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The exonuclease ERI-1 has a conserved dual role in 5.8S rRNA processing and RNAi

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Abstract

The exonuclease ERI-1 negatively regulates RNA interference (RNAi) in *Caenorhabiditis elegans* and *Schizosaccharomyces pombe*, and is required for production of some *C. elegans* endogenous small-interfering RNAs. We show that ERI-1 performs 3′ end processing of the 5.8S ribosomal RNA (rRNA) in both *C. elegans* and *S. pombe*. In *C. elegans*, two protein isoforms of ERI-1 are localized to the cytoplasm, and each has distinct functions in rRNA processing and negative regulation of RNAi.

The *C. elegans eri-1* gene encodes a 3′ to 5′ exonuclease of the DEDDh superfamily of RNase T exonucleases that was identified as a negative regulator of RNA interference (RNAi)[1]. Mutations in *eri-1* cause an enhanced RNA interference (Eri) phenotype by which double stranded RNAs (dsRNAs) that are ineffective in silencing target mRNAs in wild-type animals trigger robust silencing in the Eri mutant. In the fission yeast *S. pombe* loss of Eri1 causes increased levels of small interfering RNAs (siRNAs) corresponding to centromeric repeats and a concomitant increase in RNAi-dependent heterochromatin formation at these genomic loci[2]. Analysis of ERI-1 in *C. elegans*, human and fission yeast has shown that it can degrade the 3′ end of siRNAs and histone mRNAs *in vitro*[1-4], but *in vivo* substrates for this conserved enzyme are poorly understood.

In the course of the analysis of RNAs isolated from the *eri-1* null mutant, we observed that the 5.8S rRNA in an *eri-1* worm is longer than wild-type 5.8S rRNA (Fig. 1a). This length difference is present in all detectable 5.8S rRNA, suggesting that *eri-1* mutants have an rRNA processing defect in most, if not all, cells. The mature 5.8S, 18S, and 25-28S rRNAs in eukaryotes are generated from a 35S-47S precursor RNA via a series of processing steps mediated by multiple nucleases[5]. The activity of ERI-1 as a 3′ to 5′ exonuclease[1-4] suggests that the longer 5.8S rRNA is due to an extension of the 3′ end. RNase H cleavage and 3′ end cloning on the 5.8S rRNA of wild-type and mutant *C. elegans* indicated that all *eri-1* 5.8S rRNA is at least 1 nucleotide longer than the wild-type 5.8S rRNA specifically at the 3′ end, with a substantial fraction of *eri-1* 5.8S rRNA containing 2 to 4 additional nucleotides (Supplementary Fig. 1 online). The 5.8S processing defect was observed in two independently derived *eri-1* alleles, including another null allele (*mg388*, data not shown), and is rescued by an *eri-1* transgene (**see below**), proving that loss of *eri-1* causes the 5.8S processing defect. The 5.8S processing defect was not observed in other enhanced RNAi mutants with pleiotropies in common with *eri-1*[1,6] (data not shown).

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To characterize whether ERI-1 function in rRNA trimming is an ancient feature of this orthology group, we examined the 5.8S rRNA in the *S. pombe eril*Δ mutant and observed a length defect similar to the *C. elegans eri-1* mutant (Fig. 1b). RNase H and sequencing analysis revealed a 3′ extended 5.8S rRNA species in *eril*Δ containing from 2 to 8 additional 3′ nucleotides (Supplementary Fig. 2 online). This indicates that ERI-1 has a conserved, dual function in rRNA biogenesis and negative regulation of RNAi that has been inherited from the common ancestors of animals and fungi.

In *C. elegans*, ERI-1 forms a protein complex with Dicer (DCR-1), and *eri-1* gene activity is required for the production of a variety of *C. elegans* endogenous siRNAs. The release of DCR-1 or other RNAi factors from the production of these endogenous siRNAs in the *eri-1* mutant may be the basis of the enhanced response to exogenous dsRNAs[6,7]. Co-immunoprecipitation experiments have indicated that DCR-1 interacts specifically with one of two ERI-1 protein isoforms, ERI-1b, but does not associate with the other, ERI-1a[6]. Both ERI-1a and ERI-1b contain a conserved SAP nucleic acid binding domain[8] and 3′ to 5′ exonuclease domain[1], but ERI-1b carries an extended nematode-specific C-terminal sequence with no identifiable functional domains (Fig. 1c). To examine the role of each isoform in RNAi and rRNA biogenesis we generated transgenic *C. elegans* expressing each *eri-1* spliced-isoform in an *eri-1* null mutant background (Supplementary Fig. 3 online). Rescue of the *eri-1* enhanced RNAi phenotype was assessed using *unc-73* RNAi. Wild-type animals are unaffected by dsRNA to this neural development gene, while 99% (± 0%) of *eri-1* mutant animals display a movement defect similar to a null mutation in *unc-73* (Supplementary Fig. 3). Expression of ERI-1b in *eri-1* dramatically reduced the enhanced response to *unc-73* dsRNA to 6% (± 4%) affected, indicating that ERI-1b rescues animals to the wild-type lack of response to *unc-73* dsRNA. ERI-1a failed to rescue the enhanced RNAi response with 85% (± 10%) of transgenic animals displaying the unc-73 phenotype (Supplementary Fig. 3). Similar results were observed for each line when tested for the enhanced RNAi-dependent phenotype of *cel-1* RNAi (data not shown). Northern blot analysis indicated that ERI-1b rescued *K02E2.6* endogenous siRNA biogenesis, while ERI-1a expression failed to re-establish these siRNAs (Fig. 1d). Similar results were obtained for another *eri-1*-dependent endogenous siRNA (Supplementary Fig. 3). Expression of either ERI-1a or ERI-1b rescued 5.8S rRNA length (Fig. 1d), indicating that both isoforms can mediate rRNA processing. This rescue was robust but incomplete, as expected with expression of ERI-1 in somatic tissues from an extrachromosomal transgene array. These data show that ERI-1a mediates 5.8S rRNA processing but may not act in RNAi pathways, while ERI-1b participates in both rRNA processing and RNAi. This supports a model in which the interaction of ERI-1b with DCR-1, possibly through its extended C-terminal domain, is important for its activity in endogenous siRNA biogenesis and inhibition of exogenous RNAi.

We examined the subcellular localization of both ERI-1 isoforms by expressing C-terminal green fluorescent protein (GFP) fusions to ERI-1a and ERI-1b in the *eri-1* mutant. Rescuing GFP-tagged versions of both isoforms (Fig. 1d, and Supplementary Fig. 3) were exclusively cytoplasmic in a large variety of cell types that express the transgene (Fig. 1e). Similar results were observed for N-terminal GFP fusions to ERI-1 isoforms (data not shown). ERI-1 localization to the cytoplasm is surprising, given that rRNA processing occurs primarily in the nucleolus. However, terminal rRNA processing of the 18S is cytoplasmic [9]. Furthermore, results from *Xenopus* have suggested that processing of the 5.8S rRNA from a slightly longer pre-5.8S RNA may occur in the cytoplasm[10], and *S. pombe* Eri1 is localized to the cytoplasm2. Mammalian ERI-1 localizes to the nucleolus as well as the cytoplasm[4] (Ansel et al., co-submitted). The activity of mouse Eri-1 to trim the 5.8S rRNA on intact ribosomes, similarly to our results (see below), and its association with polysomes (Ansel et

al., co-submitted) support a role for ERI-1 5.8S rRNA processing after nuclear export of ribosomes.

Most rRNA processing and ribosome assembly steps have occurred by the time the ribosome reaches the cytoplasm. In the mature ribosome, the 3′ end of the 5.8S rRNA is paired with the 5′ end of the 25-28S rRNA[11] (Supplementary Fig. 4 online). This 5.8S/25-28S helix is reminiscent of the 3′ hairpin of the histone mRNA, and siRNA structures that are *in vitro* substrates for ERI-1[1-4]. We tested if each protein isoform could act on this structure *in vitro* using ribosomes purified from *eri-1* mutants. When recombinant ERI-1a was incubated with intact *eri-1* ribosomes a major product was generated with identical length to that of the wild-type 5.8S rRNA (Fig. 2a). In contrast, ERI-1b showed no activity *in vitro*, despite expression and purification at levels equal to ERI-1a (Fig. 2a and Supplementary Fig. 4). This is discordant with *in vivo* results indicating that ERI-1b expressed from a transgene can mediate rRNA trimming in the absence of ERI-1a expression (Fig. 1d), and may be due to the *in vitro* expressed ERI-1b enzyme lacking co-factors, such as DCR-1, which may be required for its activity. Alternatively, *in vitro* expression or assay conditions may fail to recapitulate critical aspects of *in vivo* chemistry. Similar results for enzymatic activity of ERI-1 were obtained for an oligonucleotide mimic of the 5.8S/26S structure and a synthetic siRNA (Supplementary Fig. 4 and data not shown)

Introduction of H264A and D268A mutations into the nuclease domain of *S. pombe* Eri1 disrupts the protein's exonuclease activity *in vitro*, and its negative regulation of RNAi *in vivo*[2]. To verify that ERI-1 catalyzes the 3′ end processing of the 5.8S rRNA we inserted homologous mutations into *C. elegans* ERI-1a (H317A and D321A, “ERI-1aAA”, Supplementary Fig. 4). These mutations caused a complete loss of *in vitro* nuclease activity on both purified *eri-1* ribosomes and an oligonucleotide 5.8S/26S substrate (Fig. 2b and Supplementary Fig. 4). Transgenic lines expressing either ERI-1aAA::GFP or ERI-1bAA::GFP at levels equal to the wild-type transgenes failed to rescue rRNA trimming defects of the *eri-1* mutant *in vivo* (Fig. 2c and data not shown), ERI-1bAA::GFP also failed to rescue the enhanced RNAi phenotype, or re-establish expression of endogenous siRNAs in the *eri-1* mutant (Supplementary Fig. 3).

The extensive conservation of 5.8S rRNA processing by ERI-1 in *C. elegans*, *S. pombe* and mouse (Ansel et al., co-submitted) suggests that this trimming is important to ribosome function. Despite this conservation, *eri-1* mutant *C. elegans* or *S. pombe* do not display obvious phenotypes attributable to defects in the ribosome. In contrast in mouse, loss of Eri-1 results in cell and organismal growth defects (Ansel et al., co-submitted). This distinction could reflect subtle defects in ribosome function in all three clades that are only manifest at the gross level of organismal health in the more complex biology of the mouse. Alternatively mouse ERI-1 exonuclease may process RNAs in addition to the 5.8S rRNA, just as ERI-1 in *C. elegans* and *S. pombe* regulates endogenous siRNA produced by RNA-dependent RNA polymerases, a pathway that appears to be missing in mammals. In the budding yeast *Saccharomyces cerevisiae eri-1* has been lost, along with known components of the RNAi pathway such as Dicer and Argonaute, but a CCR4-like nuclease, Ngl2p, performs analogous 3′ end trimming of the 5.8S rRNA[12]. This further supports the importance of this processing in cellular fitness.

Previously, the microRNA processor Drosha and two DEAD-box helicases were shown to act in both rRNA biogenesis and small-RNA-dependent silencing[13]. Our data exposes more extensive integration between these two cellular pathways, and suggests that future analyses may uncover additional common components and origins of these pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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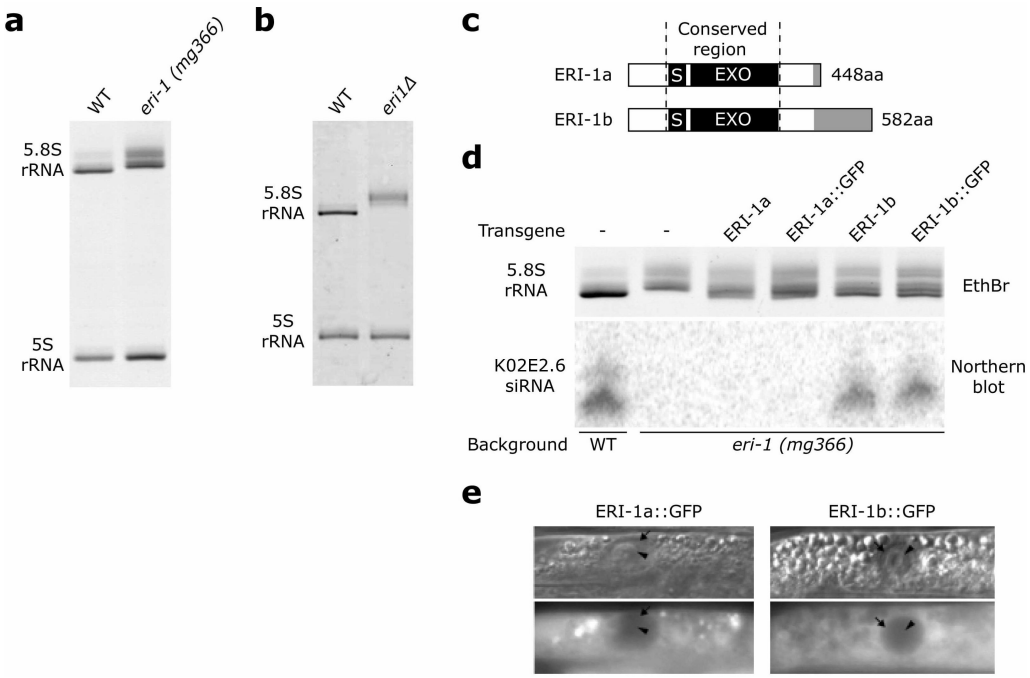

Figure 1.

eri-1 is required for 3′ end processing of the 5.8S rRNA in *C. elegans* and *S. pombe*. (**a**,**b**)Wild-type and *eri-1/eri1Δ* RNA samples from *C. elegans* (**a**) and *S. pombe* (**b**) were separated on a denaturingpolyacrylamide gel and stained with ethidium bromide. **(c**) Protein isoforms of *C. elegans* ERI-1. Location of SAP (S) nucleic acid biding domain[8], and exonuclease domain (EXO) are indicated. Gray denotes sequence unique to each isoform. **(d)** RNA from *eri-1* lines expressing each ERI-1 isoform separated on a denaturing-polyacrylamide gel and stained with ethidium bromide (EthBr), or probed for endogenous siRNAs corresponding to the K02E2.6 gene (Northern blot). (**e**) Intestinal cells expressing C-terminal GFP fusions of each ERI-1 isoform. Top: normarski, bottom: GFP fluorescence. Arrow: nuclear periphery, arrowhead: nucleolar periphery.

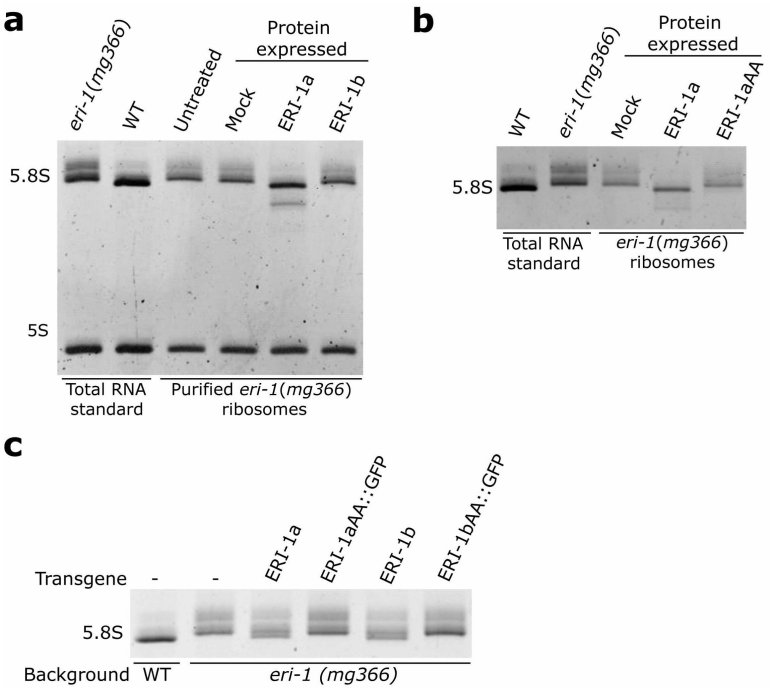

Figure 2.

ERI-1 directly processes the 5.8S rRNA in ribosomes. (**a**) Purified ribosomes from *eri-1* mutants were exposed to *in vitro* translated ERI-1a or ERI-1b (Supplementary Methods online). Total RNA from wild type and *eri-1* were run for reference. (**b**) *in vitro* translated ERI-1a or ERI-1aAA (H317A, D321A) were incubated with purified *eri-1* ribosomes. Untreated: buffer incubated ribosomes, Mock: unprogrammed reticulocyte lysate control (**c**) 5.8S rRNA of *eri-1* transgenic lines expressing the ERI-1 isoforms and point-mutants. Samples were separated on a denaturing-polyacrylamide gel and stained with ethidium bromide.