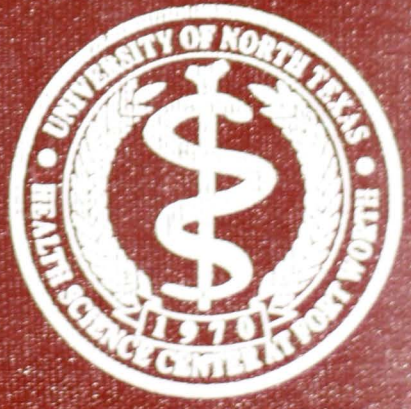
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In this dissertation project, I aimed to study checkpoint response of stationary phase yeast to DNA damage caused by basal oxidative stress. My study was focused on the regulation of Rad53 phosphorylation in different repair deficient strains of yeast. Rad53 plays decisive roles in cell cycle progression, cell death and transcriptional regulation of repair proteins to a plethora of DNA insults, including oxidative DNA damage. Rad53 activity is upregulated by phosphorylation, generating Rad53 species of various degrees of phosphorylation.

I have measured steady state levels of Rad53 phosphorylation by western blotting following SDS-polyacrylamide gel electrophoresis at different intervals in stationary phase, in various mutant backgrounds. To address the possible contribution of different repair pathways to endogenous DNA damage, I utilized two different sets of DNA repair deficient strains such as those deficient in Base excision repair (BER) and nucleotide excision repair (NER), and other set was deficient in Ku protein and NER. Interestingly, in both BERNER and Yku70rad4 strains, Rad53 phosphorylation was evident in stationary phase that is after 2 days, 4 days and 6 days but not in logarithmic phase. This covalent modification disappears after phosphatase treatment. This Rad53 modification was absent in their respective *rho*^{0} mutants, which lack mitochondrial DNA, indicating

involvement of mitochondrial ROS in this checkpoint response. We analyzed mutants of different checkpoint proteins for Rad53 phosphorylation. Exclusive involvement of Rad17, Rad50 and Mec1 kinase in Rad53 phosphorylation strongly suggests processed DNA double strand breaks as critical lesions in BERNER cells. Analysis of Yku70 and NER deficient strain showed involvement of ssDNA, which is most likely at telomeres. This study consents with the model of unrepaired oxidative base damage, which can accelerate the appearance of single stranded DNA in the vicinity of double strand breaks (DSBs) or at telomeres.

STUDIES OF CHECKPOINT RESPONSES CAUSED BY ENDOGENOUS OXIDATIVE DNA DAMAGE IN DNA REPAIR DEFICIENT SACCHAROMYCES CEREVISIAE

DISSERTATION

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By

Vaibhav Pawar, B.Tech.
Fort Worth, Texas
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Dedicated to Dr. A.P.J. Abdul Kalam, former eleventh president of India. He was engineering visionary of India and has received India's highest civilian award "Bharat Ratna". He belonged to a very modest family settled in India. I have mainly inspired by his belief in science. He firmly believes that the power vested in science can resolve society's problems. He also views science and technology as ideology free areas and believes in entrepreneurial drives. His autobiography book "Wings of fire" has been a great inspiring factor for me to pursue a scientific career. I would always like to be a student of science as him and I hope my curiosity in science will increase logarithmically over the rest of my life.

My mentor, Dr.Wolfram Siede has been instrumental in bringing out the best in me during my stay in his laboratory. I am indebted to him for his guidance. I deeply appreciate several members of his laboratory for their support and help. I would like to forward my thanks to my committee members, Dr.Rafael Alvarez-Gonzalez, Dr.Yiwei Jiang, Dr. Raghu Krishnamoorthy and Dr. Dan Dimitrijevich. I deeply appreciate their constructive criticism and efforts taken by them to teach me certain intricacies of biomedical research. This project would haven been impossible without their encouragement and guidance. Last but not least I would like to express deepest gratitude to my family and friends. My family in India has been always instrumental in my career, without their love and support I would not have successfully completed this gigantic process of Ph.D.

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LIST OF ABBREVIATIONS

AP site	Apurinic or apyrimidinic site or abasic site
APN	AP endonuclease
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3 related
BER	Base excision repair
Dnl4	DNA ligase 4
DNA	Deoxyribonucleic acid
dRpase	Deoxyribophospho diesterase
DSB	Double strand break
ETS	Electron transport system
Log phase	Logarithmic phase
MRX	Mre11/Rad50/Xrs2 complex
NER	Nucleotide excision repair

NHEJ	Non-homologous end-joining
Ntg	N-terminal glycosylase
OXPHOS	Oxidative phosphorylation
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S.cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
SOD	Super oxide dismutase
UV	Ultra violet
WT	Wild type
XP	Xeroderma pigmentousm
XPA	Xeroderma pigmentosum group A

CHAPTER I

INTRODUCTION TO THE STUDY

The introduction is divided in two broad sections. First section mainly focuses on the broader picture of my dissertation. It gives information about the role of various DNA repair pathways in the repair of oxidative DNA damage. It also includes a brief description of results, which I have included in the later part of my dissertation. The second part contains special topics, which are important in understanding certain intricacies of my dissertation. Relevant topics are explained in detail in this section.

SECTION I

All living cells appear to be capable of exiting the normal cell cycle (proliferating state) and entering an alternative (resting) state termed quiescence or G_0. Furthermore, most eukaryotic cells, whether they exist as single celled such as *Saccharomyces cerevisiae* (budding yeast) or multicellular organisms such as mammals, spend the majority of their natural lives in a quiescent

state (Gray et al., 2004). Quiescent yeast cells are commonly obtained in the laboratory by growing liquid cultures to saturation in rich media, usually for 5 to 7 days at 30°C. The term "stationary phase" has been used to describe the state of saturated liquid cultures and the state of the constituent cells. The path by which a culture of cells reaches saturation is very complex. Initially, the constituent cells derive their energy from fermentation, the process by which glucose is preferentially metabolized via glycolysis to form non-fermentable carbon compounds, particularly ethanol. During the exponential or logarithmic growth phase, the culture grows rapidly (and the constituent cells proliferate with an average doubling time of approximately 90 min at 30°C) until glucose is exhausted in the medium. This point termed as diauxic shift, at this point the cultures cease rapid growth while the constituent cells re-adjust their metabolism to utilize the non-fermentable carbon sources still present in the medium. After the diauxic shift, the cells in the culture cease proliferation after the depletion of ethanol and other non-fermentable carbon sources (Lillie & Pringle, 1980). At this point, the culture reaches stationary phase and most, if not all, of the constituent cells are quiescent (Werner-Washburne, Braun, Johnston, & Singer, 1993). All cultures having passed the diauxic shift are often classified as being in stationary phase and the constituent cells thus in quiescence. Stay of yeast in quiescence is the measure of its life span. This ability of non-dividing cells to maintain viability over time is termed as chronological life span of yeast (Longo, Liou, Valentine, & Gralla, 1999; MacLean, Harris, & Piper, 2001). The chronological life span of yeast may appear to be a starvation phase distinct from the aging of higher eukaryotic post-mitotic cells; non-dividing yeast cells are not starving (Longo & Fabrizio, 2002; Longo & Finch, 2002). Respiration is the primary source of energy in these cells and mitochondrial oxidative metabolism may be the chief source of energy

for quiescent cells. Consistent to the use of mitochondrial oxidative metabolism, the quiescent cells show production of respiration-dependent ROS (Werner-Washburne et al., 1993).

In addition to fulfill energy requirements of stationary phase cells, mitochondria are also responsible for production of ROS. Mitochondrial respiration is thought to be the major source of endogenously generated ROS (Costa & Moradas-Ferreira, 2001). It has been estimated that 1-2% of oxygen reduced by mitochondria is converted to superoxide (Turrens, 1997), with the majority produced by complex III of the electron transport chain (Cadenas, Boveris, Ragan, & Stoppani, 1977). During cellular respiration, complex I and complex II transfer electrons to coenzyme Q to produce reduced QH2. Further, to generate co-enzyme Q, complex III passes electrons from QH2 to cytochrome c. This process generates semi-quinone anions that avidly transfer electrons to molecular oxygen, thereby generating superoxide radicals. Additionally, exposure to chemical inhibitors that specifically arrest mitochondrial electron transport chain can result in increased ROS production. These ROS include active forms of oxygen such as hydroxyl and superoxide radicals, hydrogen peroxide, and singlet oxygen. The most lethal hydroxyl radicals are also generated from hydrogen peroxide though the mediation of adventitious iron or other transition metals by the process of the Fenton reaction (McCord & Fridovich, 1978). Alternatively, the superoxide radicals may be processed into hydrogen peroxide by superoxide dismutase, which may be further reduced by the metal ion catalyzed Fenton reaction to produce hydroxyl radicals. Fenton catalyzed reduction of hydrogen peroxide is believed to generate the majority of hydroxyl radicals found *in vivo*.

These highly reactive oxygen species can cause oxidative damage to virtually any bio-molecules including proteins, lipids and nucleic acids. Damage to bio-molecules other than DNA may not be significant for cellular survival as they get replenished via active transcription in stationary

phase cells. But inability to properly repair DNA damage or respond to its presence can result in a number of deleterious biological consequences, including cell death, mutation, and neoplastic transformation (Hanahan & Weinberg, 2000). DNA damage is very significant for cellular viability in stationary phase because ROS generated through cellular metabolism can induce several types of lesions, such as oxidized bases, apurinic/ apyrimidinic (AP) sites and DNA strand breaks (Dizdaroglu, 1991). ROS can react with DNA bases or can abstract hydrogen atoms from them. A wide variety of pyrimidine products can be created including thymine glycol, 5-hydroxy cytosine etc. Purine adducts are also observed such as 8-hydroxy-guanine, which can lead to mis-pairing with other nucleotides. Many types of sugar damage are also produced in DNA after ROS attack (Imlay, 2002). Some of these result in disruptions in the phosphodiester backbone, thereby giving rise to single-stranded (ss) DNA nicks of various configurations. ROS are also the source of double strand (ds) DNA breaks. The oxidative metabolism seems to be one important contributor to the generation of chromosome breaks (Karanjawala, Murphy, Hinton, Hsieh, & Lieber, 2002) and somatic mutations (Busuttil, Rubio, Dolle, Campisi, & Vijg, 2003).

One of the other important targets of ROS attack is telomeric DNA (von Zglinicki, 2002). Telomeres are specialized nucleoprotein complexes at the end of linear chromosomes. They are essential for chromosome integrity and genomic stability because they protect natural chromosome ends from degradation and end-to-end fusion. Higher eukaryotic cells show absence of telomerase and show progressive telomere shortening during cell division. This progressive telomere shortening causes irreversible loss of replicative capacity and it further leads to senescent phenotype. Recent body of work has shown that both accelerated telomere shortening and decreased replicative life span occur under a range of mild stresses. A similar

acceleration of telomere loss and reduction in proliferative lifespan is also seen in fibroblasts from subjects with Fanconi anaemia (FA), a condition that results in increased oxidative stress. Faster telomere shortening rates are also observed in certain cell strains, which show higher peroxide levels (von Zglinicki et al., 2000). Thus oxidative stress can cause different types of DNA damage at telomeres. ROS can produce nucleotide or base damage, and it could also produce telomeric double strand breaks at high frequency, which may further lead to telomere shortening.

To handle these wide varieties of DNA damage, cells have evolved a number of mechanisms to repair DNA damage (Friedberg EC et al., 2005). In *Saccharomyces cerevisiae*, these pathways include base excision repair (BER), nucleotide excision repair (NER) and DNA double strand break repair and others. Oxidized DNA bases are substrates for DNA N-glycosylases, which initiate BER pathway (Krokan, Standal, & Slupphaug, 1997; Lindahl & Wood, 1999; Memisoglu & Samson, 2000). In *S.cerevisiae*, three DNA N-glycosylases/AP lyases are involved in the repair of oxidatively damaged DNA bases; Ntg1, Ntg2 and Ogg1 (Girard & Boiteux, 1997). Ntg1 and Ntg2 are closely related to each other (Augeri, Lee, Barton, & Doetsch, 1997). Ntg1 and Ntg2 display a broad substrate specificity, releasing oxidized pyrimidines such as 5,6-dihydrothymine, 5,6-dihydrouracil, 5-hydroxy-5-methylhydantoin, 5-hydroxyuracil, 5-hydroxycytosine or thymine glycol (Alseth et al., 1999; Augeri et al., 1997; Senturker et al., 1998; You et al., 1999). Ntg1 and Ntg2 also release purine-derived lesions, 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 4,6-diamino-5-formamidopyrimidine. On the other hand, Ogg1 exhibits a narrow substrate specificity, catalyzing the removal of 8-OxoG from DNA exposed to gamma-irradiation or H2O2 (Karahalil, Roldan-Arjona, & Dizdaroglu, 1998). Finally, Ntg1, Ntg2 and Ogg1 incise DNA at AP sites via a beta-elimination reaction (Alseth et

al., 1999; Karahalil et al., 1998). In contrast to BER pathway, NER is believed to repair bulky, helix distorting lesions such as bipyrimidine dimers (Lindahl & Wood, 1999). However, several reports indicate that there is considerable overlap between the DNA excision repair pathways, BER and NER, with respect to damage processing. For example, similar to BER, NER has shown to be capable of processing oxidative lesions such as thymine glycol and 8-oxoguanine (J. J. Lin & Sancar, 1989; Reardon, Bessho, Kung, Bolton, & Sancar, 1997). In addition NER has been shown to repair abasic sites (Torres-Ramos, Johnson, Prakash, & Prakash, 2000).

DNA double strand breaks produced inside cells are repaired by either of two major pathways (Lieber, Ma, Pannicke, & Schwarz, 2003). Homologous recombination functions in somatic cells during late S and G2 of dividing cells to repair dsDNA breaks. This is a point in the cell cycle when the DNA content is 4N, and sister chromatids are positioned adjacent to one another, thereby permitting a copying of information from one sister chromatid to the other. This typically restores the information content at the break site back to normal. Non-homologous end joining (NHEJ) is another important pathway for the repair of dsDNA breaks. The NHEJ pathway is active throughout the cell cycle in all vertebrate tissues, and it is a major pathway for repair of dsDNA breaks during G0, G1 and early S phase. The NHEJ pathway simply fuses two broken ends with little or no regard for sequence homology. NHEJ starts with binding of Ku heterodimer Ku70-Ku80 (Yku70/Yku80 in *S.cerevisiae*) to the broken ends (Figure 6). Ku facilitates recruitment of Artemis-DNA-PKcs complex, which is thought to trim single stranded overhands (Lieber, Ma, Pannicke, & Schwarz, 2003). Next, the ends are covalently joined by XRCC4-DNA ligase IV complex (Lif1-Dnl4 in *S.cerevisiae).* A few nucleotides at each end of the DNA break are lost in most instances during NHEJ event (Critchlow & Jackson, 1998; Lieber, Ma, Pannicke, & Schwarz, 2003). NHEJ may also be associated with larger deletions or

insertions of filler DNA (Y. Lin & Waldman, 2001; Sargent, Brenneman, & Wilson, 1997). Ku heterodimer was initially characterized for its role in the NHEJ pathway of DNA double strand break repair, Ku is now known to associate with and function at telomeres across species (Gravel, Larrivee, Labrecque, & Wellinger, 1998; Hsu, Gilley, Blackburn, & Chen, 1999; Riha, McKnight, Griffing, & Shippen, 2001). In *Saccharomyces cerevisiae,* Ku affects several aspects of telomere biology, including the recruitment of telomerase to telomeres and telomere length homeostasis (Fisher, Taggart, & Zakian, 2004; Porter, Greenwell, Ritchie, & Petes, 1996; Stellwagen, Haimberger, Veatch, & Gottschling, 2003), the protection of telomeric ends from nucleolytic degradation and homologous recombination (Maringele & Lydall, 2002; Polotnianka, Li, & Lustig, 1998), the formation of telomeric heterochromatin, which leads to the transcriptional silencing of nearby genes (Boulton & Jackson, 1998; Laroche et al., 1998), the late firing of replication origins near telomeres, and the nuclear localization of telomeres. Therefore BER, NER and Ku proteins play important roles in the repair of almost every lesion caused by oxidative stress to DNA. And absence of any of these repair proteins may lead to significant deleterious effects inside a cell.

In all eukaryotic cells, genomic integrity is protected by surveillance mechanisms called DNA checkpoints that, when activated by DNA lesions or replication blocks, induce transcription of DNA repair genes and delay cell cycle progression in order to prevent replication and segregation of damaged DNA molecules. However, the interactions of oxidative DNA damage with cell cycle checkpoint pathways are poorly understood. Following radiation damage such as double strand breaks, DNA-damage sensors such as the MRN (Mre11/Rad50/Nbs1) complex, the PCNA-like complex, the replication factor C variant complex containing Rad17 (Rad24 in budding yeast) communicate with phosphoinositol-kinase like kinases ATM and ATR (Tel1 and

Mec1 in *S.cerevisiae* respectively). These proteins cause phosphorylation and activation of downstream acting kinases such as Rad53 (homolog of human CHEK2) and chk1 (homolog of human CHEK1) in S.cerevisiae. This signal transmission process depends on mediator proteins such as Rad9 (Sweeney et al., 2005). Activation of Rad53 is further amplified by trans-autophosphorylation (Gilbert, Green, & Lowndes, 2001). Phosphorylation of Rad53 is an easily detectable indicator of checkpoint activation in yeast and its activation is also relevant for mammalian cells.

Stationary phase of yeast is characterized by the production of respiration-dependent ROS. Stationary phase yeast is not only exposed to reactive oxygen species but would also seem to be more amenable to the accumulation of endogenous oxidative DNA damage. Oxidative DNA damage is of complex nature and can produce a wide variety of DNA lesions. To recognize these oxidative lesions various checkpoint proteins must be involved and also to repair these complex set of lesions more than one set of DNA repair pathways must be involved. Although external ROS-induced DNA damage has been intensively studied, endogenous ROS and the resulting cellular responses including DNA repair and checkpoint mechanisms have not been very well characterized. In this dissertation project, I aimed at studying the checkpoint response showed by accumulation of unrepaired DNA damage in excision repair compromised cells such as BER and NER. I have also studied how unrepaired base damage combined either with DNA double strand breaks or damage at telomere can contribute for checkpoint activation. To study effect of compromised excision repair I have employed a series of haploid yeast mutants defective in BER (*ntg1, ntg2 and apn1*), NER (*rad1* or *rad4*), BER and NER (*ntg1, ntg2, apn1 and rad1*) to determine effect of endogenous unrepaired DNA damage on checkpoint activation in stationary phase. To study checkpoint activation we monitored the well established marker of checkpoint

activation, Rad53 phosphorylation. In other set of similar experiments, I have employed Ku deficiency (*yku70* or *yku80*), NER deficiency (*rad1* or *rad4*) or both Ku and NER deficiency (*yku70rad1*) to determine combined effect of unrepaired base damage with DNA double strand breaks or damage at telomeres on checkpoint kinase phosphorylation in stationary phase.

Our results demonstrate that a combination of DNA repair pathways such as BER and NER has to be compromised to trigger Rad53 phosphorylation. This effect is dependent on mitochondrial metabolism and occurs quite specifically in extended stationary phase. We also analyzed mutants of different checkpoint proteins for Rad53 phosphorylation. Exclusive involvement of Rad17, Rad50 and Mec1 kinase in Rad53 phosphorylation strongly suggests presence of processed DNA double strand breaks as critical lesions in BERNER cells. In other set of results we found that inactivation of Ku with NER does sensitize cells for Rad53 phosphorylation in stationary phase. The effect of inactivation of Ku is much more severe than inactivation of another member of the non-homologous endjoining (NHEJ) pathway such as DNA ligase IV. After careful analysis of point mutant of Ku, we suspect that ssDNA at telomeres can combine signal with DNA double strand breaks to show Rad53 checkpoint response.

Thus, the investigated stationary phase conditions reflect the natural situation of non-growing cells, without any externally added DNA damaging agent. Repair deficiency used here is not uncommon to human cells where a decrease in DNA repair capacity is typically found with aging (Gorbunova, Seluanov, Mao, & Hine, 2007). It has been shown that terminally differentiated eukaryotic cells such as neurons, myocytes etc. are frequently characterized by reduced DNA repair activity in spite of high respiratory rates and elevated production of reactive oxygen species. DNA repair deficiencies such as those resulting from inactivation of Ku end binding protein and from certain NER mutations have been correlated with premature ageing and

frequently also with cancer (Campisi, 2003; Karanjawala & Lieber, 2004). Decline of NHEJ (non-homologous end joing) efficiency has been observed in the rat brain during ageing (Ren & de Ortiz, 2002). Therefore, it was concluded that diminished efficiency and fidelity of double strand break (DSB) repair are responsible for age related genomic instability (Gorbunova & Seluanov, 2005; Vijg & van Orsouw, 2002). Several studies also demonstrated that efficiency of nucleotide excision repair (NER) decreases with the age as the rate of removal of UV-induced DNA lesions is slower in aged humans relative to younger adults.

Also phosphorylated CHEK2 (homolog of yeast Rad53) and other markers of DNA double strand breaks (such as phosphorylated histone H2AX) are also observed in senescent human fibroblasts (d'Adda di Faganana 2003; Gire 2004; Herbig 2004; Sedelnikova 2004). Therefore, stationary phase features of yeast are similar to those observed in post-mitotic cells, suggesting that yeast can be used as an informative model for assessing the progressive biological consequences of unrepaired DNA damage in higher eukaryotes and the underlying mechanisms leading to process of aging. And these studies have developed a model of how unrepaired oxidative base damage can accelerate the appearance of single stranded DNA in the vicinity of double strand breaks (DSBs) or at telomeres. This resulted in testable concepts of how DSBs, telomere alterations and unrepaired oxidative damage can interact to trigger a regulatory response that may determine aging of post-mitotic cells.

SECTION II

In this section of introduction I describe specific and relevant topics in detail. These special topics will help to understand a broader picture of my research project.

DNA damage checkpoints

A complex network of proteins gets activated in response to unrepaired DNA damage, this network is termed as checkpoint system. Activation of checkpoint system results in cell cycle arrest in order to allow time for DNA repair (Elledge, 1996). Cell cycle arrest can occur in any phase of cell cycle, such as G1/S, G2/M or intra S phase. Alternatively, if repair attempts fail, apoptosis or senescence can be triggered through largely overlapping pathways. Checkpoint responses can lead to different consequences; they also affect transcriptional regulation of DNA repair genes, post-translational modifications of repair proteins and the intracellular redistribution of repair proteins. All these processes are known to prevent genetic instability, therefore checkpoint proteins like p53 or the ataxia telangiectasia mutated (ATM) protein are found among tumor suppressor proteins.

A large amount of data is available, which characterizes the molecular mechanisms of DNA damage response checkpoints (Friedberg et al., 2005). Basically checkpoint system is a signal transduction system consisting of DNA damage sensors, adaptors, transmitters and downstream

target proteins. These downstream target proteins are involved in mediating cell cycle progression.

DNA damage sensor proteins

Different types DNA damages involve different sensor proteins. Single stranded DNA is in general an important damage signal that is recognized by Lcd1 which forms a complex with the Mec1 protein kinase (ATRIP/ATR represent human homologs) (Figure 1). The single stranded DNA binding, heterodimeric Replication factor A (Rfa) plays an important role in this interaction and in the downstream activation events (Zou & Elledge, 2003).

DNA double strand breaks appear to be mainly recognized by the MRX (Mre11/Rad50/Xrs1) complex (termed as MRN complex in mammals N is for Nbs1) that interacts with the Tel1 protein kinase (the yeast ATM homolog) (Lee & Paull, 2005). According to recent literature, it seems that both protein kinases, ATM and ATR are needed to trigger certain downstream events following double strand breakage. Repair of DNA double strand breaks is complex process and involve action of several proteins. After initial binding of the MRN complex, its Mre11 exonuclease component appears to create single stranded DNA that in turn lead to ATRIP/ATR binding and activation (Jazayeri et al., 2006). Also, ATM/ATR dependent phosphorylation of histone H2A (gamma-H2AX) is an important factor for enhancing the double strand break signal (Rogakou, Pilch, Orr, Ivanova, & Bonner, 1998).

There are certain DNA repair proteins, which detects abnormality in chromatin and transcend the signal further to checkpoint proteins. For example, the yeast Rad14 protein (homologous to Xeroderma pigmentosum group A protein XPA) can interact with Rad17 (which is a component

transcription factor Swi6, which is basically required for G1/S transition (Sidorova & Breeden, 1997). It signals are also mediated by the downstream acing kinase Dun1, transcriptional regulators such as Crt1, which represses DNA damage inducible transcripts (Huang, Zhou, & Elledge, 1998). The mammalian Rad53 homolog CHK2 is typically involved in responses to DNA double-strand breaks and its substrates include p53, MDM2, BRCA1, CDC25A and CDC25C (Bartek, Falck, & Lukas, 2001). Thus, CHK2 plays important roles in both rapid and sustained G1/S blocks, in apoptosis, in G2 arrest and in DNA repair. CHK2 acts as a tumor suppressor gene and mutations have been associated with familial breast cancer, sporadic lung and lymphoid tumors (Bell et al., 1999, Haruki et al., 2000, Hofmann et al., 2001). Very little is known in the area of checkpoint activation following oxidative DNA damage. H2O2 and menadione can cause cell cycle arrest in yeast (Flattery-O'Brien & Dawes, 1998) but some of the observed arrest phenomena are clearly independent of the checkpoint system (Leroy, Mann, & Marsolier, 2001, Nunes & Siede, 1996). H2O2 has shown to activate Rad53 (Leroy et al., 2001). In the wild-type, this activation occurs specifically in S-phase and causes S-phase extension. It is in part dependent on the sensor and adaptor proteins Rad17, Rad9 and fully dependent on the Mec1 kinase. Rad53 phosphorylation is also found in G1 or G2 if BER (Base Excision Repair) is inactivated. It seems that efficient repair of oxidative damage may normally prevent checkpoint activation outside of S-Phase (Leroy et al., 2001). In mammalian cells, very little is known about the regulation of checkpoints following oxidative damage. G1 and G1 checkpoint arrest has shown in peroxide treated cells. This response is found absent in AT (ataxia telangiectasia) deficient cells.

Stationary phase of *Saccharomyces cerevisiae*

When starved of an essential nutrient, cells of Saccharomyces cerevisiae cease mitotic division and arrest within the G1 phase of the mitotic cell cycle. The arrested cells subsequently acquire a variety of characteristics that collectively define the stationary phase of growth (Werner-Washburne, Braun, Johnston, & Singer, 1993,Werner-Washburne, Braun, Crawford, & Peck, 1996) (Figure 2). These changes include a dramatic reduction in the overall rate of growth, an accumulation of the storage carbohydrate, glycogen, an increased resistance to a variety of environmental stresses, including heat shock, a thickening of the cell wall, and an increased ability to survive extended periods of starvation. A similar set of changes occurs when cells are starved of either a nitrogen, phosphate or carbon source (Werner-Washburne et al., 1993, Lillie & Pringle, 1980). However, it is not yet clear if the final resting state is identical in each of these instances. In particular, it has been suggested that a true stationary phase might only be reached following carbon source deprivation (Werner-Washburne et al., 1996). The above differences between G1 and stationary phase suggest that this resting state might be a distinct, out of cycle phase of growth.

Mitochondria and oxidative stress

Most amount of ATP produced in eukaryotic cells is generated through pathways localized in the mitochondria (Boveris, 1977, Costa & Moradas-Ferreira, 2001).In *Saccharomyces cerevisiae,* ATP production occurs through two major pathways: glycolysis and oxidative phosphorylation (OXPHOS). When glucose or other fermentable carbon sources are present, glycoslysis is

activated to make ATP, while mitochondrial respiration is repressed. When fermentable carbon sources are not available, the cell depends upon OXPHOS for the production of ATP.

The mitochondrial electron transport chain consists of five inner membrane bound, multimeric complexes as well as the mobile carriers, coenzyme Q and cytochrome C (Figure 3). The free energy needed to generate ATP is extracted from the oxidation of NADH and FADH2. Upon oxidation complex I and II, respectively, electrons obtained from NADH and FADH2 are transferred to ubiquinone pools. Electrons then flow from ubiquinone through complexed III and IV to the final acceptor, molecular oxygen. Electron flow is coupled to proton movement across the inner membrane in complexes I, III and IV. The resulting proton gradient is harvested by Complex V to generate ATP (Mandavilli, Santos, & Van Houten, 2002). In contrast to most other eukaryotes, the respiratory chain in Saccharomyces cerevisiae contains three NADH dehydrogenases, instead of complex I. The NADH dehydrogenases are associated with the inner mitochondrial membrane, but are not involved in proton translocation.

Living organisms are constantly exposed to oxidative stress from environmental agents and from endogenous metabolic processes (Friedberg, 2003 Hoeijmakers, 2001). Reactive oxygen species (ROS), the major mediators of oxidative stress, include active forms of oxygen such as hydroxyl ($OH^{\cdot}$) and superoxide ($O_2^{\cdot-}$) radicals, hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Costa & Moradas-Ferreira, 2001). Exogenous sources of ROS include UV and IR radiation as well as exposure to environmental chemicals such as hydrogen peroxide (Girard & Boiteux, 1997). Mitochondrial respiration is thought to be the major source of endogenously generated ROS (Costa & Moradas-Ferreira, 2001). It has been estimated that 1-2% of oxygen reduced by mitochondria is converted to superoxide (Turrens, 1997), with the majority produced by complex III of the electron transport chain (Cadenas, Boveris, Ragan, & Stoppani, 1977). During cellular

respiration, complex I and complex II transfer electrons to coenzyme Q to produced the reduced QH2. To regenerated coenzyme Q, complex III passes electrons from QH2 to cytochrome c. This process generated semiquinone anions that avidly transfer electrons to molecular oxygen, thereby generating superoxide radicals (Finkel, 2003). Additionally, exposure to chemical inhibitors that specifically arrest mitochondrial electron transport can result in increased ROS production. For example, antimycin specifically binds to the Q_N center, the site of quinine reduction, of complex III and blocks electron flow. The specific action of antimycin results in increased ROS production (Esposito, Melov, Panov, Cottrell, & Wallace, 1999, Rasmussen, Chatterjee, Rasmussen, & Singh, 2003). Saccharomyces cerevisiae is a facultative anaerobic yeast. It uses glycolysis and oxidative phosphorylation for the formation of ATP. Yeast can be maintained on fermentable carbon sources such as glucose, where it can not do respiration.

Respiration deficient mutants of yeast are referred as "petite" mutants owing to the fact that they form smaller colonies as compared to respiratory proficient "grande" cells. Petite mutants can be classified into two distinct categories: rho0 cells are completely deficient in mtDNA; whereas, rho- cells show extensive deletion of the mitochondrial genome. In rho^- mutants, the mitochondrial genome shows extensive DNA deletions (Faye et al., 1973).

Spontaneous DNA damage

Various chemical alterations of DNA can occur in cells under conditions of normal temperature, pH and metabolic activities. A consequence of normal aerobic metabolism in both prokaryotic and eukaryotic cells is the generation of ROS. ROS may lead to the oxidative damage of virtually any biomolecule including proteins, lipids, and nucleic acids. With respect to nucleic acids, superoxide and H2O2 are relatively inert in terms of their direct reactivity with DNA.

Under physiological conditions neither are able to produce DNA strand breakage or base modification (Aruoma, Halliwell, & Dizdaroglu, 1989, Brawn & Fridovich, 1981). Much of the toxicity of superoxide and hydrogen peroxide *in vivo* is thought to arise upon conversion to the highly reactive hydroxyl radical (Brawn & Fridovich, 1981). The DNA damaging hydroxyl radical is generated from hydrogen peroxide through the mediation of adventitious iron or other transition metals by the process of the Fenton reaction (McCord & Day, 1978). Alternatively, the superoxide radical may be processed into hydrogen peroxide by superoxide dismutase, which may be further reduced by the metal ion-catalyzed Fenton reaction to the hydroxyl radical. Fenton catalyzed reduction of hydrogen peroxide is believed to generate the majority of hydroxyl radicals found *in vivo* (Wang, Kreutzer, & Essigmann, 1998).

DNA repair pathways:

Base excision repair (BER) pathway of DNA repair

Base excision repair (BER) is probably the most frequently used DNA repair pathway in nature. BER involves primarily the repair of small, helix non-distorting base lesions and abasic sites (Lindahl & Wood, 1999) (Figure 4). BER is a multistep process that corrects endogenous damage to DNA caused by hydrolysis, ROS and other metabolites. The initial enzymatic event during BER forms sites in DNA without a base, called *apurinic, apyrimidinic,* or *abasic (AP)* sites. AP sites can also result from the depurination or depyrimidination of DNA following spontaneous hydrolysis of N-glycosyl bonds. The repair of AP sites requires further enzymatic processing to complete BER. The removal of AP sites is initiated by a second class of BER enzymes called *apurinic or apyrimidinic (AP) endonucleases* like *APN1*, which specifically recognize these sites in duplex DNA. AP endonucleases produce incisions or nicks in duplex

DNA by hydrolysis of the phosphodiester bond immediately 5' to the AP site. Some DNA glycosylases have an associated activity called an *AP lyase* activity like Ntg1 or Ntg2, that can cleave the DNA chain 3' to the AP site. Hydrolysis of the phosphodiester bond immediately 5' to an AP site generates a 5' terminal deoxyribose phosphate residue that is removed by yet another class of enzymes. These include exonucleases as well as enzymes with specific DNA *deoxyribophosphodiesterase (dRpase)* activity. Therefore the sequential action of a DNA glycosylase, a 5' AP endonuclease, and a DNA deoxyribophosphodiesterase can generate a single nucleotide gap in the DNA duplex during BER. The repair of double stranded DNA by BER is incomplete until the missing nucleotides are replaced by DNA synthesis and covalently joined to the parental DNA. These events are referred to as repair synthesis of DNA and DNA ligation, respectively (Memisoglu & Samson, 2000).

Nucleotide excision repair (NER) pathway of DNA repair

Nucleotide excision repair (NER) is believed to repair bulky, helix distorting lesions such as bipyrimidine UV photoproducts (Lindahl & Wood, 1999). NER is a functionally highly conserved repair pathway present in most organisms and is responsible for the correction of a wide variety of DNA lesions. NER is comprised of damage recognition followed by dual incisions 5' and 3' to the lesion site leaving a gap (size varies among different species) which is subsequently filled in by repair synthesis and completed by ligation of the DNA back bone (Figure 5). It was initially thought that the major NER substrates were bulky that induced significant distortions into duplex DNA structure. However, recent work has revealed that the scope of relevant NER substrates has expanded and also includes small, non-bulky, non-distortive base damages as well as basic sites. The elucidation of NER in *E.coli* has played a

major role in defining the mechanism of this system in other organisms. In higher organisms NER is conserved functionally but requires a larger number of proteins, including the XP-G-like proteins, and results in a larger patch size. Humans with the autosomal recessive disease xeroderma pigmentosum have defects in one of several proteins which function in NER and are prone to development of sunlight exposure-induced skin cancers and neurological problems. The nucleotide excision repair pathway generally removes bulky NDA lesions, but recent studies have implicated NER in the repair of oxidative damage. NER has been shown to be capable of processing oxidative lesions such as thymine glycol and 8-oxoguanine (J. J. Lin & Sancar, 1989; Reardon, Bessho, Kung, Bolton, & Sancar, 1997; Snowden, Kow, & Van Houten, 1990). In *S.cerevisiae* the 3' incision is produced by Rad2, while the 5' incision is produced by the Rad1/Rad10 protein complex. After the damage DNA is removed as part of an oligonucleotide, DNA polymerase fills in the gap, and DNA ligase joins the ends.

Pathways of DNA double strand break (DSB) repair

DSBs in DNA are repaired by two major mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ). During HR-mediated repair of DSB, the sister chromatid is used as a template to copy the missing information into the broken locus. In mammalian cells repair by HR is mediated by hRad51 protein with the help of other members of hRad52 epistasis group, single-strand binding protein RPA, BRCA1, BRCA2, and MRE11-RAD50-NBS1 (homologs of MRE11-RAD50-XRS2 in *S.cerevisiae*) nuclease complex. Since sister chromatids are identical to each other, DNA damage can be repaired faithfully with no genetic consequence. The NHEJ pathway simply fuses two broken ends with little or no regard for sequence homology. NHEJ starts with binding of Ku heterodimer Ku70-Ku80 (Yku70/Yku80 in

S.cerevisiae) to the broken ends (Lieber, 1999) (Figure 6). Ku facilitates recruitment of Artemis-DNA-PKcs complex, which is thought to trim single stranded overhands (Lieber, Ma, Pannicke, & Schwarz, 2003). Next, the ends are covalently joined by XRCC4-DNA ligase IV complex (Lif1-Dnl4 in *S.cerevisiae).* A few nucleotides at each end of the DNA break are lost in most instances during NHEJ event (Critchlow & Jackson, 1998; Lieber et al., 2003). NHEJ may also be associated with larger deletions or insertions of filler DNA (Gorbunova & Levy, 1997; Liang, Han, Romanienko, & Jasin, 1998; Y. Lin & Waldman, 2001a; Y. Lin & Waldman, 2001b; Sargent, Brenneman, & Wilson, 1997). Ku heterodimer was initially characterized for its role in the NHEJ pathway of DNA double strand break repair, Ku is now known to associate with and function at telomeres across species (Gravel, Larrivee, Labrecque, & Wellinger, 1998; Hsu, Gilley, Blackburn, & Chen, 1999; Riha, McKnight, Griffing, & Shippen, 2001). In *Saccharomyces cerevisiae,* Ku affects several aspects of telomere biology, including the recruitment of telomerase to telomeres and telomere length homeostasis (Fisher, Taggart, & Zakian, 2004; Porter, Greenwell, Ritchie, & Petes, 1996; Stellwagen, Haimberger, Veatch, & Gottschling, 2003), the protection of telomeric ends from nucleolytic degradation and homologous recombination (Maringele & Lydall, 2002; Polotnianka, Li, & Lustig, 1998), the formation of telomeric heterochromatin, which leads to the transcriptional silencing of nearby genes (Boulton & Jackson, 1998; Laroche et al., 1998), the late firing of replication origins near telomeres (Cosgrove, Nieduszynski, & Donaldson, 2002), and the nuclear localization of telomeres. Separation of function alleles of *S.cerevisiae YKU70 and YKU80,* which encode Yku70 (the Ku70 homolog) and Yku80 (the Ku80 homolog), respectively, have revealed that Ku is a multifunctional protein that has distinct activities not only at DSBs as compared with telomeres, but also at telomeres themselves. For example, certain *yku80* C-terminal mutations

have been shown to impair NHEJ while telomeric functions are retained. Conversely, *yku80* alleles have been identified that are proficient in NHEJ but defective in specific aspects of telomeric function, such as the *yku80-135i* allele (a five amino acid insertional mutation), which impairs solely telomere length regulation (Stellwagen et al., 2003). In some cases, function specific interactions have been identified, such as, Yku80's interaction with the RNA subunit of telomerase is required for its role in telomere length homeostasis, whereas a Yku80-Dnl4 interaction seems to be important for NHEJ (Palmbos et al., 2005).

Figure 1: Components of DNA damage signaling

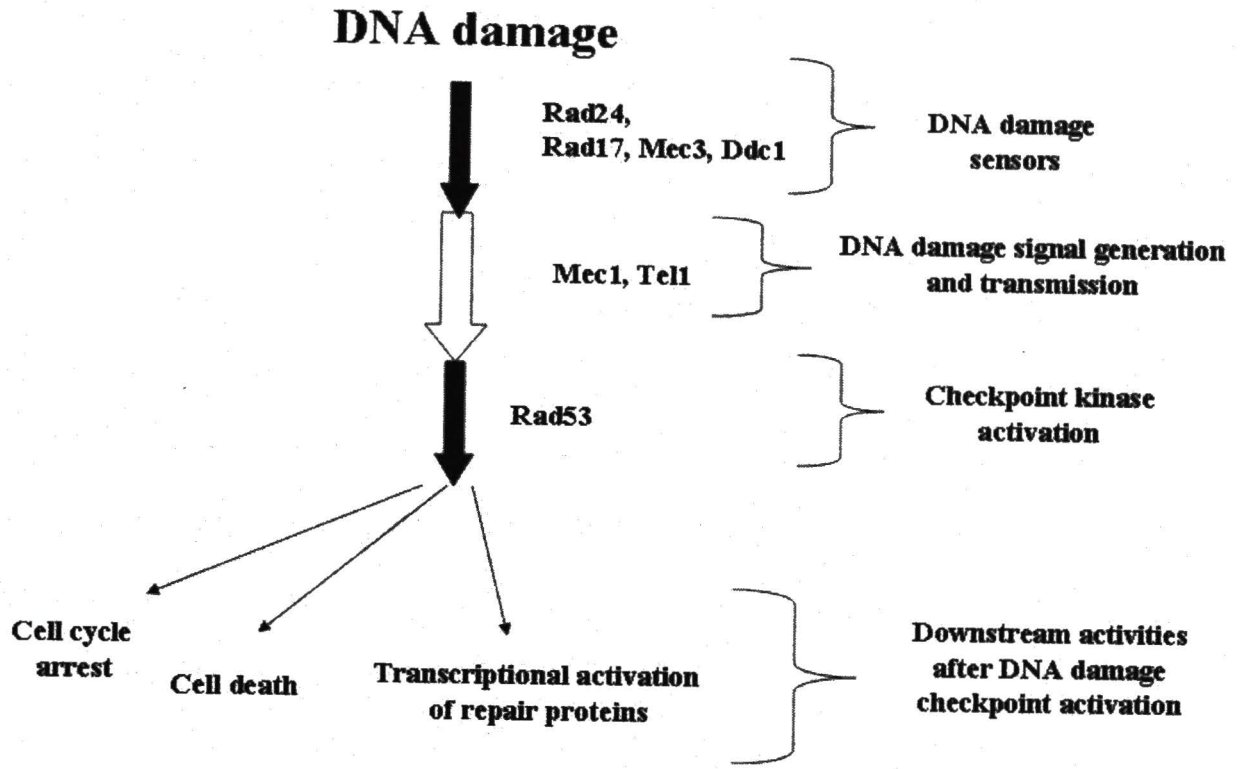
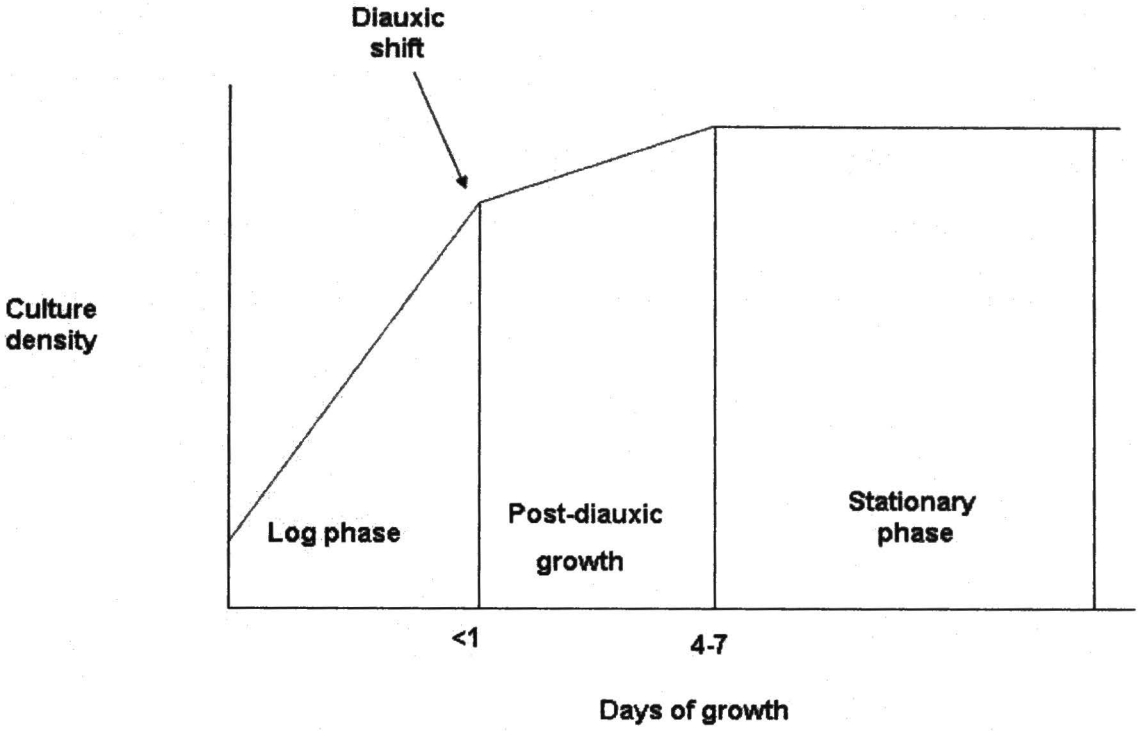


Figure 1: Components of DNA damage signaling

DNA damage checkpoint activation is a complex process and involves various proteins playing different roles. In above shown schematic a typical DNA damage signaling can be divided into various sub sections that includes, DNA damage sensors, signal generation and transmission, and activation of checkpoint kinase, and finally the downstream activities after checkpoint activation. Basically base damages can be recognized by Rad24 and a PCNA like complex that includes Rad17/Mec3/Ddc1. Single stranded DNA is in general recognized by Lcd1 which forms complex with Mec1 protein kinase. Double strand breaks appear to be mainly recognized by the MRX complex, which includes Mre11/Rad50/Xrs1 proteins respectively. MRX complex interacts with Tel1 protein kinase. DNA damage signal is further transmitted by Mec1/Tel1 kinases.

Checkpoint signaling in budding yeast is critically dependent on the phosphorylation. Mec1/Tel1 kinases phosphorylate their substrate Rad53, which itself an autocatalytic checkpoint kinase. Rad53 activation is frequently mediated by adaptor proteins such as Rad9. Rad9 undergoes Mec1/Tel1 dependent phosphorylation that results in homomeric complex formation. In budding yeast, Rad53 is involved in various events, that includes responding to different types of DNA damage and replicative stress. Checkpoint activation by Rad53 activation leads to multitude of events such as cell cycle arrest, transcription of DNA damage inducible transcripts, which further proceed to DNA repair events.

Figure 2. Growth phases exhibited by *S.cerevisiae* cultures grown on glucose based media



Modified from original publication by Herman et al. 2002

Figure 2. Growth phases exhibited by S.cerevisiae cultures grown on glucose based media

During the initial logarithmic phase of growth, the budding yeast grows by fermentation of the available glucose. When glucose becomes limiting, the cells transiently arrest growth and switch to a respiratory mode of energy production. This period of transition is known as the 'diauxic shift'. During the subsequent post-diauxic growth period, the cells grow rather slowly and utilize the ethanol that was produced during the previous period of fermentation. When this ethanol is finally exhausted, the cells enter into the true stationary phase, the growth period when the cell number is no longer increasing.

Figure 3: Electron transport and oxidative phosphorylation

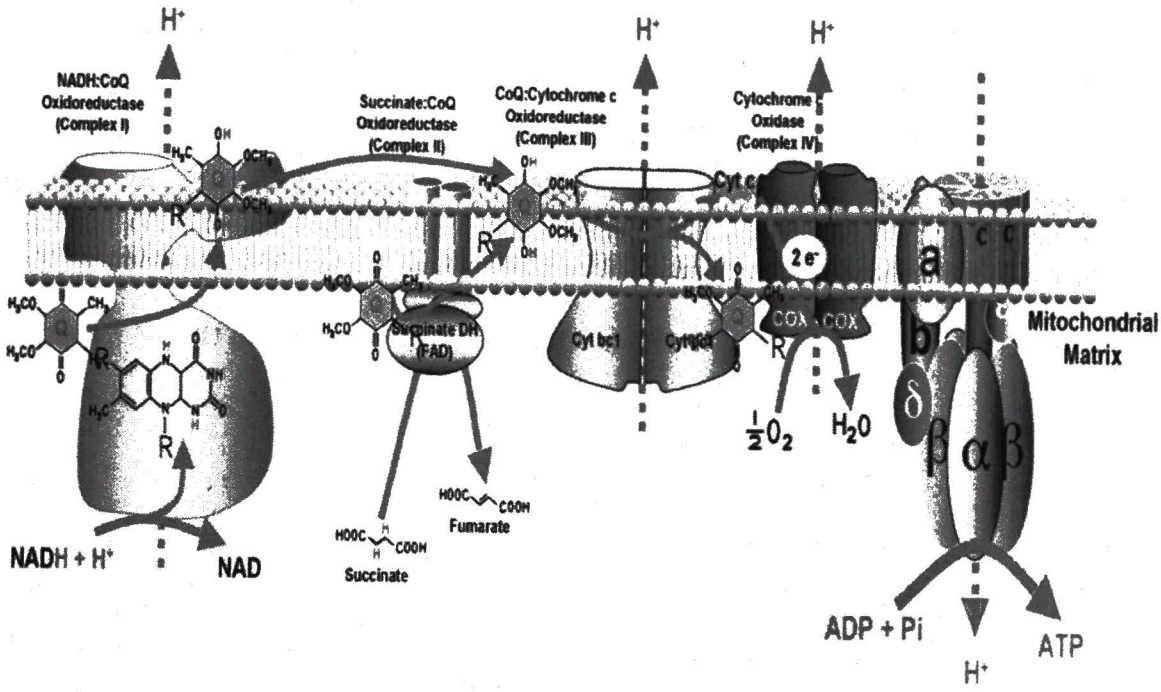


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Figure 3: Electron transport and oxidative phosphorylation

Schematic of mammalian electron transport complexes I-V. Electrons are extracted from NADH and FADH2 subsequently passed from lower to higher standard reduction potentials through the four protein complexes (complexes I-IV). Energy from the respiratory substrates is conserved in the form of an electrochemical proton gradient that is converted into chemical energy during ATP synthesis by ATP synthase (complex V). In contrast to mammalian ETC, *S.cerevisiae* contains 3 NADH dehydrogenases instead of complex I.

Figure 4: Base Excision Repair (BER) pathway of DNA repair

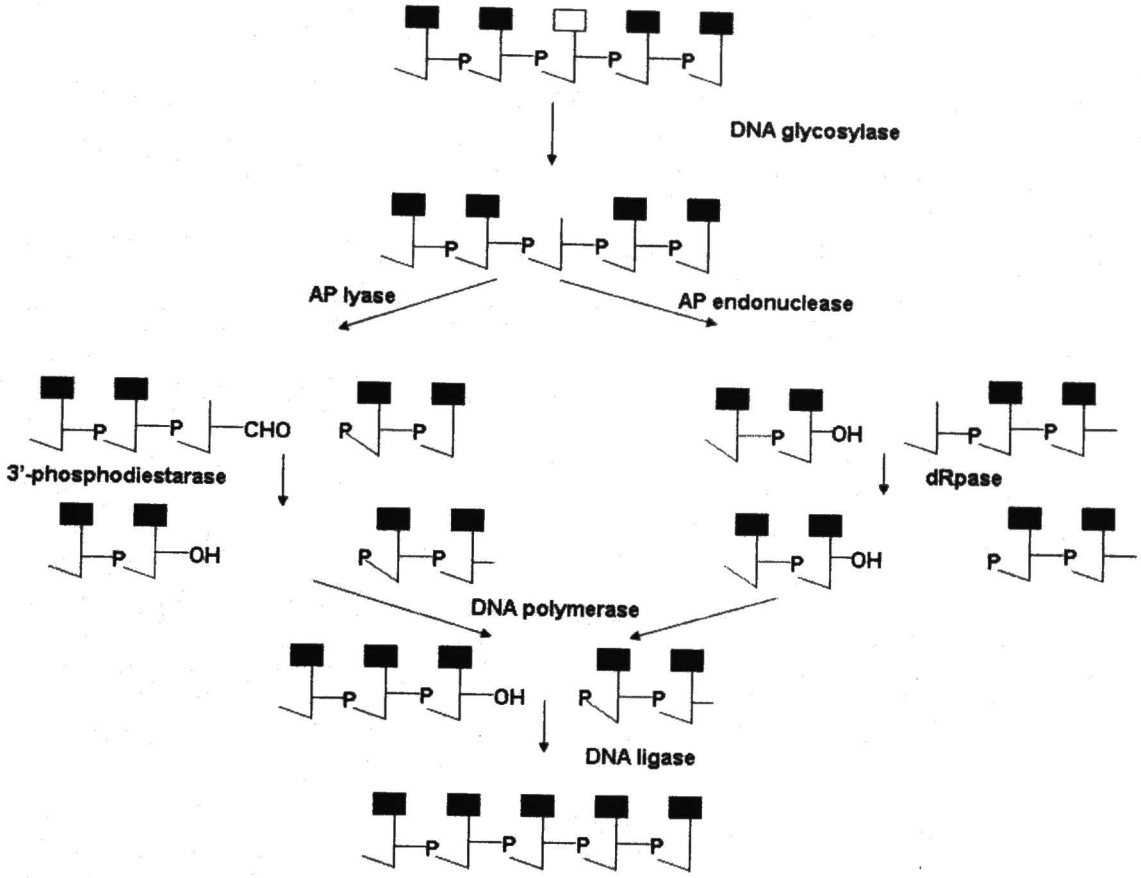

Figure 4: Base excision repair pathway

The damaged base (empty squre) is recognized by a DNA glycosylase, which cleaves the N-glycosydic bond, releasing the damaged base and generating an apurinic or apyrimidinic (AP) site. The AP site is processed by either an AP endonuclease or by an AP lyase activity associated with some DNA glycosylases. AP endonuclease cleaves 5' to the AP site, creating a "dirty" 5'-deoxynucleoside-5-phosphate residue at the 5' terminus. In contrast, AP lyase cleaves 3' to the AP site, resulting in a "dirty" 3'-4-hydroxy-2-pental residue at the 3' terminus. The modified termini, which cannot be used as substrates for DNA polymerase, must be trimmed by either DNA deoxyribophosphodiesterase (dRPase) or a 3'-phosphodiestarase to regenerate a 5'-phosphate or 3'-hydroxyl, respectively. The resulting gap is filled in by DNA polymerase, and the nick is sealed by DNA ligase to complete the repair process.

Figure 5: Nucleotide Excision Repair pathway of DNA repair

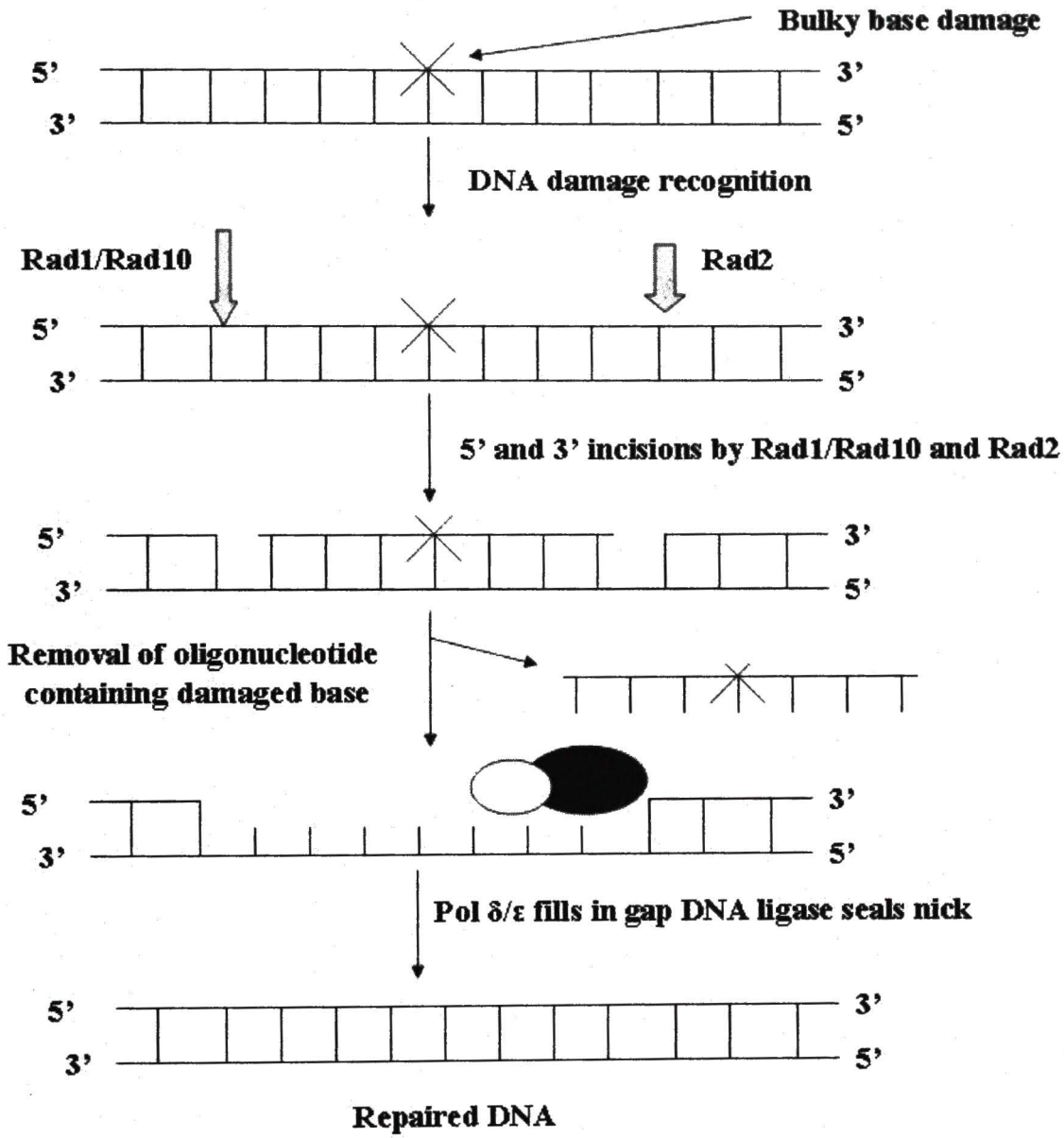

Figure 5: Nucleotide Excision Repair (NER) pathway of DNA repair

Nucleotide excision repair pathway mainly repairs bulky, helix distorting type of base modifications. In above figure base damage has marked with "X" that is process by NER pathway of DNA repair. In NER pathway DNA damage is recognized by either NER pathway proteins or damage recognition proteins. Endonucleases like Rad1/Rad10 and Rad2 incise DNA in 5' to 3' direction, which ultimately gives rise to a oligonucleotide fragment containing DNA damage. Further the DNA gap is filled by the action of Polδ/ε and DNA ligase.

Figure 6: Non-homologous end joining (NHEJ) pathway of DNA double strand break repair

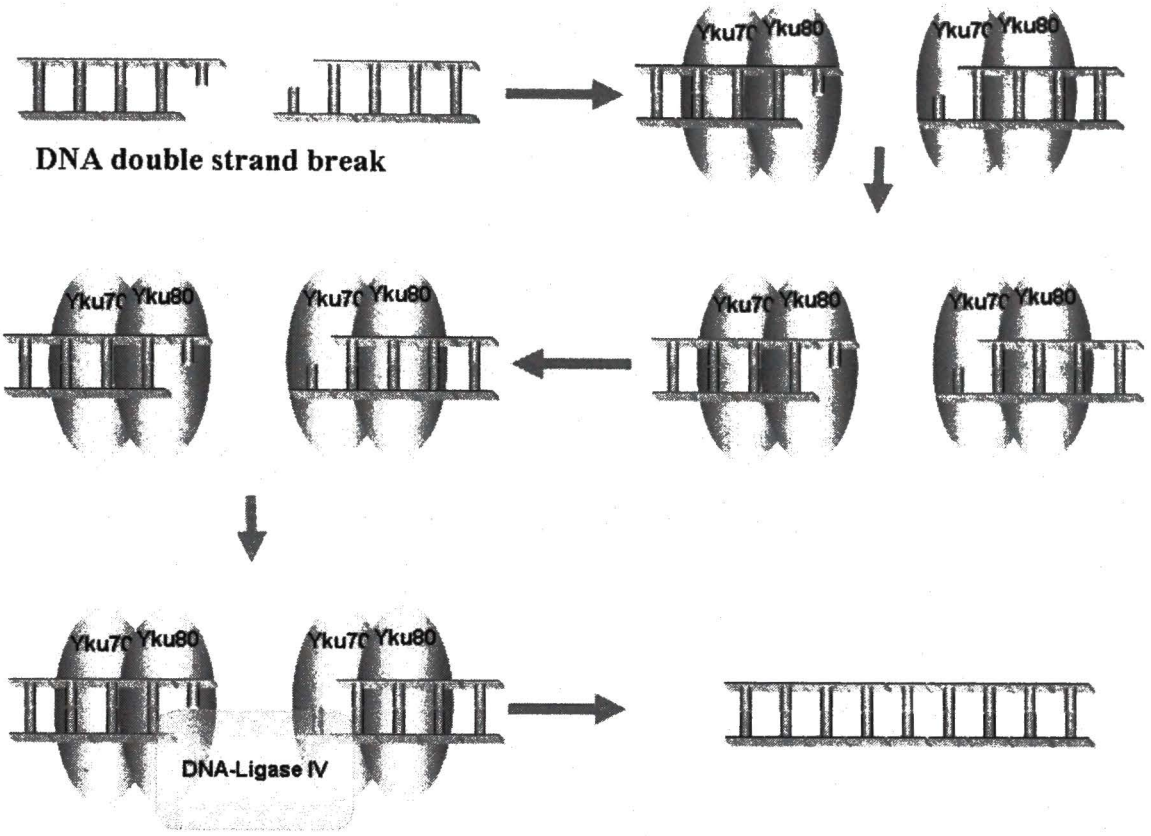

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Figure 6: Non-homologous end joining (NHEJ) pathway of DNA double strand break repair

Nonhomologous end joining (NHEJ) is formally a process of direct DNA end to end fusion that does not require strand exchanges or the availability of homologous DNA. In S.cerevisiae the Yku heterodimer (Yku70/Yku80) binds at the ends of DNA double strand breaks. If the breaks have unusual termini, which prevent ligation of DNA ends then these breaks undergo some sort of biochemical processing or trimming. In addition to single strand degradation, cleavage of loops or overhangs of a flap structure and gap filling by DNA synthesis may transpire. During these processes, the opportunity for base-pairing influences the outcome. The nucleotide structure of joints resulting from NHEJ suggests that fusion frequently occurs where microhomologies exist and as few as one to four stable base pairs can be established.

Figure 7: DNA repair deficient (BER/NER or *YKU70*) *S.cerevisiae* is prone to various types of DNA damage in stationary phase (Representative model)

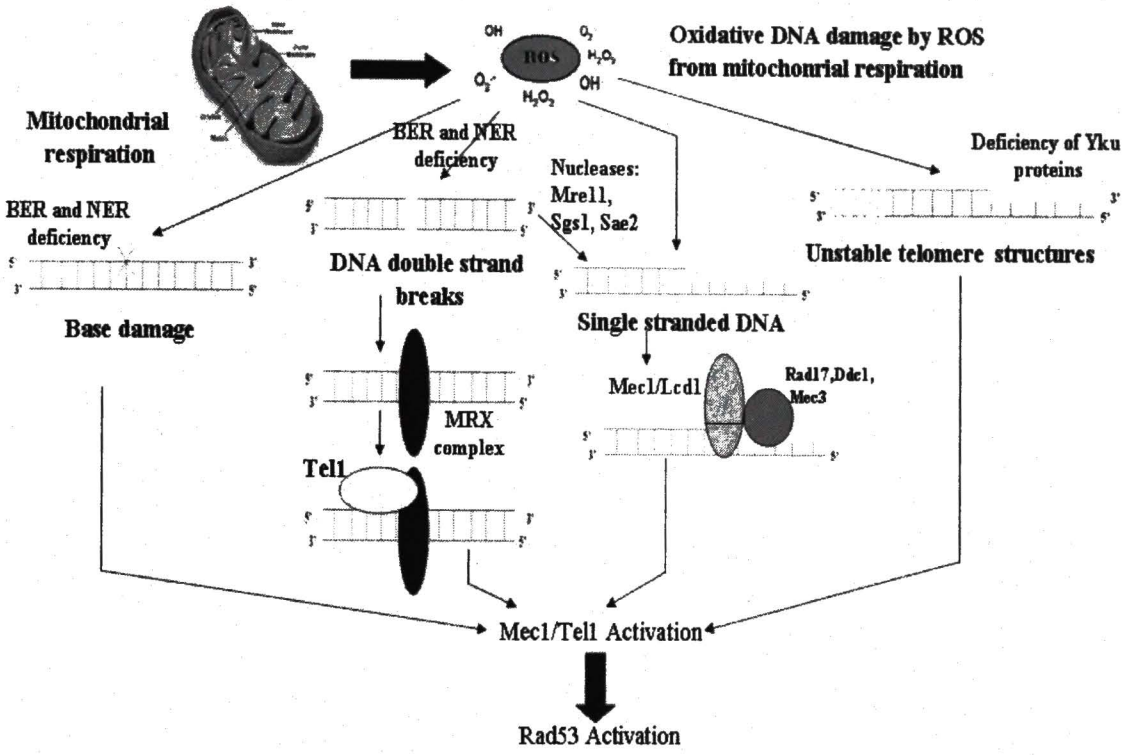

Figure 7: DNA repair deficient (BER/NER or *YKU70*) *S.cerevisiae* is prone to various types of DNA damage in stationary phase

Nuclear DNA is prone to various types of DNA damage in stationary phase *S.cerevisiae*. Oxidative phosphorylation in mitochondrial electron transport chain produces different types of ROS. ROS from mitochondrial respiration can causes multitude of DNA damages. Depicted here is base damage, single stranded DNA, DNA double strand breaks and altered telomere structures can trigger checkpoint activation in the absence of DNA repair proteins. Single stranded DNA may arise via action of certain nuclease (Mre11/Sae2/Sgs1) at double strand breaks.

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CHAPTER II

PREFACE

Can compromised DNA excision repair processes lead to checkpoint activation in stationary phase?

Oxidative DNA damage induced by external oxidizing agents has been intensively studied in the recent years. But the endogenous oxidative DNA damage and the resulting cellular responses including DNA repair and checkpoint responses have not been well understood. The main reason to study oxidative DNA damage is the prospective involvement of oxidative DNA damage in the etiologies of various human pathologies, which includes cancer and aging (Ames, 1983; Feig, Reid, & Loeb, 1994). Recent body of work suggests that oxidative DNA damage is observed in genomic DNA and its level steadily increases with age (Gorbunova & Seluanov, 2005; Karanjawala & Lieber, 2004). One of the main reasons ascribed to this observation is the

reduction in DNA repair efficiency with age. Especially if cells do not divide a large amount of unrepaired DNA damage could get accumulated in genome with aging.

When the cells are compromised for excision repair pathways like base excision repair or nucleotide excision repair there is a possibility that genomic DNA may accumulate a high amount of unrepaired DNA damage including various base modifications. This unrepaired DNA damage could cause deleterious biological consequences. Also the direct action of ROS with sugar back bone of DNA can produce a wide variety of DNA damage including single and double strand breaks. DNA double strand breaks are the most lethal DNA lesions, unrepaired strand breaks can drive cell to cell death. Therefore the recognition of particular DNA damage is very important. Here we are studying the effect of loss of both BER and NER DNA repair pathways on activation of checkpoint response, in terms of phosphorylation of Rad53. Our system does not comprise use of any external oxidizing agent and is focused on the endogenous damage caused during regular metabolic activities of cells in stationary phase. This condition more likely mimics the natural state of any living cell in higher eukaryotes because mammalian genome is always under threat of oxidative DNA damage, which causes around 10,000 hits per cell per day (Beckman & Ames, 1997). *S.cerevisiae* provides a convenient tool to study the direct effect of mitochondrial ROS on DNA damage and checkpoint activation by availability of their rho^0 mutants. rho^0 mutants lack mitochondrial DNA thus they are respiration deficient. The main aim behind this particular study was to understand relative contribution of BER and NER pathway in checkpoint activation during stationary phase. This study will provide important insights to understand how unrepaired base modification by endogenous ROS could lead to checkpoint activation. Also our analysis of checkpoint mutants will give information about the type of DNA damage such as base damage, single and double strand breaks.

Checkpoint kinase phosphorylation in response to endogenous oxidative DNA damage in repair-deficient stationary-phase *Saccharomyces cerevisiae*

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ABSTRACT

Stationary phase *Saccharomyces cerevisiae* can serve as a model for postmitotic cells of higher eukaryotes. Phosphorylation of the checkpoint kinase Rad53 was observed after more than 2 days of culture if two major pathways of oxidative DNA damage repair, base excision repair (BER) and nucleotide excision repair (NER), are inactivated. The wild type also showed a low degree of Rad53 phosphorylation when the incubation period was drastically increased. This checkpoint response as well as enhanced mutagenesis was absent in *rho*° strains, lacking detectable mitochondrial DNA, and is therefore thought to be dependent on reactive oxygen species originating from mitochondrial respiration. There was no evidence for progressive telomere shortening during stationary-phase incubation. Since Rad50 (of the MRN complex) and Mec1 (homolog of ATR) are absolutely required for the observed checkpoint response, we assume that resected random double-strand breaks are the critical lesion. Single-strand resection may be accelerated by unrepaired oxidative base damage in the vicinity of a double-strand break.

INTRODUCTION

Many studies have suggested that endogenous oxidative damage is of particular significance for the survival and functional integrity of postmitotic cells, such as neurons or cardiomyocytes, since the absence of replication may facilitate the accumulation of endogenous damage (Barzilai, 2007). Here, we studied stationary phase of budding yeast (*Saccharomyces cerevisiae)* that has already been proposed by others to be an informative model to explore chronological lifespan regulation (Chen et al., 2005; Madia et al., 2007). A yeast culture in complete medium ceases cell division after 2-3 days of growth due to nutrient limitation and enter stationary phase. This is accompanied by a change from fermentation to respiration (di-auxic switch) and thus, increased exposure to reactive oxygen species is predicted despite a general metabolic slow-down (Herker et al., 2004; Werner-Washburne et al., 1993). Since yeast can survive and grow by fermentation alone, without functional mitochondria, this eukaryotic model organism is ideally suited to determine the effects of reactive oxygen species (ROS) generated during mitochondrial metabolism. This study addresses how spontaneous endogenous DNA damage accumulating in non-dividing cells of yeast can trigger long-term activation of checkpoint pathway(s) that may correspond to senescence in mammals.

The network of eukaryotic checkpoint proteins that is activated by DNA damage improves genetic stability by allowing time for DNA repair through cell cycle arrest, regulation of damage-inducible transcripts, direct modification of repair proteins and by other mechanisms,

including apoptosis and senescence (Friedberg et al., 2005; Kastan and Bartek, 2004; Nyberg et al., 2002) . Oxidative DNA damage is of considerable importance for many aspects of human health, cancer and aging (Cooke et al., 2003; Karanjawala and Lieber, 2004). Especially for aging, numerous genetic and pharmacological experiments have lent support to the theory that ROS produced in mitochondria may limit replicative lifespan, especially in model systems (Golden et al., 2002). While still being debated, the significance of DNA damage as a causative agent is supported by the premature aging phenotype conferred by certain DNA repair mutations (Chen et al., 2007; Schumacher et al., 2008).

However, the interactions of oxidative DNA damage with the cell cycle checkpoint machinery are poorly understood. Following ionizing radiation-induced double-strand breaks (DSB), DNA-damage sensors such as the MRN complex (Mre11–Rad50–Nbs1/Xrs2 in yeast), the PCNA-like 911-complex and the replication factor C variant complex communicate with phosphoinositol-kinase like kinases ATM and ATR (Tel1 and Mec1 in *S. cerevisiae*) (Friedberg et al., 2005; Nyberg et al., 2002). These phosphorylate and consequently activate downstream acting kinases such as Rad53 and Chk1 in *S. cerevisiae*, a signal transmission step that depends on mediator proteins such as Rad9 (Sweeney et al., 2005). Activation of Rad53 is further amplified by *trans*-autophosphorylation (Gilbert et al., 2001). Phosphorylation of Rad53 is frequently used as an easily detectable indicator of checkpoint activation in yeast; its regulation is also relevant for mammalian cells. Phosphorylation of the human Rad53 homolog CHEK2 is associated with telomere-induced senescence (d'Adda di Fagagna et al., 2003; Gire et al., 2004) and with certain precancerous conditions where it acts as a barrier towards further malignant transformation (Gorgoulis et al., 2005).

We monitored Rad53 checkpoint kinase phosphorylation during continued incubation in exhausted growth medium (termed here 'extended stationary phase'). Thus, the investigated stationary-phase conditions reflect the natural situation of a non-growing cell, and the external addition of a damaging agent is avoided. During previous studies, we determined that the transcriptional profile of yeast strains deficient in both nucleotide excision repair (NER) and base excision repair (BER) resembles that of cells stressed with external oxidative agents (Evert et al., 2004). Consequently, repair-deficient cells were also used throughout the present study. This condition is not unrelated to human cells where a decrease in DNA repair capacity is typically found with aging (Gorbunova et al., 2007). The analysis presented here hints at processed double-strand breaks (DSB) resulting from oxidative damage as being essential for checkpoint activation in post-mitotic cells.

MATERIALS AND METHODS

Yeast strains:

All strains are derived from SJR751 *MAT*a *ade2-101 his3Δ200 ura3ΔNco lys2ΔBgl CAN1* (originally from Sue Jinks-Robertson). The NER-deficient derivative is deleted for *RAD1* (*rad1Δ::hisG-URA3-hisG*), the BER-deficient strain contains *ntg1Δ::LEU2 apn1Δ::HIS3 ntg2Δ::hisG-URA3-hisG rad1Δ::hisG-URA3-hisG* (Evert et al., 2004). Additional deletions were introduced using PCR-based microhomology-mediated transplacement using a plasmid-borne *KanMX4* gene (Longtine et al., 1998). To create the *MEC1* deletion, *SML1* was first deleted by *KanMX4* followed by deletion of *MEC1* using the *Hygr* marker. Construction details and primer sequences are available upon request. *Rho0* derivatives of selected strains were generated by incubation with ethidium bromide as described (Sherman et al., 1994) and verified by DAPI staining.

Detection of Rad53 and Histone 2A phosphorylation in stationary phase and following irradiation treatment:

Late-logarithmic phase cultures were diluted in fresh YPD (1% yeast extract, 2% peptone, 2% dextrose) or YPG (1% yeast extract, 2% peptone, 3% dextrose). During incubation at 30°C, about 4 x 108 cells were harvested at each time point and lysed using a TCA-based method (Foiani et al., 1994). Following SDS-PAGE, Rad53 and its phosphorylated forms were detected

by conventional Western blotting using a commercial Rad53 antibody (Santa Cruz Biotechnology). Details have been described elsewhere (Pabla et al., 2006). H2A phosphorylation was detected using a commercial phospho-specific antibody (Abcam), total H2A was detected using an antibody generously provided by Jessica Downs. Removal of bound antibodies was accomplished by using StripOBuffer (Fabgennix). Gamma irradiation was performed by exposing a portion of a stationary phase culture in a Cs137 irradiator (J. L. Shepherd and Ass.). Samples were taken 3 h after irradiation (100 Gy). In the case of UV-irradiation, a portion of the culture was irradiated as a 15 ml suspension in water (2.5 x 107 cells per ml) under constant stirring with 80 J/m2 of 254 nm UV. Cells were resuspended in their exhausted medium and incubated for 3 h before analysis.

Telomeric DNA analysis:

Yeast chromosomal DNA was extracted (Rose, 1987) and subjected to *Xho*I digest. Digested DNA was separated by agarose gel electrophoresis and Southern blotting was performed using a telomere-specific 32P-labeled single-stranded probe (5'-GTGTGGGTGTGGTGTGTGTGGGG-3') according to published protocols (Boulton and Jackson, 1996; LeBel et al., 2006).

Survival and mutagenesis assays:

Appropriate culture dilutions were plated on YPD and the fraction of colony-forming cells was determined. The fraction of canavanine-resistant mutant cells on selective media was measured as described elsewhere (Evert et al., 2004; Sherman et al., 1994). Respiration-deficient colonies were identified as small white colonies and verified by their inability to grow on media containing 3% glycerol instead of dextrose (YPG).

RESULTS

Checkpoint kinase phosphorylation during stationary phase

We have previously shown that cells with a joint defect in NER and BER exhibit dramatic growth defects, reduced plating efficiency and oxidative stress as judged by transcriptional profiling (Evert et al., 2004). BER was inactivated by deletion of the genes encoding the major *N*-DNA-glycosylases (Ntg1, Ntg2) and AP endonuclease (Apn1). A deletion of *RAD1* was used as the protoype of an NER deficiency (Evert et al., 2004). Rad1 is part of the Rad1/Rad10 nuclease that introduces a single-strand scission 5' of the UV lesion (Friedberg et al., 2005). Isogenic haploid yeast strains deficient in NER (*rad1*□), BER (*ntg1*Δ *ntg2*Δ *apn1*Δ) or both pathways (abbreviated as *ber ner*) were tested for phosphorylation of checkpoint kinase Rad53 as a function of culture age (Fig. 1 A). The appearance of multiple phosphorylated forms of Rad53 in response to DNA damage has been well documented in the literature (Lee et al., 2003). As indicated by slower mobility, phosphorylated forms of Rad53 were exclusively found in the *ber ner* strain but neither in the wild type nor in strains deficient in one repair pathway alone (Fig. 1 A). Furthermore, Rad53 modification was not or much less evident in logarithmic or early stationary phase (up to 1-2 days) but only clearly detectable after at least 2 days of incubation when no more culture growth was detectable (Fig. 1 A, see also Fig. 2 A, Fig. 3). Whereas timing and extent of phosphorylation proved to be somewhat variable between different

experiments, this principal observation was highly reproducible. When assayed for percentage of colony forming cells, a high degree of lethality was determined in the *ber ner* strain compared to single-pathway mutants during extended stationary phase (Fig. 1 B). The survival of colony forming cells during extended stationary phase was further diminished by inactivation of the MRN complex component Rad50 in the *ber ner* background (Fig. 1 B, see later discussion). Initially, we regarded the Rad53 response as dependent on cellular repair deficiency but it can in fact be detected even in the wild type if the incubation period is drastically extended. Our data indicate that an incubation period of more than 3 months is required to trigger significant Rad53 modification in a DNA-repair proficient background (Fig. 1 C).

Dependency on functional mitochondria

The presence of unrepaired oxidative DNA lesions or their derivatives may be the underlying cellular stress factor triggering the observed checkpoint response of Rad53 phosphorylation. We explored if the type of DNA damage to which Rad53 appears to respond may be caused by ROS released from mitochondria. No phosphorylated forms of Rad53 were detectable in a *rho*0 derivative of the *ber ner* strain used, even after extended incubation of this strain in stationary phase (Fig. 2 A). This respiration-deficient strain, however, is in general capable of Rad53 phosphorylation in stationary phase, as demonstrated by □-irradiation (data not shown). If respiration-proficient *ber ner* mutant cells were tested in medium containing glycerol instead of dextrose, phosphorylation of Rad53 was accelerated and already detectable in logarithmic phase (Fig. 2 A, right panel). This correlates with active mitochondrial respiration prior to stationary phase in these cultures when dependent on a non-fermentable carbon source.

Oxidative damage can be a source of mitochondrial genetic instability (Doudican et al., 2005). As a measure of mitochondrial DNA alterations, we detected the frequency of respiration-deficient colonies following plating of stationary-phase cells ("petite induction"). The increase found during extended stationary phase in the wild type was greatly accelerated in the *ber ner* strain since BER is a known pathway of mitochondrial DNA repair (Kang and Hamasaki, 2002)(Fig. 2 B).

A joint defect of BER and NER also resulted in drastically enhanced spontaneous nuclear mutability and about 100 fold higher frequencies of canavanine-resistant mutant cells were found (Evert et al., 2004). Although this difference was mostly established during logarithmic phase, the fraction of mutants among surviving cells continued to rise moderately during incubation in stationary phase, most likely indicating continuous accumulation of oxidative DNA damage (Fig. 2 C). Corresponding to a reduced oxidative DNA damage level, mutation frequencies of *rho° ber ner* cells were much lower than those of their respiration-proficient counterparts and fell within the range of the repair-proficient wild-type (Fig. 2 C).

Requirement of checkpoint proteins Rad9, Rad17, Rad50 and Mec1

The identity of the sensor proteins and checkpoint kinases required for Rad53 phosphorylation can provide important information on the molecular nature of the relevant DNA damage. We combined the BER + NER defect with chromosomal deletions of various checkpoint genes such as those encoding the DNA damage sensor Rad17 or the mediator protein Rad9 (Friedberg et al., 2005; Nyberg et al., 2002). Both deletions have a drastic effect on Rad53 phosphorylation which is greatly reduced (Fig. 3 A).

Deletion of *RAD50*, encoding a subunit of the MRN complex which plays important roles in checkpoint activation and DNA double-strand break repair (D'Amours and Jackson, 2002; Friedberg et al., 2005) has a profound effect. In the absence of functional Rad50, stationary-phase Rad53 phosphorylation is clearly absent in the *ber ner* strain (Fig. 3 A). As shown above, the survival of colony forming cells during extended stationary phase is also severely diminished by inactivation of Rad50, possibly attesting to the protective effect of checkpoint activation (Fig. 1B). The MRN complex is required for checkpoint responses to double-strand breaks but not UV (D'Amours and Jackson, 2002; Grenon et al., 2001). Gamma irradiation of the *ber ner* strain resulted in Rad53 phosphorylation or enhanced Rad53 phosphorylation, respectively, which is efficiently blocked by deletion of *RAD50* (Fig. 3 B). These cells were nevertheless able to respond to UV with Rad53 phosphorylation, even in a non-dividing state (Fig. 3 B).

Interestingly, the ATM-homologue Tel1 that is typically interacting with the MRN complex at double-strand breaks is not the critical upstream kinase since its deletion did not diminish Rad53 phosphorylation (Fig. 3 A). The ATR homologue Mec1, however, the other major kinase upstream of Rad53, was absolutely required for Rad53 phosphorylation under these conditions (Fig. 4). Its deletion was carried out in an *sml1*Δ background because of lethality of a Mec1 deletion in strains with normal ribonucleotide reductase activity. Mec1 dependency hinted at the significance of single-strand resection and we also tested the influence of Exo1 as a candidate exonuclease for converting DNA damage into a checkpoint-activating signal in the absence of repair. In a side-by-side comparison, there was some delay in the Rad53 response in stationary phase but the overall effect of an *EXO1* deletion was very minor (Fig. 4, lower panel).

H2A phosphorylation in extended stationary phase

The phosphorylation of H2A at Ser129 is one of the earliest detectable events that are triggered by DNA double strand breaks. When γH2A levels were detected during extended stationary-phase, an increase in relation to total H2A levels was found in wild type (Fig. 5). This increase was overall similar to the *ber ner* strain where a somewhat higher level was noted in logarithmic-phase cells. Interestingly, in *ber ner rho°* cells, a higher level is evident in logarithmic phase cells but no further increase occurs during stationary-phase incubation.

Telomere stability in extended stationary phase

Telomere attrition is an important causative factor in replicative aging and may also be involved in the observed checkpoint responses of non-dividing cells if telomeres are extraordinarily sensitive to spontaneous (i.e. oxidative) DNA damage. However, when measuring the overall telomere repeat length by Southern blotting, we did not detect any difference between wild-type and BER, NER and BER/NER deficient strains, irrespective of mitochondrial activity, and there was no hint at progressive shortening during stationary-phase incubation (Fig. 6).

DISCUSSION

This study examines how unrepaired spontaneous DNA damage of oxidative origin can activate the checkpoint pathway in non-dividing cells. In using extended stationary-phase in yeast as a model, we provide mechanistic insights into how post-mitotic cells may be subject to senescence.

For the externally administered oxidative agent H2O2, it has been previously demonstrated in yeast that the checkpoint system is normally not engaged unless cells are in S phase or deficient in DNA repair (Leroy et al., 2001). It has been hypothesized that it is unrepaired DNA damage or a repair intermediate accumulating under repair-deficient conditions that triggers checkpoint activation. Multiple protection and redundant repair mechanisms are in place to respond to oxidative stress in *S. cerevisiae* (Doudican et al., 2005; Huang and Kolodner, 2005). The present study was carried out mainly with cells that are defective in BER and NER. Without any external agent, phosphorylation of checkpoint kinase Rad53 was detectable, but only after approximately 2 days of incubation in stationary phase and only if both repair pathways were inactivated. Such conditions of compromised repair may be very relevant for human cells since aging is commonly accompanied by reduced DNA repair activities. This appears to be true for both nuclear NER and BER (Gorbunova et al., 2007; Intano et al., 2003; Moriwaki et al., 1996; Wang et al., 2008).

As shown for aging mitotic and post-mitotic cells in mammalian systems (Hamilton et al., 2001; Wang et al., 2008), we could correlate the accumulation of nuclear and mitochondrial DNA

damage in stationary-phase cells with mitochondrial metabolism. Reduced ROS levels are found in strains that lack mitochondrial DNA (*rho°*) and thus functional mitochondria (Rasmussen et al., 2003). Consequently, isogenic *rho°* derivatives of the strains tested here show lower nuclear mutation frequencies and no induction of Rad53 phosphorylation in stationary phase.

The observed Rad53 phosphorylation was long-term and no reduction due to adaptation was observed during extended incubation. If taken as a model for non-dividing, long-lived higher eukaryotic cells, accelerated senescence associated with unrepaired oxidative damage may be a consequence of persistent activation of the Rad53 homologue CHEK2. Additional roles of checkpoint activation in non-cycling cells can be envisioned. Presumably, checkpoint proteins may also regulate inducible repair enzymes (Bachant and Elledge, 1998) or may prevent or slow down resumption of cell cycle progression following a switch to division-promoting conditions (e.g. fresh medium in the yeast example). Inappropriate cell cycle re-entry of neurons has been implicated in several neuronal pathologies, such as Alzheimer disease (Kruman et al., 2004; McShea et al., 2007).

The studied cells are not actively progressing through the cell cycle and the possibility for secondary DNA damage is consequently severely reduced. Furthermore, it should be emphasized that it is clearly the inactivation of repair that is the precondition for accelerated checkpoint kinase phosphorylation and not its activation. It is somewhat surprising that a defect in nucleotide excision repair can predispose for checkpoint activation. If bulky base damage were essential for checkpoint activation in this system, checkpoint activation should rather be weakened since checkpoint activation by UV and other bulky base damage depends largely on nucleotide excision repair (but alternative mechanisms appear to exist) (Giannattasio et al., 2004a; Giannattasio et al., 2004b).

Using selected mutants, we addressed how the checkpoint signal is created and which class of lesion among the many types of oxidative damage may be most relevant for checkpoint activation. The specific and essential role of Rad50 in this system argues for double-strand breaks as the essential lesion. Rad53 phosphorylation was not only strictly dependent on Rad50, a member of the DSB-binding MRN complex, but also on the Mec1 kinase that requires single-stranded DNA for activation. On the other hand, Tel1 does not play a role. Tel1 has been described as stimulating strand resection at DSB of defined location (induced by HO endonuclease) through its interaction with MRN, but overall Tel1 plays only a minor role if DSB number is low and Mec1 is active (Mantiero et al., 2007).

An increase in DNA nicking in stationary-phase cells has been previously noted by applying the TUNEL assay (Madeo et al., 1999). Using pulsed-field gels we did not detect extensive double-strand breakage (data not shown). This does not exclude the presence of a few persistent double strand breaks or perhaps aberrant telomeres as critical lesions. H2A phosphorylation was indeed elevated during extended stationary phase. In our hands, the level of phosphorylated H2A was not a reliable predictor of Rad53 phosphorylation. A higher level was detectable in stationary-phase wild-type cells that do not show Rad53 phosphorylation within this time frame but its level was not notably different in repair-deficient cells. Of course, we do not know anything about the precise structure of DSB sites marked by □H2A and their half-life. However, supporting the significance of ROS-induced DSB, we did not find any increase in gamma H2A during stationary- phase incubation of *rho*° cells. Interestingly, the higher level seen in logarithmic phase *rho*° cells may reflect defective DSB repair and genetic instability in respiration-deficient yeast that had been noted previously (Huang and Kolodner, 2005).

It is informative to compare our results to recent studies in yeast involving DSB and checkpoint activation. Our results resemble those of Grenon et al. who described a dependency on Mec1 (but not Tel1) and MRN complex for checkpoint activation following strand breakage due to ectopic *Eco*RI expression both in logarithmic-phase and G1-arrested cells (Grenon et al., 2006). This contrasts with the finding that end resection and checkpoint response is dependent on Cdk1 activity following a targeted DSB and is thus greatly reduced in G1 (Ira et al., 2004). The deeply stationary phase cells of our study will be similarly low in Cdk1 activity. However, frequency as well as structure of DSB is presumably critical for these discrepancies in the literature. Unlike HO-endonuclease-induced DSB with complementary overhangs, a substantial DSB fraction introduced by ionizing radiation is indeed resected in G1 and bound by the MRN complex (Barlow et al., 2008). Ionizing-radiation induced DSB have normally non-complementary ends of complex structure and resemble those induced by ROS (von Sonntag, 2006).

In mammalian cells, a similar joint requirement for MRN complex and Mec1 homologue ATR has been demonstrated in certain checkpoint responses after double-strand breakage (Jazayeri et al., 2006). It was suggested that the endo/exonuclease activity of MRN may be required to generate single strand tracts, a known signal recognized by ATR and its interacting proteins ATRIP and RPA. Recently, CtIP and its yeast homolog Sae2 were identified as MRN interacting proteins that promote end resection (Kim et al., 2008; Sartori et al., 2007). Following H2O2 treatment, human A549 cells show recruitment of MRN to DNA damage in all cell cycle stages (Zhao et al., 2008). Persistent DSB have also been correlated with human replicative ageing (Sedelnikova et al., 2004; Seluanov et al., 2004). In summary, our interpretation that processed double-strand breaks trigger checkpoint activation by endogenous oxidative damage is substantiated by the available literature.

An essential question in need of an answer is how a higher load of unrepaired oxidative base damage, as is the case in BER+NER deficient cells, can accelerate a checkpoint activation process that involves processed DSB. Our working model is that DSB in this system are introduced directly by interaction of ROS with the sugar-phosphate backbone of DNA (von Sonntag, 2006). Thus, DSB incidence itself in stationary-phase cells is not affected by defects in BER and NER as indicated by gamma H2A levels that are similar to wild type. However, the presence of unrepaired base damage in the vicinity of a double strand break (an example of a "clustered lesion") may somehow accelerate 5'–>3' end resection, perhaps by stimulating an endonuclease activity close to the break (Fig. 7). Thus, a structure is more easily created that is prone to the activation of downstream kinases. It is also intriguing to note that in *Xenopus* extracts oligonucleotides released during MRN-dependent end resection exert a checkpoint kinase stimulating effect, at least for ATM (Jazayeri et al., 2008). The responsible exo/endonuclease(s) in our system still needs to be identified and one wonders how base damage might influence MRN endonculease activity. We already studied *Exo*1 which has at best a modest influence on checkpoint activation, suggesting the existence of several partially redundant enzymes as shown in other studies (Lam et al., 2008).

Our study concerns primarily chronological aging and not necessarily replicative aging. However, the molecular sources of aging may actually be similar and may include telomere alterations. The model outlined above may not (or not exclusively) involve random double strand breaks but also alterations at telomeres that may be extraordinarily susceptible to oxidative damage (Passos et al., 2007; Zhang et al., 2007). However, we have not detected any overall telomere shortening during extended stationary phase. We have not yet measured the exposure of single stranded DNA at telomeres; however, it should be noted that the yeast MRN complex

(which is critical in our system) is involved in checkpoint signaling at overall shortened telomeres (Viscardi et al., 2007) but not at those that exhibit more single stranded DNA (Foster et al., 2006). It is therefore unlikely that telomere alterations are critical as a signal for endogenous damage in our system.

In summary, our data indicate that resected DSB are critical for persistent checkpoint activation by endogenous oxidative damage in postmitotic cells. The greatly accelerated checkpoint activation in the absence of BER and NER may be due to enhanced resection in the presence of unrepaired oxidative base damage near a DSB. If proven, a better understanding of the interplay between damage accumulation and diminished DNA repair resulting in aging responses of post-mitotic cells can be achieved.

Figure 1.

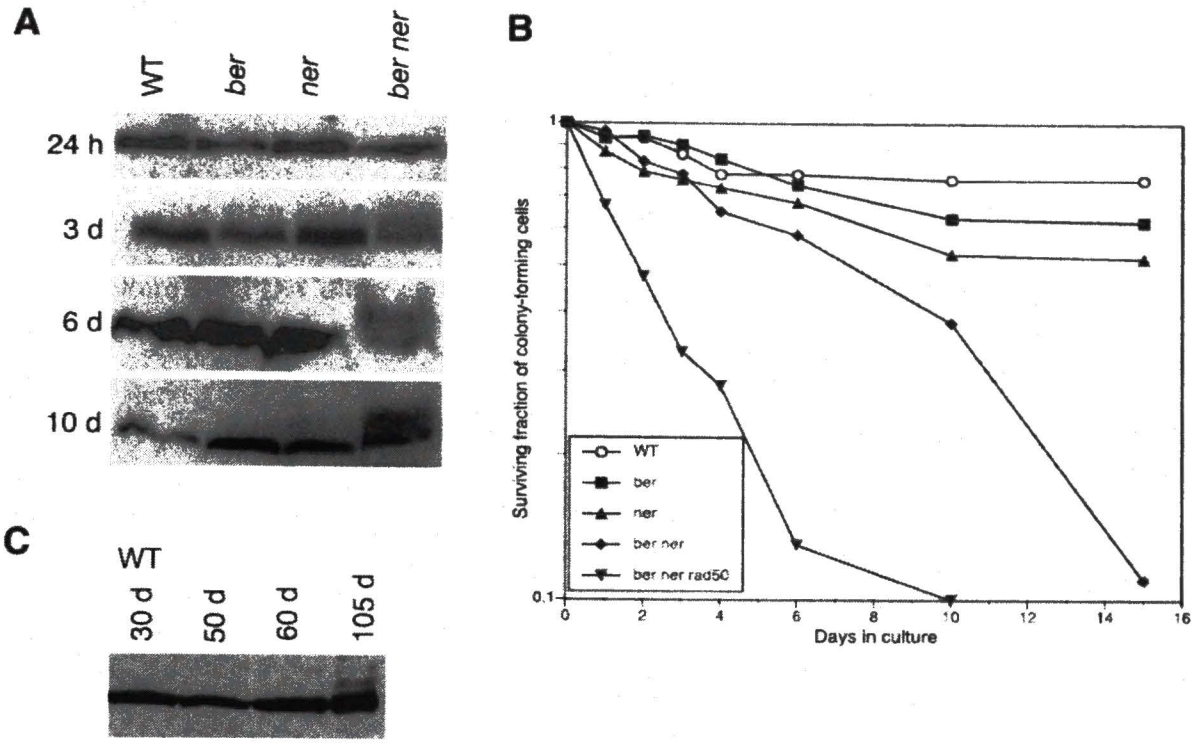
A
WT
ber
ner
ber ner
24 h
3 d
6 d
10 d
B
Surviving fraction of colony-forming cells
1
0.1
0
2
4
6
8
10
12
14
16
Days in culture
WT
ber
ner
ber ner
ber ner rad5C
C
WT
30 d
50 d
60 d
105 d

Fig. 1.

Rad53 phosphorylation, colony survival and mutagenesis during extended stationary phase in *Saccharomyces cerevisiae* DNA repair mutants (strain background SJR751).

A. Phosphorylation of Rad53 detected by altered mobility in Western blots. Extracts of wild type, NER (*rad1*Δ), BER (*ntg1*Δ *ntg2*Δ *apn1*Δ) and NER/BER deficient cells were prepared after 1-10 d of culture in YPD. Cultures were in logarithmic phase for up to 1 day of culture.

B. Survival of colony-forming cells as a function of culture age. The strains used are WT (O), *ber* (■), *ner* (= *rad1*Δ) (▲), *ber ner* (♦) and *ber ner rad50*Δ (▼). C. Rad53 phosphorylation during extended incubation of stationary-phase wild-type cells.

Figure 2.

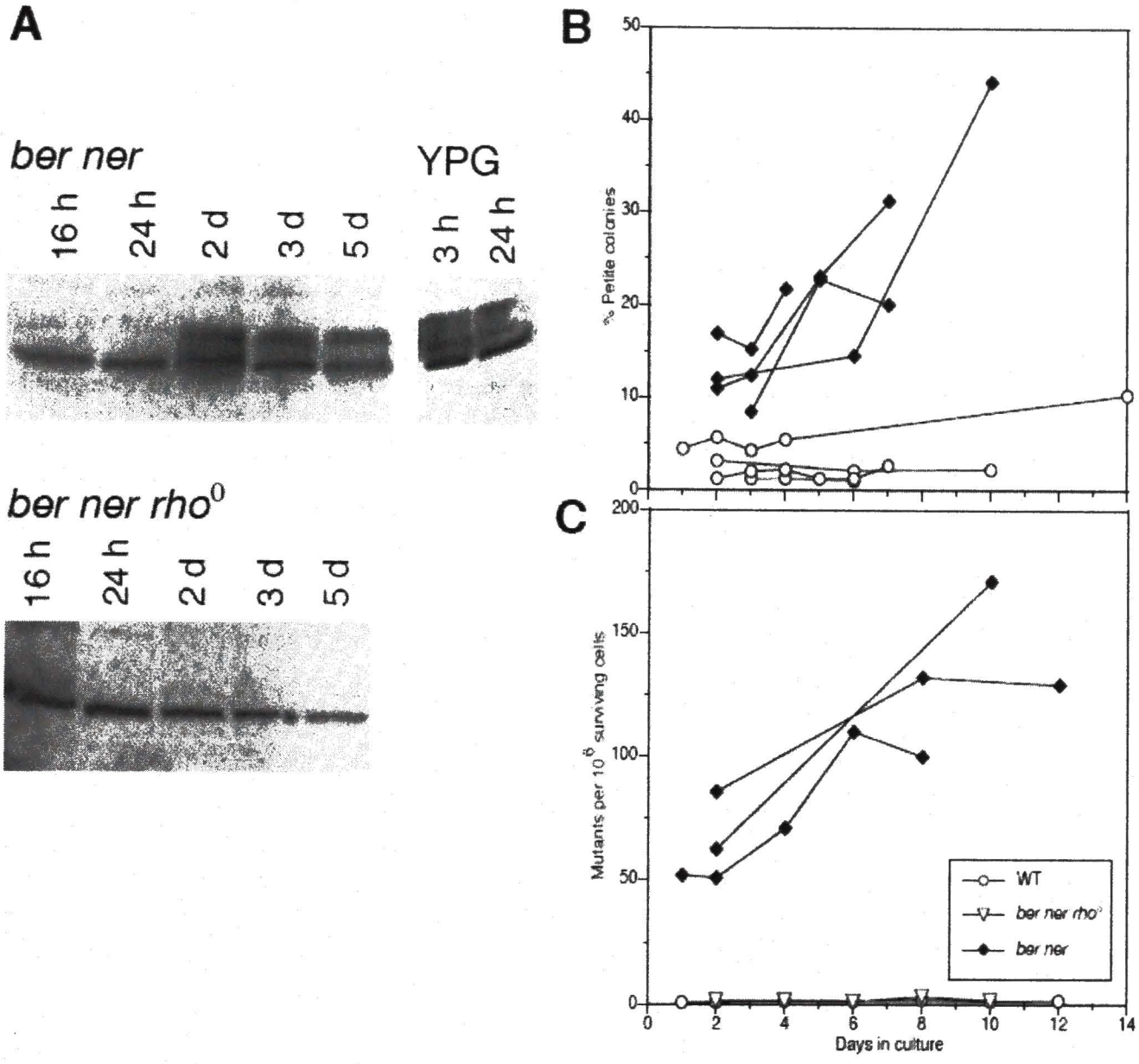
A
ber ner
16 h
24 h
2 d
3 d
5 d
YPG
3 h
24 h
ber ner rho0
16 h
24 h
2 d
3 d
5 d
B
% Petite colonies
C
Mutants per 10^6 surviving cells
WT
ber ner rho0
ber ner
Days in culture

Fig. 2.

Rad53 phosphorylation, mitochondrial and nuclear mutagenesis as a function of culture age in respiration-proficient and –deficient (= *rho*°) *ber ner* cells.

A. Rad53 phosphorylation in respiration-proficient (upper panel) and -deficient cells (lower panel). Additionally, Rad53 phosphorylation of logarithmic-phase cells in glycerol medium (YPG) (right panel) is compared to dextrose medium (all others).

B. Frequency of respiration-deficient ("petite") colonies as a function of culture age in wild-type (O) and *ber ner* (■) cells. Results of 3 and 4 independent experiments are shown.

C. Frequency of canavanine-resistant mutants among colony forming cells in wild-type (O), respiration-proficient *ber ner* (♦) and respiration-deficient *ber ner* (∇) cells during incubation in stationary phase. Results of 4 independent experiments are plotted for the *ber ner* strain.

Figure 3.

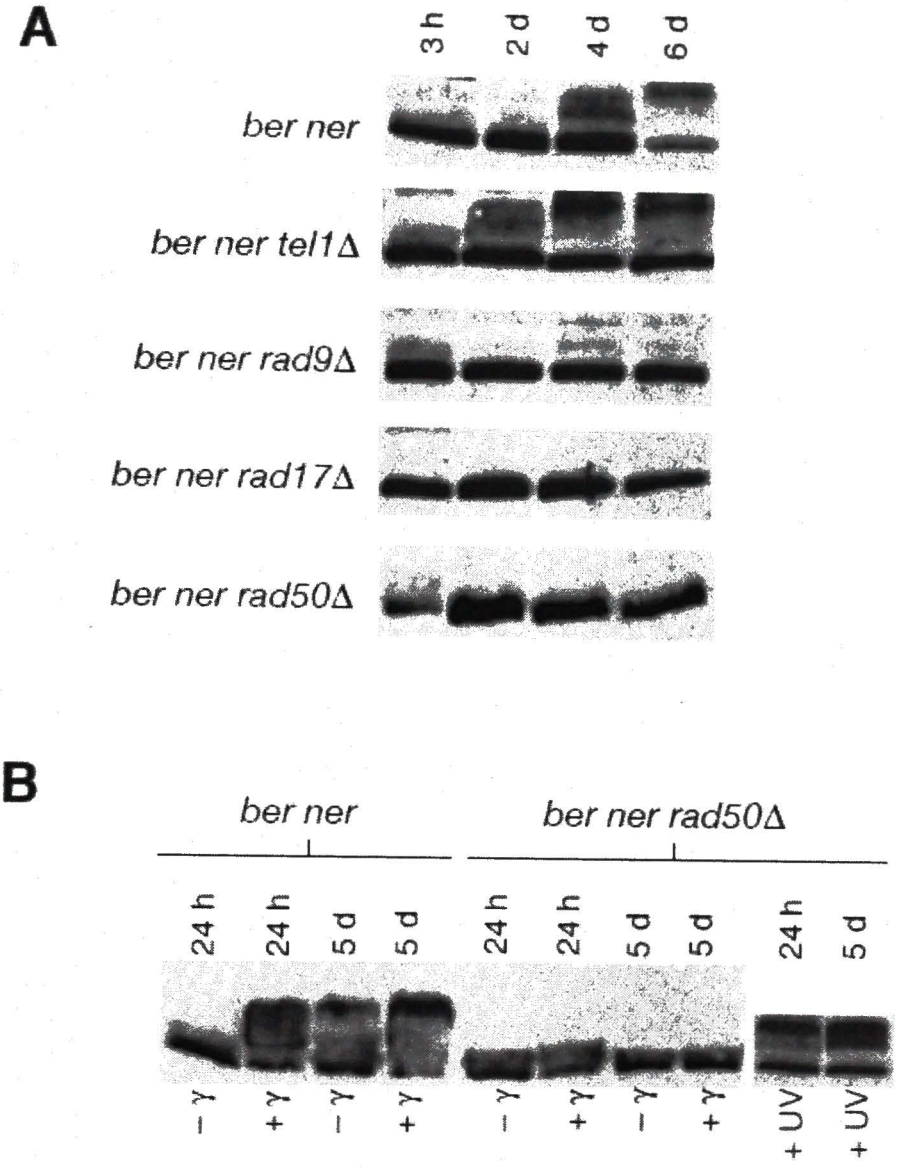
A
3 h
2 d
4 d
6 d
ber ner
ber ner tel1Δ
ber ner rad9Δ
ber ner rad17Δ
ber ner rad50Δ
B
ber ner
ber ner rad50Δ
24 h
24 h
5 d
5 d
24 h
24 h
5 d
5 d
24 h
5 d
– γ
+ γ
– γ
+ γ
– γ
+ γ
– γ
+ γ
+ UV
+ UV

Fig. 3.

Rad53 phosphorylation as a response to culture age or radiation treatment in *ber ner* cells deleted for various checkpoint and repair genes.

A. Rad53 phosphorylation in extended stationary phase of *ber ner* cells deleted for *TEL1*, *RAD9*, *RAD17*or *RAD50*.

B. Rad53 response to exogenous DNA-damaging agents (100 Gy Cs137 γ-irradiation or 80 J/m2 254 nm UV) in *ber ner* and *ber ner rad50Δ* strains is shown. Samples were withdrawn 3 h after irradiation treatment.

Figure 4.

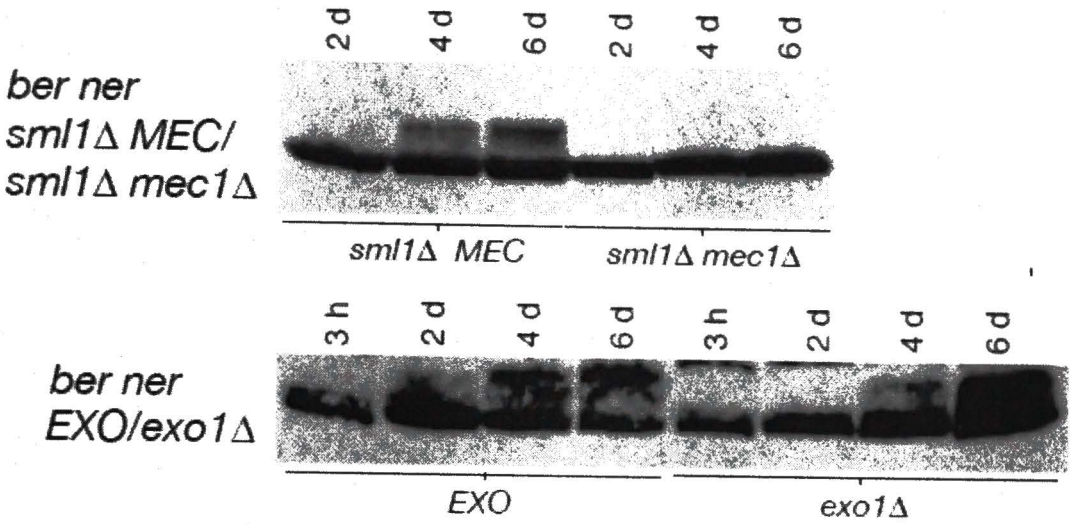
2 d
4 d
6 d
2 d
4 d
6 d
ber ner
sml1Δ MEC/
sml1Δ mec1Δ
sml1Δ MEC
sml1Δ mec1Δ
3 h
2 d
4 d
6 d
3 h
2 d
4 d
6 d
ber ner
EXO/exo1Δ
EXO
exo1Δ

Fig. 4.

Rad53 phosphorylation as a function of culture age in *ber ner* cells deleted for *MEC1* and *EXO1*. The *MEC1* deletion was examined in an *SML1* deletion background.

Figure 5.

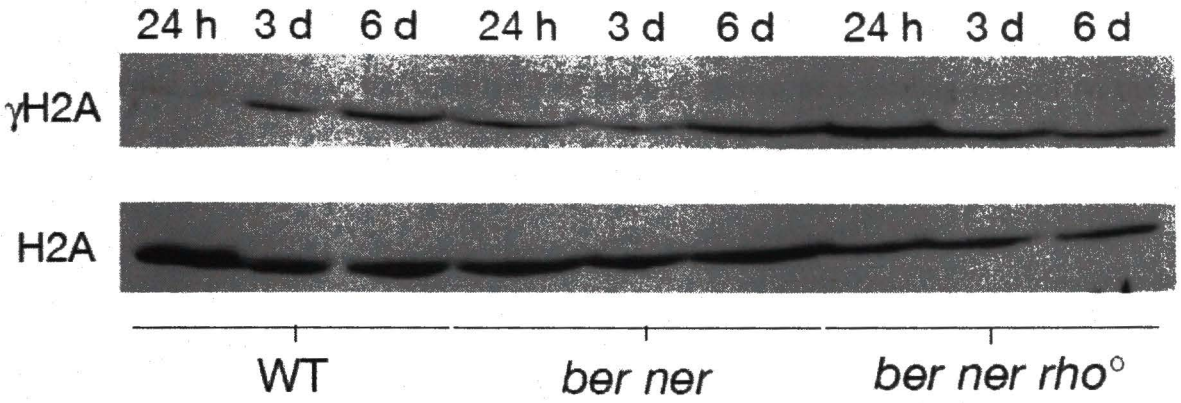

Fig. 5.

Histone H2A phosphorylation as a function of culture age in wild-type, *ber ner* and *ber ner rho°* cells. In the first row, the level of phosphorylated H2A detected by a phosphospecific antibody is shown. In the second row, the total level of H2A is shown as a loading control on the same stripped membrane.

Figure 6.

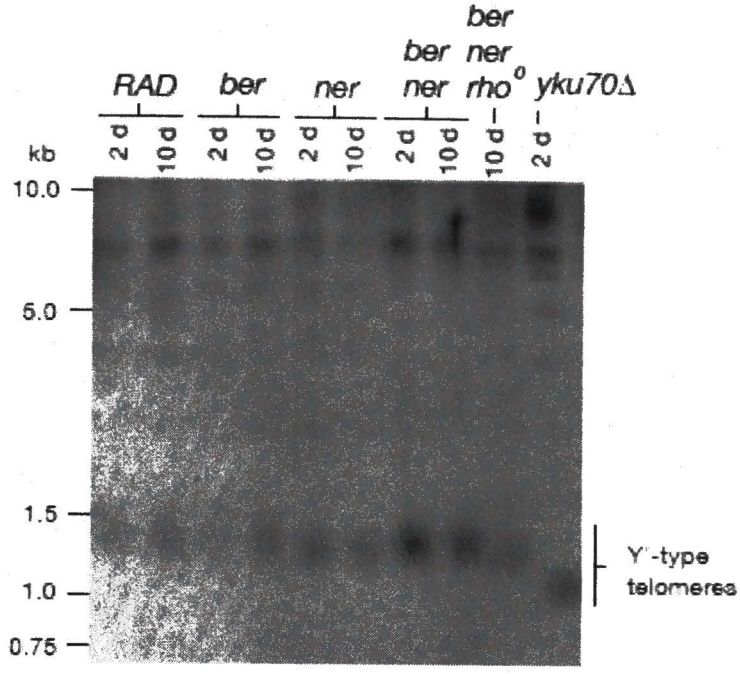
RAD
ber
ner
ber ner
ber ner rho⁰
yku70Δ
2 d
10 d
2 d
10 d
2 d
10 d
2 d
10 d
10 d
2 d
kb
10.0
5.0
1.5
1.0
0.75
Y'-type telomeres

Fig. 6.

Telomere repeat length analysis. The strains indicated were cultured in YPD for 2 or 10 days. Extracted DNA was digested with *Xho*I and probed with a telomere-specific single stranded probe. Length of Y'-type telomeres is compared in relation to molecular weight standards. *Yku70*Δ as a strain known to have shorter telomeres is included as a control.

Figure 7.

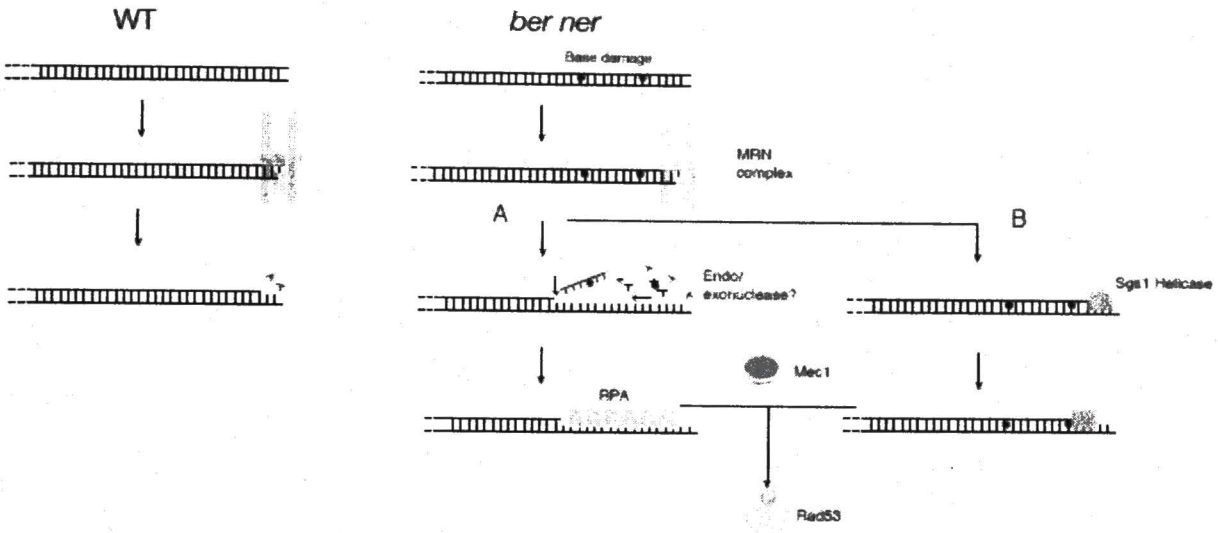
WT
ber ner
Base damage
MRN
complex
A
B
Endo/
exonuclease?
Sgs1 Helicase
Mec1
RPA
Rad53

Fig. 7.

Accelerated single-strand resection at a double strand break in the presence of unrepaired oxidative damage may be responsible for checkpoint signaling. We assume that the persistence of such damage in the vicinity of a double-strand break in BER/NER-deficient cells may stimulate a yet uncharacterized exo/endonuclease activity that depends on MRN complex binding. The exposed single-stranded DNA creates a signal that more readily leads to Rad53 phosphorylation through Mec1 activation and autophosphorylation than in the repair-proficient wild type. Alternatively, we speculate that unrepaired DNA damage may not stimulate but inhibit progression of the Sgs1 helicase that may serve as a "landing pad"for the Mec1-Lcd1 complex and thus accelerate checkpoint responses.

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CHAPTER III

PREFACE

Does unrepaired DNA damage near double stand breaks or near telomeric DNA activate checkpoint response?

In our previous study we have shown that compromised excision repair pathways may accumulate base damage and generate checkpoint response in cells. The main reason for this checkpoint activation was the presence of unrepaired base damage nearby DNA double strand breaks. Processing of unrepaired base damage near DSBs may lead to formation of ssDNA. This ssDNA may be recognized as a DNA damage signal for checkpoint activation.

The next question we have asked is does the presence of unrepaired base damage near double stand breaks or near telomeres trigger checkpoint response if we compromise DSB repair and excision repair. To study this effect I have combined Ku defect with NER deficiency. The Ku proteins play important roles in non-homologous end joining pathway of DNA double strand break repair and it also plays important roles in telomere length maintenance

(Gravel, Larrivee, Labrecque, & Wellinger, 1998; Hsu, Gilley, Blackburn, & Chen, 1999; Miyoshi, Sadaie, Kanoh, & Ishikawa, 2003; Riha, McKnight, Griffing, & Shippen, 2001). Combination of excision repair deficiency with Ku defect will accumulate unrepaired DNA damage either at double strand breaks or at telomeres. The DNA repair process at telomeric DNA is significantly less efficient compared to bulk of the genome (Petersen, Saretzki, & von Zglinicki, 1998). Therefore any damage at telomeres may lead to generation of either single strand breaks or double strand breaks and it will further generate checkpoint activation. Telomere shortening is already implicated in the aging process and age related decline in DNA double strand break repair was also reported (Mayer, Lange, Bradley, & Nichols, 1991) but very little information is available on how oxidative damage can elicit checkpoint response either through DSBs or from telomeres. Also, there is a possibility that unrepaired base damage at both DNA double strand breaks and at telomeres can collaborate together for checkpoint activation during stationary phase.

Telomere specific DNA damage generates signal for Rad53 phosphorylation in stationary phase of Ku and NER deficient *Saccharomyces cerevisiae*

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Keywords: Yeast, Stationary phase, Oxidative DNA damage, Checkpoints, Aging

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ABSTRACT

Most eukaryotic cells spend most of their life in the quiescent (G_0) state. Eukaryotic cells enter into this specialized non-dividing resting state, which is also known as stationary phase due to lack of essential nutrients or growth factors. While resting in G_0 phase, the cells are exposed to various internal and environmental stresses. Therefore, it is important to fully understand the response of cells to the stress in stationary phase. The purpose of our study is to understand the role of DNA repair pathways in stationary phase DNA damage. Here we have studied the role of Yku proteins along with nucleotide excision repair proteins in stationary phase. Yku proteins promote DNA repair as part of nonhomologous end-joining (NHEJ) pathway and they also maintain telomere homeostasis. When a Ku70 defect (*yku70*Δ) was combined with defects in nucleotide excision repair (*rad4*Δ or *rad1*Δ), phosphorylation of checkpoint kinase (Rad53) was observed in stationary phase. However, single pathway deletion mutants were incapable of Rad53 phosphorylation. Mitochondrial respiration was indispensible for Rad53 phosphorylation in stationary phase since the effect was clearly absent in *rho0* derivatives. We had suspected the occurrence of this checkpoint response is due to telomere shortening but interestingly *yku70rad4* cells did not show progressive telomere shortening during stationary phase. Neither defective NHEJ (*dnl4*) nor a mutant Ku protein defective in interaction with telomerase (*yku80-135i*) mimic the influence of a Ku deletion. However, overexpression of *TLC1* component of

telomerase abolished phosphorylation of Rad53 in stationary phase. These results suggest that oxidative DNA damage leads to phosphorylation of Rad53 in stationary phase. And the DNA damage is not result of reduced telomere length per se, but reflects an alteration of the telomere structure or excessive single stranded DNA at telomeres, which may possibly recognized as damaged DNA.

INTRODUCTION

Stationary phase of *Saccharomyces cerevisiae* (G_0) has already been considered as a viable model system for studying aging related phenomena of postmitotic cells.

S. cerevisiae cells undergo chronological aging, which refers to the total lifespan of a given cell, which includes the replicative lifespan and the time spent in a quiescent state (Longo, Liou, Valentine, & Gralla, 1999) and it is different from the replicative aging of higher eukaryotic cells (Tissenbaum & Guarente, 2002). During the initial logarithmic phase of growth, the budding yeast grows by fermentation of the available glucose. When glucose becomes limit, the cells transiently arrest growth and switch to a respiratory mode of energy production. This period of transition is known as the diauxic shift. Post-diauxic period the cells enter into the stationary phase, the growth period when the cell number does not increase but the cells get arrested in G0 and it is also characterized by the production of respiration dependent ROS. (Granot & Snyder, 1991; Werner-Washburne, Braun, Johnston, & Singer, 1993). Recently aging has been largely correlated with damage to various macromolecules. In fact cellular macromolecules are constantly exposed to both internal and external damage. Sources of extrinsic damage include UV irradiation and other environmental toxic agents, whereas intrinsic insults principally include reactive oxygen species (ROS) (Finkel & Holbrook, 2000; Marnett & Plastaras, 2001). In fact

ROS are a major source of spontaneous damage to DNA, proteins, lipids and carbohydrates. Main sources of ROS are suspected to be mitochondrial respiration and peroxisomal metabolism. There is an emerging consensus that a progressive and irreversible accumulation of oxidative damage contributes to impaired physiological function, increased incidence of disease and thus impacts the aging process. (Beckman & Ames, 1998; Kregel & Zhang, 2007). Damage to the macromolecules, which can be replaced by their fast turnover will not accumulate and thus they may not be critical for cellular life. But on the other hand, DNA is the most important information molecule of the cell and nuclear DNA mainly must last the lifetime of the cell. Therefore damage to DNA represents the most critical macromolecular damage in a cell. If the DNA damage is too severe or its accumulation exceeds its repair by DNA repair mechanisms, cellular senescence or apoptosis will occur and this may contribute to the ageing process.

One important factor that causes age-associated accumulation of DNA damage is the functional decline of DNA repair systems with age (Lombard et al., 2005; Lou & Chen, 2006). Genetic model of premature aging involves disrupted expression of Ku80 protein that plays important role in the maintenance of DNA (including telomere) integrity. Ku is a heterodimeric DNA-binding protein that functions in mammals and yeast in the repair of DNA double-strand breaks by non homologous end joining (NHEJ) (Tuteja & Tuteja, 2000a). Ku is believed to bind to DNA ends to prevent unnecessary DNA degradation, as well as to facilitate DNA ligation by juxtaposing DNA ends and recruiting DNA ligases (Bliss & Lane, 1997; Teo & Jackson, 2000a). Ku has also been shown to play an essential and direct role in telomere homeostasis. Telomeres are specialized structures at the ends of eukaryotic chromosomes that, in Sacchoromyces cerevisiae, are heterogenous C1-3A:TG1-3 repeats of ~300 base pairs. Deletion of *YKU70* or *YKU80* results in telomere shortening and loss of transcriptional silencing of genes placed near

the telomere (Boulton & Jackson, 1996a; Boulton & Jackson, 1998a; Nugent & Lundblad, 1998a; Porter, Greenwell, Ritchie, & Petes, 1996a; Tsukamoto, Kato, & Ikeda, 1997). A strong evidence for the importance of telomere damage in post-mitotic neurons of nervous system is the prominent neurodegeneration symptoms seen in patients and mice with genetic defects in proteins associated with telomeres including Ku80, ATM and NBS1. Decline of NHEJ efficiency has been observed in the rat brain during ageing (Ren & de Ortiz, 2002; Vijg & van Orsouw, 2002). Therefore, it was concluded that diminished efficiency and fidelity of double strand break (DSB) repair are responsible for age related genomic instability (Gorbunova & Seluanov, 2005; Vijg & van Orsouw, 2002).

The NER pathway is believed to repair bulky, helix distorting lesions such as bipyrimidine UV photoproducts (Lindahl & Wood, 1999). NER has also been shown to be capable of processing oxidative lesions such as thymine glycol and 8-oxoguanine (Lin & Sancar, 1989; Reardon, Bessho, Kung, Bolton, & Sancar, 1997). Several studies demonstrated that efficiency of nucleotide excision repair (NER) decreases with the age as the rate of removal of UV-induced DNA lesions is slower in aged humans relative to younger adults (Goukassian et al., 2000; Moriwaki et al., 1996). This age-associated decline was shown to result from, at least in part decreased levels of proteins that participate in the repair process (Moriwaki et al., 1996). Both Ku proteins which play important role in NHEJ and telomere maintenance and NER pathway proteins may play significant role in the regulation of ageing process. Recent studies provided evidence that links loss of Ku proteins and NER pathway proteins with DNA damage, declined stem cell functionality and ageing. Mice deficient in genomic maintenance pathways such as NER, NHEJ and telomere maintenance showed decreased stem cell functional capacity. Interestingly the importance of DSB repair, telomere maintenance and NER pathway in

determining longevity has been demonstrated convincingly in premature aging patients e.g. Werner and Cockayne syndromes. It was also suggested that increased accumulation of DNA strand breaks as well as dysfunctional telomeres and resulting premature senescence play a causative role in the Werner syndrome (WS). Furthermore, deficiency in DNA damage sensing proteins is also involved in the process of ageing. Deficiency of either ATR or ATM causes rapid premature ageing, resulting from reductions in tissue-specific stem and progenitor cells (Krishnamurthy & Sharpless, 2007). These facts glaringly show the importance of checkpoint response in controlling loss of cellular viability during aging process.

After considering all these facts together, we wanted to investigate the effect of loss of Yku and NER proteins on checkpoint activation and determine the possible mechanism behind this checkpoint response. We have employed haploid yeast mutants defective in either Yku proteins (Yku70/Yku80) or NER proteins (Rad1 or Rad4) to determine the effect of spontaneous, stationary phase DNA damage on phosphorylation of checkpoint kinase Rad53 (homolog of human Chk2). Our results demonstrate that, only double deletion mutant cells *yku70rad4* are able to phosphorylate checkpoint kinase Rad53 in stationary phase, that is 2 days after incubation in culture. The Rad53 phosphorylation is completely absent in single deletion mutants like *yku70* or *rad4* or in wild type cells. This response is only observed in rho^{+} *yku70rad4* cells and completely missing in rho^{0} derivatives. The NHEJ function of Yku proteins does not seem to play an important role in checkpoint response as shown by *dnl4rad4* deletion result. Further we have explored telomere maintenance function of Yku proteins. No progressive telomere shortening was observed in the case of *yku70rad4* cells as compared to control *yku70* cells in stationary phase. To study further function of Yku proteins, we have combined *yku80(135i)* mutant with *rad4*. *yku80(135i)* mutant lacks the telomerase interaction function of wild type

Yku80 protein. After abolishing this telomerase interaction function of Yku80, we did not observe the phosphorylation of Rad53 in *yku80(135i) rad4* cells. But interestingly, overexpression of *TLC1* component telomerase in *yku80rad4* cells completely abolished Rad53 phosphorylation. This suggests altered telomere structure or excessive single stranded character of telomeres can initiate checkpoint response in stationary phase.

MATERIALS AND METHODS

Yeast strains:

Most strains used in this study are isogenic derivatives of Sx46A (*MATa ade2 his3-532 trp1-289 ura3-52*). *Yku70* gene was deleted by *KanMX4* using PCR-based micro homology mediated transplacement, using a plasmid borne *KanMX4* gene (Longtine et al., 1998). The NER deficient derivative is deleted for *RAD1(rad1*Δ::hisG-URA3-hisG) and *RAD4 (rad4*Δ*::KanMX4). TLC1* gene was cloned in p427TEF plasmid.

Generation of *rho*^{0} strains:

Strains lacking mitochondrial DNA (mtDNA) (*rho0*) were generated by treatment of *ykurad4* with ethidium bromide. Briefly, ethidium bromide was added to a concerntration of 10ug/ml and the cells were incubated at room temperature, with agitation, for approximately 24 h in yeast extract peptone-dextrose (YPD). Following a second and third treatment with 10ug/ml ethidium bromide for 24 h, the cells were diluted (1:100). Following incubation, the cells were diluted in water and plated on YPD to obtain single colonies. The mutants were selected as cells unable to form colonies on yeast-extract peptone glycerol (YPG) plates. In *rho0* cells the loss of mtDNA was verified by 4,6-diamidino-2-phenylindole (DAPI) staining.

Detection of Rad53 phosphorylation:

Late logarithmic phase cultures were diluted in fresh YPD (1% yeast extract, 2% peptone, 2% dextrose). During incubation at 30o C, about 4 x 108 cells were harvested for each time point and lysed using a TCA-based method (Foiani, Marini, Gamba, Lucchini, & Plevani, 1994). Following SDS-PAGE, Rad53 and its phosphorylated forms were detected by conventional Western blotting using a commercial antibody (Santa Cruz Biotechnology). Details have been described elsewhere (Pabla, Pawar, Zhang, & Siede, 2006).

Telomeric DNA analysis:

Yeast chromosomal DNA was extracted as mentioned in and subjected to *XhoI* digestion. Digested DNA was separated by agarose gel electrophoresis and Southern blotting was performed using a telomere specific 32P-labeled single stranded probe (5'-GTGTGGGTGTGGTGTGTGTGGGG-3') according to protocols published in (Boulton & Jackson, 1996b; LeBel et al., 2006).

RESULTS AND DISCUSSION

Joint inactivation of Yku proteins and NER pathway proteins results in checkpoint kinase phosphorylation in stationary phase

Multiple protection and repair mechanisms are in place to respond oxidative stress in budding yeast (Doudican, Song, Shadel, & Doetsch, 2005; Huang & Kolodner, 2005). We have explored single DNA repair deficient mutants such as *yku70, rad1* for Rad53 phosphorylation in stationary phase. These single mutants along with wild type cells did not show phosphorylation of Rad53 in stationary phase that is after day 2 and onwards. But interestingly the combination of Yku deficiency with NER (*rad1*) deficiency showed Rad53 phosphorylation in stationary phase (data not shown). Rad1 is a subunit of the Rad1/Rad10 endonuclease, which has additional and distinct roles from NER (Ma, Kim, Haber, & Lee, 2003). We then combined *yku70* deletion with a disruption mutation of the *RAD4* gene, which is exclusively involved in NER to address the influence of this pathway more specifically. Similarly, *yku70rad4* deletion strain also showed phosphorylation of Rad53 in stationary phase. As indicated by slower mobility, phosphorylated forms of Rad53 were found in *yku70 rad4* cells. Rad53 modification was not observed in logarithmic phase but only detectable after at least 2 days of incubation when no more culture growth has been seen (Figure 1). Whereas timing and extent of phosphorylation

proved to be somewhat variable between different experiments, this main observation was highly reproducible. Rad53 phosphorylation appears to be from spontaneous DNA damage, which is a consequence of normal cellular processes.

Rad53 phosphorylation in *yku70rad4* cells is exclusively depend on mitochondrial respiration

Mitochondria are specialized organelles whose primary function is to synthesize ATP via oxidative phosphorylation in aerobic eukaryotes (Saraste, 1999). *Saccharomyces cerevisiae* shows increase in mitochondrial respiration during its diauxic shift. During mitochondrial respiration, electrons are transferred ultimately to oxygen after their passage through four membrane-bound complexes. Generation of ROS takes place during the transfer of electrons from one complex to other. The electron transport system is also the major endogenous source of ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. Therefore ROS produced during aerobic respiration can lead to cumulative oxidative damage and serves as a contributing factor for triggering the observed checkpoint response. To confirm the role of mitochondrial ROS in triggering Rad53 phosphorylation we have used mitochondrial deficient mutants. It has been demonstrated that rho^0 mutants of *S.cerevisiae*, which lack detectable mitochondrial DNA show reduced levels of ROS (Rasmussen, Chatterjee, Rasmussen, & Singh, 2003). rho^0 mutants of *yku70rad4* did not show Rad53 phosphorylation in stationary phase that is after 2 days or 4 days (Figure 4). This result is in contrast to the phosphorylation of Rad53 observed in stationary phase of rho^+ *yku70rad4* cells. This more likely indicates lower or no oxidative DNA damage in rho^0 cells compared to that of their *rho*+ counterparts.

Deficiency in non-homologous end joining (NHEJ) function of Yku does not seem to contribute for Rad53 checkpoint kinase phosphorylation

Multiple DNA repair pathways may co-operate to repair spontaneous DNA damage. It is logical to assume that, under conditions of compromised Yku proteins, the genomic DNA may experience DNA double strand breaks, because Ku plays important role in the repair of DNA double-strand breaks by non homologous end joining (NHEJ) (Tuteja & Tuteja, 2000b). The NHEJ pathway of double strand break (DSB) repair uses proteins to recognize, bridge, process and ligate the broken DNA strands (Karathanasis & Wilson, 2002). The first step in eukaryotic NHEJ is end binding by a Ku heterodimer composed of Ku70 and Ku80 subunits (Dudasova, Dudas, & Chovanec, 2004). In mammalian cells, the DNA dependent protein kinase catalytic subunit (DNA-PKcs) facilitates end bridging, whereas in *Saccharomyces cerevisiae*, which lacks DNA-PKcs, this activity may be provided by the Mre11-Rad50-Xrs2(MRX) complex. DNA double strand breaks caused by reactive oxygen species can have modified ends. Additional factors are also used if the DNA termini require resection at modified DNA ends to ligate the DSB ends. The final step, ligation, is catalyzed by the DNA ligase IV complex (Wilson, Grawunder, & Lieber, 1997). Like MRX complex, the Ku heterodimer has multiple cellular functions. In addition to NHEJ, it is required for telomere maintenance. Unlike Ku, the actions of the DNA ligase IV complex appear restricted to NHEJ. The DNA ligase IV complex consists of DNA ligase IV itself and XRCC4 (Dnl4 and Lif1 in yeast). Dnl4 binds strongly to Lif1 and both proteins are essential for NHEJ. Also Nej1 modulates the NHEJ activity of this complex in yeast by interacting with the Lif1 (Frank-Vaillant & Marcand, 2001).

We wanted to see if NHEJ role of Yku proteins is important in the Rad53 phosphorylation. As

Dnl4 exclusively functions in NHEJ process, we combined deletion of *DNL4* with *RAD4* (NER) deficiency. In *dnl4rad4* cells, we did not see Rad53 phosphorylation at days 2 and 4, similarly log phase cells (at 3h, 1d) also did not show Rad53 phosphorylation (Figure 3). From this result we conclude that a defect in NHEJ alone does not account for the *YKU70* deletion phenotype of predisposing single-repair pathway mutants to checkpoint activation.

Telomere length shortening does not seem to initiate for Rad53 phosphorylation

In addition to their role in NHEJ, Yku proteins has also shown to play an essential and direct role in telomere homeostasis (Boulton & Jackson, 1998b; Nugent & Lundblad, 1998b). Telomeres are specific DNA structures at the ends of chromosomes that secure genetic information by protecting chromosomes from degradation with the help of many telomere associated proteins including Yku70/80. In *Saccharomyces cerevisiae*, telomeric DNA is 250-400 bp long with a simple repeat tract C1-3A/TG1-3 (Vega, Mateyak, & Zakian, 2003). Presence of telomerase protects *S.cerevisiae* from undergoing replicative senescence. In *S.cerevisiae*, telomerase extends the G-rich strand followed by general DNA replication that fills in the opposite C-strand and, therefore, the G-rich, single stranded tail does not get detected. Telomere length is affected by many factors, and one of the important factors is the protection provided by telomere maintainace proteins. Disruption of one of Yku subunits results in a dramatic decrease in telomeric repeat length and cells do not show checkpoint kinase phosphorylation (Porter, Greenwell, Ritchie, & Petes, 1996b). But *yku70rad4* cells show Rad53 phosphorylation in stationary phase. Several explanations can be considered for this special role of Yku in stationary phase. First, both stress factors, telomere alteration and oxidative damage, may be directly connected. Unrepaired oxidative damage in the telomere region may lead to further telomere

shortening that is exacerbated by absence of Ku as a protective protein. However, our analysis of telomeric repeat length during extended stationary phase did not support this concept (Figure 4). We confirmed the shorter telomere length of Yku deficient strains but this phenotype was not aggravated by the additional NER defect and no further attrition was evident during incubation.

Telomeric DNA binding function of Ku is critical to prevent Rad53 phosphorylation

In *S.cerevisiae*, Ku's function at telomeres has been most extensively studied. Ku affects several aspects of telomere biology, including the recruitment of telomerase to telomeres and it maintains the length of telomeres (Fisher, Taggart, & Zakian, 2004; Porter, Greenwell, Ritchie, & Petes, 1996b; Stellwagen, Haimberger, Veatch, & Gottschling, 2003), protection of telomeric ends from nucleolytic degradation (Gravel, Larrivee, Labrecque, & Wellinger, 1998; Maringele & Lydall, 2002; Nugent & Lundblad, 1998b; Polotnianka, Li, & Lustig, 1998), the formation of telomeric heterochromatin, which leads to transcriptional silencing of nearby genes (Boulton & Jackson, 1998b; Laroche et al., 1998; Nugent & Lundblad, 1998b), the late firing of replication origins near telomeres, and the nuclear localization of telomeres (Laroche et al., 1998). Separation of function alleles of *S.cerevisiae YKU70* and *YKU80,* respectively, have revealed that Ku is a multifunctional protein that has distinct activities not only at DSBs as compared with telomeres, but also at telomeres themselves. *YKU80* alleles have been identified that are proficient in NHEJ but defective in specific aspects of telomeric function, such as the *yku80-135i* allele (a five amino acid insertional mutation), which impairs sole telomere length regulation (Stellwagen et al., 2003). This particular allele is absent in Yku80's specific interaction with the RNA subunit of telomerase, which is important in the telomere length maintenance. But other functions of Yku80 are intact in *yku80-135i* allele. As shown in figure 5, *yku80-135i* allele did

not show Rad53 phosphorylation in extended stationary phase indicated by day 6 and day 10. This particular result indicates that telomere length maintenance function of Ku does not seem to be important in stationary phase but other functions of Ku may play critical role. But telomere binding function of Ku seems to be critical in stationary phase. In stationary phase the telomeres are prone to oxidative DNA damage. The deficiency of NER pathway, which repairs oxidative DNA damage may exacerbate the condition of *yku70* cells. This will set stage for action of exonucleases, which can repair oxidative DNA damage. When the telomeres are unprotected by Ku proteins then there is possibility that they will become substrates for the action of certain nucleases such as Exo1, which is a mis-match repair nuclease. After 5' to 3' resection of damaged telomeres by Exo1 nuclease, telomeres will have single strand 3'-DNA overhangs. ssDNA overhangs can trigger DNA damage signal via Mec1-Lcd1 and other checkpoint proteins (Figure 7). And ultimately it will trigger Rad53 phosphorylation as a DNA damage signal.

Telomere subunit expression suppresses telomere specific checkpoint kinase phosphorylation

At 37°C, *yku70* mutant contain increased levels of ssDNA in subtelomeric sequences (Maringele & Lydall, 2002) and overexpression of *EST2* (the catalytic subunit of telomerase) or *TLC1* (the telomerase RNA component) or *EST1* (a targeting subunit of telomerase) can suppress the checkpoint response. From our observation of *yku80-135i rad4* mutant, we expected formation of ssDNA at telomeres. At 37°C, overexpression of *TLC1* can abolish Rad53 response from *yku80* cells. Therefore we wanted to see if the same process is responsible for Rad53 phosphorylation in stationary phase cells. Overexpression of *TLC1* in *yku70rad1* cells abolished Rad53 phosphorylation in stationary phase indicated by day 4 and day 6 (Figure 6). It is tempting

to speculate that the single stranded regions in the *yku70rad1* mutant initiate DNA damage signal. *TLC1* is RNA component of telomerase and it has ability to complementary bind at telomeric overhang. Binding of *TLC1* to single stranded overhangs seem to abolish the DNA damage signal from telomeres. Our data therefore lend support to models in which telomere length itself is often a poor indicator of telomere-induced crisis or checkpoint kinase phosphorylation, and suggest that other features are likely to be critical. More specifically, at least in instances where the telomeres are critically short, such as in the *yku80* mutant, it may be that components of yeast telomerase play additional telomere-specific genome-protective roles by preventing inappropriate activation of the DNA damage checkpoint pathway.

Figure 1.

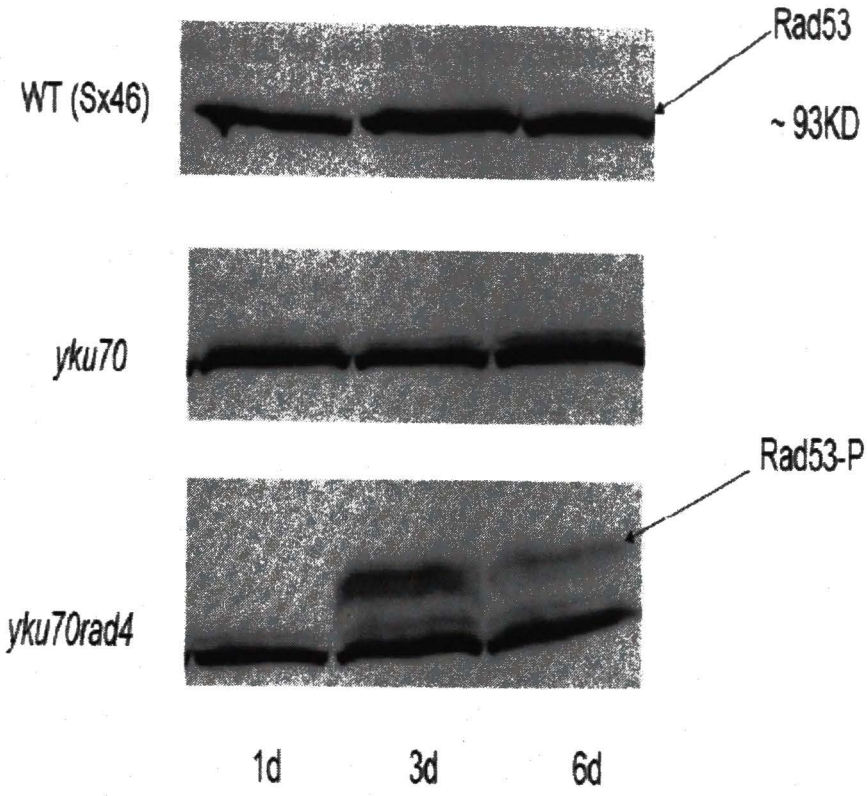

Figure 1. Rad53 phosphorylation during extended stationary phase of *Saccharomyces cerevisiae* DNA repair deficient mutants (strain background Sx46). Phosphorylation of Rad53 detected by altered mobility in Western blots. Extracts of wild type (WT), *yku70,yku70rad4* cells were prepared after 1d, 3d and 6d in YPD culture. Cultures were in logarithmic phase for up to 1 day of culture.

Figure 2.

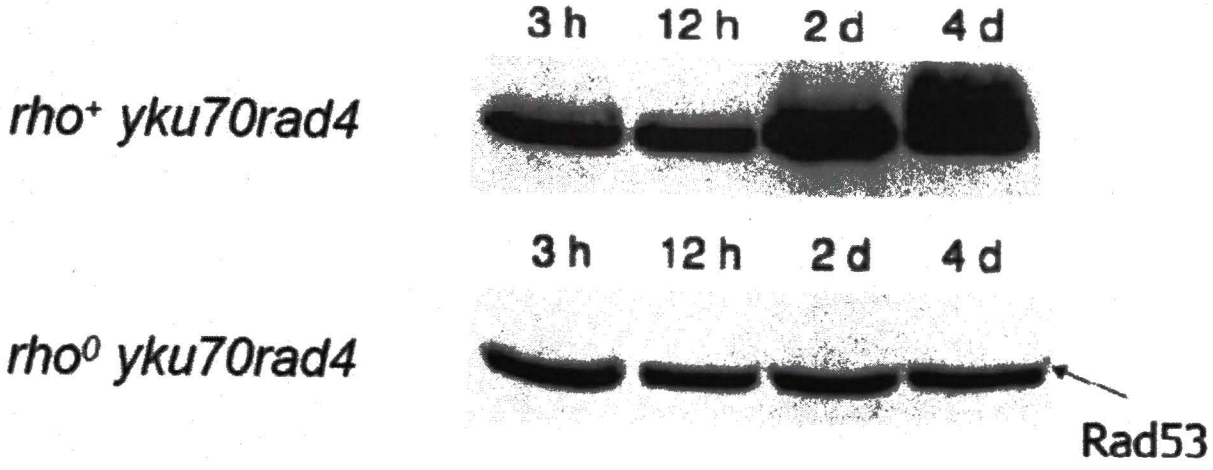

Figure 2. Rad53 phosphorylation as a function of culture age in respiration proficient *(rho+)* and –deficient (*rho0*) *yku70rad4* cells. Rad53 phosphorylation in respiration proficient (upper panel) and –deficient cells (lower panel). Extracts of *rho+* and *rho0* of *yku70rad4* cells were prepared after 3h, 12h, 2d and 4d in YPD culture.

Figure 3.

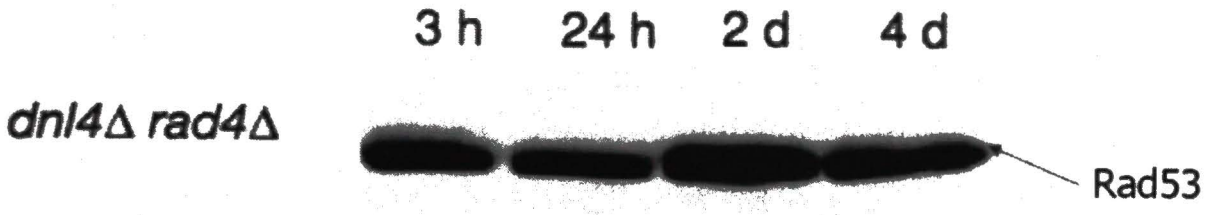

Figure 3. Rad53 phosphorylation during extended stationary phase in repair deficient *S.cerevisiae*. Extracts of *dnl4rad4* cells were prepared after 3h, 24h, 2d and 4d. Phosphorylation of Rad53 was probed using Western blot.

Figure 4.

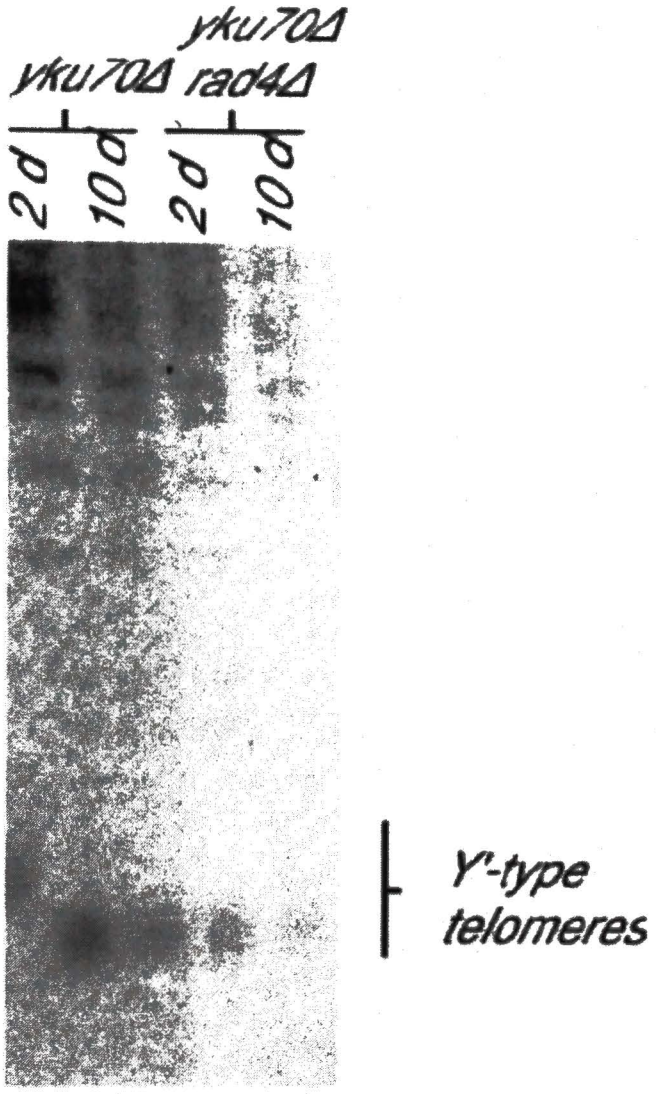

Figure 4. Telomere repeat length analysis. The strains indicated were cultured in YPD for 2 or 10 days. DNA was extracted, digested with *XhoI* and probed with a telomere specific single stranded probe. Length of Y'-type telomeres is compared in relation to molecular weight standards.

Figure 5.

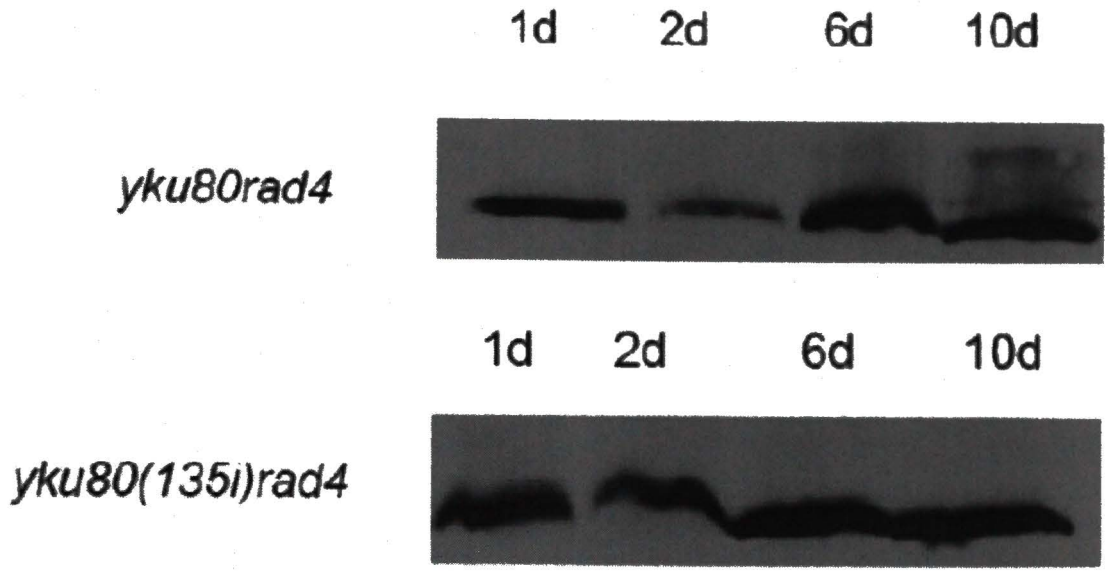

Figure 5. Rad53 phosphorylation during extended stationary phase in repair deficient *S.cerevisiae*. Extracts of *yku80rad4 and yku80(135i)rad4* cells were prepared after 1d, 2d, 6d and 10d and phosphorylation of Rad53 was probed using Western blot.

Figure 6.

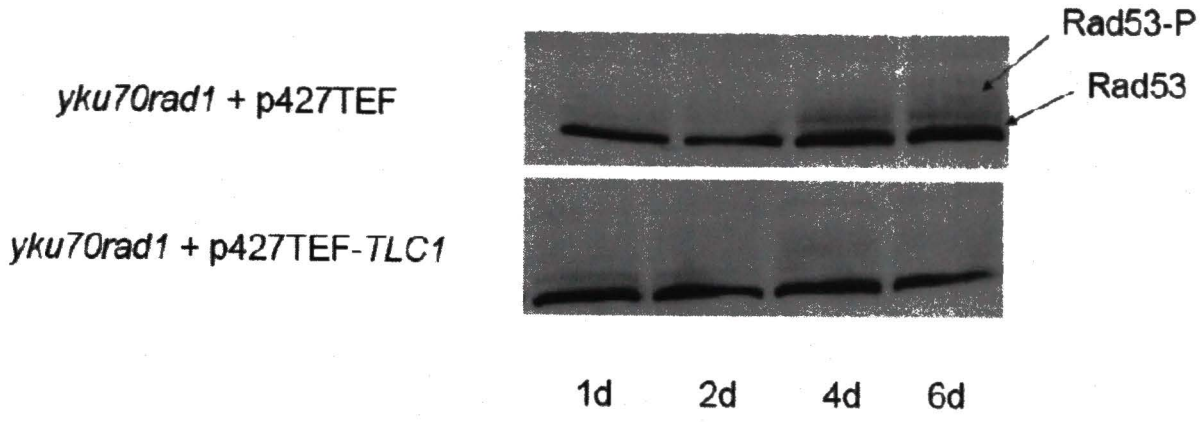
Rad53-P
Rad53
yku70rad1 + p427TEF
yku70rad1 + p427TEF-TLC1
1d
2d
4d
6d

Figure 6. Rad53 phosphorylation during extended stationary phase in *S.cerevisiae*. *TLC1* gene was overexpressed in *yku70rad1* cells using p427TEF vector. *yku70rad1* cells transformed with control p427TEF vector and vector overexpressing *TLC1* gene. Extracts were prepared after 1d, 2d, 4d and 6d in culutre. Phosphorylation of Rad53 was probed using Western blot.

Figure 7.

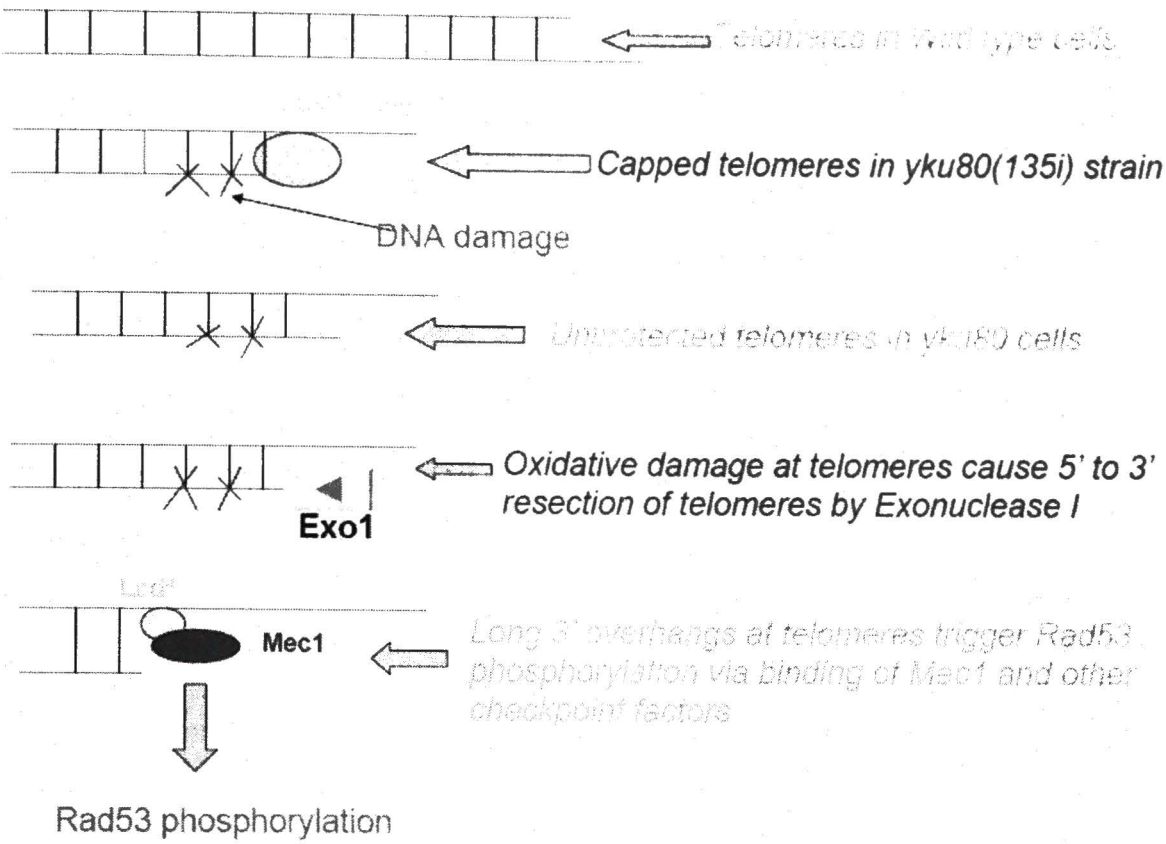
Capped telomeres in yku80(135i) strain
DNA damage
Oxidative damage at telomeres cause 5' to 3' resection of telomeres by Exonuclease I
Exo1
Mec1
Rad53 phosphorylation

Figure 7. Schematic representation of possible repair events in stationary phase cells of *yku70rad4* strain. In the absence of Ku proteins the telomeres are unprotected. In the case of *yku80(135i)* allele, telomere capping function is still intact but telomere length maintenance function is absent. Both oxidative DNA damage at telomeres and absence of telomere binding proteins both serve as a platform for the action of exonuclease to repair the damage. Further Exo1 can resect uncapped telomeres in 5' to 3' direction. Resection will lead to generation 3' DNA overhangs, which can serve as a DNA damage signal and induce Rad53 phosphorylation. This process is absent in *yku80(135i) rad4* cells, therefore they do not show Rad53 phosphorylation.

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CHAPTER IV

DISCUSSION

In this study, I have examined the activation of checkpoint pathway mainly due to unrepaired spontaneous DNA damage in stationary phase. Stationary phase is marked by absence of cell division and use of mitochondrial respiration for energy production (Werner-Washburne, Braun, Johnston, & Singer, 1993; Werner-Washburne, Braun, Crawford, & Peck, 1996)(Werner-Washburne et al., 1996). This growth arrested phase of *S.cerevisiae* most likely overlaps with the quiescent phase of post-mitotic cells (Granot & Snyder, 1991; Werner-Washburne et al., 1996). By using extended stationary-phase in yeast as a model, we provide useful insights into how post-mitotic cells may subject to senescence while their stay in quiescent phase.

Many relevant studies are available that show effect of externally used oxidizing agents on cell cycle and on DNA checkpoints. Exposure to lower concentration of H2O2 delays cell cycle progression in different phases of cell cycle including G1, S and G2 phases. But more interestingly only S phase delay in cell cycle is controlled by DNA checkpoints

(Leroy, Mann, & Marsolier, 2001) . Very little is known about the activation of checkpoints by endogenous ROS. It has been suggested that unrepaired DNA damage or repair intermediate can trigger checkpoint activation. *S.cerevisiae* comprises multiple repair pathways to repair any genotoxic stress including oxidative stress (Doudican, Song, Shadel, & Doetsch, 2005; Huang & Kolodner, 2005).

I have performed my studies using two different set of strains. But both has one common feature and that is the absence of excision repair pathway. One strain is BER and NER deficient and the other strain comprises defect in Ku and NER pathways. I have strictly carried out my studies without the use external oxidizing agent. It was very logical to conjecture that the cells will have accumulation of spontaneous damage under the conditions of compromised excision repair in both the mutant strains. Consistently we have observed various phosphorylated forms of Rad53 during stationary phase in both mutant strains, BERNER and, Ku and NER. The appearance of multiple phosphorylated forms of Rad53 in response to DNA damage has been well documented in the literature and we have demonstrated the susceptibility of these forms to phosphatase treatment elsewhere (Evert et al., 2004). Such repair deficiencies are very relevant for human cells since aging is commonly accompanied by reduced DNA repair capacity (Gorbunova, Seluanov, Mao, & Hine, 2007; Intano, Cho, McMahan, & Walter, 2003; Moriwaki, Ray, Tarone, Kraemer, & Grossman, 1996; Wang, Lukas, Yuan, & Neufeld, 2008). Rad53 phosphorylation was consistent throughout stationary phase and no reduction was observed during extended incubation.

In post-mitotic cells of higher eukaryotes, accelerated senescence is accompanied by persistent activation of CHEK2, which is a mammalian homolog of Rad53. The underlying reason for such response may be because of accumulation of unrepaired DNA damage. Role of checkpoint

activation in replicating cells is different as compared to growth arrested cells. In replicating cells activation of checkpoints either lead to arrest of cell cycle, transcriptional induction of inducible transcripts (Bai & Elledge, 1996) (Friedberg EC et al., 2005). But checkpoint activation plays slightly different functions in growth arrested cells. Presumably, checkpoint proteins may also regulate inducible repair enzymes. One of the important set of DNA damage inducible genes are RNR (ribo nucleotide reductase) genes (Pellicioli et al., 1999). Rad53 may downstream regulate transcription of RNR genes. Induction of RNR genes will further increase the dNTPs pool inside cells to facilitate the process of DNA repair. Other role of checkpoint activation may be to prevent or slow down the resumption of cell cycle progression following a switch to dividing conditions. On the similar lines inappropriate re-entry of neurons in the cell cycle has shown to be involved in neuronal pathologies such as Alzheimer disease (Kruman, 2004; McShea et al., 2007).

In both BERNER and, Ku and NER mutant strains, their *rho*^{0} derivatives do not show phosphorylaton of Rad53 in stationary phase as well as in logarithmic phase. The reason for this observation is that *rho*^{0} derivatives lack mitochondrial DNA and they are respiration deficient (Faye et al., 1973). Also reduced ROS levels have been observed in *rho*^{0} cells. Similar to aging of mitotic and post-mitotic cells in mammalian systems (Hamilton et al., 2001; Wang et al., 2008), we correlate the accumulation of nuclear DNA damage in stationary phase yeast with mitochondrial respiration.

I have studied growth arrested cells, which are not actively progressing through the cell cycle. Therefore the possibility of secondary DNA damage (such as from replication) is severely reduced. Furthermore, it should be emphasized that it is clearly the inactivation of repair that is the precondition for accelerated checkpoint kinase phosphorylation. It is somewhat surprising

that a defect in nucleotide excision repair can predispose both BER deficiency and Ku deficiency to checkpoint activation in stationary phase. If bulky base damage were essential for checkpoint activation in our system, checkpoint activation should rather be weakened since checkpoint activation by UV and other bulky base damage depends largely on nucleotide excision repair (Giannattasio et al., 2004; Giannattasio, Lazzaro, Longhese, Plevani, & Muzi-Falconi, 2004). There is a possibility that base damage may get converted in other types of DNA damage, which are completely independent of NER signaling.

To determine the type of DNA damage in BER and NER deficient mutant, we have addressed how the checkpoint signal is created and which oxidative lesion is responsible for this checkpoint activation. The specific and essential role of Rad50 in this system argues for double strand breaks as the essential lesion. In the positive control experiment we did not see Rad53 phosphorylation after gamma treatment of BERNER *rad50* cells. Rad53 phosphorylation was not only strictly dependent on Rad50, a member of double strand break–binding MRN complex but also on the Mec1 kinase that requires single-stranded DNA for activation. On the other hand, Tel1 does not play an important role in the checkpoint kinase phosphorylation. Tel1 has been demonstrated to play important role in the repair of double strand breaks induced by HO endonuclease action (Mantiero, Clerici, Lucchini, & Longhese, 2007). To detect DNA double strand breaks in BERNER cells we have used pulsed field gel elctrophoresis (PFGE). No extensive DNA double strand breaks were found in stationary phase of BERNER deficient strains by PFGE, which could have caused massive chromosomal breakage. This did not exclude the presence of few persistent DNA double strand breaks as critical lesions. Phosphorylation of H2A is a very sensitive marker to detect DNA double strand breaks (Foster & Downs, 2005). Although H2A phosphorylation was elevated during extended stationary phase, our experiment

shows that the level of phosphorylated H2A was not a reliable marker for Rad53 phosphorylation. A higher level was detectable in stationary phase wild type cells which do not show Rad53 phosphorylation within this time frame but its level was not notably different in repair-deficient cells. Currently, we do not know anything about the precise structure of sites marked by gamma H2A and their half-life. Recent studies related to DNA double strand breaks and checkpoint activation in yeast overlaps with our studies in yeast. Recent evidence supports that both Mec1 and MRN complex are required for checkpoint activation followed by induction of DNA double strand breaks by *EcoRI* (Grenon, Magill, Lowndes, & Jackson, 2006). These studies have performed in logarithmic phase and in G1 arrested cells. This contradicts with the finding that end resection and checkpoint response is dependent on Cdk1 activity following a targeted DSB at a single site and is thus greatly reduced in G1 (Ira et al., 2004). We assume that the stationary phase cells we are studying also have low in Cdk1 activity. However, amount as well as structure of DSB is presumably critical for understanding these differences in literature. Unlike DSB with complementary overhangs, a substantial fraction of double-strand breaks introduced by ionizing radiation is indeed resected in G1 and bound by the MRN complex (Barlow, Lisby, & Rothstein, 2008). In mammalian cells also a similar joint requirement for MRN complex and Mec1 homolog ATR has been demonstrated in certain checkpoint responses after DNA double strand breaks (Jazayeri et al., 2006). In this system either endonuclease or exonuclease activity of MRN complex may be required to generate single strand tracts, this signal is further recognized by ATR and its interacting proteins ATRIP and RPA. Recently, CtIP and its yeast homolog Sae2 were identified as MRN interacting proteins that promote end resection (Sartori et al., 2007). Following H2O2 treatment, human A549 cells show recruitment of MRN to DNA damage in all cell cycle stages. Also the persistent double strand breaks have

been correlated with human replicative aging (Sedelnikova et al., 2004; Seluanov, Mittelman, Pereira-Smith, Wilson, & Gorbunova, 2004). We interpret that presence of DNA double strand breaks in BERNER deficient cells trigger checkpoint activation by endogenous oxidative DNA damage.

In other mutant strain analysis, Ku and NER defect has shown Rad53 phosphorylation in stationary phase. Ku plays important roles in the repair of DNA double strand breaks by non-homologous end joining (Smith & Jackson, 1999; Tuteja & Tuteja, 2000) and it also contributes in telomere homoeostasis (Boulton & Jackson, 1998; Nugent & Lundblad, 1998).To separate Ku's role in DSB repair from telomere homeostasis, we have combined a deletion in *DNL4* with NER. Dnl4 is a DNA ligase IV and it strictly plays role in DSB repair by NHEJ process (Wilson, Grawunder, & Lieber, 1997). In contrast to *yku70rad4* strain result, we did not observe Rad53 phosphorylation in *dnl4rad4* strain. This result suggested that DNA double strand breaks are not the DNA lesions, which trigger Rad53 phosphorylation. But recent study suggests that Ku proteins play additional roles in DSB repair compared to their NHEJ partner Dnl4 (Wu, Topper, & Wilson, 2008). This led to a very important observation, when Ku proteins are present they may bind at the broken DSB ends and prevent the resection of DSBs by the action of exonucleases. This protection function will not trigger DNA damage signal from DSBs. And we think that the same case may be possible in *dnl4rad4* mutant cells.

In other functions of Ku proteins, they play very critical role in telomere homeostasis. Therefore, we tested the possibility that does unrepaired oxidative DNA damage at the telomeric region leads to further shortening of telomeres. However, our analysis of telomere repeat length during stationary phase did not observe progressive telomere length shortening in *yku70rad4* cells. This

indicated that short telomeres are not recognized as DNA damage but any structural change at telomeres may be recognized as a DNA damage substrate.

Ku extensively play multiple roles in telomere homeostasis. We have studied *yku80* functional allele (*yku80-135i*). This allele lacks Yku80's specific interaction with the RNA subunit of telomerase, which is important in the telomere length maintenance (Stellwagen, Haimberger, Veatch, & Gottschling, 2003). Combination of *yku80-(135i)* allele with *rad4* deficiency did not show Rad53 phosphorylation. This particular result concludes that telomere length maintenance function of Ku does not seem to be important in stationary phase but other functions of Ku may play critical role. In stationary phase, telomeric DNA is prone to oxidative DNA damage similar to non-telomeric DNA (von Zglinicki et al., 2000; von Zglinicki, 2002). Deficiency in excision repair combined with Ku deficiency makes the situation worse by accumulating unrepaired DNA damage at telomeres. Ku allele can bind to telomeric DNA and can protect it from oxidative DNA damage. And absence of Ku can not protect DNA from base damage. Presence of any unrepaired DNA damage at telomeres will serve as a substrate for the action of exonucleases, which may attempt to repair oxidative DNA damage. These nucleases rather than repairing oxidative DNA damage may carry 5' to 3' resection to form 3'-DNA overhangs at telomeres. These 3'-DNA overhangs can serve as a DNA damage signal for checkpoint activation. To explore the presence of 3'DNA overhangs at telomeres I had expressed *TLC1* component of telomerase. *TLC1* is RNA component of telomerase and it has ability to complementary bind to telomeric 3'-DNA overhangs (Stellwagen et al., 2003). Expression of *TLC1* component completely abolished Rad53 phosphorylation. This result suggests that DNA damage signal was indeed coming from ssDNA at telomeres. But we can not exclude the presence of low amount of DSBs at nontelomeric ends.

Similar to our finding, Rad53 phosphorylation was completely abolished after the expression of *TLC1* component in *yku70* mutant at 37°C. It has been also shown that at 37°C mitochondria activity gets disturbed and mitochondria start leaking more ROS (Davidson & Schiestl, 2001a; Davidson & Schiestl, 2001b). Our study overlaps with the Ku studies at 37°C. These studies indicate that in both cases that is at 37°C and in stationary phase telomeres are under continuous ROS attack and they show ssDNA. Telomeric ssDNA can be recognized as DNA damage and it can initiation checkpoint response. Our study supports the models in which telomere length itself is often a poor indicator of telomere-induced crisis or checkpoint response. Our study also suggests that any abnormality in telomere structure can be very critical and it can be recognized as DNA damage signal.

This study primarily concerns the chronological aging and not necessarily replicative aging. However, the molecular sources of aging may actually be similar and may include telomere alterations. Our study shows that not only double strand breaks but also alterations at telomeres may be extraordinarily susceptible to oxidative DNA damage (Passos, Saretzki, & von Zglinicki, 2007). In summary, my results indicate that both resected DSB and telomere alterations are critical for persistent checkpoint activation by endogenous oxidative DNA damage. The accelerated checkpoint activation in BERNER deficient cells may be due to enhanced resection in the presence of unrepaired oxidative base damage near a DSB. And we also postulate that presence of unrepaired oxidative base damage at telomeric DNA may enhance resection at telomeres in Ku and NER deficient cells. If these postulates are proven, a better understanding of interplay between damage accumulation and diminished DNA repair in aging can be achieved.

FUTURE DIRECTIONS

We have studied checkpoint activation in the stationary phase. This growth arrested state can be induced by using certain chemical compounds like rapamycin. The cytosolic target of rapamycin is FKBP12 (Schmelzle & Hall, 2000). This binary rapamycin-FKBP12 complex binds to and inhibits the partially redundant proteins Tor1 and Tor2 (Loewith et al., 2002). Rapamycin is an immunosuppressant drug for humans, which inhibits the proliferation of both yeast and mammalian cells and drives each into a state similar to their respective quiescent state (Schmelzle & Hall, 2000). Checkpoint studies using rapamycin will help us to find important factors, which play critical roles in stationary phase. Also study of temperature sensitive mutant that arrests in a quiescence-like state at nonpermissive temperatures, such as cdc25 will also help us finding important factors, which regulate DNA damage and checkpoint response in stationary phase (Costigan & Snyder, 1994).

We have ascribed checkpoint activation in stationary phase to mitochondrial ROS. Mitochondria are specialized organelles whose primary function is to synthesize ATP via oxidative phosphorylation (Saraste, 1999). Therefore it will be very important to study energy content of cells with and without mitochondria. Complete biochemical analysis of FADH2, NADH and ATP levels in our DNA repair deficient cells will help us to understand the effects of repair

deficiency on mitochondrial metabolism. Various mitochondrial complexes contribute for production of ROS (Bourgeron et al., 1995). Identification of a particular complex which generates ROS in stationary phase will further provide evidence for mitochondrial involvement in checkpoint activation.

In our experiments, DNA repair deficient cells show various degrees of Rad53 phosphorylation in stationary phase. We have also observed stable, steady-state Rad53 phosphorylation during stationary phase. Protein phosphorylation is a reversible reaction and certain kinases like Mec1 and/or Tel1 phosphorylate Rad53 and certain phosphatases dephosphorylate Rad53. We have a fair understanding of checkpoint activation, but very little is known about how cells deactivate the damage response. Recent evidence shows that Rad53 dephosphorylation depends on the presence of the PP2C-type phosphatases; Ptc2 and Ptc3, after repair of a persistent double strand break in G2/M (Guillemain et al., 2007; Leroy et al., 2003). Also the PP2A-like protein phosphatase; Pph3 has been shown to be required to dephosphorylate Rad53 after DNA methylation damage in S phase. Details of their possible regulation are still unknown. Our results indicate that DNA repair deficient BERNER strain show Rad53 phosphorylation throughout the stationary phase, starting from day 2. If the DNA damage gets repaired then Rad53 gets inactivated and the cells will re-enter into cell cycle. This process also termed checkpoint recovery, is required for the essential resumption of cell cycle progression. It would be interesting to study if and how BERNER deficient cells re-enter into cell cycle when the stationary phase culture is re-grown in fresh YPD medium. The absence of Rad53 inactivation in this culture will also serve as a marker for checkpoint recovery. In other scenario, if the DNA damage is not repairable then the cells will prefer another process to silence the checkpoint activation and ultimately continue their progression through cell cycle. This process is termed

checkpoint adaptation. Failure of DNA repair is responsible for checkpoint adaptation. The basic requirement for adaptation is the inactivation of Rad53 by certain phosphatases. Since we observe long-term phosphorylation of Rad53, this process may be inactive during stationary phase, perhaps due to low expression of the relevant phosphatases. It will be interesting to see the deletion of either Ptc2 or Ptc3 phosphatases interferes with re-entry of BERNER cells into the cell cycle. If the BERNER cells undergo checkpoint adaptation then the cells will re-enter into the cell cycle if re-grown in fresh YPD media but at the expense of unrepaired DNA damage. This unrepaired DNA damage will be responsible for genomic instability in BERNER cells. Therefore analysis of genomic instability in BERNER cells when their culture is re-grown in YPD will also distinguish checkpoint adaptation from checkpoint recovery.

All these study have performed *in vivo.* We think that exonuclease activity of Mre11 nuclease creates 5' to 3' DNA resection at DSBs. It would be interesting to see action of Mre11 *in vitro.* Purification of Mre11 protein and its exonuclease activity on a double stranded DNA with oxidative base damage near ends will certainly give us some important insights for its function.

In the area of Ku proteins it will be of great knowledge to see the effect of components of telomerase on telomere chromatin structure and accessibility. This will provide vital insights into how telomere capping might prevent recognition of chromosome ends as DNA double strand breaks. If oxidative damage at telomeres recruits exonucleases like Exo1 at telomeres then there is a high possibility that telomeres exhibit longer 3' overhangs. ssDNA at telomeres can be measured using primers for Y' region of telomeres. This will clearly provide evidence for the presence of ssDNA in Yku and NER deficient cells.

We have showed important role of Rad50 in Rad53 phosphorylation in stationary phase. This indicates Mre11 nuclease of MRX complex may serve as exonuclease in this system. Very recent

literature shows involvement of other nucleases like Sae2 and helicase Sgs1 and Dna2 in the 5' to 3' resection of DSBs to create ssDNA. It would be useful to explore these enzymes and their particular role in stationary phase cells.

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