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Transcription Coupled DNA Repair in *Saccharomyces Cerevisiae*: the Interplay of Facilitators and Repressors

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TRANSCRIPTION COUPLED DNA REPAIR IN *SACCHAROMYCES
CEREVISIAE*: THE INTERPLAY OF FACILITATORS AND REPRESSORS

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

by

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LIST OF ABBREVIATIONS

5-FOA	5-fluoroorotic acid
ATP	adenosine triphosphate
BER.....	base excision repair
Bpa	p-benzoyl-L-phenylalanine
CPDs	cyclobutane pyrimidine dimers
CSB.....	cockayne syndrome group B
CTR.....	C-terminal repeat
CUTs.....	cryptic unstable transcripts
DSB.....	double-strand break repair
GGR	global genomic repair
GOI	genes of interest
IP	immunoprecipitation
Mfd.....	mutation frequency decline
MMR.....	mismatch repair
MPA	mycophenolic acid
MW	molecular weight
NAT	nourseothricin
ncRNA	non-coding RNA
NER.....	nucleotide excision repair
NTS.....	nontranscribed strand
PAFc	RNA polymerase II associated factor 1 complex
PCNA.....	proliferating cell nuclear antigen
PCR.....	polymerase chain reaction

RNAP II RNA polymerase II
RNR1 ribonucleotide reductase 1
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
snRNAs small nuclear RNAs
snoRNAs small nucleolar RNAs
Swi/Snf Switch/Sucrose nonfermentable
TCA trichloroacetic acid
TCR transcription coupled repair
TFIIS transcription factor II S
TS transcribed strand
TSS transcription start site
UV ultraviolet
XPC xeroderma pigmentosum complementation group C

ABSTRACT

Nucleotide excision repair (NER) is a multi-step cellular process that removes bulky and/or helix-distorting DNA lesions, such as UV induced cyclobutane pyrimidine dimers (CPDs) and bulky chemical adducts. Transcription coupled repair (TCR) is a subpathway of NER dedicated to rapid removal of lesions in the transcribed strand of actively transcribed genes. The TCR mechanism in bacteria has been relatively well elucidated. However, TCR in eukaryotic cells appears to be extremely complicated. The exact nature of the TCR signal and the mechanism of the transcription-repair coupling have been long-standing enigmas. This dissertation focused on how the TCR repressors and facilitators interplay with RNA polymerase II (RNAP II) to carry out TCR in yeast *Saccharomyces cerevisiae*.

By site-specific incorporation of the unnatural amino acid p-benzoyl-L-phenylalanine, we mapped interactions between Spt5 and RNAP II in *S. cerevisiae*. Through its KOW4-5 domains, Spt5 extensively interacts with Rpb4/7. Spt5 also interacts with Rpb1 and Rpb2, two largest subunits of RNAP II, at the clamp, protrusion and wall domains. Deletion of Spt5 KOW4-5 domains decreases transcription elongation and derepresses TCR. Our findings suggest that Spt5 is a key coordinator for holding the RNAP II complex in a closed conformation that is highly competent for transcription elongation but repressive to TCR. We also demonstrated that E1103G mutation of Rpb1, the largest subunit of RNAP II, which promotes transcription bypass of UV-induced CPDs, increases survival of UV irradiated yeast cells but attenuates TCR. In contrast, G730D mutation of Rpb1, which abolishes transcription bypass of CPDs, enhances TCR. Our findings suggest that transcription bypass of lesions attenuates TCR but enhances cell tolerance to DNA lesions. Efficient stalling of RNAP II is essential for efficient TCR. Sen1 is an RNA/DNA helicase that has been shown to mediate termination of noncoding RNAs and some

mRNAs. Like deletion of Rad26 or Rpb9, the Sen1 N-terminal deletion (1-975 residues) increases the UV sensitivity of the GGR-deficient cells. Moreover, the Sen1 N-terminal deletion decreases TCR in *rad7Δ* and *rad7Δ rad26Δ* cells but not that in *rad7Δ rpb9Δ* cells. Our findings suggest that the N-terminal domain of Sen1 contributes to Rad26-independent TCR.

CHAPTER 1

LITERATURE REVIEW

TRANSCRIPTION COUPLED DNA REPAIR IN *SACCHAROMYCES CEREVISIAE*: INTERPLAY OF FACILITATORS AND REPRESSORS

1.1 Introduction

Cellular DNA is under constant attack from endogenous and exogenous DNA damaging agents. To deal with the deleterious DNA damage, the living organisms have evolved multiple DNA repair mechanisms, such as base excision repair (BER), double-strand break (DSB) repair, mismatch repair (MMR) and nucleotide excision repair (NER) (Friedberg, Walker et al. 2006). NER is one of the DNA repair mechanisms that can remove bulky and helix-distorting lesions which can obstruct DNA replication and transcription, such as UV induced cis-syn cyclobutane pyrimidine dimers (CPDs), 6-4 photoproducts and cancer chemotherapeutics (e.g., cisplatin) (Reardon and Sancar 2005, Hanawalt and Spivak 2008). NER has two pathways: transcription coupled repair (TCR) and global genomic repair (GGR). TCR is dedicated to rapidly removal of lesions from the transcribed strand (TS) of an actively transcribed gene (Bohr, Smith et al. 1985, Mellon, Spivak et al. 1987). GGR refers to removing lesions throughout the whole genome, including the nontranscribed strand (NTS) of actively transcribed genes (Verhage, Zeeman et al. 1994).

In the mid-1960s, NER was first discovered in bacteria (Pettijohn and Hanawalt 1964). Two decades later, the specific TCR pathway was sequentially discovered in mammalian cells (Mellon, Spivak et al. 1987), *Escherichia coli* (Mellon and Hanawalt 1989) and *Saccharomyces cerevisiae* (Smerdon and Thoma 1990). Through a “cut-and-patch” mechanism, NER machinery operates in multiple steps which include damage recognition, helix opening, lesion verification, 5' to 3' dual incision, oligonucleotide excision, gap-filling DNA synthesis, and ligation (Figure

1-1). The two NER pathways differ in the initial damage recognition step but share the later steps of the repair process. For GGR, DNA damage is recognized by specific factors, such as XPC in mammalian cells (Nospikel 2009), and Rad7, Rad16 and Elc1 in *S. cerevisiae* (Verhage, Zeeman et al. 1994, Lejeune, Chen et al. 2009). On the other hand, CSA, CSB, UVSSA and USP7 in mammalian cells (Sarasin 2012, Schwertman, Vermeulen et al. 2013) and Rad26 (homolog of mammalian CSB) in *S. cerevisiae* (Tatum and Li 2011) are involved in damage recognition step in TCR other than in GGR.

In bacteria, the molecular mechanism of TCR is relatively well characterized (Selby and Sancar 1994, Deaconescu, Chambers et al. 2006, Epshtein, Kamarthapu et al. 2014). In eukaryotes, the basic “cut-and-patch” mechanism in NER is well understood, whereas how the elongating RNAP II senses lesions and how the lesion, trapped in the elongation complex, is exposed to NER machinery during the damage recognition step in TCR remain elusive (Hanawalt and Spivak 2008). In yeast cells, a few NER factors (e.g., Rad26, Rpb9 and Sen1) which are involved in damage recognition step have been shown to have positive effect on TCR (Li and Smerdon 2002, Woudstra, Gilbert et al. 2002). Here, we call those factors “facilitators”. On the other hand, multiple NER factors, such as Spt4/5, Rpb4 and RNAP II associated factor 1 complex (PAFc), play a negative role in TCR during damage recognition step (Tatum, Li et al. 2011, Li, Giles et al. 2014). Here, we define them as “repressors”. The major functions of those “facilitator” and “repressor” are summarized in Table 1-1. In this review, we focus on the interplay of the facilitators and repressors during TCR, and summarize recent insights into the molecular mechanism of TCR in yeast cells.

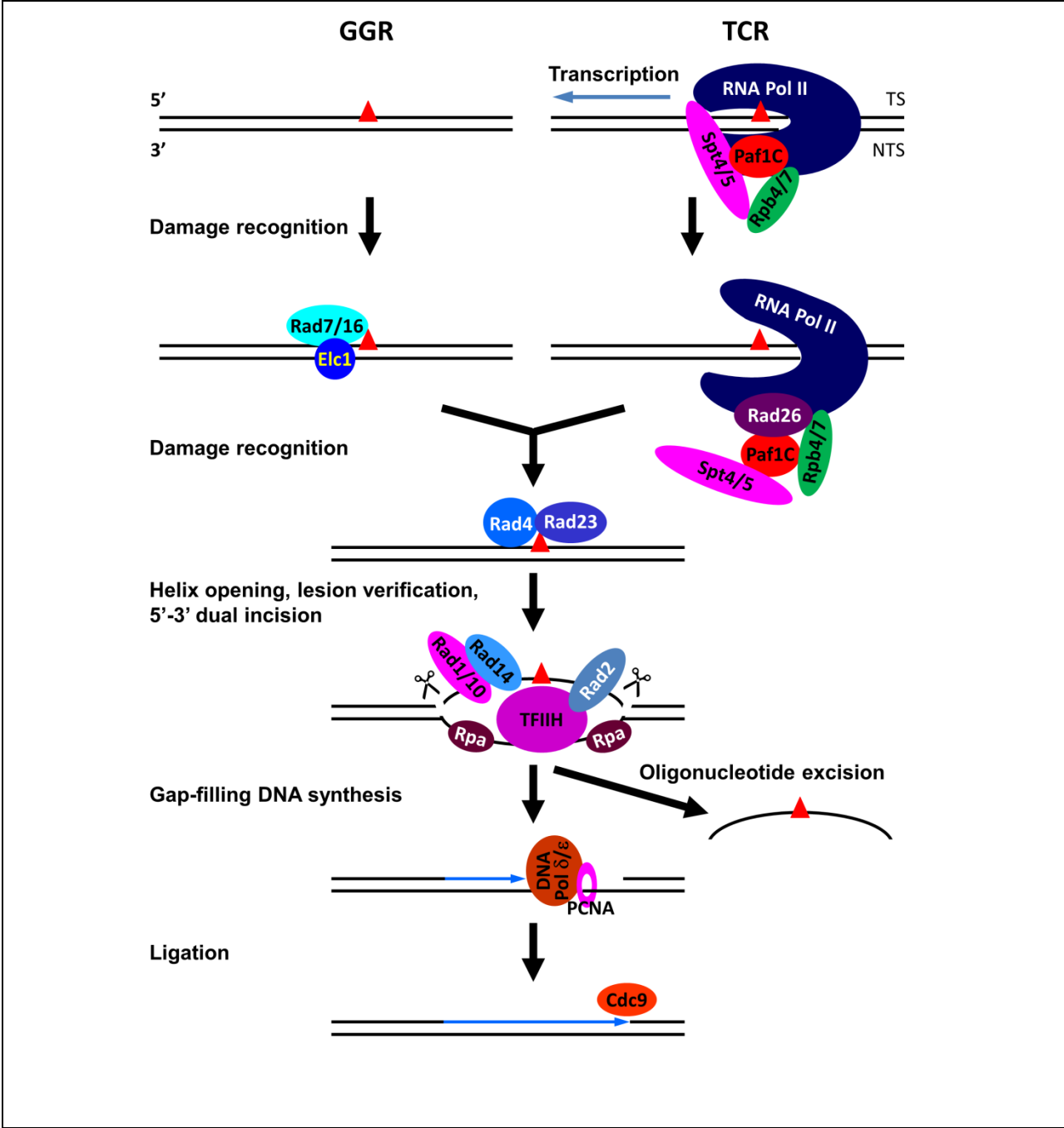


Figure 1-1. The process of NER in *S. cerevisiae*. Red triangle denotes a DNA lesion. TS, transcribed strand; NTS, nontranscribed strand. Adapted from (Tatum and Li 2011).

Table 1-1 NER facilitators and repressors and their function in yeast *S. cerevisiae*.

	Proteins	Function(s)	Reference
Facilitators	Rad26	TCR specific factor; DNA-dependent ATPase	(van Gool, Verhage et al. 1994, Prakash and Prakash 2000)
	Rpb9	Nonessential RNAP II subunit; Partially required for TCR	(Li and Smerdon 2002, Li, Ding et al. 2006)
	Sen1	ATP-dependent helicase; Contributes to Rad26-independent TCR	(Rasmussen and Culbertson 1998) (unpublished data)
Repressors	Spt4/5	Transcription elongation factors; Represses TCR	(Hartzog and Fu 2013, Li, Giles et al. 2014)
	Rpb4	Nonessential RNAP II subunit; Represses Rad26-independent TCR	(Li and Smerdon 2002, Armache, Mitterweiger et al. 2005)
	PAFc	Indirectly required for Dot1-catalyzed di- and tri-methylation of H3K79; Represses Rad26-independent TCR and marginally facilitates Rad26-dependent TCR	(Krogan, Dover et al. 2003, Tatum, Li et al. 2011)

1.2 Factors That Facilitate TCR

Although TCR in eukaryotic cells is believed to be triggered by the stalling of RNAP II at the DNA lesion site, the precise initiating signal that can elicit this pathway remains unknown (Laine and Egly 2006, Lindsey-Boltz and Sancar 2007, Hanawalt and Spivak 2008, Haines, Kim et al. 2014). The TCR mechanism in eukaryotic cells appears to be complicated and elusive, especially during the initial damage recognition step. In yeast cells, Rad26, the homolog of human CSB, plays an important role in TCR, but has no role in GGR (van Gool, Verhage et al. 1994). However, Rad26 does not seem to be the real transcription-repair coupling factor that directly recruits NER machinery, as it facilitates TCR indirectly by antagonizing the repressors and becomes solely dispensable in the absence of a repressor (Verhage, van Gool et al. 1996, Jansen, den Dulk et al. 2000, Li and Smerdon 2002, Li and Smerdon 2004). Rpb9, one nonessential subunit of RNAP II, has been found to be required for the Rad26-independent TCR (Li and Smerdon 2002, Li and Smerdon 2004, Li, Ding et al. 2006). Sen1, an ATP-dependent RNA/DNA helicase, terminates the RNAP II transcription through the Sen1-dependent termination pathway (Rasmussen and Culbertson 1998, Steinmetz, Conrad et al. 2001). Recently, we have found that the N-terminal domain of Sen1 contributes to the Rad26-independent TCR (unpublished data).

1.2.1 Rad26

Rad26, which is the homolog of human CSB and one member of the Swi/Snf family of DNA-dependent ATPases, functions in transcription elongation and TCR (van Gool, Verhage et al. 1994, Guzder, Habraken et al. 1996, Lee, Yu et al. 2001). In *E. coli*, Mfd binds to the stalled RNAP and pushes it forward to dissociate the elongation complex from DNA by using its ATPase activity (Park, Marr et al. 2002, Deaconescu, Chambers et al. 2006). In contrast, UvrD

interacts with RNAP during transcription elongation and pulls RNAP backward to expose DNA lesions through ATP hydrolysis (Epshtein, Kamarthapu et al. 2014). In consideration of its ATPase activity and the role of transcription-repair coupling factor Mfd in *E. coli*, Rad26 was thought to be the real transcription-repair coupling factor and be able to displace the stalled RNAP II by moving it forward (Park, Marr et al. 2002, Svejstrup 2002). However, there is no significant protein sequence homology between Mfd and Rad26, except for the ATPase domains. Moreover, the human CSB was shown not to be able to displace the stalled transcription complex by an in vitro study (Selby and Sancar 1997).

As Rad26 is solely or partially dispensable for TCR in the absence of Spt4, Rpb4, Spt5 CTR domain or one subunit of PAFc (Jansen, den Dulk et al. 2000, Li and Smerdon 2002, Ding, LeJeune et al. 2010, Tatum, Li et al. 2011), it is highly likely that Rad26 facilitates TCR by antagonizing the effect of those repressors.

1.2.2 Rpb9

Rpb9, the nonessential and ninth largest subunit of RNAP II, controls transcription fidelity and mediates the Rad26-independent TCR (Woychik, Lane et al. 1991, Li and Smerdon 2002, Walmacq, Kireeva et al. 2009). It also promotes ubiquitylation and degradation of Rpb1 in response to UV-induced DNA damage, but this function of Rpb9 seems to be not involved in any pathways and/or subpathways of NER (Chen, Ruggiero et al. 2007). Rpb9 resides at the tip of RNAP II jaw, and links Rpb1 and Rpb2. It is composed of N-terminal Zn1, central linker and C-terminal Zn2 domains. For the transcription elongation and TCR, the Zn1 and linker domains are essential, whereas the Zn2 domain is dispensable. For promoting the ubiquitylation and

degradation of Rpb1 after UV irradiation, Zn2 domain is indispensable, while the Zn1 and linker domains play a subsidiary role (Li, Ding et al. 2006, Chen, Ruggiero et al. 2007).

In contrast to the Rad26-dependent TCR which has equal efficiency in the coding region and the region upstream the transcription start site, Rpb9 mediated Rad26-independent TCR repairs more effectively in the coding region than that in the region immediately upstream from the transcription start site (Li and Smerdon 2002, Li and Smerdon 2004). Besides, the relative contributions of the two TCR subpathways may vary from gene to gene. For example, Rad26 seems to be solely required for TCR in *URA3* gene (Tijsterman, Verhage et al. 1997), but partially required in *RPB2* gene (Bhatia, Verhage et al. 1996, Verhage, van Gool et al. 1996, Gregory and Sweder 2001, Li and Smerdon 2002, Li and Smerdon 2002). However, Rad26 is almost dispensable for TCR in *GALI* gene, especially in the coding region, suggesting TCR in this region is operated primarily by Rpb9 mediated subpathway. The two subpathways, which are mediated by Rad26 and Rpb9 respectively, are also modulated differently by different promoter elements (Li, Chen et al. 2006).

1.2.3 Sen1

In yeast cells, Sen1 forms an Nrd1 complex through physical interactions with Nrd1 and Nab3, and functions in RNAP II termination and pre-mRNA processing (Wilson, Datar et al. 1994, Steinmetz and Brow 1996, Steinmetz, Conrad et al. 2001). It is an essential and highly conserved 5' to 3' ATP-dependent RNA/DNA helicase (MW 252 kDa) (Rasmussen and Culbertson 1998, Kim, Choe et al. 1999). The human homolog Senataxin, encoded by SETX gene, maintains genome integrity by interacting with replication and/or transcription associated factors and using its helicase activity to resolve DNA/RNA hybrids (R-loops) (Groh and Gromak

2014). Mutations in Senataxin lead to the progressive neurological diseases Ataxia-Oculomotor Apraxia 2 (AOA2) and juvenile Amyotrophic Lateral Sclerosis 4 (ALS4) (Moreira, Klur et al. 2004, Chen, Hashemi et al. 2006).

The Sen1 N-terminal domain (1-975 residues), which is nonessential for cell growth, plays an important role in protein/protein and protein/RNA interactions during termination, while the C-terminal domain, which contains conserved helicase motif, is indispensable for cell survival (Ursic, Chinchilla et al. 2004). Similar with the Rho-dependent termination mechanism in *E.coli*, Sen1 recognizes the newly synthesized RNA and dissociates the elongation complex by the energy of hydrolysis of ATP *in vitro* system (Porrua and Libri 2013). It has also been found that Sen1 distributes all along pre-mRNA transcripts in addition to binding to non-coding RNAs (ncRNAs), suggesting potential roles of Sen1 in the mRNA 3' end formation and/or termination (Creamer, Darby et al. 2011). Recently, we have found that the N-terminal domain of Sen1 contributes to the Rad26-independent TCR (unpublished data). Although how Sen1 functions in TCR is still elusive, it is rational to propose that Sen1, like Rho acts in *E. coli*, may physically interact with RNAP II, disrupt the elongation complex and eventually dissociate RNAP II to expose the DNA lesion trapped in the elongation complex. In comparison to Rad26 which plays an indirect role in TCR, Sen1 appears to be a real transcription-repair coupling factor that interacts with the stalled RNAP II directly and, much more likely, recruits NER machinery.

1.3 Factors That Repress TCR

In contrast to the above facilitators, a few repressors, such as Spt4 (Jansen, den Dulk et al. 2000), Rpb4 (Li and Smerdon 2002), Spt5 CTR (C-terminal repeat) domain (Ding, LeJeune et

al. 2010) and PAFc (Tatum, Li et al. 2011), which are generally involved in transcription elongation, have been shown to repress TCR in the absence of Rad26. Recently, structural and biochemical studies on the interactions between RNAP II and Spt5 have shed light on our understanding of the interplay between facilitators and repressors during TCR in yeast cells.

1.3.1 Spt4/5

Spt5, one member of Spt5/NusG family, is a unique RNAP-associated factor that is universally conserved across all domains of life (Harris, Kelley et al. 2003, Hartzog and Fu 2013). In eukaryotic cells, Spt5 consists of an acidic N-terminus, an NGN domain, multiple KOW domains and a CTR domain (Hartzog and Fu 2013). The NGN domain of Spt5 associates with Spt4, one zinc-binding protein (MW 11 kDa), to form a heterodimeric Spt4/5 complex. The other side of Spt5 NGN domain directly interacts with the RNAP II clamp domain (Martinez-Rucobo, Sainsbury et al. 2011). The Spt4/5 complex functions in transcription elongation, pre-mRNA processing and TCR (Ding, LeJeune et al. 2010, Hartzog and Fu 2013).

Recently, Spt5 has been found to directly interact with RNAP II clamp, protrusion and wall domains of RNAP II. The KOW4-5 domains of Spt5 also extensively interact with RNAP II stalk, a dissociable subcomplex formed by Rpb4 and Rpb7 which are two subunits of RNAP II. Interestingly, internal deletion of Spt5 KOW4-5 domains derepresses TCR even in the presence of Rad26 (Li, Giles et al. 2014). This finding suggests a coordinator role of Spt5 that holds the RNAP II complex in a closed conformation that is efficient for transcription elongation but repressive to TCR (Figure 1-2).

1.3.2 Rpb4

Rpb4, another nonessential subunit of RNAP II, forms a dissociable subcomplex with Rpb7 which is a small but essential subunit of RNAP II (Woychik and Young 1989). The Rpb4/7 subcomplex can “wedge” the clamp to a closed state if it associates with the core RNAP II, which in turn narrows the central cleft of RNAP II (Figure 1-2) (Armache, Kettenberger et al. 2003, Bushnell and Kornberg 2003). Moreover, Rpb4/7 extensively interacts with Spt5, together with the interactions between Spt5 and core RNAP II, resulting in locking the clamp in a closed state.

Deletion of Rpb4 will abolish the interaction between Rpb7 and core RNAP II, and result in the growth cessation phenotype at extreme temperatures (<12°C and >32°C) (Choder and Young 1993, Miyao, Barnett et al. 2001). Interestingly, similar to Spt4, Rpb4 deletion restores TCR in the absence of Rad26, suggesting Rpb4 represses Rad26-independent TCR (Li and Smerdon 2002). This repression of Rad26-independent TCR is high likely due to the interaction between Rpb4 and Rpb7, which further stabilizes the Spt5-RNAP II complex conformation and ultimately makes the DNA lesion trapped in the elongation complex and inaccessible for NER machinery.

1.3.3 PAFc

PAFc, which consists of Paf1, Rtf1, Cdc73, Leo1 and Ctr9 subunits in yeast, directly associates with RNAP II and chromatin, and functions in multiple cellular processes such as transcription elongation, pre-mRNA processing and histone modification (Jaehning 2010). Recently, it has been shown that the recruitment of PAFc to RNAP II complex requires the

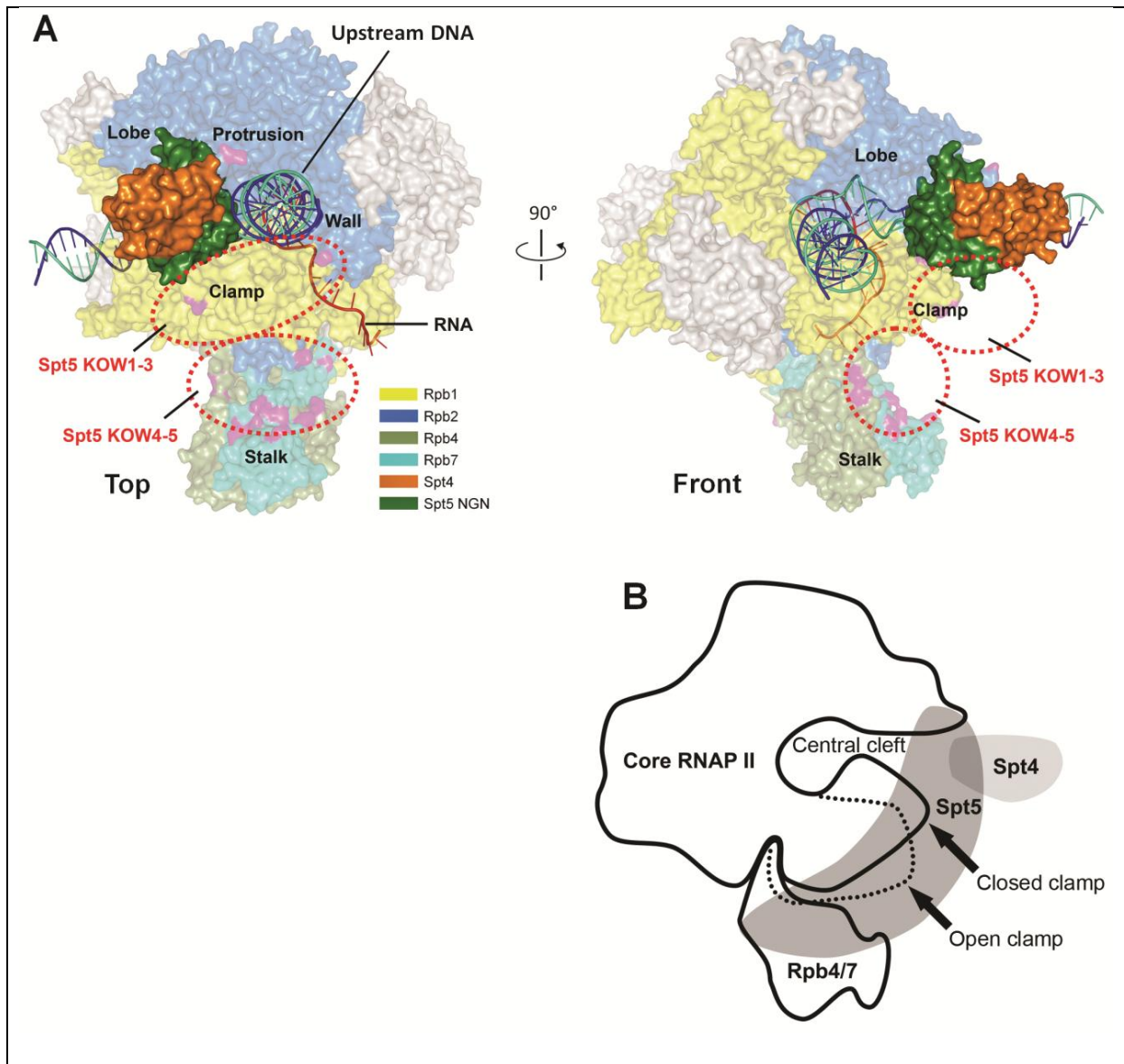


Figure 1-2. Architecture of interactions between Spt4/5 and RNAP II. (A) Locations of Spt4 and different domains of Spt5 on RNAP II. (B) Schematic cut-away view of the interaction architecture. The dashed line indicates the open clamp position observed in the absence of Rpb4/7. Based on reference (Li, Giles et al. 2014).

physical interaction between Rtf1 and Spt5 CTR domain (Mayekar, Gardner et al. 2013, Wier, Mayekar et al. 2013). Although PAFc interacts with RNAP II directly, the major functions of PAFc is independent of the transcribing RNAP II (Mueller, Porter et al. 2004). Moreover, the PAFc is required for Rad6/Bre1-dependent histone H2B lysine 123 (H2BK123) ubiquitylation (Krogan, Dover et al. 2003, Ng, Dole et al. 2003, Wood, Schneider et al. 2003), which is in turn partially required for dimethylation and solely essential for trimethylation of histone H3 lysine 79 (H3K79) by Dot1 (Shahbazian, Zhang et al. 2005, Nakanishi, Lee et al. 2009, Levesque, Leung et al. 2010).

In yeast cells, PAFc has been shown to repress Rad26-independent TCR. This repression may be achieved through physical interaction with Spt4/5. On the other hand, PAFc plays a marginal role in facilitating Rad26-dependent TCR and significantly enhances GGR (Tatum, Li et al. 2011). How PAFc facilitates Rad26-dependent TCR remains elusive. However, it is likely that PAFc facilitates the recruitment of Rad26 to the RNAP II, which in turn facilitates TCR. The facilitation of GGR by PAFc is accomplished by enabling ubiquitylation of H2BK123 by Bre1, which in turn allows the di- and tri-methylation of H3K79 by Dot1. Thus, PAFc appears to play diverse roles in different NER pathway or subpathways.

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CHAPTER 2

INSIGHTS INTO HOW SPT5 FUNCTIONS IN TRANSCRIPTION ELONGATION AND REPRESSING TRANSCRIPTION COUPLED DNA REPAIR*

2.1 Introduction

RNA polymerases (RNAPs), which carry out transcription in all living organisms, are highly conserved at the level of sequence, structure, function and molecular mechanisms (Werner and Grohmann 2011). The most studied eukaryotic RNAP is RNAP II that consists of 12 subunits (Rpb1-12). Rpb4 and Rpb7 form a dissociable stalk structure, whereas the rest of the subunits form the core RNAP II (Cramer, Armache et al. 2008). An RNAP interacts with different factors during transcription initiation and elongation (Werner and Grohmann 2011). The binding sites for initiation and elongation factors on an RNAP may overlap and the binding of the factors to RNAP is mutually exclusive, which ensures an efficient swapping of factors and may assist RNAP during promoter escape (Blombach, Daviter et al. 2013).

NusG/Spt5 family proteins are universally conserved transcription elongation factors that play pivotal roles in transcription and transcription related processes by binding to RNAP and interacting with other transcription-associated factors (Hartzog and Fu 2013). Bacterial NusG and archaeal Spt5 proteins contain an N-terminal NGN domain and a C-terminal KOW domain (Figure 2-1) (Kyrpides, Woese et al. 1996, Ponting 2002). Eukaryotic Spt5 proteins are much larger (1063 and 1087 residues in *S. cerevisiae* and humans, respectively) and more complex, consisting of an N-terminal acidic domain, an NGN domain, multiple KOW domains and a C-terminal repeat (CTR) domain (Hartzog and Fu 2013). The NGN domain of archaeal and

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eukaryotic Spt5 associates with Spt4, a relatively small zinc-binding protein (61, 102 and 117 residues in *Pyrococcus furiosus*, *S. cerevisiae* and humans, respectively), to form a heterodimeric Spt4/5 complex.

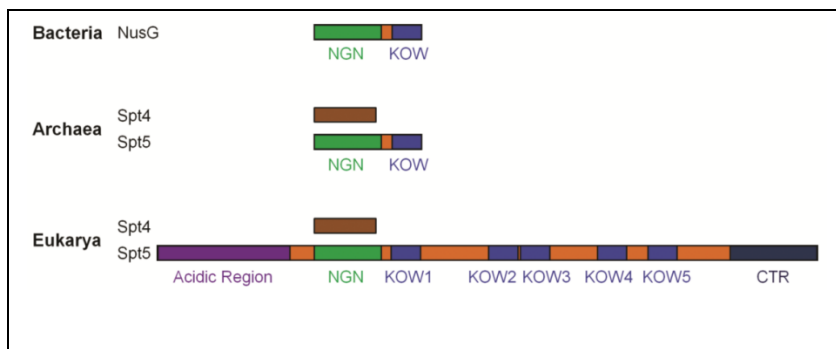


Figure 2-1. Domains of NusG in bacteria and Spt5 in archaea and eukaryotes. The NGN domain of Spt5 interacts with Spt4 in archaea and eukaryotes.

The archaeal Spt4/5 has been crystalized (Hirtreiter, Damsma et al. 2010, Klein, Bose et al. 2011) and the structural model of archaeal RNAP-Spt4/5 complex has been reconstructed based on analyses of cryo-electron microscopy single particles (Klein, Bose et al. 2011). The archaeal Spt4/5 complexed with the clamp domain of archaeal RNAP has also been crystalized (Martinez-Rucobo, Sainsbury et al. 2011). In the archaeal RNAP clamp-Spt4/5 structure, the NGN domain of Spt5 directly interacts with the RNAP clamp, whereas Spt4 interacts with the other side of the NGN domain (Martinez-Rucobo, Sainsbury et al. 2011). Furthermore, the crystal structures of the NGN domain of *S. cerevisiae* and human Spt5 bound to Spt4 have been solved (Guo, Xu et al. 2008, Wenzel, Martins et al. 2010). However, it is still very challenging to solve the structures of complete eukaryotic Spt4/5, either alone or in complex with RNAP II, presumably because the fairly large eukaryotic Spt5 proteins contain multiple disordered or unstructured regions. Due to the lack of structural information, how Spt5 functions in transcription elongation and transcription related processes in eukaryotic cells has been enigmatic.

Nucleotide excision repair (NER) is a DNA repair pathway that removes a wide variety of bulky and/or helix-distorting lesions that generally obstruct transcription, such as UV-induced cyclobutane pyrimidine dimers (CPDs) (Nospikel 2009, Tatum and Li 2011). Transcription coupled repair (TCR) is an NER subpathway dedicated to rapid removal of DNA lesions in the transcribed strand (TS) of actively transcribed genes (Hanawalt and Spivak 2008). TCR is believed to be initiated by an RNAP stalled at a lesion in the TS of a gene being transcribed. The TCR mechanism in bacteria has been elucidated in molecular details (Selby and Sancar 1993, Park, Marr et al. 2002, Deaconescu, Chambers et al. 2006, Howan, Smith et al. 2012, Epshtein, Kamarthapu et al. 2014). Mfd and UvrD, two DNA helicase/translocases, have been shown to play important roles in TCR in *E. coli*. Mfd binds to the β subunit of RNAP stalled at a lesion and displaces the complex by pushing it forward (Selby and Sancar 1993, Park, Marr et al. 2002, Deaconescu, Chambers et al. 2006, Howan, Smith et al. 2012). Concurrently, Mfd recruits UvrA to the exposed lesion site to facilitate NER (Selby and Sancar 1993). On the other hand, UvrD binds RNAP during transcription elongation and forces RNAP to backtrack along DNA, thereby exposing DNA lesions for access of NER machinery (Epshtein, Kamarthapu et al. 2014). The biochemical mechanism of TCR in eukaryotic cells is still enigmatic. In the budding yeast *S. cerevisiae*, Rad26, a DNA-stimulated ATPase that is homologous to the human CSB protein, plays an important role in TCR (van Gool, Verhage et al. 1994). However, Rad26 is dispensable for TCR in cells lacking Rpb4 (Li and Smerdon 2002) or Spt4 (Jansen, den Dulk et al. 2000). Rad26 is also partially dispensable for TCR in cells lacking the CTR domain of Spt5 (Ding, LeJeune et al. 2010) or any subunit of the 5-subunit RNAP II associated factor 1 complex (PAFc) (Tatum, Li et al. 2011). Therefore, TCR appears to be repressed by certain factors that are normally involved in transcription elongation and Rad26 facilitates TCR by antagonizing the

repression (Tatum and Li 2011). How these factors repress TCR and if and/or how they coordinate in the repression remain to be elucidated.

To gain insights into the mechanisms that underlie the functions of Spt5 in eukaryotic cells, we mapped site-specific interactions between Spt5 and RNAP II in *S. cerevisiae*. We found that Spt5 interacts with the clamp, protrusion, wall and Rpb4/7 stalk domains of RNAP II. The binding sites of Spt5 on RNAP II partially overlap with those of the transcription initiating factor TFIIE. Disruption of the interactions between Spt5 and Rpb4/7 by deleting the Spt5 domains that extensively interact with Rpb4/7 decreases transcription elongation and derepresses TCR. Our results suggest that Spt5 is a key coordinator for holding the RNAP II complex in a closed conformation that is highly competent for transcription elongation but repressive to TCR.

2.2 Materials and Methods

2.2.1 Yeast Plasmids and Strains

Plasmid pLH157 bearing the genetically engineered *E. coli* tRNA_{CUA} and tyrosyl-tRNA synthetase genes (Figure 2-2A) was obtained from Dr. Steven Hahn. Multi-copy *LEU2* plasmids bearing genes of interest (GOI) (Rpb1, Rpb2, Rpb4, Rpb7 and Spt5) with a TAG codon replacing a desired amino acid codon were created using plasmid pESC-LEU (Stratagene) as vector (Figure 2-2B). The *LEU2* gene on the original vector contains the leucine tRNA gene tRNA_{3^{Leu}}, which starts -463 nucleotides upstream of the start codon of the *LEU2* gene (Andreadis, Hsu et al. 1982). We found that the tRNA_{3^{Leu}} on the vector greatly compromises incorporation of Bpa into the protein of interest. The tRNA_{3^{Leu}} gene was therefore inactivated by removing the sequence between the HpaI and SfoI sites in the gene. To increase detection sensitivity, the Flag tag contained in the original vector was converted to 3×Flag tag by inserting

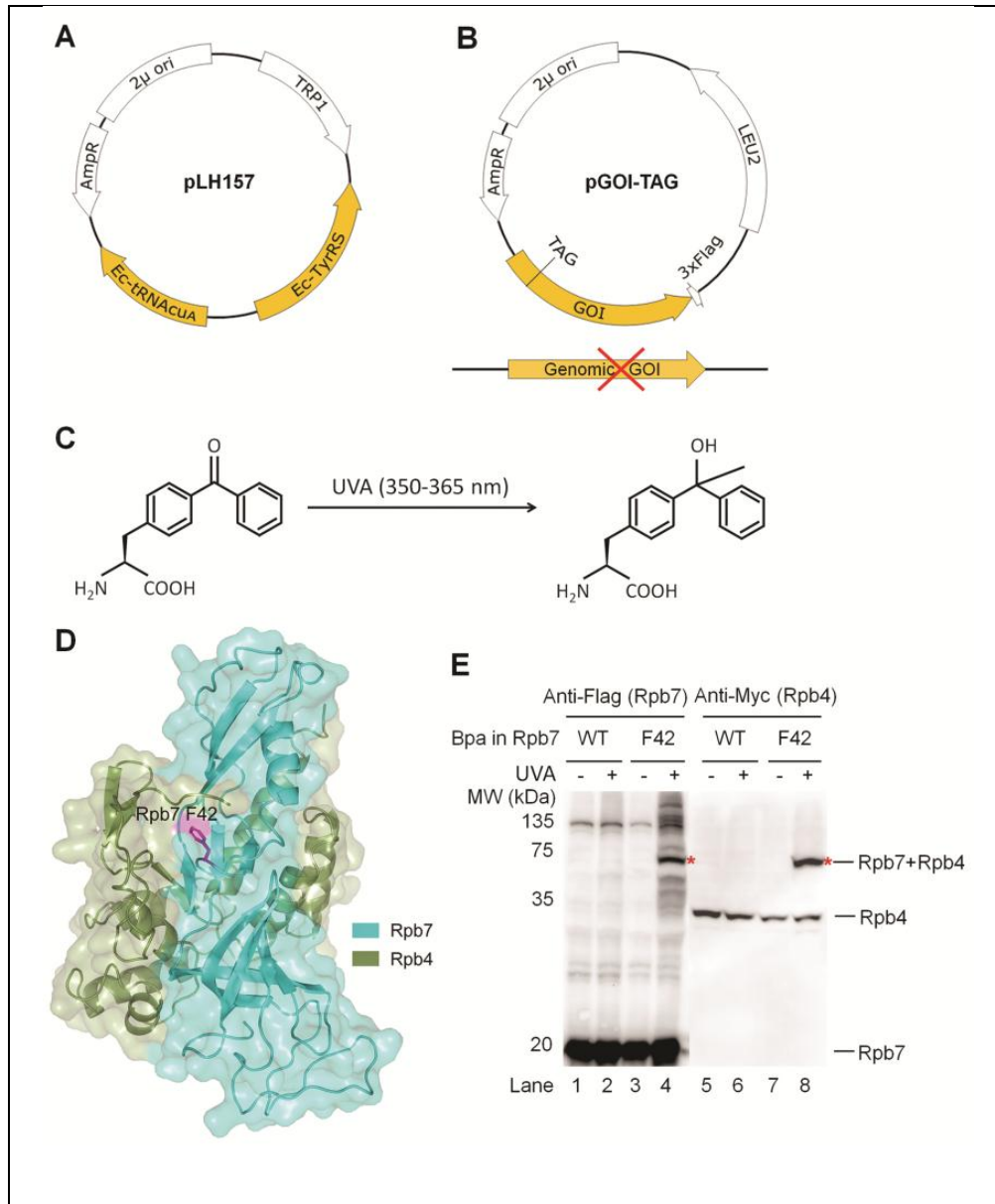


Figure 2-2. Bpa substitution of a residue of protein of interest and photo cross-linking in living yeast cells. (A) Plasmid pLH157, which contains genetically engineered *E. coli* tRNA synthetase (Ec-TyrRS) and tRNA (Ec-tRNA_{CUA}) genes for incorporating Bpa through nonsense suppression of the TAG codon. (B) Plasmid pGOI-TAG, which bears a gene of interest (GOI) with a TAG codon substituting a normal amino acid codon. This plasmid was transformed through plasmid shuffling into a yeast strain whose genomic GOI is deleted and contains pLH157. (C) Structure of Bpa. (D) Rpb4/7 subcomplex of RNAP II showing the location of Rpb7 F42 (based on PDB 1Y1W). (E) Western blots showing substitution of Rpb7 F42 with Bpa caused cross-linking to Rpb4. Red asterisks indicate bands of cross-linked 3×Flag tagged Rpb4 and 3×Myc tagged Rpb7 (Rpb4+Rpb7), which can be detected with anti-Flag and anti-Myc antibodies.

2 Flag sequences between the SacI and BglIII sites. The *GALI-10* promoter sequence on the vector was removed and replaced with the GOI encompassing their native promoters and coding sequences with a TAG codon replacing an amino acid codon of interest. All mutations were confirmed by DNA sequencing.

Plasmids pRS416-RPB1, pRS416-RPB2, pRS416-RPB4, pRS416-RPB7 and pRS416-SPT5 were created by inserting the whole respective genes including the promoter, coding sequence and 3' terminator sequences into the multiple cloning site of the single-copy centromeric *URA3* plasmid pRS416 (Sikorski and Hieter 1989). Plasmid pNAT-SPT5 Δ CTR, which encodes 3 \times Myc-tagged CTR-deleted Spt5, was created by replacing the *LEU2* gene in plasmid pSPT5/CTR Δ (Ding, LeJeune et al. 2010) with the NAT (nourseothricin) gene. Plasmids pRS415-SPT5, pRS415-SPT5 Δ KOW4 and pRS415-SPT5 Δ KOW4-5, encoding the full-length, KOW4 deleted (deleting amino acids 706-765) and KOW4-5 deleted (deleting amino acids 706-848) Spt5, respectively, were created by inserting appropriate PCR fragments of the *SPT5* gene into the EagI and BamHI sites of the single-copy centromeric *LEU2* plasmid pRS415 (Sikorski and Hieter 1989). To tag a genomic gene of interest with 3 \times Myc, plasmid p3MYC-KanMX was created by replacing the 3 \times Flag sequence in plasmid p3FLAG-KanMX (Gelbart, Rechsteiner et al. 2001) with a 3 \times Myc sequence.

All yeast strains used in this study are derivatives of BJ5465 (*MATa ura3-52 trp1 leu2 Δ 1 his3 Δ 200 pep4::HIS3 prb1 Δ 1.6R can1*) (Jones 1991). Deletion of genes were performed using procedures previously described (Li and Smerdon 2002). To delete the genomic *RPB1*, *RPB2*, *RPB4*, *RPB7* and *SPT5* genes, the cells were first transformed with plasmids pRS416-RPB1, pRS416-RPB2, pRS416-RPB4, pRS416-RPB7 and pRS416-SPT5, respectively. The 3 \times Myc

tagging of a genomic gene was achieved by using PCR fragment amplified from plasmid p3MYC-KanMX. The deletion and tagging of a gene were confirmed by PCR.

The pGOI-TAG plasmids encoding Rpb1, Rpb2, Rpb4, Rpb7 and Spt5 with a TAG codon replacing a desired amino acid codon were transformed into respective yeast strains (Table 2-1). Plasmids pRS415-SPT5, pRS415-SPT5 Δ KOW4 and pRS415-SPT5 Δ KOW4-5 were transformed into yeast strains whose genomic *SPT5* gene had been deleted and complemented with pRS416-SPT5. The transformed cells were selected with 5-fluoroorotic acid (5-FOA), which is toxic to cells with a functional *URA3* gene, to select for cells that had lost the *URA3* (pRS416) plasmids bearing the respective wild type GOI. The loss of the *URA3* plasmids and the gain of the *LEU2* plasmids in the respective transformed yeast cells were confirmed by PCR.

2.2.2 Detection of Cross-linking of Bpa-substituted Proteins

Yeast cells having plasmid pLH157 and the pGOI-TAG plasmids encoding Bpa-substituted proteins of interest were grown at 30°C in synthetic dextrose (SD) medium containing 0.5 mM Bpa (Bachem) to late log phase ($A_{600} \approx 1.0$) and harvested. The harvested cells from 15 ml of culture were washed twice with ice-cold H₂O, resuspended in 20 ml ice-cold 2% glucose, and split into two aliquots. One aliquot was kept on ice and the other was transferred into a glass petri dish (10 cm in diameter) and irradiated with 365 nm UVA for 15 min (total dose of 54 000 J/m²) on ice. The UVA source was an array of 12 fluorescent black light tubes (15W, T8 and 22 inches in length, Utilitech) mounted on a home-made wooden structure (the light tubes were 5 cm apart on the structure). The UVA source gave a dose rate of 60 J/m²/sec at a distance of 13 cm. The cells were harvested, resuspended in 400 μ l of 20% trichloroacetic acid and broken by vortexing with 400 μ l of glass beads for 30 min. The proteins were pelleted by centrifugation at 16 000 \times g for 15 min at 4°C, washed with ice-cold 80%

Table 2-1 Yeast strains used for shuffling with plasmids encoding Bpa-substituted proteins.

Strain ^a	Genotype ^b	To be shuffled plasmids	To be tested cross-linking partners
WL119	phr1Δ rpb7Δ (RPB4-3×Myc) [pRS416-RPB7, pLH157]	Rpb7	Rpb4
WL120	phr1Δ rpb7Δ (SPT5-3×Myc) [pRS416-RPB7, pLH157]	Rpb7	Spt5
WL292	phr1Δ spt5Δ (RPB4-3×Myc) [pRS416-SPT5, pLH157]	Spt5	Rpb4
WL293	phr1Δ spt5Δ (RPB7-3×Myc) [pRS416-SPT5, pLH157]	Spt5	Rpb7
WL294	phr1Δ spt5Δ [pRS416-SPT5, pLH157]	Spt5	Rpb1
WL296	phr1Δ spt5Δ (RPB2-3×Myc) [pRS416-SPT5, pLH157]	Spt5	Rpb2
WL303	rpb7Δ spt5Δ [pRS416-RPB7, pNAT-Spt5ΔCTR, pLH157]	Rpb7	CTR deleted Spt5
WL491	phr1Δ rpb2Δ (SPT5-3×Myc) [pRS416-RPB2, pLH157]	Rpb2	Spt5
WL492	phr1Δ rpb1Δ (SPT5-3×Myc) [pRS416-RPB1, pLH157]	Rpb1	Spt5
WL543	phr1Δ rpb1Δ (TFA1-3×Myc) [pRS416-RPB1, pLH157]	Rpb1	Tfa1 (TFIIE)

Table 2-1 continued

Strain ^a	Genotype ^b	To be shuffled plasmids	To be tested cross-linking partners
WL590	phr1Δ rpb4Δ (SPT5-3×Myc) [pRS416-RPB4, pLH157]	Rpb4	Spt5
WL643	phr1Δ rpb7Δ (TFA1-3×Myc) [pRS416-RPB7, pLH157]	Rpb7	Tfa1 (TFIIE)

^a All strains are isogenic to CR18 (*MATα ura3-52 trp1 his3 leu2 pep4::HIS3 rad7Δ rad26Δ*).

^b Genomic genes tagged with 3×Myc are shown in parentheses; plasmids contained in a strain are shown in brackets.

acetone, resolved on SDS-PAGE gels and subjected to Western blot. Specific SDS-PAGE conditions for Western blot detection of cross-linkings between different proteins are presented in Table 2-2. Rpb1 on the blots was detected with 8WG16 (Neoclone), which recognizes the C-terminal repeats of Rpb1. 3×Flag and 3×Myc tagged proteins were detected with anti-Flag M2 (Sigma) and anti-c-Myc (Genscript) antibodies respectively. Blots were incubated with SuperSignal[®] West Femto maximum-sensitivity substrate (Pierce) and scanned with the VersaDoc Imaging System (BioRad).

2.2.3 Detection of Co-immunoprecipitation of Spt5 and Rpb4 with Core RNAP II and Cellular Levels of Rad26

For detection of co-immunoprecipitation of Spt5 and Rpb4 with core RNAP II, 90 ml of log phase yeast cells were harvested and resuspended in 0.6 ml of chromatin preparation buffer (50 mM HEPES, pH7.8, 150 mM NaCl, 0.5% NP-40, 0.25% Triton X-100, 10% glycerol, and

Table 2-2. SDS-PAGE conditions for Western-blot detection of cross-linkings between different proteins.

Bpa-substituted protein (kD)	Cross-linking partner (kD)	SDS-PAGE condition	
		For Western-blot of Bpa-substituted protein	For Western-blot of cross-linking partner
Rpb1 (192)	Spt5 (116)	5-10%	5-10%
	Tfa1 (55)	5-10%	6-18%
Rpb2 (139)	Spt5 (116)	5-10%	5-10%
Rpb4 (25)	Spt5 (116)	6-18%	6-18%
Rpb7 (19)	Spt5 (116)	6-20%	6%
	Tfa1 (55)	10%	10%
	Rpb4 (25)	10%	10%
Spt5 (116)	Rpb1 (192)	5-12%	5-12%
	Rpb2 (139)	4-20%	4-20%
	Rpb4 (25)	8%	6-18%
	Rpb7 (19)	8%	6-18%

protease inhibitors). The cells were broken by vortexing with acid-washed glass beads, and the chromatin fractions were collected by centrifugation at $20\,000 \times g$ for 10 min at 4°C. The chromatin pellet was solubilized in 250 μ l of immunoprecipitation buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% Sodium Deoxycholate, 1mM EDTA, 10 mM NaF, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$ and protease inhibitors) by sonication with a Bioruptor (Diagenode) for 15 minutes (30 seconds on and 30 seconds off). The sample was clarified by centrifugation and the supernatant was transferred to a fresh tube. The sample was added with 1 ml of immunoprecipitation buffer and SDS to a final concentration of 0.1%. Fifty μ l of the sample was saved as “input” and the remaining was mock-immunoprecipitated (without addition of antibody) or immunoprecipitated with 4 μ g of 8WG16. The levels of Rpb1, Rpb4 and 3 \times Myc tagged Spt5 were detected with 8WG16, 2Y14 (Neoclone) and anti-Myc antibodies, respectively, on Western blots.

For detection of the cellular levels of Rad26, log phase yeast cells were harvested and the whole cell extracts were prepared using the glass beads and trichloroacetic acid method (see above). The levels of 3×Flag tagged Rad26 and Rpb1 were detected using anti-Flag and 8WG16 antibodies, respectively, on a Western blot.

2.2.4 Tests of Temperature, UVC and Mycophenolic Acid (MPA) Sensitivities

Yeast cells were grown at 30°C in SD medium to saturation, and sequential 10-fold serial dilutions were made. For temperature sensitivity test, the diluted samples were spotted onto YPD (1% yeast extract, 2% peptone and 2% dextrose) plates and incubated at 25, 30 and 37°C. For UVC sensitivity assay, the diluted samples were spotted onto YPD plates, irradiated with different doses of 254 nm UV light (from a 15W UV germicidal bulb, General Electric) and incubated at 30°C in the dark. For mycophenolate sensitivity assay, the diluted samples were spotted onto SD plates containing different concentrations of MPA and incubated at 30°C. After 3-8 days of incubation the plates were photographed.

2.2.5 Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed as described previously (Li, Chen et al. 2006). Briefly, yeast cells were grown in SD medium to late log phase ($A_{600} \approx 1.0$), cross-linked with 1% formaldehyde and lysed by vortexing with glass beads. The cell lysates were sonicated by using a Bioruptor (Diagenode) to shear the chromatin DNA to an average size of 200 bp and clarified by centrifugation at 4°C. An aliquot from each of the clarified lysates was saved as an input. The remaining lysates were immunoprecipitated with anti-Rpb1 antibody 8WG16 or mock immunoprecipitated. DNA fragments corresponding to different regions of the *RPB2* gene in the input, immunoprecipitated and mock immunoprecipitated samples were quantified in triplicates

by using real time PCR. Primers used for amplifying the different regions of the *RPB2* gene are shown in Table 2-3. The number of molecules in each immunoprecipitated sample was subtracted by that in the corresponding mock immunoprecipitated sample (generally ~ 5% of the immunoprecipitated sample) and then normalized to that in the corresponding input. Each ChIP assay was repeated three times. The levels of RNAP II association with the different regions of *RPB2* gene in cells expressing a truncated Spt5 were normalized to those in cells expressing the full-length wild type Spt5. The Student's *t*-test was used for statistical analysis.

Table 2-3. Primers used for real-time PCR quantification of *RPB2* fragments immunoprecipitated by anti-Rpb1 antibody 8WG16.

Region ^a	Primer 1 (5' → 3')	Primer 2 (5' → 3')	Size of PCR product (bp)
TSS	GGCGAACAAACAAGAA GTGAGT	ACCTGAGGAGAAGGAA TGAGTG	150
1 kb	AGGATATTCCTATTGTA ATCATATTC	AAACCCGTCTTCAACAC AAG	134
2.5 kb	ATCATGCTACAACATTT ACACATTGT	TAAAAACACACCCATA GCTTGC	149
3.9 kb	AACCAATTTGAATGTAA GGGA	AAAATCTCTCGAACGA TCGGTA	141

^a relative to the transcription start site (TSS).

2.2.6 Repair Analysis of UVC Induced CPDs

Yeast cells were grown at 30°C in SD medium to late log phase ($A_{600} \approx 1.0$), irradiated with 120 J/m² of 254 nm UV (from a 15W UV germicidal bulb, General Electric) and incubated in YPD medium 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 TS of *RPB2* gene was 3' end labeled with [α -³²P]dATP using a procedure described previously (Li and Waters 1996, Li, Waters et al. 2000). Briefly, ~ 1 μ g of total genomic DNA

was digested with DraI to release the *RPB2* fragment and incised at CPD sites with an excess amount of T4 endonuclease V. Excess copies of a biotinylated oligonucleotide, which is complementary to the 3' end of the TS of *RPB2*, were mixed with the samples. The mixtures were heated at 95°C for 5 minutes to denature the DNA and then cooled to an annealing temperature of around 50°C. The annealed molecules were attached to streptavidin magnetic beads, labeled with [α -³²P]dATP, and resolved on DNA sequencing gels. The gels were exposed to a PhosphorImager screen. The intensities of gel bands corresponding to CPD sites were quantified using Quantity One software (Bio-Rad).

2.3 Results

2.3.1 Spt5 Interacts with the Clamp, Protrusion and Wall Domains of RNAP II

Previous structural and biochemical studies have shown that the NGN domain of archaeal Spt5 directly interacts with the coiled-coil of RNAP clamp, and may have close proximity to the protrusion and lobe domains (Hirtreiter, Damsma et al. 2010, Grohmann, Nagy et al. 2011, Klein, Bose et al. 2011, Martinez-Rucobo, Sainsbury et al. 2011). To determine whether eukaryotic Spt5 interacts with RNAP II in a similar way, we used an *in vivo* site-specific cross-linking technique (Chin, Cropp et al. 2003, Chen, Warfield et al. 2007). This technique utilizes a pair of plasmids to specifically substitute a residue of a protein of interest with *p*-benzoyl-L-phenylalanine (Bpa), a photoreactive unnatural amino acid (Figure 2-2A, B and C). Upon irradiation with UVA (350-365 nm), Bpa can react with another carbon within a short distance of approximately 3 angstroms (Dorman and Prestwich 1994). In contrast to many traditional methods, which cannot distinguish direct and indirect interactions, this technique allows unambiguous detection of direct protein-protein interactions in living cells, as only if a Bpa has a

direct contact with an interacting partner can a cross-link be induced by UVA irradiation. Indeed, Bpa substitution of Rpb7 F42, which is known to interact with Rpb4, cross-linked to Rpb4 (Figure 2-2D and E). However, no cross-linking between Rpb4 and Rpb7 can be detected if Bpa substituted a residue on the surface of Rpb7 that does not contact with Rpb4 (data not shown).

We created yeast cells expressing 3×Myc-tagged Spt5 and Bpa-substituted Rpb1 or Rpb2 (Tables 2-4 and 2-5). The yeast cells were cultured in a medium containing Bpa. Whole cell extracts were prepared directly from the cells or following irradiation of the cells with UVA. The proteins were resolved on SDS-PAGE, which disrupts non-covalent protein-protein interactions and separates proteins primarily based on protein sizes, and subjected to Western blot. Bpa substitutions at Rpb1 H281 and E291, located at the coiled-coil of the RNAP II clamp, caused slower migrating bands of Spt5 upon UVA irradiation (Figure 2-3A and E; Figure 2-4A), reflecting cross-linking of Rpb1 to Spt5 at these sites. Among the Bpa substitutions of Rpb2 residues, 3 located at the RNAP II protrusion (K426, F429 and R430) were found to be lethal

Table 2-4. Cross-linking of Bpa-substituted Rpb1 to Spt5 and Tfa1. “x” not tested.

No.	Residue	Domain	Cross-linked to Spt5	Cross-linked toTfa1
1	K217	Clamp head	No	x
2	L279	Clamp core	No	x
3	H281	Clamp core	Yes	No
4	H286	Clamp core	No	Yes
5	E291	Clamp core	Yes	No
6	K688	Funnel	No	x
7	K924	Foot	No	x
8	E1167	Jaw	No	x

(Table 2-5). Viable Bpa substitutions located at the tip of the protrusion (Q433, E437), the base of the clamp (H1177) and the region of the wall (S919) that is adjacent to the clamp cross-linked to Spt5 (Figure 2-3B and E; Figure 2-4B). Note that two proteins cross-linked at different sites may migrate differently on the gel. These results indicate that Spt5 interacts with the clamp, protrusion and wall domains of RNAP II in yeast.

2.3.2 Spt5 Also Extensively Interacts with Rpb4/7

The Rpb4/7 subcomplex forms the peripheral stalk structure of RNAP II (Armache, Kettenberger et al. 2003, Bushnell and Kornberg 2003). Deletion of Rpb4 or Spt4 was shown to restore TCR in *rad26Δ* cells, indicating that these factors repress TCR in the absence of Rad26 (Jansen, den Dulk et al. 2000, Li and Smerdon 2002). We later found that the role of Spt4 in repressing TCR is indirect, by protecting Spt5 from degradation and stabilizing the interaction of Spt5 with RNAP II (Ding, LeJeune et al. 2010). However, if and/or how Spt5 and Rpb4 coordinate in repressing TCR has been unclear. We therefore determined whether Spt5 and Rpb4/7 physically interact. Indeed, Bpa substitutions at multiple sites of Rpb4 and Rpb7 caused slower migrating bands of Spt5 upon UVA irradiation (Figure 2-3C, D and E; Tables 2-6 and 2-7), reflecting cross-linking of Rpb4 and Rpb7 to Spt5 at these sites. It is intriguing to note that the sites of Rpb4 and Rpb7 that cross-link to Spt5 are distributed almost all around the cylindrical surface of the stalk structure. These results indicate that Spt5 interacts with Rpb4 and Rpb7 so extensively that it may actually wrap around the stalk structure of RNAP II. However, as transcription elongation is a dynamic process, the interactions between Spt5 and Rpb4/7 may not reflect a single static conformation in the Spt5-RNAP II complex.

Table 2-5. Cross-linking of Bpa-substituted Rpb2 to Spt5.

No.	Residue	Domain	Cross-linked to Spt5
1	Y57	Protrusion	No
2	I90	Protrusion	No
3	N103	Wall-Protrusion	No
4	V108	Wall-Protrusion	No
5	K134	Protrusion	No
6	K164	Protrusion	No
7	K277	Lobe	No
8	Q278	Lobe	No
9	V323	Lobe	No
10	K347	Lobe	No
11	K426	Protrusion	x
12	F429	Protrusion	x
13	R430	Protrusion	x
14	Q433	Protrusion	Yes
15	R434	Protrusion	No
16	E437	Protrusion	Yes
17	N881	Wall	No
18	S919	Wall	Yes
19	Y931	Wall	No
20	H1177	Clamp base	Yes

“x” not tested due to lethality of the Bpa substitution.

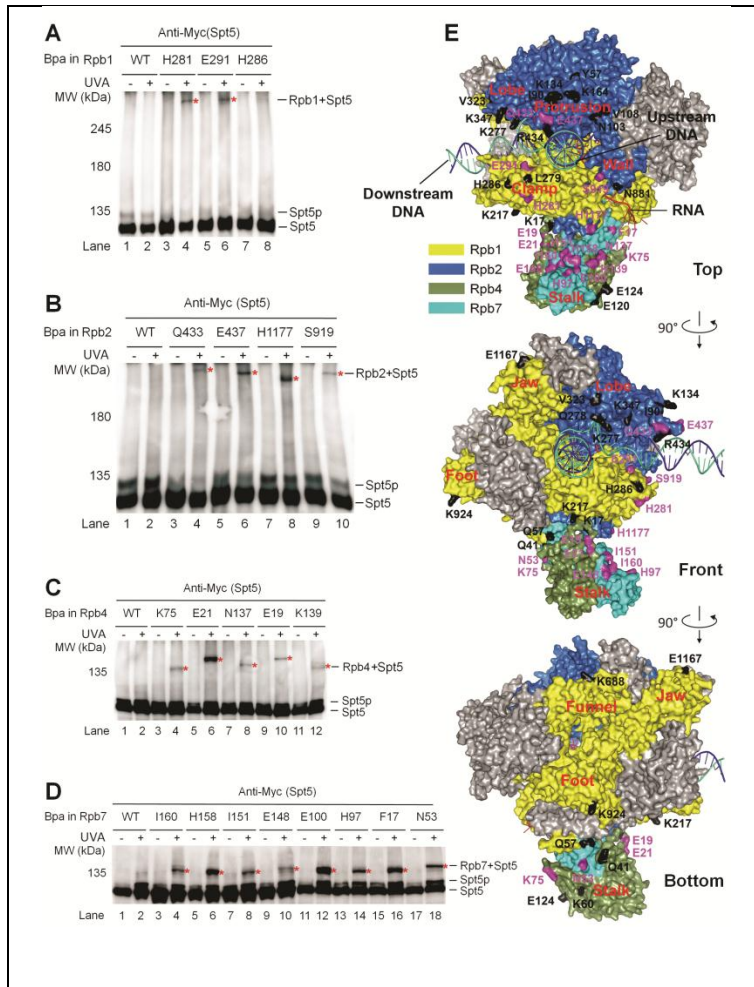


Figure 2-3. Spt5 interacts with the clamp, protrusion, wall and Rpb4/7 stalk of RNAP II. (A-D) Western blots showing cross-linking of Bpa-substituted Rpb1, Rpb2, Rpb4 and Rpb7 to Spt5. 3×Myc tagged Spt5 was detected with an anti-Myc antibody on the Western blots. Sites of Bpa substitutions are shown above the lanes of each blot. Bands of Spt5 cross-linked to Bpa-substituted proteins are marked with red asterisks. Spt5p stands for phosphorylated Spt5, which disappears following phosphatase treatment or deletion of Spt5 CTR or the *BUR2* gene (Ding, LeJeune et al. 2010). (E) Locations of Bpa-substituted residues on the surface of RNAP II. Rpb1, Rpb2, Rpb4 and Rpb7 are shown in colors as indicated. The other subunits of RNAP II are shown in gray. The template DNA, non-template DNA, and RNA transcript are shown as blue, greencyan and red ribbon structures, respectively. Residues that cross-linked to Spt5 are shown in purple and those that did not cross-link to Spt5 are shown in black. Bpa substitution at Rpb2 Y931, which did not cross-link to Spt5 is not shown, as this site is missing in the crystal structure. The RNAP II structure is based on PDB 1Y1W (Kettenberger, Armache et al. 2004), which, in addition to the protein components, reveals the locations of downstream DNA and the DNA-RNA hybrid in the transcription bubble. The locations of the non-template DNA strand in the transcription bubble and the upstream DNA duplex are based on single molecule fluorescence resonance energy transfer studies and modeling (Andrecka, Treutlein et al. 2009, Martinez-Rucobo, Sainsbury et al. 2011). See Tables 2-4 to 7 for lists of all Bpa-substituted residues in Rpb1, Rpb2, Rpb4 and Rpb7.

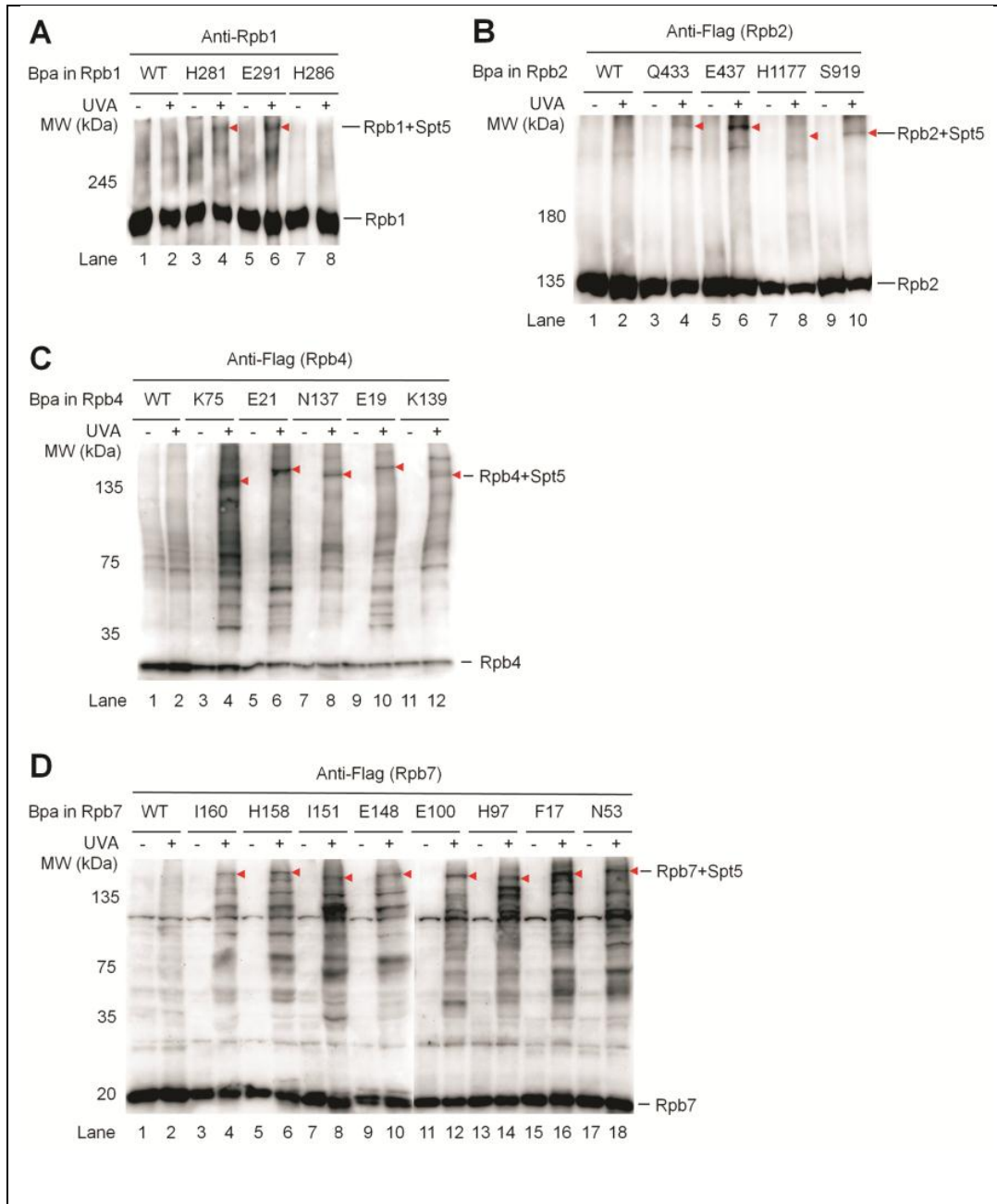


Figure 2-4. Cross-linking of Bpa-substituted Rpb1, Rpb2, Rpb4 and Rpb7 to interacting proteins. (A-D) Western blots. Sites of Bpa substitutions are shown above the lanes of each blot. Shifted bands presumably caused by cross-linking to Spt5 are marked with red arrow heads. Rpb1 was detected with antibody 8WG16. 3×Myc tagged Rpb2, Rpb4 and Rpb7 were detected with an anti-Myc antibody.

Table 2-6. Cross-linking of Bpa-substituted Rpb4 to Spt5.

No.	Residue	Cross-linked to Spt5
1	K17	No
2	E19	Yes
3	E21	Yes
4	Q41	No
5	K60	No
6	K75	Yes
7	E120	No
8	E124	No
9	N137	Yes
10	K139	Yes

Upon UVA irradiation, Bpa-substituted Rpb4 and Rpb7 showed multiple slower migrating bands on Western blots (Figure 2-4C and D). This indicates that Rpb4/7 directly interacts with other proteins in addition to Spt5. This is consistent with the fact that Rpb4/7 also interacts with various transcription factors during transcription initiation and executes some non-transcriptional activities, including mRNA transport (Choder 2004).

2.3.3 Domains of Spt5 that Interact with RNAP II and a Model of Spt4/5-RNAP II Interaction Architecture

To date, only the crystal structure of the yeast Spt5 NGN domain bound to Spt4 has been reported (Guo, Xu et al. 2008). To gain insights into the architecture of Spt5-RNAP II interactions, we generated model structures of Spt5 using I-TASSER (Zhang 2008) (Figure 2-5).

Table 2-7. Cross-linking of Bpa-substituted Rpb7 to Spt5 and Tfa1.

No.	Rpb7 Residue	Cross-linked to Spt5	Cross-linked to Tfa1
1	F17	Yes	No
2	N53	Yes	No
3	Q57	No	No
4	L62	No	No
5	H97	Yes	No
6	E100	Yes	No
7	R142	No	No
8	E148	Yes	No
9	I151	Yes	Yes
10	H158	Yes	No
11	I160	Yes	Yes

The model structures of Spt5 may be disparate from the real situation, because even the NGN domain of Spt5 in the model structures is very different from that in the real crystal structure of Spt5 NGN bound to Spt4 (Guo, Xu et al. 2008). However, the model structures provided us with certain guidance on the likely interface residues of Spt5. We substituted Bpa for Spt5 residues that are likely to be involved in interactions with other proteins (Figure 2-5 and Table 2-8). Bpa substitutions at Spt5 E367 (NGN), E608 (KOW3), K706 (KOW4) and D821 (KOW5) cross-linked to Rpb1 (Figure 2-6A and B, Figure 2-7A). Bpa substitutions at Spt5 K296, R313, N350 and D354, all located in the NGN domain, cross-linked to Rpb2 (Figure 2-6A and C, Figure 2-7B). Furthermore, Bpa substitutions at Spt5 E720, K737, K758 and K765, located in the KOW4 and KOW4-5 linker regions cross-linked to Rpb4 (Figure 2-6A and D, Figure 2-7C).

Interestingly, Bpa substitution at Spt5 E720 also cross-linked to Rpb7 (Figure 2-6A and E, Figure 2-7D), indicating that this Spt5 residue is located at the boundary between Rpb4 and Rpb7.

Table 2-8. Cross-linking of Bpa-substituted Spt5 to Rpb1, Rpb2, Rpb4 and Rpb7.

No.	Residue	Region	Cross-linked to			
			Rpb1	Rpb2	Rpb4	Rpb7
1	Q16	acidic	No	No	No	No
2	E53	acidic	No	No	No	No
3	E251	linker	No	No	No	No
4	R291	NGN	No	No	No	No
5	R293	NGN	No	No	No	No
6	K296	NGN	No	Yes	No	No
7	R313	NGN	No	Yes	No	No
8	K317	NGN	No	No	No	No
9	K318	NGN	No	No	No	No
10	E338	NGN	No	No	No	No
11	N350	NGN	No	Yes	No	No
12	D354	NGN	No	Yes	No	No
13	E367	NGN	Yes	No	No	No
14	E383	KOW1	No	No	No	No
15	Q433	KOW1	No	No	No	No
16	R458	linker (KOW1-2)	No	No	No	No
17	Q522	linker (KOW1-2)	No	No	No	No

(Table 2-8 continued)

No.	Residue	Region	Cross-linked to			
			Rpb1	Rpb2	Rpb4	Rpb7
18	R539	KOW2	No	No	No	No
19	D560	KOW2	No	No	No	No
20	E585	KOW3	No	No	No	No
21	E608	KOW3	Yes	No	No	No
22	E642	linker (KOW3-4)	No	No	No	No
23	K654	linker (KOW3-4)	No	No	No	No
24	E672	linker (KOW3-4)	No	No	No	No
25	K706	KOW4	Yes	No	No	No
26	E720	KOW4	No	No	Yes	Yes
27	K737	KOW4	No	No	Yes	No
28	K758	linker (KOW4-5)	No	No	Yes	No
29	K765	linker (KOW4-5)	No	No	Yes	No
30	K778	linker (KOW4-5)	No	No	No	No
31	K785	linker (KOW4-5)	No	No	No	No
32	Q792	linker (KOW4-5)	No	No	No	No
33	D821	KOW5	Yes	No	No	No
35	K834	KOW5	No	No	No	No
36	H843	KOW5	No	No	No	No
37	E854	Linker	No	No	No	No
38	Y1011	CTR	No	No	No	No

Like Rpb4, the CTR domain of Spt5, which can be phosphorylated by the Bur1/2 kinase complex, plays an important role in repression of TCR (Ding, LeJeune et al. 2010). We wondered if the Spt5 CTR interacts with Rpb4/7. However, Bpa substitution of residue Y1011 in the Spt5 CTR domain did not cross-link to Rpb4/7 (Table 2-8). We then tested cross-linking of Rpb7 to the CTR deleted Spt5 (residues 1-870 remaining). Bpa substitution of Rpb7 I160 is lethal when the Spt5 CTR is deleted (Table 2-9). This lethality may be due to the combination of the Bpa substitution and Spt5 CTR deletion, as this substitution is viable when the full length Spt5 is present (Table 2-7). As expected, phosphorylation can be detected in the wild type Spt5 but not the CTR deleted Spt5 (Spt5 Δ CTR) (Figure 2-6F). All the viable Bpa substitutions of Rpb7 residues normally cross-linked to the CTR deleted Spt5 (compare Figure 2-6F with Figure 2-3D). These results do not support a direct interaction between the Spt5 CTR and RNAP II. This is in agreement with previous studies showing that the CTR domain of Spt5 in human (Yamaguchi, Wada et al. 1999, Ivanov, Kwak et al. 2000) or yeast (Ding, LeJeune et al. 2010, Viktorovskaya, Appling et al. 2011) cells is not required for binding of Spt5 to RNAP II.

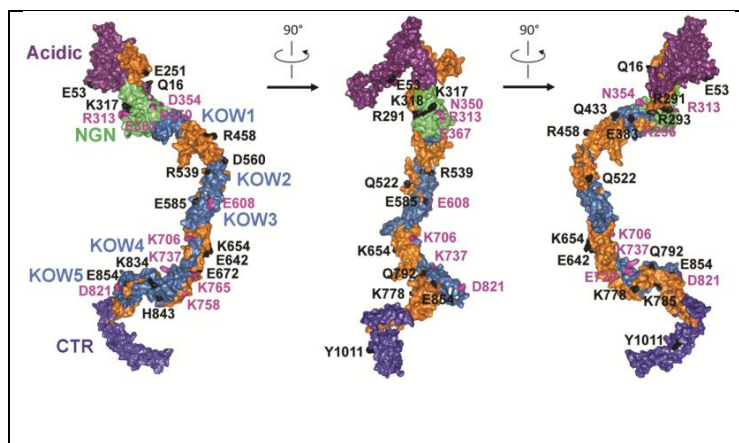


Figure 2-5. Locations of Bpa-substituted residues on a model structure of Spt5. The domains of Spt5 are shown in colors as indicated. Residues that cross-linked to RNAP II subunits are shown in magenta and those that did not cross-link to the subunits are shown in black. See Table 2-8 for a list of all Bpa-substituted residues in Spt5. The model structure of Spt5 was generated by using the I-TASSER server (<http://zhanglab.cmb.med.umich.edu/I-TASSER>).

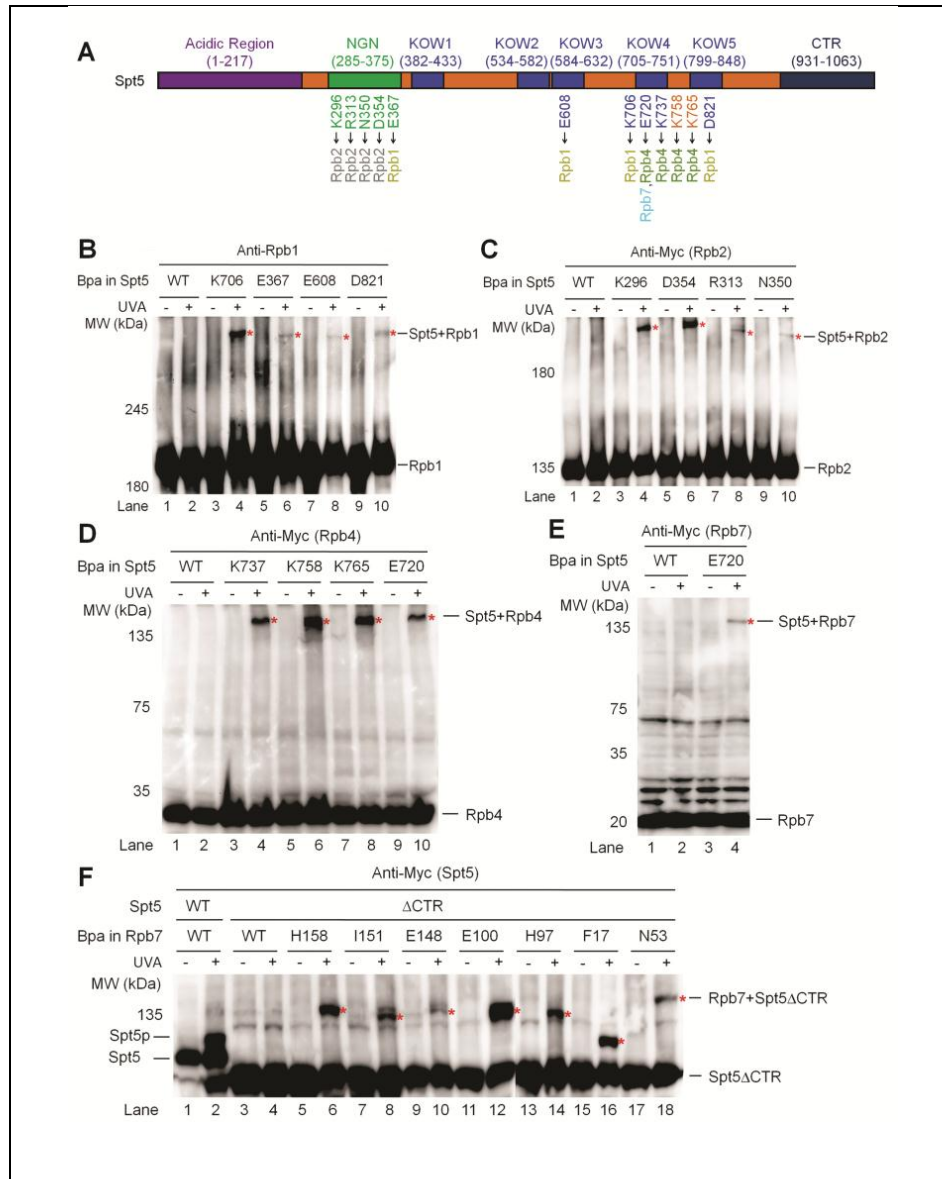


Figure 2-6. Domains of Spt5 that interact with RNAP II. (A) Schematic showing different domains of the yeast Spt5. Spt5 residues that cross-link to Rpb1, Rpb2, Rpb4 and Rpb7 are shown below the schematic. (B-E) Western blots showing cross-linking of Bpa-substituted Spt5 to Rpb1, Rpb2, Rpb4 and Rpb7. Rpb1 was detected with antibody 8WG16. 3×Myc tagged Rpb2, Rpb4 and Rpb7 were detected with an anti-Myc antibody. Sites of Bpa substitutions are shown above the lanes of each blot. Bands of cross-linked proteins are indicated with red asterisks. (F) Western blot showing cross-linking of Bpa-substituted Rpb7 to CTR-deleted Spt5 (Spt5 Δ CTR). Spt5p stands for phosphorylated Spt5, which disappears following phosphatase treatment or deletion of Spt5 CTR or the *BUR2* gene. 3×Myc tagged Spt5 was detected with an anti-Myc antibody.

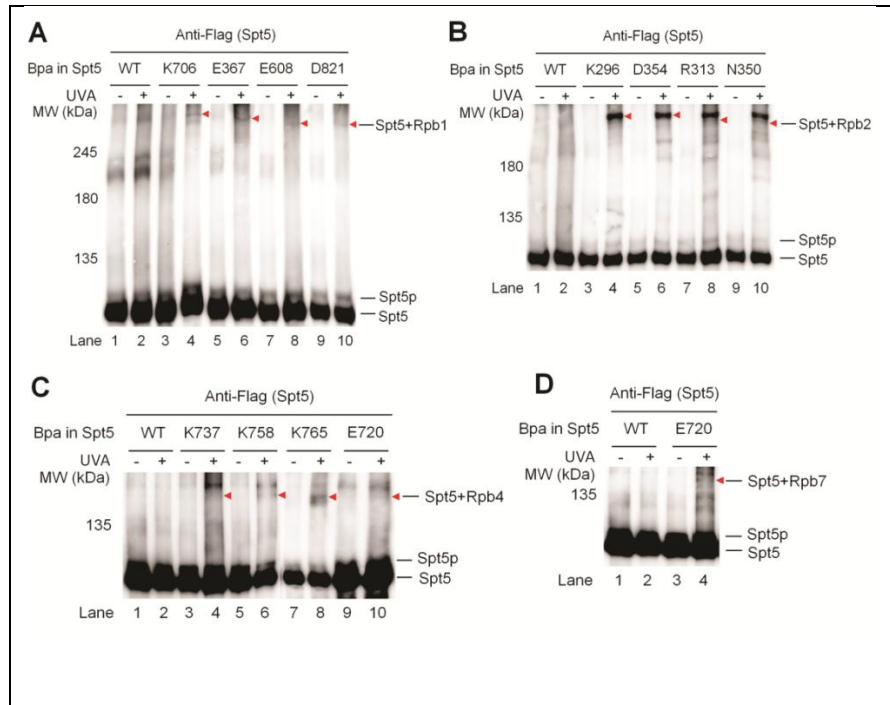


Figure 2-7. Cross-linking of Bpa-substituted Spt5 to Rpb1, Rpb2, Rpb4 and Rpb7. (A-D) Western blots. Sites of Bpa substitutions are shown above the lanes of each blot. Shifted bands presumably caused by cross-linking of Spt5 to the different RNAP II subunits are marked with red arrow heads. 3×Flag tagged Spt5 was detected with an anti-Flag antibody.

Table 2-9. Cross-linking of Bpa-substituted Rpb7 to CTR deleted Spt5.

No.	Residue	Cross-linked to CTR deleted Spt5
1	I160	x
2	H158	Yes
3	I151	Yes
4	E148	Yes
5	E100	Yes
6	H97	Yes
7	F17	Yes
8	N53	Yes

“x” not tested due to lethality of the Bpa substitution combined with the Spt5 CTR deletion.

In view of the observations that the archaeal Spt5 NGN interacts with the RNAP clamp and has close proximity to the protrusion (Martinez-Rucobo, Sainsbury et al. 2011), it is highly likely that the yeast Spt5 NGN interacts with Rpb1 through the clamp domain and with Rpb2 through the protrusion domain. Spt4 may bind to the other side of the Spt5 NGN and point away from RNAP II (Figure 2-8A and B). Based on our findings that the Spt5 KOW4 interacts with Rpb1, Rpb4 and Rpb7 (Table 2-8), this domain is likely to reside in the indentation between the Rpb1 clamp and the Rpb4/7 stalk. The Spt5 KOW5 may also reside in or be close to the indentation, as this domain cross-links to Rpb1 and the KOW4-5 linker cross-links to Rpb4. The Spt5 KOW3 may interact with the clamp region that is between the coiled coil and the base of the clamp. The KOW1 which is adjacent to the NGN and KOW2 which is adjacent to KOW3 may also reside in or be close to the region between the coiled coil and the base of the clamp, although we did not detect direct interactions of the KOW1-2 with any RNAP II subunits. It is possible that the KOW1-2 domains of Spt5 bulge away from the RNAP II surface. The acidic and CTR domains of Spt5 might not directly interact with RNAP II. This is supported by previous studies showing that the acidic and CTR domains of Spt5 are not required for binding of Spt5 to RNAP II (Yamaguchi, Wada et al. 1999, Ivanov, Kwak et al. 2000, Ding, LeJeune et al. 2010, Viktorovskaya, Appling et al. 2011). We must note that the proposed model of Spt4/5-RNAP II interaction architecture is based on non-exhaustive Bpa substitutions.

We attempted to dock model structures of different Spt5 KOW domains [obtained by using the I-TASSER server (Zhang 2008)] onto the crystal structures of elongating RNAP II, by using multiple pieces of docking software. However, none of the docking results appeared to be reasonable, including those of Spt5 KOW3, KOW4, KOW5, KOW3-4 or KOW4-5 which we found directly interact with Rpb1, Rpb4 and Rpb7. This can be due to the following: (i) the

model structures are not accurate enough and/or (ii) the crystal structures of RNAP II may be somewhat different from the dynamic structures of the enzyme in the cell.

2.3.4 Deletion of Spt5 KOW4 or KOW4-5 Domains Decreases Transcription Elongation

The Rpb4/7 stalk is easily dissociable from the 10 subunit core RNAP II *in vitro* (Armache, Kettenberger et al. 2003, Bushnell and Kornberg 2003). Association of the Rpb4/7 stalk with the core RNAP II “wedges” the clamp to the closed conformation, resulting in a narrower central cleft of the polymerase (Figure 2-8B) (Armache, Kettenberger et al. 2003, Bushnell and Kornberg 2003). The interactions of Spt5 with the Rpb4/7 stalk, clamp and protrusion of RNAP II may lock the clamp in the closed conformation and enclose the DNA being transcribed in the central cleft of the polymerase, thereby enhancing transcription elongation. To test this idea, we deleted Spt5 KOW4 (residues 706-765 removed) and KOW4-5 (residues 706-848 removed), which are involved in the interaction with Rpb4/7 (Table 2-8). These deletions, especially the KOW4-5 deletion, are expected to disrupt the interactions between Spt5 and Rpb4/7. Deletion of KOW4 or KOW4-5 did not reduce the expression of Spt5 or co-immunoprecipitation of Spt5 with RNAP II (Figure 2-9A). However, the KOW4-5 deletion caused dramatic (over 2 fold) reduction of Rpb4 co-immunoprecipitated with RNAP II (Figure 2-9A), indicating that the KOW4-5 domains of Spt5 stabilize the interaction of the Rpb4/7 stalk with the core RNAP II.

While Spt5 KOW4 deleted (*spt5ΔKOW4*) cells grew almost normally, Spt5 KOW4-5 deleted (*spt5ΔKOW4-5*) cells had growth defects especially at an elevated temperature (37°), suggesting a defect in transcription elongation (Figure 2-10A). The temperature sensitivity of *spt5ΔKOW4-5* cells is similar to that of *rpb4Δ* cells (Woychik and Young 1989). *spt5ΔKOW4-5*

cells are also somewhat more sensitive to the nucleotide depletion drug MPA than wild type cells (Hyle, Shaw et al. 2003) (Figure 2-10B). Sensitivity to a nucleotide depletion drug is often correlated with an elongation defect, although the underlying mechanism can be more

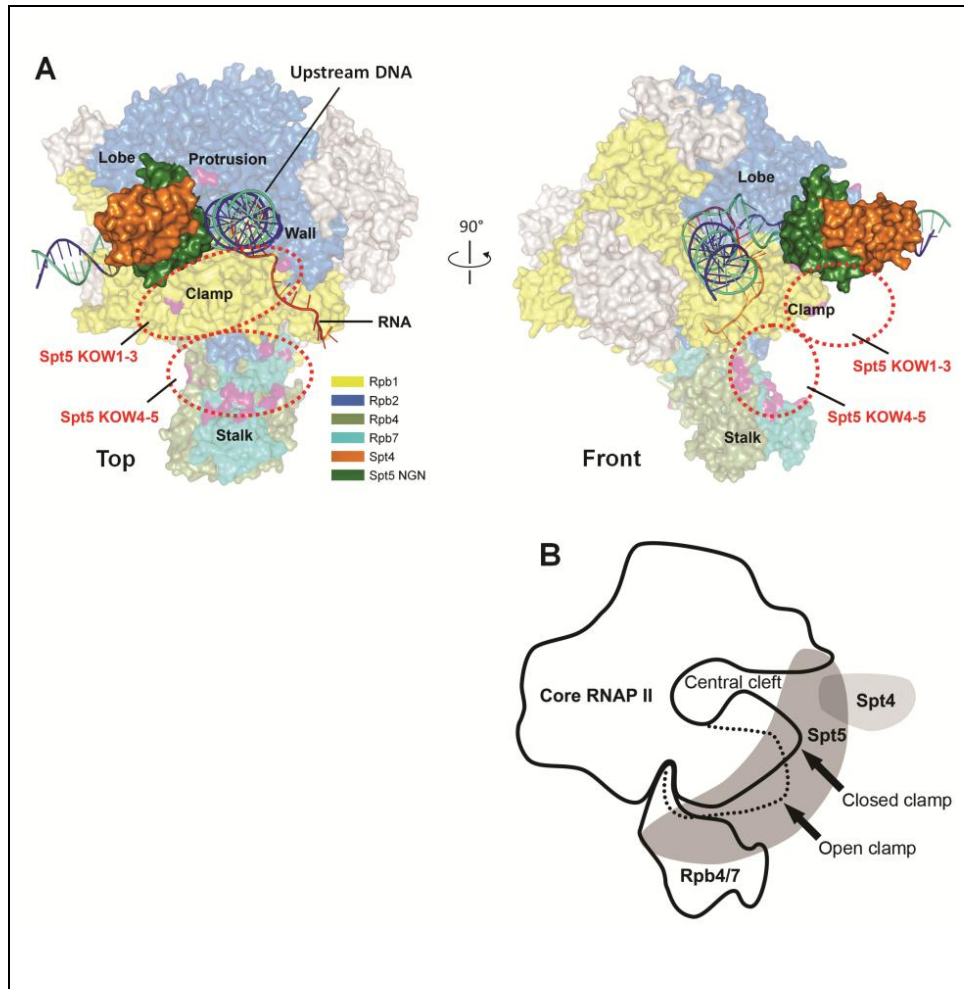


Figure 2-8. Architecture of interactions between Spt4/5 and RNAP II. (A) Locations of Spt4 and different domains of Spt5 on RNAP II. The sources of the RNAP II structures are the same as Figure 2-3E. Spt4, the NGN domain of Spt5, Rpb1, Rpb2, Rpb4 and Rpb7 are shown in colors as indicated. Other subunits of RNAP II are shown in gray. The template DNA, non-template DNA, and RNA transcript are shown as blue, greencyan and red ribbon structures, respectively. Residues of RNAP II that cross-linked to Spt5 are shown in purple. The Spt4/Spt5-NGN structure [PDB 2EXU (Guo, Xu et al. 2008)] is docked onto the RNAP II clamp based on the archaeal Spt4/5-RNAP clamp structure (Martinez-Rucobo, Sainsbury et al. 2011) and our Bpa cross-linking data. The likely locations of the Spt5 KOW domains are marked with red dashed-line ellipses. (B) Schematic cut-away view of the interaction architecture. The dashed line indicates the open clamp position observed in the absence of Rpb4/7 [PDB 1I50 (Cramer, Bushnell et al. 2001)].

complicated (Kaplan 2013). The drug sensitivity of *spt5ΔKOW4-5* cells is also similar to that of *rpb4Δ* cells (Verma-Gaur, Rao et al. 2008), indicating that Spt5 and Rpb4 may coordinate to ensure efficient transcription elongation, especially at an elevated temperature.

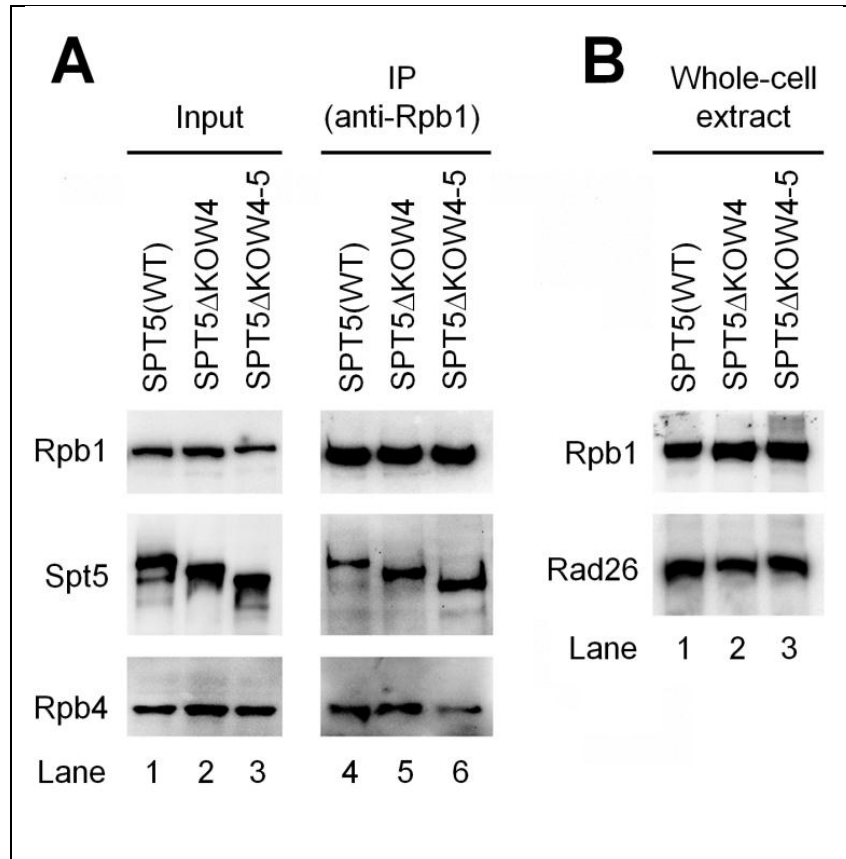


Figure 2-9. Effects of Spt5 KOW4 and KOW4-5 deletions on the association of Rpb4 with core RNAPII and the cellular levels of Rad26. (A) Deletion of Spt5 KOW4-5, but not KOW4, reduced co-immunoprecipitation of Rpb4 with core RNAP II. Western blots show the levels of Rpb1, Rpb4 and 3×Myc tagged Spt5 in chromatin fractions of whole cell extracts (input) and in samples immunoprecipitated (IP) by using the anti-Rpb1 antibody 8WG16. (B) Deletion of Spt5 KOW4 or KOW4-5 did not significantly affect the cellular levels of Rad26. Rpb1, Rpb4, 3×Myc tagged Spt5 and 3×Flag tagged Rad26 were detected with 8WG16, 2Y14, anti-Myc and anti-Flag antibodies, respectively, on the blots. The Western blots shown are representatives from 3 experiments.

We then measured the densities of RNAP II in different regions of a transcribed gene by using the chromatin immunoprecipitation (ChIP) assay. Sonicated chromatin fragments (200 bp on average) were immunoprecipitated with antibody 8WG16, which recognizes the C-terminal

repeats of Rpb1, the largest subunit of RNAP II. The immunoprecipitated fragments located at different regions of the *RPB2* gene were quantified by using real-time PCR. The RNAP II densities in different regions of the *RPB2* gene in wild type cells were normalized to 1 and those in *spt5ΔKOW4* and *spt5ΔKOW4-5* cells were represented as values relative to the wild type cells (Figure 2-10C and D). The reason for us to choose the *RPB2* gene for analysis is that we have extensively studied TCR in this RNAP II transcribed housekeeping gene (see below). The RNAP II densities in the transcription start site (TSS) in *spt5ΔKOW4* and *spt5ΔKOW4-5* cells were not significantly different from that in wild type cells (Figure 2-10D), indicating that these mutant cells have relatively normal transcription initiation. However, for an unknown reason, the RNAP II density in the 1 kb region was higher in *spt5ΔKOW4* cells than in wild type cells, which may compensate for the deficiency in transcription elongation and explain why the *spt5ΔKOW4* cells grew almost normally and were resistant to MPA (Figure 2-10A and B). Compared to wild type cells, both *spt5ΔKOW4* and *spt5ΔKOW4-5* cells showed a gradual decrease in RNAP II densities towards the 3' end of the *RPB2* gene (Figure 2-10D). It is quite unlikely that the gradual decrease of RNAP II density towards the 3' end of the *RPB2* gene in the *spt5ΔKOW4* and *spt5ΔKOW4-5* cells is caused by a gradual increase in transcription elongation rate towards the 3' end, or a gradual decrease of transcription elongation rate towards the 5' end of the gene. Rather, the gradual decrease of RNAP II density can be caused by a deficiency in transcription processivity in the absence of Spt5 KOW4 or KOW4-5.

2.3.5 Deletion of Spt5 KOW4-5 Derepresses TCR

The closed conformation of RNAP II, which can be stabilized by Spt5 and is highly competent for transcription elongation, may trap a DNA lesion in the central cleft and repress

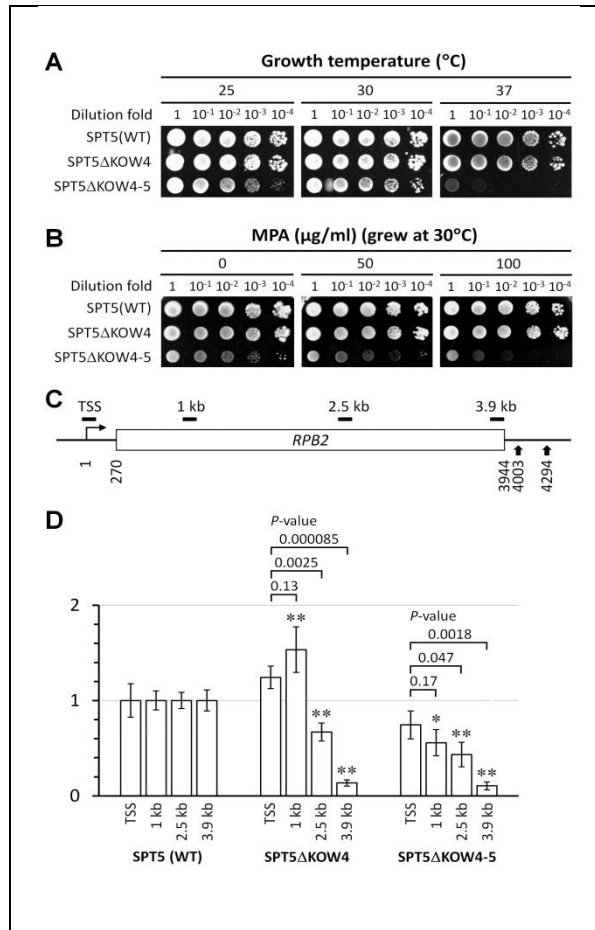


Figure 2-10. Deletion of Spt5 KOW4-5 causes defects in transcription elongation. (A) Growth of yeast cells expressing wild type (WT), KOW4 deleted (*spt5ΔKOW4*) and KOW4-5 deleted (*spt5ΔKOW4-5*) Spt5 at different temperatures. (B) Growth of the different yeast strains in the presence of different concentrations of MPA, a nucleotide depletion drug. (C) Schematic of the *RPB2* gene. Nucleotide positions are relative to the TSS. Vertical arrows at the 3' end of the gene indicate the two alternative polyadenylation sites (Yu and Volkert 2013). Short horizontal bars above the schematic indicate regions of 134-150 bp amplified by real-time PCR for quantification of ChIP fragments of the *RPB2* gene (see Table 2-3). (D) RNAP II densities in different regions of the *RPB2* gene. The RNAP II densities in the TSS, 1 kb, 2.5 kb and 3.9 kb regions of the *RPB2* gene in WT cells were normalized to 1. The RNAP II densities in the different regions of the *RPB2* gene in *spt5ΔKOW4* and *spt5ΔKOW4-5* cells are relative to those in the corresponding regions of the *RPB2* gene in WT cells. The values of RNAP II densities are represented as mean (+/- S.D.) of 3 ChIP experiments. Single asterisk (*) and double asterisks (**) denote a *P*-value of < 0.05 and < 0.01, respectively, in the Student's *t*-test between the mutant and WT cells for RNAP II densities in the corresponding regions of the *RPB2* gene. The RNAP II densities in the TSS region of the *RPB2* gene in *spt5ΔKOW4* and *spt5ΔKOW4-5* cells were not significantly different from that in the WT cells (*P*-values are 0.1 and 0.3 for the *spt5ΔKOW4* and *spt5ΔKOW4-5* cells, respectively; a *P*-value of < 0.05 is considered to be significant). Above the bars of *spt5ΔKOW4* and *spt5ΔKOW4-5* samples are shown the *P*-values of Student's *t*-test between the TSS region and the 1, 2.5 or 3.9 kb region.

TCR. If this is the case, disruption of the interactions between Spt5 and the Rpb4/7 stalk may derepress TCR. To test this idea, we analyzed the effects of Spt5 KOW4 and KOW4-5 deletions on TCR. In yeast, Rad7 and its interaction partner Rad16 are essential for global genomic repair (GGR) but play no role in TCR (Verhage, Zeeman et al. 1994). Therefore, TCR can be unambiguously analyzed in *rad7Δ* or *rad16Δ* cells. A nucleotide resolution method that uses streptavidin magnetic beads and biotinylated oligonucleotides to facilitate isolation and strand-specific end-labeling of DNA fragments of interest was used for the analysis (Li and Waters 1996, Li, Waters et al. 2000). TCR, which initiates about 40 nucleotides upstream of the TSS of the *RPB2* gene, could be seen in *rad7Δ* cells (Figure 2-11A). As expected, the additional deletion of RAD26 (*rad7Δ rad26Δ*) decreased TCR in the transcribed region of the gene (Figure 2-11, compare panels A and D), except for a region of ~ 50 nucleotides immediately downstream of the TSS (Figure 2-11D, marked with the bracket). Our results agree with previous studies showing that TCR in a short region (20 – 50 nucleotide long) immediately downstream of the TSS of a gene is rapid and less dependent on Rad26 in yeast (Tijsterman, Verhage et al. 1997, Li and Smerdon 2002) or CSA and CSB in mammalian (Tu, Bates et al. 1997, Tu, Bates et al. 1998) cells, indicating that the short region is less repressed even in the absence of Rad26, CSA or CSB. While the Spt5 KOW4 deletion slightly enhanced TCR, the Spt5 KOW4-5 deletion dramatically enhanced the repair event throughout the transcribed region of the *RPB2* gene in both *rad7Δ* and *rad7Δ rad26Δ* cells (Figure 2-11, compare panels B and C with A, and E and F with D; Figure 2-12A and B). Note that the TCR speed in *rad7Δ rad26Δ spt5ΔKOW4-5* cells was even faster than that in *rad7Δ* (*RAD26⁺* and *SPT5⁺*) cells, especially in the region ~ 50 nucleotides downstream of the TSS (Figure 2-11, compare panels A and F; Figure 2-12A and B). The enhancement of TCR by deletion of the Spt5 KOW4-5 is similar to that by deletion of Rpb4

(Li and Smerdon 2002) but more dramatic than that by deletion of Spt4 (Jansen, den Dulk et al. 2000), the Spt5 CTR (Ding, LeJeune et al. 2010) or subunits of PAFc (Tatum, Li et al. 2011). Also, in contrast to deletions of Spt4, the Spt5 CTR and subunits of PAFc, which enhance TCR only in the absence but not in the presence of Rad26, deletion of the Spt5 KOW4-5 (or Rpb4) enhances TCR in the absence or presence of Rad26. The dramatic enhancement of TCR in *spt5ΔKOW4-5* cells is not due to a change in Rad26 levels in the cell, as deletion of Spt5 KOW4 or KOW4-5 did not significantly affect the cellular levels of Rad26 (Figure 2-9B). Rather, it is likely that the full-length Spt5, by coordinating with Rpb4/7, strongly represses TCR and Rad26 can only partially antagonize its repression effect. On the other hand, Rad26 appears to be able to completely antagonize the repression effects of Spt4, the Spt5 CTR and PAFc. Spt5 (along with Spt4) is loaded to RNAP II by binding to the nascent transcript only after it reaches to 30-50 nucleotides in length (Levine 2011), which may explain why TCR in the short region immediately downstream of the TSS is not significantly repressed by Spt5.

In agreement with the enhancement of TCR, deletion of Spt5 KOW4 or KOW4-5 in *rad7Δ* or *rad7Δ rad26Δ* cells enhanced survival of yeast cells upon UVC (254 nm) irradiation, with the KOW4-5 deletion being more striking (Figure 2-12C). Also, in agreement with their highly efficient TCR, *rad7Δ rad26Δ spt5ΔKOW4-5* and *rad7Δ spt5ΔKOW4-5* cells were more UVC resistant than *rad7Δ (RAD26⁺ and SPT5⁺)* cells (Figure 2-12C). Taken together, our results indicate that the Spt5 KOW4-5 domains play a pivotal role in repression of TCR even in the presence of Rad26.

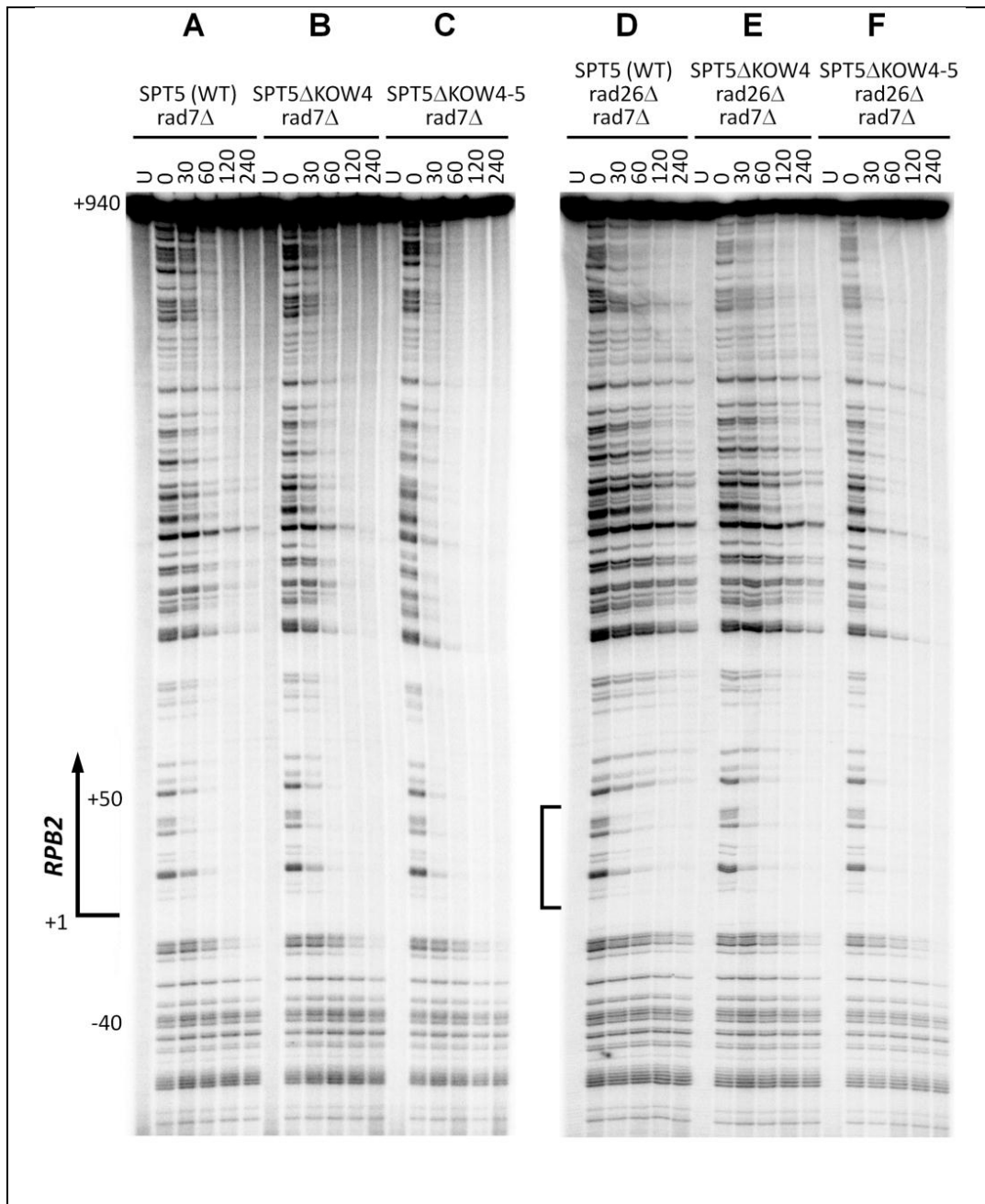


Figure 2-11. Deletion of Spt5 KOW4-5 derepresses TCR. (A-C) Sequencing gel showing TCR of CPDs in *rad7Δ* cells expressing wild type (WT), KOW4 deleted (*spt5ΔKOW4*) and KOW4-5 deleted (*spt5ΔKOW4-5*) Spt5. Unirradiated (U) and irradiated samples after different times (in minutes) of repair incubation are indicated at the top of the gel lanes. Nucleotide positions shown on the left are relative to the TSS. (D-F) Sequencing gel showing TCR of CPDs in *rad7Δ rad26Δ* cells expressing wild type, KOW4 deleted and KOW4-5 deleted Spt5. Bracket on the left of panel D indicates the region immediately downstream of the TSS where TCR is not significantly repressed even in the absence of Rad26.

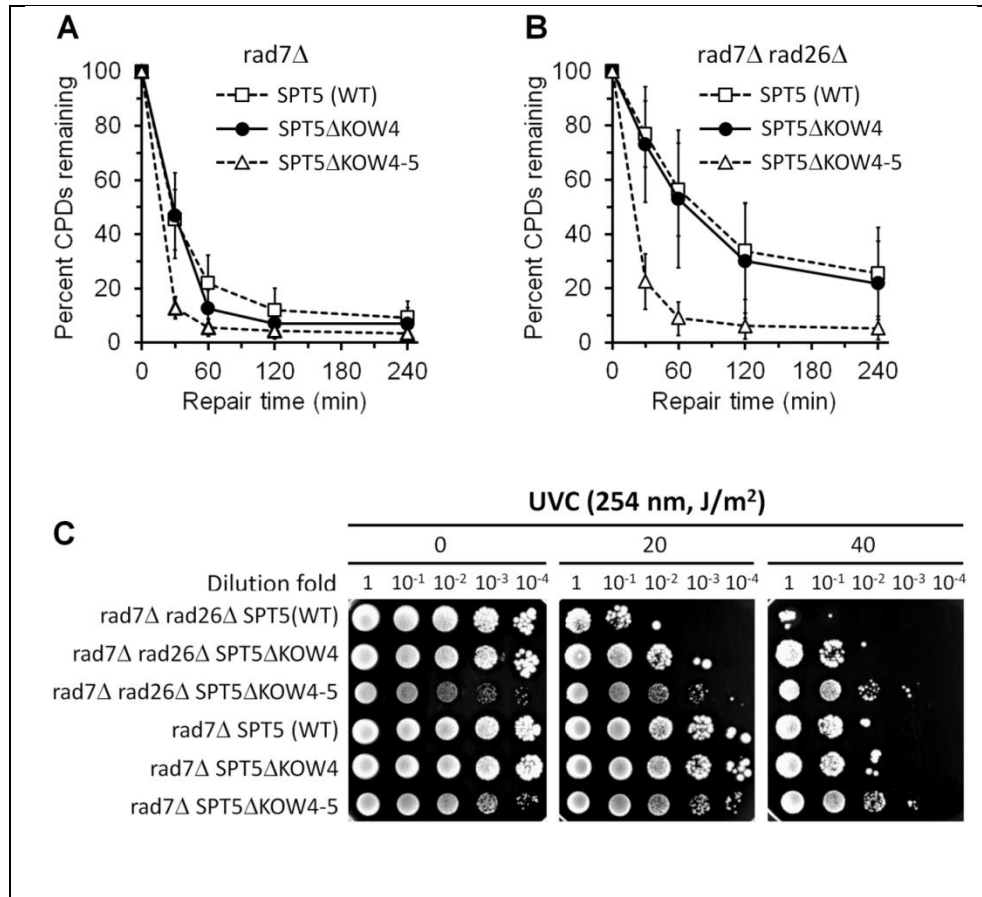


Figure 2-12. Effects of Spt5 KOW4 and KOW4-5 deletions on TCR. (A and B) Percent of CPDs remaining in the TS of the *RPB2* gene in *rad7Δ* and *rad7Δ rad26Δ* cells expressing wild type (WT), KOW4 deleted (*spt5ΔKOW4*) and KOW4-5 deleted (*spt5ΔKOW4-5*) Spt5. Data are represented as mean +/- S.D. (C) Survival of *rad7Δ* and *rad7Δ rad26Δ* cells expressing wild type, KOW4 deleted and KOW4-5 deleted Spt5 following different doses of UVC irradiation.

2.3.6 TFIIIE Interacts with the Clamp and Rpb4/7 Stalk of RNAP II Differently from Spt5, Which May Explain Why TFIIIE Does Not Significantly Represses TCR in the Short Region Immediately Downstream of the TSS

Förster resonance energy transfer analysis showed that the archaeal transcription initiation factor TFE, the homolog of eukaryotic TFIIIE alpha subunit (Tfa1), and Spt4/5 compete for binding to the RNAP clamp during the transition from transcription initiation to elongation (Grohmann, Nagy et al. 2011). Studies with hydroxyl radical-generating probes (Grunberg, Warfield et al. 2012) and cryo-electron microscopy (He, Fang et al. 2013, Murakami, Elmlund et

al. 2013) have also shown that the yeast Tfa1 interacts with the RNAP II clamp and extends to the Rpb4/7 stalk of RNAP II. Therefore, TFIIE and Spt5 may employ a similar binding strategy to enclose the DNA in the central cleft to facilitate transcription initiation and elongation, respectively. However, why is TCR in the short region immediately downstream of the TSS of a gene not significantly repressed by TFIIE? One possibility is that the interactions of RNAP II with TFIIE during transcription initiation and those with Spt5 during transcription elongation may be different. To test this idea, we mapped the sites of RNAP II that interact with Tfa1. Indeed, Bpa substitution at Rpb1 H286, located on the RNAP II clamp, cross-linked to Tfa1 (Figure 2-13A and C). However, this site did not cross-link to Spt5 (Figure 2-3A and E). On the other hand, Bpa substitutions at Rpb1 H281 and E291, which are close to H286 on the clamp, cross-linked to Spt5 (Figure 2-3A and E) but not Tfa1 (Figure 2-13A and C; Table 2-4). Bpa substitutions at Rpb7 I151 and I160, which cross-link to Spt5 (Figure 2-3D and E), also cross-linked to Tfa1 (Figure 2-13B and C). However, Bpa substitutions at Rpb7 F17, N53, H97, E100, E148 and H158, which cross-link to Spt5 did not cross-link to Tfa1 (Table 2-7). Therefore, the sites of RNAP II clamp and Rpb4/7 stalk that interact with Spt5 partially overlap with those that interact with TFIIE. This finding is consistent with the “factor swapping” mechanism that has been proposed to explain the transition from RNAP II initiation to elongation (Blombach, Daviter et al. 2013). As described above, Spt5 may wrap around the Rpb4/7 stalk, which may greatly stabilize the interaction of the stalk to the core RNAP II. The different patterns of interactions of TFIIE with RNAP II may explain why TCR is not repressed by the transcription initiation factor in the region immediately downstream of the TSS.

2.4 Discussion

2.4.1 Spt5 Is a Key Transcription Elongation Factor

Our mapping of the interactions of Spt5 with RNAP II provides insights into the functional mechanisms of this transcription elongation factor. Although the Rpb4/7 stalk is easily dissociable from the 10 subunit core RNAP II and is not essential for transcription elongation *in*

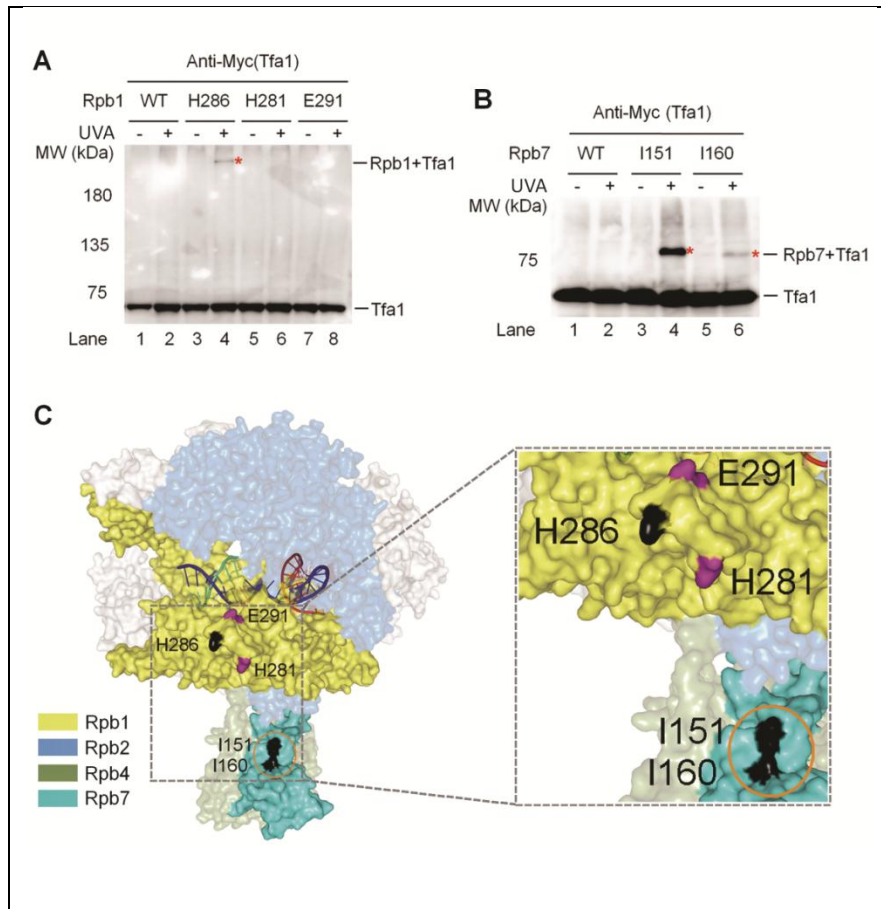


Figure 2-13. TFIIIE and Spt5 interact with the clamp and Rpb4/7 stalk of RNAP II differently. (A and B) Western blots showing cross-linking of Bpa-substituted Rpb1 and Rpb7 to Tfa1, respectively. 3×Myc tagged Tfa1 was detected with an anti-Myc antibody. Sites of Bpa substitutions are shown above the lanes of each blot. Bands of Tfa1 cross-linked to Bpa-substituted Rpb1 and Rpb7 are indicated with red asterisks. (C) Locations of Bpa-substituted Rpb1 and Rpb7 residues that cross-linked to Tfa1. Residues that cross-linked to Tfa1 are shown in black. Orange circle indicates Rpb7 residues that also cross-linked to Spt5 (see Figure 2-3D and E). The two Rpb1 residues (E291 and H281) that cross-linked to Spt5 (see Figure 2-3A and E) but not Tfa1 are shown in purple. The RNAP II structure is based on PDB 1Y1W (Kettenberger, Armache et al. 2004).

vitro, Rpb4 and Rpb7 play important roles throughout the transcription cycle *in vivo* (Jasiak, Hartmann et al. 2008, Verma-Gaur, Rao et al. 2008). Crystal structures of the 12 subunit yeast RNAP II show that Rpb4/7 interacts with the 10 subunit core RNAP II through a small “tip” of the Rpb7 wedge structure and most of the Rpb4/7 surface is not involved in the interaction (Armache, Kettenberger et al. 2003, Bushnell and Kornberg 2003). Except for wedging the RNAP II clamp to the closed conformation, association of Rpb4/7 does not cause a gross change in the structure of the core RNAP II. The interactions of Spt5 with the clamp, protrusion, wall and Rpb4/7 should stabilize the association of Rpb4/7 with the core RNAP II, thereby locking the clamp in the closed conformation and enclosing the DNA being transcribed in the central cleft of the polymerase to facilitate transcription processivity (Figure 2-8). In addition, Spt5 may directly interact with the upstream DNA and the Spt5 KOW4-5 domains may have close proximity to the RNA exiting channel and interact with the nascent transcript (Hartzog and Fu 2013). These interactions may also facilitate transcription elongation by repressing transcription pausing and backtracking (Hartzog and Fu 2013).

Unlike Spt5, which is essential for cell viability, the small zinc finger protein Spt4 is dispensable for cell survival. Spt4 binds to the NGN domain of Spt5 but may not directly interact with RNAP II (Guo, Xu et al. 2008, Martinez-Rucobo, Sainsbury et al. 2011). Spt4 protects Spt5 from degradation and stabilizes the binding of Spt5 to RNAP II (Ding, LeJeune et al. 2010). PAFc, which is also not essential for cell viability, is recruited to RNAP II complex through interaction with the CTR domain of Spt5 (Liu, Warfield et al. 2009, Zhou, Kuo et al. 2009, Tatum, Li et al. 2011, Wier, Mayekar et al. 2013). It is therefore likely that, through direct interactions with the clamp, protrusion, wall and Rpb4/7 stalk of RNAP II, Spt5 serves as a key transcription elongation factor. On the other hand, Spt4 and PAFc may serve as accessory

transcription elongation factors by interacting with the Spt5 NGN and CTR domains, respectively.

Similar to eukaryotic Spt5, archaeal Spt5 (Hirtreiter, Damsma et al. 2010, Klein, Bose et al. 2011, Martinez-Rucobo, Sainsbury et al. 2011) and bacterial NusG (Mooney, Schweimer et al. 2009) also bind to the clamp of an RNAP and may enclose the DNA being transcribed in the central cleft. However, the functional mechanism of a eukaryotic Spt5 is likely to be significantly different from that of an archaeal Spt5 or a bacterial NusG. We show here that the KOW4-5 domains of the yeast Spt5 extensively interact with Rpb4/7 and deletion of these domains significantly decreases transcription elongation. However, archaeal Spt5 or bacterial NusG has a single KOW domain and a bacterial RNAP lacks the eukaryotic Rpb4/7 counterparts. The single KOW domain of an archaeal Spt5 may not be able to reach and extensively interact with RpoF/E, the counterpart of the eukaryotic Rpb4/7. Therefore, although Spt5 and RpoF/E play important roles in transcription elongation in archaea (Werner 2013), an archaeal RNAP elongation complex may not be stabilized primarily by interactions between Spt5 and RpoF/E. In *E. coli*, the single KOW domain of NusG may interact with other transcription regulators, rather than with a subunit of RNAP (Mooney, Schweimer et al. 2009, Sevostyanova, Belogurov et al. 2011).

2.4.2 Spt5 Is a Key TCR Repressor

DNA lesions that are NER substrates are stalled at the active site of RNAP II following incorporation or misincorporation of nucleotide(s) opposite the damaged template (Brueckner, Hennecke et al. 2007, Damsma, Alt et al. 2007, Wang, Zhu et al. 2010). Our finding that Spt5 interacts with the clamp, protrusion, wall and Rpb4/7 stalk of RNAP II suggests that a DNA

lesion can be trapped in the closed elongation complex, rendering it inaccessible to the repair machinery.

We found that deletion of Spt5 KOW4-5 domains, which extensively interact with Rpb4/7, enhances TCR in *rad26Δ* and *RAD26⁺* cells. Similarly, deletion of Rpb4 also enhances TCR in *rad26Δ* and *RAD26⁺* cells (Li and Smerdon 2002). In contrast, deletion of Spt4 or subunits of PAFc enhances TCR to a lesser extent and the enhancement of TCR can only be seen in *rad26Δ* but not *RAD26⁺* cells (Jansen, den Dulk et al. 2000, Tatum, Li et al. 2011). These findings indicate that the full-length Spt5 and Rpb4 (in complex with Rpb7) are strong TCR repressors and they can repress TCR in the presence or absence of Rad26. On the other hand, Spt4 and PAFc are weaker TCR repressors and they can repress TCR only in the absence but not in the presence of Rad26. Therefore, Spt5, through direct interactions with Rpb4/7 and other domains of RNAP II, appears to play a key role in repressing TCR. Spt4 and PAFc, by interacting with the NGN and CTR domains of Spt5 respectively, may play accessory roles in repressing TCR by further stabilizing the closed elongation competent RNAP II complex. Rad26 appears to be able to completely antagonize the accessory TCR repressors but can only partially alleviate the repression of TCR by Spt5.

It has been shown recently that the *E. coli* UvrD induces backtracking of RNAP and thereby exposing DNA lesions shielded by RNAP and allowing NER enzymes to gain access to lesion sites (Epshtein, Kamarthapu et al. 2014). In contrast to UvrD, *E. coli* NusG inhibits backtracking and accelerates pause-free transcription by promoting forward translocation of RNAP (Herbert, Zhou et al. 2010). Therefore, like eukaryotic Spt5, NusG may also repress TCR in *E. coli* and UvrD may antagonize the repression by counteracting NusG. However, in view of the fact that the *E. coli* RNAP has no Rpb4/7 counterparts and the single KOW domain of *E. coli*

NusG does not directly interact with RNAP, the underlying mechanisms of TCR repression in *E. coli* and eukaryotes may be somewhat different. By binding to an upstream activating sequence, the transcription activator Fis stimulates transcription of a tRNA gene to an extremely high level and at the same time represses TCR in the gene, except for a short region immediately downstream of the TSS (Li and Waters 1997). However, the repression of TCR in *E. coli* is apparently caused by extremely high level of loading of RNAP rather than by stabilization of the transcription elongation complex. During very high level transcription in a tRNA gene in *E. coli*, an RNAP may arrive at the site of a downstream RNAP stalled at a lesion before the downstream RNAP can initiate or finish the TCR process, resulting in repression of TCR (Selby and Sancar 1994). It is also possible that UvrD may not be able to efficiently backtrack RNAP molecules densely distributed in highly transcribed genes in *E. coli*. In eukaryotic cells, however, the loading of RNAP II to a gene being transcribed does not appear to be able to reach a level that can repress TCR. The galactose-induced *GALI-10* genes are among the most highly transcribed genes by RNAP II in yeast. However, TCR occurs very rapidly in the *GALI-10* genes in *RAD26*⁺ or *rad26Δ* cells (Li and Smerdon 2002, Li and Smerdon 2004), indicating TCR is not significantly repressed in these highly transcribed genes even in the absence of Rad26. In contrast, TCR is much slower in the much more slowly transcribed *RPB2* and *URA3* genes in *rad26Δ* cells (Tijsterman, Verhage et al. 1997, Li and Smerdon 2002). However, deletion of Rpb4 or Spt4 in *rad26Δ* cells restores TCR in the *RPB2* and *URA3* genes (Jansen, den Dulk et al. 2000, Li and Smerdon 2002). These studies indicate that TCR is more repressed in the slowly transcribed genes in the absence of Rad26. Therefore, the repression of TCR in eukaryotic cells, which can be easily seen in *rad26Δ* cells, is not caused by extremely high level of loading of RNAP II but may be due to Spt5-coordinated stabilization of the transcription elongation

complex. The reason why TCR is less repressed in the highly transcribed *GALI-10* genes in *rad26Δ* cell is unknown, but may be due to a lower content of a TCR repressor in the RNAP complex engaged in highly transcribed genes.

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CHAPTER 3

TRANSCRIPTION BYPASS OF DNA LESIONS ENHANCES CELL SURVIVAL BUT ATTENUATES TRANSCRIPTION COUPLED DNA REPAIR*

3.1 Introduction

Nucleotide excision repair (NER) is a multi-step process that removes bulky and/or helix-distorting lesions, such as UV induced cis-syn cyclobutane pyrimidine dimers (CPDs) and bulky chemical adducts that generally obstruct DNA replication and transcription (Tatum and Li 2011, Scharer 2013). NER has two subpathways: transcription coupled repair (TCR) and global genomic repair (GGR). TCR is dedicated to rapid removal of lesions in the transcribed strand of actively transcribed genes. On the other hand, GGR is responsible for removal of lesions throughout the whole genome, including the nontranscribed strand of actively transcribed genes. In eukaryotic cells, the two NER subpathways rely on different proteins in the early damage recognition step but share common factors in the later steps of the repair process. CSA, CSB, UVSSA and USP7 in mammalian cells (Sarasin 2012, Schwertman, Vermeulen et al. 2013) and Rad26 (homolog of mammalian CSB) in *Saccharomyces cerevisiae* (Tatum and Li 2011) are involved in TCR but dispensable for GGR. On the other hand, XPC in mammalian cells (Scharer 2013), and Rad7, Rad16 and Elc1 in *S. cerevisiae* (Tatum and Li 2011) are specifically required for GGR but play no role in TCR.

RNA polymerase (RNAP) is an ideal proxy damage sensor for DNA damage because it has the highest selectivity of all known DNA damage recognition proteins (Lindsey-Boltz and Sancar 2007). A mammalian RNA polymerase II (RNAP II) complex stalled at a CPD has a half-

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life of ~ 20 hours, which is more stable than any complex formed between a damage-recognition protein and its substrate (Selby, Drapkin et al. 1997). TCR is generally assumed to be triggered by stalling of an RNAP at a lesion in the transcribed strand, as DNA lesions that can substantially stall RNAP are generally good substrates for TCR (Hanawalt and Spivak 2008, Vermeulen and Foustari 2013). Up to date, however, no direct evidence has been available to prove this assumption and the precise nature of the TCR signal is still enigmatic.

In *Escherichia coli*, Mfd, a DNA translocase, binds to the β subunit of a lesion-stalled RNAP, displaces the RNAP complex by pushing it forward and concurrently recruits UvrA to the exposed lesion site to facilitate TCR (Selby and Sancar 1993, Park, Marr et al. 2002, Deaconescu, Chambers et al. 2006, Howan, Smith et al. 2012). Upon binding to ATP, Mfd also binds to DNA in a manner that the DNA wraps around the protein (Selby and Sancar 1995). The DNA binding activity may anchor Mfd downstream from the transcription stop site to carry out its transcription-repair coupling function (Selby and Sancar 1995). In addition to promoting repair of lesions that stall the RNAP, Mfd may facilitate TCR downstream of the site that stalls RNAP (Haines, Kim et al. 2014). UvrD, a DNA helicase, has also been shown to play an important role in TCR in *E. coli* (Epshtein, Kamarthapu et al. 2014). Unlike Mfd, UvrD forces RNAP to backtrack, thereby exposing DNA lesions for access of the repair machinery.

How the TCR machinery gains access to a lesion initially trapped by a stalled RNA polymerase II (RNAP II) in eukaryotic cells is still unknown (Hanawalt and Spivak 2008, Vermeulen and Foustari 2013). Multiple scenarios have been suggested, including 1) ubiquitination and degradation of the largest subunit (Rpb1) of the 12 subunit (Rpb1-12) RNAP II, 2) displacement of the transcription elongation complex through forced back- or forward-tracking, and 3) remodeling of the complex without removal from the arrest site. To date,

however, none of these scenarios has been proven to be required for TCR in eukaryotic cells. In mammalian cells, CSB interacts loosely with the elongating RNAP II and stimulates transcription (Selby and Sancar 1997) but becomes more tightly bound following transcription arrest (van Gool, Citterio et al. 1997). CSB may also push the RNAP II forward, such that an additional nucleotide is incorporated opposite a CPD (Selby and Sancar 1997). However, unlike the bacterial Mfd, which displaces a lesion-stalled RNAP from the DNA, CSB does not seem to displace a lesion-stalled RNAP II (Selby and Sancar 1997). In *S. cerevisiae*, the elongating RNAP II complex, which is stabilized by Spt5 and other associated transcription elongation factors, is intrinsically repressive to TCR (Li, Giles et al. 2014). Rad26 appears to facilitate TCR by antagonizing the repression, as it becomes dispensable for TCR in the absence of Spt4 (Jansen, den Dulk et al. 2000, Ding, LeJeune et al. 2010), Rpb4 (Li and Smerdon 2002), the RNAP II associated factor 1 complex (PAFc) (Tatum, Li et al. 2011) and certain domains of Spt5 (Ding, LeJeune et al. 2010, Li, Giles et al. 2014).

An RNAP has an intrinsic capacity for transcription bypass of lesions by incorporation or misincorporation of nucleotides across the lesions (Saxowsky and Doetsch 2006, Xu, Da et al. 2014). *In vitro* studies with a DNA template containing a T-T CPD showed that the *S. cerevisiae* RNAP II catalyzes a nontemplated insertion of AMP opposite the CPD 3'-T following the A-rule, followed by a very slow and templated AMP or UMP insertion opposite the CPD 5'-T (Brueckner, Hennecke et al. 2007, Walmacq, Cheung et al. 2012). AMP incorporation opposite the CPD 5'-T enables lesion bypass, whereas UMP misincorporation opposite the 5'-T results in irreversible stalling of RNAP II (Brueckner, Hennecke et al. 2007, Walmacq, Cheung et al. 2012).

Two domains of Rpb1, the trigger loop and bridge helix, play key roles in the nucleotide addition cycle during RNA synthesis. Upon binding of a matched nucleotide, the trigger loop of RNAP II switches from an inactive open state to an active closed state (Figure 3-1). Mutations of Rpb1 near the RNAP II active center and secondary pore (through which nucleotides reach the catalytic center) have been shown to affect the fidelity of transcription in *S. cerevisiae* (Malagon, Kireeva et al. 2006, Kireeva, Nedialkov et al. 2008, Koyama, Ueda et al. 2010, Strathern, Malagon et al. 2013). Two yeast Rpb1 mutations, G730D (*rpb1G730D*) and E1103G (*rpb1E1103G*) (Figure 3-1), have been shown to affect transcription bypass of CPDs *in vitro* (Walmacq, Cheung et al. 2012). The *rpb1G730D* mutation is located in the α 21 helix, which

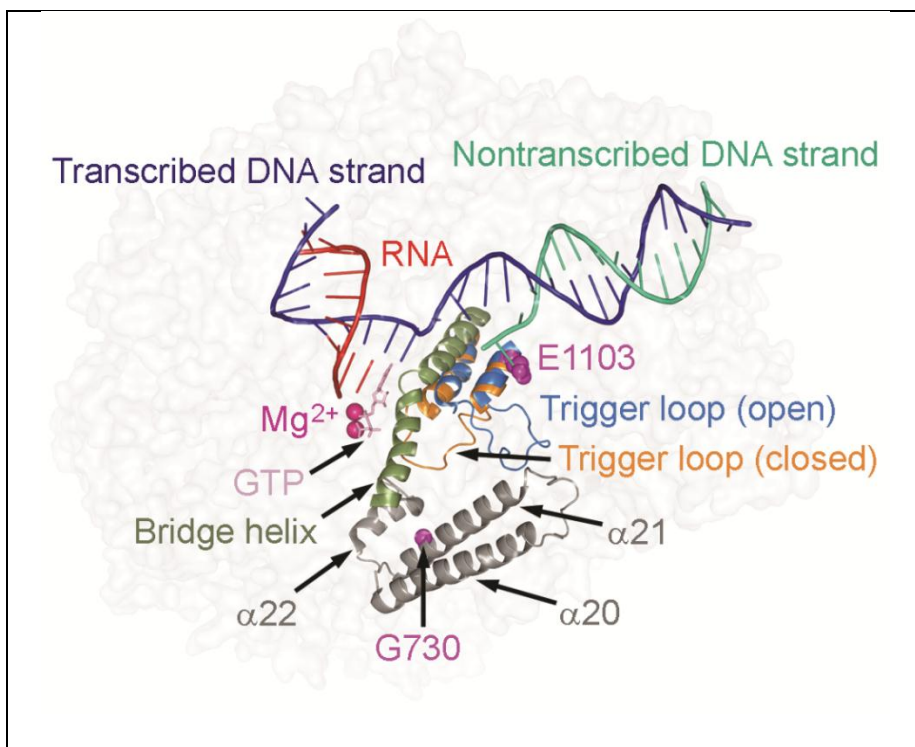


Figure 3-1. Locations of Rpb1 E1103 and G730 residues on the RNAP II structure. The RNAP II structures are based on PDB 2E2H (Wang, Bushnell et al. 2006), which has a closed trigger loop, and PDB 1Y1V (Kettenberger, Armache et al. 2004), which has an open trigger loop, and generated by using PyMOL. Indicated structures are Rpb1 trigger loop (residues 1060-1105), bridge helix (residues 810-846), and α helices 20 (residues 673-699), 21 (residues 710-735) and 22 (residues 742-748), DNA, RNA, GTP and Mg^{2+} . Other residues of RNAP II are shown in transparent light gray.

forms a part of the secondary pore and contacts the trigger loop. This mutation abolishes transcription bypass of a T-T CPD by preventing incorporation of nucleotides opposite the CPD 3'- and 5'-Ts. The *rpb1E1103G* mutation is located at the base of the trigger loop. In contrast to the *rpb1G730D* mutation, the *rpb1E1103G* mutation increases transcription bypass of T-T CPDs by enabling incorporation of 2 AMPs opposite the CPD 3'- and 5'-Ts (Walmacq, Cheung et al. 2012). The *rpb1E1103G* mutation was also observed to increase resistance of *RAD26* (wild type) cells but not *rad26Δ* cells to UV irradiation, suggesting that the increased UV resistance is dependent on Rad26-dependent TCR (Walmacq, Cheung et al. 2012). An appealing new model of TCR mechanism was therefore proposed: transcription bypass of lesions may expose the lesions to the TCR proteins after their Rad26-dependent recruitment to the lesion-stalled RNAP II and this exposure may be required for TCR (Ellenberger 2012, Walmacq, Cheung et al. 2012). However, if and/or how transcription bypass of lesions is implicated in TCR remains to be determined.

We found that increased transcription bypass of CPDs by RNAP II enhances survival of UV irradiated *S. cerevisiae* cells but attenuates TCR. The enhanced cell survival is independent of Rad26 or other NER factors. In contrast, abolition of transcription bypass of CPDs by RNAP II decreases survival of UV irradiated GGR-deficient *S. cerevisiae* cells but enhances TCR. Our results suggest that transcription bypass of lesions may enhance lesion tolerance but attenuate TCR. Efficient stalling of RNAP II is essential for efficient TCR. Our findings offer direct evidence for the long-standing but unproven notion that TCR requires sufficient stalling of an RNAP.

3.2 Materials and Methods

3.2.1 Yeast Strains and Plasmids

All yeast strains used in this study are derivatives of the wild type strain Y452 (*MAT α* , *ura3-52*, *his3-1*, *leu2-3*, *leu2-112*, *cir^o*). Deletions of *RAD16*, *RPB1*, *RAD26* and *RAD14* genes were conducted as previously described (Li and Smerdon 2002).

Plasmids pRS416-RPB1 was created by inserting the whole *RPB1* gene including the promoter, coding sequence and 3' terminator sequences into the multiple cloning site of the single-copy centromeric *URA3* plasmid pRS416 (Sikorski and Hieter 1989). Plasmid pRS415-RPB1, pRS415-RPB1E1103G and pRS415-RPB1G730D were created by inserting the whole *RPB1* gene encoding the wild type and E1103G and G730D mutant Rpb1, respectively, into the *XhoI* and *ApaI* sites of the single copy centromeric *LEU2* plasmid pRS415 (Sikorski and Hieter 1989).

Plasmids pRS415-RPB1, pRS415-RPB1E1103G and pRS415-RPB1G730D were transformed into yeast strains whose genomic *RPB1* gene was deleted and complemented with pRS416-RPB1. The transformed cells were selected with 5-fluoroorotic acid (5-FOA), which is toxic to cells with a functional *URA3* gene, to select for cells that had lost the pRS416-RPB1 plasmid.

3.2.2 Measurement of RNA Synthesis in Yeast Cells

To measure total RNA synthesis, yeast cells were grown at 30°C in synthetic dextrose medium to late log phase ($A_{600} \approx 1.0$). The harvested cells were washed twice with ice-cold H₂O, resuspended in ice-cold 2% dextrose, and split into two aliquots. One aliquot was kept on ice and the other was irradiated with 360 J/m² of 254 nm UV (by using a 15W UV germicidal bulb,

General Electric). Treatment of genomic DNA with T4 endonuclease V, followed by denaturing agarose gel analysis indicates that this dose of UV induces ~ 1 CPD per 1 kb of DNA. After addition of one tenth volume of a stock solution (6.7% yeast nitrogen base without amino acids, 1.6% yeast synthetic drop-out media supplement without uracil, 1 mM uracil), [2-¹⁴C]uracil (56.0 mCi/mmol; Moravek Biochemicals) was added to a final concentration of 2 μM. The samples were incubated at 30°C in the dark and aliquots were collected at different times of the incubation. Total RNA was isolated using a hot acidic phenol method as previously described (Collart and Oliviero 2004), and quantified by using the Qubit[®] RNA HS assay kit and a Qubit[®] 2.0 Fluorometer (Invitrogen, Life Technologies). The isolated total RNA was fractionated on formaldehyde agarose gels (Sambrook and Russell 2001), and transferred onto Hybond-N⁺ membranes (Amersham Biosciences). The membranes were exposed to Phosphorimager screens for 7 days and scanned with Molecular Imager FX (Bio-Rad).

For Northern blot analysis of galactose-induced *GAL10* transcription, yeast cells were grown at 30°C in synthetic medium containing 2% glycerol, 2% lactate and 2% ethanol to late log phase ($A_{600} \approx 1.0$). The harvested cells were washed and resuspended in ice-cold H₂O, and split into two aliquots. One aliquot was kept on ice and the other was irradiated with 120 J/m² of 254 nm UV (the dose we used for TCR analysis). The unirradiated and UV irradiated cells were pelleted by centrifugation and resuspended in YPG (1% yeast extract, 2% peptone and 2% galactose) medium and incubated at 30°C. At different times of the incubation, aliquots were collected. Total RNA was isolated, fractionated on formaldehyde agarose gels and transferred onto Hybond-N⁺ membranes. The *GAL10* transcripts on the membranes were probed with a 150 nucleotide long [α -³²P] UTP labeled riboprobe that is complementary to the 5' end of the *GAL10* transcripts.

3.2.3 Repair Analysis of UV Induced CPDs

Yeast cells were cultured at 30°C in synthetic dextrose medium to late log phase ($A_{600} \approx 1.0$), washed twice with ice-cold ddH₂O, resuspended in 2% dextrose and irradiated with 120 J/m² of 254 nm UV. After addition of one tenth volume of a stock solution (10% yeast extract, 20% peptone), the yeast samples were incubated at 30°C in the dark. At different times of the repair incubation, aliquots were collected and the genomic DNA was isolated as described previously (Li and Smerdon 2002).

The transcribed and nontranscribed strands of the *RPB2* gene were 3' end labeled with [α -³²P]dATP by using a method described previously (Li and Waters 1996, Li, Waters et al. 2000). Briefly, ~ 1 μ g of total genomic DNA was digested with DraI to release the *RPB2* fragments and incised at CPD sites with an excess amount of T4 endonuclease V (Epicentre). Excess copies of a biotinylated oligonucleotide, which is complementary to the 3' end of the transcribed or nontranscribed strand of the *RPB2* gene, were mixed with the samples. The mixtures were heated at 95°C for 5 minutes to denature the DNA and then cooled to an annealing temperature of around 50°C. The annealed molecules were attached to streptavidin magnetic beads, labeled with [α -³²P]dATP, and resolved on DNA sequencing gels. The gels were exposed to Phosphorimager screens. The intensities of gel bands corresponding to CPD sites were quantified using Quantity One (Bio-Rad).

3.2.4 UV Sensitivity Assay

Yeast cells were cultured at 30°C in synthetic dextrose medium to saturation. Sequential 10-fold dilutions of the cultures were made. For spotting assay, the diluted cells were spotted onto YPD (1% yeast extract, 2% peptone and 2% dextrose) plates and irradiated with different

doses of 254 nm UV. After 3-5 days of incubation at 30°C in the dark, the plates were photographed. For colony formation assay, the diluted cells were spread onto YPD plates and irradiated with different doses of 254 nm UV. After 3-5 days of incubation at 30°C in the dark, the colonies were counted. Three repeats were performed and the means and standard deviations were calculated.

3.2.5 Detection of Cellular Levels of Rpb1 by Western Blot.

Late log phase yeast cells were harvested and the whole cell extracts were prepared as described previously (Kushnirov 2000). Proteins were resolved on an SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Rpb1 was probed with 8WG16, which recognizes the C-terminal repeats of Rpb1. Blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce), and the protein bands were detected using ChemiDoc™ XRS+ System (Bio-Rad).

3.2.6 Measurement of RNAP II Densities on the Rpb2 Gene by Chromatin Immunoprecipitation (ChIP) Assay.

The ChIP assay was performed as described previously (Li, Chen et al. 2006). Briefly, yeast cells were grown in synthetic dextrose medium to late log phase, cross-linked with 1% formaldehyde and lysed by vortexing with glass beads. The cell lysates were sonicated by using a Bioruptor (Diagenode) to shear the chromatin DNA to an average size of 200 bp and clarified by centrifugation at 4°C. An aliquot from each of the clarified lysates was saved as an input. The remaining lysates were immunoprecipitated with the anti-Rpb1 antibody 8WG16 or mock immunoprecipitated. DNA fragments corresponding to different regions of the *RPB2* gene in the input, immunoprecipitated and mock immunoprecipitated samples were quantified in triplicates

by using real time PCR. Primers used for amplifying the different regions of the *RPB2* gene were described previously (Li, Giles et al. 2014). The number of molecules in each immunoprecipitated sample was subtracted by that in the corresponding mock immunoprecipitated sample (generally ~ 5% of the immunoprecipitated sample) and then normalized to that in the corresponding input. The levels of RNAP II association with the different regions of *RPB2* gene in cells expressing the *rpb1E1103G* and *rpb1G730D* mutants were normalized to those in cells expressing the wild type Rpb1. The Student's *t*-test was used for statistical analysis.

3.3 Results

3.3.1 *rpb1E1103G* Mutation Promotes Transcription of UV Damaged Templates, Whereas *rpb1G730D* Mutation Impairs Transcription of the Damaged Templates *in vivo*.

In vitro studies with purified yeast RNAP II have shown that the *rpb1E1103G* mutation promotes transcription bypass of CPDs, whereas the *rpb1G730D* mutation abolishes transcription bypass of the DNA lesions (Walmacq, Cheung et al. 2012). RNAP II transcription is affected by chromatin structure and regulated by a plethora of transcription elongation factors in the cell. To determine whether the Rpb1 mutations also affect transcription bypass of lesions *in vivo*, we measured RNAP II transcription by detecting incorporation of radioactive ¹⁴C uracil into total RNA in yeast cells. To prevent removal of UV induced DNA lesions, we utilized *rad14Δ* cells, which are completely deficient in NER (Tatum and Li 2011). Late log phase yeast cells were irradiated with 360 J/m² of 254 nm UV to induce ~ 1 CPD per 1 kb of DNA. Unirradiated and the UV irradiated cells were then incubated in the presence of ¹⁴C uracil. Total RNA was isolated from the cells at different times of the incubation, fractionated on

formaldehyde agarose gels and transferred onto membranes. The radioactive signals on the membranes were detected with a Phosphoimager.

The 35S (6.9 kb) unspliced rRNA precursor, which is transcribed by RNA polymerase I, and the spliced 25S (3.4 kb) and 18S (1.8 kb) rRNAs migrated as distinct bands. Small (100-200 nucleotides long) RNA species, including tRNAs and 5S rRNA, which are transcribed by RNA polymerase III, and the 5.8S rRNA, which is spliced from the 35S rRNA precursor, migrated as a diffused band at the bottom of the gels (Figure 3-2). mRNAs, which are transcribed by RNAP II and predominantly in the range of 0.3 – 5 kb (average 1.3 kb) (Hurowitz and Brown 2003), migrated as smears. ¹⁴C uracil was rapidly incorporated into the different RNA species in the unirradiated *rad14Δ RPBI* (wild type) and *rad14Δ rpb1E1103G* cells. The incorporation was slower in the unirradiated *rad14Δ rpb1G730D* cells, indicating that the mutation causes decreased transcription. The *rpb1G730D* cells grow very slowly, which is likely caused by decreased synthesis of mRNAs by RNAP II, which in turn results in decreased synthesis of other RNA species by other RNA polymerases.

UV irradiation greatly decreased incorporation of ¹⁴C uracil into RNAs. However, the *rad14Δ rpb1E1103G* cells had a higher level of incorporation of ¹⁴C uracil into mRNAs than *rad14Δ* cells, whereas the *rad14Δ rpb1G730D* cells showed very little level of the incorporation after UV irradiation (Figure 3-2G, H and I). To see how the different Rpb1 mutations affect transcription elongation of mRNAs, we calculated the ratios of signal intensities between transcripts of around 1.3 kb and those of around 0.25 kb. Transcripts of these lengths were well separated from the bands of the big rRNAs and small RNA species on the gel. A higher ratio will indicate that more shorter transcripts were elongated into longer transcripts, and thus reflect a more proficient transcription elongation. After 120 minutes of incubation, the ratios were 5.9 and

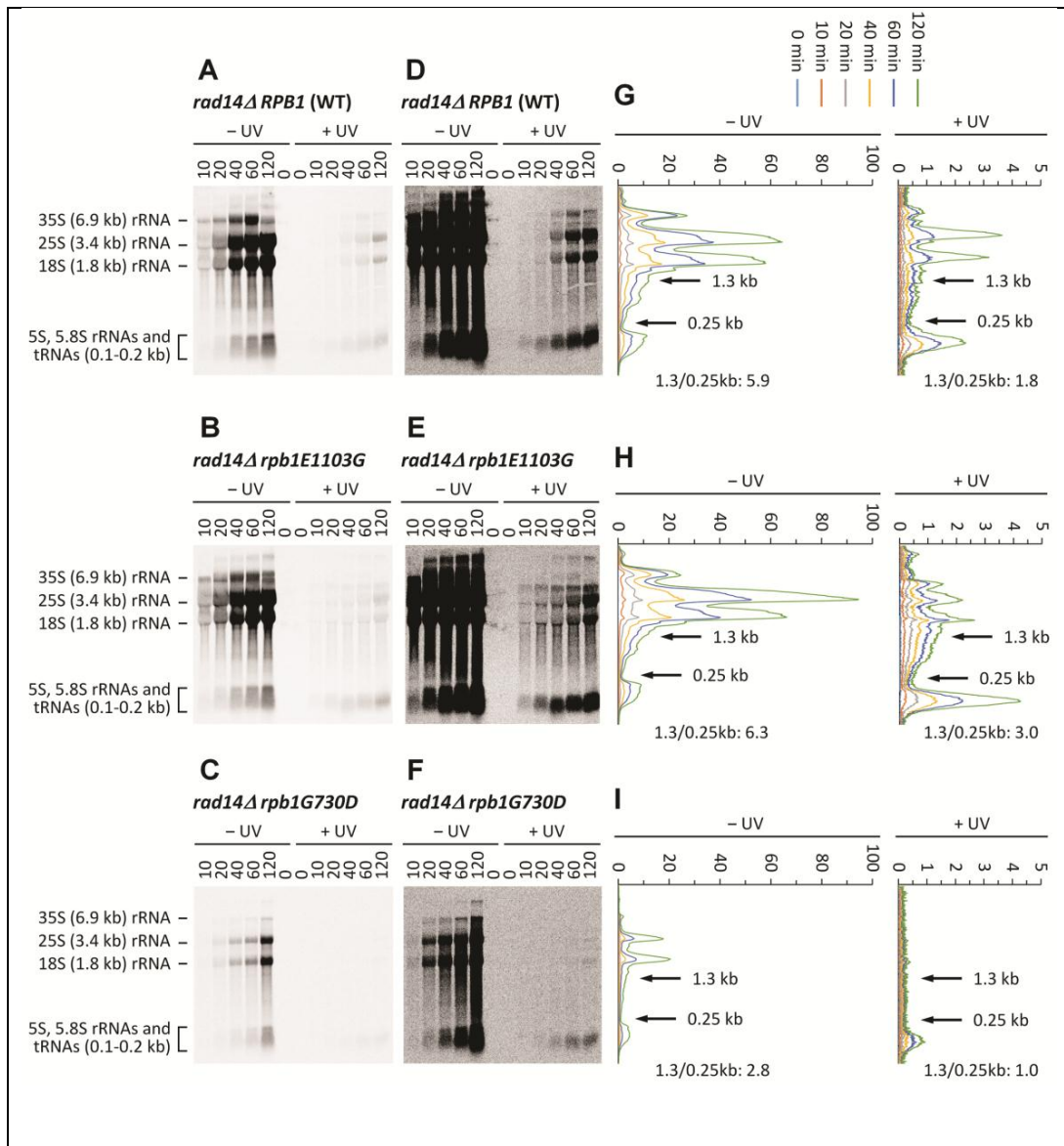


Figure 3-2. Effects of *rpb1E1103G* and *rpb1G730D* mutations on overall transcription *in vivo*. (A-C) Gel blots showing incorporation of ^{14}C uracil into different species of RNAs in UV irradiated and unirradiated *rad14\Delta* cells expressing wild type Rpb1, or *rpb1E1103G* or *rpb1G730D* mutants. Numbers on the top of the blots indicate time (min) of incubation at 30°C. rRNA and tRNA species are marked on the left of the blots. mRNAs, most of which are in the size range of 0.3 – 5 kb (average 1.3 kb) (Hurowitz and Brown 2003), migrated as smear. (D-F) Same blots as those of A-C, respectively, but with higher exposure. (G-I) Plots showing scans of ^{14}C signal intensities along the lanes of the gel blots shown in A-C, respectively. Numbers on top of the plots indicate relative signal intensity (arbitrary units). The ratio of signal intensities between the transcripts of around 1.3 kb and those of around 0.25 kb after 120 minutes of incubation is shown at the bottom of each plot.

1.8 in unirradiated and UV irradiated *rad14Δ RPB1* cells, respectively, (Figure 3-2G), indicating UV induced DNA lesions impaired transcription elongation of mRNAs. The ratios were 3.0 and 1.0 in UV irradiated *rad14Δ rpb1E1103G* and *rad14Δ rpb1G730D* cells, respectively (Figure 3-2H and I). These results suggest that the *rpb1E1103G* and *rpb1G730D* mutations respectively promotes and impairs transcription elongation of mRNAs on UV damaged templates. These results agree well with the *in vitro* observations that the Rpb1E1103G and G730D mutations respectively promote and abolish transcription bypass of DNA lesions (Walmacq, Cheung et al. 2012).

To specifically analyze the effects of the *rpb1E1103G* and *rpb1G730D* mutations on RNAP II transcription and rule out the interference by transcripts of other RNA polymerases, we measured *GAL10* transcripts in UV irradiated cells. The *GAL10* gene is not transcribed in glycerol/lactate/ethanol media, but can be rapidly induced upon switching of the cells to galactose media. Yeast cells were grown in a glycerol/lactate/ethanol medium to late log phase, irradiated with 120 J/m² of UV (the dose we used for TCR analysis) and switched to a galactose medium. For unknown reason(s), the *rpb1G730D* mutant cells grow almost as quickly as wild type cells in glycerol/lactate/ethanol or galactose media (not shown). Total RNA was isolated from the cells at different times of incubation in the galactose medium. The *GAL10* transcripts were detected on Northern blots by using a riboprobe that is complementary to the 5' end of the *GAL10* transcripts.

GAL10 transcripts could be easily detected in all the yeast cells analyzed after 10 minutes of galactose induction and leveled off after ~ 40 minutes of the induction (Figure 3-3). UV irradiation impaired the transcription of *GAL10* gene. The *rad14Δ rpb1E1103G* cells had a higher level of the *GAL10* transcripts than *rad14Δ RPB1* cells, whereas the *rad14Δ rpb1G730D*

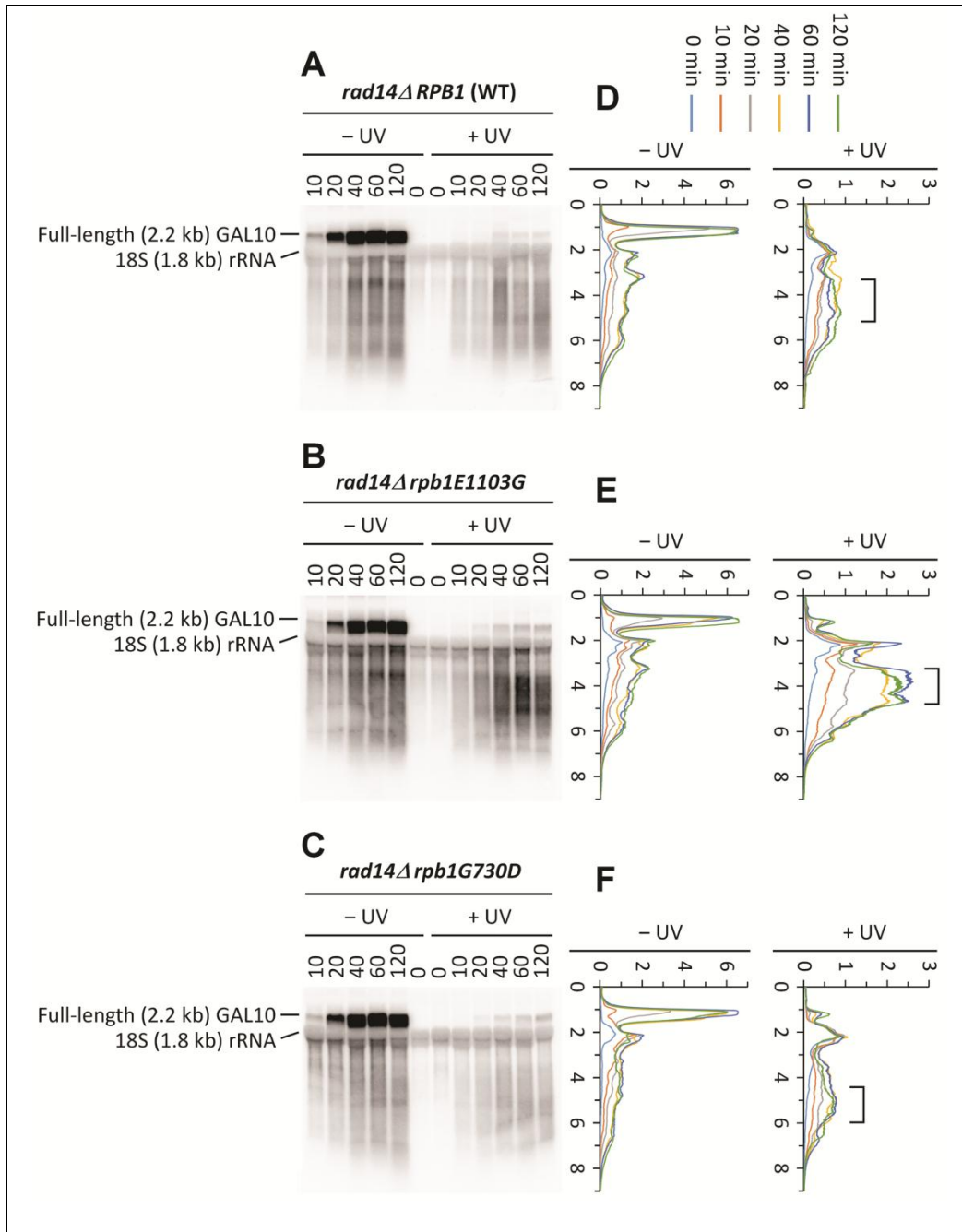


Figure 3-3. Effects of *rpb1E1103G* and *rpb1G730D* mutations on *GAL10* transcription. (A-C) Northern blots showing *GAL10* transcripts in UV irradiated and unirradiated *rad14Δ* cells expressing wild type Rpb1, or *rpb1E1103G* or *rpb1G730D* mutants. Note that the 18S rRNA has certain level of cross-hybridization with the *GAL10* riboprobe. Numbers on top of the blots indicate time (min) of incubation in galactose medium at 30°C. (D-F) Plots showing scans of signal intensities along the lanes of the blots shown in A-C, respectively. Numbers on the top of the plots indicate relative signal intensity (arbitrary units). Numbers on the left of the plots indicate relative locations on the gel (arbitrary units). Brackets on the right panels indicate the predominant *GAL10* transcripts.

cells showed a lower level of the transcripts. Also, the predominant *GAL10* transcripts were longest in the *rad14Δ rpb1E1103G* cells and shortest in the *rad14Δ rpb1G730D* cells (Figure 3-3D-E). A higher level of longer transcripts will indicate more proficient transcription elongation, and *vice versa*. Taken together, our results indicate that the *rpb1E1103G* and *rpb1G730D* mutations respectively promotes and impairs transcription elongation of the UV damaged *GAL10* gene. However, full-length *GAL10* transcripts were rare in all the cells analyzed, indicating that even the *rpb1E1103G* mutation may not enable full transcription bypass of UV lesions.

3.3.2 *rpb1E1103G* Mutation Attenuates TCR, Whereas *rpb1G730D* Mutation Enhances TCR.

To determine the implication of transcription bypass of DNA lesions in TCR, we directly measured repair of UV induced CPDs in the *RPB2* gene in *rad16Δ RPB1*, *rad16Δ rpb1E1103G* and *rad16Δ rpb1G730D* yeast cells. The reason for using *rad16Δ* cells for the measurement is to eliminate GGR so that TCR can be unambiguously analyzed. Indeed, repair could not be seen in the region of the transcribed strand that is 40 nucleotides upstream of the transcription start site (where TCR does not operate) (Figure 3-4A-F), or in the nontranscribed strand of the *RPB2* gene (Figure 3-6A-C). TCR could be easily seen in *rad16Δ RPB1* cells (Figure 3-4A). As expected, additional deletion of *RAD26* decreased TCR (Figure 3-4D). Surprisingly, the *rpb1E1103G* mutation caused attenuation of TCR in both *rad16Δ* and *rad16Δ rad26Δ* cells (Figure 3-4, compare panels A and B, and D and E; Figure 3-5). In contrast, the *rpb1G730D* mutation enhanced TCR in both *rad16Δ* and *rad16Δ rad26Δ* cells (Figure 3-4, compare panels A and C, and D and F; Figure 3-5).

Our results indicate that enhanced transcription bypass of DNA lesions attenuates TCR regardless the presence of Rad26. On the other hand, impaired transcription bypass of DNA lesions enhances Rad26-dependent and independent TCR.

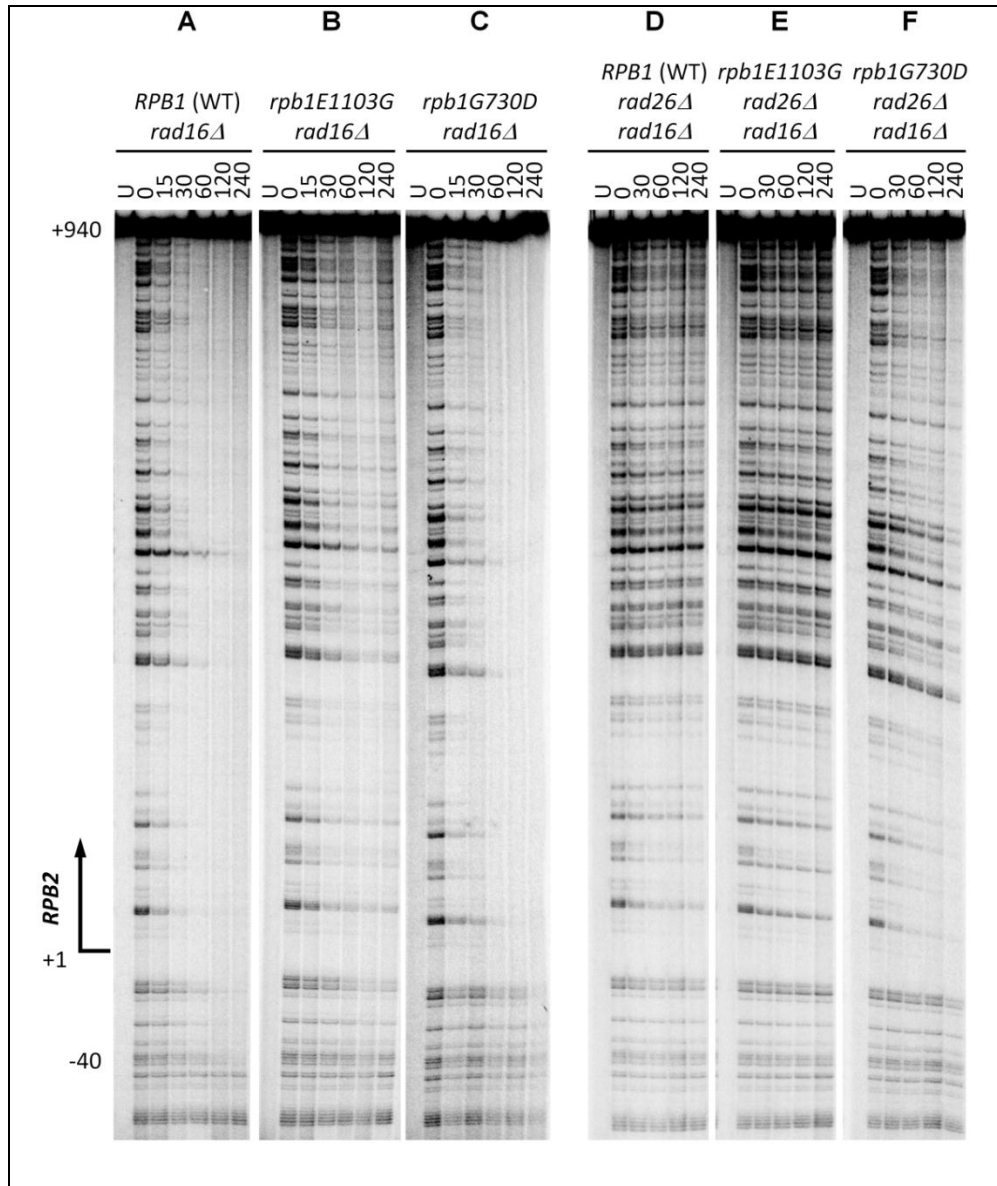


Figure 3-4. Effects of *rpb1E1103G* and *rpb1G730D* mutations on TCR. (A-C) Sequencing gel showing TCR of CPDs in *rad16Δ* cells expressing wild type Rpb1, or *rpb1E1103G* or *rpb1G730D* mutants. (D-F) Sequencing gel showing TCR of CPDs in *rad16Δ rad26Δ* cells expressing wild type Rpb1, or *rpb1E1103G* or *rpb1G730D* mutants. Unirradiated (U) and irradiated samples after different times (in minutes) of repair incubation are indicated at the top of the gel lanes. Nucleotide positions shown on the left are relative to the transcription start site.

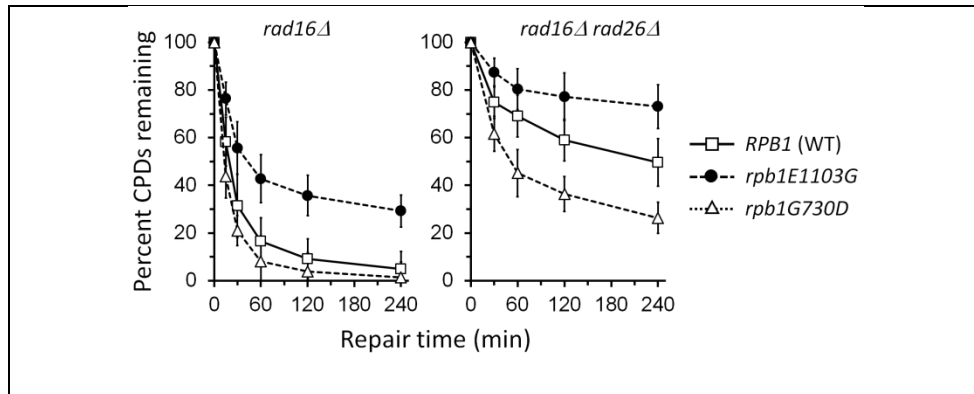


Figure 3-5. Percent of CPDs remaining in the transcribed strand of the *RPB2* gene in *rad16Δ* and *rad16Δ rad26Δ* cells expressing wild type (WT), or *rpb1E1103G* or *rpb1G730D* mutants. Data are represented as mean +/- S.D.

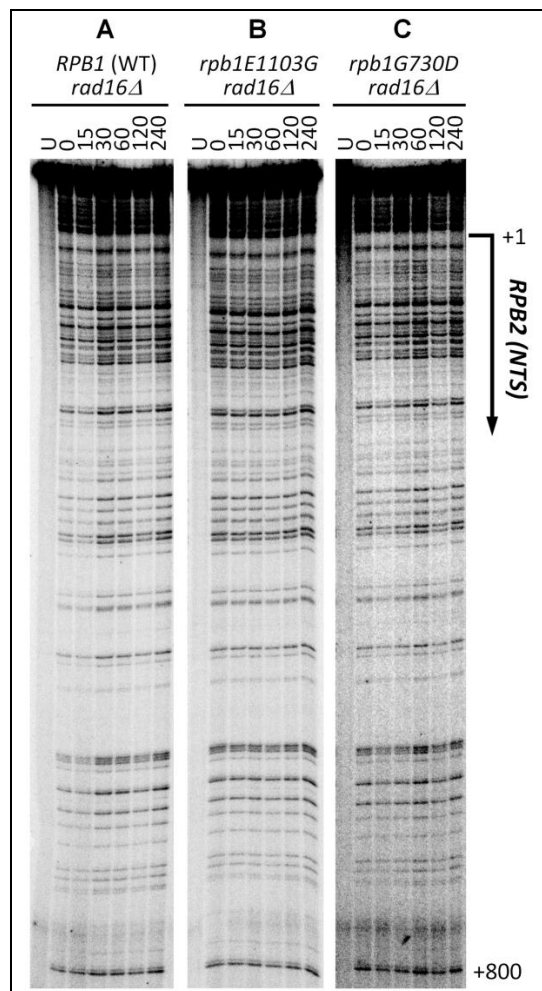


Figure 3-6. No repair of CPDs occurred in the nontranscribed strand of the *RPB2* gene in *rad16Δ* cells expressing wild type Rpb1, or *rpb1E1103G* or *rpb1G730D* mutants.

3.3.3 *rpb1E1103G* Mutation Increases Cell Resistance to UV Regardless the Presence of Any NER Subpathways, Whereas *rpb1G730D* Mutation Decreases UV Resistance only in the Absence of GGR.

The *rpb1E1103G* mutation was observed to increase UV resistance of *RAD26* cells but not that of *rad26Δ* cells, suggesting that the increased resistance is dependent on Rad26-dependent TCR (Walmacq, Cheung et al. 2012). Our direct analysis of TCR showed that the mutation actually attenuate TCR regardless of the presence of Rad26. To determine if the increased UV resistance is dependent on Rad26-dependent TCR or other subpathway of NER, we measured epistatic interactions of the *rpb1E1103G* and *rpb1G730D* mutations with *RAD26*, which plays an important role in TCR, and with *RAD16* and *RAD14*, which are essential for GGR and the entire NER, respectively. The *rpb1E1103G* mutation increases UV resistance of the otherwise wild type, *rad26Δ* (Figure 3-7A), *rad16Δ*, *rad16Δ rad26Δ* (Figure 3-7B and D) and *rad14Δ* (Figure 3-7C) cells. These results indicate that the increased UV resistance is independent of Rad26-dependent TCR, GGR or the entire NER. Rather, the increased UV resistance is likely due to enhanced lesion tolerance conferred by increased transcription bypass of the lesions. Our results do not agree with the previous report showing that the increased UV resistance of the *rpb1E1103G* mutants is dependent on Rad26 (Walmacq, Cheung et al. 2012). The reason for the discrepancy is unknown, but may be caused by strain background differences. All the strains used in this study were created in the Y452 background.

The *rpb1G730D* mutation decreases UV resistance of *rad16Δ* and *rad16Δ rad26Δ* cells (Figure 3-7B and D), but has no effect on UV resistance of wild type, *rad26Δ* (Figure 3-7A), and *rad14Δ* (Figure 3-7C) cells. These results suggest that, in the absence of Rad16 mediated GGR, abolition of transcription bypass of lesions decreases DNA lesion tolerance, although the abolition enhances TCR.

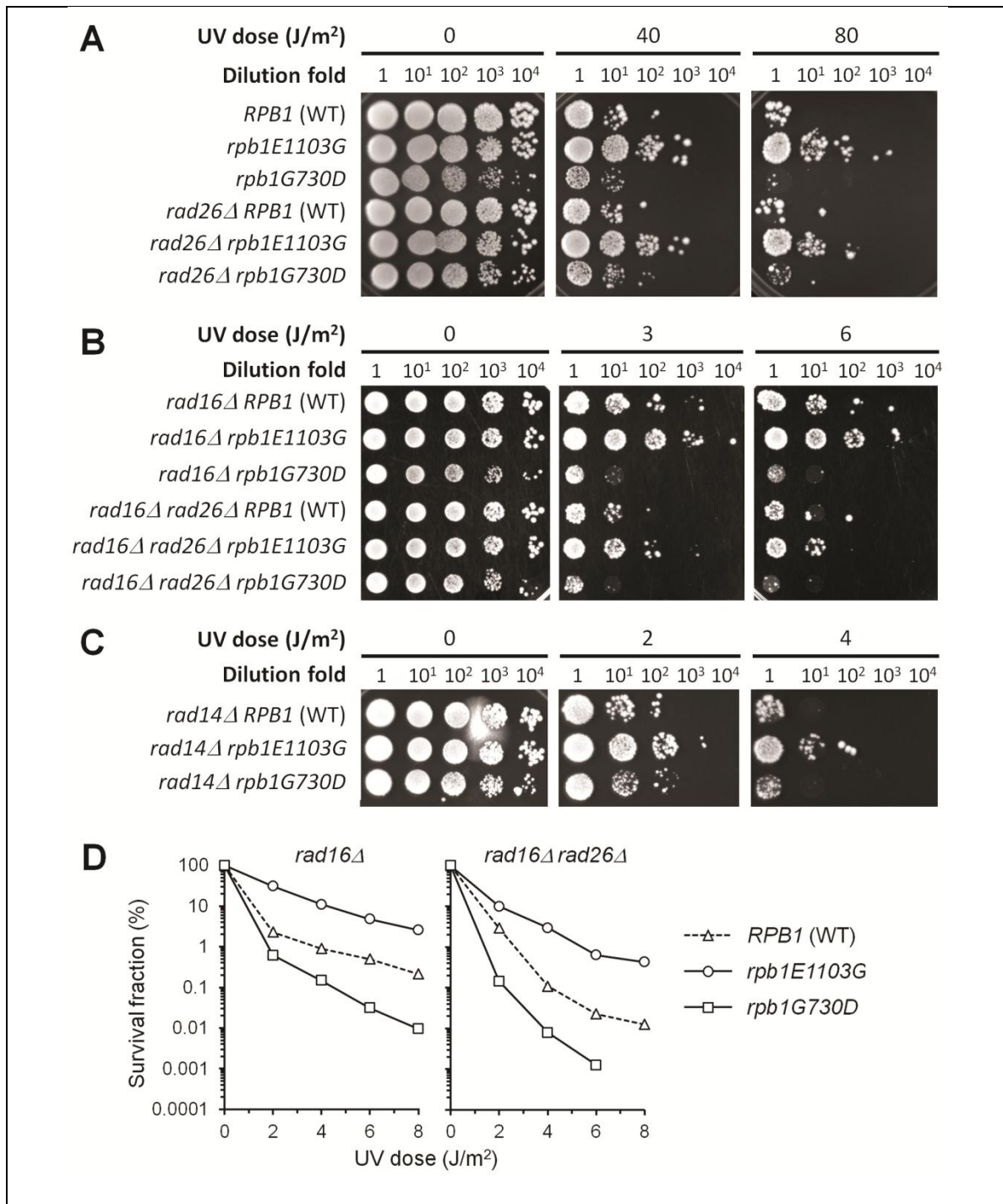


Figure 3-7. Epistatic interactions of *rpb1E1103G* and *rpb1G730D* mutations with different NER genes. (A-C) Spotting assay showing the effects of *rpb1E1103G* and *rpb1G730D* mutations on UV sensitivities of yeast cells with different NER subpathways operative. (D) Colony formation assay showing the effects of *rpb1E1103G* and *rpb1G730D* mutations on UV sensitivities of *rad16Δ* and *rad16Δ rad26Δ* cells. The values of the survival fractions are the means of 3 repeats. Standard error bars are within the symbols of the data points.

The decreased UV resistance of the *rpb1G730D* mutant may be caused not only by stalling at DNA lesions but also by lower overall transcription processivity, which may lead to aberrant transcription of multiple genes and, as a result, destabilizes cellular metabolism in general. We therefore examined the expression levels of the wild type Rpb1 and the *rpb1G730D* and *rpb1E1103G* mutants, and measured the densities of RNAP II in different regions of the *RPB2* gene. The *rpb1E1103G* and *rpb1G730D* mutants were expressed to similar levels that were ~ twice as high as the wild type Rpb1 (Figure 3-8A). The density of the *rpb1E1103G* mutant RNAP II in the 1 kb region of the *RPB2* gene was higher than that of the wild type RNAP II, indicating that this mutant RNAP II tends to pause at this region (Figure 3-8B and C). The density of the *rpb1G730D* mutant RNAP II was higher than that of the wild type RNAP II in the region around transcription start site of the *RPB2* gene. The higher expression levels of the Rpb1 mutants may enable more efficient loading of the mutant RNAP II to the promoter, leading to high densities towards the 5' end of the *RPB2* gene. The densities of *rpb1E1103G* and *rpb1G730D* mutant RNAP II decreased towards the 3' end of the *RPB2* gene (Figure 3-8C), indicating that both mutants have a deficiency in transcription processivity. Therefore, the decreased UV resistance of the *rpb1G730D* mutant may not be primarily caused by a lower overall transcription processivity, as the *rpb1E1103G* mutant has similar deficiency in transcription processivity but shows increased UV resistance.

3.4 Discussion

We showed here that increased transcription bypass of CPDs attenuates TCR but enhances cell survival, whereas abolition of the bypass enhances TCR but decreases cell survival in the absence of GGR. Our results do not support the proposition that the bypass, which may

expose the lesions, is required for TCR (Ellenberger 2012, Walmacq, Cheung et al. 2012). Rather, the bypass may actually weaken the recognition of lesions during TCR.

Transcription bypass of a DNA lesion could have serious repercussions on the cell, particularly if the lesion has miscoding properties, resulting in the insertion of incorrect nucleotides into the mRNA and generation of a mutant protein (Saxowsky and Doetsch 2006). However, the bypass may allow the completion of the ongoing mRNA synthesis to provide a steady supply of the housekeeping and repair proteins in the presence of the lesions, thereby enhancing cell survival. Therefore, the bypass may serve as a DNA damage tolerance mechanism that is alternative to TCR. The survival benefit of the damage tolerance appears to outweigh the survival deficiency of TCR attenuation caused by enhanced transcription bypass of DNA lesions.

Our results offer direct evidence for the long-standing but unproven notion that TCR requires sufficient stalling of an RNAP. Incorporation of one or two nucleotides opposite a CPD, especially misincorporation of a nucleotide opposite the 5' nucleotide of a CPD appears to be necessary to induce RNAP II stalling and potentially invoke TCR (Mei Kwei, Kuraoka et al. 2004, Brueckner and Cramer 2007, Walmacq, Cheung et al. 2012). We show here that the Rpb1 G730D mutation, which blocks RNAP II by preventing nucleotide incorporation opposite both the 3' and 5' nucleotides of a CPD, enhances TCR. This suggests that sufficient stalling of RNAP II, regardless of incorporation or misincorporation of nucleotides opposite a lesion, may be sufficient for eliciting TCR. However, transcription is regulated by a plethora of transcription elongation factors in the cell, if the Rpb1 G730D mutant RNAP II can catalyze the incorporation of nucleotides across CPDs *in vivo* has not been tested. Therefore, if the enhanced TCR caused by the Rpb1 G730D mutation requires the incorporation or misincorporation of nucleotide(s)

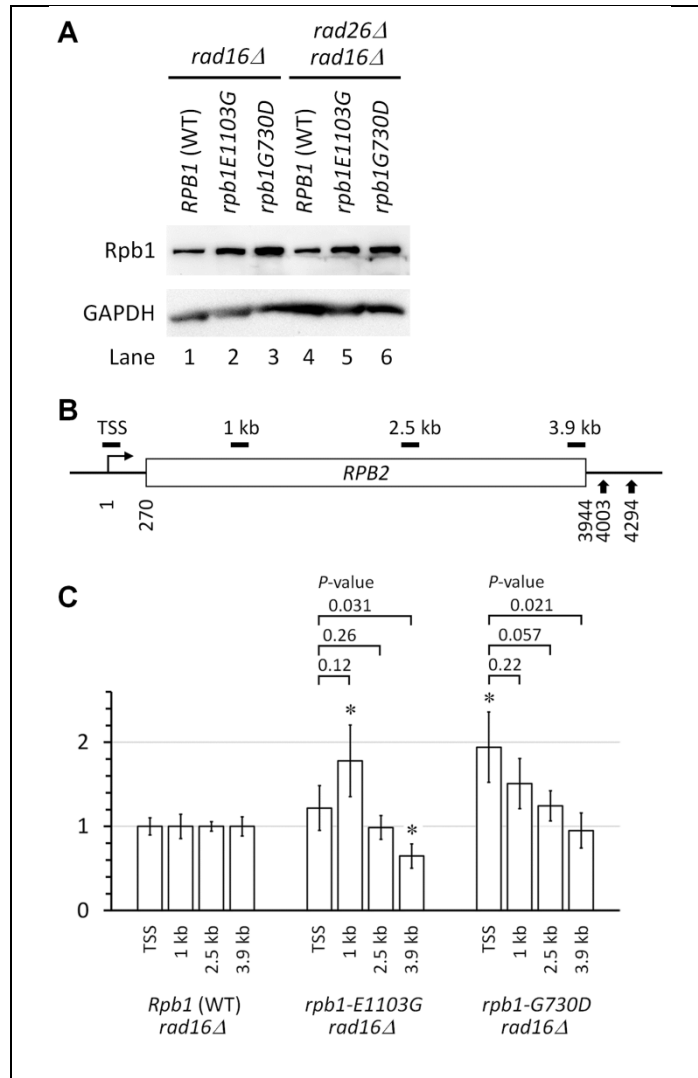


Figure 3-8. Both *rpb1E1103G* and *rpb1G730D* mutations cause a deficiency in transcription processivity. (A) Western blot showing expression levels of the wild type Rpb1 and *rpb1E1103G* and *rpb1G730D* mutants in *rad16Δ* and *rad16Δ rad26Δ* cells. (B) Schematic of the *RPB2* gene. Nucleotide positions are relative to the transcription start site (TSS). Vertical arrows at the 3' end of the gene indicate the two alternative polyadenylation sites (Yu and Volkert 2013). Short horizontal bars above the schematic indicate regions of 134-150 bp amplified by real time PCR for quantification of ChIP fragments of the *RPB2* gene (Li, Giles et al. 2014). (C) RNAP II densities in different regions of the *RPB2* gene. The RNAP II densities in the TSS, 1 kb, 2.5 kb and 3.9 kb regions of the *RPB2* gene in *RPB1* (wild type) cells were normalized to 1. The densities of *rpb1E1103G* and *rpb1G730D* mutant RNAP II in the different regions of the *RPB2* gene are relative to those in the corresponding regions of the *RPB2* gene in *RPB1* cells. The values of RNAP II densities are represented as mean (+/- S.D.) of 3 ChIP experiments. Single asterisks (*) denote a *P*-value of < 0.05, in the Student's *t*-test between the mutant and WT cells for RNAP II densities in the corresponding regions of the *RPB2* gene. Above the bars of *rpb1E1103G* and *rpb1G730D* samples are shown the *P*-values of Student's *t*-test between the TSS region and the 1, 2.5 or 3.9 kb region (a *P*-value of < 0.05 is considered to be significant).

across CPDs in living yeast cells remains to be determined. Besides enhancing TCR, stalling of RNAP II blocks ongoing transcription and may also impair replication, resulting in reduced resistance to DNA damage especially in the absence of GGR. In this case, the survival deficiency caused by impaired transcription bypass of DNA lesions outweighs the survival benefit of enhanced TCR.

Previous studies have ruled out the roles of ubiquitination and degradation of Rpb1 (Lommel, Bucheli et al. 2000, Woudstra, Gilbert et al. 2002, Chen, Ruggiero et al. 2007) and back-tracking of RNAP II by TFIIS (Verhage, Heyn et al. 1997) in TCR. A number of transcription elongation factors, such as Spt4 (Jansen, den Dulk et al. 2000), Spt5 (Ding, LeJeune et al. 2010, Li, Giles et al. 2014) and PAFc (Tatum, Li et al. 2011), which stabilize and promote forward-tracking of the RNAP II elongation complex, have been shown to repress TCR. Here we show that transcription bypass of lesions attenuates TCR, although the bypass may expose the lesions to DNA repair machinery. Taken together, these studies appear to support the model that the RNAP II complex stalled at a lesion may be remodeled, rather than removed from the stalled site, to initiate TCR in eukaryotic cells. How RNAP II complex is remodeled during TCR remains to be elucidated.

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CHAPTER 4

SEN1 N-TERMINAL DOMAIN CONTRIBUTES TO RAD26- INDEPENDENT TRANSCRIPTION COUPLED DNA REPAIR

4.1 Introduction

Gene transcription cycle is generally divided into three stages: initiation, elongation and termination. In comparison with initiation and elongation, the mechanism and regulation of termination are much less known (Kuehner, Pearson et al. 2011). In eukaryotic cells, the RNA polymerase II (RNAP II) transcribes all protein-coding genes into mRNA and some non-coding genes into non-coding RNAs (ncRNAs), such as small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and cryptic unstable transcripts (CUTs). For the termination of RNAP II transcription, there are two major pathways: the poly(A)-dependent termination and Sen1-dependent termination (Kim, Vasiljeva et al. 2006). The poly(A)-dependent pathway is mainly responsible for the termination of polyadenylated mRNA. In contrast, the Sen1-dependent pathway acts at ncRNAs. Besides the termination of ncRNAs, the Sen1-dependent pathway has been found to function in premature termination of some mRNA transcripts (Arigo, Carroll et al. 2006, Kopcewicz, O'Rourke et al. 2007, Thiebaut, Colin et al. 2008).

In *Saccharomyces cerevisiae*, Sen1 is an essential and conserved 5' to 3' ATP-dependent RNA/DNA helicase whose molecular weight is 252 kDa (Rasmussen and Culbertson 1998, Kim, Choe et al. 1999, Chen, Hashemi et al. 2006). Mutations in human senataxin, the ortholog of *S. cerevisiae* Sen1, can cause neurological disorders (Moreira, Klur et al. 2004). Sen1 physically interacts with Nrd1 (nuclear pre-mRNA down-regulation 1) and Nab3 (nuclear polyadenylated RNA-binding 3), two RNA binding proteins, to form an Nrd1 complex which mediates the RNAP II termination (Wilson, Datar et al. 1994, Steinmetz and Brow 1996, Steinmetz, Conrad et al. 2001). The N-terminal domain of Sen1 is involved in multiple protein/protein and

protein/RNA interactions, but dispensable for viability (Ursic, Chinchilla et al. 2004). The Sen1 helicase domain has been suggested to prevent the RNA:DNA hybrids formation during transcription and dissociate the elongation complex during termination by utilizing its ATPase activity (Mischo, Gomez-Gonzalez et al. 2011, Porrua and Libri 2013). Transcriptome-wide binding sites assay has shown that Sen1 not only binds to ncRNAs but also distributes all along mRNA transcripts, suggesting potential roles of Sen1 in the whole transcription cycle (Creamer, Darby et al. 2011). Recently, Sen1 has been suggested to contribute to genomic stability by regulating the expression of *RNR1* (Ribonucleotide Reductase 1) which is one subunit of the ribonucleotide reductase whose activity will be increased after DNA damage (Golla, Singh et al. 2013).

Nucleotide excision repair (NER), one highly conserved DNA repair pathway, preserves the genome integrity by removing bulky and/or helix-distorting lesions such as UV induced cyclobutane pyrimidine dimers (CPDs) (Hanawalt and Spivak 2008). Global genomic repair (GGR) is an NER subpathway that removes lesions throughout the whole genome including the nontranscribed strand (NTS) of actively transcribed genes (Verhage, Zeeman et al. 1994). Transcription coupled repair (TCR) is the other NER subpathway refers to rapid removal of DNA lesions from the transcribed strand (TS) of an actively transcribed gene (Bohr, Smith et al. 1985, Mellon, Spivak et al. 1987). The two NER subpathways share most of the NER factors during the “cut-and-patch” process, but differ in the initial damage recognition step. TCR is assumed to be triggered by the stalling of RNAP II at a DNA lesion in the TS of a gene which is being transcribed. The DNA lesion will be trapped in the elongation complex and inaccessible to the NER machinery once the elongation complex stalls at a lesion site. In *S. cerevisiae*, Rad26, which is the homolog of human CSB (Cockayne syndrome group B) protein, plays an important

role in facilitation of TCR (van Gool, Verhage et al. 1994). Rpb9, the ninth and non-essential subunit of RNAP II, also contributes to TCR by mediating Rad26-independent TCR subpathway (Li and Smerdon 2002). However, Spt4 (Jansen, den Dulk et al. 2000), Rpb4 (Li and Smerdon 2002), Spt5 CTR (C-terminal repeat) domain (Ding, LeJeune et al. 2010) and PAFc (RNAP II associated factor 1 complex) (Tatum, Li et al. 2011), which are generally involved in transcription elongation, have been shown to repress TCR in the absence of Rad26. Recently, structural and biochemical studies on the interactions between RNAP II and Spt5, which is a highly conserved transcription elongation factor, have suggested that Spt5 facilitates transcription elongation and represses TCR by holding the RNAP II in a closed conformation (Li, Giles et al. 2014). Taking all these into consideration, TCR appears to be the interplay between “facilitators” and “repressors”.

Despite our understanding of Sen1’s function in ncRNAs processing and termination, little is known about whether and/or how Sen1 plays a role in TCR. In the present study, we provide evidence that the N-terminal domain of Sen1 contributes to the Rad26-independent TCR.

4.2 Materials and Methods

4.2.1 Yeast Strains and Plasmids

All yeast strains used in this study are derivatives of BJ5465 (*MATa ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1*) (Jones 1991). The deletion or tagging of a genomic gene was achieved by using procedures as previously described (Li and Smerdon 2002, Li, Giles et al. 2014).

Plasmid pRS416-SEN1Δ1-975 was constructed by inserting the 3 × Flag-tagged and N-terminal truncated (residues 1-975) SEN1 gene including the promoter, the truncated coding

sequence followed by 3 × Flag sequences, and 3' terminator sequences into the multiple cloning site of the *URA3* plasmid pRS416. Plasmids pRS415-SEN1 and pRS415-SEN1 Δ 1-975, encoding 3 × Flag-tagged full length Sen1 and N-terminal truncated Sen1 with 3 × Flag, were created by inserting the SEN1 gene with 3 × Flag sequence and the N-terminal truncated SEN1 gene with 3 × Flag sequence into the multiple cloning site of the *LEU2* plasmid pRS415.

4.2.2 Tests of Temperature and UVC Sensitivity

Yeast cells were grown at 30°C in SD (synthetic defined) medium to saturation, and 10-fold serial dilutions were made. The diluted yeast cells were spotted onto YPD (1% yeast extract, 2% peptone and 2% dextrose) plates and air dried. For temperature sensitivity test, the plates were incubated at 30°C and 37°C. For UVC sensitivity test, the plates were irradiated with different doses of UVC (250 nm) and incubated at 30°C in the dark. After 3-5 days incubation, all the plates were photographed.

4.2.3 Repair Analysis of UVC Induced CPDs

Yeast cells were grown at 30°C in SD medium to late log phase ($A_{600} \approx 1.0$), washed twice with ice-cold ddH₂O, resuspended in 2% dextrose and irradiated with 120 J/m² of UVC. The CPDs repair incubation and total genomic DNA isolation were achieved by using previously described procedures (Li and Smerdon 2002).

The nucleotide level analysis of CPDs was conducted following a procedure described previously (Li and Waters 1996, Li, Waters et al. 2000). Briefly, the *RPB2* gene fragment was released from the genomic DNA by restriction digestion and CPD sites were cleaved by T4 endonuclease V. The *RPB2* fragments were pulled out by using biotinylated oligonucleotide and streptavidin magnetic beads. The TS fragments of *RPB2* were 3' end labeled with [α -³²P]dATP

and resolved on sequencing gel. The gel was dried and exposed to a PhosphorImager screen. The intensities of gel bands corresponding to CPD sites were quantified using Quantity One software (Bio-Rad).

4.3 Results

4.3.1 UV Sensitivity of *sen1* Δ 1-975 Mutants

The N-terminal 1-975 residues in *S. cerevisiae* Sen1 protein (2231 residues in total) has been shown to be nonessential and involved in multiple protein-protein interactions, whereas the C-terminal region (residues 976-2231) is essential and containing ATP-helicase and nuclear localization domains (Figure 4-1A) (Ursic, Chinchilla et al. 2004, Chinchilla, Rodriguez-Molina et al. 2012). Despite of its known role in Sen1-dependent termination (Hazelbaker, Marquardt et al. 2013, Porrua and Libri 2013), it remains enigmatic that whether and/or how Sen1 functions in NER.

To determine whether *sen1* is involved in NER or any subpathway of NER, we investigated epistatic interactions of the *sen1* Δ 1-975 mutation with different NER genes, which including *RAD26*, *RPB9*, *SPT4*, *RAD7* and *RAD2*. The *RAD26*, *RPB9* and *SPT4* genes are involved in TCR, whereas the *RAD7* and *RAD2* are specifically required for GGR and entire NER respectively. In comparison with strains containing wild type Sen1, the *sen1* Δ 1-975 mutation has no effect on UV sensitivity in wild type, *rad26* Δ (Figure 4-1B), *rad2* Δ (Figure 4-1C), *rad7* Δ *rpb9* Δ (Figure 4-1D), *rad7* Δ (Figure 4-1E), *rad7* Δ *rad26* Δ *spt4* Δ and *rad7* Δ *spt4* Δ (Figure 4-1F) yeast cells. Surprisingly, the *sen1* Δ 1-975 mutation increases UV sensitivity dramatically in *rad7* Δ *rad26* Δ yeast cells (Figure 4-1E). The results suggest that *sen1* Δ 1-975 mutation may not be involved in GGR or Rad26-dependent TCR, but it may play a role in Rad26-independent TCR.

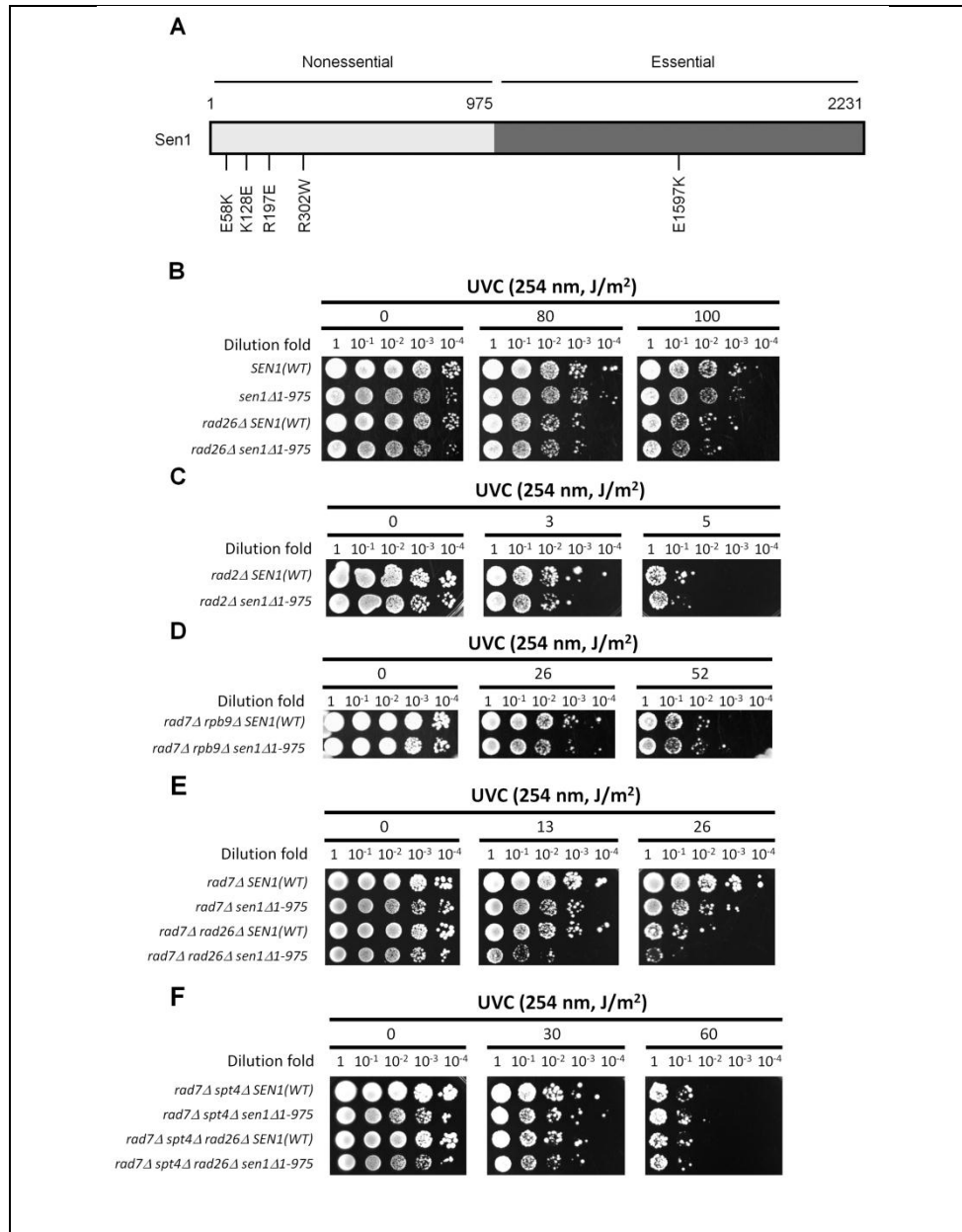


Figure 4-1. Epistatic interactions of *sen1Δ1-975* mutation with different NER genes. (A) Schematic showing nonessential N-terminal (residues 1-975) and essential C-terminal regions (residues 976-2231) of yeast Sen1 including the position of the *sen1 E1597K* point mutation. (B-F) Effects of *sen1Δ1-975* mutation on UV sensitivities of yeast cells with different NER subpathways operative.

4.3.2 *sen1Δ1-975* Mutation Attenuates TCR in the Presence or Absence of Rad26

To determine if Sen1 N-terminal domain plays a role in TCR, we measured repair of UV induced CPDs in the constitutively transcribed *RPB2* gene in *rad7ΔSEN1*, *rad7Δsen1Δ1-975*,

rad7Δrad26ΔSEN1 and *rad7Δrad26Δsen1Δ1-975* yeast cells. In yeast, Rad7 and Rad16 are essential for GGR, but dispensable for TCR. Therefore, TCR can be unambiguously analyzed if GGR is eliminated by deletion of either *RAD7* or *RAD16* gene. A nucleotide resolution method, which utilizes streptavidin magnetic beads and biotinylated oligonucleotides to facilitate isolation and strand-specific end-labeling of specific DNA fragments, was used in this CPDs repair measurement (Li and Waters 1996, Li, Waters et al. 2000). As can be seen in Figure 4-2A, TCR initiates about 40 nucleotides upstream of the transcription start site of the *RPB2* gene in *rad7ΔSEN1* cells. In agreement with previous studies, the additional deletion of *RAD26* (*rad7Δrad26Δ*) decreases TCR in the coding region of the gene (Figure 4-2C, compare panels A and C), except for a short region (about 50 nucleotides) immediately downstream of the transcription start site. Interestingly, the *sen1Δ1-975* mutation dramatically attenuates TCR in both *rad7Δ* and *rad7Δrad26Δ* cells (Figure 4-2, compare panels A and B, and D and C; Figure 4-3).

These results indicate that *sen1Δ1-975* mutation attenuates TCR in the presence or absence of Rad26. Taken together, the Sen1 is highly likely involved in Rad26-independent TCR, which is mediated by Rpb9.

4.3.3 SPT4 Deletion only Partially Eliminates the TCR Attenuation Caused by *sen1Δ1-975* Mutation

Spt4, a small zinc-binding protein, forms a Spt4/5 complex through binding to Spt5 NGN domain and stabilizes the interaction between Spt5 and RNAP II (Guo, Xu et al. 2008, Ding, LeJeune et al. 2010). Recently, Spt5 has been suggested to promote transcription elongation and

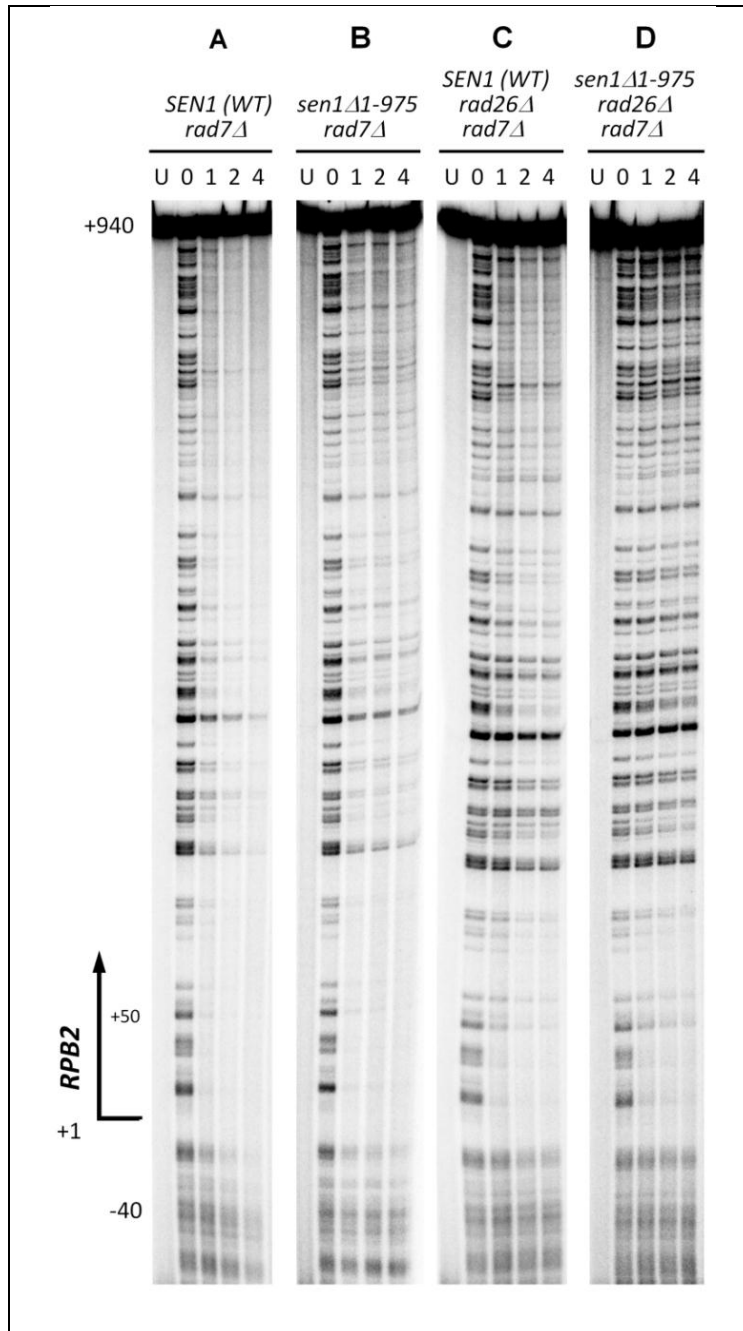


Figure 4-2. N-terminal domain (residues 1-975) truncation of yeast Sen1 attenuates TCR. (A-B) Sequencing gel showing TCR of CPDs in *rad7Δ* cells expressing wild type (WT) and N-terminal domain truncated (*sen1Δ1-975*) Sen1. (C-D) Sequencing gel showing TCR of CPDs in *rad7Δrad26Δ* cells expressing wild type and N-terminal domain truncated Sen1. (E) Sequencing gel showing TCR of CPDs in *rad7Δrad26Δ* cells expressing E1597K mutant Sen1. Unirradiated (U) and irradiated samples after different times (in hours) of repair incubation are indicated at the top of the gel lanes. Nucleotide positions shown on the left are relative to the transcription start site.

repress TCR by holding the elongating RNAP II complex in a closed conformation in yeast cells (Li, Giles et al. 2014). Rad26 appears to antagonize the TCR repression caused by some repressors, such as Spt4 (Jansen, den Dulk et al. 2000), certain domains of Spt5 (Ding, LeJeune et al. 2010, Li, Giles et al. 2014), Rpb4 (Li and Smerdon 2002) and PAFc (Tatum, Li et al. 2011), because deletion of any one of the repressors will derepress TCR to some extent in the absence of Rad26.

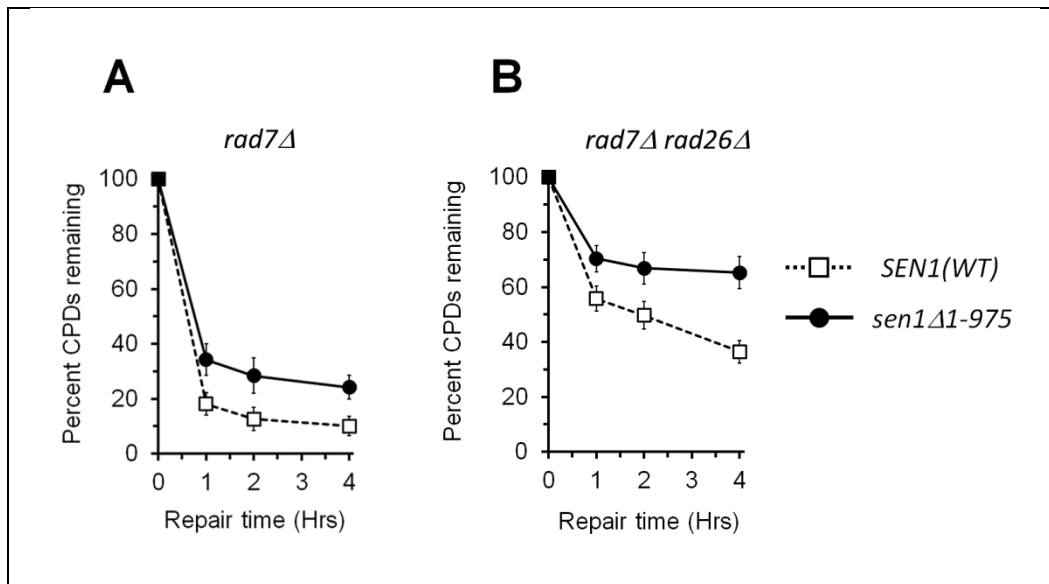


Figure 4-3. Percent of CPDs remaining in the transcribed strand of the *RPB2* gene in *rad7Δ* and *rad7Δrad26Δ* cells expressing wild type (WT) and N-terminal domain truncated (*sen1Δ1-975*) Sen1. Data are represented as mean +/- S.D.

To investigate whether deletion of *SPT4* will eliminate the TCR attenuation induced by *sen1Δ1-975* mutation, we directly measured repair of CPDs in the *RPB2* gene in *rad7Δspt4ΔSEN1*, *rad7Δspt4Δsen1Δ1-975*, *rad7Δrad26Δspt4ΔSEN1* and *rad7Δrad26Δspt4Δsen1Δ1-975* yeast cells. As expected, the additional deletion of *SPT4* restores TCR in *rad7Δ* and *rad7Δrad26Δ* cells (Figure 4-2 and 4-4, compare panels A in Figure 4-2 and C in Figure 4-4, and panels C in Figure 4-2 and A in Figure 4-4; Figure 4-5). In contrast, TCR is

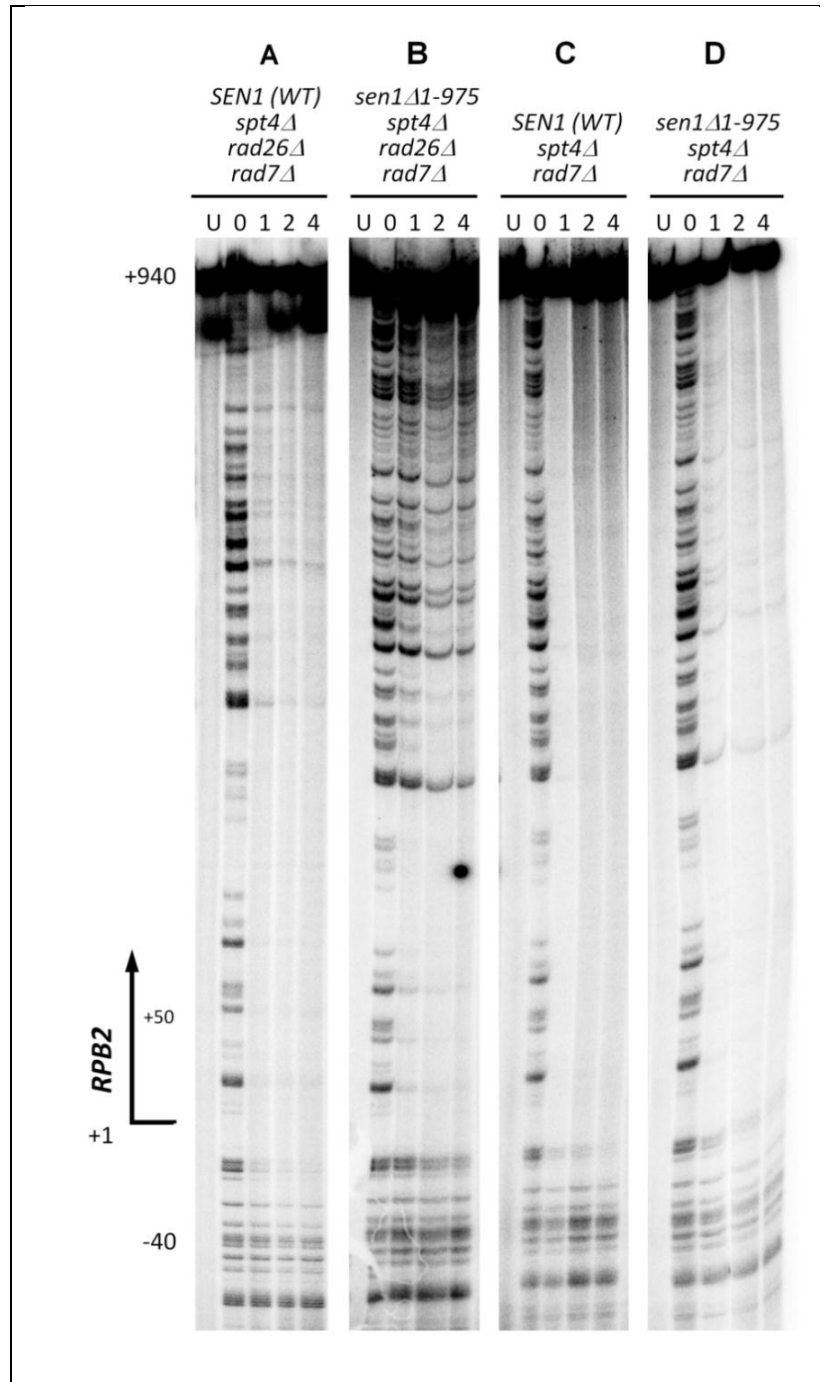


Figure 4-4. Deletion of *SPT4* partially eliminates the TCR attenuation caused by *sen1Δ1-975* mutation. (A-B) Sequencing gel showing TCR of CPDs in *rad7Δrad26Δspt4Δ* cells expressing wild type (WT) and N-terminal domain truncated (*sen1Δ1-975*) Sen1. (C-D) Sequencing gel showing TCR of CPDs in *rad7Δspt4Δ* cells expressing wild type and N-terminal domain truncated Sen1. Unirradiated (U) and irradiated samples after different times (in hours) of repair incubation are indicated at the top of the gel lanes. Nucleotide positions shown on the left are relative to the transcription start site.

not completely restored by *SPT4* deletion in either *rad7Δsen1Δ1-975* or *rad7Δrad26Δsen1Δ1-975* yeast cells (Figure 4-2 and 4-4, compare panels C in Figure 4-2 and D in Figure 4-4, and panels D in Figure 4-2 and B in Figure 4-4; Figure 4-5). Our results indicate that *SPT4* deletion only partially eliminates the TCR attenuation caused by *sen1Δ1-975* mutation, which further suggests that Sen1 N-terminal domain is involved in Rad26-independent TCR.

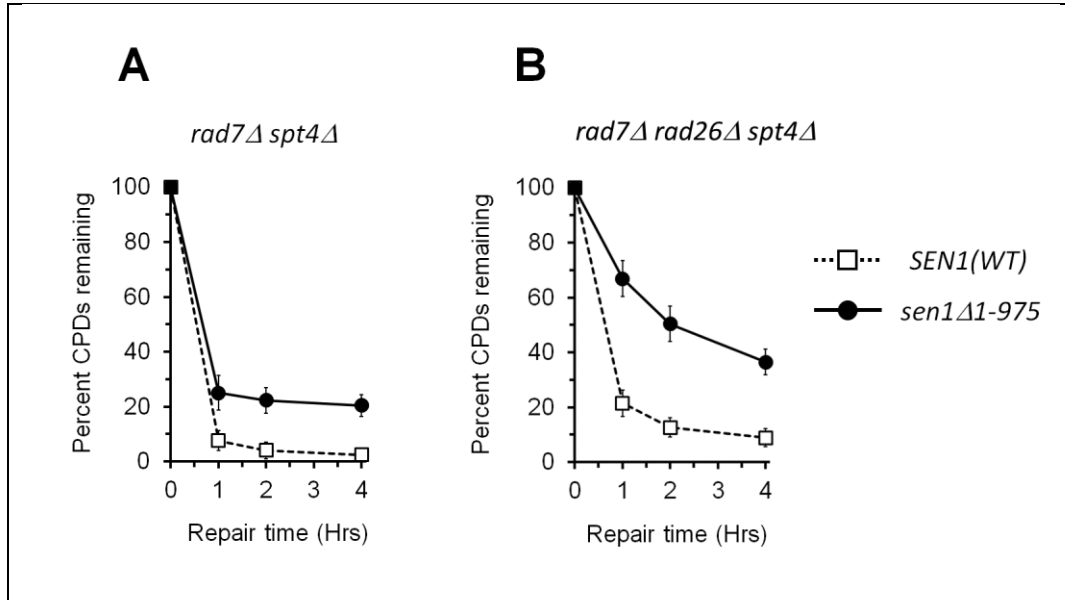


Figure 4-5. Percent of CPDs remaining in the transcribed strand of the *RPB2* gene in *rad7Δrad26Δspt4Δ* and *rad7Δspt4Δ* cells expressing wild type (WT) and N-terminal domain truncated (*sen1Δ1-975*) Sen1. Data are represented as mean \pm S.D.

4.4 Discussion

We showed here that the Sen1 N-terminal domain contribute to the Rad26-independent TCR subpathway, which is mediated by Rpb9. As discussed below, our findings have identified that Sen1 acts as a TCR “facilitator”, expanding our knowledge of Rad26-independent TCR.

Rad26-dependent and Rad26-independent TCR, two subpathways of TCR, are mediated by Rad26 and Rpb9 respectively (Li and Smerdon 2002). The Rad26 mediated TCR has equal efficiency in the coding region and the region upstream the transcription start site, whereas Rpb9 mediated TCR operates more effectively in the coding region than in the region upstream the

transcription start site (Li and Smerdon 2002, Li and Smerdon 2004). Moreover, the Rad26 mediated TCR can be either transcription coupled or transcription-independent, but Rpb9 mediated TCR is strictly transcription coupled and is much effective in highly expressed genes, such as the *GALI-10* genes (Li, Chen et al. 2006). To understand these differences between the two TCR subpathways, it is crucial to know which NER machinery proteins are involved in two distinctive TCR subpathways. Multiple proteins that facilitate transcription elongation, such as Rpb4 (Li and Smerdon 2002), Spt4(Jansen, den Dulk et al. 2000), Spt5 C-terminal repeat (CTR) domain (Ding, LeJeune et al. 2010) and PAFc (Tatum, Li et al. 2011), have been shown to only repress Rad26-independent TCR and become dispensable in the presence of Rad26. In contrast, the Sen1 protein, a transcription elongation terminator, contributes to Rad26-independent TCR serving as a “facilitator”. Thus, it appears that those proteins that benefit transcription elongation repress Rad26-independent TCR, whereas protein that impairs transcription elongation, such as Sen1, facilitates Rad26-independent TCR. Furthermore, deletion of Spt5 KOW4-5 domains, which can presumably disrupt the interaction between Spt5 and Rpb4/7, enhances TCR in the absence or presence of Rad26 suggesting that Spt5 is a key TCR repressor (Li, Giles et al. 2014). Taken together, it is highly likely Rad26 functions in TCR by antagonizing those “repressors”, and Sen1 does not antagonize those “repressors” as efficiently as Rad26 does. However, Sen1 becomes more efficient once those “repressors” are eliminated. Rad26 operates equally in the coding region and the region upstream the transcription start site, whereas Sen1 acts efficiently only in the coding region.

At present, it is largely unknown about the mechanism of the Rad26-independent TCR, mediated by Rpb9. Rpb9 links Rpb1 and Rpb2 at the very tip of the RNAP II jaw, and has three distinct domains: N-terminal Zn1, central linker and C-terminal Zn2 domains. The Zn1 and

linker domains are essential for the transcription elongation and TCR, whereas the Zn2 domain is indispensable for ubiquitylation and degradation of Rpb1 in response to UV-induced DNA damage (Li, Ding et al. 2006, Chen, Ruggiero et al. 2007). Bacterial Spt5 homologue, NusG, facilitates transcription termination through the physical interaction between its C-terminal KOW domain and Rho (Chalissery, Muteeb et al. 2011). Eukaryotic Spt5 has been found to exist in Nrd1 complex, suggesting the possible interaction of Spt5 with the termination complex (Vasiljeva and Buratowski 2006). Taking into consideration of the NusG/Spt5 family proteins' high conservation, Sen1 may directly bind to Spt5 and/or Rpb9 to function in termination and TCR. In Rad26-independent TCR, Sen1 may promote TCR by interacting with the stalled RNAP II and ultimately remodeling it to make the lesion accessible to NER machinery.

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CHAPTER 5 CONCLUDING REMARKS

5.1 Research Summary

By using budding yeast *Saccharomyces cerevisiae* as a model organism, this dissertation has focused on the structural and functional interactions between Spt5 and RNA polymerase II (RNAP II), role of translesion transcription in transcription coupled DNA repair (TCR), and function of Sen1 in TCR. The major findings are as follows:

1. Spt5 interacts with Rpb1 and Rpb2 at the clamp, protrusion and wall domains of RNAP II. Mainly through its KOW4-5 domains, Spt5 also extensively interacts with Rpb4 and Rpb7, two subunits of RNAP II that form a dissociable subcomplex. Additionally, the interaction sites of Spt5 on RNAP II partially overlap with those of transcription initiation factor TFIIE. Deletion of Spt5 KOW4 or KOW4-5 domains decreases transcription elongation and relieves repression of TCR.
2. The E1103G mutation of Rpb1, the largest subunit of RNAP II, which promotes transcription bypass of UV-induced cyclobutane pyrimidine dimers (CPDs), increases survival of UV irradiated yeast cells but attenuates TCR. The increased cell survival is independent of any NER subpathways. In contrast, G730D mutation of Rpb1, which abolishes transcription bypass of CPDs, enhances TCR.
3. The N-terminal domain (1-975 residues) of Sen1 contributes to the Rad26-independent TCR.

5.2 Concluding Remarks and Future Direction

The TCR mechanism in bacteria has been elucidated in molecular details (Selby and Sancar 1993, Park, Marr et al. 2002, Deaconescu, Chambers et al. 2006, Howan, Smith et al. 2012, Epshtein, Kamarthapu et al. 2014). However, TCR in eukaryotic cells appears to be extremely complicated. The exact nature of the TCR signal and the mechanism of the transcription-repair coupling have been long-standing enigmas. A number of TCR facilitators and repressors in yeast cells have been identified so far. Despite of the progress on TCR mechanism in recent years, how the TCR facilitators and repressors interplay with RNAP II and with NER factors to elicit the TCR signal and carry out TCR remain to be elucidated.

How TCR machinery gains access to a lesion initially trapped by a stalled RNAP II in eukaryotic cells is still unknown (Hanawalt and Spivak 2008). Multiple scenarios have been suggested, including 1) ubiquitylation and degradation of Rpb1, the largest subunit of RNAP II, 2) displacement of the transcription elongation complex through forced back- or forward-tracking, and 3) remodeling of the complex without removal from the arrest site (Hanawalt 2008). Up to date, however, none of these scenarios has been proven to be required for TCR in eukaryotic cells. However, we recently found that an RNAP II mutation that facilitates transcription bypass of DNA lesions attenuates TCR, whereas an RNAP II mutation that impairs the bypass enhances TCR. This finding suggests that efficient stalling of RNAP II at lesions, rather than transcription bypass of lesions, is essential for efficient TCR.

Future research should focus on the elucidation of the exact nature of the TCR signal and how the signal is generated, which may revolutionize the current view regarding how TCR is triggered. Moreover, how Rad26, TCR repressors and RNAP II interplay to carry out TCR and

how Sen1, an RNA/DNA helicase that is homologous to the human senataxin, functions in TCR remain to be elucidated in future studies.

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Wentao Li, a native of Shandong, China, received his Bachelor of Veterinary Medicine from Shandong Agricultural University in Tai'an, China, in July 2003. Thereafter, he attended Shanxi Agricultural University in Taigu, China, in September 2003, and received his Master of Veterinary Medicine in 2006. Wentao joined Heilongjiang Bayi Agricultural University, Daqing, China, in the fall of 2006. He then was accepted into the doctoral program at Louisiana State University School of Veterinary Medicine in Baton Rouge, Louisiana, in the spring of 2009. Wentao is currently completing his doctoral degree in Dr. Shisheng Li's DNA repair laboratory.