The Long Non-Coding RNA *lep-5* Promotes the Juvenile-to-Adult Transition by Destabilizing LIN-28

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**In Brief**
The functions of most long non-coding RNAs (lncRNAs) are unknown, despite their abundance in biological systems. Here, by characterizing *C. elegans* mutants with developmental delays, Kiontke et al. identify *lep-5*, a ~600-nt lncRNA. *lep-5* regulates developmental timing by binding to and destabilizing LIN-28, a conserved regulator of miRNA biogenesis.

**Highlights**
- *lep-5* acts in the heterochronic pathway to promote the larval-to-adult transition
- *lep-5* is a ~600 nt, highly structured lncRNA that is conserved across *Caenorhabditis*
- Like the Makorin LEP-2, *lep-5* promotes the degradation of LIN-28 protein
- *lep-5* may act as a scaffold to bring LEP-2 into close proximity with LIN-28
The Long Non-Coding RNA lep-5 Promotes the Juvenile-to-Adult Transition by Destabilizing LIN-28

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SUMMARY

Biological roles for most long non-coding RNAs (lncRNAs) remain mysterious. Here, using forward genetics, we identify lep-5, a lncRNA acting in the C. elegans heterochronic (developmental timing) pathway. Loss of lep-5 delays hypodermal maturation and male tail tip morphogenesis (TTM), hallmarks of the juvenile-to-adult transition. We find that lep-5 is a ∼600 nt cytoplasmic RNA that is conserved across Caenorhabditis and possesses three essential secondary structure motifs but no essential open reading frames. lep-5 expression is temporally controlled, peaking prior to TTM onset. Like the Makorin LEP-2, lep-5 facilitates the degradation of LIN-28, a conserved miRNA regulator specifying the juvenile state. Both LIN-28 and LEP-2 associate with lep-5 in vivo, suggesting that lep-5 directly regulates LIN-28 stability and may function as an RNA scaffold. These studies identify a key biological role for a lncRNA: by regulating protein stability, it provides a temporal cue to facilitate the juvenile-to-adult transition.

INTRODUCTION

Long non-coding RNAs (lncRNAs), once thought to be biological noise, are now appreciated as important components of many biological processes. These ≥200 nt long non-coding transcripts are associated with remarkably diverse molecular functions, including regulation of chromatin topology and modification, transcriptional activation, control of miRNA availability, and scaffolding of proteins and RNAs. lncRNAs act in diverse developmental processes including pluripotency, patterning, and differentiation and are also implicated in the pathogenesis of neurodegeneration and cancer (Cech and Steitz, 2014; Delás and Hannon, 2017; Fatica and Bozzoni, 2014; Geisler and Coller, 2013; Quinn and Chang, 2016; Ransohoff et al., 2018). While most lncRNAs function in the nucleus, some have cytoplasmic functions, e.g., TINCR brings together Staufen and mRNAs that promote epidermal differentiation (Kretz et al., 2013) and HOTAIR scaffolds the E3 ubiquitin ligases Dzip3 and Mex3b with their respective substrates Ataxin-1 and Snurportin-1 to prevent premature senescence (Yoon et al., 2013). However, biological roles and molecular functions remain unknown for the vast majority of lncRNAs, particularly for those that act in the cytoplasm.

In the nematode Caenorhabditis elegans, numerous lncRNAs have been detected by high-throughput approaches but relatively little is known about their functions or roles in biological processes (Liu et al., 2017b; Nam and Bartel, 2012; Wei et al., 2019). For another important family of ncRNAs, the microRNAs (miRNAs), essential insights came from forward genetic approaches. The first two known miRNAs in any system, lin-4 and let-7, were identified in a series of classic studies on C. elegans developmental timing mutants (Ambros, 1989; Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993). Both of these miRNAs function in the C. elegans heterochronic pathway, a mechanism that controls developmental progression through four larval stages into adulthood (Rougvie and Moss, 2013). In heterochronic mutants, certain stage-specific developmental events occur too early (“precocious” mutants) or too late (“retarded” or delayed mutants). Interestingly, regulation by non-coding RNAs figures prominently in the heterochronic pathway: in addition to lin-4 and let-7, the lin-4-related mir-237 and three other let-7-like miRNAs, mir-48, -84, and -241, also have key roles (Abbott et al., 2005; Tsialikas et al., 2017). Expression of these miRNAs in successive temporal waves during larval development keeps the activities of their targets in check until the appropriate time (Ambros, 2011).
Several key components of the heterochronic pathway, most notably the miRNA let-7 and its negative regulator LIN-28, are functionally conserved in animals (Faunes and Larraín, 2016). While lin-28 orthologs promote an immature state associated with stemness and multi-potentiality, let-7 promotes differentiation and maturation. For example, Drosophila let-7 regulates the timing of neuromuscular remodeling during metamorphosis (Caygill and Johnston, 2008; Sokol et al., 2008) and the temporal patterning of neural cell fates in the brain (Wu et al., 2012). In *C. elegans*, LIN28 promotes stem cell pluripotency (Copley et al., 2013; Zhang et al., 2016) and is genetically linked to the timing of the juvenile-to-adult transition (Faunes et al., 2017; Del Rio-Albrechtsen et al., 2016; Vadla et al., 2012). A hallmark of this transition is male tail tip morphogenesis (TTM). TTM occurs during the fourth larval stage (L4) and involves the fusion and retraction of the four tail tip hypodermal cells, hyp8-11, to generate the rounded tail tip characteristic of the adult male (Figures 1A and 1B) (Nguyen et al., 1999). In retarded *lin-41*(*gf*), let-7(*r*), and *lep-2*(*lf*) mutants, TTM is delayed or absent. This leads to the perseverance of a juvenile tail tip in adults, a phenotype called leptoderan (Lep). Conversely, TTM initiates early in *lin-41*(*lf*) and *lin-28*(*lf*) mutants, resulting in an over-retracted (Ore) phenotype in adults (Del Rio-Albrechtsen et al., 2006; Herrera et al., 2016; Vadla et al., 2012). The Lep and Ore phenotypes of these mutants result from alterations in the timing of the expression of *dmd-3*, a doublesex-family transcription factor that governs the execution of the TTM program (Mason et al., 2008; Nelson et al., 2011). While the role of the heterochronic pathway has been intensively investigated in the division and differentiation of the lateral seam cells, little is known about this pathway in other cell types or the extent to which it regulates other aspects of developmental timing. TTM provides an outstanding opportunity to address this gap.

Here, we report the identification and characterization of *lep-5*, mutations in which disrupt the onset of TTM as well as other aspects of the larval-to-adult transition. We find that *lep-5* expression is under temporal control and that it acts in the heterochronic pathway to promote the degradation of LIN-28. Surprisingly, *lep-5* acts as a lncRNA that is conserved across the *Caenorhabditis* genus. These findings highlight the role of lncRNAs as mediators of protein stability and emphasize the importance of ncRNAs in developmental timing.

**RESULTS**

**lep-5** Mutant Males Fail to Undergo Normal TTM

Using a forward genetic approach, we identified two X-linked mutants, *ny10* and *fs8*, with defects in TTM. As adult males, both mutants exhibited long, pointed (Lep) tail tips that protruded far outside the cuticular fan (Figures 1C and 1D). Other aspects of male tail anatomy appeared normal. In particular, the “anterior retraction” process, which generates the rays and fan and is mechanistically distinct from TTM (Nguyen et al., 1999; Sulston et al., 1980), was not disrupted in *lep-5* males (Figures 1C and 1D). Complementation tests showed that *ny10* and *fs8* are recessive and alleleic (see STAR Methods). We named the gene identified by these mutations *lep-5*. The Lep phenotype of *ny10* was
completely penetrant at 15°C and 25°C, whereas that of fs8 was temperature sensitive (Table 1), suggesting that fs8 is a hypomorphic allele.

Consistent with the Lep phenotype, we observed that tail tip cells in lep-5 L4 males failed to undergo normal migration and retraction. Using the adherens junction marker AJM-1::GFP, we found that some hypodermal cells remained unfused even in adult lep-5 males (Figure 1G). Thus, lep-5 influences both cell fusion and retraction, indicating that it regulates the execution of the entire TTM program (Nguyen et al., 1999).

In addition to TTM defects, we observed several mutant phenotypes that suggested a more general role for lep-5 in developmental timing. In some lep-5 males, delayed TTM occurred in adults (Figure 1H). Furthermore, 42% of lep-5 males (n = 101) and 62% of lep-5 hermaphrodites (n = 101) molted again in adulthood, a characteristic of some other developmental timing mutants (Ambros and Horvitz, 1984) (Figures 1I and S1). In males, these supernumerary molts were invariably lethal, while in hermaphrodites, they led to defects in vulva morphology (Figure S1K). The adult alae, stage-specific specializations of the lateral hypodermis, appeared normal in males, but in hermaphrodites, alae were weak or partially absent in most young adults (Figure S1). Despite this, adult seam cell numbers (n = 50 sides) and seam cell fusion were normal in lep-5 mutants of both sexes, and we found no evidence for additional seam cell divisions in adults. We conclude that lep-5 function is important for some but not all somatic features of the juvenile-to-adult transition in C. elegans.

lep-5 Alleles Identify a Previously Uncharacterized Gene

Using standard methods, we mapped lep-5 to the uncharacterized predicted gene H36L18.2 (Figure S2). Transcriptome sequencing indicates that H36L18.2 produces a mature polyadenylated RNA of ~600 nt after the removal of two introns and trans-splicing to the SL1 splice leader (Gerstein et al., 2010) (Figure 2A). We found that ny10 was a large, ~80 kb deletion encompassing 32 predicted genes (Figure S2), while fs8 was a point mutation (G23A) in the first nucleotide following the SL1 acceptor site (Figure S2C). We also engineered a lep-5 null allele, ny28, by CRISPR. While most experiments described below were carried out using ny10 and fs8, we found that lep-5(ny10) null mutants phenocopied lep-5(ny28) with respect to all phenotypes described above (Table 1). Furthermore, the lep-5(ny10) phenotypes in males and hermaphrodites are rescued by a transgene covering a region from 3,838 nt upstream to 248 nt downstream of the wild-type H36L18.2 locus (n > 50).

The mature H36L18.2 transcript has three potentially translatable regions, encoding conceptual products of 34 (ORF1), 83 (ORF2), and 106 (ORF3) amino acids. One of these (starting at M12 of ORF2) was identified as predicted ORF in the WormBase genome annotation WS250. None of the three possible translation products have detectable domains or homology to any other known proteins. Remarkably, however, the primary nucleotide sequence of lep-5 was strongly conserved in the genomes of 18 other Caenorhabditis species in the Elegans group (Kiontke et al., 2011) and less well conserved in seven more distantly related species (Figure S3). While most of these lep-5 orthologs contain potentially translatable regions, there is no detectable similarity in their potential protein products or in their positions in the predicted transcripts (Figure S4).

These findings raised the possibility that lep-5 function depends on the nucleotide sequence itself rather than coding potential. As an initial test of this idea, we asked whether expression of lep-5 orthologs from C. briggsae (Cbr-lep-5) or the more highly divergent C. angaria (Can-lep-5) could rescue the tail tip defects of C. elegans lep-5 (Cel-lep-5) mutants. Cbr-lep-5 and Can-lep-5 each have one potential ORF, but these share no coding potential with each other or with the potential ORFs in Cel-lep-5. Remarkably, we found that expression of Cbr-lep-5 completely rescued and Can-lep-5 partially rescued the Lep defect of C. elegans lep-5 mutants (Figure 2C). These results very strongly suggest that the putative ORFs of lep-5 are not required for its function. Note that polyadenylation, as observed

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*72% of males begin tail tip retraction during the L3 stage (n = 54).
Figure 2. lep-5 Is a Long Non-coding RNA

(A) The secondary structure of the lep-5 lncRNA as predicted by Turbofold. The conserved 5' stem loop (red bracket) includes the SL1 trans-spliced leader (in red). The 3' stem loop (blue bracket) and two base-paired regions forming the “central zipper” (green brackets) are also conserved. Dashed lines: boundaries of two hairpins deleted in the rescue experiments (D1 and D2). The polyA tail is omitted for clarity. For alignments, see Figure S3 and STAR Methods. See also Figures S4 and S5.

(B) The 5' region from predicted full-length structures of wild-type lep-5, lep-5/fs8 = G23A and lep-5(G23A, C30T). Asterisks indicate position 23. SL1 is in red.

(C) Rescue experiments with transgenes containing stop codons in the predicted coding region (*3 or *2 stops), the lep-5 orthologs of C. briggsae and C. angaria and constructs containing nucleotide substitutions that disrupt and restore the lep-5 secondary structure (high lep-5(G23A) = transgene at 10x concentration).

(D) Schematic of the SL1-spliced lep-5 RNA with putative ORFs shown above and selected predicted secondary structure features shown below. Boxes show the sequence of various mutant alleles compared with the wild-type allele.

(E) Male tail tip phenotype at 20°C of lep-5 mutants in which the secondary structure and/or the predicted ORFs are disrupted.
in lep-5, is a feature of many non-coding transcripts (Kopp and Mendell, 2018; Nam and Bartel, 2012).

**lep-5 Is a IncRNA with Several Prominent Secondary Structure Motifs**

To predict lep-5 secondary structure, we used Turbofold (Harmanc et al., 2011) for comparative analysis of lep-5 orthologs from 19 species in the *Elegans* group of *Caenorhabditis*. This revealed several notable features (Figures 2A, S4, and S5). First, lep-5 RNA is predicted to be highly structured, with multiple stem-loops, several prominent single-stranded regions, and a central “zipper” region. Most of the base-paired regions show high conservation; many predicted single-stranded positions are also strongly conserved, suggesting that these might serve as sites for interactions in trans (Figure 2A). Second, the extensive base-pairing, particularly in the central zipper region, suggests that the mature RNA adopts a compact structure. Third, the very 5′ end of the lep-5 RNA is predicted to fold into a 53 nt stem-loop structure that includes the 22 nt trans-spliced leader SL1. A consensus structure derived from the alignment of the 21 *Elegans*-group lep-5 genes, as well as individual Turbofold-predicted structures, indicated that three key features are conserved in all orthologs: the 5′ SL1-containing stem-loop, the central zipper region, and a stem loop near the 3′ end (Figures 2A and S4).

Interestingly, the position altered in the lep-5(fs8) mutant (G23A) lies near the tip of the 5′ stem loop and is predicted to base-pair with C30. This mutation dramatically altered the predicted structure of the 5′ region, replacing the large stem loop with several smaller double-stranded regions (Figures 2B and S3B). To ask whether base-pairing between positions 23 and 30 is important for lep-5 function, we created rescue constructs containing the G23A mutation alone and in combination with a second mutation, C30T, a compensatory change predicted to restore the 5′ stem loop (Figure 2B). While the G23A transgene had poor rescue activity, the G23A C30T double mutant transgene completely rescued the lep-5 tail tip phenotype (Figure 2C). Introducing a different mutation at this position, G23C, alone and together with its corresponding compensatory change, C30G, yielded the same pattern of results (Figure 2C). These experiments indicate that base-pairing between G23 and C30 is critical for lep-5 function, strongly supporting the existence of the 5′ stem loop in vivo.

To probe additional regions that could be important for lep-5 function, we deleted two predicted internal stem-loops from the rescue construct. “Δ1′” (ΔC482-G542) removes most of the large, well-conserved predicted stem loop near the 3′ end of lep-5 (Figure 2A). This deletion abolished rescue activity (Figure 2C). Deletion of a less well-conserved smaller internal stem loop (“Δ2′,” ΔT216-A257) reduced, but did not eliminate, rescue (Figures 2B and 2C). In contrast, introducing multiple stop codons into the putative ORF2, at codons 15, 16, and 17 (“Δ3 stops”) or at codons 25 and 26 (“Δ2 stops”), did not diminish the ability of these constructs to rescue lep-5 (Figures 2C and 2D).

To further explore the structure of the lep-5 IncRNA and to confirm the dispensability of its putative ORFs for lep-5 function, we created several new lep-5 CRISPR alleles (Figure 2D). lep-5/fs18) is a TT193AAA change that introduces a stop codon and frameshift into ORF2 but is not expected to significantly alter RNA secondary structure. These mutants were phenotypically wild-type (Figures 2D and 2E; Table 1). lep-5/fs19) mutants replace 60 nt (G476-T535) with the nucleotides CA, eliminating the 3′ stem loop and truncating ORF3, but leaving the other ORFs intact; these mutants phenocopy the lep-5(ny2B) null allele (Figures 2D and 2E; Table 1). Most tellingly, we created three mutants to disrupt the predicted central “zipper” and then restore it with predicted compensatory changes ~230 nucleotides away (Figures 2D and 2E; Table 1). In lep-5/fs21), six point mutations were introduced into the top strand of the zipper, dramatically weakening its potential to form a double-stranded region. These mutations also cause missense changes to putative ORFs 1 and 2. Separately, lep-5/fs22 introduced seven point mutations into the lower strand of the zipper, similarly disrupting it; this causes five missense changes in ORF3. Both lep-5/fs21) and lep-5/fs22) mutants had completely penetrant TTM defects that phenocopy the lep-5 null allele (Figure 2E). Thus, lep-5 function can be eliminated by two separate mutants that disrupt a central secondary-structure feature but cause no common lesion to the putative coding sequence. Finally, we introduced mutations equivalent to fs22 into fs21 to create the “double mutant” fs21fs25, which is predicted to restore the secondary structure of the lep-5 RNA but leaves extensive coding sequence changes in all putative ORFs (Figure 2D). Strikingly, lep-5/fs21fs25) mutant males were phenotypically wild-type (Figure 2E; Table 1). Thus, the integrity of the central “zipper” region of lep-5 RNA is essential for its function, and overwhelming evidence indicates that lep-5 activity is independent of the coding potential of its ORFs.

Consistent with an RNA-based function for lep-5, several previous studies have found the lep-5 RNA in ribonucleoprotein complexes in vivo. Two regions of lep-5, C335-G392 and T415-A467, were identified in high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) with the Argonaut ALG-1 (Grosswendt et al., 2014). Another region, A531-T551, was suggested to interact with the Dicer DCR-1 (Rybak-Wolf et al., 2014). Note that Dicer can bind to many classes of RNA, including IncRNAs and miRNAs, and that this interaction does not necessarily imply cleavage (Rybak-Wolf et al., 2014). In other HITS-CLIP experiments, lep-5 was found to interact with the heterochronic factor LIN-28, also through a region in its 3′ end (G434-G584) (Stefani et al., 2015).

Because of the extensive involvement of miRNAs in *C. elegans* developmental timing (Abbott et al., 2005; Lee et al., 1993; Reinhart et al., 2000) and because of *lep-5*’s association with ALG-1 and DCR-1, we considered the possibility that the full-length lep-5 transcript might serve as a precursor for one or more smaller RNAs. However, deep sequencing from multiple stages and both sexes in *C. elegans* has identified no small RNAs derived from this region (Gerstein et al., 2010; Kato et al., 2009) despite high abundance of the full-length lep-5 RNA. These results cannot exclude the possibility that very low-abundance, temporally regulated, or unstable miRNAs might be made from the lep-5 locus. However, the structure-function experiments above, together with results detailed below, are more consistent with an IncRNA-based function for lep-5.

**lep-5 IncRNA Is Expressed in a Temporal Wave and Localizes to the Cytoplasm**

To examine the lep-5 expression pattern, we fused ~4 kb of upstream sequence to GFP. This Plep-5::GFP reporter was
expressed in larvae of both sexes in several cell types including the tail tip, pharynx muscles, in neurons in the head and cloacal region, and in seam cells (arrowheads).

By qRT-PCR, *lep-5* abundance showed a similar pattern, with expression low during L1 but rising dramatically by late L2 and remaining high through early L4 (Figure 3B). *lep-5* levels were markedly reduced in *lep-5(fs8)* mutants (not shown), indicating that the fs8 mutation does not abolish trans-splicing, though it is possible that such processing is impaired.

To determine the subcellular localization of the *lep-5* IncRNA, we carried out single-molecule fluorescent in situ hybridization (smFISH). *lep-5* RNA was readily detectable in the tail tip of an L2 male (Figures 3C and 3D). Co-staining with DAPI indicated that *lep-5* RNA is predominantly, if not exclusively, cytoplasmic. Consistent with the broad expression of the transcriptional reporter, *lep-5* RNA was also detectable throughout the body, including neuronal ganglia of the head and tail (Figures 3E and 3F) and in the developing male somatic gonad (Figure 3G).

**Figure 3. lep-5 lncRNA Expression Is Temporally Regulated**

(A) Temporal expression of P*lep-5::GFP::NLS* in late L1 animals (top) through adults (bottom). Expression is observed in the tail epidermis including the tail tip cells, in pharynx muscles, in neurons in the head and cloacal region, and in seam cells (arrowheads).

(B) Temporal expression of *lep-5* IncRNA from L1 to adult as measured by qPCR. Error bars show SD.

(C and D) *lep-5* IncRNA visualized with smFISH probes in the tail tip of a wild-type (C) and *lep-5(ny10)* (D) L2 animal. *lin-44* probes were used as positive control. DAPI staining indicates nuclei.

(Figure 3A) Late L2 male. *lep-5* IncRNA visualized with smFISH probes (left) and nuclei stained with DAPI (right) showing areas of concentrated *lep-5* IncRNA expression in the ganglia in the pharynx (E) and rectal (F) region and in the somatic portion of the developing gonad (G). See also Figure S6.
first detectable in the tail tip cells in early L4 (Figure 4A) (Mason et al., 2006). By late L4, TTM is complete and tail tip expression of dmd-3 becomes undetectable. In lep-5(ny10) L4 males, dmd-3 expression was absent in the tail tip cells, while in lep-5(fs8), it was variably lost and delayed (Figure 4A; data not shown). Moreover, many lep-5 males showed aberrant expression of dmd-3 in adulthood (Figure 4A), likely accounting for the adult activation of TTM described above (Figure 1H). Thus, lep-5 regulates the timing of TTM by controlling the temporal dynamics of dmd-3 expression in the tail tip.

**lep-5 Acts in the Heterochronic Pathway**

The altered timing of TTM in lep-5 mutants, as well as the supernumerary molts and hermaphrodite alae defects, led us to consider whether lep-5 might act in the C. elegans heterochronic pathway (Rougvie and Moss, 2013). Consistent with this, we found that passage through the dauer stage, an L3 alternative used in times of stress, strongly suppressed the morphogenesis defect of lep-5 males (Table 1; Figure 4B). Suppression by dauer is a characteristic feature of several heterochronic mutants (Liu and Ambros, 1991). Furthermore, the phenotype of lep-5(ny10) mutants was enhanced by RNAi knockdown of the RISC component ain-1, which by itself causes only weak heterochronic defects but modifies the phenotypes of many heterochronic mutants (Ding et al., 2005). While ain-1(RNAi) males had no male tail defects, all ain-1(RNAi); lep-5(ny10) males displayed severe defects in anterior tail retraction and TTM (n = 28; Figure S1). ain-1(RNAi) also strongly enhanced the frequency of supernumerary molts of lep-5(ny10) adults to 100% in both sexes (n > 20). However, we observed no effect of ain-1 RNAi on seam cell development in lep-5 mutants, as all ain-1(RNAi); lep-5(ny10) adults had a normal number of seam cells (n = 27), which fused normally (n = 67), and males had normal alae (n = 38). Together, these findings indicate that lep-5 is a component of the heterochronic pathway.

We carried out several experiments to determine the regulatory relationships between lep-5 and other heterochronic genes. let-7 and its key target, the NHL/TRIM gene lin-41, regulate the timing of TTM (Del Rio-Albrechtsen et al., 2006; Mason et al., 2008). We found that lin-41(lf) and lin-41(RNAi) suppressed the lep-5 TTM phenotype (Table 1; Figures 4B and 4C). Moreover, Plin-41::GFP::lin-41 3’UTR expression, normally downregulated during L4, persisted into adulthood in lep-5 mutants (Figure S7). These observations indicate that lep-5 functions upstream of lin-41. Furthermore, 5-fold overexpression of let-7 via the transgene zals3 (Büssing et al., 2010), while causing no tail tip phenotype in a wild-type background, was able to almost completely suppress the lep-5 mutant phenotype (Figures 4B and 4C). Thus, lep-5 likely acts upstream of let-7.

The RNA-binding protein LIN-28 acts upstream of let-7 and is a central regulator of developmental progression across species. In both C. elegans and mammals, a key role of LIN-28 is to repress the biosynthesis of mature let-7 (Tsaiikas and Romer-Seibert, 2015). Consistent with the precocious developmental phenotypes of lin-28 mutants (Ambros and Horvitz, 1984; Vadla et al., 2012), these animals exhibit premature TTM (leading to over-retraction; Ote) (Herrera et al., 2016). We observed an Ore phenotype in many lin-28(RNAi) males and found that lin-28(RNAi) also strongly suppressed the lep-5 TTM phenotype (Figures 4B and 4C). Here, residual lin-28 activity may prevent complete suppression, as the penetrance of the lin-28(RNAi) TTM phenotype indicates incomplete knockdown (Herrera et al., 2016). These results suggest that lep-5 is a negative regulator of lin-28. Earlier in development, the transcription factor LIN-14 controls the progression between L1 and L2 stages by activating lin-28 (Seggerson et al., 2002). Consistent with previous findings (Herrera et al., 2016), lin-14(RNAi) males also precociously retracted their tail tips, but lin-14(RNAi) had no effect on TTM in lep-5(ny10) mutants (Figures 4B and 4C). Together, these experiments support a model in which lep-5 functions in the heterochronic pathway downstream of lin-14 and upstream of lin-28 and let-7.

**lep-5 Is Required for the Timely Decay of LIN-28**

Normal progression through larval development requires the downregulation of LIN-28 by the L3 stage (Moss et al., 1997). This allows the production of mature let-7 miRNA and has other, let-7-independent, consequences (Vadla et al., 2012; Van Wynaerberge et al., 2011). Previous work has demonstrated that the control of lin-28 mRNA stability and translation, mediated through its 3’ UTR, is an important contributor to LIN-28 downregulation (Morita and Han, 2006). To ask if lep-5 has a role in this process, we examined LIN-28 protein levels by western blot (Figure 5A). As expected, LIN-28 abundance in wild-type larvae was high in L1 and decreased significantly by L3. In lep-5 mutants, LIN-28 levels were similar to those seen in wild type during L1 but were markedly elevated in L3, indicating a defect in LIN-28 downregulation. By qRT-PCR, however, we found that the loss of lep-5 had no effect on lin-28 mRNA abundance in L3 (Figure 5B). Thus, lep-5 is necessary for the developmental decline in LIN-28 protein, but it does not affect lin-28 mRNA levels. Consistent with this function, we found that overexpression of a LIN-28::GFP fusion protein in lep-5 mutants, but not in wild type, caused lethality because of highly penetrant supernumerary adult molts in both sexes (data not shown).

Given these results, we considered two models for lep-5-mediated regulation of lin-28. First, lep-5 might be important for repressing translation of lin-28 mRNA. Such a mechanism is used by the heterochronic genesdaf-12, lin-66, and sea-2, which repress lin-28 translation through its 3’ UTR (Huang et al., 2011; Morita and Han, 2006; Seggerson et al., 2002). Alternatively, lep-5 could promote the degradation of LIN-28 protein between L1 and L3. To distinguish between these possibilities, we used a photoconvertible LIN-28::Dendra2 fusion protein to monitor the stability of LIN-28 protein during development (Herrera et al., 2016). As expected, LIN-28::Dendra2 abundance was high in L2 animals, and UV illumination converted essentially all of this fusion protein from green to red (Figure 5C). When these animals reached early L4, the pool of pre-existing (red) LIN-28::Dendra2 was nearly undetectable, and we observed no newly synthesized (green) LIN-28::Dendra2. In lep-5 mutants, LIN-28::Dendra2 levels were comparable to wild type at the L2 stage, consistent with our western blot results. Similarly, very little newly synthesized (green) LIN-28::Dendra2 was detectable in early L4 following photoconversion in L2. However, unlike wild-type larvae, lep-5 mutants exhibited significant amounts of pre-existing (red) LIN-28::Dendra2 protein in L4. Thus, the
Figure 4. *lep-5* Functions Upstream of *lin-28* in the Heterochronic Pathway to Control TTM

(A) Expression of *Pdmd-3::YFP* in wild-type and mutant male tail tips at early (e), mid (m), and late (l) L4 stage.

(B) Penetration and expressivity of tail tip phenotypes in single mutants, double mutants, and mutant and/or RNAi knockdown combinations. Left column shows results in a *lep-5* mutant background (relevant allele indicated); right column shows corresponding result in a wild-type background. PD, post dauer; HT115, RNAi control bacterial strain; asterisk, RNAi fed to L1 larvae.

(C) DIC images of representative L4 and adult male tails from the experiments summarized in (B). A rounded tail tip in L4 results from precocious TTM in L3. Adults shown for *lin-14(RNAi)* have just molted and remain surrounded by the L4 cuticle. See also Figure S7.
pool of LIN-28::Dendra2 synthesized before photoconversion was markedly more stable in \( \text{lep-5} \) mutants than in wild type. This indicates that \( \text{lep-5} \) regulates developmental timing by promoting the timely degradation of LIN-28 protein.

**\( \text{lep-5} \) RNA Associates with LIN-28 and LEP-2 \textbf{In Vivo}**

\( \text{lep-2} \), another recently identified heterochronic gene, shares many phenotypic similarities with \( \text{lep-5} \) (Herrera et al., 2016). Both mutants have severe TTM defects, both genes act in the heterochronic pathway, and both are required for the timely degradation of LIN-28. \( \text{lep-2} \) encodes the sole \( C. \text{elegans} \) Mako, a conserved but poorly understood family of proteins with RNA-binding and E3 ubiquitin-ligase capacities (Cassar et al., 2015; Gray et al., 2000; Kim et al., 2005; Lee et al., 2012; Lee et al., 2009; Liu et al., 2017a; Salvatico et al., 2010). Correspondingly, Herrera et al. (2016) hypothesized that LEP-2 might act as the E3 ligase that tags LIN-28 for proteasomal degradation. Because both LIN-28 and LEP-2 are RNA-binding proteins, we considered the possibility that they might both bind to \( \text{lep-5} \).

We therefore immunoprecipitated \textit{in vivo}-crosslinked RNA-protein complexes to ask if LEP-2 and LIN-28 bound specifically to the \( \text{lep-5} \) RNA. Using animals carrying a functional GFP::LEP-2 transgene, we carried out anti-GFP immunoprecipitation to recover GFP::LEP-2 along with any covalently-bound RNAs. After washing and crosslink reversal, we measured the recovery of \( \text{lep-5} \) RNA and a control mRNA expressed at similar levels, \( \text{cdc-42} \), by qRT-PCR and compared these to the recovery from negative-control (empty beads) immunoprecipitations. We robustly detected \( \text{lep-5} \) RNA, while \( \text{cdc-42} \) RNA was present only in trace amounts (Figure 5D). Using a similar approach, we isolated RNAs associated \textit{in vivo} with GFP::LIN-28 and with a negative control RNA-binding protein, GFP::LIN-41. Again, we found \( \text{lep-5} \) RNA, but not significant amounts of \( \text{cdc-42} \) RNA, in association with GFP::LIN-28 and with a negative control RNA-binding protein, GFP::LIN-41. Because both LIN-28 and LEP-2 are RNA-binding proteins, we considered the possibility that they might both bind to \( \text{lep-5} \).

![Figure 5. \( \text{lep-5} \) IncRNA Promotes LIN-28 Degradation](image)

(A) Detection of endogenous LIN-28 protein by western blot (actin used for a normalization control).

(B) \( \text{lin-28} \) mRNA levels determined by qPCR. Values are normalized to wild-type L1s.

(C) Analysis of a \( \text{LIN-28}::\text{Dendra2} \) fusion protein in WT (top) and \( \text{lep-5(ny10)} \) (bottom) males. Shown are neurons in the head region (pharynx and nerve ring indicated by gray lines). From left to right: green fluorescent signal before photoconversion, diminished green signal after photoconversion, red signal after photoconversion, green and red signals in the same animals 24 h later.

(D) Ratios of \( \text{lep-5} \) IncRNA and control mRNA for \( \text{cdc-42} \) in immune-precipitation of GFP-tagged proteins using anti-GFP relative to negative control IP measured by qPCR (see STAR Methods for details). For each set of triplicate experiments, the first to third quartile is represented as a box, the median as a black bar and the maximum and minimum values as whiskers.
DISCUSSION

Here, we report the discovery of a *C. elegans* IncRNA, lep-5, and demonstrate that it regulates the timing of two events in the juvenile-to-adult transition: male tail morphogenesis and the final molt. *lep-5* functions upstream of *lin-28*, a central regulator of developmental transitions—including the juvenile-to-adult transition—throughout the animal kingdom. *lep-5* promotes the timely degradation of LIN-28, an essential step for properly coordinated larval development. This regulation is likely direct and might reflect a scaffolding ability of *lep-5*, through which it may promote LIN-28 proximity to an E3 ligase, LEP-2, whose mutant phenotype is nearly identical to that of *lep-5*. The *lep-5* IncRNA is found in the cytoplasm, where LIN-28 and LEP-2 are localized and predicted to be active (Herrera et al., 2016), thus adding an important regulator to the small list of known cytoplasmic IncRNAs. To date, few IncRNAs have been characterized in *C. elegans*: *rncs-1* is thought to play a role in the response to starvation (Hellwig and Bass, 2008), and *tts-1* regulates ribosomal levels to promote the extended lifespan of insulin receptor mutants (Essers et al., 2015). A recent study identified behavioral and developmental phenotypes for 23 putative *C. elegans* IncRNAs, finding that most appear to regulate transcription or bind to endogenous miRNAs (Wei et al., 2019). These studies did not include *lep-5*, as H36L18.2 was not originally annotated as a predicted IncRNA. Our findings that *lep-5* acts cytoplasmically to regulate LIN-28 stability highlight the extensive use of non-coding RNA in the heterochronic pathway.

**lep-5** Is a IncRNA Component of the *C. elegans* Heterochronic Pathway

The delay of TTM, the presence of supernumerary molts and partially defective alae, the suppression by passage through dauer, and the enhancement by *ain-1(RNAi)* suggest that *lep-5* functions in the heterochronic pathway. The finding that *lep-5*’s key function is to promote the timely degradation of LIN-28 provides a straightforward mechanism to explain the Lep defect. Perdurance of LIN-28 function would block biogenesis of the mature *let-7* miRNA (Tsialikas and Romer-Seibert, 2015), which would then be unable to downregulate its target *lin-41*. Because *lin-41* blocks *dmd-3* activation and the onset of TTM (Del Rio-Albrechtsen et al., 2006; Mason et al., 2008), the persistence of *lin-41* in *lep-5* mutants accounts for these heterochronic defects. That *lep-5* mutants do not completely phenocopy some other mutants that disrupt LIN-28 degradation—particularly with respect to defects in seam cell lineages—strongly suggests cell-type specificity in the control of LIN-28 (see below). We believe that *lep-5* provides an instructive temporal cue for TTM, as overexpression of wild-type *lep-5* is sometimes sufficient to cause premature morphogenesis (Ore) (Figure 2C).

While multiple genes have been shown to be necessary for the proper temporal decline in *lin-28* activity, many of these (e.g., *lin-4, lin-66, daf-12*, and *sea-2*) function through the 3’ UTR of *lin-28* to repress its translation (Hochbaum et al., 2011; Huang et al., 2011; Morita and Han, 2006; Moss et al., 1997). In contrast, *lep-5*, like *ced-3* and *lep-2* (Herrera et al., 2016; Weaver et al., 2014), acts to promote LIN-28 protein degradation. The caspase CED-3, a protease best known for its role in programmed cell death, can act directly on LIN-28 (Weaver et al., 2017; Weaver et al., 2014). The Makorin LEP-2, as a putative E3 ligase, may promote LIN-28 degradation via ubiquitination (Herrera et al., 2016).

**lep-5** May Act as a Molecular Scaffold

How could the *lep-5* IncRNA regulate LIN-28 stability? In other systems, many functional roles for IncRNAs have been described, especially the regulation of transcription or chromatin state of nearby genes (Fatica and Bozzone, 2014; Geisler and Coller, 2013). We considered such a function for *lep-5* but found no obvious nearby candidate target genes. Moreover, the cytoplasmic localization of *lep-5* RNA makes this possibility unlikely. Many IncRNAs function by direct base-pairing to other RNAs (Cech and Steitz, 2014; Fatica and Bozzone, 2014; Kretz et al., 2013), but *lep-5* has no significant complementarity to any known transcribed regions of the *C. elegans* genome (BlastN e-values > 0.3) and does not harbor a target site for a relevant miRNA (see STAR Methods).

IncRNAs can also function as molecular scaffolds by recruiting factors into a functional complex (Ransohoff et al., 2018; Wang and Chang, 2011). Our results favor this hypothesis for *lep-5*. By immunoprecipitation of intact ribonucleoprotein complexes, we found that both LIN-28 and LEP-2 bind to *lep-5 in vivo*. LIN-28 possesses two RNA binding domains (Tsialikas and Romer-Seibert, 2015) and can bind RNA directly (Van Wysenberghe et al., 2011). In previous HITS-CLIP experiments, *lep-5* was found among the ~2,000 RNA species that interact with LIN-28 in vivo (Stefani et al., 2015). LEP-2 is the sole *C. elegans* Makorin (Herrera et al., 2016), a family of conserved proteins that can bind nucleic acids and act as E3 ubiquitin ligases (Arunmugam et al., 2007; Gray et al., 2000; Lee et al., 2009; Liu et al., 2017a; Salvatico et al., 2010). Because both *lep-5* and *lep-2* facilitate the degradation of LIN-28, we propose that the key function of *lep-5* is to scaffold a tripartite LEP-2–*lep-5*–LIN-28 complex. As such, *lep-5* would provide an instructive switch, allowing LEP-2 to act on LIN-28, causing it to be ubiquitinated and ultimately degraded by the proteasome (Figure 6). Such a mechanism would be similar to that of HOTAIR, an IncRNA that unites RNA-binding E3 ligases with their substrates (Yoon et al., 2013). Once LIN-28 levels decline, *pre-let-7* is able to be processed by Dicer, and the resulting increase in mature *let-7* miRNA promotes the juvenile-to-adult transition. This model accounts for the phenotypic similarity between *lep-2* and *lep-5* and explains why LEP-2, despite being nearly ubiquitously present from embryo to adult, is apparently only active during the period when *lep-5* is expressed (Herrera et al., 2016). Intriguingly, Argoaut binding is suggested to be involved in destabilizing HOTAIR RNA (Yoon et al., 2013), which, given the potential binding of ALG-1 to *lep-5* (Grosswendt et al., 2014), could also be the case here. Such predictions will be the focus of future work.

**Cell-Type Specificity in Developmental Timing Mechanisms**

In contrast to their roles in the timing of TTM and the cessation of molting, neither *lep-5* nor *lep-2* (Herrera et al., 2016) is required for stage-specific patterns of seam cell division or their terminal differentiation and fusion, both of which are canonical aspects of heterochronic control (Rougivie and Moss, 2013). This separation of phenotypes indicates that there may be cell-type-specific characteristics of heterochronic regulation. This idea
has precedent: lin-29, the terminal regulator of the larval-to-adult switch in seam cells, is not required for TTM (Ambros and Horvitz, 1984; Del Rio-Albrechtsen et al., 2006). Instead, dmd-3 fulfills this role (Mason et al., 2008). Nevertheless, given that lep-5 acts upstream of lin-28, it is surprising that lep-5 mutants display no strong defects in the seam. One possibility is that cell-type-specific mechanisms are important for regulating lin-28 activity. Additionally, different tissues may differ in their thresholds for lin-28 activity, such that the seam might be less sensitive and tail tip and body hypodermis (hyp7) more sensitive to increased lin-28 function. In any case, the identification of lep-5 and lep-2 indicate that the male tail tip provides an important and sensitive readout for studies of the heterochronic pathway, key aspects of which could be missed by focusing exclusively on seam cells. Additionally, cell-type-specific variation in the heterochronic pathway could provide important developmental and evolutionary flexibility. Indeed, tissue-specific changes in developmental timing (“heterochrony”) are thought to have important roles in morphological evolution (Gould, 1977).

**Structural Features of lep-5 RNA**

The predicted secondary structure of lep-5 features extensive base-pairing, indicating that its higher-order structure is important for its activity. Disrupting the secondary structure of the central zipper as well as the 5′ stem loop in vivo completely eliminated lep-5 function; restoring secondary structure with complementary mutations restored function. Interestingly, the predicted stem loop at the 5′ end includes the trans-spliced leader SL1. While SL1 is speculated to promote translation initiation of C. elegans mRNAs (Blumenthal, 2005), our results indicate that SL1 can also have a role in RNA secondary structure and IncRNA function. The 5′ stem loop might be an important binding site for lep-5 interactors; it is also important for RNA stability, as lep-5 levels were reduced ~5-fold in lep-5/fs8 mutants.

lep-5 IncRNA directly interacts with LIN-28 and LEP-2/Makorin. Both proteins—and even their roles in regulating developmental transitions, including the juvenile-to-adult transition—are highly conserved in animals (Abreu et al., 2013; Faunes et al., 2017; Faunes and Larraín, 2016; Gray et al., 2000; Herrera et al., 2016; Thornton and Gregory, 2012). Although one might expect this conservation to extend to lep-5, we did not find homologs outside of Caenorhabditis. It seems unlikely that Caenorhabditis evolved a special IncRNA-mediated mechanism to catalyze the LIN-28 degradation that must occur in all animals. Rather, we propose that the lep-5 primary sequence evolves rapidly and is therefore difficult to detect in more distantly related species by sequence similarity. Even identifying the C. angaria lep-5—which rescued C. elegans lep-5 mutants—required syntenic and bioinformatic analyses in addition to BlastN (see STAR Methods). In general, IncRNAs are known to evolve rapidly, and orthologs of many functionally important IncRNAs are not easily found outside closely related species (Diederichs, 2014). One explanation is that functional conservation in some IncRNAs may depend more on 3D structure than primary sequence. Considering this and the conservation of the LIN28-lep-7 regulatory module (Tsialikas and Romer-Seibert, 2015), we propose that lep-5 orthologs could indeed exist in other species but might not be identifiable based on sequence alone. Notably, recent work has shown that several IncRNAs are important for pluripotency and differentiation in mammalian stem cell systems (Flynn and Chang, 2014); the same biological processes often feature regulation by LIN28-lep-7 (Slyh-Chang and Daley, 2013). Indeed, a recently identified rodent-specific IncRNA, Ephemoner, modulates the exit of embryonic stem cells from pluripotency by regulating Lin28a; however, unlike lep-5, Ephemoner promotes Lin28a expression (Li et al., 2017). Several IncRNAs have also been implicated in the regulation of LIN28 in cancer cells, in some cases through a feedback loop involving let-7-family miRNAs (Gao et al., 2017; He et al., 2019; Peng et al., 2017; Wang et al., 2016). Our results in C. elegans indicate that IncRNA regulation of lin-28 is an anciently conserved component of mechanisms that control cell state changes and developmental progression.

**STAR METHODS**

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## STAR METHODS

### KEY RESOURCES TABLE

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<td><em>C. elegans</em> DF213 = *him-5(e1490) V; <em>zals3[let-7(+)] + myo-3::GFP</em> made by crossing CT19 with CB4088</td>
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<td><em>C. elegans</em> DF223 = *him-5(e1490) V; <em>lep-5(ny10)X; zals3[let-7(+)] + myo-3::GFP</em></td>
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<td><em>C. elegans</em> DF235 = *him-5(e1490) V; *lep-5(ny10) X; wls78[unc-119(+)] + ajm-1::GFP + scm::GFP + F58E10(+)]</td>
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<td><em>C. elegans</em> DF237 = *him-5(e1490) V; <em>mais108 [lin-28::gfp + rol-6]</em></td>
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<td><em>C. elegans</em> DF293 = *lep-2(ny23) IV; <em>him-5(e1490) V; nyEx53[lep-2::GFP::lep-2b + prf4]</em></td>
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### Oligonucleotides

See Table S1 for list of oligonucleotides

### Recombinant DNA

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### Software and Algorithms

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Douglas Portman (douglas.portman@rochester.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

These studies used the nematode *C. elegans*, which was cultured as described by Brenner (1974). Most experiments were carried out on larval and adult males, as these studies focus on a male-specific morphogenetic process. Most strains used here contained the mutation *him-5(e1490)*, which increases the frequency of spontaneous XO males among the self-progeny of XX hermaphrodites.

METHOD DETAILS

Microscopy and Phenotypic Analysis

For microscopy, worms were placed in a drop of 20 mM sodium azide or 1 mM levamisole on 5% agar pads and studied at 400x or 1000x magnification with a Zeiss Axioskop with Nomarski (DIC) and epifluorescence. Images were recorded with a C4742-95 “Orca” Hamamatsu digital camera and Openlab software, ver. 3.0.9 (Improvision). Confocal images were obtained with a Leica TCS SP8 X
microscope using the 63x objective. Images were taken as Z-stacks with 0.5 or 1 μm steps. Simple image editing was performed with ImageJ. To investigate cell-fusion phenotypes caused by *lep-5* mutations and assess the number of seam cells in adult animals, we examined strain DF235 carrying the *lep-5(ny10)* mutation, the adherens junction reporter AJM-1::GFP (Köppen et al., 2001), and the seam cell reporter pF09D12.1::GFP.

**Isolation and Mapping of *lep-5* Mutants**

The *lep-5(ny10)* deletion allele was isolated in a screen for Lep male phenotypes in which CB4088 hermaphrodites were mutagenized with trimethylpsoralen (TMP) and exposure to UV light. Using competitive genome hybridization (CGH), we identified two large deletions on X in *lep-5(ny10)* genomic DNA (see below; Figure S2A). *lep-5(fs8)* was isolated in a mutagenesis screen in which *bxs14 him-5(e1490)* hermaphrodites were mutagenized with ethylmethanesulfonate (EMS). In both screens, F1 hermaphrodites were isolated to produce the F2 generation, which were segregated. F3 male progeny of individual F2s were scored at 400x magnification for defects in ray development and tail tip morphology. Mutants were outcrossed several times with CB4088 to generate the strains used here. By SNP mapping, *fs8* was found to lie in a 2.7-Mb region that was deleted in *ny10*.

A fosmid covering one end of the *ny10* deletion rescued the *lep-5* phenotype; subsequent truncations of this region indicated that a ~3 Kb fragment containing the predicted gene H36L18.2 was sufficient for rescue (Figures S2B and S2C). By sequencing *lep-5(fs8)*, we identified a single G-to-A change (G23A) in the first nucleotide following the SL1 trans-splicing acceptor site (Figure S2C). Introducing this mutation into the 3-Kb rescuing fragment markedly reduced its activity (Figure 2B). Together, these results indicate that *lep-5* corresponds to H36L18.2.

**Array Comparative Genomic Hybridization to Map Deletions in *lep-5(ny10)***

The *ny10* allele of *lep-5* was mapped by comparative genomic hybridization by Nimblegen (Roche). Genomic DNA was isolated from the *lep-5* mutant (strain DF70) and the wild type reference strain (CB4088) using the Gentra Puregene Tissue Kit (Qiagen). The tiling arrays contained 385,179 probes with a median spacing of 167 nt covering the whole *C. elegans* genome. Reference and mutant DNA were differentially labeled with Cy3 or Cy5 dye and hybridized to the tiling array. The fluorescence intensity for Cy3 and Cy5 was measured at each probe position and the expression values were log2-normalized. To visualize the tiling array data, the ratio of the normalized expression values for each probe was plotted according to its position within the *C. elegans* genome (Figure 2A). We detected a ~24.6kb deletion on the left arm of ChrX (6,554,074 to 6,578,133 bp) and an ~86kb deletion on the right arm of ChrX (12,576,888 to 12,662,886 bp). To separate the deletions, we performed crosses with a strain that carries the homozygous strain DF135.

**Complementation Tests to Show that *fs8* and *ny10* Are Allelic**

Because *lep-5* is located on the X chromosome and the Lep phenotype is only observed in males, we used XX pseudomales to test whether *ny10* and *fs8* are dominant or recessive mutations and for genetic complementation of the two alleles. First, males from a tra-1(e1488III); him-5(e1490)V strain were crossed with hermaphrodites from a him-5(e1490)V; unc-18(e81) *lep-5(ny10)* strain. nonUnc F1 hermaphrodites were allowed to self and the nonUnc F2 pseudomales were scored for tail tip phenotypes. All of these animals had wild-type tails, demonstrating that *lep-5(ny10)* is recessive. The Unc F2 progeny from this cross were used to establish a *tra-1; him-5; unc-18* *lep-5(ny10)* strain. Hermaphrodites with this genotype were then crossed with post-dauer *lep-5(fs8)* males. nonUnc F1 hermaphrodites were allowed to self. If *fs8* and *ny10* were non-allelic, only 1/3 of the of the Tra nonUnc F2 pseudomales should display the Lep phenotype. We examined 79 such animals and found that all were Lep, indicating that both alleles are mutations in the same gene. To exclude that the proportion of hozygous *fs8* pseudomales was significantly distorted by the X chromosome non-disjunction in oocytes due to the *him-5* mutation in the background of all strains, we followed the progeny of these hermaphrodites; 48 animals were sterile, 10 gave only nonUnc progeny (and were therefore homozygous for *fs8*), 21 yielded F3 progeny of which 25% were Unc, confirming that these animals were indeed heterozygous for *fs8* and *ny10*. We concluded that *ny10* and *fs8* are alleles of *lep-5*.

**Transgenesis**

Transgenes for rescue experiments and expression constructs were made by overlap extension PCR (Nelson and Fitch, 2011) or by modification of plasmids using site directed mutagenesis. PCR products used for transgenesis were gel-purified with the Promega Wizard® SV Gel and PCR Clean-Up System. Unless otherwise noted, we used the prF4[rol-6(d)] plasmid at a concentration of 100 ng/μl as injection marker. PCR products were microinjected at a concentration of 0.5 or 1 ng/μl into the gonads of young hermaphrodites. Sequences of oligos used to generate DNA constructs for transgenesis are listed in Table S1.

**Fosmid and PCR Rescue**

Bacteria containing Geneservice fosmid clones WRM062bG06, WRM0629cE12, WRM0628aE08 and WRM0640cA10 were grown on plates overnight, resuspended in 5 ml of LB + 50 μg/ml kanamycin and plated to filter paper discs (7 mm) in 100 mm dishes containing LB + 2% agar. The fosmid identities were verified by sequencing (pCC1-forward). Fosmids were then linearized by digestion with SfiI, purified with QiAquick spin columns and injected into *lep-5(ny10)* hermaphrodites at a
concentration of 4 ng/μl. Fosmids were injected in groups of two or separately and transgenic males scored for rescue of the Lep phenotype. WRM0640ca10 was the only fosmid that rescued. Sections of its sequence, covering one or two predicted genes were generated by PCR and injected at a concentration of 1 ng/μl. A 6506 nt long PCR product obtained with primers H36l18.2_F and H36l18.2_R and covering the gene H36l18.2 rescued the phenotype of lep-5(ny10). To determine the minimal rescuing fragment for lep-5, subsequently smaller PCR products were injected into lep-5(fs8) mutants with Punc-122::GFP as injection marker.

Transgenes with secondary structure modifications of lep-5
Six different transgenes covering 3133 nt of the lep-5 transcribed and upstream sequence were made by PCR and injected into the gonads of lep-5(ny10) hermaphrodites at a concentration of 0.5 or 1 ng/μl. At least 4 lines were scored for each experiment.

1. lep-5(+), PCR fragment was amplified from N2 genomic DNA with primers KKlp5_expr-9 and KKlp5_expr-10.
2. lep-5(G23A) PCR fragment was amplified from genomic DNA of lep-5(fs8) mutants with primers KKlp5_expr-9 and KKlp5_expr-10. Because the lep-5(fs8) mutation is temperature-sensitive and the construct lep-5(G23A) showed some degree of rescue when injected at 1 ng/μl, we also injected it at a ten-fold higher concentration.
3. lep-5(G23A, C30T), Primers for A piece (forward and reverse): KKlp5_expr-1 and KKlp5_Cbr-AR used on N2 DNA.
4. lep-5(G23C), Primers for A piece: KKlp5_expr-1 and KKlp5_Cbr-R. Primers for B piece: KKlp5_Cbr-AR and KKOLp5-8a.
5. lep-5(G23C, C30G), Primers for A piece: KKlp5_expr-1 and KKlp5_Cbr-AR used on N2 DNA.
6. Pcep-lep-5::Cbr-lep-5(+). Primers for A piece (forward and reverse): KKlp5_expr-1 and KKlp5_Cbr-AR used on N2 DNA.
7. Pcep-lep-5::Can-lep-5(+). Primers for A piece (forward and reverse): KKlp5_expr-1 and KKlp5_Can-AR used on N2 DNA.

Four transgenes were made by overlap extension PCR using N2 genomic DNA as template. The modifications noted were introduced into the reverse primer of the first PCR product (A piece) and the forward primer of the second PCR product (B piece). The final product was amplified with primers KKlp5_expr-9 and KKlp5_expr-10.

Transgenes with Stop Codons and Deletions
Site-directed mutagenesis with QuikChangeXL (Agilent Technologies) was used to introduce stop codons into a plasmid containing the minimal rescuing fragment for lep-5 following the manufacturer’s instructions. This plasmid was generated by cloning a PCR product, made with attBlep-5F6 and attBlep-5R6, into the Gateway vector pDONR™P4-P1R (Life Technologies). Primers AA4,5,7F and AA4,5,7R were used to convert amino acids 4, 5 and 7 of the predicted ORF of H36l18.2 into stop codons. Amino acids 14 and 15 were converted into stop codons using primers AA14,15F and AA14,15R. The resulting plasmids were sequenced to confirm the changes and injected into hermaphrodites with Punc-122::GFP as injection marker.

To create deletion constructs, two PCR products, covering the minimal rescuing fragment for lep-5 with a gap at the intended deletion were amplified with primers that introduced an AatII restriction site at the 3′ end of the first and the 5′ end of the second piece. The PCR products were digested with AatII, gel-purified and ligated with T4 DNA ligase. Replacement of the intended sequence by the 6-bp tag GAGCTC was confirmed by sequencing. The constructs were injected into lep-5(ny10) hermaphrodites at a concentration of 10 ng/μl with Punc-122::GFP as injection marker.

To delete the endogenous lep-5 locus, genome editing with CRISPR/Cas9 (Arribere et al., 2014) was performed as described in the protocol from the Dernburg lab published on the Integrated DNA Technologies (IDT) website. Two guide RNAs were designed with ChopChop (Labun et al., 2016; Montague et al., 2014). The crRNAs, tracrRNA and CAS9 were purchased from IDT. The RNAs were reconstituted to a concentration of 200 nM in the IDT duplex buffer. 1 μl of each lep-5-specific crRNA and 2 μl of tracrRNA and 1 μl of dpy-10 crRNA and 1 μl of tracrRNA were mixed and incubated at 95 °C for 5 minutes. RNA duplexes for lep-5 and dpy-10 were mixed 1:12 and diluted to 62.5 μM. 0.5 μl RNA duplex mix was mixed with 0.5 μl of Cas9 enzyme and incubated at room temperature for 10 minutes. 1 μl RNP complex was used for 10 μl injection mix, supplemented with 0.2xTE buffer and 0.1 μM single-stranded DNA oligo to edit the dpy-10 locus. Young him-5(+) hermaphrodites were injected into both gonads and their phenotype.

Genome Editing Using CRISPR/Cas9
ny28
To delete the endogenous lep-5 locus, genome editing with CRISPR/Cas9 with dpy-10 coCRISPR (Arribere et al., 2014) was performed as described in the protocol from the Dernburg lab published on the Integrated DNA Technologies (IDT) website. Two guide RNAs were designed with ChopChop (Labun et al., 2016; Montague et al., 2014). The crRNAs, tracrRNA and CAS9 were purchased from IDT. The RNAs were reconstituted to a concentration of 200 nM in the IDT duplex buffer. 1 μl of each lep-5-specific crRNA and 2 μl of tracrRNA and 1 μl of dpy-10 crRNA and 1 μl of tracrRNA were mixed and incubated at 95 °C for 5 minutes. RNA duplexes for lep-5 and dpy-10 were mixed 1:12 and diluted to 62.5 μM. 0.5 μl RNA duplex mix was mixed with 0.5 μl of Cas9 enzyme and incubated at room temperature for 10 minutes. 1 μl RNP complex was used for 10 μl injection mix, supplemented with 0.2xTE buffer and 0.1 μM single-stranded DNA oligo to edit the dpy-10 locus. Young him-5(+) hermaphrodites were injected into both gonads and their phenotype.
offspring screened for the Rol and Dpy phenotype. Pools of 5 F1 hermaphrodites from plates with the most Rol and Dpy worms were screened for edits at the \textit{lep-5} locus by PCR with primers KK\_CR\_lep-5\_1 and and KK\_CR\_lep-5\_2. Several lines with deletion of the \textit{lep-5} locus were obtained, one of which was retained (\textit{lep-5}(ny28)). This allele deletes 572 nucleotides of the \textit{lep-5} gene, from 104 nt upstream of the transcription start site through 468 nt of the transcript, replacing this region with 7 random nucleotides and leaving 59 nt at the 3' end.

**fs18, fs19, fs21 and fs22**

To modify the endogenous \textit{lep-5} locus, CRISPR/Cas9 genome editing using \textit{dpy-10} coCRISPR was performed as described (Arribere\ et\ al.,\ 2014; Paix\ et\ al., 2015). One or two \textit{lep-5} guide RNAs were designed with CRISPOR (Haeussler\ et\ al.,\ 2016) and \textit{or ApE} software using the algorithm of Doench\ et\ al. (2014). The tracrRNA and crRNAs were purchased from Dharmacon. ssODNs and primers were made by IDT. Recombinant Cas9 (25 μg/μl) was prepared as described (Paix\ et\ al.,\ 2015). tracrRNA was reconstituted at 4 μg/μl with RNase-free Tris Buffer. Similarly, each crRNA was reconstituted at 8 μg/μl with Tris Buffer. Each ssODN was dissolved in RNase-free water at 500 ng/μl. For microinjection mixtures, we followed the published protocol (Paix\ et\ al.,\ 2015) strictly, except that the total volume of each mixture was halved from 20 μl to 10 μl. Microinjection mixtures were incubated at 37°C for 10 min before injection and used within 1h after preparation. Young \textit{him-5(+)} hermaphrodites were injected and their F1s were screened for Rol, Dpy or Dpy Rol phenotype. Pools of 3 F1 hermaphrodites from plates with the most Rol and Dpy worms were screened for edits at the \textit{lep-5} locus by PCR and restriction enzymes digest. For each allele, one or two lines were obtained and kept. All strains were backcrossed to N2 or \textit{him-5(e1490)} at least four times. Fragments containing the entire \textit{lep-5} gene, along with ~500 nt flanking regions, were confirmed by Sanger sequencing.

**fs21 fs25**

Molecularly, fs25 and fs22 are identical alleles of \textit{lep-5}. To generate fs21 fs25, the injection mix used for fs22 was injected into young \textit{him-5(+)}/V; \textit{lep-5(fs21)/oxT1015} X hermaphrodites. Non-fluorescent offspring, which must be \textit{lep-5(fs21)} homozygotes, were selected for future screening. Outcrossing and sequencing was done as for other \textit{fs} alleles.

**\textit{lep-5} Transcriptional Reporter**

A GFP reporter construct driven by the \textit{lep-5} regulatory region was generated by OES-PCR. Primers KKOLp5-1 and KKkp5-GFP-A-R were used to amplify the region upstream of the transcription start of \textit{lep-5} from N2 genomic DNA (piece A). This PCR product overlaps with piece B containing 4x NLS-GFP and the unregulated 3' UTR of \textit{let-858}. The combined piece was cloned into \textit{C. elegans} vector pCB4196 (Addgene). The final product was amplified with primers RHOLp5-7 and MN-lin-44_9, gel-purified and injected into CB4088 hermaphrodites; 8 lines were obtained.

**\textit{lep-5} IncRNA Expression via smFISH**

For smFISH, we used custom Stellaris probes from Biosearch Technologies specific for \textit{lep-5} IncRNA (22 probes) labeled with Quasar570 (excitation 548nm, emission 566nm) and for \textit{lin-44} (39 probes) labeled with Quasar670 (excitation 647nm, emission 670nm). The probes were designed by Biosearch Technologies. Sample preparation and hybridization were performed in tubes using a modification of the protocols by Ji\ and\ van\ Oudenaarden (2012) and the protocol provided by Biosearch Technologies. Briefly: mixed stage or synchronized L2 worms from strains CB4088 (WT) and DF135 (\textit{lep-5}(ny10)) were collected, washed in M9 buffer and fixed in 4% paraformaldehyde in 1x PBS for 1h at room-temperature. Fixed worms were washed twice with 1x PBS and transferred to 70% ethanol and kept on a rotating shaker at 4°C overnight. Worms were then washed with wash buffer (10% deionized formamide in 2x SSC) and hybridized in hybridization buffer (100mg/ml dextran sulfate and 10% formamide in 2x SSC) for 48 hours at 37°C in a hybridizer. The hybridization solution was washed off with wash buffer and the samples incubated in wash buffer for 30 minutes at 30°C. Worms were washed in 2x SSC and mounted in ProLong Gold with DAPI (Life Technologies) in imaging chambers as described by Ji\ and\ van\ Oudenaarden (2012). The slides were imaged on the DeltaVision Elite Imaging System using solid-state illumination with filters for DAPI, TRITC (with Quasar570 labeled probes) and Cy5 (with Quasar670 labeled probes). Images were recorded with an Evolve 512 EMCCD camera and processed using the softWoRx software package.

**Sequences of the Stellaris RNA FISH Probes**

For smFISH, we used custom Stellaris probes from Biosearch Technologies specific for \textit{lep-5} IncRNA (22 probes) labeled with Quasar570 (excitation 548nm, emission 566nm) and for \textit{lin-44} (39 probes) labeled with Quasar670 (excitation 647nm, emission 670nm). The probes were designed by Biosearch Technologies. Sample preparation and hybridization were performed in tubes using a modification of the protocols by Ji\ and\ van\ Oudenaarden (2012) and the protocol provided by Biosearch Technologies. Briefly: mixed stage or synchronized L2 worms from strains CB4088 (WT) and DF135 (\textit{lep-5}(ny10)) were collected, washed in M9 buffer and fixed in 4% paraformaldehyde in 1x PBS for 40 minutes at room-temperature. Fixed worms were washed twice with 1x PBS and transferred to 70% ethanol and kept on a rotating shaker at 4°C overnight. Worms were then washed with wash buffer (10% deionized formamide in 2x SSC) and hybridized in hybridization buffer (100mg/ml dextran sulfate and 10% formamide in 2x SSC) for 75min \textit{lin}-44 probes and 250min \textit{lep}-5 probes for 4 hours at 37°C in a hybridizer. The hybridization solution was washed off with wash buffer and the samples incubated in wash buffer for 30 minutes at 30°C. Worms were washed in 2x SSC and mounted in ProLong Gold with DAPI (Life Technologies) in imaging chambers as described by Ji\ and\ van\ Oudenaarden (2012). The slides were imaged on the DeltaVision Elite Imaging System using solid-state illumination with filters for DAPI, TRITC (with Quasar570 labeled probes) and Cy5 (with Quasar670 labeled probes). Images were recorded with an Evolve 512 EMCCD camera and processed using the softWoRx software package.
Herrera et al. (2016): L2 stage animals were examined and fluorescence in the pharynx region was captured for the green and red were investigated. The fluorescence signal of the reporter was visualized with a confocal microscope (Leica, SP8) as described in the exposed region were taken for the red and green channel to record the post-photoconversion fluorescent signal. The animal 405 nm light in 10-20 slices with ten successive scans of the z-plane with short rest periods inbetween. Sequential z-stacks of channels by sequential scans. Dendra2 photoconversion was performed by exposing a section of the pharynx to brief flashes of hemizygous male cross-progeny of \textit{lep-5} genetic background, this transgene caused bursting of hermaphrodites, and no stable line could be established. Therefore, e7 Developmental Cell

**Time-Course of \textit{lep}-5 Expression via qPCR**

Staged animals were obtained with the hatch-off method (Pepper et al., 2003) and collected 0, 12, 15, 18, 22, 26 and 41 hours after hatching at 25°C and flash-frozen. RNA was extracted using a modification of the tissue protocol for the RNAeasy micro kit from Qiagen. Frozen worms were ground with a plastic pestle fitting into a 500µl tube. When the worms began to thaw, the tube was dipped into liquid nitrogen to re-freeze. This process was repeated 5 to 6 times before buffer was added and the sample disrupted by passing it through a syringe needle. A DNase treatment was performed on-column. Wash-steps were performed according to the protocol. RNA was eluted with water and concentrations checked by NanoDrop (Thermo Scientific). cDNA was generated from 250ng total RNA using the double-prime RNA to cDNA EcoDry Premix (Takara/Clontech). qPCR was performed using the iQ SYBR Green Supermix with a two-step PCR protocol (3 min 95°C; 40x 10 sec 95°C, 60 sec 60°C; followed by melt curve analysis) on the CFX96 real time PCR detection system (BioRad). Y45F10D.4 served as reference gene (Hoogewijs et al., 2008) For primer sequences see Table S1. Unknowns were run in triplicates, standards in singles. Data were evaluated using the standard-curve method.

**RNAi Knockdown**

RNA-interference was performed by feeding \textit{E. coli} transformed with inducible RNAi vectors to mothers or larvae as previously described (Nelson et al., 2011). Bacterial strains for \textit{lin}-14 and \textit{lin}-41 RNAi were from the J. Ahringer library, and those for \textit{lin}-28 from the M. Vidal library (Source Bioscience). For \textit{lin}-14 RNAi, L4 hermaphrodites were treated and their male progeny scored as L3, L4 and adults for defects in tail tip morphogenesis. For \textit{lin}-28 and \textit{lin}-41 RNAi, L1 larvae were fed and scored as L4 and adults. Bacterial strain HT115 carrying the L4440 plasmid was used as negative control.

**\textit{lin}-28 mRNA Levels**

Staged WT (CB4088) and \textit{lep}-5\textit{(ny10)} animals were collected by 4h hatch-off. Some of the L1 were flash-frozen immediately, the rest was plated onto seeded plates and placed at 20°C. L3 larvae were collected and flash frozen 27 hours later. RNA extraction and cDNA synthesis (using 440ng RNA) were performed as described above. qPCR was performed with primers \textit{lin}-28\_FW2 and \textit{lin}-28\_RV2 and Y45F10D.4 as reference gene as above.

**Western Blot**

Western blot analysis by SDS-PAGE was performed according to standard procedure. For the L1 sample, arrested L1s were placed on food and collected a few hours later. L3 samples were collected 24 hours after plating arrested L1s at 20°C. Animals were washed twice with PBS, frozen in liquid nitrogen and ground in the presence of a protease inhibitor (Halt, Pierce), SDS buffer was added. Samples were heated to 95°C for 10 minutes and centrifuged to pellet the insoluble fraction. An aliquot was kept for Bradford assay. Approximately 10-20 µg of total protein was loaded onto a 10% Bis-Tris Bolt gel (Invitrogen). After electrophoresis and transfer to a nitrocellulose membrane, the blot was incubated overnight at 4°C with a rabbit anti-LIN-28 polyclonal antibody (gift from E. Moss; 1:5,000 dilution) and a mouse anti-actin monoclonal antibody (Sigma; 1:5,000 dilution) as a loading control. The blot was incubated 45 min in the dark with fluorescent secondary antibodies (Licor; 1:25,000) and scanned on a Licor infrared fluorescence scanner.

**Analysis of LIN-28 Degradation**

To observe LIN-28 dynamics in \textit{lep}-5 mutants, we used a \textit{Plin-28::Dendra2::lin-28\_3’UTR} reporter gene (Herrera et al., 2016). In the \textit{lep}-5 genetic background, this transgene caused bursting of hermaphrodites, and no stable line could be established. Therefore, hemizygous male cross-progeny of \textit{him}-5; \textit{nyEx56[Plin-28::Dendra2::lin-28\_3’UTR]} males and \textit{him}-5; \textit{lep}-5\textit{(ny10)} hermaphrodites were investigated. The fluorescence signal of the reporter was visualized with a confocal microscope (Leica, SP8) as described in Herrera et al. (2016); L2 stage animals were examined and fluorescence in the pharynx region was captured for the green and red channels by sequential scans. Dendra2 photoconversion was performed by exposing a section of the pharynx to brief flashes of 405 nm light in 10-20 slices with ten successive scans of the z-plane with short rest periods inbetween. Sequential z-stacks of the exposed region were taken for the red and green channel to record the post-photoconversion fluorescent signal. The animal was recovered onto a plate with food and kept at 20°C for 24 h. The worms (now L3) were remounted, and another sequential z-stack was recorded. The image stacks were analyzed with ImageJ.

**RNA co-IP with LEP-2, LIN-28 and LIN-41**

Worms were synchronized by L1 arrest and grown for 17 hours at 25°C to the L2 stage (DF237 carrying LIN-28::GFP and DF282 carrying GFP::LIN-41), or mixed stages were used (DF293 carrying GFP::LEP, DF302 carrying \textit{lep}-5::GFP). Worms were washed twice in M9 buffer and irradiated in a BioRad crosslinker with 800 mJ/cm² at 254nm. UV-treated worms were washed once in lysis buffer (20 mM Tris/Cl pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 0.5 % NP-40 [Igepal]), resuspended in an equal volume of lysis buffer supplemented with HALT protease inhibitor (Invitrogen), RNaseOUT (Thermo Fisher) and DTT and flash frozen in liquid nitrogen. At least three biological replicates were obtained for each genotype. The frozen worms were manually ground in Takara Biomasher tubes and lysed in 200 µl lysis buffer supplemented with HALT protease inhibitor and RNaseOUT. IP was performed with Chromotek.
GFP-Trap MA and Chromotek binding control magnetic agarose beads according to the guidelines by the company. The beads were equilibrated in dilution buffer (20 mM Tris/Cl pH 7.4, 150 mM NaCl, 5 mM MgCl₂) and resuspended in dilution buffer supplemented with RNase OUT, DTT and 400 μM vanadyl ribonucleoside complexes. 40-90 μl lysate was added to each kind of beads and incubated for 60 minutes at 4°C while tumbling end over end. 20% of this volume (8-18 μl) was set aside as input sample. Beads were washed twice with dilution buffer and twice with wash buffer (20 mM Tris/Cl pH 7.4, 250 mM NaCl, 1 mM MgCl₂, 0.025% SDS, 0.05% NP-40 (Igepal)). Beads and input samples were then treated for 20 minutes with Proteinase K (100μl PBS with 1μl 10% SDS and 2.5% Proteinase K (Invitrogen)) at 65°C on a Thermomixer (Eppendorf) to reverse the crosslink and digest the proteins. RNA was extracted with Trizol and BCP, precipitated overnight at -80°C after adding Glycolblue™ (Ambion), sodium acetate and isopropanol. The pellet was washed once with 70% ethanol, air dried and resuspended in 44μl water. A DNase treatment with RNase I (Ambion) was performed for 20 minutes at 37°C. The RNA was re-purified immediately using the Qiagen RNeasy Micro kit following the manufacturer’s protocol. The RNA was eluted with 12μl water. 10μl RNA was used in a 20μl cDNA synthesis reaction with the Clontech/Takara double-primed EcoDry cDNA synthesis kit, 2pg luciferase control mRNA (Promega) was added to each reaction as a control for cDNA synthesis. The cDNA was diluted 1:7 for qPCR on a BioRad CFX instrument using the BioRad iQ SYBR Green Supermix and primers for lep-5, cdc-42 and luciferase. cdc-42 was chosen as a control gene because it was present at similar levels as lep-5 (i.e., in qPCR of the input sample, the Ct values for these genes differed by no more than 1.5 except in the LIN-41::GFP strain, which showed especially high levels of lep-5). All samples were run in triplicates. The results were evaluated using the Pfaffl Method (Pfaffl, 2001) with empirical efficiencies of 2 for luciferase, 1.98 for lep-5 and 1.91 for cdc-42, comparing Ct values for GFP-trap beads (experiment) to binding control. Results for lep-5 and cdc-42 were normalized against the spiked-in luciferase to account for differences in the efficiency of the RT reaction. A moderated T-test implemented in the Limma package was performed to test for significance of the results. This test is specifically designed for expression analyses with small sample sizes (Ritchie et al., 2015).

**Identification of lep-5 Orthologs from Other Caenorhabditis Species**

Sequences of lep-5 transcripts for C. brenneri, C. elegans and C. japonica were confirmed by ESTs in WormBase. Genomic lep-5 sequences for 15 other Elegans supergroup species (Kiontke et al., 2011) were extracted from whole genomes (WormBase, and http://caenorhabditis.org) after BlastN search (Camacho et al., 2009). An unambiguous lep-5 homolog was not identified in the C. sp. 26 genome. All sequences contained two introns, which were edited out manually by assuming that their positions are homologous and that they bear the typical GT and AG motif at their 5' and 3' ends, respectively. No significant matches were found by BlastN alone in Caenorhabitis species outside of the Elegans supergroup. However, after multiple sequence alignment of a syntenic region in C. angaria and 7 Elegans supergroup species, we identified a sequence in the C. angaria genome with similarity to lep-5. A BlastN search subsequently yielded a partial C. angaria lep-5 cDNA sequence that had been previously assembled from RNA-seq reads, as part of the C. angaria genome project (Mortazavi et al., 2010). This sequence was used to design internal primers, which together with a primer complementary to SL1 and an anchored oligo(dT) primer amplified two overlapping fragments of lep-5 from C. angaria cDNA (made from total RNA with the double-primed EcoDry Premix by Takara/Clontech). The fragments were sequenced to identify the full C. angaria lep-5 transcript. This transcript was used for a BlastN search of draft genome assemblies for Caenorhabditis species outside of the Elegans supergroup (available from the laboratory of Mark Blaxter at http://download.caenorhabditis.org). This yielded lep-5 loci from C. castelli, C. sp. 38, C. plicata, and C. virilis, which we confirmed by BlastN with the C. virilis lep-5 genomic region. We used MAFFT v.7.266 (Katoh and Standley, 2013) (to align the predicted lep-5 transcripts of 19 Elegans group species (Figure S1), our lep-5 cDNA and genome sequences from C. angaria, and the four lep-5 genomic regions from non-Elegans group species. To maximize its accuracy and control the order of aligned sequences, MAFFT was run with the arguments ‘--localpair --maxiterate 16 --inputorder’. We manually edited the resulting alignment in Jalview 2.9.0b2 (Waterhouse et al., 2009) to remove trimming or poorly aligned flanking regions. Via the hmmbuild and hmmpress programs from HMMER 3.1b2 (Eddy, 2011), we converted the edited lep-5 alignment into a hidden Markov model (HMM). Searching other non-Elegans supergroup Caenorhabditis genomes with the 24-species HMM and nhmmr (Wheeler and Eddy, 2013) identified two more lep-5 loci from Caenorhabditis spp. 43 and 31. We also attempted HMM searches of non-Caenorhabditis species; although this gave various weak similarities, they were neither statistically significant nor similar to one another. We finally aligned the lep-5 sequences from all 26 Caenorhabditis species with MAFFT and visualized their alignment with Jalview (Figure S3).

**Analysis of the Secondary Structure of the lep-5 Transcript**

The processed lep-5 RNA sequence, including the SL1 trans-spliced leader, along with similar information from lep-5 orthologs from twenty Elegans group Caenorhabditis species, was analyzed using TurboFold (Harmanci et al., 2011), part of the RNAstructure package (http://rna.urmc.rochester.edu/RNAstructure.html). To model lep-5(G23A) and lep-5(G23A C30T), corresponding changes were made to all twenty orthologous sequences. The folding temperature was set at 293.15 K (20°C); default parameters were used for all other settings. Structures were drawn using VARNA (Darty et al., 2009). Nucleotide positions were color-coded for confidence level and primary sequence conservation as described in the text. VARNA linear-format representations of Turbofold structures for all species were arranged according to an unpublished phylogenetic tree for Caenorhabditis inferred by K. Kiontke using molecular data from 17 loci (RhabditinaDB, rhabditina.org), as shown in Figure S4.

Using an independent method of alignment and structure analysis produced very similar results. Specifically, we used CARNA (ver. 1.3.3, linking LocARNNA 1.9.1, Gecode 5.0.0, Vienna RNA package 2.3.2, http://rna.informatik.uni-freiburg.de) (Raden et al., 2018; Sorescu et al., 2012) and RNAalifold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAalifold.cgi). CARNA generated a
dot-plot of probabilities of basepairing which was used to generate a consensus sequence and structure, used in an iterative manner to optimize an alignment based on secondary structure conservation. This alignment was very slightly modified by hand to allow a couple additional gaps to further optimize the alignment to the average structure. This alignment was used as input to RNAalifold to generate a consensus RNA structure in which alignment positions were highlighted with regard to conservation and basepair co-variance. Although the resulting structure differed in several details from that shown in Figure 2A of this paper, there were three regions that were essentially identical: the 5' stem-loop involving SL1, the 3' stem-loop, and the central "zipper" region. Thus, very different structure-alignment methods yield essentially similar results with regard to these three important regions (highlighted red, blue and green, respectively, in Figures 2 and S4).

Search for miRNA Binding Sites in the lep-5 Transcript
The lep-5 sequence was scanned for miRNA binding sites using algorithms available on www.microrna.org, www.targetscan.org and the website of the Segal lab https://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html (Kertesz et al., 2007). High scoring miRNAs found by all three algorithms are miR-76, miR-265, miR-1830 and miR-2220. None of these miRNAs has been implicated in the heterochronic pathway.

QUANTIFICATION AND STATISTICAL ANALYSIS
For statistical analysis of the RNA co-IP experiments, we used a moderated T-test as described above in the Method Details, “RNA co-IP with LEP-2, LIN-28 and LIN-41” section.
Supplemental Information

The Long Non-Coding RNA *lep-5*

Promotes the Juvenile-to-Adult Transition

by Destabilizing LIN-28

Karin C. Kiontke, R. Antonio Herrera, Edward Vuong, Jintao Luo, Erich M. Schwarz, David H.A. Fitch, and Douglas S. Portman
Figure S1. Additional *lep-5(ny10)* phenotypes, related to Figure 1.
(A, E): Head of a hermaphrodite undergoing a supernumerary molt. (B-D) Lateral fields of hermaphrodites before the supernumerary molt. There are no alae (B), incomplete alae (C) or very weak alae (D) in the anterior part of the body; the posterior part of the body usually lacks alae. (F-H) lateral fields of hermaphrodites after the supernumerary molt: Alae are disorganized (F) or incomplete (G and H). (I, J): vulva before (I) and after initiation of the supernumerary molt (J). (K, L) vulva after completion of the supernumerary molt; the animals are Pvl and Egl. (M) Adult male tail with normal fan and Lep tail tip undergoing delayed TTM. (N) Tail of an adult male after *ain-1* RNAi treatment. The fan is narrow and the rays are short, indicating a defect in anterior retraction. (O) Tail of an adult male after *ain-1* RNAi treatment in ventral view showing extreme ectopic retraction of the tail tissue. Rays and phasmids are attached to the cuticle and appear as long processes.
Figure S2. Mapping of lep-5, related to Figure 2. (A) Rainbow plot showing the result of array comparative genomic hybridization with DNA from strain DF70 carrying the lep-5(ny10) mutation compared to DNA from the control CB4088 strain. DF70 shows two deletions in chromosome X. Subsequent analysis determined that lep-5(ny10) is associated with the right deletion. (B) The 80Kb region deleted in lep-5(ny10) contains 32 predicted genes and one pseudogene in wild-type DNA. Dashed and solid lines indicate fosmids and PCR products used in experiments to rescue the lep-5(ny10) phenotype. (C) Genomic region containing H36L18.2/lep-5 and graphic display of fragments used in rescue experiments with lep-5(fs8). A minimum length of 1970 nucleotides upstream of the transcription start site of lep-5 was required for successful rescue, indicating that this region contains important regulatory sites. The point mutation in lep-5(fs8) is marked by a red triangle.
Figure S3. Alignments for lep-5 orthologs from Caenorhabditis species; related to Figure 2. (A) ClustalOmega alignment of lep-5 sequences from 19 Caenorhabditis species. The manually added SL1 sequence is in lowercase letters. Sequencing gaps of unknown size in Caenorhabditis sp. 29 and C. sp. 32 are indicated by "N". Invariant positions are marked by asterisks. The most highly conserved regions are at the 5'- and 3'-ends of the sequence. (B) MAFFT alignment of lep-5 coding sequences, cDNA, and genomic sequences from 26 Caenorhabditis species. Sequences were identified and aligned as described in the Supplemental Experimental Procedures. The first 19 lep-5 coding sequences from the Elegans supergroup (Kiontke et al., 2011) are identical to those in Figure S3. Portions of those 19 lep-5 sequences shown here correspond to the following subsequences: C. afra, nt 1-563; C. brenneri, nt 1-517; C. briggsae, nt 1-547; C. dougertyi, nt 1-507; C. elegans, nt 1-524; C. japonica, nt 1-481; C. kamaaina, nt 1-498; C. latens, nt 1-531; C. macroperma, nt 1-511; C. nigoni, nt 1-547; C. noudraegansis, nt 1-491; C. remanei, nt 1-521; C. sinica, nt 1-527; C. sp. 28, nt 1-503; C. sp. 29, nt 1-472; C. sp. 32, nt 1-506; C. sp. 40, nt 1-521; C. tropicalis, nt 2-503; and C. wallacei, nt 1-534. The portion of the C. angaria lep-5 cDNA sequence shown corresponds to nt 1-484. Other lep-5 genomic sequences come from the following genome assemblies (given as source URLs), scaffolds within each assembly, and nucleotide coordinates within each scaffold. C. angaria: ftp://ftp.wormbase.org/pub/wormbase/releases/WS250/species/c_angaria/PRJNA51225/c_angaria.

Figure S5. Predicted structures of *lep-5* RNAs indicating base-pairing confidence, related to Figure 2. Shown are predictions for the secondary structure of full-length (A) wild-type *lep-5*, (B) *lep-5*(G23A), and (C) *lep-5*(G23A C30T). SL1 sequence is shown in red. Other nucleotide positions are colored according to base-pairing confidence as predicted by Turbifold, with yellow and blue representing low and high confidence, respectively.
Figure S6. Spatiotemporal expression of Plep-5::NLS::GFP, related to Figure 3. (DIC images left, fluorescent images right.) Expression begins to be faintly visible in late L1 animals (A). It becomes bright during L2 and remains bright until late L3 (C-F). Expression diminishes in early-mid-L4 (G, H) and is almost gone in adults (I). Expression is observed in the tail epidermis including the tail tip cells (B, F, G), in the pharynx muscles (E), nerve cells in the pharynx (E) and cloacal region (F) and in the ventral nerve cord (C, bracket). The reporter is also expressed in seam cells (arrowheads in C and D) and in the vulva cells during vulva morphogenesis (J).
Figure S7. *lin-41* expression persists into adulthood in *lep-5* mutants, related to Figure 4.
Expression of a transcriptional *Plin-41::NLS::GFP:*lin-41 3’UTR reporter in wild type and *lep-5*(*ny10*) mutant animals (left panels show DIC images for comparison). In wild type (A-E), the reporter is expressed brightly in many nuclei of the tail, including the tail tip cells in larvae as early as L2. It diminishes after the L3 stage and is restricted to few cells in late L4 and adults. (A-E, top animal in F). In *lep-5*(*ny10*) mutants (bottom animal in F, G-I), expression remains bright in adults and is even visible in older animals that have undergone an ectopic molt (I; arrow points to a ray which remains a finger-like process after molt of the fan).
Table S1. Primers, guide RNAs, and ssODN repair templates, related to STAR Methods

Rescue experiments:
pCC1-forward: ggatgtgctgcaaggcgattaagttgg (to test fosmids)
H36L18.2_F: gggcgaatagcctttgaagtgtttctgttg
H36L18.2_R: gaccagataaagtgtagctgtgagcagattatgatatcgc
lep-5F1: gtaatctgcgtcttctaggt
lep-5R1: gggcgaatagcctttga
lep-5F2: gaccagataaagtgtagctgtgagcagattatgatatcgc
lep-5F3: cttcaacaacactgtccttc
lep-5R3: tagttgaacaggctgtgtgt
lep-5F4: aagtacatgcgaacttgtgt
lep-5R4: gggcgaatagcctttga
lep-5F5: cttcaacaacactgtccttc
lep-5R5: accagcatatatgattttgca
lep-5F6: aagtacatgcgaacttgtgt
lep-5R6: accagcatatatgattttgca
attBlep-5F6: gggaccaacacttggtataagaaagttgaaagtacatgcgaacttgtgt
attBlep-5R6: gggaccaacacttggtataagaaagttgaaagtacatgcgaacttgtgt

Secondary structure modifications:
KKlp5_expr-9: caaagtacatgcgaacttgtgtgc
KKlp5_expr-10: cggtcactttggttccattgaatc
KKlp5_expr-1: cacttcaaacacaactgctcttccttatc
KKlp5_23A+30T-R: gcccatgtctttgagaaaactctgaaaattgaaaataatcgataacttaaattcg
KKlp5_23A+30T-F: aatattgaattctgatttttaaatctttctcaagacatg
KKOl5p-8a: ccattagaaaccagctgtaagc
KKlp5_23C_R: ccatgtcttttggaagcctgaaatgaaataatcgataacttaaattcg
KKlp5_23C_F: aatttaagttatcgattattttcaattttcagcgttttcccaaagacatg
KKlp5_23C+30G-R: gcccatgtctttgcgaaaacgctgaaaattgaaaataatcgataacttaaattcg
KKlp5_23C+30G-F: aatttaagttatcgattattttcaattttcagcgttttcgcaaagacatg
RHOLp5-2: gcttggtgatttagcatcacc
KKlp5_Cbr-AR: ctaagttgcccatgccatcgggaagaaccctgaaaattgaaaataatcgataacttaaattcg
KKlp5_Cbr-BF: tatattgatagtttctgatttttaaatctttctgctgctggtctgctgctg
KKlp5_Cbr-nR: ctctcgtcatggaaaacaacaaaag
KKOl5p-1: taatttagttctggtctgatttttaaatctttctgctgctggtctgctgctg
KKlp5_Can-AR: gttagatctaatgtgccaagtttgagcaagctgaaaattgaaaataatcgataacttaaattcg
KKlp5_Can-BF: cgaatttaagttatcgatttttaaatctttctgctgctgctggtctgctgctg
KKlp5_Can-nR: tccgcaagaaataatggagaatattcgg

Site-directed mutagenesis
AA4,5,7F: gcagcatctttgctacaacacttagcttcattaagtttagtttaattttgattgctatttatttaatctctcatactgtgtgtctgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg
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