AOP ID and Title:

SNAPSHOT

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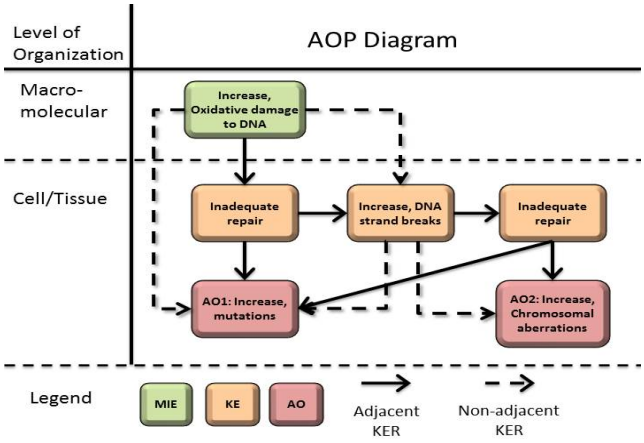
AOP 296: Oxidative DNA damage leading to chromosomal aberrations and mutations

Short Title: Oxidative DNA damage, chromosomal aberrations and mutations

Graphical Representation



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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.

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. 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. 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, superoxide dismutase, and catalase. However, 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; 8-oxodG is an accepted biomarker of oxidative stress and oxidative damage to DNA (Roszkowski et al., 2011; Guo et al., 2017).

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 replicative or translesion polymerases), 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 an intermediate. With increasing oxidative lesions and more lesions in close proximity to each other, the quality and efficiency of repair may be compromised, resulting in retention of unrepaired lesions and repair intermediates. Accumulated intermediate SSBs, along with unrepaired oxidative lesions and other intermediates like abasic sites, can interfere with repair at other damaged sites nearby and/or with the replication fork, and lead to double strand breaks (DSBs) which are more toxic and laborious 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 during excision repair, these SSBs may be converted to DSBs. 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. Importantly, this work highlights notable gaps in the empirical evidence despite the fact that this is a long-studied area in genetic toxicology. Quantifying the extent to which levels of oxidative DNA damage must increase before DNA repair processes are overwhelmed and the AOs result is required to improve our ability to predict whether this pathway is relevant to a chemical's toxicological effects.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1634	Increase, Oxidative damage to DNA (https://aopwiki.org/events/1634)	Increase, Oxidative DNA damage
2	KE	155	N/A, Inadequate DNA repair (https://aopwiki.org/events/155)	N/A, Inadequate DNA repair
3	KE	1635	Increase, DNA strand breaks (https://aopwiki.org/events/1635)	Increase, DNA strand breaks
4	AO	185	Increase, Mutations (https://aopwiki.org/events/185)	Increase, Mutations
5	AO	1636	Increase, Chromosomal aberrations (https://aopwiki.org/events/1636)	Increase, Chromosomal aberrations

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increase, Oxidative damage to DNA (https://aopwiki.org/relationships/1909)	adjacent	N/A, Inadequate DNA repair	High	Low
N/A, Inadequate DNA repair (https://aopwiki.org/relationships/1910)	adjacent	Increase, DNA strand breaks	High	Low
Increase, DNA strand breaks (https://aopwiki.org/relationships/1911)	adjacent	N/A, Inadequate DNA repair	High	Low
N/A, Inadequate DNA repair (https://aopwiki.org/relationships/164)	adjacent	Increase, Mutations	High	Low
N/A, Inadequate DNA repair (https://aopwiki.org/relationships/1912)	adjacent	Increase, Chromosomal aberrations	High	Low
Increase, Oxidative damage to DNA (https://aopwiki.org/relationships/1913)	non-adjacent	Increase, DNA strand breaks	Moderate	Low
Increase, Oxidative damage to DNA (https://aopwiki.org/relationships/1914)	non-adjacent	Increase, Mutations	High	Low
Increase, DNA strand breaks (https://aopwiki.org/relationships/1931)	non-adjacent	Increase, Mutations	High	Low
Increase, DNA strand breaks (https://aopwiki.org/relationships/1939)	non-adjacent	Increase, Chromosomal aberrations	High	Low

Stressors

Name	Evidence
Hydrogen peroxide	High
Potassium bromate	High
Ionizing Radiation	High
Cadmium chloride	High
tert-Butyl hydroperoxide	High
Reactive oxygen species	High

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 and leveraged decades of experience and research on DNA repair and genetic toxicology.

It is established and accepted that unrepaired oxidative DNA lesions, especially 8-oxodG and FapydG, are mutagenic (AO1). During DNA replication, the presence of these adducts on nucleotides leads to the formation of incorrect base pairs with incoming nucleotides without causing structural disturbance and, thus, evading mismatch repair (Cooke et al., 2003). It is well-understood that both 8-oxodG and FapydG readily form base pairs with adenine, giving rise to G to T transversions, which are predominant base substitutions caused by oxidative stress (Cadet and Wagner, 2013; Poetsch et al., 2018). 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.

The biology behind 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 to DNA strand breaks and subsequent 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.

Oxidative DNA lesions are primarily repaired by base excision repair (BER). BER is a multistep process that involves multiple enzymes including OGG1, which removes oxidized guanine bases and creates a nick 3' to the damaged site, and APE1, which removes the AP site by cleaving 5' to the AP site. A spike in BER substrates could lead to an imbalance in the initiating steps of BER, causing an accumulation of abasic sites and single strand break (SSB) intermediates (Coquerelle et al., 1995; Yang et al., 2006; Nemec et al., 2010). It is known that BER glycosylases are constitutively expressed and that APE1 is an abundant enzyme (Tell et al., 2009). Another biologically plausible way in which oxidative DNA lesions can lead to clastogenic effects is through futile cycles of MUTY-initiated BER, which removes dA opposite 8-oxodG post-replication (Hashimoto et al., 2004). Replicative polymerases may repeatedly insert dA opposite 8-oxodG, continuing the cycle of BER at the site and potentially causing an accumulation of SSBs. SSBs can turn into DSBs if they occur in close proximity to each other on opposite strands, or cause replication fork stall and collapse (Iliakis et al., 2004; Fujita et al., 2013; Mehta and Haber, 2014). If DSBs are not repaired in a timely manner, the broken ends can shift away from their original position and result in two incorrect ends being joined or loss of DNA segments, leading to structural aberrations (Obe et al., 2010; Durante et al., 2013)

Misrepair of DSBs can also lead to mutations, providing an alternate pathway to AO1, increase in mutations (Sedletska et al., 2013). Non-homologous end joining (NHEJ), the error-prone joining of two broken ends, is a faster and less labour-intensive process compared to homologous recombination (HR) which uses the homologous sequence in the homologous chromosome or sister chromatid as a template to ensure fidelity of the reconstructed strands (Mao et al., 2008a; Mao et al., 2008b). NHEJ may be preferred over HR in many instances, leading to altered sequences at the site of repair (Rodgers and McVey, 2016).The error-prone nature of DSB repair by NHEJ has been extensively studied and widely accepted. DSBs can also lead to salavage 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)

Time- and dose-response concordance:

The WOE supporting the time- and dose-response concordance of the KEs of these AOPs and the overall network 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 in vitro models, 8-oxodG has been quantified as early as 15 minutes following chemical exposure (Ballmaier and Epe, 2006). Time and concentration-response concordance in oxidative lesion formation and induction of strand breaks have been demonstrated by in vitro time course experiments, where increases in oxidative lesions was 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 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 mutation frequencies are also very limited.

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 indicates 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).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mice	Mus sp.		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10095)
rat	Rattus norvegicus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

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 oxidative DNA damage and induction of oxidative stress due to exposure and/or diseases [e.g., urinary 8-oxodG (Hanchi et al., 2017); 8-oxodG in tumour samples (Mazlumoglu et al., 2017)]. Micronucleus is also regularly quantified as a biomarker of genotoxic exposure in humans. Numerous examples of MN detection 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 exploring the effects of modulating different events in this network and measuring downstream effects have been published. These studies broadly provide strong support to the essentiality of the events to the pathway and AOs. Below we provide examples to demonstrate the effect of modulating each KE on the downstream KEs/AOs.

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 reduction in GSH was observed and the reduction was significant at all time points compared to 0h. The level of 8-oxo-dG lesions was measured at 6 and 24 hours; at both time points, there was a significant increase in oxidative DNA lesions, with a larger amount of lesions present at 24 hours. Strand breaks were also measured concurrently. While there was no observable increase in strand breaks at 6 hours, the increase at 24 hours was significant compared to control ($p<0.01$) (Beddowes et al., 2003).
- Antioxidant treatment reduces oxidative lesions and downstream strand breaks and MN (AO2)
  - A 3 hour exposure of HepG2 cells to increasing concentrations of tetrachlorohydroquinone (TCHQ) with 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 micronucleus (MN) assay at 24 hours indicated a 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/6Jp^{un}/p^{un} 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)
  - *Nth1* knock-out - FapyG and FapyA lesions were measured in the liver nuclear extracts from wild type and *Nth1*-/- mice. A significant increase in FapyG and FapyA was 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 comet assay, compared to *Ogg1*+/+ MEFs. Time series (5 – 90 minutes) immunoblotting of the genomic DNA using anti-8-oxo-dG antibody indicated a larger magnitude of increase in oxidative lesions in *Ogg1*-/- cells compared to wild type. Overall, the results demonstrate the role of Ogg1 in the generation of strand breaks as an intermediate in base excision repair 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 (indicated by % γ-H2AX positive cells) and 8-oxo-dG lesions was observed on day 7 of the exposure and following 7 days of recovery. The magnitude of increase in DSBs after the 7-day recovery was smaller in the 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, immediately following exposure; however, after 7 and 14 days of recovery, the increase was no longer significant. In *Ogg1*-/- mice, the increase in MN was significant 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.

Essentiality of Increase, DNA strand breaks (KE2)

- 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 *Aval* 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 (Tatsumi-Miyajima et al., 1993).
- Reduction in strand breaks leads to decreases in MN frequency (AO2)
  - $PCCL_3$ normal differentiated rat thyroid cells were internally irradiated by ^{131}I 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 prevented the ROS spike 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 spike in 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 at 24 and 48 hours following 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 well-understood. It is generally accepted that there exist limits 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 attempt to repair of accumulated lesions and interference of the replication fork by both unrepaired and incompletely repaired DNA lesions (e.g., repair intermediates such as abasic sites and SSBs) can lead to increase in DNA strand breaks.			
KE2 →KE1: Increase, DNA strand breaks leads to Inadequate repair	**STRONG** It is well recognized that almost all types of DNA lesions will result in recruitment of repair enzymes and factors to the site of damage, and the pathway involved in the repair of DSBs has been well-documented in a number of reviews, many of which also discuss the error-prone nature of DNA repair.			
KE1 → AO1: Inadequate repair leads to Increase, mutations	**STRONG** Numerous previous studies have demonstrated increase in mutation due to unrepaired lesions (insufficient repair) and incorrect repair (e.g., non-homologous end joining and error-prone lesion bypass), both in vitro and in vivo. The mechanisms by which these events occur are well-understood.			
KE1 → AO2: Inadequate repair leads to Increase, chromosomal aberrations	**STRONG** Chromosomal aberrations may result if DNA repair is inadequate, meaning that the double-strand breaks are misrepaired or not repaired at all. A multitude of different types of chromosomal aberrations can occur, depending on the timing and type of erroneous repair. Examples of chromosomal aberrations 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 joining of broken ends can introduce deletions, insertions, or base substitution.			
Non-adjacent MIE → KE2: Oxidative DNA lesions leads to Increase, DNA strand breaks	**MODERATE** Increase in strand breaks due to failed attempted 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 are limited.			
Non-adjacent MIE → AO1: Oxidative DNA lesions leads to Increase, mutations	**STRONG** Strong empirical evidence exist in literature demonstrating increase in mutations 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.			

	Defining Question	**High (Strong)**	**Moderate**	**Low (Weak)**
2. Support for Essentiality of KEs	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 reducing or increasing cellular ROS (via antioxidant addition or depletion) causes concordant changes in the level of strand breaks and MN.			

KE1: Inadequate repair	**STRONG** Numerous studies have investigated inadequate repair of oxidative DNA lesions by disrupting base excision repair (BER) through generating gene knock-down rodent or mammalian cell models. Modulation of the downstream KEs (i.e., DNA strand breaks, mutation, MN) by dysfunctional repair has been demonstrated in these studies.			
KE2: DNA strand breaks	**MODERATE** Theoretically, chromosomal aberrations (AO2) cannot occur unless DNA strands break. Mostly indirect evidence exists that support the essentiality of KE2 in leading to mutations (AO1).			
3. Empirical Support for KERs	**Defining Question** 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?	**High (Strong)** 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	**Moderate** 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.	**Low (Weak)** 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
MIE → KE1: Increase, oxidative DNA damage leads to inadequate repair	**MODERATE** Empirical data are available both in vitro and in vivo that demonstrate increase in oxidative DNA lesions leadings to indications of inadequate repair (i.e., increase in mutation, retention of adducts, increase in lesions despite upregulation of repair enzymes).			
KE1 → KE2: Inadequate repair leads to Increase, DNA strand breaks	**MODERATE** Limited in vivo data are available. A few In vitro studies have demonstrated a larger increase in DNA strand breaks in DNA repair-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 following a hydrogen peroxide exposure - 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.			
KE2 →KE1: Increase, DNA strand breaks leads to Inadequate repair	**MODERATE** Results from many studies indicate dose/incidence and temporal concordance between the frequency of double-strand breaks and the rate of inadequate repair. As DNA damage accumulates in organisms, 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 how error-prone NHEJ truly is, differences in responses depending on the level of exposure of a genotoxic substance, and confounding factors (such as smoking) that affect double-strand break repair fidelity.			
KE1 → AO1: Inadequate repair leads to Increase, mutations	**STRONG** Repair deficiency causing increase in mutations has been extensively demonstrated in both in vitro and in vivo models. Overexpression of repair enzymes has been shown to reduce mutation frequency following chemical exposure in vitro; these data further support the causal relationship between these two KEs.			
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 demonstrating dose and temporal concordance in the two KEs are available in both in vitro and in vivo studies. These studies used a few different chemicals and ionizing radiation as stressors.			
Non-adjacent MIE → KE2: Oxidative DNA lesions leads to Increase, DNA strand breaks	**MODERATE** Both in vitro and in vivo data are available that demonstrate dose-response concordance in oxidative DNA lesions formation and strand breaks following exposure to various stressors. However, the temporal concordance between the KEs in these results 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., Fpg comet assay vs. 8-oxodG immunodetection, comet assay vs. γ-H2AX immunodetection).			

Non-adjacent MIE → AO1: Increase, oxidative DNA lesions leads to Increase, mutations	**STRONG** This KER was demonstrated both in vitro and in vivo via knock-down of oxidative DNA damage repair protein (OGG1) and exposure to different ROS-inducing chemicals. Increase in oxidative DNA lesions followed by an increase in mutant frequency or G to T transversions was clearly shown in these studies.
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.

Quantitative Consideration

The quantitative understanding of the KERs in this AOP is overall weak. Different cell types have different baseline levels of 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 may vary by cell type.

Considerations for Potential Applications of the AOP (optional)

Genotoxicity testing is a fundamental requirement of all chemical and pharmaceutical assessments. Mutations and chromosome damage are inarguably tied to the induction of many genetic diseases including cancer. Oxidative DNA damage is a well-known genotoxic hazard and, thus, a critical endpoint in genotoxicity hazard assessment. Indeed, when the mode of genotoxic action of a chemical is uncertain, oxidative DNA damage is frequently suspected to be the cause. This AOP network provides a framework for assembling information from different mechanism based tests to determine the probability that an agent will cause genotoxicity through induction of oxidative DNA damage. Moreover, the field of applied genetic toxicology is in the midst of a paradigm shift (Dearfield et al., 2017; White and Johnson, 2016), transitioning from a strictly qualitative hazard identification approach to applications in quantitative risk assessment. AOP networks such as this one are a critical element of this paradigm change, informing how different test methods align with the measurement of adverse genotoxic outcomes. Quantitative understanding is necessary in order to be able to determine the extent of oxidative DNA lesions, and single and double strand breaks, necessary to lead to mutations and chromosomal aberrations. This AOP network documents clear gaps in quantitative understanding that must be defined in order to enhance risk assessment and predict toxicology for chemicals that induce oxidative DNA lesions. Overall, the AOP serves a variety of potential regulatory purposes including: (a) facilitating mode of action analysis for chemicals hypothesized to operate through this pathway; (b) identifying test methods and strategies that can be used to test these hypothetical AOPs for new chemicals; (c) facilitating the development of new testing strategies; and (c) highlighting gaps and uncertainties in genotoxicity modes of action.

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Appendix 1

List of MIEs in this AOP

Event: 1634: Increase, Oxidative damage to DNA (https://aopwiki.org/events/1634)

Short Name: Increase, Oxidative DNA damage

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	MolecularInitiatingEvent

Stressors

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

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

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 (enzyme that generates H_2O_2) and potassium bromate for 4 hours (Platel et al., 2011).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)
yeast	Saccharomyces cerevisiae		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=4932)

Term	Scientific Term	Evidence	Links
mouse	Mus musculus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
bovine	Bos taurus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

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.

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). 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 steady state due to endogenous redox processes. 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, or induction of ROS/NOS from reduction of endogenous molecules, as well as through release of inflammatory cell-derived oxidants, can lead to increased DNA oxidation. 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 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) (Breton et al., 2003; Zhao et al.)
  - 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)
  - 8-oxodG can be separated from digested DNA and precisely quantified using high performance liquid chromatography (HPLC) with electrochemical detection
- Mass spectrometry LC-MRM/MS (Mangal et al., 2009)
  - Liquid chromatography can also be coupled with multiple reaction monitoring/ mass spectrometry to detect and quantify 8-oxodG. Correlation between 8-oxodG measured by hOGG1-modified comet assay and LC-MS has been reported

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

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List of Key Events in the AOP

Event: 155: N/A, Inadequate DNA repair (https://aopwiki.org/events/155)

Short Name: N/A, Inadequate DNA repair

Key Event Component

Process	Object	Action
DNA repair	deoxyribonucleic acid	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:15 - Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations (https://aopwiki.org/aops/15)	KeyEvent
Aop:141 - Alkylation of DNA leading to cancer 2 (https://aopwiki.org/aops/141)	KeyEvent
Aop:139 - Alkylation of DNA leading to cancer 1 (https://aopwiki.org/aops/139)	KeyEvent
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	KeyEvent
Aop:272 - Direct deposition of ionizing energy onto DNA leading to lung cancer (https://aopwiki.org/aops/272)	KeyEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Syrian golden hamster	Mesocricetus auratus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10036)
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	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 work 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).

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 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, and 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. 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). A third form of excision repair is mismatch repair (MMR), 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) 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).

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).

DSB Repair

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, XPA 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).

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 measures 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) we developed a fluorescence-based multiplex flow-cytometric host cell reactivation assay (FM-HCR) to measures 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 through by NER, MMR, BER, NHEJ, HR and 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 ^{3}H-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 ^{3}H 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 (https://read.oecd-ilibrary.org/environment/test-no-489-in-vivo-mammalian-alkaline-comet-assay_9789264264885-en) (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 2008	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

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Event: 1635: Increase, DNA strand breaks (https://aopwiki.org/events/1635)

Short Name: Increase, DNA strand breaks

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	KeyEvent
Aop:272 - Direct deposition of ionizing energy onto DNA leading to lung cancer (https://aopwiki.org/aops/272)	KeyEvent

Stressors

Name
Ionizing Radiation
Topoisomerase inhibitors
Radiomimetic compounds

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

DNA strand breaks can occur in any eukaryotic or prokaryotic cell.

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.

DSBs 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

- Comet Assay (Single cell gel electrophoresis)
  - There are two variations of the comet assay for measuring DNA strand breaks
  - Alkaline comet assay (pH >13) (Platel et al., 2011; Nikolova et al., 2017)
    - OECD test guideline for in vivo mammalian alkaline comet assay (#489) is available (OECD, 2014)
    - Detects SSB and DSB resulting from direct-acting genotoxicants, alkali labile sites, or strand breaks that are intermediates of DNA excision repair (OECD, 2014)
  - Neutral comet assay (Anderson and Laubenthal, 2013; Nikolova et al., 2017)
    - Electrophoresis is performed in neutral pH and DNA is not denatured – mostly detects DSB
- γH2AX foci detection (Detects DSB)

  Phosphorylation of histone H2AX (γH2AX) at serine 139 is an early response to DSB; it causes chromatin decondensation and plays a critical role in recruiting repair machineries to the site of damage (Rogakou et al., 1998). γH2AX foci can be detected by immunostaining on several platforms:

  - Flow cytometry (Bryce et al., 2016); γH2AX foci counting can be high-throughput and automated using flow cytometry-based immunodetection.
  - Fluorescent microscopy (Garcia-Canton et al., 2013; Khoury et al., 2013); γH2AX foci can be counted in fluorescent microscope images. Image acquisition and foci count can be automated to increase the assay throughput
    - In-Cell Western technique (Khoury et al., 2013; Khoury et al., 2016) combines the principles of Western blotting (e.g., "blocking" to prevent non-specific antibody binding) and fluorescent microscopy for immunodetection of γH2AX foci.

- Western blotting (Revet et al., 2011); this method does not provide a quantitative measurement of γH2AX foci and is no longer commonly applied in screening for γH2AX induction.

- Pulsed field gel electrophoresis (detects DSB) (Kawashima et al., 2017)
  - Cells are embedded and lysed in agarose and fractionated by electrophoresis
  - The length of fragments can be determined by running a DNA ladder in the adjacent lane
- The TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay
  - Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase that adds deoxynucleotides to the 3'OH end of DNA strand breaks without the need for a template strand. The dUTPs incorporated at the sites of strand breaks are tagged with a fluorescent dye or a reporter enzyme to allow visualization (Loo, 2011).
  - We note that this method is typically used to measure apoptosis.

When measuring these events, it is important to distinguish between breaks that may lead to mutation or chromosomal aberrations, and those that are associated with cell death processes.

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 forced to move, forming a "comet"-like appearance	N/A
γ-H2AX Foci Quantification - Flow Cytometry	Rothkamm and Horn, 2009; Bryce et al., 2016	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 Quantification - ELISA	Ji et al., 2017	Measurement of γ-H2AX in cells by ELISA, normalized to total levels of H2AX	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	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

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List of Adverse Outcomes in this AOP

Event: 185: Increase, Mutations (https://aopwiki.org/events/185)

Short Name: Increase, Mutations

Key Event Component

Process	Object	Action
mutation	deoxyribonucleic acid	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:15 - Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations (https://aopwiki.org/aops/15)	KeyEvent
Aop:141 - Alkylation of DNA leading to cancer 2 (https://aopwiki.org/aops/141)	KeyEvent
Aop:139 - Alkylation of DNA leading to cancer 1 (https://aopwiki.org/aops/139)	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	AdverseOutcome
Aop:293 - Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	AdverseOutcome
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	AdverseOutcome
Aop:272 - Direct deposition of ionizing energy onto DNA leading to lung cancer (https://aopwiki.org/aops/272)	KeyEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
medaka	Oryzias latipes	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

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.

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.

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. Some examples are given below.

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 (TG 471). 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).

A variety of in vitro mammalian cell gene mutation tests are described in OECD's Test Guidelines 476 and 490. TG 476 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 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) 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). Development of an OECD guideline for the Pig-a assay is underway. 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). A description of the approach is found within this published TG. Further modifications to this protocol have now been made 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) or in vitro (OECD Test No. 476: In vitro Mammalian Cell Gene Mutation Test), or in bacterial cells (i.e., OECD Test No. 471) 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 address this issue. Mutations can be directly measured in humans (and other species) through the application of next-generation sequencing. Although single-molecule approaches are growing in prevalence, 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).

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 (file://ncr-a_hecsbc6s/hecsbc6/share/CCRPB/Radbiology/Vinita/AOP/assay%20summary%20table%20papers/MANY%20OTHER%20gene%20loci%20example%202.pdf) 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 (file://ncr-a_hecsbc6s/hecsbc6/share/CCRPB/Radbiology/Vinita/AOP/assay%20summary%20table%20papers/TK%20mutation%20assay%20use.pdf) et al., 2017; Liber (file://ncr-a_hecsbc6s/hecsbc6/share/CCRPB/Radbiology/Vinita/AOP/assay%20summary%20table%20papers/TK%20mutation%20assay%20protocol.pdf) 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 lethaltriflurothymidine (TFT); Only TK-/- cells are able to form colonies	Yes (No. 490)
HPRT Mutation Assay	Ayres (file://ncr-a_hecsbc6s/hecsbc6/share/CCRPB/Radbiology/Vinita/AOP/assay%20summary%20table%20papers/HPRT%20mutation%20assay%20use.pdf) 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	Kruger (file://ncr-a_hecsbc6s/hecsbc6/share/CCRPB/Radbiology/Vinita/AOP/assay%20summary%20table%20papers/MANY%20OTHER%20gene%20loci%20example%202.pdf) 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 PCR	Kraytsberg, 2005; Yauk, 2002	This PCR technique uses a single DNA template, and is often employed for detection of mutations in microsatellites, recombination studies, and generation of polonies	N/A
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)	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

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Event: 1636: Increase, Chromosomal aberrations (https://aopwiki.org/events/1636)

Short Name: Increase, Chromosomal aberrations

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	AdverseOutcome
Aop:272 - Direct deposition of ionizing energy onto DNA leading to lung cancer (https://aopwiki.org/aops/272)	KeyEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Chromosomal aberrations indicating clastogenicity can occur in any eukaryotic or prokaryotic cell. However, dose-response curves can differ depending on the cell cycle stage when the DSB agent was introduced (Obe et al., 2002).

Key Event Description

Chromosomal aberrations describe the structural damage to chromosomes that result 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 cell death (Boei et al., 1996). These events may be detectable after cell division and such damage to DNA is irreversible. Chromosomal aberrations are associated with cell death and carcinogenicity (Mitelman, 1982).

Chromosomal aberrations (CA) 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

Chromosome aberrations are typically measured after cell division.

- Micronucleus detection:
  - Micronuclei are DNA fragments that are not incorporated in the nucleus during cell division. Micronucleus induction indicates chromosomal breakage and irreversible damage.
- Traditional (microscopy-based) micronucleus assay; OECD guidelines for both in vivo (#474) and in vitro (#487) testing are available (OECD, 2014; OECD, 2016b)
- In vivo and in vitro flow cytometry-based, automated micronuclei measurements (Dertinger et al., 2004; Bryce et al., 2014)
- High content imaging (Shahane et al., 2016)
  - DNA can be stained using fluorescent dyes and micronuclei can be scored in microscope images.
- Chromosomal aberration test
  - OECD guidelines exist for both in vitro (#473) and in vivo (#475 and #483) testing (OECD, 2015; OECD, 2016a; OECD, 2016c)
  - In vitro, the cell cycle is arrested at metaphase after 1.5 cell cycle following 3-6 hour exposure
  - In vivo, the test chemically is administered as a single treatment and bone marrow is collected 18-24 hrs later (#475) while testis is collected 24-48 hrs later (#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
- Indirect measurement of clastogenicity via protein expression:
  - Flow cytometry-based quatification of γH2AX foci and p53 protein expression (Bryce et al., 2016).
  - Prediscreen Assay– In-Cell Western -based quantification of γH2AX (Khoury et al., 2013, Khoury et al., 2016)
  - 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 (Hendriks et al., 2012; Hendriks et al., 2016; Wink et al., 2014).

Assay Name	References	Description	OECD Approved Assay
Fluorescent In Situ Hybridization (FISH)	Beaton et al., 2013; Pathak et al., 2017	Fluorescent assay of condensed chromosomes that can detect CAs through chromosome painting and microscopic analysis	N/A
Cytokinesis Block Micronucleus (CBMN) Assay with Microscopy	Fenech, 2000	Cells are cultured with cytokinesis blocked, fixed to slides, and undergo MN quantification using microscopy	Yes (No.487)
CBMN with Imaging Flow Cytometry	Rodrigues et al., 2015	Cells are cultured with cytokinesis blocked, fixed in solution, and imaged with flow cytometry to quantify MN	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
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 detected in single-stranded and fluorescently-tagged DNA using a microarray plate with fixed, known DNA (or SNP) probes; 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

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1909: Increase, Oxidative DNA damage leads to N/A, Inadequate DNA repair (https://aopwiki.org/relationships/1909)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	**adjacent**	**High**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Oxidative DNA damage can occur and overwhelm the repair mechanisms in any eukaryotic and prokaryotic cell. Observation of this KER has been described in mammalian cells, yeast, and bacteria.

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 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

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).

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).

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 not until higher concentrations is repair overwhelmed (i.e., insufficient), where 8-oxo-dG significantly increases.

- AS52 Chinese hamster ovary cells (wild type and OGG1-overexpressing) 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 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 the level of OGG1 capacity was exceeded for repair of 8-oxo-dG, leading to accumulation of these lesions during this sub-chronic exposure.

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.

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Relationship: 1910: N/A, Inadequate DNA repair leads to Increase, DNA strand breaks (https://aopwiki.org/relationships/1910)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	**adjacent**	**High**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	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 H_2O_2 for varying durations
  - Higher levels of 8-oxodG were detected in *Ogg1*-/- cells compared to *Ogg1*+/+ cells after treatment with 400 µM H_2O_2 at all time points (5, 15, 30, 60, and 90 min)
    - Demonstrates insufficient removal of 8-oxo-dG in OGG1-deficient cells
  - Significantly higher % of TUNEL-positive cells ($p<0.001$) were detected in *Ogg1*+/+ cells compared to *Ogg1*-/- cells after exposure to 400 µM H_2O_2 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 H_2O_2; 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 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.
- 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).

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Relationship: 1911: Increase, DNA strand breaks leads to N/A, Inadequate DNA repair (https://aopwiki.org/relationships/1911)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	**adjacent**	**High**	**Low**
Direct deposition of ionizing energy onto DNA leading to lung cancer (https://aopwiki.org/aops/272)	**adjacent**	**Moderate**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The domain of applicability is multicellular eukaryotes (Lieber, 2008; Hartlerode & Scully, 2009) , plants (Gorbunova, 1997; Puchta, 2005), certain strains of bacteria such as *Mycobacteria*, *Pseudomonas*, *Bacillus* and *Agrobacterium* (Shuman & Glickman, 2007), and yeast (Wilson & Lieber, 1999).

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 (Liakis, 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).

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 which focussed particularly on DSBs induced by ionizing radiation and extensively detailed 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; 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.

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). 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), 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).

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) (https://docs.google.com/spreadsheets/d/1iehBBqhFFSOhgis-0U3tasQwJ50bZJPVmenWUiR4vmA/edit?usp=sharing). 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 (Dikomey & Brammer, 2000; Kuhne et al., 2000; Lobrich et al., 2000; Rothkamm & Lo, 2003; Kuhne et al., 2005; Asaithamby & Chen, 2009; Bracalente et al., 2013).

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 (Dikomey & Brammer, 2000; Kuhne et al., 2000; Lobrich et al., 2000; Rothkamm & Lo, 2003; Kuhne et al., 2005; Asaithamby & Chen, 2009; Bracalente et al., 2013), there were resulting dose-dependent increases in non-repaired DSBs (Dikomey & Brammer, 2000), DSB misrepair rates (Mcmahon et al., 2016), and misrejoined DSBs (Kuhne et al., 2000; Kuhne et al., 2005; Rydberg et al., 2005), as well as a dose-dependent decrease in the total DSB rejoining (Lobrich et al., 2000). Furthermore, only 50% of the rejoined DSBs were found to be correctly repaired (Kuhne et al., 2000; Lobrich 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 & Lo, 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 (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 (Lobrich 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:

1. 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).
2. 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).
3. 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.
4. 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).

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage suggests that DSB repair can be predicted from the presence of DSBs. In terms of DNA repair in response to radiation-induced DSBs, one study suggests that complete DNA DSB repair occurs starting at a threshold dose of 5 mGy (0.005 Gy), as measured by phosphorylation of gamma-H2AX (Lobrich et al., 2005) and presence of 53BPI foci (Asaithamby & Chen, 2009). 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).

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 (Lobrich et al., 2000; Rothkamm & Lo, 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 & Lo, 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 & Lo, 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 & Lo, 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

Not identified.

Known Feedforward/Feedback loops influencing this KER

Not identified.

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Relationship: 164: N/A, Inadequate DNA repair leads to Increase, Mutations (https://aopwiki.org/relationships/164)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations (https://aopwiki.org/aops/15)	**adjacent**	**High**	**Moderate**
Alkylation of DNA leading to cancer 2 (https://aopwiki.org/aops/141)	**adjacent**	**High**	**Moderate**
Alkylation of DNA leading to cancer 1 (https://aopwiki.org/aops/139)	**non-adjacent**	**High**	**Moderate**
Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	**adjacent**	**High**	**Low**
Direct deposition of ionizing energy onto DNA leading to lung cancer (https://aopwiki.org/aops/272)	**adjacent**	**Moderate**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The domain of applicability is multicellular eukaryotes (Lieber, 2008; Hartlerode & Scully, 2009), plants (Gorbunova, 1997; Puchta, 2005), certain strains of bacteria such as *Mycobacteria*, *Pseudomonas*, *Bacillus* and *Agrobacterium* (Shuman & Glickman, 2007), and yeast (Wilson & Lieber, 1999).

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, 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

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 AGT 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 AGT/MGMT (repair machinery – i.e., decrease in KE1) greatly reduces the incidence of mutations caused by exposure to methylating agents such as MNU and 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 ENU 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 hypothesized 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 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) (https://docs.google.com/spreadsheets/d/1iehBBqhFFSOhgis-0U3tasQwJ50bZJPVmenWUiR4vmA/edit?usp=sharing). 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. 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

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 altered 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* (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 *XPC* status. Specifically, mutation frequencies were increased in *XPC*-wild-type patients at 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.

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 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.

Inadequate repair of oxidative lesions

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 table below.

Reference	Summary
Matuo et al., 2018	Yeast cells (saccharomyces cerevisiae) exposed to high LET cardbon 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 10^9 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 10^4 cells and 0.1 point mutations per 10^4 cells (1 Gy). At 6 Gy, observation of 1.5 mutations in the HPRT gene per 10^4 cells and 0.4 point mutations per 10^4 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.

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Relationship: 1912: N/A, Inadequate DNA repair leads to Increase, Chromosomal aberrations (https://aopwiki.org/relationships/1912)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	**adjacent**	**High**	**Low**
Direct deposition of ionizing energy onto DNA leading to lung cancer (https://aopwiki.org/aops/272)	**adjacent**	**High**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The domain of applicability for this KER is multicellular eukaryotes at any stage of development, including plants (Varga & Aplan 2005; Schipler & Iliakis 2013; Manova & Gruszka 2015).

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 a 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 a 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). 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 (CTAs) that affect both sister chromatids, and chromatid-type aberrations (CSAs), affecting only one chromatid (Danford, 2012) . Examples of CTAs include chromosome-type breaks, centric ring chromosomes, and dicentric chromosomes (which have two centromeres), while CSAs 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

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 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). 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 centred 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 (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 and a translocated chromosome (Ferguson and Alt 2001; Povirk 2006). NHEJ has been shown to play a significant role in the generation of translocations (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 translocations (Simsek and Jasin 2010) , which may be due to its relatively rapid repair kinetics (Schipler and Iliakis 2013). Translocations 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, dicentric chromosomes 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) (https://docs.google.com/spreadsheets/d/1iehBBqhFFSOhgis-0U3tasQwJ50bZJPVmenWUiR4vmA/edit?usp=sharing). 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

There is strong evidence for 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 fortifying 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) .

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). Lastly, 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 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 is derived from the 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 assess the quantitative 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 comes 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

Not identified.

Known Feedforward/Feedback loops influencing this KER

Not identified.

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List of Non Adjacent Key Event Relationships

Relationship: 1913: Increase, Oxidative DNA damage leads to Increase, DNA strand breaks (https://aopwiki.org/relationships/1913)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	**non-adjacent**	**Moderate**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mice	Mus sp.		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10095)

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	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.

Key Event Relationship Description

The repair of oxidative DNA lesions produced by exposure to reactive oxygen species (ROS) involves excision repair, where 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

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 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).

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.

In vitro studies

- 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 γH2AX 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.

In vivo studies

- (Trouiller et al., 2009)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.
- Concentration concordance in mice exposed to ROS inducing titanium dioxide nanoparticles (Trouiller et al., 2009).
  - Mice were exposed to 50, 100, 250 or 500 mg/kg of 21 nm P25 TiO2 particles via drinking water for 5 days.
    - A significant increase in 8-oxodG in liver was observed at the 500 mg/kg concentration as measured by high performance liquid chromatography (other concentrations were not examined).
    - Significant increases in gamma-H2AX positive bone marrow cells were observed at all concentrations tested.
    - A significant increase in micronuclei in peripheral blood erythrocytes was observed only at the top concentration tested.

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.

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Relationship: 1914: Increase, Oxidative DNA damage leads to Increase, Mutations (https://aopwiki.org/relationships/1914)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	**non-adjacent**	**High**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mice	Mus sp.		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10095)

Life Stage Applicability

Life Stage	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 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 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.

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Relationship: 1931: Increase, DNA strand breaks leads to Increase, Mutations (https://aopwiki.org/relationships/1931)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	**non-adjacent**	**High**	**Low**
Direct deposition of ionizing energy onto DNA leading to lung cancer (https://aopwiki.org/aops/272)	**non-adjacent**	**High**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	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).

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.

In vitro studies

- 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).

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Relationship: 1939: Increase, DNA strand breaks leads to Increase, Chromosomal aberrations (https://aopwiki.org/relationships/1939)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	**non-adjacent**	**High**	**Low**
Direct deposition of ionizing energy onto DNA leading to lung cancer (https://aopwiki.org/aops/272)	**non-adjacent**	**High**	**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	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 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).

Empirical Evidence
In vitro studies demonstrating dose and temporal concordance

- 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.

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