



Degradome sequencing reveals an endogenous microRNA target in *C. elegans*



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ABSTRACT

***Caenorhabditis elegans* microRNAs (miRNAs) bind to partially complementary sequences in the 3' untranslated region of target mRNAs, resulting in translational repression through mRNA destabilization. High-throughput sequencing of RNA cleavage fragments was performed to directly detect miRNA-directed cleavage targets in adult stage *C. elegans*. From this analysis, we found that miR-249 directed the cleavage of the ZK637.6 transcript with extensive and evolutionarily conserved complementarity in nematode. In addition, expression of the ZK637.6 transcript was strongly dependent on the expression of miR-249. These findings may lead to a better understanding of miRNA-mediated gene regulation in nematodes.**

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1. Introduction

MicroRNAs (miRNAs), discovered in *Caenorhabditis elegans* [1], are non-coding RNAs with significant roles in regulating gene expression during organism development [2]. These small (~22 nt) RNAs direct post-transcriptional repression of target mRNAs in a sequence-specific manner [2]. miRNAs and their targets interact in plants and animals in generally different ways. In animals, including *C. elegans*, most miRNAs bind to partially complementary sites resulting in mRNA destabilization and translational repression. This partial complementarity usually includes the 5'-end consisting of 2-to-8 nucleotides of the miRNA, the miRNA seed region [3], and, in some cases, a 3'-compensatory site [4] that compensates for insufficient 5'-seed pairing by substantially pairing outside the seed region. A recent study suggests that some validated miRNA target sites do not contain perfect seed matches but instead exhibit complementarity between the central region of miRNA and target mRNAs [5]. Due to partial complementarity, a single miRNA can potentially repress the expression of multiple genes, which might globally influence the protein production of a particular cell. The impact of this type of miRNA regulation on an individual gene's activity is usually very marginal, often a less than

2-fold level of repression [6,7]. However, in plants, most miRNAs base pair to target genes with perfect or near-perfect complementarity, allowing RISC-mediated cleavage of the target, thus inducing a significantly more robust effect on gene regulation [8]. This finding has largely been regarded as a plant-specific phenomenon, but a few cases of miRNA-directed cleavage targets have been reported in animals [9,10]. Therefore, our initial interest was to directly detect miRNA cleavage targets in *C. elegans*, in which the miRNA-directed cleavage target has never been reported. In the present study, with the aid of degradome sequencing in *C. elegans*, a transcriptome-wide survey of miRNA-directed cleavage products [11,12], we found that miR-249 regulates a ZK637.6 pseudogene by a cleavage mechanism.

Pseudogenes, facsimiles of homologous parental genes, are generally considered nonfunctional sequences of genomic DNA because most have lost their ability to produce a functional, full-length protein [13]. It has been therefore assumed that the pseudogenes are accidental by-products in a state of mutational drift and are transcriptionally silent. Several surveys have provided the evidence for transcription of at least one-fifth of all known human pseudogenes based on tiling-array/RACE analysis and high-throughput sequencing data [13]. However, the biological and functional implications of those pseudogenes are mostly unexplored.

Motivated by these recent advances, we employed degradome sequencing to directly detect endogenous miRNA cleavage targets

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in *C. elegans*. The first discovery of miRNA-directed cleavage in *C. elegans* will serve to rapidly expand the knowledge of the miRNA-mediated gene regulation in nematodes.

2. Materials and methods

2.1. Strains of *C. elegans*

All *C. elegans* strains were maintained as previously described [14].

2.2. Degradome library construction and data analysis

Degradome library construction, sequencing and raw cleavage tag data processing were performed essentially as described [5]. More detailed procedures are available in the [Supplementary Methods](#). Raw cleavage tag data were processed as described [5]. Approximately 7.6 million 20-nt tags from wild-type adult cleavage libraries were mapped to the *C. elegans* genome (ce6), requiring a perfect match. For tags mapped to ≤ 10 genomic loci, the final tag number was normalized with the loci number. Of genome-mapping tags, approximately 70% mapped to exons of annotated genes (RefSeq with new 3' UTR annotations) [15], including rRNAs, approximately 25% mapped to intergenic regions, approximately 3% mapped to intronic regions of genes, and 0.5% mapped antisense to annotated genes. High-throughput sequencing data were deposited to NCBI GEO with the accession number GSE44336.

2.3. 5' RLM-RACE and 5' and 3' RACE-PCR

For mapping the internal cleavage site in the ZK637.6 transcript, 5' RLM-RACE was performed essentially as described by the GeneRacer™ Kit (Invitrogen). For obtaining a full-length sequence of the ZK637.6 transcript, 5' and 3' RACE-PCR was performed essentially as described by the GeneRacer™ Kit (Invitrogen). More detailed experimental procedures and primers used for the analysis are available in the [Supplementary Methods](#).

2.4. Quantitative real-time PCR

Quantitative real-time PCR was performed using the LightCycler® 480 Real-Time PCR System from Roche. More detailed experimental procedures and primers used for the analysis are available in the [Supplementary Methods](#).

2.5. TaqMan MicroRNA assay

Mature miRNA quantification was performed using TaqMan MicroRNA Assays for cel-miR-249, essentially to the manufacturer's instruction (Applied Biosystems). More detailed experimental procedures and primers used for the analysis are available in the [Supplementary Methods](#).

2.6. Small-RNA sequencing library construction and analysis

Small-RNA sequencing library construction was performed using ScriptMiner™ Small-RNA-Seq Library Preparation Kit, according to the manufacturer's instruction (Illumina). More detailed experimental procedures and primers used for the analysis are available in the [Supplementary Methods](#).

2.7. Microinjection and microscopy

Microinjection of DNA into the gonads of the adult hermaphrodites was performed essentially as described previously [16]. More

detailed experimental procedures are available in the [Supplementary Methods](#).

3. Results and discussion

3.1. miR-249-directed cleavage in *C. elegans*

Degradome sequencing was performed to directly detect small RNA-directed cleavage targets in *C. elegans*. From this approach, miRNA-directed cleavage of the ZK637.6 transcript by miR-249 was identified in adult stage *C. elegans* (Fig. 1A). miR-249 has near-perfect complementarity to a site in the ZK637.6 transcript with a single-nucleotide mismatch and a bulge on miR-249 (Fig. 1B). We noticed that the ZK637.6 transcript exhibited extensive base-pairing with the 3' region of the miR-249 to compensate for a single-nucleotide bulge in the seed region. To determine whether the cleavage of the ZK637.6 transcript is mediated by high complementarity with miR-249, we mapped the 5' ends of the ZK637.6 transcript fragments by using a modified form of 5'-RACE (rapid amplification of cDNA ends) in wild-type adult worms. The positions corresponding to the middle of the respective sequences complementary to miR-249 were markedly detected by 5'-RACE (Fig. 1B). Of the 10 RACE clones that ended in the vicinity of the miR-249 target site, nine matched between the 11th and 12th nucleotides and one matched between the 10th and 11th nucleotides in the complementary site. Although miRNAs are generally known to induce cleavage of their target RNAs between 10th and 11th nucleotides within the complementary site [9], micro-heterogeneity of the cleavage has also been reported in mammals [10] and in plants [17]. We therefore performed 5'-RACE with biological and technical replicates, as many as 213 of the 5'-RACE clones, in wild-type worms and in *mir-249* mutants. These results were compared to determine whether these respective RACE clones were significant and dependent on miR-249. We found that the cleavage of the ZK637.6 transcript between the 11th and 12th nucleotide in the complementary site was significantly dominant (143 clones out of 203 clones; 70.4%) and was clearly dependent on miR-249 because the respective cleavage tag was never found in the *mir-249* mutant (Fig. 1C and D). We also found the cleavage signals from 22nt endo-siRNAs in wild-type as well as in *mir-249* mutants, but did not find significant differences between them (Fig. 1D). To check whether such miRNA-mediated cleavage is a prevalent phenomenon in *C. elegans*, we examined the association between degradome tags and other worm miRNAs. The miRNA-mediated cleavage pathway in *C. elegans*, at least in the adult stage, was not as prevalent as that observed in human cell lines and tissues [5]. The cleavage signal was not significantly associated with near-perfect sites of miRNAs expressed in adult worms; therefore, miR-249-mediated cleavage of ZK637.6 transcript was a special case. However, we cannot exclude other possible cases of miRNA-directed cleavage in *C. elegans*. The degradome library was constructed using only adult stage worms, and therefore, our library might not reflect all possible cases. Therefore, it is expected that other possible miRNA-directed cleavages might be observed in worms under different conditions, such as worms in other life stages or faced with environmental or physical stresses, like overcrowding, limited food, or elevated temperatures [18].

3.2. Negative correlation between expression of miR-249 and the ZK637.6 transcript

We next examined whether miR-249 directs the cleavage of the ZK637.6 transcript, thereby inducing a significant change in the expression of the ZK637.6 transcript. We first employed a TaqMan MicroRNA assay to investigate the developmental stage-specific

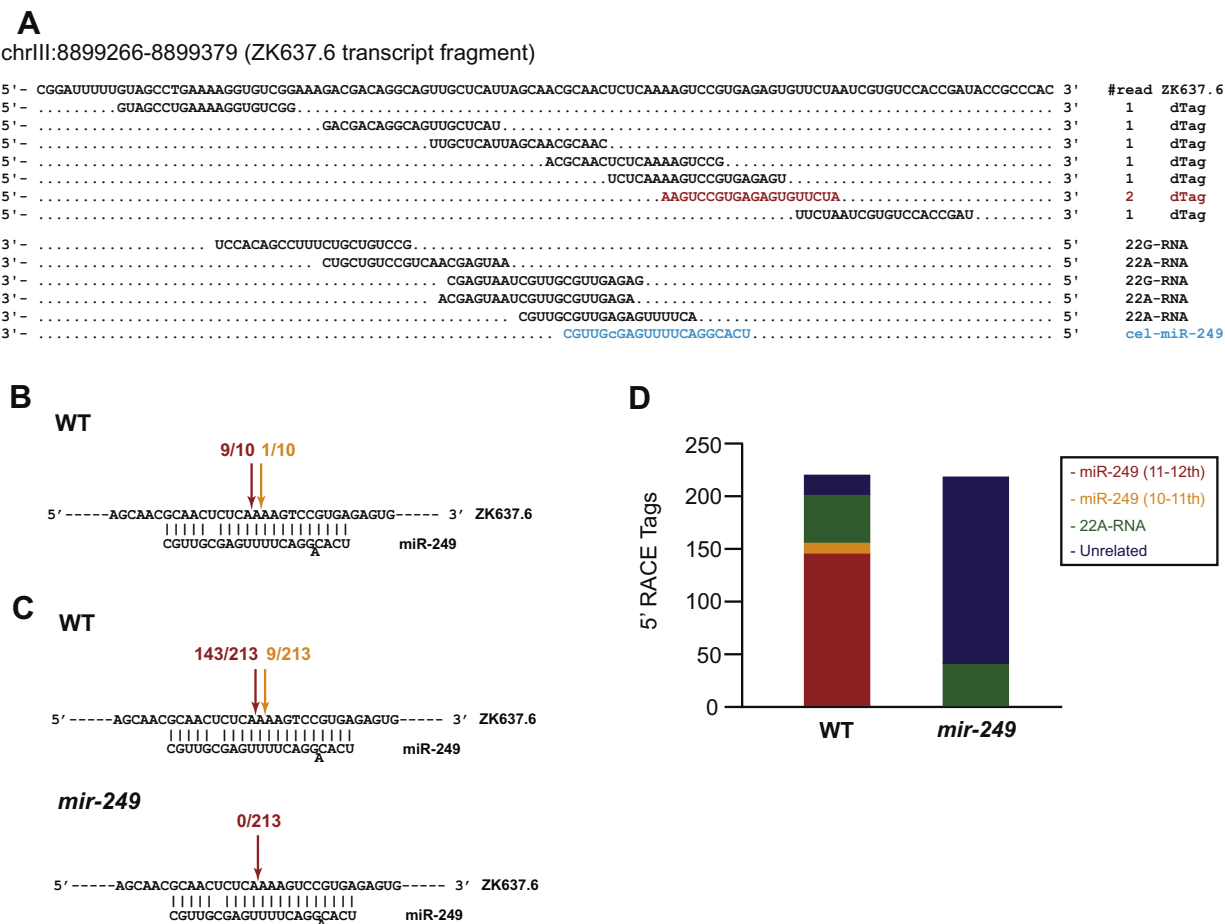


Fig. 1. MicroRNA-directed cleavage of the ZK637.6 transcript in *C. elegans*. (A) Each unique degradome tag is shown below the ZK637.6 transcript fragment, with the expected degradome tags indicated (miR-249-directed cleavage tags are red-colored) and the number of tags (dTag) listed on the right. The miR-249 mature sequence is also shown (blue-colored). (B) Detection of 5' RACE clones corresponding to the middle of the respective sequences complementary to miR-249 in wild-type worms. Fractions refer to the number of independently cloned 5' RACE products whose 5' end terminated at the indicated position (numerator) over the total number of sequenced clones (denominator). (C and D) 5'-RACE analysis in wild-type and *mir-249* mutants. Of the 213 RACE clones, of each wild-type and *mir-249* mutant, sequenced from technical and biological replicates, 152 yielded a sequence consistent with miR-249 directed cleavage (in red and orange); 44 and 39 clones were 22A-RNA directed cleavage fragments in wild-type and *mir-249* mutant, respectively (in green); and 17 and 174 unrelated clones (random degradation) in wild-type and *mir-249* mutants, respectively (in blue). In the *mir-249* mutants, no clones were consistent with miR-249-directed cleavage.

expression of miR-249. The expression of miR-249 was relatively high in early stages, especially from the L1 to L3 stages (Fig. 2A) whereas it became decreased in later larval and adult stages, which generally matched well with recent high-throughput sequencing analysis [15]. In contrast, the ZK637.6 transcript was expressed at low levels in early stages and at higher levels during the adult stage (Fig. S1). Overall, we found that miR-249 and the ZK637.6 target pair exhibited negatively correlated expression (Figs. 2A and S1). To further investigate whether the transcript level of ZK637.6 is regulated by miR-249-directed cleavage in vivo, we measured the transcript level of ZK637.6 in wild-type versus *mir-249* mutants for each developmental stage (Fig. 2B). The expression of the ZK637.6 transcript increased by approximately 4-fold in *mir-249* mutants as compared with wild-type in the adult stage (Fig. 2B). This up-regulation further increased nearly 16-fold in the L1-stage, where the miR-249 expression is relatively high. Therefore, we concluded that the expression of the ZK637.6 transcript was strongly dependent on the expression of miR-249 (Fig. 2B).

3.3. Identification and characterization of ZK637.6 and its parental gene, *asna-1*

Having established that miR-249 directed cleavage is implicated in tight regulation of the ZK637.6 transcript, we next

identified and characterized the miR-249 target gene, ZK637.6, to better understand the biological role of miR-249 targeting. The miR-249 is extensively conserved through several nematode species (Fig. S2), and its target gene, ZK637.6, is currently annotated as a pseudogene (ce6) with a predicted length of 546 nt. The 5' upstream region of ZK637.6, including the miR-249 target site, is well-conserved through several nematode species (Fig. 3). Chromatin signatures of the ZK637.6 loci support the notion that the gene is composed of a single exon structure where the H3K4me3 signal is positioned upstream of the loci (in a supposed promoter region) and the H3K36me3 signal is accumulated to the gene body of the ZK637.6 (Fig. 3). The exact sequence information for ZK637.6, particularly at the 5' and 3'-ends was not available; therefore, we identified the full-length sequence of ZK637.6 using 5' and 3' RACE-PCR. Detailed conservation and sequence information is provided (see Figs. S2–S4). Given the annotation of ZK637.6 as a pseudogene, we searched for its putative parental gene. We found the best BLAST match, *asna-1* (ZK637.5), a positive regulator of insulin secretion in *C. elegans* [19]. *asna-1* is located approximately 3 Kbp upstream of ZK637.6 and shares 87% sequence identity with ZK637.6 in a reverse-complementary relationship (Fig. S5). Following this finding, we examined whether *asna-1* is also regulated by miR-249-directed cleavage, but we were not able to detect an miR-249-directed cleavage signal for *asna-1* (Fig. S6A). In addition,

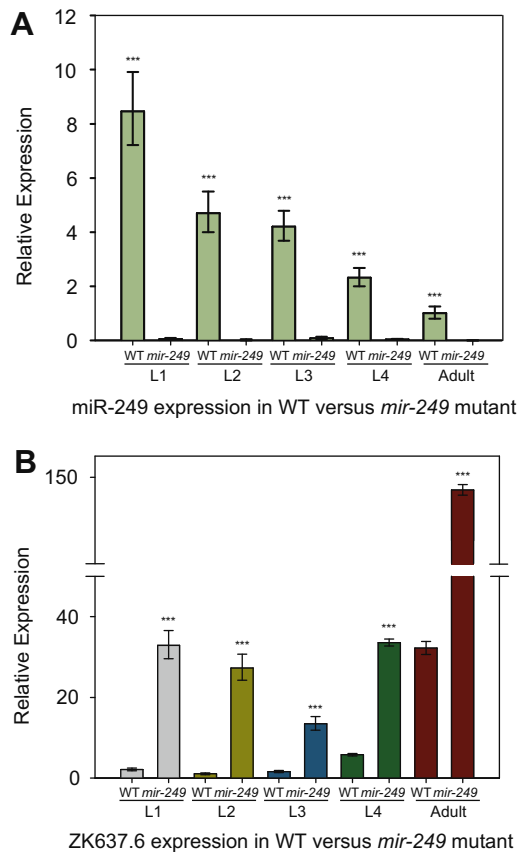


Fig. 2. The ZK637.6 transcript is strongly up-regulated in *mir-249* mutant worms. (A) Taqman qRT-PCR analysis of miR-249 expression from different developmental stages (L1, L2, L3, L4 and adult stages) in wild-type versus *mir-249* mutants). Expression of miR-249 was relatively high at early stages and gradually decreased as worms grew. There was almost no expression of miR-249 throughout the developmental stages of *mir-249* mutant worms. The *U18 snoRNA* was used as a normalizing control. Relative mean expression $\pm$ SD is shown (** $P < 0.001$, unpaired *t*-test). (B) qRT-PCR analysis of the ZK637.6 transcript expression in wild-type versus *mir-249* mutant from each developmental stage (L1, L2, L3, L4 and adult stages). Expression of the ZK637.6 transcript is up-regulated nearly 4-fold in *mir-249* mutants compared to wild-type in the adult stage. This up-regulation further increased to about 16-fold in the L1 stage. *cdc-42* was used as a normalizing control. Relative mean expression $\pm$ SD is shown (** $P < 0.001$, unpaired *t*-test).

based on the expression analysis of *asna-1* mRNA in wild-type and in *mir-249* mutants, along with the correlation with miR-249 expression (Figs. 2A and S6B), we cannot conclude that the expression of the *asna-1* mRNA is dependent on the expression of miR-249 (Fig. S6 for more detail). Thus, these results suggested that miR-249 preferentially regulates the expression of the ZK637.6 transcript but not the *asna-1* transcript.

3.4. The ZK637.6 pseudogene transcript does not act as a decoy for miRNA targeting of its parental gene, *asna-1*

A previous report suggested that a “miRNA decoy” could guard its parental gene by acting as the regulated target. This was demonstrated by a *PTENP1* pseudogene when it acted as a decoy for miRNA targeting of its parental gene, *PTEN* [20]. To investigate whether the ZK637.6 transcript could also act as an endogenous competitor, which would affect miR-249 targeting of the parental gene, we measured the transcript level of *asna-1* in ZK637.6 over-expressing worms, finding no change in *asna-1* expression (Fig. S7B). In the above study, *PTEN* was also targeted by *PTENP1*-targeting miRNAs since their miRNA seed-pairings are perfectly conserved between *PTEN* and *PTENP1*. In our case, *asna-1* was not

targeted by miR-249. There are at least two possible explanations for this observation: (i) The disruption of GC pairing at 6th nucleotide in miR-249 for the seed-matched site of the *asna-1* transcript might lead to higher base-pairing instability, resulting in loss of miR-249 targeting (Fig. S6C–D), (ii) miR-249 might act in a mutually exclusive way because the miR-249 and *asna-1* are expressed in different tissues; the expression of *mir-249::gfp* was previously observed in seam cells, head neurons and P lineage cells [21], contrary to *asna-1* whose expression was mostly observed in intestinal cells [19]. In either case, we concluded that the parental gene, *asna-1*, was not targeted by miR-249, presumably because the function of *asna-1* is involved in insulin secretion; the miRNA-directed cleavage of the *asna-1* mRNA might be so detrimental that it could be under selective pressure to escape miR-249 targeting.

3.5. The ZK637.6 transcript is not translated in vivo

We further tested whether the ZK637.6 transcript is possibly translated in vivo, to see if it serves a cellular function of its own, by employing transcriptional and translational GFP reporters (see Fig. S8 for more detail). However, the GFP signals from translational reporter were not detected. The recent study of high-throughput ribosome profiling in *C. elegans* suggests that ribosome-protected mRNA fragments (RRFs) were not aligned to the ZK637.6 transcript from L1 through L4 worms [22]. Thus, the result suggests it is highly unlikely that ZK637.6 encodes a protein in vivo.

3.6. miR-249 directed cleavage has no direct role in secondary siRNA biogenesis

Since the biogenesis of secondary siRNA is triggered by primary cleavage events of target RNA [23], by an as yet unknown mechanism [24] (Fig. S9), we presumed that the production of secondary siRNAs, derived from the ZK637.6 transcript, could be regulated by miR-249-directed cleavage, as in the case for tasi-RNAs (trans-acting siRNAs) in plant. In this sense, total small RNAs from wild-type and *mir-249* mutant adult-stage worms were subjected to small-RNA sequencing with a tobacco acid pyrophosphate (TAP) pre-treatment, allowing for the detection of secondary siRNAs carrying 5'-tri-phosphate, to investigate the possible role of miR-249-directed cleavage in secondary siRNA biogenesis. When the wild-type and *mir-249* mutant libraries data were compared, a slight difference in the level of 22G-RNAs was observed in the ZK637.6 transcript. 22G-RNAs, which are predominantly 22 nt in length with a 5' tri-phosphorylated guanine, are antisense to more than half of the annotated genes in *C. elegans* and regulate target genes both transcriptionally and post-transcriptionally [25]. 22G-RNAs are generally most abundant with respect to both the 5' and 3' ends of transcripts [25]. We noticed that two 22G-RNAs, which mapped to the 5' and 3' ends of the 5'-cleavage fragment of the ZK637.6 transcript, increased by ~44% in wild-type as compared with that in the *mir-249* mutant (Fig. S10A and S10B). Since the changes were not very apparent, these two 22G-RNAs were further subjected to northern blot analysis (Fig. S10C). The expression of the two 22G-RNA were detected in adult-stage worms; however, we did not find a significant difference between the wild-type and *mir-249* mutants (Fig. S10C). Therefore, we concluded that interaction between miR-249 and its cleavage target, ZK637.6, do not likely constitute a conserved trigger for siRNA biogenesis, as in the case for tasi-RNA pathway in plants (see Fig. S10 for detailed discussion).

4. Conclusion

This study discovered that miR-249 directs the cleavage of the ZK637.6 pseudogene transcript, thereby tightly regulating its expression. As of yet, we have not observed a physiological

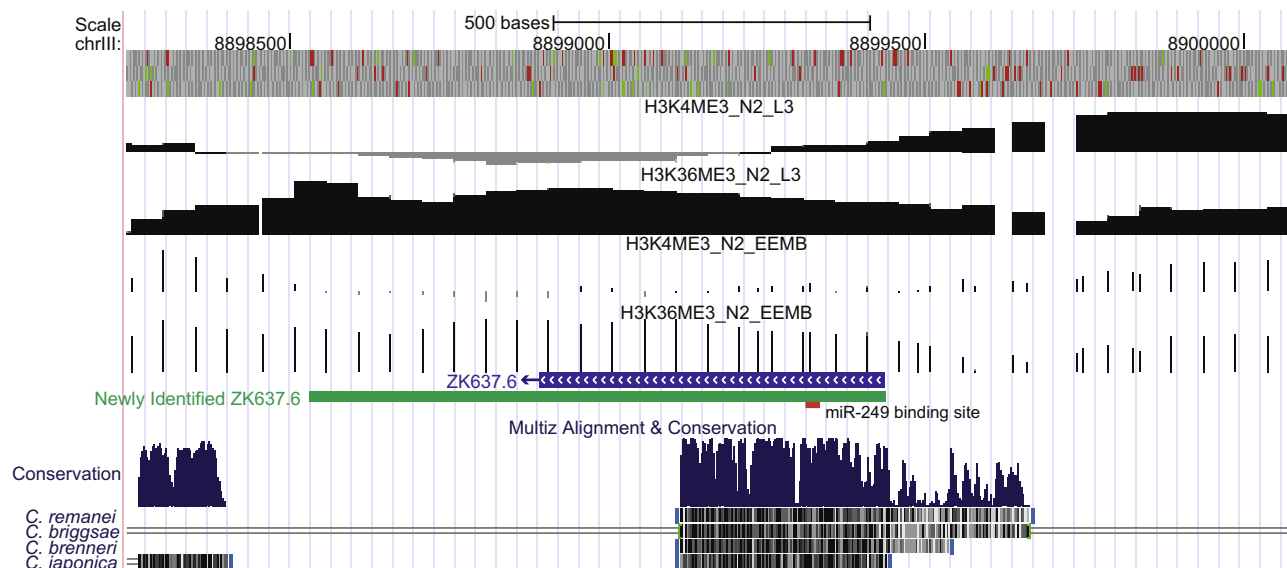


Fig. 3. Identification and characterization of ZK637.6 Conservation of the miR-249 target site is shown for several nematode species on the bottom. Profiles of H3K4me3 and H3K36me3 are visualized for ZK637.6 from embryo (N2_EEMB) and L3 (N2_L3) stage worms. Note that ZK637.6 is transcribed in the reverse direction (see the arrow). The red bar indicates the miR-249 binding site. The track of the newly identified ZK637.6 transcript is shown as a green bar.

phenotype in *mir-249* mutants [26] or in ZK637.6-over-expressing worms. The exact contribution of this cleavage event remains to be elucidated but a recent study of extinction dynamics of pseudogenes may offer a clue. In that study, pseudogenes were deleted rapidly from genomes [27], indicating that they confer deleterious effects, presumably resulting either from the dominant-negative effects of abnormal transcripts or proteins and/or from the energetic cost of transcription and translation [27,28]. Therefore, it could conceivably be hypothesized that miR-249 directed cleavage of a pseudogene can in part contribute to the process of adaptive removal of a pseudogene. At least these results indicate that the effects of pseudogene, whether they be detrimental or not, may be negated by miRNA regulation.

This is the first discovery of miRNA-directed cleavage in *C. elegans*, which will serve to rapidly expand the knowledge of miRNA functions and may provide an emerging picture of miRNA-mediated regulation of pseudogenes in *C. elegans*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.febslet.2013.02.029>.

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