Mol Biol Rep (2012) 39:3573–3583
DOI 10.1007/s11033-011-1131-5

Compromised cellular responses to DNA damage accelerate chronological aging by incurring cell wall fragility in *Saccharomyces cerevisiae*

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Received: 17 March 2011 / Accepted: 22 June 2011 / Published online: 29 June 2011

Abstract Elevated levels of reactive oxygen species (ROS) can attack almost all cell components including genomic DNA to induce many types of DNA damage. In this study, we used *Saccharomyces cerevisiae* with various mutations in a biological network supposed to prevent deleterious effects of endogenous ROS to test the effect of such a network on yeast chronological aging. Our results showed that cells with defects in cellular antioxidation, DNA repair and DNA damage checkpoints displayed a mutation rate higher than that of wild-type strain. Moreover, the chronological life span of most mutants as determined by colony formation was found to be shorter than that of wild-type cells, especially for the mutants defective in DNA replication and DNA damage checkpoints, although the observed cell number was almost the same for wild-type and mutant strains. The mutants were finally found to be more sensitive to SDS and lysing enzyme treatment, and that the degree of sensitivity was correlated with their chronological life span.

Keywords Chronological aging · DNA damage repair · Checkpoint · Genome stability · Antioxidation

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Introduction

Reactive oxygen species (ROS), such as the superoxide radical, hydroxyl radical and H_2O_2, are generated through both endogenous and exogenous sources. The majority of endogenous ROS are produced during oxidative electron transport in the mitochondrial [1]. In addition, cytosolic enzyme systems, including NADPH oxidases, and by-products of peroxisomal metabolism are also endogenous sources of ROS. Generation of ROS also occurs through exposure to numerous exogenous agents and events including ionizing radiation (IR), UV, cytokines, growth factors, chemotherapeutic drugs, environmental toxins, hyperthermia and macrophages during the inflammatory response [1, 2]. Low levels of intracellular ROS may play an important role in regulating cellular functions by activating several enzymatic cascades as well as transcriptional factors. To the contrary, elevated levels of ROS pose a significant threat to cellular integrity in terms of damage to DNA, lipids, proteins and other macromolecules. Aerobic organisms have evolved a wide array of mechanisms to antagonize the negative effects of ROS. A number of nonenzymatic and enzymatic antioxidant components that scavenge and inactivate ROS constitute the frontline defense system to maintain the cellular redox state [3]. Among others, peroxiredoxins such as Tsa1 possess the potent ability to scavenge H_2O_2 [4]. Srx1, on the other hand, has been found to recover the overoxidized Tsa1 by reducing the cysteine–sulphinic acid in Tsa1 [5]. Importantly, elimination of Tsa1 predisposes the cell to accumulating a broad spectrum of mutations including large chromosome rearrangements [6]. Therefore, to govern DNA integrality, cells are also armed with various other mechanisms to minimize, detect and repair the arising DNA lesions to preserve genome integrity. In

Saccharomyces cerevisiae, the resulting cellular responses to genotoxic stress not only include different DNA repair processes such as base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), recombination (REC) and postreplicative repair (PRR) [7], but also signaling pathways called DNA damage checkpoints which coordinate DNA metabolism with cell-cycle progression [8–10]. In the face of DNA damage, a cell can respond to amplified damage signals, either by actively halting cell cycle until it ensures that critical processes such as DNA replication or mitosis are complete or by initiating apoptosis as a last resort.

Aging yeast cells in long-term cultivation have been considered to be a model for the aging process in human postmitotic tissues [11]. A majority of these chronologically aged yeast cells die eventually exhibiting typical markers of apoptosis [12]. On the other hand, ROS have been found to be accumulated in chronologically aged yeasts and have been shown to be both necessary and sufficient for inducing apoptosis in yeast [13, 14]. A causative role for ROS in aging processes, referred to as the free radical theory of aging, proposes that ROS in biological systems attack molecules resulting in the functional decline of organ systems that eventually leads to death [5, 15]. Up to date, there has been ample evidence favoring this theory [16–19]. Similarly, yeasts with an extra copy of the alcohol dehydrogenase 1 (ADH1) gene showed increased survival in stationary phase (chronological ageing) due to induction of antioxidant enzymes such as catalase and superoxide dismutase [20]. Nevertheless, though ROS has been shown to play an important role in aging, the underlying mechanism is not completely clear. Moreover, the possible interrelationships between the broad effects of ROS on genome instability and aging are still lacking.

Here, we used *S. cerevisiae* with various mutations in a biological network supposed to prevent deleterious effects of endogenous ROS to test the response of such a network to oxidative DNA damage. The potential relationship between biological network governing DNA integrality including DNA repair and checkpoint signaling and cell aging was also studied.

Materials and methods

Strains and growth conditions

The strains used in this study were all isogenic to the S288C strain RDKY3615 (*MATa*, *ura3-52*, *leu2Δ1*, *trp1Δ63*, *his3Δ200*, *lys2ΔBgl*, *hom3-10*, *ade2Δ1*, *ade8*, and *hxt13::URA3*). The relevant genotypes of these strains were listed in Table 1. All the strains used were kindly provided by Dr. Kolodner [6, 21], except that *ctt1::TRP1*; *sod2::TRP1*; *srx1::TRP1*; *apn1::TRP apn2::HIS3* were

Table 1 *CAN1* gene mutation rates of different mutants under normal cultured conditions

Strain	Gene deleted	Relevant genotype	Can^r rate[a] (10^{-6})	WT gene function
RDKY3615	WT		1.50 ± 0.12	
SDULY01	*SOD2*	*sod2::TRP1*	35.46 ± 4.16	Superoxide dismutase
SDULY02	*CTT1*	*ctt1::TRP1*	1.78 ± 0.13[b]	Catalase
RDKY5502	*TSA1*	*tsa1::KAN*	45.1 ± 3.37	Thioredoxin peroxidase
SDULY03	*SRX1*	*srx1::TRP1*	351700 ± 55500	Sulphiredoxin
SDULY04	*APN1APN2*	*apn1::TRP apn2::HIS3*	7.74 ± 1.17	BER
RDKY3812	*MSH2*	*msh2::KAN*	20.66 ± 2.28	MMR
RDKY3636	*RAD51*	*rad51::HIS3*	14.90 ± 1.89	Recombinational repair
RDKY5517	*RAD18*	*rad18 ::KAN*	3.52 ± 0.45	Postreplication repair
RDKY3725	*MEC3*	*mec3::HIS3*	16.97 ± 2.24	Checkpoint
RDKY3633	*MRE11*	*mre11::HIS3*	5.61 ± 0.95	Recombinational repair, checkpoint
RDKY3735	*MEC1*	*sml1::KAN mec1::HIS3*	8.49 ± 1.04	Checkpoint
RDKY3731	*TEL1*	*tel1::HIS3*	2.58 ± 0.36	Checkpoint
RDKY3745	*CHK1*	*chk1::HIS3*	1.74 ± 0.09[b]	Checkpoint
RDKY3749	*RAD53*	*sml1::KAN rad53::HIS3*	6.01 ± 1.01	Checkpoint
RDKY3739	*DUN1*	*dun1::HIS3*	2.27 ± 0.32	Checkpoint
RDKY3727	*RFC5-1*	*rfc5-1*	4.10 ± 1.45	Checkpoint
RDKY4538	*DPB11-1*	*dpb11-1*	1.82 ± 0.19	Checkpoint
RDKY3813	*SGS1*	*sgs1::HIS3*	1.71 ± 0.07[b]	Checkpoint

[a] Mutation rates shown were the average of three independent experiments and were presented as ±SD

[b] Statistical analyses indicated significant differences between these mutants and WT based on Student's *t* test. $P < 0.05$

generated using gene disruption methods [22]. Yeast strains were grown in standard media including yeast extract/peptone/dextrose (YPD) medium or synthetic complete medium (SC) lacking the appropriate amino acid and incubated at 30°C. Canavanine-resistant mutants (Can^r) were selected on SC arginine dropout plates containing 60 mg/l canavanine. Transformation of yeast was performed using a standard lithium/poly (ethylene glycol) method [23].

Mutation rates

The rate of accumulation of Can^r mutations in cell populations was determined as described [6]. Briefly, yeast cells were inoculated in SC media and grown to log phase, aliquots were retrieved and plated on SC arginine dropout plates containing 60 mg/liter canavanine and YPD media respectively. The mutation frequencies were calculated as the number of mutant cells on canavanine plates divided by the total number of colonies on YPD plates. Each experiment was repeated at least three times.

Sensitivity to different concentrations of H_2O_2

Yeast cells were inoculated in SC media and grown to log phase. Cells were harvested, washed once and resuspended to the same volume in sterile water. Cells were then treated with 0, 1, 2 and 5 mM of H_2O_2 for 1 h at 30°C, harvested and washed with water. Cells were plated on YPD media. Cell viability was calculated as the percentage of cells retaining the ability to form colonies after treatment divided by those without treatment [24]. Each experiment was repeated at least three times.

Yeast chronological life span

Yeast cells were cultured with continuous shaking at 30°C in 50 ml of SC medium. Reproductive survival was quantitatively measured by plating stationary phase cells on solid YPD medium every 3 days. The numbers of colonies were counted after 36 h of growth at 30°C. Cell density (OD 600) was measured at the same time using ultraviolet spectrophotometer. The chronological life span was calculated by dividing the total cells (1OD equals 10^7 cells) by the number of colonies on YPD plates. Each experiment was repeated at least three times. To measure the metabolic activity, yeast cells ($\sim 2 \times 10^8$) were incubated with 1 ml of reaction buffer consisting of 50 μM FUN 1, 2% glucose, and 10 mM HEPES pH 7.5. The fluorescent conversion was determined using a Hitachi F-6500 fluorescence spectrophotometer with excitation wavelength at 470 nm and an emission wavelength at 535 and 580 nm. Data are expressed as the difference in the emission intensity at 580 and 535 nm over time [16].

Cell integrity assay

Yeast cells were grown in SC medium at 30°C with shaking. Aliquots of cell suspension (10 μl) were placed on cover slips and examined by using Nikon 80i microscopy with a 60 dipping objective. Images were collected with a cooled SNAP HQ^2 CCD camera. To check the integrity of cell membrane, yeast cells (1×10^7) were harvested, washed once and resuspended with wash buffer (1.2 M sorbital, 0.5 mM $MgCl_2$, 35 mM K_2HPO_4 pH 6.8). Lysing enzyme (200 μg/ml, Sigma) was added and incubated at 37°C for 2 h. The cells were then harvested, washed two times with binding buffer and resuspended with binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). 50 μl (about 1×10^6) of cell suspensions were retrieved and incubated with 2 μl of PI (Clonetech) (50 μg/ml), at room temperature for 5–15 min in the dark [25]. Samples were analyzed using a BD Biosciences FACSCalibur and WinMDI29 software.

Cell wall sensitivity to SDS and zymolase

Yeast cells (3×10^8) were harvested and quickly washed with 15 ml of H_2O. Cells were then resuspended in 10 ml of hypotonic buffer (10 mM HEPES pH 7.5) supplemented with 0.1% SDS or 0.6 mg/ml zymolase and incubated at 30°C with occasional agitation. OD600 was measured using ultraviolet spectrophotometer every 1 h [26]. Each experiment was repeated at least three times.

Results and discussion

Mutations in cellular antioxidation, DNA repair and DNA damage checkpoints increase Can^r rates in response to oxidative stress

A biological network involving not only DNA repair, but also DNA damage and replication checkpoints has been identified to prevent the deleterious effects of endogenous reactive oxygen species [10]. In accordance with previous results [6, 21], mutations in selected genes of antioxidation, DNA repair and checkpoints all resulted in elevated mutation rates as compared with the WT, though to different degrees (Table 1). Specifically, mutation rates with deletion of genes for antioxidant and DNA damage repair components were overall higher than those of deletions of DNA damage checkpoints. As compared with *chk1* and *sgs1* checkpoint mutants, the Can^r rate of *sod2*, *tsa1* and *srx1* mutants as well as *apn1apn2*, *msh2*, *rad51* mutants were especially higher than that of WT. These observations indicate that, in the absence of acute doses of external oxidants, ROS scavenging and inactivating components

such as peroxiredoxins and DNA repair system including those of MMR and BER constitute a major defense system preventing the accumulation of mutations in the genome. To further investigate the impact of exogenous H_2O_2 treatment on these mutants, exponential growing cells were treated with different concentrations of H_2O_2 (Fig. 1). Results showed that all tested mutants were more sensitive to H_2O_2 than WT displaying a lower survival rate under all concentrations of H_2O_2. Deletions of *CTT1*, *TSA1*, *SRX1* (encoding antioxidant system components); *APN1APN2* (BER), *SGS1* (encoding DNA replication stress sensors); and *MEC1*, *MRE11*, *TEL1*, *RAD53*, *DUN1* (DNA damage

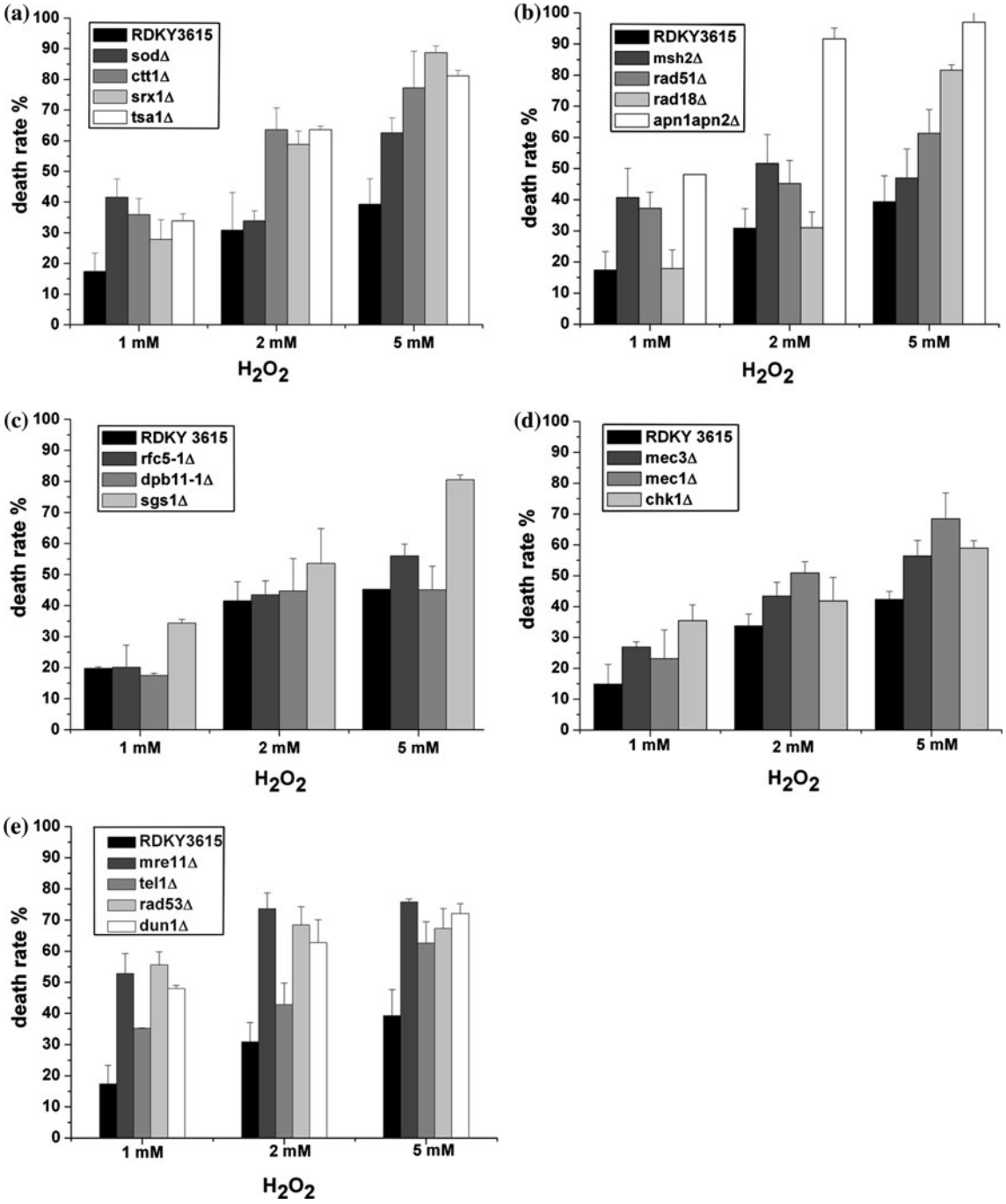

Fig. 1 Sensitivity of mutants with deletion of genes for **a** cellular antioxidant; **b** DNA damage repair; **c** S-phase DNA damage checkpoints; **d** Mec1 pathway of DNA damage checkpoints; **e** Tel1 pathway of DNA damage checkpoints, to different concentrations of H_2O_2. Yeast cells were allowed for growing to log phase in SC medium and treated with 0, 1, 2 and 5 mM of H_2O_2 for 1 h at 30°C. Death rates were the average of at least three independent experiments. *Error bars* indicate the standard deviation of at three repetitions

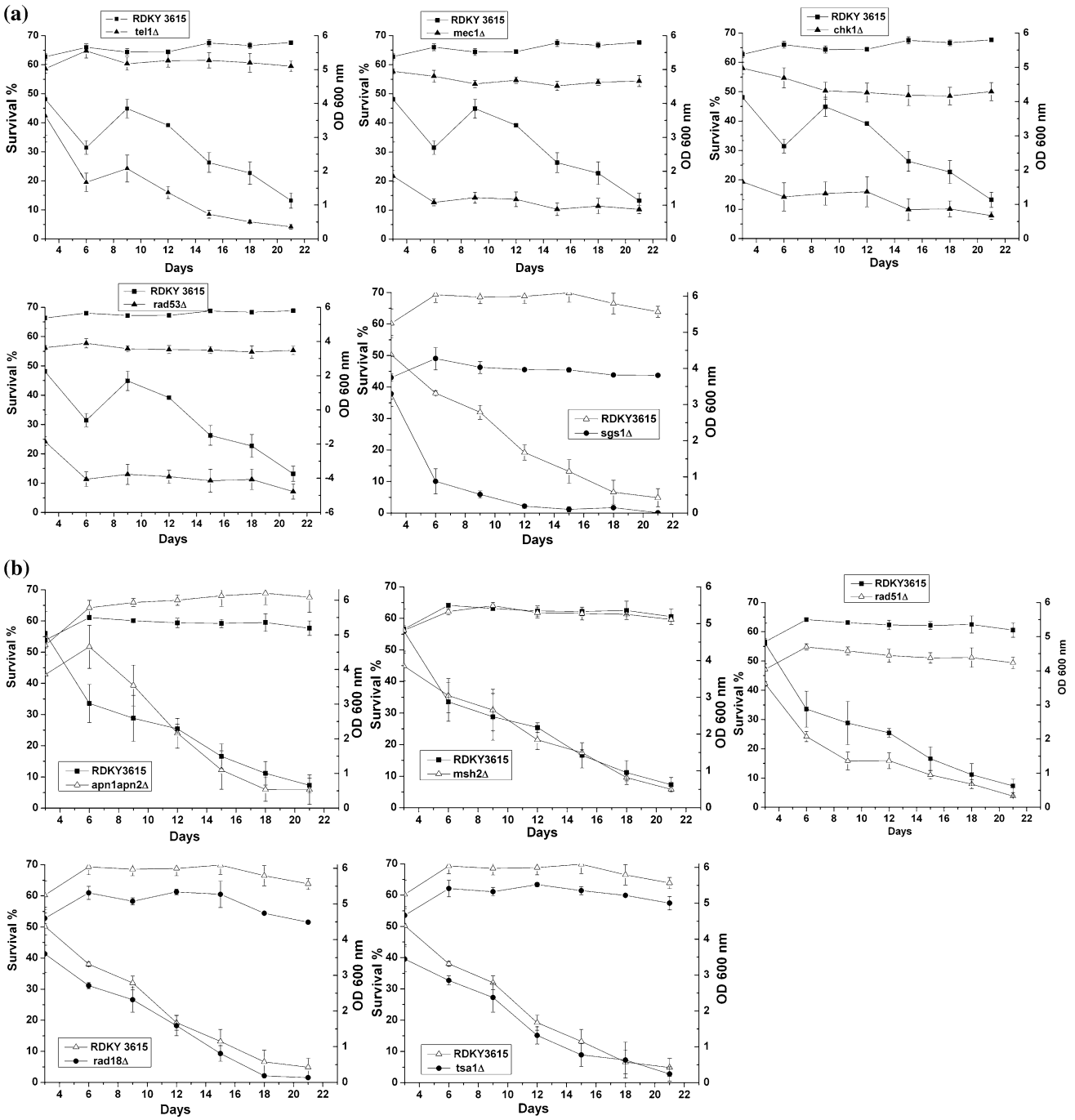

Fig. 2 Chronological life span of mutants as determined by colony formation for **a** DNA damage and DNA replication checkpoint mutants; **b** DNA repair mutants and antioxidant mutants. Yeast cells were cultured at 30°C in liquid SC medium. Reproductive survival was measured by plating stationary phase cells on solid YPD plates. The OD600 of cells was measured using ultraviolet spectrophotometer every three days. Survival rates and number of total cells were the average of three independent experiments. *Error bars* represent ±SD

and replication stress signal transduction kinases or kinases that function downstream of Mec1) inflicted a more profound effect on the survival of mutants with the lowest survival less than 10% for *apn1apn2* at 2 mM of H_2O_2. These results again indicated the importance of the identified biological network in governing DNA integrity and thus the viability under oxidative stress.

Defects in DNA damage and replication checkpoints result in shortened chronological life span

Saccharomyces cerevisiae has been used as a model system to study mechanisms controlling the aging process. To investigate the impact of compromised DNA integrity on the chronological life span of yeast, chronological life

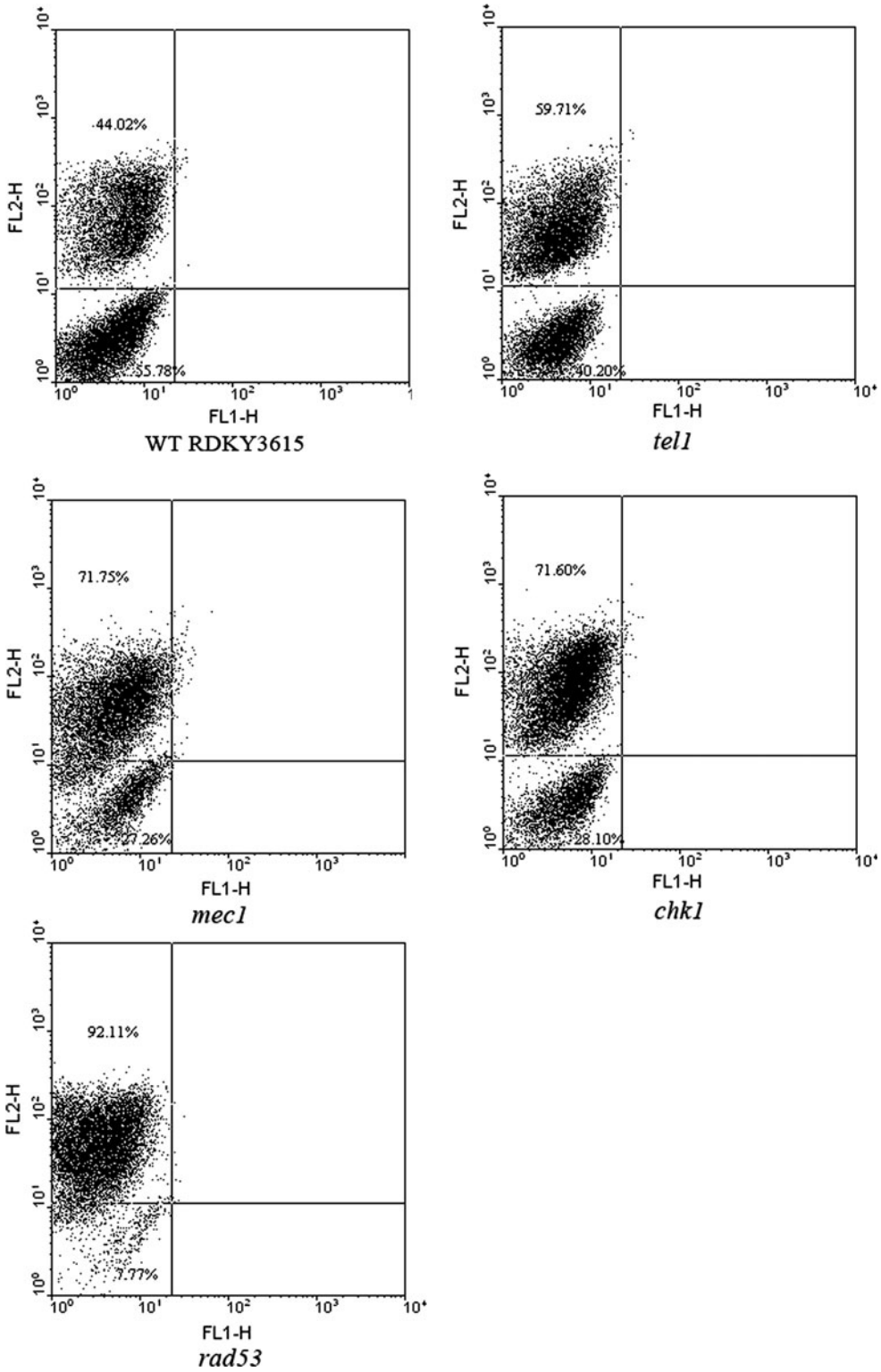

Fig. 3 FACS analysis of DNA damage checkpoint mutants on day 9 of cultivation. Yeast cells cultured for 9 days in SC medium were stained with 50 μg/ml propidium iodide (PI) before analysis by flow cytometry. The results presented were representative experiments of at least three repetitions

spans of mutants with deletions of genes involved in DNA repair of oxidative DNA as well as those checkpoint responses were measured by checking their colony forming ability. As shown in Fig. 2, the plating efficiency of mutants with deletion of DNA damage repair genes including *apn1apn2*, *msh2* and *rad18* was indistinguishable from that of WT. Deletion of *rad51* or *tsa1* resulted in moderately reduced plating efficiency (Fig. 2b). In contrast, the checkpoint mutants showed dramatically reduced plating efficiency, thus shortened chronological life span

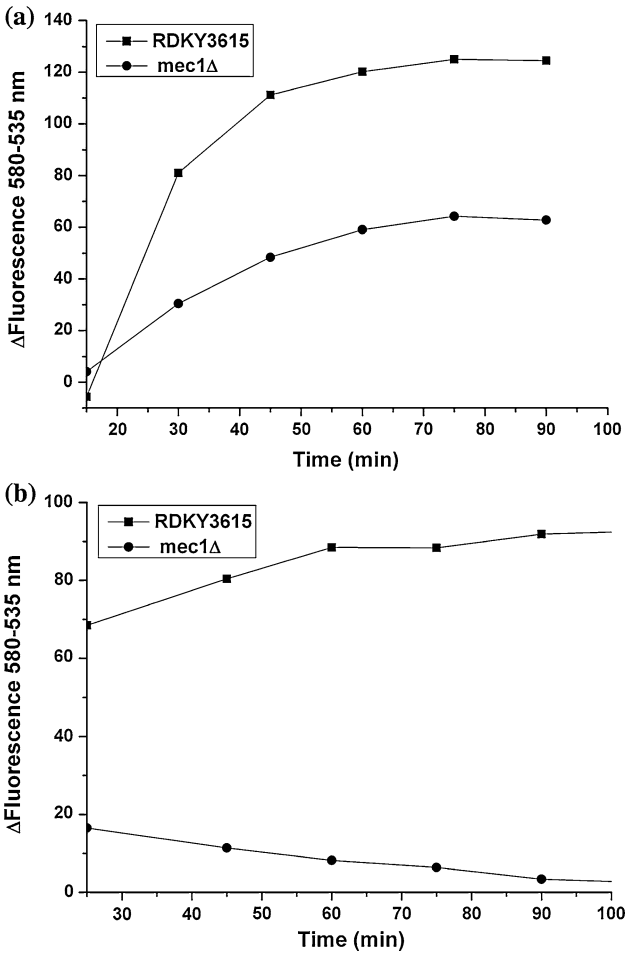

Fig. 4 Metabolic activity of *mec1* mutant cells was lower than wild-type cells cultured on day 3 (**a**) and day 9 (**b**). WT and *mec1* mutant cells were grown for 3 or 9 days in SC medium before they were incubated with FUN1. The difference between metabolized FUN1 fluorescence (living cells) and unmetabolized fluorescence (dead cells) was measured and plotted over time until stable levels were obtained. The results presented were a representative of at least three repetitive experiments

compared to WT yeast, and the most severe phenotype was observed in *sgs1* mutant (Fig. 2a). The loss of viability in chronologically aging yeast was also determined by propidium iodide (PI) staining, which is a reporter of plasma membrane integrity, and analyzed by FACS (Fig. 3). Much higher number of PI stained mutant cells including *mec1*, *chk1* and *rad53* were obtained than WT, suggesting that a higher percentage of the checkpoint mutant population had lost plasma membrane integrity. Correspondingly, results of FUN1 staining demonstrated that the metabolic activities in aged mutant cells were dramatically lower than those in WT yeast (Fig. 4). Taken together, these results suggest that, compared with potential elevated levels of unrepaired, spontaneous DNA damage resulting from the absence of specific DNA repair components, the absence of checkpoint mechanisms may have more significant effect on the aging process probably because they participate in coordinating the various important cellular processes which may include DNA replication and cell division. The relatively shorter life span of *rad51* mutant may also well be explained by its potential role in DNA replication [27].

Chronologically aged yeast cells with mutations in DNA checkpoints are morphologically intact though incapable of proliferating

Although mutant yeasts rapidly lost viability as measured by colony formation, the number of mutant cells remaining morphologically intact as determined by absorbance at OD600 or under microscope was hardly discernable from that of WT even though the more aged cells, both mutants and WT, appeared as weakly refractive "ghost" cells in phase microscopy, indicative of loss of plasma membrane integrity (Figs. 2, 5). It is well known that, while both *cdc13-1* alone and *cdc13-1mec1* rapidly lose viability as measured by colony formation, the double mutant cells apparently retain cell integrity [28]. The similarity between the above mutants and *cdc13-1mec1* double mutant indicates that the inability of the aged mutants to regrow in fresh culture may result from loss of proper surveillance and ensuing responses to guard DNA integrity by the checkpoints, and that a different cell death pathway may exist for these mutants.

Cell wall fragility may account for the shortened chronological life span in yeast cells with mutations in DNA checkpoints

It is generally accepted that elevated levels of genome DNA damage and thus the compromised DNA integrity are one of the major causes of aging [19, 29, 30]. To ask whether there is a direct link between unrepaired, spontaneous DNA damage and the aging process, we first determined the relative levels of mutations in WT and various mutants. As shown in Fig. 6, the mutation rates of yeasts with deletion of genes for DNA repair system were much higher than those in mutants with the absence of DNA damage or DNA replication checkpoints which, however, displayed much shorter life span. These results indicate that, compared to the elevated, sporadic mutations resulting from the absence of specific DNA repair system, loss of DNA damage and replication checkpoint mechanisms would be prone to have more significant effects on various cellular processes including aging. In this respect, it has been reported that there is a narrow threshold of DNA damage that needs to be exceeded for DNA damage checkpoint activation so that replication could be allowed in the presence of unrepaired DNA damage [31]. On the other hand, there would be a progression of deleterious

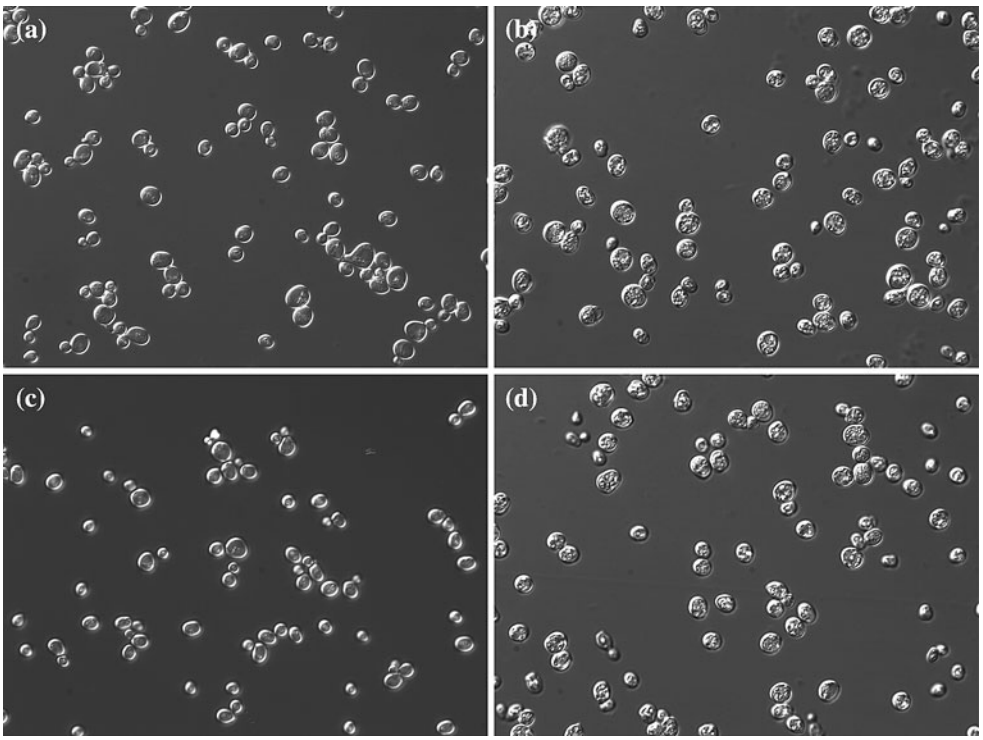

Fig. 5 Aged yeast cells remained morphologically intact. Cell images of WT (**a** and **b**) and *mec1* mutant cells (**c** and **d**) cultured in SC medium for 24 h (**a** and **c**) and 21 days (**b** and **d**) were obtained by microscopy. *Bars* 20 μm

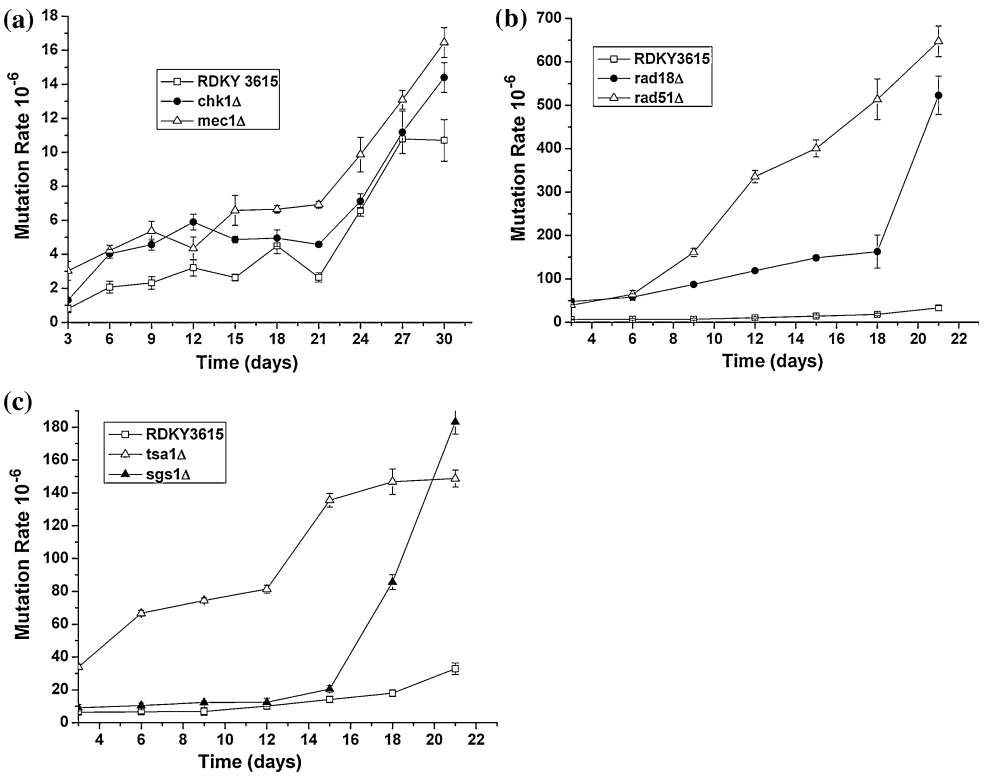

Fig. 6 Mutation rates with aging were higher in various mutants than in WT. **a** DNA damage checkpoint mutants; **b** DNA repair mutants; **c** antioxidant and DNA replication checkpoint mutants. Yeast cells were allowed for growing in SC medium. Mutator phenotypes were analyzed by the *CAN1* forward-mutation assay every 3 days. Mutation rates were the average of three independent experiments. *Error bars* represent ±SD

consequences beginning with genetic instability followed by other biological abnormalities if replication carries on in the absence of relative checkpoints.

Loss of cell wall integrity has been proposed to be one reason for the inability of a cell to continue proliferation in new environments [32]. Mutants in cell wall integrity

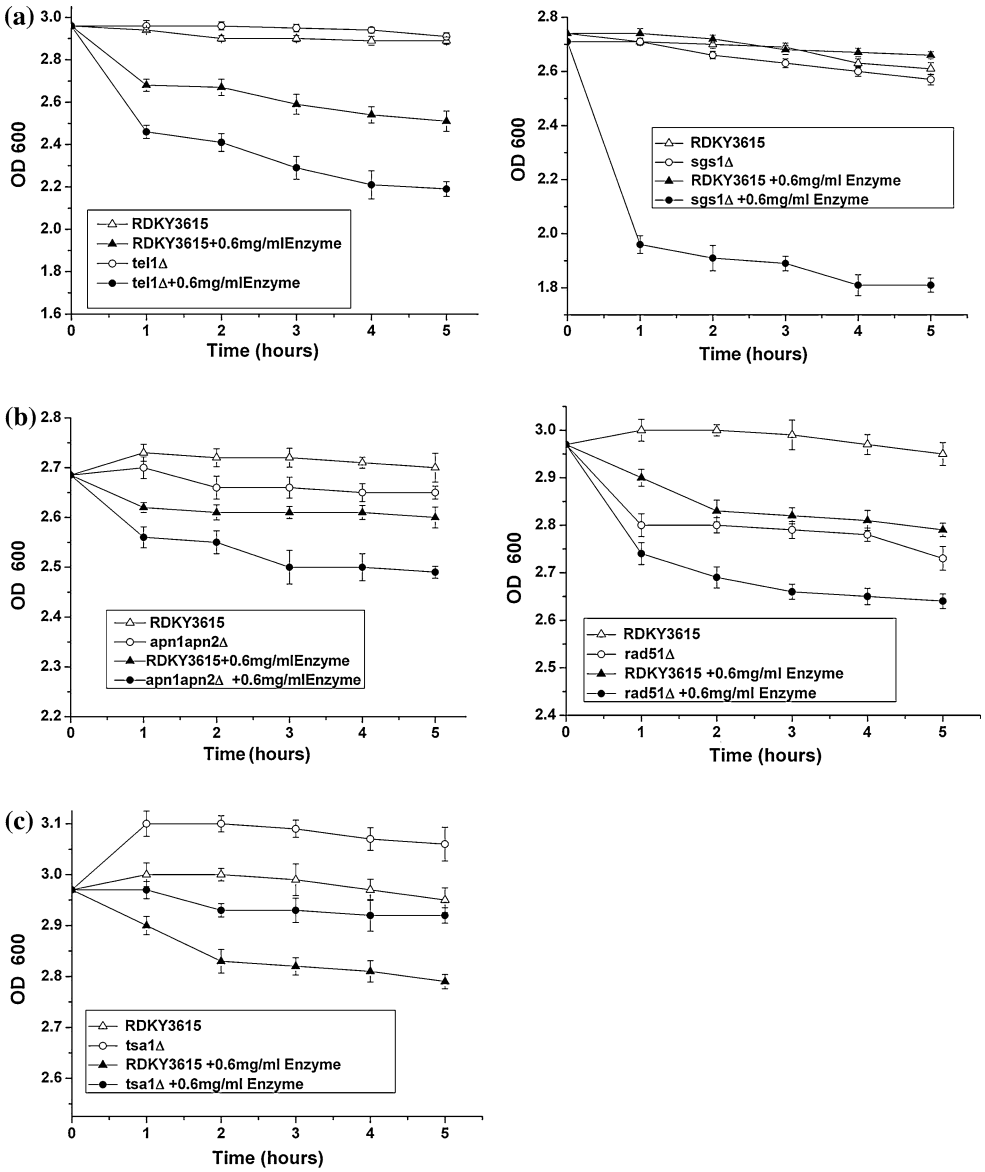

Fig. 7 Sensitivity of various mutants to lysing enzyme treatment. **a** DNA damage and DNA replication checkpoint mutants (*tel1*, *sgs1*); **b** DNA repair mutants (*apn1apn2*, *rad51*); **c** antioxidant mutants (*tsa1*). Cells entering stationary phase were treated either with 0.1% SDS or with 0.6 mg/ml of lysing enzyme by being incubated at 30°C with occasional agitation. OD600 was measured using ultraviolet spectrophotometer every 1 h. Each experiment was repeated at least three times. *Error bars* represent ±SD

pathway have thus been found to display a short life span with weakened cell wall [33]. Moreover, a direct link between DNA checkpoints and cell wall checkpoints has also been suggested based on the finding that the timely degradation of Swe1 was regulated by DNA checkpoints. Mutants with cell wall checkpoint defect were found to be more sensitive to zymolase treatment on perturbation of replication [26]. To see whether the cell wall would be affected in chronologically aged mutant cells, we tested the sensitivity of cells entering the stationary phase to SDS or lysing enzyme treatment (Fig. 7 and data not shown). In contrast to WT and mutants with DNA repair defect, mutants with deletion of genes for checkpoint components was relatively more sensitive to SDS or lysing enzyme treatment, which were in accordance with the results of chronological life span determination. These results therefore suggest that the observed shorter chronological life span of DNA damage and replication checkpoint mutants probably results from the inappropriate modulation of cell wall morphology checkpoint pathway due to the absence of DNA checkpoint activation, which then caused the abnormality in cell wall structure. The results also suggest that malfunction of cellular responses to oxidative DNA damage may not only predispose the cell to elevated DNA mutations, it may also further incur other biological abnormalities including perturbation in cell wall integrity resulting in a shortened chronological life span.

In conclusion, a previously identified biological network including cellular antioxidation, DNA repair and DNA damage checkpoints were confirmed to play an important role in preventing genomic instability in response to oxidative stress. Moreover, a potential relationship between

cellular responses to DNA damage, especially the checkpoint mechanisms and cell aging was also revealed. The chronological life span of mutants defective in DNA replication and DNA damage checkpoints was found to be shorter than that of WT cells as determined by colony formation, although the cell number was almost the same for WT and mutants. We infer that the observed incapability of mutant cells to proliferate may arise as a result of cell wall fragility due to the inappropriate modulation of the cell wall morphology checkpoint in the absence of relative DNA damage or replication checkpoints. Further study will need to establish the direct link between these two checkpoints and identify the key effectors involved.

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