2010

The roles of transcription factors in Nucleotide excision repair in yeast

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THE ROLES OF TRANSCRIPTION FACTORS IN NUCLEOTIDE EXCISION REPAIR IN YEAST

A Dissertation

Submitted to the Graduate Faculty of the
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

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May 2010

ACKNOWLEDGEMENTS

First of all, I would like to show my appreciation to my major professor, Dr. Shisheng Li, for his patient training and financial support, which have allowed me to complete all of the work presented in this dissertation. Thanks for the suggestions on my project, the opportunities to attend international academic conferences, and the help, encouragement and support during my studies. I am also grateful to my graduate advisory committee, Dr. Steven A. Barker, Dr. Shulin Li, and Dr. Henrique Cheng, for their help and instructive discussion in class and during the course of my project. Additionally, I would like to thank Dr. Beilei Ge, the Dean's representative, for her kindness and understanding of my schedule, Dr. George Strain, for his help and encouragement. I want to give special thanks to Dr. Penn for his constructive suggestion and encouragement in these years.

Next, I would like to thank our laboratory members, Ms. Christine Ruggiero, Dr. Xuefeng Chen, Dr. Runqiang Chen, Ms. Danielle Lejeune, and Mr. Wentao Li. I have benefited a lot from our discussion and cooperation. I want to express my gratitude to my family, without their love and support I could not realize this achievement. Finally, I want to thank the faculty, administration, and support staff of the Department of Comparative Biomedical Sciences.

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5-FOA……..…..……..……5-fluoroorotic acid

ADP……..…..……..……..…adenosine diphosphate

ATP……..…..……..……..adenosine triphosphate

BER……..…..……..……base excision repair

CAK……..…..……..…….cdk-activating kinase

CDK……..……..……..…..…cyclin dependent kinase

CHX……..……...……..……cycloheximide

CPDs……..……..….cyclobutane pyrimidine dimers

CS……..……..……. Cockayne syndrome

CSA……..……..…… Cockayne syndrome group A

CSB……..……..……. Cockayne syndrome group B

CTD……..……..……..C-terminal domain

CTR……..……..……C-terminal repeat

DDB……..……..…….DNA damage binding protein

DISF……..……...DRB sensitivity inducing factor

dNTP……..……..…..……deoxynucleoside triphosphate

GG-NER (GGR)……..……..……..…….global genomic nucleotide excision repair

Mfd……..……..……..……mutation frequency decline

NER……..……..…….. nucleotide excision repair

NTS……..……..…….....non-transcribed strand

PCNA…….…….…….…….proliferating cell nuclear antigen

PEI…….……..…..……polyethyleneimine

PSs…….……..progeroid syndromes

RFC……..…….……..…replication factor C

RNPII (Pol II).....……..……..……..…… RNA polymerase II

ROS……..…….……..…….reactive oxygen species

RPA…….…….....…….replication protein A

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

ssDNA……..…….…….……..single-stranded DNA

Swi/Snf…….……...……..Switch/Sucrose nonfermentable

TCA…….....…….……trichloroacetic acid

TC-NER (TCR)……..……transcription coupled nucleotide excision repair

TFIIH……..……..……..transcription factor II H

TLC…….…….….. thin-layer chromatography

TRCF……..……..……..……transcription repair coupling factor

TS……..…..……...……transcribed strand

TTD…….……..…….……Trichothiodystrophy

TTD-A……...……Trichothiodystrophy group A

XP……....……Xeroderma Pigmentosum

XPC……...……..Xeroderma Pigmentosum complementation group C

ABSTRACT

Nucleotide excision repair (NER) is a conserved DNA repair mechanism capable of removing a variety of helix-distorting lesions, such as UV-induced cyclobutane pyrimidine dimers (CPDs). NER can be grouped into two pathways: global genomic NER (GGR), which refers to repair throughout the genome, and transcription coupled NER (TCR), which refers to a repair mechanism that is dedicated to the transcribed strand (TS) of actively transcribed genes. In yeast *S. cerevisiae*, Rad7, Rad16, and Elc1 are specifically required for GGR. TCR is believed to be initiated by RNA polymerase II (Pol II) stalled at a lesion in the TS of a gene. Rad26, the yeast homolog of the human CSB protein, and RPB9, a nonessential subunit of Pol II, play important roles in TCR. However, the exact mechanisms of NER in eukaryotic cells are still elusive.

By using yeast *S. cerevisiae* as a model organism, this dissertation focused on the functional mechanisms of transcription factor Tfb5, transcription elongation factors Spt4 and Spt5, and the putative yeast transcription repair coupling factor (TRCF) Rad26 in NER, especially in TCR pathway. Tfb5, the tenth subunit of the transcription/repair factor TFIIH, is implicated in one group of the human syndrome trichothiodystrophy (TTD). We found that Tfb5 plays different roles in different NER pathways in yeast. Tfb5 is essential for GGR and Rpb9 mediated TCR. However, Tfb5 is partially dispensable for Rad26 mediated TCR, especially in GGR deficient cells. Spt4 and its interacting partner Spt5 cooperatively suppress TCR only in the absence of Rad26, regardless of the presence of Rpb9. The phosphorylation of C-terminal repeat (CTR) domain of Spt5 by the Bur kinase plays an important role in the suppression.

Immunoprecipitation results indicate that Rad26 dynamically associates with Pol II and restrains the binding of Spt4/Spt5 to Pol II. ATPase activity of Rad26 is required for facilitating TCR and for restraining the binding of Spt4/Spt5 to Pol II. Finally, we proposed that Rad26 enhances TCR by restraining the binding of suppressors Spt4/Spt5 to Pol II. These findings provide new insights into the functional mechanisms of Tfb5, Spt4/Spt5 and Rad26 in NER, especially in TCR.

CHAPTER 1

LITERATURE REVIEW
NUCLEOTIDE EXCISION REPAIR: A CONSERVED, VERSATILE AND COMPLICATED DNA REPAIR MECHANISM FROM PROKARYOTIC CELLS TO EUKARYOTIC CELLS

1.1 Introduction

The genomic DNA is constantly under threat from both exogenous (environmental factors such as X-ray and ultraviolet light) (Tessman and Kennedy, 1991) and endogenous (metabolic byproducts such as reactive oxygen species (ROS) and spontaneous hydrolysis) agents (Bjelland and Seeberg, 2003; Henle and Linn, 1997; Lindahl, 1993). The maintenance of DNA integrity in response to DNA damage is critical for cell viability, longevity and general health (Hoeijmakers, 2001; Lombard et al., 2005). Fortunately, various DNA repair mechanisms have evolved to remove different kinds of DNA damage, such as Nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), homologous recombination repair (HR) and so on (Friedberg et al., 2006). NER is one of DNA repair mechanisms capable of removing a wide variety of bulky DNA lesions, such as UV-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts [(6-4)PP] (Hanawalt et al., 1979), and adducts induced by chemotherapeutic agents (e.g. cisplatin) and alkylating agents (e.g. methylmethane sulfonate) (Damia et al., 1996; Moggs et al., 1997; Neher et al.,2010). The distinct characteristic of NER is that the damaged bases are enzymatically excised from the genome as an oligonucleotide fragment, 24-32 nucleotides in mammalian cells (Wood, 1997) and 12-13 nucleotides in *E. coli* (Savery, 2007), rather than as free bases as is the case with BER (Hollis et al., 2001). NER is the most versatile in terms of lesion recognition (Nouspikel, 2009). This repair system deals with the wide class of helix-distorting lesions that interfere with base pairing and generally obstruct transcription and normal replication (Hoeijmakers, 2001). The biological importance of NER for

human health can be clearly manifested by the existence of rare autosomal recessive human disorders, such as xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD), all associated with sensitivity to sunlight (Lehmann, 2003).

NER was found in bacteria in the mid-1960s by Philip Hanawalt and his graduate student David Pettijohn with the observation of non-semiconservative DNA synthesis during the excision of CPDs (Pettijohn and Hanawalt, 1964). Almost at the same time, the phenomena of excision repair of UV radiation-induced DNA damage was also found in mammalian cells (Rasmussen and Painter, 1964). The genomic heterogeneity of NER was observed about twenty years later. NER repairs transcribed strands of transcriptionally active genes faster than it repairs transcriptionally silent DNA. This specialized mode of NER is called transcription-coupled nucleotide excision repair (TC-NER or TCR). This process was first discovered in mammalian cells (Mellon et al., 1987), and then in *Escherichia coli* (Mellon and Hanawalt, 1989) and budding yeast *S. cerevisiae* (Smerdon and Thoma, 1990). The mode of NER which operates on the genome-wide DNA independent of transcription is referred to global genomic nucleotide excision repair (GG-NER or GGR). The difference between TCR and GGR is only in the step involving recognition of the lesion (Hanawalt, 2002). Following the recognition of the damage, these two subpathways share the same mechanisms in the following steps (Fousteri and Mullenders, 2008).

The NER process can be divided into several common steps: damage recognition and verification, dual incision, excision of the oligonucleotide fragment, repair synthesis and ligation (Friedberg et al., 2006). Although NER has been extensively studied for about fifty years, the exact molecular mechanisms remain elusive. This review will focus on the insights into the molecular mechanisms of NER in prokaryotes and eukaryotes and human NER deficiency disorders.

1.2 Nucleotide Excision Repair in Prokaryotic Cells

In the bacterium *E. coli*, both specific recognition of base damage and incision of the affected DNA strand on either side of damaged base are fulfilled by three proteins designated UvrA, UvrB, and UvrC (for "UV radiation") (van de Putte et al., 1965). These three proteins are indispensable for the excision of CPDs in *E. coli*. They interact in a sequential manner, named Uvr(A)BC endonuclease or Uvr(A)BC excinuclease (refer to both incision and excision function), although they are not associated as a stable complex during NER (Friedberg et al., 2006). Subsequently, two other genes, $uvrD^+$ (Kumura et al., 1985) and $polA^+$ (Caron et al., 1985) were found implicated in this process. The product of mfd^+ gene (for "mutation frequency decline") has been identified as a transcription repair coupling factor (TRCF) in *E. coli* (Selby et al., 1991). Protein Mfd is proposed to enhance the rate of repair by displacing stalled RNA Polymerase and the truncated transcript from DNA and recruiting the repair factors to the sites of damage (Selby and Sancar, 1993). The major functions of NER factors in *E.coli* are summarized in Table 1-1.

1.2.1 NER Factors in Prokaryotic Cells

UvrA Protein -- UvrA protein encoded by the $uvrA^+$ gene, which one of a series of coordinately regulated genes is collectively referred to as SOS genes that can be induced by DNA damage agents (Sancar et al., 1982a). UvrA protein consists of 940 amino acids with the molecular mass of 103 kDa. It is a DNA-binding protein with DNA-independent ATPase activity (Selby and Sancar, 1990a). These functional attributes are critical to its function in NER in the base damage recognition step. The amino acid sequence of UvrA protein contains two ATP binding domains, two zinc finger domains and a consensus helix-turn-helix (HtH) motif (Myles and Sancar, 1991).

Table 1-1 NER factors in *E.coli*

Factors	Functions in NER	References
UvrA	Functions as a molecular matchmaker to load UvrB onto damaged DNA in the form of $(UvrA)_2UvrB$ complex.	(Selby and Sancar, 1990a)
UvrB	Specifically recognize UV-induced damage site to form UvrB-DNA pre-incision complex.	(Selby and Sancar, 1990a; Van Houten, 1990)
UvrC	Dual incision: cut both 3' and 5' sides of recognized lesion.	(Singh et al., 2002)
UvrD	Excision: displaces both UvrC and the 12-13 nucleotides fragment containing the lesion.	(Sancar and Sancar, 1988)
Mfd	Transcription repair coupling factor (TRCF): removes stalled RNA polymerase and recruits other NER factors.	(Selby and Sancar, 1993, 1994)
Pol I	Repair synthesis and displaces UvrB protein.	(Caron et al., 1985)
DNA Ligase	Gap filling/ligation	(Burnouf et al., 2000; Lehman, 1974)

Two functional ATP binding domains of UvrA protein are located near the N-terminal and C-terminal regions of the polypeptide (Thiagalingam and Grossman, 1991). These ATP binding domains consist of a consensus amino acid sequence found in many proteins that bind and hydrolyze ATP and/or GTP, suggesting UvrA protein is a member of a superfamily of prokaryotic ATPases (Van Houten, 1990). The binding of ATP to UvrA protein is associated with a conformational change in the protein and UvrA protein becomes a dimer in the form of $1ATP/(UvrA)_2$ complex (Mazur and Grossman, 1991). HtH motif of UvrA protein consists of two α-helices and a short extended amino acid chain between them. This motif is found in hundreds of DNA-binding proteins (Aravind et al., 2005). The more C-terminal helix can fit into the major groove of DNA, thereby facilitating contact between bases and specific amino acid residues throughout the protein (Wang and Grossman, 1993). The zinc finger motifs of UvrA protein are also thought to be involved in DNA binding. The C-terminal 40 amino acids are rich in glycine residues, and this region of UvrA protein may be important for DNA damage recognition (Claassen and Grossman, 1991). The binding of UvrA protein to DNA has been

demonstrated. In the absence of ATP, the protein binds specifically to damaged DNA. However, specificity is increased in the presence of ATP hydrolysis and is only marginally inhibited by ADP (Reardon et al., 1993). Thus, ATP hydrolysis apparently increases the specificity of binding to damaged DNA but lowers the equilibrium binding constant by stimulating dissociation. The dimeric form (possibly ATP-bound) of UvrA protein can bind DNA containing various type of base damage. However, $(UvrA)_2$-DNA complexes are short-lived and dissociate rapidly (Snowden et al., 1990). Further selectivity for the binding of UvrA protein to damaged DNA is achieved by its interaction with another factor, UvrB protein (Friedberg et al., 2006). Recently, the structure of UvrA from *Bacillus stearothermophilus* indicates that the nucleotide-binding sites are formed in an intramolecular fashion and are not at the dimer interface as is typically found in other ABC ATPases (Pakotiprapha et al., 2008), a unique type of ATPase fueling transmembrane movement of a variety of molecules via many different membrane-spanning proteins (Holland and Blight, 1999). UvrA also harbors two unique domains, one of which is required for interaction with UvrB, which is regarded as the UvrB-interaction surface on UvrA (Pakotiprapha et al., 2008).

UvrB Protein -- Like the $uvrA^+$ gene, the $uvrB^+$ gene is also a member of the SOS regulated genes and is inducible by DNA damage (Selby and Sancar, 1990a). The $uvrB^+$ gene encodes a polypeptide of 673 amino acids with a molecular mass of 76 kDa. UvrB protein functions as a monomer and does not bind ATP or double-stranded DNA in isolation (Hsu et al., 1995). However, the purified protein does bind single-stranded DNA (Sancar et al., 1982b). UvrB protein interacts specifically with UvrA protein to form protein-protein and protein-DNA complexes that are important intermediates in the biochemistry of the damage-specific incision of DNA (Friedberg et al., 2006). Crystal structure of full-length UvrB protein from the thermophilic organism *Thermus thermophilus* has been reported. Domains responsible for

interaction between UvrB protein and UvrA and UvrC were identified (Sohi et al., 2000). The six helicase domains identified in UvrB protein are adjacent to the ATP-binding site, suggesting that UvrB protein undergoes motions driven by ATP hydrolysis in the presence of DNA and UvrA protein. Hence, the helicase structure and ATPase activity suggest that UvrB protein could be capable of moving along the DNA searching for damage (Theis et al., 1999). The structure of the complex between the interaction domains of *G. stearothermophilus* UvrA and UvrB has been determined. These domains are necessary and sufficient for full-length UvrA and UvrB to associate and thereby form the DNA damage-sensing complex of bacterial nucleotide excision repair (Pakotiprapha et al., 2009).

$(UvrA)_2UvrB$ complexes have a higher binding affinity for damaged DNA than does UvrA protein alone, indicating that UvrB protein plays crucial roles in the specific recognition of base damage by interacting with UvrA protein and, as a subunit of the $(UvrA)_2UvrB$ complex, with damaged DNA. Subsequently, an UvrB-DNA complex interacts with UvrC protein to generate an endonuclease activity that incises the damaged strand both 5' and 3' to sites of base damage (Friedberg et al., 2006).

UvrC Protein -- UvrC protein is the product of the $uvrC^+$ gene. Like the $uvrA^+$ and $uvrB^+$ genes, $uvrC^+$ is weakly expressed constitutively. Different from the other two *uvr* genes, $uvrC^+$ is not inducible by DNA damage (Forster and Strike, 1985). The $uvrC^+$ gene encodes a polypeptide of 610 amino acids with a calculated size of 66 kDa (Lin and Sancar, 1990).

UvrC protein has a strong affinity for the UvrB-DNA complex. The binding of UvrC protein to specific UvrB-damaged-DNA tertiary complexes is the final step in the assembly of the catalytically active damage-specific UvrABC endonuclease in *E.coli* (Friedberg et al., 2006). When UvrA, UvrB, and UvrC proteins are incubated with damaged DNA in the presence of ATP, specific incision of the DNA occurs. It has been suggested that *E.coli* UvrC protein consists of

two functional entities. The N-terminal half is required for the 3' incision, and the C-terminal half is required for the 5'incision (Van Houten and Snowden, 1993). ATP is absolutely required for 3' incision of the damaged DNA when UvrC protein added to the UvrB-DNA complex, but this triphosphate is not hydrolyzed, suggesting that nucleotide binding is required for a conformational change necessary for damage-specific incision (Singh et al., 2002). The crystal structures of both N-terminal (Truglio et al., 2005) and C-terminal (Karakas et al., 2007) domains of *Thermotoga maritima* UvrC have been determined. The N-terminal catalyzes the 3' incision reaction and shares homology with the catalytic domain of the GIY-YIG family of intron-encoded homing endonucleases. The structure reveals that the N-terminal endonuclease domain of UvrC utilizes a novel one-metal mechanism to cleave the phosphodiester bond (Truglio et al., 2005). The C-terminal contains the catalytic domain responsible for 5' incision and a helix-hairpin-helix (HhH) domain that is implicated in DNA binding (Karakas et al., 2007).

UvrD Protein (DNA Helicase II) -- UvrD protein is encoded by $uvrD^+$ gene, which also belongs to the SOS-regulated gene family and is inducible by DNA damage. Mutants defective in the $uvrD^+$ gene are more sensitive to UV radiation than wide-type cells, but not as sensitive as mutants defective in the $uvrA^+$ gene (Washburn and Kushner, 1991). UvrD protein is a single peptide of 75 kDa, with a DNA-dependent ATPase activity. UvrD protein has been identified as the same protein of a known DNA-dependent ATPase in *E.coli* was previously designated DNA helicase II (Easton and Kushner, 1983).

UvrD protein (DNA helicase II) unwinds duplex DNA with an average "step size" of 4 to 5 bp within duplex regions ranging from 10 to 40 bp. The dimeric form of UvrD protein is functional during DNA unwinding, while UvrD monomer does not support DNA helicase activity (Ali and Lohman, 1997). Following the incision, DNA helicase II displaces the damage-containing oligonucleotide fragments and UvrC protein from the post-incision complex,

leaving a UvrB-gapped-DNA complex in which the UvrB protein presumably protects the single-stranded DNA from nonspecific degradation (Orren et al., 1992). Interestingly, UvrD helicase activity can be stimulated by proteins UvrA and UvrB to unwind a range of DNA substrates. This stimulation likely plays a role in DNA strand and protein displacement by UvrD in nucleotide excision repair (Atkinson et al., 2009).

DNA Polymerase I (Pol I Protein) -- In *E.coli*, the *Pol* A^+ gene encodes DNA polymerase I (Pol I), which has 5'→3' exonuclease activity and is also involved in the repair synthesis during BER (Dianov and Lindahl, 1994). Following the displacement of UvrC protein and damage-bearing oligonucleotide fragments from the postincision complex, Pol I binds to the 3' OH terminus generated at the 5' incision and synthesizes a repair patch of 12 nucleotides (Sibghat et al., 1990). UvrB protein is not displaced during the excision reaction but apparently remains bound to the gapped DNA and released only when Pol I and substrates deoxynucleoside triphosphates (dNTPs) for repair synthesis are present. It has been suggested that DNA helicase II may translocate with Pol I in the direction of repair synthesis and facilitate the displacement of Pol I once the gap is filled (Orren et al., 1992).

DNA Ligase and Ligation Reaction -- The joining of the last newly incorporated nucleotide to the polynucleotide chain is the final post-incision biochemical event in all forms of NER. This event is catalyzed by DNA ligase. *E. coli* cells have one single DNA ligase encoded by the *ligA*$^+$ gene (Lehman, 1974), while mammalian cells have three DNA ligase-encoding genes designated *LIG1*, *LIG2*, and *LIG3* (Timson et al., 2000). The structure of T7 DNA ligase revealed two structurally conserved protein domains (Subramanya et al., 1996). The N-terminal adenylation domain of DNA ligase contains the binding site for the nucleotide cofactor and many of the conserved active site residues of the nucleotidyl transferase superfamily (Shuman and Schwer, 1995). The C-terminal domain resembles the oligonucleotide binding fold of the

single-stranded-DNA- binding proteins (Murzin, 1993). DNA ligase cannot link two molecules of single-stranded DNA or circularized single-stranded DNA. Rather, ligase seals gaps (or breaks) in double-stranded DNA molecules (Tomkinson et al., 2006).

An energy source is required to drive this reaction, in which a phosphodiester bond is created between the 3' hydroxyl group at the end of one DNA chain and the 5'-phosphate group at the end of the other. In most organisms, ATP is the energy source. In bacteria, NAD^+ plays this role (Shuman and Schwer, 1995). The chemical reaction and intermediates are well documented and are apparently conserved for both ATP- and NAD-dependent ligases. The ligation reaction comprises three energetically favorable steps (Lehman, 1974; Pascal, 2008):

1. An adenylate (AMP) group is transferred from an ATP or NAD cofactor to the side chain of a conserved active-site lysine, forming a covalent phosphoamide linkage.
2. The adenylate is transferred to the 5' end of the DNA substrate, forming a 5'-5' pyrophosphate linkage that actives the phosphate for the final step of ligation.
3. The 3' OH of a nicked DNA substrate attacks the adenylated 5' PO_4, sealing the nick and displacing the adenylate group.

Mfd (Transcription Repair Coupling Factor, TRCF) -- It is believed that transcriptional arrest caused by the presence of bulky adducts in DNA may serve as a signal for the binding of Uvr proteins, leading to preferential repair of the template strand in genes that are presumably important for cell survival (Mellon and Hanawalt, 1989). However, an *in vitro* study found that a stalled *E.coli* RNA polymerase protects the lesion from the action of the Uvr proteins and inhibits repair rather than promoting it (Selby and Sancar, 1990b). These paradoxical observations led to the discovery of the *E. coli* Mfd protein, a transcription repair coupling factor (TRCF) that overcomes the inhibitory effect of the stalled RNA polymerase and enhances the rate of strand-specific repair (Selby and Sancar, 1993).

E. coli TRCF is a 130 kDa monomeric protein encoded by the mfd^+ gene (for "mutation frequency decline") (Selby et al., 1991). The structure of Mfd protein consists of a compact arrangement of eight domains, including a translocation module similar to DNA-translocating enzyme RecG, and a region of structural similarity to *E. coli* UvrB (Selby and Sancar, 1993). Another domain that has structural similarity to the transcription elongation factor NusG plays a critical role in TRCF/RNA polymerase interactions. Comparison with the translocation module of RecG and other structural features indicates that TRCF function involves large scale conformational changes. These conformational changes, triggered through the ATP-hydrolysis cycle, are key to the translocation activity of TRCF (Deaconescu et al., 2006). Mfd protein has been shown to interact with RNA polymerase and NER component UvrA protein, has ATPase activity, bind DNA with little or no sequence specificity in an ATP-dependent manner (Savery, 2007; Selby and Sancar, 1993). Mfd protein recognizes and interacts with a stalled RNA polymerase-damaged-DNA-mRNA ternary complex, and subsequently, displaces stalled RNA polymerase and the truncated transcript and binds to DNA near the site of base damage (Selby and Sancar, 1994). Mfd protein has a binding affinity to UvrA protein, suggesting that when bound to damaged DNA, it might be especially efficient in recruiting $(UvrA)_2UvrB$ protein complexes to DNA and in facilitating the formation of a productive UvrB-DNA complex (Selby and Sancar, 1993). In addition, Mfd has been shown to reverse a "backtracked" RNA polymerase complex, which may be also relevant to the mechanism of TCR (Park et al., 2002).

1.2.2 NER Models in *E.coli*

The properties and biological functions of these proteins or protein complexes involved in NER suggest mechanism models in *E.coli* (Fig. 1-1).

GGR Model in *E.coli* -- During GGR, steps of damage recognition and verification are performed by a complex of the repair proteins $(UvrA)_2UvrB$ (Fig.1-1) (Truglio et al., 2006). This

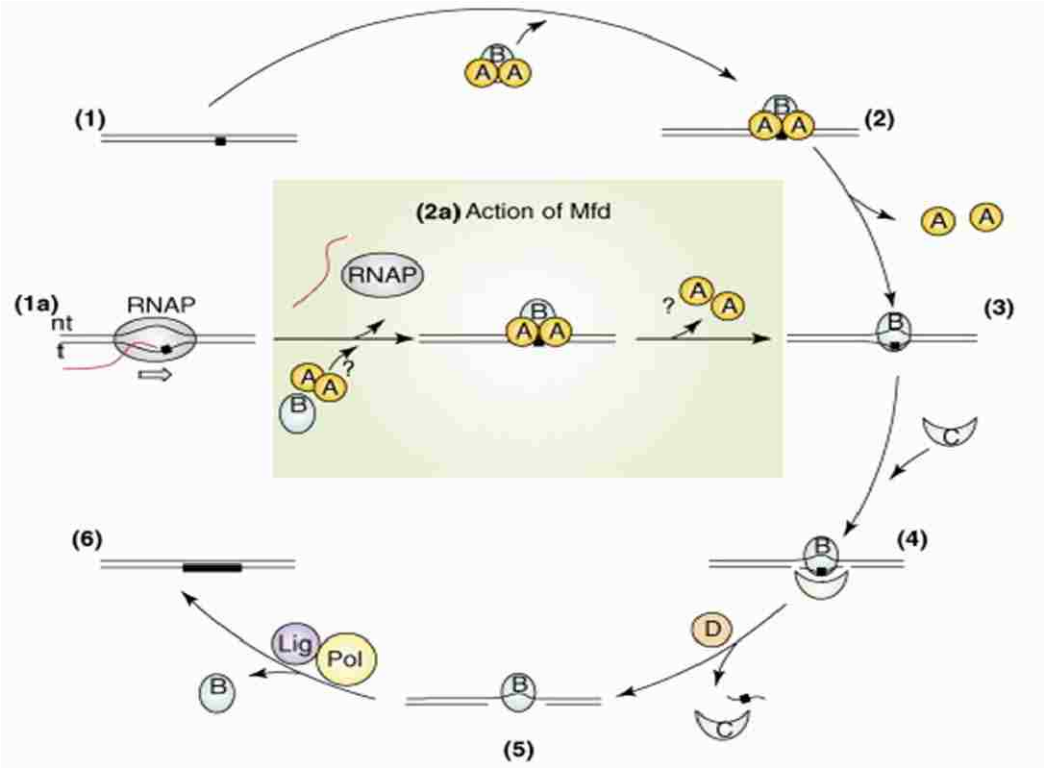

Figure 1-1 Overview of the GGR and TCR in *E. coli.* During GGR, a DNA lesion **(1)** is recognized by a complex of UvrA and UvrB. **(2)** UvrA loads UvrB onto DNA and then dissociates, leaving a stable UvrB-DNA pre-incision complex. **(3)** UvrC is recruited and cleaves the damaged strand on both sides of the lesion. **(4)** UvrD (helicase II) then displaces both UvrC and the damage-containing nucleotide. **(5)** A repair patch is synthesized and UvrB is displaced by Pol I. **(6)** DNA ligase completes the repair by sealing the nick. TCR occurs when lesions in the transcribed strand of DNA block RNAP **(1a)**. The green box summarizes the roles of Mfd **(2a)**: it removes RNAP from DNA and increases the rate of DNA repair by an undefined mechanism that might involve recruitment of UvrA or destabilization of the $(UvrA)_2UvrB$ complex. Once a stable UvrB-DNA pre-incision complex has formed, repair is thought to proceed by the same mechanism as GGR. Abbreviations: t, transcribed strand; nt, non-transcribed strand; A, UvrA protein; B, UvrB protein; C, UvrC protein; D, UvrD protein; Pol, DNA polymerase I; Lig, ligase; RNAP, RNA Polymerase. Adapted from (Savery, 2007).

complex has a higher binding affinity for damaged DNA than does UvrA protein alone, indicating that UvrB protein plays crucial roles in the specific recognition of base damage. Conversely, UvrA protein during this recognition step serves as a "molecular matchmaker" that delivers UvrB to the damaged site by formation of a transient $(UvrA)_2$UvrB-DNA complex (Sancar and Hearst, 1993). Besides UvrA protein, ATP hydrolysis is also required for the delivery of UvrB protein to sites of damage in UV-irradiated DNA (Moolenaar et al., 2000). After UvrB protein is loaded to the damaged site, UvrA dissociates rapidly from the complex, leaving a stable UvrB-DNA pre-incision complex (Savery, 2007).

Next, the UvrB-DNA pre-incision complex changes conformation by binding a nucleotide cofactor and recruits UvrC protein (Moolenaar et al., 2000). The intimate association between UvrC and UvrB generates an endonuclease activity that incises the damaged strand at both 5' and 3' sites of base damage. For the 3' incision, UvrC protein is thought to be bound to the preincision complex via an interaction between the C-terminal domain of UvrB and a homologous internal region of UvrC protein. Incisions on the 3' side of lesions precede the 5' cuts. The N-terminal half of UvrC protein is required for the 3' incision, and the C-terminal half is required for the 5' incision (Moolenaar et al., 1998).

Post-incisional events during NER include excision of the damaged nucleotide fragment, repair synthesis, and DNA ligation. UvrD (helicase II) is required for the excision of a 12–13 nucleotides section containing the damaged base or bases as well as the release of UvrC protein from post-incision complex (Orren et al., 1992). A repair patch of 12 nucleosides is synthesized by DNA polymerase I (Pol I). Although the other two DNA polymerases (Pol II and Pol III) in *E. coli* are potentially able to synthesize the repair patch, Pol I plays a primary role in this function under normal conditions (Gross and Gross, 1969; Hanawalt et al., 1979). UvrB protein is not displaced during the excision reaction but apparently remains bound to the gapped DNA and

released only when Pol I and substrates deoxynucleoside triphosphates (dNTPs) for repair synthesis are present (Orren et al., 1992). Lastly, DNA ligase completes the repair by sealing the nick at the 3′ end of the repair patch (Selby and Sancar, 1993).

TCR Model in *E.coli* -- Like GGR, UvrA, B, C, and D are also required in TCR. In addition, TCR also requires Mfd and active transcription (Selby and Sancar, 1994). For TCR, damage recognition is initiated by the stalling of an elongating RNA polymerase (RNAP) at lesions in the transcribed strands of expressed genes (Fig. 1-1). The lesion-stalled RNAP recruits the *E. coli* transcription repair coupling factor (TRCF), Mfd protein, which was shown to be necessary and sufficient for TCR by its two activities (Deaconescu et al., 2006). Mfd removes RNAP from the DNA so that it no longer acts as an obstacle to DNA repair. The interaction of Mfd with UvrA could enhance the rate of repair either by recruiting the repair proteins directly to the site of damage or by enhancing the rate of another step in the damage recognition and excision process (Deaconescu et al., 2006; Roberts and Park, 2004). The following steps of repair (incision, excision and repair synthesis) in TCR are expected to be the same as in GGR (Savery, 2007).

1.3 Nucleotide Excision Repair in Eukaryotic Cells

NER was found in eukaryotic cells shortly following the discovery of NER in *E. coli* (Rasmussen and Painter, 1964). The DNA synthesis associated with NER in mammalian cells has been demonstrated by the observation of the *unscheduled DNA synthesis*, which occurs outside of the S phase of the cell cycle (Djordjevic and Tolmach, 1967). Although the processes of NER in eukaryotes and prokaryotes are very similar, such as damage recognition and verification, dual incision, excision, repair synthesis, and gap filling, the molecular mechanisms in eukaryotic cells seem much more complicated and largely remain elusive (Fouseri and Mullenders, 2008).

The core NER factors, sets of proteins which are necessary and sufficient to carry out the NER reaction, have been identified in prokaryotic and eukaryotic cells. As mentioned above, only six polypeptides (UvrA, UvrB, UvrC, UvrD, DNA polymerase I, and DNA ligase) are required for NER process in *E. coli*. In contrast, the eukaryotic NER displays a considerably higher degree of genetic complexity. More than 30 proteins or protein complexes have been shown to be involved in NER based on the *in vitro* reconstituted study (Aboussekhra et al., 1995; Guzder et al., 1995). Some NER factors in yeast and human are listed in Table 1-2. These factors can be divided into two groups: (i) the factors necessary for damage recognition and dual DNA incision, and (ii) others required for DNA repair synthesis (Siede et al., 2006). For example, in human cells, factors which belong to the first group include XPC-hRAD23B (a dimer composed of XPC and a human homolog of Rad23), transcription factor IIH (TFIIH, consists of ten subunits), XPA (a possible homodimer), replication protein A (RPA, consists of three subunits), XPG (interacts with TFIIH), and XPF-ERCC1 (a dimer composed of XPF and excision repair cross-complementing 1 protein). The coordinated action of these six core factors is sufficient to carry out oligonucleotide excision on naked substrates *in vitro* (Riedl et al., 2003; Siede et al., 2006).

The second group of NER factors involves series of DNA replication enzymes and accessory factors. RPA may adopt a crucial function in coupling DNA incision to the subsequent DNA synthesis step by coordinating the dissociation of early incision factors with the assembly of the DNA polymerase complex. The synthesis of repair patches is also dependent on replication factor C (RFC), a pentameric matchmaker that binds to the excision gap and mediates the entry of proliferating cell nuclear antigen (PCNA), which in turn acts as a sliding clamp for DNA polymerase δ and ε (Riedl et al., 2003). Finally, the newly synthesized repair patches are ligated to the pre-existing DNA through the action of DNA ligase I (Shuck et al., 2008).

Table 1-2 Some NER factors in human and yeast *S.cerevisiae*

Human	*S.cerevisiae*	Functions
XPC	Rad4	GGR (and TCR in yeast); damage recognition (Sugasawa et al., 1998)
RAD23B	Rad23	GGR; interacts with proteasome and XPC (Hey et al., 2002)
XPA	Rad14	Stabilizes open complex; checks for damage (Ikegami et al., 1998)
RPA1,2,3	Rfa1,2,3	Stabilizes open complex (with Rad14/XPA) (Bochkarev et al., 1999)
XPG (ERCC5)	Rad2	3' incision; stabilizes full open complex (Habraken et al., 1994)
ERCC1	Rad10	5' incision (Davies et al., 1995; Gaillard and Wood, 2001)
XPF (ERCC4)	Rad1	5' incision (Newman et al., 2005)
LIG1	Cdc9	Ligation (Barnes et al., 1990)
CSA (CKN1)	Rad28	TCR, WD40 repeats (Bhatia et al., 1996)
CSB (ERCC6)	Rad26	TCR, DNA dependent ATPase (Licht et al., 2003; Troelstra et al., 1992)
DDB1, DDB2 (XPE)	Unknown	Damage recognition (Rapic-Otrin et al., 2002; Rapic-Otrin et al., 2003)
MMS19L	Mms19	Interacts with the XPB and XPD subunits of TFIIH (Lauder et al., 1996)
Unknown	Rad7	GGR; form Rad7-Rad16 complex (Verhage et al., 1994)
Unknown	Rad16	GGR; ATPase (Guzder et al., 1998; Verhage et al., 1994)
TFIIH subunits		
XPB (ERCC3)	Ssl2	3' to 5' helicase (Guzder et al., 1994; Schaeffer et al., 1993)
XPD (ERCC2)	Rad3	5' to 3' helicase (Sung et al., 1993; Sung et al., 1987)
GTF2H1 (p62)	Tfb1	Phosphorylated by a kinase (Wang et al., 1995)
GTF2H2 (p44)	Ssl1	Regulates XPD (Yoon et al., 1992)
GTF2H3 (p34)	Tfb4	Ring finger; DNA binding (Feaver et al., 1999)
GTF2H4 (p52)	Tfb2	Anchors XPB and regulates its helicase activity (Feaver et al., 2000)
GTF2H5 (TTDA)	Tfb5	Stabilization of TFIIH (Giglia-Mari et al., 2006; Ranish et al., 2004)
Cdk7	Kin28	CDK; CAK (Fisher et al., 1995)
CCNH (CycH)	Ccl1	Cyclin (Adamczewski et al., 1996)
MNAT1 (Mat1)	Tfb3	CDK assembly factor (Feaver et al., 1997; Feaver et al., 2000)

Most NER factors are conserved proteins and the gene orthologs have been identified in yeast, human and other eukaryotes (Table 1-2). Although the order of arrival/departure of each factor and the location of interactions and functions of these factors are still intensively debated, some favored models of sequential assembly and protein-protein interactions are proposed based on recent advanced studies. In this part, we will mainly talk about the NER in human and yeast cells.

1.3.1 NER Factors in Eukaryotic Cells

Yeast Rad4-Rad23 and Human XPC-RAD23B -- Yeast Rad4 is an 87 kDa protein. Rad4 protein tightly associates with Rad23 and binds to Pol II transcription factor TFIIH. Yeast Rad23 is a 42 kDa protein (Watkins et al., 1993). Several conserved domains have been identified, including an N-terminal ubiquitin-like (UbL) domain, and two copies of conserved ubiquitin-associated (UbA) domain, one in the middle and one in the C-terminal part of the protein. The UbL domain of Rad23 is necessary for maximal NER efficiency through mediating the interaction between Rad23 and the 26S proteasome. A specific interaction occurs between the Rad23 UbL domain and Rpn1 protein, a subunit of the 19S regulatory particle of the proteasome (Elsasser et al., 2002; Wang et al., 2000). The UbA domains form a large hydrophobic structure surface patch on the protein, which can bind to multiubiquitin chains (Watkins et al., 1993). In addition to Rad4 protein, Rad23 interacts with many other proteins. Rad23 protein appears to be part of the regulatory circuitry for Rad4 protein levels and may help to stabilize Rad4 by inhibiting its proteosomal degradation (Friedberg et al., 2006).

The human ortholog of Rad4 is XPC, a polypeptide of 106 kDa (Masutani et al., 1994). However, there are two human orthologs of Rad23, named RAD23A, RAD23B, respectively. Like yeast Rad23, the human RAD23A and RAD23B proteins contain UbL and UbA domains. One or more conserved regions in RAD23A and RAD23B are required for interaction with a

region in the C-terminal part of the XPC protein (Sugasawa et al., 1997). XPC is most frequently associated with RAD23B in the cell. A short peptide corresponding to the XPC-binding domain of RAD23B protein is sufficient for stimulation of NER *in vitro* (Masutani et al., 1997). XPC-RAD23B binds to UV-irradiated DNA with a considerable preference and forms a stable DNA-XPC-RAD23B complex. XPC-RAD23B binds directly to DNA on both strands around the lesion with considerable specificity to discriminate between damaged and undamaged sites (Hey et al., 2002).

Yeast and Mammalian Transcription Factor TFIIH -- TFIIH was first identified as a transcription initiation factor for Pol II, together with the factors TFIIA, TFIIB, TFIID, TFIIE, and TFIIF (Sikorski and Buratowski, 2009; Thomas and Chiang, 2006). TFIIH possesses ATP-dependent strand-separating activities that are necessary to produce open DNA complexes during Pol II transcription initiation and NER (Oksenych et al., 2009). TFIIH consists of 10 subunits in both yeast and mammalian cells (Fig. 1-2). Five of these subunits: XPB (yeast Rad25), p62 (Tfb1), p52 (Tfb2), p44 (Ssl1), and p34 (Tfb4) are tightly associated in a subcomplex called core TFIIH. XPD (Rad3) is less tightly associated with the core and mediates the binding of the CDK-activating kinase (CAK) subcomplex, which consists of three subunits, MAT1 (yeast Tfb3), CDK7 (Kin28) and cyclin H (yeast Ccl1) (Zurita and Merino, 2003). The tenth subunit (p8 or TTDA, yeast Tfb5) is an 8 kDa protein which was identified in both human and yeast cells at the same time (Giglia-Mari et al., 2004; Ranish et al., 2004).

XPB (yeast Rad25) and XPD (Rad3) are ATP-dependent DNA helicases with opposite polarity. The hydrolysis of ATP (or dATP) drives DNA helicase activity, unwinding duplex DNA to the single strand, either around the promoter, to allow transcription initiation, or around a damage site, to permit damage-specific nucleases to cleave the DNA on either side of the damage (Balajee, 2006). The 3'→ 5' helicase activity of XPB (Rad25) is essential for both transcription

and repair, whereas the XPD (Rad3) 5'→ 3' helicase activity is necessary for repair but dispensable *in vitro* for basal transcription (Chen and Suter, 2003). The kinase activity from CDK7 is able to phosphorylate numerous substrates, including both Ser5 and Ser7 C-terminal domain (CTD) of Rpb1, the largest subunit of Pol II, converting it from the initiating IIa form to the elongating IIo form (Akhtar et al., 2009). The kinase activity is also involved in the regulation of transcription and the cell cycle (Araujo et al., 2000). Interestingly, the CDK-activating kinase (CAK) subcomplex was shown to be dissociated from core TFIIH during NER, suggesting the composition of TFIIH is dynamically changing to adapt its engagement in distinct cellular processes (Coin et al., 2008).

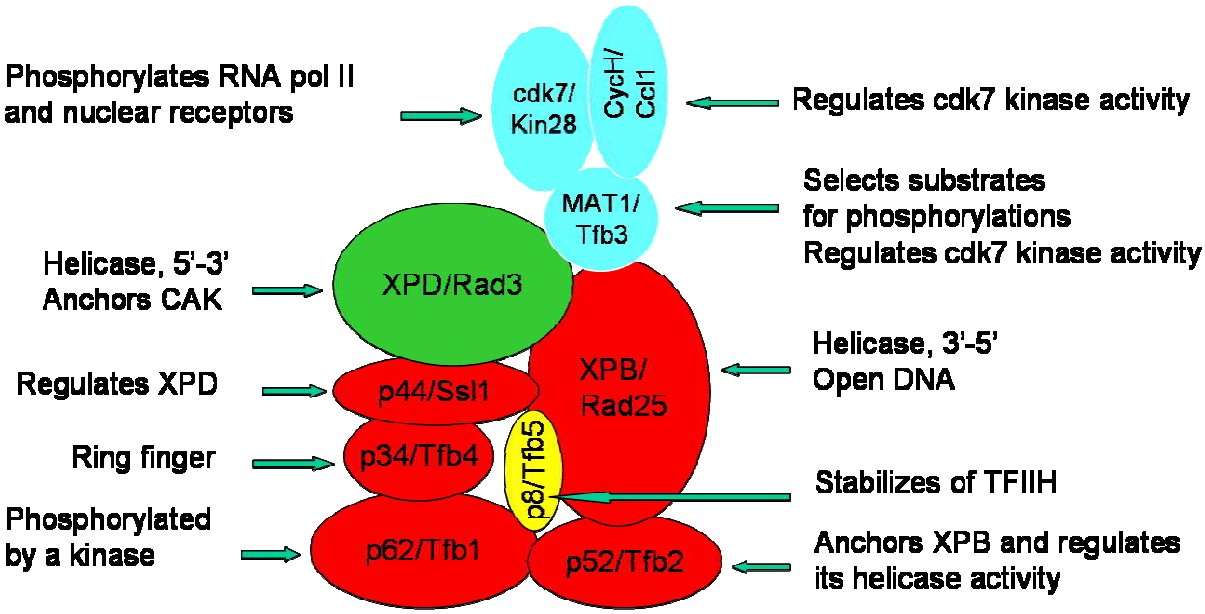

Figure 1-2 Human and yeast TFIIH composition and enzymatic functions of its subunits. The five subunits composing the core TFIIH subcomplex are in red, the three subunits of the cdk-activating kinase (CAK) subcomplex are in blue, the XPD/Rad3 subunit that bridges the two TFIIH subcomplexes is in green and p8(TTDA)/Tfb5 subunit is in yellow. Functions of each subunit were labeled on both sides. The name of each subunit is labeled in the form of human/yeast. Adapted from (Balajee, 2006).

The enzymatic activities of TFIIH are tightly controlled by interactions within the TFIIH complex or through interactions with many general and regulatory transcription factors. For example, p44 (yeast Ssl1) interacts with XPD to stimulate its helicase activity, p52 (yeast Tfb2) regulates the function of XPB through pair-wise interactions, MAT1 (Tfb3) and cyclin H (Ccl1) regulate CDK7 kinase activity, and phosphorylation of cyclin H by CDK8/cyclin C suppresses both the activation of transcription by TFIIH and the ability of TFIIH to phosphorylate RNA polymerase II (Balajee, 2006). In addition to XPB and XPD, TTDA/p8 is the third gene responsible for the photosensitive disorder of Trichothiodystrophy (TTD) (Giglia-Mari et al., 2004). It has been suggested that TTDA is involved in stabilization of TFIIH or in its protection from degradation (Vitorino et al., 2007). In yeast, Tfb5 has been shown to stabilize TFIIH through protecting the hydrophobic surface between Tfb5 and Tfb2 (Kainov et al., 2008). Moreover, Tfb5 has been shown to be required for GGR but is partially dispensable for Rad26 mediated TCR, especially in GGR deficient cells (Ding et al., 2007).

MMS19 -- MMS means methyl methanesulfonate-sensitive. The *MMS19* gene was originally found in yeast as being involved in NER. The mutant *mms19* was shown to enhance UV-induced mutagenesis (Prakash and Prakash, 1979). Genetic and biochemical evidence indicates that besides its function in NER, MMS19 also affects Pol II transcription. Thus, Mms19 protein resembles TFIIH in that it is required for both transcription and DNA repair (Lauder et al., 1996). It appears that the Mms19 protein is not directly involved in NER and Pol II transcription. Co-immunoprecipitation experiments revealed that hMMS19 directly interacts with the XPB and XPD subunits of NER-transcription factor TFIIH, suggesting that hMMS19 exerts its function in repair and transcription by interacting with the XPB and XPD helicases (Seroz et al., 2000). Alignment of the translated sequences of Mms19 from multiple eukaryotes, including mouse and human, revealed the presence of several conserved regions, including a HEAT repeat domain

near the C-terminus. The presence of HEAT repeats from higher eukaryotes can functionally complement a yeast *mms19* deletion mutant, indicating a role of Mms19 protein in the assembly of a multiprotein complex required for NER and Pol II transcription (Queimado et al., 2001).

Human XPA and Yeast Ortholog Rad14 -- Human XPA is a 31 kDa protein encoded by the *XPA* gene and was identified by phenotypic complementation of the UV radiation sensitivity of XP-A cells after transfection with total mouse genomic DNA (Friedberg et al., 2006). One feature of the XPA protein is its ability to bind DNA, with a weak preference for binding to damaged DNA (Asahina et al., 1994). XPA binds with considerably higher efficiency to partially duplex DNA than to single-stranded substrates (Asahina et al., 1994; Missura et al., 2001). The minimal DNA-binding domain of XPA protein contains a compact zinc-binding core and a more C-terminal region rich in loops (Buchko et al., 1998; Tanaka et al., 1990). Although XPA protein dose not appear to participate in the very first step of recognizing DNA damage, it is an essential component of the preincision complex that forms at a damaged site in preparation for the dual-incision reaction of eukaryotic NER (Friedberg et al., 2006).

Yeast Rad14 protein is a 29 kDa polypeptide, with 26% residual identity and 54% similarity to human XPA (Bankmann et al., 1992). Similar to human XPA, Rad14 contains a single zinc atom in the zinc finger domain and binds preferentially to UV-irradiated DNA, especially to the more helix-distorting damage site of (6-4)PP (Guzder et al., 1993).

Human and Yeast Replication Protein A (RPA) -- RPA is a eukaryotic ssDNA-binding protein composed of three subunits. In human cells, these subunits are proteins RPA1, RPA2, RPA3, with molecular masses of 70, 32, and 14 kDa, respectively (Bochkarev et al., 1997). In yeast, RPA consists of three subunits Rfa1 (70 kDa), Rfa2 (30 kDa) and Rfa3 (14 kDa) (Friedberg et al., 2006). The basic structure unit of this heterotrimeric protein is ssDNA-binding domains, which are conserved OB (for oligonucleotide/oligosaccharide-binding) fold. There are six OB-folds

between the subunits of RPA: three are in RPA1, one is in RPA2, and two are in RPA3. Four of these OB-folds bind ss-DNA and two mediate subunit interactions (Bochkarev and Bochkareva, 2004).

RPA was originally identified as a protein for DNA replication and later was found indispensable for NER machinery in both human and yeast cells (Umezu et al., 1998; Wold, 1997). RPA has been shown to be involved in the incision of damaged DNA. In a reconstituted system with purified proteins, RPA is essential for events leading to the incision reaction in both mammalian cells and yeast (Araujo et al., 2000; Prakash and Prakash, 2000). RPA interacts with several NER factors, such as XPA (or Rad14 in yeast) and is also an essential component of the preincision complex (de Laat et al., 1998b).

Yeast Rad1 and Rad10 and Human Orthologs XPF and ERCC1 -- Rad1 and Rad10 form a stable heterodimeric complex *in vitro* in the absence of damaged DNA. The deletion of either *RAD1* or *RAD10* gene will cause a defect in NER (Bardwell et al., 1994). Both the Rad10-binding domain of Rad1 and the Rad1-binding domain of Rad10 are hydrophobic, and the Rad1-Rad10 complex formation *in vitro* is stimulated at increased ionic strength (Bardwell et al., 1992). The Rad1-Rad10 complex possesses DNA endonuclease activity. Neither protein alone is endowed with this endonuclease activity. The endonuclease generates 5' phosphate and 3' OH termini and has an absolute requirement for divalent cations such as Mg^{2+}. The DNA endonuclease activity of the Rad1-Rad10 complex is specific for junctions between duplex and ssDNA which enables it to participate in DNA damage-specific incision during NER (Bardwell et al., 1994; Friedberg et al., 2006).

The human orthologs of yeast Rad1 and Rad10 are XPF (ERCC4) (104kDa) and ERCC1 (32.5kDa), respectively (de Laat et al., 1998a). Similarly, ERCC1 and XPF form a complex with structure-specific nuclease activity. It can cut DNA at junctions between a duplex and a single

strand, where the single strand moves from 5' to 3' away from the junction. This property allows the enzyme to cut the damaged strand during NER specifically on the 5' side of a lesion once the DNA double helix has been locally unwound at the site of damage (Sijbers et al., 1996). Thus, the yeast Rad1-Rad10 complex and human ERCC1-XPF complex possess one of the DNA endonucleases which are essential for the dual-incision step in NER.

Yeast Rad2 and Human XPG -- Yeast Rad2 protein is a junction-specific endonuclease. It has a polarity opposite to that of the Rad1-Rad10 endonuclease with respect to ssDNA, cleaving at junctions of duplex DNA with single strands at 3' site (Habraken et al., 1995). The C-terminal tail of Rad2 polypeptide is rich in basic amino acids, which is required for its endonuclease activity (Higgins et al., 1984). The human ortholog of Rad2 is XPG protein (133kDa) (Clarkson, 2003). XPG cleaves several types of DNA structures containing junctions between unpaired and duplex DNA, cutting the strand in which the unpaired region has a polarity moving from 3' to 5' away from the junction. Like yeast Rad2 endonuclease, XPG cleaves on the 3' side of a DNA bubble structure in an Mg^{2+}-dependent manner (O'Donovan et al., 1994).

XPG and Rad2 are members of an enzyme family that includes the FEN1 (DNase IV) group of structure-specific nucleases (Harrington and Lieber, 1994). Ethylation interference foot printing experiments indicate that XPG binds to its substrates through interaction with the phosphate backbone on one face of the helix, mainly to the dsDNA. The 3' but not the 5' single-stranded arm is necessary for DNA binding and incision activity (Friedberg et al., 2006; Hohl et al., 2003).

Yeast Rad7 and Rad16 -- Yeast Rad7 and Rad16 form a stable complex, which is specifically required for GGR, but dispensable for TCR. The deletion of either Rad7 or Rad16 will completely abolish the GGR activity (Verhage et al., 1994). The Rad7/Rad16 complex binds specifically to UV damaged DNA in an ATP-dependent manner (Guzder et al., 1998). Rad16 is a

DNA-dependent ATPase, belonging to a large family of proteins called the *SNF2/SWI2* family (Guzder et al., 1998). The observation that Rad16 has DNA-dependent ATPase activity suggested that it may be involved in some type of chromatin remodeling of the genome during NER (Friedberg et al., 2006). It has been shown recently that Rad16 mediates the ultraviolet-induced acetylation of histone H3, necessary for efficient GGR (Teng et al., 2008).

Rad7 is a 64 kDa protein. Rad7 interacts with Rad4 and has one function related to Rad23, suggesting that Rad23, Rad7, Rad16 and Rad4 proteins participate together in a biochemical pathway (Wang et al., 1997). Besides Rad7 and Rad16, the complex may also contain several other factors, such as replicating sequence binding factor 1 (Abf1) and Elc1 (elongin C) (Lejeune et al., 2009; Yu et al., 2004). Rad7 and Rad16 are also found to be in an E3 ubiquitin ligase complex (Ramsey et al., 2004; Ribar et al., 2006). The human orthologs of yeast Rad7 and Rad16 remain inconclusive.

Human DNA Damage-Binding Protein (DDB or XPE) -- DDB (also referred to as UV-DDB) is a heterodimer composing a 127 kDa subunit called DDB1 and a 48 kDa subunit called DDB2 (Keeney et al., 1993). DDB2 and XPE are identical (Rapic-Otrin et al., 2002). DDB protein can be regulated by UV radiation. In contrast with unirradiated cells, after UV irradiation, DDB becomes more tightly bound to DNA and the level of DDB2 (XPE) increases (Wakasugi et al., 2002). DDB may not be essential for NER but plays an accessory role through aiding in the recognition of DNA damage in chromatin (Tang and Chu, 2002). DDB has been shown to be a component of the cullin 4A (CUL4A) / CUL4B (CUL4B)-based ubiquitin ligases, DDB1-CUL4A (DDB2) and DDB1-CUL4B (DDB2). These enzymes ubiquitinate histone H2A in a DDB2-dependent manner and modify the chromatin structure at the sites of UV lesions to promote efficient NER (Guerrero-Santoro et al., 2008; Li et al., 2006). The yeast ortholog of human DDB has not yet been identified.

Yeast Rad26/Rad28 and Human Ortholog CSB/CSA Proteins -- In mammalian cells, Cockayne syndrome group A (CSA) and B (CSB) proteins are specifically required for TCR (Licht et al., 2003). CSA protein is a component of a cullin-containing ubiquitin E3 ligase, together with DDB1 (Groisman et al., 2003). The structural homolog of CSA in yeast is called Rad28. Both CSA and Rad28 proteins possess multiple divergent WD40 repeats, which act as sites for protein-protein interactions (Henning et al., 1995). Unlike CSA in mammalian cells, TCR or recovery of RNA synthesis after UV radiation does not depend on Rad28 function in yeast (Bhatia et al., 1996). The yeast ortholog of human CSB is Rad26. Although Rad26 plays a critical role in TCR in yeast, the UV sensitivity of *rad26* mutants cannot be seen unless in combination with the deletion of *RAD7* or *RAD16,* because the GGR and Rad26-independent TCR subpathways still occur in *rad26* mutant cells (Li and Smerdon, 2002b). CSB and Rad26 are members of Swi/Snf (Switch/Sucrose nonfermentable) protein family, in which proteins possess DNA-dependent ATPase activity and participate in chromatin remodeling and disturb DNA-protein interactions (Pazin and Kadonaga, 1997).

It has been suggested that CSA and CSB play different roles in recruitment of NER factors and assembly of NER machinery (Fousteri et al., 2006). CSB fulfills a key role as a coupling factor to attract histone acetytransferase, NER factors, and CSA-DDB1 E3-ubiquitin ligase complex, while CSA is dispensable for recruitment of NER proteins (Fousteri et al., 2006). Moreover, CSB dynamically associates with Pol IIo, and UV irradiation stabilizes this interaction (van den Boom et al., 2004). In yeast, Rad26 also plays a significant role in repairing certain regions of the repressed *GAL1-10*, *PHO5* and *ADH2* genes, especially in the core DNA of well-positioned nucleosomes (Li et al., 2007). In addition to NER, CSB-mediated chromatin remodeling is probably involved in the modulation of transcription initiation through facilitating the recruitment of TATA binding protein (TBP) and other factors to promoters after UV

irradiation (Proietti-De-Santis et al., 2006). The exact molecular mechanisms of CSB or yeast Rad26 in TCR remain elusive.

1.3.2 NER Models in Eukaryotic Cells

Recently, the order of events leading to dual incision by NER factors has been extensively investigated and some mechanism models were proposed. Similar to the assembly of NER machinery in *E. coli*, the assembly of eukaryotic NER machinery is also a multiple step process including damage recognition and verification, incision, excision, repair synthesis, and DNA ligation.

GGR Model in Eukaryotic Cells -- In GGR, the protein complexes XPC-hRAD23B (in yeast, Rad4-Rad23) and UV-DDB can recognize and bind to the damage-induced distortion and initiate GGR (Fig. 1-3). After damage recognition, the transcription factor IIH (TFIIH) is recruited to the damage site (Araujo et al., 2001). TFIIH plays an essential role in NER as two of its subunits XPB (Rad25) and XPD (Rad3) are required. These two proteins are DNA-dependent helicases and able to unwind the DNA double helix at around the lesion (Coin et al., 2007). XPB (Rad25) unwinds DNA duplex in a direction of 3' to 5', while XPD (Rad3) unwinds in a 5' to 3' direction. The combined action of XPC- hRAD23B and TFIIH creates short stretches of single stranded DNA (ssDNA) around the lesion that facilitates the recruitment of XPA and ssDNA binding protein RPA to form a pre-incision complex (Fousteri and Mullenders, 2008). The dual incision process is performed by structure-specific endonucleases XPG (Rad2) and ERCC1-XPF (Rad1-Rad10) at the 3' and 5' side of the lesion, respectively (O'Donovan et al., 1994; Sijbers et al., 1996). After the oligonucleotide (24-30 nt in length) containing the lesion has been removed, proliferating cell nuclear antigen (PCNA) is loaded by replication factor C (RFC, yeast Rfc) as is the case in DNA replication (Shuck et al., 2008). DNA polymerases δ and ε (in yeast, Pol III and Pol II, repectively) are capable of DNA repair synthesis across the gap using the undamaged

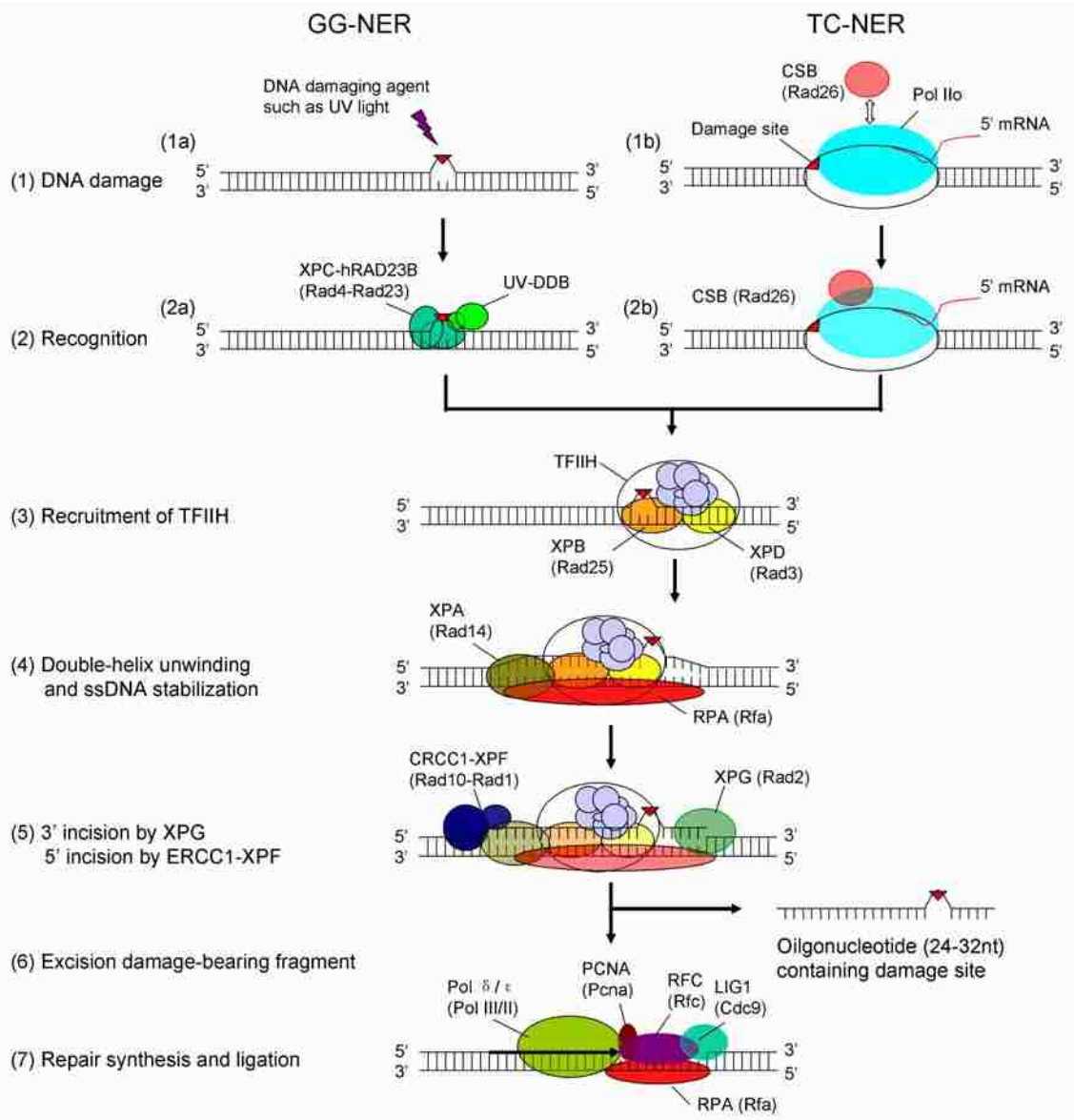

Figure 1-3 Two NER subpathways in eukaryotic cells. (1-2) Damage/distortion recognition in GGR and TCR. XPC-hRAD23B and UV-DDB complexes recognize and bind to DNA damage-mediated helix distortion and initiate GGR (2a). TCR is triggered by DNA damage-mediated blockage of RNA Polymerase IIo (2b). **(3-4)** Lesion demarcation. In the following steps, the two sub-pathways are converged. The lesion is verified and demarcated as a *bona fide* NER lesion by the concerted actions of helix opening and damage verification provided by TFIIH, XPA and RPA. **(5-6)** Dual incision. Within the pre-incision complex, ERCC1-XPF and XPG structure-specific endonucleases incise the damaged strand. **(7)** Gap filling and ligation. After dual incision around the lesion, the single strand gap is filled by DNA polymerase, PCNA and RFC, and sealed by DNA ligase III-XRCC1 in both dividing and non-dividing cells, whereas DNA polymerase and DNA ligase I are involved in dividing cells in addition to DNA polymerase and DNA ligase III-XRCC1.

strand as a template (Soria et al., 2009) ; the remaining nick can be filled by DNA ligase I (yeast Cdc9) (Fousteri and Mullenders, 2008).

TCR Model in Eukaryotic Cells -- In TCR, it is believed that the transcription elongating form of Pol IIo stalled at the damaged site is the signal for the recruitment of TCR proteins (Laine and Egly, 2006; Lindsey-Boltz and Sancar, 2007). In mammalian cells, the association between CSB and Pol IIo become more stable after encountering transcription-blocking lesions. Subsequently, CSA, TFIIH, RPA and XPA arrive at the site of damage (Fousteri et al., 2006; Maddukuri et al., 2007). In yeast, protein Rad26 was also shown to have weak binding affinity to Pol II (Jansen et al., 2002). CSB (Rad26) may work at very early step in response to the blockage of Pol II (Fousteri et al., 2006). After the damage recognition, GGR and TCR are converged in the following steps (Fig. 1-3).

How CSB/Rad26 facilitate TCR remains to be elucidated. Several models were proposed based on their Swi/Snf-like activity (DNA-dependent ATPase activity) (Svejstrup, 2002). One possibility is that the stalled Pol II (RNAPII) will be displaced by CSB/Rad26 via Swi/Snf-like activity (Fig.1-4a), like the functional mechanisms of TRCF (Mfd) in *E.coli* (Fig.1-1). However, there is little sequence homology between TRCF and CBS/Rad26 except ATPase domains. It was further demonstrated that CSB cannot dissociate a damage-stalled Pol II complex *in vitro* (Selby and Sancar, 1997b). The second model is that the damage-stalled Pol II could be retrograded by TFIIS, the transcription factor which stimulates transcript cleavage allows resumed forward translocation (Fig.1-4b) (Davie and Kane, 2000; Kettenberger et al., 2004; Saeki and Svejstrup, 2009). Another possibility is that CSB/Rad26 could alter the molecular architecture of the damage-stalled Pol II-DNA complex (Fig.1-4c). CSB indeed can enable Pol II to add an extra nucleotide when stalled at a lesion (Selby and Sancar, 1997a), suggesting that the remodeling of the Pol II-DNA interface occurs. This remodeling may involve other protein-protein interactions,

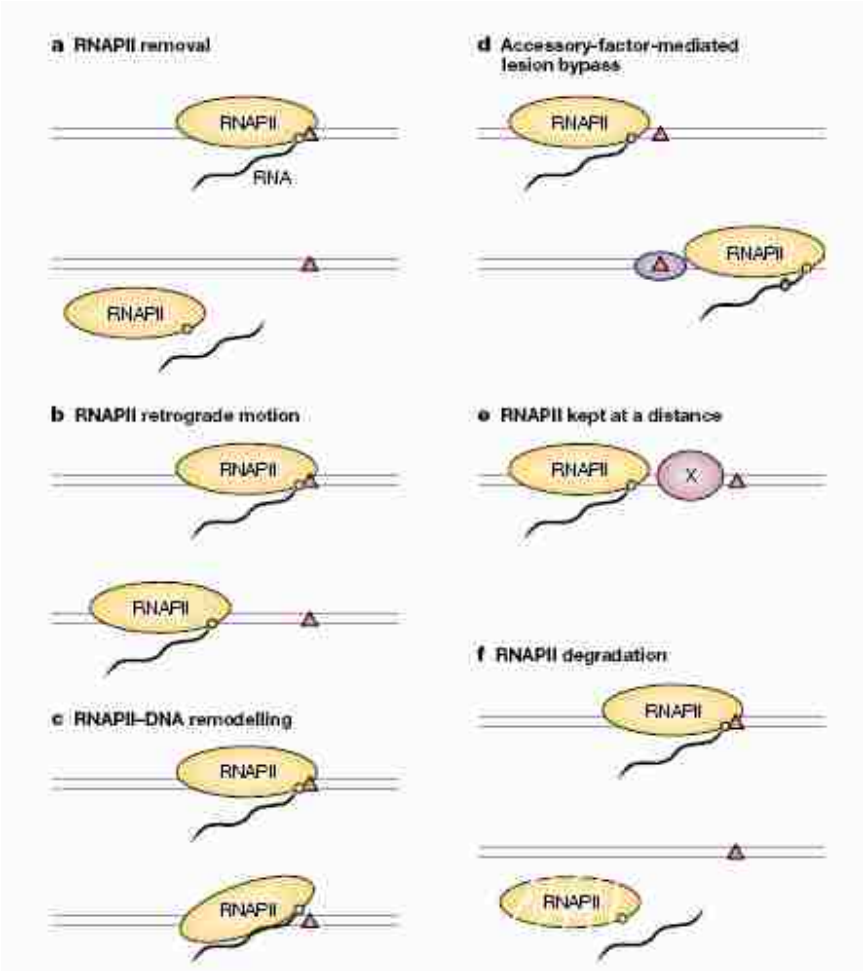

Figure 1-4 Different models for transcription-coupled repair. The models involve different modes of RNAPII displacement from the damaged DNA by CSB/Rad26 and other transcription-coupled repair (TCR) factors. **(a)** RNAPII dissociation from the site of damage. Such dissociation could be achieved by employing a transcription release factor or the Swi/Snf-like activities of CSB/Rad26. **(b)** RNAPII is moved away from the damage site. Such displacement could conceivably be achieved by Swi/Snf-like activities of CSB/Rad26, and/or the use of other activities, such as that of TFIIS. **(c)** Remodelling of the damaged DNA–RNAPII interface by CSB. **(d)** At certain lesions, bypass might be promoted by accessory factors, such as the Swi/Snf-like activity of CSB/Rad26. **(e)** Damage-binding factors arriving prior to RNAPII might ease repair by keeping the polymerase at a distance. **(f)** Degradation of RNAPII stalled at a lesion could occur. One or more of these fates of RNAPII appears to facilitate recruitment of the NER machinery to the lesion. Green circles indicate the active site of RNAPII. Red triangles denote a DNA lesion. Adapted from (Svejstrup, 2002).

such as the remodeling of interface between Pol II and TCR suppressors Spt4/Spt5 or even the displacement of Spt4/Spt5 from Pol II (Ding et al., 2009; Jansen et al., 2000). Alternative models of the fates of damage-stalled Pol II, such as accessory-factor-mediated lesion bypass (Fig.1-4d), and keeping Pol II at a distance through damage-binding factors (Fig.4-4e), might also be relevant in certain situations (Svejstrup, 2002). The finding of UV-induced Pol II ubiquitination and degradation made researchers propose the Pol II degradation model (Bregman et al., 1996; Ratner et al., 1998) (Fig. 4-4f). However, evidence accumulated that Pol II degradation does not seem to be required for TCR in yeast. Rsp5, the sole yeast ubiquitin-protein ligase that ubiquitylates Pol II, is not required for TCR (Lommel et al., 2000). Def1, a yeast protein 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 et al., 2002). Thus, the exact molecular mechanisms of TCR in eukaryotic cells are extremely complicated and remained unknown. It is possible that not all the proteins involved in TCR have been identified yet, and the molecular functions of CSB/Rad26 are not fully understood.

The orthologs of NER factors between yeast and humans may not be exactly equivalent during NER. For example: 1) XPC is dispensable for the TCR in mammalian cells (Venema et al., 1990), while its yeast homolog Rad4 is indispensable for both GGR and TCR (Prakash and Prakash, 2000), 2) CSA plays an important role in TCR in mammalian cells (Fousteri et al., 2006), while very little function can be seen for the yeast homolog Rad28 in TCR (Bhatia et al., 1996), 3) CSB is indispensable for TCR in mammalian cells (Fousteri et al., 2006), whereas TCR in yeast is not solely dependent on Rad26 (Gregory and Sweder, 2001). Substantial TCR activity can be noticed in a Rad26-independent manner, especially in the region immediately downstream of the transcription start site (Li and Smerdon, 2002a). Thus, the mechanisms of NER revealed in yeast cells need further verification before application to humans.

1.4 Nucleotide Excision Repair and Human Diseases

The importance of NER for human health can be revealed by three disorders which result from the defects of genes involved in NER, namely Xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) (Lehmann, 2003). They are all rare autosomal recessive inheritance disorders and are characterized by extreme sensitivity to sunlight (Balajee, 2006). Given the versatility of NER to repair a large number of DNA lesions and the numbers of genes involved, the clinical symptoms of NER deficiency vary considerably (Table 1-3).

Table 1-3 Diseases associated with NER deficiencies

	Xeroderma pigmentosum (XP) (Cleaver, 2005)	Cockayne syndrome (CS) (Cleaver et al., 2009)	Trichothiodystrophy (TTD) (Hashimoto and Egly, 2009)
Genes	*XPA-XPG, XPV*	*CSA, CSB, XPB, XPD, XPG*	*XPB, XPD, TTDA*
Defective pathways	NER, Translesion	TCR, Transcription	NER, Transcription
Symptoms:			
Photosensitivity	Yes	Yes	Yes
Cancer prone	Yes	No	No
Growth failure	No	Yes	Yes
Neurodegeneracy	Sometimes	Yes	Yes

1.4.1 Xeroderma Pigmentosum (XP)

XP is characterized by cutaneous symptoms in the sunlight exposed area of the skin, resulting from the defect in removal of UV-induced lesions: skin atrophy, pigmentation anomalies, and most strikingly a very high incidence of skin cancer (Cleaver, 2005). Squamous cell carcinomas, basal cell carcinomas, and melanomas may occur at the ocular surface (Kraemer et al., 1987). In addition, XP patients display a higher propensity to internal cancers, mostly of the lung or gastro-intestinal tract, suggesting the role of NER in dealing with air pollution and food carcinogens (Nouspikel, 2009). Approximately 20% of XP patients suffer from neurological

symptoms typical of progressive neurodegeneracy (Kraemer et al., 1984). Given that UV light cannot reach into the human brain, XP neurological symptoms may result from some form of endogenous DNA damage that is normally repaired by the NER pathway. It has been shown that a particular class of oxidative DNA lesions, the 8,5'-cyclopurine-2'-deoxynucleosides, are repaired by the NER pathway but not by any other known process (Brooks, 2007). The accumulation of cyclopurine in transcribed genes could be responsible for neurological diseases in XP patients (Rapin et al., 2000).

There are 8 complementation groups in XP: XP-A through XP-G, plus a variant group XP-V (Nouspikel, 2009). Factors of XP-A through XP-G play critical roles in damage recognition, double-helix unwinding, or dual incision during GGR process (Table 1-2, Figure 1-3). Mutations in these genes disable NER at the global genomic level, although TCR is still operative (Fousteri and Mullenders, 2008). XP-V encodes human DNA polymerase eta (Pol η), which shares significant amino acid sequence homology with *S. cerevisiae* Rad30 (Johnson et al., 1999; Nouspikel, 2009). Unlike other XP cells (belonging to groups XP-A to XP-G), XP-V cells carry out normal NER processes but are defective in their replication of UV-damaged DNA (Lehmann et al., 1975). Thus, it is believed that bypass polymerases which take over in XP-V cells may cause the accumulation of mutations during DNA replication (Masutani et al., 1999). The higher cancer propensity can be explained by the accumulation of mutations in the genome of actively replicating cells in XP patients.

1.4.2 Cockayne Syndrome (CS)

CS is a developmental disease, characterized by severe neurological abnormalities with white matter degeneracy, short stature, lack of subcutaneous fat, bird-like faces, tooth decay, cataracts, and a shortened life span averaging 12.5 years (Nouspikel, 2009). The majority (80%) of CS cases are caused by a defect in the *CSB* gene, while most of the remaining cases are caused

by mutations in the *CSA* gene (Stefanini et al., 1996). Besides TCR, CSB has been show to be involved in several different processes, including general transcription (Scharer, 2008), BER of some types of oxidative damage (Selby and Sancar, 1997a) and possibly chromatin remodeling (Tuo et al., 2001) (Fig. 1-5) (Newman et al., 2006). In addition, CS cells display a stronger apoptotic response to DNA damaging agents than normal cells, which in part could explain why no cancer is seen in CS patients (Stevnsner et al., 2008). Given the involvement of CSB in several different processes, it can be speculated that the participation of CSB in each pathway is somehow regulated in response to DNA damage, aging, development, and cellular differentiation (Balajee et al., 2000; Stevnsner et al., 2008).

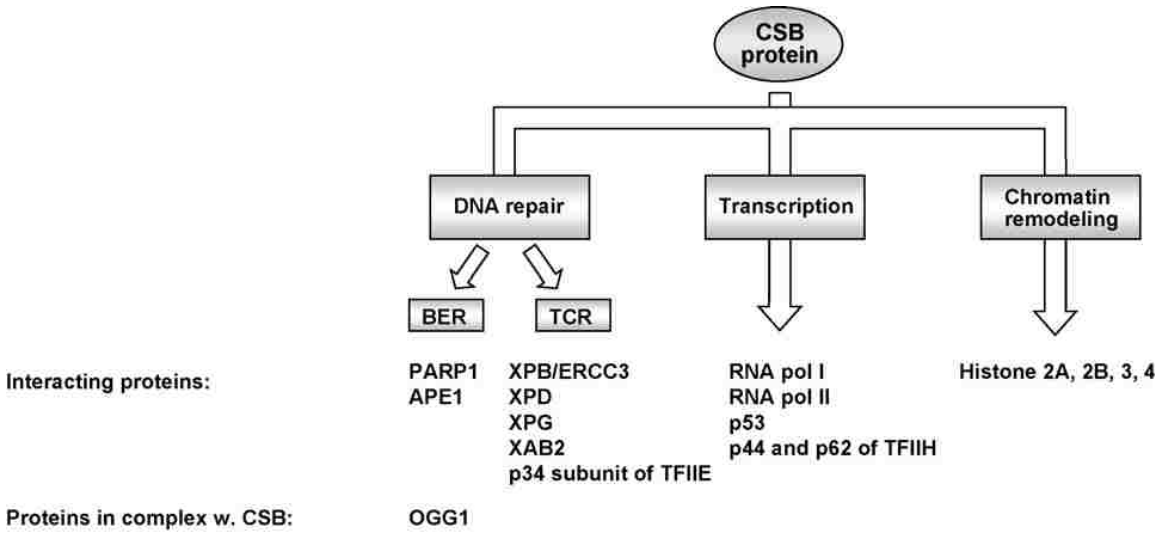

Figure 1-5 Processes in which CSB might participate. Based on proteins that have been demonstrated to interact with CSB, CSB could be involved in different processes. Adopted from (Stevnsner et al., 2008).

Besides mutations in *CSA* and *CSB* genes, three other mutations in XP genes: *XPB*, *XPD*, and *XPG* also cause severe CS with some concomitant XP symptoms (Stevnsner et al., 2008). Cells from XP/CS patients are completely deficient in NER, whether matter coupled to

transcription or not (Rapin et al., 2000). Thus, the deficiency of TCR does not account for the complex CS phenotype, since most XP patients (except XP-C and XP-E groups) are also TCR deficient, but do not suffer from typical CS symptoms (Rapin et al., 2000). It is interesting that different mutations in the same gene can cause such different clinical presentations. The panel of symptoms seems to closely correlate with the nature of the mutation. For example, point mutations in *XPG* gene that inactivate the endonuclease active site cause XP, whereas promoter mutations or mutations that yield a severely truncated and unstable XPG protein give rise to CS (Scharer, 2008). This implies that genes of XPB, XPD, and XPG probably function in several pathways, and that different mutations may impair their function in one or the other pathway (Nouspikel et al., 1997).

1.4.3 Trichothiodystrophy (TTD)

The defining feature of TTD is sulfur-deficient brittle hair caused by a reduced level of cysteine-rich matrix proteins. Associated features include mental retardation, short stature, ichthyosis, and in many cases, cutaneous photosensitivity but no increased cancer incidence (Nouspikel, 2009). TTD patients appear to be defective in NER as a consequence of alterations in one of three genes: *XPB* (Lehmann, 2003; Nouspikel, 2009), *XPD* (Weeda et al., 1997), and *TTD-A* (Stefanini et al., 1986), which all belong to subunits of TFIIH (Fig. 1-2).

The level of TFIIH was found to be substantially reduced in all TTD cells from XP-B,XP-D, and TTD-A groups (Giglia-Mari et al., 2004), suggesting TTD phenotype results from a reduced content of TFIIH, which is in turn caused by mutations that destabilize the structure of TFIIH. Given the fact that TFIIH is required for both transcription and NER, the symptoms of TTD may result from a subtle defect in transcription, besides the defect in NER (Botta et al., 2002). The reduced levels of TFIIH might be insufficient to provide adequate transcription activity. This might account for the deficiency in cysteine-rich matrix proteins in

brittle hair of TTD patients (Bootsma and Hoeijmakers, 1993). It has been shown recently that TFIIH changes subunit composition in response to UV irradiation. The detachment of CDK-activating kinase (CAK) complex from the core of TFIIH by XPA converts TFIIH from an elongation-proficient form into a repair-proficient form, suggesting TFIIH may dynamically change forms to adapt its engagement in both transcription and TCR processes (Coin et al., 2008). All the clinical features in TTD cannot be fully understood until the exact roles of TFIIH in different processes are completely deciphered.

XP, CS, and TTD are all categorized as segmental progeroid syndromes (PSs), which constitute a group of disorders characterized by clinical features mimicking physiological aging at an early age (Lehmann, 2003). Most human progeroid disorders are linked to defects in genome maintenance (Navarro et al., 2006). The study of these age-related disorders not only helps explain their severity, but also helps understand the mechanisms underlying aging.

To fully understand the mechnisms of NER, we need put the regulation of NER into the genome context and take other cellular processes such as transcription, DNA remodeling and cell cycle control into account. Some transcription factors are involved in both transcription and NER. The functional mechanisms of these factors are critical for NER regulation, especially for the coordination between TCR and transcription. This dissertation focused on the roles of yeast transcription factors Tfb5, Spt4, Spt5 and Rad26 in NER, especially in TCR.

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CHAPTER 2

TFB5 IS PARTIALLY DISPENSABLE FOR RAD26 MEDIATED TRANSCRIPTION COUPLED NUCLEOTIDE EXCISION REPAIR IN YEAST[*]

2.1 Introduction

Nucleotide excision repair (NER) is a ubiquitous DNA repair mechanism capable of removing a variety of helix-distorting lesions, including UV-induced cyclobutane pyrimidine dimers (CPDs) and photoproducts (Friedberg et al., 2006). NER is a multistep reaction and requires the coordinated action of about 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.

A specialized NER pathway, called transcription coupled NER (TC-NER, or TCR), refers to preferential repair in the transcribed strand (TS) of an actively transcribed gene. A transcribing RNA polymerase complex stalled at a DNA lesion on the TS may serve as a signal for rapidly recruiting NER machinery (Friedberg et al., 2006). Factors that are specifically required for TCR, such as Mfd, Rad26 and Cockayne syndrome complementation group A (CSA) and B (CSB) proteins, have been identified in *Escherichia coli*, yeast and human, respectively (Mellon and Hanawalt, 1989; Mellon et al., 1987; Selby and Sancar, 1993; Smerdon and Thoma, 1990; van Gool et al., 1994; van Hoffen et al., 1995). Rpb9, a non-essential subunit of RNA polymerase II (Pol II), has been shown to mediate a TCR mechanism that is independent of Rad26 in yeast (Li and Smerdon, 2002).

The genome-wide NER process is sometimes termed as global genomic NER (GG-NER, or GGR), to be distinguished from the process of TCR. In mammalian cells, xeroderma

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pigmentosum complementation group C (XPC) protein has been shown to be specifically required for GGR, but dispensable for TCR (Venema et al., 1991; Venema et al., 1990). In *Saccharomyces cerevisiae*, Rad7 and Rad16, which do not have significant sequence or structural similarity to XPC, are specifically required for GGR, but dispensable for TCR (Verhage et al., 1994). Rad7 and Rad16 are components of a complex that binds specifically to UV damaged DNA in an ATP-dependent manner, and the complex has DNA-dependent ATPase activity (Guzder et al., 1998). Besides Rad7 and Rad16, the complex may also contain several other factors, such as replicating sequence binding factor 1 (Abf1) (Reed et al., 1999; Yu et al., 2004). Recently, it was found that Rad7 and Rad16 are also components of an E3 ubiquitin ligase complex (Ramsey et al., 2004; Ribar et al., 2006).

In eukaryotic cells, most DNA sequences that were previously thought to be transcriptionally inert are actually transcribed (Struhl, 2007). Recent high resolution mapping of transcription in *S. cerevisiae* showed that a total of 85% of the genome is transcribed (David et al., 2006). Therefore, some genes or genomic sequences, which were previously supposed to be transcriptionally "silent" and were assumed to be repaired by GGR, may actually be repaired by both GGR and TCR mechanisms.

Transcription factor IIH (TFIIH) is a multi-protein complex required for both Pol II transcription and NER (Feaver et al., 1993; Schaeffer et al., 1994; Schaeffer et al., 1993; Wang et al., 1994). The role of TFIIH in transcription is mainly at the initiation stage, as it dissociates from the Pol II complex early in the transcription elongation process (Hahn, 2004). The role of TFIIH in NER can be independent of transcription, as the complex is required in reconstituted cell free reactions in the complete absence of transcription (Araujo et al., 2000; Guzder et al., 1995). It has been shown that the formation of an open DNA structure around a lesion during NER requires the ATP-dependent helicase activities of TFIIH (Evans et al., 1997a; Evans et al.,

1997b; Mu et al., 1997). TFIIH may play a unique role in TCR. In a short region around the transcription start site, the TS is preferentially repaired in the absence of Rad26 in yeast (Tijsterman et al., 1997), or CSA and CSB in human cells (Tu et al., 1997, 1998). Preferential repair in the more down stream regions of the TS, however, requires the TCR factors Rad26 in yeast (Tijsterman et al., 1997), and CSA and CSB in human cells (Tu et al., 1997, 1998). It is therefore proposed that TFIIH engaged in transcription initiation may play a direct role in TCR. Furthermore, recent studies suggest that a TCR complex may be formed without the displacement of Pol II from the DNA (Brueckner et al., 2007; Fousteri et al., 2006; Sarker et al., 2005; Tremeau-Bravard et al., 2004). TFIIH can be recruited to the TCR complex and remodel Pol II to let NER machinery gain access to the lesion on the TS (Fousteri et al., 2006; Laine and Egly, 2006; Sarker et al., 2005). Therefore, TFIIH may also play a special role in TCR when Pol II is in transcription elongation mode.

Trichothiodystrophy (TTD) is a premature aging syndrome, characterized by sulfur-deficient brittle hair and nails resulting from a reduced level of cysteine-rich matrix proteins. Associated features include progressive mental and physical retardation, ichthyosis, β-thalassaemia trait, unusual facial features, and in many cases photosensitivity (Friedberg et al., 2006). TTD can be caused by mutations in XPB and XPD, two TFIIH subunits that have ATP-dependent DNA helicase activities. The third group of TTD (TTD-A) has recently been found to be caused by mutations in Tfb5, the tenth subunit of TFIIH (Giglia-Mari et al., 2004; Ranish et al., 2004). Tfb5 is highly conserved, with a sequence identity of 28% and a sequence similarity of 56% between human and yeast (Giglia-Mari et al., 2004). In humans, the absence of Tfb5 seems to affect TFIIH stability because the steady-state level of TFIIH in TTD-A cells is about 25–30% of its wild type counterpart (Botta et al., 2002; Giglia-Mari et al., 2004; Vermeulen et al., 2000). TTD-A cells are mildly UV sensitive. A UV-induced DNA synthesis

assay, which measures overall NER synthesis, suggested that NER capacity in TTD-A cells is ~ 10% of that in wild type cells (Botta et al., 2002; Giglia-Mari et al., 2004; Vermeulen et al., 2000). In yeast, Tfb5 does not seem to affect TFIIH stability (Ranish et al., 2004). However, deletion of Tfb5 also causes mild UV sensitivity (Ranish et al., 2004; Vermeulen et al., 2000). Furthermore, it was recently shown that whole cell extracts from yeast *tfb5* cells is deficient in overall NER (Vermeulen et al., 2000). However, how Tfb5 affects NER in intact yeast cells is still not well known.

In view of the observations that Tfb5 is not absolutely required for NER (Botta et al., 2002; Giglia-Mari et al., 2004; Vermeulen et al., 2000; Zhu et al., 2007) and that TFIIH may work on different architectural complexes during different NER processes in the cells (Friedberg et al., 2006), it is possible that Tfb5 plays a different role in different NER pathways. We attempted to address this issue by analyzing the roles of Tfb5 in NER in different well-defined yeast NER mutants. We found that Tfb5 is partially dispensable for Rad26 mediated TCR, especially in GGR deficient cells. However, this TFIIH subunit is required for other NER pathways.

2.2 Materials and Methods

2.2.1 Yeast Strains

Wild type yeast strain BJ5465 (*MATa ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*) was obtained from the American Type Culture Collection. All deletion mutants were made in BJ5465 background. The cells were transformed with linearized plasmids bearing the respective genes to be deleted, with a portion of their genes replaced by the yeast URA3 or LEU2 gene as described previously (Li and Smerdon, 2002). The transformed cells were selected on SD plates containing no uracil or leucine at 30 °C. In order to introduce a

second deletion using a plasmid bearing the gene of interest replaced by the URA3 gene, the previously introduced URA3 gene that had replaced the first gene was knocked out. The URA3 knockout was done by transforming the cells with a linearized plasmid bearing a truncated (with the sequence between the sites of StuI and EcoRV removed) URA3 gene, and selecting the cells on SD plates containing 5-fluoro-orotic acid (Boeke et al., 1984). All the deletions were confirmed by PCR analysis. Nucleotides (with respect to the starting codon ATG) +214 to +1454, +58 to +2297, +11 to +366 and +15 to +202 were deleted for the *RAD7*, *RAD26, RPB9* and *TFB5* genes, respectively.

2.2.2 UV Irradiation, Repair Incubation and DNA Isolation

Yeast cells were grown at 30 °C in minimal medium containing 2% galactose to late log phase ($A_{600} \sim 1.0$), harvested, and washed twice with ice-cold water. The washed cells were resuspended in 2% galactose and irradiated with 100 J/m^2 of 254 nm UV light. One-tenth volume of a stock solution containing 10% yeast extract and 20% peptone was immediately added to the irradiated cell suspension, and the cells were incubated for various times in the dark at 30 °C before being pelleted. The pelleted cells were broken with glass beads and the genomic DNA was isolated using a hot SDS procedure as described previously (Li and Smerdon, 2002).

2.2.3 NER Analysis of UV-Induced CPDs

The gene fragments of interest were 3′-end labeled with [α-^{32}P]dATP using a procedure described previously (Li and Waters, 1996; Li et al., 2000). Briefly, ~1 μg of total genomic DNA was digested with restriction enzyme(s) to release the fragments of interest and incised at CPD sites 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 5 min 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 [(-^{32}P]dATP (Perkin-Elmer), and resolved on sequencing gels. The gels were dried and exposed to a Phosphorimager screen (Bio-Rad).

The signal intensities at gel bands corresponding to CPD sites were quantified using Quantity One software (Bio-Rad). The total signal intensity in a lane of a gel was obtained after the gel background signal was subtracted. The total signal intensity in a lane was used to normalize the loading of different lanes in a gel. The percent CPDs remaining at individual sites following different times of repair incubation were then calculated.

2.3 Results

2.3.1 Tfb5 Is Required for GGR

We examined the roles of Tfb5 in NER in yeast cells using a high resolution (nucleotide level) technique. One of the most obvious advantages for using this technique is that repair rates in different regions of a fragment can be resolved on the same gel, and therefore a small difference of repair among different regions can usually be unambiguously identified. Wild type and different NER deficient cells were cultured to late log phase, UV irradiated and incubated for different periods of time. Genomic DNA was isolated, digested with restriction enzymes to release the gene fragment of interest and incised at CPD sites with an excess amount of T4 endonuclease V (Lloyd, 2005). The incised fragments were strand specifically end labeled, resolved on DNA sequencing gels and exposed to phosphorimager screens, as previously described (Li and Waters, 1996; Li et al., 2000).

To investigate the role of Tfb5 in GGR, we first analyzed repair in the NTS of the constitutively expressed *RPB2* gene and the galactose-induced *GAL1* genes. The reason for

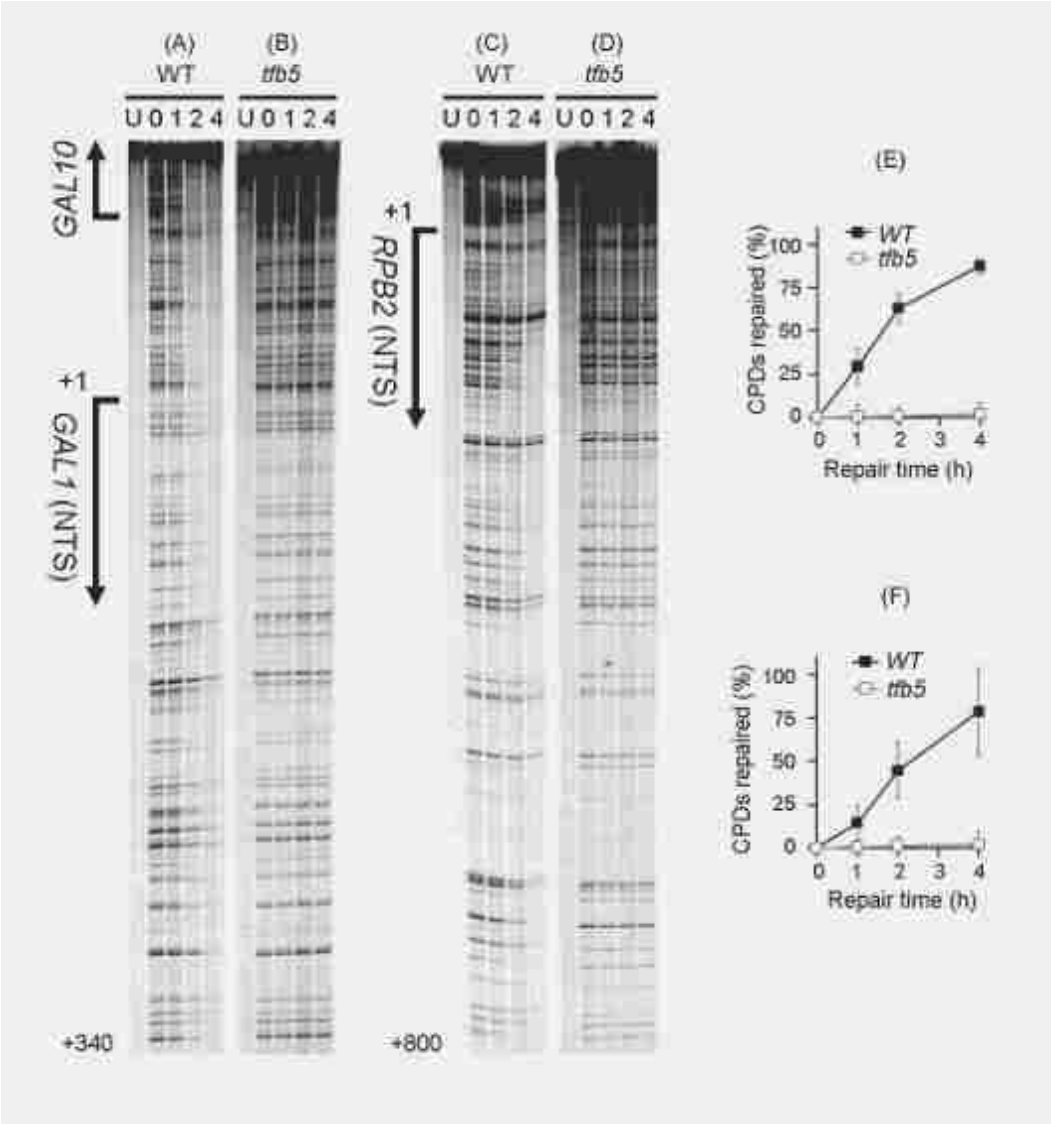

Figure 2-1 NER in the NTS of the induced *GAL1* gene and the constitutively expressed *RPB2* gene in wild type (WT) and *tfb5* cells. (A)–(D) Gels showing NER in the NTS of the two genes. (E) Plot showing percent CPDs repaired (mean ± standard deviation) in the NTS (+1 to +340) of the *GAL1* gene. The data were obtained by quantification of the gels shown in panels (A) and (B). (F) Plot showing percent CPDs repaired (mean ± S.D.) in the NTS (+1 to +800) of the *RPB2* gene. The data were obtained by quantification of the gels shown in panels (C) and (D).

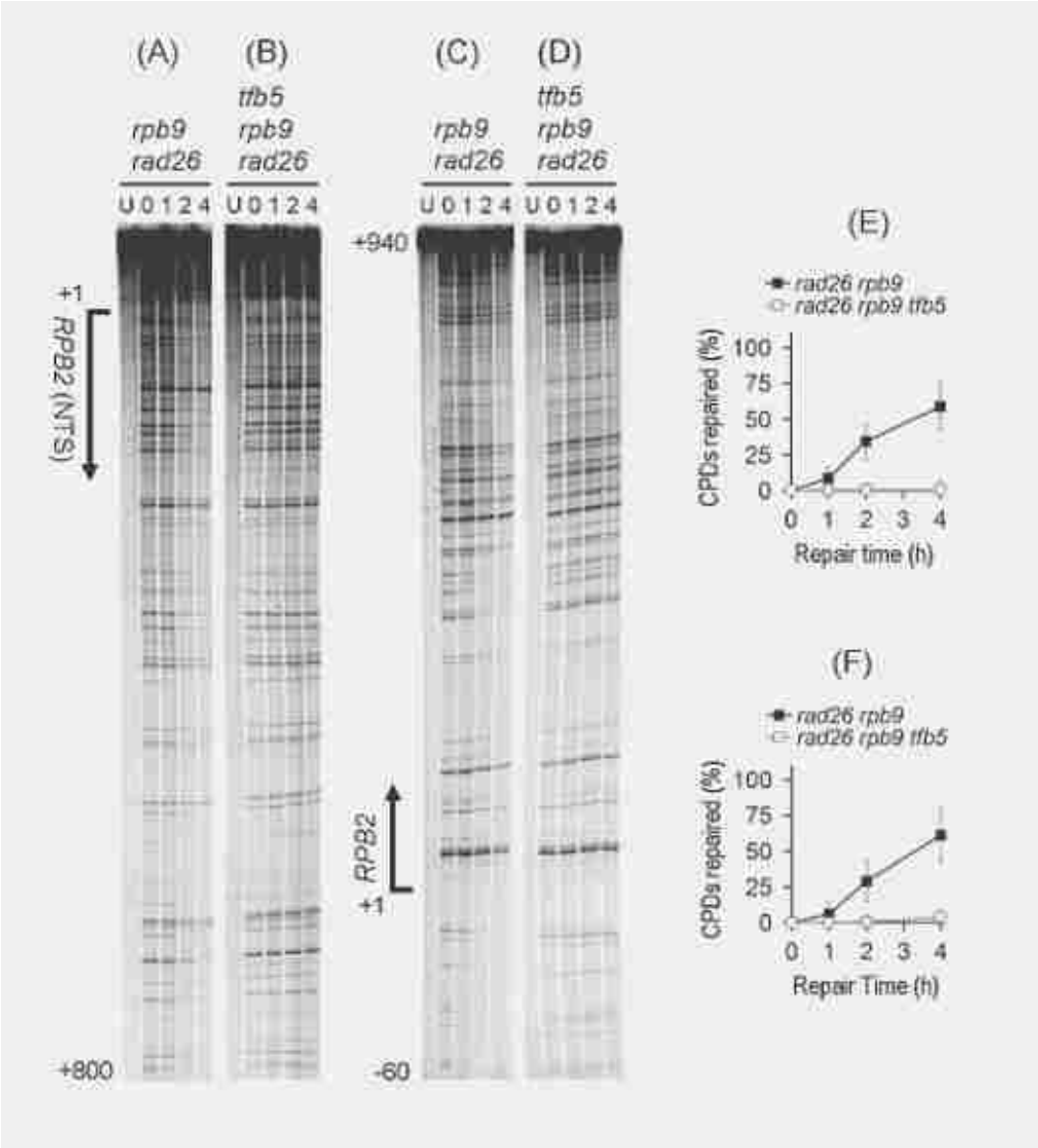

Figure 2-2 NER in the *RPB2* gene of TCR deficient *rad26 rpb9* cells. (A)–(D) Gels showing NER in the *RPB2* gene in *rad26 rpb9* and *rad26 rpb9 tfb5* cells. (E) Plot showing percent CPDs repaired (mean ± S.D.) in the NTS (+1 to +800) of the *RPB2* gene. The data were obtained by quantification of the gels shown in panels (A) and (B). (F) Plot showing percent CPDs repaired (mean ± S.D.) in the TS (+1 to +940) of the *RPB2* gene. The data were obtained by quantification of the gels shown in panels (C) and (D).

choosing these two genes for analysis is that transcriptions of these genes are well characterized. Furthermore, many studies (Li and Smerdon, 2002, 2004; Verhage et al., 1994; Verhage et al., 1996) have shown that NER in the NTS of the two actively transcribed genes is absolutely dependent on Rad7/Rad16, indicating that repair in the NTS of these genes is entirely accomplished by GGR. As expected, apparent repair occurred in wild type cells (Fig. 2-1A, C, E and F). However, no repair can be seen in the NTS of the genes in *tfb5* cells (Fig. 2-1B, D–F), indicating Tfb5 is required for GGR. To confirm this result, we analyzed the role of Tfb5 in *rad26 rpb9* cells, where TCR is completely abolished, so that GGR can be unambiguously examined (Li and Smerdon, 2002, 2004). Apparent repair occurred in both strands of the *RPB2* gene in *rad26 rpb9* cells (Fig. 2-2A, C, E and F). In fact, GGR in some sites of the gene was very efficient in the TCR deficient cells (Fig. 2-2A and C). However, no repair can be seen in any sites of the gene in *rad26 rpb9 tfb5* cells (Fig. 2-2B, D–F). Taken together, our results indicate that Tfb5 is required for GGR.

2.3.2 Tfb5 Is Required for Rpb9 Mediated TCR, but Partially Dispensable for Rad26 Mediated TCR in the Repair of Constitutively Expressed *RPB2* Gene

It has been shown that deletion of *TFB5* in yeast does not significantly affect transcription of most genes (Ranish et al., 2004). In agreement with the previous report, we found that the levels of *RPB2* transcription are similar between wild type and *tfb5* cells (not shown). Rapid repair can be seen in the TS of the *RPB2* gene in wild-type cells (Figs. 2-3A and 4A). However, only residual (~15%) repair can be seen in the coding region of the TS in *tfb5* cells during the repair incubation of 4 h (Figs. 2-3B and 2-4A). As mentioned above, no repair can be detected in the NTS of the *RPB2* gene in *tfb5* cells. These results indicate that Tfb5 is important, but not absolutely required for TCR. It has been shown that Rad7 and Rad16 are required for repairing the NTS of transcriptionally active genes (Verhage et al., 1994). Therefore,

rad7/*rad16* cells have been repeatedly used to unambiguously examine TCR (e.g., see refs. (Li and Smerdon, 2002, 2004; Teng et al., 1997; Tijsterman et al., 1997). In agreement with previous reports (Verhage et al., 1994; Verhage et al., 1996), no repair can be seen in the NTS (not shown), or in the region of the TS that is over 40 nucleotides upstream of the transcription start site (Fig. 2-3C). Interestingly, substantial (~60%) repair occurred in the coding region of the TS in *rad7 tfb5* cells during the repair incubation of 4 h (Figs. 2-3D and 2-4B). This result indicates that Tfb5 can be largely dispensable for TCR in GGR deficient *rad7* cells.

In agreement with our previous studies (Li et al., 2006; Li and Smerdon, 2002, 2004), simultaneous elimination of Rad7 (or Rad16), Rad26 and Rpb9 completely abolishes NER in yeast (Fig. 2-3I). In *rad7 rad26* cells, where only Rpb9 mediated TCR is operative (Fig. 2-3, compare panels E and I), obvious repair can be seen in the coding region of the TS, especially in the short region immediately downstream the transcription start site (Fig. 2-3E, marked by the bracket; Fig. 2-4C). It has been suggested that the rapid repair in the short region immediately downstream of the transcription start site is accomplished by TFIIH, which is associated with Pol II during transcription initiation (Tijsterman et al., 1997; Tu et al., 1997, 1998). However, no repair can be seen in the *RPB2* gene of *rad7 rad26 tfb5* cells, including the short region immediately downstream of the transcription start site (Figs. 2-3F and 2-4C). These results indicate that Tfb5 is required for Rpb9 mediated TCR throughout the *RPB2* gene.

In *rad7 rpb9* cells, where only Rad26 mediated TCR operates (Fig. 2-3, compare panels G and I), very fast repair can be seen in the coding region of the TS (Figs. 2-3G and 2-4D). Substantial repair can also be seen in the coding region of the TS in *rad7 rpb9 tfb5* cells (Figs. 2-3H and 2-4D), indicating that Tfb5 is partially dispensable for Rad26 mediated TCR in GGR deficient cells.

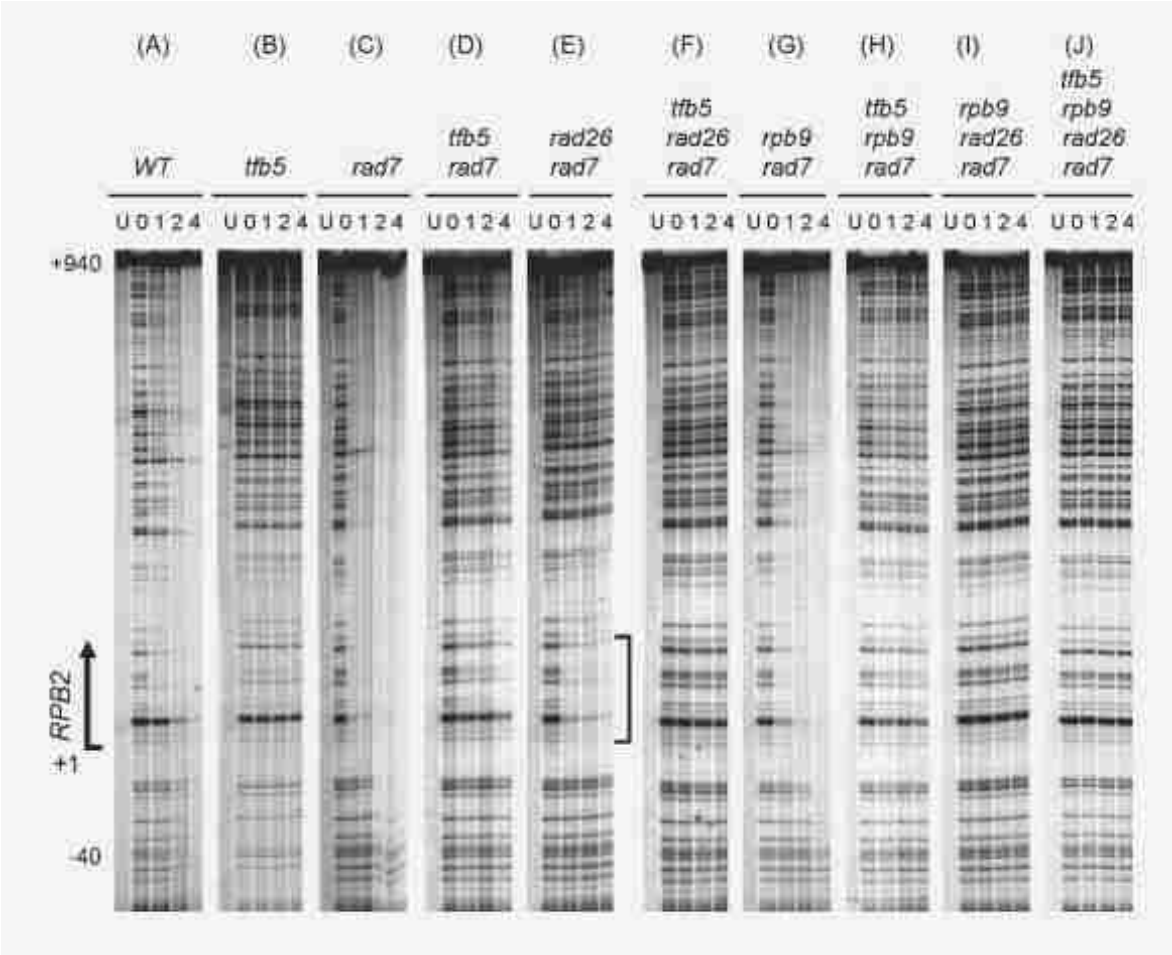

Figure 2-3 TCR in the constitutively transcribed *RPB2* gene The lanes are DNA samples from unirradiated (U) and UV irradiated cells following 0, 1, 2 and 4 h repair incubation. The arrow on the left of the gels indicates the transcription start site. The bracket on the right of panel (E) marks the region where robust Rad26-independent TCR occurs.

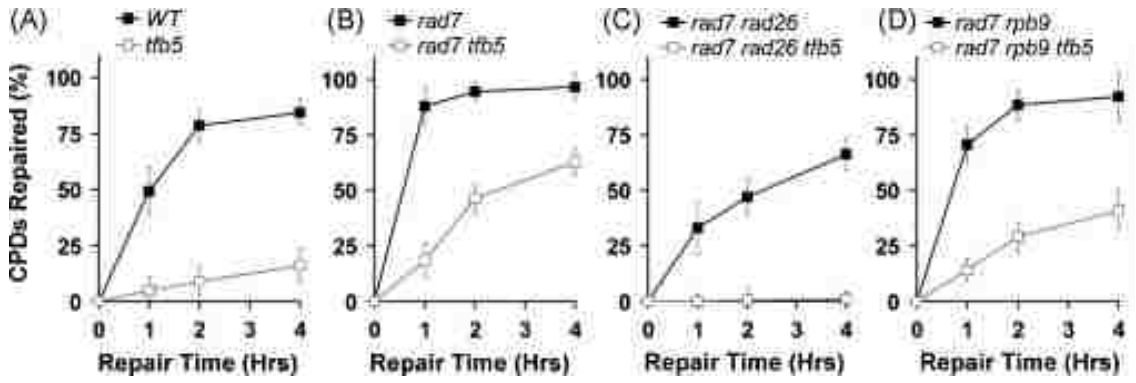

Figure 2-4 Plots showing repair of CPDs in the TS (+1 to +940) of the *RPB2* gene. The data were obtained by quantification of the gels shown in panels (A)–(H) of Figure 3. The values are shown as average (mean ± S.D.) of the percent CPDs repaired at different times of repair incubation.

2.3.3 Tfb5 Plays a Similar Role in TCR in the Induced *GAL1* Gene

To see how general the TCR trends we observed in the *RPB2* gene is, we also analyzed TCR in the *GAL1* gene, which is highly induced by galactose (Bash and Lohr, 2001; Lohr et al., 1995). In contrast to the *RPB2* gene, whose transcription level is not significantly affected by Tfb5 (see above) (Ranish et al., 2004), the *GAL1* gene showed ~5–10-fold reduction of transcription in *tfb5* cells (not shown). This level of transcriptional reduction in the *GAL1* gene agrees well with a previous report (Ranish et al., 2004). As the transcription level of the induced *GAL1* gene in wild type cells is extremely high (Lohr et al., 1995), we reasoned that a relatively high level of transcription still occurred in the induced *GAL1* gene in *tfb5* cells.

The trends of Rad26 and Rpb9 mediated TCR are similar between the *RPB2* and *GAL1* genes, except that TCR in *rad7 rad26* (or *rad16 rad26*) cells is slower in the RPB2 gene (compare Figs. 2-3E, 2-5E, 2-4C and 2-6C) (Li and Smerdon, 2002). Also, the TCR initiation site in the *GAL1* gene is at about nucleotide -180 (relative to the transcription start site) (Fig. 2-5), which is more upstream than that of the *RPB2* gene (at about nucleotide −40) (Fig. 2-3). Similar

to the TS of the *RPB2* gene, the TS of the *GAL1* gene showed substantial repair in *rad7 tfb5* (Figs. 2-5D and 2-6B) and *rad7 rpb9 tfb5* cells (Figs. 2-5H and 2-6D), but no apparent repair in *rad7 rad26 tfb5* cells (Figs. 2-5F and 2-6C). These results indicate that, in the induced *GAL1* gene, Tfb5 is required for Rpb9 mediated TCR, but partially dispensable for Rad26 mediated TCR, especially in GGR deficient cells.

2.4 Discussion

In human cells, the role of Tfb5 in NER may be achieved at least partially by stabilizing TFIIH (Vermeulen et al., 2000). In addition, the human Tfb5 may play a direct role in NER. A recent *in vitro* study showed that human Tfb5 (p8/TTD-A) triggers DNA opening by stimulating XPB ATPase activity together with the damage recognition factor XPC-hHR23B (Coin et al., 2006). In yeast, the role of Tfb5 in NER may be accomplished primarily by a direct action. It has been shown that the steady-state levels of other TFIIH subunits are not changed in *tfb5* cells (Ranish et al., 2004). We also observed that the steady-state levels of TFIIH subunits are similar between wild type and *tfb5* strains we used (not shown). Yeast Tfb5 interacts with Tfb2, another subunit of TFIIH (Zhu et al., 2007). It was proposed that the yeast Tfb5 acts as an architectural stabilizer conferring structural rigidity to the core TFIIH such that the complex is maintained in its functional architecture (Zhu et al., 2007).

Although it plays a stimulatory role (Coin et al., 2006), Tfb5 is not absolutely required for NER *in vitro*, as the core TFIIH complex (without Tfb5 and the CTD kinase subunits) is able to open DNA around a lesion (Araujo et al., 2000; Svejstrup et al., 1995). Our data presented here indicates that Tfb5 is essential for GGR *in vivo* (Fig. 2-1 and Fig. 2-2). One possibility is that the XPB ATPase activity of TFIIH needs to be stimulated by Tfb5 (Coin et al., 2006) to efficiently open the DNA structure around a lesion in the chromatin environment *in vivo*.

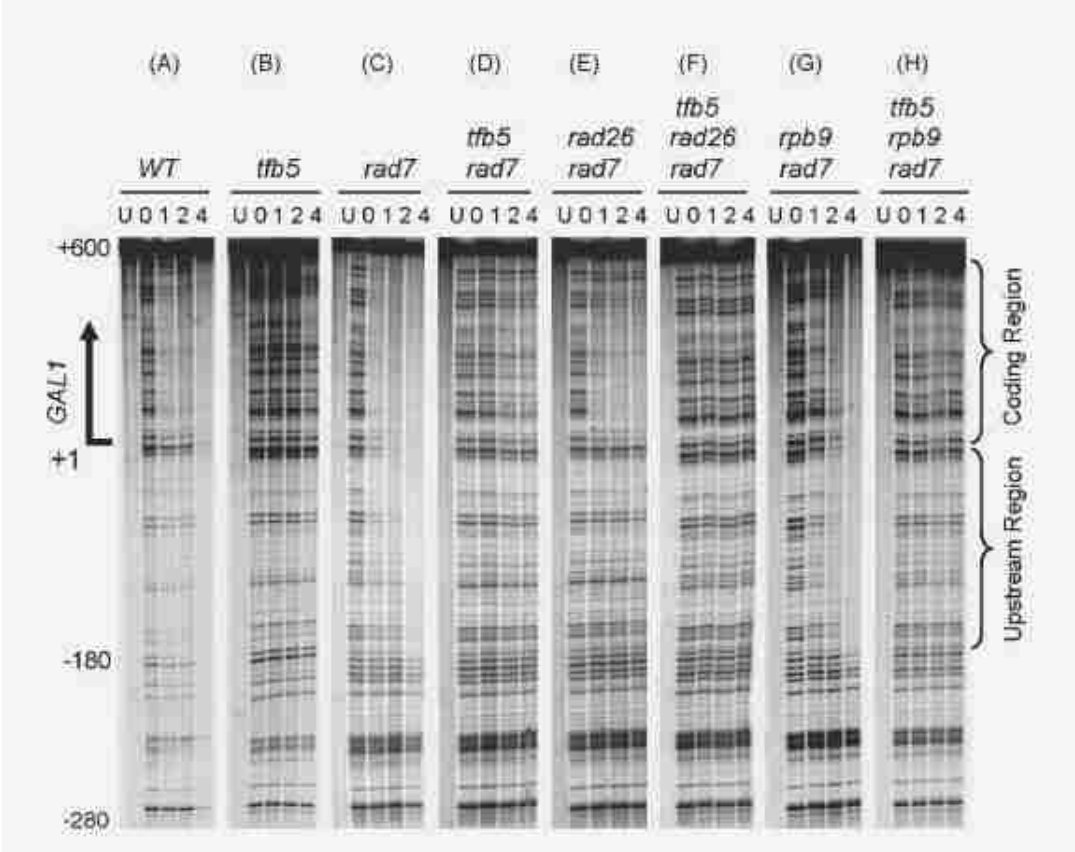

Figure 2-5 TCR in the galactose-induced *GAL1* gene The lanes are DNA samples from unirradiated (U) and UV irradiated cells following 0, 1, 2 and 4 h repair incubation. The arrow on the left of the gels indicates the transcription start site.

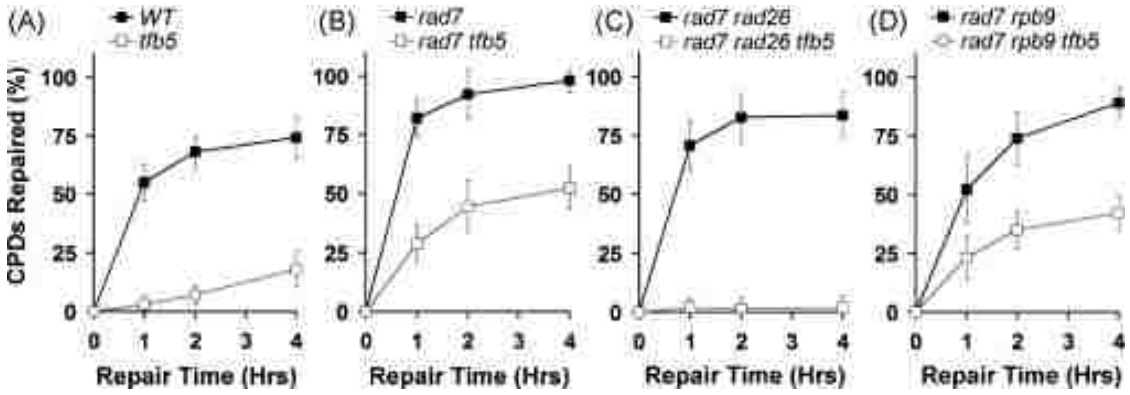

Figure 2-6 Plots showing repair of CPDs in the TS (+1 to +600) of the *GAL1* gene. The data were obtained by quantification of the gels shown in panels (A)–(H) of Figure 2-5. The values are shown as average (mean ± S.D.) of the percent CPDs repaired at different times of repair incubation.

Alternatively, the Tfb5-lacking TFIIH may not be efficiently recruited to a GGR complex in the cells. The role of Tfb5 in GGR may be similar to that of the Rad7/Rad16 complex, which is also not absolutely required for NER *in vitro*, although it dramatically stimulates the process (Boeke et al., 1984). However, the Rad7/Rad16 complex is essential for GGR *in vivo* (Verhage et al., 1994; Verhage et al., 1996).

We observed that Tfb5 is partially dispensable for Rad26 mediated TCR. During TCR, TFIIH is recruited to damaged DNA only in the presence of Rad26 (You et al., 1998). One possibility is that Tfb5-lacking TFIIH can still be efficiently recruited to a TCR complex by Rad26. The recruited Tfb5-lacking TFIIH may, to some extent, be able to open the DNA around a lesion. However, this scenario cannot explain the Rad26-independent TCR close to the transcription start site. TFIIH is essential for transcription initiation, and is obligatorily loaded to the transcription initiation complex (Hahn, 2004). TCR in the yeast URA3 gene becomes Rad26 dependent 30–40 nucleotides downstream from the transcription start site (Tijsterman et al., 1997). In the human JUN gene, TCR becomes CSA and CSB dependent at about +20 nucleotides into the coding region (Tu et al., 1997, 1998). It has been proposed that this TCR factor-independent TCR close to the transcription start site may be due to the association of TFIIH with Pol II, as TFIIH is believed to be released from Pol II 30–60 nucleotides downstream from the start site (Svejstrup, 2002). In this study, we also observed efficient TCR close to the transcription start site in the RPB2 gene of rad7 rad26 cells (Fig. 2-3E, marked with a bracket). However, simultaneous deletion of TFB5 and RAD26 genes completely abolished TCR in this region of the gene (Fig. 2-3F), indicating that the Rad26-independent TCR in this region is Tfb5 dependent. Therefore, an alternative scenario would be that Rad26 may be able to facilitate the opening of lesion-containing DNA at the transcription bubble without the participation of a fully functional (Tfb5-containing) TFIIH. Rad26 is a DNA-dependent ATPase (Guzder et al., 1996). It

also contains a DNA helicase motif, although no helicase activity can be detected (Gray et al., 1997). Recent studies in human cells or purified NER factors suggested that a TCR complex may be formed without the displacement or degradation of Pol II (Brueckner et al., 2007; Fousteri et al., 2006; Sarker et al., 2005; Tremeau-Bravard et al., 2004). Our recent results in yeast also suggest that ubiquitylation and subsequent degradation of Rpb1, the largest subunit of Pol II, is unrelated to TCR (Li et al., 2007).

We constantly observed that TCR is faster in GGR deficient *rad7*/*rad16* cells than in wild type cells (compare Fig. 2-3A and C, and Fig. 2-5A and C; see also Fig. 2-1 in ref. (Li and Smerdon, 2002)). Interestingly, we also observed that TCR is more efficient in *rad7 tfb5* cells than in *tfb5* cells (Fig. 2-3, Fig. 2-4, Fig. 2-5 and Fig. 2-6). The reason(s) for the observations remain(s) to be understood. One explanation is that the Rad7/Rad16 complex and Rad26 may compete for NER factors that are shared by different NER pathways, and elimination of Rad7/Rad16 may make more NER factors available to be recruited by Rad26. An alternative explanation is that Rad7/Rad16 may play a role in inhibiting TCR.

In contrast to Rad26 mediated TCR, Rpb9 mediated TCR appears to be dependent on Tfb5. Our recent data suggests that the transcription elongation function of Rpb9 is involved in TCR, and impairment of transcription elongation abolishes Rpb9 mediated TCR (Li et al., 2006). However, the requirement of Tfb5 for Rpb9 mediated TCR does not seem to be due to a role for Tfb5 in transcription elongation. Cells with a deficiency in transcription elongation are generally sensitive to nucleotide-depleting drugs, such as 6-azauracil and mycophenolic acid (Hyle et al., 2003). However, *tfb5* cells are not sensitive to these drugs (not shown). Therefore, without the involvement of Rad26, the Tfb5-lacking TFIIH may not be able to remodel the Pol II complex stalled at a lesion or to open the DNA round the lesion at the transcription bubble *in vivo*, resulting in deficiency in Rad26-independent TCR.

In view of the fact that Tfb5 (Giglia-Mari et al., 2004) and the NER process (Friedberg et al., 2006) are highly conserved between yeast and humans, it is reasonable to speculate that Tfb5 plays similar roles in different NER pathways in human cells. It would be very interesting to test this idea.

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CHAPTER 3

THE C-TERMINAL REPEAT DOMAIN OF SPT5 PLAYS AN IMPORTANT ROLE IN SUPPRESSION OF RAD26-INDEPENDENT TRANSCRIPTION COUPLED REPAIR*

3.1 Introduction

Nucleotide excision repair (NER) is a conserved DNA repair mechanism capable of removing a variety of helix-distorting lesions, such as UV-induced cyclobutane pyrimidine dimers (CPDs) (Friedberg et al., 2006). NER can be grouped into two pathways: global genomic repair (GGR), which refers to repair throughout the genome, and transcription coupled repair (TCR), which refers to a repair mechanism that is dedicated to the transcribed strand of actively transcribed genes (Hanawalt, 2002). In the yeast *Saccharomyces cerevisiae*, Rad7, Rad16 (Verhage et al., 1994) and Elc1 (Lejeune et al., 2009) are specifically required for GGR, but dispensable for TCR. Rad7 and Rad16 form a complex that binds specifically to UV damaged DNA in an ATP-dependent manner and has DNA-dependent ATPase activity (Guzder et al., 1998). 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 (Gillette et al., 2006; Ramsey et al., 2004). It has also been suggested that Elc1 is a component of another ubiquitin ligase complex, which contains Ela1, Cul3, and Roc1 but not Rad7 and Rad16 (Ribar et al., 2006, 2007). The role of Elc1 in GGR may not be subsidiary to that of Rad7 and Rad16 (Lejeune et al., 2009).

The mechanistic details of TCR are relatively well understood in *Escherichia coli.* The transcription-repair coupling factor Mfd targets the transcribed strand for repair by recognizing a stalled RNA polymerase and actively recruiting the NER machinery to the transcription blocking

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lesion as it dissociates the stalled RNA polymerase (Selby and Sancar, 1993). Conversely, the TCR mechanisms in eukaryotes appear to be extremely complicated [for reviews see (Fousteri and Mullenders, 2008; Hanawalt and Spivak, 2008)]. In mammalian cells, Cockayne syndrome group A (CSA) and B (CSB) proteins are specifically required for TCR, but dispensable for GGR (Lommel and Hanawalt, 1991; Troelstra et al., 1992; van Hoffen et al., 1993; Venema et al., 1990). Like its human homolog CSB, the yeast Rad26 plays an important role in TCR but is dispensable for GGR (van Gool et al., 1994). Both human CSB (Selby and Sancar, 1997b) and yeast Rad26 (Guzder et al., 1996) are DNA-stimulated ATPases and play roles in transcription elongation (Lee et al., 2001; Selby and Sancar, 1997a). However, TCR in yeast is not solely dependent on Rad26, as a substantial extent of TCR still occurs in cells lacking Rad26 (Li and Smerdon, 2002, 2004; Verhage et al., 1996). Rpb9, a nonessential subunit of RNA polymerase II (Pol II), has also been shown to play a role in TCR (Li et al., 2006; Li and Smerdon, 2002, 2004; Saunders et al., 2006).

Mutations in the *SPT4* and *SPT5* genes in yeast were originally isolated as suppressors of the Ty insertion mutations that interfere with adjacent gene transcription (Winston et al., 1984). When the Ty sequence is inserted in the upstream region of a gene, the transcription signal directs transcription from the Ty promoter and interferes with normal transcription of the adjacent gene. A mutation in *SPT4* or *SPT5* attenuates the aberrant transcription, restoring transcription from the normal site. The *SPT4* gene is dispensable (Malone et al., 1993) whereas the *SPT5* gene is essential (Swanson et al., 1991b) for cell viability. Immunoprecipitation studies showed that Spt4 and Spt5 form a complex, which physically interacts with Pol II (Hartzog et al., 1998). Yeast cells lacking Spt4 show reduced efficiency of Pol II elongation through GC-rich DNA sequences and a general decrease in Pol II processivity (Mason and Struhl, 2005; Rondon et al., 2003). These proteins are conserved eukaryotic transcription-elongation factors and are

generally required for normal development and for viral gene expression in multicellular eukaryotes (Winston, 2001). In mammalian cells, the Spt4/Spt5 complex, which is also called DRB sensitivity inducing factor (DSIF), represses transcription elongation at the elongation-recessive elongation transition (Wada et al., 1998; Yamaguchi et al., 1999). Phosphorylation of the C-terminal repeat region of Spt5 plays a key role in converting the complex from a repressor to a positive regulator of transcription (Yamada et al., 2006; Zhu et al., 2007).

Interestingly, it was shown that deletion of *spt4* alleviates the requirement of Rad26 for TCR in yeast, indicating that Spt4 suppresses Rad26-independent TCR (Jansen et al., 2000). Up to date, whether and/or how Spt5 is involved in the suppression is unclear. Like the *spt4* deletion, an Spt5 point mutation, *spt5-194*, results in Spt$^-$ phenotype (i.e., unable to suppress Ty insertion mutations) and is sensitive the nucleotide depletion drug 6-azauracil, indicating that the *spt4* deletion and the *spt5-194* mutation may cause similar deficiencies in transcription elongation (Hartzog et al., 1998; Swanson and Winston, 1992). In addition, *spt5-194* combined with an *spt4* mutation leads to synthetic lethality (Swanson and Winston, 1992). However, unlike *spt4Δ*, the *spt5-194* mutation does not suppress the UV sensitivity of *rad16Δ rad26Δ* cells (Jansen et al., 2000). This observation led to the proposition that, unlike Spt4, Spt5 may not play a role in suppressing Rad26-independent TCR or that, despite the shared phenotypes with *spt4Δ*, the specific *spt5-194* mutation may not lead to a defect in the suppression. In this paper, we present evidence that Spt4 indirectly suppresses Rad26-independent TCR by protecting Spt5 from degradation and by stabilizing the interaction of Spt5 with Pol II. We further found that 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, plays an important role in suppressing Rad26-independent TCR.

3.2 Materials and Methods

3.2.1 Yeast Strains and Plasmids

Yeast strains used in this study are listed in Table 1. Wild type yeast strain BJ5465 (*MATa ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1*) was obtained from the American Type Culture Collection. All deletion mutants were made in BJ5465 background and confirmed by PCR analysis, using procedures described previously (Li and Smerdon, 2002). *URA3*, *LEU2* and *KanMX* were used to replace the gene to be deleted. In some cases, the *URA3* gene that had replaced a gene was further knocked-out as described previously (Li and Smerdon, 2002). Nucleotides (with respect to the starting codon ATG) + 14 to + 288, + 51 to + 2400 , + 214 to + 1454, + 58 to + 2297, and + 204 to + 730 were deleted for *SPT4*, *SPT5*, *RAD7*, *RAD26,* and *BUR2* genes, respectively. Strains with their genomic genes tagged with three consecutive FLAG (3xFLAG) sequences were created using PCR products amplified from plasmid p3FLAG-KanMX, as described previously (Gelbart et al., 2001).

A plasmid overexpressing 3xFLAG tagged Spt5 under the control of the *GAL10* promoter were created using vector pESC-URA (Fig. 2A). Two consecutive FLAG sequences were inserted in-frame downstream of the FLAG sequence (downstream of the *GAL10* promoter) present in the original pESC-URA vector to create a vector encoding 3xFLAG. The *SPT5* gene coding sequence was amplified by PCR and inserted in-frame downstream of the 3xFLAG sequence to create plasmid pGAL-SPT5 (Fig. 2A).

A single-copy centromeric plasmid with the *URA3* gene as a selection marker and encoding the wild type Spt5 protein was created by using the plasmid pRS416 (Sikorski and Hieter, 1989) . The full length of the *SPT5* gene encompassing the 5’ promoter, the coding region and the 3’ terminator was amplified by PCR and inserted between the BamHI and EagI

Table 3-1 Yeast strains used in this study

Strains	Genotype[a]	Reference/Source
BJ5465	*MATa ura3-52 trp1 leu2D1 his3D200 pep4::HIS3 prb1D1.6R can1*	(Jones, 1991)
CR18	as BJ5465, but *rad7Δ rad26Δ*	(Ding et al, 2007)
CR78	as BJ5465, but *rad7Δ rad26Δ spt4::LEU2*	This study
BD4	as BJ5465, but *rad7Δ rad26Δ* (*SPT4*-3´FLAG)	This study
BD7	as BJ5465, but *rad7::URA3* (*SPT5*-3´FLAG)	This study
BD9	as BJ5465, but (*SPT5*-3´FLAG)	This study
BD10	as BJ5465, but *rad7Δ rad26Δ* (*SPT5*-3´FLAG)	This study
BD13	as BJ5465, but *spt4::LEU2* (*SPT5*-3´FLAG)	This study
BD14	as BJ5465, but *rad7Δ spt4::LEU2* (*SPT5*-3´FLAG)	This study
BD15	as BJ5465, but *rad7Δ rad26Δ spt4::LEU2* (*SPT5*-3´FLAG)	This study
BD16	as CR18, but [pGAL-SPT5]	This study
BD17	as CR78, but [pGAL-SPT5]	This study
BD21	as BJ5465, but [pGAL-SPT5]	This study
BD56	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5]	This study
BD57	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/CTRD]	This study
BD58	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/641-1063D]	This study
BD59	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/422-1063D]	This study
BD60	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/1-244D]	This study
BD61	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/1-421D]	This study
BD62	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/1-640D]	This study
BD63	as BD56, but [pRS416-SPT5] removed	This study
BD64	as BD57, but [pRS416-SPT5] removed	This study
BD94	as CR18, but *bur2::URA3*	This study
BD95	as BD64, but *bur2::URA3*	This study
BD96	as BD63, but *bur2::URA3*	This study

[a] Genomic genes tagged with 3×FLAG are indicated in parentheses; plasmids contained in strains are indicated in brackets.

sites of pRS416 to create the plasmid pRS416-SPT5. A single-copy centromeric plasmid with the *LEU2* gene as a selection marker and encoding the full-length or truncated Spt5 proteins were created by using the plasmid pRS415 (Fig. 4A and B) (Sikorski and Hieter, 1989). The promoter, full-length or truncated coding sequences, and the terminator of the *SPT5* gene, and the 3 consecutive Myc sequences (3xMyc) were amplified by PCR and ligated into pRS415 to create plasmids expressing the full-length or CTR-deleted Spt5 (Fig. 4B).

3.2.2. Shuffling of Plasmids Encoding Different Spt5 Truncates

Yeast cells were transformed with plasmid pRS416-SPT5 and the genomic *SPT5* gene is then deleted using standard procedure as described above. The specific deletion of the genomic *SPT5* gene is confirmed by PCR using primer pairs that are specific for the genomic *SPT5* gene and the plasmid-borne *SPT5* gene. pRS415-based plasmids encoding the full-length or truncated Spt5 were transformed into the yeast cells whose genomic *SPT5* gene had been deleted and complemented with pRS416-SPT5. The transformed cells were cultured in a medium containing uracil but not leucine to select for the *LEU2* plasmids and to allow the loss of the *URA3* plasmid. A centromeric plasmid generally has a loss rate of 1% per generation and shows virtually no segregation bias (Lundblad, 2004). The cultures were then spotted onto plates containing 5-fluoroorotic acid (5-FOA), which is toxic to cells with a functional *URA3* gene (Boeke et al., 1984), to select for cells that had lost plasmid pRS416-SPT5.

3.2.3 Repair Analysis of UV Induced CPDs

Yeast cells were grown at 30°C in minimal medium containing 2% glucose (SD) or 2% galactose (SG, for Spt5 overespression) to late log phase ($A_{600} \approx 1.0$), irradiated with 80 J/m^2 of 254 nm UV and incubated in YPD medium (2% peptone, 1% yeast extract and 2% glucose) or YPG medium (2% peptone, 1% yeast extract and 2% galactose) in the dark at 30°C. At different

times of the repair incubation, aliquots were removed and the genomic DNA was isolated using a hot SDS procedure as described previously (Li and Smerdon, 2002).

The gene fragments of interest were 3' end labeled with [α-^{32}P]dATP using a procedure described previously (Li and Waters, 1996; Li et al., 2000). Briefly, ~ 1 μg of total genomic DNA was digested with restriction enzyme(s) to release the fragments of interest and incised at CPD sites with an excess amount of 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 5 minutes 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), labeled with [α-^{32}P]dATP (Perkin Elmer), and resolved on sequencing gels. The gels were exposed to a Phosphorimager screen (Bio-Rad). The signal intensities at gel bands corresponding to CPD sites were quantified using Quantity One software (Bio-Rad).

3.2.4 Whole-Cell Extract Preparation and Immunoprecipitation

Yeast cells were cultured at 30°C in minimal medium containing 2% glucose or galactose (to induce a gene under the control of the *GAL10* promoter) to late log phase and harvested. For measuring cellular levels of proteins of interest, whole-cell extracts were prepared using a trichloroacetic acid (TCA) method (Chen et al., 2007). The harvested cells from a 5 ml culture were resuspended in 300 μl of 20% TCA and broken by vertexing them with acid-washed glass beads. The proteins in the lysates were pelleted by centrifugation, washed with ice-cold 80% acetone and dissolved in 100 μl of 2× SDS-PAGE gel loading buffer (Sambrook and Russell, 2001).

To examine the effect of Spt4 on Spt5 degradation, *spt4Δ* and *SPT4*$^{+}$ cells expressing 3xFLAG tagged Spt5 were cultured to late log phase. Cycloheximide (CHX), a potent protein synthesis inhibitor (Schindler and Davies, 1975), was added to the cultures to a final concentration of 500 μg/ml to completely stop protein synthesis (Chen et al., 2007). At different times following the addition of CHX, cells were harvested and whole cell extracts were prepared using the TCA method as described above.

For immunoprecipitation, the harvested cells from a 25 ml culture were washed with and resupended in 0.5 ml of IP buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1mM EGTA, 0.5% NP-40, 1% Triton X-100, 0.1% SDS, 0.2 mM PMSF and protease inhibitors) (Chen et al., 2007). The cells were broken with acid-washed glass beads, and cell debris was removed by centrifugation at 20,000 × *g* for 10 minutes at 4°C. Fifty μl of the lysate was saved as an 'input'. The remaining lysate was added with 15 μg of anti-FLAG (M2) (Sigma), anti-Myc (Sigma), 8WG16 (Neoclone) or H14 (Covance) antibodies, which recognize FLAG tag peptide, Myc tag peptide, the hypo- phosphorylated and hyper-phosphotylated C-terminal heptapeptide repeats of Rpb1, respectively (Palancade and Bensaude, 2003). The mixture was incubated at 4°C overnight with gentle rotation. Protein A- or G-coated agarose beads (Sigma) were added to the mixture and incubated at 4°C for 3 hours with gentle rotation. The beads were washed twice with IP buffer containing 0.5 M of NaCl and twice with IP buffer containing 150 mM of NaCl. Bound proteins were eluted by boiling the beads in 50 μl of 2× SDS-PAGE gel loading buffer.

3.2.5 Treatment of Immunoprecipitated Pol II Complexes with λ Phosphatase

Pol II complex was immunoprecipitated from yeast cells by using antibody H14 as described above. Protein A- or G-coated agarose beads attached with the immunoprecipitates were resuspended in 100 μl of dephosphorylation reaction buffer (50 mM HEPES, 100 mM NaCl,

2 mM DTT, 0.01 % Brij-35, 1mM $MnCl_2$). Four hundred units of λ protein phosphatase (New England Biolabs) were added to the sample. Following 30 minutes of incubation at 30°C, 100 µl of 2× SDS-PAGE gel loading buffer was added to the sample. Proteins were eluted from the beads by boiling for 5 minutes.

3.2.6 Western Blot

Proteins in whole-cell extracts, immunoprecipitation inputs, immunoprecipitated samples, or phosphatase treated samples were resolved on an SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Rpb1 was probed with 8WG16 or H14 antibodies. 3xFLAG and 3xMyc tagged proteins were probed with anti-FLAG M2 antibody (Sigma) and anti-Myc antibody (Sigma), respectively. Blots were incubated with SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce), and the protein bands were detected using a chemiluminescence scanner (Fluorchem 8800, Alpha Innotech). Band intensities were quantified using AlphaEaseFC 4.0 software.

3.2.7 Northern Blot

Yeast cells were cultured to late log phase under the same conditions as those used for NER analysis. Total RNA was isolated using a hot acidic phenol method, as described (Collart and Oliviero, 2004). The RNA was fractionated on formaldehyde–agarose gels (Sambrook and Russell, 2001), transferred onto Hybond-N^+ membranes (GE Healthcare), and hybridized with radioactive probes generated using the Prime-It® II Random Primer Labeling Kit (Stratagene).

3.2.8 UV sensitivity Assay

Yeast cells were grown at 30°C in minimal medium to saturation, 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 – 7 days in the dark prior to being photographed.

3.3 Results

3.3.1 Spt4 Protects Spt5 from Degradation and Stabilizes the Interaction of Spt5 with Pol II

It has been shown that Spt4 partially suppresses Rad26-independent TCR, as deletion of *SPT4* reinstates TCR in *rad26Δ* cells (Jansen et al., 2000). Spt4 forms a complex with Spt5 in yeast (Hartzog et al., 1998; Swanson and Winston, 1992) and human cells (Wada et al., 1998). In yeast, the *SPT4* gene is dispensable (Malone et al., 1993), whereas the *SPT5* gene is essential for cell viability (Swanson et al., 1991a). We wondered if Spt4 suppresses Rad26-independent TCR directly or through or together with Spt5. To this end, we first examined if Spt4 affects the cellular level of Spt5. Three consecutive FLAG sequences (3xFLAG) were tagged to the coding sequence of the genomic *SPT5* gene in different yeast mutants. The 3xFLAG tag did not cause any noticeable deficiency to the cells (not shown). As shown in, the cellular level of Spt5 in *spt4Δ* cells was about 1/3 of that in $SPT4^{+}$ cells, regardless of the presence of the GGR factor Rad7 or the TCR factor Rad26. However, the *SPT5* mRNA levels were similar between *spt4Δ* and $SPT4^{+}$ cells (Fig. 3-1B), indicating that the lower cellular level of Spt5 in *spt4Δ* cells was not caused by a decreased transcription of the *SPT5* gene. For an unknown reason, *SPT5* mRNA levels were somewhat higher in *rad7Δ* cells than in $RAD7^{+}$ cells (Fig. 3-1B). We then tested if Spt5 was degraded faster in *spt4Δ* cells. The level of Spt5 barely changed in $SPT4^{+}$ cells during an 8-hour incubation after protein synthesis was completely suppressed by the addition of the protein synthesis inhibitor cycloheximide (CHX) (Fig. 3-1C and D). In contrast, the Spt5 level decreased dramatically in *spt4Δ* cells under the same incubation conditions (Fig. 3-1C and D). These results indicate that Spt4 protects Spt5 from degradation.

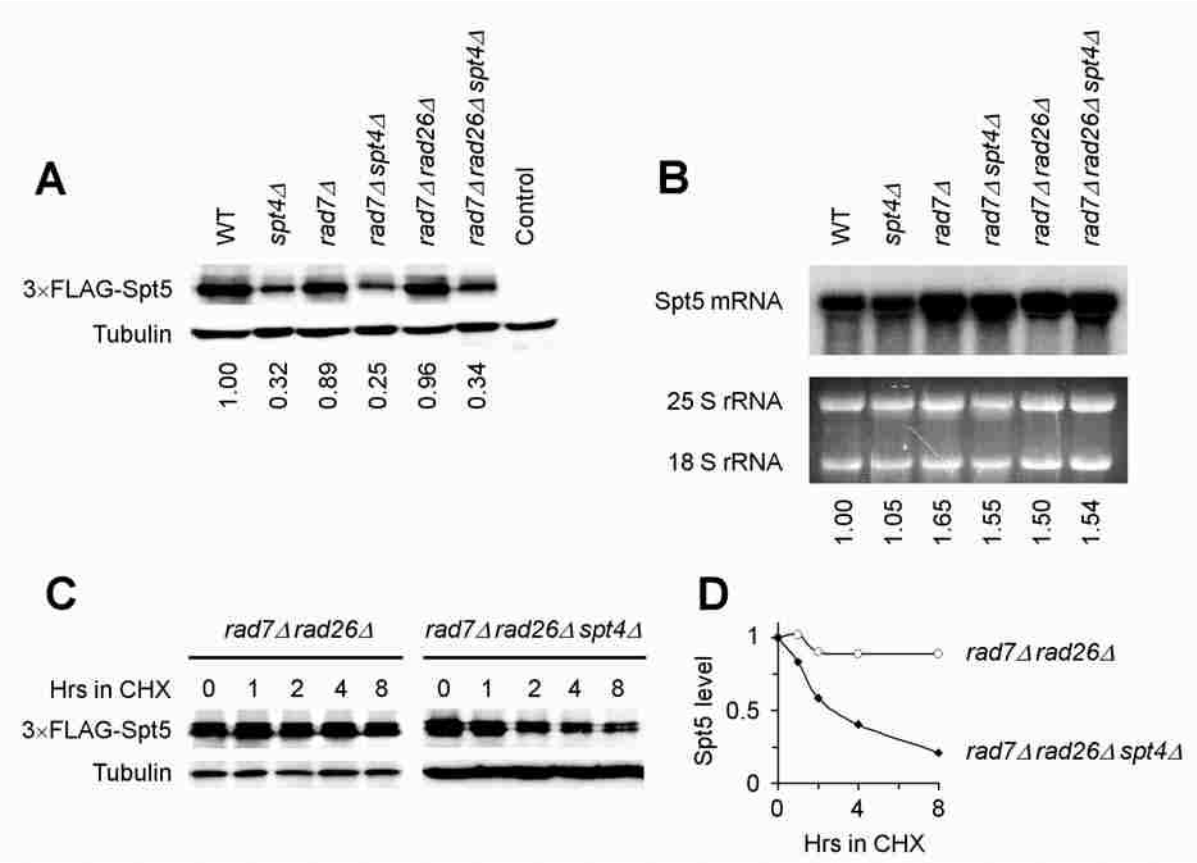

Figure 3-1 Spt4 protects Spt5 from degradation. (A) Western blot showing cellular levels of Spt5 expressed from the genomic *SPT5* gene tagged with 3xFLAG in different strains. The *control* lane contains a sample prepared from the wild type strain whose *SPT5* gene was not tagged. The tubulin bands serve as internal loading controls. The numbers at the bottom indicate relative cellular levels of the tagged Spt5 in the different strains (the level in wild type cells is set as 1). (B) Northern blot showing Spt5 mRNA levels in the different strains. The lower panel shows an ethidium bromide stained agarose gel containing the resolved total RNA before being blotted onto a membrane. The numbers at the bottom indicate relative cellular levels of Spt5 mRNA in the different strains (the level in wild type cells is set as 1). The 25 S and 18 S rRNA bands serve as internal loading controls. (C) Western blot showing levels of the tagged Spt5 at different times following the addition of cycloheximide (CHX) in *rad7Δ rad26Δ* and *rad7Δ rad26Δ spt4Δ* strains. As the steady state level of the tagged Spt5 in *spt4Δ* cells was lower than that in $SPT4^{+}$ cells, the amounts of cell extracts from *rad7Δ rad26Δ spt4Δ* cells were loaded more than those from *rad7Δ rad26Δ* cells. Tubulin bands serve as internal loading controls. (D) Plot showing relative levels of the tagged Spt5 at different times following the addition of CHX.

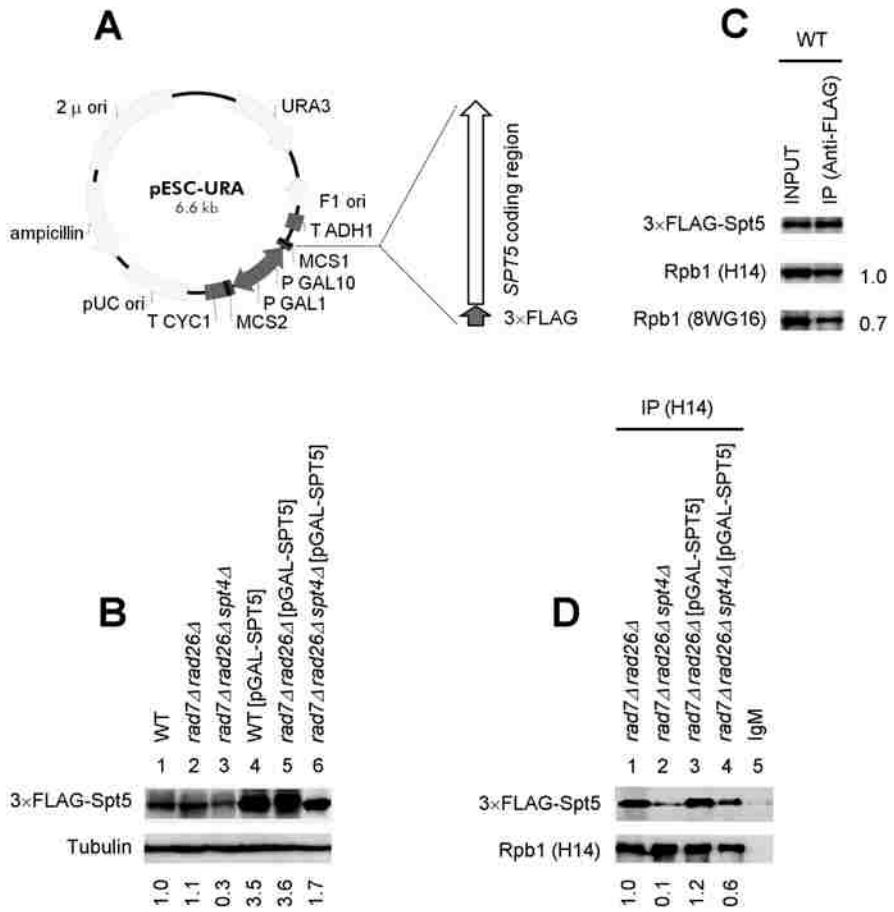

Figure 3-2 Spt4 stabilizes the interaction of Spt5 with Pol II. (A) Schematic of plasmid pGAL-SPT5 overexpressing 3xFLAG tagged Spt5. (B) Western blot showing cellular levels of 3xFLAG tagged Spt5 in different strains cultured in a galactose medium (to induce overexpression of Spt5 encoded by plasmid pGAL-SPT5). Strains containing plasmid pGAL-SPT5 are indicated in brackets. The tubulin bands serve as internal loading controls. The numbers at the bottom indicate relative cellular levels of the tagged Spt5 in the different strains (the level in wild type cells is set as 1). (C) Binding of Spt5 to hyper-phosphorylated (recognized by H14) and hypo-phosphorylated (recognized by 8WG16) Pol II. 3xFLAG tagged Spt5-associated protein complexes were immunoprecipitated from wild type cells by using an anti-FLAG (M2) antibody, and probed with antibodies H14 and 8WG16 on Western blots. Numbers on the right of the blots indicate relative levels of hyper-phosphorylated and hypo-phosphorylated Rpb1 co-immunoprecipitated with the tagged Spt5 (normalized to the respective immunoprecipitation inputs). (D) Binding of Spt5 to Pol II in different mutants. Pol II complexes were immunoprecipitated from different mutant cells cultured in a galactose medium by using the H14 antibody. Lane 5 was a mock immunoprecipitated sample by using nonspecific mouse IgM. Rpb1 and co-immunoprecipitated 3xFLAG tagged Spt5 were probed with H14 and anti-FLAG (M2) antibodies, respectively, on Western blots. Strains containing plasmid pGAL-SPT5 are indicated in brackets. Numbers underneath the blots indicate relative levels of 3xFLAG tagged Spt5 co-immunoprecipitated (normalized the immunoprecipitated Rpb1) (the level in *rad7Δ rad26Δ* cells is set as 1).

To test if overexpression of Spt5 in *spt4Δ* cells could compensate for the absence of Spt4, we created a multi-copy plasmid (pGAL-SPT5) expressing the 3xFLAG tagged Spt5 under the control of the galactose inducible *GAL10* promoter (Fig. 3-2A). The plasmid was transformed into different yeast mutant strains. Upon galactose induction, the plasmid-encoded 3xFLAG tagged Spt5 was expressed at cellular levels that were 3 – 6 times of those of the genomically encoded 3xFLAG tagged Spt5 (Fig. 3-2B, compare lanes 1 and 4, 2 and 5, and 3 and 6).

It has been shown that the Spt4/Spt5 complex is associated with Pol II (Hartzog et al., 1998). The elongation form of Pol II is hyper-phosphorylated at serines 2 and 5 of the C-terminal heptapeptide repeats ($Y_1S_2P_3T_4S_5P_6S_7$) of Rpb1, whereas the non-elongation form of Pol II is hypo-phosphorylated at the repeats (Phatnani and Greenleaf, 2006). Antibody 8WG16 recognizes the serine 2 unphosphorylated repeats, whereas H14 recognizes the serine 5 phosphorylated repeats (Palancade and Bensaude, 2003). We immunoprecipitated 3xFLAG tagged Spt5 with an anti-FLAG antibody. The presence of the hypo- and hyper-phosphorylated forms of Pol II in the immunoprecipitates were examined by using antibodies 8WG16 and H14, respectively. As can be seen in Fig. 3.2C, slightly more hyper-phosphorylated Pol II was co-immunoprecipated than hypo-phosphorylated Pol II, suggesting that Spt5 may have a slight preference for binding to the elongation form of Pol II.

To examine if Spt4 affects the binding of Spt5 to Pol II, the hypo- and hyper-phosphorylated Pol II were immunoprecipitated from different yeast mutants by using antibodies 8WG16 and H14, respectively. The level of Spt5 associated with the hyper-phosphorylated Pol II was much lower in *spt4Δ* cells than in $SPT4^+$ cells (Fig. 3-2D, compare lanes 1 and 2). Overexpression of Spt5 increased its binding to the hyper-phosphorylated Pol II, especially in *spt4Δ* cells (Fig. 3-2D, compare lanes 2 and 4). However, the level of Spt5 associated with the hyper-phosphorylated Pol II in *spt4Δ* cells overexpressing the

tagged Spt5 was still somewhat lower than that in *SPT4*$^{+}$ cells normally expressing the genomically-tagged Spt5 (Fig. 3-2D, compare lanes 1 and 4), although the cellular level of the overexpressed Spt5 in the *spt4Δ* cells is ~ 1.5 times of that in *SPT4*$^{+}$ cells normally expressing the genomically-tagged Spt5 (Fig. 3.2B, compare lanes 2 and 6). We observed similar trends of Spt5 binding to the hypo-phosphorylated Pol II (recognized by 8WG16) in the different yeast mutants (not shown). These results indicate that the decreased binding of Spt5 to Pol II (both hypo- and hyper-phosphorylated forms) in *spt4Δ* cells is due to both a lower cellular level of Spt5 and a decreased interaction between Spt5 and Pol II.

3.3.2 Overexpression of Spt5 Suppresses Rad26-Independent TCR in *spt4Δ* Cells

Next, we determined if overexpression of Spt5 can suppress Rad26-independent TCR in *spt4Δ* cells. In yeast, Rad7, Rad16 and Elc1 have been shown to be required for repairing the NTS of transcriptionlly active gene (Lejeune et al., 2009; Verhage et al., 1994; Verhage et al., 1996). Therefore, TCR can be exclusively analyzed in *rad7Δ* (or *rad16Δ* and *elc1Δ*) cells, as these cells are defective in GGR. In agreement with previous studies [e.g., (Li et al., 2006; Li and Smerdon, 2002, 2004; Ding et al., 2007)], TCR initiates ~ 40 nucleotides upstream of the transcription start site in the *RPB2* gene (Fig. 3-3A). In *rad7Δ rad26Δ* cells, little TCR can be seen in the coding region of the *RPB2* gene except for a short region immediately downstream of the transcription start site (Fig. 3-3A). In agreement with the previous report (Jansen et al., 2000), TCR rate is significantly faster in *rad7Δ rad26Δ spt4Δ* cells than in *rad7Δ rad26Δ* cells (Fig. 3-3B, C and E), indicating that Spt4 can indeed suppress Rad26-independent TCR. The TCR rate in *rad7Δ rad26Δ spt4Δ* cells overexpressing Spt5 is similar to that in *rad7Δ rad26Δ* cells (Fig. 3-3B, D and E), indicating that the overexpression can suppress Rad26-independent TCR in the absence of Spt4.

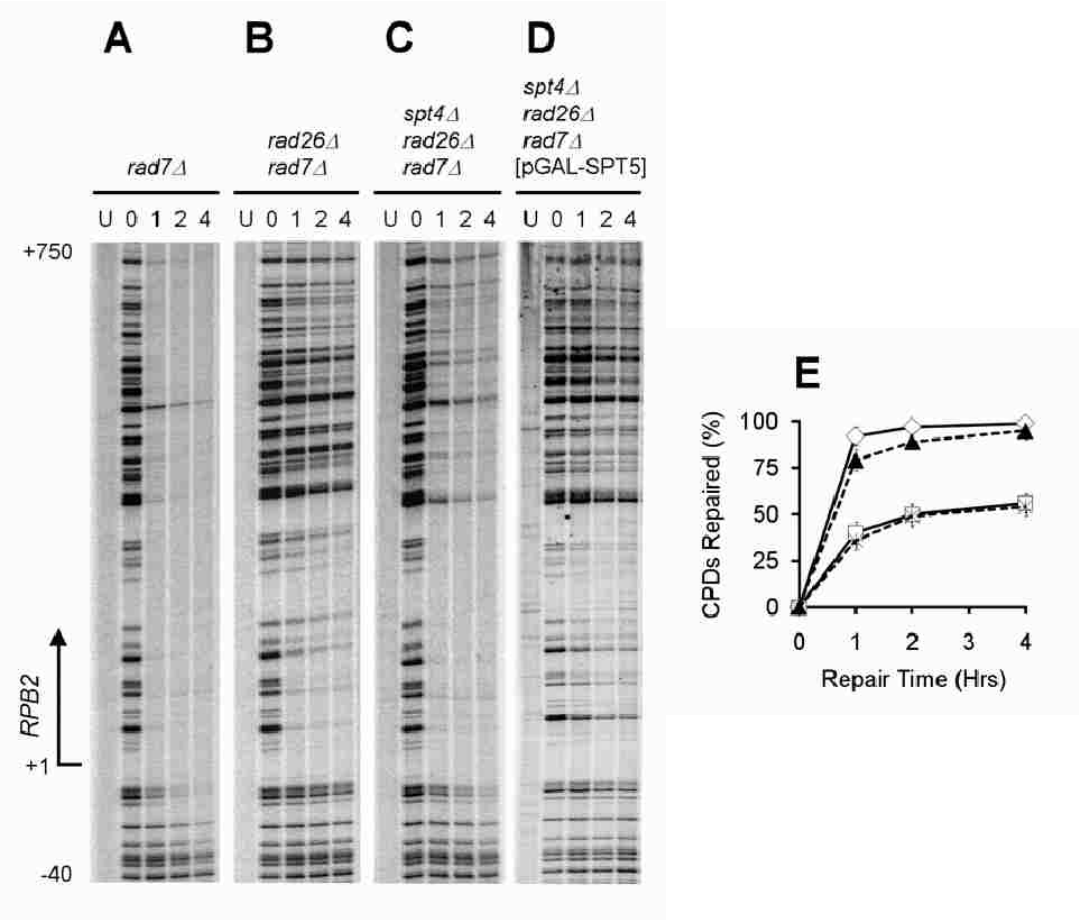

Figure 3-3 Overexpression of Spt5 suppresses TCR in *spt4Δ rad26Δ* cells. (A) – (D) DNA sequencing gels showing TCR in the *RPB2* gene in galactose cultures [to induce overexpression of Spt5 encoded by plasmid pGAL-SPT5 (Fig. 3-2A)]. Brackets at the top of panel D indicate plasmid pGAL-SPT5 contained in the strain. The lanes are DNA samples from unirradiated (*U*) and UV irradiated cells following different times (hrs) of repair incubation as indicated at the top of the gels. The arrow on the left of the gels marks the transcription start site. (D) Plot showing the mean (± standard deviation) of percent CPDs repaired in the transcribed region of the *RPB2* gene in *rad7Δ* (open diamond), *rad7Δ rad26Δ* (open square), *rad7Δ rad26Δ spt4Δ* (solid triangle) and *rad7Δ rad26Δ spt4Δ* [pGAL-SPT5] (asterisk) cells.

3.3.3 The C-terminal Repeat (CTR) Domain of Spt5 Is Dispensable for Cell Viability

Our results described above indicate that Spt5 may play a direct role in suppressing Rad26-independent TCR, whereas Spt4 may be indirectly involved in the suppression by protecting Spt5 from degradation and by stabilizing the binding of Spt5 to Pol II. We next asked which domain(s) of Spt5 is/are involved in the suppression. Based on the results of human Spt5 domain mapping (Ivanov et al., 2000; Yamaguchi et al., 1999) and the prediction with Pfam and STRING software (Finn et al., 2008; Jensen et al., 2009), yeast Spt5 consists of several distinct domains: an N-terminal acidic region, an N-terminal NusG (NGN), four KOW and the C-terminal region that contains 15 six-amino acid repeats (CTR) (Fig. 3-4A). We used a plasmid shuffling technique to map the functions of the different domains of Spt5. A series of single-copy centromeric *LEU2* [pRS415 (Sikorski and Hieter, 1989)] plasmids encoding full-length or different truncated Spt5 proteins that are tagged with 3 consecutive Myc sequences (3xMyc) and are under the control of the native Spt5 promoter were created (Fig. 3-4B). These plasmids were transformed into yeast cells whose genomic *SPT5* gene was deleted and complemented with a single-copy centromeric *URA3* [pRS416 (Sikorski and Hieter, 1989)] plasmid encoding the full-length Spt5 (pRS416-SPT5). The transformed cells were cultured in a medium containing uracil but not leucine to select for the *LEU2* plasmids and to allow the loss of pRS416-SPT5. The cultures were then spotted onto plates containing 5-fluoroorotic acid (5-FOA), which is toxic to cells with a functional *URA3* gene (Boeke et al., 1984). Therefore, only those cells that had lost the plasmid pRS416-SPT5 were able to grow on 5-FOA plates. As shown in Fig. 3.4C, cells transformed with *LEU2* plasmids encoding the full-length or the CTR-deleted Spt5 were able to grow on the 5-FOA plates, whereas those transformed with the *LEU2* plasmids encoding the other Spt5 truncates were not (Fig. 3-4C). This indicates that the CTR domain is dispensable, whereas all other domains of Spt5 examined here are essential for cell viability.

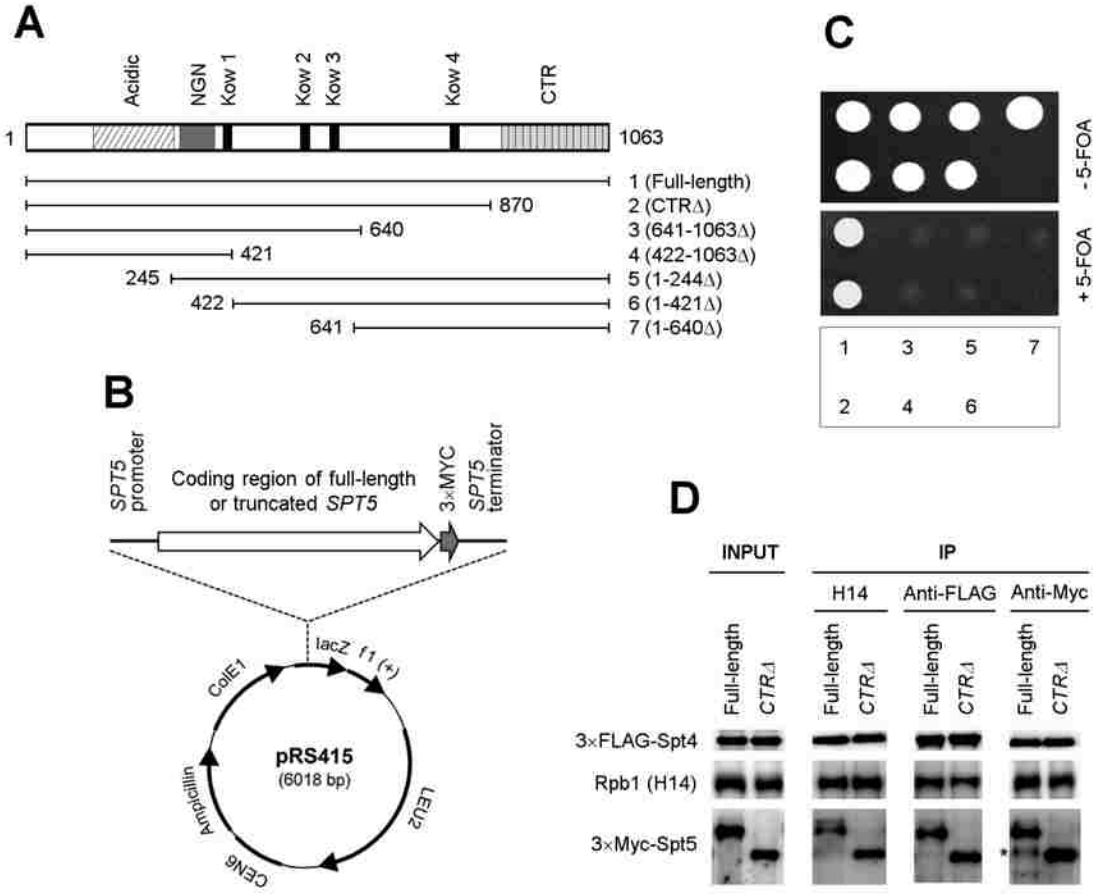

Figure 3-4 The Spt5 CTR is not essential for cell viability and does not affect the interactions of Spt5 with Spt4 and Pol II (A) Schematic of the Spt5 protein. Bars 1 – 7 underneath the schematic indicate full-length or truncated Spt5 encoded by plasmids. (B) Structure of plasmids expressing the full-length or truncated Spt5. (C) Growth of cells whose genomic *SPT5* gene was deleted and transformed with a *URA3* plasmid encoding the full-length Spt5 (pRS416-SPT5) and a *LEU2* plasmid encoding the full-length or truncated Spt5 (1 – 7, as shown in panels A and B) on 5-FOA plates. (D) Co-immunoprecipitation of the full-length or CTR-deleted Spt5 with Pol II and Spt4. Cells whose genomic *SPT5* gene was deleted and *SPT4* gene was tagged with 3xFLAG, and bearing the *LEU2* plasmid encoding the full-length or CTR-deleted Spt5 with a 3xMyc tag were cultured to log phase. Pol II, 3xFLAG tagged Spt4 and 3xMyc tagged Spt5 was immunoprecipitated from the cells with H14, anti-FLAG and anti-Myc antibodies, respectively. The levels of Pol II, 3xFLAG tagged Spt4 and 3xMyc tagged full-length or CTR-deleted Spt5 in the immunoprecipitates were probed with the respective antibodies on Western blots.

3.3.4 Deletion of the Spt5 CTR Domain Does Not Affect Its Binding to Spt4 or to Pol II

To determine the role of the Spt5 CTR domain in its bindings to Spt4 and Pol II, we conducted a series of immunoprecipitation assays. A 3xFLAG was tagged to the genomic *SPT4* gene in cells whose genomic *SPT5* gene was deleted and complemented with the single-copy centromeric *LEU2* (pRS415) plasmid encoding 3xMyc tagged full-length (WT) or CTR-deleted (CTRΔ) Spt5 (Fig. 3-4A and B). The Pol II complex, Spt4 and Spt5 were immunoprecipited by using H14, anti-FLAG and anti-Myc antibodies, respectively. Rpb1, the 3xFLAG tagged Spt4 and the 3xMyc tagged Spt5 in the immunopreciptation inputs and immunoprecipitates were detected on Western blot. Deletion of the Spt5 CTR domain did not appear to affect the cellular levels of Rpb1, Spt4 and Spt5 (Fig. 3-4D, INPUT). Furthermore, the deletion did not affect the levels of Rpb1, Spt4and Spt5 in the immunoprecipitates (Fig. 3-4D, IP), indicating that the deletion does not affect the binding of Spt5 to Spt4 or Pol II.

3.3.5 The CTR Domain of Spt5 Is Involved in Suppression of Rad26-Independent TCR

Due to the reinstatement of TCR, *rad7Δ rad26Δ spt4Δ* cells are about 10 times more UV-resistant than *rad7Δ rad26Δ* cells (Fig. 3-5). Interestingly, *rad7Δ rad26Δ spt5Δ* cells expressing the CTR-deleted Spt5 (Fig. 3-4A and B) are as UV-resistant as *rad7Δ rad26Δ spt4Δ* cells (Fig. 3-5). On the other hand, *rad7Δ rad26Δ spt5Δ* cells expressing the full-length Spt5 (Fig. 3-4A and B) are as UV-sensitive as *rad7Δ rad26Δ* cells (Fig. 3-5). This indicates that, like Spt4, the Spt5 CTR may suppress Rad26-independent TCR. We then directly analyzed TCR in *rad7Δ rad26Δ spt5Δ* cells expressing the full-length and CTR-deleted Spt5. The TCR rate in cells expressing the CTR-deleted Spt5 was significantly faster than that in cells expressing the full-length Spt5 (Fig. 3-6), indicating that the Spt5 CTR domain is indeed involved in suppression of Rad26-independent TCR.

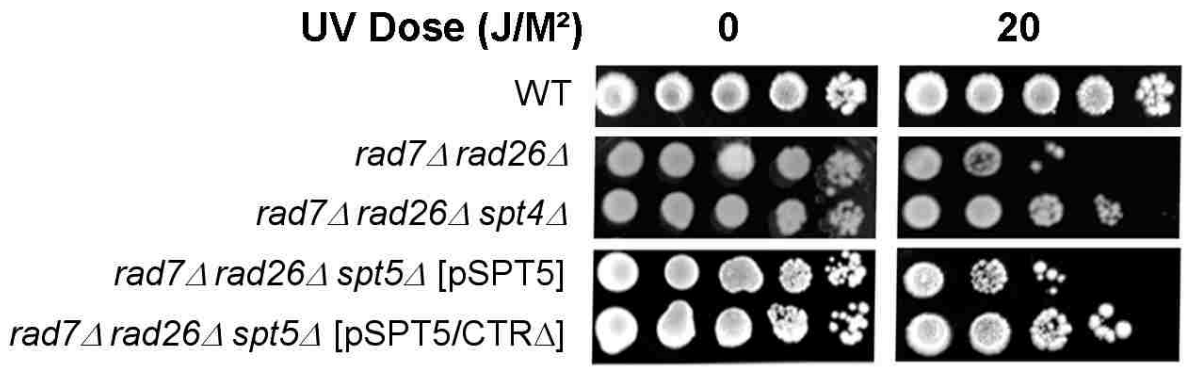

Figure 3-5 Deletion of the Spt5 CTR increases the UV resistance of *rad7Δ rad26Δ* cells to the same extent as *spt4Δ*. Saturated cultures of yeast strains were sequentially 10-fold diluted and spotted onto YPD plates. When the spots had dried, the plates were irradiated with the indicated doses of 254 nm UV light. The plates were incubated at 30°C for 3 – 5 days in the dark prior to being photographed. Strains containing a single-copy plasmid encoding the full-length (pSPT5) or the CTR-deleted (pSPT5/CTRΔ) Spt5 are indicated in brackets.

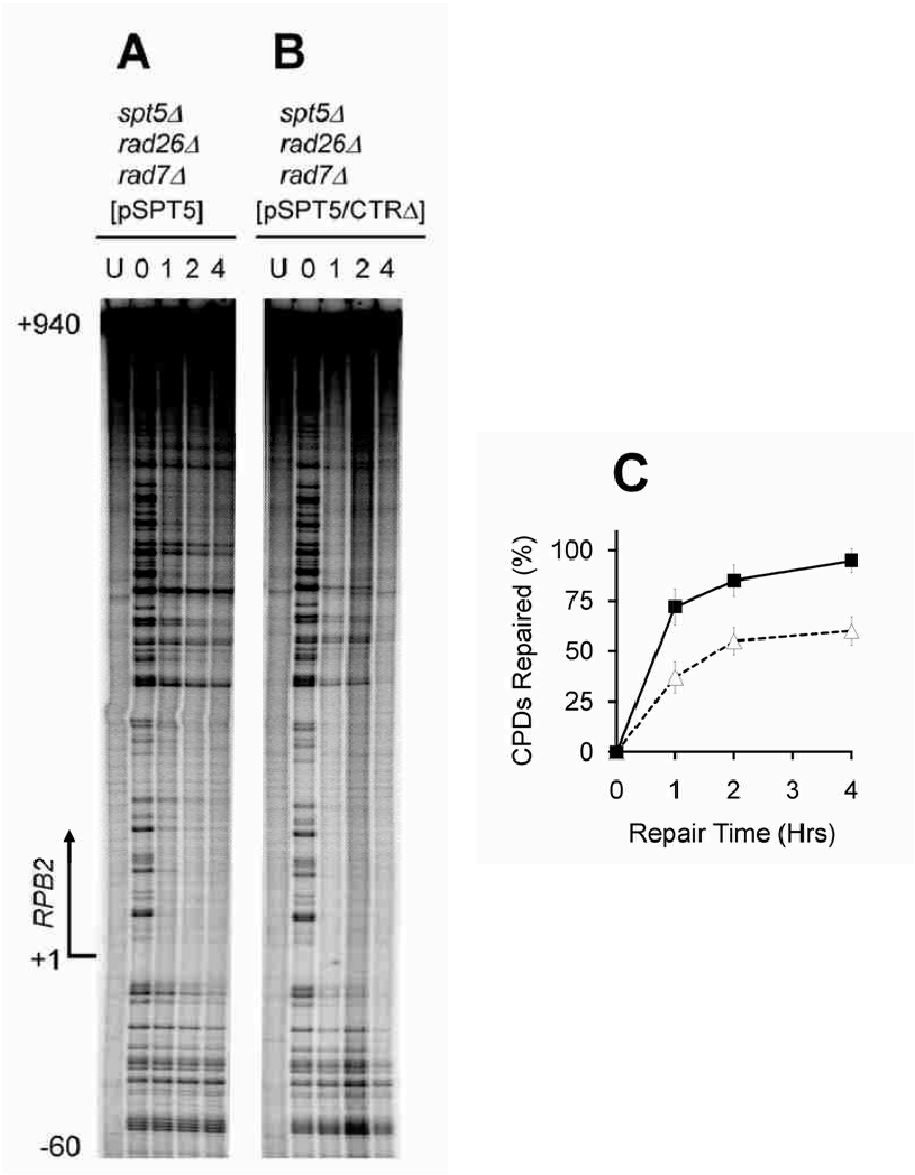

Figure 3-6 Deletion of the Spt5 CTR increases Rad26-independent TCR. (A) and (B) DNA sequencing gels showing TCR in the *RPB2* gene. Brackets at the top indicate single-copy plasmid encoding the full-length (pSPT5) or CTR-deleted (pSPT5/CTRΔ) Spt5. The lanes are DNA samples from unirradiated (*U*) and UV irradiated cells following different times (hrs) of repair incubation as indicated at the top of the gels. The arrow on the left of the gels marks the transcription start site. (C) Plot showing the mean (± standard deviation) of percent CPDs repaired in the transcribed region of the *RPB2* gene in *rad7Δ rad26Δ spt5Δ* [pSPT5] (open triangle) and *rad7Δ rad26Δ spt5Δ* [pSPT5/CTRΔ] (solid square) cells.

3.3.6 Phosphorylation of the Spt5 CTR Domain by Bur Kinase Plays a Role in Suppression of Rad26-Independent TCR

The Spt5 CTR domain contains 15 six-amino acid repeats with the consensus sequence of S-A/T-W-G-G-A/Q (Swanson et al., 1991a). The S and T residues in these repeats are potential phosphorylation sites. We noticed that the yeast Spt5 protein can show duplet bands on a Western blot, especially if the SDS-PAGE gel was run long enough (Fig. 3-7A). The slower migrating band of Spt5 tends to be much stronger in immunoprecipitated Pol II complex, suggesting that this band may be a form of Spt5 that preferably associates with Pol II. Treatment of the immunoprecipitated Pol II complex with a phosphotase eliminates the slower migrating band (Fig. 3-7A), indicating that the band is the phosphorylated Spt5. Deletion of the Spt5 CTR domain eliminates the slower migrating band (Fig. 3-7B), indicating that the phosphorylation occurs in the CTR.

In human cells, it has been shown that Spt5 can be phosphorylated by positive transcription elongation factor b (P-TEFb), a cyclin dependent kinase, composed of Cdk9 and one of three cyclin subunits, T1, T2 or K (Wada et al., 1998; Yamada et al., 2006). In yeast, two cyclin-dependent kinases are homologous to human Ctk9 (Buratowski, 2005; Wood and Shilatifard, 2006). The yeast Ctk1 has been shown to phosphorylate serine 2 of the heptapeptide repeats of the Rpb1 C-terminal domain (Keogh et al., 2003; Patturajan et al., 1999). The activity of Bur1 kinase is dependent on its cyclin partner Bur2. *bur1* and *bur2* mutations cause nearly identical spectra of phenotypes (Yao et al., 2000). However, *Bur1* is essential for cell viability, whereas *Bur2* is not. As can be seen in Fig. 3-7C, the slower migrating band reflecting the phosphorylated Spt5 cannot be detected in *bur2Δ* cells. This indicates that the Bur kinase is responsible for phosphorylation of Spt5 at the CTR, in agreement with two recent reports (Liu et al., 2009; Zhou et al., 2009).

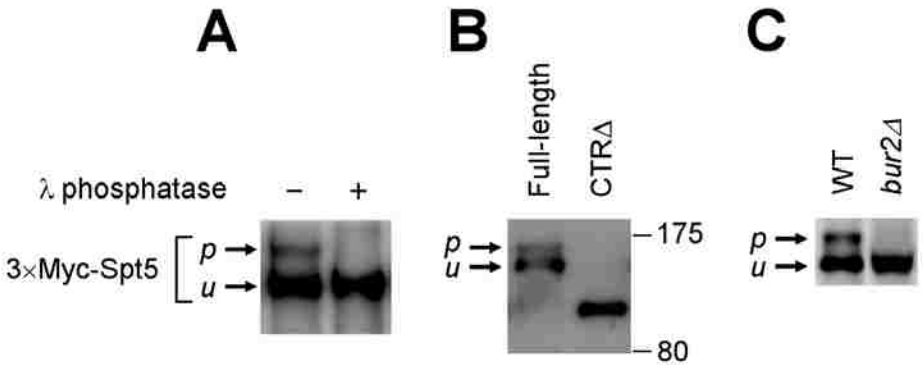

Figure 3-7 The Spt5 CTR is phosphorylated by the Bur kinase. (A) Western blot showing phosphorylation of Spt5. 3xMyc tagged Spt5 was co-immunoprecipitated with Pol II by using antibody H14, treated or mock-treated with λ phosphatase and probed with an anti-Myc antibody on a Western blot. (B) Deletion of the Spt5 CTR abolishes phosphorylation. 3xMyc tagged full-length and CTR-deleted Spt5 was co-immunoprecipitated with Pol II by using H14 antibody and probed with an anti-Myc antibody a Western blot. Numbers on the right of the blot indicate approximate positions of molecular weight standards (in kD). (C) Deletion of *bur2* abolishes Spt5 phosphorylation. 3xMyc tagged Spt5 was co-immunoprecipitated with Pol II from *BUR2*$^+$ and *bur2Δ* cells by using antibody H14, and probed with an anti-Myc antibody a Western blot. '*p*' and '*u*' on the left of each of the blots mark phosphorylated and unphosphorylated Spt5, respectivley.

We then attempted to determine if phosphorylation of the Spt5 CTR domain by the Bur kinase plays a role in suppression of Rad26-independent TCR. Although the *bur2Δ* cells grew extremely slowly (with a doubling time of ~ 8 hour as opposed to ~ 2.5 hours for *BUR2*$^+$ cells) (data not show), they showed significantly faster TCR than did the isogenic *BUR*$^+$ cells (compare Figs. 3-6A and 3-8A; Fig. 3-8C), especially during the initial hour of the repair incubation. This indicates that the Bur kinase plays a role in suppression of Rad26-independent TCR, especially during the initial period of the repair incubation. However, the TCR rate in the *burΔ* cells expressing the full-length Spt5 (*rad7Δ rad26Δ spt5Δ bur2Δ* + pSPT5) was somewhat slower than that in the isogenic *burΔ* cells expressing the CTR-deleted Spt5 (*rad7Δ rad26Δ spt5Δ bur2Δ* + pSPT5-CTRΔ), especially during the later time period of the repair incubation

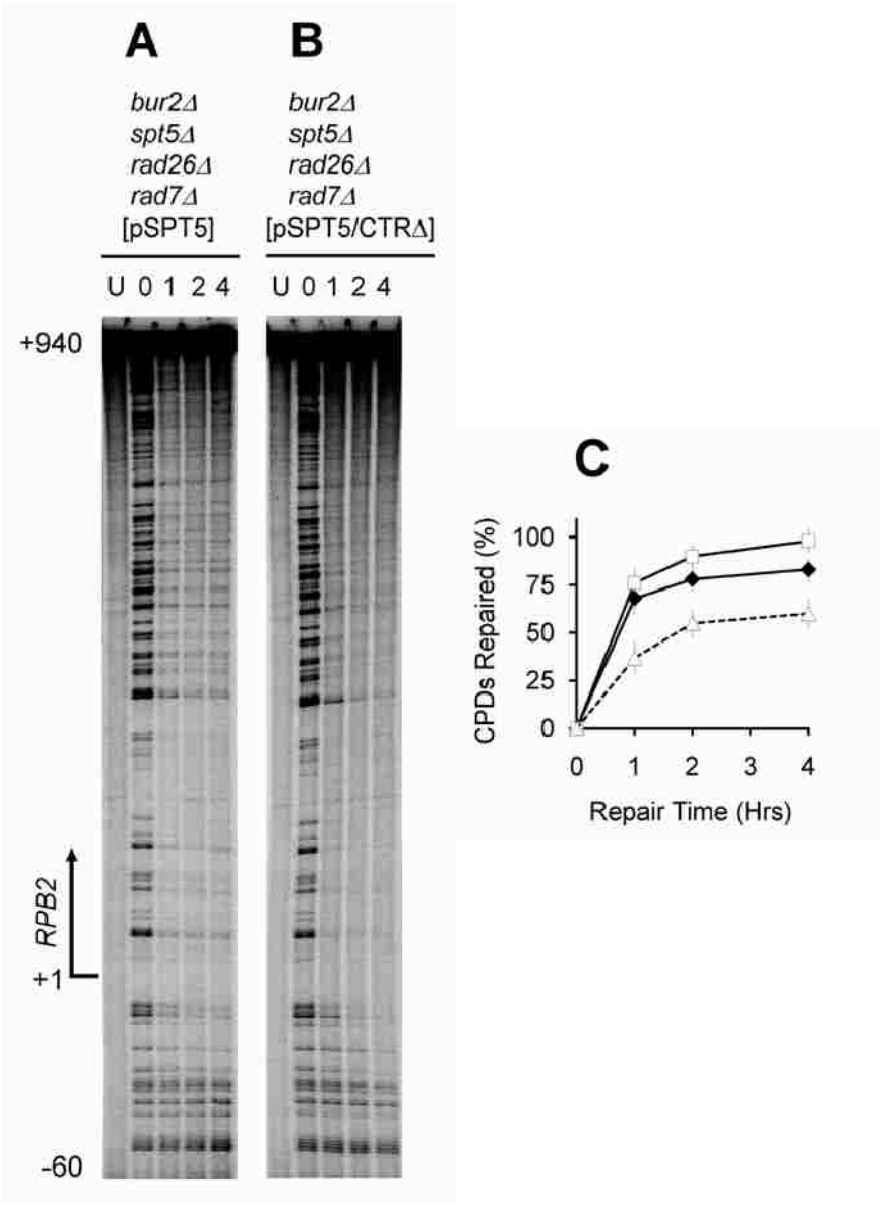

Figure 3-8 Effects of deletions of *bur2* and the Spt5 CTR on Rad26-independent TCR. (A) and (B) DNA sequencing gels showing TCR in the *RPB2* gene. Brackets at the top indicate single-copy plasmid encoding the full-length (pSPT5) or CTR-deleted (pSPT5/CTRΔ) Spt5. The lanes are DNA samples from unirradiated (*U*) and UV irradiated cells following different times (hrs) of repair incubation as indicated at the top of the gels. The arrow on the left of the gels marks the transcription start site. (C) Plot showing the mean (± standard deviation) of percent CPDs repaired in the transcribed region of the *RPB2* gene in *rad7Δ rad26Δ spt5Δ bur2Δ* [pSPT5] (solid diamond) and *rad7Δ rad26Δ spt5Δ bur2Δ* [pSPT5/CTRΔ] (open square) cells. As comparison, the repair data in *rad7Δ rad26Δ spt5Δ* [pSPT5] cells (from Fig. 3.6C) are also plotted (open triangle).

(Fig. 3-8). This indicates that phosphorylation of the Spt5 CTR domain by the Bur kinase may be partially responsible for suppression of Rad26-independent TCR. In other words, besides phosphorylation, other components of the Spt5 CTR may also play a significant role in suppression of Rad26-independent TCR.

3.4 Discussion

In this paper, we show that the nonessential CTR domain of Spt5 plays an important role in suppression of Rad26-independent TCR. We also present evidence that the interacting partner of Spt5, Spt4, indirectly suppresses Rad26-independent TCR by protecting Spt5 from degradation and by stabilizing the interaction between Spt5 and Pol II.

TCR is generally believed to be initiated by stalling of an RNA polymerase at a lesion on the transcribed strand of a gene (Hanawalt and Spivak, 2008). In principle, a high level of transcription may facilitate TCR. Indeed, the transcription elongation function of Rpb9 is involved in TCR in yeast cells (Saunders et al., 2006). The human CSB and yeast Rad26 enhance transcription elongation by Pol II (Lee et al., 2001; Selby and Sancar, 1997a). However, TCR is not always positively correlated with transcription. For example, in *E. coli* the transcription factor Fis stimulates transcription of the tRNA gene *tyrT* to a very high level and at the same time suppresses TCR in this gene (Li and Waters, 1997). It was proposed that, during very high level transcription, an RNA polymerase may arrive at the site of a downstream RNA polymerase stalled at a lesion before the downstream RNA polymerase can initiate or finish the TCR process, resulting in suppression of TCR (Selby and Sancar, 1994).

Cells carrying mutations in *SPT4* and *SPT5* genes display phenotypes associated with defects in transcription elongation (Swanson and Winston, 1992), and the gene products are thought to be involved directly in transcription elongation (Hartzog et al., 1998; Rondon et al., 2003). However, the suppression of Rad26-independent TCR by Spt4/Spt5 does not seem to be

achieved simply by stimulating transcription. First, the Spt4/Spt5 complex does not seem to stimulate Pol II transcription to a level that is high enough to suppress Rad26-independent TCR. The galactose-induced *GAL1-10* genes are among the most robustly transcribed genes by Pol II in yeast (Lohr et al., 1995). However, TCR occurs very rapidly (i.e., not suppressed) in these genes in *rad16Δ rad26Δ* (*SPT4*$^{+}$ *SPT5*$^{+}$) cells (Li and Smerdon, 2002, 2004). In contrast, TCR is much slower (i.e., largely suppressed) in the much more slowly transcribed *RPB2* gene in the same cells (Li and Smerdon, 2002, 2004). Second, *spt4* and *spt5* mutations that cause similar deficiency in transcription elongation have different effects on suppression of Rad26-independent NER. For example, the *spt5-194* mutation, which is due to S324F substitution of the Spt5 protein (Guo et al., 2008), shares similar deficiency in transcription to *spt4Δ* (Hartzog et al., 1998) or deletion of the Spt5 CTR domain (Swanson et al., 1991a). However, unlike *spt4Δ* or deletion of the Spt5 CTR, the *spt5-194* mutation does not seem to alleviate TCR in *rad16Δ rad26Δ* cells (Jansen et al., 2000).

Although the exact binding site of Spt4/Spt5 on Pol II is currently unclear, it is predicted that this site is on the Rpb4/Rpb7 subcomplex that is dissociable from the 10-subunit core Pol II (Aloy et al., 2004). In the absence of Rpb4/Rpb7, Pol II has an open confirmation, whereas in the presence of this subcomplex, Pol II assumes a closed conformation (Armache et al., 2005; Bushnell and Kornberg, 2003). Interestingly, deletion of *rpb4* also reinstates TCR in *rad26Δ* cells (Li and Smerdon, 2002). Therefore, it is likely that Spt4/Spt5 and Rpb4/Rpb7 function together to suppress Rad26-independent TCR.

In human cells, the NGN and Kow domains of Spt5 have been shown to interact with Spt4 and Pol II, respectively (Ivanov et al., 2000; Yamaguchi et al., 1999). Similar to that of the human Spt5, the NGN domain of the yeast Spt5 is involved in interaction with Spt4 (Guo et al., 2008). The CTR domain of Spt5 does not seem to be involved in these interactions. Consistent

with these studies, we found that deletion of the Spt5 CTR does not affect the interaction with either Spt4 or Pol II (Fig. 3.4D).

How does the CTR of Spt5 suppress Rad26-independent TCR? It was found recently that the Spt5 CTR is a platform for the association of proteins that promote both transcription elongation and histone modification in transcribed regions (Zhou et al., 2009). One protein complex recruited by the Spt5 CTR is PAF (Zhou et al., 2009). Interestingly, deletion of PAF also reinstates TCR in *rad16Δ rad26Δ* cells (LeJeune et al., unpublished results). PAF plays an important role for recruitment of many factors involved in transcription elongation, such as COMPASS, FACT and Rad6/Bre1 (Krogan et al., 2003; Krogan et al., 2002). Therefore, the role of the Spt5 CTR in suppressing Rad26-independent TCR-NER may be achieved by serving as a platform for assembly of a mega suppressor complex that is associated with Pol II. This mega suppressor complex may suppress Rad26-independent TCR by preventing Pol II from either efficiently 'sensing' a lesion or recruiting the NER machinery.

bur2Δ cells grow much slower than Spt5 CTR deleted cells, indicating more pleiotropic roles for the Bur kinase in transcription. In addition to phosphorylation of the Spt5 CTR, Bur2 also plays a minor role in phosphorylating serine 2 of the heptapeptide repeats of the Rpb1 CTD (Qiu et al., 2009). Phosphorylation of the Spt5 CTR by the Bur kinase may not be solely responsible for suppression of Rad26-independent TCR by the Spt5 domain, as TCR rate in *rad7Δ rad26Δ bur2Δ* cells expressing the full-length Spt5 is somewhat slower than that in the same cells expressing the CTR-deleted Spt5 (Fig. 3.8). It is possible that phosphorylation of the Spt5 CTR may enhance but not be solely responsible for the CTR to recruit other TCR suppressors, such as PAF.

In *RAD26*$^{+}$ cells, TCR does not appear to be affected by deletion of either *spt4* (Jansen et al., 2000) or the Spt5 CTR (not shown). It seems that the suppression of TCR by Spt4/Spt5 is

specifically antagonized by Rad26. Rpb9, a nonessential subunit of Pol II that also plays an important role in TCR (Li and Smerdon, 2002, 2004), does not antagonize the suppression of TCR by Spt4/Spt5 (Ding et al., unpublished results). One important question that remains to be answered is how Rad26 antagonizes the suppression of TCR by Spt4/Spt5. Rad26 (Guzder et al., 1996) and its human counterpart CSB (Selby and Sancar, 1997b) are members of the SWI2/SNF2 family of ATPases, and both are involved in transcription elongation by Pol II (Lee et al., 2001; Selby and Sancar, 1997a). Rad26 (Jansen et al., 2002) and CSB (Fousteri et al., 2006) appear to dynamically associate with Pol II, especially upon DNA damage. One explanation is that Rad26 may somehow displace Spt4/Spt5 (and possibly other suppressors) from Pol II through either competitive binding to Pol II or remodeling the Pol II complex stalled at a lesion, making Spt4/Spt5 unable to suppress TCR. We are testing this hypothesis.

3.5 References

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CHAPTER 4

RAD26 ENHANCES TRANSCRIPTION COUPLED REPAIR BY RESTRAINING THE BINDING OF SPT4/SPT5 TO RNA POLYMERASE II

4.1 Introduction

To warrant genomic stability under conditions of consistent challenges exerted by endogenous and exogenous sources, a number of DNA repair pathways have evolved (Rouse and Jackson, 2002). Nucleotide excision repair (NER) is one such repair mechanism capable of removing a wide variety of helix-distorting lesions, such as UV-induced cyclobutane pyrimidine dimers (CPDs) (Friedberg et al., 2006). NER can be divided into two pathways, global genomic NER (GGR), which refers to repair throughout the genome, and transcription coupled NER (TCR), which refers to a repair mechanism that is dedicated to the transcribed strand (TS) of actively transcribed genes (Hanawalt, 2002). In the yeast *Saccharomyces cerevisiae*, Rad7, Rad16, (Verhage et al., 1994), and Elc1 (Lejeune et al., 2009) are specifically required for GGR, but dispensable for TCR. The Rad7 and Rad16 form a complex that binds specifically to UV damaged DNA in an ATP-dependent manner and has DNA-dependent ATPase activity (Guzder et al., 1998). 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 (Gillette et al., 2006; Ramsey et al., 2004). It has also been suggested that Elc1 is a component of another ubiquitin ligase complex, which contains Ela1, Cul3, and Roc1 but not Rad7 and Rad16 (Ribar et al., 2006, 2007). The role of Elc1 in GGR may not be subsidiary to that of Rad7 and Rad16 (Lejeune et al., 2009).

The mechanistic details of TCR are relatively well understood in *Escherichia coli*. The transcription-repair coupling factor (TRCF) Mfd targets the transcribed strand for repair by recognizing a stalled RNA polymerase and actively recruiting the NER machinery to the

transcription blocking lesion as it dissociates the stalled RNA polymerase (Selby and Sancar, 1993). However, the TCR mechanisms in eukaryotes appear to be extremely complicated [for recent reviews see (Fousteri and Mullenders, 2008; Laine and Egly, 2006b; Sarasin and Stary, 2007)]. In mammalian cells, Cockayne syndrome group A (CSA) and B (CSB) proteins are specifically required for TCR, but dispensable for GGR (Troelstra et al., 1992; van Hoffen et al., 1993; Venema et al., 1990). Like its human homologue CSB, the yeast Rad26 plays an important role in TCR but is dispensable for GGR (van Gool et al., 1994). Both human CSB (Selby and Sancar, 1997b) and yeast Rad26 (Guzder et al., 1996) are SNF2-like family members, each of which contains seven ATPase motifs and possesses DNA-stimulated ATPase activity (Licht et al., 2003). It has been also shown that both human CSB and yeast Rad26 play roles in transcription elongation (Lee et al., 2001; Selby and Sancar, 1997a). Interestingly, TCR in yeast is not solely dependent on Rad26, as a substantial extent of TCR still occurs in cells lacking Rad26 (Li and Smerdon, 2002, 2004; Verhage et al., 1996). Rpb9, a nonessential subunit of RNA polymerase II (Pol II), has been shown to play a role in TCR through facilitating transcription elongation (Li et al., 2006a; Li and Smerdon, 2002, 2004; Saunders et al., 2006). However, the mechanisms of human CSB and yeast Rad26 facilitate TCR are still elusive.

Mutations in the *SPT4* and *SPT5* genes in yeast were originally isolated as suppressors of the Ty insertion mutations that interfere with adjacent gene transcription (Winston et al., 1984). Immunopreciptation study showed that Spt4 and Spt5 form a complex, which physically interacts with Pol II (Hartzog et al., 1998). These proteins are conserved eukaryotic transcription-elongation factors and are generally required for normal development and for viral gene expression in multicellular eukaryotes (Winston, 2001). In mammalian cells, the Spt4/Spt5 complex, which is also called DRB sensitivity inducing factor (DISF), represses transcription elongation at the early elongation-recessive elongation transit (Wada et al., 1998; Yamaguchi et al., 1999). One function of

yeast Spt4/Spt5 in transcription was recently shown to overcome the repressive effect of histone modification and the function of chromatin regulators in yeast (Quan and Hartzog, 2009).

Interestingly, the deletion of *spt4* has been shown to alleviate the requirement of Rad26 for TCR in yeast, indicating that Spt4 suppresses Rad26-independent TCR (Jansen et al., 2000). Our recent results indicated that Spt4 indirectly suppresses Rad26-independent TCR by protecting Spt5 from degradation and by stabilizing the interaction of Spt5 with Pol II (Ding et al., 2010). Spt5 C-terminal repeat (CTR) domain, which is dispensable for cell viability and is not involved in interactions with Spt4 and Pol II, plays an important role in the suppression. The Spt5 CTR is phosphorylated by the Bur2 kinase (Ding et al., 2010; Liu et al., 2009). Inactivation of the Bur kinase partially alleviates TCR in *rad26*Δ cells (Ding et al., 2010). Thus, Spt5 is the more direct suppressor of Rad26-independent TCR. In yeast, Rpb9 plays an important role in Rad26-independent TCR (Li and Smerdon, 2002). We wondered if Spt4/Spt5 also functionally interacts with Rpb9. If not, why does this suppression occur only in the absence of Rad26? In this study, we present evidence that Spt4/Spt5 only functionally interact with Rad26, regardless of the presence of Rpb9. In the absence of Rad26, more Spt4/Spt5, especially the phosphorylated form of Spt5 (Spt5P), can be co-immunoprecipated with Pol II, suggesting that Rad26 restrains the binding of Spt4/Spt5 to Pol II. Moreover, we found that the ATPase activity of Rad26 is required for its functions in TCR and in the displacement of Spt5P from Pol II. These results indicate that Rad26 enhances TCR by restraining the binding of suppressors (Spt4, Spt5, possibly other factors) to Pol II.

4.2 Materials and Methods

4.2.1 Yeast Strains and Plasmids

Wild type yeast strain BJ5465 (*MATa ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1*) was obtained from the American Type Culture Collection. All deletion mutants were made in

BJ5465 background and confirmed by PCR analysis, using procedures described previously (Li and Smerdon, 2002). *URA3*, *LEU2* and *KanMX* were used to replace the gene to be deleted. Nucleotides (with respect to the starting codon ATG) +14 to +288, +214 to +1454, +58 to +2297 and +11 to +366 were deleted for *SPT4*, *RAD7*, *RAD26* and *RPB9* genes, respectively. Strains with their genomic genes tagged with three consecutive FLAG (3xFLAG) sequences were created by using PCR products amplified from plasmid p3FLAG-KanMX as described previously (Gelbart et al., 2001).

A single-copy centromeric plasmid with the *URA3* gene as a selection marker and encoding the wild type Rad26 protein was created by using the plasmid pRS416 (Sikorski and Hieter, 1989). The full length of the *RAD26* gene encompassing the 5' promoter, the coding region and the 3' terminator was amplified by PCR and inserted between the SacI and ClaI sites of pRS416 to create the plasmid pRS416-RAD26. Three consecutive FLAG sequences were amplified by PCR and inserted into N-terminal of Rad26 in pRS416-RAD26 between SpeI and BanHI to create plasmids p3FRAD26. Plasmids encoding Rad26 with point mutations (K328A, T329C, Q759A, or R763A) at ATPase motifs were created using plasmids p3FRAD26 by PCR with primers containing desired mutations (see Fig. 4-5A).

4.2.2 Repair Analysis of UV Induced CPDs

Yeast cells were grown at 30°C in minimal medium containing 2% galactose (SG) to late log phase ($A_{600} \approx 1.0$), irradiated with 100 J/m^2 of 254 nm UV and incubated in YPG medium (2% peptone, 1% yeast extract and 2% galactose) in the dark at 30°C. At different times of the repair incubation, aliquots were removed and the genomic DNA was isolated using a hot SDS procedure as described previously (Li and Smerdon, 2002).

The gene fragments of interest were 3' end labeled with [α-^{32}P]dATP using a procedure described previously (Li and Waters, 1996; Li et al., 2000). Briefly, ~ 1 μg of total genomic DNA

was digested with restriction enzyme(s) to release the fragments of interest and incised at CPD sites with an excess amount of 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 5 minutes 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), labeled with [α-^{32}P]dATP (Perkin Elmer), and resolved on sequencing gels. The gels were exposed to a Phosphorimager screen (Bio-Rad). The signal intensities at gel bands corresponding to CPD sites were quantified using Quantity One software (Bio-Rad).

4.2.3 Whole-Cell Extract Preparation and Immunoprecipitation

Yeast cells were cultured at 30°C in minimal medium containing 2% glucose to late log phase ($A_{600} \approx 1.0$) and harvested immediately, or irradiated with 240 J/m^2 of 254 nm UV. The UV irradiated samples were incubated in YPD (2% peptone, 1% yeast extract and 2% glucose) at 30°C and harvested at different times during the incubation. For measuring cellular levels of proteins of interest, whole-cell extracts were prepared using a trichloroacetic acid (TCA) method (Chen et al., 2007). The harvested cells from a 5 ml culture were resuspended in 300 µl of 20% TCA and broken by vortexing them with acid-washed glass beads. The proteins in the lysates were pelleted by centrifugation, washed with ice-cold 80% acetone and dissolved in 100 µl of 2× SDS-PAGE gel loading buffer (Sambrook and Russell, 2001).

For immunoprecipitation, the harvested cells from a 50 ml culture were washed and resupended in 0.5 ml of IP buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1mM EGTA, 0.4 mM Na_4VO_3, 10 mM $Na_4P_2O_7$, 10 mM NaF, 0.5% NP-40, 1% Triton X-100, 0.1% SDS, 0.2 mM PMSF and protease inhibitors) (Chen et al., 2007). The cells were broken with

acid-washed glass beads, and cell debris was removed by centrifugation at 20,000 × *g* for 10 minutes at 4°C. Fifty μl of the lysate was saved as an 'input'. The remaining lysate was added with 15 μg of anti-FLAG M2 (Sigma), or H14 (Covance) antibodies, which recognize FLAG tagged peptide and the serine 5 hyper-phosphorylated C-terminal heptapeptide repeats of Rpb1 (Palancade and Bensaude, 2003), respectively. The mixture was incubated at 4°C overnight with gentle rotation. Protein A or G-coated agarose beads (Sigma) were added to the mixture and incubated at 4°C for 3 hours with gentle rotation. The beads were washed twice with IP buffer containing 0.5 M of NaCl and twice with IP buffer. Bound proteins were eluted by boiling the beads in 50 μl of 2× SDS-PAGE gel loading buffer (Sambrook and Russell, 2001).

To measure the binding of Spt5 to elongating Pol II, yeast cells were cross-linked by 0.1% formaldehyde for 30 min at room temperature and then stopped by addition of 125mM glycine. The harvested cells were washed twice with ice-cold TBS buffer (20 mM Tris-Cl, pH 7.4, 140 mM NaCl, 2.5mM KCl) and resuspended in 0.5 ml of lysis buffer (50 mM Hepes, pH 7.8, 150 mM NaCl, 0.5% NP-40, 1% Triton X-100, 10% glycerol). The cells were broken with acid washed beads, and the supernatant was removed by centrifugation at 20,000 × *g* for 10 minutes at 4°C. The pellet was resuspended in 0.2 ml ChIP lysis buffer (50 mM Hepes, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% Sodium Deoxycholate, 1mM EDTA, 0.4 mM Na_4VO_3, 10 mM $Na_4P_2O_7$, 10 mM NaF, 0.2 mM PMSF and protease inhibitors) and sonicated. The debris was removed by centrifugation at 4°C and the supernatant was added ChIP lysis buffer to ~0.7ml. Fifty μl of the supernatant was saved as an 'input'. The remaining supernatant was used to do immunoprecipitation as described above. For DNAase treatment in co-immunoprecipitation assay, the whole cell extract or supernatant was treated with 50U of Benzonase (Sigma) for 30 min at 37˚C in the presence of protease inhibitors prior to the addition of antibody. Finally, each

sample was resuspended into 50 µl of 2×SDS-PAGE gel loading buffer and incubated at 95˚C for 1hour after addition of 20µl of TE buffer to elute proteins and to reverse crosslinking.

4.2.4 Western Blot

Proteins in whole-cell extracts, immunoprecipitation inputs, or immunoprecipitated samples were resolved on an SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Rpb1 and 3xFLAG tagged proteins on the same blot were probed with antibodies H14 and M2, respectively. Blots were incubated with SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce), and the protein bands were detected using a chemiluminescence scanner (Fluorchem 8800, Alpha Innotech). Band intensities were quantified using AlphaEaseFC 4.0 software.

4.2.5 ATPase Assays

3xFLAG-tagged wild-type and mutant Rad26 proteins were immunoprecipitated from 50 ml of log-phase yeast cultures by using an anti-FLAG antibody (M2) and protein A-coated agarose beads (see above). After extensive washing, the agarose beads bearing the immunoprecipitated wild type or mutant Rad26 proteins were incubated with 0.5 µCi of [α-^{32}P]ATP in 20 µl of a reaction buffer (50mM Tris-HCl, pH8.0, 5mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 5% glycerol, 1 mg/ml of sonicated heat-denatured salmon sperm DNA) (Guzder et al., 1996; Li and Altman, 2001) at 37°C for 30 min. The reaction was stopped by addition of 20mM of EDTA and placed on ice. An aliquot of the reaction mixture (1µl) was spotted on a polyethyleneimine (PEI) cellulose thin-layer chromatography (TLC) plate (Sorbent Technology) and air dried. The TLC plate was developed in 750 mM potassium phosphate (pH 3.5) and exposed to a phosphorimager screen.

4.2.6 UV Sensitivity Assay

Yeast cells were grown at 30°C in minimal medium to saturation, and sequential 10-fold dilutions were made. The diluted samples were spotted onto YPD plates. When the spot had dried, the plates were irradiated with different doses of 254 nm UV light. The plates were incubated at 30°C for 2–3 days in the dark prior to being photographed.

4.3 Results

4.3.1 Spt4 Suppresses TCR Only in the Absence of Rad26, but Does Not Functionally Interact with Rpb9

It has been shown that Spt4 does not play a significant role in GGR (Jansen et al., 2000). Indeed, we observed that NER rate in the NTS of an actively transcribed gene in *spt4*Δ cells was similar to that in *SPT4*$^{+}$ cells (not shown), confirming the previous notion that Spt4 does not modulate GGR (Jansen et al., 2000). However, *spt4*Δ deletion restores TCR in *rad26*Δ (Ding et al., 2010; Jansen et al., 2000) and *rad26*Δ *rpb9*Δ (Li et al., 2006b) cells. In order to further characterize the role of Spt4 in modulating TCR, we analyzed NER in the TS of the *GAL1* gene in different mutants. These mutants were created in *rad7*Δ cells, which are deficient in GGR (Verhage et al., 1994; Verhage et al., 1996), so that the modulation of TCR by Spt4 can be specifically analyzed. As can be seen, deletion of *SPT4* did not significantly affect TCR in *rad7*Δ and *rad7*Δ *rpb9*Δ cells (Fig. 4-1, compare panels A and B, and C and D). However, in *rad7*Δ *rad26*Δ cells, deletion of *SPT4* enhanced repair dramatically (Fig. 4-1, compare panels E and F). No repair can be seen in *rad7*Δ *rad26*Δ *rpb9*Δ cells, indicating that TCR is dependent on Rad26 and Rpb9, in agreement with our previous observations (Li and Smerdon, 2002, 2004). However, additional deletion of *SPT4* in *rad7*Δ *rad26*Δ *rpb9*Δ cells restored TCR activity to a certain extent (Fig. 4-1, compare panels G and H). We also analyzed TCR in the constitutively expresses *RPB2* gene, and found *spt4* deletion does not significantly affect TCR in *RAD26*$^{+}$ cells,

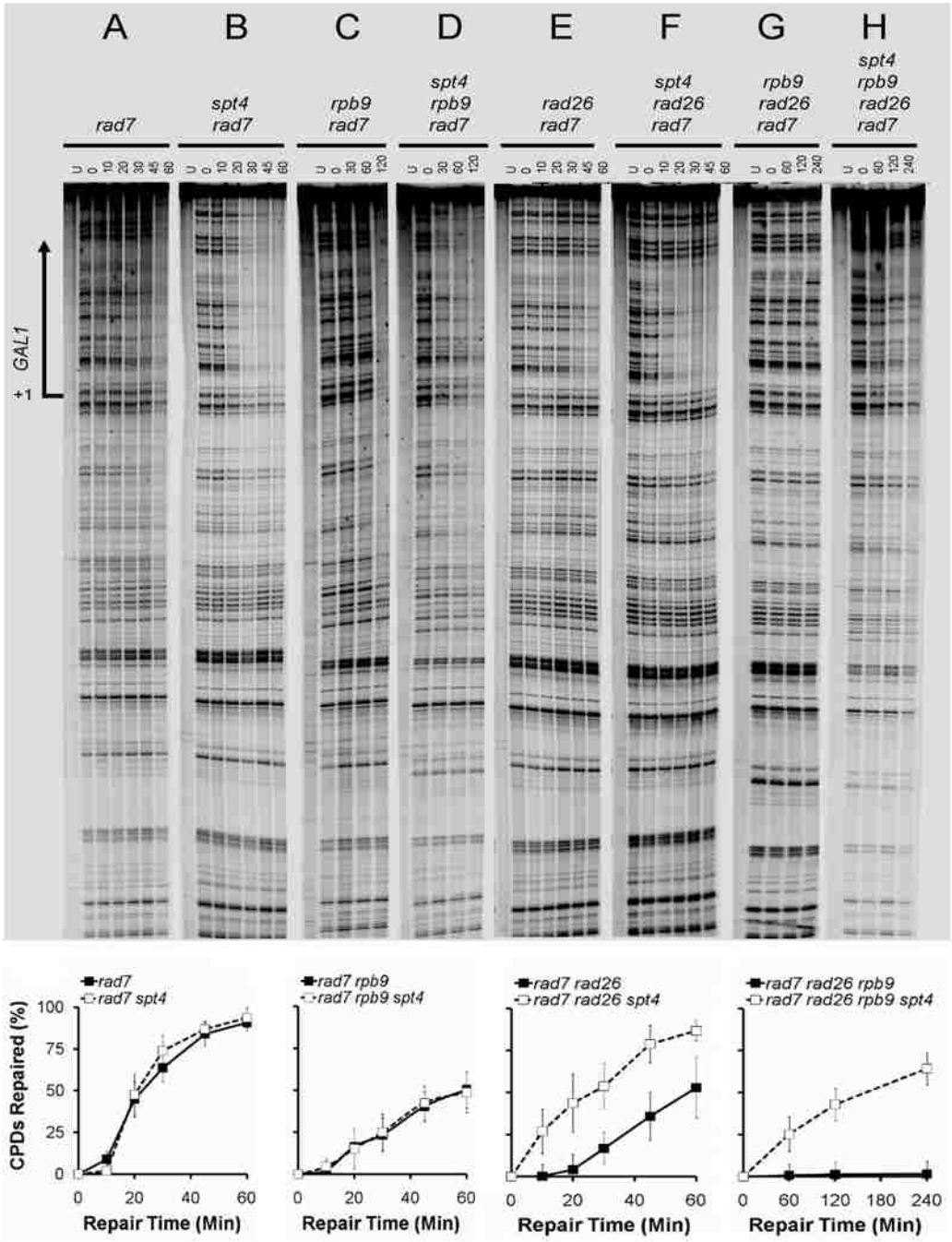

Figure 4-1 Gels showing TCR in the TS of the galactose induced *GAL1* gene. The lanes are DNA samples from unirradiated (*U*) and UV irradiated cells following different times (minutes) of repair incubation as indicated at the top of the gels. The arrow on the left of the gels marks the transcription start site. Plots at the bottom showing the mean (± standard deviation) of percent CPDs repaired in the transcribed region of *GAL1* gene in the indicated strains.

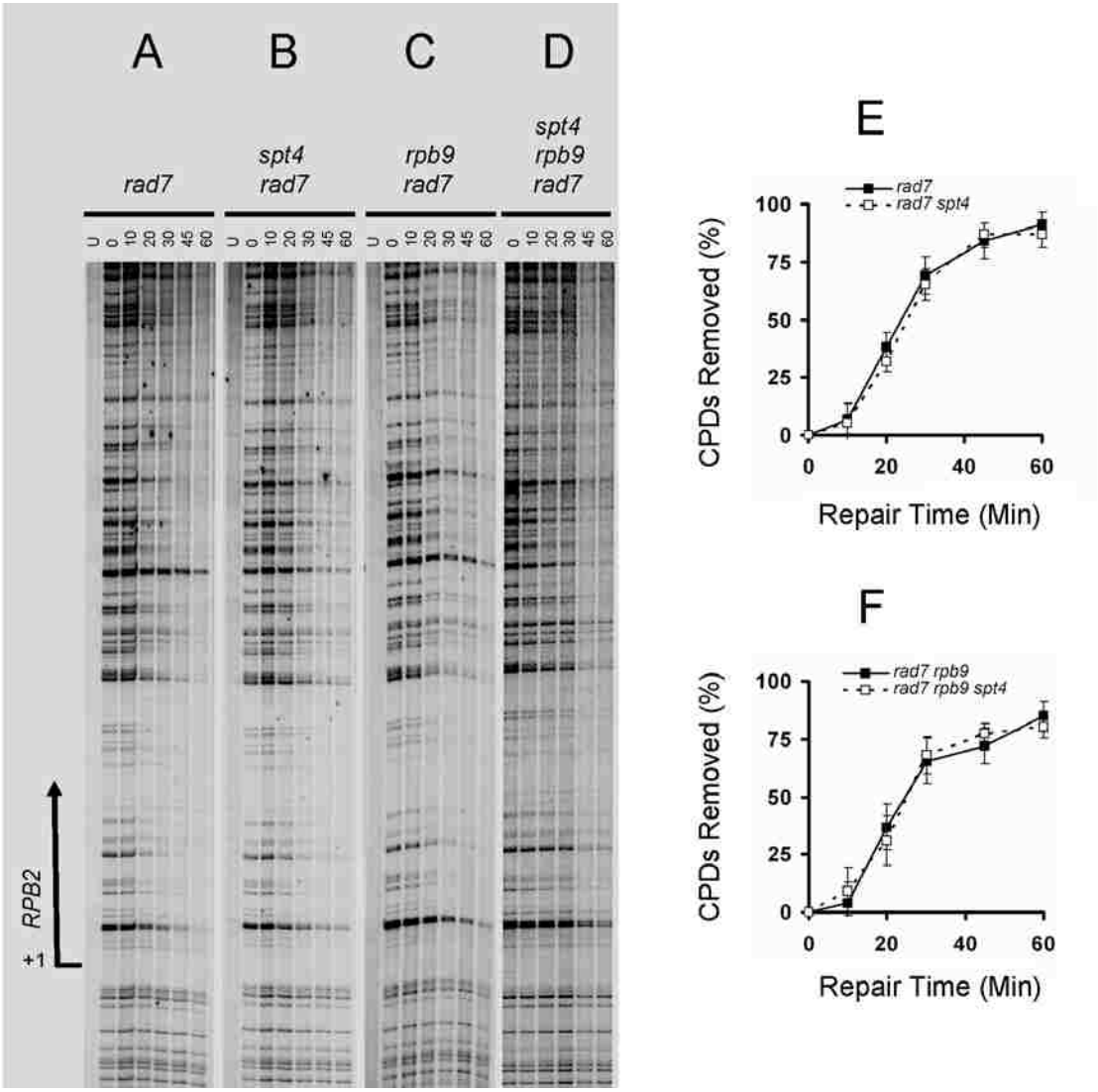

Figure 4-2 Gels showing TCR in the TS of *RPB2* gene. (A)-(D) DNA sequencing gels showing TCR in the RPB2 gene in 60 minutes repair. The lanes are DNA samples from unirradiated (*U*) and UV irradiated cells following different times (minutes) of repair incubation as indicated at the top of the gels. The arrow on the left of the gels marks the transcription start site. (E) and (F) plots showing the mean (± standard deviation) of percent CPDs repaired in the transcribed region of *RPB2* gene in the indicated strains.

regardless the presence of Rpb9 (Fig.4-2, compare panels A and B, and C and D). It has also been shown that *spt4* deletion does not significantly affect TCR in the *URA3* and *RPB2* genes in *rad16Δ* cells but significantly enhances TCR in *rad16*Δ *rad26*Δ cells (Jansen et al., 2000). Our results, together with the previous report, support the notion that Spt4 does not significantly modulate TCR in the presence of Rad26. However, when Rad26 is absent, Spt4 suppresses TCR regardless of the presence of Rpb9.

4.3.2 Rad26 Dynamically Associates with Pol II and Restrains the Binding of Spt4 and Spt5 to Pol II

Removal of Spt4 or the Spt5 CTR reinstates TCR in *rad26Δ* cells (Ding et al., 2010; Jansen et al., 2000). Interestingly, the removal does not appear to affect TCR in any $RAD26^{+}$ strains analyzed. It seems that Spt4/Spt5 suppresses TCR and Rad26 specifically antagonizes the suppression. To gain insights into the underlying mechanisms, we tested the mutual effects of Rad26 and Spt4/Spt5 in their binding to Pol II.

Compare to the cellular level of Rad26 (Fig. 4-3A, INPUT samples), only a small amount of Rad26 can be co-immunoprecipitated with Pol II (Fig. 4-3A, IP samples). Interestingly, after UV irradiation, more Rad26 can be co-immunoprecipitated with Pol II (Fig. 4-3A, IP samples). The cellular levels of Rad26 and the fractions of Rad26 associated with Pol II in *spt4Δ* cells were similar to those in wild type cells (Fig. 4-3A). Samples treated with DNAase did not affect this trend. These results indicate that protein Rad26 dynamically associates with Pol II and UV treatment facilitates this association, regardless of the presence of Spt4.

The cellular levels of Spt4 in *rad26Δ* cells were similar to that in wild type cells (Fig. 4-3B, INPUT). However, about 1/3 of the cellular Spt4 was bound to Pol II in wild type cells (Fig. 4-3B, compare INPUT and IP samples). In contrast, almost all the cellular Spt4 was associated with Pol II in *rad26Δ* cells (Fig. 4-3B, compare the INPUT and IP samples). UV

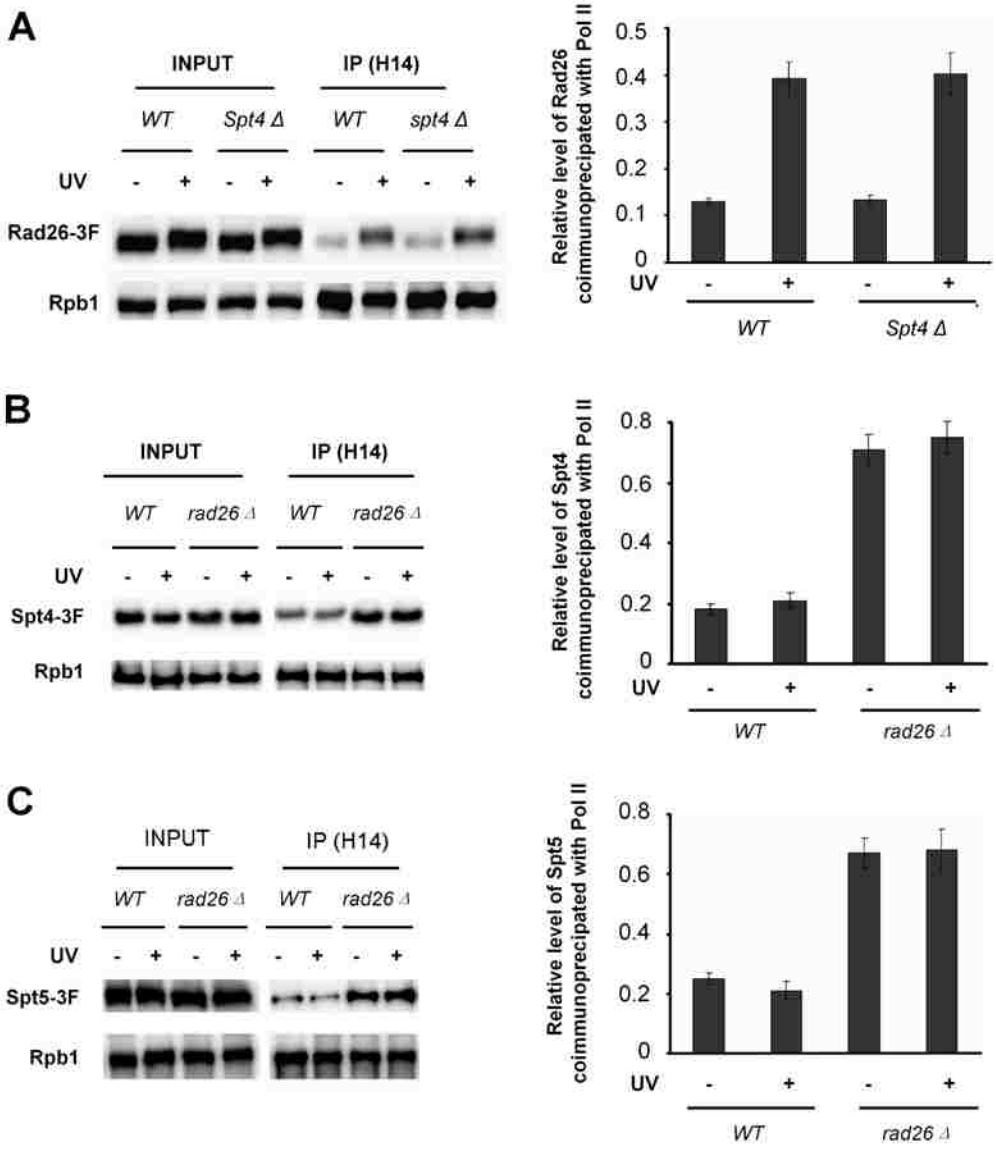

Figure 4-3 Rad26 dynamically associates with Pol II and restrains the binding of Spt4 and Spt5 to Pol II. (A) Pol II complexes were immunoprecipitated by using antibody H14 from wild-type and *spt4Δ* cells containing genomic 3xFLAG tagged Rad26 without UV irradiation or with 30 min recovery incubation after UV treatment. The level of Rad26 was probed by anti-FLAG (M2) antibody. Rpb1 was probed by H14 antibody and was set as loading control. Western blotting showing the level of 3xFLAG tagged Spt4 (Spt4-3F) (B) and 3xFLAG tagged Spt5 (Spt5-3F) (C) in wild-type and *rad26Δ* cells. Samples were treated as described in A. The plots on the right showing the relative level of Rad26, Spt4, and Spt5 can be co-immunoprecipitated with Pol II in IP samples of A, B, and C, respectively. The values shows as mean (± standard deviation) from three independent experiments and normalized with loading control.

irradiation did not affect the association of Spt4 with Pol II in either the wild type or *rad26Δ* cells (Fig. 4-3B, IP). Similarly, the level of total Spt5 associated with Pol II in *rad26Δ* was ~ 3 times that in wild type cells (Fig. 4-3C). These results indicate that Rad26 restrains the binding of Spt4 and Spt5 to Pol II, regardless of the presence of UV DNA damage.

4.3.3 Rad26 Is Not Involved in UV-Induced Dephosphorylation of Spt5

Previous studies by us (Ding et al., 2010) and others (Liu et al., 2009; Zhou et al., 2009) have shown that Spt5 can be phosphorylated at the CTR domain by the Bur kinase. We wondered if UV irradiation will affect the phosphorylation of Spt5 and the binding of Spt5 to Pol II. To this end, elongating Pol II complex was immunoprecipitated by H14 antibody in wild-type cells and rad26Δ cells that contain 3xFLAG tagged Spt5. UV irradiated and unirradiated cells were crosslinked by formaldehyde. Phosphorylated and unphosphophorylated Spt5 in immunoprecipitated samples were separated on SDS-PAGE gel by a longer electrophoresis.

In unirradiated *rad26Δ* cells, the ratio of phosphorylated to unphosphorylated Spt5 associated with Pol II was ~ 2. In contrast, in unirradiated wild type cells, the ratio was ~ 0.5 (Fig. 4-4A and B). Consistent with above results (Fig. 4-3C), the level of total (phosphorylated and unphosphorylated) Spt5 associated with Pol II in unirradiated *rad26Δ* cells was ~3 times that in wild type cells (Fig. 4-4A and C). Even the level of unphosphorylated Spt5 in unirradiated *rad26Δ* cells was also significantly higher than that in unirradiated wild type cells (Fig. 4-4A). These results suggest that Rad26 restrains the binding of both phosphorylated and unphosphophorylated Spt5 from Pol II. In *rad26Δ* cells, the high level of overall binding of Spt5 to Pol II may be easily phosphorylated, or Rad26 may interfere with the phosphorylation of Spt5 in wild type cells. Interestingly, UV irradiation caused rapid decrease in the ratios of phopsphorylated to unphosphophorylated Spt5 in both *rad26Δ* and wild type cells (Fig. 4-4A and

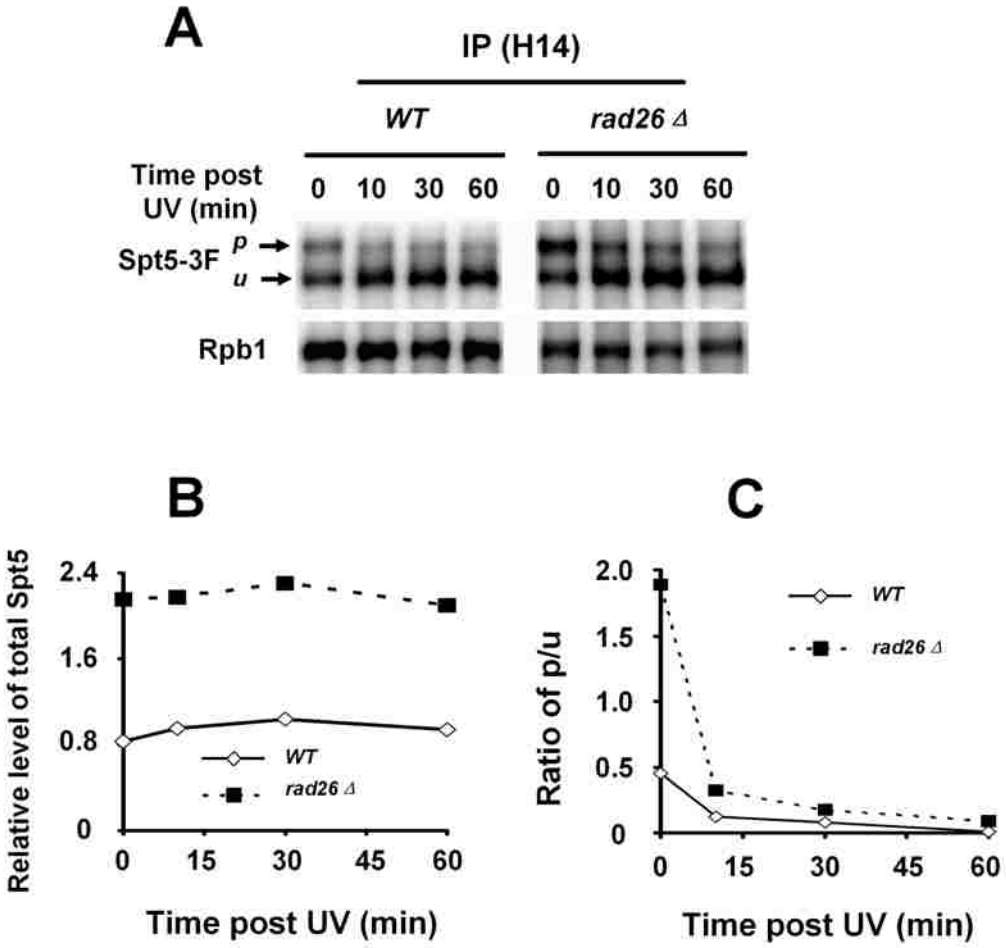

Figure 4-4 Rad26 is not involved in UV-induced dephosphorylation of Spt5. (A). Western blot showing levels of phosphorylated Spt5 and unphosphorylated Spt5 in different times after UV treatment in immunoprecipitated samples. 3xFLAG tagged Spt5 was co-immunoprecipitated with Pol II by using antibody H14 and probed by anti-FLAG antibody. Rpb1 was probed by H14 antibody and set as internal loading control. (B) Plots showing relative levels of total Spt5 in (A) (normalized by Rpb1). (C) Plots showing the ratios of phosphorylated Spt5 (*p*) to unphosphorylated Spt5 (*u*) in (A).

B). However, the irradiation did not appear to affect the total levels of Spt5 associated with Pol II (Fig. 4-4A and B). Samples treated with DNAase did not change the results (data not show). These results indicate that induction of DNA damage caused rapid dephosphorylation of Spt5 and Rad26 is not involved in the dephosphorylation.

4.3.4 The ATPase Activity of Rad26 Is Required for Facilitating TCR and for Restraining the Binding of Spt4/Spt5 to Pol II

How Rad26 restrains the binding of Spt4/Spt5 to Pol II? Given the fact that both Rad26 and Spt4/Spt5 associate with Pol II, one possibility is that Rad26 and Spt4/Spt5 competitively bind to the same site of Pol II. However, our results so far do not support this possibility. The binding of Rad26 to Pol II was obviously enhanced by UV treatment (Fig. 4-3A). On the contrary, the binding of Spt4/Spt5 did not change after UV irradiation (Fig. 4-3B and C). Although UV treatment facilitates Spt5 dephosphorylation, the level of total Spt5 in Pol II did not significantly change (Fig. 4-4D and F). On the other hand, the level of Spt5 bound to Pol II was much lower in *spt4Δ* cells than that in wild-type cells (Ding et al., 2010). However, the binding of Rad26 to Pol II did not significantly change after the deletion of *SPT4* (Fig. 4-3A). Thus, Rad26 antagonizes the binding of Spt4/Spt5 may through other approaches, such as the conformation change by allosteric effect, in stead of competitive binding.

Rad26 contains seven ATPase motifs (Fig. 4-5A) and possesses DNA-stimulated ATPase activity (Licht et al., 2003). We tested if the ATPase activity of Rad26 is required for TCR and for restraining the binding of Spt5 to Pol II. Point mutations at the Rad26 ATPase motif I (K328A T329C) or VI (Q759A R763A) (Fig. 4-5A), which did not affect the expression of Rad26 (Fig. 4-5B), appeared to completely abolish its ATPase activity, as immunoprecipitated Rad26 bearing these mutations showed ATPase activities that were similar to mock-immunoprecipitated samples (Fig. 4-5C).

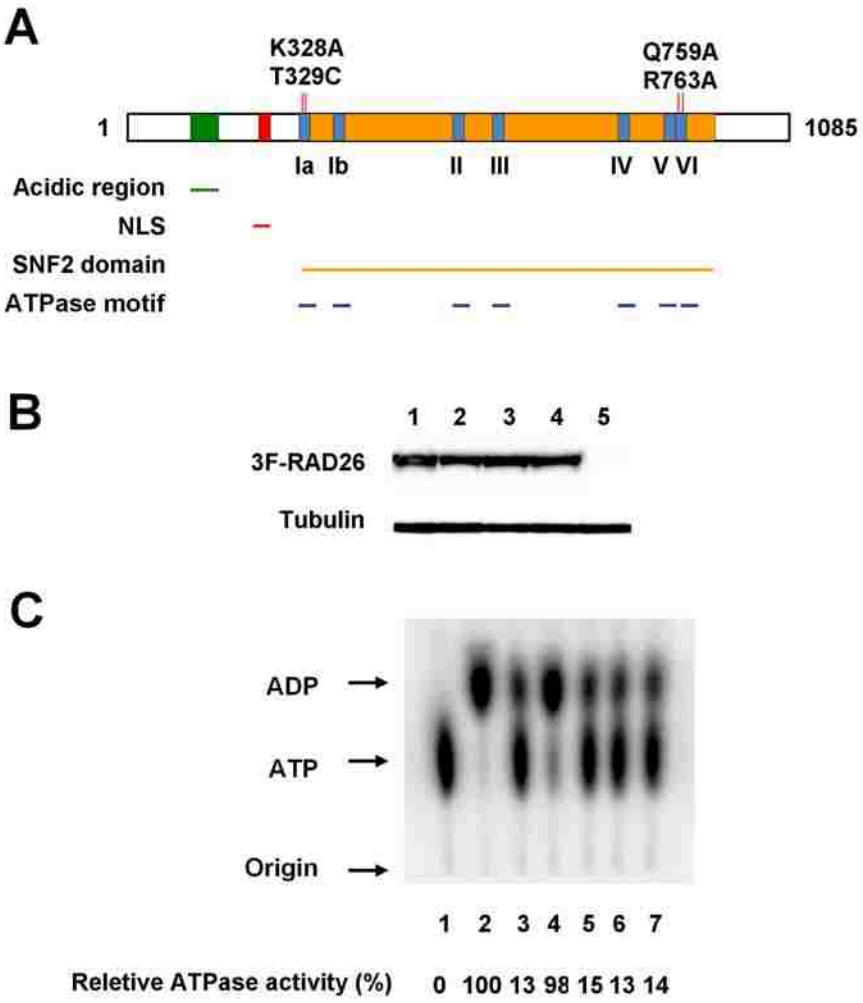

Figure 4-5 Point mutations in ATPase motifs inactivate Rad26 ATPase activity. (A) Schematic of protein Rad26. I to VI underneath indicate seven ATPase motifs. Bars underneath indicate the functional domains as labeled on the left. Positions of point mutations were labeled on the top. (B) Western blotting showing the cellular level of Rad26 in different mutants. 1. *rad7Δ rad26Δ* [3FRAD26], 2. *rad7Δ rad26Δ* [3FRAD26 K328A T329C], 3. *rad7Δ rad26Δ* [3FRAD26 Q759A R763A], 4. *rad7Δ rad26Δ* [3FRAD26 K328A T329C Q759A R763A], 5. Negative control, no 3xFLAG tagged Rad26. Tubulin was set as internal loading control. (C) Thin-layer chromatography (TLC) plate showing ATPase activity. Line 1. buffer only, 2. positive control contain known ATPase, 3. IP mock control, 4. IP sample from *rad7Δ rad26Δ* [3FRAD26], 5. IP sample from *rad7Δ rad26Δ* [3FRAD26 K328A T329C], 6. IP sample from *rad7Δ rad26Δ* [3FRAD26 Q759A R763A], 7. IP sample from *rad7Δ rad26Δ* [3FRAD26 K328A T329C Q759A R763A]. Relative ATPase activities were labeled underneath. Negative control containing buffer only was set as 0 (Line 1), and positive control containing known ATPase was set as 100 (Line 2).

GGR-deficient *rad7Δ* cells expressing the ATPase-deficient Rad26 are sensitive to UV (Fig. 4-6), and show similar rates of TCR to *rad7Δ rad26Δ* cells (Fig. 4-7, compare B to C and D, E), indicating that the ATPase activity of Rad26 is required for facilitating TCR. The levels of total (phosphorylated and unphosphorylated) Spt5 and the ratios of the phosphorylated to unphosphorylated Spt5 associated with Pol II in cells expressing the ATPase-deficient Rad26 were similar to those in *rad26Δ* cells (Fig. 4-8), indicating that the ATPase activity of Rad26 is required for restraining the binding of Spt5 to Pol II.

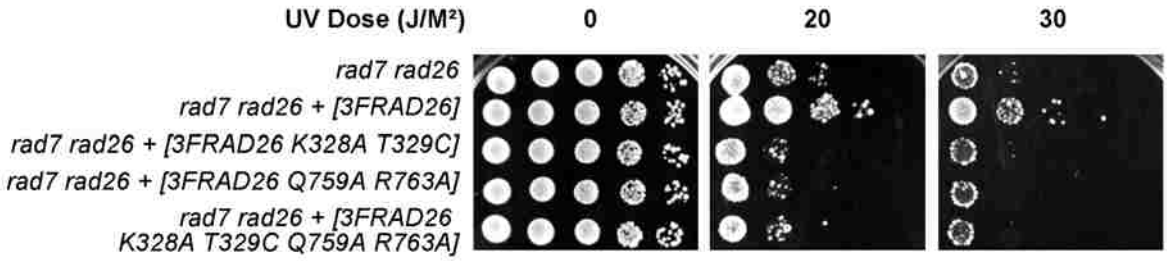

Figure 4-6 Mutations in Rad26 ATPase motifs increase the UV sensitivity. Saturated cultures of yeast strains were sequentially 10-fold diluted and spotted onto YPD plates. When the spots had dried, the plates were irradiated with the indicated doses of 254 nm UV light. The plates were incubated at 30°C for 3 – 5 days in the dark prior to being photographed. Strains containing a single-copy plasmid encoding the wild-type Rad26 or the point mutations Rad26 are indicated in brackets.

4.4 Discussion

In this study, we present evidence that Spt4/Spt5 suppresses TCR only in the absence of Rad26, regardless the presence of Rpb9. Rad26 restrains the binding of Spt4/Spt5 to Pol II. In response to UV irradiation, Spt5 is rapidly dephosphorylated, regardless of the presence of Rad26. Moreover, inactivation of Rad26 ATPase activity abolishes its functions in TCR and in restraining the binding of Spt4/Spt5 to Pol II. We propose that Rad26 facilitates TCR, at least in

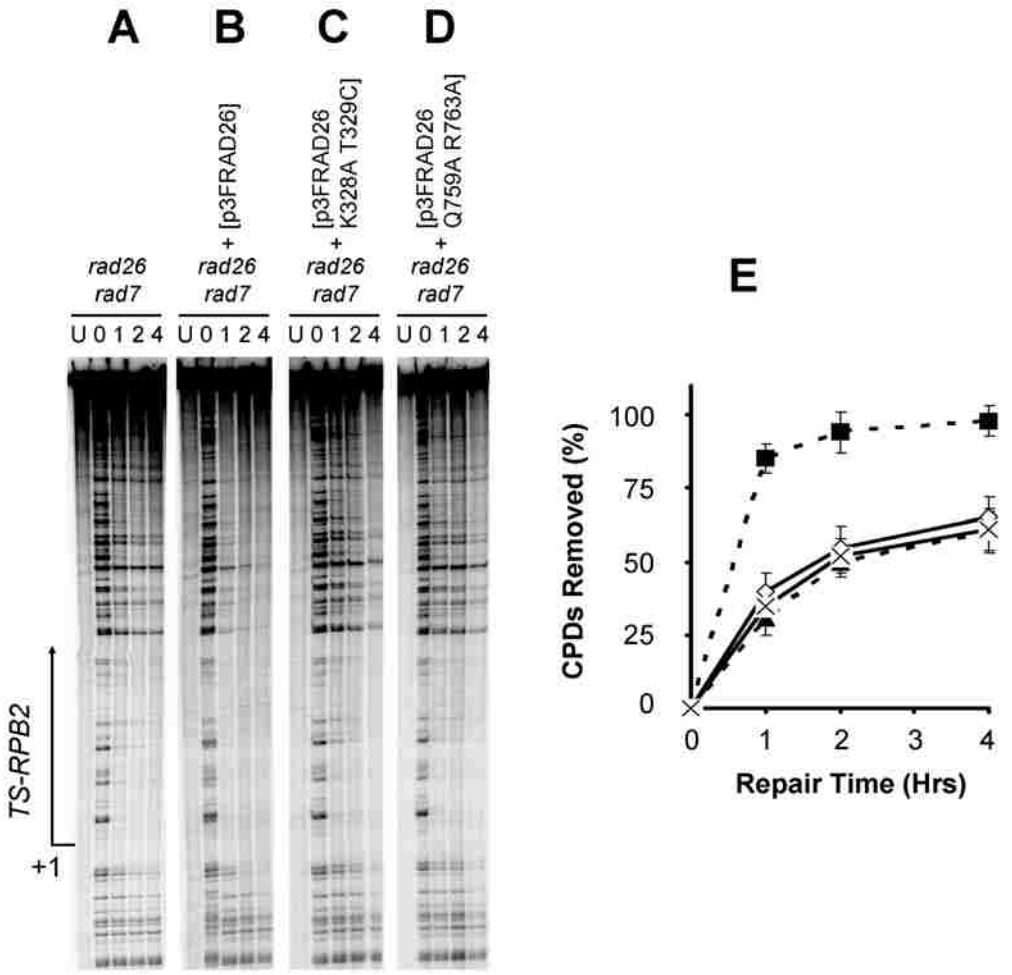

Figure 4-7 ATPase activity of Rad26 is required for facilitating TCR. (A)–(D) DNA sequencing gels showing TCR in the *RPB2* gene in different mutants. Brackets at the top indicate plasmids contained in the strain. The lanes are DNA samples from unirradiated (*U*) and UV irradiated cells following different times (hrs) of repair incubation as indicated at the top of the gels. The arrow on the left of the gels marks the transcription start site. (E) Plots showing the mean (± standard deviation) of percent CPDs repaired in the transcribed region of the *RPB2* gene in *rad7*Δ *rad26*Δ (open diamond), *rad7Δ rad26Δ* + [p3FRAD26] (solid square), *rad7*Δ *rad26*Δ + [p3FRAD26 K328A T329C] (solid triangle) and *rad7*Δ *rad26*Δ + [p3FRAD26 Q759A R763A] (asterisk) cells.

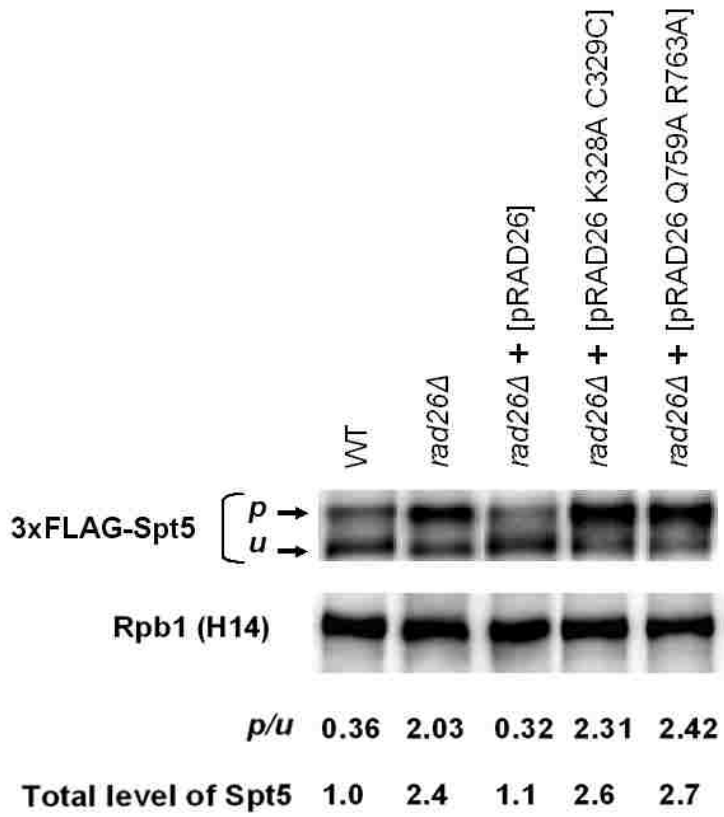

Figure 4-8 ATPase activity of Rad26 is required for restraining the binding of Spt5 to Pol II. 3xFLAG tagged Spt5 was co-immunoprecipitateted with Pol II by using H14 antibody from different strains as indicated. Spt5 was probed by anti-FLAG M2 antibody. Rpb1 was probed by H14 antibody and set as internal loading control. '*p*' and '*u*' on the left of the blot mark phosphorylated Spt5 and unphosphorylated Spt5, respectively. The ratio of phosphorylated Spt5 to unphosphorylated Spt5 (*p/u*) and the relative total levels of Spt5 were labeled underneath. The relative total levels of Spt5 were normalized by Rpb1, the level of Spt5 in wild type cells was set as 1.0.

part, through restraining the binding of the suppressors (Spt4, Spt5, possibly other factors) to Pol II by using its ATPase activity.

Although phosphorylation of the Spt5 C-terminal domain (CTR) has been shown to play an important role in the suppression of Rad26-independent TCR (Ding et al., 2010), how Spt5 suppresses TCR remains to be elucidated. One possibility is that, by binding to Pol II, Spt5 may directly suppress TCR through interfering with the access of NER factors, or changing the conformation of the Pol II complex, resulting in a deficiency in 'sensing' a lesion or a defect in recruiting NER machinery. Another possibility is that, Spt5 is indirectly involved in the suppression through mediating the recruitment of other suppressors to Pol II. It has been shown that Spt4/Spt5 complex acts as a platform in promoting the interaction between the Paf1 complex and the elongating Pol II (Qiu et al., 2006). Interestingly, the deletion of any core subunit (Paf1, Cdc73, Rtf1, Loe1, or Ctr9) of the Paf1 complex also reinstates TCR in *rad26Δ* cells (unpublished data). Thus, the possibility that Spt5 bridges TCR suppressors cannot be excluded. Given the fact that Spt4/Spt5 and Faf1 complex play very important roles in promoting transcription elongation (Mueller et al., 2004; Wada et al., 1998; Yamada et al., 2006; Zhu et al., 2007), some transcription elongation factors may be involved in both transcription and TCR processes. It will be very interesting to investigate how the functions of these factors are coordinated in different cellular processes.

It is believed that the blockage of the transcription elongation form of Pol II at damage sites serves as the initiation signal for TCR (Laine and Egly, 2006a; Lindsey-Boltz and Sancar, 2007). The human CSB protein was proposed to serve as a transcription-repair coupling factor (TRCF) and operate at early steps in the initiation of TCR (Fousteri et al., 2006). Similar to CSB, Rad26 may also act as TRCF in yeast (Svejstrup, 2002). Indeed, our results indicate that ATPase activity is required for Rad26 to mediate TCR (Figs. 4-7). However, unlike the *E. coli* TRCF

(Mfd), which displaces stopped RNA polymerase from damage sites (Savery, 2007), Rad26 displaces TCR suppressors Spt4 and Spt5 (possibly other factors) from Pol II through its ATPase activity (Figs. 4-4 and 8). Interestingly, transcription/DNA repair factor TFIIH has been shown to change subunit composition in response to UV irradiation (Coin et al., 2008). The detachment of CDK-activating kinase (CAK) complex from the core of TFIIH by XPA converts TFIIH from an elongation-proficient form into a repair-proficient form (Coin et al., 2008). Spt5 may also dynamically change forms through its C-terminal phosphorylation and dephosphorylation (Zhou et al., 2009) to adapt its engagement in transcription and TCR processes. Rad26 could regulate Spt5 functions in both transcription and TCR. Under physiological conditions, the phosohorylated Spt5 together with other transcription elongation factors promote transcription elongation through binding to Pol II (Buratowski, 2009; Liu et al., 2009). However, this transcription elongation-proficient form inhibits TCR, and needs to be switched to unphosphorylated form to release the inhibition. By using the energy from ATP hydrolysis, Rad26 might target the interface between Spt4/Spt5 and Pol II to displace Spt4/Spt5 from Pol II. The removal of Spt4/Spt5 (and other possible factors) will provide enough space or/and the signal for recruitment of NER machinery to the damage sites. After lesions are removed, the transcription machinery will be reassembled and switched back into the transcription elongation-proficient form. Consistent with this idea, a recent report (Malik et al., 2009) showed that Rad26 is recruited to the site of DNA lesion in an elongating Pol II-dependent manner, suggesting that the function of Rad26 in TCR is tightly related to transcription elongation. The function of Rad26 in TCR may be fulfilled by coordinating transcription elongation and TCR.

In human, the defect of *CSB* gene is the major cause of Cockayne syndrome (CS), a developmental disease characterized by photosensitivity, severe neurological abnormalities, short stature, and short life span (Nouspikel, 2009). These multiple clinical manifestations indicate that

CSB protein must be involved in other processes besides TCR, such as BER, transcription, and possibly chromatin remodeling (Stevnsner et al., 2008). Given that human CSB is the ortholog of the yeast Rad26, the new finding that Rad26 facilitates TCR partially through restraining the binding of Spt4/Spt5 to Pol II, provides a new avenue to understand the pathogenesis of CS. It will be very interesting to test if CSB protein has the similar function in regulation TCR and transcription.

4.5 References

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CHAPTER 5

CONCLUDING REMARKS

5.1 Research Summary

By using yeast *Saccharomyces cerevisiae* as a model organism, this dissertation was focused on the different roles of transcription factor Tfb5 in different NER pathways (Ding et al., 2007), the molecular mechanism of transcription elongation factors Spt4/Spt5 in the suppression of Rad26-independent TCR (Ding et al., 2010), and the functional mechanisms of the putative yeast transcription coupled repair factor (TCRF) candidate Rad26 in facilitating TCR. The major findings in this study are:

1. Transcription factor Tfb5, the tenth subunit of transcription/repair factor TFIIH, plays different roles in different NER pathways in yeast. Tfb5 is essential for GGR, but is not absolutely required for TCR. Tfb5 is partially dispensable for Rad26 mediated TCR, but is required for Rpb9 mediated TCR.
2. The deletion of *SPT4* alleviates TCR only in the absence of Rad26, regardless of the presence of Rpb9, suggesting Spt4 specifically suppresses Rad26-independent TCR. In another word, Rad26 specifically antagonizes this suppression.
3. Spt4 indirectly suppresses Rad26-independent TCR by protecting Spt5 from degradation and by stabilizing the interaction of Spt5 with RNA Polymerase II (Pol II). Spt5 C-terminal repeat which is dispensable for cell viability and is not involved in interactions with Spt4 and Pol II, plays an important role in the suppression.
4. Spt5 CTR is phosphorylated by Bur kinase. Inactivation of the Bur kinase partially alleviates TCR in *rad26*Δ cells, suggesting Spt5 CTR phosphorylation plays an important role in the suppression.

5. Rad26 dynamically associates with Pol II and restrains the binding of Spt4/Spt5 to Pol II. UV irradiation facilitates the association of Rad26 with Pol II and induces phosphorylated Spt5 (Spt5P) dephosphorylation.
6. ATPase activity of Rad26 is required for facilitating TCR and for restraining the binding of Spt5 to Pol II, suggesting Rad26 enhances TCR by antagonizing the binding of Spt4/Spt5 to Pol II via its ATPase activity.

5.2 Working Models

Based on these findings, we proposed the following working models to explain the functional mechanisms of Spt4, Spt5, and Rad26 in TCR. We found that Spt4 and Spt5 cooperatively suppress Rad26-independent TCR, but they play different roles in the suppression. Spt5 plays a more direct role in the suppression, whereas Spt4 is indirectly involved by stabilizing Spt5 and by enhancing the binding of Spt5 to Pol II. In the presence of Spt4, Spt4 and Spt5 form a stable complex which tightly associates with Pol II (Fig. 5-1A). The C-terminal repeat (CTR) domain of Spt5 can be phosphorylated by Bur kinase. Through binding to Pol II, Spt4 and Spt5 fulfill the suppressive function on Rad26-independent TCR (Fig. 5-1A). In the absence of Spt4, Spt5 is not stable and will be degraded. The level of Spt5 associated with Pol II is not high enough to suppress TCR any more, and then TCR is released (Fig.5-1B).

Models of functional mechanisms of Rad26 in TCR are shown in Figure 5-2. In the absence of Rad26, the Spt4/Spt5 complex tightly associates with the elongating form Pol II, in which both Ser2 and Ser5 of C-terminal heptapeptide repeats ($Y_1S_2P_3T_4S_5P_6S_7$) of Rpb1 are hyper-phosphorylated (Phatnani and Greenleaf, 2006). After Spt5 phosphorylation by Bur kinase, Spt4 and Spt5 play a positive role in transcription elongation (Chen et al., 2009; Liu et al., 2009; Zhou et al., 2009), but suppress TCR (Fig.5-2A). However, in the presence of Rad26, more than

half of Spt4/Spt5 can be displaced from Pol II by Rad26 through its ATPase activity (Figs. 4-3B and C, 4-4A and B, 5-2B). The lower level of Spt4/Spt5 in Pol II results in faster TCR. After UV irradiation, elongating Pol II will be stopped at the damage sites, which is believed to initiate TCR (Friedberg et al., 2006; Lindsey-Boltz and Sancar, 2007). In response to UV, Spt5P will be immediately dephosphorylated by unknown phosphatase(s) (Fig. 5-2B, question mark in blue), and more Rad26 will bind to Pol II (Figs. 4-3A, 5-2B). These events would be followed by NER machinery recruitment and then TCR occurs. After the removal of damage, transcription machinery could be reassembled, including Spt5 phosphorylation and the recruitment of other transcription elongation factors.

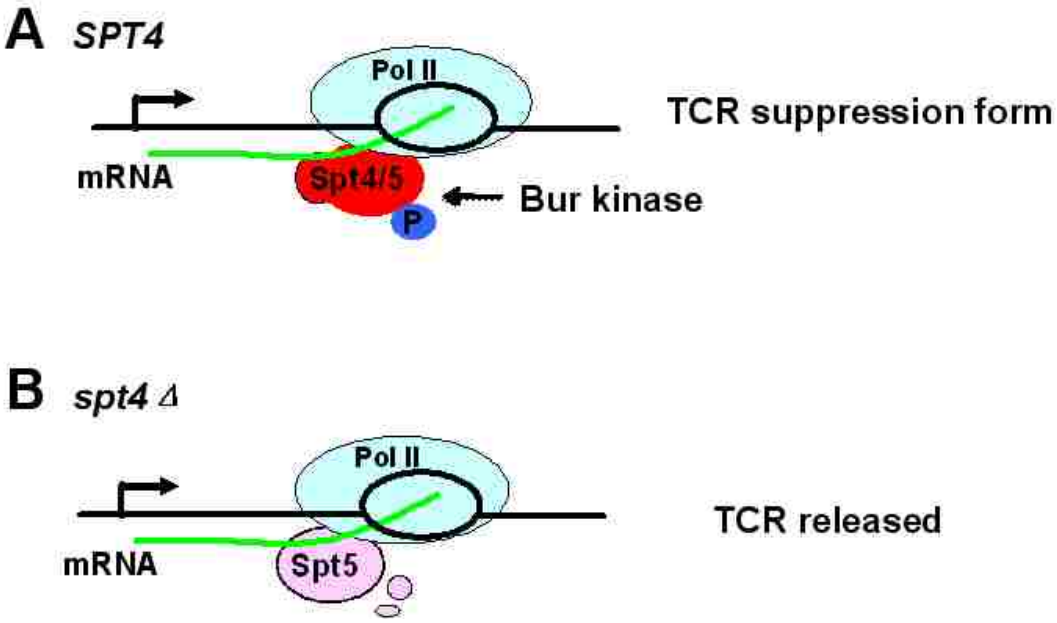

Figure 5-1 Spt4 and Spt5 cooperatively suppress TCR. (A) In the presence of Spt4, Spt4 and Spt5 form a stable complex which tightly associates with Pol II. The phosphorylation of the CTR domain of Spt5 by Bur kinase plays an important role in the suppression. (B) In the absence of Spt4, Spt5 is not stable and is degraded. The release of TCR results from less Spt5 binding to Pol II. The lighter color in red indicates lower levels of Spt5. Small circles indicate Spt5 degradation. Abbreviations: Pol II, RNA Polymerase II; P, phosphorylation.

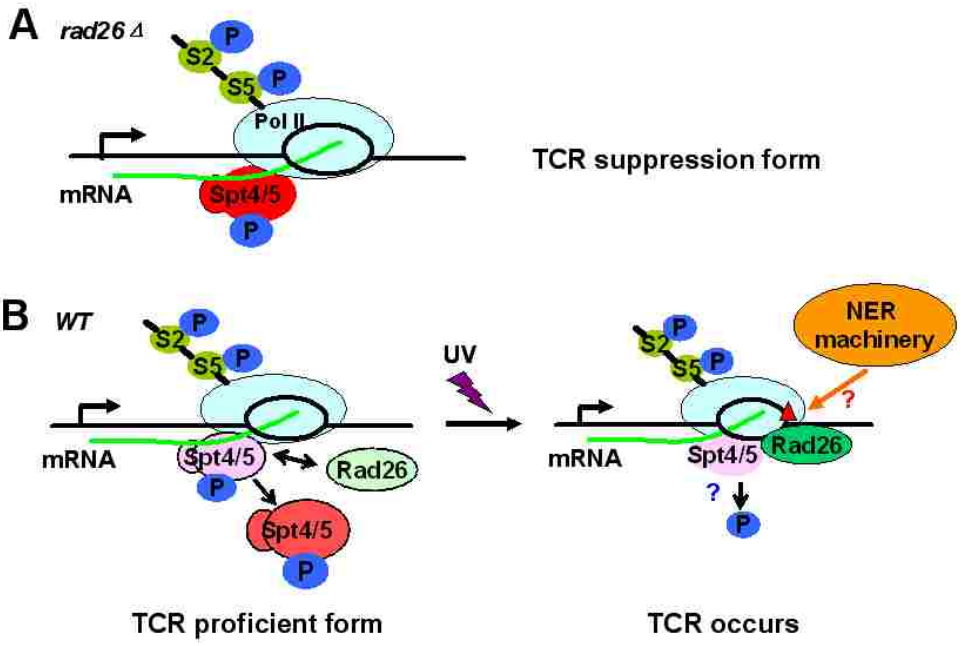

Figure 5-2 Rad26 enhances TCR by restraining the binding of Spt4/Spt5 to Pol II. (A) In the absence of Rad26, higher levels of Spt4/Spt5 bind to Pol II. The phosphorylation of Spt5 CTR facilitates transcription elongation, but suppresses TCR. (B) In wild-type cells, Rad26 dynamically associates with Pol II and restrains the binding of Spt4/Spt5 to Pol II via its ATPase activity. Less Spt4/Spt5 in Pol II forms a TCR proficient form. After UV irradiation, elongating Pol II would be stopped at the damage sites. Spt5 will be dephosphorylated and more Rad26 will bind to Pol II in response to UV. These events would be followed by NER machinery recruitment and TCR occurring. The darker colors in red and in green indicate higher protein levels of Spt4/Spt5 and Rad26, respectively. The red triangle indicates UV-induced damage site. The blue question mark indicates unidentified phosphatase(s). The red question mark indicates unknown mechanisms for NER machinery recruitment. Abbreviations: S2, Serine 2 of CTD; S5, Serine 5 of CTD; Pol II, RNA Polymerase II; P, phosphorylation.

5.3 Future Research

Although TCR has been extensively studied in eukaryotic cells for almost three decades, the exact molecular mechanisms are still unknown. The mechanisms of TCR will continue to be investigated by many researchers in the DNA repair field. Besides mechanistic studies, another aspect which needs to be focused on in this field is the pathogenesis of NER or TCR-defect

diseases, such as Xeroderma pigmentosum (XP), Trichothiodystrophy (TTD), Cockayne syndrome (CS), certain kinds of cancers, aging, and other progeroid syndromes. Based on this study and other recent reports, some research can be further carried on in the future.

5.3.1 Functional Mechanisms of Tfb5 in TFIIH and TFIIH in NER

Yeast Tfb5 plays different roles in different NER pathways (Ding et al., 2007). It would give some implication about the pathogenesis of the third group of TTD (TTD-A) if this idea was tested in humans. TFIIH has been shown to be required for both transcription initiation and NER (Hashimoto and Egly, 2009). How can TFIIH be involved in different cellular processes? In human cells, Tfb5 (p8) stimulates the ATPase but not helicase activity of XPB (Coin et al., 2006). Moreover, the CDK-activating kinase (CAK) subcomplex was shown to be dissociated from core TFIIH during NER, suggesting the composition of TFIIH is dynamically changing to adapt to functions in different processes (Coin et al., 2008). A recent report (Kainov et al.) indicated that Tfb2 bridges the Ssl2 (Rad25) helicase and Tfb5 subunit, and the Tfb5-interacting domain of Tfb2 also binds nucleic acids. Interestingly, Tfb5 triggers dissociation of nucleic acids from Tfb2, suggesting Tfb5 may facilitate a transition in TFIIH function from transcription to NER by targeting Tfb2. It will be interesting to test if Tfb5 serves as a molecular switch for the regulation of TFIIH activity and how it fulfills this regulation.

5.3.2 The Coordination Mechanisms of Spt5 in Transcription Elongation and TCR

The phosphorylation of yeast Spt5 C-terminal repeat (CTR) by Bur kinase plays important roles in both transcription elongation and the suppression of Rad26-independent TCR (Ding et al., 2010; Zhou et al., 2009). Interestingly, Spt5P is dephosphorylated in response to UV (Fig. 4-4). It seems that the events of phosphorylation and dephosphorylation play important roles in coordinating the functions of Spt5 in different cellular processes. Thus, the phosphatase(s)

of Spt5P is (are) an interesting factor(s) to be identified (Fig. 5-2B, blue question marker). Like Bur kinase (Ding et al., 2010; Keogh et al., 2003), this (these) phosphatase(s) could be involved in both transcription elongation and TCR. The Bur kinase and unidentified phosphtase(s) could act as a molecular switch to regulate Spt5 functions in these cellular processes through phosphorylation and dephosphorylation. Given that Bur kinase phosphorylates both CTR of Spt5 and C-terminal domain (CTD) Ser2 of Pol II (Liu et al., 2009; Qiu et al., 2009; Zhou et al., 2009), it is possible that Spt5 and CTD of Rpb1 are substrates of the same phosphatase(s). In yeast *S. cerevisiae*, there are several candidate phosphatases for Spt5P dephosphorylation in response to UV. One candidate is an evolutionarily conserved Pol II-binding protein called Rtr1. It was recently identified as CTD Ser5P phosphatase (Mosley et al., 2009). Rtr1 is not essential for yeast viability and the effect on Spt5P dephosphorylation cold be directly measured in *RTR1* gene deleted cells. Another CTD Ser5P phosphatase Ssu72 may be the second candidate (Krishnamurthy et al., 2004). The third candidate is Fcp1, which has CTD Ser2P phosphatase activity (Kops et al., 2002). However, unlike Rtr1, Ssu72 (Sun and Hampsey, 1996) and Fcp1(Kobor et al., 1999) are required for yeast cell viability. It would be better to measure the phosphatase activity by using techniques both *in vivo* and *in vitro*. For those essential gene products, protein purification and phosphatase activity measurement *in vitro* is required. It is possible that none of these three phosphatases is responsible for Spt5P dephosphorylation. A new phosphatase may need to be identified. These studies will provide new insights into the functional mechanisms of Spt5 in TCR and even in the transcription cycle (Buratowski, 2009).

5.3.3 Possible Cross Talk of Spt5 with Other Factors

How does Spt5 CTR suppress Rad26-independent TCR? Phosphorylation of the Spt5 CTR domain stimulates recruitment of Paf 1 complex (Liu et al., 2009), suggesting Spt5 may not be a direct suppressor on TCR. Paf1 complex also plays an important role for recruitment of

many factors involved in transcription elongation, such as COMPASS, FACT and Rad6/Bre1 (Krogan et al., 2003; Krogan et al., 2002). It will be interesting to test if these factors are involved in TCR. This study will give the indication whether the Spt5 CTR domain directly suppresses Rad26-independent TCR or just acts as a platform to recruit 'real' suppressors, which may form a mega-complex to prevent Pol II from either efficiently 'sensing' a lesion or recruiting the NER machinery.

5.3.4 Functional Mechanisms of Rad26 in TCR

Due to its ATPase activity, protein Rad26 is the most promising yeast transcription-repair coupling factor (TRCF) (Svejstrup, 2002). Indeed, ATPase activity is required for Rad26 mediated-TCR (Fig.4-7). However, unlike *E.coli* TRCF (Mfd), which displaces stopped Pol II from damage sites (Savery, 2007), Rad26 restrains the binding of suppressors Spt4/Spt5 to Pol II via its ATPase activity (Fig.4-8). Although Spt5 and Rad26 have been shown to associate with Pol II, the exact binding sites are not clear so far. It will also be interesting to identify the binding sites of Spt5 and Rad26 on Pol II. This study will clarify if competitive binding contributes to the displacement of Spt5 from Pol II besides ATPase activity. It will be also interesting and significant to test if CSB protein has a similar function in TCR in human cells. In response to UV, both yeast Rad26 and human CSB increase their binding affinity to Pol II (Fousteri et al., 2006). If the damage-stalled Pol II serves as the initial signal for TCR, the event that a higher level of Rad26 or CSB bound to Pol II could act as the TCR signal transducer for NER machinery recruitment (Fig.5-2B, red question marker). These studies are significant to understand the functional mechanisms of Rad26/CSB in TCR and the pathogenesis of TCR defects diseases such as Cockayne syndrome (CS).

5.3.5 The Effect of Human Spt4/Spt5 (DSIF) Complex on TCR

Spt4 and Spt5 are conserved transcription elongation factors. Similarly, the phosphorylation of human Spt5 CTR by positive elongation factor b (P-TEFb) is critical for processive transcription elongation (Yamada et al., 2006). It will be very interesting to test the effect of human Spt4/Spt5 on TCR. If hSpt4/Spt5 complex indeed suppresses TCR, protein Spt5 or the CTR domain peptide has the potential clinical application to treat some cancers when combined with chemical drugs (Ljungman, 2009). This could be a long-term goal for the Spt5 project.

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APPENDIX A: LETTERS OF PERMISSION

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APPENDIX B: OLIGONUCLOETIDES USED IN THIS DISSERTATION

Name	Sequence	According gene
For NER analysis		
RPB2-B	[BioTEG]ACATCATTTTGTTTTGTTTTGTTTTAAAAGCAT AATCCCAAATAATTCTGA	RPB2 (TS)
RPB2-B2	[BioTEG]CTGACATTTTTGTTTTTGTTTTTAAACACTTGA ACAATATTACCTGCAG	RPB2 (NTS)
UAS7	[BioTEG]CTGACATTTTGTTTGTTTTGTTTAGCCCCATTA TCTTAGCCTAAAAA	GAL1-10 (TS)
GAL5	[BioTEG]CTGACATTTTGTTTGTTTTGTTTAAAGTAATTA GACCAGTCCGACACAG	GAL1 (NTS)
For creation 3xFLAG or 3xMyc tagged protein		
SPT4-3F1	TTGTGGAGCTGTTGCCTCACTACAAACCGAGGGATGGC AGTCAAGTTGAGAGGGAACAAAAGCTGGAGCTC	SPT4
SPT4-3F2	AAAAAAAATTCATTCATATTATACATGTGATATCAGAA CGGAAGGTTTTAAGGGCGAATTGGGTACCG	SPT4
SPT5-3F1	ACCAAGGAAATAAGTCAAACTATGGTGGTAACAGTACA TGGGGAGGTCATAGGGAACAAAAGCTGGAGCTC	SPT5
SPT5-3F2	TTTTTTATTGATTTCTTCTTGGGTGATATTGGTTCTCCTTT TGGTGATTAAGGGCGAATTGGGTACCG	SPT5
RPB3-3F1	ATGCATCTCAAATGGGTAATACTGGATCAGGAGGGTATG ATAATGCTTGGAGGGAACAAAAGCTGGAGCT	RPB3
RPB3-3F2	TCGGTTCGTTCACTTGTTTTTTTTCCTCTATTACGCCCACT TGAGAACTATAGGGCGAATTGGGTACCG	RPB3
RPB7-F1	GTTCTATTCACGCAATCGGTAGTATCAAAGAAGATTAT TTGGGTGCTATTAGGGAACAAAAGCTGGAGCTC	RPB7
RPB7-F2	AAAGGCGAAGGCGGGCGTTCAGAAAAGCGTTGCGGAG TAACAAGTGATTAAGGGCGAATTGGGTACCG	RPB7
FW3MF	AGCTGGAGCTCGAACAGAAGTTGATTTCCGAAGAAGACC TCGAA	3MYC
RE3MF	AATTCCTGCAGTTAAAGGTCTTCTTCGGAAATCAACTTCT GTTCGTT	3MYC
FWRAD26MF	AGCTGACTAGTATGGAACAGAAGTTGATTTCCGAAGAAG ACCTCGAA	3MYC
RERAD26MF	ACTGTGGATCCAAGGTCTTCTTCGGAAATCAACTTCTGTT CGTT	3MYC
For gene deletion, truncation, point mutation and confirmation		
TFB5a	GTCAATGGATCCCGTAGCAAAATATTTTCC	TFB5
TFB5b	GCACCCAAGCTTGCTCTAGCCATTTTTTCGTT	TFB5
TFB5c	TGGATGCTCGAGAAAATCAGTAATTATTGCTCATTCAG	TFB5
TFB5d	TTTTGCGGTACCGGCTTGACCTTAAATGGT	TFB5
TFB5e	GTACTCCCAATAGAGACAAAGCCA	TFB5

Table continued

TFB5e	GTACTCCCAATAGAGACAAAGCCA	TFB5
TFB5f	GACCTGGTTATGCAGGTCATGCTC	TFB5
SPT4	CACGGTACCTCAAATAAATATATTCATGTATATAATTT	SPT4
SPT4'	TGGGGTACCGTGGAGTCACCACGTTT	SPT4
SPT4-a	GGCAAAAGCGAACGAGGTACAGTGTAAGAGATGTCTA GTGAAAGAGCCTGCCAGCTGAAGCTTCGTACGC	SPT4
SPT4-b	TTTACTCAACTTGACTGCCATCCCTCGGTTTGTAGTGAG GCAACAGCTCCAGGGCGCGAATTGGGTACCG	SPT4
SPT5-a	TGCGGATCCTTGAAGAACC TTGGTAGTTCA	SPT5
SPT5-b	CGACGGCCGGCATGTAAGCCCATCG	SPT5
SPT5-Kan1	ATTTGCTGATCCCGTAGTGGTTCCGCAGTCAACTGACAC TAAAGATGAAACCAGCTGAAGCTTCGTACGC	SPT5
SPT5-Kan2	CCTAATTGACCCTTGTAACCAGCAGAACGAATTCTTACT GTTTTGCCGAGAGGGCGAATTGGGTACCG	SPT5
SPT5-1	TCTTGCGTACAGCCAAGTGAAG	SPT5
SPT5-2	GGGACCTCCATCATCTAAGACATT	SPT5
SPT5Forward	GTCCTCGAGATGAGTGACAACTCGGACACAA	SPT5
SPT5Revers	CTATTAATTAATTAATGACCTCCCCATGTACTGTTAC	SPT5
SPT5-F1	GTCGACGTCATGAGTGACAACTCGGACACAA	SPT5
SPT5-R1	CTAAAGCTTTTAATGACCTCCCCATGTACTGT	SPT5
SPT5-R870	CTAAAGCTTTTACTGTGGAACTCTACCACGTCTATT	SPT5
SPT5-R640	CTAAAGCTTTTATGATGTAGCTGTAGTGTCGATGG	SPT5
SPT5-R421	CTAAAGCTTTTAATCCAGACGAGGAACAATTTTC	SPT5
SPT5-F245	GTCGACGTCGCTCAAAGGTTAGCGAAAGAAT	SPT5
SPT5-F422	GTCGACGTCTATGGTAAATTCGACGAAATTGA	SPT5
SPT5-F641	GTCGACGTCAGTGAATATGCGCTACATGACATA	SPT5
SPT5-I244	CTATCCGGAGTCTTCTTCTGAAGTCTTGTTCAAAT	SPT5
SPT5-I422	GTCTCCGGATATGGTAAATTCGACGAAATTGA	SPT5
SPT5-I421	CTATCCGGATTAATCCAGACGAGGAACAATTTTC	SPT5
SPT5-I244	GTCTCCGGAAGTGAATATGCGCTACATGACATA	SPT5
BUR2-3	AAGCTGTACCAAAACCAACG	BUR2
BUR2-4	TCGTCAATCACCGAATCAATCA	BUR2
BUR2-5	GTCAGGTACCAATCCACATTTGCCGTTGGT	BUR2
BUR2-6	CATGAAGCTTCACTGCTATCGGTCCATTAGTG	BUR2
BUR2-7	GTCACTCGAGTTGTGCGACGGTTATACGTTC	BUR2
BUR2-8	CATGTCTAGATGACCCTCAGATTATATGCTGCT	BUR2
DWRad26R763A	AAGCTCGAGAAGCGGCATGGAGGATTGGGCA	RAD26
UPRad26Q759A	TTTCTCGAGCTGCCATGTCAGTAGATGGGTTCCAGT	RAD26
DWRad26KT329AC	GGAGCATGCATTCAAGTTATCGCATTTATCGCA	RAD26
UPRad26KT329AC	AATGCATGCTCCCAGACCCATTTCGTCA	RAD26
KanRE	GGGATGTATGGGCTAAATGTACG	KanMX
KanFW	CCTCGACATCATCTGCCCA	KanMX

Table continued

RPB3 FW	TATTGGTACCCCAAGACATCCCAG	RPB3
RPB3 RE	AGGTGGATCCTTAACGAGCAAAAAGGAGGA	RPB3
RPB7-A	GTTGGAGCCTGCTAATCAAAGCAC	RPB7
RBP7-B	CGAACCTTCAACCTCTTCCAATAG	RPB7
RPB7-C	CGTTAGAAGACCTTGATGAACCC	RPB7

APPENDIX C: STRAINS USED IN THIS DISSERTATION

Strains	Genotype[a]	Reference/Source
WT-BJ5465	*MATa ura3-52 trp1 leu2D1 his3D200 pep4::HIS3 prb1D1.6R can1*	(1)
CR5	as BJ5465, but *tfb5::URA3*	This study
CR18	as BJ5465, but *rad7::URA3Δ rad26::URA3Δ*	This study
CR23	as BJ5465, but *rad7Δ rad26Δ rpb9::URA3Δ*	This study
CR31	as BJ5465, but *rad26Δ rpb9Δ*	This study
CR33	as BJ5465, but *rad7Δ*	This study
CR34	as BJ5465, but *rad7Δ rad26Δ tfb5::URA3*	This study
CR37	as BJ5465, but *rad26Δ rpb9 Δ tfb5::URA3*	This study
CR42	as BJ5465, but *rad7Δ tfb5::URA3*	This study
CR54	as BJ5465, but *rad26Δ rpb93Δ tfb5::URA3*	This study
CR55	as BJ5465, but *rad26Δ rpb9Δ spt4::LEU2*	This study
CR76	as BJ5465, but *rad7Δ spt4::LEU2*	This study
CR78	as BJ5465, but *rad26Δ rad7Δ spt4::LEU2*	This study
CR80	as BJ5465, but *rad7Δ rad26Δ rpb9Δ spt4::LEU*	This study
CR82	as BJ5465, but *rad7Δ rpb9::URA3*	This study
CR89	as BJ5465, but *rad7Δ rpb9Δ tfb5::URA3*	This study
CR108	as BJ5465, but *rad7Δ spt4Δ rpb9::KanMX*	This study
BD4	as BJ5465, but *rad7Δ rad26Δ* (*SPT4*-3FLAG)	This study
BD7	as BJ5465, but (*SPT5*-3´FLAG)	This study
BD9	as BJ5465, but *rad7::URA3* (*SPT5*-3FLAG)	This study
BD10	as BJ5465, but *rad7Δ rad26Δ* (*SPT5*-3FLAG)	This study
BD13	as BJ5465, but *spt4::LEU2* (*SPT5*-3FLAG)	This study
BD14	as BJ5465, but *rad7Δ spt4::LEU2* (*SPT5*-3FLAG)	This study
BD15	as BJ5465, but *rad7Δ rad26Δ spt4::LEU2* (*SPT5*-3FLAG)	This study
BD16	as CR18, but [pGAL-SPT5]	This study
BD17	as CR78, but [pGAL-SPT5]	This study
BD21	as BJ5465, but [pGAL-SPT5]	This study
BD56	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5]	This study
BD57	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/CTRD]	This study
BD58	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/641-1063D]	This study
BD59	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/422-1063D]	This study
BD60	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/1-244D]	This study
BD61	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/1-421D]	This study
BD62	as CR18, but *SPT5::KanMX* [pRS416-SPT5, pSPT5/1-640D]	This study
BD63	as BD56, but [pRS416-SPT5] removed	This study
BD64	as BD57, but [pRS416-SPT5] removed	This study
BD94	as CR18, but *bur2::URA3*	This study
BD95	as BD64, but *bur2::URA3*	This study

Table continued

BD96	as BD63, but *bur2::URA3*	This study
DB97	as BD63, but [p3FRAD26]	This study
BD98	as BD63, but [p3FRAD26 K328A T329C]	This study
BD99	as BD63, but [p3FRAD26 Q759A R763A]	This study
BD100	as BD63, but [p3FARD26 K328A T329C Q759A R763A]	This study
BD112	as BD10, but [p3MRAD26]	This study
BD113	as BD10, but [p3MRAD26 K328A T329C]	This study
BD114	as BD10, but [p3MRAD26 Q759A R763A]	This study

[a] Genomic genes tagged with 3×FLAG are indicated in parentheses; plasmids contained in strains are indicated in brackets.

1. **Jones, E. W.** 1991. Tackling the protease problem in Saccharomyces cerevisiae. Methods Enzymol **194:**428-53.

VITA

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