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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Genetic analysis of the role of RNaseH2 in preventing genome instability.

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Molecular Pathology

by

Stephanie Ruth Soltero

Committee in charge:

Professor Richard D. Kolodner, Chair
Professor James R. Feramisco, Co-Chair
Professor Xiang-Dong Fu
Professor Lawrence Goldstein
Professor Deborah Spector

2009

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The Dissertation of Stephanie Ruth Soltero is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2009

DEDICATION

To my beautiful daughter, Sophia. This is for you my baby girl. We made it this far and Oh how much farther we will go. I love you so very much!!

EPIGRAPH

Plunge boldly into the Beyond, then be free wherever you are.

- *Zen Master Shoitsu* (1202-1280)

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ACKNOWLEDGEMENTS

I am so grateful to have had the opportunity to work and grow with Richard Kolodner. He is an outstanding scientist and an amazing person. I was so very fortunate that he took me under his wing and guided me throughout my graduate school experience. His mentorship provided me with an excellent scientific education. Richard's generosity and flexibility helped me to achieve a balance between my studies and personal life. For this reason, my graduate school experience has been one of the happiest times in my life. Thank you so very much Richard!!

I would also like to thank my Thesis committee, James Feramisco, Larry Goldstein, Deborah Spector, and Xiang Dong Fu for their commitment and support. Each of them is very busy and I appreciate them for taking the time to be involved in my work.

I would like to thank James Feramisco for being easygoing, kind and supportive. His mellow spirit helped me relax during committee meetings and his kindness was very welcoming.

I would like to thank Larry Goldstein for encouraging me to think more critically about my work. I was inspired to ask more questions about my work after he told me at one of my first committee meetings, "That is the thing with you young whippersnappers these days, you simply read the

directions provided in a kit, but you don't think about why you are doing each of the steps in the protocol."

I would like to thank Deborah Spector for being a role model to me. The day I asked her to be on my committee she was moving to a new office and her daughter was helping her. I saw her as a scientist and a mother and felt inspired.

I would like to thank Xiang Dong Fu for asking the question, "What about transcription, you have only thought about replication." This question was a pivotal scientific lesson that taught me that I needed to educate myself about all of the possible interpretations of my data.

I am also so grateful for the amazing team of people that Richard has put together. The people of the Kolodner lab are some of the brightest, nicest people I have ever known. The opportunity to work with and learn from the Kolodner lab group also contributed to my overall contentment during my time as a graduate student.

In particular, I would like to thank Chris Putnam for the many talks we had about science including the talks about what my data might mean and his amazing knowledge about how to troubleshoot experiments. Chris is brilliant, patient and nice. I very much appreciated his guidance and support. Thank you, Chris!

I would also like to give special thanks to the people who helped make my research possible including Meng Er for providing me with the

rnh203 strain and Jill Harrington for providing primers and guidance to put together the *CAN1* mutation spectrum data. I would also like to thank Hans Hombauer for testing some of my strains by FACS analysis and providing me with some interesting results to pursue in the future.

In the first years when I was working on trying to purify the "currently impossible to purify" Rrm3 protein, (Thank you Virginia Zakian for validating me), both Kristina Schmidt and Dan Mazur guided me through this challenging time.

Other members of the Kolodner lab that I am also particularly grateful for their friendship and guidance include Ann Montgomery, Tikvah Hayes, Ellen Kats, Cecelia Harries, Jorrit Enserink, Scarlet Shell, Marc Mendillo, Mamata Engineer, and Hal Hoffman. Ann, thanks for all of your many contributions to the lab. Tiki, thanks for freeing up some of my time by taking care of Sophia to help me get through the final months of writing and putting together a thesis defense. Ellen, thanks for being a great friend and confidant. (I miss you at the coffee cart). Cecelia and Mamata, it was a true joy to have been pregnant and new mothers together. I am so glad we were able to share this exciting time in our lives together. Jorrit, thanks for being a great bay-mate and for your patience and help with protocols. Scarlet, thanks for being a great friend and guiding me with some of the science. Marc, thanks for being a friend. Hal, thanks for all the beach days!!

To my daughter Sophia, thank you for bringing so much love and joy into my life. You are so thoughtful and sweet. You are so very precious to me. I am a very fortunate mother to have such a lovely daughter. Thank you for always being what matters most to me! I love you so very much!!

To my family, thank you my Dad and my sisters, Christy and Melissa.

Dad, (Charlie Nardin) I appreciate your words of encouragement and expressions of how proud you are of me. It has meant so much to me. I also realize now that I am a single parent as you were at times during my developing years, that you were faced with some difficult challenges. I understand that it was not easy for you and I appreciate that you continued to provide me with a place to call home.

Christy Buboltz, what would I do without you? You have been one my best friends and support that I needed to accomplish my goals. I am so lucky we are close and I am glad we have each other. You have been there for me during all my work and personal challenges supporting me with words of encouragement. You have always believed in me and it has been during the times that I didn't believe in myself that you lift me up again and help me realize my potential. I love you so very much. Thank you for always being there for me!!

Melissa Jameson, what would I do without you? You also have been one of my best friends providing me with support and encouragement. You

have also helped me get through some really challenging times and encouraged me to believe in myself when I lost my way. Thank you for always being there for me. I love you so much!!

I would also like to thank two of my very dear friends, Jeannie Maglione and Trieva Scanlan. Both of you are my family here in San Diego. I feel so fortunate to have you as friends.

Jeannie, I would especially like to thank you for encouraging me to get myself back into school. I remember the days when we were swimming together prior to the time I went back to school. You encouraged me to just do it and see what happens. You told me to stop waiting around to try and get into a graduate program and just go for it. It is also because of you that I ended up in the Kolodner lab and I appreciate your suggestion. My life could have turned out so much differently if I didn't follow your advice!

Trieva, I want to thank you for your support and encouragement. You have helped me get through some challenging times. You have been a wonderful friend. Thanks for being there for me when I need it.

VITA

EDUCATION

2009	Ph.D., Molecular Pathology, University of California, San Diego
1997	B.S., Microbiology, San Diego State University

RESEARCH EXPERIENCE

2001-2009	Molecular Pathology graduate program, University of California, San Diego. Conducted studies on Okazaki fragment processing related functions in maintenance of genome stability in the laboratory of Dr. Richard Kolodner.
1999	Staff Research Associate II, University of California, San Diego. Investigated novel gene therapies and potential early detection tumor markers for pancreatic cancer in the laboratory of Dr. Michael Bouvet.
1997	Research Assistant, Trega Biosciences. High-throughput screening of combinatorial chemistry libraries to determine potential drug candidates
1997	Summer Internship, Chiron Technologies, Center for Gene Therapy. Investigated large scale production of recombinant retroviral vectors as gene therapy products.
1996	Summer Internship, Chiron Technologies, Center for Gene Therapy. Optimized manufacturing conditions of recombinant retroviral vectors as gene therapy products.

LEADERSHIP EXPERIENCE

2005-2006	UCSD Express to Success Yearlong Leadership Program. Participated in a one year leadership development program.

2006 Preuss School, UCSD, Mentorship. Served as a mentor for a high school senior from the Preuss School.

2005 Preuss School, UCSD, Mentorship. Served as a mentor for a high school senior from the Preuss School.

PUBLICATIONS

Soltero SR and Kolodner RD. Genetic analysis of the role of RNaseH2 in preventing genome instability. Genetics (in preparation)

Putnam C, **Soltero SR**, et.al. Bioinformatic genome-wide approach to generate an enriched list of genes that function in genome stability. (in preparation)

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POSTERS AND PRESENTATIONS

Soltero SR and Kolodner RD. 2008. Department of Cellular and Molecular Medicine seminar series. University of California, San Diego. **Talk**: Replication fork protein RNaseH2 is important for both duplication and protection of the genome.

Soltero SR and Kolodner RD. 2006. Molecular Pathology Graduate Program annual retreat, La Jolla, California. **Talk:** Replication fork protein RNaseH2 is important for both protection and duplication of the genome.

Nardin SR and Kolodner RD. 2005. Molecular Pathology Graduate Program annual retreat, La Jolla, California. **Poster:** Deletion Mutants of Okazaki Fragment Processing Genes and Implications for Roles of *rnaseH* in DNA Repair and Recombination

Nardin SR and Kolodner RD. 2004. Molecular Pathology Graduate Program annual retreat, La Jolla, California. **Poster:** Overexpression and purification of recombinant DNA helicase RRM3 protein in *Saccharomyces cerevisiae*

ABSTRACT OF THE DISSERTATION

Genetic analysis of the role of RNaseH2 in preventing genome instability

by

Stephanie Ruth Soltero

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2009

Professor Richard Kolodner, Chair

Genome instability can arise due to the accumulation of gross chromosomal rearrangements (GCRs). Specifically, translocations, deletions, and chromosome fusions are frequent events seen in

cancers with genome instability. There are multiple pathways that prevent GCRs, including S-phase cell cycle checkpoints, homologous recombination, telomere maintenance, suppression of de novo telomere addition, chromatin assembly, and mismatch repair. One view is that defects in DNA replication are one of the main causes of genome instability.

The work presented here analyzes the role of RNaseH2 in preventing genome instability. RNaseH2 is involved in resolution of RNA-DNA hybrid replication intermediates that arise during Okazaki fragment processing of lagging strand DNA replication. It has been suggested that persistence of RNA-DNA hybrids can lead to genome instability because they can become mutagenic and possibly form secondary structures.

It is known that there are pathways required to prevent the formation of DNA damage and there are also pathways required for dealing with the DNA damage once it becomes present, but that ultimately, both are required for prevention of genome instability.

RNaseH2 is thought to be involved in preventing the formation of DNA damage. The genetic analysis presented here on *rnaseh2* mutants examined what happens when there are defects in the RNaseH2 pathway thought to prevent formation of DNA damage

and in addition to that when there are also defects in the pathways that are thought to prevent the accumulation of DNA damage.

Additional work was done to survey a list of enriched genes that encode proteins with roles in genome instability to identify novel cellular functions important for maintenance of genome stability.

The results presented in this Dissertation highlight the importance of many diverse proteins that have different cellular roles important for maintaining genome stability.

CHAPTER 1

Overview of Genome instability.

1.1 GENOME INSTABILITY

Genome instability is essentially the framework of evolution. Charles Darwin stated, “It is not the strongest of the species that survive, nor the most intelligent, but the one most responsive to change.” Change is a constant challenge for all living organisms. The fundamental change essential to life is growth which starts when a single cell divides to become two cells. Cell division is essentially cell duplication of a mother cell dividing to create a duplicate daughter cell with the purpose of replicating a carbon copy of the originating genome. However, what actually happens is that the daughter cell does not always end up with an exact replica of the mother genome, but instead random changes in the genome can occur at low rates. Changes in the genome or genome instability can be helpful to survival or it can hinder survival when subject to natural selection. The work presented here focuses on genome instability.

Cell proliferation requires efficient and error-free DNA replication that depends on coordination and proper functioning of many pathways that are either linked to or act directly in DNA replication that help maintain stability of the genome. Some of the essential pathways that synchronize with DNA replication function in DNA-damage sensing, repair and cell-cycle progression to ensure with high probability stability to the genome

during cell division, thus preventing mutations and DNA rearrangements (Aguilera and Gomez-Gonzalez, 2008).

Incorporation of mutations and DNA rearrangements has been demonstrated to cause large numbers of different diseases (Bayes et al., 2003; Deininger and Batzer, 1999). In addition, ongoing genome instability, resulting in the continued accumulation of mutations, is associated with a considerable proportion of different types of cancer (Loeb, 1994).

1.2 MOLECULAR MECHANISMS SUSCEPTIBLE TO GENOME INSTABILITY

Genome instability can arise from different cellular mechanisms that are capable of producing a wide range of altered genomic substrates that vary from very minimal alterations such as single point mutations to much larger genome alterations such as chromosome rearrangements. In order to understand the cause of genome instability it is important to be familiar with the mechanisms by which genetic alterations can arise. Mechanisms that have been reported to contribute to genetic alterations are numerous including DNA replication, fragile sites, and transcription (Aguilera and Gomez-Gonzalez, 2008).

It is thought that replication impairment is the main cause of genome instability originating from DNA breaks and aberrant DNA structures that

are generated during the DNA replication process. DNA breaks and aberrant DNA structures can arise during DNA replication in a number of ways including when a replication fork encounters a single-stranded nick which could result in a double strand break if the fork passes the nick (Flores-Rozas and Kolodner, 2000).

During DNA replication the formation of aberrant DNA structures can sometimes arise from recombination processes such as formation of the DNA structure known as a Holliday junction that was first described in 1964 by Robin Holliday (Liu and West, 2004). Genome instability can also arise during DNA replication from a variety of different processes that leave behind single stranded gaps such as replication fork pausing on the leading strand that occurs when the replication fork encounters a lesion that is then followed by fork repriming downstream of the lesion (Lopes et al., 2006).

Another source of genome instability are fragile sites that are DNA sequences frequently associated with hotspots for translocations, gene amplifications, integration of exogenous DNA (Aguilera and Gomez-Gonzalez, 2008) and an increased occurrence of deletions and rearrangements (Glover and Stein, 1988). Loss of genome stability can also occur during transcription. It is known that transcription takes place on the same substrate as replication, repair and recombination (Aguilera, 2002).

One mechanism by which transcription could contribute to genome instability is through the formation of stable R-loops that then lead to DNA

breakage. Or, alternatively the formation of stable R-loops might arise during transcription coupled repair when transcription is arrested due to the presence of a DNA lesion that prompts the blocked RNA polymerase to sense the damage and load DNA repair machinery at the site of the lesion (Mellon et al., 1987).

Of the different potential mechanisms susceptible to genome instability, by far the problems associated with DNA replication have gained the most attention by researchers. Some of the problems that can arise in DNA replication that can lead to genome instability already discussed such as single stranded nicks leading to double stranded breaks, aberrant recombination structures, and single stranded gaps were mentioned to point out the outcomes of specific examples, but in fact there are many more features of DNA replication that additionally when these become problematic this also can lead to genome instability. Actually there are so many other examples that really it is impracticable to list all of them here.

In order to gain a broader view of the sources of genome instability that can arise during DNA replication a more general overview of the most common aspects of DNA replication that can be sources of genome instability are listed here and include mechanisms that involve inefficient firing of origins, a short supply of nucleotide precursors, defective DNA polymerases, defective enzymes important for DNA replication, presence of damaged DNA, defective checkpoint pathways, and even, although less

frequently when all systems are working optimally, DNA replication itself can be a source of mutations and genome rearrangements due to random errors that occur at low rates.

Indeed, it seems possible that most genome instability especially the type that leads to the development of many types of cancer can ultimately be attributed to DNA replication problems.

1.3 SUBSTRATES SUSCEPTIBLE TO GENOME INSTABILITY

The type of substrate generated during DNA replication that could possibly be a potential source of genome instability will ultimately depend on which stage of DNA replication the substrate is generated in. The types of substrates that are known to initiate replication fork arrest are strong candidates as substrates for genome stability. Some of the more common substrates that cause replication arrest include structural elements in the DNA template like fragile sites, lesions on DNA such as double strand breaks, and RNA-DNA hybrids. The field of study encompassing DNA replication and DNA repair has thus far made significant contributions to further our understanding of how the occurrence and sometimes the accumulation of some of the specific substrates mentioned can lead to genome stability.

Of all the substrates mentioned the least is known about the role of RNA-DNA hybrids in genome instability especially in the context of DNA replication. Largely, the focus of this work is on the role of RNA-DNA hybrids in genome instability.

1.4 RNA-DNA HYBRIDS AND GENOME INSTABILITY

Although less work has been done on the role of RNA-DNA hybrids in genome stability compared to most of the other substrates that were mentioned there still exists a small community of researchers focusing primarily on this topic. Interestingly, most of the work in this field have been studies investigating the role of RNA-DNA hybrids in genome instability only in the context of transcription and actually it has been suggested that the specific circumstance of RNA-DNA hybrid formation that occurs when the DNA replication and transcription machinery collide which could happen during a replication fork arrest is possibly one setting in which the RNA-DNA hybrid substrates that can give rise to genome instability are generated (Wellinger et al., 2006).

A recent review describing this phenomenon reports that transient single stranded DNA regions are formed during transcription as a consequence of DNA-strand opening, which is caused by the transient accumulation of localized negatively supercoiled DNA behind the advancing

RNA polymerase. The authors then go on to say that because the non-transcribed strand is single stranded it is more likely that it is with the double stranded transcribed strand that a RNA-DNA hybrid is formed. They suggest this could be happening when the nascent mRNA extruding from RNA polymerase might hybridize with the transcribed strand to create R-loops and that this phenomenon is facilitated by the local negative supercoiling accumulating behind RNA polymerase (Aguilera and Gomez-Gonzalez, 2008).

Acceptance of this interpretation leads one to believe that the formation of R loops that are linked to transcription and associated with genome instability would be evident in mutants that are defective in the biogenesis and processing of messenger ribonucleoprotein (mRNP) particles (Aguilera and Gomez-Gonzalez, 2008). Additionally mutants that are defective for functions involved in mRNP biogenesis steps including cleavage and polyadenylation factors known to be important for transcription elongation would also be strong candidates for playing roles in the formation of stable R-loops that can lead to genome instability.

Consistent with this hypothesis defects in proteins involved in mRNP biogenesis and related processes have been linked to maintenance of genome stability (Luna et al., 2005). One specific example involved deletion of the RNA splicing factor, ASF/SF2, which lead to the formation of stable R-loops (Li and Manley, 2005). Of particular interest related to the work in this

dissertation, the stability of the *asf/sf2*-induced R-loops was inhibited by over-expressing RNaseH (Li and Manley, 2005).

1.5 RNA-DNA HYBRIDS IN DNA REPLICATION

The current beliefs and the well characterized role of RNA-DNA hybrids in genome instability in the context of transcription do not negate the possible role of RNA-DNA hybrids in genome instability in the context of DNA replication. It is my belief that it is only due to the lack of research in this area that the role of RNA-DNA hybrids in genome instability in the context of DNA replication is not readily recognized. The formation of RNA-DNA hybrids during DNA replication is required for initiation of replication on both the leading and the lagging strand. Because the original strands of DNA are antiparallel, and only one continuous new strand can be synthesized at the 3' end of the leading strand due to the intrinsic 5'-3' polarity of DNA polymerases, the other strand must grow discontinuously in the opposite direction. The result of the discontinuous replication of the lagging strand is the production of a series of short sections of DNA called Okazaki fragments each of which consists of 8-12 nucleotides of RNA primer at its 5’ end known as the initiator RNA followed by approximately 100-150 nucleotides of DNA (Okazaki et al., 1967).

The proper processing of Okazaki fragments is necessary for cell survival. It has been reported that approximately 50,000,000 Okazaki fragments are synthesized when a human cell replicates (Stith et al., 2008). Each of the Okazaki fragments that are synthesized need to be efficiently and accurately matured into continuous lagging strands to ensure genome stability. It is thought that there are 3 different pathways that process Okazaki fragments (Figure 1-1).

One view is that the primary most efficient pathway depicted in Figure 1-1, panel A involves both RNaseH2 and Rad27. The idea is that RNaseH2 degrades all of the initiator RNA leaving behind one ribonucleotide while the structure is still an RNA-DNA hybrid, then the remaining single ribonucleotide along with 1-2 bases of DNA is displaced by the progressing DNA polymerase, creating a flap. Of importance later is that this DNA polymerase is made up of subunits, one of which is polymerase delta (*POL32).* The displaced flap is then cleaved by Rad27 (Bae et al., 2001; Chen et al., 2000; Kao and Bambara, 2003; Qiu et al., 1999).

The other pathway which some think might be less efficient involves only Rad27 is illustrated in Figure 1-1, panel B. It has been suggested that the progressing DNA polymerase displaces one ribonucleotide at a time that Rad27 cleaves, then the next ribonucleotide is displaced, followed by Rad27 cleavage, until all but one ribonucleotide of the initiator RNA remains, then

DNA polymerase displaces the single ribonucleotide along with 1-2 bases of DNA that are then cleaved by Rad27 (Kao and Bambara, 2003).

The third pathway depicted in Figure 1-1, panel C illustrates the unusual circumstance when the displaced flaps escape Rad27 cleavage and become very long and then are subsequently coated by the single stranded DNA binding protein, RPA. Binding of RPA inhibits cleavage by Rad27. So then, it is thought that resolution of this intermediate requires shortening of the flap by Dna2, such that Dna2 removes the 5' RNA and a short segment of DNA leaving behind a short flap that RPA can no longer bind and then this is subsequently cleaved by Rad27 (Kao and Bambara, 2003).

A novel role involving Mgs1 has been recently proposed that functions to prevent the formation of the long flaps that arise in the third pathway illustrated in Figure 1-1, panel C. It was found that Mgs1 physically binds with one subunit of the DNA polymerase, polymerase delta (*POL32)* and it was also shown that Mgs1 can stimulate Rad27 activity. It was suggested that Mgs1 in association with polymerase delta might increase recruitment of Rad27 causing Rad27 cleavage of flaps before they grow to a length that requires processing by Dna2 (Kim et al., 2005).

One view is that because there are approximately 50,000,000 Okazaki fragments synthesized each time a cell replicates that these structures might constitute the largest pool for potential DNA damage in the cell (Stith et al., 2008). In light of this view, one possibility exists that the

proteins important for resolution of these intermediates are important for suppression of genome instability and include RNaseH2, Rad27, Dna2, Pol32 and Mgs1.

1.6 PROTEINS THAT RESOLVE OKAZAKI FRAGMENT INTERMEDIATES ARE ALSO IMPORTANT FOR PREVENTING GENOME INSTABILITY.

Currently there is no evidence to describe the type of DNA damage that can accumulate due to the persistence of the intermediates that are formed during the Okazaki fragment processing step of DNA replication which includes resolution of both RNA-DNA hybrids and long flaps. However, it has been suggested that persistence of either RNA-DNA hybrids or long flaps can lead to the formation of secondary structures (Gordenin et al., 1997) that might possibly look like the structures illustrated in Figure 1-2. It has also been suggested that persistence of any of these types of structures might cause repeat expansion mutations, small duplication mutations and possibly may also lead to the generation of double-stranded DNA breaks that can give rise to genome instability (Stith et al., 2008).

The proteins important for processing of Okazaki fragments would be strong candidates for roles in either preventing the formation of or resolution

of the secondary structures that might form due to persistence of either RNA-DNA hybrids or long flaps that can lead to genome instability.

Some data has been collected on the roles of RNaseH2, Rad27, Dna2, Pol32 and Mgs1 in preventing genome instability. One of the common markers of genome instability is an increased rate of gross chromosomal rearrangements (GCRs). An increased rate of GCRs correlates to an accumulation of genome rearrangements that can include different types of rearrangements such as translocations, deletions, insertions and inversions. An accumulation of any of these types of rearrangements can lead to genome instability (Chen and Kolodner, 1999).

It was found that deletion of *RAD27* caused a 914 fold increase in the GCR rate compared to wild-type (Chen and Kolodner, 1999). A 20 fold increase was found in the GCR rate of a strain carrying the mutant allele, *dna2-2* of *DNA2* (Budd et al., 2006). A screen identifying weak mutator phenotypes found that *rnh203,* one of the subunits of the RNaseH2 complex displayed weak mutator characteristics (Huang et al., 2003). Deletion of *MGS1* caused a 4 fold increase in the rate of mitotic recombination (Hishida et al., 2001). Deletion of polymerase delta (*POL32*) lead to an 18 fold increase in the rate of accumulating GCRs (unpublished data).

All of this data suggests that some proteins that function in DNA replication that also process Okazaki fragments are important for suppression of genome instability. There is a fair amount of data on the roles of most of

these proteins in genome instability, except for RNaseH2. Given that not much was known about the role of RNaseH2 in genome stability I decided to pursue this topic.

1.7 RNASEH2

RnaseHs are enzymes that are involved in the degradation of the RNA in RNA/DNA hybrids. RNaseHs specifically hydrolyze RNA when annealed to a complementary DNA and are present in all living organisms (Crouch, 1998). There are two types of RNaseHs (type I and type II) that are evolutionarily conserved in mammals, yeast and bacteria (Qiu et al., 1999). RNaseHs are not essential in bacteria or yeast (Qiu et al., 1999).

Some evidence supports that RNaseHs could be essential in mammals. It was found that an *rnaseh1* null mutation leads to embryonic lethality due to defective mtDNA replication (Cerritelli et al., 2003). A study performed in *Saccharomyces cerevisiae* determined that cells without RNaseH have similar to wild-type growth phenotypes when both RNaseH1 and RNaseH2 have been eliminated due to deletion (Arudchandran et al., 2000). In the same study it was observed that transcription from *RNH201*, one of the subunits of the RNaseH2 complex, is increased in S- and late G2/M-phases, whereas transcription of *RNH1*, the gene encoding RNaseH1, is stable throughout the cell cycle (Arudchandran et al., 2000).

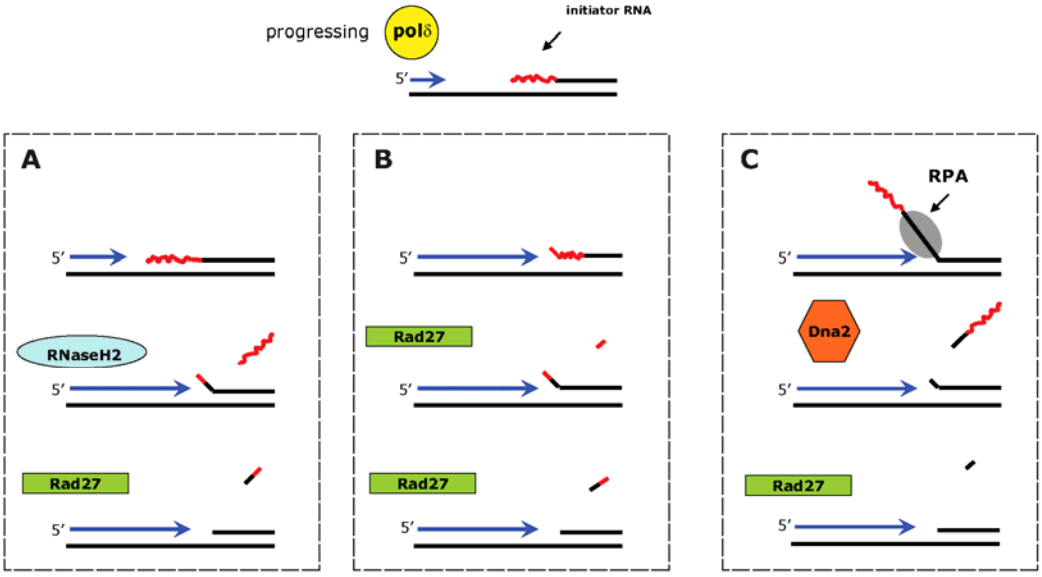

Figure 1-1: The 3 pathways of Okazaki fragment processing.
A. Primary, most efficient pathway involves RNaseH2 and Rad27
B. Alternative pathway involves only Rad27.
C. The unusual pathway when flaps escape Rad27 cleavage involves Dna2 and Rad27

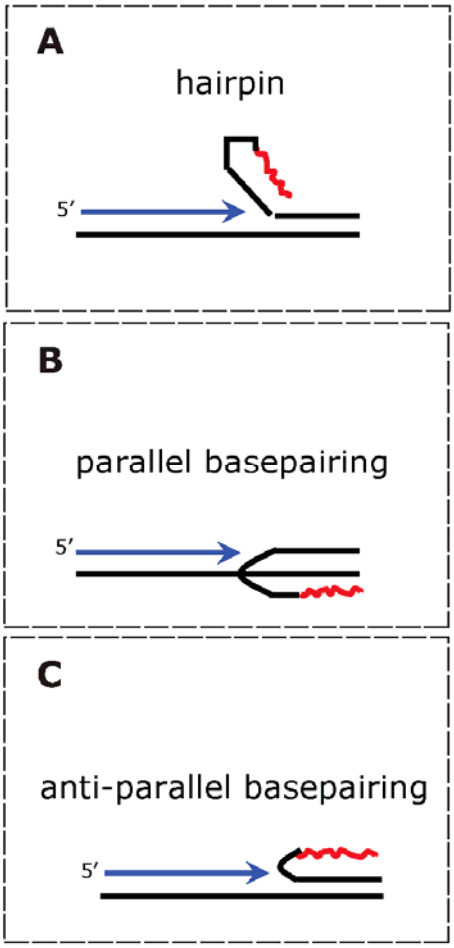

Figure 1-2: Secondary structures caused by foldbacks that might form due to persistence of RNA-DNA hybrids or long flaps.
A. A hairpin is essentially the 5’ end folding back on itself.
B. A parallel basepairing mechanism with the double strand source.
C. An anti-parallel basepairing mechanism with the newly synthesized nascent strand.

This data added more support to a suggestion made years earlier that the RNaseH2 of *Saccharomyces cerevisiae* might be more important than RNaseH1 for cells when evolutionary data for RNaseH2 and RNaseH1 generated by doing BLAST searches was compared for the two enzymes. In the three kingdoms, bacteria, eukarya, and archaea database searches detected RNaseH2, and RnaseH1 was also found in the genomes of bacteria and eukarya, but not archaea (Ohtani et al., 1999). In light of these data it has been suggested that RNaseH1 works as a housekeeping enzyme while RNaseH2 acts during DNA replication (Arudchandran et al., 2000).

Other important work in the lagging strand synthesis field was a characterization study of *Saccharomyces cerevisiae* RNaseH2 finding that it is a heterotrimeric protein complex encoded by *RNH201*, *RNH202*, and *RNH203* (Jeong et al., 2004).

1.8 RNASEH2 IN OKAZAKI FRAGMENT PROCESSING

It is thought that the primary role of RNaseH2 is to participate in what is thought to be the major pathway of Okazaki fragment processing, such that RNaseH2 endonucleolytically cleaves the initiator RNA of the RNA-DNA/DNA duplexes, namely Okazaki fragments, that are formed during DNA replication followed by removal of the last remaining single

ribonucleotide by Rad27/Fen1 (Bae et al., 2001; Chen et al., 2000; Qiu et al., 1999) as discussed above and illustrated in Figure 1-1, panel A.

A secondary role in Okazaki fragment processing has also been suggested for RNaseH2 that involves resolution of long flap intermediates. During lagging strand DNA replication, the long flap intermediates that are occasionally generated were already discussed and illustrated in Figure 1-1, panel C. The long flap intermediates are thought to be a 5' RNA primer followed by single stranded DNA that have been displaced by DNA polymerase and then are subsequently coated by RPA. As already mentioned the coating of RPA inhibits the ability of Rad27 to cleave this particular replication intermediate and additionally creates the requirement of Dna2 for proper removal of these intermediates. The role of Dna2 in this process has been suggested to explain why Dna2 is an essential protein.

It has been suggested that a possible function of RNaseH2 might come into play when the single stranded DNA polynucleotide is released from the Dna2 activity. Some think that this intermediate would still contain the 5' RNA primer and will specifically require not only a DNA exonuclease with 3'-5' directionality but also a ribonuclease activity for its complete removal. It has been suggested that RNaseH2 might fill that role, because it is known that RNaseH2 can excise single ribonucleotides embedded in double-stranded DNA, thus opening up the possibility that RNaseH2 might also excise RNA embedded in single-stranded DNA (Eder et al., 1993;

Rydberg and Game, 2002). Consistent with this hypothesis, others have suggested that RNaseH2 might also be involved in resolving structures containing RNA embedded in single stranded DNA that could be considered similar to the long flap intermediates acted upon by Dna2 that may arise by ligation of incompletely processed Okazaki fragments (Rumbaugh et al., 1997).

Alternatively, RNaseH2 might be involved in resolving the lagging strand intermediates that are generated when the long flap intermediates fold back on itself to create secondary structures (Kao and Bambara, 2003). Biochemical analyses showed that as foldbacks become larger, they become progressively more inhibitory to cleavage by Fen1 (Henricksen et al., 2000).

My hypothesis about the role of RNaseH2 in preventing genome instability is in line with these views. It is my belief that RNaseH2 is required for prevention and or removal of secondary structures that might form due to persistence of RNA-DNA hybrids and or the long flap intermediates that are generated during the Okazaki fragment processing step of DNA replication and that persistence of these structures will lead to genome instability.

1.9 RNASEH2 AND GENOME INSTABILITY

Much less is known about the role of RNaseH2 and genome stability. Based on what is known so far about Okazaki fragment defects and genome instability, we decided that characterization of RNaseH2 could very well provide valuable insights about pathways important for genome stability. Although there is little data about the role of RNaseH2 in genome instability there have been a few key studies in this area of research that have suggested RNaseH2 might be involved in suppressing genome instability.

Previous work found that a deletion mutation in *RNH203* caused a weak mutator phenotype (Huang et al., 2003; Qiu et al., 1999). Consistent with this were results from a study reporting that a deletion in *RNH201* caused weak sensitivity to EMS (Arudchandran et al., 2000). Based on these findings it was expected that deletions in any of the RNaseH2 genes would cause weak sensitivity to CPT, HU, and MMS, but actually what was found by the Brill group was no significant sensitivity to CPT, HU or MMS for any of the *rnh201, rnh202* or *rnh203* mutants (Ii and Brill, 2005). But, they did observe that all 3 *rnaseh2* mutants displayed a weak sensitivity to UV (Ii and Brill, 2005).

The same group tested different combinations of *rnh202* with *rad51, mus81* and *sgs1* and found that RNaseH2 and Rad51 act in parallel pathways to provide resistance to DNA damage induced by CPT, HU, UV or MMS,

because sensitivity to these particular DNA damaging agents was increased in these double mutants. RNaseH2 and Mus81 function together in the DNA repair pathways activated in the presence of damage induced by CPT, but not HU or MMS. RnaseH2 and Sgs1 are involved in processing DNA damage caused by HU, but not CPT, or MMS (Ii and Brill, 2005). Some of the connections between RNaseH2 and Rad51 that were made in the Brill study were consistent with those of other studies.

However, some of what was found in the Brill study contradicted previously published results. The Boeke group found growth defects by tetrad analysis that uncovered novel genetic interactions between each of the RNaseH2 subunits and Sgs1, a helicase known to be involved in DNA repair and genome stability. They also found that the synthetic growth defects arising from combining *sgs1* with *rnh201*, *rnh202*, or *rnh203* were partially reversed by *rad51* (Ooi et al., 2003).

In contrast, the Brill group determined doubling times for the mutant strains and found that the doubling time for *sgs1 rnh202* and *sgs1 rnh202 rad51* were the same. Interestingly, the Brill group also found that the *mus81 rnh202 rad51* triple mutant grew significantly slower than the *rnh202 mus81* double mutant. The combined work by these two groups of researchers has made it clear that the function of RNaseH2 is interrelated with Rad51, Mus81, and Sgs1 indicating that RNaseH2 plays a critical role in genome stability.

1.10 CLINICAL IMPLICATIONS OF RNASEH2 INVOLVEMENT IN GENOME INSTABILITY

To our benefit recent advances have been made in the understanding of the pathophysiology of Aicardi-Goutieres syndrome (AGS). In 1984, Jean Aicardi and Francoise Goutières described 8 children showing both severe brain atrophy and chronic cerebrospinal fluid lymphocytosis, with basal ganglia calcification in at least one member of each affected family, leading to rapid to death or a vegetative outcome (Stephenson, 2008).

A research study on the disorder detected a 60bp single stranded nucleotide arising during S phase of the cell cycle within the ER of cells from Trex1(-/-) mice. The same phenomenon is observed in cells from a human AGS patient (Yang et al., 2007). AGS is caused not only by mutations in the human TREX1 gene but also in any of the genes encoding the three subunits of RNaseH2 (Crow et al., 2006).

It was suggested by some that the nucleic acid species released during replication that was seen in the Trex1(-/-) mice and the cells from a human AGS patient might be from an Okazaki fragment, and thereby composed of both a 5' RNA primer and DNA. If this is true then RNaseH2 might be necessary to resolve the RNA component (Brooks et al., 2008). Defects in RNaseH2 might cause an accumulation of unresolved Okazaki

fragments. RNaseH2 defects have been reported to be part of the cause of AGS and could be one explanation of the high levels of the nucleic acid observed in the Trex1(-/-) mice and the AGS patients.

Another suggestion highlights the possibility of RNaseH2 playing a specific role in degradation of long flaps that are generated during the Okazaki fragment maturation step of DNA replication as illustrated in Figure 1-1, panel C. It was proposed that the long flaps released by Dna2 could reanneal and fold back on the single stranded DNA polynucleotide creating a secondary structure similar to a hairpin structure like the one illustrated in Figure 1-2, panel A. One view is that the foldback structure may have a specific requirement for RNaseH2 to degrade the 5' primer (Crow et al., 2006).

This view is consistent with my own belief that the role of RNaseH2 is important for prevention and or degradation of the long flaps that are occasionally formed during the Okazaki fragment maturation step of DNA replication and that persistence of the long flaps can lead to genome instability implicating RNaseH2 as a critical protein for suppression of genome instability.

Others have reported that persistence of long flap intermediates creates a cellular environment that is more susceptible to formation of secondary structures. Resolution of secondary structure is imperative because secondary structures can become mutagenic. Secondary structures

can become mutagenic if they are incorporated into the genome as damaged DNA which can lead to genome instability and even cell death.

If RNaseH2 is required to prevent and or degrade long flaps, then it is not surprising that loss of *RNASEH* in mammals has the devastating consequences of AGS in humans and embryonic lethality in mice. One explanation for this is that the long flaps formed in the Okazaki fragment maturation step of DNA replication are not resolved due to loss of RNaseH2. Persistence of these structures then leads to incorporation of DNA damage and increased genome instability causing a phenotype that is displayed as AGS in humans and embryonic lethality in mice.

Loss of RNaseH has shown less of an effect in yeast and bacteria. One explanation for this might be that there are multiple proteins in yeast and bacteria that can perform the essential function performed by RNaseH2. RNaseH1 is one such example in yeast. It is also known that there is redundancy among the Okazaki fragment processing proteins. For example, Exo1 can substitute for Rad27. It is also important to note that proteins with a primary role in Okazaki fragment processing have exhibited phenotypes that would suggest a secondary role in genome stability. It is my belief that the essential RNaseH2 activity can be compensated for by other proteins and I predict that the compensatory proteins also have important functions that suppress of genome instability.

1.11 GENOME INSTABILITY ASSAYS IN *SACCHAROMYCES CEREVISIAE.*

Genome instability leading to genome rearrangements refers to events that changes the genetic regions of DNA fragments. Increases in HR-mediated events such as unequal sister chromatid exchange (SCE) and ectopic HR between non-allelic repeated DNA fragments can result in gross chromosomal rearrangments (GCRs) such as translocations, duplications, inversions or deletions. All of these instability events that eventually lead to chromosomal rearrangements are likely the result of misrepair of DNA breaks (Lengauer et al., 1998).

Several assays have been developed over the years looking at different types of gross chromosomal rearrangements (GCRs). Other studies have utilized loss of the *URA3* marker to monitor chromosomal rearrangements that are mediated by repeat sequences, such as mating-type loci (Hiraoka et al., 2000; Umezu et al., 2002). In addition, the *CAN1* gene has been used to study mitotic recombination and chromosome loss in diploid cells (Klein, 2001). Yeast artificial chromosomes (YACs) also have been used to screen the yeast genome for possible novel regulators of gross chromosomal rearrangements (Huang and Koshland, 2003).

An assay developed several years ago in our lab has enabled us to simultaneously detect a broader spectrum of gross chromosomal

rearrangements. This assay utilizes the left arm of chromosome V containing the *CAN1* gene and a *URA3* gene inserted telomerically to *CAN1*, in a region that does not contain any other essential genes. This assay allows us to measure the rate of rearrangements on this section of chromosome V and, in combination with breakpoint mapping, allows us to detect the formation of the following types of GCRs: translocations and interstitial deletions, chromosome fusions, and terminal deletions associated with de novo telomere additions (Chen et al., 1998; Myung et al., 2001).

Using this assay, we have been able to begin to investigate the role of RNaseH2 in genome instability and to further expand our interest in the mechanisms involved in genome instability we have been able to compile a list of GCR regulators, many of which have human orthologs, with the hopes of getting insight into the mechanisms underlying genome instability.

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CHAPTER 2

Genetic analysis of the role of RNaseH2 in preventing genome instability.

2.1 INTRODUCTION

Previous work determined a role for *RNASEH2* in genome instability. Given that not much work has been done on the genetics of *RNASEH2*, my goal was to survey a carefully selected subset of DNA metabolism genes in a pathway directed manner for their potential genetic interaction with *RNASEH2* to enable me to define the basic genetics of *RNASEH2* and make predictions about how RNaseH2 is involved in genome instability. The DNA metabolism genes I surveyed are known to be the most important players in the DNA metabolism processes that define DNA repair, such as HR. Additionally some of the genes I surveyed are the most important players of DNA metabolism pathways that are intimately connected to DNA repair, such as the checkpoints.

I used synthetic lethality analysis to find the interrelated cellular processes that involve RNaseH2. This method identified 5 of the known *rnaseh2* interaction partners that were previously identified by classical genetic screens (Ii and Brill, 2005; Loeillet et al., 2005; Symington, 1998) and by high through put methods using microarray (Ooi et al., 2003; Tong et al., 2001). Additionally, I identified 18 novel genetic interactions for *rnaseh2*. The basic genetics of *RNASEH2* revealed that loss of Rnh203 requires homologous recombination, sister chromatid cohesion, chromatin

assembly/remodeling, DNA replication, intra-S checkpoint, and postreplication repair for normal growth. My results also suggested novel pathways important for suppression and generation of GCRs..

It was also determined that the synthetic lethality profile of *rnaseh2* was most similar to synthetic lethality profiles of strains carrying mutations that inactivate functions important for suppressing mutations. Taken together my results suggest that RNaseH2 plays a critical role in genome stability.

2.2 MATERIALS AND METHODS

Yeast Strains

All of the strains used in this study are *S. cerevisiae* strains that are derivatives

of S288C. Single mutant strains were made by deleting the gene of interest in RDKY3615 (*MATa ura3-52, trp1-63, his3-200, leu2-1, lys2-Bgl, hom3-10, ade2-1, ade8*, *hxt13*::*URA3*) strain by homologous recombination (HR) mediated integration of PCR fragments according to standard methods. Double and triple mutant strains were obtained by mating appropriate strains, then sporulating the resulting diploids followed by genotyping random spore clones. All of the double mutants were made by crossing the single mutants generated in RDKY3615 with either *RNH203* single mutant strain SRNY172

(*MATα ura3-52, trp1-63, his3-200, leu2-1, lys2-Bgl, hom3-10, ade2-1, ade8*, *hxt13*::*URA3, RNH203::KANMX4*) or SRNY751 (*MATα ura3-52, trp1-63, his3-200, leu2-1, lys2-Bgl, hom3-10, ade2-1, ade8*, *hxt13*::*URA3, RNH203::HIS3*). The triple mutant strain (*MATα ura3-52, trp1-63, his3-200, leu2-1, lys2-Bgl, hom3-10, ade2-1, ade8*, *hxt13*::*URA3, rad51::HIS3, rad59::TRP1, RNH203::KANMX4*) was made by crossing RDKY4427 (*MATa ura3-52, trp1-63, his3-200, leu2-1, lys2-Bgl, hom3-10, ade2-1, ade8*, *hxt13*::*URA3, rad51::HIS3, rad59::TRP1*) with SRNY172.

I found that based on the results obtained in the doubling time assay I may have been selecting for suppressors when testing the *rnh203 rad27* double mutant strains. In order to obtain accurate results for these double mutants it was necessary to obtain freshly derived haploid strains by sporulation of the appropriate diploid strain and then testing several candidate strains in doubling time experiments to determine which strains did not obtain suppressors. SRNY776, SRNY777, SRNY778, and SRNY780 (*MATα ura3-52, trp1-63, his3-200, leu2-1, lys2-Bgl, hom3-10, ade2-1, ade8*, *hxt13*::*URA3, RNH203::G418, rad27::HIS3)* did not obtain suppressors and were subsequently frozen and used successfully in other experiments. All strains were grown at 30°C. Strains and their complete genotypes are listed in Table 1. Media for propagating strains have been described previously (Chen et al., 1998).

RNH203 Mutation Spectra

The *rnh203* strain SRNY172 (*MATa ura3-52, trp1-63, his3-200, leu2-1, lys2-Bgl, hom3-10, ade2-1, ade8*, *hxt13*::*URA3, RNH203::G418*) was first streaked for single colonies on yeast extract-peptone-dextrose (YPD) plates, and then 96 individual colonies were patched onto YPD plates. The patches were replica plated onto previously described selective media without Arg/with Canavanine (Can) (Alani et al., 1994; Amin et al., 2001; Reenan and Kolodner, 1992). Can-resistant mutants were allowed to grow at 30°C for 2 days. Mutation spectra were analyzed by isolating chromosomal DNA from one Can^r mutant per patch, amplifying the *CAN1* gene by PCR and sequencing the PCR product to determine the inactivating mutation in the *CAN1* gene as has been previously described (2, 83, 84)(Das Gupta and Kolodner, 2000; Flores-Rozas and Kolodner, 1998; Marsischky et al., 1996). The PCR primer pair used for amplification of *CAN1* was CAN1FX (5'-GTTGGATCCAGTTTTTAATCTGTCGTC-3') and CAN1RX (5'-TTCGGTGTATGACTTATGAGGGTG-3'). The three primers used for sequencing *CAN1* were CAN1G (5'-CAGTGGAACTTTGTACGTCC-3'), CANSEQ3 (5'-TTCTGTCACGCAGTCCTTGG-3'), and CANSEQ5 (5'-AACTAGTTGGTATCACTGCT-3').

All DNA sequencing was performed by using an Applied Biosystems 3730XL DNA sequencer and standard chemistry. Sequence analysis was performed using Sequencher 4.2.2 (Gene Codes, Ann Arbor, MI). The rate

of each type of mutation was obtained by multiplying the total Can^r mutation rate of the *rnh203* strain by the proportion of each type of mutation found. The total Can^r rate is the rate of accumulation of mutations in cell populations as determined by fluctuation analysis by using the method of the median (Lea, 1948) as described (Marsischky et al., 1996).

Spot tests for growth analysis

A simple qualitative approach was initially used to find genes that might be genetically interacting with RNaseH2. A simple visualization of the growth of each of the double mutant strains was compared to the single mutant and wild type control strains. Strains for experiments were streaked out onto YPD plates from frozen stocks and grown at 30°C for 2-3 days. Single colonies were used to inoculate cultures that were grown overnight at 30°C in YPD, then diluted to make a cell suspension of 1.0×10^6 cells/mL, or 1.0×10^5 cells/mL and then 5 tenfold serial dilutions were made.

2 uL of each dilution was spotted in a single row of 6 spots on a YPD plate. One strain of the wild-type, *rnh203* and query single mutant strain was spotted. For the double mutant either two or three independently collected strains were spotted depending on the number of strains obtained. Plates were grown at 30°C for approximately 30 hours.

Measurement of doubling time

All strains (except *rnh203 rad27*) were streaked out onto YPD plates from frozen stocks and grown at 30°C for 2-3 days. Single colonies were used to inoculate cultures that were grown overnight at 30°C in YPD. Cultures were diluted 1:100 to obtain an accurate OD_{600} reading using the visible light setting on the spectrophotometer. 10 mLs of experimental culture was set-up at an $OD_{600} = 0.05$ by diluting the appropriate volume of the overnight culture in YPD. This was time-point zero. Cultures were grown with shaking at 30°C. After 2 hours, OD_{600} measurements were taken every 30-45 minutes until the cells reached saturation.

For a single experiment, the same batch of YPD was used for all of the strains tested on that day. Both wild-type and *rnh203* single mutant strains were included in each day's experiment. At each timepoint, 1 mL of cells was removed from the 10 mL culture and OD_{600} was measured.

Doubling times were calculated using only the data points obtained when cells were in exponential growth phase. Doubling time was determined by generating a scatter plot of OD_{600} vs. Time in excel, assigning an exponential trendline to generate an equation that fit the line $y = (A_1)e^{(A2)}$. Solve for X_1 and X_2, $X_1 = LN(0.7/A_1)/A_2$, and $X_2 = LN(1.4/A_1)/A_2$. X_2-X_1 is the doubling time that was calculated for each strain.

The doubling times reported are the average doubling times of two to four cultures that were obtained by scaling up from starter cultures that were

inoculated with independent single colonies using random spore analysis. Error bars shown are standard deviations.

Gross Chromosomal Rearrangement (GCR) Assay

The GCR strain, RDKY3615, was used as the background strain for all of the strains in this study to allow determination of the rate at which GCRs were occurring. In the RDKY3615 strain, *HXT13* (<7.5-kb telomeric to *CAN1*) was replaced with a *URA3* cassette allowing for detection of translocations, and other classes of genome rearrangements by simultaneously selecting for the loss of *CAN1* and *URA3*. Cells resistant to CAN and 5FOA have undergone a gross chromosomal rearrangement leading to a breakpoint in the region between *CAN1* and *PCM1* as well as potentially all of the DNA from *CAN1* to the telomeres (Chen and Kolodner, 1999).

The rate of accumulation of GCRs in cell populations was determined by fluctuation analysis by using the method of the median (Lea, 1948) as described (Marsischky et al., 1996). Five to seven independent cultures of either two or three independently collected strains were analyzed in each experiment. If two independent strains were obtained, then 14 independent cultures were analyzed. If three or more independent strains were obtained, then 15 independent cultures were analyzed.

Table 2-1:*Saccharomyces cerevisiae* strains used in this study.

Strains	Relevant genotype	Source
RDKY 5027	Wild-type	Chen and Kolodner, 2001
SRNY 286, SRNY 287, SRNY 594, SRNY 595	*asf1::HIS3*	This study
SRNY 289, SRNY 290, SRNY 596, SRNY 597	*asf1::HIS3 rnh203::KAN*	This study
SRNY 295, SRNY 296	*cac1::TRP1*	This study
SRNY 297, SRNY 298, SRNY 299	*cac1::TRP1 rnh203::KAN*	This study
SRNY 633	*cac2::TRP1*	This study
SRNY 628, SRNY 632	*cac2::TRP1 rnh203::KAN*	This study
SRNY 242, SRNY 243, SRNY 244	*chk1::HIS3*	This study
SRNY 245, SRNY 246, SRNY 247	*chk1::HIS3 rnh203::KAN*	This study
SRNY 710, SRNY 711, SRNY 712	*ctf18::TRP1*	This study
SRNY 706, SRNY 707, SRNY 708	*ctf18::TRP1 rnh203::KAN*	This study
SRNY 932, SRNY 937	*ctf4::KAN*	This study
SRNY 930, SRNY 931, SRNY 933, SRNY 934	*ctf4::KAN rnh203::HIS3*	This study
SRNY 217, SRNY 714, SRNY 718	*dun1::HIS3*	This study
SRNY 192, SRNY 371, SRNY 715, SRNY 716	*dun1::HIS3 rnh203::KAN*	This study
SRNY 439, SRNY 440	*esc1::HIS3*	This study
SRNY 436, SRNY 437, SRNY 438	*esc1::HIS3 rnh203::KAN*	This study
SRNY 482, SRNY 483, SRNY 577, SRNY 578	*esc2::HIS3*	This study
SRNY 479, SRNY 480, SRNY 600, SRNY 601	*esc2::HIS3 rnh203::KAN*	This study
SRNY 447, SRNY 448, SRNY 449	*esc4::HIS3*	This study
SRNY 441, SRNY 442, SRNY 443, SRNY 664	*esc4::HIS3 rnh203::KAN*	This study
SRNY 1029, SRNY 1030, SRNY 1031, SRNY 1032	*exo1::TRP1*	This study
SRNY 939, SRNY 994	*exo1::TRP1 rnh203::KAN*	This study
SRNY 645, SRNY 660, SRNY 731, SRNY 732	*hir1::HIS3*	This study
SRNY 641, SRNY 642, SRNY 646	*hir1::HIS3 rnh203::KAN*	This study
SRNY 691, SRNY 694, SRNY 695	*hir2::HIS3*	This study
SRNY 693, SRNY 697, SRNY 699	*hir2::HIS3 rnh203::KAN*	This study
SRNY 704, SRNY 705	*hst2::HIS3*	This study
SRNY 700, SRNY 702, SRNY 703	*hst2::HIS3 rnh203::KAN*	This study
SRNY 302, SRNY 303	*lig4::HIS3*	This study
SRNY 304, SRNY 305, SRNY 306	*lig4::HIS3 rnh203::KAN*	This study
SRNY 1182, SRNY 1183	*mec1::KAN sml1::TRP1*	This study
SRNY 787, SRNY 790	*mec1::KAN sml1::TRP1 rnh203::HIS3*	This study
SRNY 969, SRNY 970, SRNY 975, SRNY 977	*mec3::HIS3*	This study
SRNY 968, SRNY 971, SRNY 972, SRNY 973	*mec3::HIS3 rnh203::KAN*	This study
SRNY 963, SRNY 999, SRNY 1000	*mgs1::KAN*	This study
SRNY 990, SRNY 991, SRNY 998, SRNY 1001	*mgs1::KAN rnh203::HIS3*	This study
SRNY 985, SRNY 987	*mms4::TRP1*	This study
SRNY 986, SRNY 989*	*mms4::TRP1 rnh203::KAN*	This study
SRNY 1033, SRNY 1034, SRNY 1035, SRNY 1036	*mph1::KAN*	This study
SRNY 1002, SRNY 1003	*mph1::KAN rnh203::HIS3*	This study
SRNY 639	*mrc1::TRP1*	This study
SRNY 637, SRNY 638, SRNY 640	*mrc1::TRP1 rnh203::KAN*	This study
SRNY 337, SRNY 588, SRNY 589	*mre11::HIS3*	This study
SRNY 378, SRNY 339, SRNY 590, SRNY 591	*mre11::HIS3 rnh203::KAN*	This study
SRNY 456, SRNY 457, SRNY 580, SRNY 581	*mus81::TRP1*	This study
SRNY 453, SRNY 454, SRNY 579, SRNY 582	*mus81::TRP1 rnh203::KAN*	This study
SRNY 542, SRNY 543, SRNY 544	*pif1m-2*	This study
SRNY 539, SRNY 540, SRNY 541	*pif1m-2 rnh203::KAN*	This study
SRNY 827, SRNY 830, SRNY 831	*pol30-119::LEU2*	This study
SRNY 826, SRNY 832, SRNY 833, SRNY 834	*pol30-119::LEU2 rnh203::KAN*	This study
SRNY 625, SRNY 626, SRNY 627, SRNY 686	*pol32::TRP1*	This study
SRNY 656, SRNY 657, SRNY 658	*pol32::TRP1 rnh203::KAN*	This study
SRNY 258, SRNY 259, SRNY 260	*rad17::HIS3*	This study
SRNY 261, SRNY 262, SRNY 263	*rad17::HIS3 rnh203::KAN*	This study

All strains are isogenic to RDKY5027 except those with an *, which are isogenic to RDKY3615. Both wild-type strains have the following genotype [ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2Δbgl, hom3-10, ade2Δ1, ade8, hxt13::*URA3*] in addition to the mutations indicated. The two wild-type strains have different mating types: RDKY5027 =α and RDKY3615 = A.

Table 2-2:*Saccharomyces cerevisiae* strains used in this study.

Strains	Relevant genotype	Source
RDKY 5027	Wild-type	Chen and Kolodner, 2001
SRNY 836, SRNY 845, SRNY 846, SRNY 873	*rad18::KAN*	This study
SRNY 785, SRNY 870	*rad18::KAN rnh203::HIS3*	This study
SRNY 230, SRNY 231, SRNY 232	*rad24::HIS3*	This study
SRNY 233, SRNY 234, SRNY 235	*rad24::HIS3 rnh203::KAN*	This study
SRNY 489, SRNY 490, SRNY 491, SRNY 602	*rad27::HIS3*	This study
SRNY 770, SRNY 776, SRNY 777, SRNY 778,	*rad27::HIS3 rnh203::KAN*	This study
SRNY 838, SRNY 875, SRNY 877	*rad5::KAN*	This study
SRNY 839, SRNY 840, SRNY 841, SRNY 878	*rad5::KAN rnh203::HIS3*	This study
SRNY 525, SRNY 526, SRNY 527, SRNY 621	*rad50::HIS3*	This study
SRNY 614, SRNY 615, SRNY 616, SRNY 618	*rad50::HIS3 rnh203::KAN*	This study
SRNY 277, SRNY 807, SRNY 808	*rad51::HIS3*	This study
SRNY 360, SRNY 361, SRNY 362	*rad51::HIS3 rnh203::KAN*	This study
SRNY 199, SRNY 338, SRNY 375, SRNY 587	*rad52::HIS3*	This study
SRNY 182, SRNY 200, SRNY 374, SRNY 592	*rad52::HIS3 rnh203::KAN*	This study
SRNY 885, SRNY 886, SRNY 887, SRNY 888	*rad53::HIS3 sml1::TRP1*	This study
SRNY 851, SRNY 889	*rad53::HIS3 sml1::TRP1 rnh203::KAN*	This study
SRNY 219, SRNY 220, SRNY 221, SRNY 739	*rad55::TRP1*	This study
SRNY 222, SRNY 223, SRNY 224, SRNY 741	*rad55::TRP1 rnh203::KAN*	This study
SRNY 941	*rad57::HIS3*	This study
SRNY 939, SRNY 942, SRNY 943, SRNY 944	*rad57::HIS3 rnh203::KAN*	This study
SRNY 278, SRNY 279, SRNY 280, SRNY 735	*rad59::TRP1*	This study
SRNY 363, SRNY 364, SRNY 688, SRNY 689	*rad59::TRP1 rnh203::KAN*	This study
SRNY 536, SRNY 537, SRNY 538, SRNY 749	*rad6::HIS3*	This study
SRNY 745, SRNY 746, SRNY 747	*rad6::HIS3 rnh203::KAN*	This study
SRNY 251, SRNY 252, SRNY 253	*rad9::HIS3*	This study
SRNY 254, SRNY 725, SRNY 728, SRNY 730	*rad9::HIS3 rnh20::KAN*	This study
SRNY 228, SRNY 229, SRNY 377	*rdh54::HIS3*	This study
SRNY 225, SRNY 226, SRNY 227	*rdh54::HIS3 rnh203::KAN*	This study
SRNY 550, SRNY 551, SRNY 552	*rfa1t-33*	This study
SRNY 545, SRNY 546, SRNY 547, SRNY 666	*rfa1t-33 rnh203::KAN*	This study
SRNY 310, SRNY 311, SRNY 312	*rfc5-1::TRP1*	This study
SRNY 313, SRNY 314	*rfc5-1::TRP1 rnh203::KAN*	This study
SRNY 751	*rnh203::HIS3*	This study
SRNY 172	*rnh203::KAN*	This study
SRNY 465, SRNY 466, SRNY 467	*rrm3::TRP1*	This study
SRNY 462, SRNY 463, SRNY 464	*rrm3::TRP1 rnh203::KAN*	This study
SRNY 218, SRNY 267, SRNY 358, SRNY 661	*sgs1::HIS3*	This study
SRNY 268, SRNY 269, SRNY 270, SRNY 598	*sgs1::HIS3 rnh203::KAN*	This study
SRNY 648, SRNY 650, SRNY 652	*siz1::TRP1*	This study
SRNY 649, SRNY 651, SRNY 655	*siz1::TRP1 rnh203::KAN*	This study
SRNY 842, SRNY 843, SRNY 844, SRNY 865	*slx1::KAN*	This study
SRNY 1038, SRNY 1039	*slx1::KAN rnh203::HIS3*	This study
SRNY 431, SRNY 432	*slx4::HIS3*	This study
SRNY 429, SRNY 430	*slx4::HIS3 rnh203::KAN*	This study
SRNY 682, SRNY 683, SRNY 684, SRNY 685	*srs2::HIS3*	This study
SRNY 423, SRNY 424, SRNY 425, SRNY 668	*srs2::HIS3 rnh203::KAN*	This study
SRNY 180, SRNY 359	*tel1::HIS3*	This study
SRNY 198, SRNY 274, SRNY 275	*tel1::HIS3 rnh203::KAN*	This study
SRNY 520, SRNY 521, SRNY 522	*tof1::HIS3*	This study
SRNY 515, SRNY 516, SRNY 533	*tof1::HIS3 rnh203::KAN*	This study
SRNY 809, SRNY 812, SRNY 814, SRNY 882	*top3::KAN*	This study
SRNY 815, SRNY 883, SRNY 884	*top3::KAN rnh203::HIS3*	This study
SRNY 529, SRNY 530, SRNY 531, SRNY 608	*xrs2::HIS3*	This study
SRNY 528, SRNY 605, SRNY 606, SRNY 610	*xrs2::HIS3 rnh203::KAN*	This study
SRNY 816, SRNY 818, SRNY 819, SRNY 823	*yku70::TRP1*	This study
SRNY 820, SRNY 821, SRNY 822	*yku70::TRP1 rnh203::KAN*	This study

All strains are isogenic to RDKY5027 except those with an *, which are isogenic to RDKY3615. Both wild-type strains have the following genotype [ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2Δbgl, hom3-10, ade2Δ1, ade8, hxt13::*URA3*] in addition to the mutations indicated. The two wild-type strains have different mating types: RDKY5027 = α and RDKY3615 = A.

2.3 RESULTS

Mutation spectrum analysis of RNaseH2 deletion strain.

The previously reported *rnaseh2* weak mutator phenotype was characterized by obtaining the overall rate at which Can^r mutations accumulate in the *rnh203* strain by fluctuation analysis. The spectrum of mutations was determined by sequencing individual mutants and then subsequently calculating the rate of accumulation of each class of mutation (Table 2-3).

The results indicate that the *rnh203* strain does indeed exhibit a weak mutator phenotype. The overall Can^r mutation rate of the *rnh203* strain (5.6×10^{-7}) was 2 fold higher than the wild-type rate of (2.8×10^{-7}) and is consistent with what has been previously reported (Huang et al., 2003). It was reported that the Can^r rate for an *rnh201* strain (1.9×10^{-6}) was 6.1 fold higher than their wild-type (3.1×10^{-7}) (Qiu et al., 1999) which is higher than what I found for an *rnh203* strain. It could be the effect of defects on the different sub-units of RNaseH2 might cause them to behave slightly differently in the Can^r test, or the difference could be due to strain differences or biological variation.

To further characterize the weak mutator phenotype of *rnaseh2* mutants, I identified the classes of Can^r mutations that arose in the *rnh203*

strain by isolating chromosomal DNA from independent Can^r mutants and then sequenced the *CAN1* gene to generate a mutation spectrum.

As expected, the mutation spectrum data was consistent with a weak mutator phenotype. The data revealed that the type of mutations that arose in the *rnh203* strain were small differences similar to what was found in the wild-type strain. I did find a 2.5 fold increase in the accumulation of AT to CG base pair changes compared to the wild-type strain.

Due to the involvement of RNaseH2 in Okazaki fragment processing I predicted that the most common type of mutation in the *rnh203* strain might have been duplications similar to what was found in a *rad27* strain. It was suggested that the homology-mediated duplications of 5-108bp flanked by direct repeats of 3-12bp arising in the *rad27* mutants could be a result of a novel mutagenic process that takes place due to long range slippage errors that occur as a result of aberrant Okazaki fragment processing (Tishkoff et al., 1997).

Although I did not observe homology-mediated duplications in the *rnh203* strain, it is interesting to note that I did observe a 27bp duplication without direct repeats in the flanking sequence in the spectrum of one *rnh203* mutant. The most significant observation I made from the mutation spectrum analysis is the 4 fold increase in double base substitutions in the *rnh203* strain compared to the wild type strain. The double base changes I observed in four of the *rnh203* mutants were double base deletions in short

dinucleotide repeats consisting of either 4 or 6bp. These deletion mutations more closely resembled the mutations found in an *msh2*-like mutant. It was reported that 85% of the Can^r mutations arising in the *msh2* strain were single base deletions in short mononucleotide repeats (Marsischky et al., 1996).

In consideration of this type of deletion mutation in the context of an *rnh203* strain, then the argument that replication errors as a result of aberrant Okazaki fragment processing might lead us to believe that DNA polymerase slippage could be a viable explanation. It was determined that the occurrence of single base deletions in short mononucleotides repeats in *msh2* mutants was due to DNA polymerase slippage on templates with simple repeats. It is likely that this is also happening some of the time in the *rnh203* mutants. If the duplication mutations that arise in *rnh203* and *rad27* mutants are a result of aberrant Okazaki fragment processing that requires pathways involved in suppression of genome instability then it would make sense that duplication mutations would be seen more often in a *rad27* mutant than an *rnh203* mutant because the *rad27* deletion causes a stronger mutator phenotype than *rnh203*.

It was suggested that the duplication mutations observed in the *rad27* mutant might indicate that the replication errors or damage arising from aberrant Okazaki fragment processing could be repaired by recombination. This would have predicted that *rad27* and now also *rnh203* might have

increased rates of recombination and would either be lethal or exhibit growth defects in combination with recombination mutants. This has indeed proved true for *rad27* (Symington, 1998; Tishkoff et al., 1997) and more recently also for *rnh203* (Ii and Brill, 2005; Ooi et al., 2003). These results suggest that *rnh203* is involved in multiple pathways of mutation avoidance and is important in suppression of genome instability.

Qualitative analysis of growth reveals that RNaseH2 genetically interacts with several important genes involved in different DNA metabolism pathways

This work and previously published results on the genetics of *RNASEH2* indicates that RNaseH2 function is related in some way to genome instability and that actually it could be involved in multiple pathways of DNA repair. In order to make sense of the many complex biological processes that encompass genome instability it has helped researchers to subdivide these processes into specific pathways.

One of the aims of this study was to survey a carefully selected subset of DNA metabolism genes in a pathway directed manner for their potential genetic interaction with *RNASEH2* in order to define the basic genetics of *RNASEH2* and make predictions about how RNaseH2 is involved in genome instability. The DNA metabolism genes surveyed are known to be the most important players in the DNA metabolism processes that define

Table 2-3:Mutation spectrum analysis of RNaseH2 deficient strains.

Genotype	Mutation	Frequency (%)	Rate	Fold change relative to w.t.
Wild-type	Single BS	60/94 (63.8)	3.5×10^{-7}	
	GC to TA	19/94 (20.2)	1.1×10^{-7}	
	GC to AT	15/94 (16.0)	8.8×10^{-8}	
	GC to CG	15/94 (16.0)	8.8×10^{-8}	
	AT to TA	7/94 (7.4)	4.1×10^{-8}	
	AT to CG	2/94 (2.1)	1.2×10^{-8}	
	AT to GC	2/94 (2.1)	1.2×10^{-8}	
	Double BD	1/94 (1.1)	6.1×10^{-9}	
	-1 Frameshift	15/94 (16.0)	8.8×10^{-8}	
	+1 Frameshift	2/94 (2.1)	1.2×10^{-8}	
	Large Deletion	10/94 (10.6)	5.8×10^{-8}	
	Complex	6/94 (6.4)	3.5×10^{-8}	
rnh203 Δ	Single BS	47/95 (49.5)	2.7×10^{-7}	0.8
	GC to TA	12/95 (12.6)	7.1×10^{-8}	0.6
	GC to AT	6/95 (6.3)	3.5×10^{-8}	0.4
	GC to CG	19/95 (20.0)	1.1×10^{-7}	1.3
	AT to TA	6/95 (6.3)	3.5×10^{-8}	0.9
	AT to CG	6/95 (6.3)	3.5×10^{-8}	2.9
	AT to GC	3/95 (3.2)	1.8×10^{-8}	1.5
	Double BD	4/95 (4.2)	2.4×10^{-8}	3.9
	-1 Frameshift	19/95 (20.0)	1.1×10^{-7}	1.3
	+1 Frameshift	3/95 (3.2)	1.8×10^{-8}	1.5
	Large Deletion	9/95 (9.5)	5.3×10^{-8}	0.9
	Complex	10/95 (10.5)	5.9×10^{-8}	1.7

BS = Base Substitution including single insertions or deletions. BD = Base deletion

*The rate of each type of mutation was obtained by multiplying the total Canr mutation rate by the proportion of each mutation.

DNA repair, such as homologous recombination. Additionally some of the genes surveyed are the most important players of DNA metabolism pathways that are intimately connected to DNA repair, such as the checkpoints.

A simple test of spotting serial dilutions of cell cultures on YPD plates was done to compare the growth of *rnh203* double mutant strains to wild-type and respective single mutant controls. When the *rnh203* double mutants grew slower than both of the single mutants indicated that *RNH203* was genetically interacting with the mutant that was combined with the *RNH203* mutation. It was determined that within the subset of genes tested, the HR group had the largest number of genes that were genetically interacting with *rnaseh2* (Table 2-4).

The strong genetic interaction between *rnh203* and *sgs1* already reported in the literature (Ooi et al., 2003; Pan et al., 2006) was reproduced in my tests (Figure 2-1, Table 2-4). The discovery of *rnh203* strongly interacting with *mre11* (Figure 2-1, Table 2-4) was consistent with the genetic interaction previously reported for *rnh202* and *mre11* (Collins et al., 2007). My results were also consistent with the previous observations of *rnh202* genetically interacting weakly with *mus81* and *rad51* (Ii and Brill, 2005) I also found that *rnh203* genetically interacts weakly with *mus81* (Figure 2-1, Table 2-4) and *rad51* (Figure 2-5, Table 2-4).

It is important to note that in the growth spot test the *rad51 rnh203* double mutant appeared to have the same growth phenotype as the

rnh203 and *rad51* single mutants and the wild-type, indicating no genetic interaction (Figure 2-1). However, when the doubling time was measured for this double mutant, it was found that the *rad51 rnh203* double mutant did actually grow slightly slower than either *rad51* or *rnh203* single mutant (Figure 2-5).

This was not surprising, because it was expected that some of the weak phenotypes might not be revealed in the growth spot analysis, because of the qualitative nature of the assay. The growth defect caused by combining an *RNH203* mutation with a mutation that strongly genetically interacts with *rnh203* was much more evident in this assay.

An additional 7 novel strong genetic interactors of *rnaseh2* were discovered with the following HR genes *MMS4, MPH1, RAD50, RAD52, SLX1, TOP3,* and *XRS2* (Figure 2-1, Table 2-4) that had not been previously reported. For the HR group of genes it was found that when the *rnh203* deletion was combined with *EXO1, LIG4, RAD55, RAD57, RAD59, RDH54, SLX4, SRS2,* and *YKU70* deletion mutations that the double mutants grew the same as both single mutants and the wild-type indicating that *RNH203* does not genetically interact with these recombination genes (Figure 2-1, Table 2-4).

From the sister chromatid cohesion group, it was determined that *rnh203* interacts weakly with *ctf4* consistent with the discovery by (Pan et al., 2006) that *rnh202* and *rnh201* interact with *ctf4*. The *rnh203 ctf4* double

mutant appeared to have the same growth phenotype as the *rnh203* and *ctf4* single mutants and the wild-type, indicating no genetic interaction (Figure 2-2, Table 2-4). However, when the doubling time was measured for this double mutant, it was found that the *rnh203 ctf4* double mutant did actually grow slightly slower than either *ctf4* or *rnh203* single mutant (Figure 2-6, Table 2-4).

It is interesting to note that *rnh203 ctf18* had a normal growth phenotype indicating no genetic interaction. The work done by the Pan group (Pan et al., 2006) determined that *ctf18* interacts with *rnh201*, but not *rnh202*. The collective data so far on *RNASEH2* argues that *CTF18* differentially interacts with the subunits of the RNAseH2 complex.

This study found 3 strong genetic interactions with the genes surveyed in the chromatin remodeling group. Two of which were novel discoveries that include *rnh203* genetic interactions with *asf1* and *esc4* (Figure 2-2, Table 2-4). A genetic interaction was also found between *rnh203* and *esc2* (Figure 2-2, Table 2-4) which is consistent with what was previously determined by (Tong et al., 2001) and the results found for the *rnh202* and *rnh201* mutations (Pan et al., 2006). In contrast, *rnh203* did not interact with the other chromatin remodeling genes studied including *cac1, cac2, hir1, hir2, esc1 or hst2* (Figure 2-2, Table 2-4).

In the replication group of genes tested 2 novel interactions were found including the *rnh203* strong genetic interaction with *mgs1* and *rfa1*

(Figure 2-3, Table 2-4). The result that reveals a strong genetic interaction between *rnh203* and *rad27* (Figure 2-3, Table 2-4) is consistent with previous reports (Loeillet et al., 2005; Pan et al., 2006; Qiu et al., 1999). No genetic interactions were observed with *pif1m-2*, *pol32*, and *rrm3* (Figure 2-3, Table 2-4).

Three novel genetic interactions were found with a subset of genes involved in post-replication repair. It was determined that *rad5*, *rad6* and *rad18* all genetically interact strongly with *rnh203* (Figure 2-3, Table 2-4), but when the *rnh203* mutation is combined with either a *pol30-119* or *siz1* mutation it was determined that the resulting double mutants had normal growth (Figure 2-3, Table 2-4).

In the second largest group of genes surveyed, the checkpoint genes, two novel genetic interactions were discovered. It was found that *rnh203* genetically interacts strongly with *rad53* (Figure 2-4, Table 2-4) and *mec1* (Figure 2-9, Table 2-4). When an *rnh203* mutation was combined with *chk1*, *dun1, mec3, mrc1, rad9, rad17, rad24, rfc5, tel1*, and *tof1* mutations normal growth phenotypes were found for the double mutants indicating no genetic interaction of *rnh203* with any of these checkpoint genes (Figure 2-4, Table 2-4).

In summary, 23 non-essential genes were identified that contribute to the survival or fitness of *S cerevisiae* lacking the RNaseH2 protein. Within

this group of 23 genes, 18 novel genetic interactions were discovered (Table 2-4).

Quantitative analysis of growth confirms genetic interactions and reveals weak genetic interactions involving RNASEH2 and DNA metabolism genes.

The growth spot tests allowed were used to screen through a large group of genes to identify several genes that interact with *rnh203*. In the next set of experiments doubling times were determined for the key mutant strains to validate the results from the primary screen and also determine the severity of the defective growth phenotypes of *rnh203* double mutants. In addition, some weak genetic interactions not identified using growth spot tests were identified. Doubling times were determined for the relevant single and double mutants in the case of those found to have genetic interactions with *rnh203*. Additional genes from each of the pathways implicated were also tested to further validate results from the growth spot tests.

Doubling times were measured by growing cells in YPD and measuring cell density at incremental time points during exponential phase. Severity of the defective growth phenotypes defined the strength of genetic interactions with *rnh203*. The doubling time for the wild-type strain, (96 ± 2 min) (Figure 2-5, Figure 2-6, Figure 2-7, Figure 2-8, Figure 2-9, Figure 2-10) is consistent with what others have found for wild-type yeast strains (Hishida et al., 2006; Kaufman et al., 1998; Ooi et al., 2003; Schmidt and Kolodner,

2004; Shor et al., 2002). The *rnh203* strain had a doubling time the same as wild-type, (95 $\pm$ 2 min) (Figure 2-5, Figure 2-6, Figure 2-7, Figure 2-8, Figure 2-9, Figure 2-10), which is consistent for *rnh202* and *rnh203* mutants (Ii and Brill, 2005; Ooi et al., 2003).

This suggests that any significant difference between the double mutant growth rate and the non-*rnh203* single mutant growth rate would indicate the severity of the growth defect and subsequently the strength of the genetic interactions. Strength of genetic interactions was characterized by the difference in doubling time between the double mutant and the non-*rnh203* single mutant. The strains exhibiting a difference between 10-20 minutes were classified as weak interactions and any difference above 20 minutes was classified as a strong genetic interaction.

HR proteins are important for processing aberrant replication intermediates caused by loss of RNASEH2.

Although there are very few studies published on the genetics of *RNASEH2* there is some evidence suggesting that *RNASEH2* could very likely be involved in HR. Previous work describing the genetic interactions of *rnh203* with the *sgs1, mus81* and *rad51* is suggestive of this. Also, my work defining the mutation spectrum for *rnh203* single mutants suggests that *rnh203* mutants have higher rates of duplication mutations similar to *rad27* mutants. It has been argued that the duplication mutations found in the

Table 2-4: Summary of RNaseH2 genetic interactions.

Recombination	Growth Interaction
exo1* lig4 rad55* rad57 rad59* rdh54 slx4* srs2* yku70	none
*mus81** rad51*	weak
*mms4** *mph1** *mre11** *rad50** *rad52** *sgs1** *slx1** *top3** *xrs2**	strong

Chromatin	Growth Interaction
*cac1** *cac2* *hir1** *hir2* *hst2* *esc1*	none
*asf1** *esc2** *esc4**	strong

Sister chromatid cohesion	Growth Interaction
ctf18	none
*ctf4**	weak

Replication	Growth Interaction
*pif1m-2** *pol32** *rrm3**	none
*mgs1** *rad27** *rfa1t-33**	strong

Checkpoint	Growth Interaction
chk1 *dun1** *mec3** *mrc1** *rad9* *rad17** *rad24* *rfc5-1* *tel1** *tof1*	none
*mec1** rad53*	strong

Post replication repair	Growth Interaction
*pol30-119** *siz1*	none
*rad5** *rad6** *rad18**	strong

* represents the strains that were subsequently tested in the doubling time experiment

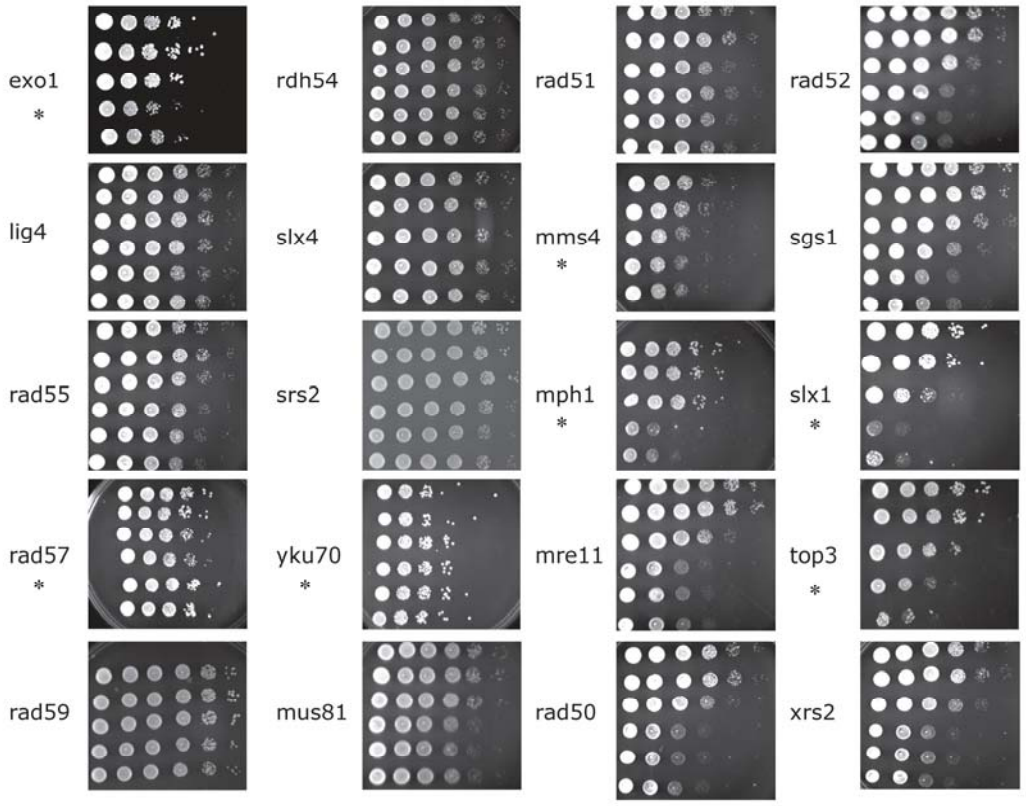

Figure 2-1: Growth spots analysis of double mutant strains carrying an *rnh203* mutation combined with recombination defects

For each plate: top row-wild-type, second row-rnh203Δ single mutant, third row-queryΔ single mutant, fourth-sixth row-rnh203Δ queryΔ double mutant. The query gene is identified just to the left of the image. Cell suspensions for serial dilutions were set up at either 1.0 X 10^6 cells/mL, or 1.0 X 10^5 cells/mL (*) followed by 5 tenfold serial dilutions.

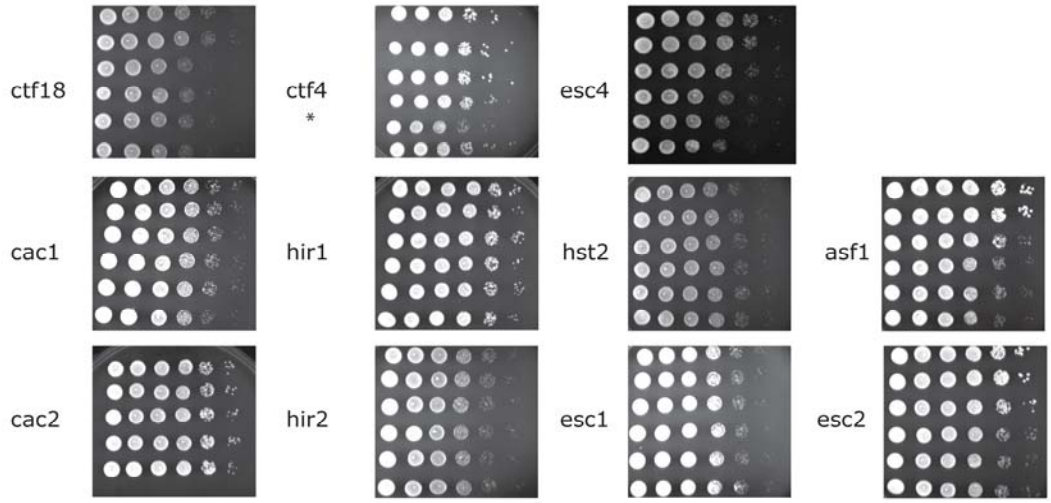

Figure 2-2: Growth spots analysis of double mutant strains carrying an *rnh203* mutation combined with sister chromatid cohesion and chromatin remodeling defects
For each plate: top row-wild-type, second row-rnh203Δ single mutant, third row-queryΔ single mutant, fourth-sixth row-rnh203Δ queryΔ double mutant. The query gene is identified just to the left of the image. Cell suspensions for serial dilutions were set up at either 1.0 X 10^6 cells/mL, or 1.0 X 10^5 cells/mL (*) followed by 5 tenfold serial dilutions.

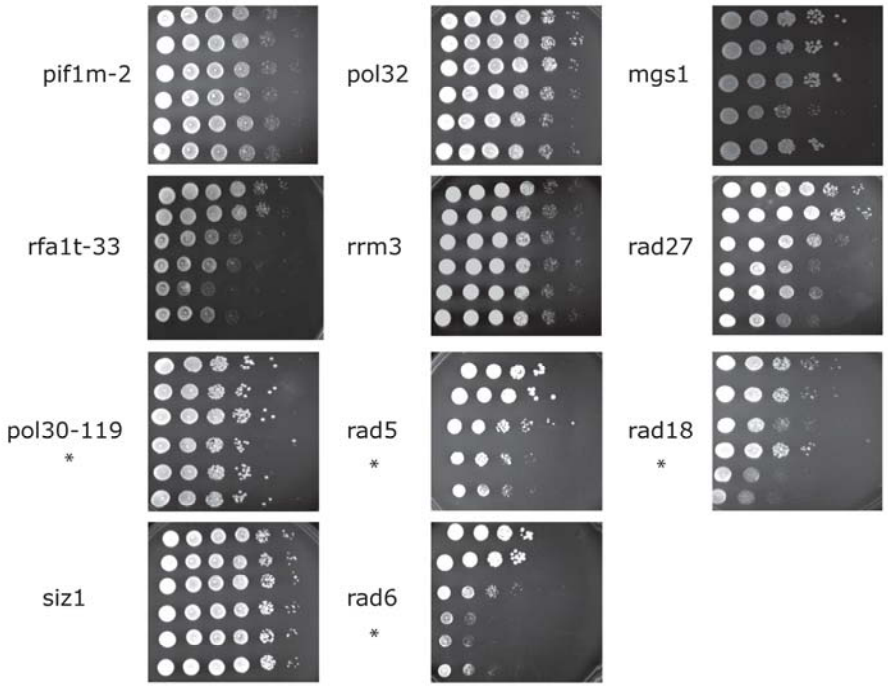

Figure 2-3: Growth spots analysis of double mutant strains carrying an *rnh203* mutation combined with DNA replication and postreplication repair defects

For each plate: top row-wild-type, second row-rnh203Δ single mutant, third row-queryΔ single mutant, fourth-sixth row-rnh203Δ queryΔ double mutant. The query gene is identified just to the left of the image. Cell suspensions for serial dilutions were set up at either 1.0 X 10^6 cells/mL, or 1.0 X 10^5 cells/mL (*) followed by 5 tenfold serial dilutions.

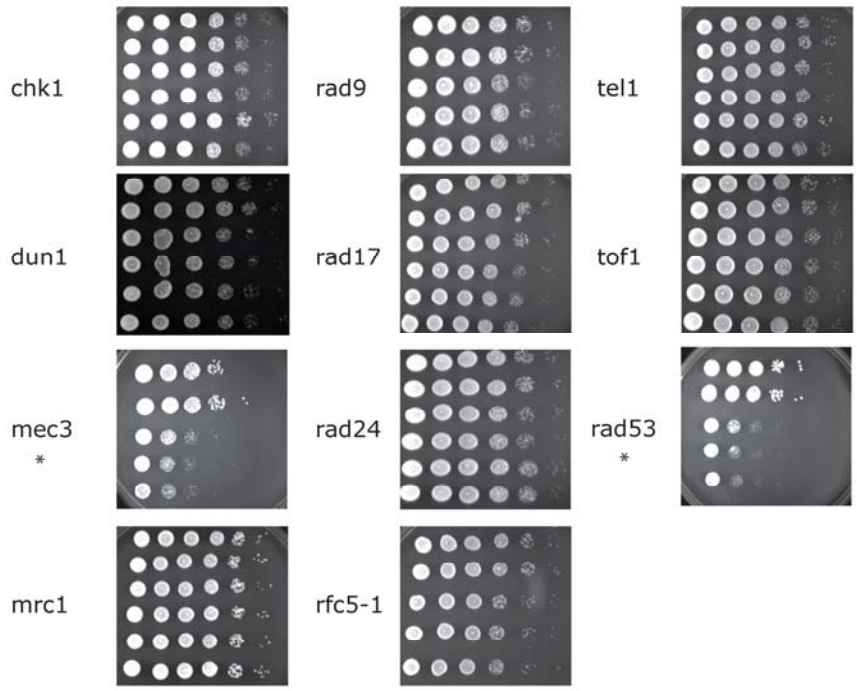

Figure 2-4: Growth spots analysis of double mutant strains carrying an *rnh203* mutation combined with checkpoint defects

For each plate: top row-wild-type, second row-rnh203Δ single mutant, third row-queryΔ single mutant, fourth-sixth row-rnh203Δ queryΔ double mutant. The query gene is identified just to the left of the image. Cell suspensions for serial dilutions were set up at either 1.0 X 10^6 cells/mL, or 1.0 X 10^5 cells/mL (*) followed by 5 tenfold serial dilutions.

rad27 strain is the result of the hyper-recombination phenotype of the *rad27* mutant. The mutation spectrum data for *rnh203* alone is not enough evidence to suggest a role in HR, but taken together with the genetic interactions with HR genes a more convincing argument develops.

That being said, it was not surprising that the doubling time tests for an *rnh203* mutation combined with HR mutations exhibited many severe growth defects in these double mutants. The growth defect found for the *rnh203 sgs1* double mutant (191 $\pm$ 16 min) (Figure 2-5), compared to the *sgs1* single mutant (115 $\pm$ 2 min) (Figure 2-5) was indicative of a much stronger genetic interaction that what others reported for either *rnh203* or *rnh202* combined with *sgs1* (Ii and Brill, 2005; Ooi et al., 2003). However, a genetic interaction between *rnh203* and *sgs1* is consistent with the literature and furthermore, the doubling time I found for the *sgs1* single mutant is consistent with what others have found (Fabre et al., 2002; Ooi et al., 2003; Schmidt and Kolodner, 2004).

The doubling times in the literature that are similar to mine are results reported on *rnh202* combined with either *mus81* or *rad51*. My result for *rnh203 rad51* (112 $\pm$ 1 min) (Figure 2-5) revealed a weak growth defect compared to the *rad51* single mutant (102 $\pm$ 7 min) (Figure 2-5) similar to what was found for *rnh202* (Ii and Brill, 2005). In this same study, a slightly more severe growth defect was observed for *rnh202 mus81* compared to what I found for *rnh203 mus81* (110 $\pm$ 1 min) (Figure 2-5) and the *mus81*

single mutant (100 ± 4 min) (Figure 2-5). However, this double mutant combination did meet my criteria for a weak genetic interaction.

The phenotype of the *rnh203 rad52* strain revealed another strong genetic interaction. A doubling time of (117 ± 10 min) (Figure 2-5) was found for the *rad52* single mutant consistent with some studies (Schmidt and Kolodner, 2004; Vance and Wilson, 2002), but less than reported in other studies (Fabre et al., 2002; Shor et al., 2002). More importantly when compared to *rnh203 rad52* (162 ± 9 min) (Figure 2-5) a strong genetic interaction is revealed.

One of the most severe growth defects found in the HR group of double mutants was with *rnh203 top3*. I found a faster doubling time for the *top3* single mutant (131 ± 28 min) (Figure 2-5) compared to other studies (Mullen et al., 2005; Shor et al., 2002). It seems likely that the large difference between my results and others could be due to strain differences or biological variation. The important point is that the *rnh203 top3* double mutant (243 ± 6 min) (Figure 2-5) grows significantly slower than the *top3* single mutant characteristic of a very strong genetic interaction.

Several additional severe growth defects were found when the *rnh203* mutation was combined with other mutations in HR genes including *mms4* with a doubling time of (110 ± 3 min) compared to *rnh203 mms4* (152 ± 16 min), *rad50* (124 ± 3 min) compared to *rnh203 rad50* (206 ± 6 min), *mph1* (97 ± 1 min) compared to *rnh203 mph1* (215 ± 23 min), *xrs2* (124 ± 4

min) compared to *rnh203 xrs2* (233 ± 24 min), *slx1* (183 ± 7 min) compared to *rnh203 slx1* (264 ± 6 min) and *mre11* (131 ± 9 min) compared to *rnh203 mre11* (242 ± 17 min) (Figure 2-5).

This data implies that cells without *RNH203* require *MUS81, RAD51, MMS4, MPH1*, *MRE11, RAD50, RAD52, SGS1, SLX1, TOP3* and *XRS2* for normal growth. The *rnh203* and HR genetic interactions provide many clues to help define the role of the RNaseH2 complex in HR. It has been proposed that toxic recombination intermediates generated during DNA replication are channeled into two different pathways one is *sgs1* dependent and the other relies on *mus81* and *srs2* (Fabre et al., 2002). It is also known that Sgs1 and Top3 function together in resolution of replication intermediates to prevent accumulation of toxic recombination products (Shor et al., 2002).

Strains carrying a mutation in *MPH1* have been shown to have similar phenotypes to *sgs1* mutants (Banerjee et al., 2008). The *SLX* and *MMS4* genes were suggested to be related to *MUS81* when they were found in a screen that identified functions for these genes in preventing genome instability via pathways that are thought to resolve the recombination intermediates that form in response to DNA damage (Mullen et al., 2001).

Combining what is known about these genes and the evidence of strong interactions of *rnh203* with *sgs1*, *top3*, and *mph1*, a moderate interaction with *mms4* and weak or no interactions with *mus81*, *srs2* or *slx4*, it seems likely that any aberrant replication intermediates that might be

generated in the absence of RNaseH2 are more dependent on Sgs1 related genome stability pathways than Mus81 related pathways.

The strong genetic interaction of *rnh203* with *slx1* does not fit this interpretation and remains puzzling. The *rnh203 slx1* double mutant was carefully genotyped to be sure it was correct and was then subjected to several rounds of re-testing and I consistently reproduced my results.

Other genetic interactions found in the HR group that can be categorized separately from either the Sgs1 or Mus81 related pathways include the MRX complex (Mre11, Rad50 and Xrs2) which is known to be involved in HR, non-homologous end joining (NHEJ) and checkpoint functions (Boulton and Jackson, 1998; Connelly and Leach, 2002; D'Amours and Jackson, 2001, 2002; Tsukamoto et al., 2001), and Rad52 which is required for all HR pathways in *Saccharomyces cerevisiae.* Both Lig4 and Yku70 are required for NHEJ.

Considering that *rnh203* strongly interacted with *sgs1* and mutants with defects in the MRX complex, moderately with *rad52* and either weakly or not at all with *rad51*, *rad59*, *lig4* or *yku70* (Figure 2-1) suggests that Rnh203, Sgs1 and MRX function is important to activate a checkpoint and this data also opens up the possibility that these HR proteins might be required to process *rnaseh2* induced DNA damage.

It seems likely that HR is not the preferred mechanism of repair of DNA damage generated in the *rnh203* mutants, and the more likely scenario

for HR involvement is carried out by HR proteins that are able to resolve more complex structures that are formed when Okazaki fragment processing is dysfunctional. A likely possibility is that recombination proteins with helicase and nuclease capabilities such as Sgs1 and the MRX complex are required to modify *rnaseh2* induced DNA damage to create substrates that can be recognized by checkpoint proteins that might possibly signal checkpoint activation. This then might be followed by processing with RAD52 dependent repair pathways or an alternative repair pathway. Ultimately, the mechanisms that are involved in processing the *rnaseh2* induced DNA damage are critical to maintain genome stability.

Sister chromatid cohesion is not important for repair of DNA damage caused by loss of RNASEH2.

It was determined whether *RNH203* involvement in suppression of genome instability could be related to sister chromatid cohesion processes. It is known that sister chromatid cohesion is intimately connected to recombination processes by bringing sister chromatids in close proximity to each other to allow for transfer of genetic material from one chromosome to the other and also that sister chromatid cohesion is required for post-replicative double strand break repair (Sjogren and Nasmyth, 2001). Both Ctf4 and Ctf18 are necessary for sister chromatid cohesion (Hanna et al., 2001).

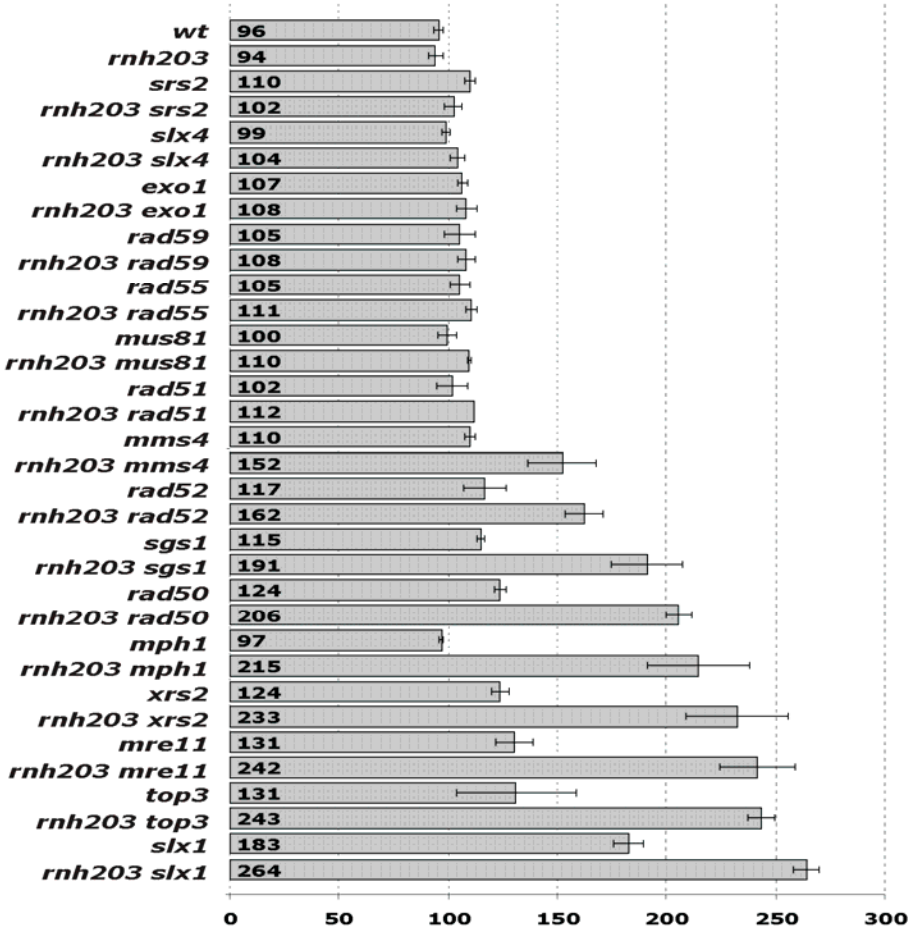

Figure 2-5: Doubling times generated for double mutant strains carrying an *rnh203* mutation combined with recombination defects

Bar graph representation of each doubling time calculated by scatter plot of OD600 vs. Time in excel. Assigned an exponential trendline to generate best fit (y = (A1)e(A2), solved for X1 and X2. X1 = LN(0.7/A1)/A2 and X2 = LN(1.4/A1)/A2. X2-X1 = doubling time. Numbers displayed on bars are average doubling times of 2-4 cultures obtained independently by random spore analysis. Error bars are standard deviations from the average

It was found that *rnh203* weakly interacts with *ctf4* when the doubling time for the *ctf4* single mutant (94 $\pm$ 1 min) was compared to the *rnh203 ctf4* double mutant (109 $\pm$ 10 min) (Figure 2-6). No interaction was found with *ctf18* (Figure 2-2) suggesting that viability of *rnh203* mutants does not depend heavily on sister chromatid cohesion or that post-replicative double strand break repair is not required in these mutants which might suggest that double strand breaks are not occurring very often in the absence of *RNASEH2*.

These results provide even more evidence to suggest that defective Okazaki fragment due to loss of *RNASEH2* generates replication errors that do not rely heavily on recombination processes for repair of errors. HR does not seem to be the preferred mechanism of repair for mutants with a defective RNaseH2 complex. The function of RNaseH2 in suppression of genome instability does not seem to involve HR pathways.

Processing of defects caused by loss of RNASEH2 involves chromatin remodeling proteins.

Based on what is known about DNA repair it was also important to find out if Rnh203 function could be tied to chromatin remodeling. The complex and multi-faceted processes that regulate chromatin structure that are taking place before, during and after DNA replication are intimately linked to DNA repair and genome instability. Cells deal with DNA damage

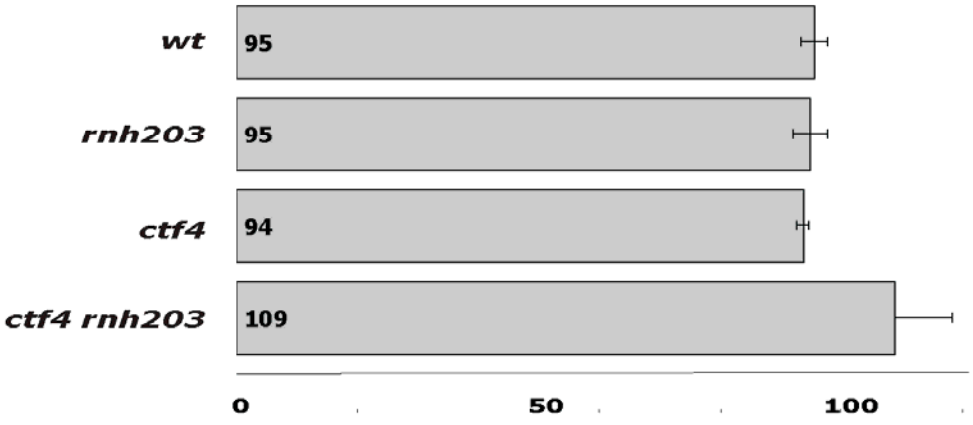

Figure 2-6: Doubling times generated for double mutant strains carrying an *rnh203* mutation combined with sister chromatid cohesion defects

Bar graph representation of each doubling time calculated by scatter plot of OD600 vs. Time in excel. Assigned an exponential trendline to generate best fit (y = (A1)e(A2), solved for X1 and X2. X1 = LN(0.7/A1)/A2 and X2 = LN(1.4/A1)/A2. X2-X1 = doubling time. Numbers displayed on bars are average doubling times of 2-4 cultures obtained independently by random spore analysis. Error bars are standard deviations from the average

at the same time or prior to the time that they replicate DNA. In order for damaged DNA to first be recognized and then repaired, access to the damage by repair machinery needs to be permitted via appropriate chromatin states.

It is known that the nucleosome is the central component of chromatin structure. The nucleosome is made up of the core histones H2A/H2B and H3/H4. Some of the proteins associated with the core histones are referred to as chaperones and include the CAF-1 complex known to associate with H2A/H2B, and the RCAF complex, known to associate with H3/H4. The proteins making up these two complexes are critical for regulating chromatin states. It is known that the tightly packed state of chromatin referred to as heterochromatin leads to gene silencing.

In the presence of DNA damage, the heterochromatin state acts as a safeguard for the cell by way of prompting the cell to trigger checkpoint functions to subsequently arrest DNA replication. Checkpoint functions are a crucial component of DNA repair and genome stability because checkpoints ensure that DNA replication is put on hold while damage is repaired. My results revealed a strong genetic interaction between *rnh203* and *asf1* when the doubling time of the single mutant *asf1* (119 $\pm$ 6 min) is compared to the double mutant *rnh203 asf1* (151 $\pm$ 5 min) (Figure 2-7). The doubling time I reported for the *cac1* single mutant (111 $\pm$ 1 min) was consistent with what others reported (Kaufman et al., 1998) and also similar to the *rnh203 cac1* double mutant (106 $\pm$ 8 min) (Figure 2-7). Asf1, anti-

silencing factor, is a chaperone protein for H3/H4 and has been shown to be central to the connection between chromatin states and the intra-S checkpoint (Kats et al., 2006).

A strong genetic interaction between *rnh203* and*, esc2* and *esc4* was also found. The primary function of both Esc2 and Esc4 has been linked to gene silencing because they were initially discovered in a screen that identified proteins capable of restoring loss of silencing in the absence of the *HMR-E* silencer (Andrulis et al., 2004). Their role in gene silencing has been further supported by their connection to the SIR protein complex which is important for silencing at the mating type loci and telomeres (Rine and Herskowitz, 1987). The BRCT motifs in Esc4 mediate Sir3 binding (Zappulla et al., 2006) and it was determined by a two-hybrid screen that Esc2 interacts with Sir2 (Cuperus and Shore, 2002).

Interestingly, one study revealed that Esc4, similar to Asf1, is central to the connection between chromatin states and the intra-S checkpoint. Esc4 contains a cluster of SQ/TQ motifs that are phosphorylated by Mec1 in response to DNA damage and the BRCT protein binding domain of Esc4 mediates the physical interaction between Esc4 and the DNA repair protein, Slx4 (Rouse, 2004).

The doubling time for the *esc4* single mutant (106 $\pm$ 1 min) was significantly faster than the *rnh203 esc4* double mutant (127 $\pm$ 10 min) (Figure 2-7). Similarly, the doubling time for the *esc2* single mutant (113 $\pm$

3 min) was significantly faster than the *rnh203 esc2* double mutant (165 ± 17 min) (Figure 2-7). Although these data do not necessarily define a role for Rnh203 in chromatin remodeling, they suggest that normal cell growth in the absence of Rnh203 function might require chromatin assembly or remodeling involving H3/H4, because of the interaction with *asf1* and also because no interactions were found with the CAF-1 complex proteins, CAC1 and CAC2 that are associated with H2A/H2B (Figure 2-2 and Figure 2-7).

The chromatin function involving Rnh203 is likely coupled to gene silencing and checkpoint function that are also likely to be important for suppression of genome instability.

RNaseH2 plays an auxiliary role in the prevention and/or creation of long flaps that are coated by RPA during Okazaki fragment processing.

DNA replication, and DNA repair and genome stability are closely connected. DNA replication relies on DNA repair for its successful completion. DNA repair deals with aberrant intermediates generated by DNA replication. Problems with DNA replication and DNA repair can lead to genome instability. Of the genes discussed thus far, most have multiple roles and have been placed into functional pathways based on what has been described in the literature as their primary role. The genes placed in the replication pathway are by far the most promiscuous set of genes in the context of functional pathways.

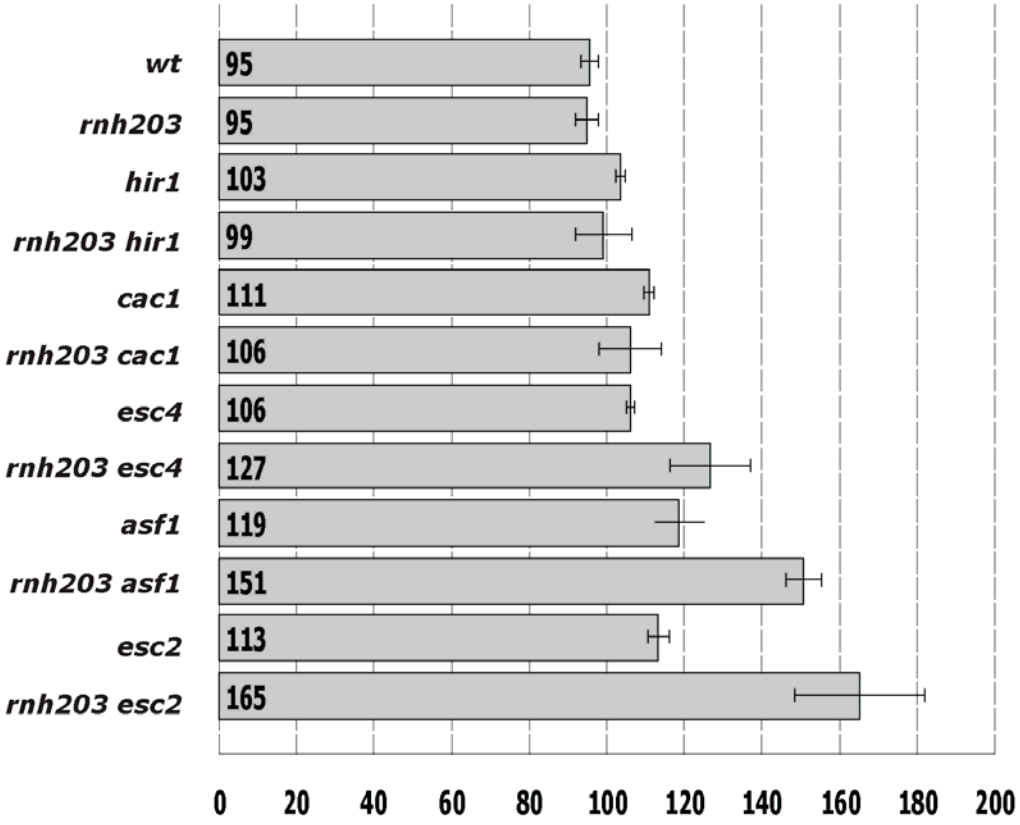

Figure 2-7: Doubling times generated for double mutant strains carrying an *rnh203* mutation combined with chromatin remodeling defects

Bar graph representation of each doubling time calculated by scatter plot of OD600 vs. Time in excel. Assigned an exponential trendline to generate best fit (y = (A1)e(A2), solved for X1 and X2. X1 = LN(0.7/A1)/A2 and X2 = LN(1.4/A1)/A2. X2-X1 = doubling time. Numbers displayed on bars are average doubling times of 2-4 cultures obtained independently by random spore analysis. Error bars are standard deviations from the average

It makes sense from an evolutionary standpoint that organisms have developed a diverse set of multi-functional proteins that are capable of accomplishing several different tasks. Protein multi-tasking is ideal for cells because it is more efficient and it also provides back-up protein resources to accomplish the essential functions needed for survival. In light of this, it is not surprising that many proteins shown to have a primary role in DNA replication have also displayed characteristics of proteins that are known to have a primary role in DNA repair and essentially are critical for suppression of genome instability.

There is an enormous amount of existing data describing the connections between DNA replication proteins and DNA repair that helps define the specific connection of *RNH203* to DNA repair and genome stability. Based on the literature I narrowed down a few candidate replication mutants that I thought might genetically interact with *rnh203*. Some of the replication mutants I tested did not show genetic interactions with *rnh203* including *rrm3*, *pol32* and the *pif1m-2*.

The Rrm3 DNA helicase is known to be required for replication fork progression through ribosomal DNA repeats and telomeric DNA, prevents replication fork pausing at many other sites, and more recently has been described to be involved in recombinational repair (Schmidt and Kolodner, 2004). The doubling time found for the *rrm3* single mutant (95 $\pm$ 3 min) (Figure 2-8) was similar to what has already been published (Schmidt and

Kolodner, 2004) and had the same growth rate as the *rnh203 rrm3* double mutant (99 ± 1 min) (Figure 2-8). Similarly, the *rnh203 pol32* and *rnh203 pif1m-2* mutants exhibited doubling times similar to their respective single mutant doubling times (Figure 2-8).

The strong growth defects detected for some of the replication double mutants revealed an interesting story. Severe growth defects were found for the *rnh203* double mutant combinations with *rfa1t-33*, *mgs1* and *rad27*. The doubling time for the single mutant *rfa1t-33* (172 ± 9 min) was faster than the double mutant *rnh203 rfa1t-33* (203 ± 11 min) by 31 minutes (Figure 2-8). The doubling time for the *mgs1* single mutant (148 ± 4 min) was slower than what has already been reported (Hishida et al., 2006), but more importantly for our analysis it was faster than the double mutant *rnh203 mgs1* (201 ± 32 min) (Figure 2-8). The most severe growth defect in the replication group was found when the single mutant *rad27* (134 ± 16 min) was compared to *rnh203 rad27* double mutant (198 ± 20 min) (Figure 2-8).

The biological mechanism with functional overlap between Rnh203, Mgs1, Rad27, Rfa1 and probably Dna2 as well is likely to be connected to the process of creating and/or preventing the long flaps that are coated by RPA during Okazaki fragment processing. The role of Mgs1 in this process has already been suggested by a group that found Mgs1 stimulated Rad27 activity. It was proposed that Mgs1 might recruit Rad27 to cleave flaps before they get long enough to be coated by RPA and then subsequently

require Dna2 for degradation (Kim et al., 2005). The implication that Rnh203 is part of the interplay between the various proteins involved in long flap cleavage is even more evident when we consider what we know about the *rfa1* mutant used in our tests.

A genetic interaction was found between *rnh203* and the *rfa1t-33* mutant. The *RFA1* mutation, *rfa1t-33* has a serine to proline change at amino acid 373 and is presumably altered in the ssDNA binding region. The ssDNA binding region of human RPA1 resides in amino acid residues 175-420 (Gomes and Wold, 1995; Lin et al., 1996), a region that is highly conserved between human RPA and yeast RFA (Erdile et al., 1991; Heyer et al., 1990; Ishiai et al., 1996). RPA binding to single stranded DNA occurs when flaps get long and is thought to be the reason Dna2 is required for degradation of these long flaps, as the RPA binding inhibits Rad27 cleavage. This particular pathway that takes place during the Okazaki fragment maturation step of DNA replication was described in more detail in Chapter 1 of the dissertation and illustrated in Figure 1-1, panel C.

It has been established that after the long flaps are processed by Dna2 the remaining substrate is cleaved by Rad27 and recently it has been suggested that Mgs1 might be involved in recruiting Rad27 to the long flaps and together with our data it appears as though RNaseH2 might also be involved in this process. Perhaps RNaseH2 functions to resolve the remaining intermediate left behind by Dna2, because it will contain both

RNA and DNA. This hypothesis coincides with what others have suggested (Crow et al., 2006) and was already described in more detail in Chapter 1 of the dissertation.

Specifically, my proposal is that RNaseH2 might be involved in resolving lagging strand intermediates that are generated when the long flap intermediates create secondary structures. Secondary structures thought to form due to faulty resolution of lagging strand intermediates has been described elsewhere (Kao and Bambara, 2003) and was also described in more detail in Chapter 1 of the thesis and illustrated in Figure 1-2. The secondary structures illustrated in Figure 1-2 are thought to be generated from long flaps prior to cleavage by Dna2 or Rad27.

The proposed RNaseH2 involvement might come into play before or after cleavage of the long flaps. The secondary structures that might form prior to cleavage would consist of flaps made up of continuous strands that contain RNA. The cleaved flaps are made up of RNA and ssDNA and perhaps would form secondary structures while still within the vicinity of the duplex by reannealing to the duplex and creating a secondary structure similar to what is illustrated in Figure 1-2 with the exception that the strands would be discontinuous. Either scenario would suggest that RNaseH2 involvement is required because of the presence of RNA. Resolution of these types of intermediates is necessary to avoid an accumulation of aberrant DNA structures in order to prevent genome instability. This would

then implicate RNaseH2 in an important role for suppression of genome instability.

Furthermore, biochemical analyses showed that as foldbacks become larger, they become progressively more inhibitory to cleavage by Fen1/Rad27 (Henricksen et al., 2000). It is my belief that RNaseH2 is required for resolution of the secondary structures that form in the long flap biosynthesis stage of Okazaki fragment maturation during DNA replication and that persistence of these structures will lead to genome instability.

The DNA damage caused by loss of RNASEH2 that triggers the SGS1 branch of the intra-S checkpoint is dependent on Mec1 and the MRX complex.

Cells have a number of checkpoint pathways that respond to DNA damage and aberrant DNA structures that are generated during the different phases of the cell cycle to arrest or slow down the cell cycle. Checkpoints allow cells the time needed to repair lesions and process aberrant structures so that cell division proceeds without DNA damage that can lead to either cell death or stable incorporation of DNA errors as mutations. There are checkpoints that act in G1 and G2 as well as two checkpoints that act in S-phase. The intra-S checkpoint slows down DNA replication and the cell cycle when damage occurs in S phase. The replication checkpoint causes cell cycle arrest and suppressed firing as late replication origins in response to depletion of the dNTP pools during S-phase. Several checkpoint proteins

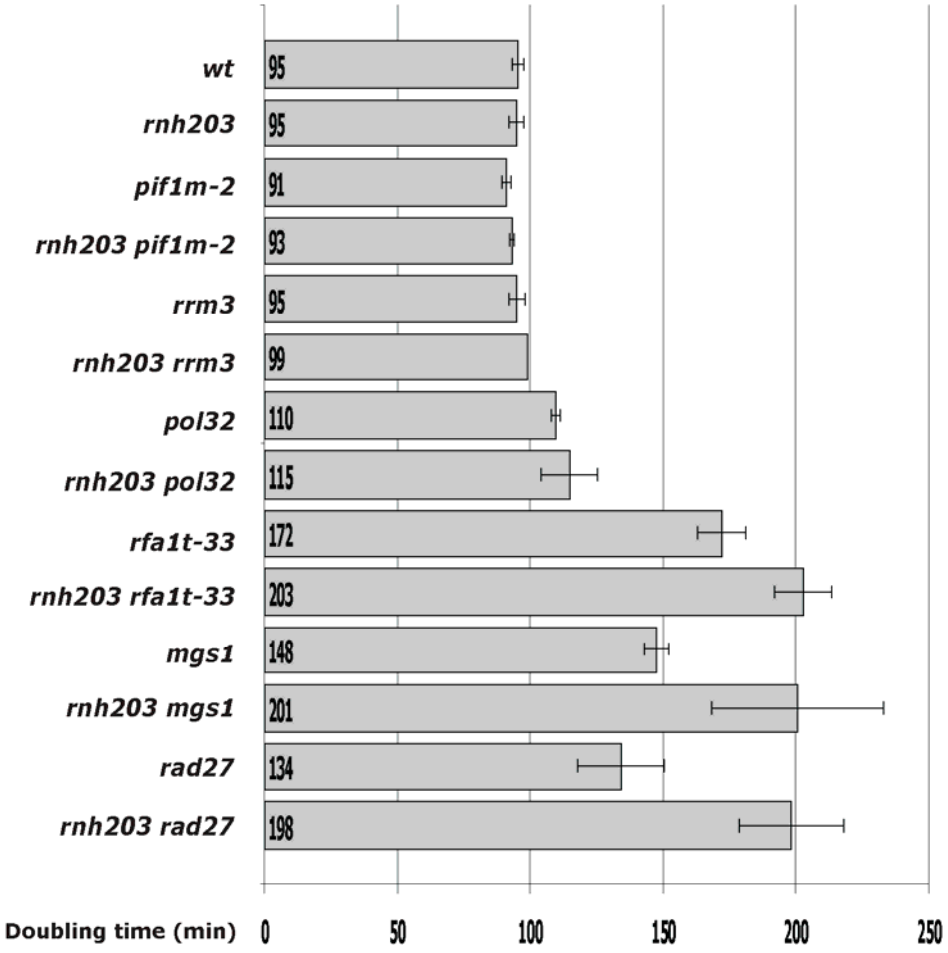

Figure 2-8: Doubling times generated for double mutant strains carrying an *rnh203* mutation combined with DNA replication defects

Bar graph representation of each doubling time calculated by scatter plot of OD600 vs. Time in excel. Assigned an exponential trendline to generate best fit (y = (A1)e(A2), solved for X1 and X2. X1 = LN(0.7/A1)/A2 and X2 = LN(1.4/A1)/A2. X2-X1 = doubling time. Numbers displayed on bars are average doubling times of 2-4 cultures obtained independently by random spore analysis. Error bars are standard deviations from the average

are known to have redundant functions and have been shown to be critical for multiple checkpoints.

In contrast, some proteins known to be involved in checkpoints have been shown to only be important in specific checkpoints. Sgs1 is one such example that is known to be important for only the intra-S checkpoint. Some of the more ubiquitous checkpoint proteins include the "RFC-like", Rad24-Rfc2-5 complex and the "PCNA-like", Rad17-Mec3-Ddc1 complex that are both critical to the G1, G2 checkpoints and the intra-S checkpoint, but is known to not be important for the replication checkpoint (Myung and Kolodner, 2002).

It was expected that the *rnh203* mutants would have an increased accumulation of DNA damage during S phase. Furthermore, the *rnaseh2* induced DNA damage was thought to most likely trigger the intra-S checkpoint based on the prediction that loss of *RNASEH2* causes defects in Okazaki fragment processing and likely an accumulation of aberrant complex DNA structures that are generated in S phase.

DNA damage checkpoints like many other biological processes require a diverse set of proteins that interact via signal transduction events such as phosphorylation to activate proteins. It is known that the intra-s checkpoint activates a phosphorylation-mediated signal transduction cascade that depends on Mec1 to activate Rad53. It has been long established that the MRX complex along with Tel1 is also required for checkpoint activation

(Nakada et al., 2003; Usui et al., 2001), but a more recent study revealed a second role for the MRX complex in checkpoint activation not requiring Tel1 that involves a double-strand break dependent Mec1 pathway that processes lesions into ssDNA (Grenon et al., 2006).

The data revealing the genetic interactions of *rnh203* with various checkpoint mutants are consistent with an S phase checkpoint involvement in response to *rnaseh2* induced DNA damage. No genetic interactions were found in double mutants carrying the *rnh203* mutation along with a "RFC-like" complex mutation defective for the Rad24 sub-unit of the complex (Figure 2-4). Additionally, no genetic interaction was found in double mutants carrying an *rnh203* mutation along with the "PCNA-like" complex mutations that cause defects in either Rad17 or Mec3 (Figure 2-9) suggesting that RNaseH2 defects do not trigger G1 or G2 checkpoints.

In line with the prediction that DNA damage caused by defective RNaseH2 triggers an-S-phase checkpoint, very strong genetic interactions were found in the double mutants carrying an *rnh203* mutation along with either a *mec1* or a *rad53* mutation (Figure 2-9). Interestingly, the genetic interaction between *mec1* and *rnh203* was the strongest genetic interaction found among all the genes that were studied. It was determined that it took 142 minutes longer for the *rnh203 mec1* double mutant (274 $\pm$ 20 min) to double compared to the *mec1* single mutant (132 $\pm$ 5 min) (Figure 2-9).

The genetic interaction between *rad53* and *rnh203* was the second strongest genetic interaction found among all the genes studied. The doubling time for the *rnh203 rad53* double mutant (272 $\pm$ 8 min) was 131 minutes longer than what was found for the *rad53* single mutant (141 $\pm$ 1 min) (Figure 2-9). The doubling time of the *rad53* single mutant was consistent with previously published results (Enserink et al., 2006).

The genetic interactions between *rnh203*, *mec1*, *rad53*, *sgs1* and the MRX complex (Figure 2-5 and Figure 2-9) provides evidence for the argument that any checkpoint involvement in response to DNA damage generated in *rnaseh2* mutants is most likely triggering the Sgs1 dependent branch of the intra-s checkpoint and checkpoint activation might require Mec1 signaling and substrate modification by the MRX complex.

The postreplication repair pathway is important for DNA damage tolerance of lesions induced by loss of RNASEH2.

In light of the results presented thus far it is clear that cells have evolved many mechanisms to respond to DNA damage to ensure survival. The postreplication repair pathway is an additional process that helps cells deal with DNA damage. The DNA postreplication repair pathway does not remove DNA damage but instead bypasses damage that is encountered. DNA damage bypass by postreplication repair is carried out by 3 separate pathways that all depend on the Rad6-Rad18 ubiquitin conjugating complex

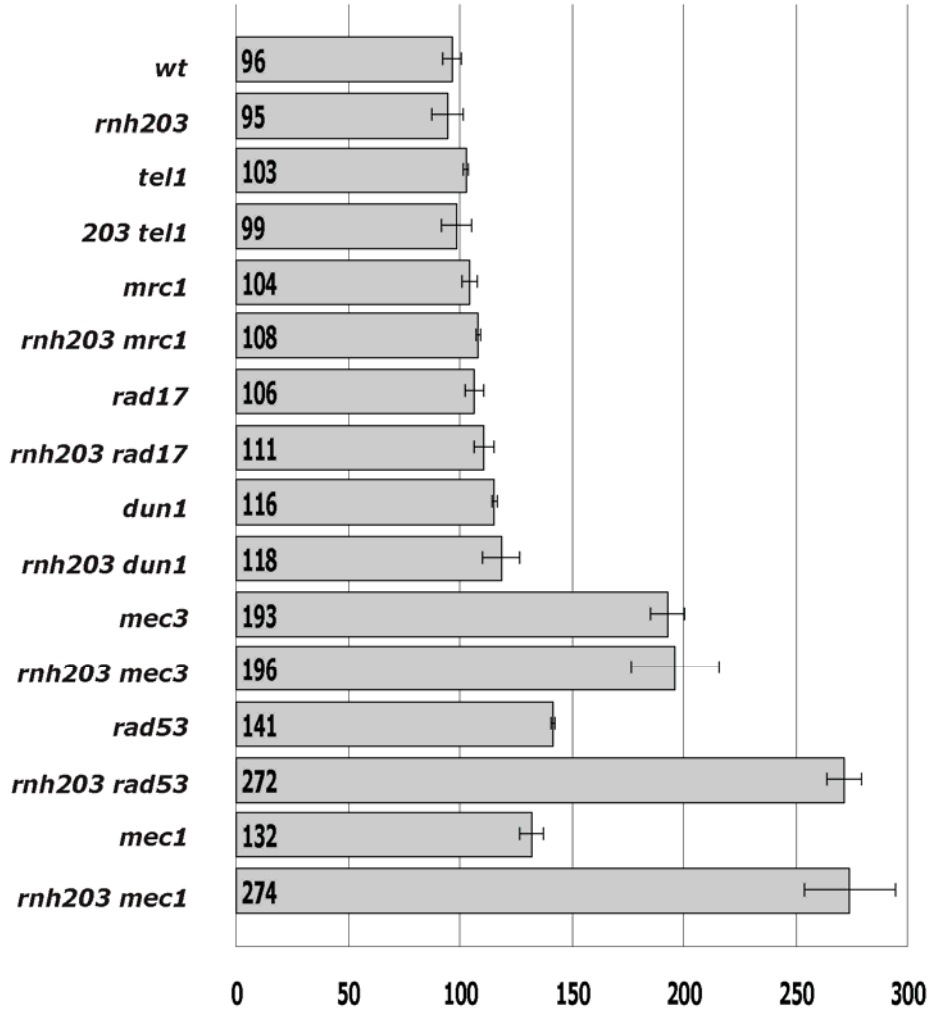

Figure 2-9: Doubling times generated for double mutant strains carrying an *rnh203* mutation combined with checkpoint defects

Bar graph representation of each doubling time calculated by scatter plot of OD600 vs. Time in excel. Assigned an exponential trendline to generate best fit (y = (A1)e(A2), solved for X1 and X2. X1 = LN(0.7/A1)/A2 and X2 = LN(1.4/A1)/A2. X2-X1 = doubling time. Numbers displayed on bars are average doubling times of 2-4 cultures obtained independently by random spore analysis. Error bars are standard deviations from the average

and PCNA. The current view is that monoubiquitation of PCNA at lys164 by the Rad6-Rad18 conjugation complex in response to DNA damage is the first step of DNA damage bypass by postreplication repair (Stelter and Ulrich, 2003).

Two of the three sub-pathways result in error-free completion of DNA replication while one sub-pathway is known to be error-prone because of incorporation of DNA lesions. The mechanism by which one of the error-free sub-pathways bypasses the DNA damage has been described to be a template switching mechanism that requires the Mms2-Ubc13 complex and Rad5 (Stelter and Ulrich, 2003). In this sub-pathway, subsequent to the monoubiquitination of PCNA, the lys164 residue of PCNA is polyubiquitinated by Ubc13 and this action has been suggested to be the signal for the DNA damage to be shuttled to this sub-pathway instead of the other sub-pathways.

In the Mms2-Ubc13 and Rad5 postreplication repair sub-pathway the replication machinery terminates synthesis at the site of the DNA lesion followed by replication restart downstream of the lesion. This process forms a gap across the lesion which is then filled in by the template switching mechanism. Template switching occurs in two steps, the first step takes place when the replication machinery stops synthesizing at the site of the DNA lesion and changes position to the newly synthesized daughter strand of the undamaged complementary sequence and subsequently uses this DNA

as the template sequence until the lesion is bypassed and once the lesion is bypassed then DNA polymerase switches back to copying the damaged template strand (Torres-Ramos et al., 2002).

The other 2 sub-pathways of postreplication repair termed translesion synthesis require specialized DNA polymerases that replicate past an otherwise replication blocking DNA lesion. The error-free translesion synthesis pathway requires polη (Rad30) and is known to replicate past different types of oxidative or ultraviolet light-induced lesions (Prakash and Prakash, 2002). The error prone translesion synthesis pathway requires polζ (Rev3) and extends primer termini opposite a variety of lesions or mismatches and is responsible for virtually all damage-induced mutagenesis (Stelter and Ulrich, 2003).

Another contributing factor to postreplication repair is an alternate modification of PCNA involving sumolyation at lys164 by Ubc9 and the SUMO-specific ligase Siz1. It has been suggested that PCNA acts as a molecular switch that in its SUMO-modified form may promote replication, whereas multi-ubiquitination of PCNA stimulates error free repair (Hoege et al., 2002).

To determine whether postreplication repair contributes to dealing with the DNA damage caused by loss of *RNASEH2,* the doubling times for double mutants carrying an *rnh203* mutation along with mutations that inactivate the most critical components of the postreplication repair

pathways were measured. First, to examine whether *rnaseh2* and *PCNA* genetically interact, the doubling time for a double mutant strain carrying an *rnh203* mutation and a *pol30-119* mutation defective for ubiquitination and sumolyation at lys164 was generated. There was almost no difference in growth rate between the *pol30-119* single mutant (102 $\pm$ 1 min) and the *rnh203 pol30-119* double mutant (109 $\pm$ 1 min) (Figure 2-10).

However, in the absence of *RNH203* it was determined that Rad5, Rad6 and Rad18 were required for normal growth. A slower doubling time for the *rad5* single mutant was found (146 $\pm$ 15 min) compared to what had been previously reported (Hishida et al., 2006), but more importantly a severe growth defect was revealed when the double mutant *rnh203 rad5* (210 $\pm$ 24 min) was compared to the single mutant (Figure 2-10). Similarly, severe growth defects were found for the single mutant *rad6* (168 $\pm$ 5 min) compared to the double mutant *rnh203 rad6* (220 $\pm$ 24 min) and also for *rad18* (131 $\pm$ 9 min) compared to *rnh203 rad18* (244 $\pm$ 18 min) (Figure 2-10).

It is interesting to note that cells lacking *MGS1*, which is also known to be involved in Okazaki fragment processing, are synthetically lethal with *rad6* and *rad18* and are synthetically sick with *rad5* (Hishida et al., 2006). The genetic interactions between *mgs1* and the postreplication repair proteins taken together with the result that Mgs1 stimulates Rad27 activity has spurred the hypothesis that in the absence of *MGS1*, processing of Okazaki

fragments is impaired and this defect can be efficiently overcome by a post-replicational repair pathway (Kim et al., 2005).

The data described thus far, might also suggest a similar scenario for RNaseH2. One possibility is that the aberrant intermediates generated in the absence of RNaseH2 due to erroneous Okazaki fragment processing are preferentially repaired by post-replication repair.

Gross chromosomal rearrangements occur in RNASEH2 defective strains.

The focus so far has been on elucidating the involvement of RNaseH2 in the various DNA repair pathways that are critical to cell survival and genome stability when DNA damage is generated due to improper Okazaki fragment maturation. The next part of the study focuses on the interplay of RNaseH2 and proteins from multiple pathways of DNA repair that function to suppress and/ or create GCRs in order to further characterize the role of RNaseH2 in DNA repair and genome stability.

It has been well established that accumulation of DNA damage can lead to genome instability and possibly cancer. One type of genome instability observed frequently is gross chromosomal rearrangements (GCRs). GCRs include translocations, deletions of chromosome arms, interstitial deletions, inversions, amplifications, chromosome end-to-end fusion, and aneuploidy (Kolodner et al., 2002). Many researchers have contributed to the extensive literature on GCRs describing the pathways

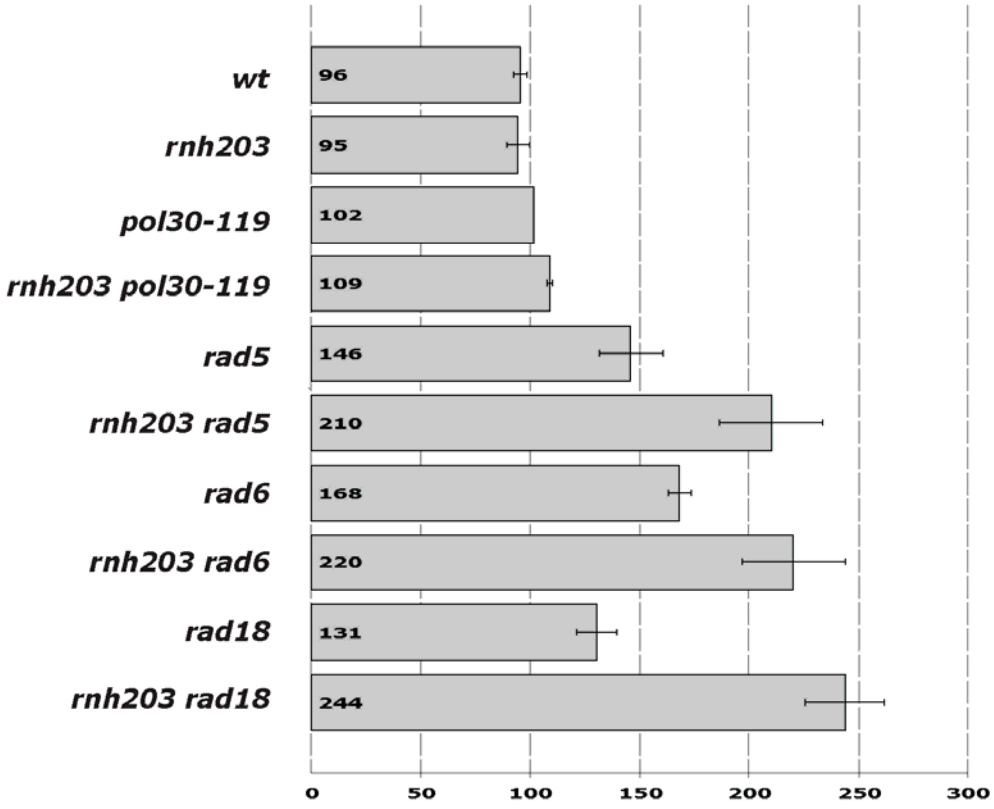

Figure 2-10: Doubling times generated for double mutant strains carrying an *rnh203* mutation combined with postreplication repair defects

Bar graph representation of each doubling time calculated by scatter plot of OD600 vs. Time in excel. Assigned an exponential trendline to generate best fit (y = (A1)e(A2), solved for X1 and X2. X1 = LN(0.7/A1)/A2 and X2 = LN(1.4/A1)/A2. X2-X1 = doubling time. Numbers displayed on bars are average doubling times of 2-4 cultures obtained independently by random spore analysis. Error bars are standard deviations from the average

involved in suppressing GCRs (Banerjee and Myung, 2004; Chan and Blackburn, 2003; Huang and Koshland, 2003; Huang and Kolodner, 2005; Kanellis et al., 2003; Lengronne and Schwob, 2002; Myung et al., 2001a; Myung et al., 2001b; Myung et al., 2001c; Myung and Kolodner, 2002; Myung et al., 2003; Pennaneach and Kolodner, 2004; Putnam et al., 2004; Smith et al., 2004; Tanaka and Diffley, 2002) and pathways required for the formation of GCRs (Hwang et al., 2005; Myung et al., 2001a; Myung et al., 2004; Pennaneach and Kolodner, 2004; Putnam et al., 2004).

The Kolodner lab in-house GCR assay was used to measure the rate of accumulation of genome rearrangements in the single *rnaseh2* mutants and also the *rnaseh2* double mutants with various DNA repair defects. A wild-type rate of (3.6×10^{-10}) was determined (Table 2-5, Table 2-6, Table 2-7, Table 2-8, Table 2-9, Table 2-10) similar to what has already been published (Chen and Kolodner, 1999; Ragu et al., 2007; Smith et al., 2005). The GCR rate of the *rnh203* single mutant was (2.0×10^{-10}) (Table 2-5, Table 2-6, Table 2-7, Table 2-8, Table 2-9, Table 2-10) and is similar to wild-type and is 3 fold less than what was found in a previous report (Huang et al., 2003). The GCR rates caused by mutations in the genes encoding the other sub-units, *rnh201* and *rnh202*, of the RNaseH2 complex were also similar to the wild-type rate (Table 2-5).

It is not uncommon that small differences in GCR rates are seen for the same mutants when comparing data among different researchers. For

example, one study reported that the *rad51* single mutant rate is (3.5×10^{-9}) (Chen and Kolodner, 1999) while a different study generated the *rad51* single mutant rate of (8.0×10^{-10}) (Schmidt and Kolodner, 2006), a four-fold difference. These small differences have been attributed to variability in the GCR assay that might partly be due to the inherent instability of biological systems.

Generally, what has become important for proper interpretation of GCR results is that within a single study the appropriate controls are performed that include generation of a single mutant rate obtained by testing multiple independently collected strains to compare with the double mutant rate. This approach was used for collection of all the GCR rates reported in this study.

Another aspect of result interpretation that actually ended up being similar to the interpretation of the doubling time results is that because the GCR rate of the *rnaseh2* mutant is the same as the wild-type rate that meant that the rate differences between the *rnh203* double mutant and the non-*rnh203* single mutants determined the impact of loss of function attributed to the interplay between Rnh203 and the query gene on suppression or generation of GCRs and ultimately genome instability.

In light of the variability of the GCR assay, my criteria for notable differences between single and double mutants required at least a 4 fold difference.

Table 2-5. Rate of accumulating GCRs in RNaseH2 defective strains.

	Wild type
Genotype	Mutation rate (Can^r 5-FOA^r)
wild-type	3.6×10^{-10} (1)
rnh201 Δ	4.7×10^{-10} (0.7)
rnh202 Δ	3.3×10^{-10} (1)
rnh203 Δ	2.0×10^{-10} (0.6)

All strains are isogenic to RDKY5027 [ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2Δbgl, hom3-10, ade2Δ1, ade8, hxt13::*URA3*]

(The numbers in parentheses are the fold increase in rate relative to that of the wild-type strain)

Loss of RNASEH2 in combination with HR defects is important for both suppression and generation of GCRs.

The results from the doubling time tests revealed that HR proteins are important for normal cell growth in the absence of *RNASEH2*. A plausible explanation for this is that aberrant replication intermediates are generated in the *rnaseh2* mutants that require recombination function for proper processing. Next, it was determined whether the rate of accumulation of GCRs would be affected in the *rnaseh2* mutants when recombination is defective. One pathway from the recombination group was found to be important for suppression of GCRs that involved SGS1.

The rate for the *sgs1* single mutant (6.9×10^{-9}) (Table 2-6) was similar to what has already been published (Huang and Kolodner, 2005; Myung et al., 2001b) and had a 12 fold higher GCR rate than the *rnh203 sgs1* double mutant (7.8×10^{-8}) (Table 2-6).

In the HR group, an additional pathway was found that is important for generation of GCRs involving SLX1. The GCR rate for the *slx1* single mutant (7.5×10^{-8}) (Table 2-6) was similar to a rate for *slx1* previously determined in the Kolodner lab (unpublished data) and was 52 fold greater than the GCR rate of the *rnh203 slx1* double mutant ($<1.5 \times 10^{-9}$) (Table 2-6). This result suggests that the formation of GCRs resulting from *slx1* defects involves RNaseH2 function.

Several weak 3 to 4 fold differences in rates between the single and double mutants were also found for *exo1, rad59, mus81, mms4* and *mre11* (Table 2-6) that are likely due to the variability of the assay. Most of the rates determined for the HR single mutants were similar or within 4 fold of what had already been published (Table 2-6) and (Banerjee and Myung, 2004; Banerjee et al., 2008; Chen and Kolodner, 1999; Huang and Kolodner, 2005; Hwang et al., 2005; Myung et al., 2001a; Myung et al., 2001b; Myung et al., 2004; Pennaneach and Kolodner, 2004; Schmidt and Kolodner, 2006; Smith et al., 2005). Additionally, the rate for *slx4* and a rate already generated for *slx4* in our lab were similar (unpublished data).

The few discrepancies between this data and what is reported in the literature are the following. The rate determined for the *mms4* single mutant (2.9×10^{-9}) (Table 2-6) is 20 fold less than reported in the literature (Smith et al., 2004). The rate for *top3* (1.5×10^{-9}) (Table 2-6) was 6 fold less than published results (Myung et al., 2001b). Given that the doubling times for the *top3* single mutant compared to what others found was also variable, this might suggest that the *top3* strain is prone to variability.

The rate for *yku70* (7.5×10^{-8}) (Table 2-6) was similar to some (Banerjee and Myung, 2004; Chen and Kolodner, 1999; Myung et al., 2001a), but 6 fold higher than what was found in one study (Motegi et al., 2006). The rate found for *rad52* (6.0×10^{-8}) (Table 2-6) was similar to some reports (Myung et al., 2001a; Myung et al., 2004) but 10 fold higher than one

study (Ragu et al., 2007). A 10 fold difference between the rate was determined for the *rad51 rad59* double mutant (1.2 X 10^{-8}) (Table 2-6) compared to (Myung et al., 2001a).

The more important thing to note is that there were not any 4 fold or greater differences between any of these single mutants and the respective *rnh203* double mutant. These results provide further evidence that defects in lagging strand synthesis due to loss of *RNASEH2* relies on Sgs1 for processing to avoid accumulation of DNA damage that leads to genome instability. Furthermore, the growth defect seen in the *rnh203 slx1* double mutant could be due to an RNaseH2 dependent cell cycle slow down that allows for proper processing of the genome rearrangements that occur in the absence of *SLX1*.

RNASEH2 defects require the chromatin functions of Esc2 for preventing genome instability.

The growth rate analysis suggested that processing of defects due to loss of *RNASEH2* involves proteins that are associated with the core histones H3/H4 and might also require a gene silencing function and/or intra-S checkpoint signaling mediated by chromatin remodeling. It was next determined if the rate of accumulation of GCRs would be affected in the *rnaseh2* mutants when different chromatin assembly/remodeling proteins were defective. For the subgroup of chromatin remodeling proteins tested,

Table 2-6. Effect of *rnh203Δ* on the rate of accumulating GCRs in different recombination defective strains.

Genotype	Wild type Mutation rate (Can^r $5\text{-}FOA^r$)	*rnh203Δ* Mutation rate (Can^r $5\text{-}FOA^r$)
Wild type	3.6×10^{-10} (1)	2.0×10^{-10} (0.5)
exo1 Δ	1.05×10^{-8} (29)	3.4×10^{-9} (9)
rad51 Δ	3.5×10^{-9} (10)	3.7×10^{-9} (10)
rad55 Δ	3.2×10^{-9} (9)	5.3×10^{-9} (15)
rad59 Δ	4.5×10^{-9} (13)	1.8×10^{-8} (50)
slx4 Δ	1.8×10^{-8} (37)	1.8×10^{-8} (38)
yku70 Δ	8.9×10^{-10} (3)	1.1×10^{-9} (3)
mus81 Δ	8.4×10^{-9} (23)	2.5×10^{-8} (69)
srs2 Δ	6.1×10^{-10} (2)	6.5×10^{-10} (2)
mms4 Δ	2.9×10^{-9} (8)	1.1×10^{-9} (3)
mph1 Δ	1.1×10^{-9} (3)	$< 1.2 \times 10^{-9}$ (3)
mre11 Δ	4.3×10^{-7} (1194)	1.4×10^{-6} (3972)
rad52 Δ	6.0×10^{-8} (167)	7.1×10^{-8} (197)
sgs1 Δ	6.9×10^{-9} (19)	7.8×10^{-8} (219)
slx1 Δ	7.5×10^{-8} (208)	$< 1.5 \times 10^{-9}$ (4)
top3 Δ	1.5×10^{-9} (4)	1.8×10^{-9} (5)
rad51 Δ rad59 Δ	1.2×10^{-8} (33)	1.6×10^{-8} (44)

All strains are isogenic to RDKY5027 [ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2Δbgl, hom3-10, ade2Δ1, ade8, hxt13::*URA3*]

only Esc2 was found to be involved in a pathway that suppresses GCRs. The rate for the *esc2* single mutant (1.7×10^{-8}) (Table 2-7) was within 5 fold of a rate already determined in the Kolodner lab (unpublished data) and was 12 fold less than the *rnh203 esc2* double mutant rate (2.1×10^{-7}) (Table 2-7).

Interestingly, one pathway was only found in the chromatin group that was important for generation of GCRs in an *rnh203* mutant requiring *CAC1*. The GCR rate for the *cac1* single mutant (3.9×10^{-8}) (Table 2-7) was within 3 fold of what has already been published (Myung et al., 2003) and was 36 fold greater than the *rnh203 cac1* double mutant rate (1.1×10^{-9}) (Table 2-7).

These results together with the doubling time results support the hypothesis that defects in Okazaki fragment processing triggers the intra-S checkpoint and regulation of chromatin states. When the pathway that combines intra-S checkpoint function and modulation of chromatin states is defective namely in *asf1*, *esc2,* and *esc4* mutants there is an increase in genome rearrangements because repair of the DNA damage is defective.

Additionally, these results suggest a novel role for RNaseH2 in suppressing the genome rearrangements generated in Cac1 defective strains. All of the other rates generated for the chromatin remodeling protein single mutants were similar or within 4 fold of what had already been published (Table 2-7) and (Banerjee and Myung, 2004) and (unpublished data).

Table 2-7. Effect of *rnh203Δ* on the rate of accumulating GCRs in different chromatin assembly/remodeling defective strains.

Genotype	Wild type Mutation rate (Can^r $5\text{-}FOA^r$)	*rnh203Δ* Mutation rate (Can^r $5\text{-}FOA^r$)
Wild type	3.6×10^{-10} (1)	2.0×10^{-10} (0.5)
asf1 Δ	1.4×10^{-8} (39)	4.1×10^{-8} (114)
cac1 Δ	3.9×10^{-8} (108)	1.0×10^{-9} (3)
esc2 Δ	1.7×10^{-8} (48)	2.1×10^{-7} (592)
esc4 Δ	1.3×10^{-8} (36)	5.9×10^{-8} (163)
hir1 Δ	7.0×10^{-10} (2)	5.2×10^{-10} (1)

All strains are isogenic to RDKY5027 [ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2Δbgl, hom3-10, ade2Δ1, ade8, hxt13::*URA3*]

Loss of RNASEH2 in combination with DNA replication defects does not lead to accumulating gross chromosomal rearrangements, but reveals that Pol32 and RNaseH2 might be involved in a novel pathway that generates GCRs.

The doubling time results point to the involvement of RNaseH2 in the prevention or creation of the long flaps that are generated during Okazaki fragment processing. The current belief about the long flaps is that they are only generated under certain conditions such as synthesis across a repeat region or in specific mutants (Andrulis et al., 2004). It seems likely that adverse conditions occur when long flaps are formed and to make things worse for the cell the long flaps themselves pose a threat to cell survival because long flaps are more prone to generating fold-back structures that if unresolved can lead to genome instability. It is known that replication errors play a role in forming genome rearrangments (Myung et al., 2001c).

Taken together with the idea that cells might be defective in long flap resolution without *RNASEH2* it was determined whether the rate of accumulation of genome rearrangements in *rnaseh2* mutants would be affected by DNA replication defects. It was determined that loss of *RNASEH2* in combination with the DNA replication defects tested did not lead to an increased rate in accumulation of GCRs suggesting that any damage that might be accumulating due to improper DNA replication and/or

defective long flap resolution is repaired or the damage is undetected by the GCR assay.

The idea that the DNA damage caused by combining an *rnaseh2* mutant with DNA replication defects is repaired is the more likely scenario because this would also provide a good explanation for the growth defect data for the *rnaseh2* mutant combinations with DNA replication defects. In line with this argument the growth defects would imply that the cell cycle slows down so that repair of the DNA damage occurs and the end result is successful error-free completion of DNA replication; hence, slow growth, but no GCRs.

None of the DNA replication pathways involving RNaseH2 tested here were involved in suppression of GCRs. However, one pathway was found that was important for generation of GCRs in an *rnh203* mutant that required Pol32. The GCR rate for the *pol32* single mutant (6.6×10^{-9}) (Table 2-8) was higher than what was previously reported (Motegi et al., 2006), To be sure the rate was correct several rounds of re-testing were performed and the same results were consistently reproduced. The GCR rate for the *pol32* single mutant was 9 fold greater than the *rnh203 pol32* double mutant rate (7.5×10^{-10}) (Table 2-8). These results suggest a novel role for RNaseH2 in suppressing the genome rearrangements generated in *pol32* defective strains.

All of the other rates that I found for the replication protein single mutants were similar or within 4 fold of what had already been published (Table 2-8) and (Banerjee and Myung, 2004; Chen and Kolodner, 1999; Huang and Kolodner, 2005; Hwang et al., 2005; Motegi et al., 2006; Myung et al., 2001a; Myung et al., 2004; Ragu et al., 2007; Schmidt and Kolodner, 2006).

Loss of RNASEH2 in combination with checkpoint defects does not lead to accumulating gross chromosomal rearrangements, but reveals that Tel1 and RNaseH2 might be involved in a novel pathway that generates GCRs.

The hypothesis that DNA damage caused by the loss of *RNASEH2* causes cell cycle slow down is supported by the result that growth defects arise in *rnh203* strains that are also defective for the central components of the intra-S checkpoint. If *rnaseh2* induced defects causes cell cycle slow down it was then expected that the proteins involved in the intra-S checkpoint pathway might be involved in suppressing GCRs.

Logically, the next thing looked at was whether the rate of accumulation of GCRs was affected in *rnh203* strains that were combined with mutations that inactivate checkpoints. Almost all of the GCR rates for the *rnh203* strains that were combined with mutations that inactivate checkpoints were either similar or within 4 fold of the rate of their respective single mutant rate (Table 2-9).

Table 2-8. Effect of *rnh203Δ* on the rate of accumulating GCRs in different DNA replication defective strains.

Genotype	Wild type Mutation rate (Can^r 5-FOA^r)	*rnh203Δ* Mutation rate (Can^r 5-FOA^r)
Wild type	3.6×10^{-10} (1)	2.0×10^{-10} (0.5)
mgs1 Δ	$< 5.3 \times 10^{-10}$ (1)	$< 1.0 \times 10^{-9}$ (3)
pif1m-2 Δ	3.1×10^{-8} (86)	2.6×10^{-8} (72)
pol32 Δ	6.6×10^{-9} (18)	7.5×10^{-10} (2)
rad27 Δ	1.1×10^{-7} (306)	2.2×10^{-7} (611)
rfa1t-33 Δ	1.2×10^{-7} (325)	8.6×10^{-8} (239)
rrm3 Δ	1.4×10^{-9} (4)	2.2×10^{-9} (6)

All strains are isogenic to RDKY5027 [ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2Δbgl, hom3-10, ade2Δ1, ade8, hxt13::*URA3*]

The one difference between single and double mutant was found to involve a pathway important for generation of GCRs in an *rnh203* mutant that required Tel1. The rate found for the *tel1* single mutant (5.9×10^{-9}) (Table 2-9) is 30 fold higher than what was found in one study (Myung et al., 2001a), but within four fold of what was found in a different report (Huang and Kolodner, 2005). Given the 8 fold suppression of rate for the *rnh203 tel1* double mutant (8.0×10^{-10}) (Table 2-9) compared to the rate for the *tel1* single mutant and also the discrepancy between my data and one of the previous reports, independently collected single mutant strains were subjected to multiple rounds of re-testing and these results were consistently generated.

It was expected that increased rates of GCRs in the *rnh203* strains combined with mutations that inactivate checkpoints would be seen. However, this was not found. A few different scenarios might explain this data. One scenario is that absence of increased rates of accumulation of GCRs could be because the DNA damage might be undergoing error free repair. Alternatively, the DNA damage might be modified in such a way that it does not generate GCRs.

The most likely scenario is that the DNA damage is successfully repaired in the checkpoint mutants and thus undetected by the GCR assay. These results also suggest a novel role for RNaseH2 in suppressing the genome rearrangements generated in *tel1* defective strains. All other rates

generated for the checkpoint single mutants were similar or within 4 fold of what had already been published (Table 2-9) and (Banerjee and Myung, 2004; Hwang et al., 2005; Myung et al., 2001a; Myung et al., 2001c; Myung et al., 2003; Myung et al., 2004).

RNASEH2 defects require a function associated with lys164 on PCNA to prevent accumulation of DNA damage that can lead to genome instability.

The results so far point to the hypothesis that the postreplication repair pathway is the preferred pathway of repair for the DNA damage induced by *rnaseh2* defects. Based on this hypothesis it was predicted there would be an increase in the rate of GCRs in an *rnh203* strain combined with mutations that inactivate postreplication repair.

GCR results determined that RNaseH2 and a function associated with lys164 on PCNA are involved in a pathway that suppresses GCRs. The rate found for the *pol30-119* single mutant (2.9×10^{-9}) (Table 2-10) was within 3 fold of what had already been published (Motegi et al., 2006) and is 18 fold less than the rate for *rnh203 pol30-119* double mutant (5.3×10^{-8}) (Table 2-10). In line with this, it was predicted that the GCR rates would also go up when the *rnh203* mutation was combined with *rad5*, *rad6*, and *rad18* mutations and possibly also a *siz1* mutation. Actually what was found though was no significant effect on the GCR rate.

Table 2-9. Effect of *rnh203Δ* on the rate of accumulating GCRs in different checkpoint defective strains.

Genotype	Wild type Mutation rate (Can^r 5-FOA^r)	*rnh203Δ* Mutation rate (Can^r 5-FOA^r)
Wild type	3.6×10^{-10} (1)	2.0×10^{-10} (0.5)
dun1 Δ	8.9×10^{-8} (247)	1.2×10^{-7} (333)
mec3 Δ	$< 2.4 \times 10^{-9}$ (6)	5.7×10^{-9} (15)
mrc1 Δ	4.3×10^{-9} (12)	2.1×10^{-9} (6)
rad17 Δ	4.9×10^{-9} (14)	6.9×10^{-9} (19)
rad53 Δ	7.4×10^{-9} (21)	5.4×10^{-9} (15)
tel1 Δ	5.9×10^{-9} (16)	8.0×10^{-10} (2)

All strains are isogenic to RDKY5027 [ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2Δbgl, hom3-10, ade2Δ1, ade8, hxt13::*URA3*]

The GCR rates found for these double mutants were all similar to the rates of their respective single mutant. The rate for the *rad5* single mutant (2.4 X 10^{-9}) and the *rnh203 rad5* double mutant (<2.6 X 10^{-9}), and the *rad6* single mutant (2.3 X 10^{-9}) and the *rnh203 rad6* double mutant (4.2 X 10^{-9}), and the *rad18* single mutant (4.0 X 10^{-9}) and the *rnh203 rad18* double mutant (1.2 X 10^{-8}), and the *siz1* single mutant (1.9 X 10^{-9}) and the *rnh203 siz1* double mutant (4.7 X 10^{-9}) are all reported in Table 2-10.

The rate for the *rad6* single mutant is within 3 fold of what was previously reported (Motegi et al., 2006). However, the rate I found for both *rad5* and *rad18* was lower than what has been reported (Hwang et al., 2005; Motegi et al., 2006; Smith et al., 2004), but similar to unpublished results (personal communication). Given the discrepancy between the results here and results found by some others, independently collected single mutant strains were subjected to multiple rounds of re-testing and the same results were consistently.

Based on the prediction that the GCR rates would increase in these mutants these results were puzzling. A few different scenarios could explain the data. First, that the absence of increased rates of accumulation of GCRs could be explained by the idea that the DNA damage is modified in such a way that it does not generate GCRs. Or alternatively, because of the very sick phenotype of the *rnh203 rad5, rnh203 rad6,* and *rnh203 rad18* double mutants that is evidenced in the growth spot and doubling time assays

(Figure 2-3 and Figure 2-10) it could be due to a high frequency of cell death that the detection limit of the GCR assay is never reached.

A high frequency of cell death in these double mutants is the most likely scenario. In part, because optimal cell densities for the assay were never reached when these double mutants were tested and this was even after incorporating different modifications to the protocol. Furthermore, this explanation would support the hypothesis that the *rnhash2* induced DNA damage is preferentially repaired by postreplication repair. If cells depend on postreplication repair to fix *rnaseh2* induced DNA damage then it would make sense that abolishing postreplication repair as would be the case in the *rnh203 rad5*, *rnh203 rad6* and *rnh203 rad18* mutants then that might presumably lead to high frequencies of cell death.

One other explanation for what could be considered as a very slight effect or no effect on the GCR rate in the double mutants that were defective for postreplication repair could be that the *rnaseh2* induced DNA damage is shuttled to alternative repair pathways, such as recombination, therefore the damage is repaired and increased GCRs then would not be detected. If this were happening then presumably it is not very efficient. An alternative repair pathway, such as recombination might take longer for the cell to process defects and this would explain why the cell cycle slows down and growth defects are seen in these double mutants.

It is known that both Rad6 and Rad18 are required for the DNA damage response related ubiquitination of lys164 (Stelter and Ulrich, 2003), and taken together with the results that include the increase in the GCR rate found for *rnh203 pol30-119,* but not an *rnh203* mutation combined with *rad5, rad6,* or *rad18* mutations suggests that *rnaseh2* induced DNA damage does not involve an ubiquitination function involving PCNA (Table 2-10). It is known that the sumolyation of lys164 on PCNA requires Siz1 (Hoege et al., 2002), and taken together with the increase in the GCR rate found for *rnh203 pol30-119,* but not *rnh203 siz1* suggests that *rnaseh2* induced DNA damage does not involve a sumolyation function involving PCNA for suppression of GCRs (Table 2-10). These results then might suggest a novel pathway involving RNaseH2 and the function taking place at lys164 of PCNA that does not involve ubiquitination or sumoylation.

Severe growth phenotypes were found in double mutant strains carrying an *rnh203* mutation that were also combined with mutations that inactivate postreplication repair defects (Figure 2-10). For the most part, normal growth phenotypes in addition to one growth phenotype that was moderate, not severe, were seen in the double mutant strains carrying an *rnh203* mutation that was combined with defects in HR (Figure 2-1, Figure 2-5). Taken together with an absence of increased rates of GCRs in the double mutant strains with an *rnh203* mutation combined with mutations that inactivate HR and a synergistic increase in the GCR rate for the *rnh203*

pol30-119 double mutant strain might suggest that postreplication repair is the preferred pathway of repair for DNA damage caused by loss of *RNASEH2*.

This idea that in the absence of postreplication repair, DNA damage is shuttled to other pathways for repair and that HR is the most likely alternative is similar to what has been described for *srs2* mutants (Reenan and Kolodner, 1992) and was also suggested as the same mode of repair in *mgs1* mutants (Kim et al., 2005). Mutations in *MGS1* are also thought to cause defects in Okazaki fragment processing leading to an accumulation of unresolved replication intermediates that leads to genome instability.

Testing triple mutant strains carrying an *rnh203* mutation combined with mutations that inactivate postreplication repair and also mutations that inactivate HR in the GCR assay would help to answer this question. However, after several attempts at using different approaches these strains have still not been obtained, which raises the possibility that these triple mutants might be synthetically lethal. If so, this would lend support to the hypothesis that *rnaseh2* defects are repaired preferentially by postreplication repair and require HR in the absence of postreplication repair for survival. However, this needs to be accurately tested using an alternative method such as tetrad dissection.

Table 2-10. Effect of *rnh203Δ* on the rate of accumulating GCRs in different postreplication repair defective strains.

Genotype	Wild type Mutation rate (Can^r 5-FOA^r)	*rnh203Δ* Mutation rate (Can^r 5-FOA^r)
Wild type	3.6×10^{-10} (1)	2.0×10^{-10} (0.5)
pol30-119Δ	2.9×10^{-9} (8)	5.3×10^{-8} (147)
rad5Δ	2.4×10^{-9} (7)	$< 2.6 \times 10^{-9}$ (7)
rad6Δ	2.3×10^{-9} (6)	4.2×10^{-9} (12)
rad18Δ	4.0×10^{-9} (11)	1.2×10^{-8} (33)
siz1Δ	1.9×10^{-9} (5)	4.7×10^{-9} (13)

All strains are isogenic to RDKY5027 [ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2Δbgl, hom3-10, ade2Δ1, ade8, hxt13::*URA3*]

Analysis of RNaseH2 synthetic lethality

The concept of using synthetic lethality as a tool to explore the function of genes is a well established method (Dobzhansky, 1946), whereas including synthetic fitness in the overall analysis of synthetic lethality screens to determine gene function has more recently emerged as a conventional method . Synthetic lethality describes any combination of two separately non-lethal mutations that leads to inviability, whereas synthetic fitness indicates a combination of two separate non-lethal mutations that confers a growth defect more severe than that of either single mutation. The interpretation is that synthetic fitness reflects an important genetic interaction, whereas synthetic lethality reflects an essential interaction (Ooi et al., 2006).

To keep the labeling of these methods consistent with the literature from now on genetic interaction methods and results will be referred to as synthetic lethality when referencing groups of genetic interactions or when appropriate a singular interaction will be referred to as synthetic fitness. Additional insights emerging from synthetic lethality data sets reveal that genes within the same pathway or complex tend to share similar synthetic lethality profiles (Tong et al., 2004; Ye et al., 2005).

With this in mind, Cytoscape (Shannon et al., 2003) and extensive literature searches that involved methods used in the next chapter that are described elsewhere (Putnam, et. al, in preparation) were used to find out

which genes shared 15 or more similar synthetic lethality partners to RNaseH2 (Fig.11, panel A). To further expand the analysis the common synthetic lethal partners among the genes with the similar profiles were listed and the prevalence of a particular gene on the list as a common interactor was also determined (Fig.11, panel B).

Three different scenarios were considered to explain the data that emerged from the synthetic lethality data sets. To better organize the discussion, the scenarios were categorized and named as follows: genetic redundancy, same substrate – same mechanism, and "the leak and the broken pump".

Analysis of RNaseH2 synthetic lethality: Genetic redundancy

Genetic redundancy has been defined a number of ways and can actually encompass many different interpretations of synthetic lethality data sets. The definition used here for genetic redundancy means that two genes or two pathways that are redundant can partially or fully substitute for the function of the other (D'Amours and Jackson, 2001). In other words, redundant genes are in the same pathway and perform the same function. The presence of genetic redundancy has been a long-established topic of study producing large collections of work describing this phenomenon including evidence that supports a hypothesis that whole-genome duplication is an important evolutionary mechanism (Wolfe and Shields, 1997).

Applying the narrowed definition of genetic redundancy used here to the *RNASEH2* synthetic lethality data set would argue that each of the genes found to have similar synthetic lethality profiles (Figure 2-11, panel A) are all in the same pathway and share the same function. Intuition alone suggests that this is not very plausible as it is highly unlikely that such a large number of genes perform the same function. Most of the genes that share synthetic lethality profiles to RNaseH2 have been well characterized and their functions are known, as demonstrated by the gene categorization according to functional pathways (Figure 2-11, panel A). Genes that share synthetic lethality profiles with RNaseH2 have many variable functions.

Analysis of RNaseH2 synthetic lethality: Same substrate – Same mechanism

The same substrate – same mechanism scenario explains the synthetic lethality data set in the context of the type of DNA damage that is generated when there are defects in the genes that share synthetic lethality profiles. The idea is that defects in genes with similar synthetic lethality profiles will produce the same type of DNA damage generating a substrate that requires the same synthetic lethal partner for proper processing. In this scenario, it would be expected that a genetic analysis of the strains carrying mutations that inactivate the genes with similar synthetic lethality profiles (Figure 2-11, panel A) would demonstrate that the DNA damage induced by the defects would require the same synthetic lethal partners for normal

growth. The synthetic lethal partners that would be expected to be required for normal growth are listed in Figure 2-11, panel B. The data reveal that many of the synthetic lethal partners are the same for genes with similar synthetic lethal profiles as is demonstrated by the prevalence of a particular gene as a common interactor (Figure 2-11, panel B).

This data then might also help predict the type of damage generated in *rnaseh2* mutants based on the same substrate-same mechanism analysis. It is known what type of damage is produced in many of the mutants that carry mutations in genes with similar synthetic lethality profiles to *RNASEH2.* Additionally, the pathways that are required to process the *rnaseh2* induced DNA damage might also be predicted because it is also known which pathways are required to process the damage induced by defects in genes that have similar synthetic lethality profiles to RNaseH2.

It is known that *sgs1* mutants have a hyperrecombination phenotype that generates replication defects, triggers checkpoints and requires HR pathways for processing/repair. The synthetic lethality profile data revealed that *SGS1* was the most similar gene to *RNASEH2* with 22 common synthetic lethal partners. Based on this, the prediction would be that *rnaseh2* mutants would also have a hyperrecombination phenotype. The *rnh203* mutation spectrum analysis found one 27bp duplication that may suggest similarity to the *rad27* mutation spectrum data (Table 2-3) The increased rate of

duplications seem in *rad27* mutants was attributed to a hyperrecombination phenotype (Tishkoff et al., 1997)

The predicted *rnaseh2* replication defects based on a *sgs1* phenotype would be expected to trigger checkpoints. The results presented in this study do indeed suggest that defects in *RNASEH2* causes checkpoint activation as was evidenced by several results in this report including the severe growth defects in the *rnh203 mec1* and *rnh203 rad53* double mutants (Figure 2-9) Furthermore, the prediction that *rnaseh2* induced defects depend on HR for processing/repair based on a *sgs1* phenotype was also in line with results found in this study as was evidenced by growth defects in an *rnh203* strain combined with mutations that inactivate HR (Figure 2-5).

Consistent with this logic it can also be predicted what type of damage will not arise due to defects in *RNASEH2*. The genes that are missing from the list of similar synthetic lethality profiles to *RNASEH2* could reveal the type of damage that will not be seen in *rnaseh2* mutants. Based on the same substrate-same mechanism analysis it would be expected that *rnaseh2* defects would not lead to the formation of stable R-loops, because of the absence of genes known to be involved in transcription functions that prevent an accumulation of the R-loop type of DNA damage, such as *BUR2, SNF2, SIN3, MED13, NOT5, SP2, CDR8,* and *KEM1* (unpublished data) and (Figure 2-11, panel A).

Analysis of RNaseH2 synthetic lethality: The leak and the broken pump

When your boat springs a leak, you can use a pump to bail it out. But when the pump breaks, it's time to find the lifejackets. Analogous logic is commonly exploited by geneticists who seek mutations at additional loci that enhance or suppress the phenotype caused by a particular mutation (Tucker and Fields, 2003). The leak and the broken pump scenario is a "real-life" example of what could be happening at the molecular level to help clarify one other interpretation of the data.

The leak and the broken pump scenario described above is a situation that involves two separate different defects that are occurring at the same time and is analogous to two individual genes acting in the same, essential pathway, with the combination of the two effects causing synthetic lethality or synthetic sickness. Or in other words, two genes have an individual function that may be either similar or distinct, and either one of the genes is required for a third intersecting pathway (Thomas, 1993).

For each of the single mutants the third intersecting pathway remains intact because the function requires one or the other, but the third intersecting pathway is inactivated in the double mutant. The synthetic lethality data demonstrates that genes with similar synthetic lethality profiles to *RNASEH2* also demonstrate a high frequency of common synthetic lethal partners (Figure 2-11, panel B) suggesting that there might be additional

intersecting pathways that require either *RNASEH2* or the synthetic lethal partners of *RNASEH2*-like genes.

It is known that defects in most of the genes that demonstrated similar synthetic lethality profiles to *RNASEH2* result in reduced capacity of repair suggests that the most probable intersecting pathway between the genes with similar synthetic lethal profiles and the common synthetic lethal partner is DNA repair. This argues that *RNASEH2* and genes involved in reducing mutations could be interfacing in a "leak and the broken pump" manner.

2.4 DISCUSSION

The data in this report raise the possibility that the absence of *RNASEH2* may impair the processing of Okazaki fragments, leading to genome instability. The first line of evidence suggesting that RNaseH2 is involved in suppression of genome instability was found from the mutation spectrum analysis of the *rnh203* strain. It was found that a mutation in *rnh203* lead to an increase in the rate of double base substitutions and a slight increase in the rate of accumulating duplications (Table 2-3)

These results were similar to what was found for *msh2* and *rad27*. It is known that Rad27 is involved in a pathway that prevents large duplications that are flanked by direct repeats (Tishkoff et al., 1997) that have been suggested to arise from long range DNA polymerase slippage

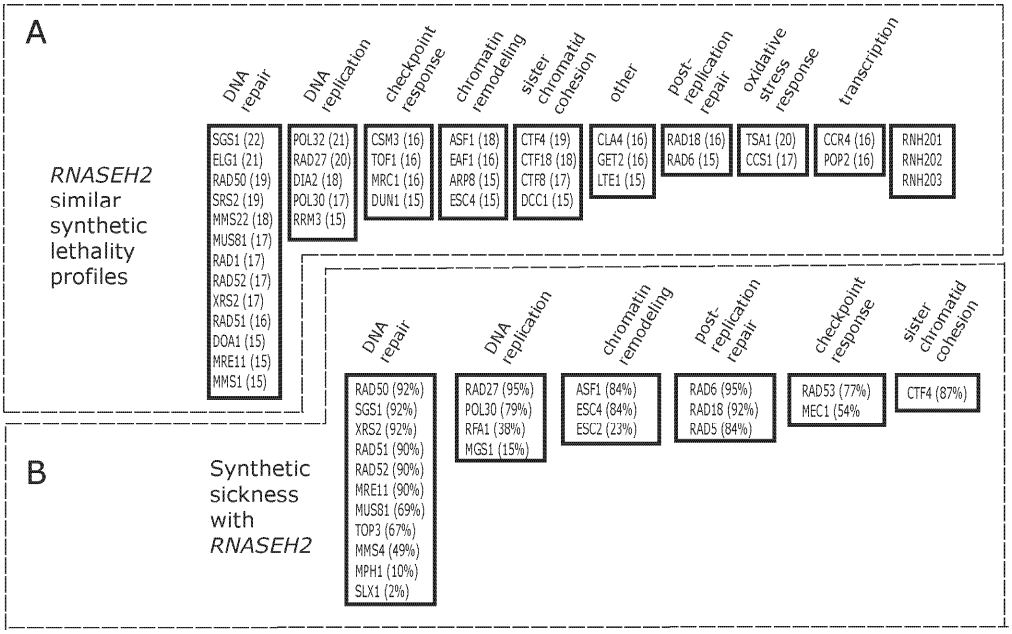

Figure 2-11: *RNASEH2* synthetic lethality profile.
A. Genes found to have 15 or more common synthetic lethality partners to RNaseH2, the number in () represents the number of common interactors with RNaseH2.
B. The synthetic lethal partners to RNaseH2, the number in () represents the % that this gene is a common interactor among genes with similar profiles to RNaseH2. Closer to 100% represents a common interactor among genes with similar profiles, genes cannot interact with themselves therefore 100% is not represented.

errors. Mutation spectrum data from *msh2* revealed that Msh2 is involved in a pathway that prevents deletions in regions of short mononucleotide repeats which was also suggested to arise from DNA polymerase slippage errors (Marsischky et al., 1996). Mutation spectrum data from *rnh203* found increased rates of deletions in regions of dinucleotide repeats and increased rates of duplications compared to the wild-type rates suggesting that RNaseH2 is also involved in a pathway that prevents errors due to long range slippage of DNA polymerase.

In order to make predictions about how *RNASEH2* is involved in genome instability, a carefully selected subset of DNA metabolism genes were surveyed in a pathway directed manner for their potential genetic interaction with *RNASEH2*. The simple test of spotting serial dilutions of cell cultures on YPD plates to compare the growth of *rnh203* double mutant strains to wild-type and respective single mutant controls identified pathways involving HR, chromatin assembly/remodeling, sister chromatid cohesion, DNA replication, checkpoints, and postreplication repair for further investigation. Doubling times were determined for the relevant single and double mutants in the case of those found to have genetic interactions with *rnh203* and additional genes from each of the pathways implicated.

The doubling time results for an *rnh203* strain that had been combined with mutations that inactivate DNA replication pathways provided many clues about the type of DNA damage that might be arising in the

rnaseh2 mutants. Severe growth defects were found in the *rnh203 mgs1*, *rnh203 rad27*, and *rnh203 rfa1t*-33 double mutants (Figure 2-8) implicating RNaseH2 in pathways important for resolution of replication intermediates generated during the Okazaki fragment maturation step of lagging strand DNA replication. One possibility is that RNaseH2 might be involved in preventing or resolving the lagging strand intermediates that are generated when long flaps create foldbacks which may lead to an accumulation of the type of secondary structures described in (Shor et al., 2002).

The data presented here support a role for RNaseH2 in a pathway required for preventing accumulation of DNA damage that is likely in the form of secondary structures arising from defects in Okazaki fragment processing and that persistence of *rnaseh2* induced DNA damage will lead to genome instability. The rest of the discussion highlights the processes required for processing *rnaseh2* induced defects for prevention of genome instability.

The doubling time tests found many severe and a few weak growth defects in an *rnh203* strain that was combined with mutations that inactivate HR including mutations in *MUS81, RAD51, MMS4, MPH1, MRE11, RAD50, RAD52, SGS1, SLX1, TOP3,* and *XRS2* (Figure 2-5). Many of these proteins known to be involved in HR have been implicated in several different functional roles. A key study found that toxic recombination intermediates that are likely to be generated by single stranded DNA resulting from

replication arrest depends on two different pathways for processing one is Sgs1 dependent and the other relies on Mus81 and Srs2 (Fabre et al., 2002). It would be expected that *rnaseh2* induced DNA damage might also arise from single stranded DNA resulting from improper resolution of long flap intermediates that likely will also lead to replication arrest.

These predictions and data thus far taken together with the severe growth defect found for the *rnh203 sgs1* double mutant and weak growth defect found for the *rnh203 mus81* double mutant and no growth defect found for the *rnh203 srs2* double mutant suggests that *rnaseh2* induced DNA damage more heavily relies on Sgs1 related pathways of repair.

Furthermore, severe growth defects were also found in an *rnh203* strain that was combined with mutations that inactivate each of the subunits of the MRX complex and additionally a moderate growth defect was found in an *rnh203* strain that was also defective for Rad52. It has long been established that the MRX complex is involved in checkpoint functions (Boulton and Jackson, 1998; Connelly and Leach, 2002; D'Amours and Jackson, 2001, 2002; Haber, 1998; Tsukamoto et al., 2001), and Rad52 is required for all HR pathways in *Saccharomyces cerevisiae.*

The growth defects found in an *rnh203* strain combined with mutations that inactivate HR suggest that *rnaseh2* induced DNA damage might trigger a checkpoint that may require Sgs1 and MRX for activation

and/or proper processing of intermediates to possibly generate a substrate recognizable by checkpoint and/or HR repair machinery.

Interestingly, it was also found that an *rnh203* strain combined with mutations in *MEC1* and *RAD53* that inactivate the central components of the S-phase checkpoint also caused severe growth defects (Figure 2-9). Involvement of Sgs1 in the S-phase checkpoint is specific to the intra-s checkpoint (Kolodner et al., 2002). Taken together with the interpretation of the results from the survey of HR proteins, especially the *rnh203 sgs1* result implies that *rnaseh2* induced DNA damage specifically activates the intra-s checkpoint. Furthermore, when *rnh203 sgs1* was tested in the GCR assay an increased GCR rate was found in the double mutant compared to each of the single mutants (Table 2-6) indicating that the pathway requiring Sgs1 for activation/maintenance of intra-s checkpoint is important for the suppression of GCRs.

Severe growth defects were also found in an *rnh203* strain that had been combined with mutations that inactivate chromatin assembly/remodeling (Asf1), and gene silencing functions (Esc2, Esc4) (Figure 2-7). Esc4 contains a cluster of SQ/TQ motifs that are phosphorylated by Mec1 in response to DNA damage (Rouse, 2004). More recently, work was done that found the increased levels of Rad53 phosphorylation known to be associated with the DNA damage response was absent in *asf1* mutants (Kats et al., 2006). Providing even further evidence

for an involvement of the intra-s checkpoint for processing of *rnaseh2* induced DNA damage.

One scenario that might explain why chromatin assembly/remodeling pathways are involved in the DNA damage response that is induced by *rnaseh2* defects which is also presumably related to the intra-s checkpoint is that the chromatin assembly/remodeling functions are regulating the checkpoint. One possibility is that the intra-S checkpoint activation might require gene silencing to signal DNA replication arrest and chromatin reassembly may be required to signal checkpoint release following proper processing of the defects caused by loss of *RNASEH2*. Furthermore, an increased GCR rate was found in the *rnh203 esc4* and *rnh203 esc2* double mutant compared to either of the respective single mutants (Table 2-7) indicating that chromatin function is important for processing of *rnaseh2* induced DNA damage for suppression of GCRs and prevention of genome instability.

It was determined that *rnaseh2* single mutants grow normally, have wild-type GCR rates and low mutation rates in the *CAN1* assay, which might suggest the existence of a pathway that can efficiently and accurately process *rnaseh2* defects. The data suggests that HR is likely not the preferred pathway of repair, because combining an *rnh203* mutation with mutations that inactivate HR did not cause increases in GCR rates. This may imply that *rnaseh2* defects are processed by a different repair pathway.

The data presented in this study implies that *rnaseh2* defects are more likely processed by postreplication repair. Postreplication repair is a repair pathway that does not remove DNA damage but instead bypasses damage that is encountered. The results from the mutation spectrum are in line with this hypothesis because the type of mutations that occurred in the *rnaseh2* mutants was suggestive of DNA polymerase slippage errors. During postreplication repair, it is known that DNA polymerase terminates synthesis at the site of the DNA lesion and then restarts downstream of the lesion creating a situation highly susceptible to slippage errors.

Furthermore, severe growth defects were found in the *rnh203 rad5, rnh203 rad6,* and *rnh203 rad18* double mutants (Figure 2-10) and an increased rate of GCRs in the *rnh203 pol30-119* double mutant compared to either respective single mutant (Table 2-10). It is known that postreplication repair depends on the Rad6-Rad18 ubiquitin conjugating complex and PCNA (Torres-Ramos et al., 2002).

A mechanism involving postreplication repair as the preferred pathway of repair has been proposed for processing DNA damage caused by defects in Mgs1. It is thought that Mgs1 functions to either resolve or prevent long flap intermediates that arise during the Okazaki fragment processing stage of DNA replication. The data presented in this study suggests that RNaseH2 is also involved in either preventing or resolving aberrant replication intermediates that may arise due to faulty Okazaki

fragment processing which may also implicate that the processing of *rnaseh2* defects is carried out by the same repair mechanism as Mgs1.

To further explore the function of *RNASEH2* in preventing genome instability the characterization of the genetic interactions was expanded to include synthetic lethality analysis. The genes that shared similar synthetic lethality profiles to *RNASEH2* were identified. The synthetic lethality partners of the *RNASEH2*-like genes were also determined. Predictions made on synthetic lethality data alone are consistent with the results found from the doubling time measurements, the mutation spectrum analysis and the GCR tests.

Some of what might be predicted from the synthetic lethality analysis include that *rnaseh2* defects are similar to HR defects that trigger the intra-s checkpoint and require HR for repair/processing. The data presented here is consistent with this prediction. Given that genes with similar synthetic lethality profiles to *RNASEH2* also demonstrated a high frequency of common synthetic lethal partners many other predictions can be made about the involvement of the DNA metabolism pathways that process *rnaseh2* defects and were already described throughout the dissertation. Synthetic lethality analysis also suggested that there are intersecting pathways requiring either *RNASEH2* or *RNASEH2*-like genes that are important for prevention of genome instability.

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CHAPTER 3

Directed survey to identify genes encoding proteins that function to prevent genome instability.

3.1 INTRODUCTION

Cells require a stable genome and protection from uncontrolled genome alterations for stable transmission of genetic information. Mutations are known to cause many different diseases and ongoing genome instability is seen in many types of cancers. Different types of genome instability have been identified including accumulation of mutations, chromosomal rearrangements and aneuploidy. One type of genome instability observed frequently is gross chromosomal rearrangements (GCRs). GCRs include translocations, deletions of chromosome arms, interstitial deletions, inversions, amplifications, and chromosome end-to-end fusion (Kolodner et al., 2002).

Several assays have been developed over the years looking at different types of gross chromosomal rearrangements (GCRs). The GCR assay developed several years ago in the Kolodner lab allows us to detect the formation of translocations, interstitial deletions, chromosome fusions, and terminal deletions associated with de novo telomere additions (Chen et al., 1998; Myung et al., 2001b). The analysis of mutant phenotypes to identify genes involved in preventing genome instability has been hampered by the tedious nature of these assays. Still many researchers have contributed to the extensive literature on GCRs describing the pathways involved in suppressing GCRs (Putnam et al., 2004; Smith et al., 2004) and pathways required for the formation of GCRs (Hwang et al., 2005; Myung et al.,

2001a; Myung et al., 2004; Pennaneach and Kolodner, 2004; Putnam et al., 2004).

This chapter describes an initial genome wide approach to identify unanticipated genes involved in genome stability that integrates the increasing amounts of phenotypic data generated for *Saccharomyces cerevisiae* mutants. The large collection of work describing genome instability support the view that the type of defects that cause genome instability are mostly due to faulty DNA repair and DNA damage responses. Furthermore, it has been well established that accumulation of DNA damage can lead to genome instability and possibly cancer. The novel genome-wide approach used to generate an enriched list of candidate genes thought to encode functions important for suppression of genome instability produced several functional clusters of genes known to be involved in the DNA damage response pathway. A partial list of genes from the enriched list were tested as single mutants in the GCR assay to gain further insights into the mechanisms underlying genome instability.

3.2 MATERIALS AND METHODS

Generation of a list of genes that are likely to function in genome instability

The method used to generate a list of genes that are likely to be involved in genome stability was generated using computational methods that involved integrating large collections of data sets is described in detail

elsewhere (Putnam, in preparation). Briefly the data sets containing genes demonstrating the ability to suppress GCR rates and data sets containing genes with increased sensitivity to various DNA damaging agents were merged to create a first iteration of the list. The second iteration expanded the list by adding in genes that were found to have similar genetic interaction networks to each of the genes from the first iteration.

Yeast Strains

All of the strains used in this study are *S. cerevisiae* strains that are derivatives of S288C. Single mutant strains were made by deleting the gene of interest in RDKY3615 (*MATa ura3-52, trp1Δ63, his3-200, leu2Δ1, lys2ΔBgl, hom3-10, ade2Δ1, ade8*, *hxt13*::*URA3*) strain by HR-mediated integration of PCR fragments according to standard methods. All strains were grown at 30°C. Strains and their complete genotypes are listed in Table 3-1. Media for propagating strains have been described previously (Chen et al., 1998).

Gross Chromosomal Rearrangement (GCR) Assay

The GCR strain, RDKY3615, was used as the background strain for all of the strains in this study to allow determination of the rate at which GCRs were occurring. In the RDKY3615 strain, *HXT13* (<7.5-kb telomeric to *CAN1*) was replaced with a *URA3* cassette allowing for detection of

translocations, and other classes of genome rearrangements by simultaneously selecting for the loss of *CAN1* and *URA3*. Cells resistant to CAN and 5FOA have undergone a gross chromosomal rearrangement leading to a breakpoint in the region between *CAN1* and *PCM1* as well as potentially all of the DNA from *CAN1* to the telomeres (Chen and Kolodner, 1999). The rate of accumulation of GCRs in cell populations was determined by fluctuation analysis by using the method of the median (Lea, 1948) as described (Marsischky et al., 1996).

For the fluctuation tests of Can^r-$5FOA^r$ mutation rates, five to seven independent cultures of either two or three independently collected strains were analyzed in each experiment. If two independent strains were obtained, then 14 independent cultures were analyzed. If three or more independent strains were obtained, then 15 independent cultures were analyzed.

3.3 RESULTS

Comprehensive data gathering reveals 945 candidate genome instability genes.

Given the availability of systematic studies using the yeast knockout collection a strategy was designed to leverage this information to generate a list of candidate genes enriched in those that function in genome stability. The data sets with the most direct functional correlation to genome stability

Table 3-1:*Saccharomyces cerevisiae* strains used in this study.

Strains	Relevant genotype	Source
RDKY 3615	Wild-type	Chen and Kolodner, 2001
JEY 2917, SRNY 1068, SRNY 1069	*cdc73::HIS3*	This study
RDKY 6485, RDKY 6486	*cdh1::HIS3*	This study
RDKY 6250, RDKY 6274	*cla4::KAN*	This study
JEY 3013, JEY 3014	*ctf19::HIS3*	This study
RDKY 6412, SRNY 1098, SRNY 1099	*ctk1::HPH*	This study
RDKY 6477, RDKY 6478	*ddc1::HIS3*	This study
SRNY 1140, SRNY 1141, SRNY 1142	*dot1::HPH*	This study
SRNY 1164, SRNY 1165, SRNY 1166	*dpb4::KAN*	This study
SRNY 1163	*dst1::KAN*	This study
RDKY 6483, RDKY 6484	*eaf1::HIS3*	This study
RDKY 6487, RDKY 6488	*hsl1::KAN*	This study
RDKY 6490, SRNY 1138, SRNY 1139	*hta1::HIS3*	This study
SRNY 992, SRNY 1059, SRNY 1060, SRNY 1061	*irc3::HIS3*	This study
SRNY 1108, SRNY 1109	*irc15::HIS3*	This study
RDKY 6393, SRNY 1102, SRNY 1103	*lge1::HIS3*	This study
SRNY 911, SRNY 2000, SRNY 2001	*mph1::KAN*	This study
RDKY 6489, SRNY 1136, SRNY 1137	*mup60::HIS3*	This study
RDKY 6640, RDKY 6641	*mup120::HIS3*	This study
RDKY 6476, SRNY 1134, SRNY 1135	*mup133::HIS3*	This study
SRNY 1167, SRNY 1168, SRNY 1169	*psy2::KAN*	This study
SRNY 1072, SRNY 1073	*sap30::HIS3*	This study
RDKY 5652, SRNY 1133	*trf4::HIS3*	This study
SRNY 1070, SRNY 1071	*ypt6::HIS3*	This study

All strains are isogenic to RDKY 3615 [ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2Δbgl, hom3-10, ade2Δ1, ade8, hxt13::*URA3*] in addition to the mutations indicated.

are the GCR studies revealing the numerous genes involved in pathways that suppress the rate of accumulating gross chromosomal rearrangements. The other data sets used that also implicate numerous genes in pathways important for genome stability are studies analyzing sensitivities to various DNA damaging agents.

The 76 genes found to suppress GCR rates and 554 genes with significant sensitivity to DNA damaging agents out of the 4,262 genes identified in 153 DNA damage sensitivity studies were merged to create the first iteration of the enriched list containing 591 genes (Figure 3-1, panel A). To identify other genes that shared phenotypes with these genes, we included genes that showed similar genetic interactions with the 591 genes from the first iteration list to create a second iteration list containing 945 genes (Figure 3-1, panel B).

The final list of 945 candidate genes were computationally categorized into functional clusters based on their genetic interaction profiles (Putnam, et. al in preparation) (Figure 3-1, panel C). Most genes from the enriched list clustered into either of the 1°, 2°, or 3° DNA repair clusters. However, functional clustering also revealed several genes from the enriched list involved in other cellular functions, such as microtubules, chromosome segregation and kinetochores suggesting that many cellular processes are important for genome stability.

Some genes from the enriched list were then screened through by testing single mutants that were not previously tested in the GCR assay for validation. The Kolodner lab in house GCR assay was used to measure the rate of accumulation of genome rearrangements in single mutants defective for genes from the enriched list including *CDC73, CDH1, CLA4, CTF19, CTK1, DDC1, DOT1, DPB4, DST1, EAF1, HSL1, HTA1, IRC15, IRC3, LGE1, MPH1, NUP60, NUP120, NUP133, PSY2, SAP30, TRF4 (PAP2)*, and *YPT6* (Table 3-2).

The enriched list contains a diverse set of genes encoding an array of cellular functions important for suppression of genome instability.

Some of the genes from the enriched list were examined for their roles in suppressing chromosomal rearrangements as single mutations in the GCR assay. A substantial number of genes from the enriched list showed an increased GCR rate greater than 5 fold compared to the wild-type rate (Putnam, et. al in preparation). Many of the genes from the enriched list have already been shown to suppress genome rearrangements suggesting that the untested genes might also be involved in pathways that suppress GCR rates and ultimately would be important for maintaining a stable genome.

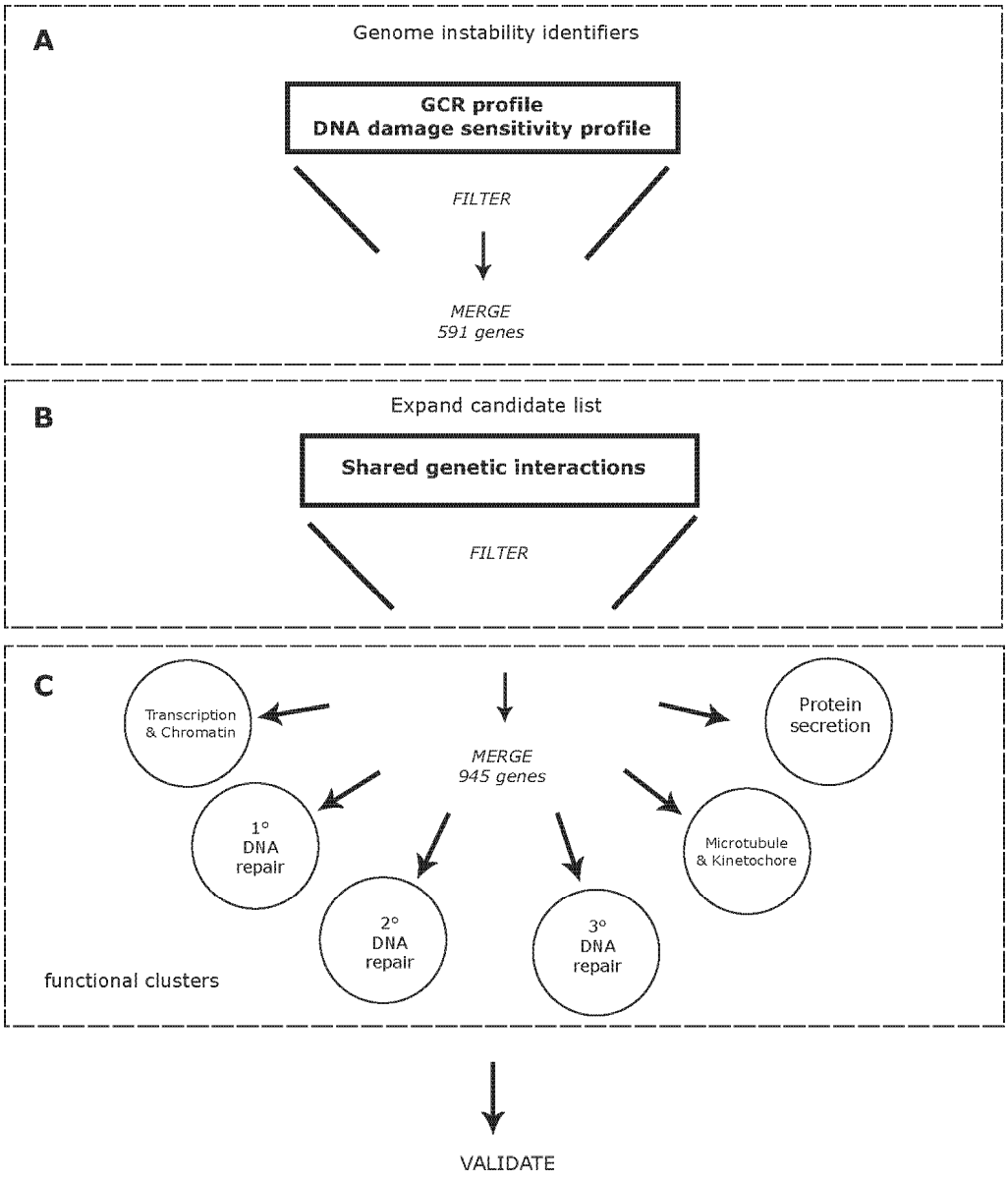

Figure 3-1 Directed survey of genome instability genes.
A) First iteration of enriched list includes GCR and DNA damage sensitivity profiles.
B) Second iteration of enriched list includes similar phenotypes to those found in first iteration based on shared genetic interactions.
C) Final set of enriched list separated by function.

Table 3-2. List of genotypes and functional connections to genome stability

Gene	Primary cellular role	Connection to DNA damage response
CDC73	methylation of histone H3	Chromatin remodeling
CDH1	activator of anaphase promoting comp	Mitotic exit
CLA4	septin ring assembly	Mitotic exit
CTF19	kinetochore assembly	Mitotic exit
CTK1	methylation of histone H3	Chromatin remodeling / BRCA1 interactor
DDC1	cell cycle checkpoint protein	RAD52 foci
DOT1	methylation of histone H3	Chromatin remodeling
DPB4	DNA replication	Chromatin remodeling
DST1	transcription elongation	Chromatin remodeling
EAF1	histone acetylase	Chromatin remodeling
HSL1	bud neck recruitment	Mitotic exit
HTA1	histone subunit (H2A)	Chromatin remodeling
IRC3	undetermined	RAD52 foci
IRC15	undetermined	RAD52 foci
LGE1	methylation of histone H3	Chromatin remodeling
MPH1	homologous recombination	DNA repair
NUP60	nuclear pore	RAD52 foci / NE translocation
NUP120	nuclear pore / mRNA transport	RAD52 foci / NE translocation / BRCA1 interactor
NUP133	nuclear pore / mRNA transport	RAD52 foci / NE translocation / BRCA1 interactor
PSY2	protein phosphatase	DNA repair
SAP30	histone deacetylase	Chromatin remodeling
TRF4 (PAP2)	RNA metabolism	RAD52 foci
YPT6	vesicular transport	none

The enriched list of genes did indeed contain genes that encode functions that are important for suppression of genome instability demonstrated by the greater than 5 fold increase in GCR rate in several of the strains carrying mutations defective for genes from the enriched list (Table 3-3). A wild-type rate (3.6×10^{-10}) (Table 3-3) was found that was similar to a previously published rate for wild-type (Chen and Kolodner, 1999).

CDH1

The most significant effect on GCR rate that found in the data set in this report which includes only some of the genes from the enriched list was a 58 fold increase in rate for the *cdh1* single mutant (2.1×10^{-8}) (Table 3-3) compared to the wild-type rate. The GCR rate found for the *cdh1* mutant was higher than what had already been reported (Ross and Cohen-Fix, 2003) most likely due to strain background differences. Cdh1 clustered into the 2° DNA repair functional cluster. Cdh1 is involved in the G2/M checkpoint pathway reportedly by regulating the ubiquitination of cyclins that act as signals to release cell cycle arrest (Ross and Cohen-Fix, 2003). An increased GCR rate in the *cdh1* mutant suggests that maintenance of a cell cycle arrest is an important DNA repair function that in its absence can lead to incorporation of genome rearrangements that can cause genome instability.

DST1

The *dst1* single mutant had a 6 fold increase in GCR rate compared to wild-type (2.2 X 10^{-9}) (Table 3-3). Dst1 clustered into the transcription/chromatin functional cluster. Dst1 is known to stimulate cleavage of nascent transcripts at stalled transcription sites in a pathway thought to be important for re-entry into the cell cycle. Interestingly, *dst1* was also shown to interact with the Swi-Snf complex known to be involved in nucleotide excision repair and double strand break repair. Dst1 has been shown to have multiple roles. In particular, an involvement in transcription might suggest that defects in transcription can also lead to genome instability.

DDC1 and *IRC15*

The GCR rate found for the *ddc1* single mutant (4.4 X 10^{-9}) (Table3-3) was a 12 fold increase compared to the wild-type rate and the *irc15* single mutant (2.8 X 10^{-9}) an 8 fold increase compared to wild-type (Table 3-3). *DDC1* clustered into the 2° DNA repair functional cluster. *IRC15* clustered into the microtubule/chromosome segregation/kinetochore functional cluster. It has been suggested that Both Ddc1 and Irc15 are involved in the primary DNA damage response as was demonstrated by identification of Ddc1 and Irc15 in a screen for mutants that increased accumulation of Rad52 foci (Alvaro et al., 2007).

It has been proposed that Ddc1 is a DNA damage sensor. Thus far, the cellular role for Irc15 is undetermined. These results taken together with the GCR result suggest that Ddc1 and Irc15 play important roles in preventing formation of genome rearrangements that can lead to genome stability possibly due to an involvement in assembly of repair centers that may act to trigger checkpoints and initiate repair functions.

NUP60 and *NUP133*

Two of the genes tested from the nuclear pore Nup84 complex demonstrated increased rates of GCRs compared to wild type, the *nup133* single mutant (3.7×10^{-9}) (Table 3-3) a 10 fold increase compared to wild-type, and the *nup60* single mutant (2.6×10^{-9}) (Table 3-3) a 7 fold increase compared to wild-type. Both *NUP60* and *NUP133* clustered into the 2° DNA repair functional cluster. The function of the Nup84 complex has been linked to several DNA repair mechanisms (Alvaro et al., 2007; Bennett et al., 2001; Bennett et al., 2008; Chang et al., 2002; Nagai et al., 2008).

An intriguing hypothesis by (Nagai et al., 2008) was the suggestion that the Nup84 complex is required to relocalize DNA damage to the nuclear envelope for proper repair. The data on the involvement of the Nup84 complex in DNA repair taken together with the increased GCR rate in mutants defective for the Nup84 complex might suggest that relocalization of

DNA damage to the nuclear envelope is important to prevent accumulation of genome rearrangements that can lead to genome instability.

Several diverse mechanisms were shown to be important for preventing genome instability including the involvement of Cdh1 in regulation of cell cycle arrest at the G2/M checkpoint. Ddc1 and Irc15 have important functions in the DNA damage response. Dst1 is thought to be involved in regulating re-entry into the cell cycle after transcription arrest, and the Nup84 complex has been implicated in functions related to DNA repair. The enriched list of genes demonstrates that there are multiple pathways involved in the suppression of genome instability requiring a diverse set of proteins.

3.4 DISCUSSION

Genes from the enriched list encode proteins that function to maintain genome stability by preventing an accumulation of GCRs.

The novel genome-wide approach identified previously unknown roles of proteins in maintenance of genome stability by generating an enriched list of genes thought to encode functions important for suppression of genome instability. This analysis implicated pathways involving *CDH1, DDC1, DST1, IRC15, NUP60* and *NUP133* as critical players involved in functions important for maintenance of genome stability.

Table 3-3. Effect of defects in genes from enriched list on the rate of accumulating GCRs

Relevant Genotype	Mutation rate (Can^r 5-FOA^r)
Wild type	3.6×10^{-10} (1)
cdh1Δ	2.1×10^{-8} (58)
ddc1Δ	4.4×10^{-9} (12)
nup133Δ	3.7×10^{-9} (10)
irc15Δ	2.8×10^{-9} (8)
nup60Δ	2.6×10^{-9} (7)
dst1Δ	2.2×10^{-9} (6)
hsl1Δ	1.9×10^{-9} (5)
mph1Δ	1.6×10^{-9} (4)
dot1Δ	1.4×10^{-9} (4)
hta1Δ	1.4×10^{-9} (4)
cdc73Δ	1.3×10^{-9} (4)
trf4Δ	8.5×10^{-10} (2)
ctf19Δ	8.3×10^{-10} (2)
dpb4Δ	4.7×10^{-10} (1)
nup120Δ	$<1.6 \times 10^{-9}$ (<4)
irc3Δ	$<1.3 \times 10^{-9}$ (<4)
ctk1Δ	$<1.0 \times 10^{-9}$ (<3)
lge1Δ	$<8.2 \times 10^{-10}$ (<2)
sap30Δ	$<8.2 \times 10^{-10}$ (<2)
cla4Δ	$<6.3 \times 10^{-10}$ (<2)
psy2Δ	$<5.4 \times 10^{-10}$ (<2)
ypt6Δ	$<5.0 \times 10^{-10}$ (<1)
eaf1Δ	$<1.7 \times 10^{-10}$ (<0.5)

All strains are isogenic to RDKY3615 [ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2Δbgl, hom3-10, ade2Δ1, ade8, hxt13::*URA3*]

The numbers in parentheses are the fold increase in rate relative to that of the wild-type strain.

Five of the genes contained in the enriched list, *IRC15, NUP60, NUP133, DDC1,* and *TRF4* had been identified in a screen that involved testing for mutations that increase *RAD52* focus formation. Single mutants defective for these genes were tested in the GCR assay and it was found that the *irc15, ddc1, nup60* and *nup133* single mutants all had increased rates of accumulating GCRs compared to the wild-type control suggesting that the proteins encoded by each of these genes have important roles in HR that help to prevent genome instability.

A novel role implicating the Nup84 complex (*NUP60, NUP120, NUP133)* in a DNA damage response pathway that shuttles DNA damage to the nuclear pore also involving the *SLX5/SLX8* ubiquitinating complex thought to target Rad52 also suggests a role for the Nup proteins in HR pathways that prevent genome instability.

DNA damage can arise due to problems with any of the numerous DNA and also RNA metabolic functions that occur normally and some of them in parallel during the different phases of the cell cycle. Cells have a number of checkpoint pathways that respond to DNA damage and aberrant DNA structures that are generated during the different phases of the cell cycle to arrest or slow down the cell cycle. There are checkpoints that act in G1 and G2 as well as two checkpoints that act in S-phase.

Cdh1 has been implicated in a role important for the G2/M checkpoint that also involves the anaphase promoting complex (APC/C). It

has been proposed that Cdh1 together with APC/C regulate the ubiquitination of Cdc28 which acts as a prerequisite for mitotic exit. The *cdh1* single mutant had an increased GCR rate compared to wild-type suggesting that problems with the G2/M checkpoint can lead to genome instability. The function of Dst1 is also thought to be intimately connected to checkpoint function by regulating re-entry into the cell cycle after transcription arrest.

Genes from the enriched list encode proteins that function to maintain genome stability by preventing an accumulation of non-GCR type of DNA damage.

Genes on the enriched list were placed on the list based on the likelihood that the proteins encoded by these genes are involved in pathways important for genome stability. Some of the genes on the list that were tested as single mutants in the GCR assay did not show increases in GCR rates compared to wild-type suggesting that some pathways involving genes from the enriched list are likely to be important for genome instability even though it was demonstrated that they do not function in suppression of GCRs. This suggests that there are other types of non-GCR DNA damage that can lead to genome instability.

Some of the genes from the enriched list tested as single mutants in the GCR assay that did not show increases in GCR rates compared to wild-

type including *CDC73, CLA4, CTF19, CTK1, DOT1, DPB4, EAF1, HSL1, HTA1, IRC3, LGE1, MPH1, NUP120, PSY2, SAP30, TRF4,* and *YPT6*. Hta1, the H2A core histone, Dpb4, a subunit of polε, the Mph1 HR protein, and Psy2 are known to be involved in the DNA damage induced phosphorylation of Rad53. Sap30 and Eaf1 are important for histone deacetylation. Ctk1, Lge1, Cdc73, and Dot1 are known to be involved in histone methylation. Hsl1 and Cla4 are thought to be involved in the G2/M checkpoint. Ctf19 has been suggested to be involved in mitotic exit.

Computational clustering implicates a variety of cellular functions important for maintenance of genome stability.

A wide assortment of proteins implicating a variety of cellular functions in maintenance of genome stability demonstrates the importance of cells possessing a diverse set of proteins that are required to process many different types of DNA damage in order to ensure genome stability.

Defects in some genes from the enriched list cause an increase in the accumulation of subnuclear RAD52 focus formation

Microscopic visualization of fluorescently tagged proteins provides a method for visualizing the relocalization of the central homologous recombination protein RAD52 into subnuclear foci. RAD52 focus formation

in response to DNA damage is a commonly used marker to identify repair complexes that are actively engaged in homologous recombination.

A genome wide analysis discovered 86 genes from a study of the DNA damage response involving a screen for diploid mutants that were capable of causing an increase in Rad52 foci (Alvaro et al., 2007). Six of the genes are from the enriched list generated in this study including *IRC15, NUP60, NUP133, DDC1, TRF4* and *IRC3* (Table 3-2).

Further analysis of the diploid mutants revealed that the different mechanisms involved that lead to the Rad52 focus formation are primarily dependent on the type of defects that the various mutants cause. The *irc15, nup60* and *nup133* diploid mutants displayed increased rates of both direct repeat and interhomology recombination, whereas the *ddc1* diploid mutants demonstrated increased rates of recombination between homologous chromosomes, but rates of sister chromatid recombination were unaffected.

The *trf4* and *irc3* diploid mutants demonstrated increased rates of Rad52 focus formation as did all the diploid mutants found in the screen, however, these two mutants fell into a class that demonstrated no alteration in the rate of any type of recombination (Alvaro et al., 2007).

This data supports the implication that *IRC15, NUP60, NUP133, DDC1, TRF4* and *IRC3* are involved in pathways important for genome stability and also demonstrates the diversity of processes that are involved in helping the cell deal with DNA damage.

Some genes from the enriched list have been implicated in a novel pathway requiring the translocation of DNA damage to the nuclear envelope.

Several studies have confirmed a functional link between the Nup84 complex and DNA repair including studies describing the typical DNA repair related phenotype for the Nup84 complex mutants that is demonstrated by an increased sensitivity to DNA damaging agents (Bennett et al., 2001; Chang et al., 2002). These results along with the increased frequency of Rad52 focus formation already described (Alvaro et al., 2007) and taken together with a more recent study that analyzed the E-MAP genetic interaction profile of the Nup84 complex has spurred the proposal for a novel mechanism involving the Nup84 complex in a DNA damage pathway.

It was suggested that the Nup84 complex is regulated by a sumolyation dependent mechanism that together with SLX5/SLX8 is involved in relocalizing the DNA damage to the nuclear envelope for repair and subsequent replication fork restart (Nagai et al., 2008). These results are consistent for a role of the Nup84 complex in a DNA damage response pathway that is likely to be important for genome instability.

Even more evidence suggesting a role for the Nup84 complex in a critical pathway needed for maintenance of genome stability was derived from the identification of NUP120 and NUP133 in a screen performed in yeast that demonstrated the ability of proteins to interact with BRCA1 over a

vast evolutionary distance. The known involvement of the BRCA1 protein in multiple DNA repair pathways in response to DNA damage (Bennett et al., 2008) further implicates a role for the Nup84 complex in a DNA damage response that is very likely to be important for genome stability particularly when taken together with the other results.

Genes from the enriched list are known to be involved in DNA repair pathways important for genome stability.

There are a large number of mechanisms responsible for the recognition and processing of damaged DNA that ensure cell survival. Many of these pathways have been described in more general terms as DNA repair pathways. Two genes from the enriched list, *MPH1* and *PSY2* are known to be involved in DNA repair. However, the precise DNA repair mechanisms utilized by these two proteins are distinct from any other mechanisms described in this work. Therefore, prompting placement into a more general DNA repair category.

MPH1 is the putative *Saccharomyces cerevisiae* homologue to FANCM which is a newly identified component of the FA (genome stability disorder) core complex known to participate in the DNA damage response (Schurer et al., 2004). Further evidence for *MPH1* involvement in a DNA damage response pathway important for genome stability was revealed by a genetic analysis study of the cellular response to DNA damage implicating a

novel role for *MPH1* in an error-free bypass pathway involving homologous recombination (Schurer et al., 2004) and another group found that *MPH1* is involved in pathways that promote formation of GCRs (Banerjee et al., 2008).

PSY2 has been placed in a DNA damage recovery pathway that alleviates cell cycle arrest. Psy2 is in a complex with Pph3 that together they directly bind activated phosphorylated Rad53, a central component of multiple S-phase checkpoints, to dephosphorylate and deactivate Rad53 to promote stalled fork restart (O'Neill et al., 2007).

These studies are consistent with a role for both *MPH1* and *PSY2* in pathways important for genome stability.

Genes from the enriched list encode functions that coordinate DNA replication and repair by modulating chromatin structure.

The complex and multi-faceted processes that regulate chromatin structure that are taking place before, during and after DNA replication are intimately linked to DNA repair. Cells deal with DNA damage at the same time that they replicate DNA. Chromatin structure is critical to maintenance of genome stability because damaged DNA needs to first be recognized and then repaired and in order for the DNA damage to be accessed by recognition and repair machinery chromatin states need to be modified to loosen the tightly compacted heterochromatin structure.

It is known that the nucleosome is the central component of chromatin structure. The nucleosome is made up of the core histones H2A/H2B and H3/H4. Hta1 from the enriched list is known to be critical for chromatin remodeling functions as it is one of the core histones of the nucleosome. The Hta1 protein encodes the *Saccharomyces cerevisiae* core histone protein H2A known to be phosphorylated by Mec1 in response to DNA damage (Downs et al., 2000).

Given that cells deal with DNA damage at the same time that they replicate DNA it was not surprising that *DPB4* was also on the enriched list. Dpb4 is a subunit of the polε DNA polymerase. polε is a repair polymerase made up of four subunits (Pol2, Dpb2, Dpb3, Dpb4) (Araki et al., 1991; Li et al., 2000; Ohya et al., 2000) that has been described to function in the S phase checkpoint as a DNA damage sensor (Navas et al., 1995).

Interestingly, it has also been proposed that polε might be involved in pathways that connect DNA replication and chromatin remodeling based on the discovery that both Dpb4 and Dpb3 when characterized were found to have histone fold motifs (Li et al., 2000) described as helix strand helix motifs first identified as structural domains in the core histones determined to be responsible for the dimerization of H2A/H2B (Arents and Moudrianakis, 1993).

These studies imply that both *HTA1* and *DPB4* encode proteins that play roles in genome stability pathways. It is likely that the pathways

involving Hta1 and Dpb4 are also connected to chromatin remodeling functions that together help maintain stability of the genome stability.

Genes from the enriched list encode proteins involved in histone specific acetylation functions that modulate chromatin structure.

Modifications of histone proteins are one other aspect of cellular regulation involving DNA repair related processes important for maintenance of genome stability. It is known that posttranslational modifications such as acetylation, methylation, phosphorylation and ubiquitination exist in the cell and some have proposed that it is the varied use of these mechanisms that contribute partly to chromatin function in maintenance of genome stability.

The regulation of transcription by way of histone acetylation is known to be associated with increased transcription and histone deacetylation is associated with repressed transcription which is partly mediated by HDAC complexes (Kuo and Allis, 1998). Of particular interest to this study is the function of Rpd3. The Rpd3 gene encodes the *Saccharomyces cerevisiae* HDAC1 protein which is known to be in a complex with Sin3 and Sap30 (Zhang et al., 1998). Sap30 is another gene from the enriched list.

An interesting study revealed a connection between histone acetylation and maintenance of genome stability. It was demonstrated that

inhibition of histone deacetylation due to loss of either Rpd3 or Sin3 in *rad9* or *mec1* checkpoint mutant strains restores a G2/M cell cycle arrest in the presence of DNA damage (Scott and Plon, 2003).

Another gene from the enriched list, *EAF1*, presented a glimpse into the acetylation side of chromatin remodeling. Eaf1 is known to be a critical component of the NuA4 histone acetylase complex (Mitchell et al., 2008) that has been implicated in the DNA damage response due to an intolerance for the replication blocking agents hydroxyurea, camptothecin and MMS (Christiansen and Westergaard, 1996; Friedberg, 1995; Merrill and Holm, 1999) and was also found as an interactor with BRCA1 in the genetic screen described earlier (Bennett et al., 2008).

A model developed in the Cote lab described the stepwise phosphorylation and acetylation of histones that directs chromatin modification during repair of double strand breaks (Utley et al., 2005). Briefly, in response to DNA damage H2A is phosphorylated, followed by recruitment of the NuA4 complex and subsequent acetylation of histone H4, then deacetylation by Rpd3, then finally phosphorylation of histone H4 to prevent reacetylation (Utley et al., 2005).

These studies imply that both *SAP30* and *EAF1* play roles in genome stability pathways and highlight the importance of acetylation/deacetylation processes of histones in chromatin remodeling functions that together help maintain stability of the genome.

Genes on the enriched list encode proteins that are involved histone specific methylation that modulate chromatin structure.

Histone methylation is one of many histone modifications known to be an important regulatory component of many DNA related processes including transcription (Martin and Zhang, 2005). Studies analyzing the cell cycle under normal conditions without the presence of DNA damage have revealed that *CTK1* (Youdell et al., 2008) and *LGE1* (Krogan et al., 2003b) both on the enriched list are involved in a pathway regulating the methylation of lysine 36 of histone H3.

Similarly, another report on a chromatin remodeling function, however distinct from the last report because data was collected in the context of a DNA damage response also involved the methylation of histone H3, but this time at lysine 79 demonstrating a requirement for Cdc73, a known subunit of the Paf1 complex, and Dot1 (Feng et al., 2002; Krogan et al., 2003a). Both Cdc73 and Dot1 are also on the enriched list. It has been suggested that chromatin remodeling mechanisms are important for maintenance of genome stability because these mechanisms allow access of repair machinery to sites of DNA damage by loosening the tightly packed heterochromatin structure.

These results imply that *CTK1, LGE1, CDC73* and *DOT1* are important for genome stability and emphasize the significance of histone

methylation processes in chromatin remodeling functions that together help maintain stability of the genome.

Genes from the enriched list encode proteins known to be involved in transcription coupled repair that together with chromatin remodeling are important for genome stability

In light of all the evidence describing connections between chromatin remodeling and genome stability pathways gathered thus far it was not surprising to find that the function of yet another gene from the enriched list *DST1* also was connected to pathways involving both chromatin remodeling and genome stability. The results from a genetic screen described a synthetic lethality between Dst1 and the Swi-Snf complex (Davie and Kane, 2000) suggesting that Dst1 could possibly play a role in the DNA damage response via the interaction with the Swi-Snf ATP dependent chromatin remodeling complex known to be involved in NER and DSBR in response to DNA damage (Osley et al., 2007).

Interestingly, the cellular roles for both Ctk1, already described above in a genome stability related histone methylation process, and Dst1 were initially defined in transcription processes and thus provoked some thought about the existence of novel pathways that might be involved in connecting transcription and chromatin remodeling to ensure genome stability.

Possible connections between transcription and genome stability have already been described for a process known as transcription coupled repair. Due to the involvement of Ctk1 and Dst1 in transcription it was also considered that transcription coupled repair could be one other important mechanism for a function critical for genome stability. The primary involvement of Ctk1 in genome stability is likely to be correlated to H3 methylation however Ctk1 was initially identified as a subunit of the Ctdk1 kinase known to phosphorylate RNA pol II (Lee and Greenleaf, 1991).

Even more evidence suggesting a role for Ctk1 in a genome stability pathway was derived from the identification of Ctk1 in the same genetic screen that identified the Nup84 complex. In brief, the known involvement of the BRCA1 protein in multiple DNA repair pathways in response to DNA damage (Bennett et al., 2008) implicates a role for Ctk1 in a DNA damage response particularly when taken together with the other results. Dst1 function could be a more direct involvement of transcription coupled repair in maintenance of genome stability as it is known that the Dst1 protein encodes the *Saccharomyces cerevisiae* transcription elongation factor S-II, which has been described to stimulate cleavage of nascent transcripts at stalled transcription arrest sites (Ubukata et al., 2003).

The involvement of Ctk1 and Dst1 in transcription factors in the possibility that transcription coupled repair could be another important function required to maintain the stability of the genome.

Genes on the enriched list encode proteins that are involved in the G2/M checkpoint that function to maintain cell cycle arrest allowing for repair of DNA damage prior to mitosis

Cells have a number of checkpoints that respond to DNA damage and aberrant DNA structures that are generated during the different phases of the cell cycle to arrest or slow down the cellular processes taking place. Checkpoints allow cells the time needed to repair lesions and process aberrant structures so that cell division proceeds without the DNA damage that can lead to either cell death or incorporation of DNA errors that in some cases can cause cancer.

Each of the checkpoints have been labeled with the same name as the phase of the cell cycle that the arrest or slow down occurs. The G2/M DNA damage checkpoint serves to prevent the cell from entering mitosis with genome DNA damage. Four genes from the enriched list *CDH1, CTF19, HSL1* and *CLA4* are thought to encode proteins that function in pathways involving the G2/M DNA damage checkpoint.

It has been suggested that Cdh1 is involved in a pathway that responds to DNA damage similarly to the assembly spindle checkpoint response which arrests the cell cycle in metaphase to prevent cells from entering anaphase. It was found that defects in Cdh1 caused compromised chromosome segregation and no degradation of Cdc28. These results

support the hypothesis that Cdh1 in combination with the anaphase promoting complex (APC/C) regulates the ubiquitination of cyclins, in particular Cdc28, which acts as a prerequisite for mitotic exit (Ross and Cohen-Fix, 2003).

Another gene from the enriched list, *CTF19*, is also thought to be important for mitotic exit and interestingly a more specific role for the protein in maintenance of genome stability has been investigated. It was demonstrated that the Ame1 and Okp1 components of the COMA complex (Ctf19, Okp1, Mcm21 and Ame1) are required for the spindle assembly checkpoint to maintain a stable arrest to allow for repair of defective microtubule attachments that are sometimes formed at kinetochores (Pot et al., 2005).

Studies analyzing the cell cycle under normal conditions without the presence of DNA damage also placed two other genes from the enriched list, Hsl1 and Cla4, in mitotic exit pathways. It is known that Swe1 is a central component of the cellular structure that is assembled prior to passage into mitosis. It was determined that Hsl1 in combination with Hsl7 regulates the localization of Swe1 to the bud neck (Asano et al., 2005), followed by a subsequent stepwise phosphorylation mechanism of Swe1 involving Cla4 (8). The involvement of Cdh1, Ctf19, Hsl1 and Cla4 in G2/M checkpoint pathways emphasizes the importance of cell cycle regulation in the presence of DNA damage to ensure genome stability. Combining all of these results

demonstrates that there are multiple pathways involved in the suppression of genome instability requiring a diverse set of proteins.

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CHAPTER 4

Summary and Future Directions

4.1 DIFFERENT TYPES OF DNA DAMAGE LEAD TO GENOME INSTABILITY

The results presented in this Dissertation highlight the importance of many diverse proteins in pathways that are important for maintaining genome stability. My work emphasizes how different types of DNA damage can accumulate due to defects in different aspects of DNA metabolism. The results suggest that defects in some pathways presented in this report are the originating source of DNA damage, and alternatively, that defects in some pathways cause an accumulation of DNA damage.

This implies that there are pathways required to prevent the formation of DNA damage and there are also pathways required for dealing with the DNA damage once it becomes present, but that ultimately, both are required for prevention of genome instability. The work presented here determined that defects that give rise to DNA damage or that abolish functions that prevent an accumulation of DNA damage can cause growth defects, an increased rate of accumulation of certain types of mutations in *CAN1,* or an increased rate of accumulation of GCRs, and sometimes these defects can lead to more than one of these phenotypes.

An enriched list of genes thought to function in genome stability was also analyzed. It was found that defects in some of the genes from the enriched list that are thought to abolish functions important for maintenance of

genome stability will sometimes cause an increased accumulation of GCRs validating their role in genome instability. However, sometimes defects in genes from the enriched list did not cause an increase in the GCR rate suggesting that defects in genes thought to be involved in genome stability can produce a non-GCR type of DNA damage (Figure 4-1).

4.2 DEFECTS IN *RNASEH2* MIGHT CAUSE AN INCREASED ACCUMULATION OF FOLDBACKS THAT LEADS TO GENOME INSTABILITY

The double base deletions found in the *rnh203* mutant occurred in regions of short dinucleotide repeats consisting of either 4 or 6 basepairs. This result was quite intriguing because these types of mutations actually resemble what is thought to be DNA polymerase slippage errors. DNA polymerase slippage errors are thought to occur in regions consisting of repeat sequence. One view is that when DNA polymerase encounters repeats it progresses along and then will skip over the repeat sequence to essentially remove the section of DNA that was skipped over.

It has been suggested that this mechanism is triggered due to the formation of secondary structures that can form as a result of aberrant Okazaki fragment processing. This has been described to be one viable explanation for the type of duplication mutations seen in a *rad27* mutant

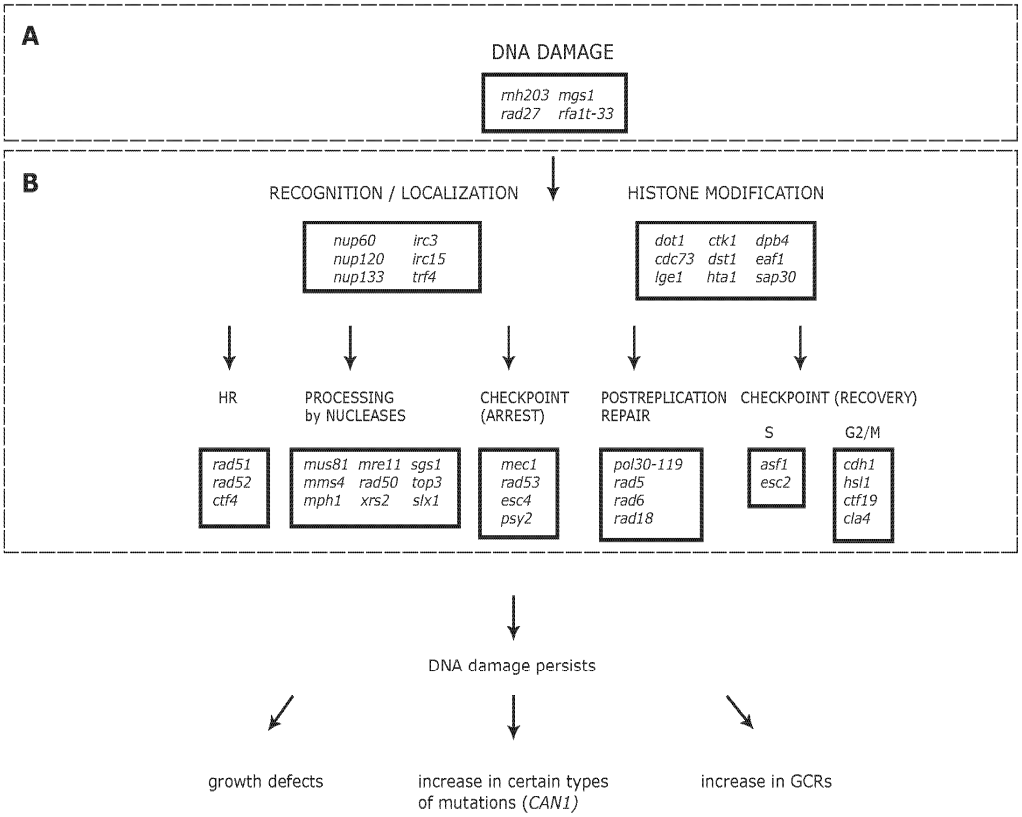

Figure 4-1: Genome instability pathways.
DNA damage persists due to defects in pathways that prevent formation or accumulation of DNA damage.
A. Some pathways prevent the formation of DNA damage
B. Some pathways prevent the accumulation of DNA damage

(Gordenin et al., 1997). The work presented in this Dissertation investigates this same hypothesis for an *rnaseh2* mutant.

Consistent with this hypothesis, the double frameshift mutations seen in the *rnh203* mutant do actually resemble DNA polymerase slippage errors. These type of errors might be accumulating in *rnaseh2* mutants because of aberrant processing of Okazaki fragment ends.

The small differences in the type of mutations found in the *CAN1* mutation spectrum experiment due to loss of RNaseH2 might indicate that RNaseH2 function could be important for suppressing certain types of mutations that can lead to genome instability.

4.3 DNA REPLICATION PROTEINS THAT CAN COMPENSATE FOR THE LOSS OF *RNASEH2* ARE INVOLVED IN LONG FLAP RESOLUTION.

The results presented in this Dissertation suggest that an accumulation of *rnaseh2* induced DNA damage activates nucleases that process replication intermediates, causes S-phase slow down, activates repair responses and checkpoint recovery pathways. The normal phenotypes of the *rnaseh2* single mutants suggest that activation of these DNA repair mechanisms leads to error-free repair (Figure 4-2). Furthermore, the results presented here

demonstrate that defects in the pathways that compensate for the loss of *RNASEH2* causes growth defects and sometimes an increased rate of GCRs.

It was found that a strain carrying an *rnh203* mutation combined with mutations in *mgs1, rad27* or *rfa1* had severe growth defects. One possibility is that these genes encode proteins involved in preventing the accumulation of mutagenic RNA-DNA hybrids and long flaps that can lead to the formation of foldback structures similar to the structures illustrated in Chapter 1 of the Dissertation (Figure 1-2).

Defects in *RAD27* leads to an increased presence of long flaps (Stith et al., 2008) because Rad27 cleaves flaps when they are short, without Rad27 flaps are more likely to be long. It has been suggested that Mgs1 plays a role in long flap biogenesis/degradation implying that defects in *MGS1* might also cause an increased accumulation of long flaps and secondary structures.

It was found that Mgs1 physically binds with DNA polymerase delta (Pol32) and Mgs1 was also shown to stimulate Rad27 activity(Kim et al., 2005) It was suggested that Mgs1 in association with DNA polymerase delta might increase recruitment of Rad27 causing Rad27 cleavage of flaps before they grow to a length that requires processing by Dna2. *MGS1* is the yeast homologue to human Werner helicase interacting protein (*WHIP)* and has single stranded DNA annealing activity (Kim et al., 2005). More specifically, it was suggested that Mgs1 might be binding to the single stranded long flaps to recruit or stabilize nucleases that are required to

process the long flaps. One possible novel role could be that Mgs1 recruits RNaseH2 to the long flaps.

RFA1 is known to bind to the single stranded flaps once they become long. The binding of Rfa1 to single stranded DNA has long been known to stabilize single stranded DNA. In cells defective for *RFA1* these regions will be highly unstable and inefficiently processed. One possibility to explain the phenotypes seen in strains carrying defects in *RAD27, MGS1,* and *RFA1* in combination with defects in *RNASEH2* is that there is an accumulation of secondary structure foldbacks in these double mutants. The accumulation of secondary structures might be triggering cell cycle slowdown to allow for repair of the foldbacks as would be evidenced by growth defects.

If the function of RNaseH2 at long flaps is critical for genome stability that suggests that a different mechanism that does not require RNaseH2 must process the long flaps, because *rnaseh2* single mutants have normal phenotypes. It seems likely that RNaseH1 in combination with Dna2 will properly degrade the long flaps but perhaps it is much less efficient which could also explain the cell cycle slow down that is demonstrated by growth defects.

4.4 DNA DAMAGE INDUCED BY DEFECTS IN *RNASEH2* TRIGGERS THE G2/M CHECKPOINT IN THE ABSENCE OF THE INTRA-S CHECKPOINT.

The results presented in this Dissertation suggest that an accumulation of *rnaseh2* induced DNA damage will cause S-phase slow down. It was found that a strain carrying an *rnh203* mutation combined with mutations that abolish the intra-s checkpoint that are defective for Mec1, Rad53 Sgs1 or Esc4 resulted in growth defects and in the case of Sgs1 and Esc4 an increased rate of accumulation of GCRs.

Mutations in *mec1, rad53, esc4,* or *sgs1* should theoretically inactivate the intra-S phase checkpoint pathway that is thought to be activated due to the presence of *rnaseh2* induced DNA damage. This might lead one to believe that without a proper checkpoint the cell cycle will not be properly maintained and the damage might be undetected. However, the results demonstrate severe growth defects in *rnh203* strains that were combined with mutations in the intra-s checkpoint which implies that the damage might be escaping the intra-s checkpoint but is likely accumulating in G2 and triggering the G2/M checkpoint which would explain the cell cycle slow down demonstrated by growth defects.

An increase in the rate of GCR accumulation in the *rnh203 esc4* and *rnh203 sgs1* double mutants was also found suggesting that there could be

additional important functions of Sgs1 and Esc4 that involves repair of *rnaseh2* induced DNA damage.

Characterization of Esc4 identified BRCT motifs in the protein that were suggested to be important for recruitment of the Slx4 DNA repair enzyme. The results presented in the Dissertation suggest that one possibility is that without Esc4 mediated Slx4 repair that *rnaseh2* induced DNA damage is accumulating in G2, triggering the G2/M checkpoint, and since it is not being repaired properly there is an accumulation of GCRs leading to genome instability.

4.5 DNA DAMAGE CAUSED BY DEFECTS IN *RNASEH2* REQUIRES PROCESSSING BY NUCLEASES

An accumulation of *rnaseh2* induced secondary structures was suggested to activate processing by nucleases that are known to function in resolution of HR intermediates. The results presented here demonstrate that defects in these pathways cause growth defects and sometimes an increased or decreased rate of GCRs. It was found that when an *rnh203* mutation was combined with mutations in *mus81, mms4, mph1, mre11, rad50, xrs2, sgs1, top3,* and *slx1* that this resulted in growth defects and in the case of *SGS1* an increased rate of accumulation of GCRs, and in the case of *SLX1* a decreased rate of GCRs.

These genes encode proteins that function as nucleases. The results indicate that *rnaseh2* induced DNA damage which might be in the form of secondary structures requires processing by these nucleases that may serve to create substrates recognizable to checkpoint machinery, or perhaps some of these nucleases are needed to modify *rnaseh2* induced DNA damage to allow recognition by repair machinery. It is likely that without processing by nucleases there will be an accumulation of substrates that activate checkpoints and repair mechanisms explaining the growth defects seen in the double mutants with an *rnh203* mutation and defects in nucleases. These results also suggest that processing of the foldbacks by the Sgs1 DNA helicase might be required to prevent an accumulation of GCRs and that RNaseH2 is involved in a novel pathway that suppresses the accumulation of GCRs generated in *slx1* mutants.

4.6 HOMOLOGOUS RECOMBINATION IS ACTIVATED TO REPAIR DNA DAMAGE INDUCED BY DEFECTS IN *RNASEH2*

The results presented in this Dissertation suggest that an accumulation of *rnaseh2* induced DNA damage which might be in the form of secondary structures will activate repair responses that lead to error-free repair (Figure 4-2). This was demonstrated by introducing defects in repair pathways that

caused growth defects. It was found that an *rnh203* mutation combined with mutations in *rad51, rad52* or *ctf4* resulted in weak growth defects.

One possibility is that HR is the less likely mechanism of repair of the *rnaseh2* induced DNA damage, because introducing defects in this pathway caused weak growth defects, but did not cause an increased accumulation of GCRs. GCR tests have been reliable for determining the type of DNA damage that requires HR for repair, albeit HR seems to be an alternative mechanism of repair for *rnaseh2* induced DNA damage.

The weak growth defects found for the *rnh203 rad51, rnh203 rad52,* and *rnh203 ctf4* double mutants might indicate that some of the damage induced by defects in *RNASEH2* is normally repaired by HR, but perhaps this repair mechanism is not relied on for most of the repairs. This could explain what might be causing the cell cycle slow down and presumably activating checkpoints.

4.7 POSTREPLICATION REPAIR IS PREFERRED PATHWAY OF REPAIR FOR DNA DAMAGE INDUCED BY DEFECTS IN *RNASEH2*

The results presented in this Dissertation suggest that an accumulation of *rnaseh2* induced DNA damage which might be in the form of secondary structures will activate repair responses that lead to error-free repair (Figure

4-2). It was found that defects in the pathways that compensate for the loss of *RNASEH2* cause growth defects and sometimes an increased rate of GCRs.

An *rnh203* mutation combined with mutations in *pol30-119, rad5, rad6,* or *rad18* resulted in growth defects, and in the case of *pol30-119* an increased rate of accumulation of GCRs. The slow growth phenotypes of an *rnh203* mutation combined with mutations that inactivate postreplication repair defects taken together with the absence of increased rates of GCRs in the strains with an *rnh203* mutation combined with mutations that inactivate HR suggests that postreplication repair might be the preferred pathway of repair for DNA damage caused by loss of *RNASEH2*.

The evidence for an increased rate of deletions within repeat regions from the mutation spectrum analysis is also in line with this hypothesis as it is known that postreplication repair uses a template switching mechanism to bypass DNA damage which creates a situation that is highly susceptible to DNA polymerase slippage.

4.8 DNA DAMAGE RESPONSE INDUCED BY DEFECTS IN *RNASEH2* RELIES ON CHECKPOINT RECOVERY FOR RE-ENTRY INTO THE CELL CYCLE

The results presented in this Dissertation suggest that an accumulation of *rnaseh2* induced DNA damage which might be in the form of secondary structures will activate checkpoint recovery pathways (Figure 4-2). It was demonstrated that defects in pathways that compensate for loss of *RNASEH2* cause growth defects and sometimes an increased rate of GCRs.

It was found that an *rnh203* mutation combined with mutations in *asf1,* or *esc2* resulted in growth defects, and in the case of *esc2* an increased rate of accumulation of GCRs. One possibility is that both Asf1 and Esc2 are involved in functions that turn off checkpoints after DNA damage has been repaired. It has been suggested that Asf1 is involved in S-phase checkpoint recovery (personal communication). Asf1 is likely to be involved in the reassembly of chromatin that might be required for re-entry into the cell cycle. The cellular role of Esc2 as of yet has not been found. If checkpoint recovery is inactivated then cells will stay stuck in arrest which would explain the growth defects in *rnh203 asf1* double mutants. An increase in rate of accumulation of GCRs in the *rnh203 esc2* double mutants was also found suggesting a novel Esc2 related function required for processing of

rnaseh2 DNA damage to prevent accumulation of GCRs that can lead to genome instability.

4.9 *RNASEH2* MODEL

The genetic analysis of the role of RNaseH2 in preventing genome instability presented in this Dissertation revealed that there are many proteins that function in a number of different mechanisms to coordinate the prevention of the formation of *rnaseh2* induced DNA damage. This work also determined that there are a number of proteins involved in functions that prevent an accumulation of *rnaseh2* induced DNA damage to ensure genome instability. The pathways involving RNaseH2 and genome stability have been summarized and presented in a model (Figure 4-3).

In summary, defects in RNaseH2 cause DNA damage that might be in the form of secondary structures. The *rnaseh2* induced DNA damage is thought to activate processing by nucleases that are known to function in processing recombination intermediates including the Sgs1/Top3 helicase complex, Mms4/Mus81 endonuclease complex, the MRX complex and the Mph1 helicase. The *rnaseh2* induced DNA damage is processed by nucleases possibly to create substrates that are recognized by checkpoint machinery.

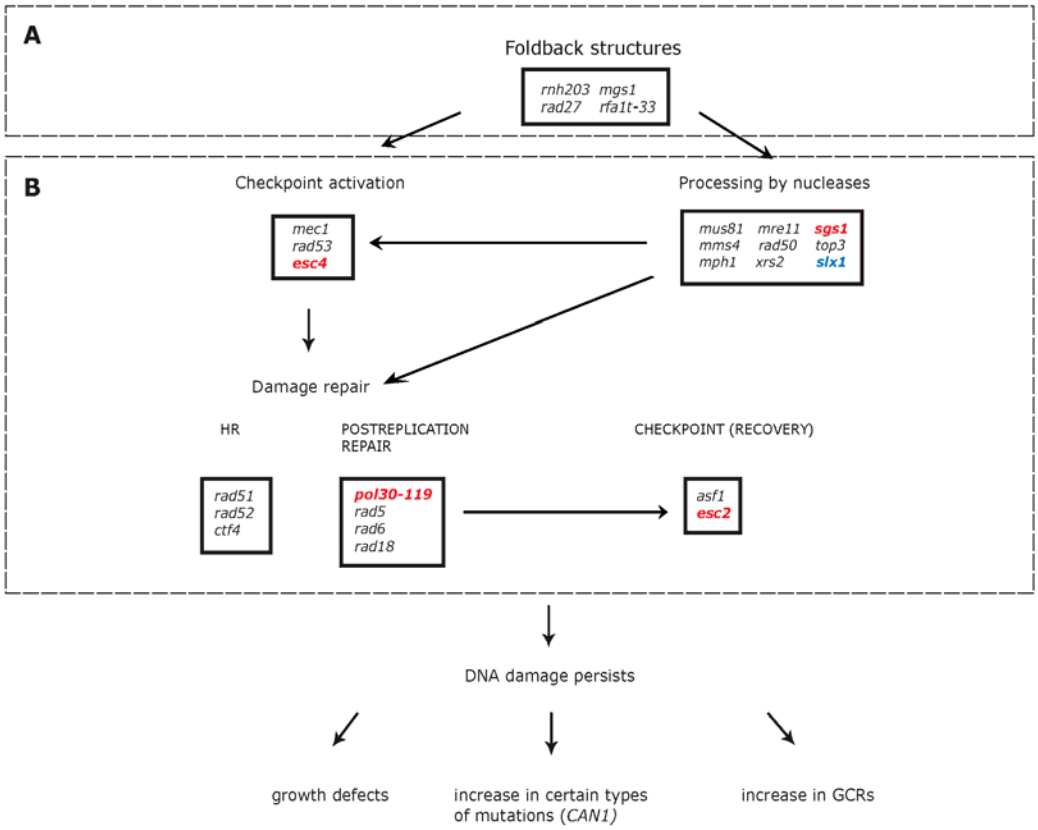

Figure 4-2: Genome instability pathways involved in an *rnaseh2* induced DNA damage response.
DNA damage persists due to defects in pathways that prevent formation or accumulation of *rnaseh2* induced DNA damage. Mutations that caused increased rates of GCRs are in red. Mutations that caused decreased rates of GCRs in blue. All mutations, except *pol30-119* caused growth defects.
A. Some pathways prevent the formation of *rnaseh2* induced DNA damage

It is then thought that the intra-s checkpoint is activated. Perhaps, an early activation step mediated by Esc4 that involves opening up of the chromatin to allow access of checkpoint machinery to the site of DNA damage. Presumably, the intra-s checkpoint activation that is triggered in response to *rnasheh2* induced DNA damage is thought to involve Sgs1, and the MRX complex to mediate Mec1 signaling that phosphorylates Rad53.

Cell cycle arrest from the checkpoint is followed by activation of repair functions. One possibility is that the *rnaseh2* induced DNA damage is processed by the postreplication repair machinery possibly using the template switching mechanism to bypass the damage which actually might coincide with the mutation spectrum data that looked like DNA polymerase slippage. The final *rnaseh2* induced DNA damage processing step is thought to involve Asf1 mediated chromatin reassembly that possibly signals the cell to re-enter the cell cycle after the damage has been repaired.

An alternative scenario was also considered involving homologous recombination which would come into play after intra-s checkpoint activation and might possibly be mediated by Esc4 and Slx4. The shuttling of the *rnaseh2* induced DNA damage from step to step is the same as was described above, except that HR would be in place of the postreplication repair step.

The mechanisms proposed in this model would explain why loss of RNaseH2 alone does not cause growth defects or an increased accumulation.

of GCRs. This model also offers up a diverse set of proteins that might be compensating for the loss of RNaseH2. Furthermore, it may also provide insights into how the function of RNaseH2 is important for suppression of genome instability

4.10 GENOME INSTABILITY HAS MANY SOURCES AND REQUIRES MANY MECHANISMS OF AVOIDANCE

The results presented in this Dissertation highlight the importance of many diverse proteins in pathways that are important for maintaining genome stability. This implies that there are pathways required to prevent the formation of DNA damage and there are also pathways required for dealing with the DNA damage once it becomes present, but that ultimately, both are required for prevention of genome instability.

4.11 FUTURE DIRECTIONS

The work presented in this Dissertation investigated the role of RNaseH2 in genome instability as a means to find out more about the mechanisms required for genome stability. As an expansion on that same topic an enriched list of candidate GCR regulators were analyzed with the hopes of gaining further insight into the mechanisms underlying genome

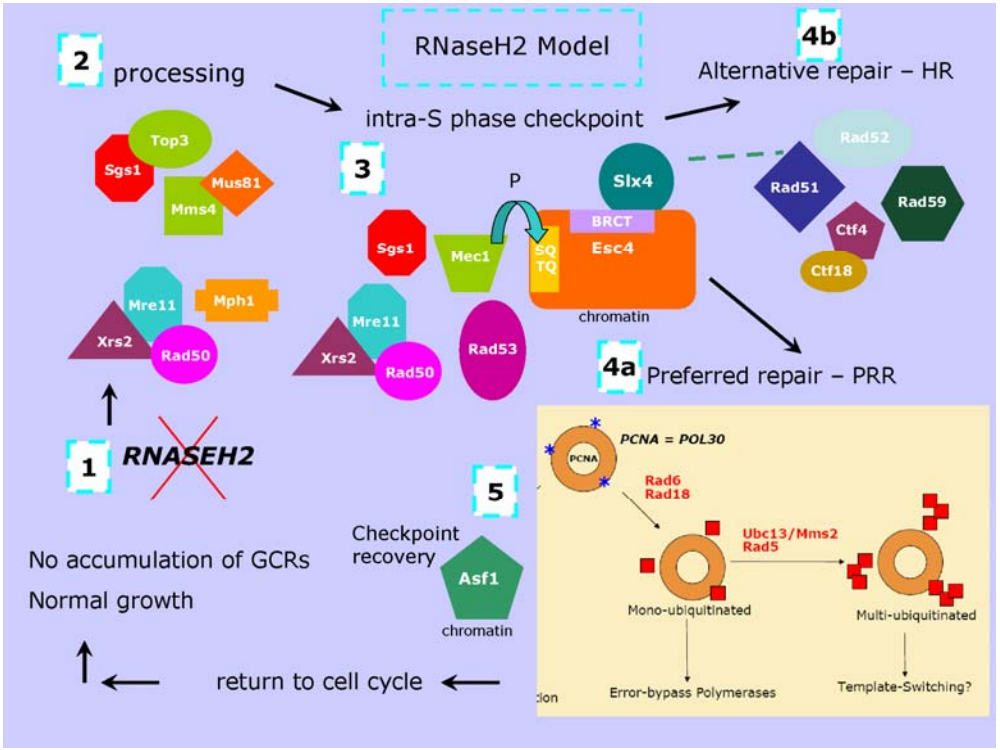

Figure 4-3: *RNASEH2* Model
Schematic of stepwise processing of *rnaseh2* induced DNA damage.
1) Loss of *RNASEH2* causes DNA damage.
2) DNA damage processed by nucleases.
3) Activation of intra-s checkpoint
4) DNA repair mechanisms process damage. Postreplication repair preferred pathway. Homologous recombination an alternative repair pathway.
5) Checkpoint release and return to cell cycle.

instability. This work defined the basic genetics of *RNASEH2* and provided many insights on how RNaseH2 might be involved in genome instability. In combination with the bioinformatic genome wide approach unanticipated genes that are involved in genome stability were identified. All of this work resulted in a large collection of diverse proteins implicating the many diverse mechanisms involved in maintenance of a stable genome.

The two surveys in this work implicating a number of proteins in pathways important for genome stability had the fortunate outcome that now some of the previously unknown basic genetics of *RNASEH2* are now known and in addition there is now an enriched list of genes that encode proteins known to be involved in genome stability. The unfortunate outcome of the approach used here is that it is deficient for insights about the specifics on the implicated mechanisms.

This has encouraged me to think about a future project that will answer specific questions about a mechanism involved in preventing the accumulation of *rnaseh2* induced DNA damage or a future project that will answer specific questions about mechanisms that have functional roles in genome stability. The number of mechanisms implicated in this work are too numerous to answer specific questions about each of them and actually most of the pathways implicated in this study are either well characterized or have been studied, except one, Esc2.

With this in mind, my future work is to focus on the specific mechanisms that involve Esc2 in processing *rnaseh2* DNA damage that leads to an accumulation of GCRs. One of the first questions I wanted to address was whether the growth defects in the *rnh203 esc2* double mutants is a result of cell death or activation of a checkpoint. I looked at cell cycling profiles by FACS analysis of the *rnh203 esc2* mutants and found a cell cycle delay in S phase suggesting that the growth defect is due to activation of a checkpoint (Figure 4-4).

In the future, I will look further into what requirements are needed to activate the checkpoint such as Rad53 activation by increased phosphorylation. I also think it would be interesting to find out whether Esc2 is involved in regulating chromatin in response to *rnaseh2* induced DNA damage. This future work would hopefully provide even more insight into the specific mechanisms that are needed to prevent genome instability especially genome instability that arises due to an accumulation of DNA replication errors.

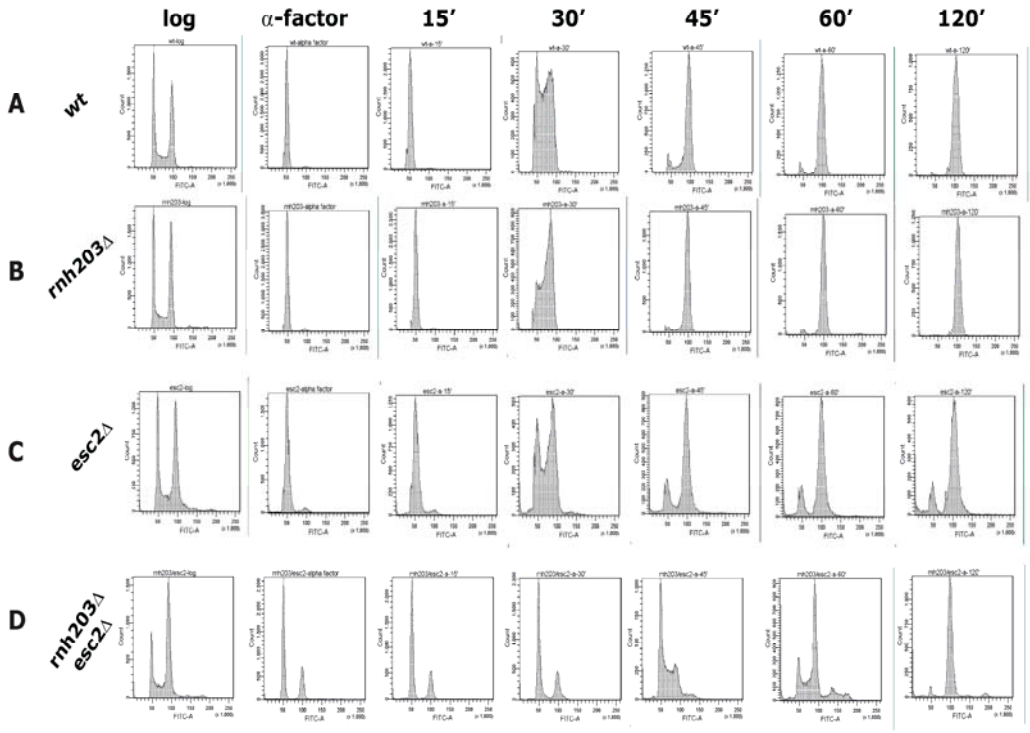

Figure 4-4: *rnh203 esc2* cell cycle profile
FACS analysis generated cell cycle profile demonstrates an S-phase delay in an *rnh203 esc2* double mutant strain.
A. Wild-type.
B. *rnh203* single mutant strain.
C. *esc2* single mutant strain.
D. *rnh203 esc2* double mutant strain.

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