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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 and activation 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 inactive. The wild type showed a low degree of Rad53 phosphorylation when the incubation period was drastically increased. In the *ber ner* strain, Rad53 phosphorylation can be abolished by inclusion of antioxidants or exclusion of oxygen. Furthermore, this modification and enhanced mutagenesis in extended stationary phase were absent in *rho*^o strains, lacking detectable mitochondrial DNA. This checkpoint response is therefore thought to be dependent on reactive oxygen species originating from mitochondrial respiration. There was no evidence for progressive overall telomere shortening during stationary-phase incubation. Since Rad50 (of the MRN complex) and Mec1 (the homolog of ATR) were 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.

Keywords

Yeast; Stationary phase; Oxidative DNA damage; Checkpoints; Aging

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1. 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 (diauxic 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 additional mechanisms, including apoptosis and senescence (Friedberg et al., 2006; 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 phosphoinositide-kinase like kinases ATM and ATR (Tel1 and Mec1 in *S. cerevisiae*) (Friedberg et al., 2006; 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, acting 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'). Although difficult to equate with withdrawal from cycle progression following differentiation of higher eukaryotic cells, the investigated stationary-phase conditions reflect a possible natural situation of a non-growing cell. 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.

2. Material and Methods

2.1. Yeast strains and plasmids

Most strains are derived from SJR751 *MATa ade2-101 his3 Δ 200 ura3 Δ Nco lys2 Δ Bgl CAN1* (originally from Sue Jinks-Robertson). The NER-deficient derivative is deleted for *RAD1* (*rad1 Δ ::hisG-URA3-hisG*), the vector used to re-introduce *RAD1* was pRS41M (Taxis and Knop, 2006). The BER-deficient strain contains *ntg1 Δ ::LEU2 apn1 Δ ::HIS3 ntg2 Δ ::hisG-URA3-hisG rad1 Δ ::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 *Hyg^r* marker. *YKU70* deletion was introduced into wild-type strain SX46 *MATa ade2 his3-532 trp1-289 ura3-52* (originally from Jasper Rhine). 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.

2.2. 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% glycerol). For anaerobic growth, cultures were overlaid with light paraffin oil and incubated without shaking. Gamma irradiation was performed by exposing portion of stationary phase cultures 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 (2.5×10^7 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.

At various time points during incubation at 30°C, about 7×10^8 cells were harvested and lysed using a TCA-based method (Foiani et al., 1994). Approximately 150 μ g protein per gel lane were loaded as measured by CB-X protein assay (G-Biosciences). Equal loading was also confirmed through cross-reacting protein bands. 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). Autophosphorylation activity of renatured, PVDF-membrane-bound Rad53 was measured as published (Pellicoli et al., 1999). 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).

2.3. Telomeric DNA analysis

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

2.4. Survival and mutagenesis assays

Appropriate culture dilutions were plated on YPD and the fraction of macrocolony-forming cells was determined. This value was confirmed by counting non colony-forming cells and microcolonies by microscopic examination 24 h after plating. 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).

3. Results

3.1. 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 prototype 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, 2005).

Isogenic haploid yeast strains deficient in NER (*rad1Δ*), BER (*ntg1Δ ntg2Δ apn1Δ*) or both pathways (abbreviated as *ber ner*) were tested for phosphorylation of checkpoint kinase Rad53 as a function of culture age (Fig. 1A). 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. 1A). 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. 1A, see also Fig. 2, Fig. 4). Whereas timing and extent of phosphorylation proved to be somewhat variable between different experiments, this principal observation was highly reproducible. By correcting the NER defect we verified that the result depended on the combined repair deficiencies. As a consequence of reintroducing Rad1 on a plasmid, Rad53 phosphorylation was abolished (Fig. 1B).

We have not been able to detect a comparable level of stationary-phase Rad53 phosphorylation in any DNA repair single pathway mutant. For example, in deletion strains of the non-homologous endjoining protein Yku70 no significant level of Rad53 modification is detectable and inactivation of the homologous recombination protein Rad52 leads to Rad53 phosphorylation primarily in logarithmic phase (Supplementary Fig. 1).

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. 1C). 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. 1C, 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. 1D).

3.2. Dependency of Rad53 phosphorylation on reactive oxygen species

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 responds may be caused by ROS released from mitochondria.

In respiration-competent *ber ner* cells, Rad53 phosphorylation could be abolished by inclusion of antioxidants (ascorbic acid, N-acetyl cysteine) but also by reducing aeration during incubation (Fig. 2A). It was necessary to prove that Rad53 phosphorylation and its prevention by antioxidants reflects its activity which was accomplished by using an autophosphorylation assay of renatured membrane-bound Rad53 (Fig. 2A, right panel). Furthermore, 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. 2B). This respiration-deficient strain, however, is in general capable of Rad53 phosphorylation in stationary phase, as demonstrated following γ -irradiation (Fig. 2B, lower right panel). 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. 2B, right panels). The wild-type does not show any Rad53 modification under the same conditions. This correlates with active mitochondrial respiration, prior to the diauxic shift of stationary phase, when cells are 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. 3A).

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 have been reported (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. 3B). Corresponding to a reduced oxidative DNA damage level, mutation frequencies of *rho*^o *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. 3B).

3.3. 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., 2006; Nyberg et al., 2002). Both deletions have a drastic effect on Rad53 phosphorylation which is greatly reduced (Fig. 4A).

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., 2006) has a profound effect. In the absence of functional Rad50, stationary-phase Rad53 phosphorylation is clearly absent in the *ber ner* strain (Fig. 4A). 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. 1C). 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. 4B). In spite of multiple repair defects and low survival, these cells were nevertheless able to respond to UV with Rad53 phosphorylation, even in stationary phase (Fig. 4B).

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. 4A). The ATR homologue Mec1, however, the other major kinase upstream of Rad53, was absolutely required for Rad53 phosphorylation under these conditions (Fig. 5). Its deletion was carried out in an *sml1Δ* 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. We 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. 5, lower panel).

3.4. 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 telomere repeat length by Southern blotting, we did not detect any difference between wild-type and BER, NER or BER/NER deficient strains for the majority of Y'-telomeres, irrespective of mitochondrial activity (Fig. 6). There was also no hint at any progressive shortening during stationary-phase incubation.

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). Without any external agent, phosphorylation and activation of checkpoint kinase Rad53 was detectable, but only after approximately 2 days of incubation in stationary phase and only if two major repair pathways, BER and NER, were inactivated. Although not amounting to complete inactivation of multiple pathways, such conditions are relevant for human cells since aging and differentiation is commonly accompanied by reduced activity of DNA repair, including nuclear BER and NER (Gorbunova et al., 2007; Intano et al., 2003; Moriwaki et al., 1996; Narciso et al., 2007; Wang et al., 2008).

As shown for aging mitotic and post-mitotic mammalian cells (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*^o) and thus functional mitochondria (Rasmussen et al., 2003). Consequently, isogenic *rho*^o derivatives of the strains tested here show lower nuclear mutation frequencies and no Rad53 phosphorylation in stationary phase. Similarly, Rad53

activation in respiration-competent cells could be prevented by antioxidants or limitation of oxygen exposure. Forcing cells to activate aerobic metabolism at an earlier culture stage by using a non-fermentable carbon source accelerated Rad53 phosphorylation.

The observed Rad53 activation was long-term and, interestingly, no reduction due to adaptation was observed during extended incubation. If taken as a model for post-mitotic higher eukaryotic cells, such persistent activation of the Rad53 homologue (CHEK2) may lead to accelerated senescence in response to unrepaired oxidative damage. Additional roles of checkpoint activation in non-cycling cells can be envisioned. For example, checkpoint proteins also regulate inducible DNA repair enzymes (Bachant and Elledge, 1998) or may prevent or slow down resumption of cell cycle progression following a switch to division-promoting conditions. Inappropriate cell cycle re-entry of neurons with unrepaired oxidative DNA damage has been implicated in several neuronal pathologies (Kruman et al., 2004; McShea et al., 2007; Nospikel and Hanawalt, 2003).

Using selected mutants, we addressed 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 DNA double-strand breaks (DSB) 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. Rad17 as part of the 9-1-1 checkpoint clamp is also critically involved in the observed response and the recognized substrate, a double-stranded/single-stranded DNA junction (Majka and Burgers, 2007), also hints at processed DSB as the critical lesion. The role of the Rad9 adaptor protein is less clear since we consistently found some residual Rad53 phosphorylation.

On the other hand, Tel1 did not play a role. Tel1 has initially 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 Mec1 is present (Mantiero et al., 2007). In mammalian cells, a joint requirement for MRN complex and Mec1 homologue ATR has similarly been demonstrated in certain checkpoint responses after double-strand breakage (Jazayeri et al., 2006). Endo/exonuclease activities dependent on MRN are required to generate single strand tracts, a known signal recognized by ATR and its interacting proteins ATRIP and RPA, and thus mediating the observed switch from ATM to ATR (Shiotani and Zou, 2009). However, a dependency of ATR on ATM/MRN (Shiotani and Zou, 2009) does not seem to be true for the yeast homologs Mec1 and Tel1 (Mantiero et al., 2007).

Published results in yeast clearly show that a joint requirement of Mec1 and the MRN complex is a hallmark of processed DSB as critical checkpoint-activating lesions. Our results resemble those of Grenon et al. who described a dependency on Mec1 and MRN complex but not Tel1 for checkpoint activation following strand breakage due to ectopic *EcoRI* expression, both in logarithmic-phase and G1-arrested cells (Grenon et al., 2006). This is somewhat in contrast with the finding that end resection and checkpoint response is dependent on Cdk1 activity following targeted DSB by HO-endonuclease 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, 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). Thus, DSB structure as well as frequency can account for these apparent discrepancies in the literature.

In summary, our interpretation that processed double-strand breaks trigger checkpoint activation by endogenous oxidative damage is substantiated by the available literature.

Persistent DSB have already been correlated with human replicative ageing (Sedelnikova et al., 2004; Seluanov et al., 2004). In stationary-phase yeast cells, an increase in DNA nicking has been previously noted using the TUNEL assay (Madeo et al., 1999) and the influence of nonhomologous endjoining on mutation distributions seemed to reflect DSB occurrence (Heidenreich et al., 2003). Although we were not able to detect extensive double-strand breakage (data not shown) the presence of a few persistent double strand breaks as critical lesions is not excluded. A higher level of phosphorylated H2A was detectable even in stationary-phase wild-type cells (without Rad53 activation) but its level was not notably different from *ber ner* cells (Supplementary Figure 2). The latter result suggests that damage frequency may not distinguish wild-type and repair-deficient cells with regard to Rad53 activation and a possible model should address differences in lesion structure instead.

How does a higher load of unrepaired oxidative base damage 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). The presence of unrepaired base damage in the vicinity of a double strand break may somehow accelerate 5'→3' end resection, perhaps by stimulating an endonuclease activity close to the break (Fig. 7). Thus, a structure is more readily created that is prone to activate downstream kinases. It is intriguing to note that in *Xenopus* extracts oligonucleotides released during MRN-dependent end resection exert a checkpoint kinase stimulating effect (Jazayeri et al., 2008). However, the responsible exo/endonuclease(s) in our system still needs to be identified since deletion of Exo1 has at best a modest influence on checkpoint activation. Nuclease redundancy has recently been uncovered by detailed studies on 5'→3' end resection at DSB in yeast (Mimitou and Symington, 2008; Zhu et al., 2008). The action of the MRN complex in conjunction with Sae2 leads to short stretches of single-stranded DNA that are extended, in a redundant fashion, by Exo1 or the complex of Sgs1 helicase and Dna2 nuclease. Intriguingly, Sgs1 can be found in a complex with Mec1 and Lcd1 (Gavin et al., 2002). Therefore, as an alternative to the model proposed above, we can also speculate that Sgs1 helicase may be inhibited by unrepaired DNA damage, serve as a nucleation center for Lcd1-Mec1 and thus lower the threshold for Rad53 activation (Fig. 7).

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, within the limits of detection, we have not detected any shortening of a significant fraction of telomeres during extended stationary phase. As exemplified by the absence of significant Rad53 phosphorylation in a *yku70* deletion mutant (Supplementary Fig. 1), shortened telomeres alone are insufficient for the observed checkpoint response. Although we have not yet measured the exposure of single stranded DNA at telomeres, the yeast MRN complex is not involved in checkpoint responses to telomeres that exhibit excessive single stranded DNA (Foster et al., 2006). It is therefore unlikely that telomere alterations are a critical signal for endogenous damage in our system.

In summary, our data indicate that resected DSB appear to be critical for persistent checkpoint activation by unrepaired endogenous oxidative damage in postmitotic cells. Altered resection in the presence of oxidative base damage near a DSB is suggested. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Rad53 phosphorylation and colony survival 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 (*rad1Δ*), BER (*ntg1Δ ntg2Δ apn1Δ*) and BER/NER deficient cells were prepared after 1–10 d of culture in YPD. Cultures were in logarithmic phase for up to 1 day of culture.

D. Comparison of Rad53 phosphorylation in BER/NER deficient cells complemented by plasmid-borne Rad1 or transformed with vector

C. Survival of colony-forming cells as a function of culture period in stationary phase. The strains used were WT (○), *ber* (■), *ner* (= *rad1Δ*) (▲), *ber ner* (◆), *rad50Δ* (◻) and *ber ner rad50Δ* (◼). Survival of logarithmic-phase overnight cultures was normalized to 1; the individual survival values in logarithmic-phase cultures were 100% (WT), 97% (*ber* or *ner*), 80% (*ber ner*), 79% (*rad50Δ*) and 65% (*ber ner rad50Δ*).

D. Rad53 phosphorylation during extended incubation of stationary-phase wild-type cells.

Fig. 2.

Rad53 phosphorylation and kinase activity as a function of culture age and condition in respiration-proficient and -deficient (= *rho*⁰) *ber ner* cells.

A. Absence of Rad53 phosphorylation in respiration-proficient *ber ner* cells in the presence of ascorbic acid (0 – 500 mM, incubated for 4 days, upper panel), under anaerobic conditions (– O₂, incubated for 6 days, lower panel) or in the presence of N-acetylcysteine (NAC, 10 mM). Rad53 kinase activity was measured by autophosphorylation in the same samples (shown are 0 and 500 mM ascorbic acid, upper right panel).

B. Comparison of Rad53 phosphorylation in respiration-proficient (upper panels) and -deficient cells (lower panels). Additionally, Rad53 phosphorylation of logarithmic-phase cells in glycerol medium (YPG) (upper right panels) is compared to dextrose medium (all others). No Rad53 modification is found in the wild type under the same conditions. Additionally, Rad53 phosphorylation was studied following γ -irradiation (100 Gy, Cs¹³⁷) in 1 day and 5 day cultures of respiration-deficient cells (lower right panel).

Fig. 3.

Genetic instability of wild-type and BER/NER-deficient yeast during extended stationary phase.

A. Frequency of respiration-deficient (“petite”) colonies as a function of culture age in wild-type (○) and *ber ner* (◆) cells. Results of 3 and 4 independent experiments are shown.

B. Frequency of canavanine-resistant mutants among colony forming cells in wild-type (○), respiration-proficient *ber ner* (◆) and respiration-deficient *ber ner rho*^o (▽) cells during incubation in stationary phase. Results of 4 independent experiments are plotted for the *ber ner* strain.

Fig. 4.

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 Cs¹³⁷γ-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.

Fig. 5. Rad53 phosphorylation as a function of culture age in *ber ner* cells deleted for *MEC1* and *EXO1*. *MEC1* deletion was examined in an *SML1* deletion background.

Fig. 6. Telomere repeat length analysis. The strains indicated were cultured in YPD for 2 or 10 days. Extracted DNA was 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. *Yku70Δ* as a strain known to have shorter telomeres is included as a control.

Fig. 7.

Altered 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 (A). 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 instead inhibit progression of a DNA processing enzyme such as the Sgs1 helicase (B) that may attract the Mec1-Lcd1 complex and thus accelerate checkpoint responses.