Choreographic dendritic arborization takes place within a defined time frame, but the timing mechanism is currently not known. Here, we report that the precisely timed lin-4-lin-14 regulatory circuit triggers an initial dendritic growth activity, whereas the precisely timed lin-28-let-7-lin-41 regulatory circuit signals a subsequent developmental decline in dendritic growth ability, hence restricting dendritic arborization within a set time frame. Loss-of-function mutations in the lin-4 microRNA gene cause limited dendritic outgrowth, whereas loss-of-function mutations in its direct target, the lin-14 transcription factor gene, cause further decline. lin-4 and let-7 regulatory circuits are expressed in the right place at the right time to set start and end times for dendritic arborization. Replacing the lin-4 upstream cis-regulatory sequence at the lin-4 locus with a late-onset let-7 upstream cis-regulatory sequence delays dendrite arborization, whereas replacing the let-7 upstream cis-regulatory sequence at the let-7 locus with an early-onset lin-4 upstream cis-regulatory sequence causes a precocious decline in dendritic growth ability. Our results indicate that the lin-4-lin-14 and the lin-28-let-7-lin-41 regulatory circuits control the timing of dendritic arborization through antagonistic regulation of the DMA-1 receptor level on dendrites. The LIN-14 transcription factor likely directly represses dma-1 gene expression through a transcriptional means, whereas the LIN-41 tripartite motif protein likely indirectly promotes dma-1 gene expression through a posttranscriptional means.

Significance

Studies on the temporal control of cell cycle progression, circadian rhythm, and segmentation have frequently converged on the concept of biological oscillators (1–4). Biological oscillators are systems of molecules with various levels of expression and activity that act as clocks that determine biological rhythms resilient to changes in external environments. Understanding molecular oscillators gives a glimpse into temporal control for the cyclical nature of these processes. However, timing control for noncyclical biological processes is less understood. Most noncyclical events in nonneuronal cells are transient, which makes it difficult to study their temporal regulation. In contrast, differentiation of neurons into complex structures occurs on longer timescales, thereby giving us a window to peek into noncyclical mechanisms that control start and end times for dendritic arborization.

Although abundant knowledge has been learned in past decades on the temporal control of cell fate specification of neurons (15–17), less is known about the timing of their wiring to give rise to complex neuronal circuits and the timing of their plasticity. The heterochronous pathways are important temporal regulators of animal development and involve a number of microRNA-regulated posttranscriptional genetic circuits, including important interactions between the lin-4 microRNA and its direct target, the lin-14 transcription factor gene (18–20), and between the let-7 microRNA and its direct target, the lin-41 tripartite motif (TRIM) protein gene (21–23). Since the discovery of the lin-4-lin-14 and the let-7-lin-41 regulatory circuits broadly expressed in the nervous system (24–26), evidences indicating a widespread role of the lin-4-lin-14 and the let-7-lin-41 regulatory circuits in timing neuronal assembly and plasticity start to emerge. The lin-4 microRNA and the LIN-14 transcription factor regulate the temporal transition of sequential events in anterior ventral microtubule (AVM) neuronal connectivity. Up-regulation of lin-4 and down-regulation of lin-14 signal the end of netrin-mediated axon pathfinding to allow synapse formation in AVM neurons (24, 27). lin-4 and lin-14 are also involved in the temporal control of axon pathfinding in other neurons (28, 29) as well as synaptic rewiring of motor neurons (30, 31). Additionally, lin-14 temporally...
regulates the onset of zig gene expression to maintain ventral nerve cord structure (32). The let-7 microRNA and the LIN-41 tripartite motif protein control the timing of a postdifferentiation event in AVM neurons (25, 33). The progressive increase of lin-7 and the progressive decrease of lin-41 contribute to a normal developmental decline in AVM axon regeneration. Both lin-4 and let-7 microRNAs regulate axodendritic polarity in the DA9 motor neuron (34). Despite advances in understanding of the temporal transition of neuronal connectivity and regeneration decline, it is still unknown how neurons set not only start but also end times of developing a complex neuronal structure. Using the CRISPR technology, we successfully manipulated the timing of lin-4 and let-7 expression by swapping their upstream cis-regulatory sequences with each other. In this way, we can turn back the clock in old neurons and turn forward the clock in young neurons to demonstrate that the two timing regulatory circuits restrict dendritic arborization within a defined time frame.

Dendritic arborization of the posterior ventral process D (PVD) nociceptive neurons takes place within a defined time period starting at the second larval (L2) stage and ending at the young adult stage during Caenorhabditis elegans development. The PVD dendritic arbor is established by a complex but well-ordered array of nonoverlapping sister dendrites. The creation of this structure involves a sequential series of branching decisions, which makes PVD neurons an ideal model system to study timing mechanisms of dendritic arborization. A 1° dendrite extends from the PVD cell body along the anterior/posterior axis at the location of the lateral nerve fascicle. Orthogonal arrays of 2°, 3°, and 4° dendritic branches envelop the animal in a manner that alternates between the dorsal/ventral and the anterior/posterior axis to produce an elaborate network of sensory processes (35, 36). The well-ordered dendrite branches are structurally likened to menorahs, which are branched candle holders. The growth of these dendrite branches depends on the DMA-1 dendrite receptor in PVD neurons (37). What timing mechanisms initiate and terminate the arborization of this complex dendritic structure? To answer these questions, we search for molecules in PVD neurons that may provide intrinsic temporal control of dendritic arborization. We identify two timing regulatory circuits that restrain the growth of PVD dendrites within a set time frame. The initial dendritic outgrowth in PVD neurons is actuated by the precisely timed lin-4-lin-14 regulatory circuit, which positively and likely directly modulates the DMA-1 dendrite receptor level through a transcriptional means. The dendritic growth is subsequently slowed down by the precisely timed lin-28-let-7-lin-41 regulatory circuit, which negatively and likely indirectly modulates the DMA-1 dendrite receptor level through a post-transcriptional means, as dendritic arborization comes to an end.

Results

lin-4 and let-7 Are Temporally Expressed in PVD Neurons during Dendrite Arborization. To identify timing mechanisms that restrict PVD dendritic arborization within a defined time period, we sought to identify molecules that are expressed in PVD neurons and whose expression coincides with start and end times of PVD dendritic arborization. We discovered lin-4 and let-7 microRNAs to be excellent candidates that fit both criteria. To understand the temporal control of lin-4 and let-7 gene expression in PVD neurons, we developed a lin-4 reporter by fusing a 1.9-kb upstream region of a mature lin-4 microRNA to the GFP gene and a let-7 reporter by fusing a 2.9-kb upstream region of mature let-7 microRNA to the GFP gene and confirmed that they both were stably expressed in PVD (Fig. 1 and SI Appendix, Fig. S1). The expression levels of these two reporters in the whole animal at different developmental stages correlated strongly with the whole animal stem-loop RT-PCR quantification of lin-4 and let-7 microRNAs, indicating that these two reporters are reliable. Using these reporters, we determined the timing of lin-4 and let-7 expression in PVD neurons during dendritic arborization (Fig. 1D–G). The lin-4 reporter was expressed highly in PVD at the late L2–the early third larval (L3) stage, when the secondary dendrites start elaborating (Fig. 1A, B, D, and F). In contrast, the let-7 reporter was expressed at relatively low levels in PVD at the L3 stage but was significantly elevated from the L4 stage onwards, when the growth of the terminal quaternary dendrites comes to an end (Fig. 1A, C, E, and G). These results indicate that lin-4 and let-7 are expressed in the right place at the right time to initiate and terminate PVD dendritic arborization, respectively.

The lin-4-lin-14 Regulatory Circuit Initiates Dendritic Outgrowth in PVD Neurons. We previously reported that the lin-4 microRNA represses the expression of the LIN-14 transcription factor to inhibit AVM axon attraction (24, 27). Further analysis of lin-4 and lin-14 reporters revealed an overlapping expression of two genes in many other neurons, including PVD, at the early L3 stage (Fig. 1B and SI Appendix, Fig. S1A–C), when PVD neurons are sending out the secondary dendrites. We showed that lin-4(e912) loss-of-function (lf) and lin-14(n355) gain-of-function (gf) mutants displayed a similar phenotype of limited dendritic outgrowth in PVD neurons (Fig. 2A–C and E). In both lin-4(e912lf) and lin-14(n355gf) mutants, the frequencies of the PVD secondary branches at the young adult stage were 30 to 40% lower compared with that in the wild type (n > 20 analyzed in each strain). In addition, PVD neurons in the majority of lin-4(e912lf) and lin-14(n355gf) mutants entirely lacked the tertiary branches even at the young adult stage, although subsets of PVD neurons in these mutants displayed a small number of tertiary branches in the area close to the cell body. These results suggest that the lin-4 microRNA may inhibit the expression of the LIN-14 transcription factor to initiate dendrite outgrowth. Consistent with this interpretation, reduced lin-14 activity caused opposite effects. In wild-type animals at the middle-L3 stage, PVD dendrites can only grow up to the tertiary branch (Fig. 2A). However, in lin-14(n179) reduction-of-function (rf) mutants at the same developmental stage, the middle-L3 stage, PVD dendrites can grow up to the quaternary branch and complete the menorah structure (Fig. 2B), suggesting precocious dendrite outgrowth. In addition, the number of overlapped tertiary dendrites caused by excessive tertiary dendrite growth was significantly higher in lin-14(n179rf) mutants than in wild-type animals at the young adult stage (Fig. 2A, D, and G and SI Appendix, Fig. S2). Thus, a reduction of function in the lin-4 gene causes precocious and excessive dendritic outgrowth. The lin-14(n179rf) mutant phenotype of excessive PVD tertiary dendrite growth can be rescued by re-expressing the lin-4 gene in PVD neurons, suggesting that lin-14 acts cell-autonomously in PVD (Fig. 2G). To further strengthen the statement that the lin-4 microRNA targets the lin-14 transcription factor to initiate dendritic outgrowth in PVD neurons, we tested whether the lin-14(n179) mutation suppresses the lin-4(e912lf) mutant phenotype of delayed dendritic outgrowth. We found that this is indeed the case (Fig. 2F). lin-4(lf); lin-14(lf) double mutants displayed the same precocious dendritic outgrowth phenotype of the lin-14(n179) single mutant rather than delayed dendritic outgrowth phenotypes of the lin-4(lf) single mutant (Fig. 2F). Taken together, our results indicate that the lin-4-lin-14 regulatory circuit initiates dendritic outgrowth in PVD neurons.
The let-7-lin-41 Regulatory Circuit Slows Down Dendrite Growth in the Final Stage of Dendrite Arborization.

We previously reported that the let-7 microRNA represses the expression of the LIN-41 tripartite motif protein to inhibit AVM axon regeneration in older neurons (25, 33). Here, we further studied the expression of let-7 and lin-41 reporters and found an overlapping expression of the two genes in many other neurons, including PVD, at the L4 stage (Fig. 1C and SI Appendix, Fig. S1 D–F), when the growth of the terminal quaternary dendrites in PVD comes to an end. We performed laser dendritomy on the primary dendrite in PVD neurons at the L3, young adult, and early developmental stage, the L3 stage (Fig. 3 A–E), suggesting that let-7 mutations may retard a normal developmental decline in dendritic growth ability. In the young adult stage, while the let-7(n2853rf) mutation significantly enhanced, the lin-41(n2914lf) mutation significantly reduced dendritic growth ability in PVD neurons (Fig. 3F). In addition, lin-41 mutations suppressed the let-7(n2853rf) mutant phenotype of enhancing dendrite growth ability at the young adult stage, suggesting that the let-7 microRNA targets the LIN-41 tripartite motif protein to inhibit dendrite growth in PVD neurons (Fig. 3F). Together, these results indicate that the let-7-lin-41 regulatory circuit slows down dendrite growth in the final stage of dendrite arborization.

lin-28 Inhibits the let-7-lin-41 Circuit to Regulate the Timing of Dendrite Arborization. Previous studies have shown that the LIN-28 RNA-binding protein blocks the maturation of the let-7 microRNA in both invertebrates and vertebrates (38–41). We found that a lin-28::GFP fosmid-based reporter, which contains the lin-28 upstream cis-regulatory, exonic, intronic, and downstream cis-regulatory sequences, is expressed in PVD neurons (Fig. 3G). To determine whether lin-28 acts upstream of let-7 to regulate the timing of PVD dendrite arborization, we first compared the endogenous LIN-41 protein level in PVD neurons between

| L2 | late | L3 | early | mid | late | L4 | early | late |
|----|------|----|-------|-----|------|----|-------|------|

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**Fig. 1.** Expression of lin-4/lin-14 and let-7/lin-41 regulatory circuits in PVD neurons. (A) The timing and steps of PVD dendrite arborization. Schematic drawings of PVD dendrites at different stages of development, late L2 to late L4. All views showing the left-side cells of each PVD pair; anterior is to the left. A single axon (gray color) emerges ventralward from the cell body before traveling anteriorly along the ventral nerve cord. The dendrite processes of PVD, at the L4 stage (Fig. 1C), when the growth of the terminal quaternary dendrites in PVD comes to an end. We performed laser dendritomy on the primary dendrite in PVD neurons at the L3, young adult, and early developmental stage, the L3 stage (Fig. 3 A–E), suggesting that let-7 mutations may retard a normal developmental decline in dendritic growth ability. In the young adult stage, while the let-7(n2853rf) mutation significantly enhanced, the lin-41(n2914lf) mutation significantly reduced dendritic growth ability in PVD neurons (Fig. 3F). In addition, lin-41 mutations suppressed the let-7(n2853rf) mutant phenotype of enhancing dendrite growth ability at the young adult stage, suggesting that the let-7 microRNA targets the LIN-41 tripartite motif protein to inhibit dendrite growth in PVD neurons (Fig. 3F). Together, these results indicate that the let-7-lin-41 regulatory circuit slows down dendrite growth in the final stage of dendrite arborization.
the wild type and lin-28(n719lf) mutants. The mNG reporter gene was knocked in the endogenous lin-41 locus to generate a mNG::lin-41 fusion gene using the CRISPR-Cas9 technology (26). We found that the lin-28(n719lf) mutation significantly reduced LIN-41 protein levels in PVD neurons compared with the wild type at the early L3 stage (Fig. 3 H and I), suggesting that lin-28 inhibits the let-7-lin-41 circuit in PVD neurons. We performed laser dendritomy on PVD primary dendrites in lin-28(n719lf) mutants at the young adult stage and observed a significantly reduced dendrite growth ability in lin-28 (n719lf) mutants compared with wild-type animals at the same stage 24 h after surgery (Fig. 3 J), a phenotype that is opposite to the let-7(n2853rf) mutant phenotype of enhanced dendrite growth ability (Fig. 3 F). Furthermore, lin-41 overexpression in PVD neurons significantly suppressed the lin-28(n719lf) mutant phenotype of reduced dendrite growth ability (Fig. 3 J). Together, these results suggest that lin-28 inhibits the let-7-lin-41 circuit to regulate the timing of PVD dendrite arborization.

Recent studies in sexually dimorphic nervous system differentiation and male tail tip morphogenesis revealed that the lep-5 long noncoding RNA (lncRNA) promotes degradation of LIN-28 (42, 43). To determine whether lep-5 plays a role in regulating the lin-28-let-7-lin-41 regulatory circuit in PVD neurons, we first analyzed the endogenous LIN-41 protein level in PVD neurons in the wild type versus lep-5(ny28lf) mutants. We found that there is no difference of LIN-41 protein levels between the wild type and lep-5(ny28lf) mutants (SI Appendix, Fig. S3 A and B). In addition, lep-5(ny28lf) mutants displayed a similar extent of dendrite growth ability in wild-type animals at the young adult stage (SI Appendix, Fig. S3C). Thus, lep-5 is unlikely to regulate the timing of PVD dendrite arborization by modulating the lin-28-let-7-lin-41 circuit.

The lin-4 to let-7 Upstream Sequence Replacement Delays Dendrite Arborization. To further support our conclusion that lin-4 and let-7 microRNAs set start and end times for PVD dendrite arborization, we manipulated the timing of their expression by swapping their upstream sequences with each other. We found that the delayed expression of lin-4 (lin-4 microRNA expressed from the let-7 upstream sequence from the L3 stage onwards) postponed dendrite arborization in PVD neurons (SI Appendix, Fig. S4). The transgene that expressed the lin-4 microRNA from the let-7 upstream sequence (let-7up::lin-4) led to retarded growth in quaternary dendrites (the final order of PVD
Developmental decline in dendrite growth ability is affected by mutations in the lin-28-let-7-lin-41 regulatory circuit. (A–D) Representative images showing the extent of dendrite regrowth 24 h after dendritomy of the primary dendrite in PVD at either the L3 or the young adult stage (4 and B) and let-7(n2853rf) mutants (C and D). PVD dendrites were visualized using the xrs37[F43H12.4::GFP] marker. Dorsal is Up; anterior is to the left. Red arrows indicate lesion sites. Scale bar, 20 μm. (E) Average PVD dendrite length regrown in wild type and let-7(n2853rf) mutants 24 h following dendritomy at different stages. Asterisks indicate cases in which later-stage animals differ from L3-stage animals at ***P < 0.001 by a Student’s t test. ***P < 0.001 by one-way ANOVA with Tukey’s test. (F) Average PVD dendrite length regrown in various strains 24 h following dendritomy at the young adult stage. Asterisks indicate cases in which let-7 or lin-41 mutants differ from wild type at ***P < 0.001 by one-way ANOVA with Tukey’s test. NS, not significant. All animals including the let-7(n2853rf) allele were raised and analyzed at 20°C. let-7(n2853rf) mutants were quantified only in those that survived bursting vulva. (G) Representative images of LIN-28 protein expression in PVD neurons in the wild type at the early L3 stage. A lin-28-GFP fosmid-based reporter was analyzed. The Pser-2::mCherry reporter was used to label PVD neurons. Scale bar, 5 μm. (H) Representative images of endogenous LIN-41 protein expression in PVD neurons in the wild type and lin-28(n719lf) mutants at the early L3 stage. Scale bar, 5 μm. (I) Quantification of LIN-41 proteins based on the mNG::LIN-41 fluorescence intensity in the PVD cell body in the wild type and lin-28(n719lf) mutants. Error bars, SEM. ***P < 0.001 by a Student’s t test. (J) Average PVD dendrite length regrown in various strains 24 h following dendritomy at the young adult stage. Error bars, SEM. ***P < 0.001 and **P < 0.01 by one-way ANOVA with Tukey’s test.

dendrites) in lin-4If mutants at the L3 molt and the early L4 stage (SI Appendix, Fig. S4 A and B). However, the growth of quaternary dendrites in these transgenic animals (lin-4(e912lf); Ex[let-7up::lin-4]) was able to catch up later in the adult stage (SI Appendix, Fig. S4C). To further strengthen this conclusion, we utilized the CRISPR-Cas9 technology to replace the endogenous lin-4 upstream sequence at the lin-4 locus with a late-onset let-7 upstream sequence (Fig. 4 A). The repair templates that have been developed recently were used to facilitate the identification of the CRISPR recombinants (44, 45) lin-4(xr70) and lin-4(xr71) in which the endogenous lin-4 upstream sequence has been replaced by a let-7 upstream sequence. We used stem-loop and TaqMan real-time RT-PCR to globally survey the temporal expression of mature lin-4 microRNA during animal development. Expression of the lin-4 microRNA in the lin-4 to let-7 upstream sequence replacement CRISPR allele was indeed delayed compared with its expression in wild-type animals (Fig. 4 A and B and SI Appendix, Fig. S5A). In these CRISPR lines, lin-4(xr70) and lin-4(xr71), we observed retarded growth of the quaternary dendrites at the early L4 stage (green dots in Fig. 4E compared with Fig. 4C of the wild type; Fig. 4G), which was able to catch up later in the adult stage (green dots in Fig. 4F compared with Fig. 4D of the wild type; Fig. 4 H–J). These results demonstrate that by manipulating the timing of let-4 expression through the lin-4 to let-7 upstream sequence replacement we can delay dendrite arborization.

The let-7 to lin-4 Upstream Sequence Replacement Precociously Inhibits Dendrite Growth Ability. Conversely, the premature expression of let-7 (let-7 microRNA expressed from the lin-4 upstream sequence from the L1 stage onward) precociously inhibited dendritic growth ability in PVD neurons (Fig. 5 A–C). We utilized the CRISPR-Cas9 technology to generate the let-7(xr67) CRISPR line in which the endogenous let-7 upstream sequence at the let-7 locus has been replaced with an early-onset lin-4 upstream sequence (Fig. 5A). Stem-loop and TaqMan real-time RT-PCR analysis showed that the expression of mature let-7 microRNA in the let-7 to lin-4 upstream sequence replacement CRISPR allele was indeed delayed compared with its expression in wild-type animals (Fig. 5 B and C and SI Appendix, Fig. S5B). In these CRISPR lines, lin-4(xr70) and lin-4(xr71), we observed retarded growth of the quaternary dendrites at the early L4 stage (green dots in Fig. 5E compared with Fig. 4C of the wild type; Fig. 4G), which was able to catch up later in the adult stage (green dots in Fig. 5F compared with Fig. 4D of the wild type; Fig. 4 H–J). These results demonstrate that by manipulating the timing of let-4 expression through the lin-4 to let-7 upstream sequence replacement we can delay dendrite arborization.

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sequence replacement CRISPR line was indeed precocious compared with its expression in wild-type animals (Fig. 5 A and B and SI Appendix, Fig. S5B). In the let-7(xr67) CRISPR line, PVD dendrite growth ability at the L3 stage was significantly lower than that in wild-type animals at the same stage (L3) and similar to that in wild-type animals at an older stage (young adult) (Fig. 5C). PVD dendrites, both proximal and distal segments to the injured site, in let-7(xr67) animals degenerated 24 h following laser dendritomcy at the D1 A stage. Because of this, we were unable to determine the dendrite growth ability at the D1 A stage (Fig. 5C). Thus, by manipulating the timing of let-7 expression through the let-7 to lin-4 upstream sequence replacement, we can precociously inhibit dendrite growth ability. Taken together, these findings support a model in which the lin-4-lin-14 regulatory circuit sets the start time, whereas the let-7-lin-41 regulatory circuit sets the end time for PVD dendrite arborization (see Fig. 7F).

**lin-14 and lin-41 Antagonistically Regulate the DMA-1 Receptor Level on PVD Dendrites.** Two transmembrane ligands, namely, SAX-7 and MNR-1, work together with LECT-2, a secreted ligand, to instruct dendrite arborization in PVD neurons through interactions with the dendrite receptor DMA-1 (37, 46–49). SAX-7, MNR-1, LECT-2, and DMA-1 form a multiprotein receptor–ligand signaling complex that directs the growth of stereotyped dendritic branches. Of these four components, only DMA-1 functions cell-autonomously in PVD neurons. One way to control dendrite growth ability during the course of PVD dendrite arborization is through the regulation of responsiveness of dendrites to growth signals, which can be accomplished by adjusting the abundance of the receptor on dendrites (50). In addition to DMA-1, HPO-30 can function cell-autonomously in PVD neurons as a coreceptor to regulate dendrite arborization by forming a signaling complex with DMA-1 (51–53). We ruled out hpo-30 as a candidate target gene of the two microRNA regulatory circuits since the level of HPO-30 proteins on dendrites was not affected by lin-14 and lin-41 mutations, suggesting hpo-30 is not regulated by
lin-14 and lin-41 (SI Appendix, Fig. S6 A and B). In contrast, the expression of DMA-1 proteins on dendrites appeared to be regulated by lin-14 and lin-41. To analyze the endogenous level of DMA proteins on PVD dendrites, we knocked in a GFP reporter after the transmembrane domain of the endogenous dma-1 gene using the CRISPR-Cas9 technology. This knock-in strain does not show any noticeable defects in PVD dendrites, suggesting that the GFP knock-in retains dma-1 gene function. The endogenous DMA-1 protein level on PVD tertiary dendrites increased in the early stage of dendrite arborization, from the early L3 to early L4 stage, and decreased subsequently in the final stage of dendrite arborization, from the early L4 to the young adult stage, which correlates with a dynamic change in dendrite growth ability during dendrite arborization (Fig. 6A). This observation, combined with our results that two microRNA regulatory circuits set start and end times for PVD dendrite outgrowth through transcriptional up-regulation of the DMA-1 protein level on dendrites (Fig. 6B).

To determine whether the lin-4-lin-14 regulatory circuit controls the timing of PVD dendrite arborization through the regulation of the DMA-1 receptor level on PVD dendrites, we analyzed the level of DMA-1 proteins on tertiary dendrites in lin-14(n179rf) mutants versus wild-type animals. We found that the endogenous level of DMA-1 proteins based on the fluorescence intensity of DMA-1::GFP was significantly increased in lin-14(n179rf) mutants (Fig. 6 C–E), consistent with their higher dendrite growth ability (Fig. 2 D and G). This result suggests that lin-14 negatively regulates dma-1 expression in PVD neurons. To understand the mechanism by which lin-14 negatively regulates the DMA-1 level on PVD dendrites, we engineered a dma-1 transcriptional reporter by inserting the SL2 trans-splice site and the mNG reporter gene into the 3’ end of the endogenous dma-1 gene using CRISPR-Cas9 technology. With the insertion of this SL2 trans-splice site between dma-1 and mNG, even though they are cotranscribed, they can be translated separately, thus allowing us to read out the transcription of the dma-1 gene based on the intensity of the mNG reporter (54). An analysis of the SL2-based dma-1 transcriptional reporter in the PVD cell body at the early L3 stage showed that dma-1 transcription was also significantly increased in the lin-14(n179rf) mutants compared with wild-type animals (Fig. 6 F–H), suggesting that lin-14 negatively regulates dma-1 expression in PVD neurons through a transcriptional means. Since the lin-14 gene encodes a transcription factor, LIN-14 is likely to directly regulate dma-1 expression. Consistent with this prediction, the chromatin immunoprecipitation sequencing (ChIP-seq) analysis showed direct binding of LIN-14 transcription factors to immediate upstream and downstream regions of the dma-1 gene and its gene body (Fig. 6 I, Left). As expected, binding of LIN-14 was not detected around the lpo-30 gene (Fig. 6 I, Right). To further strengthen the relationship between the lin-4-lin-14 regulatory circuit and dma-1 in the context of dendrite growth ability, we analyzed dendrite outgrowth in dma-1(xr50lf); lin-14(n179rf) double mutants versus lin-14(n179rf) and dma-1(xr50lf) single mutants. The dma-1(xr50lf) allele, containing a c-to-t missense mutation that results in an L/F change at aa137, shows limited PVD dendrite outgrowth similar to the null allele. The dma-1(xr50lf) mutation significantly suppressed the excessive tertiary dendrite outgrowth caused by the lin-14(n179rf) mutation (Fig. 6 J). Taken together, these results indicate that the lin-4-lin-14 regulatory circuit promotes dendrite outgrowth through transcriptional up-regulation of the DMA-1 receptor level on dendrites (Fig. 7H).

To determine whether the lin-28-lin-7-lin-41 regulatory circuit also controls the timing of PVD dendrite arborization
Fig. 6. lin-14 negatively regulates DMA-1 protein levels on PVD dendrites through a transcriptional means. (A) Average fluorescence intensity of DMA-1::GFP fusion proteins on PVD tertiary dendrites at different stages. Error bars, SEM. ** P < 0.01 and *** P < 0.001 by one-way ANOVA with Tukey's test. (B) Model of temporal control of PVD dendrite growth ability through antagonistic regulation of dma-1 by two microRNA regulatory circuits. (C and D) Representative images showing the expression of endogenous DMA-1::GFP fusion proteins on PVD dendrites in the wild type (C) and the lin-14(n179rf) mutant (D) at the early L4 stage. Arrowheads point to tertiary dendrites. Dorsal is Up; anterior is to the Left. Scale bar, 20 μm. (E) Average fluorescence intensity of DMA-1::GFP fusion proteins on PVD tertiary dendrites in the wild type versus lin-14(n179rf) mutants at the early L4 stage. Error bars, SEM. *** P < 0.001 by a Student's t test. (F and G) Representative images showing expression of the SL2-based dma-1 transcriptional reporter in PVD neurons in the wild type (F) and the lin-14(n179rf) mutant (G) at the early L3 stage. Scale bar, 5 μm. (H) Average fluorescence intensity of the SL2-based dma-1 transcriptional reporter in the PVD soma in the wild type versus lin-14(n179rf) mutants at the early L3 stage. Error bars, SEM. *** P < 0.001 by a Student's t test. (I) LIN-14 ChIP-seq binding at the dma-1 gene (Left) and the hpo-30 gene (Right). Significant LIN-14 peaks shown in red lines below the ChIP-seq tracks were called by MACS2 (69). (J) Percentages of PVD neurons at the young adult stage with excessive or limited tertiary dendrite outgrowth in various strains. All strains in C to H and J were raised at 25 °C, which is a nonpermissive temperature for the lin-14(n179) allele.
through the regulation of the DMA-1 receptor level on PVD dendrites, we analyzed the level of DMA-1 proteins on quaternary dendrites in lin-41(n2914lf) mutants versus wild-type animals. The endogenous level of DMA-1 proteins was significantly reduced in lin-41(n2914lf) mutants (Fig. 7 A–C), consistent with their lower dendrite growth ability (Fig. 3 F). This result suggests that lin-41 positively regulates the DMA-1 protein level on PVD dendrites. To understand the mechanism by which lin-41 positively regulates dma-1 expression, we analyzed expression of the SL2-based dma-1 transcriptional reporter in the PVD cell body in lin-41(ma104rf) mutants versus the wild type at the early L3 stage. The lin-41(ma104rf) mutation did not affect dma-1 transcription, suggesting that lin-41 positively regulates the DMA-1 protein level on PVD dendrites through a posttranscriptional means (Fig. 7 D–F). Considering that the known role of LIN-41, either as an RNA-binding protein or a ubiquitin ligase, is to negatively regulate target gene expression (15, 22, 25, 55), LIN-41 most likely positively regulates the DMA-1 level through an indirect mechanism such as negative regulation of a dma-1 inhibitor (Fig. 7 H). To further strengthen the relationship between the lin-28-let-7-lin-41 regulatory circuit and dma-1 in the context of dendrite growth ability, we performed laser dendritomy on the PVD primary dendrite in lin-41(ma104rf) mutants with or without dma-1 overexpression in PVD neurons at the young adult stage. dma-1 overexpression in PVD neurons significantly suppressed the reduced dendrite growth ability caused by the lin-41(ma104rf) mutation (Fig. 7 G). This result indicates that the lin-28-let-7-lin-41 regulatory circuit inhibits dendrite growth ability through posttranscriptional down-regulation of the DMA-1 receptor level on dendrites (Fig. 7 H). Taken together, our findings support a model in which the lin-4-lin-14 and lin-28-let-7-lin-41 regulatory circuits control the timing of PVD dendrite arborization through antagonistic regulation of the DMA-1 receptor level on dendrites (Fig. 7 H). The LIN-14 transcription factor likely acts directly to transcriptionally repress dma-1 expression, whereas the LIN-4 tripartite motif protein likely acts indirectly to posttranscriptionally promote dma-1 expression in PVD neurons.

A candidate gene that can act between lin-41 and dma-1 in PVD neurons is the lin-29 zinc finger transcription factor as it is a known lin-41 target gene and is expressed in PVD nuclei (SI Appendix, Fig. S7 A and C). lin-29 has been reported to be negatively regulated by lin-41 in certain cellular contexts (15, 22, 25, 55). Although the LIN-29 transcription factor is

Fig. 7. lin-41 positively regulates DMA-1 protein levels on PVD dendrites through a posttranscriptional means. (A and B) Representative images showing the expression of endogenous dma-1::GFP fusion proteins on PVD dendrites in the wild type (A) and the lin-41(n2914lf) mutant (B) at the young adult stage. Arrowheads point to quaternary dendrites. Dorsal is Up; anterior is to the Left. Scale bar, 20 μm. (C) Average fluorescence intensity of DMA-1::GFP fusion proteins on PVD quaternary dendrites in the wild type versus lin-41(n2914lf) mutants at the young adult stage. Error bars, SEM. ***P < 0.001 by a Student’s t test. (D and E) Representative images showing expression of the SL2-based dma-1 transcriptional reporter in PVD neurons in the wild type (D) and the lin-41(ma104rf) mutant (E) at the early L3 stage. Scale bar, 5 μm. (F) Average fluorescence intensity of the SL2-based dma-1 transcriptional reporter in the PVD soma in the wild type versus lin-41(ma104rf) mutants at the early L3 stage. Error bars, SEM. NS, not significant by a Student’s t test. (G) Average PVD dendrite length regrown in various strains 24 h following dendritomy of the primary dendrite at the young adult stage. Error bars, SEM. ***P < 0.001 by one-way ANOVA with Tukey’s test. (H) Model of regulation of dendritic arborization by two timing circuits. Initially, the lin-4 microRNA down-regulates lin-14 to trigger dendrite arborization. Later, the let-7 microRNA down-regulates lin-41 to slow down dendrite growth in the final stage of dendrite arborization.

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expressed in PVD nuclei strongly from the L4 stage onwards (SI Appendix, Fig. S7A), the lin-29(n333lf) mutation did not affect dma-1 transcription (SI Appendix, Fig. S7B), suggesting that lin-29 is unlikely to regulate dma-1 expression in PVD neurons. To further assess whether lin-29 acts as a dma-1 inhibitor in PVD neurons (Fig. 7H), we examined the effect of lin-29 deficiency on PVD dendrite growth ability. Using the ZIF-1-mediated protein degradation system to deplete LIN-29 proteins specifically in PVD neurons (56, 57), we found that the PVD-specific LIN-29 knockdown did not affect PVD dendrite growth ability at the young adult stage (SI Appendix, Fig. S7 C and D), further supporting the notion that lin-29 is unlikely to act between lin-41 and dma-1 to control the timing of PVD dendrite arborization.

**Discussion**

In this report, we show that two microRNA regulatory circuits are used in timing dendritic arborization in postmitotic PVD neurons, restricting dendrite growth within a defined time frame. The precisely timed lin-4–lin-14 regulatory circuit sets off initial dendrite outgrowth until the L4 stage, at which the precisely timed lin-28–let-7–lin-41 regulatory circuit decelerates dendrite growth as terminal dendrite branches are reaching final targets (Fig. 7H). These two regulatory circuits control the timing of PVD dendrite arborization through opposed regulation of the DMA-1 receptor level on PVD dendrites (Fig. 7H).

The neuronal timers that restrain dendrite arborization within a specific time window during neural circuit formation are poorly understood and remain a mystery. In this study, we uncover two microRNA regulatory circuits that temporally control the choreography of dendrite arborization in PVD neurons. Our study illustrates how neuronal timers regulate the intrinsic potential of dendrite growth. From this point forward, our goal is to describe the required timing mechanisms in dendritic arborization at a resolution that ultimately will allow us to reconstitute the process. Further dissection of genetic networks that regulate the timing of lin-4 and let-7 expression and identification of the downstream target of the LIN-41 tripartite motif protein that regulates the DMA-1 receptor level on dendrites will be indispensable to achieving this goal.

Here, we show that the lin-4–lin-14 regulatory circuit initiates PVD dendrite arborization through up-regulation of the DMA-1 receptor level on dendrites. The LIN-14 transcription factor most likely binds to the cis-regulatory region of the *dma-1* gene to negatively regulate *dma-1* transcription (Fig. 6 F–I). We also report that the lin-28–let-7–lin-41 regulatory circuit slows down PVD dendrite arborization as it comes to an end through down-regulation of the DMA-1 receptor level on dendrites. The positive regulation of the DMA-1 level by LIN-41 is likely indirect since LIN-41 is known to negatively regulate its target genes (15, 22, 25, 55). The LIN-41 tripartite motif protein is proposed to negatively regulate a *dma-1* inhibitor to posttranscriptionally promote the DMA-1 receptor level on PVD dendrites (Fig. 7 D–F and H).

In this study, we show that lin-4(e912lf), lin-14(n355gf), and *dma-1*(n50lf) mutants display a similar phenotype of limited PVD dendrite outgrowth at the young adult stage (Fig. 2 A–C, E, and Fig. 6f). The frequency of the PVD secondary branches in *dma-1*(n50lf) mutants was 35% lower compared with that in the wild type but was similar to the extent of phenotypes observed in lin-4(e912lf) and lin-14(n355gf) mutants. This observation further supports our model in which the LIN-14 transcription factor represses the *dma-1* gene expression to control the timing of PVD dendrite arborization. Furthermore, even at the young adult stage, only subsets of PVD neurons in lin-4(e912lf) and lin-14(n355gf) mutants display a small number of tertiary branches in the area close to the cell body but rarely any in the distal area. This polarity in the tertiary dendrite outgrowth seems to be less noticeable in *dma-1*(n50lf) mutants. A possible explanation is that, in lin-4(e912lf) and lin-14(n355gf) mutants, fewer DMA-1 proteins are generated in the cell body so while some tertiary dendrite outgrowth can still be supported in the area proximal to the cell body, much less DMA-1 proteins are transported to the distal area to support tertiary dendrite outgrowth.

A previous study has reported that PVD neurons display progressive morphological changes as they age into adulthood, including dendrite hyperbranching, which is disorganized and distinct from stereotyped dendrite branching in early development (58). Dendrite hyperbranching in aged neurons is likely caused by disorganization of dendrite cytoskeletons and does not reflect dendrite growth ability. Indeed, consistent with our findings, Kravtsov et al. (58) have reported a lower dendrite growth ability during the regeneration of PVD neurons in older animals than younger animals.

In the let-7(xr67) CRISPR line, the let-7 microRNA expressed from the *lin-4* upstream sequence does not block PVD dendrite growth in early development. This could be due to many different reasons. For example, the *lin-4* upstream sequence::let-7 genomic configuration may not be able to support the expression and processing of the primary let-7 microRNA as efficiently as the *lin-4* upstream sequence::lin-4 genomic configuration supports the expression and processing of the primary *lin-4* microRNA. Alternatively, between the *lin-4–lin-14* and the *let-7–lin-41* regulatory circuits, the *lin-4–lin-14* regulatory circuit could impact the DMA-1 receptor level to a larger extent than the *let-7–lin-41* regulatory circuit.

It remains to be seen whether the timing of dendrite arborization by lin-4 and let-7 microRNA regulatory circuits can be extended beyond PVD neurons, especially knowing that these two timing microRNAs are expressed broadly in many neurons in *C. elegans* (SI Appendix, Fig. S1 A–F) (24–26). Since lin-7 and lin-4 microRNAs are evolutionarily conserved, it is possible that these microRNA regulatory circuits control the timing of dendrite arborization in other organisms as well. This possibility is supported by the findings that let-7 is expressed in the nervous system in various species, including *C. elegans*, *Drosophila*, mouse, and human (21, 25, 59, 60) and that miR-125, the lin-4 homolog, functions in the *Drosophila* and mouse nervous system (61, 62). Although the timing of dendrite arborization is less understood in vertebrates, untimely dendrite arborization would likely lead to neurodevelopmental and psychiatric disorders that are frequently associated with defective dendritic arbors (63).

In this study, we discover a role for the *lin-4–lin-14* and the *lin-28–let-7–lin-41* regulatory circuits in temporal control of PVD dendrite arborization. We do not rule out the possibility that these microRNA regulatory circuits may also be involved in other differentiation events in PVD neurons. Indeed, using a PVD neuronal marker to assess its differentiation state in *lin-14*(n179gf) mutants, we found that PVD neurons in *lin-14*(n179gf) mutants exhibit precocious differentiation (SI Appendix, Fig. S8), suggesting that the timing of many differentiation events in PVD may also be controlled by the *lin-4–lin-14* regulatory circuit.

**Materials and Methods**

**Strains and Plasmids.** Strains and plasmids used in this study are listed in SI Appendix, Tables S1 and S2.
Quantification of the DMA-1::GFP Intensity. DMA-1::GFP intensities on tertiary (or quaternary) dendrite branches were quantified using ImageJ software. DMA-1::GFP intensities on different tertiary (or quaternary) dendrite branches were randomly selected in each PVD neuron, and a single focal plane that contained a selected branch in focus was used for each measurement. Overlapping regions of tertiary dendrite branches in lin-14(n179r) mutants were avoided during the analysis. For each dendrite branch, a region of interest was selected using the Line Selection Tool, and the Mean Gray Value was measured using the Measure function, followed by background subtraction. Intensity values for five branches were averaged for each PVD neuron, and the mean signal intensity and the corresponding SEM were calculated for each genotype.

ChiP-seq and Analysis. The LIN-14::GFP ChiP-seq track for dmr-1 and hpo-30 were analyzed from the dataset in Sun and Hobert (66). The C-terminally GFP-tagged lin-14 (lin-14(cc2841):lin-14::gfp) strain was used for the ChiP-seq analysis. Around 600,000 L1 animals were collected and fixed with 2% formaldehyde for 15 min at room temperature. The ChiP-seq analysis was performed as previously described (66). The immunoprecipitated DNA was purified using Ampure XP beads (A63881) and used to generate a sequencing library using the Ovation Ultralow System V2 (Tecan) according to the manufacturer’s instructions. The libraries were sequenced on Illumina NextSeq 500 machines with 75-bp single-end reads. After an initial quality check, the reads were mapped to WS220 using BWA (67) and filtered using SAMtools (68). Peaks were called using MACS2 (69) and visualized using IGV. All peaks and differential binding sites were annotated and assigned to the nearest gene using ChiPsseeker (70). Detailed descriptions of the quality check and validation of the ChiP-seq data set were described in Sun and Hobert (66). Briefly, GFP ChiP-seq from the LIN-14::GFP strain showed appreciable enrichment over input as well as GFP ChiP-seq from N2 controls. Additionally, the ChiP-seq data matched well with the expression pattern and function of LIN-14 across development, as well as validated expression change of LIN-14 targets.

Statistical Analysis. Statistical analyses were carried out by Student’s t tests, two-proportion Z-tests, or one-way ANOVA with Tukey’s or Dunnett’s tests using GraphPad Prism 7.0 or the Primer of Biostatistics software.

Data, Materials, and Software Availability. All study data are included in the article and/or SI Appendix.

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CRISPR-Cas9 Genome Editing. We generated the lin-4(xr70), lin-4(xr71), let-7(xr67), let-7(xr68), dmr-1(xr246), and dmr-1(xr80) CRISPR alleles using the CRISPR-Cas9 genome editing technology. CRISPR recombinants were identified using a self-excisng drug selection cassette as previously described (44, 45). The strategies to build the lin-4(xr70), lin-4(xr71), let-7(xr67), and let-7(xr68) alleles are illustrated in the diagrams in Figs. 4A and 5A. In our upstream sequence swap experiments, we swapped between the 2.9-kb sequence upstream of let-7 premature-microRNA (pre-miRNA) and the 1.9-kb sequence upstream of lin-4 pre-miRNA. In the upstream sequence, it contains temporal information for both driving microRNA expression and processing primary microRNA. The dmr-1(xr246)[dmr-1::gfp] allele was built by inserting a GFP reporter gene after the transmembrane domain of the endogenous dmr-1 gene. This knock-in strain does not show any noticeable defects in PVD dendrites. The dmr-1(xr80)[dmr-1::sl2::mNG] transcriptional reporter allele was built by inserting the SL2 trans-splice site and the mNG reporter gene into the 3' end of the endogenous dmr-1 gene.

Laser Dendritomy. Animals were mounted on 2% agarose pads and anesthesized with 5 mM sodium azide, the lowest possible concentration to keep adult animals immobilized. Laser dendritomy was performed on PVD primary dendrites in worms at the young adult stage unless otherwise described. Either a cavity-dumped Ti:sapphire laser oscillator (Cascade Laser, KMLabs Inc.) (65) or a MicroPoint Laser Ablation System (Andor/Oxford Instruments) was used for dendritomy. The laser pulses were tightly focused onto targeted primary dendrites using a Plan Apo VC 100×, 1.4 numerical aperture oil-immersion objective on a Nikon ECLIPSE Ti microscope. Successful laser dendritomy was confirmed by visualizing the targeted area immediately after surgery. Worms were recovered within 10 min of sodium azide treatment and placed on fresh plates with bacterial food.

Quantification of Dendrite Lengths. PVD neurons in recovered worms were imaged 24 h after dendritomy, and the dendrite regrowth was quantified. Dendrite lengths were calculated as the actual contour length between the injury site and dendrite termini measured along the cylindrical surface of each worm by tracing dendrites through a 3-dimensional image stack.
