nature structural & molecular biology

LIN-28 co-transcriptionally binds primary let-7 to regulate miRNA maturation in *Caenorhabditis elegans*

Priscilla M Van Wynsberghe[1], Zoya S Kai[1], Katlin B Massirer[2–4], Victoria H Burton[1], Gene W Yeo[2–4] & Amy E Pasquinelli[1]

The highly conserved let-7 microRNA (miRNA) regulates developmental pathways across animal phyla. Mis-expression of let-7 causes lethality in *C. elegans* and has been associated with several human diseases. We show that timing of let-7 expression in developing worms is under complex transcriptional and post-transcriptional control. Expression of let-7 primary transcripts oscillates during each larval stage, but precursor and mature let-7 miRNAs do not accumulate until later in development after LIN-28 protein has diminished. We demonstrate that LIN-28 binds endogenous primary let-7 transcripts co-transcriptionally. We further show that LIN-28 binds endogenous primary let-7 transcripts in the nuclear compartment of human ES cells, suggesting that this LIN-28 activity is conserved across species. We conclude that co-transcriptional interaction of LIN-28 with let-7 primary transcripts blocks Drosha processing and, thus, precocious expression of mature let-7 during early development.

MicroRNAs (miRNAs) function as ~22-nucleotide (nt) guide RNAs in the RNA-induced silencing complex (RISC) by binding to partially complementary sites in target mRNAs, causing inhibition of translation or destabilization[1]. Typically, mature miRNAs originate from long, capped and polyadenylated primary miRNAs (pri-miRNAs) that are transcribed by RNA polymerase II[1]. Endonucleolytic cleavage of the pri-miRNA by the RNase III enzyme Drosha in cooperation with the RNA-binding protein Pasha (also known as DGCR8) releases the ~70-nt hairpin precursor miRNA (pre-miRNA)[1]. Exportin-5 translocates the pre-miRNA to the cytoplasm, where subsequent endonucleolytic cleavage by the RNase III enzyme Dicer produces the mature miRNA that functions in the RISC complex[1,2].

Originally discovered in *C. elegans*, the let-7 miRNA is conserved across species in both sequence and temporal expression[3,4]. In *C. elegans*, let-7 regulates developmental timing and promotes cellular differentiation pathways[5,6]. The human let-7 miRNAs also have antiproliferative functions, and downregulation of let-7 levels is associated with many cancers, including those of the lung, breast and colon[5,6]. Overexpression of let-7 early in worm development causes premature adoption of adult fates, whereas cells in let-7 underexpression mutants fail to terminally differentiate at the larval-to-adult transition[4]. Thus, the level and timing of mature miRNA expression are paramount in determining organismal development.

The worm *let-7* gene encodes two nascent and one *trans*-spliced primary transcripts (**Fig. 1a**)[7]. Deletion of the 3′ splice site sequence, required for *trans* splicing, abolishes let-7 rescue activity, indicating that the splicing event or the sequence and structural changes produced by it are important for let-7 biogenesis[7]. Accumulation of mature let-7 is first observed during the third larval stage (L3) and is maintained into adulthood[4]. Recently, LIN-28 protein activity was shown to prevent premature accumulation of let-7 in the second larval stage (L2)[8]. The *lin-28* gene encodes a nucleocytoplasmic localized cold-shock domain– and zinc finger–containing protein that is conserved across animal species[9–13]. The LIN-28 protein is expressed early in worm development but is downregulated by a factor of more than 10 from L1 to L3 through the action of lin-4 miRNA and other pathways[9,14,15]. Decreases in LIN-28 protein levels coincide with mature let-7 accumulation during the L3 stage[4,14]. Likewise, opposite expression patterns for LIN-28 protein and mature let-7 miRNA have been documented in several mammalian cell types[12,16–19]. Moreover, LIN-28 has been shown to regulate the accumulation of mature let-7 miRNA in mammalian systems through multiple mechanisms, including blocked Drosha or Dicer processing and destabilization of let-7 precursor RNAs[16–22]. What determines the utilization of one mechanism versus another to regulate accumulation of mature let-7 *in vivo* has yet to be resolved.

In this study we examine the role of LIN-28 in regulating endogenous let-7 expression in a whole organism throughout development. We find that let-7 primary transcript expression is dynamic and accumulation of primary transcripts is uncoupled from pre- and mature let-7 in wild-type (WT) but not *lin-28* mutant animals. We further show that LIN-28 binds endogenous pri-let-7 in both *C. elegans* and human embryonic stem cells and that this interaction is co-transcriptional in *C. elegans*. Altogether our results suggest that LIN-28 acts co-transcriptionally at the Drosha processing step to inhibit precocious expression of let-7 during animal development.

[1]Department of Biology, University of California, San Diego, La Jolla, California, USA. [2]Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, California, USA. [3]Stem Cell Program, University of California, San Diego, La Jolla, California, USA. [4]Institute for Genomic Medicine, University of California, San Diego, La Jolla, California, USA. Correspondence should be addressed to A.E.P. (apasquin@ucsd.edu).

Received 15 May 2010; accepted 23 November 2010; published online 6 February 2011; doi:10.1038/nsmb.1986

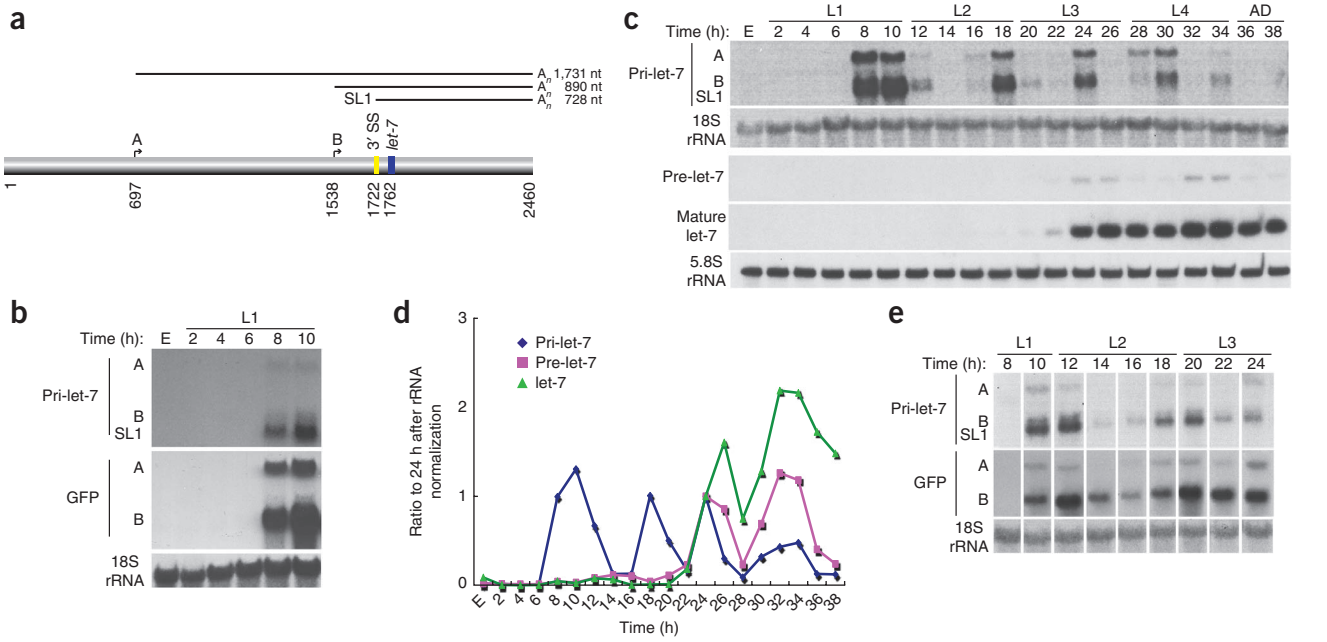


Figure 1 Expression of let-7 is transcriptionally and post-transcriptionally regulated. (**a**) Depiction of the 2,460-nt-long let-7 rescue construct with the positions of the mature let-7 sequence (blue), 3′ splice site (SS; yellow) and two start sites (A and B) and the approximate sizes of the spliced and unspliced transcripts indicated[4,7]. (**b**) Northern blot analysis of total RNA isolated from embryos (E) or synchronized plet-7_B::GFP transgenic worms. The similar-sized B and SL1 transcripts often do not clearly resolve. (**c**) Agarose or PAGE northern blot analysis of total RNA isolated from embryos (E) or synchronized WT N2 worms at larval (L) and adult (AD) stages. Representative blots from four independent experiments are shown. (**d**) Average levels of pri-, pre- and mature let-7 after normalization to 18S or 5.8S rRNA from four independent experiments. (**e**) Analysis as in **b** of total RNA isolated from synchronized plet-7_B::GFP transgenic worms. The entire blot is shown in **Supplementary Figure 1c**.

The ability of LIN-28 to interact with primary let-7 transcripts as they are being synthesized provides an efficient mechanism for blocking production of this essential miRNA in multiple organisms.

RESULTS

Uncoupling of primary and mature let-7 miRNA expression

Mature let-7 miRNA accumulates during the third larval stage (L3) of development in *C. elegans*[4,7,23,24]. Previous studies also found that the two unspliced (A and B) and one *trans*-spliced (SL1) pri-let-7 transcripts were first detected during the L3 stage, suggesting that production of mature let-7 is transcriptionally regulated[7]. However, reporter constructs consisting of green fluorescent protein (GFP) fused to sequences upstream of mature let-7 revealed potential transcriptional activity earlier than the L3 stage[23,25,26]. In agreement with this, we observed fluorescence at the end of the L1 stage in transgenic worms that express GFP fused to the pri-let-7B start site (data not shown). Detection of GFP mRNA, driven by both *let-7* promoter A and B sequences in the transgenic worms, mirrored that of endogenous let-7 primary transcripts, indicating that expression of let-7 is repressed largely at the transcriptional level from embryogenesis until the late L1 stage (**Fig. 1b** and **Supplementary Fig. 1a**).

To further investigate the possibility of uncoupled expression of let-7 primary and mature RNAs, we used northern blotting and quantitative reverse transcription PCR (qRT-PCR) to analyze the endogenous expression patterns of all three pri-let-7 isoforms as well as pre- and mature let-7 in RNA collected from embryos and every 2 h during larval development to adulthood (**Fig. 1c**,**d** and **Supplementary Figs. 1b** and **2**). Consistent with our reporter analysis, we first observed pri-let-7 during the late L1 stage (**Fig. 1c**,**d**). We detected all three pri-let-7 isoforms, and coordinate expression of these isoforms oscillated throughout development (**Fig. 1c**,**d** and **Supplementary Fig. 2**). This cycling pattern of expression was specific to pri-let-7, given that other endogenous mRNAs, such as *act-1*, maintained steady levels throughout the time course (**Supplementary Fig. 1b**). The low levels of pri-let-7 at most mid-larval time points and the slight shifts in the timing of pri-let-7 expression between experiments indicate that expression of endogenous pri-let-7 transcripts is dynamic, and that even slight changes in culture conditions can affect the rate of development and thus pri-let-7 expression (**Fig. 1** and **Supplementary Fig. 1a**,**b**). Therefore, the failure of prior studies to detect expression of let-7 primary transcripts in L1 and L2 was likely due to the analysis of only single time points at each stage[7]. GFP mRNA levels of our *let-7* promoter reporter oscillated with a frequency identical to that of endogenous pri-let-7 expression, suggesting that transcriptional mechanisms largely control the cycling pattern of pri-let-7 expression (**Fig. 1e** and **Supplementary Fig. 1c**).

Consistent with previous reports, pre- and mature let-7 RNAs were undetectable until the L3 stage, and mRNA levels of the let-7 target *lin-41* decreased concordantly with the appearance of let-7 (**Fig. 1c**,**d** and **Supplementary Fig. 1b**)[4,24]. In the L3 and L4 stages, levels of pre-let-7 oscillated in parallel to those of pri-let-7, while mature let-7 accumulated to a relatively constant level (**Fig. 1c**,**d** and **Supplementary Fig. 1b**). Taken together, our results indicate that expression of let-7 is regulated by transcriptional and post-transcriptional control mechanisms during development in *C. elegans*.

Primary let-7 processing is developmentally regulated

The detection of primary but not precursor or mature let-7 in the first two larval stages could be due to blocked Drosha processing of pri-let-7 or to destabilization of pre- or mature let-7 RNAs. To distinguish between these possibilities, we used a sensitive cloning

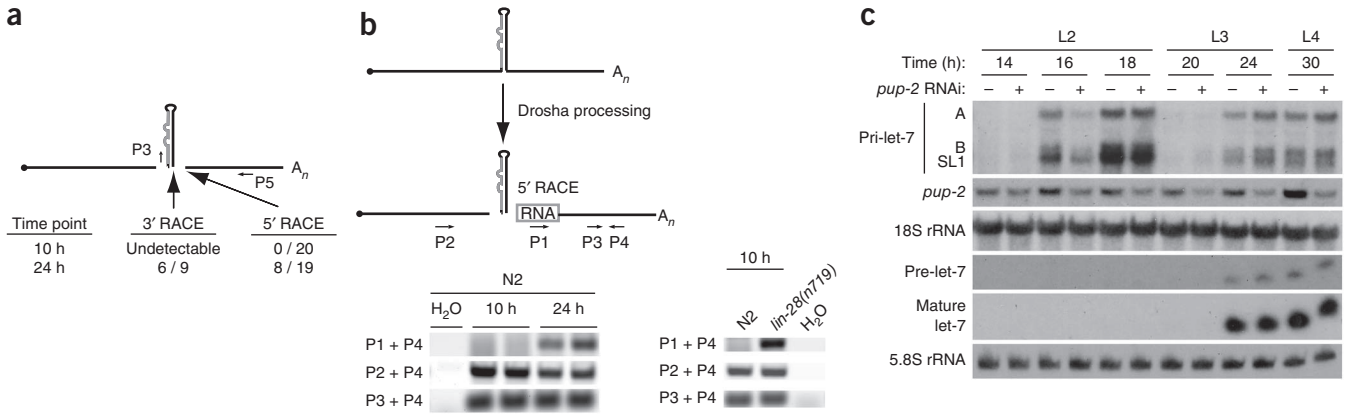


Figure 2 Developmentally regulated processing of let-7 pri-miRNA transcripts. (**a**) Depiction of expected Drosha cleavage products: 5′ flanking, let-7 hairpin precursor and 3′ flanking. The number of sequenced RACE clones that mapped to the precise 3′ and 5′ Drosha cleavage products at each time point from two independent experiments is shown. The sequences of all Drosha cleavage products are shown in **Supplementary Figure 3**. (**b**) RT-PCR analysis of two independent 5′ RACE samples from N2 (left) or N2 and *lin-28(n719)* worms (right). (**c**) Agarose and PAGE northern blot analysis of total RNA isolated from synchronized *eri-1(mg366)* RNAi-hypersensitive worms at the indicated time points after vector control (–) or *pup-2* (+) RNAi treatment. Representative blots from three independent experiments are shown.

strategy to detect potential Drosha cleavage products and/or degradation intermediates. Drosha processing is expected to release the let-7 miRNA hairpin precursor from primary transcripts, leaving specific 5′ and 3′ products comprised of flanking sequences. We assayed for these cleavage products by performing 5′ or 3′ RNA oligo ligation reactions using total RNA isolated from 10-h (L1) and 24-h (L3) time points, and then conducted standard rapid amplification of cDNA ends (RACE) cloning experiments to detect the ligation junctions. Drosha cleavage products were evident in the 24-h RNA sample, as the majority of 3′ RACE results mapped to the 3′ end of the let-7 precursor and almost half of the 5′ RACE results mapped to the expected cleavage site between the precursor and 3′ product (**Fig. 2a** and **Supplementary Fig. 3**). In contrast, no 5′ RACE products from the 10-h time point mapped to canonical Drosha cleavage sites; instead these clones may represent general degradation intermediates (**Fig. 2a** and **Supplementary Fig. 3**). The 3′ RACE of 10-h RNA samples, which was performed in parallel with RNA from the 24-h time point, yielded no products that could be cloned (**Fig. 2a**). Because we purposefully selected clones from the 5′ RACE with different-sized inserts, the identification of 8/19 clones that mapped to the Drosha cleavage site from the 24-h RNA sample is not a quantitative measure of frequency. Indeed, another 5′ RACE clone from the 24-h RNA sample mapped to the Drosha cleavage position at the 5′ end of the let-7 hairpin, likely representing a molecule in which 3′ cleavage had not yet been accomplished (**Supplementary Fig. 3**).

To further assess the presence of Drosha cleavage products within the primary transcript population of N2 (wild type, WT) worms at the 10- versus 24-h time points, we analyzed the 3′ cleavage products by PCR. 5′ RACE cDNA samples were amplified with primers corresponding to the 5′ RNA oligo linker (P1), the pri-let-7 sequence upstream of the let-7 hairpin (P2) or the pri-let-7 sequence downstream of the cleavage site (P3) and a common reverse primer (P4) (**Fig. 2b**). No amplification of the P1+P4 PCR product was detected at the 10-h time point from two independent samples, whereas consistent amplification was seen from 24-h samples (**Fig. 2b**). The P2+P4 PCR product was detected at a slightly higher level at 10 versus 24 h, whereas the P3+P4 PCR product was readily detected from all samples at both time points at similar levels (**Fig. 2b**). These differences in detection of Drosha cleavage products at 10 and 24 h indicate that processing of let-7 primary transcripts is inhibited during the first larval stages of development.

A recent study reported that RNAi inactivation of the *pup-2* poly(U) polymerase gene results in increased levels of a precursor let-7 miRNA processed from transcripts encoded by a transgene with truncated let-7 sequences driven by a heterologous promoter[8]. Using similar RNAi conditions, we also achieved an approximately 50% decrease in *pup-2* mRNA levels but did not detect substantial effects on the accumulation of let-7 RNAs (**Fig. 2c**). The strong pulse of endogenous let-7 primary transcript expression during L2 did not give rise to detectable precursor in vector control or *pup-2* RNAi samples (**Fig. 2c**). No appreciable difference in accumulation of precursor or mature let-7 miRNA during the L3 and L4 stages was observed in worms depleted of *pup-2* mRNA compared to controls (**Fig. 2c**). Similar results were also observed in the *pup-2(tm4344)* deletion strain (**Supplementary Fig. 4**). Thus, regulation of endogenous let-7 miRNA expression is independent of PUP-2 activity. Altogether our results indicate that regulation of let-7 processing occurs at a step before precursor formation in developing worms.

LIN-28 blocks early accumulation of mature let-7 miRNA

lin-28 acts upstream of let-7 in the *C. elegans* developmental timing pathway[6], and multiple mechanisms by which LIN-28 may inhibit let-7 expression have been proposed[8,16–22]. Thus, we next tested whether LIN-28 mediates post-transcriptional regulation of endogenous let-7 expression in *C. elegans*. In contrast to N2 worms, we observed accumulation of mature let-7 concordant with expression of pri-let-7 in *lin-28(n719)* putative null mutant worms (**Fig. 3a**). In RNA samples from N2 and *lin-28(n719)* worms, primary let-7 transcripts were undetectable in embryos and early L1, but by the 10-h L1 time point, unspliced pri-let-7 RNAs were apparent in both strains (**Fig. 3a**). Because *lin-28(n719)* worms develop precociously and skip the L2 stage of development[9], pri-let-7 levels at the 24-h time point in *lin-28(n719)* worms resemble the decreased levels observed in N2 at the later L4 stage (**Fig. 3a** and **Supplementary Figs. 2** and **5**). Notably, precursor and mature let-7 accumulated, whereas the SL1 *trans*-spliced primary transcript was under-represented, in *lin-28* mutants at the 10-h time point

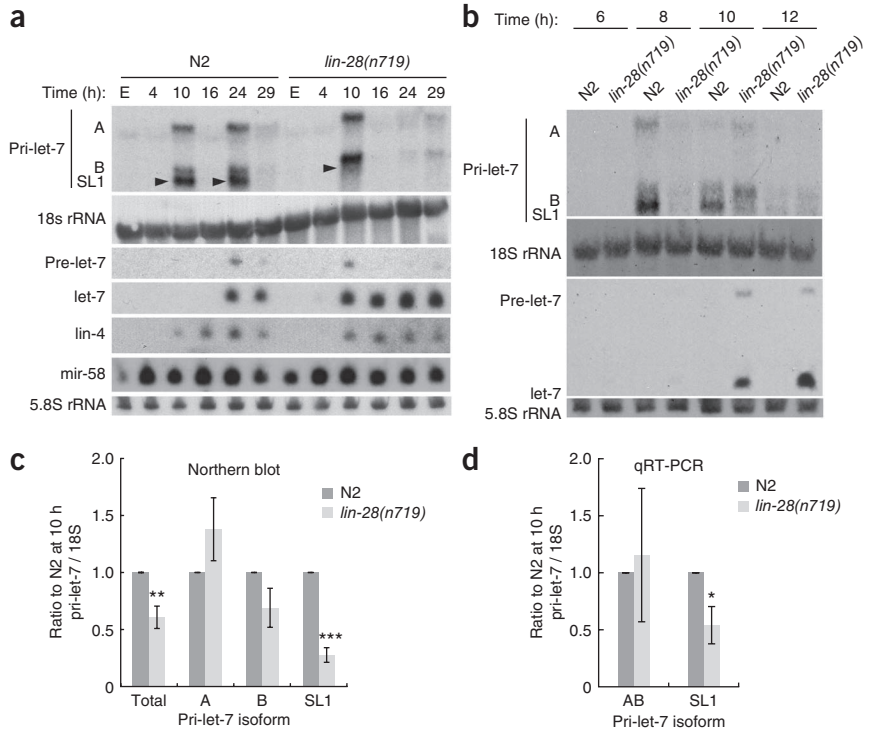


Figure 3 Regulation of let-7 processing by LIN-28. (**a**) Agarose and PAGE northern blotting analysis of total RNA isolated from WT N2 or *lin-28(n719)* embryos (E) and synchronized worms at the indicated time points. Representative blots from three independent experiments are shown. The arrowheads mark the location of the SL1 pri-let-7 transcript. (**b**) Analysis as in **a** of total RNA from late L1 and early L2. Representative blots from three independent experiments are shown. (**c**,**d**) Levels of each pri-let-7 isoform at the 10-h time point in *lin-28(n719)* relative to WT N2 worms after normalization of 18S rRNA, calculated from six independent northern blot experiments (**c**) or three independent qRT-PCR experiments (**d**) and analyzed by Student's *t*-test (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$). Error bars, s.e.m.

(**Fig. 3a**). Consistent with these results, we detected 3′ Drosha cleavage products of pri-let-7 in *lin-28(n719)* worms at the 10-h time point (**Fig. 2b**). Thus, maturation of let-7 occurs two stages earlier in *lin-28(n719)* as compared to WT worms (**Fig. 3a**). Expression of mature lin-4 and mir-58 miRNAs was unaffected in *lin-28(n719)* worms (**Fig. 3a** and **Supplementary Fig. 6**), indicating that LIN-28 has a specific role in the regulation of let-7 as opposed to a general role in miRNA biogenesis.

Closer analysis of pri-let-7 levels during the late L1 and early L2 stages revealed significantly reduced levels of total pri-let-7 during the initial peak of expression at 10 h in *lin-28(n719)* compared to WT worms (**Fig. 3b**,**c**). Furthermore, this reduction is largely accounted for by under-representation of the SL1 *trans*-spliced primary transcript isoform, as seen by both northern blotting and qRT-PCR analyses (**Fig. 3b**–**d**). The correlation between decreased pri-let-7 levels and increased pre- and mature let-7 levels in *lin-28* mutant worms suggests that LIN-28 normally functions to block processing of primary to precursor let-7 during development in *C. elegans*.

LIN-28 interacts with endogenous primary let-7 transcripts

Expression of LIN-28 protein is developmentally regulated, with strongly reduced levels by the mid-L3 stage when mature let-7 begins to accumulate[4,9,14]. Decreased LIN-28 in mammalian cells and tissues has also been linked to upregulation of mature let-7 (refs. 12,16–19). Furthermore, association of LIN-28 with let-7 primary or precursor RNAs expressed from transgenes in cell culture or synthesized *in vitro* has been shown to block processing or promote degradation of these substrates, respectively[16–22]. Because our results suggest that LIN-28 inhibits the processing step from pri- to pre-let-7, we tested whether LIN-28 binds endogenous let-7 primary transcripts in *C. elegans* by RNA immunoprecipitation (RIP). We used a strain that expresses LIN-28 tagged with GFP in the *lin-28(n719)* mutant background (PQ272); the integrated transgene fully rescues *lin-28* mutant phenotypes and is developmentally regulated like the endogenous protein, with a gradual reduction from late L1 to L3 (**Fig. 4a**,**b**)[9,14]. Extracts from 10-h late L1 transgenic worms were used for RIP experiments to test for specific association of let-7 and control RNAs with LIN-28:GFP (**Fig. 4c** and **Supplementary Fig. 7a**). Primers designed to amplify all three isoforms of pri-let-7 produced a robust signal from the anti-GFP precipitate. The unspliced A and B transcripts and the SL1 *trans*-spliced isoform were co-immunoprecipitated with LIN-28:GFP, indicating that LIN-28 does not substantially discriminate among these let-7 primary transcripts (**Fig. 4c** and **Supplementary Fig. 7a**). Sequences upstream of the A start site in the *let-7* gene could not be amplified, confirming that the PCR signals are dependent on RNA transcripts (**Fig. 4c** and **Supplementary Fig. 7a**). Additionally, signals for the abundant actin mRNA or other primary miRNA transcripts, such as pri-mir-58, were not enriched in the LIN-28:GFP immunoprecipitates (**Fig. 4c** and **Supplementary Fig. 7a**), indicating that LIN-28 specifically binds let-7 primary transcripts in *C. elegans*.

Although LIN-28 has been reported to regulate Drosha processing in mammalian embryonic stem (ES) cells, association of LIN-28 with endogenous let-7 primary transcripts has not yet been demonstrated[16,17]. To determine whether LIN-28 also binds human pri-let-7 transcripts *in vivo*, we performed RIP in the human embryonic stem cell line HUES6. As a positive control, *oct-4* mRNA was specifically detected in the LIN-28 immunoprecipitate (**Fig. 4d** and **Supplementary Fig. 7b**)[27]. Primary transcript sequences for human let-7a-1, let-7g or let-7i also were present in the anti–LIN-28 immunoprecipitation samples (**Fig. 4d** and **Supplementary Fig. 7b**). In contrast, other primary miRNA transcripts expressed in ES cells, such as pri-mir-21 and pri-mir-16-1 (ref. 28), were not enriched in the LIN-28 immunoprecipitates (**Fig. 4d** and **Supplementary Fig. 7b**). Thus, LIN-28 binds endogenous let-7 primary transcripts in worm and human cells.

To determine if LIN-28 bound pre-let-7 in addition to pri-let-7, we performed quantitative PCR (qPCR) after RIP with primers specific for pri-let-7 (pri_F and pri_R) or primers residing within the precursor sequence (pre_F and pre_R), which would amplify cDNA representing precursor and the hairpin-containing primary let-7 transcripts (**Fig. 4e**). Comparison of the LIN-28 immunoprecipitated pre- to pri-let-7 signal

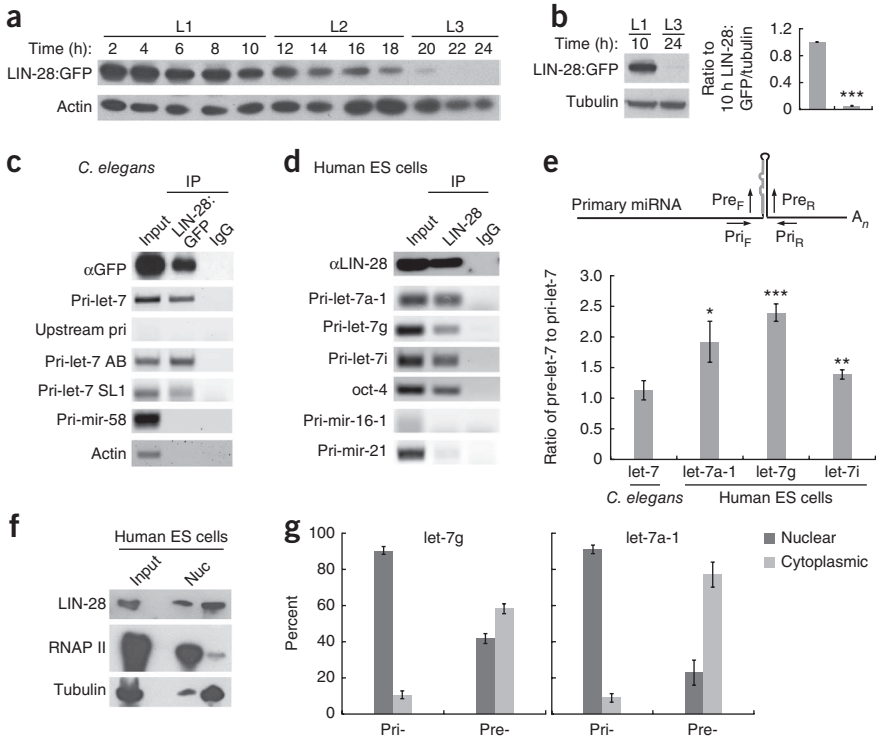
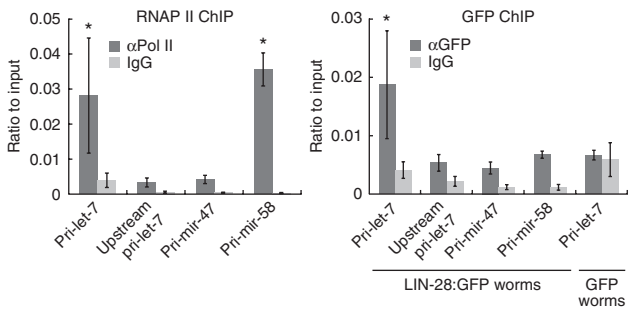


Figure 4 LIN-28 binds endogenous let-7 primary transcripts in *C. elegans* and human ES cells. (**a**,**b**) Western blot analysis of total protein isolated from PQ272 (LIN-28:GFP) worms. (**b**) Ratios of LIN-28:GFP levels to the 10-h time point after tubulin normalization, calculated from three independent experiments and analyzed by Student's *t*-test (***$P < 0.0005$). Error bars, s.e.m. (**c**) RNA immunoprecipitation (RIP) analysis of synchronized PQ272 worms collected at 10 h. Input, and LIN-28:GFP and IgG immunoprecipitates, were analyzed by western blotting or RT-PCR. α, antibody. (**d**) RIP analysis of undifferentiated HUES6 cells. Input, LIN-28 and IgG immunoprecipitates were analyzed by western blotting or RT-PCR. (**e**) qRT-PCR analysis of the worm and human cell samples from **c**,**d** to determine the levels of input or LIN-28 immunoprecipitated pri- or pre-let-7 RNAs using primers specific for pri-let-7 (pri_F and pri_R) or pre-let-7 and pri-let-7 transcripts containing the precursor hairpin (pre_F and pre_R). The ratio of precursor containing let-7 transcripts to pri-let-7 transcripts for immunoprecipitated samples after normalization to input samples for at least three independent experiments is shown, and was analyzed by Student's *t*-test (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$). Error bars, s.e.m. (**f**,**g**) RIP and western blotting analysis (**f**) or qRT-PCR analysis as in **e** (**g**) of undifferentiated HUES6 cells fractionationated into nuclear and cytoplasmic extracts. Results from three independent experiments are shown. RNAP II, RNA polymerase II.

showed no significant increase in precursor compared to primary let-7 levels in *C. elegans* (**Fig. 4e**). However, though the amount of the increase differed among the *let-7* genes, the ratio of precursor to primary for each human let-7 isoform was significantly higher than 1 (**Fig. 4e**). Thus, in *C. elegans* LIN-28 predominately interacts with endogenous let-7 primary transcripts, whereas in human ES cells LIN-28 interacts with both endogenous primary and precursor let-7 transcripts.

To determine the cellular location of LIN-28 interaction with endogenous pri- and pre-let-7, we performed RIP on fractionated HUES6 cells (**Fig. 4f**,**g**). Consistent with the results of prior studies in *C. elegans* and human cells[9,11,13,18], we detected endogenous LIN-28 in both nuclear and cytoplasmic fractions, with a greater relative distribution in the cytoplasm (**Fig. 4f**). qRT-PCR analysis of immunoprecipitated LIN-28 showed that the majority of both pri-let-7g and pri-let-7a-1 was nuclear localized (**Fig. 4g**). In contrast, immunoprecipitated pre-let-7g and pre-let-7a-1 were predominantly cytoplasmic (**Fig. 4g**). Thus, in human ES cells, LIN-28 interacts with pri- and pre-let-7 in cellular fractions consistent with the sites of Drosha and Dicer processing, respectively.



LIN-28 co-transcriptionally binds endogenous primary let-7

Our results suggest that LIN-28 negatively regulates let-7 expression at the Drosha processing step. Because Drosha processing can be co-transcriptional, we asked whether the association of LIN-28 with pri-let-7 also occurs at this step[29–31]. To test if LIN-28 binds the endogenous *let-7* gene in *C. elegans*, we performed chromatin immunoprecipitation (ChIP) experiments. Worms expressing LIN-28:GFP or GFP alone were collected at the 10-h time point in late L1 and processed to detect association of RNA polymerase II, GFP or a control IgG antibody with specific DNA sequences. qPCR was used to analyze the immunoprecipitated genomic DNA levels for multiple primary miRNAs relative to the amount of genomic DNA in the input sample. Unlike sequences for pri-mir-47 and an untranscribed region ~20 kb upstream of pri-let-7 (ref. 32), sequences for pri-let-7 and pri-mir-58 were significantly enriched for association with RNA polymerase II relative to IgG (**Fig. 5**). The *let-7* gene also showed significant association with LIN-28:GFP relative to IgG in LIN-28:GFP worms (**Fig. 5**). In contrast, no significant increase in GFP versus IgG was detected for the untranscribed regions upstream of pri-let-7, pri-mir-47 or pri-mir-58 in LIN-28:GFP worms or pri-let-7 in GFP-only worms (**Fig. 5**). Taking these observations together, we conclude that LIN-28 associates with endogenous let-7 transcripts co-transcriptionally in *C. elegans*.

Figure 5 LIN-28 binds endogenous let-7 genomic DNA. Chromatin immunoprecipitation (ChIP) analysis of synchronized PQ272 (LIN-28:GFP) or pD4792 (GFP) worms collected at 10 h. ChIP was carried out with polyclonal antibodies against RNA polymerase II (αPol II) or GFP (αGFP) or with IgG. The ratio of the indicated genomic DNA in the immunoprecipitated sample to the input sample for at least three independent experiments is shown and was analyzed by Student's *t*-test (*$P < 0.05$). Error bars, s.e.m.

DISCUSSION

The levels and timing of mature let-7 expression are critical for animal development and viability. In *C. elegans*, underexpression of let-7 late in development or overexpression of let-7 early in development causes abnormal cell fates that ultimately result in lethality[4]. In humans, inappropriate let-7 levels are found in multiple types of tumors, and in some cases, mis-expression of let-7 has been shown to have a causal role in disease progression[5]. Accordingly, multiple genes have been found that negatively, like hnRNP A1, or positively, like KSRP, regulate let-7 expression in mammalian cells[33,34]. Here we demonstrate that both transcriptional and post-transcriptional mechanisms contribute to let-7 miRNA expression during the development of *C. elegans*. Our results indicate that, early in development, LIN-28 binds and prevents processing of endogenous pri-let-7 transcripts as they are being synthesized. Downregulation of LIN-28 levels by late larval stages permits efficient processing of pri-let-7 to the precursor and mature forms.

Pri-let-7 is first detected during the late L1 stage, and its levels cycle throughout development, with peak expression coinciding with each molt early in development (**Fig. 1** and **Supplementary Figs. 1** and **2**). Identical patterns of timing and oscillation of GFP mRNA and pri-let-7 RNAs in let-7 reporter worms indicate that transcriptional control mechanisms largely regulate the pulses of pri-let-7 expression during development (**Fig. 1e** and **Supplementary Fig. 1c**). The cycling of pri-let-7 accumulation warrants caution when choosing time points to analyze pri-let-7 levels, as less than 2 h is sufficient to dramatically alter expression levels (**Fig. 1** and **Supplementary Fig. 1**). Furthermore, synchronization and the rates of worm development within a population are sensitive to slight changes in culture conditions, such as temperature and availability of food, and this is reflected in shifts in the timing of let-7 transcription (**Fig. 1** and **Supplementary Fig. 1**). Indeed, previous studies of pri-let-7 levels in staged worm samples showed varying or no pri-let-7 expression, likely because of the limited time points that were chosen for analysis[7,26].

The LIN-28 RNA binding protein is an important regulator of let-7 biogenesis across species[5,6,35]. Originally discovered as the product of a gene that regulates developmental timing in *C. elegans*[9], LIN-28 has been shown to promote stem cell fates in mammalian cells[35]. Developmental abnormalities in *lin-28* mutant worms are partially rescued by loss of let-7 expression[4]. Recent work has demonstrated that let-7 miRNA is expressed prematurely in the absence of LIN-28 activity in *C. elegans*[8]. We show that, in contrast to WT worms, in *lin-28* mutant worms the initial pulse of primary let-7 expression at the end of the first larval stage coincides with the accumulation of mature let-7 miRNA (**Fig. 3**). Thus, LIN-28 uncouples primary from mature let-7 expression early in development, and loss of this control results in premature engagement of let-7 miRNA regulatory pathways and abnormal development.

Our studies indicate that LIN-28 blocks the processing of endogenous primary let-7 transcripts. In the presence of LIN-28, neither precursor nor flanking Drosha cleavage products were detected, loss of *pup-2* activity did not affect regulation of let-7, and levels of let-7 primary transcripts diminished as precursor and mature let-7 accumulated in *lin-28* mutant worms. Additionally, LIN-28 specifically bound let-7 primary transcripts *in vivo*, and LIN-28 associated with the let-7 gene co-transcriptionally. In contrast, another study concluded that LIN-28, in conjunction with PUP-2, inhibits the processing and stability of let-7 precursor RNAs in *C. elegans*[8]. This model was based largely on the analysis of transgenic let-7 expression under the control of a heterologous promoter[8]. This construct also lacked the 3′ splice site required for generation of the SL1 isoform previously shown to be important for let-7 rescue activity[7]. Notably, endogenous primary transcript significantly decreased as mature let-7 increased in *lin-28* mutants, but this correlation was not detected in the transgenic strain[8]. As depletion of *pup-2* by RNAi was only shown to result in let-7 precursor upregulation in the transgenic strain[8], and we detected no effect on regulation of endogenous let-7 miRNA expression after RNAi treatment or in a *pup-2* mutant strain (**Fig. 2c** and **Supplementary Fig. 4**), it is possible PUP-2 helps cull excess precursor RNAs that escape the LIN-28–mediated block in primary transcript processing. In the endogenous context, there may be sufficient LIN-28 activity to fully prevent the first step of let-7 processing, but this mechanism may become limiting in cells overexpressing let-7 transcripts, resulting in the detection of additional pathways that can repress maturation of let-7 miRNA. Additionally, our findings that LIN-28 associates with let-7 co-transcriptionally and that the spliced primary transcript is particularly sensitive to LIN-28 activity suggest that natural regulation of let-7 expression may not be fully recapitulated by some transgenes.

A function for LIN-28 in repressing let-7 expression was first discovered in mammalian systems[16–19,35]. Consistent with our findings in *C. elegans*, some studies concluded that LIN-28 blocks the processing of let-7 primary transcripts in human and mouse embryonic cells[16,17]. Other reports proposed that LIN-28 binds let-7 precursors and inhibits Dicer processing and/or recruits TUT4 (also known as Zcchc11 or PUP-2 poly(U) polymerase) to catalyze 3′ end tailing, which results in destabilization of pre-let-7 RNAs[18–20,22]. We found that LIN-28 binds both primary and precursor endogenous let-7 RNAs in human ES cells, indicating that LIN-28 regulates let-7 biogenesis at multiple steps in this cell type. This ability could be required for regulation of the multiple, highly similar let-7 genes expressed in mammalian cells. In contrast, LIN-28 appears to primarily block the first step of let-7 processing during normal worm development.

Association of LIN-28 with the *let-7* gene provides an efficient mechanism for preventing processing of primary transcripts. In mammalian cells, Drosha can bind and cleave primary miRNA transcripts co-transcriptionally[29–31]. Thus, recognition of let-7 transcripts as they are being synthesized would allow LIN-28 to effectively compete with Drosha and prevent processing. A rescuing LIN-28:GFP protein shows fluorescence in the cytoplasm and occasionally in the nucleus and nucleoli of most worm cell types early in development[9]. Endogenous mammalian LIN-28 protein also has a nucleocytoplasmic distribution that fluctuates with the cell cycle[11,13]. Exit from the nucleus may be dependent on association with RNA, as mutation of both RNA binding domains renders LIN-28 entirely nuclear in mouse P19 cells[13]. We also detected LIN-28 in both the nucleus and the cytoplasm of human ES cells, and found that LIN-28 predominantly interacted with endogenous pri-let-7 in the nucleus and pre-let-7 in both the nucleus and cytoplasm (**Fig. 4f**,**g**). Taken together, the pulses of endogenous let-7 primary transcript expression may coincide with sufficient accumulation of LIN-28 in the nucleus to bind newly synthesized let-7 primary transcripts and block processing in *C. elegans*. Association of LIN-28 with let-7 RNAs may then facilitate export of the complex to the cytoplasm, where the primary transcripts are subject to general mRNA decay pathways. Recent evidence suggests that *C. elegans* let-7 primary transcripts may also undergo processing in the cytoplasm[36]. Thus, the nucleocytoplasmic distribution of LIN-28 could be poised to regulate processing of let-7 primary transcripts in either cellular compartment.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

ACKNOWLEDGMENTS

We thank J. Lykke-Andersen (Univ. California, San Diego (UCSD)) and members of the Pasquinelli laboratory for their suggestions and critical reading of this manuscript. We thank E. Moss (Univ. Medicine and Dentistry of New Jersey) for providing the plin-28:LIN-28:GFP strain, R. Gassmann and A. Desai (UCSD) for providing the polyclonal antibody to GFP, M. Li and M. David (UCSD) for sharing their real-time PCR machine, and the *Caenorhabditis* Genetics Center for worm strains. P.M.V.W. was supported by a Ruth L. Kirschstein National Research Service Award (F32GM087004) from the US National Institute of General Medical Sciences. Z.S.K. was supported by a US National Institutes of Health (NIH) Cellular and Molecular Genetics and NIH/National Cancer Institute T32 CA009523 Graduate Student Training Grants. V.H.B. was supported by a UCSD Chancellor's Undergraduate Research Scholarship. This work was funded by the NIH (GM071654), the Keck, Searle, V., Emerald and Peter Gruber Foundations (A.E.P.) and by the California Institute of Regenerative Medicine (RB1-01413) and the Stem Cell Program at UCSD (G.W.Y.).

AUTHOR CONTRIBUTIONS

A.E.P. and P.M.V. designed the project and wrote the paper; P.M.V. (all figures), Z.S.K. (**Fig. 1** and **Supplementary Fig. 1**), K.B.M. (**Fig. 4**) and V.H.B. (**Fig. 4**) performed the experiments; A.E.P. and G.W.Y. supervised the studies.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/nsmb/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Kim, V.N., Han, J. & Siomi, M.C. Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell Biol.* **10**, 126–139 (2009).
2. Bartel, D.P. MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215–233 (2009).
3. Pasquinelli, A.E. *et al.* Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* **408**, 86–89 (2000).
4. Reinhart, B.J. *et al.* The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403**, 901–906 (2000).
5. Büssing, I., Slack, F.J. & Grosshans, H. let-7 microRNAs in development, stem cells and cancer. *Trends Mol. Med.* **14**, 400–409 (2008).
6. Roush, S. & Slack, F.J. The let-7 family of microRNAs. *Trends Cell Biol.* **18**, 505–516 (2008).
7. Bracht, J., Hunter, S., Eachus, R., Weeks, P. & Pasquinelli, A.E. Trans-splicing and polyadenylation of let-7 microRNA primary transcripts. *RNA* **10**, 1586–1594 (2004).
8. Lehrbach, N.J. *et al.* LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 microRNA processing in *Caenorhabditis elegans*. *Nat. Struct. Mol. Biol.* **16**, 1016–1020 (2009).
9. Moss, E.G., Lee, R.C. & Ambros, V. The cold shock domain protein LIN-28 controls developmental timing in *C. elegans* and is regulated by the lin-4 RNA. *Cell* **88**, 637–646 (1997).
10. Moss, E.G. & Tang, L. Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites. *Dev. Biol.* **258**, 432–442 (2003).
11. Guo, Y. *et al.* Identification and characterization of lin-28 homolog B (LIN28B) in human hepatocellular carcinoma. *Gene* **384**, 51–61 (2006).
12. Balzer, E., Heine, C., Jiang, Q., Lee, V.M. & Moss, E.G. LIN28 alters cell fate succession and acts independently of the let-7 microRNA during neurogliogenesis in vitro. *Development* **137**, 891–900 (2010).
13. Balzer, E. & Moss, E.G. Localization of the developmental timing regulator Lin28 to mRNP complexes, P-bodies and stress granules. *RNA Biol.* **4**, 16–25 (2007).
14. Seggerson, K., Tang, L. & Moss, E.G. Two genetic circuits repress the *Caenorhabditis elegans* heterochronic gene lin-28 after translation initiation. *Dev. Biol.* **243**, 215–225 (2002).
15. Morita, K. & Han, M. Multiple mechanisms are involved in regulating the expression of the developmental timing regulator lin-28 in *Caenorhabditis elegans*. *EMBO J.* **25**, 5794–5804 (2006).
16. Viswanathan, S.R., Daley, G.Q. & Gregory, R.I. Selective blockade of microRNA processing by Lin28. *Science* **320**, 97–100 (2008).
17. Newman, M.A., Thomson, J.M. & Hammond, S.M. Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. *RNA* **14**, 1539–1549 (2008).
18. Heo, I. *et al.* Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol. Cell* **32**, 276–284 (2008).
19. Rybak, A. *et al.* A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. *Nat. Cell Biol.* **10**, 987–993 (2008).
20. Hagan, J.P., Piskounova, E. & Gregory, R.I. Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells. *Nat. Struct. Mol. Biol.* **16**, 1021–1025 (2009).
21. Piskounova, E. *et al.* Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28. *J. Biol. Chem.* **283**, 21310–21314 (2008).
22. Heo, I. *et al.* TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell* **138**, 696–708 (2009).
23. Johnson, S.M., Lin, S.Y. & Slack, F.J. The time of appearance of the *C. elegans* let-7 microRNA is transcriptionally controlled utilizing a temporal regulatory element in its promoter. *Dev. Biol.* **259**, 364–379 (2003).
24. Bagga, S. *et al.* Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* **122**, 553–563 (2005).
25. Esquela-Kerscher, A. *et al.* Post-embryonic expression of *C. elegans* microRNAs belonging to the lin-4 and let-7 families in the hypodermis and the reproductive system. *Dev. Dyn.* **234**, 868–877 (2005).
26. Martinez, N.J. *et al.* Genome-scale spatiotemporal analysis of *Caenorhabditis elegans* microRNA promoter activity. *Genome Res.* **18**, 2005–2015 (2008).
27. Qiu, C., Ma, Y., Wang, J., Peng, S. & Huang, Y. Lin28-mediated post-transcriptional regulation of Oct4 expression in human embryonic stem cells. *Nucleic Acids Res.* **38**, 1240–1248 (2010).
28. Suh, M.R. *et al.* Human embryonic stem cells express a unique set of microRNAs. *Dev. Biol.* **270**, 488–498 (2004).
29. Ballarino, M. *et al.* Coupled RNA processing and transcription of intergenic primary microRNAs. *Mol. Cell. Biol.* **29**, 5632–5638 (2009).
30. Morlando, M. *et al.* Primary microRNA transcripts are processed co-transcriptionally. *Nat. Struct. Mol. Biol.* **15**, 902–909 (2008).
31. Pawlicki, J.M. & Steitz, J.A. Subnuclear compartmentalization of transiently expressed polyadenylated pri-microRNAs: processing at transcription sites or accumulation in SC35 foci. *Cell Cycle* **8**, 345–356 (2009).
32. Celniker, S.E. *et al.* Unlocking the secrets of the genome. *Nature* **459**, 927–930 (2009).
33. Michlewski, G. & Caceres, J.F. Antagonistic role of hnRNP A1 and KSRP in the regulation of let-7a biogenesis. *Nat. Struct. Mol. Biol.* **17**, 1011–1018 (2010).
34. Trabucchi, M. *et al.* The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. *Nature* **459**, 1010–1014 (2009).
35. Viswanathan, S.R. & Daley, G.Q. Lin28: A microRNA regulator with a macro role. *Cell* **140**, 445–449 (2010).
36. Bussing, I., Yang, J.S., Lai, E.C. & Grosshans, H. The nuclear export receptor XPO-1 supports primary miRNA processing in *C. elegans* and *Drosophila*. *EMBO J.* **29**, 1830–1839 (2010).

ONLINE METHODS

Nematode culture and strains. *C. elegans* were grown under standard conditions[37] and synchronized by hypochlorite treatment. Starvation-arrested L1 worms were plated on OP50 bacteria, cultured at 25 °C and collected at the desired time points. The wild-type (WT) strain was N2 Bristol. The pD4792(mIs11 IV) strain expresses myo-2::GFP, pes-10::GFP and gut::GFP. plet-7B::GFP [plet-7B::GFP;pha-1(+)] expresses plet-7B::GFP and a pha-1(+) rescue construct as transgenes in a *pha-1(e2123)* background. PQ272 (lin-28(n719); plin-28::LIN-28:GFP; pRF4 (rol-6 marker)) was made by crossing *lin-28(n719)* with a strain containing stably integrated copies of rescuing LIN-28-GFP, flanked by the *lin-28* promoter and 3′ UTR[9].

RNAi treatment. Two-generation feeding RNAi experiments used the *eri-1(mg366)* RNAi hypersensitive strain as described[8].

ES culture. The hESC line HUES6 was cultured as described (http://www.mcb.harvard.edu/melton/HUES/)[38]. Briefly, cells were grown to 80% confluency on growth factor–reduced (GFR) Matrigel–coated plates (Becton Dickinson) in StemPro hESC serum-free medium (Invitrogen) before collection for RIP.

DNA constructs. plet-7B::GFP was made by PCR amplifying the *let-7* promoter (**Supplementary Table 1**) and fusing it upstream of three nuclear localization center (NLS) repeats and GFP sequence.

Northern blotting. PAGE and agarose northern blotting analysis for small (<200-nt) and larger RNA species, respectively, was performed as described[7], with the probe templates listed in **Supplementary Table 2**, and analyzed with ImageQuant software (Molecular Dynamics).

RNA ligase-mediated rapid amplification of cDNA ends (RACE). RACE was completed with the GENERACER kit (Invitrogen) and primers listed in **Supplementary Table 3** (ref. 7). For 5′ RACE, total RNA was ligated to the kit 5′ linker and reverse-transcribed with Superscript III (Invitrogen) and a pri-let-7 primer downstream of pre-let-7. PCR and nested PCR used 5′ linker and pri-let-7 sequence primers. For 3′ RACE, gel-purified, 50–100-nt, dephosphorylated RNA was ligated to a RNA linker with a 5′ phosphate group and a 3′ puromycin tag. cDNA was made as above with a primer complementary to the 3′ linker. PCR used mature let-7 and the 3′ linker primers. Nested 5′ and 3′ RACE PCR products were analyzed by gel electrophoresis (**Supplementary Table 3**) or sequenced after TOPO cloning (Invitrogen).

Western blotting. Western blotting was performed as described with mouse monoclonal antibodies against GFP (Santa Cruz), actin (MP Biomedicals), tubulin (Sigma) and RNA polymerase II (Santa Cruz) or a rabbit polyclonal antibody against LIN-28 (Abcam)[24]. The Rabbit IgG TrueBlot secondary antibody (eBioscience) was used for LIN-28 western blots.

***C. elegans* RNA immunoprecipitation (RIP).** PQ272 worms were cross-linked by UV treatment. Equal amounts of lysates were precleared before immunoprecipitation with the appropriate antibody and protein G Dynabeads (Invitrogen). Immunoprecipitated material associated with the beads was subjected to protein degradation and RNA extraction before RT-PCR with the primers listed in **Supplementary Table 4**. For further details, see **Supplementary Methods**.

ES cell RNA immunoprecipitation (RIP). Equal amounts of precleared lysates from un-cross-linked HUES6 cells were immunoprecipitated and treated as described above. For further details, see **Supplementary Methods**.

ES cell fractionation. Cell fractionation was performed as previously described[39]. For further details, see **Supplementary Methods**.

Chromatin immunoprecipitation (ChIP). ChIP was performed as described previously[24,40], with some modifications. PQ272 or pD4792 worms were cross-linked with formaldehyde. Equal amounts of sonicated worm lysates were precleared before immunoprecipitation with the appropriate antibody and protein G Dynabeads (Invitrogen). Immunoprecipitated material was eluted from the beads, reverse cross-linked and subjected to protein degradation, and DNA was then extracted. qPCR was performed with primers listed in **Supplementary Table 4**. For further details, see **Supplementary Methods**.

qPCR. qPCR was performed with SYBR green (Applied Biosystems) and 6.25 pmol of each primer (**Supplementary Table 4**) on an ABI Prism 7000 real-time PCR machine.

37. Wood, W. *The Nematode Caenorhabditis elegans* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 1988).
38. Cowan, C.A. *et al.* Derivation of embryonic stem-cell lines from human blastocysts. *N. Engl. J. Med.* **350**, 1353–1356 (2004).
39. Gondran, P., Amiot, F., Weil, D. & Dautry, F. Accumulation of mature mRNA in the nuclear fraction of mammalian cells. *FEBS Lett.* **458**, 324–328 (1999).
40. Mukhopadhyay, A., Deplancke, B., Walhout, A.J. & Tissenbaum, H.A. Chromatin immunoprecipitation (ChIP) coupled to detection by quantitative real-time PCR to study transcription factor binding to DNA in *Caenorhabditis elegans*. *Nat. Protoc.* **3**, 698–709 (2008).