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qiRNA, a new type of small interfering RNA induced by DNA damage

Heng-Chi Lee¹, Shwu-Shin Chang¹, Swati Choudhary¹, Antti P. Aalto², Mekhala Maiti^{1,3}, Dennis H. Bamford², and Yi Liu^{1,†}

¹ Department of Physiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390 USA ² Institute of Biotechnology and Department of Biological and Environmental Sciences, Biocenter, 2, P.O. Box 56, FIN-00014 University of Helsinki, Helsinki, Finland

Summary

RNA interference pathways use small RNAs to mediate gene silencing in eukaryotes. In addition to small interfering RNAs (siRNA) and microRNAs, several types of endogenously produced small RNAs play important roles in gene regulation, germ cell maintenance and transposon silencing 1–4. Production of some of these RNAs requires the synthesis of aberrant RNAs (aRNAs) or pre-siRNAs, which are specifically recognized by RNA-dependent RNA polymerases (RdRPs) to make double stranded RNA (dsRNA). The mechanism for aRNA synthesis and recognition is largely unknown. Here we show that DNA damage induces the expression of the Argonaute protein QDE-2 and a novel class of small RNAs in the filamentous fungus *Neurospora*. This class of small RNAs, named qiRNAs for their association with QDE-2, are about 20–21 nt long (several nt shorter than *Neurospora* siRNAs) with a strong preference for uridine at the 5' end and originate mostly from the ribosomal DNA locus. Production of qiRNAs requires the RdRP QDE-1, the Werner/Bloom RecQ DNA helicase homolog QDE-3 and dicers. qiRNA biogenesis also requires DNA damage-induced aRNAs as precursor, a process that is dependent on QDE-1 and QDE-3. Surprisingly, our results suggest that QDE-1 is the DNA-dependent RNA polymerase that produces aRNAs. In addition, the *Neurospora* RNAi mutants exhibit increased sensitivity to DNA damage, suggesting a role for qiRNAs in DNA damage response by inhibiting protein translation.

In the filamentous fungus *Neurospora crassa*, the RNAi pathway is essential for both dsRNA and transgene-induced gene silencing (quelling) ⁵. In the quelling pathway, QDE-1 and QDE-3 are thought to be involved in the generation of dsRNA ^{6,7}. In addition, QDE-3 was previously shown to be involved in DNA repair ⁶. It has been proposed that a repetitive transgene leads to the production of transgene-specific aRNA, which is converted to dsRNA

Correspondence to: Yi Liu¹ Correspondence and requests for materials should be addressed to Y.L. (Yi.Liu@UTSouthwestern.edu).
³Current address: Department of Cell Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390 USA

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The *Neurospora* rDNA sequence has been deposited to Genbank (FJ360521).

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by QDE-1. Two partially redundant Dicer proteins, DCL-1 and DCL-2, cleave the dsRNA into siRNAs of around 25nt in size⁸. Subsequently, the siRNAs are loaded onto the RNA-induced silencing complex (RISC), formed by the Argonaute protein QDE-2 and an exonuclease QIP^{9–11}. We previously showed that dsRNA and not siRNA transcriptionally activates *qde-2*, other RNAi components and putative antiviral genes¹².

During our study of QDE-2 regulation, we observed that supplementing histidine in the medium resulted in a significant increase of *qde-2* mRNA and QDE-2 protein levels whereas other amino acids did not (Fig. 1a & supplementary Fig. 1a). Histidine is known to inhibit DNA replication, reduce the NTP pool, and result in DNA damage in *Neurospora*^{13,14}. In addition, histidine significantly increased the mutation rate at the *mtr* locus (supplementary Fig. 1b). These results suggest that DNA damage results in the induction of *qde-2* expression. Treatment with either ethyl methanesulfonate (EMS, Fig. 1b), hydroxyurea (supplementary Fig. 1c), or methyl methanesulfonate (data not shown) also induced QDE-2 expression. Importantly, the induction of QDE-2 by histidine and other DNA damaging agents requires QDE-1, QDE-3, and the DCLs (Fig. 1b, and supplementary Fig. 1c & Fig. 1d). Furthermore, in the absence of DNA damaging agents, QDE-2 accumulates to elevated levels in DNA repair mutants that are deficient in double-stranded DNA break repair or homologous recombination repair pathways (Fig. 1c). Taken together, these results demonstrate that DNA damage activates *qde-2* expression.

Since QDE-1 and QDE-3 are involved in the generation of dsRNA and DCLs are important for maintaining the steady state level of QDE-2 posttranscriptionally¹², our results suggest that DNA damage results in production of endogenous dsRNA, which activates *qde-2* transcription. We reasoned that such dsRNA is processed into small RNAs, which then associate with QDE-2. To examine this possibility, we immunoprecipitated c-Myc tagged QDE-2 expressed in a *qde-2* null strain¹¹. The QDE-2 associated RNA was extracted and 3'-end labeled with [³²P] Cytidine bisphosphate (pCp). As shown in Fig. 2a, Myc-QDE-2 specifically associated with a group of small RNAs approximately 20–21 nt in length, which were dramatically induced after histidine or EMS treatment (data not shown). Because these small RNAs are endogenously produced and are associated with QDE-2, they were named qiRNAs for QDE-2-interacting small RNAs. The average length of qiRNAs is several nucleotides shorter than that of *Neurospora* siRNAs (Fig. 2a and supplementary Fig. 2a), which is around 25 nt⁸.

qiRNAs were cloned and sequenced. Analyses of 184 individual qiRNA sequences revealed that they indeed possess an average length of about 20–21 nt (supplementary Fig. 2b and supplementary Table 1). Similar to the piRNAs recently identified in animals¹⁵, the first nucleotide of the 5' end of qiRNAs exhibits a strong preference for U (93%) (supplementary Fig. 2c). In addition, the first nucleotide of the 3' end also prefers A (49%).

Surprisingly, the vast majority of qiRNAs (86%) originated from the ribosomal DNA (rDNA) locus (supplementary Fig. 2d), where ~200 copies of rDNA repeats form the nucleolus organizer region (NOR). Their association with QDE-2 and their 5' and 3' end nucleotide preferences suggest that qiRNAs are not non-specific rRNA degradation products. The remaining qiRNAs were mapped to intergenic regions (6.57%), open reading frames (ORFs, 4.37%) and tRNAs (1.45%).

qiRNAs from the rDNA locus correspond to both sense and antisense strands at approximately equal frequency, suggesting that the biogenesis of qiRNAs requires the formation of dsRNA (Fig. 2b). Furthermore, qiRNAs not only originate from the region corresponding to the mature rRNAs, but many of them derive from the external and internal transcribed spacer regions (ETS, ITS1 & ITS2) and the intergenic spacer regions. These

results suggest that the biogenesis of qiRNAs may require unconventional transcriptional events.

Northern blot analysis showed that the levels of 26S rDNA-specific qiRNA were undetectable under normal conditions but were dramatically induced after EMS treatment (Fig. 2c). qiRNA also accumulated to a high level in an *atm* mutant without EMS treatment. Furthermore, qiRNAs production was completely abolished in the *qde-1* and *qde-3* mutant strains (Fig. 2c), indicating that QDE-1 and QDE-3 are required for qiRNA biogenesis. In contrast, the production of qiRNA was maintained in the *qde-2* mutant. Although the sizes of qiRNAs are smaller than those of siRNAs, the production of qiRNA is abolished in a *dcl-1 dcl-2* double mutant. In addition, long RNA species accumulated in the *dcl* double mutant after DNA damage, suggesting the accumulation of long dsRNA.

To investigate the relationship between qiRNAs and aRNAs, we examined the transcript levels from the intergenic rDNA spacer regions. Quantitative RT-PCR experiments and northern blot analysis showed that RNA transcripts originating from both upstream and downstream of the transcribed rDNA region are indeed highly induced after DNA damage (Fig. 2d). In the *dcl* double mutant, aRNAs accumulated to a high level with sizes ranging from a few hundred nucleotides to ~2kb (supplementary Fig. 3), suggesting that these transcripts form dsRNA. Importantly, we found that the aRNA production was completely abolished in the *qde-3* mutant (Fig. 2e), indicating that aRNAs are the precursors of qiRNAs and that QDE-3, the RecQ DNA helicase, is required for aRNA biogenesis.

RNA polymerase I is responsible for the transcription of rRNAs. However, we found that the DNA damage-induced aRNA production was maintained in an RNA polymerase I mutant (supplementary Fig. 4a–c). Furthermore, both qRT-PCR and northern blot analyses showed that treatment of *Neurospora* with thiolutin, a potent inhibitor of RNA polymerase I, II, and III, did not affect aRNA production despite its strong inhibition of mRNA synthesis (Fig. 3a and supplementary Fig. 5a). These results suggest that the common RNA polymerases are not required for the generation of aRNAs after DNA damage.

QDE-1, the RdRP, was previously thought to specifically recognize and convert aRNA into dsRNA. To our surprise, we found that the induction of rDNA-specific aRNAs by histidine was completely abolished in the *qde-1^{ko}* mutant (Fig. 3b and supplementary Fig. 5b), indicating that QDE-1 is not only required for the production of dsRNA but also for the synthesis of aRNAs. In addition, we found that QDE-1 does not amplify small RNAs in dsRNA-mediated gene silencing (supplementary Fig. 5c, d), indicating that qiRNAs are not amplification products of primary small RNAs.

Recent structural analysis of QDE-1 has shown that its catalytic core is similar to eukaryotic DNA-dependent RNA polymerases (DdRPs) but not to viral RdRPs¹⁶. This result prompted us to examine whether QDE-1 can function as a DdRP to generate aRNAs. A c-Myc-His-tagged QDE-1 was expressed in the *qde-1^{ko}* strain and purified by affinity purification to be used in RdRP and DdRP assays. As shown in Figure 3c, QDE-1 exhibited RNA polymerase activity using both ssRNA and ssDNA as templates to generate full-length RNA products. In contrast, RNA polymerase activity was not detected using the control purification products. In addition, RNase H degraded the ³²P-labeled ssDNA-templated products of QDE-1 (Fig. 3d), indicating that they were mostly DNA/RNA hybrids. Furthermore, we found that the RNA polymerase activity of QDE-1 is not inhibited by thiolutin *in vitro* (unpublished). Together, these results demonstrate that QDE-1 can function as both an RdRP and a DdRP. The requirement of QDE-1 for aRNA synthesis suggests that QDE-1 is the RNA polymerase that generates aRNA.

To determine whether the rDNA-specific qiRNAs are functional, we immunoprecipitated Myc-QDE-2 using strains that express either the wild-type QDE-2 or QDE-2 containing a D664A mutation that abolishes its catalytic activity¹¹. As shown in Figure 4a, qiRNAs were associated with both forms of QDE-2. However, the qiRNAs associated with wild-type QDE-2 were entirely single-stranded while only double-stranded qiRNAs bound to QDE-2(D664A). This result indicates that qiRNAs are associated with an active RISC complex.

Since the majority of qiRNAs are derived from the rDNA locus, they may inhibit rRNA biogenesis and protein synthesis after DNA damage. As shown in Figure 4b, the protein synthesis rate measured by a ³⁵S labeling pulse (³⁵S-Methionine and ³⁵S-Cysteine) was significantly decreased after histidine treatment. Importantly, this decrease in protein synthesis rate was partially blocked in *qde-1* and *qde-3* mutants (*p*-values 4.6×10^{-6} and 2.2×10^{-5} , respectively). Similar results were also obtained using EMS (supplementary Fig. 6a). These results suggest that qiRNAs are involved in inhibiting protein synthesis after DNA damage.

Consistent with a role for qiRNAs in DNA damage response, a *qde-3* mutant was previously shown to be sensitive to both histidine and DNA damaging agents⁶. Furthermore, we found that both *qde-1* and the *dcl* double mutants exhibited increased sensitivity to histidine, EMS and HU treatments, although they were not as sensitive as the *atm* mutant (Fig. 4c and supplementary Fig. 6b). Taken together, these results suggest a role for the *Neurospora* RNAi pathway in DNA damage response.

After DNA damage, eukaryotic cells activate DNA repair pathways to restore DNA integrity. There are various DNA damage checkpoints initiated to arrest cell cycle progression allowing time for DNA repair¹⁷. Based on our results, we propose that the production of qiRNAs is another mechanism that contributes to DNA damage checkpoints by inhibiting protein synthesis. Results obtained from higher eukaryotic organisms also suggest the importance of rDNA-derived small RNAs. In mouse embryonic stem cells, rRNA-specific small RNAs associated with a small RNA binding protein¹⁸. In *Arabidopsis*, RNAi components are found in the nucleolus and rDNA-specific small RNAs contribute to heterochromatin formation¹⁹. The *Drosophila dicer-2* mutant exhibited disorganized nucleoli and rDNA, suggesting a role for the RNAi pathway in maintaining genome stability in the rDNA region²⁰. Like qiRNA, some of the small RNAs from higher eukaryotes are also enriched in repetitive regions of the genome¹. Our study raises the possibility that spontaneous DNA damage produced during recombination or transposon transposition could be a trigger to induce production of small RNAs. Interestingly, piRNAs from rat testes associated with rRecQ1²¹, a QDE-3 homolog. Therefore, recQ helicases may also play a role in generating primary aRNAs in other RNAi related pathways.

In fission yeast, Pol II is implicated as an RNA polymerase that generates centromeric pre-siRNA^{22,23}. In plants, the RNA polymerase IV is important for RNAi-directed transcriptional silencing^{24,25}, but its homologs are not found in fungal or animal genomes. In this study, we uncovered an unexpected role for QDE-1 as a DdRP in aRNA production in *Neurospora*. Interestingly, QDE-1 is known to interact with RPA²⁶, a ssDNA binding complex, raising the possibility that RPA may recruit QDE-1 to ssDNA *in vivo* to produce aRNAs. RDR6, an RdRP in the *Arabidopsis* RNAi pathway, was recently shown to exhibit robust DdRP activity²⁷, suggesting that DdRP activity may be a shared biochemical activity for eukaryotic RdRPs. Importantly, the aRNA production model proposed here provides an explanation for how aRNAs but not other cellular RNAs are specifically recognized by RdRPs: since the aRNA is produced by QDE-1, its close proximity makes it a preferred template for QDE-1 to make dsRNA.

METHODS SUMMARY

The *Neurospora* strains used in this study were generated previously^{11,12} or obtained from the Fungal Genetic Stock Center. For detailed strain information and molecular and biochemical protocols, please refer to the Methods.

Methods

Strains and growth conditions

The wild-type strain used in this study was FGSC 4200(a). *qde-1*, *qde-3*, and *dcl-1*; *dcl-2* double mutant were generated from our previous studies^{11,12}. DNA repair defective mutants are *atm* (FGSC11162, NCU00274.1), *mus-9* (FGSC5146, NCU11188.1), *mus-23* (FGSC8342, NCU08730.1), *mei-3* (FGSC6187, NCU2741.1), *mus-11* (FGSC5150, NCU04275.1), *telomerase* (FGSC12704, NCU2791.1), *mus-58* (FGSC11164, NCU08346.1), *chk2* (FGSC11170, NCU02814.1), *uvs-6* (FGSC4179, NCU00901.1), *mus-25* (FGSC6424, NCU11255.1), and *mus-38* (FGSC11191, NCU00942.1). These genes are homologs of *S. cerevisiae* ATM, ATR, RAD51, RAD52, telomerase, CHK1, CHK2, RAD50, RAD54 and RAD1, respectively. Liquid cultures were grown in minimal medium (1× Vogel's, 2% glucose). For liquid cultures containing QA, 0.01 M QA (pH 5.8) was added to the liquid culture medium containing 1×Vogel's, 0.1% glucose, and 0.17% arginine. For liquid culture containing DNA damaging agents or amino acids, histidine (50 to 100 µg/ml), EMS (0.2%), MMS (0.015%), HU (2mg/ml) or the indicated amino acids (50µg/ml) was added and cultures were harvested 40 hours later. For cultures treated with thiolution, 4µg/ml of the drug was added and harvested 30 hrs later.

Purification and cloning of QDE-2 associated RNA

Liquid cultures of Myc-QDE-2 expressing strain were grown in the presence or absence of histidine (100µg/ml), and harvested 40 hours after inoculation. Immunopurification of Myc-QDE-2 ribonucleoprotein complex was performed as previously described¹¹. Immunoprecipitated beads were washed 5X using the extraction buffer. The beads were then treated with 1mg/ml proteinase K at 65°C for 1 hr. QDE-2 associated RNAs were recovered by phenol/chloroform extraction and ethanol precipitation. To visualize the QDE-2 associated RNAs, 5% of the purified RNAs were labeled at 3' end with [32P] pCp by T4 RNA ligase. The labeled RNAs were resolved on 16% polyacrylamide gel before exposing to X-ray films. QDE-2 associated small RNAs were CIP treated, followed by PNK treatment in order to clone small RNAs with potential different number of phosphate at 5' ends. Small RNAs (18–26 nt) were cloned as previously described²⁸. The small RNA sequences were blasted to *Neurospora* genome using the *Neurospora crassa* database at the Broad Institute. The sequence of ribosomal DNA were generated by sequencing the plasmid pKH1 (GenBank FJ360521).

Measurement of spontaneous mutation rate

Spontaneous mutation rate at the *mtr* locus was measured as previously described²⁹. *mtr* encodes for the neutral amino acid permease in *Neurospora*. Mutations at the *mtr* locus is reflected by the resistance of the mutant strains to toxic amino acid analog p-fluorophenylalanine.

RNA analyses

Enriched low-molecular-weight RNAs were used to detect small RNAs as previously described¹¹. Sense or antisense rRNA probes were *in vitro*-transcribed using PCR template derived from 26S rDNA regions. Total RNA was used to detect aRNAs from the rDNA region. 40 µg of total RNA were separated on formaldehyde containing 1.3% agarose gel.

Probes (sense and antisense) were *in vitro*-transcribed by using PCR template derived from upstream sequence of rDNA region.

Quantitative real time PCR was performed as previously described¹². The *Neurospora* β -*tubulin* gene was used as an internal control for qRT-PCR. For thiolutin treatment experiments, the levels of mature 26S rRNA was used as the internal control. Primer sequences are available upon request.

RNA polymerase assays

A construct expressing the full-length 6His tagged c-Myc-QDE-1 was transformed into a *qde-1* mutant strain. Six grams of tissue from the c-Myc-His-QDE-1 strain or wild type strain were harvested. Cell lysates in extraction buffer (50mM HEPES pH 7.4, 100mM KCl, 10mM imidazole and 10% glycerol) were applied to Ni-NTA matrices (1ml bed volume). The matrices were then washed with 10ml of washing buffer (50mM HEPES, 100mM KCl, and 10mM imidazole) and eluted with 4 1ml elution buffer (50mM HEPES, 100mM KCl, 200mM imidazole and 20% glycerol). Equal amount of purified proteins from both the c-My-His-QDE-1 expressing strain and a control strain were used in RNA polymerase assays.

RNA polymerase reactions were performed essentially as previously described^{30,31}. The samples were subjected to gel electrophoresis using denaturing polyacrylamide (16%) TBE gels. The 175 nt ssDNA, deriving from mature 26S rRNA region, was made by boiling followed rapid chilling on ice water. ssRNA with the same sequence was made by *in vitro* transcription using T7 RNA polymerase. For RNase H treatment, the reaction products were extracted with phenol:chloroform, precipitated with NH₄OAc and ethanol, and dissolved in water. Afterwards, reaction products were supplemented with Rnase H and its reaction buffer, incubated for 30 min at +37°C, and analyzed by electrophoresis.

Measurement of general translation rate

Conidia from 7-old cultures were inoculated into petri dishes containing 1×Vogel's minimum medium with 2% glucose and were incubated at room temperature for 2 days to allow mycelial mats to form. The mycelial mats were cut into discs of equal size, which were cultured in 1×Vogel's minimum medium with 2% glucose overnight in the presence or absence of histidine or EMS (100µg/ml and 0.2%, respectively), and then metabolically labeled with 1µCi/ml EXPRE35S35S protein labeling mix (PerkinElmer) for 30 (EMS) or 60 (histidine) min. The protein extracts were then prepared. Afterwards, 50 µg of total protein was precipitated by 10% TCA on filter paper 413 (VWR) for 30 min. The filter papers were then washed with 10% TCA twice for 5 min each and dried in 1:1 ethanol/diethyl ether followed by diethyl ether. The dried filter papers were immersed in 5ml scintillation fluid and 35S signals were counted. For control cultures, the protein synthesis inhibitor cycloheximide (10 µg/ml) was added just before the labeling. The low background radioactive counts obtained from these extracts confirmed that the radioactive counts measured in our assay were due to newly synthesized proteins.

Assay for measurement of DNA damage sensitivity

Spot test was used for measuring the sensitivity of different strains to various DNA mutagens. Conidia concentration of conidia suspensions was measured and dropped onto sorbose-containing agar plates with serial indicated dilutions. The plates were incubated for 3 days at room temperature. EMS, HU, or histidine was added into agar medium at final concentration of 0.2%, 2mg/ml and 6mg/ml, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. DNA damage induces QDE-2 expression. (a) Northern and Western blot analyses showing the induction of *qde-2* mRNA and QDE-2 protein expression by histidine. (b) Western blot analysis showing that the induction of QDE-2 by EMS requires QDE-1, QDE-3 and DCLs. The labels above the lanes indicate the genotypes of the strains. (c) Western blot analyses showing high QDE-2 levels in DNA repair mutants in the absence of a DNA damaging agent.

Figure 2.

DNA damage results in the production of qiRNAs and aRNAs. (a) Enrichment of the Myc-QDE-2 associated small RNAs by immunoprecipitation. A wild-type strain that lacks the Myc-QDE-2 construct was used as a negative control. Immunoprecipitated RNA was 3' end labeled and separated on a 16% acrylamide gel. (b) Mapping of qiRNAs to an rDNA repeat. Regions encoding for the mature rRNAs are shaded. (c) Northern blot analysis of qiRNAs in the indicated strains. An RNA probe specific for the antisense 26S RNA was used. (d) qRT-PCR showing that EMS treatment results in the induction of aRNAs from the rDNA regions. The upper panel shows a schematic diagram of an rDNA repeat and the intergenic rDNA regions (U1, U2 and D1) analyzed by qRT-PCR analysis. A *dcl* double mutant was used. $n=3$; *, $P < 0.001$. Error bars, s. d. (e) qRT-PCR analysis showing the loss of EMS-induced aRNAs from the rDNA locus in the *qde-3* mutant. Results of two independent experiments are shown.

Figure 3.

QDE-1 is required for the synthesis of DNA damage-induced rDNA-specific aRNA and exhibits DdRP activity using a ssDNA template. (a) qRT-PCR results showing the levels of the rDNA-specific aRNA and several pol II transcribed genes in the wild-type strain. $n = 3$; Error bars, s. d. (b) Northern blot analysis showing the levels of aRNA in the wild-type and *qde-1^{ko}* strains. Total RNA was used. (c) *In vitro* RNA polymerase assay using a 175nt ssDNA or ssRNA template and purified Myc-QDE-1. CTL indicates a reaction using purification products from a strain without the construct. (d) The ssDNA-templated reaction products from (c) were treated with RNase H.

Figure 4.

The role of qiRNA in DNA damage response. (a) Northern blot analysis showing that the rDNA-derived qiRNAs are loaded onto an active RISC. Myc-QDE-2 or the catalytically inactive Myc-QDE-2(D664A) were immunoprecipitated and the associated RNAs were extracted. An rDNA-specific probe was used. (b) DNA damage results in the decrease of protein synthesis rate, a response that was partially blocked in the *qde-1* and *qde-3* strains. The radioactivity of ³⁵S labeled protein in various strains were used to compare the rate of total protein synthesis. n = 3; *, P < 0.0001. Error bars, s.d. (c) The *qde-1* and the *dcl* double mutants exhibit increased sensitivity to DNA damaging agents. The numbers of conidia used in the spot test are indicated.