

CORRECTION

Correction: Trans-splicing of the *C. elegans let-7* primary transcript developmentally regulates *let-7* microRNA biogenesis and *let-7* family microRNA activity (doi: 10.1242/dev.172031)

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There was an error published in *Development* (2019) **146**, dev172031 (doi:10.1242/dev.172031).

In Table S3 in the supplementary material, the genotype for strain VT3903 was incorrect.

Corrected:

maEx267 [rol-6(su1006); unc-119+, plet-7::let-7 locus without mature let-7 sequence]; let-7(ma388) X; mir-48(n4097), maIs105 V

Original:

maEx264 [unc-119+; plet-7::let-7 locus without mature let-7]; let-7(ma388) X; mir-48(n4097), maIs105 V

The online supplementary PDF has been updated.

The authors apologise for this error and any inconvenience it may have caused.

RESEARCH ARTICLE

Trans-splicing of the *C. elegans let-7* primary transcript developmentally regulates *let-7* microRNA biogenesis and *let-7* family microRNA activity

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ABSTRACT

The sequence and roles in developmental progression of the microRNA *let-7* are conserved. In general, transcription of the *let-7* primary transcript (*pri-let-7*) occurs early in development, whereas processing of the mature *let-7* microRNA arises during cellular differentiation. In *Caenorhabditis elegans* and other animals, the RNA-binding protein LIN-28 post-transcriptionally inhibits *let-7* biogenesis at early developmental stages, but the mechanisms by which LIN-28 does this are not fully understood. Nor is it understood how the developmental regulation of *let-7* might influence the expression or activities of other microRNAs of the same seed family. Here, we show that *pri-let-7* is trans-spliced to the SL1 splice leader downstream of the *let-7* precursor stem-loop, which produces a short polyadenylated downstream mRNA, and that this trans-splicing event negatively impacts the biogenesis of mature *let-7* microRNA in *cis*. Moreover, this trans-spliced mRNA contains sequences that are complementary to multiple members of the *let-7* seed family (*let-7fam*) and negatively regulates *let-7fam* function in *trans*. Thus, this study provides evidence for a mechanism by which splicing of a microRNA primary transcript can negatively regulate said microRNA in *cis* as well as other microRNAs in *trans*.

KEY WORDS: *let-7* family microRNAs, *lin-28*, Trans-splicing, Developmental timing, *Caenorhabditis elegans*

INTRODUCTION

MicroRNAs are endogenous ~22 nt RNAs that are enzymatically processed from longer primary transcripts, and that repress protein expression through imperfect base pairing with their target mRNAs. Nucleotides 2-8 of the microRNA, known as the seed, instigate target recognition through essentially complete complementarity, whereas base pairing via the non-seed nucleotides (9-22 of the microRNA) is less constrained than is seed pairing (He and Hannon, 2004). microRNAs that contain an identical seed sequence but differ in their non-seed nucleotides are classified together as a ‘family’ based on their presumed evolutionary relatedness, and their potential to act in combination on the same targets (Bartel, 2009; Ambros and Ruvkun, 2018).

The *let-7* gene was initially identified in a screen for developmental defects in *Caenorhabditis elegans* (Meneely and Herman, 1979), and later found to encode a microRNA that

promotes the differentiation of cellular fates (Reinhart et al., 2000). Orthologs of the *C. elegans let-7* microRNA are easily identified across animal phyla because of the near perfect conservation of the entire 22 nt sequence (Pasquinelli et al., 2000). In many species, *let-7* paralogs encode additional family members, including *mir-48*, *mir-84* and *mir-241* in *C. elegans*, which differ from *let-7* in some of their non-seed nucleotides. In *C. elegans*, *let-7* seed family (*let-7fam*) microRNAs function semi-redundantly to regulate stage-specific larval cell fate transitions, with *mir-48*, *mir-84* and *mir-241* primarily promoting the L2-to-L3 transition and *let-7* primarily promoting the L4-to-adult transition (Reinhart et al., 2000; Abbott et al., 2005).

In *C. elegans*, two major primary transcripts of *let-7* (*pri-let-7*) are produced, *pri-let-7 A* and *pri-let-7 B* of 1731 and 890 nucleotides, respectively. The 5' end of these transcripts can be further processed by trans-splicing with a 22-nucleotide splice leader (SL) RNA (SL1) to produce the 728 nucleotide *SL1-pri-let-7* (Bracht et al., 2004; Van Wynsberghe et al., 2011), (Fig. 1A). All three of these *pri-let-7* transcripts contain the *let-7* precursor hairpin plus additional downstream sequences, which include an element with complementarity to the *let-7fam* seed sequence that has been shown to associate *in vivo* with the Argonaute protein, ALG-1 (Zisoulis et al., 2012).

pri-let-7 transcripts are expressed at all four larval stages of *C. elegans* development (L1-L4), whereas mature *let-7* is abundantly expressed only in the L3 and L4 larval stages. Intriguingly, *pri-let-7* levels oscillate during each larval stage, peaking mid-stage and dipping during larval molts, likely as a result of underlying pulsatile transcriptional activity of the *let-7* locus (Van Wynsberghe et al., 2011; McCulloch and Rougvie, 2014; Perales et al., 2014; Van Wynsberghe et al., 2014). Why *pri-let-7* pulses with each larval stage remains unclear. However, the distinct developmental profiles of *pri-let-7* and mature *let-7*, particularly at early larval stages, indicate potent post-transcriptional inhibition of *let-7* biogenesis during the L1 and L2 stages.

LIN-28 is a conserved RNA-binding protein that can bind to and regulate a variety of RNAs (Stefani et al., 2015; Wilbert et al., 2012). In *C. elegans*, LIN-28 is expressed at early larval stages and exerts a strong inhibition of *let-7* processing (Van Wynsberghe et al., 2011). Similar to *C. elegans*, mammalian Lin28 inhibits *let-7* processing and is expressed in more pluripotent cells. Extensive studies in mammalian systems have also shown that Lin28 exerts its inhibition of *let-7* by binding to the stem-loop of either *pri-let-7* or *pre-let-7* to directly inhibit processing (reviewed by Tsalikis, 2015). Although CLIPseq data from *C. elegans* indicated that, similar to in mammals, LIN-28 could bind *pri-let-7 in vivo* to inhibit accumulation of mature *let-7* microRNA, binding occurs downstream of the *let-7* stem-loop, which suggests a different mode of regulation on *let-7*, the precise mechanism(s) of which remains unclear (Stefani et al., 2015).

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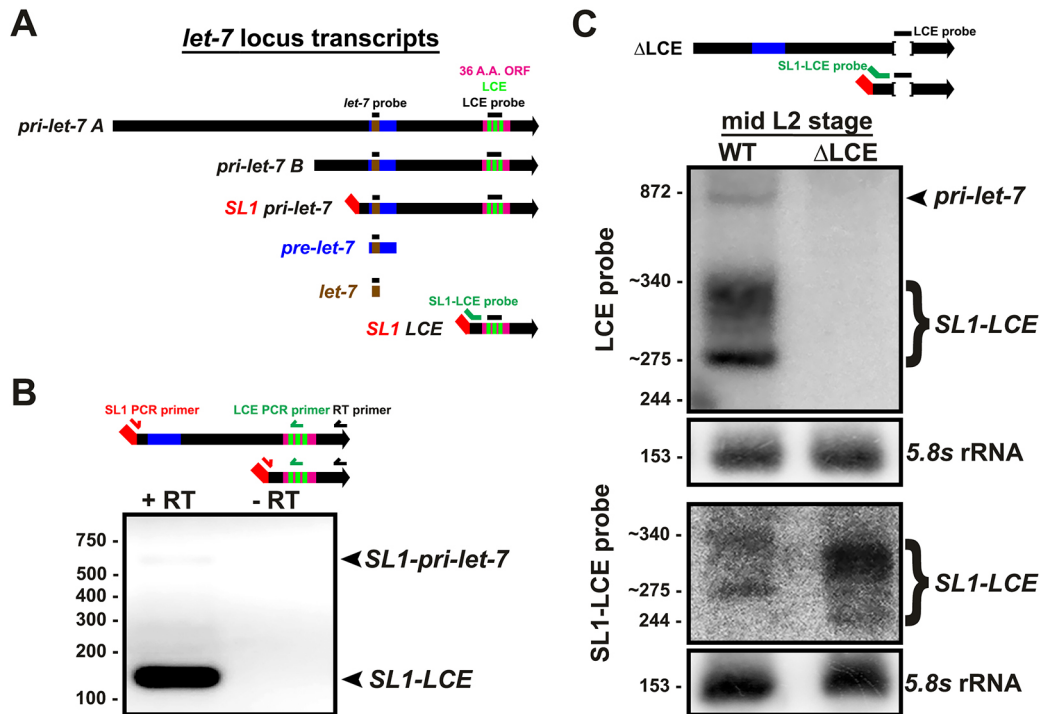


Fig. 1. The *C. elegans let-7* locus produces four transcripts. (A) Major transcripts produced from the *C. elegans let-7* locus, and the locations of probes used in the northern blots shown in C, and in subsequent figures. (B) Non-qRT-PCR analysis of total RNA from a mix-population of wild-type (WT) animals, using an SL1 forward primer and LCE reverse primer, with (+) and without (–) reverse transcriptase (RT) in the cDNA synthesis reaction. Numbers mark dsDNA ladder bands in bp. (C) Total RNA from WT and LCE-deletion mid-L2 animals (20 h after plating) analyzed using northern blotting with probes for the LCE and SL1-LCE. Numbers mark known RNA sizes in nt: 872 nt is *in vitro* transcribed *pri-let-7* B, 244 nt is *in vitro* transcribed SL1-LCE and 153 nt is 5.8s rRNA. Numbers with a tilde before them were estimated from known RNA sizes.

Trans-splicing is the act of joining two separate RNAs. In *C. elegans*, ~70% of all transcripts (including mRNAs and microRNA primary transcripts) are trans-spliced with an SL RNA. The outcomes of trans-splicing include separating the individual mRNAs of polycistronic operon transcripts, shortening the 5' untranslated regions (5' UTRs) of RNAs and changing the 5' RNA cap from monomethyl to trimethyl guanosine. There are two major classes of SL RNAs with distinct sequences: SL1 and SL2. Although there are exceptions, SL1 trans-splicing tends to be the most common 5' trans-splicing event of a primary RNA transcript and invariably results in rapid degradation of the 5' 'outtron'. SL2 trans-splicing is generally restricted to downstream open reading frames (ORFs) of operons after SL1 trans-splicing and polyadenylation of the first mRNA (Morton and Blumenthal, 2011; Blumenthal, 2012). Similar to cis-splicing, trans-splicing in *C. elegans* uses a consensus acceptor sequence of 'TTTCAG' (Graber et al., 2007). In mammalian cell culture, Lin28 has been implicated in cis-splicing through its regulation of splicing factor abundance (Wilbert et al., 2012), whereas any involvement of LIN-28 in *C. elegans* with either cis or trans-splicing remains unknown.

Here, we identify a previously undescribed trans-splicing event in *pri-let-7* that occurs downstream of the *let-7* stem-loop, and produces a short (~262 nt) mRNA that contains a 5' SL1 leader sequence, a short ORF, *let-7* complementary sequences (LCSs) and a poly-A tail. We provide evidence that LIN-28 is necessary for this splicing event, and that trans-splicing serves to negatively regulate *let-7fam* in two ways: First, by preventing precocious *let-7* expression through the degradation of the upstream outtron which contains the *let-7* precursor; second, by inhibiting *let-7fam* activity via production of an RNA that functions as a *let-7fam* sponge. Thus,

we have characterized a splicing event that involves *let-7* primary transcripts and that can regulate *let-7* biogenesis in *cis* as well as *let-7fam* activity in *trans*.

RESULTS

The *let-7* locus produces a short trans-spliced transcript that contains LCSs

As mentioned above, a previous study identified a region of *pri-let-7* in *C. elegans* that contains a *let-7* complementary element (LCE) (Zisoulis et al., 2012). We confirmed that three sites within the LCE element have complementarity to *let-7fam* microRNAs so as to permit base pairing to the *let-7*-family seed sequence plus varying degrees of 3' supplemental pairing (Fig. S1A). We also noted that an additional transcript, C05G5.7, is annotated to be transcribed from the *let-7* locus and to contain a 647 nt 5' UTR that includes the *let-7* stem-loop, a 111 nt ORF that contains the LCE, and a 79 bp 3' UTR. We also identified a potential splice acceptor (SA) sequence that is located upstream of the LCE and downstream of the *let-7* stem-loop, which could mediate SL1-trans-splicing and thereby produce a short ~262 nt transcript that contains the LCE but lacks the upstream *let-7* stem-loop (Fig. 1A, Fig. S1A). Analysis of the *let-7* locus of other *Caenorhabditis* species showed that both the trans-SA sequence and the LCE are conserved (Fig. S1A). To determine whether an ~262 nt SL1-spliced LCE transcript is expressed *in vivo*, we performed non-quantitative PCR (non-qRT-PCR) using SL1 forward and LCE reverse primers, and we observed two distinct bands. We determined the top band to be SL1-*pri-let-7*, the previously known SL1-spliced version of *pri-let-7* (with the SL1 upstream of the *let-7* stem-loop), and the bottom band to be the predicted SL1-spliced LCE transcript (hereafter referred to as

SL1-LCE) (Fig. 1B). We also determined that both *SL1-LCE* and *SL1-pri-let-7* are poly-adenylated, by generating cDNA using oligo-dT primer, followed by non-qRT-PCR (Fig. S2).

We confirmed the existence of *SL1-LCE* by northern blotting with a probe to the LCE sequence and determined that *SL1-LCE* is more abundant than *pri-let-7* at the mid-L2 stage (Fig. 1C). Interestingly, we observed a range of *SL1-LCE* lengths, from an estimated 275 to 340 nt. After probing specifically to the SL1 splice junction at the 5' end of *SL1-LCE*, we observed the same range in length, which indicates that this variation is at the 3' end, and suggests heterogeneity in poly-adenylation and/or transcriptional stop sites (Fig. 1C). To confirm that the variable length was not at the 5' end, we also performed 5' rapid amplification of cDNA ends (RACE) and observed only a single 5' terminus, at the site of the *SL1-LCE* trans-splice (Fig. S3). We also confirmed the three alternative 5' ends of *pri-let-7* (Fig. S3 and data not shown). We failed to detect the 5' end of the annotated transcript C05G5.7.

The location of the trans-SA sequence within the *let-7* locus suggests that *SL1-LCE* could be processed from *pri-let-7*. Therefore, we tested whether the expression pattern of *SL1-LCE* is also developmentally regulated, as is the case for *pri-let-7*. Using quantitative RT-PCR (qRT-PCR), we determined that, similar to *pri-let-7*, *SL1-LCE* levels pulsed in phase with the cycle of larval molts, which indicates that *SL1-LCE* expression could be driven by the same oscillatory transcriptional program as *pri-let-7* (Van Wynsberghe et al.,

2011; Perales et al., 2014). However, unlike *pri-let-7*, *SL1-LCE* stopped pulsing and remained relatively low after the L2 stage (Fig. 2A,B). These findings suggest that *SL1-LCE* is generated from *pri-let-7* by trans-splicing and that it is post-transcriptionally downregulated in conjunction with larval developmental progression.

lin-28 regulates the expression of *SL1-LCE*

Coordinated with a variety of other factors, *let-7* functions within the heterochronic pathway to ensure proper developmental timing of various cell fates, in particular the progression from larval to adult fates during the L4-to-adult transition (Reinhart et al., 2000). The high L1 and L2 expression of *SL1-LCE* suggested that the heterochronic genes that specify early larval events could promote the expression of *SL1-LCE*. Therefore, we assessed *SL1-LCE* expression in heterochronic mutants with altered temporal patterns of early larval cell fates.

lin-4 is a microRNA that is necessary for the transition from the L1 to L2 stages through its targeted repression of the *lin-14* 3' UTR. *lin-4* null [*lin-4(0)*] mutations or *lin-14* gain-of-function [*lin-14(gf)*] mutations result in aberrant upregulation of *lin-14* that retards developmental progression by continuously specifying the repetition of L1 events (Chalfie et al., 1981; Ambros and Horvitz, 1984; Ambros, 1989; Lee et al., 1993). On the other hand, *lin-14* loss-of-function [*lin-14(lf)*] results in premature developmental progression, which is characterized by a skipping of L1 events and

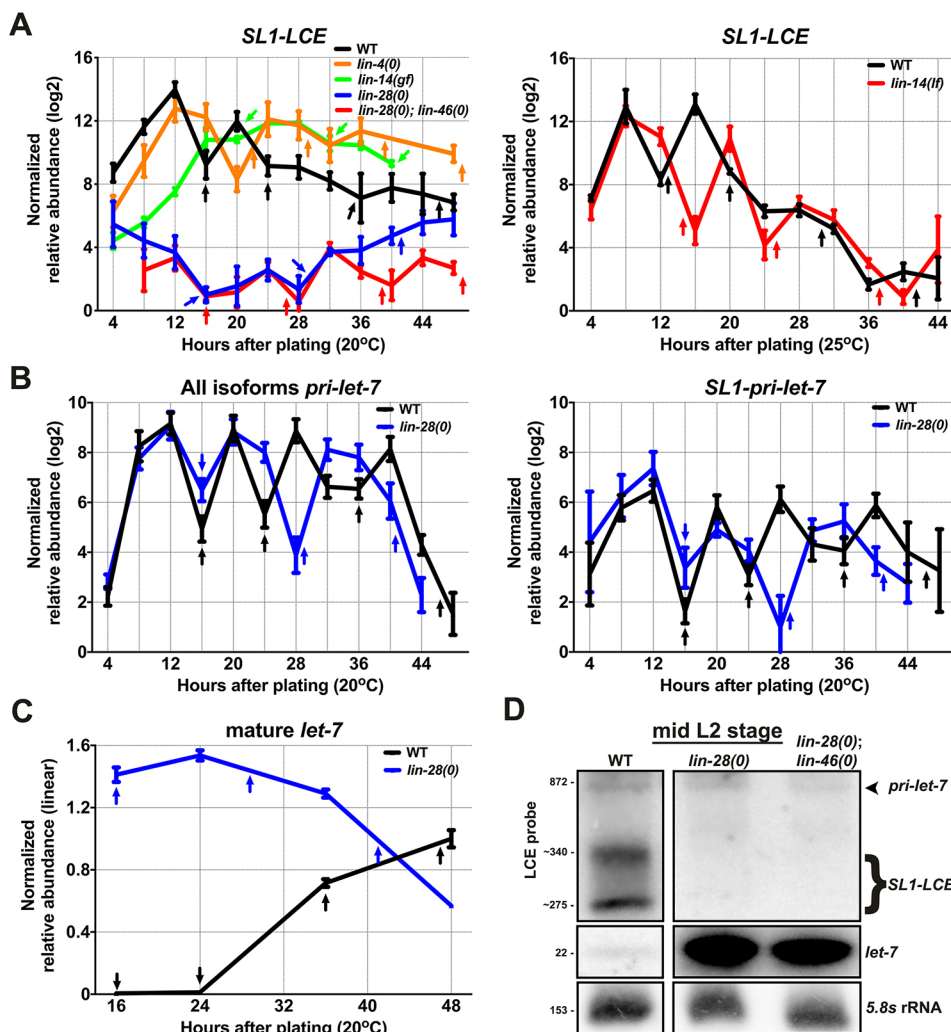


Fig. 2. *lin-28* is necessary for the expression of *SL1-LCE*.

(A) qRT-PCR analysis of *SL1-LCE* levels in samples of total RNA from staged populations of synchronously developing WT animals, and the indicated heterochronic mutants at 20°C (left) and at 25°C (right). (B) qRT-PCR analysis of the levels of all *pri-let-7* isoforms (left) and *SL1-pri-let-7* (right) in samples of total RNA from staged populations of synchronously developing WT and *lin-28(0)* animals at 20°C. $n=3$ biological replicates. (C) FirePlex miRNA analysis of *let-7* levels in samples of total RNA from staged populations of synchronously developing WT and *lin-28(0)* animals. $n=3$ biological replicates. (D) Total RNA from WT, *lin-28(0)* and *lin-28(0); lin-46(0)* mid-L2 animals (20 h after plating) analyzed using northern blotting and hybridized with probes for the LCE (top), for *let-7* (middle) or for 5.8s rRNA (bottom). Numbers mark known RNA sizes in nt: 872 nt is *in vitro* transcribed *pri-let-7-B*, 22 nt is *let-7* and 153 nt is 5.8s rRNA. Numbers with a tilde before them were estimated from known RNA sizes. Data are mean \pm s.d. Arrows mark the times of larval molts.

precocious advancement through the L2 to adult stages (Ambros and Horvitz, 1984). We observed that *SL1-LCE* levels remained high throughout development in *lin-4(0)* animals and in *lin-14(gf)* animals, whereas *SL1-LCE* levels were unchanged in *lin-14(lf)* animals (Fig. 2A), which indicates that *lin-14* promotes, but is not necessary for, *SL1-LCE* expression.

lin-28 encodes an RNA-binding protein that functions in early larval stages to regulate developmental progression from L2 to later cell fates. Loss of *lin-28* results in the skipping of L2 events, precocious advancement to the adult stage and precocious expression of *let-7* (Fig. 2C,D) (Ambros and Horvitz, 1984; Van Wynsberghe et al., 2011). We found that *lin-28(0)* animals exhibited drastically reduced *SL1-LCE* levels compared with the wild type (Fig. 2A,D). Therefore, in contrast to *lin-14*, *lin-28* appears to be essential for expression of the *SL1-LCE* transcript.

The fact that *lin-14(lf)* animals display essentially the same precocious phenotypes as those seen in *lin-28(0)* animals, yet have normal *SL1-LCE* expression, suggests that the reduced *SL1-LCE* expression in *lin-28(0)* is not an indirect consequence of precocious development. In further support of this conclusion, we observed that in *lin-28(0);lin-46(0)* animals, which are completely suppressed for precocious phenotypes but not for precocious *let-7* levels (Pepper et al., 2004; Vadla et al., 2012) (Fig. 2D), *SL1-LCE* levels are similarly reduced as in *lin-28(0)* alone (Fig. 2A,D). Taken together, this suggests that *lin-28* has a relatively direct role in promoting LCE trans-splicing.

One possible explanation for why *SL1-LCE* levels are low in *lin-28(0)* is that expression of *pri-let-7* could be reduced. To test this possibility, we measured the levels of *pri-let-7* using qRT-PCR and observed no reduced expression of *pri-let-7* in *lin-28(0)* larvae (Fig. 2B,D). This finding indicates that LIN-28 post-transcriptionally regulates the generation of *SL1-LCE*. We also noted that, despite the fact that *lin-28(0)* animals undergo only three larval stages instead of the normal four, the pulses of *pri-let-7* still coincided with each larval molt (Fig. 2B).

As mentioned previously, one *pri-let-7* isoform is SL1 trans-spliced upstream of the *let-7* stem-loop. To test whether *lin-28* loss of function also reduced this upstream trans-splicing event, we measured *SL1-pri-let-7* levels in *lin-28(0)* animals and observed no significant reduction (Fig. 2B). Put together, these data indicate that LIN-28 is essential for generating *SL1-LCE* from *pri-let-7* by specifically promoting trans-splicing at the downstream SA.

Mutations of the LCE SA result in the use of cryptic SA sequences

To determine the function of trans-splicing of the LCE transcript, we used CRISPR/Cas9 to introduce mutations of the 'TTTCAG' SL1 acceptor sequence of the LCE transcript. To our surprise, deletion of the TTTCAG sequence did not eliminate LCE trans-splicing; non-qRT-PCR and TA-cloning revealed that SL1 trans-splicing still occurred using a cryptic acceptor sequence (TTGTAG) located 27 nt upstream of the canonical TTTCAG (data not shown). qRT-PCR revealed that in wild-type animals use of this cryptic TTGTAG sequence was minimal (Fig. S4A), whereas in the animals in which the canonical TTTCAG was deleted, use of the cryptic TTGTAG was readily detectable and the expression pattern of the resulting (albeit slightly longer) *SL1-LCE* was similar to that of the normal *SL1-LCE* in wild-type animals (Fig. S4A). Furthermore, the use of this cryptic TTGTAG SA was dependent upon *lin-28* (Fig. S4A), which indicates that LIN-28 can promote LCE-proximal trans-splicing regardless of the acceptor sequence.

With the canonical TTTCAG deleted, northern blots showed a decrease in *SL1-LCE* levels and an increase in *pri-let-7* levels

(Fig. 3B), which indicates that *SL1-LCE* is trans-spliced from *pri-let-7*, and the non-canonical TTGTAG SL1 acceptor sequence is not as efficient in this context as is the wild-type TTTCAG sequence. Moreover, because this cryptic TTGTAG sequence is upstream of the canonical TTTCAG, all of the corresponding *SL1-LCE* bands were shifted up, which strengthened our interpretation that all northern blot bands that are associated with this transcript are SL1 trans-spliced (Fig. 3B).

Based on previous genomic analysis of SL1 splice sites, the consensus SL1 acceptor sequence is TTTCAG. Other acceptor sequences can be utilized, but certain nucleotides appear to remain invariant, namely T in the second position, A in the fifth position and G in the sixth position (Graber et al., 2007). There are six occurrences of the corresponding NTNNAG consensus sequence in the region between the *let-7* stem-loop and the LCE (Fig. 3A). With the aim of eliminating trans-splicing altogether in this region, we made small deletions in all six SAs using CRISPR/Cas9. Non-qRT-PCR and sequence analysis of LCE transcripts from the sixfold SA mutant (mutSA1-6) revealed that some basal level of trans-splicing still occurred, now using two far-non-canonical acceptor sequences, TTTCGG and TTCGGG, which are 1 nt apart from each other and near the original splice site location (data not shown). Compared with wild type, qRT-PCR and northern blotting of mutSA1-6 showed a reduction of approximately 20-fold in *SL1-LCE* level, as well as an increase of approximately fivefold in *pri-let-7* level in the L1 and L2 stages. (Fig. 3C,D). Interestingly, mutSA1-6 animals had no apparent heterochronic phenotype, which suggests that trans-splicing the *SL1-LCE* from *pri-let-7* is not crucial for normal development under standard laboratory conditions.

SL1-LCE trans-splicing regulates *let-7* processing

In *C. elegans*, the 5' region of an RNA that is removed by SL1 trans-splicing is called the outtron. The outtrons of trans-spliced RNAs are rarely detected, which suggests that they are rapidly degraded following trans-splicing (Morton and Blumenthal, 2011). Previously published northern blots of *pri-let-7* suggest that the outtron is below detectable levels (Bracht et al., 2004; Van Wynsberghe et al., 2011, 2014; Zisoulis et al., 2012). The resolution of our northern blots could not definitively show whether the outtron was detectable, so we employed qRT-PCR using RT primers positioned 5' or 3' of the *SL1-LCE* SA, and PCR primer pairs that flanked the *SL1-LCE* SA. cDNA primed from sequences 5' of the SA would represent both the pre-spliced *pri-let-7* as well as the post-spliced outtron, whereas cDNA primed from sequences 3' of the SA would represent only pre-spliced *pri-let-7*. Therefore, if the outtron were present at detectable levels, cDNA from sequences 5' of the SA would be more abundant than cDNA from sequences 3' of the SA. When we performed qRT-PCR to *pri-let-7*, we observed no difference in the yields of cDNA from sequences 5' versus 3' of the SA. This indicates that the outtron is undetectable by this assay, and hence relatively unstable compared with unspliced *pri-let-7* (Fig. S4B).

If trans-splicing of *SL1-LCE* produces an unstable outtron that contains unprocessed *let-7* microRNA, we hypothesized that trans-splicing of *SL1-LCE* from *pri-let-7* could have a net negative effect on the accumulation of mature *let-7* microRNA. In L1 and L2 animals, when trans-splicing is normally abundant, *let-7* microRNA levels in mutSA1-6 animals were approximately twice as high as those seen in the wild type, which indicates that trans-splicing of *SL1-LCE* negatively impacts the accumulation of mature *let-7* (Fig. 3E, Fig. S4C). Interestingly, at later developmental stages when *SL1-LCE* trans-splicing is not prevalent, *let-7* levels were

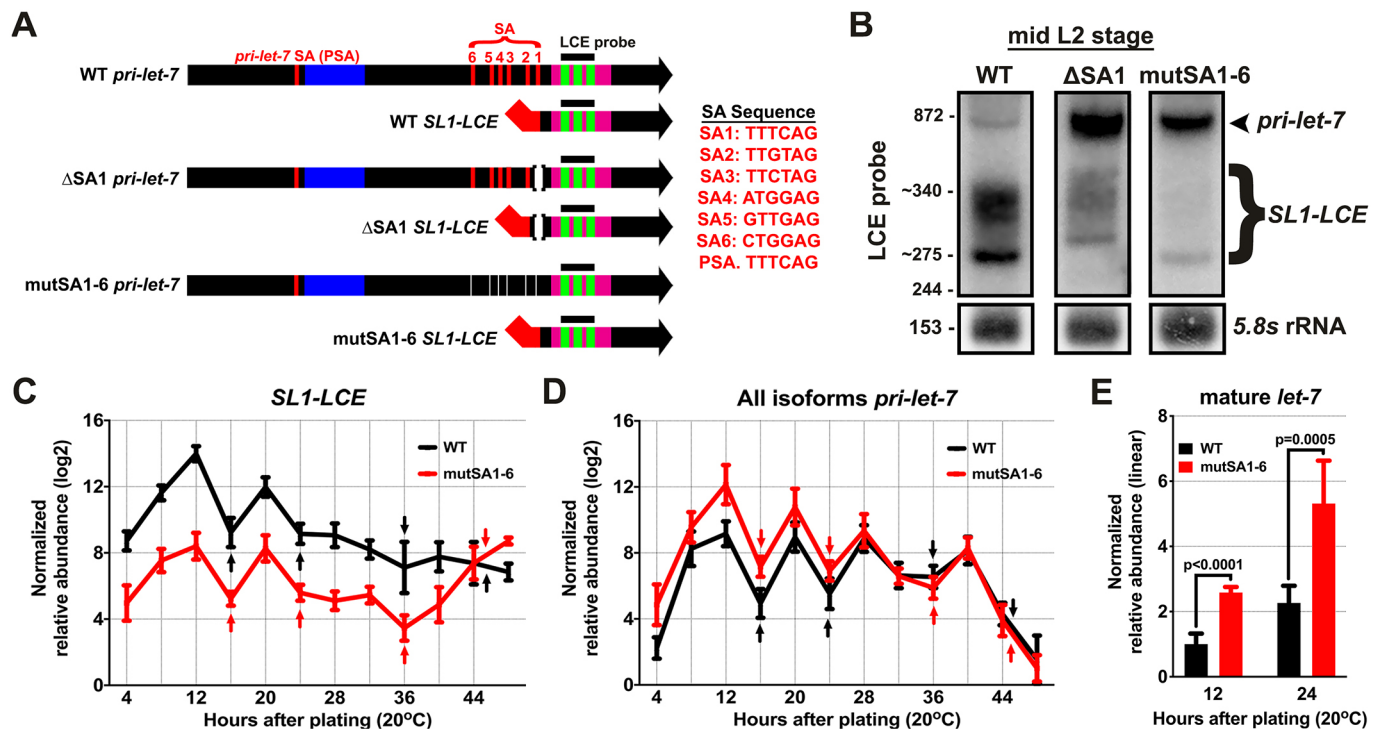


Fig. 3. Larvae with *SL1-LCE* SA site mutations display elevated levels of *pri-let-7* and mature *let-7*. (A) Locations of the NTNNAG motif *SL1* SA in the *let-7* locus and the SA mutations used in these experiments. PSA refers to the SA sequence in *pri-let-7* upstream of the *pre-let-7* stem-loop. SA1 is the position of trans-splicing that generates *SL1-LCE* in the wild type. SA2-SA6 are non-canonical SA sequences, some of which can be utilized when SA1 is mutated. (B) Total RNA from mid-L2 (20 h after plating) WT, SA1-deletion and mutSA1-6 animals, analyzed using northern blotting, hybridized with a probe for the LCE. Numbers mark known RNA sizes in nt: 872 nt is *in vitro* transcribed *pri-let-7-B*, 244 nt is *in vitro* transcribed *SL1-LCE* and 153 nt is 5.8s *rRNA*. Numbers with a tilde before them were estimated from known RNA sizes. (C) qRT-PCR analysis of the levels of *SL1-LCE* in samples of total RNA from staged populations of synchronously developing WT and mutSA1-6 animals. $n=3$ biological replicates. Note: WT *SL1-LCE* data are repeated from Fig. 2A as these experiments were performed together and used for comparison purposes. (D) qRT-PCR analysis of the levels of all *pri-let-7* isoforms in samples of total RNA from staged populations of synchronously developing WT and mutSA1-6 animals. $n=3$ biological replicates. Note: WT *pri-let-7* data are repeated from Fig. 2B as these experiments were performed together and used for comparison purposes. (E) FirePlex miRNA analysis of *let-7* levels in samples of total RNA from staged populations of synchronously developing WT and mutSA1-6 animals at the mid-L1 (12 h after plating) and late-L2 (24 h after plating) stages. $n=6$ biological replicates. Statistical significance was determined using a two-tailed Student's *t*-test. Data are mean \pm s.d. Arrows mark the times of larval molts.

reduced by ~60% to 70%, which suggests an additional positive regulatory role for sequences that overlap one or more of the SA elements that are mutated in mutSA1-6 (Fig. S4D).

The LCE functions to negatively regulate the *let-7* family

A previous study reported experiments that suggested the LCE region in *pri-let-7*, in conjunction with ALG-1, could function *in cis* to facilitate *let-7* biogenesis (Zisoulis et al., 2012). In that study, a transgene that carried a modified *let-7* locus with a deletion of 178 bp (which removed the LCE and surrounding sequences) expressed decreased levels of mature *let-7*, which suggested that the

LCE, perhaps when bound to the *let-7* RNA-induced silencing complex (miRISC), could function to promote microprocessing of *pri-let-7*. However, because this 178 bp deletion also removed sequences that are upstream of the LCE, including the *SL1*-acceptor sequence, we used CRISPR/Cas9 to create a 55 bp deletion at the endogenous *let-7* locus that removed only the LCE (Fig. 4A). This 55 bp deletion of the LCE did not result in a measurable change in *let-7* levels (Fig. 4B). We also used CRISPR/Cas9 to introduce the previously described 178 bp deletion at the endogenous *let-7* locus (Fig. 4A), and confirmed the previous (Zisoulis et al., 2012) results: an ~tenfold reduction in *let-7* levels (Fig. 4B). Based on these

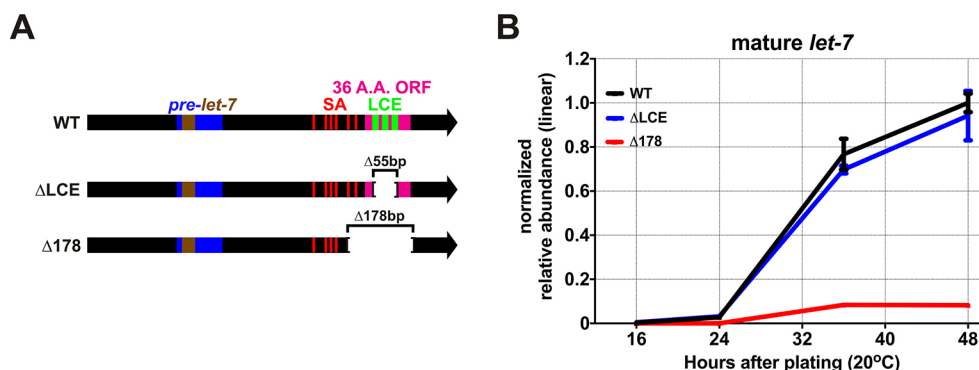


Fig. 4. LCE sequences are dispensable for normal expression of mature *let-7* microRNA. (A) Positions of the deletions in the *let-7* mutants used in these experiments. (B) FirePlex miRNA analysis of *let-7* levels in WT and LCE-deletion (Δ LCE) animals, and in animals containing a 178 bp deletion (Δ 178) that has been previously reported to reduce *let-7* biogenesis throughout development. Data are mean \pm s.d. $n=3$ biological replicates.

results, we conclude that the larger 178 bp deletion removes unknown non-LCE positive regulatory elements, and that the LCE sequences themselves do not exert a detectable positive effect on *let-7* biogenesis.

Because *SL1-LCE* contains sequences that are complementary to *let-7fam*, we hypothesized that *SL1-LCE* could function as a sponge to negatively regulate *let-7fam*. To test this, we sought to determine whether mutations that disrupt *SL1-LCE* could genetically interact with *let-7fam* in sensitized genetic backgrounds. Of the four major *let-7* family genes, only *let-7(lf)* or *mir-48(0)* mutants display overt heterochronic phenotypes in normal laboratory conditions. Loss of either *mir-48* or *let-7* results in retarded hypodermal development and an extra larval molt. In addition, *let-7(lf)* hermaphrodites burst through an improperly formed vulva (an adult lethality phenotype), and *mir-48(0)* adult hermaphrodites die because of egg retention, presumably because of their retarded hypodermal development. We hypothesized that if *SL1-LCE* were to function as a sponge for the *let-7fam* microRNAs, loss of the LCE sequences would result in increased *let-7fam* activity, which could be evidenced by suppression of *let-7(lf)* or *mir-48(0)* phenotypes.

Col-19::GFP is a reporter that is expressed in hypodermal seam cells and in the hypodermal syncytium (*hyp-7*) beginning at the L4 molt. In molting L4 animals with either the strong *let-7(lf)* allele

[*let-7(mn112)*] or the *mir-48(0)* null allele [*mir-48(n4097)*], Col-19::GFP expression in *hyp-7* is reduced by ~20-fold compared with the wild type, and is limited to the seam cells. Moreover, *let-7(mn112)* animals burst through their vulvas as young adults whereas *mir-48(n4097)* and the weaker *let-7(mg279)* allele do not burst, but survive to undergo an extra larval molt, and exhibit egg-laying defects and a reduced brood size. Using CRISPR/Cas9, we introduced a deletion of the *let-7* hairpin into the LCE deletion background and observed no suppression of the strong *let-7(lf)* phenotypes (data not shown). Therefore, we hypothesized that the phenotypes that are associated with this substantial reduction of *let-7* are too strong to be suppressed by loss of the LCE. To test whether deletion of the LCE could suppress a partial loss of function of *let-7* we used CRISPR/Cas9 to delete the LCE of the hypomorphic allele *let-7(mg279)*. The resulting *let-7(mg279 ΔLCE)* strain exhibited no apparent suppression of the *let-7(mg279)* retarded phenotypes as measured by the expression pattern of Col-19::GFP, reduced brood size, extra molting phenotype, or adult mortality (Fig. 5B, Fig. S5). Therefore, absence of the LCE did not display genetic interaction with loss of *let-7*.

By contrast, a strong genetic interaction was evident between LCE deletion and *mir-48(0)*. When the LCE deletion mutation was crossed into *mir-48(0)*, Col-19::GFP expression was restored to a

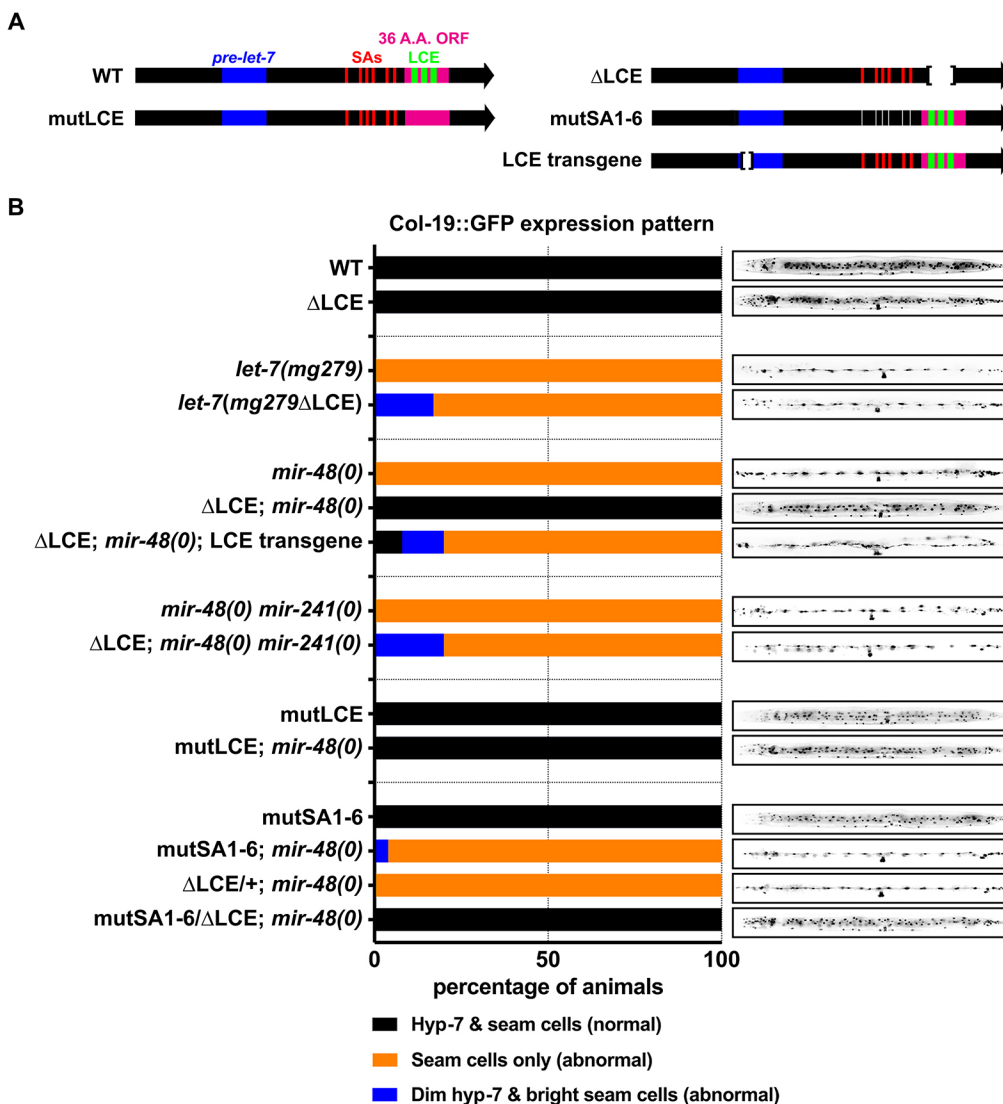


Fig. 5. Inhibition of *SL1-LCE* function, either by deletion of the LCE, or by mutations of LCE-proximal trans-splicing acceptor sequences, suppresses the retarded hypodermal phenotypes of *mir-48(0)* animals. (A) Positions of the *let-7* LCE mutations (green), SA mutants (red) and LCE transgene used in these experiments.

(B) Quantification of the Col-19::GFP phenotypes observed for each genotype in molting late L4 animals (left). From top to bottom: n=9 (WT), 10 (ΔLCE), 23 [*let-7(mg279)*], 12 [*let-7(mg279) ΔLCE*], 9 [*mir-48(0)*], 22 [ΔLCE; *mir-48(0)*], 26 [ΔLCE; *mir-48(0)*; LCE transgene], 18 [*mir-48(0) mir-241(0)*], 15 [ΔLCE; *mir-48(0) mir-241(0)*], 10 (mutLCE), 15 [mutLCE; *mir-48(0)*], 10 (mutSA1-6), 10 [mutSA1-6; *mir-48(0)*], 11 [ΔLCE/+; *mir-48(0)*] and 16 [mutSA1-6/ΔLCE; *mir-48(0)*] animals. Images (right) are of a representative molting L4 animal for each genotype.

wild-type pattern (Fig. 5B). This suggests that removal of the LCE sequences results in upregulation of the activity of one or more members of *let-7fam*. Consistent with this supposition, when a second family member, *mir-241*, was also removed, loss of the LCE failed to restore the normal timing of Col-19::GFP expression (Fig. 5B). Deletion of the LCE similarly suppressed the extra molting phenotype, restored survival and restored the brood size of *mir-48(0)*, but only partially suppressed *mir-48(0) mir-241(0)* double null phenotype (Fig. S5). To determine whether this suppression of *mir-48(0)* was due to an elevation in *let-7fam* levels, we measured *let-7fam* levels in wild-type and LCE deletion animals and observed no difference (Fig. S6). Together, these results indicated that the LCE negatively regulates *let-7fam* by modulating their activity rather than their levels.

The LCE sequence is predicted to contain a 36 amino acid ORF that is poorly conserved in other *Caenorhabditis* species (Fig. S1A) and it is therefore unlikely to perform a conserved function. Nevertheless, our 55 bp deletion of the LCE disrupts this putative ORF so it was possible that disruption of the ORF could confound the interpretation of our results. Therefore, we used CRISPR/Cas9 to mutate the three LCSs without altering the amino acid sequence of the ORF. Similar to the LCE deletion, these ‘silent’ LCS mutations did not alter *let-7* levels (data not shown), but did restore normal Col-19::GFP expression, normal survival, normal brood size and suppressed the extra molting phenotype in *mir-48(0)* animals but not in *mir-48(0), mir-241(0)* double null animals (Fig. 5B, Fig. S5).

Trans-splicing of the LCE is necessary to negatively regulate *let-7fam* microRNAs

To function as a negative regulator of *let-7fam* activity by acting as a microRNA sponge, the *SL1-LCE* transcript would presumably encounter *let-7fam* miRISC in the cytoplasm. As mentioned above, *SL1-LCE* contains a putative 36 amino acid ORF. Previously published ribosome-profiling data indicated that ribosomes locate to the LCE sequence (Michel et al., 2014), which suggests that the LCE ORF is translated, and therefore enters the cytoplasm where it could engage *let-7fam* microRNAs. To confirm that the LCE ORF can be translated *in vivo*, we generated transgenic animals that carried a transgene with the C-terminus of the LCE ORF fused to GFP and observed fluorescence in cell types that were previously reported to express *let-7fam*, including head and tail neurons, sensory neurons, ventral and dorsal nerve cords, pharynx, intestine,

hypodermis and vulva (Johnson et al., 2003; Kai et al., 2013; Martinez et al., 2008; McCulloch and Rougvie, 2014; Zou et al., 2013; Hayes et al., 2006). GFP expression appeared to be most constant and brightest in neurons, and most dynamic and dimmest in the remaining cell types. Overall, GFP expression recapitulated the temporal expression of *SL1-LCE*; however, some neurons retained bright GFP expression well into adulthood (Fig. S7).

In addition to being cytoplasmic, to function as a sponge the *SL1-LCE* transcript should be expressed at levels in molar excess of *let-7fam* microRNAs. To determine the stoichiometric ratio of *SL1-LCE* to *let-7fam* in wild-type larvae, we quantified the amount of *SL1-LCE* and *let-7fam* microRNAs in RNA samples from synchronized populations of developing larvae. We calibrated these assays using known amounts of *in vitro*-transcribed *SL1-LCE* and *pri-let-7*, and synthetic *let-7fam* microRNA oligonucleotides. The results of these quantitative assays indicated that, in whole animals, the *SL1-LCE* is in molar excess of *let-7, mir-48, mir-84* and *mir-241* during the L1 and L2 stages (Fig. 6A). Importantly, *pri-let-7* levels were not in excess of *let-7fam*, indicating that *pri-let-7*, despite containing LCE sequences, is not likely to contribute as significantly to *let-7fam* sponging as does the *SL1-LCE* transcript.

Based on its relative abundance and cytoplasmic location, our results suggest that of the two classes of LCE-containing transcripts that are produced from the *let-7* locus (*pri-let-7* and *SL1-LCE*), *SL1-LCE* is more likely to function as a sponge for *let-7fam* microRNA. This indicates that the sponging activity of the LCE would depend on trans-splicing of *SL1-LCE*. To test this supposition, we took advantage of the splice site (*mutSA1-6*) mutant animals, which have reduced *SL1-LCE* levels. Using the same calibrated quantitation as above, we determined that the reduced *SL1-LCE* level in whole *mutSA1-6* animals was less than that of *let-7fam* (Fig. 6B). Moreover, the elevation in *pri-let-7* in the *mutSA1-6* was not sufficient to put it in excess of all *let-7fam* (Fig. 6B), although we note that in *mutSA1-6*, *pri-let-7* was in excess of *mir-241* and *let-7* at the L1 peak and approximately equimolar with *let-7* at the L2 peak (Fig. 6B).

We hypothesized that, similar to deletion of the LCE sequences, the reduction in *SL1-LCE* levels in *mutSA1-6* would increase the activity of *let-7fam* and suppress *mir-48(0)* phenotypes. However, when *mutSA1-6* and *mir-48(0)* were combined, we observed no suppression (Fig. 5B, Fig. S5). This suggested that, although *mutSA1-6* causes a significant decrease in *SL1-LCE*, the remaining LCE-containing transcripts could nevertheless be functional.

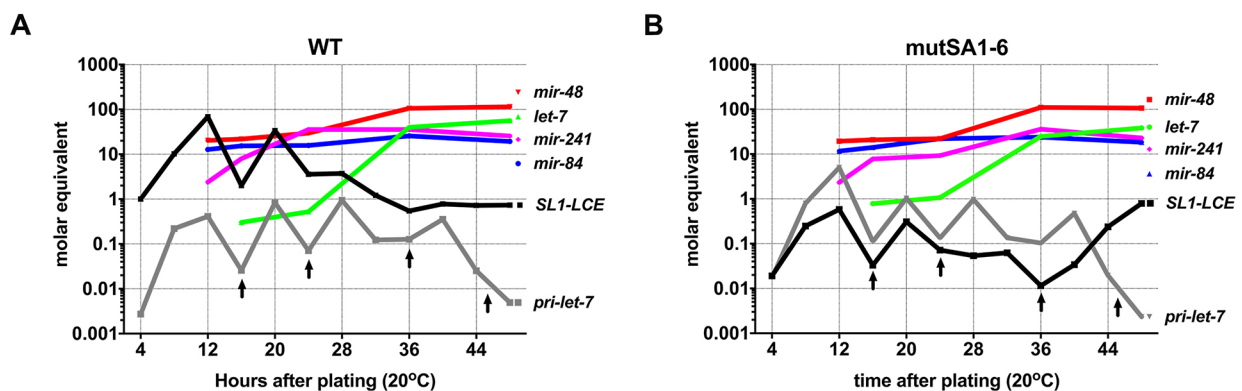


Fig. 6. The *SL1-LCE* is in molar excess relative to *let-7fam* microRNAs during early larval stages. (A,B) Relative levels (molar equivalents, normalized to *in vitro* transcribed *pri-let-7* and synthetic *let-7fam*) of *pri-let-7*, *SL1-LCE*, *mir-48*, *mir-84*, *mir-241* and *let-7*, determined using calibrated qRT-PCR (for *pri-let-7* and *SL1-LCE*) or calibrated Fireplex assay (for microRNAs), in samples of total RNA from staged populations of synchronously developing WT (A) and *mutSA1-6* (B) animals. $n=3$ biological replicates. Arrows mark the times of larval molts.

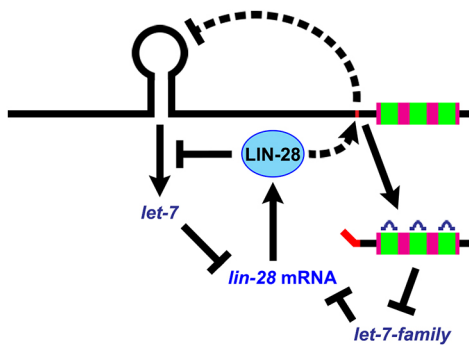


Fig. 7. A model of how LIN-28 and *let-7fam* microRNAs function in a reciprocal negative regulatory network. A summary of the genetic pathway determined by our results in which *lin-28* and *let-7fam* post-transcriptionally exert reciprocal negative feedback on each other.

Therefore, we aimed to reduce the amount of remaining *SL1-LCE* by half using *mutSA1-6/ΔLCE* trans-heterozygous animals. This further reduction in *SL1-LCE* resulted in suppression of all the heterochronic phenotypes that are associated with *mir-48(0)*, which supports the conclusion that *SL1-LCE* functions to negatively regulate *let-7* family activity (Fig. 5B, Fig. S5).

Because *SL1-LCE* levels in *mutSA1-6* whole-animal RNA extracts were significantly lower than those of *let-7fam*, we were concerned that the suppression that was observed in the *mutSA1-6/ΔLCE* trans-heterozygous animals was due to the genetic background of ΔLCE . We therefore examined $\Delta LCE/+$ animals and failed to observe any suppression of *mir-48(0)* heterochronic phenotypes, which indicates that suppression of *mir-48(0)* was due to the *mutSA1-6/ΔLCE* allelic configuration (Fig. 5B, Fig. S5). To further control for a potential genetic background origin for the suppression of *mir-48(0)* in *mutSA1-6/ΔLCE* animals, we sought to express transgenically the LCE transcript in *mir-48(0)* animals that carried the LCE deletion. If the suppression of *mir-48(0)* were due to bona fide loss of the LCE transcript, then we would expect that restoring expression of the LCE transcript from a transgene should eliminate the suppression. Accordingly, we generated a *let-7* extrachromosomal transgene that lacked the mature *let-7* sequence but still contained the LCE (Fig. 5A). When expressed in animals that lacked both *mir-48* and their endogenous LCE we observed restoration of the heterochronic phenotypes that are associated with *mir-48(0)*. Put together, these results indicate that the suppression of the *mir-48(0)* phenotypes in ΔLCE homozygotes and $\Delta LCE/mutSA1-6$ trans-heterozygotes is due to a loss or decrease in *SL1-LCE* levels.

DISCUSSION

A general property of microRNAs across diverse organisms is that they are first transcribed as longer primary transcripts, which are then enzymatically processed to produce the mature 22 nt microRNA. The multiple biogenesis steps required to generate a mature microRNA provide access for a range of transcriptional and post-transcriptional regulation. For example in *C. elegans*, HBL-1 and LIN-42 can modulate the transcriptional activity of microRNAs including *let-7* and *lin-4* (Roush and Slack, 2009; McCulloch and Rougvie, 2014; Perales et al., 2014; Van Wynsberghe et al., 2014), and LIN-28 post-transcriptionally regulates *let-7* (Van Wynsberghe et al., 2011). The negative regulation of *let-7* by LIN-28 is evolutionarily conserved. In mammals, it has been shown that LIN28 can bind to the stem-loop of *pri-let-7* and/or *pre-let-7* to directly inhibit processing by Drosha and/or Dicer (Heo et al., 2008; Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008;

Heo et al., 2009; Loughlin et al., 2012; Nam et al., 2011; Piskounova et al., 2011). In *C. elegans*, LIN-28 also appears to inhibit processing of *pri-let-7* by Drosha, although apparently not by binding the *let-7* stem loop, but rather through binding to sequences ~170 nt downstream (Stefani et al., 2015). In addition, in both *C. elegans* and mammals, the 3' UTR of *lin-28* contains sequences that are complementary to the *let-7fam*, which indicates that *let-7fam* can repress LIN-28 expression (Reinhart et al., 2000; Rybak et al., 2008). Thus, *lin-28* engages in an evolutionarily conserved reciprocal negative feedback with *let-7fam* microRNAs (Fig. 7). However, the functional significance of these *lin-28-let-7fam* regulatory interactions, and their precise mechanisms, are not fully understood.

In this study, we identified a previously undescribed RNA, *SL1-LCE*, which is trans-spliced from *C. elegans pri-let-7* downstream of the *pre-let-7* stem-loop, and which contains LCSs followed by 3' poly-A. We determined that *SL1-LCE* is highly expressed in the early larval stages, displaying an inverse expression pattern compared with *let-7*, which suggests that it could be associated with negative regulation of *let-7* biogenesis. We find that expression of *SL1-LCE* coincides with the expression of LIN-28, and is dependent on *lin-28* function, which reveals a novel regulatory circuit in which LIN-28 governs trans-splicing of *pri-let-7* to negatively impact *let-7* microprocessing (Fig. 7). The regulation of *SL1-LCE* trans-splicing by *lin-28* appears to be independent of other phenotypes that are controlled by *lin-28*, as another precocious mutant *lin-14(lf)* had no effect on trans-splicing, and *SL1-LCE* levels were low in *lin-28(0);lin-46(lf)*, in which precocious phenotypes are suppressed.

Interestingly, when we mutated the *SL1-LCE* trans-SA sequence, even in combination with mutations of nearby putative SAs, trans-splicing persisted using far non-canonical acceptor sequences. This suggests the presence of sequences in *pri-let-7* with potent splicing enhancer activity. Although the use of a far non-canonical sequence is unusual, it is not unprecedented in *C. elegans* (Aroian et al., 1993).

Our findings suggest that LIN-28 inhibits *let-7* biogenesis through the combined effects of two mechanisms. On the one hand, LIN-28 binds directly to *pri-let-7* to inhibit processing by Drosha/Pasha (Stefani et al., 2015); on the other hand, LIN-28 also promotes *SL1-LCE* trans-splicing, which results in downregulation of *pri-let-7* levels (Fig. 7). LIN-28 could be either directly regulating LCE trans-splicing through its binding to *pri-let-7*, or indirectly through other means such as regulating splicing components. In fact, in mammalian cells, LIN28 has been shown to indirectly affect alternative splicing by regulating the expression of certain splicing factors (Wilbert et al., 2012).

lin-28(0) animals exhibit greater than 100-fold elevation of *let-7* in the L1 and L2 stages, presumably as a result of a release of repression from both trans-splicing and inhibited biogenesis. When we reduced LCE trans-splicing by mutating SA1-6 in a wild-type *lin-28* background, mature *let-7* was not de-repressed as much as in *lin-28(0)*; although we observed a marked elevation in *pri-let-7*, which is consistent with a role for LCE trans-splicing in destabilizing *pri-let-7*, the attendant elevation of mature *let-7* was much more modest (only ~twofold), which apparently reflects a potent inhibition by LIN-28 of *pri-let-7* Drosha/Pasha processing.

Interestingly, although we observed an elevation of mature *let-7* levels in *lin-28(0)* larvae that exceeded 100-fold, we observed no corresponding decrease in *pri-let-7*. Apparently, in the absence of LIN-28, the destabilization of *pri-let-7* levels due to increased Drosha/Pasha processing is balanced by a commensurate stabilization of *pri-let-7* due to reduced *SL1-LCE* trans-splicing.

These observations further suggest that the level of *pri-let-7* in wild-type larvae could be subject to homeostatic regulation.

In mammals, trans-splicing is relatively rare compared with in nematodes (Lei et al., 2016). However, most human microRNA genes, including ten of the 12 genes that encode *let-7fam* members, are located within introns of mRNAs or non-coding RNAs (Rodriguez et al., 2004; Kim and Kim, 2007). Therefore, it is possible that the spliceosomal machinery could contribute to the regulation of microRNA biogenesis in contexts other than *C. elegans let-7*. In fact, interplay between microRNA microprocessing and splicing has previously been observed. For example, the microRNA processing machinery Drosha/DGCR8, as well as microRNA primary transcripts, have been observed to be associated with supraspliceosomes, and inhibition of splicing can result in the elevation of microRNAs, including *let-7* (Agranat-Tamir et al., 2014). On the other hand, there is evidence of situations in which splicing and the biogenesis of intronic microRNAs can co-occur without apparently influencing each other (Kim and Kim, 2007), which suggests that connectivity between microRNA processing and host gene splicing is likely to be subject to regulation, depending on context and circumstances.

A previous study demonstrated that the *C. elegans* microRNA Argonaute ALG-1 could bind *in vivo* to the *let-7* locus LCE suggesting that *let-7* miRISC could associate with *pri-let-7* in the nucleus and regulate *let-7* biogenesis (Zisoulis et al., 2012). In support of this idea, it was found that a transgene that contained a mutant *let-7* locus that deleted 178 bp spanning the LCE displayed a marked decrease in *let-7* biogenesis compared with wild type. When we generated the same 178 bp deletion in the endogenous *let-7* locus using genome editing, we also observed that the deletion resulted in decreased *let-7* expression, which confirmed that positive regulatory elements are contained in the 178 bp region. However, when we removed only the LCE at the endogenous locus, we observed no difference in *let-7* levels, which indicates that the putative positive elements that are contained within the 178 bp deleted region are located outside of the LCE, and that the LCE itself does not exert a detectable positive role in *let-7* biogenesis. Our finding that LCE sequences are contained in a cytoplasmic mRNA that is produced by trans-splicing from *pri-let-7* has suggested that the LCE likely functions primarily by associating with *let-7fam* microRNAs in the cytoplasm. However, we cannot rule out the possibility that LCE sequences could also interact with miRISC in the nucleus.

Our results show that LIN-28-dependent trans-splicing of the *C. elegans let-7* primary transcript can act in *cis* to negatively influence *let-7* biogenesis, and at the same time produce a trans-acting inhibitory RNA, *SL1-LCE*, which negatively regulates the activity of *let-7fam* microRNAs. We propose that *SL1-LCE* functions as a sponge for *let-7fam* microRNAs through base pairing to the *let-7fam* seed sequence. We observed that loss of the LCE suppresses *mir-48(0)* presumably by boosting the activity of the remaining *let-7-family* microRNAs. However, loss of the LCE failed to suppress *let-7(lf)* phenotypes, even in the case of a weak *let-7(lf)* mutation, *mg279*. This indicates that perhaps the particular *let-7-family* microRNA(s) that are hypothetically elevated in activity by loss of the *SL1-LCE* can substitute for *mir-48* but not *let-7*.

Interestingly, animals with deletions of the LCE or SAs displayed no overt phenotypes except in the sensitized background of *mir-48(0)*. This suggests that regulation of *SL1-LCE* production is not crucial for normal development under standard laboratory conditions, but may function to modulate *let-7* biogenesis and *let-7fam* activity in the context of ensuring robust developmental timing under stressful physiological or environmental conditions.

Analysis of RNA that was extracted from whole animals with deletions in the LCE's SA sequences indicated that the *SL1-LCE* was no longer in molar excess compared with *let-7fam*. Based on this, we would have predicted these animals to exhibit suppressed *mir-48(0)* phenotypes, but suppression was not evident unless we further reduced *SL1-LCE* levels by removing one copy of the LCE. This suggests that the change in the molar ratio of *SL1-LCE* to *let-7fam* in homozygous mutSA1-6 animals compared with wild type may not be sufficient in specific cell types to suppress the heterochronic phenotypes of *mir-48(0)*. Unfortunately, we do not know which *SL1-LCE*-expressing cell types contribute to *let-7fam* regulation, nor do we know the stoichiometry of *SL1-LCE* and *let-7fam* within the relevant cells. Furthermore, we note that mutSA1-6 animals displayed reduced levels of *let-7* at later larval stages, which could confound the detection of any suppression of *mir-48(0)*. Finally, we cannot also exclude the possibility that the *SL1-LCE* may directly or indirectly regulate the *let-7fam* in a non-molar-equivalent manner.

Employing an LCE-ORF::GFP transgenic reporter, we observed expression of the *SL1-LCE* in a variety of cell types throughout development. All of the cell types in which we observed expression have previously been reported to express members of the *let-7fam*. Although we observed weak GFP expression in cell types that are associated with the heterochronic phenotypes of *let-7fam* mutants, namely the hypodermis and vulva, we observed the strongest expression in neurons. This suggests that *SL1-LCE*-mediated regulation of *let-7fam* activity could have a neuronal component. Interestingly, all *let-7fam* members are expressed in neurons throughout development but their roles in these cells are not well understood. One of the hallmarks of *let-7fam* mutants is the expression of a supernumerary larval molt and, interestingly, neuroendocrine signaling has been shown to regulate molting in arthropods and is also thought to regulate molting in nematodes. Indeed, mutations in the *C. elegans* neuronal-expressed gene *pqn-47* (*myrf-1*) result in the reiteration of a larval molt, which demonstrates a link between neuronal signaling and the heterochronic pathway (Frand et al., 2005; Russel et al., 2011).

MATERIALS AND METHODS

Nematode methods and phenotypic analysis

C. elegans were cultured on nematode growth medium (NGM) (Brenner, 1974) and fed with *E. coli* HB101. Synchronized populations of developmentally staged worms were obtained using standard methods (Stiernagle, 2006). Unless otherwise noted, all experiments were performed at 20°C. A list of strains used in this study is in Table S3.

For heterochronic phenotype analysis, early L4 animals were picked from healthy uncrowded cultures, placed onto individual plates seeded with HB101 and observed periodically until the end of the experiment. Fluorescence microscopy was used to score Col-19::GFP expression.

Sequence alignments and target prediction

DNA and ORF alignments were performed using Clustal Omega (www.ebi.ac.uk). *let-7fam*:LCE target predictions were performed using RNAhybrid (<https://bibiserv2.cebitec.uni-bielefeld.de>).

Brood size counts

Young adult hermaphrodites were placed individually on plates seeded with HB101 and each animal was transferred daily to a fresh plate. The number of progeny produced on each plate was assessed until the animal stopped producing progeny.

RNA extraction

A population of animals was collected and flash-frozen in liquid nitrogen, and total RNA was extracted using Qiazol reagent (Qiagen) as described by McJunkin and Ambros (2017).

Northern blotting

Northern blotting was adapted from Lee and Ambros (2001). RNA samples were run on 5% urea-PAGE gels and then transferred to GeneScreen Plus Hybridization membranes (PerkinElmer) using electrophoresis. After transfer, the membranes were crosslinked with 120 mJoules of UV (wavelength of 254 nm) and baked at 80°C for 1 h. Oligonucleotide probes (Table S2) were labeled using the Integrated DNA Technologies Starfire Oligos Kit with alpha-³²P ATP and hybridized to the membranes at 37°C in 7% SDS, 0.2 M Na₂PO₄ (pH 7.0) overnight. Membranes were washed at 37°C, twice with 2× SSPE, 0.1% SDS and twice with 0.5× SSPE, 0.1% SDS. The blots were exposed on a phosphorimager screen and imaged with a Typhoon FLA7000 (GE).

Non-qRT-PCR

Samples of total RNA were pre-treated with turbo DNase (Invitrogen) (Pinto and Lindblad, 2010). cDNA was synthesized using SuperScript IV (Invitrogen) following the manufacturer's instructions, using the RT oligonucleotides 'let-7 RT' or 'oligo (dT)'. PCR was then performed using 2× PCR PreMix (Sydlabs) with the primers SL1 F and LCE R for *SL1-pri-let-7*, *SL1-LCE* and cryptic SA identifications; SL1-LCE F and LCE R for oligo (dt)-based *SL1-LCE* identification; and SL1-pri-let-7 F and pri-let-7 R for oligo (dt)-based SL1-pri-let-7 identification following the manufacturer's instructions using 1 µl of cDNA and an annealing temperature of 55°C. The products were then analyzed using electrophoresis on a 2% agarose gel, imaged, cut out and gel purified using the EZ-10 Spin Column DNA Gel Extraction Minipreps Kit (Bio Basic Canada), TA cloned using the TA Cloning Kit with pCR2.1 Vector (Invitrogen) and subjected to Sanger sequencing using the M13 reverse primer.

Quantitative PCR

cDNA was synthesized as described above using the RT oligonucleotides 'let-7 RT' and 'gpd-1 QPCR R' (for full-length *let-7* locus transcripts) or 'pri-let-7 R' and 'gpd-1 QPCR R' (for outtron detection). qPCR reactions were performed using Qiagen QuantiFast SYBR Green PCR kit following the manufacturer's instructions, using an ABI 7900HT Real Time PCR System (Applied Biosystems). With the exception of the experiments we have reported in Fig. 6, ΔCTs were calculated by normalizing samples to *gpd-1* (*GAPDH*). ΔCTs were then inverted so that greater values reflect greater RNA levels, and were normalized to set the value of the least abundant sample to one. For each biological replicate, the average of three technical replicates was used.

5' RACE

5' RACE was adapted from Pinto and Lindblad (2010) and Turchinovich et al. (2014). Samples of total RNA from late L2 animals were pre-treated with Turbo DNase (Invitrogen) (Pinto and Lindblad, 2010). Then 1.6 µl of the RNA (in H₂O) was combined with 0.5 µl of 10 µM let-7 RT oligo and 0.4 µl of 25 mM dNTPs. This mixture was incubated at 65°C for 10 min, chilled on ice, and then combined with 1.6 µl 25 mM MgCl₂, 0.6 µl 100 mM MnCl₂, 4 µl 5× First-Strand Buffer (Invitrogen), 2 µl 0.1 M dithiothreitol, 0.3 µl Ribolock (ThermoFisher) and 8 µl H₂O. This mixture was incubated at 42°C for 2 min, then 1.0 µl of SuperScript II (Invitrogen) was added and the mixture was incubated at 42°C for 30 min. Next, 2.0 µl of 5' RACE template switching oligonucleotide (10 µM) was added and the incubation was continued at 42°C for an additional 60 min. The reaction was heat inactivated at 70°C for 15 min, then diluted 1:10 and used for a standard PCR with the primers Rd1 SP and pri-let-7 R for *pri-let-7*, and Rd1 SP and LCE R for *SL1-LCE*. PCR products were TA-cloned (Invitrogen) and subjected to Sanger sequencing.

Quantitative microRNA detection

microRNAs were quantified from total RNA using FirePlex miRNA assay (Abcam) following the manufacturer's instructions. Guava easyCyte 8HT (Millipore) was used for analysis. With the exception of the Calibrated RNA quantitation experiments (below), signals (arbitrary units) were normalized using geNorm (Vandesompele et al., 2002).

Calibrated RNA quantitation

To generate T7 templates for the production of RNA standards that correspond to *pri-let-7* and *SL1-LCE*, the corresponding genomic sequences were PCR

amplified from genomic DNA using the oligonucleotides T7 pri-let-7 F and let-7 RT, and T7 SL1-LCE F and let-7 RT, respectively. T7 pri-let-7 added the T7 promoter to the *pri-let-7* PCR product, and T7 SL1-LCE added the T7 and SL1 sequences to the *SL1-LCE* PCR product. RNA from the respective PCR products was *in vitro* transcribed (IVT) using the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs) following the manufacturer's instructions, and column purified. RNA concentration and quality was measured using an Advanced Analytics Fragment Analyzer. Known amounts of the IVT RNA were then serially diluted. cDNAs from the IVT serial dilutions, and from biological samples, were synthesized as described above and subjected to qPCR. Equal amounts of total RNA were used for each biological sample, and the amounts of *pri-let-7* and *SL1-LCE* in each biological sample were calculated from the standard curve that was generated from the IVT dilutions.

Synthetic oligonucleotides of *let-7*, *mir-48*, *mir-84* and *mir-241* were ordered from Integrated DNA Technologies. Known amounts of these RNA oligonucleotides were serially diluted and subjected to FirePlex miRNA analysis, along with biological samples. Equal amounts of total RNA were used for each biological sample, and the amount of each microRNA in each biological sample were calculated from the standard curve that was generated from the synthetic microRNA dilutions.

Microscopy

Epifluorescence images were obtained using a Zeiss Imager.Z1 with a 10× objective.

Targeted genome editing by CRISPR/Cas9

Mutants were generated using CRISPR/Cas9 methods adapted from Paix et al. (2014, 2015). The germlines of young adult hermaphrodites were injected with a mix of CRISPR RNA (crRNA) that targeted the region of interest in the *let-7* locus and the 'co-CRISPR' marker *dpy-10*, trans-activating crRNA, a single-stranded oligonucleotide homologous recombination template, Cas9 protein that was prepared as described in Paix et al. (2015) and water. F1 animals that exhibited the co-CRISPR phenotype were picked, allowed to lay eggs and then genotyped using PCR. F2s were cloned from F1s that scored positively by PCR genotyping for the desired *let-7* locus modification. Homozygous F2s were then selected by PCR genotyping and subjected to Sanger sequencing for validation. All mutants were backcrossed to wild type at least thrice. See Table S1 for a list of alleles that were generated for this study along with the crRNAs used to generate them. Some crRNAs were generated using IVT (see below). crRNAs that were generated by IVT are noted in their names.

In vitro transcription of crRNAs

To produce templates for the production of crRNAs by T7 *in vitro* transcription, equal amounts of two DNA oligonucleotides (100 µM) were mixed together: The sequence of the first oligonucleotide was the reverse complement to the crRNA of interest followed by the reverse complement of the T7 promoter; the second oligonucleotide (oCN183) was complementary to the T7 promoter sequence of the first oligonucleotide. The oligonucleotide mixture annealed by rapidly heating to 95°C followed by cooling to 15°C over 10 min. Then 1.0 µl of the annealed oligonucleotide mixture was added to an IVT reaction, and transcription was carried out using the HiScribe T7 high Yield RNA Synthesis Kit following the manufacturer's instructions (New England Biolabs). The RNA product was then column purified.

Transgenic constructs

The pCN30 construct, which contains the *let-7* locus ORF tagged with GFP on its C-terminus, was constructed by cloning GFP into the *let-7* genomic rescue plasmid pZR001 (Ren and Ambros, 2015). The pCN33 construct, which contains the *let-7* locus minus the 22-nucleotide mature *let-7* sequence, was constructed from the genomic rescue plasmid pZR001 (Ren and Ambros, 2015).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.N., V.A.; Methodology: C.N., V.A.; Formal analysis: C.N., V.A.; Investigation: C.N.; Resources: V.A.; Data curation: C.N.; Writing - original draft: C.N.; Writing - review & editing: C.N., V.A.; Supervision: V.A.; Project administration: V.A.; Funding acquisition: C.N., V.A.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.172031.supplemental>

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SUPPLEMENTAL MATERIAL

Table S1. Summary of CRISPR/Cas9 alleles

| <u>Allele name</u> | <u>crRNA(s)</u> | <u>Mutation</u> |
|----------------------|--|---|
| ma388 | 5' LCE crRNA 3' LCE crRNA | X:14743758<ctgtcaccgcaaate[Δ55bp]ttggacctatTTTT>X:14743674 |
| ma403 | SA1 crRNA | X:14743856<atagtggaaatcatg[Δ14bp]aacctcatctgctgg>X:14743827 |
| ma406 | IVT 5' previously published crRNA IVT 3' previously published crRNA | X:14743877<tgctagtcttcacca[Δ178bp]acctatTTTTtaa>X:14743680 |
| ma408* | IVT mutLCE crRNA | X:14743741<caggatctcacact TtGccAcaacacctGtaTTtGatccgcatTtaTTtG attggacctatTTTT>X:14743676 |
| ma422**, ma423*** | IVT ma422 crRNA IVT ma423 crRNA | ma422 X:14743981<cggtaatgtatctgg[Δ6bp]taatctaactgatgtactgtt[Δ2bp]gtaatgtatccatg[Δ2bp]gccgttgacatttct[Δ2bp]tggtaatgatgtgcaa>X:14743888 ma423 X:14743873<agtcttcaccattgt[Δ1bp]gatagtggaaatcatgtttatttc[Δ1bp]ggggaacctcatctg>X:14743817 |
| ma485 **** | 5' LCE crRNA 3' LCE crRNA | X:14743758<ctgtcaccgcaaate[Δ55bp]ttggacctatTTTT>X:14743674 |

*made by replacing 55bp deletion of ma388 with mutated LCE; nucleotide changes are capitalized and in bold.

**ma422 was made using IVT ma422 crRNA in the background of ma409 (not used for experiments in this article) which has a 113bp deletion of SA1-2.

***ma423 was made using IVT ma423 crRNA in the background of ma422 by replacing the 113 bp deletion (ma409) with mutated SA1-2.

****ma485 was made in a *let-7(mg279)* background and is the same deletion as ma388

Table S2. Oligonucleotides used in this study

| Oligo ID | Name | Sequence |
|-----------------|-----------------|---|
| oCN66 | let-7 RT | ATCTAATTATCAAGAGCAAGTTCAAATGT |
| oCN59 | SL1 F | GGTTTAATTACCCAAGTTTGAG |
| oCN61 | LCE R | GAGGTGTTGAGGTAGAAGTGTGAG |
| oCN342 | LCE probe | GGATGAGGTAGAGGTGTTGAGGTAGGGCGGG |
| oCN343 | SL1-LCE probe | TTCCCCTCAAACCTTGGGTAATTAAACCGGCGGG |
| oCN345 | let-7 probe | AACTATAACAACCTACTACCTCAGGCGGG |
| oCN347 | 5.8s rRNA probe | GAACCAGACGTACCAACTGGAGGCCCGGCGGG |
| | oligo (dT) 20 | TTTTTTTTTTTTTTTTTTTTTTT |
| oCN70 | SL1-pri-let-7 F | GGTTTAATTACCCAAGTTTGAGGCAAG |
| oCN71 | SL1-LCE F | GGTTTAATTACCCAAGTTTGAGGGGAA |
| oCN356 | TSO 5' RACE | G TTCAGAGTTCTACAGTCCGACGATCrGrGrG |
| oCN357 | Rd1 SP | G TTCAGAGTTCTACAGTCCGACGATC |
| oCN216 | pri-let-7 F | CAAGCAGGCGATTGGTG |
| oCN63 | pri-let-7 R | CGAAGAGTTCTGTCTCCGGTAAGG |
| oCN339 | T7 SL1-LCE F | TAATACGACTCACTATAGGGGTTTAATTACCCAA GTTTGAGGGGAACCTCATCTGCTGGG |
| oCN337 | T7 pri-let-7 F | TAATACGACTCACTATAGGATTCTAGATGAGTA GCCACCTAGCAG |
| | tracrRNA | IDT Alt-R™ CRISPR tracrRNA |
| | dpy-10 crRNA | IDT Alt-R™ CRISPR crRNA /A1TR1/rGrCrUrArCrCrArUrArGrGrCrArCrCrArCrGrA rGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/A1TR2/ |
| oCN181 | 5' LCE crRNA | IDT Alt-R™ CRISPR crRNA /A1TR1/rGrGrCrUrGrUrCrArCrCrGrCrArArArUrCrArU rCrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/A1TR2/ |
| oCN182 | 3' LCE crRNA | IDT Alt-R™ CRISPR crRNA /A1TR1/rArArArArArArArUrArGrGrUrCrCrArArUr CrGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/A1TR2/ |

| | | |
|--------|--------------------------------------|---|
| oCN87 | Δ LCE HR | GCCGTCTGGCACCAAGTGGGCTGTCACCGCAA TCTTGACCTATTTTTTTTAAATTCTTCAAATAA AAAC |
| oCN180 | SA1 crRNA | IDT Alt-R™ CRISPR crRNA /A1TR1/rGrArArArUrCrArUrGrUrUrUrArUrUrUrCr ArGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/A1TR2/ |
| oCN77 | Δ SA1 HR | GCTAGTCTTCACCATTGTAGATAGTGGAAATCAT GAACCTCATCTGCTGGGCAACTACTCCAACATG CGTG |
| oCN183 | T7 promoter | TAATACGACTCACTATAG |
| oCN184 | IVT dpy-10 crRNA | CAAAACAGCATAGCTCTAAAACCTCGTGGTGCC TATGGTAGCCTATAGTGAGTCGTATTA |
| oCN198 | IVT 5' previously published crRNA | CAAAACAGCATAGCTCTAAAACCTATCTACAAT GGTGAAGACCTATAGTGAGTCGTATTA |
| oCN199 | IVT 3' previously published crRNA | CAAAACAGCATAGCTCTAAAACCGATTGGACCT ATTTTTTTCCTATAGTGAGTCGTATTA |
| oCN201 | Previously published HR | TGCAATAGTTCCAATTGCTAGTCTTCACCAACCT ATTTTTTTTAAATTCTTCAAATAAAA |
| oCN210 | IVT mutLCE crRNA | CAAAACAGCATAGCTCTAAAACAGATTTGCGGT GACAGCCCTATAGTGAGTCGTATTA |
| oCN209 | IVT mutLCE HR | GCCGTCTGGCACCAAGTGGGCTGTCACCGCAA TCATCAGGATCTCACACTTTTGCCACAACACCTG TATTTGATCCGCATTTATTTGGATTGGACCTATT TTTTTTAAATTCTTCAAATAAAAA |
| oCN239 | IVT ma422 crRNA | CAAAACAGCATAGCTCTAAAACGATACATTACC GATACAACCCTATAGTGAGTCGTATTA |
| oCN248 | mutSA3-6 HR | GAAGTGTATTCGGGAGAACTGTTGTATCGGTAAT GTATCTGGAATAATCTAATCGTATGTACTGTTGT AATGTATCCATGGCCGTTTGACATTTCTTGGTAA GATGTGCAATAGTTCCAATTGCTAGTCTT |

| | | |
|--------|-------------------|---|
| oCN266 | IVT ma423 crRNA | CAAAACAGCATAGCTCTAAAACCAAATCATCAG GATCTCACCTATAGTGAGTCGTATTA |
| oCN265 | mutSA1-2 HR | GAGGTGTTGAGGTAGAAGTGTGAGATCCTGATG ATTTGCGGTGACAGCCCACTTGGTGCCAGACGG CATTCCCTAGGCGACACGCATGTTGGAGTAGTT GCCCAGCAGATGAGGTTCCCCGAAAATAAACAT GATTTCCACTATCACAATGGTGAAGACTAGCAA TTGGA ACTATTGCACATCT |
| oCN397 | mir-241 synthetic | rUrGrArGrGrUrArGrGrUrGrCrGrArGrArArUrGrA |
| | gpd-1 QPCR F | GATGGACCAATGAAGGGAAT |
| | gpd-1 QPCR R | GTCGTACCAAGAGACGAGCTT |
| | let-7 synthetic | rUrGrArGrGrUrArGrUrArGrGrUrUrGrUrArUrArGrUr U |
| | mir-48 synthetic | rUrGrArGrGrUrArGrGrCrUrCrArGrUrArGrArUrGrCrG rA |
| | mir-84 synthetic | rUrGrArGrGrUrArGrUrArUrGrUrArArUrArUrUrGrUr ArGrA |

Table S3. *C. elegans* strains used in this study

| <u>Strain Name</u> | <u>Strain Description</u> | <u>Genotype</u> |
|--------------------|-------------------------------|--|
| MT355 | <i>lin-14(gf)</i> | <i>lin-14(n355)</i> X |
| VT965 | <i>lin-14(lf)</i> | <i>lin-14(n179)</i> X |
| VT1295 | <i>lin-28(0)/lin-28</i> null | <i>lin-28(n719)</i> I; <i>maIs105</i> V |
| VT1367 | wild type/WT | <i>maIs105</i> [<i>col-19::gfp</i>] V |
| VT3594 | <i>lin-28(0); lin-46(0)</i> | <i>lin-28(n719)</i> I; <i>lin-46(ma164)</i> , <i>maIs105</i> V |
| VT3609 | <i>lin-4(lf)</i> | <i>lin-4(e912)</i> II; <i>maIs105</i> V |
| VT3616 | Δ LCE | <i>let-7(ma388)</i> X; <i>maIs105</i> V |
| VT3666 | Δ SA1 | <i>let-7(ma403)</i> X; <i>maIs105</i> V |
| VT3669 | previously published deletion | <i>let-7(ma406)</i> X; <i>maIs105</i> V |

| | | |
|--------|--|--|
| VT3675 | Δ SA1; <i>lin-28(0)</i> | <i>let-7(ma403)</i> X; <i>lin-28(n719)</i> I; <i>maIs105</i> V |
| VT3678 | mutLCE | <i>let-7(ma408)</i> X; <i>maIs105</i> V |
| VT3718 | mutSA1-6 | <i>let-7(ma422ma423)</i> X; <i>maIs105</i> V |
| VT3719 | mutLCE; <i>mir-48(0)</i> | <i>let-7(ma408)</i> X; <i>miR-48(n4097)</i> , <i>maIs105</i> V |
| VT3720 | Δ LCE; <i>mir-48(0)</i> | <i>let-7(ma388)</i> X; <i>miR-48(n4097)</i> , <i>maIs105</i> V |
| VT3721 | Δ LCE; <i>mir-48(0)</i> <i>mir-241(0)</i> | <i>let-7(ma388)</i> X; <i>miR-48</i> <i>miR-241(nDF51)</i> , <i>maIs105</i> V |
| VT3837 | <i>mir-48(0)</i> | <i>mir-48(n4097)</i> , <i>maIs105</i> V |
| VT3838 | mutSA1-6; <i>mir-48(0)</i> | <i>let-7(ma422ma423)</i> X; <i>mir-48(n4097)</i> , <i>maIs105</i> V |
| VT3839 | <i>let-7</i> locus' ORF::GFP transgene | <i>maEx264</i> [<i>unc-119+</i> ; <i>plet-7::let-7</i> locus with ORF::GFP]; <i>unc-119(ed3)</i> III |
| VT3840 | <i>mir-48(0)</i> <i>mir-241(0)</i> | <i>mir-48</i> <i>mir-241</i> (<i>nDf51</i>), <i>maIs105</i> V |
| VT3902 | <i>let-7(mg279ΔLCE)</i> | <i>let-7(mg279ma485)</i> X; <i>maIs105</i> V |
| VT3903 | LCE transgene; Δ LCE; <i>mir-48(0)</i> | <i>maEx267</i> [<i>rol-6(su1006)</i> ; <i>unc-119+</i> , <i>plet-7::let-7</i> locus without mature <i>let-7</i> sequence]; <i>let-7(ma388)</i> X; <i>mir-48(n4097)</i> , <i>maIs105</i> V |

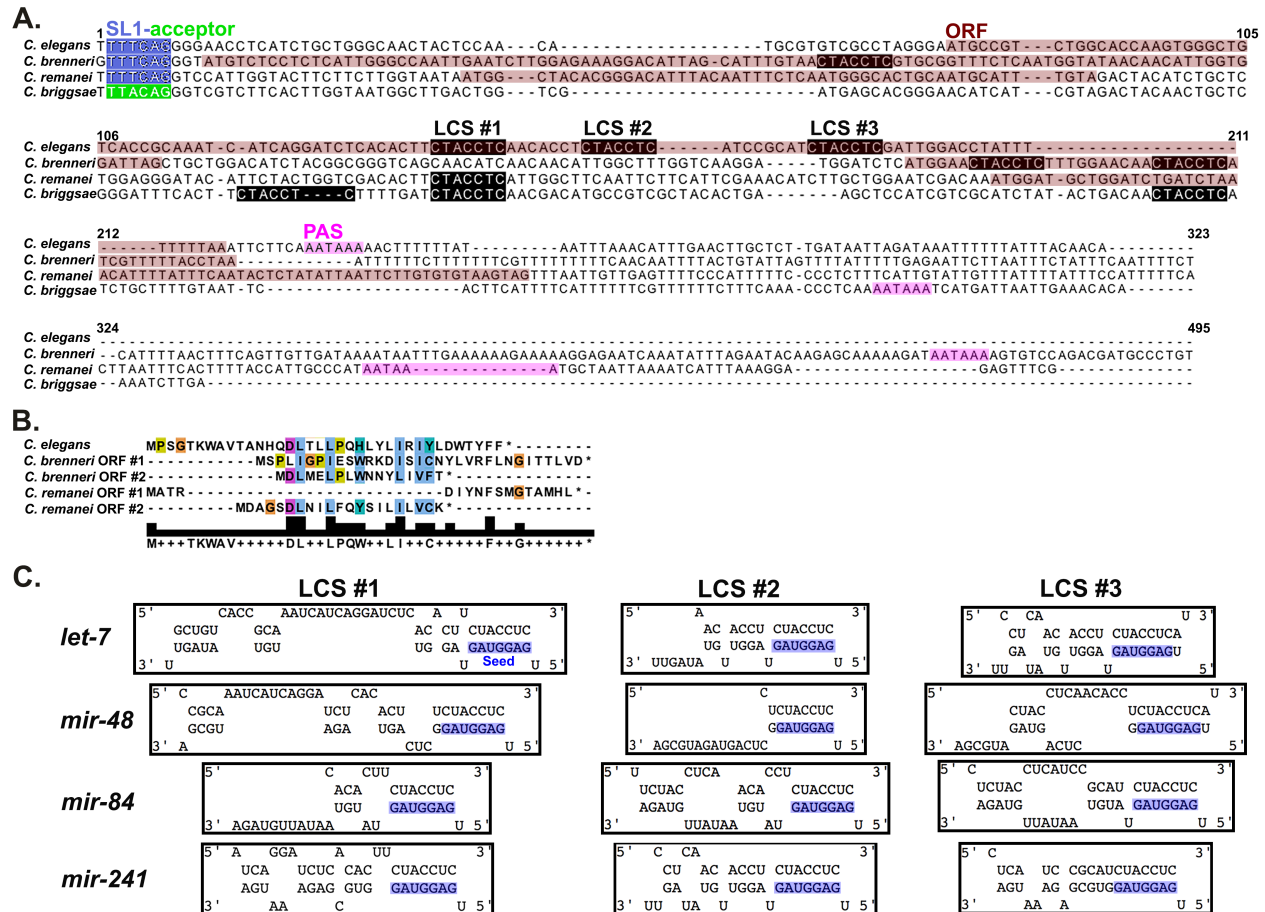


Figure S1. The LCE and SL1 splice acceptor sequences are conserved among *Caenorhabditis* species.

- (A) Genomic alignment of the regions downstream (3') from the *pre-let-7* stem-loop of four *Caenorhabditis* species' *let-7* loci. Highlighted in blue is the canonical SL1-acceptor sequence. Highlighted in green is a non-canonical SL1-acceptor sequence. Red shading marks potential open reading frames (ORF). Black shading indicates is *let-7* complementary sequence (LCS). Predicted polyadenylation signals (PAS) are shaded in pink.
- (B) Amino acid alignment of the potential ORFs of the *SL1-LCEs*.
- (C) Predicted RNA hybridization of *let-7fam* microRNAs (bottom strands; 5' to the right) with the three LCSs in *C. elegans* *SL1-LCE*. Highlighted in light blue is the seed sequence of each microRNA.

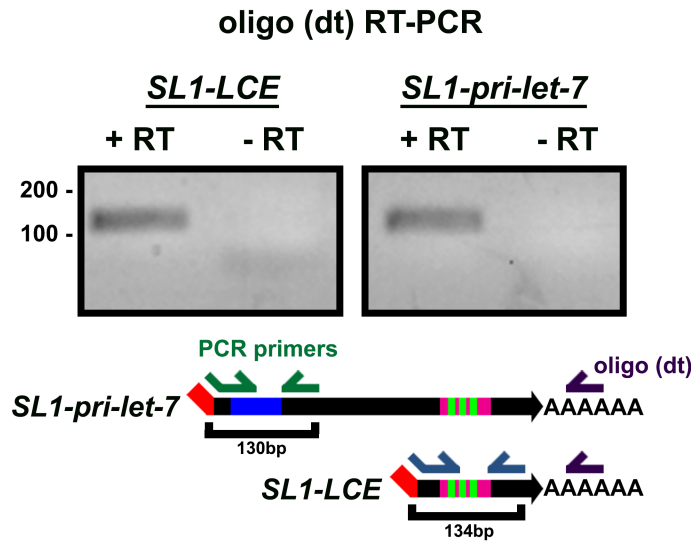


Figure S2. *let-7* locus transcripts are polyadenylated.

Non-quantitative RT-PCR of cDNA made from total RNA from a mixed-population of WT animals with (+) or without (-) RT in the cDNA synthesis step. The left panel shows products of a PCR reaction using primers (green in the diagram) specific for *SL1-pri-let-7*; the right panel shows products of a PCR reaction using primers (turquoise in the diagram) specific for *SL1-LCE*. Numbers mark dsDNA ladder bands in bp.

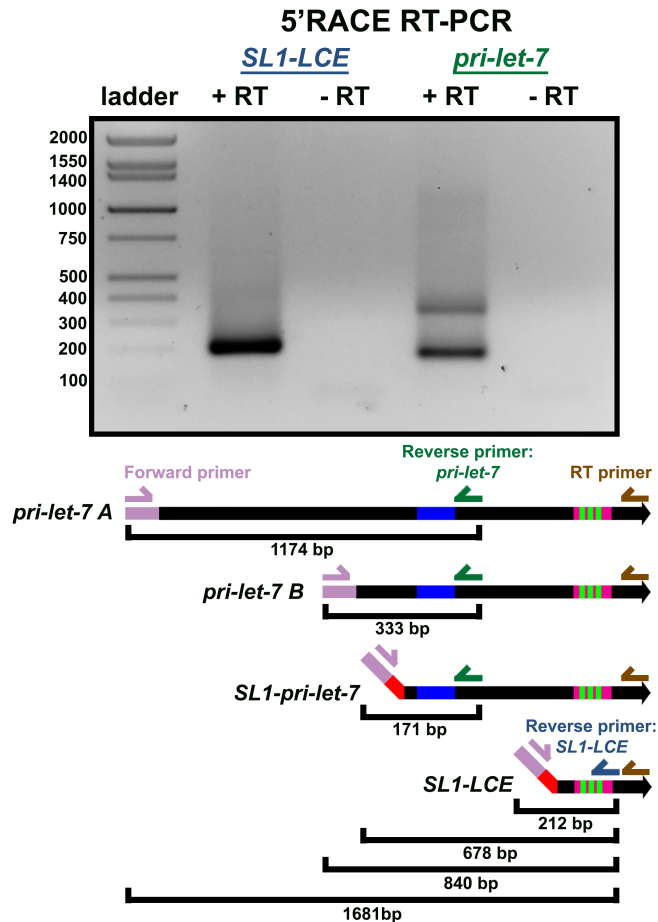


Figure S3. The *SL1-LCE* is the only detectable transcript from the *let-7* locus that does not contain *pri-let-7*.

Non-quantitative 5'RACE RT-PCR of cDNA made from total RNA from molting L2 WT animals (24 hours after plating) with (+) or without (-) RT in the cDNA synthesis step. The left two lanes show products of PCR reactions using a primer pair (turquoise and pink) that is expected to amplify all LCE containing *let-7* transcripts. Note: *SL1-LCE* is preferentially amplified because of the short product produced from *SL1-LCE* compared to the products produced from *pri-let-7* isoforms. The right two lanes show products of PCR reactions using a primer pair (green and pink) that is expected to amplify *pri-let-7* isoforms only. Note: two *pri-let-7* isoforms (*B* and *SL1*) are preferentially amplified due to their shorter product sizes. Numbers mark dsDNA ladder bands in bp.

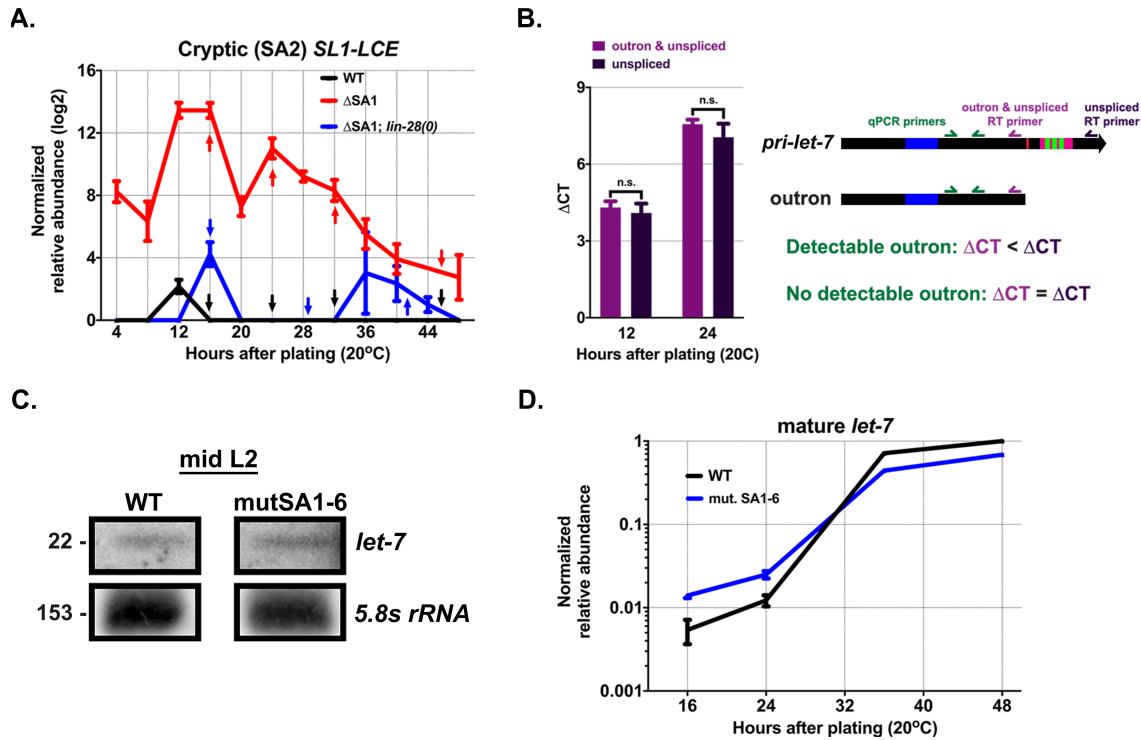


Figure S4. Deletion of the canonical splice acceptor results in the use of non-canonical splice acceptors, the *pri-let-7* outtron is not detectable in L1 and L2 larvae, and mutations that reduce LCE trans-splicing display elevated levels of mature *let-7* in the L1 and L2 stages and reduced levels in the L3 and L4 stages, compared to WT.

(A) qRT-PCR developmental profile of the levels of an *SL1-LCE* transcript

(*Cryptic(SA2)SL1-LCE*) that contains SL1 spliced to a cryptic SA sequence (TTGTAG). *Cryptic(SA2)SL1-LCE* levels were determined for samples from wild type (black), ΔSA1 (red), and doubly-mutant ΔSA1; *lin-28(0)* (blue) animals throughout development. Data are represented as mean ±SD. n's = 3 biological replicates. Arrows mark the times of larval molts.

(B) qRT-PCR analysis of cDNA synthesized from WT mid-L1 (12 hours after plating) and molting-L2 (24 hours after plating) either upstream (outtron & unspliced RT primer) or downstream (unspliced RT primer) of the canonical splice acceptor. Data are represented as mean ±SD. n = 3's biological replicates. Statistical significance was determined using a two-tailed Student's t test.

(C) Total RNA from mid-L2 (20 hours after plating) WT and mutSA1-6 animals analyzed by northern blotting with a probe for *let-7* mature microRNA. Numbers mark RNA sizes in nt.

(D) FirePlex miRNA analysis of *let-7* levels in WT and mutSA1-6 animals throughout development. Data are represented as mean \pm SD. n = 3.

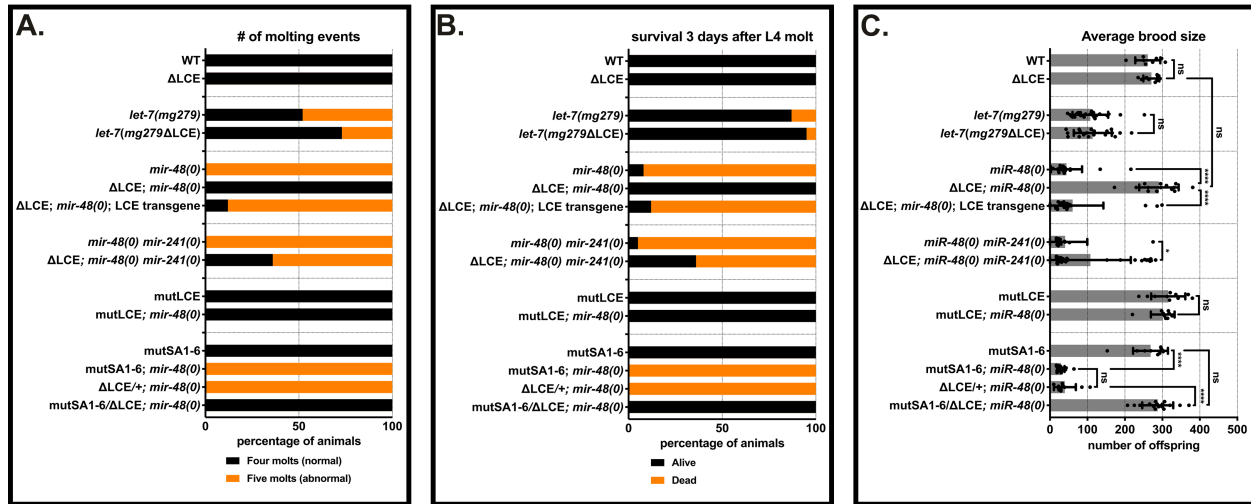


Figure S5. Inhibition of *SL1-LCE* function, either by deletion of the LCE, or by mutations of LCE-proximal trans-splicing acceptor sequences, suppresses multiple phenotypes associated with the retarded development of *mir-48(0)* animals.

Deletion (Δ LCE) or mutation (mutLCE) of the LCE or mutation in the SAs (mutSA1-6) suppresses (A) the extra molt, n's from top (WT) to bottom (mutSA1-6/ Δ LCE; *mir-48(0)*): 9, 10, 23, 12, 9, 22, 26, 18, 15, 10, 15, 10, 10, 11, and 16, (B) adult lethality, n's from top (WT) to bottom (mutSA1-6/ Δ LCE; *mir-48(0)*): 9, 10, 23, 12, 9, 22, 26, 18, 15, 10, 15, 10, 10, 11, and 16, and (C) reduced brood size of *mir-48(0)* animals, n's from top (WT) to bottom (mutSA1-6/ Δ LCE; *mir-48(0)*): 9, 9, 23, 20, 26, 14, 26, 18, 33, 9, 29, 10, 29, 11, and 16 animals. The graphs are quantifications of each respective phenotype observed for each genotype. Statistical significance was determined using a two-tailed Student's t test. P-values: ns > 0.05, * = \leq 0.05, **** \leq 0.0001.

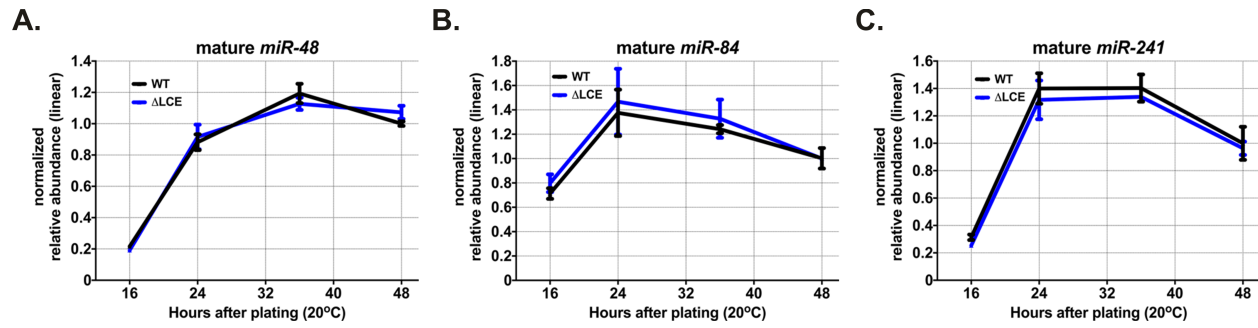


Figure S6. Deletion of LCE sequences from the *let-7* locus does not detectably change the levels of *let-7fam* microRNAs.

FirePlex miRNA analysis of (A) *mir-48*, (B) *mir-84*, and (C) *mir-241* levels in WT and LCE deletion animals throughout development. Data are represented as mean \pm SD. n's = 3 biological replicates.

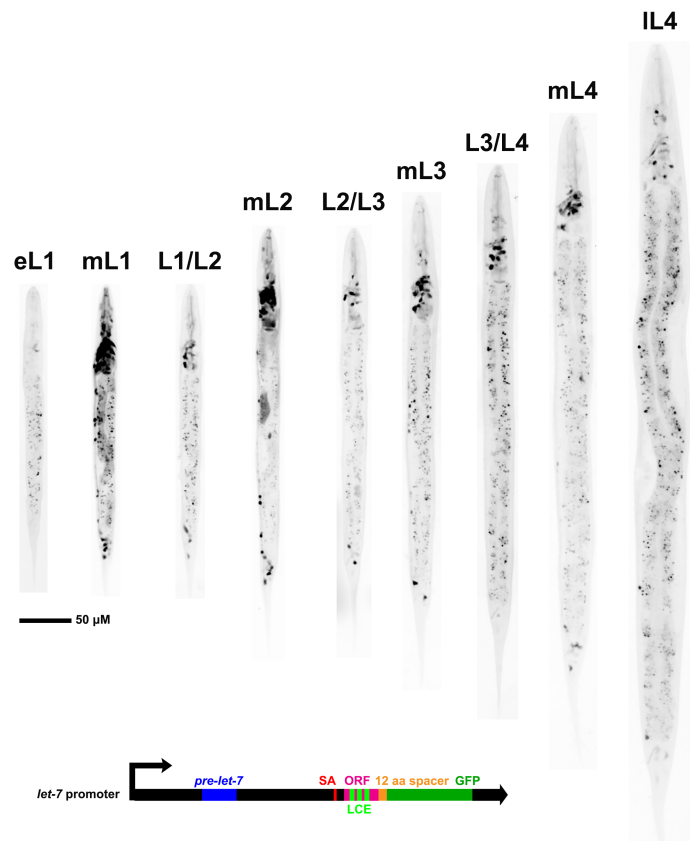


Figure S7. Temporal profile of expression of a GFP-tagged LCE ORF recapitulates that observed for *SL1-LCE*.

Express of GFP in animals carrying an *let-7* locus LCE ORF::GFP transgene throughout development. Images are of representative animals. Note: puncta observed in the intestine is from autofluorescence.