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Trans-splicing in *C. elegans* generates the negative RNAi regulator ERI-6/7

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Abstract

Mutations that enhance the response to double-stranded RNA (dsRNA) have revealed components of the RNA interference (RNAi) pathway or related small RNA pathways. To explore these small RNA pathways, we screened for *Caenorhabditis elegans* mutants displaying an enhanced response to exogenous dsRNAs. Here we describe the isolation of mutations in two adjacent, divergently transcribed open reading frames (*eri-6* and *eri-7*) that fail to complement. *eri-6* and *eri-7* produce separate pre-messenger RNAs (pre-mRNAs)are *trans*-spliced to form a functional mRNA, *eri-6/7*. *Trans*-splicing of *eri-6/7* is mediated by a direct repeat that flanks the *eri-6* gene. Adenosine to inosine editing within untranslated regions (UTRs) of *eri-6* and *eri-7* pre-mRNAs reveals a double-stranded pre-mRNA intermediate, forming in the nucleus before splicing occurs. The ERI-6/7 protein is a superfamily I helicase that both negatively regulates the exogenous RNAi pathway and functions in an endogenous RNAi pathway.

RNAi pathways act throughout phylogeny as both an experimental gene-silencing tool and a regulator of endogenous gene expression[1,2]. The endonuclease Dicer[3] and various Argonaute proteins act in specialized roles in most silencing pathways[4]. *C. elegans* Dicer interacts with negative regulators of exogenous RNAi[5], such as the RNA-dependent RNA polymerase (RdRP) RRF-3 ([ref. 6]) and the exonuclease ERI-1 ([ref. 7]). These proteins are required for the production or stability of a subset of endogenous short interfering RNA (siRNAs) suggesting a competition with the exogenous RNAi pathway for shared, rate-limiting factors[5,8].

RdRPs amplify siRNAs on mRNA templates in nematodes[9-11], fungi and plants[1]. The feed-forward nature of RNAi and the still unexplained resistance of neurons to RNAi[12–14] suggest that there are undiscovered negative regulators of RNAi. To identify such regulators, we conducted a genetic screen for mutations that confer an enhanced response to dsRNA. Here we describe the identification and characterization of adjacent genes, *eri-6* and *eri-7*, which are assembled by a dsRNA-mediated *trans*-splicing mechanism to regulate RNAi negatively. The dsRNA-dependent production of an RNAi factor suggests that there may be an autoregulatory feedback mechanism in RNAi.

Characterization of *eri* mutants

To identify negative regulators of *C. elegans* RNAi, we genetically screened for mutants having an enhanced RNAi (Eri) phenotype. These mutants, unlike wild type, exhibited an enhanced

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response to green fluorescent protein (*gfp*) RNAi, downregulating expression of a neuronally expressed *gfp* transgene (*unc-47::gfp*)[15], and an enhanced response to dsRNA targeting several non-neuronally expressed endogenous genes, including the collagen *dpy-13* ([ref. 16]), the cadherin *hmr-1* ([ref. 17]) and the transcription factor *lin-1* ([ref. 18]). Thirteen of the 44 *eri*(-) mutants were also thermosensitive sterile and showed X-chromosome non-disjunction phenotypes that define genes that encode the DCR-1-interacting proteins RRF-3, ERI-1, ERI-3 and ERI-5 ([refs 5-7]). Among the mutations not in this class, *mg379* and the independently isolated spontaneous *eri* allele *mg411* enhance RNAi and silence a transgene (*mgIs30*) that confers a rolling locomotion (Rol) phenotype (Supplementary Fig. 2, and Supplementary Tables 1 and 2). Transgene silencing and exogenous RNAi are mediated by several common factors[19], and all known *eri*(-) mutants silence transgenes[6,7]. *mg379* and *mg411*, as well as other allele combinations, fail to complement (Supplementary Table 1 and Supplementary Information). *mg411* and *mg379* were mapped to a 1.7-megabase interval. The 330 genes in the interval were assayed for a loss-of-function phenotype of transgene silencing by using dsRNAs that target each gene[20]. Two dsRNA clones induced silencing of the *mgIs30* transgene, phenocopying the *mg411* and *mg379* mutations. These dsRNAs target two adjacent, antiparallel open reading frames: C41D11.7, termed *eri-7*, and C41D11.1, termed *eri-6* (Fig. 1a). These RNAi clones are specific for each gene and do not target the adjacent gene or any other gene in the genome. DNA sequencing identified *mg411* as a missense mutation in C41D11.7 and *mg379* as a 5′ splice donor site mutation in C41D11.1. DNA sequencing of mutants isolated in the genetic screen revealed five more mutations in C41D11.7: *mg369*, *mg380*, *mg390*, *mg440* and *mg442*. An independently isolated deletion allele (*mg441*) removes *eri-6* (Supplementary Fig. 3). The transgene-silencing phenotype of mutants with either *eri-7* (*mg411*) or *eri-6*(*mg379*) alleles was rescued by transformation of a 9.1-kilobase (kb) fragment that contains only C41D11.1 and C41D11.7 (Fig. 2a, b). The hypersensitivity to RNAi by feeding conferred by the *mg411* allele was also rescued by the 9.1-kb fragment (data not shown).

Anomalous *eri-6/7* gene structure

Although the lack of complementation suggests that *eri-6*(*mg379*) and *eri-7*(*mg411*) affect a single gene, the mutations were found in ostensibly separate genes on antiparallel strands[21]. However, the syntenic region in the nematode species *C. briggsae* surprisingly revealed that *eri-6* and *eri-7* constitute a single, contiguous gene in this species (Fig. 1a). The 5′ coding region of the *C. briggsae* gene CBG03999 is homologous to *eri-6*, whereas the 3′ coding region is homologous to *eri-7*. We verified the *C. elegans* genome structure at the *eri-6* and *eri-7* loci by Southern blotting (Supplementary Fig. 4), and by PCR and sequencing analyses (data not shown). We investigated closer relatives of the *C. elegans* wild-type N2 strain—27 other natural isolates of *C. elegans*. Four of these, including an isolate from Hawaii, *C. elegans* CB4856 ([ref. 22]), show a fused gene structure similar to that of *C. briggsae* (Fig. 1b).

eri-6 in the *C. elegans* strain N2 is flanked by a nearly perfect, approximate 930-base-pair (bp) direct repeat (Fig. 1b). Within this direct repeat is a 25-bp inverted repeat (Fig. 1b and Supplementary Fig. 5). The inverted repeat may have mediated the rearrangement responsible for the difference in *eri-6* orientation relative to *eri-7* in *C. elegans* N2 compared with *C. elegans* CB4856 (Fig. 1b). This hypothesis is supported by the spontaneous allele *mg441* (Supplementary Fig. 3 and Supplementary Discussion), which deletes *eri-6* in a fashion consistent with a rearrangement having occurred through the approximate 930-bp direct repeats that flank *eri-6*.

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eri-6 and *eri-7* are *trans*-spliced

Sequencing of the expressed sequence tag (EST) yk224h9, and reverse transcription-PCR with (RT- PCR), showed that the divergently transcribed RNAs for *eri-6* and *eri-7* are assembled into one mRNA in *C. elegans* strain N2 (Fig. 1a), encoding a protein orthologous to the *C. briggsae eri-6/7* gene product. These proteins are members of the superfamily I DNA and RNA helicases (Supplementary Figs 6 and 7). Several *eri-6/7* mutations encode amino-acid substitutions in conserved motifs in the ATPase and helicase domains (Supplementary Fig. 7). A transgene containing the *eri-6* promoter, the full-length *eri-6/7* complementary DNA (cDNA) and the *eri-7* 3′ UTR rescues both the *eri-6*(*mg379*) and *eri-7*(*mg411*) phenotypes (Supplementary Table 3).

In addition to the helicase mRNA, three other *eri-6* splice variants were identified by EST analysis and 3′ rapid amplification of cDNA ends (RACE), in which *eri-6* splices to different downstream exons (Fig. 1a), encoding proteins with no homology to known proteins. Neither the *eri-6* mRNA nor the *eri-7* mRNA could be detected as the separate mRNAs predicted from the gene structure.

We considered the following two mechanisms of chimaeric RNA formation: (1) *trans*-splicing of independently transcribed pre-mRNAs; and (2) a low level of DNA inversion in particular cells that would reposition the *eri-6* and *eri-7* genes *in cis*. The genomic rearrangement model was alluring because of the observation that such inversions at the *eri-6/7* locus are apparent between and within related *Caenorhabditis* species. However, the rearrangement model is neither supported by Southern blotting (Supplementary Fig. 4) nor extensive PCR analyses designed to detect a *cis* orientation of *eri-6* and *eri-7* as a minority component of the genomic DNA of *C. elegans* N2 (Supplementary Discussion and Supplementary Fig. 8).

The generation of the *eri-6/7* mRNA by *trans*-splicing is, however, supported by multiple experimental data. The *trans*-splicing juncture of the fused *eri-6/7* mRNA uses canonical *cis* splice sites (Supplementary Fig. 9). Other *eri-6* splice variants were detected 3′ of *eri-6* and all include exons linked within 12 kb, *in cis* (Fig. 1a). Bioinformatic searches (Supplementary Discussion) of EST databases and 5′-RACE experiments did not detect distant *trans*-splicing of *eri-6* to other mRNAs. Therefore, it is likely that for *trans*-splicing to occur, the *eri-6* and *eri-7* genes need to be in close proximity. Genes can also be juxtaposed through homologous chromosome pairing during meiosis[23]. Interchromosomal *trans*-splicing of *eri-6* and *eri-7* is unlikely to efficiently occur given (1) the lack of complementation observed in *eri-7*(+)*eri-6* (*mg379*)/*eri-7*(*mg369*)*eri-6*(+) animals and (2) sequence analysis of cDNAs cloned from these *trans*-heterozygotes (Supplementary Discussion).

A prerequisite for co-transcriptional *trans*-splicing is expression of both pre-mRNAs in the same cells at the same time. *eri-7* promoter::*gfp* and *eri-6* promoter::*rfp* transgenes are expressed in overlapping patterns, with co-expression in hypodermal cells and two pairs of sensory head neurons (ASK and ASI) (Fig. 2c). The *eri-7* promoter also expresses in the somatic gonad.

The direct repeat flanking *eri-6* (Fig. 1b) could constitute the 5′-UTR of the *eri-7* pre-mRNA and, as a reverse complement copy, the *eri-6* pre-mRNA 3′- or 5′-UTR. These complementary sequences could bring the pre-mRNAs into physical contact to facilitate *trans*-splicing. To determine the pre-mRNA sequences of *eri-6* and *eri-7*, we stabilized them by attenuating splicing through RNAi inactivation of *rnp-5*, which encodes an orthologue of human RNPS1 ([ref. 21]), a member of the exon-junction complex. 5′-RACE analysis using intron-specific primers identified an *eri-7* pre-mRNA 5′ end extending 725 bp into the *eri-6/7* intergenic region (685 bp into the first direct repeat). An *eri-6* transcription start site was found at up to 100 bp 5′ of the *eri-6* start codon (Fig. 2d). We were unable to amplify the *eri-6* 3′-UTR, possibly

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because of repetitive, low complexity sequence immediately 3′ of the splice site (5′ of the second direct repeat). The splicing of *eri-6* to independent exons located 1-11 kb 3′ of the second direct repeat strongly supports an *eri-7 trans*-splicing model whereby the *eri-6* nascent transcript bears this repeat.

The significance of the second 930-bp direct repeat was tested in rescue experiments using an *eri-6/7* (9.1-kb) genomic fragment that contained this repeat, compared with an (8.2-kb) fragment that did not (Fig. 2a, b). The 9.1-kb fragment completely rescued the transgene-silencing phenotypes of both the *eri-6*(*mg379*) and *eri-7*(*mg411*) alleles, whereas the 8.2-kb fragment only weakly rescued the *eri-7* mutation. This nominal *eri-6/7* gene activity could be the result of overexpression of *eri-6* and *eri-7* from the transgenic array bypassing the need for their base pairing, or of a low level of *eri-6* transcription from an upstream start site, to include repeat sequence in the 5′-UTR that could mediate base-pairing.

Analysis of cDNA sequences derived from the *eri-7* pre-mRNA stabilized in *rnp-5* RNAi-treated animals revealed adenosine to guanosine transitions at four positions located within the direct repeat. These transitions are indicative of adenosine to inosine editing of the *eri-7* 5′-UTR by an adenosine deaminase (ADAR)[24], supporting the model that the direct repeat element flanking *eri-6* is bidirectionally transcribed to form an RNA duplex intermediate (Fig. 2e) before the two mRNAs are *trans*-spliced (Fig. 2d). Though ADARs can also edit secondary structures within single RNA strands, no such structure is predicted for the *eri-7* 5′-UTR.

To show that the *trans*-spliced mRNA is translated into a chimaeric protein, we constructed a *trans*-splicing reporter in which all but the initial four nucleotides of *eri-7* sequence are replaced by a *gfp* gene lacking a start codon (Supplementary Fig. 10). Sequencing of RT-PCR products (Fig. 3a) from GFP-expressing worms (Fig. 3b) showed that *trans*-splicing occurred from the transgene to yield an mRNA that consists of *eri-6*, the first two codons of *eri-7*, and *gfp* sequence. Western blotting showed that a protein of the size expected for the ERI-6::GFP fusion can be detected using a GFP antibody, indicating that the *eri-6* and *eri-7 trans*-spliced mRNA is likely translated into a chimaeric ERI-6/7 helicase protein (Fig. 3c).

This reporter for *eri-6/7 trans*-splicing is expressed in the amphid neuron ASK (Fig. 3b) and in the somatic gonad, but not in the hypodermis where the promoter fusions are strongly expressed. A second transgene, bearing the complete *eri-6/7* genomic region, fuses mCherry[25] to the carboxy terminus of ERI-7 (Fig. 3d-f and Supplementary Fig. 11), and shows expression in ASK and the somatic gonad. One explanation for the lack of hypodermal expression from these *trans*-splicing-dependent fusion genes is that *trans*-splicing is inefficient or inhibited in the hypodermis. Alternatively, transcription or translation in the hypodermis does not occur because of sequence in the *eri-6/7* gene or mRNA that is not present in the promoter fusion constructs. Interestingly, the *eri-6/7trans*-splicing reporter is more highly and broadly expressed when RNAi is attenuated by inactivation of the Argonaute gene *rde-1* (Supplementary Fig. 12), suggesting that the *eri-6/7* is a target of RNAi. A caveat to this result is that transgenes are generally expressed at higher levels when RNAi is defective. Quantitative RT-PCR revealed that *eri-6/7* mRNA levels are not increased in animals lacking RDE-1 (data not shown). One explanation for these data is that the dsRNA element of *eri-6/7* acts tissue-specifically as a sensor, and the changes in expression are too subtle to detect by quantitative RT-PCR on whole worms.

C. elegans has two ADAR genes: *adr-1* and *adr-2*. The *adr-1*(-); *adr-2*(-) double mutant enhances transgene silencing, suggesting that editing of dsRNAs normally inhibits their sensitivity to RNAi[26]. Perhaps in the absence of ADARs, the unedited *eri-6/7* dsRNA intermediate is a better target for the RNAi pathway, thus decreasing *eri-6/7* gene activity. However, the level of *trans*-spliced *eri-6/7* mRNA is unchanged in the *adr-1*(-); *adr-2*(-) double

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mutant as analysed by quantitative RT-PCR and by GFP fluorescence of the *trans*-splicing reporter after *adr* RNAi (data not shown), suggesting that editing of the double-stranded intermediate does not affect *eri-6/7 trans*-splicing.

Role of ERI-6/7 in RNAi

eri-6/7 alleles define a new class of Eri mutants. They lack the pleiotropic phenotypes (for example, sterility) typical of when function of the Dicer-interactors ERI-1 to ERI-5 is lost. In addition, *eri-6/7*(-) mutants do not show the characteristic synthetic multivulva phenotype that is associated with enhanced RNAi mutants of the retinoblastoma pathway[27]. We analysed the role of *eri-6/7* in small RNA pathways. Like *eri-1* alleles[7], *eri-7*(*mg369*) confers an enhanced response (over wild type) to injected double-stranded siRNAs (*unc-22*) (Supplementary Table 1); however, we did not observe a significantly increased level of siRNAs corresponding to a target gene after exposure to dsRNAs as analysed by northern blotting (data not shown). These data suggest that *eri-6/7* acts downstream of processing of the exogenous trigger-dsRNA into siRNAs. Genetic epistasis analyses (Supplementary Table 1 and data not shown) with mutants defective in RNAi (*rde-1* ([ref. 19]), *rde-4* ([ref. 28]), *rrf-1* ([ref. 11]) and *mut-7* ([ref. 29])) show that, with the exception of the somatic RdRP gene *rrf-1*, normal function of these genes is required for enhanced and normal RNAi sensitivity. RRF-1 functions in the *de novo* production of secondary siRNAs after the initial cleavage of the exogenous dsRNA into primary siRNAs by Dicer, RDE-1 and RDE-4 ([ref. 11]). Unlike *rrf-1*(-) mutants, *rrf-1*(-) *eri-7*(-) double mutants are partly proficient in somatic RNAi targeting *hmr-1* and *unc-22*, suggesting that *eri-6/7* negatively acts both in *rrf-1*-independent RNAi (through primary siRNAs) and in *rrf-1*-dependent RNAi. These data are consistent with a role of ERI-6/7 downstream of, and dependent on, the siRNA production mediated by RDE-1 and RDE-4. Null alleles of *eri-1* or *rrf-3*, when combined with *eri-6/7* null alleles, did not enhance the RNAi sensitivity compared with the single mutants, suggesting that *eri-6/7* may act in the same pathway as *eri-1* and *rrf-3* to enhance RNAi (data not shown).

Silencing in response to exogenous dsRNA is increased when certain endogenous RNAi pathways, such as the pathway dependent on *eri-1*, are defective[5]. We investigated a role for ERI-6/7 in endogenous RNAi by analysing endogenous siRNA (endo-siRNA) abundance in *eri-6/7* mutant worms by northern blotting (Fig. 4). *eri-6/7* is required for endo-siRNAs derived from the gene K02E2.6 but not for a Dicer-independent endo-siRNA matching the gene T01A4.3 (Fig. 4), which is thought to be germline-expressed[5]. ERI-6/7 protein shares these attributes with the DCR-1-interacting proteins ERI-1, RRF-3 and RDE-4 ([refs 5, 8]) (Fig. 4), as well as with the Argonaute ERGO-1 ([ref. 30]).

Conclusions

eri-6/7 encodes an RNAi factor that is assembled in *C. elegans* N2 through *trans*-splicing via a dsRNA intermediate. The *trans*-splicing of *eri-6* and *eri-7* is only observed when the two genes are on the same chromosome, suggesting that it occurs on nascent transcripts. The *eri-6/7* double-stranded, premRNA intermediate is edited by ADARs when splicing is inhibited, and may be itself regulated by RNAi.

ERI-6/7 protein is a member of the superfamily I helicases, like the RNAi factors Mov10 in mammals[31], SDE3 in *Arabidopsis*[32] and Armitage in *Drosophila*[33]. Superfamily I helicases act on either RNA or DNA. Because ERI-6/7 is required for endo-siRNA production or stability, it could function as an RNA helicase in siRNA generation. Our expression data showing exclusive or predominant cytoplasmic expression of a full-length ERI-6/7 protein fused to a fluorescent marker (Supplementary Fig. 11) suggest that it acts as an RNA helicase.

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Loss of *eri-6/7* causes enhanced exogenous RNAi. Lee *et al.* (2006) and Duchaine *et al.* (2006) have proposed that other ERI proteins bridle exogenous RNAi indirectly by competing for factors common to both the endogenous and exogenous RNAi pathways, suggesting that the normal role of ERI-6/7 is either to promote endogenous small RNA pathways or to attenuate exogenous RNAi directly. The loss of some endogenous siRNAs in *eri-6/7* mutants is consistent with the former function.

This is the first example of exon-to-exon *trans*-splicing in *C. elegans*, and only the third finding of a locus that requires such *trans*-splicing for the production of a functional protein. The other two examples were reported in *Drosophila*: *mod*(*mdg4*)[34,35] and *lola*[36]. Each of these genes encodes BTB- zinc-finger transcription factors that achieve extensive protein diversity through alternative *trans*-splicing. The requirement of proximity between the *trans*-spliced exons in these cases is not clear[35,36].

The polymorphic variation of the *eri-6/7* locus between related *Caenorhabditis* species, and especially between different isolates of the *C. elegans* species, is remarkable. The inserted repetitive sequence has likely segmented the *eri-6/7* gene into two in *C. elegans* N2 through recombinational events. The ability of the repeat to mediate homology-based *trans*-splicing, and its bidirectional promoter activity (allowing independent *eri-6* and *eri-7* expression in N2), have provided a compensatory means of bringing the two fragments together again to encode a composite *eri-6/7* mRNA. Beyond the structure of the *eri-6/7* locus, the ERI-6/7 proteins seem to have also diverged substantially between *C. elegans* and *C. briggsae*. Whereas the mean amino-acid identity between orthologues is 75% ([ref. 37]), ERI-6/7 shows only 44% identity to its fused orthologue in the related nematode *C. briggsae*. This is consistent with the rapid evolution that is seen in antiviral RNAi genes[38], and with the finding that other *C. elegans eri* genes are anti-viral[39,40].

METHODS SUMMARY

C. elegans was cultured using standard techniques[41] and fed on *Escherichia coli* OP50 or on *E. coli* HT115 harbouring a dsRNA expressing plasmid (RNAi by feeding).

C. elegans animals were mutagenized using ethylmethanesulphonate. Mutants showing an enhanced RNAi phenotype were selected after *gfp* RNAi in the F_2 and F_3 progeny of mutagenized animals carrying a transgene expressing GFP in a subset of neurons. The enhanced RNAi phenotype was further analysed using RNAi to the endogenous genes *hmr-1*, *lin-1* and *dpy-13*. In addition, several mutants were assayed for their ability to silence the transgene *mgIs30* (*lim-6::gfp*, *col-10::lacZ::lin-14*, *rol-6*(*su1006*)*::lin-14* 3′-UTR), scoring the Rol phenotype conferred by this transgene. *unc-22* siRNA injections were done as described previously[7,42].

Mutations were single nucleotide polymorphism-mapped using the Eri and transgene silencing phenotypes. Subsequently, all genes in the mapping interval were inactivated by RNAi and tested for transgene silencing. Inactivation of two genes silenced the transgene *mgIs30*; these genes were sequenced in the mutants.

Transgenic worms for rescue experiments, expression analysis as well as animals carrying the reporter for *trans*-splicing were made by microinjection of PCR fragments, cosmids or plasmids. Promoter and full-length fusions to fluorescent proteins were made by splicing by overlapping extension PCR of *gfp*, *mrfp* or *mCherry* to the promoter or in frame with the open reading frame. Western blotting of the *eri-6::gfp* reporter was done using an anti-GFP antibody.

The gene structure or *eri-6/7* in wild-type *C. elegans* N2, other *C. elegans* isolates and *C. briggsae* was confirmed by PCR, sequencing and/or Southern blotting.

eri-6/7 transcripts were analysed by EST sequencing analysis, RT-PCR, 5′- and 3′-RACE. cDNA was cloned and sequenced. Pre-mRNAs and transcriptional start sites were analysed in animals that were deficient in splicing by RNAi of *rnp-5*.

Endogenous siRNA abundance in mutant animals was assayed by northern blotting[42] using StarFire probes[5].

METHODS

Isolation of enhanced RNAi mutants

unc-47::gfp worms were mutagenized with ethyl methanesulphonate, and both the F_2 and F_3 generations were cultured on lawns of *E. coli* expressing dsRNA that targeted *gfp*. Mutagenized populations were then screened for rare individuals showing a marked decrease in the number of γ-aminobutyric acid (GABA)-ergic neurons visibly expressing GFP under the dissecting microscope, screening through approximately 50,000 haploid genomes. We identified 44 mutants that show an enhanced exogenous RNAi phenotype. To ensure isolated mutants were Eri and to test for generally enhanced RNAi, we cultured them on *E. coli* targeting endogenous genes that were expressed in a variety of tissues, asking that they be hypersensitive compared with *unc-47::gfp* worms to at least two of the following three testers: *dpy-13*, *hmr-1* and *lin-1*, which result in dumpy, embryonic lethal and sterile, and multivulva phenotypes, respectively. Thermosensitive sterility was assayed by placing L1 worms at 25 °C and scoring for unfertilized eggs in the next generation. The 31 non-sterile mutants were in at least five complementation groups.

Mapping, genetic and expression analyses

eri-6(*mg379*) and *eri-7*(*mg411*) were mapped using a highly polymorphic *C. elegans* isolate, CB4856. Recombinants generated with either outcrossed strains carrying the *mg379* or the *mg411* alleles and CB4856 were assayed for their RNAi hypersensitivity phenotype or their ability to silence a *rol-6* transgene (*mgIs30 [rol-6*(*su1006*)*::lin-14* 3′-UTR, *col-10::lacZ, lim-6::gfp]*), respectively, and subsequently genotyped for single nucleotide polymorphisms that exist between the wild-type and CB4856 strains. *mg411* was placed into a 3.8-map-unit interval on chromosome I using the transgene silencing phenotype, whereas *mg379* was placed into a 4.6-map-unit interval using Eri phenotype *lin-1*(*RNAi*). Next, *mgIs30* was used to identify genes that cause transgene silencing using feeding RNAi targeting the open reading frames in the mapped *mg411* interval. Two open reading frames tested positive: C41D11.1 and C41D11.7. Sequencing of *mg411* and *mg379* identified the following mutations: *mg411* A→T at position 33105 of C41D11, and *mg379* G→A at position 34755 of cosmid C41D11. Five additional alleles were obtained by sequencing the *eri-6/7* locus in mutants from the ethyl methanesulphonate Eri screen. An eighth, presumably spontaneous, allele (see Supplementary Discussion), *eri-6/7*(*mg441*), was isolated by mating a serendipitously obtained Eri strain in the lab with *eri-6/7* mutants to ask for non-complementation, followed by sequencing of the *eri-6/7* gene.

Rescue experiments were done with the C41D11 cosmid at 5 μg ml^{-1}, and PCR fragments at 5 μg ml^{-1}. Promoter::*gfp* or m*rfp* fusions, and the *trans*-splicing reporter construct, were injected at 10 μg ml^{-1}. Full-length ERI-6/7::mCherry fusion was injected at 5 μg ml^{-1}. DiO was used to identify dye-filling amphid sensory neurons.

Enhanced RNAi assays

unc-22, 23-bp ds-siRNA injections were done as previously described[43]. The ds-siRNA was injected at 5 mg ml^{-1}. The percentage of F_1 twitchers was scored in 330 μM levamisole. Feeding RNAi assays were done at 20 °C. For *lin-1*(*RNAi*) and *unc-73*(*RNAi*), starved L1 s were placed

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on *E. coli* expressing the dsRNA. The next generation was scored for percentage displaying Muv or Pvl (*lin-1*), or coiler (*unc-73*) phenotypes. For *cel-1(RNAi)* and *hmr-1(RNAi)*, worms that were exposed to RNAi starting at the L1 stage were scored as adults for the production of viable progeny. In complementation assays, heterozygotes of *mg369/mg379* and *mg411/tm1887* were tested for an Eri phenotype based on hypersensitivity to dsRNA targeting *hmr-1*.

cDNA analysis, 3′- and 5′-RACE

RNA was isolated from mutant worms or worms exposed to RNAi for several generations, using TRI Reagent (Molecular Research Center). RNA was treated with TurboDNase (Ambion) and cDNA was made using the RETROscript kit (Ambion). For 3″-RACE, the First Choice RLM-RACE kit (Ambion) was used; for 5″-RACE the SMART RACE kit (Clontech) was used. 3″- and 5″-RACE products were cloned using Qiagen PCR cloning kit. RT-PCR on worms expressing the *trans*-splicing reporter was done by hand-picking GFP-expressing worms for RNA isolation. RT-PCR was done using a primer in *gfp* combined with primers in *eri-6*. No PCR products were obtained with these primers using genomic DNA of GFP-expressing worms.

Western blotting

To enhance transgene expression, nematodes carrying the *trans*-splicing reporter were subjected to *rde-1* or *mut-16* RNAi by feeding. This resulted in brighter and broader expression of GFP. GFP-expressing worms from two independent transgenic lines were hand-picked into sample buffer. Nontransgenic N2 worms served as a negative control. Electrophoresis and western blotting were done according to standard methods. A monoclonal antibody to GFP (Clontech) was used at 1:10,000. A horseradish peroxidase-conjugated goat anti-mouse antibody was used as a secondary antibody.

Northern blotting

Northern blotting for endo-siRNAs was done as previously described[42] (http://chronic.dartmouth.edu/VRA/ambroslab.html) using 30 μg of RNA in 12% denaturing polyacrylamide gels. Prehybridization and hybridization were done in 7% SDS, 0.2 M Na-phosphate pH 7.0 at 35 °C. StarFire probe sequences were as described[5].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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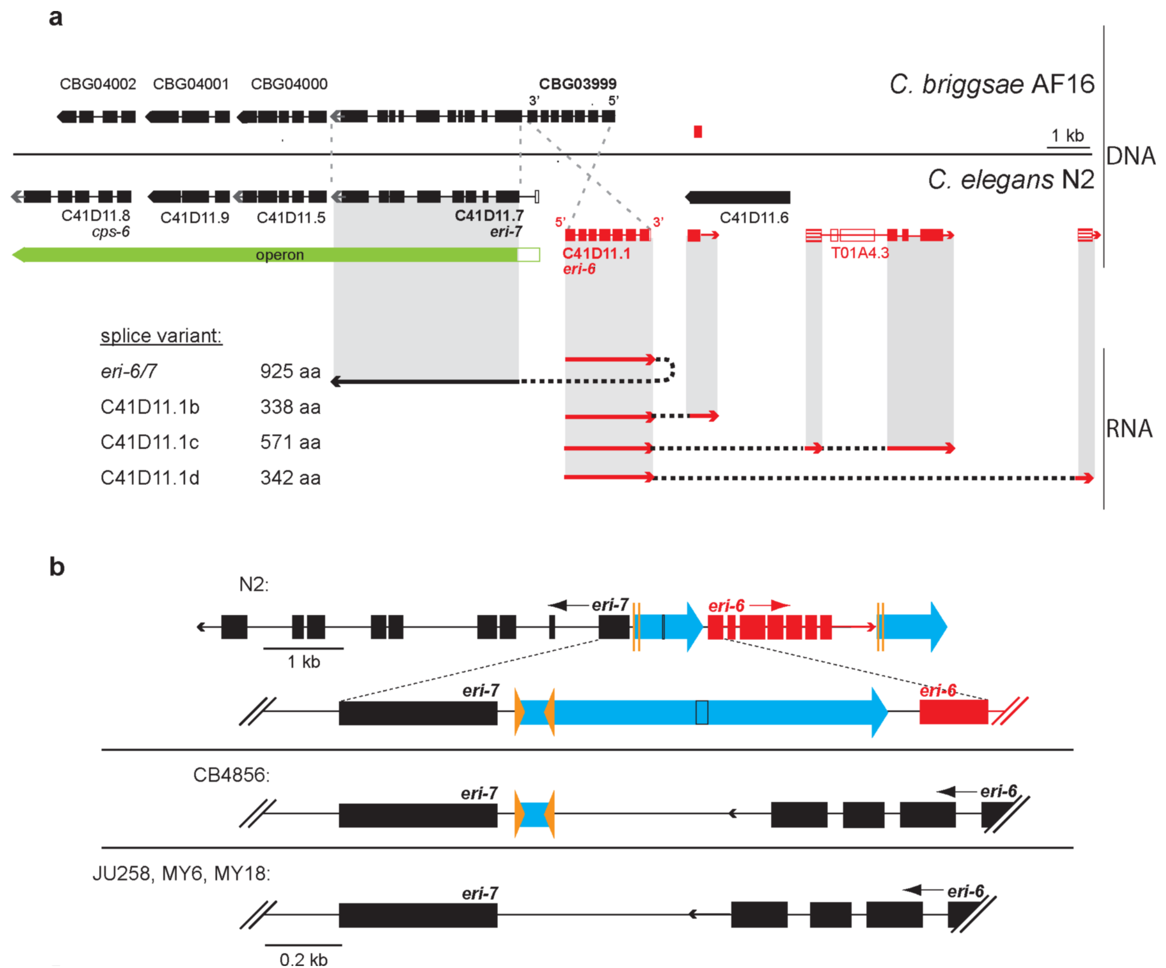

Figure 1. The *eri-6/7* locus in various strains and its gene products in *C. elegans* N2

a, Structure of *eri-6/7* in *C. elegans* N2 and the syntenic region in *C. briggsae* AF16, based on RT-PCR, 3″-RACE and WormBase[21]. The *eri-7* operon is conserved. *C. briggsae eri-6* is encoded on the opposite strand from *C. elegans* and is one gene with *C. briggsae eri-7* (CBG03999). Exons are black or red rectangles, depending on the strand on which they are encoded. Open rectangles: predicted exons[21], but unconfirmed. Striped exons: discovered experimentally. *eri-6* splices to *eri-7* 24 nucleotidesupstream of the predicted[21] *eri-6* stop codon. **b**, Relative orientation of *eri-6* and *eri-7* in four *C. elegans* isolates. Blue arrows: the approximate 930-bp direct repeat flanking *eri-6*. Orange arrowheads: 25-bp inverted repeats.

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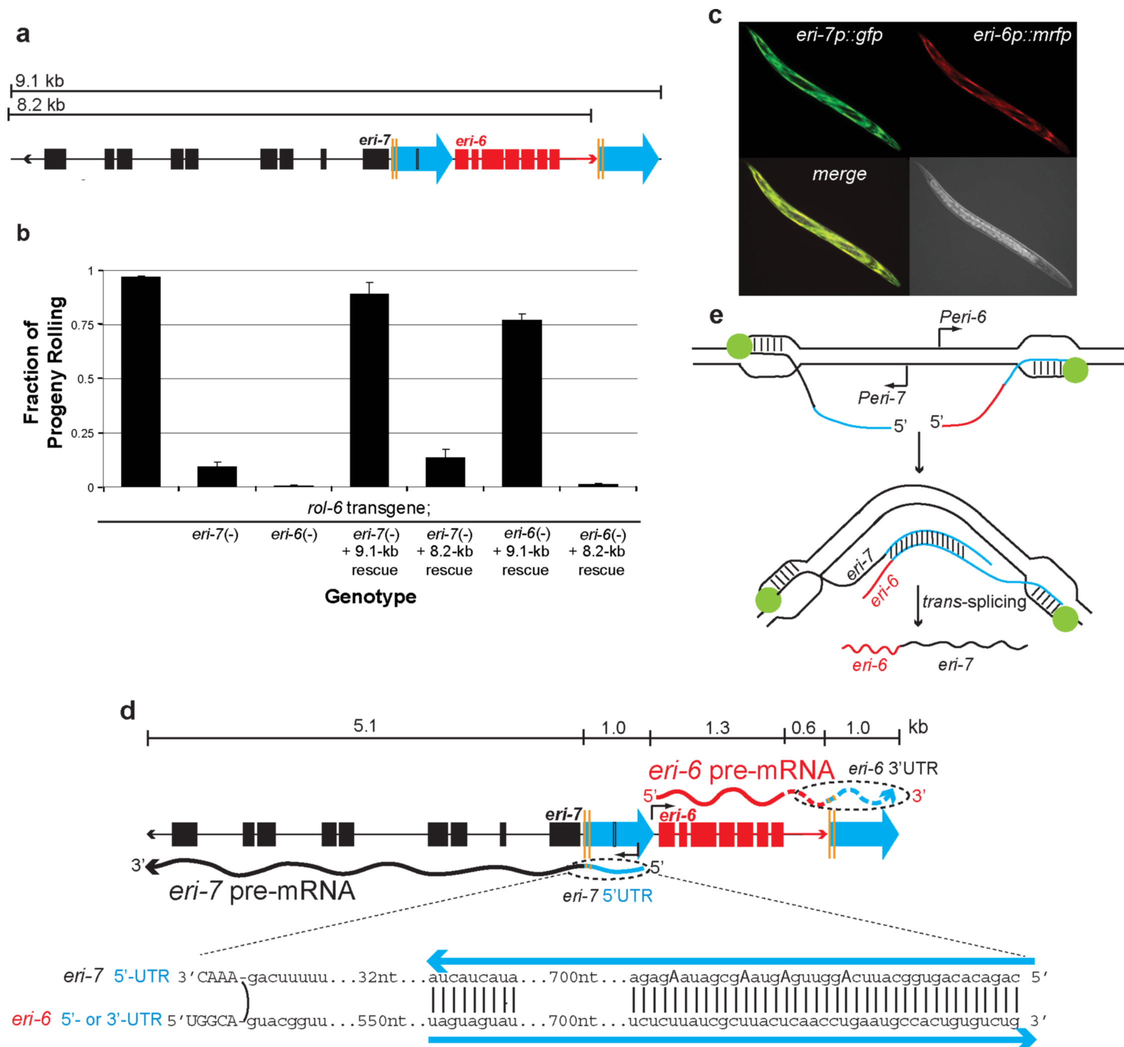

Figure 2. The *eri-6/7* mRNA is formed by local *trans*-splicing

a, Schematic of *eri-6/7* locus used for rescue: 9.1-kb fragment; and 8.2-kb fragment missing the second repeat. **b**, Rescue of *eri-6/7*(*mg411*)-induced silencing of the *mgIs30* transgene-conferred rolling phenotype. Number of broods scored = 28, 28, 18, 19, 38, 29 and 18, respectively. Multiple transgenic lines assayed. Error bars: s.e.m. **c**, The *eri-6* and *eri-7* promoters express in overlapping tissues (hypodermis and head neurons). *eri-*6 and *eri-7* promoters were fused to *rfp* and *gfp*, respectively. **d**, 5′-RACE experiments show that *eri-6* and *eri-7* are expressed as separate pre-mRNAs with sufficient nucleotide homology to base-pair, thus facilitating *trans*-splicing. *eri-7* pre-mRNA starts 775 nucleotides (nt) upstream of exon 1, whereas *eri-6* pre-mRNA starts 100 nt upstream of *eri-6* exon 1 (black arrows). Edited nucleotides (A to G indicative of A to I) in *eri-7* pre-mRNA are in bold capital letters. Blue arrows: direct repeats. Capital letters: exon sequence. Curved line: *trans*-splicing juncture. **e**, Model of *eri-6/7 trans*-splicing. *eri-6* and *eri-7* pre-mRNAs are locally co-transcribed in the same cells. The pre-mRNAs may form a dsRNA intermediate that facilitates co-transcriptional *trans*-splicing.

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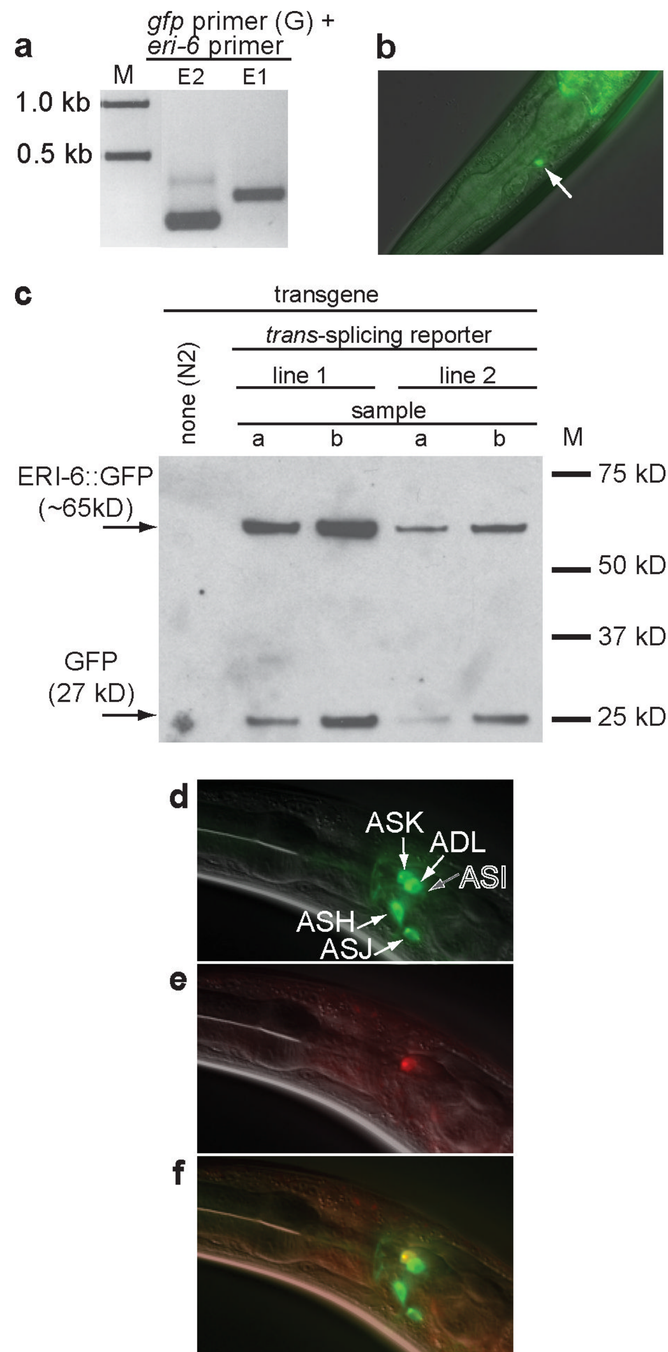

Figure 3. Chimeric ERI-6/7 protein is expressed through *trans*-splicing

a, RT-PCR on *gfp*-expressing worms using primers in *gfp* (G) and *eri-6* (E1 and E2). Sequencing of the RT-PCR products confirmed *trans*-splicing. **b**, Expression of the *trans*-splicing reporter in ASK. **c**, Western blot on animals expressing the *trans*-splicing reporter. A GFP antibody was used. The slower-migrating product (of the expected weight of ERI-6::GFP) is presumably ERI-6::GFP. The faster-migrating product may be GFP expressed using an alternative start codon. **d**, DiO fills five pairs of sensory neurons. **e**, ERI-6/7::mCherry is expressed in one amphid neuron. **f**, Merged image identifies ASK as the ERI-6/7-expressing neuron. **d, e, f**,

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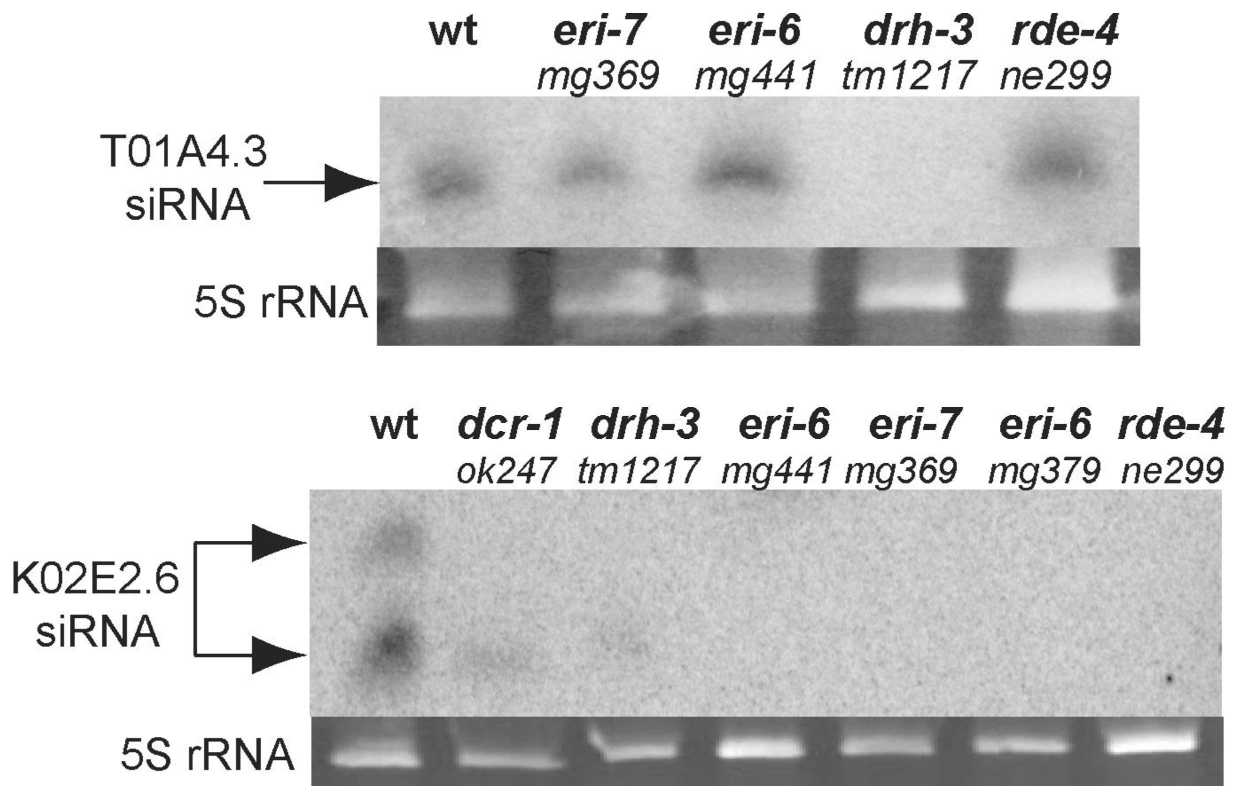

Figure 4. ERI-6/7 is required for endogenous RNAi

Shown are northern blots probed for two different endogenous siRNAs, targeting the genes T01A4.3 and K02E2.6. Mutants of additional genes known to be required for endogenous RNAi (*dcr-1*, *drh-3* and *rde-4*) are shown as controls. 5S rRNA is shown as a loading control.