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Investigation

A cohort of *Caenorhabditis* species lacking the highly conserved *let-7* microRNA

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Abstract

The *let-7* gene encodes a highly conserved microRNA with critical functions integral to cell fate specification and developmental progression in diverse animals. In *Caenorhabditis elegans*, *let-7* is a component of the heterochronic (developmental timing) gene regulatory network, and loss-of-function mutations of *let-7* result in lethality during the larval to adult transition due to misregulation of the conserved *let-7* target, *lin-41*. To date, no bilaterian animal lacking *let-7* has been characterized. In this study, we identify a cohort of nematode species within the genus *Caenorhabditis*, closely related to *C. elegans*, that lack the *let-7* microRNA, owing to absence of the *let-7* gene. Using *Caenorhabditis sulstoni* as a representative *let-7*-lacking species to characterize normal larval development in the absence of *let-7*, we demonstrate that, except for the lack of *let-7*, the heterochronic gene network is otherwise functionally conserved. We also report that species lacking *let-7* contain a group of divergent *let-7* paralogs—also known as the *let-7*-family of microRNAs—that have apparently assumed the role of targeting the LIN-41 mRNA.

Keywords: *let-7* microRNA; *Elegans* supergroup; *Elegans* group; *Japonica* group; *Caenorhabditis sulstoni*; *Caenorhabditis macrosperma*; *Caenorhabditis elegans*

Introduction

MicroRNAs are ~22 nucleotide (nt) noncoding RNAs that negatively regulate protein expression through base pairing of nts 2–8 of the microRNA (known as the microRNA seed) to complementary sequences in target mRNA 3′ UTRs. Base pairing with nts 9–22 can also contribute to target repression but non-seed base pairing is less constrained than seed pairing. Accordingly, evolutionary conservation of microRNA sequences is generally highest for nts 2–8, and less so for non-seed nts (Bartel 2009; Ambros and Ruvkun 2018).

Unlike most microRNAs, the entirety of nts 1–22 of *let-7* RNA are highly conserved across bilaterians (Figure 1A) (Pasquinelli *et al.* 2000). Why *let-7* RNA non-seed sequences are so deeply conserved remains a mystery. Alongside the deep conservation of the entire *let-7* sequence, *let-7* microRNA function is also conserved; across diverse animal phyla, *let-7* RNA expression coincides with differentiation and opposes stem cell pluripotency (Pasquinelli *et al.* 2000; Reinhart *et al.* 2000; Roush and Slack 2008; Balzeau *et al.* 2017). Accordingly, in certain contexts *let-7* RNA functions as a tumor suppressor through restricting the expression of proteins involved in cell proliferation, growth, and metabolism, among others (Balzeau *et al.* 2017). We believe that the deep conservation of *let-7* sequence holds secrets to important evolutionarily conserved molecular interactions vital to *let-7* function, and therefore, a better understanding of *let-7* conservation will reveal insights into mechanisms of microRNA function and regulation.

In most bilaterian animal clades, , including mammals and nematodes, the *let-7* gene family has been expanded into a number of related microRNAs that share the same seed sequence but differ in their non-seed nts (Roush and Slack 2008). In *Caenorhabditis elegans*, the *let-7*-family consists of *let-7* microRNA, miR-48, miR-84, miR-241, and miR-795 (Abbott *et al.* 2005). Despite the presence of multiple *let-7*-family microRNAs (which could in principle substitute for one another by seed-mediated base pairing to targets), most animals have nevertheless retained the original *let-7* microRNA with its non-seed nt sequence conserved (Pasquinelli *et al.* 2000; Roush and Slack 2008).

The *C. elegans* heterochronic gene (developmental timing) regulatory network consists of genes that either promote or restrict developmental cell fate progression. Integrated into the heterochronic pathway is the protein coding genes *lin-14*, *lin-28*, *lin-29*, *lin-41*, *lin-46*, and *hbl-1*, as well as the microRNA genes *lin-4* and the *let-7*-family (*let-7*, *mir-48*, *mir-84*, and *mir-241*). Loss-of-function mutations of genes that restrict cell fate progression, such as *lin-14*, *lin-28*, *lin-41*, or *hbl-1*, results in precocious development of the hypodermis through the skipping of larval-specific cellular events; whilst loss-of-function mutations of genes that promote cell fate progression, such as *lin-29*, *lin-46*, *lin-4*, or the *let-7*-family, results in retarded development of the hypodermis through the reiteration of larval-specific cellular divisions (Chalfie *et al.* 1981; Ambros and Horvitz 1984; Ambros 1989; Fay *et al.* 1999; Reinhart *et al.* 2000; Slack *et al.* 2000; Lin *et al.* 2003; Pepper *et al.* 2004; Abbott *et al.* 2005).

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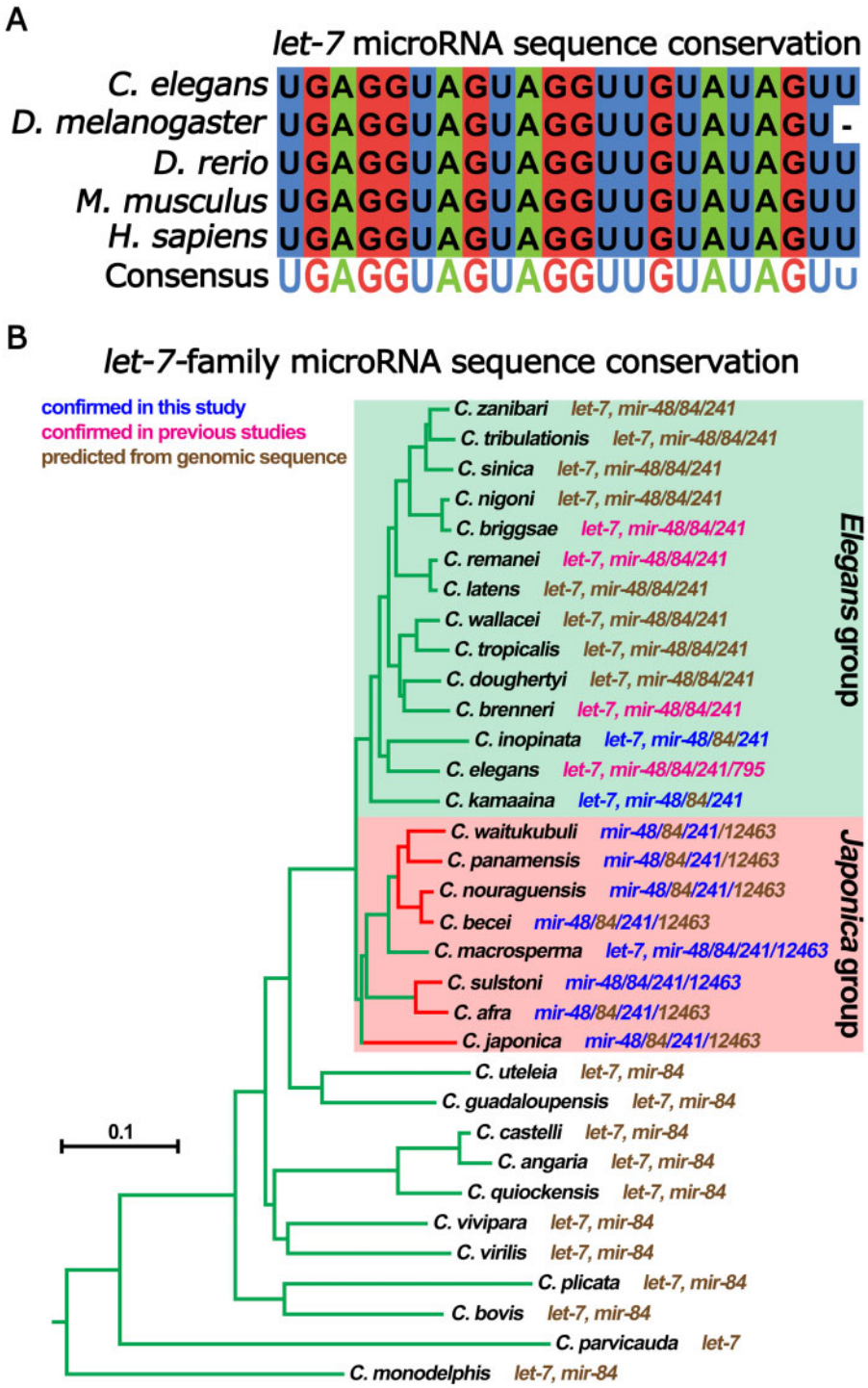

Figure 1 Conservation of the let-7 microRNA. (A) Sequence conservation of let-7 microRNA. (B) Phylogenetic tree of *Caenorhabditis* species based on phylogeny from Stevens *et al.* (2020). For each species, the makeup of their let-7-family microRNAs is indicated. let-7-family microRNAs are predicted from their genomic sequences (brown font), detection in previous studies (magenta font), or detection in this study (blue font). Green branches highlight species that are either predicted or confirmed to have *let-7*. Red branches highlight species that are either predicted or confirmed to lack *let-7*. Outlined in the green box are species recovered in the *Elegans* group. Outlined in the red boxes are species recovered in the *Japonica* group. Branch lengths are proportional to substitutions per site. Scale bar is shown.

In *C. elegans*, expression of the *lin-4* microRNA increases during the L1 and L2 stages to promote the transition from L1 cell fates to later larval cell fates through its targeted repression of synthesis of the LIN-14 and LIN-28 proteins (which promote L1 and L2 larval stage cell fates) by base-pairing to the 3′ UTRs of *lin-14* and *lin-28* mRNAs (Chalfie *et al.* 1981; Ambros and Horvitz 1984; Ambros 1989; Lee *et al.* 1993; Moss *et al.* 1997; Lim *et al.* 2003). Similarly, miR-48, miR-84, and miR-241 (miR-48/84/241), whose expressions increase during the L2 and L3 larval stages to promote transitions to later larval stages by negatively regulating LIN-14, LIN-28, and HBL-1 through base-pairing to their respective 3′ UTRs (Abrahante *et al.* 2003; Lin *et al.* 2003; Abbott *et al.* 2005; Tsialikas *et al.* 2017).

In *C. elegans*, *let-7* is required for cell cycle exit and differentiation in hypodermal cell lineages at the end of larval development, which is reflected by the dramatic up regulation of mature *let-7* microRNA in terminal larval stages (Reinhart *et al.* 2000). *let-7* function and regulation are deeply integrated into the *C. elegans* heterochronic gene regulatory network; LIN-28 posttranscriptionally restricts *let-7* microRNA biogenesis to later larval stages, and *let-7* microRNA negatively regulates the evolutionarily conserved pluripotency-promoting genes *lin-41* and *lin-28* through base-pairing to the 3′ UTRs of the LIN-41 and LIN-28 mRNAs (Reinhart *et al.* 2000; Slack *et al.* 2000; Vella *et al.* 2004; Ding and Großhans 2009; Lehrbach *et al.* 2009; Van Wynsberghe *et al.* 2011; Ecsedi *et al.* 2015; Stefani *et al.* 2015). The reciprocal direct regulation between *let-7* microRNA and LIN-28, and the direct regulation of LIN-41 mRNA by *let-7* microRNA, are evolutionarily conserved across vertebrates and invertebrates, suggesting that the unusual conservation of *let-7* sequence may be related to these conserved intimate interactions of *let-7* microRNA with the LIN-28 and LIN-41 mRNAs and with LIN-28 protein (Reinhart *et al.* 2000; Slack *et al.* 2000; Kloosterman *et al.* 2004; Vella *et al.* 2004; Schulman *et al.* 2005; Lin *et al.* 2007; Heo *et al.* 2008; Newman *et al.* 2008; Rybak *et al.* 2008; Viswanathan *et al.* 2008; Lehrbach *et al.* 2009; Nam *et al.* 2011; Van Wynsberghe *et al.* 2011; Piskounova *et al.* 2011; Stratoulias *et al.* 2014; Ecsedi *et al.* 2015; Stefani *et al.* 2015; Balzeau *et al.* 2017).

Despite the sharing of a common seed sequence and the seemingly redundant potential to regulate the same targets, the *let-7*-family microRNAs (miR-48/84/241) and *let-7* do not function interchangeably. Specifically, miR-48/84/241, as a semi-redundant cohort, primarily regulate *lin-14*, *lin-28*, and *hbl-1* during L1–3 cell fate transitions, whereas *let-7* regulates *lin-41* during a larval to adult cell fate switch (Slack *et al.* 2000; Vella *et al.* 2004; Abbott *et al.* 2005; Ecsedi *et al.* 2015; Tsialikas *et al.* 2017; Ilbay and Ambros 2019). *let-7* loss-of-function (*let-7(lf)*) mutants display phenotypes distinguishable from those of triply-mutant *mir-84(lf); mir-48(lf)mir-241(lf)* animals, as a consequence of the stage-specific de-repression of their respective targets—essentially gain-of-function of *lin-41*, or gain-of-function *lin-14/lin-28/hbl-1*, respectively (Abbott *et al.* 2005; Aeschimann *et al.* 2019). The specificity of *let-7* for regulation of *lin-41* is thought to be conferred by base pairing of 3' non-seed sequences of *let-7* microRNA to the *let-7* complementary sites in the LIN-41 mRNA 3′ UTR (Reinhart *et al.* 2000; Vella *et al.* 2004; Ecsedi *et al.* 2015), suggesting that conservation of *let-7* microRNA non-seed sequences could reflect evolutionary pressure to conserve functional distinctions between *let-7* microRNA and other *let-7*-family microRNAs.

The deep evolutionary roots of *let-7* in the heterochronic pathway, including the apparent conservation of specific targeting of LIN-41 mRNA by *let-7* microRNA suggests that hypothetical evolutionary loss of *let-7* could be expected to be accompanied by significant divergence, compared with *C. elegans*, in the functions of heterochronic genes downstream of *let-7*, such as *lin-41* and *lin-29*, and/or upstream genes, such as *lin-14*, *lin-28*, and *hbl-1*. Exploration of these questions would require the identification of species closely related to *C. elegans* that lack *let-7*.

Here, we identify a faction of *Caenorhabditis* species within the *Japonica* group, a sister group to the *Elegans* group, that lack the *let-7*gene. As far as we know, this is the first described instance of two sister clades where all known species of one clade have retained *let-7*, whereas numerous species of the sister clade do not have *let-7*. We demonstrate that for an exemplary *let-7*-lacking species, *Caenorhabditis sulstoni*, the functional architecture of the heterochronic pathway is otherwise conserved compared with *C. elegans*. Our findings indicate thatLIN-41 mRNA is apparently regulated by the remaining *let-7*-family microRNAs in most *Japonica* group species, suggesting that the heterochronic pathway can evolve to re-delegate *let-7*-family function under certain evolutionary circumstances.

Materials and methods

Nematode methods

All *Caenorhabditis* species were cultured on nematode growth medium (NGM; Brenner 1974) and fed with *Escherichia coli* HB101 except for all RNA interference (RNAi) experiments, in which *C. elegans* and *C. sulstoni* were fed *E. coli* HT115. Synchronized populations of developmentally staged worms were obtained using standard methods (Stiernagle 2006). All experiments involving *C. elegans*, unless otherwise noted, were performed at 20°C. All experiments with the other *Caenorhabditis* species, unless otherwise noted, were performed at 25°C. A list of strains used in this study is in Supplementary Table S5.

Developmental and phenotypic analyses of C. elegans, C. sulstoni, *and* Caenorhabditis macrosperma

To characterize the effects of temperature on development, synchronized populations of *C. elegans* and *C. sulstoni* were plated at 15°C, 20°C, 25°C, 30°C, 33°C, and 35°C. A synchronized population of *C. macrosperma* was plated at 25 °C. Except for *C. sulstoni* animals plated at 15 °C, developing populations plated at each respective temperature were observed every hour until animal development reached the adult stage. Following the initial 48 h of hourly observation, *C. sulstoni* animals plated at 15 °C were observed every 12 h until animals reached the adult stage. L1 alae, lethargy, cuticular molting, gonad migration, vulva development, adult alae, and oogenesis were used as markers to determine and calibrate developmental stages.

For heterochronic phenotype analyses, larvae were fed with RNAi food (as described below) starting from the L1 stage, and animals of defined developmental stages (as described above) were picked from healthy uncrowded mixed staged cultures and imaged. DIC microscopy was used to image hypodermis and alae. Fluorescence microscopy was used to image GFP-LIN-41.

For quantification of alae formation, the entire length of the animal's cuticle was observed using DIC microscopy. Alae with one or more discontinuity were scored as incomplete. Any region where alae branched into multiple directions was scored as a branch.

Microscopy

All DIC and fluorescent images were obtained using a ZEISS Imager Z1 equipped with ZEISS Axiocam 503 mono camera, and the ZEN Blue software. Prior to imaging, worms were anesthetized with 0.2 mM levamisole in M9 buffer and mounted on 2% agarose pads. Adobe Photoshop was used to adjust the brightness and contrast of the images to enhance the visualization of the DIC and fluorescent signals. All fluorescent images were taken using the same microscopy settings and a standard exposure time for each larval stage for each reporter (*C. elegans* GFP-LIN-41 and *C. sulstoni* GFP-LIN-41). Identical brightness and contrast adjustments were used for each fluorescent image.

Caenorhabditis genomes

All genomes used in this study were provided by the *Caenorhabditis* Genomes Project (CGP) (http://caenorhabditis.org (last accessed February 4, 2021)).

Syntenic comparisons

Syntenic comparisons were performed using GEvo (https://genomevolution.org/coge/GEvo.pl; Lyons and Freeling 2008) with the following algorithm settings—Alignment Algorithm: (B)LastZ: Large Regions; Word size: 8; Gap start penalty: 300; Gap extend penalty: 30; Chaining: chain and extend; Score threshold: 2000; Mask threshold: 0; Minimum High-scoring Segment Pair (HSP) length for finding overlapped features: 50.

Gene prediction

Genes predictions were performed using the AUGUSTUS web interface (http://bioinf.uni-greifswald.de/augustus/ (last accessed February 4, 2021)) with the default settings (Keller *et al.* 2011).

Identification of homologous genes

Identification of all homologs was performed using CoGeBlast and the *Caenorhabditis* Genomes Project Blast webpages (https://genomevolution.org/coge/CoGeBlast.pl (last accessed February 4, 2021); http://blast.caenorhabditis.org/ (last accessed February 4, 2021)).

Sequence alignments

Sequence alignments were performed using Clustal Omega (www.ebi.ac.uk (last accessed February 4, 2021); Madeira *et al.* 2019) and visualized using Jalview (www.jalview.org (last accessed February 4, 2021); Waterhouse *et al.* 2009).

RNA hybridization predictions and minimum free energy calculations

RNA hybridization predictions and minimum free energy (MFE) calculations were performed using RNAhybrid (https://bibiserv2.cebitec.uni-bielefeld.de (last accessed February 4, 2021); Rehmsmeier *et al.* 2004).

lin-41 phylogeny

lin-41 phylogenetic tree was generated from orthology clustering provided by the *Caenorhabditis* Genomes Project (Stevens 2020) and visualized using iTOL (https://itol.embl.de/ (last accessed February 4, 2021); Letunic and Bork 2019). The *lin-41* phylogenetic tree was rooted on the outgroup *Diploscapter coronatus*.

RNA extraction

Populations of animals were collected and flash-frozen in liquid nitrogen, and total RNA was extracted using Qiazol reagen (Qiagen) as described by Mcjunkin and Ambros (2017).

FirePlex 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.

Small RNA sequencing and *let-7*-family microRNA identification and normalization

Samples of total RNA were used to generate all small RNA libraries using a QIAseq miRNA Library Kit (Qiagen) following the manufacturer's instructions. All libraries were sequenced on an Illumina NextSeq 500 sequencer. *let-7*-family microRNAs were identified by searching for reads that contained the *let-7*-family microRNA seed sequence "GAGGTAG" at positions 2–8. *let-7*-family microRNA reads were considered legitimate if the sequence mapped to the genome and if the read was predicted to form a microRNA-like stem-loop secondary structure with adjacent genomic sequence. RNA secondary structure modeling was performed using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi (last accessed February 4, 2021); Lorenz *et al.* 2011). In most instances, reads corresponding to microRNA precursors were identified, adding additional credence to the validation of *let-7*-family microRNAs. microRNAs were quantified by normalizing the read count of a given microRNA to the total reads in that library.

GFP tagging of *C. sulstoni* LIN-41

Caenorhabditis sulstoni GFP-lin-41 was generated using CRISPR/Cas9 methods adapted from Paix *et al.* (2014, 2015) and Dokshin *et al.* (2018). The germlines of young adult females were injected with a mix of CRISPR RNA (crRNA) that targeted the 5′ end of the *lin-41* coding sequence and the "co-CRISPR" marker *dpy-10*, tracrRNA (Supplementary Table S6), a PCR-derived dsDNA HR template, Cas9 protein (Integrated DNA Technologies), and Integrated DNA Technologies duplex buffer (30 mM HEPES, pH 7.5; 100 mM potassium acetate). L4 females were picked from plates where F1 animals exhibited the co-CRISPR phenotype and mated to a single male picked from the same plate, allowed to lay eggs, and then genotyped using PCR. F2s with GFP expression were cloned from F1s that scored positively by PCR genotyping for the desired modification. Single male and single female progeny were then mated, and a homozygous line was selected by GFP expression and PCR genotyping and subjected to Sanger sequencing for validation. The mutant was then thrice backcrossed to wild type. Sequence of the *GFP::lin-41* allele generated in this study can be found in Supplementary Table S7.

Bacterial RNAi feeding strain constructions

cDNA from mixed staged *C. elegans* and *C. sulstoni* was generated from total RNA using SuperScript IV Reverse Transcriptase (ThermoFisher) and oligoDT following the manufacturer's instructions. PCR was then used to amplify portions of *C. elegans lin-41*, *C. sulstoni lin-14*, *C. sulstoni lin-28*, *C. sulstoni lin-29*, *C. sulstoni lin-41*, *C. sulstoni lin-46*, *C. sulstoni hbl-1*, and *C. sulstoni unc-22*, respectively. Primers (Supplementary Table S6) used for each respective PCR also added *KpnI* sites to each end of each PCR product except for *C. elegans lin-41*, which added a *HindIII* site to one end and a *KpnI* site to the other end. The PCR products and the T444T vector were digested with KpnI (NEB) restriction enzyme for all *C. sulstoni* genes and HindIII and KpnI for *C. elegans lin-41*. The cut T444T vector was then dephosphorylated, and the cut PCR products and the cut/dephosphorylated vector were gel purified, ligated, and transformed into TOP10 chemically competent cells. Purified plasmids were subjected to Sanger sequencing for validation and transformed into chemically competent *E. coli* HT115 cells. The T444T plasmid was a gift from Tibor Vellai (Addgene plasmid # 113081; http://n2t.net/addgene:113081 (last accessed February 4, 2021); RRID: Addgene_113081).

RNAi knockdown of heterochronic genes

RNAi by feeding *C. elegans* and *C. sulstoni* with the strains described above was conducted as described in Conte *et al.* (2015).

Caenorhabditis elegans heterochronic pathway

The *C. elegans* heterochronic pathway schematic shown in Supplementary Figure S7A was adapted from Resnick *et al.* (2010).

Data availability

All *Caenorhabditis* strains are available at the *Caenorhabditis* Genetics Center (https://cgc.umn.edu (last accessed February 4, 2021)). Reagents used in this study are available upon request.

Raw small RNA sequencing data can be found in the NCBI SRA (http://www.ncbi.nlm.nih.gov/sra (last accessed February 4, 2021)) (SUB8541612: accession numbers SAMN16816130, SAMN16816131, SAMN16816132, SAMN16816133, SAMN16816134, SAMN16816135, SAMN16816136, SAMN16816137, SAMN16816138, SAMN16816139, SAMN16816140, SAMN16816141, SAMN16816142, SAMN16816143, SAMN16816144, SAMN16816145, SAMN16816146, SAMN16816147, SAMN16816148, SAMN16816149, SAMN16816150, SAMN16816151, SAMN16816152, SAMN16816153, and SAMN16816154) under BioProject PRJNA678899. Normalized small RNA sequencing data used for Figure 3 and Supplementary Figure S4 can be found in Supplementary Table S8. The exact timing data used for Figure 3, Supplementary Figures S3 and S4 can be found in Supplementary Table S9. The RNAi quantification data used for Figures 4 and 7 can be found in Supplementary Table S10. microRNA data used for the "confirmed in previous study" sections of Figure 1A and Supplementary Table S1 were obtained from Pasquinelli *et al.* (2000), Reinhart *et al.* (2000), Lau *et al.* (2001), Lim *et al.* (2003), Ruby *et al.* (2006), de Wit *et al.* (2009), Shi *et al.* (2013), and Kozomara *et al.* (2019).

Supplementary material is available at https://doi.org/10.25387/g3.13557194.

Results

Most *Caenorhabditis* species belonging to the *Japonica* group lack *let-7* microRNA

The *let-7* microRNA was the first microRNA whose sequence and developmental function in promoting the differentiation of cell fates were shown to be conserved from nematodes to vertebrates (Figure 1A; Pasquinelli *et al.* 2000; Reinhart *et al.* 2000; Lin *et al.* 2007; Caygill and Johnston 2008; Sokol *et al.* 2008). While studying the evolution of regulatory sequences within the *let-7* genomic loci of related nematodes, we discovered that most species of the *Japonica* group of *Caenorhabditis* lack the *let-7* sequence in their genomic assemblies (Figure 1B and Supplementary Table S1). Interestingly, one exceptional *Japonica* group species, *C. macrosperma*, had *let-7* sequence in its genomic assembly, which we confirmed using PCR amplification and Sanger sequencing (Supplementary Figure S1).

To determine if the lack of *let-7* sequence in the genomic assemblies of these *Japonica* group species could reflect major genomic rearrangements and/or anomalous assembly of genomic sequence, we analyzed the genome sequences surrounding *let-7* in *C. elegans* for potential synteny to corresponding genomic sequences of all *Caenorhabditis* species predicted to lack the *let-7* sequence. We found that the genomic assemblies of all the *Caenorhabditis* species lacking *let-7* sequence contain a region syntenic to the region surrounding the *let-7* locus of *C. elegans* (representative synteny shown in Figure 2A and Supplementary Figure S2A). Except for *Caenorhabditis afra*, none of the *Caenorhabditis* species lacking *let-7* sequence exhibited any indication that loss of *let-7* sequence is associated with genomic rearrangement (Supplementary Figure S3).

The absence of the *let-7* sequence in the genomic assemblies of *Japonica* group species could reflect incomplete sequence coverage. In such cases, *let-7* microRNA could be expressed from DNA that was, for some reason, not detected by genomic sequencing. Therefore, to gather evidence, independent of genomic sequence, for whether or not *let-7* microRNA is expressed in *Japonica* group

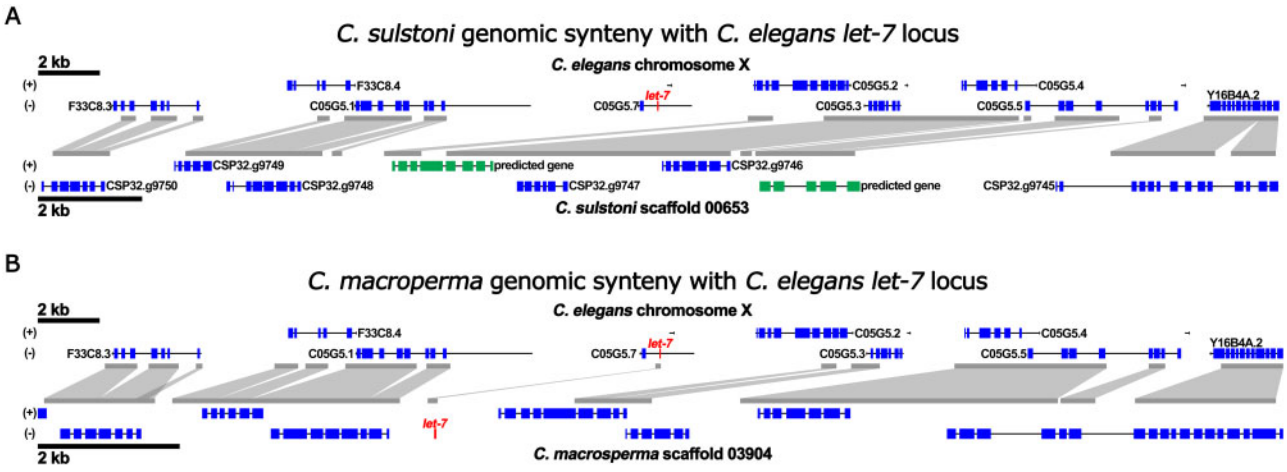

Figure 2 The genomic region containing the *let-7* sequence in *C. elegans* is syntenic to genomic regions in *C. sulstoni* and *C. macrosperma*. Synteny of a portion of *C. elegans* chromosome X containing the *let-7* sequence with *C. sulstoni* scaffold 00653 (A) and with *C. macrosperma* scaffold 03904 (B). Regions with sequence similarity are outlined in gray. *let-7* is shown in red. Annotated genes are shown in blue. Predicted genes are shown in green.

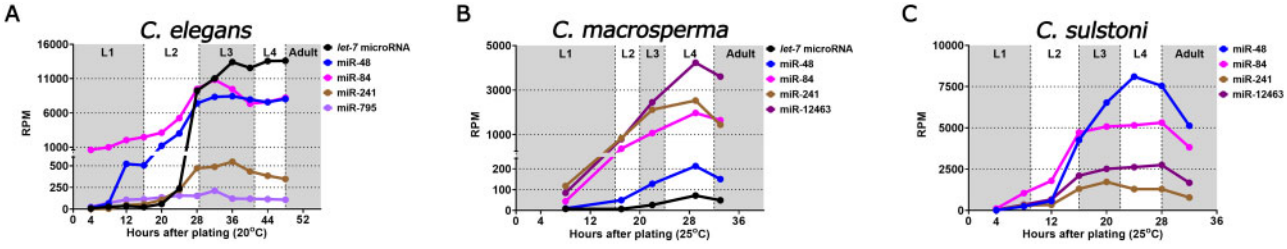

Figure 3 *let-7*-family microRNA temporal expression patterns for *C. elegans*, *C. macrosperma*, and *C. sulstoni*. Small RNA sequencing data showing expression of *let-7*-family microRNAs throughout *C. elegans* (A), *C. macrosperma* (B), and *C. sulstoni* (C) development. RPM refers to reads per million.

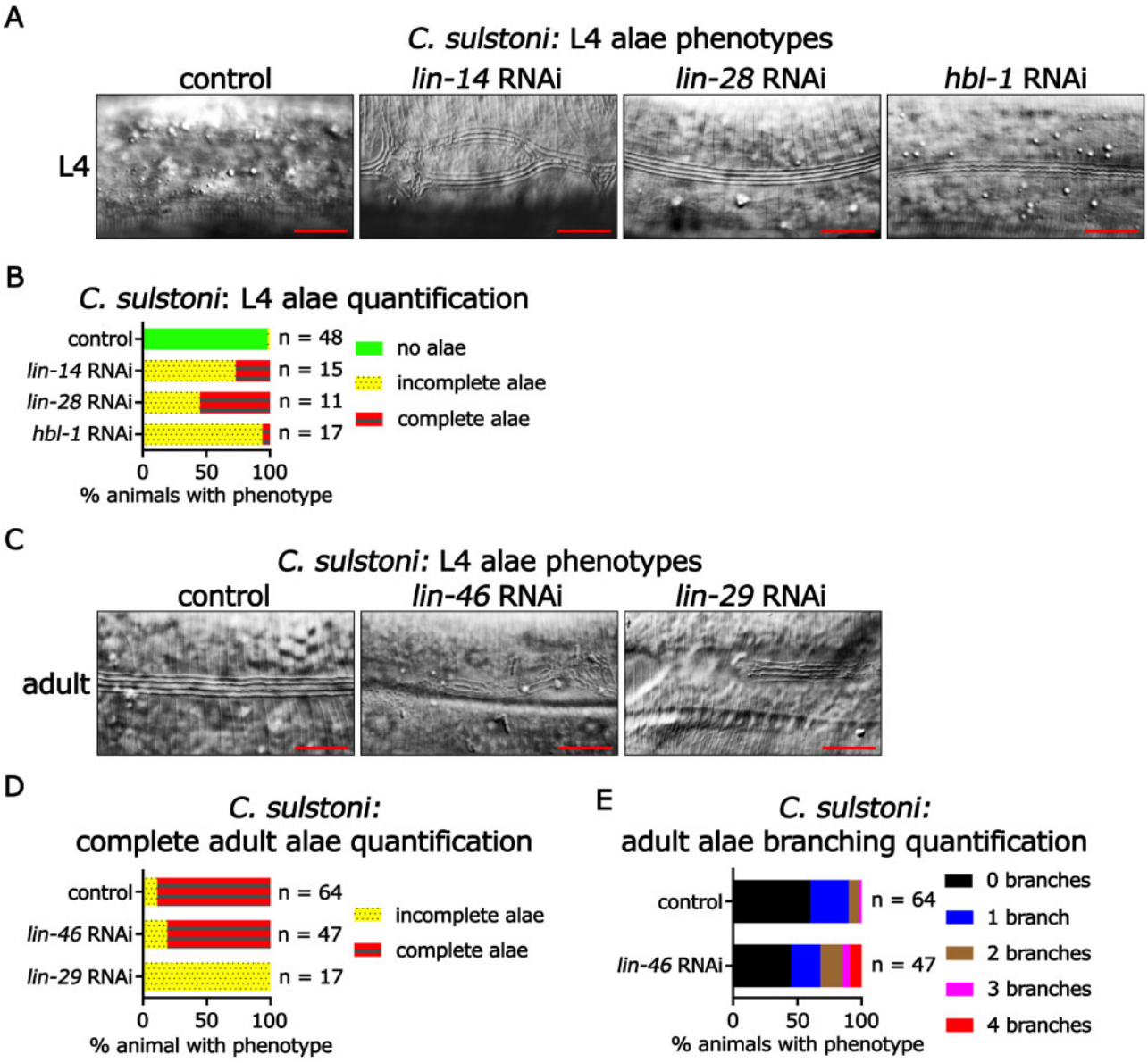

Figure 4 Heterochronic phenotypes associated with RNAi of *C. sulstoni lin-14*, *lin-28*, *hbl-1*, *lin-46*, and *lin-29*. (A) Panels from left to right: representative DIC images of *C. sulstoni* L4 hypodermis of animals fed control (empty vector), *lin-14*, *lin-28*, and *hbl-1* RNAi, respectively. (B) Quantification of observed L4 alae phenotypes in (A). Scale bars = 10 μM. (C) Panels from left to right: representative DIC images of *C. sulstoni* adult hypodermis of animals fed control (empty vector), *lin-46*, and *lin-29* RNAi, respectively. Quantification of complete adult alae (D) and adult alae branching (E) phenotypes observed in (C). Scale bars = 10 μM. Note: the control (empty vector) and *lin-46* RNAi animals used for (D) were also used for (E).

species, we profiled microRNAs using FirePlex miRNA assays in RNA samples from mixed-stage populations of eight *Japonica* species available from the *Caenorhabditis* Genetics Center (CGC), *Caenorhabditis waitukubuli, Caenorhabditis panamensis, Caenorhabditis nouraguensis, Caenorhabditis becei, Caenorhabditis macrosperma, Caenorhabditis sulstoni, C. afra*, and *Caenorhabditis japonica*.

For all seven of the *Japonica* group species that lack *let-7* in their genome assemblies as well as *C. macrosperma*, which has *let-7* sequence in its genome assembly (Figures 1B and 2B, Supplementary Figure S1 and Table S1), we failed to detect *let-7* microRNA expression by FirePlex assay. In contrast, *let-7* microRNA was readily detectable using FirePlex in mixed-staged total RNA samples from species of the closely related *Elegans* group, *Caenorhabditis inopinata, C. elegans*, and *Caenorhabditis kamaaina* (Figure 1B and Supplementary Table S1). These FirePlex data also confirmed the expression of other microRNAs, including *lin-4* microRNA and the *let-7*-family microRNAs miR-48 and miR-241 in all eight *Japonica* group species, as well as in the three *Elegans* group species (Figure 1B and Supplementary Table S1).

Caenorhabditis macrosperma expresses *let-7*

Caenorhabditis macrosperma is the one exceptional *Japonica* species in our experimental set that does contain *let-7* genomic sequence. Despite confirming the presence of the *let-7* sequence in the *C. macrosperma* genomic sequence assembly and its synteny to the *C. elegans let-7* genomic locus (Supplementary Figure S1 and Figure 2B; expanded synteny Supplementary Figure S2B), we failed to detect *let-7* microRNA expression using the FirePlex assay (see above). The FirePlex assay employs a panel of hybridization probes complementary to *C. elegans* microRNAs, which allows detection of microRNAs that precisely match the corresponding probe, or in some cases, that differ by a single internal

nt. Although the *C. macrosperma* genomic sequence assembly contains an apparent let-7-5p guide RNA sequence identical to the *C. elegans let-7* microRNA, it is possible that *let-7* microRNA was not detected by FirePlex in our *C. macrosperma* RNA samples owing to significant 5′ or 3′ end variation and/or expression levels below the limits of detection by FirePlex. Similarly, other microRNAs that were not detected in our FirePlex data (such as the *let-7* microRNA paralog miR-84, which was detected in *C. elegans* only; Figure 1B and Supplementary Table S1), could have been missed owing to low expression levels and/or significant interspecific variation in their non-seed sequences.

To definitively determine if *let-7* microRNA is expressed in *C. macrosperma*, we performed small RNA sequencing of RNA samples from each larval stage as well as early adult stage for both *C. elegans* and *C. macrosperma* (staging times shown in Supplementary Figure S4, A and B). From these data, we confirmed that *let-7* microRNA is indeed expressed in *C. macrosperma* (Figure 1B and Supplementary Table S1). Unlike *C. elegans let-7* microRNA, whose expression dramatically increases during the L3 stage and peaks at the L4 stage and accumulates to levels similar to miR-48 and miR-84, *C. macrosperma let-7* microRNA undergoes a more gradual and blunted increase in level during *C. macrosperma* development and never exceeds the level of any other *let-7*-family microRNA (Figure 3, A and B).

Small RNA sequencing also confirmed the expression of *C. macrosperma lin-4* microRNA, as well as the *let-7*-family microRNAs, miR-48, miR-241, miR-84 (which is one nt shorter and has four internal nt differences from *C. elegans* miR-84), and a novel *let-7*-family microRNA, miR-12463 (Figures 1B and 3B, Supplementary Figure S5B and Table S1). Interestingly, the *C. macrosperma mir-12463* genomic sequence is located 106 bp downstream of the *mir-84* sequence, suggesting that miR-84 and miR-12463 may be produced from a common primary transcript. Similar to the *C. elegans let-7*-family microRNAs, the developmental expression profiles of miR-48/84/241/12463 in *C. macrosperma* consist of gradual increases during the L1 to L2 stages and peaks during the L3 and L4 stages (Figure 3B). We note a relatively low expression of miR-48 in *C. macrosperma* compared with *C. elegans* miR-48 (Figure 3, A and B).

The temporal expression profile of *C. macrosperma lin-4* microRNA was similar to that of *C. elegans*, consisting of an increase in expression during early larval stages, followed by a broad peak during middle stages, and a decrease around the L4/adult transition (Supplementary Figure S5, A and B).

Robust assignment of *C. macrosperma* to the *Japonica* group

Caenorhabditis macrosperma was the only member of the *Japonica* group analyzed here to contain a *let-7* gene in its genome, implying the presence of *let-7* in a *Japonica* ancestor and loss of *let-7* during the divergence of these *Japonica* species. An alternative explanation for the exceptionalism of *C. macrosperma* as a *let-7*-containing *Japonica* species could be an erroneous phylogenic assignment of *C. macrosperma*. In this regard, *C. macrosperma* was first recovered as a member the *Japonica* group based on a comparison of selected gene segments across *Caenorhabditis* species (Kiontke *et al.* 2011; Felix *et al.* 2014). Subsequent genome-wide analyses that included additional *Caenorhabditis* species again recovered *C. macrosperma* in the *Japonica* group with maximal support (a Bayesian posterior probably of 1.0 and a bootstrap value of 100, respectively) (Stevens *et al.* 2019, 2020), indicating a high likelihood of its correct assignment.

To obtain additional evidence confirming the evolutionary affinity of *C. macrosperma* with the *Japonica* group, we examined the phylogeny of the conserved *let-7* target gene, *lin-41*. We reasoned that if the presence of a *let-7* gene reflects evolutionary affinity of *C. macrosperma* with a clade outside *Japonica*, then such hypothetical divergence from *Japonica* might be reflected in the gene-specific phylogeny of *lin-41*, a conserved *let-7*-specific target. When we constructed a *lin-41* gene tree across 33 *Caenorhabditis* species, we observed a *lin-41* phylogeny nearly identical to the species phylogeny (Supplementary Figure S6), confirming the *Japonica* affinity of *C. macrosperma lin-41*, and strongly supporting the conclusion that *C. macrosperma* is an exceptional *Japonica* group species that has retained *let-7*.

Further support for the assignment of *C. macrosperma* to the *Japonica* group comes from considering the novel *let-7*-family microRNA, miR-12463, which we initially identified in small RNA sequencing from *C. macrosperma*. BLAST search analyses of *Caenorhabditis* species genomes revealed *mir-12463* homologs to be present in all eight *Japonica* group species and in no species outside of the *Japonica* group (Figure 1B and Supplementary Table S1). This suggests that miR-12463 is a *Japonica* group specific *let-7*-family microRNA, and its presence in *C. macrosperma* supports the view that *C. macrosperma* is indeed a member of the *Japonica* group.

The *mir-84* loci in the *Japonica* group are polycistronic with novel *let-7*-family microRNAs

The identification of *mir-12463* within the *mir-84* locus of *C. macrosperma* led us to explore if there are more predicted novel *let-7*-family microRNAs in the *mir-84* loci of other *Caenorhabditis* species. To do this, we searched the genomic sequence surrounding the *mir-84* sequence for the presence of the *let-7*-family seed sequence (GAGGUAG) in all *Caenorhabditis* species. We next used *in silico* RNA folding to predict if the RNA would fold into a stem-loop structure indicative of a microRNA precursor. From this analysis, we concluded that all members of the *Japonica* group have between one and five extra *let-7*-family microRNAs in their *mir-84* loci. Interestingly, miR-84 is always the 5′ most *let-7*-family microRNA in these loci, and these additional *let-7*-family microRNAs are most likely polycistronic with miR-84 as they are no further than 221 bp apart (average of 116 ± 40 bp). We found no evidence of additional let-7-family microRNAs in the *mir-84* loci in any *Caenorhabditis* species outside of the *Japonica* group (Figure 1B and Supplementary Table S1).

Caenorhabditis sulstoni serves as a representative species lacking *let-7*

Caenorhabditis sulstoni is a gonochoristic bacteriovore that was isolated from the feces of the east African millipede *Archispirostreptus gigas* purchased at an insect market in Berlin in spring 2013 (Stevens *et al.* 2019). Based on its robust and consistent developmental trajectory and ease of experimental manipulation (including sensitivity to RNAi), we adopted *C. sulstoni* as a representative *let-7*-lacking species for our experiments. *Caenorhabditis sulstoni* grows on standard NGM plates seeded with *E. coli*. *Caenorhabditis sulstoni* larval development appears largely similar to *C. elegans*, containing six distinct phases: an embryonic stage, L1 through L4 larval stages, and adult stage. The overall organismal morphology and cellular anatomy of *C. sulstoni* males and females appear markedly similar to the corresponding sexes of *C. elegans*. Particularly, the development of the *C. sulstoni* hypodermal cell lineages, the morphologies of stage-specific cuticles, and the ontogeny of the gonad and vulva are similar to *C. elegans*.

Like *C. elegans*, *C. sulstoni* embryos can be isolated by sodium hypochlorite/sodium hydroxide treatment of populations that include gravid adults. After allowing embryos to hatch in the absence of food, populations of developmentally arrested L1 larvae (L1 diapause) are obtained. Addition of food to a population of L1 diapause larvae triggers synchronous initiation of larval development, thereby enabling the preparation of populations of developing larvae of defined larval stages for biochemical and molecular experiments. Unlike *C. elegans*, which is typically cultured between 15°C and 25°C, *C. sulstoni* can be cultured between 20°C and 30°C (Supplementary Figure S4, A and C). Interestingly, at 25°C *C. sulstoni* develop from the L1 to adulthood 13 h faster than *C. elegans*, 28 *vs* 41 h, respectively (Supplementary Figure S4, A and C). Moreover, at 30°C and 33°C (temperatures that do not support *C. elegans* development), *C. sulstoni* develops from the L1 stage to adulthood in ~23 h (Supplementary Figure S4A and C). 15°C and 33°C seem to define the low and high limits of *C. sulstoni* temperature tolerance under our culture conditions; *C. sulstoni* can develop from L1 to adulthood at 15°C or 33°C but are sterile when raised at either temperature. Populations of *C. sulstoni* grown at 15°C become asynchronous and take ~6 days for the first animals to reach adulthood (Supplementary Figure S4C).

Except for the lack of *let-7*, the heterochronic pathway is functionally conserved in *C. sulstoni*

In *C. elegans*, *let-7* functions as a significant component of the heterochronic pathway by ensuring proper developmental cell fate progression, particularly during the larval to adult cell fate transition (Supplementary Figure S6A; Reinhart *et al.* 2000). Because this hallmark microRNA of the heterochronic pathway is lacking in *C. sulstoni*, we sought to determine the status of other major components of the heterochronic pathway. As mentioned in the *Introduction* section, the protein coding genes *lin-14, lin-28, lin-46, hbl-1, lin-41*, and *lin-29* as well as the microRNA genes *lin-4, mir-48/84/241*, and *let-7* are critical components of the heterochronic pathway in *C. elegans* (Supplementary Figure S7A). In *C. elegans*, loss of *lin-14, lin-28, hbl-1*, or *lin-41* results in precocious development and the subsequent early formation of adult-specific structures including adult lateral alae (Ambros and Horvitz 1984; Fay *et al.* 1999; Slack *et al.* 2000; Abrahante *et al.* 2003; Lin *et al.* 2003). In contrast, loss of *lin-4, lin-46, mir-48/84/241*, or *lin-29* result in retarded development, characterized by incomplete formation of adult-specific structures including adult lateral alae (Chalfie *et al.* 1981; Ambros 1989; Ambros and Horvitz 1984; Pepper *et al.* 2004; Abbott *et al.* 2005).

To assess the conservation of heterochronic pathway gene function between *C. elegans* and *C. sulstoni*, we first characterized the larva-to-adult cell fate transition in the hypodermis of *C. sulstoni*. During the L4-adult transition in *C. elegans*, hypodermal seam cells, which consist of a longitudinal string of cells of either side of the animal, finish dividing, fuse with each other to form a lateral line syncytium, and produce the adult-specific cuticular structure called lateral alae, which signifies the terminal differentiation of the seam cells (Sulston and Horvitz 1977; Ambros and Horvitz 1984). Similar to *C. elegans*, in *C. sulstoni* we observed seam cell fusion and the formation for adult alae during the L4-adult transition (Figure 4, A–D).

From our FirePlex microRNA profiling, we had already confirmed the expression of *lin-4* microRNA and the *let-7*-family microRNAs miR-48 and miR-241 in *C. sulstoni* (Figure 1B and Supplementary Table S1). We sought to determine if these microRNAs are integrated into the *C. sulstoni* heterochronic network through their targeted repression of the LIN-14, LIN-28, and HBL-1 mRNA 3′ UTRs as they are in *C. elegans*. Because 3′ UTRs are not annotated in *C. sulstoni*, we used the *C. elegans* 3′ UTRs as a framework and examined sequences downstream of each respective gene's stop codon for *lin-4* and *let-7*-family microRNAs complementary sites (Supplementary Table S2). For the *C. sulstoni lin-14, lin-28*, and *hbl-1* 3′ UTR regions, predicted sites for *lin-4* and *let-7*-family microRNAs were identified, indicating conservation of the targeting of these heterochronic genes by *lin-4* and *let-7*-family microRNAs between *C. elegans* and *C. sulstoni* (Supplementary Table S2 and Figure S7B).

As mentioned previously, the FirePlex assay we used to detect *lin-4* microRNA and miR-48/241 in *C. sulstoni* employs a panel of hybridization probes complementary to *C. elegans* microRNAs, which does not allow for the detection of divergent orthologs of these microRNAs . This would include *C. sulstoni* miR-84, whichis predicted to be one nt shorter and have three internal nt differences compared with *C. elegans* miR-84.

To assay for miR-84 and to potentially identify novel *let-7*-family microRNAs in *C. sulstoni*, and to determine their temporal expression patterns during development, we performed small RNA sequencing of RNA samples from each larval stage as well as the early adult stage of *C. sulstoni*. From these data, we confirmed the expression of *C. sulstoni lin-4* microRNA, miR-48, miR-84, miR-241, and miR-12463, and found that their developmental dynamics in *C. sulstoni* are similar to *C. elegans* and *C. macrosperma* (Figure 3, A–C, Supplementary Figure S5, A–C and Table S1).

To assess the potential functional conservation of protein coding components of the heterochronic pathway, we used RNAi to knock down heterochronic gene homologs in *C. sulstoni*. Previous studies reported that loss-of-function mutations of *lin-14, lin-28*, or *hbl-1* in *C. elegans* result in precocious alae formation (Ambros and Horvitz 1984; Ambros 1989; Fay *et al.* 1999; Abrahante *et al.* 2003; Lin *et al.* 2003). Similarly, in *C. sulstoni* RNAi of *lin-14, lin-28*, or *hbl-1* caused precocious phenotypes (Figure 4, A and B) like those previously reported for *C. elegans*.

Previous studies also reported that *lin-46(lf)* results in a mild retarded phenotype manifesting as minor gaps and branches in adult alae (Pepper *et al.* 2004), and *lin-29(lf)* results in a more severe retarded phenotype manifesting as significant gaps in, or complete absence of adult alae (Ambros and Horvitz 1984; Ambros 1989). RNAi of *lin-46* or *lin-29* in *C. sulstoni* resulted in retarded phenotypes (Figure 4, C–E) like those previously reported for *C. elegans lin-46(lf)* or *lin-29(lf)* mutants.

The similarities in expression of *lin-4* and the *let-7-family*, the conservation of complementary sites in the 3′ UTRs of LIN-14, LIN-28, and HBL-1 mRNAs, and the RNAi knock down phenotypes for *lin-14, lin-28, lin-46, hbl-1*, and *lin-29* indicate that the heterochronic pathway is largely conserved between *C. sulstoni* and *C. elegans*.

Temporal regulation and function of LIN-41 are largely conserved in *C. sulstoni*

MicroRNA families are groups of microRNAs that share an identical seed sequence (nts 2–8) but differ in their non-seed sequence (Roush and Slack 2008). In principle, members of the same microRNA family can regulate the same target via seed pairing, but at the same time, differences in non-seed sequences can allow family member specificity of targeting through base-pairing of non-seed nts (Moore *et al.* 2015; Broughton *et al.* 2016; Brancati and Großhans 2018). In *C. elegans*, the *let-7*-family of microRNAs consists of *let-7* microRNA, miR-48, miR-84, miR-241, and miR-795 (Supplementary Table S1; Abbott *et al.* 2005; Roush and Slack 2008). Interestingly, *C. elegans let-7* microRNA targets the LIN-

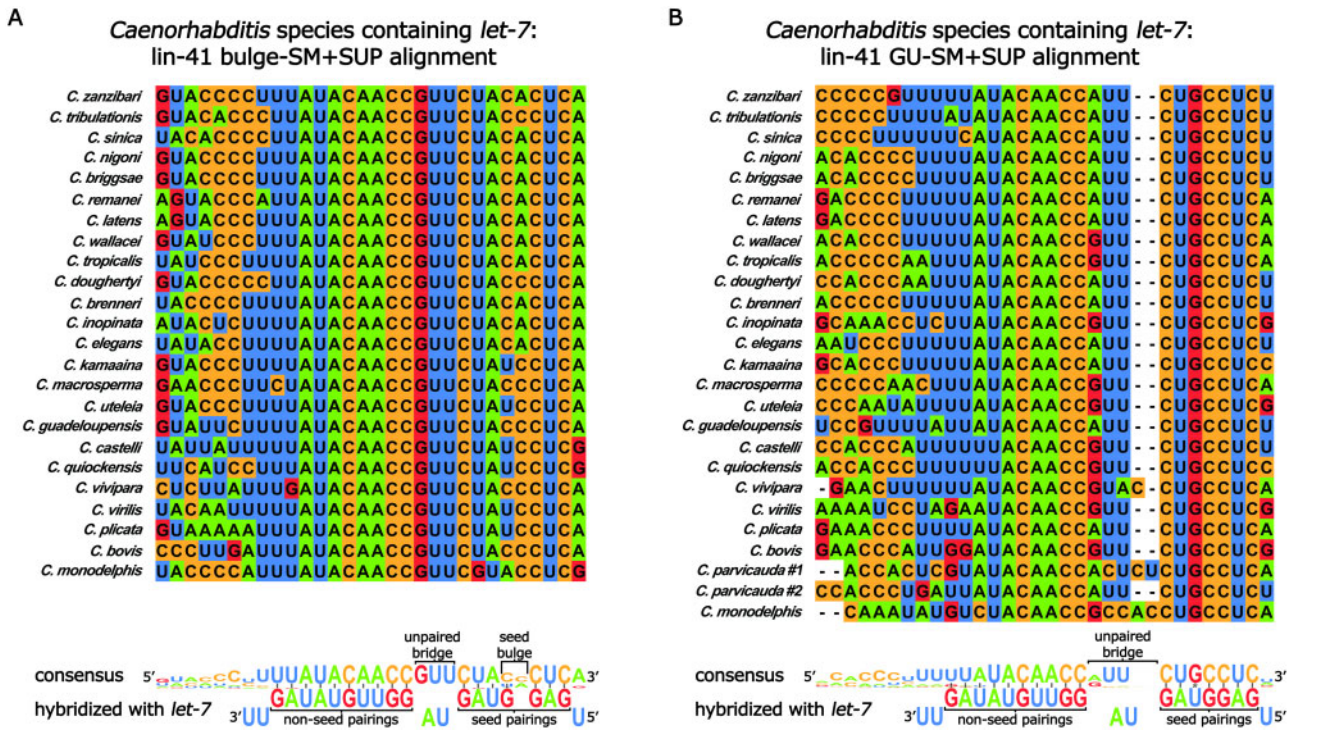

Figure 5 The *let-7* SM+SUP sites in the 3′ UTR region of *lin-41* are highly conserved in *Caenorhabditis* species that contain *let-7*. Sequence alignment of the bulge-SM+SUP site (A) and the GU-SM+SUP (B) in the 3′ UTR region of *lin-41* in *Caenorhabditis* species containing *let-7*. Shown at the bottom of each panel is the respective consensus sequence predicted hybridization with let-7 microRNA. Watson–Crick base pairing is shown with a solid line between the paired bases. The G-U base pair in the seed is shown with an asterisk between the paired bases.

41mRNA 3′ UTR via two sites that are "weakly" complementary to the *let-7*-family microRNA seed sequence (hereafter referred to as SM for "seed match"), both of which are supplemented with significant non-seed pairings to *let-7* microRNA. This base pairing configuration (hereafter referred to as SM+SUP) is thought to confer specificity for regulation of LIN-41 mRNA by *let-7* microRNA, to the exclusion of the other *let-7*-family microRNAs. Interestingly, the "weak" seed of each SM is distinct from the other: pairing of *let-7* microRNA to the first SM results in a bulged target adenine in the seed helix between g4 and g5 (hereafter referred to as the "bulge-SM"; Figure 5A); for the second SM, the seed helix contains a G-U wobble base pair at g5 (hereafter referred to as the "GU-SM"; Figure 5B; Reinhart *et al.* 2000; Vella *et al.* 2004; Ecsedi *et al.* 2015).

To gauge the phylogenetic conservation of the *let-7* microRNA SM+SUP configurations in the *lin-41* 3′ UTR regions of other related species, we aligned predicted *lin-41* SM+SUP sequences for all available *Caenorhabditis* species genomes. To identify potential *let-7*-family microRNA base paring SM+SUP sequences, we searched downstream of the stop codon of *lin-41* homologs for sequences complementary to the *let-7* microRNA seed sequence, including bulges and G-U pairings. With the exception of the species lacking *let-7*, all *Caenorhabditis* species have two "weak" SM+SUPs in their *lin-41* 3′ UTR regions, and apart from *C. parvicauda*, which has two GU-SM+SUPs, all *let-7*-containing *Caenorhabditis* species have one bulge-SM+SUP positioned 5′ of one GU-SM+SUP (Figure 5, A and B, Supplementary Tables S3 and S4). Moreover, the SM+SUPs are always in relatively close proximity to each other, with the 3′ most base-paired nt of the bulge-SM being no further than 39 nts away from the 5′ most base-paired nt of the GU-SM (Supplementary Tables S3 and S4). These results indicate that there is strong selective pressure to maintain a strict *let-7*-specific regulation of *lin-41* in *Caenorhabditis* species that express *let-7* microRNA.

One possible explanation for how the absence of *let-7* is accommodated in *Japonica* species is that one or more of the other *let-7*-family microRNAs may have adopted the role of regulating *lin-41*. To test this possibility, we examined the *lin-41* 3′ UTR regions of *let-7*-lacking genomes for sequences complementary to the remaining *let-7*-family microRNAs. Remarkably, in all seven of these genomes, we observed conservation of the GU-SM, indicating conservation of the regulation of LIN-41 mRNA by one or more *let-7*-family microRNAs, despite the absence of *let-7* itself (Figure 6, A and B and Supplementary Table S4). Interestingly, we failed to observe any significant conservation of *lin-41* 3′ UTR region sequences adjacent to the GU-SM, arguing against any conservation of supplemental non-seed pairing (SUP) by a particular *let-7*-family microRNA (Figure 6, A and B and Supplementary Table S4).

Owing to the lack of conservation of supplemental non-seed matching sequence in the *lin-41* 3′ UTR regions of *Caenorhabditis* species lacking *let-7*, we hypothesized that one or more *let-7*-family microRNAs could regulate LIN-41 mRNA via GU-SM+SUP base-pairing pattern, but the particular *let-7*-family microRNAs with the best match toLIN-41 mRNA could vary for each species. We presumed that for a *let-7*-family microRNA to assume a similar role as *let-7* microRNA in regulating LIN-41 mRNA, the MFE between that *let-7*-family microRNA and the LIN-41mRNA GU-SM+SUP should be similar to that for the interaction of *let-7* microRNA with LIN-41 mRNA in *let-7*-containing species. To test this, we calculated the MFE of base pairing for each *let-7*-lacking species' predicted *let-7*-family microRNAs to their respective *lin-41* GU-SM+SUP and compared it with the MFEs of the *let-7*-family microRNAs hybridized to the bulge-SM+SUP as well as GU-

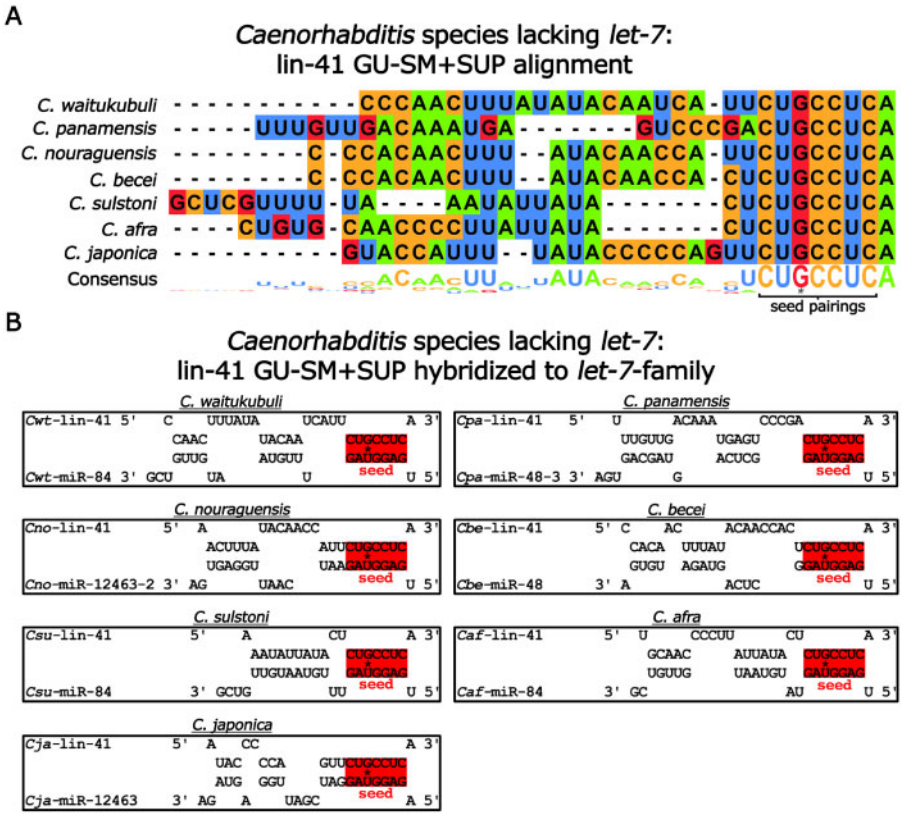

Figure 6 The SM +SUP site in the 3′ UTR region of *lin-41* are divergent amongst *Caenorhabditis* species lacking *let-7*. (A) Sequence alignment of the GU-SM+SUP in the 3′ UTR region of *lin-41* in *Caenorhabditis* species lacking *let-7*. The G-U base pair in the seed pairings is shown with an asterisk. (B) Predicted base pairing of let-7-family microRNAs with the GU-SM+SUP in the *lin-41* 3′ UTR region in *Caenorhabditis* species lacking *let-7*. Shown are the predicted base pairing of the *let-7*-family microRNA with the most favorable hybridization (lowest MFE) for each respective species. The G-U base pair in the seed pairings is shown with an asterisk.

SM+SUP in all *let-7*-containing species. In all *let-7*-containing species, the lowest (most favorable) MFE pairing of a *let-7*-family microRNA to the *lin-41* bulge-SM+SUP and GU-SM+SUP was for the interaction with *let-7* microRNA (average MFE of -27.2 ± 0.5 and -29.2 ± 1.2 kcal/mol, respectfully; Supplementary Figure S8, A and B, Tables S3 and S4). In contrast, in *let-7*-lacking species, the *let-7*-family microRNA that had the lowest MFE pairing to the *lin-41* GU-SM+SUP varied between species and was higher (less favorable) than the MFE of *let-7* microRNA with either the bulge-SM+SUP or GU-SM+SUP in species that contain *let-7* (average MFE -21.5 ± 2.1 kcal/mol; Supplementary Figure S8B and Table S4).

LIN-41 is an RNA binding protein that, in *C. elegans*, forms distinct foci in the cytoplasm particularly around the periphery of the nucleus (Figure 7A; Spike *et al.* 2014). In the hypodermis of *C. elegans*, LIN-41 is expressed during the L1 through L3 stages and is undetectable in the L4 and adult stages due to its translational repression by *let-7* microRNA (Figure 7A; Slack *et al.* 2000). The relatively weaker predicted MFE of the interaction between *let-7*-family microRNAs and *lin-41* 3′ UTR regions in species lacking *let-7* compared with *let-7* microRNA and *lin-41* 3′ UTR regions in species containing *let-7* suggests that the *let-7*-family could have a less prominent role in down regulation of LIN-41 mRNA in species lacking *let-7* than *let-7* microRNA does in species containing *let-7*. To determine whether LIN-41 protein is down regulated in a representative *let-7*-lacking species as observed in *C. elegans*, we used CRISPR to GFP-tag the N-terminus of endogenous LIN-41 in *C. sulstoni*. We observed a nearly identical expression pattern in *C. sulstoni* to what we observed in *C. elegans*: hypodermal LIN-41 is expressed in the L1 through L3 stages and is downregulated in the L4 and adult stages (Figure 7, A and B). This down regulation of LIN-41 is indicative of prominent temporal regulation similar to what is observed in *C. elegans* and could reflect the action of one or more *let-7*-family microRNAs.

In *C. elegans*, *lin-41(lf)* animals precociously produce adult alae during the L4 stage (Slack *et al.* 2000). To determine if LIN-41 could be functionally conserved in species that lack *let-7*, we used RNAi to knock down *lin-41* in *C. sulstoni*. Similar to *C. elegans*, when *lin-41* was knocked down in *C. sulstoni* we observed L4 animals with adult alae. However, precocious alae occurred in a lower percentage of animals than previously reported for *lin-41(lf)* in *C. elegans*, and generally the precocious adult alae were of rather indistinct morphology compared with bona fide adult alae (Figure 7, D and E; Reinhart *et al.* 2000; Slack *et al.* 2000; Banerjee *et al.* 2005; Nolde *et al.* 2007). The relatively low penetrance of the precocious alae phenotype could possibly reflect poor RNAi knock down of *lin-41*. To test this, we knocked down *lin-41* in our GFP-tagged strain and confirmed efficient knockdown by an absence of detectable GFP-LIN-41 fluorescence in hypodermal cells. Moreover, animals in which *lin-41* was knocked down were sterile, a hallmark of *lin-41* loss-of-function for its germline function in *C. elegans* (data not shown; Slack *et al.* 2000).

To our surprise, RNAi of *lin-41* in *C. sulstoni* caused not only precocious and morphologically abnormal alae at the L4 stage, but also caused a highly penetrant alae formation phenotype in adult stage animals, where alae were often absent, incomplete, and/or morphologically abnormal (Figure 7F). This sort of adult stage phenotype had not been reported previously for *lin-41(lf)* in

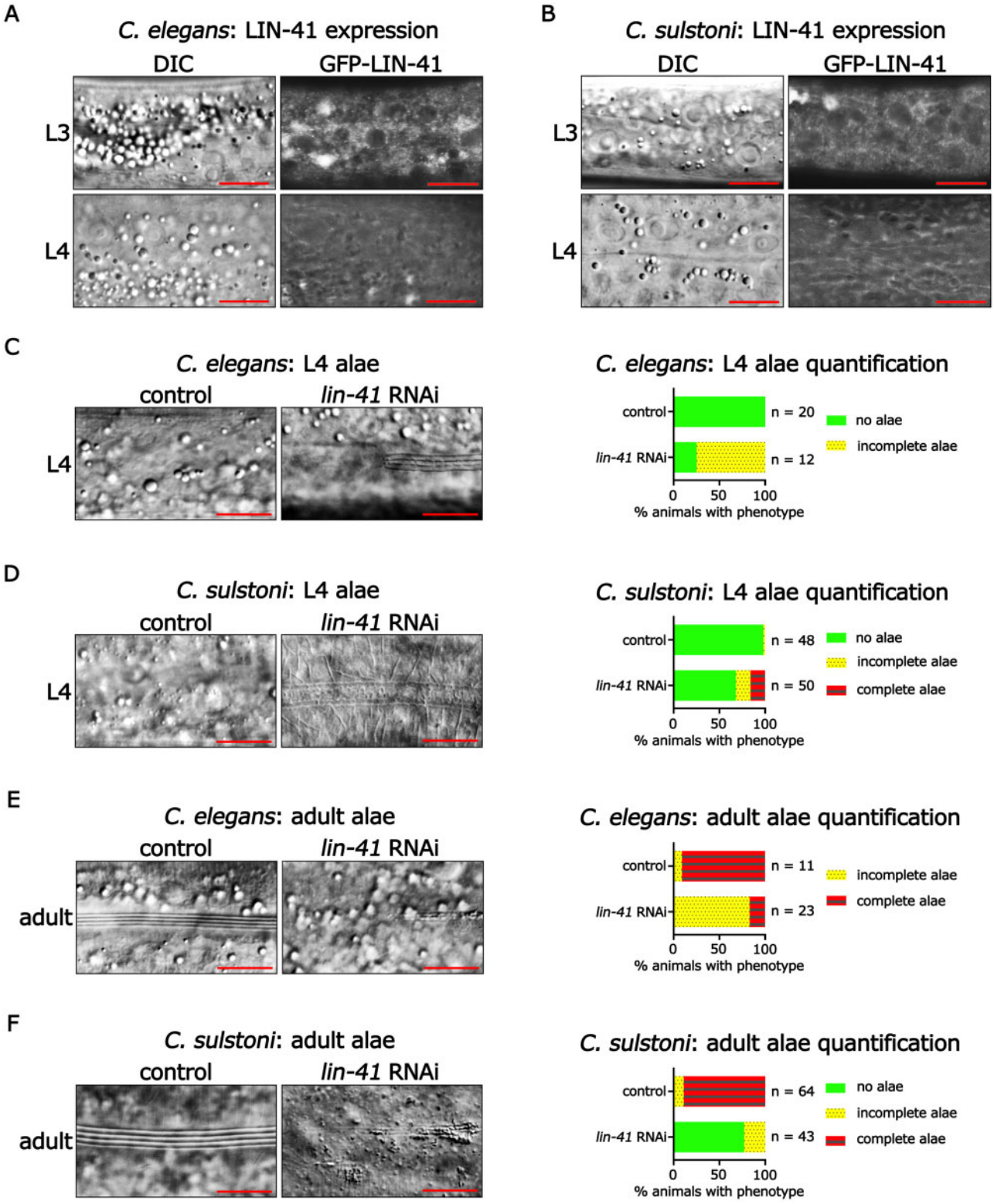

Figure 7 *lin-41* expression and function are conserved between *C. elegans* and *C. sulstoni*. (A, B) Representative DIC images (left panels) and endogenously tagged GFP-LIN-41 images (right panels) of hypodermal cells in *C. elegans* (A) and *C. sulstoni* (B) L3 (top panels) and L4 animals (bottom panels). Scale bars = 10 μM. Representative DIC images of *C. elegans* L4 hypodermis (C), *C. sulstoni* L4 hypodermis (D), *C. elegans* adult hypodermis (E), and *C. sulstoni* adult hypodermis (F) of animals fed control (empty vector) (left panels) and *lin-41* RNAi (middle panels) and quantification of alae phenotypes (graphs). Scale bars = 10 μM. Note: RNAi experiments used for Figures 4, B and D and 7, D and F were performed together. Therefore, control RNAi data used for Figure 4, B and D were also used for Figure 7, D and F.

C. elegans. The previous studies of *lin-41(lf)* in *C. elegans* employed partial loss-of-function mutations and not the high-efficiency RNAi expression vector that we used to knock down *lin-41* in *C. sulstoni* (T444T vector; Sturm *et al.* 2018). We hypothesized that the functions of *lin-41* could be conserved between *C. elegans* and *C. sulstoni*, and the discrepancies in the phenotypes previously

reported and observed here could be due to the partial function of the *lin-41* alleles used in previous studies. To test this hypothesis, we knocked down *lin-41* in *C. elegans* using the new, high-efficiency vector (T444T), and we observed similar phenotypes observed in *C. sulstoni*: L4 larvae with adult alae and incomplete/weak alae in adults (Figure 7, C–F). Interestingly, the L4 alae phenotypes observed in *C. sulstoni* were still less penetrant and weaker in appearance than what we observed in *C. elegans* (Figure 7, C and D). Moreover, the alae phenotypes observed in *C. elegans* adults appeared weaker than what we observed in *C. sulstoni* (Figure 7, E and F). Aside from these differences, our results indicate that the temporal patterning and function of LIN-41 are largely conserved between *C. elegans* and *C. sulstoni*.

Discussion

let-7 appears to be indispensable across diverse bilaterian phyla, indicating deep and pervasive evolutionary constraints on maintaining the entire 22-nt sequence of *let-7* microRNA. Moreover, *let-7*'s function in promoting cellular differentiation and repression of pluripotency are conserved as well, which could reflect some degree of conservation of orthologous targets. Chiefly among these are *lin-41* (*TRIM71* in mammals) and *lin-28* (*LIN28* in mammals) both of which encode pluripotency promoting RNA binding proteins whose expression is directly repressed by *let-7* microRNA in invertebrates and vertebrates alike (Slack *et al.* 2000; Kloosterman *et al.* 2004; Schulman *et al.* 2005; Lin *et al.* 2007; Vella *et al.* 2004; Ding and Großhans 2009; Ecsedi *et al.* 2015). Consequently, loss-of-function of *let-7* results in dysregulation of developmental progression (Reinhart *et al.* 2000; Kloosterman *et al.* 2004; Sokol *et al.* 2008; Caygill and Johnston 2008) and can lead to disease such as cancer (Balzeau *et al.* 2017). Thus, *let-7* is an important regulator of animal development and tissue homeostasis.

In this study, we characterized for the first time a cohort of animal species lacking the *let-7* microRNA sequence in their genomes. Using microRNA profiling, we confirmed the absence of mature *let-7* microRNA expression, while also confirming the conserved expression of the *let-7*-family microRNAs miR-48, miR-84, and miR-241. We also found that, except for the absence of *let-7*, the heterochronic pathway, into which *let-7* is deeply integrated in *C. elegans*, appears otherwise functionally conserved in a representative *let-7*-lacking species, *C. sulstoni*. Finally, we provide evidence that *lin-41*, a *let-7*-specific target in *C. elegans*, is regulated by other members of the *let-7*-family of microRNAs in *Caenorhabditis* species that lack *let-7*.

Our finding that *C. macrosperma* appears to be an exceptional member of the *Japonica* group that contains *let-7* raises interesting questions. Based on the current phylogenetic tree of *Caenorhabditis* species, for *C. macrosperma* to retain *let-7*, whilst the remaining members of the *Japonica* group lack *let-7*, three possibilities are suggested: (1) *C. macrosperma* does not belong in the *Japonica* group, (2) *C. macrosperma* is correctly assigned to the *Japonica* group, and gained the *let-7* sequence after it was lost in a *Japonica* ancestor, or (3) *C. macrosperma* is correctly assigned to *Japonica*, and *let-7* was lost at least three times during the evolution of the *Japonica* group.

The evidence strongly suggests that *C. macrosperma* is indeed correctly placed in the *Japonica* clade. Previous publications have routinely recovered *C. macrosperma* as a member of *Japonica* group with the most recent publications being a highly extensive, genome-wide studies with high statistical likelihood of *C. macrosperma*'s correct recovery (Kiontke *et al.* 2011; Felix *et al.* 2014; Stevens *et al.* 2019, 2020). Our results further strengthen this argument. We found that in *C. macrosperma*, the protein sequence of the *let-7*-specific target *lin-41* was most similar to *lin-41* protein sequence in other *Japonica* group species, and homologs of the novel *let-7*-family microRNA *mir-12463* that we identified in *C. macrosperma* can only be found in *Japonica* group species. Put together, these suggest that *C. macrosperma*'s recovery in the *Japonica* group is correct.

We can imagine two possible ways by which *C. macrosperma* could have gained *let-7*, (1) in a spontaneous manner, possibly by random sequence drift of a *let-7*-family paralog, or (2) through gene transfer from another *Caenorhabditis*. The first scenario is unlikely because the *let-7* genomic region in *C. macrosperma* is syntenic to the *let-7* region in *C. elegans*, arguing against spontaneous gain as no other *Japonica* group member has a *let-7*-family paralog in that syntenic region. The second scenario also seems unlikely, as there are no known examples of horizontal gene transfer between *Caenorhabditis* species. Moreover, the presence in *C. macrosperma* of *lin-41* bulge-SM+SUP and GU-SM+SUP sites matching *let-7* that are nearly identical to the *lin-41* bulge-SM+SUP and GU-SM+SUP sites in every other *let-7*-containing *Caenorhabditis* species strongly suggests that *C. macrosperma* inherited *let-7*, and its targeting of *lin-41*, from a common ancestor of the other *Japonica* species, and that *let-7* was lost during the evolution of other *Japonica* species.

A parsimonious model for loss of *let-7* in the *Japonica* clade suggests at least three independent loss events—once in the common ancestor to *C. waitukubuli*, *C. panamensis*, *C. nouraguensis*, and *C. becei*, a second loss in a common ancestor to *C. sulstoni* and *C. afra*, and a third loss in *C. japonica*. Multiple independent losses of *let-7* in the *Japonica* group, together with the dramatically lower expression level of *let-7* microRNA in *C. macrosperma* compared with the relatively robust expression of *let-7* microRNA in *C. elegans*, suggests that *let-7* became relatively dispensable in a common ancestor of these *Japonica* species. We suggest that a hallmark of the evolution of the *Japonica* group may be a reduced dependency on *let-7* for functions that are otherwise critical in most other *Caenorhabditis* species.

The extensive synteny of the *let-7* region of *C. elegans* with *C. sulstoni* suggests that loss of *let-7* was not associated with dramatic genome rearrangements in the region. Only one gene neighboring *let-7* in *C. elegans*, *C05G5.7*, also appears to have been fully lost in *C. sulstoni*. The loss of this gene might suggest a functional relationship between *let-7* and *C05G5.7*, perhaps even that loss of *C05G5.7* could functionally compensate for loss of *let-7*. Although *C05G5.7* is transcribed on the same primary transcript as *let-7* microRNA and serves as a negative regulator of *let-7* microRNA processing, nevertheless *C05G5.7* appears not to be conserved outside of *C. elegans*, and loss off *C05G5.7* does not suppress *let-7* loss-of-function (Nelson and Ambros 2019).

A key component of the heterochronic network in *C. elegans* is the RNA binding protein LIN-41. In *C. elegans*, loss of *let-7* is lethal, primarily due to the de-repression of *lin-41* (Ecsedi *et al.* 2015; Aeschimann *et al.* 2019). During wild type *C. elegans* development, LIN-41 levels decrease during the mid-to-late-L4 stage due to a sharp increase in *let-7* microRNA expression and subsequent *let-7* microRNA-mediated inhibition of LIN-41 translation. *let-7* microRNA interacts with the 3′ UTR of LIN-41 mRNA via base pairing to two non-canonical SM sites—one with a bulge adenosine of the LIN-41 mRNA in the seed helix between the g4 and g5 and the other with a G-U wobble base pair at the g5—combined with extensive SUP. In *Caenorhabditis* species that contain *let-7*, both SM+SUPs are highly conserved. In the set of *Caenorhabditis*

species that lack *let-7*, the *lin-41* 3′ UTR contains only the GU-SM, with no extensive conservation of non-seed pairings.

To be sure, the presence of a *let-7*-family SM in the 3' UTR regions of *lin-41* in species lacking *let-7* indicates that *lin-41* is likely regulated by one or more of the remaining *let-7*-family microRNAs. However, our observations suggest that *let-7*-family mediated regulation of *lin-41* in these species may be relatively less critical compared with *let-7*-containing species, where the extensive conservation of two SM+SUP sites indicates that both sites are necessary for robust regulation of LIN-41 mRNA by *let-7* microRNA. The *lin-41* 3′ UTR regions of *Caenorhabditis* species lacking *let-7* contain just one *let-7*-family SM site of relatively weak predicted binding (higher MFE) and do not exhibit evidence of conserved non-seed pairing.

In species lacking *let-7*, the particular *let-7*-family microRNA(s) predicted to regulate *lin-41* via SM+SUP pairing varied between species. miR-84 was most favorable in three species (*C. waitukubuli*, *C. sulstoni*, and *C. afra*), miR-48 was most favorable in two species (*C. panamensis* and *C. becei*), and miR-12463 was the most favorable in two species (*C. nouraguensis* and *C. japonica*; Figure 6B and Supplementary Table S4). Moreover, in some species that lack *let-7*, the *let-7*-family microRNA with the most favorable MFE was not much better than the next best MFE. For example, in *C. panamensis*, miR-48 was the most favorable with an MFE of −25.8 kcal/mol, whereas miR-84 was the next best with an MFE of −25.2 kcal/mol. This variability in which *let-7*-family microRNA is most favorable in combination with the relatively close MFEs of multiple *let-7*-family microRNAs within a species suggests that the regulation of LIN-41mRNA by the *let-7*-family microRNAs is less constrained, in terms of ortholog specificity, in *let-7*-lacking species than in *let-7*-containing species.

We found that despite the lack of *let-7* in *C. sulstoni* the temporal expression pattern and function of LIN-41 protein is largely conserved; LIN-41 levels decrease during the L4 stage of *C. sulstoni*, as is the case in *C. elegans*, and knockdown of *lin-41* results in similar heterochronic phenotypes in *C. sulstoni* and *C. elegans*. This indicates a form a compensatory evolution wherein LIN-41 in *C. sulstoni* has evolved to be regulated in a different way than LIN-41 in *C. elegans* to achieve the same functional outcome. Although our findings indicate that one or more *let-7*-family microRNA(s) may stand in for the absent *let-7* to developmentally regulate *C. sulstoni lin-41*, we cannot rule out contributions from transcriptional or other posttranscriptional mechanisms.

Indeed, this instance of compensatory evolution would not be the first example of a regulatory gene and/or pathway being replaced by a related gene/pathway. One example of gene "handoff" is the mosquito *Anopheles stephensi*, where the gene *paired*, which is required for the formation of alternating body segments in many insect species (most classically in *Drosophila melanogaster*), was lost and functionally replaced with a related gene, *gooseberry* (Jarvela *et al.* 2020). Another example is the variation in the usage of Wnt signaling as a regulator of vulva induction in nematodes—Wnt signaling is the primary inducer of vulva formation in *Pristionchus pacificus* but plays a relatively minor role in *C. elegans* vulva formation (which primarily relies on EGF signaling; Sternberg 2005; Tian *et al.* 2008).

In this study, we have identified a cohort of related *Caenorhabditis* species within the *Japonica* group that lack the (otherwise) highly conserved *let-7* microRNA. Our findings that one member of the *Japonica* group, *C. macrosperma*, has retained *let-7*, together with the pattern of species affinities within the *Japonica* group, suggests that *let-7* was lost at least three times during the evolution of the *Japonica* clade. We do not currently know what allowed for the loss of *let-7* in these species. However, it appears that in the *Japonica* group, *let-7* was seemingly released from its evolutionary-entrenched regulatory relationship with *lin-41*, thereby allowing for the regulation of *lin-41* to be taken over by the *let-7*-family. Further study is required to determine the nature of evolutionary factors that could cause an otherwise deeply conserved microRNA such as *let-7* to become dispensable.

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