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RNA interference Pathways in Filamentous Fungi

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Abstract

RNA interference is a conserved eukaryotic homology-dependent post-transcriptional gene silencing mechanism. The filamentous fungus *Neurospora crassa* is one of the first organisms used for RNAi studies. Quelling and Meiotic Silencing by Unpaired DNA (MSUD) are two RNAi related phenomena discovered in *Neurospora* and their characterizations have contributed significantly to our understanding of RNAi mechanisms in eukaryotes. More recently, a type of DNA damage-induced small RNA, microRNA-like small RNAs and Dicer-independent small silencing RNAs have been discovered in *Neurospora crassa* which can regulate gene expression. In addition, there are at least six different pathways responsible for the production of these small RNAs, indicating that this fungus is an important model system to study small RNA function and biogenesis. The RNAi studies in other filamentous fungi such as *Cryphonectria paracitica* and *Aspergillus* provide evidences that RNAi plays an important role in antiviral defense and RNAi mechanism is widely conserved in filamentous fungi, and RNAi has been commonly used as an efficient tool for studying the gene function. The discovery of the endogenous small RNAs from *M. circinelloides* further indicates the richness and complex of the RNAi field in eukaryotes.

Keywords

RNAi; quelling; meiotic silencing; microRNA; dicer-independent small RNAs; siRNA; qiRNA

Introduction

RNA interference (RNAi) is a conserved gene silencing mechanism at both post-transcriptional and transcriptional levels in eukaryotes, mediated by small silencing RNAs (sRNAs) which are small noncoding RNA molecules with various length of about 20-30 nucleotides [1-4]. RNAi includes such early phenomenal observations as co-suppression or post-transcriptional gene silencing (PTGS) in plants (1990), quelling in fungi (1992) and RNAi in animals (1998) [5-8]. In general, in the RNAi pathway, RNase III protein Dicer-like enzymes generate the small RNA duplexes from double-stranded RNA (dsRNA) precursors, then the small RNA duplexes are loaded onto the RNA-induced silencing complex (RISC) with an Argonaute family protein as the core catalytic component. Following the removal of the passenger strand of the small RNA duplex, the RISC is activated and uses the remaining strand as the guide to silence the targets [9-15]. The well

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known sRNAs functioning in RNAi pathways include at least the dicer-dependent microRNAs (miRNAs) and small interfering RNAs(siRNAs) and the dicer-independent small RNAs such as PIWI-interacting RNAs (piRNAs) (See reviews in [3-4,11,16-19]. miRNAs are processed from single-stranded RNA precursor transcripts containing hairpin structures, and can function through post-transcriptional gene silencing by mediating mRNA degradation or translational repression, or through transcriptional gene silencing involving DNA methylation or chromatin modifications. siRNAs are generated from dsRNA procursors, can act through post-transcriptional gene silencing pathways and transcriptional gene silencing pathways. piRNAs, found till now only in animals, derive mainly from repetitive elements, transposons and large piRNA clusters by matching only one DNA strand, and may function to protect germline integrity by targeting repetitive DNA and transposons involving in post-transcriptional gene silencing and transcriptional gene silencing pathways, though the functions of many piRNAs are still not known (See reviews in [3-4,11,16-19].

The filamentous fungus *Neurospora crassa* is one of the first eukaryotic model system for RNAi studies, and RNAi pathways in filamentous fungi have been mainly discovered from *Neurospora crassa*. As an excellent experimental model for more than 70 years, *Neurospora crassa* is in fact also a leading organism in RNAi studies, and studies on this simple fungus has been contributing greatly to the understanding of RNAi. Filamentous fungi include many important human and agricultural pathogens, antibiotics and other important fungal chemical producing groups, food producers such as mushrooms, symbiotics such as mycorrhizal fungi, nutrient recyclers and so on, which greatly influence our daily life. RNAi is widely present in filamentous fungi, and studies and applications of RNAi in filamentous fungi have not only been leading us to better understand how genes in these organisms function, but also been contributing significantly to find out more novel RNAi mechanisms. This review will focus on the discoveries and studies of the RNAi in *Neurospora*, then discuss the studies and the applications of RNAi in some other filamentous fungi.

Quelling/PTGS (or: RNAi in vegetative cells in *Neurospora crassa*)

Discovery of quelling

Quelling was discovered in *Neurospora crassa* by Macino group in 1992, the first transgene-induced gene silencing phenomenon described in fungi [6]. It is the second post-transcriptional gene silencing mechanism ever reported in eukaryotes, second to “co-suppression” in plants and followed by “RNAi” in animals [7]. Quelling was originally found by transforming exogenous *albino-1* (*al-1*) or *albino-3* (*al-3*) sequences into a typical orange wild type strain, which caused severe down-regulation of the endogenous *al-1* or *al-3* genes, two of the structural genes for biosynthesis of carotenoids, resulting in the albino (white) phenotype in up to 36% of the transformants [6,20-21]. This phenomenon is reversible spontaneously and progressively, as some of the albino transformants could revert back to wild type or intermediate phenotypes over a prolonged time of culture or after some subcultures, which was found to be correlated with the reduction of the copy number of the exogenous sequences integrated and accompanied by the increased steady-state mRNA levels of the corresponding endogenous genes [6]. Though quelled strains could contain only

1 to 2 ectopic integrated sequences of transgenes, both high copy number and a tandem arrangement of the transgenes seem to correlate with the successful triggering of quelling or more stable quelling [6,20-23]. It is interesting to notice that once quelling is relieved it cannot be triggered again in the reverted strains even in the presence of the ectopic sequences [6].

Quelling silences both the transgenes and the homologous endogenous genes in vegetative growth cells, with the minimum length requirement of the homologous transcribed region of 132 nt for sufficient quelling induction, though the promoter region itself is not required for and cannot induce quelling [6,20-21]. The silencing effect of quelling is posttranscriptional as the steady-state levels of the primary transcript (the precursor RNA) in quelled and non-quelled strains were unchanged though the stead-state levels of the mature mRNA of the silenced gene were reduced significantly in quelled strains [20].

al-1 mutation is generally recessive, i.e., albino phenotype should be generally observed in homokaryotic *al-1* mutants but not in heterokaryotic *al-1* mutants. However, many albino (quelled) transformants induced by trangenic *al-1* were heterokaryons, and about 95% of the forced heterokaryons created from a homokaryon of the wild type *al-1* and an albino homokaryotic transformant induced (quelled) by exogenous *al-1* were albino [20]. These results demonstrate that silencing of *al-1* by quelling is dominant in heterokaryons and is not nucleus-limited thus can act *in-trans*, indicating that quelling does not require ectopic pairing or DNA-DNA interactions and quelling is mediated by a diffusible *trans*-acting molecule [20]. This could be the first indication of the involvement of small RNAs in RNAi.

A transgene-derived sense RNA produced from promoterless *al-1* transgenes was found to be accumulated in quelled strains but absent in the reverted strains, indicating that transcription of transgenes is required for quelling, though the amount of sense transcripts did not seem to be related to the quelling establishment [20]. The observation of the unexpected sense RNA of the transgenes, which was thought to be qualitatively different from mRNA and thus is aberrant, led to the hypothesis that production of aberrant RNA in the presence of transgenes causes the post-transcriptional gene silencing, where the aberrant RNA could be recognized by an RNA-dependent RNA polymerase to produce dsRNA and then further resulting in mRNA degradation [21,23-26]. This hypothesis was proposed before Fire and Mello's discovery of RNAi [7].

As mentioned above, introduction of *al-1* transgenes could result in quelling up to 36%, i.e., still a large portion of transformants were not quelled, thus it is not sufficient for trangenes alone to induce quelling [6,26]. In fact, only transformants harbored duplicated sequences showed silencing, and high copy number and tandem insertions of the trangenes were usually correlated with quelling [21-23,26]. A stably quelled strain by transgenic *al-1* was shown to contain tandem repeats of the transgenes [21]. These could indicate that tandem repeats of trangenes is a source for aberrant RNA [26].

Mechanism of quelling and dsRNA-induced RNAi in *N. crassa*

Taking advantage of a stably quelled strain by transgenic *al-1* and using forward genetics approach by UV mutagenesis, Cogoni and Macino isolated 15 quelling defective mutants

belonging to three distinct genetic loci *qde-1*, *qde-2* and *qde-3*, which were defective in all tested transgene-induced gene silencing [21]. These three loci were further studied and the corresponding genes were cloned which encodes respectively three key components required for Quelling by Macino's group: 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) [21,27-29]. Later small RNAs of about 25 nt were found to be specifically involved in quelling, and the production of these small RNAs required *qde-1* and *qde-3*, but not *qde-2* [30]. Genes for two partially redundant Dicer proteins DCL-1 (Dicer-like-1) and DCL-2 (Dicer-like-2) were further identified and characterized by reverse genetics thanks to the release of the whole genome sequence of *N. crassa* [31-32]. In 2007 Liu lab identified QIP, an exonuclease, as another key component required for RNAi [12]. Below more detailed information will be discussed about the identification and functions of these components.

Isolation of qdes and Production of dsRNA—*qde-1* is the first gene ever cloned encoding a cellular component of the posttranscriptional gene silencing nechanism [27]. The *qde-1* insertional mutant was identified by random insertional mutagenesis on a stable quelled *al-1* transgenic strain as that mentioned above for identification of the three classes of mutants *qde-1*, *qde-2* and *qde-3*, and the functional *qde-1* gene was isolated from cosmids from *N. crassa* cosmid library, which rescued both the *qde-1* insertional mutant and the UV-mutagenized mutant [21,27]. Since QDE-1 is homologous to an RdRP encoded by a tomato cDNA, the only homologue with known function at that time, the finding of QDE-1 provided the first experimental evidence that an RdRP is involved in PTGS, which supported the model that aberrant RNA produced directly from transgenes or resulted from the presence of transgenes could be used as the template by an RdRP to lead to the production of dsRNA and finally degradation of mRNA [23,27]. The wide presence of QDE-1 homologues in plants, fungi and *C. elegans* indicate that a conserved PTGS mechanism involving RdRP may exist in all these organisms [27].

Later it was known that *qde-1* is homologous to *sgs-2* in *Arabidopsis* and *ego-1* in *C. elegans*, and both of them are required for RNAi [33-35]. The presence of an RdRP respectively to be required for quelling in fungi, PTGS in plants and RNAi in *C. elegans* (which further suggested that these three silencing phenomena are originated from one same silencing mechanism) in certain sense not only confirmed the hypothesis that in all these silencing phenomena an RdRP could play a role in the synthesis of complementary RNA and thus the production of dsRNA(a key intermediate component leading to specific RNA degradation) in the absence of direct dsRNA production from inverted repeat transgenes or exogenously provided dsRNA, but also indicated that an RdRP may function in the amplification of the dsRNA signal thus amplification of the silencing signal spreading from cell to cell [7-8,20,24,27,33,35-38]. Thus the finding of the first RdRP QDE-1 in RNA silencing somehow not only further supported such hypothesis in Fire and Mello's seminal RNAi discovery paper as the presence of a catalytic or amplification component in the silencing process, but could further provide more explainations to the observed silencing phenomena such as why the introduction of single stranded RNAs or transgenes could still induce silencing [7]. Now QDE-1 RdRP activity has been confirmed with its structure

resolved, and it is the first cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing that was shown to have RNA-dependent RNA polymerization activity in vitro [39-42]. It was originally postulated that the transcription of a transgene by RNA polymerase II produces an aberrant RNA, which is used as the substrate by QDE-1 to generate a dsRNA [42]. In fact, we know now aberrant RNA is not transcribed by common RNA polymerases (See below).

Back to early 2000, Cogoni noticed that the *qde-2* gene required for quelling is homologous to the *rde-1* gene required for the dsRNA interference in *C. elegans*, which provided the first evidence experimentally that dsRNA interference and trangene-induced PTGS have a common genetic component and indicated that they could evolve from the same ancestral mechanism, and dsRNA could be involved in quelling in fungi [29]. Soon after that, the identification and characterization of AGO1 from Arabidopsis, which is required for PTGS in plants and is homologous to QDE-2 (required for quelling) and RDE-1 (required for RNAi), provided another direct experimental evidence that PTGS in plants, quelling in fungi and RNAi in animals are related and could come from the same ancestral silencing mechanism [8]. Together with the requirement of an RdRP (SGS2, QDE-1 and EGO-1, respectively) mentioned above, PTGS, quelling and RNAi thus were linked together [8].

The isolation of the class of *qde-3* mutants prompted the identification and characterization of *qde-3* gene encoding a member of the RecQ DNA helicase family, which based on the homology could function in the quelling activation step through the DNA-DNA interaction between transgenes introduced or with an endogenous gene [28]. QDE-3 is another key protein discovered required for activation and maintenance of posttranscritional silencing, which was the first reported new function for a DNA helicase [28,43]. Though the exact role in quelling is still largely unknown, QDE-3 acts probably only upstream of the dsRNA formation, for introduction of constructs expressing dsRNA resulted in efficient gene silencing in *qde-3* mutants [28,43]. QDE-3 itself and another RecQ-type DNA helicase RecQ-2 seem to play roles in DNA repair, which are not known to have any direct relationship with the quelling function [28,44-45]. OsRecQ1, a QDE-3 homologue in rice, was found to be required for RNA silencing induced the by introduction of inverted-repeat DNA, but not for RNA silencing induced by the introduction of constructs expressing dsRNA, which is similar to QDE-3 [46]. The levels of transcripts from the plasmid of the inverted-repeat DNA constructs were significantly decreased in the *OsRecQ1* mutants compared to the wild type, indicating that OsRecQ1 is required for the efficient transcription from the plasmid construct producing inverted repeat RNA, which could be the aberrant RNA required for dsRNA formation [46]. This observation somehow may indicate or provide an experimental support for the hypothesis that QDE-3 may promote proper transcription of the transgenes or the production of aberrant RNA [46]. OsRecQ1 did not seem to influence the accumulation of some endogenous microRNAs tested and the production of the short interspersed nuclear element retroelement by small interfering RNA, which is similar to our observations of QDE-3 being not required for the production of endogenous small RNAs in Neurospora (see below), though rRecQ-1, a close homologue of QDE-3 in rats was reported to be correlated with piRNAs [42,46-47]. The efficiency of transgene induced quelling is usually around one third of the total transformants [6,42].

However, overexpression of the RdRP QDE-1 could dramatically elevate the quelling efficiency up to 92%, and furthermore, lower number of transgenes were needed to induce silencing and the quelled transformants were more stable despite progressive loss of tandemly repeated transgenes when QDE-1 was overexpressed; and the inverted-repeat constructs expressing dsRNA instead of transgenes can result in up to 80% quelling efficiency, all indicating that dsRNA production could be the limiting factor for quelling efficiency, and on the other hand, the activation and maintenance of transgene-induced silencing may depend on both the cellular amount of QDE-1 and the number of the transgenes integrated which could probably at the end influence the amount of dsRNA produced [42-43,48-49].

Though RNAi in different systems has been extensively studied, how are repetitive sequences first distinguished from endogenous genes before the dsRNA formation is not clear yet. It is believed that aberrant RNA (aRNA) synthesis and its specific recognition by RNA-dependent RNA polymerases to make double-stranded RNA are required for transgene induced RNAi, though the related mechanisms are not quite clear. In *N. crassa*, QDE-1 was proposed to specifically recognize and convert aRNA into dsRNA. By using tagged QDE-1 to immunopurify QDE-1-containing protein complexes, Nolan et al. demonstrated that QDE-1 interacts in the nucleus with RPA-1, the *Neurospora* homologue of the largest subunit of Replication Protein A which is a single-stranded DNA-binding protein important for DNA replication, repair and recombination [50]. Based on immunoprecipitation by the anti-FLAG antibody, FLAG QDE-1 was found enriched at the transgenic *al-1* locus but not the unrelated, non-silenced endogenous *actin* gene, demonstrating that QDE-1 is recruited to the transgenic locus triggering silencing [50]. The accumulation of siRNAs is DNA synthesis dependent, as hydroxyurea treatment of mycelia to inhibit DNA replication abolished siRNAs accumulation [50]. Thus it was proposed that during replication transgenes are distinguished by QDE-1 interacting with RPA-1 from endogenous genes and then targeted for silencing via dsRNA production by QDE-1[50]. A question unanswered is how RPA-1 is guided to the transgenic sequences instead of the rest of the genome [50]. One speculation is that the RecQ DNA helicase QDE-3 may be involved in this process since the RPA and the homologues of QDE-3 in other organisms have been found to be related to preventing genome instability [50]. Biochemical analyses recently demonstrated that QDE-1 is both an RdRP and a DNA-dependent RNA polymerases (DdRP) (See below qiRNA part for detailes), further indicating that QDE-3 and RPA may facilitate QDE-1 to bind to transgenic region (probably QDE-3 could resolve the complex DNA structures at the transgenic locus created upon tandem integration, and RPA could recruit QDE-1 to the resolved transgenic ssDNA), and then QDE-1 could transcribe the transgenic DNA into aberrant RNA which will be further converted into dsRNA again by QDE-1, though much more work needs to be done to find direct in vivo evidence for this hypothesis [42,50-51].

Introduction of transgene constructs expressing intron-containing self-complementary RNA, i.e., direct expressed dsRNA, could successfully bypass quelling essential genes *qde-1* and *qde-3* to induce gene silencing with high efficiency and higher quelling stability, which further supports the notion that dsRNA is a necessary intermediate for quelling and QDE-1

and QDE-3 both function in the dsRNA production pathway [43]. On the other hand, functional *qde-2* and *dcl-1 dcl-2* were still needed for quelling under direct expression of dsRNA conditions, further demonstrating that dsRNA production is upstream of the dicer processing and the QDE-2 containing RISC formation and activation [43].

Generation of siRNA—Catalanotto et al. found in *N. crassa* both sense and antisense short RNAs 25-nt in length specifically accumulated in silenced transgenic strains, demonstrating that siRNAs are involved in quelling [30]. At this time, it was known that dsRNA can be processed by Dicer into 21 to 23 nt small RNAs in *Drosophila* and antisense small RNAs with size of about 25 nt were associated with PTGS in plants, and 21 nt small RNA duplexes could induce sequence-specific RNA degradation; moreover, siRNAs were found to be associated with RISC degrading homologous mRNAs of the silencing trigger, and AGO2, a member of Argonaute proteins was found to belong to the RISC nuclease [9,52-55]. As mentioned above, a member of Argonaute family proteins is required respectively for quelling in fungi, PTGS in plants and RNAi in animals, and thus a hypothesis at this time was that similar siRNA-directed mechanism could be present in all the three major branch of eukaryotes. Catalanotto et al. further found that though requiring functional *qde-1* and *qde-3*, small RNA accumulation is not dependent on the quelling essential component functional *qde-2*, whereas QDE-2 copurified with siRNAs, indicating that QDE-2 could be a component of the siRNA-directed RISC [30]. Interestingly, besides those corresponding to transgenes, 25nt siRNAs coming from the vector of the transgene construct were also detected specifically in the silenced strains, suggesting that the chimeric transgenic transcripts (thus aberrant RNA) were recognized and converted into dsRNA by the quelling machinery including probably QDE-3 and QDE-1, and then were processed by dicer-like protein into siRNA [30].

In *N. crassa* Dicer-like proteins DCL-1 and DCL-2 process dsRNA into about 25 nt small RNAs in an energy dependent manner [31]. Double mutation of the two Dicer-like genes *dcl-1* and *dcl-2* completely abolished quelling and disrupted the processing of dsRNA into siRNA in vivo and in vitro, but both single mutants had similar quelling frequency to the wild type, and both could process dsRNA into short RNA about 25 nt, suggesting that the two Dicers are redundant, which could be why the two dicer genes escaped the earlier screening for quelling defective mutation [21,31]. However, the accumulation of siRNA was significantly reduced in the *dcl*-2 mutant compared to the level in the wild type, though that in the *dcl*-1 mutant was similar to that of the wild type, indicating the dsRNA processing activity is mostly dependent on DCL-2 [31]. These studies of the dicer function in *Neurospora* was the first demonstrating the involvement of Dicer in transgene induced gene silencing and supported the notion that the dsRNA produced by the cellular RdRP from transgenic transcripts is an essential silencing component [31].

Activation of RISC—QDE-2 is an Argonaute protein in *N. crassa*, which is among the earliest discovered protein family (QDE-2, AGO1 and RDE-1respectively) required for RNAi in fungi, animals and plants and linked together the three phenomena of co-suppression or PTGS in plants, quelling in fungi, and RNAi in animals as mentioned above [8,56]. QDE-2 is the core component of the RISC complex and is associated with siRNA

[29-30]. QDE-2 and its slicer activity are required for gene silencing and the generation of single-stranded siRNA from siRNA duplexes in vivo [12]. Mutation of the *qde-2* gene or the catalytic site of QDE-2 abolished the gene silencing and the single-stranded siRNA production. Wild type QDE-2 was associated with single-stranded siRNA, while the catalytic site mutated QDE-2 was only associated with siRNA duplex, indicating that in *N. crassa* RISC is loaded with double-stranded siRNA before the cleavage and removal of the passenger strand [12].

Another key component for quelling is QIP (QDE-2-interacting protein), an exonuclease domain containing protein which was identified by purifying QDE-2 biochemically from *N. crassa* [12]. At that time, it was known that exogenous or endogenous dsRNAs initiate the RNAi pathway conserved in plants, animals and fungi, which are then processed into about 20 to 25 nt siRNA duplexes; RISC is the RNAi effector complex with an Argonaute family protein as the catalytic core, and the siRNA duplex is loaded onto the RISC and is then separated with the passenger strand removed but the guide strand retained in the RISC and directing the mRNA target cleavage. It was found that Ago2 in *Drosophila* could bind and cleave the passenger strand of the siRNA dulex during RISC activation, and the embryo lysate from the Ago mutant could not produce single-stranded siRNA in vitro, indicating that the passenger strand removal is mediated by Argonaute family protein [57-59]. It was also proposed that an unknown factor may be involved in the removal of the cleaved passenger strand of siRNA duplex [57,60]. By utilizing a dsRNA expressing construct and a Myc-epitoped tagged QDE-2 and Myc-epitoped tagged QDE-2 (D664A), where one of the catalytic residues was mutated, Maiti et al. demonstrated in vivo that in *N. crassa* the Argonaute QDE-2 and its slicer activity are indispensible for single-stranded siRNA generation and effective silencing, which was supported by another paper at the same time showing similar function requirement of Ago2 cleavage activity during RISC activation in *Drosophila* [12,61]. Further experiments using Myc-His-epitope-tagged QDE-2, QIP, the QDE-2-interacting protein containing an exonuclease domain, was identified [12]. Mutation of the *qip* gene caused the accumulation of siRNA duplexes and impairment of gene silencing, and further analyses showed that QIP functioned as an exonuclease by removing the nicked passenger strand from the siRNA duplex in a QDE-2-dependent manner by interacting with QDE-2, which then results in RISC activation [12]. Thus QIP is not only an key player in PTGS promoting RISC activation by removing the nicked passenger strand from the siRNA duplex, it is probably the first reported exonuclease required for efficient RNAi in eukaryotes since especially the exogenuclease domain of QIP is required for its function [12]. Since PTGS is widely conserved in eukarytoes, similar function of QIP for RISC activation by removal of the passenger strand from siRNA duplexes may be present in other organisms. In *Drosophila*, an endoribonuclease C3PO was demonstrated biochemically to activate RISC by removing the cleavage products of siRNA passenger strand in an Ago2-dependent manner, which is similar to the function of QIP, indicating that similar function of QIP may be performed by different type of proteins in different systems [62]. The nick in duplex siRNA created by Ago2 cleavage could be the stimulator of C3PO RNase activity, indicating that the nuclease activity of QIP could be stimulated by the nicked siRNA duplex processed by QDE-2 [62].

Based on the above studies of QDE-2 and QIP, a model for the conserved RNAi pathway can be proposed like this [12]: dsRNA is processed into siRNA duplexes by dicer protein(s), which are loaded onto the RISC, then the Argonaute protein (QDE-2) cleaves the passenger strands of the siRNA duplexes, and an Argonaute associated protein (like the exonuclease QIP or other proteins with similar function) removes the nicked passenger strands with the guide strands remaining in the RISC complexes and activates the RISCs to silence mRNAs with homologue to the guide strands(See Figure 1).

Functions of RNAi in Neurospora—RNAi can play roles in genome defense against viruses and transposons, development regulation and chromosomal segregation [63-64] [3]. Quelling in *Neurospora* can function in silencing the transgenes by detecting and targeting the transgenetic DNA. Quelling can also act to repress the expression and expansion of transposons, for siRNAs against transposons were detected in *N. crassa*, and the transcript levels and copy number of the LINE1-like transposon *Tad* were significantly elevated in *qde-2* mutants and *Tad* transcripts accumulated significantly in the *dcl-1 dcl-2* double mutants [65-66].

RNAi is believed to be involved in heterochromation formation and/or DNA methylation in fission yeast, plants and animals, but no any of the known RNAi components in *Neurospora*, incuding three RdRPs (QDE-1, SAD-1 AND RRP-3), two Argonaute proteins (QDE-2 AND SMS-2), two dicer-like proteins (DCL-1 or SMS-3, DCL-2) and two RecQ helicases (QDE-3 and RecQ-2), is indispensable for either initiation or maintenance of heterochromatin nor DNA methylation [67]. Chicas et al. further demonstrated that transgenic siRNA production/quelling is not required for the histone H3 Lys9 methylation [65]. Thus RNAi thus far does not seem to function in heterochromation formation and DNA methylation in *Neurospora*. It was found that the transgenic *al-1* locus was hypermethylated at LysH3 in both the silenced strain and strains with mutation background of *qde-1*, *qde-2* and *qde-3* rescpectively, but mutation of the histone Lys9H3 methyltransferase gene *dim-5* caused lower efficiency of PTGS, and the silenced transformants lost rapidly the silenced phenotype and the integrated transgenic copies, and thus could not maintain the silencing [43]. The defect of *dim-5* mutants in silencing seems to be only resulted from the failing to maintain the transgene in tandem, indicating DIM-5 may play a role in maintaining the transgene in tandem [65].

Recently Cecere and Cogoni reported that the rDNA gene copy numbers in the quelling mutants *qde-1*, *qde-2* and *qde-3* are all significantly reduced, indicating that quelling may play an important role in maintaining the rDNA locus integrity and stability [68].

A dsRNA-induced transcriptional program important for RNAi

Interestingly, Choudhary et al. showed that dsRNA production can significantly induce the expression of the key RNAi components *qde-2* and *dcl-2*, while modestly induce the expression of *qde-1*, *dcl-1* and *qip* [69]. dsRNA mediated induction of QDE-2, both transcriptionally and posttranscriptionally, is indispensable for efficient RNAi, and the posttranscriptional regulation is Dicer dependent, indicating that dicers or siRNA may play a role in QDE-2 accumulation.

The transcriptional responses were regulated by dsRNA instead of siRNA, as the transcriptional activation of dsRNA-activated genes (DRAGs) was present in the *dcl* double mutant, which abolished the siRNA production [69]. Genome-wide analysis by microarray and quantitative PCR analysis demonstrated that dsRNA could activate 60 genes including additional RNAi components and homologs of antiviral and interferon-stimulated genes. The latter indicates that the dsRNA induced activation of RNAi components is part of conserved ancient host defense response to counter against viral infection and retrotransposons [69]. The signaling pathway responsible for the dsRNA response is not clear yet in *Neurospora*. Since the key genes for RNAi including *qde-2*, *dcl*, and *qde-1*, are not required for dsRNA induced transcriptional activation, and *Neurospora* does not contain the well-known mammalian dsRNA sensors PKR and Toll-like receptor 3, a novel dsRNA-sensing and transcriptional activation pathway might be present instead in *Neurospora* [69].

The DNA damage-induced qiRNA and its relationship with Quelling

It was observed that the addition of histidine in the medium but not other amino acids significantly elevated the levels of *qde-2* mRNA and QDE-2 protein [51]. Since histidine can cause DNA damage in *Neurospora*, other DNA damage agents such as ethyl methanesulphone (EMS), hydroxyurea andmethylmethanesulphonate were also tested which were shown to be able to induce QDE-2 expression. The induction of QDE-2 expression by these DNA damage regents including histidine dependends on the functional QDE-1, QDE-3 and the two Dicers. Moreover, QDE-2 levels were found increased in many DNA repair mutants compared to the wild type. Thus *qde-2* expression can be induced by DNA damage [51]. On the other hand, since dsRNA can induce the expression of QDE-2 in a dicer dependent manner, and QDE-1 and QDE-3 are required for dsRNA generation from transgenes in quelling, thus the dependence of QDE-1, QDE-3 and Dicers for QDE-2 induction by the DNA damage regents suggest that DNA damage may cause dsRNA production endogenously which results in the induction of *qde-2* expression [51,69](See also other related references above). These dsRNAs caused by DNA damage could then be processed into small RNAs by Dicers and bind to QDE-2 as those siRNAs produced in quelling.

Analysis of QDE-2-associated small RNAs using immunoprecipitated Myc-tagged QDE-2 expressed in a *qde-2* mutant background did show that a novel class of 20 to 21 nt long small RNAs significantly induced by histidine or EMS are specifically associated with Myc-QDE-2. Levels of these small RNAs were very low under normal growth conditions while were induced robustly by treating with the DNA-damaging agents histidine, hydroxyurea or EMS. These small RNAs are shorter than the regular 25 nt quelling siRNAs and were named qiRNAs for the interaction with QDE-2 [51]. Sequencing analyses showed that qiRNAs originate mainly (about 86%) from the highly repetitive rDNA locus, have a strong 5′ uridine preference and also a 3′ preference for adenine, together with the specific association with QDE-2, indicating qiRNAs are not random nonspecific degradation products [51].

qiRNA production depends on QDE-1, QDE-3 and the Dicers, for no qiRNA was detected in the *qde-1* and *qde-3* single mutants and in the *dcl-1 dcl-2* double mutants. qiRNAs are corresponding to both sense and antisense strands with similar numbers, and abundant long

RNAs accumulated in the *dcl-1 dcl-2* double mutant with DNA damage, indicating that qiRNAs are processed from long dsRNAs. Though DNA damage induces the expression of QDE-2, qiRNA production is not dependent on QDE-2. qiRNAs produced from the rDNA locus (the major qiRNA producing locus) match to not only mature rRNA regions, but also many external and internal transcribed spacer regions and the intergenic spacer regions, suggesting that qiRNAs originate from unconventional transcripts, i.e., qiRNAs require aberrant RNAs for biogenesis. qRT-PCR and northern blot analyses both demonstrated that the transcripts matching to both upstream and downstream of the transcribed rDNA region, which are aberrant RNA, were robustly induced in the wild type strain by DNA damage, while in the dicer double mutant, these aberrant transcripts accumulated abundantly with sizes from a few hundred nucleotides to about 2 kilobases, indicating that these aberrant transcripts are the precursors of the dsRNA. Treatment with a potent inhibitor (thiolutin) of RNA polymerases I,II and III could not inhibit the synthesis of aberrant RNA, further indicated that these DNA-damage induced transcripts are not conventional transcripts and do not require common RNA polymerases for the synthesis. The aberrant RNA was completely abolished in both the *qde-3* mutant and *qde-1*^{ko} mutant, which is a strong sign that the RecQ DNA helicase QDE-3 and the RdRP QDE-1are both indispensible for the synthesis of the DNA-damage-induced aberrant RNA [51]. Further studies using purified QDE-1 demonstrated that QDE-1 could generate full-length RNA products using either ssRNA or ssDNA as a template, and the products from ssDNA were mostly DNA/RNA hybrids [51]. And on the other hand, the common RNA polymerase inhibitor could not inhibit the RNA polymerase activity [51]. Thus QDE-1 is both an RdRP and a DdRP, indicating QDE-1 is the RNA polymerase to generate aRNA besides required for the production of dsRNA [51].

Besides the *qde-3* mutant sensitive to DNA damaging agents mentioned above during *qde-3* characterization and here lacking qiRNA production thus playing roles in DNA damage response, other RNAi mutants lacking qiRNA production such as *qde-1* single mutant and *dcl-1 dcl-2* double mutants were all shown to have increased sensitivity to DNA damage, all of which indicate that qiRNAs may function in the DNA-damage response by inhibiting protein translation, and RNAi/quelling in Neurospora may play roles in the DNA damage response [51].

In fact, both quelling and the DNA-damage-induced qiRNA pathway share all the key components such as QDE-1, QDE-2, QDE-3, Dicers and small RNAs (25 nt and 20-21nt respectively) and all need aberrant RNA production and dsRNA production, except the former is transgene induced and the latter is DNA damage induced, though it is not known if qiRNAs can function like transgene induced siRNAs to target the homologous mRNA through RISC with QIP to remove the nicked passenger strand for the activation. Based on the above, a common model can be proposed for transgene (and dsRNA, if expressed directly)-induced gene silencing and the DNA-damage-induced qiRNA pathway as below: Transgenes (quelling) or DNA-damage induce the synthesis of aberrant RNAs by the DdRP activity of QDE-1 facilitated by QDE-3 and RPA, where QDE-3 could probably resolve into ssDNA the complex DNA structures at the transgenic locus created upon tandem integration or caused by DNA damage, and RPA could recruit QDE-1 to the ssDNA. The aberrant RNA is then transcribed into dsRNAs by the RdRP activity of QDE-1. The Dicer proteins DCL-1

and DCL-2 cleave the dsRNAs, formed by QDE-1 or expressed directly from dsRNA-expressing constructs, into approximately 25 nt siRNA duplexes (or 20-21nt qiRNAs for DNA damage induced dsRNA), which are then loaded onto the RNA-induced silencing complex (RISC) containing QDE-2 and QIP. QDE-2 and QIP convert the siRNA duplex into the mature siRNA, resulting in RISC activation to silence targets with homology to the siRNA. qiRNA bind to the core component of QDE-2, and play roles in response to DNA damage, but it is not known if qiRNAs function through the RISC containg QDE-2 and QIP.

Meiotic Silencing by Unpaired DNA (MSUD)

Discovery of MSUD

Another PTGS mechanism discovered in *N. crassa* is the meiotic silencing by unpaired DNA (MSUD), which is similar to quelling but only present during meiosis [70-72]. *N. crassa* is generally haploid, though a transient diploid ascus cell is present when the two nuclei of opposite mating fuse (karyogamy) [73]. The diploid cell quickly goes through two rounds of meiosis and then one mitosis, resulting in eight ascospores each containing one nucleus [70,72-73]. MSUD functions in the first meiotic prophase by silencing all copies of the unpaired gene during the pairing of homologous chromosomes, though the silencing effects seem to be contained within the ascus (or asci) where the unpaired DNA is present [71-72]. In fact, MSUD originally was called meiotic transvection (or meiotic trans-sensing) though it was not clear yet at that time it is gene silencing related, referring the phenomenon that proper function of the ascospore maturation 1 gene *asm-1*+, i.e., the proper maturation of ascospores requires *asm-1*+ being in proximity or paired to its allelic counterpart in the transient diploid zygote [70-71,74-76]. Further experiments demonstrated that it is the absence of unpaired copies of *asm-1*+ is the real requirement for ascospore maturation, thus the name MSUD was the preferred name, describing the meiotic silencing by unpaired DNA of all the paired and unpaired homologues in the whole genome [71,76]. In the review of Kelly and Aramayo (2007), Meiotic trans-sensing and meiotic silencing are proposed to be two different though highly related mechanisms, with the trans-sensing scanning the presence or absence of the paired homologous genes and the presence of unpaired gene resulting in the meiotic silencing of all the copies present in the genome [77]. The view could be inferred by the observation that DNA methylation affected meiotic-sensing without influencing meiotic silencing [78].

Mechanism of MUSD

In order to figure out the silencing mechanism of MUSD, extensive screening for mutation by UV-irradiation passing through the cross in the presence of unpaired *asm-1*+ was performed [71-72]. *Sad-1*(suppressor of ascus dominance-1), a paralog of *qde-1* is the first suppressor of MSUD identified, and its mutation resulting from either UV-irradiation, RIP or deletion successfully suppresses the MSUD [71-72]. SAD-1 shares high identities with RdRPs involved in post transcriptional silencing, such as SDE-1 from *A. thaliana*, EGO-1 from *C. elegans* and QDE-1 in *N. crassa*, indicating dsRNA synthesis may be involved in MSUD [71-72]. The requirement of the RdRP SAD-1for MSUD, and the silencing of paired copies as well as the unpaired copy itself by an unpaired DNA suggest that MSUD is a post transcriptional silencing [71].

Sms-2 (suppressor of meiotic silencing-2) was identified from the Neurospora genome encoding the paralog of the Argonaute QDE-2, and was later demonstrated to be required for MSUD by analyzing the *sms-2* loss-of-function mutants [32,79].

sad-2, another dominant suppressor of meiotic silencing, is required for the proper location of SAD-1 which is important for SAD-1 function, and mutation of *sad-2* suppresses MSUD [80-81]. SAD-1 and SAD-2 colocalize in the perinuclear region, and very likely interact each other physically in vivo based on the Bimolecular fluorescene complementation (BiFC) analysis [80-81]. SAD-2 localizes in the perinuclear region even in the absence of SAD-1 but not vice vesa [81]. Thus SAD-2 is a novel protein involved in RNA silencing, and may function by recruiting SAD-1 to the perinuclear region [80].

DCL-1, one of the two dicer-like proteins in *N. crassa*, also called SMS-3 (suppressor of meiotic silencing-3, though it is not a real dominant meiotic silencing suppressor, see below), is found to be required for MSUD [32,77,82]. A homozygous cross of *dcl-1* deletion mutants is barren (but it is normal for *dcl-2* deletion mutants), which is also true for the homozygous cross of the mutants of *sad-1* and *sad-2* respectively [71,80,82]. However, though asci were observed for the homozygous cross of both the *sad-1* mutants and *sad-2* mutants, no perithecium was even seen for *dcl-1* mutants, indicating that the sexual development is defective at a much earlier stage for the *dcl-1* mutant compared with the *sad-1* and *sad-2* mutants [82]. Mutants of *sad-1* and *sad-2* respectively function as dominant suppressors of meiotic silencing, but neither the *dcl-1* deletion mutant, the *dcl-2* deletion mutant, nor the *dcl-1 dcl-2* double mutant could function as dominant suppressors of meiotic silencing [71,80,82]. By Performing the elegant experiments where *dcl-1* could be expressed at an early stage in the sexual cycle but inactivated at later stages, Alexander et al. demonstrated that the *dcl-1* deletion mutation, but not the *dcl-2* deletion mutation, suppressed the silencing of unpaired *hH1-gfp*, thus suppressed the MSUD [82]. Interestingly, quelling relies on the redundant Dicers DCL-1 and DCL-2 with DCL-2 responsible for most of the siRNA production, but here MSUD requires the *dcl-1* but not the *dcl-2* for its function [82]. Thus different sets of proteins are required for MSUD and quelling respectively, supporting the notion that two separate RNAi pathways are present in *N. crassa* [32,83]. DCL-1, SMS-2, SAD-1 and SAD-2 were demonstrated to colocalize in the perinuclear region, an interesting place where siRNAs were shown to be accumulated, indicating that the perinuclear region is an active center for MSUD and the siRNAs might be involved in the MSUD [80,82]. On the other hand, the requirement of the RdRP SAD-1, the Argonaute protein SMS-2 and the Dicer DCL-1 indicates that dsRNA and small RNAs are involved in the meiotic silencing [77,82]. Lee et al. demonstrated that only the unpaired regions with homology to the reporter transcript could trigger the meiotic silencing of the reporter gene, with better silencing effects for the greater size and the greater homology of the unpaired region, and there was an unpairing –dependent loss of a reporter transcript correlated with the induction of meiotic silencing, which support the concept that MSUD is post-transcriptional [84]. Though much work needs to be done to interpret the functioning mechanism and silencing pathway, by comparing with Quelling, a simple model below may be proposed or MSUD [77,82-83](Figure 2): unpaired DNA is a signal to initiate the transcription of an aberrant RNA from an unpaired DNA region, and the RdRP

SAD-1converts aberrant RNA into a dsRNA, which is processed by DCL-1 into small RNAs, and small RNAs are then loaded onto a RISC complex similar to that in Quelling with the Argonaute SMS-2 as the core component, which then performs the silencing function. SAD-2 may function in the MSUD pathway by recruiting SAD-1 to the proper location to perform its activity. The future finding of small RNAs associated with MSUD, especially whether there are SMS-2 binding small RNAs present during MSUD, will be a key to verify this model.

miRNA-like small RNA and Dicer-independnt sRNAs in *N. crassa*

miRNAs are small non-coding RNAs originated from single-stranded RNA precursor containing hairpin structures [64,85]. miRNAs have been found in animals, plants, and algae [86-92], and have been widely considered not present in the fungal kingdom. However, our recent studies have provided strong evidence that miRNA-like small RNAs are present in *Neurospora* and thus the miRNA mediated RNAi exist in filamentous fungi [93].

By analyzing the QDE-2-associated small RNAs produced under normal growth conditions, at least twenty-five potential miRNAs were discovered, named as microRNA-like small RNAs (milRNAs). For each milRNA, the vast majority of small RNA sequences match one arm of the hairpin (called the milRNA arm) and share a U at the 5′ ends. Furthermore, small RNAs matched to the complementary arm of the hairpin (named as milRNA*) were present but with much lower numbers. A 2nt 3′ overhang is present for some milRNA/milRNA* pairs indicating possible products of a Dicer-like enzyme. Though nearly all milRNAs from the milRNA arm share the same 5′ U position, two or more 3′ ends can be present, which is similar to the heterogeneity at 3′ termini of some miRNAs in other eukaryotes [94]. These small RNAs share many similarities with conventional miRNAs from animals and plants: these milRNAs come from highly specific stem-loop RNA precursors; most of the milRNAs require Dicer for the biogenesis; milRNAs may silence endogenous targets with imperfect complementarity like the way of the animal miRNA; and the *milR-3* milRNA production mechanism is similar to plant miRNAs.

Diverse milRNA biogenesis pathways

The four loci producing most milRNAs (*milR-1*, *milR-2*, *milR-3*, and *milR-4*) are all in intergenic regions and each had at least 9,000 reads, but the sizes of the milRNA stem loop precursors are quite flexible: for *milR-1*, *milR-3* and *milR-4*, the milRNA and milRNA* sequences are separated by more than 120 nt, but those are very close for *milR-2*. On the other hand, the size distributions of milRNAs from these four loci are also quite different, with bimodal for *milR-1* and *-4* and single peaks for *milR-2* (25 nt) and *milR-3* (19nt). All these results indicate that milRNAs from different loci may have different mechanisms of biogenesis.

Further analyses demonstrated that there are at least four different biogenesis mechanisms for milRs in Neurospora. The biogenesis of *milR-3* milRNAs requires only Dicers for pre-milRNA and milRNA processing, which is like that for plant miRNAs; while *milR-4* milRNAs biogenesis is partially Dicer-dependent. The *milR-1* production however requires Dicers DCL-1/DCL-2, the Argonaute protein QDE-2 and the exonuclease QIP; whereas, the

biogenesis of *milR-2* milRNA requires QDE-2 but is completely independent of Dicer (See Figure 3).

Northern blot analyses and cloning showed that for *milR-1* locus, the mature milRNAs are 9- and 24/25-nt, and pre-milRNAs are 33nt and 43 nt, pre-milRNA* species are about 33nt and 41nt respectively, and the pri-milRNA is the 170nt band which is indeed a transcript containing the predicted hairpin structure. The Dicers (mainly DCL-2, unpublished data) responsible for processing the pri-milRNA into pre-milRNAs, for all four smaller size products were gone while the level of the 170-nt product increased dramatically in the *dcl-1dcl-2* double mutant dcl^{DKO}. The mature milRNAs species were gone in the *qde-2* mutant, but they were not abolished in the *qde-2* (D664A) mutant (no slicer activity of QDE-2 but retaining the dsRNA binding activity in this mutant); however, the production of mature milRNA was completely abolished both in the *qip* (encoding the QDE-2 interacting protein QIP) mutant and in the *qip* mutant expressing QIP with a point-mutation of the catalytic residue (H504A), but expression of the wild-type QIP completely rescued the mature milRNA production in the *qip* mutant. On the other hand, immunoprecipiation assays showed that Myc-QDE-2 could bind the short pre-milRNA (33nt) in the *qip* mutant, and bind also two mature milRNAs as well as the 33nt pre-milRNA in the wild-type strain. All these results suggest that QDE-2 binds to pre-milRNA (the 33nt pre-milRNA, which is probably the precursor of the mature milRNAs, with the 43nt longer pre-milRNA probably being an intermediate or by-product of pri-milRNA processing) and recruits the exonuclease QIP to process pre-milRNAs into mature milRNAs, where the catalytic activity of QIP is required.

The *milR-2* locus forms perfect loop structure with both milRNA strand and milRNA* on the stem close to the loop. In fact, the 3′ ends of the mature milR-2 species (mostly around 25nt to 28 nt with the same 5′ start position) are on the loop. Northern blot analyses demonstrated three pre-milRNA species of about 33, 39, and 52 nt and a mature milRNA of∼25 nt. However, no any difference of the profiles of the pre-milRNAs and mature milRNA were observed in the *dcl-1*/*dcl-2* double mutant dcl^{DKO} compared with the wild type, demonstrating that the biogenesis of *milR-2* is Dicer-independent. Suprisingly, QDE-2 and its catalytic activity are instead required for the production of both pre-milRNAs and milRNA, for the two smaller species of pre-milRNAs disappeared but the 52-nt pre-milRNA accumulated abundantly in both the *qde-2* and *qde-2*(D664A) mutants, with a RNA band about 2 nt larger than the wild-type mature *milR-2* milRNA was observed in the mutants. For *milR-2*, *qip* mutation did not show significant influence compared with the wild type. Immunoprecipiation analysis showed that both the mature milRNA and the two shorter pre-milRNAs were associated with QDE-2. All these results suggest that the *milR-2* pri-milRNA is first processed into the pre-milRNAs by an unknown nuclease(s), and then the Argonaute QDE-2 and its catalytic activity are required for processing the pre-milRNAs into mature milRNA. The mature *milR-2* was still associated with Myc-QDE-2 in the *qde-1* and dcl^{DKO} mutants suggesting that loading of *milR-2* onto QDE-2 is also dicer-independent.

milR-2 is in fact the first known dicer-independent, but Argonaute-dependent microRNA-like small RNA for the biogenesis in eukaryotes. The discovery of dicer-independent milR-2 in *Neurospora* blurs the boundary of miRNA, as the general concept is that miRNAs are

dicer-dependent. Quite interestingly, soon after our this discovery, two papers emerged on-line which both describe the same miR-451 as a dicer-independent but Argonaute-dependent miRNA [95-96]. miR-451 practically is very close to milR-2 both for the biogenesis and on the detailed structure, which further supports milR-2 is indeed a microRNA and our discovery of the presence of dicer-independent Argonaute-dependent miRNA in fungi.

At least four different mechanisms present for milRNA biogenesis in *Neurospora*, suggests that eukaryotic miRNA biogenesis involves mechanisms much more complex than the established pathways for plants and animals. The discovery of milRNAs in *Neurospora* supports the concept that miRNA and miRNA-like small RNAs exist in all major branches of eukaryotic organisms, and the milRNA related studies in *Neurospora* will broaden the miRNA studies in other eukaryotes. No highly homologous *milR* genes outside the *Neurospora* genus were found, suggesting individual *milRs* may have evolved independently from plant and animal miRNAs and the miRNAs in fungi may evolve rapidly, as some miRNAs in plants [97].

A novel RNAse III domain-containing protein MRPL3 invloves milRNA processing

The presence of Dicer-independent mechanisms for milRNA generation led us to look for proteins responsible for Dicer-independent nuclease activity. One protein (NCU08299) homologous to the yeast mitochondrial ribosomal protein MRPL3 in the *Neurospora* genome was noticed for its possession of a putative RNAse III domain and a dsRNA recognition motif, though the RNAse III domains of MRPL3 and its homologous proteins have little sequence similarity to those of Dicers and Drosha indicative of the presence of a different family of RNAse III domain proteins. Northern blot analyses by utilizing a heterokaryotic strain that carries both wild-type and $mrpl3^{KO}$ nuclei and a strain (ds*mrpl3*) in which *mrpl3* is silenced by dsRNA demonstrated that the levels of the major mature *milR-1* milRNA and the 33nt pre-milRNAs were significantly decreased in the mutants compared to the wild type, which indicates that MRPL3 is involved in the production of *milR-1* pre-milRNAs and milRNAs by somehow collaborating with the Dicers. The mature milRNA levels of *milR-4* in the *mrpl3* mutants were also decreased to the levels similar to that in the dcl^{DKO} strain, where the milRNA production is partially dependent on Dicer. But the levels of *milR-2* and *milR-3* milRNAs and pre-milRNAs were not changed in the *mrpl3* mutants compared to the wild type, indicative of some other nuclease(s) responsible for the processing pri-*milR-2* to pre-*milR-2*s instead of MRPL3. I*n vitro* pre-*milR-4* processing activity was also significantly decreased in the silenced ds*mrpl3* strain. All in all, these results show that MRPL3 is a novel protein responsible for the Dicer-independent processing of some milRNAs in *Neurospora*.

Function of milRNA

Introducing a Myc-tagged reporter construct with *milR-1* milRNA complementary sequences into the wild-type and *qde-2* mutant strains showed that the mRNA levels and protein levels of the reporter were all higher in the *qde-2* mutants than in the wild-type strains, with the protein levels about 20 fold higher, supporting the conclusion that *milR-1* expression leads to significant silencing of its complementary target. mRNA levels of several predicted targets of the *milR* miRNA are up-regulated in the dcl^{DKO} mutant,

indicating milRNAs may regulate these genes. Furthermore, QDE-2 were found to specifically associate with these predicted miRNA targets but not with the control RNAs. All these show that milRNAs can regulate gene expression, suggesting miRNA mediated RNAi as that in animals and plants is present in fungi, though much work still needs to be done to understand the importance of these milRNAs to the growth and development.

DisiRNAs

Analyses of the *Neurospora* QDE-2-associated small RNAs by deep sequencing also revealed another novel type of small RNAs called dicer-independent small interfering RNAs (disiRNAs) [93]. This is a major group containing small RNAs symmetrically matched to both strands of DNA, which are averaged about 22nt long with a strong 5′ U preference. disiRNAs are derived from 50 loci not highly repetitive but including genes and intergenic regions with no apparent shared sequence motifs. Based on available EST data, nearly 80% of the *disiRNA* loci have overlapping sense and antisense transcripts, which suggests that these disiRNAs are likely processed from dsRNA made from naturally occurring complementary sense and antisense transcripts.

These disiRNAs do not depend QDE-1, QDE-2, nor QDE-3 for the biogenesis, and though they are very likely derived from dsRNA, their levels were not significantly changes in the *dcl-1/dcl-2* double mutant dcl^{DKO}, so Dicers are not involved in their biogenesis. Moreover, neither the double mutation of the two Argonaute genes (*qde-2 sms-2*) altered the disiRNA levels, nor the *mrpl3* mutants. These results indicate that, unlike animal piRNAs, an Argonaute-dependent maturation mechanism is not involved in disiRNA production. A heterokaryon or a knock-down strain by dsRNA for the RNT1 (an Rnase III domain containing protein in yeast) homolog in Neurospora did not show any defects on the disiRNA biogenesis. These small RNAs are not like qiRNAs either which are induced by DNA damage, derived from both strands of repetitive rDNA and dependent on QDE-1, QDE-3, and Dicers for the production [51]. None of the known RNAi pathway components in *Neurospora* influences the disiRNA production, thus an unknown novel small RNA biogenesis pathway may be present for disiRNAs. Whether these disiRNAs can function through certain RNAi pathway is not known, though all these small RNAs interact with QDE-2, the core component of the RISC, indicating that they might function via RNAi pathway (Figure 3).

RNAi in other filamentous fungi

RNAi is an antiviral defense mechanism in *Cryphonectria paracitica*

Except *N. crassa*, another excellent system for RNAi studies of filamentous fungi is the chestnut blight fungus *Cryphonectria paracitica*. Most significantly, *C. parasitica* can support the replication of members from five different RNA virus families, thus this fungus is an ideal model for studying the role of RNA silencing as an antiviral defense mechanism [98].

Though RNAi is well known in functioning as antiviral defense for plant viruses and animal viruses, no direct evidence was provided on this aspect for fungi until recently [2,99-102]. p29 is a papain-like protease encoded by the mycovirus *Cryphonectria* hypovirus 1 (CHV1),

which is similar to the plant potyvirus-encoded suppressor of RNA silencing HC-Pro [103-106]. Using the CHV1-EP713/ *C. parasitica* system, Segers et al. demonstrated that p29 suppressed the hairpin RNA-induced silencing and reversed the established RNA silencing of GFP in *C. parasitica*; and p29 also suppressed both virus-induced and agroinfiltration-induced RNA silencing and systemic spread of silencing in GFP-expressing transgenic *Nicotiana benthamiana* [104]. These results suggest that the antiviral defense mechanism of RNA silencing is conserved in both fungi and plants [104]. Segers et al. further cloned dicer-like genes *dcl-1* and *dcl-2*, which have high similarity to *dcl-1* and *dcl-2* in *Neurospora* respectively. Though the single mutants of *dcl-1* and *dcl-2* have no obvious phenotype compared to the wild type *C. parasitica*, and the single mutants of *dcl-1* don't have phenotype difference compared to the wild type after the infection of hypovirus CHV1-EP713 or reovirus MyRV1-Cp9B21, the Δ*dcl-2* and Δ*dcl-1*/Δ*dcl-2* mutant strains are highly susceptible to the infection. On the other hand, infecting the Δ*dcl-2* mutant by a hypovirus CHV1-EP713 mutant lacking the suppressor of RNA silencing p29 and the wild-type reovirus MyRV1-Cp9B21 showed elevated viral RNA levels compared to the wild type. Complementation of the Δ*dcl-2* strain with the *dcl-2* wild type copy rescued the response to virus infection to the wild type level. These results provide an direct evidence that a fungal dicer can function to regulate virus infection and RNAi plays an important role in antiviral denfense in fungi [98,104]. This is further supported by the findings that the dicer gene *dcl-2* is required for defective viral RNA production and recombinant virus vector RNA instability for hypovirus [107]; *dcl-2* expression levels are significantly increased in response to viral infection, with further more increased expression when suppressor p29 is mutated in the virus, and *dcl-2* processes hypovirus RNAs into virus-derived small interfering RNAs (vsRNAs) as part of an inducible RNA silencing antiviral response [107-108].

There are four Argonaute-like proteins (AGL1, AGL2, AGL3 and AGL4) in *C. parasitica*, but only AGL-2 is required for the antiviral defense response [109]. Similar to *dcl-2*, *agl-2* also has an increase in transcription level in response to viral infection, though not so significant [109]. The *agl2* gene is required for the increase of the virus-induced *dcl2* transcription, and a*gl2* and *dcl2* transcripts accumulated to much higher levels in response to infection by a mutant CHV1-EP713 hypovirus without p29 than to wild-type CHV1-EP713, further supporing that a virus-encoded RNA silencing suppressor suppress the RNA silencing components in fungi [109]. Like that in *N. crassa*, dsRNAs also induce significantly the expression of a*gl2* and *dcl2* (to high levels similar to that in response to p29 mutated hypovirus), which could suggest that Quelling in *N. crassa* may also function as an antiviral silencing pathway [109].

RNAi plays a role for mycovirus defense in *Aspergillus nidulans*

Inverted repeat transgene -induced RNA silencing was successfully performed in *Aspergillus nidulans*, demonstrating that RNAi is present in this model filamentous fungus [110]. Interestingly, this fungus only has one intact Dicer and Argonaute respectively which are both required for RNAi, and lacks the *N. crassa* RdRP QDE-1 homolog though contains two RdRPs in the genome with none is required for the tested RNAi [110-111]. By investigating the relationship to RNAi of the three mycoviruses with stable infections of *A. nidulans*, Hammond and Keller demonstrated that the Aspergillus virus 1816 suppressed the

inverted repeat transgene -induced RNA silencing, for the presence of this virus released the silencing phenotype and the corresponding siRNA was not detected. The mechanism for the suppression is not clear, which could involve an RNA silencing suppressor, if present in this virus. On the other hand, the virus 341-derived siRNA was detected robustly in an Argonaute mutant, showing that this virus is targeted and processed into siRNA by the RNA silencing machinery. All these results suggest that there is an antagonistic relationship between these mycoviruses and RNA silencing, and RNAi in *A. nidulans* functions in mycovirus defense [112].

RNAi and small RNA studies in *Mucor circinelloides*

In recent years, *Mucor circinelloides* has got tremendous attention on the studies of RNAi for its distance from other fungal models and the molecular tools conveniently available, with a transgene-induced RNA silencing pathway revealed and two dicer genes (*dcl1* and *dcl2*) and two RdRP genes (*rdrp1* and *rdrp2*) identified and characterized [113-116]. Self-replicative sense or inverted-repeat trangenes induced post-transcriptional gene silencing associated with both sense and antisense small RNAs, though two different size classes of small antisense siRNA of 21 nt and 25 nt respectively, were differentially accumulated during the vegetative growth of silenced transformants [114,116]. Secondary sense and antisense RNAs were detected, suggesting the amplification step is present for RNA silencing in this fungus [116]. DCL-2 is the major player in transgene-induced silencing and the production of the two classes of antisense siRNAs [114]. RdRP1 is also indicated to be involved in the transgene-induced silencing but not RdRP2 nor DCL-1 [113]. Most recently, by directly sequencing the cDNA libraries of short RNAs, four classes of endogenous short RNAs have been identified in this fungus matching to exons with fungction of regulating mRNA levels of many protein coding genes [113]. The biogenesis of the largest class of these exonic-siRNAs requires both RdRP1 and DCL-2, indicating RdRP1 converts the corresponding mRNA into dsRNA which is further processed by DCL2. A second group of exonic-siRNAs require DCL-2 and RdRP2 but not RdRP1 for the biogenesis. The third group requires both RdRP1 and RdRP2 for the biogenesis, though the two dicers seem to play redundant roles as these small RNAs are only down-regulated in the dicer double mutants. For the fourth group the small RNA production is majorly DCL-1 but not DCL-2-dependent, though both RdRPs are required for the biogenesis. Though some dicer-dependent endogeneous small RNAs were identified to map to transposons or repeats, no miRNA-like small RNA were found in this study, which could be explained by the following possibilities [113]: 1.miRNAs are not present in this fungus; 2. miRNAs are not expressed or not expressed abundantly under the test conditions; 3. miRNAs are usually Argonaute-associated, and deep sequencing of the Argonaute-binding small RNAs (enriched by Argonaute) under different growth conditions may help to answer if miRNAs are present in this fungus. The discovery of these endogenous small RNAs from *M. circinelloides* greatly enriches the understanding of RNAi in eukaryotes. The next question is to see if these small RNAs interact with rgonaute proteins, the core component of the RISC complex.

RNAi studies and application in other filamentous fungi

RNAi have been studied in some other filamentous fungi, especially after Fire and Mello's RNAi discovery [83]. A co-suppression phenomenon like that in plants was observed in

Cladosporium fulvum in 1998 [83,117] and a homology-dependent silencing was observed in *Schizophyllum commune* in 1997 [118]. Since the seminal report of RNAi in 1998 [7], gene expression suppression by utilizing a dsRNA-expressing system has been found and successfully applied in many pathogenic and non-pathogenic fungi including *Ascomycota*, *Basidiomycota*, and *Zygomycota*, such as *Magnaporthe oryzae* [119], *Sclerotinia sclerotiorum* [120], *Aspergillus fumigatus* [121-124], *Aspergillus oryzae* [119,125], *Aspergillus flavus* [126] and *Aspergillus parasiticus* [126], *Bipolaris oryzae* [127], *Colletotrichum lagenarium* [128], *Colletotrichum gloeosporioides* [129], *Coprinus cinereus* [130-131], *Fusarium solani* [132], *Fusarium graminearum*[126], *Fusarium verticillioides* [133], *Mucor circinelloides* [115-116], *Moniliophthora perniciosa* [134], *Histoplasma capsulatum* [135-136], *Cryptococcus neoformans* [137], *Schizophyllum commune* [118,138], *Coniothyrium minitans* [139], *Stagonospora nodorum* [140], *Ophiostoma floccosum* and *O. piceae* [141], *Botrytis cinerea* [142] and so on, with some of them showing the involvement of dicer in the silencing and siRNA production, suggesting RNA silencing is conserved in most of fungal species [83,143].

As more and more fungal genomes being sequenced, homologues of RdRP, Argonaute and dicer proteins in various filamentous fungi from *Ascomycota*, *Basidiomycota*, and *Zygomycota* were identified though none was found in the basidiomycete *Ustilago maydis* [83,143]. Genes involved in the RNA silencing in the ascomycete fungi usually have a *N. crassa* ortholog, including three RdRPs, two Argonautes and two dicer-like proteins [83,143]. Basidiomycete fungi have similar numbers of the protein classes involved in RNAi to the ascomycetes, though with a wider diversity and more extensive gene expansion [83,143]. In the zygomycete *Rhizopus oryzae*, five RdRP genes were identified, indicative of more entensive gene expansion [83,143].

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Perspectives

Neurospora crassa not only has two famous well characterized RNAi pathways Quelling and Meiotic Silencing by Unpaired DNA, but also can generate endogenous small RNAs which could function in different RNA silencing pathways: DNA-damage induced qiRNA; Dicer, QDE-2 and QIP-dependent milRNA-1; Dicer-dependent milR-3; Partial Dicer-dependent milR-4; Dicer-independent, Argonaute QDE-2-dependent milR-2; Dicer-independent disiRNAs. With *N. crassa* is only one member of the millions of species, filamentous fungi are among the most diverse and populated groups of eukaryotes with tremendous impact on human daily life and the ecosystem balance. Since it is widely conserved in filamentous fungi, RNAi is and will play more important roles in understanding gene function and regulation, and RNAi itself may play important function in pathogenesis in pathogenic fungi, though much work needs to be done.

Though RNAi has been got enormous attentions and the most intense studies since its discovery, there are many questions still not resolved or even not touched, especially for the filamentous fungi. Even just in the model *Neurospora*, many obvious questions related to RNAi have not been answered: How aRNA is produced? Are microRNA-like small RNAs and disiRNAs widely present in filamentous fungi? What are the functions of those miroRNA-likes small RNAs and their biogenesis mechanisms? How are the disiRNAs produced and what importance do they have to the organism's growth and development? Are there (if so, what are they) SMS-2 interacting siRNAs present for MSUD? Being the most diversified and most populated group with various habitats of eukaryotes, filamentous fungi may have more unexploited fields in RNAi compared with other eukaryotes and RNAi may play more diverse roles in them.

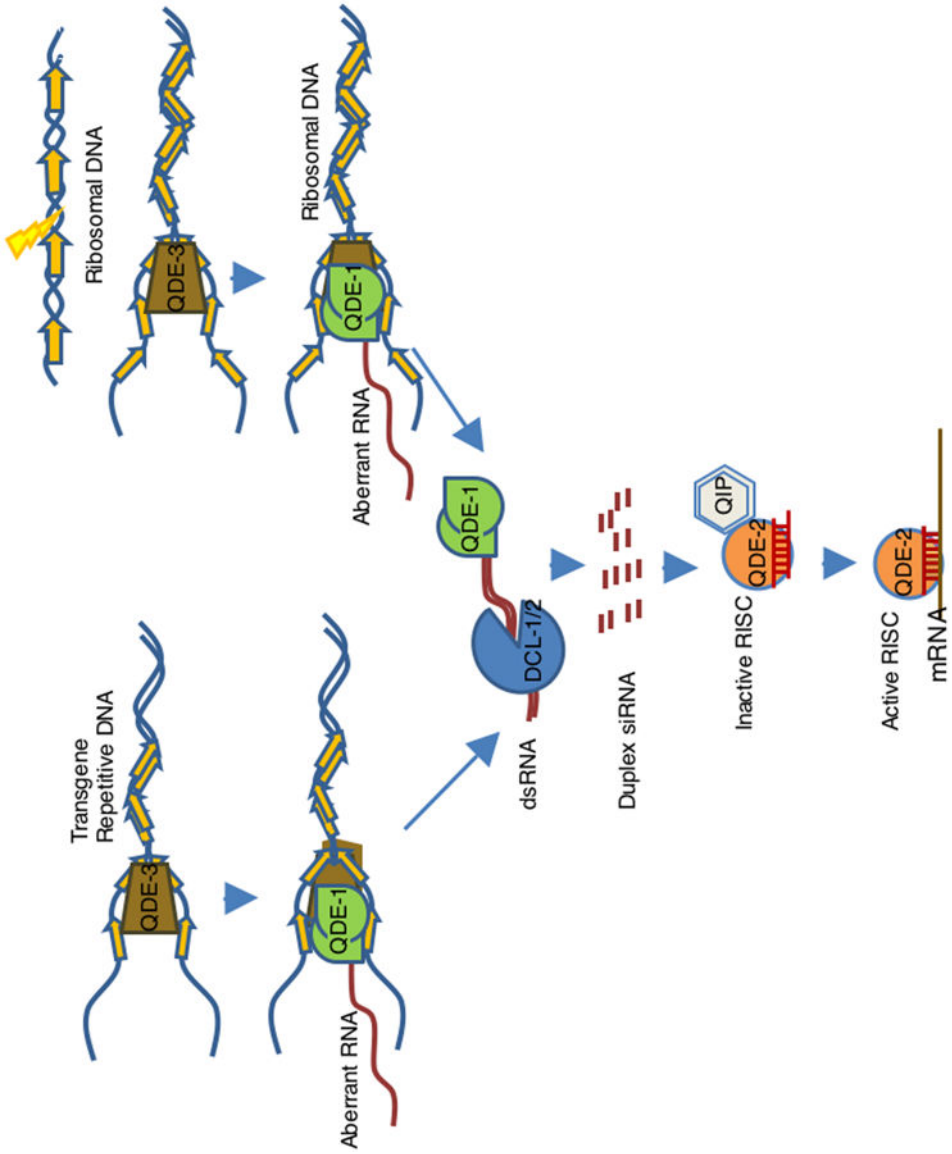

Figure 1. A model for RNAi/PTGS pathways in vegetative cells in *N. crassa*

This model puts together (a) transgene-induced PTGS (quelling), (b) dsRNA-induced PTGS and (c) DNA-damage induced qiRNA pathway. Transgenes (quelling) or DNA-damage induce the synthesis of aberrant RNAs by the DdRP activity of QDE-1 facilitated by QDE-3 and RPA, where QDE-3 could probably resolve into ssDNA the complex DNA structures at the transgenic locus created upon tandem integration or caused by DNA damage, and RPA could recruit QDE-1 to the ssDNA. The aberrant RNA is then transcribed into dsRNAs by the RdRP activity of QDE-1. The Dicer proteins DCL-1 and DCL-2 cleave the dsRNAs, formed by QDE-1 or expressed directly from dsRNA-expressing constructs, into approximately 25 nt siRNA duplexes (or 20-21nt qiRNAs for DNA damage induced dsRNA), which are then loaded onto the RNA-induced silencing complex (RISC) containing QDE-2 and QIP. QDE-2 and QIPconvert the siRNA duplex into the mature siRNA, resulting in RISC activation to silence targets with homology to the siRNA. qiRNA bind to the core

component of QDE-2, and play roles in response to DNA damage, but it is not known if qiRNAs function through the RISC containg QDE-2 and QIP.

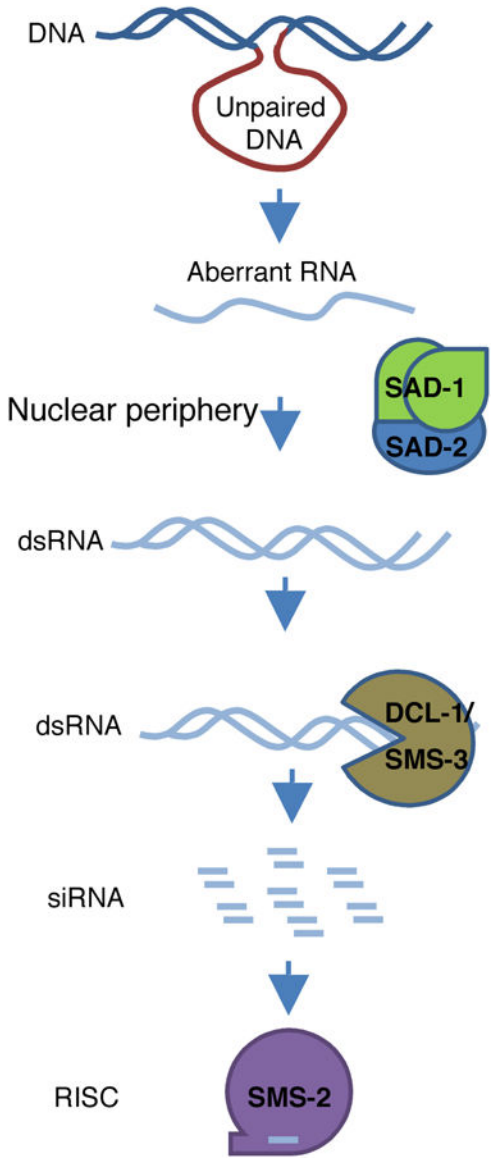

Figure 2. Meiotic silencing in *N. crassa*

During the first meiotic prophase, an unpaired DNA is a trigger for the transcription of an aberrant RNA from an unpaired DNA region, and the RdRP SAD-1converts aberrant RNA into a dsRNA, which is processed by DCL-1 into small RNAs, and small RNAs are then loaded onto a RISC complex similar to that in Quelling with the Argonaute SMS-2 as the core component, which then perform the silencing function. SAD-2 may function in the MSUD pathway by recruiting SAD-1 to the proper location to perform its activity.

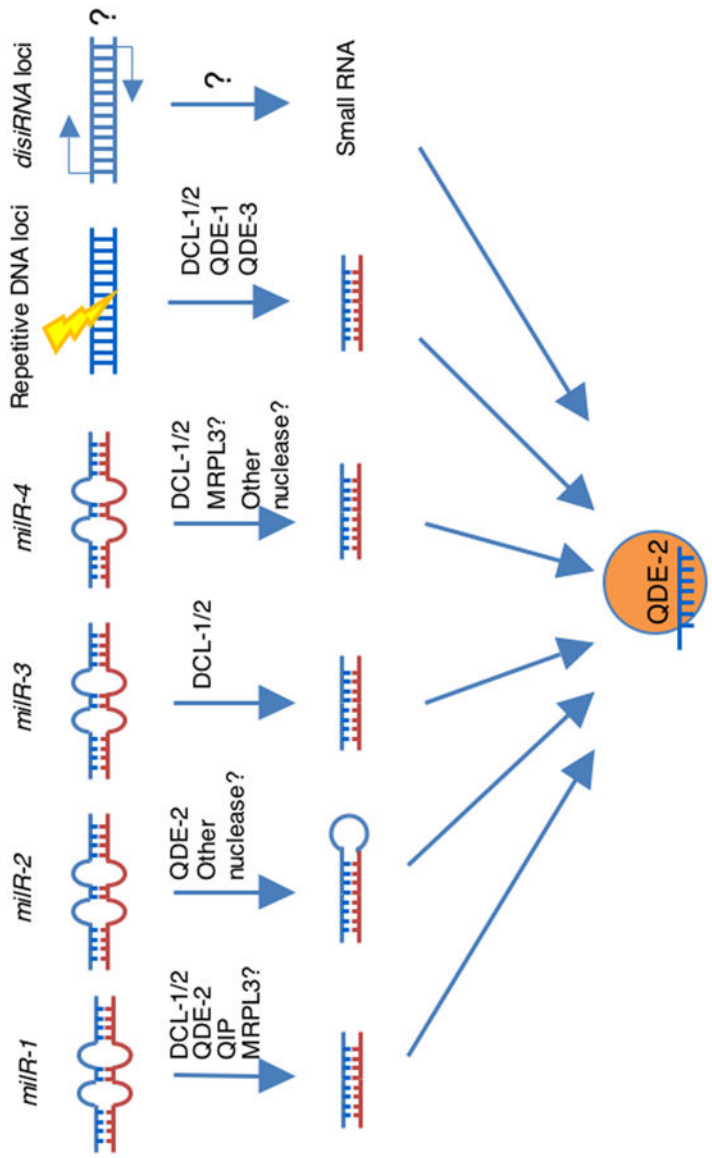

Figure 3. Novel endogenous small RNA pathways under normal growth conditions (or microRNA-like RNAi pathways and disiRNA pathway?) in *N. crassa*

microRNA-like small RNAs are present in *Neurospora* with at least four different biogenesis mechanisms: (a) Dicer, QDE-2 and QIP-dependent milRNA-1; (b) Dicer-dependent milR-3; (c) Partial Dicer-dependent milR-4; (d) Dicer-independent, Argonaute QDE-2-dependent milR-2. As in aminals and plants, miRNA mediated RNAi pathway may be present in *Neurospora*, which is here exemplified by milRNA-1. milRNA-1 is first processed from primary milRNA precursor by Dicers into pre-milRNAs, which are further processed into mature milRNA-1s by QDE-2 and QIP. MRPL3 may involve both the pre- and mature milRNA-1 production. milRNA-1 can regulate gene expression and silence the complementary targets, probably via the similar mi-RISC-like complex as that in aminals and plants, with the Argaunote QDE-2 as the core component. Another novel RNAi pathway present in Neurospora could be the (e) Dicer-independent disiRNA pathway. disiRNAs are a

novel class of QDE-2 associated small RNAs probably produced from dsRNAs, but their biogenesis do not dependent any known RNAi components.