2011

Identification of Novel Core and Accessory Factors Involved in Nucleotide Excision Repair in Yeast

Danielle Marie Tatum
Louisiana State University and Agricultural and Mechanical College

IDENTIFICATION OF NOVEL CORE AND ACCESSORY FACTORS INVOLVED IN NUCLEOTIDE EXCISION REPAIR IN YEAST

A Dissertation

Submitted to the Graduate Faculty of
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

Veterinary Medical Sciences through
the Department of Comparative Biomedical Sciences

by
Danielle Marie Tatum
B.S., University of Louisiana – Lafayette, 2001
August 2011

ACKNOWLEDGEMENTS

One of my favorite quotes comes from General George Washington who once said "Perseverance and spirit have done wonders in all ages." I consider this dissertation a wonder because I have often wondered if I would reach this point at all. As for perseverance and spirit, it has taken the efforts of not only myself but of a multitude of people surrounding me who have helped me reach this point in my academic career. While at times I may have lacked in spirit and resolve, I have never lacked in support and guidance. It is with that in mind that I compose these acknowledgements.

First and foremost, I would like to express sincere appreciation for my mentor, Dr. Shisheng Li. Thank you for the time and expertise you have contributed, for the opportunity to expand my knowledge by allowing for attendance of both national and international meetings, and for guiding and shaping my research. It is possible that I have tried your patience more than anyone else ever has, but please know that I am eternally grateful for all of the time and effort you have invested in me and that without you, I would not be where I am today.

I also owe appreciation to my graduate committee for their assistance in various aspects of my graduate career. Dr. George Strain, through your role as Graduate Advisor and now as Department Head, you have been a valuable source of advice and information throughout my time here and for that I am grateful. Dr. David Donze, you are a gifted teacher, and I have thoroughly enjoyed and greatly benefitted from your classes. I sincerely appreciate your advice, general conversation, thought provoking questions, and your willingness to give of your time to help me. Dr. Arthur Penn, the list of things to thank you for is extensive. For the sake of brevity I will not list them all, but know that you have been a second mentor to me in some ways (whether you wanted to be or not), and I sincerely value and appreciate all of the advice and

guidance you have provided during my years here. Dr. Bruce Eilts, thank you for giving of your time as the Dean's Representative on my committee. I have never thought of my research in terms of cows or their reproductive systems, so thank you for expanding my horizons and making me look at the bigger picture. Lastly, I would like to thank Dr. Shulin Li, an original member of my graduate committee who has since left the university, for his time and assistance.

A nod of appreciation must also be give to my laboratory members, Dr. Baojin Ding, Mrs. Christine Howard, Ms. Margaret Placer, and Dr. Xuefeng Chen. I have benefitted not only from your knowledge and assistance with experiments, but from your discussions and support as well. In addition to my committee and lab members, I extend appreciation to the entire faculty, administration, and staff of the Department of Comparative Biomedical Sciences for their help in navigating through my time here.

Last, but certainly not least, I owe a vast amount of gratitude to my family and friends. They have been unwavering in their love, support, and encouragement throughout my graduate career. To the close and hopefully lifelong friends I have made here in graduate school, Mr. Amandip Singh, Dr. Denada Dibra, Dr. Jeffry Cutrera, and (soon-to-be) Dr. Jeffrey Cardinale, you have been my lifeline and invaluable to me in so many ways. I cannot imagine my time here without an internal support structure like you who understands the trials and tribulations of being a graduate student. To my friends outside of school, your laughter and amazing shows of support have meant more to me than I could ever possibly convey. Without realizing it, you have proven yourselves time and time again, and I am honored to call you each friend. As for my family, how do I thank the people that have sacrificed so much for me and believed in me my entire life? Words are insufficient to tell you what you mean to me or how much I appreciate each and every time you give of yourself to help me. Mine has been a truly blessed life, as I have never lacked

in the love, support, and encouragement of my family. You believed in me when I did not, and when I wanted to quit, the thought of you and how much you have sacrificed to get me here pushed me to continue. My sincerest hope is that I have made you proud.

They say it takes a village to raise a child, and, as indicated by this extensive list of thank you's, it seems it also takes a village to raise a graduate student. Thank you to everyone for every effort you have so graciously given. Without you, this achievement would not have been possible.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS……..…ii

LIST OF TABLES.....viii

LIST OF FIGURES……..ix

LIST OF ABBREVIATIONS……..xi

ABSTRACT…….…….....xiii

CHAPTER 1 LITERATURE REVIEW
NUCLEOTIDE EXCISION REPAIR IN *SACCHAROMYCES CEREVISIAE*……..1
1.1 Introduction……...1
1.2 Nucleotide Excision Repair in *Saccharomyces cerevisiae*……..……..3
1.3 GGR in Yeast…….....……..4
1.3.1 GGR in the Context of Chromatin……..……8
1.3.2 Chromatin Remodeling and GGR……..……....9
1.3.3 Histone Modifications and GGR……...……...12
1.4 TCR in Yeast…….……17
1.4.1 The Role of Rad26 in TCR……...……19
1.4.2 Suppressors of Rad26-independent TCR…….21
1.5 References……26

CHAPTER 2
YEAST ELC1 PLAYS AN IMPORTANT ROLE IN GLOBAL GENOMIC REPAIR BUT NOT IN TRANSCRIPTION COUPLED REPAIR……..37
2.1 Introduction……..37
2.2 Materials and Methods…….39
2.2.1 Yeast Strains and Plasmids...……39
2.2.2 UV Sensitivity Assay……..……..41
2.2.3 UV Irradiation, Repair Incubation, and DNA Isolation..……..41
2.2.4 NER Analysis of UV-induced CPDs……...……41
2.2.5 UV-induced Mutagenesis……...……..42
2.2.6 Western Blot……...……..42
2.3 Results……..43
2.3.1 UV Sensitivities of *elc1* Mutants..……...43
2.3.2 The Role of Elc1 in GGR and TCR……..……...44
2.3.3 The Effect of *elc1* Deletion on UV-induced Mutagenesis…….……..48
2.3.4 Cellular Rad7 and Rad16 Levels Are Not Affected in the Absence of Elc1…….……49

2.3.5 The Role of Elc1 in GGR Can Not Be Substituted for by Overexpressing of Rad7 and Rad16 Individually or Simultaneously……50
2.4 Discussion……52
2.5 References……55

CHAPTER 3
EVIDENCE THAT THE HISTONE METHYLTRANSFERASE DOT1 MEDIATES GLOBAL GENOMIC REPAIR BY METHYLATING HISTONE H3 ON LYSINE 79….59
3.1 Introduction……59
3.2 Materials and Methods……61
3.2.1 Yeast Strains and Plasmids……..……61
3.2.2 UV Irradiation, Repair Incubation, and Genomic DNA Isolation……...61
3.2.3 NER Analysis of UV-induced CPDs at Nucleotide Resolution……..62
3.3 Results……63
3.3.1 Dot1 Is Required for GGR……63
3.3.2 Methylation of H3K79 Is Also Required for GGR……...……66
3.3.3 Bre1 and Histone H2B Lysine 123 (H2BK123) Ubiquitination Are Partially Required for GGR……..66
3.3.4 Overexpression of Rad16 Does Not Restore GGR in Cells Expressing H3K79A of H2BK123A……..68
3.3.5 Dot1 and H3K79 Methylation Do Not Play Significant Roles in TCR……68
3.4 Discussion……71
3.5 References……74

CHAPTER 4
THE ROLES OF RNA POLYMERASE II-ASSOCIATED FACTOR 1 COMPLEX IN DIFFERENT SUBPATHWAYS OF NUCLEOTIDE EXCISION REPAIR……78
4.1 Introduction……78
4.2 Materials and Methods……82
4.2.1 Yeast Strains and Plasmids……...……82
4.2.2 UV Sensitivity Assay……..…..83
4.2.3 UV Irradiation, Repair Incubation, and Genomic DNA Isolation…….…...84
4.2.4 NER Analysis of UV-induced CPDs at Nucleotide Resolution….……...84
4.2.5 Immunoprecipitation……85
4.2.6 Western Blot…...……...85
4.3 Results……..86
4.3.1 Paf1C Plays a Minor Role in Facilitating Rad26-independent TCR…...….86
4.3.2 Paf1C Suppresses Rad26-independent TCR……88
4.3.3 Paf1C Does Not Suppress Rad26-independent TCR in Cells Lacking Spt4, and *vice versa*……..90
4.3.4 The Role of Paf1C in Suppressing Rad26-independent TCR Is Not Subsidiary to That of Spt5……....91
4.3.5 The Association of Paf1C with Pol II Is Facilitated by the CTR Domain of Spt5……..92
4.3.6 Paf1C Is Not Epistatic to Either Rad26 or Spt4/Spt5……..……93
4.3.7 Paf1C Facilitates GGR, Especially in Inter-nucleosomal Linker Regions...94

4.3.8 Paf1C Facilitates GGR Through Enabling Monoubiquitination of H2BK123 by Bre1, Which in Turn Permits Di- and Tri-methylation of H3K79 by Dot1……97
4.4 Discussion……99
4.5 References……105

CHAPTER 5
CONCLUDING REMARKS……111
5.1 Research Summary……111
5.2 Concluding Remarks and Future Direction……112
5.3 References……114

APPENDIX: LETTERS OF PERMISSION……115

VITA……118

LIST OF TABLES

Table 1-1 Core NER factors, their human homolog (if known), and their function in NER…….5

Table 2-1 Frequencies of canavanine resistance (Can^R) mutations in different isogenic strains…….. 49

Table 4-1 Yeast strains used in the study of Chapter 4…….83

LIST OF FIGURES

Figure 2-1 Effect of the *elc1* deletion on UV sensitivity of otherwise wild type and GGR- and TCR-deficient cells……..…….44

Figure 2-2 The *elc1* deletion has no effect on TCR……...…….45

Figure 2-3 The *elc1* deletion results in undetectable GGR……...…….46

Figure 2-4 Plots showing repair of CPDs in the NTS of the *RPB2* gene……...……47

Figure 2-5 Rad7 and Rad16 protein levels are not increased in *elc1* cells……..……...50

Figure 2-6 Overexpression of Rad7 and/or Rad16 does not restore UV resistance of *rad26 elc1* cells……..…….51

Figure 2-7 Overexpression of Rad7 and/or Rad16 does not restore GGR in *rad26 elc1* cells.....52

Figure 3-1 Gels showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT), *dot1*Δ and H3K79A cells……..……...…65

Figure 3-2 Plot showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT), *dot1*Δ and H3K79A cells……..…….……...…66

Figure 3-3 Gels showing repair of CPDs in the NTS of the *RPB2* gene in *bre1*Δ and H2BK123A cells, and in H3K79A and H2BK123A cells transformed with a plasmid (pGALRAD16)……...67

Figure 3-4 Plot showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT), *bre1*Δ, H3K79A and H2BK123A cells, and in H3K79A and H2BK123A cells transformed with a plasmid (pGALRAD16)……..…….……....69

Figure 3-5 Gels showing repair of CPDs in the TS of the *RPB2* gene in *rad16*Δ, *rad16*Δ *dot1*Δ and *rad16*Δ H3K79A cells……..……...…70

Figure 3-6 Plot showing repair of CPDs in the TS of the *RPB2* gene in *rad16*Δ, *rad16*Δ *dot1*Δ and *rad16*Δ H3K79A cells……..……..71

Figure 4-1 Gels showing repair of CPDs in the TS of the *RPB2* gene in *rad16Δ* cells with different additional gene deletions……..…….……....87

Figure 4-2 Plot showing repair of CPDs in the TS of the *RPB2* gene in *rad16Δ* cells with different additional gene deletions……..…….……....88

Figure 4-3 Deletion of *rtf1* in *rad16Δ* and *rad16Δ rpb9Δ* cells enhances UV sensitivity…...…88

Figure 4-4 Gels showing repair of CPDs in the TS of the *RPB2* gene in *rad16Δ rad26Δ* cells with different additional gene deletions……..……..89

Figure 4-5 Plot showing repair of CPDs in the TS of the *RPB2* gene in *rad16Δ* and *rad16*Δ *rad26Δ* cells with different additional gene deletions……90

Figure 4-6 The Spt5 CTR facilitates the association of Paf1C with Pol II…….……...93

Figure 4-7 Deletion of *rtf1* in *rad16Δ rad26Δ* and *rad16Δ rad26Δ spt4Δ* cells enhances UV sensitivity……94

Figure 4-8 Gels showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT) and different mutant cells…….……96

Figure 4-9 Plot showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT) and different mutant cells…….……...….97

Figure 4-10 Plot showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT) and different mutant cells……..98

Figure 4-11 *RTF1* is epistatic to *DOT1* and *BRE1*.....99

LIST OF ABBREVIATIONS

ATP……...adenosine triphosphate

CAK……. CDK-activating kinase

CDK……... cyclin dependent kinase

CPD……cyclobutane pyrimidine dimer

CS……. Cockayne syndrome

CSA…….Cockayne syndrome group A

CSB…… Cockayne syndrome group B

CTR…….. C-terminal repeat

DDB…….. DNA damage binding protein

GGR……... global genomic repair

IP……. immunoprecipitation

MMS…… methyl methanesulfonate

NEF4……. nucleotide excision repair factor 4

NER……. nucleotide excision repair

NTS…… nontranscribed strand

PAF……..…….……..RNA polymerase II-associated factor

PCR……. polymerase chain reaction

Pol II……...RNA polymerase II

SDS-PAGE……..sodium dodecyl sulfate polyacrylamide gel electrophoresis

SWI/SNF……... Switch/Sucrose nonfermentable

TCA…… trichloroacetic acid

TCR……... transcription coupled repair

TFIIH…….. transcription factor II H

TFIIS……... transcription factor II S

TRCF…… transcription repair coupling factor

TS…… transcribed strand

TTD…….. trichothiodystrophy

TTD-A…… trichothiodystrophy group A

UAS……... upstream activating sequence

UV…… ultraviolet

XP……. xeroderma pigmentosum

XPC…… xeroderma pigmentosum complementation group C

ABSTRACT

Nucleotide excision repair (NER) is a highly conserved DNA repair mechanism which deals with a wide variety of bulky, helix-distorting lesions, such as UV-induced cyclobutane pyrimidine dimers (CPDs). NER is traditionally grouped into two pathways: global genomic repair (GGR), which is operative throughout the genome, and transcription coupled repair (TCR), which is dedicated to rapid repair of the transcribed strand of actively transcribed genes. Though most of the core NER proteins are known, the exact biochemical mechanism of eukaryotic NER remains elusive. This dissertation focused on identifying novel core and accessory factors which function in NER.

In the budding yeast *Saccharomyces cerevisiae*, GGR has previously been shown to be dependent on Rad7 and Rad16. We revealed Elc1, the yeast homolog of human elongin C, as a novel GGR-specific factor. Elc1 is required for GGR, but has no role in TCR. The precise role of Elc1 in GGR remains unknown. Dot1 is a histone methyltransferase whose sole substrate is histone H3 lysine 79 (H3K79). We identified Dot1 as another GGR-specific factor, as deletion of Dot1 or mutation of H3K79 abolishes GGR, but has no effect on TCR. H3K79 can accept up to 3 methyl groups, but Dot1 can only add one by itself. The PAF transcription elongation complex, through facilitating histone modifications, is partially required for dimethylation and fully required for trimethylation of H3K79 by Dot1. We demonstrated that through facilitating these histone modifications, PAF is partially required for GGR.

TCR is believed to be triggered by a stalled elongating RNA polymerase II (Pol II) complex. Rad26, the homolog of the human CSB gene, and Rpb9, a nonessential subunit of Pol II, play important roles in TCR. We identified a dual role for PAF in TCR. In the presence of Rad26, PAF plays a positive role, facilitating TCR. In the absence of Rad26, PAF functions as a

suppressor of TCR. PAF appears to be a part of a "megasuppressor" complex which includes Rpb4 and the Spt4/Spt5 complex, which also suppress Rad26-independent TCR. The interactions among Pol II, Rad26 and the various TCR suppressors remain to be elucidated.

CHAPTER 1

LITERATURE REVIEW
NUCLEOTIDE EXCISION REPAIR IN *SACCHAROMYCES CEREVISIAE*

1.1 Introduction

Each day organisms are faced with a barrage of genomic insults which damage and jeopardize the integrity of DNA (Lindahl and Wood 1999). DNA damage stems from both endogenous sources such as water and reactive oxygen species generated by regular cellular metabolism and exogenous sources such as sunlight, chemicals, and tobacco smoke. These DNA damaging agents can cause various types of genomic damage including base losses and modifications, strand breaks, crosslinks, bulky chemical adducts, and other DNA alterations. These genomic insults alter the chemistry of DNA and can accumulate and become mutagenic and/or cytotoxic. At the cellular level, DNA damage that is undetected or left unrepaired can result in genomic instability, apoptosis, or senescence, which can greatly affect the aging and development processes. At the level of the organism, genetic instability can predispose the organism to immunodeficiency, neurological disorders, and cancer, illustrating the need to understand the molecular basis of mutagenesis and the mechanisms of DNA repair.

In an effort to maintain the integrity of the genome, evolution has led cells to develop an elaborate DNA damage response system to counteract potentially mutagenic and cytotoxic genomic insults. This highly evolutionarily conserved system is made up of multiple DNA repair pathways, each focusing on a specific category of lesion, as well as multiple checkpoint, signal transduction, and effector systems which crosstalk with replication, transcription, recombination, and chromatin remodeling in order to control DNA damage (Harper and Elledge 2007;

Hoeijmakers 2009). The complexity of and the energetic expense dedicated by cells to this process underscores the importance of preserving genomic integrity (Hoeijmakers 2009).

One of the various DNA repair pathways cells have at their employ is the highly conserved nucleotide excision repair (NER), which is the most versatile repair mechanism in terms of lesion recognition [for a recent review, see (Nouspikel 2009)]. NER deals with a wide class of bulky, helix-distorting lesions that generally obstruct transcription and normal replication, such as UV-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts [(6-4)PP], as well as adducts and crosslinks induced by chemical agents (e.g. benzo[a]pyrene and cisplatin). NER was first discovered in bacteria in the mid-1960s by Philip Hanawalt and David Pettijohn with the observation of non-semiconservative DNA synthesis during the excision of CPDs (Pettijohn and Hanawalt 1964). Almost simultaneously, excision repair of UV-induced DNA damage was identified in mammalian cells (Rasmussen and Painter 1964).

NER is a multistep reaction which includes damage recognition, helix opening, lesion verification, dual incision of the damaged strand bracketing the lesion, removal of an oligonucleotide containing the lesion, gap-filling DNA synthesis and ligation. The distinguishing characteristic of NER is that the damaged bases are enzymatically excised from the genome as an oligonucleotide fragment of about 24-32 nucleotides in length in mammalian cells and 24-27 nucleotides in length in yeast (Prakash and Prakash 2000). The biological importance of NER for human health is illustrated by the existence of rare autosomal recessive human disorders which result from defects of genes involved in NER, namely xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD), all of which are associated with increased sensitivity to sunlight (Cleaver, Lam et al. 2009).

Although the process of NER in eukaryotes and prokaryotes share many similar features, such as damage recognition, excision, repair synthesis and ligation, the molecular mechanisms in eukaryotic cells seem much more complicated. In both prokaryotes and eukaryotes, the core NER factors, defined as the proteins which are necessary and sufficient to carry out the NER reaction, have been identified. The NER process in *Escherichia coli* is relatively well understood and requires only six proteins, whereas the NER process in eukaryotes displays a considerably higher degree of genetic complexity, requiring more than 30 proteins to reconstitute the reaction *in vitro* (Aboussekhra, Biggerstaff et al. 1995; Guzder, Habraken et al. 1995).

NER is a heterogeneous process which repairs lesions in the transcribed strands of transcriptionally active genes faster than it repairs lesions in the nontranscribed strands or transcriptionally silent regions. Based on this heterogeneity, NER is traditionally divided into two pathways: global genomic repair (GGR) and transcription coupled repair (TCR). Damage in transcriptionally silent regions and in the nontranscribed strand (NTS) of active genes is repaired by GGR, while TCR is dedicated to repairing lesions in the transcribed strand (TS) of active genes. Though TCR and GGR are generally differentiated as distinct pathways of NER, they only differ in the initial steps of DNA damage recognition. Therefore, after lesion recognition and verification, a general outline of the GGR process becomes applicable to TCR as well. A defining characteristic of NER substrates is that they cause local distortion of the DNA double helix, and in GGR, this local distortion appears to be the first structure recognized.

1.2 Nucleotide Excision Repair in *Saccharomyces cerevisiae*

Studies using the budding yeast *Saccharomyces cerevisiae* as a model organism have made major contributions in elucidating the core NER mechanism in eukaryotes and have

yielded important insights into the functions of a multitude of NER proteins (Prakash and Prakash 2000). Many cellular processes such as replication, repair, cell division, and recombination are highly conserved from lower to higher eukaryotes. Indeed, most NER factors are conserved proteins and have orthologs in humans, yeast, and other eukaryotes (Table 1). In addition to having many homologs to humans, yeast offers many advantageous features to researchers, such as having a well-defined genetic system, the ease of growth and maintenance in the laboratory, and the ability to be maintained in either a haploid or diploid state. Taken together, these features provide researchers with a powerful genetic tool to study NER.

1.3 GGR in Yeast

In *S. cerevisiae*, Rad7, Rad16, and Elc1 are specifically required for GGR (Verhage, Zeeman et al. 1994; Lejeune, Chen et al. 2009). The exact roles of these proteins in GGR are not yet clear. Rad7 and Rad16 form a complex that binds specifically to UV-damaged DNA in an ATP-dependent manner (Guzder, Sung et al. 1997). The ATP dependence of the Rad7-Rad16 complex for damage binding distinguishes this complex from Rad14, RPA, and the Rad4–Rad23 complex, which do not exhibit such dependence on ATP for damage binding (Prakash and Prakash 2000). Rad16 shares marked homology with Snf2, the catalytic subunit of the SWI/SNF chromatin remodeling complex (Bang, Verhage et al. 1992), and Snf2 contains conserved motifs found in a superfamily of ATPases thought to be involved in chromatin remodeling activities (Eisen, Sweder et al. 1995). Accordingly, the Rad7–Rad16 complex displays a DNA-dependent ATPase activity. It has been shown that this ATPase activity is inhibited when the complex comes across DNA damage (Guzder, Sung et al. 1998).

Table 1-1. Core NER factors, their human homolog (if known), and their function in NER.

S. cerevisiae	Human homolog	Function(s)	Reference
Rad4	XPC	DNA damage recognition and binding	(Guzder, Sung et al. 1998)
Rad23	hRAD23B	Interacts with and stimulates Rad4	(Guzder, Sung et al. 1998)
TFIIH	TFIIH	DNA helicase activity mediates helix opening	(Egly and Coin 2011)
Mms19	MMS19L	Stabilizes XPD subunits of TFIIH	(Kou, Zhou et al. 2008)
Rad14	XPA	Stabilizes preincision complex; lesion recognition	(Guzder, Sommers et al. 2006)
Rpa	RPA	Stabilizes open single stranded DNA; damage recognition	(Guzder, Habraken et al. 1995)
Rad2	XPG	Catalyzes 3′ incision; stabilizes open complex	(Habraken, Sung et al. 1993)
Rad10	ERCC1	Catalyzes 5′ incision; forms complex with Rad1	(Sung, Reynolds et al. 1993; Tomkinson, Bardwell et al. 1994)
Rad1	XPE	Catalyzes 5′ incision	(Sung, Reynolds et al. 1993; Tomkinson, Bardwell et al. 1994)
Rad26	CSB	TCR-specific factor; DNA-dependent ATPase	(Prakash and Prakash 2000) (van Gool, Verhage et al. 1994)
Rad7	Unknown	GGR-specific factor; forms complex with Rad16	(Verhage, Zeeman et al. 1994)
Rad16	Unknown	GGR-specific factor; DNA-dependent ATPase	(Verhage, Zeeman et al. 1994)
Elc1	Elongin C	GGR-specific factor; forms complex with Rad7 and Rad16	(Lejeune, Chen et al. 2009)

This finding led to the formation of a model which suggested that the Rad7-Rad16 complex may act as an ATP-dependent motor which translocates along the DNA in search of damage, and upon encountering a lesion, the complex is stalled, which may remodel and open damaged chromatin, thereby facilitating recruitment of other NER factors (Guzder, Sung et al. 1998) According to this model, the Rad7–Rad16 complex would arrive first on the scene of a damage site in nontranscribed regions of the genome and serve as the nucleation site for the recruitment of the other NER factors. It was also previously suggested that the ATPase activity of Rad16 generates superhelical torsion in DNA that has an altered structure due to UV-induced damage, and that this torsion is necessary for the excision of damaged bases in GGR (Yu, Owen-Hughes et al. 2004), suggesting a role for Rad16-Rad7 in the later steps of GGR.

Yeast Elc1 is a homolog of mammalian elongin C which forms a heterotrimeric complex with elongins A and B (Bradsher, Jackson et al. 1993; Aso, Lane et al. 1995). The *elc1*Δ mutation was shown to be epistatic to *rad7*Δ and *rad16*Δ mutations, but resulted in a synergistic enhancement of UV sensitivity when combined with *rad26*Δ (Ribar, Prakash et al. 2006). A study utilizing a technique which measures NER at the nucleotide level revealed that Elc1 plays an important role in GGR, as *elc1*Δ cells showed no detectable repair of CPDs in the NTS of the constitutively expressed *RPB2* gene, but no role in TCR (Lejeune, Chen et al. 2009). The role of Elc1 is not via stabilizing Rad7 or Rad16, as levels of either do not change in *elc1*Δ cells. Furthermore, the role of Elc1 does not seem to be subsidiary to that of Rad7 or Rad16, as overexpression of either or both in the absence of Elc1 did not restore GGR (Lejeune, Chen et al. 2009). The precise nature of the role of Elc1 in GGR remains unknown. Genetic studies have revealed multiple roles for this gene in separate cellular processes (Ribar, Prakash et al. 2006).

In one of these, Elc1 is a component of a ubiquitin ligase (E3) that contains Rad7 and Rad16 and is responsible for regulating the levels of Rad4 protein in response to UV damage (Ramsey, Smith et al. 2004; Gillette, Yu et al. 2006). It has also been suggested that Elc1 is a component of another ubiquitin ligase complex, which contains Ela1, Cul3, and Roc1 and is responsible for the polyubiquitylation and subsequent degradation of RNA polymerase II (Pol II) in response to DNA damage (Ribar, Prakash et al. 2006; Ribar, Prakash et al. 2007).

The TFIIH multiprotein complex, which is organized into a 7-subunit core associated with a 3-subunit CDK- activating kinase module (CAK), is involved in both Pol II-mediated transcription and NER (Egly and Coin 2011). Tfb5, the homolog of human TTD-A, is a subunit of the core TFIIH. Unlike other subunits of the core TFIIH which are required for both GGR and TCR, Tfb5 has been shown to be essential for GGR but not absolutely required for TCR, as no apparent repair can be detected in the NTS, but a certain extent of repair can be seen in the TS of either the *RPB2* or *GAL1* genes (Ding, Ruggiero et al. 2007). The effect is unlikely due to changes in the steady state levels of other TFIIH subunits, as Tfb5 does not seem to affect the stability of other TFIIH components (Ranish, Hahn et al. 2004). This may be different from human cells, where TTD-A (the homolog of yeast Tfb5) has been shown to stabilize other subunits of TFIIH (Vermeulen, Bergmann et al. 2000). Yeast Tfb5 interacts with Tfb2, another subunit of the TFIIH core (Zhou, Kou et al. 2007), and it was proposed that yeast Tfb5 acts as an architechtural stabilizer giving structural rigidity to the core TFIIH so that the complex is maintained in its functional architecture (Zhou, Kou et al. 2007). Another possibility is that the Rad25 ATPase activity of TFIIH needs to be stimulated by Tfb5 (Coin, Proietti De Santis et al. 2006) in order to efficiently unwind the double helix around a lesion in the chromatin environment *in vivo* (Ding, Ruggiero et al. 2007).

1.3.1 GGR in the Context of Chromatin

Although the core biochemical mechanism of NER is known, much remains unanswered. One of the looming questions currently being addressed is the issue of NER, especially GGR, in chromatin. As with all DNA-related processes, the NER machinery must deal with the presence of organized chromatin and the physical obstacles that it presents. How cells detect and repair lesions in diverse chromatin environments is a question that remains unanswered. Rearrangement of chromatin structure during NER was discovered more than two decades ago, however the molecular basis of chromatin dynamics during NER in eukaryotic cells is still not well understood (Gong, Kwon et al. 2005; Waters, Teng et al. 2009).

The basic repeating unit of chromatin is the nucleosome, which is comprised of 146 base pairs of DNA wrapped around an octomer of the four core histone proteins H2A, H2B, H3, and H4 (Luger, Mader et al. 1997). Most of this DNA is tightly wrapped in about 1.6 left-handed superhelical turns around the histone octamer, with linker DNA (ranging from 20 to 90 base pairs in length) separating nucleosome cores, and giving the "beads-on-a-string" appearance familiar from electron microscopy. DNA is then further compacted by the organization of nucleosomes into higher order structures, such as 30 nm fibers and the highly condensed state of chromosomes (Wolffe 1999). Adjacent nucleosomes can be arranged in various configurations which affect the accessibility of DNA, thus the DNA-nucleosome polymer must be flexible in order to allow various cellular processes such as replication, transcription, recombination, and repair (Zhang, Jones et al. 2009). The two primary mechanisms which are involved in this flexibility are histone modifications and chromatin remodeling (Palomera-Sanchez and Zurita 2011). Below we summarize new findings regarding NER and the roles of histone modifications and chromatin remodeling activities. The results of previous studies focused on elucidating these

mechanisms have been summarized in several very good recent reviews (Ataian and Krebs 2006; Altaf, Saksouk et al. 2007; Zhang, Jones et al. 2009; Palomera-Sanchez and Zurita 2011).

1.3.2 Chromatin Remodeling and GGR

The complexity of NER and the size of the repair machinery can make it difficult to imagine how DNA distorting lesions can be recognized and processed without temporary rearrangement of chromatin (Thoma 1999). Instead of utilizing specific enzymes that each recognize a specific type of lesion, NER's damage recognition factors recognize helix distortion and bind to DNA to test its local conformation (which explains the wide versatility of NER). The excision step in NER requires considerable space. About 25-30 bp of DNA are unwound in the open complex during NER (Evans, Moggs et al. 1997), and the human excision complex requires about 100 bp of DNA to excise the lesion *in vitro* (Huang and Sancar 1994). Such a complex is incompatible with the structure of the nucleosome, and the linker DNA between nucleosomes is too short to accommodate a repair complex (Thoma 1999). Thus it is obvious that *in vivo* alterations of chromatin either by the lesion itself, by the action of chromatin remodelers or histone modifications, or by DNA metabolizing processes such as transcription, are required to facilitate damage recognition and repair (Fousteri, van Hoffen et al. 2005).

Although chromatin structures can restrict the NER machinery from accessing sites of DNA damage, limited pieces of evidence have emerged recently that chromatin metabolism may also play an active role in the repair process (Waters, Teng et al. 2009). The SWI/SNF superfamily of ATP-dependent chromatin remodeling enzymes all possess an ATPase subunit which can disrupt or alter DNA-histone associations. SWI/SNF is the prototypical chromatin remodeling factor and is present in all eukaryotes (Martens and Winston 2003). Previous studies

have demonstrated that these chromatin remodeling enzymes play an important role in enabling access of the NER machinery to nucleosomal DNA [for a recent review, see (Osley, Tsukuda et al. 2007)]. As transcription disturbs chromatin structure, only the GGR ~~sub~~pathway of NER is modulated by chromatin. As DNA damage recognition is a slow and rate-limiting step in NER (Mone, Bernas et al. 2004) and it has been thought that this step required chromatin remodeling activities, an *in vivo* study examined the possible association between SWI/SNF and the DNA damage recognition complex Rad4-Rad23 in yeast (Gong, Fahy et al. 2006). Using His-tag pulldown and coimmunoprecipitation assays, this study provided evidence linking a chromatin remodeling complex with NER by demonstrating that Snf6 and Snf5, two subunits of the SWI/SNF complex in yeast, copurify with the Rad4-Rad23 heterodimer (Gong, Fahy et al. 2006). It was further shown that this association was stimulated by UV irradiation, indicating that SWI/SNF facilitates chromatin remodeling during NER and that it has a role in facilitating GGR. Based on these findings, it was postulated that Rad4-Rad23 may recruit the SWI/SNF complex to facilitate NER at damage sites *in vivo*, or that SWI/SNF may recognize and bind to another feature of damaged chromatin and aid in recruiting Rad4-Rad23 (Gong, Fahy et al. 2006). The GGR-specific factor Rad16 is also a member of the SWI/SNF family of DNA-dependent ATPases and is thought to have a role in DNA damage recognition (Prakash and Prakash 2000). Interestingly, no association was found between Snf6 and Rad16, which is surprising given that Rad16 is required for GGR. This suggests that SWI/SNF and Rad16 may operate at different stages in the repair process (Gong, Fahy et al. 2006)..

Another example of chromatin remodeling in NER comes from a recently published report which showed that the Ino80 chromatin remodeling complex promotes removal of UV lesions in regions with high nucleosome occupancy (Sarkar, Kiely et al. 2010). More

specifically, the study showed that Ino80 interacts with the early damage recognition complex of Rad4-Rad23 and was recruited to Rad4 in a UV-dependent manner. *ino80*Δ mutants were shown to be defective in both recruitment of repair factors to the damage site and restoration of nucleosome structure after repair. This suggests that Ino80 is recruited to sites of UV DNA damage through interactions with the NER machinery and is required for restoration of chromatin structure after repair (Sarkar, Kiely et al. 2010). The role of Ino80 in NER appears to be conserved in eukaryotic cells. In mammalian cells deletion of two core components of the Ino80 complex, INO80 and ARP5, significantly hampered cellular removal of UV-induced photo lesions but had no significant impact on the transcription of NER factors (Jiang, Wang et al. 2010). Loss of INO80 abolished the assembly of NER factors, suggesting that prior chromatin relaxation is important for the NER incision process.

Because transcription disturbs chromatin, only GGR is modulated by chromatin structure. Indeed, there is no correlation between the heterogeneity in NER and chromatin structure in TCR. However, chromatin remodeling activities associated with the transcription process are likely to play a role in damage recognition during TCR (Zhang, Jones et al. 2009). As mentioned previously, Rad26 is a DNA-dependent ATPase of the SWI/SNF superfamily (Guzder, Habraken et al. 1996). CSB, the human homolog of yeast Rad26, has been shown to interact with XPG (Sarker, Tsutakawa et al. 2005) and attracts repair factors and a histone acetyltransferase to the site of a damage-stalled Pol II (Newman, Bailey et al. 2006). Similar to its human homolog, Rad26 has also been found to play a role in repairing apparently transcriptionally inactive genes, a role possibly exacted through its putative chromatin remodeling activities (Bucheli, Lommel et al. 2001). However, caution needs to be exercised regarding the explanation of role of Rad26 in repairing transcriptionally repressed genes, which can be through TCR initiated by noise

transcription that commonly occurs in both strands of supposedly repressed genes (Li, Ding et al. 2007; Tatum and Li 2011).

1.3.3 Histone Modifications and GGR

From recent evidence, it is clear that GGR requires different mechanisms to relax chromatin and ultimately remove lesions (Palomera-Sanchez and Zurita 2011). In addition to chromatin remodelers, histone modifications have been implicated in various mechanisms of DNA repair. Histones are subject to a multitude of post-translational modifications including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination (Kouzarides 2007). Some of these modifications may modulate the NER process (Gong, Kwon et al. 2005; Waters, Reed et al. 2008). However, the effects of histone modifications on NER in living cells documented previously are generally quite modest and are most likely due to the alteration of chromatin compaction and/or stability. Some recent studies implicating histone modifications in the facilitation of NER are discussed below.

The functional correlation between histone hyperacetylation and efficient NER has been known for some time (Smerdon, Lan et al. 1982; Ramanathan and Smerdon 1989). More recent studies have confirmed this correlation, demonstrating reduced CPD removal in yeast cells lacking the histone acetyltransferase (HAT) Gcn5, which acetylates histone H3 on lysines 9 and 14 (H3K9 and H3K14). Furthermore, the acetylation of H3K9 and H3K14 was shown to increase throughout the genome after irradiation with UV light and resulted in more efficient GGR (Yu, Teng et al. 2005; Teng, Liu et al. 2008). This modification seems to be conserved in mammalian cells, as a recent report showed that E2F1 transcriptional factor in human cells is recruited in the chromatin at sites of UV damage and associates with GCN5 to acetylate H3K9 (Guo, Chen et al. 2011). Histone H3 in yeast has also been shown to be hyperacetylated in strains lacking the

damage recognition factors Rad4 or Rad14, indicating that H3 acetylation occurs before the repair process and is not stimulated by NER (Yu, Teng et al. 2005). However, reinstating of the acetylation level to a pre-UV state was shown to be dependent on NER (Yu, Teng et al. 2005). Additionally, UV-induced hyperacetylation of H3K9 and K14 was shown to be mediated by the GGR-specific factor Rad16 (Teng, Liu et al. 2008). Interestingly, it was demonstrated that pre-hyperacetylated regions could undergo efficient repair even in the absence of Rad16 (Teng, Liu et al. 2008), thus providing a direct link between GGR and histone acetylation. However, it remains to be elucidated whether the Rad16-independent repair is indeed GGR or TCR initiated by noise transcription that may not occur at normal conditions but takes place when the chromatin is pre-hyperacetylated. The noise transcripts can be hard to detect by traditional techniques as they are rapidly degraded after being produced (Struhl 2007). It has been postulated that histone hyperacetylation could regulate NER either directly through generating a suitable binding surface for repair proteins or indirectly through altering the compaction of nucleosomes (Irizar, Yu et al. 2010).

Much like the trend observed for gene expression, the effect of histone acetylation on repair varies according to chromatin status. In yeast, heavily compacted and suppressive regions of chromatin (i.e. heterochromatin) such as telomeres, silenced mating loci, and rDNA repeats, show reduced levels of histone H3 and H4 acetylation after UV. A recent study examined GGR of the *URA3* gene in subtelomeric regions (Irizar, Yu et al. 2010). These regions are hypoacetylated and bound by Sir proteins, which are involved in establishing silenced and heterochromatic regions in the genome. One particular Sir protein, Sir2, a NAD^+-dependent histone deacetylase (HDAC), has been shown to have a preference for removing the acetyl group from H3K9 and K14 as well as H4K16 (Imai, Armstrong et al. 2000; Landry, Sutton et al. 2000).

Repair of CPDs in these regions was shown to be slow, likely a result of the reduced histone acetylation. Furthermore, a significant increase in histone H3 and H4 acetylation after UV was shown in *sir2*Δ deletion mutants, indicating an important role for Sir2 in regulating histone acetylation in response to UV. This increase in histone acetylation resulted in improved NER efficiency, suggesting that the action of the different mechanisms that modify histones to facilitate NER may be influenced by the type of chromatin environment and the prevalence of specific factors like Sir2 in subtelomeric chromatin (Palomera-Sanchez and Zurita 2011).

In addition to histone acetylation, histone methylation also been shown to play a role in the NER process. Dot1 is a histone methyltransferase required for methylation of histone H3 lysine 79 (H3K79). *dot1*Δ mutants are sensitive to UV (Bostelman, Keller et al. 2007) and have a defect in activation of DNA damage checkpoints (Giannattasio, Lazzaro et al. 2005). Indeed, H3K79 methylation was shown to be required for efficient NER in a silenced locus of yeast (Chaudhuri, Wyrick et al. 2009). An even more recent study demonstrated Dot1 to be a novel GGR-specific factor that mediates GGR by methylating its sole substrate, H3K79 (Tatum and Li 2011). Using a nucleotide resolution method which uses streptavidin magnetic beads and biotinylated oligonucleotides to facilitate isolation and strand-specific end-labeling of DNA fragments of interest to measure GGR, the study showed that Dot1 and H3K79 methylation are required for GGR in both nucleosomal core regions and inter-nucleosomal linker regions, but play no role in TCR (Tatum and Li 2011). It was previously suggested that the role of H3K79 methylation in GGR may be via affecting expression of repair factors, such as Rad16 (Chaudhuri, Wyrick et al. 2009). However, it was shown that overexpression of Rad16 in cells whose genomic H3 genes (*HHT1* and *HHT*2) were deleted and complemented with a plasmid encoding the K79A mutant histone H3 (H3K79A) cells did not affect GGR, suggesting that the

effect of H3K79 methylation on GGR is not through regulating the expression of Rad16 (Tatum and Li 2011). It was proposed that the addition of methyl moieties to H3K79 may serve as a docking site for repair factors on the chromatin. In the absence of the methyl groups, the repair machinery may be excluded from the chromatin, including the vicinities of inter-nucleosomal linker regions (Tatum and Li 2011).

We have recently found additional evidence for the involvement of histone modifications in NER in studies involving the yeast Pol II-associated factor 1 complex (Paf1C). Paf1C is comprised of 5 subunits, namely Paf1, Rtf1, Cdc73, Leo1, and Ctr9 and interacts with Pol II and chromatin at both promoters and throughout the coding regions of genes [for a recent review, see (Jaehning 2010)]. Loss of Rtf1 or Cdc73 causes the dissociation of Paf1C from Pol II and chromatin. Paf1C has been shown to be required for monoubiquitination of H2B at lysine 123 (H2BK123) by Rad6 (an E2 ubiquitin conjugase) in complex with Bre1 (an E3 ubiquitin ligase) (Krogan, Dover et al. 2003; Ng, Dole et al. 2003; Wood, Krogan et al. 2003). The Bre1-Rad6-mediated monoubiquitination of H2BK123 is, in turn, partially required for dimethylation and fully required for trimethylation of H3K79 by Dot1 (Shahbazian, Zhang et al. 2005; Nakanishi, Lee et al. 2009; Levesque, Leung et al. 2010). Dot1 can add one methyl group to H3K79 by itself, meaning that Paf1C enables di- and trimethylation of H3K79. Although it can be associated with Pol II, Paf1C may function in enabling these histone modifications independent of Pol II, as both monoubiquitination of H2BK123 (Schulze, Jackson et al. 2009) and methylation of H3K79 (van Leeuwen, Gafken et al. 2002; Ng, Ciccone et al. 2003; Pokholok, Harbison et al. 2005) do not seem to be correlated with the transcriptional activity of a gene. Elimination of one of the PAF components (Rtf1) resulted in significantly compromised GGR, especially in inter-nucleosomal linker regions (Tatum, Placer et al. 2011). Genetic analysis

revealed an epistatic relationship between *RTF1* and *BRE1* and *DOT1*, indicating that these proteins function in the same pathway in response to UV damage. It was further demonstrated that elimination of Rtf1 in *bre1*Δ or *dot1*Δ cells did not affect GGR speed, confirming the presence of an epistatic relationship as well as indicating that the function of PAF in GGR is accomplished through enabling Bre1- and Dot1-mediated histone modifications (Tatum, Placer et al. 2011).

In addition to acetylation and methylation, studies have also provided evidence for multiple roles of histone ubiquitination in NER (Nouspikel 2011). Nucleosome stability is controlled mainly by acetylation, but also to some degree by ubiquitination. Histone H2A is constitutively ubiquitinated even in the absence of DNA damage, especially in condensed chromatin. This ubiquitination was shown to disappear rapidly after UV-induced DNA damage and reappear within 30 minutes to 2 hours (Kapetanaki, Guerrero-Santoro et al. 2006). Histones H2B, H3, and H4 are also constitutively ubiquitinated but to a much lower level (Nouspikel 2011). It was shown that ubiquitination of H3 and H4 increased within 1 hour of UV irradiation, decreased by 4 hours, and returned to original levels at 8 hours (Wang, Zhai et al. 2006). It was postulated that this may act as a means of destabilizing nucleosomes, permitting better access of the repair machinery to the site of the lesion. However, there is a lack of experimental support for this idea. In fact, *in vitro* experiments showed that ubiquitination of H3 and H4 does not cause dissociation from DNA, and *in vivo*, only about half of H3 ubiquitinated after UV-induced damage dissociated from chromatin (Bergink, Salomons et al. 2006; Wang, Zhai et al. 2006).

Evidence for UV-induced ubiquitination came from a study which used cells expressing GFP-tagged ubiquitin (Bergink, Salomons et al. 2006). These cells were UV-irradiated through a micropore filter to induce localized spots of DNA damage in the nucleus. Interestingly, after

induction of DNA damage by UV, ubiquitinated histone H2A was found to accumulate at damage sites. This ubiquitination of histone H2A was shown to be dependent on NER and occurred after incision of the damaged strand, indicating a role in the later steps of NER. Indeed, a subsequent study demonstrated that UV-induced accumulation of ubiquitinated H2A at damage sites is a part of the chromatin restoration process (Zhou, Zhu et al. 2008).

1.4 TCR in Yeast

Lesions that arrest or stall transcription by Pol II on the transcribed strand (TS) are repaired 5-10 times faster than the nontranscribed strand (NTS) by TCR (Hanawalt 1994). TCR has been shown to function in *E. coli* (Mellon and Hanawalt 1989), *S. cerevisiae* (Smerdon and Thoma 1990), and mammalian cells (Mellon, Spivak et al. 1987). While the mechanistic details of TCR in *E. coli* are relatively well understood, the mechanisms of TCR in eukaryotes appear to be extremely complicated [for reviews, see (Fousteri and Mullenders 2008; Hanawalt and Spivak 2008)].

TCR in eukaryotic cells is believed to be triggered by stalled Pol II at a lesion in the transcribed strand of a gene (Fousteri and Mullenders 2008; Hanawalt and Spivak 2008). Rad26, the yeast homolog of human CSB and a putative transcription repair coupling factor, is important for TCR but dispensable for GGR (van Gool, Verhage et al. 1994). However, TCR in yeast is not solely dependent on Rad26, as a significant amount of repair still occurs in cells lacking Rad26 (Verhage, van Gool et al. 1996; Li and Smerdon 2002; Li and Smerdon 2004). Rpb9, a nonessential subunit of Pol II, has also been shown to play a role in mediating TCR (Li and Smerdon 2002; Li and Smerdon 2004; Li, Chen et al. 2006; Li, Ding et al. 2007).

Rad26- and Rpb9- mediated TCR subpathways have been shown to have different efficiencies in different regions of a gene (Li, Chen et al. 2006). Rpb9-mediated TCR operates more effectively in the coding region than in the region upstream of the transcription start site; whereas the Rad26-mediated subpathway operates equally well in both regions (Li and Smerdon 2002; Li and Smerdon 2004). Additionally, in log phase wild type cells, the relative contributions of these two subpathways of TCR may be different from gene to gene. For the *URA3* gene, Rad26 seems to be absolutely required, except for a short region close to the transcription start site (Tijsterman, Verhage et al. 1997), indicating that TCR is accomplished primarily by the Rad26 subpathway. Rad26 is partially required for TCR in the *RPB2* gene (Bhatia, Verhage et al. 1996; Verhage, van Gool et al. 1996; Gregory and Sweder 2001; Li and Smerdon 2002), indicating that both subpathways contribute to TCR in this gene. For the *GAL1* gene, Rad26 is almost dispensable, especially in the coding region, indicating that TCR in this gene of log-phase cultures is fulfilled primarily by the Rpb9 subpathway. Rad26- and Rpb9-mediated repair are also differently modulated by different promoter elements (Li, Chen et al. 2006). The different contributions of the two subpathways of TCR in different genes may be caused by different levels of transcription. In the yeast *GAL1* gene, the efficiency of TCR mediated by Rad26 is determined by the upstream activating sequence (UAS), but not by the TATA or local sequences. However, both the UAS and TATA are necessary to confine Rad26-mediated repair to the transcribed strand of the gene. Abrogating or abolishing transcription by mutation or deletion of the TATA sequence or mutation of the UAS results in Rad26-mediated repair in both the TS and NTS of the GAL1 gene (Li, Chen et al. 2006). This suggests that Rad26-mediated repair can be either transcription-coupled, provided that a substantial level of transcription is present, or transcription-independent, if transcription is too low or absent.

However, as mentioned above, noise transcription, which cannot be easily detected by traditional techniques, may occur in both strands upon the mutation of deletion of the UAS or TATA. This unexpected noise transcription may cause Rad26-mediated repair (which is TCR) to occur in both strands. Conversely, Rpb9-mediated TCR only occurs in the transcribed strand and is efficient only if the TATA and UAS sequences are present, suggesting that TCR mediated by Rpb9 is strictly transcription coupled and is only efficient when the level of transcription is high (Li, Chen et al. 2006).

Rpb9 also plays an important role in promoting ubiquitylation and degradation of Rpb1, the largest subunit of Pol II, in response to UV damage (Chen, Ruggiero et al. 2007). Rpb9 is composed of three distinct domains: the N-terminal Zn1, the C-terminal Zn2, and the central linker. The Zn1 and linker domains are essential for both transcription elongation and TCR functions, but the Zn2 domain is almost dispensable (Li, Chen et al. 2006). The Zn2 domain is essential for Rpb9 to promote degradation of Rpb1, the largest Pol II subunit, whereas the Zn1 and linker domains play a subsidiary role in Rpb1 degradation. This function of Rpb9 seems to be unrelated to any pathways of NER, including both subpathways of TCR, and it remains to be determined how Rpb9 promotes this modification and degradation of Rpb1 (Chen, Ruggiero et al. 2007).

1.4.1 The Role of Rad26 in TCR

Like its human homolog CSB, Rad26 is a DNA-stimulated ATPase and functions in transcription elongation (Selby and Sancar 1997; Lee, Yu et al. 2001). Due to its ATPase activity, Rad26 is the most promising yeast transcription repair coupling factor (Svejstrup 2002). However, how Rad26 functions in TCR remains to be elucidated. Several models have been

proposed based on its DNA-dependent ATPase activity (Svejstrup 2002). Because other members of the Swi/Snf family are able to alter contacts between DNA and DNA-binding proteins, one possibility is that Rad26, through its Swi/Snf-like activity, may displace a stalled Pol II complex at a damage site (Svejstrup 2002). This is the case in *E. coli* where the transcription repair coupling factor Mfd, an ATP-dependent translocase, moves stalled Pol II forward from the damage site, allowing it to continue transcription (Selby and Sancar 1994; Park, Marr et al. 2002). However, other than ATPase domains, there is little structural homology between Rad26 and Mfd. Furthermore, an *in vitro* study demonstrated that CSB cannot displace Pol II stalled at a damage site (Selby and Sancar 1997).

A second model postulates that a Pol II complex stalled at a lesion may be pushed back by the general transcription factor TFIIS, which facilitates Pol II elongation through transcriptional arrest sites and stimulates transcript cleavage, allowing resumed forward translocation during normal transcription elongation (Kettenberger, Armache et al. 2003; Saeki and Svejstrup 2009). Yeast strains lacking Rad26 exhibit a synergistic increase in sensitivity to the DNA-damaging agent methyl methanesulfonate (MMS) when combined with inactivating mutations in NER, suggesting a role for Rad26 in promoting Pol II transcription elongation through damage sites in DNA (Lee, Wang et al. 2002). However, TFIIS does not seem to play any role in TCR in both yeast (Verhage, Heyn et al. 1997) and mammalian cells (Jensen and Mullenders 2010).

Alternative models addressing the fate of a damage-stalled Pol II, such as accessory-factor-mediated lesion bypass and keeping Pol II at a distance through damage-binding factors, might also be relevant in certain situations (Svejstrup 2002). The finding that Rpb1, the largest subunit of Pol II, is ubiquitinated and subsequently degraded in the CSA- and CSB-dependent

manner in response to DNA damage that blocks transcription prompted researchers to propose a model whereby Pol II degradation facilitates lesion access and repair (Bregman, Halaban et al. 1996; Ratner, Balasubramanian et al. 1998). However, a more recent report showed that CSA and CSB are not directly involved in Rpb1 ubiquitylation. The defects in Rpb1 ubiquitylation observed in CS cells are caused by an indirect mechanism: these cells shut down transcription in response to DNA damage, effectively depleting the substrate for ubiquitylation, namely elongating Pol II (Anindya, Aygun et al. 2007). Also, evidence has shown that the ubiquitination and degradation of Rpb1 do not seem to be necessary for TCR in yeast. Rsp5, the only yeast ubiquitin-protein ligase that modifies Pol II, is not required for TCR (Lommel, Bucheli et al. 2000). Furthermore, Def1, which forms a complex with Rad26 in chromatin, is required for Pol II degradation in response to DNA damage but is not required for TCR (Woudstra, Gilbert et al. 2002).

As will be discussed below, recent evidence indicates that the role of Rad26 in TCR may be entirely through indirect mechanisms, by antagonizing the actions of TCR suppressors.

1.4.2 Suppressors of Rad26-independent TCR

Recently, a number of TCR suppressors have been identified. Interestingly, in each case, the release of suppression (i.e. reinstatement of repair) is present only in cells lacking Rad26. Below is a discussion of each of the known suppressors of Rad26-independent TCR and their possible interactions.

Yeast Spt4 and Spt5 form a complex which has been shown to physically interact with Pol II (Hartzog, Wada et al. 1998). The *SPT4* gene is dispensable (Malone, Fassler et al. 1993), whereas *SPT5* is essential (Swanson, Malone et al. 1991), for cell viability. These proteins are

conserved transcription elongation factors and are generally required for normal development and viral gene expression in multicellular eukaryotes (Winston 2001). It was previously shown that deletion of *SPT4* negates the requirement of Rad26 for TCR in yeast, suggesting that Spt4 suppresses Rad26-independent TCR (Jansen, den Dulk et al. 2000). It has been further demonstrated that the suppression effect of Spt4 is indirect via protecting its interacting partner, Spt5, from degradation and by stabilizing the interaction of Spt5 with Pol II (Ding, LeJeune et al. 2010). Indeed, overexpression of Spt5 in the absence of Spt4 suppresses Rad26-independent TCR (Ding, LeJeune et al. 2010), supporting the notion that Spt4 plays an indirect role in this suppression.

Spt5 possesses a C-terminal repeat (CTR) domain, which is dispensable for cell viability and is not involved in interactions with Spt4 and Pol II (Ding, LeJeune et al. 2010). Repair analysis of *rad26Δ* cells whose genomic *SPT5* gene had been deleted and complemented with a plasmid encoding either the full length or CTR-deleted Spt5 revealed that TCR in these cells expressing the CTR-deleted Spt5 was significantly faster than in those expressing full length Spt5, indicating that the Spt5 CTR is involved in suppressing Rad26-independent TCR (Ding, LeJeune et al. 2010). Additional evidence for the role of the CTR in this suppression came from analyzing the phosphorylation state of the CTR. The CTR domain contains 15 6-amino acid repeats with the consensus sequence S(A/T)WGG(A/Q) (Swanson, Malone et al. 1991), with the serine and threonine residues being potential phosphorylation sites. It has been shown that the Spt5 CTR is phosphorylated by the Bur kinase (Liu, Warfield et al. 2009; Zhou, Kuo et al. 2009; Ding, LeJeune et al. 2010). The kinase activity of Bur1 is dependent upon its cyclin partner Bur2. Deletion of Bur1 is lethal to cells, but deletion of Bur2 is not. Additionally, *bur1*Δ and *bur2*Δ mutations result in nearly identical phenotypes (Yao, Neiman et al. 2000). Interestingly, it

was shown that deletion of Bur2 also partially alleviates the necessity for Rad26, suggesting that the phosphorylation of the Spt5 CTR may be partially responsible for suppressing TCR in the absence of Rad26 (Ding, LeJeune et al. 2010).

It is not yet clear how the CTR of Spt5 is acting to suppress Rad26-independent TCR. It was recently reported that the Spt5 CTR is a platform for the association of proteins that promote both transcription elongation and histone modifications (Zhou, Kuo et al. 2009). One such protein complex recruited by the Spt5 CTR is Paf1C (Zhou, Kuo et al. 2009). Indeed, the Rtf1 subunit of Paf1C has been shown to have extensive functional and physical connections with Spt5 (Squazzo, Costa et al. 2002). Additionally, optimal association of Paf1C with Pol II is dependent upon Spt4 (Qiu, Hu et al. 2006) and the Spt5 CTR (Tatum, Placer et al. 2011). Furthermore, recruitment of Paf1C requires the Bur-mediated phosphorylation of the CTR of Spt5 (Liu, Warfield et al. 2009). Results from our lab showed that deletion of any of Paf1C's 5 subunits in *rad26*Δ cells causes increased TCR, indicating that Paf1C too is a suppressor of Rad26-independent TCR. Furthermore, simultaneous deletion of Spt4 along with a Paf1C component in *rad26*Δ cells resulted in similar degrees of repair restoration, suggesting that these suppressors are acting through a common pathway to suppress Rad26-indepedent TCR. However, unlike Spt4, Paf1C appears to be indispensable for suppressing Rad26-independent TCR, as overexpression of Spt5 in cells lacking a Paf1C component did not affect the overall TCR rate in these cells. This suggests that both Paf1C and Spt5 are required for suppressing TCR in the absence of Rad26 and that the role of Paf1C in this suppression is not subsidiary to that of Spt5.

Rpb4 is another nonessential subunit of Pol II and forms a subcomplex with Rpb7, a small but essential subunit of Pol II (Woychik and Young 1989). This subcomplex can

dissociate from Pol II, and deletion of Rpb4 abolishes the association of Rpb7 with Pol II. Interestingly, it was shown that, like Spt4/Spt5, deletion of Rpb4 reinstates TCR in *rad26*Δ cells, indicating that Rpb4 is also a suppressor of Rad26-independent TCR (Li and Smerdon 2002).

Pol II is a globular protein with a deep central cleft (Armache, Kettenberger et al. 2003; Bushnell and Kornberg 2003). The DNA template enters and travels along this cleft to the active site. On one side of the cleft is a flexible clamp structure, which can switch between an open or closed position. The Rpb4-Rpb7 subcomplex is located downstream of the catalytic site in the center of this cleft, and its binding to the 10-subunit core Pol II pushes the clamp to the closed position (Armache, Kettenberger et al. 2003; Bushnell and Kornberg 2003).

RNA polymerases (Cramer 2002) and Spt4/Spt5 (Ponting 2002) are conserved in all three kingdoms of life: bacteria, archaea, and eukaryotes. The archaeal Spt4/Spt5 has recently been co-crystallized with the clamp domain of an archaeal polymerase (Martinez-Rucobo, Sainsbury et al. 2011). Based on this co-crystal structure, a model of the complete yeast Pol II-Spt4/Spt5 elongation complex has been proposed. This model posits that the NGN domain of Spt5 binds to the clamp of Pol II and closes the central cleft to lock nucleic acids and render the elongation complex processive and stable. The KOW1 domain of Spt5 may contact DNA and/or exiting RNA, which could possibly contribute to stability of the elongating Pol II complex and may also involve the Rpb4/Rpb7 subcomplex. The locations of the other domains of Spt5, including the CTR, are currently unpredictable (Martinez-Rucobo, Sainsbury et al. 2011). Spt4, which does not directly contact Pol II, binds to the other side of the Spt5 NGN domain and points away from the surface of Pol II. How Paf1C interacts with Pol II is currently unknown, but one point of contact between Paf1C and Pol II is thought to be an indirect one via the Rtf1 subunit of Paf1C and Spt5, an idea supported by the extensive interactions of Rtf1 and Spt5.

Structure-function analyses of Pol II elongation complexes containing a thymine-thymine CPD in the TS showed that the CPD slowly passes a translocation barrier and enters into the active site of Pol II. The 5' thymine of the CPD directs misincorporation of uridine into the elongating mRNA, which stalls the translocation of Pol II (Brueckner and Cramer 2007). All of the above findings regarding suppression of Rad26-independed repair suggest that Rpb4/Rpb7, Spt4/Spt5, and Paf1C act cooperatively and through the same pathway to exert this suppression effect. It is possible that when Rad26 is absent, a lesion becomes "locked" into the active site of a Pol II elongation complex, which is stabilized by the coordinated interactions of these suppressors with each other and with the core Pol II complex. Deletion or mutation of any of these suppressors may result in the destabilization of elongating Pol II, making it possible for TCR to occur (Tatum, Placer et al. 2011). How Rad26 affects the association of these factors with Pol II is unknown. A possible role for Rad26 in TCR may be to destabilize the Pol II elongation complex. This is supported by the evidence that indicates that Rad26 is dispensable for TCR in the absence of any of these suppression factors. This may explain why this 'megasuppressor' complex only suppresses TCR in the absence of Rad26.

As an interesting aside, it has been demonstrated that Rpb4 (Li and Smerdon 2002) and Paf1C (Tatum, Placer et al. 2011) have dual roles in TCR. Not only do they suppress Rad26-independent TCR, but they have also been shown to facilitate Rad26-dependent TCR. However, how each serves to facilitate this subpathway of TCR remains unknown. Rad26 has been shown to play a role in transcription elongation (Lee, Yu et al. 2001), leading to the possibility that Paf1C may play a positive role in TCR by cooperating with Rad26 to promote transcription elongation. The interaction of Rpb4 with other subunits of Pol II may change the conformation

of the polymerase complex, and this may, in turn, improve the interactions with Rad26 (Li and Smerdon 2002).

1.5 References

Aboussekhra, A., M. Biggerstaff, et al. (1995). "Mammalian DNA nucleotide excision repair reconstituted with purified protein components." Cell **80**(6): 859-68.

Altaf, M., N. Saksouk, et al. (2007). "Histone modifications in response to DNA damage." Mutat Res **618**(1-2): 81-90.

Anindya, R., O. Aygun, et al. (2007). "Damage-induced ubiquitylation of human RNA polymerase II by the ubiquitin ligase Nedd4, but not Cockayne syndrome proteins or BRCA1." Mol Cell **28**(3): 386-97.

Armache, K. J., H. Kettenberger, et al. (2003). "Architecture of initiation-competent 12-subunit RNA polymerase II." Proc Natl Acad Sci U S A **100**(12): 6964-8.

Aso, T., W. S. Lane, et al. (1995). "Elongin (SIII): a multisubunit regulator of elongation by RNA polymerase II." Science **269**(5229): 1439-43.

Ataian, Y. and J. E. Krebs (2006). "Five repair pathways in one context: chromatin modification during DNA repair." Biochem Cell Biol **84**(4): 490-504.

Bang, D. D., R. Verhage, et al. (1992). "Molecular cloning of RAD16, a gene involved in differential repair in Saccharomyces cerevisiae." Nucleic Acids Res **20**(15): 3925-31.

Bergink, S., F. A. Salomons, et al. (2006). "DNA damage triggers nucleotide excision repair-dependent monoubiquitylation of histone H2A." Genes Dev **20**(10): 1343-52.

Bhatia, P. K., R. A. Verhage, et al. (1996). "Molecular cloning and characterization of Saccharomyces cerevisiae RAD28, the yeast homolog of the human Cockayne syndrome A (CSA) gene." J Bacteriol **178**(20): 5977-88.

Bostelman, L. J., A. M. Keller, et al. (2007). "Methylation of histone H3 lysine-79 by Dot1p plays multiple roles in the response to UV damage in Saccharomyces cerevisiae." DNA Repair (Amst) **6**(3): 383-95.

Bradsher, J. N., K. W. Jackson, et al. (1993). "RNA polymerase II transcription factor SIII. I. Identification, purification, and properties." J Biol Chem **268**(34): 25587-93.

Bregman, D. B., R. Halaban, et al. (1996). "UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells." Proc Natl Acad Sci U S A **93**(21): 11586-90.

Brueckner, F. and P. Cramer (2007). "DNA photodamage recognition by RNA polymerase II." FEBS Lett **581**(15): 2757-2760.

Bucheli, M., L. Lommel, et al. (2001). "The defect in transcription-coupled repair displayed by a Saccharomyces cerevisiae rad26 mutant is dependent on carbon source and is not associated with a lack of transcription." Genetics **158**(3): 989-97.

Bushnell, D. A. and R. D. Kornberg (2003). "Complete, 12-subunit RNA polymerase II at 4.1-A resolution: implications for the initiation of transcription." Proc Natl Acad Sci U S A **100**(12): 6969-73.

Chaudhuri, S., J. J. Wyrick, et al. (2009). "Histone H3 Lys79 methylation is required for efficient nucleotide excision repair in a silenced locus of Saccharomyces cerevisiae." Nucleic Acids Res **37**(5): 1690-700.

Chen, X., C. Ruggiero, et al. (2007). "Yeast Rpb9 plays an important role in ubiquitylation and degradation of Rpb1 in response to UV-induced DNA damage." Mol Cell Biol **27**(13): 4617-25.

Cleaver, J. E., E. T. Lam, et al. (2009). "Disorders of nucleotide excision repair: the genetic and molecular basis of heterogeneity." Nat Rev Genet **10**(11): 756-68.

Coin, F., L. Proietti De Santis, et al. (2006). "p8/TTD-A as a repair-specific TFIIH subunit." Mol Cell **21**(2): 215-26.

Cramer, P. (2002). "Multisubunit RNA polymerases." Curr Opin Struct Biol **12**(1): 89-97.

Ding, B., D. LeJeune, et al. (2010). "The C-terminal repeat domain of Spt5 plays an important role in suppression of Rad26-independent transcription coupled repair." J Biol Chem **285**(8): 5317-26.

Ding, B., C. Ruggiero, et al. (2007). "Tfb5 is partially dispensable for Rad26 mediated transcription coupled nucleotide excision repair in yeast." DNA Repair (Amst) **6**(11): 1661-9.

Egly, J. M. and F. Coin (2011). "A history of TFIIH: Two decades of molecular biology on a pivotal transcription/repair factor." DNA Repair (Amst) **In press**.

Eisen, J. A., K. S. Sweder, et al. (1995). "Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions." Nucleic Acids Res **23**(14): 2715-23.

Evans, E., J. G. Moggs, et al. (1997). "Mechanism of open complex and dual incision formation by human nucleotide excision repair factors." Embo J **16**(21): 6559-73.

Fousteri, M. and L. H. Mullenders (2008). "Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects." Cell Res **18**(1): 73-84.

Fousteri, M., A. van Hoffen, et al. (2005). "Repair of DNA lesions in chromosomal DNA impact of chromatin structure and Cockayne syndrome proteins." DNA Repair (Amst) **4**(8): 919-25.

Giannattasio, M., F. Lazzaro, et al. (2005). "The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1 and H3 methylation by Dot1." J Biol Chem **280**(11): 9879-86.

Gillette, T. G., S. Yu, et al. (2006). "Distinct functions of the ubiquitin-proteasome pathway influence nucleotide excision repair." Embo J **25**(11): 2529-38.

Gong, F., D. Fahy, et al. (2006). "Rad4-Rad23 interaction with SWI/SNF links ATP-dependent chromatin remodeling with nucleotide excision repair." Nat Struct Mol Biol **13**(10): 902-7.

Gong, F., Y. Kwon, et al. (2005). "Nucleotide excision repair in chromatin and the right of entry." DNA Repair (Amst) **4**(8): 884-96.

Gregory, S. M. and K. S. Sweder (2001). "Deletion of the CSB homolog, RAD26, yields Spt(-) strains with proficient transcription-coupled repair." Nucleic Acids Res **29**(14): 3080-6.

Guo, R., J. Chen, et al. (2011). "GCN5 and E2F1 stimulate nucleotide excision repair by promoting H3K9 acetylation at sites of damage." Nucleic Acids Res **39**(4): 1390-7.

Guzder, S. N., Y. Habraken, et al. (1995). "Reconstitution of yeast nucleotide excision repair with purified Rad proteins, replication protein A, and transcription factor TFIIH." J Biol Chem **270**(22): 12973-6.

Guzder, S. N., Y. Habraken, et al. (1996). "RAD26, the yeast homolog of human Cockayne's syndrome group B gene, encodes a DNA-dependent ATPase." J Biol Chem **271**(31): 18314-7.

Guzder, S. N., C. H. Sommers, et al. (2006). "Complex formation with damage recognition protein Rad14 is essential for Saccharomyces cerevisiae Rad1-Rad10 nuclease to perform its function in nucleotide excision repair in vivo." Mol Cell Biol **26**(3): 1135-41.

Guzder, S. N., P. Sung, et al. (1997). "Yeast Rad7-Rad16 complex, specific for the nucleotide excision repair of the nontranscribed DNA strand, is an ATP-dependent DNA damage sensor." J Biol Chem **272**(35): 21665-8.

Guzder, S. N., P. Sung, et al. (1998). "Affinity of yeast nucleotide excision repair factor 2, consisting of the Rad4 and Rad23 proteins, for ultraviolet damaged DNA." J Biol Chem **273**(47): 31541-6.

Guzder, S. N., P. Sung, et al. (1998). "The DNA-dependent ATPase activity of yeast nucleotide excision repair factor 4 and its role in DNA damage recognition." J Biol Chem **273**(11): 6292-6.

Habraken, Y., P. Sung, et al. (1993). "Yeast excision repair gene RAD2 encodes a single-stranded DNA endonuclease." Nature **366**(6453): 365-8.

Hanawalt, P. C. (1994). "Transcription-coupled repair and human disease." Science **266**(5193): 1957-8.

Hanawalt, P. C. and G. Spivak (2008). "Transcription-coupled DNA repair: two decades of progress and surprises." Nat Rev Mol Cell Biol **9**(12): 958-70.

Harper, J. W. and S. J. Elledge (2007). "The DNA damage response: ten years after." Mol Cell **28**(5): 739-45.

Hartzog, G. A., T. Wada, et al. (1998). "Evidence that Spt4, Spt5, and Spt6 control transcription elongation by RNA polymerase II in Saccharomyces cerevisiae." Genes Dev **12**(3): 357-69.

Hoeijmakers, J. H. (2009). "DNA damage, aging, and cancer." N Engl J Med **361**(15): 1475-85.

Huang, J. C. and A. Sancar (1994). "Determination of minimum substrate size for human excinuclease." J Biol Chem **269**(29): 19034-40.

Imai, S., C. M. Armstrong, et al. (2000). "Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase." Nature **403**(6771): 795-800.

Irizar, A., Y. Yu, et al. (2010). "Silenced yeast chromatin is maintained by Sir2 in preference to permitting histone acetylations for efficient NER." Nucleic Acids Res **38**(14): 4675-86.

Jaehning, J. A. (2010). "The Paf1 complex: platform or player in RNA polymerase II transcription?" Biochim Biophys Acta **1799**(5-6): 379-88.

Jansen, L. E., H. den Dulk, et al. (2000). "Spt4 modulates Rad26 requirement in transcription-coupled nucleotide excision repair." Embo J **19**(23): 6498-507.

Jensen, A. and L. H. Mullenders (2010). "Transcription factor IIS impacts UV-inhibited transcription." DNA Repair (Amst) **9**(11): 1142-50.

Jiang, Y., X. Wang, et al. (2010). "INO80 chromatin remodeling complex promotes the removal of UV lesions by the nucleotide excision repair pathway." Proc Natl Acad Sci U S A **107**(40): 17274-9.

Kapetanaki, M. G., J. Guerrero-Santoro, et al. (2006). "The DDB1-CUL4ADDB2 ubiquitin ligase is deficient in xeroderma pigmentosum group E and targets histone H2A at UV-damaged DNA sites." Proc Natl Acad Sci U S A **103**(8): 2588-93.

Kettenberger, H., K. J. Armache, et al. (2003). "Architecture of the RNA polymerase II-TFIIS complex and implications for mRNA cleavage." Cell **114**(3): 347-57.

Kou, H., Y. Zhou, et al. (2008). "Mms19 protein functions in nucleotide excision repair by sustaining an adequate cellular concentration of the TFIIH component Rad3." Proc Natl Acad Sci U S A **105**(41): 15714-9.

Kouzarides, T. (2007). "Chromatin modifications and their function." Cell **128**(4): 693-705.

Krogan, N. J., J. Dover, et al. (2003). "The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation." Mol Cell **11**(3): 721-9.

Landry, J., A. Sutton, et al. (2000). "The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases." Proc Natl Acad Sci U S A **97**(11): 5807-11.

Lee, K. B., D. Wang, et al. (2002). "Transcription-coupled and DNA damage-dependent ubiquitination of RNA polymerase II in vitro." Proc Natl Acad Sci U S A **99**(7): 4239-44.

Lee, S. K., S. L. Yu, et al. (2001). "Requirement for yeast RAD26, a homolog of the human CSB gene, in elongation by RNA polymerase II." Mol Cell Biol **21**(24): 8651-6.

Lejeune, D., X. Chen, et al. (2009). "Yeast Elc1 plays an important role in global genomic repair but not in transcription coupled repair." DNA Repair (Amst) **8**(1): 40-50.

Levesque, N., G. P. Leung, et al. (2010). "Loss of H3 K79 trimethylation leads to suppression of Rtt107-dependent DNA damage sensitivity through the translesion synthesis pathway." J Biol Chem **285**(45): 35113-22.

Li, S., X. Chen, et al. (2006). "Modulation of Rad26- and Rpb9-mediated DNA repair by different promoter elements." J Biol Chem **281**(48): 36643-51.

Li, S., B. Ding, et al. (2007). "The roles of Rad16 and Rad26 in repairing repressed and actively transcribed genes in yeast." DNA Repair (Amst) **6**(11): 1596-606.

Li, S. and M. J. Smerdon (2002). "Nucleosome structure and repair of N-methylpurines in the GAL1-10 genes of Saccharomyces cerevisiae." J Biol Chem **277**(47): 44651-9.

Li, S. and M. J. Smerdon (2002). "Rpb4 and Rpb9 mediate subpathways of transcription-coupled DNA repair in Saccharomyces cerevisiae." Embo J **21**(21): 5921-9.

Li, S. and M. J. Smerdon (2004). "Dissecting transcription-coupled and global genomic repair in the chromatin of yeast GAL1-10 genes." J Biol Chem **279**(14): 14418-26.

Lindahl, T. and R. D. Wood (1999). "Quality control by DNA repair." Science **286**(5446): 1897-905.

Liu, Y., L. Warfield, et al. (2009). "Phosphorylation of the transcription elongation factor Spt5 by yeast Bur1 kinase stimulates recruitment of the PAF complex." Mol Cell Biol **29**(17): 4852-63.

Lommel, L., M. E. Bucheli, et al. (2000). "Transcription-coupled repair in yeast is independent from ubiquitylation of RNA pol II: implications for Cockayne's syndrome." Proc Natl Acad Sci U S A **97**(16): 9088-92.

Luger, K., A. W. Mader, et al. (1997). "Crystal structure of the nucleosome core particle at 2.8 A resolution." Nature **389**(6648): 251-60.

Malone, E. A., J. S. Fassler, et al. (1993). "Molecular and genetic characterization of SPT4, a gene important for transcription initiation in Saccharomyces cerevisiae." Mol Gen Genet **237**(3): 449-59.

Martens, J. A. and F. Winston (2003). "Recent advances in understanding chromatin remodeling by Swi/Snf complexes." Curr Opin Genet Dev **13**(2): 136-42.

Martinez-Rucobo, F. W., S. Sainsbury, et al. (2011). "Architecture of the RNA polymerase-Spt4/5 complex and basis of universal transcription processivity." Embo J **30**(7): 1302-10.

Mellon, I. and P. C. Hanawalt (1989). "Induction of the Escherichia coli lactose operon selectively increases repair of its transcribed DNA strand." Nature **342**(6245): 95-8.

Mellon, I., G. Spivak, et al. (1987). "Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene." Cell **51**(2): 241-9.

Mone, M. J., T. Bernas, et al. (2004). "In vivo dynamics of chromatin-associated complex formation in mammalian nucleotide excision repair." Proc Natl Acad Sci U S A **101**(45): 15933-7.

Nakanishi, S., J. S. Lee, et al. (2009). "Histone H2BK123 monoubiquitination is the critical determinant for H3K4 and H3K79 trimethylation by COMPASS and Dot1." J Cell Biol **186**(3): 371-7.

Newman, J. C., A. D. Bailey, et al. (2006). "Cockayne syndrome group B protein (CSB) plays a general role in chromatin maintenance and remodeling." Proc Natl Acad Sci U S A **103**(25): 9613-8.

Ng, H. H., D. N. Ciccone, et al. (2003). "Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation." Proc Natl Acad Sci U S A **100**(4): 1820-5.

Ng, H. H., S. Dole, et al. (2003). "The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B." J Biol Chem **278**(36): 33625-8.

Nouspikel, T. (2009). "DNA repair in mammalian cells : Nucleotide excision repair: variations on versatility." Cell Mol Life Sci **66**(6): 994-1009.

Nouspikel, T. (2011). "Multiple roles of ubiquitination in the control of nucleotide excision repair." Mech Ageing Dev.

Osley, M. A., T. Tsukuda, et al. (2007). "ATP-dependent chromatin remodeling factors and DNA damage repair." Mutat Res **618**(1-2): 65-80.

Palomera-Sanchez, Z. and M. Zurita (2011). "Open, repair and close again: chromatin dynamics and the response to UV-induced DNA damage." DNA Repair (Amst) **10**(2): 119-25.

Park, J. S., M. T. Marr, et al. (2002). "E. coli Transcription repair coupling factor (Mfd protein) rescues arrested complexes by promoting forward translocation." Cell **109**(6): 757-67.

Pettijohn, D. and P. Hanawalt (1964). "Evidence for Repair-Replication of Ultraviolet Damaged DNA in Bacteria." J Mol Biol **9**: 395-410.

Pokholok, D. K., C. T. Harbison, et al. (2005). "Genome-wide map of nucleosome acetylation and methylation in yeast." Cell **122**(4): 517-27.

Ponting, C. P. (2002). "Novel domains and orthologues of eukaryotic transcription elongation factors." Nucleic Acids Res **30**(17): 3643-52.

Prakash, S. and L. Prakash (2000). "Nucleotide excision repair in yeast." Mutat Res **451**(1-2): 13-24.

Qiu, H., C. Hu, et al. (2006). "The Spt4p subunit of yeast DSIF stimulates association of the Paf1 complex with elongating RNA polymerase II." Mol Cell Biol **26**(8): 3135-48.

Ramanathan, B. and M. J. Smerdon (1989). "Enhanced DNA repair synthesis in hyperacetylated nucleosomes." J Biol Chem **264**(19): 11026-34.

Ramsey, K. L., J. J. Smith, et al. (2004). "The NEF4 complex regulates Rad4 levels and utilizes Snf2/Swi2-related ATPase activity for nucleotide excision repair." Mol Cell Biol **24**(14): 6362-78.

Ranish, J. A., S. Hahn, et al. (2004). "Identification of TFB5, a new component of general transcription and DNA repair factor IIH." Nat Genet **36**(7): 707-13.

Rasmussen, R. E. and R. B. Painter (1964). "Evidence for Repair of Ultra-Violet Damaged Deoxyribonucleic Acid in Cultured Mammalian Cells." Nature **203**: 1360-2.

Ratner, J. N., B. Balasubramanian, et al. (1998). "Ultraviolet radiation-induced ubiquitination and proteasomal degradation of the large subunit of RNA polymerase II. Implications for transcription-coupled DNA repair." J Biol Chem **273**(9): 5184-9.

Ribar, B., L. Prakash, et al. (2006). "Requirement of ELC1 for RNA polymerase II polyubiquitylation and degradation in response to DNA damage in Saccharomyces cerevisiae." Mol Cell Biol **26**(11): 3999-4005.

Ribar, B., L. Prakash, et al. (2007). "ELA1 and CUL3 are required along with ELC1 for RNA polymerase II polyubiquitylation and degradation in DNA-damaged yeast cells." Mol Cell Biol **27**(8): 3211-6.

Saeki, H. and J. Q. Svejstrup (2009). "Stability, flexibility, and dynamic interactions of colliding RNA polymerase II elongation complexes." Mol Cell **35**(2): 191-205.

Sarkar, S., R. Kiely, et al. (2010). "The Ino80 chromatin-remodeling complex restores chromatin structure during UV DNA damage repair." J Cell Biol **191**(6): 1061-8.

Sarker, A. H., S. E. Tsutakawa, et al. (2005). "Recognition of RNA polymerase II and transcription bubbles by XPG, CSB, and TFIIH: insights for transcription-coupled repair and Cockayne Syndrome." Mol Cell **20**(2): 187-98.

Schulze, J. M., J. Jackson, et al. (2009). "Linking cell cycle to histone modifications: SBF and H2B monoubiquitination machinery and cell-cycle regulation of H3K79 dimethylation." Mol Cell **35**(5): 626-41.

Selby, C. P. and A. Sancar (1994). "Mechanisms of transcription-repair coupling and mutation frequency decline." Microbiol Rev **58**(3): 317-29.

Selby, C. P. and A. Sancar (1997). "Human transcription-repair coupling factor CSB/ERCC6 is a DNA-stimulated ATPase but is not a helicase and does not disrupt the ternary transcription complex of stalled RNA polymerase II." J Biol Chem **272**(3): 1885-90.

Shahbazian, M. D., K. Zhang, et al. (2005). "Histone H2B ubiquitylation controls processive methylation but not monomethylation by Dot1 and Set1." Mol Cell **19**(2): 271-7.

Smerdon, M. J., S. Y. Lan, et al. (1982). "Sodium butyrate stimulates DNA repair in UV-irradiated normal and xeroderma pigmentosum human fibroblasts." J Biol Chem **257**(22): 13441-7.

Smerdon, M. J. and F. Thoma (1990). "Site-specific DNA repair at the nucleosome level in a yeast minichromosome." Cell **61**(4): 675-84.

Squazzo, S. L., P. J. Costa, et al. (2002). "The Paf1 complex physically and functionally associates with transcription elongation factors in vivo." Embo J **21**(7): 1764-74.

Struhl, K. (2007). "Transcriptional noise and the fidelity of initiation by RNA polymerase II." Nat Struct Mol Biol **14**(2): 103-5.

Sung, P., P. Reynolds, et al. (1993). "Purification and characterization of the Saccharomyces cerevisiae RAD1/RAD10 endonuclease." J Biol Chem **268**(35): 26391-9.

Svejstrup, J. Q. (2002). "Mechanisms of transcription-coupled DNA repair." Nat Rev Mol Cell Biol **3**(1): 21-9.

Swanson, M. S., E. A. Malone, et al. (1991). "SPT5, an essential gene important for normal transcription in Saccharomyces cerevisiae, encodes an acidic nuclear protein with a carboxy-terminal repeat." Mol Cell Biol **11**(6): 3009-19.

Tatum, D. and S. Li (2011). "Evidence that the histone methyltransferase DOT1 mediates global genomic repair by methylating histone H3 on lysine 79." J Biol Chem **in press**.

Tatum, D., M. Placer, et al. (2011). "The roles of RNA polymerase II-associated factor 1 complex in different subpathways of nucleotide excision repair." Submitted.

Teng, Y., H. Liu, et al. (2008). "Saccharomyces cerevisiae Rad16 mediates ultraviolet-dependent histone H3 acetylation required for efficient global genome nucleotide-excision repair." EMBO Rep **9**(1): 97-102.

Thoma, F. (1999). "Light and dark in chromatin repair: repair of UV-induced DNA lesions by photolyase and nucleotide excision repair." Embo J **18**(23): 6585-98.

Tijsterman, M., R. A. Verhage, et al. (1997). "Transitions in the coupling of transcription and nucleotide excision repair within RNA polymerase II-transcribed genes of Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **94**(15): 8027-32.

Tomkinson, A. E., A. J. Bardwell, et al. (1994). "Purification of Rad1 protein from Saccharomyces cerevisiae and further characterization of the Rad1/Rad10 endonuclease complex." Biochemistry **33**(17): 5305-11.

van Gool, A. J., R. Verhage, et al. (1994). "RAD26, the functional S. cerevisiae homolog of the Cockayne syndrome B gene ERCC6." Embo J **13**(22): 5361-9.

van Leeuwen, F., P. R. Gafken, et al. (2002). "Dot1p modulates silencing in yeast by methylation of the nucleosome core." Cell **109**(6): 745-56.

Verhage, R., A. M. Zeeman, et al. (1994). "The RAD7 and RAD16 genes, which are essential for pyrimidine dimer removal from the silent mating type loci, are also required for repair of the nontranscribed strand of an active gene in Saccharomyces cerevisiae." Mol Cell Biol **14**(9): 6135-42.

Verhage, R. A., J. Heyn, et al. (1997). "Transcription elongation factor S-II is not required for transcription-coupled repair in yeast." Mol Gen Genet **254**(3): 284-90.

Verhage, R. A., A. J. van Gool, et al. (1996). "Double mutants of Saccharomyces cerevisiae with alterations in global genome and transcription-coupled repair." Mol Cell Biol **16**(2): 496-502.

Vermeulen, W., E. Bergmann, et al. (2000). "Sublimiting concentration of TFIIH transcription/DNA repair factor causes TTD-A trichothiodystrophy disorder." Nat Genet **26**(3): 307-13.

Wang, H., L. Zhai, et al. (2006). "Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage." Mol Cell **22**(3): 383-94.

Waters, R., S. H. Reed, et al. (2008). "Chromatin modifications and nucleotide excision repair." SEB Exp Biol Ser **59**: 189-201.

Waters, R., Y. Teng, et al. (2009). "Tilting at windmills? The nucleotide excision repair of chromosomal DNA." DNA Repair (Amst) **8**(2): 146-52.

Winston, F. (2001). "Control of eukaryotic transcription elongation." Genome Biol **2**(2): REVIEWS1006.

Wolffe, A. P. (1999). Chromatin: Structure and Function. London and New York, Academic Press.

Wood, A., N. J. Krogan, et al. (2003). "Bre1, an E3 ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter." Mol Cell **11**(1): 267-74.

Woudstra, E. C., C. Gilbert, et al. (2002). "A Rad26-Def1 complex coordinates repair and RNA pol II proteolysis in response to DNA damage." Nature **415**(6874): 929-33.

Woychik, N. A. and R. A. Young (1989). "RNA polymerase II subunit RPB4 is essential for high- and low-temperature yeast cell growth." Mol Cell Biol **9**(7): 2854-9.

Yao, S., A. Neiman, et al. (2000). "BUR1 and BUR2 encode a divergent cyclin-dependent kinase-cyclin complex important for transcription in vivo." Mol Cell Biol **20**(19): 7080-7.

Yu, S., T. Owen-Hughes, et al. (2004). "The yeast Rad7/Rad16/Abf1 complex generates superhelical torsion in DNA that is required for nucleotide excision repair." DNA Repair (Amst) **3**(3): 277-87.

Yu, Y., Y. Teng, et al. (2005). "UV irradiation stimulates histone acetylation and chromatin remodeling at a repressed yeast locus." Proc Natl Acad Sci U S A **102**(24): 8650-5.

Zhang, L., K. Jones, et al. (2009). "The molecular basis of chromatin dynamics during nucleotide excision repair." Biochem Cell Biol **87**(1): 265-72.

Zhou, K., W. H. Kuo, et al. (2009). "Control of transcriptional elongation and cotranscriptional histone modification by the yeast BUR kinase substrate Spt5." Proc Natl Acad Sci U S A **106**(17): 6956-61.

Zhou, W., P. Zhu, et al. (2008). "Histone H2A monoubiquitination represses transcription by inhibiting RNA polymerase II transcriptional elongation." Mol Cell **29**(1): 69-80.

Zhou, Y., H. Kou, et al. (2007). "Tfb5 interacts with Tfb2 and facilitates nucleotide excision repair in yeast." Nucleic Acids Res **35**(3): 861-71.

CHAPTER 2

YEAST ELC1 PLAYS AN IMPORTANT ROLE IN GLOBAL GENOMIC REPAIR BUT NOT IN TRANSCRIPTION COUPLED REPAIR*

2.1 Introduction

Nucleotide excision repair (NER) is a conserved DNA repair process that is capable of removing a large variety of helix-distorting lesions including UV-induced cyclobutane pyrimidine dimers (CPDs) and 6–4 photoproducts (Friedberg, Walker et al. 2006). NER has traditionally been grouped into two pathways: transcription coupled repair (TCR) and global genomic repair (GGR) (Hanawalt 2002). TCR is dedicated for repairing the transcribed strand (TS) of active genes and generally occurs faster than GGR, which removes lesions throughout the genome (Hanawalt 2002).

While the mechanism of TCR is relatively well understood in *Escherichia coli* (Selby and Sancar 1993) the detailed biochemical mechanism of this repair process remains largely elusive in eukaryotes (Laine and Egly 2006; Sarasin and Stary 2007; Fousteri and Mullenders 2008). It is generally thought that a stalled RNA polymerase at a DNA lesion serves as the initial signal for TCR (Hanawalt 2002). In mammalian cells, Cockayne syndrome complementation group A (CSA) and B (CSB) proteins are required for TCR (Venema, Mullenders et al. 1990; Lommel and Hanawalt 1991; Troelstra, van Gool et al. 1992; van Hoffen, Natarajan et al. 1993). In *Saccharomyces cerevisiae*, Rad26 (van Gool, Verhage et al. 1994), the homolog of human CSB, and Rpb9 (Li and Smerdon 2002; Li and Smerdon 2004; Li, Chen et al. 2006; Li, Ding et al. 2006), a non-essential subunit of RNA polymerase II (Pol II), have been shown to mediate two subpathways of TCR.

*Reprinted with the kind permission of *DNA Repair (Amst)*

The process of GGR in mammalian cells depends on xeroderma pigmentosum complementation group C (XPC) (Venema, van Hoffen et al. 1990; Venema, van Hoffen et al. 1991) and damage-specific DNA-binding proteins (DDBs) (Tang, Hwang et al. 2000). In yeast, GGR has been shown to rely on Rad7 and Rad16, which show no significant sequence or structural similarity to XPC (Verhage, Zeeman et al. 1994). Rad7 and Rad16 form a stable heterodimeric complex termed nucleotide excision repair factor 4 (NEF4) (Guzder, Sung et al. 1997). Rad16 is a member of the Swi2/Snf2 family of ATPases, and the Rad7/Rad16 complex binds specifically and preferentially to UV damaged DNA in an ATP-dependent manner (Guzder, Sung et al. 1997). The precise roles of these proteins remain unclear. One suggestion is that the Rad7/Rad16 complex acts as an ATP-dependent motor which translocates along the DNA in search of damage, and upon encountering a lesion, ATPase activity is inhibited, stopping the enzyme (Guzder, Sung et al. 1998). This stalled complex may serve to remodel and open damaged chromatin, thereby facilitating recruitment of other repair proteins and access to the lesion (Thoma 1999; Prakash and Prakash 2000). Contrary to the supposition that Rad7 and Rad16 are involved the early steps of NER, including DNA damage recognition and stimulation of incision at damage sites, it has also been posited that the Rad7/Rad16 complex instead participates in the subsequent postincision events of oligonucleotide excision and repair synthesis (Reed, You et al. 1998).

Yeast Elc1 is a homolog of mammalian elongin C which forms a heterotrimeric complex with elongins A and B (Bradsher, Jackson et al. 1993; Bradsher, Tan et al. 1993; Aso, Lane et al. 1995; Aso, Mokady et al. 1995; Aso and Conrad 1997). In mammalian cells, the elongin A, B and C complex increases the rate of transcription by suppressing Pol II pausing (Bradsher,

Jackson et al. 1993; Bradsher, Tan et al. 1993). However, in yeast, only elongins A (Ela1) and C are present, and there is no evidence of a role for this complex in transcriptional stimulation (Ramsey, Smith et al. 2004). The yeast Elc1 has been shown to be a component of a ubiquitin ligase that contains Rad7 and Rad16, and is responsible for regulating the levels of Rad4 protein in response to UV damage (Ramsey, Smith et al. 2004). It was later found that this ubiquitin ligase complex also contains Cul3, and plays an important role in ubiquitination and subsequent degradation of Rad4 (Gillette, Yu et al. 2006). The ubiquitination of Rad4, but not its subsequent degradation, was shown to facilitate NER (Gillette, Yu et al. 2006). It has also been suggested that Elc1 is a component of another ubiquitin ligase complex, which contains Ela1, Cul3, and Roc1 and is responsible for the polyubiquitylation and subsequent degradation of Pol II in response to DNA damage (Ribar, Prakash et al. 2006; Ribar, Prakash et al. 2007).

We sought to determine the roles of Elc1 in different pathways of NER and found that Elc1 has no function in TCR but plays an important role in GGR. Furthermore, we present evidence that the role of Elc1 in GGR is not subsidiary to that of Rad7 and Rad16.

2.2 Materials and Methods

2.2.1 Yeast Strains and Plasmids

Wild type yeast strains Y452 (*MAT*ˬ *ura3-52 his3-1 leu2-3 leu2-112*) and BJ5465 (*MATa ura3-52 trp1 lys2-801 leu2_1 his3_200 pep4::HIS3 prb1_1.6R can1 GAL*) were obtained from Dr. Louise Prakash and the American Type Culture Collection, respectively All deletions were made in these backgrounds and confirmed by PCR analysis using procedures described previously (Li and Smerdon 2002).

Strains with their genomic *RAD7* and *RAD16* genes tagged with three consecutive FLAG sequences (3×FLAG) were created using PCR products amplified from plasmid pFLAGKanMX,

as described previously (Gelbart, Rechsteiner et al. 2001). PCR primers were designed to include about 20 bases complementary to the tagging cassette and approximately 50 bases complementary to the gene of interest. These primers were used to amplify the dictated segment using PCR and subsequently transformed into the appropriate yeast strains. The correct integration of the tagged sequences was confirmed by PCR.

The plasmid overexpressing 3×myc tagged Rad16 was created using vector pESC-URA (Stratagene). The vector contains divergent *GAL1-10* promoters, and genes inserted downstream of the promoters can be highly induced by galactose. Two consecutive myc tag sequences were inserted in-frame downstream of the vector's native single myc sequence to create a vector for overexpressing 3×myc tagged proteins under the control of the *GAL1* promoter. The *RAD16* gene coding sequence was amplified by PCR and inserted in-frame upstream of the 3×myc sequences (between the XmaI and Sal sites) to create plasmid pRAD16-3M.

The plasmid overexpressing 3×FLAG tagged Rad7 was created using another modified version of pESC-URA. Two consecutive FLAG sequences were inserted in-frame downstream of the vector's native single FLAG sequence to create a vector for overexpressing 3×FLAG tagged proteins under the control of the *GAL10* promoter. The coding sequence of the *RAD7* gene was amplified by PCR and inserted in-frame upstream of the 3×FLAG sequences (between the SpeI and ClaI sites), yielding plasmid pRAD7-3F.

The plasmid simultaneously overexpressing 3×FLAG tagged Rad7 and 3×myc tagged Rad16 (pR16R7) was created by replacing the SpeI–PacI sequence (encompassing the FLAG sequence) in plasmid pRAD16-3Mwith the entire RAD7- 3×FLAG segment (between the SpeI and PacI sites) from pRAD7-3F.

2.2.2 UV Sensitivity Assay

Yeast cells were grown at 30°C in YPD medium (2% peptone, 1% yeast extract, 2% glucose) or minimal medium containing 2% galactose to saturation, and sequential 10-fold dilutions were made. The diluted samples were spotted onto YPD or YPG (2% peptone, 1%yeast extract, 2% galactose) plates. When the spots had dried, the plates were irradiated with different doses of 254-nm UV light. The plates were incubated at 30°C for 3–4 (on YPD plates) or 5–7 (on YPG plates) days in the dark prior to being photographed.

2.2.3 UV Irradiation, Repair Incubation, and DNA Isolation

Yeast cells were grown at 30°C in minimal medium containing 2% glucose or galactose to late log phase (*A*600 ≈1.0), harvested, and washed twice with ice-cold water. The washed cells were resuspended in ice-cold 2% glucose (for glucose cultures) or 2% galactose (for galactose cultures) and irradiated with 80 J/m^2 of 254nm UV light. One-tenth volume of a stock solution containing 10% yeast extract and 20% peptone was immediately added to the irradiated cell suspension. The cells were incubated at 30°C in the dark and aliquots were collected at different time points. Total DNA was isolated from the collected cells using a glass beads method (Li and Smerdon 2002).

2.2.4 NER Analysis of UV-induced CPDs

The gene fragments of interest were 3' end labeled with [α-^{32}P]dATP using a procedure previously described (Li and Waters 1996; Li, Waters et al. 2000). Briefly, ~1μg of genomic DNA was digested with restriction enzyme(s) to release the fragment of interest and incised at CPDs with an excess amount of purified T4 endonuclease V (Epicentre). Excess copies of biotinylated oligonucleotides, which are complementary to the 3' end of the fragments to be labeled, were mixed with the sample. The mixture was heated at 95°C for 5min to denature the

DNA and then cooled to an annealing temperature of around 50°C. The annealed fragments were attached to streptavidin magnetic beads (Invitrogen) and the other fragments were removed by washing the beads at the annealing temperature. The attached fragments were labeled with [α-^{32}P]dATP (PerkinElmer) and resolved on sequencing gels. The gels were dried and exposed to a Phosphoimager screen (BioRad). The signal intensities at gel bands corresponding to CPD sites were quantified using Quantity One software (BioRad).

2.2.5 UV-induced Mutagenesis

Cells were cultured to stationary phase in YPD medium. To select for canavanine resistant (Can^R) mutants, 100µl of saturated culture from each strain was spread on minimal medium plates containing 60 mg/L of canavanine. Plates were irradiated at various doses of 254nm UV light, and CanR colonies were counted after 5–10 days of incubation in the dark at 30°C. To measure the numbers of viable cells at the different UV doses, serial 10-fold dilutions of each saturated culture were spread onto minimal medium plates lacking canavanine. The plates were irradiated with the same doses of UV as those for selecting Can^R mutants and incubated in the dark at 30 ◦C before colonies were counted. Mutation frequencies were calculated by dividing the number of canavanine resistant colonies by the number of viable cells.

2.2.6 Western Blot

Yeast cells were cultured in minimal medium to late log phase (A_{600} ≈1.0) and pelleted by centrifugation. The cell pellet from a 5 ml culture was resuspended in 500µl of 15% TCA and broken by vortexing them with acid washed glass beads. The proteins in the cell lysates were pelleted by centrifugation at 20,000×*g* for 15 min at 4 ◦C. The protein pellet was washed with ice-cold 80% acetone and dissolved in 2×sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) gel loading buffer (Sambrook and Russell 2001). The insoluble cell debris was removed by centrifugation.

Proteins in the whole cell extracts were resolved on an SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). 3×FLAG tagged proteins were probed with an anti-FLAG antibody M2 (Sigma). As a loading control, Rpb1, the largest subunit of RNA polymerase II, was probed with antibody 8WG16, which specifically recognizes the C-terminal heptapeptide repeats of Rpb1 (Thompson, Steinberg et al. 1989). Blots were incubated with SuperSignal West Femto maximum-sensitivity substrate (Pierce), and the protein bands were detected using a chemiluminescence scanner (VersaDoc Imaging System; BioRad).

2.3 Results

2.3.1 UV Sensitivities of *elc1* Mutants

It has been shown that *ELC1* is epistatic to *RAD7*/*RAD16*, and *elc1* deletion increases UV sensitivity of *rad26* cells (Ribar, Prakash et al. 2006). In agreement with the previous report, *elc1* deletion did not enhance the UV sensitivity of otherwise wild type and GGR deficient *rad16* cells (Figure 2-1). The deletion also did not increase the UV sensitivity of *rad16 rad26* cells, where the Rpb9-mediated TCR is functional, and *rad16 rpb9* cells, where the Rad26 mediated TCR is operative (Li and Smerdon 2002; Li and Smerdon 2004). This further indicates that *ELC1* is epistatic to *RAD16*. However, the deletion significantly increased the UV sensitivities of *rad26* cells, which are partially deficient in TCR (the Rpb9 mediated TCR is still active), and *rad26 rpb9* cells, which are completely deficient in TCR (Li and Smerdon 2002; Li and Smerdon 2004) (Figure. 2-1A). Taken together, these observations support the idea that Elc1 plays a role in GGR but does not significantly affect TCR. However, the role of Elc1 in GGR may not be as significant as Rad7 and Rad16, as *elc1* single deletion mutants are not UV sensitive (Figure 2-

1A), whereas *rad7* and *rad16* cells are (Figure 2-1B) (Perozzi and Prakash 1986; Bang, Verhage et al. 1992; Verhage, Zeeman et al. 1994).

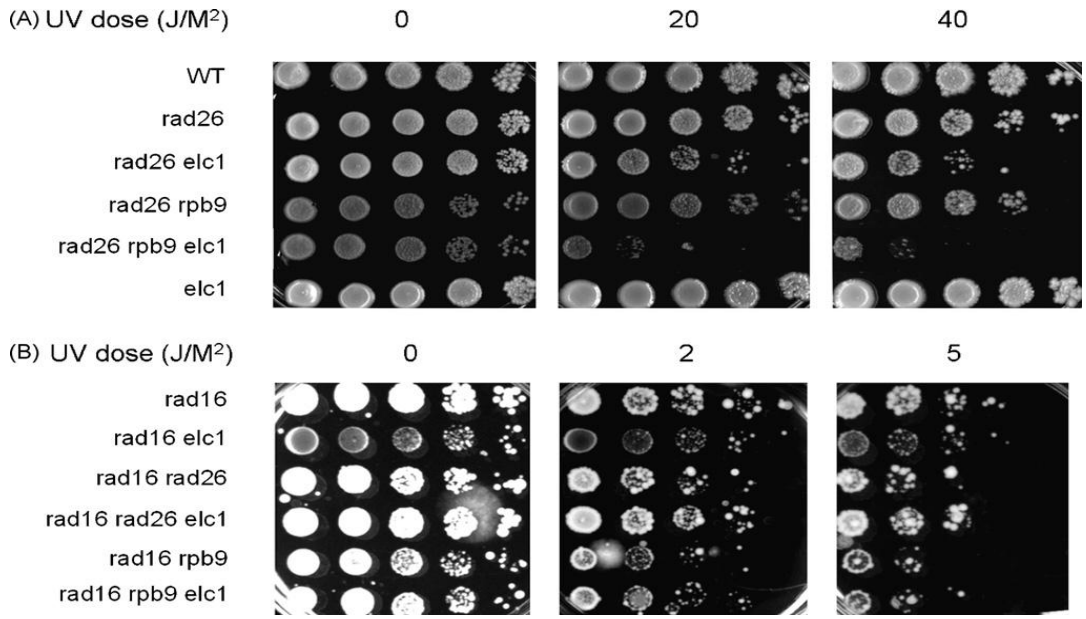

Figure 2-1. Effect of the *elc1* deletion on UV sensitivity of otherwise wild type and GGR- and TCR-deficient cells. Sequential 10-fold dilutions were made, spotted onto YPD plates, and irradiated with different doses of 254-nm UV light.

2.3.2 The Role of Elc1 in GGR and TCR

To examine to what extent Elc1 may contribute to GGR and to definitively determine whether Elc1 plays a minor role in TCR that is not reflected by the UV sensitivity test, we directly analyzed the effect of *elc1* deletion on different NER pathways. Repair of CPDs in the constitutively transcribed *RPB2* gene was measured with a high resolution method, which uses streptavidin magnetic beads and biotinylated oligonucleotides to facilitate end-labeling of DNA fragments of interest (Li and Waters 1996; Li, Waters et al. 2000). Yeast cells were cultured to late log phase ($A600 \approx 1.0$), UV irradiated, and incubated in a repair medium for varying lengths of time. Total DNA was isolated, digested with a restriction enzyme to excise the fragment of

interest, and incised at the UV-induced CPDs with an excess amount of T4 endonuclease V (Lloyd 2005). The incised fragments were strand-specifically end-labeled, resolved on a DNA sequencing gel, and exposed to a Phosphoimager screen (Li and Waters 1996; Li, Waters et al. 2000). The band intensities in the gel lane denoted "0" time repair indicate the yields of CPDs at different sites. A decrease in band intensities at respective sites indicates CPD removal and DNA repair at these sites.

The NER rates in the TS of the *RPB2* gene were similar between wild type and *elc1* and between *rad16* and *rad16 elc1* cells (Figure 2-2), indicating that Elc1 is entirely dispensable for TCR. It has been established that the NER rates in the NTS of an active gene reflect GGR (Hanawalt 2002).

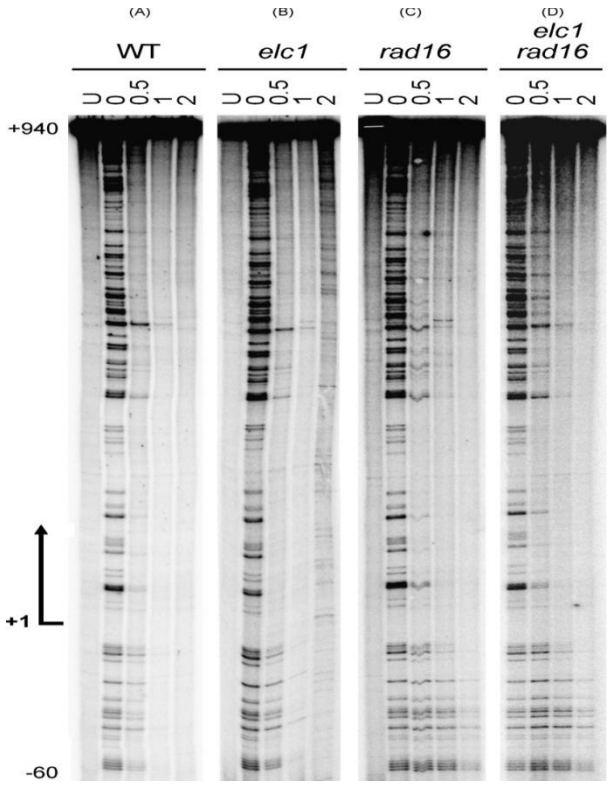

Figure 2-2. The *elc1* deletion has no effect on TCR. Gels show NER in the TS of the *RPB2* gene. The lanes are DNA samples from unirradiated (U) and UV irradiated cells following different times (hours) of repair incubation as indicated at the top of the gels. The arrow on the left of the gels marks the transcription start site.

Indeed, NER rates in the NTS of the *RPB2* gene of *rad26* and *rad26 rpb9* cells were similar to that of wild type cells (Figure 2-3, compare panels A, C and E), indicating that Rad26 and Rpb9, two factors involved in TCR (Li and Smerdon 2002; Li and Smerdon 2004), do not play a role in the repair.

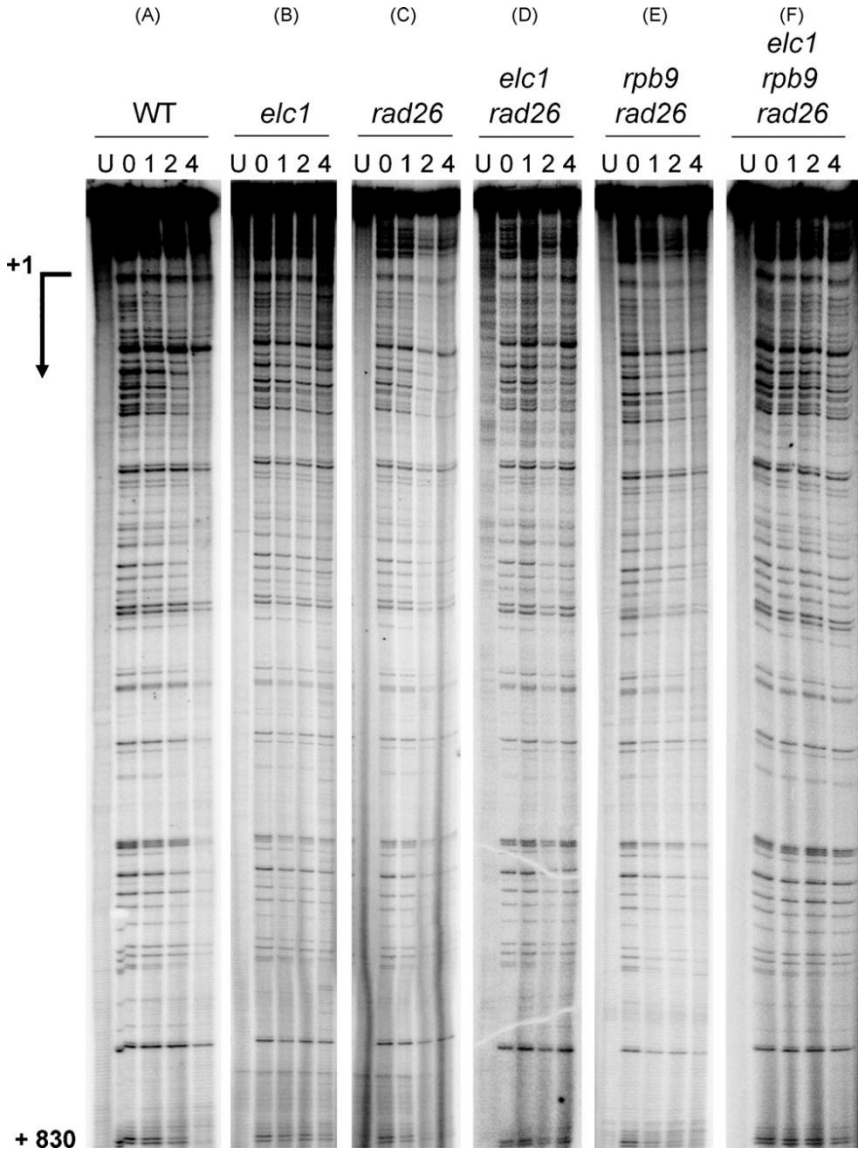

Figure 2-3. The elc1 deletion results in undetectable GGR. Gels shows NER in the NTS of the *RPB2* gene. The lanes are DNA samples from unirradiated (U) and UV irradiated cells following different times (hours) of repair incubation as indicated at the top of the gels. The arrow on the left of the gels marks the transcription start site.

In the period of 4h incubation, no obvious repair can be detected in the NTS of the *RPB2* gene in all types of cells analyzed that lack Elc1 (Figure 2-3B, D and F; Figure. 2-4), indicating that Elc1 plays an important role in GGR. This is surprising in view of the fact that *elc1* single deletion mutants are not UV sensitive, whereas the well-known GGR-deficient *rad7* and *rad16* cells are (see above). Repair in the time period of over 4h was not performed because significant cell growth occurs if UV irradiated cells are incubated in repair media for over 4h, and this obscures the fraction of repaired DNA in the samples (because of the increased fraction of newly replicated, undamaged DNA).

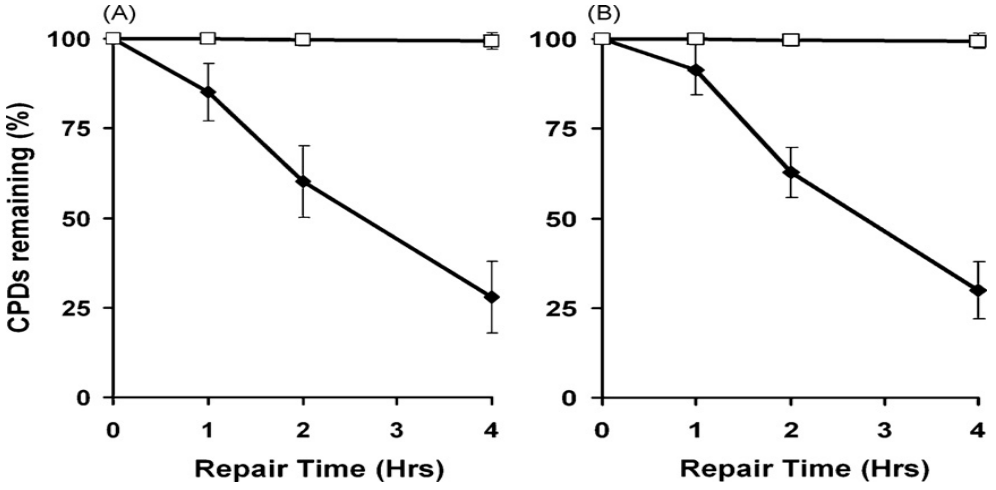

Figure 2-4. Plots showing repair of CPDs in the NTS of the *RPB2* gene. The data were obtained by quantification of the gels showing GGR in the different strains (as shown in Figure 2-3). Plot in (A) shows the average (mean±S.D.) of percent CPDs remaining at individual sites in the non-transcribed strand of the *RPB2* gene at different repair times in the cells of wild type (solid diamonds, quantified from gels as shown in Figure 2-3A) and *elc1* (empty squares, quantified from gels as shown in Figure 2-3B). Plot in (B) shows the average (mean±S.D.) of the percent CPDs remaining at individual sites in the non-transcribed strand of the *RPB2* gene at different repair times in *rad26* cells (solid diamonds, quantified from gels as shown in Figure 2-3C) and *rad26 elc1* cells (empty squares, quantified from gels as shown in Figure 2-3D). It should be noted that some minor repair may not be able to be detected due to the semi-quantitative nature of the gels.

2.3.3 The Effect of *elc1* Deletion on UV-induced Mutagenesis

We also examined the effect of *elc1* deletion on UV-induced mutagenesis. All mutations resistant to canavanine, the toxic analog of arginine, occur at the single *CAN1* gene, which encodes the arginine permease (Whelan, Gocke et al. 1979). Can^R mutants can be selected by plating cells on media containing canavanine instead of arginine (Whelan, Gocke et al. 1979). The frequencies of UV-induced Can^R mutations in *rad16* cells was over 60 times of those in wild type cells (Table 1), indicating that Rad16 plays a significant role in preventing UV-induced mutagenesis. In contrast, the mutation frequencies were not dramatically higher in *rad26* (partially deficient in TCR) and *rad26 rpb9* (completely deficient in TCR) (Li and Smerdon 2002) cells than in wild type cells (Table 1). In fact, for an unknown reason, the mutation frequency appeared to be somewhat lower in *rad26 rpb9* cells than in wild type cells (Table 1). These results indicate that Rad26 mediated TCR or the entire TCR may contribute little to the prevention of UV-induced mutations.

The frequencies of UV-induced Can^R mutations were not dramatically different between *elc1* and wild type cells and between *rad16 elc1* and *rad16* cells (Table 1), indicating that Elc1 does not dramatically affect UV-induced mutagenesis in these TCR-proficient cells. However, the UV-induced mutation frequencies in *rad26 elc1* cells were ~2–4 times of those in *rad26* cells (Table 1), indicating that Elc1 may play a minor role in preventing UV-induced mutagenesis when TCR is partially defective. Intriguingly, the UV-induced mutation frequencies were dramatically higher (~70–680 times) in *rad26 rpb9 elc1* cells than in *rad26 rpb9* cells (Table 1), indicating that Elc1 plays a critical role in preventing UV-induced mutagenesis when TCR is completely abolished.

Table 2-1. Frequencies of canavanine resistant (Can^R) mutations in different isogenic strains

UV dose (J/m2)		0	5	10	20	40	80
Wild type	Can^R frequency[a]	1.68 (0.83)	16 (1.01)	43 (19)	93 (6.2)	126 (10)	1014 (109)
elc1	Can^R frequency	0.77 (0.14)	6.6 (0.85)	41 (0.24)	122 (4.8)	285 (11)	668 (22)
	Fold increase[b]	0.46	0.42	0.96	1.31	2.27	0.66
rad16	Can^R frequency	27 (0.95)	1081 (113)	2856 (416)			
	Fold increase	16	69	67			
rad16 elc1	Can^R frequency	28 (0.97)	1048 (104)	3262 (673)			
	Fold increase	17	67	76			
rad26	Can^R frequency	9.2 (2.08)	24 (1.86)	37 (7.8)	77 (2.7)		
	Fold increase	5.45	1.55	0.86	0.83		
rad26 elc1	Can^R frequency	4.24 (0.54)	49 (9.47)	65 (20)	313 (129)		
	Fold increase	2.52	3.13	1.52	3.36		
rad26 rpb9	Can^R frequency	1.42 (0.23)	12 (2.16)	7.52 (0.64)	10 (2.18)		
	Fold increase	0.84	0.74	0.18	0.11		
rad26 rpb9 elc1	Can^R frequency	15 (1.44)	923 (19)	4909 (195)	6858 (921)		
	Fold increase	9.1	59	115	74		

[a] The frequencies (per 10^6 viable cells) of Can^R mutants are shown as the mean of at least three experiments. Numbers in parentheses are standard deviation.
[b] Relative to the frequency of Can^R mutants of wild type cells.

2.3.4 Cellular Rad7 and Rad16 Levels Are Not Affected in the Absence of Elc1

It has been shown that, in *elc1* cells, the level of Rad7 is dramatically decreased, although the level of Rad16 is normal (Ramsey, Smith et al. 2004). Furthermore, it has previously been demonstrated that the addition of the purified Rad7/Rad16 complex to a reconstituted NER reaction *in vitro* enhances incision of UV-damaged DNA (Guzder, Sung et al. 1997), indicating that Rad7/Rad16 facilitates GGR in the absence of Elc1. These observations prompted us to examine if the function of Elc1 for GGR is achieved by keeping Rad7 from degradation and therefore maintaining the level of the Rad7/Rad16 complex. To this end, we genomically tagged the Rad7 and Rad16 proteins with three consecutive FLAG sequences (3×FLAG) in wild type and different *elc1* deletion mutants. The 3×FLAGtag did not cause any noticeable phenotypic changes to the cells (not shown). Expression levels of the tagged proteins were analyzed by probing with an anti-FLAG antibody on a Western blot. In agreement with the previous report

(Ramsey, Smith et al. 2004), Rad16 levels were not significantly decreased in *elc1* and *rad7* cells (Figure 2-5A), and Rad7 levels were not significantly decreased in *rad16* cells (Figure 2-5B). Different from the previous report, however, Rad7 levels were not dramatically decreased in all *elc1* cells we analyzed (Figure 2-5B). Our results indicate that the role of Elc1 in GGR may not be achieved by maintaining the levels Rad7 and Rad16 in the cells.

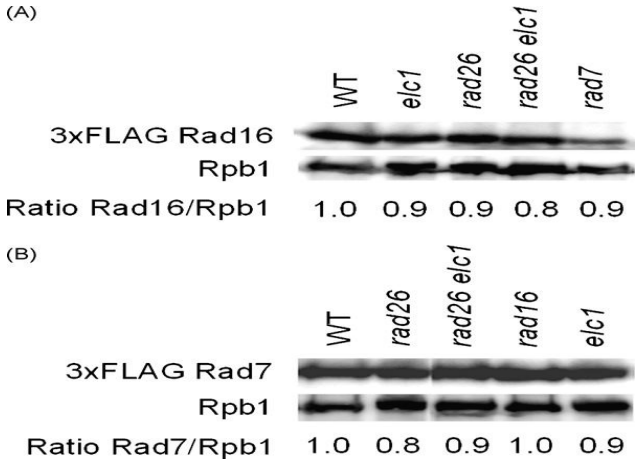

Figure 2-5. Rad7 and Rad16 protein levels are not decreased in *elc1* cells. Western blots show cellular levels of Rad7 (A) and Rad16 (B) levels in wild type and different mutant cells. Rpb1 serves as an internal loading control.

2.3.5 The Role of Elc1 in GGR Can Not Be Substituted for by Overexpressing of Rad7 and Rad16 Individually or Simultaneously

Next, we examined if overexpression of Rad7 and Rad16 individually or simultaneously could restore GGR in *elc1* cells. We surmised that if Elc1 merely served a subsidiary role, e.g., by assisting the Rad7/Rad16 complex, overexpression of the absolutely required Rad7 and/or Rad16 should be able to recover some, if not all, of the cell's capacity for GGR. To address this, we constructed multicopy plasmids overexpressing 3×myc tagged Rad16 (pRAD16-3 M) and 3×FLAG tagged Rad7 (pRAD7- 3F) under the control of the highly inducible *GAL1* and *GAL10*

promoters, respectively. The divergent *GAL1* and *GAL10* promoters share the same upstream activating sequence (UAS) and are induced identically by galactose (Lohr, Venkov et al. 1995).We also created a plasmid (pR16R7) that simultaneously overexpresses 3×FLAG tagged Rad7 and 3×myc tagged Rad16. The plasmids overexpressing the different proteins were able to complement the UV sensitivity of the cells lacking the respective protein (not shown), indicating that the proteins encoded by them are functional for GGR.

We examined the effect of the overexpressions of Rad7 and/or Rad16 on the UV sensitivity of *rad26 elc1* cells cultured in a galactose medium (to induce the tagged proteins). The reason for using *rad26 elc1*, rather than *elc1* cells, for the examination is that *elc1* deletion enhances UV sensitivity in *rad26* cells (Figure 2-1A) so that the restoration of UV resistance (and presumably GGR) can be easily observed. As shown in Figure 2-6, the overexpressions did not significantly change the UV sensitivity of *rad26 elc1* cells.

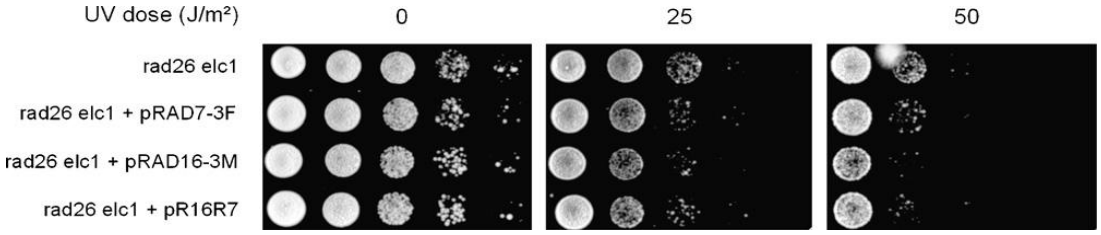

Figure 2-6. Overexpression of Rad7 and/or Rad16 does not restore UV resistance of *rad26 elc1* cells. Yeast cells containingplasmids overexpressing either 3×FLAG tagged Rad7 or 3×myc tagged Rad16, or both 3×FLAG tagged Rad7 and 3×myc tagged Rad16 and their parent strains (without plasmids) were sequentially 10-fold diluted, spotted onto agar plates containing galactose (to induce overexpression of the tagged proteins), and irradiated with different doses of 254-nm UV light.

We also directly examined the effects of Rad7 and/or Rad16 overexpressions on NER in the NTS of the *RPB2* gene in *rad26 elc1* cells. As can be seen in Figure 2-7, the overexpressions did not restore the repair. Taken together, our results indicate that the role of Elc1 in GGR cannot be substituted for by Rad7 and Rad16, even when they are overexpressed.

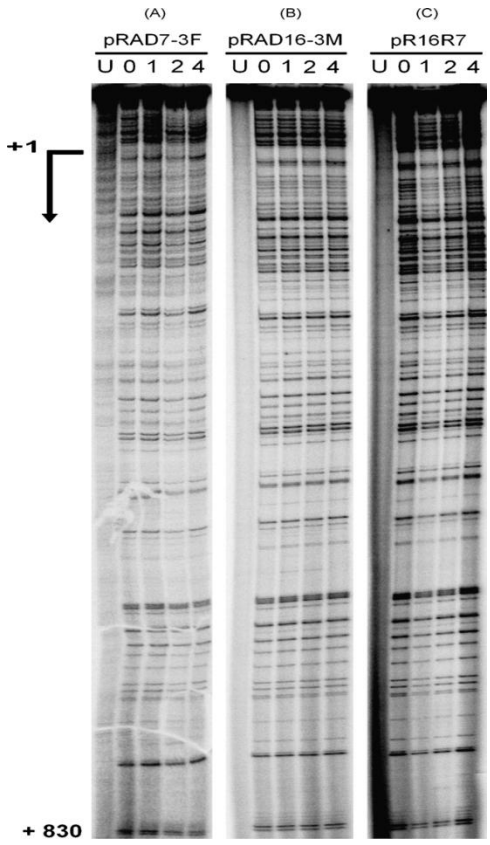

Figure 2-7. Overexpression of Rad7 and/or Rad16 does not restore GGR in *rad26 elc1* cells. Gels show NER in the NTS of the *RPB2* gene in *rad26 elc1* cells transformed with plasmids overexpressing 3×FLAG tagged Rad7 (pRAD7-3F) or 3×myc tagged Rad16 (pRAD16-3M), or both (pR16R7). Lanes labeled U are unirradiated samples. Other lanes are samples with different times (hours) of repair incubation. The arrow on the left of the gels marks the transcription start site.

2.4 Discussion

In this paper, we present the first direct evidence that Elc1 has no function in TCR but plays an important role in GGR. How Elc1 functions in GGR remains to be elucidated. One possibility is that Elc1, Rad7 and Rad16 need to be in one complex to be functional for GGR. It

was shown that Elc1, along with Cul3, Rad7 and Rad16, is present in a ubiquitin E3 ligase complex, which regulates the efficiency of NER by ubiquitylating Rad4 (Gillette, Yu et al. 2006). However, it is unlikely that the role of Elc1 in GGR is achieved primarily by ubiquitylating Rad4. First, abolishment of Rad4 ubiquitylation by mutating the Rad7 SOCS domain results in only a slight decrease in overall NER (Gillette, Yu et al. 2006), which contrasts to the undetectable GGR in *elc1* cells. Second, Rad4 is an essential NER protein, required for both TCR and GGR (Prakash and Prakash 2000), whereas Elc1 is only involved in GGR but not TCR.

It was shown that Elc1 also interacts strongly with a class of proteins that can be loosely defined as stress-responsive proteins (Jackson, Kwon et al. 2000). Some of these interacting proteins were shown to be stabilized by Elc1 (Hyman, Kwon et al. 2002). We did not observe significant changes in the levels of either Rad7 or Rad16 in *elc1* cells, indicating that Rad7 and Rad16 are not stabilized by Elc1. Furthermore, we observed that overexpression of Rad7 and Rad16 individually or simultaneously did not significantly enhance GGR in *elc1* cells, suggesting that the role of Elc1 in GGR is not achieved by stabilizing Rad7/Rad16. Therefore Rad7/Rad16 and Elc1may mainly exist in different complexes and participate in GGR independently with each other.

The ubiquitin E3 ligase complex that is involved in ubiquitylation of Pol II was proposed to contain Elc1, Ela1, Cul3, and Roc1, but not Rad7 and Rad16 (Ribar, Prakash et al. 2007). It was suggested that, in the absence of Rad26 or in the regions of the TS where Rad26- dependent TCR may be unable to act (e.g., in the promoter regions), Pol II removal upon the E3 ligase-mediated ubiquitylation may be a necessary precondition for repair to take place (Ribar, Prakash et al. 2007). This proposition perfectly explained the observed epistasis of *rad7* over *elc1*, *ela1*

and *cul3*, and the synergism between *rad7* and each of the E3 ligase components. However, in *elc1* cells, the abolition of NER in the NTS of a gene, where Pol II is not stalled by a lesion (Tornaletti, Donahue et al. 1997), cannot be explained by this proposition. Therefore, the role of Elc1 in GGR may not be achieved primarily by ubiquitylating Pol II.

In some cases, UV sensitivity of a mutant does not faithfully reflect its NER capacity, presumably due to the fact that UV sensitivity is caused by a combination of different DNA damage responses, such as NER, activation or suppression of certain genes, and checkpoint activation and recovery. The *elc1* single mutant, which is not UV sensitive but is deficient in GGR, is one of these cases. One possibility is that deletion of *elc1* may activate a DNA damage response mechanism that somehow increases the cell viability following UV irradiation, although GGR is abolished or greatly compromised. If this is the case, the activation of the DNA damage response mechanism may require either TCR or the transcription elongation functions of Rad26 and Rpb9, as *elc1* deletion only increases the UV sensitivities of *rad26* and *rad26 rpb9* cells. Our observations that *elc1* deletion does not affect UV-induced mutagenesis in TCR-proficient cells but dramatically increases the mutagenesis frequency in *rad26 rpb9* cells support this idea. It is also possible that GGR in *elc1* cells is too slow to be detected. This slow repair, alone or combined with an activation of a DNA damage response mechanism, may be able to ensure cell viability following UV irradiation. A similar slow repair mechanism has been proposed for human TTD-A (trichothiodystrophy type A) cells, which lack a functional Tfb5, the 10th subunit of TFIIH, and are mildly UV sensitive but have very little NER activity (Stefanini, Vermeulen et al. 1993; Vermeulen, Bergmann et al. 2000; Giglia-Mari, Miquel et al. 2006). Similar to human TTD-A cells, yeast cells lacking Tfb5 are also mildly UV sensitive (Zhou, Kou et al. 2007) but have very little NER activity (Ding, Ruggiero et al. 2007).

2.5 References

Aso, T. and M. N. Conrad (1997). "Molecular cloning of DNAs encoding the regulatory subunits of elongin from Saccharomyces cerevisiae and Drosophila melanogaster." Biochem Biophys Res Commun **241**(2): 334-40.

Aso, T., W. S. Lane, et al. (1995). "Elongin (SIII): a multisubunit regulator of elongation by RNA polymerase II." Science **269**(5229): 1439-43.

Aso, T., N. Mokady, et al. (1995). "Assignment of a human gene encoding the 110-kDa subunit of general transcription factor elongin (SIII) to chromosome 1p36.1." Genomics **30**(2): 393-4.

Bang, D. D., R. Verhage, et al. (1992). "Molecular cloning of RAD16, a gene involved in differential repair in Saccharomyces cerevisiae." Nucleic Acids Res **20**(15): 3925-31.

Bradsher, J. N., K. W. Jackson, et al. (1993). "RNA polymerase II transcription factor SIII. I. Identification, purification, and properties." J Biol Chem **268**(34): 25587-93.

Bradsher, J. N., S. Tan, et al. (1993). "RNA polymerase II transcription factor SIII. II. Functional properties and role in RNA chain elongation." J Biol Chem **268**(34): 25594-603.

Ding, B., C. Ruggiero, et al. (2007). "Tfb5 is partially dispensable for Rad26 mediated transcription coupled nucleotide excision repair in yeast." DNA Repair (Amst) **6**(11): 1661-9.

Fousteri, M. and L. H. Mullenders (2008). "Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects." Cell Res **18**(1): 73-84.

Friedberg, E. C., G. C. Walker, et al. (2006). DNA Repair and Mutagenesis. Washington D.C., ASM Press.

Gelbart, M. E., T. Rechsteiner, et al. (2001). "Interactions of Isw2 chromatin remodeling complex with nucleosomal arrays: analyses using recombinant yeast histones and immobilized templates." Mol Cell Biol **21**(6): 2098-106.

Giglia-Mari, G., C. Miquel, et al. (2006). "Dynamic interaction of TTDA with TFIIH is stabilized by nucleotide excision repair in living cells." PLoS Biol **4**(6): e156.

Gillette, T. G., S. Yu, et al. (2006). "Distinct functions of the ubiquitin-proteasome pathway influence nucleotide excision repair." Embo J **25**(11): 2529-38.

Guzder, S. N., P. Sung, et al. (1997). "Yeast Rad7-Rad16 complex, specific for the nucleotide excision repair of the nontranscribed DNA strand, is an ATP-dependent DNA damage sensor." J Biol Chem **272**(35): 21665-8.

Guzder, S. N., P. Sung, et al. (1998). "The DNA-dependent ATPase activity of yeast nucleotide excision repair factor 4 and its role in DNA damage recognition." J Biol Chem **273**(11): 6292-6.

Hanawalt, P. C. (2002). "Subpathways of nucleotide excision repair and their regulation." Oncogene **21**(58): 8949-56.

Hyman, L. E., E. Kwon, et al. (2002). "Binding to Elongin C inhibits degradation of interacting proteins in yeast." J Biol Chem **277**(18): 15586-91.

Jackson, T., E. Kwon, et al. (2000). "Novel roles for elongin C in yeast." Biochim Biophys Acta **1491**(1-3): 161-76.

Laine, J. P. and J. M. Egly (2006). "When transcription and repair meet: a complex system." Trends Genet **22**(8): 430-6.

Li, S., X. Chen, et al. (2006). "Modulation of Rad26- and Rpb9-mediated DNA repair by different promoter elements." J Biol Chem **281**(48): 36643-51.

Li, S., B. Ding, et al. (2006). "Evidence that the transcription elongation function of Rpb9 is involved in transcription-coupled DNA repair in Saccharomyces cerevisiae." Mol Cell Biol **26**(24): 9430-41.

Li, S. and M. J. Smerdon (2002). "Rpb4 and Rpb9 mediate subpathways of transcription-coupled DNA repair in Saccharomyces cerevisiae." Embo J **21**(21): 5921-9.

Li, S. and M. J. Smerdon (2004). "Dissecting transcription-coupled and global genomic repair in the chromatin of yeast GAL1-10 genes." J Biol Chem **279**(14): 14418-26.

Li, S. and R. Waters (1996). "Nucleotide level detection of cyclobutane pyrimidine dimers using oligonucleotides and magnetic beads to facilitate labelling of DNA fragments incised at the dimers and chemical sequencing reference ladders." Carcinogenesis **17**(8): 1549-52.

Li, S., R. Waters, et al. (2000). "Low- and high-resolution mapping of DNA damage at specific sites." Methods **22**(2): 170-9.

Lloyd, R. S. (2005). "Investigations of pyrimidine dimer glycosylases--a paradigm for DNA base excision repair enzymology." Mutat Res **577**(1-2): 77-91.

Lohr, D., P. Venkov, et al. (1995). "Transcriptional regulation in the yeast GAL gene family: a complex genetic network." Faseb J **9**(9): 777-87.

Lommel, L. and P. C. Hanawalt (1991). "The genetic defect in the Chinese hamster ovary cell mutant UV61 permits moderate selective repair of cyclobutane pyrimidine dimers in an expressed gene." Mutat Res **255**(2): 183-91.

Perozzi, G. and S. Prakash (1986). "RAD7 gene of Saccharomyces cerevisiae: transcripts, nucleotide sequence analysis, and functional relationship between the RAD7 and RAD23 gene products." Mol Cell Biol **6**(5): 1497-507.

Prakash, S. and L. Prakash (2000). "Nucleotide excision repair in yeast." Mutat Res **451**(1-2): 13-24.

Ramsey, K. L., J. J. Smith, et al. (2004). "The NEF4 complex regulates Rad4 levels and utilizes Snf2/Swi2-related ATPase activity for nucleotide excision repair." Mol Cell Biol **24**(14): 6362-78.

Reed, S. H., Z. You, et al. (1998). "The yeast RAD7 and RAD16 genes are required for postincision events during nucleotide excision repair. In vitro and in vivo studies with rad7 and rad16 mutants and purification of a Rad7/Rad16-containing protein complex." J Biol Chem **273**(45): 29481-8.

Ribar, B., L. Prakash, et al. (2006). "Requirement of ELC1 for RNA polymerase II polyubiquitylation and degradation in response to DNA damage in Saccharomyces cerevisiae." Mol Cell Biol **26**(11): 3999-4005.

Ribar, B., L. Prakash, et al. (2007). "ELA1 and CUL3 are required along with ELC1 for RNA polymerase II polyubiquitylation and degradation in DNA-damaged yeast cells." Mol Cell Biol **27**(8): 3211-6.

Sambrook, J. and D. W. Russell (2001). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press.

Sarasin, A. and A. Stary (2007). "New insights for understanding the transcription-coupled repair pathway." DNA Repair (Amst) **6**(2): 265-9.

Selby, C. P. and A. Sancar (1993). "Molecular mechanism of transcription-repair coupling." Science **260**(5104): 53-8.

Stefanini, M., W. Vermeulen, et al. (1993). "A new nucleotide-excision-repair gene associated with the disorder trichothiodystrophy." Am J Hum Genet **53**(4): 817-21.

Tang, J. Y., B. J. Hwang, et al. (2000). "Xeroderma pigmentosum p48 gene enhances global genomic repair and suppresses UV-induced mutagenesis." Mol Cell **5**(4): 737-44.

Thoma, F. (1999). "Light and dark in chromatin repair: repair of UV-induced DNA lesions by photolyase and nucleotide excision repair." Embo J **18**(23): 6585-98.

Thompson, N. E., T. H. Steinberg, et al. (1989). "Inhibition of in vivo and in vitro transcription by monoclonal antibodies prepared against wheat germ RNA polymerase II that react with the heptapeptide repeat of eukaryotic RNA polymerase II." J Biol Chem **264**(19): 11511-20.

Tornaletti, S., B. A. Donahue, et al. (1997). "Nucleotide sequence context effect of a cyclobutane pyrimidine dimer upon RNA polymerase II transcription." J Biol Chem **272**(50): 31719-24.

Troelstra, C., A. van Gool, et al. (1992). "ERCC6, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes." Cell **71**(6): 939-53.

van Gool, A. J., R. Verhage, et al. (1994). "RAD26, the functional S. cerevisiae homolog of the Cockayne syndrome B gene ERCC6." Embo J **13**(22): 5361-9.

van Hoffen, A., A. T. Natarajan, et al. (1993). "Deficient repair of the transcribed strand of active genes in Cockayne's syndrome cells." Nucleic Acids Res **21**(25): 5890-5.

Venema, J., L. H. Mullenders, et al. (1990). "The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA." Proc Natl Acad Sci U S A **87**(12): 4707-11.

Venema, J., A. van Hoffen, et al. (1991). "Xeroderma pigmentosum complementation group C cells remove pyrimidine dimers selectively from the transcribed strand of active genes." Mol Cell Biol **11**(8): 4128-34.

Venema, J., A. van Hoffen, et al. (1990). "The residual repair capacity of xeroderma pigmentosum complementation group C fibroblasts is highly specific for transcriptionally active DNA." Nucleic Acids Res **18**(3): 443-8.

Verhage, R., A. M. Zeeman, et al. (1994). "The RAD7 and RAD16 genes, which are essential for pyrimidine dimer removal from the silent mating type loci, are also required for repair of the nontranscribed strand of an active gene in Saccharomyces cerevisiae." Mol Cell Biol **14**(9): 6135-42.

Vermeulen, W., E. Bergmann, et al. (2000). "Sublimiting concentration of TFIIH transcription/DNA repair factor causes TTD-A trichothiodystrophy disorder." Nat Genet **26**(3): 307-13.

Whelan, W. L., E. Gocke, et al. (1979). "The CAN1 locus of Saccharomyces cerevisiae: fine-structure analysis and forward mutation rates." Genetics **91**(1): 35-51.

Zhou, Y., H. Kou, et al. (2007). "Tfb5 interacts with Tfb2 and facilitates nucleotide excision repair in yeast." Nucleic Acids Res **35**(3): 861-71.

CHAPTER 3

EVIDENCE THAT THE HISTONE METHYLTRANSFERASE DOT1 MEDIATES GLOBAL GENOMIC REPAIR BY METHYLATING HISTONE H3 ON LYSINE 79*

3.1 Introduction

Nucleotide excision repair (NER) is a highly conserved DNA repair mechanism that removes a wide variety of bulky, helix-distorting lesions that generally obstruct transcription and normal replication, such as UV-induced cyclobutane pyrimidine dimers (CPDs) (Friedberg, Walker et al. 2006). Transcription coupled repair (TCR) is a specialized NER pathway that is dedicated to rapid repair in the transcribed strand (TS) of active genes and is believed to be initiated by an RNA polymerase stalled at a lesion in the TS (Hanawalt and Spivak 2008). The genome-wide NER, which includes repair in the nontranscribed strand (NTS) of actively transcribed genes, is termed global genomic repair (GGR) to be distinguished from TCR. The two NER pathways share most of the common repair factors and differ only in the damage recognition step (Friedberg, Walker et al. 2006).

In the budding yeast *Saccharomyces cerevisiae*, Rad7, Rad16, and Elc1 are specifically required for GGR (Verhage, Zeeman et al. 1994; Lejeune, Chen et al. 2009). The exact roles of these proteins in GGR are not yet clear. The Rad7/Rad16 complex may act as an ATP-dependent motor which translocates along the DNA in search of damage, and upon encountering a lesion, the complex is stalled, which may remodel and open damaged chromatin, thereby facilitating recruitment of other NER factors (Guzder, Sung et al. 1998). Elc1 has been shown to be a component of a ubiquitin ligase that contains Rad7 and Rad16 (Gillette, Yu et al. 2006). It has also been suggested that Elc1 is a component of another ubiquitin ligase complex, which

*Reprinted with the kind permission of *J. Biol. Chem.*

contains Ela1, Cul3, and Roc1 but not Rad7 and Rad16 (Ribar, Prakash et al. 2007). The role of Elc1 in GGR does not seem to be subsidiary to that of Rad7 and Rad16 (Lejeune, Chen et al. 2009).

The molecular basis of chromatin dynamics during NER in eukaryotic cells is still not well understood (Gong, Kwon et al. 2005; Waters, Teng et al. 2009). The basic repeating component of chromatin is the nucleosome, which is comprised of 146 base pairs of DNA wrapped around an octomer of the four core histone proteins H2A, H2B, H3, and H4 (Luger, Mader et al. 1997). Histones are subject to a multitude of post-translational modifications including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination (Kouzarides 2007). Some of these modifications may modulate the NER process (Gong, Kwon et al. 2005; Waters, Teng et al. 2009). However, the effects of histone modifications on NER in living cells documented so far are generally quite modest and are most likely due to the alteration of chromatin compaction and/or stability. It has been unknown if the NER, especially GGR, machinery relies on a specific histone modification to gain access to a lesion in the chromatin.

Dot1 is a histone methyltransferase required for methylation of histone H3 lysine 79 (H3K79) (van Leeuwen, Gafken et al. 2002). *dot1* mutants are sensitive to UV (Bostelman, Keller et al. 2007) and have a defect in activation of DNA damage checkpoints (Giannattasio, Lazzaro et al. 2005). In this paper, we present evidence that Dot1, by methylating H3K79, plays a pivotal role in GGR but is entirely dispensable for TCR. Our studies identified a novel GGR-specific NER factor and unveiled the critical link between a covalent histone modification and GGR.

3.2 Materials and Methods

3.2.1 Yeast Strains and Plasmids

Wild type yeast strains used were Y452 (*MATα ura3-52 his3-1 leu2-3 leu2-112)* and BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). *bre1*Δ cells (*MATa his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 bre1::Kan*) were from Open Biosystems. Cells expressing mutant H3K79A and H2BK123A, and their isogenic wild type strains YBL574 and FY406 (Nakanishi, Sanderson et al. 2008) were kindly provided by Dr. Ali Shilatifard (Stowers Institute for Medical Research). *DOT1* and *RAD16* deletions were created using procedures previously described (Li and Smerdon 2002).

pGAL-RAD16, a plasmid encoding 3×myc tagged Rad16 under the control of the *GAL1* promoter, was created using vector pESC-URA (Stratagene). Two consecutive myc tag sequences were inserted in-frame downstream of the vector's native single myc sequence. The *RAD16* gene coding sequence was inserted in-frame upstream of the 3×myc sequences. The plasmid can complement the deletion of the genomic *RAD16* gene for GGR, indicating that the plasmid-encoded Rad16 is functional.

3.2.2 UV Irradiation, Repair Incubation and Genomic DNA Isolation

Yeast cells were grown at 30°C in minimal media containing 2% glucose or 2% galactose (to induce a gene under the control of the *GAL1* promoter) to late log phase ($A_{600} \approx 1.0$), washed twice with ice-cold water, resuspended in ice-cold 2% glucose (for glucose cultures) or 2% galactose (for galactose cultures), and irradiated with 80 J/m^2 of 254 nm UV light. One-tenth volume of a stock solution containing 10% yeast extract and 20% peptone was added to the irradiated cell suspension. The cells were incubated at 30°C in the dark to allow them to repair

their DNA and aliquots were collected at different time points. Genomic DNA was isolated from the cells as described previously (Li and Smerdon 2002).

3.2.3 NER Analysis of UV-induced CPDs at Nucleotide Resolution

To measure the induction and repair of CPDs at individual sites in each strand of the *RPB2* gene, we used the method that allows for strand-specific 'fishing out' and labeling of a DNA fragment of interest (Li and Waters 1996; Li and Waters 1997; Li, Waters et al. 2000). Briefly, ~ 1 μg of each of the total genomic DNA samples was digested with *Dra*I to release the *RPB2* gene fragment of 1144 bp, which bears the 197 bp sequence upstream and the 947 bp sequence downstream of the transcription start site of the gene. The CPDs induced (in samples of '0' hr repair) or remaining (in samples of different times of repair incubation) were converted to single-strand breaks by treatment with an excess amount of T4 endonuclease V, which specifically cleaves the DNA at CPD sites (Lloyd 2005). Two biotinylated oligonucleotides were then used to specifically 'fish out' and label the TS and NTS of the *RPB2* gene fragment, respectively. One pmol of one of the oligonucleotides was mixed with each of the samples. The mixtures were heated at 95°C for 5 min to denature the DNA and then cooled to an annealing temperature of around 50°C, to hybridize one strand of the *RPB2* fragment to the respective biotinylated oligonucleotide. One hundred μg of streptavidin magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen) was added to each of the mixtures to capture the strand of the *RPB2* fragment hybridized to the biotinylated oligonucleotide. The other unwanted genomic DNA fragments were washed away by incubating the beads in TE (10 mM tris-HCl, 1 mM EDTA, pH8.0) at the annealing temperature (50°C). The fragments captured on the magnetic beads were 3'-end labeled with [α-^{32}P]dATP and Sequnase Version 2 (US Biochemicals). The labeled fragments were eluted from the magnetic beads with a DNA sequencing gel loading buffer at

50°C and resolved on sequencing gels. The gels were dried and exposed to a Phosphoimager screen (Bio-Rad). The signal intensities at gel bands corresponding to CPD sites were quantified by using Quantity One software (Bio-Rad). The percent CPDs remaining at individual sites after different times of repair incubation were calculated and the times required for repairing 50% of CPDs ($T_{1/2}$) were obtained by either linear or second order polynomial regression.

3.3 Results

3.3.1 Dot1 Is Required for GGR

It has been established that NER rates in the NTS of an active gene reflect GGR (Friedberg, Walker et al. 2006). In theory, NER in either strand of an absolutely repressed gene may also reflect GGR. However, "noise" transcription commonly occurs in both strands of supposedly repressed genes in eukaryotic cells (Struhl 2007). The "noise" transcription cannot be detected by traditional ways but may be able to initiate a certain level of TCR, which can be confused with GGR (Li, Ding et al. 2007). Active transcription from the TS of a gene may prevent "noise" transcription from the NTS. Therefore, NER in the NTS of an actively transcribed gene may reflect GGR better than that in either strand of a repressed gene. Our previous studies have shown that NER in the NTS of the constitutively transcribed *RPB2* gene is absolutely dependent on the GGR-specific factors Rad7, Rad16 and Elc1, and therefore appears to exclusively reflect GGR (Li, Ding et al. 2007; Lejeune, Chen et al. 2009).

In order to determine the role of Dot1 in GGR, we measured repair of UV-induced CPDs in the NTS of the *RPB2* gene in wild type and *dot1Δ* cells. A nucleotide resolution method which uses streptavidin magnetic beads and biotinylated oligonucleotides to facilitate isolation and strand-specific end-labeling of DNA fragments of interest was used for the measurement (Li and Waters 1996; Li, Waters et al. 2000). The yeast cells were cultured to late log phase, UV

irradiated, and incubated in a repair medium for various lengths of time. Total genomic DNA was isolated, digested with a restriction enzyme to release the *RPB2* fragment, and incised at the UV-induced CPDs with an excess amount of T4 endonuclease V (Lloyd 2005). The NTS of the restricted *RPB2* gene fragment was 'fished out', radioactively labeled at the 3' end, and resolved on a DNA sequencing gel. The band intensities in the gel lane of "0" time repair indicate the yields of CPDs at these sites, and a decrease in band intensities at respective sites indicates repair of the damage (Figure 3-1).

In wild type cells, CPDs were repaired at different rates at different sites in the NTS of the *RPB2* gene (Figures 3-1A and 3-2). The repair rates correlated generally well with nucleosome positioning, being slowest in the central regions of nucleosomal core DNA and fastest in the inter-nucleosomal linker regions (Figures 3-1A and 3-2). This indicates that a nucleosome structure inhibits GGR, in agreement with previous reports [e.g., (Wellinger and Thoma 1997; Li and Smerdon 2004)]. In *dot1Δ* cells, no obvious repair can be seen in the same sequence in the period of 4 hrs (Figures 3-1B and 3-2), indicating that Dot1 plays an important role in GGR throughout the NTS, including the inter-nucleosomal linker regions. A longer time of repair incubation was not carried out because 1) most NER events in the yeast occur in the initial hrs of repair incubation, and 2) significant cell growth may occur after 4 hrs of repair incubation, which may obscure the fraction of repaired DNA in the samples.

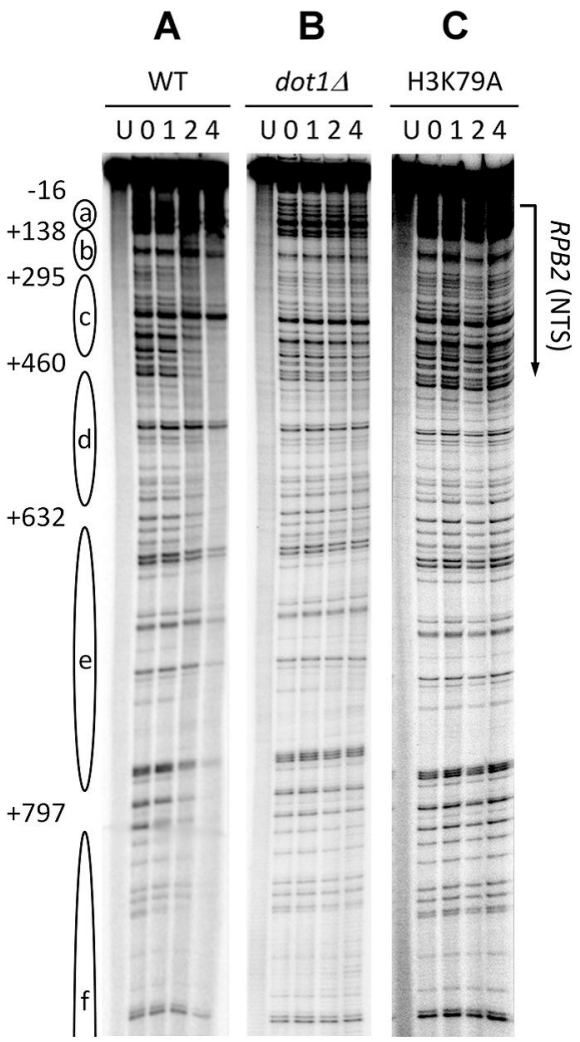

Figure 3-1. Gels showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT), *dot1*Δ and H3K79A cells. The lanes are DNA samples from unirradiated (U) and UV irradiated cells following different times (hrs) of repair incubation. Ovals on the left represent positioned nucleosomes. Numbers on the left indicate nucleotide positions (relative to the transcription start site) at the centers of nucleosome linker regions, which is based on the systematic reference map of nucleosome positions across the yeast genome (Jiang and Pugh 2009). The arrow on the right indicates the transcription start site.

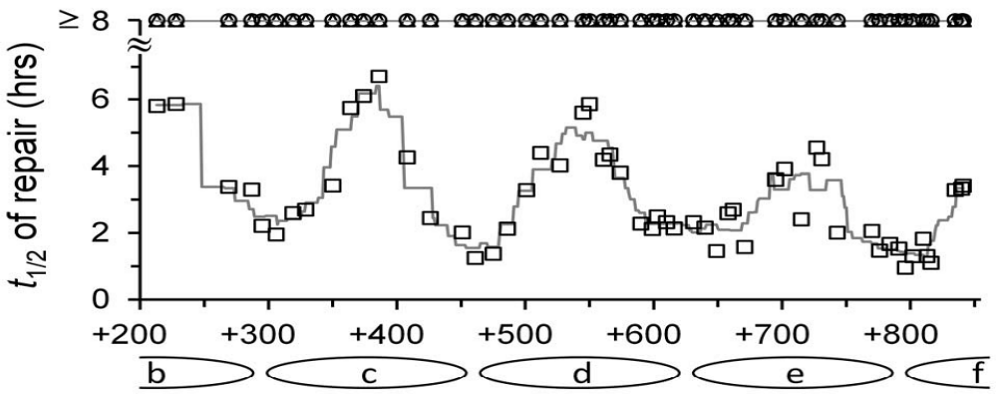

Figure 3-2. Plot showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT), *dot1*Δ and H3K79A cells. The times (hrs) required for repairing 50% (*t*1/2) of CPDs at individual sites along the NTS of the *RPB2* gene in WT (squares), *dot1*Δ (triangles) and H3K79A (circles) cells are plotted. The $t_{1/2}$ values > 4 h were obtained by extrapolation of regression of the data obtained from 0, 1, 2 and 4 h of repair. The gray lines are smoothed *t*1/2 values, which were carried out by averaging the individual $t_{1/2}$ values at continuous intervals of 40 nt where the 40-nt brackets were ramped along the DNA by 1 nt. Ovals at the bottom represent nucleosome positions along the *RPB2* gene region analyzed. Nucleotide positions are numbered from the transcription start site of the gene.

3.3.2 Methylation of H3K79 Is Also Required for GGR

Dot1 has been shown to be required for methylation of H3K79 (van Leeuwen, Gafken et al. 2002). To determine if the role of Dot1 in GGR is accomplished by methylating H3K79, we measured GGR in cells whose genomic histone H3 genes (*HHT1* and *HHT2*) were deleted and complemented with a plasmid encoding the K79A mutant histone H3 (H3K79A) (Nakanishi, Sanderson et al. 2008). Like *dot1Δ* cells, the H3K79A mutant cells showed no repair in any sites of the NTS of the *RPB2* gene (Figures 3-1C and 3-2), indicating that Dot1 may mediate GGR by methylating H3K79.

3.3.3 Bre1 and Histone H2B Lysine 123 (H2BK123) Ubiquitination Are Partially Required for GGR

Dot1 catalyzes mono-, di- and tri-methylation of H3K79 (van Leeuwen, Gafken et al. 2002). Mono-ubiquitination of H2BK123, which is catalyzed by the ubiquitin E3 ligase Bre1,

has been shown to be partially required for di-methylation and absolutely required for tri-methylation but is dispensable for mono-methylation of H3K79 (Shahbazian, Zhang et al. 2005; Levesque, Leung et al.). To determine if the methylation states of H3K79 affect GGR, we analyzed *bre1Δ* cells and those whose genomic histone H2B genes (*HTB1* and *HTB2*) were deleted and complemented with a plasmid encoding the K123A mutant histone H2B (H2BK123A) (Nakanishi, Sanderson et al. 2008). As can be seen in Figures 3-3A, 3-3B and 3-4, GGR was still apparent but significantly compromised in these mutant cells. These results indicate that 1) tri-methylation of H3K79 may contribute to but is not absolutely required for GGR, and 2) lower levels of methylation (mono- and di-methylation) at the K79 also promote GGR.

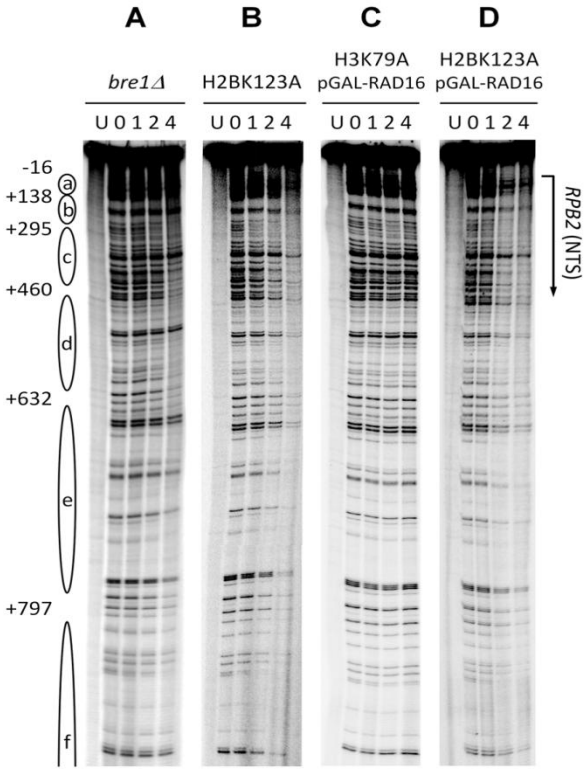

Figure 3-3. Gels showing repair of CPDs in the NTS of the *RPB2* gene in *bre1*Δ and H2BK123A cells, and in H3K79A and H2BK123A cells transformed with a plasmid overexpressing Rad16 (pGALRAD16). Labels are the same as those shown in Figure 3-1.

3.3.4 Overexpression of Rad16 Does Not Restore GGR in Cells Expressing H3K79A or H2BK123A Mutant Histones

Loss of Dot1 or H3K79 methylation have been shown to have no or only a very minor effect on genome-wide transcription levels (Hughes, Marton et al. 2000). Also, histone H3 K4R and K79R mutations (H3K4,79R), which prevent methylation at both K4 and K79, do not affect expression of all NER genes tested (Chaudhuri, Wyrick et al. 2009). However, there was a 50% decrease in the *RAD16* mRNA in the H3K4,79R mutant cells after UV irradiation compared to ~ 2-fold increase in wild type cells (Chaudhuri, Wyrick et al. 2009). To address the possibility that the deficient GGR we observed was due to lower levels of Rad16, we transformed H3K79A and H2BK123A cells with a plasmid bearing the *RAD16* gene (pGAL-RAD16) tagged with 3×myc under the control of the *GAL1* promoter. Upon galactose induction, the Rad16 protein was overexpressed more than 10-fold. However, the overexpression did not affect GGR (Figures 3-3 and 3-4), indicating that the effects of H3K79 methylation and H2BK123 ubiquitination on GGR are not caused by lower levels of Rad16.

3.3.5 Dot1 and H3K79 Methylation Do Not Play Significant Roles in TCR

To determine if Dot1 and H3K79 methylation also play roles in TCR, we measured repair of CPDs in the TS of the *RPB2* gene in *rad16Δ* cells lacking Dot1 or expressing the H3K79A mutant. The reason for using *rad16Δ* cells is that these cells are deficient in GGR (Verhage, Zeeman et al. 1994), so that TCR can be unambiguously determined. Following restriction digestion to release the *RPB2* fragment and incision at the CPDs with T4 endonuclease V, the TS of the *RPB2* gene fragment was fished out, radioactively labeled at the 3' end, and resolved on a DNA sequencing gel.

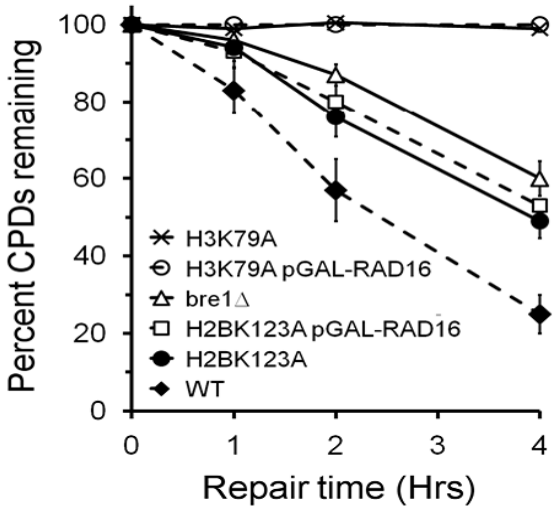

Figure 3-4. Plot showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT), *bre1*Δ, H3K79A and H2BK123A cells, and in H3K79A and H2BK123A cells transformed with a plasmid overexpressing Rad16 (pGAL-RAD16). The values shown are means (± S.E.) of percent CPDs remaining at individual sites in the NTS of the *RPB2* gene at different repair times.

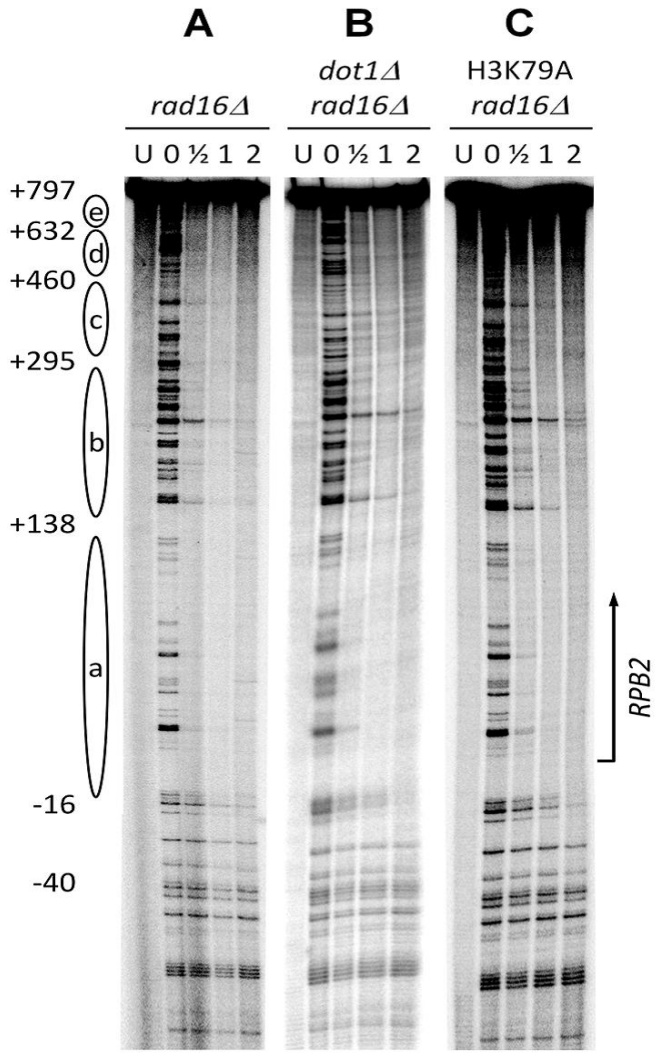

Figure 3-5. Gels showing repair of CPDs in the TS of the *RPB2* gene in *rad16*Δ, *rad16*Δ *dot1*Δ and *rad16*Δ H3K79A cells. The lanes are DNA samples from unirradiated (U) and UV irradiated cells following different times (hrs) of repair incubation. Ovals on the left represent positioned nucleosomes. Numbers on the left indicate nucleotide positions (relative to the transcription start site) at the centers of nucleosome linker regions, which is based on the systematic reference map of nucleosome positions across the yeast genome (Jiang and Pugh 2009). The arrow on the right indicates the transcription start site.

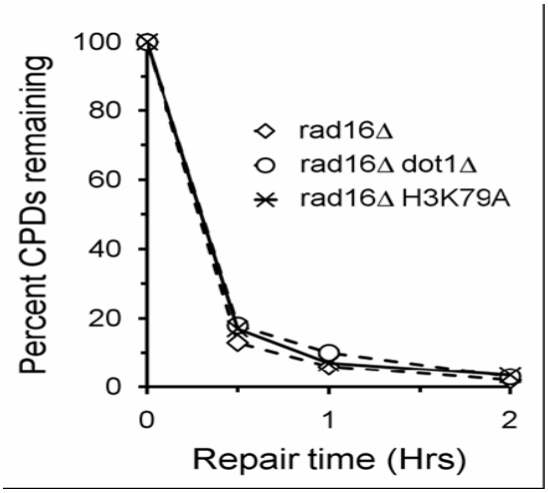

Figure 3-6. Plot showing repair of CPDs in the TS of the *RPB2* gene in *rad16*Δ, *rad16*Δ *dot1*Δ and *rad16*Δ H3K79A cells. The values shown are means of percent CPDs remaining at individual sites in the coding region of the TS of the *RPB2* gene at different repair times. The standard error bars are within the symbols.

As can be seen in Figures 3-5 and 3-6, rapid TCR, which initiates ~ 40 nucleotides upstream of the transcription start site of the *RPB2* gene occurred in *rad16Δ, rad16Δ dot1Δ* and *rad16Δ* H3K79A cells, indicating that Dot1 and H3K79 methylation do not play a significant role in TCR. In agreement with previous reports [e.g., (Wellinger and Thoma 1997; Li and Smerdon 2004)], TCR in these cells was not significantly modulated by nucleosome positioning (Figure 3-5).

3.4 Discussion

In this paper, we present evidence that Dot1 and H3K79 methylation are required for GGR but dispensable for TCR. Dot1 and H3K79 methylation have been shown to be required for important aspects of DNA damage checkpoint activation (Giannattasio, Lazzaro et al. 2005). The roles of Dot1 and H3K79 methylation in GGR are unlikely to be achieved indirectly by activating the DNA damage checkpoint. First, *dot1*Δ strains largely share the checkpoint defects of *bre1*Δ strains, implying that the checkpoint role of Bre1 (through mono-ubiquitination of H2BK123) is mostly manifested through its ability to permit di- and tri-methylation of H3K79,

although deleting *SET1* (and thus blocking histone H3 K4 methylation) as well as *DOT1* is required to replicate the full checkpoint defect of *bre1*Δ strains (Giannattasio, Lazzaro et al. 2005). However, although compromised, GGR is still apparent in *bre1Δ* and H2BK123A mutant cells, indicating that di- and tri-methylation of H3K79 contributes to but is not absolutely required for GGR. Second, cells lacking *MEC1*, which plays the most important role in the checkpoint activation in the yeast (Siede, Allen et al. 1996), have little defect in GGR (not shown). However, introduction of mutations to *mec1Δ* cells that disrupt H2BK123 ubiquitination or H3K79 methylation significantly decrease or abolish GGR, respectively (not shown), indicating that the histone modifications play much more important roles in GGR than the checkpoint activation.

Although chromatin structures can restrict the NER machinery from accessing sites of DNA damage, limited pieces of evidence have emerged recently that chromatin metabolism may also play an active role in the repair process (Waters, Teng et al. 2009). For example, acetylation of histone H3 K9 and/or K14 by the acetyltransferase Gcn5 facilitates GGR (Yu, Teng et al. 2005; Teng, Liu et al. 2008). Also, SWI/SNF, an ATP dependent chromatin remodeling complex, has been shown to be recruited to UV damaged chromatin DNA (Gong, Fahy et al. 2006). However, the effects of chromatin modifications/remodeling on NER in living cells documented so far are generally quite modest. In sharp contrast, H3K79 methylation by Dot1 appears to play a pivotal role in GGR. K79 of the two histone H3 molecules contained in a nucleosome are located at the top and bottom surfaces of the nucleosome disk and most likely regulate interactions with exogenous proteins (White, Suto et al. 2001). The K79 with methyl moieties may serve as a docking site for the GGR machinery on the chromatin. In the absence of the methyl moieties, the GGR machinery may be excluded from the chromatin, including the

vicinities of inter-nucleosomal linker regions. Indeed, all GGR-specific factors identified so far, including Rad7, Rad16 (Li, Ding et al. 2007; Lettieri, Kraehenbuehl et al. 2008), Elc1 (Lejeune, Chen et al. 2009) and Dot1, are required not only for repair in nucleosome core regions but also in inter-nucleosomal linker DNA.

Lesion processing by NER factors has been shown to be required for activation of the checkpoints in response to UV radiation (Giannattasio, Lazzaro et al. 2004). It is therefore reasonable to suggest that the roles of Dot1 and H3K79 methylation in the DNA damage checkpoint activation (Giannattasio, Lazzaro et al. 2005) may be indirectly achieved by their mediation of GGR. This explanation agrees with the observation that Dot1 and H3K79 methylation are epistatic to *RAD1*, which is essential for NER (both GGR and TCR) (Bostelman, Keller et al. 2007).

About 90% of all histone H3 are methylated in the yeast (van Leeuwen, Gafken et al. 2002; Ng, Ciccone et al. 2003). H3K79 methylation is ~ 10-fold lower (but still 8- to 10-fold higher than background) at all Sir-dependent silenced regions, such as the telomeric and silent mating-type loci, but not at other transcriptionally repressed regions, such as the *TSL1* gene and the promoters of the repressed *SUC2* and *INO1* genes (Ng, Ciccone et al. 2003). Indeed, most genes have nucleosomes modified at H3K79; there was little correlation between the relative levels of H3K79 methylation at genes and transcriptional activity (Ng, Ciccone et al. 2003; Pokholok, Harbison et al. 2005). The widespread feature of H3K79 methylation makes it ideal for mediating GGR. Telomeres (Rochette and Brash 2010) and centromeres (Capiaghi, Ho et al. 2004) are refractory to NER, which may be partly due to hypomethylation of H3K79.

A previous study showed that yeast cells expressing K79R mutant histone H3 have impaired NER at the transcriptionally silent mating-type locus *HML*, while maintaining nearly

normal NER in the constitutively expressed *RPB2* gene and transcriptionally repressed *GAL10* gene (Chaudhuri, Wyrick et al. 2009). This study collectively measured NER in both strands of the different loci (i.e., did not distinguish the two strands), which may have missed the detection of a repair defect in the NTS of the *RPB2* gene. There is evidence that GGR and TCR compete for common NER factors; specific elimination of GGR may enhance the rate of TCR in the cell (Li, Ding et al. 2007). The observation that H3K79 methylation does not affect overall NER in the repressed *GAL10* gene agrees with our results, which indicate that a defect in GGR does not cause a significant slowdown of overall NER in the repressed *GAL1-10* genes, presumably due to enhanced TCR mediated by "noise" transcription at the repressed loci [(Li, Ding et al. 2007) and data not shown].

In summation, we identified a novel GGR-specific NER factor (Dot1) and unveiled a critical link between a histone modification (H3K79 methylation) and the GGR process. These findings may open up new avenues of research regarding the fascinating mechanisms of how chromatin is actively engaged in NER.

3.5 References

Bostelman, L. J., A. M. Keller, et al. (2007). "Methylation of histone H3 lysine-79 by Dot1p plays multiple roles in the response to UV damage in Saccharomyces cerevisiae." DNA Repair (Amst) **6**(3): 383-95.

Capiaghi, C., T. V. Ho, et al. (2004). "Kinetochores prevent repair of UV damage in Saccharomyces cerevisiae centromeres." Mol Cell Biol **24**(16): 6907-18.

Chaudhuri, S., J. J. Wyrick, et al. (2009). "Histone H3 Lys79 methylation is required for efficient nucleotide excision repair in a silenced locus of Saccharomyces cerevisiae." Nucleic Acids Res **37**(5): 1690-700.

Friedberg, E. C., G. C. Walker, et al. (2006). DNA Repair and Mutagenesis. Washington D.C., ASM Press.

Giannattasio, M., F. Lazzaro, et al. (2004). "Physical and functional interactions between nucleotide excision repair and DNA damage checkpoint." Embo J **23**(2): 429-38.

Giannattasio, M., F. Lazzaro, et al. (2005). "The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1 and H3 methylation by Dot1." J Biol Chem **280**(11): 9879-86.

Gillette, T. G., S. Yu, et al. (2006). "Distinct functions of the ubiquitin-proteasome pathway influence nucleotide excision repair." Embo J **25**(11): 2529-38.

Gong, F., D. Fahy, et al. (2006). "Rad4-Rad23 interaction with SWI/SNF links ATP-dependent chromatin remodeling with nucleotide excision repair." Nat Struct Mol Biol **13**(10): 902-7.

Gong, F., Y. Kwon, et al. (2005). "Nucleotide excision repair in chromatin and the right of entry." DNA Repair (Amst) **4**(8): 884-96.

Guzder, S. N., P. Sung, et al. (1998). "The DNA-dependent ATPase activity of yeast nucleotide excision repair factor 4 and its role in DNA damage recognition." J Biol Chem **273**(11): 6292-6.

Hanawalt, P. C. and G. Spivak (2008). "Transcription-coupled DNA repair: two decades of progress and surprises." Nat Rev Mol Cell Biol **9**(12): 958-70.

Hughes, T. R., M. J. Marton, et al. (2000). "Functional discovery via a compendium of expression profiles." Cell **102**(1): 109-26.

Jiang, C. and B. F. Pugh (2009). "A compiled and systematic reference map of nucleosome positions across the Saccharomyces cerevisiae genome." Genome Biol **10**(10): R109.

Kouzarides, T. (2007). "Chromatin modifications and their function." Cell **128**(4): 693-705.

Lejeune, D., X. Chen, et al. (2009). "Yeast Elc1 plays an important role in global genomic repair but not in transcription coupled repair." DNA Repair (Amst) **8**(1): 40-50.

Lettieri, T., R. Kraehenbuehl, et al. (2008). "Functionally distinct nucleosome-free regions in yeast require Rad7 and Rad16 for nucleotide excision repair." DNA Repair (Amst) **7**(5): 734-43.

Levesque, N., G. P. Leung, et al. (2010). "Loss of H3 K79 trimethylation leads to suppression of Rtt107-dependent DNA damage sensitivity through the translesion synthesis pathway." J Biol Chem **285**(45): 35113-22.

Li, S., B. Ding, et al. (2007). "The roles of Rad16 and Rad26 in repairing repressed and actively transcribed genes in yeast." DNA Repair (Amst) **6**(11): 1596-606.

Li, S. and M. J. Smerdon (2002). "Rpb4 and Rpb9 mediate subpathways of transcription-coupled DNA repair in Saccharomyces cerevisiae." Embo J **21**(21): 5921-9.

Li, S. and M. J. Smerdon (2004). "Dissecting transcription-coupled and global genomic repair in the chromatin of yeast GAL1-10 genes." J Biol Chem **279**(14): 14418-26.

Li, S. and R. Waters (1996). "Nucleotide level detection of cyclobutane pyrimidine dimers using oligonucleotides and magnetic beads to facilitate labelling of DNA fragments incised at the dimers and chemical sequencing reference ladders." Carcinogenesis **17**(8): 1549-52.

Li, S. and R. Waters (1997). "Induction and repair of cyclobutane pyrimidine dimers in the Escherichia coli tRNA gene tyrT: Fis protein affects dimer induction in the control region and suppresses preferential repair in the coding region of the transcribed strand, except in a short region near the transcription start site." J Mol Biol **271**(1): 31-46.

Li, S., R. Waters, et al. (2000). "Low- and high-resolution mapping of DNA damage at specific sites." Methods **22**(2): 170-9.

Lloyd, R. S. (2005). "Investigations of pyrimidine dimer glycosylases--a paradigm for DNA base excision repair enzymology." Mutat Res **577**(1-2): 77-91.

Luger, K., A. W. Mader, et al. (1997). "Crystal structure of the nucleosome core particle at 2.8 A resolution." Nature **389**(6648): 251-60.

Nakanishi, S., B. W. Sanderson, et al. (2008). "A comprehensive library of histone mutants identifies nucleosomal residues required for H3K4 methylation." Nat Struct Mol Biol **15**(8): 881-8.

Ng, H. H., D. N. Ciccone, et al. (2003). "Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation." Proc Natl Acad Sci U S A **100**(4): 1820-5.

Pokholok, D. K., C. T. Harbison, et al. (2005). "Genome-wide map of nucleosome acetylation and methylation in yeast." Cell **122**(4): 517-27.

Ribar, B., L. Prakash, et al. (2007). "ELA1 and CUL3 are required along with ELC1 for RNA polymerase II polyubiquitylation and degradation in DNA-damaged yeast cells." Mol Cell Biol **27**(8): 3211-6.

Rochette, P. J. and D. E. Brash (2010). "Human telomeres are hypersensitive to UV-induced DNA Damage and refractory to repair." PLoS Genet **6**(4): e1000926.

Shahbazian, M. D., K. Zhang, et al. (2005). "Histone H2B ubiquitylation controls processive methylation but not monomethylation by Dot1 and Set1." Mol Cell **19**(2): 271-7.

Siede, W., J. B. Allen, et al. (1996). "The Saccharomyces cerevisiae MEC1 gene, which encodes a homolog of the human ATM gene product, is required for G1 arrest following radiation treatment." J Bacteriol **178**(19): 5841-3.

Struhl, K. (2007). "Transcriptional noise and the fidelity of initiation by RNA polymerase II." Nat Struct Mol Biol **14**(2): 103-5.

Teng, Y., H. Liu, et al. (2008). "Saccharomyces cerevisiae Rad16 mediates ultraviolet-dependent histone H3 acetylation required for efficient global genome nucleotide-excision repair." EMBO Rep **9**(1): 97-102.

van Leeuwen, F., P. R. Gafken, et al. (2002). "Dot1p modulates silencing in yeast by methylation of the nucleosome core." Cell **109**(6): 745-56.

Verhage, R., A. M. Zeeman, et al. (1994). "The RAD7 and RAD16 genes, which are essential for pyrimidine dimer removal from the silent mating type loci, are also required for repair of the nontranscribed strand of an active gene in Saccharomyces cerevisiae." Mol Cell Biol **14**(9): 6135-42.

Waters, R., Y. Teng, et al. (2009). "Tilting at windmills? The nucleotide excision repair of chromosomal DNA." DNA Repair (Amst) **8**(2): 146-52.

Wellinger, R. E. and F. Thoma (1997). "Nucleosome structure and positioning modulate nucleotide excision repair in the non-transcribed strand of an active gene." Embo J **16**(16): 5046-56.

White, C. L., R. K. Suto, et al. (2001). "Structure of the yeast nucleosome core particle reveals fundamental changes in internucleosome interactions." Embo J **20**(18): 5207-18.

Yu, Y., Y. Teng, et al. (2005). "UV irradiation stimulates histone acetylation and chromatin remodeling at a repressed yeast locus." Proc Natl Acad Sci U S A **102**(24): 8650-5.

CHAPTER 4

THE ROLES OF RNA POLYMERASE II-ASSOCIATED FACTOR 1 COMPLEX IN DIFFERENT SUBPATHWAYS OF NUCLEOTIDE EXCISION REPAIR

4.1 Introduction

Almost all cellular organisms are equipped with multiple DNA repair pathways to contend with constantly occurring DNA damage caused by endogenous and exogenous DNA damaging agents (Friedberg, Walker et al. 2006). Nucleotide excision repair (NER) is a DNA repair pathway that removes a wide variety of bulky, helix-distorting lesions that generally obstruct transcription and normal replication, such as UV-induced cyclobutane pyrimidine dimers (CPDs). NER is a multistep reaction that requires the coordinated action of over 30 proteins implicated in damage recognition, helix opening, lesion verification, dual incision of the damaged strand bracketing the lesion, removal of an oligonucleotide containing the lesion, gap-filling DNA synthesis, and ligation. Transcription coupled repair (TCR) is an NER pathway dedicated to rapid repair in the transcribed strand (TS) of actively transcribed genes. Global genomic repair (GGR) is the other NER pathway that removes lesions throughout the genome including the nontranscribed strand (NTS) of actively transcribed genes. The two NER pathways share most of the common NER factors but differ in the damage recognition step.

TCR is believed to be initiated by an RNA polymerase stalled at a lesion in the TS of active genes (Hanawalt and Spivak 2008). However, TCR in eukaryotic cells appears to be extremely complicated and the biochemical mechanism of the process is still largely unknown. In the budding yeast *Saccharomyces cerevisiae*, Rad26, a DNA-stimulated ATPase that is homologous to the human CSB protein (Guzder, Habraken et al. 1996), plays an important role in TCR (van Gool, Verhage et al. 1994). However, TCR is not solely dependent on Rad26, as a substantial extent of TCR still occurs in *rad26Δ* cells (Verhage, van Gool et al. 1996; Li and Smerdon 2002;

Li and Smerdon 2004). Rpb9, a nonessential subunit of the 12-subunit (Rpb1-12) RNA polymerase II (Pol II), has been shown to be required for Rad26-independnet TCR (Li and Smerdon 2002). Interestingly, the Rad26-independent TCR has been shown to be suppressed by at least 3 proteins, namely Rpb4 (Li and Smerdon 2002), Spt4 (Jansen, den Dulk et al. 2000), and Spt5 (Ding, LeJeune et al. 2010). Rpb4 is another nonessential Pol II subunit that forms a subcomplex with Rpb7. The Rpb4-Rpb7 subcomplex is associated with the core Pol II through a "wedge" structure on Rpb7, "pushing" the clamp of the 10-subunit core Pol II to a closed position (Armache, Kettenberger et al. 2003; Bushnell and Kornberg 2003). Spt4 forms a complex with Spt5, which physically interacts with Pol II (Hartzog, Wada et al. 1998). It was found recently that the role of Spt4 in suppressing Rad26-independent TCR is indirectly achieved by protecting Spt5 from degradation and by stabilizing the interaction of Spt5 with Pol II (Ding, LeJeune et al. 2010). Furthermore, the C-terminal repeat (CTR) domain of Spt5, which contains 15 copies of a six-amino acid sequence that can be phosphorylated by the Bur kinase, is responsible for suppressing Rad26-independent TCR (Ding, LeJeune et al. 2010).

Rad7, Rad16 (Verhage, Zeeman et al. 1994) and Elc1 (Lejeune, Chen et al. 2009) are specifically required for GGR in yeast. The exact roles of these proteins in GGR are not yet clear. It has been proposed that the Rad7-Rad16 complex may act as an ATP-dependent motor which translocates along the DNA in search of damage, and upon encountering a lesion, the complex is stalled, which may remodel and open damaged chromatin, thereby facilitating recruitment of other NER factors (Guzder, Sung et al. 1998). Elc1 has been shown to be a component of an E3 ubiquitin ligase that contains Rad7 and Rad16 (Gillette, Yu et al. 2006). This E3 ubiquitin ligase has been shown to ubiquitinate Rad4, an essential NER factor required for both GGR and TCR. Optimal NER correlates with the ubiquitination of Rad4, but not its

subsequent degradation (Gillette, Yu et al. 2006). Elc1 has also been suggested to be a component of another ubiquitin ligase complex, which contains Ela1, Cul3, and Roc1 but not Rad7 and Rad16, and is required for ubiquitination and degradation of Rpb1, the largest subunit of Pol II (Ribar, Prakash et al. 2007).

The basic repeating component of chromatin in eukaryotic cells is the nucleosome, which is comprised of 146 base pairs of DNA wrapped around a protein octomer containing two molecules of each of the four core histones H2A, H2B, H3, and H4 (Luger, Mader et al. 1997). Although the packaging of DNA in chromatin can restrict the NER machinery, especially the GGR machinery, from accessing sites of DNA damage, limited pieces of evidence have emerged recently that chromatin metabolism may also play an active role in the repair process (Waters, Teng et al. 2009). For example, acetylation of histone H3 on lysine 9 and/or 14 by the acetyltransferase Gcn5 facilitates GGR (Yu, Teng et al. 2005; Teng, Liu et al. 2008). Also, SWI/SNF, an ATP dependent chromatin remodeling complex, has been shown to be recruited to chromatin upon induction of DNA damage by UV (Gong, Fahy et al. 2006). A critical piece of evidence indicating the active engagement of chromatin in GGR is the recent discovery that methylation of histone H3 lysine 79 (H3K79), catalyzed by the histone methyltransferase Dot1, is required for GGR in yeast (Tatum and Li 2011). K79 of the two histone H3 molecules contained in a nucleosome are located at the top and bottom surfaces of the nucleosome disk and most likely regulate interactions with exogenous proteins (White, Suto et al. 2001). It was proposed that the methylated H3K79 may serve as a docking site for the GGR machinery on the chromatin (Tatum and Li 2011).

The highly conserved Pol II-associated factor 1 complex (Paf1C), which is abundant in simple and complex eukaryotic cells, directly interacts with Pol II and chromatin at both

promoter regions and throughout the coding regions of genes [for a recent review, see (Jaehning 2010)]. In yeast, Paf1C is composed of Paf1, Rtf1, Cdc73, Leo1 and Ctr9 whereas in human cells, Paf1C also contains Ski8. Paf1C may interact with Pol II through Rtf1 and Cdc73 in yeast, because when either of them is lost, the rest of Paf1C components still associate with each other, but no longer interact with Pol II or chromatin and relocalize as a unit from the nucleus to the nucleolus. Paf1C is involved in a variety of cellular processes, including transcription elongation, 3'-processing of mRNAs, and modification of chromatin. Genome-wide gene expression analyses have shown that Paf1C affects transcription of a small number of yeast genes, among which are many cell wall biosynthetic genes and a subset of cell cycle-regulated genes, but no NER genes (Chang, French-Cornay et al. 1999; Porter, Washburn et al. 2002).

Although Paf1C binds to Pol II, the major functions of Paf1C may be independent of Pol II. Indeed, loss of Rtf1 or Cdc73, which results in loss of Paf1 factors from chromatin and from the Pol II complex, has little phenotypic consequence (Mueller, Porter et al. 2004). Also, loss of Paf1, which results in severe phenotypes and reduced amounts of other Paf1C components, has little effect on the abundance or chromatin distribution of Pol II (Mueller, Porter et al. 2004). Furthermore, Paf1C has been shown to be required for Bre1-catalyzed monoubiquitination of histone H2B lysine 123 (H2BK123) (Krogan, Dover et al. 2003; Ng, Dole et al. 2003; Wood, Schneider et al. 2003), which is in turn the prerequisite for Dot1-catalyzed di- and tri-methylation of histone H3 lysine 79 (H3K79) (Shahbazian, Zhang et al. 2005; Nakanishi, Lee et al. 2009; Levesque, Leung et al. 2010). However, these histone modifications are not specifically limited to the transcribed regions of the genome (van Leeuwen, Gafken et al. 2002; Ng, Ciccone et al. 2003; Pokholok, Harbison et al. 2005; Schulze, Jackson et al. 2009), supporting the idea

that a fraction of Paf1C that is not associated with Pol II is able to promote these histone modifications.

In this paper, we show that Paf1C plays diverse roles in different NER pathways or subpathways. We also present evidence that the different roles of Paf1C in the different NER pathways or subpathways are manifested through different mechanisms.

4.2 Materials and Methods

4.2.1 Yeast Strains and Plasmids

Genes that were deleted individually or combinatorially in yeast cells are shown in Table 4-1. The *bre1Δ* strain (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bre1::Kan*) and its isogenic wild type BY4741 strain were purchased from Open Biosystems. Cells expressing mutant H3K79A and H2BK123A histones, and their isogenic wild type strains YBL574 and FY406 (Nakanishi, Sanderson et al. 2008) were kindly provided by Dr. Ali Shilatifard (Stowers Institute for Medical Research). Gene deletions in cells expressing the mutant histones and in the wild type strain Y452 (*MATα ura3-52 his3-1 leu2-3 leu2-112*) was created using procedures previously described (Li and Smerdon 2002). Cells with their genomic *PAF1* gene tagged with three consecutive FLAG (3×FLAG) sequences were created using PCR products amplified from plasmid p3FLAG-KanMX, as described previously (Gelbart, Rechsteiner et al. 2001).

A multi-copy plasmid (pGAL-SPT5) overexpressing 3×FLAG tagged Spt5 under the control of the *GAL10* promoter and single-copy centromeric plasmids encoding the full-length or the CTR-deleted Spt5 were created as described previously (Ding, LeJeune et al. 2010).

Table 4-1. Genes deleted individually or combinatorially in yeast cells

Genes	Encoded proteins	Known interactions and/or functions
PAF1	Paf1C	Binds to Pol II
RTF1		Required for efficient expression of a small number of genes
CDC73		
LEO1		Required for Bre1-catalyzed monoubiquitination of H2BK123
CTR9		Indirectly required for Dot1-catalyzed di- and tri-methylation of H3K79
RAD26	DNA-stimulated ATPase	Partially required for TCR
RPB9	nonessential Pol II subunit	Partially required for TCR
RPB4	nonessential Pol II subunit	Suppresses Rad26-independent TCR
SPT4	Spt4-Spt5 transcription elongation factor	Binds to Pol II
SPT5		Suppresses Rad26-independent TCR
RAD16	ATPase	Forms complex with Rad7, and possibly with Elc1 as well Required for GGR
BRE1	E3 ubiquitin ligase	Forms heterodimer with Rad6 Catalyzes monoubiquitination of H2BK123 Required for Dot1-catalyzed di- and tri-methylation of H3K79 Partially required for GGR
DOT1	Histone methyltransferase	Catalyzes mono-, di- and tri- methylation of H3K79 Required for GGR

4.2.2 UV Sensitivity Assay

Yeast cells were grown at 30°C in YPD medium (2% peptone, 1% yeast extract, 2% glucose), and sequential 10-fold dilutions were made. The diluted samples were spotted onto YPD plates. When the spots had dried, the plates were irradiated with different doses of 254 nm UV light. The plates were incubated at 30°C for 3-4 days in the dark prior to being photographed.

4.2.3 UV Irradiation, Repair Incubation and Genomic DNA Isolation

Yeast cells were grown at 30°C in minimal media containing 2% glucose or 2% galactose (to induce a gene under the control of the *GAL1* promoter) to late log phase($A_{600} \approx 1.0$), washed twice with ice-cold water, resuspended in ice-cold 2% glucose (for glucose cultures) or 2% galactose (for galactose cultures), and irradiated with 80 J/m^2 of 254 nm UV light. One-tenth volume of a stock solution containing 10% yeast extract and 20% peptone was added to the irradiated cell suspension. The cells were incubated at 30°C in the dark to allow them to repair their DNA and aliquots were collected at different time points. Genomic DNA was isolated from the cells as described previously (Li and Smerdon 2002).

4.2.4 NER Analysis of UV-induced CPDs at Nucleotide Resolution

The induction and repair of CPDs at individual sites in each strand of the *RPB2* gene were measured using a method we developed previously (Li and Waters 1996; Li and Waters 1997; Li, Waters et al. 2000). Briefly, ~ 1μg of genomic DNA was digested with *Dra*I to release the *RPB2* fragment and incised at CPDs with an excess amount of purified T4 endonuclease V (Epicentre). Two biotinylated oligonucleotides were then used to specifically 'fish out' and label the TS and NTS of the *RPB2* gene fragment, respectively. One pmol of one of the oligonucleotides was mixed with each of the samples. The mixtures were heated at 95°C for 5 min to denature the DNA and then cooled to an annealing temperature of around 50°C. One hundred μg of streptavidin magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen) was added to each of the mixtures to capture the strand of the *RPB2* fragment hybridized to the biotinylated oligonucleotide. The other unwanted genomic DNA fragments were washed away. The fragments captured on the magnetic beads were 3'-end labeled with [α-^{32}P]dATP and Sequnase Version 2 (US Biochemicals). The labeled fragments were resolved on sequencing

gels, which were then dried and exposed to a Phosphoimager screen (Bio-Rad). The signal intensities at gel bands corresponding to CPD sites were quantified by using Quantity One software (Bio-Rad). The percent CPDs remaining at individual sites after different times of repair incubation were calculated and the times required for repairing 50% of CPDs ($t_{1/2}$) were obtained by either linear or second order polynomial regression.

4.2.5 Immunoprecipitation

Yeast cells were cultured at 30°C in minimal medium to late log phase and harvested. The cells harvested from a 25 ml culture were washed with and resuspended in 0.5 ml of immunoprecipitation buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.4 mM Na_4VO_3, 10 mM $Na_4P_2O_7$, 10 mM sodium fluoride, 0.5% Nonidet P-40, 1% Triton X-100, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors) (Chen, Ruggiero et al. 2007). The cells were broken with acid-washed glassed beads, and cell debris was removed by centrifugation at 20,000 × *g* for 10 min at 4°C. Fifty µl of the lysate was saved as "input". The remaining lysate was added with 15 µg of 8WG16 (Neoclone), which recognizes the C-terminal heptapeptide repeats of Rpb1, the largest subunit of Pol II (Thompson, Steinberg et al. 1989). The mixture was incubated at 4°C overnight with gentle rotation. Protein A-coated agarose beads (Sigma) were added to the mixture and incubated at 4°C for 3 h with gentle rotation. The beads were washed twice with immunoprecipitation buffer containing 0.5 M NaCl and twice with immunoprecipitation buffer containing 0.15 M NaCl. Bound proteins were eluted by boiling the beads in 50 µl of 2 × SDS-PAGE gel loading buffer.

4.2.6 Western Blot

Immunoprecipitation inputs and immunoprecipitates were resolved on an SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Rpb1 and

3×FLAG tagged proteins were probed with 8WG16 and anti-FLAG M2 antibodies (Sigma), respectively. Blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce), and the protein bands were detected using ChemiDoc™ XRS+ System (Bio-Rad). Band intensities were quantified using Quantity One software (Bio-Rad).

4.3 Results

4.3.1 Paf1C Plays a Minor Role in Facilitating Rad26-dependent TCR

To determine the role of Paf1C in TCR, we deleted genes encoding Paf1C components in *rad16Δ* cells, which are deficient in GGR (Verhage, Zeeman et al. 1994), so that TCR can be unambiguously analyzed. Yeast cells were cultured to late log phase, UV irradiated, and incubated in a repair medium for various lengths of time. Total genomic DNA was isolated, digested with a restriction enzyme to release a fragment of the constitutively expressed *RPB2* gene, and incised at the CPDs with an excess amount of T4 endonuclease V (Lloyd 2005). The TS of the *RPB2* gene fragment was 'fished out' with a biotinylated oligonucleotide and streptavidin magnetic beads, radioactively labeled at the 3' end, and resolved on a DNA sequencing gel. As can be seen in Figures 4-1A and 4-2, rapid TCR, which initiates ~ 40 nucleotides upstream of the transcription start site of the *RPB2* gene, occurred in *rad16Δ* cells. The TCR rates were marginally but reproducibly slower in *rad16Δ* cells lacking a Paf1C component (Figures 4-1B, 4-1C and 4-2; data not shown), indicating that Paf1C plays a little role in facilitating TCR.

In yeast, TCR is dependent on Rad26 and Rpb9 (Li and Smerdon 2002). To determine whether the minor role of Paf1C in facilitating TCR is dependent on Rad26 or Rpb9, or both, we analyzed TCR in *rad16Δ rpb9Δ* and *rad16Δ rpb9Δ rtf1Δ* cells where only Rad26-dependent TCR is operative. The TCR rate was also marginally but reproducibly slower in the *rad16Δ*

rpb9Δ rtf1Δ cells than in the *rad16Δ rpb9Δ* cells (Figures 4-1D, 4-1E and 4-2), indicating that the little role of Paf1C in facilitating TCR is dependent on Rad26.

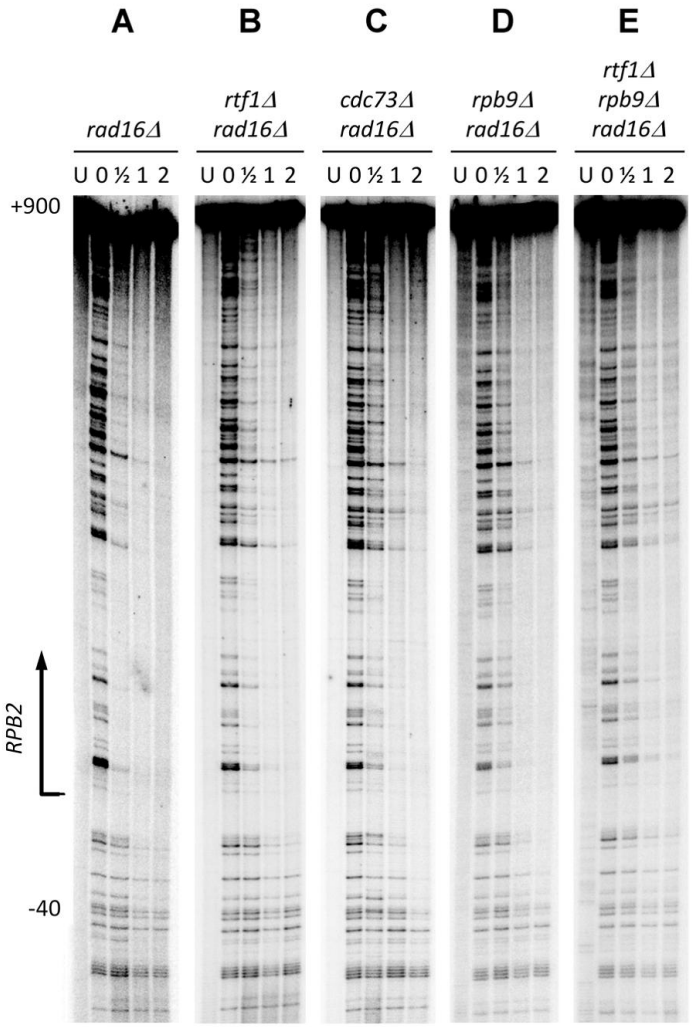

Figure 4-1. Gels showing repair of CPDs in the TS of the *RPB2* gene in *rad16Δ* cells with different additional gene deletions. The lanes are DNA samples from unirradiated (U) and UV irradiated cells following different times (hrs) of repair incubation. Numbers on the left indicate nucleotide positions relative to the transcription start site (shown as the arrow).

Additional deletion of *RTF1* in *rad16Δ* and *rad16Δ rpb9Δ* cells caused increased UV sensitivity (Figure 4-3), indicating that Paf1C is not epistatic to Rad16 or Rpb9 and supporting the notion that Paf1C facilitates Rad26-dependent TCR. However, the effect of the *RTF1* deletion on UV sensitivity appeared to be greater than would be expected from the marginal

deficiency in Rad26-dependent TCR caused by the deletion. It is therefore likely that, besides the minor role in facilitating Rad26-dependent TCR, Paf1C may function in other repair and/or DNA damage tolerance pathway(s).

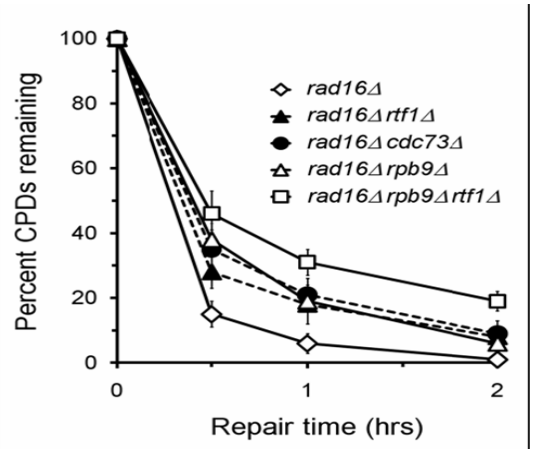

Figure 4-2. Plot showing repair of CPDs in the TS of the *RPB2* gene in *rad16Δ* cells with different additional gene deletions. The values shown are means (± S.E.) of percent CPDs remaining at individual sites in the coding region of the TS of the *RPB2* gene at different repair times.

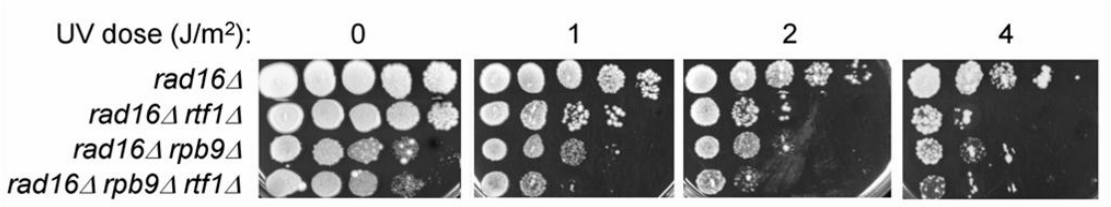

Figure 4-3. Deletion of *rtf1* in *rad16Δ* and *rad16Δ rpb9Δ* cells enhances UV sensitivity. Saturated cultures of yeast strains were sequentially 10-fold diluted and spotted onto YPD plates. When the spots had dried, the plates were irradiated with the indicated doses of 254 nm UV light. The plates were incubated at 30°C for 3-4 days in the dark prior to being photographed.

4.3.2 Paf1C Suppresses Rad26-independent TCR

We then asked what role Paf1C may play in Rad26-independent TCR. Surprisingly, elimination of a Paf1C component in *rad16Δ rad26Δ* cells, where only Rad26-independent TCR is operative, resulted in enhanced repair (Figure 4-4, compare panels B – E with A; Figure 4-5), indicating that Paf1C suppresses Rad26-independent TCR.

In agreement with our previous studies (Li and Smerdon 2002; Li and Smerdon 2004), no repair can be seen in *rad16Δ rad26Δ rpb9Δ* cells (Figures 4-4F and 4-5). Additional elimination of a Paf1C component did not result in restoration of TCR in *rad16Δ rad26Δ rpb9Δ* cells (Figures 4-4G and 4-5). These results indicate that Paf1C suppresses Rad26-independent TCR that is absolutely dependent on Rpb9.

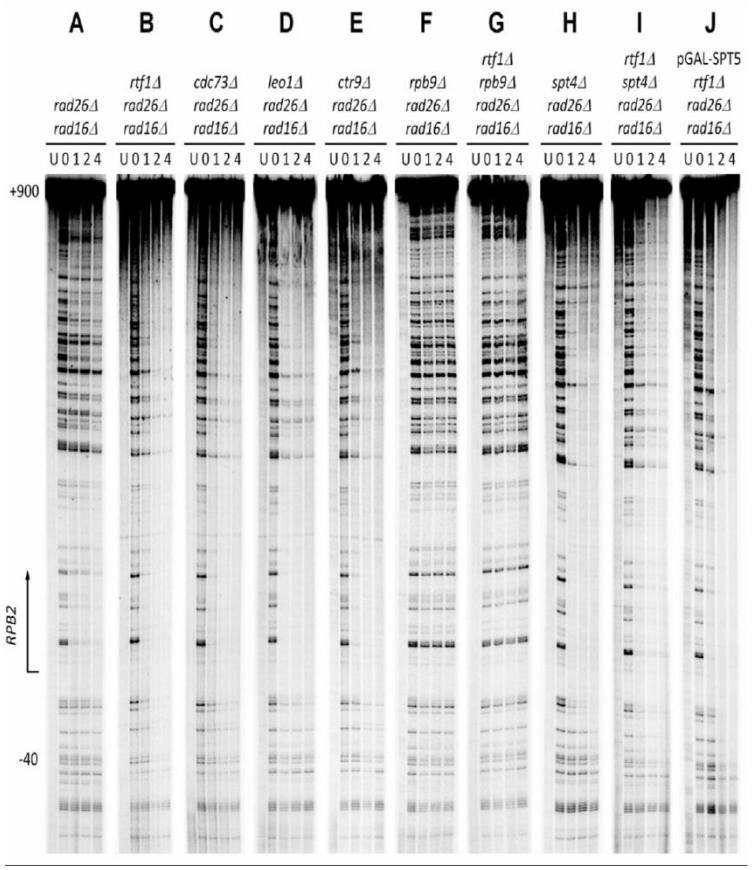

Figure 4-4. Gels showing repair of CPDs in the TS of the *RPB2* gene in *rad16Δ rad26Δ* cells with different additional gene deletions. The lanes are DNA samples from unirradiated (U) and UV irradiated cells following different times (hrs) of repair incubation. Numbers on the left indicate nucleotide positions relative to the transcription start site (shown as the arrow).

Having found the role of Paf1C in suppressing Rad26-independent TCR, we wanted to elucidate how this might occur. Several factors, such as Rpb4 (Li and Smerdon 2002), Spt4 and Spt5 (through its CTR domain) (Jansen, den Dulk et al. 2000; Ding, LeJeune et al. 2010) have

been shown to also suppress Rad26-independent TCR. We therefore attempted to investigate the functional interactions between Paf1C and these factors. We found that deletion of *RPB4* and a Paf1C gene is synthetically lethal for the cell (not shown), which prevented us from further exploring the functional interaction between Paf1C and Rpb4. We therefore turned to Spt4 and Spt5.

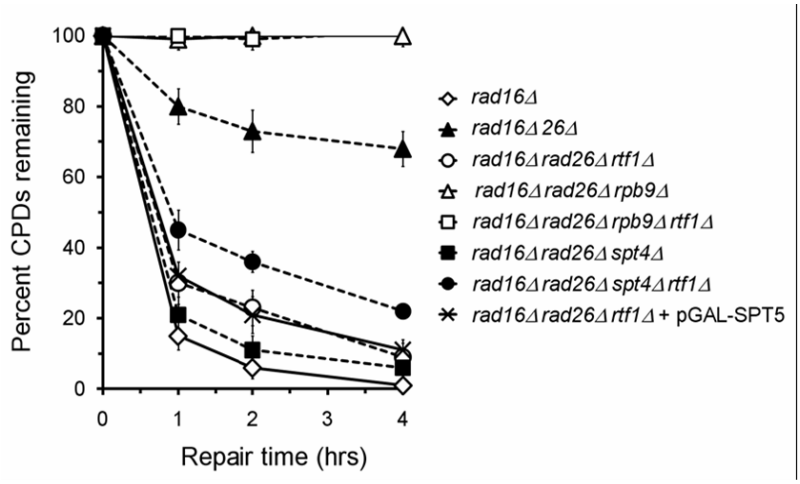

Figure 4-5. Plot showing repair of CPDs in the TS of the *RPB2* gene in *rad16Δ* and *rad16Δ rad26Δ* cells with different additional gene deletions. The values shown are means (± S.E.) of percent CPDs remaining at individual sites in the coding region of the TS of the *RPB2* gene at different repair times.

4.3.3 Paf1C Does Not Suppress Rad26-independent TCR in Cells Lacking Spt4, and *vice versa*

In agreement with a previous report (Jansen, den Dulk et al. 2000), TCR was more rapid in *rad16Δ rad26Δ spt4Δ* cells than in *rad16Δ rad26Δ* cells (Figure 4-4, compare panels A and H; Figure 4-5). We wondered if Paf1C and Spt4 suppress Rad26-independent TCR through a common pathway. If they do, Paf1C will not suppress Rad26-independent TCR in cells lacking Spt4, and *vice versa*. Indeed, the TCR rate in *rad16Δ rad26Δ* cells lacking both a Paf1C component and Spt4 was slightly slower, rather than faster, than those lacking either a Paf1C component or Spt4 (Figure 4-4, compare panel I with panels B-E and H; Figure 4-5). In other

words, elimination of a Paf1C component did not further release Rad26-independent TCR in cells lacking Spt4, and *vice versa*. This indicates that the roles of Paf1C and Spt4 in suppressing Rad26-independent TCR are likely to be through a common pathway. The slight slowdown of TCR in *rad16Δ rad26Δ* cells lacking both a Paf1C component and Spt4 may be attributed to the fact that both Paf1C and Spt4 function in transcription elongation, and elimination of both may synergistically cause a transcriptional elongation defect.

4.3.4 The Role of Paf1C in Suppressing Rad26-independent TCR Is Not Subsidiary to That of Spt5

Spt4 forms a complex with Spt5 (Hartzog, Wada et al. 1998). Unlike Spt4 which is dispensable, Spt5 is essential for cell viability (Swanson, Malone et al. 1991). The different roles of Spt4 and Spt5 in cell viability may be due to the proposition that Spt5 directly interacts with Pol II and plays a more fundamental role in transcription, whereas Spt4 is associated with Pol II through interaction with Spt5 (Martinez-Rucobo, Sainsbury et al. 2011). We recently found that Spt4 actually suppresses Rad26-independent TCR indirectly by protecting Spt5 from degradation and by stabilizing the interaction of Spt5 with Pol II (Ding, LeJeune et al. 2010). Indeed, overexpression of Spt5 suppresses Rad26-independent TCR in cells lacking Spt4 (Ding, LeJeune et al. 2010). To test whether the role of Paf1C in suppressing Rad26-independent TCR is also subsidiary to that of Spt5, we overexpressed Spt5 in *rad16Δ rad26Δ rtf1Δ* cells. The overexpression did not affect the TCR rate in these cells (Figure 4-4, compare panel J with panel B; Figure 4-5), indicating that Paf1C is indispensable for suppressing Rad26-independent TCR even when an excess amount of Spt5 is present. In other words, both Paf1C and Spt5 are required for suppressing Rad26-independent TCR and the role of Paf1C in the suppression is not subsidiary to that of Spt5.

4.3.5 The Association of Paf1C with Pol II Is Facilitated by the CTR Domain of Spt5

We have found that the CTR domain of Spt5, which is dispensable for cell viability and is not required for interactions with Spt4 and Pol II, is responsible for suppressing Rad26-independent TCR (Ding, LeJeune et al. 2010). Previous studies by others have indicated that Spt5 and Paf1C have extensive genetic and physical interactions (Squazzo, Costa et al. 2002). We wondered whether the Spt5 CTR is responsible for recruiting Paf1C to Pol II, thereby forming a larger complex that suppresses Rad26-independent TCR. We created yeast strains whose genomic *SPT5* gene is deleted and complemented with a plasmid encoding the full-length or CTR-deleted Spt5. Three consecutive FLAG sequences (3×FLAG) were tagged to the coding sequence of the genomic *PAF1* gene in these yeast cells. The 3×FLAG tag did not cause any noticeable deficiency to the cells (not shown). We immunoprecipitated the Pol II complex with antibody 8WG16, which recognizes the C-terminal heptapeptide repeats of Rpb1, the largest subunit of Pol II (Thompson, Steinberg et al. 1989). The presence of Pol II and 3×FLAG tagged Paf1 in the immunoprecipitates were examined on a Western blot by using the anti-Rpb1 (8WG16) and an anti-FLAG antibody, respectively. The level of 3×FLAG tagged Paf1 in cells expressing the CTR-deleted Spt5 is ~ 1.6 fold that in cells expressing the full-length Spt5 (Figure 4-6, Input), presumably due to an unknown compensation mechanism in the absence of the Spt5 CTR. The 3×FLAG tagged Paf1 coimmunoprecipitated with Pol II in cells expressing the CTR-deleted Spt5 is ~ 30% of that in cells expressing the full-length Spt5 (Fig. 6, IP), indicating that the Spt5 CTR facilitates the association of Paf1C with Pol II.

In view of the observations that Paf1C is not subsidiary to Spt5 in suppressing Rad26-independent TCR and that the association of Paf1C with Pol II is facilitated by the Spt5 CTR, it is likely that a complex which includes at least Spt4-Spt5 and Paf1C is responsible for the suppression.

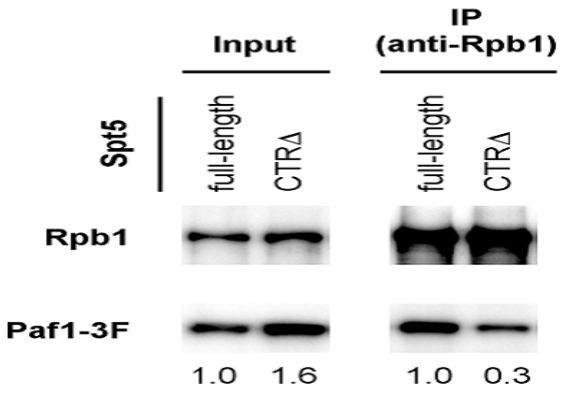

Figure 4-6. The Spt5 CTR facilitates the association of Paf1C with Pol II. Yeast cells whose genomic *PAF1* gene was tagged with 3×FLAG and whose genomic *SPT5* gene was deleted and complemented with a plasmid encoding either the full-length or CTR-deleted Spt5 were cultured to late log phase. Pol II complexes were immunoprecipited from these cells with antibody 8WG16 (anti-Rpb1). The immunoprecipitation input and immunoprecipitates (IP) were subjected to Western blot and the presence of Rpb1 and 3×FLAG-tagged Paf1 (Paf1-3F) on the blot were detected with 8WG16 and anti-FLAG antibodies, respectively. Numbers underneath the blot indicate relative levels of 3×FLAG tagged Paf1 (normalized to the level of Rpb1) (the level in cells expressing the full-length Paf1 is set as 1).

4.3.6 Paf1C Is Not Epistatic to Either Rad26 or Spt4/Spt5

In line with a role for Spt4 and the Spt5 CTR in suppressing Rad26-independent TCR, elimination of either Spt4 (Jansen, den Dulk et al. 2000; Ding, LeJeune et al. 2010) or the Spt5 CTR (Ding, LeJeune et al. 2010) in *rad16Δ rad26Δ* (or *rad7Δ rad26Δ*) cells restores the UV resistance of these cells to the level of *rad16Δ* (or *rad7Δ*) cells. However, elimination of Spt4 or the Spt5 CTR in *RAD26*$^{+}$ cells does not affect UV sensitivity, which agrees with the observations that Spt4 and the Spt5 CTR do not affect GGR and Rad26-dependent TCR (Jansen, den Dulk et al. 2000; Ding, LeJeune et al. 2010). However, instead of restoring UV resistance, additional

elimination of any Paf1C component in *rad16Δ rad26Δ* cells enhanced UV sensitivity (Figure 4-7; *rad16Δ rad26Δ* cells with other Paf1C components eliminated not shown). Also, elimination of Paf1C components enhanced the UV sensitivity of *rad16Δ rad26Δ spt4Δ* cells (Figure 4-7). These results indicate that Paf1C is not epistatic to either Rad26 or Spt4/Spt5, and besides having a role in suppressing Rad26-independent TCR together with Spt4/Spt5, Paf1C functions in other repair and/or DNA damage tolerance pathway(s).

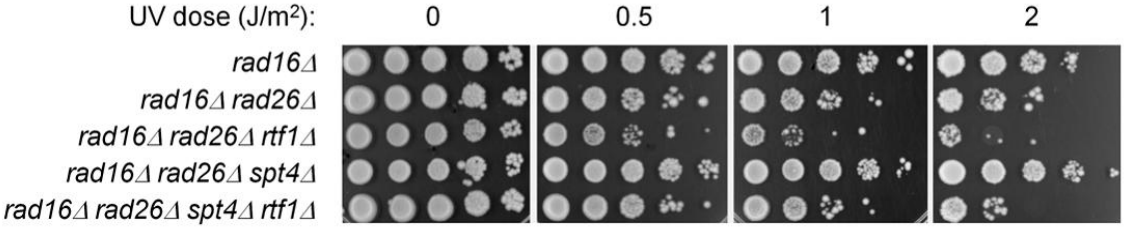

Figure 4-7. Deletion of *rtf1* in *rad16Δ rad26Δ* and *rad16Δ rad26Δ spt4Δ* cells enhances UV sensitivity. Saturated cultures of yeast strains were sequentially 10-fold diluted and spotted onto YPD plates. When the spots had dried, the plates were irradiated with the indicated doses of 254 nm UV light. The plates were incubated at 30°C for 3-4 days in the dark prior to being photographed.

4.3.7 Paf1C Facilitates GGR, Especially in Inter-nucleosomal Linker Regions

To determine if Paf1C plays a role in GGR, we measured repair of UV-induced CPDs in the NTS of the *RPB2* gene. In principle, NER in either strand of a repressed gene may also reflect GGR. However, "noise" transcription, which commonly occurs in both strands of repressed genes in eukaryotic cells (Struhl 2007), may be able to initiate a certain level of TCR, which can be confused with GGR (Li, Ding et al. 2007). Indeed, apparent NER which is dependent on Rad26 still occurs in all repressed genes (e.g., *GAL1-10*, *PHO5* and *ADH2*) tested in GGR-deficient *rad16Δ* cells (Li, Ding et al. 2007). The "noise" transcripts cannot be detected by traditional ways as they are rapidly degraded after being produced in the cell (Struhl 2007).

Active transcription from the TS of a gene may prevent "noise" transcription from the NTS. Therefore, NER in the NTS of an actively transcribed gene may reflect GGR better than that in either strand of a repressed gene. We have found that NER in the NTS of the *RPB2* gene is absolutely dependent on the GGR-specific factors Rad7, Rad16 and Elc1, and thus appears to exclusively reflect GGR (Li, Ding et al. 2007; Lejeune, Chen et al. 2009).

In wild type cells, CPDs were repaired at different rates at different sites in the NTS of the *RPB2* gene (Figures 4-8A and 4-9). The repair rates generally correlated with nucleosome positioning, being slowest in the central regions of nucleosomal core DNA and fastest in the inter-nucleosomal linker regions (Figures 4-8A and 4-9). This indicates that nucleosome structure inhibits GGR, in agreement with previous reports [e.g., (Wellinger and Thoma 1997; Li and Smerdon 2004)]. In cells lacking a Paf1C component, GGR was still apparent but significantly compromised (Figure 4-8, compare panel A with panels B and C; Figure 4-9). Indeed, in the inter-nucleosomal linker regions, the repair was about two times slower in Paf1C-eliminated cells than in wild type cells (Figures 4-8 and 4-9). We noticed, however, the difference of the repair speeds between Paf1C-eliminated and wild type cells in nucleosomal core regions was not as dramatic as in inter-nucleosomal linker regions, which could be due to the fact that GGR was quite slow in nucleosomal core DNA even in wild-type cells (Figures 4-8 and 4-9). Taken together, our results indicate that Paf1C facilitates GGR, especially in inter-nucleosomal linker regions.

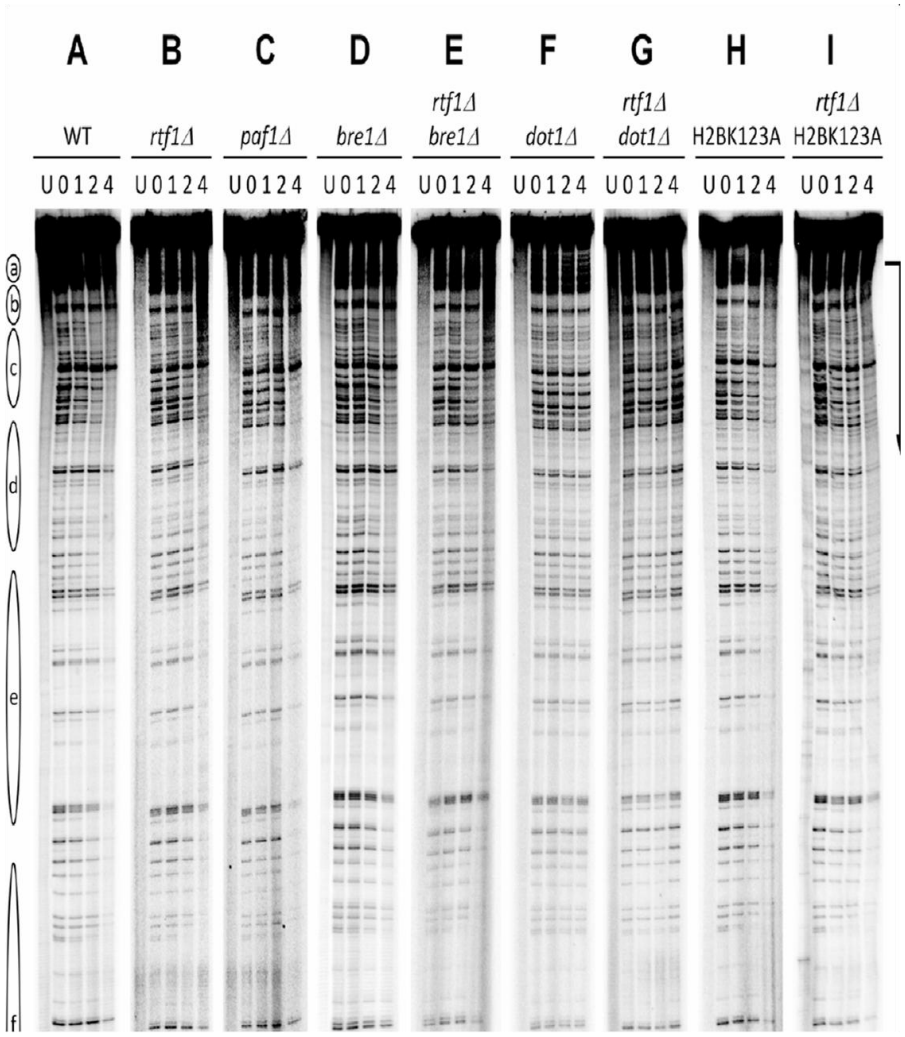

Figure 4-8. Gels showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT) and different mutant cells. The lanes are DNA samples from unirradiated (U) and UV irradiated cells following different times (hrs) of repair incubation. Ovals on the left represent positioned nucleosomes. Numbers on the left indicate nucleotide positions (relative to the transcription start site) at the centers of nucleosome linker regions, which is based on the systematic reference map of nucleosome positions across yeast genome (Jiang and Pugh 2009). The arrow on the right indicates the transcription start site.

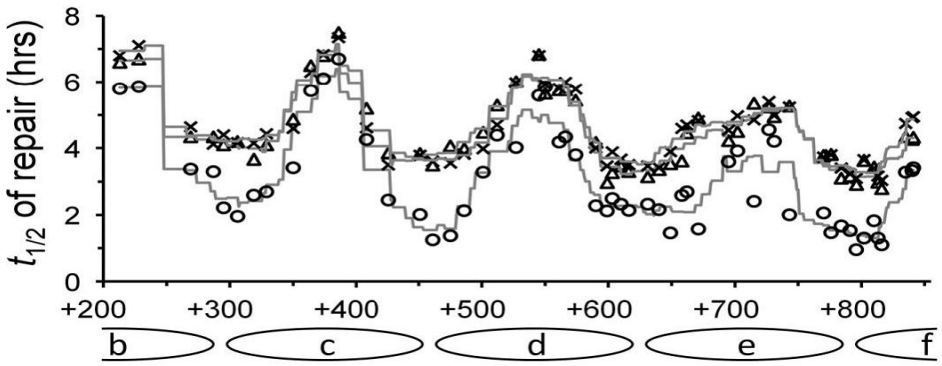

Figure 4-9. Plot showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT) and different mutant cells. The times (hrs) required for repairing 50% ($t_{1/2}$) of CPDs at individual sites along the NTS of the *RPB2* gene in WT (circles), *rtf1Δ* (triangles) and *rtf1Δ bre1Δ* (crosses) cells are plotted. The $t_{1/2}$ values > 4 h were obtained by extrapolation of regression of the data obtained from 0, 1, 2 and 4 h of repair. The gray lines are smoothed $t_{1/2}$ values for each of the mutant strains, which were carried out by averaging the individual $t_{1/2}$ values at continuous intervals of 40 nt where the 40-nt brackets were ramped along the DNA by 1 nt. Ovals at the bottom represent nucleosome positions along the *RPB2* gene region analyzed. Nucleotide positions are numbered from the transcription start site of the gene.

4.3.8 Paf1C Facilitates GGR Through Enabling Monoubiquitination of H2BK123 by Bre1, Which in Turn Permits Di- and Tri-methylation of H3K79 by Dot1

Paf1C is required for monoubiquitination of H2BK123 by Bre1 (an E3 ubiquitin ligase) in complex with Rad6 (an E2 ubiquitin conjugase) (Krogan, Dover et al. 2003; Ng, Dole et al. 2003; Wood, Schneider et al. 2003). This function of Paf1C appears to be achieved by direct and selective interaction with Bre1 (Kim and Roeder 2009). H2BK123 monoubiquitination is in turn partially required for di-methylation and absolutely required for tri-methylation of H3K79 by Dot1 (Shahbazian, Zhang et al. 2005; Nakanishi, Lee et al. 2009; Levesque, Leung et al. 2010). However, H2BK123 ubiquitination does not affect mono-methylation of H3K79 by Dot1. Therefore, Paf1C indirectly enables the di- and tri-methylation of H3K79, but not the mono-methylation of H3K79.

We recently found that Dot1 mediates GGR by methylating H3K79 (Tatum and Li 2011). We also found that Bre1 and H2BK123 ubiquitination are partially required for GGR, indicating

that 1) tri-methylation of H3K79 may contribute to but is not absolutely required for GGR, and 2) lower levels of methylation (mono- and di-methylation) on the K79 also promote GGR (Tatum and Li 2011).

Similar to deletion of a Paf1C gene, deletion of *BRE1* significantly decreased GGR rates, especially in the inter-nucleosomal linker regions (Figure 4-8, compare panels B-D; Figure 4-10A). Cells with both *BRE1* and a Paf1C gene deleted showed similar GGR to those deleted with either of the genes (Figure 4-8, compare panel E with B and D; Figures 4-9 and 4-10A). In agreement with our previous results (Tatum and Li 2011), deletion of *DOT1* abolished GGR (Figure 4-8F). Deletion of a Paf1 gene did not affect GGR in *dot1Δ* cells (Figure 4-8F and G). These results indicate that Paf1C may facilitate GGR trough the same pathway as Bre1 and Dot1.

Similar to *bre1Δ* cells, cells exclusively expressing the H2BK123A mutant histone showed compromised GGR (Figures 4-8H and 4-10B). Deletion of a Paf1C gene in the H2BK123A mutant cells did not significantly affect GGR (Figure 4-8H and I; Figure 4-10B). Also, similar to *dot1Δ* cells, cells exclusively expressing the H3K79A mutant histone were completely defective in GGR (Figure 4-10B; gels not shown). Deletion of a Paf1C gene in the H3K79A mutant cells did not affect GGR (Figure 4-10B).

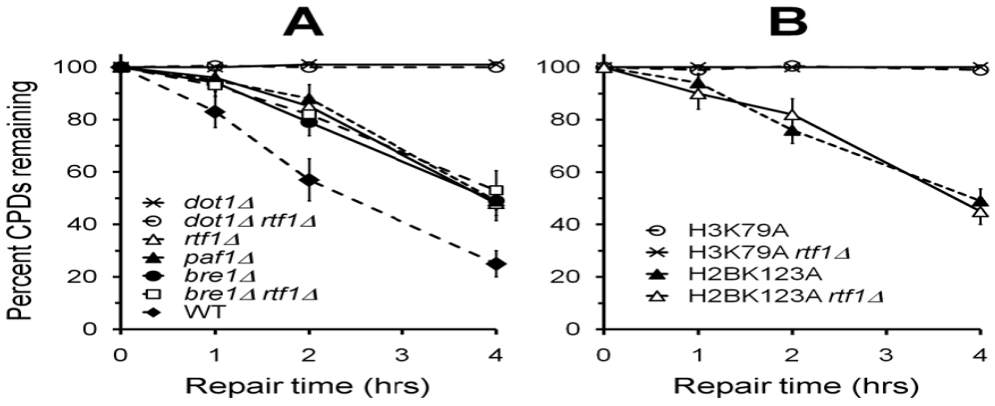

Figure 4-10. Plot showing repair of CPDs in the NTS of the *RPB2* gene in wild type (WT) and different mutant cells. The values shown are means (± S.E.) of percent CPDs remaining at individual sites in the NTS of the *RPB2* gene at different repair times.

The UV sensitivity of *rft1Δ* cells is similar to that of *bre1Δ* or *dot1Δ* cells, being about 10 times more sensitive than wild-type cells (Figure 4-11). Although Dot1 is required and Bre1 is partially required for GGR, *dot1Δ* cells are not significantly more UV sensitive than *bre1Δ* cells (Figure 4-11), reflecting the fact that the UV sensitivity may not always faithfully reflect repair capacity. Indeed, previous studies have shown that elimination of the GGR-specific factor Elc1 (Lejeune, Chen et al. 2009) or the TCR-specific factor Rad26 (van Gool, Verhage et al. 1994; Li and Smerdon 2002) in otherwise wild type cells does not cause any detectable UV sensitivity. Combined deletions of *RTF1* with either *BRE1* or *DOT1* did not result in additional UV sensitivity relative to the single mutants (Figure 4-11), indicating that *RTF1* is epistatic to *BRE1* and *DOT1*.

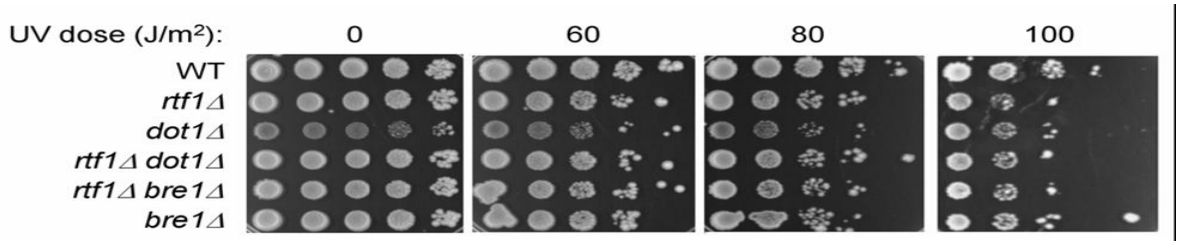

Figure 4-11. *RTF1* is epistatic to *DOT1* and *BRE1*. Saturated cultures of yeast strains were sequentially 10-fold diluted and spotted onto YPD plates. When the spots had dried, the plates were irradiated with the indicated doses of 254 nm UV light. The plates were incubated at 30°C for 3-4 days in the dark prior to being photographed.

Taken together, our results indicate that Paf1C facilitates GGR through enabling monoubiquitination of H2BK123 by Bre1, which in turn permits di- and tri-methylation of H3K79 by Dot1.

4.4 Discussion

As different NER pathways/subpathways can be superimposed in the same gene, it can be challenging to dissect the multiple functions of a factor in the different NER pathways/subpathways, especially if the factor acts positively in one NER pathway/subpathway

but negatively in another. We have successfully dissected the different functions of Paf1C in the different NER pathways/subpathways. This successful dissection can be attributed to two technological aspects. First, genes that are specifically required for different NER pathways/subpathways have been identified and it is possible to create desirable combinations of deletions of these genes in haploid yeast cells. Second, nucleotide-level NER analysis methods are available, which allow for unambiguous comparison of the repair rates among different sites/regions in the same DNA fragment. To the best of our knowledge, among the NER-modulating factors documented so far, Paf1C has the most diverse functions in the different NER pathways/subpathways.

How Is the Minor Role of Paf1C in Facilitating Rad26-dependent TCR Achieved?

We found that Paf1C plays a minor role in facilitating Rad26-dependent TCR. It is unlikely that the minor role is manifested by promoting expression of common NER factor(s) that is/are shared by the TCR and GGR machineries. Indeed, Paf1C also facilitates GGR, especially in inter-nucleosomal linker regions (see more discussion below). However, the facilitation of GGR by Paf1C is achieved by enabling ubiquitination of H2BK123 by Bre1 and di- and tri-methylation of H3K79 by Dot1, and these histone modifications have been shown to play no role in TCR (including Rad26-dependent TCR) (Tatum and Li 2011). Also, genome-wide gene expression analyses have shown that Paf1C affects transcription of a small subset of yeast genes, among which are many cell wall biosynthetic genes and a subset of cell cycle-regulated genes, but no NER genes (Chang, French-Cornay et al. 1999; Porter, Washburn et al. 2002).

It has been shown that, in the absence of DNA damage, Rad26 associates with the coding sequence of a gene in a transcription-dependent manner (Malik, Chaurasia et al. 2009). Induction of DNA damage does not appear to cause more recruitment of Rad26 to an actively transcribed

gene. It is therefore likely that Rad26 is intrinsically associated with Pol II, thereby 'priming' the transcription machinery competent for TCR. The minor role of Paf1C in facilitating Rad26-dependent TCR may be achieved by enhancing the "priming". We found that a small amount of Rad26 indeed coimmunoprecipitates with Pol II. However, the coimmunoprecipitation is not affected by the presence of Paf1C and the association of Paf1C with Pol II is not affected by the presence of Rad26 (not shown). Therefore, if and/or how Paf1C enhances the priming remains to be determined. Rad26 plays a role in transcription elongation (Lee, Yu et al. 2001). An alternative possibility is that Paf1C and Rad26 may cooperate to a certain extent to promote transcription elongation, which in turn facilitates TCR.

How Does Paf1C Suppress Rad26-independent TCR?

Pol II is a globular enzyme with a deep central cleft (Armache, Kettenberger et al. 2003; Bushnell and Kornberg 2003). The DNA template enters and travels along the base of this cleft to the active site. On one side of the cleft is a flexible clamp structure. Binding of the Rpb4-Rpb7 subcomplex to the 10-subunit core Pol II "pushes" the clamp to the closed position (Armache, Kettenberger et al. 2003; Bushnell and Kornberg 2003). RNA polymerases (Cramer 2002) and Spt4/Spt5 (Ponting 2002) from all three kingdoms of life (bacteria, archaea, and eukaryotes) are conserved. The Spt4-Spt5 complex has not been co-crystallized with an intact RNA polymerase (Martinez-Rucobo, Sainsbury et al. 2011). However, an archaeal Spt4-Spt5 has been co-crystallized with the clamp domain of an archaeal RNA polymerase (Martinez-Rucobo, Sainsbury et al. 2011). Based on the archaeal co-crystal structure, a model of the complete yeast Pol II-Spt4-Spt5 elongation complex has been proposed. This model suggests that the NGN domain of Spt5 binds to the Pol II clamp and closes the central cleft to lock nucleic acids and render the elongation complex stable and processive. Spt4 binds to the other side of the NGN

domain of Spt5 and points away from the Pol II surface. The KOW1 domain of Spt5 may contact DNA and/or exiting RNA, and such contacts could contribute to Pol II elongation complex stability and may also involve the Rpb4-Rpb7 subcomplex. The locations of other domains(KOW2-4 and the CTR) of Spt5 are currently unpredictable (Martinez-Rucobo, Sainsbury et al. 2011). How Paf1C interacts with Pol II is presently unknown. However, the interaction may be at least partially through Sp4-Spt5. Paf1C and Spt5 have been shown to have extensive genetic and physical interactions (Squazzo, Costa et al. 2002). Furthermore, optimal association of Paf1C with Pol II is dependent on Spt4 (Qiu, Hu et al. 2006) and the Spt5 CTR (Fig. 6).

Structure-function analyses of Pol II elongation complexes containing a T-T CPD in the TS showed that the CPD slowly passes a translocation barrier and enters the Pol II active site. The 5'-T of the CPD then directs uridine misincorporation into the elongating mRNA, which stalls the translocation of Pol II (Brueckner and Cramer 2007). Our results indicate that both Paf1C and the Spt4-Spt5 complex are required for suppressing Rad26-independent TCR and the two complexes may exert the suppression through a common pathway. It is therefore likely that, in the absence of Rad26, a lesion is 'locked' in the active center of a Pol II elongation complex, which is stabilized by the coordinated interactions of Spt4-Spt5, Rpb4-Rpb7 and Paf1C with each other and with the core Pol II. Elimination of any of these factors may destabilize the Pol II elongation complex, making it possible for TCR to take place. The role of Rad26 in TCR may be achieved indirectly by destabilizing the Pol II elongation complex, as in the absence of any of these TCR suppressors Rad26 is dispensable for TCR. This model may explain why Spt4-Spt5, Rpb4-Rpb7 and Paf1C suppress TCR only in the absence of Rad26. However, it remains to be

understood how Rad26 destabilizes the Pol II elongation complex and how TCR takes place in the absence of both Rad26 and the TCR suppressors.

Although Paf1C and Spt4-Spt5 may suppress Rad26-independent TCR through a common pathway, Paf1C appears to be more "peripheral" for the suppression. First, TCR is somewhat slower in *rad16Δ rad26Δ* cells lacking a Paf1C component than in *rad16Δ rad26Δ spt4Δ* cells (Figs. 4 and 5). Second, elimination of Spt4 restores TCR not only in cells lacking Rad26, but also, to a certain extent, in cells lacking both Rad26 and Rpb9 [(Li, Ding et al. 2006 and data not shown)]. Therefore, both Rad26 and Rpb9 appear to facilitate TCR indirectly rather than by directly recruiting NER factors. However, elimination of a Paf1C component restores TCR only in cells lacking Rad26, but not in cells lacking both Rad26 and Rpb9 (Figs. 4 and 5). The Spt4-Spt5 complex appears to interact with Pol II more directly and more tightly than Paf1C, as the association of Paf1C with Pol II is dependent on Spt4 and the Spt5 CTR whereas the association of Spt4-Spt5 with Pol II is not affected by Paf1C [(Qiu, Hu et al. 2006); this study and data not shown]. Therefore, elimination of Spt4 may cause more destabilization of the Pol II elongation complex than Paf1C, thereby allowing a higher extent of restoration of TCR in *rad26Δ* and *rad26Δ rpb9Δ* cells.

How Does Paf1C Facilitate GGR?

Our results indicate that Paf1C facilitates GGR through enabling ubiquitination of H2BK123 by Bre1 and di- and tri-methylation of H3K79 by Dot1. Paf1C is a transcription elongation factor and travels along with Pol II. How can Paf1C facilitate GGR in inactive genes or intergenic regions? In fact, although Paf1C is essential for H2BK123 ubiquitination, which is in turn the prerequisite for di- and tri-methylation of H3K79 by Dot1, these histone modifications are not specifically limited to the transcribed regions of the genome. Indeed, ~

90% of all histone H3 are methylated on K79, and the relative levels of H3K79 methylation have little correlation with the transcriptional activity of a gene (van Leeuwen, Gafken et al. 2002; Ng, Ciccone et al. 2003; Pokholok, Harbison et al. 2005). Also, the levels of H2BK123 ubiquitination do not seem to be correlated with the transcriptional activity of a gene (Schulze, Jackson et al. 2009). The wide-spread feature of these histone modifications make them ideal for mediating GGR. It is possible that a fraction of Paf1C that is not associated with Pol II is able to promote the histone modifications. There is evidence that Paf1C has functions independent of Pol II (Mueller, Porter et al. 2004). As discussed above, Spt4 and the Spt5 CTR are important for the association of Paf1C with Pol II. However, unlike elimination of a Paf1C component, elimination of Spt4 or the Spt5 CTR does not cause any defect in GGR. This supports the notion that Paf1C may enable the histone modifications and facilitate GGR independently of Pol II. However, it remains to be elucidated how methylation of H3K79 is engaged in GGR.

Epistatic Interactions of Paf1C with NER-modulating Factors

Although it plays a minor role in facilitating Rad26-dependent TCR, suppresses Rad26-independent (Rpb9-dependent) TCR along with Spt4-Spt5, and facilitates GGR, Paf1C is not epistatic to Rad26, Rpb9, Spt4 or Rad16 in terms of UV resistance (Figures 4-3 and 4-7). Therefore, besides modulating different NER pathways or subpathways, Paf1C must have an additional function that confers the cells with additional UV resistance. The additional function is likely the activation of DNA damage checkpoints, which enhances the resistance of cells to DNA damage (Harrison and Haber 2006). Cells lacking Paf1C, Dot1 and Bre1 have been shown to have similar defect in activation of DNA damage checkpoints (Giannattasio, Lazzaro et al. 2005; Wysocki, Javaheri et al. 2005), implying that the roles of these factors in checkpoint activation are mostly manifested through their common ability to directly or indirectly permit di-

and tri-methylation of H3K79. Therefore, the epistatic relationship among Paf1C, Dot1 and Bre1 can be explained by their common effects on di- and tri-methylation of H3K79, which play roles not only in GGR but also in checkpoint activation.

4.5 References

Armache, K. J., H. Kettenberger, et al. (2003). "Architecture of initiation-competent 12-subunit RNA polymerase II." Proc Natl Acad Sci U S A **100**(12): 6964-8.

Brueckner, F. and P. Cramer (2007). "DNA photodamage recognition by RNA polymerase II." FEBS Lett **581**(15): 2757-2760.

Bushnell, D. A. and R. D. Kornberg (2003). "Complete, 12-subunit RNA polymerase II at 4.1-A resolution: implications for the initiation of transcription." Proc Natl Acad Sci U S A **100**(12): 6969-73.

Chang, M., D. French-Cornay, et al. (1999). "A complex containing RNA polymerase II, Paf1p, Cdc73p, Hpr1p, and Ccr4p plays a role in protein kinase C signaling." Mol Cell Biol **19**(2): 1056-67.

Chen, X., C. Ruggiero, et al. (2007). "Yeast Rpb9 plays an important role in ubiquitylation and degradation of Rpb1 in response to UV-induced DNA damage." Mol Cell Biol **27**(13): 4617-25.

Cramer, P. (2002). "Multisubunit RNA polymerases." Curr Opin Struct Biol **12**(1): 89-97.

Ding, B., D. LeJeune, et al. (2010). "The C-terminal repeat domain of Spt5 plays an important role in suppression of Rad26-independent transcription coupled repair." J Biol Chem **285**(8): 5317-26.

Friedberg, E. C., G. C. Walker, et al. (2006). DNA Repair and Mutagenesis. Washington D.C., ASM Press.

Gelbart, M. E., T. Rechsteiner, et al. (2001). "Interactions of Isw2 chromatin remodeling complex with nucleosomal arrays: analyses using recombinant yeast histones and immobilized templates." Mol Cell Biol **21**(6): 2098-106.

Giannattasio, M., F. Lazzaro, et al. (2005). "The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1 and H3 methylation by Dot1." J Biol Chem **280**(11): 9879-86.

Gillette, T. G., S. Yu, et al. (2006). "Distinct functions of the ubiquitin-proteasome pathway influence nucleotide excision repair." Embo J **25**(11): 2529-38.

Gong, F., D. Fahy, et al. (2006). "Rad4-Rad23 interaction with SWI/SNF links ATP-dependent chromatin remodeling with nucleotide excision repair." Nat Struct Mol Biol **13**(10): 902-7.

Guzder, S. N., Y. Habraken, et al. (1996). "RAD26, the yeast homolog of human Cockayne's syndrome group B gene, encodes a DNA-dependent ATPase." J Biol Chem **271**(31): 18314-7.

Guzder, S. N., P. Sung, et al. (1998). "The DNA-dependent ATPase activity of yeast nucleotide excision repair factor 4 and its role in DNA damage recognition." J Biol Chem **273**(11): 6292-6.

Hanawalt, P. C. and G. Spivak (2008). "Transcription-coupled DNA repair: two decades of progress and surprises." Nat Rev Mol Cell Biol **9**(12): 958-70.

Harrison, J. C. and J. E. Haber (2006). "Surviving the breakup: the DNA damage checkpoint." Annu Rev Genet **40**: 209-35.

Hartzog, G. A., T. Wada, et al. (1998). "Evidence that Spt4, Spt5, and Spt6 control transcription elongation by RNA polymerase II in Saccharomyces cerevisiae." Genes Dev **12**(3): 357-69.

Jaehning, J. A. (2010). "The Paf1 complex: platform or player in RNA polymerase II transcription?" Biochim Biophys Acta **1799**(5-6): 379-88.

Jansen, L. E., H. den Dulk, et al. (2000). "Spt4 modulates Rad26 requirement in transcription-coupled nucleotide excision repair." Embo J **19**(23): 6498-507.

Jiang, C. and B. F. Pugh (2009). "A compiled and systematic reference map of nucleosome positions across the Saccharomyces cerevisiae genome." Genome Biol **10**(10): R109.

Kim, J. and R. G. Roeder (2009). "Direct Bre1-Paf1 complex interactions and RING finger-independent Bre1-Rad6 interactions mediate histone H2B ubiquitylation in yeast." J Biol Chem **284**(31): 20582-92.

Krogan, N. J., J. Dover, et al. (2003). "The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation." Mol Cell **11**(3): 721-9.

Lee, S. K., S. L. Yu, et al. (2001). "Requirement for yeast RAD26, a homolog of the human CSB gene, in elongation by RNA polymerase II." Mol Cell Biol **21**(24): 8651-6.

Lejeune, D., X. Chen, et al. (2009). "Yeast Elc1 plays an important role in global genomic repair but not in transcription coupled repair." DNA Repair (Amst) **8**(1): 40-50.

Levesque, N., G. P. Leung, et al. (2010). "Loss of H3 K79 trimethylation leads to suppression of Rtt107-dependent DNA damage sensitivity through the translesion synthesis pathway." J Biol Chem **285**(45): 35113-22.

Li, S., B. Ding, et al. (2006). "Evidence that the transcription elongation function of Rpb9 is involved in transcription-coupled DNA repair in Saccharomyces cerevisiae." Mol Cell Biol **26**(24): 9430-41.

Li, S., B. Ding, et al. (2007). "The roles of Rad16 and Rad26 in repairing repressed and actively transcribed genes in yeast." DNA Repair (Amst) **6**(11): 1596-606.

Li, S. and M. J. Smerdon (2002). "Rpb4 and Rpb9 mediate subpathways of transcription-coupled DNA repair in Saccharomyces cerevisiae." Embo J **21**(21): 5921-9.

Li, S. and M. J. Smerdon (2004). "Dissecting transcription-coupled and global genomic repair in the chromatin of yeast GAL1-10 genes." J Biol Chem **279**(14): 14418-26.

Li, S. and R. Waters (1996). "Nucleotide level detection of cyclobutane pyrimidine dimers using oligonucleotides and magnetic beads to facilitate labelling of DNA fragments incised at the dimers and chemical sequencing reference ladders." Carcinogenesis **17**(8): 1549-52.

Li, S. and R. Waters (1997). "Induction and repair of cyclobutane pyrimidine dimers in the Escherichia coli tRNA gene tyrT: Fis protein affects dimer induction in the control region and suppresses preferential repair in the coding region of the transcribed strand, except in a short region near the transcription start site." J Mol Biol **271**(1): 31-46.

Li, S., R. Waters, et al. (2000). "Low- and high-resolution mapping of DNA damage at specific sites." Methods **22**(2): 170-9.

Lloyd, R. S. (2005). "Investigations of pyrimidine dimer glycosylases--a paradigm for DNA base excision repair enzymology." Mutat Res **577**(1-2): 77-91.

Luger, K., A. W. Mader, et al. (1997). "Crystal structure of the nucleosome core particle at 2.8 A resolution." Nature **389**(6648): 251-60.

Malik, S., P. Chaurasia, et al. (2009). "Rad26p, a transcription-coupled repair factor, is recruited to the site of DNA lesion in an elongating RNA polymerase II-dependent manner in vivo." Nucleic Acids Res **38**(5): 1461-77.

Martinez-Rucobo, F. W., S. Sainsbury, et al. (2011). "Architecture of the RNA polymerase-Spt4/5 complex and basis of universal transcription processivity." Embo J **30**(7): 1302-10.

Mueller, C. L., S. E. Porter, et al. (2004). "The Paf1 complex has functions independent of actively transcribing RNA polymerase II." Mol Cell **14**(4): 447-56.

Nakanishi, S., J. S. Lee, et al. (2009). "Histone H2BK123 monoubiquitination is the critical determinant for H3K4 and H3K79 trimethylation by COMPASS and Dot1." J Cell Biol **186**(3): 371-7.

Nakanishi, S., B. W. Sanderson, et al. (2008). "A comprehensive library of histone mutants identifies nucleosomal residues required for H3K4 methylation." Nat Struct Mol Biol **15**(8): 881-8.

Ng, H. H., D. N. Ciccone, et al. (2003). "Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation." Proc Natl Acad Sci U S A **100**(4): 1820-5.

Ng, H. H., S. Dole, et al. (2003). "The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B." J Biol Chem **278**(36): 33625-8.

Pokholok, D. K., C. T. Harbison, et al. (2005). "Genome-wide map of nucleosome acetylation and methylation in yeast." Cell **122**(4): 517-27.

Ponting, C. P. (2002). "Novel domains and orthologues of eukaryotic transcription elongation factors." Nucleic Acids Res **30**(17): 3643-52.

Porter, S. E., T. M. Washburn, et al. (2002). "The yeast pafl-rNA polymerase II complex is required for full expression of a subset of cell cycle-regulated genes." Eukaryot Cell **1**(5): 830-42.

Qiu, H., C. Hu, et al. (2006). "The Spt4p subunit of yeast DSIF stimulates association of the Paf1 complex with elongating RNA polymerase II." Mol Cell Biol **26**(8): 3135-48.

Ribar, B., L. Prakash, et al. (2007). "ELA1 and CUL3 are required along with ELC1 for RNA polymerase II polyubiquitylation and degradation in DNA-damaged yeast cells." Mol Cell Biol **27**(8): 3211-6.

Schulze, J. M., J. Jackson, et al. (2009). "Linking cell cycle to histone modifications: SBF and H2B monoubiquitination machinery and cell-cycle regulation of H3K79 dimethylation." Mol Cell **35**(5): 626-41.

Shahbazian, M. D., K. Zhang, et al. (2005). "Histone H2B ubiquitylation controls processive methylation but not monomethylation by Dot1 and Set1." Mol Cell **19**(2): 271-7.

Squazzo, S. L., P. J. Costa, et al. (2002). "The Paf1 complex physically and functionally associates with transcription elongation factors in vivo." Embo J **21**(7): 1764-74.

Struhl, K. (2007). "Transcriptional noise and the fidelity of initiation by RNA polymerase II." Nat Struct Mol Biol **14**(2): 103-5.

Swanson, M. S., E. A. Malone, et al. (1991). "SPT5, an essential gene important for normal transcription in Saccharomyces cerevisiae, encodes an acidic nuclear protein with a carboxy-terminal repeat." Mol Cell Biol **11**(6): 3009-19.

Tatum, D. and S. Li (2011). "Evidence that the histone methyltransferase DOT1 mediates global genomic repair by methylating histone H3 on lysine 79." J Biol Chem **in press**.

Teng, Y., H. Liu, et al. (2008). "Saccharomyces cerevisiae Rad16 mediates ultraviolet-dependent histone H3 acetylation required for efficient global genome nucleotide-excision repair." EMBO Rep **9**(1): 97-102.

Thompson, N. E., T. H. Steinberg, et al. (1989). "Inhibition of in vivo and in vitro transcription by monoclonal antibodies prepared against wheat germ RNA polymerase II that react with the heptapeptide repeat of eukaryotic RNA polymerase II." J Biol Chem **264**(19): 11511-20.

van Gool, A. J., R. Verhage, et al. (1994). "RAD26, the functional S. cerevisiae homolog of the Cockayne syndrome B gene ERCC6." Embo J **13**(22): 5361-9.

van Leeuwen, F., P. R. Gafken, et al. (2002). "Dot1p modulates silencing in yeast by methylation of the nucleosome core." Cell **109**(6): 745-56.

Verhage, R., A. M. Zeeman, et al. (1994). "The RAD7 and RAD16 genes, which are essential for pyrimidine dimer removal from the silent mating type loci, are also required for repair of the nontranscribed strand of an active gene in Saccharomyces cerevisiae." Mol Cell Biol **14**(9): 6135-42.

Verhage, R. A., A. J. van Gool, et al. (1996). "Double mutants of Saccharomyces cerevisiae with alterations in global genome and transcription-coupled repair." Mol Cell Biol **16**(2): 496-502.

Waters, R., Y. Teng, et al. (2009). "Tilting at windmills? The nucleotide excision repair of chromosomal DNA." DNA Repair (Amst) **8**(2): 146-52.

Wellinger, R. E. and F. Thoma (1997). "Nucleosome structure and positioning modulate nucleotide excision repair in the non-transcribed strand of an active gene." Embo J **16**(16): 5046-56.

White, C. L., R. K. Suto, et al. (2001). "Structure of the yeast nucleosome core particle reveals fundamental changes in internucleosome interactions." Embo J **20**(18): 5207-18.

Wood, A., N. J. Krogan, et al. (2003). "Bre1, an E3 ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter." Mol Cell **11**(1): 267-74.

Wood, A., J. Schneider, et al. (2003). "The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p." J Biol Chem **278**(37): 34739-42.

Wysocki, R., A. Javaheri, et al. (2005). "Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9." Mol Cell Biol **25**(19): 8430-43.

Yu, Y., Y. Teng, et al. (2005). "UV irradiation stimulates histone acetylation and chromatin remodeling at a repressed yeast locus." Proc Natl Acad Sci U S A **102**(24): 8650-5.

CHAPTER 5

CONCLUDING REMARKS

5.1 Research Summary

Using the budding yeast *Saccharomyces cerevisiae* as a model organism, this dissertation has focused on identifying and characterizing novel core and accessory repair factors in the eukaryotic nucleotide excision repair pathway. The major findings of this body of work are as follows:

1. Yeast Elc1, the homolog of mammalian elongin C, is required for GGR, but has no detectable role in TCR. The role of Elc1 in GGR is not subsidiary to that of Rad16 or Rad7.
2. Dot1 and methylation of histone H3K79, the sole substrate of Dot1, are required for GGR.
3. Bre1 and Bre1-mediated monoubiquitination of histone H2BK123 are partially required for GGR.
4. The transcription elongation complex PAF plays different roles in different subpathways of NER. These diverse roles are as follows:
    a. PAF facilitates GGR through enabling monoubiquitination of H2BK123 by Bre1, which in turn permits di- and tri-methylation of Dot1.
    b. PAF facilitates Rad26-dependent TCR through an as-yet-unidentified mechanism.
    c. PAF suppresses Rad26-independent TCR. This suppression is dependent upon, but not subsidiary to, the suppression role of Spt5.

5.2 Concluding Remarks and Future Direction

Although most, if not all, core NER factors have been identified and extensively characterized, new accessory factors which modulate GGR and/or TCR are continuously being identified. It is not only important to identify these factors, but also to uncover the role they play (i.e. facilitation or suppression), how they exact their functions, and the interactions they have with other repair proteins in order to gain a more holistic understanding of the repair process. Furthermore, current understanding of NER in living yeast cells is limited to either genome-overall-level or to certain very limited regions of the genome. The detailed DNA damage distribution and NER kinetics in the vast majority of the genomes are still virtually unknown. This illustrates the need for a high-resolution, genome-wide assessment of damage, repair, and repair kinetics. Only then will we be able to paint a complete picture and have full understanding of this repair mechanism that has thus far proven elusive.

In bacteria, most NER enzymes are induced by the SOS response after DNA damage (Janion 2008), but this does not seem to be the case in higher organisms. By and large, NER in eukaryotes seems to be modulated by posttranslational modifications and protein-protein interactions, not translational induction of genes encoding repair factors (Nouspikel 2011). This seems intuitive, as DNA damage (such as CPDs) can impede transcription, making it a safer choice to rely on the activation of present enzymes rather than on their damage-induced synthesis. Many posttranslational modifications of histones, including ones not discussed in this dissertation, have been shown to have important functions in NER. These modifications operate in a concerted manner to coordinate a plethora of tasks such as damage signaling, opening/relaxing chromatin to allow repair factors access to damage sites, activating the DNA damage cell cycle checkpoint, facilitating lesion identification, and restoring the chromatin to its

original state once the repair process is complete. Our discovery that H3K79 methylation is required for GGR (Tatum and Li 2011) unveiled a critical link between chromatin modification and the repair process. However, it remains to be understood as to whether the methylation indeed serves as a docking site for the NER machinery or the modification is indirectly involved in GGR.

Though progress has been made in recent years regarding chromatin dynamics in NER, many questions remain unanswered. Many studies attempting to elucidate the roles of histone modifications during NER have focused only on specific histone tail residues or single modifications, yet there may be many other modifications involved in the NER process (Palomera-Sanchez and Zurita 2011). While informative, this provides us with only a narrow glimpse into the cellular response to genomic insult and lacks the broader scope of examining the changes to histones throughout the entire genome in response to DNA damage. This underscores the need for a genome-wide analysis to monitor the responses of the DNA damage-induced histone modifications that occur in all of the chromosomes and how these different modifications crosstalk. Until then, continued efforts to decipher the encrypted code of these modifications will provide a much clearer understanding of the tightly regulated mechanisms of NER and its crosstalk with other processes such as DNA damage-induced checkpoint activation. These future findings could prove to be valuable clinically, as they may be advantageous targets for chemotherapeutics or treatment of other diseases related to genomic instability.

The TCR mechanism in eukaryotic cells remains largely mysterious. The interactions among Pol II, Rad26 and the various known and possibly unknown TCR suppressors remain to be elucidated and are the major key to unlocking this mystery. Determining the exact binding site of Rad26 on Pol II would provide valuable insight into the antagonistic effect of Rad26 on

the suppression of Rad26-independent TCR. Furthermore, Rad26 does not seem to be a true transcription-repair-coupling factor and may facilitate TCR indirectly rather than by directly recruiting NER factors, as in the absence of a suppressor, Rad26 can be entirely dispensable. It is therefore likely that either Pol II is intrinsically proficient in mediating TCR or a true transcription-repair-coupling factor has not been discovered. These different possibilities remain to be addressed.

5.3 References

Janion, C. (2008). "Inducible SOS response system of DNA repair and mutagenesis in Escherichia coli." Int J Biol Sci **4**(6): 338-44.

Nouspikel, T. (2011). "Multiple roles of ubiquitination in the control of nucleotide excision repair." Mech Ageing Dev.

Palomera-Sanchez, Z. and M. Zurita (2011). "Open, repair and close again: chromatin dynamics and the response to UV-induced DNA damage." DNA Repair (Amst) **10**(2): 119-25.

Tatum, D. and S. Li (2011). "Evidence that the histone methyltransferase DOT1 mediates global genomic repair by methylating histone H3 on lysine 79." J Biol Chem **in press**.

APPENDIX: LETTERS OF PERMISSION

ELSEVIER LICENSE
TERMS AND CONDITIONS

May 19, 2011

This is a License Agreement between Danielle M Tatum ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Danielle M Tatum
Customer address	Comparative Biomedical Sciences Baton Rouge, LA 70803
License number	2664920698335
License date	May 09, 2011
Licensed content publisher	Elsevier
Licensed content publication	DNA Repair
Licensed content title	Yeast Elc1 plays an important role in global genomic repair but not in transcription coupled repair
Licensed content author	Danielle LeJeune, Xuefeng Chen, Christine Ruggiero, Shannon Berryhill, Baojin Ding, Shisheng Li
Licensed content date	1 January 2009
Licensed content volume number	8
Licensed content issue number	1
Number of pages	11
Start Page	40
End Page	50
Type of Use	reuse in a thesis/dissertation
Portion	full article

Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	The roles of RNA polymerase II-associated transcription elongation factors in nucleotide excision repair in yeast
Expected completion date	Jun 2011
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD

VITA

Danielle Tatum was born in Morgan City, Louisiana, In October 1978. After attending Berwick High School in Berwick, Louisiana, she attended the University of Louisiana-Lafayette, where she majored in biological sciences and minored in chemistry. In December 2001, Danielle received her Bachelor of Science degree. Danielle relocated to Dallas, Texas, in 2002 where she began working as microbiologist for Silliker, Inc. In 2004, Danielle moved back home to Louisiana and worked as a Research Associate I in the lab of Dr. Arthur Penn at the Louisiana State University School of Veterinary Medicine. In 2005 she was accepted into the doctoral program in the School of Veterinary Medicine's Department of Comparative Biomedical Sciences under the mentorship of Dr. Inder Sehgal where her work focused on investigating mechanisms for prostate cancer metastasis. In 2006, Danielle changed advisors and began a research program investigating DNA repair mechanisms in the laboratory of Dr. Shisheng Li, where she is currently completing her doctoral degree.

PUBLICATIONS

Tatum, D. and Li, S. (2011) "Nucleotide excision repair in yeast" DNA Repair. Storici F, editor. Intech Open Access Publisher, 2011 (*in press*)

Tatum, D., Placer, M., and Li, S. (2011) The RNA polymerase II-associated factor 1 complex plays diverse roles in different subpathways of nucleotide excision repair. J Biol Chem. (*Under review*)

Tatum, D. and Li, S. (2011) Evidence that the histone methyltransferase DOT1 mediates global genomic repair by methylating histone H3 on lysine 79. J Biol Chem 286: 17530-5.

Ding, B., **LeJeune, D**., and Li, S. (2010) The C-terminal repeat of Spt5 plays an important role in suppression of Rad26-independent transcription coupled repair. J Biol Chem 285: 5317-26.

LeJeune, D., Chen, X., Ruggiero, C., Berryhill, S., Ding, B., and Li, S. (2009) Yeast Elc1 plays an important role in global genomic repair but not transcription coupled repair. DNA Repair 8: 40-50.

Chen, X., Ding, B., **LeJeune, D**., and Li, S. (2009) Rpb1 sumoylation in response to UV radiation or transcriptional impairment in yeast. PLoS One *4:e5267.*

Li, S., Ding, B., **LeJeune, D**., Ruggiero, C., Chen, X., and Smerdon, MJ. (2007) The roles of Rad16 and Rad26 in repairing repressed and actively transcribed genes in yeast. *DNA Repair* 6: 1596-606.

LeJeune, D., Hassanuzzaman, H., Pitcock, A., Francis, J., and Sehgal, I. (2006) The superoxide scavenger TEMPOL induces urokinase receptor (uPAR) expression in human prostate cancer cells. *Molecular Cancer* 5: 21.

Pitcock, A., Hassanuzzaman, H., **LeJeune, D**., Guggilam, A., Francis, J., and Sehgal, I. (2006) Increased reactive oxygen species are associated with low density and migration in a metastatic human prostate cancer cell line. *International Journal of Cancer Research* 2: 188-198.