OECD Series on Adverse Outcome Pathways No. 29

Oxidative DNA damage leading to chromosomal aberrations and mutations

Eunnara Cho,
Ashley Allemang,
Marc Audebert,
Vinita Chauhan,
Stephen Dertinger,
Giel Hendriks,
Mirjam Luijten,
Francesco Marchetti,
Sheroy Minocherhomji,
Stefan Pfuhler,
Daniel J. Roberts,
Kristina Trenz,
Carole L. Yauk

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OECD

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Foreword

This Adverse Outcome Pathway (AOP) on Oxidative DNA damage leading to chromosomal aberrations and mutations has been developed under the auspices of the OECD AOP Development Programme, overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), which is an advisory group under the Working Party of the National Coordinators for the Test Guidelines Programme (WNT) and the Working Party on Hazard Assessment (WPHA).

The AOP has been reviewed for compliance with the AOP development principles following the EAGMST coaching approach. The scientific review was subsequently conducted by the scientific journal Environmental and Molecular Mutagenesis (EMM), following the OECD AOP review principles outlined in the Guidance Document on the scientific review of AOPs. This AOP was endorsed by the WNT and the WPHA on 17 February 2023

Through endorsement of this AOP, the WNT and the WPHA express confidence in the scientific review process that the AOP has undergone and accept the recommendation of the EAGMST that the AOP be disseminated publicly. Endorsement does not necessarily indicate that the AOP is now considered a tool for direct regulatory application.

The OECD's Chemicals and Biotechnology Committee agreed to declassification of this AOP on May 5th, 2023.

This document is being published under the responsibility of the OECD's Chemicals and Biotechnology Committee.

The outcome of the scientific review is publicly available in the AOP-Wiki at the following link: [scientific review].

Authors:

Eunnara Cho[1,2], Ashley Allemang[3], Marc Audebert[4], Vinita Chauhan[5], Stephen Dertinger[6], Giel Hendriks[7], Mirjam Luijten[8], Francesco Marchetti[1,2], Sheroy Minocherhomji[9, 12], Stefan Pfuhler[3], Daniel J. Roberts[7], Kristina Trenz[10], Carole L. Yauk[2, 11 *]

[1] Environmental Health Science and Research Bureau, Health Canada, Ottawa, ON, Canada

[2] Department of Biology, Carleton University, Ottawa, ON, Canada

[3] The Procter & Gamble Company, Mason, OH, United States

[4] Toxalim, INRAE, Toulouse, France

[5] Consumer and Clinical Radiation Protection Bureau, Health Canada, Ottawa, ON, Canada

[6] Litron Laboratories, Rochester, NY, United States

7 Toxys, B.V., Netherlands

[8] Centre for Health Protection, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

[9] Amgen, Thousand Oaks, CA, United States

[10] Boehringer-Ingelheim Pharma GmbH Co.KG, Biberach, Germany[11] Department of Biology, University of Ottawa, Ottawa, ON, Canada

[12] Current address: Eli Lilly & Company, Indianapolis, IN, United States

*Corresponding author: Carole Yauk (carole.yauk@uottawa.ca)

Abstract

This adverse outcome pathway (AOP) network describes the linkage between oxidative DNA damage and irreversible genomic damage (chromosomal aberrations and mutations). Both endpoints are of regulatory interest because irreversible genomic damage is associated with various adverse health effects such as cancer and heritable disorders.

Mutagens are genotoxic substances that alter the DNA sequence and this includes single base substitutions, deletion or addition of a single base or multiple bases of DNA, and complex multi-site mutations. Mutations can occur in coding and non-coding regions of the genome and can be functional or silent. The site and type of mutation will determine its consequence. Clastogens are genotoxic substances that cause DNA single- and double-strand breaks that can result in deletion, addition, or rearrangement of sections in the chromosomes. As with mutagens, the type and extent of chromosome modification(s) determine cellular consequences.

The molecular initiating event (MIE) of this AOP is increase in oxidative DNA damage, indicated by increases in oxidative DNA lesions. DNA in any cell type is susceptible to oxidative damage due to endogenous (e.g., aerobic respiration) and exogenous (i.e., exposure to oxidants) oxidative insults. Although this is the MIE for this AOP network, we note that there are numerous upstream key events (KE) that can also lead to DNA oxidation. Thus, we expect this AOP to be expanded upstream, and to be incorporated into a variety of AOP networks. Generally, cells are able to tolerate and readily repair oxidative DNA lesions by basal repair mechanisms. However, excessive damage can override the basal repair capacity and lead to inadequate repair of oxidative damage (KE1). Mutations (AO1) can arise from incorrect repair following oxidative damage (KE1), where incorrect bases are inserted opposite lesions during DNA replication. Insufficiently or incompletely repaired oxidative DNA lesions can also lead to DNA strand breaks (KE2) that, if insufficiently repaired (KE1), may result in chromosome aberrations (AO2) and/or mutations (AO1) following DNA replication.

Support for this AOP is strong based on extensive understanding of the mechanisms involved in this pathway, evidence of essentiality of certain KE (i.e., studies using reactive oxidative species scavengers and modulating DNA repair enzymes), and a robust set of studies providing empirical support for many of the KERs.

We anticipate that this AOP will be of widespread use to the regulatory community as oxidative DNA damage is considered an important contributor to the adverse health effects of many environmental toxicants. Importantly, the AOP points to critical research gaps required to establish the quantitative associations and modulating factors that connect KEs across the AOP, and highlights the utility of novel test methods in understanding and evaluating the implications of oxidative DNA damage.

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Background

This AOP network describes oxidative damage to DNA (MIE) leading to mutations (AO1) and chromosomal aberrations (AO2). The AOP summarizes the evidence supporting how increases in oxidative DNA lesions can overwhelm DNA repair mechanisms, causing an accumulation of unrepaired lesions and/or repair intermediates. Failure to resolve oxidative DNA damage can lead to permanent alterations to the genome. Increases in reactive oxygen and nitrogen species (RONS) that can lead to oxidative DNA lesions is a broad characteristic of many xenobiotics and indeed, is noted as one of the 'key characteristics of carcinogens' (Smith et al., 2016). Moreover, oxidative stress is often suspected to be the cause of DNA damage by substances whose mechanism of genotoxicity is uncertain [e.g., glyphosate (Kier and Kirkland, 2013; Benbrook, 2019), monosodium glutamate (Ataseven et al., 2016)]. Thus, this AOP network will serve as a key tool in mechanism-based genotoxic hazard identification and assessment.

Oxidative stress describes an imbalance of oxidants and antioxidants in the cell. Oxidative stress can occur when free radicals overwhelm the antioxidant capacity under certain physiological conditions, such as inflammation due to diseases, and also through exogenous stressors that are oxidants and/or induce ROS and other free radicals. Excess oxidants can occur following exposure to agents that: (a) generate free radicals and other RONS, (b) deplete cellular antioxidants, and/or (c) have oxidizing properties (Parke, 1982). Furthermore, there are substances that can induce inflammatory responses and in turn cause oxidative stress as a secondary effect (Gustafson et al., 2016). The effects of oxidative stress in the cell are broad; all biomolecules are susceptible to damage by oxidizing agents. Oxidative stress and associated damage to cellular components have been implicated in various diseases, including neurodegenerative diseases, cardiovascular diseases, diabetes, and different cancers (Liguori et al., 2018).

Free radicals and other RONS are continuously generated as by-products of endogenous redox reactions (e.g., oxidative phosphorylation in the mitochondria, NADPH oxidation to NADP+ by NADPH oxidase) at steady state. The steady state concentration of oxidants is essential for cellular functions (e.g., as secondary signalling molecules) and is tightly regulated by endogenous antioxidants such as glutathione, and antioxidant enzymes such as superoxide dismutase and catalase. There are many protective barriers against oxidative damage to the genome within the cell. Compartmentalization is one of the ways in which DNA is protected from exposure to ROS and other free radicals generated by various biological processes that occur in different parts of the cell. Examples include CYP450 enzyme activity in the cytoplasmic endoplasmic reticulum and oxidative phosphorylation in the mitochondria (Dan Dunn et al., 2015; Veith and Moorthy, 2019).

Exogenous sources such as ionizing radiation, ultraviolet (UV) radiation, and certain compounds can directly or indirectly generate reactive species, causing oxidative stress. Oxidizing compounds can also directly cause oxidative damage to cellular components (Liguori et al., 2018). The nitrogenous bases of the DNA are susceptible to oxidation by both endogenous and exogenous oxidants (Berquist and Wilson III, 2012). Oxidizing agents cause a wide range of oxidative DNA lesions. In addition to strand breaks due to direct RONS attack on the phosphate backbone, the nitrogenous bases can be modified in various ways by free radicals and other reactive species. If these lesions are left unrepaired or the attempt at repair fails, mutations and strand breaks can occur, permanently altering the DNA sequence. All nitrogenous bases are susceptible to oxidative damage, however, to different extents. A variety of DNA lesions caused by RONS are described within this

AOP (Cooke et al., 2003). Notably, guanine is most readily damaged by RONS and other oxidants due to its low reduction potential. Indeed, 8-oxoG is the most abundant oxidative DNA lesion and has been extensively studied. Consequently, chromosomal regions containing a higher GC content are more susceptible to oxidative base modifications. For example, human telomeres, which are constituted by TTAGGG repeats and a single-stranded G-rich 3' overhang, are known to be comparatively more sensitive to oxidative damage than other regions in the genome and accumulate 8-oxodG lesions that eventually lead to telomere shortening and genomic instability (Petersen et al., 1998; Bolzan, 2012; Fouquerel et al., 2019). The repair mechanisms and consequences of oxidative damage to telomeres are active areas of research.

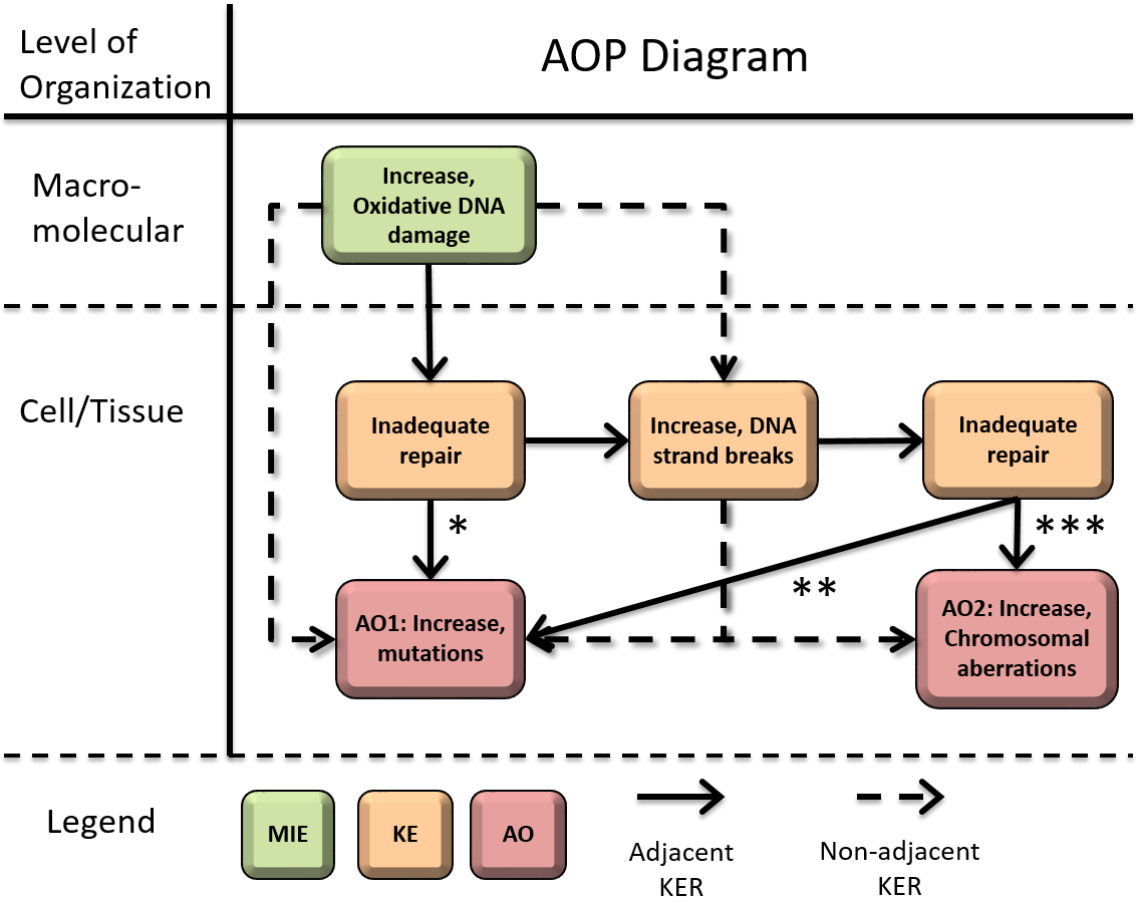
Within this AOP network, we mainly focus on 8-oxo-dG as oxidative DNA damage representing the MIE, for practicality. The fate of guanine lesions has been most extensively researched and well understood (Roszkowski et al., 2011; Whitaker et al., 2017; Cadet et al., 2017; Markkanen, 2017). Also, 8-oxodG is an accepted biomarker of oxidative stress and oxidative damage to DNA both *in vitro* and *in vivo* (Cooke et al., 2008; Roszkowski et al., 2011; P. Li et al., 2014; Guo et al., 2017). Several different detection methods for 8-oxo-dG are commercially available and, thus, are easy to access (e.g., immunodetection, comet assay). We note that 8-oxo-dG is not a terminal product of oxidative damage; 8-oxo-dG can be further oxidized to additional mutagenic lesions such as spiroiminodihydantoin and guanidinohydantoin (Jena and Mishra, 2012). However, as with many other oxidative lesions on pyrimidines and adenine, these guanine lesions are estimated to be small fractions compared to 8-oxo-dG (Yu et al., 2005; Cooke et al., 2008).

The pathway to mutations (AO1) from oxidative DNA lesions can either proceed (a) directly to mutation through replication of unrepaired oxidized DNA bases (insertion of an incorrect nucleotide by a replicative or translesion polymerase), or (b) indirectly through the creation of strand breaks that can be misrepaired to introduce mutations (Taggart et al., 2014; Rodgers and McVey, 2016). Strand breaks can arise during attempted repair of oxidative DNA lesions. Oxidative base damage is predominantly repaired by base excision repair (BER), and by nucleotide excision repair (NER) to a lesser extent (Whitaker et al., 2017). In the excision repair pathways, single strand breaks (SSB) are transiently introduced as repair intermediates. With increasing oxidative lesions and more lesions in close proximity to each other, the quality and efficiency of repair may be compromised, resulting in persistent unrepaired lesions and repair intermediates. Accumulated repair intermediates such as SSBs, oxidized bases, and abasic sites can interfere with proximal excision repair and/or impede replication fork elongation, leading to double strand breaks (DSBs), which are more toxic and difficult to repair (Yang et al., 2006; Sedletska et al., 2013; Ensminger et al., 2014). Furthermore, if a SSB is introduced nearby another SSB on the opposite strand prior to or during excision repair, these SSBs may be converted to DSBs. Some studies suggest that multiple DNA lesions within one or two helical turns can increase the rate of DSB formation (Cannan and Pederson, 2017). Insufficiently repaired DSBs (incorrect or lack of rejoining) can permanently alter the DNA sequence (e.g., insertion, deletion, translocations), and cause both mutations (AO1) and structural chromosomal aberrations (AO2) (Rodgers and McVey, 2016). These processes are described in more detail within the AOP.

Overall, we anticipate that this AOP network will provide a key sub-network that will be relevant to many future AOPs. However, we note that the AOs herein, increased mutations and chromosomal aberrations, are regulatory endpoints of concern in and of themselves. This AOP also provides a template for designing testing strategies for RONS-induced genetic effects. Despite the fact that this is a long-studied area in genetic toxicology, this work highlights notable gaps in the empirical evidence linking adjacent KEs. For example, the extent to which the levels of oxidative DNA damage must increase before DNA repair

processes are overwhelmed leading to an AO is currently poorly understood, and may vary based on the test system. Hence, further data are needed to improve our ability to predict whether this pathway is relevant to a chemical's toxicological effects.

Graphical representation



*** Relevant repair pathway for Event 155: Excision repair**
**** Relevant repair pathway for Event 155: Error-prone double strand break repair (NHEJ)**
***** Relevant repair pathway for Event 155: Error-prone double strand break repair (NHEJ)**
"Inadequate DNA repair" (KE 155) is shown twice in this flow diagram to emphasize that "Increase, DNA strand breaks" (KE 1635) is required for the progression to AO2 "Increase, Chromosomal aberrations" (KE 1636), and that different repair pathways are involved in repairing different types of DNA damage.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Type	Event ID	Title	Short name
MIE	1634	Increase, Oxidative damage to DNA	Increase, Oxidative DNA damage
KE	155	Inadequate DNA repair	Inadequate DNA repair
KE	1635	Increase, DNA strand breaks	Increase, DNA strand breaks
AO	185	Increase, Mutations	Increase, Mutations
AO	1636	Increase, Chromosomal aberrations	Increase, Chromosomal aberrations

Key Event Relationships

Title	Adjacency	Evidence	Quantitative Understanding
Increase, Oxidative DNA damage leads to Inadequate DNA repair	adjacent	High	Low
Inadequate DNA repair leads to Increase, DNA strand breaks	adjacent	High	Low
Increase, DNA strand breaks leads to Inadequate DNA repair	adjacent	High	Low
Inadequate DNA repair leads to Increase, Mutations	adjacent	High	Low
Inadequate DNA repair leads to Increase, Chromosomal aberrations	adjacent	High	Low
Increase, Oxidative DNA damage leads to Increase, DNA strand breaks	non-adjacent	Moderate	Low
Increase, Oxidative DNA damage leads to Increase, Mutations	non-adjacent	High	Low
Increase, DNA strand breaks leads to Increase, Mutations	non-adjacent	High	Low
Increase, DNA strand breaks leads to Increase, Chromosomal aberrations	non-adjacent	High	Low

Prototypical Stressors

Name
Hydrogen peroxide
Potassium bromate
Ionizing Radiation

Cadmium chloride
tert-Butyl hydroperoxide
Reactive oxygen species
Hydroquinone
4-Nitroquinoline 1-oxide

Overall Assessment of the AOP

Biological plausibility:

Overall, the biological plausibility of this AOP network is strong. This network was developed by a team of experts within the Health and Environmental Sciences Institute's Genetic Toxicology Technical Committee who have decades of experience in research on DNA repair and genetic toxicology.

Oxidative DNA lesions are primarily repaired by base excision repair (BER). BER is a multistep process that involves multiple enzymes including 8-oxoguanine DNA glycosylase 1 (OGG1), which removes oxidized guanine bases and creates a nick 3' to the damaged base, and human apurinic/apyrimidinic (AP) endonuclease (APE1), which then removes the resulting abasic site by cleaving 5' to the damaged base. It is known that BER glycosylases are constitutively expressed and that APE1 is an abundant enzyme (Tell et al., 2009). A spike in BER substrates could lead to an imbalance in the initiating steps of BER, causing an accumulation of abasic sites and other repair intermediates (e.g., SSBs) that can lead to the AOs described herein (Coquerelle et al., 1995; Yang et al., 2006; Nemec et al., 2010). Another suspected and biologically plausible mechanism by which oxidative DNA lesions can lead to clastogenic effects is through futile cycles of mutY DNA glycosylase (MUTYH)-initiated BER, which removes dA inserted opposite 8-oxodG during replication. MUTYH is readily available at the replication foci to initiate BER if needed but can initiate BER post-replication as well (Hayashi et al., 2002; Nakamura et al., 2021). During BER, following the removal of dA by MUTY and APE1, polymerases such as pol β and pol κ could re-insert dA opposite 8-oxodG, rendering the repair process futile. Such cyclical rounds of BER may cause an accumulation of repair intermediates such as SSBs in the newly synthesized strand (Hashimoto et al., 2004; Oka and Nakabeppu, 2011) SSBs can turn into DSBs if they occur in close proximity to each other on opposite strands (Iliakis et al., 2004; Fujita et al., 2013; Mehta and Haber, 2014). If DSBs are not repaired in a timely manner, the broken ends may diffuse away from their original position and result in genetic translocation where incorrect ends are joined, or loss of DNA segments, leading to structural aberrations (AO2) (Obe et al., 2010; Durante et al., 2013).

Error-prone repair of DSBs (KE1 Description section 3) can also lead to mutations, providing an alternate pathway to AO1, an increase in mutations (Sedletska et al., 2013). Non-homologous end joining (NHEJ), the error-prone joining of two broken ends, is a faster process compared to homologous recombination (HR), which uses the homologous sequence in the sister chromatid or homologous chromosome as a template to ensure fidelity of the reconstructed strands (Mao et al., 2008a; Mao et al., 2008b). The preference for the use of sister chromatids versus homologous chromosomes in HR depends on the stage of cell cycle in which the DSB occurs. NHEJ may be preferred over HR in many instances, especially under stress, leading to altered sequences at the site of repair (Rodgers and McVey, 2016). HR is mostly restricted to S and G2 phases of the cell cycle and, while NHEJ can occur at all stages of the cell cycle, it mostly occurs in G1, when the sister chromatids have not yet been synthesized (Brandsma and van Gent, 2012).

The structure of the site of a DSB and end resection can determine the repair pathway and the repair outcome. Typically, breaks with single stranded overhangs are processed by end resection and proceed to HR or error-prone homology-based annealing (i.e, single strand annealing and alternative end-joining). DSBs with blunt ends are more likely to be rejoined by NHEJ (Ceccaldi et al., 2016). The error-prone nature of DSB repair by NHEJ has been extensively studied and widely accepted. Under stress by exogenous or endogenous sources

(e.g., xenobiotics), DSBs can also lead to mutagenic salvage DNA repair pathways such as break-induced replication (BIR) and microhomology-mediated break-induced replication (MMBIR) which are linked to mutagenesis, chromosomal rearrangemnts, and genomic instability (Sakofsky et al., 2015; Kramara et al., 2018).

It is established and accepted that unrepaired oxidative DNA lesions, especially 8-oxodG and FapydG (a common oxidative DNA lesion involving opening of the imidazole ring), are mutagenic (AO1). During DNA replication, the presence of these unrepaired adducts (KE1: Inadequate repair) on nucleotides leads to incorrect base pairing with incoming nucleosides. This occurs without causing structural disturbance leading to evasion of mismatch repair (Cooke et al., 2003). It is well-understood that both 8-oxodG and FapydG readily base pair with adenine, giving rise to G to T transversions, which are the predominant base substitutions caused by oxidative stress (Cadet and Wagner, 2013; Poetsch et al., 2018).

The underlying biology of the KERs leading to chromosomal aberrations (AO2) is more complex. There are a variety of biologically plausible mechanisms that link inadequate repair of oxidative DNA lesions (KE1; see section 2) of KE Description) to DNA strand breaks (KE2), which, if insufficiently repaired (KE1; see section 3) of KE Description), can cause chromosomal aberrations. Mechanistically, these pathways are well understood (Yang et al., 2006; Nemec et al., 2010; Markkanen, 2017). However, empirical evidence supporting the occurrence of these events is limited in the current literature.

Telomeres, which are rich in GC, are especially susceptible to oxidative damage. 8-oxodG in telomeres causes the replicative DNA polymerase δ to stall because pol δ is not proficient in extending past dC inserted opposite 8-oxodG (Markkanen et al. 2012). Thus, an accumulation of 8-oxodG in telomeres can increase the risk of replication fork collapsing due to stalling (Fouquerel et al. 2019). Furthermore, oxidized dNTPs such as 8-oxoGTPs inserted in the telomere by the telomerase terminates the elongation process as the telomerase is incapable of extending past an 8-oxodG (Fouquerel et al. 2016). Both scenarios can result in the loss and shortening of telomeres. Damage to telomeres can trigger the DNA damage response and be treated in the same manner as DSBs, in which unprotected ends are fused with other available ends leading to structural aberrations (i.e., chromosomal fusions, bridges, micronuclei) (Barnes et al., 2019; Fouquerel et al., 2019).

Time- and dose-response concordance:

The WOE supporting the time- and dose-response concordance of KEs leading to the AOs is between moderate and strong.

The MIE (increase in oxidative DNA lesions) can be measured shortly following exposure to stressors. In cell-free systems and cell-based *in vitro* models, 8-oxodG has been quantified as early as 15 minutes following chemical exposure (Ballmaier and Epe, 2006). Oxidative lesion formation and induction of strand breaks have been demonstrated by time course experiments, where increases in oxidative lesions were detected at earlier time points and at lower concentrations than strand breaks following exposure to various oxidative stress-inducing chemicals [e.g., Ballmaier and Epe (2006), Deferme et al. (2013)]. Mutations (AO1) and chromosomal aberrations (AO2) must be measured after replication and cell division; therefore, these endpoints are only detected at much later time points than the MIE and KEs. Due to the vastly different sensitivities and dynamic ranges of the methodologies detecting the events in these AOPs, it is difficult to demonstrate concordance in concentration-response between the upstream events and AO.

Uncertainties, inconsistencies, and data gaps:

Currently, quantitative understanding of the amount of oxidative lesions that lead to the two AOs of this AOP network, mutations and chromosomal aberrations, is very limited. Very few studies have specifically investigated the extent of chromosomal aberrations induced by different levels of oxidative DNA lesions. Quantitative studies of different oxidative DNA lesions corresponding to mutation frequencies are also very limited. In order to increase the quantitative understanding of the KERs and to facilitate predictive toxicology, studies are needed to investigate the quantity of 8-oxodG in addition to the endogenous levels required to overwhelm DNA repair and lead to chromosomal aberrations and mutations. We note that the mutagenicity of 8-oxodG has been most extensively studied, while other oxidative DNA lesions have been studied to a lesser extent. In addition, oxidative DNA base modifications such as 8-oxoG also appear to play a role in modulating gene expression and serve as epigenetic markers (Ba and Boldogh, 2018; Bordin et al. 2021). Thus, the interplay of the regulatory roles of oxidized DNA bases and DNA damage response, and its influence on the toxicity of oxidative DNA lesions must be considered.

Quantitative understanding of the relationships comes primarily from studies that modulate levels of oxidative DNA damage through manipulation of repair enzyme activity. In these studies, conflicting observations have been made following modulation of OGG1, the primary repair enzyme for 8-oxodG lesions. While OGG1 protected against DSB formation and cytotoxicity of certain compounds (e.g., methyl mercury, bleomycin, hydrogen peroxide), DSBs were exacerbated by the presence of OGG1 in some other cases (e.g., ionizing radiation, conflicting results for hydrogen peroxide) (Ondovcik et al., 2012; Wang et al., 2018). Available literature indicate that the effect of inadequate repair of oxidative lesions manifests differently for different stressors; it has been suggested that these discrepancies may be due to the difference in proximity of lesions to each other (clustered lesions vs. single lesions) (Yang et al., 2004; Yang et al., 2006).

This AOP network primarily describes oxidative damage to the nuclear DNA (nDNA). However, we must acknowledge that oxidative damage occurs also in the deoxynucleotide triphosphate (dNTP) pool and mitochondrial DNA (mtDNA). Due to mtDNA's location, it is more susceptible to oxidative damage than nDNA. Indeed, crosstalk exists between the nucleus and mitochondria during oxidative stress (Cha et al., 2015; Saki and Prakash, 2017). BER maintains both mitochondrial and nuclear genomic integrity (Cha et al., 2015). Oxidized dNTPs, especially 8-oxodGMP, can be inserted into both genomes during replication and excision repair, resulting in mismatches and impediment of the repair of existing damage, respectively; both scenarios can directly lead to inadequate DNA repair, contributing to the progression of the AOP network (Colussi et al., 2002; Russo et al., 2004; Caglayan et al., 2017). Moving forward, KEs addressing oxidative damage to the dNTP pool and mtDNA are necessary to build a more complete map of oxidative stress-related genotoxicity and to expand the AOP network to other related AOs.

Domain of Applicability

Life Stage Applicability

Life stage	Evidence
All life stages	

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human	Homo sapiens		NCBI
mice	Mus sp.		NCBI
rat	Rattus norvegicus		NCBI
fish	fish		

Sex Applicability

Sex	Evidence
Unspecific	

Theoretically, this AOP is relevant to any cell type in any organism at any life stage. Regardless of the type of cell or organism, DNA is susceptible to oxidative damage and repair mechanisms exist to protect the cell against permanent chromosomal damage. Generally, DNA repair pathways are highly conserved among eukaryotic organisms (Wirth et al., 2016). Base excision repair (BER), the primary repair mechanism for oxidative DNA lesions, and associated glycosylases are highly conserved across eukaryotes (Jacobs and Schar, 2012). DNA strand break repair pathways such as homologous recombination (HR) and non-homologous end joining (NHEJ) are shared among eukaryotes as well. Induction of chromosomal aberrations and mutations following oxidative DNA damage has been studied in both eukaryotic and prokaryotic cells. Notably, the KEs of this AOP have been measured in rodent models (i.e., rat and mouse) and mammalian cells in culture (e.g., TK6 human lymphoblastoid cells, HepG2 human hepatic cells, Chinese hamster ovary cells) (Klungland et al., 1999; Arai et al., 2002; Platel et al., 2009; Platel et al., 2011; Deferme et al., 2013).

The occurrence of oxidative DNA damage and chromosomal aberrations are well-established events in humans. Micronucleus and 8-oxodG have been quantified in various tissues and fluids as part of occupational health and biomonitoring studies. Detection of 8-oxodG is typically used as a measure of oxidiative DNA damage to link exposure and/or diseases to oxidative stress [e.g., urinary 8-oxodG (Hanchi et al., 2017); 8-oxodG in tumour samples (Mazlumoglu et al., 2017)]. Micronuclei (MN) are also regularly quantified as a biomarker of genotoxicant exposure or genotoxic stress in humans. Numerous examples of detecting MN in different human tissues (e.g., lymphocytes, buccal cells, urothelial cells) are available in the current literature (Li et al., 2014; Dong et al., 2019; Alpire et al., 2019). Mutations also have been measured in human samples of diverse cell types (Ojha et al., 2018; Zhu et al., 2019; Liljedahl et al., 2019). As such, observations of the MIE and the two AOs of this AOP have been extensively documented in humans.

Essentiality of the Key Events

A large number of studies have been published that explore the effects of KE modulation on downstream effects. These studies broadly provide strong support to the essentiality of the events within the AOP. Below are examples demonstrating the effects of KE modulation on downstream events.

Essentiality of Increase, oxidative DNA damage (MIE)

- GSH depletion increases 8-oxo-dG (MIE), and DNA strand breaks (KE2)
    - HepG2 human hepatocytes were treated with 1 mM buthionine sulphoximine (BSO), a GSH-depleting agent, for 4, 8, and 24 hours. Time-dependent statistically significant reduction in GSH was observed at all time points when compared to baseline. The level of 8-oxo-dG lesions was measured 6 and 24 hours after BSO exposure and, at both time points, there was a statistically significant increase in oxidative DNA lesions. A higher magnitude of lesions were present at 24 hours and with statistically significant increases in strand breaks (measured via comet assay) as compared to control ($p<0.01$) (Beddowes et al., 2003).
- Antioxidant treatment reduces oxidative lesions, downstream strand breaks, and MN induction (AO2)
    - A 3 hour exposure of HepG2 cells to increasing concentrations of tetrachlorohydroquinone (TCHQ) with a 5 mM N-acetylcysteine (NAC: a radical scanvenger and precursor to glutathione) pre-treatment reduced the amount of cellular ROS (measured by DCFH-DA assay), 8-oxodG, and strand breaks induced by TCHQ measured immediately following exposure. The MN assay at 24 hours indicated a statistically significant decrease in MN at the highest concentration (Dong et al., 2014).
    - Reduction of 8-oxo-dG levels following NAC treatment was also observed in embryos isolated from C57BL/6Jpun/pun mice treated with NAC via drinking water; NAC significantly reduced the number of 8-oxo-dG in the treatment group (Reliene et al., 2004). In human blood mononuclear cells collected in clinical studies, 72-hour NAC treatment significantly reduced the number of MN in the cells. Together, these data support the correlation between the levels of ROS, 8-oxo-dG, and MN frequency (Federici et al., 2015).

Essentiality of Inadequate DNA repair (KE1)

- The effect of inadequate DNA repair on lesion accumulation and strand breaks (KE2)
    - Endonuclease III-like protein 1 (Nth1) knock-out *in vivo* - FapyG and FapyA lesions were measured in liver nuclear extracts from wild type and Nth1-/- mice. Statistically significant increases in FapyG and FapyA were observed in Nth1-/- mice. These results demonstrate insufficient repair leading to accumulation of unrepaired oxidative lesions (Hu et al., 2005).
    - Ogg1 knock-out *in vitro* - In Ogg1-/- mouse embryonic fibroblasts (MEF) treated with 400 µM hydrogen peroxide for 30 minutes, there were significantly fewer strand breaks measured by alkaline comet assay, compared to Ogg1+/+ MEFs. Time series (5 – 90 minutes)

immunoblotting of the genomic DNA using anti-8-oxo-dG antibodies indicated a larger magnitude of oxidative lesions in Ogg1-/- cells compared to wild type. Overall, these results demonstrate the role of Ogg1 in the generation of strand breaks during BER following oxidative DNA damage (Wang et al., 2018).

- The effect of inadequate DNA repair on MN induction (AO2)
    - Ogg1 knock-out *in vivo* - In Ogg1-deficient mice exposed to silver nanoparticles (AgNPs) for seven days, a significant increase (compared to Ogg1+/+) in double strand breaks (by assessing the increase in phosphorylation of histone-2AX, a marker of DNA DSBs) and 8-oxo-dG lesions were observed at the end of treatment and after 7 days of recovery. The magnitude of increase in DSBs after the 7-day recovery was smaller in wild type. Levels of MN were measured in erythrocytes at the same time points. Increases in MN frequency were significant in wild type (compared to untreated control) on day 7, but not after 7 and 14 days of recovery. In Ogg1-/- mice, the increase in MN was significantly higher on day 7 compared to Ogg1+/+ mice and untreated Ogg1-/- mice and remained significant 7 and 14 days after the exposure (Nallanthighal et al., 2017). Thus, the DNA damage was retained in repair deficient mice leading to persistent clastogenic effects.
- The effect of inadequate DNA repair on mutations (AO1)
    - Suzuki et al. (2010) knocked-down BER-initiating glycosylases (OGG1, NEIL1, MYH, NTH1) in HEK293T human embryonic kidney cells transfected with plasmids that were either positive or negative for 8-oxodG. The resulting changes in mutant frequencies were measured. Compared to the negative control, all knock-downs caused the mutant frequency to increase in 8-oxodG plasmid-containing cells. Moreover, G:C to T:A transversion frequency increased in all analyzed cells. MYH knock-down decreased A:T to C:G transversion frequency of A paired to 8-oxo-dG; the latter result supports the futile MYH-initiated BER model for the repair of 8-oxo-dG opposite A (Suzuki et al., 2010). Overall, these findings support the essential role of DNA repair in mitigating the mutagenic effects of oxidative DNA lesions.

<u>Essentiality of Increase, DNA strand breaks (KE2)</u>

- Double strand breaks leading to mutations (AO1)
    - Tatsumi-Miyajima et al. (1993) analyzed different mutations arising from the repair of DSBs induced by a restriction endonuclease, AvaI, in five different human fibroblast cell lines transfected with plasmids containing the AvaI restriction site in the supF gene. Cells containing non-digested plasmids (negative control) produced spontaneous supF mutation frequencies between 0.197 and 2.49 x10-3. In cells containing Ava1-digested plasmids, the number of supF mutants increased, indicated by the rejoining fidelity ((total colonies-supF mutants)/total colonies) between 0.50-0.86. Hence, up to 50% of the colonies were mutated at the AvaI restriction site due to erroneous repair of DSBs induced by the endonuclease.(Tatsumi-Miyajima et al., 1993).

- Reduction in strand breaks leads to decreases in MN frequency (AO2)
    - Differentiated rat thyroid cells (PCCL3) were internally irradiated by 131I treatment and externally irradiated by 5 Gy X-rays, with or without NAC pre-treatment. Cellular ROS and strand breaks were measured at different time points after irradiation. NAC pre-treatment abrogated ROS induced by both internal and external irradiation at 30 min. The level of ROS was also significantly lower in the NAC-treated cells compared to the non-treated cells at later time points (2, 24, and 48 hours). Moreover, the induction of strand breaks at 30 min was also prevented by NAC pre-treatment and there was a reduction in strand breaks compared to the non-treated cells at later time points as well. Finally, the induction of MN measured 24 and 48 hours after irradiation was significantly lower in NAC-treated cells compared to non-treated cells (Kurashige et al., 2017).

Weight of Evidence Summary

	Defining Question	**High (Strong)**	**Moderate**	**Low (Weak)**
1. Support for biological plausibility	Is there a mechanistic relationship between KEup and KEdown consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance.	KER is plausible based on analogy to accepted biological relationships, but scientific understanding is incomplete	Empirical support for association between KEs, but the structural or functional relationship between them is not understood.
MIE → KE1: Increase, oxidative DNA damage leads to inadequate repair	**STRONG** The repair mechanisms for oxidative DNA damage have been extensively studied and are well-understood. It is generally accepted that limits exist on the amount of oxidative DNA damage that can be managed by these repair mechanisms.			
KE1 → KE2: Inadequate repair leads to Increase, DNA strand breaks	**STRONG** It is well-established that failed attempts to repair accumulated oxidative lesions and replication fork stalling by both unrepaired and incompletely repaired DNA lesions (e.g., repair intermediates such as abasic sites and SSBs) lead to an increase in DNA strand breaks.			
KE2 →KE1: Increase, DNA strand breaks leads to Inadequate repair	**STRONG** It is well recognized that the pathways involved in the repair of DSBs is error-prone. In addition to errors induced by NHEJ, all repair mechanisms have a capacity limit; if the number of strand breaks exceed the repair capacity of the cell, unrepaired SSBs and DSBs may accumulate.			
KE1 → AO1: Inadequate repair leads to Increase, mutations	**STRONG** Numerous studies (cell-based and *in vivo*) have demonstrated increases in mutation due to unrepaired oxidative DNA lesions (insufficient repair) and incorrect repair (e.g., non-homologous end joining and error-prone lesion bypass). The mechanisms by which these events occur are well-understood.			
KE1 → AO2: Inadequate repair leads to	**STRONG** Chromosomal aberrations may result if DNA repair is inadequate, meaning that the DSBs are misrepaired or not repaired at all. A large variety of different chromosomal			

Increase, chromosomal aberrations	aberrations can occur, depending on the timing (i.e., cell cycle) and type of inadequate repair. Examples include copy number variants, deletions, translocations, inversions, dicentric chromosomes, nucleoplasmic bridges, nuclear buds, micronuclei, centric rings, and acentric fragments. A multitude of publications are available that provide details on how these various chromosomal aberrations are formed in the context of inadequate repair.
Non-adjacent: KE2 →AO1: Increase, DNA strand breaks leads to Increase, mutations	**STRONG** Mechanisms of DNA strand break repair have been extensively studied. It is accepted that non-homologous end joining of DSBs can introduce deletions, insertions, translocations, or base substitution.
Non-adjacent MIE → KE2: Oxidative DNA lesions leads to Increase, DNA strand breaks	**MODERATE** Increase in strand breaks due to failed repair of oxidative DNA lesions is an accepted mechanism for the clastogenic effects of oxidative damage. Concurrent increases in the two KEs have been observed in previous studies. However, data that demonstrate a causal relationship, in accordance with the Bradford-Hill criteria for causality, are limited.
Non-adjacent MIE → AO1: Oxidative DNA lesions leads to Increase, mutations	**STRONG** Strong empirical evidence exists in literature demonstrating increases in mutation frequency due to increase in oxidative DNA lesions. Notably, mutagenicity of 8-oxodG, the most abundant oxidative DNA lesion, has been extensively studied and is well-known to cause G to T transversions.
Non-adjacent KE2→AO2: Increase, DNA strand breaks leads to Increase, chromosomal aberrations	**STRONG** DNA strands breaks must occur for chromosomal aberrations to occur. Increase in strand breaks, especially DSBs, may increase the risk of inadequate repair (lack of repair or misrepair) of the damage, leading to translocations, inversions, insertions, and deletions.

2. Support for Essentiality of KEs	**Defining Question**	**High (Strong)**	**Moderate**	**Low (Weak)**
	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
MIE: Increase, oxidative DNA damage	**MODERATE** Studies have demonstrated that indirectly reducing or increasing the amount of oxidative DNA lesions by modulating cellular ROS levels (via antioxidant addition or depletion) causes concordant changes in the levels of strand breaks and MN.			
KE1: Inadequate	**STRONG** Numerous studies have investigated inadequate BER of oxidative DNA lesions by			

<table>
<tr><td>repair</td><td colspan="4">disrupting BER through generating gene KO rodent or mammalian cell models. Modulation of the downstream KEs (i.e., DNA strand breaks, mutation, MN induction) by dysfunctional BER has been demonstrated in these studies.</td></tr>
<tr><td>KE2: DNA strand breaks</td><td colspan="4">MODERATE
Theoretically, chromosomal aberrations (AO2) cannot occur unless DNA strand breaks occur. Predominantly, indirect evidence exists that support the essentiality of KE2 in leading to mutations (AO1).</td></tr>
<tr><td rowspan="2">3. Empirical Support for KERs</td><td>Defining Question</td><td>High (Strong)</td><td>Moderate</td><td>Low (Weak)</td></tr>
<tr><td>Does empirical evidence support that a change in KEup leads to an appropriate change in KEdown?
Does KEup occur at lower doses and earlier time points than KE down and is the incidence of KEup> than that for KEdown?
Inconsistencies?</td><td>Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors.
No or few critical data gaps or conflicting data</td><td>Demonstrated dependent change in both events following exposure to a small number of stressors.
Some inconsistencies with expected pattern that can be explained by various factors.</td><td>Limited or no studies reporting dependent change in both events following exposure to a specific stressor; and/or significant inconsistencies in empirical support across taxa and species that don’t align with hypothesized AOP</td></tr>
<tr><td>MIE → KE1: Increase, oxidative DNA damage leads to inadequate repair</td><td colspan="4">MODERATE
Empirical in vitro and in vivo data demonstrate that increases in oxidative DNA lesions lead to indications of inadequate repair (i.e., increases in mutation, retention of adducts, increases in lesions despite upregulation of repair enzymes).</td></tr>
<tr><td>KE1 → KE2: Inadequate repair leads to Increase, DNA strand breaks</td><td colspan="4">MODERATE
Limited in vivo data are available. A few In vitro studies have demonstrated a larger increase in DNA strand breaks in BER-defective cells compared to wildtype cells, following various oxidative stresse-inducing chemical exposures.
In certain cases, as demonstrated by Wang et al. (2018), knock-down of OGG1 (BER-initiating glycosylase) reduced the amount of DNA strand breaks that formed after exposure to hydrogen peroxide - mostly likely due to the reduction in the incidences of incomplete repair. As such, deficiency in different DNA repair proteins can have varying effects on downstream strand breaks; inadequate repair may manifest differently for different stressors.</td></tr>
<tr><td>KE2 →KE1: Increase, DNA strand breaks leads to Inadequate repair</td><td colspan="4">MODERATE
Results from many studies indicate dose/incidence and temporal concordance between the frequency of DSBs and the rate of inadequate repair. As DNA damage accumulates in cells, the incidence of inadequate DNA repair activity (in the form of non-repaired or misrepaired DSBs) also increases. Uncertainties in this KER include controversy surrounding the error rate of NHEJ, differences in responses depending on genotoxicant exposure levels and confounding clinical factors (such as smoking) that affect DSB repair fidelity.</td></tr>
<tr><td>KE1 → AO1: Inadequate repair leads to Increase, mutations</td><td colspan="4">STRONG
Repair deficiency causing increases in mutations has been extensively demonstrated in both in vitro and in vivo. Overexpression of repair enzymes has been shown to reduce mutation frequency following chemical exposure in vitro, which further supports the causal relationship between these two KEs.</td></tr>
</table>

KE1 → AO2: Inadequate repair leads to Increase, chromosomal aberrations	**MODERATE** There is little empirical evidence available that directly examines the dose and incidence concordance between DNA repair and CAs within the same study. Similarly, there is not clear evidence of a temporal concordance between these two events. More research is required to establish empirical evidence for this KER.
Non-adjacent: KE2 →AO1: Increase, DNA strand breaks leads to Increase, mutations	**MODERATE** Evidence from *in vitro* and *in vivo* studies demonstrating dose and temporal concordance of the two KEs are available. These investigations utilized various stressors such as chemicals and ionizing radiation.
Non-adjacent MIE → KE2: Oxidative DNA lesions leads to Increase, DNA strand breaks	**MODERATE** Both *in vitro* and *in vivo* data are available that the demonstrate dose-response concordance of oxidative DNA lesions formation and strand breakage following exposure to various stressors. However, the temporal concordance between the KEs is not strong; there are discrepancies in the temporal sequence of events that appear to be dependent on the endpoint used to measure the KE (i.e., formamidopyrimidine DNA glycosylase (Fpg) comet assay vs. 8-oxodG immunodetection, comet assay vs. ɣ-H2AX immunodetection).
Non-adjacent MIE → AO1: Increase, oxidative DNA lesions leads to Increase, mutations	**STRONG** This KER was demonstrated by knocking out oxidative DNA damage repair protein (OGG1) and exposure to different ROS-inducing chemicals *in vitro* and *in vivo*. It is clear that an increase in oxidative DNA lesions is followed by an increase in mutant frequency or G to T transversions.
Non-adjacent KE2→AO2: Increase, DNA strand breaks leads to Increase, chromosomal aberrations	**MODERATE** Temporal concordance is clear in both *in vitro* and *in vivo* data. However, due to the differences in the methods used to measure strand breaks and chromosomal aberrations, the concentration-response of these events often appear to be discordant.

Please also refer to AOP #272 “Direct deposition of ionizing energy onto DNA leading to lung cancer”, which shares numerous KE and KERs.

Quantitative Consideration

The quantitative understanding of the KERs in this AOP is overall weak. Different cell types have different baseline levels of antioxidants and antioxidant enzymes, as well as different oxidative DNA lesion repair capacity. For example, Nishioka et al. (1999) demonstrated difference in the expression level of OGG1 mRNA across different human tissues (Nishioka et al., 1999). Thus, the quantity of oxidative DNA lesions required to overwhelm the repair mechanisms and lead to chromosomal damage or mutations differ by cell type. Furthermore, antioxidant and DNA repair capacities differ by individual *in vivo* and are influenced by factors such as age and the disease state of the individual; for example, DNA repair ability and antioxidant enzyme activities are known to decline with

age in humans (Liguori et al., 2018; Kozakiewicz et al., 2019; Chen et al., 2020). Such are modulating factors for the AOP progression. Thus, we note that different thresholds exist for the amount of oxidative DNA damage that leads to the AO, depending on the individual and the modulating factors affecting the individual.

Modulating Factors

As discussed above, there are various modulating factors for this AOP, including genetic polymorphisms in DNA repair and antioxidant response-associated genes in individuals and the metabolic competency (i.e., phase 2 xenobiotic metabolism) of the cell line used. For *in vitro* experiments, a critical consideration is the concentration at which genomic effects are measured, as ROS production is expected to be highly elevated at overtly cytotoxic concentrations (i.e., generally >50% cytotoxicity in the context of genotoxicity testing). Mutations and chromosomal aberrations occurring above certain levels of cytotoxicity are not considered relevant to *in vivo* outcomes. As such, *in vitro* genotoxicity testing guidelines should be consulted for specific recommendations for selecting the top chemical exposure concentrations.

Considerations for Potential Applications of the AOP

Genotoxicity testing is a fundamental requirement for all chemical and pharmaceutical safety assessments. Although there are established guidelines for in vitro tests, the current standard in vitro genotoxicity assays provide limited mechanistic information and suffer from high sensitivity and low specificity, potentially leading to unnecessary follow up work. The field is moving towards the use of more biologically relevant in vitro models and tests that inform on mechanisms that cause the observed apical outcome (Whitwell et al., 2015). There is also a movement away from the notion that genotoxicity testing is only valuable to inform potential hazards and identify genotoxic mechanisms of carcinogenesis, as many mechanisms of genotoxicity only operate once a critical exposure threshold is satisfied. Indeed, there is increasing use of genotoxicity data in deriving points of departure to inform risk assessments (Klapacz and Gollapudi, 2020; Luijten et al., 2020; White et al., 2020). Overall, understanding the biological mechanisms (i.e., the MIEs and KEs) that lead to genotoxic outcomes is essential to the risk assessment process (Dearfield et al., 2017), especially if the mechanism is not biologically active at clinically relevant exposures. This AOP network provides a framework for assembling information from different mechanism-based tests to determine the probability that an agent induces oxidative DNA damage, which can be used to demonstrate that the agent itself is not DNA reactive. For example, analysis of a chemical using the Bradford-Hill criteria aligned against this AOP network could be used to determine if the chemical's primary mode of action is via ROS-induction, rather than an artefact of over-exposure (e.g., cytotoxicity). In the case of a true ROS-driven mechanism, levels of oxidative DNA damage and likely cell cycle delay should occur at concentrations below those that induce chromosomal aberrations and mutations.

Oxidative DNA damage is a long-established mechanism of inducing genotoxicity and, thus, a useful endpoint in assessing genotoxicity risk. From a human health perspective, there is an increasing understanding (and acceptance) of the fact that genomic damage such as mutations, in and of themselves, are adverse (e.g., germ cell mutations) (Heflich et al., 2020). However, a chemical that induces oxidative DNA damage will demonstrate a threshold concentration, below which it does not induce measurable genotoxicity. This is due to the various pathways of endogenous DNA repair and enzymatic reductions that prevent progression of oxidative lesions to adverse genotoxicity outcomes, facilitating

quantification of safe permissible exposures. Hence, once it is demonstrated for a chemical that this AOP is operable, a quantitative assessment of in vivo genotoxicity data (e.g., micronuclei or mutations) could be used to assess risk, provided the study design is appropriate (White and Johnson, 2016; Dearfield et al., 2017; White et al., 2020).

Overall, AOP networks (such as this one) can inform how different testing methods, including fit-for-purpose mechanistic assays, should be used to quantitatively relate KEs to specific adverse genotoxic outcomes. Presently, this AOP network documents clear gaps in the quantitative understanding of genomic damage induced by oxidative DNA lesions that when filled, will enhance risk assessment and predictive toxicology for chemicals that induce oxidative DNA lesions. In conclusion, AOPs like this can be applied in regulatory assessment of chemicals to (a) facilitate mode of action analysis of chemicals to hypothesize potential molecular initiating events; (b) identify test methods and strategies for use with untested chemicals to link them to the appropriate AOP(s); (c) highlight knowledge gaps and uncertainties in genotoxic MOAs; (d) facilitate the development of new ‘all-in-one’ testing strategies, where MOA and apical endpoints are measured concomitantly; and (e) support a non-linear risk assessment when direct DNA-reactivity is not empirically supported.

References

Alpire, M., C. Cardoso, C. Seabra Pereira, and D. Ribeiro (2019), "Genomic instability in Buccal mucosal cells of children living in abnormal conditions from Santos-Sao Vicente Estuary", Int J Environ Health Res, 1:1-7.

Arai, T., V.P. Kelly, O. Minowa, T. Noda, and S. Nishimura (2002), "High accumulation of oxidative DNA damage, 8-hydroxyguanine, in Mmh/Ogg1 deficient mice by chronic oxidative stress", Carcinogenesis, 23:2005-2010.

Ataseven, N., C. Yuzbasioglu A., and F. Unal (2016), "Genotoxicity of monosodium glutamate", Food Chem Toxicol, 91:8-18.

Ba, X., and I. Boldogh (2018), "8-Oxoguanine DNA glycosylase 1: Beyond repair of the oxidatively modified base lesions", Redox Biol,14:669-678. .

Ballmaier, D. and B. Epe (2006), "DNA damage by bromate: Mechanism and consequences", Toxicol, 221:166-171.

Barnes R., E. Fouquerel, and P. Opresko (2019), "The impact of oxidative DNA damage and stress on telomere homeostasis", Mech Ageing Dev, 177: 37–45.

Beddowes, E., S. Faux, and J.K. Chipman (2003), "Chloroform, carbon tetrachloride and glutathione depletion induce secondary genotoxicity in liver cells via oxidative stress", Toxicol, 187:101-115.

Benbrook, C.M. (2019), "How did the US EPA and IARC reach diametrically opposed conclusions on the genotoxicity of glyphosate-based herbicides?", Envrion Sci Eur, 31:2.

Berquist, B. and D. Wilson III (2012), "Pathways for Repairing and Tolerating the Spectrum of Oxidative DNA Lesions", Cancer Lett, 327:61-72.

Bordin, D. L., Lirussi, L., and H. Nilsen (2021), "Cellular response to endogenous DNA damage: DNA base modifications in gene expression regulation", DNA Repair (Amst), 99:103051.

Bolzan, A. D. (2012), "Chromosomal aberrations involving telomeres and interstitial telomeric sequences", Mutagenesis, 27: 1-15.

Brandsma, I. and D. C. van Gent (2012), "Pathway choice in DNA double strand break repair: observations of a balancing act", Genome Integr, 3:9.

Cadet, J. and J.R. Wagner (2013), "DNA Base Damage by Reactive Oxygen Species, Oxidizing Agents, and UV Radiation", Cold Spring Harb Perspect Biol, 5:a012559.

Caglayan M, J. K. Horton, D. P. Dai , D. F. Stefanick, and S. H. Wilson. (2017). "Oxidized nucleotide insertion by pol β confounds ligation during base excision repair". Nature Communications 8.

Cannan, W. J. and D. S. Pederson (2017), "Mechanisms and Consequences of Double-strand DNA Break Formation in Chromatin", J Cell Physiol, 231:3-14.

Ceccaldi, R., B. Rondinelli, and A.D. D'Andrea (2016), "Repair Pathway Choices and Consequences at the Double-Strand Break", Trends Cell Biol, 26:52-64.

Cha, M., D. G. Kim, and I. Mook-Jung. (2015). "The role of mitochondrial DNA mutation on neurodegenerative diseases". Exp Mol Med 47:e150.

Chen Y, A. Geng, W. Zhang, Z. Qian, X. Wan , Y. Jiang, and Z. Mao (2020), "Fight to the bitter end: DNA repair and aging", Ageing Res Rev, 64:101154.

Colussi, C., E. Parlanti, P. Degan, M. Crescenzi, E. Dogliotti, and M. Bignami (2002). "The Mammalian Mismatch Repair Pathway Removes DNA 8-oxodGMP Incorporated from the Oxidized dNTP Pool". Curr Biol 12:912-918.

Cooke, M., M. Evans, M. Dizdaroglu, and J. Lunec (2003), "Oxidative DNA damage: mechanisms, mutation, and disease", FASEB J, 17:1195-1214.

Coquerelle, T., J. Dosch, and B. Kaina (1995), "Overexpression of N-methylpurine-DNA glycosylase in Chinese hamster ovary cells renders them more sensitive to the production of chromosomal aberrations by methylating agents - a case of imbalanced DNA repair ", Mutat Res, 336:9-17.

Dan Dunn, J., L. A. Alvarez, X. Zhang, and T. Soldati (2015), "Reactive oxygen species and mitochondria: A nexus of cellular homeostasis", Redox biol, 6: 472–485.

Dearfield, K., B. Gollapudi, J. Bemis, R. Benz, G. Douglas, R. Elespuru, G. Johnson, D. Kirkland, M. LeBaron, A. Li, F. Marchetti, L. Pottenger, E. Rorije, J. Tanir, V. Thybaud, J. van Benthem, C.L. Yauk, E. Zeiger, and M. Luijten (2017), "Next Generation Testing Strategy for Assessment of Genomic Damage: A conceptual framework and considerations", Environ Mol Mutagen, 58:264-283.

Deavall, D., E. Martin, J. Hornet, and R. Roberts (2012), "Drug-Induced Oxidative Stress and Toxicity", J Toxicol, 2012:645460.

Deferme, L., J.J. Briede, S.M. Claessen, D.G. Jennen, R. Cavill, and J.C. Kleinjans (2013), "Time series analysis of oxidative stress response patterns in HepG2: A toxicogenomics approach ", Toxicol, 306:24-34.

Dong, H., D. Xu, L. Hu, L. Li, E. Song, and Y. Song (2014), "Evaluation of N-acetyl-cysteine against tetrachlorobenzoquinoneinduced genotoxicity and oxidative stress in HepG2 cells", Food Chem Toxicol, 64:291-297.

Dong, J., J. Wang, Q. Qian, G. Li, D. Yang, and C. Jiang (2019), "Micronucleus assay for monitoring the genotoxic effects of arsenic in human populations: A systematic review of the literature and meta-analysis", Mutat Res, 780:1-10.

Durante, M., J.S. Bedford, D.J. Chen, S. Conrad, M.N. Cornforth, A.T. Natarajan, D. van Gent, and G. Obe (2013), "From DNA damage to chromosome aberrations: Joining the break", Mutat Res, 756:5-13.

Ensminger, M., L. Iloff, C. Ebel, T. Nikolova, B. Kaina, and M. Lobrich (2014), "DNA breaks and chromosomal aberrations arise when replication meets base excision repair", J Cell Biol, 206:29.

Federici, C., K. Drake, C. Rigelsky, L. McNelly, S. Meade, S. Comhair, S. Erzurum, and M. Aldred (2015), "Increased Mutagen Sensitivity and DNA Damage in Pulmonary Arterial Hypertension", Am J Respir Crit Care Med, 192:219-228.

Fouquerel, E., J. Lormand, A. Bose, H. T. Lee, G. S. Kim, J. Li, Sobol, B. D. Freudenthal, S. Myong, and P. L. Opresko (2016), "Oxidative guanine base damage regulates human telomerase activity", Nat Struct Mol Biol, 23: 1092–1100.

Fouquerel, E., R. P. Barnes, S. Uttam, S. C. Watkins, M. P. Bruchez, and P. L. Opresko (2019), "Targeted and Persistent 8-Oxoguanine Base Damage at Telomeres Promotes Telomere Loss and Crisis", Mol Cell, 75: 117-130.

Fujita, M., H. Sasanuma, K. Yamamoto, H. Harada, A. Kurosawa, N. Adachi, M. Omura, M. Hiraoka, S. Takeda, and K. Hirota (2013), "Interference in DNA Replication Can Cause Mitotic Chromosomal Breakage Unassociated with Double-Strand Breaks", PLoS One, 8:e60043.

Gedik, C., S. Boyle, S. Wood, N. Vaughan, and A.R. Collins (2002), "Oxidative stress in humans: validation of biomarkers of DNA damage", Carcinogenesis, 23:1441-1446.

Guo, C., P. Ding, C. Xie, C. Ye, M. Ye, C. Pan, X. Cao, S. Zhang, and S. Zheng (2017), "Potential application of the oxidative nucleic acid damage biomarkers in detection of diseases", Oncotarget, 8:75767-75777.

Gustafson, H. H., D. Holt-Casper, D. W. Grainger, and H. Ghandehari, (2015), "Nanoparticle Uptake: The Phagocyte Problem", Nano today, 10(4): 487–510.

Hashimoto, K., Y. Tominaga, Y. Nakabeppu, and M. Moriya (2004), "Futile short-patch DNA base excision repair of adenine:8-oxoguanine mispair", Nucleic Acids Res, 32:5928-5934.

Hayashi, H., Y. Tominaga, S. Hirano, A. E. McKenna, Y. Nakabeppu, and Y. Matsumoto (2002), "Replication-associated repair of adenine:8-oxoguanine mispairs by MYH", Curr Biol, 12:335–339.

Heflich R. H., G. E. Johnson, A. Zeller, F. Marchetti, G. R. Douglas, K. L. Witt, B. B. Gollapudi, and P. A. White (2020), "Mutation as a Toxicological Endpoint for Regulatory Decision-Making", Environ Mol Mutagen 61:34-41.

Hanchi, M., L. Campo, E. Polledri, L. Olgiati, D. Consonni, D. Saidane-Mosbahi, and S. Fustinoni (2017), "Urinary 8-Oxo-7,8-Dihydro-2'-Deoxyguanosine in Tunisian Electric Steel Foundry Workers Exposed to Polycyclic Aromatic Hydrocarbons", Ann Work Expo Health, 61:333-343.

Hu, J., N.C. de Souza-Pinto, K. Haraguchi, B. Hogue, P. Jaruga, M.M. Greenberg, M. Dizdaroglu, and V. Bohr (2005), "Repair of formamidopyrimidines in DNA involves different glycosylases: role of the OGG1, NTH1, and NEIL1 enzymes", J Biol Chem, 280:40544-40551.

Iliakis, G., H. Wang, A.R. Perrault, W. Boecker, B. Rosidi, F. Windhofer, W. Wu, J. Guan, G. Terzoudi, and G. Pantelias (2004), "Mechanisms of DNA double strand break repair and chromosome aberration formation", Cytogenet Genome Res, 104:14-20.

Jena, N. R. and P. C. Mishra. (2012). "Formation of ring-opened and rearranged products of guanine: Mechanisms and biological significance". Free Rad Biol Med 53:81-94.

Kier, L.D. and D. Kirkland (2013), "Review of genotoxicity studies of glyphosate and glyphosate-based formulations", Crit Rev Toxicol, 43:283-315.

Klapacz, J. and B. B. Gollapudi (2020), "Considerations for the Use of Mutation as a Regulatory Endpoint in Risk Assessment", Environ Mol Mutagen 61:84-93.

Klungland, A., I. Rosewell, S. Hollenbach, E. Larsen, G. Daly, B. Epe, E. Seeberg, T. Lindahl, and D. Barnes (1999), "Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage", Proc Natl Acad Sci USA, 96:13300-13305.

Kozakiewicz, M., M. Kornatowski, O. Krzywińska, and K. Kędziora-Kornatowska (2019), "Changes in the blood antioxidant defense of advanced age people", Clin Interv Aging, 14: 763–771.

Kramara, J., B. Osia, and A. Malkova (2018), "Break-Induced Replication: The Where, The Why, and The How", Trends Genet, 34:518-531.

Kurashige, T., M. Shimamura, and Y. Nagayama (2017), "N-Acetyl-l-cysteine protects thyroid cells against DNA damage induced by external and internal irradiation", Radiat Environ Biophys, 56:405-412.

Li, P., Y. Gu, S. Yu, Y. Li, J. Yang, and G. Jia (2014), "Assessing the suitability of 8-OHdG and micronuclei as genotoxic biomarkers in chromate-exposed workers: a cross-sectional study", BMJ Open, 4:e005979.

Liguori, I., G. Russo, F. Curcio, G. Bulli, L. Aran, D. Della-Morte, G. Gargiulo, G. Testa, F. Cacciatore, D. Bonaduce, and P. Abete (2018), "Oxidative stress, aging, and diseases", Clin Interv Aging, 13:757-772.

Liljedahl, E., K. Wahlberg, C. Liden, M. Albin, and K. Broberg (2019), "Genetic variants of filaggrin are associated with occupational dermal exposure and blood DNA alterations in hairdressers", Sci Total Environ, 653:45-54.

Luijten, M., N S. Ball, K. Dearfield, B. B. Gollapudi, G. E. Johnson, F. Madia, L. Peel, S. Pfuhler, R. S. Settivari, W. Ter Burg, P. A. White, and J. van Benthem (2020), "Utility of a next generation framework for assessment of genomic damage: A case study using the industrial chemical benzene", Environ Mol Mutagen, 61:94-113.

Mao, Z., M. Bozzella, A. Seluanov, and V. Gorbunova (2008a), "Comparison of nonhomologous end joining and homologous recombination in human cells", DNA Repair, 7:1765-1771.

Mao, Z., M. Bozzella, A. Seluanov, and V. Gorbunova (2008b), "DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells", Cell Cycle, 7:2902-2906.

Markkanen, E., B. Castrec, G. Villani, and U. Hübscher (2012), "A switch between DNA polymerases δ and λ promotes error-free bypass of 8-oxo-G lesions", Proc Natl Acad Sci USA, 109: 20401–20406.

Markkanen, E. (2017), "Not breathing is not an option: How to deal with oxidative DNA damage", DNA Repair, 59:82-105.

Mazlumoglu, M.R., O. Ozkan, H.H. Alp, E. Ozyildirim, F. Bingol, O. Yoruk, and O. Kuduban (2017), "Measuring Oxidative DNA Damage With 8-Hydroxy-2'-Deoxyguanosine Levels in Patients With Laryngeal Cancer", Ann Otol Rhinol Laryngol, 126:103-109.

Mehta, A. and J. Haber (2014), "Sources of DNA Double-Strand Breaks and Models of Recombinational DNA Repair", Cold Spring Harb Perspect Biol, 6:a016428.

Nakamura T., K. Okabe, S. Hirayama, M. Chirifu, S. Ikemizu, H. Morioka, Y. Nakabeppu and Y. Yamagata (2021), "Structure of the mammalian adenine DNA glycosylase MUTYH: insights into the base excision repair pathway and cancer", Nucleic Acids Res, 49:7154–7163.

Nallanthighal, S., C. Chan, T. Murray, A. Mosier, N. Cady, and R. Reliene (2017), "Differential effects of silver nanoparticles on DNA damage and DNA repair gene expression in Ogg1-deficient and wild type mice", Nanotoxicol, 11:996-1011.

Nemec, A., S. Wallace, and J. Sweasy (2010), "Variant base excision repair proteins: Contributors to genomic instability", Seminars Cancer Biol, 20:320-328.

Obe, G., C. Johannes, and S. Ritter (2010), "The number and not the molecular structure of DNA double-strand breaks is more important for the formation of chromosomal aberrations: A hypothesis", Mutat Res, 701:3-11.

Ojha, J., I. Dyagil, S. Finch, R. Reiss, A. de Smith, S. Gonseth, M. Zhou, H. Hansen, A. Sherborne, J. Nakamura, P. Bracci, N. Gudzenko, M. Hatch, N. Babkina, M.P. Little, V.V. Chumak, K. Walsh, D. Bazyka, J. Wiemels, and L. Zablotska (2018), "Genomic characterization of chronic lymphocytic leukemia (CLL) in radiation-exposed Chornobyl cleanup workers", Environ Health, 17:43.

Oka, S. and Y. Nakabeppu (2011), "DNA glycosylase encoded by MUTYH functions as a molecular switch for programmed cell death under oxidative stress to suppress tumorigenesis", Cancer Sci, 102:677-682.

Ondovcik, S.L., L. Tamblyn, J.P. McPherson, and P. Wells (2012), "Oxoguanine Glycosylase 1 (OGG1) Protects Cells from DNA Double-Strand Break Damage Following Methylmercury (MeHg) Exposure", Toxicol Sci, 128:272-283.

Parke, D. V. (1982), "Mechanisms of chemical toxicity--a unifying hypothesis", Regul Toxicol Pharmacol, 2(4):267-86.

Petersen, S., G. Saretzki, and T. von Zglinicki (1998), "Preferential accumulation of single-stranded regions in telomeres of human fibroblasts", Exp Cell Res, 239:152-60.

Platel, A., F. Nesslany, V. Gervais, and D. Marzin (2009), "Study of oxidative DNA damage in TK6 human lymphoblastoid cells by use of the *in vitro* micronucleus test: Determination of No-Observed-Effect Levels", Mutat Res, 678:30-37.

Poetsch, A., S. Boulton, and N. Luscombe (2018), "Genomic landscape of oxidative DNA damage and repair reveals regioselective protection from mutagenesis", Genome Biol, 19:215.

Reliene, R., E. Fischer, and R. Schiestl (2004), "Effect of N-Acetyl Cysteine on Oxidative DNA Damage and the Frequency of DNA Deletions in Atm-Deficient Mice", Cancer Res, 64:5148-5153.

Rodgers, K. and M. McVey (2016), "Error-prone repair of DNA double-strand breaks", J Cell Physiol, 231:15-24.

Roszkowski, K., W. Jozwicki, P. Blaszczyk, A. Mucha-Malecka, and A. Siomek (2011), "Oxidative damage DNA: 8-oxoGua and 8-oxodG as molecular markers of cancer", Medical Science Monitor, 17:329-333.

Russo, M. T., M. F. Blasi, F. Chiera, P. Fortini, P. Degan, P. Macpherson, M. Furuichi, Y. Nakabeppu, P. Karran , G. Aquuilina, and M. Bignami. (2004). "The Oxidized Deoxynucleoside Triphosphate Pool Is a Significant Contributor to Genetic Instability in Mismatch Repair-Deficient Cells". Mol Cell Biol 24:465-474.

Saki M, Prakash A. 2017. DNA Damage Related Crosstalk Between the Nucleus and Mitochondria. Free Radic Biol Med 107:216-227.

Sakofsky, C.J., S. Ayyar, A. Deem, W.H. Chung, G. Ira, and A. Malkova (2015), "Translesion Polymerases Drive Microhomology-Mediated Break-Induced Replication Leading to Complex Chromosomal Rearrangements", Mol Cell, 60:860-872.

Sapounidou, M., Ebbrell, D.J., Bonnell, M.A., Campos, B., Firman, J.W., Gutsell, S., Hodges, G., Roberts, J., Cronin, M.T.D. (2021), "Development of an Enhanced Mechanistically Driven Mode of Action Classification Scheme for Adverse Effects on Environmental Species", Environ Sci Technol. 55(3):1897-1907.

Sedletska, Y., J.P. Radicella, and E. Sage (2013), "Replication fork collapse is a major cause of the high mutation frequency at three-base lesion clusters", Nucleic Acids Res, 41:9339-9348.

Shah, A., K. Gray, N. Figg, A. Finigan, L. Starks, and M. Bennett (2018), ". Defective Base Excision Repair of Oxidative DNA Damage in Vascular Smooth Muscle Cells Promotes Atherosclerosis", Circulation, 138:1446-1462.

Shih, W., C. Chang, H. Chen, and K. Fan (2018), "Antioxidant activity and leukemia initiation prevention in vitro and in vivo by N-acetyl-L-cysteine", Oncol Lett, 16:2046-2052.

Smith, M.T., K.Z. Guyton, C.F. Gibbons, J.M. Fritz, C.J. Portier, I. Rusyn, D.M. DeMarini, J.C. Caldwell, R.J. Kavlock, P.F. Lambert, S.S. Hecht, J.R. Bucher, B.W. Stewart, R.A. Baan, V.J. Cogliano, and K. Straif. (2016), "Key Characteristics of Carcinogens as a Basis for Organizing Data on Mechanisms of Carcinogenesis", Environ Health Perspect. 2016 Jun;124(6):713-21.

Suzuki, T., H. Harashima, and H. Kamiya (2010), "Effects of base excision repair proteins on mutagenesis by 8-oxo-7,8-dihydroguanine (8-hydroxyguanine) paired with cytosine and adenine", DNA Repair, 9:542-550.

Taggart, D., S. Fredrickson, V. Gadkari, and Z. Suo (2014), "Mutagenic Potential of 8-Oxo-7,8-dihydro-2′-deoxyguanosine Bypass Catalyzed by Human Y-Family DNA Polymerases", Chem Res Toxicol, 27:931-940.

Tatsumi-Miyajima, J., T. Yagi, and H. Takebe (1993), "Analysis of mutations caused by DNA double-strand breaks produced by a restriction enzyme in shuttle vector plasmids propagated in ataxia telangiectasia cells", Mutat Res, 294:317-323.

Tell, G., F. Quadrifoglio, C. Tiribelli, and M.R. Kelley (2009), "The Many Functions of APE1/Ref-1: Not Only a DNA Repair Enzyme", Antioxid Redox Signal, 11:601-619.

Veith, A. and B. Moorthy, (2018), "Role of cytochrome P450s in the generation and metabolism of reactive oxygen species", Curr opin toxicol, 7: 44–51.

Wang, R., C. Li, P. Qiao, Y. Xue, X. Zheng, H. Chen, X. Zeng, W. Liu, I. Boldogh, and X. Ba (2018), "OGG1-initiated base excision repair exacerbates oxidative stress-induced parthanatos", Cell Death and Disease, 9:628.

Whitaker, A., M. Schaich, M.S. Smith, T. Flynn, and B. Freudenthal (2017), "Base excision repair of oxidative DNA damage: from mechanism to disease", Front Biosci, 22:1493-1522.

White, P. A., A. S. Long, and G. E. Johnson (2020), "Quantitative Interpretation of Genetic Toxicity Dose-Response Data for Risk Assessment and Regulatory Decision-Making: Current Status and Emerging Priorities", Environ Mol Mutatgen, 61:66-83.

White, P.A. and G.E. Johnson (2016), "Genetic toxicology at the crossroads-from qualitative hazard evaluation to quantitative risk assessment", Mutagenesis, 31:233-237.

Whitwell, J., R. Smith, K. Jenner, H. Lyon, D. Wood, J. Clements, K. Aschcroft-Hawley, B. B. Gollapudi, D. Kirkland, E. Lorge, S. Pfuhler, J. Tanir, and V. Thybaud (2015), "Relationships between p53 status, apoptosis and induction of micronuclei in different human and mouse cell lines *in vitro:* implications for improving existing assays", Environ Mutatgen, 789-790:7-27.

Wirth, N., C.N. GrosBuechner, C. Kisker, and I. Tessmer (2016), "Conservation and Divergence in Nucleotide Excision Repair Lesion Recognition", J Biol Chem, 291:18932-18946.

Yang, N., A. Chaudry, and S. Wallace (2006), "Base excision repair by hNTH1 and hOGG1: A two edged sword in the processing of DNA damage in gamma-irradiated human cells", DNA Repair, 5:43-51.

Yang, N., H. Galick, and S. Wallace (2004), "Attempted base excision repair of ionizing radiation damage in human lymphoblastoid cells produces lethal and mutagenic double strand breaks", DNA Repair, 3:1323-1334.

Yu, H., L. Venkatarangan, J. Wishnok, and S. R. Tannenbaum. (2005). "Quantitation of Four Guanine Oxidation Products from Reaction of DNA with Varying Doses of Peroxynitrite". Chem Res Toxicol 18:1849-1857.

Zhu, F., Y. Zhang, L. Shi, C. Wu, S. Chen, H. Zheng, and D. Song (2019), "Gene mutation detection of urinary sediment cells for NMIBC early diagnose and prediction of NMIBC relapse after surgery", Medicine, 98:e16451.

Appendix 1 - MIE, KEs and AO

List of MIEs in this AOP

Event: 1634: Increase, Oxidative damage to DNA

Short Name: Increase, Oxidative DNA damage

Key Event Component

Process	Object	Action
regulation of response to reactive oxygen species	reactive oxygen species	occurrence

AOPs Including This Key Event

AOP Name	Role of event in AOP
Oxidative DNA damage, chromosomal aberrations and mutations	Molecular Initiating Event
Energy deposition leading to population decline via DNA oxidation and follicular atresia	Key Event
Energy deposition leading to population decline via DNA oxidation and oocyte apoptosis	Key Event
Deposition of energy leading to cataracts	Key Event

Biological context

Level of Biological Organization
Molecular

Cell term
eukaryotic cell

Organ term
organ

Stressors

Name
Hydrogen peroxide
Potassium bromate
Ionizing Radiation
Sodium arsenite
Reactive oxygen species

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human and other cells in culture	human and other cells in culture	Moderate	NCBI
yeast	Saccharomyces cerevisiae	Low	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	Low	NCBI
bovine	Bos taurus	Low	NCBI
human	Homo sapiens	High	NCBI
rabbit	Oryctolagus cuniculus	Low	NCBI

Life Stage Applicability

Life stage	Evidence
All life stages	High

Sex Applicability

Term	Evidence
Unspecific	Moderate

Taxonomic applicability: Theoretically, DNA oxidation can occur in any cell type, in any organism. Oxidative DNA lesions have been measured in mammalian cells (human, mouse, calf, rat) in vitro and in vivo, and in prokaryotes.

Life stage applicability: This key event is not life stage specific (Mesa & Bassnett, 2013; Suman et al., 2019).

Sex applicability: This key event is not sex specific (Mesa & Bassnett, 2013).

Evidence for Perturbation by Prototypic Stressor

H_2O_2 and $KBrO_3$ – A concentration-dependent increase in oxidative lesions was observed in both Fpg- and hOGG1-modified comet assays of TK6 cells treated with increasing concentrations of glucose oxidase (an enzyme that generates H_2O_2) and potassium bromate for 4 h (Platel et al., 2011).

Evidence indicates that oxidative DNA damage is also induced by X-rays (Bahia et al., 2018), ^{60}Co γ-rays, ^{12}C ions, α particles, electrons (Georgakilas, 2013), UVB (Mesa and Bassnett, 2013), γ-rays, ^{56}Fe ions (Datta et al., 2012), and protons (Suman et al., 2019).

Key Event Description

The nitrogenous bases of DNA are susceptible to oxidation in the presence of oxidizing agents. Oxidative adducts form mainly on C5 and to a lesser degree on C6 of thymine and cytosine, and on C8 of guanine and adenine. Guanine is most prone to oxidation due to its low oxidation potential (Jovanovic and Simic, 1986). Indeed, 8-oxo-2'-deoxyguanosine (8-oxodG)/8-hydroxy-2'-deoxyguanosine (8-OHdG) is the most abundant and well-studied oxidative DNA lesion in the cell (Swenberg et al., 2011). It causes an A(anti):8-oxo-G(syn) mispair instead of the normal C(anti):8-oxo-G(syn) pair. This pairing does not cause large structural changes to the DNA backbone, and therefore remains undetected by the

polymerase's proofreading mechanism. Consequently, one of the daughter strands will have an AT pair instead of the correct GC pair after replication (Markkanen, 2017).

Formamidopyrimidine lesions on guanine and adenine (FaPyG and FaPyA), 8-hydroxy-2'-deoxyadenine (8-oxodA), and thymidine glycol (Tg) are other common oxidative lesions. We refer the reader to reviews on this topic to see the full set of potential oxidative DNA lesions (Whitaker et al., 2017). Oxidative DNA lesions are present in the cell at a steady state due to endogenous redox processes (Swenberg et al., 2010). Under normal conditions, cells are able to withstand the baseline level of oxidized bases through efficient repair and regulation of free radicals in the cell. However, direct chemical insult from specific compounds, or induction of reactive oxygen species (ROS) from the reduction of endogenous molecules, as well as through the release of inflammatory cell-derived oxidants, can lead to increased DNA oxidation, a state known as oxidative stress (Turner et al., 2002; Schoenfeld et al., 2012; Tangvarasittichai and Tangvarasittichai, 2019). Furthermore, although cells do possess repair mechanisms to deal with oxidative DNA damage, sometimes the repair intermediates can interfere with genome function or decrease stability of the genome. This creates a balancing act between when it is best to repair damage and when it is best to leave it (Poetsch, 2020a).

This KE describes an increase in oxidative lesions in the nuclear DNA above the steady-state level. Oxidative DNA damage can occur in any cell type with nuclear DNA under oxidative stress.

How it is Measured or Detected

Relative Quantification of Oxidative DNA Lesions

- Comet assay (single cell gel electrophoresis) with Fpg and hOGG1 modifications (Smith et al., 2006; Platel et al., 2011)
    - Oxoguanine glycosylase (hOGG1) and formamidopyrimidine-DNA glycosylase (Fpg) are base excision repair (BER) enzymes in eukaryotic and prokaryotic cells, respectively
    - Both enzymes are bi-functional; the glycosylase function cleaves the glycosidic bond between the ribose and the oxidized base, giving rise to an abasic site, and the apurinic/apymidinic (AP) site lyase function cleaves the phosphodiester bond via β-elimination reaction and creates a single strand break
    - Treatment of DNA with either enzyme prior to performing the electrophoresis step of the comet assay allows detection of oxidative lesions by measuring the increase in comet tail length when compared against untreated samples.
- Enzyme-linked immunosorbant assay (ELISA) (Dizdaroglu et al., 2002; Breton et al., 2003; Xu et al., 2008; Zhao et al. 2017)
    - 8-oxodG can be detected using immunoassays, such as ELISA, that use antibodies against 8-oxodG lesions. It has been noted that immunodetection of 8-oxodG can be interfered by certain compounds in biological samples.

Absolute Quantification of Oxidative DNA Lesions

- Quantification of 8-oxodG using HPLC-EC (Breton et al., 2003; Chepelev et al., 2015; Drake et al., 2019 ; Chiorcea-Paquin, 2022)
    - 8-oxodG can be separated from digested DNA and precisely quantified using high performance liquid chromatography (HPLC) with electrochemical detection

- Liquid chromatography can also be coupled with different mass spectrometry-based methods to detect and quantify oxidative lesions. Correlation between lesions measured by hOGG1-modified comet assay and LC-MS has been reported (Andries et al., 2021; Mangal et al., 2009).
    - Multiple reaction monitoring/ mass spectrometry (MRM/MS) (Mangal et al., 2009)
    - Liquid chromatography-nanoelectrospray ionization-tandem mass spectrometry (Ma et al., 2016)
    - Ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (Sambiagio et al., 2021)

Gas chromatography-mass spectrometry (GC-MS)

- DNA is hydrolyzed to release either free bases or nucleosides and then undergoes derivatization in order to increase their volatility. Finally, samples run through a gas chromatograph and then a mass spectrometer. The mass spectrometer results are used to determine oxidative DNA damage by identifying modified bases or nucleosides (Dizdaroglu, 1994).

Sequencing assays

- Various markers are used to detect and highlight sites of DNA damage; the result is then processed and sequenced. This category encompasses a wide range of assays such as snAP-seq, OGG1-AP-seq, oxiDIP-seq, OG-seq, and click-code-seq (Yun et al., 2017; Wu et al., 2018; Amente et al., 2019; Poetsch, 2020b).

- We note that other types of oxidative lesions can be quantified using the methods described above.

References

Amente, S. et al. (2019), "Genome-wide mapping of 8-oxo-7,8-dihydro-2'-deoxyguanosine reveals accumulation of oxidatively-generated damage at DNA replication origins within transcribed long genes of mammalian cells", *Nucleic Acids Research 2019*, Vol. 47/1, Oxford University Press, England, https://doi.org/10.1093/nar/gky1152

Andries, A., Rozenski, J., Vermeersch, P., Mekahli, D., & Van Schepdael, A. (2021). Recent progress in the LC-MS/MS analysis of oxidative stress biomarkers. Electrophoresis, 42(4), 402–428. https://doi.org/10.1002/elps.202000208

Bahia, S. et al. (2018), "Oxidative and nitrative stress-related changes in human lens epithelial cells following exposure to X-rays", *International journal of radiation biology*, Vol. 94/4, England, https://doi.org/10.1080/09553002.2018.1439194

Breton J, Sichel F, Bainchini F, Prevost V. (2003). Measurement of 8-Hydroxy-2′-Deoxyguanosine by a Commercially Available ELISA Test: Comparison with HPLC/Electrochemical Detection in Calf Thymus DNA and Determination in Human Serum. Anal Lett 36:123-134.

Cabrera, M. P., R. Chihuailaf and F. Wittwer Menge (2011), "Antioxidants and the integrity of ocular tissues", *Veterinary medicine international*, Vol. 2011, SAGE-Hindawi Access to Research, United States, https://doi.org/10.4061/2011/905153

Cadet, J. et al. (2012), "Oxidatively generated complex DNA damage: tandem and clustered lesions", *Cancer letters*, Vol. 327/1, Elsevier Ireland Ltd, Ireland. https://doi.org/10.1016/j.canlet.2012.04.005

Chepelev N, Kennedy D, Gagne R, White T, Long A, Yauk C, White P. (2015). HPLC Measurement of the DNA Oxidation Biomarker, 8-oxo-7,8-dihydro-2'-deoxyguanosine, in Cultured Cells and Animal Tissues. Journal of Visualized Experiments 102:e52697.

Chiorcea-Paquim A. M. (2022). 8-oxoguanine and 8-oxodeoxyguanosine Biomarkers of Oxidative DNA Damage: A Review on HPLC-ECD Determination. Molecules (Basel, Switzerland), 27(5), 1620. https://doi.org/10.3390/molecules27051620

Collins, A. R. (2014), "Measuring oxidative damage to DNA and its repair with the comet assay", *Biochimica et biophysica acta. General subjects*, Vol. 1840/2, Elsevier B.V., https://doi.org/10.1016/j.bbagen.2013.04.022

Datta, K. et al. (2012), "Exposure to heavy ion radiation induces persistent oxidative stress in mouse intestive", *PloS One*, Vol. 7/8, Public Library of Science, United States, https://doi.org/10.1371/journal.pone.0042224

Dizdaroglu, M. (1994), "Chemical determination of oxidative DNA damage by gas chromatography-mass spectrometry", *Methods in Enzymology*, Vol. 234, Elsevier Science & Technology, United States, https://doi.org/ 10.1016/0076-6879(94)34072-2

Dizdaroglu, M. et al. (2002), "Free radical-induced damage to DNA : mechanisms and measurement", *Free radical biology & medicine*, Vol. 32/11, United States, pp. 1102-1115

Drake, Danielle M et al. "Measurement of the Oxidative DNA Lesion 8-Oxoguanine (8-oxoG) by ELISA or by High-Performance Liquid Chromatography (HPLC) with Electrochemical Detection." Methods in molecular biology (Clifton, N.J.) vol. 1965 (2019): 313-328. doi:10.1007/978-1-4939-9182-2_21

Eaton, J. W. (1995), "UV-mediated cataractogenesis: a radical perspective", *Documenta ophthalmologica*, Vol. 88/3-4, Springer, Dordrecht, https://doi.org/10.1007/BF01203677

Fletcher, A. E. (2010), "Free radicals, antioxidants and eye diseases: evidence from epidemiological studies on cataract and age-related macular degeneration", *Ophthalmic Research*, Vol. 44/3, Karger international, Basel, https://doi.org/10.1159/000316476

Georgakilas, A. G et al. (2013), "Induction and repair of clustered DNA lesions: what do we know so far?", Radiation Research, Vol. 180/1, *The Radiation Research Society*, United States, https://doi.org/10.1667/RR3041.1

Jose, D. et al. (2009). "Spectroscopic studies of position-specific DNA "breathing" fluctuations at replication forks and primer-template junctions", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 106/11, https://doi.org/10.1073/pnas.0900803106

Jovanovic S, Simic M. (1986). One-electron redox potential of purines and pyrimidines. J Phys Chem 90:974-978.

Kruk, J., K. Kubasik-Kladna and H. Y. Aboul-Enein (2015), "The role oxidative stress in the pathogenesis of eye diseases: current status and a dual role of physical activity", *Mini-reviews in medicinal chemistry*, Vol. 16/3, Bentham Science Publishers Ltd, Netherlands, https://doi.org/10.2174/1389557516666151120114605

Lee, J. et al. (2004), "Reactive oxygen species, aging, and antioxidative nutraceuticals", *Comprehensive reviews in food science and food safety*, Vol. 3/1, Blackwell Publishing Ltd, Oxford, https://doi.org/10.1111/j.1541-4337.2004.tb00058.x

Ma, B., Jing, M., Villalta, P. et al. (2016), Simultaneous determination of 8-oxo-2'-deoxyguanosine and 8-oxo-2'-deoxyadenosine in human retinal DNA by liquid chromatography nanoelectrospray-tandem mass spectrometry. Sci Rep 6: 22375. https://doi.org/10.1038/srep22375

Mangal D, Vudathala D, Park J, Lee S, Penning T, Blair I. (2009). Analysis of 7,8-Dihydro-8-oxo-2′-deoxyguanosine in Cellular DNA during Oxidative Stress. Chem Res Toxicol 22:788-797.

Markkanen, E. (2017), "Not breathing is not an option: How to deal with oxidative DNA damage", *DNA repair*, Vol. 59, Elsevier B.V., Netherlands, https://doi.org/10.1016/j.dnarep.2017.09.007

Mesa, R. and S. Bassnett (2013), "UV-B induced DNA damage and repair in the mouse lens", *Investigative ophthalmology & visual science*, Vol. 54/10, the Association for Research in Vision and Ophthalmology, United States, https://doi.org/10.1167/iovs.13-12644

Pendergrass, W. et al. (2010), "X-ray induced cataract is preceded by LEC loss, and coincident with accumulation of cortical DNA, and ROS; similarities with age-related cataracts", *Molecular vision*, Vol. 16, Molecular Vision, United States, pp. 1496-1513

Platel A, Nesslany F, Gervais V, Claude N, Marzin D. (2011). Study of oxidative DNA damage in TK6 human lymphoblastoid cells by use of the thymidine kinase gene-mutation assay and the *in vitro* modified comet assay: Determination of No-Observed-Genotoxic-Effect-Levels. Mutat Res 726:151-159.

Poetsch, Anna R. (2020a), "The genomics of oxidative DNA damage, repair, and resulting mutagenesis", *Computational and structural biotechnology journal 2020*, Vol. 18, Elsevier B.V., Netherlands https://doi.org/10.1016/j.csbj.2019.12.013

Poetsch, A. R. (2020b), "AP-Seq: A method to measure apurinic sites and small base adducts genome-wide", The Nucleus, Springer US, New York, Sacca, S. C. et al. (2009), "Gene-environment interactions in ocular diseases", *Mutation research – fundamental and molecular mechanisms of mutagenesis*, Vol. 667/1-2, Elsevier, Amsterdam, https://doi.org/10.1016/j.mrfmmm.2008.11.002

Sambiagio, N., Sauvain, J.-J., Berthet, A., Auer, R., Schoeni, A., & Hopf, N. B. (2020), Rapid Liquid Chromatography—Tandem Mass Spectrometry Analysis of Two Urinary Oxidative Stress Biomarkers: 8-oxodG and 8-isoprostane. *Antioxidants*, 10: 38 https://doi.org/10.3390/antiox10010038

Schoenfeld, M. P. et al. (2012), "A hypothesis on biological protection from space radiation through the use of new therapeutic gases as medical counter measures", *Medical gas research*, Vol. 2/1, BioMed Central Ltd, India, https://doi.org/10.1186/2045-9912-2-8

Smith C, O'Donovan M, Martin E. (2006). hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. Mutagenesis 21:185-190.

Stohs, S. J. (1995), "The role of free radicals in toxicity and disease", *Journal of Basic and Clinical Physiology and Pharmacology*, Vol. 6/3-4, Freund Publishing House Ltd, https://doi.org/10.1515/JBCPP.1995.6.3-4.205

Suman, S. et al. (2019), "Fractionated and acute proton radiation show differential intestinal tumorigenesis and DNA damage and repair pathway response in ApcMin/+

mice", *International Journal of Radiation Oncology*, Biology, Physics, Vol. 105/3, Elsevier Inc, https://doi.org/10.1016/j.ijrobp.2019.06.2532

Swenberg J, Lu K, Moeller B, Gao L, Upton P, Nakamura J, Starr T. (2011). Endogenous versus Exogenous DNA Adducts: Their Role in Carcinogenesis, Epidemiology, and Risk Assessment. Toxicol Sci 120:S130-S145.

Tangvarasittichai, O and S. Tangvarasittichai (2018), "Oxidative stress, ocular disease, and diabetes retinopathy", *Current Pharmaceutical Design*, Vol. 24/40, Bentham Science Publishers, https://doi.org/10.2174/1381612825666190115121531

Turner, N. D. et al. (2002), "Opportunities for nutritional amelioration of radiation-induced cellular damage", *Nutrition*, Vol. 18/10, Elsevier Inc, New York, https://doi.org/10.1016/S0899-9007(02)00945-0

Whitaker A, Schaich M, Smith MS, Flynn T, Freudenthal B. (2017). Base excision repair of oxidative DNA damage: from mechanism to disease. Front Biosci 22:1493-1522.

Wu, J. (2018), "Nucleotide-resolution genome-wide mapping of oxidative DNA damage by click-code-seq", *Journal of the American Chemical Society 2018*, American Chemical Society, United States https://doi-org.proxy.bib.uottawa.ca/10.1021/jacs.8b03715

Xu, X. et al. (2008). "Fluorescence recovery assay for the detection of protein-DNA binding", *Analytical Chemistry*, Vol. 80/14, https://doi.org/10.1021/ac8007016

Zhao M, Howard E, Guo Z, Parris A, Yang X. (2017). p53 pathway determines the cellular response to alcohol-induced DNA damage in MCF-7 breast cancer cells. PLoS One 12:e0175121.

List of Key Events in the AOP

Event: 155: Inadequate DNA repair

Short Name: Inadequate DNA repair

Key Event Component

Process	Object	Action
DNA repair	deoxyribonucleic acid	functional change

AOPs Including This Key Event

AOP Name	Role of event in AOP
Alkylation of DNA leading to heritable mutations	KeyEvent
DNA alkylation -> cancer 2	KeyEvent
DNA alkylation -> cancer 1	KeyEvent
Oxidative DNA damage, chromosomal aberrations and mutations	KeyEvent
Deposition of energy leading to lung cancer	
Alkylation of DNA leading to reduced sperm count	KeyEvent
Bulky DNA adducts leading to mutations	KeyEvent
Ionizing Radiation-Induced AML	KeyEvent
DNA damage and metastatic breast cancer	KeyEvent
Deposition of energy leading to cataracts	KeyEvent

Stressors

Name
Ionizing Radiation
UV radiation
Hydrogen peroxide
Potassium bromate
Menadione
Hydroxyurea
N-ethyl-N-nitrosourea

Biological context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	Moderate	NCBI
Syrian golden hamster	Mesocricetus auratus	Moderate	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life stage	Evidence
All life stages	High

Sex Applicability

Term	Evidence
Unspecific	High

The retention of adducts has been directly measured in many different types of eukaryotic somatic cells (in vitro and in vivo). In male germ cells, work has been done on hamsters, rats and mice. The accumulation of mutation and changes in mutation spectrum has been measured in mice and human cells in culture. Theoretically, saturation of DNA repair occurs in every species (prokaryotic and eukaryotic). The principles of this process were established in prokaryotic models. Nagel et al. (2014) have produced an assay that directly measures DNA repair in human cells in culture.

NHEJ is primarily used by vertebrate multicellular eukaryotes, but it also been observed in plants. Furthermore, it has recently been discovered that some bacteria (Matthews et al., 2014) and yeast (Emerson et al., 2016) also use NHEJ. In terms of invertebrates, most lack the core DNA-PK_{cs} and Artemis proteins; they accomplish end joining by using the RA50:MRE11:NBS1 complex (Chen et al., 2001). HR occurs naturally in eukaryotes, bacteria, and some viruses (Bhatti et al., 2016).

Taxonomic applicability: Inadequate DNA repair is applicable to all species, as they all contain DNA (White & Vijg, 2016).

Life stage applicability: This key event is not life stage specific as any life stage can have poor repair, though as individuals age their repair process become less effective (Gorbunova & Seluanov, 2016).

Sex applicability: There is no evidence of sex-specificity for this key event, with initial rate of DNA repair not significantly different between sexes (Trzeciak et al., 2008).

Evidence for perturbation by a stressor: Multiple studies demonstrate that inadequate DNA repair can occur as a result of stressors such as ionizing and non-ionizing radiation, as well as chemical agents (Kuhne et al., 2005; Rydberg et al., 2005; Dahle et al., 2008; Seager et al., 2012; Wilhelm, 2014; O'Brien et al., 2015).

Key Event Description

DNA lesions may result from the formation of DNA adducts (i.e., covalent modification of DNA by chemicals), or by the action of agents such as radiation that may produce strand breaks or modified nucleotides within the DNA molecule. These DNA lesions are repaired through several mechanistically distinct pathways that can be categorized as follows:

1. **Damage reversal** acts to reverse the damage without breaking any bonds within the sugar phosphate backbone of the DNA. The most prominent enzymes associated with

damage reversal are photolyases (Sancar, 2003) that can repair UV dimers in some organisms, and O6-alkylguanine-DNA alkyltransferase (AGT) (Pegg 2011) and oxidative demethylases (Sundheim et al., 2008), which can repair some types of alkylated bases.

2. **Excision repair** involves the removal of a damaged nucleotide(s) through cleavage of the sugar phosphate backbone followed by re-synthesis of DNA within the resultant gap. Excision repair of DNA lesions can be mechanistically divided into:

   a) Base excision repair (BER) (Dianov and Hübscher, 2013), in which the damaged base is removed by a damage-specific glycosylase prior to incision of the phosphodiester backbone at the resulting abasic site.

   b) Nucleotide excision repair (NER) (Schärer, 2013), in which the DNA strand containing the damaged nucleotide is incised at sites several nucleotides 5' and 3' to the site of damage, and a polynucleotide containing the damaged nucleotide is removed prior to DNA resynthesis within the resultant gap.

   c) Mismatch repair (MMR) (Li et al., 2016) which does not act on DNA lesions but does recognize mispaired bases resulting from replication errors. In MMR the strand containing the misincorporated base is removed prior to DNA resynthesis.

   The major pathway that removes oxidative DNA damage is base excision repair (BER), which can be either monofunctional or bifunctional; in mammals, a specific DNA glycosylase (OGG1: 8-Oxoguanine glycosylase) is responsible for excision of 8-oxoguanine (8-oxoG) and other oxidative lesions (Hu et al., 2005; Scott et al., 2014; Whitaker et al., 2017). We note that long-patch BER is used for the repair of clustered oxidative lesions, which uses several enzymes from DNA replication pathways (Klungland and Lindahl, 1997). These pathways are described in detail in various reviews e.g., (Whitaker et al., 2017).

3. **Single strand break repair (SSBR)** involves different proteins and enzymes depending on the origin of the SSB (e.g., produced as an intermediate in excision repair or due to direct chemical insult) but the same general steps of repair are taken for all SSBs: detection, DNA end processing, synthesis, and ligation (Caldecott, 2014). Poly-ADP-ribose polymerase1 (PARP1) detects and binds unscheduled SSBs (i.e., not deliberately induced during excision repair) and synthesizes PAR as a signal to the downstream factors in repair. The X-ray repair cross-complementing protein 1 (XRCC1) complex is then recruited to the site of damage and acts as a scaffold for proteins and enzymes required for repair. Depending on the nature of the damaged termini of the DNA strand, different enzymes are required for end processing to generate the substrates that DNA polymerase β (Polβ; short patch repair) or Pol δ/ε (long patch repair) can bind to synthesize over the gap. Synthesis in long-patch repair displaces a single stranded flap which is excised by flap endonuclease 1 (FEN1). In short-patch repair, the XRCC1/Lig3α complex joins the two ends after synthesis. In long-patch repair, the PCNA/Lig1 complex ligates the ends. (Caldecott, 2014).

4. **Double strand break repair (DSBR)** is necessary to preserve genomic integrity when breaks occur in both strands of a DNA molecule. There are two major pathways for DSBR: homologous recombination (HR), which operates primarily during S phase in dividing cells, and nonhomologous end joining (NHEJ), which can function in both dividing and non-dividing cells (Teruaki Iyama and David M. Wilson III, 2013).

In higher eukaryotes such as mammals, NHEJ is usually the preferred pathway for DNA DSBR. Its use, however, is dependent on the cell type, the gene locus, and the nuclease platform (Miyaoka et al., 2016). The use of NHEJ is also dependent on the cell cycle; NHEJ is generally not the pathway of choice when the cell is in the late S or G2 phase of the cell cycle, or in mitotic cells when the sister chromatid is directly adjacent to the double-strand break (DSB) (Lieber et al., 2003). In these cases, the HR pathway is commonly used for repair of DSBs. Despite this, NHEJ is still used more commonly than HR in human cells. Classical NHEJ (C-NHEJ) is the most common NHEJ repair mechanism, but alternative NHEJ (alt-NHEJ) can also occur, especially in the absence of C-NHEJ and HR.

The process of C-NHEJ in humans requires at least seven core proteins: Ku70, Ku86, DNA-dependent protein kinase complex (DNA-PK_{cs}), Artemis, X-ray cross-complementing protein 4 (XRCC4), XRCC4-like factor (XLF), and DNA ligase IV (Boboila et al., 2012). When DSBs occur, the Ku proteins, which have a high affinity for DNA ends, will bind to the break site and form a heterodimer. This protects the DNA from exonucleolytic attack and acts to recruit DNA-PK_{cs}, thus forming a trimeric complex on the ends of the DNA strands. The kinase activity of DNA-PK_{cs} is then triggered, causing DNA-PK_{cs} to auto-phosphorylate and thereby lose its kinase activity; the now phosphorylated DNA-PK_{cs} dissociates from the DNA-bound Ku proteins. The free DNA-PK_{cs} phosphorylates Artemis, an enzyme that possesses 5'-3' exonuclease and endonuclease activity in the presence of DNA-PK_{cs} and ATP. Artemis is responsible for 'cleaning up' the ends of the DNA. For 5' overhangs, Artemis nicks the overhang, generally leaving a blunt duplex end. For 3' overhangs, Artemis will often leave a four- or five-nucleotide single stranded overhang (Pardo et al., 2009; Fattah et al., 2010; Lieber et al., 2010). Next, the XLF and XRCC4 proteins form a complex which makes a channel to bind DNA and aligns the ends for efficient ligation via DNA ligase IV (Hammel et al., 2011).

The process of alt-NHEJ is less well understood than C-NHEJ. Alt-NHEJ is known to involve slightly different core proteins than C-NHEJ, but the steps of the pathway are essentially the same between the two processes (reviewed in Chiruvella et al., 2013). It is established, however, that alt-NHEJ is more error-prone in nature than C-NHEJ, which contributes to incorrect DNA repair. Alt-NHEJ is thus considered primarily to be a backup repair mechanism (reviewed in Chiruvella et al., 2013).

In contrast to NHEJ, HR takes advantage of similar or identical DNA sequences to repair DSBs (Sung and Klein, 2006). The initiating step of HR is the creation of a 3' single strand DNA (ss-DNA) overhang. Combinases such as RecA and Rad51 then bind to the ss-DNA overhang, and other accessory factors, including Rad54, help recognize and invade the homologous region on another DNA strand. From there, DNA polymerases are able to elongate the 3' invading single strand and resynthesize the broken DNA strand using the corresponding sequence on the homologous strand.

Fidelity of DNA Repair

Most DNA repair pathways are extremely efficient. However, in principal, all DNA repair pathways can be overwhelmed when the DNA lesion burden exceeds the capacity of a given DNA repair pathway to recognize and remove the lesion. Exceeded repair capacity may lead to toxicity or mutagenesis following DNA damage. Apart from extremely high DNA lesion burden, inadequate repair may arise through several different specific mechanisms. For example, during repair of DNA containing O6-alkylguanine adducts, AGT irreversibly binds a single O6-alkylguanine lesion and as a result is inactivated (this

is termed suicide inactivation, as its own action causes it to become inactivated). Thus, the capacity of AGT to carry out alkylation repair can become rapidly saturated when the DNA repair rate exceeds the de novo synthesis of AGT (Pegg, 2011).

A second mechanism relates to cell specific differences in the cellular levels or activity of some DNA repair proteins. For example, xeroderma pigmentosum group A (XPA), a DNA damage recognition and repair factor, is an essential component of the NER complex. The level of XPA that is active in NER is low in the testes, which may reduce the efficiency of NER in testes as compared to other tissues (Köberle et al., 1999). Likewise, both NER and BER have been reported to be deficient in cells lacking functional p53 (Adimoolam and Ford, 2003; Hanawalt et al., 2003; Seo and Jung, 2004). A third mechanism relates to the importance of the DNA sequence context of a lesion in its recognition by DNA repair enzymes. For example, 8-oxoguanine (8-oxoG) is repaired primarily by BER; the lesion is initially acted upon by a bifunctional glycosylase, OGG1, which carries out the initial damage recognition and excision steps of 8-oxoG repair. However, the rate of excision of 8-oxoG is modulated strongly by both chromatin components (Menoni et al., 2012) and DNA sequence context (Allgayer et al., 2013) leading to significant differences in the repair of lesions situated in different chromosomal locations.

DNA repair is also remarkably error-free. However, misrepair can arise during repair under some circumstances. DSBR is notably error prone, particularly when breaks are processed through NHEJ, during which partial loss of genome information is common at the site of the double strand break (Iyama and Wilson, 2013). This is because NHEJ rejoins broken DNA ends without the use of extensive homology; instead, it uses the microhomology present between the two ends of the DNA strand break to ligate the strand back into one. When the overhangs are not compatible, however, indels (insertion or deletion events), duplications, translocations, and inversions in the DNA can occur. These changes in the DNA may lead to significant issues within the cell, including alterations in the gene determinants for cellular fatality (Moore et al., 1996).

Activation of mutagenic DNA repair pathways to withstand cellular or replication stress either from endogenous or exogenous sources can promote cellular viability, albeit at a cost of increased genome instability and mutagenesis (Fitzgerald et al., 2017). These salvage DNA repair pathways including Break-induced Replication (BIR) and Microhomology-mediated Break-induced Replication (MMBIR). BIR repairs one-ended DSBs and has been extensively studied in yeast as well as in mammalian systems. BIR and MMBIR are linked with heightened levels of mutagenesis, chromosomal rearrangements and ensuing genome instability (Deem et al., 2011; Sakofsky et al., 2015; Saini et al., 2017; Kramara et al., 2018). In mammalian genomes BIR-like synthesis has been proposed to be involved in late stage Mitotic DNA Synthesis (MiDAS) that predominantly occurs at so-called Common Fragile Sites (CFSs) and maintains telomere length under conditions of replication stress that serve to promote cell viability (Minocherhomji et al., 2015; Bhowmick et al., 2016; Dilley et al., 2016).

Misrepair may also occur through other repair pathways. Excision repair pathways require the resynthesis of DNA and rare DNA polymerase errors during gap resynthesis will result in mutations (Brown et al., 2011). Errors may also arise during gap resynthesis when the strand that is being used as a template for DNA synthesis contains DNA lesions (Kozmin and Jinks-Robertson, 2013). In addition, it has been shown that sequences that contain tandemly repeated sequences, such as CAG triplet repeats, are subject to expansion during gap resynthesis that occurs during BER of 8-oxoG damage (Liu et al., 2009).

How it is Measured or Detected

There is no test guideline for this event. The event is usually inferred from measuring the retention of DNA adducts or the creation of mutations as a measure of lack of repair or incorrect repair. These 'indirect' measures of its occurrence are crucial to determining the mechanisms of genotoxic chemicals and for regulatory applications (i.e., determining the best approach for deriving a point of departure). More recently, a fluorescence-based multiplex flow-cytometric host cell reactivation assay (FM-HCR) has been developed to directly measure the ability of human cells to repair plasmid reporters (Nagel et al., 2014).

Indirect Measurement

In somatic and spermatogenic cells, measurement of DNA repair is usually inferred by measuring DNA adduct formation/removal. Insufficient repair is inferred from the retention of adducts and from increasing adduct formation with dose. Insufficient DNA repair is also measured by the formation of increased numbers of mutations and alterations in mutation spectrum. The methods will be specific to the type of DNA adduct that is under study.

Some EXAMPLES are given below for alkylated DNA.

DOSE-RESPONSE CURVE FOR ALKYL ADDUCTS/MUTATIONS: It is important to consider that some adducts are not mutagenic at all because they are very effectively repaired. Others are effectively repaired, but if these repair processes become overwhelmed mutations begin to occur. The relationship between exposure to mutagenic agents and the presence of adducts (determined as adducts per nucleotide) provide an indication of whether the removal of adducts occurs, and whether it is more efficient at low doses. A sub-linear DNA adduct curve suggests that less effective repair occurs at higher doses (i.e., repair processes are becoming saturated). A sub-linear shape for the dose-response curves for mutation induction is also suggestive of repair of adducts at low doses, followed by saturation of repair at higher doses. Measurement of a clear point of inflection in the dose-response curve for mutations suggests that repair does occur, at least to some extent, but reduced repair efficiency arises above the breakpoint. A lack of increase in mutation frequencies (i.e., flat line for dose-response) for a compound showing a dose-dependent increase in adducts would imply that the adducts formed are either not mutagenic or are effectively repaired.

RETENTION OF ALKYL ADDUCTS: Alkylated DNA can be found in cells long after exposure has occurred. This indicates that repair has not effectively removed the adducts. For example, DNA adducts have been measured in hamster and rat spermatogonia several days following exposure to alkylating agents, indicating lack of repair (Seiler et al., 1997; Scherer et al., 1987).

MUTATION SPECTRUM: Shifts in mutation spectrum (i.e., the specific changes in the DNA sequence) following a chemical exposure (relative to non-exposed mutation spectrum) indicates that repair was not operating effectively to remove specific types of lesions. The shift in mutation spectrum is indicative of the types of DNA lesions (target nucleotides and DNA sequence context) that were not repaired. For example, if a greater proportion of mutations occur at guanine nucleotides in exposed cells, it can be assumed that the chemical causes DNA adducts on guanine that are not effectively repaired.

Direct Measurement

Nagel et al. (2014) developed a fluorescence-based multiplex flow-cytometric host cell reactivation assay (FM-HCR) to measure the ability of human cells to repair plasmid reporters. These reporters contain different types and amounts of DNA damage and can be used to measure repair by NER, MMR, BER, NHEJ, HR and O-6-Methylguanine-DNA-Methyltransferase (MGMT).

Please refer to the table below for additional details and methodologies for detecting DNA damage and repair.

Assay Name	References	Description	DNA Damage/Repair Being Measured	OECD Approved Assay
Dose-Response Curve for Alkyl Adducts/ Mutations	Lutz 1991 Clewell 2016	Creation of a curve plotting the stressor dose and the abundance of adducts/mutations; Characteristics of the resulting curve can provide information on the efficiency of DNA repair	Alkylation, oxidative damage, or DSBs	N/A
Retention of Alkyl Adducts	Seiler 1997 Scherer 1987	Examination of DNA for alkylation after exposure to an alkylating agent; Presence of alkylation suggests a lack of repair	Alkylation	N/A
Mutation Spectrum	Wyrick 2015	Shifts in the mutation spectrum after exposure to a chemical/mutagen relative to an unexposed subject can provide an indication of DNA repair efficiency, and can inform as to the type of DNA lesions present	Alkylation, oxidative damage, or DSBs	N/A
DSB Repair Assay (Reporter constructs)	Mao et al., 2011	Transfection of a GFP reporter construct (and DsRed control) where the GFP signal is only detected if the DSB is repaired; GFP signal is quantified using fluorescence microscopy or flow cytometry	DSBs	N/A
Primary Rat Hepatocyte DNA Repair Assay	Jeffrey and Williams, 2000 Butterworth et al., 1987	Rat primary hepatocytes are cultured with a 3H-thymidine solution in order to measure DNA synthesis in response to a stressor in non-replicating cells; Autoradiography is used to measure the amount of 3H incorporated in the DNA post-repair	Unscheduled DNA synthesis in response to DNA damage	N/A
Repair synthesis measurement by ^{3}H-thymine incorporation	Iyama and Wilson, 2013	Measure DNA synthesis in non-dividing cells as indication of gap filling during excision repair	Excision repair	N/A

Comet Assay with Time-Course	Olive et al., 1990 Trucco et al., 1998	Comet assay is performed with a time-course; Quantity of DNA in the tail should decrease as DNA repair progresses	DSBs	Yes (No. 489)
Pulsed Field Gel Electro-phoresis (PFGE) with Time-Course	Biedermann et al., 1991	PFGE assay with a time-course; Quantity of small DNA fragments should decrease as DNA repair progresses	DSBs	N/A
Fluorescence - Based Multiplex Flow-Cytometric Host Reactivation Assay (FM-HCR)	Nagel et al., 2014	Measures the ability of human cells to repair plasma reporters, which contain different types and amounts of DNA damage; Used to measure repair processes including HR, NHEJ, BER, NER, MMR, and MGMT	HR, NHEJ, BER, NER, MMR, or MGMT	N/A
Alkaline Unwinding Assay with Time Course	Nacci et al., 1991 Thyagarajan et al., 2007 Moreno-Villanueva et al., 2009	DNA is stored in alkaline solutions with DNA-specific dye and allowed to unwind following removal from tissue, increased strand damage associated with increased unwinding. Samples analyzed at different time points to compare remaining damage following repair opportunities	DSBs	N/A
Sucrose Density Gradient Centrifugation with Time Course	Larsen et al., 1982	Strand breaks alter the molecular weight of the DNA piece. DNA in alkaline solution centrifuged into sugar density gradient, repeated set time apart. The less DNA breaks identified in the assay repeats, the more repair occurred	SSBs	N/A
y-H2AX Foci Staining with Time Course	Mariotti et al. 2013 Penninckx et al. 2021	Histone H2AX is phosphorylated in the presence of DNA strand breaks, the rate of its disappearance over time is used as a measure of DNA repair	DSBs	N/A
Alkaline Elution Assay with Time Course	Larsen et al. 1982	DNA with strand breaks elute faster than DNA without, plotted against time intervals to determine the rate at which strand breaks repair	SSBs	N/A
53BP1 foci Detection with Time Course	Penninckx et al. 2021	53BP1 is recruited to the site of DNA damage, the rate at which its level decreases over time is used to measure DNA repair	DSBs	N/A

References

Adimoolam, S. & J.M. Ford (2003), "p53 and regulation of DNA damage recognition during nucleotide excision repair" *DNA Repair* (Amst), 2(9): 947-54.

Allgayer, J. et al. (2013), "Modulation of base excision repair of 8-oxoguanine by the nucleotide sequence", *Nucleic Acids Res*, 41(18): 8559-8571. Doi: 10.1093/nar/gkt620.

Beranek, D.T. (1990), "Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents", *Mutation Research*, 231(1): 11-30. Doi: 10.1016/0027-5107(90)90173-2.

Bhatti, A. et al., (2016), "Homologous Recombination Biology.", *Encyclopedia Britannica*.

Bhowmick, R., S. et al. (2016), "RAD52 Facilitates Mitotic DNA Synthesis Following Replication Stress", *Mol Cell*, 64:1117-1126. Doi: 10.1016/j.molcel.2016.10.037.

Biedermann, A. K. et al. (1991), "SCID mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair", *Cell Biology*, 88(4): 1394-7. Doi: 10.1073/pnas.88.4.1394.

Boboila, C., F. W. Alt & B. Schwer. (2012), "Classical and alternative end-joining pathways for repair of lymphocyte-specific and general DNA double-strand breaks." *Adv Immunol*, 116, 1-49. doi:10.1016/B978-0-12-394300-2.00001-6

Bronstein, S.M. et al. (1991), "Toxicity, mutagenicity, and mutational spectra of N-ethyl-N-nitrosourea in human cell lines with different DNA repair phenotypes", *Cancer Research*, 51(19): 5188-5197.

Bronstein, S.M. et al. (1992), "Efficient repair of O6-ethylguanine, but not O4-ethylthymine or O2-ethylthymine, is dependent upon O6-alkylguanine-DNA alkyltransferase and nucleotide excision repair activities in human cells", *Cancer Research*, 52(7): 2008-2011.

Brown, J.A. et al. (2011), "Efficiency and fidelity of human DNA polymerases λ and β during gap-filling DNA synthesis", *DNA Repair (Amst).*, 10(1):24-33.

Butterworth, E. B. et al., (1987), A protocol and guide for the in vitro rat hepatocyte DNA-repair assay. *Mutation Research*. 189, 113-21. Doi: 10.1016/0165-1218(87)90017-6.

Caldecott, K. W. (2014), "DNA single-strand break repair", Exp Cell Res, 329(1): 2-8.

Chen, L. et al., (2001), Promotion of DNA ligase IV-catalyzed DNA end-joining by the Rad50/Mre11/Xrs2 and Hdf1/Hdf2 complexes. *Mol Cell*. 8(5), 1105-15.

Chiruvella, K. K., Z. Liang & T. E. Wilson, (2013), Repair of Double-Strand Breaks by End Joining. *Cold Spring Harbor Perspectives in Biology*, 5(5):127-57. Doi: 10.1101/cshperspect.a012757.

Dahle, J., et al. (2008), "Overexpression of human OGG1 in mammalian cells decreases ultraviolet A induced mutagenesis", Cancer Letters, Vol.267, Elsevier, Amsterdam, https://doi.org/10.1016/j.canlet.2008.03.002.

Deem, A. et al. (2011), "Break-Induced Replication Is Highly Inaccurate.", *PLoS Biol*. 9:e1000594. Doi: 10.1371/journal.pbio.1000594.

Dianov, G.L. & U. Hübscher (2013), "Mammalian base excision repair: the forgotten archangel", *Nucleic Acids Res.*, 41(6):3483-90. Doi: 10.1093/nar/gkt076.

Dilley, R.L. et al. Greenberg (2016), "Break-induced telomere synthesis underlies alternative telomere maintenance", *Nature*, 539:54-58. Doi: 10.1038/nature20099.

Douglas, G.R. et al. (1995), "Temporal and molecular characteristics of mutations induced by ethylnitrosourea in germ cells isolated from seminiferous tubules and in spermatozoa of lacZ transgenic mice", *Proceedings of the National Academy of Sciences of the United States of America*, 92(16):7485-7489. Doi: 10.1073/pnas.92.16.7485.

Fattah, F. et al., (2010), Ku regulates the non-homologous end joining pathway choice of DNA double-strand break repair in human somatic cells. PLoS Genet, 6(2), doi:10.1371/journal.pgen.1000855

Fitzgerald, D.M., P.J. Hastings, and S.M. Rosenberg (2017), "Stress-Induced Mutagenesis: Implications in Cancer and Drug Resistance", Ann Rev Cancer Biol, 1:119-140. Doi: 10.1146/annurev-cancerbio-050216-121919.

Gorbunova, V. and A. Seluanov. (2016), "DNA double strand break repair, aging and the chromatin connection", Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, Vol.788/1-2, Elsevier, Amsterdam, http://dx.doi.org/10.1016/j.mrfmmm.2016.02.004.

Hammel, M. et al., (2011), XRCC4 protein interactions with XRCC4-like factor (XLF) create an extended grooved scaffold for DNA ligation and double strand break repair. J Biol Chem, 286(37), 32638-32650. doi:10.1074/jbc.M111.272641.

Hanawalt, P.C., J.M. Ford and D.R. Lloyd (2003), "Functional characterization of global genomic DNA repair and its implications for cancer", *Mutation Research*, 544(2-3): 107–114.

Harbach, P. R. et al., (1989), "The in vitro unscheduled DNA synthesis (UDS) assay in rat primary hepatocytes", *Mutation Research*, 216(2):101-10. Doi:10.1016/0165-1161(89)90010-1.

Iyama, T. and D.M. Wilson III (2013), "DNA repair mechanisms in dividing and non-dividing cells", *DNA Repair*, 12(8): 620– 636.

Jeffrey, M. A.& M. G. Williams, (2000), "Lack of DNA-damaging Activity of Five Non-nutritive Sweeteners in the Rat Hepatocyte/DNA Repair Assay", *Food and Chemical Toxicology*, 38: 335-338. Doi: 10.1016/S0278-6915(99)00163-5.

Köberle, B. et al. (1999), "Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours", *Curr. Biol.*, 9(5):273-6. Doi: 10.1016/s0960-9822(99)80118-3.

Kozmin, S.G. & S. Jinks-Robertson S. (2013), "The mechanism of nucleotide excision repair-mediated UV-induced mutagenesis in nonproliferating cells", *Genetics*, 193(3): 803-17. Doi: 10.1534/genetics.112.147421.

Kramara, J., B. Osia, and A. Malkova (2018), "Break-Induced Replication: The Where, The Why, and The How", *Trends Genet*, 34:518-531. Doi: 10.1016/j.tig.2018.04.002.

Kuhne, M., G. Urban and M. Lo (2005), "DNA Double-Strand Break Misrejoining after Exposure of Primary Human Fibroblasts to CK Characteristic X Rays, 29 kVp X Rays and 60Co γ Rays", Radiation. Research, Vol.164/5, Radiation Research Society, Indianapolis, https://doi.org/10.1667/RR3461.1.

Larsen, K.H. et al. (1982), "DNA repair assays as tests for environmental mutagens: A report of the U.S. EPA gene-tox program", Mutation Research, Vol.98/3, Elsevier, Amsterdam, https://doi.org/10.1016/0165-1110(82)90037-9.

Li Z, A. H. Pearlman, and P. Hsieh (2016), "DNA mismatch repair and the DNA damage response", *DNA Repair (Amst)*, 38:94-101.

Lieber, M. R., (2010), "The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway." *Annu Rev Biochem*. 79:181-211. doi:10.1146/annurev.biochem.052308.093131.

Lieber, M. R. et al., (2003), "Mechanism and regulation of human non-homologous DNA end-joining", *Nat Rev Mol Cell Biol*. 4(9):712-720. doi:10.1038/nrm1202.

Liu, Y. et al. (2009), "Coordination between polymerase beta and FEN1 can modulate CAG repeat expansion", J. Biol. Chem., 284(41): 28352-28366. Doi: 10.1074/jbc.M109.050286.

Mao, Z. et al., (2011), "SIRT6 promotes DNA repair under stress by activating PARP1", *Science*. 332(6036): 1443-1446. doi:10.1126/science.1202723.

Mariotti, L.G. et al. (2013), "Use of the γ-H2AX Assay to Investigate DNA Repair Dynamics Following Multiple Radiation Exposures", PLoS ONE, Vol.8/11, PLoS, San Francisco, https://doi.org/10.1371/journal.pone.0079541.

Matthews, L. A., & L. A. Simmons, (2014), "Bacterial nonhomologous end joining requires teamwork", *J Bacteriol*. 196(19): 3363-3365. doi:10.1128/JB.02042-14.

Menoni, H. et al. (2012), "Base excision repair of 8-oxoG in dinucleosomes", *Nucleic Acids Res.* ,40(2): 692-700. Doi: 10.1093/nar/gkr761.

Minocherhomji, S. et al. (2015), "Replication stress activates DNA repair synthesis in mitosis", *Nature*, 528:286-290. Doi: 10.1038/nature16139.

Miyaoka, Y. et al., (2016), "Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing", *Sci Rep*, 6, 23549. doi:10.1038/srep23549/.

Moore, J. K., & J. E. Haber, (1996), "Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae", *Molecular and Cellular Biology*, 16(5), 2164–73. Doi: 10.1128/MCB.16.5.2164.

Moreno-Villanueva, M., Pfeiffer, R., Sindlinger, T. et al. (2009), "A modified and automated version of the 'Fluorimetric Detection of Alkaline DNA Unwinding' method to quantify formation and repair of DNA strand breaks", *BMC Biotechnol,* 9, 39 https://doi.org/10.1186/1472-6750-9-39

Nacci, D. et al. (1992), "Application of the DNA alkaline unwinding assay to detect DNA strand breaks in marine bivalves", Marine Environmental Research, Vol.33/2, Elsevier BV, Amsterdam, https://doi.org/10.1016/0141-1136(92)90134-8.

Nagel, Z.D. et al. (2014), "Multiplexed DNA repair assays for multiple lesions and multiple doses via transcription inhibition and transcriptional mutagenesis", *Proc. Natl. Acad. Sci. USA*, 111(18):E1823-32. Doi: 10.1073/pnas.1401182111.

O'Brien, J.M. et al. (2015), "Sublinear response in lacZ mutant frequency of Muta™ Mouse spermatogonial stem cells after low dose subchronic exposure to N-ethyl-N-nitrosourea", *Environ. Mol. Mutagen.,* 56(4): 347-55. Doi: 10.1002/em.21932.

Olive, L. P., J. P. Bnath & E. R. Durand, (1990), "Heterogeneity in Radiation-Induced DNA Damage and Repairing Tumor and Normal Cells Measured Using the "Comet" Assay", *Radiation Research*. 122: 86-94. Doi: 10.1667/rrav04.1.

Pardo, B., B. Gomez-Gonzalez & A. Aguilera, (2009), "DNA repair in mammalian cells: DNA double-strand break repair: how to fix a broken relationship*", Cell Mol Life Sci*, 66(6), 1039-1056. doi:10.1007/s00018-009-8740-3.

Pegg, A.E. (2011), "Multifaceted roles of alkyltransferase and related proteins in DNA repair, DNA damage, resistance to chemotherapy, and research tools", *Chem. Res. Toxicol.*, 4(5): 618-39. Doi: 10.1021/tx200031q.

Penninckx, S. et al. (2021), "Quantification of radiation-induced DNA double strand break repair foci to evaluate and predict biological responses to ionizing radiation", NAR Cancer, Vol.3/4, Oxford University Press, Oxford, https://doi.org/10.1093/narcan/zcab046.

Rydberg, B. et al. (2005), "Dose-Dependent Misrejoining of Radiation-Induced DNA Double-Strand Breaks in Human Fibroblasts: Experimental and Theoretical Study for High- and Low-LET Radiation", Radiation Research, Vol.163/5, Radiation Research Society, Indianapolis, https://doi.org/10.1667/RR3346.

Sancar, A. (2003), "Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors", *Chem Rev.*, 103(6): 2203-37. Doi: 10.1021/cr0204348.

Saini, N. et al. (2017), "Migrating bubble during break-induced replication drives conservative DNA synthesis", *Nature*, 502:389-392. Doi: 10.1038/nature12584.

Sakofsky, C.J. et al. (2015), "Translesion Polymerases Drive Microhomology-Mediated Break-Induced Replication Leading to Complex Chromosomal Rearrangements", *Mol Cell*, 60:860-872. Doi: 10.1016/j.molcel.2015.10.041.

Schärer, O.D. (2013), "Nucleotide excision repair in eukaryotes", *Cold Spring Harb. Perspect. Biol.*, 5(10): a012609. Doi: 10.1101/cshperspect.a012609.

Scherer, E., A.A. Jenner and L. den Engelse (1987), "Immunocytochemical studies on the formation and repair of O6-alkylguanine in rat tissues", *IARC Sci Publ.*, 84: 55-8.

Seager, A., Shah, U., Mikhail, J., Nelson, B., Marquis, B., Doak, S., Johnson, G., Griffiths, S., Carmichael, P., Scott, S., Scott, A., Jenkins, G. (2012), Pro-oxidant Induced DNA Damage in Human Lymphoblastoid Cells: Homeostatic Mechanisms of Genotoxic Tolerance, Toxicol Sci, 128:387-397.

Seiler, F., K. Kamino, M. Emura, U. Mohr and J. Thomale (1997), "Formation and persistence of the miscoding DNA alkylation product O6-ethylguanine in male germ cells of the hamster", *Mutat Res.*, 385(3): 205-211. Doi: 10.1016/s0921-8777(97)00043-8.

Seo, Y.R. and H.J. Jung (2004), "The potential roles of p53 tumor suppressor in nucleotide excision repair (NER) and base excision repair (BER)", *Exp. Mol. Med.*, 36(6): 505-509. Doi: 10.1038/emm.2004.64.

Shelby, M.D. and K.R. Tindall (1997), "Mammalian germ cell mutagenicity of ENU, IPMS and MMS, chemicals selected for a transgenic mouse collaborative study", *Mutation Research*, 388(2-3): 99-109. Doi: 10.1016/s1383-5718(96)00106-4.

Sundheim, O. et al. (2008), "AlkB demethylases flip out in different ways", *DNA Repair (Amst).*, 7(11): 1916-1923. Doi: 10.1016/j.dnarep.2008.07.015.

Sung, P., & H. Klein, (2006), "Mechanism of homologous recombination: mediators and helicases take on regulatory functions", *Nat Rev Mol Cell Biol*, 7(10), 739-750. Doi:10. 1038/nrm2008.

Thyagarajan, B., Anderson, K. E., Lessard, C. J., Veltri, G., Jacobs, D. R., Folsom, A. R., & Gross, M. D. (2007), "Alkaline unwinding flow cytometry assay to measure nucleotide excision repair", *Mutagenesis*, 22(2): 147–153. https://doi.org/10.1093/mutage/gel071

Trucco, C., et al., (1998), “DNA repair defect i poly(ADP-ribose) polymerase-deficient cell lines”, Nucleic Acids Research. 26(11): 2644–2649. Doi: 10.1093/nar/26.11.2644.

Trzeciak, A.R. et al. (2008), “Age, sex, and race influence single-strand break repair capacity in a human population”, Free Radical Biology & Medicine, Vol. 45, Elsevier, Amsterdam, https://doi.org/10.1016/j.freeradbiomed.2008.08.031.

White, R.R. and J. Vijg. (2016), “Do DNA Double-Strand Breaks Drive Aging?”, Molecular Cell, Vol.63, Elsevier, Amsterdam, http://doi.org/10.1016/j.molcel.2016.08.004.

Wilhelm, T. et al. (2014), "Spontaneous slow replication fork progression elicits mitosis alterations in homologous recombination-deficient mammalian cells.", Proc. Natl. Acad. Sci. 111(2):763-768. doi:10.1073/pnas.1311520111.

Wyrick, J.J. & S. A. Roberts, (2015), “Genomic approaches to DNA repair and mutagenesis”, DNA Repair (Amst). 36:146-155. doi: 10.1016/j.dnarep.2015.09.018.

van Zeeland, A.A., A. de Groot and A. Neuhäuser-Klaus (1990), "DNA adduct formation in mouse testis by ethylating agents: a comparison with germ-cell mutagenesis", *Mutat. Res.*, 231(1): 55-62.

Event: 1635: Increase, DNA strand breaks

Short Name: Increase, DNA strand breaks

AOPs Including This Key Event

AOP Name	Role of event in AOP
Oxidative DNA damage, chromosomal aberrations and mutations	KeyEvent
Deposition of energy leading to lung cancer	KeyEvent
Alkylation of DNA leading to reduced sperm count	KeyEvent
Deposition of energy leading to population decline via DSB and follicular atresia	KeyEvent
Deposition of energy leading to population decline via DSB and apoptosis	KeyEvent
Deposition of energy leading to cataracts	KeyEvent

Stressors

Name
Ionizing and non-ionizing Radiation
Oxidizing agents
Topoisomerase inhibitors
Radiomimetic compounds

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human and other cells in culture	human and other cells in culture		NCBI

Life Stage Applicability

Life stage	Evidence
All life stages	High

Sex Applicability

Term	Evidence
Unspecific	High

Taxonomic applicability: DNA strand breaks are relevant to all species, including vertebrates such as humans, that contain DNA (Cannan & Pederson, 2016).

Life stage applicability: This key event is not life stage specific as all life stages display strand breaks. However, there is an increase in baseline levels of DNA strand breaks seen in older individuals though it is unknown whether this change due to increased break induction or a greater retention of breaks due to poor repair (White & Vijg, 2016).

Sex applicability: This key event is not sex specific as both sexes display evidence of strand breaks. In some cell types, such as peripheral blood mononuclear cells, males show higher levels of single strand breaks than females (Garm et al., 2012).

Evidence for perturbation by a stressor: There are studies demonstrating that increased DNA strand breaks can result from exposure to multiple stressor types including ionizing & non-ionizing radiation, chemical agents, and oxidizing agents (EPRI, 2014; Hamada, 2014; Cencer et al., 2018; Cannan & Pederson, 2016; Yang et al., 1998).

Key Event Description

DNA strand breaks can occur on a single strand (SSB) or both strands (double strand breaks; DSB). SSBs arise when the phosphate backbone connecting adjacent nucleotides in DNA is broken on one strand. DSBs are generated when both strands are simultaneously broken at sites that are sufficiently close to one another that base-pairing and chromatin structure are insufficient to keep the two DNA ends juxtaposed. As a consequence, the two DNA ends generated by a DSB can physically dissociate from one another, becoming difficult to repair and increasing the chance of inappropriate recombination with other sites in the genome (Jackson, 2002). SSB can turn into DSB if the replication fork stalls at the lesion leading to fork collapse.

Strand breaks are intermediates in various biological events, including DNA repair (e.g., excision repair), V(D)J recombination in developing lymphoid cells and chromatin remodeling in both somatic cells and germ cells. The spectrum of damage can be complex, particularly if the stressor is from large amounts of deposited energy which can result in complex lesions and clustered damage defined as two or more oxidized bases, abasic sites or strand breaks on opposing DNA strands within a few helical turns. These lesions are more difficult to repair and have been studied in many types of models (Barbieri et al., 2019 and Asaithamby et al., 2011). DSBs and complex lesions are of particular concern, as they are considered the most lethal and deleterious type of DNA lesion. If misrepaired or left unrepaired, DSBs may drive the cell towards genomic instability, apoptosis or tumorigenesis (Beir, 1999).

How It Is Measured or Detected

Please refer to the table below for details regarding these and other methodologies for detecting DNA DSBs.

Assay Name	References	Description	OECD Approved Assay
Comet Assay (Single Cell Gel Eletrophoresis - Alkaline)	Collins, 2004; Olive and Banath, 2006; Platel et al., 2011; Nikolova et al., 2017	To detect SSBs or DSBs, single cells are encapsulated in agarose on a slide, lysed, and subjected to gel electrophoresis at an alkaline pH (pH >13); DNA fragments are forced to move, forming a "comet"-like appearance	Yes (No. 489)
Comet Assay (Single Cell Gel Eltrophoresis - Neutral)	Collins, 2014; Olive and Banath, 2006; Anderson and Laubenthal, 2013; Nikolova et al., 2017	To detect DSBs, single cells are encapsulated in agarose on a slide, lysed, and subjected to gel electrophoresis at a neutral pH; DNA fragments, which are not denatured at the neutral pH, are	N/A

		forced to move, forming a "comet"-like appearance	
γ-H2AX Foci Quantification - Flow Cytometry (standard and imagining flow cytometry)	Rothkamm and Horn, 2009; Bryce et al., 2016 Lee et al., 2019	Measurement of γ-H2AX immunostaining in cells by flow cytometry, normalized to total levels of H2AX	N/A
γ-H2AX Foci Quantification - Western Blot	Burma et al., 2001; Revet et al., 2011	Measurement of γ-H2AX immunostaining in cells by Western blotting, normalized to total levels of H2AX	N/A
γ-H2AX Foci Quantification - Microscopy	Redon et al., 2010; Mah et al., 2010; Garcia-Canton et al., 2013	Quantification of γ-H2AX immunostaining by counting γ-H2AX foci visualized with a microscope	N/A
γ-H2AX Foci Detection - ELISA and flow cytometry	Ji et al., 2017; Bryce et al., 2016	Detection of γ-H2AX in cells by ELISA, normalized to total levels of H2AX; γH2AX foci detection can be high-throughput and automated using flow cytometry-based immunodetection.	N/A
Pulsed Field Gel Electrophoresis (PFGE)	Ager et al., 1990; Gardiner et al., 1985; Herschleb et al., 2007; Kawashima et al., 2017	To detect DSBs, cells are embedded and lysed in agarose, and the released DNA undergoes gel electrophoresis in which the direction of the voltage is periodically alternated; Large DNA fragments are thus able to be separated by size	N/A
The TUNEL (Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling) Assay	Loo, 2011	To detect strand breaks, dUTPs added to the 3'OH end of a strand break by the DNA polymerase terminal deoxynucleotidyl transferase (TdT) are tagged with a fluorescent dye or a reporter enzyme to allow visualization (We note that this method is typically used to measure apoptosis)	N/A
In Vitro DNA Cleavage Assays using Topoisomerase	Nitiss, 2012	Cleavage of DNA can be achieved using purified topoisomerase; DNA strand breaks can then be separated and quantified using gel electrophoresis	N/A
PCR assay	Figueroa-González & Pérez-Plasencia, 2017	Assay of strand breaks through the observation of DNA amplification prevention. Breaks block Taq polymerase, reducing the number of DNA templates, preventing amplification	N/A
Sucrose density gradient centrifuge	Raschke et al. 2009	Division of DNA pieces by density, increased fractionation leads to lower density pieces, with the use of a sucrose cushion	N/A
Alkaline Elution Assay	Kohn, 1991	Cells lysed with detergent-solution, filtered through	N/A

		membrane to remove all but intact DNA	
Unwinding Assay	Nacci et al. 1992	DNA is stored in alkaline solutions with DNA-specific dye and allowed to unwind following removal from tissue, increased strand damage associated with increased unwinding	N/A

References

Ager, D. D. et al. (1990). “Measurement of Radiation- Induced DNA Double-Strand Breaks by Pulsed-Field Gel Electrophoresis.” Radiat Res. 122(2), 181-7.

Anderson, D. & Laubenthal J. (2013), “Analysis of DNA Damage via Single-Cell Electrophoresis. In: Makovets S, editor. DNA Electrophoresis. Totowa.”, NJ: Humana Press. p 209-218.

Asaithamby, A., B. Hu and D.J. Chen. (2011) Unrepaired clustered DNA lesions induce chromosome breakage in human cells. Proc Natl Acad Sci U S A 108(20): 8293-8298 .

Barbieri, S., G. Babini, J. Morini et a l (2019). . Predicting DNA damage foci and their experimental readout with 2D microscopy: a unified approach applied to photon and neutron exposures. Scientific Reports 9(1): 14019

Bryce, S. et al. (2016), “Genotoxic mode of action predictions from a multiplexed flow cytometric assay and a machine learning approach.”, Environ Mol Mutagen. 57:171-189. Doi: 10.1002/em.21996.

Burma, S. et al. (2001), “ATM phosphorylates histone H2AX in response to DNA double-strand breaks.”, J Biol Chem, 276(45): 42462-42467. doi:10.1074/jbc.C100466200

Cannan, W.J. and D.S. Pederson (2016), "Mechanisms and Consequences of Double-Strand DNA Break Formation in Chromatin.", Journal of Cellular Physiology, Vol.231/1, Wiley, New York, https://doi.org/10.1002/jcp.25048.

Cencer, C. et al. (2018), “PARP-1/PAR Activity in Cultured Human Lens Epithelial Cells Exposed to Two Levels of UVB Light”, Photochemistry and Photobiology, Vol.94/1, Wiley-Blackwell, Hoboken, https://doi.org/10.1111/php.12814.

Charlton, E. D. et al. (1989), “Calculation of Initial Yields of Single and Double Stranded Breaks in Cell Nuclei from Electrons, Protons, and Alpha Particles.”, Int. J. Radiat. Biol. 56(1): 1-19. doi: 10.1080/09553008914551141.

Collins, R. A. (2004), “The Comet Assay for DNA Damage and Repair. Molecular Biotechnology.”, Mol Biotechnol. 26(3): 249-61. doi:10.1385/MB:26:3:249

EPRI (2014), Epidemiology and mechanistic effects of radiation on the lens of the eye: Review and scientific appraisal of the literature, EPRI, California.

Figueroa-González, G. and C. Pérez-Plasencia. (2017), “Strategies for the evaluation of DNA damage and repair mechanisms in cancer”, Oncology Letters, Vol.13/6, Spandidos Publications, Athens, https://doi.org/10.3892/ol.2017.6002.

Garcia-Canton, C. et al. (2013), "Assessment of the in vitro p-H2AX assay by High Content Screening asa novel genotoxicity test.", Mutat Res. 757:158-166. Doi: 10.1016/j.mrgentox.2013.08.002

Gardiner, K. et al. (1986), "Fractionation of Large Mammalian DNA Restriction Fragments Using Vertical Pulsed-Field Gradient Gel Electrophoresis.", Somatic Cell and Molecular Genetics. 12(2): 185-95.Doi: 10.1007/bf01560665.

Garm, C. et al. (2012), "Age and gender effects on DNA strand break repair in peripheral blood mononuclear cells", Aging Cell, Vol.12/1, Blackwell Publishing Ltd, Oxford, https://doi.org/10.1111/acel.12019.

Hamada, N. (2014), "What are the intracellular targets and intratissue target cells for radiation effects?", Radiation research, Vol. 181/1, The Radiation Research Society, Indianapolis, https://doi.org/10.1667/RR13505.1.

Herschleb, J. et al. (2007), "Pulsed-field gel electrophoresis.", Nat Protoc. 2(3): 677-684. doi:10.1038/nprot.2007.94

Iliakis, G. et al. (2015), "Alternative End-Joining Repair Pathways Are the Ultimate Backup for Abrogated Classical Non-Homologous End-Joining and Homologous Recombination Repair: Implications for the Formation of Chromosome Translocations.", Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2(3): 677-84. doi: 10.1038/nprot.2007.94

Jackson, S. (2002). "Sensing and repairing DNA double-strand breaks.", Carcinogenesis. 23:687-696. Doi:10.1093/carcin/23.5.687.

Ji, J. et al. (2017), "Phosphorylated fraction of H2AX as a measurement for DNA damage in cancer cells and potential applications of a novel assay.", PLoS One. 12(2): e0171582. doi:10.1371/journal.pone.0171582

Kawashima, Y. (2017), "Detection of DNA double-strand breaks by pulsed-field gel electrophoresis.", Genes Cells 22:84-93. Doi: 10.1111/gtc.12457.

Khoury, L. et al. (2013), "Validation of high-throughput genotoxicity assay screening using cH2AX in-cell Western assay on HepG2 cells.", Environ Mol Mutagen, 54:737-746. Doi: 10.1002/em.21817.

Khoury, L. et al. (2016), "Evaluation of four human cell lines with distinct biotransformation properties for genotoxic screening.", Mutagenesis, 31:83-96. Doi: 10.1093/mutage/gev058.

Kohn, K.W. (1991), "Principles and practice of DNA filter elution", Pharmacology & Therapeutics, Vol.49/1, Elsevier, Amsterdam, https://doi.org/10.1016/0163-7258(91)90022-E.

Lee, Y., Wang, Q., Shuryak, I. et al. (2019), "Development of a high-throughput γ-H2AX assay based on imaging flow cytometry", Radiat Oncol, 14: 150 https://doi.org/10.1186/s13014-019-1344-7

Loo, DT. (2011), "In Situ Detection of Apoptosis by the TUNEL Assay: An Overview of Techniques. In: Didenko V, editor. DNA Damage Detection In Situ, Ex Vivo, and In Vivo. Totowa.", NJ: Humana Press. p 3-13.doi: 10.1007/978-1-60327-409-8_1.

Mah, L. J. et al. (2010), "Quantification of gammaH2AX foci in response to ionising radiation.", J Vis Exp(38). doi:10.3791/1957.

Nacci, D. et al. (1992), "Application of the DNA alkaline unwinding assay to detect DNA strand breaks in marine bivalves", Marine Environmental Research, Vol.33/2, Elsevier BV, Amsterdam, https://doi.org/10.1016/0141-1136(92)90134-8.

Nikolova, T., F. et al. (2017), "Genotoxicity testing: Comparison of the γH2AX focus assay with the alkaline and neutral comet assays.", Mutat Res 822:10-18. Doi: 10.1016/j.mrgentox.2017.07.004.

Nitiss, J. L. et al. (2012), "Topoisomerase assays. ", Curr Protoc Pharmacol. Chapter 3: Unit 3 3.

OECD. (2014). Test No. 489: "In vivo mammalian alkaline comet assay." OECD Guideline for the Testing of Chemicals, Section 4 .

Olive, P. L., & Banáth, J. P. (2006), "The comet assay: a method to measure DNA damage in individual cells.", Nature Protocols. 1(1): 23-29. doi:10.1038/nprot.2006.5.

Platel A. et al. (2011), "Study of oxidative DNA damage in TK6 human lymphoblastoid cells by use of the thymidine kinase gene-mutation assay and the *in vitro* modified comet assay: Determination of No-Observed-Genotoxic-Effect-Levels.", Mutat Res 726:151-159. Doi: 10.1016/j.mrgentox.2011.09.003.

Raschke, S., J. Guan and G. Iliakis. (2009), "Application of alkaline sucrose gradient centrifugation in the analysis of DNA replication after DNA damage", Methods in Molecular Biology, Vol.521, Humana Press, Totowa, https://doi.org/10.1007/978-1-60327-815-7_18.

Redon, C. et al. (2010), "The use of gamma-H2AX as a biodosimeter for total-body radiation exposure in non-human primates.", PLoS One. 5(11): e15544. doi:10.1371/journal.pone.0015544

Revet, I. et al. (2011), "Functional relevance of the histone γH2Ax in the response to DNA damaging agents." Proc Natl Acad Sci USA.108:8663-8667. Doi: 10.1073/pnas.1105866108

Rogakou, E.P. et al. (1998), "DNA Double-stranded Breaks Induce Histone H2AX Phosphorylation on Serine 139." , J Biol Chem, 273:5858-5868. Doi: 10.1074/jbc.273.10.5858

Rothkamm, K. & Horn, S. (2009), "γ-H2AX as protein biomarker for radiation exposure.", Ann Ist Super Sanità, 45(3): 265-71.

White, R.R. and J. Vijg. (2016), "Do DNA Double-Strand Breaks Drive Aging?", Molecular Cell, Vol.63, Elsevier, Amsterdam, http://doi.org/10.1016/j.molcel.2016.08.004.

Yang, Y. et al. (1998), "The effect of catalase amplification on immortal lens epithelial cell lines", Experimental Eye Research, Vol.67/6, Academic Press Inc, Cambridge, https://doi.org/10.1006/exer.1998.0560.

List of Adverse Outcomes in this AOP

Event: 185: Increase, Mutations

Short Name: Increase, Mutations

Key Event Component

Process	Object	Action
mutation	deoxyribonucleic acid	increased

AOPs Including This Key Event

AOP Name	Role of event in AOP
Alkylation of DNA leading to heritable mutations	KeyEvent
DNA alkylation -> cancer 2	KeyEvent
DNA alkylation -> cancer 1	KeyEvent
RONS leading to breast cancer	AdverseOutcome
Increased DNA damage leading to breast cancer	AdverseOutcome
Oxidative DNA damage, chromosomal aberrations and mutations	AdverseOutcome
Deposition of energy leading to lung cancer	KeyEvent
Bulky DNA adducts leading to mutations	AdverseOutcome
DNA damage and metastatic breast cancer	KeyEvent
Deposition of energy leading to cataracts	KeyEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
Mus musculus	Mus musculus	High	NCBI
medaka	Oryzias latipes	Moderate	NCBI
rat	Rattus norvegicus	High	NCBI
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life stage	Evidence
All life stages	High

Sex Applicability

Term	Evidence
Unspecific	High

Taxonomic applicability: Mutations can occur in any organism and in any cell type, and are the fundamental material of evolution. The test guidelines described above range from analysis from prokaryotes, to rodents, to human cells in vitro. Mutations have been measured in virtually every human tissue sampled in vivo.

Life stage applicability: This key event is not life stage specific as all stages of life have DNA that can be mutated; however, baseline levels of mutations are seen to increase with age (Slebos et al., 2004; Kirkwood, 1989).

Sex applicability: This key event is not sex specific as both sexes undergo mutations. Males have a higher mutation rate than females (Hedrick, 2007).

Evidence for perturbation by a stressor: Many studies demonstrate that increased mutations can occur as a result of ionizing radiation (Sankaranarayanan & Nikjoo, 2015; Russell et al., 1957; Winegar et al., 1994; Gossen et al., 1995).

Key Event Description

A mutation is a change in DNA sequence. Mutations can thus alter the coding sequence of genes, potentially leading to malformed or truncated proteins. Mutations can also occur in promoter regions, splice junctions, non-coding RNA, DNA segments, and other functional locations in the genome. These mutations can lead to various downstream consequences, including alterations in gene expression. There are several different types of mutations including missense, nonsense, insertion, deletion, duplication, and frameshift mutations, all of which can impact the genome and its expression in unique ways.

Missense mutations are the substitution of one base in the codon with another. This change is significant because the three bases in a codon code for a specific amino acid and the new combination may signal for a different amino acid to be formed. Nonsense mutations also result from changes to the codon bases, but in this case, they cause the generation of a stop codon in the DNA strand where there previously was not one. This stop codon takes the place of a normal coding triplet, preventing its translation into an amino acid. This will cause the translation of the strand to prematurely stop. Both missense and nonsense mutations can result from substitutions, insertions, or deletions of bases (Chakarov et al. 2014).

Insertion and deletion mutations are the addition and removal of bases from the strand, respectively. These often accompany a frameshift mutation, as the alteration in the number of bases in the strand causes the frame of the base reader to shift by the added or reduced number, altering the amino acids that are produced if that number is not devisable by three. Codons come in specific orders, sectioned into groups of three. When the boundaries of which three bases are included in one group are changed, this can change the whole transcriptional output of the strand (Chakaroy et al. 2014).

Mutations can be propagated to daughter cells upon cellular replication. Mutations in stem cells (versus terminally differentiated non-replicating cells) are the most concerning, as these will persist in the organism. The consequence of the mutation, and thus the fate of the cell, depends on the location (e.g., coding versus non-coding) and the type (e.g., nonsense versus silent) of mutation.

Mutations can occur in somatic cells or germ cells (sperm or egg).

How It Is Measured or Detected

Mutations can be measured using a variety of both OECD and non-OECD mutagenicity tests. Listed below are common methods for detecting the KE, however there may be other comparable methods that are not listed.

Somatic cells: The Salmonella mutagenicity test (Ames Test) is generally used as part of a first tier screen to determine if a chemical can cause gene mutations. This well-established test has an OECD test guideline (OECD TG 471, 2020). A variety of bacterial strains are used, in the presence and absence of a metabolic activation system (e.g., rat liver microsomal S9 fraction), to determine the mutagenic potency of chemicals by dose-response analysis. A full description is found in Test No. 471: Bacterial Reverse Mutation Test (OECD, 2016).

A variety of in vitro mammalian cell gene mutation tests are described in OECD's Test Guidelines 476 (2016) and 490 (2015). TG 476 (2016) is used to identify substances that induce gene mutations at the hprt (hypoxanthine-guanine phosphoribosyl transferase) gene, or the transgenic xprt (xanthine-guanine phosphoribosyl transferase) reporter locus. The most commonly used cells for the HPRT test include the CHO, CHL and V79 lines of Chinese hamster cells, L5178Y mouse lymphoma cells, and TK6 human lymphoblastoid cells. The only cells suitable for the XPRT test are AS52 cells containing the bacterial xprt (or gpt) transgene (from which the hprt gene was deleted).

The new OECD TG 490 (2015) describes two distinct in vitro mammalian gene mutation assays using the thymidine kinase (tk) locus and requiring two specific tk heterozygous cells lines: L5178Y tk+/-3.7.2C cells for the mouse lymphoma assay (MLA) and TK6 tk+/- cells for the TK6 assay. The autosomal and heterozygous nature of the thymidine kinase gene in the two cell lines enables the detection of cells deficient in the enzyme thymidine kinase following mutation from tk+/- to tk-/-.

It is important to consider that different mutation spectra are detected by the different mutation endpoints assessed. The non-autosomal location of the hprt gene (X-chromosome) means that the types of mutations detected in this assay are point mutations, including base pair substitutions and frameshift mutations resulting from small insertions and deletions. Whereas, the autosomal location of the transgenic xprt, tk, or gpt locus allows the detection of large deletions not readily detected at the hemizygous hprt locus on X-chromosomes. Genetic events detected using the tk locus include both gene mutations (point mutations, frameshift mutations, small deletions) and large deletions.

The transgenic rodent mutation assay (OECD TG 488, 2020) is the only assay capable of measuring gene mutation in virtually all tissues in vivo. Specific details on the rodent transgenic mutation reporter assays are reviewed in Lambert et al. (2005, 2009). The

transgenic reporter genes are used for detection of gene mutations and/or chromosomal deletions and rearrangements resulting in DNA size changes (the latter specifically in the lacZ plasmid and Spi- test models) induced in vivo by test substances (OECD, 2009, OECD, 2011; Lambert et al., 2005). Briefly, transgenic rodents (mouse or rat) are exposed to the chemical agent sub-chronically. Following a manifestation period, genomic DNA is extracted from tissues, transgenes are rescued from genomic DNA, and transfected into bacteria where the mutant frequency is measured using specific selection systems.

The Pig-a (phosphatidylinositol glycan, Class A) gene on the X chromosome codes for a catalytic subunit of the N-acetylglucosamine transferase complex that is involved in glycosylphosphatidyl inositol (GPI) cell surface anchor synthesis. Cells lacking GPI anchors, or GPI-anchored cell surface proteins are predominantly due to mutations in the Pig-a gene. Thus, flow cytometry of red blood cells expressing or not expressing the Pig-a gene has been developed for mutation analysis in blood cells from humans, rats, mice, and monkeys. The assay is described in detail in Dobrovolsky et al. (2010). The Mammalian Erythrocyte Pig-a Gene Mutation Assay was published as TG 470 in 2022 (OECD TG 470, 2022). In addition, experiments determining precisely what proportion of cells expressing the Pig-a mutant phenotype have mutations in the Pig-a gene are in progress (e.g., Nicklas et al., 2015, Drobovolsky et al., 2015). A recent paper indicates that the majority of CD48 deficient cells from 7,12-dimethylbenz[a]anthracene-treated rats (78%) are indeed due to mutation in Pig-a (Drobovolsky et al., 2015).

Germ cells: Tandem repeat mutations can be measured in bone marrow, sperm, and other tissues using single-molecule PCR. This approach has been applied most frequently to measure repeat mutations occurring in sperm DNA. Isolation of sperm DNA is as described above for the transgenic rodent mutation assay, and analysis of tandem repeats is done using electrophoresis for size analysis of allele length using single-molecule PCR. For expanded simple tandem repeat this involved agarose gel electrophoresis and Southern blotting, whereas for microsatellites sizing is done by capillary electrophoresis. Detailed methodologies for this approach are found in Yauk et al. (2002) and Beal et al. (2015).

Mutations in rodent sperm can also be measured using the transgenic reporter model (OECD TG 488, 2020). A description of the approach is found within this published TG. Further modifications to this protocol have been made as of 2022 for the analysis of germ cells. Detailed methodology for detecting mutant frequency arising in spermatogonia is described in Douglas et al. (1995), O'Brien et al. (2013); and O'Brien et al. (2014). Briefly, male mice are exposed to the mutagen and killed at varying times post-exposure to evaluate effects on different phases of spermatogenesis. Sperm are collected from the vas deferens or caudal epididymis (the latter preferred). Modified protocols have been developed for extraction of DNA from sperm.

A similar transgenic assay can be used in transgenic medaka (Norris and Winn, 2010).

Please note, gene mutations that occur in somatic cells in vivo (OECD Test. No. 488, 2020) or in vitro (OECD Test No. 476: In vitro Mammalian Cell Gene Mutation Test, 2016), or in bacterial cells (i.e., OECD Test No. 471, 2020) can be used as an indicator that mutations in male pre-meiotic germ cells may occur for a particular agent (sensitivity and specificity of other assays for male germ cell effects is given in Waters et al., 1994). However, given the very unique biological features of spermatogenesis relative to other cell types, known exceptions to this rule, and the small database on which this is based, inferring results from somatic cell or bacterial tests to male pre-meiotic germ cells must be done with caution.

That mutational assays in somatic cells may predict mutations in germ cells has not been rigorously tested empirically (Singer and Yauk, 2010). The IWGT working group on germ cells specifically addressed this gap in knowledge in their report (Yauk et al., 2015) and recommended that additional research addresses this issue. Mutations can be directly measured in humans (and other species) through the application of next-generation sequencing. While the most robust approach to measure mutation using next-generation sequencing today requires clonal expansion of the mutation to a sizable proportion (e.g., sequencing tumours; Shen et al., 2015), or analysis of families to identify germline derived mutations (reviewed in Campbell and Eichler, 2013; Adewoye et al., 2015), single-molecule and single-cell approaches are growing in prevalence (Olafsson and Anderson, 2021; Marchetti et al., 2023).

Please refer to the table below for additional details and methodologies for measuring mutations.

Assay Name	**References**	**Description**	**OECD Approved Assay**
Assorted Gene Loci Mutation Assays	Tindall et al., 1989; Kruger et al., 2015	After exposure to a chemical/mutagen, mutations can be measured by the ability of exposed cells to form colonies in the presence of specific compounds that would normally inhibit colony growth; Usually only cells -/- for the gene of interest are able to form colonies	N/A
TK Mutation Assay	Yamamoto et al., 2017; Liber et al., 1982; Lloyd and Kidd, 2012	After exposure to a chemical/mutagen, mutations are detected at the thymidine kinase (TK) loci of L5178Y wild-type mouse lymphoma TK (+/-) cells by measuring resistance to lethal triflurothymidine (TFT); Only TK-/- cells are able to form colonies	Yes (No. 490)
HPRT Mutation Assay	Ayres et al., 2006; Parry and Parry, 2012	Similar to TK Mutation Assay above, X-linked HPRT mutations produced in response to chemical/mutagen exposure can be measured through colony formation in the presence of 6-TG or 8-azoguanine; Only HPRT-/- cells are able to form colonies	Yes (No. 476)
Salmonella Mutagenicity Test (Ames Test)	OECD, 1997	After exposure to a chemical/mutagen, point mutations are detected by analyzing the growth capacity of different bacterial strains in the presence and absence of various metabolic activation systems	Yes (No. 471)
PIG-A / PIG-O Assay	OECD, 2022 Kruger et al., 2015; Nakamura, 2012; Chikura, 2019	After exposure to a chemical/mutagen, mutations in PIG-A or PIG-O (which decrease the biosynthesis of the glycosylphosphatidylinositol (GPI) anchor protein) are assessed by the colony-forming capabilities of cells after *in vitro* exposure, or by flow cytometry of blood samples after *in vivo* exposure	N/A
Single Molecule	Kraytsberg &	This PCR technique uses a single DNA template,	N/A

PCR	Khrapko, 2005; Yauk, 2002	and is often employed for detection of mutations in microsatellites, recombination studies, and generation of polonies	
ACB-PCR	Myers et al., 2014 (Textbook, pg 345-363); Banda et al., 2013; Banda et al., 2015; Parsons et al., 2017	Using this PCR technique, single base pair substitution mutations within oncogenes or tumour suppressor genes can be detected by selectively amplifying specific point mutations within an allele and selectively blocking amplification of the wild-type allele	N/A
Transgenic Rodent Mutation Assay	OECD 2013; Lambert 2005; Lambert 2009	This *in vivo* test detects gene mutations using transgenic rodents that possess transgenes and reporter genes; After *in vivo* exposure to a chemical/mutagen, the transgenes are analyzed by transfecting bacteria with the reporter gene and examining the resulting phenotype	Yes (No. 488)
Conditionally inducible transgenic mouse models	Parsons 2018 (Review)	Inducible mutations linked to fluorescent tags are introduced into transgenic mice; Upon exposure of the transgenic mice to an inducing agent, the presence and functional assessment of the mutations can be easily ascertained due to expression of the linked fluorescent tags	N/A
Error-Corrected Next Generation Sequencing (NGS)	Salk 2018 (Review) Marchetti et al., 2023	This technique detects rare subclonal mutations within a pool of heterogeneous DNA samples through the application of new error-correction strategies to NGS; At present, few laboratories in the world are capable of doing this, but commercial services are becoming available (e.g., Duplex sequencing at TwinStrand BioSciences)	N/A

References

Adewoye, A.B. et al. (2015), "The genome-wide effects of ionizing radiation on mutation induction in the mammalian germline", *Nat. Commu.*, 6:6684. Doi: 10.1038/ncomms7684.

Ayres, M. F. et al. (2006), "Low doses of gamma ionizing radiation increase hprt mutant frequencies of TK6 cells without triggering the mutator phenotype pathway", *Genetics and Molecular Biology*. 2(3): 558-561. Doi:10.1590/S1415-4757200600030002.

Banda M, Recio L, and Parsons BL. (2013), "ACB-PCR measurement of spontaneous and furan-induced H-ras codon 61 CAA to CTA and CAA to AAA mutation in B6C3F1 mouse liver", *Environ Mol Mutagen*. 54(8):659-67. Doi:10.1002/em.21808.

Banda, M. et al. (2015), “Quantification of Kras mutant fraction in the lung DNA of mice exposed to aerosolized particulate vanadium pentoxide by inhalation”, *Mutat Res Genet Toxicol Environ Mutagen*. 789-790:53-60. Doi: 10.1016/j.mrgentox.2015.07.003

Campbell, C.D. & E.E. Eichler (2013), "Properties and rates of germline mutations in humans", *Trends Genet*., 29(10): 575-84. Doi: 10.1016/j.tig.2013.04.005

Chakarov, S. et al. (2014), “DNA damage and mutation. Types of DNA damage”, BioDiscovery, Vol.11, Pensoft Publishers, Sofia, https://doi.org/10.7750/BIODISCOVERY.2014.11.1.

Chikura, S. et al. (2019), “Standard protocol for the total red blood cell Pig-a assay used in the interlaboratory trial organized by the Mammalian Mutagenicity Study Group of the Japanese Environmental Mutagen Society”, *Genes Environ*. 27:41-5. Doi: 10.1186/s41021-019-0121-z.

Dobrovolsky, V.N. et al. (2015), "CD48-deficient T-lymphocytes from DMBA-treated rats have de novo mutations in the endogenous Pig-a gene. CD48-Deficient T-Lymphocytes from DMBA-Treated Rats Have De Novo Mutations in the Endogenous Pig-a Gene", Environ. Mol. Mutagen., (6): 674-683. Doi: 10.1002/em.21959.

Douglas, G.R. et al. (1995), "Temporal and molecular characteristics of mutations induced by ethylnitrosourea in germ cells isolated from seminiferous tubules and in spermatozoa of lacZ transgenic mice", *Proceedings of the National Academy of Sciences of the United States of America*, 92(16): 7485-7489. Doi: 10.1073/pnas.92.16.7485.

Gossen, J.A. et al. (1995), "Spontaneous and X-ray-induced deletion mutations in a LacZ plasmid-based transgenic mouse model", Mutation Research, 331/1, Elsevier, Amsterdam, https://doi.org/10.1016/0027-5107(95)00055-N.

Hedrick, P.W. (2007), “Sex: Differences In Mutation, Recombination, Selection, Gene Flow, And Genetic Drift”, Evolution, Vol.61/12, Wiley, Hoboken, https://doi.org/10.1111/j.1558-5646.2007.00250.x.

Kirkwood, T.B.L. (1989), “DNA, mutations and aging”, Mutation Research, Vol.219/1, Elsevier B.V., Amsterdam, https://doi.org/10.1016/0921-8734(89)90035-0

Kraytsberg,Y. & Khrapko, K. (2005), “Single-molecule PCR: an artifact-free PCR approach for the analysis of somatic mutations”, *Expert Rev Mol Diagn*. 5(5):809-15. Doi: 10.1586/14737159.5.5.809.

Krüger, T. C., Hofmann, M., & Hartwig, A. (2015), “The in vitro PIG-A gene mutation assay: mutagenicity testing via flow cytometry based on the glycosylphosphatidylinositol (GPI) status of TK6 cells”, *Arch Toxicol*. 89(12), 2429-43. Doi: 10.1007/s00204-014-1413-5.

Lambert, I.B. et al. (2005), "Detailed review of transgenic rodent mutation assays", *Mutat Res.*, 590(1-3):1-280. Doi: 10.1016/j.mrrev.2005.04.002.

Liber, L. H., & Thilly, G. W. (1982), “Mutation assay at the thymidine kinase locus in diploid human lymphoblasts”, *Mutation Research*. 94: 467-485. Doi:10.1016/0027-5107(82)90308-6.

Lloyd, M., & Kidd, D. (2012), “The Mouse Lymphoma Assay. In: Parry J., Parry E. (eds) Genetic Toxicology, Methods in Molecular Biology (Methods and Protocols), 817. Springer, New York, NY.

Marchetti, F., Cardoso, R., Chen, C. L., Douglas, G. R., Elloway, J., Escobar, P. A., Harper, T., Jr, Heflich, R. H., Kidd, D., Lynch, A. M., Myers, M. B., Parsons, B. L., Salk, J. J.,

Settivari, R. S., Smith-Roe, S. L., Witt, K. L., Yauk, C., Young, R. R., Zhang, S., & Minocherhomji, S. (2023). Error-corrected next-generation sequencing to advance nonclinical genotoxicity and carcinogenicity testing. Nature reviews. Drug discovery, 10.1038/d41573-023-00014-y. Advance online publication. https://doi.org/10.1038/d41573-023-00014-y

Myers, M. B. et al., (2014), "ACB-PCR Quantification of Somatic Oncomutation", *Molecular Toxicology Protocols, Methods in Molecular Biology*. DOI: 10.1007/978-1-62703-739-6_27

Nakamura, J. et al., (2012), "Detection of PIGO-deficient cells using proaerolysin: a valuable tool to investigate mechanisms of mutagenesis in the DT40 cell system", *PLoS One*.7(3): e33563. Doi:10.1371/journal.pone.0033563.

Nicklas, J.A., E.W. Carter and R.J. Albertini (2015), "Both PIGA and PIGL mutations cause GPI-a deficient isolates in the Tk6 cell line", Environ. Mol. Mutagen., 6(8):663-73. Doi: 10.1002/em.21953.

Norris, M.B. and R.N. Winn (2010), "Isolated spermatozoa as indicators of mutations transmitted to progeny", Mutat Res., 688(1-2): 36–40. Doi: 10.1016/j.mrfmmm.2010.02.008.

O'Brien, J.M. et al.(2013), "No evidence for transgenerational genomic instability in the F1 or F2 descendants of Muta™Mouse males exposed to N-ethyl-N-nitrosourea", *Mutat. Res*., 741-742:11-7. Doi: 10.1016/j.mrfmmm.2013.02.004.

O'Brien, J.M. et al. (2014), "Transgenic rodent assay for quanitifying male germ cell mutation frequency", *Journal of Visual Experimentation*, Aug 6;(90). Doi: 10.3791/51576.

O'Brien, J.M. et al. (2015), "Sublinear response in lacZ mutant frequency of Muta™ Mouse spermatogonial stem cells after low dose subchronic exposure to N-ethyl-N-nitrosourea", *Environ. Mol. Mutagen.*, 6(4): 347-355. Doi: 10.1002/em.21932.

OECD (2022), Test No. 470: Mammalian Erythrocyte Pig-a Gene Mutation Assay, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/4faea90e-en.

OECD (2020), Test No. 471: Bacterial Reverse Mutation Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.

OECD (2016), Test No. 476: In vitro Mammalian Cell Gene Mutation Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.

OECD (2009), Detailed Review Paper on Transgenic Rodent Mutation Assays, Series on Testing and Assessment, N° 103, ENV/JM/MONO 7, OECD, Paris.

OECD (2020), Test No. 488: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.

OECD (2016), Test. No. 490: In vitro mammalian cell gene mutation mutation tests using the thymidine kinase gene, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.

OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.

Olafsson, S., & Anderson, C. A. (2021), "Somatic mutations provide important and unique insights into the biology of complex diseases", Trends in genetics : TIG, 37(10): 872–881. https://doi.org/10.1016/j.tig.2021.06.012

Parry MJ, & Parry ME. 2012. Genetic Toxicology Principles and Methods. Humana Press. Springer Protocols.

Parsons BL, McKim KL, Myers MB. 2017. Variation in organ-specific PIK3CA and KRAS mutant levels in normal human tissues correlates with mutation prevalence in corresponding carcinomas. Environ Mol Mutagen. 58(7):466-476. Doi: 10.1002/em.22110.

Parsons BL. Multiclonal tumor origin: Evidence and implications. *Mutat Res*. 2018. 777:1-18. doi: 10.1016/j.mrrev.2018.05.001.

Russell, W.L. et al. (1957), "Radiation Dose Rate and Mutation Frequency.", Science, Vol.128/3338, American Association for the Advancement of Science, Washington, https://doi.org/10.1126/science.128.3338.1546.

Salk JJ, Schmitt MW, &Loeb LA. (2018), "Enhancing the accuracy of next-generation sequencing for detecting rare and subclonal mutations", *Nat Rev Genet*. 19(5):269-285. Doi: 10.1038/nrg.2017.117.

Sankaranarayanan, K. & H. Nikjoo (2015), "Genome-based, mechanism-driven computational modeling of risks of ionizing radiation: The next frontier in genetic risk estimation?", Mutation Research, Vol.764, Elsevier, Amsterdam, https://doi.org/10.1016/j.mrrev.2014.12.003.

Shen, T., S.H. Pajaro-Van de Stadt, N.C. Yeat and J.C. Lin (2015), "Clinical applications of next generation sequencing in cancer: from panels, to exomes, to genomes" *Front. Genet.*, 6: 215. Doi: 10.3389/fgene.2015.00215.

Singer, T.M. and C.L. Yauk CL (2010), "Germ cell mutagens: risk assessment challenges in the 21st century", *Environ. Mol. Mutagen.*, 51(8-9): 919-928. Doi: 10.1002/em.20613.

Slebos, R.J.C. et al. (2004), "Mini-and microsatellite mutations in children from Chernobyl accident cleanup workers", Mutation Research/Genetic Toxicology and Environmental Mutagenesis, Vol.559/1-2, Elsevier, Amsterdam, https://doi.org/10.1016/j.mrgentox.2004.01.003.

Tindall, R. K., & Stankowski Jr., F. L. (1989), "Molecular analysis of spontaneous mutations at the GPT locus in Chinese hamster ovary (AS52) cells", *Mutation Research*, 220, 241-53. Doi: 10.1016/0165-1110(89)90028-6.

Waters, M.D. et al. (1994), "The performance of short-term tests in identifying potential germ cell mutagens: a qualitative and quantitative analysis", *Mutat. Res.*, 341(2): 109-31. Doi: 10.1016/0165-1218(94)90093-0.

Winegar, R.A. et al. (1994), "Radiation-induced point mutations, deletions and micronuclei in lacI transgenic mice", Mutation Research, Vol.307/2, Elsevier, Amsterdam, https://doi.org/10.1016/0027-5107(94)90258-5.

Yamamoto, A. et al. (2017), "Radioprotective activity of blackcurrant extract evaluated by in vitro micronucleus and gene mutation assays in TK6 human lymphoblastoid cells", *Genes and Environment.* 39: 22. Doi: 10.1186/s41021-017-0082-z.

Yauk, C.L. et al. (2002), "A novel single molecule analysis of spontaneous and radiation-induced mutation at a mouse tandem repeat locus", Mutat. Res., 500(1-2): 147-56. Doi: 10.1016/s0027-5107(02)00005-2.

Yauk, C.L. et al. (2015), "Approaches for Identifying Germ Cell Mutagens: Report of the 2013 IWGT Workshop on Germ Cell Assays", *Mutat. Res. Genet. Toxicol. Environ. Mutagen.*, 783: 36-54. Doi: 10.1016/j.mrgentox.2015.01.008.

Yeat and J.C. Lin. 2015. Clinical applications of next generation sequencing in cancer: from panels, to exomes, to genomes. *Front. Genet*., 6: 215. Doi: 10.3389/fgene.2015.00215.

Event: 1636: Increase, Chromosomal aberrations

Short Name: Increase, Chromosomal aberrations

AOPs Including This Key Event

AOP Name	Role of event in AOP
Oxidative DNA damage, chromosomal aberrations and mutations	AdverseOutcome
Deposition of energy leading to lung cancer	KeyEvent
Deposition of energy leading to cataracts	KeyEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stages

Life stage	Evidence
All life stages	High

Sex Applicability

Term	Evidence
Unspecific	High

Taxonomic applicability: CAs are possible in nucleated cells of any species (Ferguson-Smith, 2015).

Life stage applicability: This key event is not life stage specific as subjects of all ages have chromosomes that can be improperly structured. However, older individuals have naturally higher baseline levels of CAs (Vick et al., 2017). Individuals born with stable type aberrations will retain them throughout their lifetime (Gardner et al., 2011).

Sex applicability: This key event is not sex specific, with both sexes experiencing chromosomal breaks at comparable rates (Kašuba et al., 1995).

Evidence for perturbation by a stressor: Many studies have provided evidence to support increased CAs occurring as a result of exposure to ionizing radiation (Franken et al., 2012; Cornforth et al., 2002; Loucas et al., 2013).

Key Event Description

Structural chromosomal aberrations describe the damage to chromosomes that results from breaks along the DNA and may lead to deletion, addition, or rearrangement of sections in the chromosome. Chromosomal aberrations can be divided in two major categories: chromatid-type or chromosome-type depending on whether one or both chromatids are involved, respectively. They can be further classified as rejoined or non-rejoined aberrations. Rejoined aberrations include translocations, insertions, dicentrics and rings, while unrejoined aberrations include acentric fragments and breaks (Savage, 1976). Some of these aberrations are stable (i.e., reciprocal translocations) and can persist for many years (Tucker and Preston, 1996). Others are unstable (i.e., dicentrics, acentric fragments) and decline at each cell division because of clonogenic inactivation (Boei et al., 1996). These events may be detectable after cell division and such damage to DNA is irreversible. Chromosomal aberrations are associated with clonogenic inactivation and carcinogenicity (Mitelman, 1982).

Chromosomal aberrations (CAs) refer to a missing, extra or irregular portion of chromosomal DNA. These DNA changes in the chromosome structure may be produced by different double strand break (DSB) repair mechanisms (Obe et al., 2002).

There are 4 main types of CAs: deletions, duplications, translocations, and inversions. Deletions happen when a portion of the genetic material from a chromosome is lost. Terminal deletions occur when an end piece of the chromosome is cleaved. Interstitial deletions arise when a chromosome breaks in two separate locations and rejoins incorrectly, with the center piece being omitted. Duplications transpire when there is any addition or rearrangement of excess genetic material; types of duplications include transpositions, tandem duplications, reverse duplications, and displaced duplications (Griffiths et al., 2000). Translocations result from a section of one chromosome being transferred to a non-homologous chromosome (Bunting and Nussenzweig, 2013). When there is an exchange of segments on two non-homologous chromosomes, it is called a reciprocal translocation. Inversions occur in a single chromosome and involve both of the ends breaking and being ligated on the opposite ends, effectively inverting the DNA sequence.

A fifth type of CA that can occur in the genome is the copy number variant (CNV). CNVs, which may comprise greater than 10% of the human genome (Shlien et al., 2009; Zhang et al., 2016; Hastings et al., 2009), are deletions or duplications that can vary in size from 50 base pairs (Arlt et al., 2012; Arlt et al., 2014; Liu et al., 2013) up into the megabase pair range (Arlt et al., 2012; Wilson et al., 2015; Arlt et al., 2014; Zhang et al., 2016). CNV regions are especially enriched in large genes and large active transcription units (Wilson et al., 2015), and are of particular concern when they cause deletions in tumour suppressor genes or duplications in oncogenes (Liu et al., 2013; Curtis et al., 2012). There are two types of CNVs: recurrent and non-recurrent. Recurrent CNVs are thought to be produced through a recombination process during meiosis known as non-allelic homologous recombination (NAHR) (Arlt et al., 2012; Hastings et al., 2009). These recurrent CNVs, also called germline CNVs, could be inherited and are thus common across different individuals (Shlien et al., 2009; Liu et al., 2013). Non-recurrent CNVs are believed to be

produced in mitotic cells during the process of replication. Although the mechanism is not well studied, it has been suggested that stress during replication, in particular stalling replication forks, prompt microhomology-mediated mechanisms to overcome the replication stall, which often results in duplications or deletions. Two models that have been proposed to explain this mechanism include the Fork Stalling and Template Switching (FoSTeS) model, and the Microhomology-Mediated Break-Induced Replication (MMBIR) model (Arlt et al., 2012; Wilson et al., 2015; Lee et al., 2007; Hastings et al., 2009).

CAs can be classified according to whether the chromosome or chromatid is affected by the aberration. Chromosome-type aberrations (CSAs) include chromosome-type breaks, ring chromosomes, marker chromosomes, and dicentric chromosomes; chromatid-type aberrations (CTAs) refer to chromatid breaks and chromatid exchanges (Bonassi et al., 2008; Hagmar et al., 2004). When cells are blocked at the cytokinesis step, CAs are evident in binucleated cells as micronuclei (MN; small nucleus-like structures that contain a chromosome or a piece of a chromosome that was lost during mitosis) and nucleoplasmic bridges (NPBs; physical connections that exist between the two nuclei) (El-Zein et al., 2014). Other CAs can be assessed by examining the DNA sequence, as is the case when detecting copy number variants (CNVs) (Liu et al., 2013).

OECD defines clastogens as 'any substance that causes structural chromosomal aberrations in populations of cells or organisms'.

How it is Measured or Detected

CAs can be detected before and after cell division. Widely used assays are described in the table below, however there may be other comparable methods that are not listed.

Assay	References	Description	OECD-approved assay
Premature Chromosome Condensation (PCC)	Prasanna et al., 2000; Okayasu et al., 2019	Cells are exposed to mitosis-promoting factors (MPF) following cell fusion, causing the chromosomes to condense prematurely. In another approach, cells are exposed to protein phosphatase inhibitors, such as type 1 and 2A protein phosphatases, also causing premature chromosome condensation.	N/A
Chromosomal G-banding	Schwatz, 1990	Use of Giesma dye to stain chromosomal bands, abnormalities determined by the presence of altered morphology	N/A
Fluorescent In Situ Hybridization (FISH)	Beaton et al., 2013; Pathak et al., 2017	Fluorescent assay of metaphase chromosomes that can detect CAs through chromosome painting and microscopic analysis	N/A
Micronuclei (MN) Assay via Microscopy *in*	OECD, 2016a	Micronuclei are scored in vitro using microscopy	Yes (No. 487)

vitro			
Cytokinesis Block Micronucleus (CBMN) Assay with Microscopy in vitro	Fenech, 2000; OECD, 2016a	Cells are cultured with cytokinesis blocking agent, fixed to slides, and undergo MN quantification using microscopy.	Yes (No.487)
Micronucleus (MN) Assay by Microscopy in vivo	OECD, 2016b	Cells are fixed on slides and MN are scored using microscopy. Red blood cells can also be scored for MN using flow cytometry (see below)	Yes (No. 474)
CBMN with Imaging Flow Cytometry	Rodrigues et al., 2015	Cells are cultured with cytokinesis blocking agent, fixed in solution, and imaged with flow cytometry to quantify MN	N/A
Flow cytometry detection of MN	Dertinger et al., 2004; Bryce et al., 2007; OECD 2016a, 2016b	In vivo and in vitro flow cytometry-based, automated micronuclei measurements are also done without cytokinesis block. MN analysis in vivo is performed in peripheral blood cells to detect MN in erythrocytes and reticulocytes.	Yes (No.487; No. 474)
High-throughput biomarker assays (indirect measures to confirm clastogenicity)	Bryce et al. 2014, 2016, 2018 Khoury et al., 2013, Khoury et al., 2016) Hendriks et al., 2012, 2016; Wink et al., 2014	Multiplexed biomarkers can be measured by flow cytometry are used to discern clastogenic and aneugenic mechanisms for MN induction. Flow cytometry-based quantification of γH2AX foci and p53 protein expression (Bryce et al., 2016). Prediscreen Assay– In-Cell Western - based quantification of γH2AX Green fluorescent protein reporter assay to detect the activation of stress signaling pathways, including DNA damage signaling including a reporter porter that is associated with DNA double strand breaks.	N/A
Dicentric Chromosome Assay (DCA)	Abe et al., 2018	Cells are fixed on microscope slides, chromosomes are stained, and the number of dicentric chromosomes are quantified	N/A
High-content and high-throughput imaging	Shahane et al., 2016	DNA can be stained using fluorescent dyes and micronuclei can be scored high-throughput microscopy image analysis.	N/A
Chromosomal aberration test	OECD, 2016c; 2016d; 20l16e	In vitro, the cell cycle is arrested at metaphase after 1.5 cell cycle following 3-6 hour exposure	Yes. In vitro (No. 473)

		In vivo, the test chemical is administered as a single treatment and bone marrow is collected 18-24 hrs later (TG 475), while testis is collected 24-48 hrs later (TG 483). The cell cycle is arrested with a metaphase-arresting chemical (e.g., colchicine) 2-5 hours before cell collection. Once cells are fixed and stained on microscope slides, chromosomal aberrations are scored	In vivo (No. 475 and No. 483)
Array Comparative Genomic Hybridization (aCGH) or SNP Microarray	Adewoye et al., 2015; Wilson et al., 2015; Arlt et al., 2014; Redon et al., 2006; Keren, 2014; Mukherjee, 2017	CNVs are most commonly detected using global DNA microarray technologies; This method, however, is unable to detect balanced CAs, such as inversions	N/A
Next Generation Sequencing (NGS): Whole Genome Sequencing (WGS) or Whole Exome Sequencing (WES)	Liu, 2013; Shen, 2016; Mukherjee, 2017	CNVs are detected by fragmenting the genome and using NGS to sequence either the entire genome (WGS), or only the exome (WES); Challenges with this methodology include only being able to detect CNVs in exon-rich areas if using WES, the computational investment required for the storage and analysis of these large datasets, and the lack of computational algorithms available for effectively detecting somatic CNVs	N/A

References

Abe, Y et al. (2018), "Dose-response curves for analyzing of dicentric chromosomes and chromosome translocations following doses of 1000 mGy or less, based on irradiated peripheral blood samples from five healthy individuals", *J Radiat Res*. 59(1), 35-42. doi:10.1093/jrr/rrx052

Adewoye, A.B.et al. (2015), "The genome-wide effects of ionizing radiation on mutation induction in the mammalian germline", *Nat. Commun*. 6:66-84. doi: 10.1038/ncomms7684.

Arlt MF, Wilson TE, Glover TW. (2012), "Replication stress and mechanisms of CNV formation", *Curr Opin Genet Dev*. 22(3):204-10. doi: 10.1016/j.gde.2012.01.009.

Arlt, MF. Et al. (2014), "Copy number variants are produced in response to low-dose ionizing radiation in cultured cells", *Environ Mol Mutagen*. 55(2):103-13. doi: 10.1002/em.21840.

Beaton, L. A. et al. (2013), "Investigating chromosome damage using fluorescent in situ hybridization to identify biomarkers of radiosensitivity in prostate cancer patients", Int J Radiat Biol. 89(12): 1087-1093. doi:10.3109/09553002.2013.825060

Boei, J.J., Vermeulen, S., Natarajan, A.T. (1996), "Detection of chromosomal aberrations by fluorescence in situ hybridization in the first three postirradiation divisions of human lymphocytes", Mutat Res, 349:127-135. Doi: 10.1016/0027-5107(95)00171-9.

Bonassi, S. (2008),"Chromosomal aberration frequency in lymphocytes predicts the risk of cancer: results from a pooled cohort study of 22 358 subjects in 11 countries", *Carcinogenesis.* 29(6):1178-83. doi: 10.1093/carcin/bgn075.

Bryce SM, Bemis JC, Avlasevich SL, Dertinger SD. In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity. Mutat Res. 2007 Jun 15;630(1-2):78-91. doi: 10.1016/j.mrgentox.2007.03.002. Epub 2007 Mar 19. PMID: 17434794; PMCID: PMC1950716.

Bryce, S. et al. (2014), "Interpreting In VitroMicronucleus Positive Results: Simple Biomarker Matrix Discriminates Clastogens, Aneugens, and Misleading Positive Agents", Environ Mol Mutagen, 55:542-555. Doi:10.1002/em.21868.

Bryce, S. et al.(2016), "Genotoxic mode of action predictions from a multiplexed flow cytometric assay and a machine learning approach", Environ Mol Mutagen, 57:171-189. Doi: 10.1002/em.21996.

Bryce SM, Bernacki DT, Smith-Roe SL, Witt KL, Bemis JC, Dertinger SD. Investigating the Generalizability of the MultiFlow ® DNA Damage Assay and Several Companion Machine Learning Models With a Set of 103 Diverse Test Chemicals. Toxicol Sci. 2018 Mar 1;162(1):146-166. doi: 10.1093/toxsci/kfx235. PMID: 29106658; PMCID: PMC6059150.

Bunting, S. F., & Nussenzweig, A. (2013), "End-joining, translocations and cancer", Nature Reviews Cancer.13 (7): 443-454. doi:10.1038/nrc3537

Cornforth, M.N., S.M. Bailey, and E.H. Goodwin. (2002), "Dose Responses for Chromosome Aberrations Produced in Noncycling Primary Human Fibroblasts by Alpha Particles, and by Gamma Rays Delivered at Sublimating Low Dose Rates", Radiation Research, Vol.158, Radiation Research Society, Indianapolis, https://doi.org/10.1667/0033-7587(2002)158[0043:DRFCAP]2.0.CO;2.

Curtis, C. et al. (2012), "The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups", Nature. 486(7403):346-52. doi: 10.1038/nature10983.

Dertinger, S.D. et al.(2004), "Three-color labeling method for flow cytometric measurement of cytogenetic damage in rodent and human blood", Environ Mol Mutagen, 44:427-435. Doi: 10.1002/em.20075.

El-Zein, RA. Et al. (2014), "The cytokinesis-blocked micronucleus assay as a strong predictor of lung cancer: extension of a lung cancer risk prediction model", Cancer Epidemiol Biomarkers Prev. 23(11):2462-70. doi: 10.1158/1055-9965.EPI-14-0462.

Fenech, M. (2000), "The in vitro micronucleus technique", Mutation Research. 455(1-2), 81-95. Doi: 10.1016/s0027-5107(00)00065-8

Ferguson-Smith, M.A. (2015), "History and evolution of cytogenetics", Molecular Cytogenetics, Vol.8/19, Biomed Central, London, https://doi.org/10.1186/s13039-015-0125-8.

Franken, N.A.P. et al. (2012), "Relative biological effectiveness of high linear energy transfer alpha-particles for the induction of DNA-double-strand breaks, chromosome aberrations and reproductive cell death in SW-1573 lung tumour cells", Oncology Reports, Vol.27, Spandidos Publications, Athens, https://doi.org/10.3892/or.2011.1604.

Gardner, R.M., G.R. Sutherland, and L.G. Shaffer. (2011), "Chapter 1: Elements in Medical Cytogenetics" in Chromosome abnormalities and genetic counseling (No. 61), Oxford University Press, USA, pp.7-15.

Griffiths, A. J. F., Miller, J. H., & Suzuki, D. T. (2000), "An Introduction to Genetic Analysis", 7th edition. New York: W. H. Freeman. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21766/

Hagmar, L. et al. (2004), "Impact of types of lymphocyte chromosomal aberrations on human cancer risk: results from Nordic and Italian cohorts", Cancer Res. 64(6):2258-63.

Hastings PJ, Ira G & Lupski JR. (2009), "A microhomology-mediated break-induced replication model for the origin of human copy number variation". PLoS Genet. 2009 Jan;5(1): e1000327. doi: 10.1371/journal.pgen.1000327.

Hendriks, G. et al. (2012), "The ToxTracker assay: novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals", Toxicol Sci, 125:285-298. Doi: 10.1093/toxsci/kfr281.

Hendriks, G. et al. (2016), "The Extended ToxTracker Assay Discriminates Between Induction of DNA Damage, Oxidative Stress, and Protein Misfolding", Toxicol Sci, 150:190-203. Doi: 10.1093/toxsci/kfv323.

Kašuba, V., et al. (1995), "Chromosome aberrations in peripheral blood lymphocytes from control individuals", Mutation Research Letters, Vol.346/4, Elsevier, Amsterdam, https://doi.org/10.1016/0165-7992(95)90034-9.

Keren, B. (2014),"The advantages of SNP arrays over CGH arrays", Molecular Cytogenetics.7(1):I31. Doi: 10.1186/1755-8166-7-S1-I31.

Khoury, L., Zalko, D., Audebert, M. (2016), "Evaluation of four human cell lines with distinct biotransformation properties for genotoxic screening", Mutagenesis. 31:83-96. Doi: 10.1093/mutage/gev058.

Khoury, L., Zalko, D., Audebert, M. (2013), "Validation of high-throughput genotoxicity assay screening using cH2AX in-cell Western assay on HepG2 cells", Environ Mol Mutagen, 54:737-746. Doi: 10.1002/em.21817.

Lee JA, Carvalho CM, Lupski JR. (2007). "Replication mechanism for generating nonrecurrent rearrangements associated with genomic disorders", Cell. 131(7):1235-47. Doi: 10.1016/j.cell.2007.11.037.

Liu B. et al. (2013). "Computational methods for detecting copy number variations in cancer genome using next generation sequencing: principles and challenges", Oncotarget. 4(11):1868-81. Doi: 10.18632/oncotarget.1537.

Loucas, B.D., et al. (2013), "Chromosome Damage in Human Cells by Gamma Rays, Alpha Particles and Heavy Ions: Track Interactions in Basic Dose-Response Relationships", Radiation Research, Vol.179/1, Radiation Research Society, Indianapolis, https://doi.org/10.1667/RR3089.1.

Mitelman, F. (1982), "Application of cytogenetic methods to analysis of etiologic factors in carcinogenesis", IARC Sci Publ, 39:481-496.

Mukherjee. S. et al. (2017), "Addition of chromosomal microarray and next generation sequencing to FISH and classical cytogenetics enhances genomic profiling of myeloid malignancies, Cancer Genet. 216-217:128-141. doi: 10.1016/j.cancergen.2017.07.010.

Obe, G. et al. (2002), "Chromosomal Aberrations: formation, Identification, and Distribution", Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 504(1-2), 17-36. Doi: 10.1016/s0027-5107(02)00076-3.

Savage, J.R. (1976), "Classification and relationships of induced chromosomal structual changes", J Med Genet, 13:103-122. Doi: 10.1136/jmg.13.2.103.

Shahane SA, Nishihara K, Xia M. High-Throughput and High-Content Micronucleus Assay in CHO-K1 Cells. Methods Mol Biol. 2016;1473:77-85. doi: 10.1007/978-1-4939-6346-1_9. PMID: 27518626.

OECD (2016a), *Test No. 487: In Vitro Mammalian Cell Micronucleus Test*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264264861-en.

OECD (2016a), Test No. 487: In Vitro Mammalian Cell Micronucleus Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264264861-en.

OECD. (2016b), "Test No. 474: Mammalian Erythrocyte Micronucleus Test. OECD Guideline for the Testing of Chemicals, Section 4."Paris: OECD Publishing.

OECD. (2016c), "In Vitro Mammalian Chromosomal Aberration Test 473."

OECD. (2016d). Test No. 475: Mammalian Bone Marrow Chromosomal Aberration Test. OECD Guideline for the Testing of Chemicals, Section 4. Paris: OECD Publishing.

OECD. (2016e). Test No. 483: Mammalian Spermatogonial Chromosomal Aberration Test. Paris: OECD Publishing.

Okayasu, R. and C. Liu. (2019), "G1 premature chromosome condensation (PCC) assay", Methods in molecular biology, Humana Press, Totowa, https://doi.org/10.1007/978-1-4939-9432-8_4.

Pathak, R., Koturbash, I., & Hauer-Jensen, M. (2017), "Detection of Inter-chromosomal Stable Aberrations by Multiple Fluorescence In Situ Hybridization (mFISH) and Spectral Karyotyping (SKY) in Irradiated Mice", J Vis Exp(119). doi:10.3791/55162.

Prasanna, P. G. S., N. D. Escalada and W. F. Blakely (2000), "Induction of premature chromosome condensation by a phosphatase inhibitor and a protein kinase in unstimulated human peripheral blood lymphocytes: a simple and rapid technique to study chromosome aberrations using specific whole-chromosome DNA hybridization probes for biological dosimetry", Mutation Research, Vol. 466/2, Elsevier B.V., Amsterdam, https://doi/org/10.1016/S1383-5718(00)00011-5

Redon, R. et al. (2006), "Global variation in copy number in the human genome", Nature. 444(7118):444-54. 10.1038/nature05329.

Rodrigues, M. A., Beaton-Green, L. A., & Wilkins, R. C. (2016), "Validation of the Cytokinesis-block Micronucleus Assay Using Imaging Flow Cytometry for High Throughput Radiation Biodosimetry", Health Phys. 110(1): 29-36. doi:10.1097/HP.0000000000000371

Schwartz, G. G. (1990), "Chromosome aberrations. Biological Markers in Epidemiology (BS Hulka, TC Wlwosky, and JD Griffith, Eds.)", Oxford University Press, Oxford, pp.147-172.

Shahane S, Nishihara K, Xia M. (2016), "High-Throughput and High-Content Micronucleus Assay in CHO-K1 Cells", In: Zhu H, Xia M, editors. High-Throughput Screening Assays in Toxicology. New York, NY: Humana Press. p 77-85.

Shen.TW, (2016),"Concurrent detection of targeted copy number variants and mutations using a myeloid malignancy next generation sequencing panel allows comprehensive genetic analysis using a single testing strategy", Br J Haematol. 173(1):49-58. doi: 10.1111/bjh.13921.

Shlien A, Malkin D. (2009), "Copy number variations and cancer", Genome Med. 1(6):62. doi: 10.1186/gm62.

Tucker, J.D., Preston, R.J. (1996), "Chromosome aberrations, micronuclei, aneuploidy, sister chromatid exchanges, and cancer risk assessment", Mutat Res, 365:147-159.

Vick, E. et al. (2017), Age-related chromosomal aberrations in patients with diffuse large B-cell lymphoma, American Society of Hematology, https://doi.org/10.1182/blood.V130.Suppl_1.1571.1571

Wilson, TE. et al. (2015), "Large transcription units unify copy number variants and common fragile sites arising under replication stress", Genome Res. 25(2):189-200. doi: 10.1101/gr.177121.114.

Wink, S. et al. (2014), "Quantitative high content imaging of cellular adaptive stress response pathways in toxicity for chemical safety assessment", Chem Res Toxicol, 27:338-355.

Zhang N, Wang M, Zhang P, Huang T. 2016. Classification of cancers based on copy number variation landscapes. Biochim Biophys Acta. 1860(11 Pt B):2750-5. doi: 10.1016/j.bbagen.2016.06.003.

Appendix 2 – Key Event Relationships

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1909: Increase, Oxidative DNA damage leads to Inadequate DNA repair

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations	adjacent	High	Low
Deposition of energy leading to occurrence of cataracts	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human	Homo sapiens	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI
rat	Rattus norvegicus	Low	NCBI

Life Stage Applicability

Term	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

This KER is plausible in all life stages, sexes, and organisms with DNA. The majority of the evidence is from in vivo mice studies of all ages with no specification on sex. No in vitro evidence was found to support the relationship.

Key Event Relationship Description

Oxidative DNA lesions are present in the cell at steady state due to low levels of reactive oxygen species (ROS) and other free radicals generated by endogenous processes involving redox reactions. The most prominent examples of oxidative DNA lesions include 7, 8-dihydro-8oxo-deoxyGuanine (8-oxo-dG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPydG), and thymidine glycol (Tg). Under homeostatic conditions, cells are able to regulate the level of free radicals and readily repair oxidized

DNA bases using basal repair mechanisms to prevent irreversible damage (Swenberg et al., 2011). Oxidative DNA lesions are mainly repaired by base excision repair (BER) initiated by DNA glycosylases such as oxoguanine glycosylase 1 (OGG1), endonuclease III homologue 1 (NTH1), and Nei-like DNA glycosylases (NEIL 1/2), which detect and remove damaged bases. Abasic sites are then cleaved by endonucleases or lyases, resulting in transient single-strand breaks (SSB) that enter either short-patch or long-patch repair. Nucleotide excision repair (NER) is also involved in repairing oxidized bases to a lesser extent (Shafirovich et al., 2016). Increase in free radicals or exposure to oxidizing agents can increase the level of oxidative DNA lesions and overwhelm the repair pathways, compromising the quality of repair. If the repair mechanisms are compromised, oxidative lesions may accumulate (insufficient repair) and cause incorrect base pairing during replication or incomplete repair (indicated by accumulation of repair intermediates) (Markkanen, 2017).

Evidence Supporting this KER

Overall Weight of Evidence: Moderate

Inadequate repair of oxidative lesions is indicated by an increase in oxidative lesions above background, activation of repair enzymes, increase in repair intermediates (abasic sites and single strand breaks), and incorrect base insertion opposite lesion during replication (lesion bypass by translesion DNA synthesis).

Biological Plausibility

The mechanism of repair of oxidative DNA lesions in humans is well-established and numerous literature reviews are available on this topic (Berquist and Wilson III, 2012; Cadet and Wagner, 2013). As described above, oxidative DNA lesions are mostly repaired via BER and, to a lesser extent, NER. Previous studies have reported thresholded dose-response curves in oxidative DNA damage and attributed these observations to exceeded repair capacity at the inflection point on the curve (Gagne et al., 2012; Seager et al., 2012). In vivo, increase and accumulation of oxidative DNA lesions despite the activation of BER have been observed following chemical exposures, demonstrating insufficient repair of oxidative DNA lesions past a certain level (Ma et al., 2008).

OGG1 and NTH1, the glycosylases that initiate the BER of 8-oxo-dG and thymine glycol (Tg) lesions, respectively, are bifunctional, containing both glycosylase and lyase activities. The glycosylase removes the oxidized guanine by cleaving the glycosidic bond, giving rise to an apurinic site. The lyase then cleaves the phosphodiester bond 5’ to the AP site; a transient SSB is created for further processing in BER (Delaney et al., 2012). Abasic sites created by OGG1 and other glycosylases are also processed by apuric/apyrimidinic endonucleases (APE1) to create the 5’ nick (Allgayer et al., 2016). The repair process can be inhibited when non-DSB oxidative DNA damage results in altered nuclease or glycosylase activity, making the area resistant to repair following radiation exposure (Georgakilas et al., 2013).

Previous studies have demonstrated that an imbalance in any one of the multiple steps of BER can lead to an accumulation of repair intermediates and failed repair. Given that OGG1 is relatively slower in releasing its catalytic product than other glycosylases, it is highly likely that a disproportionate increase in oxidative DNA lesions compared to the level of available OGG1 would lead to an imbalance between lesions and the initiating step

of BER (Brenerman et al., 2014). Accumulation of oxidative lesions would be observed as a result. Moreover, studies have reported accumulation of SSB due to OGG1 and NTH1 overexpression, demonstrating that the imbalanced lyase activity generates excessive SSB intermediates (Yang et al., 2004; Yoshikawa et al., 2015; Wang et al., 2018).

Increases in oxidative lesions may produce more lesions and repair intermediates in close proximity to each other. Previous studies in mammalian cell extracts have reported reduction in repair efficiency when oxidative lesions are in tandem or opposite each other. For example, OGG1 showed reduced binding to 8-oxo-dG near an AP site incision. Furthermore, the OGG1-8-oxo-dG complex has been observed to hinder the repair of neighbouring AP site incision, delaying the completion of BER; this interaction between BER enzymes has been suggested to cause an accumulation of oxidative lesions and repair intermediates (Pearson et al., 2004; Budworth et al., 2005; Bellon et al., 2009; Yoshikawa et al., 2015; Sharma et al., 2016).

If oxidative lesions persist in the genome due to insufficient repair, incorrect base insertion opposite unrepaired oxidative DNA lesions may occur during replication. This is a well-established event. For example, 8-oxo-dG and FaPydG, the two most prominent oxidative DNA lesions, are able to form base pairs with dATP, giving rise to G:C→T:A transversions after subsequent DNA synthesis (Freudenthal et al., 2013; Gehrke et al., 2013; Markkanen, 2017). Replicative DNA polymerases such as DNA polymerase α, δ, and ε (pol α, δ, ε) have a poor ability to extend the DNA strand past 8-oxo-dG:dCTP base pairs and may cause replication to stall or incorrectly insert dATP opposite 8-oxo-dG (Hashimoto et al., 2004; Markkanen et al., 2012). In stalled replication forks, repair polymerases may be recruited to perform translesion DNA synthesis (TLS). Human Y-family DNA polymerases (Rev 1, pol κ, ι, and η) are DNA repair polymerases mainly involved in TLS in stalled replication forks. However, TLS is not free of error and its accuracy differs for each repair polymerase. For example, it is known that pol κ and η perform TLS across 8-oxo-dG and preferentially insert dATP opposite the lesion, generating G:C→T:A transversions. The error-prone nature of bypassing unrepaired oxidative lesions has been described in many previous studies and reviews (Greenberg, 2012; Maddukuri et al., 2014; Taggart et al., 2014; Shah et al., 2018). There is also risk associated with repairing the lesions, that the process could lead to increased genomic instability and mutation potential. A balance needs to be achieved between the risk posed by repair and that by residual oxidative damage (Poetsch, 2020).

Repair by OGG1 requires 8-oxo-dG:dC base pairing, thus, it is unable to repair 8-oxo-dG:dA mispairing in newly synthesized strands. The repair of 8-oxo-dG:dA base pairs post-replication is performed by MUT Y homologue, MYH, an adenine DNA glycosylase. However, the removal of dA instead of the damaged guanine may lead to futile cycles of BER because: 1) another dA is often inserted opposite the lesion, or 2) BER ligases have a poor ability of ligating the 3'end of dC opposite 8-oxo-dG (Hashimoto et al., 2004; Caglayan and Wilson, 2015). Accumulated 8-oxo-dG may be more resistant to repair post-replication due to this futile BER.

Empirical Evidence

Example in vitro studies demonstrating dose and temporal concordance, or essentiality

- Human normal hepatocytes (HL-7702) were exposed to N,N-dimethylformamide for 24 hours at increasing concentrations (C. Wang et al., 2016)

- Concentration-dependent increase in ROS was observed; the increase was statistically significant compared to control at all concentrations (6.4, 16, 40, 100 mM)
- No significant increase in 8-oxodG was observed until the highest two concentrations (40 and 100 mM) indicating insufficient repair at these concentrations
- Significant up-regulation of excision repair genes (XRCC2 and XRCC3) occurred at 6.4 and 16 mM, below the concentrations that significantly induced 8-oxodG, supporting sufficient DNA repair at these low concentrations.
- These results demonstrate that repair is sufficient at low concentrations (rapidly removing 8-oxodG) and DNA repair is only overwhelmed at higher concentrations (i.e., insufficient), where 8-oxo-dG significantly increases.

- AS52 Chinese hamster ovary cells (wild type and OGG1-overexpressing (OGG1+)) were exposed to varying doses of ultraviolet A (UVA) radiation (Dahle et al., 2008)
  - Formamidopyrimidine glycosylase (Fpg)-sensitive sites were quantified using alkaline elution after increasing repair times (0, 1, 2, 3, 4 h) following 100 kJ/m^2 UVA irradiation
  - OGG1-overexpressing AS52 cells (OGG1+): Fpg-sensitive sites reduced to 71% within half an hour and down to background levels at 4h
  - Wild type AS52 cells: at 4h, 70% of the Fpg-sensitive sites remained, indicating accumulation of oxidative lesions
  - The above results demonstrated that excess OGG1 was able to prevent the accumulation of oxidative lesions, while the amount of OGG1 in wild type was insufficient to handle the amount of lesions induced by the same magnitude of UVA irradiation.
  - Mutations in the *Gpt* gene was quantified in both wild type and OGG1+ cells by sequencing after 13-15 days following 400 kJ/m^2 UVA irradiation
    - G:C→T:A mutations in UVA-irradiated OGG1+ cells were completely eliminated (thus, repair was sufficient when repair overexpressed).
    - G:C→T:A mutation frequency in wild type cells increased from 1.8 mutants/million cells to 3.8 mutants/million cells following irradiation – indicating incorrect repair or lack of repair of accumulated 8-oxo-dG.
    - The above result also demonstrates the essentiality of 8-oxo-dG formation in the oxidative DNA damage-induced G to T transversion mutations.

- HL-60 human leukemia cells were irradiated with X-rays at a rate of 0.5 Gy/min for increasing durations (i.e., increasing doses). 8-OHdG levels were quantified by HPLC as number of 8-OHdG per 10^6 deoxyguanosine (Li et al., 2013)
  - No increase in 8-OHdG was observed up to 2 Gy (sufficient repair at low doses), above which the level of lesions increased linearly up to 20 Gy (insufficient repair)
  - This thresholded dose-response curve, indicative of overwhelmed repair processes, was also observed in mouse liver in the same study described below.

In vivo studies demonstrating dose or time concordance

- Two groups of 5-week-old C57BL/6J mice were exposed to increasing doses of X-rays at a rate of 0.5 Gy/min (200 kV, 12 mA). The livers were collected from one group immediately after exposure and urine samples were collected over 24 hours following irradiation in the second group of mice (Li et al., 2013).
    - 8-OHdG in the mouse liver DNA were quantified by HPLC and expressed as 8-OHdG per 10^6 deoxyguanosine
    - Between 0 and 0.5 Gy, no increase in lesions was observed
    - Between 0.5 and 30 Gy, a linear dose-response in 8-OHdG was observed
    - The thresholded dose-response curve was concordant in the urine samples; no increase in urinary 8-OHdG (8-OHdG/creatinine (ng/mg)) was observed between 0 and 0.1 Gy but between 0.1 and 5 Gy, the number of lesions increased linearly with dose

- Male Sprague-Dawley rats were fed 0.5 mmol aniline/kg/day for 30 days. Genomic DNA, nuclear extracts, and mitochondrial extracts were collected from spleen tissues (Ma et al., 2008).
    - 8-OHdG was quantified using enzyme-linked immunosorbent assay (ELISA) on digested genomic DNA. There was a significant 2.8-fold increase in lesions in aniline-fed rats than in control rats.
    - Both the nuclear extracts and mitochondrial extracts were tested for OGG1 activity, where 1.32-fold and 1.15-fold increase in enzyme activity (both significant; $p<0.05$) were observed in the respective extracts of aniline-treated rats.
    - The OGG1 enzyme content in the extracts was detected using Western blotting; the increase in OGG1 content in aniline-treated rats was consistent with the OGG1 activity assay.
    - Despite the increase in OGG1 enzyme content and activity, the quantity of 8-OHdG increased.

    - Together, these results demonstrate that repair is sufficient at low concentrations because 8-oxodG adducts are rapidly removed. At higher concentrations, 8-oxo-dG begins to significantly increase indicating repair is overwhelmed (i.e., insufficient).

- Two groups of C57BL/6J mice received lens-specific irradiation in vivo with 3 mJ/cm2 UVB a week apart, with one group being sacrificed 7 days after exposure and the other sacrificed immediately. Immunofluorescence was used to observe cyclobutane pyrimidine dimers (CPD) (Mesa & Bassnett, 2013).
    - Exposed lenses showed a 25% decrease in cyclobutane pyrimidine dimer levels seven days post-exposure. Oxidative damage was not measured.

Uncertainties and Inconsistencies

Although the dual functionality of OGG1 as a glycosylase and lyase has been widely accepted and demonstrated experimentally, there are studies showing that the cleavage of phosphodiester bond 5' to the lesion is mainly performed by apurinic endonuclease 1 (APE1) (Allgayer et al., 2016; R. Wang et al., 2018). In some cases, APE1 may be the main factor driving the accumulation of BER intermediates. Some studies suggest that OGG1 is involved in the repair of non-transcribed strands and is not required for transcription-coupled repair of 8-oxo-dG; Le Page et al. reported efficient repair of 8-oxo-dG in the

transcribed sequence in *Ogg1* knockout mouse cells (Le Page et al., 2000). Moreover, the repair of 8-oxo-dG is also affected by the neighbouring sequence; the position of the lesions may have a negative effect on repair efficiency (Pastoriza-Gallego et al., 2007). We note that the study by Allgayer et al. was investigating the fate and effect of 8-oxo-dG during transcription; repair mechanism may vary by situation and availability of repair enzymes at the time.

Quantitative Understanding of the Linkage

The precise relationship between levels of oxidative DNA lesions and when repair can be considered inadequate have not been fully defined; this relationship will very likely differ between cell types and tissues and, thus, difficult to define. There are computational models of repair kinetics of 8-oxo-dG.

Sokhansanj and Wilson III [2004] applied a quantitative model of BER and the literature value for the rate of formation of endogenous 8-oxo-dG to investigate the rate of clearance of BER repair intermediates (Sokhansanj and Wilson III, 2004).

- The BER model used Michaelis-Menten enzyme kinetics and included the activities of OGG1, AP lyases, polymerases, and ligases.
- The model assumed the formation rate of endogenous oxidative lesions to be 500 8-oxo-dG/day
- Based on the above, it was estimated that following a sudden spike in 8-oxo-dG up to 20,000 8-oxo-dG/cell, the total level of repair intermediates would return to baseline within 4000 seconds (less than 1 hour)
    - This model also assumed that OGG1 was available in excess
- When APE1 (AP site endonuclease) is present, glycosylase reaction kinetics of OGG1(a bifunctional glycosylase/lyase) was observed to increase
    - Suggested to be due to the coordinated action of the two enzymes
- A 10-fold reduction in OGG1 kinetics led to 10-fold increase in 8-oxo-dG, while no other repair intermediates increased.

Known modulating factors
N/A
Known Feedforward/Feedback loops influencing this KER
N/A

References

Allgayer, J., Kitsera, N., Bartelt, S., Epe, B., Khobta, A. (2016), Widespread transcriptional gene inactivation initiated by a repair intermediate of 8-oxoguanine, Nucleic Acids Res, 44:7267-7280.

Bellon, S., Shikazono, N., Cunniffe, S., Lomax, M., O'Neill, P. (2009), Processing of thymine glycol in a clustered DNA damage site: mutagenic or cytotoxic, Nucleic Acids Res, 37:4430-4440.

Berquist, B., Wilson III, D. (2012), Pathways for Repairing and Tolerating the Spectrum of Oxidative DNA Lesions, Cancer Lett, 327:61-72.

Brenerman, B., Illuzzi, J., Wilson III, D. (2014), Base excision repair capacity in informing healthspan, Carcinogenesis, 35:2643-2652.

Budworth, H., Matthewman, G., O'Neill, P., Dianov, G. (2005), Repair of Tandem Base Lesions in DNA by Human Cell Extracts Generates Persisting Single-strand Breaks, J Mol Biol, 351:1020-1029.

Cadet, J., Wagner, J.R. (2013), DNA Base Damage by Reactive Oxygen Species, Oxidizing Agents, and UV Radiation, Cold Spring Harb Perspect Biol, 5:a012559.

Caglayan, M., Wilson, S. (2015), Oxidant and environmental toxicant-induced effects compromise DNA ligation during base excision DNA repair, DNA Repair, 35:85-89.

Dahle, J., Brunborg, G., Svendsrud, D., Stokke, T., Kvam, E. (2008), Overexpression of human OGG1 in mammalian cells decreases ultraviolet A induced mutagenesis, Cancer Lett, 267:18-25.

Delaney, S., Jarem, D., Volle, C., Yennie, C. (2012), Chemical and Biological Consequences of Oxidatively Damaged Guanine in DNA, Free Radic Res, 46:420-441.

Freudenthal, B., Beard, W., Wilson, S. (2013), DNA polymerase minor groove interactions modulate mutagenic bypass of a templating 8-oxoguanine lesion., Nucleic Acids Res, 41:1848-1858.

Gagne, J., Rouleau, M., Poirier, G. (2012), PARP-1 Activation— Bringing the Pieces Together, Science, 336:678-279.

Gehrke, T., Lischke, U., Gasteiger, K., Schneider, S., Arnold, S., Muller, H., Stephenson, D., Zipse, H., Carell, T. (2013), Unexpected non-Hoogsteen–based mutagenicity mechanism of FaPy-DNA lesions, Nat Chem Biol, 9:455-461.

Greenberg, M. (2012), Purine Lesions Formed in Competition With 8-Oxopurines From Oxidative Stress, Acc Chem Res, 45:588-597.

Georgakilas, A., P. O'Neill, and R. Stewart. (2013), "Induction and repair of clustered DNA lesions: What do we know so far?", Radiation Research, Vol.180/1, Radiation Research Society, Indianapolis, https://doi.org/10.1667/RR3041.1.

Hashimoto, K., Tominaga, Y., Nakabeppu, Y., Moriya, M. (2004), Futile short-patch DNA base excision repair of adenine:8-oxoguanine mispair, Nucleic Acids Res, 32:5928-5934.

Kozbenko, T. et al. (2022), "Deploying elements of scoping review methods for adverse outcome pathway development: a space travel case example", International Journal of Radiation Biology, 1–12. https://doi.org/10.1080/09553002.2022.2110306

Le Page, F., Klunglund, A., Barnes, D., Sarasin, A., Boiteux, S. (2000), Transcription coupled repair of 8-oxoguanine in murine cells: The Ogg1 protein is required for repair in nontranscribed sequences but not in transcribed sequences, Proc Natl Acad Sci USA, 97:8397-8402.

Li, Y., Song, M., Kasai, H., Kawai, K. (2013), Generation and threshold level of 8-OHdG as oxidative DNA damage elicited by low dose ionizing radiation, Genes Environ, 35:88-92.

Ma, H., Wang, J., Abdel-Rahman, S., Boor, P., Firoze, M. (2008), Oxidative DNA damage and its repair in rat spleen following subchronic exposure to aniline, Toxicol Appl Pharmacol, 233:247-253.

Maddukuri, L., Ketkar, A., Eddy, S., Zafar, M., Eoff, R. (2014), The Werner syndrome protein limits the error-prone 8-oxo-dG lesion bypass activity of human DNA polymerase kappa, Nucleic Acids Res, 42:12027-12040.

Markkanen, E. (2017), Not breathing is not an option: How to deal with oxidative DNA damage, DNA Repair, 59:82-105.

Markkanen, E., Castrec, B., Vilani, G., Hubscher, U. (2012), A switch between DNA polymerases δ and λ promotes error-free bypass of 8-oxo-G lesions, Proc Natl Acad Sci USA, 27:931-940.

Mesa, R. and S. Bassnett. (2013), "UV-B-induced DNA damage and repair in the mouse lens", Investigative Ophthalmology and Visual Science, Vol.54/10, Association for Research in Vision and Ophthalmology, Rockville, https://doi.org/10.1167/iovs.13-12644.

Pastoriza-Gallego, M., Armier, J., Sarasin, A. (2007), Transcription through 8-oxoguanine in DNA repair-proficient and Csb−/Ogg1− DNA repair-deficient mouse embryonic fibroblasts is dependent upon promoter strength and sequence context, Mutagenesis, 22:343-351.

Pearson, C., Shikazono, N., Thacker, J., O'Neill, P. (2004), Enhanced mutagenic potential of 8-oxo-7,8-dihydroguanine when present within a clustered DNA damage site, Nucleic Acids Res, 32:263-270.

Poetsch, A. (2020), "The genomics of oxidative DNA damage, repair, and resulting mutagenesis", Computational and Structural Biotechnology Journal, Vol.18, Elsevier, Amsterdam, https://doi.org/10.1016/j.csbj.2019.12.013.

Seager, A., Shah, U., Mikhail, J., Nelson, B., Marquis, B., Doak, S., Johnson, G., Griffiths, S., Carmichael, P., Scott, S., Scott, A., Jenkins, G. (2012), Pro-oxidant Induced DNA Damage in Human Lymphoblastoid Cells: Homeostatic Mechanisms of Genotoxic Tolerance, Toxicol Sci, 128:387-397.

Shafirovich, V., Kropachev, K., Anderson, T., Li, Z., Kolbanovskiy, M., Martin, B., Sugden, K., Shim, Y., Min, J., Ceacintov, N. (2016), Base and Nucleotide Excision Repair of Oxidatively Generated Guanine Lesions in DNA, J Biol Chem, 291:5309-5319.

Shah, A., Gray, K., Figg, N., Finigan, A., Starks, L., Bennett, M. (2018), . Defective Base Excision Repair of Oxidative DNA Damage in Vascular Smooth Muscle Cells Promotes Atherosclerosis, Circulation, 138:1446-1462.

Sharma, V., Collins, L., Chen, T., Herr, N., Takeda, S., Sun, W., Swenberg, J., Nakamura, J. (2016), Oxidative stress at low levels can induce clustered DNA lesions leading to NHEJ mediated mutations, Oncotarget, 7:25377-25390.

Sokhansanj, B., Wilson III, D. (2004), Oxidative DNA damage background estimated by a system model of base excision repair, Free Rad Biol Med, 37:433-427.

Swenberg, J., Lu, K., Moeller, B., Gao, L., Upton, P., Nakamura, J., Starr, T. (2011), Endogenous versus Exogenous DNA Adducts: Their Role in Carcinogenesis, Epidemiology, and Risk Assessment, Toxicol Sci, 120:S130-S145.

Taggart, D., Fredrickson, S., Gadkari, V., Suo, Z. (2014), Mutagenic Potential of 8-Oxo-7,8-dihydro-2′-deoxyguanosine Bypass Catalyzed by Human Y-Family DNA Polymerases, Chem Res Toxicol, 27:931-940.

Wang, C., Yang, J., Lu, D., Fan, Y., Zhao, M., Li, Z. (2016), Oxidative stress-related DNA damage and homologous recombination repairing induced by N,N-dimethylformamide , J Appl Toxicol, 36:936-945.

Wang, R., Li, C., Qiao, P., Xue, Y., Zheng, X., Chen, H., Zeng, X., Liu, W., Boldogh, I., Ba, X. (2018), OGG1-initiated base excision repair exacerbates oxidative stress-induced parthanatos, Cell Death and Disease, 9:628.

Yang, N., Galick, H., Wallace, S. (2004), Attempted base excision repair of ionizing radiation damage in human lymphoblastoid cells produces lethal and mutagenic double strand breaks, DNA Repair, 3:1323-1334.

Yoshikawa, Y., Yamasaki, A., Takatori., K., Suzuki, M., Kobayashi, J., Takao, M., Zhang-Akiyama, Q. (2015), Excess processing of oxidative damaged bases causes hypersensitivity to oxidative stress and low dose rate irradiation, Free Radic Res, 49:1239-1248.

Relationship: 1910: Inadequate DNA repair leads to Increase, DNA strand breaks

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations	adjacent	High	Low
Alkylation of DNA leading to reduced sperm count	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human	Homo sapiens		NCBI
mouse	Mus musculus		NCBI
rat	Rattus norvegicus		NCBI

Life Stage Applicability

Term	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

This KER applies to any cell type that has DNA repair capabilities.

Key Event Relationship Description

Inadequate repair of DNA damage includes incorrect repair (i.e., incorrect base insertion), incomplete repair (i.e., accumulation of repair intermediates such as strand breaks, stalled replications forks, and/or abasic sites), and absent repair resulting in the retention of DNA damage.

It is well-established that DNA excision repair pathways require DNA strand breakage for removing the damaged sites; for example, base excision repair (BER) of oxidative lesions involves removal of oxidized bases by glycosylases followed by cleavage of the DNA strand 5' from the abasic site. If the repair process is disrupted at this point, repair intermediates including single strand breaks (SSB) may persist in the DNA. A SSB can turn into a double strand break (DSB) if it occurs sufficiently close to another SSB on the opposite strand. SSBs can be converted into DSBs when helicase unwinds the DNA strands during replication. Furthermore, SSBs and abasic sites can act as replication blocks causing the replication fork to stall and collapse, giving rise to DSBs (Minko et al., 2016; Whitaker et al., 2017).

The two most common DSB repair mechanisms are non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is may favoured over HR and has also been shown to be 10^4 times more efficient than HR in repairing DSBs (Godwin et al., 1994; Benjamin and Little, 1992). There are two subtypes of NHEJ: canonical NHEJ (C-NHEJ) or alternative non-homologous end joining (alt-NHEJ). During C-NHEJ, broken ends of DNA are simply ligated together. In alt-NHEJ, one strand of the DNA on either side of the break is resected to repair the lesion (Betermeir et al., 2014). Although both repair mechanisms are error-prone (Thurtle-Schmidt and Lo, 2018), alt-NHEJ is considered more error-prone than C-NHEJ (Guirouil-Barbat et al., 2007; Simsek and Jasin, 2010). While NHEJ may prevent cell death due to the cytotoxicity of DSBs, it may lead to mutations and genomic instability downstream.

Evidence Supporting this KER

Biological Plausibility

1. DNA strand breaks generated due to faulty attempted repair

Excision repair pathways require the induction of SSB as part of damage processing. Increases in DNA lesions may lead to the accumulation of intermediate SSB. Attempted excision repair of lesions on opposite strands can turn into DSBs if the two are in close proximity (Eccles et al., 2010). Generation of DSBs has been observed in both nucleotide excision repair (NER) and BER (Ma et al., 2009; Wakasugi et al., 2014).

Previous studies have demonstrated that an imbalance in one of the multiple steps of BER can lead to an accumulation of repair intermediates and failed repair. It is highly likely that a disproportionate increase in oxidative DNA lesions compared to the level of available BER glycosylases leads to an imbalance between lesions and the initiating step of BER (Brenerman et al., 2014). Accumulation of oxidative lesions, abasic sites, and SSBs generated from OGG1, NTH1, and APE1 activities would be observed as a result. Moreover, studies have reported accumulation of SSB due to OGG1- and NHT1-overexpression (Yang et al., 2004; Yoshikawa et al., 2015; Wang et al., 2018). BER repair intermediates have been observed to interfere with transcription as well (Kitsera et al., 2011). While overexpression may lead to imbalanced lyase activities that generate excessive SSB intermediates, deficiency of these enzymes is also known to cause an accumulation of oxidative lesions that could lead to strand breaks downstream. Hence, both the overexpression and deficiencies of repair enzymes can lead to strand breaks due to excessive activity or inadequate repair, respectively.

2. DNA strand breaks generated due to replication stress caused by accumulated DNA lesions

Retention of DNA lesions (i.e., damaged bases and SSB) can interfere with the progression of the replication fork. Thymidine glycol is an example of an oxidative DNA lesion that acts as a replication block (Dolinnaya et al., 2013). Persistent replication fork stalling and dissociation of replication machinery are known to cause the replication fork to collapse, which generates highly toxic DSBs (Zeman and Cimprich, 2014; Alexander and Orr-Weaver, 2016). Fork stalling also increases the risk of two replication forks colliding with each other, generating DSBs.

In addition, the replication fork can collide with SSBs generated during BER, hindering the completion of repair and giving rise to DSBs (Ensminger et al., 2014).

Empirical Evidence

In vitro studies with empirical evidence are shown below for select DNA repair pathways. These studies build in elements of essentiality (modulation of DNA repair), as well as dose and incidence concordance. The primary evidence is essentiality, where repair is genetically modulated in some way. Because multiple lines of evidence are considered within individual studies, we present the data by source of evidence (in vitro versus in vivo) rather than by type of empirical evidence (dose, incidence, or temporal concordance; essentiality) to avoid repetitive use of the same studies.

Inadequate repair of oxidative lesions

- Concentration concordance of strand breaks in repair-deficient and –proficient cells (insufficient repair) (Wu et al., 2008)
    - In a study using A549 human adenocarcinoma cells, DNA strand breaks in hOGG1-proficient and hOGG1-deficient cells were compared following exposure to increasing concentrations of bleomycin.
    - Strand breaks were measured as DNA migration length in alkaline comet assay after 3 hours of exposure to six increasing concentrations (0.05, 0.25, 0.5, 1, 5, and 10 mg/L).
    - Concentration-dependent increase in strand breaks was observed in both cell types; however, at all concentrations significantly more strand breaks ($p<0.05$) were present in the hOGG1-deficient cells than in the proficient cells, demonstrating insufficient repair of oxidative lesions leading to DNA strand breaks.
    - Thus, this evidence supports the essentiality of inadequate DNA repair as a modulator of the downstream KE.
- Incomplete OGG1-initiated base excision repair (BER) leads to DNA strand breaks (Wang et al., 2018):
    - In a study using mouse embryonic fibroblasts (MEF), Ogg1+/+ and Ogg1-/- cells were treated with increasing concentrations of H2O2 for varying durations
      Higher levels of 8-oxodG were detected in Ogg1-/- cells compared to Ogg1+/+ cells after treatment with 400 µM H2O2 at all time points (5, 15, 30, 60, and 90 min)
        - Demonstrates insufficient removal of 8-oxo-dG in OGG1-deficient cells
    - Significantly more strand breaks, as indicated by the higher % of TUNEL-positive cells ($p<0.001$), were detected in Ogg1+/+ cells compared to Ogg1-/- cells after exposure to 400 µM H2O2 for 3 hours
        - Both cell types showed a very similar increase in DNA strand breaks at lower concentrations (50, 100, and 200 µM) and there was no significant difference between Ogg1+/+ and Ogg1-/- cells at these concentrations – this suggests that up to a certain level of oxidative damage, OGG1-initiated BER does not exacerbate strand breaks but when oxidative stress is excessive (at 400µM in this study), OGG1-initiated BER is compromised and leads to increased strand breaks (incomplete repair)

- Finally, DNA strand breaks in both cell types were measured using both alkaline and neutral comet assay after a 30- minute exposure to 400µM H2O2; while there was an increase in the olive tail moment (indicating DNA strand breaks) in both cell types compared to the control, the increase of strand breaks in Ogg1+/+ cells was significantly larger than in Ogg1-/- cells in both assays ($p<0.001$)

Inadequate repair of alkylated DNA

- Interference of N-methylpurine DNA glycosylase (MPG)-initiated BER by replication leading to strand breaks (Ensminger et al., 2014)
  - A549 human alveolar basal epithelial cells were exposed to increasing concentrations of methylmethane sulfonate (MMS) for 1 hour and replicating cells were labeled using a thymidine analogue, 5-ethynyl-2'-desoxyuridine (EdU).
  - In S-phase cells, MMS concentration-dependent increase in γH2AX foci was detected (70 foci/cell at the highest concentration). In contrast, γH2AX foci were not detected in G1- and G2-phase cells until the highest concentration (15 foci/cell).
  - MPG-depleted cells in S-phase showed no significant increase in γH2AX foci, while the control cells showed significant MMS concentration-dependent increases.
  - These results suggest interference of MPG-initiated BER by replication, leading to DSBs, and that the depletion of MPG decreases the probability of strand breaks in S-phase (evidence of essentiality of 'inadequate repair' to KEdown).

Inadequate mismatch repair

- Incomplete/incorrect mismatch repair (MMR) leads to DNA strand breaks (Peterson-Roth et al., 2005):
  - MLH1 (MMR protein)-deficient and -proficient HCT116 human colon cancer cells were treated with 30µM K_2CrO_4 (DNA crosslinking, Cr adducts, protein-DNA crosslinking, DNA oxidation) for 3, 6, and 12 hours and γH2AX foci (biomarker of DNA DSB) were scored by fluorescence microscopy
  - At 6 and 12 hours, MLH1+ cells had higher percentage of γH2AX foci than MLH1- cells
  - The futile repair model of MMR suggests that strand breaks arise from MMR attempting repeatedly to repair the newly synthesized strand opposite adducts in S and G2 phases; approximately 80% of the γH2AX-positive MLH1+ cells were in G2 phase 12 hours after a 3-hour exposure to 20 µM Cr(VI), while the level was five times lower in MLH1- cells, suggesting that the MMR-induced DSB occurred following DNA synthesis; this supports the futile repair model and demonstrates inadequate repair

Inadequate Repair of DSBs

- Rydberg et al. [2005] exposed GM38 primary human dermal fibroblasts to increasing doses of linear electron transfer (LET) radiation of helium and iron ions (Rydberg et al., 2005).
  - The cells were allowed to recover for 16 hours following irradiation.

  - Unrepaired DSBs were measured after recovery using PFGE.
  - There was a dose-dependent increase in unrepaired DSBs due to both ion exposures.
  - Increase in persistent unrepaired DSBs with increasing dosage indicates exceeded repair capacity.

- DSB repair was also monitored by measuring γH2AX foci 0.05 - 24 hours after irradiation.
  - DSBs decreased over time and less than 1 foci per cell on average remained in MRC-5 cells 24hours after 0.02, 0.2 and 2 Gy exposures.
  - Repair was slower in 180BR cells, particularly for the 2 Gy exposure, where 20 foci per cell remained after 24 h.
  - A follow-up study by the same group, found similar results for MRC-5 and 180BR cells exposed to 0.02 and 0.2 Gy of X-rays (Kühne et al., 2004).

- Rothkamm and Löbrich (2003) exposed MRC-5 primary human lung fibroblasts (repair-proficient) and 180BR DNA ligase IV-deficient human fibroblasts to 10 and 80 Gy of X-rays (Rothkamm and Lobrich, 2003).
  - DNA ligase IV deficiency results in impaired NHEJ
  - DSB repair was monitored using PFGE by measuring the % of DSBs remaining after 0.25, 2, and 24 h following irradiation.
  - DSBs decreased over time and, eventually, less than 10% of the DSBs remained in MRC-5 cells after 24h following both 80 and 10 Gy exposures.
  - Repair was noticeably slower in 180BR cells, where the clearance of DSBs was hindered and approximately 40 and 20% of the DSBs remained at 24 hours following 80 and 10 Gy exposures, respectively.
  - The above demonstrates defective DNA repair leading to persistent DSBs.

Uncertainties and Inconsistencies

- A variety of confounding factors and genetic characteristics (i.e., SNPs) may modulate which repair pathways are invoked and the degree to which they are inadequate. These have yet to be fully defined.
- Both protective and damaging effects of OGG1 against strand breaks have been described in the literature. As demonstrated in the section above, the effect of OGG1-deficiency (BER-initiating enzyme) is observed to be different in different cell types; Wang et al. (2018) demonstrated strand breaks exacerbated by excessive OGG1 activity, while Wu et al. (2008) and Shah et al. (2018) demonstrated increased strand breaks due to lack of repair in mammalian cells in culture (Shah et al., 2018; Wu et al., 2008; Wang et al., 2018). Cell cycle and replication may influence the effect of DNA repair on exacerbating strand breaks.
- Dahle et al. (2008) exposed wild type and OGG1-overexpressing Chinese hamster ovary cells, AS52, to UVA. While OGG1-overexpression prevented the accumulation of Fpg-sensitive lesions (e.g., 8-oxo-dG and FaPyG) that were observed in wild type cells 4 hours after irradiation, there was no difference in the amount of strand breaks in the two cell types at 4h (Dahle et al., 2008).
- A recent study suggests that the NHEJ may be more accurate than previously thought (reviewed in Betermier et al., 2014). The accuracy of NHEJ may be dependent on the structure of the termini. The termini processing rather than the NHEJ itself is thus argued to be error-prone process (Betemier et al., 2014).

References

Alexander, J., Orr-Weaver, T. (2016), Replication fork instability and the consequences of fork collisions from rereplication, Genes Dev, 30:2241-2252.

Brenerman, B., Illuzzi, J., Wilson III, D. (2014), Base excision repair capacity in informing healthspan, Carcinogenesis, 35:2643-2652.

Dahle, J., Brunborg, G., Svendsrud, D., Stokke, T., Kvam, E. (2008), Overexpression of human OGG1 in mammalian cells decreases ultraviolet A induced mutagenesis, Cancer Lett, 267:18-25.

Dolinnaya, N., Kubareva, E., Romanova, E., Trikin, R., Oretskaya, T. (2013), Thymidine glycol: the effect on DNA molecular structure and enzymatic processing, Biochimie, 95:134-147.

Eccles, L., Lomax, M., O'Neill, P. (2010), Hierarchy of lesion processing governs the repair, double-strand break formation and mutability of three-lesion clustered DNA damage, Nucleic Acids Res, 38:1123-1134.

Ensminger, M., Iloff, L., Ebel, C., Nikolova, T., Kaina, B., Lobrich, M. (2014), DNA breaks and chromosomal aberrations arise when replication meets base excision repair, J Cell Biol, 206:29.

Kitsera, N., Stathis, D., Luhnsdorf, B., Muller, H., Carell, T., Epe, B., Khobta, A. (2011), 8-Oxo-7,8-dihydroguanine in DNA does not constitute a barrier to transcription, but is converted into transcription-blocking damage by OGG1, Nucleic Acids Res, 38:5926-5934.

Kühne, M., E. Riballo, N. Rief, K. Rothkamm, P. Jeggo, & M. Löbrich (2004), "A Double-Strand Break Repair Defect in ATM-Deficient Cells Contributes to Radiosensitivity", Cancer Res, 64(2): 500-508.

Ma, W., Panduri, V., Sterling, J., Van Houten, B., Gordenin, D., Resnick, M. (2009), The Transition of Closely Opposed Lesions to Double-Strand Breaks during Long-Patch Base Excision Repair Is Prevented by the Coordinated Action of DNA Polymerase and Rad27/Fen1 , Mol Cell Biol, 29:1212-1221.

Minko, I., Jacobs, A., de Leon, A., Gruppi, F., Donley, N., Harris, T., Rizzo, C., McCullough, A., Lloyd, R.S. (2016), Catalysts of DNA Strand Cleavage at Apurinic/Apyrimidinic Sites, Sci Rep, 6.

Peterson-Roth, E., Reynolds, M., Quievryn, G., Zhitkovich, A. (2005), Mismatch Repair Proteins Are Activators of Toxic Responses to Chromium-DNA Damage, Mol Cell Biol, 25:3596-3607.

Rothkamm, K., Lobrich, M. (2003), Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses, Proc Natl Acad Sci USA, 100:5057-5062.

Rydberg, B., Cooper, B., Cooper, P., Holley, W., Chatterjee, A. (2005), Dose-Dependent Misrejoining of Radiation-Induced DNA Double-Strand Breaks in Human Fibroblasts: Experimental and Theoretical Study for High- and Low-LET Radiation, Radiat Res, 163:526-534.

Shah, A., Gray, K., Figg, N., Finigan, A., Starks, L., Bennett, M. (2018), . Defective Base Excision Repair of Oxidative DNA Damage in Vascular Smooth Muscle Cells Promotes Atherosclerosis, Circulation, 138:1446-1462.

Wakasugi, M., Sasaki, T., Matsumoto, M., Nagaoka, M., Inoue, K., Inobe, M., Horibata, K., Tanaka, K., Matsunaga, T. (2014), Nucleotide Excision Repair-dependent DNA

Double-strand Break Formation and ATM Signaling Activation in Mammalian Quiescent Cells, J Biol Chem, 289:28730-28737.

Wang, R., Li, C., Qiao, P., Xue, Y., Zheng, X., Chen, H., Zeng, X., Liu, W., Boldogh, I., Ba, X. (2018), OGG1-initiated base excision repair exacerbates oxidative stress-induced parthanatos, Cell Death and Disease, 9:628.

Whitaker, A., Schaich, M., Smith, M.S., Flynn, T., Freudenthal, B. (2017), Base excision repair of oxidative DNA damage: from mechanism to disease, Front Biosci, 22:1493-1522.

Wu, M., Zhang, Z., Che, W. (2008), Suppression of a DNA base excision repair gene, hOGG1, increases bleomycin sensitivity of human lung cancer cell line, Toxicol App Pharmacol, 228:395-402.

Yang, N., Galick, H., Wallace, S. (2004), Attempted base excision repair of ionizing radiation damage in human lymphoblastoid cells produces lethal and mutagenic double strand breaks, DNA Repair, 3:1323-1334.

Yoshikawa, Y., Yamasaki, A., Takatori., K., Suzuki, M., Kobayashi, J., Takao, M., Zhang-Akiyama, Q. (2015), Excess processing of oxidative damaged bases causes hypersensitivity to oxidative stress and low dose rate irradiation, Free Radic Res, 49:1239-1248.

Zeman, M., Cimprich, K. (2014), Causes and Consequences of Replication Stress, Nat Cell Biol, 12:2-9.

Relationship: 1911: Increase, DNA strand breaks leads to Inadequate DNA repair

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations	adjacent	High	Low
Deposition of energy leading to lung cancer	adjacent	Moderate	Moderate
Deposition of energy leading to occurrence of cataracts	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Term	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

This KER is plausible in all life stages, sexes, and organisms with DNA. The majority of the evidence is from in vivo adult mice with no specification on sex, and in vitro human models that do not specify sex.

Key Event Relationship Description

The maintenance of DNA integrity is essential for genomic stability; for this reason cells have multiple response mechanisms that enable the repair of damaged DNA. Thus when DNA double strand breaks (DSBs) occur, the most detrimental type of lesion, the cell will initiate repair machinery. These mechanisms are not foolproof, and emerging evidence suggests that closely spaced lesions can compromise the repair machinery. The two most common DSB repair mechanisms are non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is initiated in G1 and early S phases of the cell cycle (Lieber et al., 2003) and is preferentially used to repair DSB damage (Godwint et al., 1994), as it is rapid and more efficient than HR (Lliakis, 1991; Jeggo, 1998; Mao et al., 2008). In higher-order eukaryotes such as humans, NHEJ is the favoured DNA repair mechanism because of the large non-coding regions within the genome. NHEJ can occur through one of two subtypes: canonical NHEJ (C-NHEJ) or alternative non-homologous end joining (alt-NHEJ). C-NHEJ, as the name suggests, simply ligates the broken ends back together. In contrast, alt-NHEJ occurs when one strand of the DNA on either side of the

break is resected to repair the lesion (Bétermier et al., 2014). Both repair mechanisms are error-prone, meaning insertions and deletions are sometimes formed due to the DSBs being repaired imperfectly (Thurtle-Schmidt and Lo, 2018). However, alt-NHEJ is considered more error-prone than C-NHEJ, as studies have shown that it more often leads to chromosomal aberrations (Zhu et al., 2002; Guirouilh-Barbat et al., 2007; Simsek & Jasin, 2010). HR is mostly operative during S and G2 phases because of the presence of the sister chromatid that can be used as template for repair (Van Gent et al 2001). Because of the reliance on the undamaged sister chromatid to repair the DSB, HR is less error-prone than NEHJ. Nevertheless, defects in HR are known to contribute to genomic instability and the formation of chromosomal aberrations (Deans et al 2000)

There is extensive evidence that DNA repair capacity can be overwhelmed or saturated in the presence of high numbers of strand breaks. This is demonstrated by decades of studies showing dose-related increases in chromosomal exchanges, chromosomal breaks and micronuclei following exposure to double-strand break inducers. Inadequate repair not only refers to overwhelming of DNA repair machinery, but also the use of repair mechanisms that are error-prone (i.e., misrepair is considered inadequate repair).

Evidence Supporting this KER

Biological Plausibility

The biological rationale linking increased DNA DSB formation with inadequate DSB repair is supported strongly by literature. This is evident from the number of review articles that have been published on the subject. Of particular relevance is a recent review that focuses particularly on DSBs induced by ionizing radiation and extensively details the processes involved in repairing DSBs, including discussions of entire pathways and individual proteins involved in DNA repair (Thompson, 2012). Multiple other shorter reviews are also available on the subject, which cover such topics as: the mechanisms of DSB formation and repair, how to quantify these two events, and the biological consequences of unrepaired or misrepaired DNA damage (van Gent et al., 2001; Khanna & Jackson, 2001; Vignard et al., 2013; Moore et al., 2014; Rothkamm et al., 2015; Chang et al., 2017; Lobrich and Jeggo, 2017; Sage and Shikazono, 2017). A brief overview of the biological plausibility of this KER is given below; for more detail, please consult the above-cited reviews.

When confronted with DSBs, there are two common repair pathways employed by the cell: homologous recombination (HR) and non-homologous end-joining (NHEJ). In HR, a homologous sequence on a sister chromatid is used as a template, ensuring that no sequence information is lost over the course of repair (e.g., Ferguson & Alt, 2001; van Gent et al., 2001; Jeggo & Markus, 2015; Schipler & Iliakis, 2013). Due to being inherently error-prone, NHEJ is commonly used in repairing DSBs in multicellular eukaryotic organisms, especially in humans (Feldmann et al., 2000). Due to being inherently error-prone, this repair process is used to generate genetic variation within antigen receptor axons through VDJ recombination, a process that leads to the careful breakage and repair of DNA (Murakami & Keeney, 2008; Malu et al., 2012). Genetic variation is also often generated during the repair of highly toxic DSB lesions. Repair to these DSB sites normally triggers cell cycle delay. NHEJ is most active in the following order of the cell cycle: G1 > S > G2/M (Mao et al., 2008). Since most somatic mammalian cells are in the G1 pre-replicative phase, DSBs also usually appear in this phase and thus are often repaired using the error-prone NHEJ (Jeggo et al., 1995).

The two broken ends of DNA DSBs are bridged by overlapping single-strand microhomology termini (Anderson, 1993; Getts & Stamato, 1994; Rathmell & Chu, 1994; Jeggo et al., 1995; Miller et al., 1995; Kirchgessner et al., 1995). The microhomology termini are ligated only when complementary base pairs are overlapped and, depending on where this match is found on the termini, it can lead to deletions and other rearrangements. With increasing DSBs, the probability of insufficient or incorrect repair of these breaks increases proportionately. It has been suggested that clustered DNA damage is less easily repairable than any other form of DNA damage (United Nations, 2000; Stenerlöw et al., 2000). With multiple lesions in close proximity within a damaged cluster, the probability of misrepair is high. This leads to an increased number of misrepaired termini (Goodhead et al., 1994; Goodhead, 1980; Tsao, 2007; Blakely, 2012), as the presence of multiple damage sites interferes with the ability of the repair enzymes to recognize and bind to the DNA accurately (Harrison et al., 1999; Tsao, 2007).

Empirical Evidence

Empirical data obtained for this KER strongly supports the idea that an increase in DNA DSBs will increase the frequency of inadequate DSB repair. The evidence presented below is summarized in table 4, here (click link). Much of the evidence comes from work with radiation stressors, which directly cause DNA DSBs in the genome (Pinto & Prise, 2005; Dong et al., 2017) in a dose-dependent fashion (Aufderheide, 1987; Frankenburg-Schwager et al., 1994; Rydberg et al., 1994; Durante et al., 1998; Dikomey & Brammer, 2000; Kuhne et al., 2000; Lobrich et al., 2000; Baumstark-Khan et al., 2003; Rothkamm & Lobrich, 2003; Kuhne et al., 2005; Asaithamby & Chen, 2009; Bracalente et al., 2013). This is a very data-rich area and it is not possible to summarize all of the evidence. However, some examples of key studies are provided below. We also direct the reader to the key event relationships 1939 (DNA strand breaks leading to chromosomal aberrations) and 1931 (DNA strand breaks leading to mutations).

The formation of DSBs by ionizing radiation, the repair process, the various methods used to analyze this repair process, and the biological consequences of unrepaired or misrepaired DNA damage are reviewed in Sage & Shikazono (2017).

Dose and Incidence Concordance

There is evidence in the literature suggesting a dose/incidence concordance between the occurrence of DSBs and the incidence of inadequate DNA repair upon exposure to radiation. Inadequate DNA repair appears to occur at the same radiation dose as DSBs. Visually, immunofluorescence has demonstrated a colocalization of DNA repair proteins with DSB foci in response to a radiation stressor (Paull et al., 2000; Asaithamby & Chen, 2009; Dong et al., 2017). In studies examining cellular responses to increasing doses of radiation, which is known to evoke a dose-dependent increase in DNA DSBs (Aufderheide, 1987; Durante et al., 1998; Dikomey & Brammer, 2000; Kuhne et al., 2000; Löbrich et al., 2000; Rothkamm & Lobrich, 2003; Kuhne et al., 2005; Asaithamby & Chen, 2009; Bracalente et al., 2013), there were resulting dose-dependent increases in non-repaired DSBs (Aufderheide, 1987; Rydberg et al., 1994; Dikomey & Brammer, 2000; Baumstark-Khan et al., 2003), DSB misrepair rates (Mcmahon et al., 2016), and misrejoined DSBs (Durante et al., 1998; Kuhne et al., 2000; Kuhne et al., 2005; Rydberg et al., 2005), as well as a dose-dependent decrease in the total DSB rejoining (Löbrich et al., 2000). Furthermore, only 50% of the rejoined DSBs were found to be correctly repaired (Kuhne et al., 2000; Löbrich et al., 2000); 24 hours after being irradiated with an 80 Gy dose of alpha particles, this frequency of misrejoining increased to and remained constant at 80% (Kuhne et al.,

2000). Furthermore, delivering radiation doses in fractionated increments also showed a dose-dependent change in the percentage of misrejoinings, such that larger fractionated doses (for example, 2 x 40 Gy) had a higher rate of DSB misrejoining than smaller fractionated doses (for example, 4 x 10 Gy) (Kuhne et al., 2000).

Temporal Concordance

There is evidence suggesting a time concordance between DSBs and DNA repair. DSBs and DNA repair have both been observed within minutes to hours of radiation exposure (Paull et al., 2000; Rothkamm & Lobrich, 2003; Pinto & Prise, 2005; Asaithamby & Chen, 2009).

Essentiality

There is evidence from inhibition studies and knock-out/knock down studies suggesting that there is a strong relationship between DSBs and DNA repair. When an inhibitor of a DNA repair protein was added to cells prior to exposure to a radiation stressor, DNA repair foci were not formed post-irradiation (Paull et al., 2000), and there were significant increases in DSBs at 6 hours and 12 hours after the radiation treatment (Dong et al., 2017). Similarly, there have been several knock-out/knock-down studies in which cells lacking a DNA repair protein have been exposed to a radiation stressor. As a result, DSBs were found to persist in these cells longer than in the wild-type cells (Coquerelle et al., 1987; Rothkamm and Lo, 2003; Bracalente et al., 2013; Mcmahon et al., 2016; Dong et al., 2017), and there was an increase in incorrectly rejoined DSBs (Löbrich et al., 2000). In one striking example, a human cell line lacking DNA ligase IV had DSBs that were still present approximately 240 - 340 hours post-irradiation (Mcmahon et al., 2016). Interestingly, there were also increased levels of DSBs in these cells prior to being exposed to a radiation stressor (Paull et al., 2000). Similarly, a study examining DSB repair kinetics after irradiation found that DSBs persisted for a longer time period in two repair-deficient mouse strains relative to a repair-proficient mouse strain; this pattern was found in lymphocytes, as well as tissues from the brains, lungs, hearts and intestines of these mice (Rube et al., 2008). The roles of various DNA repair proteins in the context of DSBs are highlighted in reviews by Chang et al. (2001) and Van Gent et al. (2001) with discussions focussing on the consequences of losing some of these proteins in cells, mice and humans (Van Gent et al., 2001)

Uncertainties and Inconsistencies

Uncertainties and inconsistencies in this KER are as follows:

- There is controversy surrounding how error-prone NHEJ truly is. Recent studies suggest that the process may be quite accurate (reviewed in (Bétermier et al. 2014)). The accuracy of NHEJ may actually be dependent on the structure of the termini. Thus, the termini processing rather than the NHEJ mechanism itself is argued to be the error-prone process (Bétermier et al. 2014).
- There may be different cellular responses associated with low-dose radiation exposure and high-dose radiation exposure; these differences may also be dependent on a DSB threshold being exceeded prior to initiation repair. It has been suggested that DNA repair may not be activated at low doses of radiation exposure in order to prevent the risk of mutations from error-prone repair mechanisms (Marples 2004).
- DSB repair fidelity varies in terms of confounding factors and the genetic characteristics of individuals (Scott 2006). For example, individuals who smoke have

a 50% reduction in the mean level of DSB repair capacity relative to the non-smokers; this is due to an increased methylation index in smokers. A higher methylation index indicates more inactivation of gene expression. It is thus possible that expression of DNA repair proteins in smokers is decreased due to increased methylation of the genes encoding for repair proteins. In terms of individual genetics, single nucleotide polymorphisms (SNPs) within the MRE11A, CHEK2, XRCC3, DNA-PKcs, and NBN repair genes have been highly associated with the methylation index (Leng et al. 2008). SNPs can critically affect the function of these core proteins, varying the fidelity of DNA repair from person to person.

- Cells containing DNA damaged may be eliminated by apoptotic pathways, therefore not undergo repair, alternatively evidence has also shown that damaged cells can propagate due to lack of detection by repair machinery (Valentin 2005).
- The focus of this KER was on DSBs because there is lack of data to support that single strand breaks (SSBs) lead to inadequate repair. Multiple SSBs can lead to DSBs. Thus, DSBs are the focus as they can drive the cell towards genomic instability, apoptosis or tumorigenesis. Further quantitative evidence to define the extent of SSBs leading to DSBs and the relationship with repair is necessary.
- Ercc2+/- mice have a mutation in a gene involved in the nucleotide excision repair (NER) pathway, leading to DNA repair deficiency. However, when compared to wild type mice Ercc2+/- mice had fewer DNA strand breaks. This was true of both central and peripheral lens cells, as well as 4 and 24 h after irradiation (60Co γ-rays, 0.3, 0.063 Gy/min) (Barnard et al., 2021).
- DNA damage repair times can vary depending on the stressors that instigate the DNA damage. For example, it has been found that some types of radiation i.e., high linear energy transfer (LET) increases the amount of time required to repair DNA breaks (Aufderheide, 1987; Frankenburg-Schwager et al., 1994; Rydberg et al., 1994; Baumstark-Khan et al., 2003; Tsao, 2007; Blakely, 2012), however Stenerlöw et al. (2000) found that repair half-times were independent of LET.

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage suggests that DSB repair can be predicted from the presence of DSBs. The following tables provide representative examples of the relationship, unless otherwise indicated, all data is statistically significant. In terms of DNA repair in response to radiation-induced DSBs, studies suggest that under *in vitro* conditions, low doses of ionizing radiation (e.g., 5 mGy) leads to a reduced DSB repair capacity compared to relatively higher doses (e.g., 100, 500 mGy). The relationship between radiation dose and the number of γ-H2AX foci, which indicates the presence of DSBs, measured after a 5 h incubation following irradiation was non-linear in human lymphocytes and primary fibroblasts (Rothkamm & Lobrich, 2003; Lobrich et al., 2005); 5 h after 500 mGy-irradiation, a 90% reduction in γ-H2AX foci occurred, while only 50% of the induced γ-H2AX foci were removed following a 5 mGy exposure. However, the rate of DSB repair *in vivo* was not observed to be affected by dose and the relationship was linear; the number of γ-H2AX foci detected in human lymphocytes 0.5, 1, 2.5, 5, and 24h post-irradiation by various doses (157 – 1514 mGy*cm) via computerized tomography (CT) examination corresponded to dose (Lobrich et al., 2005). In this study, the levels of γ-H2AX foci were back to the baseline levels in all individuals after 24h. It must be noted that the resolution of γ-H2AX foci does not necessarily indicate error-free repair of DSBs. After a 10 Gy dose of radiation, approximately 10 - 15% of DSBs were found to be misrepaired (Mcmahon et al., 2016); at a dose of 80 Gy, the relative percentage of DSBs incorrectly repaired was

estimated at 50 - 60% (Kuhne et al., 2000; Lobrich et al., 2000; Mcmahon et al., 2016). Twenty-four hours post-irradiation, this rate increased to approximately 80% for alpha particle irradiation at 80 Gy and remained constant until the end of the assay (10 days) (Kuhne et al., 2000).

Dose Concordance

Reference	Experiment Description	Result
Rydberg et al., 1994	In vitro. Human VA13 lung fibroblast and GM38A skin fibroblast cells were exposed to neon ions (425 MeV/u, 1 – 5 Gy/min, 80 Gy), iron ions (600 MeV/u, 1 – 5 Gy/min, 50 Gy), and X rays (425 MeV/u, 1 – 2 Gy/min, 80 Gy) to induce DNA strand breaks. Initial breaks after exposure were measured via the fraction of activity released (FAR) assay referring to the fraction of radiolabeled DNA released on PFGE gels, with an increased FAR value indicating an increased number of breaks. Repair was measured using the FAR assay after a period of incubation.	Exposure to X-rays, neon, and iron ions led to a 90, 70, and 50% FAR increase relative to control respectively, indicating the highest level of breaks in samples exposed to X-rays. Four h later, 15, 20, and 73% of the DNA strand breaks had not been repaired.
Kuhne et al., 2000	In vitro. Human lung fibroblast cells were exposed to X-rays (23 Gy/min) at doses from 0 - 320 Gy. Following this, both correct (measured via hybridization assay), and total (measured via FAR assay) breaks remaining were measured. Therefore, allowing for calculation of the amount of misrepaired breaks.	Cells exposed to 0 - 320 Gy X-rays displayed an approximately linear increase in DSBs. This led to a gradual increase in the % DSBs misrejoined, which began to plateau after 80 Gy at a misrejoining frequency of 50%.
Baumstark-Khan et al., 2003	In vitro. Bovine LECs were exposed to X-rays (5 Gy/min, 0 to 50 Gy), 16O (3.4, 8.7 MeV/u, 230.5 to 642.9 Gy), 40Ar (2.7, 6.2, 10.5, 19.3 MeV/u, 0 to 190 Gy), 132Xe (5.4, 10.1, 16.5 MeV/u, 0 to 80 Gy), 208Pb (3.0, 6.8, 15.4 MeV/u, 0 to 50 Gy), 238U (1.5, 1.9, 2.6, 4.0 MeV/u, 0 to 150 Gy), 48Ti (4.8, 5.6, 14.3 Me V/u, 0 to 150 Gy). This led to the induction of both SSBs and DSBs, whose repair was measured using a method similar to the hydroxyapatite chromatography of alkaline unwound DNA.	Irradiation below 10 000 keV/µm led to above 90% rejoining of SSBs and DSBs within 24 hours. At LETs above 10 000 keV/µm the rejoining capacity varied depending on the original level of damage. After irradiation with 238U (LET ~ 15 700 000 – 16 300 keV/µm) the extrapolated rejoining capacity as t -> ∞ ranged from 50 to 100%. After irradiation with 208Pb (LET ~ 14 100 – 14 300 keV/µm) the extrapolated rejoining capacity as t -> ∞ ranged from 18 to 30%. 48Ti was an exception; with an LET of 1440 keV/µm, the expected rejoining capacity reached only 65% rather than above 90% as t -> ∞.
Aufderheide, 1987	In vitro. Bovine lens epithelial cells (LECs) were exposed to 238U (5, 10, 20 x 106	Bovine LECs exposed to 21 x 106 P/cm2 84Kr displayed a 1.3x

	P/cm2), 132Xe (3, 5, 7, 12, 20 x 106 P/cm2), 84Kr (9, 21 x 106 P/cm2), 40Ar (24 x 106 P/cm2), 16O (80 x 106 P/cm2), and X-rays (20, 40, 200 Gy). The radiation exposure induced DNA breaks were measured using the DNA unwinding method described by Rydberg (1975). The DNA then underwent a period of repair incubation lasting between 5 to 40 h, after which any remaining DNA damage was measured using the same method as before.	increase in DNA breaks and a 5% decrease in the level of breaks repaired compared to cells exposed to 9 x 106 P/cm2.
Stenerlöw et al., 2000	In vitro. Human skin fibroblast cells were exposed to 100 Gy of photons (60Co, < 0.5 keV/um), nitrogen ions (80, 125, 175, 225 keV/um), and helium ions (40 keV/um), resulting in the formation of DSBs. Their number was calculated by fragment analysis, based upon the fraction of DNA less than 5.7 Mbp, under the assumption that the breaks were evenly distributed. DNA repair was also measured via fragment analysis.	Exposure to increasing LET of radiation at 100 Gy led to increasing DSBs, in general, with about 600 DSBs/Gbp after γ-ray irradiation and about 700 DSBs/Gbp after 225 keV/um nitrogen ion irradiation. A dose of 100 Gy also led to decreased repair at increased LET. About 20-22 h after γ-ray irradiation, 4% of DSBs were unrepaired, while 20-22 h after 225 keV/um nitrogen ion irradiation, 12% of DSBs were unrepaired.

Incidence Concordance

No studies were found.

Time Concordance

Reference	Experiment Description	Result
Durante et al., 1998	In vitro. Human, male, lymphocyte cells were exposed to either iron ions (140 keV/μm, 2 Gy), or carbon ions (42 keV/μm, 5 Gy) to induce DNA strand breaks. Misrepair was measured by producing chromosome spreads and evaluating them using a microscope and the PAINT classification code.	Exposure to 2 Gy iron particles resulted in about 0.45 breaks/cell, of which 50% were repaired 10 h later. However, there were 0.1 translocations/cell, 0.08 incomplete exchanges/cell, 0.075 complex exchanges/cell, and 0.07 dicentrics/cell. Exposure to 5 Gy carbon ions resulted in 1.15 breaks/cell, of which 25% were repaired 10 h later. However, there were 0.35 translocations/cell, 0.28 incomplete exchanges/cell, 0.43 complex exchanges/cell, and 0.29 dicentrics/cell.
Rydberg et al., 1994	In vitro. Human VA13 lung fibroblast and GM38A skin fibroblast cells were exposed to	In GM38A cells, exposure to 80 Gy of all three radiation types led to

	neon ions (425 MeV/u, 1 – 5 Gy/min, 80 Gy), iron ions (250, 400, 600 MeV/u, 1 – 5 Gy/min, 50 Gy), and X rays (425 MeV/u, 1 – 2 Gy/min, 80 Gy) to induce DNA breaks. Their repair was measured using pulsed-field gel electrophoresis and determining the amount of DNA released from the gel plug (fraction of activity released – FAR).	DNA breaks. Repair was observed between 0.5 and 4 h after this. The most breaks remained after exposure to iron ions (75% of breaks remained), 25 – 42% remained after neon exposure, and only 15 – 20% remained after X ray irradiation.

Response-response relationship

There is evidence of a response-response relationship for DNA repair of radiation-induced DSBs. The frequency of DSBs has been shown to increase linearly with radiation dose (Löbrich et al., 2000; Rothkamm & Lobrich, 2003; Kuhne et al., 2005; Asaithamby & Chen, 2009). For DNA repair, increasing doses of a radiation stressor were found to cause a linear-quadratic relationship between the radiation dose and the number of misrejoined DSBs per cell (Kuhne et al., 2005). Interestingly, the relationships between radiation and DNA repair were found to vary depending on the type of radiation. There was a more linear response between radiation dose and the number of misrejoined DSBs for high LET particles relative to a more curvilinear relationship for lower LET particles (Rydberg et al., 2005). Additionally, a linear relationship was defined for low dose-rate radiation and the number of non-repaired DNA DSBs, but a linear-quadratic equation was described for high dose-rate radiation (Dikomey & Brammer, 2000).

Time-scale

Data from temporal response studies suggests that DSB repair may occur within 15 - 30 minutes of a DSB-inducing radiation stressor (Paull et al., 2000; Rothkamm & Lobrich, 2003; Pinto & Prise, 2005; Dong et al., 2017), with foci documented as early as 3-5 minutes post-irradiation (Asaithamby & Chen, 2009). The majority of DSB repair has been reported to occur within the first 3 - 6 hours following DSB induction (Rothkamm & Lobrich, 2003; Pinto & Prise, 2005; Asaithamby & Chen, 2009; Dong et al., 2017), with complete or near-complete DSB repair within 24 hours of the radiation stressor (Dikomey & Brammer, 2000; Lobrich et al., 2000; Rothkamm & Lobrich, 2003; Asaithamby & Chen, 2009; Mcmahon et al., 2016). In one 48-hour time-course experiment for DSB repair using two different types of radiation, the following repair progression was found at 30 minutes, 1 hour, 3 hours, 24 hours and 48 hours, respectively: 40 - 55%, 55 - 70%, 85%, 97 - 98% and 98% repair for X-rays and 30%, 45 - 50%, 65 - 70%, 85 - 90% and 90 - 96% repair for alpha particles (Pinto & Prise, 2005). Twenty-four hours post-irradiation, the frequency of DSB misrejoining was found to remain constant at approximately 80% for the 10 days that the DSB repair was monitored (Kuhne et al., 2000).

Known modulating factors

Modulating Factor	Details	Effects on the KER	References
Linear energy transfer (LET)	Increased LET	As the LET of the stressor increases, the amount of misrepaired and unrejoined DSBs also increases. One possible explanation for this is that DSB free ends are closer together at higher LETs, making it easier for misrepair to occur. Furthermore, higher LET stressors produce more complex, clustered breaks which also increasing repair difficulty. At very high LET values (over 10 000 keV/um), no significant DNA repair is detected.	Aufderheide, 1987; Rydberg et al., 1994; Durante et al., 1998; Kuhne et al., 2000; Stenerlöw et al., 2000; Baumstark-Khan et al., 2003; Tsao, 2007; Mukherjee et al., 2008; Blakely, 2012; Hamada, 2017
Oxygen	Decreased oxygen levels	Cells in an anoxic environment will rejoin DNA breaks more quickly than those in an oxic environment because oxygen can attach to the broken ends of DNA, fixing the damage and making it unrepairable.	Frankenburg-Schwager et al., 1994

Known Feedforward/Feedback loops influencing this KER

Not identified.

References

Anderson, C.W. 1993, "DNA damage and the DNA-activated protein kinase.", Trends Biochem. Sci. 18(11):433–437. doi:10.1016/0968-0004(93)90144-C.

Antonelli, A.F. et al. (2015), "Induction and Repair of DNA DSB as Revealed by H2AX Phosphorylation Foci in Human Fibroblasts Exposed to Low- and High-LET Radiation: Relationship with Early and Delayed Reproductive Cell Death", Radiat. Res. 183(4):417-31, doi:10.1667/RR13855.1.

Asaithamby, A. & D.J. Chen (2009), "Cellular responses to DNA double-strand breaks after low-dose c-irradiation.", Nucleic Acids Res. 37(12):3912–3923. doi:10.1093/nar/gkp237.

Aufderheide, E. (1987), “Heavy ion effects on cellular DNA: strand break induction and repair in cultured diploid lens epithelial cells”, International journal of radiation biology and related studies in physics, chemistry and medicine, Vol. 51/5, Taylor & Francis, London, https://doi.org/10.1080/09553008714551071

Barnard, S. G. R. (2018), “Dotting the eyes: mouse strain dependency of the lens epithelium to low dose radiation-induced DNA damage”, International Journal of Radiation Biology, Vol. 94/12, https://doi.org/10.1080/09553002.2018.1532609

Barnard, S. G. R. (2021), “Radiation-induced DNA damage and repair in lens epithelial cells of both Ptch1(+/-) and Ercc2(+/-) mutated mice”, Radiation Research, Vol. 197/1, Radiation Research Society, United States, https://doi.org/10.1667/RADE-20-00264.1

Baumstark-Khan, C. et al. (2003), “Induction and repair of DNA strand breaks in bovine lens epithelial cells after high LET irradiation”, Advances in Space Research, Vol. 31/6, Elsevier Ltd, England, https://doi.org/10.1016/S0273-1177(03)00095-4

Bétermier, M., P. Bertrand & B.S. Lopez (2014), "Is Non-Homologous End-Joining Really an Inherently Error-Prone Process?", PLoS Genet. 10(1). doi:10.1371/journal.pgen.1004086.

Blakely, E. A. (2012), “Lauriston S. Taylor lecture on radiation protection and measurements: what makes particle radiation so effective?”, Health Physics, Vol. 103/5, Health Physics Society, United States, https://doi.org/10.1097/HP.0b013e31826a5b85

Bracalente, C. et al. (2013), "Induction and Persistence of Large g H2AX Foci by High Linear Energy Transfer Radiation in DNA-Dependent protein kinase e Deficient Cells.", Int. J. Radiat. Oncol. Biol. Phys. 87(4). doi:10.1016/j.ijrobp.2013.07.014.

Bradley, M. O., and K. W. Kohn (1979), “X-ray induced DNA double strand break production and repair in mammalian cells as measured by neutral filter elution”, in Nuclei Acids Research, Vol. 7/3, Oxford University Press, England, http://dx.doi.org/ 10.1093/nar/7.3.793

Chang, H.H.Y. et al. (2017), "Non-homologous DNA end joining and alternative pathways to double - strand break repair.", Nat. Publ. Gr. 18(8):495–506. doi:10.1038/nrm.2017.48.

Dalke, C. et al. (2018), “Lifetime study in mice after acute low-dose ionizing radiation: a multifactorial study with special focus on cataract risk”, Radiation and Environmental Biophysics, Vol. 57/2, Springer Berlin Heidelberg, Berlin/Heidelberg, https://doi.org/10.1007/s00411-017-0728-z

Deans, B., Griffin, C. S., Maconochie, M. & Thacker, J. (2000), Xrcc2 is required for genetic stability, embryonic neurogenesis and viability in mice. EMBO J. 19, 6675–6685.

Dikomey, E. & I. Brammer (2000), "Relationship between cellular radiosensitivity and non-repaired double-strand breaks studied for di Ú erent growth states, dose rates and plating conditions in a normal human broblast line.", Int. J. Radiat. Biol., 76(6). doi:10.1080/09553000050028922.

Dong, J. et al. (2017), "Inhibiting DNA-PKcs in a non-homologous end-joining pathway in response to DNA double-strand breaks.", Oncotarget. 8(14):22662–22673. doi: 10.18632/oncotarget.15153.

Dubrova, Y.E. et al. (2002), "Elevated Minisatellite Mutation Rate in the Post-Chernobyl Families from Ukraine.", Am. J. Hum. Genet. 71(4):801–809. doi:10.1086/342729.

Durante, M. et al. (1998), “Rejoining and misrejoining of radiation-induced chromatin breaks. IV. Charged particles”, Radiation Research, Vol. 149/5, Radiation Research Society, Oak Brook, https://doi.org/10.2307/3579784

Feldmann, E. et al. (2000), "DNA double-strand break repair in cell-free extracts from Ku80-deficient cells: implications for Ku serving as an alignment factor in non-homologous DNA end joining.", Nucleic Acids Res. 28(13):2585–2596. doi:10.1093/nar/28.13.2585.

Ferguson, D.O. & F.W. Alt (2001), "DNA double strand break repair and chromosomal translocation: Lessons from animal models.", Oncogene, 20(40):5572–5579. doi: 10.1038/sj.onc.1204767.

Frankenburg-Schwager, M. et al. (1994), “Half-life values for DNA double-strand break rejoining in yeast can vary by more than an order of magnitude depending on the irradiation conditions”, International Journal of Radiation Biology, Vol. 66/5, Informa UK Ltd, London, https://doi.org/10.1080/09553009414551591

van Gent D.C., J.H.J. Hoeijmakers & R. Kanaar (2001), "Chromosomal stability and the DNA double-stranded break connection.", Nat. Rev. Genet. 2(3):196–206. doi:10.1038/35056049. http://www.ncbi.nlm.nih.gov/pubmed/11256071.

Getts, R.C. & T.D. Stamato (1994), "Absence of a Ku-like DNA end binding activity in the xrs double-strand DNA repair-deficient mutant.", J. Biol. Chem. 269(23):15981–15984.

Godwin, A.R. et al. (1994), "Spontaneous and restriction enzyme-induced chromosomal recombination in mammalian cells.", PNAS 91(December):12554–12558. doi: 10.1073/pnas.91.26.12554

Goodhead, D.T. (1994), "Initial events in the cellular effects of ionizing radiations: clustered damage in DNA.", Int. J. Radiat. Biol. 65(1):7–17. doi:10.1080/09553009414550021. http://www.ncbi.nlm.nih.gov/pubmed/7905912.

Goodhead, D.T. et al. (1980), "Mutation and inactivation of cultured mammalian cells exposed to beams of accelerated heavy ions. IV. Biophysical interpretation.", Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 37(2):135–67. doi:10.1080/09553008014550201.

Gorbunova, V. 1997, "Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions.", Nucleic Acids Res. 25(22):4650–4657. doi:10.1093/nar/25.22.4650.

Guirouilh-Barbat, J. et al. (2007), "Defects in XRCC4 and KU80 differentially affect the joining of distal nonhomologous ends.", Proc Natl Acad Sci. 104(52):20902–20907. doi:10.1073/pnas.0708541104.

Grudzenski, S. et al. (2010), "Inducible response required for repair of low-dose radiation damage in human fibroblasts.", Proc. Natl. Acad. Sci. USA. 107(32): 14205-14210, doi:10.1073/pnas.1002213107.

Guirouilh-barbat, J. et al. (2014), "Is homologous recombination really an error-free process?", Front Genet. 5:175. doi:10.3389/fgene.2014.00175.

Hamada, N. (2017), “Ionizing radiation sensitivity of the ocular lens and its dose rate dependence”, International Journal of Radiation Biology, Vol. 93/10, Taylor & Francis, England, https://doi.org/10.1080/09553002.2016.1266407

Harrison, L., Z. Hatahet & S.S. Wallace (1999), "In vitro repair of synthetic ionizing radiation-induced multiply damaged DNA sites 1 1Edited by J. H. Miller.", J. Mol. Biol. 290(3):667–684. doi:10.1006/jmbi.1999.2892.

Hartlerode, A.J. & R. Scully (2009), "Mechanisms of double-strand break in somatic mammalian cells.", Biochem J. 423(2):157–168. doi:10.1042/BJ20090942.Mechanisms.

Jeggo, P.A. (1998), "DNA breakage and repair.", Adv. Genet. 38:185–218. doi:DOI: 10.1016/S0065-2660(08)60144-3. doi: DOI: 10.1016/S0065-2660(08)60144-3.

Jeggo, P.A. & L. Markus (2015), "How cancer cells hijack DNA double-strand break repair pathways to gain genomic instability.", Biochem. J., 471(1):1–11. doi:10.1042/BJ20150582.

Khanna, K.K. & S.P. Jackson (2001), "DNA double-strand breaks: signaling , repair and the cancer connection.", 27(march):247–254. doi: 10.1038/85798.

Kirchgessner, C. et al. (1995), "DNA-dependent kinase (p350) as a candidate gene for the murine SCID defect.", Science (80-). 267(5201):1178–1183. doi:10.1126/science.7855601.

Kozbenko, T. et al. (2022), "Deploying elements of scoping review methods for adverse outcome pathway development: a space travel case example", International Journal of Radiation Biology, 1–12. https://doi.org/10.1080/09553002.2022.2110306

Kuhne, M., K. Rothkamm & M. Lobrich (2000), "No dose-dependence of DNA double-strand break misrejoining following a -particle irradiation.", Int. J. Radiat. Biol. 76(7):891-900

Kuhne, M., G. Urban & M. Lo (2005), "DNA Double-Strand Break Misrejoining after Exposure of Primary Human Fibroblasts to CK Characteristic X Rays, 29 kVp X Rays and 60Co γ Rays", Radiat. Res., 164(5):669–676. doi:10.1667/RR3461.1.

de Lara, C.M. et al. (2001), "Dependence of the Yield of DNA Double-Strand Breaks in Chinese Hamster V79-4 Cells on the Photon Energy of Ultrasoft X Rays.", Radiation Research. 155(3):440-8. doi:10.1667/0033-7587(2001)155[0440:DOTYOD]2.0.CO;2.

Leng, S. et al. (2008), "Public Access NIH Public Access. PLoS One.", 32(7):736–740. doi:10.1371/journal.pone.0178059.

Lieber, M.R. (2008), "The mechanism of human nonhomologous DNA End joining.", J Biol Chem. 283(1):1–5. doi:10.1074/jbc.R700039200.

Lobrich, M. and P. Jeggo, (2017), A Process of Resection-Dependent Nonhomologous End Joining Involving the Goddess Artemis., Trends Biochem Sci. 42(9): 690-701. doi: 10.1016/j.tibs.2017.06.011.

Lobrich, M. et al. (2000), "Joining of Correct and Incorrect DNA Double-Strand Break Ends in Normal Human and Ataxia Telangiectasia Fibroblasts.", 68(July 1999):59–68. doi:DOI: 10.1002/(SICI)1098-2264(200001)27:1<59::AID-GCC8>3.0.CO;2-9.

Lobrich, M. et al. (2005), "In vivo formation and repair of DNA double-strand breaks after computed tomography examinations.", Proc. Natl. Acad. Sci. 102(25):8984–8989. doi:10.1073/pnas.0501895102.

Lett, J. T. (1996), "Experimental models for cellular radiation targets: LET, RBE and radioprotectors", Advances in Space Research, Vol. 18/1, Elsevier Ltd, England, https://doi.org/10.1016/0273-1177(95)00786-E

Malu, S. et al. (2012), "Role of non-homologous end joining in V(D)J recombination.", Immunol. Res. 54(1–3):233–246. doi:10.1007/s12026-012-8329-z.

Mao, Z. et al. (2008), "DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells.", Cell Cycle. 7(18):2902–2906. doi:10.4161/cc.7.18.6679.

Marples, B. (2004), "Is low-dose hyper-radiosensitivity a measure of G2-phase cell radiosensitivity?", Cancer Metastasis Rev. 23(3–4):197–207. doi:10.1023/B:CANC.0000031761.61361.2a.

McMahon, S.J. et al. (2016), "Mechanistic Modelling of DNA Repair and Cellular Survival Following Radiation-Induced DNA Damage.", Nat. Publ. Gr.(April):1–14. doi:10.1038/srep33290.

Miller, R.C. et al. (1995), "The Biological Effectiveness of Radon-Progeny Alpha Particles.", Radiat. Res. 142(1):61–69. doi:10.2307/3578967.

Moore, S., F.K.T. Stanley & A.A. Goodarzi (2014), "The repair of environmentally relevant DNA double strand breaks caused by high linear energy transfer irradiation – No simple task.", DNA repair (Amst), 17:64–73. doi: 10.1016/j.dnarep.2014.01.014.

Mukherjee, B. et al. (2008), "Modulation of the DNA-damage response to HZE particles by shielding", DNA Repair, Vol. 7/10, Elsevier B.V, Amsterdam, https://doi.org/10.1016/j.dnarep.2008.06.016

Murakami, H. & S. Keeney (2008), "Regulating the formation of DNA double-strand breaks in meiosis.", Genes Dev. 22(3):286–292. doi:10.1101/gad.1642308.

Paull, T.T. et al. (2000), "A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage.", Curr. Biol. 10(15):886–895. doi:10.1016/S0960-9822(00)00610-2

Pinto, M. & K. Prise (2005), "Evidence for Complexity at the Nanometer Scale of Radiation-Induced DNA DSBs as a Determinant of Rejoining Kinetics Evidence for Complexity at the Nanometer Scale of Radiation-Induced DNA DSBs as a Determinant of Rejoining Kinetics.", Radiat. Res. 164(1):73-85 doi:10.1667/RR3394.

Puchta, H. (2005), "The repair of double-strand breaks in plants: Mechanisms and consequences for genome evolution.", J. Exp. Bot. 56(409):1–14. doi:10.1093/jxb/eri025.

Thurtle-Schmidt, D.M. & T-W. Lo (2018), "Molecular biology at the cutting edge: A review on CRISPR/CAS9 gene editing for undergraduates.", Biochem. Mol. Biol. Educ. 46(2):195–205. doi:10.1002/bmb.21108.

Rathmell, W,K. & G. Chu (1994), "Involvement of the Ku autoantigen in the cellular response to DNA double-strand breaks.", Proc. Natl. Acad. Sci. 91(16):7623–7627. doi:10.1073/pnas.91.16.7623.

Rogakou, E.P. et al. (1999), "Megabase Chromatin Domains Involved in DNA Double-Strand Breaks In Vivo.", J. Cell Biol, 146(5):905-16. doi: 10.1083/jcb.146.5.905.

Rothkamm, K. et al. (2015), "Review DNA Damage Foci: Meaning and Significance.", Environ. Mol. Mutagen., 56(6):491-504, doi: 10.1002/em.21944.

Rothkamm, K. & M. Lobrich (2003), "Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses.", PNAS, 100(9):5057-62. doi:10.1073/pnas.0830918100.

Rube, C.E. et al. (2008), "Cancer Therapy: Preclinical DNA Double-Strand Break Repair of Blood Lymphocytes and Normal Tissues Analysed in a Preclinical Mouse Model: Implications for Radiosensitivity Testing.", Clin. Cancer Res., 14(20):6546–6556. doi:10.1158/1078-0432.CCR-07-5147.

Rydberg, B. (1975), "The rate of strand separation in alkali of DNA of irradiated mammalian cells", Radiation Research, Vol. 178/2, United States, pp. 190-197

Rydberg, B. et al. (1994), "DNA double-strand breaks induced by high-energy neon and iron ions in human fibroblasts. I. Pulsed-filed gel electrophoresis method", Radiation Research, Vol. 139/2, Radiation Research Society, Oak Brook, https://doi.org/10.2307/3578657

Rydberg, B. et al. (2005), "Dose-Dependent Misrejoining of Radiation-Induced DNA Double-Strand Breaks in Human Fibroblasts: Experimental and Theoretical Study for High- and Low-LET Radiation.", Radiat. Res. 163(5):526–534. doi:10.1667/RR3346.

Sage, E. & N. Shikazono (2017), "Free Radical Biology and Medicine Radiation-induced clustered DNA lesions: Repair and mutagenesis.", Free. Radic. Biol. Med. 107(December 2016):125–135. doi:10.1016/j.freeradbiomed.2016.12.008.

Schipler, A. & G. Iliakis (2013), "DNA double-strand – break complexity levels and their possible contributions to the probability for error-prone processing and repair pathway choice.", Nucleic Acids Res., 41(16):7589–7605. doi:10.1093/nar/gkt556.

Scott, B. (2006), "Stochastic Thresholds: A Novel Explanation of Nonlinear Dose-Response Relationships for Stochastic Radiobiological Effects.", Dose-Response, 3(4):547–567. doi:10.2203/dose-response.003.04.009.

Shuman, S. & M.S. Glickman (2007), "Bacterial DNA repair by non-homologous end joining.", Nat. Rev. Microbiol. 5(11):852–861. doi:10.1038/nrmicro1768.

Sidjanin, D. et al. (1993), "DNA damage and repair in rabbit lens epithelial cells following UVA radiation, Taylor & Francis, Vol. 12/9, Informa UK Ltd, Lisse, https://doi.org/10.3109/02713689309020382

Simsek, D. & M. Jasin (2010), "HHS Public Access.", 118(24):6072–6078. doi:10.1002/cncr.27633.

Stenerlöw, E. H. et al. (2000), "Rejoining of DNA fragments produced by radiations of different linear energy transfer", International Journal of Radiation Biology, Vol. 76/4, Informa UK Ltd, London, https://doi.org/10.1080/095530000138565

Sutherland, B.M. et al. (2000), "Clustered DNA damages induced in isolated DNA and in human cells by low doses of ionizing radiation.", J. of Rad. Res. 43 Suppl(S):S149-52. doi: 10.1269/jrr.43.S149

Thompson, L.H. (2012), "Recognition, signaling, and repair of DNA double-strand breaks produced by ionizing radiation in mammalian cells : The molecular choreography.", Mutat Res., 751(2):158–246. doi: 10.1016/j.mrrev.2012.06.002.

Tsao, D. et al. (2007), "Induction and processing of oxidative clustered DNA lesions in 56Fe-ion-irradiated human monocytes", Radiation Research, Vol.168/1, United States, https://doi.org/10.1667/RR0865.1

Valentin J. (2005), "Low-dose Extrapolation of Radiation-related Cancer Risk.", Ann. ICRP, 35(4):1-140

Vignard, J., G. Mirey & B. Salles (2013), "Ionizing-radiation induced DNA double-strand breaks: A direct and indirect lighting up.", Radiother. Oncol. 108(3):362–369. doi:10.1016/j.radonc.2013.06.013.

Ward, J. F. (1988), "DNA Damage Produced by Ionizing Radiation in Mammalian Cells: Identities, Mechanisms of Formation, and Reparability.", Prog. Nucleic Acid Res. Mol. Biol. 35(C):95–125. doi:10.1016/S0079-6603(08)60611-X.

Wilson, T.E. & M.R. Lieber (1999), "Efficient Processing of DNA Ends during Yeast Nonhomologous End Joining.", J. Biol. Chem. 274(33):23599–23609. doi:10.1074/jbc.274.33.23599.

Zhu, C. et al. (2002), "Unrepaired DNA breaks in p53-deficient cells lead to oncogenic gene amplification subsequent to translocations.", Cell. 109(7):811–21. doi:10.1016/s0092-8674(02)00770-5.

Relationship: 164: Inadequate DNA repair leads to Increase, Mutations

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations	adjacent	High	Moderate
Alkylation of DNA leading to cancer 2	adjacent	High	Moderate
Alkylation of DNA leading to cancer 1	non-adjacent	High	Moderate
Oxidative DNA damage leading to chromosomal aberrations and mutations	adjacent	High	Low
Deposition of energy leading to lung cancer	adjacent	Moderate	Moderate
Bulky DNA adducts leading to mutations	adjacent		
Alcohol Induced DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	High	High
Deposition of energy leading to occurrence of cataracts	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Term	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

This KER is plausible in all life stages, sexes, and organisms with DNA. The majority of the evidence is from in vivo adult mice and male human, and mice in vitro models.

All organisms, from prokaryotes to eukaryotes, have DNA repair systems. Indeed, much of the empirical evidence on the fundamental principles described in this KER are derived from prokaryotic models. DNA adducts can occur in any cell type with DNA, and may or may not be repaired, leading to mutation. While there are differences among DNA repair systems across eukaryotic taxa, all species develop mutations following excessive burdens of DNA lesions like DNA adducts. Theoretically, any sexually reproducing organism (i.e., producing gametes) can also acquire DNA lesions that may or may not be repaired, leading to mutations in gametes.

Key Event Relationship Description

Insufficient repair results in the retention of damaged DNA that is then used as a template during DNA replication. During replication of damaged DNA, incorrect nucleotides may be inserted, and upon replication these become 'fixed' in the cell. Further replication propagates the mutation to additional cells.

For example, it is well established that replication of alkylated DNA can cause insertion of an incorrect base in the DNA duplex (i.e., mutation). Replication of non-repaired O4 thymine alkylation leads primarily to A:T→G:C transitions. Retained O6 guanine alkylation causes primarily G:C→A:T transitions.

For repairing DNA double strand breaks (DSBs), non-homologous end joining (NHEJ) is one of the repair mechanisms used in human somatic cells (Petrini et al., 1997; Mao et al., 2008). However, this mechanism is error-prone and may create mutations during the process of DNA repair (Little, 2000). NHEJ is considered error-prone because it does not use a homologous template to repair the DSB. The NHEJ mechanism involves many proteins that work together to bridge the DSB gap by overlapping single-strand termini that are usually less than 10 nucleotides long (Anderson, 1993; Getts & Stamato, 1994; Rathmell & Chu, 1994). Inherent in this process is the introduction of errors that may result in mutations such as insertions, deletions, inversions, or translocations.

Evidence Supporting this KER

Overall Weight of Evidence: High

Biological Plausibility
If DNA repair is able to correctly and efficiently repair DNA lesions introduced by a genotoxic stressor, then no increase in mutation frequency will occur.

For example, for alkylated DNA, efficient removal by O^6-alkylguanine DNA alkyltransferase will result in no increases in mutation frequency. However, above a certain dose AGT becomes saturated and is no longer able to efficiently remove the alkyl adducts. Replication of O-alkyl adducts leads to mutation. The evidence demonstrating that replication of unrepaired O-alkylated DNA causes mutations is extensive in somatic cells and has been reviewed (Basu and Essigmann 1990; Shrivastav et al. 2010); specific examples are given below.

It is important to note that not all DNA lesions will cause mutations. It is well documented that many are bypassed error-free. For example, N-alkyl adducts can quite readily be bypassed error-free with no increase in mutations (Philippin et al., 2014).

Inadequate repair of DSB

Collective data from tumors and tumor cell lines has emerged that suggests that DNA repair mechanisms may be error-prone (reviewed in Sishc et al., 2017) (Sishc & Davis, 2017). NHEJ, the most common pathway used to repair DSBs, has been described as error-prone. The error-prone nature of NHEJ, however, is thought to be dependent on the structure of the DSB ends being repaired, and not necessarily dependent on the NHEJ mechanism itself (Bétermier et al., 2014). Usually when perfectly cohesive ends are formed

as a result of a DSB event, ligase 4 (LIG4) will have limited end processing to perform, thereby keeping ligation errors to a minimum (Waters et al., 2014). When the ends are difficult to ligate, however, the resulting repair may not be completed properly; this often leads to point mutations and other chromosomal rearrangements. It has been shown that approximately 25 - 50% of DSBs are misrejoined after exposure to ionizing radiation (Löbrich et al., 1998; Kuhne et al., 2000; Lobrich et al., 2000). Defective repair mechanisms can increase sensitivity to agents that induce DSBs and lead eventually to genomic instability (reviewed in Sishc et al., (2017)).

Activation of mutagenic DNA repair pathways to withstand cellular or replication stress either from endogenous or exogenous sources can promote cellular viability, albeit at a cost of increased genome instability and mutagenesis (Fitzgerald et al., 2017). These salvage DNA repair pathways including, Break-induced Replication (BIR) and Microhomology-mediated Break-induced Replication (MMBIR). BIR repairs one-ended DSBs and has been extensively studied in yeast as well as in mammalian systems. BIR and MMBIR are linked with heightened levels of mutagenesis, chromosomal rearrangements and ensuing genome instability (Deem et al., 2011; Sakofsky et al., 2015; Saini et al., 2017; Kramara et al., 2018). In mammalian genomes BIR-like synthesis has been proposed to be involved in late-stage Mitotic DNA Synthesis (MiDAS) that predominantly occurs at so-called Common Fragile Sites (CFSs) and maintains telomere length under s conditions of replication stress that serve to promote cell viability (Minocherhomji et al., 2015; Bhowmick et al., 2016; Dilley et al., 2016).

Empirical Evidence

INSUFFICIENT REPAIR OF ALKYLATED DNA

Evidence in somatic cells

Empirical evidence to support this KER is primarily from studies in which synthetic oligonucleotides containing well-characterized DNA lesions were genetically engineered in viral or plasmid genomes and subsequently introduced into bacterial or mammalian cells. Mutagenicity of each lesion is ascertained by sequencing, confirming that replication of alkylated DNA (i.e., unrepaired DNA) causes mutations in addition to revealing the important DNA repair pathways and polymerases involved in the process. For example, plasmids containing O6-methyl or O6-ethylguanine were introduced into AGT deficient or normal Chinese hamster ovary cells (Ellison et al. 1989). Following replication, an increase in mutant fraction to 19% for O6-methylguanine and 11% for O6-ethylguanine adducts was observed in AGT deficient cells versus undetectable levels for control plasmids. The relationship between input of alkylated DNA versus recovered mutant fractions revealed that a large proportion of alkyl adducts were converted to mutations in the AGT deficient cells (relationship slightly sublinear, with more adducts than mutations). The primary mutation occurring was G:C-A:T transitions. The results indicate that replication of the adducted DNA caused mutations and that this was more prevalent with reduced repair capacity. The number of mutations measured is less than the unrepaired alkyl adducts transfected into cells, supporting that insufficient repair occurs prior to mutation. Moreover, the alkyl adducts occur prior to mutation formation, demonstrating temporal concordance.

Various studies in cultured cells and microorganisms have shown that the expression of O^6-methylguanine DNA methyltransferase (AGT/MGMT) (repair machinery – i.e., decrease in DNA strand breaks) greatly reduces the incidence of mutations caused by exposure to methylating agents such as *N*-methyl-*N*-nitrosourea (MNU) and *N*-methyl-*N*'-nitro-*N*-

nitrosoguanidine (MNNG) (reviewed in Kaina et al. 2007; Pegg 2011). Thomas et al. (2013) used O6-benzylguanine to specifically inhibit MGMT activity in AHH-1 cells. Inhibition was carried out for one hour prior to exposure to MNU, a potent alkylating agent. Inactivation of MGMT resulted in increased MNU-induced HPRT (hypoxanthine-guanine phosphoribosyltransferase) mutagenesis and shifted the concentrations at which induced mutations occurred to the left on the dose axis (10 fold reduction of the lowest observed genotoxic effect level from 0.01 to 0.001 µg/ml). The ratio of mutants recovered in DNA repair deficient cells was 3-5 fold higher than repair competent cells at concentrations below 0.01 µg/ml, but was approximately equal at higher concentrations, indicating that repair operated effectively to a certain concentration. Only at this concentration (above 0.01 µg/ml when repair machinery is overwhelmed and repair becomes deficient) do the induced mutations in the repair competent cells approach those of repair deficient. Thus, induced mutation frequencies in wild type cells are suppressed until repair is overwhelmed for this alkylating agent. The mutations prevented by MGMT are predominantly G:C-A:T transitions caused by O6-methylguanine.

Evidence in germ cells

That saturation of repair leads to mutation in spermatogonial cells is supported by work using the OECD TG488 rodent mutation reporter assay in sperm. A sub-linear dose-response was found using the lacZ MutaMouse assay in sperm exposed as spermatogonial stem cells, though the number of doses was limited (van Delft and Baan 1995). This is indirect evidence that repair occurs efficiently at low doses and that saturation of repair causes mutations at high doses. Lack of additional data motivated a dose-response study using the MutaMouse model following both acute and sub-chronic *N*-ethyl-*N*-nitrosourea (ENU), a strong DNA alkylator, exposure by oral gavage (O'Brien et al. 2015). The results indicate a linear dose-response for single acute exposures, but a sub-linear dose-response occurs for lower dose sub-chronic (28 day) exposures, during which mutation was only observed to occur at the highest dose. This is consistent with the expected pattern for dose-response based on the hypothetical AOP. Thus, this sub-linear curve for mutation at low doses following sub-chronic ENU exposure suggests that DNA repair in spermatogonia is effective in preventing mutations until the process becomes overwhelmed at higher doses.

Mutation spectrum: Following exposure to alkylating agents, the most mutagenic adducts to DNA in pre-meiotic male germ cells include O6-ethylguanine, O4-ethylthymine and O2-ethylthymine (Beranek 1990; Shelby and Tindall 1997). Studies on sperm samples collected post-ENU exposure in transgenic rodents have shown that 70% of the observed mutations are at A:T sites (Douglas et al. 1995). The mutations observed at G:C base pairs are almost exclusively G:C-A:T transitions, presumably resulting from O6-ethylguanine. It is proposed that the prevalence of mutations at A:T basepairs is the result of efficient removal of O6-alkylguanine by AGT in spermatogonia, which is consistent with observation in human somatic cells (Bronstein et al. 1991; Bronstein et al. 1992). This results in the majority of O6-ethylguanine adducts being removed, leaving O4- and O2-ethylthymine lesions to mispair during replication. Thus, lack of repair predominantly at thymines and guanines at increasing doses leads to mutations in these nucleotides, consistent with the concordance expected between diminished repair capabilities at these adducts and mutation induction (i.e., concordance relates to seeing these patterns across multiple studies, species and across the data in germ cells and offspring).

Inadequate repair of oxidative DNA lesions: In vitro studies

- AS52 Chinese hamster ovary cells (wild type and OGG1-overexpressing) were exposed to 400 kJ/m^2 UVA radiation (Dahle et al., 2008).
    - Mutations in the gpt gene were quantified in both wild type and OGG1+ cells by sequencing after 13-15 days following 400 kJ/m^2 UVA irradiation
        - G:C-A:T mutations in UVA-irradiated OGG1+ cells were completely eliminated
        - G:C-A:T mutation frequency in wild type cells increased from 1.8 mutants/million cells to 3.8 mutants/million cells following irradiation – indicating incorrect repair or lack of repair of accumulated 8-oxo-dG
        - Elevated levels of OGG1 was able to prevent G:C-A:T mutations, while the OGG1 levels in wild type cells was insufficient, leading to an increase in mutants (demonstrates inadequate repair leading to mutations)
- Xeroderma pigmentosum complementation group A (XPA) knockout (KO) and wild type TSCER122 human lymphoblastoid cells were transfected with TK gene-containing vectors with no adduct, a single 8-oxo-dG, or two 8-oxo-dG adducts in tandem (Sassa et al., 2015).
    - XPA is a key protein in nucleotide excision repair (NER) that acts as a scaffold in the assembly the repair complex.
    - Mutation frequency was determined by the number of TK-revertant colonies
    - Control vector induced a mutation frequency of 1.3% in both WT and XPA KO
    - Two 8-oxo-dG in tandem on the transcribed strand were most mutagenic in XPA KO, inducing 12% mutant frequency compared to 7% in WT
    - For both XPA KO and WT, G:C-A:T transversion due to 8-oxo-dG was the most predominant point mutation in the mutants
    - The lack of a key factor in NER leading to increased 8-oxo-dG-induced transversions demonstrates insufficient repair leading to increase in mutations

Inadequate repair of oxidative DNA lesions: In vivo studies in mice

- Spontaneous mutation frequencies in the liver of Ogg1-deficient (-/-) Big Blue mice was measured at 10 weeks of age (Klungland et al., 1999).
    - Mutation frequencies were 2- to 3-fold higher in the *Ogg1*-/- mice than in wild type
    - Of the 16 base substitutions detected in *Ogg1* -/- mutant plaques analyzed by sequencing, 10 indicated G:C-A:T transversions consistent with the known spectrum of mutation
    - The results support that insufficient repair of oxidized bases leads to mutation.
- *Ogg1* knockout (*Ogg1*-/-) in C57BL/6J mice resulted in 4.2-fold and 12-fold increases in the amount of 8-oxo-dG in the liver compared to wild type at 9 and 14 weeks of age, respectively (Minowa et al., 2000).
    - In these mice, there was an average of 2.3-fold increase in mutation frequencies in the liver (measured between 16-20 weeks)
        - 57% of the observed base substitutions were G:C-A:T transversions, while 35% in wild type mice corresponded to this transversion.
        - Approximately 70% of the increase in mutation frequency was due to G to T transversions.

- Concordantly, KBrO3 treatment resulted in a 2.9-fold increase in mutation frequency in the kidney of *Ogg1* -/- mice compared to KBrO3-treated wild type (Arai et al., 2002).
    - G:C-A:T transversions made up 50% of the base substitutions in the *Ogg1-/-* mice.
- Heterozygous *Ogg1* mutants (*Ogg1*+/-) retained the original repair capacity, where no increase in 8-oxo-dG lesions was observed in the liver at 9 and 14 weeks (Minowa et al., 2000).
    - This observation was consistent even after KBrO3 treatment of the mice (Arai et al., 2002).
- From these results, we can infer that OGG1 proteins are present in excess and that one functional copy of the gene is sufficient in addressing endogenous and, to a certain degree, chemical-induced oxidative DNA lesions.

Inadequate Repair of DSB

Empirical data obtained for this KER moderately supports the idea that inadequate DNA repair increases the frequency of mutations. The evidence presented below related to the inadequate repair of DSBs is summarized in table 5, here (click link). The review article by Sishc & Davis (2017) provides an overview of NHEJ mechanisms with a focus on the inherently error-prone nature of DSB repair mechanisms, particularly when core proteins of NHEJ are knocked-out. Another review also provides an overview of DSB induction, the repair process and how mutations may result, as well as the biological relevance of misrepaired or non-repaired DNA damage (Sage & Shikazono, 2017).

Dose and Incidence Concordance

There is evidence in the literature suggesting a dose/incidence concordance between inadequate DNA repair and increases in mutation frequencies. Evidence presented below related to the dose-response of mutation frequencies is summarized in table 2, here (click link). In response to increasing doses from a radiation stressor, dose-dependent increases in both measures of inadequate DNA repair and mutation frequency have been found. In an analysis that amalgamated results from several different studies conducted using in vitro cell-lines, the rate of DSB misrepair was revealed to increase in a dose-dependent fashion from 0 - 80 Gy, with the mutation rate also similarly increasing from 0 - 6 Gy (Mcmahon et al., 2016). Additionally, using a plant model, it was shown that increasing radiation dose from 0 - 10 Gy resulted in increased DNA damage as a consequence of inadequate repair. Mutations were observed 2 - 3 weeks post-irradiation (Ptácek et al., 2001). Moreover, increases in mutation densities were found in specific genomic regions of cancer samples (namely promoter DNAse I-hypersensitive sites (DHS) and 100 bp upstream of transcription start sites (TSS)) that were also found to have decreased DNA repair rates attributable to inadequate nucleotide excision repair (NER) (Perera et al., 2016).

Interestingly, mutation rates have been shown to increase as the required DNA repair becomes more complex. Upon completion of DSB repair in response to radiation and treatment with restriction enzymes, more mutations were found in cases where the ends were non-complementary and thus required more complex DNA repair (1 - 4% error-free) relative to cases where ends were complementary (34 - 38% error-free) (Smith et al., 2001).

Temporal Concordance

There is evidence in the literature suggesting a time concordance between the initiation of DNA repair and the occurrence of mutations. For simple ligation events, mutations were not evident until 12 - 24 hours, whereas DSB repair was evident at 6 -12 hours. For complex ligation events, however, mutations and DSB repair were both evident at 12 - 24 hours. As the relative percent of DNA repair increased over time, the corresponding percent of error-free rejoining decreased over time in both ligation cases, suggesting that overall DNA repair fidelity decreases with time ((Smith et al., 2001).

Essentiality

Inadequate DNA repair has been found to increase mutations above background levels. There is evidence from knock-out/knock-down studies suggesting that there is a strong relationship between the adequacy of DNA repair and mutation frequency. In all examined cases, deficiencies in proteins involved in DNA repair resulted in increased mutation frequencies relative to wild-type cases. There were significant decreases in the frequency and accuracy of DNA repair in cell lines deficient in LIG4 (DNA ligase 4, a DNA repair protein) (Smith et al., 2003) and Ku80 (Feldmann et al., 2000). Rescue experiments performed with these two cell lines further confirmed that inadequate DNA repair was the cause of the observed decreases in repair frequency and accuracy (Feldmann et al., 2000; Smith et al., 2003). In primary Nibrin-deficient mouse fibroblasts, there was increased spontaneous DNA damage relative to wild-type controls, suggestive of inadequate DNA repair. Using the corresponding Nibrin-deficient and wild-type mice, in vivo mutation frequencies were also found to be elevated in the Nibrin-deficient animals (Wessendorf et al., 2014). Furthermore, mutation densities were differentially affected in specific genomic regions in cancer patients depending on their Xeroderma pigmentosum group C (XPC) gene status. Specifically, mutation frequencies were increased in XPC-wild-type patients at DNase I-hypersensitive site (DHS) promoters and 100 bp upstream of TSS relative to cancer patients lacking functional XPC (Perera et al., 2016). Lastly, in a study using WKT1 cells with less repair capacity, radiation exposure induced four times more mutations in these cells than in TK6 cell, which had a normal repair capacity (Amundson and Chen, 1996).

Uncertainties and Inconsistencies

Repair of alkylated DNA

There were no inconsistencies in the empirical data reviewed or in the literature relating to biological plausibility. Much of the support for this KER comes predominantly from data in somatic cells and in prokaryotic organisms. We note that all of the data in germ cells used in this KER are produced exclusively from ENU exposure. Data on other chemicals are required. We consider the overall weight of evidence of this KER to be strong because of the obvious biological plausibility of the KER, and documented temporal association and incidence concordance based on studies over-expressing and repressing DNA repair in somatic cells.

Repair of oxidative lesions

- Thresholded concentration-response curve of mutation frequency was observed in AHH-1 human lymphoblastoid cells after treatment with pro-oxidants (H_2O_2 and $KBrO_2$) known

to cause oxidative DNA damage (Seager et al., 2012), suggesting that cells are able to tolerate low levels of DNA damage using basal repair. However, increase in 8-oxo-dG lesions and up-regulation of DNA repair proteins were not observed under the same experimental condition.

- Mutagenicity of oxidative DNA lesions other than 8-oxo-dG, such as FaPydG and thymidine glycol, has not been as extensively studied and there are mixed results regarding the mutagenic outcome of these lesions.

Repair of double strand breaks

- One review paper found that DNA DSBs are repaired more efficiently at low dose (≤0.1 Gy) compared to high dose (>1 Gy) X-rays, but delayed mutation induction and genomic instability have also been demonstrated to occur at low doses (<1 cGy) of ionizing radiation (Preston et al., 2013).

Overall

- Mutation induction is stochastic, spontaneous, and dependent on the cell type as well as the individual's capability to repair efficiently (NRC, 1990; Pouget & Mather, 2001).

Quantitative Understanding of the Linkage

Thresholds for mutagenicity indicate that the response at low doses is modulated by the DNA repair machinery, which is effectively able to remove alkylated DNA at low doses [Gocke and Muller 2009; Lutz and Lutz 2009; Pozniak et al. 2009]. Kinetics of DNA repair saturation in somatic cells is described in Muller et al. [Muller et al. 2009].

For O-methyl adducts, once the primary repair process is saturated, in vitro data suggest that misreplication occurs almost every time a polymerase encounters a methylated guanine [Ellison et al. 1989; Singer et al. 1989]; however, it should be noted that this process can be modulated by the flanking sequence. This conversion of adducts to mutations also appears to be reduced substantially in vivo [Ellison et al. 1989]. The probability of mutation will also depend on the type of adduct (e.g., O-alkyl adducts are more mutagenic than N-alkyl adducts; larger alkyl groups are generally more mutagenic, etc.). Overall, a substantive number of factors must be considered in developing a quantitative model.

<u>Inadequate repair of oxidative lesions</u>

The relationship between the quantity/activity of repair enzymes such as OGG1 in the cell and the quantity of oxidative lesions need to be better understood to define a threshold on the quantity of oxidative lesions exceeding basal repair capacity. Moreover, the proportion of oxidative lesions formed that lead to mutation versus strand breaks is not clearly understood.

Mutations resulting from oxidative DNA damage can occur via replicative polymerases and translesion synthesis (TLS) polymerases during replication, and during attempted repair. However, an in vitro study on TLS in yeast has shown that bypass of 8-oxo-dG by TLS polymerases during replication is approximately 94-95% accurate. Therefore, the mutagenicity of 8-oxo-dG and other oxidative lesions may depend on their abundance, not

on a single lesion (Rodriguez et al., 2013). Applicability of this observation in mammalian cells needs further investigation. Information on the accuracy of 8-oxo-dG bypass in mammalian cells is limited.

The most notable example of mutation arising from inadequate repair of DNA oxidation is G to T transversion due to 8-oxo-dG lesions. Previous studies have demonstrated higher mutation frequency of this lesion compared to other oxidative lesions; for example, Tan et al. (1999) compared the mutation rate of 8-oxo-dG and 8-oxo-dA in COS-7 monkey kidney cells and reported that under similar conditions, 8-oxo-dG was observed to be four times more likely to cause base substitution (Tan et al., 1999).

Inadequate Repair of DSB

Quantitative understanding of this linkage is derived from the studies that examined DSB misrepair rates or mutation rates in response to a radiation stressor. In general, combining results from these studies suggests that increased mutations can be predicted when DNA repair is inadequate. At a radiation dose of 10 Gy, the rate of DSB misrepair was found to be approximately 10 - 15% (Lobrich et al., 2000); this rate increased to 50 - 60% at a radiation exposure of 80 Gy (Kuhne et al., 2000; Lobrich et al., 2000; McMahon et al., 2016). For mutation rates in response to radiation across a variety of models and radiation doses, please refer to the example table below.

Reference	Summary
Matuo et al., 2018	Yeast cells (saccharomyces cerevisiae) exposed to high LET carbon ions (25 keV/um) and low LET carbon ions (13 keV/um) between 0-200 Gy induces a 24-fold increase overbaseline of mutations (high LET) and 11-fold increase over baseline mutations (low LET).
Nagashima et al., 2018	Hamster cells (GM06318-10) exposed to x-rays in the 0-1 Gy. Response of 19.0 ± 6.1 mutants per 109 survivors.
Albertini et al., 1997	T-lymphcytes isolated from human peripheral blood exposed to low LET gamma-rays (0.5-5 Gy) and high LET radon gas (0-1 Gy). Response of 7.0x10-6 mutants/Gy (Gamma-rays 0-2 Gy), 54x10-6 mutants/Gy (Gamma-rays 2-4 Gy) and 63x10-6 mutants/Gy (0-1 Gy).
Dubrova et al., 2002	Observation of paternal ESTR mutation rates in CBAH mice following exposure to acute low LET X-rays (0-1 Gy), chronic low LET gamma-rays (0-1 Gy) and chronic high LET neutrons (0-0.5 Gy). Modelled response of y = mx + C, values of (m,C): X-rays: (0.338, 0.111), Gamma-rays: (0.373±0.082, 0.110), Neutrons: (1.135±0.202, 0.136).
McMahon et al., 2016	Study of HPRT gene in Chinese hamster cells following exposure to radiation of 1-6 Gy. Observation of 0.2 mutations in HPRT gene per 104 cells and 0.1 point mutations per 104 cells (1 Gy). At 6 Gy, observation of 1.5 mutations in the HPRT gene per 104 cells and 0.4 point mutations per 104 cells.

Response-response relationship

Inadequate Repair of DSB

There is evidence of a response-response relationship between inadequate DNA repair and increased frequency of mutations. When exposed to a radiation stressor, there was a positive relationship between the radiation dose and the DSB misrepair rate, and between

the mutation rate and the radiation dose (Mcmahon et al., 2016). Similarly, there was a negative correlation found between NER and the mutation densities at specific genomic regions in cancer patients. Specifically, inadequate NER resulted in more mutations in the promoter DHS and the TSS, but normal NER at DHS flanking regions resulted in fewer mutations (Perera et al., 2016).

Time-scale

Inadequate Repair of DSB

Two studies were used to provide data regarding the time scale of DNA repair and the appearance of mutations. In a study using plants, DNA damage was evident immediately following radiation with 30 Gy of radiation; 50% of repairs were complete by 51.7 minutes, 80% by 4 hours, and repair was completed by 24 hours post-irradiation. Although no mutational analysis was performed during the period of repair, irradiated plants were found to have increased mutations when they were examined 2 - 3 weeks later (Ptácek et al., 2001). Both DNA repair and mutation frequency were examined at the same time in a study comparing simple and complex ligation of linearized plasmids. In this study, repaired plasmids were first detected between 6 - 12 hours for simple ligation events and between 12 - 24 hours for more complex ligation events; this first period was when the most error-free rejoining occurred in both cases. After this initial period of repair until its completion at 48 hr, repair became increasingly more erroneous such that mutations were found in more than half of the repaired plasmids at 48 hr regardless of the type of required ligation (Smith et al., 2001).

Known modulating factors
Not identified.

Known Feedforward/Feedback loops influencing this KER
Not identified.

References

Albertini, R.J. et al. (1997), "Radiation Quality Affects the Efficiency of Induction and the Molecular Spectrum of HPRT Mutations in Human T Cells", 148(5 Suppl):S76-86.

Amundson, S.A. & D.J. Chen (1996), "Ionizing Radiation-Induced Mutation of Human Cells With Different DNA Repair Capacities.", Adv. Space Res. 18(1-2):119-126.

Anderson, C.W. 1993, "DNA damage and the DNA-activated protein kinase.", Trends Biochem. Sci. 18(11):433–437. doi:10.1016/0968-0004(93)90144-C.

Arai, T., Kelly, V.P., Minowa, O., Noda, T., Nishimura, S. (2002), High accumulation of oxidative DNA damage, 8-hydroxyguanine, in Mmh/Ogg1 deficient mice by chronic oxidative stress, Carcinogenesis, 23:2005-2010.

Basu, A.K. and J.M. Essigmann (1990), "Site-specific alkylated oligodeoxynucleotides: Probes for mutagenesis, DNA repair and the structure effects of DNA damage", *Mutation Research*, 233: 189-201.

Beranek, D.T. (1990), "Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents", *Mutation Research*, 231(1): 11-30.

Bétermier, M., P. Bertrand & B.S. Lopez (2014), "Is Non-Homologous End-Joining Really an Inherently Error-Prone Process?", PLoS Genet. 10(1). doi:10.1371/journal.pgen.1004086.

Bhowmick, R., S. Minocherhomji & I.D. Hickson (2016), "RAD52 Facilitates Mitotic DNA Synthesis Following Replication Stress", Mol. Cell., 64(6):1117-1126.

Dahle, J., Brunborg, G., Svendsrud, D., Stokke, T., Kvam, E. (2008), Overexpression of human OGG1 in mammalian cells decreases ultraviolet A induced mutagenesis, Cancer Lett, 267:18-25.

Deem, A. et al. (2011), "Break-Induced Replication Is Highly Inaccurate", PLoS Biol., 9(2):e1000594, doi: 10.1371/journal.pbio.1000594.

Dilley, R.L. et al. (2016), "Break-induced telomere synthesis underlies alternative telomere maintenance", Nature, 539:54-58.

Douglas, G.R., J. Jiao, J.D. Gingerich, J.A. Gossen and L.M. Soper (1995), "Temporal and molecular characteristics of mutations induced by ethylnitrosourea in germ cells isolated from seminiferous tubules and in spermatozoa of lacZ transgenic mice", *Proc. Natl. Acad. Sci. USA*, 92(16): 7485-7489.

Dubrova, Y.E. et al. (2002), "Elevated Minisatellite Mutation Rate in the Post-Chernobyl Families from Ukraine.", Am. J. Hum. Genet. 71(4): 801-809.

Ellison, K.S., E. Dogliotti, T.D. Connors, A.K. Basu and J.M. Essigmann (1989), "Site-specific mutagenesis by O6-alkyguanines located in the chromosomes of mammalian cells: Influence of the mammalian O6-alkylguanine-DNA alkyltransferase", *Proc. Natl. Acad. Sci. USA*, 86: 8620-8624.

Feldmann, E. et al. (2000), "DNA double-strand break repair in cell-free extracts from Ku80-deficient cells : implications for Ku serving as an alignment factor in non-homologous DNA end joining.", Nucleic Acids Res. 28(13):2585–2596.

Fitzgerald, D.M., P.J. Hastings, and S.M. Rosenberg (2017), "Stress-Induced Mutagenesis: Implications in Cancer and Drug Resistance", Ann. Rev. Cancer Biol., 1:119-140, doi: 10.1146/annurev-cancerbio-050216-121919.

Getts, R.C. & T.D. Stamato (1994), "Absence of a Ku-like DNA end binding activity in the xrs double-strand DNA repair-deficient mutant.", J. Biol. Chem. 269(23):15981–15984.

Gocke, E. and L. Muller (2009), "In vivo studies in the mouse to define a threhold for the genotoxicity of EMS and ENU", *Mutat. Res.*, 678, 101-107.

Gorbunova, V. (1997), "Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions.", Nucleic Acids Res. 25(22):4650–4657. doi:10.1093/nar/25.22.4650.

Hartlerode, A.J. & R. Scully (2009), "Mechanisms of double-strand break in somatic mammalian cells.", Biochem J. 423(2):157–168. doi:10.1042/BJ20090942.Mechanisms.

Kaina, B., M. Christmann, S. Naumann and W.P. Roos (2007), "MGMT: Key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents", *DNA Repair*, 6: 1079–1099.

Klungland, A., Rosewell, I., Hollenbach, S., Larsen, E., Daly, G., Epe, B., Seeberg, E., Lindahl, T., Barnes, D. (1999), Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage, Proc Natl Acad Sci USA, 96:13300-13305.

Kramara, J., B. Osia & A. Malkova (2018), "Break-Induced Replication: The Where, The Why, and The How", Trends Genet. 34(7):518-531, doi: 10.1016/j.tig.2018.04.002.

Kuhne, M., K. Rothkamm & M. Lobrich (2000), "No dose-dependence of DNA double-strand break misrejoining following a -particle irradiation.", Int. J. Radiat. Biol. 76(7):891-900

Lieber, M.R. (2008), "The mechanism of human nonhomologous DNA End joining.", J Biol Chem. 283(1):1–5. doi:10.1074/jbc.R700039200.

Little, J.B. (2000), "Radiation carcinogenesis.", Carcinogenesis 21(3):397-404 doi:10.1093/carcin/21.3.397.

Lobrich, M. et al. (2000), "Joining of Correct and Incorrect DNA Double-Strand Break Ends in Normal Human and Ataxia Telangiectasia Fibroblasts.", 68(July 1999):59–68. doi:DOI: 10.1002/(SICI)1098-2264(200001)27:1<59::AID-GCC8>3.0.CO;2-9.

Mao Z, Bozzella M, Seluanov A, Gorbunova V. 2008. DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells. Cell Cycle. 7(18):2902–2906. doi:10.4161/cc.7.18.6679.

Matuo Y, Izumi Y, Furusawa Y, Shimizu K. 2018. Mutat Res Fund Mol Mech Mutagen Biological e ff ects of carbon ion beams with various LETs on budding yeast Saccharomyces cerevisiae. Mutat Res Fund Mol Mech Mutagen. 810(November 2017):45–51. doi:10.1016/j.mrfmmm.2017.10.003.

Mcmahon SJ, Schuemann J, Paganetti H, Prise KM. 2016. Mechanistic Modelling of DNA Repair and Cellular Survival Following Radiation-Induced DNA Damage. Nat Publ Gr.(April):1–14. doi:10.1038/srep33290.

Minocherhomji, S. et al. (2015), "Replication stress activates DNA repair synthesis in mitosis", Nature, 528(7581):286-290.

Minowa, O., Arai, T., Hirano, M., Monden, Y., Nakai, S., Fukuda, M., Itoh, M., Takano, H., Hippou, Y., Aburatani, H., Masumura, K., Nohmi, T., Nishimura, S., Noda, T. (2000), Mmh/Ogg1 gene inactivation results in accumulation of 8-hydroxyguanine in mice, Proc Natl Acad Sci USA, 97:4156-4161.

Muller, L., E. Gocke, T. Lave and T. Pfister (2009), "Ethyl methanesulfonate toxicity in Viracept – A comprehensive human risk assessment based on threshold data for genotoxicity", *Toxicology Letters*, 190: 317-329.

Nagashima, H. et al. (2018), "Induction of somatic mutations by low-dose X-rays : the challenge in recognizing radiation-induced events.", J. Radiat. Res., Na 59(October 2017):11–17. doi:10.1093/jrr/rrx053.

NRC (1990), "Health Effects of Exposure to Low Levels of Ionizing Radiation", (BEIR V).

O'Brien, J.M., A. Williams, J. Gingerich, G.R. Douglas, F. Marchetti and C.L. Yauk CL. (2013), "No evidence for transgenerational genomic instability in the F1 or F2 descendants of Muta™Mouse males exposed to N-ethyl-N-nitrosourea", *Mutat. Res.*, 741-742:11-7

O'Brien, J.M., M. Walker, A. Sivathayalan, G.R. Douglas, C.L. Yauk and F. Marchetti (2015), "Sublinear response in lacZ mutant frequency of Muta™ Mouse spermatogonial

stem cells after low dose subchronic exposure to N-ethyl-N-nitrosourea", Environ. Mol. Mutagen., 56(4): 347-55.

Pegg, A.E., (2011), "Multifaceted roles of alkyltransferase and related proteins in DNA repair, DNA damage, resistance to chemotherapy, and research tools", *Chem. Res. Toxicol.*, 24(5): 618-639.

Perera, D. et al. (2016), "Differential DNA repair underlies mutation hotspots at active promoters in cancer genomes.", Nature 532, 259-263.

Petrini, J.H.J., D.A. Bressan & M.S. Yao (1997), "The RAD52 epistasis group in mammalian double strand break repair.", Semin Immunol. 9(3):181–188. doi:10.1006/smim.1997.0067

Philippin, G., J. Cadet, D. Gasparutto, G. Mazon, R.P. Fuchs (2014), "Ethylene oxide and propylene oxide derived N7-alkylguanine adducts are bypassed accurately in vivo", *DNA Repair (Amst)*, 22:133-6.

Pouget, J.P. & S.J. Mather (2001), "General aspects of the cellular response to low- and high-LET radiation.", Eur. J. Nucl. Med. 28(4):541–561. doi:10.1007/s002590100484

Preston, R. et al. (2013), "Uncertainties in estimating health risks associated with exposure to ionising radiation", Journal of Radiological Protection, Vol.33/3, IOP Publishing, Bristol, https://doi.org/10.1088/0952-4746/33/3/573.

Ptácek, O. et al. (2001), "Induction and repair of DNA damage as measured by the Comet assay and the yield of somatic mutations in gamma-irradiated tobacco seedlings.", Mutat Res. 491(1-2):17–23

Puchta, H. (2005), "The repair of double-strand breaks in plants: Mechanisms and consequences for genome evolution.", J. Exp. Bot. 56(409):1–14. doi:10.1093/jxb/eri025

Pzoniak, A., L. Muller, M. Salgo, J.K. Jone, P. Larson and D. Tweats (2009), "Elevated ethyl methansulfonate in nelfinavir mesylate (Viracept, Roche): overview", *Aids Research and Therapy*, 6: 18.

Rathmell, W.K. & G. Chu (1994), "Involvement of the Ku autoantigen in the cellular response to DNA double-strand breaks.", Proc. Natl. Acad. Sci. 91(16):7623–7627. doi:10.1073/pnas.91.16.7623

Rodriguez, G.P., Song, J.B., Crouse, G.F. (2013), In Vivo Bypass of 8-oxodG, PLoS Genetics, 9:e1003682.

Sage, E. & N. Shikazono (2017), "Free Radical Biology and Medicine Radiation-induced clustered DNA lesions : Repair and mutagenesis ☆.", Free Radic. Biol. Med. 107(December 2016):125–135. doi:10.1016/j.freeradbiomed.2016.12.008

Saini, N. et al. (2017), "Migrating bubble during break-induced replication drives conservative DNA synthesis", Nature, 502:389-392.

Sakofsky, C.J. et al. (2015), "Translesion Polymerases Drive Microhomology-Mediated Break-Induced Replication Leading to Complex Chromosomal Rearrangements", Mol. Cell, 60:860-872.

Sassa, A., Kamoshita, N., Kanemaru, Y., Honma, M., Yasui, M. (2015), Xeroderma Pigmentosum Group A Suppresses Mutagenesis Caused by Clustered Oxidative DNA Adducts in the Human Genome, PLoS One, 10:e0142218.

Seager, A., Shah, U., Mikhail, J., Nelson, B., Marquis, B., Doak, S., Johnson, G., Griffiths, S., Carmichael, P., Scott, S., Scott, A., Jenkins, G. (2012), Pro-oxidant Induced DNA

Damage in Human Lymphoblastoid Cells: Homeostatic Mechanisms of Genotoxic Tolerance, Toxicol Sci, 128:387-397.

Shelby, M.D. and K.R. Tindall (1997), "Mammalian germ cell mutagenicity of ENU, IPMS and MMS, chemicals selected for a transgenic mouse collaborative study. Mutation Research 388(2-3):99-109.

Shrivastav, N., D. Li and J.M. Essignmann (2010), "Chemical biology of mutagenesis and DNA repair: cellular response to DNA alkylation", *Carcinogenesis*, 31(1): 59-70.

Shuman, S. & M.S. Glickman (2007), "Bacterial DNA repair by non-homologous end joining.", Nat. Rev. Microbiol. 5(11):852–861. doi:10.1038/nrmicro1768.

Singer, B., F. Chavez, M.F. Goodman, J.M. Essigman and M.K. Dosanjh (1989), "Effect of 3' flanking neighbors on kinetics of pairing of dCTP or dTTP opposite O6-methylguanine in a defined primed oligonucleotide when Escherichia coli DNA polymerase I is used", *Proc. Natl. Acad. Sci. USA*, 86(21): 8271-8274.

Sishc-Brock J. & A.J. Davis (2017), "The role of the core non-homologous end joining factors in carcinogenesis and cancer.", Cancers (Basel). 9(7). doi:10.3390/cancers9070081.

Smith, J. et al. (2001), "The influence of DNA double-strand break structure on end-joining in human cells.", Nucleic Acids Res. 29(23):4783–4792

Smith, J. et al. (2003), "Impact of DNA ligase IV on the ® delity of end joining in human cells.", Nucleic Acids Res., 31(8):2157-67. doi:10.1093/nar/gkg317

Tan, X., Grollman, A., Shibutani, S. (1999), Comparison of the mutagenic properties of 8-oxo-7,8-dihydro-2'-deoxyadenosine and 8-oxo-7,8-dihydro-2'-deoxyguanosine DNA lesions in mammalian cells, Carcinogenesis, 20:2287-2292.

Thomas, A.D., G.J. Jenkins, B. Kaina, O.G. Bodger, K.H. Tomaszowski, P.D. Lewis, S.H. Doak and G.E. Johnson (2013), "Influence of DNA repair on nonlinear dose-responses for mutation", *Toxicol. Sci.*, 132(1): 87-95.

van Delft, J.H. and R.A. Baan (1995), "Germ cell mutagenesis in lambda lacZ transgenic mice treated with ethylnitrosourea; comparison with specific-locus test", *Mutagenesis*, 10(3): 209-214.

Waters, C.A. et al. (2014), "The fidelity of the ligation step determines how ends are resolved during nonhomologous end joining.", Nat Commun. 5:1–11. doi:10.1038/ncomms5286.

Wessendorf P. et al. (2014), "Mutation Research / Fundamental and Molecular Mechanisms of Mutagenesis Deficiency of the DNA repair protein nibrin increases the basal but not the radiation induced mutation frequency in vivo.", Mutat. Res. - Fundam. Mol. Mech. Mutagen. 769:11–16. doi:10.1016/j.mrfmmm.2014.07.001.

Wilson, T.E. & M.R. Lieber (1999), "Efficient Processing of DNA Ends during Yeast Nonhomologous End Joining.", J. Biol. Chem. 274(33):23599–23609. doi:10.1074/jbc.274.33.23599.

Relationship: 1912: Inadequate DNA repair leads to Increase, Chromosomal aberrations

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations	adjacent	High	Low
Deposition of energy leading to lung cancer	adjacent	High	Low
Deposition of energy leading to occurrence of cataracts	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
rat	Rattus norvegicus	Low	NCBI
mouse	Mus musculus	Low	NCBI
human	Homo sapiens	Low	NCBI

Life Stage Applicability

Term	Evidence
All life stages	Low

Sex Applicability

Sex	Evidence
Unspecific	Low

This KER is plausible in all life stages, sexes, and organisms with chromosomes. The majority of the evidence is from in vitro fetal human male models. No in vivo evidence was found to support the relationship.

Key Event Relationship Description

Cells are exposed to many insults, both endogenous and exogenous, that may cause damage to their DNA. In response to this constant threat, cells have accordingly evolved many different pathways for repairing DNA damage (Pfeiffer & Goedecke, 2000; Hoeijmakers, 2001; Jeggo & Markus, 2015; Rode et al., 2016). When confronted with double strand breaks (DSBs), there are two common repair pathways employed by the cell: homologous recombination (HR) and non-homologous end-joining (NHEJ). In HR, a homologous sequence on the sister chromatid is used as a template, ensuring that no sequence information is lost over the course of repair (Ferguson & Alt, 2001; van Gent et al., 2001; Hoeijmakers, 2001; Jeggo & Markus, 2015; Schipler & Iliakis, 2013; Venkitaraman, 2002). However, this method of DNA repair may result in a loss of an allele leading to heterozygosity. This may occur if a non-homologous chromosome with an erronous sequence is used as the template instead of the homologous chromosome, thus leading to a loss of genetic information (Ferguson & Alt, 2001). Despite this possible error, HR is generally considered to be one of the more accurate methods of DNA repair because it does make use of a template (van Gent et al., 2001; Schipler & Iliakis, 2013; Venkitaraman,

2002). NHEJ, however, does not use a template and is generally described as being error-prone. This repair process allows for the direct religation of broken DNA ends without using template DNA as a guide (van Gent et al., 2001; Ferguson & Alt, 2001; Hoeijmakers, 2001; Venkitaraman, 2002; Schipler & Iliakis, 2013; Jeggo & Markus, 2015; Rode et al., 2016). In lieu of a template, NHEJ utilizes rapid repair kinetics to religate the broken ends before they have time to diffuse away from each other (Schipler & Iliakis, 2013), thus fitting two 'sticky' DNA ends back together (Danford, 2012). There is not, however, an inherent quality control check; as such, sections of DNA may be gained or lost, or the wrong ends may be rejoined (Schipler & Iliakis, 2013). There are two versions of this error-prone DNA repair: classical or canonical NHEJ (c-NHEJ), and alternative NHEJ (alt-NHEJ) (Schipler & Iliakis, 2013). It is not well understood when or why one pathway is selected over another (Venkitaraman, 2002; Schipler & Iliakis, 2013). It has been proposed that the phase of the cell cycle may influence repair pathway choice (Ferguson & Alt, 2001; Vodicka et al., 2018); for instance, HR is generally more common than NHEJ when sister chromatids are available in S and G2 phases of the cell cycle (Hoeijmakers, 2001; Venkitaraman, 2002). If both HR and c-NHEJ are compromised, alt-NHEJ, which is slower and more error-prone than c-NHEJ, is thought to be the stand-by repair mechanism (Schipler & Iliakis, 2013).

If these repair processes are not able to properly and adequately repair the DNA, this may lead to the formation of chromosomal aberrations (CAs). CAs are defined as abnormalities in the chromosome structure, often due to losses or gains of chromosome sections or the entire chromosomes itself (van Gent et al., 2001; Durante & Cucinotta, 2008). These abnormalities can take many different forms and can be classified according to several different schemes. CAs can be defined as breaks, which occur when DSBs are not rejoined, or as exchanges, where the presence of multiple DSBs results in misrejoining of the DNA ends (Danford, 2012; Registre et al., 2016). CA classes can be further subdivided into chromosome-type aberrations (CSAs) that affect both sister chromatids, and chromatid-type aberrations (CTAs), affecting only one chromatid (Danford, 2012). Examples of CSAs include chromosome-type breaks, centric ring chromosomes, and dicentric chromosomes (which have two centromeres), while CTAs refer to chromatid-type breaks and chromatid exchanges (Hagmar et al., 2004; Bonassi et al., 2008). Other types of CAs that may occur include micronuclei (MN; small nucleus-like structures containing chromosome fragments enclosed by a nuclear membrane (Fenech & Natarajan, 2011; Doherty et al., 2016)), nucleoplasmic bridges (NPBs; a stretch of chromatin enclosed by a nuclear membrane that is attached to two centromeres (Fenech & Natarajan, 2011; Russo et al., 2015)), nuclear buds (NBUDs; a MN that is still connected to the nucleus by nucleoplasmic material (Fenech & Natarajan, 2011)), and copy number variants (CNVs; base pair to megabase pair deletions or duplications of chromosomal segments (Russo et al., 2015)). CAs may also be classified as stable aberrations (translocations, inversions, insertions and deletions) and unstable aberrations (dicentric chromosomes, acentric fragments, centric rings and MN) (Hunter & Muirhead, 2009; Qian et al., 2016).

Evidence Supporting this KER

Overall Weight of Evidence: Low

Biological Plausibility

There is strong biological plausibility for a relationship between inadequate repair of DNA damage and a corresponding increase in CAs. This is evident in a variety of reviews on the

topic (van Gent et al., 2001; Hoeijmakers, 2001; Povirk, 2006; Weinstock et al., 2006; Lieber et al., 2010; Rode et al., 2016).

The two most common methods used to repair DSBs, which are one of the most dangerous types of DNA lesions, are HR and NHEJ. Mechanisms for these two methods of DNA repair are well-established and have been thoroughly reviewed (Van Gent et al. 2001; Hoeijmakers 2001; Lieber et al. 2010; Jeggo and Markus 2015; Sishc and Davis 2017). Briefly, HR requires a template DNA strand to repair damage and thus facilitates the invasion of the damaged strand with matching sequences on homologous chromosomes or sister chromatids (Ferguson and Alt 2001; van Gent et al. 2001; Hoeijmakers 2001; Jeggo and Markus 2015; Schipler and Iliakis 2013; Venkitaraman 2002). Proteins involved in the HR pathway include the RAD50 proteins, MRE11, BRCA1, and BRCA2 (Ferguson and Alt 2001; van Gent et al. 2001; Hoeijmakers 2001; Jeggo and Markus 2015; Venkitaraman 2002). In contrast to this relatively accurate form of DNA repair (van Gent et al. 2001; Schipler and Iliakis 2013; Venkitaraman 2002), NHEJ is more error-prone. It does not require a template to guide repair, but simply re-ligates broken DNA ends back together (Van Gent et al. 2001; Ferguson and Alt 2001; Hoeijmakers 2001; Lieber et al. 2010; Schipler and Iliakis 2013; Jeggo and Markus 2015; Rode et al. 2016; Sishc and Davis 2017) Proteins used during NHEJ include the DNA-PK complex (encompassing Ku70, Ku80 and DNA-PK_{cs}), and the XRCC4-DNA ligase IV complex (Ferguson & Alt, 2001; van Gent et al., 2001; Hoeijmakers, 2001; Jeggo & Markus, 2015; Sishc & Davis, 2017).Interestingly, NHEJ is used in the biological V(D)J recombination process because its error-prone mechanism allows immune cells to develop a wide range of unique receptors for antigen detection (Ferguson & Alt, 2001; van Gent et al., 2001; Lieber, 2010).

Damaged DNA in the form of DSBs can follow three possible outcomes: the DSB is rejoined accurately, with no changes made to the genome; the DSB is left unrepaired and the broken ends diffuse away from each other; or the DSB is repaired incorrectly such that the repaired version is different from the original version (Danford, 2012). These latter two errors in repair (the complete absence of repair or inaccurate repair) could arise due to interruptions to the repair process that allow time for the broken ends to move away from each other before they can be rejoined, mis-rejoining of the wrong DNA ends, or post-repair alterations that modify the junction point and lead to nucleotide losses (Schipler and Iliakis 2013). Unrepaired DSBs are the direct origin of micronuclei and unrepaired chromosomes correlated with radiosensitivity (Foray et al., 2016). Errors occurring during repair may be particularly detrimental if they interrupt or modify key genes, or if chromosome structures are created that cannot undergo proper mitosis (Schipler and Iliakis 2013).

The classic model of CA formation has centered around misrepair of DSBs. Exposing DNA to an endogenous or exogenous DSB-inducing agent directly results in DSBs, which may either persist or be misrepaired by inadequate repair mechanisms; in the event of this erroneous repair, CAs often eventually result (Bignold, 2009; Danford, 2012; Schipler & Iliakis, 2013). Another model has been proposed that suggests CAs may actually be due to failure of enzymes that tether the DNA strands during the repair of enzyme-induced breaks in the DNA; the various pathways in the cell would likely employ assorted tethering enzymes. The numerous types of CAs would thus result from different kinds of tethering errors (Bignold 2009).

The type of CA that results may be dependent on the timing of inadequate repair. For example, DSBs may result in CSAs or CTAs depending on when during the cell cycle the DSB was incurred. DSBs that are not repaired before DNA duplication in the S-phase will be replicated and result in CTAs. If DSBs are incurred after the S-phase and are improperly repaired, CSAs will result (Danford, 2012; Registre et al., 2016; Vodicka et al., 2018). Similarly, CNVs are thought to be induced during the DNA replication phase. Although the mechanism is not well studied, it has been suggested that stress during replication, in particular stalling replication forks, prompt microhomology-mediated mechanisms to overcome the replication stall, which often results in duplications or deletions. Two models that have been proposed to explain this mechanism include the Fork Stalling and Template Switching (FoSTeS) model, and the Microhomology-Mediated Break-Induced Replication (MMBIR) model (Lee et al. 2007; Hastings et al. 2009; Arlt et al. 2012; Arlt et al. 2014; Wilson et al. 2015).

The type of CA may also be dependent on the type of erroneous repair that occurs. Deletions or chromosome breaks may occur when DSBs are left unrepaired (Danford 2012). Deletions may also occur when nucleotides are removed at the junctions (Schipler and Iliakis 2013) or when the wrong DNA ends are religated (Venkitaraman 2002). Ligation of the incorrect ends of DNA DSBs may also lead to translocations or dicentrics (Ferguson & Alt, 2001; Lieber, 2010; Povirk, 2006; Venkitaraman, 2002). This type of error may occur when there are two or more DSBs in close proximity to each other that are misrejoined, thus resulting in the exchange of genetic material between two chromosomes (Ferguson and Alt 2001; Povirk 2006). NHEJ has been shown to play a significant role in the generation of chromosomal exchanges (Lieber 2010; Povirk 2006; Weinstock et al. 2006). Evidence for this comes from analysis of breakpoint junctions, which typically have little to no chromosomal homology when NHEJ repair is used (Povirk 2006; Weinstock et al. 2006); this was demonstrated in studies using translocation reporters (reviewed in Weinstock et al., 2006). There are, however, two types of NHEJ. c-NHEJ has been shown to suppress exchanges (Simsek and Jasin 2010), which may be due to its relatively rapid repair kinetics (Schipler and Iliakis 2013). Chromosomal exchanges are thus suggested to originate more often from alt-NHEJ (Simsek and Jasin 2010; Zhang and Jasin 2011; Schipler and Iliakis 2013).

NHEJ is also thought to mediate the formation of other types of CAs. Based on analysis of breakpoint junctions in lung adenocarcinoma samples where reciprocal inversions were found between genes *RET* and *KIF5B/CCDC6*, the majority of the inversions were thought to be induced by NHEJ (Mizukami et al. 2014). Chromothripsis, which refers to a single event that results in a massive number of CAs localized to a single or very few chromosomes (Russo et al. 2015; Leibowitz et al. 2015; Rode et al. 2016), may also be linked to NHEJ. The single catastrophic event sparking chromothripsis likely induces a large quantity of DSBs, essentially shattering the chromosome(s). These DSBs are then processed mainly by the error-prone NHEJ, which results in a large number of CAs, including chromosomal rearrangements, CNVs, and loss of heterozygosity (Leibowitz et al. 2015; Rode et al. 2016).

Fusing two broken chromosomes may lead to the formation of dicentric chromosomes, which are characterized by the presence of two centromeres. Dicentrics may also be formed by telomere-to-telomere end fusions (Fenech and Natarajan 2011; Rode et al. 2016). Telomeres, composed of TTAGGG repeats, are important structures that protect the ends of chromosomes and ensure accurate replication (Ferguson and Alt 2001; Hoeijmakers 2001; Vodicka et al. 2018); these nucleoprotein structures are shortened (Vodicka et al. 2018) by approximately 100 base pairs after each division, and are only replenished in cell

types expressing the enzyme telomerase (Hoeijmakers 2001). If the telomeres become critically short, they can be mistaken for broken DNA ends by DNA repair machinery, and thus may be 'repaired' by fusing the ends of two chromosomes together (Ferguson and Alt 2001; Vodicka et al. 2018).

Dicentrics can also contribute to other types of CAs. During mitosis, the two centromeres of a dicentric chromosome may be pulled to opposite ends of the cell by mitotic spindle (Ferguson and Alt 2001; Fenech and Natarajan 2011; Leibowitz et al. 2015; Rode et al. 2016). Because the ends of the chromosomes are fused, this can lead to the formation of an anaphase chromatin bridge between the daughter cells (Russo et al. 2015; Leibowitz et al. 2015; Rode et al. 2016). If this bridge persists beyond anaphase, it may become enclosed in a nucleoplasmic membrane along with the nucleus, thus generating a NPB (Fenech and Natarajan 2011). Eventually, however, these bridges do break (Ferguson and Alt 2001; Fenech and Natarajan 2011; Russo et al. 2015; Leibowitz et al. 2015; Rode et al. 2016); the break is nearly always uneven, meaning that one daughter cell will be missing genetic material and one will have extra genetic material (Fenech and Natarajan 2011). These fragments, with their 'sticky' ends from the break, may further propagate the formation of CAs by being ligated inappropriately to another chromosome. Thus the cycle, known as the breakage-fusion-bridge (BFB) cycle, is propagated and further contributes to chromosomal instability (Ferguson and Alt 2001; Fenech and Natarajan 2011; Russo et al. 2015; Leibowitz et al. 2015; Rode et al. 2016).

MN may also be formed during this BFB cycle. When the anaphase bridges break, the remaining chromosome fragments may be packaged by a nuclear membrane into its own mini nucleus, thus, forming an MN. MN may also enclose acentric chromosome fragments, chromatid fragments, or even entire chromosomes that were not properly segregated during mitosis (Fenech and Natarajan 2011; Doherty et al. 2016). Similar to MN in structure are NBUDs; the only difference between these two structures is that NBUDs are still attached to the nucleus by nucleoplasmic material. A NBUD is formed if there is amplified DNA that needs to be removed; this amplified material is often segregated from the other DNA at the periphery of the nuclear membrane and excluded from the nucleus by budding, resulting in a NBUD. Additionally, NBUDs may also result from NPB breakages (Fenech and Natarajan 2011).

Empirical Evidence

There is moderate empirical evidence supporting the relationship between inadequate DNA repair and the frequency of CAs. The evidence presented below is summarized in table 6, here (click link). Several reviews discuss evidence that associates these two events (Ferguson and Alt 2001; van Gent et al. 2001; Sishc and Davis 2017; Venkitaraman 2002). Overall, however, there is weak empirical evidence available supporting a dose and incidence concordance, little empirical evidence supporting a temporal concordance, and strong empirical evidence supporting essentiality for this KER.

Dose and Incidence Concordance

There is weak empirical evidence available that directly examines the dose and incidence concordance between DNA repair and CAs within the same study. There are, however, studies that use an ionizing radiation stressor to examine dose concordance of either inadequate DNA repair in response to radiation exposure, or CA frequencies in response to irradiation. In an analysis that amalgamated results from several different studies conducted using *in vitro* experiments, the rate of DSB misrepair was revealed to increase in a dose-

dependent fashion from 0 - 80 Gy (Mcmahon et al. 2016). Similarly, there was a clear correlation between radiation dose (i.e., increasing amounts of energy deposition) between 0 - 10 Gy and different clastogenic endpoints (Thomas et al. 2003; Tucker et al. 2005A; George et al. 2009; Arlt et al. 2014; Balajee et al. 2014; Lin et al. 2014; Suto et al. 2015; Mcmahon et al. 2016) . Overall, this suggests that exposure to radiation may increase both inadequate repair of DNA damage and the frequency of CAs in a dose-dependent fashion. More studies, however, are required to better assess the dose and incidence concordance of this KER.

Temporal Concordance

Temporal concordance between inadequate DNA repair and CA frequency is not well established. One study using cells pretreated with a DNA-PK inhibitor and irradiated with gamma rays found that DNA repair and MN were evident when they were assessed at 3 hours post-irradiation and 24 hours post-irradiation, respectively (Chernikova et al. 1999). This study does therefore suggest that there may be temporal concordance between these two events. Other radiation-based studies examining these two events separately, however, do not provide clear evidence of temporal concordance between DNA repair and CA frequency.

Essentiality

Numerous studies demonstrate that simply knocking-out one gene involved in DNA repair, without any other added stressor, is enough to increase the frequency of CAs in several types of cells (Karanjawala et al. 1999; Patel et al. 1998; Wilhelm et al. 2014). Further strengthening this relationship, addition of a DSB-inducing stressor to these DNA repair knock-out cells also significantly increases CA levels relative to wild-type cells receiving the same treatment (Cornforth and Bedford 1985; Simsek and Jasin 2010; Lin et al. 2014; Mcmahon et al. 2016). Essentiality is also supported by looking at patients with the recessive genetic disorder ataxia-telangiectasia (AT), in which mutations in the gene encoding the ATM protein results in defects in DNA damage repair signaling. One recent study showed that in comparison to control patients, patients with AT had increased levels of several types of CAs. Upon exposure to a DSB-inducing stressor such as ionizing radiation, these patients showed further increases in these aberrations as well as a significant increase in the levels of complex aberrations as compared to controls (Bucher et al. 2021).

Inhibitor studies have also found similar results. Two strains of wild-type cells that were treated with hydroxyurea, which is known to inhibit DNA repair, both had increased CAs relative to untreated wild-type cells (Wilhelm et al. 2014). Similarly, immortalized myeloid cell lines, cells from patients with myeloid leukemia, and cells from healthy donors were all found to have dose-dependent decreases in ligation efficiency after being treated with increasing doses of antibodies against various NHEJ proteins (Heterodimer et al. 2002). In addition, cells that were pretreated with DNA-PK inhibitor wortmannin prior to being irradiated were found to have not only increased levels of MN, but also decreased rates of DNA rejoining (Chernikova et al. 1999). A study by White et al. (2010) reported similar results under ATM and DNA-PK inhibition, where IR-exposed human lung cells treated for 1 hour with a reversible inhibitor of either enzyme exhibited an elevated level of Cas at all tested doses of IR, compared to the non-inhibited, IR-exposed cells 48 hours post-exposure. These findings demonstrated that even a transient inhibition of ATM or DNA-PK can sufficiently disrupt DNA damage repair and lead to CAs (White et al., 2010).

Functional defects in the factors involved in NER due to mutations or knock-down/out have shown concordant results that are supportive of this KER. For example, UV61 Chinese hamster ovary cells (homogolous to human Cockayne syndrome group B cells), which have a defective ERCC6 gene, are incapable of repairing UV-induced cyclobutane pyrimidine dimers due to the compromised transcription-coupled NER (TCR). Following UV exposure, a significantly higher percentage of TCR-defective UV61 cells contained Cas than another Chinese hamster ovary cell line that is TCR-proficient (Proiettis de Santis et al., 2001). Down-regulation of xeroderma pigmentosum group A–complementing protein (XPA) by RNA interference (RNAi) in human bladder cancer cells was observed to significantly increase the baseline frequency of MN, nucleoplasmic bridges, and nuclear buds, while overexpression of XPA by transfection in the same cell line reduced these levels below that in the control cells (Zhi et al., 2017). Both studies support the essentiality of inadequate repair in the occurence of chromosomal aberrations.

A rescue experiment provided further evidence of the essential role DNA repair plays in relation to CA frequencies. Inhibition of NHEJ through knocking out either Ku70 or Xrcc4 resulted in higher CA frequencies in the form of translocations; when Xrcc4 was transiently expressed in Xrcc4-/- cells, translocations were significantly decreased by 5-fold (Simsek and Jasin 2010). This provides strong evidence that the NHEJ repair pathway plays an important role in the formation of CAs, specifically translocations.

Uncertainties and Inconsistencies

Uncertainties in this KER are as follows:

1. In an experiment using both wild-type and *Ku70*-/- cells, knock-down of alt-NHEJ protein CtIP resulted in significantly decreased translocations in both cell types. When CtIP expression was rescued, translocation frequencies in these cells also returned to normal levels. This however, is opposite to results obtained in a similar study, where knock-out of Ku70 or Xrcc4 led to increased translocation frequency, and Xrcc4 rescue experiments resulted in decreased translocations (Simsek and Jasin 2010). It should be noted that alt-NHEJ is thought to be the major repair pathway responsible for generating translocations (Simsek and Jasin 2010; Zhang and Jasin 2011; Schipler and Iliakis 2013).
2. There is currently discussion regarding the accuracy of HR relative to NHEJ. Traditionally HR has been considered the more accurate type of DNA repair, while NHEJ is classically described as error-prone. There is emerging evidence, however, suggesting that HR may in fact be a mutagenic process. Evidence supporting HR as an error-prone repair pathway has been reviewed (Guirouilh-barbat et al. 2014).

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage is lacking. Most data are derived from studies that examined DSB misrepair rates or CA rates in response to a radiation stressor. In terms of inadequate DNA repair, the rate of DSB misrepair was found to be approximately 10 - 15% at 10 Gy of radiation (Lobrich et al. 2000); this rate increased to 50 - 60% at a radiation exposure of 80 Gy (Kuhne et al. 2000; Lobrich et al. 2000; Mcmahon et al. 2016). It is not known, however, how this rate of inadequate repair directly relates to CA frequency. Overall, more studies are required that directly assess this relationship.

Response-response relationship
Studies directly examining the response-response relationship between inadequate repair and CA frequency are lacking. One study examined both DNA repair and CA frequency in cells exposed to DNA-PK inhibitor wortmannin. There was a negative, approximately linear relationship between DNA repair and increasing wortmannin dose, and a positive, approximately linear relationship between MN frequency and increasing wortmannin dose; this suggests that as adequate DNA repair declines, CA frequency increases (Chernikova et al. 1999). More studies are required, however, that directly quantify the response-response relationship between inadequate DNA repair and CAs.

Time-scale
The time scale between inadequate DNA repair and the increased frequency of CAs has not been well-established. Most data come from studies that assess only one of these events in relation to a radiation stressor rather than assessing the timing of the events relative to each other. More studies are thus required that directly assess this relationship.

Known modulating factors
DNA repair is a modulating factor in this KER. The progression from “Inadequate DNA repair” to “Increase, Chromosomal aberrations” only occurs when "Increase, DNA strand breaks" (KE 1635) precedes "Inadequate DNA repair", which indicates that DNA strand breaks could not be repaired.

Known Feedforward/Feedback loops influencing this KER
Not identified.

References

Arlt, M.F. et al. (2014), "NIH Public Access", 55(2):103–113. doi:10.1002/em.21840.

Arlt, M.F., T.E. Wilson & T.W. Glover (2012), "Replication Stress and Mechanisms of CNV Formation.", Curr. Opin. Genet. Dev. 22(3):204–210. doi:10.1016/j.gde.2012.01.009.

Balajee, A.S. (2014), "Multicolour FISH analysis of ionising radiation induced micronucleus formation in human lymphocytes.", Mutagenesis, 29(6):447–455. doi:10.1093/mutage/geu041.

Bignold, L.P. (2009), "Mechanisms of clastogen-induced chromosomal aberrations : A critical review and description of a model based on failures of tethering of DNA strand ends to strand-breaking enzymes.", Mutat. Res. 681:271–298. doi:10.1016/j.mrrev.2008.11.004.

Bonassi, S. (2008), "Chromosomal aberration frequency in lymphocytes predicts the risk of cancer: results from a pooled cohort study of 22 358 subjects in 11 countries.", Carninogenesis, 29(6):1178–1183. doi:10.1093/carcin/bgn075.

Bucher, M. et al., (2021), Analysis of chromosomal aberrations and γH2A.X foci to identify radiation-sensitive ataxia-telangiectasia patients., Mutat Res., Jan-Feb;861-862

Chernikova, S.B., R.L. Wells & M.M. Elkind (1999), "Wortmannin Sensitizes Mammalian Cells to Radiation by Inhibiting the DNA-Dependent Protein Kinase-Mediated Rejoining of Double-Strand Breaks.", Radit. Res. 151(2):159–166. doi: 10.2307/3579766.

Cornforth, M. & J. Bedford (1985), "On the Nature of a Defect in Cells from Individuals with Ataxia-Telangiectasia.", Science 227(4694):1589–1591. doi:10.1126/science.3975628.

Danford, N. (2012), "The Interpretation and Analysis of Cytogenetic Data.", Methods Mol. Biol. 817:93-120, doi:10.1007/978-1-61779-421-6.

Doherty, A., S.M. Bryce & J.C. Bemis (2016), "The In Vitro Micronucleus Assay.", Elsevier Inc.

Durante, M. and F. Cucinotta. (2008), “Heavy ion carcinogenesis and human space exploration”, Nature Reviews Cancer, Vol.8/6, Nature Portfolio, Berlin, https://doi.org/10.1038/nrc2391.

Fenech, M. & A.T. Natarajan (2011), "Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. 26(1):125–132. doi:10.1093/mutage/geq052.

Ferguson, D.O. & F.W. Alt (2001), "DNA double strand break repair and chromosomal translocation: Lessons from animal models.", Oncogene, 20(40):5572–5579. doi: 10.1038/sj.onc.1204767.

Foray, N., M. Bourguignon and N. Hamada. (2016), “Individual response to ionizing radiation”, Mutation Research - Reviews in Mutation Research, Vol.770, Elsevier, Amsterdam, https://doi.org/10.1016/j.mrrev.2016.09.001.

van Gent D.C., J.H.J. Hoeijmakers & R. Kanaar (2001), "Chromosomal stability and the DNA double-stranded break connection.", Nat. Rev. Genet. 2(3):196–206. doi:10.1038/35056049.

George, K.A. et al. (2009), "Dose Response of γ Rays and Iron Nuclei for Induction of Chromosomal Aberrations in Normal and Repair-Deficient Cell Lines Dose Response of c Rays and Iron Nuclei for Induction of Chromosomal Aberrations in Normal and Repair-Deficient Cell Lines." Radit. Res., 171(6):752–763. doi:10.1667/RR1680.1.

Guirouilh-barbat, J. et al. (2014), "Is homologous recombination really an error-free process?", Front Genet. 5:175. doi:10.3389/fgene.2014.00175.

Hagmar, L. et al. (2004), "Impact of Types of Lymphocyte Chromosomal Aberrations on Human Cancer Risk: Results from Nordic and Italian Cohorts.", Cancer Res. 64(6):2258–2263. doi: 10.1158/0008-5472.CAN-03-3360.

Hastings, P.J., G. Ira & J.R. Lupski (2009), "A Microhomology-Mediated Break-Induced Replication Model for the Origin of Human Copy Number Variation.", 5(1). doi:10.1371/journal.pgen.1000327.

Heterodimer, K. et al. (2002), "Myeloid Leukemias Have Increased Activity of the Nonhomologous End-Joining Pathway and Concomitant DNA Misrepair that Is Dependent on the Ku70/86 Heterodimer.", Cancer Res. 62(10):2791-7.

Hunter, N. & C.R. Muirhead (2009), "Review of relative biological effectiveness dependence on linear energy transfer for low-LET radiations Review of relative biological effectiveness dependence.", J. Radiol. Prot. doi:10.1088/0952-4746/29/1/R01.

Jeggo, P.A. & L. Markus (2015), "How cancer cells hijack DNA double-strand break repair pathways to gain genomic instability.", Biochem. J., 471(1):1–11. doi:10.1042/BJ20150582.

Karanjawala, Z.E. et al. (1999), "The nonhomologous DNA end joining pathway is important for chromosome stability in primary fibroblasts.", Curr. Biol. 9(24):1501-4. doi: 10.1016/S0960-9822(00)80123-2.

Kozbenko, T. et al. (2022), "Deploying elements of scoping review methods for adverse outcome pathway development: a space travel case example", International Journal of Radiation Biology, 1–12. https://doi.org/10.1080/09553002.2022.2110306

Kuhne, M., K. Rothkamm & M. Lobrich (2000), "No dose-dependence of DNA double-strand break misrejoining following a -particle irradiation.", Int. J. Radiat. Biol. 76(7):891-900

Lee, J.A., C.M.B. Carvalho & J.R. Lupski (2007), "A DNA Replication Mechanism for Generating Nonrecurrent Rearrangements Associated with Genomic Disorders.", Cell. 131(7):1235–1247. doi:10.1016/j.cell.2007.11.037.

Leibowitz, M.L., C. Zhang & D. Pellman (2015), "Chromothripsis: A New Mechanism for Rapid Karyotype Evolution.", Annu. Rev. Genet. 49:183-211, doi:10.1146/annurev-genet-120213-092228.

Lieber, M.R. et al. (2010), "Nonhomologous DNA End Joining (NHEJ) and Chromosomal Translocations in Humans.", Subcell. Biochem., 50:279-96 doi:10.1007/978-90-481-3471-7.

Lin, Y. et al. (2014), "Differential Radiosensitivity Phenotypes of DNA-PKcs Mutations Affecting NHEJ and HRR Systems following Irradiation with Gamma-Rays or Very Low Fluences of Alpha Particles.", PLoS One. 9(4):2–11. doi:10.1371/journal.pone.0093579.

Lobrich, M. et al. (2000), "Joining of Correct and Incorrect DNA Double-Strand Break Ends in Normal Human and Ataxia Telangiectasia Fibroblasts.", 68(July 1999):59–68. doi:DOI: 10.1002/(SICI)1098-2264(200001)27:1<59::AID-GCC8>3.0.CO;2-9.

Manova, V. & D. Gruszka (2015), "DNA damage and repair in plants - from models to crops.", Front Plant Sci. 6(October):885. doi:10.3389/fpls.2015.00885.

McMahon, S.J. et al. (2016), "Mechanistic Modelling of DNA Repair and Cellular Survival Following Radiation-Induced DNA Damage.", Nat. Publ. Gr.(April):1–14. doi:10.1038/srep33290.

Mizukami, T. et al. (2014), "Molecular Mechanisms Underlying Oncogenic RET Fusion in lung adenocarcinoma", J. Thorac. Oncol. 9(5):622–630. doi:10.1097/JTO.0000000000000135.

Patel, K.J. et al. (1998), "Involvement of Brca2 in DNA Repair.", Mol. Cell. 1(3):347-57. doi: 10.1016/S1097-2765(00)80035-0.

Pfeiffer, P. & W. Goedecke (2000), "Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations.", Mutagenesis 15(4):289-302. doi: http://dx.doi.org/10.1093/mutage/15.4.289.

Povirk, L.F. (2006), "Biochemical mechanisms of chromosomal translocations resulting from DNA double-strand breaks.", DNA Repair (Amst.) 5(9-10):1199–1212. doi:10.1016/j.dnarep.2006.05.016.

Proietti De Santis, L., C. L. Garcia, A. S. Balajee, G. T. Brea Calvo, L. Bassi, & F. Palitti (2001), "Transcription coupled repair deficiency results in increased chromosomal

aberrations and apoptotic death in the UV61 cell line, the Chinese hamster homologue of Cockayne's syndrome B", Mutat Res, 485(2): 121–132.

Qian, Q. et al. (2016), "Effects of Ionising Radiation on Micronucleus Formation and Chromosomal Aberrations in Chinese.", Radiat. Prot. Dosimetry 168(2):197–203. doi: 10.1093/rpd/ncv290

Registre, M., R. Proudlock & N. Carolina (2016), "The In Vitro Chromosome Aberration Test.", Elsevier Inc. Genetic Toxicology Testing, pp.207-267. doi: 10.1016/B978-0-12-800764-8.00007-0.

Rode, A. et al. (2016), "Chromothripsis in cancer cells: An update.", Int. J. Cancer, 2333:2322–2333. doi:10.1002/ijc.29888.

Russo, A. et al. (2015), "Review Article Genomic Instability: Crossing Pathways at the Origin of Structural and Numerical Chromosome Changes.", Envrion. Mol. Mutagen. 56(7):563-580. doi:10.1002/em.

Schipler, A. & G. Iliakis (2013), "DNA double-strand – break complexity levels and their possible contributions to the probability for error-prone processing and repair pathway choice.", Nucleic Acids Res., 41(16):7589–7605. doi:10.1093/nar/gkt556.

Simsek, D. & M. Jasin (2010), "Alternative end-joining is suppressed by the canonical NHEJ component Xrcc4/ligase IV during chromosomal translocation formation", Nat. Struct. Mol. Bio. 17(4):410–416. doi:10.1038/nsmb.1773.

Sishc, B.J. & A.J. Davis (2017), "The Role of the Core Non-Homologous End Joining Factors in Carcinogenesis and Cancer.", Cancers (Basel), 9(7) pii E81, doi:10.3390/cancers9070081.

Suto, Y. et al. (2015), "Construction of a cytogenetic dose – response curve for low-dose range gamma-irradiation in human peripheral blood lymphocytes using three-color FISH", Mut. Res. / Gen. Tox. and Environ. Mut. 794:32–38.

Thomas, P., K. Umegaki & M. Fenech (2003), "Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay.", Mutagenesis, 18(2):187-194, doi:10.1093/mutage/18.2.187.

Tucker, J.D. et al. (2005), "Persistence of Chromosome Aberrations Following Acute Radiation: I, PAINT Translocations, Dicentrics, Rings, Fragments, and Insertions.", Environ. Mol. Mutagen, 45(2-3):229-249. doi:10.1002/em.20090.

Varga, T. & P.D. Aplan (2005), "Chromosomal aberrations induced by double strand DNA breaks.", DNA Repair (Amst). 4(9):1038–1046. doi:10.1016/j.dnarep.2005.05.004.

Venkitaraman, A.R. (2002). "Cancer susceptibility and the Functions of BRCA1 and BRCA2.", Cell 108(2):171–182.

Vodicka, P. et al. (2018), "Genetic variation of acquired structural chromosomal aberrations.", Mutat. Res. Gen. Tox. En. 836(May):13–21. doi:10.1016/j.mrgentox.2018.05.014.

Weinstock, D.M. et al. (2006), "Modeling oncogenic translocations: Distinct roles for double-strand break repair pathways in translocation formation in mammalian cells.", DNA Repair (Amst.) 5(9-10):1065–1074. doi:10.1016/j.dnarep.2006.05.028.

White, J. S., S. Choi, & C. J. Bakkenist (2010), "Transient ATM kinase inhibition disrupts DNA damage-induced sister chromatid exchange", Sci Signal, 3(124): ra44. https://doi.org/10.1126/scisignal.2000758.

Wilhelm, T. et al. (2014), "Spontaneous slow replication fork progression elicits mitosis alterations in homologous recombination-deficient mammalian cells.", Proc. Natl. Acad. Sci. 111(2):763-768. doi:10.1073/pnas.1311520111.

Wilson, J.W. et al. (2015), "The effects of extremely low frequency magnetic fields on mutation induction in mice.", Mutat Res - Fundam Mol Mech Mutagen. 773:22–26. doi:10.1016/j.mrfmmm.2015.01.014.

Zhang, Y. & M. Jasin (2011), "An essential role for CtIP in chromosomal translocation formation through an alternative end-joining pathway.", Nat Publ Gr. 18(1):80–84. doi:10.1038/nsmb.1940.

Zhi, Y., H. Ji, J. Pan, P. He, X. Zhou, H. Zhang, Z. Zhou, & Z. Chen (2017), "Downregulated XPA promotes carcinogenesis of bladder cancer via impairment of DNA repair", Tumour Biol, 39(2): 1010428317691679.

List of Non Adjacent Key Event Relationships

Relationship: 1913: Increase, Oxidative DNA damage leads to Increase, DNA strand breaks

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations	non-adjacent	Moderate	Low
Deposition of energy leading to occurrence of cataracts	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human	Homo sapiens	Moderate	NCBI
mice	Mus sp.		NCBI
rat	Rattus norvegicus	Low	NCBI

Life Stage Applicability

Term	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate
Male	Low

This KER is plausible in all life stages, sexes, and organisms with DNA. The majority of the evidence is from in vivo male rats and human male adolescent in vitro models.

Key Event Relationship Description

The repair of oxidative DNA lesions produced by exposure to reactive oxygen species (ROS) involves excision repair, where a damaged base is removed by glycosylases, a strand break is generated 5' to the apurinic/apyrimidinic (AP) site by lyases and endonucleases, and finally, a new strand is synthesized across the break. Although these strand breaks are mostly transient under normal conditions, elevated levels of oxidative DNA lesions can increase the early AP lyase activities generating a higher number of SSBs that can be more persistent (Yang et al., 2004; Yang et al., 2006). These SSBs can exacerbate the DNA damage by interfering with the replication fork causing it to collapse, and ultimately becoming double strand breaks (DSBs).

Evidence Supporting this KER

Overall Weight of Evidence: Low

Biological Plausibility

The mechanism of repair of oxidative DNA damage in humans is well-established and numerous literature reviews are available on this topic (Berquist and Wilson III, 2012; Cadet and Wagner, 2013). Oxidative DNA damage is mostly repaired via base excision repair (BER) and via nucleotide excision repair (NER) to a lesser extent. With an increase in oxidative DNA lesions, the more glycosylase and lyase activities occur, introducing SSBs at a higher rate than at homeostasis. It is highly plausible that an increase in SSBs also increases the risk for DSBs, which are more difficult to repair accurately. Previous studies have reported thresholded dose-response curves in oxidative DNA damage and attributed these observations to failed repair at the inflection point on the curve, *thus allowing strand breaks to accumulate* (Gagne et al., 2012; Seager et al., 2012). When DNA bases sustain oxidative damage via ROS through base oxidation or deletion, this creates small nicks in the DNA strand (Cannan & Pederson, 2016). The bases guanine and adenine are most vulnerable to oxidative damage due to their low oxidation potentials (Fong, 2016). The mechanism of repair, BER, will work to fix these SSBs. If there are multiple SSBs close together in space and time, there will be many sites of BER occurring close together that can cause strain on the strand and result in the conversion of the SSBs to DSBs prior to completion of repair (Cannan & Pederson, 2016).

Empirical Evidence

The studies collected frequently address both dose and temporal concordance within a single study. Thus, we have not split out these types of empirical data by sub-headings. Instead, we indicate what evidence is available both in vitro and in vivo.

<u>In vitro studies</u>

- Concentration concordance in the formation of oxidative DNA lesions and strand breaks in HepG2 cells treated with nodularin (ROS-inducing substance (Bouaicha and Maatouk, 2004)) (Lankoff et al., 2006):
    - A concentration-dependent increase in oxidative lesions and strand breaks was observed after 6, 12, and 24h of treatment using Fpg-modified and regular comet assays, respectively.
        - At 6h, the increase in oxidative lesions was significant at 2.5, 5, and 10 µg/mL, while the increase strand breaks was significant at 5 and 10 µg/mL.
        - At 12 and 24 h, the increase in lesions was significant from 1 µg/mL and above, while significant increase in strand breaks occurred from 2.5 µg/mL and above.
    - At all time points, significant increase in oxidative DNA lesions occurred at a lower concentration than DNA strand breaks.
    - These results demonstrate the concentration concordance in the formation of oxidative DNA lesions and DNA strand breaks.
- Concentration and temporal concordance in human glioblastoma LN-229 cells treated with artesunate, a ROS inducing agent (Berdelle et al., 2011).
    - Concentration and time dependent increases in oxidative lesions were observed using the +Fpg comet test and immunofluorescence staining of 8-oxo-dG.

        - Significant increases in oxidative lesions were observed in cells treated with 25 µg/ml after 6 and 24 hours of treatment, but not 2 and 4 hours, using the + Fpg comet. No increases were observed using - Fpg comet.
        - Concentration-dependent increases in oxidative lesions were observed at the 24 hour timepoint using the +Fpg comet (50 and 75 µg/ml).
        - Oxidative lesions were also measured using immunofluorescence staining of 8-OxodG. Significant increases in oxidative lesions were observed at 6 and 8 hours of continuous treatment with 15 ug/ml artesunate, but not 1 and 4 hours.
            - Upon removal of test chemical, 8-OxodG levels decreased, returning to negative control level after 6 hours.
    - Significant increases in strand breaks as measured by ɣH2AX were observed 2 and 10 hours after treatment (15 µg/ml).

- Deferme et al. (2013) exposed HepG2 cells to 100 µM menadione, 200 µM tert butylhydroperoxide, and 50 µM hydrogen peroxide for increasing durations (30 min, 1, 2, 4, 6, 8, 24 h). The temporal profiles of strand breaks and oxidative lesions were analyzed. The results shown below demonstrate incidence and temporal concordance in oxidative lesion formation and strand breaks (Deferme et al., 2013).
    - Strand breaks were measured by alkaline comet assay.
    - Oxidative DNA lesions were measured by Fpg-modified comet assay
    - Menadione: strand breaks and oxidative lesions increased in a time-dependent manner from 30 min to 4h, when both reached their maximum. The tail moment values of fpg-digested comets were significantly higher than those of no-fpg comets at 1, 2, and 4h, indicating that the induction of oxidative lesions was significant at these time points. After 4h, both strand breaks and oxidative lesions gradually decreased.
    - Tert butylhydroperoxide: From 30 min to 1h, both strand breaks and oxidative lesions increased and gradually decreased from 2 to 24h. Oxidative lesion induction was significant at both 30min and 1h.
    - Hydrogen peroxide: The highest amount of strand breaks and oxidative lesions occurred at 30 min. From 1h onward, the levels of both decreased. Notably, the induction of oxidative lesions was significant at 30min and also at 1h, despite the decrease from 30min.

- Rat alveolar epithelial type II cells (AECII) were isolated from neonatal Wistar rats within 24h of birth and cultured. Cells were then incubated under either normorxic conditions (21% O_2 and 5% CO_2) or hyperoxic conditions (90% O_2 and 5% CO_2) for 12, 24, 48 or 72h (Jin et al., 2015).
    - Time-dependent increases in 8-oxodG were detected by ELISA under hyperoxic conditions**;** the level of 8-oxodG at 24h was significantly higher than at 12h (p-value <0.05), and the level had further increased significantly when measured at 48h (p-value <0.05) and remained constant until 72h.
    - At all time points, the level of 8-oxodG in hyperoxic cells was signficantly higher than in normoxic cells.
    - Time-dependent increases in DNA strand breaks were also observed in hyperoxic cells in the alkaline comet assay. The Olive tail moment in hyperoxic cells was significantly higher than in normoxic cells at all time points. However, the time-dependent increase in strands breaks in hyperoxic cells was statistically significant only at 72h (p-value <0.01).
    - No change was observed in the level of DNA strands breaks or 8-oxodG in normoxic cells across all time points.

In vivo studies

- Concentration concordance in Wistar rats orally exposed to ochratoxin A (OTA) and fumonisin B1 (FB1), ROS inducing agents (Domijan et al., 2006).
    - Kidney cells of male Wistar rats were examined using the comet assay +/- Fpg after oral exposure to OTA for 15 days (5ng, 0.05 mg, 0.5 mg/kg b.w.) or FB1 for 5 days (200 ng, 0.05 mg, 0.5 mg/kg b.w.).
        - Significant increases in oxidative lesions were observed using +Fpg comet at all concentrations tested of both OTA and FB1
        - Significant increases were observed in strand breaks using the standard comet assay at all concentrations of both OTA and FB1.

Uncertainties and Inconsistencies
As demonstrated by the Domijan et al paper, results can be complicated by mixed MOA's. The comet results were positive with and without Fpg suggesting oxidative stress is not the only mechanism.

Quantitative Understanding of the Linkage

Alimited number of studies explored the quantitative correlation between oxidative DNA lesions and DNA strand breaks. There are computational models availabe that describe this relationship. Spassova et al. (2015) developed a simulated kinetic model of $KBrO_3$-induced oxidative DNA damage based on Michaelis-Menten enzyme kinetics to study the effect of BER on the shape of the dose-response curve of 8-oxo-dG lesions and strand breaks (Spassova et al., 2015).

- Both time and concentration dependence of the responses were explored.
- The time course simulation of a sustained exposure at various concentrations produced a sharp increase in 8-oxo-dG immediately following exposure.
    - The authors attributed this accumulation to lagged, inefficient repair.
- This increase was later followed by a steep decrease in 8-oxo-dG lesions, accompanied by a linear increase in SSBs.
    - The repair of adducts by BER, both successful and failed, are responsible for the decrease of 8-oxo-dG; the SSBs are generated as a result of repair failure.
- Moreover, the concentration-response model of 8-oxo-dG showed a thresholded curve, where no DNA damage was observed at low concentrations due to effective repair up to a certain concentration of $KBrO_3$ indicating insufficient repair at the inflection point.

Known Feedforward/Feedback loops influencing this KER
N/A

References

Berdelle, N., Nikolova, T., Quiros, S., Efferth, T., Kaina, B. (2011), Artesunate Induces Oxidative DNA Damage, Sustained DNA Double-Strand Breaks, and the ATM/ATR Damage Response in Cancer Cells, Mol Cancer Ther, 10:2224-2233.

Berquist, B., Wilson III, D. (2012), Pathways for Repairing and Tolerating the Spectrum of Oxidative DNA Lesions, Cancer Lett, 327:61-72.

Bouaicha, N., Maatouk, I. (2004), Microcystin-LR and nodularin induce intracellular glutathione alteration, reactive oxygene species production and lipid peroxidation in primary cultured rat hepatocytes, Toxicol Lett, 148:53-63.

Cadet, J., Wagner, J.R. (2013), DNA Base Damage by Reactive Oxygen Species, Oxidizing Agents, and UV Radiation, Cold Spring Harb Perspect Biol, 5:a012559.

Cannan, W. and D. Pederson. (2016), "Mechanisms and consequences of double-strand DNA break formation in chromatin", Journal of Cell Physiology, Vol.231/1, Wiley, Hoboken, https://doi.org/10.1002/jcp.25048.

Deferme, L., Briede, J.J., Claessen, S.M., Jennen, D.G., Cavill, R., Kleinjans, J.C. (2013), Time series analysis of oxidative stress response patterns in HepG2: A toxicogenomics approach , Toxicol, 306:24-34.

Domijan, A., Zeljezic, D., Kopjar, D., Peraica, M. (2006), Standard and Fpg-modified comet assay in kidney cells of ochratoxin A- and fumonisin B(1)-treated rats, Toxciol, 222:53-59.

Fong, C.W. (2016), "Platinum anti-cancer drugs: Free radical mechanism of Pt-DNA adduct formation and anti-neoplastic effect", Free Radical Biology and Medicine, Vol.95/June 2016, Elsevier, Amsterdam, https://doi.org/10.1016/j.freeradbiomed.2016.03.006.

Gagne, J., Rouleau, M., Poirier, G. (2012), PARP-1 Activation— Bringing the Pieces Together, Science, 336:678-279.

Jin, L., Yang, H., Fu, J., Xue, X., Yao, L., Qiao, L. (2015), Association between oxidative DNA damage and the expression of 8-oxoguanine DNA glycosylase 1 in lung epithelial cells of neonatal rats exposed to hyperoxia, Mol Med Rep, 11: 4079-4086.

Kozbenko, T. et al. (2022), "Deploying elements of scoping review methods for adverse outcome pathway development: a space travel case example", International Journal of Radiation Biology, 1–12. https://doi.org/10.1080/09553002.2022.2110306

Lankoff, A., Wojcik, A., Fessard, V., Meriluoto, J. (2006), Nodularin-induced genotoxicity following oxidative DNA damage and aneuploidy in HepG2 cells, Toxicol Lett, 164:239-248.

Seager, A., Shah, U., Mikhail, J., Nelson, B., Marquis, B., Doak, S., Johnson, G., Griffiths, S., Carmichael, P., Scott, S., Scott, A., Jenkins, G. (2012), Pro-oxidant Induced DNA Damage in Human Lymphoblastoid Cells: Homeostatic Mechanisms of Genotoxic Tolerance, Toxicol Sci, 128:387-397.

Spassova, M., Miller, D., Nikolov, A. (2015), Kinetic Modeling Reveals the Roles of Reactive Oxygen Species Scavenging and DNA Repair Processes in Shaping the Dose-Response Curve of KBrO3-Induced DNA Damage, Oxid Med Cell Longev, 2015:764375.

Yang, N., Chaudry, A., Wallace, S. (2006), Base excision repair by hNTH1 and hOGG1: A two edged sword in the processing of DNA damage in gamma-irradiated human cells, DNA Repair, 5:43-51.

Yang, N., Galick, H., Wallace, S. (2004), Attempted base excision repair of ionizing radiation damage in human lymphoblastoid cells produces lethal and mutagenic double strand breaks, DNA Repair, 3:1323-1334.

Relationship: 1914: Increase, Oxidative DNA damage leads to Increase, Mutations

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations	non-adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human	Homo sapiens		NCBI
rat	Rattus norvegicus		NCBI
mice	Mus sp.		NCBI

Life Stage Applicability

Term	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

DNA in any cell type is susceptible to oxidative damage due to endogenous (e.g., aerobic respiration) and exogenous (i.e., exposure to oxidants) oxidative insults. Resulting increase in mutation frequency has been described in both eukaryotic and prokaryotic cells.

Key Event Relationship Description

Oxidative DNA lesions such as 7, 8-dihydro-8oxo-deoxyGuanine (8-oxo-dG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPydG) are mutagenic because if they are not repaired they are able to form base pairs with dATP instead dCTP during replication. This can lead to permanent changes in the DNA sequence that is inherited by daughter cells with subsequent replication. G:C→T:A transversions are the most abundant base substitution attributed to oxidative DNA lesions (Cadet and Wagner, 2013).

Evidence Supporting this KER

Biological Plausibility

Mutagenicity of oxidative DNA lesions has been extensively studied; incorrect base insertion opposite unrepaired oxidative DNA lesions during replication is a well-established event.

For example, 8-oxo-dG and FaPydG, the two most prominent oxidative DNA lesions, are able to form base pairs with dATP, giving rise to G:C→T:A transversions with subsequent DNA synthesis (Gehrke et al., 2013; Freudenthal et al., 2013; Markkanen, 2017). Replicative DNA polymerases such as DNA polymerase α, δ, and ε (pol α, δ, ε) have a poor ability to extend the DNA strand past 8-oxo-dG:dCTP base pairs and may cause replication to stall or incorrectly insert dATP opposite 8-oxo-dG (Hashimoto et al., 2004; Markkanen et al., 2012). In stalled replication forks, repair polymerases may be recruited to perform translesion DNA synthesis (TLS). Human Y-family DNA polymerases (Rev 1, pol κ, ι, and η) are DNA repair polymerases mainly involved in TLS for stalled replication forks. However, TLS is not free of error and its accuracy differs for each repair polymerase. For example, it is known that pol κ and η perform TLS across 8-oxo-dG and often insert dATP opposite the lesion, generating G:C→T:A transversions. The error-prone nature of bypassing unrepaired oxidative lesions has been described in many previous studies and reviews (Greenberg, 2012; Maddukuri et al., 2014; Taggart et al., 2014; Sha et al., 2017).

Empirical Evidence

In vitro studies

- Concentration-dependent increase in oxidative lesions observed in TK6 human lymphoblastoid cells exposed to $KBrO_3$ and glucose oxidase (GOx; enzyme that produces H_2O_2) for 1 hour; increase in mutation frequency measured in TK assay after 14 days (3 days in non-selective medium and 11 days in selective medium) following 1 hour exposure corresponded with the concentration-response observed in oxidative lesions (Platel et al., 2011).
    - NOGEL could be determined in TK assay (KBrO3: 1.75 mM; bleomycin: 0.6µM; GOx: 1.17x10-5 units/mL) but not in the Fpg-modified comet assays (First statistically significant concentrations: KBrO3: 1 mM; bleomycin: 0.5µM; GOx: 1.08x10-5 units/mL)
        - These results indicate that statistically significant increases in oxidative lesions (measured in Fpg comet assay) occur at lower concentrations of the above three stressors than mutations measured by the Tk gene mutation assay at a later time point (after 14-day recovery)
        - Demonstrates concentration concordance in oxidative DNA lesions and mutation

In vivo studies

- Klungland et al. (1999) measured and compared the level of 8-oxodG in the liver of OGG1-null Big Blue mice and *Ogg1*+/+ Blue Blue mice at 13-15 weeks of age. (Klungland et al., 1999).
    - The amount of 8-oxodG in the OGG1-null mice was 1.7-fold higher than in wild-type mice at the time of measurement.
    - Spontaneous mutation frequencies in the liver of OGG1 null (*Ogg1* -/-) Big Blue mice and wild type (*Ogg1* +/+) Big Blue mice were measured at 10 and 20 weeks of age:
- At 10 weeks, mutation frequency increased by 2- to 3-fold in OGG1 -/- mice compared to the wild type mice. No further increase was observed at 20 weeks.
- Of the 16 base substitutions detected in *Ogg1* -/- mutant plaques analyzed by sequencing, 10 indicated G→T transversions.

- This study demonstrates that increased levels of oxidative DNA damage in the null mice was concordant with increased incidence of mutations.
- Unfried et al. (2002) measured the level of 8-oxodG and mutations in the omenta of rats exposed to crocidolite asbestos for various durations (Unfried et al., 2002).
    - Statistically significant increases in 8-oxodG were observed compared to control after 10 and 20 weeks of exposure.
    - The number of G→T transversions after 4, 12, and 24 weeks of exposure was significantly higher compared to control and G→T transversions were the most prominent base substitution in these samples.
    - This mutation spectrum supports that oxidative DNA lesions were the source of mutations.
- Five-week-old male gpt delta mice were given drinking water containing 85 ppm sodium arsenite for 3 weeks and sacrificed 2 weeks after administration was stopped (Takumi et al., 2014).
    - The gpt mutation assay and 8-OHdG quantification was performed using genomic DNA isolated from the liver
    - Significantly higher levels of 8-OHdG were observed in the arsenite group ($1.15/10^6$ dG) compared to the control group ($0.86/10^6$ dG)
    - Elevated mutation frequency was observed in the arsenite group with an average of 1.10×10^{-5}, compared to that of the control group (0.71×10^{-5})
    - G:C→T:A made up 46% of the mutations in the arsenite-fed mice
    - These data demonstrating a positive correlation between incidence of oxidative lesions in DNA and elevation in mutation frequency support that these events are associated, and the mutation spectrum further suggest that mutations were the result of oxidative lesions.

Uncertainties and Inconsistencies

The provided empirical evidence examined only the quantities of 8-oxo-dG and related the observed mutations to this oxidative lesion; the level of overall DNA oxidation is inferred from the level of 8-oxo-dG present. It is unclear how other oxidative DNA lesions such as FapyG, FapyA, and thymidine glycol contribute to the mutation spectra and frequencies.

References

Cadet, J., Wagner, J.R. (2013), DNA Base Damage by Reactive Oxygen Species, Oxidizing Agents, and UV Radiation, Cold Spring Harb Perspect Biol, 5:a012559.

Freudenthal, B., Beard, W., Wilson, S. (2013), DNA polymerase minor groove interactions modulate mutagenic bypass of a templating 8-oxoguanine lesion., Nucleic Acids Res, 41:1848-1858.

Gehrke, T., Lischke, U., Gasteiger, K., Schneider, S., Arnold, S., Muller, H., Stephenson, D., Zipse, H., Carell, T. (2013), Unexpected non-Hoogsteen–based mutagenicity mechanism of FaPy-DNA lesions, Nat Chem Biol, 9:455-461.

Greenberg, M. (2012), Purine Lesions Formed in Competition With 8-Oxopurines From Oxidative Stress, Acc Chem Res, 45:588-597.

Hashimoto, K., Tominaga, Y., Nakabeppu, Y., Moriya, M. (2004), Futile short-patch DNA base excision repair of adenine:8-oxoguanine mispair, Nucleic Acids Res, 32:5928-5934.

Klungland, A., Rosewell, I., Hollenbach, S., Larsen, E., Daly, G., Epe, B., Seeberg, E., Lindahl, T., Barnes, D. (1999), Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage, Proc Natl Acad Sci USA, 96:13300-13305.

Maddukuri, L., Ketkar, A., Eddy, S., Zafar, M., Eoff, R. (2014), The Werner syndrome protein limits the error-prone 8-oxo-dG lesion bypass activity of human DNA polymerase kappa, Nucleic Acids Res, 42:12027-12040.

Markkanen, E. (2017), Not breathing is not an option: How to deal with oxidative DNA damage, DNA Repair, 59:82-105.

Markkanen, E., Castrec, B., Vilani, G., Hubscher, U. (2012), A switch between DNA polymerases δ and λ promotes error-free bypass of 8-oxo-G lesions, Proc Natl Acad Sci USA, 27:931-940.

Platel, A., Nesslany, F., Gervais, V., Claude, N., Marzin, D. (2011), Study of oxidative DNA damage in TK6 human lymphoblastoid cells by use of the thymidine kinase gene-mutation assay and the *in vitro* modified comet assay: Determination of No-Observed-Genotoxic-Effect-Levels, Mutat Res, 726:151-159.

Sha, Y., Minko, I., Malik, C., Rizzo, C., Lloyd, R.S. (2017), Error-Prone Replication Bypass of the Imidazole Ring-Opened Formamidopyrimidine Deoxyguanosine Adduct, Envrion Mol Mutatgen, 58:182-189.

Taggart, D., Fredrickson, S., Gadkari, V., Suo, Z. (2014), Mutagenic Potential of 8-Oxo-7,8-dihydro-2′-deoxyguanosine Bypass Catalyzed by Human Y-Family DNA Polymerases, Chem Res Toxicol, 27:931-940.

Takumi, S., Aoki, Y., Sano, T., Suzuki, T., Nohmi, T., Nohara, K. (2014), In vivo mutagenicity of arsenite in the livers of gpt delta transgenic mice , Mutat Res, 760:42-47.

Unfried, K., Schurkes, C., Abel, J. (2002), Distinct Spectrum of Mutations Induced by Crocidolite Asbestos: Clue for 8-Hydroxydeoxyguanosine-dependent Mutagenesis in Vivo, Cancer Res, 62:104.

Relationship: 1931: Increase, DNA strand breaks leads to Increase, Mutations

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations	non-adjacent	High	Low
Deposition of energy leading to lung cancer	non-adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Term	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

DNA strand breaks and subsequent mutations can occur in any eukaryotic and prokaryotic cell. Any DNA strand break has potential to cause alterations in DNA sequence (e.g., deletions and insertions), whether it is due to insufficient or faulty repair.

Key Event Relationship Description

DNA single strand breaks (SSB) are generally repaired rapidly and efficiently. However, if left unrepaired, SSBs can interfere with replication and cause the replication fork to collapse resulting in double strand breaks (DSB). Multiple SSBs in close proximity to each other can also give rise to DSBs. DSBs can be repaired virtually error-free by homologous recombination (HR), which uses DNA sequence in the homologous chromosome or sister chromatid as a template for new strand synthesis (Polo and Jackson, 2011). Alternatively, the broken ends may be joined to other sites in the genome regardless of homology via non-homologous end joining (NHEJ), irreversibly altering the DNA sequence (deletion, addition, rearrangement). Because HR is a more time-consuming and labour-intensive process, larger proportions of DSBs are repaired via NHEJ than via HR (Mao et al., 2008a; Mao et al., 2008b).

Alterations in DNA sequence can also occur from structural damage to the chromosomes; observations of micronucleus indicate chromosomal aberrations and that a permanent loss of DNA segments has occurred.

Evidence Supporting this KER

The mechanisms by which strand breaks lead to mutations are very well studied and understood. Thus, we provide a small selection of empirical evidence below supporting this KER; i.e., we did not undertake and exhaustive literature search.

Biological Plausibility

The error-prone nature of DSB repair in eukaryotes has been described in numerous reviews. In mammalian and yeast cells, both HR and NHEJ can lead to alteration in DNA sequence; insertions, deletions, and translocations can arise from NHEJ and base substitutions can occur during the repair synthesis of HR (Hicks and Haber, 2010; Bunting and Nussenzweig, 2013; Byrne et al., 2014; Rodgers and McVey, 2016; Dwivedi and Haber, 2018; Hanscom and McVey, 2020).

Empirical Evidence

The mechanisms by which strand breaks lead to mutations are very well studied and understood. Thus, we provide a small selection of empirical evidence below supporting this KER; i.e., we did not undertake and exhaustive literature search.

<u>In vitro studies</u>

- Strand breaks and mutation frequencies were measured in TK6 cells after exposure to bleomycin and glucose oxidase (enzyme that generates H_2O_2) for 1 hour (Platel et al., 2011).
    - Concentration-dependent increases in strand breaks were measured using the alkaline comet assay.
    - At the same concentrations, mutation frequencies measured by TK gene mutation assay also showed a concentration-dependent increasing trend.
    - No Observed Genotoxic Effect Level was determined in TK assay (bleomycin: 0.6µM; GOx: $1.17x10^{-5}$ units/mL) while it couldn't be identified in comet assay, indicating that every tested concentration induced an increase in strand breaks (First statistically significant concentration: bleomycin: 1.5 µM; GOx: $1.08x10^{-5}$ units/mL).

- Spassova et al. (2013) combined the alkaline comet assay data from Luan et al. (2007) and Tk gene mutation assay data from Harrington-Brock et al. (2003) (Spassova et al., 2013).
    - Luan et al. treated TK6 cells with $KBrO_3$ for 4 hours and performed alkaline comet assay to measure strand breaks.
    - Harrington-Brock et al. treated L5178Y/$Tk^{+/-}$ mouse lymphoma cells with $KBrO_3$ for 4 hours and measured the Tk mutant frequency after a 13-day incubation.
    - Spassova et al. (2013) found no significant differences between the two experiments in regression analysis, thus, combined the datasets (same concentration range was used in both studies)
    - In both comet assay and Tk mutation assay, concentration-dependent increase in response was observed.
    - These results demonstrate the occurrence of DNA strand breaks followed by increase in mutations.

- Indirect measurement of mutations by measuring misrejoined DSBs in vitro
    - Rydberg et al. (2005) exposed GM38 human primary dermal fibroblasts to increasing doses of X-rays and linear electron transfer (LET) by nitrogen, helium, and iron ions.
    - DSBs were measured by pulsed field gel electrophoresis (PFGE)
        - Dose-dependent increase in DSBs was observed immediately following irradiation.
    - Misrejoining of ends was monitored using the Hybridization assay:
        - DNA is digested using a restriction enzyme and fractionated by PFGE.
        - ^{32}P-labeled probe for a 3.2-Mbp *NotI* restriction fragment is then used in Southern blotting to detect intact restriction fragments.
        - Failure to reconstitute the restriction fragment indicates incorrect joining of ends following DSBs and *altered DNA sequence.*
    - After 16 h of recovery following irradiation, Rydberg et al. observed a radiation dose-dependent increase in misrejoined DSBs in all four treatment groups.
    - A similar study by Kuhne et al. (2005) reported concordant results (Kuhne et al., 2005):
    - Subsequently, there was a dose-dependent increase in misrejoined DSBs 24h post irradiation.
    - Increasing doses of X-rays and γ rays immediately induced DSBs in primary human fibroblasts in a dose-dependent manner.
    - Alterations in the restriction fragment due to irradiation indicate changes in the DNA sequence (i.e., shorter fragments would suggest loss of DNA sequence), thus, induction of mutations (Rydberg et al., 2005; Kuhne et al., 2005).
    - These results demonstrate the concentration and temporal concordance in strand breaks leading to mutations.

- In a study by Kuhne et al. (2000), irradiated normal human fibroblasts were examined for both DSBs and the percentage of misrejoined DSBs (Kuhne et al., 2000).
    - Increasing doses of alpha-particle radiation from 0 – 80 Gy resulted in a linear, dose-dependent increase in the number of DSBs per mega base pair, as measured by the FAR assay.
    - Using X-ray radiation, the percentage of misrejoined DSBs were found to increase approximately linearly from 0 – 40 Gy doses per fraction. By 80 Gy, the rate of misrejoining plateaued at approximately 50%, and this plateau was maintained at X-ray doses between 80 and 320 Gy.
    - Overall, these results provide indirect evidence suggesting that elevated numbers of DSBs may lead to the formation of increasingly more mutations, as indicated by the corresponding increased number of misrejoined DSBs.

- Dikomey et al. (2000) performed a study using normal human skin fibroblasts that were irradiated with 200 kVp X-rays at doses ranging from 0 – 180 Gy, and then were examined for DSBs immediately following irradiation, and for non-repaired DSBs 24 hours after radiation exposure (Dikomey and Brammer, 2000).
    - As measured by constant field gel electrophoresis, there was a dose-dependent increase in the number of DSBs after exposure to X-rays doses of 0 – 80 Gy.
    - The number of non-repaired DSBs also increased with increasing radiation dose from 0 – 180 Gy. After 30 Gy, there were more non-repaired DSBs when cells were exposed to radiation with a high dose-rate (4 Gy/min) relative to those exposed to radiation with a low dose-rate (0.4 Gy/min).
    - These results suggest that there are increasing DSBs with increasing radiation dose, and that there are also an increasing number of DSBs that are not repaired

with increasing radiation dose. This is important as non-repaired DSBs may result in mutations in the genome.

- Both lung and dermal fibroblasts were irradiated with 80 kV X-rays at 23 Gy/min, and analyzed for the number of DSBs and the percentage of correctly rejoined DSBs in a study by (Lobrich et al., 2000).
    - Results from the FAR assay showed a linear increase in the number of DSBs in all cell lines for radiation doses ranging from 0 – 80 Gy.
    - After being irradiated with 80 Gy of X-rays, approximately 50% of the DSBs were correctly rejoined, as measured by the hybridization assay.
    - A dose-dependent increase in the number of rearrangements per mega base pair was found in cells irradiated with 0 – 80 Gy of X-rays.
    - The results of this study provide evidence of dose concordance, as the number of DSBs and the number of rearrangements both increase with increasing radiation dose.

In vivo studies

- Strand breaks and mutation frequencies were measured in the leaves of *Nicotiana tabacum* var. xanthi after the seedling plants were irradiated with 0 – 10 Gy doses of gamma-ray radiation (Ptacek et al., 2001).
    - DNA strand breaks in the leaves were measured using the Comet assay immediately following irradiation. Results of this assay showed a linear, dose-dependent increase in strand breaks, which were resolved by 24 hour post-irradiation.
    - Mutations in the leaves were measured when the seedling plants put out their 6^{th} or 7^{th} true leaves following irradiation. Similar to results found for radiation-induced strand breaks, there was a corresponding dose-dependent increase in the number of mutations per radiation dose.
    - These results demonstrate a dose concordance between DNA strands breaks and mutation frequency, and suggest a time concordance.

Uncertainties and Inconsistencies

In Kuhne et al. (2005) and Rydberg et al. (2005) studies provided above, mutation was not directly measured. The PFGE and hybridization assay detects a 3.2-Mbp restriction fragment from chromosome 21. Deviation of DNA restriction fragments from the 3.2-Mbp mark during electrophoresis suggests occurrence of breakage and failed reconstruction in this segment of chromosome 21; induction of mutations can be inferred from the change in the size of the restriction fragments. The remaining 22 chromosomes are not considered. This method may not be sensitive enough to detect small base changes.

Cell cycle can influence the repair pathway of DSBs and, thus, the risk of incorrect rejoining of broken ends. In G1 phase, NHEJ may be favoured, while in S, G2, or M phase, both HR and NHEJ have been observed to be active in repair (Mao et al., 2008b).

References

Bunting, S. & A. Nussenzweig (2013), "End-joining, Translocations and Cancer", Nat Rev Cancer, 13:443-454.

Byrne, M. et al. (2014), "Mechanisms of oncogenic chromosomal translocations", Ann. N.Y. Acad. Sci., 1310:89-97.

Dikomey, E. & I. Brammer (2000), "Relationship between cellular radiosensitivity and non-repaired double-strand breaks studied for different growth states, dose rates and plating conditions in a normal human Ž fibroblast line.", Int. J. Radiat. Biol., 76:773-781.

Dwivedi, G. & J.E. Haber (2018), "Assaying Mutations Associated With Gene Conversion Repair of a Double-Strand Break", Methods Enzymiol., 601:145-160.

Hanscom, T., & M. McVey (2020), “Regulation of Error-Prone DNA Double-Strand Break Repair and Its Impact on Genome Evolution”. Cells, 9(7): 1657. https://doi.org/10.3390/cells9071657

Hicks, W. & J.E. Haber (2010), "Increased Mutagenesis and Unique Mutation Signature Associated with Mitotic Gene Conversion", Nat. Rev. Cancer, 329:82-84.

Kuhne, M., K. Rothkamm & M. Lobrich (2000), "No dose-dependence of DNA double-strand break misrejoining following a -particle irradiation.", Int. J. Radiat. Biol. 76(7):891-900

Kuhne, M., G. Urban & M. Lo, (2005), "DNA Double-Strand Break Misrejoining after Exposure of Primary Human Fibroblasts to C K Characteristic X Rays, 29 kVp X Rays and Co g-Rays.", Radiation Research. 164(5):669-676. doi:10.1667/RR3461.1.

Lobrich, M. et al. (2000), "Joining of Correct and Incorrect DNA Double-Strand Break Ends in Normal Human and Ataxia Telangiectasia Fibroblasts.", 68(July 1999):59–68. doi: 10.1002/(SICI)1098-2264(200001)27:1<59::AID-GCC8>3.0.CO;2-9.

Mao, Z. et al. (2008a), "Comparison of nonhomologous end joining and homologous recombination in human cells.", DNA Repair, 7:1765-1771.

Mao, Z. et al. (2008b), "DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells.", Cell Cycle, 7:2902-2906.

McMahon, S.J. et al. (2016), "Mechanistic Modelling of DNA Repair and Cellular Survival Following Radiation-Induced DNA Damage.", Nat. Publ. Gr.(April):1–14. doi:10.1038/srep33290.

Platel, A. et al. (2011), "Study of oxidative DNA damage in TK6 human lymphoblastoid cells by use of the thymidine kinase gene-mutation assay and the *in vitro* modified comet assay: Determination of No-Observed-Genotoxic-Effect-Levels.", Mutat. Res., 726:151-159.

Polo, S. & S. Jackson (2011), "Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications.", Genes Dev., 25:409-433.

Ptacek, O. et al. (2001), "Induction and repair of DNA damage as measured by the Comet assay and the yield of somatic mutations in gamma-irradiated tobacco seedlings.", Mutat. Res., 491:17-23.

Rodgers, K. & M. McVey (2016), "Error-prone repair of DNA double-strand breaks.", J. Cell. Physiol., 231:15-24.

Rothkamm, K. & M. Lobrich (2003), "Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses.", PNAS, 100(9):5057-62. doi:10.1073/pnas.0830918100.

Rydberg, B. et al. (2005), "Dose-Dependent Misrejoining of Radiation-Induced DNA Double-Strand Breaks in Human Fibroblasts: Experimental and Theoretical Study for High- and Low-LET Radiation.", Radiat. Res. 163(5):526–534. doi:10.1667/RR3346.

Spassova, M. et al. (2013), "Dose-Response Analysis of Bromate-Induced DNA Damage andMutagenicity Is Consistent With Low-Dose Linear,Nonthreshold Processes", Environ. Mol. Mutagen., 54:19-35.

Relationship: 1939: Increase, DNA strand breaks leads to Increase, Chromosomal aberrations

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations	non-adjacent	High	Low
Deposition of energy leading to lung cancer	non-adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Term	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

DNA strand breaks and subsequent chromosomal aberrations can occur in any eukaryotic and prokaryotic cell.

Key Event Relationship Description

DNA strand breaks (single and double) can arise from endogenous processes (e.g., topoisomerase reaction, excision repair, and VDJ recombination) and exogenous insults (e.g., replications stressors, ionizing radiation, and reactive oxygen species). Single strand breaks (SSBs) are generally repaired rapidly without error. However, multiple SSBs in close proximity to each other and interference of replication by unrepaired SSBs can lead to double strand breaks (DSB). DSB are more difficult to repair and are more toxic than SSB (Kuzminov, 2001). DSBs may lead to chromosomal breakages that may permanently alter the structure of chromosomes (i.e., chromosomal aberrations) and cause loss of DNA segments.

Evidence Supporting this KER

Biological Plausibility

DNA strand breaks are a necessity for chromosomal aberrations to occur. However, not all strand breaks lead to clastogenic events as most of them is repaired rapidly by a variety of different repair mechanisms. DNA DSBs are the critical damage because they lead to chromosome breakage. It is well-understood that unrepaired DSBs can lead to chromosomal aberrations. Studies have demonstrated DSBs leading to irreversible structural damage; for example, treatment of cultured cells with replication stress-inducing agents such as hydroxyurea induced micronuclei that are positive for gamma-H2AX, a marker of DSBs (Xu et al., 2010). The link between DSBs and the importance of DSB repair processes, such as non-homologous end joining (NHEJ) and homologous recombination (HR), in preventing chromosomal aberrations/genomic instability is extensively discussed in literature and many reviews are available (van Gent et al., 2001; Ferguson and Alt, 2001; Hoeijmakers, 2001; Iliakis et al., 2004; Povirk, 2006; Weinstock et al., 2006; Natarajan and Palitti, 2008; Lieber et al., 2010; Mehta and Haber, 2014; Ceccaldi et al., 2016; Chang et al., 2017; Sishc and Davis, 2017; Brunet and Jasin, 2018).

In addition, attempted repair of DSBs can lead to chromosomal aberrations such as translocations; NHEJ is a recognized source of oncogenic translocations in human cancers (Ferguson and Alt, 2001; Weinstock et al., 2006; Byrne et al., 2014; Brunet and Jasin, 2018), and a contributor to the carcinogenic process (Hoeijmakers, 2001; Sishc and Davis, 2017). Other types of chromosomal aberrations can serve as indicators of genomic instability that can contribute to a variety of adverse health effects including neurodegeneration (Madabhushi et al., 2014).

Empirical Evidence

<u>In vitro studies demonstrating dose and temporal concordance</u>

- In the 2009 and 2011 studies by Platel et al. TK6 cells were exposed to bleomycin and glucose oxidase (H_2O_2-generating enzyme) for 1 hour at increasing concentrations (Platel et al., 2009; Platel et al., 2011).
    - Concentration-dependent increase in DNA strand breaks was measured using the alkaline comet assay 1 hr post-exposure
        - First statistically significant concentration: bleomycin: 0.5 µM; GOx: $1.08x10^{-5}$ units/mL
        - NOEL could not be defined, indicating that there was response at every tested concentration.
    - MN frequency was measured 23 hours post exposure; concentration-dependent increase in MN frequency was observed and NOEL was identified.
        - NOEL: bleomycin: 0.023 µM; GOx: $1.78x10^{-5}$ units/mL
        - All concentrations above the NOEL induced significant increases in MN frequency.
    - Thus, the data demonstrate temporal concordance for both stressors; lack of concordance in the concentration at which response for bleomycin occurs is likely due to differences in detection sensitivities between these assays.

- Strand breaks and chromosomal breakage were measured in V79 cells with the comet assay and the MN test after exposure to hyperbaric oxygen at 3 bar for different periods of time (Rothfuss et al., 1999).
    - Stand breaks were observed in the comet assay after treatment of 3 bar hyperbaric oxygen starting at treatment times of 30 mins. The effect increased constantly up to 180 min.
    - The MN frequency was measured 20 h post treatment and showed increasing numbers of MN starting at treatment times of 30 mins, being clearly increased at treatment times of 60 min up to 180 min.
    - These data demonstrate both dose- and temporal concordance in DNA strand breaks observed by comet assay and MN frequency.

- Lymphoblastoid cell lines were investigated with the comet assay and the MN test using gamma irradiation of 1 and 2 Gy (Trenz et al., 2003). Pulsed field gel electrophoresis was used additionally to investigate the occurrence of strand breaks (Trenz et al., 2005).
    - Strand breaks were shown in the comet assay in all cell lines tested, immediately after treatment with 1 and 2 Gy.
    - 40 h post treatment the cell lines were prepared for MN analysis: an increase in MN frequency was shown in all cell lines after treatment with 1 and 2 Gy.
    - Thus, the data demonstrate both temporal and dose concordance.

- Watters et al. (2009) treated mouse embryonic fibroblasts (MEFs) with bleomycin for 4 hours and conducted comparative investigations using the H2AX assay, the comet assay and the MN test (Watters et al., 2009).
    - The occurrence of DNA DSB was shown with the gamma-H2AX assay immediately following exposure. The number of foci increased up to 0.1 µg/ml; however, it was not statistically significant until 1 µg/ml and above.
    - The comet assay showed a continuous increase in tail moment immediately following exposure, showing more than 2-fold increase at 10 µg/ml, but did not reach statistical significance.
    - Significant increases in MN frequency was observed 26h post exposure (~1.5 cycles) at concentrations of 0.1µg/ml and above.
    - These data support temporal concordance; lack of concordance in the dose at which the endpoints reach statistical significance is likely the rest of different sensitivities of these assays.

- Using bleomycin as a stressor, Kawaguchi et al. monitored DNA strand breaks in TK6 human lymphoblastoid cells with the comet assay/modified comet assay using DNA repair inhibitors and monitored clastogenic events with the MN test after a treatment period of 2h (Kawaguchi et al., 2010).
    - In the regular alkaline comet assay an increase in DNA strand breaks was observed immediately following the 2h exposure, reaching significance at 12.5 µg/mL, and in the modified AraC/HU version at 6.25 µg/ml.
    - A statistically significant increase in MN frequency was observed 24 h after treatment at 5 µg/mL.
    - This provides support for temporal-concordance and the lack of dose-concordance is consistent with the increased sensitivity of the MN assay relative to the comet assay.

- Wild type and N-methylpurine DNA glycosylase (MPG)-deficient (*Mpg*-/-) Mouse embryonic fibroblasts (MEFs) were treated with increasing concentrations of methyl methane sulfonate (MMS) (0.5, 1, 1.5, 2.5 mM) for 1 hour (Ensminger et al., 2014).

- DSBs were measured as the number of γH2AX foci immediately following the exposure.
- There was a concentration-dependent increase in DSBs in wild type MEFs, and the increase was significantly larger in wild type compared to *Mpg*-/- cells at every concentration.
- Chromosomal aberrations (breaks and translocations) were monitored in metaphase spreads 24h following 1h 1 mM MMS treatment.
- At 1 mM MMS, the amount of chromatid breaks and translocations was significantly larger in wild type cells, compared to *Mpg*-/- cells, concordant with the observations in DSBs.
- The results support that increases in DSBs lead to increases in chromosomal aberrations.

- Dertinger et al. (2019) exposed TK6 cells to 34 diverse genotoxic chemicals over a range of concentrations for 24 hrs (Dertinger et al., 2019). At 4 and 24 hr time points cell aliquots were evaluated with the MultiFlow assay, which includes the gH2AX biomarker. At the 24 hr time point, remaining cells were evaluated with the in vitro MicroFlow assay, which includes %MN measurements.
  - Benchmark dose analyses were conducted to estimate Point of Departure values for MN and gamma-H2AX responses.
  - In vitro MN and gamma-H2AX BMD confidence intervals for 18 clastogens were graphed on cross system plots. Good correlations were observed for 24 hr MN and 24 hr gamma-H2AX (shown), as well as 24 hr MN and 4 hr gamma-H2AX (not shown).
  - Thus, the data demonstrate both temporal and dose concordance for these endpoints.

- Isolated lymphocytes and whole blood samples taken from four healthy, adult males were exposed to gamma-ray radiation at 20 cGy/minute at doses ranging from 0 – 50 cGy. Immediately following irradiation, DNA strand breaks were assessed using the comet assay and chromosomal aberrations were examined by cytogenetic analysis (Sudprasert et al., 2006).
  - In irradiated lymphocytes, there were dose-dependent increases in the number of DNA strand breaks, with significant increases in strand breaks evident from 5 – 50 cGy doses.
  - Irradiated whole blood samples showed significantly increased strand breaks by 10 cGy, but this level stayed relatively stable from 10 - 50 cGy.
  - Analysis of chromosomal aberrations in irradiated whole blood samples indicated dose-dependent increases in deletions and dicentric chromosomes across 50 cGy, with more deletions detected than dicentrics. All doses (5 – 50 cGy) showed significantly more aberrations than unirradiated controls.
  - The results of this study support dose concordance and are suggestive of time concordance.

- In a study by Chernikova et al. 1999, PL61 cells were exposed to radiation sensitizer/DNA repair inhibitor wortmannin prior to gamma-ray irradiation, and then analyzed for DSBs and micronuclei (indicative of chromosomal aberrations) (Chernikova et al., 1999).
  - DSB experiments were performed with cells treated with 25 µM of wortmannin + radiation, and with cells exposed only to radiation. In both cases, there was a linear, dose-dependent increase in the number of DSBs across radiation doses ranging from 0 – 60 Gy, as measured by the FAR assay. Wortmannin treatment did not affect the number of DSBs that were formed.

  - In terms of DNA repair, however, cells irradiated with 45 Gy of gamma-rays showed a dose-dependent decline in the percentage of DNA repair with increasing wortmannin concentrations from 0 – 25 µM.
  - Furthermore, cells treated with wortmannin + 2 Gy of radiation demonstrated a dose-dependent increase in the number of micronuclei from 0 – 25 µM of wortmannin.
  - Overall, the results of this study suggest that as the number of DSBs increase and repair processes are inhibited, there is a corresponding increase in the number of chromosomal aberrations. Thus the data demonstrate dose concordance and essentiality.

- Iliakis, et al. (2019) studied the relationship between DSB damage and chromosomal aberrations using an experimental model that mimics the clustered DNA DSB damage induced by high linear energy transfer (LET) radiation (Iliakis et al., 2019). Chinese hamster ovary cells and human retinal epithelial cells were engineered to carry I-SceI meganuclease recognition sites at specific locations in order to generate specific DSB clustered damage. Cells were then transfected with plasmids expressing I-SceI to induce the DNA breakages. Twelve hours or 24 hours post-transfection, cells were analyzed by immunofluorescence microscopy for DSBs, and by cytogenetic analysis for chromosome translocations.
  - DSBs were increased in all cells transfected with the endonuclease relative to cells from the same cell lines that underwent a mock transfection.
  - Chromosomal translocations were also elevated in cell lines transfected with an endonuclease, with increasing chromosomal translocations found in cells with increasing DSB cluster damage.
  - This study shows an association between DSB cluster damage and chromosomal translocation incidence.

In vivo studies

- Sprague-Dawley rats were dosed with different genotoxic compounds at select concentrations (methotrexate, cisplatin, chlorambucil, and cyclophosphamide) and blood samples were collected at different time points following the dosing (6, 12, 24, 36, 48, 72, and 96 hours post dosing) (Mughal et al., 2010).
  - Peripheral blood lymphocytes were isolated for comet assay and peripheral blood erythrocytes were used to measure MN at each time point.
    - Different comet assay parameters such as tail length, moment, olive tail moment, and % tail DNA were compared to MN frequency
    - All comet assay parameters had a positive correlation to MN frequency demonstrated in all chemical treatments.
    - DNA tail length and % tail DNA showed visible increases in strand breaks at early time points (6 and 12h), while the increase in MN frequency was not observed until after 12-24 h.
    - This early response at 6 h was not observed in tail moment or olive tail moment; these two paramenters did not show as strong of a response as tail length and % tail DNA to all four chemical treatments.
  - The results suggest temporal concordance in strand breaks measured by comet assay and induction of MN, where strand breaks are observed earlier than MN.

- C57BL/6 mice were irradiated with increasing doses of X-rays (1.1, 2.2, 4.4 Gy) at rate of 1.03 Gy/min (acute high dose) and 0.31 cGy/min (low dose rate). Lymphocytes were

isolated and collected 24h and 7 days from the start of irradiation (different mice were used for each time point) (Turner et al., 2015).

- γH2AX measured at 24h showed a dose-dependent increase in DSBs in both acute and low dose rate exposed mice.
  - The level of DSBs due to the acute dose treatment was significantly higher than due to the low dose rate treatment at 1.1 and 2.2 Gy.
- MN frequency was also measured 24h and 7 days post exposure;
  - At both time points and in both treatment groups, MN frequency increased with dose from 1.1 and 2.2 Gy. However, there was no further increase at 4.4 Gy
  - There was no statistical difference in the two treatment groups

Overall, the above data demonstrate that when strand breaks occur there is an increase in MN frequency, which is indicative of chromosomal aberrations. There is a clear temporal-concordance but dose-concordance is not always consistent due to differences in assay sensitivity.

Uncertainties and Inconsistencies

As described above, statistically significant increases in MN occur, in some cases, at lower concentrations than strand breaks measured with the comet assay (Platel et al., 2001; Watters et al., 2009; Kawaguchi et al., 2010). The two assays measure different endpoints at different time points; the MN test may appear to be more sensitive than the comet assay but it is difficult to directly compare these two assays.

Mughal et al. (2010) study compared different parameters of comet assay (tail moment, length, and % tail DNA) to MN frequency. Depending on the parameter, the observation of increase in strand breaks varied. For example, % tail DNA would show a visible increase in strand breaks at one concentration; however, no change would be observed in the tail moment calculated using the same data. Use of different parameters in presenting comet assay data may add subjectivity to the results that are reported in certain papers.

Rossner Jr. et al. exposed human embryonic lung fibroblasts (HEL12469) to 1, 10, and 25 µM of benzo[a]pyrene (B[a]P) for 24 hours and measured DSB (γH2AX immunodetection by Western blotting) and translocations (by fluorescence in situ hybridization of chromosomes 1, 2, 4, 5, 7, 17) (Rossner Jr. et al., 2014).

- Increases in γH2AX were observed only at 25 µM B[a]P (~2.5 fold increase) after the 24h exposure.
- Translocations were quantified and expressed as the genomic frequency of translocations per 100 cells (F_G/100)
- All concentrations of B[a]P induced an elevated frequency of translocations compared to the DMSO control (DMSO: ~0.19/100; 1 µM: ~0.53/100 cells; 10 µM: ~0.33/100; 25 µM: ~0.39/100)

In this study, the increase in translocations was detected at concentrations that did not induce an increase in γH2AX signal. This observation of the discordant relationship between γH2AX and translocations may be due to the differences in assay sensitivity. In addition, immunodetection by Western blotting cannot precisely measure small changes in protein content.

References

Brunet, E. & M. Jasin (2018), "Induction of chromosomal translocations with CRISPR-Cas9 and other nucleases: Understanding the repair mechanisms that give rise to translocations.", Adv. Exp. Med. Biol. 1044:15-25.

Byrne, M. et al. (2014), "Mechanisms of oncogenic chromosomal translocations.", Ann. N.Y. Acad. Sci., 1310:89-97.

Ceccaldi, R., B. Rondinelli & A.D. D'Andrea (2016), "Repair Pathway Choices and Consequences at the Double-Strand Break.", Trends Cell Biol. 26(1):52-64.

Chang, H. et al. (2017), "Non-homologous DNA end joining and alternative pathways to double-strand break repair.", Nature Rev. Mol. Cell. Biol., 18:495-506.

Chernikova, S.B., R.L. Wells & M. Elkind (1999), "Wortmannin Sensitizes Mammalian Cells to Radiation by Inhibiting the DNA-Dependent Protein Kinase-Mediated Rejoining of Double-Strand Breaks.", Radiat. Res., 151:159-166.

Collins, A.R. et al. (2008), "The comet assay: topical issues.", Mutagenesis, 23:143-151.

Dertinger, S.D. et al. (2019), "Predictions of genotoxic potential, mode of action, molecular targets, and potency via a tiered multiflow® assay data analysis strategy.", Environ. Mol. Mutagen., 60(6):513-533

Ensminger, M. et al. (2014), "DNA breaks and chromosomal aberrations arise when replication meets base excision repair.", J. Cell Biol., 206:29.

Ferguson, D.O. & F.W. Alt (2001), "DNA double strand break repair and chromosomal translocation: Lessons from animal models.", Oncogene 20(40):5572–5579.

Hoeijmakers, J.H. (2001), "Genome maintenance mechanisms for preventing cancer.", Nature, 411:366-374.

Iliakis, G. et al. (2019), "Defined Biological Models of High-LET Radiation Lesions.", Radiat. Protect Dosimet., 183:60-68.

Iliakis, G. et al. (2004), "Mechanisms of DNA double strand break repair and chromosome aberration formation.", Cytogenet. Genome Res. 104:14-20.

Kawaguchi, S. et al. (2010), "Is the comet assay a sensitive procedure for detecting genotoxicity?.", J. Nucleic Acids, 2010:541050.

Kuzminov, A. (2001), "Single-strand interruptions in replicating chromosomes cause double-strand breaks.", Proc. Natl. Acad. Sci. USA 95:8241-8246.

Lieber, M. et al. (2010), "Nonhomologous DNA End Joining (NHEJ) and Chromosomal Translocations in Humans.", Subcell Biochem., 50:279-296.

Madabhushi, R., Pan, L., Tsai, L. (2014) "DNA damage and its links to neurodegeneration.", Neuron, 83(2):266-282. doi: 10.1016/j.neuron.2014.06.034.

Mehta, A. & J. Haber (2014), "Sources of DNA Double-Strand Breaks and Models of Recombinational DNA Repair.", Cold Spring Harb. Perspect Biol., 6:a016428.

Mughal, A. et al. (2010), "Micronucleus and comet assay in the peripheral blood of juvenile rat: Establishment of assay feasibility, time of sampling and the induction of DNA damage.", Mutat. Res. Gen. Tox. En., 700:86-94.

Natarajan, A.T & F. Palitti (2008), "DNA repair and chromosomal alterations.", Mutat. Res., 657:3-7.

Platel, A. et al. (2011), "Study of oxidative DNA damage in TK6 human lymphoblastoid cells by use of the thymidine kinase gene-mutation assay and the *in vitro* modified comet assay: Determination of No-Observed-Genotoxic-Effect-Levels.", Mutat. Res., 726:151-159.

Platel, A. et al. (2009), "Study of oxidative DNA damage in TK6 human lymphoblastoid cells by use of the *in vitro* micronucleus test: Determination of No-Observed-Effect Levels.", Mutat. Res., 678:30-37.

Povirk, L. (2006), "Biochemical mechanisms of chromosomal translocations resulting from DNA double-strand breaks.", DNA Repair 5:1199-1212.

Rogakou, E.P. et al. (1999), "Megabase chromatin domains involved in DNA double-strand breaks in vivo.", J. Cell Biol., 146:905-916.

Rossner, Jr. P et al. (2014), "Nonhomologous DNA end joining and chromosome aberrations in human embryonic lung fibroblasts treated with environmental pollutants.", Mutat. Res., 763-764:28-38.

Rothfuss, A. et al. (1999), "Evaluation of mutagenic effects of hyperbaric oxygen (HBO) in vitro.", Environ. Mol. Mutagen., 34:291-296.

Sishc, B.J. & A.J. Davis (2017), "The Role of the Core Non-Homologous End Joining Factors in Carcinogenesis and Cancer.", Cancers (Basel), 9(7): pii E82.

Sudprasert, W., P. Navasumrit & M. Ruchirawat (2006), "Effects of low-dose gamma radiation on DNA damage, chromosomal aberration and expression of repair genes in human blood cells.", Int. J. Hyg. Environ.-Health, 206:503-511.

Trenz, K., J. Landgraf & G. Speit (2003), "Mutagen sensitivity of human lymphoblastoid cells with a BRCA1 mutation.", Breast Cancer Res. Treat., 78:69-79.

Trenz, K., P. Schutz & G. Speit (2005), "Radiosensitivity of lymphoblastoid cell lines with a heterozygous BRCA1 mutation is not detected by the comet assay and pulsed field gel electrophoresis.", Mutagenesis, 20:131-137.

Turner, H.C. et al. (2015), "Effect of Dose Rate on Residual c-H2AX Levels and Frequency of Micronuclei in X-Irradiated Mouse Lymphocytes.", Radiat. Res., 183:315-324.

van Gent, D., J.H. Hoeijmakers & R. Kanaar (2001), "Chromosomal Stability and the DNA Double-Stranded Break Connection.", Nature 2:196-206.

Watters, G.P. et al. (2009), "H2AX phosphorylation as a genotoxicity endpoint.", Mutat. Res., 670:50-58.

Weinstock, D. et al. (2006), "Modeling oncogenic translocations: Distinct roles for double-strand break repair pathways in translocation formation in mammalian cells.", DNA Repair 5:1065-1074.

Xu, B. et al. (2010), "Replication Stress Induces Micronuclei Comprising of Aggregated DNA Double-Strand Breaks.", PLoS One, 6:e18618.