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Cell autonomous specification of temporal identity by *Caenorhabditis elegans* microRNA *lin-4*

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Abstract

MicroRNAs provide developing systems with substantial flexibility in posttranscriptional gene regulation. Despite advances made in understanding microRNA structure and function, the relationships between their site-of-synthesis and site-of-action ("autonomy" versus "non autonomy") remain an open question. Given the well-defined role of microRNA *lin-4* in a reproducible series of time-specific developmental switches, *lin-4* is an excellent candidate for understanding whether microRNAs and the resulting heterochronic regulatory pathway have the potential to act cell autonomously. By monitoring temporal development and reporter activity in animals where *lin-4* is modulated, we have demonstrated that *lin-4* acts cell autonomously to specify temporal identity. This work (i) provides an example of cell autonomy in microRNA functions, and (ii) reveals a cell autonomous component of temporal regulation in *C. elegans*.

Keywords

C. elegans; *lin-4*; Cell autonomy; Temporal regulation; MicroRNAs

Introduction

The proper development of a multicellular organism requires the timely differentiation of various cell types aided by environmental cues and cellular interactions. The nematode *Caenorhabditis elegans* goes through four larval stages, with the development of different tissues following a strict cell fate specification program that is reflected during the choreographed program (Sulston and Horvitz, 1977). Heterochronic mutations in *C. elegans* affect the temporal regulation of development, often resulting in retarded or precocious larval stage transitions (Ambros and Horvitz, 1984). *lin-4*, a 21-nucleotide microRNA, was first identified as a regulator of temporal development with a key role in the L1-to-L2 transition (Lee et al., 1993). A lack of *lin-4* causes the failure of some developmental transitions, resulting in a retarded phenotype where cells retain characteristics of an earlier cell fate (Ambros and Horvitz, 1984; Liu and Ambros, 1989). As such, the *lin-4* mutant lacks certain adult somatic features including the adult alae and vulva (Arasu et al., 1991; Chalfie et al., 1981; Feinbaum and Ambros, 1999; Lee et al., 1993; Wightman et al., 1993).

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The discovery of *lin-4* through genetic means provided an exemplary case of what has since been recognized as a major class of eukaryotic regulatory effectors (Lee et al., 1993). MicroRNAs, short regulatory 21–24 nucleotide RNAs that repress target mRNAs by binding to imperfect complementary sites, are important in diverse processes ranging from limb morphogenesis to hematopoiesis (Bushati and Cohen, 2007). Despite microRNAs having such significant roles, the exact details of how and where they function are still unclear. In fact, the majority of microRNAs still have unknown functions; in particular, overt phenotypes have been missing in knockouts of a large subset of *C. elegans* microRNAs (Alvarez-Saavedra and Horvitz, 2010; Miska et al., 2007). Studies reporting the expression profiles of microRNAs have shown that different microRNAs have different spatial and temporal expression patterns, suggesting that they may function in the specific tissues in which they are expressed (Lagos-Quintana et al., 2002; Martinez et al., 2008). Detection of microRNAs in microvesicles has also been recently reported, raising the possibility that microRNAs may be transferred from cell to cell (Valadi et al., 2007; Yuan et al., 2009). Determining the locale of microRNA action will be crucial in understanding how microRNAs exact their functions.

Given the broad role of *lin-4* in the developmental switch from L1 to L2 in *C. elegans*, it is of considerable interest that the *lin-4* promoter is active as transgene arrays in many different tissues at late L1 stage (Esquela-Kerscher et al., 2005). Considering that *C. elegans* development involves both cell autonomous and non-cell autonomous mechanisms, the activity of *lin-4* and the fundamental setting of temporal identity could be either localized or general. One model would be that *lin-4* acts in a central “timing headquarters” to cue a global transition into L2 after *lin-4* expression is initiated at late L1 stage. In this case, *lin-4* need not act in more than one cell, as a distinct signal could be sent out (RNA, protein, or small molecule) to coordinate developmental timing throughout the animal. It is likewise plausible that *lin-4* itself could act as a diffusible signal, broadcasting a decision made at a central regulatory site throughout the animal. Alternatively, *lin-4* might act independently to set cell fate in the cells of the numerous tissues that execute temporal-specific developmental processes. Each of these possibilities has some precedent in observations of gene silencing due to exogenously added double-stranded RNA (dsRNA). DsRNA-triggered silencing has been shown to spread throughout the animal following localized administration of externally-produced dsRNA while localized expression of dsRNA-producing transgenes has been shown to produce silencing effects that can in some (but not all) cases affect distant cells (Fire et al., 1998; Tabara et al., 1998; Timmons et al., 2003; Winston et al., 2002). Genetic and biochemical characterization of spreading in RNA interference (RNAi) has resulted in the identification of a specific uptake mechanism, including a dsRNA transmembrane channel (Feinberg and Hunter, 2003; Winston et al., 2002). Although a dedicated cell-to-cell transport mechanism for microRNAs has not been described, it is certainly possible that a related or distinct pathway mediates cellular spreading of microRNAs or microRNA precursors.

In this work, we monitor temporal development and reporter activity following tissue and cell specific *lin-4* expression and depletion of *lin-4* activity. The substantial tissue and cell autonomy observed in all assays is indicative of localized function by *lin-4* in setting developmental identity in *C. elegans*.

Materials and Methods

Plasmids and Strains Used

Plasmid	Description
pC1	*pha-1(+)* (Granato et al., 1994)

pHZ018	*lin-4(+)*
pHZ054	*pmyo-3::yfp::lin-14_3′UTR*
pHZ081	*pcol-10::lin-4::let-858_3′UTR*
pHZ082, pHZ146	*pREAR::lin-4::unc-54_3′UTR*
pHZ126	*phlh-8::lin-4::unc-54_3′UTR*
pHZ149	*pREAR::DsRed::unc-54_3′UTR*
pJKL464.1	*phlh-8::gfp::unc-54_3′UTR*
pPD102.07	*pREAR::gfp::unc-54_3′UTR*
pPD117.01	*pmec-7::gfp*
pPD133.47	*pmyo-3::cfp::lacZ::unc-54_3′UTR*
pPD162.21	*pmyo-3::dsRed::let-858_3′UTR*
pPD190.99	*pmyo-3::gfp::lin-14_3′UTR*
pPD191.37, pPD191.39	*pcol-10::gfp::lin-14_3′UTR*
pPD191.45	*pcol-10::gfp::unc-54_3′UTR*
pPD192.32	*pcol-10::gfp::let-858_3′UTR*
pRF4	*rol-6(su1006)* (Mello et al., 1991)

Strain	Description
PD4454	*ayIs2* IV (Harfe et al., 1998a)
PD5102	*pha-1(e2123ts)* III; *rde-1(ne300)* V
PD5325	*pha-1(e2123ts)* III; *rde-1(ne300)* V; *ccEx5325[pC1; pPD162.21; pPD190.99]*
PD7119	*lin-4(e912)/mC6* II
PD7120	*lin-4(e912)/mC6* II; *ayIs2* IV
PD7133	*lin-4(e912)/mC6* II; *pha-1(e2123ts)* III; *rde-1(ne300)* V; *ccEx5325[pC1; pPD162.21; pPD190.99]*
PD7143	*lin-4(e912)/mC6* II; *pha-1(e2123ts)* III; *rde-1(ne300)* V
PD7175	*lin-4(e912)/mC6* II; *pha-1(e2123ts)* III; *rde-1(ne300)* V; *ccEx7175[pC1; pPD133.47; pHZ054]*
PD8168	*ccEx8168[pRF4; pREAR::gfp]*

Microscopy

Animals were immobilized in 1.0 mM levamisole and mounted on 2% agar pads for imaging with a Nikon Eclipse E600 microscope. The following filter sets were used for fluorescence imaging: 96341M CY GFP (CFP), 96345M YEL GFP HQ (YFP), 96342M EN GFP HQ LP (GFP) and 96321M TRITC HQ (DsRed), with image intensities balanced for clarity.

Generation of reporter strains responsive to *lin-4*

lin-4 responsive reporter constructs used in this study each consist of a characterized promoter fused to *gfp* or *yfp* that has the *lin-14_3′UTR* region and flanking sequences appended to the 3′ end. In initial experiments, we observed some *lin-14_3′UTR*-independent background silencing of transgenes in later stage larvae and adults. This age-dependent “background” silencing is a common occurrence with *C. elegans* transgenes, and can be overcome by carrying out experiments in a genetic background in which RNAi is impaired but temporal regulation appears normal. Experiments with reporter constructs were therefore carried out in an *rde-1(ne300)* background.

PD5325 was generated by the co-injection of pC1 (50 ng/ul), *pmyo-3::DsRed::let-858_3′UTR* (pPD162.21, 20 ng/ul) and *pmyo-3::gfp::lin-14_3′UTR* (pPD190.99, 3 ng/ul) into PD5102 [*pha-1(e2123ts)* III*; rde-1(ne300)* V]. PD5325 animals express both DsRed and GFP in the bodywall muscles of larvae, with GFP (but not DsRed)

downregulated in adults by endogenous *lin-4*. This transgenic array, cc*Ex5325[pC1; pPD162.21; pPD190.99]*, was crossed into PD7143 [*lin-4(e912)/mC6* II; *pha-1(e2123ts)* III; *rde-1(ne300)* V] to generate PD7133. GFP is downregulated in PD7133 heterozygous adults [*lin-4(e912)/mC6*] whereas PD7133 *lin-4(e912)* homozygous adults retain GFP expression in the bodywall muscles.

PD7175 was generated by the co-injection of pC1 (45 ng/ul), *pmyo-3::cfp::lacZ::unc-54_3′UTR* (pPD133.47, 50 ng/ul) and *pmyo-3::yfp::lin-14_3′UTR* (pHZ054, 5 ng/ul) into PD7143. PD7175 animals express both CFP and YFP in the bodywall muscles of larvae, with CFP persisting through adult stages. YFP is downregulated in PD7175 heterozygous adults [*lin-4(e912)/mC6*] by endogenous *lin-4* whereas PD7175 *lin-4(e912)* homozygous adults retain YFP expression in the bodywall muscles.

Transgene-based tissue specific expression of *lin-4*

Posterior bodywall muscles—A posterior-acting enhancer segment combined with a truncated *myo-2* promoter (*pREAR*) was used to drive expression in the posterior bodywall muscles (Krause et al., 1994). PD8168 expresses GFP (*pREAR::gfp*, pPD102.07) in the pharynx and posterior bodywall muscles of the worm. The *gfp* sequence was replaced by *lin-4* pre-microRNA sequence to make pHZ082 and pHZ146 (*pREAR::lin-4*). pHZ146 also has an additional deletion in the *myo-2* promoter to remove sequences responsible for pharyngeal expression. A co-injection marker, pHZ149 (*pREAR::DsRed::unc-54_3′UTR*), was made by replacing *lin-4* pre-microRNA sequence in pHZ146 with *DsRed* sequence. pHZ082 (25 ng/ul) and pRF4 (75 ng/ul) were co-injected into PD7133 to express *lin-4* in the posterior bodywall muscles. PD7133 *lin-4(e912)* homozygous adult rollers carrying *Ex[pRF4; pHZ082]* from three transgenic lines (Lines 55.2.2.1, 55.2.2.2, 55.1.2.1) were assayed for the absence of GFP in these cells.

pHZ146 (17 ng/ul), pRF4 (42 ng/ul) and pHZ149 (42 ng/ul) were co-injected into PD7175 to express *lin-4* in the posterior bodywall muscles. Transgenic PD7175 worms were identified by their expression of DsRed in the posterior bodywall muscles. Transgenic PD7175 *lin-4(e912)* homozygous adults carrying *Ex[pRF4; pHZ146; pHZ149]* from three transgenic lines (Lines 158.2.1.1, 159.1.1.1 and 159.2.1.1) were assayed for absence of YFP in the posterior bodywall muscles.

Sex muscle precursors—PD4454 [*ayIs2* IV] expresses *pegl-15::gfp* in adult vm1 vulval muscles (Harfe et al., 1998a). PD4454 was crossed into PD7119 [*lin-4(e912)/mC6* II] to obtain PD7120 [*lin-4(e912)/mC6* II; *ayIs2* IV]. A *phlh-8::lin-4* (pHZ126) fusion was constructed by replacing *gfp* sequence in pJKL464.1 with *lin-4* pre-microRNA sequence. pHZ126 (25 ng/ul) and pRF4 (75 ng/ul) were co-injected into PD7120 to express *lin-4* in the sex muscle precursors. PD7120 *lin-4(e912)* homozygous adult rollers carrying *Ex[pRF4; pHZ126]* from three transgenic lines (Lines 124.3.1.1, 124.3.2.1, 124.3.4.1) were assayed for the presence of *pegl-15::gfp* and rescue of vulvaless phenotype.

Hypodermal cells—A *pcol-10::lin-4* (pHZ081) fusion was constructed by replacing *gfp* sequence in *pcol-10::gfp* (pPD192.32) with *lin-4* pre-microRNA sequence. pHZ081 (1 ng/ul) and pC1 (99 ng/ul) were co-injected into PD7143 to express *lin-4* in hypodermal tissue. PD7143 *lin-4(e912)* homozygous adults carrying *Ex[pC1; pHZ081]* from four transgenic lines (Lines 128.1.1.1, 128.1.1.2, 128.1.1.3, 128.1.1.4) were assayed for adult alae and vulva formation using differential interference contrast (DIC) microscopy.

To look at changes in *pegl-15::gfp* expression, pHZ081 (3 ng/ul) and *pmec-7::gfp* (pPD117.01, 97 ng/ul) were co-injected into PD7120. The *pmec-7::gfp* construct drives *gfp* expression in the touch receptors and serves as a co-injection marker (Hamelin et al., 1992).

Transgenic PD7120 *lin-4(e912)* homozygous F1 adults with *gfp*-expressing touch cells were assayed for adult alae and vulva formation. Only transgenic worms with restored alae formation or vulva formation were further assayed for *pegl-15::gfp* expression.

Tissue specific overexpression of *lin-14 3′UTR* "sponge" experiments

Lines 5195, 5203 and 5204 were made by co-injection of pC1 (50 ng/ul), pRF4 (25 ng/ul) and *pcol-10::gfp::lin-14_3′UTR* (pPD191.39, 5 ng/ul) into PD5102. Lines 5205 and 5206 were made by co-injection of pC1 (50 ng/ul), pRF4 (25 ng/ul) and *pcol-10::gfp::unc-54_3′UTR* (pPD191.45, 5 ng/ul) into PD5102. Transgenic adult rollers from each of these lines (Lines 5159, 5203, 5204, 5205, 5206) were assayed for GFP expression in the seam cells. Only transgenic adult rollers with *gfp*-expressing seam cells were further assayed for adult alae formation using DIC microscopy.

pRF4 (95 ng/ul) and pPD191.37 (*pcol-10::gfp::lin-14_3′UTR*, 5 ng/ul) were co-injected into PD4454. PD4454 adult rollers carrying *Ex[pRF4; pPD191.37]* from five lines (Lines 143.1.5.1, 144.1.1.1, 144.1.6.1, 144.2.5.1, 139.1.8.1) were assayed for presence of GFP in vm1 vulval muscles and adult alae formation.

Adult rollers from Lines 5195, 139.1.8.1 and 144.1.1.1 with mosaic character were identified by presence of broken adult alae using DIC microscopy. These animals were then assayed for a correlation between *gfp*-expressing seam cells and adult alae formation.

lin-4(+) (pHZ018, 67 ng/ul) and pPD117.01 (267 ng/ul) were co-injected into adult rollers from Lines 139.1.8.1 and 144.1.1.1. Uninjected adult rollers (*Ex[pRF4; pcol-10::gfp::lin-14_3′UTR]*) and transgenic adult rollers (*Ex[pRF4; pcol-10::gfp::lin-14_3′UTR]; Ex[lin-4(+); pPD117.01])* with *gfp*-expressing touch cells were assayed for adult alae formation.

Results

Cell autonomy of *lin-4* in posterior bodywall muscles

The combination of *lin-4* gene activity in the bodywall musculature, and our ability (using defined muscle-gene promoter elements) to engineer expression of RNAs in a subset of body muscles yields opportunities to examine autonomy of *lin-4* repression of a target construct in that tissue (Esquela-Kerscher et al., 2005; Krause et al., 1994). We first established strains that expressed reporters that are responsive to *lin-4* regulation in the bodywall muscles. This required a defined bodywall muscle promoter (*myo-3*; Okkema et al., 1993), a readily assayed reporter construct (*gfp*; Chalfie et al., 1994), a 3′UTR regulated by *lin-4* (*lin-14*; Lee et al., 1993; Wightman et al., 1993), and conditions for introducing the DNA that allow regulation of the transgene. We found that introduction of the *pmyo-3::gfp::lin-14_3′UTR* construct at low molar concentration (with an excess of the selectable marker *pha-1* [*pha-1(+)*] (Granato et al., 1994)) produced transgenic lines for which expression is extinguished in later larval stages and adults. The loss of expression corresponds with the endogenous expression and role of *lin-4. lin-4* expression is first detected in L1 larvae, with expression increased through the subsequent developmental stages and maintained at high levels in adults (Feinbaum and Ambros, 1999). This *lin-14_3′UTR* GFP reporter, as well as selectable marker *pha-1* [*pha-1(+)*] and *pmyo-3::DsRed::let-858_3′UTR* (Matz et al., 1999), were co-injected to generate a transgenic line (PD5325) that expressed GFP at early but not late larval stages, with DsRed activity persisting through adult stages (Fig. 1A). DsRed expression in the bodywall muscles serves as a control for the expression of the extrachromosomal array in these cells. In order to verify that the decreased GFP expression in PD5325 is due to *lin-4* regulation, the extrachromosomal array was crossed into a balanced strain with the *lin-4(e912)* mutation.

The resulting transgenic *lin-4(e912)* homozygotes (PD7133) maintain high GFP expression even as adults (Fig. 1A), whereas the PD7133 heterozygotes have a similar GFP expression profile as the PD5325 animals. The maintained GFP expression in the *lin-4(e912)* mutants indicates that the GFP reporter is responsive to *lin-4*.

To test cell autonomy of *lin-4* target repression in bodywall muscles, we expressed *lin-4* in a subset of bodywall muscles of the reporter strain, PD7133. We made use of a posterior-acting hybrid promoter "*pREAR*" derived from 12 copies of a sequence (−2745 to −2761 from ATG; Krause et al., 1994) upstream of the *hlh-1* driving a minimal *myo-2* promoter. The *pREAR* promoter provides a highly specific pattern consisting of the posterior bodywall muscles (two in each quadrant). The activity of this hybrid promoter is illustrated in Supplemental Fig. 1, where GFP expression is driven in the posterior bodywall muscles. A deriviative construct was produced by replacing *gfp* with the *lin-4* pre-microRNA sequence. The *pREAR::lin-4* construct was co-injected with pRF4 (*rol-6(su1006)*, which produces a distinctive rolling phenotype) into the *lin-14_3′UTR* reporter strain PD7133, and GFP expression of *lin-4(e912)* homozygous adults carrying both transgenes were examined (Table 1A). 63% of injected transgenic animals showed GFP downregulation in the posterior bodywall muscles (Fig. 1B). Other (more anterior) bodywall muscles maintained high GFP expression. This suggests that *lin-4* is able to downregulate a target construct (*gfp*) in the bodywall muscles in a cell autonomous manner.

As a complementary approach to examining localized *lin-4* effects in muscle, we generated a transgenic strain (PD7175) that expresses a distinguishable pair of fluorophore reporters (CFP and YFP) in bodywall muscles. In this strain, the YFP reporter (Miyawaki et al., 1997) is responsive to *lin-4* regulation and the CFP reporter (Heim et al., 1994) acts as a control for the expression of the extrachromosomal array. For better visualization of the bodywall muscles, both control CFP and *lin-4* responsive YFP were nuclear localized, allowing downregulation of the *lin-14_3′ UTR* construct (YFP) to be readily visualized in adults (Fig. 1C, Supplemental Fig. 2A). No YFP downregulation was observed in animals lacking *lin-4* activity (Fig. 1C, Supplemental Fig. 2B). We produced *lin-4* in a posterior bodywall muscles-specific manner in such animals, using the expression system above. This intervention resulted in a highly cell-specific suppression of reporter expression in a pattern corresponding to expression of the *lin-4* effector (Table 1B, Fig. 1D). Combined with the results from paired GFP/DsRED expression, these results suggest an ability of *lin-4* to modulate target activity in a cell specific manner within the bodywall musculature.

Tissue autonomy of *lin-4* in lineages generating vulval muscles

We examined the specification of an additional lineally-related group of muscle cells (the vulval musculature) as a further test for autonomous activity of *lin-4*. Animals lacking *lin-4* activity show a spectrum of specific defects in the lineages that produce the vulval musculature (Ambros and Horvitz, 1984; Chalfie et al., 1981). The availability of integrated GFP reporter strains to follow the development of sex muscles and characterized promoters for driving expression in these cells provided a functional developmental assay for testing tissue autonomy of *lin-4*. *pegl-15::gfp* provides an excellent marker for the vm1 vulval musculature in these experiments, with these cells being the major site of expression in late larvae and adults (Harfe et al., 1998a). PD4454 animals expressing *pegl-15::gfp* in adult vm1 vulval muscles were crossed into the *lin-4(e912)* background to determine if the absence of *lin-4* would cause an observable change in *pegl-15::gfp* expression pattern. In otherwise-wildtype *pegl-15::gfp* adults, GFP is observed in the four vm1 vulval muscles (Fig. 2A; Harfe et al., 1998a). By contrast, GFP is mostly absent in the PD7120 [*lin-4(e912)* II; *pegl-15::gfp* IV] adults (Fig. 2B). When GFP is detected in the *lin-4(e912)* animals, expression is usually very faint and limited to one or two cells. Thus, *lin-4* activity can be

functionally assayed during vulval muscle development through examination of *pegl-15::gfp* expression pattern.

Using the absence of *pegl-15::gfp* expression in *lin-4(e912)* mutants as a functional assay for lin-4 activity, we wanted to test if restoring *lin-4* expression in the lineage producing vulval muscles would restore specification of these cells. Vulval muscles are derived from undifferentiated sex myoblasts produced postembryonically by the M lineage (Sulston and Horvitz, 1977). Specific expression in the M lineage can be engineered using the promoter for the *C. elegans Twist* homolog *hlh-8* (Harfe et al., 1998b). A *phlh-8::lin-4* fusion was constructed to express *lin-4* in undifferentiated sex muscles. Early *lin-4* expression in the undifferentiated precursor cells was desired since the exact temporal requirements for *lin-4* in these cells are unclear. The *phlh-8::lin-4* construct was co-injected with pRF4 into PD7120 [*lin-4(e912)/mC6* II; *pegl-15::gfp* IV] animals, and transgenic *lin-4(e912)* adult rollers were examined for *pegl-15::gfp* expression and vulva formation. As shown in Table 2, a high percentage of transgenic animals have detectable GFP compared to uninjected control animals. GFP expression in the *phlh-8::lin-4* rescued animals was also observed in four cells, similar to patterns for this reporter in wildtype animals (Fig. 2C). Despite the restoration of *pegl-15::gfp* expression in the musculature normally associated with a functioning vulva, the transgenic animals continued to lack the ectodermally-derived specializations that form vulval hypodermis (Table 2). These results show that specific *lin-4* expression in sex muscle precursors was sufficient to restore *pegl-15::gfp* expression but was insufficient to restore proper vulva formation. This suggests that *lin-4* can promote specific aspects of the adult developmental pattern if expressed in the lineally-corresponding groups of precursor cells.

Tissue autonomy of *lin-4* in hypodermal tissues

Much of the characterized phenotypic role of *lin-4* relates to patterning of the hypodermal tissues that make up the surface of animals (Ambros, 1989; Liu et al., 1995). We used a specific cuticle patterning mark (the adult alae) to initially assess autonomy of *lin-4* in hypodermal tissues. Adult alae comprise of three protruding ridges that form longitudinally across the lateral margins of the animal, in an area where cuticular components have been secreted by mature hypodermal seam cells (Cox et al., 1981). A lack of adult alae in *lin-4(e912)* mutants indicates that *lin-4* is required for this aspect of hypodermal patterning (Chalfie et al., 1981). Expression of *plin-4::gfp* in the hypodermal tissues is consistent with the possibility that *lin-4* expression may be required in these cells (Esquela-Kerscher et al., 2005). Using a *col-10* promoter that drives expression in the hypodermal cells, a *pcol-10::lin-4* fusion was constructed to express *lin-4* in the hypodermal cells (Cox and Hirsh, 1985). This DNA was co-injected with *pha-1(+)* into PD7143 [*lin-4(e912)/mC6* II; *pha-1(e2123ts)* III; *rde-1(ne300)* V] and *lin-4(e912)* mutants with the transgene were analyzed for presence of adult alae and vulva formation. As shown in Table 3A, the majority of transgenic animals had restored adult alae formation (Fig. 3A). Although the populations of completely vulvaless animals were substantially reduced (Table 3A), a fully functional vulva was rarely formed in the rescued animals. Instead, we frequently observed a protruding pseudovulva or bursting vulva that prevents successful egg-laying (Fig. 3B). Since both hypodermal and gonadal cell types contribute towards vulva formation, it is not surprising that full rescue of the vulvaless phenotype was not observed (Kimble, 1981).

To test whether hypodermal expression of *lin-4* also rescues the defect in *pegl-15::gfp* expression pattern, the *pcol-10::lin-4* construct was injected into the *pegl-15::gfp* strain PD7120 (a *pmec-7::gfp* construct was used to identify transformed animals). Compared to uninjected controls, the transgenic *lin-4(e912)* animals did not restore vulval muscle *pegl-15::gfp* expression (Table 3B). We confirmed in these animals that the *pcol-10::lin-4* construct was active, as only transgenic animals with either restored adult alae formation or

partial vulva formation were considered. These results suggest that *lin-4* acts tissue autonomously in hypodermal tissue.

Mosaic expression of *lin-14_3′UTR* "sponge" in seam cells corresponds with cell autonomous loss of adult alae

Titration of functional microRNA by "sponging" of a complementary sequence has been shown in several systems to produce a specific loss of microRNA activity (Ebert et al., 2007; Hutvagner et al., 2004; Loya et al., 2009). To evaluate such an approach for *C. elegans*, we first overexpressed a target downregulated by *lin-4* in the hypodermal tissues of wildtype animals (*pcol-10::gfp::lin-14_3′UTR)* to test whether tissue specific overexpression of a *lin-4* target might result in a tissue specific *lin-4* deficient phenotype (lack of adult alae). In cases where target GFP was expressed at a sufficiently high level in the hypodermal tissues (and thus GFP appeared unregulated with loss of *lin-4* activity), we might expect to observe a lack of adult alae in transgenic worms that express GFP. Conversely, transgenic worms that have lost GFP expression (due to either loss or downregulation of the extrachromosomal array) might be expected to have normal adult alae formation.

Adult transgenic worms produced with high concentrations of the *pcol-10::gfp::lin-14_3′UTR* plasmid were examined for adult alae formation. As shown in Supplemental Table 1A, more than half of these animals had no adult alae, consistent with loss or reduction of *lin-4* activity. In contrast, all uninjected animals formed adult alae. As a control to ensure that the absence of adult alae was due to the *lin-14_3′UTR*, a *pcol-10::gfp::unc-54_3′UTR* construct was overexpressed in wildtype animals. The *unc-54_3′UTR* is not known to contain any *lin-4* binding sites, hence *pcol-10::gfp::unc-54_3′UTR* should not reduce endogenous *lin-4* activity in wildtype animals. Indeed, adult alae are present in transgenic animals with *gfp*-expressing hypodermal tissues (Supplemental Table 1A and Supplemental Fig. 3A). These results indicate that the overexpressed *lin-14_3′UTR* is responsible for the lack of adult alae.

It was also critical to test whether the absence of adult alae after *lin-14_3′UTR* hypodermal overexpression in the transgenic animals was due to titration of *lin-4* activity. To specifically test this, we expressed *lin-4* in these animals. If adult alae formation could be restored with *lin-4* expression in these animals, this would suggest that loss of adult alae was due to loss of *lin-4* activity. As shown in Supplemental Table 1B, adult alae formation was restored in transgenic animals expressing *lin-4*, consistent with a loss of adult alae in animals overexpressing *lin-14_3′UTR* as a result of reduction in *lin-4* activity.

To determine whether the effects of *pcol-10::gfp::lin-14_3′UTR* overexpression was tissue specific, *pcol-10::gfp::lin-14_3′UTR* was also overexpressed in *pegl-15::gfp* strain PD4454. Transgenic animals with no adult alae, an indication of reduction in *lin-4* activity in the hypodermal tissues, were assayed for *pegl-15::gfp* expression. The hypodermal reduction in *lin-4* activity by *pcol-10::gfp::lin-14_3′UTR* did not affect wildtype *pegl-15::gfp* expression pattern (Supplemental Table 1C, Supplemental Fig. 3B), suggesting that the effects of *lin-14_3′UTR* hypodermal overexpression (loss of *lin-4* activity) are tissue specific.

To examine the ability of the overexpressed 3′UTR to act in individual cells, we first looked for transgenic animals carrying *pcol-10::gfp::lin-14_3′UTR* that had non-continuous adult alae. Non-continuous alae indicate that some of the hypodermal seam cells were able to terminally differentiate and form adult alae. Hence, broken adult alae are observed when there is mosaicism in cell fates among the hypodermal seam cells. If *lin-4* acts in a cell autonomous manner, we would expect to see an inverse correlation between *gfp*-expressing seam cells (loss of *lin-4* activity) and presence of adult alae (wildtype *lin-4* activity) in these

mosaic animals. As shown in Table 4, 87% of *gfp*-expressing seam cells in visibly mosaic animals show a coincidental localized loss of adult alae. An example in which a transgene-expressing seam cell is flanked by non-expressing seam cells is shown in Fig. 4; adult alae were absent above the middle *gfp*-expressing seam cell, whereas adult alae were formed above the surrounding non-*gfp*-expressing seam cells. Additional examples of such autonomy are pictured in Supplemental Fig. 3C. These results indicate strong cell autonomy in the activities of the overexpressed *lin-14_3′UTR*.

Discussion

The goal of this study was to test whether a global temporal regulator, microRNA *lin-4*, can exert a cell autonomous function in *C. elegans* development. In addition to the importance in understanding how developmental timing in *C. elegans* is regulated, this question has substantial implications in the manner in which microRNAs function. Although previous studies have defined numerous distinct expression patterns for microRNAs, additional observations (e.g. of potential microRNAs transport in microvesicles) have suggested the possibility for microRNAs to act directly in broad sets of cells that extend beyond the sites of synthesis (Lagos-Quintana et al., 2002; Martinez et al., 2008; Valadi et al., 2007; Yuan et al., 2009). Considering *lin-4*′s requirement in diverse developmental transitions of various tissues, this microRNA provides an excellent assay for microRNA autonomy, as well as “autonomous” versus “global” coordination of developmental timing decisions.

As an initial assay for autonomy, we generated reporters expressed in the bodywall muscles of *C. elegans* that can be downregulated by *lin-4*. Following expression of *lin-4* in the posterior bodywall muscles of *lin-4(e912)* mutants carrying the *lin-4* responsive reporter, we observed that the reporter was selectively downregulated in these cells. Downregulation was not observed in neighboring (more anterior) bodywall muscles and global *lin-4(e912)* phenotypes were not rescued. We then extended the analysis of autonomy to a number of tissues where specific *lin-4* phenotypes were observed at a developmental level. The hypodermal seam cells fail to execute the adult program in *lin-4(e912)* mutants and an adult cuticle pattern is not observed (Chalfie et al., 1981).

Similarly, the sex muscle precursors in these mutants do not specify their final cell fates as evident by the lack of *pegl-15::gfp* expression, a marker for vm1 vulval muscles. In experiments where we specifically express *lin-4* in the hypodermal tissues or sex muscle precursors in *lin-4(e912)* mutants, only the specific phenotypes associated with these tissues were rescued; *lin-4(e912)* defects associated with other tissues were not rescued. The successful transition to adult fate of the specific tissues expressing *lin-4*, regardless of the lack of proper development in the surrounding tissues, suggests that the decision for development to proceed can be made independently in numerous distinct developing tissues during key ontological transitions.

We used a complementary strategy, employing a microRNA sponge (Ebert et al., 2007; Hutvagner et al., 2004; Loya et al., 2009) to examine the effects of isolated loss of *lin-4* function. With hypodermal overexpression of *lin-14_3′UTR* (containing known *lin-4* binding sites) in wildtype animals, tissue autonomy was evident from the loss of adult alae (but not of underlying mesodermal pattern) in animals with extensive expression of the sponge. In cases where expression of *lin-14_3′UTR* was highly mosaic, precise cell autonomy of *lin-14_3′UTR* (as well as *lin-4* activity) was demonstrated by the ability of a single isolated sponge-expressing cell to show a characteristic *lin-4* defective phenotype. Taken together, the results strongly suggest a cell autonomous role in specification of temporal identity during development.

From our study, *lin-4* appears to act cell autonomously in the various tissues and cells that we have assayed. It is possible that *lin-4* may act non-cell autonomously to regulate developmental timing in other groups of cells that were not tested here. Even if this was true, the cell autonomous activity of *lin-4* in temporal specification demonstrates that *lin-4* does not coordinate developmental transitions of *C. elegans* in an anatomically-centralized fashion. Although *lin-4* appears to act cell autonomously, there could be upstream regulatory machinery in the heterochronic pathway that might act from a single focal center. Indeed, the remarkable ability of *C. elegans* grown under diverse conditions (e.g. different temperatures and dauer formation) to carry out developmental events in concerted groups is consistent with a cellular or tissue "center" of temporal identity that considers both environmental and inherent genetic inputs (Butcher et al., 2007; Hirsh et al., 1976; Klass and Hirsh, 1976).

It is of considerable interest to compare siRNAs and microRNAs for their ability to act outside of the original cells that synthesized them. At first consideration, the processes may appear to behave in opposite ways, with external RNA capable of generating a well-known systemic response while our results in this study failed to detect systemic spreading of *lin-4* effects (Fire et al., 1998). Systemic responses to RNAi, however, are more effective for externally-provided dsRNA triggers delivered initially to an extracellular or shared cellular space (Timmons et al., 2003). Sharing of RNAi responses between cells requires an additional export step that is evidently dependent on additional machinery and cues (Jose et al., 2009; Timmons et al., 2003). By analogy with systemic siRNA effects dependent on regulated aspects of the transport machinery, it is conceivable that dedicated mechanisms to allow spreading of microRNAs from sites of synthesis might exist and be inducible in specific biological processes. Conversely, a difference in the "autonomy" of siRNA and microRNA functions could conceivably be one way in which the two mechanisms have evolved to distinguish their biological roles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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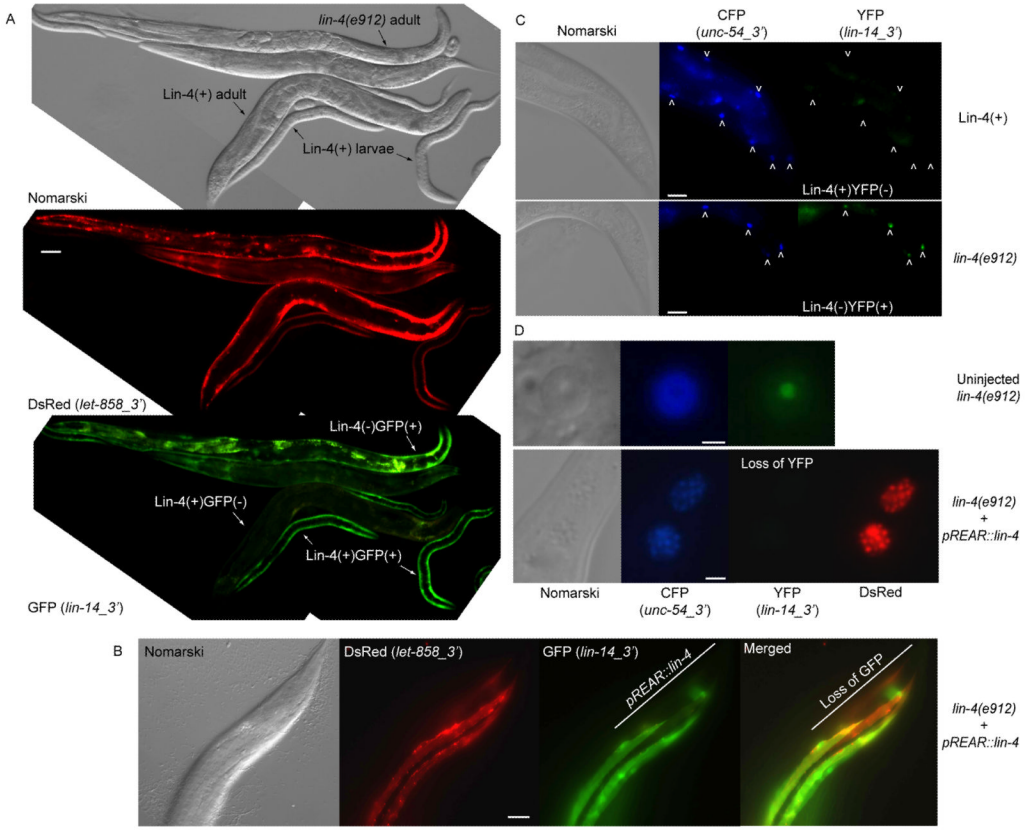

Fig. 1. Cell autonomy of *lin-4* in posterior bodywall muscles

Strains expressing fluorescent reporters that are responsive to *lin-4* regulation in the bodywall muscles are shown. (A) PD5325 and PD7133 [*lin-4(e912)/mC6* II; *ccEx5325*] animals express DsRed (*let-858_3′UTR*) and *lin-4* responsive GFP (*lin-14_3′UTR*) in the bodywall muscles. GFP is downregulated by *lin-4* in PD5325 adults (Lin-4(+) adult). GFP is not downregulated in PD5325 young larvae (Lin-4(+) larvae) and PD7133 *lin-4(e912)* adults (Lin-4(−) adult) where *lin-4* is either lowly expressed or absent. Scale bar represents 50 uM. (B) Expression of *pREAR::lin-4* in *lin-4(e912) ccEx5325* animals causes GFP downregulation in the posterior bodywall muscles, but not in the more anterior bodywall muscles. Images are showing the posterior end of the animal. Scale bar represents 20 uM. (C) PD7175 animals express nuclear localized CFP (*unc-54_3′UTR*) and *lin-4* responsive YFP (*lin-14_3′UTR*) in the bodywall muscles. YFP is downregulated by endogenous *lin-4* in PD7175 *lin-4(e912)/mC6* adults (top row) but not PD7175 *lin-4(e912)* mutants (bottom row). CFP is expressed in both PD7175 *lin-4(e912)/mC6* and *lin-4(e912)* animals. The posterior end of the animal is shown in these images and arrowheads point to nuclei of bodywall muscles. Scale bars represent 50 uM. (D) The bodywall muscle nuclei at the posterior end of the animal are shown in these images. In uninjected control *lin-4(e912) ccEx7175* adults (top row), both CFP and YFP are expressed. In contrast, YFP is downregulated by *lin-4* in posterior bodywall muscles of *lin-4(e912) ccEx7175* animals expressing *pREAR::lin-4* (bottom row). For this experiment, a co-injection marker (*pREAR::DsRed*) with identical expression signals to the *lin-4* expression construct was used. This marker was a direct indicator for the presence and activity of the extrachromosomal array in the relevant cells. Scale bars represent 2 uM.

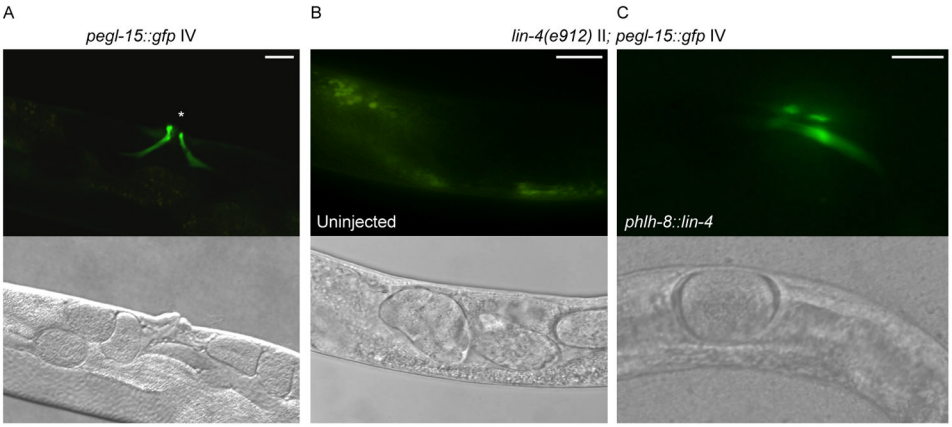

Fig. 2. Tissue autonomy of *lin-4* in lineages generating vulval muscles

Scale bars represent 25 uM. (A) *pegl-15::gfp* is a marker for the vm1 vulval musculature and is expressed in the four vm1 muscles of PD4454 [*ayIs2* IV] adults. Only two *gfp*-expressing cells were visible in this focal plane. The asterisk denotes the vulva. (B) *pegl-15::gfp* expression is absent in [*lin-4(e912)* II; *ayIs2* IV] adults that carry the integrated *pegl-15::gfp* marker. These animals are also vulvaless. (C) The vulval muscles are derived from undifferentiated sex muscles and the *hlh-8* promoter drives expression in these precursor cells. Expression of *phlh-8::lin-4* in [*lin-4(e912)* II; *ayIs2* IV] mutants restores *pegl-15::gfp* expression pattern but not vulva formation. Four *gfp*-expressing cells were observed in this animal.

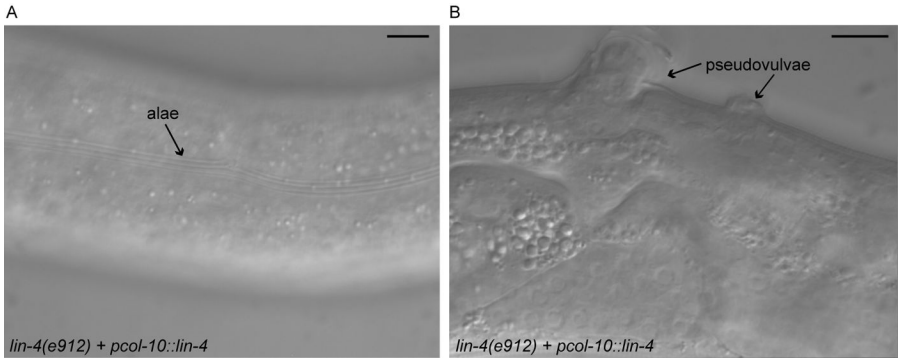

Fig. 3. Tissue autonomy of *lin-4* in hypodermal tissues

Scale bars represent 20 uM. (A) The *col-10* promoter drives hypodermal expression. Expression of *pcol-10::lin-4* restores adult alae formation in transgenic *lin-4(e912)* adults. (B) Vulva formation is also partially restored.

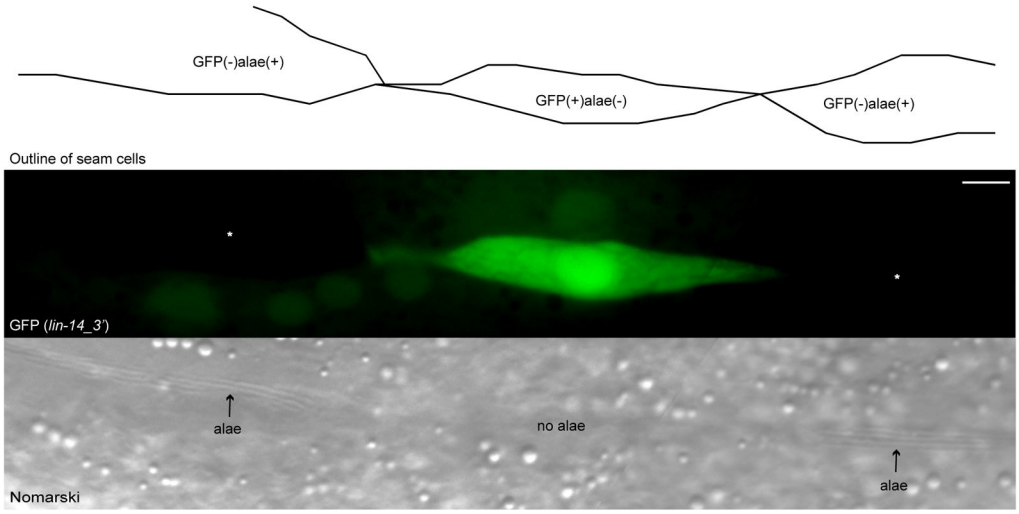

Fig. 4. Mosaic hypodermal expression of *lin-14_3′UTR* “sponge” corresponds with cell autonomous loss of adult alae

A *lin-14_3′UTR* “sponge”, *pcol-10::gfp::lin- 14_3′UTR*, was expressed in the hypodermal tissues of wildtype animals to titrate endogenous *lin-4* activity. Overexpression of “sponge” results in an absence of adult alae, which is a characteristic loss-of-*lin-4* phenotype in the hypodermal tissues. In transgenic animals showing mosaicism in cell fates of seam cells (determined by presence of broken alae), expression of *pcol-10::gfp::lin-14_3′UTR* correlates inversely with adult alae formation. An example is shown here. There is no adult alae formation above the *gfp*- expressing seam cell in the center, whereas adult alae are formed above the flanking non- *gfp*-expressing seam cells. Asterisks denote neighbouring seam cells. Scale bar represents 5 uM.

Table 1

Cell autonomous downregulation of *lin-14_3′UTR* fluorescent reporters by expression of *lin-4* in posterior bodywall muscles.

Expression construct	n (lines)	% showing GFP downregulation
Uninjected	25	0
pREAR::lin-4		
Line 55.2.2.1	18	72
Line 55.2.2.2	19	74
Line 55.1.2.1	20	45
Total	57 (3)	63

(A) Posterior bodywall muscle expression of *lin-4* downregulates a *lin-14_3′UTR* GFP reporter construct in a cell specific manner.

Expression construct	n (lines)	% showing YFP downregulation
Uninjected	25	8
pREAR::lin-4		
Line 158.2.1.1	49	74
Line 159.1.1.1	29	93
Line 159.2.1.1	21	90
Total	99 (3)	83

(B) Posterior bodywall muscle expression of *lin-4* downregulates a *lin-14_3′UTR* YFP reporter construct in a cell specific manner.

Table 2

Targeted expression of *lin-4* in mesodermal lineages rescues mesodermal but not hypodermal defects in a *lin-4(e912)* background.

Expression construct	n (lines)	% with *pegl-15::gfp*	% vulvaless
Uninjected	87	21	100
phlh-8::lin-4			
Line 124.3.1.1	48	94	100
Line 124.3.2.1	26	96	100
Line 124.3.4.1	43	95	100
Total	117 (3)	95	100

Table 3

Targeted expression of *lin-4* in hypodermal tissues rescues hypodermal but not mesodermal defects in a *lin-4(e912)* background.

Expression construct	n (lines)	% vulvaless	% with adult alae
Uninjected	30	100	0
pcol-10::lin-4			
Line 128.1.1.1	46	1	91
Line 128.1.1.2	39	4	100
Line 128.1.1.3	43	0	98
Line 128.1.1.4	47	0	96
Total	175 (4)	3	96

(A) Rescue of hypodermal defects in vulval formation and cuticle patterning by directed expression of *lin-4* in hypodermal tissues.

Expression construct	n	% with *pegl-15::gfp*
Uninjected	87	21
pcol-10::lin-4	20	30

(B) Directed hypodermal expression of *lin-4* fails to rescue a mesodermal defect.

Table 4

Cell-by-cell correspondence between hypodermal expression of *lin-14_3′UTR* “sponge” and loss of adult alae in mosaic animals.

Seam cells	n	% with adult alae	% with no adult alae
non-*gfp*-expressing	71	87	13
gfp-expressing	164	23	77

Only animals with broken adult alae (indicative of mosaicism) were considered.