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## Stimulation of Sister Chromatid Exchanges and Mutation by Aflatoxin B<sub>1</sub>-DNA Adducts in *Saccharomyces cerevisiae* Requires *MEC1* (ATR), *RAD53*, and *DUN1*

Michael Fasullo<sup>1,2,\*</sup>, Mingzeng Sun<sup>1</sup>, and Patricia Egner<sup>3</sup>

<sup>1</sup>Ordway Research Institute, Center for Medical Sciences, 150 New Scotland Avenue, Albany, New York 12209

<sup>2</sup>Department of Biomedical Sciences, State University of New York at Albany, 150 New Scotland Avenue, Albany, New York 12209

<sup>3</sup>Bloomberg School of Public Health, Johns Hopkins University, 615 North Wolfe Street, Baltimore, Maryland 21205

### Abstract

The hepatocarcinogen aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is a potent recombinagen but weak mutagen in the yeast *Saccharomyces cerevisiae*. AFB<sub>1</sub> exposure induces DNA damage-inducible genes, such as *RAD51* and those encoding ribonucleotide reductase (*RNR*), through a *MEC1* (ATR homolog)-dependent pathway. Previous studies have indicated that *MEC1* is required for both AFB<sub>1</sub>-associated recombination and mutation, and suggested that AFB<sub>1</sub>-DNA adducts are common substrates for recombination and mutagenesis. However, little is known about the downstream effectors of *MEC1* required for genotoxic events associated with AFB<sub>1</sub> exposure. Here we show that AFB<sub>1</sub> exposure increases frequencies of *RAD51*-dependent unequal sister chromatid exchange (SCE) and activates Rad53 (*CHK2*). We found that *MEC1*, *RAD53*, and *DUN1* are required for both AFB<sub>1</sub>-associated mutation and SCE. Deletion of *SML1*, which encodes an inhibitor of *RNR*, did not suppress the *DUN1*-dependent requirement for AFB<sub>1</sub>-associated genetic events, indicating that higher dNTP levels could not suppress the *dun1* phenotype. We identified AFB<sub>1</sub>-DNA adducts and show that approximately the same number of adducts are obtained in both wild type and *rad53* mutants. Since *DUN1* is not required for UV-associated mutation and recombination, these studies define a distinct role for *DUN1* in AFB<sub>1</sub>-associated mutagenesis and recombination. We speculate that AFB<sub>1</sub>-associated DNA adducts stall DNA replication, a consequence of which can either be mutation or recombination.

### Keywords

aflatoxin B<sub>1</sub>; DNA adducts; mutation recombination; cell-cycle checkpoint; *Saccharomyces cerevisiae*

### INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) strongly correlates with exposure to aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and to hepatitis B and C virus [1]. The correlation of the p53 Ser249 mutation in HCC associated with AFB<sub>1</sub> exposure is consistent with the idea that AFB<sub>1</sub> is a

\*Correspondence to: Ordway Research Institute, Center for Medical Sciences, 150 New Scotland Avenue, Albany, New York 12209..

strong liver carcinogen because it is a mutagen [2-4]. A current hypothesis is that recurrent regeneration after injury renders liver cells more susceptible to carcinogen-associated DNA damage [5]. Thus, active DNA replication may enhance AFB<sub>1</sub>-associated genotoxic effects.

AFB<sub>1</sub>-associated mutagenesis is correlated with the number of AFB<sub>1</sub>-associated DNA adducts. The highly unstable 8,9-dihydro-8-(*N*<sup>7</sup>-guanyl)-9-hydroxyafatoxin B1 (AFB<sup>1</sup>-*N*<sup>7</sup>-Gua) adduct converts to both a formamidopyrimidine (FAPY) and an apurinic site [6]. The FAPY structures are highly stable, stall replication, and are poor substrates for the *Escherichia coli* polV translesion polymerase [6]. However, introduction of oligonucleotides containing AFB<sub>1</sub>-associated FAPY structures in *E. coli* generates mutations, similar to those observed in liver carcinomas, at or near the sites of the AFB<sub>1</sub>-DNA adducts [6]. These studies suggest that AFB<sub>1</sub>-associated FAPY structures are indeed mutagenic adducts.

Considering that DNA repair mechanisms are similar in yeast *Saccharomyces cerevisiae* and higher eukaryotes, yeast is a useful organism to study how genetic factors can increase AFB<sub>1</sub>-associated genotoxic effects, including mutations and homologous recombination events, such as gene conversion events, and translocations. Observations that *rad51* mutants, defective in recombinational repair, exhibit enhanced AFB<sub>1</sub>-associated mutagenesis implies that a common DNA adduct stimulates both recombination and mutagenesis [7]. These observations suggest that DNA damage tolerance pathways are important in triggering AFB<sub>1</sub>-associated recombination and mutation; however, how recombinational or mutagenic pathways are selected is unclear.

These AFB<sub>1</sub>-associated genotoxic responses occur in the context of a global genome response to AFB<sub>1</sub>-associated DNA damage. The global transcription response to AFB<sub>1</sub> includes an induction of recombinational repair, mismatch repair, and nucleotide excision repair genes in nongrowing cells [8], while genes involved in nucleotide metabolism and recombination, such as *RNR* and *RAD51*, are induced in growing cells [9]. Cells exposed to AFB<sub>1</sub> exhibit an extended S phase and a fivefold decline in histone transcripts, suggesting that AFB<sub>1</sub>-associated DNA adducts trigger a replication block and a checkpoint response [9]. This is supported by the observation that *MEC1* (ataxia telangiectasia and Rad3 related, ATR) is required for both AFB<sub>1</sub>-associated recombination [8] and mutation [9].

Genes that are involved in the S phase checkpoint include *MEC1* (ATR), *RAD53*, and *DUN1*. *RAD53* is required for replication delay [10]. *DUN1* induces *RNR* (ribonucleotide reductase) transcription by inactivating the Crt1 repressor [11] and activates Rnr enzymatic activities by phosphorylating and thereby inactivating the Sml1 inhibitor [12]. While *RAD53* is required for DNA damage-associated homologous recombination [13], *DUN1* is neither required for UV or X-ray associated homologous recombination [14].

We determined whether *MEC1*, *RAD53*, and *DUN1* were involved in either AFB<sub>1</sub>-associated mutation or sister chromatid exchange (SCE). Based on the identification of AFB<sub>1</sub>-associated DNA adducts in yeast, we speculate that these DNA adducts initiate the checkpoint activation. We observed that *MEC1*, *RAD53*, and *DUN1* were required for both AFB<sub>1</sub>-associated mutation and SCE. However, we observed that *DUN1* was not required for UV-associated SCE or mutation. We therefore suggest that distinct genetic requirements for AFB<sub>1</sub>-associated and UV-associated genotoxic effects.

## MATERIALS AND METHODS

### Strains and Media

The genotypes of yeast strains used in this study are listed in Table 1. Strains for measuring SCE and mutation are derived from the canavanine sensitive strain YB163, which contains

*his3* recombination substrates in tandem at *TRP1* [15]. Mutants containing *sml1::KanMX mec1-Δ::TRP1*, *sml1::KanMX*, *rad53-Δ::LEU2*, and *dun1::KanMX* were generated by one-step gene disruption [16] using PCR amplified gene fragments and selecting for Kan<sup>R</sup> or the appropriate auxotrophic selection. The primers used for amplifying these gene fragments are listed in the “Yeast Deletion Database” (<http://www-deletion.-stanford.edu>). The *dun1::kanMX sml1::URA3* double mutant was made by genetic cross. Ura<sup>-</sup> derivatives of the *rad51* mutant YB205 [17] and *dun1::kanMX sml1::URA3* strains were made by selecting for 5-fluorouracil (FOA) resistance. Plasmid pCS316 [18], containing cytochrome P450 1A2 (CYP1A2) and hOR, was introduced into wild type and checkpoint mutants by selecting for Ura<sup>+</sup>. pJW730, containing *DUN1*, was introduced into *dun1* mutants by selecting for Trp<sup>+</sup> transformants.

Standard media were used for the culture of yeast cells. YP (yeast extract, peptone), YPD (YP, dextrose), SC (synthetic complete, dextrose), SC-LEU (SC lacking leucine), SC-TRP (SC lacking tryptophan), SC-URA (SC lacking uracil), and FOA media are described by Burke et al. [19]. Media to select for canavanine resistance contain SC-ARG (synthetic complete lacking arginine) and 60 µg/mL canavanine (CAN) sulfate.

### Measuring DNA Damage-Associated Recombination and Mutation Events

To measure AFB<sub>1</sub>-associated genotoxic events, log phase yeast cells ( $A_{600}=0.5-1$ ) were exposed to indicated doses of AFB<sub>1</sub>, previously dissolved in DMSO. Cells were maintained in nutrient media (SC-URA) during the carcinogen exposure. To measure UV-associated events, log phase cells ( $A_{600}=0.5-1$ ) were washed and resuspended in sterile H<sub>2</sub>O and then exposed to indicated doses of UV (260 nM, 2J/m<sup>2</sup>/s). After the exposure, cells were washed twice in H<sub>2</sub>O, and then plated on SC-HIS or SC-ARG CAN to measure SCE or mutation frequency, respectively. An appropriate dilution was inoculated on YPD to measure viability.

### Detection and Quantification of DNA Adducts

To measure the AFB<sub>1</sub>-associated DNA adducts in yeast, we used liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) [20]. Log phase cultures of yeast expressing human CYP1A2 (pCS316) were exposed to 50 µM AFB<sub>1</sub> for 4 h. Because standard protocols for isolating yeast DNA involve alkaline buffers, rendering the highly unstable AFB<sub>1</sub> N<sup>7</sup>-Gua DNA adducts labile, we have modified the “smash-and-grab” protocol [21] so that we are using a neutral buffer containing 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 2% Triton X-100, 1% SDS, pH 7. DNA was then isolated from two independent samples of yeast cells. The DNA adducts were identified and measured by high performance liquid chromatography and tandem mass spectroscopy (LC-ESI/MS/MS) after acid hydrolysis [20].

### Determining Rad53 (Chk2) Activation

Activation of Rad53 was determined by Western blots. Cells were inoculated in SC-URA medium. Log phase cells ( $A_{600}=0.5-1$ ) were concentrated threefold in SC-URA and exposed to 50 µM AFB<sub>1</sub> for 4 h. After washing cells twice in H<sub>2</sub>O, aliquots were plated directly on SC-HIS to measure recombination, appropriately diluted and plated on YPD to measure viability. Protein extracts were prepared as previously described by Foiani et al. [22], separated on 10% acrylamide/0.266% bis-acrylamide gels for Rad53 detection, and transferred to nitrocellulose membranes. Rad53 was detected by Western blotting using goat anti-Rad53 (yC-19, Santa Cruz, Biotechnology, Santa Cruz, CA). The secondary antibody used was anti-goat IgG-HRP (Santa Cruz).

## RESULTS

### AFB<sub>1</sub>-Associated SCE Requires *RAD51*

AFB<sub>1</sub> efficiently stimulates homologous recombination in diploid yeast expressing human CYP1A2 and CYP1A1 [8]. Since, compared to wild type, AFB<sub>1</sub>-associated mutation frequencies increase in *rad51* haploid mutants [7], which are defective in homologous recombination, we investigated whether there was a common genetic control for AFB<sub>1</sub>-associated SCE and mutagenesis in yeast.

We measured mutation and unequal SCE in yeast strains (Table 1) expressing human CYP1A2 by selecting for Can<sup>R</sup> or His<sup>+</sup>, respectively (Figure 1). The spontaneous frequency of SCE in wild type and the *rad51* mutant was  $2 \times 10^{-5}$ , consistent with previous results [23], while the spontaneous mutation frequency was 10-fold higher ( $5 \times 10^{-5} / 5 \times 10^{-6}$ ) in the *rad51* mutant, compared to wild type. Previous experiments indicated 100-fold and 20-fold increase in translocation and gene conversion frequencies, respectively, in wild-type log-phase cells that were maintained in phosphate buffer while exposed to AFB<sub>1</sub> [18]. We detected no AFB<sub>1</sub>-associated SCE in wild-type log-phase cells that were maintained in phosphate buffer while exposed to AFB<sub>1</sub> (data not shown). We exposed log phase wild-type and *rad51* cells to AFB<sub>1</sub> for 4 h in growth (SC-URA) medium, as performed by Guo et al. [7]. While we detected a significant threefold and eightfold stimulation of SCE in wild-type cells after exposure to 15 and 50  $\mu\text{M}$  AFB<sub>1</sub>, respectively, we detected no significant stimulation of SCE in *rad51* cells. However, we did detect a higher net frequency of Can<sup>R</sup> mutants after exposure to 50  $\mu\text{M}$  AFB<sub>1</sub> ( $35 \times 10^{-6}$ ,  $n=4$ ) in *rad51* cells, compared to wild type ( $17 \times 10^{-6}$ ,  $n=4$ ), consistent with a previous study [7]. Thus, AFB<sub>1</sub> exposure stimulates *RAD51*-dependent SCE recombination in haploid cells that are maintained in growth medium at concentrations previously observed to stimulate both gene conversion events and directed translocations in diploid cells.

### *MEC1*-Dependent Rad53 Activation Correlates With AFB<sub>1</sub>-Associated SCE

Considering that *RNR2* and *DUN1* expression is induced in actively growing yeast cells exposed to AFB<sub>1</sub> [9], we next determined whether the *RAD53*-dependent signaling pathway that activates Dun1 was *MEC1*-dependent by following Rad53 phosphorylation after AFB<sub>1</sub> exposure (Figure 2). To obtain sufficient protein and minimize AFB<sub>1</sub> amounts, concentrated log phase cells ( $\sim 5 \times 10^7$  cells/mL) were exposed to AFB<sub>1</sub>. Similar to previous experiments, we detected greater than fourfold increase in recombination in wild-type cells. By western blots, we measured Rad53 (Chk2) phosphorylation in *mec1*, *dun1*, and wild-type cells after 4 h of AFB<sub>1</sub> exposure. Rad53 activation occurred in the *dun1* mutant, but was reduced or abolished in the *mec1-21* (missense) or *mec1-Δ* (null) mutants, respectively, confirming the requirement for the *MEC1* pathway as a response to DNA damaging agents [24]. These studies thus indicate that AFB<sub>1</sub> exposure coincidentally stimulates recombination and checkpoint activation.

### AFB<sub>1</sub>-Associated SCE and Mutation Require *MEC1*, *RAD53*, and *DUN1*

We then determined whether *MEC1*, *RAD53*, and *DUN1* gene functions were required for both AFB<sub>1</sub>-associated SCE and mutation. We found no increase in AFB<sub>1</sub>-associated SCE in *mec1*, *rad53*, and *dun1* mutants (Figure 3, left). The *DUN1* requirement was surprising, considering that UV-associated SCE is *DUN1*-independent [14]. Interestingly, while *rad53* and *mec1* mutants exhibit similar viability after AFB<sub>1</sub> exposure, we observed that *dun1* mutants were the most sensitive to AFB<sub>1</sub> (Figure 3, right). These studies indicate that AFB<sub>1</sub>-associated SCE requires *RAD53*, *DUN1*, and *MEC1*.

We then determined whether, similar to SCE recombination, AFB<sub>1</sub>-associated mutation also required *MEC1*, *RAD53*, and *DUN1* by measuring Can<sup>R</sup> mutants. The spontaneous frequency of Can<sup>R</sup> in wild type was  $5 \times 10^{-6}$ , similar to Guo et al. [7]. After exposure to 100  $\mu\text{M}$  AFB<sub>1</sub>,

we observed approximately sixfold increase in mutation frequency in wild type. However, in *dun1*, *mec1*, and *rad53* mutants, we observed at most a twofold increase in the frequencies of Can<sup>R</sup> mutants (Figure 3, center). We observed a 30-fold increase in the frequencies of UV-associated Can<sup>R</sup> in wild type and the *dun1* mutant after cells were exposed to 120 J/m<sup>2</sup> (data not shown, *n*=3). Thus, similar to SCE, we observed that AFB<sub>1</sub>-associated mutation requires *MEC1*, *RAD53*, and *DUN1*.

To determine that *dun1* phenotypes are recessive we introduced the *DUN1*-containing plasmid (pJW730) in the *dun1* strain by selecting for Trp<sup>+</sup> transformants. We then measured unequal SCE, mutation, and survival after the Trp<sup>+</sup> transformants were exposed to 100 μM AFB<sub>1</sub>. Compared to spontaneous frequencies, we observed fourfold ( $22 \times 10^{-6} / 5 \times 10^{-6}$ , *n*=3) and fivefold ( $9 \times 10^{-5} / 1.9 \times 10^{-5}$ , *n*=3) increase in mutation and recombination frequencies, respectively. Compared to the *dun1* mutant, we observed an approximately twofold increase in percent survival (51%/28%). These data indicate that extrachromosomal expression of *DUN1* suppresses *dun1* phenotypes.

*DUN1* has multiple functions including induction of RNR, regulation of the G2 checkpoint, and stabilization of stalled replication forks [25]. Since the *dun1* DNA damage sensitivity can be suppressed by mutation in the negative regulator of RNR, *SML1* [12], we determined whether the *dun1* mutation and recombination phenotypes could also be suppressed in a *dun1 sm11* mutant. We introduced CYP1A2 (pCS316) into a *dun1 sm11* strain and then measured survival, mutation, and recombination frequencies after exposure to 100 μM AFB<sub>1</sub>. The *dun1 sm11* mutant was as resistant (76% survival, *n*=5) as wild type. Compared to the spontaneous frequencies, we observed less than a twofold increase in the AFB<sub>1</sub>-associated frequencies of recombination [ $(8.3 \pm 5.4) / (6.5 \pm 5.4) \times 10^{-5}$ , *n*=4] and mutation [ $(13.7 \pm 4.5) / (8.7 \pm 4.2) \times 10^{-6}$ , *n*=5], respectively. We conclude that increasing deoxynucleotide triphosphate (dNTP) levels per se is insufficient to suppress the *dun1* defect in stimulating AFB<sub>1</sub>-associated recombination and mutation. However, considering that *sm11* mutations do suppress AFB<sub>1</sub>-associated lethality in *dun1*, higher dNTP levels increase survival after AFB<sub>1</sub> exposure.

### AFB<sub>1</sub>-N<sup>7</sup>-Gua and AFB<sub>1</sub>-FAPY DNA Adducts Can be Detected and Measured

We speculate that the AFB<sub>1</sub>-DNA adducts, such as the N<sup>7</sup>-Gua and FAPY-AFB<sub>1</sub> adducts, stall DNA replication. We therefore set out to detect AFB<sub>1</sub>-DNA adducts in both wild type and checkpoint mutants [20]. Log phase cultures of yeast expressing human CYP1A2 were exposed to 50 μM AFB<sub>1</sub> for 4 h. DNA was then isolated from two independent samples of yeast cells, and subjected to acid hydrolysis. Both the AFB<sub>1</sub>-N<sup>7</sup>-Gua and AFB<sub>1</sub>-FAPY adducts were identified and measured by LC-ESI/MS/MS (Figure 4). The data indicate that the level of AFB<sub>1</sub> adducts are essentially the same for both the wild type and the *rad53* mutant (Table 2). We deduced the number of adducts to be ~1-2 for every 1000 kb. Thus, for each yeast genome equivalent, ~15-30 AFB<sub>1</sub> adducts are generated under these conditions.

## DISCUSSION

AFB<sub>1</sub> is a strong recombinagen but weak mutagen in yeast [8,18]. Higher AFB<sub>1</sub>-associated mutation frequencies in a haploid *rad51* mutant, compared to wild type [7], suggest that a common DNA lesion could stimulate either SCE or mutation. AFB<sub>1</sub> exposure stimulates SCE in lymphocytes and rat hepatoma lines (for review, see [26]); however, no similar studies have been done in yeast. Since AFB<sub>1</sub>-associated mutation requires *MEC1* [7] and RNR induction results from AFB<sub>1</sub> exposure [6], we determined whether AFB<sub>1</sub>-associated genotoxicity requires the *MEC1* (ATR), *RAD53*, *DUN1*-mediated pathway involved in increasing dNTP levels after DNA damage exposure. The major conclusion of this article is that both recombination and mutation require *MEC1*, *RAD53*, and *DUN1*; however, higher dNTP levels per se do not circumvent the *DUN1* requirement, indicating that other checkpoint pathway

functions, such as stabilizing replication forks, may be required for AFB<sub>1</sub>-associated genotoxic effects. We speculate that AFB<sub>1</sub>-associated mutation and SCE occur when the DNA replication apparatus encounters AFB<sub>1</sub>-DNA adducts.

The studies thus extended previous work indicating that *MEC1* and *RAD53* are required for mutagenesis [7] by showing that SCE and mutagenesis are jointly regulated. We observed that AFB<sub>1</sub>-associated Rad53 activation correlated with higher frequencies of SCE, and detected AFB<sub>1</sub>-N<sup>7</sup>-Gua and FAPY DNA adducts that have been previously suggested to impede DNA replication [6]. Indeed although AFB<sub>1</sub>-N<sup>7</sup>-guanine adducts are unstable, they were more abundant than FAPY DNA adducts after 4 h AFB<sub>1</sub> exposure, indicating that the former is present in growing cells. The protracted presence of these adducts, which are poor templates for DNA polymerases [6] may explain why cells exposed to AFB<sub>1</sub> exhibit elongated S phases [9]. The AFB<sub>1</sub>-associated Rad53 activation could also result from stalled replication forks [27]. We do not know, however, whether recombination precedes mutation, as suggested by observations that Rev1 (error-prone polymerase) accumulates during late S and G2 phases of the cell-cycle [28,29]. Further studies are necessary to determine whether defects in translesion synthesis lead to more AFB<sub>1</sub>-associated SCE events.

The *DUN1*-mediated checkpoint pathway for both AFB<sub>1</sub> mutation and recombination is not required for UV-associated mutation and SCE [14], but is required for mutagenesis in DNA replication mutants [30]. One possibility is that translesion polymerases bypass UV lesions, such as thymidine dimers [31], more easily compared to AFB<sub>1</sub>-associated DNA lesions. We speculate that the *DUN1* requirement for AFB<sub>1</sub>-associated events result from *DUN1* function in stabilizing DNA replication forks at replication blocks [25]. *DUN1* may be involved in repressing translation of *RAD5* RNA transcripts, and *RAD5* overexpression sensitizes cells to replication blocks [32]. *RAD5* is also required for template switching events, indicating a possible function at stalled replication forks [33]. Further experiments are necessary to determine whether AFB<sub>1</sub> specific adducts stall DNA polymerase.

The genetic requirements of AFB<sub>1</sub>-associated SCE are distinct from those for X-ray associated SCE, which is *DUN1*-independent [14], suggesting that the initiating lesion is not a double-strand break. Standard protocols to measure X-ray associated SCE use log-phase cells incubated in H<sub>2</sub>O (nutrient-depleted media), not nutrient medium, during the radiation exposure. These observations strengthen the idea that AFB<sub>1</sub>-associated SCE occurs as a consequence of the DNA replication fork encountering the AFB<sub>1</sub>-associated DNA lesion.

Although other types of AFB<sub>1</sub>-associated genotoxic events, such as translocations, require *MEC1* [8], we do not know whether the AFB<sub>1</sub>-DNA adducts that initiate SCE are the same as those that initiate translocations and gene conversion events [8,18]. Fifteen DNA repair genes were induced in cells exposed to AFB<sub>1</sub> in phosphate buffer [8], while only *RAD51* and *RAD54* were induced in cells exposed in nutrient media [9]. It will be important to determine whether different genotoxic events following AFB<sub>1</sub> exposure are the consequence of the induction of different DNA repair genes or of different DNA adducts. Further experiments are also necessary to determine whether other AFB<sub>1</sub>-associated recombination events require *RAD53* and *DUN1*.

In conclusion, we have found that both AFB<sub>1</sub>-associated recombination and mutation require *MEC1*, *RAD53*, and *DUN1* and coincide with Rad53 activation. Although AFB<sub>1</sub>-DNA adducts have been measured in yeast [34], this is the first study to positively identify specific AFB<sub>1</sub>-DNA adducts in yeast, and indicate that the unstable AFB<sub>1</sub>-N<sup>7</sup>-Gua can be detected after a 4 h exposure time. Future studies may reveal how individual adducts are repaired and contribute to AFB<sub>1</sub>-associated genotoxic effects in yeast. Our results may have some interesting implications for AFB<sub>1</sub>-associated genotoxicity in humans. For example, liver injury, resulting

in increased liver cell proliferation, correlates with a higher incidence of HCC. We speculate that the DNA replication in the presence of AFB<sub>1</sub>-DNA adducts may correspond to a higher level of AFB<sub>1</sub>-associated genotoxicity in mammalian cells.

## ACKNOWLEDGMENTS

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## Abbreviations

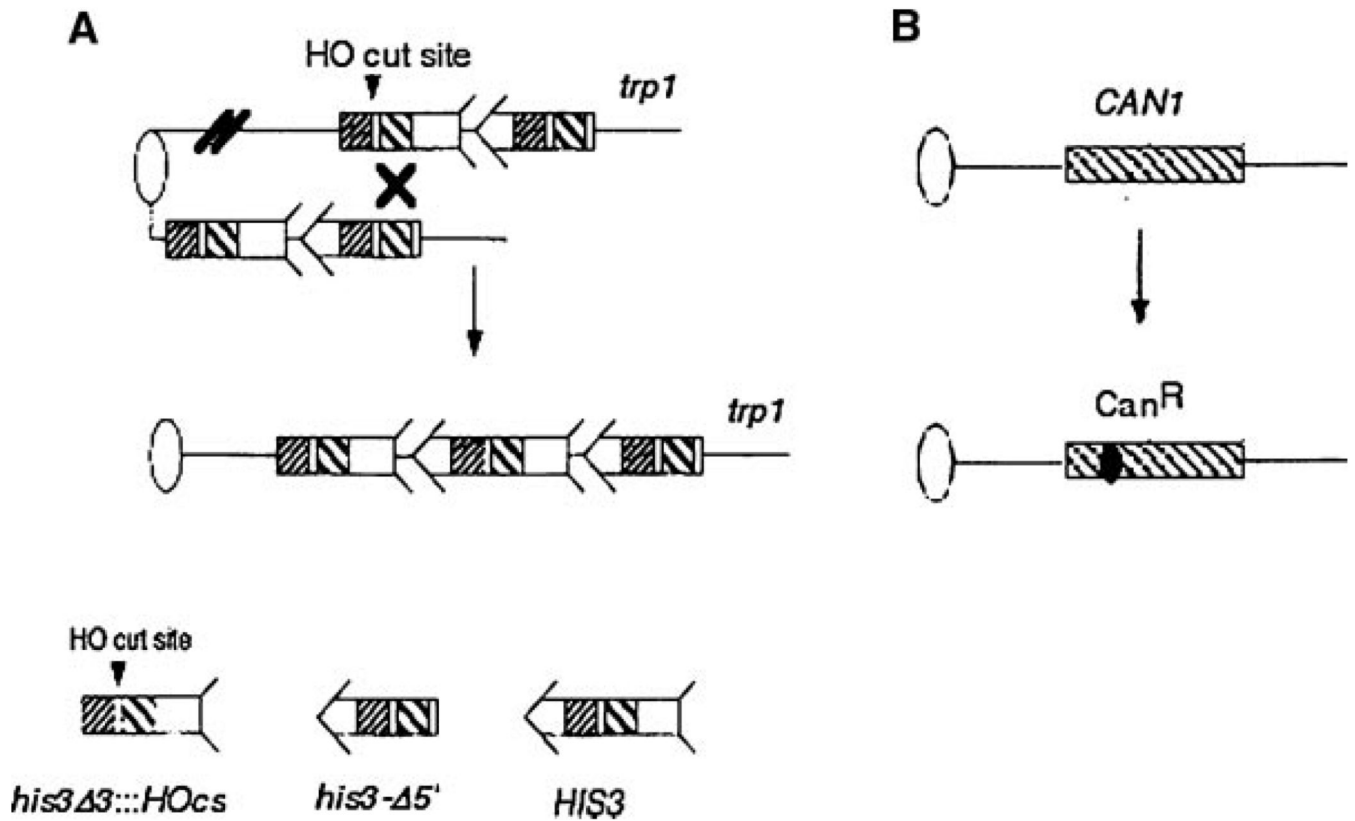
HCC, hepatocellular carcinoma; AFB<sub>1</sub>, aflatoxin B<sub>1</sub>; AFB<sub>1</sub>-*N*<sup>7</sup>-Gua, 8,9-dihydro-8-(*N*<sup>7</sup>-guanyl)-9-hydroxyaflatoxin B<sub>1</sub>; FAPY, formamidopyrimidine; RNR, ribonucleotide reductase; ATR, ataxia telangiectasia and Rad3 related; SCE, sister chromatid exchange; CYP1A2, cytochrome P450 1A2; LC-ESI/MS/MS, liquid chromatography-electrospray ionization tandem mass spectrometry; dNTP, deoxynucleotide triphosphate.

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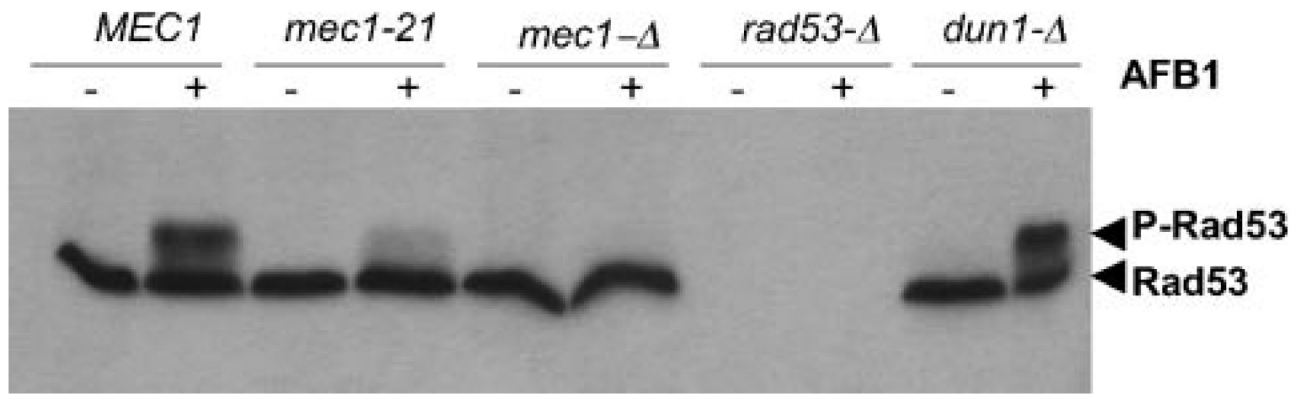
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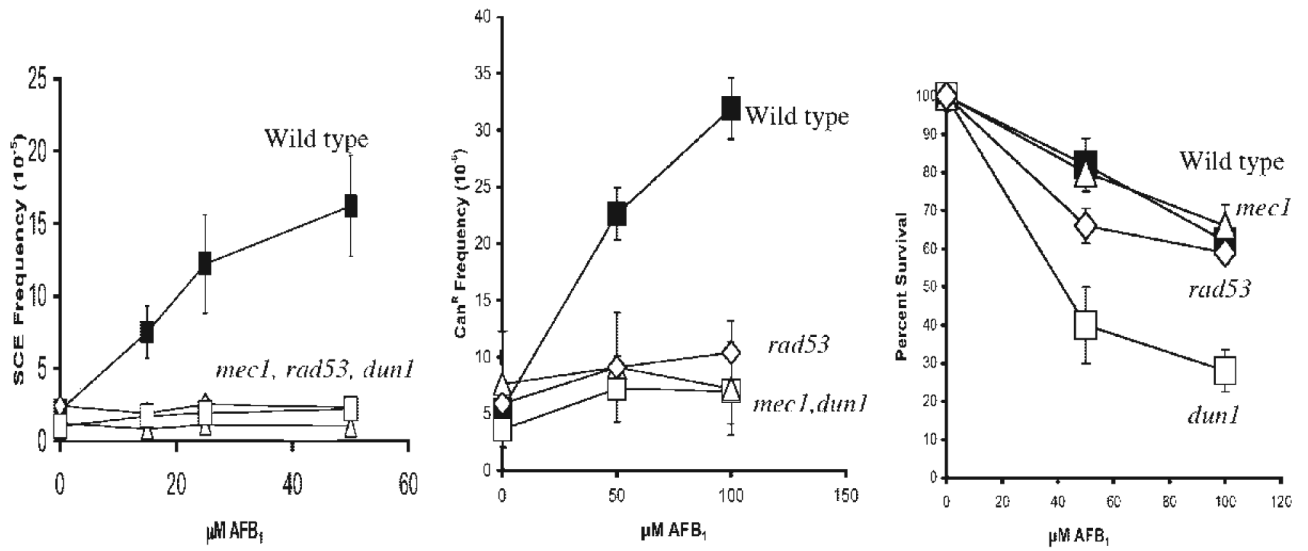
**Figure 1.**

Recombination and mutation assay used in this study. The oval represents the centromere and the single line represents duplex DNA. For simplicity, the left arm of chromosome IV is not shown. (A) Unequal sister chromatid recombination is monitored by selecting for His<sup>+</sup> prototrophs that result from recombination between the juxtaposed, truncated *his3* fragments. The *his3-Δ3'* lacks the 3' sequences (arrow head), while the *his3-Δ5'* lacks to promoter sequences (feathers). Both *his3* fragments are located with the amino acid reading frames oriented to the centromere. The *his3* fragments share a total of 450 bp sequence homology. (B) The mutation assay is a forward mutation assay by selecting for canavanine resistance.

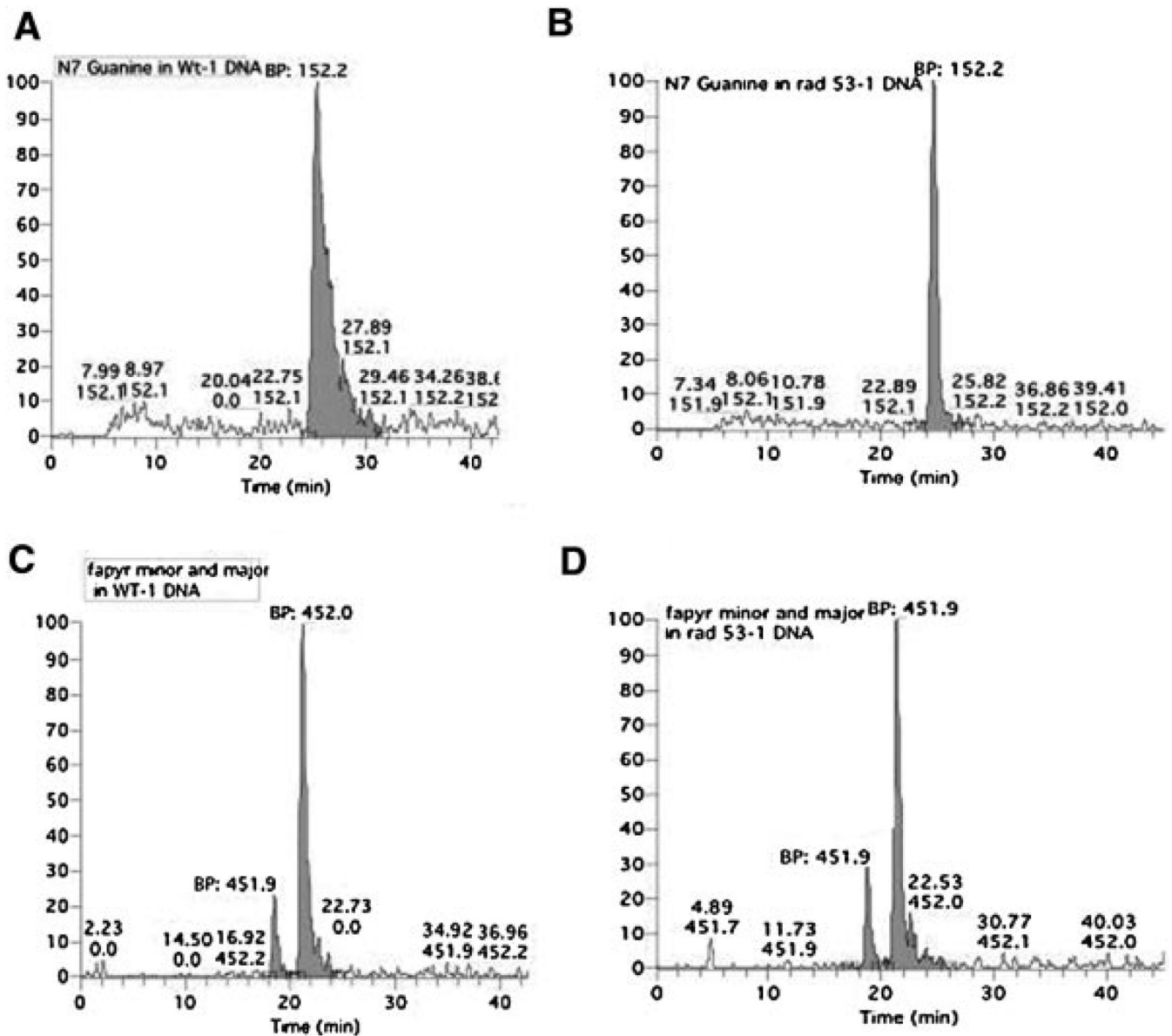


**Figure 2.**

AFB<sub>1</sub>-associated Rad53 activation occurs in wild-type and *dun1* cells but not in *mec1* null mutants. Cells were exposed to 50 μM AFB<sub>1</sub> for 4 h and Rad53 activation was measured in the wild type (YB163), *dun1* (YB387), *mec1-21* (YB385), and *mec1* null (YB386) mutants. The arrow points to phosphorylated Rad53.



**Figure 3.** Genetic requirements for AFB<sub>1</sub>-associated mutagenesis and recombination. Mutation frequency is plotted against AFB<sub>1</sub> concentration. The total exposure time is 4 h. Survival is plotted against AFB<sub>1</sub> concentration. The solid black square is wild type (YB163), the open black square is *dun1*(YB387), the open triangle is *mec1*(YB86), and the open diamond is *rad53* (YB353). Genotypes are given next to the symbols.



**Figure 4.** *N*<sup>7</sup>-Gua AFB<sub>1</sub> and FAPY AFB<sub>1</sub> adducts present in wild type (YB163) and *rad53* (YB230) strains. AFB<sub>1</sub> adducts were identified by LC-ESI/MS/MS. Relative abundance is plotted against time of detection; adduct quantities were computed from shaded areas under base peaks. DNA from both wild type (A,C) and *rad53* (B,D) strains were obtained after cells were exposed to 50 μM AFB<sub>1</sub> for 4 h. Base peak (BP) m/z 152.2 (A,B) corresponds with AFB<sub>1</sub>, while m/z 452 (C,D) corresponds with major and minor FAPY-AFB<sub>1</sub> adducts.

**Table 1**

Yeast Strains\*

Strain	Genotype	Plasmid	Source
YB353	MAT $\alpha$ ura3-52 his3- $\Delta$ 200 ade2-n trp1- $\Delta$ 1 gal3 <sup>-</sup> leu2-3,112 GAL1::his3- $\Delta$ 5' trp1::his3- $\Delta$ 3'::HOcs lys2 <sup>'</sup> (leaky) sml1::KanMX rad53::LEU2	pCS316	This laboratory
YB163	MAT $\alpha$ -inc ura3-52his3- $\Delta$ 200 ade2-101 lys-801 trp1- $\Delta$ 1 gal3- trp1 ::[his3- $\Delta$ 3'::HOcs, his3- $\Delta$ 5']		This laboratory
YB384	MAT $\alpha$ rad51 leu2- $\Delta$ 1	pCS316	This laboratory
YB368	MAT $\alpha$ -inc mec1-21 leu2		This laboratory
YB385	MAT $\alpha$ -inc mec1-21 leu2	pCS316	pCS316 introduced in YB368
YB327	MAT $\alpha$ -inc mec1- $\Delta$ ::TRP1 sml1::KanMX		This laboratory
YB386	MAT $\alpha$ -inc mec1- $\Delta$ ::TRP1 sml1::KanMX	pCS316	pCS316 introduced in YB327
YB230	MAT $\alpha$ -inc leu2- $\Delta$ 1 mec2-1(rad53)::LEU2		This laboratory
YB387	MAT $\alpha$ -inc dun1::KanMX	pCS316	
YB388	MAT $\alpha$ -inc dun1::KanMX	pCS316 + pJW730	pJW730 in YB387
YB389	MAT $\alpha$ -inc dun 1::KanMX sml1::URA3		
YB390	MAT $\alpha$ -inc dun 1::KanMX sml1::ura3	pCS316	pCS316 introduced in a FOA <sup>R</sup> derivative of YB389

\* Strains listed below YB163 have the same genotype as YB163 unless indicated.

**Table 2**  
Concentrations of AFB<sub>1</sub>-DNA Adducts in Yeast DNA

Yeast genotype (strain) <sup>a</sup>	AFB <sub>1</sub> -N <sup>7</sup> -Gua adducts/ mg DNA <sup>b</sup> (nmol)	FAPY adducts/mg DNA <sup>c</sup> (nmol)	Ratio <sup>d</sup>
Wild type (YB163)	$4 \times 10^{-3}$	$1.1 \times 10^{-3}$	4:1
<i>rad53</i> (YB320)	$4.5 \times 10^{-3}$	$2.3 \times 10^{-3}$	2:1

<sup>a</sup> Relevant genotype respect to *RAD53*, both strains derived from S288c. See Table 1 for complete genotype.

<sup>b</sup> AFB<sub>1</sub>-N<sup>7</sup> Gua, 8,9-dihydro-8-(N<sup>7</sup>-guanyl)-9-hydroxyafatoxin B1; *n* = 2.

<sup>c</sup> FAPY, formamidopyrimidine; FAPY determined for one sample of each genotype.

<sup>d</sup> Ratio = major AFB<sub>1</sub>-N<sup>7</sup>-Gua adduct: FAPY adduct.