the ribosome.51,54,55 Despite the fact that its precise mechanisms of action are still unclear, however, RACK1 is required for proper miRNA function in all of these models. Here, we show that depletion of C. elegans RACK-1 increases the levels of the let-7 miRNA and suppresses the retarded heterochronic phenotypes related to let-7, suggesting that, at least with respect to let-7-mediated gene regulation, RACK1 may also play a negative role in the miRNA pathway.

**RACK1 acts in miRNA biogenesis**

Two previous reports described roles for RACK1 that are independent of miRNA biogenesis. Otsuka et al. observed that a few miRNAs displayed impaired silencing upon RACK1 knockdown with no detectable changes in their overall levels.54
However, lower amounts of these miRNAs were detected in Ago2-containing complexes, leading the authors to propose that RACK1 functions after miRNA maturation and is required to load mature miRNAs into miRISCs.\textsuperscript{54} Jannot et al. observed that depletion of RACK1 impaired miRNA regulation and reduced the amount of Argonautes associated with the polysome.\textsuperscript{55} They proposed that RACK1, as an Argonaute-interacting protein, facilitates the recruitment of miRISC to the ribosome.

Interestingly, Jannot et al. observed retarded developmental defects in C. elegans which were proposed to stem from lower miRISC function as well as an unexpected increase in the levels of let-7 miRNA upon rack-1(RNAi).\textsuperscript{51} The accumulation of let-7 was proposed to be due to the stabilization of miRISCs caused by impaired interactions between miRNAs and their targets. This is based on the observation that extensive complementarity between miRNAs and their targets can trigger miRNA tailing,\textsuperscript{56} 3′-to-5′ trimming and destabilization in Drosophila.\textsuperscript{78} However, it is unclear whether this mechanism exists in C. elegans and, if it does, whether the complementarity of major miRNA-target interactions is extensive enough to broadly trigger such miRNA degradation. Also contrary to the authors’ explanation, the binding of miRNAs to their targets has been reported to protect miRNAs from the 5′-to-3′ exonuclease activity in C. elegans,\textsuperscript{79} leading to a prediction that impaired miRNA-target interaction caused by rack-1(RNAi) should reduce the abundance of miRNAs. Taken together, it seems unlikely that enhanced stabilization is the primary mechanism for let-7 accumulation with rack-1(RNAi).\textsuperscript{51} Here, our results show that reduction of RACK1 increased the levels of pre-let-7 as well as its mature form. When Dicer was reduced, a vast synergistic accumulation of pre-let-7 was observed with rack-1(RNAi), suggesting that the increase in let-7 may stem from a biogenesis step(s) upstream of Dicer.

We have not observed clear retarded heterochronic phenotypes in wild-type animals with rack-1(RNAi), which were previously reported by Jannot et al.\textsuperscript{51} but did find significant suppression of the retarded terminal cell differentiation in let-7(n2853) and mir-48 mir-241(nDf51) animals. One straightforward explanation is that the accumulation of let-7 caused by rack-1(RNAi) was sufficient to compensate for impaired miRNA function or synthesis in these mutants, in spite of possibly reduced recruitment of miRISCs to the polysome as Jannot et al. have proposed.\textsuperscript{51} Thus, this leads to the possibility that RACK1 may be involved in 2 distinct mechanisms in the miRNA pathway, i.e., miRNA biogenesis and miRISC function, each with different sensitivities to reduction of RACK1. Here we cannot completely exclude the possibility that depletion of pleiotropic RACK1 influences cell cycle exit in let-7(n2853) and mir-48 mir-241(nDf51) animals through parallel pathways. However, since we have observed no alteration of the lin-29(n333) phenotypes with rack-1(RNAi), it is likely that the effect of rack-1(RNAi) acts at least in part through the heterochronic gene pathway.

In Arabidopsis, RACK1 was also reported to function in miRNA biogenesis.\textsuperscript{55} However, in contrast to our finding, rack1 mutations reduced the levels of a large number of miRNAs with few exceptions. Some pri-miRNAs accumulated to higher levels in rack1 mutants, suggesting that RACK1 affects the processing and transcription/stability of certain pri-miRNAs.\textsuperscript{55} Moreover, aberrant non-canonical miRNAs were more abundant in rack1 mutants, indicating that RACK1 also ensures precise processing.\textsuperscript{55} Plant pri-miRNAs and pre-miRNAs are cleaved in the nucleus by the same RNase III enzyme Dicer-LIKE 1 (DCL1).\textsuperscript{80} Therefore, Arabidopsis RACK1 may be required for steps upstream of or with DCL1 to affect pri-miRNA processing or stability. In this study, we found that C. elegans RACK-1 acts upstream of Dicer. Transcription of the let-7 promoter or the levels of pri-let-7 were not altered with rack-1(RNAi), leading us to suggest that C. elegans RACK-1 functions between pri-let-7 and pre-let-7 processing and may not affect pri-let-7 stability. Since the machinery of miRNA biogenesis is distinct in animals and plants, it is possible that RACK1 also works with miRNAs in different ways. Although the mechanism is still unclear, our results strongly support the possibility that in addition to miRISC functions, RACK1 may also play an important role in miRNA biogenesis.

**RACK1 and Argonautes**

We began to investigate the role for RACK-1 in miRNA function after detecting a physical interaction between RACK-1 and a C. elegans Argonaute, ALG-1.\textsuperscript{57} While we have not addressed where in the cell C. elegans RACK-1 affects miRNA biogenesis in this work, Arabidopsis RACK1 has been shown to regulate pri-miRNA processing in the nucleus.\textsuperscript{55} Thus, it is likely that free rather than ribosome-bound RACK1 is involved in the regulation of miRNA biogenesis. Interestingly, Speth et al. have shown that Arabidopsis RACK1 and AGO1 are part of a common complex outside the ribosome, and that these proteins co-localize in the nucleus and the cytoplasm.\textsuperscript{55} We also detected C. elegans RACK-1 in both the nucleus and the cytoplasm (Fig. S4). Differences in localization and binding partners may explain the distinct functions of RACK1 in miRNA biogenesis and miRISC function. Ribosome-bound RACK1 may help recruit miRISCs to the ribosome, while free RACK1, perhaps with associated Argonautes, may play roles in miRNA biogenesis in the nucleus and/or the cytoplasm.

C. elegans ALG-1/2 and mammalian Ago2 appear to be associated with miRNA maturation.\textsuperscript{38,81,82} Mammalian Ago2 also has been shown to encounter pre-miRNA in the nucleus and cytoplasm at a step earlier than Dicer and possibly facilitate access of pre-miRNAs to Dicer.\textsuperscript{55} In addition, it has been reported that the slicing activity of both human and C. elegans Argonautes cleaves pri-miRNA hairpins to produce processing intermediates.\textsuperscript{84,85} Moreover, a recent finding in C. elegans has revealed that let-7-bound ALG-1 enters the nucleus and promotes pri-let-7 processing.\textsuperscript{46} Taken collectively, miRNA-specific Argonautes seem to not only repress mRNA activity, but also to mediate pre-miRNA and pri-miRNA processing. RACK1 might play a role in these processes by shuttling Argonautes to particular intracellular sites. On the other hand, RACK1 has been shown to promote or suppress the activities of bound enzymes (for a review, see ref. 46), leading to the possibility that RACK1 also regulates the activity of Argonautes in these processes. An observation that Arabidopsis RACK1 affects the cleavage accuracy of AGO1 may support this notion.\textsuperscript{55} However, since RACK1 interacts with and modulates
the localizations and functions of numerous proteins, additional investigation will be necessary to clarify which effector(s) RACK1 employs to modulate miRNA biogenesis.

In summary, our studies have uncovered a novel role for RACK-1 in the *C. elegans* let-7 miRNA pathway. Reduction of RACK-1 causes increased levels of pre-let-7 and let-7 miRNAs and, in turn, suppression of retarded heterochronic phenotypes in the late larval stages and at the L/A switch. Since both let-7 and RACK1 are involved in the regulation of many biological processes as well as several kinds of cancers, the mechanisms and implications of this finding will be the focus of future studies.

**Materials and Methods**

**Nematode strains and culture conditions**

Wild-type N2, MT7626 let-7(n2853) X, SP231 mnDp1(X;V)+/V; unc-3(e151) let-7(mn112) X, MT333 lin-29(n333) II, MT13669 mir-48 mir-241(nDf51) V, VC3013 rack-1(ok3676) IV, EM101 lin-41(bx37) I; him-5(e1490) V and EM106 lin-41(bx42) I; him-5(e1490) V were provided by the *Caenorhabditis* Genetics Center (CGC). RN1123 hbl-1(mg285) wls79[najm-1::gfp; scm-1::gfp] X and JR667 unc-119(e2498::Te1) III; wls51[sm::gfp; unc-119(+)] V were kindly provided by Dr Ryusuke Niwa. To visualize seam cell nuclei in mutants, wls51 was crossed into MT7626, MT13669, and MT3669. To generate rack-1(ok3676); mir-48 mir-241(nDf51) wls51, wls51, and mir-48 mir-241(nDf51) were crossed into VC3013. To generate wls51; hbl-1(mg285), wls51 was crossed into RN1123, and animals expressing wls51 but not wls79 were selected and homozygosed. mals105[coil-19::gfp] V was kindly provided by Dr Victor Ambros and Dr Helge Grosshans, and was crossed into MT7627 and MT13669. PQ462 pler-7B::gfp was kindly provided by Dr Priscilla Van Wynsberghe and Dr Amy E Pasquinelli. Worms were maintained under standard conditions at 15 °C or 20 °C and synchronized by standard hypochlorite treatment.97,88

**RNA interference**

Feeding RNAi experiments were performed at 20 °C using *E. coli* HT115 bacteria expressing RNAi constructs from the Ahringer library.98 Synchronized L1 animals were placed onto RNAi feeding plates and harvested at the indicated stage or time. For Figures 2 and 6C, and D and Figure S3, synchronized L1 parental animals (P0) were cultured on NGM plates seeded with OP50 *E. coli* until L4 and then transferred to RNAi feeding plates.87 Adult P0 were used for the hypochlorite treatment and synchronized L1 animals (F1) were placed on plates with the same RNAi expressing bacteria until harvested. In double-knockdown experiments, the bacteria food consisted of 50% expressing one RNAi construct and 50% expressing the other. For a fair comparison, single knockdown of each gene in this set of experiments was diluted by 50% with bacteria expressing the empty vector L4440 (Figs. 5, 6C, and D; Figs. S2 and S3). For RNAi experiments with let-7(mn112) animals, the SP231 strain carrying unc-3(e151) let-7(mn112) and a balanced translocation was used.98 Starved L1 animals were placed onto RNAi plates and grown to L3. Homozygous unc-3(e151) let-7(mn112) mutants exhibiting the uncoordinated (Unc) phenotype were transferred to new RNAi plates, and the vulva-bursting phenotype was scored at the L/A switch.

**Antibodies and western blotting**

Nematode lysates were prepared as previously described.90 Samples were separated by 10% Bis-Tris acrylamide gels and transferred onto PVDF membranes for western blotting. The polyclonal antibody against *C. elegans* RACK-1 amino acids 264–282 (IWLEDKKEEELKPEIAS) was raised in rabbits by LTK BioLaboratories, and unprocessed immune serum was used at a 1:5000 dilution to detect RACK-1 as a single band. Actin was detected by monoclonal mouse β-Actin antibody (A00702, GenScript, 1:10000 dilution). Horseradish peroxidase-conjugated anti-rabbit (211-032-171, Jackson ImmunoResearch Labs, 1:10000 dilution) or anti-mouse (115-035-174, Jackson ImmunoResearch Labs, 1:10000 dilution) light-chain-specific secondary antibodies were used for signal detection by ECL Prime (GE Healthcare). Images were acquired by UVP BioSpectrum 500 imaging system.

**RNA extraction and northern blot analysis**

Total RNA was isolated by modifying previously described protocols.92 Twenty (20) μl of packed animals was washed with M9 buffer and mixed with 1 ml of TRIzol (Invitrogen). The mixtures were then placed into a 2-ml screw-cap tube that was preloaded with 0.5 ml 1.0 mm Zirconia beads (11079110xx, BioSpec Products) and homogenized by FastPrep-24 homogenizer (MP Biomedicals) at 4 °C for 4 rounds of 30 s at a speed of 6.5 m/s with 1 min intervals. After a 5 min centrifugation at 13000 rpm, the supernatant was collected, and a TRIzol extraction was performed according to the manufacturer’s instructions. For detection of miRNAs, total RNA samples, 10 μg per lane, were separated by 12% polyacrylamide gels (8 M urea, Acrylamide/ Bis 19:1) and transferred onto Hybond-N+ membranes (GE Healthcare). RNA was crosslinked to the membrane by 254 nm UV light irradiation (120000 microjoules/cm2) and baking the membrane at 80 °C for 1 h. Short RNA probes complementary to miRNAs or U6 snRNA were prepared by in vitro transcription using T7 RNA polymerase as previously described (For oligonucleotide sequences, see Supplementary Information).92 Hybridization was performed out at 55 °C in 0.36 M Na2HPO4, 0.14 M NaH2PO4, 1 mM EDTA, 10% SDS, 25% Formamide, and 0.1 mg/ml salmon sperm DNA. Washes were done at 55 °C twice in the low stringency buffer (4× SSPE and 4% SDS) and once in the high stringency buffer (0.1x SSC and 0.1% SDS). Radioactive signals were detected by a storage phosphor image plate and Typhoon Trio Variable Mode Imager (GE Healthcare).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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