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# Genotoxic Assays for Measuring P450 Activation of Chemical Mutagens

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## Abstract

This review discusses using yeast as a model organism for studying the biological effects of P450-mediated metabolism of xenobiotics. We discuss the challenges of testing the safety of thousands of chemicals currently introduced into the market place, the limitations of the animal systems, the advantages of model organisms, and the humanization of the yeast cells by expressing human cytochrome P450 (CYP) genes. We discuss strategies in utilizing multiple genetic endpoints in screening chemicals and yeast strains that facilitate phenotyping CYP polymorphisms. In particular, we discuss yeast mutants that facilitate xenobiotic import and retention and particular DNA repair mutants that can facilitate in measuring genotoxic endpoints and elucidating genotoxic mechanisms. New directions in toxicogenetics suggest that particular DNA damaging agents may interact with chromatin and perturb gene silencing, which may also generate genetic instabilities. By introducing human CYP genes into yeast strains, new strategies can be explored for high-throughput testing of xenobiotics and identifying potent DNA damaging agents.

**Keywords:** cytochrome P450 polymorphisms, genotoxins, budding yeast, recombination assays

## 1. Introduction

Genotoxins are generally referred to as chemical agents that cause DNA damage, which, in turn, can initiate recombination or mutation events or chromosome loss [1]. While mutagens and recombinagens are genotoxic, not all genotoxins are directly mutagenic [2]. Genotoxic exposure has been correlated to birth defects [3], cardiovascular disease [4], carcinogenesis [5], and accelerated aging [6]. Public health depends on minimizing exposure to genotoxic chemicals. Nonetheless, thousands of chemicals have yet to be tested, and new chemicals are annually synthesized. Federal agencies mandate that all chemicals be tested for safety before being introduced into the marketplace [7]. Generally, this testing has involved rapid screens for bacterial mutagenesis, micronuclei assays or comet assays for testing DNA fragmentation, and animal testing for determining carcinogenicity. Animal testing is often expensive and time-consuming and has increasingly raised ethical concerns. While microbial plate assays, such as the Ames test [8], have been standard in identifying chemical mutagens, some chemicals that test negative in

the Ames assays are carcinogenic, while others that test positive in the Ames assays are not carcinogens [8, 9]. Many chemicals are not genotoxic per se but require metabolic bioactivation [10]. The bioactivated compound is generally a highly reactive intermediate in a pathway which renders hydrophobic compounds more hydrophilic to facilitate excretion. While bioactivation does occur in specific animal models, toxicity outcomes differ depending on the species [11]. Thus, there is a need for metabolic competent cell-based assays that can measure multiple genotoxic endpoints.

Bioactivation occurs by phase I and phase II enzymes; phase I enzymes generally hydroxylate compounds so that phase II enzymes can conjugate larger molecules, facilitating the export and excretion of the modified compound. Phase I enzymes include cytochrome P450 monooxygenases (CYPs), which compose a superfamily of over 50 genes, and catalyze the formation of highly reactive electrophiles and epoxides, as intermediates in xenobiotic metabolism [12, 13]. Up to 80% of all bioactivations require CYPs [14]. For catalytic efficiency, the CYP proteins must be reduced by oxidoreductases, which are colocalized with CYPs in the endoplasmic reticulum (ER [15]).

*Saccharomyces cerevisiae* (budding yeast) is an excellent eukaryotic model organism for studying the genotoxicity of xenobiotics, including pharmaceuticals, pesticides, insecticides, and suspected carcinogens. Similar to bacterial organisms, yeast strains are easy to culture, grow rapidly, and can be manipulated genetically, rendering it possible to perform high-throughput analysis [16]. Many yeast genes are similar to human genes, and approximately 30% of can be functionally replaced by the human orthologue [17, 18]. DNA repair pathways and genes are also similar [17, 16]. Mitochondrial genotoxicity can also be measured [19]. Thus, identifying genotoxins and understanding their mechanisms in budding yeast can elucidate whether similar mechanisms occur in human cells.

However, yeasts also have some disadvantages. First, yeast cells contain a cell wall that blocks entry to carcinogens, and higher chemical concentrations are required in yeast than in mammalian organisms to observe similar genotoxic endpoints [1, 20]. Second, yeast lacks some functions of mammalian cells; while there are many yeast genes that have human homologs, other human DNA repair genes, such as p53, BRCA1, and BRCA2, have no corresponding yeast homologs. Nonetheless, the ability to modify the yeast genome has enabled yeast biologists to enhance carcinogen uptake and retention in cells [20, 21].

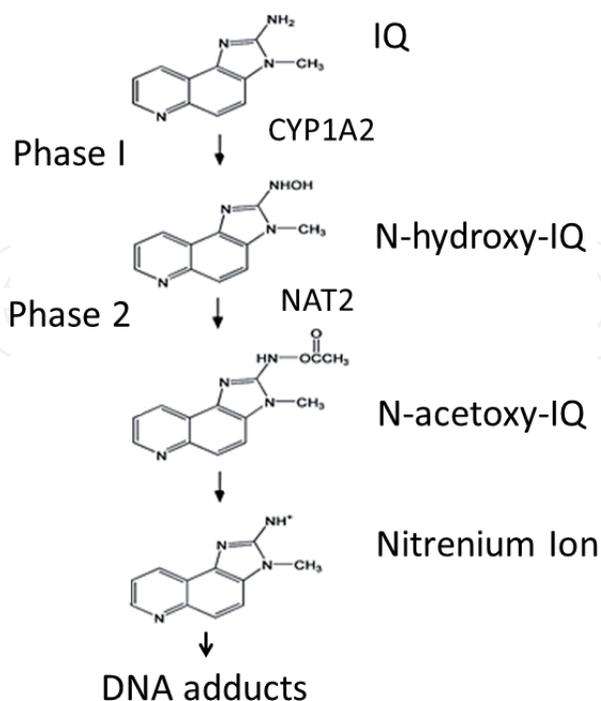
Engineered yeast strains enable high-throughput screens for identifying genotoxins among the thousands of novel synthetic chemicals, circumventing the limitations and reducing the escalating costs of animal tests. By expressing specific human cytochrome P450 genes in the engineered strains, tissue-specific metabolic activation can be simulated. Besides identifying genotoxins, engineered yeast strains can elucidate genotoxic mechanisms by measuring multiple genetic alterations, as well as DNA and organelle damage. Future engineering of yeast strains may identify additional human metabolic genes that can confer resistance to P450-activated genotoxins.

This review will address (1) characterization of human CYPs that activate the majority of carcinogens, (2) yeast vectors that have been engineered to express these CYPs, (3) plate and reporter assays that have been used to detect CYP-dependent activated compounds in yeast, (4) chemicals which have been identified and mechanistic insights that have been garnered by utilizing yeast genetics, (5) studies that have phenotypes P450 polymorphisms, (6) comparisons with other model eukaryotes, and (7) future directions in guiding genotoxic assays.

## 2. Phase I and phase II enzymes that bioactivate xenobiotics

While 57 CYPs have been identified, approximately 80% of all bioactivation is mediated by just 7 CYPs: CYP1A1, 1A2, 1B1, 2A13, 2A6, 2E1, and 3A4 [22]. Xenobiotic chemicals that are activated by these CYPs include polycyclic aromatic hydrocarbons (PAHs), aryl- and heterocyclic amines (HAAs), and nitrosamines, as well as small molecules such as benzene, naphthalene, and furans [22]. Examples of CYP-activated xenobiotics include tobacco carcinogens, industrial solvents, and food carcinogens, including the most potent liver carcinogen, aflatoxin B1 (AFB1). The importance of individual CYPs is underscored by observations that particular knockout mice are more resistant to environmental carcinogens, for example, fewer tumors arise in *Cyp1b1*<sup>-/-</sup> and *Cyp2a5*<sup>-/-</sup> knockout mice after exposure to 7,12-dimethylbenz[a]anthracene [23] and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [24], respectively.

Phase II enzymes include glutathione S-transferases, N-acetyl transferases, epoxide hydrolases, glucuronidases, and sulfotransferases. They serve to both inactivate highly reactive intermediates that are formed by phase I enzymes and conjugate larger molecules onto the products of phase I reactions to facilitate export and excretion [25]. While some phase II enzymes, such as glutathione S-transferases (GSTs), may inactivate epoxide intermediates, other phase II enzymes, such as N-acetyl transferases (NATs), may facilitate the conversion of hydroxylated heterocyclic aromatic amines to highly nitrenium ions (**Figure 1** [25]). For example, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) is hydroxylated by CYP1A2 in the liver [26]. Further modification by NAT2 acetylates the hydroxylated product, resulting in an unstable intermediate yielding a reactive nitrenium ion; this nitrenium ion reacts with DNA yielding deoxyguanine DNA adducts [25, 26]. Pathways by which activated HAAs drive colon carcinogenesis are unclear. One notion is that reactive IQ metabolites can also be glucuronidated in the liver and excreted into



**Figure 1.**

*Phase 1 and phase 2 metabolism of IQ. CYP1A2 generates the formation of N-hydroxy IQ. NAT2 generates an unstable molecule, N-acetoxy-IQ, which then generates a reactive nitrenium ion. The reactive nitrenium ion interacts with DNA to form adducts, particularly at the C8 and C2 positions of guanine. Figure was adapted from Kim and Guengerich [25].*

the colon. Gut microbial glucuronidases then cleave the glucose and reactivate the compound leading to genotoxicity in the colon [27]. An alternative notion is that IQ is bioactivated to a mutagen in situ in the colon. Expression of multiple mammalian phase I and phase II enzymes may be important in rendering some compounds genotoxic; examples include CYP1A1 and epoxide hydrolase in the activation of benzo[a]pyrene and CYP2E1 and SULT1A1 for the activation of 2,5-dimethylfuran (DMF) and furfuryl alcohol (FFA) [5, 22].

When compounds are substrates for multiple CYPs or phase II enzymes, products of varying toxicity can be generated; examples of substrates include estradiol, N-acetyl-*p*-aminophenol (APAP or acetaminophen), and AFB1. In the case of estradiol, CYP1A2 and CYP3A4 predominately hydroxylate estradiol in the 2' position generating 2' hydroxyestradiol [28, 29], while CYP1B1 hydroxylates estradiol in the 4' position generating 4' hydroxyestradiol; further modification of 4'-hydroxyestradiol by peroxidases generates a highly reactive form that generates DNA adducts, while 2' hydroxyestradiol can be detoxified [29]. In the case of acetaminophen, most acetaminophen is converted to nontoxic forms by sulfotransferases and glucuronidases; CYP2E1 converts acetaminophen to N-acetyl-*p*-benzoquinone imine (NAPQI), which depletes glutathione levels, causes oxidative stress, and is highly toxic in the liver [30]. Since CYP2E1 is induced by alcohol, the combination of alcohol and acetaminophen can be lethal [29]. Regarding AFB1, AFB1 is metabolized by CYP1A2 and CYP3A4 into a toxic epoxide intermediate, while the extrahepatic CYP1A1 can also convert AFB1 to AFM, which is still genotoxic but not as carcinogenic [31]. Because expression and inducibility of CYPs vary among individuals and multiple CYPs are expressed in tissues, it can be difficult to identify which CYP(s) generates the activated genotoxin.

Yeast presents advantages in deciphering which human CYPs can metabolize genotoxins. First, the three endogenous yeast CYPs largely function to synthesize ergosterol or dityrosine synthesis [32, 33]. Second, expression of CYPs in yeast can be regulated by inducible promoters or by copy number, mitigating potential toxic effects of their expression [34, 35]. Considering that CYP proteins locate to the yeast ER, the entire CYP cDNA can be expressed without truncating the sequence that encodes the N-terminus, as it is necessary for efficient CYP expression in *Escherichia coli* [36]. Third, although CYPs are differentially degraded [37], they are sufficiently stable to activate carcinogens for extended time duration, circumventing problems of transient or variable expression observed in cultures derived from cryopreserved hepatocytes. Based on genotoxic endpoints that can be easily scored, it is possible to phenotype CYP polymorphisms and to determine whether their substrate specificities are altered.

## 2.1 Mammalian CYP expression in budding yeast

Yeast has been an attractive organism for the expression of heterologous proteins and useful for characterizing biochemical properties of mammalian cytochrome P450 properties. Its success at producing large quantities of human proteins, such as human insulin [38], has largely been due to an advanced understanding of both the transcriptional and translational machinery of eukaryotic gene expression, including well-characterized transcriptional promoters and terminators [39]. Constitutive promoters for expression include *ADH1*, *GAPDH*, *PGK1*, *TPI*, *ENO*, *PYK1*, and *TEF*, while inducible promoters include *GAL1-10*, *CUP1*, *PHO5*, and *ADH* [40]. Expression can be further amplified by high-copy-number vectors or modulated by single-copy vectors, which have been well-described in the literature [34]. Oeda et al. [41] expressed rat CYP2E1cDNA using the constitutive *ADH1* promoter and the phosphoglycerol kinase (pGK) terminator using a high-copy-number vector.

The ability to bind carbon monoxide (CO) confirmed the presence of the recombinant protein. Characterization of human CYP3A4 produced in yeast underscored its broad substrate specificity [42, 43]. Additional in vitro studies involved expressing either human or rat cDNAs of CYP1A1, CYP2E1, CYP1A2, and CYP1B1 and confirming their biochemical properties [43]. A list of CYPs that have been expressed in yeast is shown in **Table 1**.

While inserting mammalian cDNA into expression vector by standard molecular techniques of subcloning can be tedious, many mammalian CYP cDNAs are now commercially available in gateway compatible DNA vectors. Gateway compatible vectors contain small segments of DNA, referred to as attP and attB sites, which

CYP gene	Expression vector	Enzymatic assays	Carcinogen activation	Genotoxic assays/ biosensor reporter	References
CYP1A1	pSB229, pRS424 CYP1A1	EROD <sup>1</sup>	BaP-DHD <sup>4</sup>	HR <sup>9</sup> , mutation, growth curves	[46, 50, 51]
			AFB1 <sup>5</sup>	HR, mutation, growth curves Yfp-Rad51 foci	[46, 50, 51]
			IQ <sup>6</sup>	Growth curves	[51]
CYP1A2	pCS316, pAAH5N	EROD, MROD <sup>2</sup> , LND <sup>3</sup>	AFB1	HR, mutation Yfp-Rad51 foci	[43, 50]
			MeIQx <sup>7</sup>	HR	[87]
			IQ	HR	[87]
CYP1A2/ NAT2	pGP100	MROD, SMZ assay	IQ, MeIQx, MeIQ	HR	[87]
CYP1B1	pYES2, pAG24	EROD	AFB1, BaP-DHD		[111]
CYP2A6	pAAH5N	LND			[43]
CYP2B6	pAAH5N, pESC-URA3	LND, 7-ethoxycoumarin- 3-carbonitrile deethylation	N-nitrodimethyl amine		[43]
			AFB1	RAD54- GFP	[71]
CYP2C8	pAAH5N	LND			[43]
CYP2C9	pAAH5N	Lauric acid (omega-1)- hydroxylation			[43]
CYP2C18	pAAH5N	LND			[43]
CYP2D6	pAAH5N, pESC-URA3	LND, debrisoquine 4-hydroxylation, ethoxycoumarin- 3-carbonitrile deethylation		RAD54- GFP	[71]

CYP gene	Expression vector	Enzymatic assays	Carcinogen activation	Genotoxic assays/ biosensor reporter	References
CYP2E1	pAAH5N	Lauric acid (omega-1)-hydroxylation			[43]
CYP3A4	pAAH5N, pMA34, pESC-URA3	Diclorofenac, testosterone 6 $\beta$ -hydroxylation, ethoxycoumarin- 3-carbonitrile deethylation	AFB1	HR, growth curves RAD54-GFP, RNR3-GFP	[67, 71]
			IQ		[67]
			BaP <sup>8</sup>	RAD54-GFP	[71]

<sup>1</sup>Ethoxyresorufin deethylase (EROD).

<sup>2</sup>Methoxyresorufin demethylase (MROD).

<sup>3</sup>Lidocaine N-deethylation (LND).

<sup>4</sup>Benzo[a]pyrene 7,8, dihydrodiol (BaP-DHD).

<sup>5</sup>Aflatoxin B1 (AFB1).

<sup>6</sup>2-Amino-3-methylimidazo-[4,5-f]quinoline (IQ).

<sup>7</sup>2-Amino-3,8-dimethylimidazo-[4,5-f]quinoxaline (MeIQx).

<sup>8</sup>Benzo[a]pyrene,

<sup>9</sup>homologous recombination (HR).

**Table 1.**

Human P450 genes that have been expressed in yeast.

flank the insert and are substrates for site-specific recombinases [44]. CYP cDNAs inserted into donor vectors can then be transferred into recipient yeast expression vectors by mixing the appropriate DNAs with recombinases; these reagents are commercially available and eliminate protocols using restriction enzymes and ligase. Recipient yeast expression vectors include multi-copied vectors as well as inducible and constitutive promoters [44]. An additional mechanism to increase CYP expression is to enhance translation of mRNA; Kozak sequences can be inserted into DNA sequences that encode mRNA upstream untranslated regions (UTR) [45].

## 2.2 Assays for detecting CYP expression

Enzymatic assays to measure CYP activity have often relied on converting non-fluorescent substrates into fluorescent products or measuring products by high-performance liquid chromatography (HPLC). Fluorescent products can be measured in a 96-well plate on a plate reader. The assay mix involves NADPH or a NADPH-regenerating system, such as glucose dehydrogenase; the pH is critical so the assay mix must be carefully buffered [46]. Microsome preparations of cytochrome P450s from yeast involve lysing cells using glass beads, centrifugation to remove debris, and precipitating microsomes using NaCl and polyethylene glycol [47]. These microsome fractions can be further concentrated by ultracentrifugation and stored at  $-80^{\circ}\text{C}$  for extended time periods. Activity measurements are expressed as picomole of product per minute per mg protein; more precise measurements of CYP protein concentration can be obtained by measuring absorbance at a 450nm wavelength after the sample has been exposed to CO.

To optimize mammalian enzyme activity in yeast cells, it is necessary to co-express the CYP, human oxidoreductase (hOR), and cytochrome B (cytB)

oxidoreductase [48]. Because yeasts contain endogenous oxidoreductases [49], the overexpression of the hOR is not a requirement for expression of all CYPs but generally does enhance CYP activity. For example, expression of hOR is required to measure CYP1A1 but not CYP1A2 activity [49, 50]. Other investigators have shown that the insertion of hOR directly in the genome is sufficient to obtain extracts to monitor the activity of CYP1A1 and CYP3A4 [51, 52].

### 3. Genotoxic assays

To be proven positive, the genotoxic effects must be dose dependent and reproducible. Examples of genotoxic agents include those that directly bind to or modify DNA, induce reactive oxygen species (ROS), and inhibit topoisomerases and other proteins involved in DNA metabolism. These genotoxic agents can cause a multiplicity of DNA insults, including DNA base modifications, DNA adducts, cross-links, and single- and double-strand breaks. Different DNA damage insults can quantitatively result in different biological endpoints. For example, a single double-strand break is sufficient to initiate genome rearrangements and trigger cell cycle arrest [53], while other types of DNA damage, such as particular cross-links and abasic sites, are effectively tolerated by DNA replication bypass pathways (for reviews, [54]). These replication bypass pathways include template switching and error-free polymerase switch mechanisms that may not trigger cell cycle arrest or a DNA damage response [54]. Thus, there is a need for measurements of multiple genotoxic endpoints to accurately assess the biological effect of any genotoxin.

Genotoxic endpoints include direct measurements of DNA damage and DNA adducts, reporter assays that detect transcriptional induction of DNA damage-inducible genes, growth assays for monitoring fitness [55], and plate assays for detecting recombination and mutations. Reporter assays involve yeast strains that contain a DNA damage-inducible promoter linked to a protein tag whose fluorescence or activity can be readily detected. Examples of proteins whose activity can be readily measured include lacZ, encoding  $\beta$ -galactoside, and GUS encoding  $\beta$ -glucuronidase (reviewed in [1]). Signaling assays have been successfully employed for high-throughput analysis using 96-well plate platforms and flow cytometry. The plate assays can elucidate endpoints of genotoxicity, while reporter assays can identify a chemical as a genotoxic assay and establish minimum concentrations in which a chemical may have an effect. Plate assays have been successful in measuring multiple genotoxic endpoints, including mutation [56, 57], homologous recombination [2], retrotransposition [58], and gross chromosomal rearrangements [59]. Plate assays involve inoculating engineered yeast strains on selective media, and after an incubation period, selected colonies can be counted and viability can be measured on nonselective media.

Direct assays to measure DNA strand breaks include chromosomal DNA integrity by pulse-field electrophoresis [60] and by single-cell comet assays [61]. Pulse-field electrophoresis has been successfully used to monitor repair of radiation-induced double-stranded DNA and the integrity of rDNA. Single-cell comet assays involve exposing cells to chemical agents, embedding them in agarose, subjecting them to an electric field, and staining for DNA [61]. Fragmented DNA migrates faster in an electric field, and the fragmented DNA appears as a "tail" [62]. Chemical DNA adducts, such as AFB1-N7-guanine adducts, can be detected using high-performance liquid chromatography, mass spectrometry (LC/MS-MS) after cells have been lysed and DNA has been extracted and acid hydrolyzed [63, 64].

### 3.1 Reporter assays

Reporter assays with fluorescent readouts are useful in detecting cells that have been exposed to genotoxins that induce DNA damage. Fluorescence can be monitored using 96-well plates, rendering it possible to perform high-throughput analysis. Fluorescent cells can also be imaged using flow cytometry platforms, such as the Amnis Image Stream [65], which can also measure cell type, DNA content, and cell cycle stages. DNA damage reporters include *RAD54*-GFP [66], *RNR3*-GFP [67], and *HUG1*-GFP [68]. These fusions have been widely used because signal-to-noise ratio is very low. *RAD54* is a DNA repair gene that functions in recombinational repair of double-strand breaks; GreenScreen assay (GSA) utilizes the *RAD54*-GFP reporter in high-throughput screens [69]. The RadarScreen assay uses a *RAD54*  $\beta$ -galactosidase reporter construct in which  $\beta$ -galactosidase cleaves the substrate into galactose and luciferin [70]. *HUG1* encodes an inhibitor of ribonucleotide reductase, while *RNR3* encodes the large subunit of ribonucleotide reductase that is specifically induced because of DNA damage [71]. The fluorescent markers can be enhanced using yEGFP, engineered for yeast codon bias. While *RNR3* and *RAD54* promoters have been extensively used, promoters for *PLM2*, encoding a putative transcription factor, and for *DIN7*, encoding a mitochondrial nuclease, have been recently reported to be more inducible for detection of genotoxins [1]. These studies indicate that there are robust reporters with sensitive readouts for screening genotoxins.

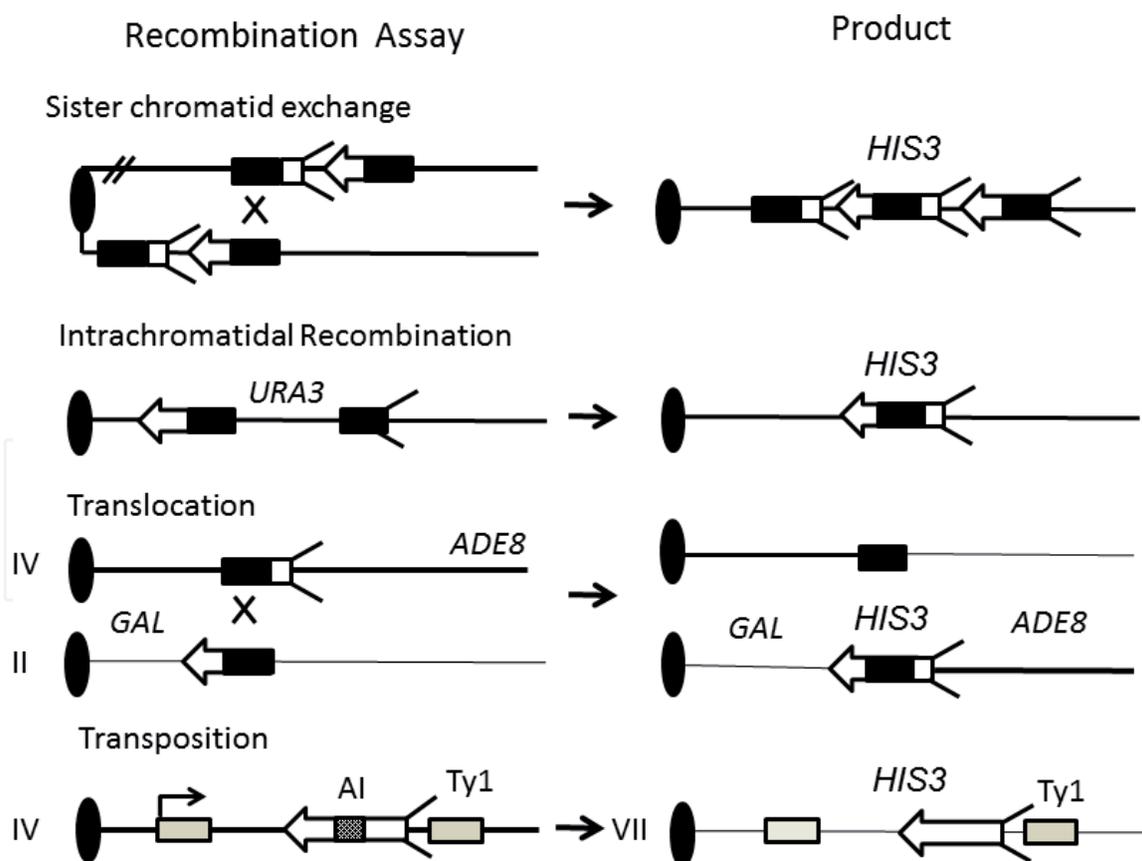
Genotoxins that inhibit histone deacetylases, such as Sir2, can be detected using reporters that detect expression of the silent mating-type locus (*HML*). In a strain containing one such fluorescent reporter, the cre recombinase gene was placed within the *HML* locus, while loxP recombination sequences were positioned flanking an intervening sequence that occluded a promoter from transcribing the GFP reporter [72]. Transient expression of cre triggers recombination at the loxP sites and excision of the intervening sequence, thus allowing the promoter to transcribe GFP. While this assay does not directly measure genotoxicity, inhibition of Sir2 can trigger rDNA instability [73].

### 3.2 Plate assays for detecting recombination, mutation, and microsatellite instabilities

Plate assays that detect mutation and recombination endpoints consist of selections or screens for prototrophic or drug resistance markers. Several genotoxic endpoints can be determined by color phenotypes. For example, Ade<sup>+</sup> colonies are white, while *ade2* and *ade3* colonies are red. Recombination between two non-revertible *ade2* and *ade3* alleles can be observed by visualizing colony sectors [74]. A similar scheme can also be employed for detecting mutations that affect the *SUP4-o* function in suppressing *ade2* nonsense alleles [75]. By choosing different prototrophic selections for individual assays, combinations of these assays in a single strain can facilitate measurements for multiple genotoxic endpoints, including mutation and recombination. Typically, the strains are diploids. Prototype strains included D7, which allows for measuring multiple number of mutation event [57]. For designing strains to detect DNA damage-associated homologous recombination between heteroalleles or between repeated sequences (ectopic recombination), gene editing and one-step gene replacement [76] rendered it possible to position non-revertible markers in tandem, on homologs or on nonhomologous chromosomes. For example, *ade2-a* and *ade2-n* alleles can be used for measuring intrachromatid gene conversion and recombination between homologs [74]. By deleting the entire wild-type gene, and positioning overlapping gene fragments at preselected

positions in the yeast genome, frequencies of rare recombinants can be selected. For example, *his3* gene fragments, *his3*- $\Delta 3'$  and *his3*- $\Delta 5'$ , can be positioned at predetermined positions in the genome and His<sup>+</sup> recombinants for measuring frequencies of unequal sister chromatid exchange (SCE), translocations, and intrachromatid deletions [77]. Schiestl et al. [2] used a diploid strain that contained a *his3* deletion on one chromosome and a disrupted *HIS3* gene to measure intrachromatid recombination; this strain has also been referred to as the “DEL” assay. The complete deletion of *HIS3*, *his3*- $\Delta 200$ , also enabled a selection for monitoring Ty1 transposition. Boeke et al. [78] inserted an artificial intron in an inverted orientation within *HIS3* contained within Ty1 so that His<sup>+</sup> cells could only result when retrotransposition of Ty1 occurred. Transposition of *HIS3*, as well as chromosomal rearrangements generated by recombination between *his3* fragments, can be physically characterized by pulse-field gel electrophoresis (Figure 2).

A plate assay that detects gross chromosomal rearrangements was devised in haploid strains. This assay involved two drug selection markers, *CAN1* and *URA3*, where the *URA3* gene was inserted near *CAN1* on the right arm of chromosome V at the *HXT13* locus; the right arm of chromosome V is not required for viability [79]. *CAN1* encodes the arginine permease gene and confers sensitivity to the arginine analog, canavanine, while *URA3* confers sensitivity to the drug 5-fluoro-orotic acid (FOA). Since the frequencies of spontaneous Can<sup>R</sup> FOA<sup>R</sup> is extremely low,  $3.5 \times 10^{-10}$  [79], most Can<sup>R</sup> FOA<sup>R</sup> selected colonies contain gross chromosomal rearrangements, in which deletions, translocations, or multiple rearrangements have



**Figure 2.** Recombination assays that are used in detecting DNA damage-associated recombination. The *HIS3* gene is shown with an arrow and feathers. The fragment that lacks the 3' end is shown without the arrow; the fragment that lacks the 5' end is shown without the arrow. The two fragments share approximately 300 bp of homology. The Roman numerals represent different chromosomes. The oval represents the centromere. For simplicity, the left arm of the chromosomes is not shown. In the transposition assay, an artificial intron (AI) is inserted in the *HIS3* so that it is in the opposite orientation as the *HIS3* promoter. For *HIS3* to be expressed, the Ty1 element must first be transcribed, the AI spliced from the mRNA, and the mRNA reverse transcribed and integrated into the chromosome.

occurred that conferred resistance to both drugs. However, because drug sensitivity is dominant,  $\text{Can}^R \text{FOA}^R$  recombinants are detected in haploid strains.

By combining different gene fragments and alleles, as well as drug-resistant markers, multiple genotoxic endpoints, including heteroallelic recombination, unequal SCE, translocations, and mutation, can be measured within a single strain. As an example, Fasullo et al. [64] designed a haploid strain useful in measuring frequencies of DNA damage-associated mutations and unequal SCE after exposure to AFB1. A useful diploid strain was also engineered for measuring frequencies of DNA damage-associated homolog recombination between heteroalleles and ectopic recombination between gene fragments on nonhomologous chromosomes [64]. While these plate assays can elucidate genotoxic endpoints, their noise-to-signal ratio can vary, depending on the frequencies of spontaneous events. While frequencies of spontaneous mutations at *CAN1* are relatively low,  $10^{-6}$ , the frequencies of spontaneous recombinants can vary from  $10^{-4}$  to  $10^{-10}$  [79]. Higher frequencies of spontaneous recombination are generally associated with intrachromosomal events, while lower frequencies of spontaneous recombination are associated with ectopic recombination between gene fragments on nonhomologous chromosomes. The lower the spontaneous frequency infers the higher the signal-to-noise ratio; thus, DNA damage-associated recombinants may be identified at exposures to lower concentrations of genotoxins.

While there are a multitude of plate assays for detecting nuclear genotoxic stress, there are fewer assays for detecting mitochondrial genotoxic stress. In part this is due to few auxotrophic markers, the high copy number (50–100) of mitochondrial DNA, and random segregation of mitochondria in mitosis [80]. Nonetheless, mitochondrial deficient yeast can be detected by the petite colony phenotype and the color phenotype of  $\text{Ade}^-$  mutants that appear pink or white in contrast to red on YPD media that is limiting in adenine [81]. In addition, Sia et al. [82] constructed a mitochondrial reporter gene *arg8(m)*. This reporter has poly(AT) or poly(GT) out-of-frame insertions within the coding sequence so that  $\text{Arg}^+$  prototrophs can be selected resulting from microsatellite instability.

While the plate and reporter assays are useful for detecting genotoxins and elucidating their mechanisms, yeast lacks many metabolic activities found in metabolically competent mammalian cells. Some protocols to activate carcinogens use rat S9 fractions, which may produce more metabolites than human CYPs, [83–85]. To mitigate this deficiency, human CYPs have been introduced into the strains for both plate assays and reporter assays. For example, Bui et al. [71] expressed CYP1A2, CYP2C9, CYP3A4, and CYP2D6 in a strain that monitors RAD54-GFP. Sengstag et al. [50] and Fasullo et al. [64, 67] have expressed CYP1A1, CYP1A2, and CYP3A4 in strains that monitor translocations, mutations, and unequal SCE. Guo et al. [86] have introduced CYP1A2 into multiple yeast mutants to determine AFB1 resistance. Paladino et al. [87] have expressed CYP1A2 and NAT2 to activate a variety of heterocyclic aromatic amine in strains to measure homology-directed translocations. Both CYP-containing reporter strains and plate assay strains expand the repertoire of chemicals that can be tested by high-throughput analysis.

#### **4. Chemicals that test positive in the yeast strains**

Overall, thousands of chemicals have been tested using either one or both plate and reporter-based assays [1]. Van Gompel et al. [69] report on the screening of 2698 proprietary compounds and pharmaceuticals using the GreenScreen assay; of these compounds, approximately 7% of those 164 that test positive are also positive in the Ames assays, demonstrating that agents that test positive represent

overlapping groups. Screens of industrial, environmental, and food carcinogens have used multitude tester strains, including the “DEL” and transposition assays [88]. Chemical agents include those that directly inflict DNA damage, induce ROS, inhibit DNA metabolic function, and alter histone modification. Metallic nanoparticles also test positive in several assays although their mechanism of action has yet to be determined [89]. Whereas almost all chemicals that test positive in plate assays will also test positive in reporter assays, the converse is not necessarily true. These results demonstrate that several reporter assays are capable of high-throughput screening and can identify multiple compounds that test positive in additional genotoxic assays.

Several agents that cause direct DNA damage, such as base pair damage, cross-links, DNA adducts, or DNA strand breaks, test positive in reporter assays and may test positive in one or more of the plate assays [90]. For example, alkylating agents, such as methyl methane sulfonate (MMS), increase frequencies of mutations, recombination, gross chromosomal rearrangements (GCRs), and retrotransposition. Interestingly, alkylating agents also test positive in enhancing expression of the silent mating-type locus *HML* [91]. Other types of alkylating agents, such as 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) and ethyl methane sulfonate (EMS), test positive in reporter and mutation assays; however, frequencies of DNA damage-associated sister chromatid exchange events are only modestly increased [58, 64, 67]. Cross-linking agents, such as cis-platinum and other UV-mimetic agents, also tend to be positive in a broad range of reporter assays, including those for retrotransposition and homologous recombination [90]. Finally, radiomimetic agents that cause strand breaks, such as zeocin and bleomycin, test positive in many reporter assays as well as assays for gross chromosomal rearrangements and translocations [59]. These studies demonstrate that while a subset of alkylating agents manifest broad genotoxicity, frequencies of DNA damage associated with GCRs and translocations manifest the highest increase after exposure to agents that cause double-strand breaks.

Chemical agents that inhibit DNA metabolic and repair functions are often genotoxic. These include camptothecin, which inhibits topoisomerase I and causes single-strand breaks and replication fork collapse, and hydroxyurea, which blocks DNA replication by inhibiting ribonucleotide reductase and thus depleting deoxynucleotides [92, 93]. Other metabolic inhibitors include those that inhibit dihydrofolate reductase, and result in uracil incorporation also tests positive in a broad range of plate assays, including those for sister chromatid recombination, heteroallelic recombination, and translocations.  $\text{Cd}^{2+}$  exposure inhibits mismatch repair [94] and is also genotoxic [95]. These studies indicate that genotoxins include chemicals that may directly inhibit critical enzymes in DNA metabolism.

While chemicals are individually screened in many plate and reporter assays, combination of chemicals can also enhance DNA damage or enhance mutagenesis. An example includes intercalating agents, such as acridine and bleomycin; the insertion of acridine in the DNA helix facilitates bleomycin access to the minor groove and subsequent strand breakage [96]. In addition, by inhibiting mismatch repair,  $\text{Cd}^{2+}$  exposure facilitates the mutagenesis by alkylating agents [94]. These studies indicate that combinations of genotoxins can accelerate genome instability.

Mitochondria are particularly prone to DNA intercalating agents, and agents that cause oxidative damage, and reduce or cause imbalance to deoxynucleotide pools [97]. ROS-associated damage in the mitochondrial genome, associated with oxidative phosphorylation, is not repaired by nucleotide excision repair (NER) but by base excision repair (BER) [1]. In addition, mitochondrial DNA is circular and therefore is more prone to DNA intercalating agents that can cause topological stress, such as ethidium bromide and acridine compounds [98]. Several fluorescent

CYP allele	Amino acid substitution	Enzyme assay	Disease association	Genotoxic endpoints	Reference
CYP1A1*2C	1462V (near heme binding site)	EROD <sup>1</sup>	Lung, prostate and breast cancer	Rad51 foci, growth curves, HR <sup>4</sup>	[51]
CYP1A1*4	T461N (near heme binding site)	EROD	Endometrial and lung cancer	Rad51 foci, growth curves, HR	[51]
CYP1A2*5	C406Y	MROD <sup>2</sup>	ND	Rad51 foci, growth curves, HR	[63]
CYP1A2*3	D348N	MROD	ND	Rad51 foci, growth curves, HR	[63]
CYP1A2*4	I386F	MROD	ND	Rad51 foci, growth curves, HR	[63]
CYP1B1*7	R48G; A119S; L432V; A443G	BaP-DHD epoxidation <sup>3</sup>	L432V has an increased risk for prostate and lung	NT	[110]
CYP1B1*12	G61E	BaP-DHD epoxidation	Glaucoma	NT	[110]
CYP1B1*18	G365W	BaP-DHD epoxidation	Glaucoma	NT	[110]
CYP1B1*23	P437L	BaP-DHD epoxidation	Glaucoma	NT	[110]
CYP2E1		Benzene hydroxylation		NT	[111, 112]
CYP2E1*2	<b>R76H</b>	Benzene hydroxylation	Bladder cancer (reduced risk in Asian population)	NT	[111, 112]
CYP2E1*3	V389I	Benzene hydroxylation	Bladder cancer (reduced risk in Asian population)	NT	[111, 112]
CYP2E1*4	V179I	Benzene hydroxylation	Bladder cancer	NT	[111, 112]

<sup>1</sup>Ethoxyresorufin deethylase (EROD).

<sup>2</sup>Methoxyresorufin demethylase (MROD).

<sup>3</sup>Benzo[a]pyrene 7,8, dihydrodiol (BaP-DHD).

<sup>4</sup>Homologous recombination (HR).

ND = not determined, NT = not tested.

**Table 2.**

*Cytochrome P450 polymorphisms expressed in yeast.*

dyes can also induce mitochondrial DNA damage [1]. Replication of mitochondrial DNA depends on a single polymerase, DNA polymerase  $\gamma$  [99]. Therefore, chemicals that inhibit mitochondrial DNA polymerase, such as dideoxynucleoside antiretrovirals, are often genotoxic [100]. Thus, yeast screens that detect mitochondrial DNA damage are useful in screening off-target effects on antiretroviral agents.

While many carcinogens are directly genotoxic, others require metabolic activation. The list of CYPs expressed in yeast and chemical agents that are activated are listed in **Table 2**. The agents tested include polyaromatic hydrocarbons (BaP-DHD), mycotoxins (AFB1), and heterocyclic aromatic amines (2-amino-3,8-dimethylimidazo-[4,5-f]quinoxaline (MeIQ<sub>x</sub>), 2-amino-3,4-dimethylimidazo-[4,5-f]quinoline (MeIQ), and 2-amino-3-methylimidazo-[4,5-f]quinoline (IQ)). Bui et al. [71] introduced human CYPs into strains to measure induction of GFP using the reporter *RAD54-GFP*. Li et al. [52] used a sensitive fluorimetric assay to measure inhibition of secreted dextranase; the assay consists of strains expressing *Lipomyces kononenkoae* amylase, CYP3A4, and hOR [52]. The amylase-expressing strain detected AFB1 at 2 ng/ml and the T-2 mycotoxin [52].

Activation of these compounds has also been determined by measuring DNA recombination and mutation; DNA adducts have been detected after AFB1 and BaP-DHD exposure. Frequencies of mutations and recombination may be differentially elevated by CYP-activated genotoxins. For example, CYP1A1 and CYP1A2 activation of AFB1 in yeast results in a 20–50-fold increase in the stimulation of recombination but only a fivefold increase in mutation frequency [50]. However, CYP1A1-mediated activation of BaP-DHD results in a higher activation of mutation but somewhat diminished activation of recombination [50]. Because the background frequency is so low, the CYP1A2-expressing strains containing the translocation assay have been particularly useful in detecting the DNA damage-associated recombinants [50].

## 5. Yeast mutants that exhibit enhanced phenotypes after genotoxin exposure

Various gene mutations can increase the signal-to-noise ratio. Typically, these mutations are encoded in cells lacking cell wall components, nucleotide or base excision repair genes, and xenobiotic transporters. Strains that lack cell wall components and xenobiotic transporters include *pdr5*, *snq2*, *cwp1*, *cwp2* [1]. Strains that lack NER and BER genes include those mutated in *rad1* and *mag1*, respectively. Several strains also contain deletions in *yap1* [101], a gene that encodes a transcription factor that confers transcriptional induction among antioxidant genes, such as *TRX2*, and is required for H<sub>2</sub>O<sub>2</sub> and Cd resistance [102]. Several strains have been designed so that multiple genes are mutated to enhance the genotoxic signal. Wei et al. [103] have used a septuplet deletion mutant (*snq2*, *prd5*, *cwp1*, *cwp2*, *yap1*, *rad1*, *mag1*) in combination with an integrated HUG1- $\gamma$ EGFP reporter as a very sensitive detection for multiple chemicals. Deletion of NER genes has been successful in enhancing DNA damage-associated recombination after exposure to BaP-DHD and AFB1 [104].

While deleting DNA repair genes may enhance signal-to-noise ratios for reporter assays and some recombination and mutation assays, particular DNA repair defects may decrease frequencies of DNA damage-associated recombination in particular plate assays. For example, blocking nonhomologous end joining (NHEJ) may increase homologous recombination initiated by double-strand breaks in haploid strains, while decreasing DSB-associated translocations [105]; the likely explanation is that competing DNA repair pathways for recombination are differentially favored for homologous vs. NHEJ. *Rad1* mutations may confer lower DNA damage-associated recombination in assays, such as the “DEL” assay [106]. The *rad1* mutants are defective in the Rad1/Rad10 nuclease; this nuclease cleaves 3' blocked termini and is important in single-strand annealing mechanisms. However, other *rad* mutants that are deficient in NER, such as *rad4*, may be suitable for observing both enhanced DNA damage-associated recombination and mutations [67].

One strategy has been to use DNA repair mutants that are knocked out in multiple DNA repair pathways to assess the genotoxicity of chemicals. For example, *rad4 rad51* double mutants, which are deficient in both NER and in recombinational repair, are synergistically more sensitive to UV and many chemical UV-mimetic agents. By introducing CYP genes into the *rad4rad51*, the strain also becomes synergistically more sensitive to AFB1 as well as extremely sensitive to other PAHs and HAAs [51, 67].

Mechanistic insights into how genotoxic agents stimulate chromosomal instability are also gained from studies of checkpoint genes. For example, deleting the RAD9-mediated checkpoint which triggers G2 arrest confers higher levels of DNA damage-associated recombination (translocations) after cells are exposed to X-rays and radiomimetic agents that cause breaks such as MMS and bleomycin. Enhanced translocation frequencies are also observed after *rad9* cells are exposed to camptothecin, an inhibitor of topoisomerase I. On the other hand, *rad9* deletion does not confer higher levels of recombination associated with agents, such as 4-nitroquinoline oxide (4-NQO), that cause bulky DNA damage [107]. These observations suggest that agents that stimulate DNA break formation may be identified if they enhance recombination in *rad9* mutants.

## 6. Phenotyping CYP polymorphisms in budding yeast

The CYP genes are highly polymorphic, and particular polymorphisms have been identified as risk factors for cancer [13, 22, 108] and glaucoma [109]. While yeast strains are useful in elucidating the genotoxicity of P450-activated carcinogens, yeast strains are also useful in characterizing human CYP polymorphisms. CYP1A1, CYP1A2, CYP1B1, and CYP2E1 polymorphisms have been studied in yeast [51, 63, 110–112]. The polymorphisms can be characterized in a number of ways: (1) substrate specificity, (2) activity with a defined substrate, (3) genotoxic endpoints, and (4) DNA adducts. For example, CYP1A2 polymorphisms have different affinities for heterocyclic aromatic amines; these polymorphisms have been also characterized by their ability to bioactivate aflatoxin B1. Activity assays have been performed for polymorphisms in CYP2E1, CYP1B1, CYP1A1, and CYP1A2 [51, 63, 110, 111]. In general activity assays agree with those performed when assays are performed in other model systems, such as *E. coli* [113].

Several CYP1A1 polymorphisms are present in a significant percentage of the population and may be risk factors for lung and breast cancer. For example, CYP1A1 I462V and CYP1A1 T461N have been correlated to have higher incidence of lung, breast, and endometrial cancer [114, 115]. A plausible hypothesis is that CYP1A1 I462V and CYP1A1 T461N are more active in converting breast- and lung-associated carcinogens into genotoxins. However, another model suggests that CYP1A1 is protective, since CYP1A1 knockout mice actually have a higher incidence of carcinogen-associated cancer [10]. Freedland et al. [51] measured multiple genotoxic endpoints in yeast strains expressing CYP1A1 I462V after exposure to multiple carcinogens and interestingly found a reduced level of bioactivation. This is consistent with a model that CYP1A1 may actually be protective and compete with other CYPs that convert carcinogens into active genotoxins [10].

## 7. Implications for higher eukaryotes

The ability to perform high-throughput screening to identify genotoxins using yeast strains containing sensitive reporter facilitates the identification of chemicals that merit more detailed and expensive studies. While yeast reporter strains can

be useful for high-throughput identification of genotoxins, yeast plate assays and genetics can elucidate mechanisms. Genotoxins that stimulate recombination and retrotransposition in yeast are likely to stimulate genetic instability in higher eukaryotes. Indeed, many recombinagens that have tested positive in yeast also test positive in higher eukaryotes. An excellent example is AFB1, which is also a recombinagen in human cell lines [116].

## 8. Conclusions and future directions

Yeast assays for detecting genotoxins and identifying genotoxic mechanisms are urgently needed to screen a multitude of industrial chemicals, pesticides, and pharmaceuticals. These assays have already been successful in screening thousands of chemicals, aiding in our understanding of genotoxic mechanisms. These assays have been further empowered by the technology to introduce human phase I and phase II metabolism in yeast cells. While the reporter assays enable high-throughput studies for rapid identification of genotoxins, the multitude of plate assays enables mechanistic studies to elucidate genotoxic mechanisms. The future challenge is to combine many of the reporters and plate assays so that both the screening and the mechanistic studies can be expedited.

Currently, the mechanisms of many chemical agents, which increase cancer risk, are unknown. Of particular interests are many small-molecule toxicants present in industrial workplace or which are extensively used in agriculture. How exposure to mixtures of these chemicals increases genotoxicity will be important in assessing risk factors to human health.

## Acknowledgements

The author was supported by NIH grants R21ES015954, F33ES021133, and R15E023685.

## Conflict of interest

The author declares no conflict of interest.

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