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Diverse Pathways Generate MicroRNA-like RNAs and Dicer-Independent Small Interfering RNAs in Fungi

Heng-Chi Lee,[1,8] Liande Li,[1,8] Weifeng Gu,[2,3,8] Zhihong Xue,[1] Susan K. Crosthwaite,[4] Alexander Pertsemlidis,[5] Zachary A. Lewis,[6] Michael Freitag,[7] Eric U. Selker,[6] Craig C. Mello,[2,3] and Yi Liu[1,*]
[1]Department of Physiology, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
[2]Howard Hughes Medical Institute
[3]Program in Molecular Medicine
University of Massachusetts Medical School, Worcester, MA 01605, USA
[4]Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
[5]McDermott Center for Human Growth and Development, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
[6]Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229, USA
[7]Center for Genome Research and Biocomputing, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, USA
[8]These authors contributed equally to this work
*Correspondence: yi.liu@utsouthwestern.edu
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SUMMARY

A variety of small RNAs, including the Dicer-dependent miRNAs and the Dicer-independent Piwi-interacting RNAs, associate with Argonaute family proteins to regulate gene expression in diverse cellular processes. These two species of small RNA have not been found in fungi. Here, by analyzing small RNAs associated with the *Neurospora* Argonaute protein QDE-2, we show that diverse pathways generate miRNA-like small RNAs (milRNAs) and Dicer-independent small interfering RNAs (disiRNAs) in this filamentous fungus. Surprisingly, milRNAs are produced by at least four different mechanisms that use a distinct combination of factors, including Dicers, QDE-2, the exonuclease QIP, and an RNase III domain-containing protein, MRPL3. In contrast, disiRNAs originate from loci producing overlapping sense and antisense transcripts, and do not require the known RNAi components for their production. Taken together, these results uncover several pathways for small RNA production in filamentous fungi, shedding light on the diversity and evolutionary origins of eukaryotic small RNAs.

INTRODUCTION

RNA interference (RNAi) is a conserved eukaryotic mechanism in which small RNAs mediate both posttranscriptional and transcriptional gene regulation (Ambros, 2004; Buhler and Moazed, 2007; Ghildiyal and Zamore, 2009; Hannon, 2002). Various types of small RNAs, with sizes ranging from 20 to 30 nt, guide Argonaute family proteins to RNA targets to regulate expression of diverse genes. Based on whether or not their biogenesis is dependent on the double-stranded RNA (dsRNA)-specific RNase III ribonuclease Dicer, the known eukaryotic small RNAs can be classified into Dicer-dependent and Dicer-independent groups. The Dicer-dependent group of small silencing RNAs includes microRNAs (miRNAs) and various small interfering RNAs (siRNAs), such as exo-siRNAs, endo-siRNAs, and natsiRNAs (Ghildiyal and Zamore, 2009). miRNAs arise from discrete loci and are processed by Dicer-like enzymes from stem-loop RNA precursors (Ambros et al., 2003; Bartel, 2004). Since the discovery of the first miRNA in *C. elegans*, miRNAs have been found in animals, plants, and algae (Grimson et al., 2008; Lagos-Quintana et al., 2001; Lee and Ambros, 2001; Lee et al., 1993; Llave et al., 2002; Molnar et al., 2007; Zhao et al., 2007), but not in fungi. The absence of fungal miRNAs and the differences between animal and plant miRNAs led to the conclusion that miRNA pathways evolved independently in plants and animals (Jones-Rhoades et al., 2006).

Piwi-interacting RNAs (piRNAs) physically associate with the Piwi clade of Argonaute proteins and are highly enriched in germline cells (Aravin et al., 2006; Batista et al., 2008; Das et al., 2008; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Vagin et al., 2006). The biogenesis of piRNAs is Dicer independent. piRNAs originate mostly from repetitive DNA and frequently match only one strand of DNA, suggesting that they arise from single-stranded RNA (ssRNA). Production of piRNA requires Piwi-related proteins, and a "piRNA ping-pong" amplification mechanism was proposed to account for the production of secondary piRNA from primary piRNA via the slicer activity of Piwi-related proteins (Brennecke et al., 2007; Gunawardane et al., 2007). However, such an amplification mechanism does not appear to be involved in piRNA production in *C. elegans* (Batista et al., 2008; Das et al., 2008). To date, no Dicer-independent small RNAs have been found to associate with Argonaute proteins outside of the animal kingdom.

Previous studies of small RNAs in fungi have been restricted to siRNAs, which are absent from the budding yeast *Saccharomyces cerevisiae* but have been identified in the fission yeast

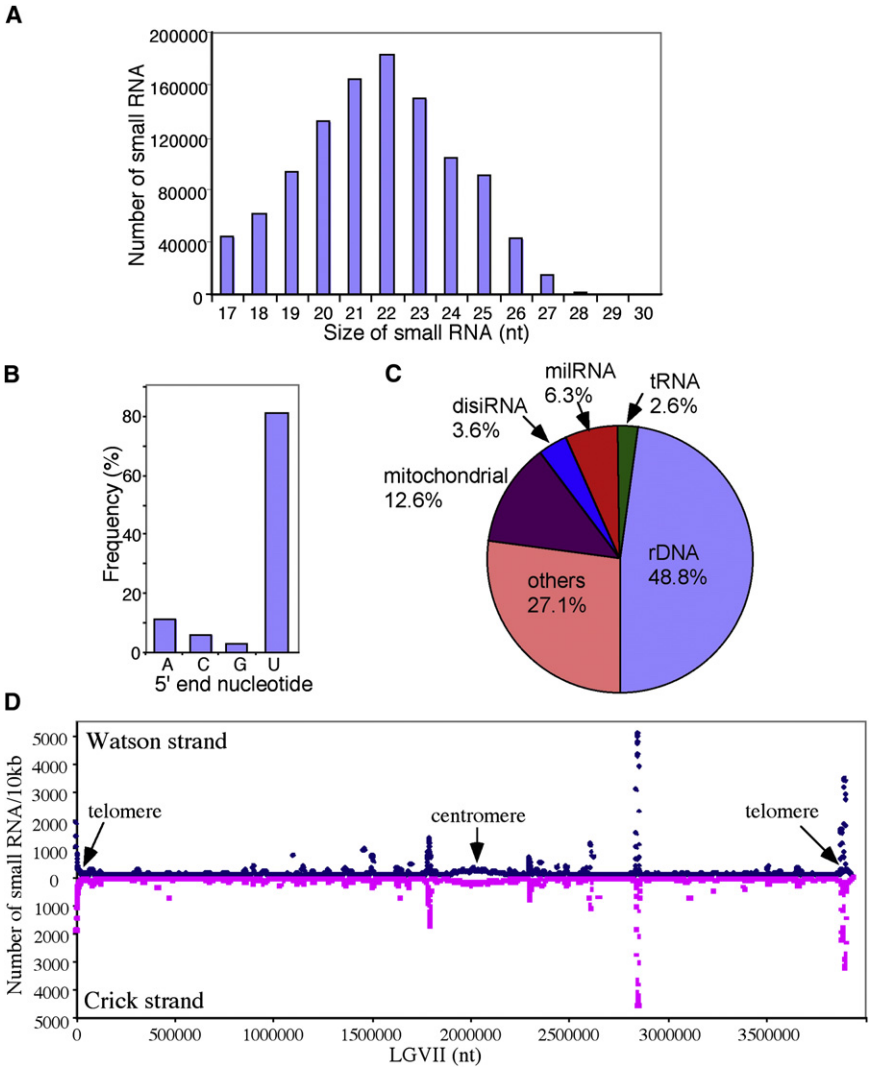

Figure 1. Characterization of QDE-2-Associated Small RNAs

(A) Size distribution of QDE-2-associated small RNAs.

(B) Nucleotide frequency of the 5′ end of small RNAs.

(C) A pie graph showing the relative abundance of different classes of small RNA.

(D) Small RNA distribution on both strands of linkage group VII (LGVII). The numbers of small RNAs/10 kb in 1 kb sliding windows are plotted. The locations of subtelomeric and centromeric sequences are indicated.

Schizosaccharomyces pombe, in budding yeast *Saccharomyces castellii* and *Candida albicans*, and in filamentous fungi (Buhler and Moazed, 2007; Catalanotto et al., 2006; Drinnenberg et al., 2009). In the filamentous fungus *Neurospora crassa*, transgene-induced posttranscriptional gene silencing (quelling) requires QDE-1 (QUELLING DEFICIENT-1, an RNA-dependent RNA polymerase), QDE-2 (an Argonaute protein), QDE-3 (a RecQ DNA helicase homologous to the Werner/Bloom Syndrome proteins), and DCL-1/DCL-2 (two partially redundant Dicer proteins) (Catalanotto et al., 2000, 2004; Choudhary et al., 2007; Cogoni and Macino, 1999a, 1999b). The *Neurospora* Dicers are essential for generating siRNA from dsRNA in vitro and in vivo (Catalanotto et al., 2004).

Neurospora Dicer proteins cleave dsRNA into approximately 25 nucleotide (nt) siRNAs, which are then loaded onto the RNA-induced silencing complex (RISC) containing the core components QDE-2 and QIP (Maiti et al., 2007). We previously showed that QIP, an exonuclease, interacts with QDE-2 and removes the nicked passenger strand from the siRNA duplex, resulting in RISC activation (Maiti et al., 2007). Recently, we identified a class of *Neurospora* QDE-2-interacting siRNAs (qiRNAs) that are induced after DNA damage (Lee et al., 2009). qiRNAs originate from the highly repetitive rDNA locus, and their biogenesis requires QDE-1, QDE-3, and Dicers, but not QDE-2. Here we report that analyses of the *Neurospora* QDE-2-associated small RNAs uncovered several additional types of small RNAs, including miRNA-like small RNAs (milRNAs) and dicer-independent small interfering RNAs (disiRNAs), and five different small RNA biogenesis pathways in this filamentous fungus.

RESULTS

Analyses of *Neurospora* QDE-2-Associated Small RNAs by Deep Sequencing

To comprehensively examine small RNA species in *N. crassa*, we immunoprecipitated c-Myc-tagged QDE-2 and isolated its associated RNAs. Myc-QDE-2 specifically associated with small RNAs of around 21 nt in length (data not shown). cDNA libraries of QDE-2-associated small RNAs (17–30 nt) were sequenced using an Illumina/Solexa Genome Analyzer. We obtained 2,180,272 small RNA sequences that match the current *Neurospora* genome assembly (accession number GSE21175). Most of the small RNAs identified were 19–25 nt long, with the peak at 21–22 nt, and had a strong preference (82.2%) for 5′U (Figures 1A and 1B and see Table S1 available online). The relative abundance and average sizes of different classes of small RNAs are shown in Figure 1C. Although low levels of small RNAs were found throughout the genome (~6/1 kb DNA), sequences corresponding to some genomic regions were highly enriched in our small RNA library, as exemplified by the small RNA distribution across linkage group VII (LG VII) (Figure 1D). Many of those regions, ranging from a few kilobases to more than 20 kb, and including subtelomeric regions, centromeric region, and many other disiRNA regions (see below), were represented by nearly equal numbers of sense and antisense small RNAs. A second set of loci was characterized by small RNAs

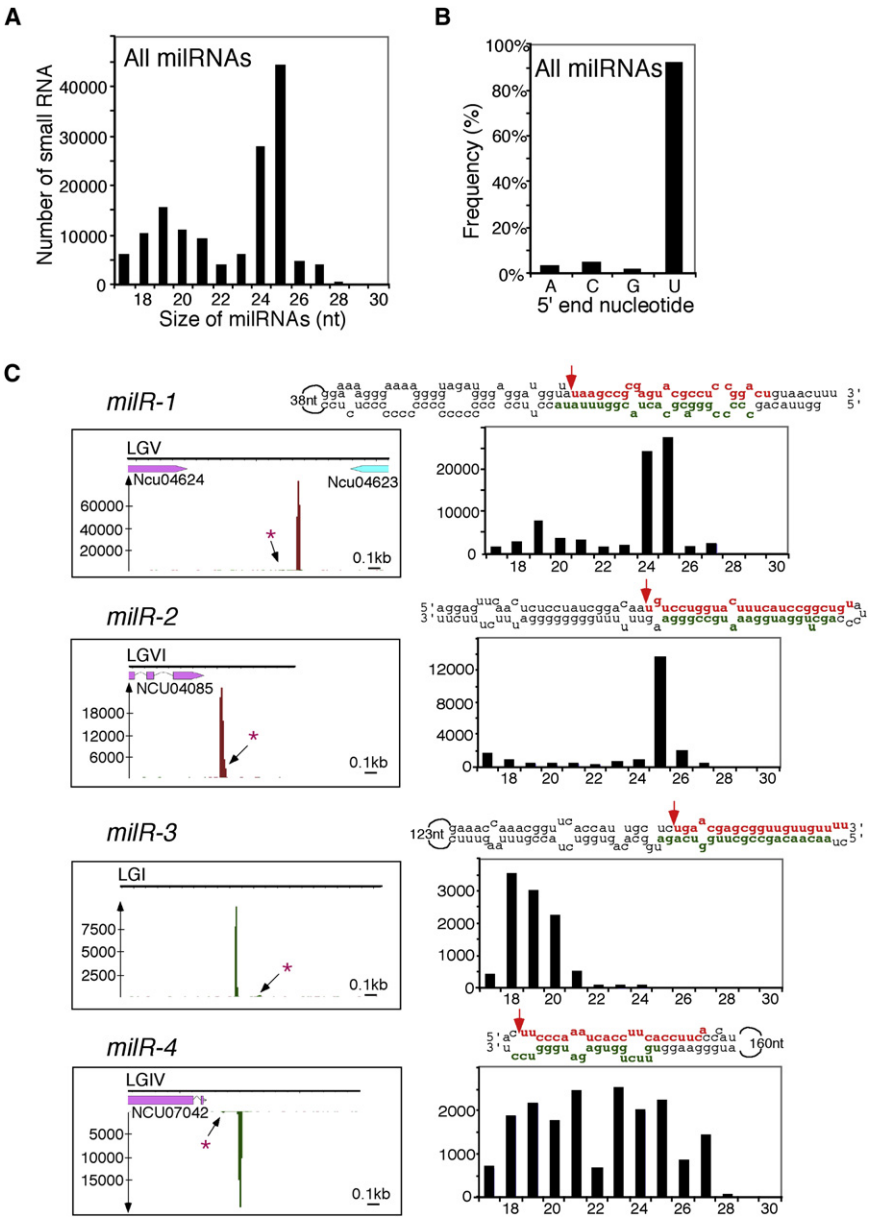

Figure 2. Characterization of QDE-2-Associated milRNAs

(A) Size distribution of the total population of the *Neurospora* milRNAs.

(B) Nucleotide frequency of the 5′ end of milRNAs.

(C) Chromosomal and size distributions of small RNAs and predicted structures of pre-milRNAs of the *milR-1*, *milR-2*, *milR-3*, and *milR-4* loci. In the chromosomal histograms, the numbers of small RNAs (reads in 10 nt nonoverlapping windows), the linkage group, and locations of the milRNA* sequences (arrows with *) are indicated. The major peak in each histogram is the milRNA peak. An RNA more than 20 nt spanned three separate windows, resulting in three peaks in the histogram. For each *milR*, the milRNA sequence and its paired milRNA* sequence (if available) are labeled in red and green, respectively. The position of the 5′ end shared by multiple milRNA species is indicated on the predicted secondary structure by red arrows.

that match only one DNA strand. These loci were considerably smaller (~100–300 nt) and were almost exclusively located in intergenic regions. The different characteristics and distribution of small RNAs suggest the existence of distinct classes of small RNAs in *Neurospora*.

Potential miRNA-like Genes in *Neurospora*

The strand-specific small RNAs suggest the existence of miRNA-like genes. To identify potential miRNA-like genes, the following criteria were used: First, we identified loci that were highly enriched for small RNA (>240 hits/1 kb, top 0.3% small RNA-producing loci of the genome). Second, we focused on loci that produce small RNA predominantly from one strand (>10-fold difference). Third, we analyzed the predicted ability of these RNAs to form hairpin structures with the flanking sequences, a conserved feature of animal and plant miRNAs. Finally, we checked if the most abundant small RNA species reside in the stem region of the hairpin structure. In this manner, we identified 25 potential miRNA-like loci (Table S2). Several individual loci produced multiple, mostly overlapping small RNA species with the same 5′ end. These small RNA producing loci are named *milRs* for miRNA-like RNA genes. Even though these small RNAs and miRNAs from animals and plants share many similarities, they are not named miRNAs and are instead called miRNA-like RNAs (milRNAs) because some of them are produced by similar but not identical mechanisms as those for conventional miRNAs (see below).

A total of 138,022 small RNAs from *milR* loci were sequenced. The size distribution of milRNAs was bimodal, peaking at 25 and 19 nt, with very strong preference for U at their 5′ termini (91.3%) (Figures 2A and 2B). The distinct size distribution of milRNAs compared to that of the total small RNA population suggests the existence of different biogenesis mechanisms in *Neurospora*.

The chromosomal and size distribution of milRNAs, as well as the predicted milRNA precursor (pre-milRNA) structure of the four loci that produce most milRNAs (*milR-1*, *milR-2*, *milR-3*, and *milR-4*), are illustrated in Figure 2C. All four loci are in intergenic regions, and each was represented by at least 9000 reads. In every case, the vast majority of small RNA sequences correspond to one arm of the hairpin (the milRNA arm) and share a common U at the 5′ ends. In addition, small RNAs (milRNA*) matched to the complementary arm of the hairpin were also sequenced, but at much lower frequencies. Several

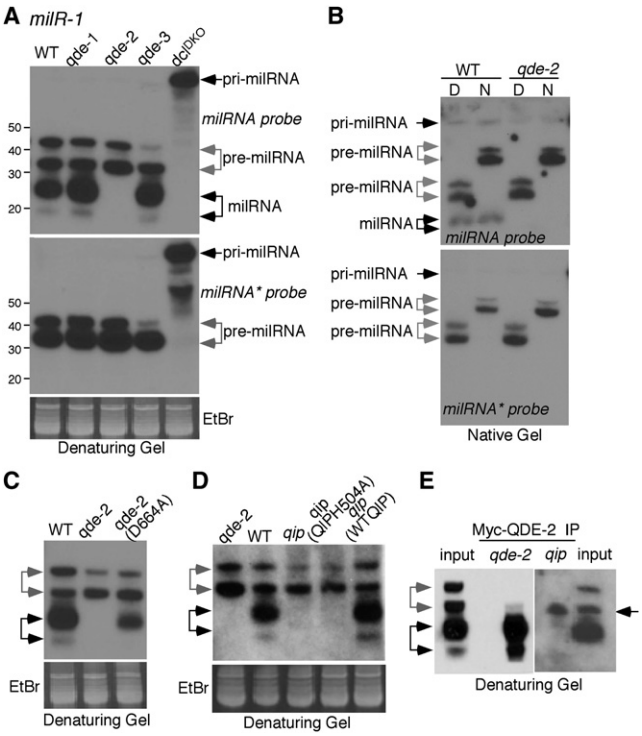

Figure 3. Dicers, QDE-2, and QIP Are Required for the Biogenesis of *milR-1* milRNAs

(A) Northern blot analyses of small RNA samples showing the levels of *milR-1* pri-milRNAs, pre-milRNAs, and milRNAs in the indicated strains in denaturing gels. The top and middle panels show northern blots probed with the milRNA- or milRNA*-specific StarFire probes, respectively. The ethidium bromide-stained denaturing gel in the bottom panel shows equal loading of RNA. The sizes of a RNA ladder are shown at left.

(B) Northern blot analyses of small RNA samples showing the expression of *milR-1* pri-milRNAs, pre-milRNAs, and milRNAs in native gels. "D" indicates that RNA samples were denatured by boiling. "N" indicates the use of native and undenatured RNA samples.

(C) Northern blot analysis of small RNA samples in the *qde-2* mutants.

(D) Northern blot analysis of small RNA samples showing that QIP and its catalytic activity are required for *milR-1* milRNA biogenesis. *qip* (WT QIP) and *qip* (QIPH504A) are *qip* strains that expresses the wild-type QIP or QIP with its catalytic residue mutated.

(E) Northern blot analysis showing the association of Myc-QDE-2 with *milR-1* mature milRNAs and pre-milRNA. Myc-QDE-2 was immunoprecipitated with c-Myc antibody from the Myc-QDE-2 strain in *qde-2* or *qip* mutant background.

milRNA/milRNA* pairs have a 2 nt 3′ overhang, suggesting they may be products of a Dicer-like enzyme (Figure 2C). Although almost all milRNAs from the milRNA arm share the same 5′U position, they have two or more 3′ ends. Similar heterogeneity at 3′ termini has been reported for miRNAs in other eukaryotes (Ruby et al., 2006). Notably, the sizes of the milRNA stem-loop precursors are flexible in *Neurospora*: the milRNA and milRNA* sequences of *milR-1*, *milR-3*, and *milR-4* are separated by more than 120 nt, whereas they are very close for *milR-2*. In addition, the size distributions of milRNAs from these four loci showed different profiles: bimodal for *milR-1* and *-4* and single peaks for *milR-2* (25 nt) and *milR-3* (19 nt). These results suggest that milRNAs from the different *milR* loci are generated by different mechanisms.

Dicer, QDE-2, and QIP-Dependent Mechanism for *milR-1* milRNAs Biogenesis

Under our normal growth conditions, *milR-1* is the most prolific small RNA-producing locus in the *Neurospora* genome. Of >76,000 small RNAs sequenced from this locus, 27,000 were 25 nt long, 23,000 were 24 nt, and 7,000 were 19 nt, all starting with a common 5′U. Northern blot analyses of small RNAs from different strains were performed under denaturing or native conditions. A probe specific for the most abundant milRNA species detected five bands in the wild-type strain, with approximate sizes of 19, 24/25, 33, 43, and ~170 nt in the denaturing gels (Figure 3A, top panel). The 19 and 24/25 nt bands match the size distribution of milRNAs sequenced from *milR-1*, indicating that they are the mature milRNA products. Interestingly, all four lower bands were lost in the *dcl-1/dcl-2* double mutant (dcl^{DKO}), while the level of the ~170 nt band increased dramatically. This result indicates that the four smaller RNA species are processed from the long primary-milRNA (pri-milRNA) by Dicer, and that the two intermediate RNAs (33 and 43 nt) are likely the precursor of milRNAs (pre-milRNAs). Consistently, northern blot analysis of total RNA showed that the pri-milRNA was barely detectable in the wild-type strain but was detected at high levels in the dcl^{DKO} mutant (Figure S1A). In addition, the levels of mature *milR-1* in the *qde-1* and *qde-3* mutants were similar to that of wild-type, indicating that the RdRP and RecQ helicases are not required for *milR-1* milRNA production (Figure 3A, top). In contrast, the two mature milRNAs species were completely eliminated in the *qde-2* mutant, suggesting that this Argonaute protein is required for *milR-1* milRNA biogenesis.

Because of the small sizes of the pre-milRNAs (33 and 43 nt), we hypothesize that they are dsRNA with the loop region of the pri-milRNA cleaved. The cloning and sequencing of the pri-milRNA and pre-milRNA species confirmed our prediction (Figure S1B). First, the pri-milRNA (170 nt) is indeed a transcript containing the predicted hairpin structure. Second, two pre-milRNAs are derived from part of the stem region with the loop region removed. Consistent with this conclusion, a probe specific for the milRNA* strand detected three bands in denaturing gel, corresponding to pri-milRNA and the two pre-milRNA* species (33 and 41 nt), but the mature milRNA* was not detected, most likely due to its low level (Figure 3A, bottom). Furthermore, results of northern blot analyses using a native gel further demonstrated the duplex nature of the pre-milRNA (Figure 3B). RNA samples denatured by boiling exhibited a *milR-1* small RNA profile similar to that shown in Figure 3A, but with native RNA samples; the two pre-milRNA species in the wild-type and *qde-2* strains showed reduced gel mobilities, consistent with them being dsRNA. In contrast, mobilities of the mature milRNA were identical under both conditions, indicating that they are single stranded.

The requirement of QDE-2 for *milR-1* milRNAs production prompted us to examine whether the slicer activity of QDE-2 is necessary for milRNA biogenesis. We previously showed that a mutation of QDE-2 in its DDH motif (D664A) completely abolished its slicer activity (Maiti et al., 2007). The production of milRNAs was

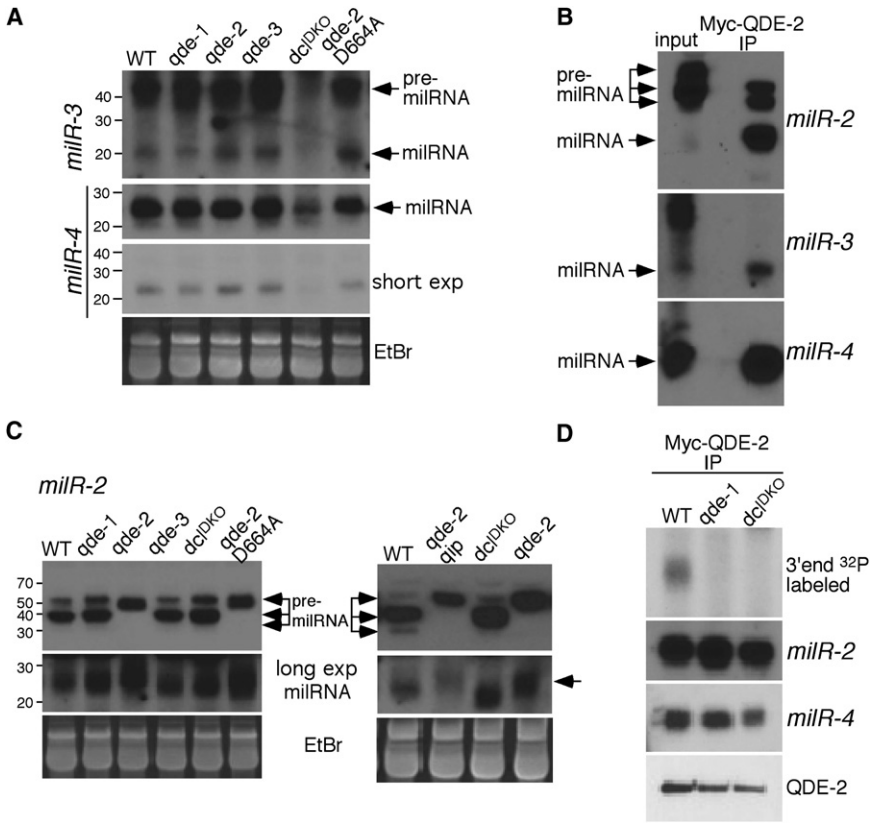

Figure 4. Distinct milRNA Biogenesis Mechanism for *milR-2*, *milR-3*, and *milR-4*

(A) Northern blot analyses of small RNA samples showing the production of pre-milRNAs and milRNA of *milR-3* and *milR-4* in different strains.

(B) Northern blot analyses of Myc-QDE-2-associated small RNAs. Myc-QDE-2 was immunoprecipitated with c-Myc antibody from the *qde-2*, Myc-QDE-2 strain.

(C) Northern blot analyses of small RNA samples showing that the production of pre-milRNAs and milRNA of *milR-2* requires QDE-2 but is independent of Dicers. The top and middle panels show the *milR-2* pre-milRNAs and milRNA, respectively. A longer exposure of the milRNA panels is shown. Gels for panels at right were subjected to a longer electrophoresis time to show size differences in milRNAs.

(D) Analyses of Myc-QDE-2-associated small RNAs in the wild-type, *qde-1*, and *dcl*^{DKO} strains. A construct that expresses Myc-QDE-2 was introduced into the indicated strains. In the top panel, the Myc-QDE-2-associated total small RNAs were 3′ end labeled with ^{32}P. In the middle and bottom panels, northern blot results of the Myc-QDE-2-associated small RNAs show that *milR-2* and *milR-4* milRNAs are associated with QDE-2 in the *dcl*^{DKO} strain.

reduced but, surprisingly, not abolished in the *qde-2* (D664A) mutant (Figure 3C), indicating that the slicer activity of QDE-2 is not required for milRNA generation. Since QDE-2(D664A) retains its ability to bind duplex sRNA (Maiti et al., 2007), this suggests that QDE-2 may bind to pre-milRNA and recruit another factor to processe the pre-milRNAs into mature milRNAs.

We previously showed that the exonuclease QIP interacts with QDE-2 and removes the nicked passenger strand of the siRNA duplex (Maiti et al., 2007). Thus, we examined the role of QIP in *milR-1* milRNA biogenesis. The mature milRNA was completely abolished in the *qip* mutant as in the *qde-2* mutant (Figure 3D). The function of QIP in milRNA processing requires its exonuclease activity, since expression of QIP protein with a point mutation of its catalytic residue (H504A) (Maiti et al., 2007) in the *qip* mutant did not rescue *milR-1* milRNA production (Figure 3D). In contrast, expression of the wild-type QIP fully restored the mature milRNA profile in the *qip* mutant. Since QDE-2 still can bind small RNA duplex in the *qip* mutant (Maiti et al., 2007), this result also suggests that the lack of mature *milR-1* milRNAs in the *qde-2* mutant is not due to the role of QDE-2 in stabilizing milRNA.

Immunoprecipiation assays showed that MycQDE-2 associated with the short pre-milRNA in the *qip* mutant but also bound two mature milRNAs in the wild-type strain (Figure 3E). These results indicate that the 33 nt pre-milRNA duplex is the precursor of the mature milRNAs, whereas the long pre-milRNA may be an intermediate or by-product of pri-milRNA processing.

Based on our results, we propose the following model for *milR-1* processing: (1) *milR-1* pri-milRNA is first processed by Dicer to generate double-stranded pre-milRNAs without the loop, and (2) QDE-2 binds to pre-milRNA and (3) recruits the exonuclease QIP to process the pre-milRNAs into mature milRNAs (Figure S1C).

Complete or Partial Dicer Dependence for *milR-3* and *milR-4* milRNA Biogenesis

We found that Dicer is also important for the generation of *milR-3 and milR-4* milRNAs (Figure 4A). In the *dcl*^{DKO} mutant, both the pre-milRNA and mature milRNA of *milR-3* were abolished. Although the level of the mature milRNA of *milR-4* was significantly reduced in the *dcl*^{DKO} mutant, surprisingly it was not eliminated. Unlike *milR-1*, QDE-2 is not involved in the generation of mature milRNAs for *milR-3* and *-4*. Furthermore, only the mature milRNAs of *milR-3* and *-4*, not their pre-milRNAs, were associated with QDE-2 (Figure 4B), consistent with their QDE-2-independent biogenesis. Thus, the biogenesis of *milR-3* milRNA resembles miRNA maturation in plants. In contrast, the production of *milR-4* milRNA is only partially dependent on Dicer, indicating involvement of another nuclease.

A Dicer-Independent, QDE-2-Dependent Mechanism for Biogenesis of *milR-2* milRNA

The *milR-2*-specific probe revealed pre-milRNA species of approximately 33, 39, and 52 nt and a mature milRNA of ~25 nt (Figure 4C). The signals for pre-milRNAs were much stronger than that of the milRNA. Surprisingly, the production of the pre-milRNAs and mature milRNA occurred in the *dcl*^{DKO} mutant,

indicating that the biogenesis of *milR-2* milRNA is Dicer independent. In contrast, QDE-2 and its catalytic activity were required for the production of both pre-milRNAs and milRNA, as shown by the disappearance of the two smaller species of pre-milRNAs and the accumulation of the 52 nt form in the *qde-2* and *qde-2*(D664A) mutants. An RNA band ~2 nt larger than the wild-type mature *milR-2* milRNA was observed in the *qde-2* mutants. Unlike *milR-1*, the generation of *milR-2* pre-milRNAs and mature milRNAs did not require QIP (data not shown). Furthermore, both its mature milRNA and the two shorter pre-milRNAs were associated with QDE-2 (Figure 4B). These results suggest that the *milR-2* pri-milRNA is processed into the pre-milRNAs by an unidentified nuclease(s). Afterwards, QDE-2 associates with the pre-milRNAs, and its slicer activity is involved in generation of mature milRNA.

Because Dicers are known to participate in the process of siRNA loading onto Argonaute proteins in other organisms (Liu et al., 2003), we examined whether the Dicer-independent milRNAs of *milR-2* and *milR-4* are loaded onto QDE-2 in a dicer-independent manner. We expressed Myc-QDE-2 in wild-type, *qde-1*, and dcl^{DKO} strains and immunoprecipitated Myc-QDE-2. The total levels of Myc-QDE-2-associated small RNAs (3′ end-labeled with ^{32}P) were significantly reduced in the *qde-1* and dcl^{DKO} mutants (Figure 4D, top panel), showing that most of the small RNAs produced in *Neurospora* are QDE-1 and Dicer dependent. Interestingly, both the mature milRNAs of *milR-2* and *milR-4* were found to be associated with Myc-QDE-2 in the *qde-1* and dcl^{DKO} mutants (Figure 4D, lower panels), indicating that loading of these milRNAs onto QDE-2 does not require Dicer.

These results further establish the existence of Dicer-independent processing of small RNAs in *Neurospora*. Since QIP is known to facilitate the formation of single-stranded siRNA-containing active RISC complex by removing the passenger strand, we tested whether QIP is also responsible for miRNA RISC maturation (Maiti et al., 2007). QDE-2-associated mature *milR-4* remained single stranded in the *qip* mutant (Figure S2A), suggesting a *qip*-independent mechanism for milRNA RISC activation. This result indicates that the activations of siRNA and milRNA RISC are mechanistically distinct processes in *Neurospora*. Recent reports also suggested that the activation mechanisms of siRNA and miRNA RISC are different (Kawamata et al., 2009; Liu et al., 2009; Maiti et al., 2007).

Because the QDE-2-associated small RNAs were cloned after CIP and PNK treatments, we examined the 5′ phosphate polarity of milRNAs. As shown in Figure S2B, most of the milRNAs could be ligated to a 22 nt 5′ adaptor, indicating that they contain 5′ monophosphate.

Taken together, our results reveal the existence of miRNA-like genes, or *milRs*, in *Neurospora*. Importantly, these four milRNAs and their precursors are produced by four distinct mechanisms, uncovering surprisingly diverse pathways for small RNA generation in this filamentous fungus.

milRs Mediate Gene Silencing in *Neurospora*

For most candidate *milR* loci, the corresponding milRNAs and their predicted secondary structures are conserved in two other *Neurospora* species: *N. discreta* and *N. tetrasperma* (data not shown). For *milR-1*, the sequences of both the milRNA and milRNA* strands are highly conserved, but nucleotides in the loop region between them are not (Figure S3A), suggesting functional importance of *milR-1* in the genus *Neurospora*.

To determine whether *Neurospora* miRNAs can mediate gene silencing, we created a Myc-tagged reporter construct carrying in-frame five *milR-1* milRNA complementary sequences in the middle of the open reading frame. This construct was introduced into the wild-type and *qde-2* mutant strains. Protein levels of the reporter were ~20-fold higher in the *qde-2* mutants than in the wild-type strains (Figure 5A), indicating that *milR-1* expression results in robust silencing of its complementary target. Quantitative RT-PCR analysis showed that the levels of the reporter mRNA in the *qde-2* mutant was only modestly higher than that in the wild-type strain (Figure 5B), suggesting that *milR-1* milRNA may mostly mediate gene silencing by repression of translation.

No perfect matches for any of the major *milR* miRNA sequences were found elsewhere in the *N. crassa* genome, which suggests that, like animal miRNAs, milRNAs may target imperfectly complementary sequences. We therefore predicted putative milRNA targets using miRmate, a mammalian miRNA target prediction program (Du et al., 2009; Younger et al., 2009). The mRNA levels of several predicted targets (Figure 5C) are upregulated in the dcl^{DKO} mutant (Figure 5D), suggesting they are regulated by milRNAs. In addition, the mRNA levels of these predicted targets were also elevated in the *qde-2* mutant (Figure S3).

To demonstrate the specificity of miRNA action on these predicted target genes, we generated a *milR-1* knockout strain ($milR\text{-}1^{KO}$) in which the production of *milR-1* milRNAs was eliminated (Figure S3B). As expected, the mRNA levels of NCU07311 and NCU09469, two predicted *milR-1* target genes, were elevated in the $milR\text{-}1^{KO}$ and *qde-2* strains (Figure S3C). In contrast, although the mRNA level of NCU05770, a predicted *milR-4* target, was upregulated in the *qde-2* strain, it did not change in the $milR\text{-}1^{KO}$ strain. These results suggest that the *milR-1* milRNA downregulates the expression of its predicted target genes.

To further test if those mRNA are targets of milRNAs, Myc-QDE-2 was immunoprecipitated and levels of the associated RNAs were measured. Specific association between QDE-2 and these predicted miRNA targets was observed, whereas QDE-2 did not bind to several control RNAs specifically (Figure 5E). Among these genes, NCU05770 encodes a peroxidase, NCU09469 contains an EF hand and an ubiquitin binding domain, and both NCU07311 and NCU03313 are fungal-specific genes. Except for NCU05770, which has the milRNA target site in its 3′UTR, the other target genes have the milRNA target sites located within their ORFs. Taken together, our results show that *milRs* regulates gene expression in *Neurospora*.

MRPL3, an RNase III Domain-Containing Protein, Is Involved in Dicer-Independent Processing of Some milRNAs

The evidence of Dicer-independent mechanisms for milRNA generation prompted us to look for Dicer-independent nuclease activity. Because of the similarities between milRNAs and conventional miRNAs, we carried out pre-miRNA processing assays

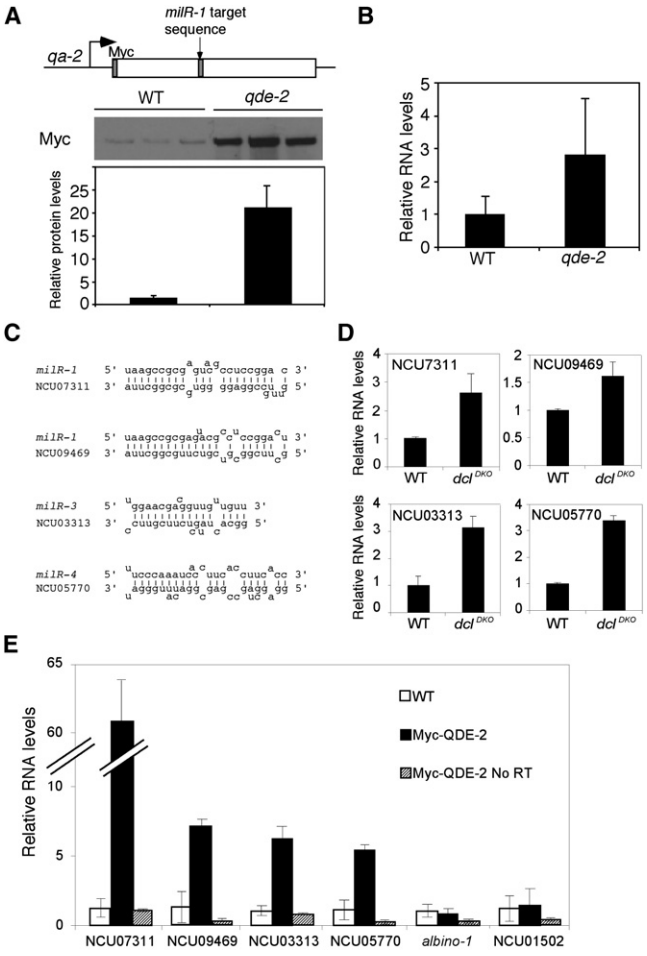

Figure 5. *milRs* Mediate Gene Silencing in *Neurospora*

(A) *milR-1* milRNA mediates gene silencing of a Myc-tagged reporter gene with *milR-1* target sites. The top diagram shows the construct that carries the *milR-1* target sequences in the ORF. The middle and bottom panels show western blot results with c-Myc antibody. Three independent transformants of the wild-type and *qde-2* strains were used. Error bars indicate standard deviations in this figure.

(B) qRT-PCR results showing the mRNA levels of the reporter construct in wild-type and *qde-2* strains. The error bars indicate standard deviations from independent experiments.

(C) Base pairing between *milRs* and their predicted targets.

(D) qRT-PCR results showing the mRNA levels of predicted milRNA targets in the wild-type and dcl^{DKO} mutant strains.

(E) qRT-PCR analysis of MycQDE-2-immunoprecipitated RNAs showing the association of Myc-QDE-2 with predicted *milR* targets. Myc-QDE-2 was immunoprecipitated with c-Myc antibody from the Myc-QDE-2-expressing strain. A wild-type strain was used as a control.

using *Neurospora* extracts and the *Drosophila let-7* pre-miRNA (Jiang et al., 2005). *Neurospora* extracts from both the wild-type and dcl^{DKO} strains exhibited similar miRNA processing activity, indicating Dicer-independent activity (Figure 6A). Interestingly, the size of the processed *let-7* miRNA in the *Neurospora* extracts was ~1–2 nt larger than that produced by the recombinant *Drosophila* Dicer-1/Loqs complex (Jiang et al., 2005). In contrast, it was previously shown that the *Neurospora* Dicers are essential for generating siRNA from dsRNA in vitro and in vivo (Catalanotto et al., 2004).

In an attempt to identify the protein responsible for the observed activity, we searched for putative RNase III domain-containing proteins. In addition to the two Dicers, one protein (NCU08299) homologous to the yeast mitochondrial ribosomal protein MRPL3 possesses a putative RNase III domain and a dsRNA recognition motif (Figure 6B). Although MRPL3 homologs were identified as part of the mitochondrial ribosomal protein large subunit (Smits et al., 2007), their function is unknown. MRPL3 homologs are found in most eukaryotic organisms; MRPL44 is the human homolog. The RNase III domains of MRPL3 proteins show little sequence similarity to those of Dicers and Drosha, suggesting that they belong to different families of RNase III domain proteins. *mrpl3* is apparently an essential gene in *Neurospora*, since no homokaryotic deletion strain could be obtained, but a heterokaryotic strain that carries a mixture of wild-type and $mrpl3^{KO}$ nuclei showed reduced *mrpl3* mRNA level (Figure 6C).

Examination of the levels of milRNA by northern blot analyses revealed that the levels of the major mature *milR-1* milRNA and the 33 nt pre-milRNAs were markedly reduced in the $mrpl3^{KO(het)}$ strain (Figure 6D), suggesting that MRPL3 may collaborate with Dicer to generate *milR-1* pre-milRNAs and milRNAs. For *milR-4*, whose milRNA production is partially dependent on Dicer, the mature milRNA in the *mrpl3* strain was also reduced to a level similar to that in the dcl^{DKO} strain. However, the levels of *milR-2* and *milR-3* milRNAs and pre-milRNAs were not altered in the *mrpl3* mutant compared to wild-type (data not shown). In addition, milRNA levels for *milR-1* and *milR-4* were also reduced in a strain (ds*mrpl3*) in which *mrpl3* is silenced by dsRNA (Figure 6E). Furthermore, pre-*let-7* processing activity was reduced in the ds*mrpl3* strain in vitro, as indicated by the decreased level of *let-7* and increased level of pre-*let-7* (Figure 6F). Finally, the pre-*milR-4* processing activity was also reduced in the ds*mrpl3* strain in vitro (Figure 6G). Together, these results suggest that MRPL3 may be important for the Dicer-independent biogenesis of some *Neurospora* milRNAs.

Dicer-Independent Small Interfering RNAs

A major group of *Neurospora* small RNAs are comprised of small RNAs matched symmetrically to both strands of DNA. We have previously shown that the DNA damage-induced qiRNAs originating from both strands of rDNA are dependent on QDE-1, QDE-3, and Dicers. In addition to the rDNA repeats, we identified 50 small RNA-enriched loci in which nearly equal amounts of small RNAs matched both DNA strands (Table S3). Unlike rDNA, these loci are not repetitive; rather, they include genes and intergenic regions and have no apparent sequence motifs. Small RNAs resulting from these loci averaged 22 nt in length, with a strong 5′U preference (94%) (Figures 7A and 7B). The small RNAs from these loci are not induced by DNA damage (data not shown), indicating they are not produced by the same mechanism as qiRNAs. We named these regions *disiRNA* loci because their characteristics are distinct from those of all known types of small RNAs (see below).

Notably, disiRNAs appear to originate from loci that produce overlapping sense and antisense transcripts. Figures 7C and

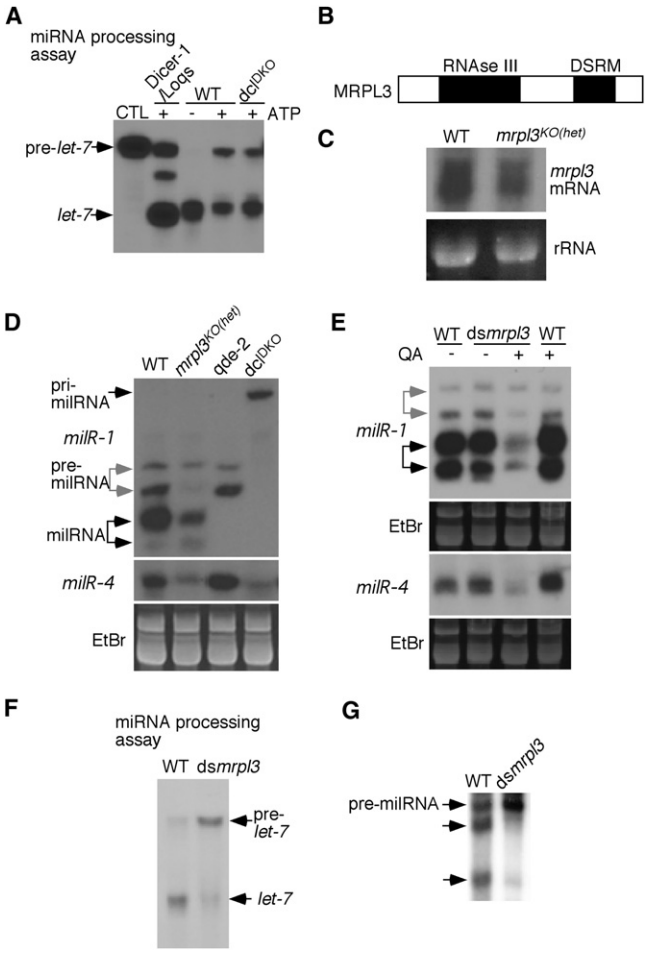

Figure 6. MRPL3 Is Important for the Biogenesis of *milR-1* and *milR-4* milRNAs

(A) miRNA processing assay using *Drosophila* pre-*let-7* shows the in vitro miRNA processing activity in the wild-type and dcl^{DKO} extracts.

(B) A diagram showing the domain structure of MRPL3.

(C) Northern blot analysis showing the reduced level of *mrpl3* mRNA in the $mrpl3^{KO(het)}$ strain.

(D and E) Northern blot analyses showing the levels of milRNAs in the indicated strains. ds*mrpl3* is a strain in which the expression of *mrpl3*-specific dsRNA is induced by QA.

(F) miRNA processing assay showing the reduced level of in vitro pre-*let7* processing activity in the ds*mrpl3* strain.

(G) In vitro processing assay for *milR-4* pre-milRNA. Universally labeled *milR-4* pre-milRNA was incubated with extracts of the indicated strains. The positions of the pre-milRNA and two processed products are labeled with arrows.

7D show the chromosomal distribution of small RNAs originating from three *disiRNA* loci. *disiRNA-6* is one of the most abundant *disiRNA* loci, with close to 7000 overlapping sense and antisense disiRNAs covering a 15 kb region. This region contains two predicted *Neurospora* genes, NCU00911 and NCU00910. The majority of the small RNAs are located between NCU00911 and NCU00912. The overlapping sense and antisense disiRNAs arising from this locus suggest that disiRNAs is derived from dsRNA. Consistent with this notion, overlapping sense and antisense ESTs (red arrows) were found downstream of NCU00911.

The *qde-2* gene is one of the *disiRNA* loci (*disiRNA-36*); small RNAs cover the entire gene, although much lower levels of small RNAs corresponding to the neighboring gene were also found (Figure 7D). Several ESTs antisense to *qde-2* have been found in the *Neurospora* genome database.

The distribution of small RNAs in the *disiRNA-37* locus peaks between two convergently transcribed genes in close proximity. As several overlapping sense and antisense ESTs were identified, there may be a role for convergent transcription in disiRNA production. A survey of the available EST sequences complementary to the 50 *disiRNA* loci showed that out of the 44 loci for which ESTs were found, 32 have overlapping sense and antisense ESTs, and another 3 are likely to have overlapping sense and antisense ESTs. Thus, based on the limited EST data available, nearly 80% of the *disiRNA* loci produce overlapping sense and antisense transcripts. These results suggest that disiRNAs are likely derived from dsRNA made from naturally occurring complementary sense and antisense transcripts.

We examined expression of disiRNAs from seven loci in different RNAi mutant strains (Figure 7E). Unlike qiRNAs, disiRNA biogenesis is independent of QDE-1, QDE-2, and QDE-3. Surprisingly, despite evidence that disiRNAs are derived from dsRNA, their levels were not affected in the dcl^{DKO} mutant, indicating that Dicers are not involved in generating disiRNAs. In addition, we found that disiRNA levels were not altered in a strain that has both *Neurospora* Argonaute genes deleted (*qde-2 sms-2*) or in other known RNAi mutants or *mrpl3* mutants (Figure 7E). These results indicate that, unlike animal piRNAs, an Argonaute-dependent maturation mechanism is not involved in disiRNA production. It was recently shown that in some budding yeast species, a second RNT1 (contains RNase III domain similar to Dicer)-like protein acts as the noncanonical Dicer protein to generate siRNA from long hairpin RNAs (Drinnenberg et al., 2009). However, *Neurospora* has only one gene encoding for an RNT1 homolog, and it is essential for cell survival. In addition, its knockdown by dsRNA did not affect disiRNA production (data not shown). Together, these results indicate that the biogenesis of disiRNAs does not require known RNAi components and suggest another Dicer-independent small RNA biogenesis pathway.

DISCUSSION

In this study, we conducted a genome-wide survey of Argonaute-bound small RNA population from *Neurospora* and identified two groups of small RNAs, milRNAs and disiRNAs. Our discovery of *milRs* in *Neurospora* demonstrates that miRNA and milRNAs exist in all major branches of eukaryotic organisms, and our functional studies of *milRs* suggest that miRNA-like gene-regulatory mechanisms evolved early in the eukaryotic lineage. In addition, our demonstration of Dicer-independent biogenesis of some *Neurospora milRs* and disiRNAs suggests that other dsRNA-specific nucleases, e.g., other putative RNase III enzymes, participate in the biogenesis of milRNAs. This raises the possibility that milRNAs may be produced in eubacteria and archea, which lack homologs of Dicer, but encode Argonaute-like proteins (Song et al., 2004).

Neurospora lacks sequence homologs of Drosha and Pasha, which generate pre-miRNAs from pri-miRNAs in animals.

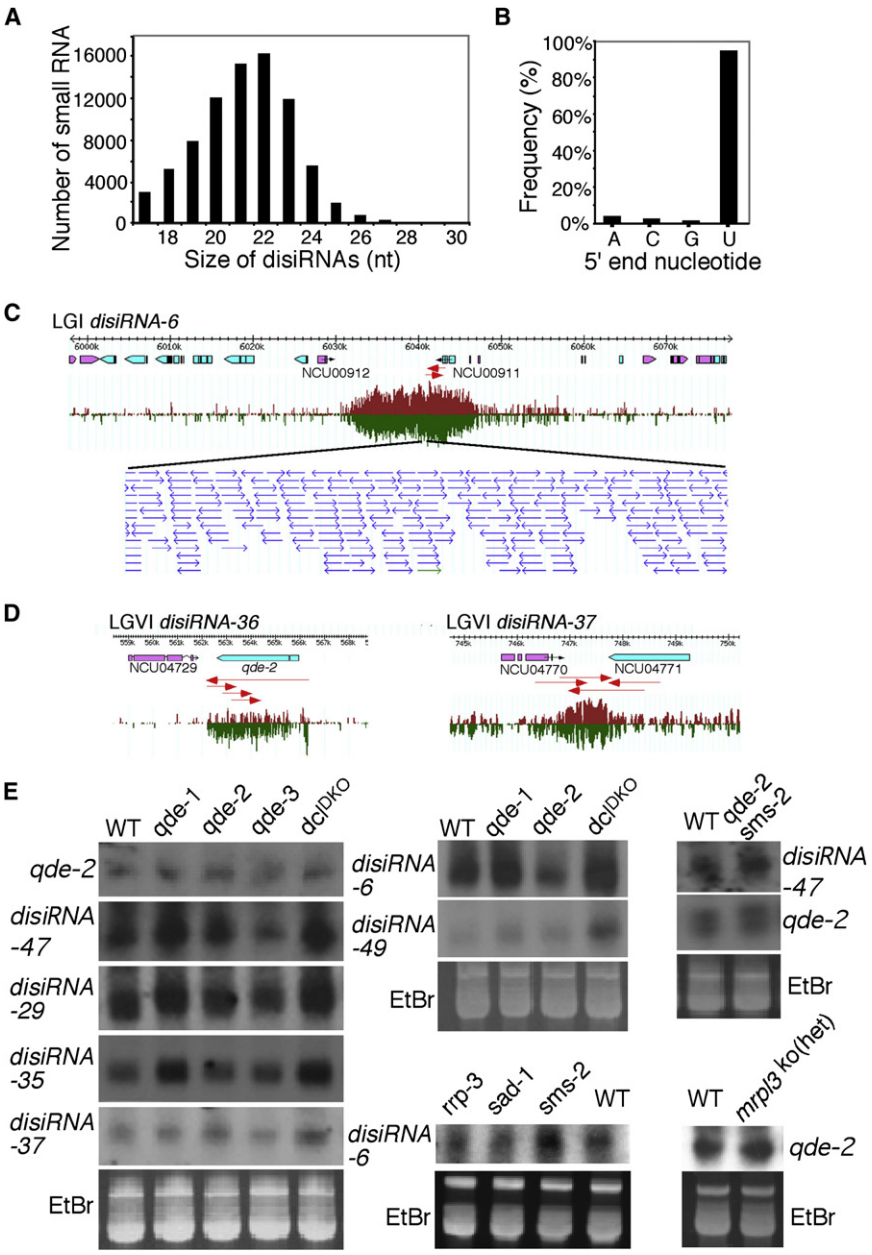

Figure 7. Characterization of disiRNAs

(A) Size distribution of disiRNAs.

(B) Nucleotide frequency of 5′ end of disiRNAs.

(C) Distribution of small RNAs at the *disiRNA-6* locus. The middle panel shows the small RNA distribution matched to either Watson (red) or Crick (green) strands of DNA. Numbers of small RNAs (logarithmic scale) in 10 nt sliding windows are shown. The bottom panel shows the small RNA hits of both directions in a small representative region of the locus. The red arrows indicate the overlapping sense and antisense ESTs identified.

(D) Chromosomal distribution of small RNAs at the *disiRNA-36* and *disiRNA-37* loci. The red arrows indicate the identified sense and antisense ESTs of *qde-2* and overlapping convergent ESTs in the *disiRNA-37* locus.

(E) Northern blot analyses of small RNA samples showing the levels of different disiRNAs in the indicated strains.

Despite some differences between the *Neurospora* milRNAs and miRNAs in animal and plants in their biogenesis mechanisms, they share remarkable similarities. First, as miRNAs, the *Neurospora* milRNAs arise from highly specific stem-loop RNA precursors. Second, most of the milRNAs examined in this study require Dicer for their production. Third, like the animal miRNAs, milRNAs appear to silence endogenous targets with mismatches.

A major finding of this study is the discovery of diverse pathways for small RNA biogenesis. Our examination of four *milRs* uncovered surprisingly diverse mechanisms for milRNA production: only the biogenesis of *milR-3* milRNAs resembles that in plants, which require only Dicers for pre-milRNA and milRNA processing (Jones-Rhoades et al., 2006). For *milR-1*, the production milRNAs requires Dicer, QDE-2, QIP, and MRPL3. The production of *milR-4* milRNAs is partially Dicer dependent and requires MRPL3. On the other hand, the biogenesis of *milR-2* milRNA requires QDE-2 and its catalytic activity but is completely independent of Dicer. The mechanistic diversity observed for milRNA biogenesis in *Neurospora* stands in stark contrast with the well-defined miRNA biogenesis pathways in animals and plants and suggests that eukaryotic small RNAs' generation mechanisms are more diverse than previously thought.

BLAST searches did not identify highly homologous *milR* genes outside the *Neurospora* genus, suggesting individual *milRs* have evolved independently from plant and animal miRNAs. However, mechanisms similar to those used in *Neurospora* milRNA production may be involved in the biogenesis of some types of small RNAs in animals and plants. For example, Argonaute-dependent maturation of *milR-1* and *-2* milRNAs is similar to that of animal piRNAs. In addition, we identified a role of Argonaute in small RNA biogenesis, where QDE-2 functions as an adaptor through binding to pre-milRNA and recruiting its associated QIP exonuclease to produce mature *milR-1*. Maturation of animal piRNAs may also involve recruitment of a nuclease to trim the 3′ ends of the Piwi-associated piRNA precursors (Ghildiyal and Zamore, 2009).

We identified the putative ribonuclease MRPL3, which has an RNase III domain, as an important component in milRNA biogenesis. Although the exact biochemical function of MRPL3

in milRNAs' biogenesis is not known, its predicted RNase III domain suggests that it may function as a hairpin-specific RNase that processes pri- or pre-milRNAs. The eukaryotic homologs of *Neurospora* MRPL3 have unknown functions but are present in the large subunit of the mitochondrial ribosomes. Thus, our results suggest a potential link between mitochondrial ribosomal function and milRNA biogenesis.

Our discovery of QDE-2-associated disiRNAs in *Neurospora* demonstrates that Dicer-independent small RNAs exist in the fungal kingdom. The different characteristics of *Neurospora* disiRNAs and animal piRNAs suggest that they are two distinct classes of small RNAs. First, disiRNAs originate from nonrepetitive parts of the genome and are matched to overlapping sense and antisense strands of DNA. Second, most of the disiRNA-producing loci yield overlapping sense and antisense transcripts, suggesting that disiRNAs arise from dsRNA templates. In this aspect, disiRNAs are similar to endo-siRNAs and natsiRNAs in animals and plants (Ghildiyal and Zamore, 2009). Since Dicers are essential for the generation of siRNA from dsRNA made from long hairpin RNA transcripts in *Neurospora* (Catalanotto et al., 2004; Choudhary et al., 2007), it is likely that dsRNA formed by sense and antisense transcripts can be recognized by an unidentified ribonuclease in *Neurospora*. Third, unlike the animal piRNAs, the biogenesis of disiRNAs does not require the Argonaute proteins. Furthermore, their independence of QDE-1 or other RDRPs suggests that disiRNAs are not secondary small RNAs amplified by RdRPs. Taken together, these results indicate that although disiRNAs share similarities to piRNAs and endo-siRNAs, they are produced by a different small RNA biogenesis pathway. Since the submission of this manuscript, a class of Dicer-independent primal RNAs was reported to contribute heterochromatin formation in *S. pombe* (Halic and Moazed, 2010).

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions

The wild-type strain used in this study was FGSC 4200(a). Mutant strains including *qde-1*, *qde-2*, *qde-2* (D664A), *qde-3*, *qip*, and a *dcl-1 dcl-2* double mutant (dcl^{DKO}) were generated previously (Choudhary et al., 2007; Maiti et al., 2007). Myc-tagged protein-expressing strains, including Myc-QDE-2, Myc-QIP, and Myc-QIP (H504A) strains, were also created previously (Maiti et al., 2007). The homokaryotic $milR\text{-}1^{KO}$ strain was generated by replacing the entire *milR-1* pri-milRNA with a *hph* resistance cassette by homologous recombination. $mrpl\text{-}3^{KO}$ (heterokaryon, FGSC#18364) was obtained from the Fungal Genetic Stock Center. The ds*mrpl3* strain was created by transforming a wild-type strain with a plasmid that can inducibly express an inverted repeat specific for *mrpl3* ORF (Cheng et al., 2005). Liquid cultures were grown in minimal medium (1× Vogel's, 2% glucose) at room temperature. 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.

Purification and Cloning of QDE-2-Associated sRNAs

Immunopurification of Myc-QDE-2 ribonucleoprotein complex was performed as previously described (Lee et al., 2009). Small RNAs from the QDE-2 IP were purified on 15% PAGE/7 M urea gels using 18–26 nt synthetic RNA markers, followed by overnight elution with elution buffer containing 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 0.3 M NaCl and precipitation with 20 μg glycogen and 1 volume of isopropanol. RNAs were incubated with 1 U/μl calf intestinal phosphatase (NEB) and 1 U/μl SUPERase•In in buffer 3 (NEB) at 37°C for 1 hr, extracted twice with phenol, and precipitated with ethanol. RNA was incubated with 10 μM of preadenylated DNA oligo (AppCTGTAGGCACCATCAAT/ddC/), 1 unit/μl of SUPERase•In, 10% DMSO, and 2 unit/μl T4 RNA ligase (Takara Bio Inc.) in 10 μl ligation buffer (50mM Tris-Cl [pH 7.5], 10 mM $MgCl_2$, 0.06 μg/μl BSA, 10 mM DTT). The 3′-ligated RNAs were phosphorylated with 1 unit/μl polynucleotide kinase in buffer 3 containing 1 unit/μl SUPERase•In and 2 mM ATP at 37°C for 1 hr, extracted with phenol, precipitated with ethanol, and 5′ ligated with 2 unit/μl T4 RNA ligase and 30 μM 5′ linker GTTCTACAGTCCGACGAT C in 10 μl 1× buffer containing 1 unit/μl SUPERase•In, 0.1 μg/μl BSA, and 10% DMSO. cDNA was synthesized using SuperScript III with RT oligo ATTGATGGTGCCTACAG. The cDNA was PCR amplified with oligos containing Illumina/Solexa linkers.

Small RNA Analyses

All small RNA analyses were performed with custom Perl (5.8.6) scripts on a chromosome genome assembly based on *N. crassa* assembly 7 (Lewis et al., 2009) (available upon request from M.F.), rDNA sequence (Lee et al., 2009), and Version 3 gene predictions for the *N. crassa* assembly 7 release including mitochondria sequence (Broad Institute). Annotations for genes, structural RNAs, and repeats in Version 3 gene predictions were mapped and converted to sequence coordinates in the chromosome genome assembly. Solexa reads containing a perfect match to the first 6 nt of the 3′ linker were used to extract the inserted sequences. Inserts 17–30 nt long were used to identify perfect matches to the genome. To calculate small RNA reads derived from a single genomic locus, the small RNA reads were normalized using its total number of matched genomic loci (repeat-normalized reads). The density of small RNA was presented in two ways: using a nonoverlapping 10 nt sliding window along the Watson or Crick strand of each chromosome (Figure 2C), and using a 10,000 nt window moving 1000 nt each step along the Watson or Crick strand of each chromosome (Figure 1D).

Identification of *milRs* and *disiRNA* Loci

To identify *milRs* and disiRNAs, structural RNAs such as tRNA or rRNA were first excluded. The following criteria were used to identify *milR* loci: (1) Small RNAs were highly enriched at the loci for a density over 240 reads/kbp, which resides in the top 0.3% most-enriched loci of the genome. The median loci in the genome contain ~6 hits/kbp. (2) Small RNAs were predominantly derived from one strand (at least a 10-fold difference between two DNA strands). (3) Small RNAs form a stem-loop structure (hairpin) with flanking sequences, and the most abundant species of the small RNA resides in the stem region. Secondary structures of small RNAs and their flanking sequences (up to 200 nt) were examined by mfold.

disiRNA loci were identified as regions in which small RNAs are derived from both strands with similar abundance and the small RNA density is significantly than the background (>70 hits/kb, which consists of the top 1% loci in the genome).

RACE and Sequencing of Pre-*milR-1* and Pri-*milR-1*

Small RNAs with sizes of 30–60 nt and 100–200 nt were fractioned from wild-type and $dicer^{DKO}$ mutant samples, respectively, using the MirVana kit followed by gel purification. The RNAs were cloned and the cDNA libraries were made without CIP and PNK treatments. To identify the 5′ and 3′ ends of pre-*milR-1* and pri-*milR-1*, the intermediates were amplified using a *milR-1*-specific primer paired with one of the primers corresponding to the 5′ or 3′ linker sequence. Since PsiI and BspEI sites partially overlapped with the 5′ and 3′ ends of mature milRNA, respectively, the specific *milR-1*-amplified products are identified by their sensitivity to PsiI or BsPE1 digestions and were subjected to DNA sequencing. At least three amplified products of each species were sequenced.

Northern Blot Analyses and qPCR Assay

Small RNA (12.5–25 μg) was separated on a 16% denaturing polyacrylamide gel Urea and transferred onto a Hybond-NX membrane (GE Healthcare). An RNA ladder (Ambion) was used as a size marker. Crosslinking of RNA to Hybond-NX was performed using a carbodiimide-mediated crosslinking method at 60°C for 2 hr as described (Pall et al., 2007), followed by baking

at 80°C for 2 hr. Hybridization was performed according to the manufacturer's instructions in ULTRAhyb-Oligo hybridization buffer (Ambion) for StareFire (IDT)-labeled DNA probes, or in ULTRAhyb hybridization buffer (Ambion) for T7-transcribed riboprobes. The Starfire probe sequences and primer sequences for PCR fragments used in T7 transcribed riboprobes was described in the Supplemental Experimental Procedures. Northern blot and qRT-PCR analyses using total RNA were performed as previously described (Cheng et al., 2001; Choudhary et al., 2007).

Strains that Express the *milR-1*-Targeting Reporter Protein

To obtain the fragment containing five *milR-1* milRNA sequences, we designed two complementary primers with BanI sites at both 5′ and 3′ ends (5′-ggtgccATAAGCCGCGAGTACGCCTCCGGACTGTAAggtgcc-3′; 5′-ggcaccTTACAGTCCGGAGGCGTACTCGCGGCTTATggcacc-3′). The two primers were annealed, digested with BanI enzyme, and self-ligated to create concatamers. The concatamers were gel purified and cloned into TOPO-pCRII (Invitrogen). Sequencing validated TOPO-pCRII_milr-1 with five *milR-1* miRNA complementary sequences, which was cloned into the EcoRI site of qaMyc-QDE-2, resulting in the insertion of five *milR-1* miRNA-targeting sequences within the Myc-QDE-2 ORF. The insertion localizes in the Piwi domain and should result in a nonfunctional protein. The reporter construct was introduced to the wild-type strain and the *qde-2*^{rip} strain at the *his-3* locus. The expression of the reporter is induced by the presence of quinic acid.

Target Prediction for *milRs*

Neurospora genes and the regions 1 kb of the 3′ ends were used for the search of *milR* targets by miRmate, a mammalian miRNA target prediction algorithm (Du et al., 2009; Younger et al., 2009). miRmate considers complementarity (allowing the G-U base pairing) based on variable weighting of positions across the miRNA, so as to encourage (but not require) complete complementarity at the seed region (positions 2–7 of the miRNA) and mismatches or insertions in the central bulge at positions 9–11 of the miRNA (Lewis et al., 2005).

Pre-miRNA Cleavage Assay

A 61 nt synthetic *Drosophila* pre-let-7 RNA, 5′-UGAGGUAGUAGGUUGUAUAGU AGUAAUUACACAUCAUACUAUACAAUGUGCUAGCUUUCUU-3′, was kindly provided by Dr. Qinghua Liu and labeled at the 5′ end by PNK. For pre-milRNA of *milR-4*, the predicted hairpin sequence was PCR amplified and T7 transcribed in the presence of ^{32}P-UTP. Labeled pre-RNA was gel purified before it was applied in the in vitro pre-milRNA processing assays as previously described (Jiang et al., 2005). Cell extracts (30 μg total protein) from different strains were used in the assays.

ACCESSION NUMBERS

The NCBI accession number for the deep sequencing data reported in this study is GSE21175.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, and Supplemental Experimental Procedures and can be found with this article at doi:10.1016/j.molcel.2010.04.005.

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