THE FUNCTION AND MECHANISM OF RNA INTERFERENCE IN NEUROSPORA

APPROVED BY SUPERVISORY COMMITTEE

Yi Liu, Ph.D. (Mentor)

Micheal White, Ph.D. (Chair)

Dean Smith, Ph.D.

Qinghua Liu, Ph.D.

DEDICATION

To my father Wei-Chung Lee and my wife Ying-Chun Huang

THE FUNCTION AND MECHANISM OF RNA INTERFERENCE IN NEUROSPORA

by

HENG-CHI LEE

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2008

Copyright

by

HENG-CHI LEE, 2008

All Rights Reserved

ACKNOWLEDGEMENT

I first want to thank my mentor, Dr. Yi Liu, for his guidance and support throughout the years. He has led me through a memorable scientific journey by participating in every aspect during my pursuit for a Ph.D.; ranging from sharing hypotheses, designing experiments, troubleshooting results and publishing our work. His passion in science, insightful scientific suggestions and affection on people has been instrumental and critical to my success.

I want to thank the people in our lab for their generosity, time and efforts, which has made our research a rewarding experience. I want to thank Dr. Mekhala Maiti for setting the groundwork of the RNAi project in the lab. I also want to thank Dr. Ping Cheng for providing scientific insights and teaching me all kinds of techniques. I am thankful to Dr. Swati Choudhary, Suzy Chang, Dr. Guocun Huang and Dr. Joonseok Cha for their helpful discussion and collaboration in various projects. I am grateful to have Patsy Tucker, Lixin Wang and Hai-Yan Yuan, who provided excellent administrative and technical support. I am thankful to several past and current colleagues in our lab, including Dr. Qiyang He, Dr. Qun He, Dr. Jinhu Guo, Dr. Shaojie Li, Dr. Chi-Tai Tang and Dr. Liande Li. They have made the lab a great place to work and have always kindly shared ideas and materials with me.

My research was greatly benefited from collaborations with several other laboratories. I am indebted to Dr. Qinghua Liu for providing scientific advice and sharing novel techniques with us. I want to thank Dr. Craig Mello, Dr. Weifeng Gu and Dr. Xiang Liu for their collaborations and support in small RNA cloning projects. I thank Dr. Feng Jiang for her help in establishing several RNAi in vitro assays, to Dr. Bamford and Antti Aalto for their collaboration on QDE-1 projects, to Dr. Jen-Chieh Chuang and Dr. Joyce Reppa for their assistance in quantitative RT-PCR assays. I also want to thank the *Neurospora* community and *Neurospora* genome projects for providing strains and protocols.

I am indebted to my dissertation committee members: Dr. Michael White, Dr. Dean Smith and Dr. Qinghua Liu for their continuous support. They have provided critical insights for my projects and given invaluable advice on my scientific presentation and writing skills.

None of this would have been possible without the support from my family. My father Wei-Chung Lee has always been the greatest mentor of my life. He has taught me the value of family, mind, music and love, which are far more important then money or my success. I can never express enough appreciation for my wife Ying-Chun Huang. She has provided me more love, caring and support than one can ever imagine. I am also very fortunate to have a very cute and sweet beagle, Bagel. He has never stopped showing his love and has always provided different perspectives of life.

THE FUNCTION AND MECHANISM OF RNA INTERFERENCE IN NEUROSPORA

HENG-CHI LEE, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2008

Mentor: YI LIU, Ph.D.

RNA interference (RNAi) is a conserved gene silencing mechanism important for various biological processes, including developmental timing, genome defense, and heterochromatin formation. RNAi is triggered by double stranded RNA (dsRNA), which is processed by Dicer to siRNA. siRNA is loaded onto RNA-induced silencing complex (RISC), in which an Argonaute family protein, guided by a siRNA, mediates the cleavage of homologous RNAs. In the filamentous fungus *Neurospora*, we show that dsRNA not only trigger RNAi, it also transcriptionally activates several key components of RNAi pathway,

including *qde-2* (an Argonaute) and *dcl-2* (a Dicer). A genome wide identification of dsRNA activated genes suggests that RNAi is part of a broad ancient host-defense response against viral and transposon infections.

Our research on *qde-2* regulation also suggests a role of RNAi during DNA damage. We show that DNA damage induces *qde-2* expression, and the purification of QDE-2 bound RNAs identifies a novel class of small RNAs named qiRNAs. qiRNAs are averaged 21 nt in length and are mostly derived from ribosomal DNA (rDNA) locus. Importantly, qiRNA biogenesis requires RNAi components and RNAi mutants exhibit increased sensitivity to DNA damage, suggesting a role for qiRNAs during DNA repair. Further analysis suggests that the qiRNA contributes to the DNA damage checkpoints by inhibiting protein translation after DNA damage.

To trigger RNAi against transgenes, it has been proposed that transgene-specific aberrant RNA (aRNA) is made and converted into dsRNA by RNA dependent RNA polymerase (RdRP). How aRNA is produced and specifically recognized by RdRP is not known. We show that QDE-1, a RdRP is also the DNA-dependent RNA polymerase (DdRP) that produces aRNA from ssDNA. QDE-1 is recruited to ssDNA by Replication Protein A (RPA) and QDE-3 (an RecQ helicase), both of them are also essential for aRNA production. Moreover, QDE-1 can produce dsRNA from ssDNA, a process facilitated by RPA. Our results provide a molecular mechanism of aRNA production in RNAi pathways.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Lee HC*, Aalto AP*, Chang SS, Huang G, Fisher D, Cha J, Poranen MM, Bamford DH, and Yi Liu. (2008). An RNA-dependent RNA polymerase acts as a DNA-dependent RNA polymerase to generate aberrant transcripts and dsRNA in the RNAi pathway, a process requiring Replication Protein A and a RecQ DNA helicase (under review)

Lee HC, Chang SS, Choudhary S, Maiti M, and Liu Y. (2008). qiRNA, a novel type of small interference RNAs induced by DNA damage. (Revised)

Choudhary S*, Lee HC*, Maiti M*, He Q, Cheng P, Liu Q, and Liu Y. (2007).
A double-stranded RNA response program important for RNA interference efficiency.
Mol Cell Biol. 27(11):3995-4005

Maiti M, Lee HC and Liu Y. (2007). QIP, a putative exonuclease, interacts with the Neurospora Argonaute protein and facilitates conversion of duplex siRNA into single strands. *Genes & Dev.* 21(5):590-600

He Q, Cha J, He Q, Lee HC, Yang Y, and Liu Y. (2006). CKI and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the Neurospora circadian negative feedback loop.
Genes & Dev. 20(18):2552-65.

* equal contribution.

LIST OF FIGURES

LIST OF TABLES

LIST OF DEFINITIONS

AGO Argonaute

aRNA aberrant RNA

ATM ataxia telangiectasia mutated

BLM Bloom symdrome

cDNA complementary DNA

ChIP Chromatin Immunoprecipitation

DCL dicer-like

DdRP DNA-dependent RNA polymerase

DNA deoxyribonucleic acid

DRAG dsRNA-activated gene

dsDNA double-stranded DNA

dsRNA double-stranded RNA

EMS ethyl methanesulfonate

his histidine

hph hygromycin

HSP heat shock protein

HU hydroxyurea

IFN Interferon

ISG Interferon-stimulated gene

KO knock out

miRNA micro RNA

mRNA messenger RNA

MSUD meiotic silencing by unpaired DNA

Mx myxovirus resistance

NOR nucleolar organizer region

nt nucleotide

NTP nucleotide triphosphate

ORF open reading frame

PAGE poly-acrylamide gel electrophoresis

PAZ Piwi Argonaut and Zwille

PCR Polymerase chain reaction

PEG Poly-ethylene glycol

piRNA PIWI interacting RNA

PIWI P-element induced wimpy testis

Pol Polymerase

PVDF polyvinylidene difluoride

QA quinic acid

QDE quelling deficient

QIP QDE-2 interacting protein

qiRNA qde-2 associated RNA

qRT-PCR Quantitative Real Time-PCR

rasiRNA repeat associated small interferring RNA

rDNA ribosomal DNA

RdRP RNA-dependent RNA polymerase

RIG-I retinoic-acid-inducible gene I

RIP repeat-induced point mutation

RISC RNA-induced silencing complex

RNA Ribonucleic acid

RNAi RNA interference

RPA Replication Protein A

rRNA ribosomal RNA

siRNA small interferring RNA

ssDNA single-stranded DNA

ssRNA single-stranded RNA

TLR toll-like receptor

tRNA transfer RNA

WRN Werner syndrome

CHAPTER ONE

INTRODUCTION

The discovery of RNA interference (RNAi) opened several unexplored fields of biological and biomedical research, including the mechanism of RNAi and functions of small non-coding RNAs (Mello, 2007). Studies in last decade revealed that RNAi pathways and small RNAs (such as miRNA and piRNA) play important roles in development, genome defense and stem cell maintenance (Carrington and Ambros 2003; Verdel and Moazed 2005; Klattenhoff and Theurkauf 2008). Furthermore, RNAi-based technologies provide powerful tools to study gene function systematically and promising therapeutic strategies to silence genes that cause diseases (Vastenhouw et al. 2003; Hannon and Rossi 2004; Nguyen et al. 2008).

1.1 The discovery of RNA interference

The first RNA interference (RNAi) phenomenon was reported in 1990 in plant (Napoli et al. 1990). In the attempts to overexpress chalcone synthase, a key enzyme responsible for pigmentation of flower in *Petunia*, they found several transgenic plants exhibit loss of pigmentation phenotype. This mysterious phenomenon was named "Cosuppression" for its feature to trigger silencing of both the transgene and the endogenous cognate gene. A similar phenomenon "Quelling" was also observed in the

filamentous fungi *Neurospora* in 1992 (Romano and Macino 1992). The molecule triggers these silencing processes was finally identified in research performed in *C. elegans*. In 1995, Guo and Kemphues first made an unexpected observation that injection of either sense or antisense RNAs to worms both led to silence of homologous genes (Guo and Kemphues 1995). Through a series of extensive and careful analysis, Dr. Mello and Dr. Fire published their landmark discovery in 1998 describing double-stranded RNAs, but not sense or antisense RNA, are the actual trigger of this potent gene silencing response (Fire et al. 1998). Similar silencing pathways triggered by dsRNA were later identified in almost all eukaryotic organisms from fungi to human (Hammond et al. 2001).

1.2 The mechanism of RNA interference

Double stranded RNAs (dsRNAs) come from various endogenous or exogenous sources to trigger RNA interference of homologous sequences. (Figure 1). An RNAse III-like enzyme, Dicer, specific recognizes dsRNA and cleaves them into siRNA (Bernstein et al. 2001). siRNA is short dsRNA duplex of 21-25 nt in length with 2 nt 3' overhang (Zamore et al. 2000). siRNAs produced by Dicer is then loaded onto an RNA induced silencing complex (RISC), where Argonaute protein functions as the catalytic core of the complex (Hammond et al. 2001a).

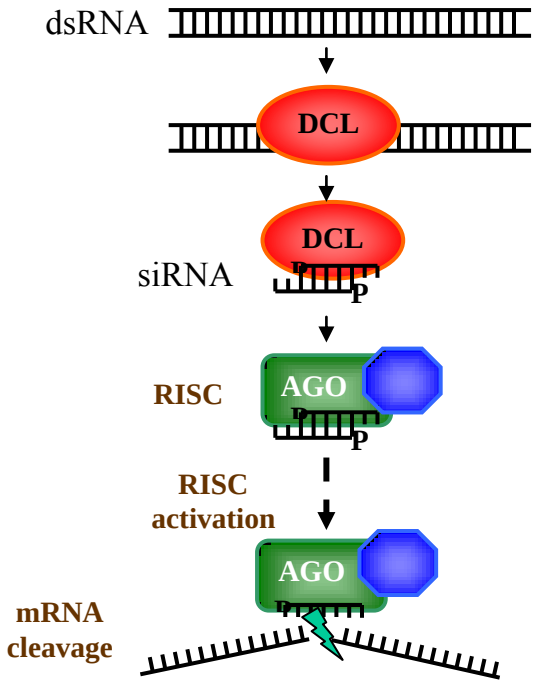


Figure 1. The mechanism of RNAi interference triggered by dsRNA. A cartoon shows that Dicer (DCL) cleaves dsRNA into siRNAs, and Argonaute (AGO) uses siRNA as a guide to cleave complementary mRNA.

The structural and biochemical studies identified the PAZ and PIWI domains as two functionally critical domains in Argonaute proteins (Rand et al. 2004; Song et al. 2004). The PAZ domain is involved in recognizing and binding the 3' overhang of siRNAs. The PIWI domain, which its structure resembles RNAse H, uses single stranded siRNA as a guide and cleaves complementary RNAs to trigger silencing of target genes (Figure 2).

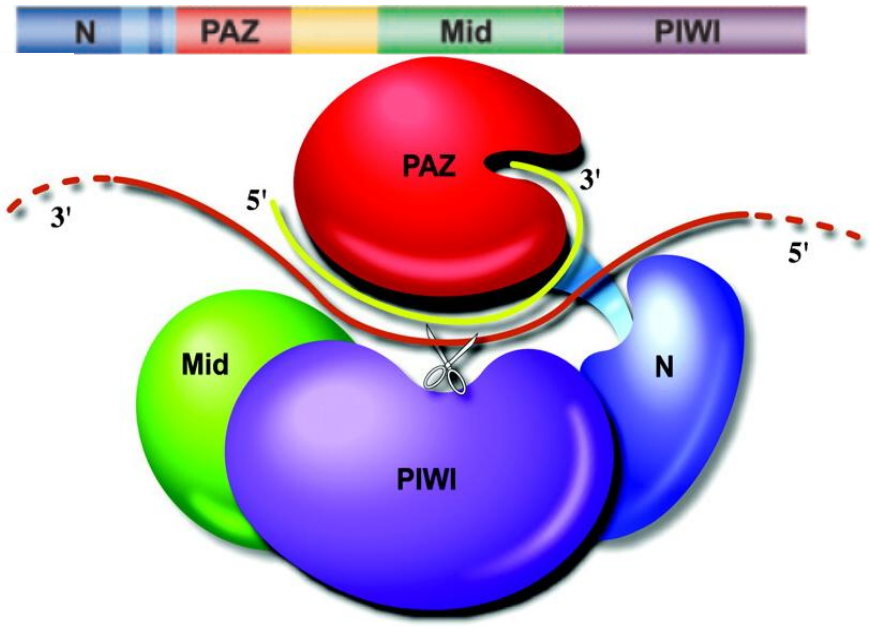


Figure 2. Argonaute, the slicer of RISC complex. A schematic (top) shows the order of domains in an Argonaute protein. A model (bottom) shows the functions of PAZ and PIWI domain in 3' siRNA recognition and mRNA cleavage, respectively. (Model adapted fromSong et al. 2004)

1.3 The biological functions of RNA interference

The RNAi-based pathway plays important roles in a wide variety of biological processes. RNAi serve as a genome defense mechanism to silence intruding viruses and transposons. In *C. elegans* and *Drosophila*, DNA or RNA virus infections trigger the production of virus-specific siRNAs, which results in the inhibition of viral replcation (Li et al. 2002; Ding et al. 2004). Transposon is also silenced in the germ line by the RNAi pathway in *C. elegans* and mammals (Sijen and Plasterk 2003; Klattenhoff and

Theurkauf 2008). The suppression of transposon transposition or replication serves as a protective mechanism to maintain the genome integrity.

In addition to silence targets post-transcriptionally, siRNAs can also induce transcriptional silencing in plants, fruit fly and mammals (Wassenegger and Pelissier 1998; Morris 2008). In *Arabidopsis*, siRNA induces transcriptional silencing by triggering DNA methylation, a process called RNA-directed DNA methylation (RdDM). In addition to modulate DNA methylation, siRNA can also trigger histone modification. The formation of heterochromatin at centromeric region in *S. pombe* requires RNAi machinery (White and Allshire 2008). To induce heterochromatin formation, Ago1 (Argonaute), Chp1 (a chromodomain containing protein), and Tas3 (a novel protein) form the RITS (RNA-induced initiation of transcriptional gene silencing) complex (Verdel et al. 2004), which use siRNA as a guide to trigger histone 3 lysine 9 methylation.

In addition to siRNA, microRNA (miRNA) represents another class of small RNA produced by Dicer. miRNA are endogenously encoded and evolutionally conserved small RNAs functions in gene-specific translational repression (Carrington and Ambros 2003). miRNAs are cleaved products of pre-miRNA, which forms a hairpin-like structure and therefore recognized by Dicer (Figure 3). miRNAs play a major role in controlling various developmental processes, such as flowering in plants and blood vessel in mammals (Terzi and Simpson 2008; Wang et al. 2008). In addition to serve as developmental cues, miRNAs also function in stress response, such as response against osmotic pressure in plants and stress-dependent cardiac growth in mice (Sunkar and

Zhu 2004; van Rooij et al. 2007). With hundreds of human miRNA identified, it has been estimated that about 30% of human genes are under controlled by miRNAs. (Lewis et al. 2005)

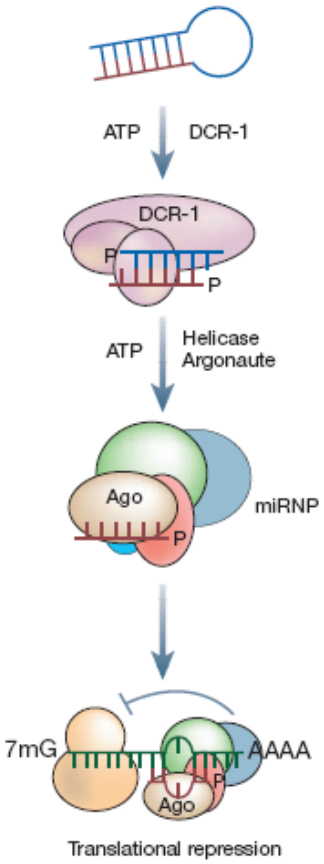


Figure 3. Biogenesis of miRNA and its role in translational repression.

A cartoon shows that Dicer (DCL) cleaves pre-miRNA into miRNAs, while Argonaute (Ago) uses miRNA as a guide to trigger the translational repression of target genes.

(Figure adapted from Meister and Tuschl, 2004)

Recently a novel class of small RNAs, named PIWI-interacting RNAs (piRNA), are reported in worms, fruit fly and mammals (Girard et al. 2006; Lau et al. 2006; Vagin et al. 2006). Surprisingly, the biogenesis of piRNA does not require Dicer,

suggesting a distinct mechanism for piRNA production. Phylogenetic analysis of Argonaute proteins reveals two major clades: an AGO clade and a PIWI clade. While Argonautes of AGO clade bind siRNA or miRNA, Argonaute of PIWI clade specifically binds piRNA. These dicer-independent piRNAs are mostly derived from transposons or several distinct loci of the genomes. piRNAs play a major role in silencing transposons and likely a role in stem cell maintenance, as mutations of PIWI clade genes in mice cause infertile phenotype. (Deng and Lin 2002; Aravin et al. 2006).

1.4 RNA interference in the filamentous fungi *Neurospora*

The filamentous fungus *Neurospora* is one of the first model organisms used to study RNAi (Romano and Macino 1992). Through a genetic screen conducted in *Neurospora*, Macino and colleagues identified the first cellular RNAi component in any organism in 1999 (Cogoni and Macino 1999). *Neurospora* can be easily cultured in large quantity for biochemical purification and is also a great model system for genetic studies, as *Neurospora* maintains haploid throughout its vegetative stage (Borkovich et al. 2004). Gene-specific knockout or transgenic strain can be efficiently made in Neurospora. Furthermore, thousands of gene specific knockout strains have been made by a *Neurospora* genome project, which allows rapid characterization of interested genes (Dunlap et al. 2007).

Quelling is an RNAi-based posttranscriptional gene silencing mechanism in *Neurospora*. Quelling was first reported in 1992 by Dr. Macino and colleagues, which

they found that the transformation of multiple copies of a transgene into *Neurospora* leads to the silencing of both the transgene and the endogenous genes with sequence homology (Romano and Macino 1992). The gene silencing by quelling acts at the post-transcriptional level, as the nuclear nascent transcripts remain at similar levels, while cytoplasmic transcripts are decreased in quelled strains (Cogoni et al. 1996).

Three quelling-deficient genes named *qde-1*, *qde-2*, and *qde-3* were previously identified by a genetic screen (Cogoni and Macino 1999a; Cogoni and Macino 1999b; Catalanotto et al. 2000). QDE-1 (an RNA-dependent RNA polymerase) and QDE-3 (a RecQ DNA helicase) are thought to function upstream of the RNAi pathway and are likely involved in the generation of transgene-specific dsRNA. Consistent with this notion, QDE-1 and QDE-3 are not required for the production of siRNA and gene silencing if dsRNA is made from an exogenous hairpin RNA (Catalanotto et al. 2004). In addition, *Neurospora* has two partially redundant Dicer proteins, DCL-1 and DCL-2. DCL-2 is responsible for ~ 90% of the siRNA-generating activity. DCL-1 and DCL-2 cleave dsRNA into siRNA of around 25nt in length (Catalanotto et al. 2004). Afterwards, the duplex form of siRNA is loaded onto RISC complex, where QDE-2 forms the catalytic core (Maiti et al. 2007).

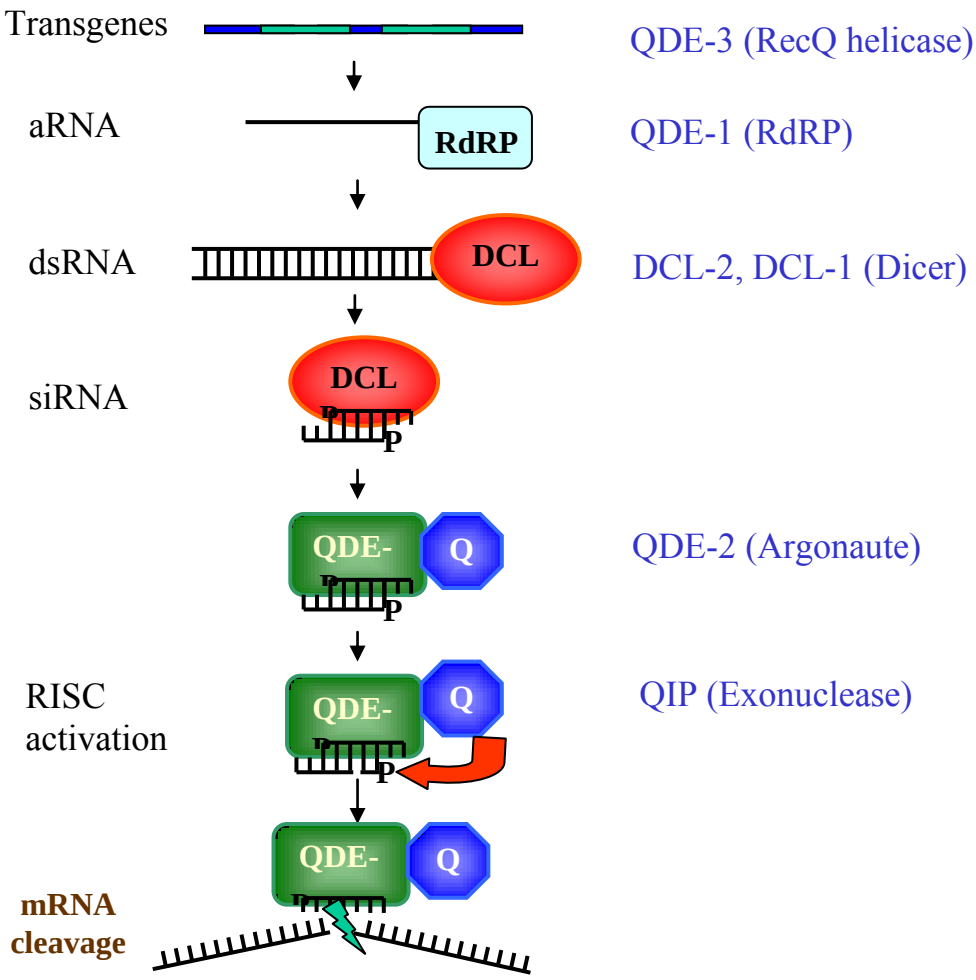


Figure 4. Quelling, a RNAi-based post transcriptional gene silencing pathway in the filamentous fungus *Neurospora.*

A model presents the mechanism of quelling induced by transgenes. Gene names involved in Quelling are shown in blue.

Our recent study shows that QDE-2 and a QDE-2 interacting exonuclease, QIP, are required for generating active RISC complex, which contains single stranded

siRNA (Maiti et al. 2007). As shown in figure 4, QDE-2 first cleaves the passenger strand of the siRNA duplex to create a nicked duplex. QIP, an exonuclease that interacts with QDE-2, then removes the nicked passenger strand of siRNA, resulting in the activation of RISC (Maiti et al. 2007). The active RISC can then use single stranded siRNA as a guide to cleave complementary target RNAs.

During meiosis, *Neurospora* possess another RNAi-based pathway to silence potential parasitic DNA. This pathway silences unpaired DNA region during meiosis after karyogamy and therefore was named Meiotic Silencing by Unpaired DNA, or MSUD (Aramayo and Metzenberg 1996). *sad-1* (an RNA dependent RNA polymerase), *dcl-1* (an Dicer), and *sms-2* (an Argonaute) are required for MSUD (Shiu et al. 2001; Lee et al. 2003; Alexander et al. 2008).

1.5 Summary

The identifications of piRNAs, miRNAs and endogenous siRNAs have demonstrated the complexity of small RNAs world and reveal their key functions in regulating various biological processes. However, no miRNA or piRNA-like small RNA was previously identified in fungus or simple eukaryotes. Therefore, the origin of different types of small RNAs is unclear. In Chapter 2, I describe our identification of a novel type of small RNA, named qiRNA for QDE-2 Interacting

small RNA. qiRNA is dramatically produced when cell encounters DNA damage. The biogenesis and the function of qiRNAs will be discussed.

In chapter 3, I describe our efforts to understand the biogenesis and the nature of the aberrant RNA (also called pre-siRNA). The production of aberrant RNA is thought to be a critical step in initiating RNAi against transgenes, transposon or viruses. Once aberrant RNA is made, RNA dependent RNA polymerase (RdRP) can convert aberrant RNA into dsRNA and trigger silencing. Consistent with this notion, RdRP are required for defense against transgene or viruses in various model organisms. However, it is not clear which RNA polymerase is responsible for production of aberrant RNA and how aberrant RNA was specifically recognized by RdRP over other cellular RNAs..

Depsite our current knowedge of RNAi pathways, very little is known on how the RNAi components are regulated. In Chapter 4, we showed that dsRNA is a trigger to activate transcription of several key RNAi genes such as *qde-2* (Argonaute) and *dcl-2* (Dicer). A genome-wide identification of dsRNA activated genes further reveal a dsRNA induced transcriptional program in *Neurospora*. Possible functions of this dsRNA response program in defense against transposon and virus will be discussed.

1.6 Bibliography

Alexander, W.G., N.B. Raju, H. Xiao, T.M. Hammond, T.D. Perdue, R.L. Metzenberg, P.J. Pukkila, and P.K. Shiu. 2008. DCL-1 colocalizes with other components of the MSUD machinery and is required for silencing. *Fungal Genet Biol* 45: 719-27.

Aramayo, R. and R.L. Metzenberg. 1996. Meiotic transvection in fungi. *Cell* 86: 103-13.

Aravin, A., D. Gaidatzis, S. Pfeffer, M. Lagos-Quintana, P. Landgraf, N. Iovino, P. Morris, M.J. Brownstein, S. Kuramochi-Miyagawa, T. Nakano, M. Chien, J.J. Russo, J. Ju, R. Sheridan, C. Sander, M. Zavolan, and T. Tuschl. 2006. A novel class of small RNAs bind to MILI protein in mouse testes. *Nature* 442: 203-7.

Bernstein, E., A.A. Caudy, S.M. Hammond, and G.J. Hannon. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409: 363-6.

Borkovich, K.A., L.A. Alex, O. Yarden, M. Freitag, G.E. Turner, N.D. Read, S. Seiler, D. Bell-Pedersen, J. Paietta, N. Plesofsky, M. Plamann, M. Goodrich-Tanrikulu, U. Schulte, G. Mannhaupt, F.E. Nargang, A. Radford, C. Selitrennikoff, J.E. Galagan, J.C. Dunlap, J.J. Loros, D. Catcheside, H. Inoue, R. Aramayo, M. Polymenis, E.U. Selker, M.S. Sachs, G.A. Marzluf, I. Paulsen, R. Davis, D.J. Ebbole, A. Zelter, E.R. Kalkman, R. O'Rourke, F. Bowring, J. Yeadon, C. Ishii, K. Suzuki, W. Sakai, and R. Pratt. 2004. Lessons from the genome sequence of Neurospora crassa: tracing the path from genomic blueprint to multicellular organism. *Microbiol Mol Biol Rev* 68: 1-108.

Carrington, J.C. and V. Ambros. 2003. Role of microRNAs in plant and animal development. *Science* 301: 336-8.

Catalanotto, C., G. Azzalin, G. Macino, and C. Cogoni. 2000. Gene silencing in worms and fungi. *Nature* 404: 245.

Catalanotto, C., M. Pallotta, P. ReFalo, M.S. Sachs, L. Vayssie, G. Macino, and C. Cogoni. 2004. Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in Neurospora crassa. *Mol Cell Biol* 24: 2536-45.

Cogoni, C., J.T. Irelan, M. Schumacher, T.J. Schmidhauser, E.U. Selker, and G. Macino. 1996. Transgene silencing of the al-1 gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. *Embo J* 15: 3153-63.

Cogoni, C. and G. Macino. 1999a. Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. *Nature* 399: 166-9.
-. 1999b. Posttranscriptional gene silencing in Neurospora by a RecQ DNA helicase. *Science* 286: 2342-4.

Deng, W. and H. Lin. 2002. miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. *Dev Cell* 2: 819-30.

Ding, S.W., H. Li, R. Lu, F. Li, and W.X. Li. 2004. RNA silencing: a conserved antiviral immunity of plants and animals. *Virus Res* 102: 109-15.

Dunlap, J.C., K.A. Borkovich, M.R. Henn, G.E. Turner, M.S. Sachs, N.L. Glass, K. McCluskey, M. Plamann, J.E. Galagan, B.W. Birren, R.L. Weiss, J.P. Townsend, J.J. Loros, M.A. Nelson, R. Lambreghts, H.V. Colot, G. Park, P. Collopy, C. Ringelberg, C. Crew, L. Litvinkova, D. DeCaprio, H.M. Hood, S. Curilla, M. Shi, M. Crawford, M. Koerhsen, P. Montgomery, L. Larson, M. Pearson, T. Kasuga, C. Tian, M. Basturkmen, L. Altamirano, and J. Xu. 2007. Enabling a community to dissect an organism: overview of the Neurospora functional genomics project. *Adv Genet* 57: 49-96.

Fire, A., S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 391: 806-11.

Girard, A., R. Sachidanandam, G.J. Hannon, and M.A. Carmell. 2006. A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature* 442: 199-202.

Guo, S. and K.J. Kemphues. 1995. par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* 81: 611-20.

Hammond, S.M., S. Boettcher, A.A. Caudy, R. Kobayashi, and G.J. Hannon. 2001a. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293: 1146-50.

Hammond, S.M., A.A. Caudy, and G.J. Hannon. 2001b. Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev Genet* 2: 110-9.

Hannon, G.J. and J.J. Rossi. 2004. Unlocking the potential of the human genome with RNA interference. *Nature* 431: 371-8.

Klattenhoff, C. and W. Theurkauf. 2008. Biogenesis and germline functions of piRNAs. *Development* 135: 3-9.

Lau, N.C., A.G. Seto, J. Kim, S. Kuramochi-Miyagawa, T. Nakano, D.P. Bartel, and R.E. Kingston. 2006. Characterization of the piRNA complex from rat testes. *Science* 313: 363-7.

Lee, D.W., R.J. Pratt, M. McLaughlin, and R. Aramayo. 2003. An argonaute-like protein is required for meiotic silencing. *Genetics* 164: 821-8.

Lewis, B.P., C.B. Burge, and D.P. Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120: 15-20.

Li, H., W.X. Li, and S.W. Ding. 2002. Induction and suppression of RNA silencing by an animal virus. *Science* 296: 1319-21.

Maiti, M., H.C. Lee, and Y. Liu. 2007. QIP, a putative exonuclease, interacts with the Neurospora Argonaute protein and facilitates conversion of duplex siRNA into single strands. *Genes Dev* 21: 590-600.

Meister, G. and T. Tuschl. 2004. Mechanisms of gene silencing by double-stranded RNA. *Nature* 431: 343-9.

Mello, C.C. 2007. Return to the RNAi world: rethinking gene expression and evolution. *Cell Death Differ* 14: 2013-20.

Morris, K.V. 2008. RNA-mediated transcriptional gene silencing in human cells. *Curr Top Microbiol Immunol* 320: 211-24.

Napoli, C., C. Lemieux, and R. Jorgensen. 1990. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell* 2: 279-289.

Nguyen, T., E.M. Menocal, J. Harborth, and J.H. Fruehauf. 2008. RNAi therapeutics: an update on delivery. *Curr Opin Mol Ther* 10: 158-67.

Rand, T.A., K. Ginalski, N.V. Grishin, X. Wang, S. Petersen, and F. Du. 2004. Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity

Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Proc Natl Acad Sci U S A* 101: 14385-9.

Romano, N. and G. Macino. 1992. Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. *Mol Microbiol* 6: 3343-53.

Shiu, P.K., N.B. Raju, D. Zickler, and R.L. Metzenberg. 2001. Meiotic silencing by unpaired DNA. *Cell* 107: 905-16.

Sijen, T. and R.H. Plasterk. 2003. Transposon silencing in the Caenorhabditis elegans germ line by natural RNAi. *Nature* 426: 310-4.

Song, J.J., S.K. Smith, G.J. Hannon, and L. Joshua-Tor. 2004. Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* 305: 1434-7.

Sunkar, R. and J.K. Zhu. 2004. Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *Plant Cell* 16: 2001-19.

Terzi, L.C. and G.G. Simpson. 2008. Regulation of flowering time by RNA processing. *Curr Top Microbiol Immunol* 326: 201-18.

Vagin, V.V., A. Sigova, C. Li, H. Seitz, V. Gvozdev, and P.D. Zamore. 2006. A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* 313: 320-4.

van Rooij, E., L.B. Sutherland, X. Qi, J.A. Richardson, J. Hill, and E.N. Olson. 2007. Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* 316: 575-9.

Vastenhouw, N.L., S.E. Fischer, V.J. Robert, K.L. Thijssen, A.G. Fraser, R.S. Kamath, J. Ahringer, and R.H. Plasterk. 2003. A genome-wide screen identifies 27 genes involved in transposon silencing in C. elegans. *Curr Biol* 13: 1311-6.

Verdel, A., S. Jia, S. Gerber, T. Sugiyama, S. Gygi, S.I. Grewal, and D. Moazed. 2004. RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* 303: 672-6.

Verdel, A. and D. Moazed. 2005. RNAi-directed assembly of heterochromatin in fission yeast. *FEBS Lett* 579: 5872-8.

Wang, S., A.B. Aurora, B.A. Johnson, X. Qi, J. McAnally, J.A. Hill, J.A. Richardson, R. Bassel-Duby, and E.N. Olson. 2008. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Dev Cell* 15: 261-71.

Wassenegger, M. and T. Pelissier. 1998. A model for RNA-mediated gene silencing in higher plants. *Plant Mol Biol* 37: 349-62.

White, S.A. and R.C. Allshire. 2008. RNAi-mediated chromatin silencing in fission yeast. *Curr Top Microbiol Immunol* 320: 157-83.

Zamore, P.D., T. Tuschl, P.A. Sharp, and D.P. Bartel. 2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101: 25-33.

CHAPTER TWO

QDE-2 INTERACTING SMALL RNA (QIRNA), A NOVEL TYPE OF SMALL RNA INDUCED BY DNA DAMAGE

2.1 Introduction

RNA interference (RNAi) is a conserved eukaryotic gene regulation mechanism that uses small interfering RNAs to trigger posttranscriptional or transcriptional gene silencing (Buhler and Moazed, 2007; Hannon, 2002; Tomari and Zamore, 2005). In addition to the microRNAs, recent studies have uncovered the existence of several types of endogenous small RNAs (20-30 nt) in animals and plants, including endogenous siRNAs, Piwi-interacting RNAs (piRNAs), rasiRNAs and 21U RNAs (Ambros and Chen, 2007; Aravin et al., 2007; Baulcombe, 2004; Ghildiyal et al., 2008; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Ruby et al., 2006). These small RNAs frequently originate from mobile and repetitive elements of the genome and play important roles in germ cell maintenance, gene regulation and transposon silencing. Differences exist among these classes of small RNAs. For example, piRNAs differ from siRNA in sizes and are produced in pathway independent of Dicer (Lin, 2007). How these small RNAs are originated and regulated are largely unknown. Despite the

conservation of the RNAi pathways, whether similar types of small RNAs exist in the lower eukaryotes has been largely unexplored.

In this chapter I describe our identification of a novel class of small RNA induced by DNA damaging conditions. This class of small RNAs, named qiRNAs for their association with QDE-2, are about 20-21 nt long (several nt shorter than *Neurospora* siRNAs) with a strong preference for uridine at the 5' end and are mostly originated from the ribosomal DNA (rDNA) locus. Production of qiRNAs depends on the RNA-dependent RNA polymerase QDE-1 and RecQ helicase QDE-3. qiRNA biogenesis also requires the DNA damage-induced aberrant transcripts, a process that is dependent on the Warner/Bloom RecQ DNA helicase homolog QDE-3. The *Neurospora* RNAi mutants exhibit increased sensitivity to DNA damage, suggesting a role for qiRNAs in DNA damage response. Our results further suggest that the production of qiRNA is a mechanism that contributes to the DNA damage checkpoints by inhibiting protein translation after DNA damage.

2.2 Materials and Methods

2.2.1 Strains and growth conditions

The wild-type strain used in this study was FGSC 4200(a). *qde-1*, *qde-3*, and *dcl-1*; *dcl-2* double mutant were generated from our previous studies (Choudhary

et al., 2007; Maiti et al., 2007), and the method is described in 4.2.2. Mutants and knock-out strains for genes involved in DNA repairs were ordered from Fungal Genetic Stock Center (FGSC). The following strains were used: *atm* (FGSC11162, NCU00274.1), *mus-9* (FGSC5146, NCU11188.1) (Kazama et al., 2008), *mus-23* (FGSC8342, NCU08730.1) (Watanabe et al., 1997), *mei-3* (FGSC6187, NCU2741.1) (Kato and Inoue, 2006), *mus-11* (FGSC5150, NCU04275.1) (Sakuraba et al., 2000), *telomerase* (FGSC12704, NCU2791.1), *mus-58* (FGSC11164, NCU08346.1) (Wakabayashi et al., 2008), *chk2* (FGSC11170, NCU02814.1) (Pregueiro et al., 2006), *uvs-6* (FGSC4179, NCU00901.1) , (Kafer, 1983), *mus-25* (FGSC6424, NCU11255.1) (Suzuki et al., 2005), and *mus-38* (FGSC11191, NCU00942.1) (Hatakeyama et al., 1998). These genes are homologs of *S. cerevisiae* ATM, ATR, RAD51, RAD52, teleomerase. CHK1, CHK2, RAD50, RAD54 and RAD1, respectively. Liquid cultures were grown in minimal medium (1x Vogel's, 2% glucose) (Davis and deSerres, 1970). For liquid cultures containing QA, 0.01 M QA (pH 5.8) was added to the liquid culture medium containing 1xVogel's, 0.1% glucose, and 0.17% arginine. For liquid culture containing DNA damaging agents, histidine (50 to 100 μg/ml) or EMS (ethyl methanesulfonate, 0.2%) was added and cultures were harvested 40 hours later. For cultures containing amino acids, the indicated amino acid (50 μg/ml) were added and cultures were harvested 40 hours later.

2.2.2 Measurement of spontaneous mutation rate

Spontaneous mutation rate at the *mtr* locus was measured with protocol adapted from previous studies (Chary et al., 1994; Stadler et al., 1991). *mtr* encodes for the neutral amino acid permease in *Neurospora*. Mutations at the *mtr* locus is reflected by the resistance of the mutant strains to toxic amino acid analog p-fluorophenylalanine (FPA). $5x10^6$ conidia of 7-day old culture were plated on sorbose-containing medium with FPA (50 μg/ml) plates in the presence or absence of histidine (100 μg/ml). Colonies were counted after 5-days of growth at 30^oC. 200 conidia of each strain were also plated on medium without FPA to determine viability. *mtr* mutation rates were determined by correcting for viability.

2.2.3 Purification and cloning of QDE-2 associated RNA

Immunopurification of Myc-QDE-2 ribonucleoprotein complex was performed as previously described (Maiti et al., 2007). Liquid cultures of Myc-QDE-2 expressing strain were grown in the presence or absence of histidine (100 μg/ml), and harvested 40 hours after inoculation. Cell extracts were prepared in a buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1% NP40, 1mM DTT, protease inhibitors, and 100 U/mL RNase inhibitor (RNaseOut,

Invitrogen). Cell extracts were then pre-cleaned by incubation with 20 μL of Protein G Sepharose (GE Healthcare) for 1 h at 4°C. For immunoprecipitation of Myc-QDE-2, the pre-cleaned extracts were incubated first with 5μg Myc monoclonal antibody (Clone 9E10, Roche) overnight at 4°C and followed with 25 μL of Protein G Sepharose for 2 h at 4°C. Immunoprecipitated beads were washed five times using the extraction buffer. The beads were then resuspended in 150 μL proteinase K buffer and treated with 1mg/ml proteinase K (RNA grade, Invitrogen) at 65°C for 1 hr. QDE-2 associated RNAs were recovered by phenol/chloroform extraction and ethanol precipitation. To visualize the QDE-2 associated RNAs, 5% of the purified RNAs were labeled at 3' end with [32P] pCp (PerkinElmer) by T4 RNA ligase (Ambion). The labeled RNAs were resolved on 16% polyacrylamide gel before exposing to X-ray films. Small RNAs were cloned using a procedure modified from Lau et al. (Lau et al., 2001). In brief, QDE-2 associated small RNAs were CIP (calf intestinal phosphatase) treated, followed by PNK treatment in order to clone small RNAs with potential different number of phosphate at 5' ends. Trace amounts of radio-labeled 18 to 24 nt siRNAs were then mixed with QDE-2 associated small RNAs or total RNAs. After resolved on 16% denaturing gel, RNAs around 18 to 26 nt were recovered. The recovered RNAs were ligated to 3' adaptor (miRNA linker1, IDT) in the absence of ATP, and then ligated to 5' adaptor (5' miRNA linker, IDT). The ligated products with expected sizes were recovered and reverse transcribed to

cDNAs. The cDNAs were digested with BanI (NEB) and concatamerized with T4 DNA ligase (NEB). The concatamers were cloned and sequenced and small RNA sequences were blasted to *Neurospora* genome using the *Neurospora crassa* database at the Broad Institute. The sequence of ribosomal DNA were generated by sequencing the plasmid pKH1 (containing a rDNA repeat, generously provided by Eric Selker) (Rountree and Selker, 1997). The rDNA sequence has been submitted to GenBank (FJ360521).

2.2.4 Enrichment of Low-molecular-weight small RNA and Northern blot analyses

Enriched low-molecular-weight RNAs were used to detect small RNAs. To enrich low-molecular-weight RNAs, 250 μg total RNAs were incubated in buffer containing 5% PEG-8000 and 0.5M NaCl at 4°C for 30 min. After centrifugation at 12000g for 10 min, the supernatants containing low-molecular-weight RNAs were ethanol precipitated. 25 μg of the low molecular weight RNAs were run on urea-containing 16% polyacrylamide and transferred onto Hybond N^+ membrane (Amersham Biosciences). Sense rRNA probes were *in vitro*-transcribed by MAXIscript T7 kit (Ambion) using PCR template derived from 26S rDNA regions. The probes were partially hydrolysed in solution containing 80mM $NaHCO_3$ and 120 mM Na_2CO_3 at 60 °C for 3 hours before using for hybridization.

Total RNA was used to detect the aberrant transcripts from the rDNA region. Total RNAs was extracted as previously described (Aronson et al., 1994). 40 μg of total RNA were run on formaldehyde containing 1.3% agarose gel and transferred onto Hybond N^+ membrane. Probe was also *in vitro*-transcribed by using PCR template derived from upstream sequence of rDNA coding region.

2.2.5 Western Blot analysis

Protein extraction, quantification and Western blot analysis were performed as previously described (Cheng et al., 2001; Garceau et al., 1997). Equal amounts of total protein (50 μg) were loaded into each lane of a 7.5% SDS PAGE gel. After electrophoresis, proteins were transferred onto a PVDF membrane. The western blot analyses were performed using standard protocol (Garceau et al., 1997) and the blots were developed by chemiluminescence (ECL, Amersham Pharmacia). QDE-2 antibody was used in 1:3000 dilution and was generated by Dr. Maiti. The method of making QDE-2 specific antibody was described in a previous study (Choudhary et al., 2007)

.

2.2.6 Quantitative real time-PCR analysis

Quantitative real-time PCR (qRT-PCR) was performed with an Applied Biosystems Prism 7900HT sequence detection system using a previously described protocol (Kurrasch et al., 2004). Briefly, total RNA was purified using

an RNeasy mini kit (QIAGEN) and treated with DNase I (0.6 units; Roche). Equal amounts of DNase-treated RNAs (2 μg) were reverse transcribed with SuperScript II (Invitrogen) using random hexamers. cDNAs (50 ng) were mixed in 10 μl qRT-PCR mix with 5 μl SYBR Green PCR Master Mix (Applied Biosystems) and 150 nM primers. Each reaction was duplicated, and non-reverse-transcribed samples were used as controls. Gene-specific primers were designed using Primer Express software (Perkin-Elmer Life Sciences), and each primer pair was validated by cDNA template titration to ensure similar amplification kinetics and a single melting point of quantitative PCR products.

Neurospora β-tubulin gene (NCU04054.1) was used as an internal control for qRT-PCR. Three rDNA specific pairs of primer, U1 (GCGCAATCGACCTTTGGA / TCTCATACAGGCCCGTCGTAC), U2 (CTGAGAAGGTGCGACCCAGT / TTAAAAATCGACTCACGGCCAC), and D1 (GTTGCTTTCCAGCTCCTACTGG / CCTTTACTCAACCACATAGC) were used to detect transcript derived from intergenic regions of the rDNA locus.

2.2.7 Measurement of general translation rate

Conidia from 7-old cultures were inoculated into petri dishes containing 1xVogel's minimum medium with 2% glucose and were incubated at room temperature for 2 days without shaking to allow mycelial mats to form. The mycelial mats were cut into discs of equal size. The discs were cultured in

1xVogel's minimum medium with 2% glucose overnight in the presence or absence of histidine or EMS (100μg/ml and 0.2%, repectively), and then metabolically labeled with 1μCi/ml EXPRE35S35S protein labeling mix (PerkinElmer) for 30 (EMS) or 60 (histidine) min. The protein extracts were then prepared as previously described (Cheng et al., 2001). Afterwards, 50 μg of total protein was precipitated by 10% TCA on filter paper 413 (VWR) for 30 min. The filter papers were then washed with 10% TCA (Trichloroacetic acid) twice for 5 min each and dried in 1:1 ethanol / diethyl ether followed by diethyl ether. The dried filter papers were immersed in 5ml scintillation fluid and 35S signals were counted. For control cultures, the protein synthesis inhibitor cycloheximide (10 μg/ml) was added just before the labeling. The low background radioactive counts obtained from these extracts confirmed that the radioactive counts measured in our assay were due to newly synthesized proteins.

2.2.8 Assay for measurement of DNA damage sensitivity

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

2.3 Results

2.3.1 DNA damage induces the Argonaute QDE-2 expression

During our study of QDE-2 expression, we observed that supplementing histidine in the culture medium resulted in a significant increase of *qde-2* mRNA and QDE-2 protein levels (Figure 5A). The induction of QDE-2 by histidine requires QDE-1 (RdRP), QDE-3 (RecQ helicase), and the DCLs (Figure 5B). Since QDE-1 and QDE-3 are involved in the generation of dsRNA and DCLs are important for maintaining the steady state level of QDE-2 posttranscriptionally (Choudhary et al., 2007), these results suggest that histidine results in production of endogenous dsRNA, which activates *qde-2* transcription. Induction of QDE-2 was not observed when culture medium was supplemented with other amino acids (Figure 1C), suggesting that the effect of histidine on QDE-2 expression is specific and not due to a general amino acid effect.

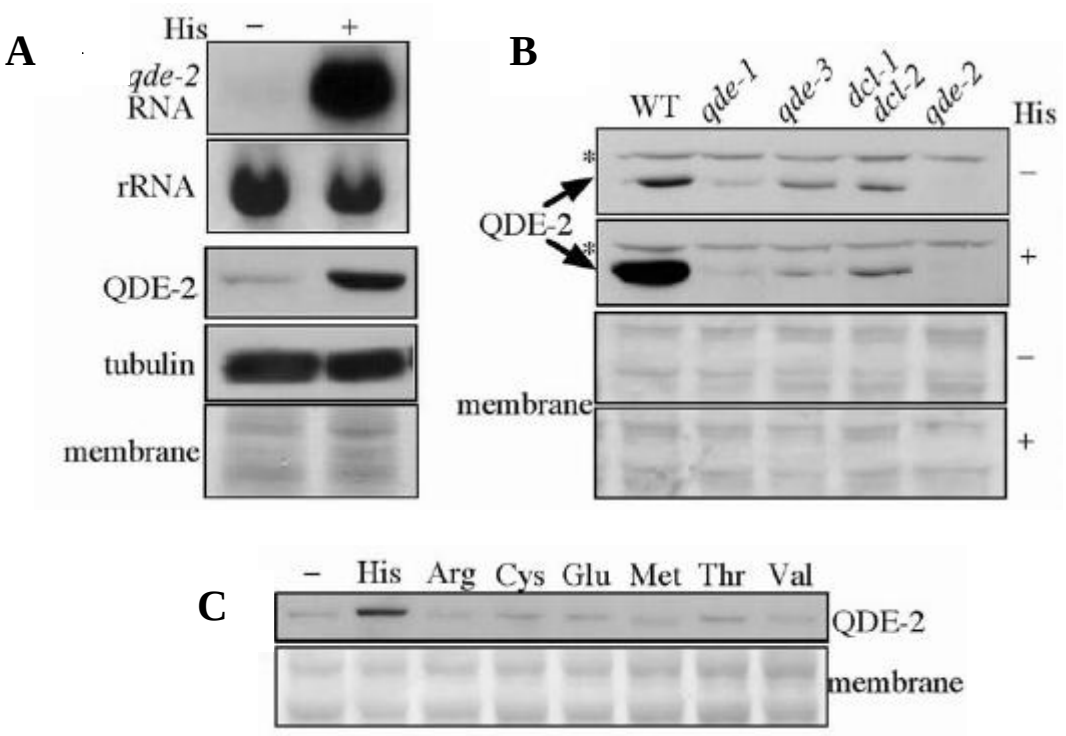
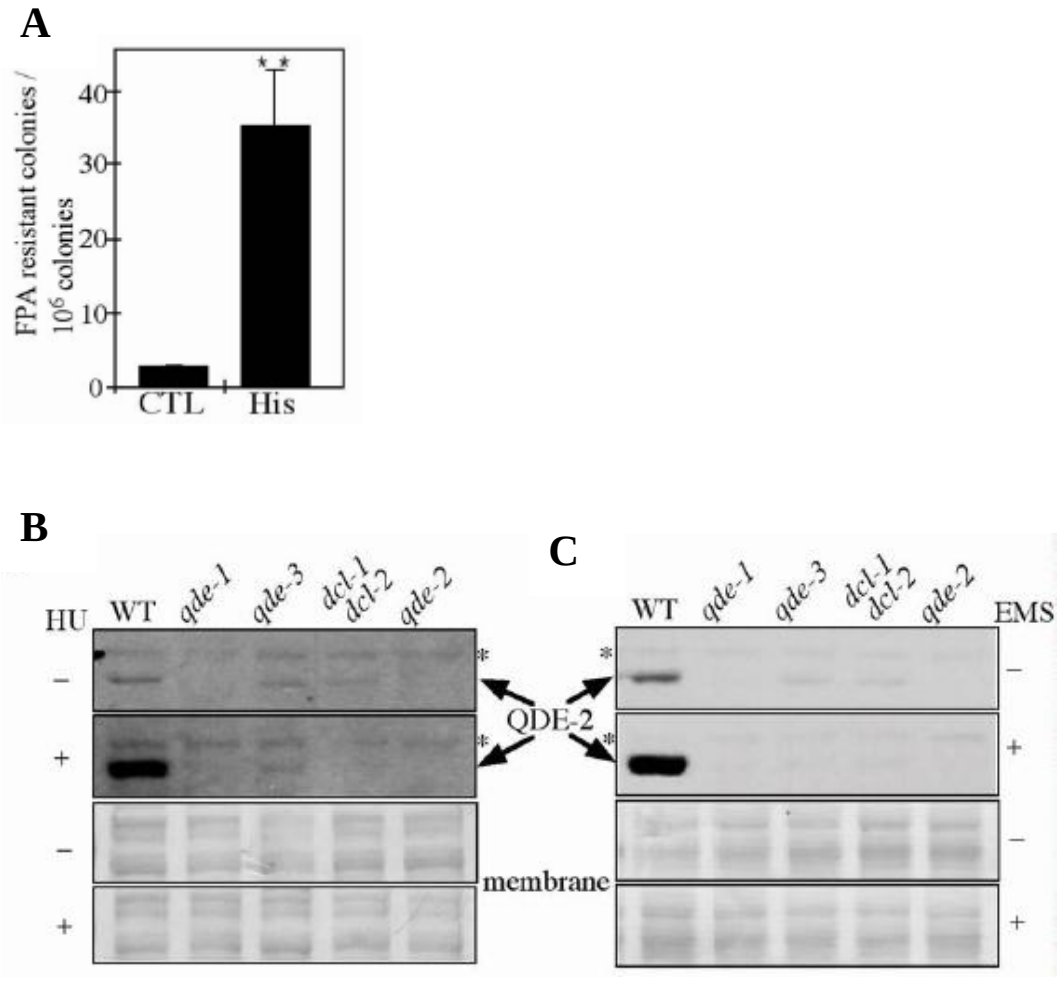


Figure 5. Histidine induces QDE-2 expression. (a) Northern and Western blot analyses showing the induction of *qde-2* mRNA and QDE-2 protein expression by histidine. rRNA was used as a loading control for RNA. Tubulin protein and amido black stained protein membrane were used as controls for Western blot. (b) Western blot analysis showing that the induction of QDE-2 by histidine requires QDE-1, QDE-3 and DCLs. The labels above the lanes indicate the genotypes of the strains used. The asterisk indicates a non-specific cross-reacting band recognized by our QDE-2 antibody. The amido black stained protein membrane shows the protein loading in the gel. (c) Western blot analysis showing the induction of QDE-2 by histidine but not by other amino acids. 50 μg/ml of each amino acid was used.

Histidine is long known to inhibit *Neurospora* cell growth and most *Neurospora* DNA repair mutants exhibit hypersensitivity to histidine (Sakuraba et al., 2000; Schroeder, 1970). In addition, histidine treatment was shown to inhibit DNA replication, reduce NTP pool, and result in DNA nicks or breaks and genome instability in *Neurospora* (Howard and Baker, 1988; Srivastava et al., 1988). These results suggest that histidine is a DNA mutagen for *Neurospora*. To further demonstrate the DNA damaging effects of histidine, we measured the mutation frequency in a wild-type *Neurospora* strain exposed to histidine treatment. As shown in Figure 6A, the presence of histidine significantly increased the mutation rate at the *mtr* locus encoding the neutral amino acid permease. Together with previous studies, this result indicates that histidine treatment can result in DNA damage in *Neurospora*.

The genome toxicity effects of histidine was proposed to be its ability to directly inhibit ribonucleotide reductase[25]. Consistent with this notion, treatment with hydroxyurea (HU), a specific inhibitor of ribonucleotide reductase, also resulted in the induction of QDE-2 in a QDE-1 and QDE-3 dependent manner (Figure 6B). Chemical mutagens, such as ethyl methanesulfonate (EMS, Figure 6C) and methyl methanesulfonate (data not shown) also induce QDE-2 expression and the activation is dependent upon QDE-1, QDE-3, and DCL. Furthermore, in the absence of DNA damage agents, QDE-2 accumulates to high levels in DNA

repair mutants that are deficient in double-stranded DNA break repair or homologous recombination repair pathway (Figure 6D). Thus, spontaneously occurring and unrepaired DNA mutations also induce QDE-2 expression. Taken together, these results demonstrate that DNA damage result in the activation QDE-2 expression.



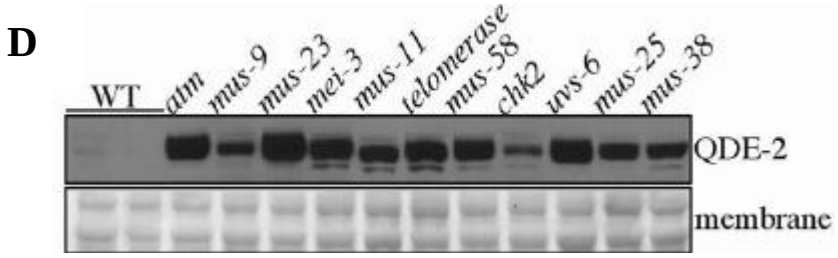
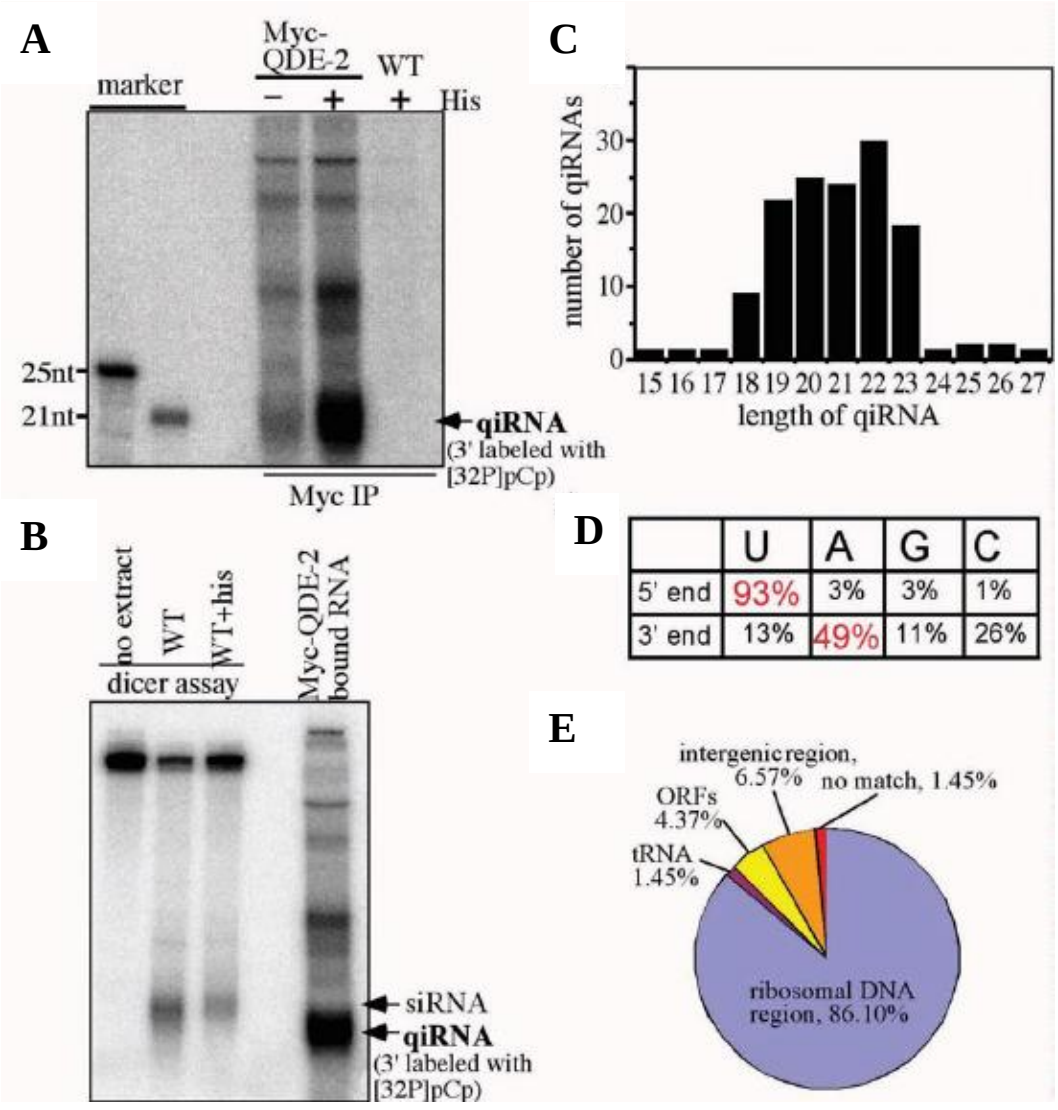


Figure 6. DNA mutagens induce QDE-2 expression (A) Mutation frequency of a wildtype strain is increased by histidine treatment. Mutation of the neutral amino acid permease gene *mtr* results in p-fluorophenylalanine (FPA) resistance. Statistical significance was determined by Student's t test, n = 3; **, p = 0.01. The error bars indicate standard deviations. (B & C) Western blot analyses showing the induction QDE-2 expression by hydroxy urea (HU) (B)and EMS (C) requires QDE-1, QDE-3 and DCLs. (D) Western blot analyses showing high QDE-2 levels in DNA repair mutants in the absence of DNA damage agent.

2.3.2 DNA damage induces QDE-2 Interacting RNA (qiRNA)

The requirement of QDE-1 and QDE-3 for QDE-2 induction suggests the generation of endogenous dsRNA after DNA damage. We reasoned that such dsRNA is processed into small RNAs, which associate with QDE-2, the Argonaute protein. To examine this possibility, we immunoprecipitated c-Myc tagged QDE-2 expressed in a *qde-2* knock-out strain (Maiti et al., 2007). The

associated RNA was extracted and 3'-end labeled with [^{32}P] Cytidine bisphosphate (pCp) by T4 RNA ligase. As shown in Figure 7A, Myc-QDE-2 specifically associated with a group of small RNAs approximately 20-21 nt in length, which were dramatically induced after histidine treatment. Similar results were also obtained with EMS treatment (data not shown).



	U	A	G	C
5' end	93%	3%	3%	1%
3' end	13%	49%	11%	26%

Figure 7. DNA damage results in the production of QDE-2 associated qiRNAs. (A) Enrichment of the Myc-QDE-2 associated small RNAs by immunoprecipitation. A wild-type strain that lacks the Myc-QDE-2 construct was used as the negative control. Immunoprecipitated RNA was 3'end labeled with ^{32}P pCp and separated on a 16% polyacrylamide gel. ^{32}P labeled oligo-nucleotides of indicated length were used as markers. (B) Side-by-side comparison of the sizes of qiRNAs and siRNA (generated by dicer assay). Note that qiRNA was 3'end labeled with ^{32}P pCp so that the actual size of qiRNA was 1 nucleotide smaller. (C) Size distribution of cloned and sequenced qiRNAs. (D) 5' and 3' end nucleotide composition of qiRNAs. (E) Genome distribution of qiRNAs.

Because these small RNAs are endogenously produced and are associated with QDE-2, they were named qiRNAs for QDE-2-Interacting small RNAs. The average size of qiRNAs is several nucleotides smaller than *Neurospora* siRNA, which is around 25 nt (Catalanotto et al., 2004). Direct comparison of qiRNA and the *Neurospora* siRNA confirmed the size difference of these two types of small RNAs (Figure 7B). To understand the nature of the DNA-damage induced qiRNAs, they were cloned and subjected to conventional sequencing. Analyses of 184 individual qiRNA sequences revealed that they indeed have an average length of about 20-21 nt (Figure 7C). Similar to the piRNAs and 21U RNAs recently identified in animals (Lin, 2007), the first nucleotide of the 5' end of qiRNAs

exhibits a strong preference for U (93%) (Figure 7D). In addition, the first nucleotide of the 3' end of qiRNA also prefers A (49%).

2.3.3 qiRNAs are mostly originated from ribosomal DNA locus

Sequence analyses of these qiRNAs also revealed that the vast majority of qiRNAs (86%) originated from the ribosomal DNA (rDNA) locus (Figure 7E), where ~200 copies of rDNA repeats form the nucleolus organizer region (NOR) (Krumlauf and Marzluf, 1980). The remaining qiRNAs were mapped to intergenic regions (6.57%), open reading frames (ORFs, 4.37%) and tRNAs (1.45%). Due to the small number of qiRNA sequences analyzed, their genome coverage is limited.

It was surprising that most of the qiRNAs are from the rDNA locus. Small RNAs with sequence corresponding to rRNAs were normally treated as non-specific degradation products in previous small RNA cloning experiments. Because of their specific association with QDE-2 and their 5' and 3' end nucleotide preferences, it is very unlikely that qiRNAs were due to nonspecific RNA degradation. We also purified and cloned small RNAs from total *Neurospora* RNA by size fractionation (not by affinity purification) using the same protocol and found that the small RNAs matching to rRNAs lack 5' and 3'end nucleotide preferences, suggesting that small RNAs purified by size fractionation are mostly RNA degradation products (data not shown). Our results

presented below further indicate that qiRNAs are produced by a specific pathway and are not non-specific degradation products of rRNAs.

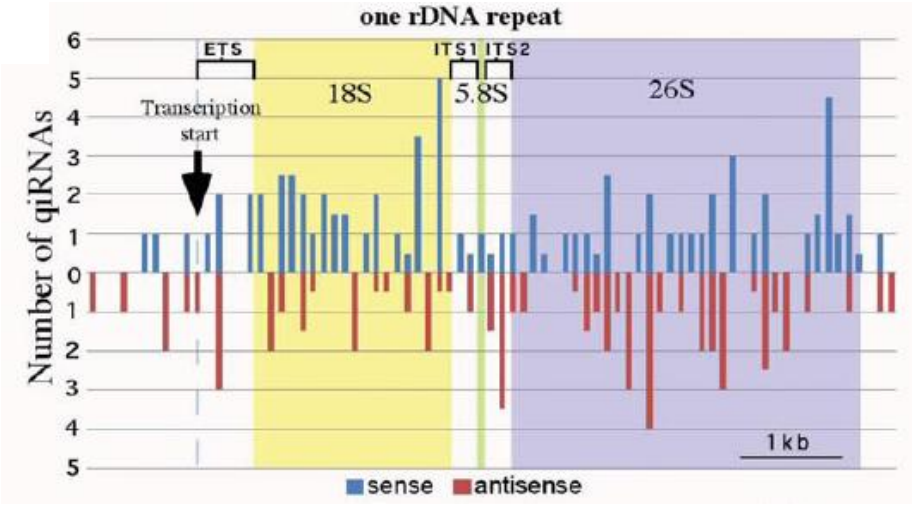


Figure 8. Mapping of rDNA-derived qiRNAs to the sense and antisense strands of an rDNA repeat. The regions encoding for the mature rRNAs are colored, while the transcribed and untranscribed spacer regions of the rDNA are labeled. Transcription state site of the pre-rRNA is indicated.

To map the location and orientation of rDNA specific qiRNAs, we sequenced one repeat of the rDNA gene. Our results showed that the qiRNAs from the rDNA locus correspond to both sense and antisense strands at approximately equal frequency, suggesting that the biogenesis of qiRNAs requires the formation of dsRNA (Figure 8. Furthermore, qiRNAs not only originate from

the region corresponding to the mature rRNAs (18S, 5.8S and 26S), many of them derive from the external and internal transcribed spacer regions (ETS, ITS1 & ITS2) and the untranscribed spacer regions (upstream of the rRNA transcription start site and downstream of the end of rRNA). The production of qiRNAs from the untranscribed rDNA spacer regions suggests that the biogenesis of qiRNAs may require unconventional transcriptional events.

2.3.4 Production of qiRNA requireds QDE-1 (RdRP), QDE-3 (RecQ helicase) and Dicers

To confirm the production of qiRNAs by DNA damage, we examined the production of rDNA-derived qiRNA by Northern blot analysis. As shown in Figure 9, the level of 26S rDNAspecific qiRNA was undetectable under normal conditions but was dramatically induced when the culture was treated with EMS. In addition, qiRNA accumulated to an elevated level in the *atm* mutant (impaired in DSB repair) without EMS treatment. These results indicate that qiRNAs are induced by DNA damage. Similar results were also obtained for 18S and 5.8S rDNA probes (data not shown). Furthermore, the production of qiRNA was completely abolished in the *qde-1* and *qde-3* mutant strains (Figure 9), indicating that these proteins are involved in the biogenesis of qiRNA. In contrast, the production of qiRNA was maintained in the *qde-2* mutant strain. Although the sizes of qiRNAs are smaller than that of siRNA, we surprisingly found that the

production of qiRNA is abolished in the *dcl-1 dcl-2* double mutant (Figure 9). In addition, long RNA species accumulated in the *dcl* mutant after DNA damage and resulted in a smear of RNA signals, suggesting the rDNA-specific long dsRNA accumulated in the absence of DCLs. Since the biogenesis of piRNA and rasiRNA in animals are independent of Dicer (Aravin et al., 2006; Brennecke et al., 2007; Grivna et al., 2006; Gunawardane et al., 2007; Lau et al., 2006), this result indicates the possibility that in *Neurospora*, qiRNA is produced from a distinct mechanism.

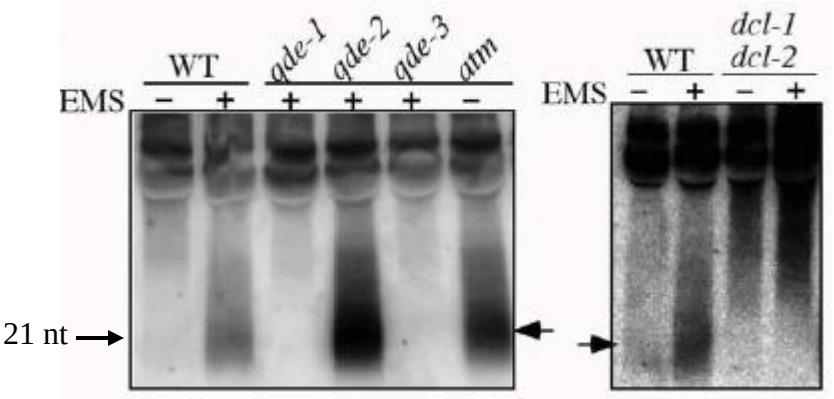


Figure 9. qiRNA biogenesis requires *qde-1*, *qde-3* and *dicers*. 16% urea-containing polyacrylamide gel was used. An RNA probe specific for the antisense 26S RNA was used.

2.3.5 DNA damage induces the production of aberrant rRNA transcript from intergenic rDNA regions

As mentioned earlier, qiRNAs are also derived from the intergenic untranscribed rDNA region, indicating that unconventional transcription may occur to produce aberrant transcripts at the rDNA locus upon DNA damage. To detect those aberrant transcripts, we examine transcripts levels from the intergenic DNA spacer regions because of their low background transcription. Quantitative RT-PCR experiments and Northern blot analysis showed that RNA transcripts originating from both upstream and downstream of the transcribed rDNA region

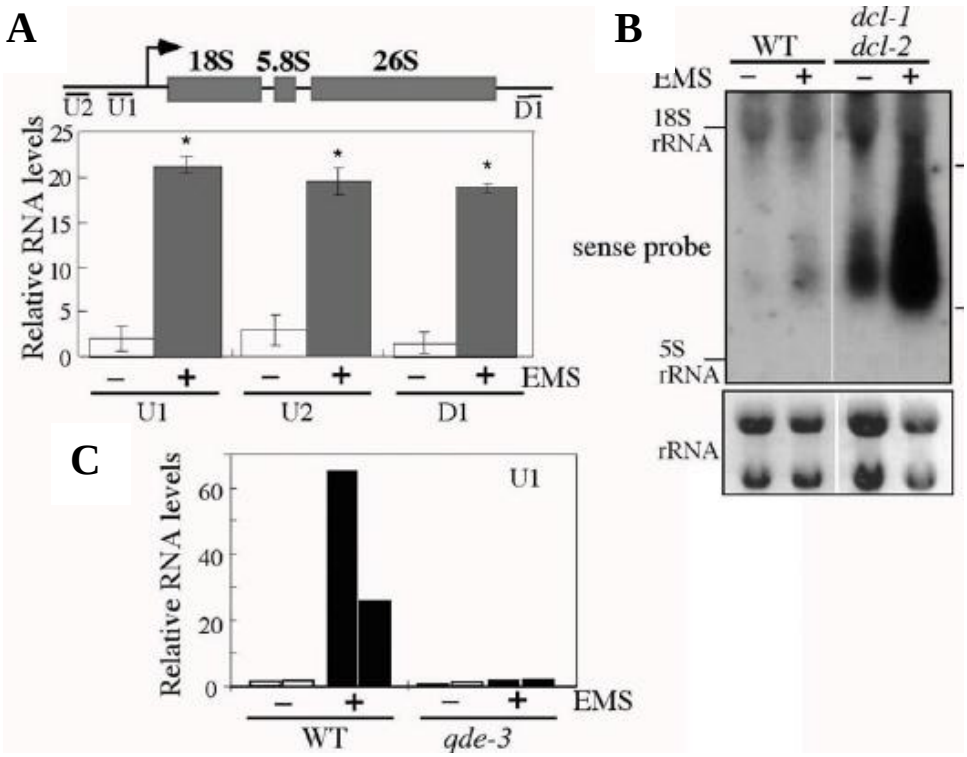


Figure 10. DNA damage induces aberrant transcription at rDNA locus.
(A) qRT-PCR showing that EMS treatment results in the induction of aberrant transcripts from the rDNA regions. The upper panel shows the structure of an rDNA repeat and the intergenic rDNA regions (U1, U2 and D1) analyzed by qRT-PCR analysis. The *dcl* double mutant was used. Statistical significance was determined by Student's t test, n = 3; *, $P < 0.01$. The error bars indicate standard deviations. (B) Northern blot analysis of total RNA showing the induction of rDNA specific transcripts after EMS treatment. Total RNA was separated by a 1.3% agarose gel and a RNA probe specific for intergenic rDNA region was used. The position of the mature 5S and 18S rRNA are indicated. (C) qRT-PCR analysis showing the loss of EMS-induced aberrant transcripts from the rDNA locus in the *qde-3* mutant. Results of two independent experiments were shown.

are indeed induced under conditions that cause DNA damage (Figure 10A-B). In the *dcl* mutant, aberrant transcripts accumulated to a high level with sizes ranging from a few hundreds nucleotides to ~2kb (Figure 10B), suggesting that these transcripts forms dsRNA and are processed by Dicer.

As both QDE-1 and QDE-3 are implicated in producing dsRNA in the quelling pathway, the loss of qiRNA biogenesis in *qde-1* and *qde-3* mutants suggests that QDE-1 and QDE-3 are responsible for generating DNA damage-induced dsRNA. The DNA damage-induced aberrant transcripts from rDNA

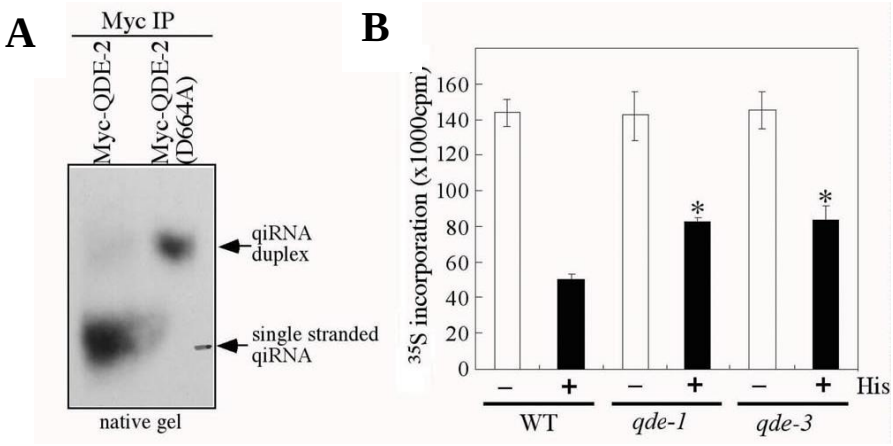
regions are likely converted into dsRNA by QDE-1 (an RdRP). Importantly, we found that the production of the aberrant transcripts was completely abolished in the *qde-3* mutant (Figure 10C), indicating that qiRNAs production is the downstream products of the aberrant transcripts. This result also demonstrates that QDE-3, the RecQ helicase, is required for the generation of aberrant RNAs.

2.3.6 qiRNA contributes to the inhibition of protein synthesis after DNA damage

To examine whether the rDNA-specific qiRNAs associated with active RISC complex, we immunoprecipitated Myc-QDE-2 using strains that expresses either the wild-type QDE-2 or QDE-2 containing a D664A mutation that abolishes its catalytic activity (Maiti et al., 2007). As shown in Figure 11A, the histidine-induced rDNA-derived qiRNAs associated with both forms of QDE-2. However, the qiRNAs associated with wild-type QDE-2 were entirely single-stranded while only double-stranded qiRNAs bound to QDE-2(D664A). This result indicates that qiRNAs are loaded onto the RISC complex as duplexes and the passenger strand can be efficiently processed by QDE-2 and QIP to generate active RISC complex (Maiti et al., 2007).

Since the majority of qiRNAs are derived from the rDNA locus, the single-stranded qiRNA-containing RISC complexes should be able to inhibit rRNA

biogenesis. As a result, the general protein translation rate should decrease after DNA damage. As shown in Figure 11B, the protein synthesis rate measured by a $_{35}$S labeling pulse (containing both ^{35}S-Methionine and ^{35}S-Cysteine) was significantly decreased after histidine treatment. Importantly, such a decrease in protein synthesis rate was partially blocked in the *qde-1* and *qde-3* mutants (*p*-values $4.6\text{x}10^{-6}$ and $2.2\text{x}10^{-5}$, respectively). Similar results were also obtained with EMS treatment (Figure 11C). These results suggest that qiRNAs are involved in inhibiting protein synthesis after DNA damage.



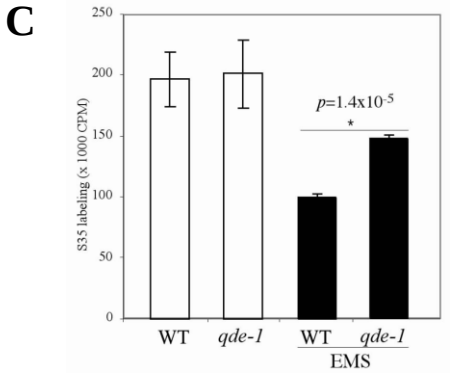


Figure 11. DNA damage leads to inhibition of protein synthesis, a response that is partially blocked in *qde-1* and *qde-3* strain.
(A) Northern blot analysis showing that the rDNAderived qiRNAs are loaded onto QDE-2 as double-stranded and then processed into singlestranded to generate active RISC. Myc-QDE-2 or the catalytic inactive Myc-QDE2 (D664A) were immunoprecipitated and the associated RNAs were extracted. An rDNA-specific probe was used. (B&C) DNA damage induced by Histidine (B) or EMS (C) results in the decrease of protein synthesis rate, a response that was partially blocked in the *qde-1* and *qde-3* strains. The counts of ^{35}S labeled protein in various strains were used to compare the rate of total protein synthesis. Statistical significance was determined by Student's t test, n = 3; *, $P < 0.0001$.

2.3.7 RNAi mutants are hypersensitive to DNA damaging agents

Consistent with a role of qiRNA production in DNA repair response, *qde-3* mutant was previously shown to be sensitive to both histidine and DNA damage agents (Cogoni and Macino, 1999b; Kato and Inoue, 2006). Furthermore, we found that both *qde-1* and the *dcl* double mutants exhibited increased sensitivity to histidine, EMS and HU treatments, although they were not as sensitive as the *atm* mutant (Figure 12). Taken together, these results suggest a role for the *Neurospora* RNAi pathway in responding to conditions that result in DNA damage.

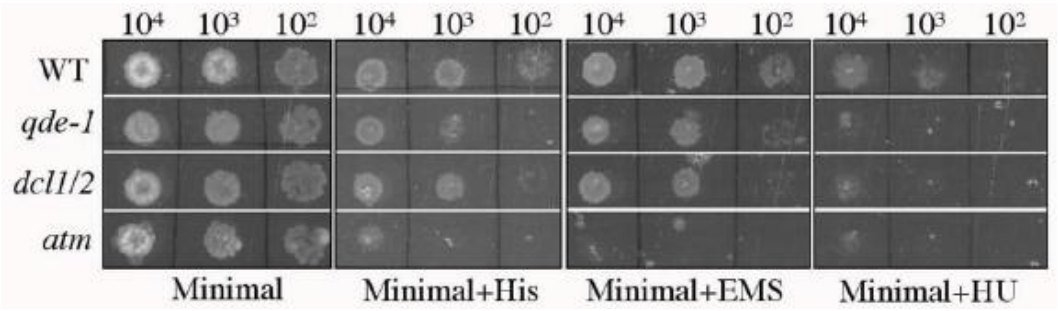


Figure 12. *qde-1* and the *dcl-1 ; dcl-2* double mutants exhibit increased sensitivity to DNA damage agents.
The numbers of conidia, strains, and mutagen used in the spot test are indicated.

2.4 Discussion

Mechanisms that maintain genome integrity are crucial for cell survival and cell functions in unicellular and multicellular organisms. DNA damage, if not repaired, can lead to gene mutations, genome instability, cancer or cell death. After incurring DNA damage, eukaryotic cells activate DNA repair pathways to restore DNA integrity. In addition, cells also activate various DNA damage checkpoints to arrest cell cycle progression to allow time for DNA repair. These checkpoints include the G1/S checkpoint, the intra-S checkpoint, G2/M

checkpoints and the inhibition of RNA (mRNA and rRNAs) transcription (Kruhlak et al., 2007; Sancar et al., 2004). Our results suggest a role of small RNAs in DNA damage response. Based on these results, we propose that the production of qiRNA is likely another mechanism that contributes to the DNA damage checkpoints by inhibiting protein synthesis after DNA damage. Consistent with this notion, the damage sensitivity of the RNAi mutants is not as severe as the *atm* mutant. Thus, qiRNAs may not be directly involved in repairing DNA, but provide a mechanism to help optimal cell survival under DNA damaging conditions. It is also possible that qiRNA may also participate other unknown aspects of DNA damage response to maintain genome integrity.

In this study, we identified qiRNA as a novel type of DNA damage-induced small RNAs that originate mostly from the rDNA locus in *Neurospora*. Why are most of the qiRNAs from the rDNA region? In *Neurospora*, all rDNAs, except for some 5S rDNAs, are arranged as a series of tandem repeat units at NOR (Butler and Metzenberg, 1989; Butler and Metzenberg, 1990). In *Neurospora*, the NOR is a site of frequent chromosome breakage probably due to its repetitive nature (Butler, 1992), suggesting that the NOR as a fragile site *in vivo*. Thus, it is possible that DNA damage agents may preferentially causes the breakage of the NOR and consequently triggers production of rDNA-specific aberrant RNAs. qiRNAs also originate from randomly distributed ORFs and intergenic regions. Thus, random chromosomal damage is a likely cause for the

production of aberrant RNAs and qiRNAs. Consistent with this hypothesis, we found that DNA damage leads to the production of aberrant RNA from non-coding region of rDNA locus. Like qiRNA, piRNA and rasiRNA from higher eukaryotes are also enriched in repetitive regions of the genome, our study raises the possibility that spontaneous DNA damage produced during recombination or transposon transposition could be a trigger to induce production of endogenous small RNAs. In addition, qiRNA biogenesis requires DNA damage-induced aberrant transcription, a process that involves the RecQ DNA helicase QDE-3, the *Neurospora* homolog of the Warners/Bloom Syndome protein. Interestingly, piRNAs from rat testes associated with rRecQ1 (Lau et al., 2006), a QDE-3 homolog. The requirement of QDE-3 in aberrant RNA production in *Neurospora* suggests that in mammals, rRecQ1 may also play a role in generating primary aberrant RNAs, which are used as the precursor for piRNAs in mammals.

Although rRNA specific small RNAs were frequently found in small RNA cloning experiments, they were normally treated as non-specific degradation products due to the abundance of rRNAs. Our results presented here demonstrate that rDNA-specific small RNAs are an important part of the endogenously produced small RNAs generated by the RNAi pathway. Evidence in higher organisms also suggests the importance of rDNA-derived small RNAs. In mouse embryonic stem cells, rRNA-specific small RNAs were identified by their interaction with a small RNA binding protein (Calabrese and Sharp, 2006). In

Arabidopsis, RNAi components are localized in the nucleolus and the rDNA-specific small RNAs contribute to the heterochromatin formation in the rDNA locus (Pontes et al., 2006). In *Drosophila*, the *dicer-2* mutant displayed disorganized nucleoli and rDNA, suggesting a role for the RNAi pathway in maintaining genome stability in the rDNA region (Peng and Karpen, 2007). Thus, qiRNA-like small RNAs may also exist in higher eukaryotes to function in the DNA repair responses.

2.5 Bibliography

Ambros, V., and Chen, X. (2007). The regulation of genes and genomes by small RNAs. Development *134*, 1635-1641.

Aravin, A., Gaidatzis, D., Pfeffer, S., Lagos-Quintana, M., Landgraf, P., Iovino, N., Morris, P., Brownstein, M. J., Kuramochi-Miyagawa, S., Nakano, T., *et al.* (2006). A novel class of small RNAs bind to MILI protein in mouse testes. Nature *442*, 203-207.

Aravin, A. A., Sachidanandam, R., Girard, A., Fejes-Toth, K., and Hannon, G. J. (2007). Developmentally regulated piRNA clusters implicate MILI in transposon control. Science *316*, 744-747.

Aronson, B., Johnson, K., Loros, J. J., and Dunlap, J. C. (1994). Negative feedback defining a circadian clock: autoregulation in the clock gene *frequency*. Science *263*, 1578-1584.

Baulcombe, D. (2004). RNA silencing in plants. Nature *431*, 356-363.

Brennecke, J., Aravin, A. A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., and Hannon, G. J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. Cell *128*, 1089-1103.

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

Butler, D. K. (1992). Ribosomal DNA is a site of chromosome breakage in aneuploid strains of Neurospora. Genetics *131*, 581-592.

Butler, D. K., and Metzenberg, R. L. (1989). Premeiotic change of nucleolus organizer size in Neurospora. Genetics *122*, 783-791.

Butler, D. K., and Metzenberg, R. L. (1990). Expansion and contraction of the nucleolus organizer region of Neurospora: changes originate in both proximal and distal segments. Genetics *126*, 325-333.

Calabrese, J. M., and Sharp, P. A. (2006). Characterization of the short RNAs bound by the P19 suppressor of RNA silencing in mouse embryonic stem cells. RNA *12*, 2092-2102.

Catalanotto, C., Pallotta, M., ReFalo, P., Sachs, M. S., Vayssie, L., Macino, G., and Cogoni, C. (2004). Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in Neurospora crassa. Mol Cell Biol *24*, 2536-2545.

Chary, P., Dillon, D., Schroeder, A. L., and Natvig, D. O. (1994). Superoxide dismutase (sod-1) null mutants of Neurospora crassa: oxidative stress sensitivity, spontaneous mutation rate and response to mutagens. Genetics *137*, 723-730.

Cheng, P., Yang, Y., Heintzen, C., and Liu, Y. (2001). Coiled-coil domain mediated FRQ-FRQ interaction is essential for its circadian clock function in *Neurospora*. EMBO J *20*, 101-108.

Choudhary, S., Lee, H. C., Maiti, M., He, Q., Cheng, P., Liu, Q., and Liu, Y. (2007). A double-stranded-RNA response program important for RNA interference efficiency. Mol Cell Biol *27*, 3995-4005.

Cogoni, C., and Macino, G. (1999). Posttranscriptional gene silencing in Neurospora by a RecQ DNA helicase. Science *286*, 2342-2344.

Davis, R. L., and deSerres, D. (1970). Genetic and microbial research techniques for *Neurospora crassa*. Meth Enzymol *27A*, 79-143.

Garceau, N., Liu, Y., Loros, J. J., and Dunlap, J. C. (1997). Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. Cell *89*, 469 - 476.

Ghildiyal, M., Seitz, H., Horwich, M. D., Li, C., Du, T., Lee, S., Xu, J., Kittler, E. L., Zapp, M. L., Weng, Z., and Zamore, P. D. (2008). Endogenous siRNAs derived from transposons and mRNAs in Drosophila somatic cells. Science *320*, 1077-1081.

Girard, A., Sachidanandam, R., Hannon, G. J., and Carmell, M. A. (2006). A germline-specific class of small RNAs binds mammalian Piwi proteins. Nature *442*, 199-202.

Grivna, S. T., Beyret, E., Wang, Z., and Lin, H. (2006). A novel class of small RNAs in mouse spermatogenic cells. Genes Dev *20*, 1709-1714.

Gunawardane, L. S., Saito, K., Nishida, K. M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H., and Siomi, M. C. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in Drosophila. Science *315*, 1587-1590.

Hannon, G. J. (2002). RNA interference. Nature *418*, 244-251.

Hatakeyama, S., Ito, Y., Shimane, A., Ishii, C., and Inoue, H. (1998). Cloning and characterization of the yeast RAD1 homolog gene (mus-38) from Neurospora crassa: evidence for involvement in nucleotide excision repair. Curr Genet *33*, 276-283.

Howard, C. A., and Baker, T. I. (1988). Relationship of histidine sensitivity to DNA damage and stress induced responses in mutagen sensitive mutants of Neurospora crassa. Curr Genet *13*, 391-399.

Kafer, E. (1983). Epistatic Grouping of Repair-Deficient Mutants in Neurospora: Comparative Analysis of Two uvs-3 Alleles, uvs-6 and Their mus Double Mutant Strains. Genetics *105*, 19-33.

Kato, A., and Inoue, H. (2006). Growth defect and mutator phenotypes of RecQ-deficient Neurospora crassa mutants separately result from homologous recombination and nonhomologous end joining during repair of DNA double-strand breaks. Genetics *172*, 113-125.

Kazama, Y., Ishii, C., Schroeder, A. L., Shimada, H., Wakabayashi, M., and Inoue, H. (2008). The Neurospora crassa UVS-3 epistasis group encodes homologues of the ATR/ATRIP checkpoint control system. DNA Repair (Amst) *7*, 213-229.

Kruhlak, M., Crouch, E. E., Orlov, M., Montano, C., Gorski, S. A., Nussenzweig, A., Misteli, T., Phair, R. D., and Casellas, R. (2007). The ATM repair pathway inhibits RNA polymerase I transcription in response to chromosome breaks. Nature *447*, 730-734.

Krumlauf, R., and Marzluf, G. A. (1980). Genome organization and characterization of the repetitive and inverted repeat DNA sequences in Neurospora crassa. J Biol Chem *255*, 1138-1145.

Kurrasch, D. M., Huang, J., Wilkie, T. M., and Repa, J. J. (2004). Quantitative real-time polymerase chain reaction measurement of regulators of G-protein signaling mRNA levels in mouse tissues. Methods Enzymol *389*, 3-15.

Lau, N. C., Lim, L. P., Weinstein, E. G., and Bartel, D. P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science *294*, 858-862.

Lau, N. C., Seto, A. G., Kim, J., Kuramochi-Miyagawa, S., Nakano, T., Bartel, D. P., and Kingston, R. E. (2006). Characterization of the piRNA complex from rat testes. Science *313*, 363-367.

Lin, H. (2007). piRNAs in the germ line. Science *316*, 397.

Maiti, M., Lee, H. C., and Liu, Y. (2007). QIP, a putative exonuclease, interacts with the Neurospora Argonaute protein and facilitates conversion of duplex siRNA into single strands. Genes Dev *21*, 590-600.

Pontes, O., Li, C. F., Nunes, P. C., Haag, J., Ream, T., Vitins, A., Jacobsen, S. E., and Pikaard, C. S. (2006). The Arabidopsis chromatin-modifying nuclear siRNA pathway involves a nucleolar RNA processing center. Cell *126*, 79-92.

Pregueiro, A. M., Liu, Q., Baker, C. L., Dunlap, J. C., and Loros, J. J. (2006). The Neurospora checkpoint kinase 2: a regulatory link between the circadian and cell cycles. Science *313*, 644-649.

Rountree, M. R., and Selker, E. U. (1997). DNA methylation inhibits elongation but not initiation of transcription in Neurospora crassa. Genes Dev *11*, 2383-2395.

Ruby, J. G., Jan, C., Player, C., Axtell, M. J., Lee, W., Nusbaum, C., Ge, H., and Bartel, D. P. (2006). Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans. Cell *127*, 1193-1207.

Sakuraba, Y., Schroeder, A. L., Ishii, C., and Inoue, H. (2000). A Neurospora double-strand-break repair gene, mus-11, encodes a RAD52 homologue and is inducible by mutagens. Mol Gen Genet *264*, 392-401.

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

Schroeder, A. L. (1970). Ultraviolet-sensitive mutants of Neurospora. II. Radiation studies. Mol Gen Genet *107*, 305-320.

Srivastava, V. K., Pall, M. L., and Schroeder, A. L. (1988). Deoxyribonucleoside triphosphate pools in Neurospora crassa: effects of histidine and hydroxyurea. Mutat Res *200*, 45-53.

Stadler, D., Macleod, H., and Dillon, D. (1991). Spontaneous mutation at the mtr locus of Neurospora: the spectrum of mutant types. Genetics *129*, 39-45.

Suzuki, K., Kato, A., Sakuraba, Y., and Inoue, H. (2005). Srs2 and RecQ homologs cooperate in mei-3-mediated homologous recombination repair of Neurospora crassa. Nucleic Acids Res *33*, 1848-1858.

Tomari, Y., and Zamore, P. D. (2005). Perspective: machines for RNAi. Genes Dev *19*, 517-529.

Wakabayashi, M., Ishii, C., Inoue, H., and Tanaka, S. (2008). Genetic analysis of CHK1 and CHK2 homologues revealed a unique cross talk between ATM and ATR pathways in Neurospora crassa. DNA Repair (Amst).

Watanabe, K., Sakuraba, Y., and Inoue, H. (1997). Genetic and molecular characterization of Neurospora crassa mus-23: a gene involved in recombinational repair. Mol Gen Genet *256*, 436-445.

CHAPTER THREE

QDE-1 IS BOTH A RNA-DEPENDENT AND DNA-DEPENDENT RNA POLYMERASE IN THE RNA INTERFERENCE PATHWAY

3.1 Introduction

RNAi play an important role in genome defense against transgenes, transposon and virus. (Li et al., 2002; Napoli et al., 1990; Romano and Macino, 1992; Sijen and Plasterk, 2003). To trigger RNA, it has been proposed that specific aberrant RNA (aRNA) is made from those parasitic and pathogenic DNAs. In fungus, worm and plant, cellular RNA-dependent RNA polymerase (RdRP) can recognize aRNAs and use them as templates to generate dsRNAs, which induce gene silencing. In consistent with this model, mutation of genes encoding RdRPs fail to silence transgenes, and viruses (Dalmay et al., 2000; Muangsan et al., 2004). However, it is unclear which RNA polymerase makes the aRNA. Furthermore, how does RdRPs recognize aRNA specifically over other cellular RNAs is also unclear.

In plants, the DNA-dependent RNA polymerase IV (Pol IV) is important for siRNA production and RNAi-directed transcriptional silencing (Herr et al., 2005; Onodera et al., 2005). However, Pol IV homologs are not found in fungal or animal genomes. In fission yeast, mutants of the RNA polymerase II (Pol II) subunits show loss of centromeric siRNA and RNAi-dependent heterochromatin formation at the centromeric repeat regions, probably by coupling transcription

with transcriptional silencing machinery (Buhler and Moazed, 2007; Djupedal et al., 2005; Kato et al., 2005). It is not known whether Pol II plays a similar role in posttranscriptional silencing and in the silencing of other chromosomal regions. Furthermore, there is no direct evidence showing either Pol IV or Pol II is required for aberrant RNA production.

In the filamentous fungus *Neurospora crassa*, quelling is an RNAi-based, posttranscriptional silencing mechanism triggered by multiple copies of transgenes during vegetative growth (Catalanotto et al., 2006). In the quelling pathway, QDE-1 (QUELLING DEFICIENT-1,an RdRP), QDE-2 (an Argonaute protein), QDE-3 (a RecQ DNA helicase homologous to the Werner/Bloom Syndrome protein), and two partially redundant Dicer proteins (DCL-1 and DCL-2) are required for silencing (Catalanotto et al., 2000, 2002; Catalanotto et al., 2004; Cogoni and Macino, 1999a, b; Maiti et al., 2007). It has been proposed that the introduction of a transgene leads to the production of transgene-specific aRNA transcripts, which are then specifically recognized and converted to dsRNA by QDE-1 (Catalanotto et al., 2006; Cogoni and Macino, 1999a). The mechanism of aRNA production and the role of QDE-3 in this process are not known.

To understand the nature of the mysterious aRNA, we studied the aRNA and dsRNA production at ribosomal DNA (rDNA) locus after DNA damage. Recently, we found that DNA damage induces the expression of the Argonaute

protein QDE-2 and a novel class of small RNAs named qiRNAs for their association with QDE-2 (See Chapter 2). qiRNAs originate mostly from the rDNA locus, and their production depends on QDE-1, QDE-3 and Dicers. In addition, qiRNA biogenesis also requires the DNA damage-induced aRNA from the rDNA locus. In this chapter, I describe our finding that RdRP QDE-1 is required for the production of aRNA from the rDNA locus. Recombinant QDE-1 also exhibits strong activity using ssDNA as template. Our results suggest that QDE-1, in addition to being an RdRP that converts ssRNA into dsRNA, is also a DNA-dependent RNA polymerase (DdRP) that generates its own ssRNA template from ssDNA. Our data also suggest that QDE-1 is recruited to ssDNA by Replication Protein A (RPA) and the RecQ DNA helicase QDE-3. Like QDE-1 and QDE-3, RPA is required for qiRNA and aRNA production and quelling. More over, RPA promotes the synthesis of dsRNA by QDE-1 from ssDNA templates by preventing the formation of DNA/RNA hybrids. Together, these results established a mechanism for the generation of aRNA and provide an explanation for how aRNA is specifically recognized by RdRPs.

3.2 Materials and Methods

3.2.1 Strains and growth conditions

A wild type strain of *Neurospora crassa* (FGSC4200) was used in this study unless otherwise mentioned. *un-18* mutant and *rpa-3* (NCU01460.3) knock-

out strain were obtained from Fungal Genetic Stock Center (FGSC). ds*al-1* knock down strain was made previously (Choudhary et al.,2007; Maiti et al., 2007). A similar strategy was used to create knock-down strains of ds*rpa-1* (NCU03606.3) and ds*rpa-2* (NCU07717.3). Transgenic strains expressing c-Myc or FLAG tagged proteins were created by plasmid transformation into *his-3* locus by electroporation. Strains co-expressing both c-Myc and FLAG tagged proteins were generated by cotransformation of plasmid containing the FLAG tagged gene with plasmid (pBT6) containing benomyl-resistance into the strains expressing the c-Myc-tag protein.

For the expression of inverted repeats and tagged proteins in *Neurospora*, 0.01 M QA (pH 5.8) was added to the liquid culture medium containing 1xVogel's, 0.1% glucose, and 0.17% arginine. DNA damage was induced by the addition of either histidine (100 μg/ml) or EMS (0.2%) and samples were harvested 40 hours later.

3.2.2 Construction of plasmid expressing FLAG and c-Myc tagged proteins

A FLAG (3xFLAG) tag containing plasmid was a kind gift from Dr. James Chen at UT Southwestern. The FLAG tag was subcloned downstream of the *qa-2* promoter, creating plasmid qa-3FLAG. Full length *qde-1* or *rpa-1* genes were then inserted in frame following the FLAG tag. To create c-Myc-tagged proteins, full length- genes of *qde-1*, *qde-3* or *rpa-1* were inserted into a c-Myc tag

containing plasmid described previously (He et al., 2005a). Both FLAG and Myc containing plasmids also contain sequences encoding 6 histidines, allowing purification with Ni-NTA matrices (QIAGEN).

3.2.3 Quantitative real time PCR analysis (qRT-PCR)

qRT-PCR experiments were performed as described in Chapter 2. Specific primer pairs were used to detect intergenic transcripts from the rDNA promoter region (CTGAGAAGGTGCGACCCAGT / TTAAAAATCGACTCACGGCCAC) and mature 26S rRNA transcript (GAAGTCGGCAAAATAGATCCGT / TACCCAACCCTTAGAGCCAATC).

3.2.4 RNA extraction and Northern blot analysis

Total RNA extraction, enrichment of low molecular weight small RNA and Northern blot were performed as described in Chapter 2.

3.2.5 Expression and purification of RdRPs

Recombinant QDE-1 WT, QDE-1ΔN, QDE-1ΔN^{DA}, and RdRP of bacteriophage φ6 were expressed and purified as previously described (Laurila et al., 2005; Makeyev and Bamford, 2000). pEM41, pEM69 and pEM56 expressing QDE-1 WT, QDE-1ΔN and QDE-1ΔN^{DA}, respectively, each with a carboxy terminal His tag, were introduced into *Saccharomyces cerevisiae* strain INVSc1

(Invitrogen). The recombinant proteins were expressed at 28ºC for 22h and purified to near homogeneity. Wild-type recombinant φ6 P2 was expressed in *Escherichia coli* BL21(DE3) strain (Novagen) containing the plasmid pEMG2 (Poranen et al., 2008) at 20ºC for 15 h and purified to near homogeneity as previously described (Makeyev and Bamford, 2000). The purified proteins were stored in 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.13 % Triton X-100, 100 mM NaCl, and 62.5 % glycerol at -20ºC.

The full length 6His tagged c-Myc-His-QDE-1 from *Neurospora* was partially purified by Ni-NTA matrices (QIAGEN). In brief, six grams of tissue from c-Myc-His-QDE-1 expressing strain or wild type strain were harvested. Cell lysates in extraction buffer (50mM HEPES pH 7.4, 100mM KCl, 10mM imidazole and 10% glycerol) were applied to the Ni-NTA matrices (1ml bed volume). The matrices were then washed with 10ml of washing buffer (50mM HEPES, 100mM KCl, and 10mM imidazole) and eluted with 4 1ml elution buffer (50mM HEPES, 100mM KCl, 200mM imidazole and 20% glycerol). Equal amount of partially purified proteins from both the c-My-His-QDE-1 expressing strain and a control strain were applied for RdRP assays.

3.2.6 Template RNAs and DNAs

The single-stranded 176 nt DNA oligonucleotide was purchased from biomers.net, and its sequence corresponds to nts 726-901 of pEGFP-C1

(Clontech). For the synthesis of ssRNA of the same sequence, the 176 nt ssDNA was PCR-amplified. The resulting PCR product was purified and used as a template for T7 transcription reaction. The DNA template was then degraded with DNaseI (Promega), and the ssRNA was gel purified. For some experiments, the 175 nt ssDNA, deriving from mature 26S rRNA region, was made by asymmetric PCR (Curaba and Chen, 2008) and used in DdRP assay. ssRNA with the same sequences was made by T7 transcription as described above. The primer sequences used to create the templates are available upon request. The genomic ssDNAs of bacteriophages φX174 and M13mp18 were purchased from New England Biolabs.

3.2.7 RNA polymerase assays

RNA polymerase reactions were performed essentially as described (Makeyev and Bamford, 2000, 2002). The samples were subjected to gel electrophoresis using native agarose gel or denaturing polyacrylamide (6% or 16%) TBE gels. Radioactivity was detected by phosphorimaging and analyzed by densitometry with TINA software (Raytest Isotopenme geräte GmbH). For nuclease assays of reaction products, RNasin® Ribonuclease Inhibitor (Promega) was omitted from the reactions, and the reaction products were extracted with phenol:chloroform, precipitated with NH_4OAc and ethanol, and dissolved in water. Subsequently, equal amounts of reaction products were supplemented with

0.1 U/μl of RQ1 DNase (Promega), 0.5 U/μl of RNase H (Fermentas) or 0.01 U/μl of RNase V1 (Ambion), and their respective 1x reaction buffers, incubated for 30 min at 37ºC, quenched with loading buffer and analyzed by electrophoresis. For reactions with RPA, the ssDNA template was first incubated with RPA at 37ºC for 10 min before adding recombinant QDE-1.

3.2.8 Protein extraction and Western blot analysis

Protein extraction, quantification, immunoprecipitation (IP) and Western blot analysis were performed as previously described (Cheng et al., 2001; Maiti et al., 2007). Equal amounts of total protein (50 μg), and nuclear and cytoplasmic proteins (15μg) were separated in SDS PAGE and transferred onto PVDF membrane. Nuclear and cytoplasmic protein extracts were prepared as previously described (Luo et al., 1998). For Western blot, a monoclonal c-Myc antibody (Roche 9E10), anti-FLAG M2 antibody (Sigma) and anti-α-tubulin (Sigma T9026) were used. For immunoprecipiation, anti-c-Myc and anti-FLAG antibodies were used at 1:1000, and 1:300 dilutions, respectively.

3.2.9 Chromatin immunoprecipitation assay (ChIP)

The ChIP assay was performed as previously described (He et al., 2005b) except that 1:1000 dilution of c-Myc antibody was used for immunoprecipitation. PCR reactions were as follows: 4 min at 94°C, 26–29 cycles of 94°C (15 sec),

60°C (30 sec), and 72°C (1 min). PCR products were resolved by 2% agarose gels. Primer sequences are available upon request. Each ChIP experiment was independently performed three times and the bindings were quantified with NIH image.

3.2.10 Quelling assay

Neurospora strains were co-transformed with 0.5 μg pBSK*al-1* plasmid (contains a truncated *abino-1 (al-1)* gene) and 0.5 μg pBT6 plasmid. The benomyl-resistant colonies were picked and grown on slants. The colors of around 100 transformants from each strain were observed for comparing silencing (quelling) efficiency of the *al-1* gene.

3.3 Results

3.3.1 QDE-1 but not RNA polymerase I is required for the generation of the rDNA-specific aRNA transcripts

Most of the qiRNAs are produced from the rDNA locus (Chapter 2). Under normal growth conditions, the un-transcribed rDNA spacer regions are silent and produce few transcripts. However, when the cells are subjected to conditions causing DNA damage, these regions become transcriptionally active and produce aRNAs (Chapter 2). Although aRNAs are produced from the entire rDNA

regions, the low basal transcription levels from the un-transcribed rDNA spacer regions allow us to determine aRNA expression levels under different conditions. Since DNA-dependent RNA polymerase I is responsible for the transcription of rRNAs, we first examined its role in the production of aberrant transcripts from the rDNA locus. *un-18* is a temperature-sensitive *Neurospora* mutant due to a point mutation (G976D) in the second-largest subunit of RNA polymerase I (Onai et al., 1998). At restrictive temperature (32ºC), the *un-18* mutant exhibited reduced 26S rRNA levels compared to the wild-type strain (Figure 13B). As in the wild-type strain, the presence of histidine, a DNA mutagen for *Neurospora*, further reduced the levels of rRNA in the mutant. However, the histidine treatment resulted in similar induction of aRNA from upstream of the rDNA transcription start site in both strains (Figure 13C). These results indicate that RNA polymerase I is not required for the generation of the rDNA-specific aRNAs after DNA damage.

The DNA damage-induced aRNAs accumulate to high levels in a dicer mutant (Figure 10B), indicating that aRNAs are converted to dsRNA. Consistent with this notion, QDE-1 is required for the production of qiRNA (Figure 9). QDE-1 was previously thought to specifically recognize and convert aRNA into dsRNA. If so, we would expect that the production of aRNA should be

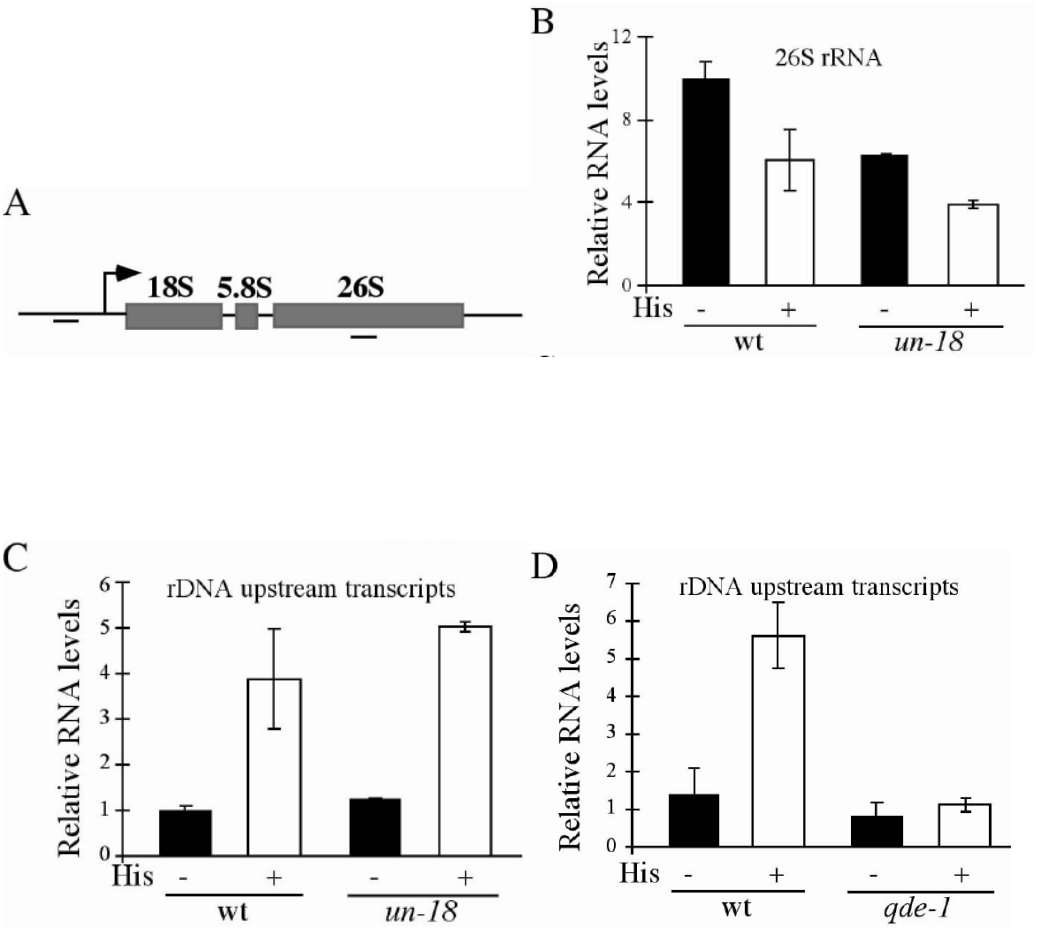


Figure 13. QDE-1 but not PolI is required for the synthesis of DNA-damage induced rDNA specific aRNA.

(A) A schematic diagram of a single rDNA repeat. Regions examined by qRT-PCR are indicated by bars. The transcriptional start site is marked with an arrow. (B, C &D) qRT-PCR results showing the levels of transcripts under normal or DNA damaged (histidine treated) condition. (B) The levels of matured 26S rRNAs and (C) DNA damage-induced aberrant transcripts in the wild-type and *un-18* strains. (D) The levels of DNA damage-induced aberrant transcripts in the *qde-1*^{ko} mutant.

maintained in a *qde-1* knock-out mutant (*qde-1*^{ko}). To our surprise, we found that the induction of the rDNA-specific aRNA by histidine was completely abolished in the *qde-1*^{ko} mutant (Figure 13D). This result indicates that QDE-1 is not only required for the production of dsRNA but also for the synthesis of the primary transcripts.

In addition, the production of rDNA-specific aRNA by histidine was also completely abolished in the *qde-3*^{ko} mutant (Figure 10C), suggesting QDE-3 may function together with QDE-1 in the production of aRNA.

3.3.2 QDE-1 and QDE-3 are recruited to the rDNA locus when qiRNA is induced

The requirement of QDE-1 and QDE-3 in aRNA production suggests that they play a direct role in aRNA transcription. If so, both proteins should be localized to the nucleus. To test this, a construct expressing either c-Myc-His-tagged QDE-1 or QDE-3 was transformed into a wild-type strain and the cellular localization of the fusion proteins was examined by fractionation followed by Western blot analyses using a monoclonal c-Myc specific antibody. As shown in Figure 14A, Myc-His-QDE-3 was only found in the nuclear fraction and Myc-His-QDE-1 was substantially enriched in the nucleus. In addition, a Dicer-specific fusion protein Myc-His-DCL-2 was also found to localize in the nuclear fraction.

The absence of the cytoplasmic protein tubulin in the nuclear extracts indicates that our nuclear preparation was free of cytoplasmic contamination.

We then performed a chromatin-immunprecipitation (ChIP) assay to examine whether QDE-1 and QDE-3 can bind to the rDNA locus when aRNA is induced. Indeed, we found that histidine treatment significantly induced the binding of both Myc-His-QDE-1 and Myc-His8 QDE-3 to the rDNA spacer region (Figure 14B & C). This result suggests that both proteins are recruited to the rDNA locus when the production of aberrant transcripts is induced.

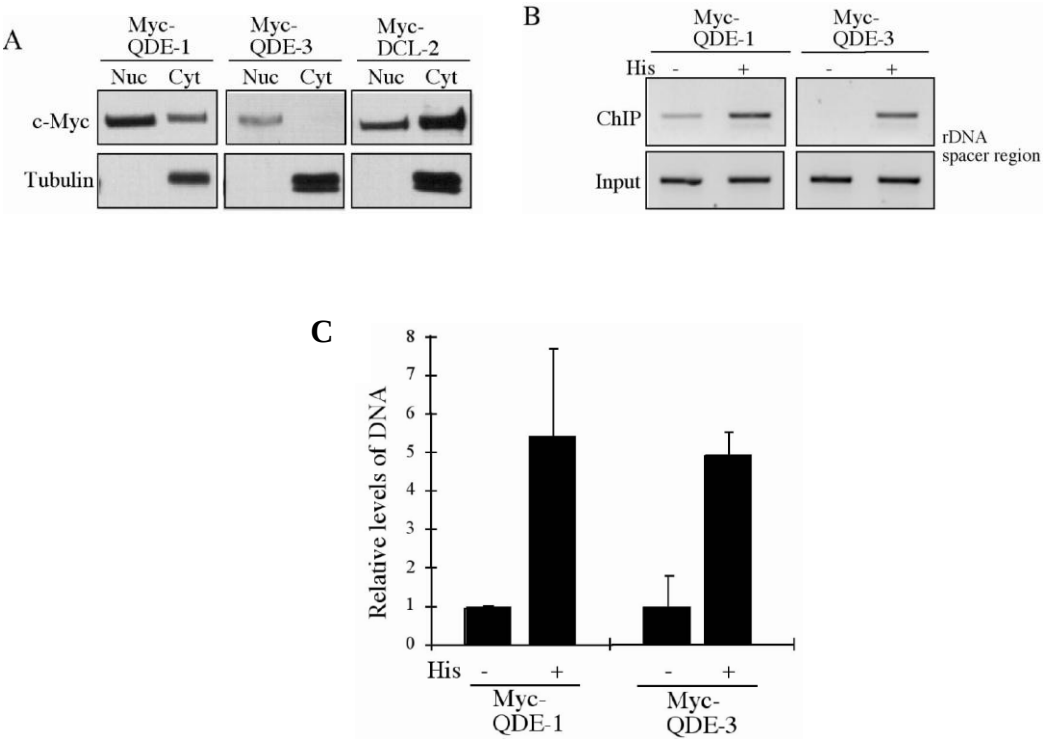


Figure 14. QDE-1 and QDE-3 are recruited to rDNA after DNA damage.
(A) Western blot analyses of c-Myc-his-QDE-1, c-Myc-his-QDE-3 and c-Myc-his-DCL-2 in nuclear (Nuc) and cytoplasmic (Cyt) fractions of wild type *Neurospora* transformed with one of the c-Myc-his fusion protein expression constructs (indicated above the panels). Tubulin was used as a cytoplasmic specific marker (lower panels). (B) Chromatin immunoprecipitation (ChIP) assays of QDE-1 and QDE-3 binding to rDNA spacer region after DNA damage (above). Densitometric analysis of the ChIP results from three independently performed experiments (below). The levels of binding are normalized by the input signals.

3.3.3 QDE-1 can use ssDNA as a template to generate DNA/RNA hybrids

The nuclear localization of QDE-1 and its requirement in aRNA production suggest that it is directly involved in the synthesis of aRNA. Previous structural analysis of QDE-1 has shown that the catalytic core of QDE-1 is structurally similar to eukaryotic DNA-dependent RNA polymerases (Salgado et al., 2006). During the analyses of the recombinant catalytically active C-terminal portion of QDE-1 (QDE-1ΔN, residues 376-1402) by gel-shift and surface plasmon resonance assays, we found that QDE-1ΔN can bind to ssRNA, but surprisingly, it can also bind to single-stranded DNA (ssDNA) at comparable affinities in a manner independent of its catalytic activity (data not shown). In

contrast, no binding was detected between QDE-1ΔN and double-stranded nucleic acids. Together, these results suggest that QDE-1 may be able to use ssDNA as a template to synthesize RNA transcripts. To determine whether QDE-1 possesses DdRP activity, we designed a synthetic 176 nt ssDNA oligonucleotide corresponding to a region of enhanced green fluorescent protein sequence. A ssRNA of the same length and sequence was used as the control template. As shown in Figure 15A, QDE-1ΔN can use both ssRNA and ssDNA as templates to synthesize radioactively labeled products in a reaction mixture containing all the four ribonucleotides and trace amounts of P^{32} UTP. In contrast, the bacteriophage φ6 RdRP can only use ssRNA as a template. When the same amounts of templates were used, the ssDNA-templated activity of QDE-1 was approximately 1.5 times higher than the ssRNA-templated activity (Figure 15A). In addition, the DdRP activity of QDE-1ΔN was completely abolished when its conserved catalytic residue was mutated (QDE-1ΔN D1011A) (Makeyev and Bamford, 2002). Furthermore, QDE-1ΔN was inactive when dsDNA was used as the template (data not shown). To determine the nature of the ssDNA-templated reaction products by QDE-1, we subjected it to several different nuclease treatments (Figure 15B). RNase H, which cleaves the RNA strand of a DNA/RNA hybrid, degraded the P^{32} labeled products and shifted the ethidium bromide stained band to template length. RQ1 DNase, which degrades DNA, shifted the majority of the labelled products to the template-length. RNase V1, which cleaves

base-paired DNA or RNA, degraded the product completely. These results indicate that the products of ssDNA-templated activity of QDE-1 are mostly DNA/RNA hybrids. As expected, the ssRNAtemplated products of QDE-1ΔN were dsRBA, as neither RQ1 DNase nor RNase H had any effect on the products, while RNase V1 treatment resulted in their complete degradation (Figure 15B, right panels).

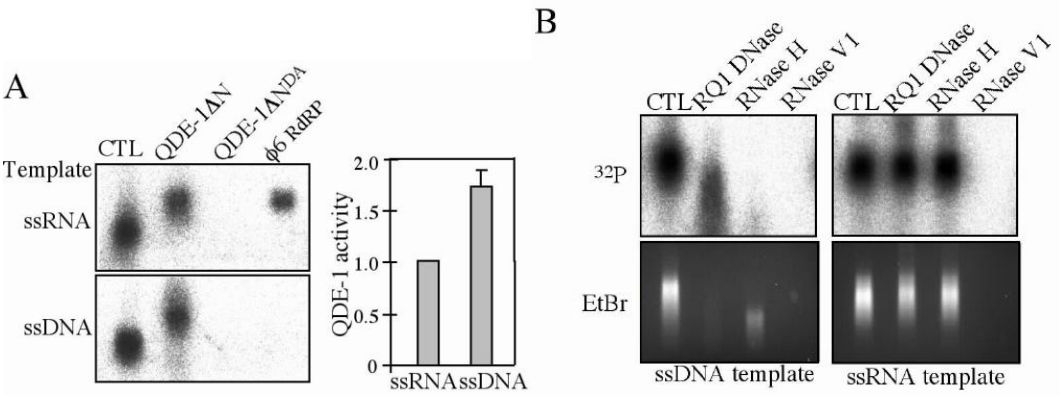


Figure 15. Recombinant QDE-1 exhibits both RdRP and DdRP activities. (A) *In vitro* RNA polymerase assay using the 176 nt ssDNA or ssRNA templates and recombinant QDE-1ΔN, catalytically inactive QDE-1ΔN^{DA} or bacteriophage φ6 RdRP. P^{32} –labeled reaction products were resolved on a 3% TBE agarose gel. CTL lane contains the corresponding 5'-terminally ^{32}P labeled template. The relative DdRP and RdRP activity of QDE-1 was compared side-by-side and the densitometric analysis of four independent experiments is shown. The DdRP activity is normalized by corresponding RdRP activity, which is set to 1. (B) Characterization of the QDE-1ΔN products from DdRP and RdRP assay by various nucleases treatment.

We then examined whether the full-length QDE-1 expressed in *Neurospora* also displayed DdRP activity. The Myc-His-QDE-1 construct was transformed into the *qde-1*^{ko} strain. The resulting transformants rescued the low QDE-2 phenotype in the *qde-1*^{ko} strain (data not shown), indicating that Myc-His-QDE-1 can function as the endogenous protein. To examine the biochemical activity of Myc-His-QDE-1, it was partially purified from *Neurospora* by affinity purification and used in RdRP and DdRP assays. As shown in Figure 16A, Myc-His-QDE-1 exhibited RNA polymerase activity using both ssRNA and ssDNA as templates. In contrast, no RNA polymerase activity was detected using the control purification products from a wild-type strain without the Myc-His-QDE-1 construct. In addition, Myc-His-QDE-1 can only use ssDNA but not dsDNA as a template (Figure 16B). Furthermore, when using ssDNA as a template, Myc-His-QDE-1 produced both high molecular weight and low molecular weight products (Figure 16C). The high molecular weight products were sensitive to RNase H, and thus are mostly DNA/RNA hybrids. Interestingly, the low molecular weight products were resistant to the RNase H treatment, suggesting that they were either ssRNA or dsRNA (further examined below). Together, these results demonstrate that QDE-1 can function as both an RdRP and DdRP and suggest that QDE-1 is the RNA polymerase that generates the aberrant transcripts.

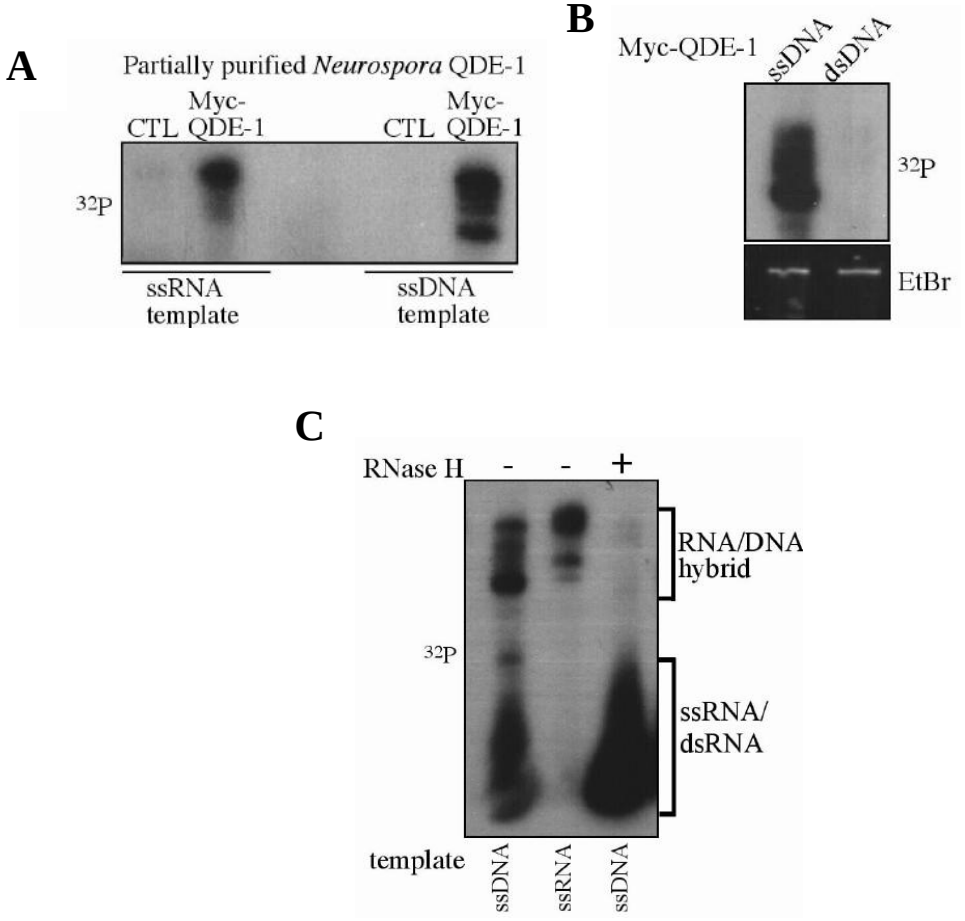


Figure 16. *In vivo* partially purified QDE-1 exhibits both RdRP and DdRP activities. (A) A 175nt ssDNA or ssRNA was used as a template. CTL indicates a reaction in which c-Myc-his-QDE-1 was replaced with a corresponding purification product from a strain without the c-Myc-His-QDE-1 construct. In (B), equal amounts of ssDNA or dsDNA were applied. (C) The reaction products from (A) were treated with RNase H. The products were resolved in 6% urea containing polyacrylamide gel.

3.3.4 RPA interacts with QDE-1 and is required both for the generation of aberrant transcripts and for quelling

If QDE-1 functions as the RNA polymerase that generates the aberrant transcripts *in vivo*, it has to be recruited to ssDNA where the aberrant transcripts are expressed. Consistent with this notion, QDE-1 and rDNA associate when rDNA-specific aberrant transcripts are induced (Figure 14B). Previously, QDE-1 was found to interact with RPA (Nolan et al., 2008), but the role of RPA in gene silencing and in QDE-1 function is not known. RPA is a ssDNA binding protein complex and is a major component involved in DNA replication, repair and recombination pathways (Fanning et al., 2006; Zou et al., 2006). It is a conserved eukaryotic heterotrimeric complex, consisting of RPA1 (RPA70 in mammals), RPA2 (RPA32) and RPA3 (RPA14), that prevents ssDNA from damage, secondary structure formation and re-annealing in DNA processing pathways. The QDE-1-RPA interaction suggests that RPA may be the protein that recruits QDE-1 to ssDNA. If so, RPA should be required for the production of aRNA and qiRNA. To test this hypothesis, we sought to generate *rpa* mutants in *Neurospora*. The knock-outs of *rpa-1* or *rpa-2* are lethal, but the knock-out of *rpa-3*, the smallest subunit of RPA, is viable and does not cause severe growth and developmental defects. To obtain *rpa-1* and *rpa-2* knock-down strains, we introduced constructs that can express an inverted repeat specific for *rpa-1* (ds*rpa-1*) or *rpa-2* (ds*rpa-2*) into a wild-type strain. We first measured the

induction of QDE-2 by histidine, an indication of endogenous dsRNA generation. As shown in Figure 17A, the induction of QDE-2 was completely abolished in two independent *rpa3*$_{ko}$ mutants, and the levels of QDE-2 in the *rpa-3*^{ko} mutants were lower than the basal wild-type level. Also, in the ds*rpa-1* and ds*rpa-2* knockdown strains, the induction of QDE-2 by histidine was mostly abolished. In addition, qiRNA and aRNA expression at the rDNA region were both completely absent in the *rpa3* ^{ko} mutant (Figure 17B & C). Therefore, like QDE-1 and QDE-3, RPA is required for the generation of qiRNA and the rDNA-specific aberrant transcripts. These results further suggest that QDE-1 is recruited by RPA to ssDNA to function as an RNA polymerase, generating the initial transcripts in the RNAi pathway. In addition to their role in qiRNA production, QDE-1 and QDE-3 are also important components in the quelling pathway. The similarity between the qiRNA biogenesis pathway and the quelling pathway prompted us to examine the role of RPA in quelling by transforming a truncated *al-1* gene into the *rpa-3*^{ko} and *qde-3*$_{ko}$ strains. As shown in Table1, the quelling efficiency of the *rpa3*^{ko} strain is significantly lower than that of the wild-type strain and is comparable to that of the *qde-3*^{ko} strain. Both *qde-1* and *qde-3* strains exhibited low levels of quelling but were not completely deficient using our quelling protocol, probably due to the generation of inverted repeats after transformation (data not shown). These results demonstrate that, like QDE-1 and QDE-3, RPA is an essential component in the quelling pathway.

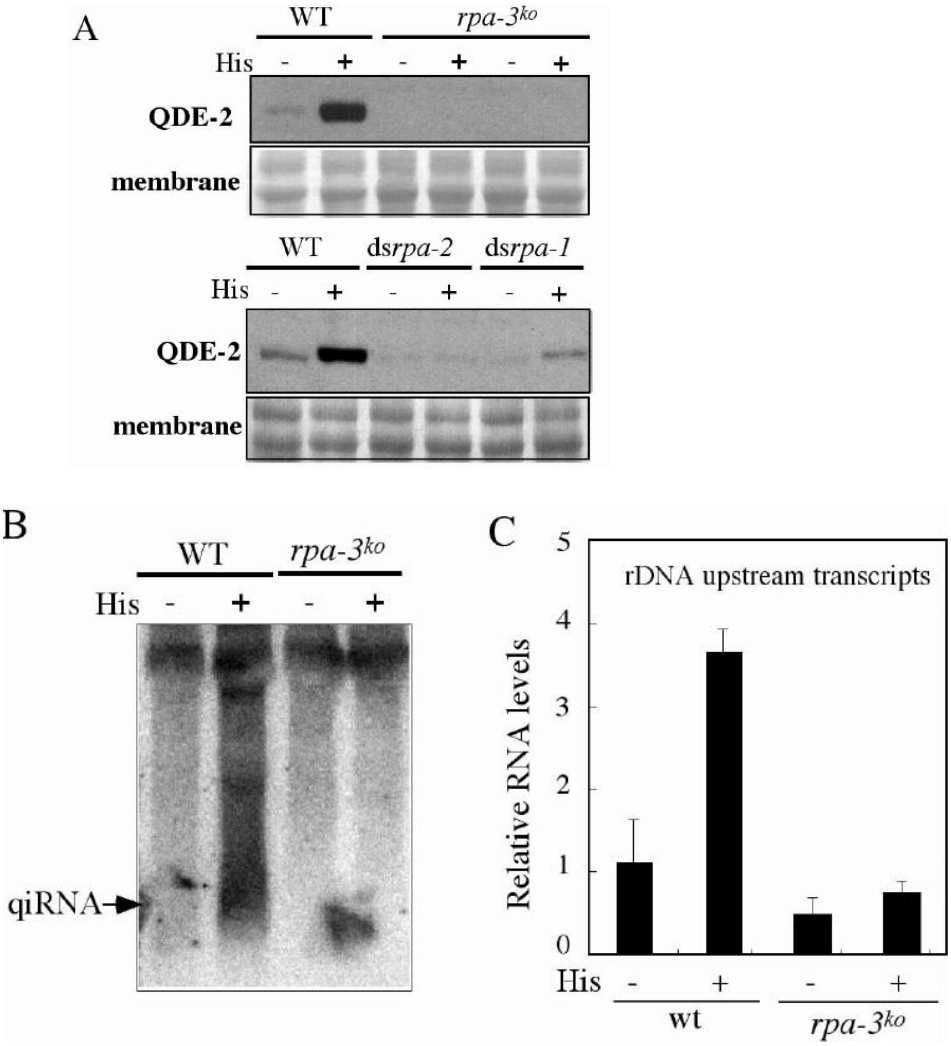


Figure 17. RPA is required for aRNA and qiRNA production.

(A) Western blot analyses of QDE-2 induction by histidine in wild type (WT), *rpa-3* knock-out (*rpa-3*^{ko}), *rpa-1* knock-down (ds*rpa-1*) and *rpa-2* knock-down (ds*rpa-1*) strains. (B) Northern blot analysis of the qiRNA biogenesis in the wild type (WT) and *rpa-3*^{ko} strains after histidine treatment. (C) qRT-PCR showing the production of DNA damage-induced aberrant rDNA transcript after histidine treatment in wild type and *rpa-3*^{ko} strains.

quelling assay

	no silencing (orange)	*al-1* silenced (yellow & white)
WT	69% (58)	31% (26)
qde-3	93.2% (83)	6.8% (7)
rpa-3	91.2% (82)	8.8% (8)

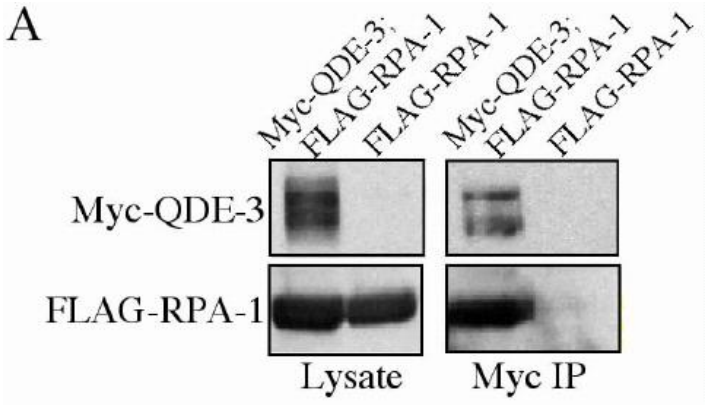
Table 1. RPA is required for quelling.

The results of the quelling assay showing the silencing efficiency of *albino-1 (al-1)* gene in the wild-type, *qde-3*^{ko} and *rpa-3*^{ko} strains. Percentage and actual numbers (shown in parenthesis) of *al-1* silenced transformants are shown.

3.3.5 The interaction between QDE-1 and RPA requires QDE-3

RPA participates in many DNA metabolic processes, but qiRNA is preferentially produced only after treatments with DNA damaging agents, suggesting that the interaction between QDE-1 and RPA requires additional factor(s). In addition, the generation of ssDNA should also be a critical step in this process. QDE-3 and its eukaryotic homologs such as human BLM and WRN and yeast SGS1 play important roles in genome maintenance and DNA replication (Bachrati and Hickson, 2003, 2008; Cogoni and Macino, 1999b; Kato et al., 2004; Wu and Hickson, 2006). These QDE-3 homologs display ATP dependent 3'–5'

DNA helicase activity to unwind duplex DNA and are recruited to damaged replication forks after treatment with DNA damaging agents or a blockade of replication. In addition, it has been shown that RPA interacts with BLM and WRN proteins and that RPA stimulates their DNA helicase activity (Bachrati and Hickson, 2008; Brosh et al., 2000; Choudhary et al., 2006). To understand the function of QDE-3 in aRNA production, we examined its interaction with RPA in *Neurospora*. A c-Myc-His-tagged QDE-3 and a FLAG-His-tagged RPA-1 constructs were co-transformed into wild-type *Neurospora*. As shown in Figure 18A, Myc-His-QDE-3 was found to interact specifically with FLAG-His-RPA-1 by immunoprecipitation (IP) assay suggesting that QDE-3 and RPA work together to generate and maintain ssDNA after DNA damage. Consistent with earlier results (Nolan et al., 2008), we found that QDE-1 interacts with RPA-1 in the wild-type *Neurospora* strain. Importantly, this interaction was abolished in the *qde-3*^{ko} strain, indicating that QDE-3 is required for recruiting QDE-1 to RPA (Figure 18B). This result also provides a molecular explanation for the induction of aRNA and qiRNA after treatments with DNA damaging agents. Although RPA is involved in normal DNA metabolism, it can only interact with QDE-1 when QDE-3 is present at the damaged DNA loci.



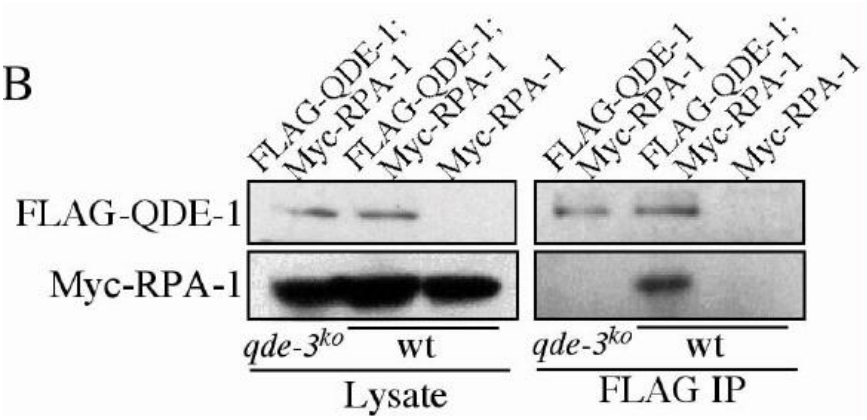


Figure 18. The interaction between QDE-1 and RPA-1 requires QDE-3.
A c-Myc-His-tagged QDE-3 and a FLAG-His-tagged RPA-1 constructs were co-transformed into wild-type *Neurospora* (A and B) and *qde-3*^{ko} (B) strains. (A) Immunoprecipitation (IP) with a c-Mycspecific antibody showing the interaction of c-Myc-His-QDE-3 with FLAG-His-RPA-1. (B) IP showing the interaction between FLAG-His-QDE-1 and c-Myc-His-RPA-1, and the disappearance of this interaction in the *qde-3*^{ko} strain. The antibodies used are indicated on the left, the strains below and the fusion protein constructs above the panels.

3.3.6 QDE-1 can initiate internally from ssDNA templates and can produce dsRNA directly from ssDNA

Since free ssDNA ends are rare *in vivo* and mostly protected by proteins, we were wondering if QDE-1 can initiate RNA synthesis internally from ssDNA. In addition, since QDE-1 generates ssRNA from ssDNA, we wondered whether QDE-1 can use the ssRNAs it produces as templates to generate dsRNA. To test these possibilities, we performed RNA polymerase assays using long circular ssDNA templates (genomic ssDNA of bacteriophage M13mp18 or φX174, 5.4 and 7.2kb respectively) and QDE-1ΔN. As shown in Figure 19 (the CTL lanes), QDE-1ΔN exhibited robust DdRP activity for both circular templates and the band corresponding to ssDNA templates disappeared. In addition, treatment of M13 ssDNA template with Exonuclease I, which degrades linear ssDNA, prior to the reactions did not affect the activity of QDE-1. These results demonstrate that QDE-1 can indeed initiate RNA synthesis internally from ssDNA templates. There are two types of products made by QDE-1 using these circular ssDNA templates as indicated by the prominent high molecular weight bands and the low molecular weight smear (Figure 19). The primary high molecular weight QDE-1 products were degraded by both RQ1 DNase and RNase H. Furthermore, the RNase H treatment resulted in the reappearance of the ssDNA template band, indicating that the products of the QDE-1 catalyzed reaction are mostly DNA/RNA hybrids. In contrast, the low molecular weight smear products were

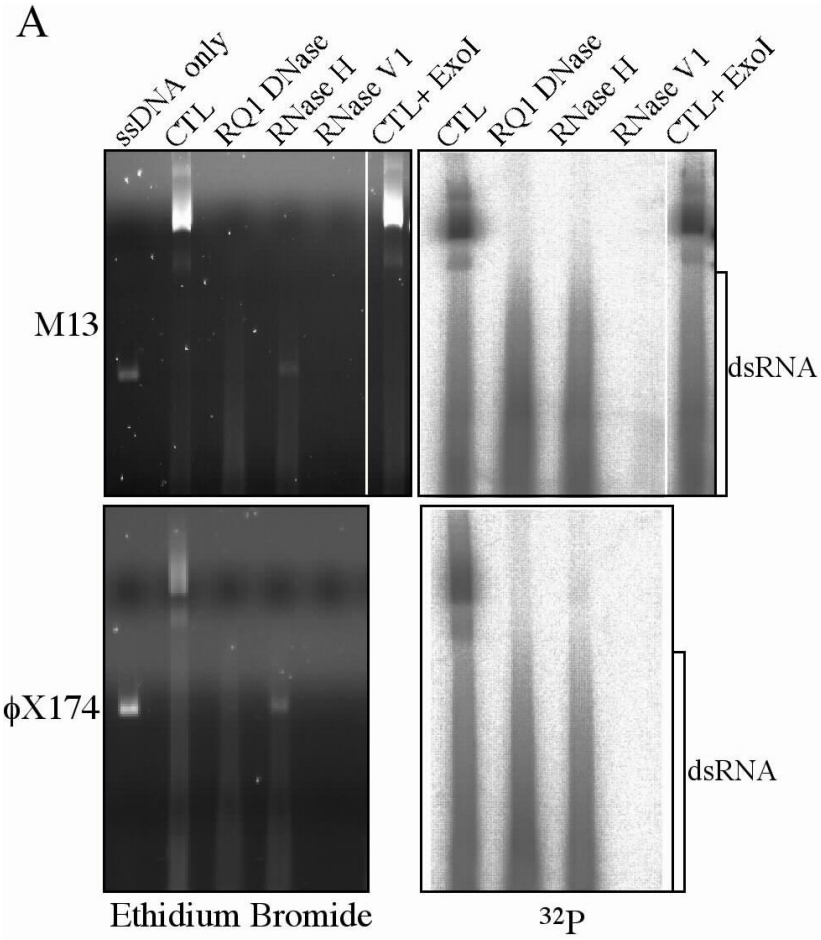


Figure 19. QDE-1 can initiates polymerization internally from ssDNA and produces dsRNA directly from ssDNA. *In vitro* DdRP assay using circular bacteriophage M13 or X174 ssDNA as templates. The nature of the products were characterized by various nucleases treatment and resolved in 0.6% native agarose gel.

resistant to both RQ1 DNase and RNase H but were completely degraded by RNase V1. Since RNase V1 degrades base-paired RNA or DNA, these results demonstrate that the low molecular weight smear products are mostly dsRNA.

These data also indicate that some of the short nascent RNAs can dissociate from ssDNA, providing free ssRNA for dsRNA synthesis by QDE-1, resulting in dsRNA products of variable lengths. Thus, QDE-1 can directly generate dsRNA from ssDNA templates.

3.3.7 RPA promotes the ability of QDE-1 to produce dsRNA by preventing the formation of DNA/RNA hybrids

Although QDE-1 can produce some dsRNA from ssDNA, the majority of the QDE-1 products are DNA/RNA hybrids, which can prevent the generation of dsRNA. Thus, for robust dsRNA production, a mechanism must exist to unwind the nascent DNA/RNA hybrids or to prevent their formation. Since RPA binds to ssDNA, we examined its effect on QDE-1 RNA polymerase activity. In addition, we were interested to know whether QDE-1 can use RPAbound ssDNA as a template. Different concentrations of human RPA complex were incubated with ssDNA (a 175nt template**)** before the addition of recombinant full-length QDE-1. The reaction products were separated by a denaturing polyacrylamide gel. As shown in Figure 20, without RPA or at a low RPA concentration (10nM), the QDE-1 products were very similar to the ones in Figure 16C, which were mostly DNA/RNA hybrids. However, when RPA was increased to 35 nM, the DNA/RNA hybrid bands decreased and a high molecular weight band appeared. At 70 nM of RPA, the DNA/RNA hybrid bands completely disappeared and the

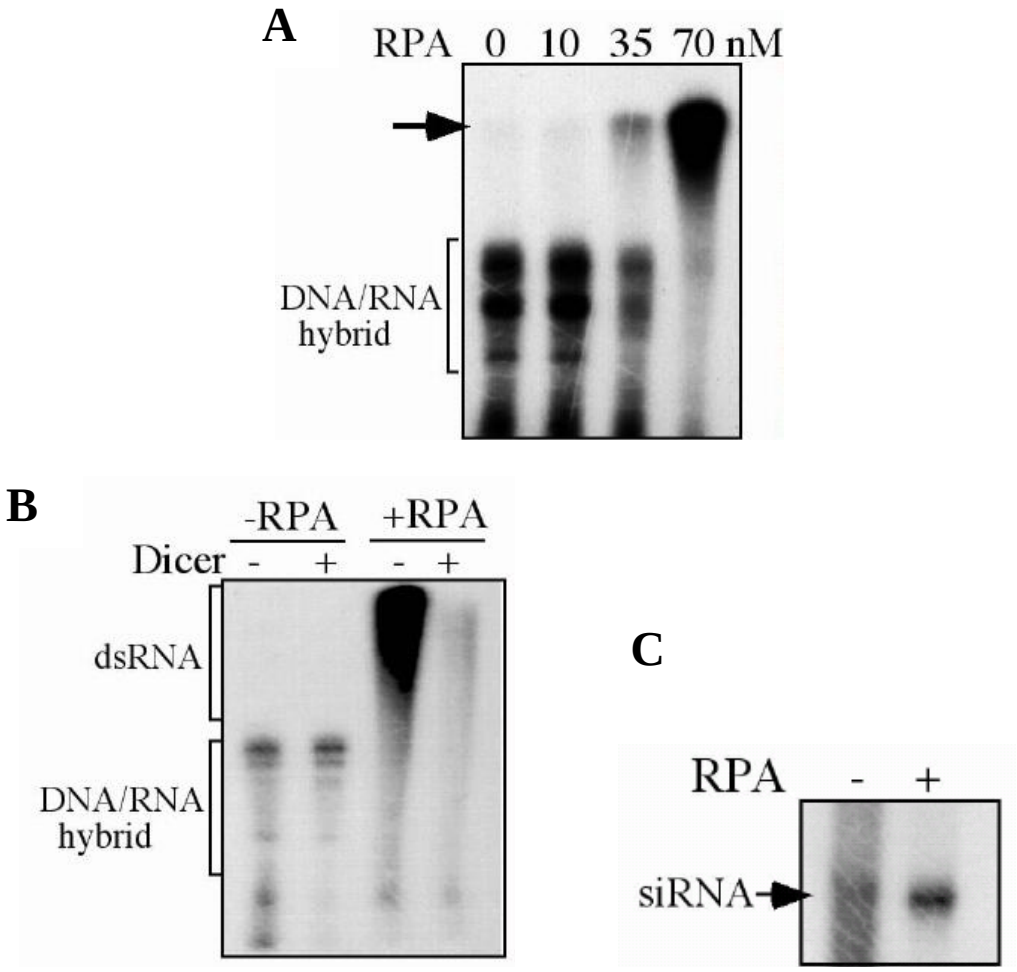


Figure 20. ssDNA-directed synthesis of dsRNA by QDE-1 is promoted by RPA.

(A) *In vitro* DdRP assay with various amounts of RPA complex. The ss175nt ssDNA was preincubated with RPA before adding QDE-1, and the products were resolved by 6% urea containing polyacrylamide gel. (B&C) The QDE-1 RdRP products with 0 or 70nM RPA were treated with recombinant Dicer and resolved in (B) 6% or (C) 16% urea containing polyacrylamide gel

high molecular weight forms became the only major QDE-1 products. This result demonstrates that QDE-1 can use RPA-bound ssDNA as template and RPA can regulate the RNA polymerase activity of QDE-1.

It was previously shown that for ssRNA templates *in vitro*, QDE-1 can utilizes the 3' end of the ssRNA to create dsRNAs in which the complementary strands are covalently attached to each other, as a result of a mode of initiation called back priming (Makeyev and Bamford, 2002). The high molecular weight products detected in the denaturing polyacrylamide gel suggest that they are most likely dsRNA molecules synthesized by back-priming initiation. To confirm this, the QDE-1 products synthesized in the presence or absence of RPA were subjected to Dicer treatment (recombinant *Drosophila* Dicer-2 purified from Sf9 insect cells). As shown in Figure 20B, Dicer treatment removed the vast majority of the high molecular weight QDE-1 products but the DNA/RNA hybrids were unaffected. Note that some of the small QDE-1 products made in the absence of RPA were also removed by Dicer. Subsequently, the Dicer-treated products were separated in a 16% polyacrylamide gel to detect the presence of siRNAs. As shown in Figure 20C, the QDE-1 products synthesized in the presence of RPA resulted in a significant increase of siRNA level after the Dicer treatment. Together, these results demonstrate that RPA promotes the ability of QDE-1 to synthesize dsRNA by preventing the formation of DNA/RNA hybrids or by dissociating the hybrids.

3.4 Discussion

The production of aRNA and its recognition and conversion to dsRNA by RdRP is the initial step in many RNAi-related pathways. The genetic and biochemical results presented here suggest that in *Neurospora*, QDE-1 acts both as a DdRP and an RdRP to generate the aRNA and the subsequent dsRNA necessary to initiate the RNAi pathway (Figure 21).

This conclusion is supported by several lines of evidence. i) QDE-1 but not Pol I is required for aRNA production from the rDNA locus after treatment with DNA damaging agents ii) QDE-1 and QDE-3 are recruited to the rDNA locus when aRNA is induced iii) Both the recombinant QDE-1 and the QDE-1 purified from *Neurospora* exhibit robust DdRP activity using ssDNA but not dsDNA templates, and the DdRP activity of QDE-1 is higher than its RdRP activity. iv) QDE-1 can directly generate dsRNA from ssDNA templates. Therefore, although we cannot exclude the possibility that other RNA polymerases such as Pol II or Pol III also contribute to the rDNA specific aRNA production, the evidence suggests that QDE-1 should be the major RNA polymerase involved in this process. QDE-1 can use ssDNA but not dsDNA as a template, suggesting that QDE-1 needs to be recruited to ssDNA *in vivo*. The requirement of RPA (the QDE-1-interacting ssDNA-binding protein complex) in aRNA and qiRNA production and the interaction between RPA and QDE-1 suggest that QDE-1 is recruited to ssDNA by RPA.

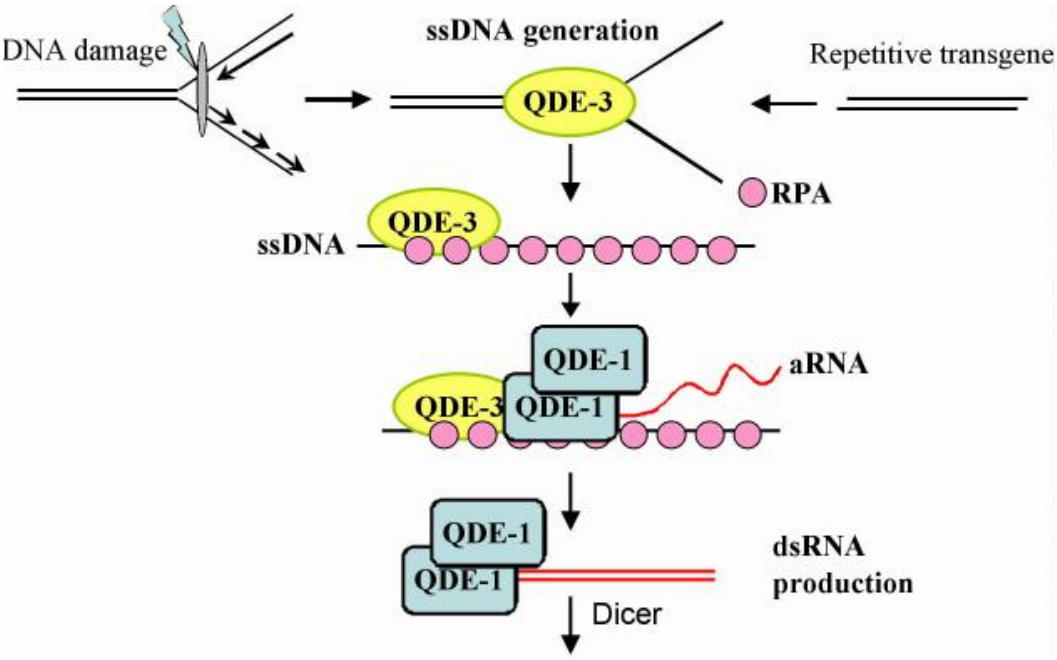


Figure 21. A proposed model for the biogenesis of aRNA and dsRNA after DNA damage.

Abnormal DNA structures, which can occur after DNA damage, replication fork stall or at a locus with repetitive transgenes, are recognized by QDE-3, which may produce ssDNA. RPA then binds to ssDNA and recruits QDE-1 together with QDE-3. QDE-1 produces aberrant ssRNA using ssDNA as a template. RPA blocks the formation of DNA/RNA hybrids so that the free ssRNA is converted to dsRNA by the RdRP activity of QDE-1. dsRNA can be recognized and cleaved by dicer, triggering RNAi.

In addition, like their mammalian homologs, RPA and QDE-3 apparently associate with each other *in vivo*. Since RPA can stimulate the DNA helicase

activity of QDE-3 homologs in mammals (Bachrati and Hickson, 2008; Brosh et al., 2000; Choudhary et al., 2006), it is likely that in *Neurospora*, QDE-3, with the help of RPA, is responsible for the generation of ssDNA template for QDE-1. Furthermore, the loss of QDE-1-RPA interaction in the *qde-3*^{ko} mutant indicates that QDE-3 mediates this interaction. The involvement of QDE-3 in this process suggests that although RPA is involved in many aspects of DNA metabolism and ssDNA is generated during normal DNA replication, QDE-1 is only recruited to the ssDNA locus if both RPA and QDE-3 are present. This conclusion is further supported with the observation that qiRNA and aRNA production is abolished in the *qde-3*^{ko} mutant. Our results also provide a molecular explanation for how aRNA but not other cellular RNA is specifically recognized by QDE-1. In plants and yeast, it was proposed that RdRPs recognize their specific templates through the recruitment of RdRP by Argonaute-siRNA complexes although it is not clear whether RdRPs are also required for the production of primary siRNAs (Buhler and Moazed, 2007). However, QDE-2 is not required for the production of aRNA and qiRNA, suggesting a novel mechanism of aRNA recognition by QDE-1 (Lee et al.).

The results presented here suggest that since the aRNA is generated by QDE-1, the close proximity and the relatively high ssRNA-binding ability of QDE-1 (data not shown) allow it to use the nascent aRNA as a template to make dsRNA. Thus, there is no need for a mechanism to specifically recruit QDE-1 to

aRNA. Notably, the recombinant QDE-1 forms a homodimer (Salgado et al., 2006) and may have a free active site on "standby" to convert the nascent ssRNA into dsRNA. Therefore, the specificity between QDE-1 and aRNA is determined by their shared location and by the biochemical activity of QDE-1. Although most of the *in vitro* DdRP products of QDE-1 are RNA/DNA hydrids, some ssRNA can be generated, as indicated by the generation of dsRNA from ssDNA, suggesting that some of the nascent RNA transcripts can dissociate from the ssDNA template. More importantly, we showed that RPA can prevent the formation of DNA/RNA hybrids and that it strongly promotes dsRNA production by QDE-1 from ssDNA. Therefore, RPA has a dual role in the small RNA production process: recruiting QDE-1 to ssDNA and blocking the formation of DNA/RNA hybrids. Interestingly, it was recently shown that the human QDE-3 homolog BLM can unwind RNA/DNA hybrids to release free ssRNAs (Popuri et al., 2008). Thus, QDE-3 could also potentially contribute to the unwinding of the RNA/DNA hybrid *in vivo*.

Our results reveal the mechanism for the production of aRNA and dsRNA in the RNAi pathway in *Neurospora*. Although we use the DNA damage-induced aRNA as a model, the mechanism of aRNA production is most likely shared by quelling, a post-transcriptional silencing mechanism against transgenes. We show that, like QDE-1 and QDE-3, RPA are also required for quelling. In addition, QDE-1 is recruited to the transgene locus upon quelling (Nolan et al., 2008). In

Arabidopsis, the RdRP RDR6, which plays an important role in RNAi pathways, has been shown to have a strong DdRP activity on ssDNA and it cannot distinguish between RNAs with or without a cap or poly(A) tail *in vitro* (Curaba and Chen, 2008). In addition, a mutation of RPA2 in *Arabidopsis* impairs transcriptional gene silencing at certain loci (Kapoor et al., 2005). These results suggest that RDR6 and RPA may play a similar role in plants as in *Neurospora*. Recent studies have discovered several types of endogenous small RNAs (20-30 nt) in animals and plants, including endogenous siRNAs, Piwi-interacting RNAs (piRNAs), rasiRNAs and 21U RNAs (Ambros and Chen, 2007; Batista et al., 2008; Baulcombe, 2004; Ghildiyal et al., 2008; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Lin, 2007; Ruby et al., 2006). Like qiRNA in *Neurospora*, many of these small RNAs originate from repetitive elements of the genome. Interestingly, piRNAs from rat testes were found to be associated with rRecQ1 (Lau et al., 2006), a QDE-3 homolog. The requirement of QDE-3 in aRNA production in *Neurospora* suggests that rRecQ1 may have the same function in generating primary aRNAs, which are used as the precursor for piRNAs in mammals. Although RdRP homologs are not found in insects or mammals, it is possible that the highly conserved RPA works together with a RecQ DNA helicase to recruit other RNA polymerase(s) to generate the primary RNA transcripts in the RNAi pathway.

3.5 Bibliography

Ambros, V., and Chen, X. (2007). The regulation of genes and genomes by small RNAs. Development *134*, 1635-1641.

Bachrati, C.Z., and Hickson, I.D. (2003). RecQ helicases: suppressors of tumorigenesis and premature aging. Biochem J *374*, 577-606.

Bachrati, C.Z., and Hickson, I.D. (2008). RecQ helicases: guardian angels of the DNA replication fork. Chromosoma *117*, 219-233.

Batista, P.J., Ruby, J.G., Claycomb, J.M., Chiang, R., Fahlgren, N., Kasschau, K.D., Chaves, D.A., Gu, W., Vasale, J.J., Duan, S., *et al.* (2008). PRG-1 and 21U-RNAs interact to form the piRNA complex required for fertility in C. elegans. Mol Cell *31*, 67-78.

Baulcombe, D. (2004). RNA silencing in plants. Nature *431*, 356-363.

Brosh, R.M., Jr., Li, J.L., Kenny, M.K., Karow, J.K., Cooper, M.P., Kureekattil, R.P., Hickson, I.D., and Bohr, V.A. (2000). Replication protein A physically interacts with the Bloom's syndrome protein and stimulates its helicase activity. J Biol Chem *275*, 23500-23508.

Buhler, M., and Moazed, D. (2007). Transcription and RNAi in heterochromatic gene silencing. Nature structural & molecular biology *14*, 1041-1048.

Catalanotto, C., Azzalin, G., Macino, G., and Cogoni, C. (2000). Gene silencing in worms and fungi. Nature *404*, 245.

Catalanotto, C., Azzalin, G., Macino, G., and Cogoni, C. (2002). Involvement of small RNAs and role of the qde genes in the gene silencing pathway in Neurospora. Genes Dev *16*, 790-795.

Catalanotto, C., Nolan, T., and Cogoni, C. (2006). Homology effects in Neurospora crassa. FEMS Microbiol Lett *254*, 182-189.

Catalanotto, C., Pallotta, M., ReFalo, P., Sachs, M.S., Vayssie, L., Macino, G., and Cogoni, C. (2004). Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in Neurospora crassa. Mol Cell Biol *24*, 2536-2545.

Cheng, P., Yang, Y., Heintzen, C., and Liu, Y. (2001). Coiled-coil domain mediated FRQ-FRQ interaction is essential for its circadian clock function in *Neurospora*. EMBO J *20*, 101-108.

Choudhary, S., Doherty, K.M., Handy, C.J., Sayer, J.M., Yagi, H., Jerina, D.M., and Brosh, R.M., Jr. (2006). Inhibition of Werner syndrome helicase activity by benzo[a]pyrene diol epoxide adducts can be overcome by replication protein A. J Biol Chem *281*, 6000-6009.

Choudhary, S., Lee, H.C., Maiti, M., He, Q., Cheng, P., Liu, Q., and Liu, Y. (2007). A double-stranded RNA response program important for RNAi efficiency. Mol Cell Biol, 3994-4005.

Cogoni, C., Irelan, J.T., Schumacher, M., Schmidhauser, T.J., Selker, E.U., and Macino, G. (1996). Transgene silencing of the al-1 gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. Embo J *15*, 3153-3163.

Cogoni, C., and Macino, G. (1999a). Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. Nature *399*, 166-169.

Cogoni, C., and Macino, G. (1999b). Posttranscriptional gene silencing in Neurospora by a RecQ DNA helicase. Science *286*, 2342-2344.

Curaba, J., and Chen, X. (2008). Biochemical activities of Arabidopsis RNA-dependent RNA polymerase 6. J Biol Chem *283*, 3059-3066.

Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D. C. (2000). An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell *101*, 543-553.

Djupedal, I., Portoso, M., Spahr, H., Bonilla, C., Gustafsson, C.M., Allshire, R.C., and Ekwall, K. (2005). RNA Pol II subunit Rpb7 promotes centromeric transcription and RNAi-directed chromatin silencing. Genes Dev *19*, 2301-2306.

Fanning, E., Klimovich, V., and Nager, A.R. (2006). A dynamic model for replication protein A (RPA) function in DNA processing pathways. Nucleic Acids Res *34*, 4126-4137.

Ghildiyal, M., Seitz, H., Horwich, M.D., Li, C., Du, T., Lee, S., Xu, J., Kittler, E.L., Zapp, M.L., Weng, Z., *et al.* (2008). Endogenous siRNAs Derived from Transposons and mRNAs in Drosophila Somatic Cells. Science.

Girard, A., Sachidanandam, R., Hannon, G.J., and Carmell, M.A. (2006). A germline-specific class of small RNAs binds mammalian Piwi proteins. Nature *442*, 199-202.

Grivna, S.T., Beyret, E., Wang, Z., and Lin, H. (2006). A novel class of small RNAs in mouse spermatogenic cells. Genes Dev *20*, 1709-1714.

He, Q., Cheng, P., He, Q., and Liu, Y. (2005a). The COP9 signalosome regulates the Neurospora circadian clock by controlling the stability of the SCFFWD-1 complex. Genes & Dev *19*, 1518-1531.

He, Q., Shu, H., Cheng, P., Chen, S., Wang, L., and Liu, Y. (2005b). Light-independent phosphorylation of WHITE COLLAR-1 regulates its function in the *Neurospora* circadian negative feedback loop. J Biol Chem *280*, 17526-17532.

Herr, A.J., Jensen, M.B., Dalmay, T., and Baulcombe, D.C. (2005). RNA polymerase IV directs silencing of endogenous DNA. Science *308*, 118-120.

Kapoor, A., Agarwal, M., Andreucci, A., Zheng, X., Gong, Z., Hasegawa, P.M., Bressan, R.A., and Zhu, J.K. (2005). Mutations in a conserved replication protein suppress transcriptional gene silencing in a DNA-methylation-independent manner in Arabidopsis. Curr Biol *15*, 1912-1918.

Kato, A., Akamatsu, Y., Sakuraba, Y., and Inoue, H. (2004). The Neurospora crassa mus-19 gene is identical to the qde-3 gene, which encodes a RecQ homologue and is involved in recombination repair and postreplication repair. Curr Genet *45*, 37-44.

Kato, H., Goto, D.B., Martienssen, R.A., Urano, T., Furukawa, K., and Murakami, Y. (2005). RNA polymerase II is required for RNAi-dependent heterochromatin assembly. Science *309*, 467-469.

Lau, N.C., Seto, A.G., Kim, J., Kuramochi-Miyagawa, S., Nakano, T., Bartel, D.P., and Kingston, R.E. (2006). Characterization of the piRNA complex from rat testes. Science *313*, 363-367.

Laurila, M.R., Salgado, P.S., Makeyev, E.V., Nettelship, J., Stuart, D.I., Grimes, J.M., and Bamford, D.H. (2005). Gene silencing pathway RNA-dependent RNA

polymerase of Neurospora crassa: yeast expression and crystallization of selenomethionated QDE-1 protein. Journal of structural biology *149*, 111-115.

Li, H., Li, W. X., and Ding, S. W. (2002). Induction and suppression of RNA silencing by an animal virus. Science *296*, 1319-1321.

Lee, H.-C., Chang, S.-S., Choudhary, S., Maiti, M., and Liu, Y. qiRNA, a novel type of small interference RNA induced by DNA damage. submitted.

Lin, H. (2007). piRNAs in the germ line. Science *316*, 397.

Luo, C., Loros, J.J., and Dunlap, J.C. (1998). Nuclear localization is required for function of the essential clock protein FREQUENCY. EMBO J *17*, 1228 - 1235.
Maiti, M., Lee, H.C., and Liu, Y. (2007). QIP, a putative exonuclease, interacts with the Neurospora Argonaute protein and facilitates conversion of duplex siRNA into single strands. Genes Dev *21*, 590-600.

Makeyev, E.V., and Bamford, D.H. (2000). Replicase activity of purified recombinant protein P2 of double-stranded RNA bacteriophage phi6. Embo J *19*, 124-133.

Makeyev, E.V., and Bamford, D.H. (2002). Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. Mol Cell *10*, 1417-1427.

Muangsan, N., Beclin, C., Vaucheret, H., and Robertson, D. (2004). Geminivirus VIGS of endogenous genes requires SGS2/SDE1 and SGS3 and defines a new branch in the genetic pathway for silencing in plants. Plant J *38*, 1004-1014.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell *2*, 279-289.

Nolan, T., Cecere, G., Mancone, C., Alonzi, T., Tripodi, M., Catalanotto, C., and Cogoni, C. (2008). The RNA-dependent RNA polymerase essential for post-transcriptional gene silencing in Neurospora crassa interacts with replication protein A. Nucleic Acids Res *36*, 532-538.

Onai, K., Katagiri, S., Akiyama, M., and Nakashima, H. (1998). Mutation of the gene for the second-largest subunit of RNA polymerase I prolongs the period

length of the circadian conidiation rhythm in Neurospora crassa. Mol Gen Genet *259*, 264-271.

Onodera, Y., Haag, J.R., Ream, T., Nunes, P.C., Pontes, O., and Pikaard, C.S. (2005). Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. Cell *120*, 613-622.

Popuri, V., Bachrati, C.Z., Muzzolini, L., Mosedale, G., Costantini, S., Giacomini, E., Hickson, I.D., and Vindigni, A. (2008). The Human RecQ helicases, BLM and RECQ1, display distinct DNA substrate specificities. J Biol Chem *283*, 17766-17776.

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

Ruby, J.G., Jan, C., Player, C., Axtell, M.J., Lee, W., Nusbaum, C., Ge, H., and Bartel, D.P. (2006). Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans. Cell *127*, 1193-1207.

Salgado, P.S., Koivunen, M.R., Makeyev, E.V., Bamford, D.H., Stuart, D.I., and Grimes, J.M. (2006). The structure of an RNAi polymerase links RNA silencing and transcription. PLoS Biol *4*, e434.

Sijen, T., and Plasterk, R. H. (2003). Transposon silencing in the Caenorhabditis elegans germ line by natural RNAi. Nature *426*, 310-314.

Wu, L., and Hickson, I.D. (2006). DNA helicases required for homologous recombination and repair of damaged replication forks. Annu Rev Genet *40*, 279-306.

Zou, Y., Liu, Y., Wu, X., and Shell, S.M. (2006). Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses. Journal of cellular physiology *208*, 267-273.

CHAPTER FOUR

Activation of the RNAi components as part of the double-stranded RNA induced immune response in *Neurospora*

4.1 Introduction

The production of double-stranded RNA (dsRNA) in eukaryotic cells, generally as the result of viral replication or the transcription of transposable elements and repetitive DNA sequences, is known to elicit two types of cellular defense responses. In the first type of response, dsRNA is recognized and cleaved by the RNase III enzyme Dicer to yield 20 to 25 nucleotide short interfering RNA (siRNA) duplexes (Carmell and Hannon, 2004; Meister and Tuschl, 2004). The production of siRNA initiates RNA interference (RNAi), also known as posttranscriptional gene silencing. The siRNAs are loaded onto the RNA-induced silencing complex (RISC), of which an Argonaute (Ago) family protein forms the catalytic core, and guide the RISC to recognize and cleave homologous mRNA or result in the transcriptional silencing of the homologous DNA locus (Liu et al., 2004; Rand et al., 2004; Sigova et al., 2004; Sontheimer, 2005; Verdel et al., 2004) Dicer and the Argonaute proteins are the central components of the RNAi pathway.

The RNAi pathway plays important roles in silencing transposon and viruses in animal and plant cells and is a defense system against viruses and

transposons (Ding et al., 2004; Li et al., 2002; Lu et al., 2005; Sijen and Plasterk, 2003; Wilkins et al., 2005). Components of the RNAi pathway probably cleave viral dsRNA and degrade viral and transposon mRNAs. Consistent with this notion, many viral genomes are known to encode inhibitors of the RNAi pathway (Carrington et al., 2001; Ding et al., 2004; Lucy et al., 2000). In vertebrates, dsRNA and siRNA are also known to trigger the transcription-based antiviral interferon (IFN) response (Haller et al., 2006; Karpala et al., 2005; Sledz et al., 2003). In mammalian cells, dsRNA is recognized by dsRNA sensors such as MDA-5 (RIG-I like helicase) and Toll-like receptor 3 (Takeuchi and Akira, 2008). This leads to the activation of the IFN-regulatory transcription factors and NF-κB, which in turn results in the expression of the IFNs. The expression of IFNs then activates the transcription of hundreds of IFN-stimulated genes (ISGs) through the JAK-STAT pathway (Der et al., 1998). Many of the ISGs encode proteins with antiviral activities, including PKR and myxovirus (influenza virus) resistance (Mx) proteins (Goodbourn et al., 2000; Haller et al., 2006; Pavlovic et al., 1995; Peng et al., 2006). Thus, the IFN response plays a crucial role in antiviral immunity in vertebrates.

Although the basic mechanism of RNAi is now fairly well understood, little is known regarding the regulation of the RNAi components. It is also not known whether there is cross talk between the transcription-based antiviral response and the posttranscription-based RNAi pathway. In addition, although the

dsRNA-induced IFN response has been well characterized, it has been documented only in vertebrate systems, and the evolutionary root of the response is not known.

To understand the regulation of the RNAi pathway, we examined the responses of the RNAi components after the induction of dsRNA. Our results showed that dsRNA induced the transcription of both *qde-2* and *dcl-2*, two of the central components of the RNAi pathway. A genome wide approach was then used to further identify the dsRNA response program. Using microarray and quantitative PCR (qPCR) analyses, we identified 60 dsRNA-activated genes (DRAGs) in *Neurospora*. Functional classification of the DRAGs suggests that the induction of RNAi components is a part of a broad host defense response against viral infection and transposons in this filamentous fungus. Finally, we show that RNAi pathway indeed plays an important role against retrotransposon in *Neurospora*, suggesting an ancient role of RNAi in genome defense.

This chapter is adapted from ***Choudhary, S., Lee, H. C., Maiti, M., He, Q., Cheng, P., Liu, Q., and Liu, Y. (2007). A double-stranded-RNA response program important for RNA interference efficiency. Mol Cell Biol 27, 3995-4005***.

4.2 Materials and Methods

4.2.1 Strains and growth conditions

The wild-type strain used was either FGSC987 (*A*; obtained from Fungal Genetic Stock Center) or 87-3 (*bd; a*). Either FGSC462 (*his-3; A*) or 301-6 (*bd; his-3; A*) was the host strain used for the insertion of the *his-3*-targeting constructs. The following mutant strains (in the wild-type or *his-3* background) were created by Dr. Maiti and the methods are described in 4.2.2: *dcl-1*^{ko}, *dcl-2*^{rip}, *dcl-1*^{ko}*; dcl-2*^{rip}, *qde-1* ^{ko}, *qde-2*^{rip} and *qde-3* ^{ko}. The mutants in the *his-3* background were used as the host strains for *his-3*-targeting constructs. Culture conditions were the same as those described previously (Aronson et al., 1994). Mycelium mats were inoculated in shaking flasks with 50 ml medium. For liquid cultures containing quinic acid (QA), 0.01MQA (pH 5.8) was added to the liquid culture medium containing 1x Vogel's medium, 0.1% glucose, and 0.17% arginine (Cheng et al., 2001). The cultures were grown in the presence of QA for 2 days unless otherwise indicated. For liquid cultures containing histidine, a final concentration of 0.5 mg/ml was used.

4.2.2 Creation of mutant strains

The *Neurospora qde-2* and *dcl-2* genes were disrupted by a repeat-induced point mutation (RIP) (Cambareri et al., 1989). The PCR fragment containing the entire *qde-2* or *dcl-2* open reading frame (ORF) and its 3' untranslated region (3.3

kb for *qde-2* and 5.1 kb for *dcl-2*) was cloned into pDE3BH and introduced into the *his-3* locus of a wild-type strain (7088, *his-3; a*) by electroporation (Margolin, 1999). A positive transformant was crossed with a wild-type strain. DNA sequencing was performed to identify the strains in which the endogenous *qde-2/dcl-2* ORF was mutated with multiple premature stop codons. The gene replacement method was used to disrupt the *Neurospora qde-1*, *qde-3*, and *dcl-1* genes. A PCR fragment containing the entire ORF and 3' untranslated region of the gene (*qde-1/qde-3/dcl-1*) was cloned into pDE3BH, resulting in pQDE-1/pQDE-3/pDCL-1.

To make the disruption construct, a hygromycin resistance gene (*hph*) fragment containing promoter and terminator sequences was inserted into the XbaIPvuII site of pQDE-1, the PvuII site of pQDE-3, or the BamHI site of pDCL-1. A PCR fragment containing the gene replacement cassette was introduced into a wild-type strain by electroporation to select for hygromycin-resistant transformants (200 μg/ml hygromycin). PCR was performed to identify strains carrying the *hph* fragment at the endogenous locus. Positive transformants were crossed with a wild-type strain, and sexual spores were picked individually and germinated on slants containing hygromycin. Southern blot analysis was performed to confirm the *qde-1*^{ko}, *qde-3*^{ko}, and *dcl-1*^{ko} strains. A *dcl-1*^{ko} strain was crossed with a *dcl-2*^{rip} strain to generate a *dcl-1*^{ko} *dcl-2*^{rip} double mutant. All

mutants were crossed with a *his-3* strain to obtain mutants in the *his-3* background;

these mutants were used for all *his-3*-targeting transformations.

4.2.4 Creation of dsRNA strains

dsRNA constructs were created by Dr. Cheng and Dr. Maiti and the were described previously (Cheng et al., 2005). The following regions from *al-1*, *frq*, and *frh* were cloned into pDE3BH.qa in reverse and forward orientations, respectively: *al-1*, bp 1322 to 1942 and bp 1412 to 1942; *frq*, bp 669 to 2309 and bp 791 to 1252; and *frh*, bp 2087 to 2703 and bp 2189 to 2703. The first nucleotide of each ORF is counted as 1. The resulting plasmids that contain the inverted repeats under the control of the *qa-2* promoter were targeted to the *his-3* locus of a wild-type strain (301-6 *bd his-3 A*) and other *his-3* RNAi mutant strains by transformation.

4.2.5 Northern and Western blot analysis

Northern and Western Blot analysis were performed as described in Chapter 2.

4.2.6 Quantitative real time-PCR analysis (qRT-PCR)

qRT-PCR experiments were performed as described in Chapter 2.

4.2.7 Microarray Analysis

Neurospora oligonucleotide microarray chips were obtained from the FGSC. The microarray (created by the *Neurospora* Genome Project) consists of 10,526 predicted ORFs, which covers nearly all predicted genes in *Neurospora*. The chips were postprocessed and UV cross-linked as suggested by the manufacturer (GAPS II coated slides; Corning). Microarray experiments, including aminoallyl cDNA synthesis, CyDye conjugation, and array hybridization, were performed using Pronto Plus Indirect systems (Promega). Cy5 and Cy3 (CyDye; Amersham Biosciences) were used to label experimental and control cDNAs, respectively. Microarray spot analysis and acquisition were performed using a GenePix4000B scanner with GenePix6 software (Axon Instruments). GeneTraffic software (Stratagene) was used for normalization and further data analysis.

4.3 Results

4.3.1 Induction of *qde-2* (Argonaute) mRNA expression by dsRNA

QDE-2, an Argonaute protein, is a core component of the *Neurospora* RNAi pathway (Maiti et al., 2007). We hypothesized that its expression might be regulated. Indeed, Dr. Choudhary (a formal graduate student in the lab) observed that productions of dsRNA against different genes all lead to an increase in QDE-2 protein and mRNA expression (Figure 22).

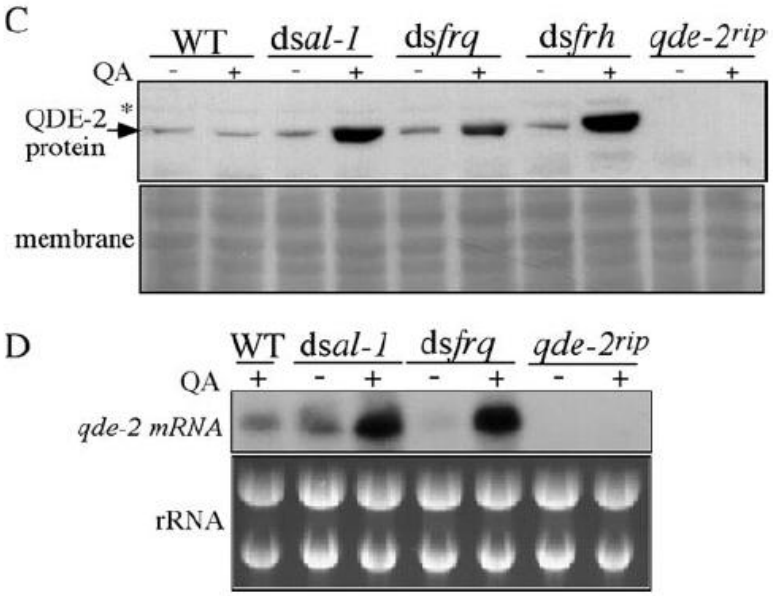


Figure 22. Induction of *qde-2* expression by dsRNA expression.
Western blot (A) and Northern blot (B) analyses showing the induction of the QDE-2 protein and *qde-2* mRNA, respectively, in strains with dsRNA constructs. The production of hairpin like dsRNA is under the control of quinic acid (QA) promoter. Addition of quinic acid into medium induces the production of dsRNA. The liquid cultures were grown for 2 days (with/without QA) before harvesting. (Experiment performed by Dr. Choudhary)

4.3.2 Induction of DCL-2 (Dicer) by dsRNA

The induction of *qde-2* expression by dsRNA prompted us to examine whether the other key component of the RNAi pathway, DCL-2, is also regulated by dsRNA. Although the functions of DCL-1 and DCL-2 are partially redundant, DCL-2 is responsible for more than 90% of the *Neurospora* Dicer activity (Catalanotto et al., 2004). As shown in Fig. 23A, *dcl-2* mRNA was strongly induced by the production of dsRNA in two strains with different dsRNA constructs, indicating that dsRNA also transcriptionally activates *dcl-2* expression. Examination of the DCL-2 protein showed that the production of dsRNA also led to a significant increase in DCL-2 protein levels (Fig. 23B). In contrast, the presence of QA had no effect on *dcl-2* or DCL-2 levels in the wild-type strain. Interestingly, the kinetics of DCL-2 induction by dsRNA were significantly delayed compared to those of QDE-2; DCL-2 levels did not peak until 24 h after

the addition of QA. These data suggest that the induction of *dcl-2* by dsRNA is a secondary response rather than an immediate response as presumed for *qde-2*.

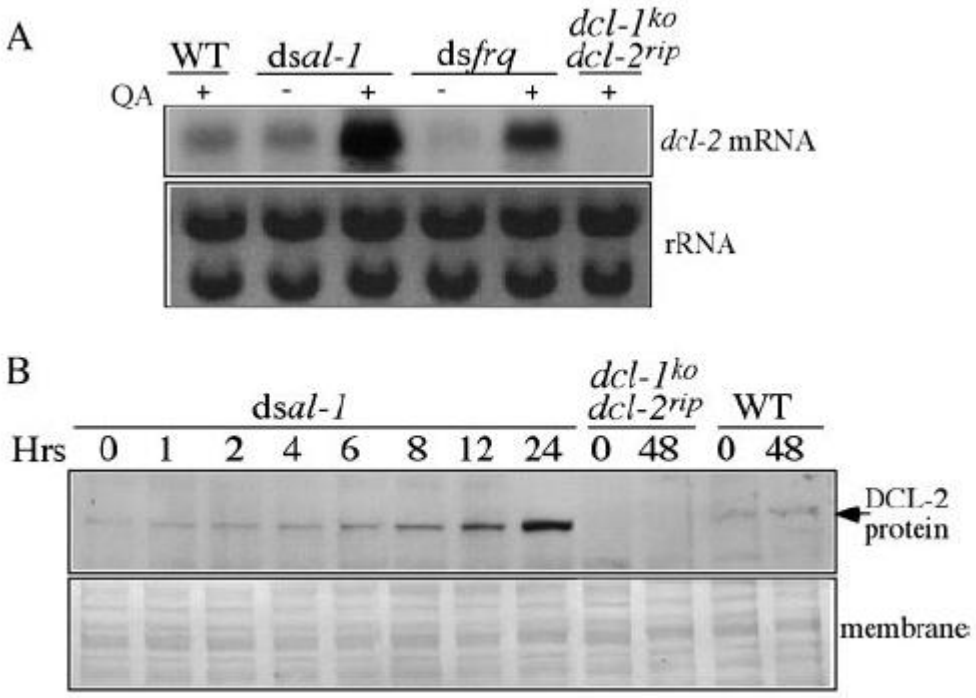


Figure 23. Induction of *dcl-2* expression by dsRNA expression.
Northern blot analysis showing the induction of *dcl-2* mRNA in strains with dsRNA constructs. (B) Western blot analysis showing the induction of DCL-2 protein by dsRNA. The number of hours indicates the time after the addition of QA. (Northern blot performed by Dr. Choudhary)

4.3.3 Genome-wide search revealed that additional RNAi components and genes homologous to host defense responses are induced by dsRNA

The observation that *qde-2* and *dcl-2* were induced by dsRNA indicates the existence of a transcription-based dsRNA response program in *Neurospora*. To understand the function of this response, we carried out a genome-wide study to identify other dsRNA activated genes (DRAGs) in *Neurospora* by microarray and qRT-PCR analyses. To identify genes that are immediately activated by dsRNA, cultures of the ds*al-1* strain were treated with QA for 6 h. The wild-type cultures treated with QA were used as the controls. As shown in Figure 24A, as expected, *qde-2* mRNA was dramatically induced in the ds*al-1* cultures. In contrast, the qRT-PCR analysis showed that the levels of *alpha-actin* and *qa-2* (a QA-inducible gene) mRNAs were comparable in both strains (Figure 24B), indicating that the QA responses were similar in both strains. Thus, activation of genes in the ds*al-1* strain should be due to the production of dsRNA. In addition to the microarray experiments, genes known to be involved in the RNAi machinery and some genes with low signal levels in the microarray experiments were analyzed by qRT-PCR.

The microarray experiments showed that the expression of the vast majority of *Neurospora* genes is not activated by dsRNA. To generate the list of genes induced by dsRNA, only genes that exhibited an average of more than a 1.5-fold increase in the ds*al-1* groups and also showed a >1.3-fold increase in all triplicate samples were included. In addition, genes with a weak signal (the signal values are less than 1.5-fold of the background value) were excluded.

To confirm the microarray results, more than half of the identified DRAGs were examined by qRT-PCR analysis, which is a more sensitive and quantitative method than the microarray assay (Kurrasch et al., 2004). In addition to the genes identified by microarray experiments, genes known to be involved in the RNAi machinery and some genes with low signal levels in the microarray experiments were also analyzed by qRT-PCR. qRT-PCR results showed that more than 90% of the genes that we identified by the microarray analysis were up-regulated.

The classification of the DRAGs based on their known or putative functions revealed that they belong to the major functional groups (Table 2).

(i) RNAi machinery.

As expected, *qde-2* was identified as a DRAG by microarray experiments. Although *dcl-2* was not found to be significantly induced in the microarray analysis, qRT-PCR revealed that its level was doubled after the induction of dsRNA (Figure 24C), suggesting that *dcl-2* is not a gene that is

TABLE 2. dsRNA activated genes (DRAGs)

Category and GenBank accession no.	Gene description	Fold induction	
		Microarray	qPCR
RNAI components and regulation			
NCU08435.1	*rrp-3* (RNA-dependent RNA polymerase)	38.53	29.9
NCU04730.1	*qde-2* (Argonaute)	3.66	5.8
NCU06766.1	*dicer-2*	1.23	2.1
NCU07534.1	*qde-1* (RNA-dependent RNA polymerase)	ND	1.6
NCU08270.1	*dicer-1*	ND	1.5
NCU00076.1	*qip* (QDE-2-interacting protein)	ND	1.9
Interferon stimulated and antiviral genes			
NCU04935.1	IFN-induced Mx protein	4.74	237.1
NCU05693.1	IFN-induced Mx protein	ND	5.7
NCU08973.1	IFN-induced Mx protein	ND	5.7
NCU08359.1	Cytidine deaminase	3.85	NA
NCU04491.1	6-16 family (ISG12 domain)	1.94	3.0
NCU04490.1	6-16 family (ISG12 domain)	2.05	2.0
NCU04489.1	6-16 family (ISG12 domain)	2.48	5.0
NCU04488.1	6-16 family (ISG12 domain)	2.15	2.8
NCU04486.1	6-16 family (ISG12 domain)	ND	1.7
RNA/DNA binding and regulation			
NCU07036.1	3′–5′ exonuclease (Rnase D-like)	42.26	213.5
NCU04472.1	RNA helicase	3.98	39.7
NCU09495.1	*set-6* (SET domain containing)	14.37	79.7
NCU06125.1	CCR4/NOT complex *sub1*	2.01	1.8
NCU00582.1	Cryptochrome	1.98	NA
NCU01871.1	DNA replication licensing factor Mcm7	1.67	1.9
Stress response			
NCU09602.1	HSP70	1.99	1.8
NCU03288.1	HSP70-like	ND	1.8
NCU04142.1	HSP80	2.26	NA
NCU00704.1	Cu/Zn superoxide dismutase	ND	3.8
NCU02623.1	Mitochodrial hypoxia-induced protein	1.78	2.3
NCU00754.1	Multidrug resistance protein (membrane)	2.25	2.2
NCU03732.1	DNAJ-like (HSP70 cochaperone)	ND	1.7
NCU03556.1	Peroxisomal membrane protein PMP47B	2.34	NA
NCU04802.1	Peroxisome membrane protein PMP30	1.70	NA

Category and GenBank accession no.	Gene description	Fold induction	
		Microarray	qPCR
Protein degradation			
NCU09309.1	20S proteasome subunit PRE2	1.5	1.5
NCU02840.1	26S regulatory subunit YTA3	1.50	1.5
Metabolism			
NCU09873.1	Phosphoenolpyruvate carboxykinase	2.31	NA
NCU06836.1	Acetyl-CoA synthetase	2.28	NA
NCU04923.1	Glycerol dehydrogenase	2.03	NA
NCU07263.1	Carnitine/acylcarnitine carrier	2.19	NA
NCU08002.1	Carnitine acetyltransferase	1.90	NA
NCU01611.1	Carnitine acetyltransferase FacC	1.96	NA
NCU08561.1	Succinate-fumarate transporter	2.11	NA
NCU05627.1	Related to sugar transporter	2.35	NA
NCU07853.1	Urate oxidase (uricase)	2.10	NA
NCU08434.1	Methionine synthase	2.09	NA
NCU03139.1	*his-3*	4.78	4.6
Unknown function			
NCU00947.1	116 aa	10.43	473.5
NCU06289.1	Unknown	11.06	117.5
NCU04436.1	BTB domain containing	2.41	28
NCU05628.1	Similarity to RNase H	15.00	188.1
NCU05629.1	Unknown	ND	3.3
NCU05631.1	Unknown	4.44	17.1
NCU08351.1	81 aa	434.58	NA
NCU04197.1	CipC protein	7.35	NA
NCU06294.1	P-loop containing	6.10	NA
NCU07257.1	Fungus specific	3.89	NA
NCU05881.1	DUF500 containing	2.35	NA
NCU09650.1	74 aa	2.30	NA
NCU07352.1	Fungus specific	2.26	NA
NCU02884.1	220 aa	2.12	NA
NCU10028.1	Hormone-induced membrane protein	1.97	NA
NCU05927.1	GTP-binding protein LepA	1.58	NA
NCU04487.1	Unknown	2.13	10.1

[a] Numbers indicate the increase (*n*-fold) in mRNA levels after 6 h of QA treatment in the d*sal-1* strain in the microarray or qPCR experiment. ND, not detectable; NA, not performed; aa, amino acids.

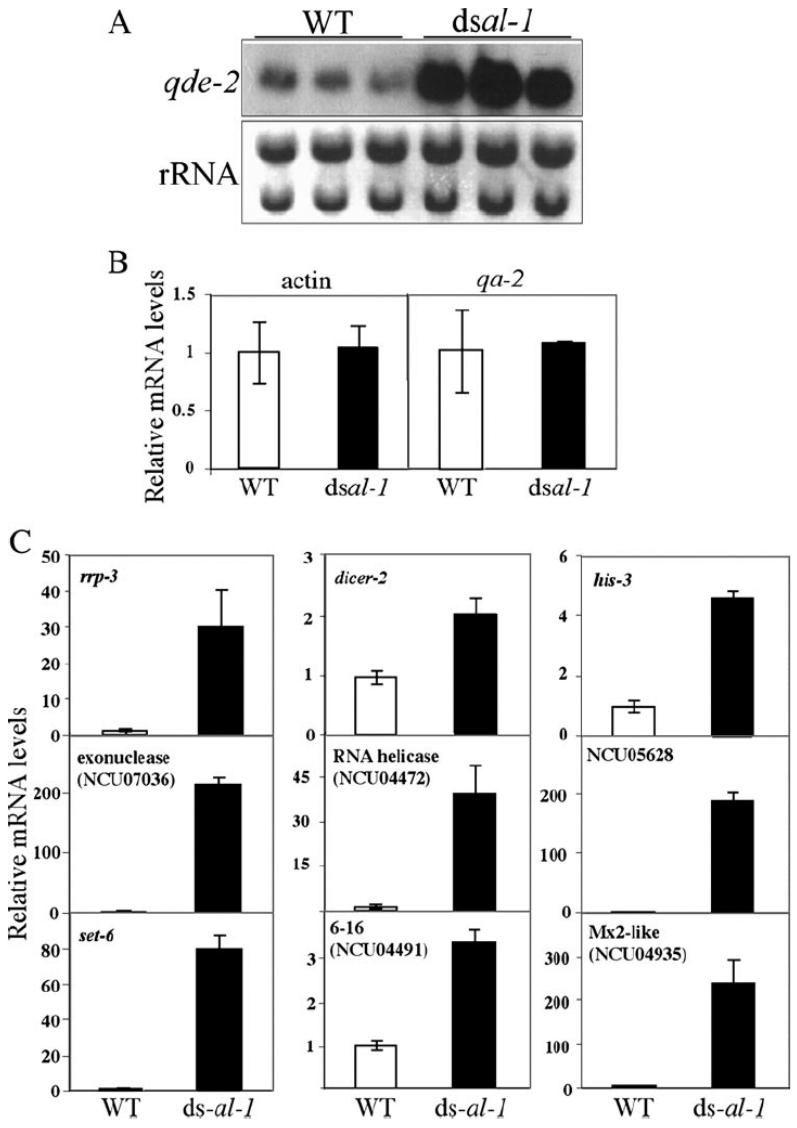


Figure 24. Genome-wide identification of DRAGs.

QA was added to wild-type (WT) and ds*al-1* triplicate cultures for 6 h before their harvest. (A) Northern blot analysis of *qde-2* for samples used in the microarray experiments. (B and C) qRT-PCR analysis of gene expression.

immediately activated by dsRNA. In addition, *qde-1* and *dcl-1*, but not *qde-3,* were found to be modestly induced by dsRNA in the qRT-PCR analysis. *qip*, a recently identified exonuclease that interacts with QDE-2 and facilitates singled-stranded siRNA production in the RISC complex, was also up-regulated by dsRNA. Thus, most known components of the *Neurospora* RNAi pathway are induced by dsRNA. Surprisingly, *rrp-3*, which encodes one of the three RNA-dependent RNA polymerases in *Neurospora* (Borkovich et al., 2004), is the most highly induced DRAG (~30-fold induction) in this group of genes. This result suggests that RRP-3, a homolog of QDE-1, may play a role in the formation of viral and retrotransposon dsRNA (Nolan et al., 2005).

(ii) IFN-stimulated and antiviral genes.

Genes with homology to mammalian ISGs form a major class of DRAGs. *Neurospora* has four genes that are homologous to the mammalian myxovirus (influenza virus) resistance (Mx) proteins, and three are strongly induced by dsRNA; one (NCU04935.1) was induced >200-fold. Mx proteins are conserved large GTPases with homology to dynamin. They inhibit viral growth by interfering with virus replication, and their induction by IFNs in mammals is an important part of the antiviral response (Goodbourn et al., 2000; Haller et al., 2006). The induction of Mx genes by dsRNA in *Neurospora* and mammals suggests a conserved dsRNA response from fungi to mammals and indicates the

potential importance of Mx proteins in antiviral defense systems. IFN-induced 6-16 family proteins are small proteins containing the IGS12 domain with unknown functions in mammals (Martensen and Justesen, 2004). *Neurospora* has five genes encoding 6-16 family proteins (NCU04486.1, NCU04488.1, NCU04489.1, NCY04490.1, and NCU04491.1) that are clustered in a single chromosomal locus. Interestingly, all six genes in this locus (the five 6-16 family protein genes and NCU04487.1) are significantly induced by dsRNA, whereas genes flanking this region (NCU04492.1 and NCU04485.1) are not (data not shown).

iii) RNA/DNA binding and regulation.

The RNA/DNA binding and regulation DRAGs include a 3'-5' exonuclease (NCU07036.1), an RNA helicase (NCU04472.1), and *set-6* (NCU09495.1); all were dramatically induced by dsRNA (from 40- to 200-fold). The exonuclease belongs to the RNase D family. Mut-7, an RNase D-like protein, is involved in transposon silencing in *Caenorhabditis elegans* (Ketting et al., 1999). The RNA helicase induced by dsRNA belongs to the *superkiller-2* (*ski-2*) subfamily of helicases. In yeast, *ski-2* is part of the host defense system that represses the propagation of dsRNA viruses by working with the exosome complex to degrade viral RNA (Wickner, 1996). In addition, several RNA helicases, including RIG-I, are involved in the antiviral response or the RNAi pathway in animals (Cook et al., 2004; Takeuchi and Akira, 2008; Tijsterman et al., 2002). *set-6*, which

encodes one of the SET-domain-containing proteins in *Neurospora* (Borkovich et al., 2004)

, was induced ~80-fold by dsRNA. SET domains are a signature of lysine protein methyltransferases and are found in histone methyltransferases. In *Neurospora*, DIM-5, a SET-domain-containing protein, is a histone 3 Lys9 histone methyltransferase (Tamaru and Selker, 2001). Although the function of *set-6* is not known, its strong induction by dsRNA suggests that it may have a role in chromatin remodeling in response to dsRNA expression.

(iv) Stress response and protein degradation.

Several heat shock proteins (HSPs) and one DNAJ-like cochaperone are up-regulated upon dsRNA induction in *Neurospora*. DRAGs involved in stress responses include a Cu/Zn superoxide dismutase, a multidrug resistance protein, and proteins involved in peroxisome function. In addition, two genes involved in regulating proteasome function were induced by dsRNA. In mammals, HSPs and proteasomal subunits are also known to be induced after viral infections or by IFNs (Der et al., 1998; Phillips et al., 1991). The induction of these genes in *Neurospora* suggests a stress response for dsRNA production.

(v) Metabolism.

DRAGs involved in metabolism include genes involved in fatty acid and carbohydrate metabolism and transport, such as phosphoenolpyruvate carboxykinase, acetyl coenzyme A (acetyl-CoA) synthetase, two carnitine acetyltransferase, and carnitine/acylcarnitine carrier. Interestingly, phosphoenolpyruvate carboxykinase is an ISG in mammals (Der et al., 1998). Fatty acid metabolism has been shown to play a role in hepatitis C virus replication, and acetyl-CoA synthetase, an enzyme involved in fatty acid metabolism, was found to be up-regulated upon hepatitis C virus infection in mammals (Kapadia and Chisari, 2005). The *his-3* gene, which encodes the histidinol dehydrogenase, a key enzyme involved in the histidine biosynthesis pathway, was also significantly induced by dsRNA.

(vi) Genes with unknown functions.

There are 17 DRAGs that are genes with unknown functions. Among these genes, NCU05628.1 (induced ~188-fold) has similarity to RNase H, which is structurally similar to the PIWI domain of the Argonaute proteins (Rand et al., 2004; Song et al., 2004). Interestingly, two of its neighboring genes (NCU05629.1 and NCU05631.1) are also significantly induced by dsRNA. Several DRAGs that encode small proteins of unknown function are some of the most highly induced

genes identified in our experiments. One of them, NCU08351.1 (81 amino acids), was the most highly induced DRAG in the microarray analysis.

4.3.4 QDE-2 represses the replication of retrotransposon TAD

To directly test if RNAi serves as as a defense mechanism against transposon, TAD retrotransposon (a non-LTR class retrotransposon) was transformed into *his-3* locus of WT or *qde-2* ^{rip} background. Therefore, the transformants will have a single TAD transposon. The transformants were then transferred weekly to fresh slants, allowing multiple replications of *Neurospora*.

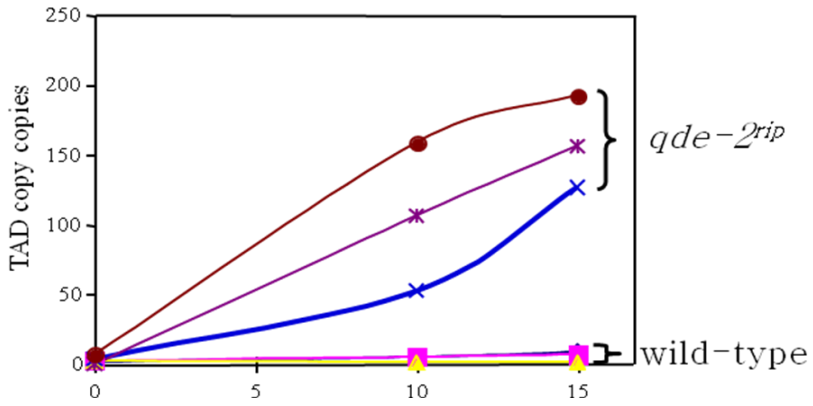


Figure 25. QDE-2 represses the replication of TAD retrotransposon.

Three independent TAD transformants from WT or *qde-2* background were transferred weekly on fresh slants. TAD copies number in the genomic DNA were measured by quantitative PCR.

The copy number of TAD retrotransposon in the genome was measured by quantitative PCR. After 15 weeks, TAD number was accumulated to around ~ 150 copies per genome in *qde-2*^{rip} background, compared to only about average 10 copies per genome in WT strains. These results demonstrate that RNAi pathway was required for inhibiting the replication of retrotransposon in *Neurospora.*

4.4 Discussion

In this chapter, we showed that the production of dsRNA leads to the induction of expression of *Neurospora qde-2*, *dcl-2*, two of the key components of the RNAi pathway. A genome-wide search for dsRNA-activated genes further identified 60 genes that are activated by dsRNA, including several additional RNAi components. The induction of antiviral genes and homologs of the mammalian ISGs in *Neurospora* suggests an evolutionarily conserved response against viral invasion and retrotransposons. To our knowledge, this is the first report of the dsRNA-induced transcription-based defense response in a nonvertebrate organism. In addition, this is also the first report of a regulatory mechanism that controls the expression of RNAi components.

What are the physiological roles of such a response? dsRNA can be generated from several sources in eukaryotic cells. First, viral infections can be a major source of dsRNA since dsRNA is an intermediate of viral replication. Second, active retrotranspons can also generate dsRNA. Although the exact mechanisms of dsRNA production may be different, siRNA with sequences corresponding to retrotransposons has been detected in *Neurospora* and animals, suggesting the production of dsRNA (Nolan et al., 2005; Sijen and Plasterk, 2003). Third, eukaryotic cells can generate endogenous dsRNA or hairpin RNA, for example, as precursors of microRNA (miRNA) or from repetitive sequences. In *Neurospora*, no confirmed miRNA has been reported. Our data suggest that the induction of QDE-2 and DCL-2 is a regulatory mechanism that can significantly increase the efficiency of the RNAi pathway and, therefore, the removal of dsRNA. The removal of dsRNA is achieved by two mechanisms. First, dsRNA is detected by DCLs and is cleaved into siRNA. Second, guided by siRNA, QDE-2 along with other components of the RISC complex will lead to the destruction of RNA template for the production of dsRNA.

The basal levels of QDE-2 and DCL-2 are low in the wild-type strain and in dsRNA strains before the induction of dsRNA. After the induction of dsRNA expression, both QDE-2 and DCL-2 were induced ~10-fold. Since the *qa-2* promoter is not a very strong promoter (Cheng et al., 2001), these results suggest that the levels of endogenous dsRNA or miRNA are low in *Neurospora*. Thus, it is

likely that the dsRNA-activated RNAi pathway is an inducible defense response triggered by dsRNA produced due to viral infection or active transposons. Consistent with this hypothesis, the RNAi pathway has been shown to play important roles in silencing transposons and inhibiting viral invasion in animals and plants (Ding et al., 2004; Li et al., 2002; Lu et al., 2005; Sijen and Plasterk, 2003; Wilkins et al., 2005). In addition, several identified DRAGs are genes that encode proteins with potential antiviral and transposon-silencing activities.

Although no virus has been reported to infect laboratory *Neurospora* strains, it is likely that viral infection is a serious threat to *Neurospora* survival in nature. In fact, viruses (both dsRNA and single-stranded RNA) are known to infect filamentous fungi (Hillman et al., 2004). Our data also suggests that RNAi play an important role in defense against retrotransposon. Recently, it was also shown that QDE-2 and DCLs are important for transposon silencing in *Neurospora* (Nolan et al., 2005)**.** Taken together, out data indicates that virus or transposon-derived dsRNA will leads to the activation of DRAGs expression and RNAi to silence those parasite DNAs.

In addition to the induction of genes in the RNAi pathway, dsRNA also led to the activation of ~50 additional genes. Although the physiological importance of the activation of these genes has not been established, functional classification of the DRAGs suggests that their activation is a part of a broad immune-like response against the production of dsRNA. First, homologs of the mammalian

ISGs, including the three Mx, five 6-16 family, HSP, and phosphoenolpyruvate carboxykinase genes, are DRAGs in *Neurospora*. Second, like the genes in the RNAi pathway, some of the DRAGs, including the Mx, RNA helicase, and exonuclease genes, have been shown to play important roles in the antiviral and transposon-silencing processes in other organisms. The similarities between the mammalian IFN response and the dsRNA response in *Neurospora* suggest that they may have similar functions with a common evolutionary link. Third, the genes involved in stress responses are a major class of DRAGs, suggesting that the presence of dsRNA is regarded as stress in *Neurospora*. Finally, some of the most highly induced DRAGs, such as the exonuclease, RNA helicase, and *set-6* genes, have putative roles in RNA processing and chromatin remodeling, suggesting potential novel processes controlling dsRNA in *Neurospora*.

The signaling pathway that triggers the transcription-based response remains to be identified. Our results showed that genes involved in RNAi, such as *qde-2*, *dcl*, and *qde-1*, are not required for transcriptional activation by dsRNA (data not shown). In mammals, PKR and Toll-like receptor 3 are important dsRNA sensors that activate the IFN pathway through IFN-regulatory transcription factors and NF-κB. The lack of *Neurospora* homologs for these genes suggests a novel dsRNA-sensing and transcriptional activation pathway in this fungal organism.

4.5 Bibliography

Aronson, B. D., Johnson, K. A., Loros, J. J., and Dunlap, J. C. (1994). Negative feedback defining a circadian clock: autoregulation of the clock gene frequency. Science *263*, 1578-1584.

Borkovich, K. A., Alex, L. A., Yarden, O., Freitag, M., Turner, G. E., Read, N. D., Seiler, S., Bell-Pedersen, D., Paietta, J., Plesofsky, N., *et al.* (2004). Lessons from the genome sequence of Neurospora crassa: tracing the path from genomic blueprint to multicellular organism. Microbiol Mol Biol Rev *68*, 1-108.

Cambareri, E. B., Helber, J., and Kinsey, J. A. (1994). Tad1-1, an active LINE-like element of Neurospora crassa. Mol Gen Genet *242*, 658-665.

Cambareri, E. B., Jensen, B. C., Schabtach, E., and Selker, E. U. (1989). Repeat-induced G-C to A-T mutations in Neurospora. Science *244*, 1571-1575.

Carmell, M. A., and Hannon, G. J. (2004). RNase III enzymes and the initiation of gene silencing. Nat Struct Mol Biol *11*, 214-218.

Carrington, J. C., Kasschau, K. D., and Johansen, L. K. (2001). Activation and suppression of RNA silencing by plant viruses. Virology *281*, 1-5.

Catalanotto, C., Pallotta, M., ReFalo, P., Sachs, M. S., Vayssie, L., Macino, G., and Cogoni, C. (2004). Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in Neurospora crassa. Mol Cell Biol *24*, 2536-2545.

Cheng, P., He, Q., He, Q., Wang, L., and Liu, Y. (2005). Regulation of the Neurospora circadian clock by an RNA helicase. Genes Dev *19*, 234-241.

Cheng, P., Yang, Y., and Liu, Y. (2001). Interlocked feedback loops contribute to the robustness of the Neurospora circadian clock. Proc Natl Acad Sci U S A *98*, 7408-7413.

Choudhary, S., Lee, H. C., Maiti, M., He, Q., Cheng, P., Liu, Q., and Liu, Y. (2007). A double-stranded-RNA response program important for RNA interference efficiency. Mol Cell Biol *27*, 3995-4005.

Cook, H. A., Koppetsch, B. S., Wu, J., and Theurkauf, W. E. (2004). The Drosophila SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification. Cell *116*, 817-829.

Der, S. D., Zhou, A., Williams, B. R., and Silverman, R. H. (1998). Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A *95*, 15623-15628.

Ding, S. W., Li, H., Lu, R., Li, F., and Li, W. X. (2004). RNA silencing: a conserved antiviral immunity of plants and animals. Virus Res *102*, 109-115.

Goodbourn, S., Didcock, L., and Randall, R. E. (2000). Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J Gen Virol *81*, 2341-2364.

Haller, O., Kochs, G., and Weber, F. (2006). The interferon response circuit: induction and suppression by pathogenic viruses. Virology *344*, 119-130.

Hillman, B. I., Supyani, S., Kondo, H., and Suzuki, N. (2004). A reovirus of the fungus Cryphonectria parasitica that is infectious as particles and related to the coltivirus genus of animal pathogens. J Virol *78*, 892-898.

Kapadia, S. B., and Chisari, F. V. (2005). Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. Proc Natl Acad Sci U S A *102*, 2561-2566.

Karpala, A. J., Doran, T. J., and Bean, A. G. (2005). Immune responses to dsRNA: implications for gene silencing technologies. Immunol Cell Biol *83*, 211-216.

Ketting, R. F., Haverkamp, T. H., van Luenen, H. G., and Plasterk, R. H. (1999). Mut-7 of C. elegans, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell *99*, 133-141.

Kurrasch, D. M., Huang, J., Wilkie, T. M., and Repa, J. J. (2004). Quantitative real-time polymerase chain reaction measurement of regulators of G-protein signaling mRNA levels in mouse tissues. Methods Enzymol *389*, 3-15.

Li, H., Li, W. X., and Ding, S. W. (2002). Induction and suppression of RNA silencing by an animal virus. Science *296*, 1319-1321.

Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., Hammond, S. M., Joshua-Tor, L., and Hannon, G. J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science *305*, 1437-1441.

Lu, R., Maduro, M., Li, F., Li, H. W., Broitman-Maduro, G., Li, W. X., and Ding, S. W. (2005). Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. Nature *436*, 1040-1043.

Lucy, A. P., Guo, H. S., Li, W. X., and Ding, S. W. (2000). Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. Embo J *19*, 1672-1680.

Maiti, M., Lee, H. C., and Liu, Y. (2007). QIP, a putative exonuclease, interacts with the Neurospora Argonaute protein and facilitates conversion of duplex siRNA into single strands. Genes Dev *21*, 590-600.

Margolin, B. S., Freitag, M., and Selker, E.U (1999). Improved plasmids for gene targeting at the *his-3* locus of *Neurospora crassa* by electroporation. Fungal Genet Newsl *44*, 24-36.

Martensen, P. M., and Justesen, J. (2004). Small ISGs coming forward. J Interferon Cytokine Res *24*, 1-19.

Meister, G., and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. Nature *431*, 343-349.

Nolan, T., Braccini, L., Azzalin, G., De Toni, A., Macino, G., and Cogoni, C. (2005). The post-transcriptional gene silencing machinery functions independently of DNA methylation to repress a LINE1-like retrotransposon in Neurospora crassa. Nucleic Acids Res *33*, 1564-1573.

Pavlovic, J., Arzet, H. A., Hefti, H. P., Frese, M., Rost, D., Ernst, B., Kolb, E., Staeheli, P., and Haller, O. (1995). Enhanced virus resistance of transgenic mice expressing the human MxA protein. J Virol *69*, 4506-4510.

Peng, G., Lei, K. J., Jin, W., Greenwell-Wild, T., and Wahl, S. M. (2006). Induction of APOBEC3 family proteins, a defensive maneuver underlying interferon-induced anti-HIV-1 activity. J Exp Med *203*, 41-46.

Phillips, B., Abravaya, K., and Morimoto, R. I. (1991). Analysis of the specificity and mechanism of transcriptional activation of the human hsp70 gene during infection by DNA viruses. J Virol *65*, 5680-5692.

Rand, T. A., Ginalski, K., Grishin, N. V., and Wang, X. (2004). Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. Proc Natl Acad Sci U S A *101*, 14385-14389.

Sigova, A., Rhind, N., and Zamore, P. D. (2004). A single Argonaute protein mediates both transcriptional and posttranscriptional silencing in Schizosaccharomyces pombe. Genes Dev *18*, 2359-2367.

Sijen, T., and Plasterk, R. H. (2003). Transposon silencing in the Caenorhabditis elegans germ line by natural RNAi. Nature *426*, 310-314.

Sledz, C. A., Holko, M., de Veer, M. J., Silverman, R. H., and Williams, B. R. (2003). Activation of the interferon system by short-interfering RNAs. Nat Cell Biol *5*, 834-839.

Song, J. J., Smith, S. K., Hannon, G. J., and Joshua-Tor, L. (2004). Crystal structure of Argonaute and its implications for RISC slicer activity. Science *305*, 1434-1437.

Sontheimer, E. J. (2005). Assembly and function of RNA silencing complexes. Nat Rev Mol Cell Biol *6*, 127-138.
Takeuchi, O., and Akira, S. (2008). MDA5/RIG-I and virus recognition. Curr Opin Immunol *20*, 17-22.

Tamaru, H., and Selker, E. U. (2001). A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature *414*, 277-283.

Tijsterman, M., Ketting, R. F., Okihara, K. L., Sijen, T., and Plasterk, R. H. (2002). RNA helicase MUT-14-dependent gene silencing triggered in C. elegans by short antisense RNAs. Science *295*, 694-697.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S. I., and Moazed, D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. Science *303*, 672-676.

Wickner, R. B. (1996). Double-stranded RNA viruses of Saccharomyces cerevisiae. Microbiol Rev *60*, 250-265.

Wilkins, C., Dishongh, R., Moore, S. C., Whitt, M. A., Chow, M., and Machaca, K. (2005). RNA interference is an antiviral defence mechanism in Caenorhabditis elegans. Nature *436*, 1044-1047

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Function and regulation of RNAi pathway in *Neurospora*

The functions of small RNAs in lower eukaryotes remain largely unexplored. Our discovery of DNA damage-induced small qiRNAs provides a novel link between DNA damage response and RNA interference pathway in *Neurospora* (Chapter 2). We show that qiRNAs are mostly derived from rDNA regions and play a role to inhibit protein translation after DNA damage. Importantly, RNAi mutants are hypersensitive to DNA mutagens, suggesting a role of qiRNA in DNA damage response or DNA repair.

It will be exciting to further explore the function of qiRNAs and other small RNAs in *Neurospora* in the future. The recent delelopments of deep sequencing technologies, such as 454 or Solexa sequencing (Hafner et al., 2008), will allow us to identify millions of small RNAs under normal or DNA damaged condition. Small RNAs which are rare or have a lower basal level can then be identified. Indeed, our preliminary results from Selexa sequencing suggest the existence of several other types of endogenous small RNAs, such as siRNA, miRNA and other types of small RNAs (data not shown). Characterization of those small RNAs will futher increase our understanding toward the mechanism and function of RNAi pathway.

It is previously not clear if RNAi pathways are regulated. The identification of RNAi as part of dsRNA-induced transcriptioanl response suggests an evolutionary cross-talk between RNAi pathway and an interferon-like response (Chapter 3). Our data suggests that dsRNAs, which can be generated from virus or transposon replication, will trigger both pathways. The induction of dsRNA-activated genes (DRAGs) will increase the levels of RNAi machinery to achieve efficient removal of dsRNA and target RNA. Indeed, our data showed the induction of QDE-2 is required for efficient silencing of its target (Choudhary et al., 2007). We also show that RNAi play an important role in controlling retrotransposon replication.

The lack of RIG-I or TLR3 homologue in *Neurospora* suggests a distinct dsRNA sensing pathway between *Neurospora* and mammals. Therefore, it will be interesting to identify the components responsible for dsRNA sensing, sinaling, and transcriptional activation in the future.

5.2 Mechanism of RNAi in *Neurospora*

The biogenesis of aberrant RNA (aRNA) is proposed to be the precursor of dsRNA in various RNAi pathways. The nature and biogenesis of aRNA are not understood. Our research shows that QDE-1, a RNA dependent RNA polymerase, also functions as a DNA dependent RNA polymerase to make aRNA using ssDNA as templates (Chapter 4). Afterwards, QDE-1 can use aRNA made by

itself as a template to produce dsRNA and trigger RNAi. Furthermore, our data suggests that QDE-1 is likely recruited to ssDNA by RPA and QDE-3 (a RecQ helicase). Consistent with this model, we demonstrate that both QDE-3 and RPA is important for aRNA production. We also show that the interaction between RPA and QDE-1 is dependent on QDE-3. Therefore, QDE-3 seems to function upstream of RPA and QDE-1 in the RNAi pathway by recognizing and unwinding "aberrant DNA" structure. However, it is unclear how QDE-3 recognizes aberrant DNAs and if other factors are involved this process. Therefore, further experiments are required to fully understand the nature of aberrant DNA and the roles of QDE-3 in the RNAi pathway. Taken together, our model provides a possibly conserved mechanism to trigger RNAi against transgenes, DNA virus and other repetitive sequences.

5.3 Bibliography

Choudhary, S., Lee, H. C., Maiti, M., He, Q., Cheng, P., Liu, Q., and Liu, Y. (2007). A double-stranded-RNA response program important for RNA interference efficiency. Mol Cell Biol *27*, 3995-4005.

Hafner, M., Landgraf, P., Ludwig, J., Rice, A., Ojo, T., Lin, C., Holoch, D., Lim, C., and Tuschl, T. (2008). Identification of microRNAs and other small regulatory RNAs using cDNA library sequencing. Methods *44*, 3-12