

# Defining a genotoxic profile with mouse embryonic stem cells

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## Abstract

Many genotoxins are found in the environment from synthetic to natural, yet very few have been studied in depth. This means we fail to understand many molecules that damage DNA, we do not understand the type of damage they cause and the repair pathways required to correct their lesions. It is surprising so little is known about the vast majority of genotoxins since they have potential to cause disease from developmental defects to cancer to degenerative ailments. By contrast, some of these molecules have commercial and medical potential and some can be weaponized. Therefore, we need a systematic method to efficiently generate a genotoxic profile for these agents. A genotoxic profile would include the type of damage the genotoxin causes, the pathways used to repair the damage and the resultant mutations if repair fails. Mouse embryonic stem (ES) cells are well suited for identifying pathways and mutations. Mouse ES cells are genetically tractable and many DNA repair mutant cells are available. ES cells have a high mitotic index and form colonies so experiments can be completed quickly and easily. Furthermore, ES cells have robust DNA repair pathways to minimize genetic mutations at a particularly vulnerable time in life, early development when a mutation in a single cell could ultimately contribute to a large fraction of the individual. After an initial screen, other types of cells and mouse models can be used to complement the analysis. This review discusses the merging field of genotoxic screens in mouse ES cells that can be used to discover and study potential genotoxic activity for chemicals commonly found in our environment.

**Keywords:** DNA repair, genotoxin, mutation, DNA damage, mouse embryonic stem cell

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## DNA repair pathways

Specific DNA repair pathways have evolved to correct a wide range of genetic lesions to preserve genome integrity.<sup>1</sup> A comprehensive presentation of these DNA repair pathways is beyond the scope of this review; therefore, we refer the reader to the following excellent reviews. In general, pathways that correct base lesions and single-strand breaks (SSB) include a variety of excision repair pathways like base excision repair (BER),<sup>2,3</sup> nucleotide excision repair (NER)<sup>4</sup> and mismatch repair (MMR).<sup>5</sup> Of these, BER prominently repairs reactive oxygen species-induced DNA damage, NER repairs ultraviolet (UV) light-induced lesions and MMR corrects replication-associated lesions. Even though these pathways are specific for correcting certain lesions there is some overlap. There are also repair pathways specific for correcting DNA double-strand breaks (DSBs) that include homologous recombination (HR), non-homologous end joining (NHEJ) and single strand annealing (SSA). HR is an error-free pathway that utilizes the sister chromatid as a template during S/G<sub>2</sub> phases.<sup>6</sup> NHEJ joins ends together without a template and

functions during both G<sub>1</sub> and S phases.<sup>7</sup> There are two NHEJ pathways, classical (cNHEJ) and alternative (aNHEJ).<sup>8</sup> Only, the classical pathway rejoins ends with high fidelity whereas the alternative pathway is error prone.<sup>9,10</sup> SSA joins repeats that flank a DSB and removes the intervening sequences via 3' flap removal.<sup>11</sup> Finally, specific pathways exist to address replication forks that stall when they collide with DNA damage. The Fanconi anemia pathway has proven critical for suppressing stalled replication in conjunction with HR.<sup>12–14</sup> There are also post-replication repair pathways that do not repair but instead bypass lesions at stalled replication forks with the goal of preventing replication fork collapse. There are two branches to postreplication repair that are best understood from work with the budding yeast *Saccharomyces cerevisiae*.<sup>15,16</sup> The first is translesion synthesis (TLS) that bypasses lesions simply by changing a high fidelity replicative polymerase to a TLS polymerase. The second is error free-postreplication repair (EF-PRR) that bypasses lesions via template switch or annealing of the blocked nascent replication strand to the complementary sister chromatid strand. There are also

a host of modifying proteins like helicases, topoisomerase, polymerases and nucleases that are important for a variety of repair pathways. Together, these pathways are essential for maintaining the genome against a multitude of genotoxins capable of causing a diversity of lesions.

## Genotoxic compounds

There are many natural genotoxic agents from both exogenous (outside the organism) and endogenous (produced by the organism) sources. Exogenous sources give rise to many agents that cause genetic damage. These agents are everywhere in our water, air and soil.<sup>17</sup> Some well-known exogenous agents include UV light and ionizing radiation.<sup>18,19</sup> Common endogenous agents are reactive oxygen species (ROS), by-products of oxygen metabolism that are produced in the mitochondria and peroxisomes. ROS include superoxide, hydrogen peroxide and hydroxy radicals. ROS are important for many physiological processes acting as second messengers for cell signaling.<sup>20,21</sup> However, ROS are also highly reactive due to unpaired electrons that react with multiple biomolecules including DNA. These reactions could cause a wide range of genetic damage,<sup>22</sup> mostly base lesions and DNA SSBs but also an occasional DNA DSB. In addition, genome maintenance pathways normally cause DNA damage during intermediate steps of their function. For example, single strand gaps and breaks are normally found at replication forks. DSBs may be generated by site-specific endonucleases important for V(D)J [Variable(Diverse)Joining] recombination for assembling antigen receptor genes.<sup>23</sup> Furthermore, a spontaneous process like cytosine deamination could lead to damage. Thus, the genome is constantly damaged from a variety of agents and metabolic processes.<sup>22</sup>

There are also synthesized molecules that can act as genotoxins. Some of these man-made genotoxins are used as anticancer chemotherapeutics.<sup>24</sup> These synthesized compounds could cause unique lesions that our DNA repair pathways did not evolve to repair. As a result a combination of pathways may address the lesion. For example, some alkylating agents cause DNA interstrand crosslinks (ICLs)<sup>25</sup> that are extremely toxic because they tether complementary DNA strands together to disrupt replication and transcription. As a result crosslinking agents are particularly toxic to highly proliferating cells. This endows crosslinking agents as powerful anticancer therapeutics<sup>25</sup> as well as effective weapons of mass destruction as seen during World War 1 and in Kurdistan.<sup>26</sup> No simple pathway exists to remove ICLs. Instead a complicated integration of multiple pathways is needed that includes Fanconi anemia, HR, NER and TLS.<sup>27,28</sup> Thus, comprehensive analyses of these genotoxins are needed to understand the pathways utilized to remove complicated lesions.

## The embryonic stem cell and small molecule libraries

To define the genotoxic profile of a specific compound, a mutant mouse embryonic stem (ES) cell library that

**Table 1** Mutant ES cell library

| Control cells | Mutant cells           | Mutations         | Function        |
|---------------|------------------------|-------------------|-----------------|
| AB1.1         | Msh2                   | −/−               | MMR             |
| AB2.2         | Brca2                  | Exon 27 deletion  | HR              |
|               | Blm                    | 88% decrease      | Helicase/HR     |
|               | Recql5                 | −/−               | Helicase/HR     |
|               | Trex2                  | −/−               | Exonuclease/RF  |
|               | FancB                  | Exon 2 deletion   | ICLR/RF         |
|               | Rad51 <sup>K133A</sup> | Dominant negative | HR              |
|               | Rad51 <sup>K133R</sup> | Dominant negative | HR              |
|               | Top3β                  | +/−               | Topoisomerase   |
|               | Top3α                  | +/−               | Topoisomerase   |
| B44           | Xpa                    | −/−               | NER             |
|               | Xpc                    | −/−               | NER             |
| J1            | Ku70                   | −/−               | cNHEJ           |
| TC1           | H2AX                   | −/−               | DDR/HR          |
|               | Cernunnos              | −/−               | cNHEJ           |
|               | Brca1                  | BRCT deletion     | DDR/HR/NHEJ     |
|               | Sirt1                  | −/−               | DAC             |
| IB10          | Rad18                  | −/−               | Lesion bypass   |
| E14 (IB10)    | Rad52                  | −/−               | HR              |
|               | Rad54                  | −/−               | HR              |
|               | Mus81                  | −/−               | Endonuclease/HR |
|               | Ercc1                  | −/−               | NER/HR/ICLR     |
|               | Rev1                   | BRCT deletion     | TLS             |
| R1            | Rtel1                  | −/−               | HR/telomere     |

−/−, homozygote mutant; +/−, heterozygote mutant; RF, replication fork; cNHEJ, classical nonhomologous end joining; DDR, DNA damage response; HDAC, histone deacetylase; FISH, fluorescence *in situ* hybridization; HR, homologous recombination; TLS, translesion synthesis; MMR, mismatch repair; NER, nucleotide excision repair; ICLR, interstrand crosslink repair; DAC, deacetylase

encompasses most DNA repair pathways can be used. The DNA repair mutant cells in our library are listed in Table 1. Included are cells ablated for NER (Xpa,<sup>29</sup> Xpc<sup>30</sup>) MMR (Msh2),<sup>31</sup> TLS (Rev1),<sup>32</sup> error-free postreplication repair (Rad18)<sup>33</sup> and Fanconi anemia (Fancb).<sup>34</sup> There are also cells deleted for NHEJ (Ku70)<sup>35</sup> and compromised for NHEJ (Cernunnos).<sup>36,37</sup> Complete ablation of HR is cell lethal;<sup>38</sup> therefore, we use null cells for several genes that contribute to but are not essential for HR (*H2ax*,<sup>39</sup> *Rad52*,<sup>40</sup> *Rad54*<sup>41</sup>). In addition, we have cells that are partially defective for essential proteins that include a deletion of *Brca2* exon 27<sup>42</sup> and deletion of *Brca1* exon 11.<sup>43</sup> There are cells that express dominant-negative *Rad51* point mutants defective for ATP binding (K133A) and ATP hydrolysis (K133R).<sup>44</sup> We also use cells defective for HR regulation that include mutations in the helicases *Rtel1*,<sup>45,46</sup> *Blm*<sup>47</sup> and *Recql5*.<sup>48</sup> These helicases reduce crossing over but also have other functions. We also have a variety of cells defective for general purpose proteins like histone deacetylase (*Sirt1*),<sup>49</sup> topoisomerases (*top3α* and *top3β*),<sup>50</sup> endonucleases (*Mus81*<sup>51</sup> and *Ercc1*<sup>52</sup>) and exonucleases (*Trex2*).<sup>53</sup> These proteins have the potential to function in multiple pathways and we encourage reading the referenced papers for a more detailed description of their function. Thus, we have a library of genetically altered mouse ES cells that can be used to establish a genotoxic profile for test chemicals.

**Table 2** Small molecule DNA repair inhibitors

| Inhibitor              | Target                                                  | Function                                     |
|------------------------|---------------------------------------------------------|----------------------------------------------|
| LY-294002              | DNA-PKcs                                                | NHEJ                                         |
| NU-7026                | DNA-PKcs                                                | NHEJ                                         |
| Vanillin               | DNA-PKcs                                                | NHEJ                                         |
| KU55933                | ATM                                                     | DDR                                          |
| KU 60019               | ATM                                                     | DDR                                          |
| CRT0044876             | APE1                                                    | BER                                          |
| Methoxyamine           | AP blocker                                              | BER                                          |
| Nicotinamide           | SIRT6                                                   | Deacetylase                                  |
| O6-Benzyl guanine      | MGMT                                                    | Alkyl removal                                |
| NU 1025                | PARP                                                    | ssDNA break<br>repair, lpBER,<br>aNHEJ       |
| NU1064                 | PARP                                                    | ssDNA break<br>repair, lpBER,<br>aNHEJ       |
| NU 1085                | PARP                                                    | ssDNA break<br>repair, lpBER,<br>aNHEJ       |
| Mirin                  | MRN complex                                             | DSB repair, DDR                              |
| Camptothecin (CPT)     | Type 1 topoisomerase                                    | Supercoiling                                 |
| ICRF-193               | Type 2 topoisomerase                                    | Supercoiling                                 |
| Etoposide              | Type 2 topoisomerase                                    | Supercoiling                                 |
| Hydroxyurea (HU)       | Ribonucleotide<br>reductase                             | DNA replication                              |
| Aphidicolin            | DNA polymerases<br>( $\alpha$ , $\delta$ , $\epsilon$ ) | DNA replication                              |
| L82                    | DNA ligase 1                                            | DNA replication,<br>excision repair          |
| L67                    | DNA ligases 1&3                                         | DNA replication,<br>excision repair          |
| L189                   | DNA ligases 1, 3, 4                                     | DNA replication,<br>excision repair,<br>NHEJ |
| Colcemid               | Microtubules                                            | Mitosis                                      |
| Trichostatin A         | Histone deacetylase                                     | Chromatin,<br>Transcription                  |
| 5-Aza-2'-deoxycytidine | DNA methyltransferases                                  |                                              |

ssDNA, single-strand DNA; lpBER, long patch BER; NHEJ, non-homologous end joining

A library of small molecule inhibitors (Table 2)<sup>54</sup> will complement the cell library and broaden the analytical scope. Of particular importance are the inhibitors to BER since there are no adequate mutant cells to represent this pathway because it is essential for cell survival. To impair BER we use small molecules that include an AP (apurinic/aprimidinic) blocker (Methoxyamine)<sup>55,56</sup> and an APE1 (AP endonuclease 1) inhibitor (CRT0044876). Both block the early and essential endonuclease step that immediately follows deglycosylation.<sup>3</sup> We also have PARP inhibitors (NU 1025, NU 1064, NU 1085) that block the repair of SSBs.<sup>54</sup> There is O-6-methylguanine-DNA methyltransferase inhibitor (MGMT: O6-benzyl guanine)<sup>57</sup> that impairs demethylation. Other small molecule inhibitors include those that impair the DNA damage response kinases DNA-PKcs (LY294002, NU7026, vanillin)<sup>58,59</sup> and ATM (KU 0055933, KU 60019).<sup>60</sup> An inhibitor to the Mre11 nuclease (mirin)<sup>61</sup> disables the MRE11-Rad50-NBS1 complex and prevents nucleic process of DNA strands during HR. There are inhibitors to DNA ligases that include L82, L67, L189.<sup>62</sup> There are also inhibitors to type 1 topoisomerases (camptothecin),<sup>63</sup> type 2 topoisomerases (ICRF-193, etoposide)<sup>64,65</sup>

that impair a variety of processes including DNA replication (camptothecin)<sup>66</sup> and DNA decatenation (ICRF-193).<sup>67</sup> Small molecules that cause replication fork stalling include inhibitors to ribonucleotide reductase called hydroxyurea (HU)<sup>68</sup> and a DNA polymerase called aphidicolin.<sup>69</sup> Inhibitor to microtubules in mitosis includes colcemid.<sup>70</sup> There is an inhibitor to the deacetylase SIRT6 (nicotinamide)<sup>71</sup> that is important for DNA repair and telomere structure<sup>72</sup> and an inhibitor to histone deacetylases (trichostatin A).<sup>73</sup> There is an inhibitor to DNA methyltransferase 1 (5-Aza-2'-deoxycytidine)<sup>74</sup> that also generates DSBs.<sup>75</sup> Thus, the small molecule library can complement the mouse ES cell library to establish a genotoxic profile for chemicals.

## Defining the genotoxic profile for a test chemical

### Discover the threshold levels of toxicity in control cells

The first step is to determine the physiologically relevant concentrations of the test chemical (TC) for control cells. For this experiment we use the protocol described for our genotoxic screen.<sup>76</sup> This is a cell proliferation assay that measures the fraction of cells that survive after one week in tissue culture. A survival fraction is the number of cells exposed to the TC divided by the number of cells exposed to only the vehicle used to suspend the TC (usually water, ethanol or dimethylsulfoxide). Please refer to our previous publication for a detailed protocol.<sup>76</sup> Cells are seeded on day 0 in the wells of a 24-well plate. The TC or vehicle is added the next day using a wide range of concentrations (low nanomolar to millimolar) with 10-fold intervals. Since the intervals are so large there will likely be a gap between complete cell survival and no survival. Then a second and perhaps a third experiment will be needed to fill in this gap. The goal is to find about four concentrations of TC that reduce survival by ~1–99.9%. Thus, this initial experiment establishes working concentrations for the TC.

In order to determine the length of time the TC remains toxic in tissue culture, a series of pulse chase time courses should be performed. For this experiment a fixed concentration of cells is seeded onto 14 wells of a 24-well plate. This allows cells exposed to TC and vehicle to be counted every day for seven days. The TC or vehicle is added to seven wells the day after seeding using a single concentration that reduces the cell number by ~90% as determined from the first experiment. Twenty-four hours later the media is replaced without TC or vehicle. Then for the next seven days one TC and one vehicle well are counted every day to determine the survival fraction. This time course will determine the length of time needed to see a decrease in cell number. In addition to cell number, apoptosis and cellular proliferation can be measured by staining with Annexin V (recognizes phosphatidyl serine proteins that are exposed to the outer cell membrane in early apoptosis) and with bromodeoxyuridine (BrdU) incorporation, respectively. Fluorescence-activated cell sorting analysis is used for quantitation. For example, we showed cell number declined

approximately three days after a 24 h pulse of the type 1 topoisomerase inhibitor camptothecin.<sup>76</sup> In addition, there was an increase in apoptosis and a decrease in proliferation that preceded the decline in cell number. A series of pulse chase-time courses can be performed to determine the length of time the TC remains toxic by altering the length of the pulse.

A one-month time course can be formed to observe the consequences of long-term exposure to low-dose TCs. To avoid confluency (cells at such a high density there is no more room for them to proliferate), passage cells at a specific time interval as determined by the previous experiment. For example, if TC remains toxic for all seven days, then count and re-seed cells at the original concentration in fresh media with the TC on days 7, 14, 21 and 28 (if the TC is cytotoxic for only six days, then this will be done every sixth day and so on). For this long-term experiment a low concentration of TC is used that reduces cell number by only 1–5% which may be more relevant to lifetime environmental exposure. Cells from these experiments are available for analysis as DNA damage and mutations as described below.

### Discover the threshold levels of toxicity in mutant cells

Next determine the threshold levels of sensitivity for the cells shown in Table 1 by performing one-week dose response curves as described above and in our previous publications.<sup>76,77</sup> Mutant cells should always be compared with control cells of the same strain since there is some variation between different strains.

As compared with control cells, any gene defect that causes increased sensitivity (fewer cells) or resistance (more cells) to TC should be considered important. Increased sensitivity indicates direct involvement in repairing TC-induced lesions while increased resistance indicates a DNA damage response designed to remove cells or engagement of a pathway that forms toxic repair intermediates. For example, cells defective for HR would be hypersensitive to an agent that causes DSBs since HR repairs DSBs. However, these same cells could also be resistant to an agent that increases DNA supercoiling or DNA catenation since these conditions would need to be resolved to complete HR.<sup>44</sup> Thus, both outcomes are indicators of pathway involvement in addressing TC-induced lesions.

Expose cells to the TC and simultaneously to the small molecule inhibitors shown in Table 2 to further evaluate the DNA repair pathways required to repair TC-induced lesions. This is especially important for essential pathways like BER, since BER-deletion is cell lethal and BER-null cells do not exist. Combinatorial exposure has been described in detail in our previous publication using DNA-PK<sub>cs</sub> inhibitors in combination with TSA (the TC).<sup>78</sup> For this experiment, compare cells exposed to vehicle + small molecule inhibitor to cells exposed to TC with and without the small molecule inhibitor. The dose of small molecule inhibitor should be at the threshold of toxicity (~5% reduction in cell number) while the dose to the TC should vary as in the previously determined dose response curve. These assays will identify DNA repair proteins that

**Table 3** Functional assays

| Assay                 | Function                                                   |
|-----------------------|------------------------------------------------------------|
| Dose response         | Measure cell survival & identify important repair pathways |
| BrdU                  | Cell proliferation/S phase entry                           |
| Annexin V             | Cell death                                                 |
| Foci                  | Protein nuclear localization                               |
| Western               | Protein levels and modifications                           |
| Protein fractionation | Protein localization                                       |
| iPOND                 | Protein dynamics at the replication fork                   |
| SCEs                  | crossovers                                                 |
| Microfiber analysis   | Replication fork integrity                                 |
| Three-color FISH      | Chromosomal breaks and rearrangements                      |
| SKY                   | Translocations, deletions, insertions                      |
| I-Sce1-GFP            | Define DSB repair pathways                                 |
| Aprt                  | LOH mechanisms                                             |

DSB, double-strand break; LOH, loss of heterozygosity; SCE, sister chromatid exchange; BrdU, bromodeoxyuridine

respond to TC-induced DNA damage or alterations and increase the breadth of the mutant cell screen.

Changes in sensitivity (increased or decrease cell number) must be verified. A common approach for verification is to express the cDNA of the mutant gene to rescue the phenotype. Another possible approach is to use RNA interference in control cells to knockdown the gene of interest. Verification is essential and should be done immediately to avoid perusing an experimental artifact.

### Functional analysis that defines the genotoxic profile in control and relevant mutant cells

Next perform a comprehensive functional analysis using a variety of assays (Table 3) for those mutant cells (and their controls) that exhibit increased sensitivity or resistance to the TC. There is a word of caution because the dose response curve can have false negatives since a defect in DNA repair does not always change cell survival to a genotoxin, yet may increase DNA damage and mutations. Therefore, the investigator might want to perform some of the functional assays even if there is no evidence of function from the dose response curve. For all assays both control and relevant DNA repair mutant cells should be observed after exposure to the TC or vehicle.

Protein foci formation can be used to analyze the localizations and levels of multiple replication fork maintenance and DNA repair proteins after exposure to the TC. A time course will reveal if certain events described below occur or if they are delayed or modified. Analysis of foci formation can be done for H2B, FANCD2, BLM, PCNA, RAD51, MRE11, H2AX, and 53Bp1 in mouse cells. H2B is a histone and can serve as a control since H2BS14P forms foci.<sup>79</sup> PCNA is a DNA sliding clamp and its detection will evaluate lesion bypass.<sup>15</sup> RAD51 evaluates replication fork restart and HR.<sup>80</sup> MRE11 evaluates end resection required for RAD51 loading.<sup>81</sup> MRE11-independent RAD51 loading indicates replication fork restart while MRE11-dependent RAD51 loading indicates HR-mediated DSB repair.<sup>82</sup> H2AX is a histone phosphorylated in response to DSBs ( $\gamma$ -H2AX).<sup>39</sup>  $\gamma$ -H2AX recognizes DNA DSBs,<sup>83</sup>

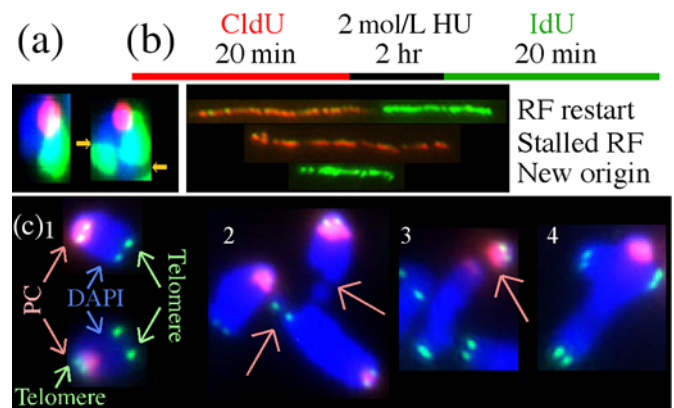


recruits DNA repair proteins to DNA damage<sup>84</sup> and controls recombination between sister chromatids.<sup>85,86</sup>  $\gamma$ -H2AX early loading indicates single-strand gaps at stalled replication forks.<sup>82</sup> In addition,  $\gamma$ -H2AX and 53BP1 evaluates DSB repair at collapsed replication forks by choosing HR versus NHEJ, respectively.<sup>87</sup> FANCD2 and BLM evaluate the Fanconi anemia pathway and HR regulation. Thus, foci formation will identify areas of DNA damage and evaluate the integrity of DNA repair.

Western analysis, protein fractionation and iPOND (isolation of proteins on nascent DNA) will reveal the levels and modifications of genome maintenance proteins before and after exposure to the TC. The same proteins mentioned for foci formation are also amenable to these approaches. Western will measure the protein level and modifications like phosphorylation and ubiquitination that can be critical for pathway determination. This is particularly valuable for those proteins that do not form foci like the cNHEJ proteins, Ku70 and Ku80. Protein fractionation can measure the protein level on chromatin.<sup>44</sup> iPOND can measure the protein dynamics at active and stalled replication forks and measure chromatin maturation.<sup>82</sup> This analysis identifies proteins bound to the nascent DNA strand during replication and detects proteins on ssDNA immediately adjacent to the nascent strand. Thus, Western and iPOND are effective assays to measure protein levels in the cell, chromatin and replication fork after exposure to the TC.

Sister chromatid exchanges (SCEs) will assess the impact the TC has on chromosomal crossovers during replication. A single chromatid break initiates an SCE. The nascent strand at the break then invades her complementary strand on the sister chromatid. During this process, sister chromatids are reciprocally exchanged.<sup>88</sup> HR-mediated repair of DSBs induce SCE.<sup>89–93</sup> Therefore, SCE levels directly correlate with HR activity.<sup>47,94,95</sup> Other pathways, besides HR, may also induce SCEs but the nature of these pathways are not fully understood.<sup>96</sup> To measure SCEs the nascent DNA strand is labeled with BrdU that appears green while the template strand is labeled with DAPI (4',6-diamidino-2-phenylindole) and appears blue.<sup>96</sup> After SCE, the green and blue will be present in both arms in a reciprocal manner (Figure 1a). This SCE assay will address HR and possibly other pathways that recombine sister chromatids during replication.

A microfiber analysis can be used to test if the TC impacts replication fork restart.<sup>80</sup> Some genotoxins cause damage that stall replication forks. These stalled forks may collapse and form a one-end DSB. To test for replication fork restart, the nascent DNA strand is labeled with two nucleotides, CldU and IdU that can be differentially identified with unique antibodies followed with fluorescent secondary antibodies (Figure 1b). Thus, the nascent strand can be visualized before and after a genotoxic insult. For example, the CldU and IdU strands can be labeled red and green, respectively. After labeling the cells are lysed and fibers (DNA strands) are prepared on a slide. This will allow detection of replication fork restart (red followed by green), a stalled replication fork (red only) and a new origin of replication (green only). If the



**Figure 1** Functional analysis. (a) Sister chromatid exchange. No sister chromatid exchange (SCE) (left) and one SCE (right). The arrows point to the junction. BrdU (bromodeoxyuridine) is green. Pericentromere is red. DAPI (4',6-diamidino-2-phenylindole) stains the long chromosome arm blue. (b) Microfiber analysis. (c) Three-color fluorescence *in situ* hybridization. The pericentromere (PC) is red, the telomeres are green and DAPI stains the long chromosome arms blue. 1. Normal unaltered chromosomes. 2. Chromatid breaks (arrows). 3. Isochromatid break in the pericentromere (arrow). 4. Radial structures that show multiple chromosomes are joined together

TC stalls replication forks then there will be a decrease in replication fork restart.

A microfiber analysis can also be used to test if the TC impacts nascent strand integrity during replication.<sup>13</sup> Stalled replication forks might lead to degeneration of the nascent strand. Thus, analysis of microfibers can similarly be used to detect nascent strand length as described for replication fork restart except only IdU is used to label the nascent strand. Again cells are cultured in IdU, then the TC is added, cells lysed, fibers made and the green strand is measured. Both control and relevant DNA repair mutant cells should be observed to evaluate the TC and the DNA repair/replication fork maintenance pathways that it affects.

Metaphase spreads are used to reveal chromosomal breaks and rearrangements after exposure to the TC. We discuss two types of metaphase spread techniques: three-color FISH (fluorescence *in situ* hybridization) and SKY (spectral karyotyping).<sup>97</sup> Three-color FISH is a fast and cost-effective way to detect chromosomal breaks and rearrangements (Figure 1c). Defects in replication cause one-side DSBs that appear as a broken chromatid. In addition, there may be breaks in both chromatids (isochromatid breaks) that suggest failed strand exchange intermediates. Three-color FISH can also detect any chromosomal alteration that changes the number of telomeres and pericentromeres, for example, telomere deletions, dicentrics and radials. For three-color FISH, the metaphase spreads are stained with a telomere probe (green), a major satellite repeat probe in the pericentromere (red) and DAPI (stains the long chromosome arms blue).<sup>98</sup> Three-color FISH is advantageous over SKY because it is much faster and cheaper permitting the analysis of many more metaphase spreads. However, chromosomal alterations that do not change the number of telomeres or pericentromeres are difficult to observe with three-color FISH. To detect these alterations, SKY is advantageous over three-color FISH

since every chromosome is identified with a colored probe. Therefore, SKY is ideal for detecting translocations, insertions and deletions for all chromosomes. A combination of these two techniques will evaluate the structural integrity of chromosomes after exposure to the TC.

### Cells that contain repair and mutation reporters

There are multiple types of mouse ES cells that contain a reporter designed to detect specific genome maintenance events. These reporter cells can evaluate the specific genome maintenance event after exposure to the TC. Thus, these reporter cells might be useful to test after analysis of the libraries and assays described in Tables 1–3. Reporters are typically available in wild-type cells with only a limited number in mutant cells. Therefore, the reporter may not be in the desired mutant background. Thus, one would have to either place the reporter in the desired mutant cells or mutate the desired gene in cells with the reporter. Alternatively, RNA interference techniques can be used to knockdown the gene. Regardless, the impact the TC has on the reporter can be useful even in control cells.

A series of reporters can be used to evaluate the TC's impact on DSB repair pathways. These reporters contain an I-SceI-site and the eGFP (enhance green fluorescent protein) reporter. I-SceI is an intron-encoded endonuclease present in the mitochondria of *S. cerevisiae*.<sup>99</sup> It is useful for generating a single DNA DSB in mammalian cells because of its large 18 bp recognition sequence.<sup>100,101</sup> A comprehensive series of reporters were designed with the I-SceI site and eGFP that are able to distinguish different DSB repair pathways including HR, SSA, cNHEJ and aNHEJ.<sup>9,10,96</sup> For all reporters, DSB repair is detected by the correction of a defective eGFP during the repair of an I-SceI-induced DSB. Thus, one can easily evaluate the effect the TC has on DSB repair pathways. In addition, many of these reporters are in both wild type and mutant cells since they are easily targeted to the *Pim1* locus (a gene important for embryonic development but not for genome maintenance). Thus, one can easily generate mutant cells with these reporters.

An elegant system was designed to detect loss of heterozygosity (LOH),<sup>102</sup> a potential cancer-causing mechanism.<sup>103</sup> A variety of mechanisms cause LOH including recombination, non-disjunction (chromosomal separation failure at mitosis) and point mutations. Mouse ES cells were designed to detect LOH at the endogenous *Aprt* (adenine phosphoribosyltransferase) locus.<sup>102</sup> *Aprt* is a member of the adenine salvage pathway; therefore, cells disrupted for *Aprt* function can be selected in 2'6'-diaminopurine (DAP) while cells that maintain *Aprt* function can be selected in alanosine or azaserine.<sup>104</sup> These LOH reporter ES cells are heterozygous for *Aprt* and were derived from a 128XC3HF1 cross. Therefore, a series of PCR reactions can identify each chromosome so the investigator can distinguish the potential events that cause LOH (loss of *Aprt* function). These events include mitotic recombination, gene conversion and non-disjunction. Using these cells, ionizing radiation was shown to induce LOH

primarily by mitotic recombination with lesser contributions from deletions, gene conversions, point mutations and epigenetic inactivation.<sup>102</sup> Unfortunately this reporter system is not present in DNA repair mutant cells so RNA interference may be the best approach to reduce the levels of those proteins important for suppressing TC-induced LOH. Thus, these cells coupled with RNA knockdown make an excellent model for testing LOH.

Point mutations are more difficult to observe than chromosomal breaks and rearrangements since they cannot be seen with metaphase spreads. However, there are reporters that can be used to detect point mutations. One of the best reporters is the above described *Aprt* gene since point mutations also cause LOH.<sup>102</sup> ES cells derived from the 128XC3HF1 cross can be used to measure point mutations using the same PCR strategy to observe both chromosomal *Aprt* copies. If both copies are still present and there is no recombination or gene conversion, then sequence analysis will detect base changes. The *Aprt* reporter is amenable to sequencing since the entire genome sequence is less than 2.5 kb. Thus, the *Aprt* locus is ideal for detection of a variety of pathways that cause LOH including simple point mutations.

### Conclusion

Here we describe a systematic approach to identify the DNA repair pathways required to correct damage caused by a genotoxin of interest. We first identify the threshold levels of genotoxin needed to suppress cell number in a proliferation assay. Then we use a series of libraries to identify important pathways needed to correct the TC-induced damage. Functional assays are then used to measure the dynamics of DNA repair and chromosomal integrity. Finally, reporter cells are used to fine-tune this analysis. The above described approach is comprehensive and a good first step to identify the types of repair pathways used to correct TC-induced DNA damage and the types of damage/mutations that result if the lesions are not corrected.

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