THE ROLE OF PC4 IN OXIDATIVE STRESS

A Dissertation Presented

By

LIJIAN YU

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ABSTRACT

Oxidative stress is a cellular condition where cells are challenged by elevated levels of reactive oxygen species (ROS) that are produced endogenously or exogenously. ROS can damage vital cellular components, including lipid, protein, DNA and RNA. Oxidative damage to DNA often leads to cell death or mutagenesis, the underlying cause of various human disease states. Previously our laboratory discovered that human PC4 gene can prevent oxidative mutagenesis in the bacterium *Escherichia coli* and that the yeast homolog *SUB1* has a conserved function in oxidation protection. In this thesis I examined the underlying mechanisms of PC4's oxidation protection function. My initial efforts to examine the predicted role of *SUB1* in transcription-coupled DNA repair essentially negated this hypothesis. Instead, results from our experiments suggest that PC4 and yeast *SUB1* can directly protect genomic DNA from oxidative damage. While testing SUB1's role in double strand DNA break (DSB) repair, I found the *sub1Δ* mutant resects DSB ends rapidly but still ligates chromosomal breaks effectively, suggesting that DSB resection is not inhibitory to nonhomologous end-joining, an important DSB repair pathway. Finally, in the course of studying transcription recovery after UV damage, I found UV induces a longer form of *RPB2* mRNA and demonstrated that this is caused by alternative polyadenylation of the *RPB2* mRNA and that alternative polyadenylation contributes to UV resistance. Based on results of preliminary experiments, I propose that UV activates an alternative RNA polymerase to transcribe RNA POL II mRNA, a novel

mechanism to facilitate recovery from inhibition of transcription resulting from UV damage. The hypothetical polymerase switch may account for the UV-induced alternative polyadenylation of the *RPB2* mRNA.

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LIST OF ABBREVIATIONS

3’-UTR	3’-untranslated region
BIR	break induced replication
bp	base pairs
cDNA	complementary DNA
CPD	cyclobutane pyrimidine dimer
CS	Cockayne syndrome
CTD	C-terminal domain
DCFH-DA	2’,7’-dichlorfluorescein-diacetate
DSB	double strand DNA break
dsDNA	double strand DNA
GGR	global genome repair
HR	homologous recombination
MCO assay	metal-ion catalyzed oxidation assay
MMEJ	micro-homology mediated end joining
mRNA	messenger RNA

NADPH	nicotinamide adenine dinucleotide phosphate
NER	nucleotide excision repair
NHEJ	nonhomologous end-joining
NTS	non-transcribed strand
PFGE	pulse field gel electrophoresis
RACE	rapid amplification of cDNA ends
ROS	reactive oxygen species
SOD	superoxide dismutase
SSA	single strand annealing
SSB	single strand DNA break
ssDNA	single strand DNA
TCR	transcription-coupled repair
TEV	T4 endonuclease V
TS	transcribed strand
UV	ultraviolet
XP	Xeroderma Pigmentosum

CHAPTER I

Introduction

Chapter I - Introduction

We, as human beings, get half of our genetic information from our mother and the other half from our father, as the most precious gift. This genetic information is stored in the linear sequence of DNA and is used to determine who we are. Unfortunately, DNA is subject to numerous hostile attacks every moment of the day from the very beginning of our life (Ciccia & Elledge, 2010). The sources of the attacks are chemicals, radiation, sunlight, and various intermediates of oxygen metabolism. The damage either misrepresents or voids the genetic information. Fortunately, the majority of the DNA damage is reversed to its intact state by intricate DNA repair systems that exist in each cell in our body (Wood et al, 2005). The importance of the DNA repair pathways is manifested by the plethora of human diseases associated with mutations in DNA repair genes, including hereditary genetic disorders, neurodegenerative diseases, cancers, and the aging process (Lombard et al, 2005; Lehmann, 2003; David et al, 2007).

In this thesis, I continued and extended a project initiated by Wang *et al.* (Wang et al, 2004) to investigate the role of human PC4 and its yeast *SUB1* homolog in oxidative stress. I initially tested the role of *SUB1* in DNA repair, including transcription-coupled repair and double strand break repair. While results from those experiments do not answer the original question, they led to some interesting discoveries that are discussed in

chapter II and IV. In chapter III, I present evidence that PC4 possesses an intrinsic antioxidant activity that prevents DNA oxidation.

1. DNA repair pathways

The double helix structure of DNA consists of the DNA bases and the DNA backbones that are two strings of deoxyriboses connected by phosphodiester bonds. Every component in this structure can be damaged by various agents, resulting in different DNA damages (Sancar et al, 2004). For example, UV irradiation can excite adjacent pyrimidine bases, giving rise to cyclobutane pyrimidine dimers (CPDs) or 6-4 photoproducts. 8-oxoguanine and thymine glycol are the common oxidized DNA bases caused by reactive oxygen species. γ-radiation generates double strand DNA breaks as well as a large number of single strand DNA breaks. It appears that different DNA repair pathways primarily target repair to specific types of DNA damage (de Laat et al, 1999). For example, glycosylases remove modified DNA bases to initiate base excision repair (Sung & Demple, 2006). Bulky DNA lesions such as CPDs are repaired by nucleotide excision repair. Double strand breaks are repaired by homologous recombination or nonhomologous end-joining, depending on the cellular context (Weterings & Chen, 2008). In the next sections I introduce the two repair systems most relevant to my studies: Nucleotide excision repair and double strand break repair.

1.1. Nucleotide excision repair (NER)

Human genes that are required for NER were originally discovered in patients who suffer from a rare recessive photosensitive syndrome called xeroderma pigmentosum (XP), a condition manifested by extreme photosensitivity in the skin and predisposition to skin cancer (de Laat et al, 1999; Cleaver & Bootsma, 1975). The most studied lesions in NER are UV-induced cyclobutane pyrimidine dimers (CPDs), although NER is capable of repairing other bulky chemical adducts and some forms of oxidative DNA damage (de Boer & Hoeijmakers, 2000; Ischenko & Saparbaev, 2002; Klungland et al, 1999). Seven NER genes were isolated and cloned, designated from XPA to XPG. The NER reaction starts with DNA damage recognition by XPC. XPA verifies the damage and recruits other repair factors. XPB and XPD are components of the general transcription factor TFIIH. They function as helicases to separate the DNA strands at the site of the lesion. After bidirectional unwinding around the lesion, XPG and the XPF/ERCC1 complex excise a fragment of 24-30 nucleotides containing the lesion (Huang et al, 1992). Finally the single strand DNA gap is filled by RPA, RFC, PCNA, and polymerase δ and ε and ligated by DNA ligase I.

While DNA damage in the whole genome is repaired by NER at a relatively slow pace, NER quickly repairs DNA damage in the actively transcribed genes, more particularly on the transcribed strand (or template strand) of the DNA, a phenomenon called transcription-coupled repair (TCR) (Bohr et al, 1985; Hanawalt & Spivak, 2008; Lainé & Egly, 2006). Hence the NER pathway acting on the transcriptionally silent genomic regions is designated global genome repair (GGR). It appears that GGR and

TCR differ only at the damage recognition step. While GGR uses XPC to recognize DNA damage and initiate repair, TCR does not require XPC and is triggered by stalled RNA polymerase II at the damage site (Tornaletti, 2005). A defect in TCR is manifested by a distinct disorder designated Cockayne syndrome (CS). For example, CSA and CSB, two essential genes for TCR, are often found mutated in CS patients. While the function of CSA is not clear, CSB appears to push RNA polymerase II through the DNA damage or remove it from the DNA, presumably for other NER factors to gain access to the lesion (Selby & Sancar, 1997; Svejstrup, 2003; Woudstra et al, 2002). In addition to CSA and CSB, the XP genes XPG, XPB, and XPD appears to play a role in TCR besides their nuclease and helicase activities.

GGR and TCR are conserved in most organisms from bacteria to human. For example, the yeast *RAD26* gene was identified based on its sequence similarity to the CSB gene and is required for TCR in yeast (van Gool et al, 1994; Lee et al, 2001, 2002). The *RAD7* and *RAD16* genes appear to be functionally equivalent to XPC, required for GGR but not TCR in yeast. Notably, the relative contributions of these two NER pathways vary in different organisms. Unlike human CS patients, the yeast *rad26Δ* mutant is not sensitive to UV treatment (van Gool et al, 1994; Verhage et al, 1996). GGR appears to be very efficient in yeast and compensates for the defect in TCR in the *rad26Δ* mutant. Therefore the *rad26Δ* mutant becomes UV sensitive only when GGR is inactivated by the *rad16Δ* or *rad7Δ* mutation.

1.2. Double strand break repair

Double strand DNA breaks (DSBs) can be generated by ionizing radiation, chemicals like camptothecin, reactive oxygen species, faulty replication, or during V(D)J recombination and antibody class switching (Hiom, 2010). Regardless of the generating source, a DSB may causes the abrupt end to two important cellular processes: transcription and replication. Clearly DSBs must be repaired at any cost. Two mechanisms are used to repair a DSB in all eukaryotic cells from yeast to human. The most intuitive approach is to put directly the two ends of the break back together, a process designated nonhomologous end-joining (NHEJ). In yeast, the key NHEJ factors include the Yku70/Yku80 complex, the MRX complex comprised of Mre11, Rad50 and Xrs2, ligase Dnl4 that is complexed with Lif1, and the regulatory factor Nej1. In NHEJ, a DSB is recognized and bound by Yku70/80, which recruits other NHEJ factors, and eventually ligated by Dnl4 (See Figure 1.2). The MRX complex contains 5'→3' nuclease activity. However, this nuclease activity of MRX seems dispensable for NHEJ, and the function of MRX is to bridge the DSB ends (Chen et al, 2001; Dudásová et al, 2004). The molecular function of Nej1 remains ambiguous, although its binding to Lif1 that is complexed with Dnl4 suggests it modulates the ligase activity (Daley et al, 2005). Because NHEJ is the direct ligation of DNA ends without requiring correct sequence information, it does not guarantee the accuracy of the repair. This is especially true when the DNA ends contain damaged DNA bases and are not directly ligatable. Processing of the ends can lead to loss of DNA bases in the joint after repair. In the extreme case, large stretches of DNA can be deleted and homologous sequences of 8-10 base pairs can be

used to direct the ligation, a process called microhomology-mediated end joining (MMEJ) (Ma et al, 2003). However, MMEJ occurs only at a low frequency and is independent of the KU complex and Dnl4.

In contrast to NHEJ that forcibly ligates the DNA ends, homologous recombinational repair (HR) analyzes the break and searches the whole genome for a homologous DNA sequence that can be used to "copy and patch" the break in an error-free style (See Figure 1.3). HR depends on genes in the *RAD52* group: *RAD51*, *RAD52*, *RAD55*, *RAD57*, *RAD59*, *RAD54*, *RAD50*, and the MRX complex that is shared with the NHEJ pathway (Krogh & Symington, 2004; van den Bosch et al, 2002). To analyze the break, the HR pathway first resects the DNA ends in the 5' to 3' direction, exposing the 3'-single strand DNA (ssDNA) end. Biochemical and genetic analysis of the yeast resection system reveals that it requires the MRX complex, nucleases Dna2, Exo1 and Sae2, the helicase Sgs1, and RPA to stabilize the emerging ssDNA.(Mimitou & Symington, 2011; Cejka et al, 2010; Niu et al, 2010). After the 3'-ssDNA is exposed, Rad51 replaces RPA on the ssDNA to form the Rad51-ssDNA filament that is able to search and invade the homologous sequence in the genome. Subsequently DNA replication copies the homologous DNA sequence that covers the gap in the DNA break. Because a template is used to repair the DNA break, HR is considered error-free. However, if substantial homology exists in the two ssDNA ends, annealing can occur between the two ends, resulting in deletion of one of the repeats and any intervening DNA, a process called Single Strand Annealing (SSA) (See Figure 1.4) (Krogh & Symington, 2004). Because Rad51 is not required for SSA (Krogh & Symington, 2004),

this process avoids the step of homology search and might represent aborted homologous recombination repair.

An obvious complication of multiple repair pathways for a single type of DNA damage is the need to decide which repair pathway to use and avoid conflicts between repair systems. In some cases, only one pathway can be used and there is no need to choose pathways if the cell can sense the situation. For example, one-ended double strand breaks can occur during replication fork collapse. NHEJ does not operate on one-ended breaks and HR is used to resume the replication in a process called break induced replication (BIR). In contrast, chromosomal beaks that occur in a haploid yeast cell during G1 phase often cannot be repaired by HR. It therefore appears to be useful to regulate HR and NHEJ during the cell cycle, encouraging NHEJ in G1 phase and HR in S and G2 phase (Helleday et al, 2007). In mammalian cells, however, NHEJ is predominant even in G1 phase because of the existence of a large number of repetitive sequences that may mislead the homology search if HR is used (Kao et al, 2005). Because ssDNA is required for HR but not for NHEJ, break resection is regarded as the regulatory step that enables HR but disallows NHEJ. However, DNA break resection does not appear to immediately reject NHEJ. For example, prior to NHEJ, damaged DNA ends that are not directly ligatable indeed require resection to remove the damaged bases (Bahmed et al, 2011; Quennet et al, 2011). On the other hand, it would be life-saving to reinstate NHEJ if the attempt to find the homologous sequences required by HR fails.

2. Interaction of DNA repair with transcription

Transcription is the process that reads information from template DNA and produces RNA. Therefore transcription is a process strongly affected by DNA damage. DNA damage can block the elongating polymerase (Mei Kwei et al, 2004), resulting in transcription inhibition (Reagan & Friedberg, 1997; Rockx et al, 2000) or transcription-coupled repair (Hanawalt & Spivak, 2008), a specialized form of NER that repairs the template strand of the DNA as discussed above. In those cases where DNA damage does not block transcription, it can cause transcription mutagenesis (Marietta & Brooks, 2007), producing aberrant proteins. In general, genomic domains with active transcription are repaired more efficiently than silent regions (Nouspikel et al, 2006), suggesting transcription may increase the accessibility for DNA repair factors.

Recent reports also show that DNA damage affects other processes in eukaryotic transcription. Alternative splicing is the process that selectively connects exons in the pre-mRNA to form mature mRNA (Nilsen & Graveley, 2010). 65% of human genes are affected by alternative splicing (Kim et al, 2007). DNA damage has been shown to affect the splicing of a broad range of pre-mRNA in fly and in human (Marengo & Wassarman, 2008; Muñoz et al, 2009). Similarly alternative polyadenylation is a ubiquitous process affecting most human genes. DNA damage has been shown to affect the polyadenylation site selection for the tropoelastin mRNA (Schwartz et al, 1998). Although the effect of DNA damage on alternative polyadenylation was not further studied, it may be a more general phenomenon.

3. DNA damage response

The mechanism by which cells sense DNA damage is still not completely clear (Yang, 2006). ssDNA coated with the RPA proteins seems to be critical in the early steps of damage detection (Zou & Elledge, 2003). Mammalian kinases ATM and ATR (yeast homologs are Tel1 and Mec1) are also recruited to the damage site. ATM and ATR phosphorylate a specific histone variant H2AX around the lesion, leading to the emergence of repair foci that are observable in a light microscope (Dellaire & Bazett-Jones, 2007). Notably, many DNA repair proteins also localize to these repair foci, including ATM, Rad51, Mre11, Rad50, NBS1, 53BP1, MDC1, etc (Dellaire & Bazett-Jones, 2007; Sak & Stuschke, 2010). Thus the repair foci may function to increase the local concentrations of the repair factors and reflect altered chromatin structure.

Some other substrates of ATM and ATR include kinases Chk1, Chk2 (yeast homolog of Rad53), P53, etc. They serve as signal transducers, eliciting a broad range of cellular responses (Branzei & Foiani, 2006; Sancar et al, 2004; Zhou & Elledge, 2000). For example, a checkpoint can be activated to arrest cell cycle progression, gaining extra time for the repair process before the cell enters a new round of DNA replication or mitosis. Another example is that the transcription of some DNA repair genes is enhanced after DNA damaging treatments (Alseth et al, 1999; Das et al, 2005).

4. Oxidative stress and the cellular defense

Oxidative stress is caused by reactive oxygen species (ROS) in the cells. Because ROS can be generated by the electron transport reactions in mitochondria, oxidative stress is a ubiquitous and a constant threat to each cell in our body (Valko et al, 2006). It was estimated that every human cell generates 1.5×10^5 ROS molecules per day (Beckman & Ames, 1997). Other sources that produce ROS include UV irradiation from the sunlight, X-rays and γ-rays, peroxisomes, and inflammation (Besaratinia et al, 2007; Circu & Aw, 2010). The primary ROS is the superoxide anion ($O_2^{\bullet -}$) that is produced by one electron transfer to molecular oxygen (O_2). After further reduction, $O_2^{\bullet -}$ produces many other ROS. Among them, hydrogen peroxide (H_2O_2) is the most stable and the hydroxyl radical (HO•) is the most reactive. ROS can potentially damage every cellular component, including lipid, protein, RNA and DNA. For example, oxidative damage can cause protein aggregation (Squier, 2001) and damage many protein side chains: arginyl residues can be converted to glutamylsemialdehyde residues, prolyl to pyroglutamyl/glutamyl, cysteinyl to -S-S- disulfide, lysyl to -α-Aminoadipylsemialdehyde, methionyl to methionylsulfoxide, tyrosyl to tyrosyl-tyrosyl cross-links, histidyl to asparaginyl/aspartyl (Stadtman, 1990). The most studied target of oxidative damage, however, is DNA, because oxidative DNA damage causes many human diseases including neurodegenerative diseases, cancer, and ageing (Barzilai & Yamamoto, 2004; Huang & Kolodner, 2005; Valko et al, 2006; Grzelak et al, 2006; Buonocore et al, 2010). ROS attacks both the DNA bases and the deoxyribose backbone, resulting in more than 100 different oxidative products, including single strand breaks,

double strand breaks, base losses or modifications, and DNA cross-links (Valko et al, 2006). An example is the oxidized guanine, 7,8-dihydro-8-oxoguanine (also known as 8-oxoG) . 8-oxoG spontaneously occurs at the frequency of about 1-10 per million DNA base pairs and causes G:C to T:A transversion. 8-oxoG has been used as a biomarker to assess cellular oxidative stress (David et al, 2007), although it is still technically challenging to measure it directly (Collins et al, 1996; ESCODD, 2003; Collins et al, 2004).

The first line of defense against oxidative stress is antioxidants that scavenge ROS. There are a variety of antioxidants in the cell, ranging from non-enzymatic small molecules to enzymatic proteins (Valko et al, 2006). Small molecule antioxidants are usually present at high concentrations and function as redox buffers to maintain the cytosol and nucleus in a reducing state. Examples are vitamin C, vitamin E, NADPH, and glutathione. The tripeptide glutathione (GSH) is the most important non-enzymatic molecule that provides the pool of active thiol groups (-SH) for glutaredoxin to reduce the disulphide bonds (GSSG) in oxidized proteins (Go & Jones, 2010). Enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutaredoxin, peroxiredoxin, thioredoxin/thioredoxin reductase, glutathione reductase and others. Superoxide dismutase binds to metal ions such as Cu, Zn, Mn and catalyzes the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2**.** Catalase converts H_2O_2 to molecular oxygen and water. Peroxiredoxin was first discovered in yeast (Kim et al, 1988, 1989; Rand & Grant, 2006; Trotter et al, 2008). It decomposes organic and inorganic peroxides by oxidizing its cysteine residues. Thioredoxin reduces peroxiredoxin and other proteins by donating electrons from its

cysteine residues. The disulphide bond in the oxidized thioredoxin is reduced back to the thiol form by thioredoxin reductase which uses electrons from NADPH. Glutathione reductase uses NADPH to catalyze the reduction of disulphide glutathione (GSSG) back to glutathione.

If the quantity of ROS exceeds the capacity of the antioxidant system, ROS can damage various cellular components. As listed above, many protein oxidations can be reversed by the antioxidant enzymes. Alternatively, oxidative damage to proteins and lipids are minimized by the turn over of protein and lipid. DNA damage, however, has to be repaired for faithful replication to occur. The importance of DNA repair in combating oxidative DNA damage is manifested by the multitude of DNA repair enzymes dedicated to repair oxidative DNA damage. For example, cells from bacteria to human use three different enzymes to minimize the mutagenic effect of 8-oxoguanine. OGG1 directly removes 8-oxoG from DNA, MYH excises adenine in the 8-oxoguanine:A mispair, and MTH1 hydrolyzes the 8-oxodGTP pool (Halliwell & Aruoma, 1991; Klungland & Bjelland, 2007). Although base excision repair has been studied extensively in the repair of oxidative base modifications, other repair pathways including nucleotide excision repair and double strand break repair have been implicated in the repair of more complicated oxidative DNA damage (D'Errico et al, 2008; Gopalakrishnan et al, 2010; Steinboeck et al, 2010).

5. Human genes that prevent oxidative mutagenesis in bacteria

Studies have shown that human genes can function in bacteria to repair or protect genomic DNA (Chen et al, 1989; Samson et al, 1991; Bonanno et al, 2002; Takao et al, 2009). Our laboratory previously established an assay to screen for human genes that can prevent G:C-> T:A transversion in bacteria (Wang et al, 2004). As discussed above, 8-oxoguanine is highly mutagenic and causes G:C->T:A transversions. In this assay, a point mutation is positioned in the active site codon of the *lac*Z gene in *E. coli* that inactivates the gene and only a G:C->T:A transversion can revert the mutation. Revertant cells carrying a functional *lac*Z gene will turn X-Gal blue, resulting in blue microcolonies within the white bacterial colony. Because the GC→TA transversion is characteristic of oxidative mutagenesis, the number of blue microcolonies represent the frequency of the oxidative mutation events. As shown in Figure 1.1A, wild type bacteria only have a few oxidative mutation events manifested by the low number of blue microcolonies, while the repair deficient mutant *mutM mutY* exhibits a large number of mutations (Figure 1.1B). Over-expression of the bacterial 8-oxoG repair gene MutM (Figure 1.1C) or the human 8-oxoG repair gene OGG1 (Figure 1.1D) suppresses most of the oxidative mutations in the *mutM mutY* background. Among the vast number of human genes screened, PC4 is able to completely suppress oxidative mutagenesis in the *mutM mutY* background (Figure 1.1E).

PC4 was first identified in 1994 and was named PC4 because of its function as a positive coactivator in the *in vitro* transcription system (Kretzschmar et al, 1994; Ge & Roeder, 1994). PC4 activates transcription at low concentrations and inhibits

transcription at high concentrations (Ge & Roeder, 1994; Werten et al, 1998b; Fukuda et al, 2003). This transcription regulation activity of PC4 might be based on its direct interaction with the transcription factor TFIIA and its nonspecific binding to double strand DNA (dsDNA) and single strand DNA (ssDNA) (Kaiser et al, 1995; Werten et al, 1998b). PC4 appears to have a higher affinity for binding to ssDNA. The crystal structure of PC4-ssDNA complexes has been solved, showing PC4 forms homodimers and interacts with 5-nucleotide regions in two opposing ssDNA strands (Werten & Moras, 2006; Mortusewicz et al, 2008). Among the 127 amino acid residues of PC4, the amino terminus contains two serine rich domains and a lysine rich domain which appear to be required for its transcription activation function. On the carboxyl terminus there is a single strand DNA binding domain that is dispensable for transcription activation and is suggested to be involved in oxidation protection (Wang et al, 2004). Recently PC4 was shown to be a chromatin-associated protein and induces chromatin condensation (Das et al, 2006). Mortusewicz *et al.* also showed that PC4 is recruited to sites of DNA damage (Mortusewicz et al, 2008).

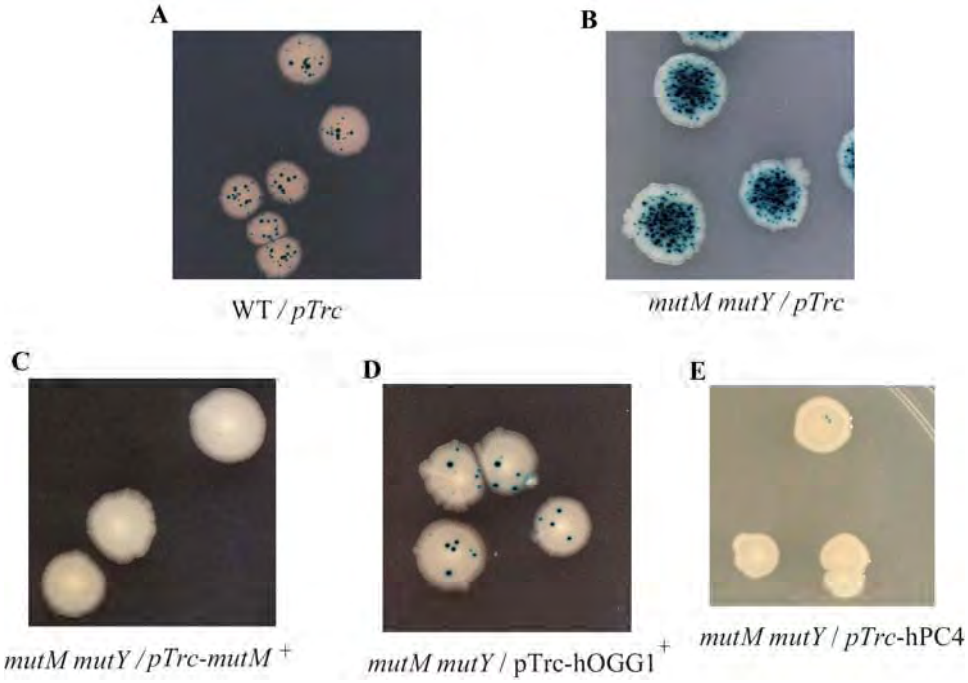
The homolog of PC4 in yeast *Saccharomyces cerevisiae* is designated as *SUB1* for its ability to suppress TFIIB mutations (Knaus et al, 1996; Henry et al, 1996). In the conserved region, *SUB1* and PC4 share 47% identity. Like PC4, *SUB1* appears to be able to activate the transcription of some genes but inhibit some others (Knaus et al, 1996; Koyama et al, 2008). *SUB1* appears to be part of the transcription machinery: it directly interacts with the TFIIB subunit and is present throughout the transcription process from transcription initiation to termination (Knaus et al, 1996; Calvo & Manley, 2005).

Interestingly, *SUB1* was reported to play a role in the transcription by RNA polymerase III (Tavenet et al, 2009; Rosonina et al, 2009). Wang *et al.* showed that the *sub1Δ* mutant exhibits elevated mutagenesis and peroxide sensitivity (Wang et al, 2004).

6. Figures and legends

Figure 1.1 The papillation assay demonstrates that PC4 can prevent oxidative mutagenesis in bacteria. Microcolonies within the white bacterial colony represent oxidative mutation events because a point mutation is positioned in the active site of the *lacZ* gene in *E. coli* and the GC→TA transversion is required to revert this point mutation to reactivate the *lacZ* gene. **A**: Wild type strain with the empty expression vector exhibits a few spontaneous oxidative mutagenesis events, **B**: The *mutM mutY* double mutant strain is highly mutagenic. **C**: Expression of the bacterial DNA repair gene $mutM^{+}$ suppresses oxidative mutagenesis in the *mutM mutY* double mutant. **D**: The human DNA repair gene hOGG1 suppresses oxidative mutagenesis in the *mutM mutY* double mutant when overexpressed. **E**: cDNA of PC4 when expressed in the *mutM mutY* double mutant suppresses oxidative mutagenesis. Pictures provided by Dr. Volkert. Experimental details see reference (Wang et al, 2004).

Figure 1.1


A
WT / pTrc
B
mutM mutY / pTrc
C
mutM mutY / pTrc-mutM +
D
mutM mutY / pTrc-hOGG1 +
E
mutM mutY / pTrc-hPC4

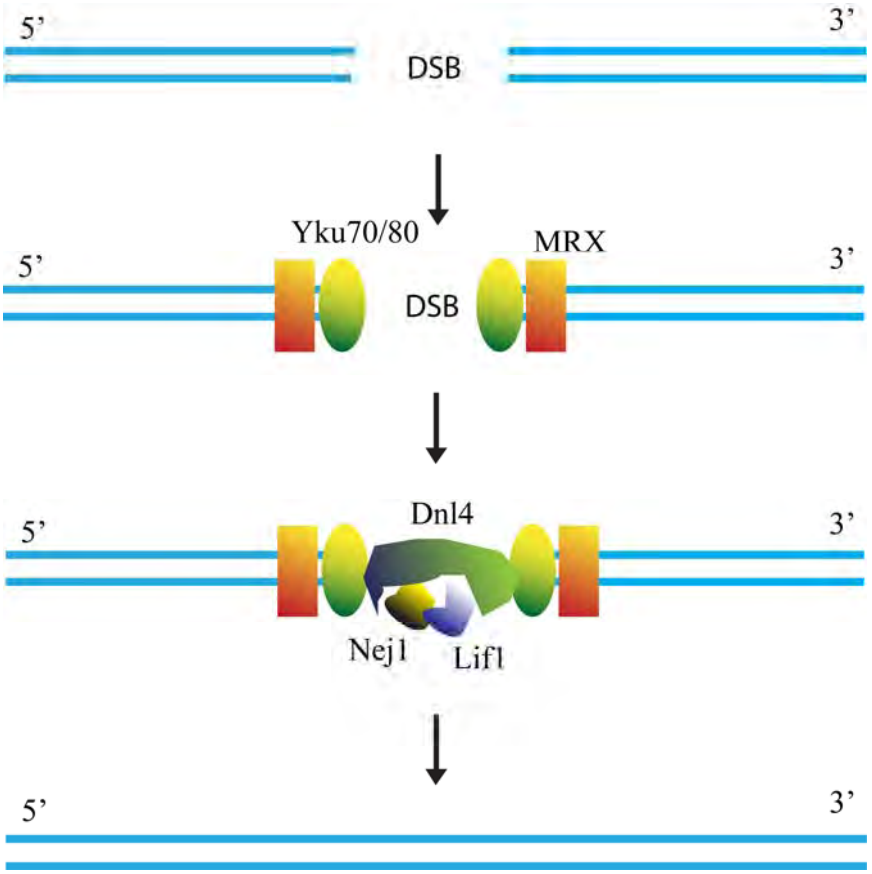
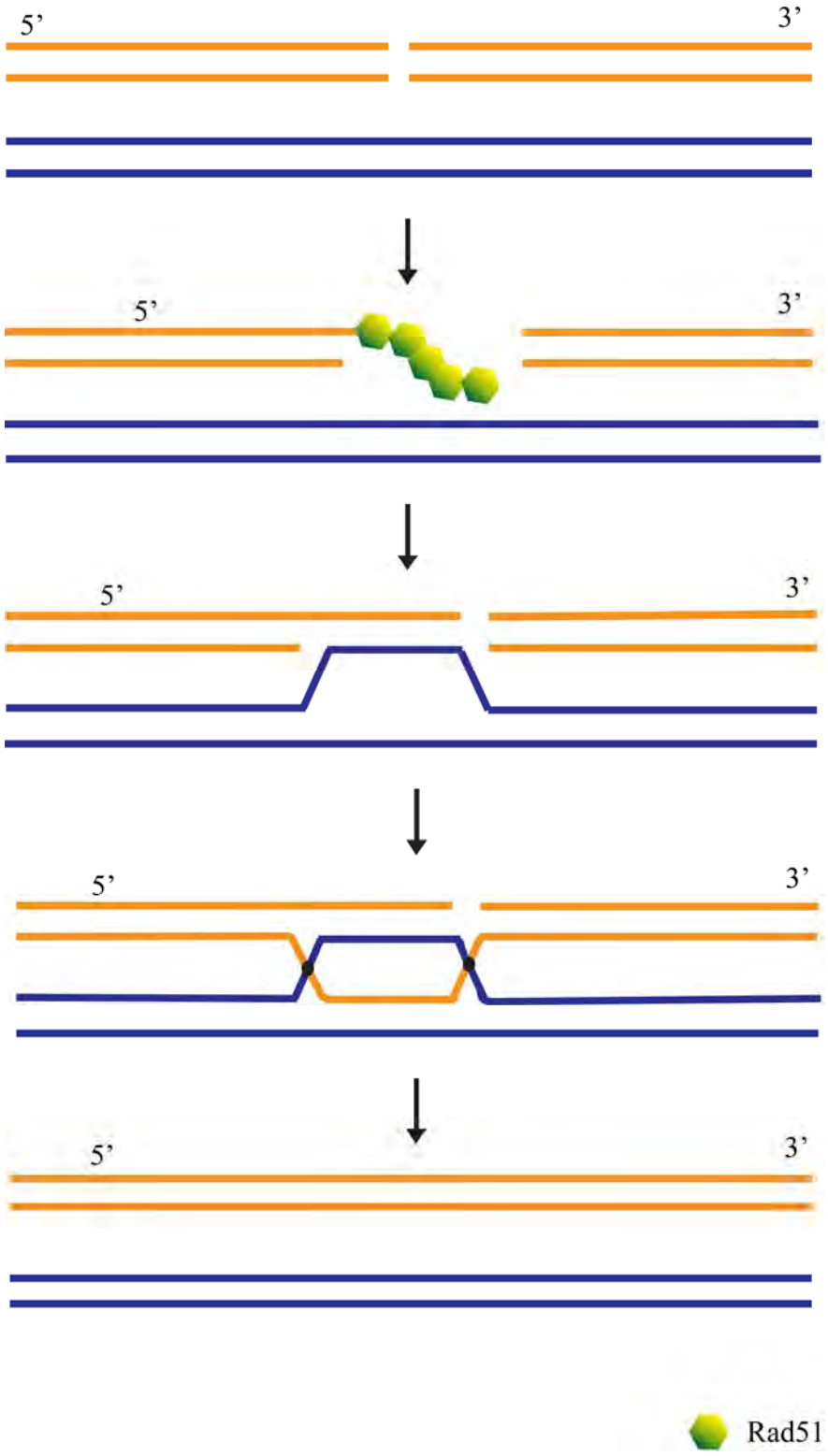


Figure 1.2 The NHEJ repair pathway in yeast. The DSB is first recognized and bound by the Yku70/80 complex and the MRX complex. The DNA ligase Dnl4 which is complexed with Nej1 and Lif1 is recruited to ligate the DNA break.

Figure 1.3 The homologous recombination repair pathway in yeast. A DSB is resected to generate single strand DNA which is subsequently coated with Rad51 proteins. The Rad51-ssDNA filament searches for homologous sequences in the genome and invade the double helix of the homologous sequence. DNA synthesis and resolution of the Holliday junction result in repair of the DNA break.

Figure 1.3



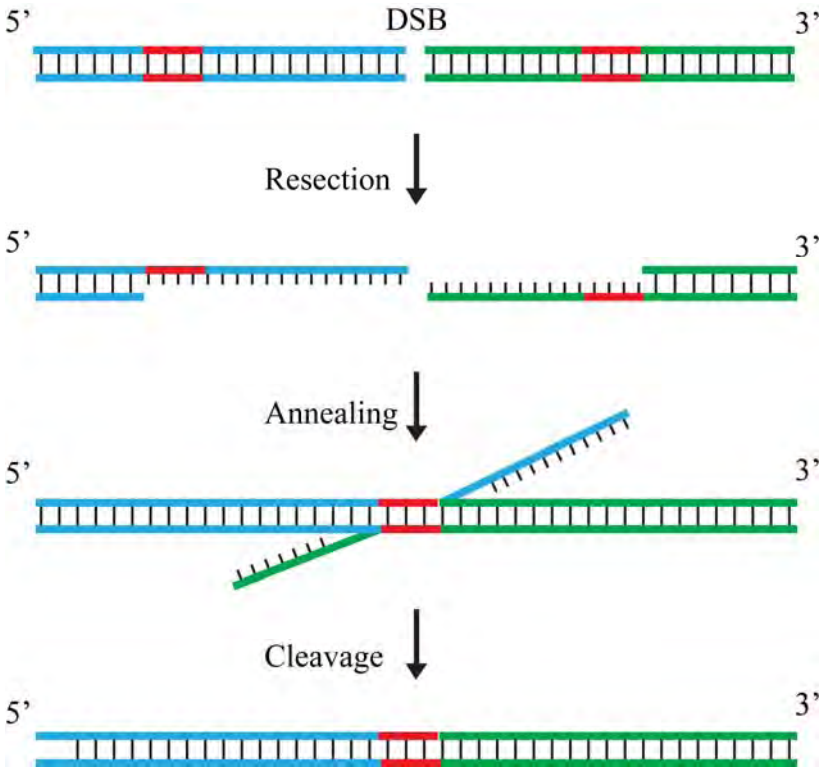


Figure 1.4 The single strand annealing pathway. The DNA fragments drawn in the red color located in both sides of the double strand break represent homologous sequences. After DNA resection produces single strand DNA at the DSB,the two homologous sequences anneal and the protruding single-strand DNA fragments are cleaved, leading to loss of information in this DSB repair pathway.

Chapter II

The role of PC4 in DNA repair

1. *SUB1* is not required for transcription-coupled DNA repair

1.1 Introduction

The function of PC4 in DNA repair is suggested by multiple lines of evidence. The bacterial DNA is protected from oxidation when PC4 is expressed (Wang et al, 2004), suggesting PC4 protects DNA from oxidative damage or increases repair of DNA damage. Although bacteria have a quite different cellular environment from human cells, human DNA repair genes have been found to repair DNA damage in bacteria (Chen et al, 1989; Samson et al, 1991; Bonanno et al, 2002; Wang et al, 2004; Takao et al, 2009). More recently, Mortusewicz *et al.* showed that PC4 accumulates at DNA damage sites and predicted it detects DNA damage and initiates the DNA repair cascade (Mortusewicz et al, 2008). The DNA-damage inducing agents used in their assays include hydroxyurea, hydrogen peroxide, and near-UV laser irradiation and the DNA damage produced can be double strand DNA breaks, single strand DNA breaks, oxidative DNA damage, UV damage, or a mixture of these. The accumulation of PC4 at DNA damage sites suggests that PC4 might be recruited to DNA repair foci to repair the DNA damage. The fact that the PC4 foci can be induced by various DNA damaging agents suggests that PC4 might repair different types of DNA damage. Another line of evidence for a role of PC4 in DNA repair is that PC4 directly interacts with the DNA repair protein XPG (Wang et al, 2004). XPG is required for both sub-pathways of nucleotide excision repair: the global genome repair and the transcription-coupled repair (Hanawalt & Spivak, 2008). PC4

appears to be recruited by and displaces XPG from double strand DNA that contains an unpaired region (Wang et al, 2004).

If PC4 functions in DNA repair pathways, it is very tempting to hypothesize that its repair activity is coupled to the transcription process where PC4 is actively involved. PC4 directly interacts with transcription factor TFIIA and stimulates transcription (Ge & Roeder, 1994; Kaiser et al, 1995). Work in yeast showed that the yeast homolog Sub1 stimulates transcription in vivo and interacts with the transcription factor TFIIB (Knaus et al, 1996). Sub1 has been shown to associate with the transcription machinery throughout the transcription process (Calvo & Manley, 2005). Moreover, besides XPG, PC4 directly interacts with the polyadenylationfactor CstF64 (Calvo & Manley, 2001) and CstF64 has been shown to be required for transcription-coupled repair of UV induced DNA damage (Mirkin et al, 2008). Therefore we hypothesized that PC4 might be involved in transcription-coupled DNA repair.

The transcription-coupled repair hypothesis and the oxidation protection function of PC4 prompted to a further conjecture, that is PC4 repairs oxidative DNA damage by transcription-coupled repair. Whether oxidative DNA damage can be repaired by transcription-coupled repair, however, remains elusive since the retractions of a series of publications on this topic (Hanawalt & Spivak, 2008). A direct assay to test transcription-coupled repair of oxidative damage is currently unavailable because of technical difficulties in inducing high amounts of oxidative DNA damage in cells and spurious oxidation of DNA samples during handling.

Other evidence that PC4 might be involved in transcription-coupled DNA repair comes from the UV sensitivity data of the yeast *sub1Δ* mutant. The *sub1Δ* mutant is not sensitive to UV irradiation compared to wild type, but becomes sensitive when global genomic repair is impaired by deleting the essential *RAD16* gene (Figure 2.1, right panel). This phenotype of the *sub1Δ* mutant mimics that of the *rad26Δ* mutant (Figure 2.1, left panel). As discussed in the introduction (Chapter I), *RAD26* is required for transcription-coupled repair in yeast and the *rad26Δ* mutant is UV sensitive only in the *rad16Δ* background. These results made two important suggestions. First, *SUB1* may be a component of the transcription-coupled repair pathway in yeast, in line with our hypotheses that PC4 may function in transcription-coupled repair. Second, we can test the potential role of *SUB1* in transcription-coupled repair by investigating repair of UV damage, which is technically possible (Hanawalt & Spivak, 2008).

1.2 The *sub1Δ* mutant is proficient in transcription-coupled repair of UV damage

Transcription-coupled repair (TCR) is a nucleotide excision repair pathway that is triggered when the transcription machinery stalls at DNA damage sites (Lainé & Egly, 2006). It preferentially removes the DNA lesions from the template strand. DNA lesions on the template strand of transcribing genes, therefore, disappear faster than lesions on the non-template strand. The TCR assay we used measures the UV-induced DNA damage on each strand of the highly-transcribed *RPB2* gene at different times after the cells are irradiated with UV. In this assay, cyclobutane pyrimidine dimers (CPDs), a

common form of UV induced DNA damage, are converted to single strand DNA breaks by digesting the genomic DNA with T4 endonuclease V, a DNA repair enzyme that specifically recognizes and nicks the DNA strand at CPDs. After electrophoresis on alkaline agarose gels, DNA strands of *RPB2* gene that contained CPDs will be cut and migrate below the 3.4kb *RPB2* DNA band (NruI digestion fragment of genomic DNA). As shown in Figure 2.2A, immediately after UV irradiation (time 0), the *RPB2* DNA without TEV digestion migrates as a single band at top of the gel. After digestion with TEV, however, the damage containing *RPB2* DNA has many single strand DNA breaks evidenced by extensive smearing of the DNA on the gel, indicating UV induces CPD in DNA. The non-transcribed strand (NTS) and the transcribed strand (TS) start with the same level of CPDs as shown in Figure 2.2A, time 0. From 15 to 120 minutes after UV irradiation, the amount of CPD on the non-transcribed strand does not decrease significantly (Figure 2.2A, upper panel). However, damage completely disappears from the transcribed strand as evidenced by reduced nicking (Figure 2.2A, lower panel). This suggests wild type cells possess efficient transcription-coupled repair activity. Figure 2.2B shows that the preferential removal of CPDs from the transcribed strand (TS) of *RPB2* does not occur in the *rad26Δ* mutant. This result confirms that *RAD26* is an essential gene for transcription-coupled repair in yeast. Figure 2.2C shows that CPDs on the transcribed strand (TS) of *RPB2* are as quickly repaired in the *sub1Δ* mutant as in wild type (Figure 2.2A), suggesting *SUB1* is not required for transcription-coupled repair of UV damage.

The results of these experiments clearly ruled out a role of *SUB1* in transcription-coupled repair of UV-induced DNA damage. We did not directly test if *SUB1* is required for transcription-coupled repair of oxidative DNA damage. As discussed above, whether oxidative DNA damage can be repaired by transcription-coupled repair remains controversial. The key to this question would be whether oxidative DNA damage blocks transcription elongation similar to UV-induced DNA damage which causes greater helix distortion in the DNA. 8-oxoguanine, a common oxidative DNA damage, has been reported not to block transcription (Kuraoka et al, 2007) but to block transcription when converted to other DNA repair intermediates (Kitsera et al, 2011; Charlet-Berguerand et al, 2006). 8-oxoG is repaired by TCR in bacteria (Brégeon et al, 2003) but not in mammalian cells (Thorslund et al, 2002). If transcription-coupled DNA repair is elicited by stalled transcription machinery and is independent of the damage types, then our results can be generalized to suggest that *SUB1* does not repair DNA damage including oxidative damage in the transcription-coupled fashion.

Next I tested if *SUB1* is required for repair of transcription-silent regions in the genome, i.e. Global Genome Repair (GGR). Yeast has two mating types, the **a** type and the **α** type (Astell et al, 1981). The mating types are determined by the DNA sequence in the mating type locus: *MAT**a*** for the **a** type and *MAT**α*** for the **α** type. Transcription is active on the mating type locus. Interestingly, yeast cells have an extra copy of the mating type locus in the genome: *HML* (Hidden *MAT* Left) carries the MATα and *HMR* (Hidden *MAT* Right) the *MATa*. Transcription on those hidden copies is strictly suppressed (Nasmyth, 1982). I determined the repair of UV damage in the transcription

silent *HML* region in a *MATα* yeast cell by measuring the amount of UV-induced cyclobutane pyrimidine dimers (CPD) in that region following UV treatment. Similar to the TCR assay, T4 endonuclease V (TEV) is used to convert CPD to single strand DNA breaks. The DNA is digested with HaeII and electrophoresed on alkaline agarose gels before probing with the *MATα*-specific probe. The transcription-active *MATα* locus is repaired at a higher rate than the transcription-silent *HML* locus (Figure 2.3A, wild type), confirming that transcription-coupled repair preferentially removes UV damage on the actively transcribed genes. Over the time of 4 hours, the *HML* locus is repaired in the *sub1Δ* mutant at a rate similar to that in wild type (Figure 2.3B), suggesting *SUB1* is not required for global genome repair. In contrast, repair of *HML* in the *rad16Δ* mutants (Figure 2.3 C and D) is almost completely abolished, because *RAD16* is required for global genome repair (Verhage et al, 1996).

1.3 *SUB1* is not required for transcription recovery

Transcription-coupled repair occurs when the transcription machinery stalls at bulky DNA lesions in the DNA template . After DNA damage is repaired, however, transcription needs to recover. Therefore transcription recovery and transcription-coupled repair may be two epistatic events in the cells after UV treatment. Since we have ruled out a role of *SUB1* in transcription-coupled repair, we tested if *SUB1* is required for transcription recovery after UV damage. We measured the level of *RPB2* mRNA in the cells at different times after UV treatment. As expected, both wild type and the *sub1Δ* mutant have reduced amounts of *RPB2* mRNA at 15 minutes after UV treatment (Figure

2.4A), suggesting transcription inhibition by UV damage. However, *RPB2* mRNA increases at 45 and 60 minutes in the *sub1Δ* mutant as well as in wild type (Figure 2.4A), suggesting transcription recovers normally in the *sub1Δ* mutant. Northern blots show that the *PEX11* mRNA also recovers normally in the *sub1Δ* mutant after UV treatment (Figure 2.4B).

Although *SUB1* appears not to be required for transcription recovery after UV damage, I noted that the *RPB2* gene encodes two mRNA species and transcription of the long form is preferentially induced by UV (Figure 2.4A). Subsequent experiments demonstrated that this is caused by UV-induced polyadenylation switching of the *RPB2* mRNA and that work is presented in chapter IV of this thesis.

2. *SUB1* is involved in Double Strand DNA break repair

2.1 Introduction

We showed above that *SUB1* is not required for repair of UV damage, either by transcription-coupled repair or global genome repair. In 2009, Batta *et al.* showed that PC4 stimulates the ligation of DNA fragments *in vitro* (Batta et al, 2009). This suggests a role of PC4 in double strand DNA break repair, particularly in the nonhomologous end-joining pathway (NHEJ). Therefore we tested if *SUB1* is involved in double strand break repair *in vivo* in yeast. The work presented below shows that the *sub1Δ* mutant resects DNA ends quickly, leading to loss of linear plasmids, but ligates chromosomal breaks efficiently. An interesting conclusion is that rapid resection of DNA breaks in the *sub1Δ* mutant does not inhibit NHEJ, a DSB repair pathway that is generally regarded incapable of operating on the resected DNA breaks (Zierhut & Diffley, 2008; Wu et al, 2008; Longhese et al, 2010; Mimitou & Symington, 2011).

2.2 DNA break resection does not inhibit nonhomologous end joining in the *Saccharomyces cerevisiae sub1Δ* mutant

The work presented in this section is currently under peer review by DNA REPAIR:

Lijian Yu, Michael Volkert. DNA break resection does not inhibit nonhomologous end joining in the *Saccharomyces cerevisiae sub1Δ* mutant. (Submitted to DNA REPAIR, May, 2011)

Summary

Eukaryotic cells repair double strand DNA breaks (DSBs), either by homologous recombination (HR), or nonhomologous end-joining (NHEJ). HR repair requires a 3'-single strand DNA end that is produced by extensive 5' → 3' resection of the DNA breaks. In contrast, break resection and single strand DNA overhangs are not required for NHEJ. It has been generally accepted that DNA break resection is a regulatory step in DSB repair, channeling the repair pathway into HR but not NHEJ. We found the yeast *sub1Δ* mutant resects chromosomal DNA breaks at an elevated rate, but uses NHEJ repair more efficiently than wild type, suggesting DNA resection is not inhibitory to NHEJ. Additionally, we provide evidence that resected chromosomal DNA in wild type and the *sub1Δ* mutant, but not the *yku70Δ* mutant, can be repaired by NHEJ. This suggests NHEJ repair of resected DNA is not unique to the *sub1Δ* mutant and that the NHEJ deficiency of the *yku70Δ* mutant may be unrelated to increased resection. Based on these results, we propose that DNA break resection is not a regulatory step in determining the pathway choice between HR or NHEJ in the cells.

Introduction

Double strand DNA breaks are generated pathologically by DNA damaging agents or physiologically during lymphocyte development (Lieber, 2010). Without proper handling, DSBs lead to chromosomal instability or cell death. Two distinct mechanisms have been identified to repair DSBs in all eukaryotic cells from yeast to human. The direct ligation of two DSB ends, called Nonhomologous End-Joining, depends on the NHEJ factors Yku70/Yku80, Dnl4, Lif1, Nej1, and the MRX complex in yeast (Daley et al, 2005). As DSB ends joined by NHEJ may include deletion or addition of DNA bases at the junction, NHEJ is considered error-prone. This is especially true when the DSB sites require end-processing prior to ligation. In contrast, Homologous Recombination uses homologous sequences in the genome as a template to restore the chromosomal break and is error-free (Krogh & Symington, 2004). Genes in the *RAD52* epistasis group are required for HR repair in all cell types. In yeast, HR repair is initiated with detection of the DNA breaks by the MRX complex, followed by the 5' → 3' resection orchestrated by MRX, Sae2, Sgs1, Exo1, Dna2, and others. (Mimitou & Symington, 2011). DNA break resection is a key step in HR repair that produces the single strand DNA (ssDNA) ends required for formation of the presynaptic Rad51-ssDNA filament (Sinha & Peterson, 2008) and for ATR-mediated checkpoint activation (Zou & Elledge, 2003). The MRX complex, required for both HR and NHEJ, works with Sae2 to initiate DNA resection (Niu et al, 2010; Cejka et al, 2010). However, the nuclease activity of Mre11 is not required for DNA resection (Llorente & Symington, 2004), suggesting a stimulatory role of the MRX complex in resection. The NHEJ repair pathway appears inhibitory to DNA

resection, as Ku and other NHEJ factors protect DSB from 5’ →3’ resection (Zhang et al, 2007; Longhese et al, 2010).

How cells make the choice between HR and NHEJ to repair a DSB is gaining increasing attention. The decision making appears fairly rational: haploid yeast cells tends to choose NHEJ to repair a DSB in G1 phase but HR in G2/S phase because the extra copy of genetic information is available (Huertas, 2010). DNA break resection has been regarded as the key process in determining whether NHEJ or HR carries out the repair. It is generally believed that once resection is started, cells are destined to use HR to repair the DSB and NHEJ is no longer an option (Zierhut & Diffley, 2008; Wu et al, 2008; Longhese et al, 2010; Mimitou & Symington, 2011). Evidence is primarily based on the observation that transformed plasmids with a resected DSB are repaired less efficiently by NHEJ (Daley & Wilson, 2005; Frank-Vaillant & Marcand, 2002). However, as shown below, the results of plasmid assays may not be applicable to in vivo repair processes working on chromosomal breaks. Additionally, inhibition of chromosomal resection is not itself sufficient to restore KU association with DSB or NHEJ proficiency, suggesting NHEJ can be regulated through processes other than DSB resection (Zhang et al, 2009).

Recently chromatin proteins have been found to impact DSB resection. For example, H2AX prevents CtIP-mediated DNA resection in murine lymphocytes (Helmink et al, 2011) and yeast H2A is required for DNA resection (van Attikum et al, 2004) as well as NHEJ (Downs et al, 2000). PC4 is a multifunctional protein involved in

transcription (Ge & Roeder, 1994; Kretzschmar et al, 1994), DNA damage repair (Mortusewicz et al, 2008; Wang et al, 2004), and replication (Pan et al, 1996). Recently Batta et al. showed that PC4 is a chromatin associated protein (Das et al, 2006) that stimulates NHEJ (Batta et al, 2009). In this study we investigate the role of *SUB1*, the yeast homolog of PC4, in DSB repair. We found that the *sub1Δ* mutant exhibits rapid resection of DSB ends, similar to the *yku70Δ* mutant. However, rapid DSB resection in the *sub1Δ* mutant does not reduce NHEJ proficiency, suggesting NHEJ is capable of functioning on resected DNA. Thus, we propose that cells do not use DNA resection as a means of choosing between NHEJ and HR repair.

Results

Rapid DSB resection in the *sub1Δ* mutant

Human PC4 is involved in double strand DNA break repair (Batta et al, 2009), forms repair foci at sites of induced DNA damage (Mortusewicz et al, 2008), and is a chromatin-associated protein involved in chromatin condensation (Das et al, 2006). Recently the chromatin protein H2AX has been reported to prevent DNA break resection (Helmink et al, 2011). Here we asked if PC4's yeast homolog *SUB1* prevents DSB resection similar to H2AX. To test this, we used HO endonuclease to induce a chromosomal break and measured the ssDNA produced by end resection. As illustrated in Figure 2.5a, HO cuts the 1881bp StyI-digested chromosome III fragment and generates a shorter 717bp fragment that is detected by the labeled probe on a Southern blot. Because StyI does not cut ssDNA, the 717bp fragment disappears as DNA resection progresses beyond the first StyI site and the DNA is instead cut at the next StyI site. Figure 2.5b shows that HO induces the DSB successfully within half an hour and DSB resection causes loss of the 717bp fragment 2 hours after the DSB induction. We compared DSB resection of the *sub1Δ* mutant with wild type and the *yku70Δ* mutant. These results confirmed that the *yku70Δ* mutant resects DSB ends more rapidly than wild type as reported previously (Figure 2.5c) (Longhese et al, 2010; Clerici et al, 2008). Importantly, we found the *sub1Δ* mutant resects DSB at an increased rate, similar to that seen in the *yku70Δ* mutant (figure 2.5d).

sub1Δ mutant inefficiently recovers linearized plasmids

Batta *et al* reported that human PC4 stimulates ligation of plasmid DNA (Batta et al, 2009), suggesting a similar role for *SUB1* in yeast. To test this, we transformed yeast cells with plasmid pMV1328 that is linearized by the restriction enzyme NcoI (Figure 2.6a) and measured the efficiency of plasmid recovery which requires NHEJ repair. Because DNA sequences flanking the NcoI-created DSB have no homology in the yeast genome, cells circularize the plasmid primarily by direct ligation. Figure 2.6b shows that the plasmid ligation efficiency in the *sub1Δ* mutant is greatly reduced, indicating either defective NHEJ or loss of plasmids due to extensive resection (see below). Similarly the *yku70Δ* mutant has reduced ligation efficiency as reported previously (Boulton & Jackson, 1996). We found 94% of the repaired plasmids in the *yku70Δ* mutant contain mutations that inactivated the KanMX6 gene, indicating a shift toward mutagenic NHEJ pathway (Figure 2.6c). In contrast, the *sub1Δ* mutant generates fewer mutagenic ligations compared to the *yku70Δ* mutant (Figure 2.6c), suggesting rapid DSB resection shared by the *sub1Δ* and *yku70Δ* mutants results in increased mutagenic ligations only in the *yku70Δ* mutant. Interestingly, it appears that *SUB1* but not *YKU70* is required for the recovery of plasmids that contain a blunt-ended DSB, since ligation of blunt-ended DNA is increased relative to wild type in the *yku70Δ* mutant (Figure 2.6d and references (Boulton & Jackson, 1996; Hegde & Klein, 2000)), but decreased in the *sub1Δ* mutant (Figure 2.6d).

To test if rapid DNA resection contributes to the reduced ligation efficiency in the *sub1Δ* mutant, we attempted to reduce the resection rate in the *sub1Δ* mutant by using the *exo1Δ sgs1 Δ* mutant background. EXO1 is an exonuclease and SGS1 is a helicase involved in DSB resection in *S. cerevisiae* (Huertas, 2010). Figure 2.7a and 2.7b show that the *exo1Δ sgs1 Δ* double mutant and the *exo1Δ sgs1 Δ sub1Δ* triple mutant have decreased DSB resection rates compared to wild type as shown in Figure 2.5b. The *exo1Δ sgs1 Δ* mutant exhibits a slightly reduced ligation efficiency and as expected the *sub1Δ* has very poor ligation efficiency. Interestingly, the triple mutant *exo1Δ sgs1 Δ sub1Δ* improves the ligation efficiency considerably compared to the *sub1Δ* mutant, suggesting DNA resection contributes to the reduced ligation efficiency in the *sub1Δ* mutant.

Efficient joining of chromosomal breaks in the *sub1Δ* mutant

Yeast mutants that are deficient in NHEJ usually exhibit reduced ability to ligate chromosomal breaks (Daley et al, 2005; Lieber, 2010). Therefore we tested if the *sub1Δ* mutant can ligate chromosomal breaks efficiently. We used the donorless strain (JKM179, (Haber, 2002)) in which the repair of DSBs produced by HO induction can not be repaired by homologous recombination and is dependent upon NHEJ. Table 2.1 shows that the *yku70Δ* mutant is defective in joining chromosomal breaks. Unexpectedly, the *sub1Δ* mutant survives the chromosomal breaks better than wild type, suggesting efficient NHEJ repair of chromosomal DSBs. From Figure 2.5 we have learned that the *sub1Δ* mutant exhibits rapid resection on the induced chromosomal break. Taken together, we conclude that rapid DSB resection in the *sub1Δ* mutant does not prevent the chromosomal

beaks from being repaired by NHEJ. Furthermore, 2 hours after resection most DSB ends have been resected extensively in both wild type (Figure 2.5b) and the *sub1Δ* mutant (Figure 2.5d). However, both wild type and the *sub1Δ* mutant have nearly 40% survival 2 hours after DSB induction. This suggests that many resected DSBs can be repaired by NHEJ. Thus, the reduced ability of the *yku70Δ* mutant to survive chromosomal breaks (Table 2.1) might be caused by a defect in NHEJ repair that is unrelated to the rapid DSB resection.

To test if rapid resection of the *sub1Δ* mutant causes mutagenic ligations as reported for the *yku70Δ* mutant (Boulton & Jackson, 1996; Guirouilh-Barbat et al, 2004), we used the suicide deletion assay in which precise ligation of chromosomal ends removes a 3kb interstitial DNA fragment carrying the I-SceI gene inactivating it and, if the repair is accurate, reconstructing a functional *ADE2* gene (Karathanasis & Wilson, 2002; Wilson, 2002). Figure 2.8a shows that the *sub1Δ* mutant efficiently joins the chromosomal breaks, confirming the results in table 2.1. In the *yku70Δ* mutant nearly 50% of the chromosomal joints are mutated during repair, but in the *sub1Δ* mutant and wild type the majority of the chromosome breaks are precisely ligated since the Ade2 gene function is restored (Figure 2.8b). This suggests that rapid resection does not promote mutagenic ligation in the *sub1Δ* mutant.

To directly quantify the ligation events in the suicide deletion assay, we used quantative real time PCR analysis (RT-PCR) to measure the ligation products formed after induction of the chromosomal breaks. The *yku70Δ* mutant shows no detectable

ligations, while the *sub1Δ* mutant and the wild type control produce increasing amounts of ligation products within the first 5 hours after DSB induction (figure 2.8c). This confirms that DSB resection does not by itself reduce efficiency of NHEJ. Restriction digestion and sequence analysis confirmed that the ligation products from the *sub1Δ* mutant and wild type are not mutagenic (data not shown).

Discussion

DNA end resection has long been regarded as the key step in DSB repair, endorsing HR repair but rejecting NHEJ (Zierhut & Diffley, 2008; Wu et al, 2008; Longhese et al, 2010; Mimitou & Symington, 2011). Here we use the *sub1Δ* mutant to show that rapid resection does not reduce the proficiency of NHEJ. Furthermore, we show evidence that both wild type cells and the *sub1Δ* mutant repairs resected DNA via NHEJ. Thus, we propose DSB resection is not a regulatory process that channels the repair pathway into HR. It is clear that DNA resection is required for HR repair, because ssDNA produced by HR enables loading of recombinational proteins and homology search (Krogh & Symington, 2004). However, our data indicate that NHEJ can still operate after DSB ends are resected. It remains to be determined if NHEJ is constitutively active on the DSBs after the end is resected, or if NHEJ is resumed following the failure of homology search. Our results also suggest that loss of NHEJ in the *yku70Δ* mutant is not solely the result of rapid DSB resection, but suggests an additional role for Ku proteins in NHEJ.

The plasmid repair assay has been used extensively to study NHEJ in yeast cells (Haber, 2002; Daley et al, 2005; Valencia et al, 2001; Downs et al, 2000). Our data

suggest that caution must be taken when interpreting the results from plasmid assays as they may not apply to the repair of chromosomal breaks. In fact similar results have been seen in the yeast *rad9Δ* mutant. Rad9 is a chromatin-binding protein that signals cell cycle arrest in response to DNA damage (Hammet et al, 2007). It was reported that the *rad9Δ* mutant has rapid DSB resection (Lazzaro et al, 2008), reduced plasmid ligation efficiency (de la Torre-Ruiz & Lowndes, 2000), but normal NHEJ repair of chromosomal DSBs (Daley et al, 2005). Because *SUB1*'s human homolog PC4 has been shown to be a chromatin binding protein like Rad9 (Das et al, 2006), *SUB1* may have a similar role to *RAD9* in DNA resection and NHEJ repair, or Rad9 may regulate Sub1. However, unlike *RAD9*, *SUB1* appears not to be involved in checkpoint activation (manuscript submitted).

It is also interesting to note that rapid DSB resection in the *sub1Δ* mutant does not increase mutagenic NHEJ events as observed in the *yku70Δ* mutant. This suggests that the KU complex is essential to maintain the fidelity of NHEJ and cells do not automatically use micro-homology mediated NHEJ (MM-NHEJ) on resected DNA.

Using the *exo1Δ sgs1Δ* double mutant that is defective in DSB resection, we showed that rapid DSB resection in the *sub1Δ* mutant might account for the apparent low ligation efficiency observed in the plasmid assay. Rapid DNA resection in the *sub1Δ* mutant may have caused loss of essential sequences of linear plasmid DNA upon entry into cells during transformation. In fact, it has been observed that DNA resection in the Ku-deficient cells may cause excess degradation of extrachromosomal DNA (Liang & Jasin, 1996). In contrast, bidirectional resection of chromosomes begins at the DSB and

will not cause chromosomal loss because the 3'-strands are not degraded by exonucleases approaching from the 5' ends. The resected DNA can be filled by DNA polymerase allowing completion of NHEJ-mediated repair, because both HO and I-SceI produce 3' sticky ends with 4 base pair overhangs, making it possible that resected ssDNA may anneal at the 4 base pair overlaps and subsequently enable the 5'-3' DNA synthesis to fill in the ssDNA gaps. Additionally, DNA ends of chromosomal breaks are held together by the nuclear matrix, but naked plasmid DNA is not (Lisby et al, 2003a; Kaye et al, 2004). This difference may make the NHEJ machinery function differently on plasmid DSBs versus chromosomal breaks. It is not clear why the *sgs1Δ exo1Δ* double knockout does not fully rescue the plasmid ligation deficiency of the *sub1Δ* mutant. Residual resection may contribute to loss of linear plasmids. Alternatively other properties of Sub1 may affect the stability of the linear plasmid. For example, we have found that Sub1 functions to protect DNA from oxidative damage and that the mutant has increased intracellular ROS (manuscript submitted). Elevated levels of oxidative stress might reduce the stability of the transformed DNA in the cells.

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3. Materials and methods

Yeast strains. Yeast strains used in section 2.2 are listed in Table 2.2. MVY150 (wild type), MVY151 (*rad26Δ*), MVY169 (*sub1Δ*) are used in the TCR assay. MVY150 (wild type), MVY169 (*sub1Δ*) , MVY154 (*rad16Δ*), and MVY348 (*rad16Δ sub1Δ*) are examined for repair of the mating type locus. The PCR based gene replacement method was used to create the yeast knock out strains (Baudin et al, 1993). Yeast media was prepared as described (Adams et al, 1997).

Transcription-coupled repair assay. MVY150 (wild type), MVY169 (*sub1Δ*), and MVY151 (*rad26Δ*) are grown over night at 30°C to mid log phase, UV irradiated, cultured for different times, and subjected to the TCR assay (see Appendix I for a detailed protocol).

Repair of the mating type locus. MVY150 (wild type), MVY169 (*sub1Δ*), MVY154 (*rad16Δ*), and MVY348 (*rad16Δ sub1Δ*) are grown at 30°C to mid log phase and irradiated with UV at 1.72 J/m^2/s for 42 seconds. After cultured in the YPD medium for indicated times, genomic DNA is exacted and subjected to the Southern analysis similar to the TCR assay in Appendix I, except HaeII is used to digest the genomic DNA and a dsDNA probe specific to the mating type locus is used to probe the nylon membrane.

Transcription recovery. MVY154 (*rad16Δ*) and MVY348 (*rad16Δ sub1Δ*) are irradiated with UV at 1.71J/m^2/s for 42 seconds. Yeast total RNA is extracted after culturing the cells in the YPD medium for different times and subjected to Northern analysis as described (He et al, 2003).

Plasmid ligation assay. The yeast transformation procedure is as described in Knop et al. (Knop et al, 1999). Briefly, cells are grown to log phase, sonicated briefly, made transformation-competent and used immediately or stored at -80°C. 100-200ng DNA is used in each transformation. After transformation, cells are plated directly onto drop-out media or incubated in YPD medium (1% yeast extract, 2% peptone, 2% glucose) for 2 hours before plating onto YPD containing 200μg/ml G418.

The plasmid religation assay is similar to the assay described by Schär et al. (Schär et al, 1997). Linearized plasmid DNA is produced by digesting pMV1328 with NcoI or NruI followed by gel-purification. Both linearized and circular plasmid are used to transform the yeast cells. Colonies are counted 3-4 days after transformation. The plasmid ligation efficiency is calculated as the transformation efficiency of the linearized plasmid divided by the transformation efficiency of the circular plasmid.

To quantify the ratio of mutagenic ligation events, yeast cells are transformed with NcoI-linearized pMV1328, selected on the leucine drop-out plates, and then streaked on YPD agar medium containing 200μg/ml G418 to test KanMX function. Colonies that are Leu+ but G418-sensitive are counted toward mutagenic ligation events.

HO induction and cell survival. Wild type and the mutant cells are incubated in YEP-raffinose (1% yeast extract, 2% peptone, 2% raffinose) to log phase (OD_{600}<0.8) and sonicated briefly. Half of each culture is supplemented with 2% galactose to induce the HO endonuclease for indicated times. Both the induced and uninduced cells are diluted in

water and plated onto YPD agar medium to count the number of viable cells. The presence of glucose in the YPD medium suppresses HO expression.

Suicide deletion assay. The suicide deletion assay is performed as described in Karathanasis and Wilson (Karathanasis & Wilson, 2002). Briefly, wild type and mutant cells are incubated in drop-out medium lacking uracil for 3 days to reach stationary phase. Cells are then sonicated briefly and diluted in water. Dilutions are plated on synthetic complete agar media with glucose as the carbon source (SC-glucose) to count the total cell number or on synthetic complete agar media with galactose as the carbon source (SC-galactose) to induce I-SceI and select for cells that repair the induced chromosome break. Survivors on SC-galactose exhibit a white color if the *ADE2* gene is restored by NHEJ repair, or a red color if the repair is inaccurate. The frequency of mutagenic ligation is the number of red colonies divided by the total number of colonies on the SC-galactose plates.

Quantitative real-time PCR. Wild type and mutant cells are incubated in uracil drop-out media with raffinose as carbon source (SC-Ura-raffinose) for 4 days, diluted 1:10 in SC-Ura-raffinose and cultured overnight. 2% galactose is added to cultures to induce I-SceI. After the addition of galactose, equal volumes of cells are taken every hour for 7 hours. Yeast genomic DNA is extracted from the cells using a rapid phenol-glass beads method as described (Sugawara & Haber, 2006). Quantitative real-time PCR is performed as described Wu et al. (Wu et al, 2005). Briefly the Stratagene Brilliant SYBR Green QPCR Master Mix was used in a 20μL reaction volume. Primers SDf

(CGGACAAAACAATCAAGTATGG) and SDr (GTTTGCTGCCTCAACAATCA) are used at 200nM concentration to amplify a 126bp ligation product that is produced only after repair. Equal loading of the template DNA is confirmed by quantitating the genomic DNA on agarose gels. The reaction is heated to 95°C for 10 minutes followed by 45 cycles of 95°C for 30 seconds, 55°C for 20 seconds, and 72°C for 20 seconds. The abundance of the ligation products at time 0 after induction is normalized to 1. Therefore, the relative abundance at other time points is calculated as $2^{-(Ct-C0)}$ where Ct and C0 are the threshold cycle values at time point t and time point 0.

DSB resection assay. The resection assay is similar to the method used by other laboratories (Zierhut & Diffley, 2008; Papamichos-Chronakis et al, 2006; Kegel et al, 2001; Mimitou & Symington, 2008) with modifications. Briefly, chromosomal DSBs are induced as described above in the "HO induction and cell survival" section and yeast genomic DNA is extracted using the phenol-glass beads method (Sugawara & Haber, 2006). Equal amounts of genomic DNA are digested with StyI overnight, resolved by alkaline gel electrophoresis, neutralized, transferred to Zeta-Probe blotting membranes (Bio-Rad, Hercules, CA), and probed for the *PDR5* gene and the mating type locus as described in Figure 2.5a.

4. Figures and legends

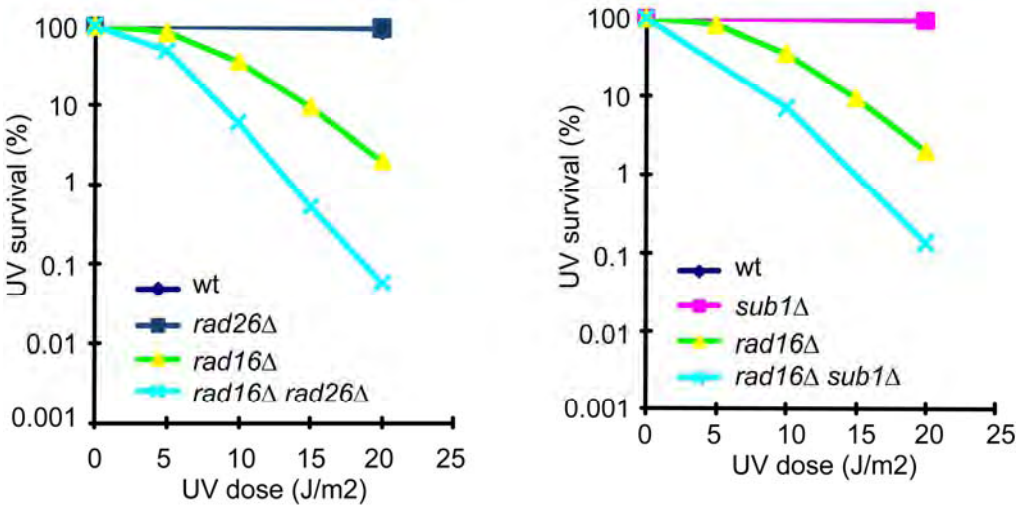
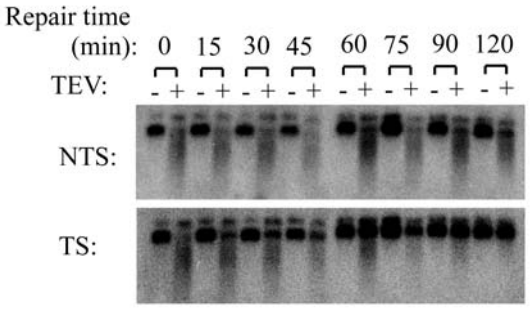


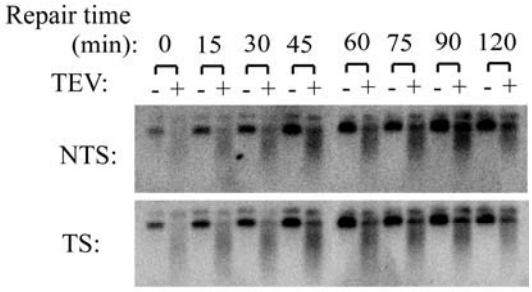
Figure 2.1 The UV sensitivity of the *sub1Δ* mutant mimics that of the *rad26Δ* mutant. Both the *sub1Δ* mutant and the *rad26Δ* mutant are not sensitive to UV treatment. They become sensitive when the *RAD16* gene is knocked out. *RAD16* is an essential gene in global genomic repair pathway. Data were provided by Dr. Volkert.

Figure 2.2. *SUB1* is not required for transcription-coupled repair of UV-induced DNA damage. Cells are irradiated with UV at 1.89J/m^2/s for 40 seconds and cultured in YPD medium for indicated times. Genomic DNA is digested with NruI and with or without T4 endonuclease V (TEV), separated on alkaline agarose gels, transferred onto nylon membranes, and probed with strand-specific RNA probes for the *RPB2* genomic locus. UV-induced CPDs in DNA causes smearing of the TEV-treated DNA band on the membranes. **A.** Repair in wild type (MVY150). CPDs disappear quickly from the transcribed strand (TS) of *RPB2* but not from the non-transcribed strand (NTS), suggesting wild type cells have efficient transcription-coupled repair (TCR). **B.** TCR is impaired in the *rad26Δ* mutant (MVY151). CPDs are removed slowly from both the transcribed and non-transcribed DNA strands in the the *rad26Δ* mutant. **C.** CPDs disappear quickly from the TS DNA strand in the *sub1Δ* mutant (MVY169), suggesting that *SUB1* is not required for TCR.

A. Wild type



B. *rad26*Δ



C. *sub1*Δ

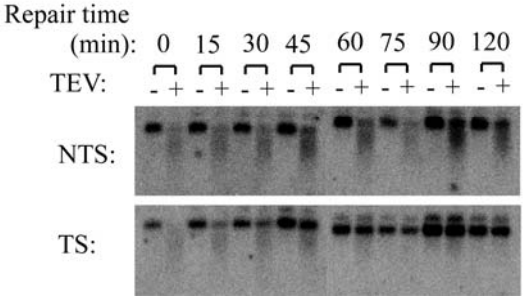
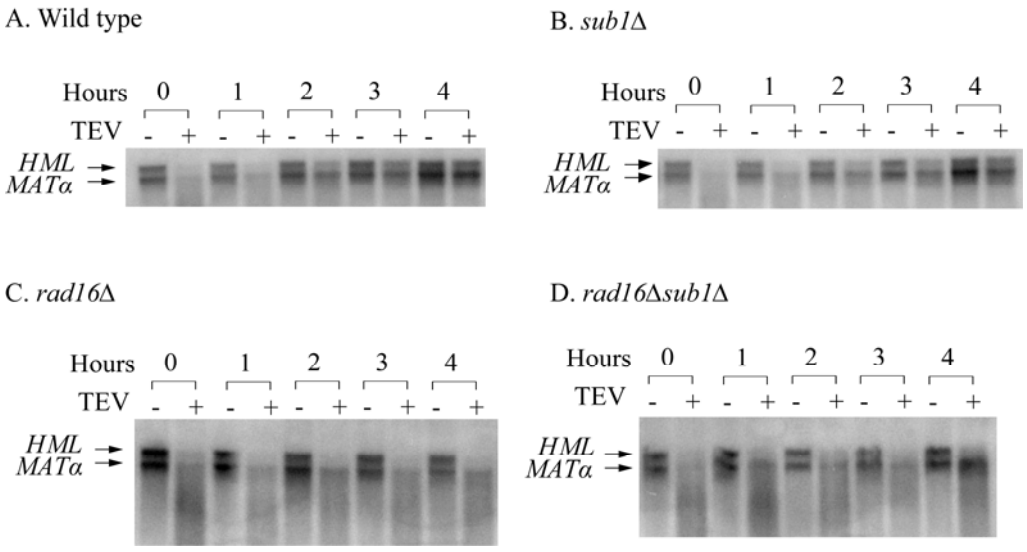


Figure 2.3 *SUB1* is not required for repair of transcription silent regions in the genome. MVY150 (wild type) and its derivative MVY169 (*sub1Δ*), or MVY154 (*rad16Δ*) and its *sub1Δ* derivative (*rad16Δ sub1Δ*), are irradiated with UV at 1.72J/m^2/s for 42 seconds and cultured in the YPD medium for indicated times. Genomic DNA is then extracted, digested with HaeII, and separated on alkaline agarose. After the DNA is transferred to a nylon membrane, the membrane is probed with a ^{32}P-labeled DNA probe specific for the mating type locus sequence. **A** and **B**: The *HML* and *MATα* loci are repaired within 4 hours in both wild type and the *sub1Δ* mutant, suggesting *SUB1* is not required for repair of the mating type loci. **C** and **D**: When *RAD16* is deleted, repair of the transcription-silent *HML* region is impaired, but *MATα* is repaired in both *rad16Δ* and *rad16Δ sub1Δ*, suggesting *SUB1* is not required for repair of *MATα*.

Figure 2.3


A. Wild type
Hours 0 1 2 3 4
TEV - + - + - + - + - +
HML
MATα
B. sub1Δ
Hours 0 1 2 3 4
TEV - + - + - + - + - +
HML
MATα
C. rad16Δ
Hours 0 1 2 3 4
TEV - + - + - + - + - +
HML
MATα
D. rad16Δsub1Δ
Hours 0 1 2 3 4
TEV - + - + - + - + - +
HML
MATα

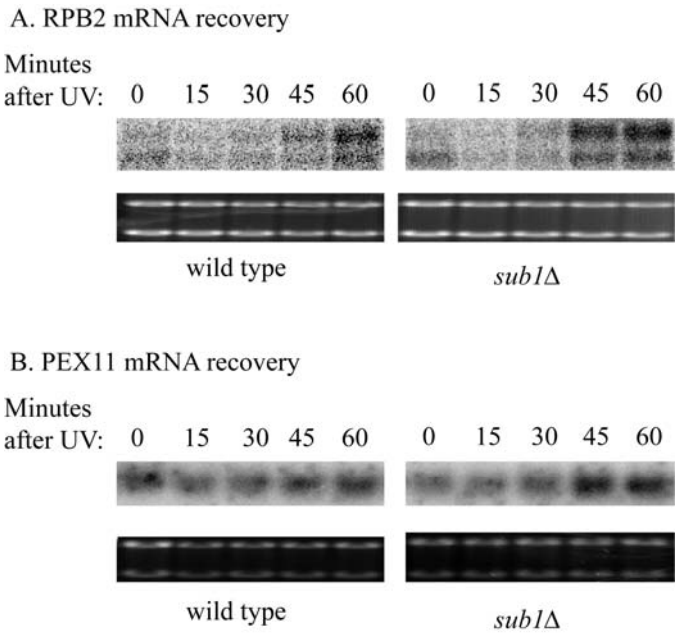
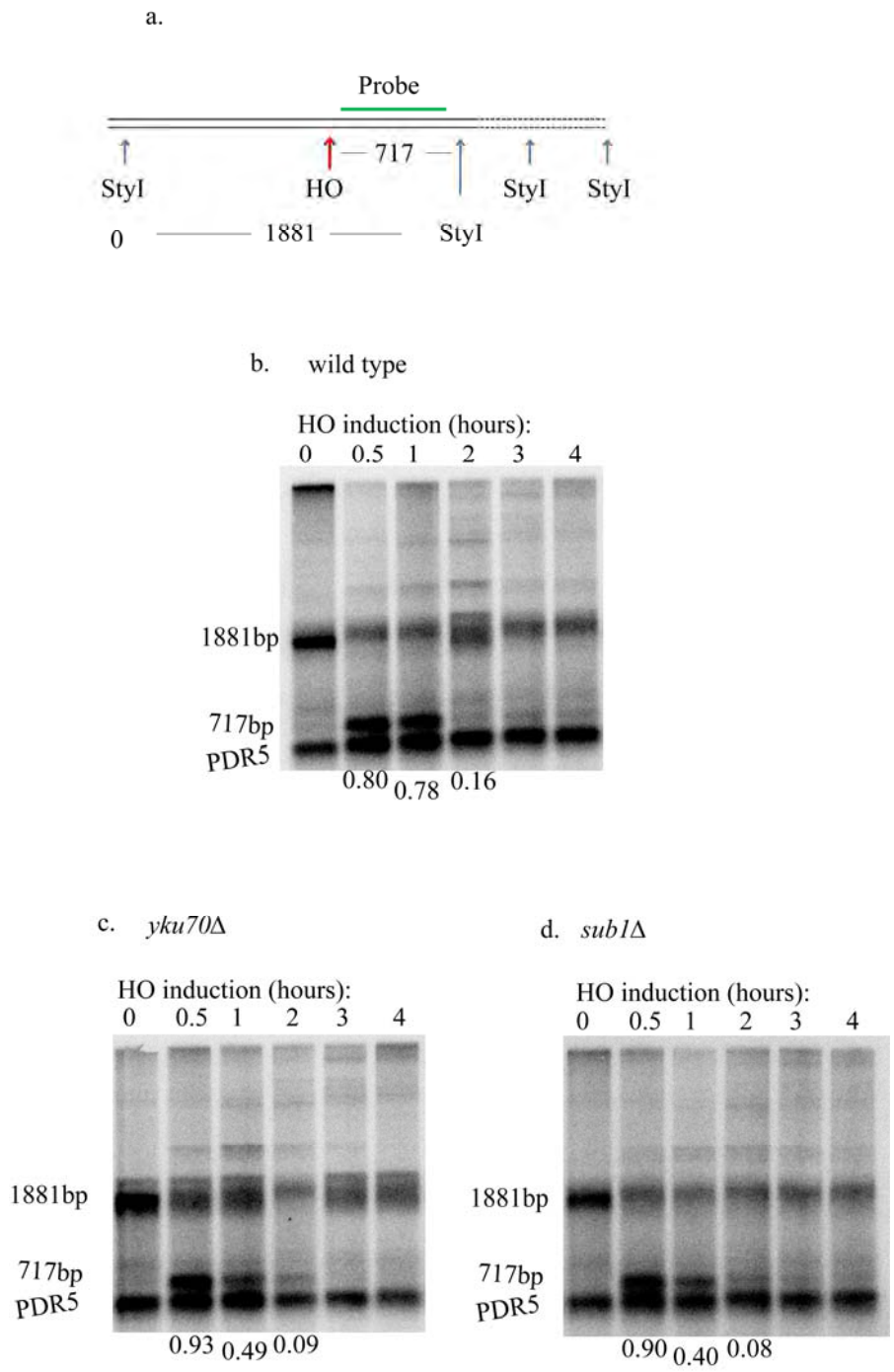


Figure 2.4. transcription recovery after UV damage. Cells are treated with UV at 1.71J/m^2/s for 42 seconds and cultured in the YPD medium for different times. Yeast total RNA is extracted using the hot-phenol method and subjected to Northern analysis (upper panels) (He et al, 2003). Ribosomal RNA is shown as a loading control (lower panels). **A**. mRNA for the *RPB2* gene is probed on the membrane. *RPB2* exhibits two forms of mRNA and the transcription is initially inhibited by UV damage (compare lanes of 15 minutes and 30 minutes). Both wild type (MVY154) and the *sub1Δ* mutant recover the *RPB2* mRNA level within an hour, suggesting *SUB1* is not required for transcription recovery following UV damage. **B**. The *PEX11* mRNA is probed. The deletion of *SUB1* does not appear to affect transcription inhibition of this transcript either.

Figure 2.5. DSB resection is more rapid in the *sub1 Δ* and *yku70Δ* mutants than in wild type. a. Schematic representation of chromosome III in yeast JKM179 (Haber, 2002). StyI digestion yields the 1881bp DNA fragment that is cut in middle by HO generating the shorter 717bp DNA fragment. StyI does not cut ssDNA generated by DSB resection, leading to a gradual loss of the 717bp fragment after DSB induction. The position of the probe and additional StyI sites are indicated. **b**. Analysis of DSB resection in wild type (MVY610) after DSB is induced for indicated times. The positions of the 1881bp band and the 717bp band are indicated. The PDR5 gene is shown as the loading control. The ratio between the intensities of the 717bp DNA and PDR5 gene is calculated and indicated under corresponding lanes. **c**. Analysis of DSB resection in the *yku70 Δ* mutant (MVY614), details as in b. **d**. Analysis of DSB resection in the *sub1Δ* mutant (MVY617), details as in b.

Figure 2.5


a.
Probe
717
StyI
HO
StyI
StyI
0
1881
StyI
b. wild type
HO induction (hours):
0 0.5 1 2 3 4
1881bp
717bp
PDR5
0.80 0.78 0.16
c. yku70Δ
HO induction (hours):
0 0.5 1 2 3 4
1881bp
717bp
PDR5
0.93 0.49 0.09
d. sub1Δ
HO induction (hours):
0 0.5 1 2 3 4
1881bp
717bp
PDR5
0.90 0.40 0.08

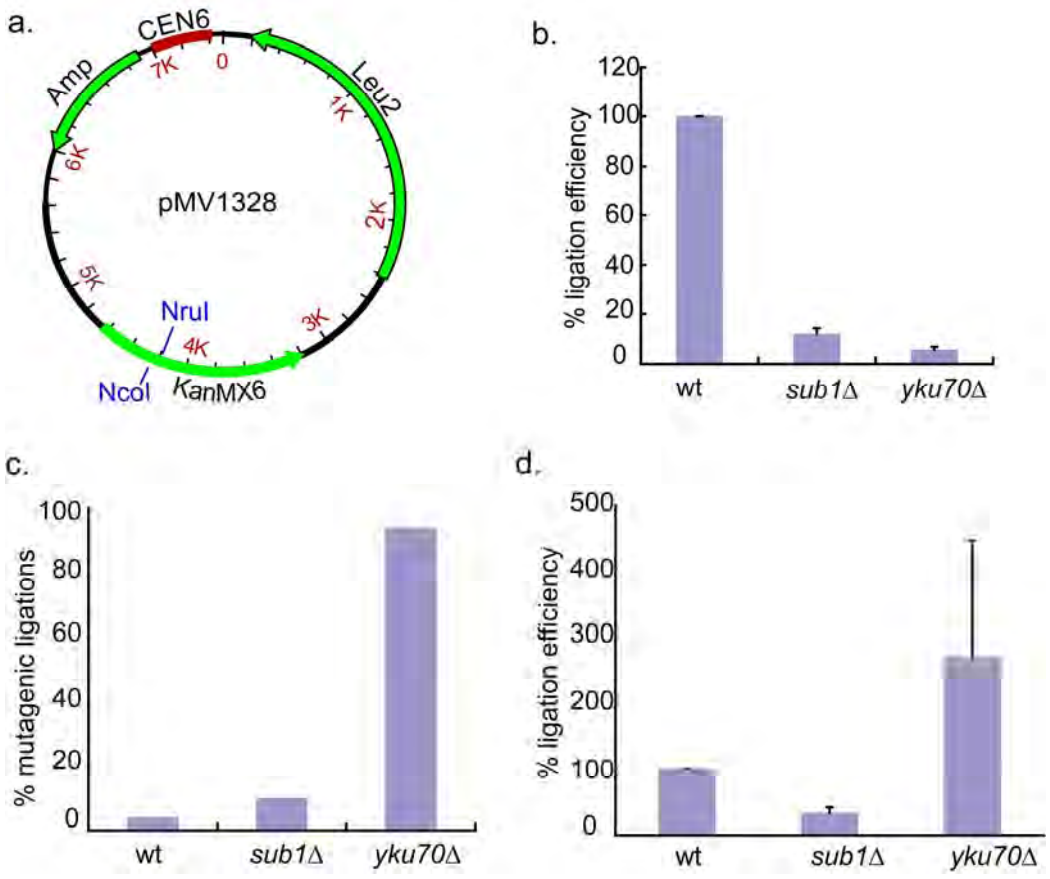


Figure 2.6. SUB1 is required for plasmid ligation. wt: MVY101, *sub1Δ*: MVY105, *yku70Δ*: MVY601 **a,** Map of the yeast plasmid pMV1328 used in the ligation assay. The unique NcoI and NruI restriction sites in the KanMX6 gene are indicated. **b,** Ligation of NcoI-digested pMV1328 in the cells. Competent cells are transformed with DNA and plated on Leu-dropout medium. The ligation efficiency of wild-type is normalized to 100%. Undigested plasmids are used to determine relative transformation efficiencies. **c,** Frequency of mutagenic ligations in transformants obtained in **b**. **d,** Similar to **b** except that blunt-ended NruI-linearized pMV1328 is used. Error bars represent standard deviations.

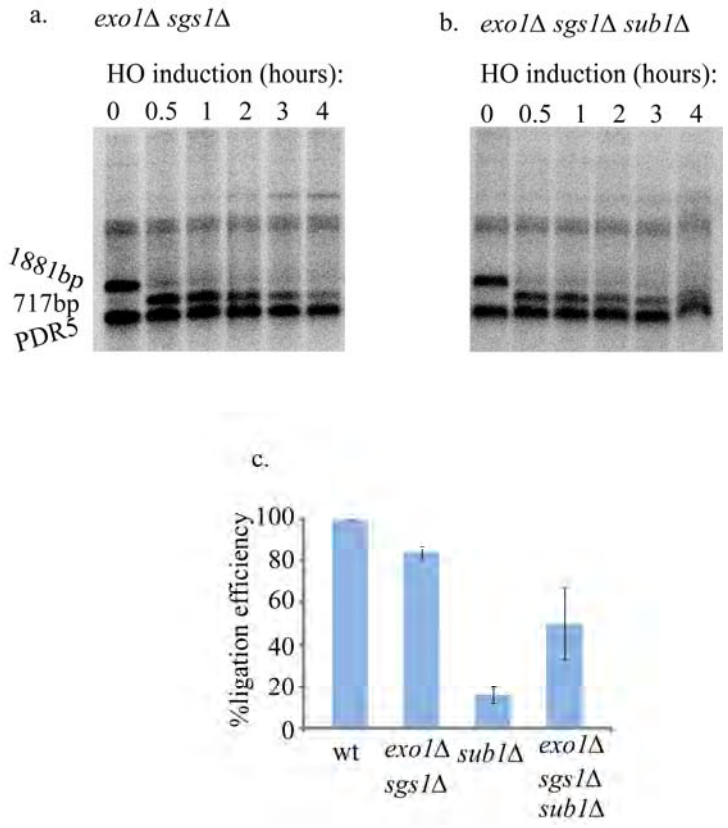


Figure 2.7. Slow resection alleviates poor plasmid recovery in the *sub1Δ* mutant. wt: MVY816, *exo1Δsgs1Δ*: MVY817, *sub1Δ*: MVY840, *exo1Δsgs1Δsub1Δ*: MVY823. **a.** The *exo1Δ sgs1Δ* double mutant exhibits slow DSB resection compared to wild type shown as in Figure 2.5b. See Figure 2.5 for experimental design and figure details. **b.** The *exo1Δ sgs1Δ sub1Δ* triple mutant has slow DSB resection similar to its parental strain. **c.** Recovery of NcoI-digested pMV1328 in cells. Experiments are performed as described in Figure 2.6b.

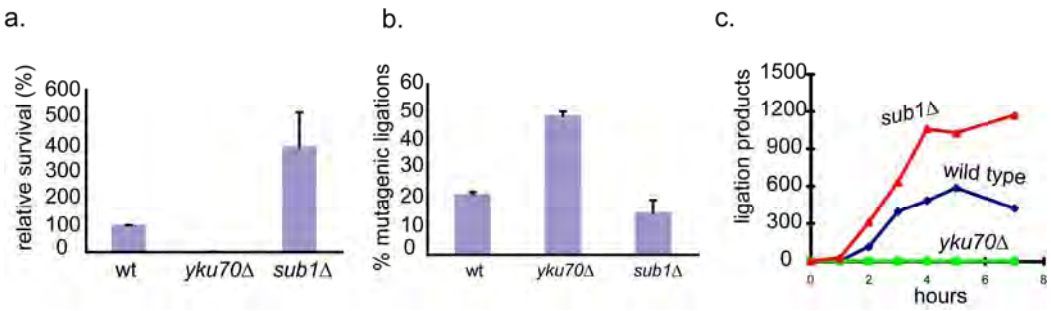


Figure 2.8. The *sub1Δ* mutant exhibits efficient and precise joining of chromosome breaks in the suicide deletion assay. In this assay, two I-SceI induced chromosomal breaks are repaired by NHEJ and precise ligation reconstructs a functional *ADE2* gene. wt:MVY665, *yku70Δ*: MVY666, *sub1Δ*: MVY667. **a.** The *sub1Δ* mutant survives chromosomal breaks better than wild type, indicating an efficient repair by NHEJ. Cell survivals are normalized to wild type. **b.** The *sub1Δ* mutant does not have increased mutagenic ligation events as seen in the *yku70Δ* mutant. **c.** RT-PCR shows that the *sub1Δ* mutant and wild type generate ligation products quickly after DSB induction. In comparison, the *yku70Δ* mutant does not yield detectable ligation products.

Tables

Table 2.1. Yeast cell survival after chromosomal DSB is induced for various times. wild type: MVY610, *yku70Δ*: MVY614, *sub1Δ*: MVY617. a: Difference between *yku70Δ* and wild type is significant. $p<0.05$, $n=3$; b: Difference between *sub1Δ* and wild type is not significant. $p=0.08$, $n=3$.

strains	no induction	1 hour	2 hours	3 hours
wild type	100%	43%	37%	25%
yku70Δ	100%	2%	0.44%	0.33% [a]
sub1Δ	100%	54%	45%	40% [b]

Table 2.2. Yeast strains used in section 2.2.

Strain	Original name, genotype (annotation)	Reference
MVY101	FY833, *MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202*	(Wu et al, 1999)
MVY105	MVY101 with *sub1Δ*::*hisG*	(Wu et al, 1999)
MVY601	MVY101 with *yku70Δ*::*HIS3*	this study
MVY610	JKM179, *MATα hoΔ hmlΔ*::*ADE1 hmrΔ*::*ADE1 ade1-100 leu2,3-112 lys5 trp1Δ*::hisG *ura3-52 ade3*::GAL-HO	(Haber, 2002)
MVY614	MVY610 with *yku70Δ*::*URA3*	this study
MVY617	MVY610 with *sub1Δ*::*TRP1*	this study
MVY816	JKM139, MATa *hoΔ hmlΔ*::*ADE1 hmrΔ*::*ADE1 ade1-100 leu2,3-112 lys5 trp1Δ*::hisG *ura3-52 ade3*::GAL-HO	(Haber, 2002)
MVY817	yGI199, MVY816 with *exo1Δ*::*TRP1 sgs1Δ*::*KanMX*	(Zhu et al, 2008)
MVY823	MVY 817 with *sub1Δ*::*URA3*	this study
MVY840	MVY816 with *sub1Δ*::*TRP1*	this study
MVY665	YW714, *MATa*, ade2::*SD2-*::*URA3 his3D1 leu2D0 LYS2 MET15 ura3D0*	(Karathanasis & Wilson, 2002)
MVY666	YW713, *MATα, ade2::SD2-::URA3 his3D1 Leu2D0 LYS2 MET15 ura3D0 yku70::kanMX4*	(Karathanasis & Wilson, 2002)
MVY667	MVY665 with *sub1::KanMX4*	this study

Chapter III

PC4 protects DNA from oxidative damage

The work presented in this chapter is a manuscript in preparation:

Lijian Yu, Hong Ma, Michael Volkert. PC4 protects DNA from oxidative damage.

Dr. Hong Ma performed the UV sensitivity experiments in Figure 3.3c, the Rad53 immunoblot experiments in Figure 3.4b, and the ROS quantification experiments in Figure 3.6a. Lijian Yu performed all other experiments.

Summary

Reactive Oxygen Species (ROS) are by-products of aerobic metabolism and can damage all cellular components. Oxidative damage may lead to cell death or mutagenesis if the target is nuclear DNA. Previously we found that the human PC4 gene can prevent oxidative mutagenesis in bacteria. In this study we found PC4 and its yeast homolog Sub1 possess antioxidant activity and protect nuclear DNA from oxidative damage. We demonstrate that yeast *SUB1* is induced by oxidative stress and that the *sub1Δ* mutant is sensitive to peroxide treatment due to increased oxidative DNA damage. When expressed in yeast, PC4 reduces intracellular ROS and confers resistance to peroxide treatment. Furthermore we showed that PC4 directly protects DNA from oxidation *in vitro*. Because PC4 is an abundant nuclear protein in human cells, its novel antioxidant activity may play an important role in maintaining genomic stability.

Introduction

All aerobic organisms from bacteria to humans reduce molecular oxygen to produce energy generating Reactive Oxygen Species (ROS) as by-products (Buonocore et al, 2010). ROS can damage cellular components including lipids, proteins, RNA and DNA, triggering a variety of disease including cancers, neurodegenerative diseases and aging (Duracková, 2010; Valko et al, 2006). To combat ROS, cells contain numerous antioxidant defenses to prevent cellular damage (Go & Jones, 2010).

The cell nucleus is a more reducing environment than the cytoplasm (Go & Jones, 2010), however oxidative DNA damage still occurs and numerous pathways repair oxidative DNA damage (Slupphaug et al, 2003). To date no antioxidant proteins have been reported to function exclusively in the nucleus, or to protect DNA specifically, although some isoforms of cytoplasmic antioxidant proteins have been found in the nucleus (Go & Jones, 2010; Lukosz et al, 2010).

PC4 is an abundant nuclear protein with only 127 amino acids that was isolated from HeLa cell nuclear extracts and shown to enhance transcription *in vitro*, a function requiring the serine and lysine rich N terminal domain (amino acids 1-40) (Kretzschmar et al, 1994). It is also a DNA binding protein and PC4 binds to both double strand DNA (dsDNA) and single strand DNA (ssDNA), without apparent sequence specificity but with higher affinity to partially unpaired dsDNA and ssDNA (Kaiser et al, 1995; Werten et al, 1998a). This function requires the carboxyl-terminal ssDNA-binding activity (amino acids 63-91) (Kretzschmar et al, 1994; Wang et al, 2004). The Yeast Sub1 protein

is highly homologous to PC4 in the conserved DNA binding and dimerization domains (47% identity) and Sub1's active role in transcription, which depends on its homologous N-terminal domain, is suggested by its interactions with yeast transcription factor TFIIB and its stimulation of GCN4 and HAP4 promoters when over-expressed in yeast (Henry et al, 1996; Knaus et al, 1996). Similar to PC4, Sub1 binds nonspecifically to ssDNA and dsDNA with higher affinity to ssDNA (Henry et al, 1996).

PC4 was also identified in a screen for human genes that can prevent oxidative mutagenesis in bacteria (Wang et al, 2004). Tests on yeast showed that the yeast homolog *SUB1* is required for hydrogen peroxide resistance. The recruitment of PC4 to DNA repair sites by XPG protein suggests a possible role of PC4 in DNA repair (Wang et al, 2004). However, its role in cellular oxidative stress is not clear.

We confirm that PC4 complements the peroxide sensitivity of the yeast *sub1Δ* mutant, indicating a conserved function between Sub1 and PC4 in preventing oxidative killing. We show that the *SUB1* gene is induced by oxidative stress and *sub1Δ* mutants suffer from increased DNA damage and elevated ROS levels. We also demonstrate that purified PC4 protein prevents oxidative DNA damage *in vitro*.

Results

Yeast *SUB1* gene encodes a nuclear protein and is induced by oxidative stress. The nuclear localization of Sub1 is inferred from its sequence homology to PC4 and its role in transcription regulation (www.yeastgenome.org). To determine if Sub1 exhibits the expected nuclear localization, we fused the Green Fluorescent Protein (GFP) gene to the *SUB1* gene. Sub1-GFP appears to distribute evenly and exclusively in the nucleus, whether it is driven from its own promoter on the chromosome (Figure 3.1a), or overexpressed from the methionine promoter (Figure 3.1b).

Consistent with many other ROS resistance genes (Kim et al, 1989; Salmon et al, 2004), we found the *SUB1* mRNA levels increase considerably after treatment with 5mM H_2O_2 (Figure 3.2), whereas the *PDR5* control shows reduced mRNA levels, presumably due to DNA or RNA damage.

Both PC4 and Sub1 protect yeast cells from oxidative killing. Previously Wang et al. showed that deletion of the *SUB1* gene in yeast causes hypersensitivity to peroxide treatment and that truncated PC4, which contains amino acids 40-127, protects the *sub1Δ* mutant from peroxide treatment (Wang et al, 2004). Here we found that truncated and full length PC4 both complement the peroxide sensitivity of the *sub1Δ* mutant (Figure 3.3), suggesting that the oxidation protection functions of PC4 and *SUB1* are interchangeable in yeast. The ability of the truncated form of PC4 to fully complement the *sub1Δ* mutant suggests that transcription regulation is not required for the oxidation resistance function of PC4, since the deleted segments are required for transcription activation (Kretzschmar

et al, 1994). This is also consistent with our previous finding that this same truncated form of PC4 prevents oxidative mutagenesis when expressed in bacteria (Wang et al, 2004), as it is highly unlikely that a human gene will specifically regulate bacterial oxidative resistance genes.

***SUB1* is not required for checkpoint activation in response to oxidative stress.** We have previously noted that the *sub1Δ* mutant has a longer doubling time than wild type, suggesting cell cycle delays in the *sub1Δ* mutants. Because yeast mutants deficient in cell cycle checkpoint activation are hypersensitive to oxidative stress (Leroy et al, 2001), we investigated cell cycle progression of the *sub1Δ* mutant using FACS analysis. Figure 3.4a shows that the *sub1Δ* mutant, like the wild type, can be synchronized by alpha factor in G1 phase and will initiate chromosomal replication after α factor removal. Nonetheless, the *sub1Δ* mutant shows a broader shoulder between the 1N and 2N peaks (compare wild type and the *sub1Δ* mutant at 30min - 60min after α factor release), indicating unsynchronized DNA replication, possibly accounting for the *sub1Δ* mutant's longer doubling time. Importantly, in the presence of 0.8 mM hydrogen peroxide, both the wild type and the *sub1Δ* mutant arrest the cell cycle, suggesting the *sub1Δ* mutant, like wild type, can respond to oxidative damage and arrest the cell cycle. Notably, the duration of the *sub1Δ* mutant's arrest in G1 phase is longer than wild type, suggesting that the *sub1Δ* mutant may generate more DNA damage after treated with the same amount of peroxide.

To confirm that checkpoint activation is functional in the *sub1Δ* mutant, we measured Rad53 phosphorylation, an indicator of checkpoint activation (Sanchez et al,

1996). In the absence of DNA damaging treatments, Rad53 is not phosphorylated in either the *sub1Δ* mutant or wild type (Figure 3.4b). However, when treated with UV irradiation or hydrogen peroxide, Rad53 from the wild type and *sub1Δ* mutant strains migrates slower on the PAGE gel, indicating phosphorylation of Rad53 and checkpoint activation, demonstrating that the *sub1Δ* mutant responds to DNA damage and arrests its cell cycle.

Elevated levels of oxidative damage to DNA in the *sub1Δ* mutant. The FACS analysis indicates that the *sub1Δ* mutant might suffer from more oxidative damage after peroxide treatment. Considering the nuclear localization of Sub1, we tested if the *sub1Δ* mutant has more single strand DNA breaks (SSBs) after peroxide treatment. SSBs are common DNA lesions caused by ROS (Imlay & Linn, 1988) and can be visualized by the alkaline comet assay (Azevedo et al, 2011). Figure 3.5a shows that yeast chromosomal DNA migrates out of the nucleus in an electric field, forming a comet like shape with a bright nuclear center. After treating the cells with peroxide, more DNA migrates out of the nucleus diminishing its intensity and the DNA becomes more diffuse (compare left panels with right panels in Figure 3.5a), indicating SSBs are generated by peroxide treatment. Comets of the *sub1Δ* mutant are essentially devoid of their nuclei and are more diffused than those of wild type after peroxide treatment, indicating peroxide produces more oxidative DNA damage in the *sub1*Δ mutant.

To confirm the implications of the comet assay, we used alkaline gel electrophoresis to measure peroxide-induced SSBs (Figure 3.5b). Under the conditions

used, large fragments of genomic DNA cluster and migrate as a single high molecular weight band very near the top of the gel (Figure 3.5b). When cells are exposed to peroxide and the DNA is harvested immediately after treatment, the dose dependent decrease in full-length, undamaged DNA is greater in the *sub1Δ* mutant than in wild type. This is most apparent at the highest dose used (10mM peroxide), indicating that the same peroxide dose produces more SSBs in the *sub1Δ* mutant than wild type.

Double strand DNA breaks (DSBs) are a more lethal form of oxidative DNA damage, which can be produced by ROS directly, or possibly from SSBs during DNA replication. We used Pulse Field Gel Electrophoresis (PFGE) to test if peroxide produces more DSBs in the *sub1Δ* mutant (Figure 3.5c). DSBs generated by peroxide treatment cause a reduction in intensity of the full-length chromosome bands seen on the gel. Since one DSB per chromosome results in its loss from the full-length band, the largest chromosomes are more vulnerable to damage, because they are larger targets. Figure 3.5c demonstrates that the *sub1Δ* mutant contains more DSBs than the wild type, especially at the highest doses of peroxide.

The increase in DNA damage can be the result of greater production or less repair of DNA damage during oxidative challenge to the cells. In order to test the SSB repair activity of the *sub1Δ* mutant, cells are treated with peroxide, washed twice with fresh medium, and incubated to allow repair to occur. Figure 3.5d demonstrates that peroxide treatment damaged the majority of the genomic DNA in both the wild type and the *sub1*Δ mutant. Since both strains repair most of the SSBs within 30 minutes, this result suggests

both wild type and *sub1Δ* mutant strains have a robust SSB repair activity. The *sub1Δ* mutant appears to have more SSBs after 30 minutes of repair which could result from more initial SSBs at the same dose (Figure 3.5b and compare Figure 3.5d lanes 2 and 6)

The major repair pathways of DSB in yeast include Nonhomologous End-Joining (NHEJ) and Homologous Recombination (HR). However, the *dnl4Δ* mutant that is deficient in NHEJ is not sensitive to peroxide (Lijian Yu, data not published), making it unlikely that NHEJ deficiency is the reason why the *sub1Δ* mutant is oxidation sensitive. This conclusion is further supported by the observation that the *sub1*Δ mutant shows normal levels of NHEJ (Table 2.1) when this is tested in a strain that can repair HO sites only by NHEJ due to deletion of the silent mating type loci (Haber, 2002). Therefore we tested if Sub1 plays a role in the recombinational repair that is dependent on Rad52. We compared the peroxide sensitivity of *sub1Δ* and *rad52Δ* mutants with a *sub1Δ rad52Δ* double mutant. Figure 3.5e shows that the *sub1Δ* mutant is more sensitive than the *rad52Δ* mutant and the double mutant is more sensitive than either of the single mutants, indicating that the peroxide sensitivities are additive and that Sub1 does not function in the Rad52-dependent homologous recombination pathway. The lack of a role in recombination repair is also consistent with the lack of γ-ray sensitivity (J. Westmoreland and M. A. Resnick, personal communication). Collectively these data indicate that the increased DSB in peroxide-treated *sub1Δ* mutants is not caused by reduced DSB repair activity. Rather, it is more likely that peroxide produces more oxidative damage in the *sub1Δ* mutant.

PC4 and Sub1 protect DNA by reducing ROS. To test if the observed higher levels of oxidative DNA damage in the *sub1Δ* mutant is produced by higher levels of ROS, we used DCFH-DA stain to quantify intracellular ROS (Davidson et al, 1996). Figure 3.6a shows that the *sub1Δ* mutant has elevated ROS levels compared to wild type after peroxide treatments. In fact, the *sub1Δ* mutant has higher ROS levels without any treatment, suggesting chronic elevated levels of ROS (Figure 3.6a). This may account for the increased doubling time of the *sub1Δ* mutant, seen even in the absence of peroxide treatment. Because PC4 and the PC4-CTD (residues 40-127) can complement the *sub1Δ* mutant, we tested the ROS levels in the *sub1Δ* mutant expressing PC4-CTD and found it reduces ROS in peroxide-treated and untreated cells.

Because the PC4-CTD mutant lacks sequences required for its transcription regulation function, we reasoned that PC4 and Sub1 do not reduce ROS through transcription regulation in yeast. Rather, it is more likely that PC4 directly reduces ROS in the nucleus. This possibility was tested *in vitro* using the Metal ion Catalyzed Oxidation (MCO) assay (Park & Floyd, 1994). This assay uses the mixture of Fe^{3+} and DTT in the presence of oxygen to produce ROS, particularly H_2O_2 and hydroxyl radicals, producing SSBs and 8-hydroxy-2'-deoxyguanosine in the DNA (Park & Floyd, 1994). Introduction of SSBs converts the supercoiled pUC19 substrate DNA to relaxed and linear forms and causes further degradation as damage levels increase. Figure 3.6b shows that in pUC19 DNA relaxation occurs as a function of exposure time and extensive degradation of the plasmid is seen at 60 minutes (Figure 3.6b). Purified PC4 protects the DNA from oxidation in a dose dependent manner (Figure 3.6c,d, e). At 20ng/ul, PC4

completely blocks the oxidation of the plasmid DNA. Based on the protein and DNA concentrations used, only about one third of the bases can potentially be bound by PC4. This demonstrates that purified PC4 protein can prevent oxidative DNA damage. In comparison, BSA provides no protection of plasmid DNA even at much higher concentrations (Figure 3.6e, lane 8).

Discussion

In summary, we found that the human PC4 protein and its yeast homolog Sub1 have a novel antioxidant activity to protect genomic DNA from oxidative damage. The yeast *sub1Δ* mutant is sensitive to peroxide and displays increased levels of ROS and oxidative DNA damage after peroxide treatment. PC4 is able to protect DNA from oxidative damage in bactera, in yeast, and *in vitro*. Therefore the antioxidant activity of PC4 appears to be intrinsic to this small protein, and we predict that it plays a role in protecting the human genome. Its high abundance further suggests that it may be a key factor controlling nuclear redox homeostasis (Werten et al, 1998b).

PC4 family members have been proposed to function in many processes: They are recruited to transcription complexes (Kretzschmar et al, 1994; Kaiser et al, 1995), DNA repair complexes (Wang et al, 2004; Mortusewicz et al, 2008), and possibly double strand breaks (Mortusewicz et al, 2008; Batta et al, 2009) and replication complexes (Pan et al, 1996). Since PC4 binds to DNA with a strong preference for unpaired double-stranded DNA regions and single-stranded DNA (Kaiser et al, 1995; Werten et al, 1998a, 1998b), and our results indicate that it prevents oxidative DNA damage, this suggests a common function for this family of proteins in all of these processes. In each case the DNA is devoid of histones and other potentially protective proteins and is partially unwound, exposing ssDNA regions, and/or unpaired dsDNA regions. The function for PC4 in all of these processes may be to prevent oxidative damage when DNA is most vulnerable to attack by oxidative agents.

The precise chemical mechanism of oxidation protection by PC4 remains to be determined. PC4 shares some weak similarities in function and structure to the bacterial Dps protein, a well-conserved oxidation resistance protein found only in prokaryotes. Dps binds DNA nonspecifically and protects DNA from oxidation through three modes of actions: DNA shielding, iron sequestration, and its ferroxidase activity (Calhoun & Kwon, 2011; Zhao et al, 2002). DNA shielding might play a role in PC4's antioxidant activity because PC4 binds to DNA nonspecifically. However the stoichiometry suggests shielding cannot be the only function, since the co-crystal of PC4 with DNA indicates a single molecule of PC4 can bind to an 8 base loop of ssDNA, or 8 bases of unpaired dsDNA (Werten & Moras, 2006) and full protection from peroxide damage can be attained *in vitro* at a ratio of 1 PC4 molecule to 12 base pairs (Figure 3.6e, lane 6). The observation that PC4 reduces intracellular ROS in yeast further indicates that PC4 plays a more direct role in protecting the DNA.

Oxidative stress is the underlying cause of cancers and many other diseases. Because PC4 is a potent antioxidant protein that specifically protects nuclear DNA, it may play a pivotal role in cancer prevention. Mutations in PC4 may reduce its ability to prevent oxidative DNA damage. In fact, studies have shown that PC4 maps to chromosome locus 5p13 where loss of heterozygosity frequently occurs in bladder and lung tumors (Kannan & Tainsky, 1999), suggesting a potential protective role for PC4 against cancers.

Acknowledgements

We thank Michael Hampsey, James Haber, and Johannes Hegemann for yeast strains and plasmids, Duane Jenness for α-factor, Kenan Murphy and Jen-Yeu Wang for strain construction and Martin Marinus, Anita Fenton , Feng He, Yahui Kong and Hang Cui for technical advice and assistance. This work was funded in part by NIH grant CA100122.

Materials and Methods

Yeast strains and plasmids. Yeast strains used in this study are listed in Table 3.1. All yeast knockout strains were created by PCR based gene replacement methods (Baudin et al, 1993; Adams et al, 1997).

Peroxide sensitivity. Cells are inoculated in 5ml synthetic complete medium or drop out media (for strains carrying plasmids) (Adams et al, 1997) at 30°C for 2 days, diluted to $1x10^6$ cells/ml with fresh media, and cultured at room temperature overnight. When the cell densities reach $1x10^7$cells/ml, cells are briefly sonicated and resuspended to $1x10^7$cells/ml in PBS or YPD liquid medium (Figure 3.5e) with indicated amounts of hydrogen peroxide. After incubation at 30°C for 30 min with agitation, cells are washed twice with PBS to remove H_2O_2, diluted in water, and plated onto YPD agar plates and incubated for two days at 30°C.

UV sensitivity.

To test the UV sensitivity of the *sub1Δ* mutant, cells are cultured to mid log phase in YPD liquid medium at 30°C, resuspended in fresh YPD liquid medium to an OD_{600} of 1, sonicated, cultured for another hour, and then resuspended in PBS to OD_{600} of 0.8. Then cells are UV irradiated for the indicated times under constant agitation. A germicidal UV light source is used to irradiate the cells (GE G15T8, 15watts, 1.71J/m^2/s). After irradiation, cells are diluted in water and plated onto YPD agar plates to form colonies. Plates are counted after 2 days.

Analysis of peroxide induced *SUB1* gene expression. Cells are grown in 20ml YPD liquid medium at 30°Covernight, resuspended in 75ml YPD liquid medium and incubated for 4 hrs. Cell density is adjusted to an OD_{600} of 0.8 and hydrogen peroxide is added at the indicated concentrations. RNA is extracted and measured by northern blotting as described elsewhere (He & Jacobson, 1995).

Western blot analysis of Rad53 phosphorylation. MVY105 (*sub1Δ)* and MVY101 (wild type) cells are inoculated in 5ml YPD liquid medium and grown at 30C overnight. Cells are then diluted to an OD_{600} of 0.3 in 15ml YPD liquid medium and grown for 4 hours at 30C. Cells are either treated by adding 5mM hydrogen peroxide to the culture and incubated at 30°C for 30 minutes, or cells are resuspended in PBS to an OD_{600} of 0.8 and UV irradiated for 20 seconds at 1.71 J/m^2/s, followed by incubation in YPD liquid medium at 30°C for 30 minutes. Cells are then washed once with water and protein extracts are prepared as described (He & Jacobson, 1995). After separation on a 8% SDS-

PAGE gel and transfer onto a PVDF membrane, proteins are immuno-detected using the Rad53 antibody (yC-19, Santa Cruz Biotechnology, CA) after 1:500 dilution using Western blot protocols as described (Haghnazari & Heyer, 2004a).

FACS analysis. Cell cycle progression was monitored as described (Lisby et al, 2003b). MVY105 (*sub1*Δ) and MVY101(wild type) are cultured at 30°C to an OD_{600} of 0.25 in 30ml YPD liquid medium buffered with 50mM sodium succinate (pH=5.0). Synchronization at G1 phase is achieved by adding 3µM α-factor and culturing at 30°C for 2-2.5 hours. After washing off the residual α-factor, cells are cultured at 30°C in pre-warmed YPD with or without 0.8mM hydrogen peroxide. Samples are taken at different times and fixed in 70% ethanol overnight at 4°C. Cells are then washed with PBS, sonicated, resuspended RNase (0.25mg/ml in PBS) and incubated overnight at 37°C. After washing off RNase, cells are stained for 30 minutes with propidium iodide (16 µg/ml in PBS) and analyzed by the Flow Cytometry Core Facility in UMASS Worcester. Data is analyzed using the FlowJo software (Tree Star Inc, Ashland, OR).

Analysis of SSB damage by the comet assay. The yeast comet assay is as described (Olive & Banáth, 2006) with modifications. MVY105 (*sub1Δ*) and MVY101 (wild type) strains are incubated in YPD liquid medium at 30°C overnight to early log phase. Cells are resuspended in 1ml YPD liquid medium at OD_{600} of 0.2 with or without hydrogen peroxide at the concentration indicated and incubated at 4°C for 10 minutes. Cells are then washed and resuspended in 1ml of digestion buffer (1M sorbitol, 0.1M EDTA, pH=7.5). Then 50 µL of cells and 50 µL of 15 mg/ml Zymolyase 20T (Seikagaku

Corporation, Japan) are mixed with 400 µL of 1% low melting agarose gel in the digestion buffer and maintained at 37°C. After incubating at 37°C for 5 min, 50 µL of the mixture is transferred onto a microscope slide precoated with 1% agarose, covered with cover slips, followed by further incubation for 30 min at 30°C to digest the cell wall. Then the cover slips are removed and the cells are lysed at 4°C for 1 hour in lysis solution (50 mM EDTA, 1M NaCl, 30mM NaOH, 0.1% sarcosyl, pH 12.3). After lysis, the slides are transferred into an electrophoresis tank filled with electrophoresis buffer (30 mM NaOH, 10 mM EDTA, pH 12.4). After 30 minutes of denaturation, electrophoresis is performed at 0.5 V/cm for 7 minutes. Then, the slides are washed once with 75% ethanol and once with 95% ethanol, neutralized in 10 mM Tris (pH 7.5) for 10minutes, dried, stained with CYBR gold (Invitrogen, Carlsbad, CA), and photographed on a Nikon Eclipse E800 fluorescence microscope at a magnification of 10 x 40.

Analysis of SSB damage by alkaline gel electrophoresis. MVY150 (wild type) and MVY169 (*sub1*Δ) are grown to early log phase, sonicated, $2x10^8$ cells are then treated with hydrogen peroxide as indicated (Figure 3.5b and 3.5d) in YPD liquid medium at room temperature for 30 minutes. After treatment, cells are washed once with YPD and once with the digestion buffer (1 M sorbitol, 0.1 M EDTA, pH 7.5) and resuspended in 1ml of digestion buffer supplemented with 25 µL, 15 mg/ml Zymolyase 20T (Seikagaku Corporation, Japan). After digestion at 37°C for 1-3 hours, cells are lysed and denatured as in the comet assay described above, followed by electrophoresis in 0.8% agarose gel in 30 mM NaOH and 1 mM EDTA at 3 V/cm for 1.5 hours. The gel is then neutralized for

45 minutes in neutralization buffer (1M Tris, 1.5 M NaCl), stained with CYBR gold, and visualized (KODAK Gel Logic 2000).

For repair of SSB (Figure 3.5d), cells are treated with 10 mM hydrogen peroxide, washed with YPD liquid medium and incubated in fresh YPD liquid medium at 30°Cfor the indicated times. Cells are then prepared and electrophoresed as described above.

Pulsed-Field Gel Electrophoresis. Pulsed-field gel electrophoresis is as described by Herschleb et al. with modifications (Herschleb et al, 2007). MVY101 (wild type) and MVY105 (*sub1Δ*) are grown to log phase and treated with peroxide at the specified concentrations for 10 minutes. Then the cell wall is removed by zymolyase treatment to form spheroplasts. The spheroplasts are mixed with equal volume of 2% low-melting agarose and injected into gel plug casting molds (Bio-Rad). The gel plugs are then digested overnight at 50°C in NDSK solution (0.5M EDTA, 1% N-laurylsarcosine, 1mg/ml Proteinase K, pH=9.5). Pulsed-field gel electrophoresis is performed on a Bio-Rad CHEF-DR II apparatus according to the manufacture's instructions. The gel plugs containing the chromosomal DNA are electrophoresed in 0.5X TBE at 14°C for 24 hours with 6V/cm voltage using an initial/final switching time of 60/120 seconds.

ROS measurements. 2,7-Dichlorofluorescin diacetate (FH-DA) is used to measure yeast intracellular ROS as described (Davidson et al, 1996). Briefly, cells are cultured in synthetic complete medium, or the same medium lacking uracil to maintain plasmids. Cells were incubated for two days, diluted to $1x10^5$ cells/ml in YPD liquid medium and cultured at 30°C overnight. Cells are then diluted to $1x10^7$cells/ml in YPD liquid medium

and cultured at 30°C for 4 hours. Cells are then washed once with PBS, pre-loaded with the DCFH-DA stain, and treated with indicated amount of hydrogen peroxide. Samples are loaded into a black 96-well plate and read using a fluorescence plate reader (SpectraMax GeminiXS, Molecular Devices, Sunnyvale, CA) at the excitation wavelength of 485nm and emission wavelength of 530±10nm.

PC4 purification. Recombinant PC4 was purified from the *E. coli* strain MV4996 expressing full-length PC4 (pMV801) as described previously (Ge et al, 1996) with modifications. Briefly, MV4996 is incubated in LB liquid medium with 100 µg/ml ampicillin and induced with IPTG (1 mM) for 3 hours. Cells are then resuspended in BC300 (Ge et al, 1996), sonicated, and the cell lysate loaded onto a Heparin-Sepharose column. After washing extensively with BC300, PC4 is eluted with BC500 (without EDTA) and loaded into a P11 phosphocellulose column (Whatman Inc. Piscataway, NJ), washed with 10 column volumes of BC500 (without EDTA), and eluted with BC1000 (without EDTA). The final PC4 eluate is dialyzed against 500ml of 25% glycerol for 3 hours, followed by dialysis against 1 L of 25% glycerol overnight. The dialyzed protein is quickly frozen in liquid nitrogen and then stored at -80°C. The concentration of PC4 is determined by Commassie blue staining of the purified PC4 protein with the BSA standard electrophoresed in SDS-PAGE gels.

MCO assay for oxidative damage. The MCO assay is as described previously (Park & Floyd, 1994) with some modifications. The reaction contains 5µL of 100µM $FeCl_3$, 5µL of 100mM DTT, 5µL of 200mM Hepes (pH=7.0), 2.5µL of 230ng/µL pUC19 DNA, and

water to a final volume of 50µL. $FeCl_3$ is added in the last step to initiate the reactions. PC4 (200ng/µL stock) or BSA (500 ng/µL stock) is added as indicated (Figure 3.6e). Reactions are incubated at 37°C for one hour unless otherwise indicated. After the incubation, 2µL of 0.5M EDTA is added to stop the reactions and 52µL phenol (pH=8.0) is added to remove the proteins. After centrifugation, 10µL of the aqueous fraction is loaded onto the agarose gel and electrophoresed at 5V/cm for 30minutes. The gel is then stained with ethidium bromide and photographed (KODAK Gel Logic 2000).

Table 3.1. Yeast strains used in this study.

Strain	Original name, genotype (annotation)	Reference
MVY101	FY833, *MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202*	(Wu et al, 1999)
MVY105	MVY101 with *sub1Δ*::*hisG*	(Wu et al, 1999)
MVY115	MVY105 with pMV1340 (PC4-CTD (a.a.40-127) in p426GPD)	this study
MVY150	W303-1B, *MATα ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura3-1*	lab strain
MVY169	MVY150 with *sub1Δ*::*TRP1*	this study
MVY383	MVY150 with *rad52Δ*::*URA3*	this study
MVY384	MVY169 with *rad52Δ*::*URA3*	this study
MVY610	JKM179, *MATα hoΔ hmlΔ*::ADE1 *hmrΔ*::*ADE1 ade1-100 leu2,3-112 lys5 trp1Δ*::hisG *ura3-52 ade3*::*GAL-HO*	(Haber, 2002)
MVY617	MVY610 with *sub1Δ*::*TRP1*	this study
MVY653	MVY105 with pMV1327 (*GFP-SUB1* fusion in pUG36)	this study
MVY809	MVY610 with *SUB1::SUB1-GFP*	this study
MVY832	MVY105 with pMV1345 (PC4 full length in p426GPD)	this study

Figures and legends

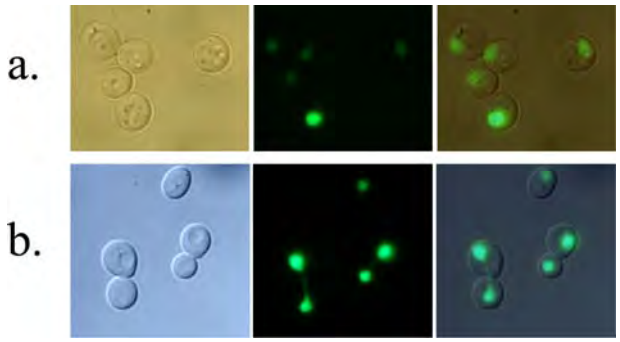


Figure 3.1 Nuclear localization of Sub1 shown by *GFP* fusions using fluorescence microscopy. Left panels: Phase contrast; middle: *GFP*; right: merged images. **a.** The *GFP* gene is fused to the chromosomal *SUB1* gene. **b.** The *GFP-SUB1* fusion gene transcribed from the methionine promoter of plasmid pMV1327. Shown are two yeast cells undergoing mitosis.

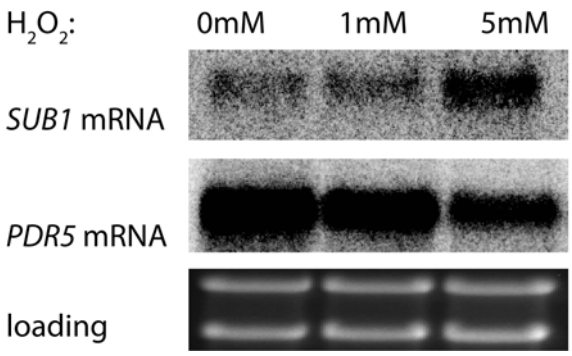


Figure 3.2 Expression of *SUB1* induced by oxidative stress. Northern blot analysis of yeast mRNA extracted from wile type cells (MVY150) treated with peroxide at the indicated concentrations. The rRNA (lower panel) is shown as loading control.

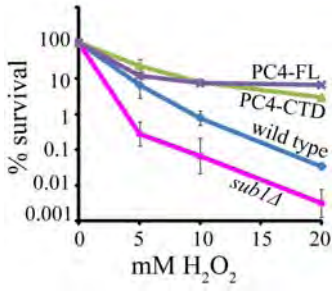


Figure 3.3 The *sub1Δ* mutant is hypersensitive to peroxide and PC4 complements the peroxide sensitivity. *sub1Δ* (MVY105), wild type (MVY101), PC4-CTD (a.a. 40-127) complemented *sub1Δ* mutant (MVY115), PC4 complemented *sub1Δ* mutant (MVY832).

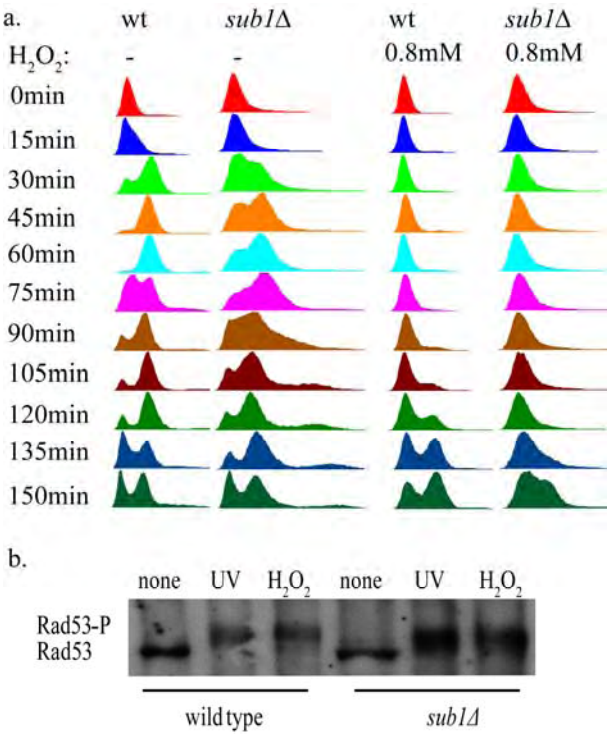


Figure 3.4 *SUB1* is not required for checkpoint activation. **a**. Cell cycle progression of the *sub1Δ* mutant (MVY105) and the wild type (MVY101). Shown are flow cytometry analysis of the genomic DNA content of the cells. Cells are arrested at G1 phase by α factor and released with or without hydrogen peroxide in the media as indicated. **b**. Rad53 phosphorylation status in the wild type (MVY101) and the *sub1Δ* mutant (MVY105) after 34J/m^2 UV irradiation (UV), 5mM peroxide treatment (H_2O_2) , or no treatment (none).

Figure 3.5 Peroxide produces more oxidative DNA damage in the *sub1Δ* mutant. **a.** SSB analysis by alkaline comet assay (wild type, MVY101; sub1Δ, MVY105). Shown are representative microscopic pictures of the yeast comets. **b.** Measurement of SSB by alkaline gel electrophoresis of genomic DNA from cells treated with different amounts of hydrogen peroxide. **c.** Measurement of DSBs by PFGE. Cells (wild type, MVY101; *sub1*Δ, MVY105) are treated with different amounts of hydrogen peroxide as indicated and chromosomes are separated by PFGE. w: wild type, m: *sub1Δ* mutant. **d.** Repair of peroxide induced SSBs in the wild type and *sub1Δ* mutant. Cells are treated with 10mM peroxide and genomic DNA separated on alkaline gel as in 5b. pre: pre-treatment. **e.** Peroxide sensitivity of the wild type (MVY150), the *sub1Δ* mutant (MVY169), the *rad52Δ* mutant (MVY383), and the *rad52Δ sub1Δ* double mutant (MVY384). Representative data of 3 repeats showing similar trends are shown.

Figure 3.5

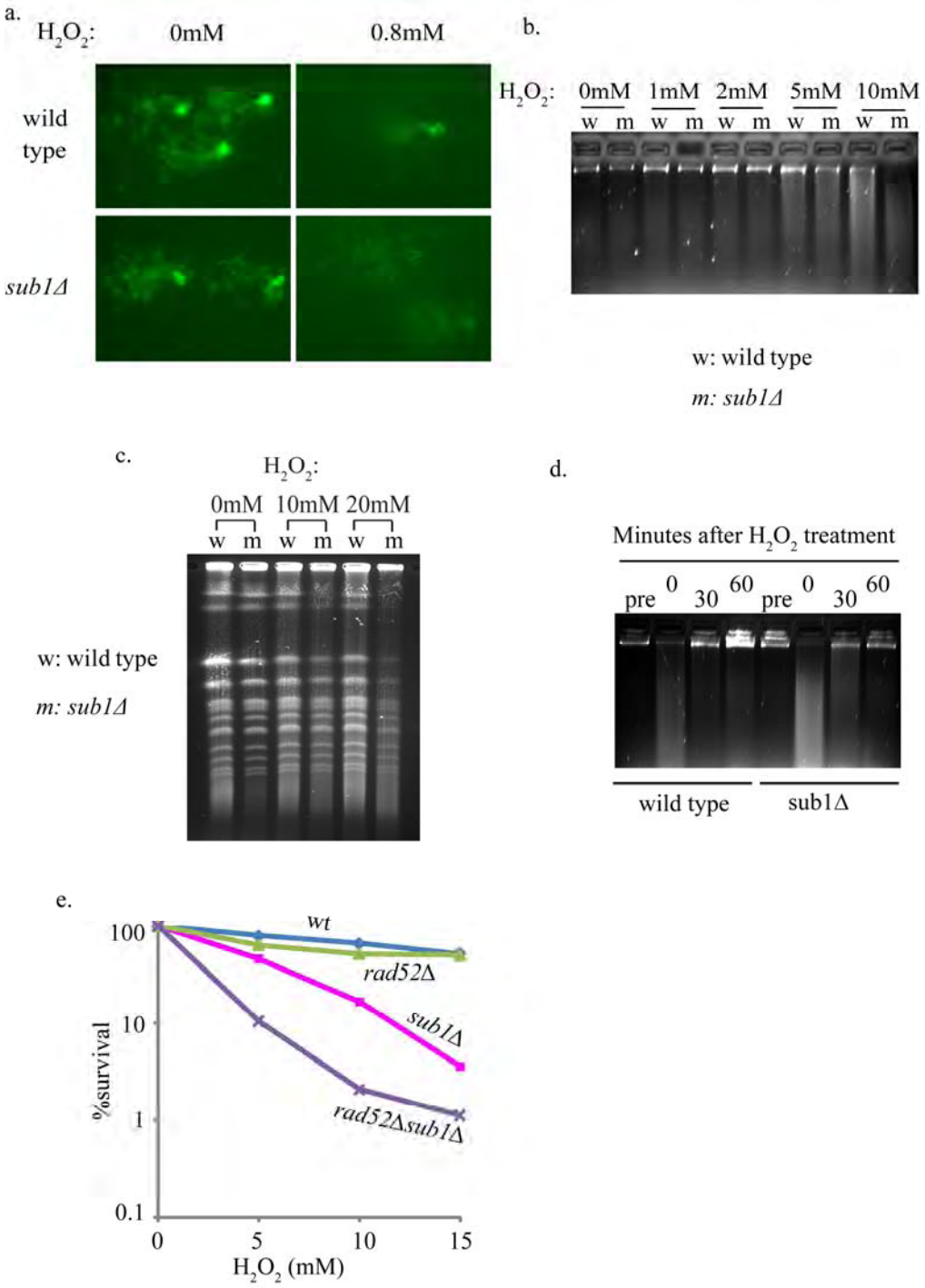
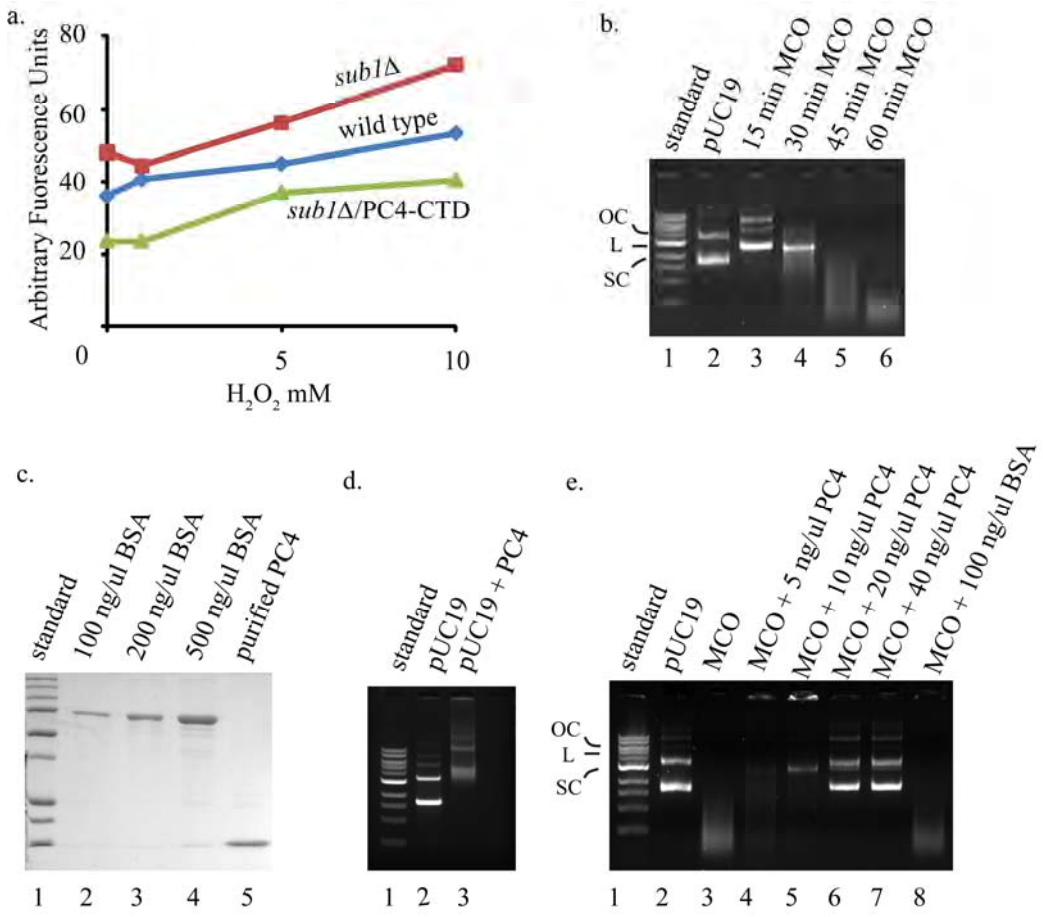

a.
H2O2:
0mM
0.8mM
wild type
sub1Δ
b.
H2O2:
0mM 1mM 2mM 5mM 10mM
w m w m w m w m w m
w: wild type
m: sub1Δ
c.
H2O2:
0mM 10mM 20mM
w m w m w m
w: wild type
m: sub1Δ
d.
Minutes after H2O2 treatment
pre 0 30 60 pre 0 30 60
wild type
sub1Δ
e.
100
10
1
0.1
%survival
0
5
10
15
H2O2 (mM)
wt
rad52Δ
sub1Δ
rad52Δsub1Δ

Figure 3.6 PC4 protects DNA from ROS induced oxidation directly. **a.** Intracellular ROS induced by peroxide treatment in the wild type (MVY101), *sub1Δ* mutant (MVY105), and PC4-CTD complemented *sub1Δ* mutant (MVY115). Cells are loaded with DCFH-DA stain, treated with indicated amount of hydrogen peroxide, and fluorescence read in a plate reader. Shown are representative data of six independent experiments. **b.** Oxidation of the pUC19 plasmid by the MCO system. Lanes are: 1: NEB 1kb DNA standard; 2: pUC19 DNA; 3-6: pUC19 DNA oxidized by the MCO system for 15, 30, 45, 60 minutes. SC: supercoil form; L: Linear form; OC: open-circle form. **c.** **N**on-tagged PC4 is purified to homogeneity. Lanes are 5µL of: 1: Bio-Rad precision plus all blue protein standard; 2: 100ng/µL BSA; 3: 200ng/µL BSA; 4: 500ng/µL; 5: purified PC4, estimated concentration 200ng/µL. **d.** Purified PC4 binds to DNA, indicating it is functional. Lanes: 1: NEB 1kb DNA standard; 2: pUC19 DNA; 3: pUC19 DNA plus PC4. **e.** PC4 protects DNA from oxidation in vitro. Lanes as: 1: NEB 1kb DNA standard; 2: pUC19 DNA; 3: pUC19 oxidized by the MCO system; 4-7: pUC19 oxidized in the presence of PC4 at concentrations: 5ng/µL, 10ng/µL, 20ng/µL, 40ng/µL; 8: pUC19 oxidized in the presence of 50ng/µL BSA. SC: supercoil form; L: linear form; OC: open-circle form.

Figure 3.6


a.
Arbitrary Fuorescence Units
80
60
40
20
0
5
10
H2O2 mM
sub1Δ
wild type
sub1Δ/PC4-CTD
b.
standard
pUC19
15 min MCO
30 min MCO
45 min MCO
60 min MCO
OC
L
SC
1 2 3 4 5 6
c.
standard
100 ng/ul BSA
200 ng/ul BSA
500 ng/ul BSA
purified PC4
1 2 3 4 5
d.
standard
pUC19
pUC19 + PC4
1 2 3
e.
standard
pUC19
MCO
MCO + 5 ng/ul PC4
MCO + 10 ng/ul PC4
MCO + 20 ng/ul PC4
MCO + 40 ng/ul PC4
MCO + 100 ng/ul BSA
OC
L
SC
1 2 3 4 5 6 7 8

Chapter IV

UV induced polyadenylation switching in yeast

During completion of this manuscript, additional experiments to measure mRNA stability were conducted. These experiments are preliminary and suggest that a UV-induced RNA polymerase switching is the underlying cause of alternative polyadenylation of the *RPB2* gene. Because the preliminary nature of these results, they are presented in the appendices and discussed in chapter V.

Summary

Most human genes are transcribed into messenger RNAs that contain a 3'-polyadenosine tail, and the majority are alternatively polyadenylated. Alternative polyadenylation appears to play important roles in embryonic development and cancer suppression. In this report, we demonstrate that UV irradiation induces alternative polyadenylation in yeast. In UV-irradiated yeast cells the transcription machinery proceeds through the first polyadenylation site of the *RPB2* gene which encodes the second largest subunit of RNA polymerase II and stops at the second site, generating a longer form of mRNA. Replacing the *RPB2* 3'-UTR with the 3'-UTR of the *CYC1* or *GCN4* gene disrupts the polyadenylation switch and sensitizes yeast cells to UV killing, suggesting alternative polyadenylation plays an important role in cell survival after UV damage.

Introduction

Transcription termination in eukaryotic cells involves cleavage of the newly transcribed messenger RNA (mRNA) at specific sites and addition of adenosine (A) residues to the 3'-end of the newly synthesized mRNA, a process called polyadenylation (Lutz, 2008). Surprisingly, more than half of human genes have multiple polyadenylation signals and are subject to alternative polyadenylation (Lutz, 2008; Ozsolak et al, 2010), suggesting that alternative polyadenylation plays an important role in increasing transcript diversity. The selective use of different polyadenylation signals may influence efficiencies of transcription and translation, mRNA stability, and nuclear export of the mature mRNA (Millevoi & Vagner, 2010; Proudfoot & O'Sullivan, 2002; Moore, 2005). Notably, alternative polyadenylation has been reported to be a highly regulated process during embryonic development (Ji et al, 2009), cancerous transformation (Mayr & Bartel, 2009), and neuronal synapse development (Flavell et al, 2008).

DNA damage induced by environmental stimuli or endogenous insults is a major threat to cell survival and perturbs cellular transcription in many ways. For example, DNA damage can promote the transcription of specific genes (Fu et al, 2008), block the progression of the transcription machinery on DNA lesions and trigger transcription-coupled repair that is dedicated to remove DNA lesions preferentially from the template strand (Hanawalt & Spivak, 2008), inhibit transcription (Reagan & Friedberg, 1997), and regulate alternative splicing (Muñoz et al, 2009; Marengo & Wassarman, 2008). Recently,

Kleiman *et al.* showed that mRNA 3'-end cleavage can be inhibited by UV damage *in vitro* (Cevher et al, 2010; Mirkin et al, 2008; Kleiman & Manley, 2001) and this inhibition might be critical for cancer suppression (Kleiman & Manley, 2001). UV induced alternative polyadenylation has previously been reported in mammalian cells. However, no functional significance was demonstrated to result from the switch, nor was the process itself studied further (Schwartz et al, 1998).

Alternative polyadenylation in yeast is probably as pervasive as in human because it has been estimated that 72.1% yeast genes contain multiple polyadenylation sites (Ozsolak et al, 2010). However, only a few genes have been studied to determine the effects of alternative polyadenylation. Examples are *CBP1, APE2,* and *RNA14* where transcription terminates within the coding sequences when yeast cells are shifted from anaerobic growth to aerobic growth (Mayer & Dieckmann, 1991; Sparks & Dieckmann, 1998), and *SUA7* where two non-truncated transcripts are produced with different length and the longer form is reduced by heat shock or starvation (Hoopes et al, 2000). Various factors involved in mRNA cleavage and polyadenylation have been reported to affect poly(A) site selection (Seoane et al, 2009; Kim Guisbert et al, 2007). However, whether UV irradiation induces alternative polyadenylation in yeast has not been studied.

Transcription of most genes in yeast is catalyzed by RNA polymerase II which is a hetero-12-mer complex consisting of two large subunits, *RPB1*and *RPB2,* and 10 small subunits (Woychik & Young, 1990; Ishihama et al, 1998). During the course of studying transcription recovery after UV-induced DNA damage, we found two mRNA species

which are encoded by the *RPB2* gene. UV damage inhibits transcription of the short species and increases transcription of the long species. In this study we demonstrate that the two mRNA species are the products of alternative polyadenylation and production of the long *RPB2* mRNA increases cellular survival of UV damage.

Results

UV damage induces alternative polyadenylation of the *RPB2* mRNA. The *RPB2* gene in yeast has been used as a target to study transcription-coupled DNA repair (TCR) due to its high constitutive transcription rate and large size (van Gool et al, 1994; Sweder & Hanawalt, 1994; Verhage et al, 1996). It has been demonstrated that UV damage on the transcribed strand of *RPB2* gene is removed more rapidly than damage on the non-transcribed strand ((van Gool et al, 1994; Sweder & Hanawalt, 1994; Verhage et al, 1996) and Chapter II). However, transcription recovery of the *RPB2* mRNA following repair of the UV damage has not been investigated. To test this, we irradiated wild-type yeast cells with UV and monitored the *RPB2* mRNA levels in the cells by Northern blot. We found that the *RPB2* mRNA level declines within 15 minutes after UV damage, suggesting transcription inhibition by DNA damage or decreased mRNA stability (Figure 4.1A). At 30 minutes, however, the *RPB2* mRNA level increases and two different *RPB2* mRNA species are produced. The long form is at a low level prior to UV treatment and increases after UV irradiation (Figure 4.1A).

Alternative splicing is rare in *S. cerevisiae* and the *RPB2* gene appears not to contain introns ((Davis et al, 2000; Pleiss et al, 2007) and www.yeastgenome.org). Therefore, the different *RPB2* mRNA species might be the result of alternative transcription initiation or alternative polyadenylation. To test transcription initiation of the *RPB2* gene, we performed Rapid Amplification of cDNA Ends (RACE) (Scotto-Lavino et al, 2006a) to determine the 5'-end of the *RPB2* mRNA. We found *RPB2* has only one transcription initiation site located 270 base pairs (bp) upstream of the translation initiation codon ATG (Figure 4.1B). Subsequently, we used 3'-RACE (Scotto-Lavino et al, 2006b) to examine the 3'-end of the *RPB2* mRNA and found two transcription termination sites that are 287bp apart in the 3'- untranslated region (3'UTR) of the *RPB2* gene (Figure 4.1B). Thus, *RPB2* appears to be alternatively polyadenylated.

To confirm that UV treatment induces the polyadenylation switching, we inserted the 1.5kb *KanMX* gene between the two polyadenylation sites of *RPB2* and treated the mutants with UV. If UV induces the transcription machinery to bypass the first polyadenlyation site and cleave at the second site, post-UV transcription in the mutants is expected to bypass the first polyadenylation site and produce a long polycistronic mRNA. Figure 4.1C shows that two independent clones of the mutants with the *KanMX* insertion transcribed the polycistronic mRNA much higher levels after UV treatment. This clearly indicates the two *RPB2* mRNA species in wild type are produced by alternative polyadenylation and that UV triggers the preferential use of the second polyadenylation site.

Yeast polyadenylation signals do not contain highly conserved sequences and are poorly defined (Zhao et al, 1999). We tested if the sequence elements for the UV-inducible polyadenylation switch reside in the 3'- UTR of the *RPB2* gene. We inserted the 3'-UTR of the *RPB2* gene after the *URA3* gene coding sequence, replacing its 3'-UTR. *URA3* does not use alternative polyadenylation and is normally transcribed as a single mRNA (Buckholz & Cooper, 1983). Figure 4.1D shows that when the *RPB2* 3'-UTR is attached to the *URA3* coding sequence, two mRNA transcripts differing by about 300 bp are produced, indicating that both *RPB2* polyadenylation sites are used. This suggests that the *RPB2* 3'-UTR contains sequence elements sufficient to direct alternative polyadenylation of other genes. However, UV irradiation does not result in a switch to preferential production of the longer form of the *URA3* mRNAs (Figure 4.1D). This suggests that additional sequence elements upstream of the *RPB2* 3'-UTR are required for the UV-induced polyadenylation switch from the first to the second site even though both sites are functional.

UV induced alternative polyadenylation is independent of transcription-coupled DNA repair. Transcription-coupled DNA repair (TCR) is triggered by blocked RNA polymerases and rapidly removes DNA lesions from the template strand of transcribing genes. It has been shown that TCR is required for transcription recovery after DNA damage (Reagan & Friedberg, 1997).Therefore we tested if the preferential production of the longer form of *RPB2* mRNA after UV damage is dependent on TCR. We treated yeast cells with UV and compared *RPB2* mRNA recovery in wild type and in the TCR deficient *rad26Δ* mutant (van Gool et al, 1994). Figure 4.2 shows that wild type cells

recover mRNA transcription within 30 minutes and that the long *RPB2* mRNA is preferentially transcribed. In comparison, the *rad26Δ* mutant recovers transcription of both forms of *RPB2* mRNA at a reduced rate, indicating that TCR is required for general transcription recovery after UV damage. However, the long *RPB2* mRNA is still preferentially produced in the *rad26Δ* mutant, suggesting the process of alternative polyadenylation is not affected by the defect in TCR once the DNA damage is repaired by other DNA repair pathways, e.g. global genome repair (Svejstrup, 2002).

Alternative polyadenylation of *RPB2* mRNA increases cellular UV resistance. To test if UV-induced alternative polyadenylation of *RPB2* is physiologically important or just a passive alteration in the transcription process, we replaced the 3'-UTR of the *RPB2* gene with 3'-UTRs of two other genes that do not exhibit alternative polyadenylation, *CYC1* (Muhlrad & Parker, 1999) and *GCN4* (Irniger et al, 1991). Figure 4.3A shows that transcription of the *RPB2* gene carrying either the *CYC1* or *GCN4* terminators yields only one species both in unirradiated and UV-irradiated cells. When the 3'-UTR of the *GCN4* gene replaces the *RPB2* terminator, levels of the *RPB2* transcripts increase after UV treatment. Nonetheless, both the *RPB2-CYC1* and *RPB2-GCN4* mutants exhibit UV sensitivity at low UV doses compared to the parental strain (Figure 4.3B), suggesting that alternative polyadenylation of the *RPB2* gene is an adaptive response to UV damage that increases cell survival.

Discussion

Alternative polyadenylation has been found to be a ubiquitous process occurring on the majority of human genes, contributing to embryonic development, cancer prevention, and neural circuitry formation (Lutz, 2008; Ji et al, 2009; Mayr & Bartel, 2009; Flavell et al, 2008). UV induced polyadenylation switching has previously been observed in human cells (Schwartz et al, 1998). We demonstrate that alternative polyadenylation is induced by UV damage in yeast, suggesting that this process is a conserved response to UV damage in eukaryotes. Moreover, we also demonstrate that alternative polyadenylation of the *RPB2* gene of yeast contributes modestly to cellular resistance to UV damage, especially at low doses, demonstrating that the process is physiologically important and contributes to cellular survival.

UV irradiation is known to cause DNA damage that blocks transcription elongation and reduces the level of available transcription complexes in the cell (Svejstrup, 2003). Our results show that alternative polyadenylation increases the abundance of the *RPB2* mRNA. The increased the mRNA level may allow increased production of Rpb2 protein and higher levels of functional RNA polymerase II complexes. Alternatively, the longer *RPB2* mRNA may have additional beneficial properties that may help cells recover from DNA damage, e.g. increased mRNA stability and/or higher translation efficiency.

In addition to its novel physiological role, the UV-induced switch from preferential polyadenylation at site 1 to site 2 appears to be regulated by a mechanism that is yet to be determined. Kleiman *et al.* previously showed that UV irradiation suppresses 3’-cleavage

of a pre-mRNA *in vitro* (Cevher et al, 2010; Mirkin et al, 2008; Kleiman & Manley, 2001). The UV induced alternative polyadenylation observed in this study may include molecular events that weaken the effect of the first polyadenylation signal of *RPB2* and increase the use of the second polyadenylation signal. However, the hypothesis that UV irradiation induces suppression of mRNA cleavage at specific polyadenylation sites does not explain why the *RPB2* 3'-UTR attached to the *URA3* gene does not also respond to UV irradiation. Therefore signals upstream in *RPB2* appear to be required for UV-induced polyadenylation switching. Our results suggest that the upstream signals in *RPB2* and the UV-induced modifications of the transcription machinery cooperatively determine the use of the downstream polyadenylation sites. Although our data suggest that the DNA repair process that is triggered by the blocked transcription machinery is not required for the UV-induced polyadenylation switching, it remains to be determined if transcription arrest induced by UV damage indeed plays a role in this process. Thus, UV-induced polyadenylation switching involves a complicated crosstalk between environmental stimuli, the transcription machinery, and the transcribed genes. Elucidation of this mechanism may facilitate our understanding of the alternative polyadenylation events involved in embryonic development and cancer suppression (Ji et al, 2009; Mayr & Bartel, 2009).

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Materials and Methods

Yeast strains and plasmids.

Yeast strains used in this study are listed in Table 4.1 and the construction details of key strains are described below. All primers used in this study are listed in Table 4.2. Yeast transformation methods are as described (Knop et al, 1999). All plasmids were sequenced to confirm that they contain no mutations.

To construct plasmid pMV1352, which contains the *URA3* gene followed by the *RPB2* 3'-UTR, we first amplified the *URA3* gene from plasmid pRS416 (Sikorski & Hieter, 1989) using primers SacUra and BamUra, then inserted the *URA3* DNA into plasmid pMV1351 between the SacII and BamHI restriction sites. Plasmid pMV1351 was derived from pRS315 (Sikorski & Hieter, 1989) by inserting the *RPB2* 3'-UTR DNA which was amplified by PCR from the yeast genome using primers BamRPB2-4653 and SalRPB2-5148, then inserting the PCR fragment into the BamHI and SalI sites of the

vector. Plasmid pMV1352 was used to transform yeast strain MVY150 to construct strain MVY838.

To construct yeast strains MVY818 and MVY819, which have the *KanMX* gene inserted into the chromosomal *RPB2* 3'-UTR between poly A site 1 and poly A site 2 (Figure 4.1), we assembled three DNA fragments; the *KanMX6* gene, which was obtained as a XmaI SacII fragment from plasmid pFA6a-KanMX6 (Wach et al., 1997), and two PCR products produced from the downstream region of the *RPB2* gene and its 3' UTR using primer pairs. One PCR product was produced using primers KpnI-up500-f and up500-Xma-r, and the second, using primers SacII-down500-f and down500-SacI-r. These three fragments were then assembled together with KpnI and SacI digested pBluescript II SK plasmid to produce plasmid pMV1343, which carries the *KanMX6* gene flanked by the two 500 bp *RPB2* targeting sequences. This fragment was then released as a single linear DNA fragment of 2630 bp using SnaBI and KpnI. After gel purification, yeast cells were transformed with this fragment and *KanMX6* carrying clones were selected by G418 resistance. Such clones carry the *KanMX6* gene between the two Poly A sites shown in Figure 4.1.

To replace the *RPB2* 3'UTR with the *CYC1* 3' UTR sequences, we first amplified by PCR 500 bp of *RPB2* DNA using primers Kpn-RPB2-4131 and Xma-Xho-RPB2 and used this fragment to replace the KpnI-XmaI fragment of pMV1343 to produce plasmid pMV1346. We then amplified the *CYC1* terminator sequence from the yeast genome using primers Xho-CYC1 and Bgl-CYC1-1586 and inserted this PCR product into

pMV1346 to insert the *CYC1* terminator to produce pMV1347. Digestion with SnaB1 and KpnI releases a 2739 bp fragment containing the C-terminal region of *RPB2*, followed by the *CYC1* terminator, the *KanMX6* to allow selection of recombinants and the downstream 500 bp RBP2 targeting sequence contain *RPB2* poly A site 2. This fragment was then used to transform yeast strain MVY150 to replace the chromosomal *RPB2* termination regions to produce strain MVY836.

pMV1348 was constructed in a manner identical to that of pMV1347 except that the *GCN4* terminator region was amplified using primers Xho-GCN4 and Bgl-GCN4-2081 and inserted into pMV1346 instead of the *CYC1* sequences. After release of the SnaBI KpnI fragment carrying the 2-500bp targeting regions that flank the *GCN4* terminator and the *KanMX6* gene, it was used to transform yeast strain MVY150 to produce MVY837.

UV irradiation and Northern analysis

Yeast cells in mid log phase are suspended in PBS at an OD_{600} reading of 0.8, irradiated with UV at 1.71J/m^2/s for 42 seconds or mock treated, resuspended in YPD medium (Adams et al, 1997) and cultured for 30 minutes or indicated times, and collected and frozen on dry ice. Total yeast RNA is extracted using the hot phenol method and analyzed using the Northern analysis as described elsewhere (He & Jacobson, 1995). The Random Primed DNA labeling kit (Roche Applied Science, Indianapolis, IN) is used to synthesize the ^{32}P-labeled *RPB2* probe (the template DNA is a purified RPB2 fragment

from 1075 bp to 2133 bp in the ORF) and the *URA3* probe (the template DNA is a purified URA3 fragment from 206 bp to 824 bp in the ORF).

5'-RACE assay

The 5'-RACE assay is performed as described (Scotto-Lavino et al, 2006a). Briefly, 400ng of DNase-treated total yeast RNA is reverse transcribed by the SuperscriptIII reverse transcriptase (Invitrogen, Carlsbad, CA) using primer RPB2-13r, digested by RNase H, purified using the QIAQuick PCR purification kit (QIAGEN, Valencia, CA), polyadenylated by terminal transferase (NEB, Ipswich, MA). The resulting polyadenylated cDNA is subjected to two rounds of PCR amplification with the first round using primers RPB2-14R and RACE1 and the second round using primers anchorP and RPB2-17R. The final PCR product is gel purified and sequenced using primer RPB2-17R to determine the 5' transcription start sites.

3'-RACE assay

The 3'-RACE assay is performed as described (Scotto-Lavino et al, 2006b). Briefly, cDNA is prepared as in the 5'-RACE assay except that primer RACE1 is used in reverse transcription and no polyadenylation step is included. Primers RPB2-12 and anchorP are used to PCR amplify the 3'-UTR of the *RPB2* gene from the cDNA. The

PCR product contains two DNA fragments that are gel purified and sequenced using primer RPB2-13 to determine the polyadenylation sites.

UV sensitivity assay

Yeast cells are cultured in YPD medium to mid log phase (OD_{600}=0.8), resuspended in PBS, and irradiated with UV at 1.71J/m^2/s for different times. After irradiation, cells are diluted in water, plated onto YPD, and colonies are counted after 2 days.

Table 4.1. Yeast strains used in this study.

Strain	Original name, genotype (annotation)	Reference
MVY150	W303-1B, *MATα ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura3-1*	(van Gool et al, 1994)
MVY151	MGSC102, MVY150 with *rad26 ⊿:HIS3*	(van Gool et al, 1994)
MVY818	MVY150 with the *KanMX* insertion in *RPB2 3'-UTR*, #1	this stuy
MVY819	MVY150 with te *KanMX* insertion in *RPB2 3'-UTR*, #2	this study
MVY836	MVY150 with 3'-UTR of *CYC1* inserted after *RPB2* ORF	this study
MVY837	MVY150 with 3'-UTR of *GCN4* inserted after *RPB2* ORF	this study
MVY838	MVY150 with pMV1352 (*URA3* ORF + *RPB2* 3'-UTR)	this study

Table 4.2. Primers used in this study.

SacUra	GCGCCCGCGGTGCACCATACCACAGCTTTT
BamUra	CGGCGGATCCTTAGTTTTGCTGGCCGCA
BamRPB2-4653	GCGCGGATCCGATCGTTCGAGAGATTTT
SalRPB2-5148	CGGCGTCGACCTTTTTGCAGTCTTCAATCC
KpnI-up500-f	CGGCGGTACCGACACATGGTGGATGACAAGA
up500-Xma-r	GCGCCCCGGGTTGGTAAAATGCGAAACAAGG
SacII-down500-f	GCCACCGCGGCGGTGTTCATTTTGGAACAA
down500-SacI-r	GACGGAGCTCCATTGGGTAGATTGGCTTCAG
Xho-CYC1	CGGCCTCGAGACAGGCCCCTTTTCCTTTG
Bgl-CYC1-1586	GCGCAGATCTCGTCCCAAAACCTTCTCAAG
Kpn-RPB2-4131	CGGCGGTACCCCTCTCCTTTCACGGACATT
Xma-Xho-RPB2	GCGCCCCGGGCTCGAGTTAAAAATCTCTCGAACGATCGGTATATAAACG
Xho-GCN4	CGGCCTCGAGTTTCATTTACCTTTTATTTTATATTTTTTATTTCATTCTCG
Bgl-GCN4-2081	GCGCAGATCTGCAACGCGTCTGACTTCTAA
RPB2-13r	GGTGGAATCCTCGCAAATAA
RPB2-14r	AAAGCGGATATAACAGCCCA
RACE1	GCTCGATGTGCACTGCTTTTTTTTTTTTTTT
anchorP	GCTCGATGTGCACTGC
RPB2-17r	GCACTTTCATCCTCGAATCC
RPB2-12	GCTGATGACAGTTATCGCG
RPB2-13	GCCGCGAAGTTATTATTCCAAG

Figures and legends

Figure 4.1 UV damage induces alternative polyadenylation of *RPB2* mRNA. **A.** Northern blot analysis showing *RPB2* mRNA recovery after UV-irradiation. After UV treatment, yeast cells (MVY150) are incubated in growth media for the indicated times. Letters L and S indicate positions of the long and short species of *RPB2* mRNAs. pre: RNA from cells before UV treatment. The 30 min lane is duplicated on the right at a lighter exposure to reduce noise. Ribosomal RNA is shown as a loading control. **B.** Genomic DNA sequence of the 3' and 5' UTRs of the *RPB2* locus. The start and stop codons of *RPB2* gene are in the bold font and the coding sequence (ATG---TGA) is omitted and marked in bold by its start and stop codons. The polyA site 1 and polyA site 2 were determined by 3'-RACE. **C.** UV irradiation induces yeast cells to transcribe the long polycistronic mRNA when the 1.5kb *KanMX6* gene is inserted between the polyA site1 and polyA site2 as shown in panel B. The insertion site is marked by the filled triangle in panel B and 106 bp DNA following the insertion site is replaced by the *KanMX6* gene. MVY818 and MVY819 are two individual clones with the *KanMX6* insertion. L: Long form of the *RPB2* mRNA, S: Short form of the *RPB2* mRNA, P: position of the RPB2-KanMX polycistronic mRNA. **D.** The 3'-UTR of *RPB2* gene contains sequence elements for alternative polyadenylation. The 3'-UTR of the *RPB2* gene is inserted after the *URA3* ORF in plasmid pMV1352. Yeast cells with pMV1352 (MVY838) are treated or mock-treated with UV. Northern blots are used to analyze the *URA3* mRNA. Ribosomal RNA is shown as loading control.

Figure 4.1


A
minutes after UV:
pre 0 5 8 10 15 30 30
L
S
rRNA
B
GTAATTCATG CAATAAGGAA CTGACCACTA CTTTTAGCAA
CCATAAAAGC TAGTGATTTG TCTAAGTAGG GAGCAGCTTT
transcription start
ATAAAGTTAG GGTACTTGGA TATTGCACTC ATTCCTTCTC
CTCAGGTTTA CAGATAGTTC GAATAATTTT TGCTTATCGT
ATATTTCTTA AAGAAGAACC CAACAGTAAG GAACAAACAT
AAGGAACACG ACAGCGGAAT AAGACACTGT AGAAGCTCCG
TATTATAAAA ATTAGGACCA GAAAAAGAAC ACAAACAAGT
ATAGAAAAAT AATTATAAGG ATG----TAA GAAATAATGT
TGTAACTCTA AGAAAGTAAA ATAATAAAAA ACATTTCATT
TGTGCTGATC TTGCCAAAAA AAGGGTAAAC AAGTAGGCAA
polyA site1
TAGTAATAAA ATAAGAATGA AGCATTTCTT TTAATAAGCA
GAGAGAACTT TCAAGCATTC AAAAAACACA AAAATAATGA
TGAATAAAAT GGGACTCACA GTGTTTATTT ATAGTCCTTG
TTTCGCATTT TACCAAATTA AAATTTAATT TTATCAAGAC
ATATTAATGA ACTTTAGCGG AAAACCTTAA ATATCCAAGT
CATAAATAAT GACTTATGTA AAAAGTTATA AGAAAAATAT
ACGGTGTTCA TTTTGGAACA ATGTTGCTGT TACACACTGT
polyA site2
TAAATATATT TAGTATTCAT AAAAGAAAGA
C
strains: MVY150 MVY818 MVY819
UV: - + - + - +
P
L
S
D
UV: - +
rRNA

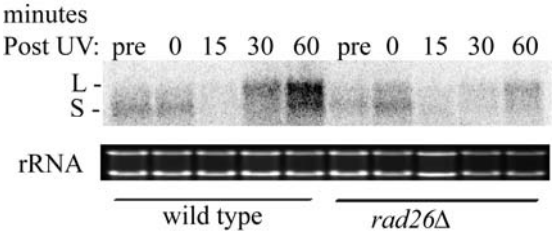


Figure 4.2 Deficiency in transcription-coupled DNA repair (TCR) delays recovery of *RPB2* mRNA transcription after UV damage, but does not prevent preferential transcription of the long *RPB2* mRNA. Wild type (MVY150) and the TCR deficient mutant (*rad26Δ*, MVY151) are treated or mock-treated (pre) with UV and incubated in culture medium for indicated times. Northern blot analysis is used to detect the *RPB2* mRNA. Ribosomal RNA is shown as a loading control.

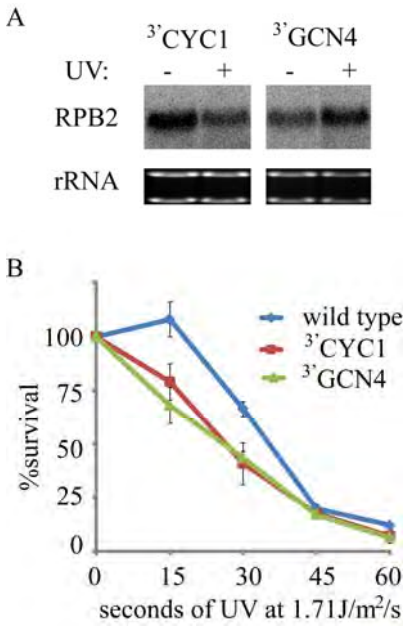


Figure 4.3 Replacing the *RPB2* 3'-UTR causes low-dose UV sensitivity. The *RPB2* 3'-UTR is replaced with either 3'-UTR of the *CYC1* gene (MVY836) or that of the *GCN4* gene (MVY837). Strain MVY836 is indicated as $^{3'}$*CYC1* and MVY837 as $^{3'}$*GCN4*. **A.** Northern blot analysis of the *RPB2* mRNA in MVY836 and MVY837 after UV treatment (1.71J/m^2/s for 42 seconds) or mock treatment. Ribosomal RNA is shown as loading control. **B.** UV sensitivity of MVY150 (wild type), MVY836 ($^{3'}$*CYC1*), and MVY837 ($^{3'}$*GCN4*).

Chapter V

General discussion and future directions

Reactive oxygen species (ROS) are constantly produced during aerobic metabolism. Cell survival and faithful reproduction depend on the intricate oxidative defense system and the DNA repair system. A breach in these systems may lead to various human diseases from cancer to aging. Therefore it is important to understand how these systems combat the effects of ROS in humans. For this purpose our laboratory has previously established a useful assay to identify human genes that can prevent oxidative mutagenesis. PC4 was one of the genes that was found to effectively suppress oxidative mutagenesis. Initial experiments by Wang *et al.* showed that the yeast homolog *SUB1* is important for the cell to survive oxidative stress and human PC4 and yeast *SUB1* appear to be interchangeable in oxidation protection in yeast. It was possible that PC4 and *SUB1* repairs oxidative DNA damage by participating in the transcription-coupled DNA repair pathway. The purpose of this thesis was to elucidate how cells use PC4 or Sub1 to protect themselves from ROS attacks.

Because of its interaction with the transcription machinery and NER proteins, the role of PC4 in transcription-coupled repair (TCR) was studied by testing the requirement of *SUB1* for preferential removal of UV damage on the transcribed strand of the *RPB2* DNA. The result was clear, *SUB1* is not required for TCR of UV damage. Although we cannot completely rule out the possibility that PC4 repairs oxidative DNA damage by using TCR, our result suggests that this role is less likely. Furthermore, we present evidence that PC4 possesses an intrinsic antioxidant activity and prevents DNA oxidation. This evidence may explain the oxidation protection function of PC4 in various conditions. We predict

that PC4 is an important component of the human ROS defense system, and we will be seeking its molecular basis in future experiments.

We also characterized the role of *SUB1* in double strand break (DSB) repair. The puzzle that different DSB repair assays reveal different requirements for *SUB1* was reconciled by the discovery that the *sub1Δ* mutant resects DNA ends rapidly which results in destruction of plasmids but not chromosomes. DNA break resection is an important phenomenon in DSB repair. The identification of *SUB1* as a new player in DNA break resection is clearly going to change our current view of DNA resection and DSB repair. An example is the conclusion we presented in chapter II that resection does not inhibit nonhomologous end-joining as we previously thought.

While studying the requirement of *SUB1* in transcription recovery, I found UV induces alternative polyadenylation of the yeast *RPB2* gene. While the evidence presented in chapter IV is observational, in the following sections I present more preliminary data and propose a mechanistic model to illustrate the cellular events that occur during transcription recovery.

1. PC4's antioxidant activity

In chapter III of this thesis, I presented evidence that PC4 possesses antioxidant activity. The antioxidant activity of PC4 provides an explanation for the antimutagensis activity of PC4 in *E. coli*. That is, it could reduce spontaneous ROS in *E. coli* and prevent the genomic DNA from oxidation. The high abundance of PC4 suggests that PC4 might

be important to maintain the redox homeostasis in the nucleus. Additionally, because PC4 nonspecifically binds to ssDNA and dsDNA, it might be recruited to the nucleosome-depeleted genomic regions during various nuclear processes, providing localized protection for genomic DNA. As we have observed that the yeast *sub1Δ* mutant is highly sensitive to oxidative stress and exhibits increased mutagenesis (Wang et al, 2004), it is expected that PC4 protects the human genome from oxidative damage as well. Therefore it will be interesting to knock down PC4 in human cell lines and determine if the cells become sensitive to oxidative stress. Because oxidative stress induces genomic instability and contributes to cancer formation, it might be interesting to analyze cancer genomics databases (Chin et al, 2011; Stratton, 2011) to determine if mutations in PC4 are associated with cancer.

Another immediate and important question is the molecular mechanism of PC4's antioxidant property. From the in vitro assay, PC4 appears to protect DNA without assistance from other antioxidant molecules. Therefore there are two possible mechanisms. The first is that PC4 chelates iron ions to suppress ROS production. To test this possibility, we can determine the metal ion affinity of PC4 in future experiments. The second possilibity is that PC4 donates electrons from its amino acid residues to reduce ROS. If this hypothesis is true, then the DNA binding property of PC4 may increase the effectiveness of the antioxidant activity of PC4 by recruiting more PC4 to the vicinity of DNA. To identify the critical amino acids involved in oxidation protection, comparative mass spectrometry can be used to determine the residue changes in PC4

after protein oxidation. Special attention should be paid to the conserved residues between PC4 and its yeast homolog Sub1.

PC4 appears to be toxic when over-expressed in bacteria. In the papillation assay, bacteria of high titer are required to be plated in order to obtain a few colonies (See Figure 1.1). However, re-testing of individual clones that were still capable of suppressing oxidative mutagenesis in the *mutM mutY E. coli* strain showed that these clones grow normally, indicating that mutations in PC4 have been selected that inactivate its toxicity but not its antioxidant activity. In the preliminary experiments I have sequenced 3 single clones and found that they all contain large deletions in the PC4 coding sequence. In the clone named "WC2", DNA sequences encoding amino acids beyond 71 are missing and a short random sequence is attached, which terminates the coding sequence (Appendix A). Because it has already been shown that the amino terminus a.a. 1-39 are not required for the oxidation protection function of PC4 (Chapter III and (Wang et al, 2004)), this essentially narrows down the functional domain for PC4's antioxidant activity to be within a.a. 40-70. Furthermore, only 6 amino acids are conserved between PC4 and Sub1 within that short range: two serines, an aspartic acid, a phenylalanine, a glycine, and a lysine (Appendix A). Further mutational analysis of this domain is expected to shed light on the molecular mechanism of PC4's antioxidant activity.

2. Sub1's role in DNA break resection

End resection of double strand DNA breaks (DSBs) is an important step in DNA repair. It generates the ssDNA that is required for homologous recombination and DNA damage response. In Chapter II I showed that the *sub1Δ* mutant is deficient in repair of DSBs in plasmid DNA but proficient in repair of chromosomal DSBs. This apparent discrepancy appears to be caused by rapid resection of the DNA ends in the *sub1Δ* mutant. The *ykuΔ* mutant is the only other known yeast mutant that resects DNA ends more rapidly than normal. However, unlike the *sub1Δ* mutant, the *yku70Δ* mutant is deficient in both plasmid and chromosomal DSB repair. Clearly, rapid resection in the sub1Δ mutant is not caused by transcriptional repression of the Ku proteins, because lack of *YKU70* expression should affect all DSB repair, not just that of plasmid, and NHEJ is mutagenic in the *yku70Δ* mutant but not in the *sub1Δ* mutant. Although the molecular events that cause rapid resection in the *sub1Δ* mutant need to be determined, the *sub1Δ* mutant exhibits three features described in Chapter II: rapid resection, potent NHEJ, and error free ligation. Two important aspects of NHEJ can be drawn from these features of the *sub1Δ* mutant. The first is that rapid resection does not inhibit NHEJ in the *sub1Δ* mutant. This conclusion inevitably challenges the generally accepted concept that DNA resection channels DSB repair into homologous recombination repair (HR). Nonetheless, other recent discoveries support my conclusion. For example, resection of topoisomerase-induced DSB is not only non-inhibitory for NHEJ, but also is required for NHEJ (Quennet et al, 2011). The second aspect of NHEJ in the *sub1Δ* mutant is that DSB resection does not cause mutagenic ligation. Previously rapid resection was only seen in the *ykuΔ* mutants and NHEJ in these mutants are highly mutagenic. My results indicate

that the Ku complex is critical for fidelity of NHEJ and ssDNA ends produced by resection does not necessarily lead to mutagenic ligation or microhomology mediated end joining (MMEJ).

The next question to ask would be if rapid resection in the *sub1Δ* mutant increases the efficiency of homologous recombination? The efficiency of HR has not been directly determined in the *sub1Δ* mutant, but the efficiency of single strand annealing (SSA) has been analyzed (Appendix B). The *sub1Δ* mutation does not appear to increase the SSA efficiency between two 90bp repeats located on both sides of the induced DSB. SSA is different from normal recombinational repair in that it does not have the homology search step. So other assays that directly test the HR efficiency in the *sub1Δ* mutant are warranted in future experiments. The other caveat of the SSA assay depicted in Figure 6.2 (Appendix B) could be the asymmetric distribution of the two 90bp repeats. While one of the repeats is located directly adjacent to the I-SceI site, the other is located several kilo-bases away. It is unknown if this would affect the SSA efficiency. So other SSA assays are needed to confirm the current results.

The molecular mechanism that leads to rapid resection in the *sub1Δ* mutant is very important for our understanding of the resection process. Formally we cannot rule out the possibility that Sub1 acts as a transcription cofactor to regulate the resection pathways. However, it seems that a direct involvement of Sub1 in the resection process is more likely. The first reason is that PC4 is recruited to the DNA damage sites and forms visible foci. Based on the similarity between Sub1 and PC4, Sub1 is expected to be recruited to DSBs. Both PC4 and Sub1 are high-affinity DNA binding proteins (Wang et

al, 2004). The tight-binding of PC4/Sub1 to DNA may become a physical obstruction for the DNA resection enzymes, therefore preventing the DNA ends from being resected. The other evidence supporting a direct role of PC4 in resection is that expression of truncated PC4 complements the *sub1Δ* mutant and restores NHEJ in the plasmid ligation assay (Appendix C). Because the truncated PC4 lacks the amino-terminal domain that is required for transcription regulation, it is unlikely that PC4 inhibits resection by transcription regulation. It is more likely that PC4 prevents DNA resection and therefore avoids plasmid loss. However, the rate of DSB resection needs to be determined directly in the PC4-complemented *sub1Δ* mutant in future experiments. Another possible approach is to use the *in vitro* resection system to test if the presence of purified PC4 suppresses DNA resection. To this end it would be best to collaborate with the Stephen Kowalczykowski lab or the Grzegorz Ira lab because they have already established the *in vitro* resection systems (Cejka et al, 2010) (Niu et al, 2010).

Because DSB resection is tightly regulated within cell cycles, another future experiment is to determine how the state of the cell cycle affects DSB resection in the *sub1Δ* mutant. A single DSB is not resected in G1 phase in the haploid yeast cell. If Sub1 protects DNA ends from resection, a *SUB1* deletion might allow the ends to be resected during G1 phase. Thus I propose to determine if the *sub1Δ* mutant resects DSBs in G1 phase. Synchronization of the *sub1Δ* cells in G1 phase can be achieved by adding α factor to the *MAT**a*** cell culture. Alternatively, the *sub1Δ* mutant might resect DSB ends more rapidly in the G2/M phase. Nocodazole can be used to arrest yeast cells in the G2/M phase.

3. UV induced polyadenylation switching

Work presented in Chapter IV demonstrates that UV induces polyadenylation switching on the yeast *RPB2* gene. This is the first report that alternative polyadenylation can be induced by UV irradiation in yeast. Increasing evidence suggests that alternative polyadenylation is a crucial cellular process that contributes to embryonic development, neural plasticity, and carcinogenesis (Ji et al, 2009; Mayr & Bartel, 2009; Flavell et al, 2008). My work suggests that alternative polyadenylation may be an important cellular response to UV irradiation because disruption of the polyadenylation switching of the *RPB2* gene causes UV sensitivity to the cells.

RPB2 is an essential gene that encodes the second largest subunit of the RNA polymerase II in yeast (Appendix D). A potential beneficial feature of the long form *RPB2* mRNA could be that it is more stable. Therefore, I tested the decay rate of the *RPB2* mRNAs by using the temperature sensitive *rpb1-1* strain (Nonet et al, 1987; Scafe et al, 1990). Rpb1 is the largest subunit of mRNA polymerase II in yeast. After shifting to non-permissive temperature at 37°C, transcription in *rpb1-1* immediately shuts down and levels of all mRNA start to diminish according to their respective decay rates. This is a well established system and has been used by many laboratories to determine mRNA half lives (He et al, 2008; Coller, 2008; Marín-Navarro et al, 2011; Parker et al, 1991). The control mRNAs of the *SUB1* gene and the *YRA1* gene were shown to be degraded almost immediately after inactivating RNA polymerase II at 37°C (Appendix E). Quite surprisingly, the long form of the *RPB2* mRNA increases in abundance over time after shifting the *ts* strain to non-permissive temperature, while the short form decreases

quickly and synthesis never resumes. This suggests that the transcription of the long *RPB2* mRNA is independent of RNA polymerase II, or alternatively that POL II transcription is leaky in the *rpb1-1* mutant at non-permissive temperature and the increase of abundance in the longer *RPB2* mRNA may be caused by increased stability of the longer *RPB2* mRNA.

If the *RPB2* gene is indeed transcribed independently of POL II in the *rpb1-1* mutant, the relevant effect of UV irradiation may be to induce POL II inhibition as well. In fact it is known that RNA polymerase II is blocked by UV-induced DNA damage and the stalled POL II complexes are subjected to proteasomal degradation (Ratner et al, 1998; Ribar et al, 2006, 2007). POL II independent transcription prompts the model that POLII mRNAs are synthesized by another RNA polymerase when POL II is inactivated as illustrated in Appendix F. Following UV irradiation or temperature shift of the *rpb1-1* mutant, transcription is inhibited and cells enter state "B" where POL II is inactivated and all mRNAs start to decay (Figure 6.5 in Appendix F). Cells can not easily move back to state "A" because they not only need enough POL II to recover mRNA synthesis but they also need more POLII mRNA to make enough POL II. If the POL II mRNA is in fact damaged by UV, the production of POL II proteins would be even more severe. As my current working model suggests (Appendix F), another RNA polymerase may replace POL II and transcribe the mRNAs for POL II subunits after transcription inhibition, potentially producing sufficient amounts of POL II mRNA to be translated into new POL II complexes, moving the cell into state "C". Then new POL II subunits are translated from the POL II mRNAs and assembled into new POL II complexes,

moving the cell into state “D”. Once enough POL II complexes are available in state “D”, mRNA transcription by POL II can resume and all other mRNA can again be transcribed. Eventually the cell can recover from transcription inhibition and enter the normal cellular state “A”.

An important prediction about this model is that mRNA of other subunits of POL II should also be transcribed independently of POL II. Yeast POL II includes 12 subunits, and most of them are essential (Appendix D). Rpb1 is the largest and Rpb2 the second. Therefore I tested if *RPB1* can be transcribed after shifting the *rpb1-1* strain to non-permissive temperature. As expected, the mRNA level of *RPB1* increases after heat inactivating POL II (Appendix E).

The critical question to be addressed in the feed-back synthesis model is to determine what polymerase transcribes the POL II mRNA during POL II inhibition. There are only 3 known RNA polymerases in yeast and all other eukaryotes: RNA polymerase I, II, and III (Ishihama et al, 1998). They are often designated POL I, POL II, and POL III. POL I transcribes ribosomal RNA (rRNA), POL II transcribes mRNA, and POL III transcribes transfer RNA (tRNA) and 5S rRNA. In order to dissect the roles of these RNA polymerases, I propose to use transcription inhibitors α-amanitin and thiolutin to confirm that the *RPB2* mRNA can be synthesized in the absence of a functional POL II and test if *RPB2* mRNA synthesis is dependent on the other two polymerases. α-amanitin only inhibits POL II transcription at low concentrations and thiolutin inhibits transcription by all three polymerases, I, II, and III (Bushnell et al, 2002; Brueckner & Cramer, 2008; Coller, 2008; Raha et al, 2010; Tipper, 1973). In these experiments we

expect that the *RPB2* mRNA will be transcribed in the presence of α-amanitin but will most likely be inhibited by thiolutin unless the fourth unknown RNA polymerase exists.

Interestingly, more than a decade ago Ishihama *et al.* discovered multiple Reb1/Reb2 binding sites in the promoters of most of the genes that encode the POL II subunits (Jansma et al, 1996). Reb1 is a POL I enhancer binding protein (Morrow et al, 1989)(Wang et al, 1990). Deletion of these Reb1 binding site in *RPB1* and *RPB2* greatly reduces transcription of the genes for the POL II subunits. Therefore I predict that POL I is most likely the RNA polymerase that transcribes the mRNA for POL II subunits. A null mutation in POL I has been made viable by expressing the rRNA gene from a POL II promoter (Gadal et al, 1997; Buck et al, 2002; Cioci et al, 2003; Nogi et al, 1991). I propose to use these POL I null mutants to test if the UV induced polyadenylation switching and the feed-back synthesis of POL II mRNA is driven by POL I transcription. Additionally, the lack of Reb1 and Reb2 sites should reduce production of the large form of *RPB2* mRNA after UV treatment.

The cellular signals that drive the polyadenylation switching and the POL II -independent POL II mRNA transcription are a question to be answered in the future. A possible source of signal could be the UV-induced DNA damage response. POL II is known to be blocked by UV induced DNA damage such as CPD (Mei Kwei et al, 2004). In the case of *rpb1-1*, elongating RNA polymerases in the *rpb1-1* strain could be inactivated *in situ* on the DNA template after shifting to the non-permissive temperature, mimicking the situation where polymerases are blocked by UV-induced DNA damage. Preliminary data suggested that *RAD53*, the gene required for the DNA damage response,

is not required for triggering polyadenylation switching (Appendix G). Moreover, peroxide and MMS treatments are known to induce the DNA damage response [(Leroy et al, 2001; Conde et al, 2010; Haghnazari & Heyer, 2004b) and Chapter III] but do not effectively trigger polyadenylation switching on the *RPB2* gene (Appendix H). Taken together, our results suggest that the DNA damage response is not the trigger for polyadenylation switching and POL II independent mRNA synthesis. Rather, it appears more likely that blocked transcription or the reduced number of POL II complexes are likely events that trigger the feed-back synthesis of POL II mRNA. Both stalled POL II on DNA and heat-denatured POL II might be targeted to ubiquitin-dependent degradation. Therefore it would be of interest to test if the ubiquitin-dependent degradation pathway is involved in signaling the POL II independent mRNA synthesis.

Chapter VI

Appendices

Appendix A. Important residues for PC4's antioxidant activity.

PC4 appears to be toxic to bacteria because few cells survive when PC4 is induced in the papillation assay (Wang et al, 2004). The few bacteria that do survive in the papillation assay seem to contain mutations because they grow normally when PC4 is induced. I sequenced 3 single clones from bacterial colonies that remained suppressed, based on the white colony phenotype. They are named WC1, WC2, WC3. WC2 suppressed mutagenesis in the *mutM mutY* strain upon retransformation. It has a large 3' deletion in the coding sequence of PC4 and suppressed mutagenesis. Its protein sequence is shown in Figure 6.1. When aligned with PC4, we found residue 1-70 are identical while the rest is missing in WC2, suggesting a.a. 1-70 is sufficient for PC4's antioxidant activity. As Sub1 appears to conserve the antioxidant activity of PC4 (Chapter III), Sub1 is also included in the alignment for comparison. The "CON" lines show conserved residues between Sub1 and PC4. Residues conserved in all of the three proteins are highlighted in the bold font. These residues may be critical amino acids for the antioxidant activity of PC4 and Sub1. Sub1 sequence after the conserved region is not shown.

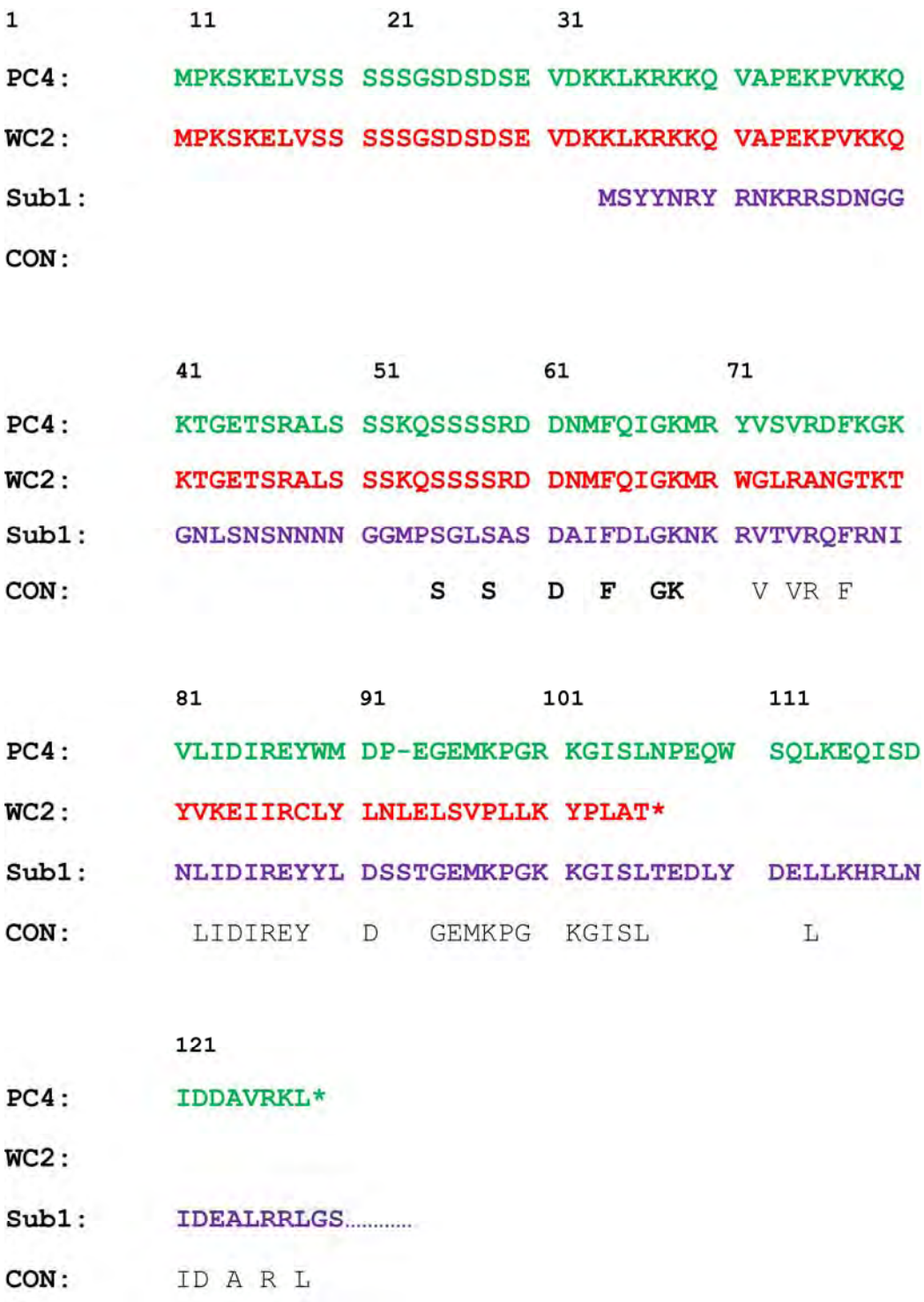


Figure 6.1 Protein sequence alignment of PC4, WC2, and Sub1. See previous page for details.

Appendix B. Analysis of single strand annealing (SSA) in the *sub1Δ* mutant.

Rationale: SSA is the process where homologous regions located on both sides of the double strand break anneal and any intervening sequences are deleted. It is a special form of homologous recombination repair and it depends on *RAD52* but not *RAD51*.

Because the yeast *sub1Δ* mutant produces single strand DNA (ssDNA) ends rapidly by DNA end resection, these ssDNA ends might stimulate SSA. Therefore I tested if the *sub1Δ* mutant has an increased frequency of SSA. I used the yeast strain FRO-830 to test the SSA efficiency. FRO-830 is a gift from Francesca Storici (Storici et al, 2006; Storici & Resnick, 2006). As shown in Figure 6.2, the GSHU CORE cassette is inserted into the *LYS2* gene between two 90bp repeats. When the I-SceI endonuclease is induced by galactose it cuts the chromosome at the I-SceI recognition site as depicted. DSB resection can expose the two 90bp repeats. When the repeats anneal, they will cause deletion of the GSHU CORE cassette, leading to a functional *LYS2* gene.

Methods: wild type (MVY802, i.e. FRO-830) and the *sub1Δ* derivative (MVY804) were grown in YEP-raffinose at 30°C overnight to mid log phase. The culture was incubated with 2% galactose at 30°C for 90 minutes to induce DSBs. Cells were plated onto lysine dropout medium to quantify SSA events and onto Uracil dropout medium to quantify direct ligation events. Uninduced cells were plated onto YPD to measure total cell numbers.

Results:

The percentage of cells that performed SSA or direct ligation are shown in Table 6.1. The *sub1Δ* mutant does not appear to have an increased SSA activity.Note: The percent of SSA and ligation does not add up to 100%. This is presumably caused by different plating efficiencies on YPD medium versus galactose containing minimum medium.

Table 6.1 Efficiencies of SSA and direct ligation in wild type and the *sub1Δ* mutant.

	SSA	**ligation**
wild type	7.8%	98%
sub1Δ	5.4%	106%

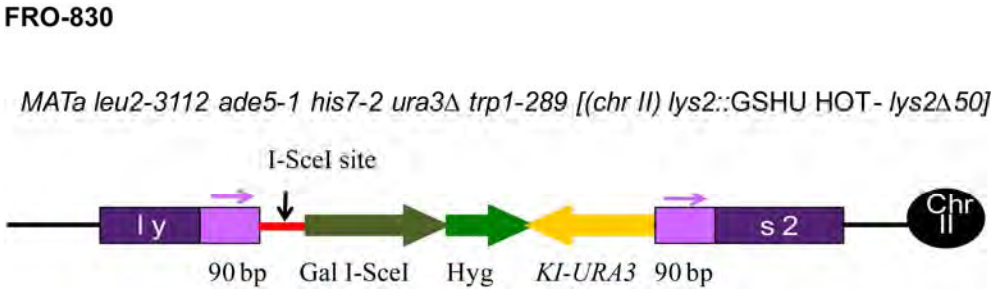


Figure 6.2 Schematic drawing of the SSA system. The GSHU CORE consists of the I-SceI gene under the Gal promoter, the hygromycin resistance gene, and the *URA3* gene from *Kluyveromyces lactis* (*KlURA3*). Drawing is used by permission from Dr. Francesca Storici (Georgia Institute of Technology).

Appendix C. PC4-CTD (a.a. 40-127) complements the *sub1Δ* mutant in the plasmid ligation assay

Rationale: To test if PC4 complements the *sub1Δ* mutant in the plasmid ligation assay, I compared the plasmid ligation efficiencies of wild type (MVY101), the *sub1Δ* mutant (MVY105), and the *sub1Δ* mutant with PC4-CTD (a.a. 40-127) expressed under a *GPD* promoter (MVY115). PC4-CTD (a.a. 40-127) lacks the amino terminal domains that are required for its transcription regulation function. It has been shown that it complements the *sub1Δ* mutant in peroxide sensitivity (Chapter III and (Wang et al, 2004)).

Methods: Competent yeast cells are transformed with plasmid pRS315 or the plasmid linearized by BamHI, and plated on the leucine drop out medium to select transformants. Cells that ligate the linear plasmid by NHEJ will retain the *LEU2* gene and survive on the selection media. Ligation efficiency is calculated as the transformation efficiency obtained using the linear plasmid divided by the transformation efficiency using the circular plasmid. Ligation efficiencies of wild type are normalized to 100% in each experiment.

Results: The ligation efficiency of the PC4-expressing sub1Δ mutant (MVY115) is almost twice of that of wild type, whereas that of the *sub1Δ* mutant is greatly reduced. (See Figure 6.3).

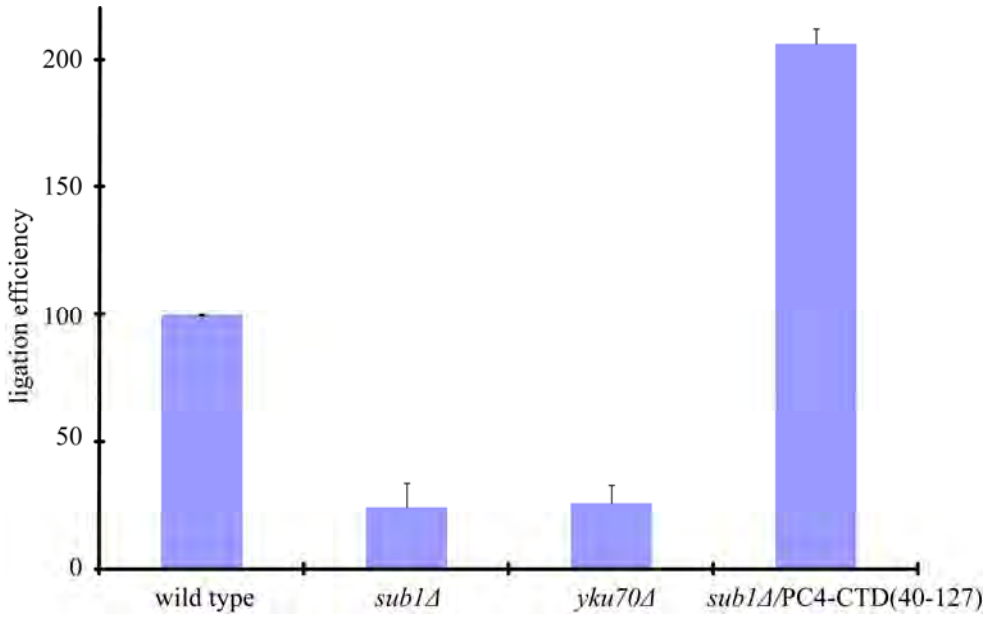


Figure 6.3 PC4-CTD (40-127) complements the *sub1Δ* mutant in plasmid ligation.

(For details see: methods in Appendix C).

Appendix D. Subunits of RNA polymerase II in yeast.

Table 6.2 Yeast POL II subunits.

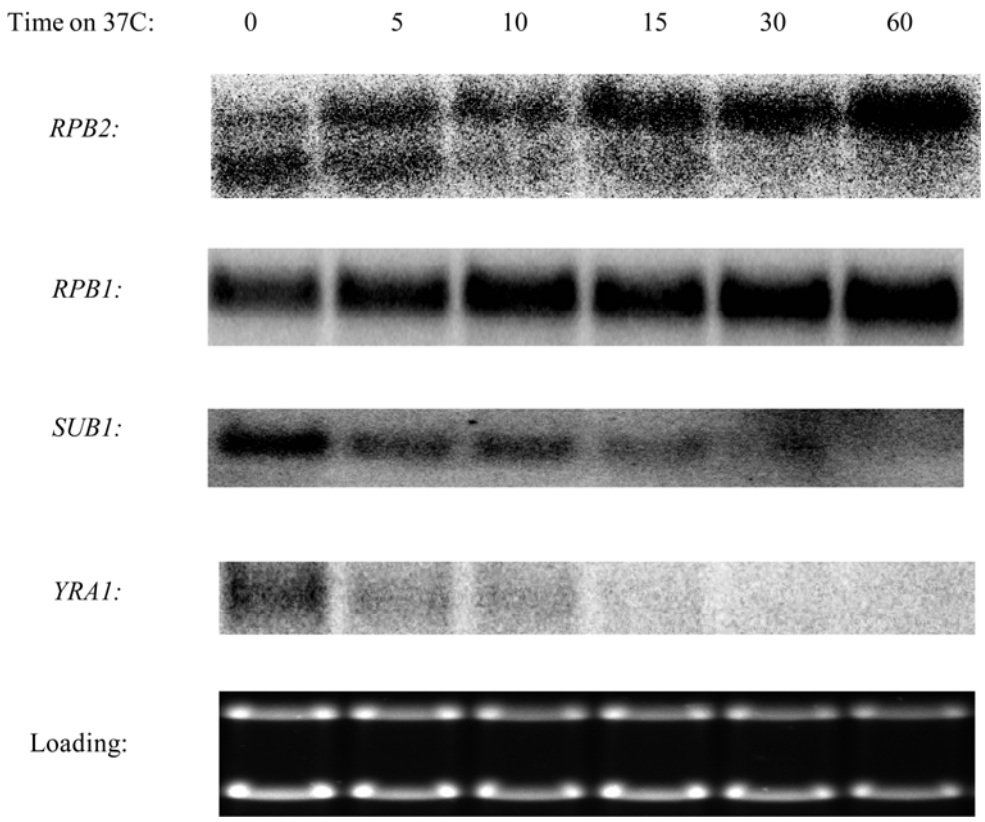
Gene	Size (kDa)	deletion viability
RPB1	220	inviable
RPB2	150	inviable
RPB3	45	inviable
RPB4	32	conditional
RPB5	27	inviable
RPB6	23	inviable
RPB7	17	viable
RPB8	14	inviable
RPB9	13	conditional
RPB10	10	inviable
RPB11	14	inviable
RPB12	8	conditional

References: (Woychik & Young, 1990; Ishihama et al, 1998)

Appendix E. mRNA transcription in the *rpb1-1*mutant at non-permissive temperature.

Figure 6.4. mRNA transcription in the *rpb1-1* mutant after shifting to the non-permissive temperature 37°C. Ribosomal RNA is shown as the loading control. *rpb1-1* cells were cultured to mid-log phase at 23°C, then shifted to 37°C to inactivate POL II. Levels of *SUB1* and *YRA1* mRNA decreased immediately after the temperature shift, confirming that POL II dependent transcription had been inhibited. The *RPB2* gene exhibits two forms of mRNA, the shorter form is present at a higher level at time 0. After the temperature shift, the shorter form disappeared quickly but the longer form increased robustly over time. Thus transcription of the long *RPB2* mRNA is independent of RNA polymerase II.*RPB1* mRNA, which encodes another POL II subunit, also continued to increase in levels after the temperature shift. However, unlike *RPB2*, no differences in polyadenylation were evident in the *RPB1* message.

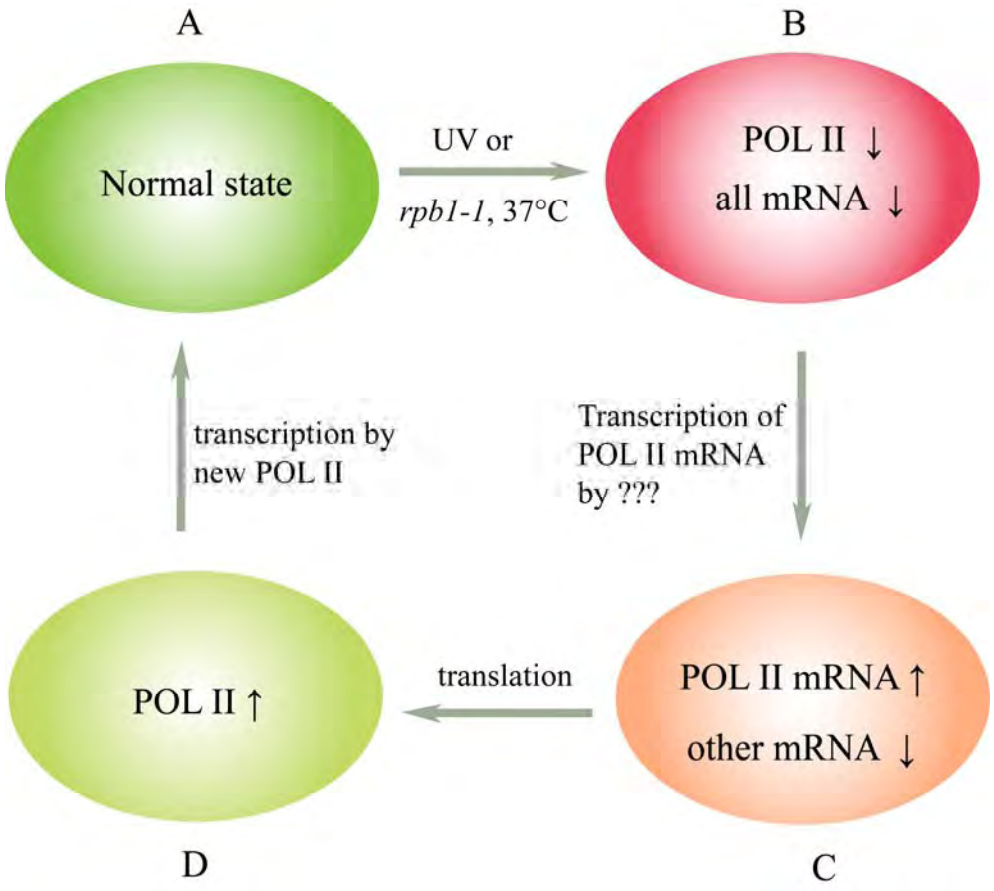
Figure 6.4



Appendix F. The feed-back synthesis model

Figure 6.5. The feed-back synthesis model to describe transcription recovery following transcription inhibition. After UV irradiation, or shifting the *rpb1-1* strain to non-permissive temperature, 37°C, the number of POL II complexes in the cell dramatically drops due to transcription-blocking DNA damage or heat inactivation of the largest POL II subunit Rpb1. Subsequently, the levels of all mRNAs including the mRNAs for the POL II subunits start to decrease, a situation described in state B. In order to move back to state A, the cell needs to regenerate its POL II pool, which depends on the translation of the mRNAs of POL II subunits. However, because the mRNA levels are low, the cell will demand more POL II mRNAs, which depends on efficient transcription by POL II. In order to exit this negative cycle of requesting POLII or POL II mRNA, cells use another as yet unidentified RNA polymerase to synthesize the mRNAs of POL II, leading to state C. Once in state C, cells translate the POL II mRNA and assemble the subunits into functional POL II holoenzymes, leading to state D. Finally, cells in state D use the newly synthesized POL II to transcribe all other mRNA and the cells return to the normal state A. Overall, this model hypothesize that cells use the reduced number of POL II as a feed-back signal to synthesize more POL II, for the cells to recover from transcription inhibition.

Figure 6.5


A
B
Normal state
UV or
rpb1-1, 37°C
POL II ↓
all mRNA ↓
transcription by
new POL II
Transcription of
POL II mRNA
by ???
POL II ↑
translation
POL II mRNA ↑
other mRNA ↓
D
C

Appendix G. Rad53 is not required for polyadenylation switching.

Rationale: *RAD53* is required for inducing the DNA damage response in yeast cells. I used the *rad53Δ* mutant (MVY496, isogenic to MVY150 or the W303 strain) to test if the DNA damage response is required for UV induced polyadenylation switching of the *RPB2* gene. If the DNA damage response is required to signal the polyadenylation switching, we would not expect to see polyadenlyation switching of the *RPB2* gene in the UV-irradiated *rad53Δ* mutant cells.

Methods: MVY496 was grown to mid log phase, resuspended in PBS to an OD_{600} reading of 0.8, then irradiated with UV at 1.7J/m^2/s for 42 seconds. Cells were then collected and cultured in YPD liquid medium at 30°C. After 0, 15, and 30 minutes, cells were collected and yeast total RNA was extracted and analyzed by Northern analysis.

Results: The long form of *RPB2* mRNA was preferentially synthesized 30 minutes after UV treatment in the *rad53Δ* mutant, suggesting that the DNA damage response is not required for polyadenylation switching of the *RPB2* gene.

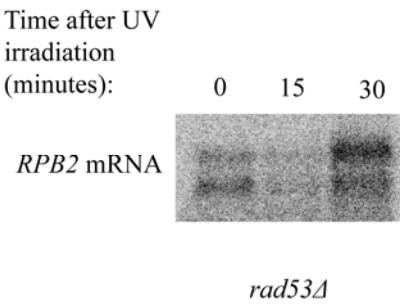


Figure 6.6. Polyadenylation switching occurs in the *rad53Δ* mutant after UV treatment. The *rad53Δ* mutant cells (MVY496) were treated with UV at 1.71J/m^2/s for 42 seconds and immediately cultured in YPD liquid medium for indicated times. Yeast total RNA was then extracted and subjected to Northern analysis. The *RPB2* gene exhibited two distinct bands due to alternative polyadenylation as described in Chapter IV. Transcription was initially inhibited by UV (see the lane of 15 minutes), then recovers after 30 minutes. The long form of *RPB2* mRNA was preferentially synthesized, suggesting alternative polyadenylation is induced in the *rad53Δ* mutant by UV.

Appendix H. H_2O_2 and MMS do not effectively induce polyadenylation switching.

Rationale: Peroxide and MMS can damage DNA and are known to induce the DNA damage response (Leroy et al, 2001; Conde et al, 2010; Haghnazari & Heyer, 2004b). Here I tested if peroxide and MMS can induce polyadenylation switching. I treated yeast cells with different concentrations of MMS or peroxide and analyzed the *RPB2* mRNA by Northern blot.

Methods: Yeast strain MVY150 was grown in 50ml YPD to mid-log phase and 5 ml of cells (OD_{600}=2.4) was saved as the pretreatment sample. For H_2O_2 treatment, H_2O_2 was added to 5 ml of cells (OD_{600}=2.4) to final concentrations of 1, 5, 10mM. The cells were then incubated at 30°C for 30 minutes. For MMS treatment, MMS was added to 5ml of cells (OD_{600}=2.4) to final MMS concentrations of 0.05%, 0.1%, and 0.2%. Then after incubation at 30°C for 30 minutes, the yeast total RNA was extracted by using the hot phenol method and subsequently subjected to the Northern analysis (He & Jacobson, 1995).

Results: As shown in Figure 6.7, transcription of the long form of *RPB2* mRNA does not increase in MMS and peroxide treated cells at various concentrations. These results suggest that the DNA damage response does not trigger polyadenylation switching in the *RPB2* gene.

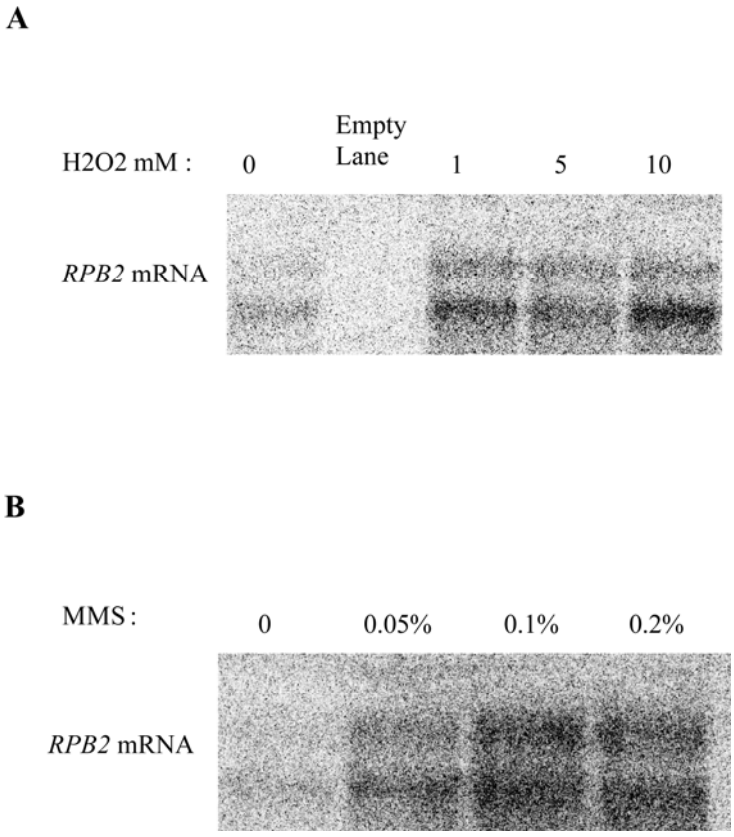


Figure 6.7 Peroxide and MMS do not induce polyadenylation switching of the *RPB2* gene effectively. A. The level of the long form of *RPB2* mRNA is not induced to exceed that of the short form by 1mM, 5mM, and 10mM H_2O_2. 5mM H_2O_2 has been shown to induce a robust DNA damage response (Figure 3.4b). B. Similarly, MMS does not induce polyadenylation switching effectively.

Appendix I. The protocol of the transcription-coupled repair assay.

[Prepare cells]

1. Equilibrate 1 liter of sterile PBS in 4°C refrigerator.

2. Inoculate yeast cells overnight to OD_{600}<=1.6 ($1x10^8$ cells/ml). Measure OD. (Note: Do not use saturated culture. They don't form spheroplasts during zymolyase digestion)

3. Rinse the Pyrex dish with 70% EtOH and irradiate with UV for over 5 minutes. Leave the UV light ON till all irradiations are finished. Record the intensity of the UV light. (Note: Stabilizing the UV light is important to maintain a consistent irradiation condition)

4. Collect the cells and resuspend in sterile and cold PBS to **OD=0.7** (total volume 80~200ml) in the Pyrex dish. (Note: the cell density is an important parameter and should be kept constant. Cell density greatly affects how much UV each individual cell receives.)

5. Turn off the light and work in dark till step 16.

6. Irradiate the cells in PBS for 42 second ($75J/m^2$). Turn on the rotary platform (set at 2.5) during irradiation. (Note: 40~45 seconds of UV irradiation is the appropriate

dosage range. In this dosage range, the transcribed strand can be repaired completely in an hour, while the non-transcribed strand will repair ~70% of the damage so we can see repair kinetics in both strands. Lower dosage produces smaller amount of CPD and the repair in both strands could be too quick to be characterized. If very high UV dosage is used (say 135J/m^2), only TCR will be seen (because TCR is very efficient) and GGR will be overwhelmed.

7. Take 15ml of culture for each repair time point (say: 0, 15min, 30min, 45min, 60min, 90min) in 15ml centrifuge tubes.

8. collect the cells and resuspend in 10ml culture media (say: YPD). Wrap the tubes with aluminum foil.

9. Store the tube for time point 0 on ice. Roll other tubes at 30°C for different repair times and store them on ice afterwards.

10. Collect the cells by centrifugation.

[Prepare DNA]

11. Resuspend the cells in 2ml of 0.9M sorbitol, 0.1M Na_2EDTA (PH7.5).
    (Note: store the buffer at 4°C)

12. Add 50μL of a 15mg/ml solution of Zymolyase 20T (0.1ml at 2mg/ml also works).
    Incubate for 30 minutes at 37°C (on a rotary roller).

13. Collect the cells by centrifugation briefly. Discard the supernatant.

14. Resuspend the cell pellet in 3ml of 50mM Tris-Cl (pH7.4), 20mM Na_2EDTA.

15. Add 300µl of 10% SDS and mix.
16. Incubate for 30 minutes at 65°C. Shake occasionally to dissolve clumps.
17. Add 900µL of 5M potassium acetate and store on ice for 1 hour or over night (overnight preferred).
18. Centrifuge in JA12 rotor at 5000 rpm for 20 minutes at 4°C. If large clumps are seen, re-centrifuge at 7000rmp for 10minutes to clear debris.
19. Transfer the supernatant to a fresh plastic centrifuge tube and add 7/3 volumes of ethanol at room temperature. Mix and centrifuge in a JA12 rotor at 5000 rpm for 15 minutes at room temperature. Discard the supernatant.
20. Discard the supernatant, drain the liquid, resuspend the pellet in 400 µl of TE.
21. Transfer to a 1.5ml centrifuge tube.
22. Add 30µL of NaOAc and 950µL of 100% EtOH. Mix by inversion. Centrifuge at 12000g in a tabletop centrifuge for 10 minutes. Discard the supernatants. Wash with 500µL of 70% ethanol and centrifuge at 12000g for 5 minutes. Discard the supernatant. Wash with 70% ethanol. Vacuum dry for 7 minutes.
23. Resuspend the precipitate in 200µl of TE (PH=8) (This may take a long time, don't use 50°C to facilitate the dissolving). Store at 4°C.
24. Measure the relative DNA concentrations by spectrometer at the 260nm absorbance . The DNA concentration should be about 50ng/µL. (Note: for any strain, we can assume the concentration of the DNA with medium A260 reading is 50ng/µL, and other concentrations can be derived from their A260 readings)

[Digest DNA]

25. Restriction digest the DNA with NruI (for *RPB2* gene). E.g.:

    3µg genome DNA in 60µL + 0.6 NruI + 74 H_2O+ 15 NEB3

    Shake occasionally, 37°C for 2 hours.

26. Ethanol precipitate the DNA. Vacuum dry for 7 minutes.
27. Resuspend the DNA in $TE_{7.4}$ to concentrations of 150-200ng/µL.
28. Add 1-1.5µL of T4 endo V (NEB) or 0.25µL of T4 endo V (Epicentre) to 0.5µg (3µL) restriction-digestion product, e.g.:

    3µL DNA + 1µL T4-buffer + 1µL BSA + 4µL H_2O + 1µL T4EV (NEB) Mix thoroughly. Incubate at 37°C for 30minutes. Freeze at -20°C or run gel directly.

[Alkaline gel electrophoresis]

29. Make fresh 0.8% agarose with correct amount of agarose and water and let it cool down in 50°C water bath (Do this step the day before running the gel is more convenient).

30. Add NaOH and EDTA to the gel according to the table below:

(The B1 gel cast needs 50ml gel to hold 17µL samples with B1-14 (1.5mm) comb)

Final Conc	Stock	Volume of Stock		
		100ml (2 B1)	25ml (B1A)	50ml (B1)
0.03N	10 N NaOH	300µL	75µL	150µL
1mM	0.5M EDTA pH8.0	200µL	50µL	100µL

Let solidify for about 30 minutes.

31. Each lane use 0.5µg DNA (10µL). Mix thoroughly the samples with 6X alkaline loading buffer (extra NaOH added) (Rule of thumb: 10µL sample+ 4µL loading buffer). Load all the samples into the wells. Run gel at 60V for 3 hours (if B1 gel).
32. Rinse the gel, treat the gel in 0.25N HCl for 20 minutes (depurinate).
33. Rinse the gel, treat the gel in 0.5N NaOH for 30 minutes (denature).
34. Rinse the gel, neutralize the gel by soaking the gel in neutralizing solution for 30 minutes. (1 M Tris.Cl [pH7.6], 1.5 M NaCl).
35. (optional) Dilute Vistra Green 1:10000 in TE buffer or TAE buffer (pH7-8.5), soak the gel for 10-20 minutes. Take pictures under UV. (Staining won't affect subsequent steps)

[Transfer DNA to Membrane]

36. The procedures should be performed according to the manufacture's instructions.
37. Use 10X SSC as transfer buffer, transfer the DNA onto nylon membrane (Zeta-Probe blotting membranes from BioRad is the tested working membrane. Membrane from Amersham is no good: it cannot be stripped).
38. After the transfer, mark the membrane with pencil for information.
39. Stain the gel with Ethidium Bromide and check transfer efficiency.
40. Cross-link the DNA to the membrane (better to be damp) by placing the (damp) DNA-side up on the filter paper in the UV crosslinker and UV irradiate.
41. The membrane is now ready to hybridize, or it can be stored dry at 2-8°C.

[Hybridize the membrane]

Prepare the probes according to the attached protocol.

42. Boil the 100μg/ml Salmon Sperm DNA for 10 minutes before use.

43. Prepare the prehybridization buffer (tRNA can be omitted if DNA probe is used):

	15ml	20ml	30ml	40ml	60ml
preBuffer1	15ml	20ml	30ml	40ml	60ml
10mg/ml salmon sperm DNA	300µL	400µL	600µl	800µL	1.2mL
10mg/ml tRNA	37.5µL	50µL	75µL	100µL	150µL

44. Prepare the hybridization buffer (tRNA can be omitted if DNA probe is used):

	15ml	20ml	30ml	40ml	60ml
PreBuffer2	15ml	20ml	30ml	40ml	60ml
10mg/ml salmon sperm DNA	150µL	200µL	300ml	400µL	600µL
10mg/ml tRNA (final 25µg/ml)	37.5µL	50µL	75µL	100µL	150µL

45. Wet the membrane using 6XSSC if it is dry.
46. Slide the membrane into the roller bottle, add 15ml hybridization buffer, prehybidize at 42°C for 2 hours or over night. (Don't overlap membranes. Don't use the nylon mesh.)
47. Add RNA probe ($0.5\text{-}2x10^6$ incorporated counts per ml of hybridization buffer) to 15ml hybridization buffer. Replace the prehybridization buffer with the probe containing buffer.
48. Hybridize at 42°C overnight.

[Wash and detect]

49. [2XSSC +0.1%SDS], 100ml in plastic boxes, RT, 2X5min (room temperature).
50. **Prewarm** [0.1%SSC+0.1%SDS] to 58°C, 50ml in roller bottles, 58°C, 2 x 15min. (60°C can be used if the probe is new)
51. Use phosphoimager to image the membrane.

[Deprobe the membrane if necessary]

51. Incubate membrane at 45°C for 30 min in 0.4M NaOH (2ml 10M NaOH + 48ml ddwater)
52. Transfer to a solution of:

ddH_2O	39.25ml ddH_2O
0.1x SSPE	0.25ml 20X SSPE
0.1% SDS	0.5ml 10% SDS
0.2M Tris-HCl	10ml of 1M Tris pH 7.5
	total 50ml

53. Incubate for 15min at 45°C.
54. Wrap in Saran Wrap.
55. check deprobe overnight.
56. Store at 4°C.

Random primed DNA labeling

1. Mix 2μL template DNA and 7μL water.
2. Boil for 10 min, chill on ice.
3. add in order:

1). dAGTTP	3μL
2). reaction mix (vial 6)	2μL
3). 50μCi dCTP (10μCi/μL)	5μL
4). Klenow enzyme (vial 7)	1-2μL

4. Mix, bump, 37°C for 1 hour.
5. Use G-50 column (for DNA) to remove single nucleotides.
    a) Resuspend the columns gently.
    b) Remove the caps of the columns, and the pinings of the columns to drain the columns for 2 minutes.
    c) Spin in SwingBucket centrifuge at 1100g for 2 min, place the columns in a new collection tube.
    d) Apply the reaction mixture (60μL) to the center of the columns.
    e) Spin at 1100g for 5 min, the flow through is the purified sample.
6. Boil or 95°C for 10min before transferring to the hybridization bottle.

Note to make template DNA: Gel extract the preferred fragment. Use 50-100ng of the DNA template (the Roche protocol recommends 25ng template though).

In vitro transcription RNA labeling

1. Add in order:

T7 probe:

1). Transcription 5X buffer	4μL
2). DTT, 100mM	2μL
3). RNasin inhibitor	1μL
4). rAGUTP, 2.5mM each	4μL
5). 100μM rCTP	2.4μL
6). T7 probe template	1μL
7). [α-^{32}P] rCTP (10μCi/μL)	5μL
8). T7 RNA polymerase	1μL

T3 probe:

1). Transcription 5X buffer	4μL
2). DTT, 100mM	2μL
3). RNasin inhibitor	1μL
4). rAGUTP, 2.5mM each	4μL
5). 100μM rCTP	2.4μL
6). T3 probe template	1μL
7). [α-^{32}P] rCTP (10μCi/μL)	5μL
8). T3 RNA polymerase	1μL

2. Mix, bump, 37°C for 1hour.
3. Remove the DNA template:
    a) Add 1μL RQ1 DNase to each reaction
    b) Incubate at 37°C for 15min.
    c) Add 1μL 0.5M EDTA to stop the reaction, add 40μL water.
4. Remove unincorporated nucleotides:
    1. resuspend the column, open the caps and snap the pinings to drain.
    2. 1100g fro 2min in SwingBucket centrifuge.
    3. Use a new collection tube, apply the sample to the center of the column.

4. 1100g for 5 min.

Note: No need to heat denature the RNA probe.

Note: to make the template:

Linearize the vector.

Extract with phenol:chloroform:isoamyl alcohol.

Ethanol precipitate.

Resuspend in TE. (0.2-1.0mg/ml)

Formula to make 6X alkaline loading buffer (1ml final volume)

Final Concentration	Stock Concentration	Volume of Stock
300mM NaOH	10N NaOH	30µL
6mM EDTA, pH8.0	0.5M EDTA	12µL
18% Ficoll	Powder	0.18gm
0.15%Brom cresol Green	Powder	0.0015gm
0.25% Xylene cyanol FF	Powder	0.0025gm
Water		Fill tube to 1ml

Add 26µL of 10N NaOH to 1000µL 6X alkaline loading buffer before use. This will denature the sample completely.

Formula to make alkaline running buffer:

Final concentration	Stock	Volume of stock	
		1L final volume	2L final volume
30mM	10 N NaOH	3mL	6ml
1mM	0.5 M EDTA, pH8.0	2ml	4ml

Formula to make 20X SSC:

Dissolve 175.3g of NaCl and 88.2g of sodium citrate in 800ml of H_2O. Adjust the pH to 7.0 with HCl. Adjust the volume to 1 liter with H_2O. Sterilize by autoclaving.

Salmon Sperm DNA:

Prepare the 10mg/ml salmon sperm DNA by pressing through 16~21 gauge needle 5 times. Aliquot and freeze.

100X Denhardt's Reagent:

10g Ficoll 400

10g BSA,

10g Polyvinylpyrrolidone

add ddH_2O to 500ml,

mix (don't filter), store at -20°C.

preBuffer 1	500ml:
250ml of formamide	50% formamide
125ml of 20X SSPE	5x SSPE
25ml of 20%SDS	1% SDS
50ml of 100X Denhardt's	10x
50ml of Water	

preBuffer 2	500ml:
250ml of formamide	50% formamide
125ml of 20X SSPE	5x SSPE
25ml of 20%SDS	1% SDS
10ml of 100X Denhardt’s	2X
90ml of water	

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