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

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The *let-7* microRNA (miRNA) regulates cell cycle exit and terminal differentiation in the *C. elegans* heterochronic gene pathway. Low expression of *let-7* results in retarded vulva and hypodermal cell development in *C. elegans* and has been associated with several human cancers. Previously, the versatile scaffold protein receptor for activated C kinase 1 (RACK1) was proposed to facilitate recruitment of the miRNA-induced silencing complex (miRISC) to the polysome and to be required for miRNA function in *C. elegans* and humans. Here, we show that depletion of *C. elegans* RACK-1 by RNAi increases *let-7* miRNA levels and suppresses the retarded terminal differentiation of lateral hypodermal seam cells in mutants carrying the hypomorphic *let-7*(*n2853*) allele or lacking the *let-7* family miRNA genes *mir-48* and *mir-241*. Depletion of RACK-1 also increases the levels of precursor *let-7* miRNA. When Dicer is knocked down and pre-miRNA processing is inhibited, depletion of RACK-1 still leads to increased levels of pre-*let-7*, suggesting that RACK-1 affects a biogenesis mechanism upstream of Dicer. No changes in the activity of the *let-7* promoter or the levels of primary *let-7* miRNA are associated with depletion of RACK-1, suggesting that RACK-1 affects *let-7* miRNA biogenesis at the post-transcriptional level. Interestingly, *rack-1* knockdown also increases the levels of a few other precursor miRNAs. Our results reveal that RACK-1 controls the biogenesis of a subset of miRNAs, including *let-7*, and in this way plays a role in the heterochronic gene pathway during *C. elegans* development.

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 transcripts (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), 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 mRNA 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),[17-19] 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,[28-32] 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.[27-29,32] In humans, more than a thousand miRNAs have been found and predicted to regulate greater than

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60% of protein-encoding genes.[33] 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

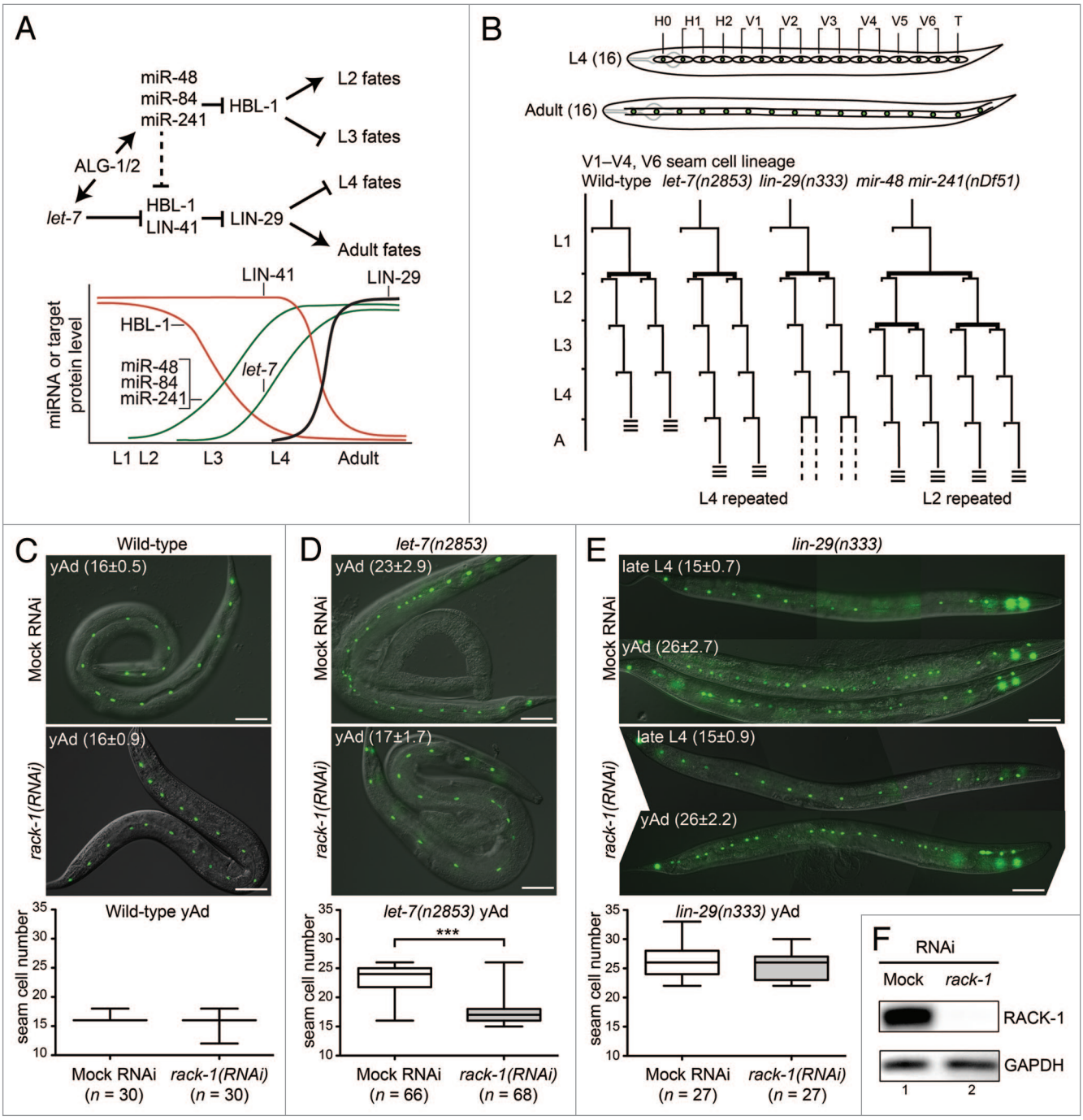


Figure 1. For figure legend, see page 1997.

proper miRNA functions in *C. elegans* and humans by playing a crucial role in recruitment of miRISCs to the ribosome.[51] 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.[51] 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.[54] They have proposed that RACK1 functions after miRNA maturation and is required to load mature miRNAs into miRISCs. In *Arabidopsis*, mutations in *rack1* genes reduce the levels of a large number of miRNAs.[55] Speth et al. have shown that RACK1 is associated with AGO1 in a complex outside the ribosome and affects the accumulation and processing precision of some primary miRNAs.[55] 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-associating 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*.[58] 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.[9,10] 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.[9] The *let-7*(*mn112*) allele harbors a deletion in the *let-7* locus that completely prevents the production of the mature *let-7* miRNA.[9] *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.[9,10] 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.[61,62] 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 *hbl-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 expression. miR-48, miR-84, and miR-241 may also contribute to the repression of HBL-1 and LIN-41 in the late larval stage (dashed 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*(*nDf51*) 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 cells continue to divide after the L4 molt and maintain larval characteristics. In *mir-48 mir-241*(*nDf51*) animals, seam cells 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 *wIs51*[*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.

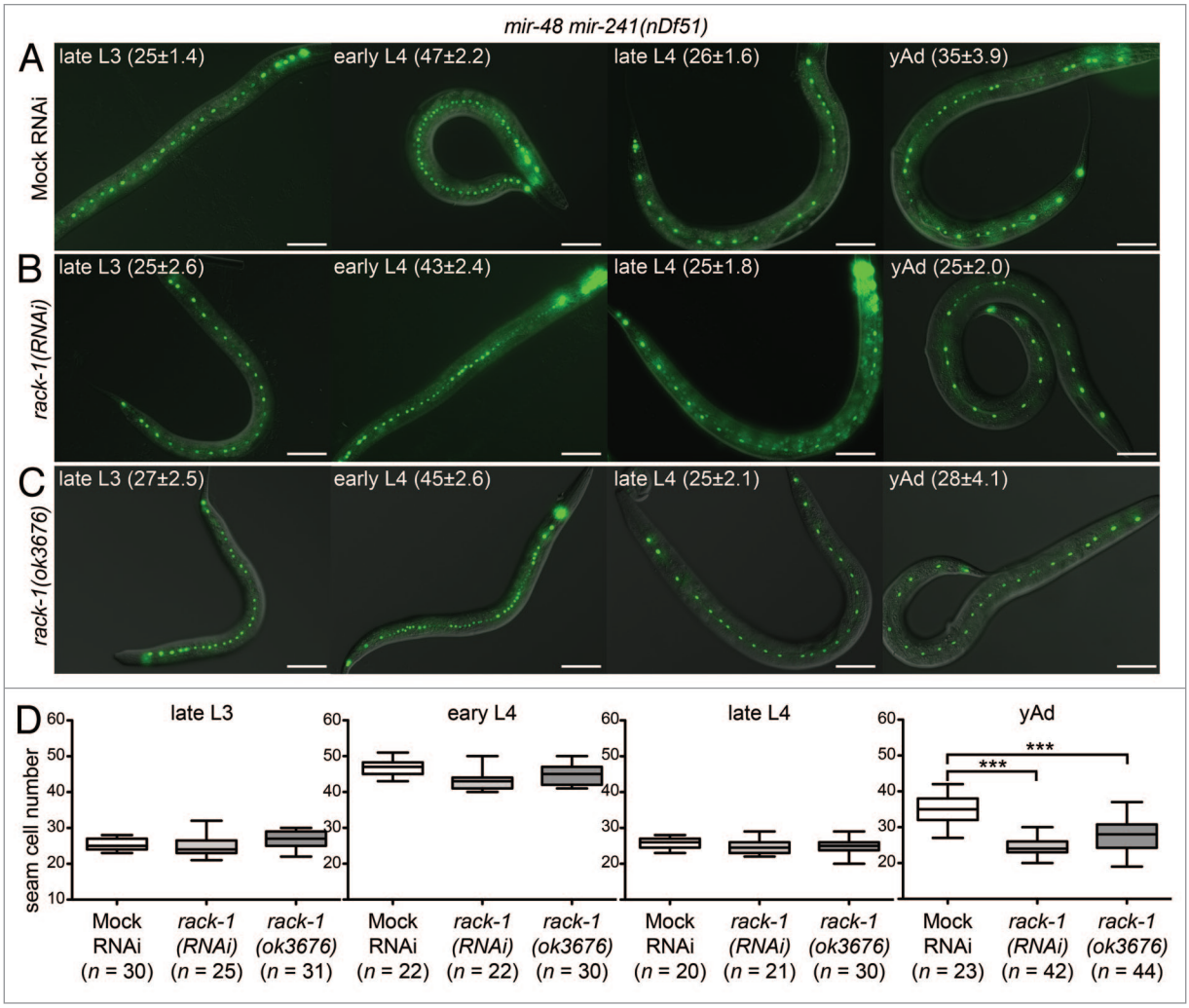


Figure 2. Depletion of RACK-1 suppresses the retarded terminal differentiation of seam cells in *mir-48 mir-241*(*nDf51*) animals. *mir-48 mir-241*(*nDf51*) animals carrying the *wIs51*[*scm::gfp*] transgene were monitored for the seam cell proliferation patterns from late L3 to young adulthood following depletion of RACK-1. (**A**) Mock RNAi; (**B**) *rack-1*(*RNAi*). Feeding RNAi was initiated in L4-stage parental (P_0) 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 that were 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.

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*.[9-13] *lin-29* encodes an adult-specific transcription

factor that promotes adult cell fates (depicted in **Fig. 1A**).[17-19,63] 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**).[64] We scored seam cell nuclei using a nuclear seam cell marker *scm::gfp* encoded by *wIs51* as previously described.[58,65] 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.[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**).[9] Interestingly, *rack-1*(*RNAi*) suppressed the reiterated seam cell divisions (average 17 ± 1.7 [± SD] vs. 23 ± 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**).[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**).

Table 1. Suppression of *let-7* mutation lethality

Genotype	RNAi[a]	Suppression (% viable animals)[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

[a]Synchronized L1 animals were subjected to feeding RNAi at 20 °C. The RNAi vector L4440 was used for mock experiments. [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(X;V)/+ V; unc-3(e151) let-7(mn112) X was used and only Unc animals homologous for the let-7(mn112) allele were scored.

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.[14] They begin to be expressed during the L2 stage, repressing HBL-1 and mediating the L2-to-L3 transition (depicted in **Fig. 1A**).[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**).[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.[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**).[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.[64] 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.[9,10,12,13] We monitored seam cell fusion using the *ajm-1::gfp* reporter of *wIs79* as previously described.[66] Although we did not observe significant precocious seam cell fusion in *rack-1*(*RNAi*) wild-type animals (data not

shown), we found that depletion of RACK-1 enhanced the precocious 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**). *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.

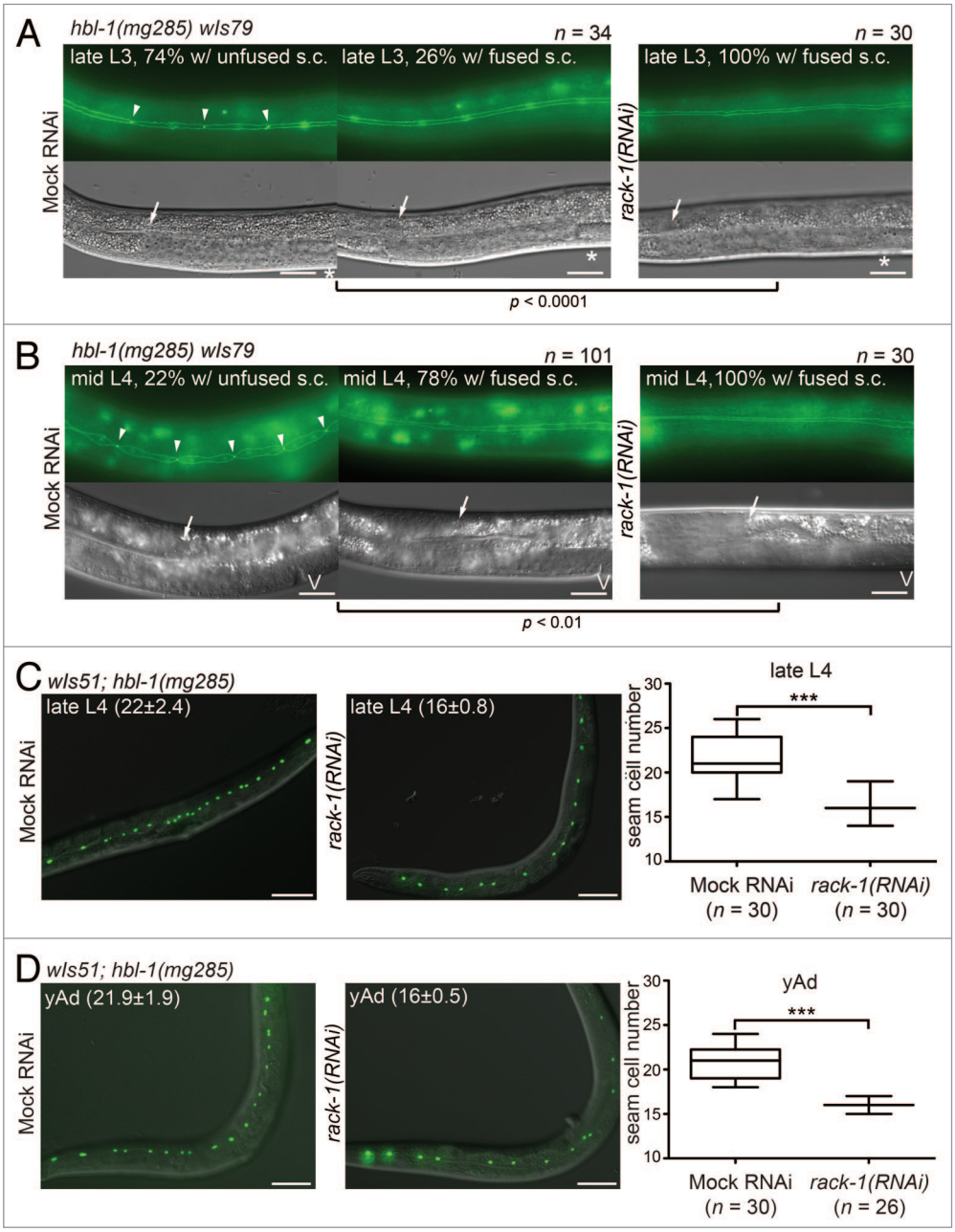


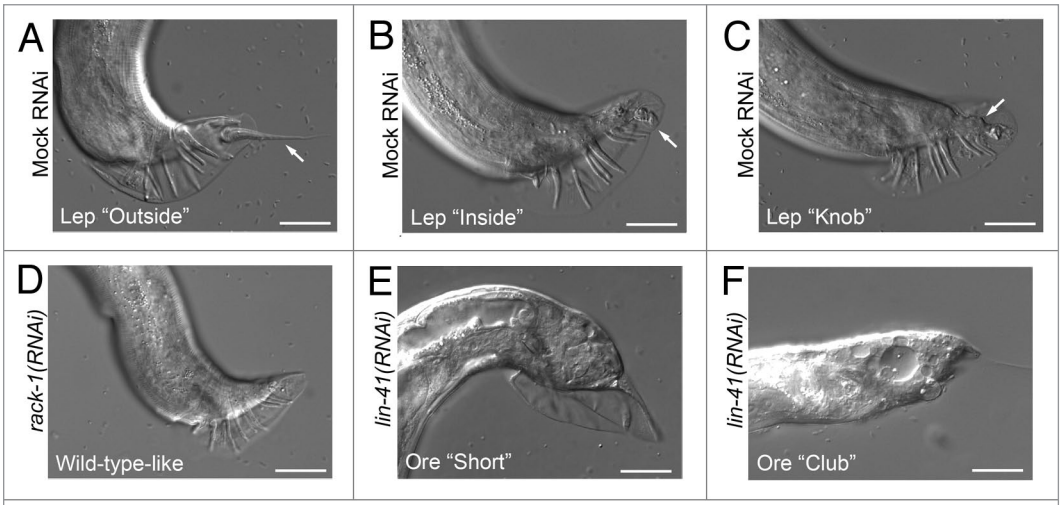
Figure 3. Depletion of RACK-1 enhances the precocious heterochronic phenotypes in *hbl-1*(*mg285*) animals. *rack-1*(*RNAi*) increased the percentage of (**A**) late-L3 and (**B**) mid-L4 *hbl-1*(*mg285*) animals exhibiting precocious seam cell fusion. Upper panel: fluorescence images of *hbl-1*(*mg285*) *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.

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.[67,68] 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

"leptoderan" (Lep) tails.[69] 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**).[69] 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.[69] In this work, under mock RNAi, most *lin-41*(*bx37*) and *lin-41*(*bx42*) males displayed a leptoderan (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.[69] 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.[69] 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



G

	Lep (%)			Wild-type-like (%)	Ore (%)		LSI	*n*		
	Outside	Inside	Knob		Short	Club				
lin-41(bx37)										
Mock RNAi	86	2	6	6	0	0	**89**	192	$p < 0.0001$	$p < 0.0001$
rack-1(RNAi)	43	6	9	42	0	0	**50**	196		
lin-41(RNAi)	3	5	9	71	11	1	**9**	150		
lin-41(bx42)										
Mock RNAi	89	6	3	2	0	0	**94**	202	$p < 0.0001$	$p < 0.0001$
rack-1(RNAi)	56	11	13	20	0	0	**68**	200		
lin-41(RNAi)	5	5	17	51	15	7	**14**	200		

Figure 4. *rack-1*(*RNAi*) suppresses the retarded male tail morphogenesis in *lin-41* gain-of-function mutants. (**A–C**) Lep male tail phenotypes of *lin-41*(*bx37*). Depending on the level of protrusion or the shape of tail tips (indicated by arrows), the phenotypes are categorized into one of 3 classes ("outside", "inside", and "knob"). (**D**) A representative image of a wild-type-like phenotype in *lin-41*(*bx37*) animals grown under *rack-1*(*RNAi*). (**E**) A representative image of the Ore (over-retracted) "Short" male tail phenotype in *lin-41*(*bx37*); *lin-41*(*RNAi*) animals, showing a clearly truncated tail tip but with a fan present. (**F**) A representative image of the Ore (over-retracted) "Club" male tail phenotype in *lin-41*(*bx37*); *lin-41*(*RNAi*) animals, showing a clearly truncated tail tip without a fan and/or with other severe defects. Scale bars represent 50 μm. (**G**) Percentage of adult male animals in *lin-41*(*bx37*) and *lin-41*(*bx42*) exhibiting retarded tail tip morphogenesis following RNAi with the indicated feeding vectors. LSI, Lep Severity Index = (%knob + [2 × %inside] + [3 × %outside]) / 3. Brackets indicate a statistically significant difference calculated through a chi-square test comparing distributions of Lep and wild-type phenotypes between RNAi experiments. *n*, the number of animals assayed.

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 *maIs105*[*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

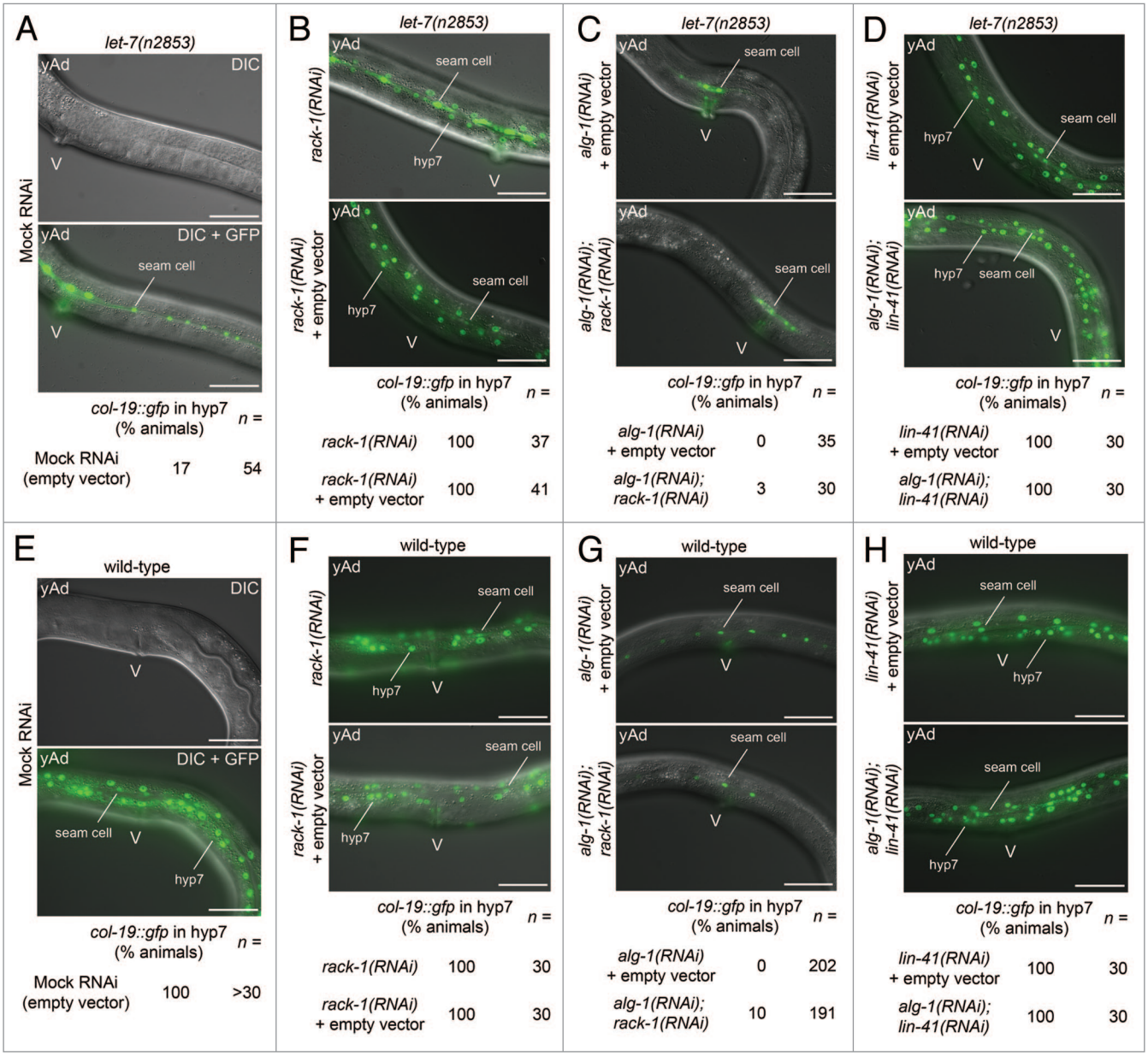


Figure 5. Depletion of RACK-1 enhances *col-19* expression in *let-7*(*n2853*) adult animals, and this effect may require the Argonaute protein ALG-1. (**A–D**) *mals105*[*col-19::gfp*]; *let-7*(*n2853*) animals were synchronized at L1 and subjected to RNAi. *col-19::gfp* expression was scored in young 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 knockdown for a fair comparison of results from single and double RNAi experiments. (**E–H**) *mals105*[*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

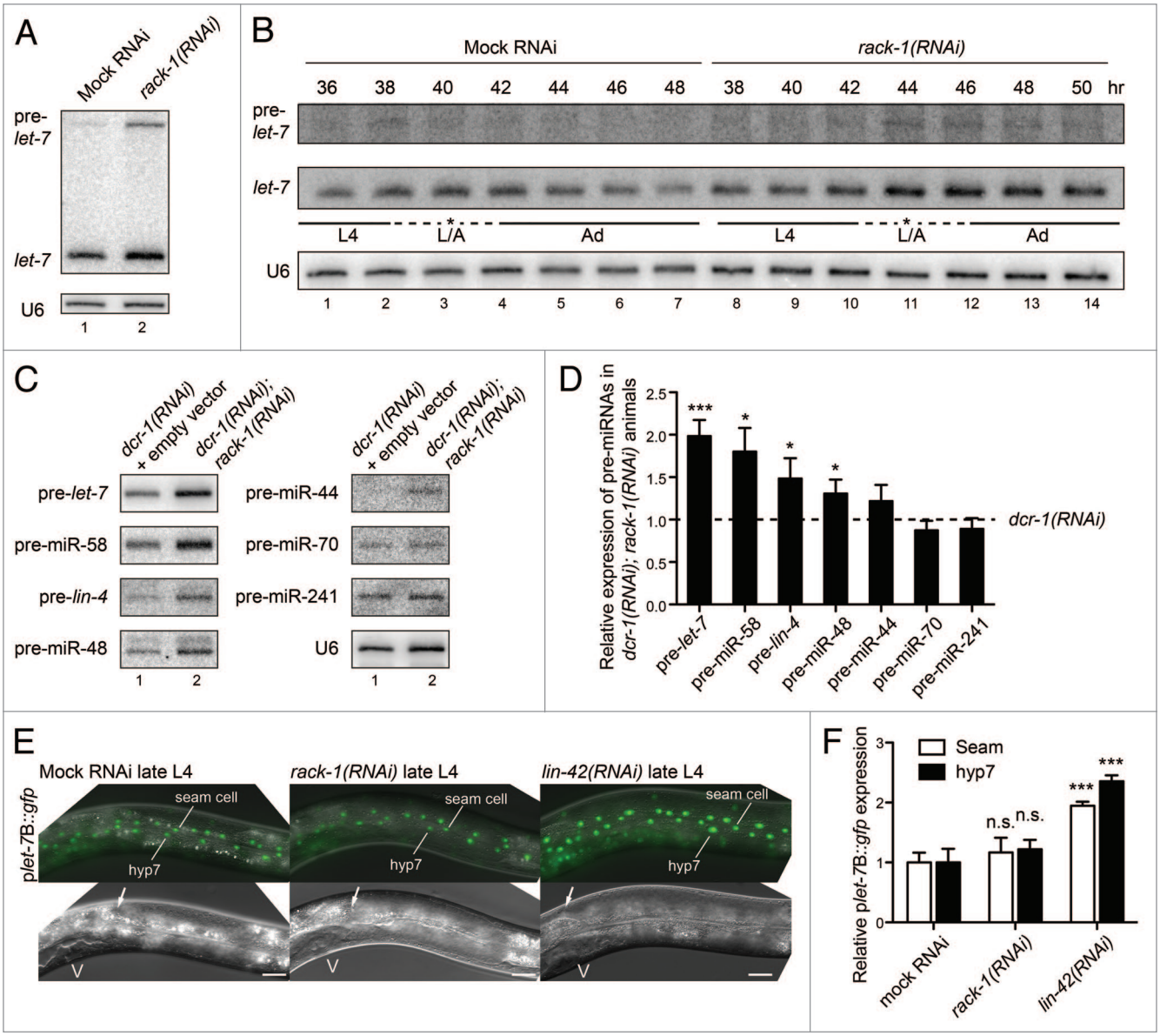


Figure 6. Depletion of RACK-1 increases the levels of *let-7* and pre-*let-7* miRNAs. (**A**) The levels of *let-7* and pre-*let-7* miRNAs were analyzed by northern blot analysis of RNA samples purified from synchronized *rack-1*(*RNAi*) and mock RNAi animals at the L/A switch. (**B**) Oscillating levels 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 p*let-7*B::*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 *rack-1*(*RNAi*) and examined the results.[70] 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 efficacies of *rack-1*(*RNAi*) or *alg-1*(*RNAi*) (**Fig. S2**). Moreover, *let-7*(*n2853*) animals displayed *col-19::gfp* expression in hyp7 under the diluted *rack-1*(*RNAi*) as when the RNAi was undiluted (**Fig. 5B**). Interestingly, *alg-1*(*RNAi*) predominantly reduced the net effect of *rack-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*).[58] 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 *rack-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 *rack-1*(*RNAi*) enhances *let-7* function by increasing the number of miRISCs, since the protein levels of ALG-1 were unchanged in *rack-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 *rack-1* was knocked down. We detected an increase in *let-7* in *rack-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.[51] As a consequence, our earlier observation that *rack-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 *rack-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.[51] 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**).[58,72] We clearly detected a 2-fold increase in pre-*let-7* levels with *rack-1*(*RNAi*) when *dcr-1* was knocked down (**Fig. 6C and D**; **Fig. S3**), suggesting that *rack-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 p*let-7*B*::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 Ahringer *F47F6.1* RNAi construct,[70] and detected significantly increased expression of p*let-7*B*::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,[75,76] 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 p*let-7*B*::gfp* expression when *rack-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 *rack-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.[54] Jannot et al. observed that depletion of RACK1 impaired miRNA regulation and reduced the amount of Argonautes associated with the polysome.[51] 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*).[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, 3′-to-5′ trimming and destabilization in *Drosophila*.[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*,[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*). 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.,[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.[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.[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.[55] Moreover, aberrant non-canonical miRNAs were more abundant in *rack1* mutants, indicating that RACK1 also ensures precise processing.[55] Plant pri-miRNAs and pre-mRNAs are cleaved in the nucleus by the same RNase III enzyme DICER-LIKE 1 (DCL1).[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.[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.[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 colocalize in the nucleus and the cytoplasm.[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.[58,81,82] 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.[83] In addition, it has been reported that the slicing activity of both human and *C. elegans* Argonautes cleaves pre-miRNA hairpins to produce processing intermediates.[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.[86] 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.[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*) *wIs79*[*ajm-1::gfp; scm-1::gfp*] *X* and JR667 *unc-119*(*e2498::Tc1*) *III*; *wIs51*[*scm::gfp; unc-119*(+)] *V* were kindly provided by Dr Ryusuke Niwa. To visualize seam cell nuclei in mutants, *wIs51* was crossed into MT333, MT7626, and MT13669. To generate *rack-1*(*ok3676*)*; mir-48 mir-241*(*nDf51*) *wIs51*, *wIs51,* and *mir-48 mir-241*(*nDf51*) were crossed into VC3013. To generate *wIs51; hbl-1*(*mg285*), *wIs51* was crossed into RN1123, and animals expressing *wIs51* but not *wIs79* were selected and homozygosed. *maIs105*[*col-19::gfp*] *V* was kindly provided by Dr Victor Ambros and Dr Helge Grosshans, and was crossed into MT7627 and MT13669. PQ462 p*let-7*B*::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.[87,88]

RNA interference

Feeding RNAi experiments were performed at 20 °C using *E. coli* HT115 bacteria expressing RNAi constructs from the Ahringer library.[70] 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 (P_0) were cultured on NGM plates seeded with OP50 *E. coli* until L4 and then transferred to RNAi feeding plates.[87] Adult P_0 were used for the hypochlorite treatment and synchronized L1 animals (F_1) 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.[9,89] 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 polyacrylamide gels and transferred onto PVDF membranes for western blotting. The polyclonal antibody against *C. elegans* RACK-1 amino acids 264–282 (IWDLEDKKEIEELKPEIAS) 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, 1:1000 dilution). Horseradish peroxidase-conjugated anti-rabbit (211-032-171, Jackson ImmunoResearch Labs, 1:10 000 dilution) or anti-mouse (115-035-174, Jackson ImmunoResearch Labs, 1:10 000 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.[91] Twenty (20) μl of packed animals were 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 (11079110zx, 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 13 000 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 (120 000 microjoules/cm^2) 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 Na_2HPO_4, 0.14 M NaH_2PO_4, 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 (0.1× 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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Supplemental Materials

Supplemental materials may be found here:
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References

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116:281-97; PMID:14744438; http://dx.doi.org/10.1016/S0092-8674(04)00045-5
2. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 2010; 79:351-79; PMID:20533884; http://dx.doi.org/10.1146/annurev-biochem-060308-103103
3. Pasquinelli AE. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet 2012; 13:271-82; PMID:22411466
4. Ameres SL, Zamore PD. Diversifying microRNA sequence and function. Nat Rev Mol Cell Biol 2013; 14:475-88; PMID:23800994; http://dx.doi.org/10.1038/nrm3611
5. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993; 75:843-54; PMID:8252621; http://dx.doi.org/10.1016/0092-8674(93)90529-Y
6. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 1993; 75:855-62; PMID:8252622; http://dx.doi.org/10.1016/0092-8674(93)90530-4
7. Moss EG, Lee RC, Ambros V. The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. Cell 1997; 88:637-46; PMID:9054503; http://dx.doi.org/10.1016/S0092-8674(00)81906-6
8. Feinbaum R, Ambros V. The timing of lin-4 RNA accumulation controls the timing of postembryonic developmental events in Caenorhabditis elegans. Dev Biol 1999; 210:87-95; PMID:10364429; http://dx.doi.org/10.1006/dbio.1999.9272
9. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 2000; 403:901-6; PMID:10706289; http://dx.doi.org/10.1038/35002607
10. Slack FJ, Basson M, Liu Z, Ambros V, Horvitz HR, Ruvkun G. The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol Cell 2000; 5:659-69; PMID:10882102; http://dx.doi.org/10.1016/S1097-2765(00)80245-2
11. Vadla B, Kemper K, Alaimo J, Heine C, Moss EG. lin-28 controls the succession of cell fate choices via two distinct activities. PLoS Genet 2012; 8:e1002588; PMID:22457637; http://dx.doi.org/10.1371/journal.pgen.1002588
12. Abrahante JE, Daul AL, Li M, Volk ML, Tennessen JM, Miller EA, Rougvie AE. The Caenorhabditis elegans hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. Dev Cell 2003; 4:625-37; PMID:12737799; http://dx.doi.org/10.1016/S1534-5807(03)00127-8
13. Lin S-Y, Johnson SM, Abraham M, Vella MC, Pasquinelli A, Gamberi C, Gottlieb E, Slack FJ. The C elegans hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. Dev Cell 2003; 4:639-50; PMID:12737800; http://dx.doi.org/10.1016/S1534-5807(03)00124-2
14. Abbott AL, Alvarez-Saavedra E, Miska EA, Lau NC, Bartel DP, Horvitz HR, Ambros V. The let-7 MicroRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in Caenorhabditis elegans. Dev Cell 2005; 9:403-14; PMID:16139228; http://dx.doi.org/10.1016/j.devcel.2005.07.009
15. Li M, Jones-Rhoades MW, Lau NC, Bartel DP, Rougvie AE. Regulatory mutations of mir-48, a C. elegans let-7 family MicroRNA, cause developmental timing defects. Dev Cell 2005; 9:415-22; PMID:16139229; http://dx.doi.org/10.1016/j.devcel.2005.08.002
16. Vella MC, Choi E-Y, Lin S-Y, Reinert K, Slack FJ. The C. elegans microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3'UTR. Genes Dev 2004; 18:132-7; PMID:14729570; http://dx.doi.org/10.1101/gad.1165404
17. Ambros V. A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell 1989; 57:49-57; PMID:2702689; http://dx.doi.org/10.1016/0092-8674(89)90171-2
18. Liu Z, Kirch S, Ambros V. The Caenorhabditis elegans heterochronic gene pathway controls stage-specific transcription of collagen genes. Development 1995; 121:2471-8; PMID:7671811
19. Rougvie AE, Ambros V. The heterochronic gene lin-29 encodes a zinc finger protein that controls a terminal differentiation event in Caenorhabditis elegans. Development 1995; 121:2491-500; PMID:7671813
20. Moss EG. Heterochronic genes and the nature of developmental time. Curr Biol 2007; 17:R425-34; PMID:17550772; http://dx.doi.org/10.1016/j.cub.2007.03.043
21. Roush S, Slack FJ. The let-7 family of microRNAs. Trends Cell Biol 2008; 18:505-16; PMID:18774294; http://dx.doi.org/10.1016/j.tcb.2008.07.007
22. Nimmo RA, Slack FJ. An elegant miRror: microRNAs in stem cells, developmental timing and cancer. Chromosoma 2009; 118:405-18; PMID:19340450; http://dx.doi.org/10.1007/s00412-009-0210-z
23. Ambros V. MicroRNAs and developmental timing. Curr Opin Genet Dev 2011; 21:511-7; PMID:21530229; http://dx.doi.org/10.1016/j.gde.2011.04.003
24. Mondol V, Pasquinelli AE. Let's make it happen: the role of let-7 microRNA in development. Curr Top Dev Biol 2012; 99:1-30; PMID:22365733; http://dx.doi.org/10.1016/B978-0-12-387038-4.00001-X
25. Rougvie AE, Moss EG. Developmental transitions in C. elegans larval stages. Curr Top Dev Biol 2013; 105:153-80; PMID:23962842; http://dx.doi.org/10.1016/B978-0-12-396968-2.00006-3
26. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Müller P, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 2000; 408:86-9; PMID:11081512; http://dx.doi.org/10.1038/35040556
27. Wu L, Belasco JG. Micro-RNA regulation of the mammalian lin-28 gene during neuronal differentiation of embryonal carcinoma cells. Mol Cell Biol 2005; 25:9198-208; PMID:16227573; http://dx.doi.org/10.1128/MCB.25.21.9198-9208.2005
28. Lee YS, Kim HK, Chung S, Kim K-S, Dutta A. Depletion of human micro-RNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the down-regulation of putative targets during differentiation. J Biol Chem 2005; 280:16635-41; PMID:15722555; http://dx.doi.org/10.1074/jbc.M412247200
29. Schulman BRM, Esquela-Kerscher A, Slack FJ. Reciprocal expression of lin-41 and the microRNAs let-7 and mir-125 during mouse embryogenesis. Dev Dyn 2005; 234:1046-54; PMID:16247770; http://dx.doi.org/10.1002/dvdy.20599
30. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, Horvitz HR, Kauppinen S, Plasterk RHA. MicroRNA expression in zebrafish embryonic development. Science 2005; 309:310-1; PMID:15919954; http://dx.doi.org/10.1126/science.1114519
31. Wulczyn FG, Smirnova L, Rybak A, Brandt C, Kwidzinski E, Ninnemann O, Strehle M, Seiler A, Schumacher S, Nitsch R. Post-transcriptional regulation of the let-7 microRNA during neural cell specification. FASEB J 2007; 21:415-26; PMID:17167072; http://dx.doi.org/10.1096/fj.06-6130com
32. Rybak A, Fuchs H, Smirnova L, Brandt C, Pohl EE, Nitsch R, Wulczyn FG. A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. Nat Cell Biol 2008; 10:987-93; PMID:18604195; http://dx.doi.org/10.1038/ncb1759
33. Friedman RC, Farh KK-H, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009; 19:92-105; PMID:18955434; http://dx.doi.org/10.1101/gr.082701.108
34. Alvarez-Garcia I, Miska EA. MicroRNA functions in animal development and human disease. Development 2005; 132:4653-62; PMID:16224045; http://dx.doi.org/10.1242/dev.02073
35. Büssing I, Slack FJ, Grosshans H. let-7 microRNAs in development, stem cells and cancer. Trends Mol Med 2008; 14:400-9; PMID:18674967; http://dx.doi.org/10.1016/j.molmed.2008.07.001
36. Medina PP, Slack FJ. microRNAs and cancer: an overview. Cell Cycle 2008; 7:2485-92; PMID:18719380; http://dx.doi.org/10.4161/cc.7.16.6453
37. Kato M, Slack FJ. microRNAs: small molecules with big roles - C. elegans to human cancer. Biol Cell 2008; 100:71-81; PMID:18199046; http://dx.doi.org/10.1042/BC20070078
38. Stefani G, Slack FJ. Small non-coding RNAs in animal development. Nat Rev Mol Cell Biol 2008; 9:219-30; PMID:18270516; http://dx.doi.org/10.1038/nrm2347
39. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ. RAS is regulated by the let-7 microRNA family. Cell 2005; 120:635-47; PMID:15766527; http://dx.doi.org/10.1016/j.cell.2005.01.014
40. Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, Wilson M, Wang X, Shelton J, Shingara J, et al. The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res 2007; 67:7713-22; PMID:17699775; http://dx.doi.org/10.1158/0008-5472.CAN-07-1083

41. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J, et al. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 2007; 131:1109-23; PMID:18083101; http://dx.doi.org/10.1016/j.cell.2007.10.054
42. Bousquet M, Harris MH, Zhou B, Lodish HF. MicroRNA miR-125b causes leukemia. Proc Natl Acad Sci U S A 2010; 107:21558-63; PMID:21118985; http://dx.doi.org/10.1073/pnas.1016611107
43. Klusmann J-H, Li Z, Böhmer K, Maroz A, Koch ML, Emmrich S, Godinho FJ, Orkin SH, Reinhardt D. miR-125b-2 is a potential oncomiR on human chromosome 21 in megakaryoblastic leukemia. Genes Dev 2010; 24:478-90; PMID:20194440; http://dx.doi.org/10.1101/gad.1856210
44. Mochly-Rosen D, Khaner H, Lopez J. Identification of intracellular receptor proteins for activated protein kinase C. Proc Natl Acad Sci U S A 1991; 88:3997-4000; PMID:1850844; http://dx.doi.org/10.1073/pnas.88.9.3997
45. Ron D, Chen CH, Caldwell J, Jamieson L, Orr E, Mochly-Rosen D. Cloning of an intracellular receptor for protein kinase C: a homolog of the beta subunit of G proteins. Proc Natl Acad Sci U S A 1994; 91:839-43; PMID:8302854; http://dx.doi.org/10.1073/pnas.91.3.839
46. Adams DR, Ron D, Kiely PA. RACK1, A multifaceted scaffolding protein: Structure and function. Cell Commun Signal 2011; 9:22; PMID:21978545; http://dx.doi.org/10.1186/1478-811X-9-22
47. Ceci M, Gaviraghi C, Gorrini C, Sala LA, Offenhäuser N, Marchisio PC, Biffo S. Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. Nature 2003; 426:579-84; PMID:14654845; http://dx.doi.org/10.1038/nature02160
48. Nilsson J, Sengupta J, Frank J, Nissen P. Regulation of eukaryotic translation by the RACK1 protein: a platform for signalling molecules on the ribosome. EMBO Rep 2004; 5:1137-41; PMID:15577927; http://dx.doi.org/10.1038/sj.embor.7400291
49. Sengupta J, Nilsson J, Gursky R, Spahn CMT, Nissen P, Frank J. Identification of the versatile scaffold protein RACK1 on the eukaryotic ribosome by cryo-EM. Nat Struct Mol Biol 2004; 11:957-62; PMID:15334071; http://dx.doi.org/10.1038/nsmb822
50. Coyle SM, Gilbert WV, Doudna JA. Direct link between RACK1 function and localization at the ribosome in vivo. Mol Cell Biol 2009; 29:1626-34; PMID:19114558; http://dx.doi.org/10.1128/MCB.01718-08
51. Jannot G, Bajan S, Giguère NJ, Bouasker S, Banville IH, Piquet S, Hutvagner G, Simard MJ. The ribosomal protein RACK1 is required for microRNA function in both C. elegans and humans. EMBO Rep 2011; 12:581-6; PMID:21525958; http://dx.doi.org/10.1038/embor.2011.66
52. Kouba T, Rutkai E, Karásková M, Valášek L. The eIF3c/NIP1 PCI domain interacts with RNA and RACK1/ASC1 and promotes assembly of translation preinitiation complexes. Nucleic Acids Res 2012; 40:2683-99; PMID:22123745; http://dx.doi.org/10.1093/nar/gkr1083
53. Li S, Esterberg R, Lachance V, Ren D, Radde-Gallwitz K, Chi F, Parent J-L, Fritz A, Chen P. Rack1 is required for Vangl2 membrane localization and planar cell polarity signaling while attenuating canonical Wnt activity. Proc Natl Acad Sci U S A 2011; 108:2264-9; PMID:21262816; http://dx.doi.org/10.1073/pnas.1013170108
54. Otsuka M, Takata A, Yoshikawa T, Kojima K, Kishikawa T, Shibata C, Takekawa M, Yoshida H, Omata M, Koike K. Receptor for activated protein kinase C: requirement for efficient microRNA function and reduced expression in hepatocellular carcinoma. PLoS One 2011; 6:e24359; PMID:21935400; http://dx.doi.org/10.1371/journal.pone.0024359
55. Speth C, Willing E-M, Rausch S, Schneeberger K, Laubinger S. RACK1 scaffold proteins influence miRNA abundance in Arabidopsis. Plant J 2013; 76:433-45; PMID:23941160; http://dx.doi.org/10.1111/tpj.12308
56. Speth C, Laubinger S. RACK1 and the microRNA pathway: Is it déjà-vu all over again? Plant Signal Behav 2014; 9:e27909; PMID:24521556; http://dx.doi.org/10.4161/psb.27909
57. Chan S-P, Slack FJ. Ribosomal protein RPS-14 modulates let-7 microRNA function in Caenorhabditis elegans. Dev Biol 2009; 334:152-60; PMID:19627982; http://dx.doi.org/10.1016/j.ydbio.2009.07.011
58. Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell 2001; 106:23-34; PMID:11461699; http://dx.doi.org/10.1016/S0092-8674(01)00431-7
59. Grosshans H, Johnson T, Reinert KL, Gerstein M, Slack FJ. The temporal patterning microRNA let-7 regulates several transcription factors at the larval to adult transition in C. elegans. Dev Cell 2005; 8:321-30; PMID:15737928; http://dx.doi.org/10.1016/j.devcel.2004.12.019
60. Ding XC, Slack FJ, Grosshans H. The let-7 microRNA interfaces extensively with the translation machinery to regulate cell differentiation. Cell Cycle 2008; 7:3083-90; PMID:18818519; http://dx.doi.org/10.4161/cc.7.19.6778
61. Hurschler BA, Harris DT, Grosshans H. The type II poly(A)-binding protein PABP-2 genetically interacts with the let-7 miRNA and elicits heterochronic phenotypes in Caenorhabditis elegans. Nucleic Acids Res 2011; 39:5647-57; PMID:21415013; http://dx.doi.org/10.1093/nar/gkr145
62. Bossé GD, Rüegger S, Ow MC, Vasquez-Rifo A, Rondeau EL, Ambros VR, Grosshans H, Simard MJ. The decapping scavenger enzyme DCS-1 controls microRNA levels in Caenorhabditis elegans. Mol Cell 2013; 50:281-7; PMID:23541767; http://dx.doi.org/10.1016/j.molcel.2013.02.023
63. Ambros V, Horvitz HR. Heterochronic mutants of the nematode Caenorhabditis elegans. Science 1984; 226:409-16; PMID:6494891; http://dx.doi.org/10.1126/science.6494891
64. Sulston JE, Horvitz HR. Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev Biol 1977; 56:110-56; PMID:838129; http://dx.doi.org/10.1016/0012-1606(77)90158-0
65. Koh K, Rothman JH. ELT-5 and ELT-6 are required continuously to regulate epidermal seam cell differentiation and cell fusion in C. elegans. Development 2001; 128:2867-80; PMID:11532911
66. Mohler WA, Simske JS, Williams-Masson EM, Hardin JD, White JG. Dynamics and ultrastructure of developmental cell fusions in the Caenorhabditis elegans hypodermis. Curr Biol 1998; 8:1087-90; PMID:9768364; http://dx.doi.org/10.1016/S0960-9822(98)70447-6
67. Nguyen CQ, Hall DH, Yang Y, Fitch DH. Morphogenesis of the Caenorhabditis elegans male tail tip. Dev Biol 1999; 207:86-106; PMID:10049567; http://dx.doi.org/10.1006/dbio.1998.9173
68. Sulston JE, Albertson DG, Thomson JN. The Caenorhabditis elegans male: postembryonic development of nongonadal structures. Dev Biol 1980; 78:542-76; PMID:7409314; http://dx.doi.org/10.1016/0012-1606(80)90352-8
69. Del Rio-Albrechtsen T, Kiontke K, Chiou S-Y, Fitch DHA. Novel gain-of-function alleles demonstrate a role for the heterochronic gene lin-41 in C. elegans male tail tip morphogenesis. Dev Biol 2006; 297:74-86; PMID:16806150; http://dx.doi.org/10.1016/j.ydbio.2006.04.472
70. Fraser AG, Kamath RS, Zipperlen P, Martinez-Campos M, Sohrmann M, Ahringer J. Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature 2000; 408:325-30; PMID:11099033; http://dx.doi.org/10.1038/35042517
71. Van Wynsberghe PM, Kai ZS, Massirer KB, Burton VH, Yeo GW, Pasquinelli AE. LIN-28 co-transcriptionally binds primary let-7 to regulate miRNA maturation in Caenorhabditis elegans. Nat Struct Mol Biol 2011; 18:302-8; PMID:21297634; http://dx.doi.org/10.1038/nsmb.1986
72. Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev 2001; 15:2654-9; PMID:11641272; http://dx.doi.org/10.1101/gad.927801
73. Kai ZS, Finnegan EF, Huang S, Pasquinelli AE. Multiple cis-elements and trans-acting factors regulate dynamic spatio-temporal transcription of let-7 in Caenorhabditis elegans. Dev Biol 2013; 374:223-33; PMID:23201578; http://dx.doi.org/10.1016/j.ydbio.2012.11.021
74. Jeon M, Gardner HF, Miller EA, Deshler J, Rougvie AE. Similarity of the C. elegans developmental timing protein LIN-42 to circadian rhythm proteins. Science 1999; 286:1141-6; PMID:10550049; http://dx.doi.org/10.1126/science.286.5442.1141
75. Tennessen JMJ, Gardner HFH, Volk MLM, Rougvie AEA. Novel heterochronic functions of the Caenorhabditis elegans period-related protein LIN-42. Dev Biol 2006; 289:30-43; PMID:16300753; http://dx.doi.org/10.1016/j.ydbio.2005.09.044
76. Abrahante JE, Miller EA, Rougvie AE. Identification of heterochronic mutants in Caenorhabditis elegans. Temporal misexpression of a collagen:green fluorescent protein fusion gene. Genetics 1998; 149:1335-51; PMID:9649524
77. Van Wynsberghe PM, Finnegan EF, Stark T, Angelus EP, Homan KE, Yeo GW, Pasquinelli AE. The Period protein homolog LIN-42 negatively regulates microRNA biogenesis in C. elegans. Dev Biol 2014; PMID:24699545; http://dx.doi.org/10.1016/j.ydbio.2014.03.017
78. Ameres SL, Horwich MD, Hung J-H, Xu J, Ghildiyal M, Weng Z, Zamore PD. Target RNA-directed trimming and tailing of small silencing RNAs. Science 2010; 328:1534-9; PMID:20558712; http://dx.doi.org/10.1126/science.1187058
79. Chatterjee S, Fasler M, Büssing I, Grosshans H. Target-mediated protection of endogenous microRNAs in C. elegans. Dev Cell 2011; 20:388-96; PMID:21397849; http://dx.doi.org/10.1016/j.devcel.2011.02.008
80. Park W, Li J, Song R, Messing J, Chen X. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Curr Biol 2002; 12:1484-95; PMID:12225663; http://dx.doi.org/10.1016/S0960-9822(02)01017-5
81. Tops BBJ, Plasterk RHA, Ketting RF. The Caenorhabditis elegans Argonautes ALG-1 and ALG-2: almost identical yet different. Cold Spring Harb Symp Quant Biol 2006; 71:189-94; PMID:17381296; http://dx.doi.org/10.1101/sqb.2006.71.035
82. O'Carroll D, Mecklenbrauker I, Das PP, Santana A, Koenig U, Enright AJ, Miska EA, Tarakhovsky A. A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway. Genes Dev 2007; 21:1999-2004; PMID:17626790; http://dx.doi.org/10.1101/gad.1565607

83. Tan GS, Garchow BG, Liu X, Yeung J, Morris JP 4th, Cuellar TL, McManus MT, Kiriakidou M. Expanded RNA-binding activities of mammalian Argonaute 2. Nucleic Acids Res 2009; 37:7533-45; PMID:19808937; http://dx.doi.org/10.1093/nar/gkp812
84. Diederichs S, Haber DA. Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. Cell 2007; 131:1097-108; PMID:18083100; http://dx.doi.org/10.1016/j.cell.2007.10.032
85. Bouasker S, Simard MJ. The slicing activity of miRNA-specific Argonautes is essential for the miRNA pathway in C. elegans. Nucleic Acids Res 2012; 40:10452-62; PMID:22904066; http://dx.doi.org/10.1093/nar/gks748
86. Zisoulis DG, Kai ZS, Chang RK, Pasquinelli AE. Autoregulation of microRNA biogenesis by let-7 and Argonaute. Nature 2012; 486:541-4; PMID:22722835
87. Brenner S. The genetics of Caenorhabditis elegans. Genetics 1974; 77:71-94; PMID:4366476
88. Emmons SW, Klass MR, Hirsh D. Analysis of the constancy of DNA sequences during development and evolution of the nematode Caenorhabditis elegans. Proc Natl Acad Sci U S A 1979; 76:1333-7; PMID:286315; http://dx.doi.org/10.1073/pnas.76.3.1333
89. Meneely PMP, Herman RKR. Lethals, steriles and deficiencies in a region of the X chromosome of Caenorhabditis elegans. Genetics 1979; 92:99-115; PMID:574105
90. Chan S-P, Ramaswamy G, Choi EY, Slack FJ. Identification of specific let-7 microRNA binding complexes in Caenorhabditis elegans. RNA 2008; 14:2104-14; PMID:18719242; http://dx.doi.org/10.1261/rna.551208
91. Van Wynsberghe PM, Chan S-P, Slack FJ, Pasquinelli AE. Analysis of microRNA expression and function. Methods Cell Biol 2011; 106:219-52; PMID:22118279; http://dx.doi.org/10.1016/B978-0-12-544172-8.00008-6
92. Sambrook J, Russell DW. Synthesis of single-stranded RNA probes by in vitro transcription. CSH Protoc 2006; 2006:pdb.prot3847; PMID:22485332