THE MECHANISM OF SMALL INTERFERING RNA BIOGENESIS IN *NEUROSPORA CRASSA*

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DEDICATION

To my father Peiyi Zhang and mother Guifen Jiang.

THE MECHANISM OF SMALL INTERFERING RNA BIOGENESIS IN *NEUROSPORA* CRASSA

by

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RNA interference is a well-conserved post-transcriptional gene silencing mechanism that regulates various biological processes including development, genome defense, and heterochromatin formation. In filamentous fungus *Neurospora crassa*, quelling is an RNAi-related phenomenon that post-transcriptionally silences repetitive DNA and transposon. We previously identified a type of DNA damage-induced small RNA called qiRNAs that originate from ribosomal DNA. Ribosomal DNA cluster remains the only highly repetitive sequences in *Neurospora* genome. To understand how small RNAs are generated from repetitive DNA, we carried out a genetic screen to identify genes required for qiRNA biogenesis. Factors directly involved in homologous

recombination (HR) and chromatin remodeling factors required for HR are essential for qiRNA production. HR is also required for quelling, and quelling is also the result of DNA damage, indicating that quelling and qiRNA production share a common mechanism. These results suggest that DNA damage triggered HR-based recombination allows the RNAi pathway to recognize repetitive DNA to produce small RNA.

The involvement of chromatin remodeling factors indicates that siRNA biogenesis is regulated in the chromatin level. From our systematic knock out library screen, we identified a novel component, RTT109, which is required for both qiRNA and quelling-induced small RNA production. RTT109 is a fungal-specific histone acetyltransferase (HAT) for histone H3 on lysine 56 and its catalytic activity is required for its function in small RNA production. Furthermore, we show that RTT109 is required for homologous recombination and H3K56Ac is enriched around double strand break, which overlaps with RAD51 binding. Taken together, our results suggest that H3K56 acetylation is required for small RNA production through its role in homologous recombination.

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PRIOR PUBLICATIONS

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LIST OF ABBREVIATION

AGO – Argonauate

aRNA – Aberrant RNA

ATP – Adenosine triphosphate

CHS – Chalcone synthase

DCL – Dicer-like protein

DDRP – DNA-dependent RNA polymerase

DNA – Deoxyribonucleic acid

DSB – Double strand break

dsRNA – Double stranded RNA

EDTA – Ethylenediamine tetra acetic acid

FGFC – Fungal genetics stock center

HAT – Histone acetyltransferase

HCL – Hydrochloric acid

HDAC – Histone deacetylase

HEPES – 4-(2-hydroxyethyl)-1-piperizine-ethane sulfonic acid

HR – Homologous recombination

kB – kilobase

kDa – kilodalton

miRNA – MicroRNA

milRNA – MicroRNA-like RNA

mM – Millimolar

mRNA – Messenger RNA

MSUD – Meiotic silencing by unpaired DNA

NHEJ – Non-homologous end joining

nt – Nucleotide

ORF – Open reading frame

PAGE – Poly-acrylamide gel electrophoresis

PAZ – Piwi-Argonaute-Zwile

PCR – Polymerase chain reaction

PEG – Poly-ethylene glycol

PTGS – Post-translational gene silencing

QA – Quinic acid

QDE – Quelling deficient

QIP – QDE-2 interacting protein

qiRNA – QDE-2 interacting RNA

qRT-PCR – Quantitative real-time PCR

RdRP – RNA dependent RNA polymerase

RISC – RNA induced silencing complex

RNA – Ribonucleic acid

RNAi – RNA interference

RNaseIII – Ribonuclease III

rDNA – Ribosomal DNA

rRNA – Ribosomal RNA

SAD – Suppressor of ascus dominance

SDS – Sodium dodecyl sulfate

siRNA – Small interfering RNA

SSC – Sodium chloride-sodium citrate

ssDNA – Single stranded DNA

TBE – Tris-Borate-EDTA

TE – Tris-EDTA

UTP – Uridine triphosphate

UTR – Untranslated region

CHAPTER ONE

INTRODUCTION

RNA interference (RNAi) is a post transcriptional/transcriptional gene silencing mechanism, which is well conserved across eukaryotes (Fire et al. 1998; Carthew and Sontheimer 2009). Over the last two decades, various types of small non-coding RNA classes have been identified, including small interfering RNA (siRNA), microRNA (miRNA), and PIWI-interacting RNA (piRNA) (Carthew and Sontheimer 2009; Ghildiyal and Zamore 2009; Kim et al. 2009; Moazed 2009; Thomson and Lin 2009). A large number of studies have revealed unexpected diversity in their biogenesis pathways and regulatory mechanisms. (Chapman and Carrington 2007; Siomi and Siomi 2009). These small non-coding RNAs play critical roles in both somatic and germline cells to regulate many genes and protect the genome from reproduction of invasive DNA (Hsieh and Fire 2000; Carthew and Sontheimer 2009). Furthermore, studies of RNA interference not only shed important insights to understand the mechanism of underling physiological processes but also provide us powerful experimental tools in biological research and clinical applications (Hannon 2002; Paddison et al. 2004; Reynolds et al. 2004; Judge et al. 2005).

1.1 The discovery of RNA interference

RNA interference phenomenon was first reported in plant in 1990 (Napoli et al. 1990). In an attempt to overexpress chalcone synthase (CHS) in pigmented petunia by transformation, it was unexpectedly found that forty-two percent of plants exhibited loss of pigment phenotype. This so called “co-suppression” phenomenon resulted in the

reduced level of mRNA from both endogenous and exogenous loci (Napoli et al. 1990). Similar to co-suppression, a "quelling" phenomenon was reported in *Neurospora* in 1992 (Romano and Macino 1992). In 1995, Guo and Kemphues discovered that the injection of either sense or antisense RNAs to worm led to silencing of homologous genes (Guo and Kemphues 1995). The idea of RNA interference (RNAi) was first introduced in 1998 as a phenomenon triggered by double-stranded RNA (dsRNA) that results in the silencing of the genes complementary to the dsRNA (Fire et al. 1998; Mello and Conte 2004). It turned out that quelling and co-suppression were both RNAi-related mechanism (Catalanotto et al. 2000) and studies in Neurospora has made fundamental contributions to mechanism of RNAi (add a few reference here, Chang et al., Annual review & Dang et al, review). Intensive follow-up studies using tools based on genetics, biochemistry, and crystallography have revealed that RNAi pathways use small noncoding RNAs to regulate diverse cellular, developmental, and physiological processes (Chang et al. 2012). Among identified small RNAs, there are basically three major types: siRNA, miRNA, and piRNA. Each class of sRNAs is produced differently and has diverse functions. In this chapter, I will mainly focus on the mechanisms and functions of small interfering RNAs in *Neurospora crassa*. Because RNAi pathways are conserved across eukaryotes, conclusions drawn from studies performed in *Neurospora* shed important light onto the small RNA biogenesis mechanism in eukaryotes (Carthew and Sontheimer 2009).

1.2 Quelling: its biogenesis and function

In 1992, quelling was discovered by transforming carotenoid biosynthesis genes, *albino-1* and *albino-3* (*al-1* and *al-3*), which resulted in silencing of endogenous *al-1* and

al-3 genes, as indicated by the albino/pale yellow phenotype and a large reduction in mRNA level in around 30% of the transformants (Romano and Macino 1992). This silencing phenotype was found to be spontaneously and progressively revert to wild type or intermediate phenotypes over a prolonged culture time (Romano and Macino 1992; Fulci and Macino 2007). The length of a transgene needs to be longer than ~132nt in order to have silencing effect. The initiation of quelling is independent of promoter sequences or other specific sequences, suggesting that it is likely to be triggered by some aberrant DNA structure. Furthermore, it should be noted that there is a strong correlation between the copy number of transgenes and the silencing efficiency. In fact, progressive phenotypic reversion of quelled strains correlates with the reduction of the number of the ectopic integrated sequences (Romano and Macino 1992; Cogoni et al. 1994; Cogoni et al. 1996).

Quelling was proposed to be mediated by RNA based on several observations. First, mutations in carotenoid genes are typically recessive; however, most of the *al-1* quelled strains were heterokaryons and were dominant over wild type strains, indicating a diffusible, trans-acting molecule is involved in quelling (Cogoni et al. 1996). Second, a sense RNA derived from exogenous promoter-less *al-1* transgene was specifically found in quelled strain but absent in the reverted strains, suggesting that the transcription of transgenes is involved in the quelling pathway (Cogoni et al. 1996). In addition, it was shown that quelling act in post-transcriptional level because the amount of precursor mRNA was about the same in both quelled and non-quelled strains, whereas the amount of mature mRNA was largely reduced in quelled strains (Cogoni et al. 1996). These findings together led to the hypothesis that the production of aberrant RNA (aRNA) in

the presence of multi-copies of a transgene causes post-transcriptional gene silencing in a trans-acting manner. This was one of the earliest studies suggesting that aRNA transcript is involved in gene silencing mechanism. Several components involved in quelling pathway have been identified by either forward- or reverse-genetic approaches (Cogoni and Macino 1997; Catalanotto et al. 2004; Dang et al. 2011). Mutational analysis led to the isolation of three complementation groups of quelling-defective (*qde*) mutants, called *qde-1*, *qde-2*, and *qde-3*. Studies of these three genes together with findings from other organisms established the general frame work of quelling pathway as one of the RNA interference related mechanism which is conserved across eukaryotes (Cogoni and Macino 1997). QDE-1 is the first RNAi component ever identified, which is a putative protein homologous to RNA-dependent RNA polymerase (RdRP). The requirement of an RdRP in quelling demonstrates that dsRNA is a necessary intermediate to trigger gene silencing (Cogoni and Macino 1999a).

QDE-3 belongs to the RecQ helicase family. RecQ helicases are typically involved in homologous recombination, DNA replication and DNA repair (Cogoni and Macino 1999b). It has been reported that QDE-3 and another RecQ helicase homolog, RecQ2, play roles in DNA repair in *Neurospora* (Pickford et al. 2003; Kato et al. 2004). We have reasoned that quelling is not due to specific sequences since a large variety of DNA fragments can induce quelling without promoters. Thus, it is likely that certain specific aberrant chromatin structure of repetitive transgenes facilitates the production of siRNA. The involvement of QDE-3 in quelling pathway suggests that repetitive transgenes can be recognized and processed by QDE-3 to produce aberrant DNA structure which can further promote siRNA production. Interestingly, OsRecQ1, a RecQ

helicase homolog in rice, was later found to be required for inverted-repeat induced RNA silencing (Chen et al. 2008). rRecQ-1, a homologue of QDE-3 in rats was reported to be associated with piRNA-binding complex (Lau et al. 2006).

QDE-1 and QDE-3 function in the upstream of quelling pathway, very likely to be involved in the production of aberrant RNA, because inverted repeat-containing transgene can bypass QDE-1 and QDE-3 to produce dsRNA which lead to gene silencing (Li et al. 2010). One important question is that how QDE-1 and/or QDE-3 produce RNA from DNA template. In plant, it has been demonstrated that RNA Pol IV and Pol V are required for the production of non-coding RNA which is the precursor of endogenous siRNA (Wierzbicki et al. 2008). In pombe, Pol II is the RNA polymerase to produce centromeric siRNA precursor (Djupedal et al. 2005; Schramke et al. 2005). Crystal structure of QDE-1 revealed that its catalytic core is similar to eukaryotic DNA-dependent RNA polymerase (DdRP)(Salgado et al. 2006). This information give rise to a possibility that QDE-1 might function as a DdRP. Recently our lab has demonstrated that QDE-1 is indeed both DdRP and RdRP which utilize ssDNA and ssRNA as templates to produce DNA/RNA hybrid and dsRNA, respectively (Lee et al. 2010a). Interestingly, the DdRP activity of QDE-1 is even higher than its RdRP activity *in vitro*. This is consistent with the observation that QDE-1 is not involved in the amplification and production of secondary small RNAs, unlike some RdRPs in some other organisms (Lee et al. 2009). QDE-1 has been shown to associate with RPA-1, which is the largest subunit of Replication Protein A, involved in DNA replication, recombination and repair (Nolan et al. 2008; Lee et al. 2010a). RPA can also strongly promote dsRNA production by QDE-1 from ssDNA by preventing the formation of

DNA/RNA hybrid. These results suggest that RPA plays a dual role in the production of aRNA: recruiting QDE-1 to ssDNA and blocking the formation of DNA/RNA hybrid. Another major question for quelling is that how exactly repetitive transgenes are recognized and distinguished from other genomic loci to produce aRNA? Repeat induced gene silencing has been reported in almost all the model systems, including fungi, plants, *C. elegans*, *Drosophila*, and mammals (Hsieh and Fire 2000). It is likely that repetitive sequences result in some aberrant structures via DNA replication of recombination mediated by QDE-1 and RPA. These aberrant DNA structures somehow can be recognized by QDE-1 to produce aRNA. However, the exact mechanism was largely unclear, and it becomes the main question for my thesis project.

Upon the production of dsRNA precursor, two RNase III domain-containing proteins, Dicer-like-1 (DCL-1) and DCL-2, process it into about 25-nt small RNAs in an ATP-dependent manner (Catalanotto et al. 2004). The elimination of both dicer genes completely abolished quelling and the processing of dsRNA into a siRNA form. However, single mutants displayed the quelling frequencies comparable to that of the wild-type strain, indicating that these two Dicers are functionally redundant. In vitro assay suggested that DCL-2 might be a major dsRNA processing enzyme because *dcl-2* mutant but not *dcl-1* mutant showed reduced Dicer activity (Catalanotto et al. 2004).

QDE-2, an Argonaute homolog in *Neurospora*, can associate with siRNA duplex and form an inactive RISC complex (Catalanotto et al. 2000; Catalanotto et al. 2002). In consistent with this, the dsRNA production in gene silencing depends on functional QDE-1 and QDE-3 but does not depend on functional QDE-2, indicating that QDE-2 functions in the downstream step of the gene silencing pathway (Catalanotto et al. 2002).

In order to activate RISC complex and execute gene silencing, passenger strand of the siRNA duplex needs to be removed. We showed that QDE-2 and its slicer activity are required for single stranded siRNA production and gene silencing in vivo (Maiti et al. 2007). This provides the first in vivo evidence that Argonaute is involved in generating single-stranded siRNA and RISC activation. However, QDE-2 alone is not sufficient to remove the passenger strand. Biochemical purification of QDE-2 led to the identification of a QDE-2 interacting protein (QIP), which is putative exonuclease (Maiti et al. 2007). Further in vitro experiments have shown that QIP can cleave and remove the nicked passenger strand from siRNA duplex in a QDE-2 dependent manner. Thus, QIP plays a critical role in dsRNA-induced gene silencing and is also the first identified eukaryotic exonuclease required for efficient RNAi. These results indicate that cleavage and removal of passenger strand from siRNA duplex is an essential step in dsRNA-induced gene silencing. Consistently, a *Drosophila* ribonuclease, C3PO (component 3 promoter of RISC), promotes RISC activation by removing siRNA passenger strand cleavage products (Liu et al. 2009).

Taken together, a model for the *Neurospora* quelling pathway (Fig. 1) was proposed: repetitive transgenes form aberrant DNA structures mediated by QDE-3 and RPA. These aberrant DNA will be recognized and transcribed by QDE-1 to produce aberrant RNA and then dsRNA. The dsRNA precursor will be processed into siRNA duplexes by Dicer proteins, which are then loaded onto the RISC. QDE-2 cleaves and removes the passenger strand with the help of QIP to form an active RISC associated with single-stranded siRNA, resulting in gene silencing.

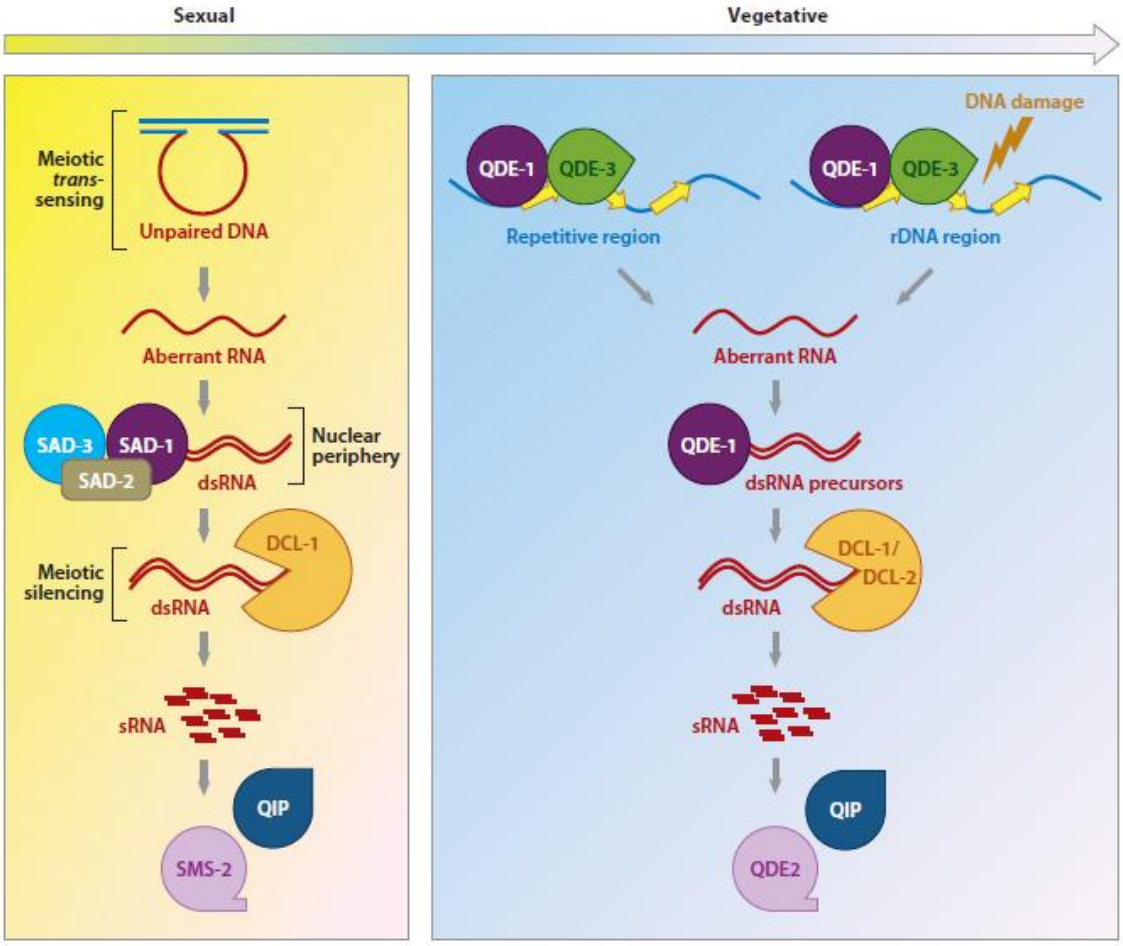

Figure 1. Models for RNAi-related pathways in Neurospora.
A cartoon shows the biogenesis of MSUD pathway during sexual stage (Left), and quelling or qiRNA pathway during vegetative stage (Right).

(Figure adapted from Shwu-Shin Chang et al. 2013)

Quelling is triggered in the presence of repetitive transgenes in *Neurospora* genome, which indicates a possible function in genome defense by suppressing transposon activity. However, due to the RIP process during the sexual cycle (Galagan and Selker 2004), most of the repetitive genes have been mutated, resulting in no

functional transposons in most of the *Neurospora* strains examined. Despise the scarse detection of transposons in *Neurospora*, an African exotic strain was studied and detected a functional LINE-like transposon, Tad (Kinsey 1990). Nolan *et al*. introduced this Tad transposon into the *Neurospora* laboratory strain and showed that repression of its activity requires QDE-2 and Dicer, but not QDE-1 or QDE-3 (Nolan et al. 2005). These results suggest that transposition of Tad may generate inverted repeats that form dsRNA without the requirement of QDE-1 and QDE-3.

1.3 qiRNA, a type of DNA damage-induced siRNA

In addition to quelling-induced siRNA, another class of endogenous siRNA during vegetative stage was detected under DNA damage condition (Lee et al. 2009). Because this type of small RNAs was associated with QDE2, it was termed as QDE-2-interacting small RNA, short for qiRNA (Lee et al. 2009). qiRNAs have a strong preference of 5' uridine and 3' adenine. Deep sequencing analysis showed that most of the qiRNAs (~90%) originate from ribosomal DNA (rDNA) cluster. It should be stressed here that rDNA remains the only highly repetitive region in the *Neurospora* genome due to the existence of repeat-target gene silencing/mutation mechanisms, such as MSUD and RIP (Aramayo and Metzenberg 1996; Selker 2002).

Interestingly, qiRNAs are produced not only from the transcribed region of rDNA but also the untranscribed intergenic spacer regions, indicating that qiRNA originate from aRNA precursors via unconventional transcription. Northern blot revealed that the size of aRNA ranges approximately from 500bp~2kb. The production of qiRNA does not dependent on Pol I, Pol II, or Pol III. However, genetic study showed that qiRNA

biogenesis requires QDE-1, QDE-3, and Dicers, as similar to quelling pathway (Lee et al. 2009). These results further suggest that qiRNA is specifically made by RNAi machinery but not resulted from non-specific rRNA degradation. In the *dicer-1*/*dicer-2* double knock-out strain, aRNA was accumulated, indicating that Dicers function in the processing of aRNA into small dsRNA (Lee et al. 2009). *qde-1* and *qde-3* mutants abolish the production aRNA, indicating that the RecQ helicase and the RdRP/DdRP QDE-1 are required for the biogenesis of DNA damage induced aRNA. This result is consistent with the notion that QDE-1 is a DNA-dependent RNA polymerase which generates single strand aRNA from genomic loci (Salgado et al. 2006; Lee et al. 2009; Lee et al. 2010a).

qiRNA can be induced by a large variety of DNA damage agents, including hydroxyurea (HU), camptothecin (CPT), histidine, methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS), which cause DNA damage through different ways (Lee et al. 2009; Zhang et al. 2013). Moreover, mutant strains that are deficient in DNA damage repair or check point pathways showed the elevated level of qiRNA without additional DNA damage agent treatment (Lee et al. 2009). Because all these external or internal cues to induce qiRNA will ultimately cause double strand break, these results suggest that DSB is a trigger for qiRNA induction. Since the discovery of qiRNA, DNA damage-induced sRNA was also discovered in different organisms (Francia et al. 2012; Michalik et al. 2012; Wei et al. 2012; Sharma and Misteli 2013), suggesting that DNA damage is a shared trigger for small RNA production in eukaryotes. However, how DNA damage is sensed and promotes the production of aRNA was largely unclear.

Like quelling siRNA functions at post transcriptional level by degrading mRNA with active RISC complex, qiRNA derived from rDNA locus can also inhibit rRNA production and proteins synthesis after DNA damage. This inhibition effect was partially rescued in the *qde-1* and *qde-3* mutant (Lee et al. 2009). Consistent with the role of qiRNA in the DNA damage response, QDE-3 was previously shown to play a role in DNA damage repair and response (Cogoni and Macino 1999b; Pickford et al. 2003; Kato et al. 2004). Furthermore, QDE-1 and Dicer mutants also showed increased sensitivity to DNA damage agents' treatment, suggesting that qiRNA may provide another mechanism that contributes to DNA damage checkpoints by inhibiting protein synthesis (Lee et al. 2009). In *Arabidopsis*, RNAi components are found to be enriched in the nucleolus and rDNA-derived siRNAs facilitate heterochromatin formation (Pontes et al. 2006). Furthermore, the *Drosophila* RNAi deficient mutants displayed disorganized nucleoli and rDNA (Peng and Karpen 2007). These reports suggest it is a conserved mechanism across eukaryotes that rDNA- or other endogenous repetitive sequences-derived siRNA might function to maintain genome integrity and stability.

1.4 Summary

The small RNA biogenesis pathways have been intensively studied in recent years and we have proposed a model for the production of aRNA and sRNA. However, how aRNAs are transcribed from repetitive genomic loci is largely unknown. QDE-1 and QDE-3 alone are apparently not sufficient to recognize and drive transcription from specific loci. In chapter 2, I aim to answer this question by starting with a large scale screen using the *Neurospora* knock out library. We identified homologous recombination

components as new players in the *Neurospora* RNAi pathway. Moreover, we elucidate the mechanistic relationship between quelling and qiRNA pathways. Our results demonstrate that quelling and qiRNA are mechanistically similar and both of them result from DNA damage.

In chapter 3, I further investigate the mechanism underlying the homologous recombination based siRNA biogenesis pathway. We found aRNA production is regulated at the chromatin level. An H3K56 specific histone acetyltransferase, RTT109, was discovered to be required in the quelling and qiRNA pathway. My results showed that it function through the involvement of homologous recombination. Taken together, our results provide an explanation for how repetitive DNA sequences give rise to small RNAs and lead to gene silencing.

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CHAPTER TWO

HOMOLOGOUS RECOMBINATION AS A MECHANISM TO RECOGNIZE REPETITIVE DNA SEQUENCES IN AN RNAI PATHWAY

2.1 Introduction

RNA interference (RNAi) is a mechanism of gene silencing conserved from fungi to mammals (Catalanotto et al. 2006; Buhler and Moazed 2007; Ghildiyal and Zamore 2009). RNAi is an ancient genome defense mechanism that silences transposons and viral invasion (Sijen and Plasterk 2003; Siomi et al. 2008; Wang et al. 2010; Chang et al. 2012). Consistent with this role of RNAi, a significant portion of eukaryotic small RNAs, including siRNA and the piwi-interacting small RNAs (piRNAs), are produced from repetitive DNA loci in fungi, plants, and animals and target active transposon sequences or their relics (Siomi et al. 2008; Ghildiyal and Zamore 2009). How small RNAs are specifically produced from repetitive DNA loci is not clear.

As described in chapter one, quelling is triggered by multiple copies of transgenes and produces transgene-specific siRNA (Catalanotto et al. 2006). In the quelling pathway, QDE-1 (Quelling-Deficient-1) first acts as a DNA-dependent RNA polymerase (DdRP) to produce aberrant RNA (aRNA) from the repetitive transgene loci and then uses its RNA-dependent RNA polymerase (RdRP) activity to convert aRNA into dsRNA (Cogoni and Macino 1999a; Lee et al. 2010). This process requires the RecQ DNA helicase QDE-3 and the single-stranded DNA binding complex RPA (Cogoni and Macino 1999b; Nolan et al. 2008; Lee et al. 2010). dsRNA is then processed by Dicer proteins to produce siRNA, which is then loaded onto the Argonaute protein QDE-2 to mediate post-transcriptional gene silencing (Maiti et al. 2007). In most *Neurospora* strains, the ribosomal DNA locus has the only repetitive DNA sequences. The potent silencing effect of quelling on

repetitive transgenes suggests that it is an anti-transposon response. Consistently, the quelling pathway suppresses the replication of a functional transposon (Nolan et al. 2005).

We previously discovered that DNA damage induces the expression of QDE-2 and a class of small RNAs named qiRNAs for their association with QDE-2 (Cecere and Cogoni 2009; Lee et al. 2009; Lee et al. 2010). qiRNAs originate from the rDNA locus, which contains ~ 200 copies of rDNA repeat, and their production depends on QDE-1, QDE-3, Dicers, and RPA. qiRNA levels are very low under normal growth conditions. DNA damage-induced small RNAs were recently also discovered in *Arabidopsis*, fly, and mammals (Francia et al. 2012; Michalik et al. 2012; Wei et al. 2012), suggesting that DNA damage is a common trigger for small RNA production in eukaryotes.

How does the quelling pathway specifically target repetitive DNA sequences? What is the mechanistic link between quelling and the DNA damage-induced qiRNA production? In this chapter, we show that the homologous recombination (HR) process is essential for qiRNA production and quelling. We further show that the qiRNA and the quelling-induced siRNA share the same biogenesis mechanism. Together, our results suggest that *Neurospora* uses HR to distinguish repetitive DNA loci from the rest of the genome.

2.2 Materials and methods

2.2.1 Strains and growth conditions

A wild-type strain of *Neurospora crassa* (FGSC4200) was used in this study unless otherwise indicated. Mutant *Neurospora* strains are listed in Table 1. The *Neurospora* knock-out mutant strains used in this study were created by the *Neurospora* Functional

Genomics Project and obtained from the Fungal Genetic Stock Center (Colot et al. 2006). Liquid culture medium contained 1xVogel's and 2% glucose.

Table1

The Neurospora mutant strains used in this study

Mutants	Function Category	FGSC #	NCU#
qde-1	siRNA Pathway, DdRP/RdRP	FGSC11156	NCU07534
qde-3	siRNA Pathway, HR	FGSC12505	NCU08598
rad51 (*mei-3*)	HR	FGSC12433	NCU02741
rad52 (*mus-11*)	HR	FGSC16079	NCU04275
rad54 (*mus-25*)	HR	FGSC6424	NCU11255
srs2 (*mus-50*)	Negative Regulator of HR	FGSC11737	NCU04733
ku70 (*mus-51*)	NHEJ	FGSC20277	NCU08290
ku80 (*mus-52*)	NHEJ	FGSC15968	NCU00077
pnpk	Base Excision Repair	FGSC12525	NCU08151
msh3	DNA mismatch repair	FGSC12523	NCU08115
rad18 (*uvs-2*)	Post Replication Repair	FGSC11444	NCU05210
rad5 (*mus-41*)	Post Replication Repair	FGSC12066	NCU09516
atm (mus-21)	Check Point Pathway	FGSC11162	NCU00274
atr (mus-9)	Check Point Pathway	FGSC5146	NCU11188
chk2	Check Point Pathway	FGSC11170	NCU02814
chd1	Chromatin Remodeling	FGSC14805	NCU03060
swr1	Chromatin Remodeling	FGSC11398	NCU09993
isw1	Chromatin Remodeling	FGSC11780	NCU03875
swi-3	Replication Protection	FGSC19229	NCU01858
mrc1	Replication Protection	FGSC15718	NCU04321
mcl1	Replication Protection	FGSC12518	NCU08484

Liquid cultures were grown in minimal medium (1X Vogel's, 2% glucose). For liquid cultures containing QA, 10^{-3} M QA, pH 5.8, was added to the liquid culture medium containing 1 X Vogel's, 0.1% glucose, and 0.17% arginine. For liquid culture containing DNA damage agents to induce qiRNA production, histidine (1 mg/ml) or the indicated concentrations of HU, were added and cultures were collected 48 hrs later(Lee et al. 2009). For race tube assays containing QA, no glucose was added to the medium.

2.2.2 RNA northern blot analyses

Total RNA extraction, enrichment of small sized RNA, and northern blots were performed as previously described(Maiti et al. 2007). RNA probes were made using the MAXIscript T7 kit (Ambion) from a T7 promoter on a PCR product template. To make small RNA probes, the labeled RNA product was hydrolyzed by adding hydrolysis solution (80 mM $NaHCO_3$, 120 mM Na_2CO_3) to the probe at 60 ^oC for 3 hrs. The sample was then neutralized by adding 3 M NaOAc. aRNA probes were made to detect intergenic transcripts from the rDNA region (upstream of rRNA coding region). Primers used were rDNA2F (5'- ACTTTTGTAATGTCTTGCCCCC-3') and T7 rDNA2R (5'- TAATACGACTCACTATAGGG CCCTACAGCGTCCACTCAAACT-3'). qiRNA probes were made to specifically detect anti-sense small RNA from the 26S rDNA region. Primers were T7 rDNA-2 (5'- TAATACGACTCACTATAGGG TGAAGGACCGAAGTGGGGAAAGG-3') and rDNA-1 (5'- GTCTTCTTTCCCCGCTGATTCTG-3'). *al-1* siRNA probes were used to specifically detect antisense small RNA from the *al-1* gene. The two primers were *al-1-1* for (5'-CTTCCGCCGCTACCTCTCGTGG-3') and *al-1-2* rev (5'-CCCTTTGTTGGTGGCGTTGATG-3').

2.2.3 Homologous recombination assay

The HR assay was performed essentially as described (Ishibashi et al. 2006). The *Neurospora* strains were grown for 7-10 days before harvesting the conidia in 1 M sorbitol. The *Eco*RI linearized *bar*-containing plasmid (pGS1-1KR, contains *bar* gene flanked by 1 kb of homologous sequence of *mtr*) was incubated with the conidial suspension on ice for 30 min. The fragment was transformed into *Neurospora* strains by

electroporation. The transformed conidia were plated onto low nitrogen-containing top agar containing 0.4 mg/ml bialaphos. The bialaphos-resistant transformants were picked onto bialaphos containing slants, and resistant transformants were further selected on FPA-containing slants. The HR rate was calculated as the ratio between the FPA-resistant colonies to the total bialaphos-resistant colonies.

2.2.4 Quelling assay

Quelling assays were performed as previously described with minor modifications (Cogoni et al. 1996). The wild-type and the mutant strains used for quelling assays were grown for 7-10 days before harvesting the conidia in 1 M sorbitol. A mixture of 2 mg pBSK*al-1* (carrying the *al-1* fragment) and 0.5 mg of pBT6 (a benomyl resistant gene-containing plasmid) was incubated with the conidial suspension for 4-5 hrs at 4°C. The plasmids were transformed into *Neurospora* by electroporation. The benomyl resistant transformants were picked onto minimal slants and visually inspected to identify the completely quelled (white), partially quelled (yellow), or non-quelled (orange) strains.

2.2.5 2-D gel electrophoresis

Samples were grown for 2 days in 1 L liquid medium. Samples were then collected and resuspended in 20 ml of cold water and 1 ml of trioxalen (SIGMA, dissolved in 100% EtOH at 200 μg/ml) for 10 min in a petri dish. The petri dish was then exposed to 365 nm UV light for 10 min and incubated without UV light for another 10 min. This step was repeated twice, and then DNA was extracted. Total DNA was digested by EcoRI overnight. About 10 μg of total EcoRI DNA fragments were used for 2-D gel electrophoresis. The 2-D gel electrophoresis was performed as described previously

(Brewer and Fangman 1987; Zou and Rothstein 1997). A DNA probe was made using the Rediprime II DNA Labeling System (Amersham). The DNA template was 1.5 kb long and amplified by primers rDNA26F (5'- GGAAGCGTTTGTGACCAG-3') and rDNA26R (5'- CCAACCCTTAGAGCCAAT-3'). HR intermediates from the signal in the region denoted by the open arrow in Figure 8 were quantified using the ImageQuant 5.2 program. Data were normalized to the amount of total linear DNA in the right lower corner of the image.

2.2.6 Chromatin immunoprecipitation assay

Conidia of strains of interest were grown in 150 mm x 15 mm plates with 50 ml of liquid media for 2 days to form mats. *Neurospora* discs cut from mat were inoculated into liquid culture. The discs were grown on shakers for 40 hrs at room temperature. The discs were fixed in the culturing media containing 1% formaldehyde for 15 minutes with shaking. Glycine was added to 125 mM, and samples were incubated for 5 min. The discs were transferred to washing buffer (50 mM HEPES, pH 7.5, 137 mM NaCl) for 5 minutes with shaking and harvested under vacuum. Protein extracts were prepared by adding lysis buffer (50 mM HEPES, pH 7.5, 137 nM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS) containing protease inhibitors. DNA was sheared by sonication to approximately 500-1000 bp fragments (DNA fragment sized was confirmed on agarose gels). Sonication was performed with 3 cycles of 25 pulses with duty cycle 40 and output control 4. The samples of 2 mg of protein were pre-cleared with 40 µl of slurry equilibrated with Gamma Sepharose beads for 2 hrs at 4 °C with rotation. The blocking beads were resuspended, and the lysate was transferred to a new tube. The myc antibody was added to the pre-cleared protein lysate and incubated for another 2 hrs

with rotation. No antibody was added to the control sample. A 40-µl slurry of Gamma Sepharose beads was subsequently added to each sample. Samples were incubated for another 1 hr at 4 °C with rotation. The beads were washed with Low Salt Immune Complex Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl) for 5 min on a shaker, with High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCl, 500 mM NaCl) for 5 min on a shaker, with LNDET Buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) for 5 min, and finally twice with 1XTE (1 mM EDTA, 10 mM Tris, pH 8.0). Chelex beads (100 µl, 10% beads) were added to the sample, and samples were vortexed briefly. The samples were boiled at 94 °C for 10 min and then chilled on ice. The supernatants were diluted 1 to 2.5 before use as templates for qPCR analysis. PCR primers used here correspond to the inserted region of *al-1* gene in pBSK*al-1*: qal-1F (5'-AAGGTGTTGGACGCTTTGGT-3') and qal-1R (5'-GTACTTGACGCCCATCCTCTCT-3').

2.3 Results

2.3.1 Homologous recombination components are identified to be essential for qiRNA production.

Treatment of *Neurospora* with DNA damage agents, such as histidine, hydroxyurea (HU), or ethyl methanesulfonate (EMS), induces the production of qiRNA and induction of QDE-2 protein expression (Lee et al. 2009). In addition, the DNA damage-induced QDE-2 expression is abolished in mutants defective for qiRNA production and quelling (Lee et al. 2009; Lee et al. 2010). To identify additional components of qiRNA and

quelling pathways, we reasoned that mutants deficient in DNA damage-induced QDE-2 expression would also be defective in qiRNA and quelling pathways. Therefore, using the available *Neurospora* knockout mutants (Colot et al. 2006), we carried out a comprehensive QDE-2 western blot analysis-based screen to identify mutants with impaired histidine-induced QDE-2 expression. Among the more than 2,000 *Neurospora* knockout mutants examined, we found that a knock-out mutant of *rad52* (also called *mus-11*) was deficient in the histidine-induced QDE-2 expression (Fig. 2A).

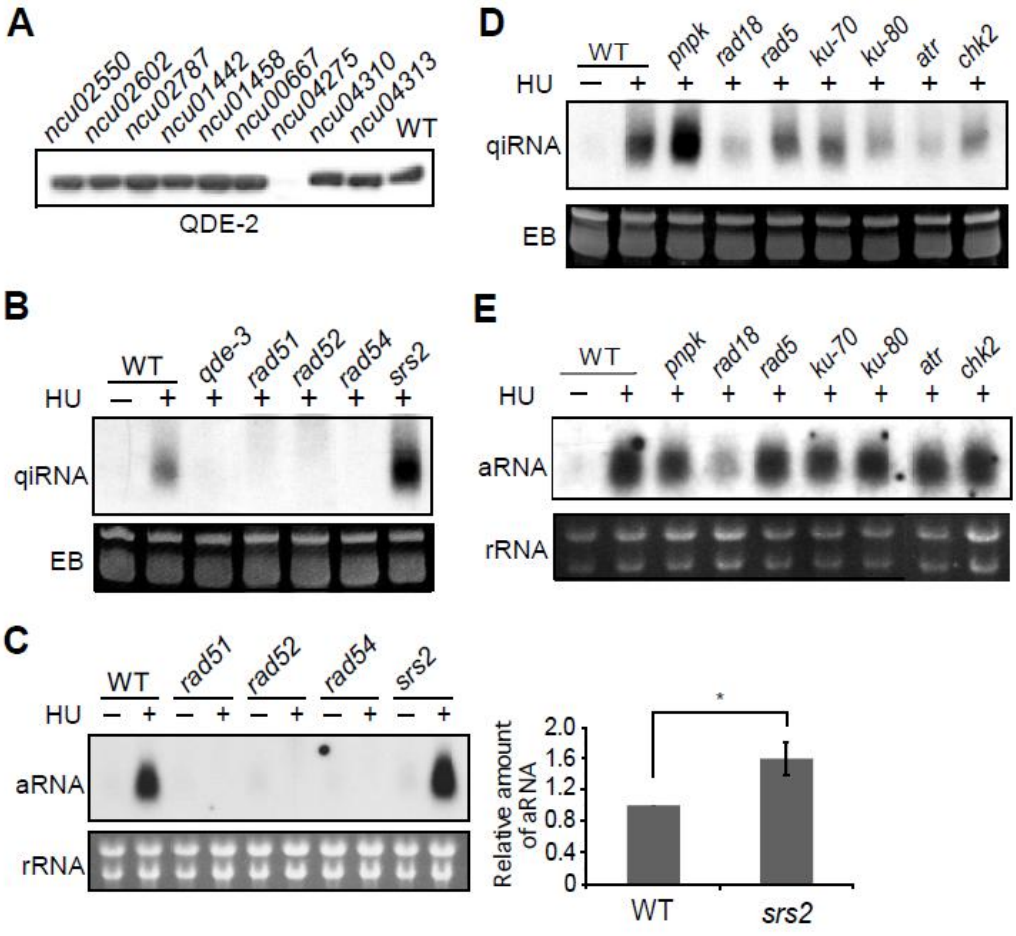

Figure 2. Homologous recombination is required for qiRNA and aRNA production.
(**A**) QDE-2 western blot analysis of a panel of knock out strains. All the strains were grown in histidine for 2 days. (**B**) & (**C**) Northern blot analysis of qiRNA and aRNA products from mutants deficient in HR pathways. Samples were collected 2 days after 1 mg/ml HU treatment. Ethidium bromide stained membranes were used as loading controls. Right panel in (**C**), quantification of the relative HU-induced aRNA levels in wild-type and *srs2* knock out strains. n=3; * P<0.01; error bars indicate s.d. (**D**) & (**E**) Northern blot analysis of qiRNA and aRNA products in different damage repair and checkpoint mutants.

RAD52 is an essential component of the eukaryotic HR pathway (Sancar et al. 2004). In addition to RAD52, RAD51 and RAD54 are also essential for HR. Thus, we examined the HU-induced qiRNA production in the *rad51* (*mei-3*), *rad52*, and *rad54* (*mus-25*) mutants. As shown by the small RNA northern blot in Figure 2B, HU treatment at 1 mg/ml, a concentration that still permits cell growth, resulted in the induction of qiRNA in a wild-type strain, but such a response was abolished in the *qde-3*, *rad51*, *rad52*, and *rad54* mutants. We then examined the expression of the rDNA-specific aRNA (sizes ranging from 0.5 to 2 kb), the precursor of qiRNA and found that the HU-induced aRNA was also abolished in the HR mutants (Figure 2C). In addition, the production of aRNA in the *rad54* mutants could be rescued by a transgene that expresses c-Myc-tagged RAD54 (Figure 3A). *srs2* (*mus-50)* encodes a DNA helicase that inhibits the HR process by dislodging RAD51 from the RAD51-nucleoprotein filament (Sung and Klein 2006). As expected, we found that knock-out of *srs2* in *Neurospora* resulted in elevated levels of HU-induced qiRNA, aRNA, and QDE-2 (Fig. 2B, C, 3B).

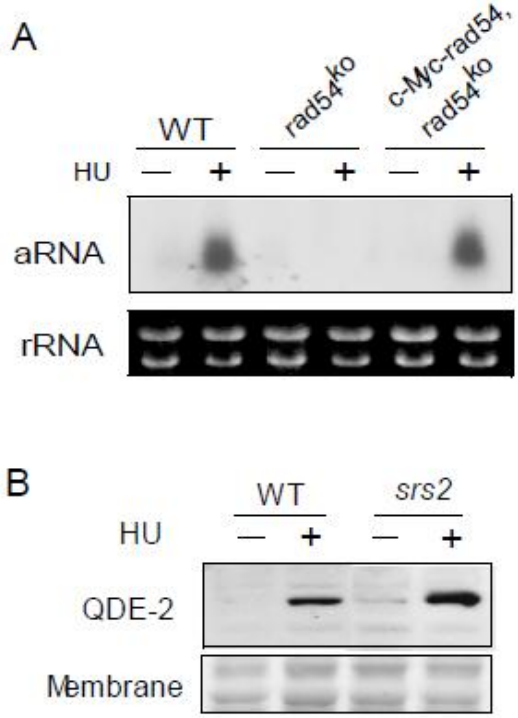

Figure 3. *rad54* phenotype complementation.
(A) Expression of Myc-tagged RAD54 restores the DNA damage-induced aRNA expression in the *rad54* mutant.
(B) QDE-2 western blot analysis showing the induction of QDE-2 by HU. Strains were grown in 1mg/ml HU.

Because HR is a part of the DNA repair and recombination processes, we examined the HU-induced qiRNA and aRNA production in a series of *Neurospora* DNA repair and checkpoint pathway mutants (Fig. 2 D and E, and data not shown). These mutants included *rad18* (post-replication repair), *rad5* (post-replication repair), *ku70/mus-51* and *ku80/mus-52* (non-homologous end joining), *atr/mus-9* and *chk2* (checkpoint), *pnpk* (single-strand break and base excision repair), and *msh3* (mismatch repair). In contrast to the HR mutants, the HU-induced qiRNA and aRNA could still been produced in these mutants. Moreover, the *qde-1* and *qde-3* mRNA levels in these mutants and in the HR mutants were comparable to those in the wild-type strain, indicating that the phenotype observed in the HR mutants is not due to low QDE-1 or QDE-3 levels (Figure 4). These

results indicate that HR, but not other DNA repair and checkpoint pathways, is required for qiRNA synthesis in a step upstream of aRNA production.

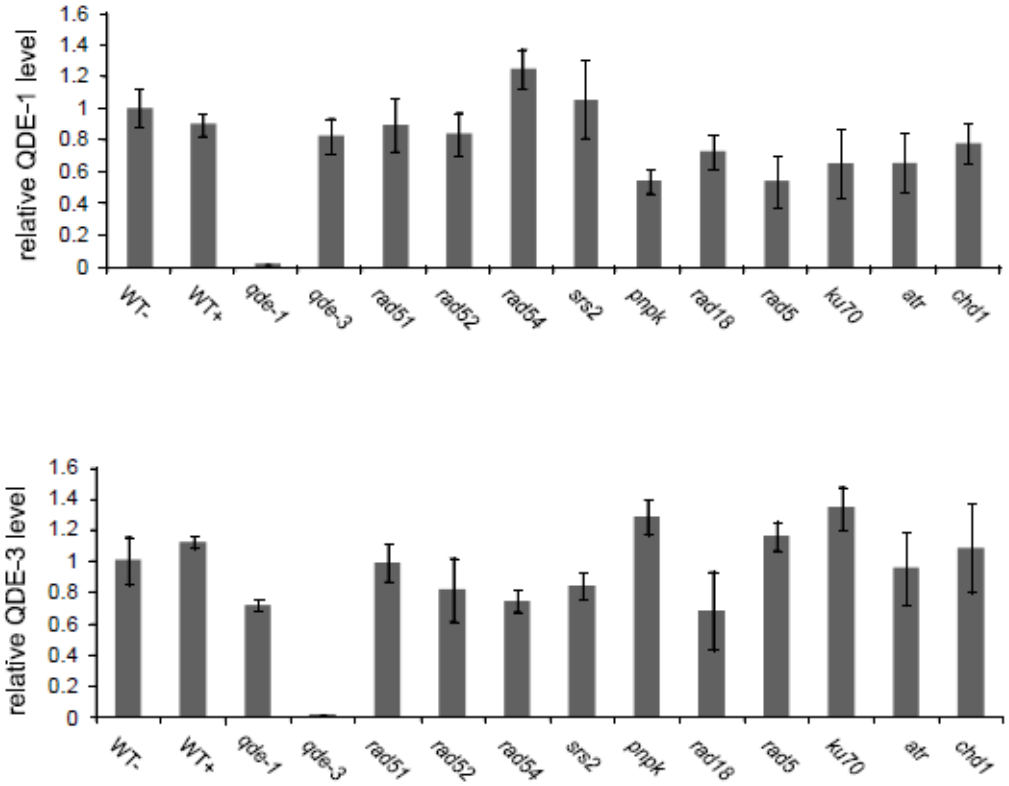

Figure 4. QDE-1 and QDE-3 levels remain similar in different mutants. qRT-PCR analysis showing relative expression level of QDE-1 and QDE-3 in the indicated mutants. The first lane is wild type under normal growth condition. All the rest strains were grown under 1mg/ml HU treatment. QDE-1 and QDE-3 level were normalized to actin level. N=3; error bars indicate s.d.

2.3.2 Chromatin remodelers are required for qiRNA production

In our screen, several genes encoding for ATP-dependent chromatin-remodeling enzymes were also found to be required for the histidine-induced QDE-2 expression (Fig. 5A and data not shown). These genes encode the *Neurospora* homologs of *swr1*, *iswi*, and *chd1* (NCU09993, NCU03875, and NCU03060, respectively). As shown in Figure 5B, both qiRNA and aRNA production induced by HU were completely or mostly abolished in the *swr1*, *iswi*, and *chd1* mutants, indicating the important roles of these proteins in qiRNA biogenesis. These results further suggest that qiRNA and aRNA production is regulated at the chromatin level.

These chromatin-remodeling enzymes utilize ATP to alter histone-DNA contacts, causing changes in the status of chromatin by moving and restructuring nucleosomes (Clapier and Cairns 2009). Chromatin remodeling factors INO80 and SWR1 have been previously reported to be associated with DNA repair processes and INO80 is involved in HR process (Vignali et al. 2000; van Attikum et al. 2007). ISW1 is involved in replication initiation and promotes replication fork progression (Vincent et al. 2008). Because HR is required for qiRNA biogenesis, we hypothesized that these enzymes are also involved in HR. Thus, we examined the recombination rates at the *methyltryptophan resistance* (*mtr*) locus in different *Neurospora* strains by transforming cells with a construct containing the bialaphos-resistance gene (*bar)* that can disrupt the *mtr* gene by HR (Ishibashi et al. 2006). The targeting of *bar* gene into the *mtr* gene results in transformants that are resistant to both bialaphos and the amino acid analog *p*-fluorophenylalanine (FPA). The wild-type strain has a recombination rate of approximately 20% (Figure 5C), which is a typical HR rate in *Neurospora*. As expected,

the HR rates of the *ku80* strain and the *rad51* strain are nearly 100% and 0%, respectively. Consistent with a role of SRS2 in suppressing HR, the *srs2* mutant exhibited ~50% HR rate. On the other hand, the HR rates were very low or completely abolished in the *swr1*, *isw1*, and *chd1*mutants. These results suggest that these chromatin-remodeling enzymes are required for qiRNA production due to their non-redundant roles in HR and that the chromatin-remodeling factors act collaboratively to regulate chromatin status during qiRNA production process.

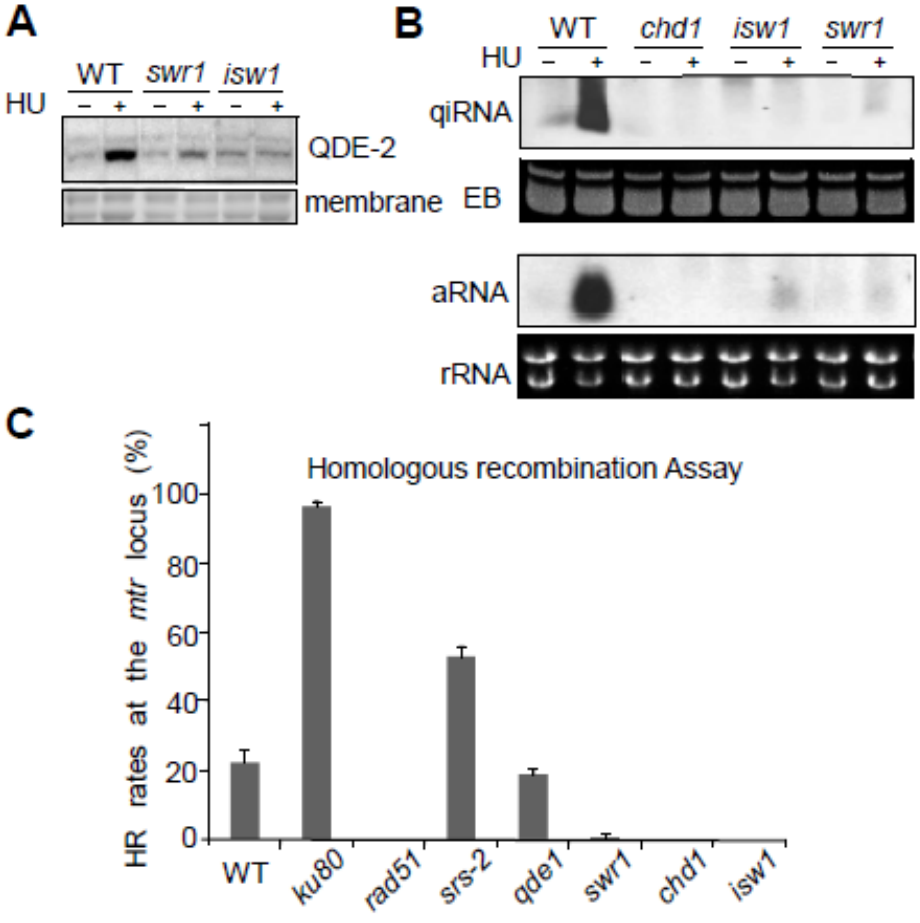

Figure 5. ATP-dependent chromatin remodelers are involved in qiRNA pathway. **(A)** QDE-2 western blot analysis of indicated strains. Cultures were grown in histidine for 2 days. (**B**) Northern blot analysis showing qiRNA and aRNA production in different strains after 2 days of treatment. (**C**) HR assays showing the HR rates of the indicated strains. n≧3; error bar indicates s.d.

2.3.3 qiRNA production requires DNA replication

HR is the predominant DNA double strand break (DSB) repair pathway during S and G2 phases when replicated sister chromatin provides a template for HR-dependent repair (Sancar et al. 2004). The accumulation of recombination intermediates correlates with the accumulation of replication intermediates, indicating that HR activity occurs most frequently during DNA synthesis (Zou and Rothstein 1997). To determine whether qiRNA production requires DNA replication, we treated the wild-type *Neurospora* with different concentrations of HU. At low concentrations of HU (0.5 and 1 mg/ml), qiRNA and aRNA production were induced, whereas at 8 mg/ml HU, a concentration at which DNA replication is completely blocked (Srivastava et al. 1988), qiRNA and aRNA production were abolished (Fig. 6A &B). Similarly, treatment of wild-type cells with EMS also led to the induction of qiRNA at low concentrations but a blockade of qiRNA synthesis at a high concentration (Fig. 6A). A similar HU dose-dependent response was also observed in an *atm* mutant (Fig. 6B). These results suggest that qiRNA biogenesis requires DNA replication even though qiRNA production is induced by modest DNA damage that can result in partial replication inhibition.

To further confirm our conclusion, we created *Neurospora* strains in which one of the essential components for DNA replication, proliferating cell nuclear antigen (PCNA), can be silenced by quinic acid (QA) inducible *pcna*-specific dsRNA (Cheng et al. 2005). PCNA is a processivity factor that forms a complex with DNA polymerase and acts as a clamp that tethers DNA polymerase to DNA template (Kelman 1997). Figure 6C shows the race tube results that compared the growth phenotypes of the wild-type strain and two independent *pcna* knock-down strains (ds*pcna*). In the presence of QA, the cell growth of the ds*pcna* strains was dramatically inhibited, indicating that DNA replication was inhibited by the silencing of *pcna* (Fig. 6C). In the presence of QA, the HU-induced qiRNA production was completely abolished in the ds*pcna* strains (Fig. 6D). To examine whether the inhibition of DNA replication leads to a non-specific loss of all small RNA, we examined the levels of an siRNA at different concentrations of HU and found that *albino-1* (*al-1*) siRNA (produced from a double-stranded *al-1* hairpin RNA) was expressed at similar levels at all concentrations of HU tested (Fig. 6E). This result suggests that HU treatment does not affect the stability of small RNA. Together, these results suggest that qiRNA biogenesis requires DNA replication and occurs during the S phase of the cell cycle.

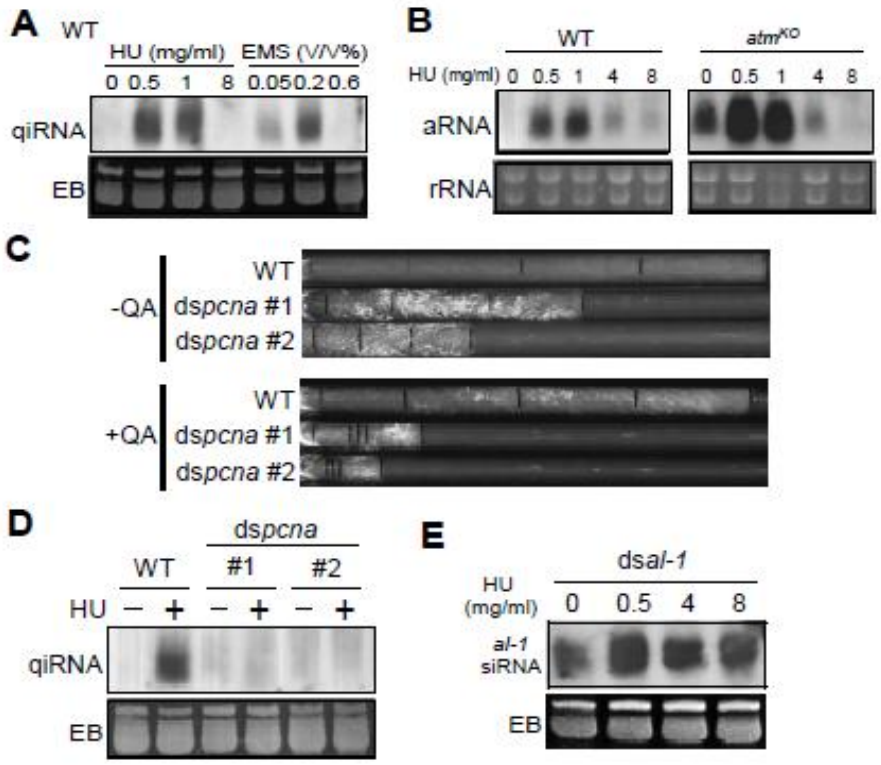

Figure 6. DNA replication is required for qiRNA biogenesis.
(**A**) Northern blot analysis showing the levels of qiRNA in the wild-type strain grown in the indicated concentrations of HU and EMS. (**B**) Northern blot analysis showing aRNA production in different strains. (**C**) Race tube analysis showing the growth of indicated strains in race tubes. ds*pcna* #1 and #2 strains are two independent dsRNA knock down strains in which *pcna* dsRNA expression can be induced by QA ($1x10^{-3}$ M). The black lines indicate cell growth fronts that were marked every 24 hours. (**D**) Northern blot analysis showing elimination of qiRNA production in the ds*pcna* strains. Culture media contain 10^{-3} M QA. (**E**) Northern blot analysis showing the levels of *al-1* siRNA in the ds*al-1* strain that expresses *al-1* specific dsRNA.

2.3.4 rDNA-specific recombination intermediates accumulate upon DNA damage.

The requirements for HR and the RecQ DNA helicase QDE-3 in qiRNA production raised the possibility that recombination of rDNA repeats results in production of an

"aberrant" DNA structure that acts as a trigger for qiRNA production. To test this hypothesis, we subjected unsynchronized *Neurospora* cultures to HU treatment, extracted genomic DNA, and performed 2-D electrophoresis assays. In the absence of HU, little or no replication intermediates of rDNA could be observed (Fig. 7). The HU treatment resulted in the accumulation of rDNA-specific replication intermediates in both the wild-type and *srs2* strains, which are represented as an "arc" in the 2-D electrophoresis assay (Brewer and Fangman 1987). In addition, a low level of recombination intermediates (indicated by an open arrow), which are typically represented as a spike above the replication intermediates on the 2-D gels (Brewer and Fangman 1987; Zou and Rothstein 1997), appeared for the HU-treated wild-type culture. Consistent with a negative role for SRS2 in the HR process, the levels of both replication intermediates and recombination intermediates were further increased in the *srs2* mutant. This result suggests that HU treatment results in the formation of recombination-mediated DNA structures, which are likely the trigger for qiRNA production.

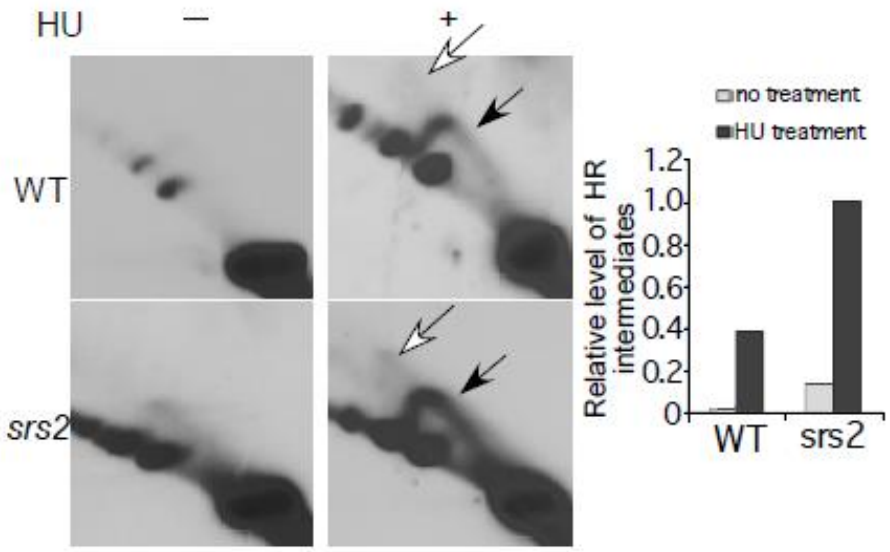

Figure 7. HR intermediates are induced upon DNA damage.
2-D gel electrophoresis assay of wild-type strain and *srs2* mutant strain showing the accumulation of replication and HR intermediates upon HU treatment. HU concentration was 1 mg/ml. Solid and open arrows indicate the replication and recombination intermediates, respectively. The quantification of the 2-D gel electrophoresis assay is shown. The relative amounts of total HR intermediates were normalized to the result from the *srs2* mutant treated with HU.

2.3.5 Quelling requires HR and is also induced by DNA damage

Quelling-triggered gene silencing and siRNA production occur under normal growth conditions and do not require DNA damage agent treatment. However, the similarity between quelling and qiRNA biogenesis suggest that these two phenomena maybe mechanistically the same. To test this hypothesis, we performed quelling assays by transforming *Neurospora* with an *al-1* transgene. As shown in Figure 8A, 29% of the wild-type transformants exhibited quelling, as indicated by the change of conidia color

from orange to yellow or white. In contrast, very low quelling efficiency was observed in the *qde-3*, *rad51*, and *rad54* strains, indicating that like QDE-3, the HR components are required for quelling.

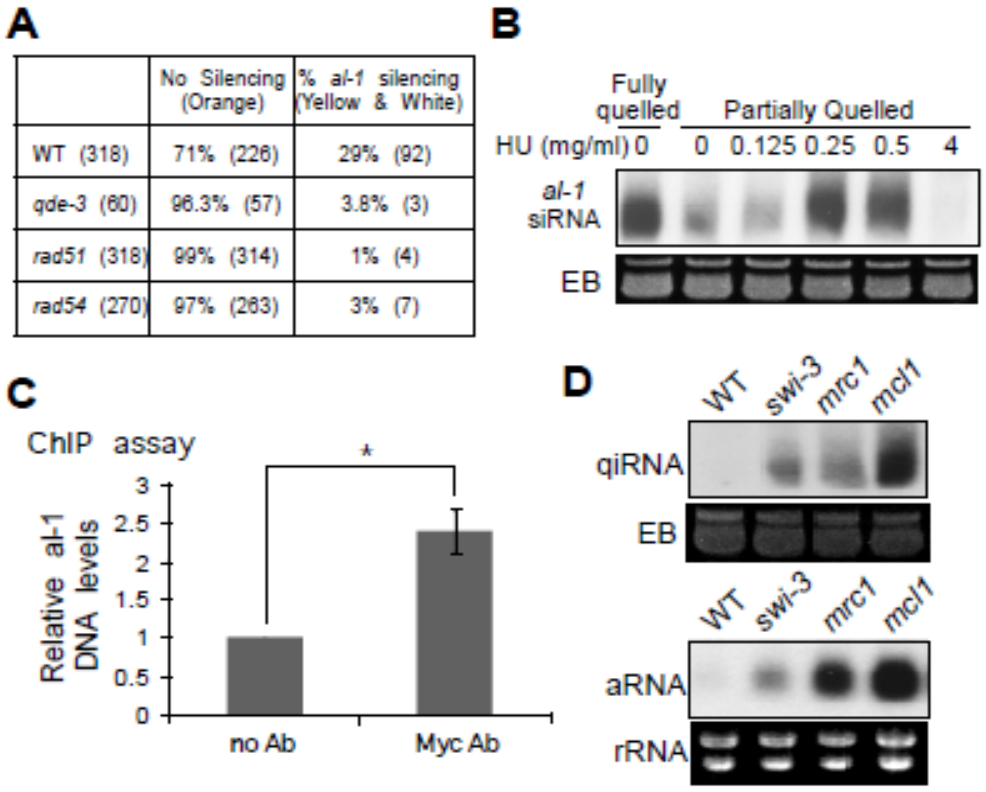

Figure 8. Quelling and qiRNA pathways are mechanistically the same.
(**A**) Quelling efficiency of the indicated strains. The numbers in the parentheses indicate the total number of strains tested, un-quelled strains, or quelled strains. (**B**) Northern blot analysis showing that *al-1*-specific siRNA was induced by HU treatment in a partially *al-1* quelled strain. A fully quelled strain was used as a control. (**C**) ChIP-qPCR analysis using a monoclonal c-Myc antibody showing the enrichment of Myc-RAD51 at the quelled *al-1* locus in a quelled strain expressing Myc-RAD51. n=4; $P<0.01$; error bars indicate s.d. (**D**) Northern blot analysis showing that the levels of qiRNA and aRNA in the indicated mutant strains in the absence of DNA damage treatment.

Because repetitive DNA is known to be a major cause of genome instability due to recombination (Bzymek and Lovett 2001; Vader et al. 2011), we hypothesized that quelling is also caused by DNA damage due to repetitive transgene. Thus, we examined whether the quelling-triggered siRNA, like qiRNA production, is triggered by DNA damage and requires DNA replication. We reasoned that a fully quelled strain might already be subject to high levels of DNA replication stress at the quelled locus and that further DNA damage treatment would not further promote the production of siRNA. Therefore, a partially *al-1* quelled wild-type transformant (yellow conidia) was isolated and was treated with HU at different concentrations. As shown in Figure 8B, the level of *al-1* siRNA was induced to a level that was similar to that of a fully quelled strain at low concentrations of HU, but the production of siRNA was completely abolished at 4 mg/ml HU. As expected, 0.5mg/ml of HU treatment of the partially quelled strain resulted in a decrease of *al-1* mRNA to a level that was comparable that of the fully quelled strain (Figure 9). These results suggest that the quelling-triggered siRNA production is also the result of DNA damage and requires DNA replication.

To determine whether the HR pathway is directly involved in quelling, we expressed c-Myc-tagged RAD51 in an *al-1* quelled strain and performed a chromosome immunoprecipitation (ChIP) assay using a monoclonal c-Myc antibody. As shown in Figure 8C, a significant enrichment of Myc-RAD51 was observed at the *al-1* transgene locus. Taken together, these results indicate that quelling and qiRNA biogenesis share the same pathway and that HR is also an essential step in quelling.

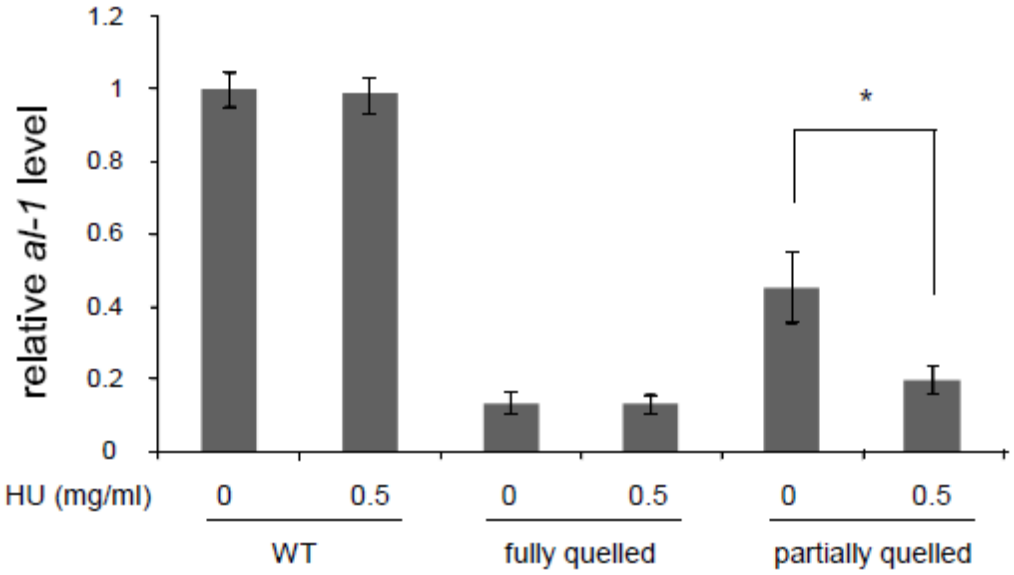

Figure 9. al-1 level in fully and partially quelled strains.
qRT-PCR analysis showing the relative al-1 level in the indicated strains. al-1 mRNA level was normalized to actin level n=3; P<0.01; error bar indicates s.d.

2.3.6 rDNA locus is protected to prevent qiRNA production under normal growth DNA

Why is qiRNA only induced after DNA damage treatment, whereas the quelling-triggered siRNA is produced under normal growth conditions? The eukaryotic rDNA region consists of several hundreds of copies of tandem rDNA repeats that each contains a potential origin of DNA replication. Because of its highly repetitive nature, hyper-recombination can occur at rDNA locus, resulting in genome instability and chromosomal rearrangements. Previous studies have shown that several mechanisms, including rDNA transcriptional silencing and regulation of rDNA replication, prevent rDNA hyper-recombination and maintain rDNA copy numbers (Calzada et al. 2005;

Huang et al. 2006). During rDNA replication, the intergenic replication fork barrier sites in rDNA repeats can stall replication forks unidirectionally so that replication of the rDNA repeats occurs in the same direction as rDNA transcription. The stalled replication forks are protected and maintained by a fork protection complex, which prevents the collapse of the replication fork and is important for maintaining genome stability at the rDNA region. In yeast, the components of this complex have shown to be important for the maintenance and progression of replication fork at the rDNA replication barrier sites (Krings and Bastia 2004; Mohanty et al. 2006).

We hypothesized that mechanisms that suppress rDNA recombination during replication inhibit qiRNA production under normal growth conditions. The *Neurospora swi3* (NCU01858), *mrc1* (NCU04321), and *mcl1* (NCU08484) genes encode the homologs of the fission yeast Swi3p, Mrc1p, and Mcl1p, respectively, which are part of the fork protection complex. To test our hypothesis, we examined the production of qiRNA and aRNA in the *swi3*, *mrc1*, and *mcl1* mutants. As shown in Figure 9D, the levels of both qiRNA and aRNA were high even in the absence of DNA damage agent treatment in these mutant strains. In the fission yeast, SWI3 is important for the replication fork arrest in the rDNA region and deletion of *swi3* resembles the HU-induced replication stress at the rDNA region (Krings and Bastia 2004). Quelling assay results showed that these replication fork protection mutants have normal quelling efficiency (Table 2), suggest that these factors do not have a major impact on repetitive transgenes. These results suggest that the difference between qiRNA production and quelling is that the rDNA locus is normally actively protected from HR but the repetitive transgene loci are not, thus, quelling occurs under normal growth conditions.

Table 2
Quelling assay showing the silencing efficiency of *al-1* gene in the wild type strain and mutants deficient in replication fork protection complex

	% No Silencing (orange)	% *al-1* silencing (yellow & white)
WT	71.7% (86)	28.3% (34)
swi-3	70.6% (113)	29.4% (47)
mrc1	72.3% (117)	27.7% (43)
mcl1	71.6% (118)	28.4% (42)

2.4 Discussion

In this study, we identified HR as an essential process for the DNA damage-induced qiRNA production and quelling. Because qiRNA and quelling are both produced from repetitive DNA loci and the normally protected rDNA locus is the only highly repetitive DNA locus in the *Neurospora* genome, our results suggest that HR is a mechanism that can distinguish repetitive foreign DNA from the rest of the genome. Consistent with this conclusion, the quelling pathway has been shown to suppress transposon proliferation in *Neurospora* (Nolan et al. 2005).

Our study provides important insights into the mechanism of small RNA production from repetitive DNA loci. Even though qiRNA and the quelling-induced siRNA may appear to be triggered by different cues, we showed that the upstream mechanism for their production is the same and that both are results of DNA damage. First, the biogenesis of both types of small RNA require the same set of components, including QDE-1, QDE-3, Dicers, RPA, and the HR components. Second, as qiRNA, the transgene-induced siRNA can also be induced by DNA damage and requires DNA replication. Repetitive DNA is known to be a major source of genome instability in different organisms due to homologous recombination (Bzymek and Lovett 2001; Vader

et al. 2011). Therefore, even under normal growth conditions, repetitive transgenes lead to DNA replication stress or double-stranded breaks, resulting in the production of transgene-specific siRNA.

Third, in mutants that are deficient in maintaining fork stability and progression in rDNA locus, qiRNA levels are high without DNA damage treatment (Fig. 9D). This result suggests that the rDNA locus is normally protected to maintain its stability and to suppress DNA recombination.

aRNA is the precursor of dsRNA. The production of aRNA and dsRNA is catalyzed by the DdRP/RdRP QDE-1 (Lee et al. 2010). How does QDE-1 recognize the quelled locus and rDNA? How does DNA damage trigger the production of qiRNA and siRNA? Our genetic screening results indicate that only the HR process, but not other DNA repair or checkpoint point pathways, is required for qiRNA production. After DSBs, the Rad51-coated single stranded DNA, with the help of Rad54, invades the sister chromatin to form recombination intermediates. Even though how repetitive DNA is recognized by the quelling machinery is not clear, our results suggest a model in which DNA damage promotes the formation of "aberrant" forms of HR- intermediates of repetitive DNA, which are recognized by QDE-3, the *Neurospora* homolog of the BLM/Werner helicase. Together with RPA, they recruit QDE-1 to produce aRNA and dsRNA (Lee et al. 2010). Consistent with this model, HU treatment leads to the accumulation of rDNA-specific recombination intermediates. This model provides an explanation for why small RNAs are specifically produced at repetitive DNA loci: only the repetitive transgenes and rDNA array provide abundant donor sequences for HR. In addition, BLM helicases have been

shown to be recruited to DNA damage sites and play an important role in resolving aberrant recombination intermediates (De Muyt et al. 2012).

Repeat-associated small RNAs have been found in almost all eukaryotes. Since our report of qiRNA, DNA damage-induced small RNAs have been demonstrated in *Arabidopsis*, *Dosophila* and mammals (Francia et al. 2012; Michalik et al. 2012; Wei et al. 2012), suggesting that DNA damage is a common trigger for small RNA production in eukaryotes. Because of the conservation of eukaryotic RNAi pathways and the fact that repetitive DNA can be a trigger for DNA damage, our results suggest that homologous recombination is also likely to be involved in small RNA production processes in other organisms.

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CHAPTER THREE

H3K56 ACETYLATION IS REQUIRED FOR QUELLING-INDUCED SMALL RNA PRODUCTION THROUGH ITS ROLE IN HOMOLOGOUS RECOMBINATION

3.1 Introduction

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism conserved from fungi to mammals (Catalanotto et al. 2006; Buhler and Moazed 2007; Ghildiyal and Zamore 2009; Zhang et al. 2013). Despite its divergent roles in regulating gene expression, RNAi has been considered as an ancient genome defense mechanism that silences viral invasion and transposons (Sijen and Plasterk 2003; Siomi et al. 2008; Li et al. 2010; Wang et al. 2010; Chang et al. 2012). Consistent with this role, many organisms have developed a mechanism to produce small RNAs from repetitive DNA sequences that result from transposon replication or foreign DNA incorporation (Napoli et al. 1990; Romano and Macino 1992; Hsieh and Fire 2000).

Chapter two showed that both quelling and qiRNA are result from DNA damage/replication stress (Zhang et al. 2013). DNA damage agent treatment can induce quelling-induced siRNA in the quelled strain. Similar to qiRNA, it was recently shown in plants and animals that DNA damage is a common trigger to induce small non-coding RNAs around double strand break (DSB) (Francia et al. 2012; Michalik et al. 2012; Wei et al. 2012). In the context of quelling and qiRNA pathways, we showed that homologous recombination (HR) pathway is required for quelling and qiRNA production. This leads to the hypothesis that HR serves a mechanism to recognize repetitive DNA and initiates aRNA transcription under DNA damage condition (Zhang et al. 2013). However, the mechanistic detail of this HR-based siRNA biogenesis pathway is not clear. A number of

chromatin remodelers are also required for both HR and siRNA pathway in *Neurospora* (Zhang et al. 2013), indicating that a favorable chromatin environment is essential for this siRNA biogenesis pathway (Zhang et al. 2013).

In this project, by systematically screening the *Neurospora* knock-out mutants, we identify RTT109, a histone acetyltransferase for histone H3 on lysine 56, as a new component in the qiRNA production and quelling pathway. In addition, we demonstrate that RTT109 and its histone acetyltransferase activity are required for HR and qiRNA production and quelling pathway.

3.2 Material and Methods

3.2.1 Strains and growth conditions

A wild-type strain of *Neurospora crassa* (FGSC4200) was used in this study. The *Neurospora* knockout mutant strains used in this study were obtained from the Fungal Genetic Stock Center (Colot et al. 2006). Liquid cultures were grown in minimal medium (1X Vogel's, 2% glucose). For liquid cultures containing QA, 10^{-3} M QA (pH 5.8), was added to the culture medium containing 1 X Vogel's, 0.1% glucose, and 0.17% arginine. To induce qiRNA production, histidine (1 mg/ml) or the indicated concentrations of hydroxyurea (HU), were added and cultures were collected 48 hrs later (Lee et al. 2009).

3.2.2 Protein and RNA analyses

Protein extraction, quantification, and western blot analysis were performed as previously described (Cheng et al. 2005; Zhang et al. 2013). Nuclear protein extracts were prepared as previously described (Luo et al. 1998). Equal amounts of total protein (40μg) were loaded in each lane, and after electrophoresis, proteins were transferred onto

PVDF membrane and western blot analysis was performed. Antibodies against H3K56Ac, H3-K27Ac and γH2AX were purchased from Active Motif. Antibodies against H3-K9Ac and histone H3 were purchased from Abcam. Immunoprecipitation followed by mass-spectrometry was performed based on protocol described previously (Cheng et al. 2005) to identify RTT109-interacting proteins. The resulting MS files were searched against NCBI-nr protein sequence databases for protein identification.

Total RNA extraction, enrichment of small sized RNA, and northern blots were performed as previously described (Maiti et al. 2007). RNA probes were made using the MAXIscript T7 kit (Ambion) from a T7 promoter on a PCR product template. To make small RNA probes, the labeled RNA product was hydrolyzed by adding hydrolysis solution (80 mM $NaHCO_3$, 120 mM Na_2CO_3) to the probe at 60 ^oC for 3 hrs. The sample was then neutralized by adding 3 M NaOAc. RNA probes were made to detect intergenic transcripts from the rDNA region (Zhang et al. 2013).

3.2.3 Quelling assay

Quelling assays were performed as previously described with minor modifications (Cogoni et al. 1996). The wild-type and the mutant strains used for quelling assays were grown for 7-10 days before harvesting the conidia in 1 M sorbitol. A mixture of 2 mg pBSK*al-1* (carrying the *al-1* fragment) and 0.5 mg of pBT6 (a benomyl resistant gene-containing plasmid) was incubated with the conidial suspension for 4-5 hrs at 4℃. The plasmids were transformed into *Neurospora* by electroporation. The benomyl resistant transformants were picked onto minimal slants and visually inspected to identify the completely quelled (white), partially quelled (yellow), or non-quelled (orange) strains.

3.2.4 Assay for DNA damage sensitivity

A spot test was used for measuring the sensitivity of different strains to various DNA mutagens. The conidia concentration of conidia suspensions was measured and dropped onto sorbose-containing agar plates with indicated serial dilutions. The plates were incubated for 3 days at room temperature. HU, CPT, histidine, or EMS was added into agar medium at a final concentration of 1mg/ml, 0.1ug/ml, 6μg/ml and 0.2%, respectively. The description of the expression I-Scel in *Neurospora* will be described elsewhere.

3.2.5 Homologous recombination assay

The HR assay was performed essentially as described (Ishibashi et al. 2006). The *Neurospora* strains were grown for 7-10 days before harvesting the conidia in 1 M sorbitol. The *Eco*RI linearized *bar*-containing plasmid (pGS1-1KR, contains *bar* gene flanked by 1 kb of homologous sequence of *mtr*) was incubated with the conidial suspension on ice for 30 min. The fragment was transformed into *Neurospora* strains by electroporation. The transformed conidia were plated onto low nitrogen-containing top agar containing 0.4 mg/ml bialaphos. The bialaphos-resistant transformants were picked onto bialaphos containing slants, and resistant transformants were further selected on FPA-containing slants. The HR rate was calculated as the ratio between the FPA-resistant colonies to the total bialaphos-resistant colonies.

3.2.6 Chromatin immunoprecipitation assay

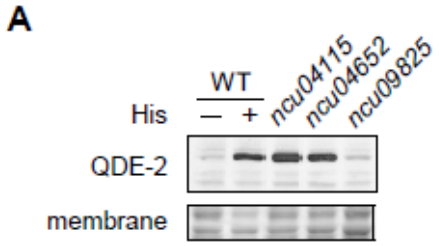
ChIP experiment was performed as previously described (Zhang et al. 2013). Double strand break (DSB) was induced by culture in liquid media containing QA for 2 days. The tissues were fixed in the culturing media containing 1% formaldehyde for 15 minutes with shaking. After fixation and washing, cell lysates were subject to sonication with 3

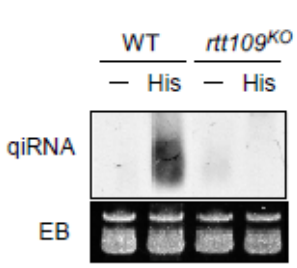
cycles of 25 pulses with duty cycle 40 and output control 4. The samples of 2 mg of protein were pre-cleared with 40 μl of slurry equilibrated with Gamma Sepharose beads for 2 hrs at 4 ℃ with rotation. The blocking beads were resuspended, and the lysate was transferred to a new tube. The monoclonal c-Myc antibody or antibody specific for H3K56ac was used to detect enrichment of Myc-RAD51 or H3K56Ac around DSB. The ChIP samples were diluted 1 to 2.5 fold before use as templates for qPCR analysis. Specific primers detecting *al-1* gene and *am-1* gene were used in qPCR analysis (Zhang et al. 2013).

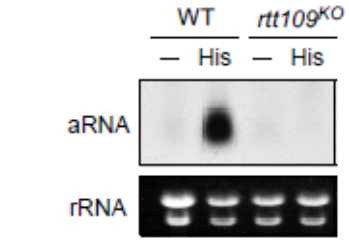
3.3 Result

3.3.1 RTT109 is required for qiRNA and quelling pathway

We sought to characterize the mechanism of the DNA damage-induced qiRNA production by carrying a systematic genetic screen using the *Neurospora* knockout library (Zhang et al. 2013). In addition to the homologous recombination components and chromatin remodelers that are characterized previously (Zhang et al. 2013), we also found that the knockout strain of NCU09825 was deficient in the histidine-induced QDE-2 expression (Fig. 10A). Sequence analysis showed that the predicted protein encoded by NCU09825 shares a strong sequence homology across the entire open reading frame with the RTT109 protein in budding yeast.







D

	No Silencing (Orange)	% *al-1* silencing (Yellow & White)
WT (160)	71% (114)	29% (46)
qde-3 (80)	96.3% (77)	3.8% (3)
rtt109 (204)	98% (200)	2% (4)

Figure 10. RTT109 is required for qiRNA and quelling pathway.
A, QDE-2 western blot analysis of a panel of *Neurospora* knock-out strains, including *rtt109* (NCU09825), from the unbiased screen. All the strains were grown in histidine (1 mg/ml) for 2 days. His, histidine. *B* & *C*, Northern blot analysis of qiRNA and aRNA products from the wild-type strain and *rtt109* mutant. Cultures were treated with 1 mg/ml histidine for 2 days. Ethidium bromide stained membranes were used as loading controls. *D*, Quelling efficiency of the indicated strains. The numbers in the parentheses indicate the number of strains examined after the quelling assays.

The yeast RTT109 has been demonstrated to be a bona fide histone acetyltransferase for histone H3 on lysine 56 both *in vivo* and *in vitro* (Schneider et al. 2006; Driscoll et al. 2007; Han et al. 2007a). Its HAT activity is largely dependent on its association with either of two histone chaperons, VPS75 and ASF1 (Driscoll et al. 2007; Han et al. 2007a; Tsubota et al. 2007). Interestingly, even though RTT109 is structurally similar to its

functional counterpart in flies and humans, p300/CBP, they share no sequence homology (Tang et al. 2008; Das et al. 2009). H3K56Ac catalyzed by RTT109 plays an important role in DNA damage response/repair as mutants that are deficient in H3K56Ac are sensitive to genotoxic stress (Masumoto et al. 2005; Han et al. 2007a; Han et al. 2007b). This sensitivity is at least partly due to its role in the deposition of newly synthesized histones and histone replacement during DNA replication and repair (Chen et al. 2008; Li et al. 2008). However, little is known about the function of RTT109 beside its role in DNA damage response.

To investigate the phenotype of RTT109 in qiRNA and quelling pathway, we first examined the damage-induced qiRNA production in the *rtt109*^{KO} mutant. Figure 10B shows that histidine treatment induced the qiRNA production in the wild type strain, but such a response was abolished in the *rtt109* mutant. Then, we examined the expression of the rDNA-specific aRNA, the precursor of qiRNA, and found that the histidine-induced aRNA production was also abolished in the *rtt109* mutant (Fig. 10C), indicating that RTT109 is required for qiRNA synthesis in a step that is upstream of aRNA production. Previous studies show that quelling pathway and damage-induced qiRNA pathway is mechanistically similar (Romano and Macino 1992; Lee et al. 2010; Zhang et al. 2013). Thus, we asked if RTT109 is also required for the quelling pathway. To test this, we performed quelling assay after transformation of cells with an *al-1* transgene. As shown in Figure 10D, 29% of the wild-type transformants exhibited quelling, as indicated by the change of conidia color from orange to yellow or white. In contrast, quelling efficiency in *rtt109* strain is only 2%, which is to that in *qde-3* strain, indicating that like QDE-3,

RTT109 is required for quelling. Taken together, these results suggest that RTT109 is a novel component involved in qiRNA and quelling-induced siRNA biogenesis pathway.

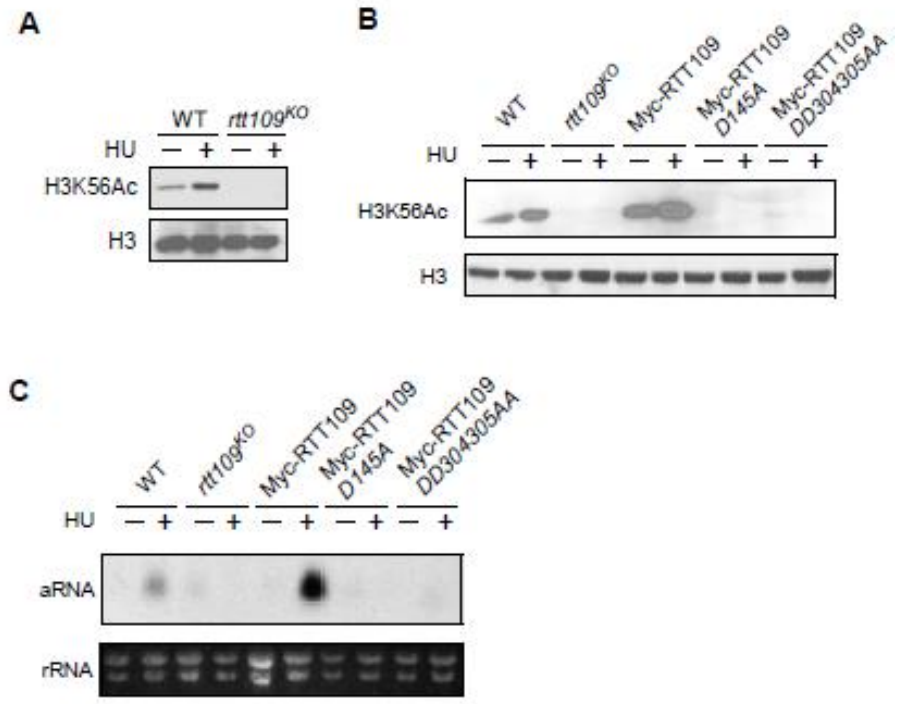


Figure 11. HAT activity of RTT109 is required for qiRNA production.
A, H3K56Ac western blot of the wild-type and *rtt109*^{KO} strains. Cultures were grown in media with/without 1 mg/ml HU for 2 days. H3 western blot was used as a loading control. *B*, Western blot analysis showing the level of H3K56Ac in the indicated strains. *C*, Northern blot analysis showing the level of the rDNA-specific aRNA in the indicated strains. Cultures were grown in media with/without 1 mg/ml HU treatment for 2 days.

3.3.2 HAT activity of RTT109 is required for qiRNA production

To examine whether RTT109 is a HAT for histone H3 on lysine 56 in *Neurospora*, western blot against H3K56Ac was performed in both wild-type and *rtt109* strains. Figure 11A shows that H3K56Ac was abolished in the *rtt109* mutant. This global loss of H3K56Ac was restored when a construct that expresses the c-Myc tagged RTT109 was transformed into the *rtt109*^{KO} strain. On the other hand, point mutations of the conserved catalytic asparate residues (Han et al. 2007a) to alanines (D145A and DD304305AA) complete abolished H3K56Ac. These results indicate that RTT109 is an essential HAT for H3K56Ac in *Neurospora*.

To examine whether the HAT activity of RTT109 is required for siRNA biogenesis in *Neurospora*, we examined the production of rDNA-specific aRNA. As shown in Figure 11C, while the wild-type RTT109 was able to rescue the HU-induced aRNA production, the RTT109 D145A and D304A, D305A mutants were deficient in this response. This result indicates that RTT109 acts as a HAT in the qiRNA production pathway.

HU treatment resulted in an increase of H3K56ac level (Figure 12A &B). Figure 12C also showed that the production of aRNA in Myc-RTT109 strain was higher compared to the wild-type strain. This is correlated with the higher level of H3K56Ac in the Myc-RTT109 strain (Fig. 11B), suggesting that H3K56Ac level might be a limiting factor in qiRNA production.

3.3.3 VPS75 and ASF1 are required for qiRNA production

To better understand how RTT109 functions, we sought to identify RTT109 interacting proteins by immunoprecipitation of Myc-tagged RTT109 from *Neurospora*

and followed by mass spectrometry (Fig. 12A). One of RTT109-interacting proteins was identified to the *Neurospora* homolog of the yeast VPS75, which has been shown to be in the same complex as RTT109 and is important for H3K56Ac both *in vivo* and *in vitro* (Tsubota et al. 2007). It was previously reported that another histone chaperon ASF1 is also essential for H3K56Ac (Driscoll et al. 2007; Han et al. 2007b; Tsubota et al. 2007). To examine, whether VPS75 and ASF1 act together with RTT109, we examined the H3K56Ac levels in the *Neurospora vps75* and *asf1* knock-out mutants. We found that both ASF1 and VPS75 are required for H3K56Ac (Fig. 12B). Furthermore, the production of HU-induced aRNA was also dramatically reduced in the *asf1* and *vps75* mutants (Fig. 12C). These results further support that the importance of H3K56 acetylation in the qiRNA biogenesis pathway.

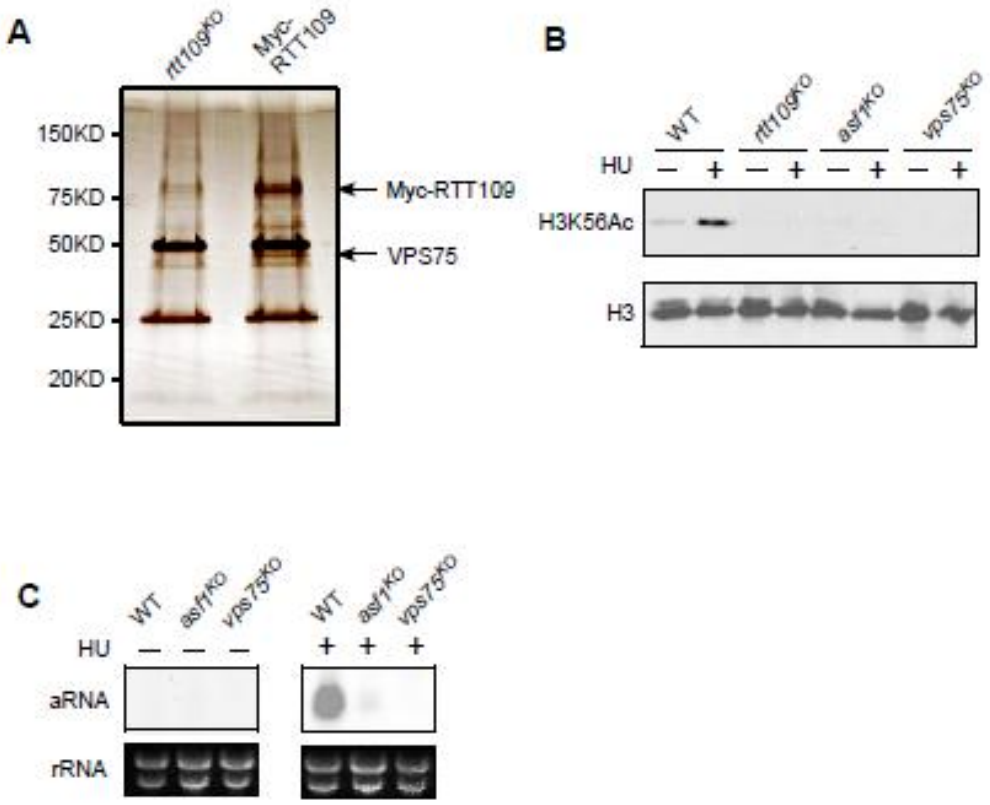


Figure 12. VPS75 and ASF1 are required for qiRNA production.
A, Silver-stained SDS PAGE gel showing the affinity purified Myc-RTT109 products. Cell extracts of the *rtt109*^{KO} and *rtt109*^{KO} strain expressing Myc-RTT109 were used for purification. *B*, Western blot showing the levels of H3K56Ac in the indicated strains. Cultures were grown with/without 1mg/ml HU for 2 days. *C*, Northern blot analysis showing the level of aRNA in the indicated strains.

3.3.4 RTT109 is involved in homologous recombination

To understand how RTT109 is involved in the qiRNA and quelling pathway. We first examined the physiological function of RTT1090 in *Neurospora*. It was previously reported in yeast that the *rtt109* mutant is sensitive to DNA damage (Schneider et al. 2006; Driscoll et al. 2007; Han et al. 2007a) and in the *rtt109* mutant, the basal level of RAD52 foci is increased (Han et al. 2007a). Consistent with previous findings, we found that the *Neurospora rtt109* mutant is also very sensitive to DNA damage agent treatment (Fig. 13A), including HU, camptothecin (CPT), histidine, and ethyl methanesulfonate (EMS), suggesting a role for H3K56Ac in the DNA damage response or repair.

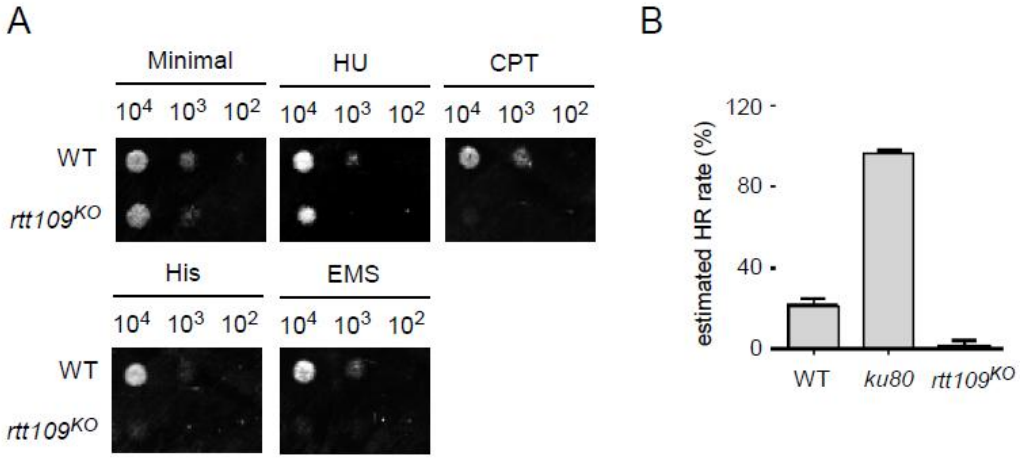


Figure 13. RTT109 is required for homologous recombination.
A, A spot test showing the *rtt109* mutant is more sensitive to DNA damage agents, including HU, CPT, Histidine and EMS. The numbers of conidia used in the spot test are indicated. *B,* Homologous recombination assays showing the HR rates of the indicated strains. n=3; error bar indicates s.d.

There are two major mechanisms to repair damaged DNA in *Neurospora*, the homologous recombination (HR) and the non-homologous end joining (NHEJ) pathways. Since we previously showed that HR is required for both quelling- and damage-induced siRNA production (Zhang et al. 2013), we hypothesized that H3K56Ac catalyzed by RTT109 is involved in HR. To test this hypothesis, we examined homologous recombination rate at the *methyltryptophan resistance* (*mtr*) locus in different *Neurospora* strains by transforming cells with a construct containing the bialaphos-resistance gene (*bar*) that can disrupt the *mtr* gene by homologous recombination (Ishibashi et al. 2006). The targeting of *bar* gene into the *mtr* gene by HR will result in transformants that are resistant to both bialaphos and the amino acid analog p-fluorophenylalanine (FPA). As shown in Figure 13B, the wild-type strain has a recombination rate of approximately 20%, which is a typical HR rate in *Neurospora*. As expected, the HR rate was nearly 100% in a *ku80* knockout mutant in which the NHEJ pathway is abolished and HR becomes the dominant repair pathway. As expected, the HR rate was dramatically reduced in the *rtt109* mutant. This result indicates that RTT109 and H3K56Ac play a critical role in the HR process.

To understand how H3K56Ac responds to DNA damage agent treatment, we treated the wild-type *Neurospora* cells with different concentration of HU for 2 days. We observed a global induction H3K56Ac after HU treatment (Fig. 14A). However, the level of H3K56Ac did not further increase at high concentrations of HU treatment. Since qiRNA production will be abolished under high concentration of HU treatment where DNA replication is completely stopped (Zhang et al. 2013), this result suggests that although H3K56Ac is required but not sufficient to result in qiRNA production.

Next, we examined how H3K56Ac functions in the process of homologous recombination. H3K56Ac has been proposed to function in nucleosome assembly/disassembly (Chen et al. 2008; Li et al. 2008). A recent study revealed that H3K56ac can replace H2A.Z with H2A by altering the substrate specificity of a chromatin remodeling complex (Watanabe et al. 2013). Because γH2AX formation around the double strand break (DSB) site is the first step of DNA damage response/repair (van Attikum and Gasser 2009). We first examined if H3K56Ac will affect γH2AX formation. As expected, γH2AX in the wild-type strain was barely detectable without DNA damage and was induced after treatment by CPT, a topoisomerase I inhibitor (Fig. 14B). The basal levels of γH2AX were higher in the *rtt109* and *vps75* mutants compared to wild-type strain, indicating a higher basal level of DNA damage or replication stress in these mutants. However, γH2AX can still be further induced in the presence of CPT in the *rtt109* and *vps75* mutants (Fig. 14B). This result suggests that H3K56Ac is required in the homologous recombination process in a step that is downstream of γH2AX formation.

Then, we examined that whether H3K56Ac is enriched around DSB during homologous recombination. We used a *Neurospora* strain with an I-SceI cutting site flanked by *al-1* sequence at the *his-3* locus was generated (Yang et al., unpublished). In this strain, FLAG-tagged ISCEI and Myc-RAD51 was co-expressed under the control of quinic acid (QA) inducible promoter. Thus, a DSB will be created by FLAG-ISCEI at the *al-1* site in the presence of QA. We examined the enrichment of Myc-RAD51 around the DSB by chromatin immunoprecipitation (ChIP) assay. As shown in Figure 14C, a significant enrichment of Myc-RAD51 was observed around *al-1* DSB site (~10-fold).

This result confirmed that I-SceI site was cut and homologous recombination did take place after DSB. Similarly, we also detected a significant enrichment of H3K56Ac around the *al-1* DSB flanking region but not at a control (*am-1*) locus (Fig. 14D). The correlation between the enrichment of H3K56Ac and Myc-RAD51 around the DSB site further suggests that H3K56Ac facilitates HR process.

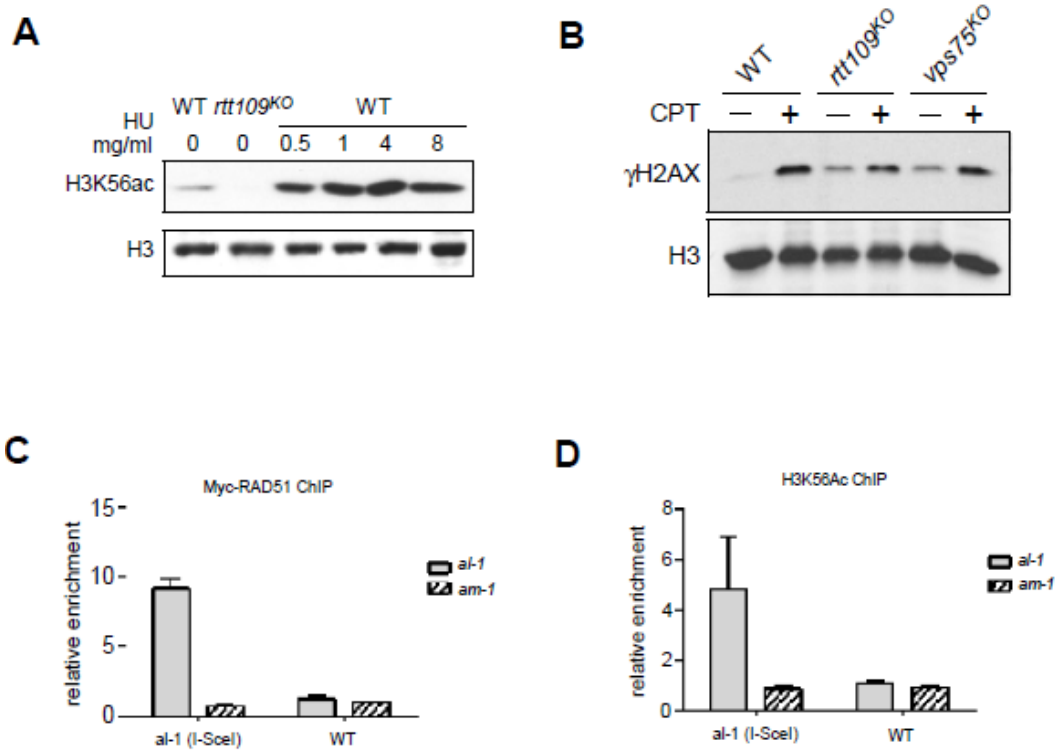


Figure 14. H3K56Ac and Myc-RAD51 is enriched around DSB.
A, Western blot analysis showing the level of H3K56Ac in the *rtt109* and wild-type strains grown in indicated concentration of HU. *B*, Western blot analysis showing the level of H2AX in the indicated strains. H2AX was examined after cultures were treated with 0.1 g/ml CPT treatment for 2 days. *C*, ChIP-qPCR analysis showing the enrichment of MycRAD51 around the I-SceI-induced DSB, which is flanked by *al-1* sequence. Primer set detecting *am-1* gene is used as a negative control *D*, ChIP assays showing the H3K56Ac enrichment around the DSB following the same procedure in (*C*). n=3; error bar indicate s.d.

3.4 Discussion

Quelling-induced small RNAs and DNA damage induced qiRNAs are produced in *Neurospora* during vegetative stage from repetitive DNA loci (Li et al. 2010; Chang et al. 2012). We previously showed that homologous recombination process are essential for the production of these small RNAs and is likely the mechanism to distinguish the repetitive sequences from the rest of the genome. All the key components in HR are required for siRNA pathway, including RAD51, RAD52, RAD54 and RPA (Cogoni and Macino 1999; Lee et al. 2010; Zhang et al. 2013). Consistent with this model, several chromatin remodelers that are required for the small RNA production are also required for HR (Zhang et al. 2013). These results suggest that HR among the repetitive DNA sequences allow the formation of appropriate chromatin structures that are specifically recognized by the RNAi pathway to produce aRNA and siRNA. In this study, we identified the H3K56 acetyltransferase, RTT109, as a new component of the quelling and qiRNA pathway. Further supporting the critical role of HR in repetitive DNA -induced small RNA production, we showed that RTT109 is required for homologous recombination in *Neurospoa*. Our results not only identified a HAT in a small RNA biogenesis pathway but also suggest that H3K56ac is required for the homologous recombination process in eukaryotic organisms.

Previous studies in yeast have shown that the histone chaperons VPS75 and/or ASF1 stimulate the H3K56ac activity of RTT109 (Schneider et al. 2006; Driscoll et al. 2007; Han et al. 2007a; Tsubota et al. 2007). Consistent with these studies (Han et al. 2007a; Tsubota et al. 2007), we found that RTT109 interacts with VPS75 in *Neurospora* (Fig. 12A) and both ASF1 and VPS75 are critical for H3K56Ac in *Neurospora*. In addition,

qiRNA production is also impaired in the *asf1* and *vps75* mutants, further supporting the role of H3K56Ac in siRNA biogenesis.

Defects in the H3K56ac result in increased sensitivity to genotoxic stress that cause DNA damage during replication (Masumoto et al. 2005; Driscoll et al. 2007; Han et al. 2007a). It has been proposed that H3K56Ac participates in the replication- and repair-coupled nucleosome assembly (Chen et al. 2008; Li et al. 2008). Similarly, we also showed that the *rtt109* mutant is sensitive to DNA damage. Furthermore, we showed that the *rtt109* mutant is deficient in HR, suggesting that the DNA damage sensitivity of the *rtt109* mutant is due to impaired HR-dependent DNA repair pathway. This observation is not quite in line with previous reports, which showed that RTT109 or ASF1 is not required for HR at mating type switching locus induced by HO endonuclease (Ramey et al. 2004; Chen et al. 2008). One of the explanations is that H3K56Ac is important for both NHEJ and HR, the overall repair efficiency is reduced by knocking out RTT109. However, the DSB repair assay performed in yeast was done in survived yeast cells which have been successfully repaired. In another word, even though the HR rate might be lowered, there are still some residual HR events that can be detected. Therefore, we need to test our hypothesis in yeast again by using the same principle we did in *Neurospora*. Furthermore, the global level of H3K56Ac is increased after DNA damage treatment (Fig. 14A). Importantly, we showed that H3K56ac occurs at a define DBS locus that is correlated with RAd51 enrichment (Figure 14C-D). It has been shown that H3K56ac promotes rapid nucleosomes replacement or facilitates the deposition of histone in the context of transcription, DNA replication, and repair (Chen et al. 2008; Kaplan et al. 2008; Li et al. 2008; Watanabe et al. 2013). Because several chromatin remodelers

have been shown to play a role in HR (Zhang et al. 2013), it is possible that H3K56Ac may also participate in the chromatin remodeling process to create a favorable chromatin environment for HR to proceed. However, the exact function of H3K56Ac in HR remains unclear. I have tried to obtain a strain that has a defined DSB in the *rtt109* mutant background to examine whether RTT109 is required for RAD51 loading onto the single-stranded DNA after DSB. However, we were not successful after repeated attempts, probably because the *rtt109* mutant is very sensitive to DNA damage (Fig. 13A). In addition, I also tried to examine if RTT109 will influence the H3 density around DSB due to its possible function in nucleosome assembly/disassembly. However, the H3 enrichment by ChIP assay did not show robust change upon DSB induction. This is probably because the nucleosome remodeling is so asynchronous and dynamic that ChIP can only capture a small proportion of cells that are undergoing nucleosome remodeling at a time.

Since our discovery of qiRNA, it is recently shown that DNA damage can also induce small non-coding RNAs around double strand break (DSB) in both plants and animals (Francia et al. 2012; Michalik et al. 2012; Wei et al. 2012). Thus, a common pathway may be responsible for the DNA damage-induced small RNA production in fungi, plants and animals. Because H3K56Ac is involved in both DNA damage repair and small RNA biogenesis pathways, our study uncovers a possible mechanistic link between DNA damage response and small RNA production.

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CHAPTER FOUR

CONCLUSION AND FUTURE DIRECTIONS

4.1 The upstream biogenesis mechanism of siRNA in *Neurospora*

Repeat-induced gene silencing mechanism has been reported in many organisms, including fission yeast, *Neurospora*, plant, *Drosophila*, human, etc. (Hsieh and Fire 2000). The mechanism how repetitive DNA is recognized and therefore produces small RNA is not clear. Our study here provides a novel insight that HR might serve as a mechanism to distinguish repetitive sequences from the rest of the genome. This HR-based biogenesis must be regulated at the chromatin level, provided with the evidence that chromatin remodeling factors and HAT protein are required for the production of aRNA. One of the questions remains is that what step along HR will initiate the production of aRNA. Homologous recombination can be dissected into several steps, from DNA end resection, synaptic complex formation, to D-loop formation and extension (Sung and Klein 2006). We have previously reported that QDE-1 and QDE-3 interact with RPA (Lee et al. 2010). It has been shown that RecQ helicase can physically associate with RAD51 (Braybrooke et al. 2003). RecQ helicase is involved in D-loop extension (Bernstein et al. 2010). In addition, RPA was shown to bind on the displaced D-loop (Wang and Haber 2004). All these information together prompts us hypothesize that the D-loop formation might be the step where aRNA is produced because all the upstream RNAi components are brought into close proximity in the D-loop (Figure 15). The RPA-bound displaced D-loop might serve as a template for QDE-1 to transcribe aRNA. Of course, this hypothesis needs to be experimentally tested before any conclusion can be drawn. One of the future experiments could be to set up an

in vitro reconstitution assay with the presence of artificial D-loop and a mixture of required RNAi components to examine if aRNA can be produced.

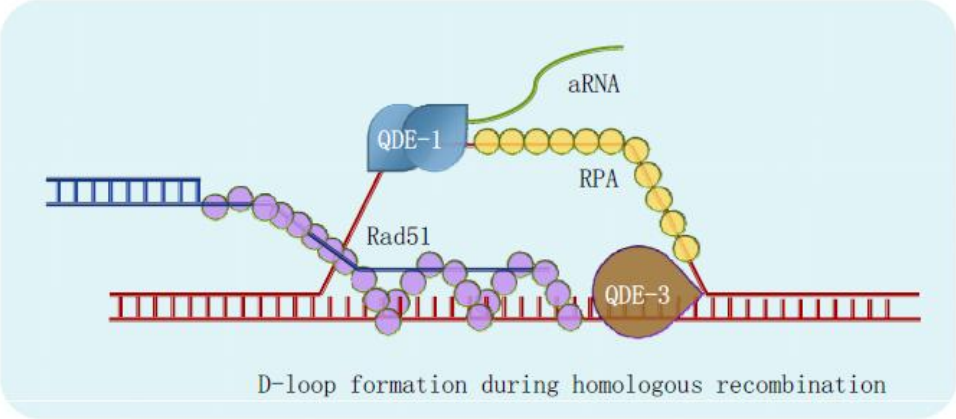

Figure 15. A hypothetical model showing the production of aRNA. In the context of D-loop extension during homologous recombination, rad51 biding filament will be annealed to the homologous target sequences. QDE-3 might be involved in D-loop extension. RPA-bound ssDNA can recruit QDE-1 to produce aRNA.

Our study also suggests that both qiRNA and quelling-induced small RNAs are result from DNA damage. Their biogenesis pathways are mechanistically similar. One of the puzzles is that why quelling can occur under normal growth condition whereas qiRNA cannot. We have shown that rDNA cluster is protected by DNA replication fork protection complex to prevent the production of aRNA. It is possible that those transgenic insertion sites in quelled strains are naturally under DNA replication stress. Because these sites, or so called fragile sites, are prone to form DSB, therefore foreign DNA fragments will have more chance to insert there. Another postdoc in the lab, Dr. Qiuying Yang, is currently examining the existence of fragile sites in *Neurospora* and testing this

hypothesis. From her preliminary and unpublished data, it is suggestive that DSB or fragile site does promote the production of site-specific small RNAs. This is consistent with recent findings from other organisms that DSB is the trigger for the production of small non-coding RNA (Francia et al. 2012; Wei et al. 2012).

4.2 Function of quelling/damage-induced siRNA

siRNA is considered as a genome defense mechanism to prevent foreign DNA invasion. It has been shown that QDE-2 and Dicer are required to suppress the Tad transposon activity in *Neurospora* laboratory strain (Nolan et al. 2005). qiRNAs mostly originate from rDNA region, therefore it should presumably target to ribosomal RNA through RNAi machinery. In fact, the global translation efficiency in *Neurospora* is reduced upon DNA damage and can be restored by knocking out RNAi components. An interesting idea is that qiRNA serves as a part of the check point mechanism by slowing the overall translation to allow cells to repair damage (Lee et al. 2009). Recently, increasing evidences have shown that DNA damage can trigger the production of small RNAs from damage site. It has been shown in *Arabidopsis* and human cells that about ~21 nt small RNAs are produced specifically around DSB sites (Wei et al. 2012). In addition, DICER and DROSHA-dependent small RNAs have been shown to be produced specifically from specific damage sites (Francia et al. 2012). What is the function of these damage induced sRNAs? It has been shown that RNAi components including Dicer and RdRP are required for efficient DNA damage repair probably through homologous recombination (Wei et al. 2012). It has also been proposed that damage-induced small RNAs are involved in DNA damage response foci formation (Francia et al. 2012). These

results indicate that small RNAs play a critical role in DNA damage response. However, more experimental evidences are needed to draw further conclusions.

In order to examine if qiRNA has additional functions other than slowing translation speed. First, I examined the copy number of rDNA before and after DNA damage. Because repetitive rDNA gives rise to qiRNA, it is possible that the copy number of rDNA cluster is regulated by qiRNA. However, the copy number of rDNA has no significant change under different condition or strains. Second, I examined if qiRNAs direct DNA methylation because centromeric sRNAs in fission yeast and sRNAs from plant can guide DNA methylation through RNAi components (Mathieu and Bender 2004; Zilberman et al. 2004). Intriguingly, DNA damage can induce a robust methylation (about 20 fold by MeDIP, confirmed by southern blot analysis) across the whole rDNA region including transcribed and untranscribed intergenic spacer regions. However, this rDNA specific methylation is not Dicer and QDE-1 dependent, indicating that DNA damage-induced methylation and qiRNA production are two parallel and independent events. Interestingly, this damage induced methylation is dependent on a functional QDE-3. It remains to be studied that how DNA damage induced rDNA specific methylation with the help of QDE-3 and what is the function of this type of methylation.

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