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The Period protein homolog LIN-42 negatively regulates microRNA biogenesis in *C. elegans*

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ABSTRACT

MicroRNAs (miRNAs) are small RNAs that post-transcriptionally regulate gene expression in many multicellular organisms. They are encoded in the genome and transcribed into primary (pri-) miRNAs before two processing steps that ultimately produce the mature miRNA. In order to generate the appropriate amount of a particular miRNA in the correct location at the correct time, proper regulation of miRNA biogenesis is essential. Here we identify the Period protein homolog LIN-42 as a new regulator of miRNA biogenesis in *Caenorhabditis elegans*. We mapped a spontaneous suppressor of the normally lethal *let-7(n2853)* allele to the *lin-42* gene. Mutations in this allele (*ap201*) or a second *lin-42* allele (*n1089*) caused increased mature let-7 miRNA levels at most time points when mature let-7 miRNA is normally expressed. Levels of pri-let-7 and a let-7 transcriptional reporter were also increased in *lin-42 (n1089)* worms. These results indicate that LIN-42 normally represses pri-let-7 transcription and thus the accumulation of let-7 miRNA. This inhibition is not specific to let-7, as pri- and mature levels of lin-4 and miR-35 were also increased in *lin-42* mutants. Furthermore, small RNA-seq analysis showed widespread increases in the levels of mature miRNAs in *lin-42* mutants. Thus, we propose that the period protein homolog LIN-42 is a global regulator of miRNA biogenesis.

Introduction

MicroRNAs (miRNAs) are small ~22 nucleotide (nt) RNAs that post-transcriptionally regulate gene expression (Pasquinelli, 2012). By imperfectly binding to target mRNAs, miRNAs mediate target degradation and translation inhibition of a large number of genes (Huntzinger and Izaurralde, 2011; Pasquinelli, 2012). Thus, miRNAs are important regulators of basic cellular and developmental processes, and misregulation of miRNA expression has been associated with a multitude of biological effects, including disease (Abbott, 2011; Sayed and Abdellatif, 2011).

Though functional as small RNAs, miRNAs are originally encoded as long primary transcripts in intergenic or intragenic regions of the genome (Finnegan and Pasquinelli, 2013). Transcription by RNA polymerase II yields primary (pri-) miRNA transcripts that are subsequently capped and polyadenylated (Finnegan and Pasquinelli, 2013; Resnick et al., 2010). The Microprocessor complex, composed of the RNase III enzyme Drosha and the RNA binding protein DGCR8 (also known as Pasha) excises the ~70 nt precursor (pre-) miRNA hairpin from the pri-miRNA (Finnegan and Pasquinelli, 2013; Resnick et al., 2010). Following export of the pre-miRNA to the cytoplasm, a second RNase III enzyme, Dicer, removes an ~22 nt duplex consisting of the mature miRNA and its complementary star strand (also called the passenger strand) (Finnegan and Pasquinelli, 2013; Resnick et al., 2010). In a small number of cases, the miRNA duplex can be immediately excised from specially structured, debranched introns (mirtrons) after Dicer cleavage (Westholm and Lai, 2011). Regardless of its source, after Dicer cleavage the mature miRNA is loaded onto Argonaute to form the miRNA-induced silencing complex (miRISC) (Aalto and Pasquinelli, 2012). Using the mature miRNA as a guide, miRISC downregulates target gene expression (Huntzinger and Izaurralde, 2011; Pasquinelli, 2012). Consequently, the amount of mature miRNA associated with miRISC can dictate the level of target gene downregulation, and proper regulation of mature miRNA levels is crucial for appropriate target gene expression (Pasquinelli, 2012). Underlying this importance is the finding that each step in miRNA

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biogenesis is subject to regulation (Finnegan and Pasquinelli, 2013; Resnick et al., 2010). Recently, a miRNA has even been found to directly regulate the processing of its own primary transcript (Zisoulis et al., 2012). Some proteins regulate biogenesis of a specific miRNA while others act globally to regulate a particular step in the biogenesis of multiple miRNAs (Finnegan and Pasquinelli, 2013).

Many miRNAs, their targets, and their regulators are conserved (Finnegan and Pasquinelli, 2013). Originally discovered in *Caenorhabditis elegans*, both the mature sequence and temporal expression pattern of the let-7 miRNA is conserved across many eukaryotic species, including humans (Pasquinelli et al., 2000; Reinhart et al., 2000). Let-7 is an important member of the heterochronic pathway that regulates developmental timing in *C. elegans* (Ambros, 2011; Mondol and Pasquinelli, 2012; Resnick et al., 2010; Sokol, 2012). Under-expression of let-7 in *C. elegans* contributes to delayed or blocked cell differentiation and ultimately results in retarded development and a bursting vulva phenotype (Reinhart et al., 2000). In humans, low levels of let-7 expression are also associated with loss of the differentiated state and breast, colon and lung cancer (Mondol and Pasquinelli, 2012; Sayed and Abdellatif, 2011). In *C. elegans* pri-let-7 levels oscillate throughout development, initiating with transcription at the end of the first larval stage (L1) (Kai et al., 2013; Van Wynsberghe et al., 2011b). In contrast, pre- and mature let-7 are not detectable until L3 (Van Wynsberghe et al., 2011b). This uncoupling of pri- and pre-let-7 expression is due to regulation by the heterochronic pathway member and RNA binding protein LIN-28, which co-transcriptionally inhibits pri-let-7 processing by Drosha (Van Wynsberghe et al., 2011b). In mammalian cells LIN-28 also binds pri- and pre-let-7 to inhibit processing (Thornton and Gregory, 2012).

In addition to LIN-28, inhibition of many different genes can suppress or enhance let-7 developmental phenotypes in *C. elegans* (Grosshans et al., 2005; Hayes and Ruvkun, 2006; Lu et al., 2009; Parry et al., 2007; Reinhart et al., 2000). However, since many of these genes are also members of the heterochronic pathway they could be indirect biogenesis regulators or targets of let-7. One heterochronic gene whose inhibition can suppress retarded development and bursting phenotypes in let-7 mutant worms is *lin-42* (Abrahante et al., 1998; Banerjee et al., 2005; Hayes and Ruvkun, 2006; Reinhart et al., 2000; Tennessen et al., 2006). The *lin-42* gene encodes a Period protein homolog based on sequence homology and the rhythmic expression of both *lin-42* mRNA and protein (Jeon et al., 1999; Monsalve et al., 2011; Tennessen et al., 2006). Period proteins function as transcriptional repressors to control circadian rhythms (Hardin, 2005; Yu and Hardin, 2006). In *C. elegans*, circadian regulation of multiple behavioral rhythms, including locomotion, olfaction, defecation and molting, has been observed (Migliori et al., 2011; Olmedo et al., 2012; Temmerman et al., 2011; van der Linden et al., 2010). Consistent with its homology to period proteins, *lin-42* is essential for maintaining proper molting rhythms and for entry into an alternative third larval stage called dauer (Monsalve et al., 2011; Tennessen et al., 2010).

Here we identify a new allele of *lin-42*, *lin-42(ap201)*, which also suppresses lethal *let-7* phenotypes. To understand the mechanism by which *lin-42* suppresses *let-7* vulval bursting and reduced progeny numbers, we analyzed the effect of *lin-42* on mature let-7. We found that let-7 levels were significantly increased and that levels of the let-7 target *lin-41* were significantly decreased in *lin-42* mutant worms. Furthermore, this increase in mature let-7 levels occurred at most time points throughout development when mature let-7 is normally expressed. Our results indicate that transcription of let-7 is elevated in *lin-42* mutants, leading to increased substrates for processing and, hence, mature product. Additionally, levels of pri-lin-4 and pri-miR-35 were increased in *lin-42* mutants relative to WT worms, suggesting a general effect of *lin-42* on miRNA transcription. *lin-42* appears to have a broad influence over mature miRNA accumulation, as we found that the majority of miRNAs increased in abundance in *lin-42* mutants compared to WT worms. Together these results suggest that *lin-42* globally regulates miRNA biogenesis by acting as a transcriptional repressor of primary miRNA production in *C. elegans*. Because *lin-42* and many miRNAs have homologs in higher eukaryotes, these results also suggest that in addition to repressing mRNA transcription, Period proteins may control circadian rhythms by regulating miRNA accumulation.

Materials and methods

Nematode strains and culture conditions

The following *C. elegans* strains were used: wild type (WT) N2 Bristol, Hawaiian (HA) (CB4856), *let-7(n2853)* (MT7626), *let-7(n2853);lin-42(ap201)* (PQ63), *lin-42(n1089)* (MT2257), *lin-42(ap201)* (PQ62), plet-7B::GFP (PQ462), and plet-7B::GFP;*lin-42(n1089)* (PQ520). The pD4792(mIs11 IV) strain expresses myo-2::GFP, pes-10::GFP and gut::GFP. Worms were maintained at 15 °C or 20 °C and synchronized by standard hypochlorite treatment. Starvation-arrested L1 worms were plated on OP50 at 25 °C and collected at the appropriate time point. Larval stages correspond to the timing of development for N2 WT worms based on previously published time course analyses of worm development and molting at 25 °C (Jeon et al., 1999; Zisoulis et al., 2012), as *lin-42* mutants develop somewhat asynchronously (Monsalve et al., 2011).

Mapping

lin-42(ap201) hermaphrodites were crossed with HA males and F1 cross progeny were singled. Dumpy F2 progeny were singled and lysed in genotyping buffer [50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.45% Nonidet P-40, 0.45% Tween, and 0.01% (w/v) gelatin] by incubation at 65 °C for 1 h. SNP maps of F2 progeny were analyzed as previously described (Wicks et al., 2001).

RNA analyses

Total RNA was extracted from synchronized, staged worm populations using TRIzol reagent (Life Technologies), and analyzed by PAGE northern blotting (<200 nt) or agarose northern blotting (>200 nt) as previously described (Van Wynsberghe et al., 2011a). Probe templates are listed in Supplementary Table 1. Exposed Typhoon Phosphorimager screens were scanned on a Typhoon Trio PhosphorImager (GE Healthcare) and band signals were quantified with ImageQuant software. For qRT-PCR analyses, RNA was extracted as above and cDNA synthesis was completed as previously described with random oligos or oligo dT (Van Wynsberghe et al., 2011a). qPCR was performed with SYBR Green (Applied Biosystems) and 6.25 pmol of each primer (Supplementary Table 1) on an ABI Prism 7000 or 7900 real time PCR machine.

Small RNA sequencing

Total RNA (1 ug) from embryo or L4 stage worms was used for cloning with the TruSeq Small RNA kit (Illumina), including synthetic RNA oligo spike-in controls (control_22: CAUUCUCCUAUCGCUUCAGCUU and control_33: CAGAUAUGUCGAUUCGCAAUGU) (Hafner et al., 2011). Two independent libraries of each strain and time point were sequenced with an Illumina Genome Analyzer IIx and computationally processed to remove adapter sequences and annotate reads according to miRBase version 18. Only miRNAs with 10 or more reads in all experiments were used

for analyses. The total number of reads for each miRNA was normalized to the average number of reads for the two control oligos in each sequencing sample.

Results

lin-42 regulates let-7 biogenesis

let-7(n2853) mutant worms exhibit a lethal bursting phenotype after the L4 molt when grown at 25 °C (Reinhart et al., 2000). This severe phenotype can be suppressed by reduced activity of particular genes in the heterochronic and other pathways (Grosshans et al., 2005; Reinhart et al., 2000). We identified a spontaneous suppressor of the *let-7* bursting phenotype and mapped the mutation through SNP–SNP genotyping (Supplementary Fig. 1) (Wicks et al., 2001). Through sequence analysis we localized this mutation to a 363 nt deletion surrounding exon 8 of the *lin-42* gene (Fig. 1A). Sequence analysis of the cDNA and genomic DNA from this *lin-42* mutant found that this deletion removes exon 8 in the *lin-42a* and *lin-42b* isoforms, while the *lin-42c* isoform is likely unaffected by this mutation. In addition, this deletion introduces a frameshift that eliminates expression of wild-type exon 9 in the *lin-42a* and *lin-42b* isoforms. Though most of the LIN-42A amino acid sequence is encompassed in the C terminal region of LIN-42B, the first exon of LIN-42A is unique and LIN-42A is independently transcribed (Fig. 1A) (Tennessen et al., 2006). LIN-42 is a known member of the heterochronic pathway, and *lin-42* loss of function has previously been shown to rescue

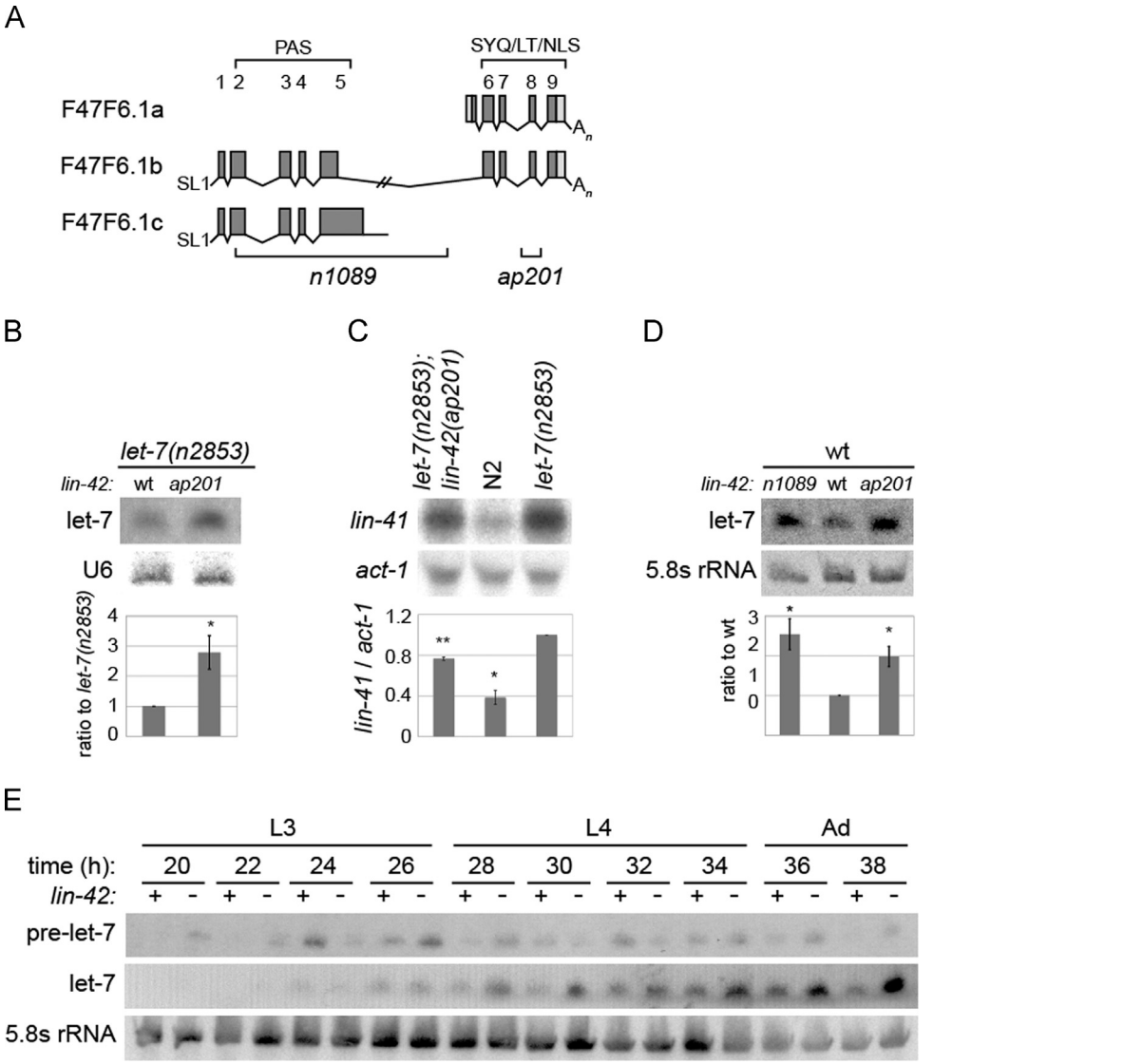


Fig. 1. *lin-42* negatively regulates accumulation of mature *let-7*. (A) Depiction of the *lin-42* gene, based on WormBase. These three isoforms were previously described as *lin-42d*, *lin-42c* and *lin-42a*, respectively (Tennessen et al., 2006). Conserved domains and amino acids, and alleles are respectively marked above and below the gene diagrams. The *let-7(n2853)* suppressor mutation (*ap201*) was mapped to the C terminal region of LIN-42. PAS, protein interaction domain; SYQ, conserved region containing multiple ser, tyr and gln amino acids; LT, conserved region containing multiple leu and thr amino acids; NLS, nuclear localization signal. (B) RNA was extracted from mid-L4 *let-7(n2853)* or *let-7(n2853);lin-42(ap201)* worms and analyzed by PAGE northern blotting. The levels of mature let-7 after U6 RNA normalization relative to *let-7(n2853)* worms were calculated from three independent experiments and analyzed by Student's *t*-tests (*, $p < 0.05$). Error bars show s.e.m. (C) RNA was extracted from L3 stage *let-7(n2853)*, *let-7(n2853);lin-42(ap201)* or WT N2 worms and analyzed by agarose northern blotting. The levels of *lin-41* mRNA after *act-1* mRNA normalization relative to *let-7(n2853)* worms were calculated from three independent experiments and analyzed by Student's *t*-tests (*, $p < 0.05$; **, $p < 0.005$). Error bars show s.e.m. (D) RNA was extracted from mid-L4 WT N2, *lin-42(ap201)*, or *lin-42(n1089)* mutant worms and analyzed by PAGE northern blotting. The levels of mature let-7 after 5.8s rRNA normalization relative to WT N2 worms were calculated from three independent experiments and analyzed by Student's *t*-tests (*, $p < 0.05$). Error bars show s.e.m. (E) PAGE northern blot analysis of total RNA isolated from synchronized WT N2 (+) or *lin-42(ap201)* (−) worms at larval (L) or adult (Ad) stages. A representative blot from two independent experiments is shown.

bursting and retarded phenotypes of *let-7* mutant worms (Abrahante et al., 1998; Banerjee et al., 2005; Hayes and Ruvkun, 2006; Reinhart et al., 2000; Tennessen et al., 2006).

The *n2853* allele of *let-7* is a temperature-sensitive mutation of the fifth nucleotide in the mature let-7 sequence that decreases mature let-7 levels more than 5 fold compared to WT (Bagga et al., 2005; Chatterjee and Grosshans, 2009; Reinhart et al., 2000; Zisoulis et al., 2012). This decrease has been attributed to reduced stability of the mature miRNA as well as loss of an auto-regulatory feedback loop that promotes processing of primary let-7 (Chatterjee and Grosshans, 2009; Zisoulis et al., 2012). To test if rescue of *let-7(n2853)* phenotypes by the *lin-42(ap201)* mutation was associated with a change in let-7 expression, we analyzed mature let-7 levels in the fourth larval stage (L4) at 25 °C in *let-7(n2853)* and *let-7(n2853);lin-42(ap201)* worms (Fig. 1B and Supplementary Fig. 2). We found that *let-7(n2853)* levels were increased ~2.5 fold in the mutant *lin-42* background (Fig. 1B). This increase in let-7(n2853) levels coincided with a small (~25%), but significant decrease in mRNA levels of the let-7 target, *lin-41*, in *let-7(n2853);lin-42(ap201)* worms relative to *let-7(n2853)* worms (Fig. 1C). Similarly to *let-7(n2853)* levels, WT let-7 levels were increased ~2 fold in *lin-42(ap201)* compared to WT N2 worms (Fig. 1D). This effect was not specific to the *lin-42(ap201)* allele, since WT let-7 levels increased ~2.5 fold in *lin-42(n1089)* versus WT N2 worms (Fig. 1D). WT *let-7* levels also increased ~1.5 fold in worms treated with RNAi against the N or the C terminus of *lin-42* mRNA compared to a vector control (data not shown). In WT worms, mature let-7 is first apparent by northern blotting during L3 (Esquela-Kerscher et al., 2005; Reinhart et al., 2000; Van Wynsberghe et al., 2011b). To determine if the effect of *lin-42* on let-7 accumulation was specific to the mid-L4 stage, we analyzed let-7 levels throughout multiple larval and young adult stages of development. The appearance of mature let-7 was generally concordant at the L3 stage in both WT and *lin-42(ap201)* worms. However, by the L4 stage, mature let-7 levels were higher at all time points in *lin-42(ap201)* and this effect continued into adulthood (Fig. 1E).

Higher levels of mature let-7 in *lin-42* mutants could be due to increased transcription of the *let-7* gene or enhanced processing of the let-7 primary or precursor RNAs. To discern among these possibilities, we analyzed the RNAs involved in let-7 biogenesis. Primary let-7 is first expressed in the late L1 stage and oscillates throughout development (Kai et al., 2013; Van Wynsberghe et al., 2011b). Thus we analyzed pri-let-7 levels at two-hour intervals from embryogenesis to adulthood at 25 °C. Similar to the effects on mature let-7, we did not detect precocious expression of primary let-7 in *lin-42(ap201)* or *lin-42(n1089)* worms relative to WT N2 (Fig. 2A). Instead, we observed subtle, consistent shifts in the timing of the peaks of pri-*let-7* expression in *lin-42* mutant compared to WT worms (Fig. 2A). Early peaks of pri-let-7 expression in both WT and *lin-42* mutant worms are clear and coincide with the first and second molts, while later peaks of pri-let-7

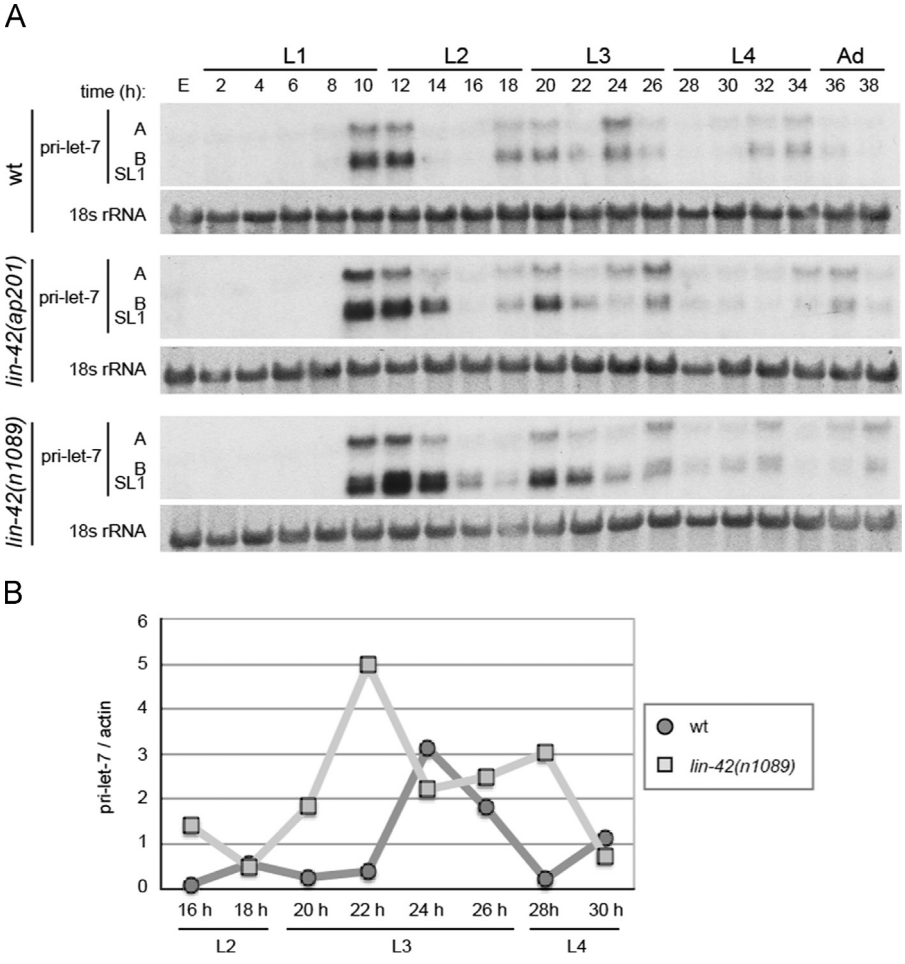


Fig. 2. Effect of *lin-42* on primary let-7 expression. (A) Agarose northern blot analysis of total RNA isolated from embryos (E) or synchronized WT N2, *lin-42(ap201)*, or *lin-42(n1089)* worms at larval (L) or adult (Ad) stages. Representative blots from three independent experiments are shown. The two pri-let-7 transcripts A and B are 1731 and 890 nt respectively while the trans-spliced SL1-pri-let-7 transcript is 728 nt. (B) Total RNA was isolated from synchronized WT N2 or *lin-42(n1089)* worms at larval (L) stages. Levels of primary let-7 and actin mRNA were analyzed by qPCR after reverse transcription with random primers. Representative analyses from three independent experiments are shown.

expression are less distinct in *lin-42* mutant worms compared to WT worms (Fig. 2A). The somewhat shifted cycling pattern of pri-let-7 in the *lin-42* mutants may be attributed to the previously described asynchronous development in the absence of wild type *lin-42* activity (Monsalve et al., 2011). Quantitative real time PCR (RT-PCR) analysis corroborated this altered pattern in pri-*let-7* levels in *lin-42(n1089)* versus WT N2 worms (Fig. 2B and Supplementary Fig. 3A). Moreover, this analysis indicated that pri-let-7 levels were similar or increased at almost all time points throughout development in *lin-42(n0189)* versus WT N2 worms (Fig. 2B and Supplementary Fig. 3A). The timing of let-7 precursor detection was unaltered in *lin-42* mutants and no consistent difference in pre-let-7 levels was observed in the mutants versus WT (Fig. 1E), which likely reflects efficient Dicer processing to the mature form. Taken together, the data suggest that increased production of primary let-7 to enter the processing pathway contributes to the greater accumulation of mature let-7 in *lin-42* mutants compared to WT worms.

To discern whether *lin-42* was acting as a transcriptional or post-transcriptional regulator of pri-*let-7* expression, we utilized an integrated reporter that expresses GFP from the *let-7* promoter (Kai et al., 2013). Consistent with prior results, GFP expression was undetectable at the 8 h L1 time point in WT N2 or *lin-42(n1089)* worms (Fig. 3A) (Kai et al., 2013). By L2, GFP was readily detectable in several cell types in both strains and continued to be expressed until adulthood. Once expressed, GFP was consistently higher in *lin-42(n1089)* relative to WT N2 worms, and this effect was most pronounced at the L4 stage (Fig. 3A). Accordingly, quantitative real time PCR analysis showed that GFP mRNA levels were significantly increased at 28 h (L4) in *lin-42(n1089)* compared to WT N2 worms (Fig. 3B). This effect was specific to the *let-7* promoter, since there was no change in GFP mRNA levels in *lin-42(n1089)* worms relative to WT N2 worms that expressed GFP from a *myo-2* promoter (Fig. 3B).

lin-42 globally regulates miRNA biogenesis

Some proteins, like LIN-28, specifically regulate *let-7* biogenesis (Thornton and Gregory, 2012), while other proteins are important, general regulators of miRNA biogenesis (Finnegan and Pasquinelli, 2013). To test which category LIN-42 belongs to, we analyzed the effect of decreased *lin-42* activity on other miRNAs in *C. elegans*. We found that lin-4, miR-35 and miR-58 mature miRNA levels were increased in *lin-42(ap201)* relative to WT N2 worms

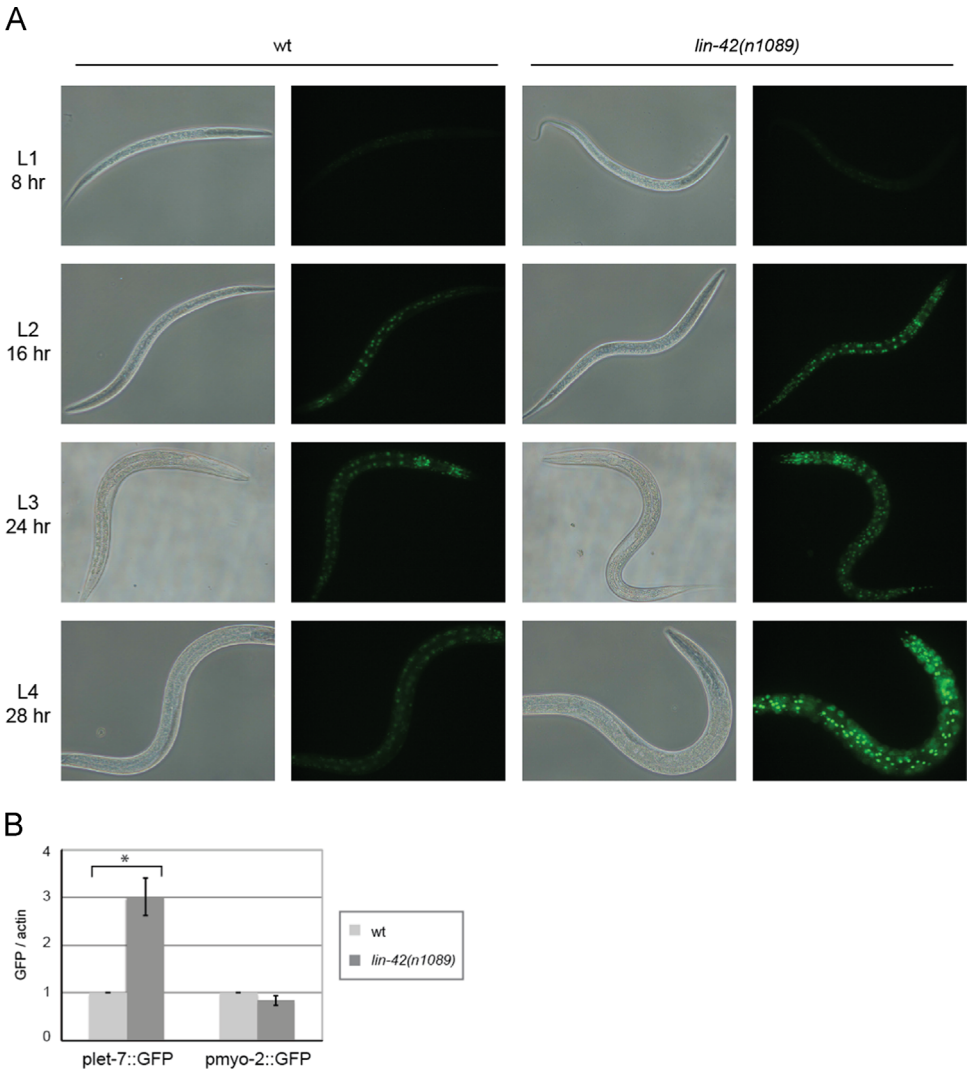


Fig. 3. Effect of *lin-42* on let-7 transcription. (A) Representative images of WT or *lin-42(n1089)* worms expressing the plet-7::GFP reporter throughout development. Fluorescent micrographs were captured under equivalent exposure times. (B) Total RNA was isolated from WT N2 or *lin-42(n1089)* worms expressing the plet-7::GFP reporter or pmyo-2::GFP promoter during the L4 stage (28 h). The level of GFP after actin mRNA normalization relative to WT N2 worms at 28 h were calculated from at least 3 independent experiments and analyzed by Student's *t*-tests (*, $p < 0.05$). Error bars show s.e.m.

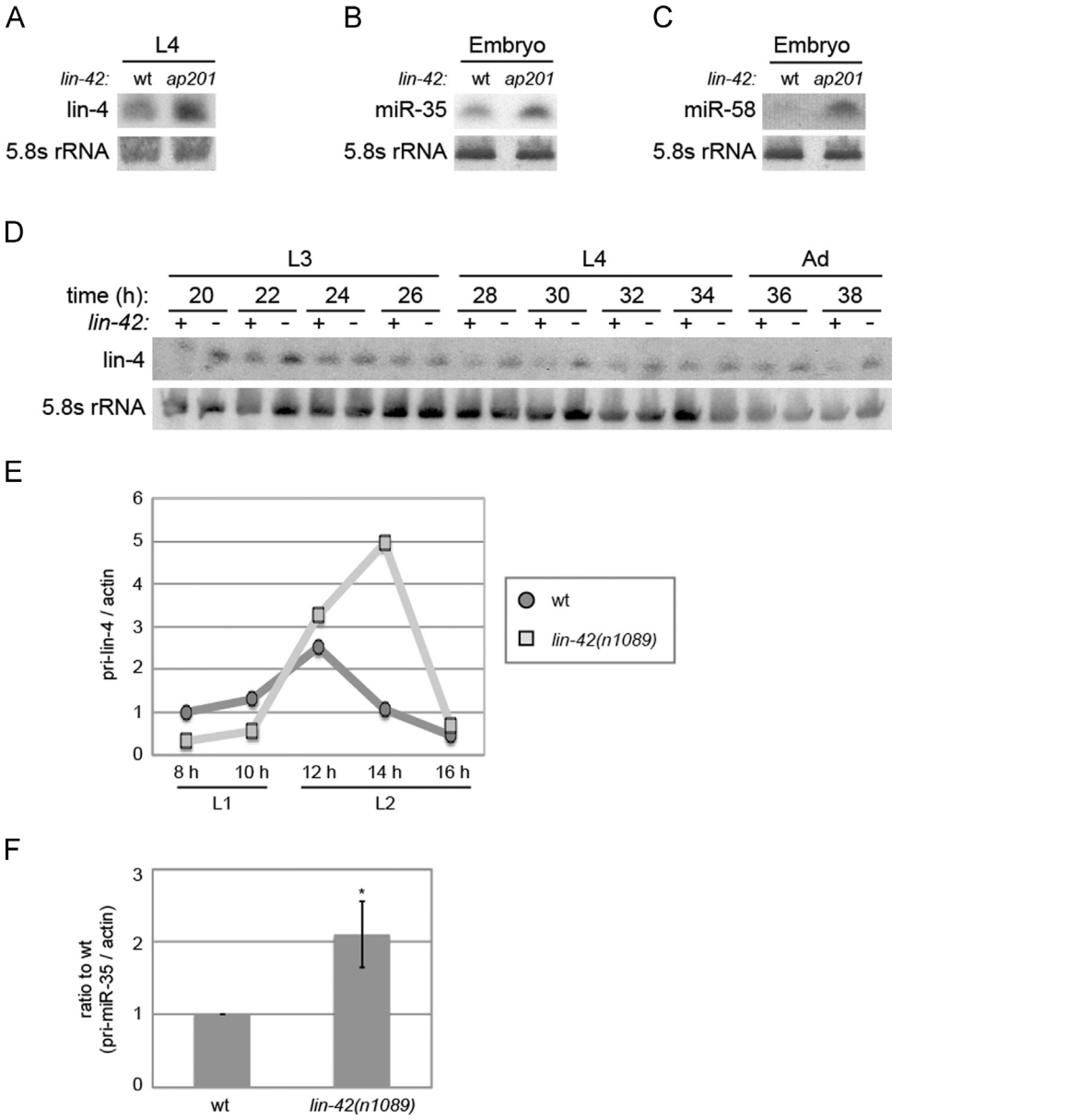
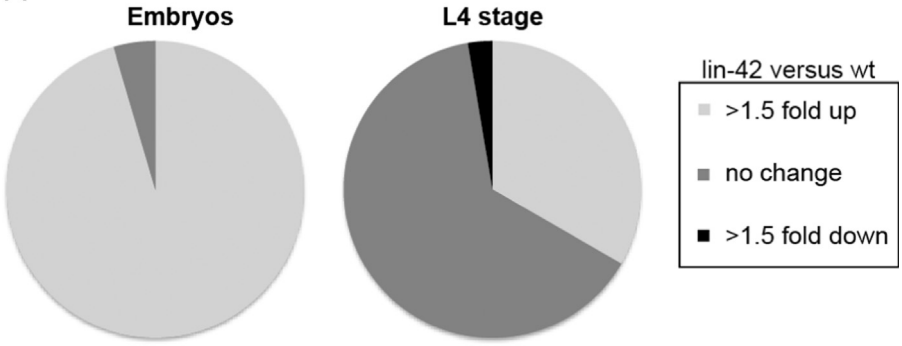


Fig. 4. LIN-42 negatively regulates other microRNAs. (A–C) Total RNA was isolated from synchronized WT N2 or *lin-42(ap201)* worms at the indicated stages and analyzed by PAGE northern blotting for lin-4 (A), miR-35 (B), and miR-58 (C). (D) PAGE northern blot analysis of total RNA isolated from synchronized WT N2 or *lin-42(ap201)* worms at larval (L) or adult (Ad) stages. Representative blot from two independent experiments is shown. (E) Total RNA was isolated from synchronized WT N2 or *lin-42(n1089)* worms at larval (L) stages. Levels of pri-lin-4 and actin mRNA were analyzed by qPCR after reverse transcription with random primers. Representative analyses from three independent experiments are shown. (F) Total RNA was isolated from egg stage N2 or *lin-42(n1089)* worms. The level of pri-miR-35 after actin mRNA normalization relative to WT N2 worms was calculated from three independent experiments and analyzed by Student's *t*-tests (*, $p < 0.05$). Error bars show s.e.m.

(Fig. 4A–C). Notably, miR-35 and miR-58 were analyzed in embryos and the results indicate that this developmental stage is also sensitive to the effect of *lin-42* on miRNA accumulation. Like let-7, mature lin-4 levels were increased at most time points tested throughout development (Fig. 4D). The similarities were not limited to mature miRNAs as the levels of pri-lin-4 and pri-miR-35 were also increased in *lin-42(n1089)* compared to WT N2 worms in L2 and embryo stages, respectively (Fig. 4E, F and Supplementary Fig. 3B).

To determine how ubiquitously *lin-42* affects miRNA accumulation in *C. elegans*, we performed quantitative small RNA cloning and sequencing in WT N2 and *lin-42(n1089)* worms at egg and mid-L4 stages. Control RNA oligos were spiked into the total small RNA cloning reactions and used to normalize the sequencing results (Hafner et al., 2011). We found that many miRNAs increased by at least 1.5-fold in the embryo or L4 stage in *lin-42* mutants compared to WT worms (Fig. 5A and Supplementary Table 2). In total, 169 of 177 miRNAs present in eggs (95%) and 63 of 189 miRNAs present in L4 stage worms (33%) were upregulated by more than 1.5-fold in *lin-42(n1089)* worms compared to WT (Fig. 5A). Zero or only 5 miRNAs (3%) were downregulated in *lin-42 (n1089)* worms in embryonic or L4 stage worms, respectively (Fig. 5A). The effect of *lin-42* was not specific to the location of the miRNA in the genome, as the majority of intragenic and intergenic miRNAs and mirtrons showed increased miRNA levels in the absence versus the presence of *lin-42*. Consistent with our northern analyses (Figs. 1B and 4A–C), we found that miR-35 and miR-58 levels were upregulated in *lin-42(n1089)* embryos and that let-7 and lin-4 levels were upregulated in *lin-42(n1089)* L4 stage worms (Supplementary Table 2). These results were also validated by qRT-PCR (data not shown). In addition, miR-35, let-7 and lin-4 family members were also upregulated in *lin-42(n1089)* embryos

A



B

Embryos		L4 stage	
miRNA	lin-42/wt	miRNA	lin-42/wt
cel-miR-35-3p	**1.93**	cel-let-7-3p	8.25
cel-miR-35-5p	1.60	**cel-let-7-5p**	**1.71**
cel-miR-36-3p	**2.70**	cel-miR-241-3p	2.40
cel-miR-36-5p	1.43	**cel-miR-241-5p**	**2.09**
cel-miR-37-3p	**2.07**	cel-miR-48-3p	1.84
cel-miR-37-5p	1.32	**cel-miR-48-5p**	**1.44**
cel-miR-38-3p	**2.56**	cel-miR-84-3p	2.08
cel-miR-38-5p	1.91	**cel-miR-84-5p**	**1.44**
cel-miR-39-3p	**2.14**	cel-lin-4-3p	4.31
cel-miR-39-5p	1.92	**cel-lin-4-5p**	**1.74**
cel-miR-40-3p	**1.67**	cel-miR-237-3p	7.71
cel-miR-40-5p	2.22	**cel-miR-237-5p**	**4.05**
cel-miR-41-3p	**1.61**		
cel-miR-41-5p	1.28		
cel-miR-42-3p	**1.82**		
cel-miR-42-5p	1.53		

Fig. 5. Widespread up-regulation of miRNAs in *lin-42* mutants. (A) Distribution of miRNAs up- (light gray) or down- (black) regulated by more than 1.5-fold or not changed in either direction by more than 1.5-fold (dark gray) based on the average of two independent small RNA cloning and sequencing experiments from embryo and L4 stage wildtype (N2) and *lin-42(n1089)* mutant worms. (B) Fold increase in members of the miR-35 family of miRNAs in embryos and the let-7 and lin-4 families of miRNAs in L4 stage *lin-42(n1089)* compared to N2 worms. Bold indicates the major guide strand miRNA.

or L4 stage worms (Fig. 5B). Interestingly, an increase in the guide strand miRNA was often associated with an increase in the star strand miRNA, which would be expected if *lin-42* affects primary transcript production (Fig. 5B and Supplementary Table 2). Thus, *lin-42* appears to generally repress the expression of miRNAs in *C. elegans*.

Discussion

Here we demonstrate a new function of the Period protein homolog LIN-42 in globally regulating miRNA expression at the transcriptional level in *C. elegans*. In the absence of LIN-42, mature let-7 miRNA levels increase concordantly with pri-let-7 levels throughout development (Figs. 1 and 2). LIN-42 mediates this effect through the let-7 promoter as GFP mRNA and protein levels, when placed under the control of the let-7 promoter, increase in the absence of LIN-42 (Fig. 3). LIN-42 also inhibits the accumulation of pri-lin-4 and pri-miR-35 in larval stage worms and embryos, respectively (Fig. 4). Indeed, almost all miRNAs in embryos and many miRNAs expressed at the L4 stage were increased in the absence of LIN-42 (Fig. 5). These results uncover a new pathway that Period proteins may utilize to regulate rhythmic processes.

In order for a functional miRNA to be expressed, it must first be transcribed as a primary transcript, processed into an ~70 nt hairpin precursor miRNA, exported to the cytoplasm and further processed to the 22 nt mature miRNA (Finnegan and Pasquinelli, 2013; Resnick et al., 2010). Further complicating miRNA biogenesis is the fact that each step in this pathway is subject to regulation. Some regulators act only on specific miRNAs, while others, as we have found for LIN-42, act globally on multiple miRNAs. Though most known regulators of let-7 biogenesis also regulate other miRNAs, some proteins specifically target let-7 production. For example, HBL-1 inhibits let-7 transcription in *C. elegans* (Roush and Slack, 2009), while LIN-28 inhibits pri- and pre-let-7 processing in *C. elegans* and mammalian cells (Thornton and Gregory, 2012). Mature let-7 also specifically regulates pri-let-7 processing by binding to a let-7 complementary site in pri-let-7 (Zisoulis et al., 2012). In contrast, the tumor suppressor protein p53 affects transcription and processing of multiple primary miRNAs, including pri-let-7, in specific, stress-induced conditions (Saleh et al., 2011). Many other proteins, including hnRNP A1, p68, p72, BRCA1 and TDP-43, also affect processing of primary miRNAs like let-7 (Buratti et al., 2010; Gregory et al., 2004; Kawai and Amano, 2012; Michlewski and Caceres, 2010; Salzman et al., 2007). Processing of precursor miRNAs, including pre-let-7, is often affected by changes in Dicer levels. PACT, TRBP, RBM3, MCPIP1 and let-7 have all been shown to affect Dicer mRNA levels, and thus pre-miRNA expression (Chakravarthy et al., 2010; Forman et al., 2008; Lee et al., 2006; Paroo et al., 2009; Pilotte et al., 2011; Suzuki et al., 2011). Some proteins, including KSRP, ADAR1 and ATM9 via phosphorylation

of KSRP affect both pri- and pre-miRNA processing (Nemlich et al., 2013; Trabucchi et al., 2009; Zhang et al., 2011). In contrast, Ago2, DCS-1, XRN1 and XRN2 regulate the stability of mature miRNAs like let-7 (Bosse et al., 2013; Chatterjee et al., 2011; Chatterjee and Grosshans, 2009; Diederichs and Haber, 2007; Grishok et al., 2001). Our results suggest that LIN-42 globally regulates primary miRNA production by acting as a transcriptional repressor. LIN-42 mRNA and protein levels peak during the intermolt and decrease at the molt (Jeon et al., 1999; Tennessen et al., 2006), while our results suggest that pri-let-7 levels peak at the intermolt early in development. However, the continued presence of the pri-let-7 oscillatory pattern in lin-42 mutant worms, suggests that though LIN-42 is important for regulating pri-let-7 levels, LIN-42 is not the main factor driving pri-let-7 oscillation.

LIN-42 is a member of the heterochronic pathway in *C. elegans* that controls developmental timing in multiple cell types, including hypodermal seam cells (Resnick et al., 2010). Throughout larval development seam cells exhibit characteristic cell division patterns. Prior to completion of the fourth molt seam cells terminally differentiate, fuse and generate a cuticular ridge structure called alae (Resnick et al., 2010). The absence of *lin-42*, either by RNAi or mutation, causes precocious seam cell fusion and alae formation, precocious distal tip cell migration in the gonad, a slight dumpy phenotype, and occasional bursting or bagging (Abrahante et al., 1998; Tennessen et al., 2006). These lin-42 mutant phenotypes are likely due, at least in part, to the over-expression of many diverse types of miRNAs (Fig. 5). The finding that most miRNAs are regulated by LIN-42 in embryos suggests that in addition to post-embryonic development *lin-42* also plays an important role in embryogenesis (Fig. 5). The heterochronic phenotypes exhibited by *lin-42* mutant worms are likely mostly affected by the overexpression of let-7, a member of the heterochronic pathway (Resnick et al., 2010). *lin-42* loss of function has previously been shown to rescue bursting and retarded alae formation in *let-7* mutant worms (Abrahante et al., 1998; Banerjee et al., 2005; Hayes and Ruvkun, 2006; Reinhart et al., 2000; Tennessen et al., 2006), and our results suggest that the *lin-42(ap201)* allele also rescues bursting in *let-7* mutant worms in part by increasing let-7 levels and thus decreasing *lin-41* levels (Figs. 1, 4 and Supplementary Fig. 1). Besides bursting, *lin-42* mutants suppress abnormal seam cell development in *lin-46* or *daf-12* mutant worms (Tennessen et al., 2006). Loss of *lin-42* also enhances precocious alae formation in *lin-58*, *hbl-1* or *lin-41* mutant worms, and suppresses retarded alae formation in either *lin-4(−)*, *let-7(−)* or *lin-14(gf)* mutant worms (Abrahante et al., 1998; Banerjee et al., 2005; Hayes and Ruvkun, 2006; Tennessen et al., 2006). However, mutations in *lin-4(−)*, *let-7(−)* or *lin-14(gf)* also dampen the precocious phenotype of *lin-42* mutant worms, suggesting a complex relationship between *lin-42* and the heterochronic pathway (Abrahante et al., 1998; Tennessen et al., 2006). Loss of *lin-29* suppresses precocious alae phenotypes in *lin-42* mutants, suggesting that *lin-42* acts upstream of *lin-29* in the heterochronic pathway (Abrahante et al., 1998).

LIN-42 is expressed in almost all somatic cell nuclei throughout development including hypodermal, vulval, intestinal, muscle, neuronal, sex myoblasts, somatic gonad and distal tip cells (Jeon et al., 1999; Tennessen et al., 2006). Such a wide expression pattern suggests that LIN-42 functions in multiple diverse pathways or a few pathways conserved among multiple cell types. The former hypothesis is supported by the finding that in addition to its role in the heterochronic pathway, LIN-42 is also essential for proper molting in *C. elegans* (Monsalve et al., 2011). *lin-42(ok2385)* animals, which contain a large deletion in *lin-42a* and the C terminus of *lin-42b*, exhibit asynchronous molts and increased lethargy that is enhanced during later larval stages (Monsalve et al., 2011). LIN-42 is also important for proper dauer entry (Tennessen et al., 2010). *lin-42* mutant animals are hypersensitive to changes in DAF-12 hormonal signaling and are thus more likely to enter dauer when exposed to stresses like high temperature (Tennessen et al., 2010).

LIN-42 shares sequence homology with the PAS domain and at the SYQ and LT amino acid regions of the core circadian clock gene Period (Jeon et al., 1999; Tennessen et al., 2006). The PAS domain mediates protein–protein interactions and contains a cytoplasmic localization domain, while the LT region contains a predicted nuclear localization signal (Jeon et al., 1999; Tennessen et al., 2006). In addition, *lin-42* mRNA and protein levels oscillate in accordance with the molting cycle (Jeon et al., 1999; Monsalve et al., 2011; Tennessen et al., 2006). Endogenous *lin-42* mRNA and protein levels (isoforms A and B) peak during the intermolt, while other reports of a transcriptional gene fusion show peak LIN-42A expression during the molts (Jeon et al., 1999; Monsalve et al., 2011; Tennessen et al., 2006). We find that pri-let-7 and pri-lin-4 levels peak in association with the molt early during development (Figs. 2 and 4). This expression pattern directly opposes the endogenous lin-42 cycling pattern (Jeon et al., 1999; Tennessen et al., 2006), as expected from LIN-42's inhibitory effect on primary miRNA transcription. However, since pri-let-7 and pri-lin-4 levels still oscillate throughout development in *lin-42* mutant worms there are likely other mechanisms that control primary miRNA oscillation patterns.

The periodic cycling of the *C. elegans* molting phases is coupled to the sleep-like state called lethargus, and both of these events are regulated by LIN-42 (Monsalve et al., 2011; Raizen et al., 2008). Besides molting, *C. elegans* exhibit many other rhythmic behaviors including locomotion, defecation, olfaction and osmotic stress resistance (Liu and Thomas, 1994; Olmedo et al., 2012; Temmerman et al., 2011). In addition to LIN-42, many homologs of circadian clock components have been identified in *C. elegans* (Banerjee et al., 2005). Period proteins in *Drosophila* participate in circadian rhythm regulation by binding the Clock protein to inhibit dimerization of the Clock-Cycle transcriptional activator, thereby repressing transcription (Hardin, 2005).

Our results also suggest that LIN-42 inhibits transcription of multiple genes. Specifically, our results suggest that LIN-42 globally represses transcription of primary miRNAs. Since LIN-42 affects the levels of multiple miRNAs, it is possible that LIN-42 indirectly regulates the transcription of these primary miRNAs by inhibiting a general transcriptional activator, as occurs in *Drosophila* and has been proposed previously for *C. elegans* (Tennessen et al., 2006). However, our data are not inconsistent with the idea that LIN-42 could activate a general transcriptional repressor or directly interact with a common sequence motif in the promoters of these primary miRNAs to inhibit pri-miRNA transcription. Additionally, we find that LIN-42 negatively regulates intragenic miRNAs and mirtrons, suggesting that LIN-42 may also regulate the non-miRNA host gene of these targets. However, many intragenic miRNAs, like lin-4 (Bracht et al., 2010), are expressed independently of their host gene, suggesting that LIN-42 could act directly or indirectly on the independent promoters of these genes to inhibit their transcription. Further studies will be needed to elucidate the mechanism by which LIN-42 globally represses primary miRNA transcription.

The fact that miRNAs regulate diverse processes in multiple cell types is consistent with the diverse locations and functions of LIN-42. miRNAs have been shown to regulate both core components of circadian clocks and the output pathways of these clocks (Mehta and Cheng, 2013). Our results suggest a new role for Period proteins in the regulation of miRNA expression to potentially control rhythmic processes across species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.03.017.

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