Mechanism of siRNA production from repetitive DNA

Qiuying Yang, Qiaohong Anne Ye, and Yi Liu

Department of Physiology, The University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA

RNAi is a conserved genome defense mechanism in eukaryotes that protects against deleterious effects of transposons and viral invasion. Repetitive DNA loci are a major source for the production of eukaryotic small RNAs, but how these small RNAs are produced is not clear. Quelling in *Neurospora* is one of the first known RNAi-related phenomena and is triggered by the presence of multiple copies of transgenes. Here we showed that DNA tandem repeats and double-strand breaks are necessary and, when both are present, sufficient to trigger gene silencing and siRNA production. Introduction of a site-specific double-strand break or DNA fragile site resulted in homologous recombination of repetitive sequences, which is required for gene silencing. In addition to siRNA production, the quelling pathway also maintains tandem repeats by regulating homologous recombination. Our study identified the mechanistic trigger for siRNA production from repetitive DNA and established a role for siRNA in maintaining genome stability.

[*Keywords*: RNA interference; quelling; double-strand break; homologous recombination; repetitive sequences]

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RNAi is a gene silencing mechanism conserved from fungi to mammals (Baulcombe 2004; Catalanotto et al. 2006; Buhler and Moazed 2007; Ghildiyal and Zamore 2009). The RNAi-related pathways are dependent on siRNAs generated from dsRNA by Dicer cleavage. An Argonaute (Ago) family protein associates with siRNAs to mediate post-transcriptional or transcriptional gene silencing. In eukaryotes, a major function of RNAi is to act as a host defense mechanism against transposons and viral invasion (Napoli et al. 1990; Sijen and Plasterk 2003; Siomi et al. 2008; Wang et al. 2010; Chang et al. 2012). Consistently, a significant portion of eukaryotic small RNAs are produced from repetitive DNA loci in fungi, plants, and animals and silence transposon sequences (Siomi et al. 2008; Ghildiyal and Zamore 2009). How these small RNAs are specifically produced from repetitive DNA loci is not clear.

Quelling, a post-transcriptional gene silencing mechanism in the filamentous fungus *Neurospora crassa*, was one of the first RNAi-related phenomena characterized (Romano and Macino 1992; Cogoni et al. 1996; Catalanotto et al. 2006; Chang et al. 2012). Similar to the process of cosuppression in plants, quelling is achieved by random transformation of a transgene, which results in the silencing of expression of homologous genes post-transcriptionally (Napoli et al. 1990; Romano and Macino 1992). Because the silencing triggered by quelling is associated with the presence of multiple copies of a transgene and is transient due to the loss of transgene or reduction of transgene copy numbers, it was proposed that quelling is triggered by repetitive transgenes. Most *Neurospora* strains lack highly repetitive DNA sequences except for the ribosomal DNA (rDNA), suggesting that quelling is a genome defense mechanism against transposon replication. Supporting this conclusion, the RNAi pathway was shown to suppress proliferation of a LINE-like transposon in *Neurospora* (Nolan et al. 2005).

In the quelling pathway, quelling-deficient-1 (QDE-1) is a dual-functional enzyme with both DNA-dependent RNA polymerase (DdRP) and RNA-dependent RNA polymerase (RdRP) activities (Cogoni and Macino 1999a; Lee et al. 2010). QDE-1 first acts as a DdRP to produce single-stranded aberrant RNA (aRNA) from the repetitive DNA loci and then uses its RdRP activity to convert aRNA into dsRNA. The RecQ DNA helicase QDE-3 and the ssDNA-binding complex RPA are required for this process (Cogoni and Macino 1999b; Nolan et al. 2008; Lee et al. 2010). After dsRNA is processed by Dicer, the resulting siRNAs are loaded onto the Ago protein QDE-2 to mediate post-transcriptional gene silencing of homologous RNAs (Catalanotto et al. 2004; Maiti et al. 2007).

Corresponding author: yi.liu@utsouthwestern.edu
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We previously discovered that treatment of *Neurospora* with a DNA damage-inducing agent results in expression of a class of small RNAs, named qiRNAs, from the rDNA locus, which contains ~200 copies of rDNA (Lee et al. 2009, 2010). Similar to quelling-induced siRNA synthesis, the production of qiRNAs also requires QDE-1, QDE-3, Dicer, and RPA. More recently, we showed that the homologous recombination (HR) process is required for qiRNA production and quelling (Zhang et al. 2013, 2014). Because repetitive sequences can provide abundant donor sequences for HR, our results suggested that HR is a mechanism that allows the genome to distinguish repetitive DNA sequences from nonrepetitive sequences. Since our discovery of qiRNA, DNA damage-induced small RNAs have been reported in *Arabidopsis*, *Drosophila*, and mammals (Francia et al. 2012; Michalik et al. 2012; Wei et al. 2012), suggesting that DNA damage is a common trigger for small RNA production in eukaryotes.

What is the genomic feature of repetitive DNA required for gene silencing, and what is the mechanism that allows the specific production of siRNAs from the repetitive DNA loci? Do siRNAs have additional functions other than gene silencing? In this study, we sought to determine the mechanism of siRNA production from repetitive DNA. We demonstrate here that DNA tandem repeats and a double-strand break (DSB) are both required for siRNA production. In addition, we show that the RNAi pathway regulates HR of repetitive sequences. This study thus identified the trigger for siRNA production from repetitive DNA and established a role for siRNAs in maintaining genome stability.

Results

Quelling is associated with tandem transgene repeats

Quelling in *Neurospora* is triggered by the transformation of multiple copies of a transgene and results in the silencing of the homologous endogenous gene through the RNAi pathway (Romano and Macino 1992; Cogoni et al. 1996; Fulci and Macino 2007). Quelling efficiency correlates with transgene copy numbers, and silencing is frequently unstable due to loss of the transgene. To investigate the genomic nature of the quelling phenomenon, we carried out quelling assays by randomly transforming a truncated *albino-1* (*al-1*) fragment-containing plasmid, pBSK*al-1*, into a *qde-1*^{ko} strain in which QDE-1 expression can be induced by the addition of quinic acid (QA). The inducible expression of QDE-1 allowed us to identify true quelled strains because random transformation may also yield inverted *al-1* repeats, from which dsRNA and siRNA can be produced independently of QDE-1 and QDE-3 (Goldoni et al. 2004).

Pronounced silencing of the endogenous *al-1* gene was observed in the presence of QA in ~30% of the resulting transformants, as indicated by their white conidia and hyphae (Fig. 1A, top panel). Modest silencing was observed in the absence of QA because of low QDE-1 expression due to the leakiness of the *qa-2* promoter. Similar to earlier reports (Romano and Macino 1992; Cogoni et al. 1996), Southern blot analysis of *al-1* revealed that in addition to the endogenous *al-1* gene, multiple copies of *al-1* transgenes were integrated into the genome. In addition, there were ladder-like multiple *al-1*-specific signals in each quelled strain (Fig. 1A, bottom panel). In contrast, in the unquelled strains from the same transformation, the *al-1* transgene copy numbers were low, and only one or two *al-1* transgene-specific bands were observed. Quantitative PCR (qPCR) analyses of *al-1* copy numbers from 27 independently generated quelled strains showed that there were an average of 14 copies of *al-1*, ranging from five to 38 copies, in each strain. Because *Neurospora* is multinucleated and not all nuclei have the transgene, the actual transgene copy number in silenced nuclei should be higher than this estimate.

Microconidia were purified to obtain homokaryotic progenies of quelled transformants. As shown in Figure 1B, *al-1* silencing was observed in most of the progenies, but silencing was lost in some due to the loss of *al-1* transgene. Although the ladder-like *al-1* transgene signals remained in the strains with the silencing phenotype, the signal patterns varied from progeny to progeny. Together, these results are consistent with the conclusion that quelling requires multiple copies of a transgene and indicate that the transgene and its copy numbers are highly unstable.

How are multiple copies of *al-1* transgenes arranged in the genome? Although it was long assumed that the transgene copies might form tandem repeats (Cogoni et al. 1996), the presence of multiple *al-1* transgene signals also suggested the possibility that the copies are integrated in many different loci of the genome. To determine the locations of the transgene copies, we pooled individually isolated genomic DNA from 43 independently generated *al-1* quelled strains and performed high-throughput whole-genome deep sequencing (Fig. 1C). The total sequencing reads obtained were predicted to cover >99% of the genome for every strain. Despite such a high coverage, there were only a total of 61 *al-1* genomic integration sites (1.4 per strain) identified. These sites appear to be randomly distributed in the genome and do not appear to have any preference for genome context. This result strongly suggests that most of the transgenes in each strain form tandem repeats at one integration site and are not integrated at multiple genomic loci. Because HR is required for quelling (Zhang et al. 2013, 2014), the ladder-like *al-1* signals in the quelled strains are therefore likely due to HR events among transgene repeats, which would result in different transgene copy numbers in different nuclei.

Tandem repeats are not sufficient for silencing but can trigger silencing after DNA damage agent treatment

Quelling assays are normally performed by using a random non-HR-based transformation method, which cannot control the integration site, copy number, or nature of the repeat of the transgene. To determine whether the tandem repeat itself is sufficient to trigger quelling, we generated a series of constructs that contained one (1x*al-1*)

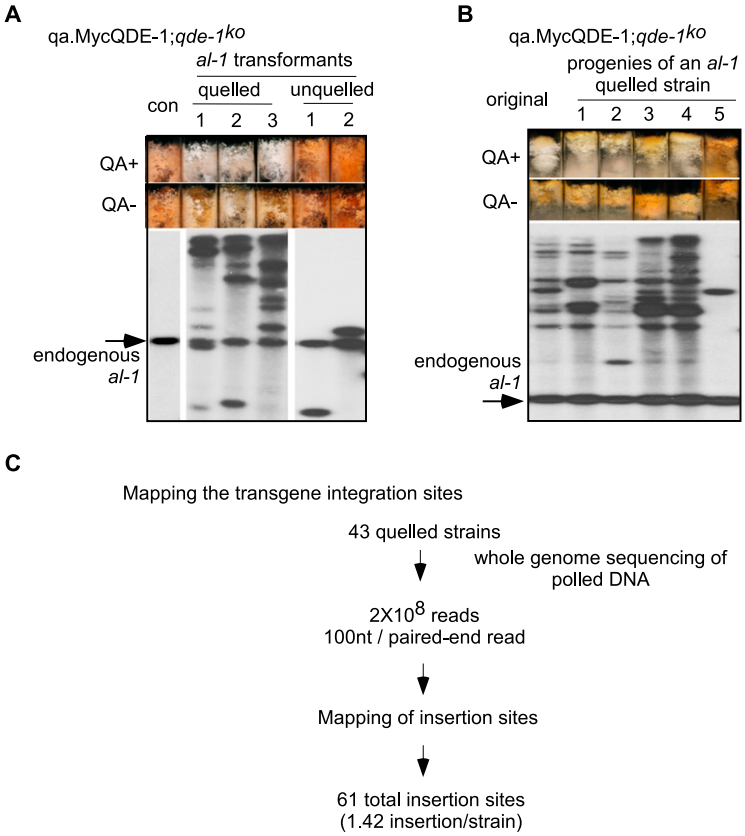


Figure 1. Tandem repeats of the *al-1* transgene are associated with quelling. (*A*) Multiple copies of the *al-1* transgene are associated with quelling. (*Top* panel) Slants of the *qde-1*^{ko}; MycQDE-1 strains after transformation with the *al-1* transgene. The quelled transformants display white/yellow conidia and aerial hyphae in the presence of QA due to silencing of the *al-1* gene. The unquelled transformants are orange as is the control strain (con), which is the host strain without *al-1* transformation. The addition of QA induces expression of MycQDE-1. (*Bottom* panel) Southern blot analysis using an *al-1* fragment as the probe shows the profiles of transgenes in the indicated strains. The arrow indicates the endogenous *al-1* signal. (*B*) Progenies of a quelled strain have different quelling phenotypes and transgene profiles. (*C*) Schematic diagram showing transgene integration sites identified by whole-genome sequencing.

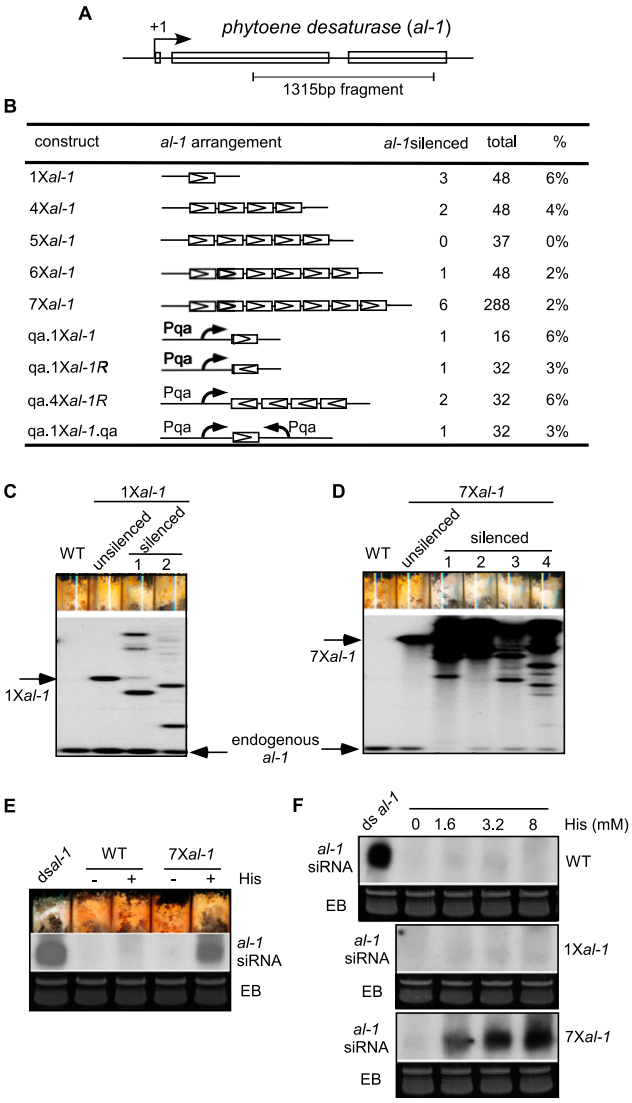
to seven (7x*al-1*) tandem repeats of a promoter-less 1.3-kb *al-1* ORF fragment (Fig. 2A). In addition, constructs that have the *qa-2*-inducible promoter that can drive sense, antisense, or convergent transcription of the *al-1* fragment were also generated. These constructs were individually targeted to the *his-3* locus of a wild-type strain, which, in most but not all cases, resulted in single-copy integration of the construct by HR. Surprisingly, no *al-1* silencing was observed in a vast majority of the transformants (Fig. 2B), demonstrating that tandem repeats (up to seven) by themselves were not sufficient to trigger silencing.

Silencing was observed in a very low percentage of the transformants (Fig. 2B–D). To determine the genomic nature of these silenced transformants, Southern blot analyses were performed for a few randomly selected strains that were transformed with constructs containing one or seven copies of the *al-1* repeat. In contrast to the unsilenced strains, which, as expected, only have a single site and single-copy integration of the indicated *al-1* construct at the *his-3* locus, ladder-like *al-1* signals were seen in the silenced transformants (Fig. 2C,D, bottom panels), indicating that the silencing in these strains was due to nonhomologous integration of the constructs that form multiple repeats of the construct. These results indicate that the low frequency of gene silencing is due to low frequency of non-HR of the transgene in the *his-3* targeting transformation method.

We previously showed that treatment of *Neurospora* with a DNA-damaging agent triggers production of qiRNAs from a repetitive rDNA locus (Lee et al. 2009, 2010). To determine whether DNA damage can trigger the production of siRNA from tandem repeats at a non-rDNA locus, we treated a strain that contains a single copy of 7x*al-1* with histidine, a known DNA-damaging agent in *Neurospora* that inhibits ribonucleotide reductase (Pendyala and Wellman 1975; Lee et al. 2009). As shown in Figure 2E, siRNA was not observed in the wild-type strain or the 7x*al-1* strain without histidine treatment, but siRNA production was induced by histidine in the 7x*al-1* strain. Consistent with the siRNA production, silencing of *al-1* was also observed in the 7x*al-1* strain, as indicated by the color change of the conidia and aerial hyphae after histidine treatment. Histidine induced siRNA production in a dose-dependent manner in the 7x*al-1* strain but failed to induce siRNA in strains with a single copy of *al-1* repeat (Fig. 2F). These results indicate that DNA damage treatment can specifically trigger siRNA production from a locus with tandem repeats.

A DNA DSB in an al-1 tandem repeat triggers silencing

Histidine is a mild DNA-damaging agent that results in replication stress by inhibiting nucleotide biosynthesis in *Neurospora* (Pendyala and Wellman 1975; Lee et al. 2009). To determine the nature of DNA damage that triggers siRNA production, we introduced a cleavage site for the homing endonuclease I-SceI (TAGGGATAACAGGGT



construct	al-1 arrangement	al-1silenced	total	%
1X*al-1*		3	48	6%
4X*al-1*		2	48	4%
5X*al-1*		0	37	0%
6X*al-1*		1	48	2%
7X*al-1*		6	288	2%
qa.1X*al-1*	Pqa	1	16	6%
qa.1X*al-1R*	Pqa	1	32	3%
qa.4X*al-1R*	Pqa	2	32	6%
qa.1X*al-1*.qa	Pqa Pqa	1	32	3%

Figure 2. Tandem repeats are not sufficient for silencing but can trigger silencing after DNA damage agent treatment. (*A*) A diagram showing the *al-1* fragment used to generate *al-1* tandem repeat constructs. (*B*) A table showing the *al-1*-containing *his-3* targeting constructs and the summary of *al-1* silencing results of the transformants for each construct. (*C*,*D*, *top* panels) Slants of the transformants from the 1x*al-1* (*C*) and 7x*al-1* (*D*) transformants. (*Bottom* panels) Southern blot analyses using the *al-1* fragment as the probe, showing the profiles of transgenes in the indicated strains. (WT) A wild-type control strain. The low levels of the endogenous *al-1* bands in some lanes are due to unequal loading of DNA samples. (*E*, *top* panel) Slants of the indicated strains in the presence or absence of histidine. (*Middle* panel) Small RNA Northern blot analysis results showing the production of *al-1*-specific siRNA production in the indicated strains. (*Bottom* panel) Ethidium bromide-stained gel showing the RNA loading in the indicated samples. (*F*) Small RNA Northern blot analysis results showing the production of siRNA in a histidine dose-dependent manner in the 7x*al-1* strain but not in the other strains. Ethidium bromide-stained gels are shown *below* each Northern blot. The ds*al-1* strain produces *al-1* siRNA by expression from an *al-1* inverted repeat.

AAT) into one of the *al-1* tandem repeats at the *his-3* locus (Fig. 3A). The yeast enzyme I-SceI expressed very poorly in *Neurospora*, likely due to the strong codon biases of the *Neurospora* genome (Zhou et al. 2013). To express I-SceI in *Neurospora*, we codon-optimized the *I-SceI* gene based on *Neurospora* codon preferences. Because there is no endogenous I-SceI site in the *Neurospora* genome, the expression of I-SceI results in a DSB only at the introduced I-SceI site.

To determine whether the expressed I-SceI can create a site-specific DSB in *Neurospora*, we first examined a strain [1x*al-1*(3XI-SceI)] that has one *al-1* repeat and three concatenated I-SceI recognition sites. PCR of the *al-1* region spanning the I-SceI sites (Fig. 3B) and DNA sequencing showed that the expression of I-SceI specifically led to a reduction in the length of the I-SceI-containing fragment in the 1x*al-1*(3XI-SceI) strain, but not in the strain lacking I-SceI expression, due to loss of one or two I-SceI sites as a result of DNA repair. This result demonstrated that the codon-optimized I-SceI expressed in *Neurospora* results in a DSB at the introduced site.

We then examined the effect of I-SceI expression in strains that carry one, two, or seven tandem *al-1* repeats with an I-SceI site. I-SceI expression did not cause detectable *al-1* silencing or siRNA production in strains with only one or two repeats, although in the 2x*al-1*(I) (I stands for I-SceI site) strains, an *al-1* transgene band that corresponds to the size of one *al-1* repeat was observed (Fig. 3C–E). In the 7x*al-1*(I) strains, the I-SceI expression led to *al-1* silencing (as indicated by the yellow or white aerial hyphae and conidia), *al-1* siRNA production, and the appearance of ladder-like *al-1* transgene signals in Southern blot (Fig. 3F,G). The ladder-like *al-1* transgene signals are similar to those seen in the quelled strains. The ladder-like transgene signals are due to HR events of tandem repetitive sequences, which result in a population of nuclei with different repeat numbers.

To further confirm our conclusion, we introduced an artificial DNA fragile site (FR) that contains multiple (CGG)n trinucleotides (n = 133) next to the *al-1* tandem repeats (Fig. 4A; Fu et al. 1991). As expected, the presence of the fragile site resulted in *al-1* silencing in ~35% of the transformants, which all exhibited ladder-like *al-1* transgene signals (Fig. 4B [top panel, lanes 1–6], D). A probe specific for the regions covering the fragile site (probe FR) (Fig. 4B, bottom panel) showed that all of these strains retained fragile sites of various lengths. The transformants that lacked *al-1* silencing had lost either the fragile site or the *al-1* repeat (Fig. 4B, lanes 7,8).

Because the length of the trinucleotide repeats in the fragile site correlate with replication stalling and DSBs (Eichler et al. 1994; Jarem et al. 2010), we examined the effects of fragile sites with fewer CGG repeats: FR-S (n = 33) and FR-M (n = 67). No detectable silencing and no ladder-like *al-1* transgene signals were observed in the FR-S strain (Fig. 4C, left). On the other hand, weak but detectable *al-1* silencing and weak ladder-like *al-1* transgene signals were seen in the FR-M strains (Fig. 4C,D).

To confirm the presence of a DSB due to the presence of the fragile site, we examined the accumulation of γH2A,

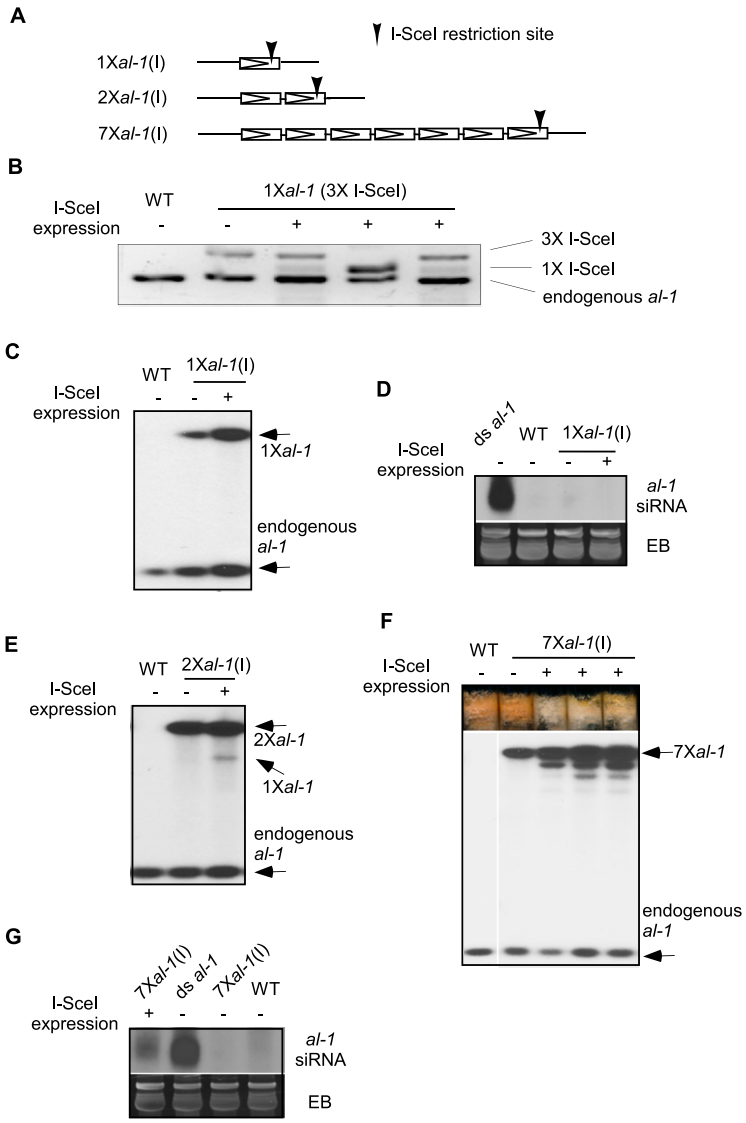


Figure 3. I-SceI-induced DSB in the *al-1* tandem repeat triggers gene silencing and siRNA production. (*A*) Schematic diagrams showing the *al-1 his-3* targeting construct and the location of the I-SceI site. (*B*) PCR amplification of the 1x*al-1* sequence spanning the 3XI-SceI cut site from strains containing a 1x*al-1*(3XI-SceI) construct with/without I-SceI expression. The expression of I-SceI was achieved by introducing an I-SceI expression construct into the indicated strains. (*C*) Southern blot analysis results using an *al-1* probe, showing the 1x*al-1*(I) strain with/without I-SceI expression. (*D*) Small RNA Northern blot analysis showing the lack of siRNA production from the 1x*al-1*(I) strain. (*E*) Southern blot analysis results using an *al-1* probe of the 2x*al-1*(I) strain with/without I-SceI expression. (*F*, *top* panel) Photo of the slants showing that I-SceI induced *al-1* silencing in the 7x*al-1*(I) strains. (*Bottom* panel) Southern blot analysis results using an *al-1* probe of the 7x*al-1*(I) strain with/without I-SceI expression. (*G*) Small RNA Northern blot analysis showing the induction of siRNA production by I-SceI expression from the 7x*al-1*(I) strain. The ethidium bromide-stained gels show equal loading of RNA samples. The ds*al-1* and wild-type (WT) strains served as positive and negative siRNA controls, respectively.

a marker of DSBs (Lobrich et al. 2010), near the fragile site by chromatin immunoprecipitation (ChIP). As expected, there was significant enrichment of γH2A near the fragile site in the 7x*al-1-FR* strain (Fig. 4E), confirming the presence of DSBs. Similarly, significant enrichment of γH2A was also found at the *al-1* transgene site in previously obtained quelled strains (Fig. 4F), indicating that quelling is also a result of DSBs. Together, these results further confirmed the requirement of DSBs in triggering siRNA production from the tandem repetitive sequences. In addition, the tight correlation between silencing and the presence of various transgene HR products further supports the role of HR in the small RNA production.

A comparison of the colors of silenced strains revealed that the presence of the longest fragile site (n = 133) resulted in more robust *al-1* silencing than did the I-SceI-induced DSB (Figs. 3F, 4B). This difference is likely due to the fact that the repair of the I-SceI cleavage can result in deletion or mutation of the I-SceI site. As a result, the effect of I-SceI would be transient.

DSB-induced siRNA production profile at the tandem repeat region

To evaluate the siRNA production profile after DSB induction, we carried out small RNA sequencing in strains with and without tandem repeats and mapped the small RNA reads to the genome. No siRNA production was observed in the wild-type strain or strains with only one copy of the *al-1* fragment with or without the I-SceI expression at the *his-3* locus (Fig. 5A), indicating that DSB alone is not sufficient to trigger small RNA production. In contrast, in the strain containing 7x*al-1* at the *his-3* locus, siRNA production was observed at the transgene site only when I-SceI was expressed (Fig. 5B). Similarly, the presence of the fragile site next to the 7x*al-1* repeats also resulted in robust siRNA production at the transgene site (Fig. 5C).

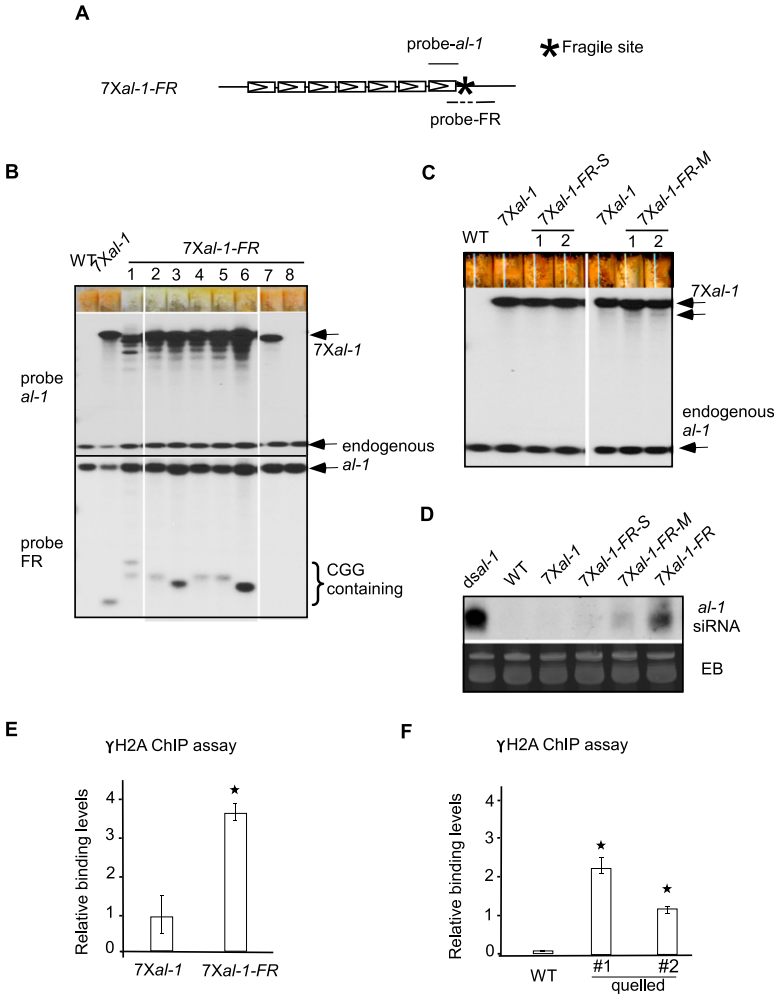


Figure 4. Artificial fragile site in an *al-1* tandem repeat triggers silencing. (*A*) A schematic diagram of the 7x*al-1* construct with the fragile site (FR). (*B*, *top* panel) Photo of the slants of the indicated strains showing *al-1* silencing in the 7x*al-I-FR* transformants. (*Middle* panel) Southern blot analysis results using the *al-1* probe for the indicated strains. (*Bottom* panel) Southern blot analysis results using a probe to the fragile site. (*C*) Silencing phenotypes and *al-1* genomic profiles in strains with a 7x*al-1-FR-S* or 7x*al-1-FR-M* construct at the *his-3* locus. Wild-type and 7x*al-1* strains without the fragile site served as negative controls. (*D*) Northern blot analysis showing the production of siRNAs in the 7x*al-1-FR* transformants. The ethidium bromide-stained gel shows equal loading of RNA samples. The ds*al-1* and wild-type (WT) strains served as positive and negative siRNA controls, respectively. (*E*,*F*) ChIP assays using the phospho-γH2A antibody showing the association of γH2A at the repetitive *al-1* region in 7x*al-1-FR* transformants (*left*) or the *al-1* quelled strains (*right*). (⋆) $P < 0.05$.

At the *his-3* locus, the DSB-induced small RNA reads (each read was mapped only once to the genome) covered the entire *al-1* repeat and were also found in ≤3-kb flanking regions, indicating that siRNA production spread outside the repetitive regions. In contrast, the reads mapped to the endogenous *al-1* locus were solely limited to the repeated *al-1* region, indicating that siRNAs were only produced from the repetitive transgene locus, and the mapped reads at the endogenous *al-1* locus were false reads. Together, these results further demonstrated the requirement of both DSBs and tandem DNA repeats in siRNA generation.

The siRNA production pathway regulates HR of the repetitive DNA

The production of DSB-induced siRNA from the repetitive DNA prompted us to examine whether the RNAi pathway has an additional role in genome stability. To test this, we introduced the 7x*al-1-FR* construct into *dicer*^{dko}, *qde-1*^{ko}, and *qde-2*^{ko} strains. Dicer and QDE-1 are required for the production of siRNA, whereas the Ago QDE-2 is required for siRNA-mediated gene silencing. As shown in Figure 6A, silencing from the 7x*al-1-FR* construct was completely abolished in the *dicer*^{dko}, *qde-1*^{ko}, and *qde-2*^{ko} strains, indicating their essential roles in this pathway. As expected, *al-1* siRNA production was abolished in the *dicer*^{dko} and *qde-1*^{ko} strains.

Southern blot analyses of the obtained transformants carrying the 7x*al-1-FR* in the wild-type and mutant backgrounds were compared. In the wild-type background, ~35% of the transformants exhibited *al-1* silencing and showed the expected pattern of *al-1* transgene: the presence of the predicted full-length 7x*al-1* band with a ladder of bands corresponding to the reduced *al-1* copy number (Fig. 6B, lanes 1–4). For strains with no detectable *al-1* silencing (for example, Fig. 6B, lanes 5–8), the patterns of the *al-1* transgene ladders were abnormal. The patterns had one to three major transgene bands of various sizes and lacked the ladder-like signal seen in the silenced strains. This result suggests that the 7x*al-1* tandem repeats subjected to DSB are unstable and can be lost due to recombination among the repeats. In contrast to wild-type transformants, almost none of the 7x*al-1-FR* transformants of the *dicer*^{dko}, *qde-1*^{ko}, and *qde-2*^{ko} strains exhibited the expected transgene pattern, and

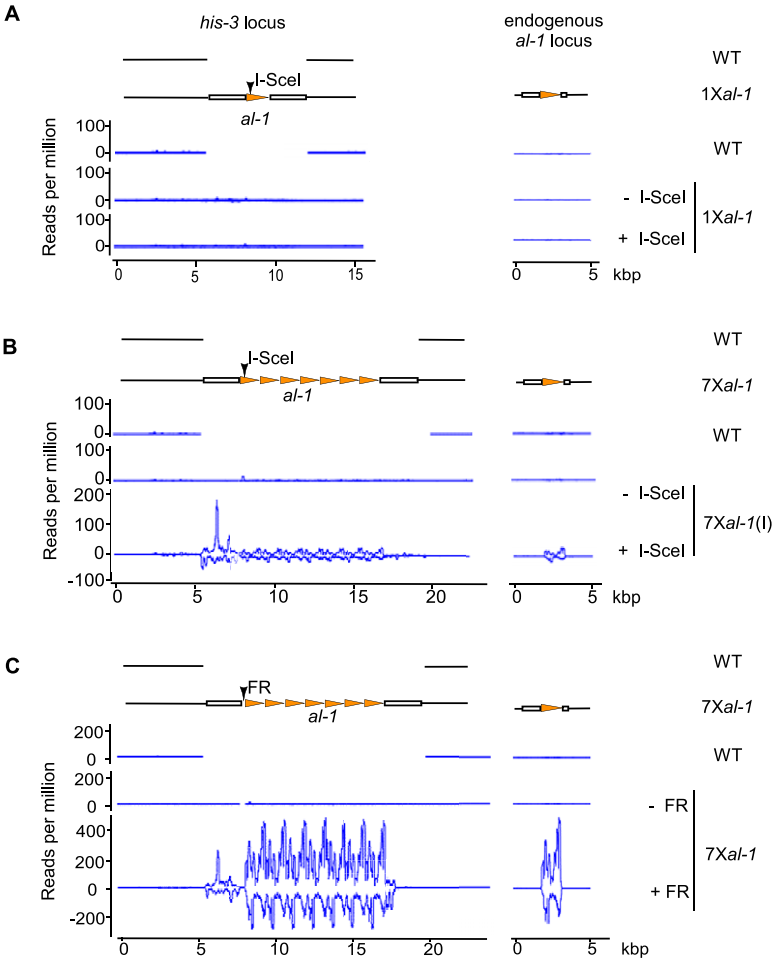


Figure 5. Small RNA sequencing reveals siRNA production profiles from the tandem repeats after DSB. Histograms showing siRNAs of the indicated strains mapped to the *al-1* transgene at the *his-3* locus and the endogenous *al-1* locus. Each repeat of the *al-1* fragment is indicated by one yellow triangle. The *Y*-axis represents the number of normalized small RNA reads per million sequences. Plus (+) and minus (−) values represent the reads of small RNAs derived from sense and antisense strands, respectively. The *X*-axis indicates the position (in kilobases) of the regions analyzed. + I-SceI and − I-SceI indicate with or without I-SceI expression, respectively. + FR and − FR indicate with or without the fragile site in the construct. (*A*) siRNA distributions in the wild type (WT) and the strain with one copy of the *al-1*(I) fragment at the *his-3* locus. (*B*) The comparison of siRNA distribution between the wild type and the strain with 7x*al-1*(I) at the *his-3* locus. (*C*) siRNA distributions in the wild type and the strains with 7x*al-1* or 7x*al-1-FR* at the *his-3* locus.

almost all showed the abnormal transgene patterns, suggesting rapid loss/abnormal repair of *al-1* repeats in these quelling mutant strains (Fig. 6C–F).

To further confirm these results, we also compared the genomic profiles of the 7x*al-1* with an I-SceI site after I-SceI expression in the wild-type strain and *qde-1*^{ko} mutant. As shown in Figure 6G, the expression of I-SceI resulted in the ladder-like 7x*al-1* transgene signals in most of the wild-type transformants. In the *qde-1*^{ko} transformants, however, the transgene profiles were abnormal, and the ladder-like signals disappeared (Fig. 6H). Together, these results strongly suggest that the quelling pathway maintains the tandem repeats and regulates the recombination of the repetitive sequences.

Removal of the nonhomologous end-joining (NHEJ) repair pathway rescues the abnormal genomic profile of al-1 tandem repeats in a quelling mutant

HR and NHEJ are two major pathways for DSB repair (Sancar et al. 2004). In *Neurospora*, Ku80 is one of the essential components required for NHEJ (Ishibashi et al. 2006). To determine whether the abnormal genomic profiles of the *al-1* tandem repeats in the quelling mutants are due to elevated NHEJ events after DNA damage, we created a *ku80*^{ko},*qde-1*^{ko} double mutant and introduced the 7x*al-1-FR* construct at the *his-3* locus by HR. In contrast to the *qde-1*^{ko} single mutant, most of the *ku80*^{ko},*qde-1*^{ko} transformants displayed the full-length 7x*al-1* bands with ladder-like signals (Fig. 6I) despite the lack of *al-1* silencing (data not shown). This result suggests that the abnormal genomic profiles of the tandem repeats are mostly due to NHEJ, and the DSB-induced siRNA promotes HR events of the tandem repeats.

Discussion

Repetitive DNA loci are a major genomic source for small RNA production in eukaryotes. In this study, using *Neurospora* as a model system, we demonstrated that tandem repeats and DSBs are both necessary and, when both are present, sufficient to trigger gene silencing and siRNA production. Furthermore, we showed that the RNAi pathway helps to maintain the repetitive sequences in the genome by promoting HR. These results uncovered the mechanistic trigger of siRNA production from repetitive DNA and established a role for RNAi in maintaining genome stability.

Consistent with previous studies (Romano and Macino 1992; Cogoni et al. 1996), we showed that quelling in

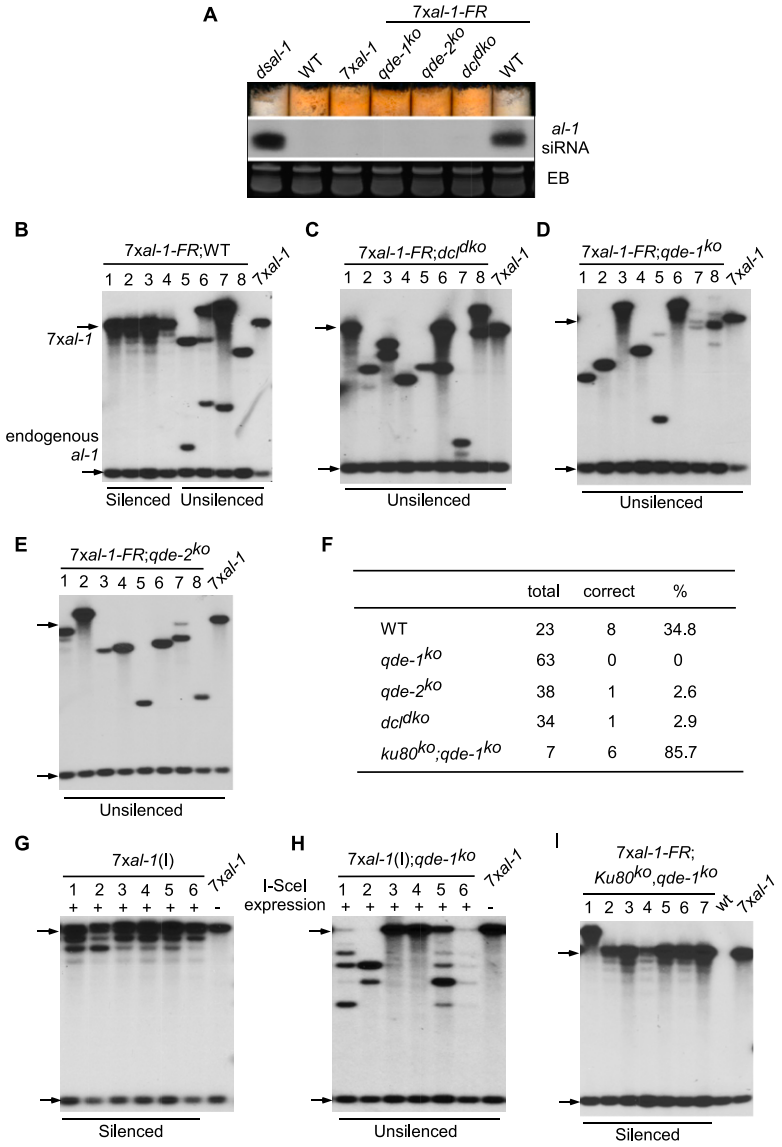


Figure 6. The quelling pathway contributes to the maintenance of tandem repeats by regulating HR. (*A*) Phenotypes of the 7x*al-1-FR*-containing strains in the wild-type (WT) and the quelling mutant backgrounds. (*Top*) Photo of slants. (*Middle*) *al-1* siRNA Northern blot analysis results. (*Bottom*) Ethidium bromide-stained gel. The ds*al-1* and wild-type (WT) strains served as positive and negative siRNA controls, respectively. (*B–E*) Southern blot analysis results showing the genomic profiles of the 7x*al-1-FR* transgene in wild-type (*B*), dcl^{dko} (*C*), $qde\text{-}1^{ko}$ (*D*), and $qde\text{-}2^{ko}$ (*E*) backgrounds. (*F*) A table summarizing the *al-1* Southern blot analysis results on the 7x*al-1-FR* transgene profiles in the indicated strains. (*G*,H) *al-1* Southern blot analysis results showing the genomic profiles of the 7x*al-1*(I) transgene after I-SceI induced DSBs in wild-type (*G*) and $qde\text{-}1^{ko}$ (*H*) strains. (*I*) *al-1* Southern blot analysis results showing the genomic profiles of the 7x*al-1-FR* transgene in the $ku80^{ko}$,$qde\text{-}1^{ko}$ double mutant.

Neurospora is tightly associated with multiple copies of a transgene. The transient nature of quelling is due to loss or reduction of transgene copy number. Whole-genome sequencing of the quelled strains indicated that the multiple copies of a transgene form tandem repeats at a single site in the genome, indicating that the formation of tandem repeats rather than transgene integration at multiple sites is required for quelling. However, by generation of a series of strains with defined repeat numbers at a defined genetic locus, we showed that the tandem repeat alone is not sufficient for gene silencing and siRNA production (Fig. 2).

By introducing a defined DSB or a DNA fragile site at the tandem repeat region, we demonstrated that a DSB is necessary and sufficient for gene silencing and siRNA production (Figs. 3–5). These results demonstrate that a DSB in the tandem repeat locus is the initial trigger for the activation of the quelling pathway (Fig. 7). In quelled strains and in tandem repeat-containing strains with gene silencing, Southern blots exhibited ladder-like transgene signals. The bands correspond to different transgene copy numbers due to HR events between individual repeat sequences. Supporting this conclusion, we previously showed that the HR process is required for quelling (Zhang et al. 2013, 2014). Thus, HR of the tandem repeat region may result in recombination intermediates that can be specifically recognized by the quelling pathway, likely QDE-3, a putative RecQ DNA helicase homologous to the Werner/Bloom syndrome proteins (Cogoni and Macino 1999b). QDE-3 may resolve the recombination intermediates into ssDNA using its DNA helicase activity and may recruit ssDNA-binding protein RPA and the dual-functional enzyme QDE-1 to the site (Lee et al. 2010). QDE-1 uses its DdRP and RdRP activities to first produce ssRNA and then dsRNA to activate the downstream RNAi pathway. As a result, this pathway ensures that siRNAs are specifically produced from the repetitive DNA loci, which can be formed by transposon replication, but not from nonrepetitive parts of the genome.

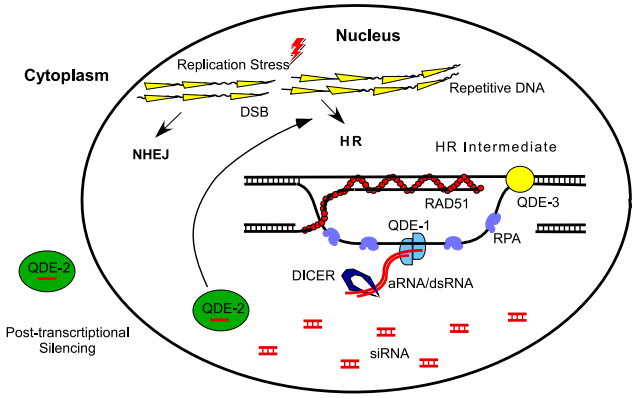


Figure 7. A model showing the proposed mechanism of repeat-induced siRNA production. Replication stress or DNA damage triggered DSBs in the tandem-repetitive genome locus. DSBs result in HR of repetitive sequences. The recombination intermediates of the HR process can be specifically recognized by the quelling pathway, likely QDE-3, which may resolve the recombination intermediates into ssDNA and recruit RPA and QDE-1. QDE-1 uses its DdRP and RdRP activities to first produce ssRNA and then dsRNA to activate the downstream RNAi pathway. The nuclear QDE-2 associates with siRNAs and promotes the HR process between the repetitive sequences. The cytoplasmic siRNA-associated QDE-2 results in post-transcriptional gene silencing.

In addition to the mechanism of small RNA production, our study also uncovered a role for the RNAi pathway in maintaining genome stability by regulating the HR process. In mutants that are deficient in RNAi or siRNA production, the ladder-like transgene signals were absent, and the tandem repeats in the genome were not maintained after DSB as a result of rapid loss of the repeats (Fig. 6). This observation is similar to the hyper-recombination phenotype of yeast *rad51* (Aguilera 1995; Fasullo et al. 2001). Importantly, such a defect of the RNAi mutant was rescued when the NHEJ pathway was blocked (Fig. 6I). These results suggest that the siRNAs induced by DSBs are involved in maintaining the repeats and promoting the HR process between the repetitive sequences or suppressing NHEJ events. Consistent with this observation, it was previously observed that the quelling mutants have reduced rDNA copy numbers (Cecere and Cogoni 2009). Consistent with such a nuclear role of RNAi, we found that a significant amount of QDE-2 protein resides in the nucleus (Supplemental Fig. S1). Together, these results suggest that in addition to post-transcriptional gene silencing, siRNAs produced from repeat regions also help maintain genome stability.

How does the RNAi pathway regulate the DNA repair process? In fission yeast, plants, and animals, small RNAs mediate epigenetic modifications of chromatin to cause transcriptional gene silencing (Buhler and Moazed 2007; Zhang and Zhu 2011; Castel and Martienssen 2013). However, the quelling pathway and epigenetic regulation of chromatin appear to be independent of each other in *Neurospora* (Chicas et al. 2004, 2005; Freitag et al. 2004). Furthermore, we failed to detect significant levels of DNA/histone H3K9 methylation and H3K27 methylation of the tandem repeats at the *his-3* locus (Supplemental Fig. S2; data not known). These results suggest that siRNAs may not act by regulating the chromatin modifications. Because of the requirement of the Ago QDE-2 in maintaining the tandem repeats, it is likely that siRNA brings QDE-2 to the nascent transcript after DSB formation. In mammalian cells, it was very recently shown that AGO-2 interacts with RAD51 and is important for the localization of RAD51 to DSB sites (Gao et al. 2014). Thus, siRNA may target the HR machinery to the DSB to promote HR. On the other hand, it was recently shown in yeast that RNA transcripts can mediate HR of chromatin (Keskin et al. 2014). Thus, QDE-2 may also target RNA transcripts made from the repetitive region to regulate their role in HR.

Repeat-associated small RNAs are found in almost all eukaryotes. In addition, DNA damage-induced small RNAs have been reported in *Arabidopsis*, *Drosophila*, and mammals (Francia et al. 2012; Michalik et al. 2012; Wei et al. 2012), suggesting that DNA damage may be a common trigger for small RNA production in eukaryotes. Interestingly, in plants and mammalian cells, the reporter constructs used for the detection of small RNA production after DSB formation have two tandemly arranged repeats flanking the I-SceI sites (Wei et al. 2012). Thus, as in *Neurospora*, the presence of repeats and the HR process may also be required in the production of siRNAs in other organisms.

Repetitive DNA is known to be a major source of genome instability in various organisms due to hyper-recombination events as a result of replication stress (Bzymek and Lovett 2001; Vader et al. 2011). In *Neurospora*, the repetitive rDNA locus is a site of frequent chromosome breakage (Butler 1992), and we previously showed that the replication process is required for qiRNA production (Zhang et al. 2013). Here we demonstrated that a tandem repeat alone resulted in siRNA production when subjected to replication stress treatment. Thus, repetitive DNA is sensitive to replication stress, which can lead to DSBs and siRNA production. Dicers have been shown to be involved in maintaining the genome stability and rDNA integrity in *Drosophila* and fission yeast (Peng and Karpen 2007; Castel et al. 2014). Therefore, small RNA and RNAi components appear to have a broad role in maintaining genome stability in eukaryotes.

Materials and methods

Strains, growth conditions, and constructs

The wild-type strain used in this study was FGSC 4200(a). Quelled transformants were obtained by cotransformation of a mixture of 2 μg of pBSKal-1 (an *al-1* fragment-containing plasmid) and 0.5 μg of pBT6 (a benomyl-resistant gene-containing plasmid) to the *qde-1*^{ko}; qa.MycQDE-1 strain. The *qde-1*^{ko}; qa.MycQDE-1 strain was generated previously (Lee et al. 2010). The benomyl-resistant transformants were picked onto minimal slants supplemented with QA to identify the quelled (white or yellow conidia and aerial hyphae) transformants. A liquid medium of 1× Vogel's, 0.1% glucose, and 0.17% arginine with or without 0.01 M QA (pH 5.8) was used for liquid cultures. For

liquid cultures containing histidine, a final concentration of 0.5 mg/mL was used.

The *his-3* locus targeting vector pDE3dBH was used to construct the tandem repetitive *al-1* plasmids. ds*al-1*, wild-type (301-6, *his-3*), *qde-1^{ko}* (*his-3*), *qde-2^{ko}* (*his-3*), and *dicer^{dko}* (*his-3*) strains were generated previously (Choudhary et al. 2007; Maiti et al. 2007). The *qde-1^{ko} Ku80^{ko} his-3* strain was generated in this study by crossing *qde-1^{ko}* and *Ku80^{ko} his-3* strains. The *his-3* locus targeting plasmids were introduced into the indicated strains. The (CGG)n fragile sites were created by self-annealing PCR reaction as described previously (Samadashwily et al. 1997) and were ligated to a blunt-end T vector. The (CGG)n fragments of different lengths were selected and inserted into the pDE3dBH.nx*al-1*constructs at the EcoRI site.

ChIP assay

Anti-γH2A (phospho-S129) and anti-H3K9me3 antibodies (ab15083 and ab8898, Abcam) were used to perform immunoprecipitation assays. The wild-type extract was used as control. qPCR was carried out to quantify the levels of DNA in the precipitates. The relative DNA-binding levels of ChIP assays were determined by comparing the relative enrichment of the chromatin-immunoprecipitated DNA between the quelled strain and a wild-type strain. Relative enrichment of the chromatin-immunoprecipitated DNA was normalized to the actual *al-1* copy numbers in each quelled strain or transformant. *actin* levels in the chromatin-immunoprecipitated DNA were used to normalize the loading in different samples.

Southern blot analyses

Genomic DNA was extracted and purified as previously described (Mohn et al. 2009). Approximately 10 μg of genomic DNA was used in the Southern blot analyses. PstI (absent in the *al-1* fragment) was used to digest the genomic DNA for *al-1* pattern detection. BamHI and EcoRV were used to digest the genomic DNA for (CGG)n length detection. The digested DNA was separated on a 0.8% agarose gel by electrophoresis and then transferred onto a Hybond N$^+$ (GE Healthcare) membrane. Prehybridization, hybridization, and washing of the blot were performed as recommended by the manufacturers. Probes were labeled with ^{32}P by PCR using dCTP [α-^{32}P] from PerkinElmer. The primers used in labeling the probes were probe_al_F, GCGTTACTTCAAGACGGATCGCA; probe_al_R, CATCAACGCCACCAACAAAGGG; probe_a_F, TTTGGCCCAC GACTTCTTC; probe_a_R, CATCAACGCCACCAACAAAG; probe_b_F, GGGAAACGCCTGGTATCTTTA; and probe_b_R, TCAGATCTGCCGGTCTCCCTATAG.

Northern blot and qPCR assays

Small RNA was prepared and enriched from total RNA using 5% polyethylene glycol (MW8000) and 500 mM NaCl as previously described (Maiti et al. 2007). RNA concentration was measured using a NanoDrop (Thermo Scientific). Small RNA was separated by electrophoresis through 16% polyacrylamide, 7 M urea, and 0.5× tris-borate EDTA (TBE) gels. Equal amounts of small RNA (20 μg) were loaded in each lane. UV-cross-linking was used to fix small RNAs to a Hybond N$^+$ membrane (GE Healthcare). siRNA hybridization was carried out as described previously (Yang et al. 2013). qPCR was performed using iTaq SYBR Green Supermix with ROX (Bio-Rad) as previously described (Choudhary et al. 2007).

Genome sequencing

Whole-genome sequencing was performed by The McDermott Center Next-Generation Sequencing Core at University of Texas Southwestern. Genomic DNA was randomly sheared into small fragments using Covaris E210, and paired-end 2 × 100-base-pair (bp) sequencing was performed on Illumina HiSeq. After DNA sequencing, the cleaned reads were mapped to the reference *Neurospora* genome sequence (http://www.broadinstitute.org/annotation/genome/neurospora/GenomeDescriptions.html#Neurospora_crassa_OR74A) using SAMtools (Li et al. 2009). Reads that could not fully map to either the *Neurospora* genome or plasmid used for transformation were aligned against the *Neurospora* genome and plasmid using NCBI-BLAST with parameters for short sequences to determine the junction sites of transgene insertion.

Small RNA sequencing

Small RNA sequencing was performed by The McDermott Center Next-Generation Sequencing Core at University of Texas Southwestern. The cleaned reads were aligned to the *Neurospora* genome as well as the introduced plasmid sequences. Small RNA densities in the regions of interest were calculated and plotted within 100-bp sliding windows with a step size of 1 bp using R (http://www.R-project.org). Sequence data from this study are available through the NCBI Short Read Archives (accession no. SRP051839)

Genomic DNA extraction and methylated DNA immunoprecipitation (MeDIP) assay

Genomic DNA was extracted as previously described (Mohn et al. 2009). For MeDIP assays, 5–10 μg of genomic DNA was sonicated into small fragments, and 1 μg of the 5-methylcytosine monoclonal antibody (Active Motif, catalog no. 39649) was used to perform the MeDIP assay as previously described (Pomraning et al. 2009). MeDIP samples were analyzed with qPCR. Relative enrichment of DNA was calculated as the ratio of MeDIP sample to input, and ratios were normalized to the actual *al-1* copy numbers in each quelled strain or transformant. *actin* levels were used to normalize the loading in different samples.

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References

Aguilera A. 1995. Genetic evidence for different RAD52-dependent intrachromosomal recombination pathways in *Saccharomyces cerevisiae*. *Curr Genet* **27:** 298–305.

Baulcombe D. 2004. RNA silencing in plants. *Nature* **431:** 356–363.

Buhler M, Moazed D. 2007. Transcription and RNAi in heterochromatic gene silencing. *Nat Struct Mol Biol* **14:** 1041–1048.

Butler DK. 1992. Ribosomal DNA is a site of chromosome breakage in aneuploid strains of *Neurospora*. *Genetics* **131:** 581–592.

Bzymek M, Lovett ST. 2001. Instability of repetitive DNA sequences: the role of replication in multiple mechanisms. *Proc Natl Acad Sci* **98:** 8319–8325.

Castel SE, Martienssen RA. 2013. RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. *Nat Rev Genet* **14:** 100–112.

Castel SE, Ren J, Bhattacharjee S, Chang A, Sánchez M, Valbuena A, Antequera F, Martienssen RA. 2014. Dicer

promotes transcription termination at sites of replication stress to maintain genome stability. *Cell* **159:** 572–583.
Catalanotto C, Pallotta M, ReFalo P, Sachs MS, Vayssie L, Macino G, Cogoni C. 2004. Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in *Neurospora crassa*. *Mol Cell Biol* **24:** 2536–2545.
Catalanotto C, Nolan T, Cogoni C. 2006. Homology effects in *Neurospora crassa*. *FEMS Microbiol Lett* **254:** 182–189.
Cecere G, Cogoni C. 2009. Quelling targets the rDNA locus and functions in rDNA copy number control. *BMC Microbiol* **9:** 44.
Chang SS, Zhang Z, Liu Y. 2012. RNA interference pathways in fungi: mechanisms and functions. *Annu Rev Microbiol* **66:** 305–323.
Chicas A, Cogoni C, Macino G. 2004. RNAi-dependent and RNAi-independent mechanisms contribute to the silencing of RIPed sequences in *Neurospora crassa*. *Nucleic Acids Res* **32:** 4237–4243.
Chicas A, Forrest EC, Sepich S, Cogoni C, Macino G. 2005. Small interfering RNAs that trigger posttranscriptional gene silencing are not required for the histone H3 Lys9 methylation necessary for transgenic tandem repeat stabilization in *Neurospora crassa*. *Mol Cell Biol* **25:** 3793–3801.
Choudhary S, Lee HC, Maiti M, He Q, Cheng P, Liu Q, Liu Y. 2007. A double-stranded-RNA response program important for RNA interference efficiency. *Mol Cell Biol* **27:** 3995–4005.
Cogoni C, Macino G. 1999a. Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. *Nature* **399:** 166–169.
Cogoni C, Macino G. 1999b. Posttranscriptional gene silencing in *Neurospora* by a RecQ DNA helicase. *Science* **286:** 2342–2344.
Cogoni C, Irelan JT, Schumacher M, Schmidhauser TJ, Selker EU, Macino G. 1996. Transgene silencing of the al-1 gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. *EMBO J* **15:** 3153–3163.
Eichler EE, Holden JJ, Popovich BW, Reiss AL, Snow K, Thibodeau SN, Richards CS, Ward PA, Nelson DL. 1994. Length of uninterrupted CGG repeats determines instability in the FMR1 gene. *Nat Genet* **8:** 88–94.
Fasullo M, Giallanza P, Dong Z, Cera C, Bennett T. 2001. *Saccharomyces cerevisiae* rad51 mutants are defective in DNA damage-associated sister chromatid exchanges but exhibit increased rates of homology-directed translocations. *Genetics* **158:** 959–972.
Francia S, Michelini F, Saxena A, Tang D, de Hoon M, Anelli V, Mione M, Carninci P, d'Adda di Fagagna F. 2012. Site-specific DICER and DROSHA RNA products control the DNA-damage response. *Nature* **488:** 231–235.
Freitag M, Lee DW, Kothe GO, Pratt RJ, Aramayo R, Selker EU. 2004. DNA methylation is independent of RNA interference in *Neurospora*. *Science* **304:** 1939.
Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJ, Holden JJ, Fenwick RG Jr, Warren ST, et al. 1991. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell* **67:** 1047–1058.
Fulci V, Macino G. 2007. Quelling: post-transcriptional gene silencing guided by small RNAs in *Neurospora crassa*. *Curr Opin Microbiol* **10:** 199–203.
Gao M, Wei W, Li MM, Wu YS, Ba Z, Jin KX, Liao YQ, Adhikari S, Chong Z, Zhang T, et al. 2014. Ago2 facilitates Rad51 recruitment and DNA double-strand break repair by homologous recombination. *Cell Res* **24:** 532–541.
Ghildiyal M, Zamore PD. 2009. Small silencing RNAs: an expanding universe. *Nat Rev Genet* **10:** 94–108.
Goldoni M, Azzalin G, Macino G, Cogoni C. 2004. Efficient gene silencing by expression of double stranded RNA in *Neurospora crassa*. *Fungal Genet Biol* **41:** 1016–1024.
Ishibashi K, Suzuki K, Ando Y, Takakura C, Inoue H. 2006. Nonhomologous chromosomal integration of foreign DNA is completely dependent on MUS-53 (human Lig4 homolog) in *Neurospora*. *Proc Natl Acad Sci* **103:** 14871–14876.
Jarem DA, Huckaby LV, Delaney S. 2010. AGG interruptions in (CGG)(n) DNA repeat tracts modulate the structure and thermodynamics of non-B conformations in vitro. *Biochemistry* **49:** 6826–6837.
Keskin H, Shen Y, Huang F, Patel M, Yang T, Ashley K, Mazin AV, Storici F. 2014. Transcript-RNA-templated DNA recombination and repair. *Nature* **515:** 436–439.
Lee HC, Chang SS, Choudhary S, Aalto AP, Maiti M, Bamford DH, Liu Y. 2009. qiRNA is a new type of small interfering RNA induced by DNA damage. *Nature* **459:** 274–277.
Lee HC, Aalto AP, Yang Q, Chang SS, Huang G, Fisher D, Cha J, Poranen MM, Bamford DH, Liu Y. 2010. The DNA/RNA-dependent RNA polymerase QDE-1 generates aberrant RNA and dsRNA for RNAi in a process requiring replication protein A and a DNA helicase. *PLoS Biol* **8:** e1000496.
Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25:** 2078–2079.
Lobrich M, Shibata A, Beucher A, Fisher A, Ensminger M, Goodarzi AA, Barton O, Jeggo PA. 2010. γH2AX foci analysis for monitoring DNA double-strand break repair: strengths, limitations and optimization. *Cell Cycle* **9:** 662–669.
Maiti M, Lee HC, Liu Y. 2007. QIP, a putative exonuclease, interacts with the *Neurospora* Argonaute protein and facilitates conversion of duplex siRNA into single strands. *Genes Dev* **21:** 590–600.
Michalik KM, Bottcher R, Forstemann K. 2012. A small RNA response at DNA ends in *Drosophila*. *Nucleic Acids Res* **40:** 9596–9603.
Mohn F, Weber M, Schubeler D, Roloff TC. 2009. Methylated DNA immunoprecipitation (MeDIP). *Methods Mol Biol* **507:** 55–64.
Napoli C, Lemieux C, Jorgensen R. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2:** 279–285.
Nolan T, Braccini L, Azzalin G, De Toni A, Macino G, Cogoni C. 2005. The post-transcriptional gene silencing machinery functions independently of DNA methylation to repress a LINE1-like retrotransposon in *Neurospora crassa*. *Nucleic Acids Res* **33:** 1564–1573.
Nolan T, Cecere G, Mancone C, Alonzi T, Tripodi M, Catalanotto C, Cogoni C. 2008. The RNA-dependent RNA polymerase essential for post-transcriptional gene silencing in *Neurospora crassa* interacts with replication protein A. *Nucleic Acids Res* **36:** 532–538.
Pendyala L, Wellman AM. 1975. Effect of histidine on purine nucleotide synthesis and utilization in *Neurospora crassa*. *J Bacteriol* **124:** 78–85.
Peng JC, Karpen GH. 2007. H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability. *Nat Cell Biol* **9:** 25–35.

Pomraning KR, Smith KM, Freitag M. 2009. Genome-wide high throughput analysis of DNA methylation in eukaryotes. *Methods* **47:** 142–150.

Romano N, Macino G. 1992. Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Mol Microbiol* **6:** 3343–3353.

Samadashwily GM, Raca G, Mirkin SM. 1997. Trinucleotide repeats affect DNA replication in vivo. *Nat Genet* **17:** 298–304.

Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem* **73:** 39–85.

Sijen T, Plasterk RH. 2003. Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi. *Nature* **426:** 310–314.

Siomi MC, Saito K, Siomi H. 2008. How selfish retrotransposons are silenced in *Drosophila* germline and somatic cells. *FEBS Lett* **582:** 2473–2478.

Vader G, Blitzblau HG, Tame MA, Falk JE, Curtin L, Hochwagen A. 2011. Protection of repetitive DNA borders from self-induced meiotic instability. *Nature* **477:** 115–119.

Wang X, Hsueh YP, Li W, Floyd A, Skalsky R, Heitman J. 2010. Sex-induced silencing defends the genome of *Cryptococcus neoformans* via RNAi. *Genes Dev* **24:** 2566–2582.

Wei W, Ba Z, Gao M, Wu Y, Ma Y, Amiard S, White CI, Rendtlew Danielsen JM, Yang YG, Qi Y. 2012. A role for small RNAs in DNA double-strand break repair. *Cell* **149:** 101–112.

Yang Q, Li L, Xue Z, Ye Q, Zhang L, Li S, Liu Y. 2013. Transcription of the major *Neurospora crassa* microRNA-like small RNAs relies on RNA polymerase III. *PLoS Genet* **9:** e1003227.

Zhang H, Zhu JK. 2011. RNA-directed DNA methylation. *Curr Opin Plant Biol* **14:** 142–147.

Zhang Z, Chang SS, Xue Z, Zhang H, Li S, Liu Y. 2013. Homologous recombination as a mechanism to recognize repetitive DNA sequences in an RNAi pathway. *Genes Dev* **27:** 145–150.

Zhang Z, Yang Q, Sun G, Chen S, He Q, Li S, Liu Y. 2014. Histone H3K56 acetylation is required for quelling-induced small RNA production through its role in homologous recombination. *J Biol Chem* **289:** 9365–9371.

Zhou M, Guo J, Cha J, Chae M, Chen S, Barral JM, Sachs MS, Liu Y. 2013. Non-optimal codon usage affects expression, structure and function of clock protein FRQ. *Nature* **495:** 111–115.