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Report

Conserved Ribonuclease, Eri1, Negatively Regulates Heterochromatin Assembly in Fission Yeast

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Summary

RNA interference (RNAi) is a conserved silencing mechanism that has widespread roles in RNA degradation, translational repression, and the epigenetic control of chromatin structure [1]. In fission yeast, heterochromatin assembly requires RNAi machinery and is initiated by small interference RNAs (siRNAs) derived from heterochromatic regions and by the RNA-induced transcriptional silencing (RITS) complex [2–7]. Although recent studies have been successful in uncovering the functions of effector complexes in the RNAi pathway [4, 5, 8–10], exactly how heterochromatic siRNAs are processed and function in assembling heterochromatin remains unclear. In this study we focused on a conserved ribonuclease, Eri1, which was originally identified as a negative regulator of RNAi in *C. elegans* [11], and show the importance of the Eri1 protein in RNAi-mediated heterochromatin assembly in fission yeast. Eri1 specifically degrades double-stranded siRNAs through two functional domains and represses the accumulation of cellular siRNAs in vivo. Deletion of *eri1*$^+$ causes an increase in siRNAs associated with the RITS complex and enhances heterochromatic silencing, which is accompanied by increased levels of histone H3-K9 methylation and the Swi6 protein. Our findings suggest that the fission yeast Eri1 controls the accumulation of heterochromatic siRNAs and negatively regulates the RNAi-mediated heterochromatin assembly.

Results and Discussion

Eri-1 is a conserved siRNA-degrading ribonuclease (RNase) that was originally identified as a negative regulator of RNAi in *C. elegans* (*Ce*) [11]. It was also recently reported that *Ce* Eri-1 is involved in the accumulation of a set of endogenous siRNAs [12, 13]. These results suggest that Eri-1 has a diverse role in the metabolism of a distinct species of small RNAs. To study the mechanisms underlying siRNA-mediated heterochromatin formation, we focused on the function of the Eri-1 ortholog in *Schizosaccaromyces pombe*. A database search for *Ce* Eri-1 orthologs predicted a single *S. pombe* candidate that is encoded by SPBC30B4.08 [11]. Subsequent analyses of its cDNAs revealed an uncharacterized second exon, and the resultant 313 amino acid protein contained conserved SAP and DEMDh exonuclease (EXO) domains, each of which showed more than 30% identity to those of *Ce* Eri-1 (Figure 1A) [11]. These results indicate that SPBC30B4.08 encodes an Eri-1 ortholog in *S. pombe,* and we therefore named it *eri1*$^+$ (Eri1).

A previous study had shown that human and *Ce* Eri-1 possess enzymatic activity for degrading 3′ overhangs of double-stranded (ds) siRNA in vitro [11]. To characterize the activities of the *S. pombe* (*Sp*) Eri1, we prepared recombinant Eri1 as a GST fusion protein (GST-Eri1) and incubated ^{32}P-labeled synthetic oligonucleotides with GST-Eri1 (Figures 1B and 1C). We found that GST-Eri1, but neither GST alone nor RNaseT1, the control for single-strand specific RNase, degraded 23 nt dsRNA with 2 nt 3′ overhangs to the level of short oligonucleotides (Figure 1C). GST-Eri1 also degraded the RNA moiety of an RNA-DNA hybrid, but not single-stranded (ss) DNA. These results indicate that *Sp* Eri1 has RNase activity that degrades siRNA-like dsRNAs. Interestingly, this activity is distinct from the previously observed *Ce* Eri-1 activity [11]. Taking into account the fact that both human and *Ce* Eri-1 have activity for degrading at least a base into the double-stranded region of siRNA substrates, we think it is likely that the appropriate conditions may permit Eri-1-family proteins in other species to degrade dsRNAs, as found in *Sp* Eri1.

To further dissect the functions of conserved domains, we separately expressed each domain as a GST-fusion protein (GST-SAP and GST-EXO) (Figure 1B). Although full-length GST-Eri1 efficiently degraded the substrate, we found that neither GST-SAP nor GST-EXO degraded dsRNA (Figure 1D), suggesting that both domains are required for Eri1's dsRNA-degrading activity. Although GST-EXO could not efficiently degrade the dsRNA, an excess amount of GST-EXO partially degraded it (Figure S1A in the Supplemental Data available with this article online), indicating that the EXO domain functions in a catalytic role in the Eri1 enzymatic activity. Because the SAP domain was originally defined as a DNA binding motif [14], we performed electrophoretic mobility shift assays and investigated whether the SAP domain binds dsRNAs. In this assay, GST-SAP but not GST or GST-EXO efficiently bound the ^{32}P-labeled dsRNA probe (Figure 1E). Unlabeled dsRNA or RNA-DNA hybrid, a substrate of Eri1, clearly competed with this binding, but ssRNA, dsDNA, and ssDNA did so only negligibly (Figure S1B). Taken together, these results suggest a molecular mechanism in which the Eri1 recognizes dsRNAs by its N-terminal SAP domain and degrades the substrate by using its C-terminal catalytic domain.

In an attempt to determine the subcellular compartment in which *Sp* Eri1 functions, we expressed the FLAG-tagged functional Eri1 (Eri1-F; Figure S2A) and observed its localization by indirect immunofluorescence analyses. The majority of the Eri1-F protein

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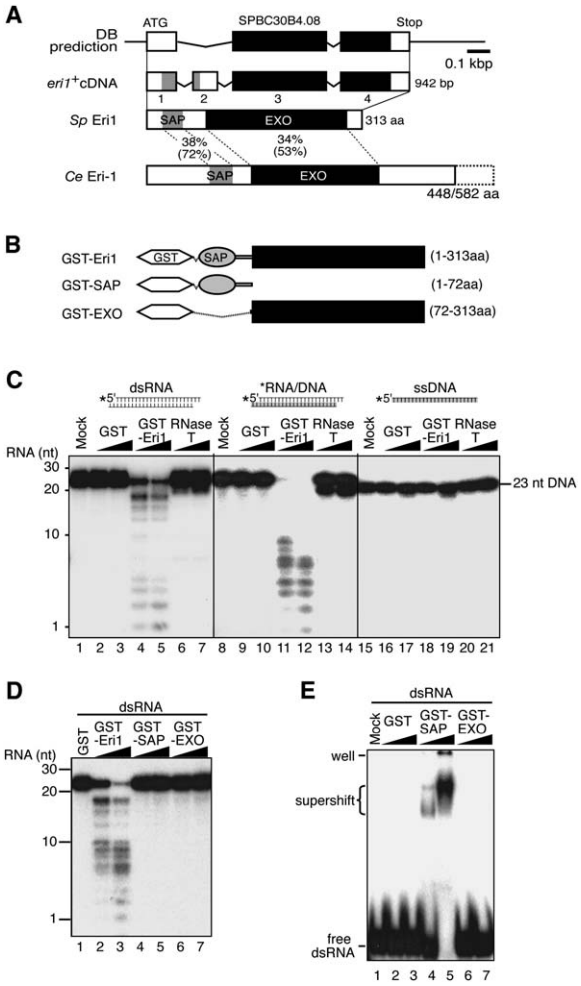

Figure 1. Eri1 Is a Double-Strand siRNA-Degrading Enzyme

(A) The top two drawings show the gene structure at the SPBC30B4.08 locus as predicted by "*Schizosaccaromyces pombe* GeneDB" (http://www.genedb.org/genedb/pombe/). The three predicted exons are shown as boxes. The cDNA structure of the *eri1*$^+$ gene determined by the cloning of *eri1*$^+$ cDNA is shown in the second drawing. The lower two drawings show the structures of *S. pombe* Eri1 (*Sp* Eri1) and *Caenorhabditis elegans* Eri-1 (*Ce* Eri-1: accession numbers AAK39278 and AAK39277). Gray and black boxes represent the conserved "SAP" and "DEMDh exonuclease (EXO)" domains, respectively. The identities (similarities) of the SAP and EXO domains between *Sp* Eri1 and *Ce* Eri-1 are also shown.

(B) Schematic drawing of Eri1 (GST-Eri1), Eri1-N-terminal (GST-SAP), and Eri1-C-terminal (GST-EXO) proteins prepared from *E. coli* as GST-fusion proteins and used in (C)–(E).

(C) Ribonuclease (RNase) activity of Eri1 in vitro. GST, GST-Eri1, or RNaseT1 was incubated with 23 nt double-stranded RNA (dsRNA), RNA/DNA heteroduplex, or single-stranded DNA (ssDNA) at 37ºC for 1 hr. The DNA strands are shown as darker lines. The 5′-end-labeled oligo nucleotides are denoted with an asterisk (*). The reaction lacking recombinant proteins were defined as "Mock".

(D) N- and C-terminal domains of Eri1 are required for dsRNA-degrading activity. Equal amounts of GST, GST-Eri1, GST-SAP, or GST-EXO were used in an RNase assay.

(E) dsRNA binding activity of the SAP domain. Five or 25 pmol GST, GST-SAP, or GST-EXO was incubated with 10 pmol dsRNA and resolved by native PAGE. The reaction lacking recombinant proteins were defined as "Mock".

localized to the cytoplasm (Figure S2B). Similar localization patterns were observed by subcellular fractionation analyses (Figure S2C). These results suggest that *Sp* Eri1 localizes predominantly to the cytoplasm, which is consistent with the previous observation of *Ce* Eri-1 [11].

Taken together, the conserved structure, RNase activity, and cellular localization of *Sp* Eri1 implied its function in siRNA-mediated cellular processes in fission yeast. RNAi machinery in fission yeast is required for the initiation or formation of heterochromatin at centromeres and the mating-type loci [2, 3]. To test whether *eri1*$^+$ is involved in heterochromatic gene silencing, we deleted the *eri1*$^+$ gene and observed silencing of a *ura4*$^+$ reporter gene inserted within the central region (*cnt1::ura4*$^+$), the innermost repeats (*imr1R::ura4*$^+$), the outer repeats (*otr1R::ura4*$^+$), or the mating-type *K*-region (*Kint::ura4*$^+$) (Figure 2A) [15, 16]. Interestingly, we found that the *eri1*$^+$ deletion (*eri1*Δ) did not affect normal cellular growth on the non-selective medium (Figure 2B, AA), but it did cause a decrease in cells expressing *imr1R::ura4*$^+$, *otr1R::ura4*$^+$, or *Kint::ura4*$^+$ reporter gene (Figure 2B, AA-Ura). Furthermore, we did not see this effect in RNAi-deficient strains (*dcr1*Δ, *ago1*Δ, or *rdp1*Δ), which alleviated *otr1R::ura4*$^+$ silencing [2] (Figure 2C). These results suggest that *eri1*Δ enhanced silencing at these heterochromatic regions and also show that this silencing enhancement depends on the RNAi machinery. It should be noted that *eri1*Δ had similar effects on endogenous centromeric transcripts. Quantitative RT-PCR analyses revealed that centromeric transcripts derived from *dh* repeats were decreased by the *eri1*Δ mutation in an RNAi-factor-dependent manner (Figure 2D).

We next sought to determine whether the enzyme activity is critical to Eri1's function in vivo. We confirmed that two alanine-exchange mutations (H264A and D268A) in the EXO domain (*eri1AA*) impaired the dsRNA-degrading activity of GST-Eri1 in vitro (Figures 3A and 3B), and we then introduced this *eri1AA* mutation into the endogenous *eri1*$^+$ gene with a sequence for the C-terminal FLAG tag, which enabled us to verify the levels of FLAG-tagged functional Eri1 (*eri1-F*) and mutant Eri1 (*eri1AA-F*) proteins (Figure 3C). In contrast to the wild-type-level silencing of *eri1-F* cells, *eri1AA-F* mutant cells showed enhanced *otr1R::ura4*$^+$ silencing, as observed in *eri1*Δ cells (Figure 3D). These results suggest that Eri1 negatively regulates heterochromatic silencing through its enzymatic activity.

To further examine Eri1's role in heterochromatic silencing, we tested whether overproduction of Eri1 leads to silencing derepression at these heterochromatic regions. We found, however, that the induction of *eri1*$^+$ expression by the *nmt1* promoter caused a severe growth defect, and short-time induction did not clearly change the *otr1R::ura4*$^+$ silencing (Figure S3A; data not shown). We think that the growth defect might be attributable to the fact that Eri1 could degrade cellular RNAs, which are not physiological targets of the Eri1 protein.

In fission yeast, heterochromatin is marked by the methylation of Histone H3 at lysine-9 (H3-K9me), and this modification becomes a target of Swi6 to form silent heterochromatin [17]. To test whether the heterochromatin structure is affected by *eri1*Δ, we performed chromatin immunoprecipitation (ChIP) assays by using antibodies against Swi6 and H3-K9me. In the ChIP assay,

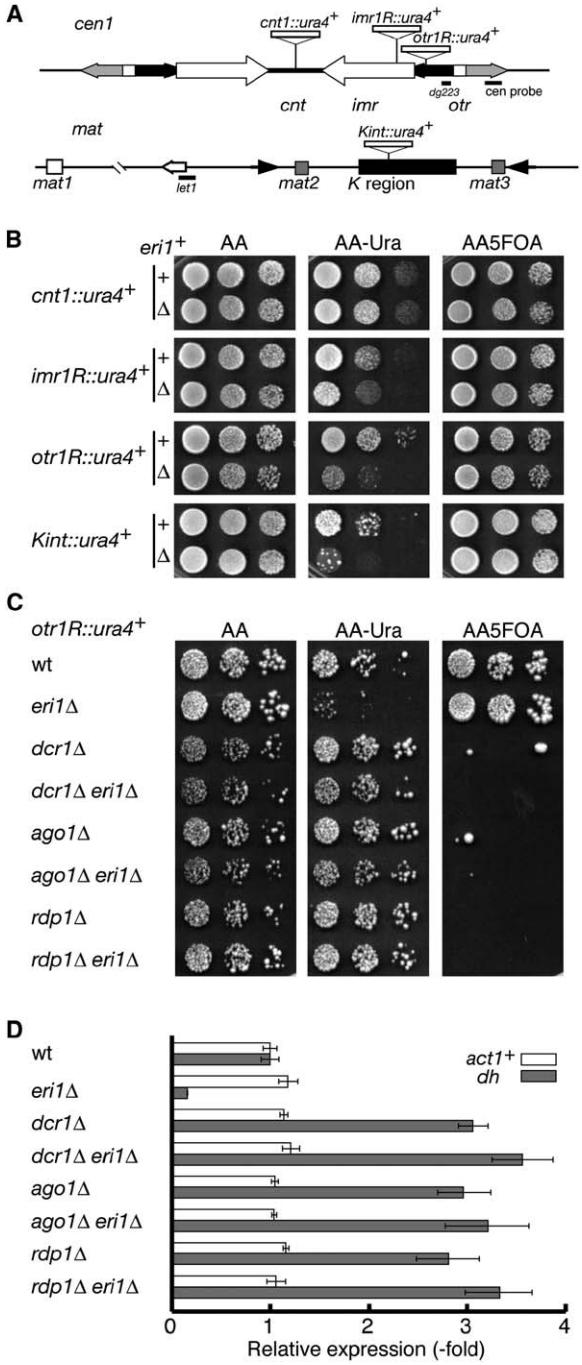

Figure 2. The *eri1⊿* Mutation Enhances Heterochromatic Silencing in *S. pombe* in an RNAi-Pathway-Dependent Manner

(A) Schematic diagram of *centoromere1* (*cen1*) and the mating-type loci (*mat*). The positions of the silencing reporter gene *ura4*$^+$, ChIP fragment of *dg* repeats (*dg223*), and *let1*$^+$ locus as well as the probe used for the northern analysis of long transcripts from the *otr* repeats are indicated by black bars. The relative lengths of *cen1* and *mat* loci are not drawn to scale.

(B) Silencing enhancement by the *eri1⊿* mutation. A 10-fold serial dilution of cultures of the indicated strains carrying the *ura4*$^+$ gene in the *cen1* (*cnt1, imr1R*, *or otr1R*) or *mat* locus were spotted onto AA medium, AA medium lacking uracil (AA-Ura), and AA medium containing 5FOA (AA5FOA) (counter selector for *ura4*$^+$ cells).

(C) RNAi-dependent enhancement of heterochromatic silencing by the *eri1⊿* mutation. Ten-fold serial dilutions of cultures of the indicated strains carrying *otr1R::ura4*$^+$ were spotted onto AA, AA-Ura, or AA5FOA medium.

(D) Effects of the *eri1⊿* mutation on centromeric transcripts. Real-time RT-PCR analyses were performed, and the expression level of *act1*$^+$ (white bar) and *dh* (gray bar) transcripts of each mutant strain was compared with its expression level in wild-type cells. Error bars represent the standard error of the mean.

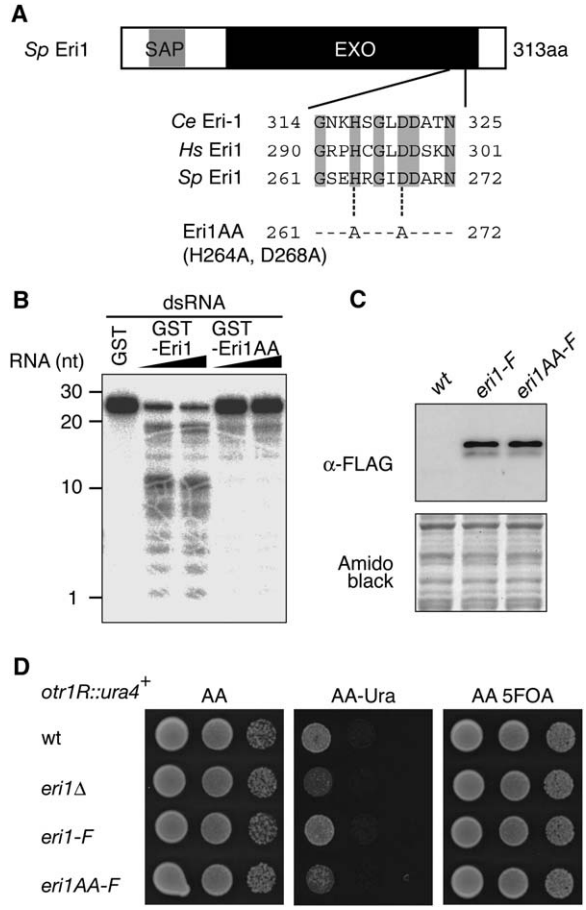

Figure 3. Ribonuclease Activity Is Required for Eri1 Function In Vivo

(A) Schematic diagram of the Eri1AA mutant protein carrying the amino acid changes in the conserved exonuclease (EXO) domain. The conserved amino-acid residues in the putative catalytic center of the EXO domain are shown in gray boxes. *Ce*: *C. elegans*, *Hs*: *Homo sapiens,* and *Sp*: *S. pombe*.

(B) The H264A and D268A mutations impaired the RNase activity of the GST-Eri1 protein in vitro. The RNase assay was performed with GST, GST-Eri1, and GST-Eri1AA recombinant proteins as in Figure 1C.

(C) The H264A and D268A mutations did not affect expression levels of Eri1-F proteins. FLAG-tagged Eri1 proteins in the indicated strains were detected by western analysis with anti-FLAG antibody. As a loading control, the Amido-black stained blot of the corresponding area is shown.

(D) A 10-fold serial dilution of cultures of the indicated strains carrying *otr1R::ura4*$^+$ were spotted onto AA, AA-Ura, or AA5FOA medium as in Figure 2B.

we found that the levels of Swi6 and H3-K9me at *otr1R::ura4*$^+$ and *imr1R::ura4* loci in *eri1⊿* cells were clearly higher than in wild-type cells (Figure 4A; also Figure S4). As reported previously, the enrichment of *otr1R::ura4*$^+$ was absent in *dcr1⊿* [2], and in this background

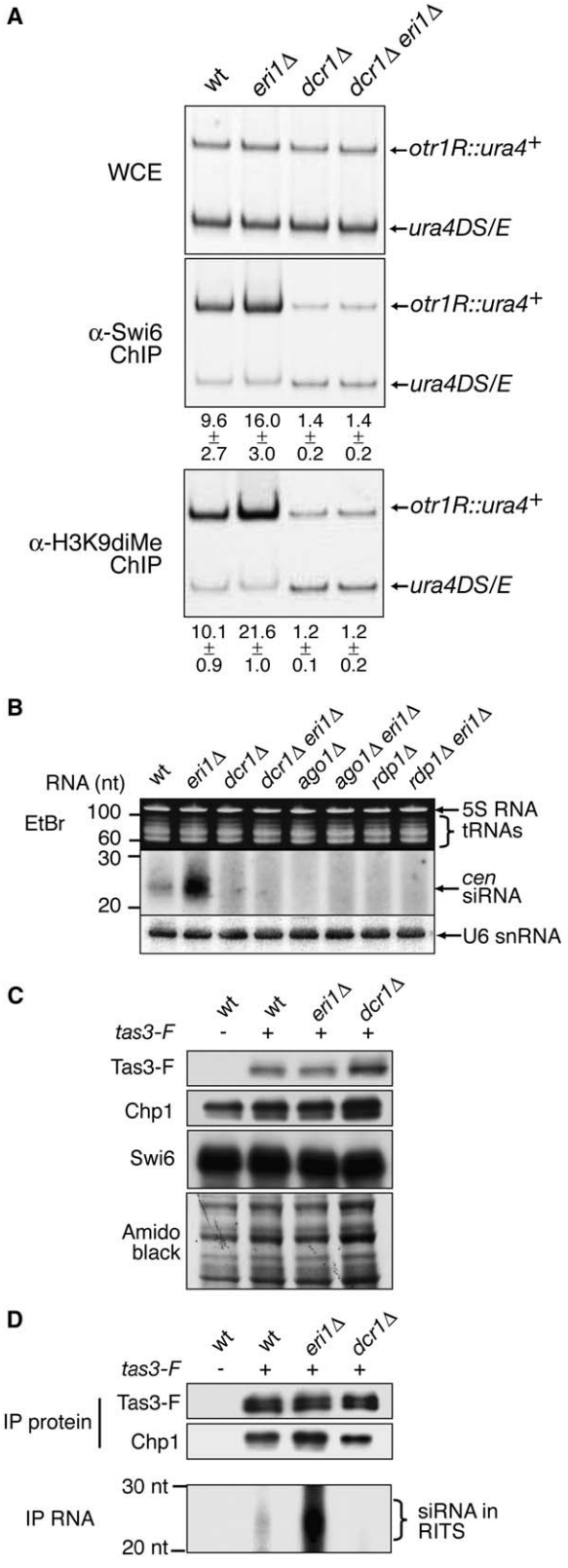

Figure 4. The *eri1⊿* Mutant Affects the Heterochromatin Structure and the RITS Complex

(A) Heterochromatin structure alteration by the *eri1⊿* mutation. Chromatin immunoprecipitation (ChIP) assays were performed with DNA isolated from anti-Swi6 or anti-H3K9diMe immunoprecipitates. Purified DNAs from the indicated strains were used as a template for the PCR amplification of *otr1R::ura4*$^+$ and control *ura4* mini gene (*ura4-DS/E*). The *otr1R::ura4*$^+$ signal enrichments relative to the *ura4-DS/E* signal in the ChIP results were normalized to the WCE signals and are shown beneath each lane with the standard error of the mean.

(B) Effects of the *eri1⊿* mutation on *cen* siRNAs. Centromeric siRNAs and U6 snRNA were detected by northern-blot analysis with siRNA-oligo and U6-antisense DNA probes, respectively. Ethidium-bromide-stained RNAs are also shown as loading control.

(C and D) the *eri1⊿* mutation increased the amount of siRNA associated with the RITS complex. Chp1, Swi6, and Tas3 (Tas3-F) proteins in whole-cell extracts prepared from the indicated strains were detected by immunoblotting with anti-FLAG, anti-Chp1, and anti-Swi6 antibodies, respectively. As a control, the Amido-black stained blot of corresponding area is shown (C). The RITS complex in each indicated strain was purified by anti-FLAG immunoprecipitation of Tas3-F. Tas3-F and Chp1 proteins in the immunoprecipitates were detected by western analysis as shown in (D). siRNAs associated with the RITS complex were detected by autoradiography of 3′-end radiolabeled RNAs in the immunoprecipitates.

eri1⊿ did not affect the levels of Swi6 or H3-K9me (Figure 4A). From these results, we concluded that the *eri1⊿* mutation enhances heterochromatin silencing, at least in part, by affecting the heterochromatin structure through the RNAi pathway. Intriguingly, although we showed that centromeric *dh* transcripts were decreased in *eri1⊿* cells (Figure 2D), we were unable to detect clear changes in the levels of Swi6 and H3-K9me at the centromeric *dg* repeat region *(dg223)* by the *eri1⊿* mutation (Figure S4B). This is presumably because heterochromatin was fully established at centromeric-repeat regions in both wild-type and *eir1*Δ cells and is also due to the limitation of ChIP analyses in assessing strengthened heterochromatin structure by the *eri1* deletion.

We next investigated whether *eri1⊿* affects the in vivo abundance of small RNAs corresponding to the centromere repeats (*cen* siRNAs) by northern analysis. Remarkably, the amount of *cen* siRNAs detected in the *eri1⊿* cells was considerably greater than in wild-type cells (Figure 4B). In contrast, *eri1⊿* did not affect the level of *cen* siRNAs in the cells lacking each of the RNAi factors required for generating siRNAs [4, 5, 9, 10, 18]. Furthermore, small RNAs derived from the *otr1R::ura4*$^+$ locus also emerged in *eri1⊿* cells (Figure S3B). These results suggest that Eri1 negatively regulates the accumulation of heterochromatic siRNAs generated by the RNAi pathway.

Small heterochromatic RNAs are associated with an effector complex, RITS, which contains Ago1, Chp1, and Tas3 [4], and are thought to help anchor RITS to chromatin, nascent transcripts, or both [5, 9]. To test whether the *eri1⊿* directly affects siRNAs associating with RITS, we purified the RITS complex by immunoprecipitating the FLAG-tagged functional Tas3 protein (Tas3-F; data not shown) and analyzed the associated small RNAs. Although the *eri1⊿* mutation did not affect the levels of Tas3-F, Chp1, or Swi6 (Figure 4C) and the purified RITS complex contained comparable levels of Tas3-F and Chp1, we found that the amount of copurified siRNAs greatly increased in *eri1⊿* mutant cells (Figure 4D). These results suggest that *eri1*$^+$ negatively regulates siRNA accumulation and thereby downregulates the level of siRNA-containing active RITS complex.

Our findings provide insight into the regulatory mechanism of RNAi-mediated heterochromatin formation and

the functional involvement of Eri1 in the RNAi pathway. We show that Eri1 targets endogenous heterochromatic siRNAs and negatively regulates heterochromatin formation. These results are consistent with the original observation in *C. elegans* that the Eri-1 negatively regulates the RNAi pathway by degrading exogenous siRNAs [11], supporting the idea that Eri-1 family proteins have a conserved function as a negative regulator of the RNAi pathway. Recent findings have uncovered a further role for *Ce* Eri-1; in this role, Eri-1 physically interacts with Dicer and is required for the production of a specific class of endogenous siRNAs [12, 13]. Although the precise mechanisms underlying Eri-1's involvement in the production of endogenous siRNAs remain to be identified, it is possible that *Sp* Eri1 also plays a role in the processing or production of siRNAs that are distinct from heterochromatic siRNAs. The interaction between Eri-1 and Dicer appears to be important when one considers the physiological function of the Eri-1 family protein. Although we have not yet detected any interactions between *Sp* Eri1 and Dcr1 (our unpublished observation), it is likely that Eri1 specifically targets and degrades siRNAs that are processed by Dcr1. When one considers that the RNAi pathway is potentially capable of targeting any kind of cellular mRNAs or chromatin, the Eri-1 family proteins may function in controlling the amounts of cellular siRNAs and thereby suppress unfavorable or over-driven RNAi systems involved in the RNA degradation or in heterochromatin formation. Through this study we found that although *eri1*Δ affects heterochromatin formation in the nucleus, Eri1 mainly localized to the cytoplasm (Figure S2). This conserved localization suggests that Eri-1-family proteins mainly function in the cytoplasm as siRNA-processing enzymes. Currently, the means by which cytoplasmic Eri1 regulates nuclear heterochromatin remains unclear. One possibility is that a small fraction of Eri1 functions in the nucleus and targets siRNAs in the vicinity of heterochromatin.

Previous studies have suggested that siRNAs produced by Dcr1 are incorporated into the RITS complex to guide it to homologous heterochromatic regions and that the tethering of RITS and RDRC to chromatin also couples the generation of siRNAs and heterochromatin assembly [4, 5, 9, 10]. Our finding that the *eri1*Δ mutation causes an increase in siRNAs associated with RITS and enhances heterochromatin formation (Figures 2 and 4) suggest that the levels of cellular siRNAs are correlated with the amount of siRNA-containing active RITS that facilitates heterochromatin assembly. As in Figure 4A and Figure S4, this enhanced heterochromatin assembly in *eir1*Δ cells may permit the efficient spreading of heterochromatin into marker genes from centromeric-repeat regions in which heterochromatin has been fully established. Alternatively, the *eri1*Δ mutation results in accumulation of siRNAs from marker genes and may facilitate the RNA-mediated heterochromatin formation at marker gene loci (Figure S3B). From this point of view, Eri1 may suppress excess accumulation of siRNAs and regulate the initial targeting step of RITS, presumably in order to stably maintain RNAi-mediated heterochromatin formation *in cis*, whereas the excess amount of activated RITS may act *in trans* in other chromatin regions with sequence homology to siRNAs—consistent with the above-mentioned idea mentioned involving the conserved role of Eri-1 family proteins.

Supplemental Data

Supplemental Data including four figures, one table, and Supplemental Experimental Procedures are available with this article online at http://www.current-biology.com/cgi/content/full/16/14/1459/DC1/.

Acknowledgments

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