Homologous recombination is required for recovery from oxidative DNA damage

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Homologous recombination and oxidative DNA damage

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ABSTRACT

We have been studying the genetic events, including chromosome loss, chromosome rearrangements and intragenic point mutations, that are responsible for the deletion of a URA3 marker in a loss of heterozygosity (LOH) assay in the yeast Saccharomyces cerevisiae. With this assay, we previously showed that homologous recombination plays an important role in genome maintenance in response to DNA lesions that occur spontaneously in normally growing cells. Here, to investigate DNA lesions capable of triggering homologous recombination, we examined the effects of oxidative stress, a prominent cause of endogenous DNA damage, on LOH events. Treatment of log-phase cells with H₂O₂ first caused growth arrest and then, during the subsequent recovery, chromosome loss and various chromosome rearrangements were induced more than 10-fold. Further analysis of the rearrangements showed that gene conversion was strongly induced, approximately 100 times more frequently than in untreated cells. Consistent with these results, two diploid strains deficient for homologous recombination, rad52Δ/rad52Δ and rad51Δ/rad51Δ, were sensitive to H₂O₂ treatment. In addition, chromosome DNA breaks were detected in H₂O₂-treated cells using pulsed-field gel electrophoresis. Altogether, these results suggest that oxidative stress induced recombinogenic lesions on chromosomes, which then triggered homologous recombination leading to chromosome rearrangements, and that this response contributed to the survival of cells afflicted by oxidative DNA damage. We therefore conclude that homologous recombination is required for the recovery of cells from oxidative stress.
INTRODUCTION

Genetic alterations are more complex in diploid cells than in haploid cells. In particular, alterations at the chromosome level that involve the loss of an essential gene, such as chromosome loss and rearrangement, can only be studied in diploid cells because they are lethal in haploids. In humans, chromosome aberrations are often observed in cancer cells as well as in normal cells of elderly individuals, and are implicated in both cancer development and the aging process (Tischfield, 1997; Lengauer et al., 1998; Rao et al., 2017). Thus, it is clearly important to understand the mechanisms and causes leading to chromosome aberrations.

To gain an overview of chromosome aberrations at the molecular level, we are utilizing the diploid yeast *Saccharomyces cerevisiae* (Hiraoka et al., 2000; Ajima et al., 2002; Umezu et al., 2002; Watanabe et al., 2002; Yoshida et al., 2003). As shown in Fig. 1, we have developed a loss of heterozygosity (LOH) assay method to analyze the genetic events responsible for loss of a heterozygous *URA3* marker on chromosome III, which makes the cell resistant to 5-fluoro-orotic acid (5-FOA). Loss of the *URA3* marker is caused by several genetic alterations including chromosome loss, various chromosome rearrangements and intragenic point mutations, and, to classify these events, two additional markers on the same chromosome are utilized to monitor how chromosomes are rearranged in three different phenotypes: 5-FOA' Leu⁻ Ade⁻ (Class A), 5-FOA' Leu⁺ Ade⁻ (Class B) and 5-FOA' Leu⁺ Ade⁺ (Class C). In addition to determining the frequencies of each class, chromosome structure in LOH clones can be physically analyzed by pulsed-field gel electrophoresis (PFGE) and PCR, in which aberrant-sized chromosome III derivatives are readily detected (Hiraoka et al., 2000). We can define these events at the
nucleotide level, when necessary, by determining the fusion points of aberrant-sized chromosomes using a PCR-based method to quantify the ploidy at a series of loci along chromosome III (Umezu et al., 2002).

By exploiting these molecular genetic methods, we have determined the factors involved in spontaneous genetic alterations arising in normally growing cells. The total LOH frequency of wild-type cells was \(1-2 \times 10^{-4}\) and the analysis of LOH events revealed that homologous recombination is involved in processes leading to LOH in multiple ways, including allelic recombination, chromosome size alteration, and a particular kind of chromosome loss (Hiraoka et al., 2000). Analysis of the fusion points of more than 80 aberrant chromosomes revealed that all of them had breakpoints within repetitive sequences scattered over the genome, such as Ty1, indicating that homologous recombination is a leading process in chromosome size alteration (Umezu et al., 2002). In recombination-deficient mutants, on the other hand, LOH events were significantly increased up to frequencies of \(3-5 \times 10^{-3}\), and mostly involved chromosome loss, indicating that homologous recombination also plays an important role in proper chromosome maintenance (Yoshida et al., 2003). Taken together, these results strongly suggest that spontaneous DNA lesions capable of triggering homologous recombination occur at a strikingly high frequency throughout the genome during normal cell growth; the majority of lesions are repaired through sister chromatid recombination, but occasionally this process leads to LOH events.

To characterize DNA lesions triggering homologous recombination, here we studied the effects of oxidative stress on LOH events. Although previous studies by others have shown that
radical oxygen species are involved in chromosome rearrangements in yeast, the assays used can
detect a narrower range of rearrangements than our LOH assay. One group analyzed events called
gross chromosomal rearrangements in haploid cells (Chen et al., 1998; Ragu et al., 2007), and the
other identified a specific deletion between a tandem repeat on a chromosome in diploids
(Brennan et al., 1994). We therefore anticipated that our LOH assay would yield an overview of
the mutagenic effects of oxidative stress. Yeast cells were treated with H₂O₂, which in the cells
generates highly reactive hydroxyl radicals that have direct effects on most cellular constituents
including DNA (Dawes, 2006). The effects of oxidative damage on DNA and nucleotides are well
defined and cause specific point mutations (Nakatsu and Sekiguchi, 2006; Rasmussen, 2006). We
demonstrate that chromosome alterations as well as point mutations were induced by the
oxidative treatment, and that gene conversion-type rearrangements, the least erroneous
rearrangements among LOH events, were especially increased.

MATERIALS AND METHODS

Media  Media for yeast strains included complex glucose (YPD), synthetic complete (SC) and
various drop-out media, and were prepared as previously described (Rose et al., 1990). 5-FOA
plates were prepared as described (Rose et al., 1990) and depleted of leucine and/or adenine
sulfate where indicated.

Strains  All yeast strains used in this study are derivatives of YKU23 (MATα lys2Δ202 leu2Δ1
ura3-52 his3Δ200 ade2Δ::hisG) and YKU34 (MATα lys2Δ202 ura3-52 trp1Δ63 ade2Δ::hisG)
III-205::URA3 III-314::ADE2) with the S288c background (Hiraoka et al., 2000). III-205::URA3 signifies that the URA3 fragment was inserted at a locus 205 kb from the left end of chromosome III. Similarly, III-314::ADE2 denotes that the ADE2 fragment was inserted at 314 kb. Nucleotide coordinates are as given in the Saccharomyces Genome Database (http://www.yeastgenome.org).

Derivatives of rad52Δ and rad51Δ mutations from YKU23 are YMO2 and YMO6, respectively, and those from YKU34 are YMO9 and YMO8, respectively (Yoshida et al., 2003). Diploid cells were constructed by mating between haploid a cells and α cells, and selection on SC medium depleted of uracil, leucine, adenine, histidine and tryptophan just before use. Diploid strains are designated RD301 (wild-type), RD304 (rad52Δ/rad52Δ) and RD305 (rad51Δ/rad51Δ) (Hiraoka et al., 2000; Yoshida et al., 2003).

**H₂O₂ or methyl methansulfonate (MMS) treatment**  
H₂O₂ or MMS treatment was performed as previously described (Ragu et al., 2007) with minor modifications. Haploid (YKU34) or diploid cells were grown to mid-logarithmic phase at 30 °C in SC medium depleted of uracil, leucine and adenine, and about 1 × 10⁷ cells were then collected, washed twice with distilled water and suspended in distilled water at 1 × 10⁷ cells/ml, after which H₂O₂ or MMS was added to the indicated concentration. After being incubated for 60 min at 30 °C, the cells were washed twice with distilled water and analyzed. Survival of the H₂O₂-treated cells was determined on YPD plates after incubation for 4 days at 30 °C.

**Analysis of cell growth and 5-FOA⁺ conversion after H₂O₂ treatment**  
H₂O₂-treated cells

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were inoculated in YPD medium and incubated at 30 °C with vigorous shaking. At the indicated
time points, aliquots of cells were removed, diluted, and spread on YPD and 5-FOA plates.
Colonies were counted after incubation for 4 days at 30 °C. At least three independent
experiments were performed to determine the frequencies of 5-FOAr clones.

LOH assay  LOH assay was performed basically as previously described (Hiraoka et al., 2000;
Yoshida et al., 2003) with minor modification. H2O2-treated cells were inoculated in YPD
medium, and incubated at 30 °C with vigorous shaking for 22 h. After appropriate dilution, the
cells were spread on YPD, 5-FOA, 5-FOA leucine-depleted, and 5-FOA leucine- and
adenine-depleted plates, and incubated at 30 °C for 3-5 days. At least three independent
experiments were performed to determine the frequencies of LOH. The frequency of FOA' Leu−
clones was determined by subtracting the mean frequency of FOA' Leu+ clones from that of
FOA' clones. Similarly, the frequency of FOA' Leu+ Ade− clones was determined by subtracting
the mean frequency of FOA' Leu+ Ade+ clones from that of FOA' Leu+ cells.

PCR procedures to classify Class C events  Yeast genomic DNA was purified using InstaGene
Matrix (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Two μl of the DNA
preparation was used in a 20-μl PCR reaction. PCR was performed under standard conditions
with Ex Taq DNA polymerase (Takara, Tokyo, Japan) to determine genetic events of Class C
clones as previously described (Hiraoka et al., 2000). Briefly, intrachromosomal deletion between
the MAT and HMR loci was detected by PCR with primers encompassing these loci.
Semi-quantitative PCR using primers encompassing the *URA3*-inserted locus (*III*-205) was performed to distinguish intragenic point mutation and gene conversion-type rearrangements. Genomic DNA of the former generated PCR products from both intact *III*-205 and *URA3*-inserted *III*-205 loci, while that of the latter generated a product only from the intact *III*-205 locus.

**PFGE**  PFGE analysis of chromosomes was performed as previously described (Hiraoka et al., 2000). Agarose plugs of chromosomal DNA were prepared using a CHEF yeast genomic DNA plug kit (Bio-Rad) according to the manufacturer’s instructions. The plugs were made using cells from equal volumes of culture. Electrophoresis was carried out with 1% PFGE-certified agarose (Bio-Rad) in 0.5 × TBE buffer at 14 °C, using a CHEF Mapper XA pulsed-field electrophoresis system (Bio-Rad). Chromosomes were separated at 6 V/cm of pulses angled at 120 °. Switch time was linearly increased from 24.03 sec to 1 min 33.69 sec for 29 h 57 min for standard analysis (Fig. 6B). Higher resolution around chromosome XII was obtained by altering the switch time linearly from 24.03 sec to 3 min 48.48 sec for 28 h 59 min (Fig. 6A). After electrophoresis, the gel was stained with ethidium bromide and destained with water. Gel images were acquired with the CCD camera of a Dolphin-View image system (Wealtec, Sparks, NV).

**RESULTS**

**Diploid cells are more resistant to oxidative stress than haploid cells**  To examine whether oxidative DNA damage could be a cause of the spontaneous homologous recombination that we
detected previously with our LOH analysis (Hiraoka et al., 2000; Umezu et al., 2002), yeast cells were first exposed to oxidative stress using H$_2$O$_2$, which is known to be produced endogenously during normal cellular metabolism (Friedberg et al., 2006). Within the cells, H$_2$O$_2$ is converted in the Fenton reaction to the hydroxyl radical, which is one of the most reactive oxygen radicals and causes serious damage to most cellular constituents including DNA (Dawes, 2006; Nakatsu and Sekiguchi, 2006). In Fig. 2, the survival curves against H$_2$O$_2$ are compared for diploid and haploid cells. The results showed that diploid cells were approximately 10-fold more resistant to H$_2$O$_2$ than haploids, suggesting that some recovery processes available only in diploid cells contributes to cell survival. Another oxidant called menadione, which also generates hydroxyl radicals in the cells (Nutter et al., 1992), yielded similar survival curves to those against for H$_2$O$_2$, with diploids again clearly more resistant than haploids (data not shown), suggesting that the difference between diploid and haploid cells was associated with their recovery from damage caused by hydroxyl radicals. Since one repair mechanism that operates only in diploids is recombination between homologous chromosomes, we next tested whether chromosome rearrangements were indeed induced in these cells during their recovery from H$_2$O$_2$ treatment.

**Oxidative stress causes cell growth arrest and increases LOH emergence**   During the above experiments, we noticed that H$_2$O$_2$ treatment arrested cell growth, presumably by activating DNA damage checkpoints. Fig. 3A shows the growth curves of yeast cells after treatment with various concentrations of H$_2$O$_2$. The duration of cell growth arrest was prolonged in a dose-dependent manner. Whereas treatment with 32 mM H$_2$O$_2$ delayed cell growth for several hours, and that
with 8 mM for about one hour.

We therefore next examined if and when LOH convertants emerged in the populations after H₂O₂ treatment (Fig. 3B). The total LOH frequency in untreated cells, providing a baseline of spontaneous LOH in normally growing cells, increased depending on cell growth up to nearly 10⁻⁴, consistent with our previous results (Hiraoka et al., 2000). LOH frequency in H₂O₂-treated cells increased in a dose-dependent manner; when the cells were treated with 32 mM H₂O₂, the emergence of LOH convertants was more than 10-fold higher than that in untreated cells. A comparison of the induction of LOH (Fig. 3B) with the population growth curves (Fig. 3A) indicated that LOH clones began to emerge above the basal level of the untreated cells after cells started to grow again. When cells were treated with 32 mM H₂O₂, it took several hours after the treatment until LOH clones emerged more frequently than the spontaneous level, and afterward the frequency of LOH increased depending on cell growth, suggesting that the recovery process from oxidative damage was accompanied by genetic alterations observed as LOH.

In Table 1 and Fig. 4, LOH clones induced by various concentrations of H₂O₂ were further classified into three groups of genetic events (Classes A, B and C) according to their phenotypes (Fig. 1). For untreated cells, half of the events were Class B interchromosomal rearrangements, and most of the others were Class A chromosome loss, in agreement with our previous findings for spontaneous LOH (Hiraoka et al., 2000). For LOH events induced by H₂O₂, although the frequencies of all three classes increased, the degree of induction and the dose dependency of frequencies varied among classes. Class C was most highly induced among the three and the frequency increased in a dose-dependent manner. While Class C events that occurred
spontaneously without H₂O₂ treatment accounted for about 10% of total LOH, treatment with 32 mM H₂O₂ increased their proportion to around 25%, and the frequency was 30-fold higher than that of untreated cells. The induction of Class B interchromosomal recombination events was also dose-dependent. When the cells were treated with 32 mM H₂O₂, the frequency of Class B was 11-fold higher than that of untreated cells, about one-third of the induction level of Class C. On the other hand, Class A chromosome loss events were induced 6- to 9-fold by H₂O₂ and the dose dependency of their induction was unclear. These results led us to consider whether one specific genetic event within Class C might be particularly induced by H₂O₂.

We therefore further defined Class C clones and classified them into the following three genetic events by PCR-based analysis: a) an intrachromosomal 94-kb deletion between the MATα and HMR loci encompassing the URA3 marker, Hawthorne deletion (Hawthorne, 1963); b) allelic interchromosomal rearrangements in which only URA3 among the three markers was lost (gene conversion-type); and c) point mutations within the URA3 marker (Fig. 1). Among spontaneous Class C LOH events analyzed in our previous work, MATα-HMR deletion was the most common, followed by gene conversion-type rearrangements, while point mutations were rare (Hiraoka et al., 2000). H₂O₂ treatment significantly changed the distribution of these events within Class C. Table 2 shows the classification of Class C clones obtained from untreated and 32 mM H₂O₂-treated cells. Although all three events were induced by H₂O₂ treatment, the contributions of individual events differed from those in spontaneous LOH. The vast majority of the events induced by H₂O₂ were gene conversion-type rearrangements and their frequency was almost 100 times that in untreated cells. The magnitude of this increase indicated that gene conversion was
the most highly induced type of LOH event in all three Classes. This allelic interchromosomal recombination results in the restoration of a normal-sized chromosome with internal exchange between homologous chromosomes, and hence could be defined as the least erroneous type of rearrangement among LOH events. It is notable that spontaneous gene conversion in our assay depends on both RAD51 and RAD52 genes, and hence presumably resulted from a conventional type of homologous recombination reaction involving both of these genes (Yoshida et al., 2003).

**Mutant strains defective in homologous recombination are more sensitive to oxidative stress**

Next, we examined the effects of the recombination genes RAD51 and RAD52 on survival after H₂O₂ treatment (Fig. 5). rad52Δ/rad52Δ mutant cells, known to be totally deficient in homologous recombination (Symington, 2002; Mehta and Haber, 2014), were sensitive to H₂O₂, displaying 30-40% survival relative to wild-type cells. The homozygous deletion mutant of RAD51, which encodes the key enzyme responsible for homology search and strand exchange in yeast (Symington, 2002; Mehta and Haber, 2014), was also more sensitive to H₂O₂ than wild type. These strains were also treated with menadione and showed similar patterns of sensitivity to those obtained with H₂O₂ (data not shown). These results suggest that homologous recombination plays an important part in the recovery process and consequent survival of cells damaged by hydroxyl radicals.

**Oxidative stress causes strand breaks on chromosomal DNA**

Finally, we directly analyzed chromosomal DNA within H₂O₂-treated cells by PFGE to examine if visible DNA lesions were
induced by the treatment (Fig. 6). As a control agent, cells were also treated with MMS, which is known to cause strand breaks in DNA (Schwartz, 1989). As shown in Fig. 6A, when the cells were treated with H₂O₂, chromosome bands became smeared in a dose-dependent manner and the intact bands of longer chromosomes became faint at the higher concentrations. The longest chromosome, Ch XII, became almost invisible at the highest concentration 500 mM. This result is consistent with the notion that a longer chromosome has a higher possibility to acquire the DNA lesion. These results indicate that DNA double strand breaks were introduced by the treatment. In the time-course experiments shown in Fig. 6B, smeared chromosome bands were observed up to six hours after H₂O₂ treatment, corresponding to the growth-arrested cell population (Fig. 3A). The extent of degradation of chromosome DNA proceeded up to four hours after treatment; presumably reflecting the occurrence of double strand breaks within the cells entering S phase (see Discussion). On the other hand, within the cells surviving growth arrest that were sampled at 22 h after treatment, chromosomal DNA appeared to have become intact again. DNA double strand breaks are well-known recombinogenic lesions (Symington, 2002; Mehta and Haber, 2014), and the breaks detected here may be the trigger events for the observed chromosome rearrangements induced by H₂O₂ treatment.

**DISCUSSION**

In living cells, genomic DNA is continuously assaulted, with various DNA lesions occurring endogenously even in normal growth, and the efficient repair of these lesions is essential to protect genome integrity (Friedberg et al., 2006; Rasmussen, 2006) In this context, homologous recombination serves as an important pathway, as shown by the fact that its
inactivation results in loss of chromosomes at a high frequency (Yoshida et al., 2003). Although the majority of recombination is precise and accompanied by no genetic changes, it is occasionally inaccurate, leading to chromosome rearrangements (Kuzminov, 1999; Mehta and Haber, 2014). These observations indicate that DNA lesions triggering recombination occur at a high frequency during mitotic growth. Several lines of evidence suggest that, in our assay, most LOH events occur in S-phase, implying that recombinogenic lesions arise during DNA replication (Watanabe et al., 2002; Yoshida et al., 2003). When the replication machinery encounters DNA damage in regions lacking coding information, the machinery collapses or stalls, leaving replication-induced lesions on both strands, namely two-stranded lesions (Kuzminov, 1999; Symington, 2002; Mehta and Haber, 2014). These lesions can be repaired using an undamaged sister chromatid as a donor for homologous recombination, and this kind of role for recombination in replication restart is widely accepted (Kuzminov, 1999; Symington, 2002; Mehta and Haber, 2014). However, the nature of the original DNA damage that collapses or stalls the replication machinery has not yet been clarified. Here, we focused on oxidative stress as a trigger of homologous recombination, as it is one of the leading causes of DNA damage in normally growing cells (Friedberg et al., 2006).

Among the reactive oxygen species produced in normal cellular metabolism, hydroxyl radicals are the most prominent and active, and are known to directly react with DNA and nucleotides, which could lead to recombinogenic lesions on chromosomal DNA (Friedberg et al., 2006; Nakatsu and Sekiguchi, 2006; Rasmussen, 2006). To generate hydroxyl radicals in yeast cells, we utilized H$_2$O$_2$ and also, in some cases, menadione. H$_2$O$_2$ treatment of cells caused
growth arrest, presumably by activating DNA damage checkpoints, and the process of recovery from the arrest was accompanied by genetic alterations which were isolated as LOH events. Point mutations were induced by H$_2$O$_2$ treatment about 30-fold more frequently than in untreated cells (Table 2), indicating that H$_2$O$_2$ treatment directly caused DNA and/or nucleotide damage within the treated cells. In addition, physical analysis of chromosomal DNA within H$_2$O$_2$-treated cells revealed that many double strand breaks were generated in the arrested cells, as previously shown by others (Ribeiro et al., 2006; Azevedo et al., 2011). These double strand breaks could either have been caused directly by hydroxyl radicals or have resulted from replication-induced strand breaks triggered by oxidative DNA damage generated by the hydroxyl radicals. In either case, the resulting DNA double strand breaks are well known to induce homologous recombination reactions (Symington, 2002; Mehta and Haber, 2014).

Consistent with the idea that double strands breaks are recombinogenic lesions, various chromosome rearrangements were observed at high frequency within H$_2$O$_2$-treated cells (Table 1 and 2), among which gene conversion-type rearrangements were most highly increased, to about 100-fold their frequency in untreated cells. As the gene conversion-type rearrangements of our LOH assay could result from several mechanisms of homologous recombination, the reason for this particular induction of gene conversion is, as yet, unclear. However, it cannot be explained simply by two crossing-over events across the $URA3$ marker on the observed chromosome, which may be triggered when multiple DNA lesions are generated by H$_2$O$_2$ treatment, because allelic interchromosomal recombination of Class B (crossing-over type) was not increased to a level comparable to that of the gene conversion type. It is noteworthy that, in our previous studies of
spontaneous LOH events, gene conversion-type rearrangements were absolutely dependent on both *RAD51* and *RAD52* genes (Yoshida et al., 2003). The requirement of *RAD51* strongly suggests that gene conversion-type rearrangements in our LOH assay, at least spontaneous ones, are presumably produced by a conventional type of homologous recombination reaction involving homology search and strand exchange by Rad51 protein. In the case of gene conversion-type rearrangements, the resulting rearranged chromosome maintains its overall original structure, other than in the region around the *URA3* marker, and hence may be defined as the least erroneous rearrangement within LOH events, compared both with more drastic rearrangements like translocations and also with allelic crossing over. Cells may possess a regulatory mechanism to control homologous recombination in such a way as to suppress genome instability if possible, even when responding to a massive dose of recombinogenic DNA lesions, as in the case of the H2O2 treatment in this study. In the absence of such a mechanism, the same repair process might result in more extensive genetic alterations, such as aberrant chromosomes and chromosome loss, and then lead to devastating outcomes including cancer and some genetic diseases.

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REFERENCES


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Table 1. Classification of LOH induced by H$_2$O$_2$ treatment

<table>
<thead>
<tr>
<th>H$_2$O$_2$ (mM)</th>
<th>Class A</th>
<th>Class B</th>
<th>Class C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.9±0.7</td>
<td>5.4±1.1</td>
<td>1.1±0.2</td>
<td>10.4±0.9</td>
</tr>
<tr>
<td>0.5</td>
<td>24.5±6.0</td>
<td>27.9±3.2</td>
<td>10.2±1.6</td>
<td>62.6±10.0</td>
</tr>
<tr>
<td>2</td>
<td>28.4±7.5</td>
<td>33.3±3.5</td>
<td>12.9±1.4</td>
<td>74.6±12.3</td>
</tr>
<tr>
<td>8</td>
<td>29.5±5.7</td>
<td>39.8±3.1</td>
<td>17.4±1.2</td>
<td>86.8±8.7</td>
</tr>
<tr>
<td>16</td>
<td>33.9±13.6</td>
<td>44.5±11.1</td>
<td>24.1±4.0</td>
<td>102.6±9.2</td>
</tr>
<tr>
<td>32</td>
<td>29.8±3.3</td>
<td>58.6±17.1</td>
<td>32.3±7.6</td>
<td>120.7±16.4</td>
</tr>
</tbody>
</table>

LOH clones were classified into the indicated three classes according to their phenotypes as shown in Fig. 1. Indicated are the mean frequencies with standard deviations determined from at least three independent experiments. In parenthesis, fold increase relative to untreated cells is indicated.
Table 2. Determination of Genetic events accompanied with giving rise to Class C LOH clones

<table>
<thead>
<tr>
<th>Class C event</th>
<th>0 mM H₂O₂</th>
<th></th>
<th>32 mM H₂O₂</th>
<th></th>
<th>Fold increase in frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio (%)</td>
<td>Frequency (× 10⁻⁵)</td>
<td>Ratio (%)</td>
<td>Frequency (× 10⁻⁵)</td>
<td></td>
</tr>
<tr>
<td>Point mutation</td>
<td>6.6</td>
<td>0.07</td>
<td>6.7</td>
<td>2.2</td>
<td>31</td>
</tr>
<tr>
<td>Gene conversion</td>
<td>26.7</td>
<td>0.29</td>
<td>86.6</td>
<td>27.9</td>
<td>97</td>
</tr>
<tr>
<td>Deletion</td>
<td>66.7</td>
<td>0.74</td>
<td>6.7</td>
<td>2.2</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>1.1</td>
<td>100</td>
<td>32.3</td>
<td>29</td>
</tr>
</tbody>
</table>

LOH clones of Class C obtained from untreated and 32 mM H₂O₂-treated cells were analyzed by PCR-based methods as described in Materials and Methods to determine genetic causes of LOH. Thirty clones were analyzed for H₂O₂-treated cells and 15 for untreated cells.
Legends to Figures

Fig. 1. LOH assay. Classification of genetic alterations leading to functional inactivation of the $URA3$ marker in strain RD301. Chromosomes $III$ in parent strain RD301 (top) and their possible alteration in 5-FOA$^r$ convertants (bottom) are illustrated with relative positions of the three markers used for the analysis. The 5-FOA$^r$ convertants are classified with their indicated phenotypes (Class A-C) and with alteration patterns of chromosome $III$ (a-c). The segments of chromosome $III$ originally harboring the markers are shown by open bars and those of the homologous chromosome $III$ are shown by shaded bars with their centromeres as circles. A solid bar in Class B-a indicates a chromosome segment translocated to the marked chromosome $III$ from another chromosome. The $URA3$ insert at $III$-205 is indicated by an open triangle, the $ADE2$ insert at $III$-314 is shown by solid triangles, and the positions of intrinsic $LEU2$ loci are indicated by vertical lines, which are marked with a cross for the $leu2$ allele. A point mutation inactivating the $URA3$ insert is shown by a cross on the open triangle.

Fig. 2. Diploid cells were more resistant to H$_2$O$_2$ than haploid cells. Log-phase cultures of wild-type diploid or haploid cells were treated with the indicated concentrations of H$_2$O$_2$, and survival was determined as described in Materials and Methods. Viability was determined in experiments repeated at least three times. Error bars represent standard deviation.

Fig. 3. H$_2$O$_2$ treatment induced cell growth arrest and LOH convertants emerged during the recovery process. Wild-type diploid cells of log-phase cultures were treated with the indicated
concentrations of \( \text{H}_2\text{O}_2 \) for 60 min at 30 °C, and then cultured in YPD medium without \( \text{H}_2\text{O}_2 \) at 30 °C. At the indicted time points, the number of viable cells (A) and the frequency of LOH clones (B) in the culture were determined as described in Materials and Methods. Similar experiments were also performed with 2 mM and 16 mM \( \text{H}_2\text{O}_2 \) (data not shown).

**Fig. 4.** Oxidative stress induces all three classes of LOH events, but especially Class C. The cumulative bar graphs plot the results shown in Table 1. Frequencies of LOH Classes A-C in cells treated at with the indicated concentration of \( \text{H}_2\text{O}_2 \) are incorporated into each bar, which consequently indicates the total LOH frequency.

**Fig. 5.** Recombination-deficient diploid cells are sensitive to \( \text{H}_2\text{O}_2 \). Survival curves without or after \( \text{H}_2\text{O}_2 \) treatment were determined for wild-type (wt/wt), \( \text{rad51}\Delta/\text{rad51}\Delta \) and \( \text{rad52}\Delta/\text{rad52}\Delta \) diploid strains, respectively, as described in Materials and Methods. The strains used were RD301 (wt/wt), RD305 (\( \text{rad51}\Delta/\text{rad51}\Delta \)) and RD304 (\( \text{rad52}\Delta/\text{rad52}\Delta \)). Viability was determined in experiments repeated at least three times. Error bar represent standard deviation.

**Fig. 6.** \( \text{H}_2\text{O}_2 \) treatment induces DNA strand breaks on chromosomes. DNA within cells treated with \( \text{H}_2\text{O}_2 \) or MMS was analyzed by PFGE as described in Materials and Methods. Cells from equal volumes of culture were used to make PFGE-plugs for each experiment shown in (A) and (B). The ethidium bromide-stained gels are shown. The identity of the chromosome (Ch) bands is indicated to the right. (A) Cells were treated with \( \text{H}_2\text{O}_2 \) or MMS at the indicated concentration for
60 min at 30 °C. (B) Cells were treated with the indicated concentration of H$_2$O$_2$ or MMS for 60 min at 30 °C, and, for 64 mM H$_2$O$_2$-treated cells, subsequently incubated in fresh rich medium for the indicated time period.
RD301  5-FOA<sup>s</sup> Leu<sup>+</sup> Ade<sup>+</sup>

Class A
(5-FOA<sup>r</sup> Leu<sup>-</sup> Ade<sup>-</sup>)
- a. chromosome loss

Class B
(5-FOA<sup>r</sup> Leu<sup>+</sup> Ade<sup>-</sup>)
- a. translocation unequal crossing over
- b. crossing over

Class C
(5-FOA<sup>r</sup> Leu<sup>+</sup> Ade<sup>+</sup>)
- a. intrachromosomal deletion
- b. local gene conversion
- c. intragenic point mutation

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Viability

H₂O₂ (mM)

O Diploid
△ Haploid
**A**

CFU (× 10^7 cells/ml)

- ● 0 mM
- □ 8 mM
- ◇ 32 mM

**B**

5-FOA^r frequency (× 10^-6)

- ● 0 mM
- □ 8 mM
- ◇ 32 mM

Time after H₂O₂ treatment (h)