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Yap1 and Skn7 genetically interact with Rad51 in response to oxidative stress and DNA double-strand break in *Saccharomyces cerevisiae*

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ABSTRACT

Reactive oxygen species (ROS)-mediated DNA adducts as well as DNA strand breaks are highly mutagenic leading to genomic instability and tumorigenesis. DNA damage repair pathways and oxidative stress response signaling have been proposed to be highly associated, but the underlying interaction remains unknown. In this study, we employed mutant strains lacking Rad51, the homolog of *E. coli* RecA recombinase, and Yap1 or Skn7, two major transcription factors responsive to ROS, to examine genetic interactions between double-strand break (DSB) repair proteins and cellular redox regulators in budding yeast *Saccharomyces cerevisiae*. Abnormal expression of *YAP1* or *SKN7* aggravated the mutation rate of *rad51* mutants and their sensitivity to DSB- or ROS-generating reagents. Rad51 deficiency exacerbated genome instability in the presence of increased levels of ROS, and the accumulation of DSB lesions resulted in elevated intracellular ROS levels. Our findings suggest that evident crosstalk between DSB repair pathways and ROS signaling proteins contributes to cell survival and maintenance of genome integrity in response to genotoxic stress.

1. Introduction

The generation of DNA lesions leading to cell death is the fundamental strategy underlying cancer treatment by radiotherapy and certain chemotherapies [1]. Although a substantial proportion of DNA damage that makes cells lethal is attributed to single-strand breaks (SSBs) and double-strand breaks (DSBs), oxidative damages produced by endogenous reactive oxygen species (ROS) and oxidizing chemicals are also significant sources of detrimental base modifications and mutagenesis that might eventually lead to potential tumorigenesis [2–4].

DNA damage repair pathways and oxidative stress signaling have been proposed to be highly associated and exhibit mutual causality. The oxidative DNA adducts resulting from ROS typically includes modified bases, abasic DNA sites and occasional SSBs, all of which are considered non-lethal but highly mutagenic, and are efficiently processed by base excision repair (BER) and to a lesser extent nucleotide excision repair (NER) [4–6]. Conversely, it has also been reported that increased DNA damage per se causes increases in intracellular ROS [7,8]. BER- and NER-defective cells are genetically unstable and highly mutagenic with greatly increased ROS levels [7,9]. DNA damage signaling triggered by non-oxidative alkylating agent is mediated by Yap1, a transcription activator specifically involved in oxidative stress response and redox homeostasis in yeast, suggesting that ROS signaling is interconnected with the DNA damage response [10]. It has been posited, however, that complex and lethal lesions such as DSBs are not easily induced by DNA oxidation unless simultaneous attack of DNA by a very high concentration of hydroxyl radicals causes two neighboring SSBs elaborately in close proximity [4].

It has been reported that the ROS-generating xenobiotic phenytoin increases both the DNA oxidation and homologous recombination (HR) required for DSB repair in a Chinese hamster ovary (CHO) cell line, and that the rate of DNA recombination in yeast cells stimulated by the human leukemogen benzene is diminished by *N*-acetylcysteine, a free radical scavenger, supporting the idea that ROS-induced DNA damage could be recombinogenic [11,12].

Yeast has been used as a prominent model organism to assess the biological consequences of cytotoxicity mediated by ROS and unrepaired DNA damage in higher organisms. HR-mediated DSB repair pathways mediated through Rad52 epistasis group proteins in yeast are highly conserved in all forms of complex life studied to date [13]. Studies delineating the relationships between proteins in pathways of ROS signaling and of HR in yeast would provide useful clues for the identification of novel candidate therapeutic targets in human cancers and for understanding their mechanisms of action [14–16].

A global genetic analysis of synthetic fitness or lethality defect (SFL) interactions in yeast revealed that mutations in five genes required for oxidative stress response (*TSA1*, *SOD1*, *LYS7*, *SKN7*, and *YAP1*) impaired growth of HR pathway mutants; interestingly, all of these genes play a significant role in suppression of mutagenesis [17,18]. Among these, Tsa1, the most potent peroxiredoxin that scavenges H_2O_2, is also the most significant contributor to genome stability with a severe mutator phenotype seen in the *tsa1* mutant [17]. Defects in Rad51-mediated DSB repair by HR and Rad6-mediated postreplicative repair (PRR) cause synthetic lethality in the absence of Tsa1 [19]. Moreover, SOD1 (Superoxide dismutase 1) inhibition has been proposed as a promising approach for selective killing of cancer cells and synthetic lethal interaction between yeast *rad54* and *sod1* has been shown to be conserved within a human colorectal cancer (CRC) context based on the observation that DNA damage resulting from an increase in ROS following SOD1 inhibition persists within *RAD54B*-deficient cells and induces apoptosis [20,21]. Despite the results above, the underlying mechanism of action by which ROS signaling and DSB repair through HR are interconnected remains unclear. Instead of creating *rad51 sod1* and *rad51 tsa1* double mutants that are lethal in aerobic growth conditions, we introduce *RAD51* deletions into mutant strains that lack Yap1 or Skn7, which are two major transcription factors that modulate large oxidative stress response regulons including the *TSA1*, *TRX2*, and *TRR1* genes in yeast [22]. Cells inactive in either Yap1 or Skn7 are hypersensitive to H_2O_2 and superoxide generators such as paraquat [23,24]. Induction of many antioxidant genes by oxidative stress is nearly equally affected by deletion of *YAP1* or *SKN7*, although a *yap1 skn7* double mutant has almost the same phenotype as does either single mutant, with no additional effect of mutation of the second gene [25,26].

Here, we report that Rad51, a crucial player of recombinational repair, acts in concert with Yap1 and Skn7 in cellular responses to genome instability caused by both DNA damage and oxidative stress. Aberrant expression of *YAP1* or *SKN7* aggravates mutation rates and sensitivity of *rad51* mutants to DSB- and ROS-generating reagents. Rad51 deficiency contributes to elevated accumulation of DSB lesions in response to increased ROS levels, and the ensuing genomic instability increases the intracellular ROS levels, leading to relocalization of Yap1 to the nucleus. Collectively, these results suggest a specific crosstalk between DSB repair pathways and ROS signal transducing proteins, contributing to cell survival and maintenance of genome stability in the presence of genotoxic stress.

2. Materials and methods

2.1. Yeast strains, plasmids and growth media

All of the strains used in this study are isogenic derivatives of *S. cerevisiae* BY4741 (*MAT*a *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) obtained from the Yeast Knockout (YKO) collection (YSC1053 glycerol stock, Thermo Scientific) unless noted otherwise. The strains with C-terminally GFP-fused proteins were constructed by oligonucleotide-directed in-frame tagging method as previously described [27]. The genotypes of all strains are listed in Supplementary Table 1. Yeast cell cultures and treatments with DNA damaging agents, ROS-generating reagents, and antioxidants were all performed in standard rich YEPD media or minimal SD media supplemented with the required amino acids. All

cultures were incubated at 30 ºC. Overexpression plasmids of the wild-type *YAP1* and *SKN7* genes were created using pRS426, which includes an *ADH1* promoter [28]. The ORF regions of C-terminally TAP-tagged *YAP1* and *SKN7* were PCR-amplified and cloned into the *Spe*I/*Xho*I and *Hin*dIII/*Xho*I sites of pRS426ADH, respectively, and transformed into WT yeast and each mutant strain. Overexpression of Yap1 and Skn7 was confirmed by Western blot analysis.

2.2. Drug sensitivity analysis

The drug sensitivity of yeast cells was measured via spotting assays. For these assays, cells were grown overnight at 30 ºC, and the culture was re-inoculated into fresh media and grown in 5 ml YEPD or SD liquid media to reach approximately $3–4×10^7$ cells/ml, then diluted 10-fold serially and spotted in rows onto YEPD or SD plates containing the selected chemicals. The plates were incubated for 2–3 days at 30 ºC and then photographed. For experiments involving exposure to the ROS/DSB-inducing agents, paraquat (PQ), hydrogen peroxide (H_2O_2), phleomycin (PHL), hydroxyurea (HU), or methyl methanesulfonate (MMS) were included in the plates before spotting.

2.3. Measurement of cell viability and mutation frequency

Yeast cells were inoculated into 5 ml of YEPD media and grown overnight at 30 ºC. On the following day, cells were diluted into 5 ml of fresh media to an optical density at 600 nm (OD_{600}) of ~0.2 and incubated with shaking for 6 h. PHL or H_2O_2 was added at the indicated final concentrations, followed by incubation with shaking for 2 h. The number of cells was estimated for each population using a hemocytometer, and the viability and mutation rate of cells were determined. To determine viability under several conditions, a colony forming unit (CFU) assay was used. One hundred cells from different cultures were plated onto three YEPD plates. The plates were incubated at 30 ºC for 2–3 days and then colonies were counted. The rate of spontaneous mutations as a result of genome instability caused by drug treatment was determined by a forward-mutation assay that detects mutations in the *CAN1* gene [29]. The yeast *CAN1* gene encodes an arginine permease that is normally required for arginine uptake but is also able to transport canavanine, a toxic arginine analog. Cells with loss-of-function mutations in the *CAN1* gene locus can form colonies on canavanine-containing SD plates. Yeast cells were treated with PHL or H_2O_2 for 4 h at the indicated concentrations and then were grown on plates with or without 60 µg/ml canavanine. Spontaneous mutation rates were determined by counting CFUs after incubation for 3–4 days at 30 ºC. All rates represent the average of three independent experiments and error bars indicate the standard deviation.

2.4. Fluorescence microscopy

Fluorescence microscopy was carried out on a Nikon Eclipse Ti inverted microscope. Image analysis was performed using NIS-Elements AR3.1 microscopy software (Nikon) in order to determine the percentage of cells with subnuclear foci or predominately nuclear fluorescence. To detect nuclear foci of Rad52-GFP or Yap1-GFP, yeast cells were treated with PHL or H_2O_2, and then further incubated with shaking for the indicated time periods in YEPD before being photographed. At least 100 cells were counted at least three times for each measurement.

2.5. Measurement of intracellular ROS level

Yeast cells were grown to mid-log phase and diluted to an OD_{600} of ~0.2. After treatments with MMS, PHL, or H_2O_2 for 2 h, H2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) (excitation/emission: 492–495/517–527 nm, ThermoFisher Scientific) a cell-permeant indicator of ROS detection, was added at a final concentration of 5 µg/ml,

and then cells were incubated with shaking for 2 h at 30 °C. Intracellular ROS levels were determined using a BD FACS Canto II flow cytometer (Becton Dickinson) as previously described [30]. A baseline of zero (background level of fluorescence) was set based on the maximum value of control sample without the ROS indicator. The cells with higher ROS level than background were counted and converted into a percentage [31]. Trypan blue staining was performed to discriminate between viable and non-viable cells presenting high-ROS signals.

2.6. Gel electrophoresis and Western blot analysis

To detect TAP-tagged Yap1 and phosphorylated Rad53, cell extracts were prepared by suspending cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.2% NP-40) including protease and, only for phosphorylated Rad53 detection, phosphatase inhibitors (WSE-7420, ATTO), followed by bead-beating. Extracts were centrifuged at 1600g for 10 min at 4 °C, and the supernatants were subjected to SDS-PAGE. Western blot analysis was performed by standard methods with anti-Rad53 antibody (ab104232, Abcam), HRP-conjugated anti-rabbit secondary antibody (sc-2004 HRP, Santa Cruz Biotechnology), and anti-Pgk1 antibody (ab154613, Abcam) as loading control.

2.7. Cell cycle progression analysis and doubling time determination

Exponentially growing cells under normal conditions or with drug treatments were examined by light microscopy to count and classify them into G1 (unbudded), S (small budded), or G2/M cell cycle phase (large budded). The ImageJ program developed at the NIH was used for image processing and analysis. All cell cycle experiments were performed at least three times with at least 300 cells. For determination of doubling times of yeast cells, cultures in liquid YEPD media were used. OD_{600} of yeast cultures measured by spectrophotometry at different time points during the exponential growth phase was plotted and the time required to duplicate the optical density was calculated.

3. Results

3.1. Genetic interactions between ROS response and DSB repair pathway

To study the genetic interactions between oxidative stress response and DNA damage repair pathway by HR, we constructed combined mutant strains lacking Yap1 or Skn7, two crucial transcription factors that cooperate in response to redox stress signals, and Rad51, a recombinase required for homology search and strand exchange during HR [18,32], followed by examination of the sensitivities of these strains to various ROS-generating chemicals and DNA damaging agents. The *yap1* and *skn7* mutants were sensitive to PQ, a superoxide radical generator, and H_2O_2 but not to DNA damaging agents such as HU, MMS, and PHL (Fig. 1A). The *rad51* mutant was sensitive to all tested DNA-damaging agents and also showed sensitivity to PQ equal to that of *yap1* or *skn7* mutant and considerable sensitivity to a relatively high dose of H_2O_2, indicating that oxidative stress causes DSB lesions that require a Rad51-mediated HR pathway for repair. Interestingly, the *rad51 yap1* and *rad51 skn7* double mutants displayed much more sensitive phenotypes than either of single mutants in response to both oxidative stresses and DNA damaging agents and revealed more than just additive effects in their sensitivities suggesting those two damage response pathways are not independent (Fig. 1A). These findings can be interpreted to indicate that Yap1 and Skn7 are involved in the response to DSBs or the repair of such in conjunction with Rad51. When the growth of each mutant strain was investigated under normal condition, the estimated doubling times of *rad51 yap1* and *rad51 skn7* double mutants were much longer than that of WT about ~35% and ~50%, respectively (Fig. 1B). The double mutant cells also showed

large-budded shapes with the mother and a similarly-sized daughter under normal growth condition, indicative of transient cell cycle arrest at G2/M phase (Supplemental Fig. 1). These phenotypes of double mutants seem to emerge synthetically since they are not shown in any of single mutants. These observations suggest that Yap1 and Skn7, the ROS response signaling factors, genetically interacts with HR-mediated DSB repair protein, Rad51.

3.2. Rad51 deficiency exacerbates genomic instability in response to increased ROS

To investigate the biological functioning and maintenance of genome stability of each mutant strain in the context of various drug treatments, we measured changes in viability and mutation rates when cells were treated with PHL or H_2O_2 up to the concentrations at which each mutant was barely viable (Fig. 2). The viability of *yap1* or *skn7* mutants in the presence of PHL was not much different from that of WT cells, but Rad51-deficient mutants were sensitive to transient PHL treatment at a concentration about five-fold lower than that required to affect WT cells (Fig. 2A and B). The *yap1* and *skn7* mutants showed greater sensitivity to H_2O_2 treatment than did WT cells, as was expected [19,20], but they became even more sensitive when Rad51 was compromised (Fig. 2C and D), suggesting that accumulation of DSBs is induced in response to exogenous ROS and becomes even more severe in the absence of Yap1 or Skn7.

The spontaneous mutation rates of *yap1* and *skn7* mutant strains, as determined by counting canavanine-resistant colonies, were ~3 times higher than those of WT cells (Fig. 2E and F), which is consistent with the findings of a previous report [13]. Mutation rates greatly increased up to ~100 fold in both *yap1* and *skn7* mutants as well as in WT cells upon administration of 80 μg/ml PHL. However, the mutation rates of *yap1* and *skn7* mutants, which were 3 times higher than those of WT cells, were largely unaltered regardless of drug concentration (Fig. 2E). This implies that the elevated spontaneous mutation rates observed in *yap1* and *skn7* mutants are independent of PHL-mediated DSB formation, but instead are probably due to oxidative DNA adducts primarily processed by BER [10]. The mutation rate of *rad51* mutants was ~20 times higher than that of WT cells but was not altered by elevated concentrations of PHL, whereas *rad51 yap1* and *rad51 skn7* double mutants displayed additive phenotypes of each single mutant, with slight increases as a result of PHL treatment (Fig. 2F). These results indicate that PHL-induced mutagenesis is mainly dependent on Rad51-mediated repair by HR, and that accumulated DSBs might lead to higher sensitivity of cells to aberrant redox status specifically in the absence of Yap1 or Skn7. This also seems consistent with the data shown in Fig. 2G and H, as a gradual increase in mutation rate of *rad51* mutants was observed with elevated H_2O_2 concentration and the spontaneous mutation rate was even higher in *rad51 yap1* and *rad51 skn7* double mutants. Collectively, these results suggest that deficiency of Rad51 renders cells more sensitive to ROS challenge and to DSB lesions specifically when Yap1 or Skn7 is compromised.

3.3. Overexpression of YAP1 or SKN7 adversely affects the growth of rad51 mutant cells

The observations described above prompted us to investigate the effects of Yap1 and Skn7 overexpression. Previous studies have revealed that *YAP1* overexpression enhances resistance of yeast cells to a variety of toxic compounds, including ROS such as H_2O_2, *t*-butyl hydroperoxide, and thiol oxidants [19,33]. Surprisingly, however, we found that overexpression of *YAP1* or *SKN7* via the multicopy plasmid pRS426ADH aggravated the sensitivity of *rad51* mutants to H_2O_2, PHL, and MMS (Fig. 3A). Overexpression of *YAP1* almost completely rescued the impaired growth of *yap1* mutants following H_2O_2 treatment and alleviated the sensitivity of the *rad51 yap1* strain, albeit to a lesser extent, suggesting that elevated *YAP1* expression adversely
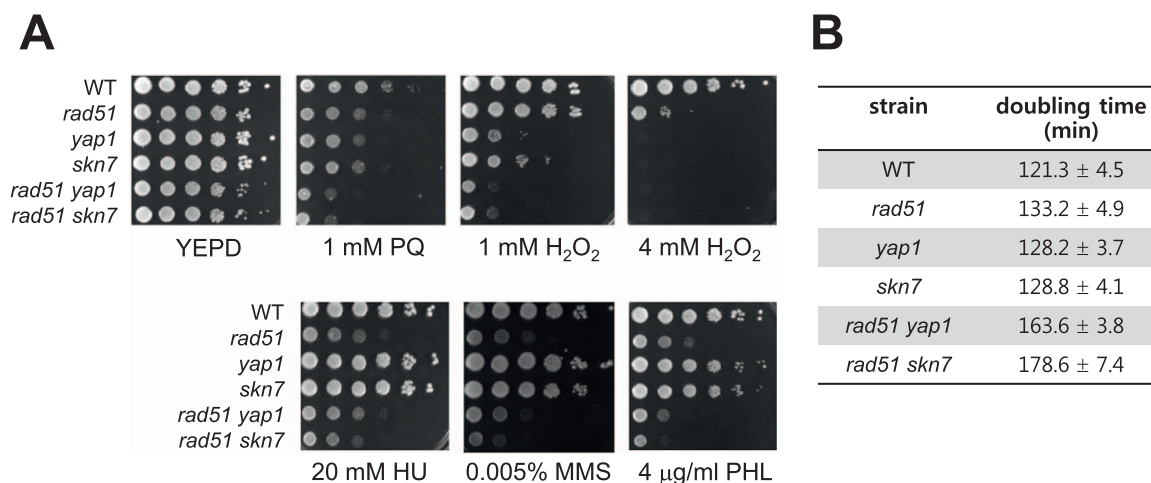
Fig. 1. ROS response signaling pathway genetically interacts with HR-mediated DSB repair pathway. (A) 10-fold serial dilutions of the indicated strains were spotted on YEPD media in the presence of drugs and incubated for 2 days at 30 °C. For the exposure to ROS- or DSB-generating reagents, paraquat (PQ), hydrogen peroxide (H_2O_2), hydroxyurea (HU), methyl methanesulfonate (MMS), or phleomycin (PHL) was added in the plates before spotting. (B) Doubling times of the indicated strains were calculated by spectrometry during the exponential growth phase. Averages and standard deviations from three independent experiments are presented.
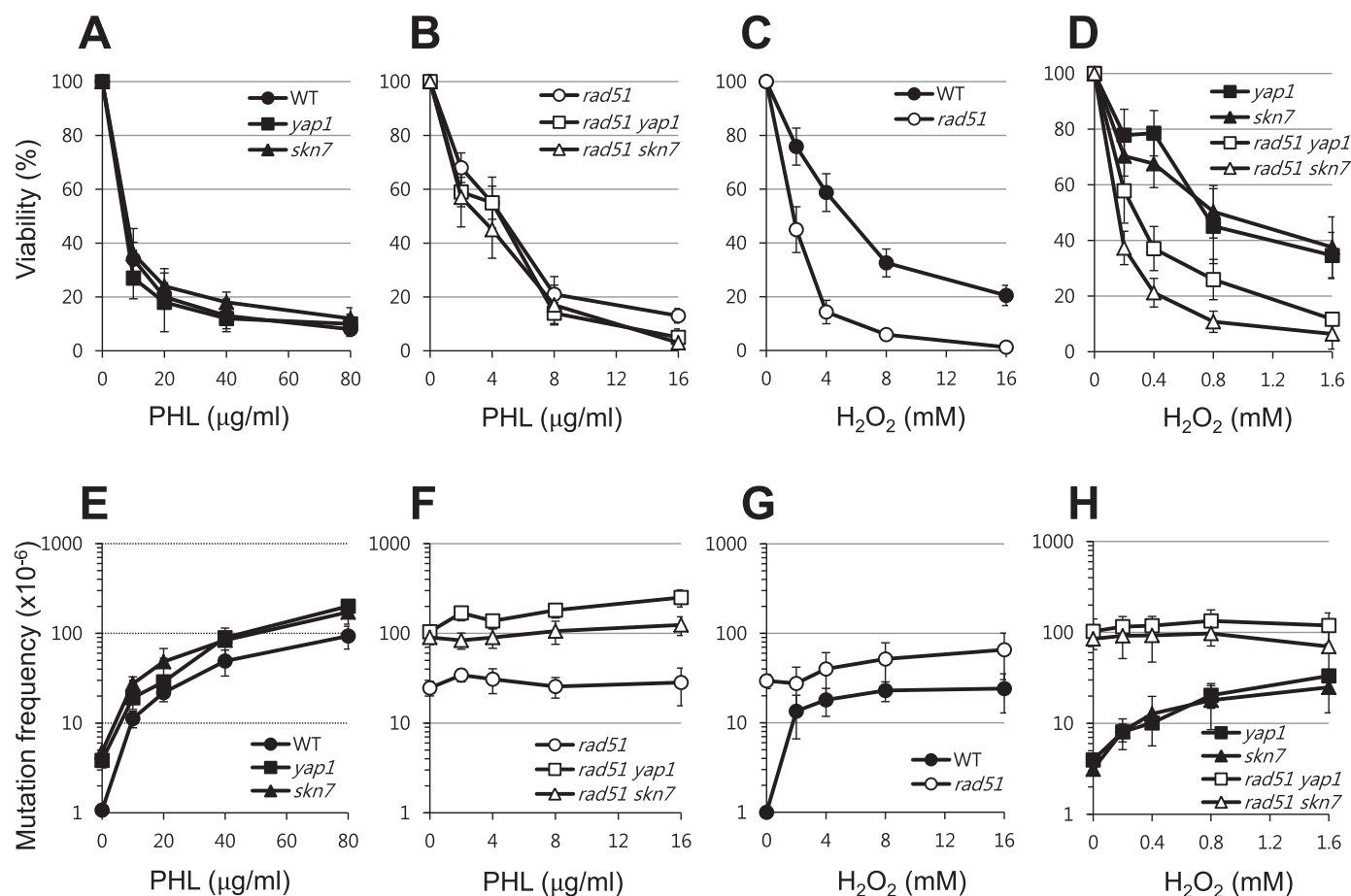

Fig. 2. The sensitivity and mutation rate of *yap1* or *skn7* mutant strains are affected by Rad51 deficiency in response to ROS- or DSB-inducing reagent. The sensitivity of the indicated strains to PHL (A and B) or H_2O_2 (C and D) was measured at various concentrations. The percentage is the ratio of viable colonies arising after drug exposure vs. mock treatment. The mutation frequency was measured by *CAN1* forward-mutation assay in response to PHL (E and F) or H_2O_2 (G and H) at the concentration used in (A and B) or (C and D), respectively. Results are shown as means with standard deviations from three independent experiments.

affects yeast cell growth. A similar result of complementation was observed when *SKN7* was overexpressed in cells lacking Skn7. However, it is likely that overexpressed Yap1 cannot suppress the H_2O_2 sensitivity of *skn7* mutants, and vice versa (Fig. 3A), supporting the previous observation that Yap1 and Skn7 proteins interact physically and cooperate on the promoters of many oxidative stress response

genes [22]. Most notably, the growth of *rad51* mutant cells was strongly inhibited when *YAP1* was overexpressed even in the absence of drug treatment (Fig. 3A). Western blot analysis revealed that the expression level of Yap1 out of multicopy plasmid in *rad51* mutant cells was ~4 folds higher than those of control strains (Fig. 3B). These data point to the possibility that a genetic interaction exists between
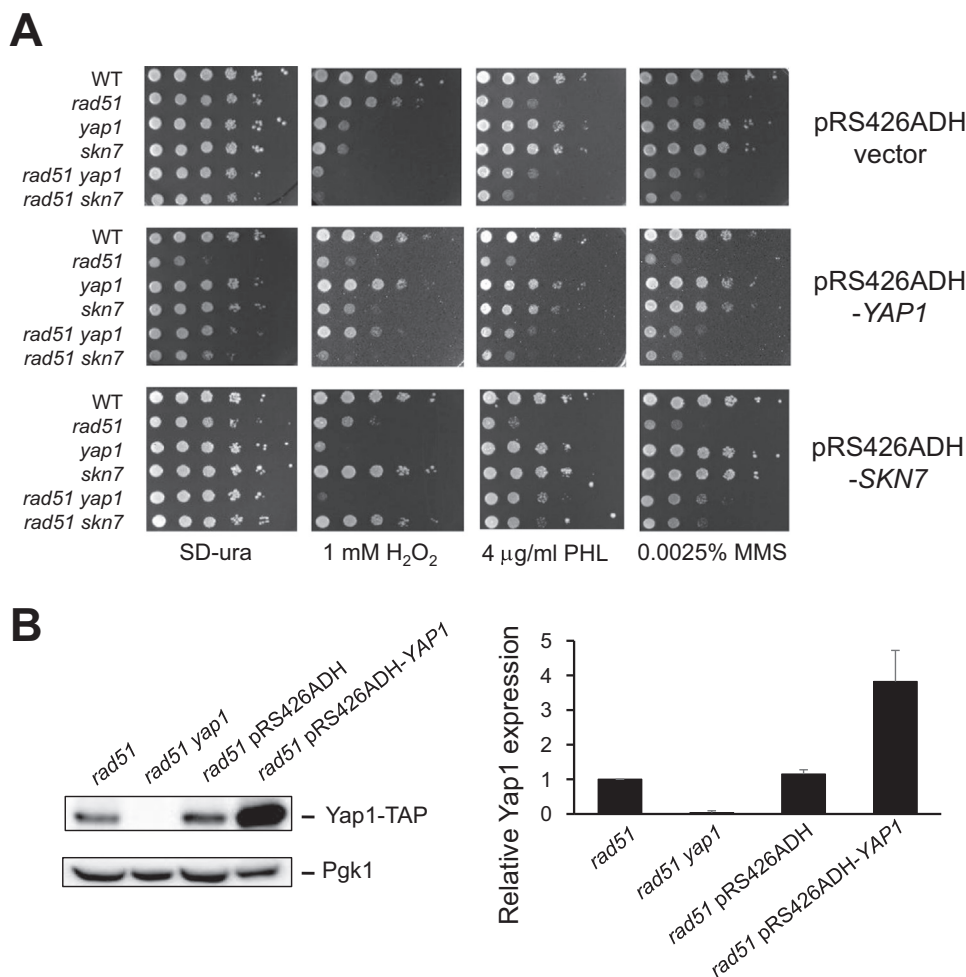
Fig. 3. Overexpression of *YAP1* or *SKN7* inhibits the growth of *rad51* mutant. (A) The indicated strains with *YAP1*- or *SKN7*-overexpression plasmids were spotted in 10-fold serial dilutions on SD media containing H$_2$O$_2$, PHL, or MMS, followed by incubation for 3 days at 30 °C. (B) The expression level of Yap1 was analyzed by Western blotting with antibodies against TAP tag and Pgk1 as internal control. Relative intensity ratio of Yap1/Pgk1 was determined by densitometry. Data are representative of three independent experiments.

the Rad51-mediated genome maintenance pathway and intracellular redox regulating factors including Yap1 and Skn7 although the underlying mechanisms are not fully understood.
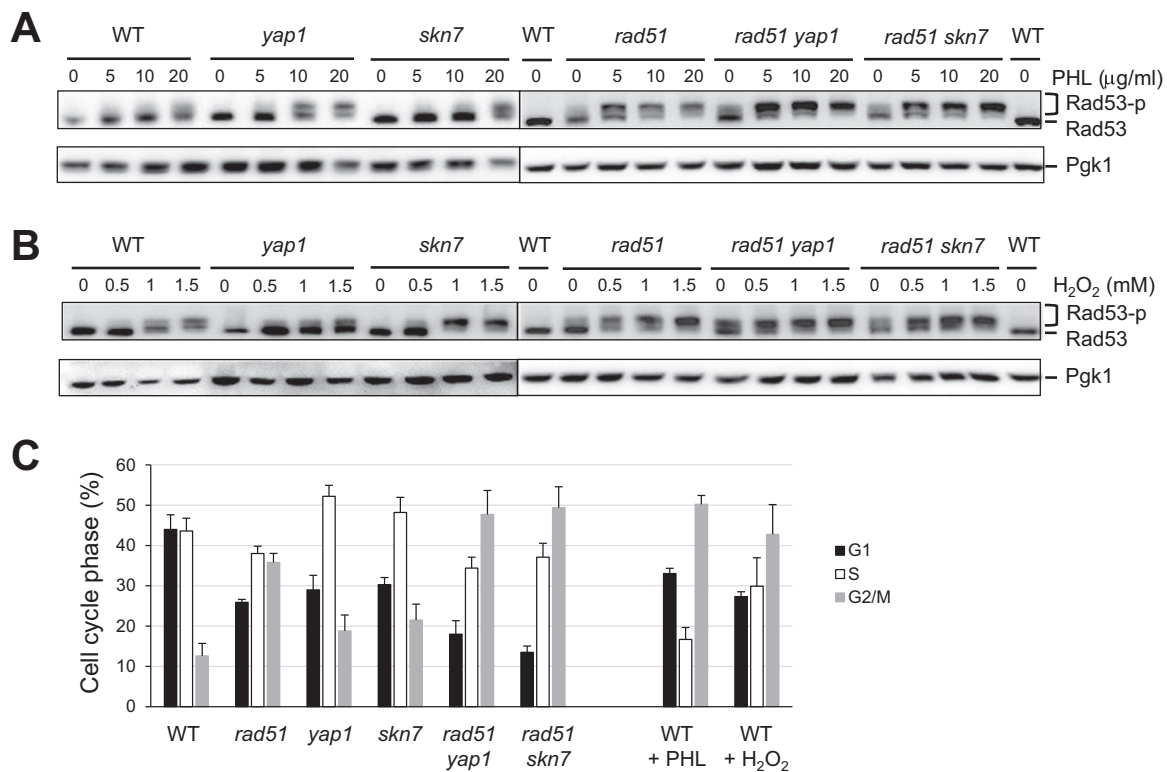
3.4. Yap1 and Skn7 are involved in prevention of DNA damage checkpoint signaling with Rad51

In yeast, the occurrence of DNA damage or a block in replication activates a Mec1-dependent cell cycle checkpoint that pauses cell cycle progression and provides additional time for cells to repair the damage prior to resuming the cell cycle [34]. In this surveillance mechanism, the phosphorylation of the Rad53 effector kinase plays a key role in transducing the checkpoint signal to ensure genomic integrity [35,36].

To further investigate the involvement of Yap1 and Skn7 in protection from DSB-induced cytotoxicity, we analyzed DNA damage checkpoint activation in various mutant strains by measuring the phosphorylation level of the Rad53 kinase. In WT cells, phosphorylation of Rad53 was observed when cells were treated with 20 µg/ml PHL (Fig. 4A). In contrast, phosphorylated Rad53 began to be detectable in *yap1* mutant cells at PHL concentration of 10 µg/ml with similar intensity to unmodified Rad53 band. In *skn7* mutant, the mobility shift of Rad53 at low concentration was equivalent to that of WT cells, but the intensity of the shifted band was stronger than that from WT cells exposed to 20 µg/ml PHL. However, *rad51 yap1* and *rad51 skn7* double mutants displayed much higher band upshifts than did *rad51* single mutant in response to a low concentration of PHL (Fig. 4A). A similar result was also shown when cells were exposed to H$_2$O$_2$

(Fig. 4B), suggesting that Yap1 and Skn7 prevent the accumulation of DNA damage induced by DSB-inducing agents as well as ROS challenges. Moreover, in *rad51 yap1* and *rad51 skn7* double mutants, phosphorylated Rad53 level was higher even under mock treatment conditions than it was in each single mutant, indicating that the accumulation of spontaneous DNA damages rendering cells more sensitive to exogenous insult can be ascribed in part to a deficiency of Yap1 or Skn7 (Fig. 4A and B). Based on the observation by Pawar *et al.* [37] that Rad53 phosphorylation is barely detectable in normally growing cells even lacking either BER or NER pathway that might contain large amount of endogenously-induced ROS, our data suggest that lack of Yap1 or Skn7 is involved in the accumulation of DSB damages, not just oxidative DNA damages in *rad51* mutant background.

These results raise the question as to whether damage checkpoint activation resulted from lack of Yap1 or Skn7 is distinguishable from that in the Rad51-deficient strain, since it has been reported that yeast cells exposed to ROS can undergo G1 arrest and apoptotic cell death, whereas unrepaired DSBs mostly induce checkpoint-mediated G2/M arrest [30,38–40]. To address this question, the cell cycle phase distribution of exponentially growing cells was determined. WT cells under normal conditions chiefly occupied the G1 (> 40%) and S phases (> 40%), while *rad51* mutant cells showed a decreased proportion of G1 phase cells and an approximately 3-fold increase in G2/M phase cells (~36%) compared to WT (~12%) (Fig. 4C), indicating that Rad51-deficient cells progress more slowly through the G2/M phase, probably due to accumulation of unrepaired DSB lesions [41]. The *yap1* or *skn7*

Fig. 4. Yap1 and Skn7 are involved in the suppression of DNA damage checkpoint activation with Rad51. (A and B) Western blot shows the phosphorylation status of Rad53 kinase in the indicated strains. Pgk1 served as loading control. Cells were untreated or treated with PHL (A) or H_2O_2 (B) for 2 h prior to analysis. (C) Graph shows the percentage of the indicated strain cells in G1, S, or G2/M phase during normal exponential growth. WT cells treated with 20 μg/ml PHL or 2 mM H_2O_2 for 4 h were presented as positive controls.

mutant cells mainly occupied the S phase (~50%) rather than the G1 or G2/M phase, which is quite different from either WT or the *rad51* mutant strain. Inspiringly, both *rad51 yap1* and *rad51 skn7* double mutants had significant percentages of their population in the G2/M phase (~50%), in which arrest likely occurred by prolonged checkpoint activation with swollen dumbbell-shaped cell morphology (Figs. 4C and 1C). The proportions of both double mutant cells accumulated in G2/M phase under normal condition are similar to those of WT cells treated with PHL or H_2O_2 (Fig. 4C). Taken together, these results imply that both Yap1 and Skn7 are involved in the prevention of DNA damage checkpoint signaling in concert with the HR-associated repair protein Rad51.
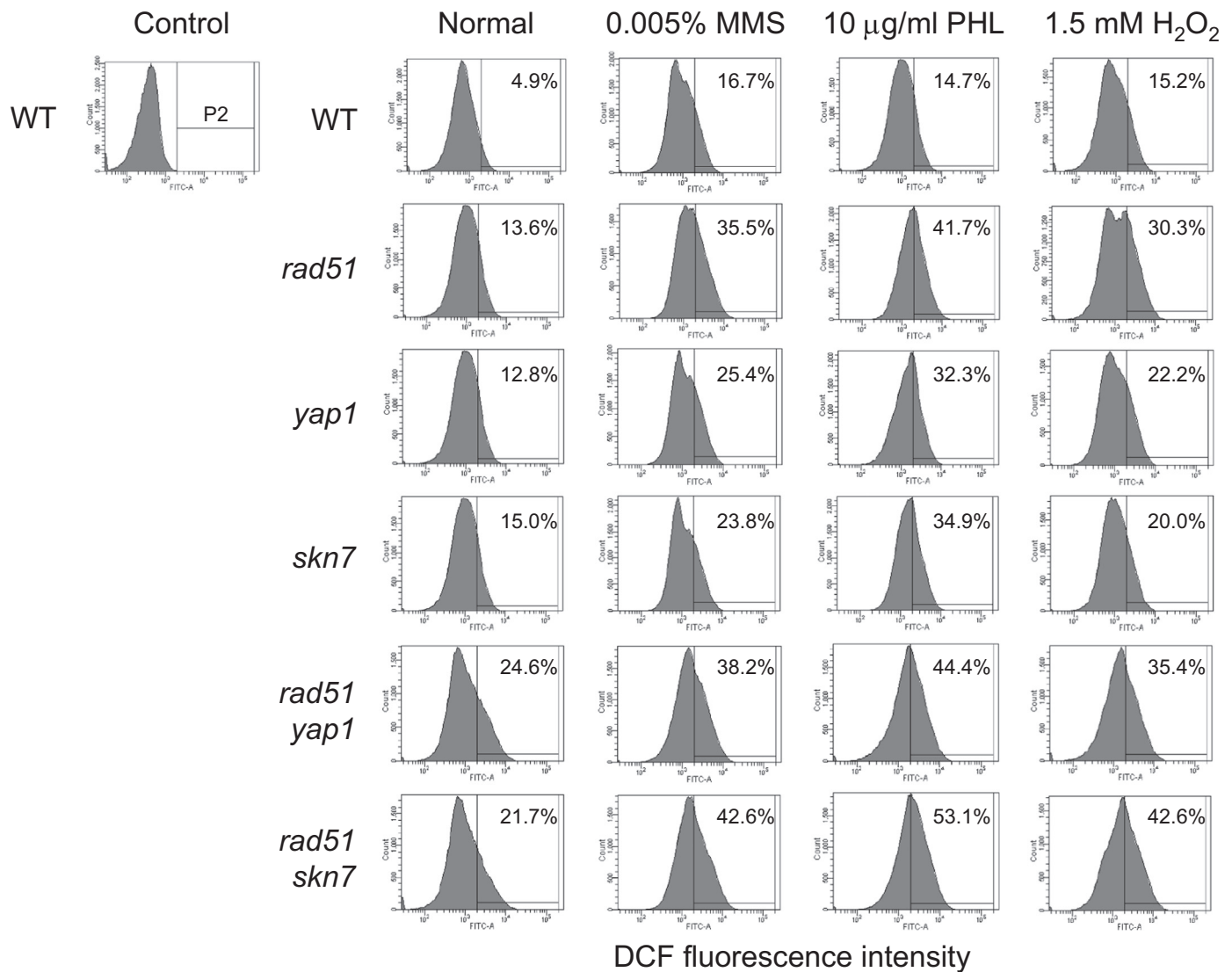
3.5. Accumulation of DSB lesions increases intracellular ROS levels

An important issue in the present work was to determine whether or not DSB lesions mediate any alteration in intracellular ROS levels. To detect intracellular ROS production, we treated cells with or without MMS, PHL, or H_2O_2 and employed flow cytometry using H_2DCFDA, a fluorescent probe that reacts with several ROS including H_2O_2, hydroxyl radicals, and peroxynitrite [42]. The percentage of the cell population emitting higher than background level of fluorescence was referred to as the P2%. WT cells had a P2 of ~5% under normal conditions (Fig. 5). As anticipated, we detected a 3-fold increase in fluorescence of WT cells (15.2%) after a 2 h exposure to 1.5 mM H_2O_2. When cells were treated with exogenous DSB-inducing reagents, *yap1* and *skn7* mutants as well as WT showed significant increases in ROS levels of approximately 2–3 fold in response to 0.005% MMS and 10 μg/ml PHL. Intriguingly, the *rad51* mutant displayed a similar level of endogenous ROS (~14%) to *yap1* or *skn7* mutant in normal conditions, but displayed more drastic increase in fluorescence (~42%) than *yap1* and *skn7* mutants when treated with MMS (~25%) and PHL (~35%), implying that accumulation of unrepaired DSB lesions could raise ROS levels in cells (Fig. 5). The drug-induced

ROS levels of *rad51 yap1* and *rad51 skn7* double mutants as well as spontaneously generated ones were higher than those of each single mutant. This observation is consistent with our results of drug sensitivity spotting analysis and regarding checkpoint activation in this study (Figs. 1A and 4). To rule out any possibility that increased fluorescent signals were attributed to the increase of dead cells, we performed trypan blue exclusion test and found that the number of dead cells did not increase significantly under normal conditions in either single or double mutants with elevated ROS level and the proportion of high-ROS cells does not reflect the number of cells that lost viability (Supplemental Fig. 2 and Supplemental Fig. 3). In summary, these results strongly suggest that overabundant DSB lesions especially in the absence of Rad51 lead to high intracellular levels of ROS, which are induced more significantly when combined with a lack of Yap1 or Skn7.

3.6. DSB repair proteins and ROS-responsive transcription factors participate in genetic crosstalk contributing to cell survival and genome integrity

Common to DSB repair process through HR is the relocalization of Rad52, a mediator of Rad51 nucleofilament formation for homolog search, from a diffuse distribution throughout the nucleus to sub-nuclear foci at certain broken chromosome ends [43,44]. Therefore, Rad52 foci represent the positions of DSB lesions actively engaged in repair events [45]. Yap1 is mainly found in the cytoplasm under ordinary conditions; however, in response to oxidative stress it localizes to the nucleus following conformational change by disulfide bond formation [46,47]. Fluorescence microscopy monitoring GFP-tagged fusion proteins was utilized to determine whether Rad52 gathers to DSB sites forming foci and whether Yap1 is activated and imported into the nucleus in response to PHL and H_2O_2 (Fig. 6). In WT cells, fewer than 10% cells showed Rad52 foci in the nucleus under normal condition; when cells were exposed to 1 mM H_2O_2, a significant

Fig. 5. Accumulation of DSB lesions raises the intracellular ROS level. The intracellular ROS level was determined by flow cytometry analysis in the indicated strains. Cells were mock treated or treated with MMS, PHL, or H_2O_2 for 2 h prior to ROS detection. P2 percentage indicates the proportion of cells with an increased production of ROS, emitting above the background levels of DCF fluorescence. Averages from three independent experiments are presented.

increase to 25% of cells containing foci was observed within 1 h and this increased proportion was common to all types of mutants as well as WT (Fig. 6A and C), verifying that exogenous H_2O_2 treatment results in genomic instability due to DSB formation. As anticipated, the proportion of cells with Rad52 foci was higher when exposed to PHL in a dose-dependent manner (Fig. 6A and D). More importantly, cells lacking Yap1 or Skn7 showed twice as many cells (16–18%) with spontaneously occurring foci than did WT cells (~9%) even without exogenous treatment, and the difference became larger in the case of *rad51 yap1* or *rad51 skn7* double mutant, which displayed much higher percentages of foci-containing cells (~35%) than did the *rad51* single mutant (~22%) (Fig. 6C and D). To further examine whether the accumulated DSBs in double mutants are directly from increased intracellular ROS or due to increased SSBs that are being converted into DSBs during replication, we pretreated cells with 15 μg/ml nocodazole for 4 h to arrest them in G2/M phase and these cells would not carry out replication while foci are detected. All strains arrested by nocodazole showed no significant difference in the level of spontaneous or H_2O_2-induced Rad52-GFP foci formation compared to those of each strain with no G2/M arrest (Supplemental Fig. 4 and Fig. 6C). This result supports that DSBs could be generated directly from increased ROS accumulation.

Next, we observed Yap1 relocalization into the nucleus within 15 min after exposure to 0.5 mM H_2O_2 in ~70% of the cell population with nuclear fluorescence, consistent with a previous report [46]. A similar proportion of nuclear Yap1 relocalization in response to H_2O_2 treatment was seen with all of the mutants tested (Fig. 6B and E). Slight but statistically meaningful increases in nuclear Yap1 were also observed following administration of 5−10 μg/ml of PHL to mutants as well as WT cells (Fig. 6B and F). Even when not exposed to any DNA-damaging agents, the spontaneous accumulation of Yap1 in the nucleus in Rad51-compromised cells (~17%) was about twice that in WT cells (~8%) (Fig. 6E and F). The *rad51 skn7* double mutant cells also exhibited a much higher proportion of cells (~24%) with nuclear Yap1 than did *skn7* single mutant cells (~15%) under no-treatment conditions, supporting the idea that DSB damage and its repair protein, Rad51, are associated with Yap1 nuclear localization and probably its activation as well. We conclude that Yap1 and Skn7 play significant roles not only in transduction of ROS-mediated stress signals, but also in protection from DSB damage-associated genomic instability in concert with Rad51. Rad51, reciprocally, is highly involved in accumulation of intracellular ROS and the ensuing activation of Yap1 transcription factor.
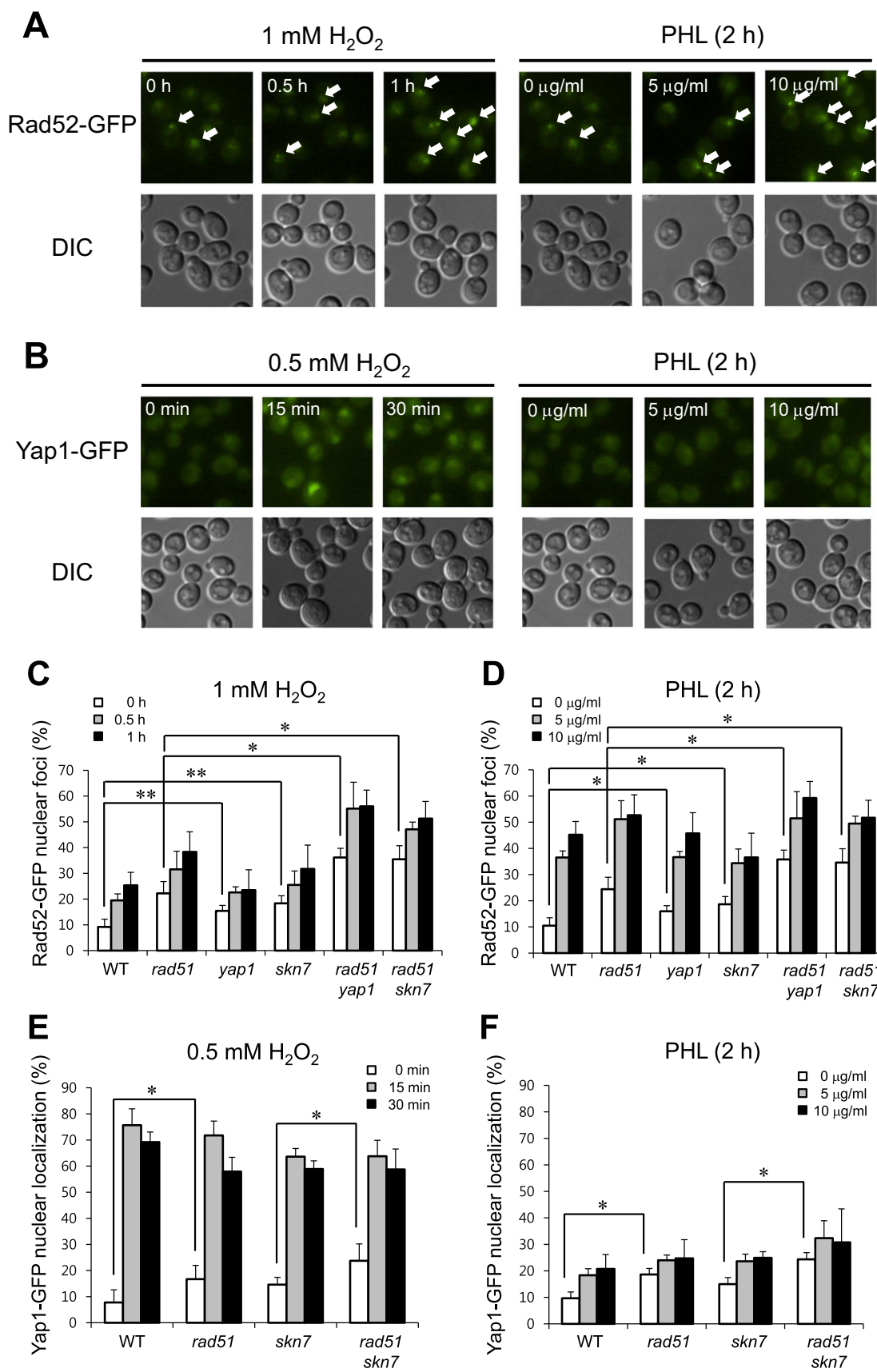
Fig. 6. Pathways of DSB repair and ROS response signaling have genetic crosstalk for cell survival and maintenance of genome integrity. (A) Spontaneous or induced Rad52-GFP foci (white arrows) were photographed in WT cells under H_2O_2 or PHL treatment. (B) Spontaneous or induced Yap1-GFP nuclear localizations were photographed in WT cells under H_2O_2 or PHL treatment. DIC, differential interference contrast. The proportion of cells containing spontaneous or induced Rad52-GFP foci was analyzed in the indicated strains under H_2O_2 (C) or PHL treatment (D). The proportion of cells showing spontaneous or induced Yap1-GFP nuclear localization was analyzed in the indicated strains under H_2O_2 (E) or PHL treatment (F). Standard deviations are derived from three independent experiments (*$p < 0.05$, **$p < 0.01$, Student's t-test).

4. Discussion

Previous studies have claimed that DNA damage itself gives rise to an increase in cellular ROS based on the observation that yeast cells deficient in BER pathway alone or in conjunction with NER pathway show elevated ROS levels and increased chromosomal aberrations [8,9]. The elevated ROS levels in BER-defective cells were diminished in a *yap1* mutant background despite the lack of any increase in chromosomal aberrations, revealing an epistatic link between Yap1 and BER pathway. Yap1 is considered to be a damage responder for DNA lesions that are primarily processed by BER but not by NER [10]. However, it remains undetermined whether such a biological connection also exists between ROS-responsive regulators and damage repair proteins specifically in charge of HR, which is an indispensable pathway for the repair of DSBs, the most dangerous type of DNA damage.

Rad51 is one of the most important members of the *RAD52* epistasis group that are involved in the repair of DSBs. *Rad51* deletion is not lethal because DSBs can be repaired by multiple HR pathways, among which Rad51 is only crucial for synthesis-dependent strand annealing (SDSA), but not for break-induced replication (BIR) or single-strand annealing (SSA) [48,49]. It has been determined by high-throughput analysis that *RAD51* has ~110 synthetic lethal genetic interactions, among which at least 25 genes are responsible for DNA replication or repair processes. However, only two of these genes, *SOD1* and *TSA1*, are implicated in intracellular redox regulation as functional antioxidant enzymes [14,15]. Unusually, those two genes have already been reported to play a significant role in mutation suppression [13]. These synthetic lethal relations between HR and redox regulatory pathways have recently begun to be exploited to identify novel drug targets for anticancer therapy, as in a study showing that inhibition of SOD1 resulted in DSB-mediated selective apoptosis of *RAD54B*-deficient human cancer cells [17].

We employed a series of yeast null strains devoid of Rad51 and/or either of two transcription factors, Yap1 and Skn7, responsible for the expression of major mutation suppressors to determine the causal relationship between DSB repair and oxidative stress responses in yeast. The *rad51* mutant was more sensitive to PQ and H_2O_2 than were WT cells and showed enhanced formation of Rad52 nuclear foci in response to H_2O_2 treatment, clearly indicating that ROS induce DSB lesions in chromosomes in a dose-dependent manner (Fig. 1 and Fig. 6C). The hypothesis that ROS are a major source of endogenous DNA damage is also supported by the recent observation that anaerobic growth conditions decrease mutation rates and gross chromosomal rearrangements (GCRs) with reduced oxygen metabolism [50]. Sensitivity to endogenous ROS and spontaneous transport of Yap1 into the nucleus greatly increased in *rad51 skn7* double mutants relative to those in the *skn7* single deletion mutant (Figs. 1A and 6E), pointing to the possibility that Rad51 plays as yet unidentified roles, likely in general ROS scavenging or rendering tolerance to ROS plethora in the cell. It is also plausible that accumulation of unrepaired DSBs due to the loss of Rad51 activity is intimately linked to the generation of ROS, which could serve as signaling molecules to protect cells from further exogenous insults or to activate checkpoint pathways to provide time to allow thorough repair of damage (Fig. 4). Low levels of ROS can be involved in various cell signaling pathways, although high levels are detrimental to cells [51]. Interestingly, human XRCC3, one of the five Rad51 paralogs that play a crucial role in HR, has cysteine residues that can potentially be exposed to ROS and can be oxidized by UVA photosensitization, suggesting that alteration in redox status might be involved in the regulation of HR pathway [52]. *RAD51* in yeast is not essential, while homozygous loss of Rad51 in mice causes early embryonic lethality [53]. Rad51 in mammals interacts with a set of proteins such as the tumor-suppressor p53, Brca1, and Brca2, all of which lack clear orthologs in yeast [54]. These results indicate that Rad51 might have multiple structural variations and distinctive mechanisms that are activated and function in concert with a variety of different regulatory factors.

Yap1 and Skn7 are two main transcription factors in yeast that are implicated in the control of a large oxidative stress response regulon governing the expression of several hundreds of genes with antioxidant and oxidoreductase activities. Strains that are inactivated in either of these regulators are hypersensitive to H_2O_2 [19,20]. It has been reported that overexpression of *YAP1* renders cells resistant to *t*-butyl hydroperoxide and partially rescues the hypersensitive phenotype of *skn7* mutant to peroxide stress, and that overexpression of *SKN7* can suppress the mutant phenotype of defective cell wall biosynthesis [18,55]. Contrary to previous reports, however, we discovered that overexpression of *YAP1* or *SKN7* exacerbates the sensitivity of *rad51* mutant to both ROS- and DSB-generating agents, and that Yap1 overproduction significantly inhibits cell growth in the absence of Rad51 (Fig. 3). These observations strongly suggest that an excess of Yap1 renders genetic requirement of Rad51 for normal growth of yeast cells, probably due to the generation of excessive DSB lesions, in agreement with our hypothesis that genetic interactions exist between HR-mediated maintenance of genome integrity and intracellular redox signaling.

Ragu et al. [56] recently identified *trr1* deletion as a suppressor of the lethality of a *rad51 tsa1* double mutant. They claimed that deregulation of the thioredoxin redox system due to the loss of Trr1 contributes to constitutive Yap1 accumulation in the nucleus and subsequent suppression of *rad51*- and *tsa1*-induced mutagenesis achieved by overexpression of Yap1-controlled oxidoreductases and reduction of dNTP levels [56]. These results suggest that excessive Yap1 activity affects the pool of intracellular dNTP, a large amount of which is required for proper repair of accumulated DSB lesions in *rad51* mutant cells. The questions of whether and how the viability of HR-deficient strains is affected by the expression levels of redox regulators need to be further addressed. Additionally, a growing body of evidence suggests the existence of genetic links between cellular redox regulators and DNA damage checkpoint signaling proteins. ATM kinase is considered to be a sensor of oxidative stress that is directly activated by H_2O_2, and the checkpoint response mediated by Mec1, a yeast ATM homolog, is impaired by the altered cellular redox state in Sod1-deficient cells [57,58]. Moreover, Tsang et al. [59] have reported that Dun1 kinase, a downstream effector of Mec1, interacts with Sod1 and regulates its activity through phosphorylation, providing the first evidence of genetic and physical interaction between them.

In summary, our study reveals novel functions of Yap1 and Skn7, two transcription factors responsible for oxidative stress response, which are highly associated with the Rad51-mediated HR pathway for repair of chromosomal DSB lesions. Loss of Rad51 more highly contributes to genomic instability and sensitivity to genotoxic stresses in the absence of Yap1 or Skn7, and the accumulation of DSB lesions increases intracellular ROS levels. Our findings provide a framework for further research on this topic to lead to a comprehensive understanding of genetic crosstalk between two major cellular damage response pathways, which could be applicable to the development of new translational medicines for anticancer therapy.

Conflict of interests

The authors have no conflict of interests to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2016.11.005.

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