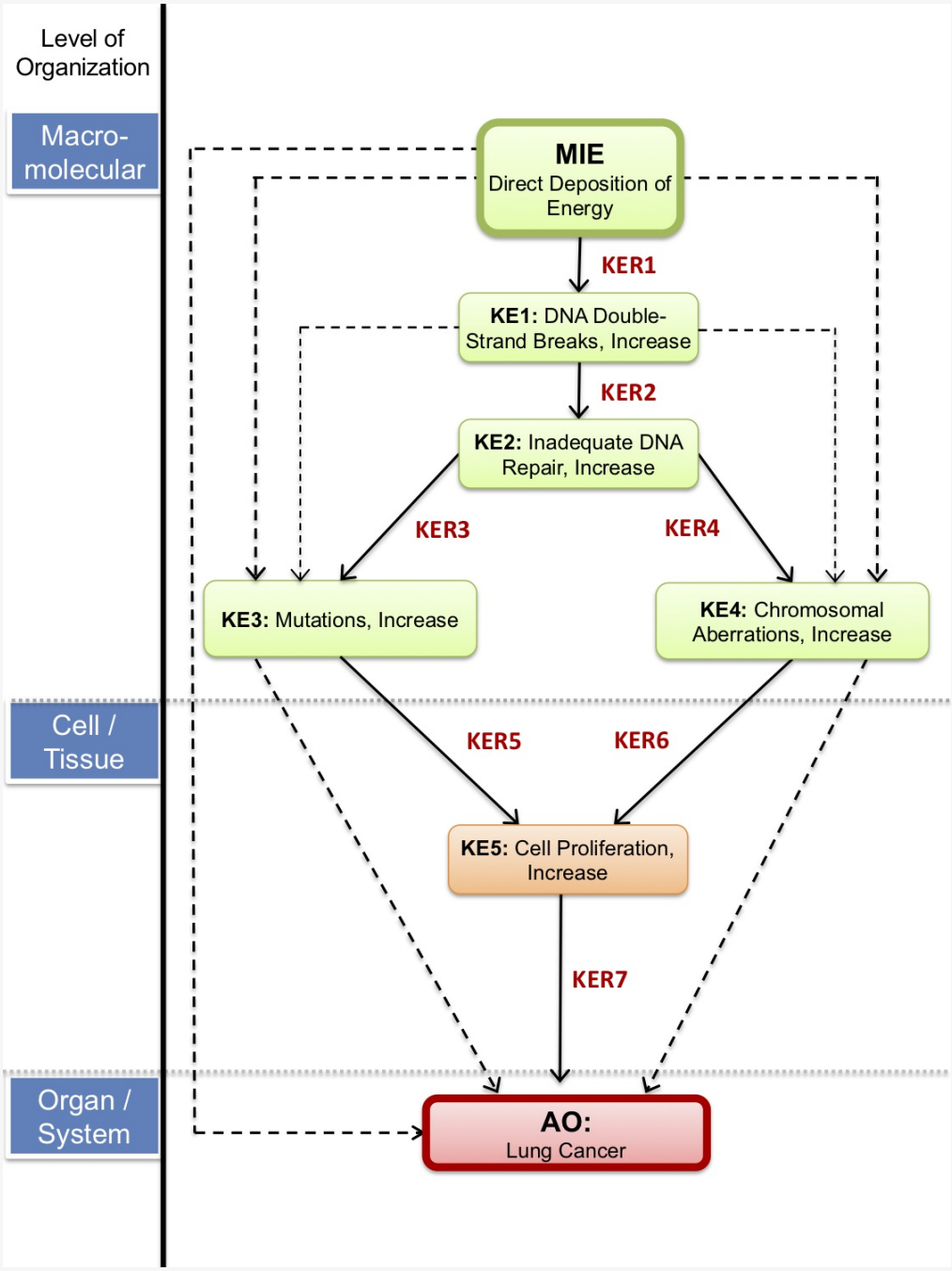
AOP ID and Title:

AOP 272: Deposition of energy leading to lung cancer
Short Title: Deposition of energy leading to lung cancer

Graphical Representation



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Abstract

Despite its widespread recognition in chemical toxicology, the adverse outcome pathway (AOP) framework has not been fully explored in the radiation field to guide relevant research and subsequent risk assessment. Development of a radiation relevant AOP is described here using a case example of lung cancer. Lung cancer is a major public health problem world-wide, causing the deaths of an estimated 1.5 million people annually; it imposes a major health-care burden. Numerous environmental factors are known contributors including both chemical (e.g.. asbestos, air pollution and arsenic) and radiation stressors (e.g.. radon gas). Radon gas is the second leading cause of lung cancer in North America. Evidence suggests that environmental and indoor radon exposure constitutes a significant public health problem. The mechanism of lung cancer development from exposure to radon gas is unclear. Data suggest that cytogenetic damage from radon decay progeny may be an important contributor. This AOP defines a path to cancer using key events related to DNA damage response and repair. The molecular initiating event (MIE) which represents the first chemical interaction with the cell is identified as the deposition of ionizing energy. Energy deposited onto a cell can lead to multiple ionization events to targets such as DNA. This energy will break DNA double strands (KE1) and initiate double strand break (DSB) repair machinery. In higher eukaryotes, this occurs through non-homologous end joining (NHEJ) which is a quick and efficient, but error-prone process (KE2). If DSBs occur in regions of the DNA transcribing critical genes, then mutations (KE3) generated through faulty repair may alter the function of these genes or may cause chromosomal aberrations (KE4), resulting in genomic instability. These events will alter the functions of many gene products and impact cellular pathways such as cell growth, cell cycling, and apoptosis. With these alterations, cell proliferation (KE5) will be promoted by escaping the regulatory control and form hyperplasia in lung epithelial cells, leading eventually to lung cancer (AO) induction and metastasis . The overall weight of evidence for this AOP is strong. The uncertainties and inconsistencies surrounding this AOP are centred on dose-response relationships associated with dose, dose-rates and radiation quality. The proposed AOP will act as a case example to motivate more researchers in the radiation field to use the AOP framework to effectively exchange knowledge and identify research gaps in the area of low dose risk assessment.

Background

According to the World Cancer Research Fund, lung cancer is a disease that poses a significant healthcare burden world-wide. (https://www.wcrf.org/dietandcancer/cancer-trends/worldwide-cancer-data (https://www.wcrf.org/dietandcancer/cancer-trends/worldwide-cancer-data)). It is the most commonly diagnosed cancer with the highest incidence of occurrence on a global scale (excluding non-melanoma skin cancers). It is a multi-faceted disease exhibiting various genetic lesions and involving the accumulation of multiple molecular abnormalities over time. It is responsible for 1.5 million deaths annually. There is convincing evidence to show that smoking is an important risk modulating factor to lung cancer development. This risk is increased by age at which one starts, the total number of years and number of cigarettes smoked/day. Studies highlight smoking leads to the largest (relative) increases for small cell carcinoma and squamous cell carcinoma and (Sobue et al., 1999 and Janssen-Heijnen et al., 2001). Other risk factors include lack of physical activity, genetic mutations, dietary factors, asbestos, air pollution (de Groot et al., 2012). Although the link between smoking and lung cancer has been well-established, environmental and indoor radiation exposure are also significant contributors. Risk assessment measures for defining acceptable exposure levels of radiation exposure still remain uncertain; including the scientific research to support the justifications. This is partially due to the assumption of a non-threshold and linear model at low doses with no consideration that cellular/tissue effects of low dose radiation exposure remain poorly understood.

Efforts were focused on developing a simple, unidirectional AOP to lung cancer using predominantly available data from radiation studies. Decades of research suggest that energy in the form of ionizing radiation can break DNA molecules. In vitro mutagenicity studies suggest that alterations in genes in the form of mutations, chromosomal aberrations and micronuclei formation may be important for cancer cell differentiation/proliferation and eventually neoplastic transformation (Harris, 1987). The MIE was selected to be “deposition of energy” as it is the initial measurable interaction at the macro-molecular level within an organism that can lead to a perturbation that initiates the AOP. The term accurately defines the initiating phenomena that manifest from any type of radiation insult (e.g. alpha- and beta-particles, photons, neutrons and heavy ions) and is distinguishable from chemical-based initiation events. Although the “deposition of energy” is itself a physical phenomenon (not biological) it is essential to describe the causal relationship between radiation insults and the stochastic onset of associated downstream biological damage. Historically, this relationship has been empirically observed and reported in the form of dose-response data. In addition, this MIE encapsulates the known varieties of radiation and their differing physical properties while still adhering to the stressor agnostic principles of the AOP framework.

This AOP has brought together molecular and cellular based research in the radiation realm and defined a modular, simplistic path towards lung cancer. It has used data–rich key events to a classic targeted response onto a cell that is applicable to multiple radiation stressors (e.g. X-rays, gamma rays, alpha particles, beta particles, heavy ions, neutrons) and well supported thorough empirical evidence. The proposed AOP is not the only route to lung cancer it is likely to be one linear path in a network of multiple pathways that may include other critical events. This hypothetical AOP will be networked to AOP-296, AOP- 322, AOP-293, AOP-294 and AOP-303 forming a larger network of KEs related inflammation, apoptosis, and oxidative stress, providing a more complete path to lung cancer. This AOP is also a case example of how existing evidence from radiation stressors can stregthen empirical evidence surrounding key events that may be non-radiation specific and vice versa. By using a radiation centric molecular initiating event (MIE), networks can be developed for multiple adverse outcomes distinct to a radiation response. As different radiation stressors can trigger the MIE, the AOP will have wide applicability.

It is our goal, with the development of this AOP to motivate radiation researchers to use this framework for bringing together research data, exchanging knowledge, identifying priority areas and better co-ordinating research in the low-dose ionizing radiation field.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1686	Deposition of Energy	Energy Deposition
2	KE	1635	Increase, DNA strand breaks	Increase, DNA strand breaks
3	KE	155	Inadequate DNA repair	Inadequate DNA repair
4	KE	185	Increase, Mutations	Increase, Mutations

Sequence	Type	Event ID	Title	Short name
5	KE	1636	Increase, Chromosomal aberrations	Increase, Chromosomal aberrations
6	KE	870	Increase, Cell Proliferation	Increase, Cell Proliferation
	AO	1556	Increase, lung cancer	Increase, lung cancer

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Deposition of Energy	adjacent	Increase, DNA strand breaks	High	High
Increase, DNA strand breaks	adjacent	Inadequate DNA repair	Moderate	Moderate
Inadequate DNA repair	adjacent	Increase, Mutations	Moderate	Moderate
Inadequate DNA repair	adjacent	Increase, Chromosomal aberrations	High	Low
Increase, Mutations	adjacent	Increase, Cell Proliferation	High	Low
Increase, Chromosomal aberrations	adjacent	Increase, Cell Proliferation	Moderate	Low
Increase, Cell Proliferation	adjacent	Increase, lung cancer	High	Low
Deposition of Energy	non-adjacent	Increase, Mutations	High	High
Deposition of Energy	non-adjacent	Increase, Chromosomal aberrations	High	High
Deposition of Energy	non-adjacent	Increase, lung cancer	Moderate	Moderate
Increase, DNA strand breaks	non-adjacent	Increase, Mutations	High	Low
Increase, DNA strand breaks	non-adjacent	Increase, Chromosomal aberrations	High	Low
Increase, Mutations	non-adjacent	Increase, lung cancer	High	Low
Increase, Chromosomal aberrations	non-adjacent	Increase, lung cancer	Moderate	Moderate

Stressors

Name	Evidence
Ionizing Radiation	High

Overall Assessment of the AOP

Considerable mechanistic dose-response data has been generated in the radiation field, particularly in the area of clastogenic lesions. This data has been compiled and captured in this AOP in the most simplified, modular path to lung cancer from a molecular initiating event of deposited energy onto DNA. This AOP is supported through KERs for which there is biological plausibility and available empirical evidence. Although it is clear that our proposed AOP is not the only route to the AO, it does represent a classic targeted response of radiation insult on a cell. The empirical evidence to support this pathway is strong and probabilistic. As per AOP conventions, the pathway does not describe every mechanism and alteration that is ultimately involved in radiation-associated carcinogenesis. Instead, KEs that are routinely measured using modern and conventional assays are described. For this reason, not all of the KEs that are hallmarks of cancer (i.e. evasion, angiogenesis etc.) are mapped out, but as they are critical events they can be developed separately, and possibly networked in later. This AOP will be the first to use a MIE that is radiation-specific and therefore can act as a foundational AOP to build networks of radiation-specific responses. Networks can evolve to multiple AOs with additional KEs that incorporate non-targeted effects, immune and adaptive responses, in parallel.

While this AOP is applicable to other types of radiation-induced cancers, lung cancer was selected as the AO due to its relevance to radon risk assessment and its broader applicability to the chemical field. Lung cancer is a major public health problem world-wide, killing an estimated 1.5 million people annually (https://www.wcrf.org/dietandcancer/cancer-trends/worldwide-cancer-data). Although smoking is the leading cause of lung cancer, numerous environmental sources are also important contributors including radon, asbestos, air pollution and arsenic (Hubaux et al., 2012). Some of these stressors can act synergistically to increase risk, particularly among smokers. It has been shown that the histological lung profile of individuals that are smokers is quite different from non-smokers exposed to high radon levels (Kreuzer et al., 2000). This is in part due to the complexity of each stressor, in terms of its interaction with cells at the molecular level. As radon is the second leading cause of cancer, distinguishing its mode of action at the cellular level from smoking becomes important. Environmental and indoor radon exposures are significant contributors to lung cancer and risk assessment measures for defining acceptable exposure levels of radon exposure still remain uncertain, including the scientific research to support the justification of these levels (Samet et al., 2000 and 2006). This is partially due to the assumption of a non-threshold and linear model with no consideration that cellular/tissue effects of low dose radiation exposure remain poorly understood (Ruhm et al., 2016; Shore et al., 2018).

Despite the decades of research in the area of radiation and DNA damage, a major challenge in developing this AOP was in finding the required components (i.e. essentiality, temporal, incidence and dose concordance) to provide strong empirical evidence to help support the KERs. Across all KERs, studies were lacking that used a broad dose-range. Most studies conducted analysis at one time-point and there were limited studies that supported the essentiality criteria. This was particularly evident for the KERs of inadequate repair to mutations/CA and mutations/CA to cellular proliferation. The non-adjacent KERs (i.e. DDOE to CA or DDOE to mutations), were generally more well supported. Furthermore, no single study encompassed all the KERs proposed in this AOP. In addition, there were considerable discordant results across KEs simply due to the MIE, as its outcome is dependent on factors such as cell type, dose, dose-rate, and radiation quality. These factors can influence the amount and type of damage, which in turn can affect the probability to drive a path forward to cancer. The principle knowledge gap arose from the lack of data in the form of essentiality studies, using inhibitors and knock-in genes. Furthermore, for a number of KERs, there was minimal dose-response and temporal response data in well-conducted animal studies. There is also a range of uncertainty on how confounders such as lifestyle, health status, and radiosensitivities affect an individual's path to the AO. Additional KEs may need to be added in parallel as our knowledge in these areas becomes better understood. These challenges can drive research priorities in the future.

An overall assessment of this AOP shows that there is strong biological plausibility and moderate empirical evidence to suggest a qualitative link between the deposition of energy on DNA to the final AO of lung cancer. This evidence has been derived predominately from decades of research using laboratory studies and through mathematical simulations of cell-based models. These studies have shown both dose- and temporal-response relationships for select KEs. The quantitative thresholds to initiate each of the KEs have been shown to vary with factors such as the cell type, dose-rate of exposure and radiation quality. Thus, an absolute amount of deposited energy (MIE) to drive a KE forward to a path of cancer is not yet definable. This is particularly relevant to low doses and low dose-rates of radiation exposure where the biology is interplayed with conflicting concepts of hormesis, hypersensitivity and the linear no threshold theory. Furthermore, due to the stochastic nature of the MIE, it remains difficult to identify specific threshold values of DSBs needed to overwhelm the DNA repair machinery to cause "inadequate" DNA repair leading to downstream genetic abnormalities and eventually cancer. With a radiation stressor, a single hit to the DNA molecule could drive a path forward to lung cancer; however this is with low probability. Studies have shown that more than 1 hit is required for tumorigenesis (reviewed in Loeb et al. 2003). Conversely, at much higher doses, a cell will induce apoptosis and may not be driven to cancer induction. Although empirical modeling of cancer probability vs. mean radiation dose and time to lethality, does provide a good visualization of the effective thresholds (Raabe et al., 2011), practically, there is still considerable uncertainty surrounding the connection of biologically contingent observations and stochastic energy deposition. Future work may focus on developing more precise quantitative and predictive models to help address these types of uncertainties.

This foundational AOP will initiate the building of networks, and feedback loops that will further the essential events towards lung cancer, including genome alterations, oxidative stress, and metabolomics effectors. This will require efforts from the larger radiation community. As the empirical evidence to support these areas becomes stronger, a better representation of events leading to lung cancer will emerge. By identifying uncertainties and inconsistencies in the literature, research can be directed to address knowledge gaps, which can later help refine the pathway. It is our goal, with this AOP to motivate radiation researchers to use this framework for bringing together research data, exchanging knowledge and identifying research priority areas in the low-dose ionizing radiation field. The current version of this AOP was developed by a team of researchers with backgrounds primarily in AOP development, carcinogenesis, radiobiology, radiation physics and biomolecular epidemiology. However, due to the importance of radiation epidemiology in the international radiological protection system and its underlying assumptions, it seems essential to strengthen the epidemiological aspects of this AOP, a specific area of future improvement.

Long-term, this AOP alongside others in the radiation field will help to identify key events common to chemical stressors and multiple adverse outcomes, which will be important to help refine risk assessment. In all, by building more radiation-relevant AOPs, the AOP framework will have a bigger role in supporting the system of radiological protection.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	High

This AOP is relevant to mammals (Eymin & Gazzeri, 2009; Barron et al., 2014; Kurgan et al., 2017). The pathway leading to the development of lung cancer often occurs during adulthood but may be applicable at earlier life stages (Liu et al., 2015) and is independent of sex. In humans, however, genetic abnormalities/mutations suggestive of lung cancer risk seem to be influenced by ethnicity (Lloyd et al., 2013), smoking history (Lim et al., 2009; Sanders & Albitar, 2010; Paik et al., 2012; Lloyd et al., 2013; Cortot et al., 2014; Minina et al., 2017, Cahoon et al., 2017), age (Lloyd et al., 2013), sex (Lim et al., 2009; Cortot et al., 2014) and genotype (Lim et al., 2009; Sanders & Albitar, 2010; Kim et al., 2012; Paik et al., 2012; Leng et al. 2013; Cortot et al., 2014; Minina et al., 2017). Evidence supporting this AOP comes primarily from studies using bacterial DNA (Sutherland et al., 2000; Jorge et al., 2012), human fibroblast cells (Rothkamm & Lo, 2003; Kuhne et al., 2005; Rydberg et al., 2005a), mice (Duan et al., 2008; Zhang & Jasin, 2011), hamsters (Bracalente et al., 2013; Lin et al., 2014), lung cancer cell lines (Sato, Melville B. Vaughan, et al. 2006; Kurgan et al., 2017; Tu et al., 2018), and tissue samples (both with and without lung cancer) Sun et al., 2016; Tu et al., 2018 Warth et al., 2014.

Essentiality of the Key Events

	Defining Question	Strong	Moderate	Weak
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: **Deposition of Energy**	**Evidence for Essentiality of KE: Strong** This event is difficult to test for essentiality as deposition of energy is a physical stressor and cannot be blocked/decreased using chemicals. However, there are a number of antioxidant studies demonstrating that treatment with various antioxidants prior to irradiation decreases the number of radiation-induced DSBs (results summarized in a review by Kuefner et al. 2015; Smith et al. 2017).
KE1: **Double-Strand Breaks, Increase**	**Evidence for Essentiality of KE: Weak** A variety of different studies demonstrate that organisms with compromised DNA repair tend to have an increased incidence of DSBs. Inhibition studies have shown that addition of a DNA repair antagonist results in significant increases in DSBs at 6 and 12 hours post-irradiation (Dong et al. 2017). Similarly, knock-outs/knock-downs of DNA repair proteins also results in persisting DSBs post-irradiation (Rothkamm and Lo 2003; Bracalente et al. 2013; Mcmahon et al. 2016; Dong et al. 2017), with one DNA ligase IV-deficient human cell line showing DSBs 240 - 340 hours after radiation exposure (Mcmahon et al. 2016). Studies by Tatsumi-Miyajima et al., (1993) note the increased rate of supF mutation frequencies following the use of a restriction enzyme, *AvaI,* which induces DSBs in different human fibroblast cell lines transfected with plasmids containing the *AvaI* restriction site. Kurashige et al. (2017) have demonstrated a decrease in MN frequency following the reduction in DSBs by regulating NAC pre-treatment.
KE2: Inadequate DNA Repair, Increase	**Evidence for Essentiality of KE: Strong** There is extensive evidence to demonstrate the essentiality of inadequate repair to downstream events. Studies show that inhibiting DNA repair results in a lack of DNA repair foci post-irradiation (Paull et al. 2000), while cells deficient in ATM (involved in DNA repair) show increased levels of incorrectly rejoined DSBs (Lobrich et al. 2000; Bucher et al. 2021). Similarly, chromosomal aberrations were more frequent after inhibition of various proteins involved in DNA repair (Chernikova et al. 1999; Heterodimer et al. 2002; Wilhelm et al. 2014). Furthermore, when knock-out cell lines (i.e., knock-out of genes involved in DNA repair to increase the incidence of 'inadequate' repair) were examined for genomic abnormalities, increased incidence of chromosomal aberrations were clearly evident (Karanjawala et al. 1999; Cornforth and Bedford 1994; Patel et al. 1998; Simsek and Jasin 2010; Lin et al. 2014; Wilhelm et al. 2014; Mcmahon et al. 2016). Deficiencies in proteins involved in DNA repair also resulted in altered mutation frequencies relative to wild-type cases (Amundson and Chen 1996; Feldmann et al. 2000; Smith et al. 2003; Wessendorf et al. 2014; Perera et al. 2016). Mutation frequency increased following 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 (Suzuki et al., 2010). Moreover, G:C to T:A transversion frequency increased in all analyzed cells. Nallanthighal et al. (2017) demonstrated that inadequate DNA repair impacts MN induction in irradiated Ogg1-deficienct mice (compared to Oggff1+/+ mice).
KE3: Mutations, Increase	**Evidence for Essentiality of KE: Strong** Numerous studies show a strong correlation between inadequate DNA repair and mutation incidence, as altered mutation frequencies were evident when there were deficiencies in the proteins involved in DNA repair (Amundson and Chen 1996; Feldmann et al. 2000; Smith et al. 2003; Wessendorf et al. 2014; Perera et al. 2016). Mutations in several different genes, including tumour suppressor gene TP53, have also been shown to increase cell proliferation rates (Hundley et al. 1997; Lang et al. 2004; Ventura et al. 2007; Welcker and Clurman 2008; Duan et al. 2008; Geng et al. 2017; Li and Xiong 2017); mutant or absent TP53 has likewise been implicated in carcinogenesis (Iwakuma and Lozano 2007; Muller et al. 2011; Kim and Lozano 2018). In terms of lung cancer specifically, there are many different studies showing that mutations in TP53, KRAS, and EGFR are associated with lung carcinogenesis. The conceptual 'removal' or 'blocking' of these mutations using conditional knock out models, inducible mutation models, and treatment with various antagonizing and agonizing compounds has been observed to reverse or prevent lung tumourigenesis in vivo (Roth et al. 1996; Fisher et al. 2001; Ventura et al.

	2007; Iwakuma and Lozano 2007; Jia et al. 2016; Luo et al. 2019, Krasinski 2012). The lung tumourigenesis process was also observed to be expedited by exposure of Gprc5a knock-out mice to a known pulmonary carcinogen; this resulted in more somatic mutations and an increased tumour burden in a much shorter time frame relative to unexposed mice (Fujimoto et al. 2017).
KE4: Chromosomal Aberrations, Increase	**Evidence for Essentiality of KE: Weak** Many studies using a model with inadequate DNA repair (in the form of knock-out cell lines and DNA repair inhibitor studies) demonstrated that chromosomal aberrations were significantly increased when DNA repair was inadequate (Karanjawala et al.; Patel et al. 1998; Deniz Simsek and Jasin 2010; Lin et al. 2014; Wilhelm et al. 2014; Mcmahon et al. 2016, Cornforth 1994). The presence of chromosomal aberrations, particularly gene fusions and translocations, has also been associated with high rates of cellular proliferation (Li et al. 2007; Soda et al. 2007; Guarnerio et al. 2016).There also is support for the essentiality of CAs in the induction of cancer. There were significant increases in CAs (micronuclei, nucleoplasmic bridges and nuclear buds) in peripheral blood lymphocyte cultures after addition of a known pulmonary carcinogen to the cells (Lloyd et al. 2013). Furthermore, introduction of the BCR/ABL translocation in mice resulted in chronic myelogenous leukemia; this was accomplished by lethally irradiating the mice and performing a bone marrow transplant with cells that contained a retrovirus carrying the BCR/ABL translocation (Pear et al. 1998). Furthermore, tumour-inducing A549 cells, which are deficient in TSCL1 due to a loss of heterozygosity at chromosome 11, can induce detectable tumours within 3 weeks of injection; transfection of these A549 cells with genes to correct the TSCL1 deficiency and subsequent injection into mice results in fewer and slower-growing tumours (Kuramochi et al. 2001).
KE5: **Cell Proliferation, Increase**	**Evidence for Essentiality of KE: Strong** Rates of cellular proliferation have been shown to be increased when there are mutations in key genes associated with cell cycle control, including tumour suppressor gene TP53 (Hundley et al. 1997; Lang et al. 2004; Ventura et al. 2007; Welcker and Clurman 2008; Duan et al. 2008; Geng et al. 2017; Li and Xiong 2017). Cells transformed with various oncogenic mutations that suppressed tumour suppressor genes and enhanced activity of proto-oncogenes also showed increased cellular proliferation rates in the form of higher tumour volumes (Sato et al. 2017). Addition of inhibitors that blocked the pro-proliferative signaling pathway associated with KRAS and EGFR in these oncogenically-transformed cells resulted in lower rates of cellular proliferation (Sato et al. 2017). Similarly, several specific chromosomal gene fusions and translocations have also been associated with increasing the rate of cellular proliferation (Li et al. 2007; Soda et al. 2007; Guarnerio et al. 2016). In cancer cells known to harbor the Philadelphia chromosome (a translocation heavily implicated in the pathogenesis of acute lymphoblastic leukemia), addition of an ERB inhibitor resulted in decreased cellular proliferation rates in the cancer cells (Irwin et al. 2013). In another experiment where human ovarian cancer cells were treated with estrogen, there was an increase in the levels of micronuclei and a corresponding increase in the proliferation rates; addition of an antagonist maintained micronuclei frequencies and cell proliferation rates at control cell levels (Stopper et al. 2003). Cellular proliferation rates were decreased using both in vitro and in vivo carcinogenic models exposed to anti-cancer compounds, which highlights the importance of high cellular proliferation for carcinogenesis (Kassie et al. 2008; Lv et al. 2012; Wanitchakool et al. 2012; Pal et al. 2013; Warin et al. 2014; Tu et al. 2018). Genetic manipulations of genes involved in proliferation also resulted in modified cellular proliferation rates (Lv et al. 2012; Sun et al. 2016).

Weight of Evidence Summary

	Defining Question	***Strong***	***Moderate***	***Weak***
Support for Biological Plausibility of KERs	*Is there a mechanistic relationship between KE_{up} and KE_{down} consistent with established biological knowledge?*	*Extensive understanding of the KER based on extensive previous documentation and broad acceptance; Established mechanistic basis*	*KER is plausible based on analogy to accepted biological relationships, but scientific understanding is not completely established*	*There is empirical support for statistical association between KEs, but the structural or functional relationship between them is not understood*

Deposition of Energy (MIE) --> Double-Strand Breaks, Increase (KE1)	***Evidence for Biological Plausibility of KER:*** **Strong** It is well established that ionizing radiation can cause various types of DNA damage including single-strand and double-strand breaks (DSBs) (reviewed in Lomax et al. 2013). In particular, there is evidence for the deposition of energy and a resulting increase in DSBs (Ward 1988; Terato and Ide 2005; Goodhead 2006; Hada and Georgakilas 2008; Asaithamby and Chen 2011; Okayasu 2012; Lomax et al. 2013; Moore et al. 2014; Desouky et al. 2015; Sage and Shikazono 2017; Chadwick 2017; Franken et al., 2012; Frankenberg et al., 1999; Rydberg et al., 2002; Belli et al., 2000). Structural damage from the deposited energy can induce chemical modifications in the form of breaks to the phosphodiester backbone of both strands of the DNA. (Joiner 2009). DSBs are also often formed by indirect interactions with radiation through water molecules. Energy deposited on water molecules by radiation results in the production of reactive oxygen species that can then damage the DNA (Ward 1988; Desouky et al. 2015; Maier et al. 2016).
Deposition of Energy (MIE) --> Mutations, Increase (KE3)	***Evidence for Biological Plausibility of KER:*** **Strong** Many studies across a variety of different models provide evidence that deposition of energy by ionizing radiation results in increased mutation frequencies (Russell et al. 1957; Winegar et al. 1994; Gossen et al. 1995; Suzuki and Hei 1996; Albertini et al. 1997; Dubrova et al. 1998; Dubrova et al. 2000; Canova et al. 2002; Dubrova et al. 2002; Dubrova and Plumb 2002; Masumura et al. 2002; Somers et al. 2004; Burr et al. 2007; Ali et al. 2012; Adewoye et al. 2015; Wilson et al. 2015; Bolsunovsky et al. 2016; Mcmahon et al. 2016; Matuo et al. 2018; Nagashima et al. 2018; Wu et al., 1999; Hei et al., 1997; Nagasawa and Little, 1999; Barnhart and Cox, 1979; Thacker at al., 1982; Zhu et al., 1982; Metting et al., 1992; Schwartz et al., 1991; Chen et al., 1984). Radiation-specific mutational signatures have been identified in a variety of radiation-induced tumours (Sherborne et al. 2015; Behjati et al. 2016), and there is extensive evidence that radiation increases germline mutations in both mice (Dubrova et al. 1998; Dubrova et al. 2000; Dubrova et al. 2002; Somers et al. 2004; Barber et al. 2009; Ali et al. 2012; Adewoye et al. 2015; Wilson et al. 2015) and humans (Dubrova et al. 2002; Dubrova and Plumb 2002).
Deposition of Energy (MIE) --> Chromosomal Aberrations, Increase (KE4)	***Evidence for Biological Plausibility of KER:*** **Strong** Extensive and diverse data from human, animal and *in vitro*-based studies show ionizing radiation induces a rich variety of chromosomal aberrations (Bauchinger et al. 1994; Schmid et al. 2002; Thomas et al. 2003; Maffei et al. 2004; Tucker et al. 2005a; Tucker et al. 2005b; George et al. 2009; Meenakshi and Mohankumar 2013; Santovito et al. 2013; Arlt et al. 2014; Balajee et al. 2014; Han et al. 2014; Vellingiri et al. 2014; Suto et al. 2015; Adewoye et al. 2015; Cheki et al. 2016; Mcmahon et al. 2016; Morishita et al. 2016; Qian et al. 2016; Basheerudeen et al. 2017; Meenakshi et al. 2017; Abe et al. 2018; Jang et al. 2019; Puig et al., 2016; Barquinero et al., 2004; Curwen et al., 2012; Testa et al., 2018; Franken et al., 2012; Cornforth et al., 2002; Loucas et al., 2013; Nagasawa et al., 1990a; Nagasawa et al., 1990b; Edwards et al., 1980; Themis et al., 2013; Schmid et al., 1996; Mestres et al., 2004; Bilbao et al., 1989; Mill et al., 1996; Brooks, 1975; Tawn and Thierens, 2009; Durante et al., 1992; Hamza and Mohankumar, 2009; Takatsuji and Sasaki, 1984; Moquet et al., 2001; Purrott et al., 1980; duFrain et al., 1979).The mechanism leading from deposition of energy to chromosomal aberrations has been described in several reviews (Smith et al. 2003; Christensen 2014; Sage and Shikazono 2017). Other evidence derives from studies examining the mechanism of copy number variant formation (Arlt et al. 2014) and induction of radiation-induced chromothripsis (Morishita et al. 2016).
Double-Strand Breaks, Increase (KE1) --> Inadequate DNA Repair, Increase (KE2)	***Evidence for Biological Plausibility of KER:*** **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 (Van Gent et al. 2001; Hoeijmakers 2001a; Khanna and Jackson 2001; Lieber et al. 2003; San Filippo et al. 2008; Lieber et al. 2010; Polo and Jackson 2011; Schipler and Iliakis 2013; Vignard et al. 2013; Betermier et al. 2014; Mehta and Haber 2014; Moore et al. 2014; Rothkamm et al. 2015; Jeggo and Markus 2015; Chang et al. 2017; Lobrich and Jeggo 2017; Sage and Shikazono 2017) Error-prone repair processes are particularly important when DSBs are biologically induced and repaired during V(D)J recombination of developing lymphocytes(Jeggo et al. 1995; Malu et al. 2012) and during meiotic divisions to generate gametes (Murakami and Keeney 2008).
	Evidence for Biological Plausibility of KER: **Strong**

Inadequate DNA Repair, Increase (KE2) --> Mutations, Increase (KE3)	Decades of research have shown that DNA repair pathways are error prone and can cause mutations inherently (such as the error prone NHEJ) (Sishc and Davis 2017). This error-prone repair, however, may be due more to the structure of the DSB ends rather than the repair machinery; more complex breaks require more processing, increasing the likelihood that there will be errors in the DNA sequence upon completion of repair (Betermier et al. 2014; Waters et al. 2014). After being exposed to ionizing radiation, approximately 25 – 50% of double-strand breaks have been shown to be incorrectly repaired (Löbrich et al. 1998; Kuhne et al. 2000; Lobrich et al. 2000).
Inadequate DNA Repair, Increase (KE2) --> Chromosomal Aberrations, Increase (KE4)	***Evidence for Biological Plausibility of KER:*** **Strong** DSBs are repaired by non-homologous end joining (NHEJ) and homologous recombination (HR). HR uses a template DNA strand to repair DNA damage, while the more error-prone NHEJ simply religates broken ends back together without the use of a template (van Gent et al. 2001; Hoeijmakers 2001; Jeggo and Markus 2015; Sishc and Davis 2017). Chromosomal aberrations may result if DNA repair is inadequate, meaning that the double-strand breaks are misrepaired or not repaired at all (Bignold, 2009; Danford, 2012; Schipler & Iliakis, 2013). 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 (Ferguson and Alt 2001; Venkitaraman 2002; Povirk 2006; Weinstock et al. 2006; Denis Simsek and Jasin 2010; Lieber et al. 2010; Fenech and Natarajan 2011; Danford 2012; Schipler and Iliakis 2013; Mizukami et al. 2014; Russo et al. 2015; Leibowitz et al. 2015; Rode et al. 2016; Vodicka et al. 2018).
Mutations, Increase (KE3) --> Cell Proliferation, Increase (KE5)	***Evidence for Biological Plausibility of KER:*** **Strong** It is clearly documented that when enough mutations accumulate in critical genes associated with cell cycling or proliferation, there is potential for uncontrollable cell proliferation to occur, which in some cases leads to carcinogenesis (Bertram 2001; Vogelstein and Kinzler 2004; Panov 2005, Lee and Muller 2010). In fact, one of the hallmarks of cancer is sustained proliferative signalling, and one of the enabling characteristics of this increased proliferation is genomic instability/mutations (Hanahan and Weinberg 2011). Thus mutations are particularly dangerous if they occur in proteins controlling the cell cycle checkpoint for entry into proliferation, such as RB and p53 (Lee and Muller 2010). Activating mutations in proto-oncogenes (Bertram 2001; Vogelstein and Kinzler 2004; Larsen and Minna 2011; Lee and Muller 2010) Lee and Muller 2010, inactivating mutations in tumour suppressor genes (Bertram 2001; Vogelstein and Kinzler 2004; Lee and Muller 2010; Fernandez-Antoran et al. 2019) and inactivating mutations in caretaker/stability genes (Vogelstein and Kinzler 2004; Hanahan and Weinberg 2011) are all associated with abnormal increases the rate of cellular proliferation.
Chromosomal Aberrations, Increase (KE4) --> Cell Proliferation, Increase (KE5)	***Evidence for Biological Plausibility of KER:*** **Strong** Chromosomal aberrations are formed when there is inadequate DNA repair (Bignold 2009; Danford 2012; Schipler and Iliakis 2013) or errors during mitosis (Levine and Holland 2018). Chromosomal aberrations have been shown to increase cell proliferation when the aberrations result in the activation of proto-oncogenes (Bertram 2001; Vogelstein and Kinzler 2004), the inactivation of tumour suppressor genes (Bertram 2001; Vogelstein and Kinzler 2004),, or the modification of caretaker/stability genes (Vogelstein and Kinzler 2004). Reviews documenting the contribution of CAs to cellular proliferation and/or cancer development (which implies high rates of cellular proliferation) are available (Mes-Masson and Witte 1987; Bertram 2001; Vogelstein and Kinzler 2004; Ghazavi et al. 2015; Kang et al. 2016). The link between chromosomal instability (CIN), which describes the rate of chromosome gains and losses, and cancer development has also been reviewed (Thompson et al. 2017; Gronroos 2018; Targa and Rancati 2018; Lepage et al. 2019).
	Evidence for Biological Plausibility of KER: **Strong** The means by which dysregulation of cell proliferation promotes the transformation of normal to carcinogenic cells has been heavily reviewed (Pucci et al. 2000; Bertram 2001; Panov 2005; Eymin and Gazzeri 2009; Hanahan and Weinberg 2011; Larsen and Minna 2011). The cell cycle is essential in controlling cellular proliferation rates, and requires a series of checkpoints to be passed before the cell can fully commit to the process of cell division (Pucci et al. 2000; Bertram 2001; Eymin and Gazzeri 2009; Hanahan and Weinberg 2011). One of the most important checkpoints requires the proper functioning of p53, RB, CDK4 and CDK6. The tumour suppressor p53 plays a particularly important role in stopping the

Cell Proliferation, Increase (KE5) --> Lung Cancer, Increase (AO)	cell cycle when there is DNA damage, and for triggering apoptosis when damage is too severe to be repaired (Bertram 2001; Hanahan and Weinberg 2011; Larsen and Minna 2011). Telomeres also play a role in controlling cell proliferation; when the telomeres become too short to protect the coding DNA, the cell enters into a state of replicative senescence (Bertram 2001; Hanahan and Weinberg 2011). All of these processes play a role in controlling the rate of cellular proliferation within a cell. Cancer may occur when these processes became dysregulated such that cells begin to proliferate at excessively high rates. High rates of proliferation are in fact one of the strongest hallmarks of cancer (Hanahan and Weinberg 2011), and uncontrolled proliferation can be accomplished through sustained proliferative signalling through activation of proto-oncogenes (Bertram 2001; Vogelstein and Kinzler 2004; Eymin and Gazzeri 2009; Hanahan and Weinberg 2011; Larsen and Minna 2011), evading growth suppressors and resisting cell death through suppression of tumour suppressor genes (Bertram 2001; Vogelstein and Kinzler 2004; Eymin and Gazzeri 2009; Hanahan and Weinberg 2011; Larsen and Minna 2011), and overcoming replicative senescence through expression of the telomere-lengthening enzyme telomerase (Bertram 2001; Panov 2005; Hanahan and Weinberg 2011; Larsen and Minna 2011). In lung cancer specifically, commonly activated proto-oncogenes include *EGFR, ERBB2, MYC, KRAS, MET, CCND1, CDK4* and *BCL2*, while commonly inactivated tumour suppressor genes are *TP53, RB1, STK11, CDKN2A, FHIT, RASSF1A,* and *PTEN* (Larsen and Minna 2011). Telomerase is also activated in nearly all small cell lung cancer (SCLC) cases, and in over three-quarters of non-small cell lung cancer (NSCLC) cases (Panov 2005; Larsen and Minna 2011).
Double-Strand Breaks, Increase (KE1) --> Mutations, Increase (KE3)	***Evidence for Biological Plausibility of KER:*** **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. In mamalian and yeast cells, both HR and NHEJ can lead to alteration in DNA sequence (Hicks & Haber, 2010; Butning & Nussenzweig, 2013; Byrne et al., 2014; Rodgers & McVey, 2016; Dwivedi & Haver, 2018).
Double-Strand Breaks, Increase (KE1) --> Chromosomal Aberrations, Increase (KE4)	***Evidence for Biological Plausibility of KER:*** **Strong** DNA strand breaks must occur for chromosomal aberrations to occur. Studies have shown DSBs leading to irreversible damage. The links between DSBs and the role DSB repairs has in preventing chromosomal aberrations is widely discussed, with several reviews available: (van Gent et al., 2001; Ferguson & Alt, 2001; Hoeijmakers, 2001; Iliakis et al., 2004; Povirik, 2006; Weinstock et al., 2006; Natarajan & Palitti, 2008; Lieber et al., 2010; Mehta & Haber, 2014; Ceccaldi et al., 2016; Chang et al., 2017; Sishc & Davis, 2017; Brunet & Jasin, 2018).
Mutations, Increase (KE3) --> Lung Cancer, Increase (AO)	***Evidence for Biological Plausibility of KER:*** **Moderate** There is strong biological plausibility for the relationship between mutations and lung cancer. Bioinformatics studies have identified unique mutation signature profiles associated with specific types of cancer, including lung adenocarcinoma, lung squamous cell carcinoma and lung small cell carcinoma (Alexandrov et al. 2013; Jia et al. 2014; George et al. 2015). Moreover, mutations/genome instability have been implicated as one of the 'enabling characteristics' underlying the hallmarks of cancer (Hanahan and Weinberg 2011). Mutations are thought to promote tumourigenesis by modifying the expression of tumour suppressor genes, proto-oncogenes, and caretaker/stability genes in such a way that promotes cell proliferation and/or suppresses apoptosis (Vogelstein and Kinzler 2004; Panov 2005; Sanders and Albitar 2010; Hanahan and Weinberg 2011; Larsen and Minna 2011). Commonly mutated genes in lung cancer include *TP53, KRAS* and *EGFR*. Mutations in these genes, along with known lung cancer driver mutations, are thought to promote tumourigenesis by stimulating pro-proliferation signalling pathways such as the PI3K-AKT-mTOR pathway and RAS-REF-MEK pathway (Varella-garcia 2009; Sanders and Albitar 2010; Larsen and Minna 2011McCubrey 2006).
	Evidence for Biological Plausibility of KER: **Moderate** Chromosomal aberrations, encompassing chromosome-type aberrations, chromatid-type aberrations, micronuclei, and nucleoplasmic bridges, have all been found to be predictive of cancer risk in various human cohorts (Bonassi et al. 2000; Smerhovsky et al. 2002; Hagmar et al. 2004; Norppa et al. 2006;

Chromosomal Aberrations, Increase (KE4) --> Lung Cancer, Increase (AO)	Boffetta et al. 2007; Bonassi et al. 2008; Lloyd et al. 2013; El-zein et al. 2014; Vodenkova et al. 2015; El-zein et al. 2017). Specific categories of CAs, including CNVs (Wrage et al. 2009; Shlien and Malkin 2009; Liu et al. 2013; Mukherjee et al. 2016; Zhang et al. 2016; Ohshima et al. 2017) and gene rearrangements (Bartova et al. 2000; Trask 2002; Sanders and Albitar 2010; Sasaki et al. 2010; Mao et al. 2011), have also been associated with cancer development. Chromosomal aberrations promote tumourigenesis through the alteration of pathways controlling cellular growth and apoptosis (Albertson et al. 2003; Sanders and Albitar 2010). The chromosomal aberration burden may be increased by factors such as aberrant centromeres, telomerase deficiencies paired with poor cell surveillance (Albertson et al. 2003), ionizing radiation (Hei et al. 1994; Weaver et al. 1997; Weaver et al. 2000), and the interplay between non-clonal and clonal CAs (Heng, Bremer, et al. 2006; Heng, Stevens, et al. 2006).
Deposition of Energy (MIE) --> Lung Cancer, Increase (AO)	***Evidence for Biological Plausibility of KER: Strong*** The deposition of energy, particularly by radon gas, has been associated heavily with lung cancer (Axelson 1995; Jostes 1996; Beir 1999; Kendall and Smith 2002a; Al-Zoughool and Krewski 2009; Robertson et al. 2013). Deposition of energy that triggers lung carcinogenesis in particular is thought to enter the body through inhalation (Beir 1999; Kendall and Smith 2002b). The inhaled particles are thought to deposit on lung tissue and decay, producing ionizing radiation (Axelson 1995; Beir 1999; Kendall and Smith 2002b; Al-Zoughool and Krewski 2009) that can direct the cell towards carcinogenesis (Axelson 1995; Beir 1999; Robertson et al. 2013). The process of radiation-induced carcinogenesis often follows three steps: initiation, promotion and progression. Initiation refers to the interaction between the radiation and the cell, and results in irreversible genetic changes. Promotion occurs when non-carcinogenic promoter is added to the initiated cells such that it synergistically increases oncogenesis, often through receptor-mediated epigenetic changes. Progression occurs at the point when the cells convert from benign to malignant, and is associated with rapid growth and further accumulation of genomic aberrations (NRC 1990; Pitot 1993).

	Defining Question	***Strong***	***Moderate***	***Weak***
Support for Empirical Evidence of KERs	*Does empirical evidence support that a change in KE_{up} leads to an appropriate change in KE_{down}? Does KE_{up} occur at lower doses and earlier time points than KE_{down} and is the incidence of KE_{up} > than that for KE_{down}?* *Inconsistencies?*	*Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors (Extensive evidence for temporal, dose-response and incidence concordance); No or few critical data gaps or conflicting data*	*Demonstrated dependent change in both events following exposure to a small number of specific stressors; Some evidence inconsistent with expected pattern that can be explained by factors such as the experimental design, technical considerations, differences between laboratories, etc.*	*Limited or no studies reporting dependent change in both events following exposure to a specific stressor (i.e. endpoints never measured in the same study or not at all); And/or significant inconsistencies in empirical support across taxa and species that don't align with expected pattern for hypothesized AOP*

Deposition of Energy (MIE) --> Double-Strand Breaks, Increase (KE1)	***Evidence for Empirical Support of KER:*** **Strong** Evidence exists for dose/incidence and temporal concordance between deposition of energy and the resultant formation of DNA double-strand breaks. With increasing ionizing radiation, there is an increase in the frequency of double-strand breaks (Aufderheide et al., 1987; Charlton et al. 1989; Sidjanin, 1993; Reddy et al., 1998; Frankenberg et al., 1999; Rogakou et al. 1999; Belli et al., 2000; Sutherland et al. 2000; Lara et al. 2001; Rydberg et al., 2002; Baumstark-Khan et al., 2003; Rothkamm and Lo 2003; Rogers et al., 2004; Kuhne et al. 2005; Sudprasert et al. 2006; Rube et al. 2008; Beels et al. 2009; Grudzenski et al. 2010; Liao et al., 2011; Franken et al., 2012; Bannik, 2013; Antonelli et al. 2015; Flegal et al., 2015; Markiewicz et al., 2015; Shelke and Das, 2015; Chadwick, 2017; Hamada, 2017; Allen et al., 2018; Cencer et al., 2018; Bains, 2019; Barnard, 2019; Ahmadi et al., 2021; Barnard, 2021). However, dose-rate and radiation quality play a crucial role in determining the degree of DNA damage. Temporally, DSBs have been evident at 3 - 30 minutes post-irradiation (Rogakou et al. 1999; Rothkamm and Lo 2003; Rube et al. 2008; Beels et al. 2009; Kuefner et al. 2009; Grudzenski et al. 2010; Antonelli et al. 2015; Cencer et al., 2018). A significant proportion of the DSBs are resolved within 5 hours of radiation (Kleiman, 1990; Sidjanin, 1993; Rogakou et al. 1999; Rube et al. 2008; Kuefner et al. 2009; Grudzenski et al. 2010; Bannik, 2013; Markiewicz et al., 2015; Shelke and Das 2015; Cencer et al., 2018), with a return to baseline levels by 24 hours in most cases (Aufderheide et al., 1987;

	Baumstark-Khan et al., 2003; Rothkamm and Lo 2003; Rube et al. 2008; Grudzenski et al. 2010; Bannik et al., 2013; Antonelli et al., 2015; Markiewicz et al., 2015; Russo et al., 2015; Dalke, 2018; Bains, 2019; Barnard, 2019; Ahmadi et al., 2021).
Deposition of Energy (MIE) --> Mutations, Increase (KE3)	***Evidence for Empirical Support of KER:*** **Strong** Evidence exists for dose/incidence concordance between deposition of energy by ionizing radiation and a corresponding dose-dependent increase in mutation frequency (Suzuki and Hei 1996; Schmidt and Kiefer 1998; Kraemer et al. 2000; Canova et al. 2002; Bolsunovsky et al. 2016; Mcmahon et al. 2016; Matuo et al. 2018; Nagashima et al. 2018). The linear energy transfer of the radiation (Dubrova and Plumb 2002; Matuo et al. 2018), whether the radiation is chronic or acute (Russell 1958), the radiation type (Schmidt and Kiefer 1998; Masumura 2002), and the tissue being irradiated (Masumura 2002, Gossen 1995) all affect this dose-dependent increase. Temporally, it is well established that an increased incidence of mutations is reported after the deposition of energy by radiation (Winegar 1994, Gossen 1995, Albertini 1997, Dubrova 2002A, Matuo 2018, Canova 2002, Nagashima 2018, Masumura 2002, Russell 1958). Most of these studies, however, span over days and weeks, thus making it difficult to pinpoint exactly when mutations occur. Several studies report the manifestation of mutations within 2 - 3 days of irradiation (Winegar 1994, Masumura 2002, Gossen 1995), with an increased mutation frequency still elevated at 14 (Winegar 1994) and 21 days (Gossen 1995) after radiation exposure. At low doses (<1 Gy) the induction of mutations in cells has been observed for high-LET radiation such as alpha particles (Wu et al., 1999; Hei et al., 1997; Nagasawa and Little, 1999; Barnhart and Cox, 1979; Thacker at al., 1982; Zhu et al., 1982; Metting et al., 1992; Schwartz et al., 1991; Chen et al., 1984; Albertini et al., 1997).
Deposition of Energy (MIE) --> Chromosomal Aberrations, Increase (KE4)	***Evidence for Empirical Support of KER:*** **Strong** Results from many studies indicate dose/incidence and temporal concordance between the deposition of energy and the increased frequency of chromosomal aberrations. There is strong evidence of a dose-dependent increase in a wide range of chromosomal aberrations in response to increasing radiation dose (Schmid 2002, Hande et al. 2003, Thomas 2003, Jang 2019, Abe 2018, Suto 2015, McMahon 2016, Tucker 2005A, Tucker 2005B, Arlt 2014, McMahon 2016, Balajee 2014,George 2009, Maffei 2004, Qian 2015; Puig et al., 2016; Barquinero et al., 2004; Curwen et al., 2012; Testa et al., 2018; Franken et al., 2012; Cornforth et al., 2002; Loucas et al., 2013; Nagasawa et al., 1990a; Nagasawa et al., 1990b; Edwards et al., 1980; Themis et al., 2013; Schmid et al., 1996; Mestres et al., 2004; Bilbao et al., 1989; Mill et al., 1996; Brooks, 1975; Tawn and Thierens, 2009; Durante et al., 1992; Hamza and Mohankumar, 2009; Takatsuji and Sasaki, 1984; Moquet et al., 2001; Purrott et al., 1980; duFrain et al., 1979). Temporally, it is well-established that chromosomal aberrations occur after exposure to radiation (Schmid 2002, Thomas 2003, Balajee 2014, Arlt 2014, George 2009, Suto 2015, Basheerudeen 2017, Tucker 2005A, Tucker 2005B, Abe 2018, Jang 2019), though the exact timing is difficult to pinpoint because most assays take place hours or days after the radiation exposure. One notable study did, however, document the presence of chromosomal aberrations within the first 20 minutes of irradiation, with the frequency increasing sharply until approximately 40 minutes, followed by a plateau (McMahon 2016). By 7 days post-irradiation, the frequencies of most chromosomal aberrations had declined (Tucker 2005A, Tucker 2005B). It should be noted that chromosomal aberrations induced by ionizing radiation are dependent on dose, dose-rate, and radiation type (Bender et al., 1988; Guerrero-Carbajal et al., 2003; Day et al., 2007, Suzuki 1996, Hande et al. 2003).
Double-Strand Breaks, Increase (KE1) --> Inadequate DNA Repair, Increase (KE2)	***Evidence for Empirical Support of KER:*** **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 in adequate DNA repair activity (in the form of non-repaired or misrepaired DSBs) also increases (Dikomey 2000, McMahon 2016, Kuhne 2005, Rydberg 2005, Kuhne 2000, Lobrich 2000). DNA damage and its ensuing repair also follow a very similar time course, with both events documented within minutes of a radiation stressor (Pinto 2005, Rothkamm 2003, Asaithambly 2009, Dong 2017, Paull 2000). Uncertainties in this KER include controversy surrounding how error-prone NHEJ truly is (Betemier 2014), differences in responses depending on the level of exposure of a genotoxic substance (Marples 2004), and confounding factors (such as smoking) that affect double-strand break repair fidelity (Scott 2006, Leng 2008).
Inadequate DNA Repair, Increase (KE2) --> Mutations, Increase (KE3)	***Evidence for Empirical Support of KER:*** **Moderate** There are several studies that indicate a dose/incidence concordance between inadequate DNA repair and an increased frequency of mutations. Inadequate DNA repair (Ptácek et al. 2001; Mcmahon et al. 2016) and mutation frequencies (Mcmahon et al. 2016) have both been found to increase in a dose-dependent fashion with increasing doses of a radiation stressor. Moreover, specific genomic regions with inadequate DNA repair rates also were found to have increased mutation densities in cancer samples (Perera et al. 2016). Increased

	mutation frequencies have also been demonstrated in cases where more complex DNA repair is required (Smith et al. 2001). According to the results of this study, evidence of repaired DNA was present prior to the detection of mutations in cases of simple repair, whereas these two events occurred together at a later time point when more complex repair was required (Smith et al. 2001).
Inadequate DNA Repair, Increase (KE2) --> Chromosomal Aberrations, Increase (KE4)	***Evidence for Empirical Support of KER:*** **Moderate** There is little empirical evidence available that directly examines the dose and incidence concordance between DNA repair and CAs within the same study. However, comparison of results from studies that measure either radiation-induced DNA repair or radiation-induced chromosomal aberrations demonstrate that the rate of double-strand break misrepair increases in a dose-dependent fashion with radiation doses between 0 - 80 Gy (Mcmahon et al. 2016), as does the incidence of chromosomal aberrations between doses of 0 - 10 Gy (Thomas et al. 2003; Tucker et al. 2005a; Tucker et al. 2005b; George et al. 2009; Arlt et al. 2014; Balajee et al. 2014; Han et al. 2014; Suto et al. 2015; Mcmahon et al. 2016). Similarly, there is not clear evidence of a temporal concordance between these two events. One study examining DNA repair and micronuclei in irradiated cells pre-treated with a DNA repair inhibitor found that both repair and micronuclei were present at 3 hours and 24 hours post-irradiation. This suggests that there may be temporal concordance (Chernikova et al. 1999). More research, however, is required to establish empirical evidence for this KER.
Mutations, Increase (KE3) --> Cell Proliferation, Increase (KE5)	***Evidence for Empirical Support of KER:*** **Moderate** There is little empirical evidence available that assesses the dose and incidence concordance between mutation frequency and cellular proliferation rates. The correlation between these two events is clear in human epidemiology studies examining the incidence between mutations in specific genes, such as *TP53* and *BRCA1*, and the proliferative status of human tumours (M Jarvis et al. 1998; Schabath et al. 2016). Another study introducing oncogenic mutations into mouse lung epithelial cells demonstrated that the addition of multiple oncogenic mutations to the cells resulted in increased tumour volumes over 40 days (suggestive of cell proliferation); in contrast, cells containing only one of these mutations did not show significant changes in tumour volumes (Sato et al. 2017). Unsurprisingly, there is also little empirical evidence available supporting a temporal concordance between these two events. One review explores the timing between these two events by comparing the somatic mutation theory of cancer and the stem cell division theory of cancer. In the somatic mutation theory, it is suggested that mutations accumulate and result in increased rates of cellular proliferation; the stem cell theory, however, states that high proliferation in stem cells allows the accumulation of mutations (López-lázaro 2018). More research is thus required to establish empirical evidence for this KER.
Chromosomal Aberrations, Increase (KE4) --> Cell Proliferation, Increase (KE5)	***Evidence for Empirical Support of KER:*** **Moderate** There is little empirical evidence available that assesses the dose and incidence concordance between chromosomal aberration frequency and cellular proliferation rates. There are several reviews available that discuss the structure and function of specific human cancer-associated chromosomal aberrations, including *BCR-ABL1*, *ALK* fusions, and *ETV6-RUNX1* (Mes-Masson and Witte 1987; Ghazavi et al. 2015; Kang et al. 2016). There was no identified evidence supporting dose and incidence concordance. Details from a study where estrogen-responsive cancer cells were treated with estrogen suggested the possibility of a temporal concordance, as both micronuclei levels and proliferation rates were higher in the estrogen-treated cells at 140 and 216 hours post-treatment (Stopper et al. 2003). Overall, however, more empirical evidence is required to support this KER.
Cell Proliferation, Increase	***Evidence for Empirical Support of KER:*** **Moderate** There is some empirical evidence of a dose and incidence concordance between cell proliferation and lung carcinogenesis. In a few experiments, rodent lungs exposed to various carcinogens showed increased levels of proliferation and developed squamous metaplasia (Zhong et al. 2005) or full-blown tumours (Kassie et al. 2008). Furthermore, nude mice injected with carcinogenic human NSCLC cells also developed tumours within a few weeks of the injection (Pal et al. 2013; Warin et al. 2014; Sun et al. 2016; Tu et al. 2018)(Sun 2016, Pal 2013, Tu 2018, Warin 2014). In terms of temporal concordance between these two events, studies are also limited. Multiple tumour xenograft experiments found that nude mice injected with NSCLC cells develop detectable tumours within two weeks of inoculation, which continued to increase in size over time (Sun 2016,

(KE5) --> Lung Cancer, Increase (AO)	Pal 2013, Tu 2018, Warin 2014). Examination of lung squamous metaplasia after 14 weeks of exposure to high levels of tobacco smoke showed increased cell proliferation markers in comparison to lungs from rats exposed to filtered air (Zhong et al. 2005). Similarly, lung tumours from mice that received carcinogens NNK and BaP orally over 4 weeks were also found to express proliferation markers when examined 27 weeks after the start of the experiment (Kassie et al. 2008).
Double-Strand Breaks, Increase (KE1) --> Mutations, Increase (KE3)	***Evidence for Empirical Support of KER:*** **Moderate** There is some evidence demonstrating dose and temporal concordance between the two KEs, both in-viv and in-vitro. These studies used a variety of sources of ionizing radiation as stressors. The types of radiation testing this relationship include X-rays, gamma-rays, alpha particles and heavy ions. Example studies include: (in vitro) Rydberg et al., 2005; Kuhne et al., 2005, 2000; Dikomey et al., 2000; Lobrich et al., 2000, (in vivo) Ptacek et al., 2001. For a discussion of chemical stressors affecting this relationship, see AOP 296.
Double-Strand Breaks, Increase (KE1) --> Chromosomal Aberrations, Increase (KE4)	***Evidence for Empirical Support of KER:*** **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 dose-response of these events often appear to be discordant. Examples of studies relating the links between DSBs and chromosomal aberrations include an in-vitro study of gamma-radiated lymphoblasted cell lines (Trenz et al. 2003) isolated lymphocytes and whole blood samples (Sudpresert et al., 2006) and PL61 cells (Chernikova et al., 1999). Source of high linear energy transfer have also been probed, see Iliakis et al. (2019).
Mutations, Increase (KE3) --> Lung Cancer, Increase (AO)	***Evidence for Empirical Support of KER:*** **Moderate** Evidence for dose/incidence concordance comes from studies with similar radiological and biological conditions that assessed either the relationship between radiation exposure and mutations, or radiation exposure and cancer. Using various *in vitro* models, there was a dose-dependent relationship found for mutation induction and radiation dose (Suzuki and Hei 1996; Weaver et al. 1997; Canova et al. 2002), and for oncogenic transformations and radiation dose (Hei et al. 1994; Miller et al. 1995; Miller et al. 1999). Analyses of lung cancer incidences in radon-exposed rats and uranium miners echo these results (Monchaux et al. 1994; Lubin et al. 1995; Ramkissoon et al. 2018). Likewise, administration of a known pulmonary carcinogen to *Gprc5a* knock-out mice resulted in an increased rate of tumourigenesis and increased mutation accumulation relative to saline-treated mice (Fujimoto et al. 2017). Increasing the number of mutations *in vitro* and *in vivo* resulted in cells becoming increasingly oncogenic (Sato, Melville B Vaughan, et al. 2006; Sasai et al. 2011) and mice sporting a faster rate of lung tumourigenesis (Fisher et al. 2001; Kasinski and Slack 2012), respectively. In terms of temporal concordance, there is some evidence from separate studies indicating that mutations precede tumourigenesis (Hei et al. 1994; Lubin et al. 1995; Hei et al. 1997; Miller et al. 1999; Fujimoto et al. 2017) , particulary in Cre-inducible models where Cre expression must be induced for the mutations to be expressed (Fisher et al. 2001; Kasinski and Slack 2012).
Chromosomal Aberrations, Increase (KE4) --> Lung Cancer, Increase (AO)	***Evidence for Empirical Support of KER:*** **Moderate** Evidence for dose/incidence concordance comes from epidemiological studies of radon-exposed uranium miners that found there was an increased CA load with increasing radon exposure (Smerhovsky et al. 2002), and an increased risk of lung cancer with increased cumulative radon exposure (Tirmarchel et al. 1993; Smerhovsky et al. 2002; Vacquier et al. 2008; Walsh et al. 2010). *In vivo* and *in vitro* studies have also shown a dose-dependent increase in CAs in lung and non-lung cell lines (Nagasawa et al. 1990; Deshpande et al. 1996; Yamada et al. 2002; Stevens et al. 2014) and lung cells of rodents with increasing radiation dose (A.L. Brooks et al. 1995; Khan et al. 1995; Werner et al. 2017), and a dose-dependent increase in oncogenic transformation in non-lung cells lines (Robertson et al. 1983; Miller et al. 1996) and in rodent lung tumours with increasing radiation dose (Monchaux et al. 1994; Yamada et al. 2017) Furthermore, there are several published reviews that provide evidence for associations between radon exposure and the appearance of CAs, and radon exposure and the incidence of lung cancer (Jostes 1996; Al-Zoughool and Krewski 2009; Robertson et al. 2013). Likewise, more CAs were found to accumulate in larger tumours (To et al. 2011) and in increasingly more oncogenic lung tissue lesions (Thibervile et al. 1995; Wistuba et al. 1999). There is also evidence for temporal concordance as, the time gap between radiation exposure and the increased incidence of CAs is hours to days (Nagasawa et al. 1990; A.A.L. Brooks et al. 1995; Deshpande et al. 1996; Yamada et al. 2002; Stevens et al. 2014; Werner et al. 2017), while the time gap between radiation exposure and the

	development of oncogenic transformations or lung tumours is weeks, months or years (Robertson et al. 1983; Tirmarchel et al. 1993; Miller et al. 1996; Pear et al. 1998; Kuramochi et al. 2001; Yamada et al. 2017).
Deposition of Energy (MIE) --> Lung Cancer, Increase (AO)	***Evidence for Empirical Support of KER:*** **Moderate** There is strong evidence of the relationship between radiation exposure and lung carcinogenesis in human epidemiological studies that assess radon exposure and the risk of lung cancer. Results from numerous studies assessing indoor residential radon exposure and outdoor radon exposure in miners suggest that there is a positive association between cumulative radon exposure and lung cancer risk (Lubin et al. 1995; Hazelton et al. 2001; Darby et al. 2005; Krewski et al. 2005; Krewski et al. 2006; TAl-Zoughool and Krewski 2009; Torres-Durán et al. 2014; Kreuzer et al. 2015; Sheen et al. 2016; Rodríguez-Martínez et al. 2018; Ramkissoon et al. 2018; Rage et al. 2020). Several *in vitro* studies showed that cells could be induced to obtain oncogenic characteristics through radiation exposure (Hei et al. 1994; Miller et al. 1995). Likewise, irradiation of rats at radon levels comparable to those experienced by uranium miners resulted in a dose-dependent increase in lung carcinoma incidence (Monchaux et al. 1994). There is also evidence of temporal concordance, as the oncogenic characteristics of the radon-exposed cells were not evident until weeks after the irradiation (Hei et al. 1994; Miller et al. 1995), while tumours took months to years to grow (Hei et al. 1994; Monchaux et al. 1994). In humans, the risk of lung cancer was also found to increase with increasing time since exposure (Hazelton et al. 2001) at a mean time of 15 years (Aßenmacher et al. 2019) and with longer periods of exposure (Lubin et al.1995).

Quantitative Consideration

There is strong biological plausibility and empirical evidence to suggest a qualitative link between the deposition of energy on DNA to the final adverse outcome of lung cancer. This evidence has been derived predominately from laboratory studies and through mathematical simulations using cell-based models. The studies show both dose and temporal-response relationships for a select KEs. The quantitative thresholds to initiate each of the KEs are not definitive and have been shown to vary with factors such as the cell type, dose-rate of exposure and radiation quality. Thus, an absolute amount of deposited energy (MIE) to drive a key event forward to a path of cancer is not yet definable. This is particularly relevant to low doses and low dose-rates of radiation exposure where the biology is interplayed with conflicting concepts of hormesis, hypersensitivity and the linear no threshold theory. Furthermore due to the stochastic nature of the MIE, it remains difficult to identify specific threshold values of DSBs needed to overwhelm the DNA repair machinery to cause “inadequate” DNA repair leading to downstream genetic abnormalities and eventually cancer. With a radiation stressor, a single hit to the DNA molecule could drive a path forward to lung cancer; however this is with low probability. Empirical modeling of cancer probability vs. mean radiation dose and time to lethality, does provide a good visualization of the effective thresholds (Raabe 2011). However, in general there is considerable uncertainty surrounding the connection of biologically contingent observations and stochastic energy deposition.

Raabe OG. Toward improved ionizing radiation safety standards. Health Phys 101: 84–93; 2011.

	Defining Question	***Strong***	***Moderate***	***Weak***
Support for Quantitative Understanding of KERs	*What is the extent to which a change in KE_{down} can be predicted from KE_{up}? What is the precision with which uncertainty in the prediction of KE_{down} can be quantified? What is the extent to which known modulating factors or feedback mechanisms can be accounted for? What is the extent to which the relationships can be reliably generalized across the applicability domain of the KER?*	*Change in KE_{down} can be precisely predicted based on a relevant measure of KE_{up}; Uncertainty in the quantitative prediction can be precisely estimated from the variability in the relevant KE_{up} measure; Known modulating factors and feedback/ feedforward mechanisms are accounted for in the quantitative description; Evidence that the quantitative relationship between the KEs generalizes across the relevant applicability domain of the KER*	*Change in KE_{down} can be precisely predicted based on relevant measure of KE_{up}; Uncertainty in the quantitative prediction is influenced by factors other than the variability in the relevant KE_{up} measure; Quantitative description does not account for all known modulating factors and/or known feedback/ feedforward mechanisms; Quantitative relationship has only been demonstrated for a subset of the overall applicability domain of the KER*	*Only a qualitative or semi-quantitative prediction of the change in KE_{down} can be determined from a measure of KE_{up}; Known modulating factors and feedback/ feedforward mechanisms are not accounted for; Quantitative relationship has only been demonstrated for a narrow subset of the overall applicability domain of the KER*
	Evidence for Quantitative Understanding of KER: Strong The vast majority of studies examining energy deposition and incidence of DSBs suggest a positive, linear			

Deposition of Energy (MIE) --> Double-Strand Breaks, Increase (KE1)	relationship between these two events (Aufderheide et al., 1987; Sidjanin, 1993; Frankenberg et al., 1999; Sutherland et al. 2000; Lara et al. 2001; Baumstark-Khan et al., 2003; Rothkamm and Lo 2003; Kuhne et al. 2005; Rube et al. 2008; Grudzenski et al. 2010; Bannik et al., 2013; Shelke and Das 2015; Antonelli et al. 2015; Dalke, 2018). Predicting the exact number of DSBs from the deposition of energy, however, appears to be highly dependent on the biological model, the type of radiation and the radiation dose range, as evidenced by the differing calculated DSB rates across studies (Charlton et al. 1989; Rogakou et al. 1999; Sutherland et al. 2000; Lara et al. 2001; Rothkamm and Lo 2003; Kuhne et al. 2005; Rube et al. 2008; Grudzenski et al. 2010; Antonelli et al. 2015) .
Deposition of Energy (MIE) --> Mutations, Increase (KE3)	***Evidence for Quantitative Understanding of KER: Strong*** Most studies indicate a positive, linear relationship between the radiation dose and the mutation frequency (Russell et al. 1957; Albertini et al. 1997; Canova et al. 2002; Dubrova et al. 2002; Nagashima et al. 2018). In order to predict the number of mutations induced by a particular dose of radiation, parameters such as the type of radiation, the radiation's LET, and the type of model system being used should be taken into account (Albertini et al. 1997; Dubrova et al. 2002; Matuo et al. 2018; Nagashima et al. 2018). Predicting the mutation frequency at particular time-points, however, would be very difficult owing to our limited time scale knowledge.
Deposition of Energy (MIE) --> Chromosomal Aberrations, Increase (KE4)	***Evidence for Quantitative Understanding of KER: Strong*** Most studies indicate a positive, linear-quadratic relationship between the deposition of energy by ionizing radiation and the frequency of chromosomal aberrations (Schmid et al. 2002; Suto et al. 2015; Abe et al. 2018; Jang et al. 2019). Equations describing this relationship were given in a number of studies (Schmid et al. 2002; George et al. 2009; Suto et al. 2015; Abe et al. 2018; Jang et al. 2019), with validation of the dose-response curve performed in one particular case (Suto et al. 2015). In terms of time scale predictions, this may still be difficult owing to the often-lengthy cell cultures required to assess chromosomal aberrations post-irradiation. For translocations in particular, however, one study defined a linear relationship between time and translocation frequency at lower radiation doses (0 - 0.5 Gy) and a linear quadratic relationship at higher doses (0.5 - 4 Gy) (Tucker et al. 2005b).
Double-Strand Breaks, Increase (KE1) --> Inadequate DNA Repair, Increase (KE2)	***Evidence for Quantitative Understanding of KER:*** **Moderate** According to studies examining DSBs and DNA repair after exposure to a radiation stressor, there was a positive linear relationship between DSBs and radiation dose (Lobrich et al. 2000; Rothkamm and Lo 2003; Kuhne et al. 2005; Asaithamby and Chen 2009), and a linear-quadratic relationship between the number of misrejoined DSBs and radiation dose (Kuhne et al. 2005) which varied according to LET (Rydberg et al. 2005b) and dose-rate (Dikomey and Brammer 2000) of the radiation. Overall, 1 Gy of radiation may induce between 35 and 70 DSBs (Dubrova et al. 2002; Rothkamm and Lo 2003), with 10 - 15% being misrepaired at 10 Gy (Mcmahon et al. 2016) and 50 - 60% being misrepaired at 80 Gy (Lobrich et al. 2000; Mcmahon et al. 2016). Twenty-four hours after radiation exposure the frequency of misrepair appeared to remain relatively constant around 80%, a rate that was maintained for the next ten days of monitoring (Kuhne et al. 2000).
Inadequate DNA Repair, Increase (KE2) --> Mutations, Increase (KE3)	***Evidence for Quantitative Understanding of KER: Moderate*** Positive relationships have been reported between radiation stressor and inadequate DNA repair, radiation stressor and mutation frequency (Mcmahon et al. 2016), and inadequate DNA repair and mutation frequency (Perera et al. 2016). It has been found that 10 - 15% of DSBs are misrepaired at 10 Gy (Mcmahon et al. 2016) and 50 - 60% at 80 Gy (Lobrich et al. 2000; Mcmahon et al. 2016), with mutation rates varying from 0.1 - 0.2 mutation per 10^4 cells at 1 Gy and 0.4 - 1.5 mutation per 10^4 cells at 6 Gy (Mcmahon et al. 2016).
Inadequate DNA Repair, Increase (KE2) --> Chromosomal Aberrations, Increase (KE4)	***Evidence for Quantitative Understanding of KER: Weak*** A direct quantitative understanding of the relationship between inadequate DNA repair and chromosomal aberrations has not been established. However, some data has been generated using studies from radiation stressor studies. 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). It is not known, however, how this rate of misrepair relates to chromosomal aberration frequency. Results from one study using a DNA repair inhibitor suggested that as adequate DNA repair declines, the chromosomal aberration frequency increases (Chernikova et al. 1999).

	The time scale between inadequate repair and chromosomal aberration frequency has also not been well established.
Mutations, Increase (KE3) --> Cell Proliferation, Increase (KE5)	***Evidence for Quantitative Understanding of KER: Weak*** Quantitative understanding of the relationship between these two events has not been well established. There are, however, some studies that have examined how cellular proliferation changes over time in the presence of mutations. In cells harbouring mutations in critical genes, higher proliferation rates were evident by the fourth day in culture (Lang et al. 2004; Li and Xiong 2017) and higher rates of population doublings were evident by passage 7 (Li and Xiong 2017) relative to wild-type cells. DNA synthesis (which could be indicative of cellular proliferation) was higher in p53-/- cells than in wild-type cells for the first 6 days of culture, and increased to drastically higher levels in the knock-out cells until the end of the experiment at day 10 (Lang et al. 2004). *In vivo*, mice injected with oncogenically-transformed cells containing multiple mutations had detectable tumour growth by 10 - 12 days post-inoculation. These volumes continued increasing over the 40-day experiment (Sato et al. 2017).
Chromosomal Aberrations, Increase (KE4) --> Cell Proliferation, Increase (KE5)	***Evidence for Quantitative Understanding of KER: Weak*** Quantitative understanding of the relationship between these two events has not been well established. . Although studies that directly assessed the time scale between chromosomal aberrations and cell proliferation rates were not identified, differences in cellular proliferation rates for cells with different CA-related manipulations or treatments were evident within the first 3 days of culture (Stopper et al. 2003; Li et al. 2007; Soda et al. 2007; Irwin et al. 2013; Guarnerio et al. 2016).
Cell Proliferation, Increase (KE5) --> Lung Cancer, Increase (AO)	***Evidence for Quantitative Understanding of KER: Weak*** Quantitative understanding of the relationship between these two events has not been well established. Human non-carcinogenic cells are thought to undergo 50 – 70 cell divisions before the telomeres can no longer support cell division (Panov 2005); this number would presumably be higher in cancer cells, but quantitative data was not able to be identified. There are some studies available, however, that provide some details regarding the timing between these two events. *In vitro* experiments using lung cancer cell lines demonstrated that expression levels of key proteins involved in the regulation of the cell cycle and/or proliferation were modified by chemical inhibitors within the first 48 hours of treatment (Lv et al. 2012; Wanitchakool et al. 2012; Pal et al. 2013; Sun et al. 2016). *In vivo* studies using xenograft nude mice found that tumours were detected within two weeks of NSCLC-cell inoculation, and continued to grow over the experimental period (Pal et al. 2013; Warin et al. 2014; Sun et al. 2016; Tu et al. 2018). Differences in tumour growth rates between mice treated with an anti-cancer drug and those left untreated were also evident within 13 - 27 days (Pal et al. 2013; Sun et al. 2016; Tu et al. 2018), with significant differences in cell proliferation markers and tumour numbers or sizes at time of harvest (22 days - 27 weeks) (Kassie et al. 2008; Pal et al. 2013; Warin et al. 2014; Sun et al. 2016; Tu et al. 2018).
Double-Strand Breaks, Increase (KE1) --> Mutations, Increase (KE3)	***Evidence for Quantitative Understanding of KER: Weak*** There is overall limited quantitiative understanding of the relationship between DSBs and increased mutation rates. McMahon et al., 2016 compiled data from multiple studies spanning different human and mouse cell lines to model the IR dose-dependent increase in mutation rate. However, further quantitiative studies into this relationship are required to provide a better quantitiative understanding.
Double-Strand Breaks, Increase (KE1) --> Chromosomal Aberrations, Increase (KE4)	***Evidence for Quantitative Understanding of KER: Weak*** Similarly to the non-adjacent relationship above (KE1 -> KE4), there is overall limited quantitiative understanding of the relationship between DSBs and increased rates of chromosomal aberrations. McMahon et al., 2016 compiled data from multiple studies spanning different human and mouse cell lines to model the IR dose-dependent increase in the rate of chromosomal aberrations. However, further quantitiative studies into this relationship are required to provide a better quantitiative understanding.

Mutations, Increase (KE3) --> Lung Cancer, Increase (AO)	***Evidence for Quantitative Understanding of KER: Weak*** Finding studies addressing the quantitative relationship between mutations and cancer directly was particularly challenging. However, many studies indicated that there was a positive, dose-dependent increase in mutations with increasing radiation dose (Suzuki and Hei 1996; Canova et al. 2002). A similar positive, dose-dependent relationship was found for the oncogenic transformations in cell and the radiation dose (Miller et al. 1995), and the incidence of lung cancer in rats and their cumulative radon exposure (Monchaux et al. 1994). Epidemiological studies examining lung cancer in radon-exposed uranium miners found a positive, linear relationship between lung cancer and cumulative radon exposure (Lubin et al. 1995; Ramkissoon et al. 2018). In terms of time-scale, mutations were evident in 2 weeks following irradiation (Hei et al. 1997), whereas oncogenic transformations took 7 weeks to develop following radiation exposure (Miller et al. 1999). *In vivo* models with injected tumour cells, inherent mutations, exposure to carcinogens, or Cre-induced mutations showed tumour growth months after exposure to the tumour-inducing insult (Hei et al. 1994; Fisher et al. 2001; Kasinski and Slack 2012; Fujimoto et al. 2017).
Chromosomal Aberrations, Increase (KE4) --> Lung Cancer, Increase (AO)	***Evidence for Quantitative Understanding of KER: Moderate*** There is evidence of a positive, linear relationship between radiation dose and CAs (Nagasawa et al. 1990; A.L. Brooks et al. 1995; Khan et al. 1995; Yamada et al. 2002; Stevens et al. 2014), radiation dose and oncogenic transformations (Miller et al. 1996), as well as radon exposure and the risk of lung cancer mortality (Tirmarchel et al. 1993; Walsh et al. 2010). The latter relationship was found to be exponentially modified, however, by factors such as the age at median exposure, the time since median exposure, and the radon exposure rate (Walsh et al. 2010). Equations defining these relationships were derived in a number of different studies (Tirmarchel et al. 1993; A.L. Brooks et al. 1995; Khan et al. 1995; Miller et al. 1996; Girard et al. 2000; Yamada et al. 2002; Walsh et al. 2010; Stevens et al. 2014). In terms of time scale, micronuclei were documented in cells of the rodent lung as early as 0.2 days (Khan et al. 1995), and were found to persist for days to weeks (Khan et al. 1995; Deshpande et al. 1996; Werner et al. 2017). Oncogenic transformations, on the other hand, took weeks to develop (Robertson et al. 1983; Miller et al. 1996), while lung tumours took months or years to develop following radiation exposure (Tirmarchel et al. 1993; Yamada et al. 2017). Delivery of an agent carrying a cancer-related CA resulted in tumour growth within 21 - 31 days of its injection into mice (Pear et al. 1998; Kuramochi et al. 2001).
Deposition of Energy (MIE) --> Lung Cancer, Increase (AO)	***Evidence for Quantitative Understanding of KER: Moderate*** Quantitative understanding has been well-established for this KER. According to current Canadian guidelines developed by Health Canada, annual residential radon levels should not exceed 200 Bq/m^3. Similarly, the WHO recommends that the national annual residential radon levels not exceed 100 Bq/m^3 where possible; if there are geographic or national constraints that make this target unachievable, the national standard should not be higher than 300 Bq/m^3 (World Health Organization - Radon Guide 2009). Positive relationships between radon exposure and lung cancer have been established using *in vitro* models (Miller 1995), *in vivo* models(Monchaux et al. 1994) and results from human epidemiological studies (Lubin et al. 1995; Hazelton et al. 2001; Darby et al. 2005; Krewski et al. 2005; Krewski et al. 2006; Rodríguez-Martínez et al. 2018; Ramkissoon et al. 2018). Unsurprisingly, oncogenic transformation in cells were found weeks after radiation exposure (Miller et al. 1995), sizable tumours developed months after irradiation in mice (Hei et al. 1994) and lung cancer was found years after exposure in humans (Lubin et al. 1995; Darby et al. 2005; Torres-Durán et al. 2014; Rodríguez-Martínez et al. 2018; Ramkissoon et al. 2018).

Quantification of AOP KERs

The development of quantitative AOPs (qAOPs) has been demonstrated in other fields such as chemical toxicology (Zgheib et al., 2019) and similar objectives are warranted for AOPs with ionizing radiation stressors. The quantification of an AOP can help expedite the development of an AOP by reducing the original long-form and qualitative nature of an AOP to tables and graphs that summarize particular features e.g. dose ranges considered, radiation types included etc. Quantification is achieved by extracting numerical information from the underlying supporting evidence of KERs. The quantification of four key event relationships (KERs) from this AOP has been completed. The KERs which have been quantified are as follows:

1. Energy deposition leads to Increase, DNA strand breaks (Ad-KER1)
2. Energy deposition leads to Increase, mutations (NAd-KER1)
3. Energy deposition leads to Increase, Chromosomal aberrations (NAd-KER2)
4. Energy deposition leads to Increase, lung cancer (NAd-KER7)

For each of the KERs listed above, all relevant publications from those used to support the AOP were considered for quantification. In some cases, the measure of dose-response featured in one publication could not be reconciled with the measure adopted by another. For example, in the study of energy deposition leading to an increase in DNA strand breaks, Sudprasert et al. (2006) use a measure of *olive moment* from the Comet assay technique, whereas Sutherland et al. (2000) measure the relative site frequency compared to a benchmark instance of DNA damage. Due to variations such as these, not all studies that contribute qualitatively to supporting the weight of

evidence of a given KER is eligible for quantification. In the case of the four KERs considered above, the most common measure of response across studies was adopted ensure the largest data sample possible. These response measured were as follows (in same order for each KER listed above):

1. Ad-KER1 - DNA DSBs / cell
2. NAd-KER1 - Mutations / 10^6 cells
3. NAd-KER2 - Chromosomal aberrations / 100 cells
4. NAd-KER7 - Relative risk (RR) of lung cancer

The quantification of these four key event relationships (KERs) from this AOP has been completed as detailed in Stainforth et al., 2021. The process of quantification first involves digitizing data from publications. Results provided from tables were used directly. For figures (e.g. scatter or bar-charts) information was obtained by using the WebPlotDigitizer-4.2 authored by Rohatgi (2019). Full information of all quantified studies and respective references can be found in Tables 1-7, here.

The two dominant radiation types featured in the AOP are from photon and alpha-particle sources, see Table 1 below. Upstream KERs describing Ad-KER1, NAd-KER1 and NAd-KER2 are respectively composed of datasets with 298, 176 and 629 data points with 59%, 39% and 57% from photon sources and 35%, 52% and 42% from alpha-particle sources. The AO (NAd-KER7) is 100% characterized by radon (alpha-particle emitter) with a total of 33 data points.

A graphical representation of the four quantified KERs is shown in Figure 1. This AOP is best documented for alpha-particles but could potentially support further data relevant to lung cancer incidence from photon radiation sources. The scope of the AOP could be extended with additional data from proton and heavy ion sources. This would encapsulate research areas such as space-travel where galactic radiation is predominantly composed of protons, and to a lesser extent, heavy ions (Chancellor et al., 2014). Overall, Figure 1 and Table 1 demonstrate how reviewing supporting empirical evidence through a quantitative *lens* reduces the description of an AOP to tables and graphs that can be used to identify inconsistencies and potential missing information across KERs and radiation types.

	Radiation quality			
	Photons	**Protons**	**Alpha-particles**	**Heavy ions**
KER	**Values of dose, response, time and dose rate quoted as [minimum, maximum, average]**			
	Dose [Gy]			
Ad-KER1	[1.2×10^{3}, 80, 7.9]	[0.5, 0.5, 0.5]	[0.1, 713, 203]	[0.5, -, -]
NAd-KER1	[1.7×10^{-5}, 14, 2.4]	[1.24, 3.74, 2.5]	[3.4×10^{-5}, 2.4, 0.6]	[10, 20, 11.8]
NAd-KER2	[6.3×10^{-4}, 10, 1.8]	N/A	[4.3×10^{-4}, 6.9, 0.7]	[0.15, 1.5, 0.7]
NAd-KER7	[4.8×10^{-2}, 2.63, 0.9]	N/A	[7.89×10^{-3}, 10.1, 0.63]	N/A
	Response measures [DNA DSBs / cell (Ad-KER1), Mutant frequency / 10^6 cells (NAd-KER1), CAs / 100 cells (NAd-KER2), Increase in lung cancer RR [%] (NAd-KER7)]			
Ad-KER1	[5×10^{-3}, 2.8×10^{3}, 244]	[0.34, 10.1, 5.3]	[1.3, 3×10^{4}, 9.31×10^{3}]	[0.4, 8.8, 4.3]
NAd-KER1	[0.3, 1.9×10^{3}, 148]	N/A	[1.7, 3.8×10^{3}, 279]	[0.4, 19.4, 4]
NAd-KER2	[0.01, 584, 44.8]	N/A	[0.08, 314, 34.9]	[13.2, 138, 5.7]
NAd-KER7	[2.7, 166, 64.4]	N/A	[-17.9, 942, 84.4]	N/A
	Time [hours (Ad-KER1), days (Ad-KER1, NAd-KER2), years (NAd-KER7)]			
Ad-KER1	[0.02, 72, 10.6]	[0.03, 24, 6.5]	[0.02, 24, 0.5]	[0.25, 24, 6.5]
NAd-KER1	[6.9×10^{-4}, 67, 5.3]	[6.9×10^{-4}, -, -]	[6.94×10^{-4}, 6, 1.4]	[6.94×10^{-4}, 2, 0.1]

NAd-KER2	[$6.9x10^{-4}$, 56, 1.2]	N/A	[$6.94x10^{-4}$, 362, 23.6]	[$6.94x10^{-4}$, -, -]
NAd-KER7	[40, -, -]	N/A	[5.7, 39.0, 18.5]	N/A
	Dose rate [Gy/min]			
Ad-KER1	[0.03, 2, 0.9]	N/A	[0.08, 100, 51.5]	N/A
NAd-KER1	[$1.1x10^{-6}$, 1.2, 0.5]	N/A	[$2x10^{-3}$, 3.6, 1.3]	[1, 5, 4.8]
NAd-KER2	[$1.7x10^{-3}$, 5.9, 0.9]	N/A	[$5.3x10^{-6}$, 2.3, 0.4]	[0.5, -, -]
NAd-KER7	[$2.27x10^{-9}$, $1.25x10^{-7}$, $4.15x10^{-8}$]	N/A	[$7.7x10^{-10}$, $3.4x10^{-6}$, $1.8x10^{-7}$]	N/A
	% data points for KER dataset with valid dose and response values (number of data points)			
Ad-KER1	59 (177)	3 (8)	35 (105)	3 (8)
NAd-KER1	40 (75)	3 (6)	48 (91)	9 (17)
NAd-KER2	56 (344)	0 (0)	43 (262)	1 (10)
NAd-KER7	12 (6)	0 (0)	88 (44)	0 (0)

Table 1: Summary of the quantified datasets from four KERs of the AOP. Data is categorized by both KER and radiation type. Values of dose, response measure, time since irradiation and dose rate are quoted in terms of '[minimum, maximum, average]' values. 'N/A' denotes fields where there was no data. The final set of rows denote the percentages of dose-response data of a given KER associated with a given radiation type.

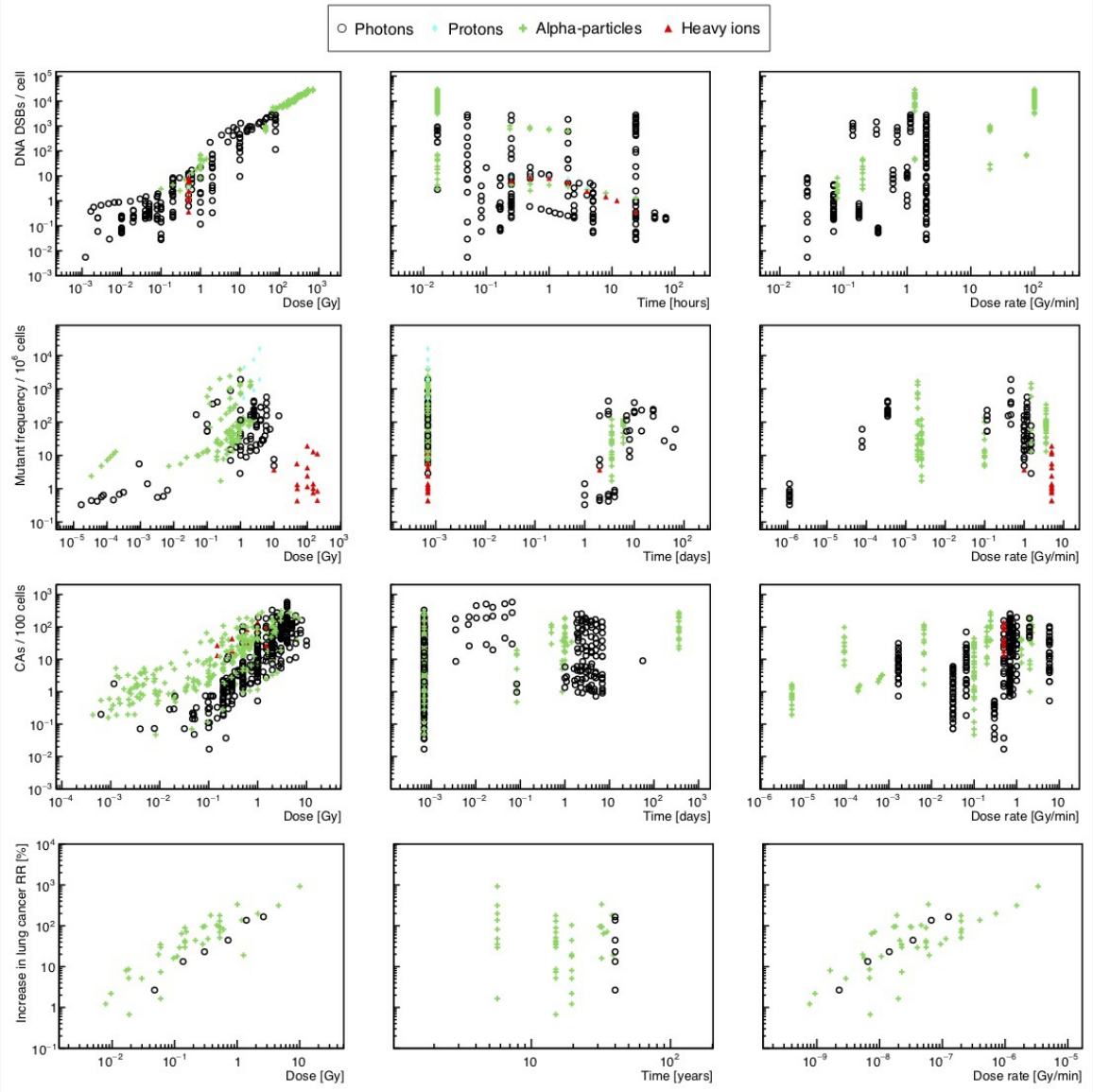

Figure 1: Quantified datasets of the four KERs in graphical form. Each row of plots represents a KER in the following order from top to bottom: Ad-KER1, NAd-KER1, NAd-KER2 and NAd-KER7. The response measure for each KER is shown along the y-axis of each plot, and from left to right the dose, time and dose rate along the x-axes respectively.

Shown in Figure 2 below is a comparison between the two dominat radiation sources; alpha-particles (green) and photon radiation (back). For each of the response measures shown in Figure 2, different symbols denote different end-points or variants of the response as measured for each KER. In the case of chromsomal aberrations (bottom-left) there is a distinct difference in the response of different chromsomal aberration types among a given radiation type e.g. for alpha-particles PCC rings (solid stars) can be 10-100 times less abundant than dicentric chromsomal types (solid circles).

While these differences and variations are embraced by the standard AOP construction, it should be questioned if the quantitative form of these variations is of use for constructing predictive models, and whether such an application is limited only to those of direct response-response relationships where the level of variation may be reduced. Even then, such response-response relationships would need to account for radiation type effects between each KE e.g. differing cell survival rates and the fraction of total DNA damage attributable to single strand breaks (SSBs), DSBs and complex/clustered damage. These are both very different between photon and alpha-particle sources (Franken et al., 2012; Nikjoo et al., 2001). This ultimately constrains any quantitative formalism of an AOP to be radiation type specific.

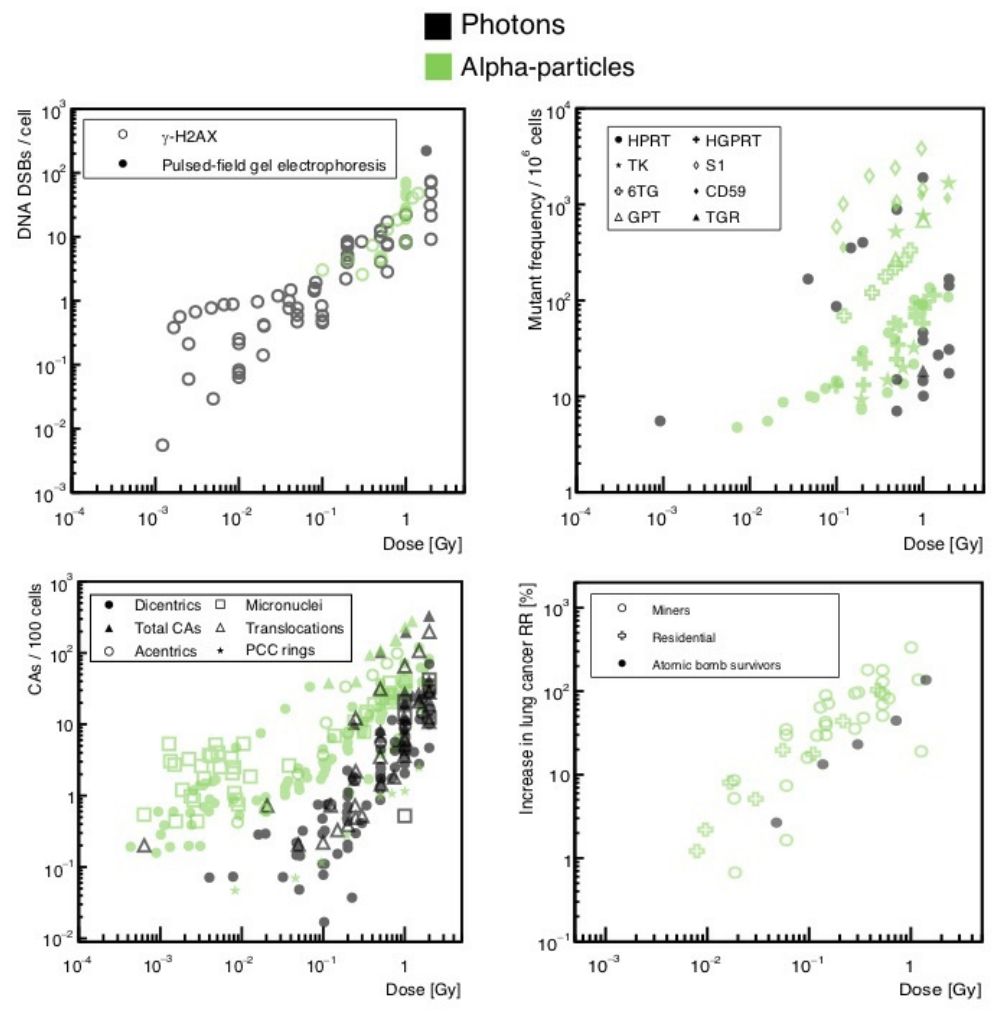

Figure 2: Quantified dose-response of the four KERs in graphical form. Data is focussed on the comparison between photon and alpha-particle radiation types, in addition to the response variants for each type of response. Data is evaluated for the low-dose range of 0-2 Gy for time periods following exposure < 60 minutes for Ad-KER1 (top-left), NAd-KER1 (top-right), and NAd-KER2 (bottom-left). No restriction on the time value for data points plotted for NAd-KER7 (bottom-right) has been made.

Stainforth, R. et al., (2021), Challenges in the quantificaton approach to a radiation relevant adverse outcome patway for lung cancer. Int J Radiat Biol. 97(1):85-101.

Considerations for Potential Applications of the AOP (optional)

At present the AOP framework is not readily used to support regulatory decision-making in radiation protection practices. The goal of developing this AOP is to bring attention to the framework to the radiation community as an effective means to organize knowledge, identify gaps and co-ordinate research. We have used lung cancer as the case example due to its relevance to radon risk assessment and broadly because it can be represented as a simplified targeted path with a molecular initiating event that is specific to a radiation insult. From this AOP, more complex networks can form which are relevant to both radiation and chemical exposure scenarios. Furthermore, as mechanistic knowledge surrounding low dose radiation exposures becomes clear, this information can be incorporated into the AOP. By developing this AOP, we have supported the necessary efforts highlighted by the international and national radiation protection agencies such as, the United Nations Scientific Committee on the Effects of Atomic Radiation, International Commission of Radiological Protection, International Dose Effect Alliance and the Electric Power Research Institute Radiation Program to consolidate and enhance the knowledge in understanding of low dose radiation exposures from the cellular to organelle levels within the biological system.

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Appendix 1

List of MIEs in this AOP

Event: 1686: Deposition of Energy

Short Name: Energy Deposition

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:272 - Deposition of energy leading to lung cancer	MolecularInitiatingEvent
Aop:432 - Deposition of Energy by Ionizing Radiation leading to Acute Myeloid Leukemia	MolecularInitiatingEvent
Aop:386 - Deposition of ionizing energy leads to leading to population decline via inhibition of photosynthesis	MolecularInitiatingEvent
Aop:387 - Deposition of ionising energy leading to population decline via mitochondrial dysfunction	MolecularInitiatingEvent
Aop:388 - Deposition of ionising energy leading to population decline via programmed cell death	MolecularInitiatingEvent
Aop:435 - Deposition of ionising energy leads to population decline via pollen abnormal	MolecularInitiatingEvent

AOP ID and Name	Event Type
Aop:216 - Deposition of energy leading to population decline via DNA strand breaks and follicular atresia	MolecularInitiatingEvent
Aop:238 - Deposition of energy leading to population decline via DNA strand breaks and oocyte apoptosis	MolecularInitiatingEvent
Aop:311 - Deposition of energy leading to population decline via DNA oxidation and oocyte apoptosis	MolecularInitiatingEvent
Aop:299 - Deposition of energy leading to population decline via DNA oxidation and follicular atresia	MolecularInitiatingEvent
Aop:441 - Ionizing radiation-induced DNA damage leads to microcephaly via apoptosis and premature cell differentiation	MolecularInitiatingEvent
Aop:444 - Ionizing radiation leads to reduced reproduction in Eisenia fetida via reduced spermatogenesis and cocoon hatchability	MolecularInitiatingEvent
Aop:470 - Deposition of energy leads to vascular remodeling	MolecularInitiatingEvent
Aop:473 - Energy deposition from internalized Ra-226 decay lower oxygen binding capacity of hemocyanin	MolecularInitiatingEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	MolecularInitiatingEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

It is well documented that ionizing radiation((eg. X-rays, gamma, photons, alpha, beta, neutrons, heavy ions) leads to energy deposition on the atoms and molecules of the substrate. Many studies, have demonstrated that the type of radiation and distance from source has an impact on the pattern of energy deposition (Alloni, et al. 2014). High linear energy transfer (LET) radiation has been associated with higher-energy deposits (Liamsuwan et al., 2014) that are more densely-packed and cause more complex effects within the particle track (Hada and Georgakilas, 2008; Okayasu, 2012ab; Lorat et al., 2015; Nikitaki et al., 2016) in comparison to low LET radiation. Parameters such as mean lineal energy, dose mean lineal energy, frequency mean specific energy and dose mean specific energy can impact track structure of the traversed energy into a medium (Friedland et al., 2017). The detection of energy deposition by ionizing radiation can be demonstrated with the use of fluorescent nuclear track detectors (FNTDs). FNTDs used in conjunction with fluorescent microscopy, are able to visualize radiation tracks produced by ionizing radiation (Niklas et al., 2013; Kodaira et al., 2015; Sawakuchi and Akselrod, 2016). In addition, these FNTD chips can quantify the LET of primary and secondary radiation tracks up to 0.47 keV/um (Sawakuchi and Akselrod, 2016). This co-visualization of the radiation tracks and the cell markers enable the mapping of the radiation trajectory to specific cellular compartments, and the identification of accrued damage (Niklas et al., 2013; Kodaira et al., 2015). There are no known chemical initiators or prototypes that can mimic the MIE.

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	Low

Energy can be deposited into any substrate, both living and non-living; it is independent of age, taxa, sex, or life-stage.

Taxonomic applicability: This MIE is not taxonomically specific.

Life stage applicability: This MIE is not life stage specific.

Sex applicability: This MIE is not sex specific.

Key Event Description

Deposition of energy refers to events where subatomic particles or electromagnetic waves of sufficient energy cause ionization in the media through which they transverse (Beir, et al. 1999). The resulting energy can cause the ejection of electrons from atoms and molecules, thereby resulting in their ionization and the breakage of chemical bonds and ionizing atoms and molecules. The energy of these subatomic particles or electromagnetic waves ranges from 124 KeV to 5.4 MeV, and is dependent on the source and type of radiation (Zyla et al., 2020). Not all electromagnetic radiation is ionizing; as the incident radiation must have sufficient energy to free electrons from the atom or molecule's electron orbitals. The energy can induce direct and indirect ionization events and can be via internal (injections, inhalation, injection) or external exposure. Direct ionization is the principal path where charged particles interact with DNA to cause a biological damage. Photons, which are electromagnetic waves can also cause direct ionization. Indirect ionization produces free radicals of other molecules, specifically water, which can transform to damage critical targets such as DNA (Beir, et al. 1999; Balagamwala et al., 2013). There are no chemical mimetics or prototypes of energy deposition. Given the fundamental nature of energy deposition by nuclei, nucleons or elementary particles in material, this process is universal to all biological contexts. It is a phenomenon dictated by radioactive decay laws. As such chemical initiators are also not applicable to this MIE.

Energy deposition is influenced by the linear energy transfer (LET) (Hall and Giaccia, 2018 UNSCEAR, 2020). High LET refers to energy above 10 keV µm-1 which produces more complex, dense structural damage than low LET radiation (below 10 keV µm-1). Low-LET particles produce sparse ionization events such as photons (X- and gamma rays), as well as high-energy protons. Low LET radiation travels farther into tissue but deposits smaller amounts of energy, whereas high LET radiation, which includes heavy ions, alpha particles and high-energy neutrons, does not travel as far but deposits larger amounts of energy into tissue at the same absorbed dose. The biological effect of the deposition of energy can be modulated by varying dose and dose rate of exposure, such as acute, chronic, or fractionated exposures (Hall and Giaccia, 2018).

How it is Measured or Detected

Assay Name	References	Description	OECD Approved Assay
Monte Carlo Simulations (Geant4)	Douglass et al., 2013; Douglass et al. 2012	Monte Carlo simulations are based on a computational algorithm that mathematically models the deposition of energy into materials.	N/A
Fluorescent Nuclear Track Detector (FNTD)	Sawakuchi, 2016; Niklas, 2013; Koaira et al., 2015	FNTDs are biocompatible chips with crystals of aluminium oxide doped with carbon and magnesium; used in conjuction with fluorescent microscopy, these FNTDs allow for the visualization and the linear energy transfer (LET) quantification of tracks produced by the deposition of energy into a material.	N/A
Tissue equivalent proportional counter (TEPC)	Straume et al, 2015	Measure the LET spectrum and calculate the dose equivalent.	N/A

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List of Key Events in the AOP

Event: 1635: Increase, DNA strand breaks

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	KeyEvent
Aop:272 - Deposition of energy leading to lung cancer	KeyEvent
Aop:322 - Alkylation of DNA leading to reduced sperm count	KeyEvent
Aop:216 - Deposition of energy leading to population decline via DNA strand breaks and follicular atresia	KeyEvent
Aop:238 - Deposition of energy leading to population decline via DNA strand breaks and oocyte apoptosis	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

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

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

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

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

Key Event Description

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

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

How it is Measured or Detected

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

Assay Name	References	Description	OECD Approved Assay
Comet Assay (Single Cell Gel Eletrophoresis - Alkaline)	Collins, 2004; Olive and Banath, 2006; Platel et al., 2011; Nikolova et al., 2017	To detect SSBs or DSBs, single cells are encapsulated in agarose on a slide, lysed, and subjected to gel electrophoresis at an alkaline pH (pH >13); DNA fragments are forced to move, forming a "comet"-like appearance	Yes (No. 489)
Comet Assay (Single Cell Gel Eltrophoresis - Neutral)	Collins, 2014; Olive and Banath, 2006; Anderson and Laubenthal, 2013; Nikolova et al., 2017	To detect DSBs, single cells are encapsulated in agarose on a slide, lysed, and subjected to gel electrophoresis at a neutral pH; DNA fragments, which are not denatured at the neutral pH, are 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	Redon et al.,	Quantification of γ-H2AX immunostaining by	N/A

Quantification - Microscopy	2010; Mah et al., 2010; Garcia-Canton et al., 2013	counting γ- H2AX foci visualized with a microscope	
γ-H2AX Foci Detection - ELISA and flow cytometry	Ji et al., 2017; Bryce et al., 2016	Detection of γ-H2AX in cells by ELISA, normalized to total levels of H2AX; γH2AX foci detection can be high-throughput and automated using flow cytometry-based immunodetection.	N/A
Pulsed Field Gel Electrophoresis (PFGE)	Ager et al., 1990; Gardiner et al., 1985; Herschleb et al., 2007; Kawashima et al., 2017	To detect DSBs, cells are embedded and lysed in agarose, and the released DNA undergoes gel electrophoresis in which the direction of the voltage is periodically alternated; Large DNA fragments are thus able to be separated by size	N/A
The TUNEL (Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling) Assay	Loo, 2011	To detect strand breaks, dUTPs added to the 3'OH end of a strand break by the DNA polymerase terminal deoxynucleotidyl transferase (TdT) are tagged with a fluorescent dye or a reporter enzyme to allow visualization (We note that this method is typically used to measure apoptosis)	N/A
In Vitro DNA Cleavage Assays using Topoisomerase	Nitiss, 2012	Cleavage of DNA can be achieved using purified topoisomerase; DNA strand breaks can then be separated and quantified using gel electrophoresis	N/A
PCR assay	Figueroa-González & Pérez-Plasencia, 2017	Assay of strand breaks through the observation of DNA amplification prevention. Breaks block Taq polymerase, reducing the number of DNA templates, preventing amplification	N/A
Sucrose density gradient centrifuge	Raschke et al. 2009	Division of DNA pieces by density, increased fractionation leads to lower density pieces, with the use of a sucrose cushion	N/A
Alkaline Elution Assay	Kohn, 1991	Cells lysed with detergent-solution, filtered through membrane to remove all but intact DNA	N/A
Unwinding Assay	Nacci et al. 1992	DNA is stored in alkaline solutions with DNA-specific dye and allowed to unwind following removal from tissue, increased strand damage associated with increased unwinding	N/A

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Event: 155: Inadequate DNA repair

Short Name: Inadequate DNA repair

Key Event Component

Process	Object	Action
DNA repair	deoxyribonucleic acid	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:15 - Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations	KeyEvent
Aop:141 - Alkylation of DNA leading to cancer 2	KeyEvent
Aop:139 - Alkylation of DNA leading to cancer 1	KeyEvent
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations	KeyEvent
Aop:272 - Deposition of energy leading to lung cancer	KeyEvent
Aop:322 - Alkylation of DNA leading to reduced sperm count	KeyEvent
Aop:397 - Bulky DNA adducts leading to mutations	KeyEvent
Aop:432 - Deposition of Energy by Ionizing Radiation leading to Acute Myeloid Leukemia	KeyEvent
Aop:443 - Alcohol Induced DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	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

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Moderate	NCBI
Syrian golden hamster	Mesocricetus auratus	Moderate	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

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).

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

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

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

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

Key Event Description

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

1. **Damage reversal** acts to reverse the damage without breaking any bonds within the sugar phosphate backbone of the DNA. The most prominent enzymes associated with damage reversal are photolyases (Sancar, 2003) that can repair UV dimers in some organisms, and O6-alkylguanine-DNA alkyltransferase (AGT) (Pegg 2011) and oxidative demethylases (Sundheim et al., 2008), which can repair some types of alkylated bases.
2. **Excision repair** involves the removal of a damaged nucleotide(s) through cleavage of the sugar phosphate backbone followed by re-synthesis of DNA within the resultant gap. Excision repair of DNA lesions can be mechanistically divided into:

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

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

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

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

3. **Single strand break repair (SSBR)** involves different proteins and enzymes depending on the origin of the SSB (e.g.,

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

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

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

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

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

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

Fidelity of DNA Repair

Most DNA repair pathways are extremely efficient. However, in principal, all DNA repair pathways can be overwhelmed when the DNA lesion burden exceeds the capacity of a given DNA repair pathway to recognize and remove the lesion. Exceeded repair capacity may lead to toxicity or mutagenesis following DNA damage. Apart from extremely high DNA lesion burden, inadequate repair may arise through several different specific mechanisms. For example, during repair of DNA containing O6-alkylguanine adducts, AGT irreversibly binds a single O6-alkylguanine lesion and as a result is inactivated (this is termed suicide inactivation, as its own action causes it to become inactivated). Thus, the capacity of AGT to carry out alkylation repair can become rapidly saturated when the DNA repair rate exceeds the de novo synthesis of AGT (Pegg, 2011).

A second mechanism relates to cell specific differences in the cellular levels or activity of some DNA repair proteins. For example, 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).

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).

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

How it is Measured or Detected

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

Indirect Measurement

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

Some EXAMPLES are given below for alkylated DNA.

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

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

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

Direct Measurement

Nagel et al. (2014) 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 (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	Nagel et al., 2014	Measures the ability of human cells to repair plasma reporters, which contain different types and amounts of DNA damage; Used to measure repair processes including HR, NHEJ, BER, NER, MMR, and MGMT	HR, NHEJ, BER, NER, MMR, or MGMT	N/A

(FM-HCR)				
Alkaline Unwinding Assay with Time Course	Nacci et al. 1991	DNA is stored in alkaline solutions with DNA-specific dye and allowed to unwind following removal from tissue, increased strand damage associated with increased unwinding. Samples analyzed at different time points to compare remaining damage following repair opportunities	DSBs	Yes (For DSBs)
Sucrose Density Gradient Centrifugation with Time Course	Larsen et al. 1982	Strand breaks alter the molecular weight of the DNA piece. DNA in alkaline solution centrifuged into sugar density gradient, repeated set time apart. The less DNA breaks identified in the assay repeats, the more repair occurred	SSBs	N/A
y-H2AX Foci Staining with Time Course	Mariotti et al. 2013 Penninckx et al. 2021	Histone H2AX is phosphorylated in the presence of DNA strand breaks, the rate of its disappearance over time is used as a measure of DNA repair	DSBs	N/A
Alkaline Elution Assay with Time Course	Larsen et al. 1982	DNA with strand breaks elute faster than DNA without, plotted against time intervals to determine the rate at which strand breaks repair	SSBs	N/A
53BP1 foci Detection with Time Course	Penninckx et al. 2021	53BP1 is recruited to the site of DNA damage, the rate at which its level decreases over time is used to measure DNA repair	DSBs	N/A

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Event: 185: Increase, Mutations

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	KeyEvent
Aop:141 - Alkylation of DNA leading to cancer 2	KeyEvent
Aop:139 - Alkylation of DNA leading to cancer 1	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer	AdverseOutcome
Aop:293 - Increased DNA damage leading to increased risk of breast cancer	AdverseOutcome
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations	AdverseOutcome
Aop:272 - Deposition of energy leading to lung cancer	KeyEvent
Aop:397 - Bulky DNA adducts leading to mutations	AdverseOutcome
Aop:443 - Alcohol Induced DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	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
medaka	Oryzias latipes	Moderate	NCBI
rat	Rattus norvegicus	High	NCBI
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

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

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

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

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

Key Event Description

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

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

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

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

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

How it is Measured or Detected

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

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

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

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

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

The transgenic rodent mutation assay (OECD TG 488, 2020) is the only assay capable of measuring gene mutation in virtually all tissues in vivo. Specific details on the rodent transgenic mutation reporter assays are reviewed in Lambert et al. (2005, 2009). The transgenic reporter genes are used for detection of gene mutations and/or chromosomal deletions and rearrangements resulting in DNA size changes (the latter specifically in the lacZ plasmid and Spi- test models) induced in vivo by test substances (OECD, 2009, OECD, 2011; Lambert et al., 2005). Briefly, transgenic rodents (mouse or rat) are exposed to the chemical agent sub-chronically. Following a manifestation period, genomic DNA is extracted from tissues, transgenes are rescued from genomic DNA, and transfected into bacteria where the mutant frequency is measured using specific selection systems.

The Pig-a (phosphatidylinositol glycan, Class A) gene on the X chromosome codes for a catalytic subunit of the N-acetylglucosamine transferase complex that is involved in glycosylphosphatidyl inositol (GPI) cell surface anchor synthesis. Cells lacking GPI anchors, or GPI-anchored cell surface proteins are predominantly due to mutations in the Pig-a gene. Thus, flow cytometry of red blood cells expressing or not expressing the Pig-a gene has been developed for mutation analysis in blood cells from humans, rats, mice, and monkeys. The assay is described in detail in Dobrovolsky et al. (2010). 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, 2020). A description of the approach is found within this published TG. Further modifications to this protocol have been made as of 2022 for the analysis of germ cells. Detailed methodology for detecting mutant frequency arising in spermatogonia is described in Douglas et al. (1995), O'Brien et al. (2013); and O'Brien et al. (2014). Briefly, male mice are exposed to the mutagen and killed at varying times post-exposure to evaluate effects on different phases of spermatogenesis. Sperm are collected from the vas deferens or caudal epididymis (the latter preferred). Modified protocols have been developed for extraction of DNA from sperm.

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

Please note, gene mutations that occur in somatic cells in vivo (OECD Test. No. 488, 2020) or in vitro (OECD Test No. 476: In vitro Mammalian Cell Gene Mutation Test, 2016), or in bacterial cells (i.e., OECD Test No. 471, 2020) can be used as an indicator that mutations in male pre-meiotic germ cells may occur for a particular agent (sensitivity and specificity of other assays for male germ cell effects is given in Waters et al., 1994). However, given the very unique biological features of spermatogenesis relative to other cell types, known exceptions to this rule, and the small database on which this is based, inferring results from somatic cell or bacterial tests to male pre-meiotic germ cells must be done with caution. That mutational assays in somatic cells may predict mutations in germ cells has not been rigorously tested empirically (Singer and Yauk, 2010). The IWGT working group on germ cells specifically addressed this gap in knowledge in their report (Yauk et al., 2015) and recommended that additional research 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
		After exposure to a chemical/mutagen, mutations	

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

	2017		
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

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	AdverseOutcome
Aop:272 - Deposition of energy leading to lung cancer	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	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
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

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

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

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

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

Key Event Description

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

Chromosomal aberrations (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

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

Assay	References	Description	OECD-approved assay
Premature Chromosome Condensation (PCC)	Prasanna et al., 2000; Okayasu et al., 2019	Cells are exposed to mitosis-promoting factors (MPF) following cell fusion, causing the chromosomes to condense prematurely. In another approach, cells are exposed to protein phosphatase inhibitors, such as type 1 and 2A protein phosphatases, also causing premature chromosome condensation.	N/A
Chromosomal G-banding	Schwatz, 1990	Use of Giesma dye to stain chromosomal bands, abnormalities determined by the presence of altered morphology	N/A
Fluorescent In Situ Hybridization (FISH)	Beaton et al., 2013; Pathak et al., 2017	Fluorescent assay of metaphase chromosomes that can detect CAs through chromosome painting and microscopic analysis	N/A
Micronuclei (MN) Assay via Microscopy *in vitro*	OECD, 2016a	Micronuclei are scored in vitro using microscopy	Yes (No. 487)
Cytokinesis Block Micronucleus (CBMN) Assay with Microscopy in vitro	Fenech, 2000; OECD, 2016a	Cells are cultured with cytokinesis blocking agent, fixed to slides, and undergo MN quantification using microscopy.	Yes (No.487)
Micronucleus (MN) Assay by Microscopy in vivo	OECD, 2016b	Cells are fixed on slides and MN are scored using microscopy. Red blood cells can also be scored for MN using flow cytometry (see below)	Yes (No. 474)
CBMN with Imaging Flow Cytometry	Rodrigues et al., 2015	Cells are cultured with cytokinesis blocking agent, fixed in solution, and imaged with flow cytometry to quantify MN	N/A
Flow cytometry detection of MN	Dertinger et al., 2004; Bryce et al., 2007; OECD 2016a, 2016b	In vivo and in vitro flow cytometry-based, automated micronuclei measurements are also done without cytokinesis block. MN analysis in vivo is performed in peripheral blood cells to detect MN in erythrocytes and reticulocytes.	Yes (No.487; No. 474)
		Multiplexed biomarkers can be measured by flow cytometry are used to discern	

High-throughput biomarker assays (indirect measures to confirm clastogenicity)	Bryce et al. 2014, 2016, 2018 Khoury et al., 2013, Khoury et al., 2016) Hendriks et al., 2012, 2016; Wink et al., 2014	clastogenic and aneugenic mechanisms for MN induction. Flow cytometry-based quantification of γH2AX foci and p53 protein expression (Bryce et al., 2016). Prediscreen Assay– In-Cell Western - based quantification of γH2AX Green fluorescent protein reporter assay to detect the activation of stress signaling pathways, including DNA damage signaling including a reporter porter that is associated with DNA double strand breaks.	N/A
Dicentric Chromosome Assay (DCA)	Abe et al., 2018	Cells are fixed on microscope slides, chromosomes are stained, and the number of dicentric chromosomes are quantified	N/A
High content imaging	Shahane et al., 2016	DNA can be stained using fluorescent dyes and micronuclei can be scored high-throughput microscopy image analysis.	N/A
Chromosomal aberration test	OECD, 2016c; 2016d; 20l16e	In vitro, the cell cycle is arrested at metaphase after 1.5 cell cycle following 3-6 hour exposure In vivo, the test chemical is administered as a single treatment and bone marrow is collected 18-24 hrs later (TG 475), while testis is collected 24-48 hrs later (TG 483). The cell cycle is arrested with a metaphase-arresting chemical (e.g., colchicine) 2-5 hours before cell collection. Once cells are fixed and stained on microscope slides, chromosomal aberrations are scored	Yes. In vitro (No. 473) In vivo (No. 475 and No. 483)
Array Comparative Genomic Hybridization (aCGH) or SNP Microarray	Adewoye et al., 2015; Wilson et al., 2015; Arlt et al., 2014; Redon et al., 2006; Keren, 2014; Mukherjee, 2017	CNVs are most commonly detected using global DNA microarray technologies; This method, however, is unable to detect balanced CAs, such as inversions	N/A
Next Generation Sequencing (NGS): Whole Genome Sequencing (WGS) or Whole Exome Sequencing (WES)	Liu, 2013; Shen, 2016; Mukherjee, 2017	CNVs are detected by fragmenting the genome and using NGS to sequence either the entire genome (WGS), or only the exome (WES); Challenges with this methodology include only being able to detect CNVs in exon-rich areas if using WES, the computational investment required for the storage and analysis of these large datasets, and the lack of computational algorithms available for effectively detecting somatic CNVs	N/A

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Event: 870: Increase, Cell Proliferation

Short Name: Increase, Cell Proliferation

Key Event Component

Process	Object	Action
cell proliferation	epithelial cell	increased
cell proliferation	mesothelial cell	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:136 - Intracellular Acidification Induced Olfactory Epithelial Injury Leading to Site of Contact Nasal Tumors	KeyEvent
Aop:303 - Frustrated phagocytosis-induced lung cancer	KeyEvent
Aop:272 - Deposition of energy leading to lung cancer	KeyEvent
Aop:409 - Frustrated phagocytosis leads to malignant mesothelioma	KeyEvent
Aop:420 - Aryl hydrocarbon receptor activation leading to lung cancer through sustained NRF2 toxicity pathway	KeyEvent
Aop:432 - Deposition of Energy by Ionizing Radiation leading to Acute Myeloid Leukemia	KeyEvent
Aop:451 - Interaction with lung resident cell membrane components leads to lung cancer	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	KeyEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic applicability: Cell proliferation is a central process supporting development, tissue homeostasis and carcinogenesis, each of which occur in all vertebrates. This key event has been observed nasal tissues of rats exposed to the chemical initiator vinyl acetate. In general, cell proliferation is necessary in the biological development and reproduction of most organisms. This KE is thus relevant and applicable to all multicellular cell types, tissue types, and taxa.

Life stage applicability: This key event is not life stage specific (Fujimichi and Hamada, 2014; Barnard et al., 2022).

Sex applicability: This key event is not sex specific (Markiewicz et al., 2015).

Evidence for perturbation by a stressor: There is a large body of evidence supporting the effectiveness of ionizing radiation, UV, and mechanical wounding as stressors for increased cell proliferation. These stressors can be subdivided into X-rays (van Sallmann, 1951; Ramsell and Berry, 1966; Richards, 1966; Riley et al., 1988; Riley et al., 1989; Kleiman et al., 2007; Pendergrass et al., 2010; Fujimichi and Hamada, 2014, Markiewicz et al., 2015; Bahia et al., 2018), 60Co γ-rays (Hanna and O'Brien, 1963; Barnard et al., 2022; McCarron et al., 2021), 137Cs γ-rays (Andley and Spector, 2005), neutrons (Richards, 1966; Riley et al., 1988; Riley et al., 1989), 40Ar (Worgul et al., 1986), 56Fe (Riley et al., 1989), UVB (Söderberg et al., 1986; Andley et al., 1994; Cheng et al., 2019), UVC (Trenton and Courtois, 1981), and mechanical wounding (Riley et al., 1989).

Key Event Description

Throughout their life, cells replicate their organelles and genetic information before dividing to form two new daughter cells, in a process known as cellular proliferation. This replicative process is known as the cell cycle and is subdivided into various stages notably, G1, S, G2, and M in mammals. G1 and G2 are gap phases, separating mitosis and DNA synthesis. Differentiated cells typically remain in G1 however; quiescent cells reside in an optional phase just before G1, known as G0.

Progression through the cycle is dependent on sufficient nutrient availability to provide optimal nucleic acid, protein, and lipid levels, as well as sufficient cell mass. To this end, the cell cycle is mediated by three major checkpoints: the restriction (R) point, or G1/S checkpoint, controlling entry into S phase, the G2/M checkpoint, controlling entry into mitosis, and one more controlling entry into cytokinesis. If conditions are ideal for division, cells will pass the restriction point (G1/S) and begin the activation and expression of genes used for duplicating centrosomes and DNA, eventually leading to proliferation (Cuyàs et al., 2014).

Various protein complexes, known as cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs) regulate passage through each phase by activating and inhibiting specific processes (Lovicu et al., 2014). The CDKs are responsible for controlling progression through the cell cycle. They promote DNA synthesis and mitosis, and therefore cell division (Barnum & O'Connell, 2014). Furthermore, growth factors are required to stimulate cell division, but after passing through the restriction point at G1 they are no longer necessary (Lovicu et al., 2014).

In the context of cancer, one hallmark is the sustained and uncontrolled cell proliferation (Hanahan et al., 2011, Portt et al., 2011). When cells in the lung epithelium obtain a growth advantage due to mutations in critical genes that regulate cell cycle progression, they may begin to proliferate excessively, resulting in hyperplasia and potentially leading to the development of a tumour (Hanahan et al., 2011). It has been hypothesized that stressors such as radiation can result in cell inactivation and the replacement of these cells can initiate clonal expansion (Heidenreich adn Paretzke et al., 2008).

Sustained atrophy/degeneration olfactory epithelium under the influence of a cytotoxic agent leads to adaptive tissue remodeling. Cell types unique to olfactory epithelium, e.g. olfactory neurons, sustentacular cells and Bowmans glands, are replaced by cell types comprising respiratory epithelium or squamous epithelium.

How it is Measured or Detected

Two common methods of measuring cell proliferation in vivo are the use of Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) labeling (Pera, 1977), and Ki67 immunostaining (Grogan, 1988). BrdU is a synthetic analogue of the nucleoside Thymidine. BrDu is incorporated into DNA synthesized during the S1 phase of cell replication and is stable for long periods. Labeling of dividing cells by BrdU is accomplished by infusion, bolus injection, or implantation of osmotic pumps containing BrdU for a period of time sufficient to generate measureable numbers of labeled cells. Tissue sections are stained immunhistochemically with antibodies for BrdU and labeled cells are counted as dividing cells. Ki67 is a cellular marker of replication not found in quiescent cells (Roche, 2015). Direct immunohistochemical staining of cells for protein Ki67 using antibodies is an alternative to the use of BrdU, with the benefit of not requiring a separate treatment (injection for pulse-labeling). Cells positive for Ki67 are counted as replicating cells. Replicating cell

number is reported per unit tissue area or per cell nuclei (Bogdanffy, 1997). Listed below are common methods for detecting the KE, however there may be other comparable methods that are not listed.

Assay Name	References	Description	OECD Approved Assay
CyQuant Cell Proliferation Assay	Jones et al., 2001	DNA-binding dye is added to cell cultures, and the dye signal is measured directly to provide a cell count and thus an indication of cellular proliferation	N/A
Nucleotide Analog Incorporation Assays (e.g. BrdU, EdU)	Romar et al., 2016, Roche; 2013	Nucleoside analogs are added to cells in culture or injected into animals and become incorporated into the DNA at different rates, depending on the level of cellular proliferation; Antibodies conjugated to a peroxidase or fluorescent tag are used for quantification of the incorporated nucleoside analogs using techniques such as ELISA, flow cytometry, or microscopy	Yes (No. 442B)
Cytoplasmic Proliferation Dye Assays	Quah & Parish, 2012	Cells are incubated with a cytoplasmic dye of a certain fluorescent intensity; Cell divisions decrease the intensity in such a way that the number of divisions can be calculated using flow cytometry measurements	N/A
Colourimetric Dye Assays	Vega-Avila & Pugsley, 2011; American Type Culture Collection	Cells are incubated with a dye that changes colour following metabolism; Colour change can be measured and extrapolated to cell number and thus provide an indication of cellular proliferation rates	N/A

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List of Adverse Outcomes in this AOP

Event: 1556: Increase, lung cancer

Short Name: Increase, lung cancer

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:272 - Deposition of energy leading to lung cancer	AdverseOutcome

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization

Organ
Level of Biological Organization

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Lung cancer and subsequent metastasis occurs in multicellular eukaryotic vertebrate organisms that have lungs.

Key Event Description

Abnormally high levels of cell proliferation in the lungs may eventually culminate in the formation of malignant tumours and thus lung cancer. The term lung cancer refers to all malignant neoplasms arising from the bronchial, bronchiolar, and alveolar epithelium (Keshamouni et al., 2009). The cellular origin(s) of lung cancer remains largely unknown. It has been speculated that different tumour histopathological subtypes arise from distinct cells of origin localized in defined microenvironments. Histological characteristics of lung cancers, as defined by light microscopy, have led to the categorization of lung cancers into four main subtypes: small cell carcinoma, adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Beasly et al., 2005). These histological subtypes are grouped under one of the two umbrella terms used to describe lung cancers: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The term SCLC refers to small cell carcinoma. The term NSCLC, which represents approximately 85% of all lung cancers (Molina et al., 2008), encompasses squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. These three tumour types are grouped together due to similarities in their prognosis and management (Keshamouni et al., 2009); patients with NSCLC often have poor prognoses and low 5-year survival rates due to the high metastatic potential of the tumours (Spira and Ettinger, 2004; Herbst et al., 2008). Some of the most common sites for lung cancer metastasis are the other lobe of the lungs, skeleton, adrenal glands, liver, and brain (Simon et al., 2015).

How it is Measured or Detected

Assay Name	Reference	Description	OECD Approved Assay
Computed Tomography (CT) Scans: CT, High-Resolution CT (HRCT), and Positron Emission Tomography-CT (PET-CT)	Bach et al., 2012; Ollier et al., 2014	CT scans are described as a 3D X-ray; They provide cross-sections of organs/tissues/bones, and can thus be used to detect tumours	N/A
Magnetic Resonance Imaging (MRI)	Khalil et al., 2016; Wu et al., 2011	This technique uses magnetic fields and radio waves (NOT ionizing radiation) to generate a picture of organs, and can thus be used to detect tumours	N/A
Sputum Analysis	Hubers et al., 2013	Sputum is collected and analyzed for a variety of markers, including mutations in *KRAS* and *TP53*, specific RNA/protein biomarkers, and chromosomal aberrations	N/A
Bronchoscopy: Conventional		Bronchoscope (usually with a camera) is passed down through	

White Light Bronchoscopy, Autofluorescence Bronchoscopy (AFB), and Endobronchial Ultrasonography (EBUS)	Ikeda et al., 2007	the throat to the lungs to provide a visual of the respiratory tract; Traditionally, visualization has been performed using conventional white light, but new technologies have also allowed for visualization using fluorescence and ultrasound technologies	N/A
Transbronchial Needle Aspiration	Navani et al., 2015; Aziz, 2012	A needle is used to aspirate a tissue sample from a lesion of suspected lung cancer for analysis	N/A
Analysis of Volatile Organic Compounds in the Breath	Zhou et al., 2017	Volatile organic compounds, which may act as lung cancer biomarkers, are collected from the breath and quantified (mostly using mass spectrometry)	N/A
Cell Transformation Assays	Redpath et al., 1987	Measurement of the tumourigenicity of a tumour/biopsy sample by analyzing changes in cell physiology and morphology in response to tumour-inducing radiation or chemicals	Yes (No. 231)
Rodent Two-Year Cancer Bioassays (Carcinogenicity Studies)	Matsumo, 2012; Nambiar, 2014; Maronpot, 2015	Animals are exposed to a possible carcinogen for a long period of time (often two years), allowing for long-term cancer-related studies	Yes (No. 451)
Window Chamber Models	Moeller, 2004; Schafer, 2014; Chen, 2016	Window chambers are implanted into the animal to observe tumour progression in living animals using imaging techniques such as *in vivo* microscopy, MRI or nuclear imaging	N/A
Xenograft Assays	Wang, 2018; Shi, 2017; Jin, 2018; Wang, 2017; Zhou, 2012	Tumour cells (usually human) are grown *in vitro* and injected into animals to induce tumour growth and/or to test the tumourigenicity of the injected cells	N/A

Regulatory Significance of the AO

At present the AOP framework is not readily used to support regulatory decision-making in radiation protection practices.The goal of developing this AOP is to bring attention to the framework as an effective means to organize knowledge and identify gaps associated with the mechanistic understanding of low dose radiation exposures. We have used lung cancer as the case example due to its relevance to both radiation and chemical risk assessment. This AOP will help build the concept of an “all hazards” approach to risk assessment, as it will be the first with a molecular initiating event that is specific to a radiation insult. This in turn could serve to identify networks that are critical to both radiation and chemical exposure scenarios and contribute to prioritizing co-exposures of relevance to risk assessment. By developing this AOP, we will support the necessary efforts highlighted by the international and national radiation protection agencies such as, the United Nations Scientific Committee on the Effects of Atomic Radiation, International Commission of Radiological Protection, International Dose Effect Alliance and the Electric Power Research Institute Radiation Program to consolidate and enhance the knowledge in understanding the mechanisms of low dose radiation exposures from the cellular to organelle levels within the system.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1977: Energy Deposition leads to Increase, DNA strand breaks

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deposition of energy leading to lung cancer	adjacent	High	High
Deposition of energy leading to population decline via DNA strand breaks and follicular atresia	adjacent	High	
Deposition of energy leading to population decline via DNA strand breaks and oocyte apoptosis	adjacent		
Deposition of energy leading to occurrence of cataracts	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
bovine	Bos taurus	Low	NCBI
rabbit	Oryctolagus cuniculus	Low	NCBI
Pig	Pig	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

This KER is plausible in all life stages, sexes, and organisms with DNA. The majority of the evidence is from In vivo adult mice and human In vitro models that do not specify the sex.

Key Event Relationship Description

Direct deposition of ionizing energy refers to imparted energy interacting directly with the DNA double helix and producing randomized damage. This can be in the form of double strand breaks (DSBs), single-strand breaks, base damage, or the crosslinking of DNA to other molecules (Smith et al., 2003; Joiner, 2009; Christensen, 2014; Sage and Shikazono, 2017). Among these, the most detrimental type of DNA damage to a cell is DSBs. They are caused by the breaking of the sugar-phosphate backbone on both strands of the DNA double helix molecule, either directly across from each other or several nucleotides apart (Ward, 1988; Iliakis et al., 2015). This occurs when high-energy subatomic particles interact with the orbital electrons of the DNA causing ionization (where electrons are ejected from atoms) and excitation (where electrons are raised to higher energy levels) (Joiner, 2009). The number of DSBs produced and the complexity of the breaks is highly dependent on the amount of energy deposited on and absorbed by the cell. This can vary as a function of the dose-rate (Brooks et al., 2016) and the radiation quality which is a function of its linear energy transfer (LET) (Sutherland et al., 2000; Nikjoo et al., 2001; Jorge et al., 2012). LET describes the amount of energy that an ionizing particle transfers to media per unit distance (Smith et al., 2003; Okayasu, 2012a; Christensen et al., 2014). High LET radiation, such as alpha particles, heavy ion particles, and neutrons, can deposit larger quantities of energy within a single track than low LET radiation, such as γ-rays, X-rays, electrons, and protons (Kadhim et al., 2006; Franken et al., 2012; Frankenberg et al., 1999; Rydberg et al., 2002; Belli et al., 2000; Antonelli et al., 2015). As such, radiation with higher LETs tends to produce more complex, dense structural damage, particularly in the form of clustered damage, in comparison to lower LET radiation (Nikjoo et al., 2001; Terato and Ide, 2005; Hada and Georgakilas, 2008; Okayasu, 2012a; Lorat et al., 2015; Nikitaki et al., 2016). Thus, the complexity and yield of clustered DNA damage increases with ionizing density (Ward, 1988; Goodhead, 2006). However, clustered damage can also be induced even by a single radiation track through a cell.

While the amount of DSBs produced depends on the radiation dose (see dose concordance), it also depends on several other factors. As the LET increases, the complexity of DNA damage increases, decreasing the repair rate, and increasing toxicity (Franken et al., 2012; Antonelli et al., 2015).

Evidence Supporting this KER

Overall Weight of Evidence for this KER: High

Biological Plausibility

The biological rationale linking the direct deposition of energy on DNA with an increase in DSB formation is strongly supported by numerous literature reviews that are

available on this topic (J .F. Ward, 1988; Lipman, 1988; Hightower, 1995; Terato & Ide, 2005; Goodhead, 2006; Kim & Lee, 2007; Asaithamby et al., 2008; Hada & Georgakilas, 2008; Jeggo, 2009; Clement, 2012; Okayasu, 2012b; Stewart, 2012; M. E. Lomax et al., 2013; EPRI, 2014; Hamada, 2014; Moore et al., 2014; Desouky et al., 2015; Ainsbury, 2016; Foray et al., 2016; Hamada & Sato, 2016; Hamada, 2017a; Sage & Shikazono, 2017; Chadwick, 2017). Ionizing radiation can be in the form of high energy particles (such as alpha particles, beta particles, or charged ions) or high energy photons (such as gamma-rays or X-rays). Ionizing radiation can break the DNA within chromosomes both directly and indirectly, as shown through using velocity sedimentation of DNA through neutral and alkaline sucrose gradients. The most direct path entails a collision between a high-energy particle or photon and a strand of DNA.

Additionally, excitation of secondary electrons in the DNA allows for a cascade of ionization events to occur, which can lead to the formation of multiple damage sites (Joiner, 2009). As an example, high-energy electrons will traverse a DNA molecule in a mammalian cell within 10^{-18} s and 10^{-14} s, resulting in 100,000 ionizing events per 1 Gy dose in a 10 µm cell (Joiner, 2009). The amount of damage can be influenced by factors such as the cell cycle stage and chromatin structure. It has been shown that in more condensed, packed chromatin structures such as those present in intact cells and heterochromatin, it is more difficult for the DNA to be damaged (Radulescu et al., 2006; Agrawala et al., 2008; Falk et al., 2008; Venkatesh et al., 2016). In contrast, DNA damage is more easily induced in lightly-packed chromatin such as euchromatin and nucleoids, (Radulescu et al., 2006; Falk et al., 2008; Venkatesh et al., 2016).

Of the possible radiation-induced DNA damage types, DSB is considered to be the most harmful to the cell, as there may be severe consequences if this damage is not adequately repaired (Khanna & Jackson, 2001; Smith et al., 2003; Okayasu, 2012a; M. E. Lomax et al., 2013; Rothkamm et al., 2015).

A considerable fraction of DSBs can also be formed in cells through indirect mechanisms. In this case, deposited energy can split water molecules near DNA, which can generate a significant quantity of reactive oxygen species in the form of hydroxyl free radicals (Ward, 1988; Wolf, 2008; Desouky et al., 2015; Maier et al., 2016, Cencer et al., 2018; Bains, 2019; Ahmadi et al., 2021). Estimates using models and experimental results suggest that hydroxyl radicals may be present within nanoseconds of energy deposition by radiation (Yamaguchi et al., 2005). These short-lived but highly reactive hydroxyl radicals may react with nearby DNA. This will produce DNA damage, including single-strand breaks and DSBs (Ward, 1988; Sasaki, 1998; Desouky et al., 2015; Maier et al., 2016). DNA breaks are especially likely to be produced if the sugar moiety is damaged, and DSBs occur when two single-strand breaks are in close proximity to each other (Ward, 1988).

Empirical Evidence

Empirical data strongly supports this KER. The evidence presented below is summarized in table 1. The types of DNA damage produced by ionizing radiation and the associated mechanisms, including the induction of DSBs, are reviewed by Lomax et al. (2013) and documents produced by international radiation governing frameworks (Valentin, 1998; UNSCEAR, 2000). Other reviews also highlight the relationship between the deposition of energy by radiation and DSB induction, and discuss the various methods available to detect these DSBs (Terato & Ide, 2005; Rothkamm et al., 2015; Sage & Shikazono, 2017). A visual representation of the time frames and dose ranges probed by the dedicated studies discussed here is shown in Figures 1 & 2 below.

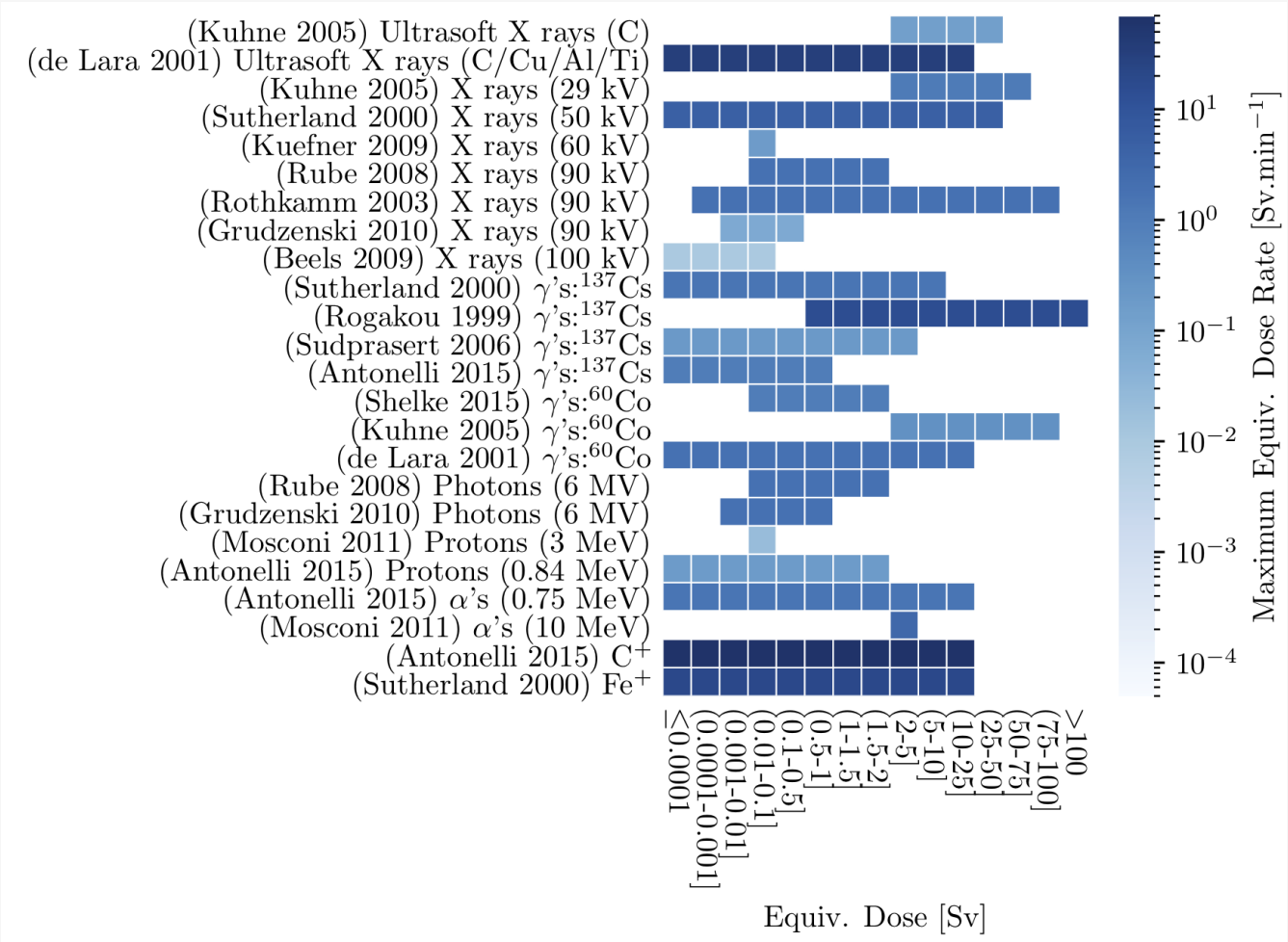

Figure 1: Plot of example studies (y-axis) against equivalent dose (Sv) used to determine the empirical link between direct deposition of energy and DSBs. The z-axis denotes the equivalent dose rate used in each study. The y-axis is ordered from low LET to high LET from top to bottom.

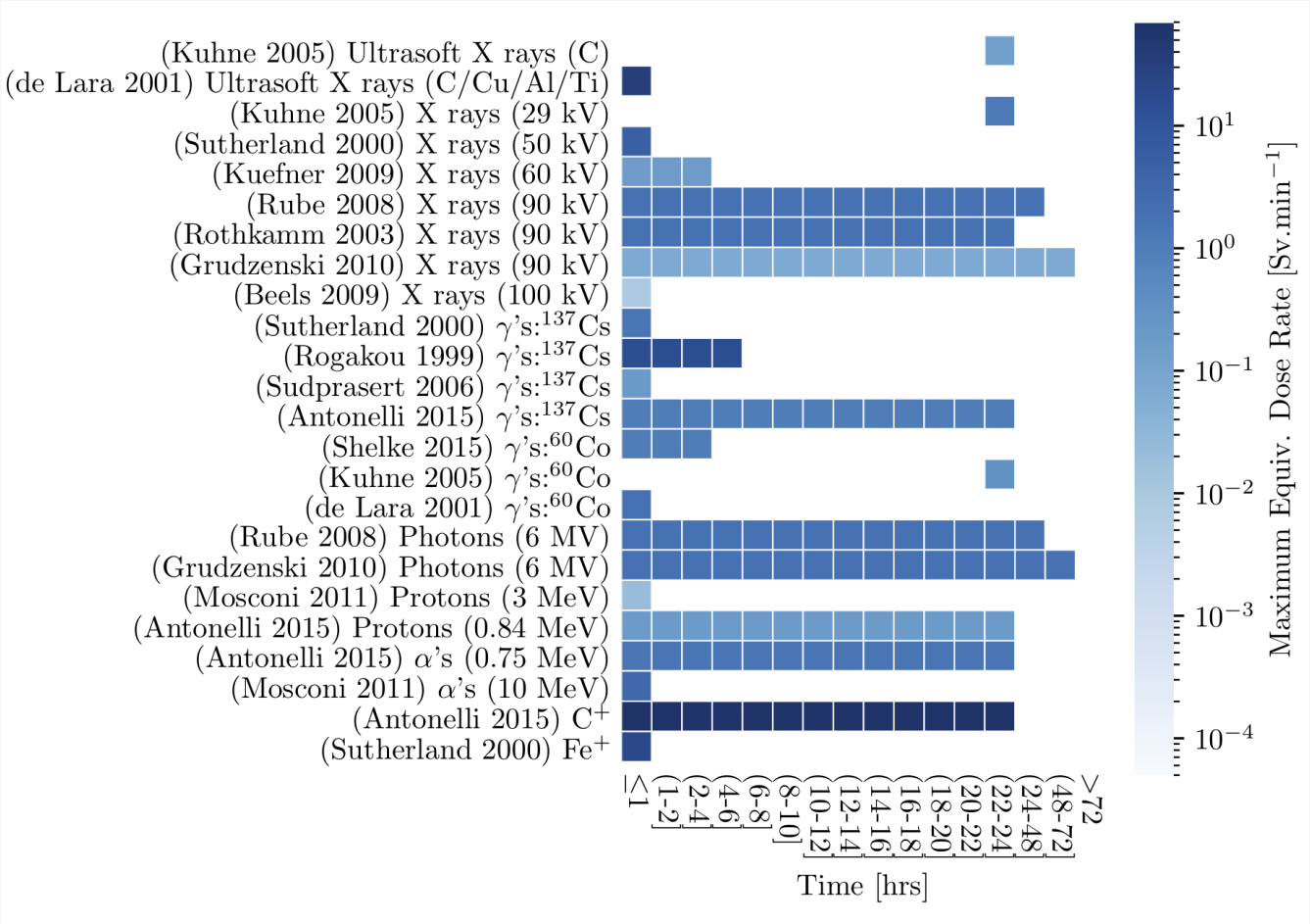

Figure 2: Plot of example studies (y-axis) against time scales used to determine the empirical link between direct deposition of energy and DSBs. The z-axis denotes the equivalent dose rate used in each study. The y-axis is ordered from low LET to high LET from top to bottom.

Dose Concordance

There is evidence in the literature suggesting a dose concordance between the direct deposition of energy by ionizing radiation and the incidence (Grudzenski et al., 2010) of DNA DSBs. Results from in vitro (Aufderheide et al., 1987; Sidjanin, 1993; Bucolo, 1994; Frankenberg et al., 1999; Rogakou et al., 1999; Belli et al., 2000; Sutherland et al., 2000; Lara et al., 2001; Rydberg et al., 2002; Baumstark-Kham et al., 2003; Rothkamm and Lo, 2003; Long, 2004; Kuhne et al., 2005; Sudprasert et al., 2006; Beels et al., 2009; Grudzenski et al., 2010; Liao, 2011; Franken et al., 2012; Bannik et al., 2013; Shelke & Das, 2015; Antonelli et al., 2015; Markiewicz et al., 2015; Allen, 2018; Dalke, 2018; Bains, 2019; Ahmadi et al., 2021), in vivo (Reddy, 1998; Sutherland et al., 2000; Rube et al., 2008; Beels et al., 2009; Grudzenski et al., 2010; Markiewicz et al., 2015; Barnard, 2018; Barnard, 2019; Barnard, 2022), ex vivo (Rube et al., 2008; Flegal et al., 2015) and simulation studies (Charlton et al., 1989) suggest that there is a positive, linear, dose-dependent increase in DSBs with increasing deposition of energy across a wide range of radiation types (iron ions, X-rays, ultrasoft X-rays, gamma-rays, photons, UV light, and alpha particles) and radiation doses (1 mGy - 100 Gy) (Aufderheide et al., 1987; Sidjanin, 1993; Frankenberg et al., 1999; Sutherland et al., 2000; de Lara et al., 2001; Baumstark-Khan et al., 2003; Rothkamm & Lo, 2003; Kuhne et al., 2005; Rube et al., 2008; Grudzenski et al., 2010; Bannik et al., 2013; Shelke & Das, 2015; Antonelli et al., 2015; Dalke, 2018). DSBs have been predicted to occur at energy deposition levels as low as 75 eV (Charlton et al., 1989).

Time Concordance

There is evidence suggesting a time concordance between the direct deposition of energy and the incidence of DSBs. A number of different models and experiments have provided evidence of ionizing radiation-induced foci (IRIF), which can be used to infer DSB formation seconds (Mosconi et al., 2011) or minutes after radiation exposure (Rogakou et al., 1999; Rothkamm and Lo, 2003; Rube et al., 2008; Beels et al., 2009; Kuefner et al., 2009; Grudzenski et al., 2010; Antonelli et al., 2015).

Essentiality

Deposition of energy is essential for DNA strand breaks. They can also be caused through other routes, such as oxidative stress (Cadet et al., 2012), but under normal physiological conditions deposition of energy is necessary. This was tested through many studies using various indicators such as 53BP1 foci/cell, γH2AX foci/cell, DNA migration, and the amount of DNA in tails for the comet assay. Various organisms such as humans, mice, rabbits, guinea pigs, and cattle were used. They showed that

without the deposition of energy, there was only a negligible amount of DNA strand breaks (Aufderheide et al., 1987; Sidjanin, 1993; Bucolo, 1994; Reddy, 1998; Rogers, 2004; Bannik et al., 2013; Dalke, 2018; Bains, 2019; Barnard, 2019; Barnard, 2021).

Uncertainties and Inconsistencies

Uncertainties and inconsistencies in this KER are as follows:

- Studies have shown that dose-rates (Brooks et al., 2016) and radiation quality (Sutherland et al., 2000; Nikjoo et al., 2001; Jorge et al., 2012) are factors that can influence the dose-response relationship.
- Low-dose radiation has been observed to have beneficial effects and may even invoke protection against spontaneous genomic damage (Feinendegen, 2005; Day et al., 2007; Feinendegen et al., 2007; Shah et al., 2012; Nenoi et al., 2015; Dalke, 2018). This protective effect has been documented in in vivo and in vitro, as reviewed by ICRP (2007) and UNSCEAR (2008) and can vary depending on the cell type, the tissue, the organ, or the entire organism (Brooks et al., 2016).
- Depositing ionizing energy is a stochastic event; as such this can influence the location, degree and type of DNA damage imparted on a cell. As an example, studies have shown that mitochondrial DNA may also be an important target for genotoxic effects of ionizing radiation (Wu et al., 1999).

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage suggests that DSBs can be predicted upon exposure to ionizing radiation. This is dependent on the biological model, the type of radiation and the radiation dose. In general, 1 Gy of radiation is thought to result in 3000 damaged bases (Maier et al., 2016), 1000 single-strand breaks, and 40 DSBs (Ward, 1988; Foray et al., 2016; Maier et al., 2016) . The table below provides representative examples of the calculated DNA damage rates across different model systems, most of which are examining DNA DSBs.

Dose Concordance

The following tables provide representative examples of the relationship, unless otherwise indicated, all data is significantly significant.

Reference	Experiment Description	Result
Ward, 1988	In vitro. Cells containing approximately 6 pg of DNA were exposed to 1 Gy.	Under the assumption of 6 pg of DNA per cell. 60 eV of energy deposited per event over a total of 1 Gy. Deoxyribose (2.3 pg/cell): 14,000 eV deposited, 235 events. Bases (2.4 pg/cell): 14.7 keV deposited, 245 events. Phosphate (1.2 pg/cell): 7,300 eV deposited, 120 events. Bound water (3.1 pg/cell): 19 keV deposited, 315 events. Inner hydration shell (4.2 pg/cell): 25,000 eV deposited 415 events.
Charlton, 1989	In-silico. A computer simulation/model was used to test various types of radiation with doses from 0 to 400 eV (energy deposited) on the amount of DNA damage produced.	Simulated dose-concordance prediction of increase in number of DSBs/54 nucleotide pairs as direct deposition of energy increases in the range 75-400 eV. In the range 100 - 150 eV: 0.38 DSBs/54 nucleotide pairs and at 400 eV: ~0.80 DSBs per 64 nucleotide pairs.
Sutherland, 2000	In vitro. Human cells were exposed to ^{137}Cs γ-rays (0 – 100 Gy, 0.16 – 1.6 Gy/min). The frequency of DSBs was determined using gel electrophoresis.	Using isolated bacteriophage T7 DNA and 0-100 Gy of γ radiations, observed a response of 2.4 DSBs per megabase pair per Gy.
Rogakou et al., 1999	In vitro. Normal human fibroblasts (IMR90) and human breast cancer cells (MCF7 were exposed to 0.6 and 2 Gy ^{137}Cs γ-rays delivered at 15.7 Gy/min. The number of DSBs were determined by immunoblotting for γ-H2AX.	Radiation doses of 0.6 Gy & 2 Gy to normal human fibroblasts (IMR90) and MCF7 cells resulted in 10.1 & 12.2 DSBs per nucleus on average (0.6 Gy), respectively; increasing to 24 & 27.1 DSBs per nucleus (2 Gy).
Kuhne et al., 2005	In vitro. Primary human skin fibroblasts (HSF2) were exposed to 0 – 70 Gy ^{60}Co γ-rays (0.33 Gy/min), X-rays (29 kVp, 1.13 Gy/min), and CKX-rays (0.14 Gy/min). The number of DSBs were determined with pulsed-field gel electrophoresis.	γ-ray and X-ray irradiation of primary human skin fibroblasts (HSF2) at 0 - 70 Gy. γ-rays: (6.1 ± 0.2) x 10-9 DSBs per base pair per Gy, X-rays: (7.0 ± 0.2) x 10-9 DSBs per base pair per Gy. CKX -rays: (12.1 ± 1.9) x 10-9 DSBs per base pair per Gy.
Rothkamm, 2003	In vitro. Primary human fibroblast cell lines MRC-5 (lung), HSF1 and HSF2 (skin), and180BR (deficient in DNA ligase IV) were exposed to 1 mGy – 100 Gy X-rays (90 kV). Low doses were delivered at 6 – 60 mGy/min and high doses were delivered at 2 Gy/min. The number of DSBs were determined with pulsed-field gel electrophoresis.	X-ray irradiation of primary human fibroblasts (MRC-5) in the range 1 mGy - 100 Gy, 35 DSBs per cell per Gy.
Grudzenski et al, 2010	In vitro. Primary human fibroblasts (HSF1) and C57BL/6NCrl adult mice were exposed to X-rays (2.5 – 200 mGy, 70 mGy/min), and photons (10 mGy – 1 Gy, 2 Gy/min (100 mGy and 1 Gy), and 0.35 Gy/min (10 mGy)). γ-H2AX immunofluorescence was observed to determine DSBs.	X-rays irradiating primary human fibroblasts (HSF1) in the range 2.5 - 100 mGy yielded a response of 21 foci per Gy. When irradiating adult C57BL/6NCrl mice with photons a response of 0.07 foci per cell at 10 mGy was found. At 100 mGy the response was 0.6 foci per cell and finally, at 1 Gy; 8 foci per cell.
de Lara, 2001	In vitro. Chinese hamster cells (V79-4) were exposed to 0 – 20 Gy of ^{60}Co γ-rays (2 Gy/min), and ultrasoft X-rays (0.7 – 35 Gy/min): carbon-K shell (0.28 keV), copper L-shell (0.96 keV), aluminum K-shell (1.49 keV), and titanium K-shell (4.55 keV). The number of DSBs were determined with pulsed-field gel electrophoresis.	V79-4 cells irradiated with γ-rays and ultrasoft X-rays (carbon K-shell, copper L-shell, aluminium K-shell and titanum K-shell) in the range 0 - 20 Gy. Response (DSBs per Gy per cell): γ-rays: 41, carbon K-shell: 112, copper L-shell: 94, aluminum K-shell: 77, titanium K-shell: 56.
Rübe et al., 2008	In vivo. Brain, lung, heart and small intestine tissue from adult SCID, A-T, BALB/c and C57BL/6NCrl mice; Whole blood and isolated lymphocytes from BALB/c and C57BL/6NCrl mice were exposed to 0.1 – 2 Gy of photons (whole body irradiation, 6 MV, 2 Gy/min) and X-rays (whole body irradiation, 90 kV, 2 Gy/min). γ-H2AX foci were determined with immunochemistry to measure DSBs.	Linear dose-dependent increase in DSBs in the brain, small intestine, lung and heart of C57BL/6CNrl mice after whole-body irradiation with 0.1 - 1.0 Gy of radiation. 0.8 foci per cell (0.1 Gy) and 8 foci per cell (1 Gy).
Antonelli et	In vitro. Primary human foreskin fibroblasts (AG01522) were exposed to 0 – 1 Gy of ^{136}Cs γ-rays (1 Gy/min), protons (0.84 MeV, 28.5 keV/um), carbon	Linear dose-dependent increase in the number of DSBs from 0 - 1 Gy for γ-

al., 2015	ions (58 MeV/u, 39.4 keV/um), and alpha particles (americium-241, 0.75 MeV/u, 0.08 Gy/min, 125.2 keV/um). γ-H2AX foci were determined with immunochemistry to measure DSBs.	rays and alpha particles as follows: γ-rays: 24.1 foci per Gy per cell nucleus, alpha particles: 8.8 foci per Gy per cell nucleus.
Barnard et al., 2019	In vivo. 10-week-old female C57BL/6 mice were whole-body exposed to 0.5, 1, and 2 Gy of 60Co γ-rays at 0.3, 0.063, and 0.014 Gy/min. 53BP1 foci were determined via immunofluorescence.	Central LECs showed a linear increase in mean 53BP1 foci/cell with the maximum dose and dose-rate displaying a 78x increase compared to control. Peripheral LECs and lower dose rates displayed similar results, with slightly fewer foci.
Ahmadi et al., 2021	In vitro. Human LEC cells were exposed to 137Cs γ-rays at doses of 0, 0.1, 0.25, and 0.5 Gy and dose rates of 0.065 and 0.3 Gy/min. DNA strand breaks were measured using the comet assay.	Human LECs showed a gradual increase in the tail from the comet assay with the maximum dose and dose-rate displaying a 3.7x increase compared to control. Lower dose-rates followed a similar pattern with a lower amount of strand breaks.
Hamada et al., 2006	In vitro. Primary normal human diploid fibroblast (HE49) cells were exposed to 0.1, 0.5, and 4 Gy X-rays at 240 kV with a dose rate of 0.5 Gy/min. The number of H2AX foci/cell, which represented DNA strand breaks, was determined 6 – 7 minutes after irradiation through fluorescence microscopy.	Cells displayed a linear increase in the number of H2AX foci/cell, with the maximum dose displaying a 125x increase compared to control.
Dubrova & Plumb, 2002		At 1 Gy observe 70 DSBs, 1000 single-strange breaks and 2000 damaged DNA bases per cell per Gy.

Time Concordance

Reference	Experiment Description	Result
Rogakou et al., 1999	In vitro. Normal human fibroblasts (IMR90), human breast cancer cells (MCF7), human astrocytoma cells (SF268), Indian muntjac Muntiacus muntjak normal skin fibroblasts, Xenopus laevisA6 normal kidney cells, Drosophila melanogaster epithelial cells, and Saccharomyces cerevisae were exposed to 0.6, 2, 20, 22, 100, and 200 Gy 137Cs γ-rays. Doses below 20 Gy were delivered at 15.7 Gy/min and other doses were delivered in 1 minute. DNA breaks were visualized using γ-H2AX antibodies and microscopy.	DSBs were present at 3 min and persisted from 15 - 60 min.
Hamada & Woloschak, 2017	In vitro. human LECs were exposed to 0.025 Gy X-rays at 0.42 – 0.45 Gy/min. 53BP1 foci were measured via indirect immunofluorescence.	In cells immediately exposed to 0.025 Gy, the level of 53BP1 foci/cell increased to 3.3x relative to control 0.5 h post-irradiation.
Hamada et al., 2006	In vitro. Primary normal human diploid fibroblast (HE49) cells were exposed to 0.1, 0.5, and 4 Gy (deposition of energy) at 240 kV with a dose rate of 0.5 Gy/min. The number of H2AX foci/cell, which represented DNA strand breaks, was determined through fluorescence microscopy.	In cells immediately exposed to 0.5 Gy, 11% of cells had 18 foci six min post-irradiation, compared to 90% of controls having 0 foci.

Response-response relationship

There is evidence of a response-response relationship between the deposition of energy and the frequency of DSBs. In studies encompassing a variety of biological models, radiation types and radiation doses, a positive, linear relationship was found between the radiation dose and the number of DSBs (Aufderheide et al., 1987; Sidjanin, 1993; Frankenberg et al., 1999; Sutherland et al., 2000; de Lara et al., 2001; Baumstark-Khan et al., 2003; Rothkamm & Lo, 2003; Kuhne et al., 2005; Rube et al., 2008; Grudzenski et al., 2010; Bannik et al., 2013; Shelke & Das, 2015; Antonelli et al., 2015; Hamada, 2017b; Dalke, 2018). There were, however, at least four exceptions reported. When human blood lymphocytes were irradiated with X-rays in vitro, a linear relationship was only found for doses ranging from 6 - 500 mGy; at low doses from 0 - 6 mGy, there was a quadratic relationship reported (Beels et al., 2009). Secondly, simulation studies predicted that there would be a non-linear increase in DSBs as energy deposition increased, with a saturation point at higher LETs (Charlton et al., 1989). Furthermore, primary normal human fibroblasts exposed to 1.2 – 5 mGy X-rays at 5.67 mGy/min showed a supralinear relationship, indicating at low doses, the DSBs are mostly due to radiation-induced bystander effects. Doses above 10 mGy showed a positive linear relationship (Ojima et al., 2008). Finally, in the human lens epithelial cell line SRA01/04, DNA strand breaks appeared immediately after exposure to UVB (0.14 J/cm2) and were repaired after 30 minutes. They then reappeared after 60 and 90 minutes. Both were once again repaired within 30 minutes. However, the two subsequent stages of DNA strand breaks did not occur when exposed to a lower dose of UVB (0.014 J/cm2) (Cencer et al., 2018).

Time-scale

Data from temporal response studies suggests that DSBs likely occur within seconds to minutes of energy deposition by ionizing radiation. In a variety of biological models, the presence of DSBs has been well documented within 10 - 30 minutes of radiation exposure (Rogakou et al., 1999; Rube et al., 2008; Beels et al., 2009; Kuefner et al., 2009; Grudzenski et al., 2010; Antonelli et al., 2015); there is also evidence that DSBs may actually be present within 3 - 5 minutes of irradiation (Kleiman, 1990; Rogakou et al., 1999; Rothkamm & Lo, 2003; Rube et al., 2008; Grudzenski et al., 2010; Cencer et al., 2018). Interestingly, one study that focussed on monitoring the cells before, during and after irradiation by taking photos every 5, 10 or 15 seconds found that foci indicative of DSBs were present 25 and 40 seconds after collision of the alpha particles and protons with the cell, respectively. The number of foci were found to increase over time until plateauing at approximately 200 seconds after alpha particle exposure and 800 seconds after proton exposure (Mosconi et al., 2011).

After the 30 minute mark, DSBs have been shown to rapidly decline in number. By 24 hours post-irradiation, DSB numbers had declined substantially in systems exposed to radiation doses between 40 mGy and 80 Gy (Aufderheide et al., 1987; Baumstark-Khan et al., 2003; Rothkamm & Lo, 2003; Rube et al., 2008; Grudzenski et al., 2010; Bannik et al., 2013; Markiewicz et al., 2015; Russo et al., 2015; Antonelli et al., 2015; Dalke, 2018; Bains, 2019; Barnard, 2019; Ahmadi et al., 2021), with the sharpest decrease documented within the first 5 h (Kleiman, 1990; Sidjanin, 1993; Rogakou et al., 1999; Rube et al., 2008; Kuefner et al., 2009; Grudzenski et al., 2010; Bannik, 2013; Markiewicz et al., 2015; Shelke and Das, 2015; Cencer et al., 2018). Interestingly, DSBs were found to be more persistent when they were induced by higher LET radiation (Aufderheide et al., 1987, Baumstark-Khan et al., 2003; Antonelli et al., 2015).

Known modulating factors

Modulating Factor	Details	Effects on the KER	References
Nitroxides	Increased concentration	Decreased DNA strand breaks.	DeGraff et al., 1992; Citrin & Mitchel, 2014
5-fluorouracil	Increased concentration	Increased DNA strand breaks.	De Angelis et al., 2006; Citrin & Mitchel, 2014
Thiols	Increased concentration	Decreased DNA strand breaks.	Milligan et al., 1995; Citrin & Mitchel, 2014
Cisplatin	Increased concentration	Decreased DNA break repair.	Sears & Turchi; Citrin & Mitchel, 2014

Known Feedforward/Feedback loops influencing this KER

Not identified.

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

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

Key Event Relationship Description

The maintenance of DNA integrity is essential for genomic stability; for this reason cells have multiple response mechanisms that enable the repair of damaged DNA. Thus when DNA double strand breaks (DSBs) occur, the most detrimental type of lesion, the cell will initiate repair machinery. These mechanisms are not foolproof, and emerging evidence suggests that closely spaced lesions can compromise the repair machinery. The two most common DSB repair mechanisms are non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is initiated in G1 and early S phases of the cell cycle (Lieber et al., 2003) and is preferentially used to repair DSB damage (Godwint et al., 1994), as it is rapid and more efficient than HR (Lliakis, 1991; Jeggo, 1998; Mao et al., 2008). In higher-order eukaryotes such as humans, NHEJ is the favoured DNA repair mechanism because of the large non-coding regions within the genome. NHEJ can occur through one of two subtypes: canonical NHEJ (C-NHEJ) or alternative non-homologous end joining (alt-NHEJ). C-NHEJ, as the name suggests, simply ligates the broken ends back together. In contrast, alt-NHEJ occurs when one strand of the DNA on either side of the break is resected to repair the lesion (Bétermier et al., 2014). Both repair mechanisms are error-prone, meaning insertions and deletions are sometimes formed due to the DSBs being repaired imperfectly (Thurtle-Schmidt and Lo, 2018). However, alt-NHEJ is considered more error-prone than C-NHEJ, as studies have shown that it more often leads to chromosomal aberrations (Zhu et al., 2002; Guirouilh-Barbat et al., 2007; Simsek & Jasin, 2010). HR is mostly operative during S and G2 phases because of the presence of the sister chromatid that can be used as template for repair (Van Gent et al 2001). Because of the reliance on the undamaged sister chromatid to repair the DSB, HR is less error-prone than NEHJ. Nevertheless, defects in HR are known to contribute to genomic instability and the formation of chromosomal aberrations (Deans et al 2000)

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

Evidence Supporting this KER

Biological Plausibility

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

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

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

Empirical Evidence

Empirical data obtained for this KER strongly supports the idea that an increase in DNA DSBs will increase the frequency of inadequate DSB repair. The evidence presented

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

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

Dose and Incidence Concordance

There is evidence in the literature suggesting a dose/incidence concordance between the occurrence of DSBs and the incidence of inadequate DNA repair upon exposure to radiation. Inadequate DNA repair appears to occur at the same radiation dose as DSBs. Visually, immunofluorescence has demonstrated a colocalization of DNA repair proteins with DSB foci in response to a radiation stressor (Paull et al., 2000; Asaithamby & Chen, 2009; Dong et al., 2017). In studies examining cellular responses to increasing doses of radiation, which is known to evoke a dose-dependent increase in DNA DSBs (Aufderheide, 1987; Durante et al., 1998; Dikomey & Brammer, 2000; Kuhne et al., 2000; Löbrich et al., 2000; Rothkamm & Lo, 2003; Kuhne et al., 2005; Asaithamby & Chen, 2009; Bracalente et al., 2013), there were resulting dose-dependent increases in non-repaired DSBs (Aufderheide, 1987; Rydberg et al., 1994; Dikomey & Brammer, 2000; Baumstark-Khan et al., 2003), DSB misrepair rates (Mcmahon et al., 2016), and misrejoined DSBs (Durante et al., 1998; Kuhne et al., 2000; Kuhne et al., 2005; Rydberg et al., 2005), as well as a dose-dependent decrease in the total DSB rejoining (Löbrich et al., 2000). Furthermore, only 50% of the rejoined DSBs were found to be correctly repaired (Kuhne et al., 2000; Löbrich et al., 2000); 24 hours after being irradiated with an 80 Gy dose of alpha particles, this frequency of misrejoining increased to and remained constant at 80% (Kuhne et al., 2000). Furthermore, delivering radiation doses in fractionated increments also showed a dose-dependent change in the percentage of misrejoinings, such that larger fractionated doses (for example, 2 x 40 Gy) had a higher rate of DSB misrejoining than smaller fractionated doses (for example, 4 x 10 Gy) (Kuhne et al., 2000).

Temporal Concordance

There is evidence suggesting a time concordance between DSBs and DNA repair. DSBs and DNA repair have both been observed within minutes to hours of radiation exposure (Paull et al., 2000; Rothkamm & 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 (Coquerelle et al., 1987; Rothkamm and Lo, 2003; Bracalente et al., 2013; Mcmahon et al., 2016; Dong et al., 2017), and there was an increase in incorrectly rejoined DSBs (Löbrich et al., 2000). In one striking example, a human cell line lacking DNA ligase IV had DSBs that were still present approximately 240 - 340 hours post-irradiation (Mcmahon et al., 2016). Interestingly, there were also increased levels of DSBs in these cells prior to being exposed to a radiation stressor (Paull et al., 2000) . Similarly, a study examining DSB repair kinetics after irradiation found that DSBs persisted for a longer time period in two repair-deficient mouse strains relative to a repair-proficient mouse strain; this pattern was found in lymphocytes, as well as tissues from the brains, lungs, hearts and intestines of these mice (Rube et al., 2008). The roles of various DNA repair proteins in the context of DSBs are highlighted in reviews by Chang et al. (2001) and Van Gent et al. (2001) with discussions focussing on the consequences of losing some of these proteins in cells, mice and humans (Van Gent et al., 2001)

Uncertainties and Inconsistencies

Uncertainties and inconsistencies in this KER are as follows:

- There is controversy surrounding how error-prone NHEJ truly is. Recent studies suggest that the process may be quite accurate (reviewed in (Bétermier et al. 2014)). The accuracy of NHEJ may actually be dependent on the structure of the termini. Thus, the termini processing rather than the NHEJ mechanism itself is argued to be the error-prone process (Bétermier et al. 2014).
- There may be different cellular responses associated with low-dose radiation exposure and high-dose radiation exposure; these differences may also be dependent on a DSB threshold being exceeded prior to initiation repair. It has been suggested that DNA repair may not be activated at low doses of radiation exposure in order to prevent the risk of mutations from error-prone repair mechanisms (Marples 2004).
- DSB repair fidelity varies in terms of confounding factors and the genetic characteristics of individuals (Scott 2006). For example, individuals who smoke have a 50% reduction in the mean level of DSB repair capacity relative to the non-smokers; this is due to an increased methylation index in smokers. A higher methylation index indicates more inactivation of gene expression. It is thus possible that expression of DNA repair proteins in smokers is decreased due to increased methylation of the genes encoding for repair proteins. In terms of individual genetics, single nucleotide polymorphisms (SNPs) within the MRE11A, CHEK2, XRCC3, DNA-PKcs, and NBN repair genes have been highly associated with the methylation index (Leng et al. 2008). SNPs can critically affect the function of these core proteins, varying the fidelity of DNA repair from person to person.
- Cells containing DNA damaged may be eliminated by apoptotic pathways, therefore not undergo repair, alternatively evidence has also shown that damaged cells can propagate due to lack of detection by repair machinery (Valentin 2005).
- The focus of this KER was on DSBs because there is lack of data to support that single strand breaks (SSBs) lead to inadequate repair. Multiple SSBs can lead to DSBs. Thus, DSBs are the focus as they can drive the cell towards genomic instability, apoptosis or tumorigenesis. Further quantitative evidence to define the extent of SSBs leading to DSBs and the relationship with repair is necessary.
- Ercc2+/- mice have a mutation in a gene involved in the nucleotide excision repair (NER) pathway, leading to DNA repair deficiency. However, when compared to wild type mice Ercc2+/- mice had fewer DNA strand breaks. This was true of both central and peripheral lens cells, as well as 4 and 24 h after irradiation (60Co γ-rays, 0.3, 0.063 Gy/min) (Barnard et al., 2021).
- DNA damage repair times can vary depending on the stressors that instigate the DNA damage. For example, it has been found that some types of radiation i.e., high linear energy transfer (LET) increases the amount of time required to repair DNA breaks (Aufderheide, 1987; Frankenburg-Schwager et al., 1994; Rydberg et al., 1994; Baumstark-Khan et al., 2003; Tsao, 2007; Blakely, 2012), however Stenerlöw et al. (2000) found that repair half-times were independent of LET.

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage suggests that DSB repair can be predicted from the presence of DSBs. The following tables provide representative examples of the relationship, unless otherwise indicated, all data is significantly significant. 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 the presence of γ-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).

Dose Concordance

Reference	Experiment Description	Result
Rydberg et al., 1994	In vitro. Human VA13 lung fibroblast and GM38A skin fibroblast cells were exposed to neon ions (425 MeV/u, 1 – 5 Gy/min, 80 Gy), iron ions (600 MeV/u, 1 – 5 Gy/min, 50 Gy), and X rays (425 MeV/u, 1 – 2 Gy/min, 80 Gy) to induce DNA strand breaks. Initial breaks after exposure were measured via the fraction of activity released (FAR) assay, with an increased FAR value indicating an increased number of breaks. Repair was measured using the FAR assay after a period of incubation.	Exposure to X-rays, neon, and iron ions led to a 90, 70, and 50% FAR increase relative to control respectively, indicating the highest level of breaks in samples exposed to X-rays. Four h later, 15, 20, and 73% of the DNA strand breaks had not been repaired.
Kuhne et al., 2000	In vitro. Human lung fibroblast cells were exposed to X-rays (23 Gy/min) at doses from 0 - 320 Gy. Following this, both correct (measured via hybridization assay), and total (measured via FAR assay) breaks remaining were measured. Therefore, allowing for calculation of the amount of misrepaired breaks.	Cells exposed to 0 - 320 Gy X-rays displayed an approximately linear increase in DSBs. This led to a gradual increase in the % DSBs misrejoined, which began to plateau after 80 Gy at a misrejoining frequency of 50%.
Baumstark-Khan et al., 2003	In vitro. Bovine LECs were exposed to X-rays (5 Gy/min, 0 to 50 Gy), 16O (3.4, 8.7 MeV/u, 230.5 to 642.9 Gy), 40Ar (2.7, 6.2, 10.5, 19.3 MeV/u, 0 to 190 Gy), 132Xe (5.4, 10.1, 16.5 MeV/u, 0 to 80 Gy), 208Pb (3.0, 6.8, 15.4 MeV/u, 0 to 50 Gy), 238U (1.5, 1.9, 2.6, 4.0 MeV/u, 0 to 150 Gy). This led to the induction of both SSBs and DSBs, whose repair was measured using a method similar to the hydroxyapatite chromatography of alkaline unwound DNA.	Irradiation below 10 000 keV/µm led to almost 100% rejoining of SSBs and DSBs. At LETs above 10 000 keV/µm the rejoining capacity varied depending on the original level of damage. After irradiation with 238U (LET ~ 20 000 keV/µm) rejoining capacity as t -> ∞ ranged from 50 to 100%. After irradiation with 208Pb (LET ~ 18 000 keV/µm) rejoining capacity as t -> ∞ ranged from 15 to 28%. 48Ti was an exception, with an LET of 1440 keV/µm that resulted in a rejoining capacity of only 65% rather than almost 100% as t -> ∞.
Aufderheide, 1987	In vitro. Bovine lens epithelial cells (LECs) were exposed to 238U (5, 10, 20 x 106 P/cm2), 132Xe (3, 5, 7, 12, 20 x 106 P/cm2), 84Kr (9, 21 x 106 P/cm2), 40Ar (24 x 106 P/cm2), 16O (80 x 106 P/cm2), and X-rays (20, 40, 200 Gy). The radiation exposure induced DNA breaks were measured using the DNA unwinding method described by Rydberg (1975). The DNA then underwent a period of repair incubation lasting between 5 to 40 h, after which any remaining DNA damage was measured using the same method as before.	Bovine LECs exposed to 21 x 106 P/cm2 84Kr displayed a 1.3x increase in DNA breaks and a 5% decrease in the level of breaks repaired compared to cells exposed to 9 x 106 P/cm2.
Stenerlöw et al., 2000	In vitro. Human skin fibroblast cells were exposed to 100 Gy of photons (60Co, < 0.5 keV/um), nitrogen ions (80, 125, 175, 225 keV/um), and helium ions (40 keV/um), resulting in the formation of DSBs. Their number was calculated by fragment analysis, based upon the fraction of DNA less than 5.7 Mbp, under the assumption that the breaks were evenly distributed. DNA repair was also measured via fragment analysis.	Exposure to increasing LET of radiation at 100 Gy led to increasing DSBs, in general, with about 600 DSBs/Gbp after γ-ray irradiation and about 700 DSBs/Gbp after 225 keV/um nitrogen ion irradiation. A dose of 100 Gy also led to decreased repair at increased LET. About 20-22 h after γ-ray irradiation, 4% of DSBs were unrepaired, while 20-22 h after 225 keV/um nitrogen ion irradiation, 12% of DSBs were unrepaired.

Incidence Concordance

No studies were found.

Time Concordance

Reference	Experiment Description	Result
Durante et al., 1998	In vitro. Human, male, lymphocyte cells were exposed to either iron ions (140 keV/µm, 2 Gy), or carbon ions (42 keV/µm, 5 Gy) to induce DNA strand breaks. Misrepair was measured by producing chromosome spreads and evaluating them using a microscope and the PAINT classification code.	Exposure to 2 Gy iron particles resulted in about 0.45 breaks/cell, of which 50% were repaired 10 h later. However, there were 0.1 translocations/cell, 0.08 incomplete exchanges/cell, 0.075 complex exchanges/cell, and 0.07 dicentrics/cell. Exposure to 5 Gy carbon ions resulted in 1.15 breaks/cell, of which 25% were repaired 10 h later. However, there were 0.35 translocations/cell, 0.28 incomplete exchanges/cell, 0.43 complex exchanges/cell, and 0.29 dicentrics/cell.
Rydberg et al., 1994	In vitro. Human VA13 lung fibroblast and GM38A skin fibroblast cells were exposed to neon ions (425 MeV/u, 1 – 5 Gy/min, 80 Gy), iron ions (250, 400, 600 MeV/u, 1 – 5 Gy/min, 50 Gy), and X rays (425 MeV/u, 1 – 2 Gy/min, 80 Gy) to induce DNA breaks. Their repair was measured using pulsed-field gel electrophoresis and determining the amount of DNA released from the gel plug	In GM38A cells, exposure to 80 Gy of all three radiation types led to DNA breaks. Repair was observed between 0.5 and 4 h after this. The most breaks remained after exposure to iron ions (75% of breaks remained), 25 – 42% remained after

	(fraction of activity released – FAR).	neon exposure, and only 15 – 20% remained after X ray irradiation.

Response-response relationship

There is evidence of a response-response relationship for DNA repair of radiation-induced DSBs. The frequency of DSBs has been shown to increase linearly with radiation dose (Löbrich et al., 2000; Rothkamm & 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

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

Known Feedforward/Feedback loops influencing this KER

Not identified.

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

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

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

Key Event Relationship Description

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

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

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

Evidence Supporting this KER

Overall Weight of Evidence: High

Biological Plausibility

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

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

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

Inadequate repair of DSB

Collective data from tumors and tumor cell lines has emerged that suggests that DNA repair mechanisms may be error-prone (reviewed in Sishc et al., 2017) (Sishc & Davis, 2017). NHEJ, the most common pathway used to repair DSBs, has been described as error-prone. The error-prone nature of NHEJ, however, is thought to be dependent on the structure of the DSB ends being repaired, and not necessarily dependent on the NHEJ mechanism itself (Bétermier et al., 2014). Usually when perfectly cohesive ends are formed as a result of a DSB event, ligase 4 (LIG4) will have limited end processing to perform, thereby keeping ligation errors to a minimum (Waters et al., 2014). When the ends are difficult to ligate, however, the resulting repair may not be completed properly; this often leads to point mutations and other chromosomal rearrangements. It has been shown that approximately 25 - 50% of DSBs are misrejoined after exposure to ionizing radiation (Löbrich et al., 1998; Kuhne et al., 2000; Lobrich et al., 2000). Defective repair mechanisms can increase sensitivity to agents that induce DSBs and lead eventually to genomic instability (reviewed in Sishc et al., (2017)).

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

Empirical Evidence

INSUFFICIENT REPAIR OF ALKYLATED DNA

Evidence in somatic cells

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

Various studies in cultured cells and microorganisms have shown that the expression of O^6-methylguanine DNA methyltransferase (AGT/MGMT) (repair machinery – i.e., decrease in DNA strand breaks) greatly reduces the incidence of mutations caused by exposure to methylating agents such as 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 hypothetical AOP. Thus, this sub-linear curve for mutation at low doses following sub-chronic ENU exposure suggests that DNA repair in spermatogonia is effective in preventing mutations until the process becomes overwhelmed at higher doses.

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

Inadequate repair of oxidative DNA lesions: In vitro studies

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

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

- Spontaneous mutation frequencies in the liver of Ogg1-deficient (-/-) Big Blue mice was measured at 10 weeks of age (Klungland et al., 1999).
  - Mutation frequencies were 2- to 3-fold higher in the *Ogg1*-/- mice than in wild type
  - Of the 16 base substitutions detected in *Ogg1* -/- mutant plaques analyzed by sequencing, 10 indicated G:C-A:T transversions consistent with the known spectrum of mutation
  - The results support that insufficient repair of oxidized bases leads to mutation.
- *Ogg1* knockout (*Ogg1*-/-) in C57BL/6J mice resulted in 4.2-fold and 12-fold increases in the amount of 8-oxo-dG in the liver compared to wild type at 9 and 14 weeks of age, respectively (Minowa et al., 2000).
  - In these mice, there was an average of 2.3-fold increase in mutation frequencies in the liver (measured between 16-20 weeks)
    - 57% of the observed base substitutions were G:C-A:T transversions, while 35% in wild type mice corresponded to this transversion.
    - Approximately 70% of the increase in mutation frequency was due to G to T transversions.
  - Concordantly, KBrO3 treatment resulted in a 2.9-fold increase in mutation frequency in the kidney of *Ogg1* -/- mice compared to KBrO3-treated wild type (Arai et al., 2002).
    - G:C-A:T transversions made up 50% of the base substitutions in the *Ogg1*-/- mice.
  - Heterozygous *Ogg1* mutants (*Ogg1*+/-) retained the original repair capacity, where no increase in 8-oxo-dG lesions was observed in the liver at 9 and 14 weeks (Minowa et al., 2000).
    - This observation was consistent even after KBrO3 treatment of the mice (Arai et al., 2002).
  - From these results, we can infer that OGG1 proteins are present in excess and that one functional copy of the gene is sufficient in addressing endogenous and, to a certain degree, chemical-induced oxidative DNA lesions.

Inadequate Repair of DSB

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

Dose and Incidence Concordance

There is evidence in the literature suggesting a dose/incidence concordance between inadequate DNA repair and increases in mutation frequencies. Evidence presented below related to the dose-response of mutation frequencies is summarized in table 2, here (click link). In response to increasing doses from a radiation stressor, dose-dependent increases in both measures of inadequate DNA repair and mutation frequency have been found. In an analysis that amalgamated results from several different studies conducted using in vitro cell-lines, the rate of DSB misrepair was revealed to increase in a dose-dependent fashion from 0 - 80 Gy, with the mutation rate also

similarly increasing from 0 - 6 Gy (Mcmahon et al., 2016). Additionally, using a plant model, it was shown that increasing radiation dose from 0 - 10 Gy resulted in increased DNA damage as a consequence of inadequate repair. Mutations were observed 2 - 3 weeks post-irradiation (Ptácek et al., 2001). Moreover, increases in mutation densities were found in specific genomic regions of cancer samples (namely promoter DNAse I-hypersensitive sites (DHS) and 100 bp upstream of transcription start sites (TSS)) that were also found to have decreased DNA repair rates attributable to inadequate nucleotide excision repair (NER) (Perera et al., 2016).

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

Temporal Concordance

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

Essentiality

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

Uncertainties and Inconsistencies

Repair of alkylated DNA

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

Repair of oxidative lesions

- Thresholded concentration-response curve of mutation frequency was observed in AHH-1 human lymphoblastoid cells after treatment with pro-oxidants (H_2O_2 and $KBrO_2$) known to cause oxidative DNA damage (Seager et al., 2012), suggesting that cells are able to tolerate low levels of DNA damage using basal repair. However, increase in 8-oxo-dG lesions and up-regulation of DNA repair proteins were not observed under the same experimental condition.
- Mutagenicity of oxidative DNA lesions other than 8-oxo-dG, such as FaPydG and thymidine glycol, has not been as extensively studied and there are mixed results regarding the mutagenic outcome of these lesions.

Repair of double strand breaks

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

Overall

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

Quantitative Understanding of the Linkage

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

For O-methyl adducts, once the primary repair process is saturated, in vitro data suggest that misreplication occurs almost every time a polymerase encounters a methylated guanine [Ellison et al. 1989; Singer et al. 1989]; however, it should be noted that this process can be modulated by 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 example 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 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: Inadequate DNA repair leads to Increase, Chromosomal aberrations

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	Low

Sex Applicability

Sex	Evidence
Unspecific	Low

This KER is plausible in all life stages, sexes, and organisms with chromosomes. The majority of the evidence is from in vitro fetal human male models. No in vivo evidence was found to support the relationship.

Key Event Relationship Description

Cells are exposed to many insults, both endogenous and exogenous, that may cause damage to their DNA. In response to this constant threat, cells have accordingly evolved many different pathways for repairing DNA damage (Pfeiffer & Goedecke, 2000; Hoeijmakers, 2001; Jeggo & Markus, 2015; Rode et al., 2016). When confronted with double strand breaks (DSBs), there are two common repair pathways employed by the cell: homologous recombination (HR) and non-homologous end-joining (NHEJ). In HR, a homologous sequence on the sister chromatid is used as a template, ensuring that no sequence information is lost over the course of repair (Ferguson & Alt, 2001; van Gent et al., 2001; Hoeijmakers, 2001; Jeggo & Markus, 2015; Schipler & Iliakis, 2013; Venkitaraman, 2002). However, this method of DNA repair may result in a loss of an allele leading to heterozygosity. This may occur if a non-homologous chromosome with an erronous sequence is used as the template instead of the homologous chromosome, thus leading to a loss of genetic information (Ferguson & Alt, 2001). Despite this possible error, HR is generally considered to be one of the more accurate methods of DNA repair because it does make use of a template (van Gent et al., 2001; Schipler & Iliakis, 2013; Venkitaraman, 2002) . NHEJ, however, does not use a template and is generally described as being error-prone. This repair process allows for the direct religation of broken DNA ends without using template DNA as a guide (van Gent et al., 2001; Ferguson & Alt, 2001; Hoeijmakers, 2001; Venkitaraman, 2002; Schipler & Iliakis, 2013; Jeggo & Markus, 2015; Rode et al., 2016). In lieu of a template, NHEJ utilizes rapid repair kinetics to religate the broken ends before they have time to diffuse away from each other (Schipler & Iliakis, 2013), thus fitting two ‘sticky’ DNA ends back together (Danford, 2012). There is not, however, an inherent quality control check; as such, sections of DNA may be gained or lost, or the wrong ends may be rejoined (Schipler & Iliakis, 2013). There are two versions of this error-prone DNA repair: classical or canonical NHEJ (c-NHEJ), and alternative NHEJ (alt-NHEJ) (Schipler & Iliakis, 2013). It is not well understood when or why one pathway is selected over another (Venkitaraman, 2002; Schipler & Iliakis, 2013). It has been proposed that the phase of the cell cycle may influence repair pathway choice (Ferguson & Alt, 2001; Vodicka et al., 2018); for instance, HR is generally more common than NHEJ when sister chromatids are available in S and G2 phases of the cell cycle (Hoeijmakers, 2001; Venkitaraman, 2002). If both HR and c-NHEJ are compromised, alt-NHEJ, which is slower and more error-prone than c-NHEJ, is thought to be the stand-by repair mechanism (Schipler & Iliakis, 2013).

If these repair processes are not able to properly and adequately repair the DNA, this may lead to the formation of chromosomal aberrations (CAs). CAs are defined as abnormalities in the chromosome structure, often due to losses or gains of chromosome sections or the entire chromosomes itself (van Gent et al., 2001; Durante & Cucinotta, 2008). These abnormalities can take many different forms and can be classified according to several different schemes. CAs can be defined as breaks, which occur when DSBs are not rejoined, or as exchanges, where the presence of multiple DSBs results in misrejoining of the DNA ends (Danford, 2012; Registre et al., 2016). CA classes can be further subdivided into chromosome-type aberrations (CSAs) that affect both sister chromatids, and chromatid-type aberrations (CTAs), affecting only one chromatid (Danford, 2012) . Examples of CSAs include chromosome-type breaks, centric ring chromosomes, and dicentric chromosomes (which have two centromeres), while CTAs refer to chromatid-type breaks and chromatid exchanges (Hagmar et al., 2004; Bonassi et al., 2008). Other types of CAs that may occur include micronuclei (MN; small nucleus-like structures containing chromosome fragments enclosed by a nuclear membrane

(Fenech & Natarajan, 2011; Doherty et al., 2016)), nucleoplasmic bridges (NPBs; a stretch of chromatin enclosed by a nuclear membrane that is attached to two centromeres (Fenech & Natarajan, 2011; Russo et al., 2015)), nuclear buds (NBUDs; a MN that is still connected to the nucleus by nucleoplasmic material (Fenech & Natarajan, 2011)), and copy number variants (CNVs; base pair to megabase pair deletions or duplications of chromosomal segments (Russo et al., 2015)). CAs may also be classified as stable aberrations (translocations, inversions, insertions and deletions) and unstable aberrations (dicentric chromosomes, acentric fragments, centric rings and MN) (Hunter & Muirhead, 2009; Qian et al., 2016).

Evidence Supporting this KER

Overall Weight of Evidence: Low

Biological Plausibility

There is strong biological plausibility for a relationship between inadequate repair of DNA damage and a corresponding increase in CAs. This is evident in a variety of reviews on the topic (van Gent et al., 2001; Hoeijmakers, 2001; Povirk, 2006; Weinstock et al., 2006; Lieber et al., 2010; Rode et al., 2016).

The two most common methods used to repair DSBs, which are one of the most dangerous types of DNA lesions, are HR and NHEJ. Mechanisms for these two methods of DNA repair are well-established and have been thoroughly reviewed (Van Gent et al. 2001; Hoeijmakers 2001; Lieber et al. 2010; Jeggo and Markus 2015; Sishc and Davis 2017). Briefly, HR requires a template DNA strand to repair damage and thus facilitates the invasion of the damaged strand with matching sequences on homologous chromosomes or sister chromatids (Ferguson and Alt 2001; van Gent et al. 2001; Hoeijmakers 2001; Jeggo and Markus 2015; Schipler and Iliakis 2013; Venkitaraman 2002). Proteins involved in the HR pathway include the RAD50 proteins, MRE11, BRCA1, and BRCA2 (Ferguson and Alt 2001; van Gent et al. 2001; Hoeijmakers 2001; Jeggo and Markus 2015; Venkitaraman 2002). In contrast to this relatively accurate form of DNA repair (van Gent et al. 2001; Schipler and Iliakis 2013; Venkitaraman 2002), NHEJ is more error-prone. It does not require a template to guide repair, but simply re-ligates broken DNA ends back together (Van Gent et al. 2001; Ferguson and Alt 2001; Hoeijmakers 2001; Lieber et al. 2010; Schipler and Iliakis 2013; Jeggo and Markus 2015; Rode et al. 2016; Sishc and Davis 2017) Proteins used during NHEJ include the DNA-PK complex (encompassing Ku70, Ku80 and DNA-PK_{cs}), and the XRCC4-DNA ligase IV complex (Ferguson & Alt, 2001; van Gent et al., 2001; Hoeijmakers, 2001; Jeggo & Markus, 2015; Sishc & Davis, 2017).Interestingly, NHEJ is used in the biological V(D)J recombination process because its error-prone mechanism allows immune cells to develop a wide range of unique receptors for antigen detection (Ferguson & Alt, 2001; van Gent et al., 2001; Lieber, 2010).

Damaged DNA in the form of DSBs can follow three possible outcomes: the DSB is rejoined accurately, with no changes made to the genome; the DSB is left unrepaired and the broken ends diffuse away from each other; or the DSB is repaired incorrectly such that the repaired version is different from the original version (Danford, 2012). These latter two errors in repair (the complete absence of repair or inaccurate repair) could arise due to interruptions to the repair process that allow time for the broken ends to move away from each other before they can be rejoined, mis-rejoining of the wrong DNA ends, or post-repair alterations that modify the junction point and lead to nucleotide losses (Schipler and Iliakis 2013). Unrepaired DSBs are the direct origin of micronuclei and unrepaired chromosomes correlated with radiosensitivity (Foray et al., 2016). Errors occurring during repair may be particularly detrimental if they interrupt or modify key genes, or if chromosome structures are created that cannot undergo proper mitosis (Schipler and Iliakis 2013).

The classic model of CA formation has centered around misrepair of DSBs. Exposing DNA to an endogenous or exogenous DSB-inducing agent directly results in DSBs, which may either persist or be misrepaired by inadequate repair mechanisms; in the event of this erroneous repair, CAs often eventually result (Bignold, 2009; Danford, 2012; Schipler & Iliakis, 2013) . Another model has been proposed that suggests CAs may actually be due to failure of enzymes that tether the DNA strands during the repair of enzyme-induced breaks in the DNA; the various pathways in the cell would likely employ assorted tethering enzymes. The numerous types of CAs would thus result from different kinds of tethering errors (Bignold 2009).

The type of CA that results may be dependent on the timing of inadequate repair. For example, DSBs may result in CSAs or CTAs depending on when during the cell cycle the DSB was incurred. DSBs that are not repaired before DNA duplication in the S-phase will be replicated and result in CTAs. If DSBs are incurred after the S-phase and are improperly repaired, CSAs will result (Danford, 2012; Registre et al., 2016; Vodicka et al., 2018). Similarly, CNVs are thought to be induced during the DNA replication phase. Although the mechanism is not well studied, it has been suggested that stress during replication, in particular stalling replication forks, prompt microhomology-mediated mechanisms to overcome the replication stall, which often results in duplications or deletions. Two models that have been proposed to explain this mechanism include the Fork Stalling and Template Switching (FoSTeS) model, and the Microhomology-Mediated Break-Induced Replication (MMBIR) model (Lee et al. 2007; Hastings et al. 2009; Arlt et al. 2012; Arlt et al. 2014; Wilson et al. 2015).

The type of CA may also be dependent on the type of erroneous repair that occurs. Deletions or chromosome breaks may occur when DSBs are left unrepaired (Danford 2012). Deletions may also occur when nucleotides are removed at the junctions (Schipler

and Iliakis 2013) or when the wrong DNA ends are religated (Venkitaraman 2002). Ligation of the incorrect ends of DNA DSBs may also lead to translocations or dicentrics (Ferguson & Alt, 2001; Lieber, 2010; Povirk, 2006; Venkitaraman, 2002). This type of error may occur when there are two or more DSBs in close proximity to each other that are misrejoined, thus resulting in the exchange of genetic material between two chromosomes (Ferguson and Alt 2001; Povirk 2006). NHEJ has been shown to play a significant role in the generation of chromosomal exchanges (Lieber 2010; Povirk 2006; Weinstock et al. 2006). Evidence for this comes from analysis of breakpoint junctions, which typically have little to no chromosomal homology when NHEJ repair is used (Povirk 2006; Weinstock et al. 2006); this was demonstrated in studies using translocation reporters (reviewed in Weinstock et al., 2006). There are, however, two types of NHEJ. c-NHEJ has been shown to suppress exchanges (Simsek and Jasin 2010) , which may be due to its relatively rapid repair kinetics (Schipler and Iliakis 2013). Chromosomal exchanges are thus suggested to originate more often from alt-NHEJ (Simsek and Jasin 2010; Zhang and Jasin 2011; Schipler and Iliakis 2013) .

NHEJ is also thought to mediate the formation of other types of CAs. Based on analysis of breakpoint junctions in lung adenocarcinoma samples where reciprocal inversions were found between genes *RET* and *KIF5B/CCDC6*, the majority of the inversions were thought to be induced by NHEJ (Mizukami et al. 2014). Chromothripsis, which refers to a single event that results in a massive number of CAs localized to a single or very few chromosomes (Russo et al. 2015; Leibowitz et al. 2015; Rode et al. 2016), may also be linked to NHEJ. The single catastrophic event sparking chromothripsis likely induces a large quantity of DSBs, essentially shattering the chromosome(s). These DSBs are then processed mainly by the error-prone NHEJ, which results in a large number of CAs, including chromosomal rearrangements, CNVs, and loss of heterozygosity (Leibowitz et al. 2015; Rode et al. 2016).

Fusing two broken chromosomes may lead to the formation of dicentric chromosomes, which are characterized by the presence of two centromeres. Dicentrics may also be formed by telomere-to-telomere end fusions (Fenech and Natarajan 2011; Rode et al. 2016). Telomeres, composed of TTAGGG repeats, are important structures that protect the ends of chromosomes and ensure accurate replication (Ferguson and Alt 2001; Hoeijmakers 2001; Vodicka et al. 2018); these nucleoprotein structures are shortened (Vodicka et al. 2018) by approximately 100 base pairs after each division, and are only replenished in cell types expressing the enzyme telomerase (Hoeijmakers 2001). If the telomeres become critically short, they can be mistaken for broken DNA ends by DNA repair machinery, and thus may be ‘repaired’ by fusing the ends of two chromosomes together (Ferguson and Alt 2001; Vodicka et al. 2018).

Dicentrics can also contribute to other types of CAs. During mitosis, the two centromeres of a dicentric chromosome may be pulled to opposite ends of the cell by mitotic spindle (Ferguson and Alt 2001; Fenech and Natarajan 2011; Leibowitz et al. 2015; Rode et al. 2016). Because the ends of the chromosomes are fused, this can lead to the formation of an anaphase chromatin bridge between the daughter cells (Russo et al. 2015; Leibowitz et al. 2015; Rode et al. 2016). If this bridge persists beyond anaphase, it may become enclosed in a nucleoplasmic membrane along with the nucleus, thus generating a NPB (Fenech and Natarajan 2011). Eventually, however, these bridges do break (Ferguson and Alt 2001; Fenech and Natarajan 2011; Russo et al. 2015; Leibowitz et al. 2015; Rode et al. 2016); the break is nearly always uneven, meaning that one daughter cell will be missing genetic material and one will have extra genetic material (Fenech and Natarajan 2011). These fragments, with their ‘sticky’ ends from the break, may further propagate the formation of CAs by being ligated inappropriately to another chromosome. Thus the cycle, known as the breakage-fusion-bridge (BFB) cycle, is propagated and further contributes to chromosomal instability (Ferguson and Alt 2001; Fenech and Natarajan 2011; Russo et al. 2015; Leibowitz et al. 2015; Rode et al. 2016) .

MN may also be formed during this BFB cycle. When the anaphase bridges break, the remaining chromosome fragments may be packaged by a nuclear membrane into its own mini nucleus, thus, forming an MN. MN may also enclose acentric chromosome fragments, chromatid fragments, or even entire chromosomes that were not properly segregated during mitosis (Fenech and Natarajan 2011; Doherty et al. 2016). Similar to MN in structure are NBUDs; the only difference between these two structures is that NBUDs are still attached to the nucleus by nucleoplasmic material. A NBUD is formed if there is amplified DNA that needs to be removed; this amplified material is often segregated from the other DNA at the periphery of the nuclear membrane and excluded from the nucleus by budding, resulting in a NBUD. Additionally, NBUDs may also result from NPB breakages (Fenech and Natarajan 2011).

Empirical Evidence

There is moderate empirical evidence supporting the relationship between inadequate DNA repair and the frequency of CAs. The evidence presented below is summarized in table 6, here (click link). Several reviews discuss evidence that associates these two events (Ferguson and Alt 2001; van Gent et al. 2001; Sishc and Davis 2017; Venkitaraman 2002). Overall, however, there is weak empirical evidence available supporting a dose and incidence concordance, little empirical evidence supporting a temporal concordance, and strong empirical evidence supporting essentiality for this KER.

Dose and Incidence Concordance

There is weak empirical evidence available that directly examines the dose and incidence concordance between DNA repair and CAs within the same study. There are, however, studies that use an ionizing radiation stressor to examine dose concordance of

either inadequate DNA repair in response to radiation exposure, or CA frequencies in response to irradiation. In an analysis that amalgamated results from several different studies conducted using *in vitro* experiments, the rate of DSB misrepair was revealed to increase in a dose-dependent fashion from 0 - 80 Gy (Mcmahon et al. 2016). Similarly, there was a clear correlation between radiation dose (i.e., increasing amounts of energy deposition) between 0 - 10 Gy and different clastogenic endpoints (Thomas et al. 2003; Tucker et al. 2005A; George et al. 2009; Arlt et al. 2014; Balajee et al. 2014; Lin et al. 2014; Suto et al. 2015; Mcmahon et al. 2016) . Overall, this suggests that exposure to radiation may increase both inadequate repair of DNA damage and the frequency of CAs in a dose-dependent fashion. More studies, however, are required to better assess the dose and incidence concordance of this KER.

Temporal Concordance

Temporal concordance between inadequate DNA repair and CA frequency is not well established. One study using cells pretreated with a DNA-PK inhibitor and irradiated with gamma rays found that DNA repair and MN were evident when they were assessed at 3 hours post-irradiation and 24 hours post-irradiation, respectively (Chernikova et al. 1999). This study does therefore suggest that there may be temporal concordance between these two events. Other radiation-based studies examining these two events separately, however, do not provide clear evidence of temporal concordance between DNA repair and CA frequency.

Essentiality

~~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 strengthening this relationship, addition of a DSB-inducing stressor to these DNA repair knock-out cells also significantly increases CA levels relative to wild-type cells receiving the same treatment (Cornforth and Bedford 1985; Simsek and Jasin 2010; Lin et al. 2014; Mcmahon et al. 2016). Essentiality is also supported by looking at patients with the recessive genetic disorder ataxia-telangiectasia (AT), in which mutations in the gene encoding the ATM protein results in defects in DNA damage repair signaling. One recent study showed that in comparison to control patients, patients with AT had increased levels of several types of CAs. Upon exposure to a DSB-inducing stressor such as ionizing radiation, these patients showed further increases in these aberrations as well as a significant increase in the levels of complex aberrations as compared to controls (Bucher et al. 2021).

Inhibitor studies have also found similar results. Two strains of wild-type cells that were treated with hydroxyurea, which is known to inhibit DNA repair, both had increased CAs relative to untreated wild-type cells (Wilhelm et al. 2014). Similarly, immortalized myeloid cell lines, cells from patients with myeloid leukemia, and cells from healthy donors were all found to have dose-dependent decreases in ligation efficiency after being treated with increasing doses of antibodies against various NHEJ proteins (Heterodimer et al. 2002). In addition, cells that were pretreated with DNA-PK inhibitor wortmannin prior to being irradiated were found to have not only increased levels of MN, but also decreased rates of DNA rejoining (Chernikova et al. 1999). A study by White et al. (2010) reported similar results under ATM and DNA-PK inhibition, where IR-exposed human lung cells treated for 1 hour with a reversible inhibitor of either enzyme exhibited an elevated level of CAs at all tested doses of IR, compared to the non-inhibited, IR-exposed cells 48 hours post-exposure. These findings demonstrated that even a transient inhibition of ATM or DNA-PK can sufficiently disrupt DNA damage repair and lead to CAs (White et al., 2010).

Functional defects in the factors involved in NER due to mutations or knock-down/out have shown concordant results that are supportive of this KER. For example, UV61 Chinese hamster ovary cells (homogolous to human Cockayne syndrome group B cells), which have a defective ERCC6 gene, are incapable of repairing UV-induced cyclobutane pyrimidine dimers due to the compromised transcription-coupled NER (TCR). Following UV exposure, a significantly higher percentage of TCR-defective UV61 cells contained CAs than another Chinese hamster ovary cell line that is TCR-proficient (Proiettis de Santis et al., 2001). Down-regulation of xeroderma pigmentosum group A–complementing protein (XPA) by RNA interference (RNAi) in human bladder cancer cells was observed to significantly increase the baseline frequency of MN, nucleoplasmic bridges, and nuclear buds, while overexpression of XPA by transfection in the same cell line reduced these levels below that in the control cells (Zhi et al., 2017). Both studies support the essentiality of inadequate repair in the occurence of chromosomal aberrations.

A rescue experiment provided further evidence of the essential role DNA repair plays in relation to CA frequencies. Inhibition of NHEJ through knocking out either Ku70 or Xrcc4 resulted in higher CA frequencies in the form of translocations; when Xrcc4 was transiently expressed in Xrcc4-/- cells, translocations were significantly decreased by 5-fold(Simsek and Jasin 2010) . This provides strong evidence that the NHEJ repair pathway plays an important role in the formation of CAs, specifically translocations.

Uncertainties and Inconsistencies

Uncertainties in this KER are as follows:

1. In an experiment using both wild-type and *Ku70*-/- cells, knock-down of alt-NHEJ protein CtIP resulted in significantly

decreased translocations in both cell types. When CtIP expression was rescued, translocation frequencies in these cells also returned to normal levels. This however, is opposite to results obtained in a similar study, where knock-out of Ku70 or Xrcc4 led to increased translocation frequency, and Xrcc4 rescue experiments resulted in decreased translocations (Simsek and Jasin 2010). It should be noted that alt-NHEJ is thought to be the major repair pathway responsible for generating translocations (Simsek and Jasin 2010; Zhang and Jasin 2011; Schipler and Iliakis 2013).

2. There is currently discussion regarding the accuracy of HR relative to NHEJ. Traditionally HR has been considered the more accurate type of DNA repair, while NHEJ is classically described as error-prone. There is emerging evidence, however, suggesting that HR may in fact be a mutagenic process. Evidence supporting HR as an error-prone repair pathway has been reviewed (Guirouilh-barbat et al. 2014).

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage is lacking. Most data are derived from studies that examined DSB misrepair rates or CA rates in response to a radiation stressor. In terms of inadequate DNA repair, the rate of DSB misrepair was found to be approximately 10 - 15% at 10 Gy of radiation (Lobrich et al. 2000); this rate increased to 50 - 60% at a radiation exposure of 80 Gy (Kuhne et al. 2000; Lobrich et al. 2000; Mcmahon et al. 2016). It is not known, however, how this rate of inadequate repair directly relates to CA frequency. Overall, more studies are required that directly assess this relationship.

Response-response relationship

Studies directly examining the response-response relationship between inadequate repair and CA frequency are lacking. One study examined both DNA repair and CA frequency in cells exposed to DNA-PK inhibitor wortmannin. There was a negative, approximately linear relationship between DNA repair and increasing wortmannin dose, and a positive, approximately linear relationship between MN frequency and increasing wortmannin dose; this suggests that as adequate DNA repair declines, CA frequency increases (Chernikova et al. 1999). More studies are required, however, that directly quantify the response-response relationship between inadequate DNA repair and CAs.

Time-scale

The time scale between inadequate DNA repair and the increased frequency of CAs has not been well-established. Most data come from studies that assess only one of these events in relation to a radiation stressor rather than assessing the timing of the events relative to each other. More studies are thus required that directly assess this relationship.

Known modulating factors

DNA repair is a modulating factor in this KER. The progression from “Inadequate DNA repair” to “Increase, Chromosomal aberrations” only occurs when "Increase, DNA strand breaks" (KE 1635) precedes "Inadequate DNA repair", which indicates that DNA strand breaks could not be repaired.

Known Feedforward/Feedback loops influencing this KER

Not identified.

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Relationship: 1978: Increase, Mutations leads to Increase, Cell Proliferation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deposition of energy leading to lung cancer	adjacent	High	Low
Deposition of energy leading to occurrence of cataracts	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The domain of applicability pertains to all multicellular organisms, as cell proliferation and death regulate tissue homeostasis (Pucci et al. 2000).

Key Event Relationship Description

Mutations are defined as changes in the DNA sequence, which could occur in the form of deletions, insertions, missense mutations, nonsense mutations or frameshift mutations (Bertram, 2001; Danesi et al., 2003; Lodish, 2000). Elevated mutation frequencies may impact cellular activities by activating or inhibiting essential processes that control the natural course of cell proliferation (Bertram, 2001; Vogelstein and Kinzler, 2004; Lodish, 2000). Increased rates of cellular proliferation may arise due to mutations that activate proto-oncogenes, which results in sustained signaling for cell growth (Bertram, 2001; Vogelstein and Kinzler, 2004; Larsen and Minna, 2011; Lodish, 2000) and due to mutations that inactivate tumour suppressor genes (TSGs), resulting in the removal of cell cycle inhibition and/or decreased cell death signaling (Bertram, 2001; Vogelstein and Kinzler, 2004; Lodish, 2000). Mutations altering gene expression or protein activity can enable cells to escape growth inhibition by increasing resistance to apoptosis, or other inhibitory signals, or by escape of cell cycle checkpoints. Alternatively, mutations can stimulate growth by activating proliferative pathways such as EGFR.

Evidence Supporting this KER

Biological Plausibility

There is a strong biological plausibility for a relationship between increasing mutation frequencies and increasing cellular proliferation. This relationship is especially evident when examining the molecular biology of carcinogenesis. It is well-known that exposure of cells to a DNA-damaging agent, such as ionizing radiation, may result in damage to the DNA that manifests as genomic instability, including mutations. If enough mutations accumulate in critical genes, cells may begin to proliferate uncontrollably. This, alongside other events, may eventually result in tumourigenesis and cancer (reviewed in Bertram, 2001; Vogelstein and Kinzler, 2004; Panov, 2005; Lodish, 2000). In fact, one of the hallmarks of cancer is sustained proliferative signalling, and one of the enabling characteristics of this increased proliferation is genomic instability/mutations (Hanahan and Weinberg, 2011).

For a mutation to occur, damaged DNA must be passed on to the next generation (Bertram, 2001). To prevent the propagation of erroneous DNA, there are specific cell cycle checkpoints that must be passed before DNA replication and mitosis can proceed. One of the most important checkpoints for committing to cell proliferation occurs during late G1 (Bertram, 2001; Lodish, 2000). This checkpoint is managed by retinoblastoma protein (RB), transcription factor E2F, and transcription factor p53. In a resting cell, RB is tightly bound to E2F; when growth factor signals are present, proteins are activated that phosphorylate RB, resulting in a conformation change and the release of E2F. This transcription factor then initiates transcription of genes required for DNA synthesis and thus cell proliferation. If there is damage to the DNA, p53 is upregulated and binds to unphosphorylated RB, thereby preventing the dissociation of RB and E2F (Bertram, 2001). This gives the cell enough time to repair the damaged DNA prior to DNA replication, and thus minimizes the propagation of the DNA errors. Existing mutations in the checkpoint genes, however, may compromise this process. For example, if mutations in p53 render it non-functional, damaged DNA will not be stopped at the checkpoint and will continue to be synthesized, despite the damage. Accumulation of mutations in this manner may affect genes that impact cell proliferation rates (Bertram, 2001; Lodish, 2000). There are three categories of genes that, if mutated, may allow for uncontrolled cell proliferation: proto-oncogenes, TSGs, and caretaker/stability genes.

Proto-oncogenes are defined as genes that, when activated, promote cellular proliferation (Bertram, 2001; Lodish, 2000); they have been likened to the gas pedal of the car (Vogelstein and Kinzler, 2004). These genes are particularly dangerous if they are rendered abnormally active by gain-of-function (GOF) mutations; this may result in cellular proliferation being aberrantly activated (Bertram, 2001; Vogelstein and Kinzler,, 2004; Larsen and Minna 2011; Lodish, 2000). Two common examples of mutated proto-oncogenes that contribute to increased cell proliferation rates are *EGFR* and *KRAS*. The *EGFR* gene encodes the epidermal growth factor receptor (EGFR), a trans-membrane protein with tyrosine kinase activity. Binding of growth factors to EGFRs results in receptor dimerization, autophosphorylation, and propagation of pro-proliferative signals to the nucleus (Danesi et al., 2003; Santos et al., 2010; Larsen and Minna, 2011; NIH, 2018 EGFR). *KRAS* is responsible for making the KRAS protein, which is a G-protein with GTPase activity that is used in the RAS/MAPK signalling pathway. When a signal that promotes cellular growth is detected, KRAS binds to GTP and activates downstream signalling molecules, thus facilitating signal propagation to the nucleus (Adjei, 2001; Panov, 2005; Jancik et al., 2010; NIH, 2018 KRAS). Mutations that render these receptors constitutively active would thus result in increased rates of cellular proliferation (Sanders and Albitar, 2010).

TSGs, which are analogous to the brakes in a car (Vogelstein and Kinzler, 2004; Lodish, 2000), are genes that negatively regulate cellular growth by preventing proliferation and in some cases, promoting apoptosis (Bertram, 2001; Vogelstein and Kinzler, 2004; Panov, 2005; Sanders and Albitar, 2010; Lodish, 2000). Many of the cell cycle checkpoint proteins and proteins controlling cell death are TSGs (Bertram, 2001; Lodish, 2000). Loss-of function (LOF) mutations that result in the inactivation of these TSGs may thus promote cellular proliferation (Bertram, 2001; Vogelstein and Kinzler, 2004; Lodish, 2000). A common example of a mutated TSG is *TP53*, which encodes the p53 protein. As mentioned above, p53 is a cell checkpoint protein that delays replication when damaged DNA is present; if damage is severe enough, p53 may also

activate an apoptotic pathway (Bertram, 2001; Danesi et al., 2003; Panov, 2005; Larsen and Minna, 2011; Lodish, 2000, NIH 2018c). Inactivating mutations in p53 thus allow for unhindered progression through the cell cycle, resulting in higher cell proliferation rates (Danesi et al., 2003; Fernandez-Antoran et al., 2019).

Finally, caretaker/stability genes encode for proteins involved in the detection, repair and prevention of DNA damage (Vogelstein and Kinzler 2004; Hanahan and Weinberg 2011). Genes involved in mismatch repair (MMR), nucleotide excision repair (NER) and base-excision repair (BER) pathways are examples of caretaker/stability genes (Vogelstein and Kinzler, 2004). Mutations in these genes may compromise aspects of DNA repair—the detection of damage, the initiation of repair, the repair process itself, or the removal of mutagens that could possibly damage DNA—thus allowing for more mutations to accumulate in the genome than usual (Hanahan and Weinberg, 2011). Although all genes may suffer from increased mutation rates when caretaker/stability genes are improperly functioning, mutations in TSGs and proto-oncogenes are the main contributors to increased cellular proliferation (Vogelstein and Kinzler, 2004). Caretaker/stability genes are similar to TSGs in that disruption of both alleles must occur for the gene function to be compromised (Vogelstein and Kinzler, 2004; Hanahan and Weinberg, 2011).

Empirical Evidence

There is moderate empirical evidence supporting the relationship between mutations and the cellular proliferation. The evidence presented below is summarized in table 7, here (click link). There are some available reviews that provide evidence for this relationship in the context of carcinogenesis (Welcker 2008, Kim 2018, Iwakuma 2007, Muller 2011), as one of the hallmarks of this disease is high levels of cellular proliferation (Hanahan and Weinberg 2011). Another review article explores the relationship between mutation accumulation and cellular proliferation through discussion of the stem cell division theory of cancer, and how it compares to the somatic mutation theory of cancer (López-lázaro 2018). Overall, however, there is little empirical evidence available supporting dose and incidence concordance, little empirical evidence supporting temporal concordance, and strong empirical evidence supporting essentiality for this KER. Some evidence from human epidemiology association and genetic studies also provides support for this KER.

Dose and Incidence Concordance

There are few studies available that assess the dose and incidence concordance between mutations and cell proliferation. One study providing dose information on this particular relationship analyzed the effect of sequentially adding mutations to mouse lung epithelial cells. Addition of mutations in the form of LT (suppression of p53 and pRB) or Kras(G12V) (an activated oncogene) on their own to lung epithelial cells did not increase tumour volume, but a combination of these genetic manipulations resulted in increasing tumour volume (suggestive of increased cell proliferation) over 40 days. The same results for LT and EGFR(ex19del) genetic manipulations were also achieved. This suggests that addition of multiple mutations increases cell proliferation (Sato et al. 2017). More studies, however, are required to directly assess this particular aspect of the relationship between mutations and cellular proliferation.

Time Concordance

Few studies are available that study the time concordance between mutations and cell proliferation. The timing between these two events is explored in a review that discusses theories of carcinogenesis. The somatic mutation theory of cancer states that accumulation of mutations results in higher rates of cellular proliferation, which eventually leads to cancer. A component of the stem cell division theory of cancer also states that an increased mutation burden may elevate rates of stem cell divisions in late carcinogenesis; however, a high frequency of stem cell division in the initial stages of cancer development is thought to be a key factor that contributes to mutation accumulation (López-lázaro 2018). More research is thus required to definitively determine whether mutations occur prior to increased rates of cellular proliferation.

Essentiality

There is strong evidence for the essentiality component of this KER. Numerous studies indicate that cellular proliferation is increased in biological systems with genetically manipulated TSGs and/or proto-oncogenes. It is important to note that uncontrolled cellular proliferation is a hallmark of human cancers (Hanahan and Weinberg 2011); the Catalogue of Somatic Mutations in Cancer (COSMIC) includes over 136,000 coding mutations in over 500,000 tumour samples (83 major cancer genes and 49 fusion gene pairs) and this number is continually increasing (Forbes et al. 2011). The managers of COSMIC note that key amongst all of these genes is *TP53*. Several review articles that focussed on genetic manipulations of *TP53* demonstrated that mutant or knocked-out p53 increased carcinogenesis across a variety of biological systems (Iwakuma and Lozano 2007; Muller et al. 2011; Kim and Lozano 2018). Furthermore, a number of studies that measured cellular proliferation directly found that both cells and mice lacking p53 had increased rates of cell proliferation (Hundley et al. 1997; Lang et al. 2004; Ventura et al. 2007; Duan et al. 2008; Li and Xiong 2017), in addition to modifications to the cell cycle such that more cells were found in the S- and G2/M phases and less in the G1 phase (Hundley et al. 1997). Some p53 mutations, including 515A, may also result in increased cellular proliferation (Lang et al. 2004). Further underlining the importance of p53 in controlling cellular proliferation, restoration of p53 in a p53-/- mouse model resulted in a significant size reduction in 7 out of 10 tumours, with some tumours disappearing altogether (Ventura et al. 2007).

Manipulations to other genes have also been shown to affect cellular proliferation. A review article centred on the tumour suppressor FBW7, which is a ubiquitin ligase that plays a role in degrading proto-oncogene products and thus controlling cellular proliferation, demonstrated that mutations to *FBW7* may contribute to carcinogenesis (Welcker and Clurman 2008). Knock-out of prostate SPOP (an E3 ubiquitin ligase adaptor commonly mutated in primary prostate adenocarcinoma) in $Spop^{fl/fl}$*;PBCre(+)* mice resulted in prostates with significantly higher masses, significantly more cellular proliferation, and increased expression of c-MYC protein relative to prostates from $Spop^{fl/fl}$*;PBCre(-)* controls with normal prostate SPOP expression. Furthermore, there was a strong inverse correlation between c-MYC activity and SPOP mRNA levels in two independent prostate cancer patient cohorts, suggesting that c-MYC upregulation in the absence of SPOP may be responsible for the increased cellular proliferation (Geng et al. 2017). Similarly, mouse embryonic fibroblasts lacking Cul9, a scaffold protein for assembly of E3 ubiquitin ligases, had an increased cellular proliferation rate and an increased number of cells in the S-phase of the cell cycle relative to wild-type controls. Cul9 mutant cells also showed similar cellular proliferation rates to Cul9-/- cells. In contrast, Arf-/- cells, p53-/- cells, and Cul9-/-p53-/- double knock-out cells had significantly higher cellular proliferation rates relative to the Cul9-/- and Cul9 mutant cells; all of these mutant cells, however, showed increased proliferation relative to wild-type cells (Li and Xiong 2017).

Inhibitor studies further highlight the role of mutations in increasing cellular proliferation. Mouse lung epithelial cells transformed with both Large T-antigen (LT; suppresses TSGs p53 and pRB) and activated oncogene Kras(G12V) or EGFR(ex19del) resulted in increased tumour volumes, which is suggestive of cell proliferation. Increasing concentrations of MEK inhibitor, which blocks the signalling pathway downstream of both Kras and EGFR, caused declines in cell number in the two transformed cell lines and in the parental lung epithelial cells. An EGFR inhibitor, which blocks signalling downstream of EGFR but upstream of Kras, had no effect on the transformed cells with activated Kras, but caused rapid declines in cell proliferation of transformed cells with activated EGFR. Altogether, these inhibitor studies suggest that the activated oncogene has an important role in promoting high rates of cell proliferation (Sato et al. 2017).

Human epidemiology association and genetic studies

Association studies in humans clearly show the correlation between mutations in specific genes and the proliferative status of human tumours. Human lung adenocarcinoma tumours were assessed for mutational status of *KRAS, TP53* and *STK11,* and cellular proliferation levels were measured in the mutant tumours relative to the wild-type tumours. Overall, mutations in *TP53* were associated with significantly increased proliferation levels regardless of the mutational status of *KRAS.* In contrast, mutations in *STK11*, either alone or in combination with *KRAS* mutations, were not associated with increased proliferation (Schabath et al. 2016). Assessment of breast cancer tumours demonstrated that those with low BRCA1 expression displayed increased cellular proliferation relative to those with high BRCA1 expression, as measured by nuclear Ki-67 levels (Jarvis et al. 1998).

Uncertainties and Inconsistencies

Uncertainties in this KER are as follows:

1. The location of the mutation will be critical in determining the downstream effects. This can also be modulated by an individual's susceptibility (Loewe and Hill 2010).
2. Although activating mutations in oncogenes such as RAS and MYC may induce abnormally high rates of cellular proliferation, extremely high levels of these proteins may actually lead to the opposite—cells may enter into a state of senescence and cease proliferation (Hanahan and Weinberg 2011).\
3. Cellular proliferation may be impacted by circadian cycles, such that disruptions to this natural circadian rhythm may also affect the cell cycle (Shostak 2017).

Quantitative Understanding of the Linkage

Data establishing a quantitative understanding between mutation frequency and cellular proliferation was not identified. More research is required to establish the quantitative relationship between these two events.

Response-response relationship

Data establishing a response-response relationship between mutation frequency and cellular proliferation was not identified. More research is required to establish the response-response relationship between these two events.

Time-scale

Although the time scale is not well-established for this KER, there are a few studies that have examined how cellular proliferation changes overtime in the presence of mutations. In Cul9-/- mouse embryonic fibroblasts, a higher proliferation rate relative to Cul9+/+ cells was evident by 3 days in culture (Li and Xiong 2017). A similar relationship was observed in mouse embryonic fibroblasts with p53 manipulations. Increased proliferation in p53-/-, p53 515A/+ and p53 515A/515A relative to p53+/- and p53+/+ cells was present by the fourth day in culture (Lang et al. 2004). Examination of population doublings in various cell lines found that Cul9-/- and Cul9 mutant cells had higher population doublings than wild-type cells by approximately passage 7; Arf-/-, p53-/-, and Cul9-/-p53-/- cells, however, displayed even higher rates of population doublings by passage 6 (Li and Xiong 2017). Additionally, tumour growth in mice inoculated with lung epithelial cells engineered with LT (suppresses p53 and pRB) and an activated oncogene (either EGFR or KRAS) was monitored over 40 days post-injection. Relative to mice inoculated with either LT-lung epithelial cells or activated oncogene-lung epithelial cells, mice inoculated cells containing both mutations had detectable tumours by approximately day 10 - 12 post-injection; the volumes of these tumours continued increasing until the end of the experiment (Sato et al. 2017).

There were also differences in the rate of DNA synthesis over time, which could possibly indicate higher rates of cell division. In all cell types examined (p53-/-, p53+/- and p53+/+, p53 515A/+, and p53 515A/515A), DNA synthesis declined over the first 6 days in culture, though the mutant p53 lines always had higher synthesis rates than p53-/-, p53+/- and p53+/+ cells. During culture days 6 - 10, DNA synthesis in the mutant p53 lines drastically increased, while the other p53 lines remained at the same relatively low level of synthesis (Lang et al. 2004).

Known Feedforward/Feedback loops influencing this KER

Proliferation increases the likelihood that existing DNA damage will result in mutation and creates new mutations through errors in replication.

It is generally accepted that proliferation increases the risk of mutation and cancer (Preston-Martin, Pike et al. 1990). DNA damage that has not been completely or correctly repaired when a cell undergoes mitosis can be fixed in the genome permanently as a mutation, to be propagated to future daughter cells. Incomplete DNA repair can also cause additional DNA damage when encountered by replicative forks. Therefore, in the presence of any DNA damage (and there is a background rate of damage in addition to any other genotoxic stimuli) mutations will increase with cell division (Kiraly, Gong et al. 2015). Mutation-prone double strand breaks can also arise from replicative stress in hyperplastic cells including hyperplasia arising from excess growth factor stimulation (Gorgoulis, Vassiliou et al. 2005). This relationship between proliferation and mutation is thought to drive a significant portion of the risk of cancer from estrogen exposure since breast cells proliferate in response to estrogen or estrogen plus progesterone and risk increases with cumulative estrogen exposure (Preston-Martin, Pike et al. 1990).

Not all proliferating tissue shows replicative stress and DSBs - tissue with a naturally high proliferative index like colon cells don't show any sign of damage (Halazonetis, Gorgoulis et al. 2008). Additional factors are therefore required beyond replication for damage and mutation from replicative stress, but replication is essential for the expression of these factors.

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NIHa, EGFR gene epidermal growth factor receptor Normal. 2018. Genetics Home Reference EGFR gene.

NIHb, KRAS proto-oncogene, GTPase Normal. 2018. Genetics Home Reference KRAS gene.

NIHc, TP53 gene tumor protein p53 Normal. 2018. Genetics Home Reference TP53 gene.

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Relationship: 1979: Increase, Chromosomal aberrations leads to Increase, Cell Proliferation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deposition of energy leading to lung cancer	adjacent	Moderate	Low
Deposition of energy leading to occurrence of cataracts	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The domain of applicability pertains to all multicellular organisms, as cell proliferation and death regulate tissue homeostasis (Pucci et al., 2000).

Key Event Relationship Description

CAs are defined as abnormalities in the chromosome structure, often due to losses or gains of chromosome sections or the entire chromosomes itself, or chromosomal rearrangements (van Gent et al., 2001). These aberrant structures can come in a multitude of different forms. Types of CAs include: inversions, insertions, deletions, translocations, dicentric chromosomes (chromosomes that contain two centromeres, often resulting from telomere end fusions (Fenech & Natarajan 2011; Rode et al., 2016), centric ring chromosomes, acentric chromosome fragments, micronuclei (MN; small nucleus-like structures containing entire chromosomes or chromosome fragments (Fenech & Natarajan, 2011; Doherty et al., 2016), nucleoplasmic bridges (NBPs; a corridor of nucleoplasmic material containing chromatin that is attached to both daughter cell nuclei), nuclear buds (NBUDs; small MN-type structures that are still connected to the main nucleus (Fenech & Natarajan, 2011), and copy number variants (CNVs; deletions or duplications of chromosome segments (Russo et al., 2015).

If these CAs affect genes involved in controlling the cell cycle, this may result in increased cellular proliferation. There are three types of genes that, if modified, may result in high rates of proliferation: proto-oncogenes, tumour suppressor genes (TSGs), and caretaker/stability genes (Vogelstein & Kinzler, 2004; Hanahan & Weinberg, 2011). Furthermore, gene fusions that result from CAs have also been implicated in augmenting cellular proliferation (Sanders & Albitar, 2010; Ghazavi et al., 2015; Kang et al., 2016).

Evidence Supporting this KER

Biological Plausibility

There is a strong biological plausibility for a relationship between CAs and rates of cellular proliferation. This is particularly emphasized in the context of carcinogenesis, as high cellular proliferation is a known hallmark of cancer, and an enabling characteristic of increased proliferation is genomic instability (Hanahan & Weinberg, 2011).Topical reviews are available documenting the contribution of CAs to cellular proliferation and/or cancer development (Mes-Masson & Witte, 1987; Bertram, 2001; Vogelstein & Kinzler, 2004; Ghazavi et al. ,2015; Kang et al., 2016). The link between chromosomal instability (CIN), which describes the rate of chromosome gains and losses, and cancer development has also been well documented (Thompson et al., 2017; Gronroos, 2018; Targa & Rancati, 2018; Lepage et al., 2019).

Many CAs are thought to be formed through two main mechanisms: inadequate repair of DNA damage, and errors in mitosis. If there is damage to the DNA that the cell is unable to properly repair, the unrepaired lesion may translate into a CAs (Bignold, 2009; Danford, 2012; Schipler & Iliakis, 2013); the type of resulting CA is often influenced by the cell cycle stage when the damage occurred (Danford, 2012; Registre et al., 2016; Vodicka et al., 2018), and the type of erroneous repair (Ferguson & Alt, 2001; Povirk, 2006; Bignold, 2009; Danford, 2012; Schipler & Iliakis, 2013). Errors made during repair may be particularly detrimental if they interrupt or modify critical genes, or if chromosome structures are created that cannot undergo mitosis (Schipler & Iliakis, 2013). Similarly, errors in mitosis that prevent chromosomes from being properly segregated may also lead to CAs. These errors could be due to by improper timing of centrosome separation, the presence of extra centrosomes, inappropriate mitotic spindle assembly and attachment to kinetochores (found on the centromeres), and incorrect sister-chromatid cohesion (Levine & Holland, 2018).

The presence of CAs in cells may be particularly detrimental if they alter the rate of cellular proliferation by affecting genes that control the cell cycle, namely proto-oncogenes, TSGs (Bertram, 2001; Vogelstein & Kinzler, 2004) or caretaker/stability genes (Vogelstein & Kinzler, 2004). Proto-oncogenes are genes that, when activated, promote cellular proliferation. CAs that increase activation of these genes may aberrantly boost cell cycling and therefore increase proliferation (Bertram, 2001; Vogelstein & Kinzler, 2004). Activation of proto-oncogenes have also been implicated in the cancer stem cell theory of carcinogenesis (Vicente-duen et al., 2013). Examples or proto-oncogenes include *EGFR* and *KRAS* (Sanders & Albitar, 2010). TSGs refer to genes that actively suppress cell proliferation and, in some cases, promote apoptosis (Bertram, 2001; Vogelstein & Kinzler, 2004; Sanders & Albitar, 2010). If these genes are silenced by CAs, this may remove cell cycle checkpoints, thus allowing for unhindered cellular proliferation and decreased apoptosis (Bertram, 2001; Vogelstein & Kinzler, 2004). Common TSGs are *TP53* and *RB* (Hanahan & Weinberg, 2011). Lastly, caretaker/stability genes are those involved in the prevention and detection of DNA damage, and the instigation and completion of the required DNA repair (Vogelstein & Kinzler, 2004; Hanahan & Weinberg, 2011). If the function of these caretaker/stability genes is affected by CAs, this may result in genome-wide inadequate DNA repair, which in turn may result in genetic damage to TSGs or proto-oncogenes (Vogelstein & Kinzler, 2004). Genes involved in mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) are all examples of caretaker/stability genes (Vogelstein & Kinzler, 2004).

There are also other CAs commonly associated with cancer. In prostate cancer, truncated TSGs such as *TP53, PTEN, BRCA1*, and *BRCA2* are a result of chromosomal rearrangements (Mao et al., 2011). Similarly, chromosomal inversions were found to be responsible for just over half of the *RET* gene fusions associated with lung adenocarcinoma samples (Mizukami et al., 2014).

Empirical Evidence

There is moderate empirical evidence supporting the relationship between CAs and the cellular proliferation. The evidence presented below is summarized in table 8, here (click link). There are some available reviews that provide evidence for this relationship in the context of carcinogenesis, as high levels of cellular proliferation is one of the hallmarks of cancer (Hanahan & Weinberg, 2011). Many of these reviews focus especially on the structure and function of specific cancer-associated CAs (Mes-Masson & Witte, 1987; Ghazavi et al., 2015; Kang et al., 2016). Another interesting review discusses transgenic mouse models that have contributed to our understanding of how oncogenes and TSGs promote carcinogenesis in a variety of tissues (Fowlis & Balmain, 1992). Overall, however, there is a lack of empirical evidence available supporting dose and incidence concordance, little empirical evidence supporting temporal concordance, but strong empirical evidence supporting essentiality for this KER.

Dose and Incidence Concordance

Not identified.

Temporal Concordance

There were no studies identified that directly assessed the temporal concordance between CA and increasing rates of cellular proliferation. In a study examining MN frequency and cell proliferation in estrogen-responsive cancer cells treated with estradiol, both MN levels and proliferation rates were higher in estradiol-treated cells relative to controls at 140 and 216 hours post-treatment (Stopper et al., 2003). This suggests that both events are increased at the same time points in response to the estradiol. More work is required, however, to directly assess the temporal concordance between CA frequency and cell proliferation rates.

Essentiality

Much of the evidence for essentiality stems from studies of gene fusions produced by chromosomal translocations and the corresponding impact on cellular proliferation rates. One such gene fusion, *JAFZ1-JJAZ1*, has been identified in endometrial stromal sarcomas. The role of this relatively unknown translocation was evaluated using knock-down and knock-in experiments. When wild-type *JJAZ1* was disabled by siRNA, HEK 293 cells expressing the *JAFZ1-JJAZ1* fusion were found to have an increased rate of cellular proliferation (Li et al., 2007). Similarly, the role of the *EML4-ALK* fusion gene was examined in IL-3 dependent BA/F3 cells. These cells were transfected with a plasmid carrying only *CD8*, or *CD8* in combination with *ALK, EML4-ALK*, or mutant *EML4-ALK* (which contained a lysine to methionine mutation in the kinase domain). In all cases, cell proliferation was found to increase linearly over 7 days in the presence of IL-3; in the absence of IL-3, all cells died by day 3 of culture, with the exception, however, of cells carrying *EML4-ALK*. Only cells with *EML4-ALK* were able to maintain a positive, linear growth in both the presence and absence of IL-3. Addition of a JAK2 inhibitor to these *EML4-ALK* cells resulted in a dose-dependent decline in cellular proliferation, such that at a dose of 10 um of inhibitor, cells numbers declined steadily until death at day 5. This is in contrast to the *CD8*-expressing cells exposed to the same inhibitor doses, in which there was only a very slight decline in cellular proliferation rates (Soda et al. 2007). Both of these studies provide evidence that translocations increase proliferation rates in cells.

In addition to causing gene fusions, translocations may also lead to the production of circular RNA fusion products (f-CircRNA), which can be studied to further understand the link between CAs and cellular proliferation. For example, f-CircPR has been associated with the *PML-RARα* translocation, f-CircM9 has been associated with the *MLL-AF9* translocation, and expressions of f-CircPR or f-CircM9 were both found to increase cell proliferation rates in mouse embryonic fibroblasts. Inhibition of these f-CircRNAs, either through addition of silencing shRNA or by using a mutant non-circularizing f-CircRNA, resulted in decreased rates of cell proliferation (Guarnerio et al., 2016). These results again indicate that there is a relationship between CAs and increased cellular proliferation.

Other experiments provide evidence that CAs can increase cellular proliferation using cancer cells. Using two human Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph^+ALL) cell lines (both of which contain the *BCR-ABL* translocation), cellular proliferation was studied by cell counting and by analyzing levels of phosphorylated ErbB2. ErbB2 is a member of the ERB receptor tyrosine kinase family that is involved in pro-proliferative signalling, and it is known to be expressed in cells from ALL patients. Cell proliferation rates were found to decline in a dose-dependent fashion when treated with either an ErbB family tyrosine kinase inhibitor, or a more specific ErbB1/ErbB2 tyrosine kinase inhibitor. Furthermore, treatment with the ErbB family inhibitor also resulted in significant decreases in phosphorylated ErbB2 (Irwin et al., 2013). In another set of experiments using estrogen receptor-positive human ovarian cancer cells, treatment of cells with estrogen were found to have significantly increased levels of MN and significantly increased proliferation rates relative to vehicle-treated control cells; furthermore, there were more cells in S-phase and fewer in the G2/M phases of the cell cycle relative to controls. These results were specific to estrogen-response cells, as treatment of estrogen receptor-negative human ovarian cancer cells with estrogen did not result in any changes to MN or cell proliferation. Furthermore, addition of an estrogen antagonist to estrogen-responsive cells maintained MN frequencies and cell proliferation at control levels (Stopper et al., 2003).

Human Epidemiology Association and Genetic Studies

Very often, CAs result in gene fusions. A gene fusion occurs when two genes not normally in close proximity to each other are juxtaposed; this may result in altered expression of one or both genes, or an altered gene product (Mitelman, 2005). There are several well-known gene fusions implicated in carcinogenesis that are associated with increased cellular proliferation. One well-characterized gene fusion is the Philadelphia chromosome, also known as the *BCR-ABL1* fusion. This gene fusion is formed by a translocation between chromosome 9 and 22, and is commonly found in chronic myelogenous leukemia (CML) (Mes-Masson and Witte 1987; Kang et al. 2016), as well as acute lymphoblastic leukemia (ALL) (Ghazavi et al., 2015). The protein created from *BCR-ABL1* has elevated tyrosine kinase activity, and was shown to increase activation of cellular proliferation pathways (Ghazavi et al., 2015; Kang et al., 2016) including JAK2/STAT, PI3K-AKT, and MAPK/ERK (Kang et al., 2016). Another common gene fusion partner is *ALK*, which is a receptor tyrosine kinase involved in the PI3K-AKT signalling pathway. Very often, *ALK* gene fusions result in upregulated *ALK* expression, and a resulting increase in pro-proliferative signalling in the PI3K-AKT pathway. In non-small cell lung cancer, the *ALK-EML4* gene fusion is particularly common (Sanders & Albitar, 2010). Similarly, *ETV6-RUNX1* is the most common fusion gene in B-cell precursor acute lymphoblastic leukemia (BCP-ALL), and is thought to initiate leukemogenesis (Ghazavi et al., 2015).

Uncertainties and Inconsistencies

Uncertainties in this KER are as follows:

1. A study using peripheral blood lymphocytes isolated from head and neck cancer patients found significantly increased CAs (including chromosome-type aberrations, chromatid-type aberrations, dicentric chromosomes, aneuploidy, MN, NPBs and NBUDs) relative to healthy controls. In the lymphocytes from these same cancer patients, however, the cell proliferation rates were significantly decreased (George et al., 2014).
2. Characterization of 20 different ameloblastomas, which are benign tumours associated with the jaw, found low CAs frequencies and low rates of cellular proliferation (Jääskeläinen et al., 2002).

Quantitative Understanding of the Linkage

Quantitative understanding has not been well-established for this KER. There were no studies identified that documented a response-response relationship between CA frequency and cell proliferation rates, and a severe lack of time scale-oriented studies. Overall, more research is required to establish a quantitative understanding of this KER.

Response-response relationship

Not established.

Time-scale

Studies that directly assessed the time scale between CAs and cellular proliferation were not identified. However, differences in cellular proliferation rates for cells with different CA-related manipulations or treatments were evident within the first 3 days of culture (Stopper et al., 2003; Li et al., 2007; Soda et al., 2007; Irwin et al., 2013; Guarnerio et al., 2016). More studies are required, however, to formulate a detailed time scale relating these two events.

Known modulating factors

Not established.

Known Feedforward/Feedback loops influencing this KER

Not established.

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Relationship: 1980: Increase, Cell Proliferation leads to Increase, lung cancer

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deposition of energy leading to lung cancer	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The domain of applicability for this KER is mammals.

Key Event Relationship Description

Cell proliferation is a process that occurs in normal healthy cells, allowing for tissue growth and repair. It is controlled by the cell cycle, which contains specific and highly controlled checkpoints that must be passed before the cell can undergo DNA synthesis and mitosis (Pucci et al., 2000; Bertram, 2001; Eymin & Gazzeri, 2009). In cases where there are cells that contain severely damaged DNA or that are unneeded, regulatory mechanisms may arrest pro-proliferative signals and instead direct the cell cycle towards apoptosis (programmed cell death) (Portt et al., 2011). Proliferation may also be halted if the protective telomeres capping the ends of chromosomes become too short to support DNA replication; this causes cells to either enter into a state of replicative senescence (Bertram, 2001; Panov, 2005; Hanahan & Weinberg, 2011) or to undergo apoptosis (Hanahan & Weinberg, 2011). The cell cycle thus plays an important role in balancing cell proliferation with cell death to maintain homeostasis (Pucci et al., 2000; Bertram, 2001; Panov, 2005; Portt et al., 2011).

Dysregulation of the cell cycle may lead to abnormally high rates of cellular proliferation. This may occur through upregulation of pro-proliferative signalling, downregulation of anti-proliferative signaling (including alterations to proteins controlling cell cycle checkpoints), increasing resistance to pro-apoptotic signalling, and evasion of replicative senescence (Bertram, 2001; Panov, 2005; Hanahan & Weinberg, 2011). As these pro-proliferative events accumulate and cellular proliferation rates increase, cells may become increasingly tumourigenic. High rates of cellular proliferation may thus lead to the development of cancer; if these processes occur in the lung specifically, the end result may be lung cancer (Panov, 2005; Eymin & Gazzeri, 2009; Sanders & Albitar, 2010; Larsen & Minna, 2011).

Evidence Supporting this KER

Biological Plausibility

There is a strong biological plausibility for the relationship between cell proliferation and lung cancer. This is heavily supported by the multitude of research examining the general mechanistic control of cell proliferation, and the ways in which dysregulation of cell proliferation promotes the transformation of normal cells to carcinogenic ones (Pucci et al. 2000; Bertram 2001; Panov 2005; Eymin and Gazzeri 2009; Hanahan and Weinberg 2011; Larsen and Minna 2011). In this section, an overview cell proliferation processes will be provided, followed by a discussion of how these control mechanisms are modified to increase cell proliferation rates in carcinogenesis.

Cell proliferation rates are controlled by the cell cycle. The cell cycle consists of five phases: G0, G1, S, G2, and M. G0 is described as the quiescent stage, where cells are inactive in terms of cellular proliferation. The cell exits G0 and enters G1, when growth signals are initiated. G1 is known as a gap phase, where the cell begins to prepare for DNA synthesis. In the S-phase, DNA is replicated and identical sister chromatids are formed in preparation for cell division. Another gap phase, known as G2, follows DNA synthesis; during G2, cell organelles are duplicated as the cell prepares to divide. Mitosis occurs during the M-phase, which culminates in cytokinesis and the production of two genetically-identical daughter cells (Pucci et al. 2000; Eymin and Gazzeri 2009).

Progression through the cell cycle is highly regulated and very tightly controlled, as there is a very specific and time-sensitive order of events that must occur to ensure proper cell division (Pucci et al. 2000; Bertram 2001; Eymin and Gazzeri 2009; Hanahan and Weinberg 2011). As such, there are several key check-points that must be passed before the cell can proceed into the next phase of the cell cycle. One of the most important checkpoints is between G1 and S, known as the restriction point; it is the ‘point of no return’ in terms of DNA synthesis. This check point is controlled by RB (Pucci et al. 2000; Bertram 2001; Eymin and Gazzeri 2009), a protein that decides whether the cell cycle progresses by integrating intra- and extra-cellular signals (Hanahan and Weinberg 2011). In its unphosphorylated state, RB binds tightly to the transcription factor E2F and thus prevents transcription of genes required for DNA synthesis. When growth signals are received by the cell, this activates the transcription of cyclin-D and cyclin-dependent kinase (CDK) 4 and CDK6. Binding of cyclin-D with CDK4 or CDK6 allows activation of the kinase function, which results in the phosphorylation of RB. Phosphorylated RB releases E2F, allowing for the transcription of genes required not only for DNA synthesis, but also for maintaining the phosphorylated state of RB throughout the DNA synthesis process (Pucci et al. 2000; Bertram 2001; Panov 2005; Eymin and Gazzeri 2009).

The protein product of *TP53*, p53, also plays an important role in controlling the cell cycle. This tumour suppressor protein is responsible for DNA quality control and for monitoring stresses within the cell. If DNA damage is detected (Bertram 2001; Panov 2005; Hanahan and Weinberg 2011; Larsen and Minna 2011) or if cellular supplies (such as nucleotides, oxygen or glucose) are inadequate (Bertram 2001; Hanahan and Weinberg 2011), p53 is upregulated. Even in the presence of growth signals, p53 inhibits RB phosphorylation and prevents activation of E2F (Bertram 2001), thereby halting the cell cycle. This cell cycle arrest provides the DNA repair machinery time to repair the damaged DNA before the process of cell division is resumed. If the damage is too severe, p53 can trigger cell death through the process of apoptosis (Bertram 2001; Hanahan and Weinberg 2011; Larsen and Minna 2011).

Apoptosis is a non-inflammatory process of programmed cell death that is used to remove heavily damaged, defective, or unneeded cells. This process is homeostatically balanced with cell proliferation, thus allowing the organism to adapt to and change with its environment as required (Pucci et al. 2000; Bertram 2001; Panov 2005; Portt et al. 2011). A higher proportion of pro-apoptotic compared to anti-apoptotic factors will trigger a cell to undergo apoptosis (Hanahan and Weinberg 2011; Portt et al. 2011). This programmed cell death can be initiated by an intrinsic pathway mediated by cytochrome C release from the mitochondria, or by an extrinsic pathway mediated by death receptors on the plasma membrane. After initiation of apoptosis, a sequential cascade of caspase activations eventually leads to the characteristic hallmarks of apoptosis, including DNA and nuclear fragmentation, and break-down of cellular components (Panov 2005; Hanahan and Weinberg 2011; Portt et al. 2011). Key regulators of apoptosis include p53 and Bcl-2, while the main executors are the caspases (Panov 2005; Hanahan and Weinberg 2011).

In addition to cell cycle checkpoints and apoptosis, cell proliferation is also limited by telomere length. Telomeres are six-nucleotide repeats found on the ends of chromosomes that protect coding DNA from damage (Bertram 2001; Ferguson and Alt 2001; Panov 2005; Vodicka et al. 2018). After each round of replication, however, telomeres become progressively shorter due to the unidirectionality (5’-3’) of the replication machinery (Bertram 2001; Panov 2005). Eventually, the telomeres become too short to support cellular proliferation (Bertram 2001; Ferguson and Alt 2001; Hanahan and Weinberg 2011; Vodicka et al. 2018). In this case, DNA repair machinery may fuse the short telomeres (mistaken for damaged DNA) to form dicentric chromosomes (Ferguson and Alt 2001; Vodicka et al. 2018). The short telomeres may also trigger the cell to enter into a state of replicative senescence in which cell division is no longer supported (Bertram 2001; Hanahan and Weinberg 2011), or to undergo apoptosis (Hanahan and Weinberg 2011). In contrast, germ cells and stem cells are able to infinitely divide; this is due to their expression of the enzyme telomerase, which maintains telomere length (Bertram 2001). Most somatic cells, however, do not express telomerase and are thus limited in their replicative potential (Bertram 2001; Panov 2005; Hanahan and Weinberg 2011).

All of these processes play a role in controlling the rate of cellular proliferation in cells. In general, cellular proliferation is balanced with cell death to maintain homeostasis within an organism. If any of the above processes become aberrantly regulated such that cells begin to proliferate at excessively high rates, this may result in cancer. High rates of proliferation are considered one of the most dominant characteristics of cancer cells (Bertram 2001; Eymin and Gazzeri 2009; Hanahan and Weinberg 2011). In fact, several of the identified hallmarks of cancer are processes that relate to increases in proliferation. These hallmarks, as stated by Hanahan 2011, include: sustained proliferative signalling, evading growth suppressors, resisting cell death, and enabling replicative immortality (Hanahan and Weinberg 2011).

Sustained proliferative signalling allows cancer cells to carry out pro-proliferative activities even in the absence of external growth signals (Eymin and Gazzeri 2009; Hanahan and Weinberg 2011). This may be achieved by abnormally activated proto-oncogenes which stimulate cell proliferation and thus are able to increase the level of

pro-proliferative signalling within the cell (Bertram 2001; Vogelstein and Kinzler 2004; Hanahan and Weinberg 2011; Larsen and Minna 2011). The mechanisms by which proto-oncogenes enhance proliferative signaling include: increased expression of growth factor receptors on the cell surface, increased production of ligands for growth factor receptors, constitutive activation of downstream pro-proliferative signalling molecules (Bertram 2001; Hanahan and Weinberg 2011), or structurally modified growth factor receptors that activate downstream pathways even in the absence of ligand binding (Hanahan and Weinberg 2011). In lung cancer specifically, several commonly activated proto-oncogenes include *EGFR, ERBB2, MYC, KRAS, MET, CCND1, CDK4* and *BCL2* (Larsen and Minna 2011).

As cells transition from normal to tumourigenic, cellular proliferation can be further enhanced by evading growth suppressors and resisting cell death (Eymin and Gazzeri 2009; Hanahan and Weinberg 2011). This is often achieved by genetic alterations that inactivate tumour suppressor genes (TSGs). TSGs encode proteins, often involved in cell cycle checkpoints, which limit cell proliferation and promote apoptosis (Harris 1996; Bertram 2001; Vogelstein and Kinzler 2004). Two of the most common TSGs inactivated in cancer include *RB1* (Vogelstein and Kinzler 2004; Hanahan and Weinberg 2011) and *TP53* (Harris 1996; Vogelstein and Kinzler 2004; Hanahan and Weinberg 2011). Inactivation of *RB1* (and therefore decreased levels of RB) allows for uncontrolled proliferation by removing the restriction checkpoint in the cell cycle, thus allowing cells to easily pass from G1 to S (Bertram 2001; Hanahan and Weinberg 2011; Larsen and Minna 2011). In a similar fashion, inactivation of *TP53* (and therefore decreased p53) removes DNA quality control, meaning that cells with damaged DNA are able to continue with cell proliferation unhindered (Bertram 2001; Panov 2005; Hanahan and Weinberg 2011; Larsen and Minna 2011). Loss of the pro-apoptotic p53 as well as downregulation of other pro-apoptotic factors, coupled with the upregulation of anti-apoptotic factors such as Bcl-2, further promotes cell proliferation by increasing the cell's resistance to apoptotic pathways (Hanahan and Weinberg 2011; Portt et al. 2011). In terms of lung cancer, TSGs that are commonly inactivated include not only *TP53* and *RB1,* but also *STK11, CDKN2A, FHIT, RASSF1A,* and *PTEN* (Larsen and Minna 2011).

Lastly, cancer cells often accumulate genetic abnormalities that allow them to overcome replicative senescence. These immortalized cancer cells are thus capable of dividing an infinite number of times. Immortalization is most often achieved in tumour cells through activation of telomerase. Expression of telomerase allows telomeres to be regenerated upon DNA replication, which prevents cells from undergoing replicative senescence or apoptosis from critically shortened telomeres (Bertram 2001; Panov 2005; Hanahan and Weinberg 2011; Larsen and Minna 2011). In lung cancer specifically, telomerase has been found to be activated in nearly all small cell lung cancer (SCLC) cases, and in over three-quarters of non-small cell lung cancer (NSCLC) cases (Panov 2005; Larsen and Minna 2011).

Empirical Evidence

There is moderate empirical evidence supporting the relationship between increased cellular proliferation and lung cancer. The evidence presented below is summarized in table 9, here (click link). There are several lung cancer-specific reviews available that discuss the various molecular mechanisms by which abnormal cell proliferation occurs in cells, and how this leads to carcinogenesis of the lungs (Panov 2005; Eymin and Gazzeri 2009; Sanders and Albitar 2010; Larsen and Minna 2011). Furthermore, one of the hallmarks of cancer is high levels of cellular proliferation (Hanahan and Weinberg 2011), thus aberrant cell proliferation and lung tumourigenesis will inevitably be linked. Overall, however, there is a weak empirical evidence available supporting dose, incidence and temporal concordance, and strong empirical evidence supporting essentiality for this KER.

Dose and Incidence Concordance

There are not limited studies available that assess the dose/incidence concordance between cell proliferation and lung carcinogenesis. In a few experiments, rodent lungs exposed to various carcinogens showed increased levels of proliferation and developed squamous metaplasia (Zhong et al. 2005) or full-blown tumours (Kassie et al. 2008). Furthermore, nude mice injected with carcinogenic human NSCLC cells also developed tumours within a few weeks of the injection (Pal et al. 2013; Warin et al. 2014; Sun et al. 2016; Tu et al. 2018). More studies, however, are required to further explore the dose/incidence concordance between these two events.

Temporal Concordance

Studies examining temporal concordance between increased cellular proliferation rates and lung carcinogenesis are also lacking. Multiple tumour xenograft experiments found that nude mice injected with NSCLC cells develop detectable tumours within two weeks of inoculation, which continued to increase in size over time (Pal et al. 2013; Warin et al. 2014; Sun et al. 2016; Tu et al. 2018). This tumour growth necessarily suggests a high rate of cell proliferation. Accordingly, examination of lung squamous metaplasia after 14 weeks of exposure to high levels of tobacco smoke showed increased cell proliferation markers in comparison to lungs from rats exposed to filtered air (Zhong et al. 2005). Similarly, lung tumours from mice that received carcinogens NNK and BaP orally over 4 weeks were also found to express proliferation markers when examined 27 weeks after the start of the experiment (Kassie et al. 2008). Although these studies do suggest that increased rates of proliferation occur prior to and during tumour development, more research is required to more firmly establish temporal concordance between these two events.

Essentiality

Much of the evidence for essentiality is derived from studies where anti-tumourigenic compounds were applied to *in vitro* and *in vivo* NSCLC models. Application of suspected anti-cancer compound cleistanthoside A tetraacetate (CAT) to lung cancer cells resulted in changes to the cell cycle such that there were fewer cells involved in proliferative cell cycle phases; there were also corresponding declines in levels of the G1/S checkpoint proteins cyclin-D1, CDK4 and CDK6 (Wanitchakool et al. 2012). Likewise, treatment of two NSCLC cell lines with histone demethylase inhibitor pargyline resulted in significant decreases in cell proliferation rates (Lv et al. 2012). In a similar fashion, treatment of EGFR- and VEGFR2-over expressing NSCLC cells with EGFR/VEGFR2 inhibitor delphinidin resulted in significant decreases in cell proliferation markers *in vitro. In vivo* delphinidin treatment of xenograft nude mice inoculated with these NSCLC cells accordingly led to decreased cell proliferation and dose-dependent decreases in tumour volume (Pal et al. 2013). Corresponding *in vitro* and *in vivo* results were found in NSCLC models treated with taurine, an amino acid thought to be protective against tumourigenesis. Not only were *in vitro* cell proliferation rates decreased in taurine-treated NSCLC cells, but anti-apoptotic Bcl-2 levels were decreased and pro-apoptotic PUMA and Bax levels were increased. When xenograft nude mice inoculated with tumour-promoting NSCLC cells were treated with either taurine, exogenous PUMA, or a combination of taurine and PUMA, there were significant *in vivo* declines in cell proliferation, tumour volume and tumour weight; the largest declines, however, were found in mice treated with both taurine and exogenous PUMA (Tu et al. 2018). In another experiment involving NSCLC xenograft nude mice, treatment of mice with 6-shogaol (6S; a component of dry ginger) or its metabolite cysteine-conjugated 6S (M2) resulted in decreases in cell proliferation, tumour volumes and tumour weights (Warin et al. 2014). Other experiments were performed using healthy mice that ingested carcinogens NNK and BaP over 4 weeks, and were then treated orally with suggested tumour suppressor indole-3-carbinol (I3C). Regardless of whether I3C treatment started halfway through the carcinogenic treatment period (10 - 112 µmol/g diet) or after completion of the 4 week carcinogenic paradigm (112 µmol/g diet), there were significant decreases in cell proliferation and in the number of tumours per mouse (Kassie et al. 2008).

Other evidence for the association between cell proliferation and carcinogenesis comes from studies involving genetic manipulations. NSCLC cells transfected with a vector

to silence abnormally expressed histone demethylase LSD1 resulted in decreased cell proliferation *in vitro*. In contrast, transfection of these cells with a vector to overexpress LSD1 led to increased *in vitro* proliferation rates (Lv et al. 2012). NSCLC cells and tumours have also been shown to have increased levels of ZIC5, which belongs to a family of transcription factors thought to play a role in regulation of the cell cycle during periods of high proliferation. Knock-down of ZIC5 by transfecting NSCLC cells with ZIC5-silencing RNA resulted in decreased cell proliferation and decreased clone formation *in vitro*. In xenograft nude mice inoculated with NSCLC cells carrying the ZIC5-silencing RNA, there were also *in vivo* declines in tumour growth and in tumour cell proliferation relative to mice inoculated with non-manipulated NSCLC cells (Sun et al. 2016).

Uncertainties and Inconsistencies

Uncertainties in this KER are as follows:

1. Inconsistencies in results were observed in studies using radiation as a stressor.The dose threshold for the onset of proliferation and lung cancer induction varies with radiation quality, individual cell sensitivity, and confounding factors (Taylor 2013). The latter two are also be true for chemical carcinogens (Malhotra et al., 2016).

Quantitative Understanding of the Linkage

Quantitative understanding has not been well-established for this KER. In terms of human non-carcinogenic cells, 50 - 70 cell divisions are thought to be possible before telomeres become too short to support further cell division (Panov 2005); this cell division number would presumably increase in carcinogenic cells. There were no studies, however, that documented a response-response relationship between cell proliferation rates and lung carcinogenesis, and a severe lack of time scale-oriented studies. Overall, more research is required to establish a quantitative understanding of this KER.

Response-response relationship

Not identified.

Time-scale

Studies that directly assessed the time scale between increased cellular proliferation and lung carcinogenesis are lacking. There are some studies, however, that provide details regarding the timing between these two events. *In vitro* experiments using lung cancer cell lines demonstrated that expression levels of key proteins involved in the regulation of the cell cycle and/or proliferation were modified by chemical inhibitors within the first 48 hours of treatment. Delphinidin caused changes in the expression levels of EGFR, pEGFR, VEGFR2 and pVEGFR2 within the first 3 hours (Pal et al. 2013), and pargyline decreased LSD1 levels within 6 hours of treatment (Lv et al. 2012). Delphinidin-induced changes to the expression of PI3K/p110, PI3K/p85, pAKT, pERK1/2, pJNK1/2, pp38, PCNA and cyclin-D1 were documented within 48 hours of treatment (Pal et al. 2013). Similarly, CAT application led to significant declines in cell cycle checkpoint proteins cyclin-D1, CDK4 and CDK6 by 36 hours post-treatment (Wanitchakool et al. 2012). Additionally, changes to the cell cycle were evident within 24 - 48 hours of CAT treatment (Wanitchakool et al. 2012), and within 48 hours of ZIC5 knockdown with silencing RNA (Sun et al. 2016). ZIC5 knockdown also caused declines in cell proliferation by 96 hours post-transfection, and declines in clone formation after 2 weeks (Sun et al. 2016). Overall, these *in vitro* studies demonstrate that modifications to both cell cycle regulation and cell proliferation rates in cancer cells can be affected within hours to days of a perturbance.

In vivo studies also provide information regarding the timescale between cell proliferation and tumourigenesis. Tumours in xenograft nude mice were detected within two weeks of NSCLC-cell inoculation (Pal et al. 2013; Warin et al. 2014; Sun et al. 2016; Tu et al. 2018), with one study showing tumour detection as early as 1 week post-inoculation (Warin et al. 2014).Tumours continued to grow over the experimental period until time of harvest (Pal et al. 2013; Warin et al. 2014; Sun et al. 2016; Tu et al. 2018). Differences in tumour growth rates between treated and untreated mice were evident within 13 -16 days of delphinidin treatment (Pal et al. 2013), 3 weeks of ZIC5 knock-down (Sun et al. 2016), and by 27 days of either taurine, PUMA or taurine and PUMA treatment (Tu et al. 2018). At the time of xenograft nude mouse tumour harvest (which varied between 22 days and 27 weeks), there were significant differences in markers of cell proliferation and tumour size or number in mice exposed to anti-cancer compounds and their respective controls (Kassie et al. 2008; Pal et al. 2013; Warin et al. 2014; Sun et al. 2016; Tu et al. 2018). In non-xenograft mice exposed to a high levels of tobacco smoke, increased markers of cell proliferation and the incidence of airway squamous metaplasia was evident upon sacrifice after 14 weeks of constant tobacco smoke exposure (Zhong et al. 2005).

Known modulating factors

Ingestible materials, such as wine and vitamin E, may be capable of modulating cell proliferation and thus tumourigenesis. Treatment of NSCLC cells with wine at low doses was found to inhibit proliferation of the cells, suggesting that wine may have an anti-tumourigenic effect (Barron et al. 2014). Vitamin E exposure has also been associated with anti-tumourigenesis by inducing apoptosis in proliferating endothelial cells and thus decreasing angiogenesis. This is significant, as angiogenesis is required to support tumour development (Dong et al. 2007).

Known Feedforward/Feedback loops influencing this KER

Usually, non-cancerous cells are stimulated by growth factors originating from other cell types. For cancer cell lines, cell proliferation rates can be increased by autocrine signalling. Some cancer cells acquire the ability to produce both the growth factors and the required receptors, thus allowing the cell to respond to its own growth signals, and further stimulate more cell proliferation (Hanahan and Weinberg 2011).

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List of Non Adjacent Key Event Relationships

Relationship: 1981: Energy Deposition leads to Increase, Mutations

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deposition of energy leading to lung cancer	non-adjacent	High	High
Deposition of energy leading to occurrence of cataracts	non-adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence

Unspecific **Sex** High **Evidence**

The domain of applicability applies to single-celled organisms such as bacteria and yeast, eukaryotic cells, and multi-cellular organisms such as fish, mice and humans.

Key Event Relationship Description

Energy can be deposited on biomolecules from various forms of radiation. Radiation with high linear energy transfer (LET) tends to produce more complex, dense structural damage than low LET radiation; both, however, can lead to detrimental damage within a cell (Hada & Georgakilas, 2008; Okayasu, 2012; Lorat et al., 2015; Nikitaki et al., 2016). The DNA is particularly susceptible to damage which can be in the form of mutations. Mutations may occur in germ cells or somatic cells; mutations in germ stem and progenitor cells are often of the greatest concern, as they may persist and be propagated to offspring. Regardless of the cell type, there are several different categories of mutations including: missense, nonsense, insertion, deletion, duplication, and frame-shift mutations. These mutations can present with different downstream effects which are not predictable but can potentially initiate a path to carcinogenesis.

Evidence Supporting this KER

Biological Plausibility

The biological rationale for linking direct deposition of energy by ionizing radiation to mutation induction is strong. The structural and functional relationships in this KER contribute sufficiently to the overall biological plausibility.

There are numerous studies that demonstrate, using various model systems, an increase in mutation frequency in response to radiation exposure (Russell et al., 1957; Winegar et al., 1994; Gossen et al., 1995; Suzuki & Hei 1996; Albertini et al., 1997; Dubrova et al., 1998; Kraemer et al., 2000; Dubrova, Plumb, et al., 2000; Canova et al., 2002; Dubrova et al., 2002; Dubrova & Plumb, 2002; Masumura et al., 2002; Somers et al., 2004; Burr et al., 2007; Ali et al., 2012; Bolsunovsky et al., 2016; Mcmahon et al., 2016; Matuo et al., 2018; Nagashima et al., 2018; Wu et al., 1999; Hei et al., 1997; Nagasawa and Little, 1999; Barnhart and Cox, 1979; Thacker at al., 1982; Zhu et al., 1982; Metting et al., 1992; Schwartz et al., 1991; Chen et al., 1984; Albertini et al., 1997). The process of mutation induction by radiation is initiated when cells are exposed to ionizing radiation. These high-energy waves or particles interact with the genetic material in the nucleus, damaging the DNA and triggering a cascade of signalling events and activities aimed at repairing the damage. This process, however, may result in not only the repair of the DNA, but also the formation of mutations (Sankaranarayanan & Nikjoo, 2015). Of note, radiation is not likely to impact only one gene; more often than not, the random nature of energy deposition by radiation results in mutations to many genes and genomic sites clustered in the same area (Sankaranarayanan & Nikjoo, 2015; Adewoye et al., 2015). Many of the radiation-induced mutations have been documented as deletions (Gossen et al., 1995; Behjati et al., 2016), often of differing sizes in a number of different genes (Sankaranarayanan & Nikjoo, 2015). The mechanism for radiation-induced mutations is thought to be similar to the process for spontaneously-occurring mutations, as the structure of radiation-induced mutations examined at expanded simple tandem repeat (ESTR) loci was not found to differ from the structure of spontaneous mutations (Dubrova, 2005). Moreover, exposure to radiation may produce specific mutational signatures. Two ionizing radiation-specific mutational signatures were found when 12 radiation-induced secondary tumours across 4 different tumour types underwent whole-genome sequencing and bioinformatics processing. In particular, these radiation-exposed tumours were significantly enriched in small deletions and balanced inversions. These results were validated when the same mutational signatures were observed in radiation-exposed but not radiation-naïve prostate tumours from a previously-published dataset (Behjati et al., 2016). Similarly, another study examining mutations present in radiation-induced tumours of *Nf1* heterozygous and wild-type mice revealed three distinctive mutational signatures. Interestingly, these signatures were found in all of the tumours regardless of its histology or of the animal's genotype. Moreover, these signatures were still present after removal of the 33 most mutated samples from the analysis, after analysis of only the non-synonymous substitutions, and after analysis of only the synonymous substitutions (though the third mutational signature could not be extracted in this last analysis group) (Sherborne et al. 2015). There were also common cellular pathways that were found to be frequently mutated in the tumours of these mice. In sarcomas from mice of both genetic backgrounds (*Nf1* heterozygous and wild-type), the top two pathways harbouring mutations were those influencing cellular assembly and organization, and those involved in cellular function and maintenance. Additionally, Ras pathways were commonly mutated in tumours from both genetic backgrounds. Specific to wild-type sarcomas, mutations were also found in cell cycle and cell signalling pathways (Sherborne et al., 2015). Supporting the finding that different genetic backgrounds in mice do not affect mutational signatures in tumours (Sherborne et al., 2015), there also does not appear to be strain-specific differences in ESTR mutational frequencies in response to radiation. One study examined five different strains of male mice that were irradiated and mated to unirradiated females at least 4 weeks post-irradiation. Although there was a difference in doubling doses between strains, the ESTR mutations themselves were not significantly different. Furthermore, there were no significant differences found between strains in terms of germline mutation induction (Dubrova, 2005).

Germline mutations have been further interrogated in studies examining the effects of radiation exposure on germ cells. There is evidence from mouse studies suggesting that the germ cells of radiation-exposed males have elevated ESTR mutations and that the offspring of these irradiated males inherit more ESTR mutations as a result of the germline mutations (Dubrova et al., 1998; Dubrova, Bersimbaev, et al., 2000; Dubrova & Plumb, 2002; Somers et al., 2004; Barber et al., 2009; Ali et al., 2012; T.E. Wilson et al., 2015). This was reviewed by Somers et al. (2006). Interestingly, *in utero* irradiation of embryos at day 12 resulted in increased ESTR mutations across several tissue types in males and females; however, only the offspring of the irradiated males showed an elevated ESTR mutation rate (Barber et al., 2009). On a genome-wide scale, the offspring of irradiated males were found to have significantly more clustered single nucleotide variants (SNVs) and insertion/deletion events compared to offspring from unirradiated fathers (Adewoye et al., 2015).

Human studies have also shown correlations in radiation exposure and increased germline mutations. This relationship was assessed in families exposed accidently to high doses of ionizing radiation after the Chernobyl accident in Ukraine, and in families living in close proximity to the Semipalatinsk nuclear test site in Kazakhstan. In both cases, germline mutations were evaluated using eight hypervariable minisatellite probes. In the Chernobyl study, the paternal mutation rate in the exposed group was significantly increased by 1.6-fold relative to an unexposed control group; there was, however, no significant difference in the maternal germline mutation rates between the exposed group and the unexposed control group (Dubrova et al., 2002C). In the Semipalatinsk study, analysis of families living in the affected region over three generations found that germline mutations in the first and second generation were significantly increased relative to unexposed families living in a low-radiation area. Overall, the germline mutation rate in the families exposed to radiation from this test site was doubled (Dubrova, Bersimbaev, et al., 2000).

Empirical Evidence

Overall, there is strong supporting evidence that direct deposition of energy increases the frequency of mutations. The evidence presented below is summarized in table 2, here (click link). In general, exposure to ionizing radiation has been documented to elevate mutation frequencies in a number of different studies spanning different models and cell types (Russell et al., 1957; Winegar et al., 1994; Gossen et al., 1995; Suzuki & Hei, 1996; Albertini et al., 1997; Kraemer et al., 2000; Canova et al., 2002; Dubrova & Plumb, 2002; Masumura et al., 2002; Bolsunovsky et al., 2016; Mcmahon et al., 2016; Matuo et al., 2018; Nagashima et al., 2018; Wu et al., 1999; Hei et al., 1997; Nagasawa and Little, 1999; Barnhart and Cox, 1979; Thacker at al., 1982; Zhu et al., 1982; Metting et al., 1992; Schwartz et al., 1991; Chen et al., 1984; Albertini et al., 1997) . Furthermore, several reviews outline evidence of the relationship specifically between radon gas exposure and mutation frequency (Jostes, 1996; Robertson et al., 2013;

ICRP, 2005). At low doses (<1 Gy) the induction of mutations in cells has been observed for high-LET radiation such as alpha particles (Wu et al., 1999; Hei et al., 1997; Nagasawa and Little, 1999; Barnhart and Cox, 1979; Thacker at al., 1982; Zhu et al., 1982; Metting et al., 1992; Schwartz et al., 1991; Chen et al., 1984; Albertini et al., 1997).

Figure 1: *Plot of example studies (y-axis) against equivalent dose (Sv) used to determine the empircal link between direct deposition of energy and increased cell mutation rates. The z-axis denotes the equivalent dose rate used in each study. The y-axis is ordered from low LET to high LET from top to bottom.*

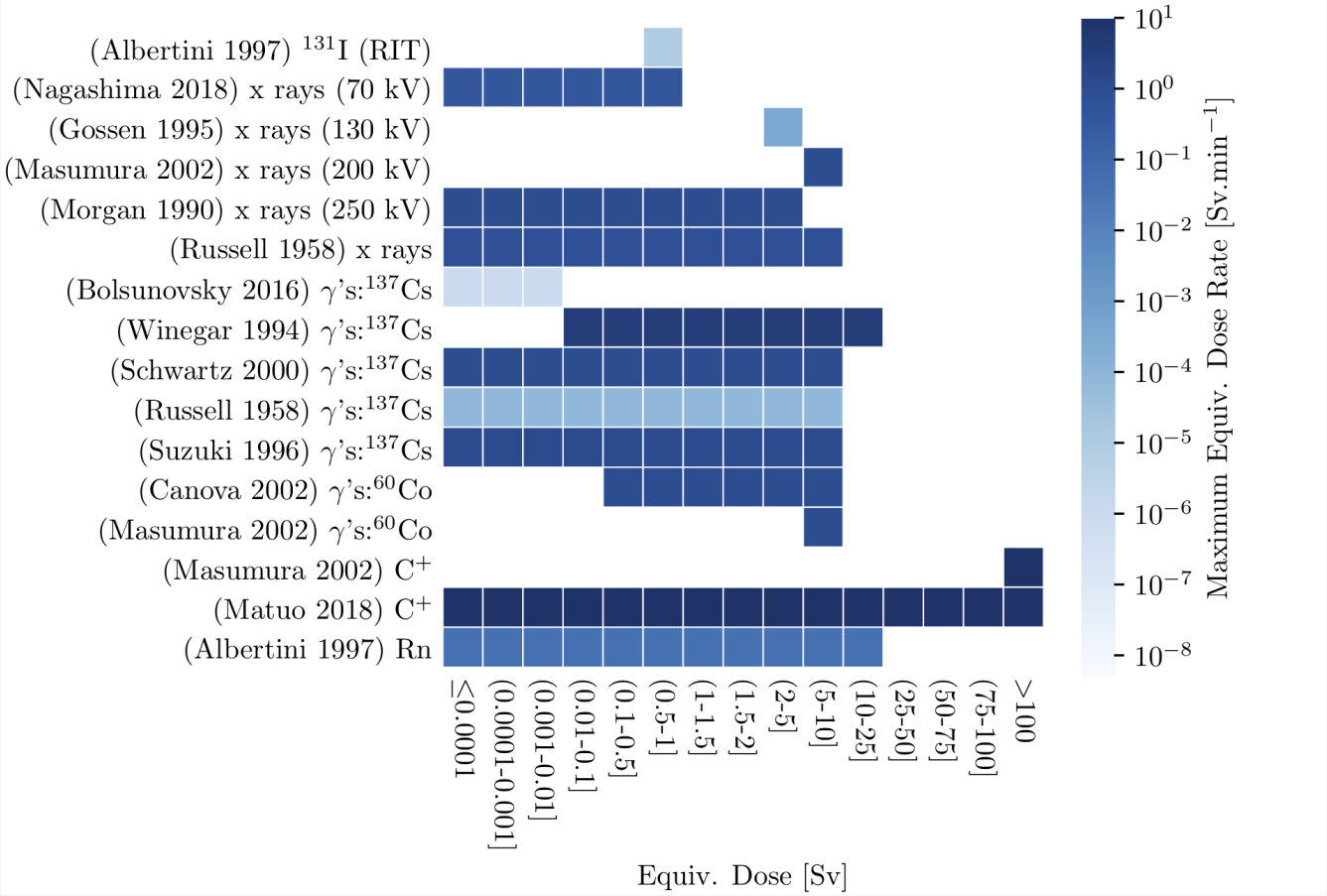
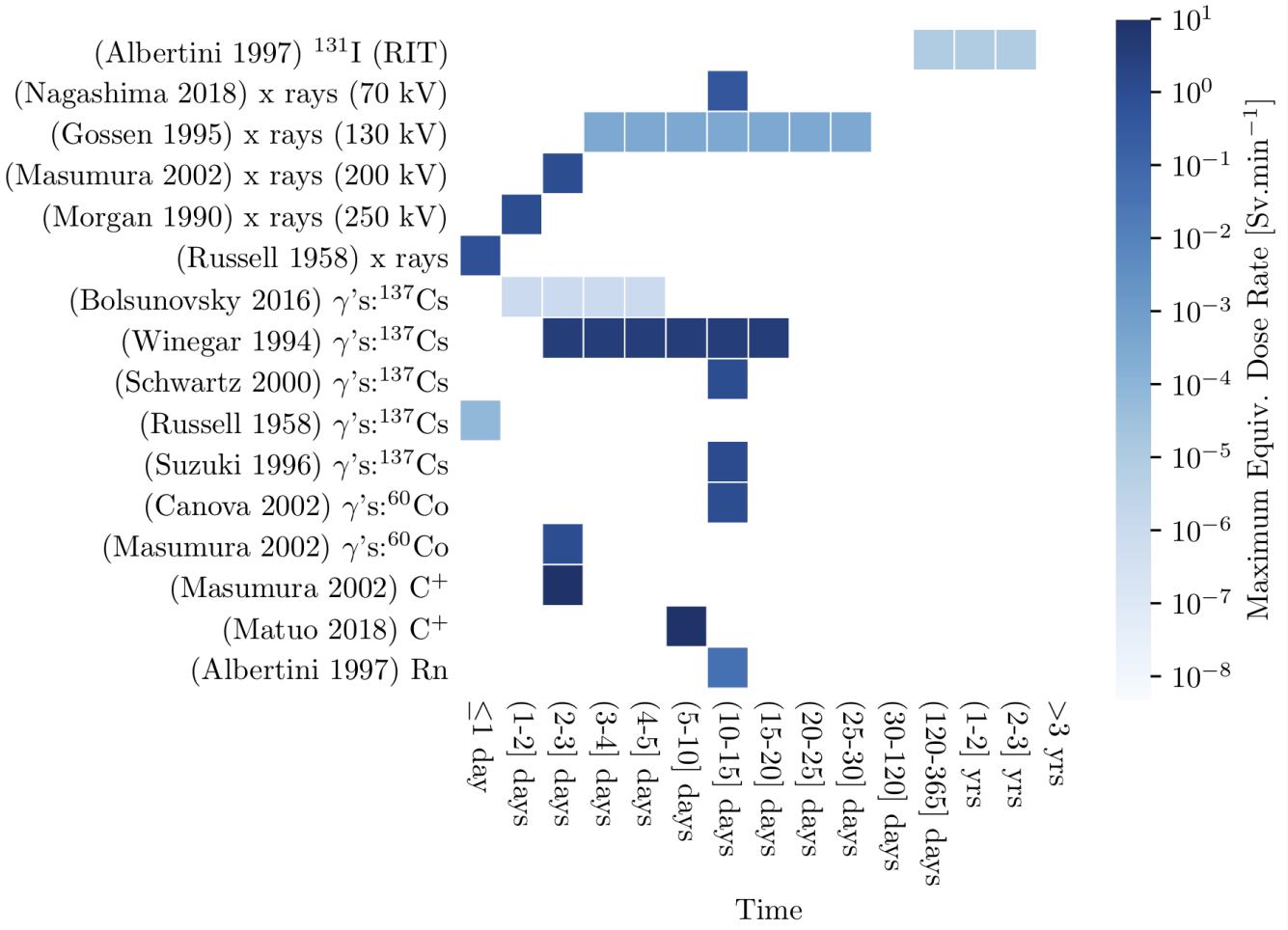


Figure 2: *Plot of example studies (y-axis) against time scales used to determine the empircal link between direct deposition of energy and and increased cell mutation rates. The z-axis denotes the equivalent dose rate used in each study. The y-axis is ordered from low LET to high LET from top to bottom.*



Dose and Incidence Concordance

It is clear that increasing doses of ionizing radiation is concordant with increased incidence of mutations (see table under ***Quantitative Understanding of the Linkage***). Extensive evidence from *in vitro* studies using human cells (Suzuki & Hei 1996; Canova et al., 2002), animal cells (Schmidt and Kiefer, 1998; Canova et al., 2002; Mcmahon et al., 2016; Nagashima et al., 2018), hybrid cell lines (Kraemer et al., 2000), yeast cells (Matuo et al., 2018), and bacteria (Bolsunovsky et al., 2016) demonstrates this concordance. *In vivo* studies using mice have also found a dose-dependent increase in mutations across several different types of radiation (Russell et al., 1957; Dubrova & Plumb 2002).

This relationship between radiation exposure and mutation incidence is impacted by several different factors. Higher LET radiation, such as high LET carbon ions and neutrons, were found to induce more mutations in comparison to radiation of a lower LET, including low LET carbon ions, gamma-rays and X-rays (Dubrova & Plumb, 2002; Matuo et al., 2018). Similarly, more mutations were present in the gametes of mice exposed to acute X-rays compared to those exposed to chronic gamma-rays (Russell et al., 1957). The tissue being irradiated may also have a role in determining mutant frequency, as whole body irradiation of mice led to a significant increase in mutations (mostly deletions) of the spleen, liver, lung and kidneys (Gossen et al., 1995; Masumura et al., 2002), but not the testis (Masumura et al., 2002). Furthermore, the specific kind of mutation may be dependent on the type of radiation. In one study, irradiation of the liver with carbon ions resulted in a significant increase in deletion mutations, while irradiation with X-rays or gamma-rays resulted in a significant increase in point mutations (Masumura et al., 2002).

Temporal Concordance

Temporal concordance is well established. As described above, energy deposition happens immediately upon radiation exposure, with an increased incidence of mutations documented days or weeks after irradiation (Russell et al., 1957; Winegar et al., 1994; Gossen et al., 1995; Albertini et al., 1997; Canova et al., 2002; Dubrova & Plumb, 2002; Masumura et al., 2002; Matuo et al., 2018; Nagashima et al., 2018).

Essentiality

Not identified.

Uncertainties and Inconsistencies

Uncertainties and inconsistencies in this KER are as follows:

1. In a review paper describing the role ionizing radiation plays in elevating mutation frequency in the germline and therefore genetic risk, Sankaranarayanan & Nikjoo (2015) stated that most radiation-induced mutations tended to be deletions. In contrast, an examination of ESTR loci mutations in offspring and their irradiated fathers

found that the ESTR mutations tended to be gains more often than losses (Dubrova ,2005). This may, however, highlight a characteristic specific to ESTR mutations rather than mutations in general.

2. In a study examining the long-term of effects of *in utero* radiation exposure, males irradiated at embryonic day 12 showed significant increases in both somatic and germline ESTR mutations as adults, and produced offspring with significantly elevated ESTR mutations in their sperm (Barber et al., 2009). In contrast, male mice exposed to radiation during their neonatal days (6 - 8 days old) or pubertal stage (18 - 25 days) did not have increased mutations in adult spermatozoa, as mutant frequencies that were present in spermatogenesis stages immediately after radiation returned to normal levels later in the spermatogenesis process (Xu et al., 2008).
3. Factors such as dose, dose-rate, tissue type and radiation quality can influence mutation rate induction (Hooker et al., 2004; Rydberg et al., 2005; Day et al., 2007; Okudaira et al., 2010; Brooks et al., 2016).

Quantitative Understanding of the Linkage

Below are representative examples of the mutation frequency rates across different studies. Overall, a quantitative understanding of this linkage suggests that mutation rates can be predicted and are dependent on the type and dose of radiation exposure.

Reference	Summary
Matuo et al., 2018	Study of impact of high and low LET radiation (high LET: carbon ions, 25 keV/um, low LET: carbon ions, 13 keV/um) in the dose range of 0 - 200 Gy incident on Saccharomyces cerevisiae (yeast cells). Found a 24-fold increase over baseline of mutations from high LET radiation and an 11-fold increase for low LET radiation.
Nagashima et al, 2018	Study of X-rays incident on GM06318-10 hamster cells in the dose range of 0-1 Gy. Found a calculated mutation rate of 19.0 ± 6.1 mutants per 10^4 survivors per Gy.
Albertini et al., 1997	Study of T-lyphocytes from human peripheral blood exposed to low LET gamma-rays and high LET radon gas. Doses in the range 0.5 - 5 Gy (gamma-rays) and 0 - 1 Gy (radon gas). The calculated mutation rate was as follows: gamma-rays (0-2 Gy): 7.0×10^{-6} mutants / Gy, gamma-rays (2-4 Gy): 54.0×10^{-6} mutants / Gy, radon gas (0-1 Gy): 63.0×10^{-6} mutants / Gy.
Dubrova and Plumb 2002	Study of paternal ESTR mutation rates in CBA/H mice. Mice exposed to acute low LET X-rays, chronic low LET gamma-rays and chronic high LET neutrons. X-rays in the 0 - 1 Gy dose (D) range, gamma-rays: 0 - 1 Gy & neutrons: 0 - 0.5 Gy. Calculated mutation rate (y) (of the form y = a + bD) as follows: X-rays (a, b := 0.111, 0.338), gamma-rays (a,b := 0.110, 0.373 ± 0.082), neutrons (a, b := 0.136, 1.135 ± 0.202).
McMahon et al., 2016	Study across various studies of the HPRT gene in chinese hamster cells exposed to doses in the range of 1 - 6 Gy. Found 0.2 mutations in HPRT gene per 10^4 cells and 0.1 point mutations per 10^4 cells (1 Gy). At higher doses (6 Gy) observed 1.5 mutations per 10^4 cells and 0.4 point mutations per 10^4 cells.

Response-response relationship

There is evidence of a positive response-response relationship between the radiation dose and the frequency of mutations (Russell et al., 1957; Suzuki & Hei, 1996; Albertini et al., 1997; Kraemer et al., 2000; Canova et al., 2002; Dubrova & Plumb, 2002; J.W. Wilson et al., 2015; Bolsunovsky et al., 2016; Mcmahon et al., 2016; Nagashima et al., 2018) . Most studies found that the response-response relationship was linear (Russell et al., 1957; Albertini et al., 1997; Canova et al., 2002; Dubrova et al., 2002; Nagashima et al., 2018). There were however, two exceptions. In a study using normal human bronchial epithelial cells irradiated with 1 - 6 Gy of gamma-rays, the relationship between the number of induced *HPRT* mutants and the radiation dose was described as non-linear (Suzuki & Hei, 1996) Similarly, in a study examining *HPRT* mutations in isolated peripheral blood T-lymphocytes irradiated with low LET gamma-rays, the slope of the line from 0 - 2 Gy differed from the slope at the 2 - 4 Gy interval; thus this was described as two different linear relationships or an overall linear-quadratic relationship (Albertini et al., 1997). In a study with V79 Chinese hamster cells, a curvilinear response was also seen as a result of x-ray response while a linear response was seen for Am-241 alpha-particle exposure (Schmidt and Keifer, 1998).

Time-scale

The time scale relationship between radiation exposure and the frequency of mutations is not well defined. Most studies look for manifestation of mutations days or weeks after irradiation, making it particularly difficult to pinpoint exactly when the mutations first occur. Analysis of various organs from mice after *in vivo* radiation found that mutations were present at 2 days (Winegar et al., 1994; Masumura et al., 2002) and 3 days (Gossen et al., 1995)(Gossen, 1995) post-exposure. Mutations were still present at 7 days and 14 days (Winegar et al., 1994), and 10 days and 21 days (Gossen, 1995) following irradiation. One study documented a doubling in the number of mutations from 7 to 14 days (Winegar et al., 1994) while the other reported a two-fold decrease from 3 to 21 days (Gossen et al., 1995).

An attempt to better define this time scale relationship was made in a study using *Salmonella typhimurium* bacteria. This study was designed to determine how mutation frequency was affected by constant cesium-137 gamma-ray radiation exposure at defined dose rates of 67.8 uGy/hour, 3.2 uGy/hour, and 0.6 uGy/hour; these mutation frequencies were compared to a control group exposed to background radiation levels (0.09 uGy/hour). Mutation frequencies were evaluated after 24, 48, 72 and 96 hours of constant exposure. At 24 hours, the 67.8 uGy/hour, 3.2 uGy/hour and 0.6 uGy/hour mutant frequencies were significantly higher than background exposure controls. Interestingly, however, these levels were decreased at 48 hours and continued to decline gradually towards control frequencies over time. This decline was proposed to be due to an elimination of the highly mutated cells, leaving behind an increasing number of cells that had adapted to the radiation and were thus more equipped for survival (Bolsunovsky et al., 2016). Other studies are required to build a more complete understanding of this timeline.

Known modulating factors

There are several factors that have been documented to affect the relationship between direct deposition of energy and increased mutation frequency. The sex, age, and use of adaptive dosing have been demonstrated to affect the radiation-induced mutations present in offspring. In contrast to male mice, female mice that were irradiated in utero (Barber et al., 2009) or as adults (Ali et al., 2012)(Ali, 2012) did not produce offspring with increased ESTR mutations. This suggests that radiation-induced mutations are only heritable through the paternal line. As such, the age of the father may affect the mutant frequency in the offspring, as increased mutations were present in spermatogenic cells of older male mice relative to younger males both at baseline levels and post-irradiation (Xu et al., 2012). Lastly, the use of 'adaptive' radiation dosing, or giving a very small dose 24 hours prior to the full radiation dose, may also affect offspring's mutational frequency. In male mice who received adaptive dosing relative to males who received only the full radiation dose, there were significant decreases in germline mutation frequencies and in the rate of paternal mutations in their offspring (Somers et al., 2004) .

The radiation-mutation relationship may also be impacted by the genetics of the organism, as the genotype appears to play an important role in determining how the biological system responds to radiation. In yeast with inactivated *rad50* or *rad52*, the radiation-induced mutation frequency was significantly increased relative to wild-type yeast (Matuo et al., 2018). *Msh2* knock-out mice (Burr et al., 2007) and medaka fish (Otozai et al., 2014) both had significantly increased baseline mutation frequencies relative to wild-type animals. Irradiation, however, did not change this mutation rate from baseline for these *Msh2* knock-out animals (Burr et al., 2007; Otozai et al., 2014). Similarly, *BRCA2* knock-out embryos had significantly elevated baseline mutation rates relative to wild-type littermates; however, *in utero* radiation was found to increase the mutation rate of all genotypes. Thus irradiated *BRCA2* knock-out embryos also had a significantly increased mutation frequency relative to wild-type embryos by approximately three-fold (Tutt et al., 2002). Finally, baseline mutation levels in *p53* knock-out medaka fish did not differ from wild-types; however, *p53* knock-out fish exposed to radiation were found to have a 24-fold increase in mutation frequency relative to unirradiated *p53* knock-out fish (Otozai et al., 2014). Construction of a dose response curve found the following mutation rates for wild-type, *Msh2* knock-out, *p53* knockout, and *Msh2/p53* double knock-out medaka fish, respectively: $1.1x10^{-4}$ mutations/allele/Gy, $1.1x10^{-4}$ mutations/allele/Gy, $4.3x10^{-4}$ mutations/allele/Gy, and $5.6x10^{-4}$ mutations/allele/Gy (Otozai et al., 2014).

Finally, factors such as dose, dose-rate, tissue type and radiation quality can influence mutation rate induction (Suzuki & Hei ,1996; Hooker et al., 2004; Rydberg et al., 2005; Day et al., 2007; Okudaira et al., 2010; Brooks et al., 2016).

Known Feedforward/Feedback loops influencing this KER

Not identified.

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Relationship: 1982: Energy Deposition leads to Increase, Chromosomal aberrations

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deposition of energy leading to lung cancer	non-adjacent	High	High
Deposition of energy leading to occurrence of cataracts	non-adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

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

Key Event Relationship Description

Energy can be deposited on biomolecules from various forms of radiation in a randomized manner. Radiation with high linear energy transfer (LET) tends to produce more complex, dense structural damage than low LET radiation; both, however, can lead to detrimental damage within a cell (Bauchinger and Schmid 1998; Evans et al., 2001; Hada and Georgakilas 2008; Okayasu 2012; Lorat et al. 2015; Nikitaki et al. 2016). The DNA is particularly susceptible to damage in the form of DNA strand breaks. This damaged DNA can lead to aberrations/rearrangements in chromosomes and chromatids. Examples of chromosome-type aberrations include chromosome-type breaks, ring chromosomes, and dicentric chromosomes, while chromatid-type aberrations refer to chromatid-type breaks and chromatid exchanges (Hagmar et al. 2004; Bonassi et al. 2008). Other types of CAs that may occur in response to radiation include micronuclei (MN), nucleoplasmic bridges (NPBs), and copy number variants (CNVs). 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 and Muirhead 2009; Zölzer et al. 2013; Qian et al. 2016).

Evidence Supporting this KER

Overall Weight of Evidence: High

Biological Plausibility

The biological plausibility for this KER is strong, as there is a broad mechanistic understanding of the process CA induction from deposited energy in the form of radiation, which is widely accepted. This has especially been demonstrated in humans and adult mammals. Reviews have been published that provide details regarding the relationships between radiation of different LETs and the relative effectiveness of CA induction (Hunter and Muirhead 2009), ionizing radiation and genomic instability (Smith et al. 2003), and low dose ionizing radiation and chromosomal translocations (Tucker 2008). When ionizing radiation comes into contact with a cell, it is able to deposit energy through ionization and excitation of molecules, which results in the freeing of electrons. These electrons have enough energy to break chemical bonds; thus if the high-energy electrons come into contact with DNA, they may break DNA bonds and cause damage in the form of double-strand breaks, single-strand breaks, base damage, or the crosslinking of DNA to other molecules. This damage should trigger DNA repair. If the enzymatic repair, however, is incorrect or incomplete, this could push the cell towards apoptotic pathways. However, the repair processes may lead to asymmetrical exchanges in the chromosomes that are not removed from the cell and can propagate in the form of aberrations. Radiation-damaged cells display accumulated CAs in the form of chromosomal rearrangements, genetic amplifications and/or MN (Smith et al. 2003; Christensen 2014; Sage and Shikazono 2017).

CNVs may also be generated through deposition of energy by ionizing radiation. Due to the structural similarities between CNVs that are radiation-induced, chemically-induced, and spontaneously-occurring, all CNVs are likely produced by a similar mechanism. The chemicals, aphidicolin and hydroxyurea, are known inducers of DNA replication stress. This suggests that radiation-induced CNVs are also formed through a similar replication-dependent mechanism(Arlt et al. 2014). Additionally, CNVs may affect germline cells. In fact, there was a significant 8-fold increase in de novo CNVs in the progeny of irradiated male mice, regardless of whether the radiation affected post-meiotic sperm or pre-meiotic sperm. The majority of these CNVs were found to be large deletions, often more than 1000 kB (Adewoye et al. 2015).

Empirical Evidence

Evidence supporting the formation of CAs from the direct deposition of energy in the form of ionizing radiation is strong. Of this data, humans are the most common organisms, adults are the most common life stage, and α particles and X rays are the most common stressors studied. There is also more in vivo data compared to in vitro, however most of the human studies are in vitro (Worgul et al., 1989; Tao et al., 1993; Loucas and Geard, 1994; Tao et al., 1994; Bauchinger and Schmid 1998; Durante et al., 1998; Williams et al., 1999; Belkacémi et al., 2000; Belkacémi et al., 2001; Evans et al., 2001; Schmid et al. 2002; Hande et al., 2003; Thomas et al. 2003; Maffei et al. 2004; Tucker et al., 2004; Hande et al., 2005; Tucker et al. 2005a; Tucker et al. 2005b; Wolf et al., 2008; George et al. 2009; George et al., 2010; Blakely, 2012; Balajee et al. 2014; George et al. 2014; Han et al. 2014; Vellingiri et al. 2014; Suto et al. 2015; Adewoye et al. 2015; Cheki et al. 2016; Foray et al., 2016; McMahon et al. 2016; Morishita et al. 2016; Qian et al. 2016; Basheerudeen et al. 2017; Meenakshi et al. 2017; Abe et al. 2018; Dalke et al., 2018; Bains et al., 2019; Jang et al. 2019; Udroiu et al., 2020; Puig et al., 2016; Barquinero et al., 2004; Curwen et al., 2012; Testa et al., 2018; Franken et al., 2012; Cornforth et al., 2002; Loucas et al., 2013; Nagasawa et al., 1990a; Nagasawa et al., 1990b; Edwards et al., 1980; Themis et al., 2013; Schmid et al., 1996; Mestres et al., 2004; Bilbao et al., 1989; Mill et al., 1996; Brooks, 1975; Tawn and Thierens, 2009; Durante et al., 1992; Hamza and Mohankumar, 2009; Takatsuji and Sasaki, 1984; Moquet et al., 2001; Purrott et al., 1980; duFrain et al., 1979).

Figure 1: *Plot of example studies (y-axis) against equivalent dose (Sv) used to determine the empircal link between direct deposition of energy and increased rates of chromosomal aberrations. The y-axis is ordered from low LET to high LET from top to bottom.*

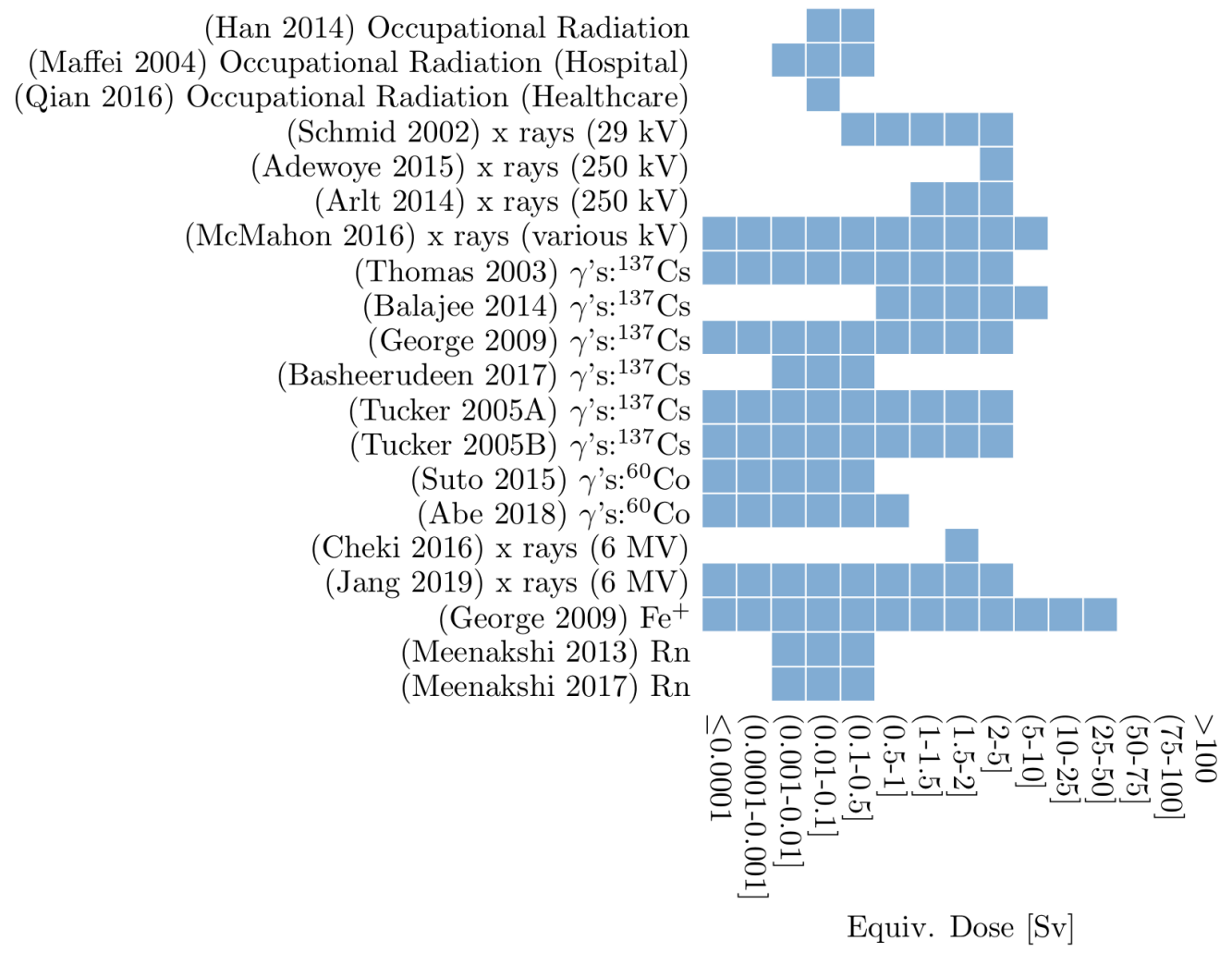
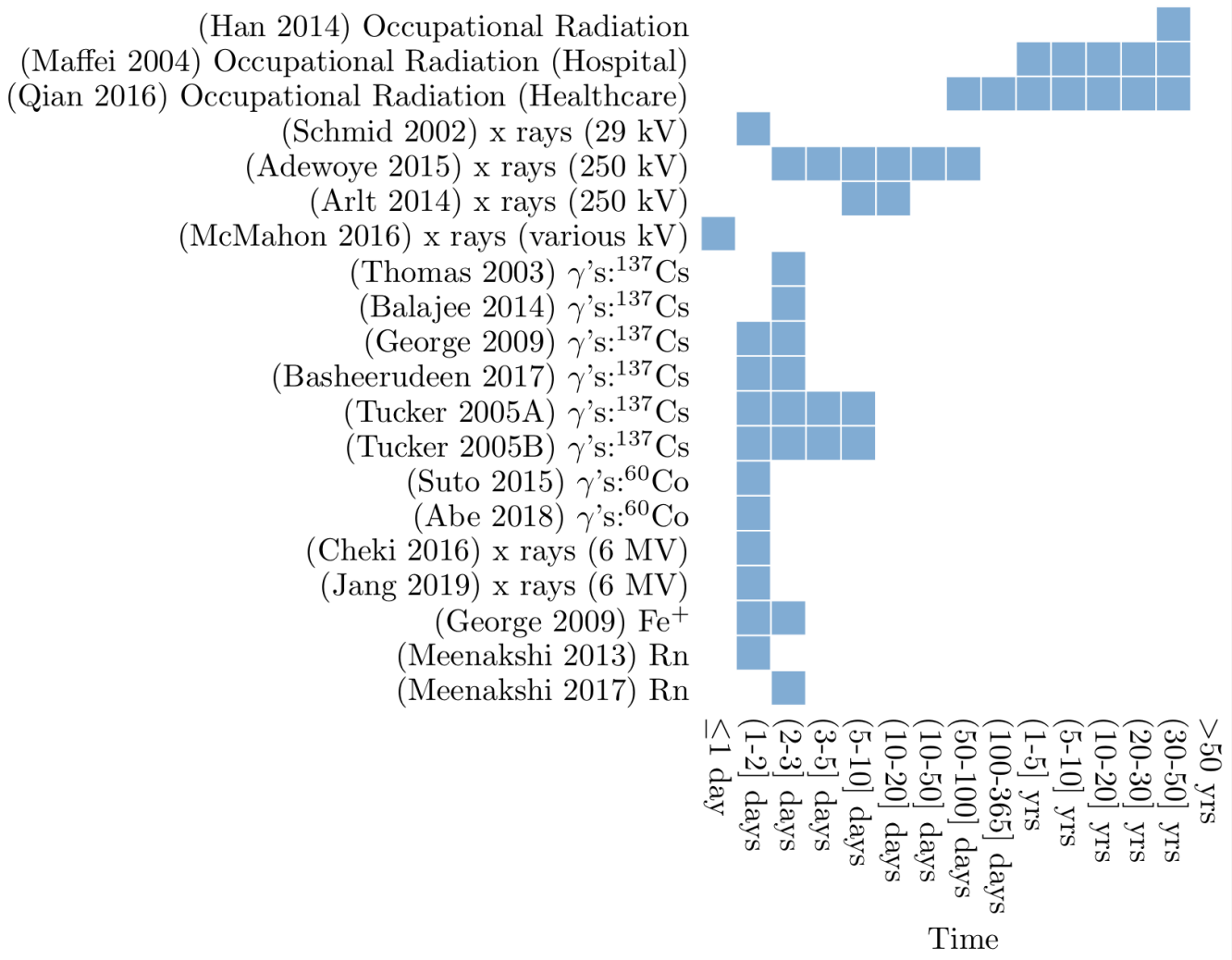


Figure 2: *Plot of example studies (y-axis) against time over which studies were conducted for a temporal response used to determine the empircal link between direct deposition of energy and increased rates of chromosomal aberrations. The y-axis is ordered from low LET to high LET from top to bottom.*



Human relevance

Several human epidemiological studies have provided evidence of both dose/incidence and temporal concordance in terms of deposition of energy by ionizing radiation and resultant CAs. In a study involving 34 health professionals occupationally exposed to radiation, there was a significant increase in the number of chromosome breaks and aberrant cells relative to a group of 35 unexposed professionals from the same hospital. Furthermore, when the exposed group was broken into two groups based on the levels of radiation exposure (those with an effective dose of ≤50 mSv and those with an effective dose of >50 mSv), there was a dose-dependent increase in aberrant cells, chromosome breaks and chromatid breaks such that the higher exposure group had significantly elevated aberrations relative to controls for all three parameters scored (Maffei et al. 2004). In a similar study involving 1,392 radiation healthcare workers in the city of Tangshan in 2010, there was a significant increase in CA and MN in exposed workers relative to unexposed healthy controls. Furthermore, there were significant, dose-dependent increases in the CA rate and the MN rate when the exposed workers were split into groups according to effective dose, ranging from <10 mSv up to >50 mSv. There was also a time-dependent increase in CA and MN rate, such that workers with longer exposure times had significantly increased CAs and MNs. Exposure times ranged from less than 10 years to greater than 20 years (Qian et al. 2016). A final study with 31 plutonium and reactor workers found that those exposed to high doses of Pu had a frequency of complex translocations that was 2.9% higher and a frequency of simple translocations that was 5.1% higher compared to unexposed workers. They also found that workers exposed to γ-rays had a frequency of complex translocations that was only 0.21% higher than that of controls, and a frequency of simple translocations that was 4% higher than controls (Hande et al., 2005).

Dose Concordance

There is a clear correlation between radiation dose (i.e., increasing amounts of energy deposition) and different clastogenic endpoints including dose-dependent increases in: dicentric aberrations(Schmid et al. 2002; Thomas et al. 2003; Tucker et al. 2005A; Suto et al. 2015; Mcmahon et al. 2016; Abe et al. 2018; Jang et al. 2019), centric rings (Tucker et al. 2005a) (Schmid et al. 2002; Thomas et al. 2003; Tucker et al. 2005A), acentric fragments (Loucas and Geard, 1994; Schmid et al. 2002; Thomas et al. 2003) , translocations (Hande et al., 2005; Tucker et al. 2005A; Tucker et al. 2005B; Suto et al. 2015; Abe et al. 2018; Jang et al. 2019), CNVs (Arlt et al. 2014), large deletions (Mcmahon et al. 2016), NPBs (Thomas et al. 2003), MNs (Tao et al., 1993; Tao et al., 1994; Thomas et al. 2003; Balajee et al. 2014), fragmented nuclei (Tao et al., 1993), strand breaks (Durante et al., 1998) and CAs in general (Williams et al., 1999; Evans et al., 2001; Hande et al., 2003; George et al., 2009; Mcmahon et al. 2016) (George et al. 2009).

Interestingly, MN structural complexity was likewise demonstrated to be dose-dependent between 1 and 10 Gy. MN were found to contain fragments from two or more different chromosomes at and above 2 Gy; between 5 and 10 Gy, MN contained material from 3 - 5 different chromosomes. These results suggest that MN formation appears to become increasingly more complex with higher doses of radiation due to the increasing number of acentric fragments and the resultant fusion of these fragments (Balajee et al. 2014). Of note, the photon energy of the radiation has an effect on the relationship between direct deposition of energy and the resulting CAs. Specifically, dicentric aberration frequency in human peripheral blood lymphocytes was observed to change with voltage of the ionizing radiation. As the X-ray voltage decreased from 60 kV to 10 kV, there was an increase in the number of dicentric aberrations (Schmid et al. 2002).

Time Concordance

Temporal concordance is well established. Energy deposition happens immediately upon radiation exposure, with an increased incidence of CAs documented minutes, hours or days after irradiation (Loucas and Geard, 1994; Durante et al., 1998; Schmid et al. 2002; Thomas et al. 2003; Tucker et al. 2005A; Tucker et al. 2005B; George et al. 2009; Meenakshi and Mohankumar 2013; Arlt et al. 2014; Balajee et al. 2014; Suto et al. 2015; Cheki et al. 2016; Mcmahon et al. 2016; Basheerudeen et al. 2017; Meenakshi et al. 2017; Abe et al. 2018; Jang et al. 2019).

Essentiality

Radiation exposure has been found to increase levels of CAs above background levels and lead to more complex damage, highlighting the essentiality of deposition of energy (Tao et al., 1994; Durante et al., 1998; Williams et al., 1999; Evans et al., 2001; Schmid et al., 2002; Hande et al., 2005; Wolf et al., 2008; George et al., 2009; Suto et al., 2015; Abe et al., 2018; Dalke et al., 2018; Bains et al., 2019; Jang et al., 2019; Udroiu et al., 2020). In the absence of radiation, there are minimal amounts of CAs. For example, abnormal karyotypes are half as likely in non-irradiated cells compared to irradiated cells (Wolf et al., 2008; Zhou et al., 2016). Since deposited energy initiates events immediately, the removal of deposited energy, a physical stressor, also supports the essentiality of the key event. Studies that do not deposit energy are observed to have no downstream effects.

Uncertainties and Inconsistencies

Uncertainties and inconsistencies in this KER are as follows:

- An individual's response to radiation can be affected by a large variety of factors. Many of them cannot be controlled in a study, therefore leading to inconsistencies in results (Bender et al., 1988).
- When an organism is exposed to an initial low radiation dose followed by a higher dose, it can initiate an adaptive response, therefore decreasing the resulting damage. Day et al. also found this to be applicable when a low radiation dose was followed by an even lower dose (2007).

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage suggests that CA frequency can be predicted from the radiation, as per the representative examples provided below. When predicting this relationship, the characteristics of the radiation and the model system should be taken into account (Smith et al. 2003; Hunter and Muirhead 2009). It is widely accepted that the deposition of energy, at all doses, results in immediate ionization events, followed by downstream events. The following tables provide representative examples of the relationship, unless otherwise indicated, all data is significantly significant.

Dose Concordance

Reference	Experiment Description	Result
Suto et al., 2015	In vitro. Human peripheral blood lymphocytes were exposed to 60Co γ-rays at 0 – 300 mGy and 5 mGy/sec. The number of dicentrics and translocations in chromosomes 1, 2, and 4 were determined using three differentially colored chromosome painting probes.	Study of human peripheral blood lymphocytes from a healthy donor subjected to γ-ray radiation in the dose (D) range of 0 - 300 mGy found a calculated CA rate (y) of dicentrics, translocations and dicentrics+translocations (of the quadratic form, y = a + bD + bD^2) found - dicentrics + translocations (a,b,c := 0.0023 ± 0.0003, 0.0015 ± 0.0058, 0.0819 ± 0.0225), dicentrics (a,b,c := 0.0004 ± 0.0001, 0.0008 ± 0.0028, 0.0398 ± 0.0117), translocations (a,b,c := 0.0019 ± 0.0003, 0.0008 ± 0.0028, 0.0398 ± 0.0117).
Abe et al., 2018	In vitro. Human mononuclear blood cells from five donors (four males aged 23, 35, 44, and 55 years old, and a 33-year-old female) were exposed to 60Co γ-rays at 0 – 1000 mGy. The dose rate in the irradiator was 26.6 mGy/min with an additional 6.42 mGy to the sample while entering and leaving the irradiation source. None of the subjects had a history of radiotherapy, smoking, or chemotherapy. The number of dicentrics and translocations in chromosomes 1, 2, and 4 were determined using Giemsa staining and Centromere-FISH staining.	Study of human mononuclear blood cells from healthy donors; analyzed for dicentric chromosomes. Exposure to γ-ray doses (D) in the 0 - 1000 mGy range. Quadratic form fit for the CA rate in Giemsa staining and Centromere-FISH staining cases (y) (of the form y = a + bD + cD^2) found to be: Giemsa staining: (a,b,c := 0.0013 ± 0.0005, 0.0067 ± 0.0071, 0.0313 ± 0.0091), Centromere-FISH staining (a,b,c := 0.0010 ± 0.0004, 0.0186 ± 0.0081, 0.0329 ± 0.0104). Study of mononuclear blood cells from healthy donors; analyzed for translocations. Exposure to γ-ray doses (D) in the 0 - 1000 mGy range. Quadratic form fit for the CA rate (y) before and after donor age adjustment (of the form y = a + bD + cD^2) found to be: before donor age adjustment: (a,b,c := 0.0053 ± 0.0009, 0.259 ± 0.0127, 0.0826 ± 0.0161), after donor age adjustment (a,b,c := 0.0015 ± 0.0009, 0.0049 ± 0.0155, 0.1033 ± 0.0223).
Jang et al., 2019	In vitro. Human peripheral blood lymphocytes from four different donors (two males, 36 and 22 years old, and two females, 28 and 24 years old) were exposed to 6 MV X-rays at 0-5 Gy and 0.5 Gy/min. None of the subjects had a history of chemotherapy, smoking, or radiotherapy. The number of dicentrics and translocations in chromosomes 1, 2, and 4 were determined using Giemsa staining and chromosome painting, respectively.	Human peripheral blood lymphocytes studied from healthy donors. Lymphocytes irradiated with X-rays in a dose (D) range 0 - 5 Gy. Calculated CA rate from dicentrics or translocations (y) (of the form y = a + bD + cD^2). Dicentrics, (a,b,c := 0.0011 ± 0.0004, 0.0119 ± 0.0032, 0.0617 ± 0.0019). Translocations, (a,b,c := 0.0015 ± 0.0004, 0.0048 ± 0.0024, 0.0237 ± 0.0014).
Schmid et al., 2002	In vitro. Human peripheral blood lymphocytes from one male donor were exposed to 29 kV X-rays at 0.115 – 2.194 Gy and at 0.009 – 0.140 Gy/min. CAs were determined with fluorescence plus Giemsa staining.	Study of various X- and γ-ray types irradiating peripheral human blood lymphocytes, analyzed dicentrics and acentrics (10, 29, 60, 220 kV X-rays & Cs-137, Co-60 γ-rays). See Schmid et al. (2002) for details on equations.
George et al., 2009	In vitro. HF19 normal primary lung fibroblasts, AT primary fibroblasts, NSB1-deficient primary fibroblasts, M059K glioblastoma cells, and M059J glioblastoma cells were exposed to iron nuclei (1000 MeV/nucleon, 0.2 – 0.5 Gy/min, average LET of 151 keV/um), and 137Cs γ-rays (0.2 – 1 Gy/min) at 0 – 3 Gy. CAs in chromosomes 1, 2, 4, and 11 were determined using FISH staining.	γ-rays and iron nuclei irradiating HF19 normal primary lung fibroblasts; Ataxia telangiectasia (AT) primary fibroblasts; NSB1-deficient primary fibroblasts (Nijmegen breakage syndrome); M059K glioblastoma cells & M059J glioblastoma cells (lack DNA-dependent protein kinase activity). Dose range of 0 - 3 Gy. See Table 5 & 6 of George et al. (2009) for details on equations.
Wolf et al. 2008	In vivo. Female, C57BL/6 mice received head-only exposure to 11 Gy soft X-rays. Mice were either 2 or 26 months-old at irradiation. 6 were irradiated and 6 were non-irradiated controls. Chromosomal aberrations were detected using Wright-trypsin G-banding.	Irradiation of eight-week-old mice with 11 Gy of X-rays increased the number of abnormal chromosomes from 15 (control) to 27. Irradiation of 26-month-old mice with 11 Gy of X-rays increased the number of abnormal chromosomes from 0 (control) to 30). However, the significance of these results was not indicated.
Williams et al., 1999	In vitro. Human lymphocyte cells were exposed to Fe ions (0 – 1 Gy, 1 GeV incident energy), 137Cs photons (0 – 7.5 Gy, 83 cGy/min), and protons (5 Gy, 250 keV incident energy). Chromosomal aberrations were identified using	CAs rose from 0 to 0.001, 0.004, 0.014, and 0.017 after exposure to 1 Gy 137Cs, 1 Gy Fe, 5 Gy 137Cs, and 5 Gy energetic photons respectively.

	Leishman's stain.	
Evans et al., 2001	In vitro. TK6 and WTK1 human lymphoblastoid cells were exposed to 56Fe ions (0 – 2.25 Gy, 1087 MeV/nucleon) and 137Cs γ-rays (0 – 4 Gy, 0.87 – 0.92 Gy/min). CAs were assessed using Giemsa staining.	Exposure to higher doses resulted in increased amounts of aberrant cells. For example, in TK6 cells after 56Fe irradiation, 0% of cells were aberrant in the control, 19% were aberrant after 0.63 Gy, and 66% were aberrant after 2.25 Gy. Additionally, 56Fe ions induced increased numbers of aberrant cells when compared to 137Cs γ-rays.
Hande et al., 2005	In vivo. Lymphocytes from 31 Russian reactor and plutonium workers were exposed to plutonium ions and γ-rays. Highly exposed plutonium workers received 1.1 Gy of Pu and 1.5 Gy of γ-rays. Moderately exposed plutonium workers received 0.19 Gy of Pu and 0.19 Gy of γ-rays. Reactor workers received only 2.3 Gy of γ-rays. Values are averages. CAs were detected using the mFISH assay.	The amount of interchromosomal complex translocations were 2.9, 0.23, and 0.21% higher than controls in workers highly exposed, moderately exposed, and exposed to only γ-rays, respectively. The amount of interchromosomal simple translocations were 5.1, 1.5, and 4% higher than controls in workers highly exposed, moderately exposed, and exposed to only γ-rays, respectively.
Durante et al., 1998	In vitro. Human male lymphocyte cells were exposed to carbon ions (5 Gy, 290 meV/nucleon, 42 keV/µm), neon ions (7 Gy, 400 MeV/nucleon, 31 keV/µm), hydrogen ions (1, 3, 5, 7 Gy, 0.4 keV/µm), and iron ions (0.5, 0.75, 1, 2, Gy, 1000 MeV/nucleon, 140 keV/µm). Chromosomal aberrations were detected using DAPI-counterstaining the PAINT classification.	As the dose increased from 0 to 7 Gy, the number of DNA breaks per human male lymphocyte cell also increased from 0 to 1.2. This included data using iron ions, hydrogen ions, carbon, and neon.
Tao et al., 1994	In vivo. 90 – 110-day old B6CF1/Anl mice received irradiation to the anterior 2/3 of the body with 60Co γ-rays (10 – 40 cGy), 20Ne (670 MeV/amu, 25 keV/um), 56Fe (600 MeV/amu, 193 keV/um), 93Nb (600 MeV/amu, 464 keV/um), 139La (593 MeV/um, 953 keV/um). All stressors except 60Co delivered 10, 20, 40, 80, 160, and 320 cGy. Doses were delivered to the anterior 2/3 of the body. CAs were detected using a modified Feulgen method 64 weeks post-exposure.	Mouse lenses exposed to all radiation types showed increased MN number per whole mount at increasing doses from 10 to 160 cGy. For example, irradiation from 56Fe particles led to a MN number of 10 after 10 cGy and an MN number of 100 after 160 cGy.
Bains et al., 2019	In vitro. Human LECs were exposed to X-rays at 0, 0.001, 0.01, 0.02, 0.1, 1, and 2 Gy. Doses of 0.1, 1.0, and 2.0 had a dose rate of 0.58 Gy/min. 0.001, 0.01 and 0.02 Gy at 0.022 Gy/min. The γ-H2AX assay was used to determine the number of telomere dysfunction induced foci (TIF).	At 30 min after irradiation, the number of TIF/human lens epithelial cell remained almost 0 after exposure to 0, 0.001, and 0.01 Gy. It then rose quickly to 4.75 TIF/cell at 2 Gy.
Udroiu et al., 2020	In vitro. Human LECs were exposed to X-rays at 25, 51.25, 135, 235, and 300 mGy at 0.51, 0.15, and 0.228 Gy/min. The micronuclei frequency was measured with a cytokinesis-blocked micronucleus assay.	In human LECs exposed to 25 – 300 mGy the micronuclei frequency increased steadily, reaching 2.4x control at the maximum dose.
Dalke et al., 2018	In vivo. 10-week-old, mixed sex B6C3F1 and B6RCF1 hybrid mice received whole-body exposure to 0.063, 0.125, and 0.5 Gy 60Co γ-rays at 0.063 Gy/min. CAs were assessed using Giemsa staining.	In heterozygous Ercc2 mutants, 12 months after irradiation, the number of aberrations/cell rose from 0.1 (control) to 0.82 (0.5 Gy). There was also a slightly smaller increase after 18 months where the number of chromosomal aberrations/cell rose from 0.1 (control) to 0.3 (0.5 Gy). In wild type mice the largest increase occurred after 12 months where the number of aberrations/cell rose from 0.12 (control) to 0.32 (0.5 Gy).

Time Concordance

Reference	Experiment Description	Result
Tucker et al., 2004	In vivo. 7-week-old female C57BL/6 mice transgenic for lacZ received whole-body exposure to 1 Gy of 26Fe ions at 1 Gy/min. Aberrations on chromosomes 1, 2, 3, and 8 were determined with a FISH assay.	Lymphocytes from mice exposed immediately to 1 Gy of iron ions had 19 translocations per 100 cells, 14 acentric fragments per 100 cells, and 10 dicentric chromosomes per 100 cells at 1 week post-irradiation.
Tao et al., 1994	In vivo. 90 – 110-day old B6CF1/Anl mice received irradiation to the anterior 2/3 of the body with 60Co γ-rays (10 – 40 cGy), 20Ne (670 MeV/amu, 25 keV/um), 56Fe (600 MeV/amu, 193 keV/um), 93Nb (600 MeV/amu, 464 keV/um), 139La (593 MeV/um, 953 keV/um). All stressors except 60Co delivered 10, 20, 40, 80, 160, and 320 cGy. Doses were delivered to the anterior 2/3 of the body. CAs were detected using a modified Feulgen method.	Mouse lenses exposed immediately to all radiation types showed increased MN number per whole mount at increasing doses from 10 to 160 cGy at 64 weeks post-irradiation. For example, irradiation from 56Fe particles led to a MN number of 10 after 10 cGy and an MN number of 100 after 160 cGy at 64 weeks post-irradiation.
Tao et al., 1993	In vivo. 90 – 110-day old female B6CF mice received irradiation to the anterior 2/3 of the body with 10, 20, 40, 80, 160, and 320 cGy of protons: 250 MeV, LET 0.4 keV/um. 20Ne: 670 MeV/amu, LET 25 keV/um. 56Fe: 600 MeV/amu, LET 183 keV/um. 56Fe: 350 MeV/amu, LET 219 keV/um. 93Nb: 600 MeV/amu, LET 464 keV/um. 139La: 593 MeV/amu, 953 keV/amu. Fragmented nuclei (FN) and MN were detected using a modified Feulgen staining method or haematoxylin and eosin staining.	In mice immediately irradiated with iron ions, the number of FN in the whole mount of lens cells increased from 1.5 FN (control) to 11 FN at 160 cGy after 64 weeks. The number of FN in the meridional rows increased from 0 (control) to 6 FN at 160 cGy after 64 weeks as well. The number of MN on the whole mount also increased from 0 to 100 at 160 cGy after 64 weeks. Similar changes were also observed with the other radiation types.
Belkacémi et al., 2001	In vitro. Bovine lens cells were exposed to 10 Gy at 2 Gy/min from a linear accelerator. The Hoechst 33342 fluorescence was used to measure chromosomal aberrations.	In lens cells immediately irradiated with X-rays, Hoechst 33342 fluorescence increased 13%, 25%, and 32% above controls at 24, 72, and 96 h post-irradiation, respectively.
Bains et al., 2019	In vitro. Human LECs were exposed to X-rays at 0, 0.001, 0.01, 0.02, 0.1, 1, and 2 Gy. Doses of 0.1, 1.0, and 2.0 had a dose rate of 0.58 Gy/min. 0.001, 0.01 and 0.02 Gy at 0.022 Gy/min.	In human LECs immediately exposed in vitro to 0.02 Gy, the number of telomere dysfunction-induced foci (TIF)/cell increased to 3x control 30 min post-irradiation.
Dalke et al., 2018	In vivo. 10-week-old, mixed sex B6C3F1 and B6RCF1 hybrid mice received whole-body exposure to 0.063, 0.125, and 0.5 Gy 60Co γ-rays at 0.063 Gy/min. CAs were assessed using Giemsa staining.	In mice immediately exposed to 0.125-0.5 Gy of γ-rays, CAs were observed after 12 months, increasing to a maximum of 0.82 CAs/cell at 0.5 Gy.

Response-response relationship

There is evidence of a positive response-response relationship between the radiation dose and the frequency of CAs (Tao et al., 1993; Tao et al., 1994; Durante et al., 1998; Williams et al., 1999; Belkacémi et al., 2001; Evans et al., 2001; Schmid et al. 2002; Thomas et al. 2003;Tucker et al., 2004; Hande et al., 2005; Tucker et al. 2005a; Tucker et al. 2005b; Wolf et al., 2008; George et al. 2009; Arlt et al. 2014; Balajee et al. 2014; Suto et al. 2015; Mcmahon et al. 2016; Abe et al. 2018; Dalke et al., 2018; Bains et al., 2019; Jang et al. 2019; Udroiu et al., 2020). Most studies found that the response-response relationship was linear-quadratic (Schmid et al. 2002; Suto et al. 2015; Foray et al., 2016; Abe et al. 2018; Jang et al. 2019). One study, however, reported different results when CAs were examined across five cell lines that had been irradiated with either iron nuclei or γ-rays. For complex aberrations in three types of fibroblasts (two of which were deficient in DNA repair), the best fit was a quadratic relationship for both γ-rays and iron ions; for simple aberrations induced by iron ions in these cells, there was a linear relationship found. In two tumor cell lines, a linear response was defined for simple aberrations for both types of radiation, while the response for complex aberrations was not well-defined by the models that were evaluated (George et al. 2009).

Time-scale

The time scale relationship between radiation exposure and the frequency of CAs has been examined. Most studies search for CAs hours, days, weeks, or even years after exposure to radiation (Loucas and Geard, 1994; Durante et al., 1998; Schmid et al. 2002; Thomas et al. 2003; Tucker et al., 2004; Tucker et al. 2005a; Tucker et al. 2005b; George et al. 2009; Meenakshi and Mohankumar 2013; Arlt et al. 2014; Balajee et al. 2014; Han et al. 2014; Suto et al. 2015; Cheki et al. 2016; Mcmahon et al. 2016; Basheerudeen et al. 2017; Meenakshi et al. 2017; Abe et al. 2018; Jang et al. 2019) ; this makes it particularly difficult to identify CA induction in relation to the deposition of energy by ionizing radiation. There is an account, however, of CAs appearing within 20 minutes of irradiation, with levels peaking at 40 min and plateauing for the remainder of the experiment (up to 100 min) (Mcmahon et al. 2016). CAs have also been documented 2 - 3 h after radiation exposure, with frequency being shown to increase slightly at 24 h (Basheerudeen et al. 2017). CA frequency begins to decrease after exposure, but not all aberrations are repaired (Loucas and Geard, 1994; Durante et al., 1998; Tucker et al., 2004). This process also appears to depend on LET, with strand breaks induced by radiation with a lower LET able to be repaired quicker than those induced by a higher LET (Durante et al., 1998). Furthermore, a study examining CAs in human blood samples for 2 - 7 days following irradiation with γ-rays found that CAs were present at the 2-day mark, but had declined by day 7 (Tucker et al. 2005a; Tucker et al. 2005b) to suspected asymptotic minimum levels (Tucker et al. 2005b). For translocations specifically, the relationship between time and translocation frequency was found to be linear at low doses (0 - 0.5 Gy) and linear quadratic at higher doses (0.5 - 4 Gy) (Tucker et al. 2005b). The sharpest decline over the 7 days was found in dicentrics, acentric fragments, and ring chromosomes (Tucker et al. 2005a).

Interestingly, in vivo radiation exposure has been shown to induce long-lasting CAs in a relatively short time-frame. When lymphocytes from patients undergoing an interventional radiology procedure were compared pre-procedure and 2-3 h post-procedure, there were significant increases in chromatid-type aberrations, chromosome-type aberrations, dicentrics and MN in post-procedure lymphocytes)(Basheerudeen et al. 2017). Similarly, lymphocytes from subjects exposed to radiation 32-41 years prior to blood collection were found to have significantly increased chromosome-type aberrations (acentric fragments, dicentrics and translocations) and MN relative to unexposed controls (Han et al. 2014). Taken together, the results from these two studies suggest that CAs are not only induced within mere h of radiation exposure, but that these radiation-induced CAs may also endure for several decades.

Known modulating factors

Modulating Factor	Details	Effects on the KER	References
Sex	Females	Females were found to have increased aberrant cells and chromosome breaks relative to males.	Maffei et al., 2004
Age	Increased or decreased age	Increases in age were associated with increased CAs. However, it has also been found that young organisms are more sensitive to radiation. One possible explanation for this is that dividing cells are more radiosensitive than those that are quiescent.	Blakely, 2000; Blackely, 2012; Santovito et al., 2013; Vellingiri et al., 2014
Smoking	Smoking status	Smoking was found to increase chromosomal damage. Chromosome breaks were found to be significantly increased in smokers relative to non-smokers. Likewise, blood samples from smokers that were exposed to radon gas had lymphocytes with significantly increased dicentric aberrations, acentric fragments, chromatid breaks, MN, and NPBs relative to lymphocytes from non-smokers also exposed to radon gas.	Maffei et al., 2004; Meenakshi and Mohankumar 2013; Meenakshi et al., 2017
Hyperthermia	Increased temperature	In cells exposed to hyperthermic conditions (41°C for 1 h) followed by radiation (4 Gy), there were significant increases in chromosomal translocations and chromosomal fragments at 1 and at 24 h post-exposure, respectively, as compared to cells exposed only to radiation.	Bergs et al., 2016
DNA ligase IV	Presence	DNA ligase IV helps prevent DNA degradation and increase accurate DNA rejoining, therefore decreasing chromosome breaks and radiation-induced MN.	Smith et al., 2003; Foray et al., 2016
Genetic syndromes	Cockayne syndrome, AT-like disorder, Nijmegen breakage syndrome, Bloom's syndrome, xeroderma pigmentosum, Fanconi anemia, and ataxia telangiectasia	The presence of one of these conditions can increase the number of CAs.	Bender et al., 1988; Foray et al., 2016
Antioxidants or antigenotoxic agents	Increased concentration, examples include dimethyl sulfoxide (DMSO)	The compounds can help decrease the frequency of CAs after irradiation.	Yang, 1999; Kim and Lee, 2007

Known Feedforward/Feedback loops influencing this KER

Not identified.

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Relationship: 1983: Energy Deposition leads to Increase, lung cancer

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deposition of energy leading to lung cancer	non-adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

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 organisms that possess lungs.

Key Event Relationship Description

Ionizing energy can traverse matter to induce biological damage. Tissue regions and cell types that are within depths of the traversable energy particles then have a higher likely hood of becoming transformed into malignant tumours (NRC 1990; Axelson 1995; Jostes 1996; NRC 1999; Kendall and Smith 2002; Al-Zoughool and Krewski 2009; Robertson et al. 2013). This multistep process is initiated by ionizations within the cell (L.E. Smith et al. 2003; Christensen 2014). If these ionizations hit DNA molecules, DNA damage is incurred, possibly in the form of double-strand breaks (DSBs) (J. Smith et al. 2003; Okayasu 2012; Lomax et al. 2013; Rothkamm et al. 2015). Inadequately repaired DNA damage could further lead to mutations and chromosomal aberrations (CAs), which often accumulate in the cell and disrupt the cellular dynamic. If these aberrations affect critical genes involved in the control of cell-cycle checkpoints it can promote uncontrolled cellular proliferation. An abnormally high rate of proliferation in cells of the respiratory tract can lead to lung tumourigenesis (Bertram 2001; Vogelstein and Kinzler 2004; Panov 2005; Hanahan and Weinberg 2011). Radon gas exposure at high levels is especially linked to carcinogenesis of the lung (Axelson 1995; Miller et al. 1996; NRC 1999; Kendall and Smith 2002; Al-Zoughool and Krewski 2009; Robertson et al. 2013).

Evidence Supporting this KER

Biological Plausibility

There is strong biological plausibility for the association between the direct deposition of energy by ionizing radiation and lung cancer incidence. The majority of the evidence is drawn from studies using radon gas as the stressor. Radon, a radioactive noble gas, is considered to be the second leading cause of lung cancer, behind smoking (Robertson et al. 2013; Rodríguez-Martínez et al. 2018; Axelson 1995; Miller et al. 1996; NRC 1999; Kendall and Smith 2002; Al-Zoughool and Krewski 2009; Robertson et al. 2013). Deposited energy from radiation in the form of particles can enter the body most often through inhalation (NRC 1999; Kendall and Smith 2002). These particles can deposit onto lung tissue and decay, producing harmful radiation (Axelson 1995; NRC 1999; Kendall and Smith 2002; Al-Zoughool and Krewski 2009). The radiation can ionize molecules within the cell and initiate the process of lung cancer. There are numerous reviews available detailing the molecular biology involved in lung carcinogenesis (Zabarovsky et al. 2002; Danesi et al. 2003; Massion and Carbone 2003; Panov 2005; Sher et al. 2008; Brambilla and Gazdar 2009; Eymin and Gazzeri 2009; Sanders and Albitar 2010; Larsen and Minna 2011; Santos et al. 2011) and discussing potential therapeutic options for lung cancer patients (Danesi et al. 2003; Massion and Carbone 2003; Sher et al. 2008; Eymin and Gazzeri 2009; PhD and MD 2011; Santos et al. 2011). Briefly there are three cellular steps: initiation, promotion and progression (reviewed by Gilbert 2009). Initiation refers to the interaction between the cell and the cancer-inducing agent, in this case ionizing radiation. The end-result of this interaction is irreversible genetic change(s) (NRC 1990; Pitot 1993). This, in turn, may lead to malfunctions in various pathways and, as the cell continues cycling, increasing genomic

instability (NRC 1990). The promotion phase occurs when a promotor is applied to the irradiated cells and reversibly alters gene expression in an epigenetic fashion (NRC 1990; Pitot 1993), often by binding to its respective receptor (Pitot 1993). The promotor is not carcinogenic if applied alone, but it is capable of enhancing the oncogenic effect of the radiation (NRC 1990). For example, phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is often used as a promotor and was shown to enhance the oncogenic effects of X-ray radiation when applied to C3H/10T½ cells in culture (Kennedy et al. 1978). In some cases, if the dose of the initiator is high enough, the promotion phase may be bypassed altogether (NRC 1990; Pitot 1993). The final irreversible stage of carcinogenesis is progression, which can be boosted by radiation exposure. This is defined as the point at which the benign tumour becomes malignant due to an accumulation of genetic abnormalities, including mutations and chromosomal aberrations. At this point, the tumour grows rapidly due to high rates of cell proliferation, and the levels of genomic instability continue to increase (NRC 1990; Pitot 1993).

Empirical Evidence

There is strong empirical evidence supporting the relationship between direct deposition of energy by ionizing radiation and the development of lung cancer. The evidence presented below is summarized in table 12, here (click link). Biologically based mechanistic models of carcinogenesis have been developed that describe the complex process of maliginancy (Ruhme et al. 2017; Luebeck et al. 1999; Zaballa 2016, Eidemuller 2012; Heidenreich 2012; Jacob 2007, Hazelton 2006; Brugmans 2004 and Heidenreich 2000). There is a vast number of reviews that provide evidence of this association (Axelson 1995; Jostes 1996; NRC 1999; Kendall and Smith 2002; Al-Zoughool and Krewski 2009; Robertson et al. 2013; Sheen et al. 2016; Chadwick, 2017; Clement et al. 2010; UNSCEAR 2019). Overall, there is strong empirical evidence available supporting dose and incidence concordance, strong epidemiological data, and strong support for temporal concordance.

Figure 1: *Plot of studies (y-axis) against equivalent dose (Sv) used to determine the empircal link between direct deposition of energy and increased rates of lung cancer. The y-axis is ordered from low LET to high LET from top to bottom.*

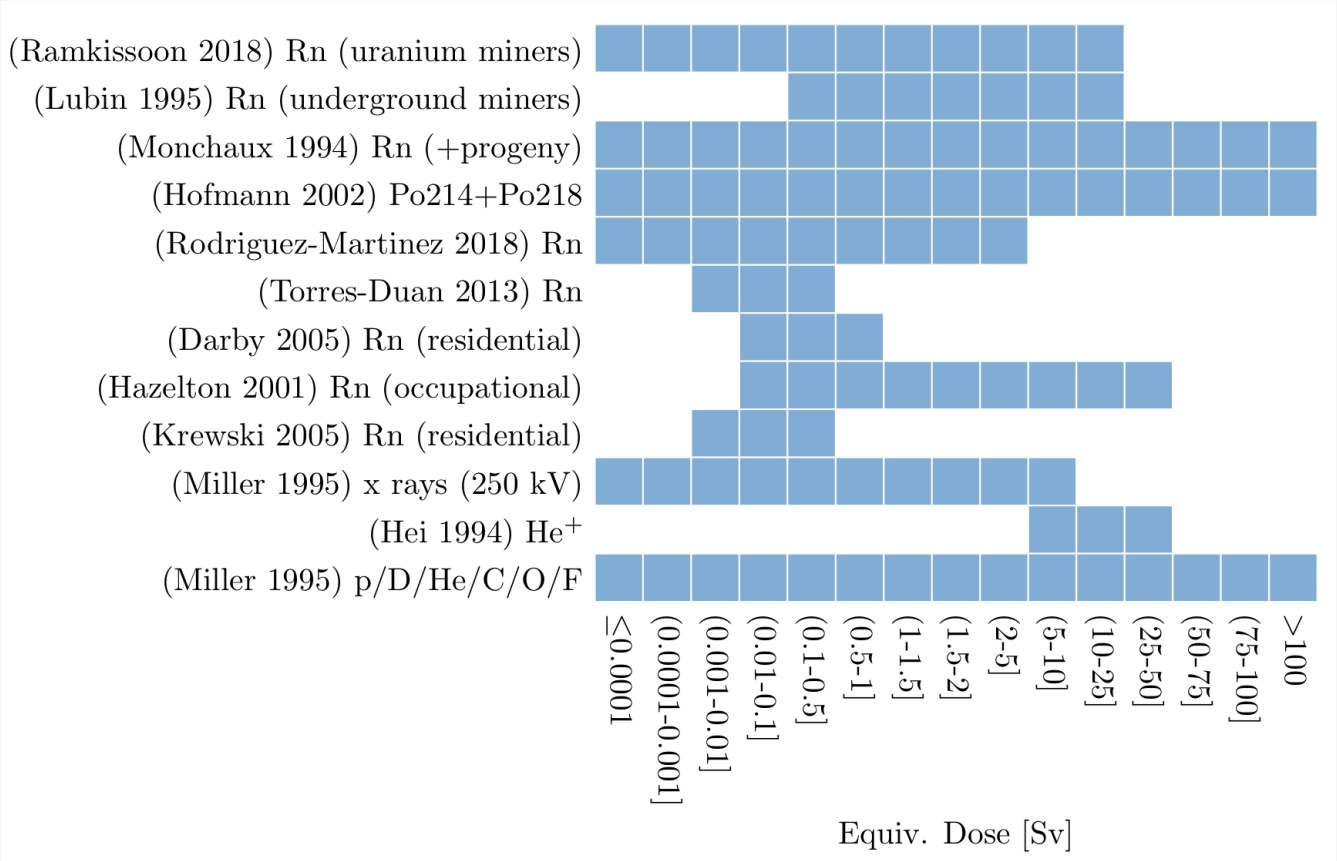
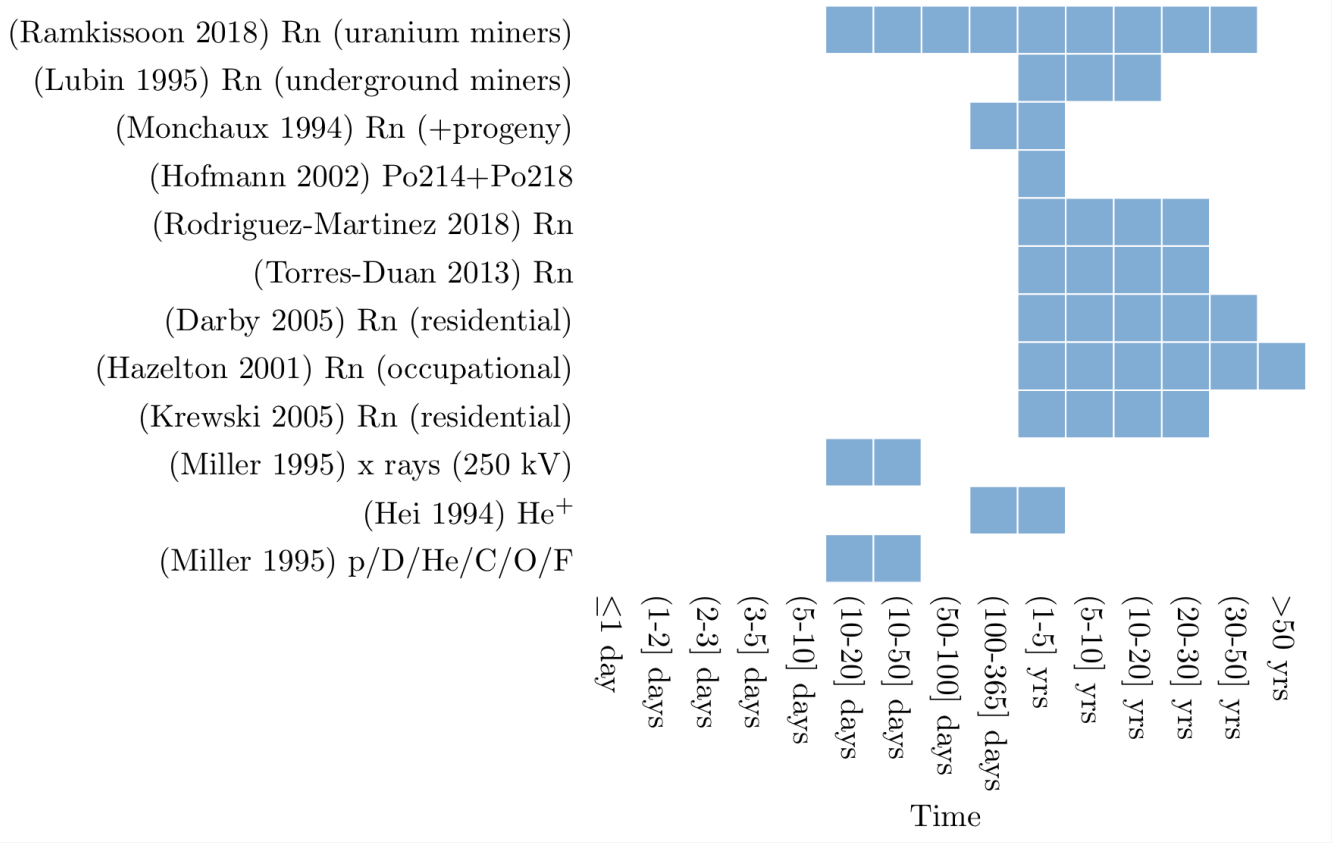


Figure 2: Plot of studies (y-axis) against time over which studies were conducted for a temporal response used to determine the empircal link between direct deposition of energy and increased rates of lung cancer. The y-axis is ordered from low LET to high LET from top to bottom.



Dose and Incidence Concordance

There are numerous studies available that provide evidence supporting a dose/incidence concordance between the direct deposition of energy by ionizing radiation and the incidence of lung cancer. Several *in vitro* studies showed that cells could be induced to obtain oncogenic characteristics through radiation exposure (Hei et al. 1994; Miller et al. 1995); these oncogenic transformations also increased in a dose-dependent manner in some cases (Miller et al. 1995). Likewise, irradiation of rats at levels comparable to those experienced by uranium miners (25 - 3000 WLM of radon and its progeny) resulted in a dose-dependent increase in lung carcinoma incidence (Monchaux et al. 1994). Furthermore, a simulation model was developed that predicted an increased probability of a lung tumour after exposure to radiation doses ranging from 0 - 800 WLM (Hofmann et al. 2002). Munley et al. (2011) exposed bi-transgenic CCSP-rtTA/Ki-ras mice 80–160 mGy and showed staistically significant increase in the frequency of lung cancer incidence with a higher number in females. Evidence also exists for a dose-dependent linear relationship between excess relative risk of lung cancer and exposure to other non-radon forms of ionizing radiation such as that from survivors of the atomic bombs at Hiroshima and Nagasaki (Cahoon et al., 2017).

Human Epidemiological Studies

Indoor Radon Exposure

Indoor radon levels and lung cancer risk has been studied to establish a relationship between indoor radon exposure and lung carcinogenesis. The World Health Organization (WHO) recommends residential radon levels to be 100 Bq/m^3 or less. This is based on the results of the two most relevant, large-scale residential radon studies, encompassing populations across Europe (Darby et al. 2005) and North America (Krewski et al. 2005; Krewski et al. 2006). These studies found a positive association between indoor radon exposure and lung cancer risk (Darby et al. 2005; Krewski et al. 2005; Krewski et al. 2006). A systematic review of never-smokers exposed to residential radon encompassing 14 studies across the world suggested a possible increased risk of lung cancer. There were, however, inconsistencies in results across studies (Torres-Durán et al. 2014), predominately due to confounders and dosimetric calculations.. In a review published by Sheen et al. (2016), analysis of results from 24 case-control studies examining residential radon exposure and lung cancer risk also found conflicting results. A portion of the studies did, however, show a significantly increased risk in subjects exposed to higher radon concentrations.

Outdoor Radon Exposure

In terms of outdoor radon exposure, most data is derived from studies of lung cancer in miners occupationally exposed to radon. Overall, there seems to be a positive association between radon exposure and lung cancer risk. This was evident in a cohort of uranium miners who worked in mines in Ontario, Canada between 1954 and 1996 (Ramkissoon et al. 2018); in tin miners from Yunnan, China followed from 1976 - 1988 (Hazelton et al. 2001); and in a pooled analysis of underground miners of various nationalities from 11 cohort studies (Lubin et al. 1995); in a recent cohort study of pooled uranium miners analysis, also known as PUMA (Rage et al., 2020). Furthermore, a section of a review paper that examined epidemiological studies of radon-exposed miners found a positive, statistically significant association between radon exposure and lung cancer risk in all studies examined (Al-Zoughool and Krewski 2009, UNSCEAR 2006). In one study looking at the histopathology of lung carcinoma in a cohort of German uranium miners, it was shown that the relative frequency of specifically SqCC was associatd with increased expousre to radon (Kreuzer et al., 2000). This was confirmed in a later study, which showed increased relative frequency of both SqCC and SCLC when compared to AC (Taeger et al., 2006).

Indoor and Outdoor Radon Exposure

The association between lung cancer risk, and radon exposure was further fortified by a large systematic review that synthesized results from 16 studies of both indoor and outdoor exposure. This systematic review analysed results from studies of miners, pooled population studies and case control studies. Overall, there was a positive association found between radon exposure and SCLC risk (Rodríguez-Martínez et al. 2018). Results from these epidemiological studies suggest that radon exposure is linked to lung carcinogenesis, whether people are exposed indoors or outdoors.

Temporal Concordance

There is empirical evidence supporting that the direct deposition of energy by ionizing radiation precedes the development of lung cancer. In cells that were oncogenically transformed by irradiation, the oncogenic characteristics were detected weeks after the irradiation (Hei et al. 1994; Miller et al. 1995). Similarly, tumours induced in nude mice by inoculation with oncogenic cells took months to grow (Hei et al. 1994), while lung cancer in rats exposed to radon were not detected for months to years (Monchaux et al. 1994). In humans, the risk of lung cancer was also found to increase with increasing time since exposure (Hazelton et al. 2001; Cahoon et al., 2017) and with longer periods of exposure (Lubin et al. 1995). In addition, the lung cancer simulation model developed by Hofmann (2002) was based on an average radiation exposure time of 4 years. One study using distributed lag non-linear models (DLNMs) showed that risk onset began at about 2 years following initial exposure to radon in uranium miners, with the mean lag period being 15 years (Aßenmacher et al., 2019).

Essentiality

Not identified.

Uncertainties and Inconsistencies

Uncertainties and inconsistencies in this KER are as follows:

1. Studies have shown that dose-rates (Brooks et al. 2016) and radiation quality (Nikjoo et al. 1997; Sutherland et al. 2000; Jorge et al. 2012) are factors that can influence the dose-response relationship.
2. Low-dose radiation has been observed to have beneficial effects and may even invoke protection against spontaneous genomic damage and induced mutations (Feinendegen 2005; Day et al. 2007; Feinendegen et al. 2007; Shah et al. 2012; Nenoi et al. 2015).
3. Deposition of ionizing energy is a stochastic event; as such, the nucleus is not the only region that may be affected by radiation exposure. *In vitro* evidence has shown that ionizing radiation may also cause genotoxic effects when deposited in the cytoplasm (Wu et al. 1999).
4. When analyzing the relationship between radiation exposure and lung cancer in miners, other confounding carcinogen exposures, including silica, diesel engine exhaust, arsenic and tobacco, should also be accounted for (Cocco et al. 1994; Hazelton et al. 2001; Cao et al. 2017).
5. There are inherent difficulties in measuring radon exposures in the general public. Residential radon levels are measured using alpha trackers, but people all have different lifestyles and spend differing amounts of time in their home. Furthermore, it is very common for people to move from home to home. These factors challenge the ability to accurately estimate an individual's radon exposure and thus to extrapolate this to lung cancer risk (Axelson 1995; Robertson et al. 2013).
6. While some of the epidemiological studies summarized in a systemic review by Torres-Duran et al (2014) showed an association between residential radon exposure and lung cancer, others did not. This is a result of uncertainties in dosimetric considerations, radon exposure levels, confounders such as smoking
7. There has been controversy surrounding the ICRP-reported dose coefficients being used to estimate risk from radon exposure. These coefficients were different across several ICRP reports and thus gave different estimates of risk for an identical radon exposure scenario. A report by Muller (2016) highlighted these controversies and summarized the results of a radon workshop addressing the situation (Müller et al. 2016).
8. A paper by Zarnke (2019) critiques the conclusions drawn by the BEIR VI report regarding radon exposure and health effects. Based upon the authors' analyses, radon exposure in the home is not linked to lung cancer, and may in fact be protective against smoking-induced lung cancer.

Quantitative Understanding of the Linkage

There are many epidemiological studies available that provide quantitative data linking radiation exposure with lung cancer risk, incidence and/or death. Results from several of these studies are summarized in the table below.

Reference	Summary
UNSCEAR, 2000	Study of general population and its exposure to low LET radiation with a dose of 1 Sv. Study found a lifetime risk estimate for solid cancer mortality of 9% for men and 13% for women.
EPA, 2003	Study of the general population (USA) exposed to residential levels of radon found a lung cancer deaths linked at 14.3% (in 1995) at a risk per unit of radon exposure as $5.38x10^{-4}$ per WLM. Two further studied sampled from strictly non- and smoking-populations for similar levels of residential radon exposure. From the smoking population the risk per unit radon exposure was higher, $9.68x10^{-4}$ per WLM compared to the non-smoking population; $1.67x10^{-4}$ per WLM.
Darby, 2005	Study covering 13 European cohorts who were exposed to residential levels of radon found that lung cancer risks increases by 8.4% per 100 Bq/m^3.
Krewski, 2006	7 North American cohorts were studied who were exposed to residential levels of radon. It was found that the odds ratio, OR(x) = 1 + 0.00096x. The odds ratio for subjects living in 1-2 residences with 20+ years of radon monitoring. OR(x) = 1 + 0.00176x.
Grundy et al., 2017	Study of the general population of Alberta (Canada) exposed to residential levels of radon in the range of 71.0 Bq/m3 (Alberta mean). Study found that overall, lung cancer deaths linked to radon were 16.6% (324 excess attributable cases). Ever smoker lung cancer deaths linked to radon: 15.6% (274 excess attributable cases). and never smoking lung cancer deaths linked to radon were: 24.8% (48 excess attributable cases).
Peterson et al., 2013	A study of the general population in Ontario exposed to residential levels of radon (Ontario mean: 43 Bq/m3) found the % of lung cancer deaths linked to radon as being 13.6% (847 cases). Ever smoker lung cancer deaths linked to radon: 15.6% (274 excess attributable cases). Never smoker lung cancer deaths linked to radon: 24.8% (48 excess attributable cases).
	Study of the general population in Sweden exposed to residential levels of radon. Study found relative risk

Lagarde et al., 2001	of lung cancer at different exposure levels: 50 Bq/m3: 1.08 with a confidence interval of 0.8-1.5. 80 Bq/m3: 1.18 with a confidence interval of 0.9 - 1.6. 140 Bq/m3: 1.44 with a confidence interval of 1.0 - 2.1. Overall excess relative risk: 10% per 100 Bq/m3.
Torres-Duran et al., 2014	Study of the general population (obtained through a systemic review of 14 studies) of residential radon exposure *TABLE 2*
Al-Zoughool and Krewski, 2009	*TABLE 2*

Response-response relationship

Overall, studies suggest that there is a positive relationship between radiation exposure and lung cancer risk. A direct basis for the link has been provided by epidemiological studies in miners occupationally exposed to radon (UNSCEAR 2006, Lubin et al. 1995; Ramkissoon et al. 2018). In a study of tin miners exposed to radon, there was an increasing risk of lung cancer with increasing radon exposure (Hazelton et al. 2001). This positive relationship has likewise also been found in residential radon studies (Darby et al. 2005; Krewski et al. 2005; Krewski et al. 2006). A large systemic review encompassing miner cohort studies, pooled population studies, and case-control studies showed a strong association between residential radon concentration and lung cancer (Rodríguez-Martínez et al. 2018). Mechanistic in vitro (Miller et al. 1995) and in vivo (Monchaux et al. 1994) experimental models also provide data to support this relationship.

Time-scale

There is some quantitative data available regarding the time scale between radiation exposure and the development of lung cancer. *In vitro* oncogenic transformations were evident 6 weeks after cells were irradiated with X-rays or charged particles of varying LETs (Miller et al. 1995). Similarly, irradiated, tumourigenic bronchial epithelial cells were able to induce tumour growth within 13 weeks of injection into nude mice; tumours reached a size of 0.6 - 0.7 cm by 6 months post-inoculation. In comparison, unirradiated implanted cells did not induce tumour growth (Hei et al. 1994). Epidemiology studies also suggest that lung cancers are detected years after exposure to radiation (Lubin et al. 1995; Darby et al. 2005; Torres-Durán et al. 2014; Rodríguez-Martínez et al. 2018; Ramkissoon et al. 2018). Exposure to radon for longer periods of time predicts an increased relative risk of lung cancer; this risk increased with increasing duration of exposure over 5, 10 and 20 years (Lubin et al. 1995). In a study of tin miners, there were sharp increases in risk at approximately 40 years since first exposure and approximately 40 years since last exposure (Hazelton et al. 2001).

Known modulating factors

There are several agents, summarized in the NRC 1990 report, that may affect radiation-mediated oncogenic transformations/carcinogenesis. Some agents can enhance the effects of radiation to increase the accumulation of oncogenic characteristics. These include hydroxyurea and 12-O-tetradecanoyl-phorbol-acetate (TPA) (NRC 1990). The effects of hydroxyurea were seen within 11 hours of treatment (Hahn et al. 1986), while the effects of TPA were evident both immediately following irradiation, and up to 96 hours post-irradiation (Kennedy et al. 1978). Other agents may reduce the effectiveness of radiation-induced malignant transformations. Suppressors of radiation-mediated oncogenic transformations include antipain (a protease inhibitor), selenium, and 5-aminobenzamide. Hormone levels may also have an effect on the radiation-carcinogenesis relationship. For example, high levels of thyroid hormone T3 worked synergistically with radiation to enhance oncogenic characteristics, while low T3 levels antagonized the effects of radiation (NRC 1990). Studies have also discussed sex as a modulating factor to radon induced lung cancer. Kim et al. 2016 reported that the proportion of lung cancer deaths induced by radon was slightly higher in females but after stratifying for smoking, the attributable risk of lung cancer death was similar between gender. A review analyzing sex differences of radiation response, generally found that the excess relative risk for lung cancer was higher in females than males when workers were exposed to plutonium at the Mayak nuclear facility (Narendran et al. 2019). Similarily, a higher excess relative risk for lung cancer was found in females after Japanese atomic bomb exposure (Cahoon et al. 2017; Ozasa et al. 2012)

Known Feedforward/Feedback loops influencing this KER

Not identified.

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Relationship: 1931: Increase, DNA strand breaks leads to Increase, Mutations

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

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 processes, such as non-homologous end joining (NHEJ) and homologous recombination (HR), in preventing chromosomal aberrations/genomic instability is extensively discussed in literature and many reviews are available (van Gent et al., 2001; Ferguson and Alt, 2001; Hoeijmakers, 2001; Iliakis et al., 2004; Povirk, 2006; Weinstock et al., 2006; Natarajan and Palitti, 2008; Lieber et al., 2010; Mehta and Haber, 2014; Ceccaldi et al., 2016; Chang et al., 2017; Sishc and Davis, 2017; Brunet and Jasin, 2018).

In addition, attempted repair of DSBs can lead to chromosomal aberrations such as translocations; NHEJ is a recognized source of oncogenic translocations in human cancers (Ferguson and Alt, 2001; Weinstock et al., 2006; Byrne et al., 2014; Brunet and Jasin, 2018), and a contributor to the carcinogenic process (Hoeijmakers, 2001; Sishc and Davis, 2017). Other types of chromosomal aberrations can serve as indicators of genomic instability that can contribute to a variety of adverse health effects including neurodegeneration (Madabhushi et al., 2014).

Empirical Evidence

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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Relationship: 1984: Increase, Mutations leads to Increase, lung cancer

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deposition of energy leading to lung cancer	non-adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High

The domain of applicability applies to mammals, including rodents and humans.

Key Event Relationship Description

A mutation occurs when there is a change in the DNA sequence. In some cases, mutations are silent and do not cause any functional changes in the cell; in other cases, mutations may have catastrophic consequences. If these errors occur in genes implicated in critical regulatory pathways such as DNA repair mechanisms, cell-cycle checkpoints, apoptosis, or telomere length genes, then the cells are generally more susceptible to carcinogenesis (Chen et al. 1990; Hei et al. 1994; Kronenberg et al. 1995; Zhu et al. 1996, NRC 1999). The result of disrupting these regulatory pathways is ultimately the abnormal accumulation of malignant cells that may lead to cancer. Lung cancer in particular may occur if catastrophic mutations occur in cells of the lung.

Evidence Supporting this KER

Biological Plausibility

The biological rationale for linking mutations to lung cancer is strongly supported by the literature. Numerous studies and reviews are available on this topic.

There is evidence that mutation patterns may be specific to cancer type. Results from large bioinformatics-based studies have suggested that each cancer may have a characteristic mutation fingerprint. Twenty-one mutation signatures were detected upon analysis of approximately 7000 samples with nearly 5 million mutations across 30 different cancer categories, with each cancer type displaying a different profile of mutation signatures (Alexandrov et al. 2013). Similarly, analysis of approximately 2100 genomes across 9 different cancers also identified numerous mutation signatures that, in combination, were able to differentiate between cancer types (Jia et al. 2014). Lung

adenocarcinoma and lung squamous cell carcinoma, for example, shared two of the same mutational signatures, but were ultimately found to have different overall profiles; lung adenocarcinoma had four mutation signatures, and lung squamous cell carcinoma had three (Alexandrov et al. 2013; Jia et al. 2014). Likewise lung small cell carcinoma had only two signatures, one of which was associated with smoking and was shared with both lung adenocarcinoma and lung squamous cell carcinoma (Alexandrov et al. 2013). There were also 39 significant associations found between mutational signatures and driver mutations upon analysis of nearly 8000 cancer exomes across 26 types of cancer, suggesting that the mutation signatures may be informative as to biological processes occurring in cancer (Poulos et al. 2018).

Mutations are thought to be at the heart of many of the features associated with tumours. In a report on the hallmarks of cancer by Hanahan and Weinburg (2011), the six original hallmarks were identified as sustained proliferative signalling, evading growth receptors, activating invasion and metastasis, enabling replicative memory, inducing angiogenesis, and resisting cell death; two new emerging hallmarks of cancer were identified as deregulating cellular energetics and avoiding immune destruction. One of the 'enabling characteristics' proposed to be underlying these key cancer hallmarks was genome instability/mutations (Hanahan and Weinberg 2011). This suggests that many of the processes involved in tumourigenesis are facilitated by accumulating mutations that confer a survival advantage to the cells, allowing for the development of cancer (Vogelstein and Kinzler 2004; Hanahan and Weinberg 2011). Cancer thus arises from a large accumulation of genetic abnormalities over time, rather than one single detrimental mutation (Vogelstein and Kinzler 2004); these abnormalities may occur at the level of the nucleotides, the chromosomes, or the transcriptome (Larsen and Minna 2011). Many of the cancer-enabling mutations are found in tumour suppressor genes (TSGs), proto-oncogenes, or caretaker/stability genes (Vogelstein and Kinzler 2004; Larsen and Minna 2011).

TSGs have been compared to the brakes in a car (Vogelstein and Kinzler 2004), as they are genes that typically prevent proliferation and, in some cases, promote apoptosis. They thus play an important role in negatively regulating cellular growth. This preventative function is especially important in situations where DNA is damaged, as the products of TSGs will stop the cell from undergoing mitosis and may even initiate apoptotic pathways in order to avoid the propagation of damaged DNA. Mutations that reduce the activity of or completely inactivate TSGs may thus promote tumourigenesis by removing cell proliferation checkpoints and blocking apoptotic pathways (Vogelstein and Kinzler 2004; Panov 2005; Sanders and Albitar 2010). For TSGs to contribute to cancer development, however, generally both copies of the allele must be disrupted (Vogelstein and Kinzler 2004; Larsen and Minna 2011); this typically occurs through the loss of an entire chromosomal segment containing one allele and an inactivating or activity-reducing mutation that occurs in the second allele (such as missense mutations in a critical residue, mutations that produce a truncated protein, or deletions/insertions) (Vogelstein and Kinzler 2004). In lung cancer, some of the commonly inactivated TSGs include *TP53, RB1, STK11, CDKN2A, FHIT, RASSF1A* and *PTEN* (Larsen and Minna 2011).

If TSGs are the brakes for cellular proliferation, proto-oncogenes have been described as the gas pedal (Vogelstein and Kinzler 2004). Mutations in proto-oncogenes that render these genes constitutively or abnormally active may result in high rates of cellular proliferation,, thus supporting tumourigenesis (Vogelstein and Kinzler 2004; Larsen and Minna 2011). These mutations could be in the form of chromosomal translocations, gene amplifications, or mutations that affect critical segments for activity regulation. In contrast to TSGs, an activating mutation in one allele is often adequate to increase proliferation rates in the cell (Vogelstein and Kinzler 2004). Thus mutations in proto-oncogenes are frequently found in cancers, particularly in solid tumours such as non-small cell lung carcinoma (NSCLC) (Danesi et al. 2003). Some commonly activated proto-oncogenes in lung cancer include *EGFR, ERBB2, MYC, KRAS, MET, CCND1, CSK4, MET,* and *BCL2* (Larsen and Minna 2011) .

Overall, TSGs and proto-oncogenes are similar in that they both increase the number of tumour cells through increasing proliferation, decreasing cell death, or by increasing angiogenesis in the area (thus enabling nutrient delivery) (Vogelstein and Kinzler 2004). In addition, mutations to caretaker/stability genes may also play a role in promoting cancer. These genes function differently from TSGs and proto-oncogenes in tumourigenesis in that they facilitate the accumulation of mutations. In normal situations, caretaker/stability genes are involved in the detection, repair and prevention of DNA damage (Vogelstein and Kinzler 2004; Hanahan and Weinberg 2011). Genes involved in mismatch repair (MMR), nucleotide excision repair (NER) and base-excision repair (BER) pathways are examples of caretaker/stability genes (Vogelstein 2004). Mutations in these genes may compromise aspects of DNA repair—the detection of damage, the initiation of repair, the repair process itself, or the removal of mutagens that could possibly damage DNA—thus allowing for more mutations to accumulate than usual (Hanahan and Weinberg 2011). All genes across the genome are equally susceptible to gaining increased mutations when caretaker/stability genes are not functioning properly; however, only mutations that affect TSGs and proto-oncogenes contribute to tumourigenesis (Vogelstein and Kinzler 2004). Similar to TSGs, generally both alleles of the caretaker/stability genes must be disrupted for the gene function to be lost (Vogelstein and Kinzler 2004; Hanahan and Weinberg 2011).

According to the COSMIC database (https://cancer.sanger.ac.uk/cosmic) the TSG *TP53* and the proto-oncogenes *KRAS* and *EGFR* are identified as the top three mutations found in lung cancer. Numerous epidemiological reports and analyses of lung tumours have confirmed this finding, as have many studies involving *in vitro* and *in vivo* manipulation of these genes.

The transcription factor *TP53* is amongst the most commonly mutated TSGs in not only lung cancer (Varella-garcia 2009; Sanders and Albitar 2010; George et al., 2015) (COSMIC database, https://cancer.sanger.ac.uk/cosmic), but also human cancers in general (Iwakuma 2007, Kim 2018, Hollstein 1991). *TP53*, which produces the protein p53, plays a role in controlling cell cycling and promoting apoptosis in times of cellular or genotoxic stress (Danesi et al. 2003; Vogelstein and Kinzler 2004; Panov 2005; Iwakuma and Lozano 2007; Varella-garcia 2009; Larsen and Minna 2011; Cortot et al. 2014; Kim and Lozano 2018). It acts as a checkpoint for passing into the G2 phase of the cell cycle in order to prevent cells with damaged DNA from undergoing mitosis (Danesi et al. 2003; Panov 2005). If DNA damage is detected by p53 at this checkpoint, p53 is responsible for arresting the cell and either activating genes responsible for DNA repair or activating apoptotic pathways (Panov 2005; Larsen and Minna 2011). Mutations in *TP53* that disrupt the function of p53 thus allow for unrestricted cellular proliferation and promotion of tumourigenesis (Iwakuma and Lozano 2007; Kim and Lozano 2018), as cells are no longer stopped at the G2 checkpoint. Loss of this checkpoint also supports tumourigenesis by allowing potentially damaged DNA to be used in mitosis, thus increasing the likelihood of mutation accumulation (Danesi et al. 2003). The function of p53 may be disrupted by complete deletion of *TP53*, but it is more often affected by mutations, especially missense rather than null mutations. In the case of mutant p53 production, the altered protein may be able to bind to different partners and alter the expression of different genes, thus displaying a gain-of-function phenotype (Kim and Lozano 2018).

Mutations in *TP53* are very common in lung cancer (Varella-garcia 2009), occurring in more than two-thirds of patients (Massion and Carbone 2003). In one study that looked at the genomes of small cell lung cancer (SCLC) samples, 100% of those without chromotrhipsis were found to have mutations in the TP53 gene (George et al., 2015). Mutant *TP53 is* especially common in smokers and in aggressive tumours (Varella-garcia 2009). It is thought that loss of p53 function is an early occurrence in lung cancer, and may be associated with deregulation of telomerase activity (Danesi et al. 2003). Low levels of $p14^{arf}$, which is the product of *CDKN2A* (Cortot et al. 2014), another commonly mutated TSG in NSCLC (Sanders and Albitar 2010), and SCLC (George et al., 2015), may further exacerbate the cellular consequences of a mutated p53. Normally, $p14^{arf}$ plays a role in stabilizing and activating p53; tumourigenesis is thus particularly encouraged when mutations are present that cause not only the downregulation of $p14^{arf}$ and/or p53, but also the upregulation of proto-oncogenes (Cortot et al. 2014).

KRAS is one of the most commonly mutated members of the RAS family in lung cancer (Varella-garcia 2009; Sanders and Albitar 2010). Mutations in this gene have been reported in at least 20% of NSCLC cases (Massion and Carbone 2003; Sanders and Albitar 2010; Cortot et al. 2014; Wang et al. 2018), and are most frequently found in lung adenocarcinomas (Massion and Carbone 2003). *KRAS* is classified as a proto-oncogene and encodes a G-protein that plays an important role in signal transduction, especially in differentiation, proliferation and survival pathways (Varella-garcia 2009). When a signal that promotes cellular growth is detected, KRAS, which is attached to the inner side of the cellular membrane, is activated and binds to GTP. Using its inherent GTPase activity to hydrolyze GTP to GDP, KRAS interacts with its downstream partner, Raf 1, before returning to an inactive state. The signal, meanwhile, is propagated all the way to the nucleus by downstream kinases, eventually leading to the activation and translocation of MAPK to the nucleus to stimulate pro-proliferation activities. Mutations in *KRAS* may result in GTPase errors such that GTP remains bound to KRAS (Panov 2005) and the protein remains constitutively active, thus extending pro-proliferative signalling indefinitely (Panov 2005; Varella-garcia 2009). Mutated *KRAS* may also play a role in mediating cell invasion through epithelial mesenchymal transition (EMT), as seen in cases of NSCLC (Wang et al. 2018). This is supported by a study in which *KRAS* was identified as a cancer driver in cell invasion, as well as pathways related to hypoxia, inducing angiogenesis, and blocking apoptosis (Cava et al. 2018).

EGFR is classified as a receptor tyrosine kinase and a proto-oncogene. When activated by phosphorylation, EGFR plays an important role in stimulating cellular proliferation and survival using the RAS-REF-MEK and PI3K-AKT-mTOR pathways (Danesi et al. 2003; Varella-garcia 2009; Sanders and Albitar 2010). When inactive, these receptors exist in monomeric form; upon binding of a ligand, receptors will homo- or hetero- dimerize to active the tyrosine kinase domain. This leads to autophosphorylation, and a downstream signalling cascade that eventually results in pro-proliferative activities in the nucleus (Danesi et al. 2003). Mutations affecting this pathway may support tumourigenesis (Danesi et al. 2003; Sanders and Albitar 2010) by increasing cellular proliferation, inducing angiogenesis, stimulating metastasis and inhibiting apoptosis (Danesi et al. 2003). In lung cancer specifically, *EGFR* mutations have been found in approximately one third of adenocarcinoma patients (Cai et al. 2013; Cortot et al. 2014). In a study composed only of non-smoker NSCLC patients, *EGFR* mutations were likewise present in nearly half of the patients (Kim et al. 2012). Most *EGFR* mutations result in overexpression of EGFR (Varella-garcia 2009). In general, lung cancer patients with mutations resulting in the amplification of *EGFR* have a more negative prognosis (Varella-garcia 2009; Sanders and Albitar 2010).

Cancers are also known to obtain specific driver mutations that play a major role in tumourigenesis and help to drive carcinogenic pathways. Driver mutations allow for the continued aberrant signalling by mutated proteins, and as such, they sustain tumour growth. In NSCLC, important driver mutations include rearrangements in *ALK, RET,* and *ROS1*; mutations in *AKT1, BRAF, DDR2, EGFR, HER2, KRAS, MEK1, NRAS, PIK3CA,* and *PTEN*; and amplifications in *FGFR1* and *MET*. In general, the majority of NSCLC tumours harbour only one of these driver mutations (Larsen and Minna 2011).

The presence of these mutations may affect several signalling pathways associated with cancer development. Examples include the PI3K-AKT-mTOR pathway and RAS-REF-MEK pathway. Mutations affecting factors involved in the PI3K-AKT-mTOR pathway tend to result in increased cell proliferation, growth and survival; thus mutations that cause constitutive or uncontrolled activation of this pathway may result in tumour growth (Varella-garcia 2009; Sanders and Albitar 2010; Larsen and Minna 2011). For example, activating *PIK3CA* mutations and inactivating *PTEN* mutations are associated with increased activity of the PI3K-AKT-mTOR pathway(Sanders and Albitar 2010; Larsen and Minna 2011); the opposite effects are shown when *PIK3CA* is inhibited (Kang et al., 2005; Cheng et al., 2014). Activity of this signalling pathway can also be stimulated by interactions involving IGF1R, PDGF, EGFR, EGF, TNF-alpha, PI3Ks, PDK-1 and Akt/PKB (Varella-garcia 2009). Specifically in lung cancer, this pathway is thought to be activated relatively early in the pathogenesis process (Larsen 2011). Similarly, activity of the RAS-REF-MEK pathway helps to direct cell growth, differentiation, and prevent apoptosis (McCubrey et al., 2006). This pathway functions through activated receptor tyrosine kinases, which allow RAS GTPases to bind GTP and ultimately activate MEK and ERK signalling cascades. Alterations to this pathway, such as the presence of activating *KRAS* mutations that cause irreversible binding of GTP and thus increased signalling activity, may result in tumour formation (Sanders and Albitar 2010)(; McCubrey et al., 2006). This pathway is often found to be activated in lung cancer, especially when *KRAS* obtains activating mutations (Larsen and Minna 2011).

Empirical Evidence

There is moderate empirical evidence supporting the relationship between the frequency of mutations and the incidence of lung cancer. The evidence presented below is summarized in table 10, here (click link). There is little empirical evidence available supporting a dose and incidence concordance, some empirical evidence supporting a temporal concordance, and strong empirical evidence supporting essentiality. Several review papers provide summaries of the relationship between these two key events. Genetic abnormalities found in lung cancer that result in genomic instability are discussed by Massion (2003). Several radon-specific review papers are also available that discuss available evidence for the link between radon exposure, mutation induction and lung carcinogenesis across a variety of models (Jostes 1996; Robertson et al. 2013).

Dose and Incidence Concordance

There is a lack of empirical evidence to show dose and incidence concordance between mutations and lung cancer, particularly in the field of ionizing radiation. As described above, numerous studies are available that highlight mutation signatures in different tumours with strong evidence linking specific mutations to cancer incidence, radiation exposure to mutation frequency, and radiation exposure to cancer incidence. However, there is a lack of studies that assess whether increasing doses of a stressor, such as ionizing radiation, translate into dose-dependent changes in mutation frequencies and dose-dependent changes in cancer incidences.

Attempts were thus made to identify studies using similar radiological and biological conditions that assessed either mutation frequency or cancer incidence independently. Using this strategy, two studies were found that addressed the link between mutations and cancer with increasing doses of radiation. In these two complementary studies, a microbeam system was used to precisely and selectively expose the nuclei of cells to a specific number of alpha particles. Upon exposure to 1 - 8 individual alpha particles, there was a dose-dependent increase in the number of $S1^{-}$ mutations in hybrid hamster-human cells (A_L) (Hei et al. 1997). This correlated well with a study conducted by Miller et al., that showed an increase in the frequency of oncogenic transformations in mouse fibroblasts (C3H10T1/2) within that same dose range (Miller et al. 1999).

Likewise, side-by-side comparisons of other studies using comparable radiation doses and biological systems also provide evidence of a dose-dependent relationship between mutation frequency and oncogenic potential. Exposure of two different cell lines, Chinese hamster embryonal lung fibroblasts and normal human bronchial epithelial cells, to gamma-ray radiation at doses between 0 and 6 Gy resulted in dose-dependent increases in mutations in both cell types (Suzuki and Hei 1996; Canova et al. 2002). Radiation in the range of 0 and 6 Gy can thus induce dose-dependent increases in mutation frequencies; *in vitro* treatment with similar radiation doses can also evoke cancer-like changes. For example, exposure of bronchial epithelial cells to 0.3 or 0.6 Gy of radiation from helium-4 ions resulted in cells with tumour-like characteristics (Hei

et al. 1994)). Similarly, C3H10T1/2 fibroblasts exposed to several types of ions at varying LETs displayed dose-dependent increases in oncogenic transformations between 0 - 1 Gy for all radiation conditions tested (Miller et al. 1999).

Analyses of lung cancer incidences in radon-exposed rats and humans equally echo these results. There was a dose-dependent increase in lung cancer incidence in rats exposed to radon and radon progeny at levels of 25 - 3000 working level months (WLM) (Monchaux et al. 1994). (One WLM is calculated based on 170 hours of exposure to one working level (WL), and 1 WL refers to the equivalent of 1.3 x10^5 MeV of alpha particle energy in 1 L of air.) Damage from 1 WLM is thought to be equivalent to 0.8 - 10.0 mGy (Jostes 1996), which corresponds to 100 - 1250 WLM/Gy; thus 25 WLM is equivalent to 0.02 - 0.25 Gy, and 3000 WLM is equivalent to 2.4 - 30 Gy. In epidemiological studies of uranium miners exposed to radon radiation within this exposure range, there was a dose-dependent increase in the relative risk of lung cancer with increasing cumulative radon exposure (Lubin et al. 1995; Ramkissoon et al. 2018).

Further support for this relationship can be derived from a study using a known tobacco carcinogen, NNK. Exposure of *Gprc5a* knock-out mice to NNK increased both the somatic mutation burden and the rate of tumourigenesis in the lungs of these mice relative to saline-treated controls (Fujimoto et al. 2017).

There are also several studies showing that successive addition of mutations *in vitro* or *in vivo* result in increased oncogenic potential. The sequential accrual of mutations in *TP53*, *KRAS*, and *EGFR* to immortalized human bronchial epithelial cells resulted in cells that were increasingly more oncogenic (Sato et al. 2006). In a similar study using small airway epithelial cells, the accumulation of *hTERT, CDK4, p53* and *KRAS* manipulations plus the addition of manipulations to either *PIK3KA, CYCLIN-D1*, or *LKB2* was successful in producing fully malignant cells (Sasai et al. 2011). Furthermore, *in vivo* mouse models that required Cre to induce mutations selectively in the lungs also found a relationship between the induction of mutations and lung tumours. In bitransgenic mice engineered such that ingestion of doxycycline (a tetracycline analog) induced expression of mutant *K-Ras4b* in type II pneumocytes of the lung, addition of a second mutation (specifically a constitutive deletion in either *TP53* or *Ink4A/Arf*) resulted in a faster rate of lung tumourigenesis (Fisher et al. 2001). Similarly, a faster rate of lung tumourigenesis was also achieved when higher intratracheal doses of Cre-carrying adenoviruses were delivered to the lungs of transgenic mice with Cre-inducible mutations in *KRAS* and *TP53* (Kasinski and Slack 2012).

Temporal Concordance

There is some empirical evidence of temporal concordance between mutations and lung cancer incidence. Mutations have been shown to occur prior to lung tumourigenesis, but the exact period of time between mutation incidence and cancer development is difficult to pinpoint and appears to be affected by a variety of different factors. Results from a number of different studies, however, are in general agreement that the accumulation of oncogenic traits *in vitro* occurs weeks after mutations are induced (Miller et al. 1995; Hei et al. 1997; Miller et al. 1999), while *in vivo* tumourigenesis is not evident for weeks, months or even years after mutations are induced/accumulated (Hei et al. 1994; Monchaux et al. 1994; Lubin et al. 1995; Fisher et al. 2001; Kasinski and Slack 2012; Fujimoto et al. 2017).

Essentiality

In contrast to sparse studies indicating dose and incidence concordance between mutations and cancer, there are many different studies showing essentiality of mutations for the induction of lung cancer, especially for mutations in *TP53*, *KRAS*, and *EGFR*. The conceptual 'removal' or 'blocking' of these mutations using conditional knock-out models, inducible mutation models, and treatment with various antagonizing and agonizing compounds has been observed to reverse or prevent lung tumourigenesis *in vivo*.

In general, there are strong links between mutations in *TP53* and tumourigenesis. A review of results from experiments involving *in vivo* p53 mouse models found that mice with dysfunctional/null p53 resulted in more tumourigenesis than mice with functional or semi-functional p53. The results of these studies demonstrate that mutant or absent p53 has a key role in promoting tumour growth (Iwakuma and Lozano 2007). Restoration of p53 function may cause tumour regression, as evidenced in a study using a conditional *TP53* knock-out mouse model. All of the mice in the study had confirmed tumour growth, and restoring p53 function by tamoxifen injection decreased tumour size in 7 of 10 tumours by 46 - 100% (Ventura et al. 2007).

Similar results were found in a Phase 1 clinical trial specifically examining lung cancer. Seven NSCLC patients with metastatic or recurring lung tumours that had been unresponsive to previous treatments and that harboured mutations in *TP53* were included in the study. The clinical trial was designed to examine the effect of delivering functional, wild-type p53 to the tumours by direct injection of a retroviral vector carrying p53 into the tumour, with the goal of restoring p53 function. All patients showed evidence of gene transfer. Four weeks post-treatment, tumours in six of these patients showed increased apoptosis; furthermore, the tumour had also regressed in three patients and stabilized in three patients (Roth et al. 1996).

Mutations in *TP53* have also been examined alongside *KRAS* mutations. In one particular study, transgenic mice were engineered such that ingestion of doxycycline (an analogue of tetracycline) induced mutant *K-Ras4b* expression specifically in type II pneumocytes of the lung. Activation of this mutant *K-Ras4b*, in turn, resulted in lung tumourigenesis two months later. Addition of a constitutive deletion in *TP53* or *Ink4A/Arf* resulted in a faster rate of tumourigenesis, such that tumours were present one month after the activation of mutant *K-Ras4b*. Analogous to the above studies where restoration of normal p53 resulted in tumour regression, the tumour growth in this study was reversed when doxycycline was withdrawn and the expression of mutant *K-Ras4b* was effectively stopped; the same regression was observed both with and without the constitutive deletion (Fisher et al. 2001).

Likewise, lung tumours resulting from mutations in *TP53* and/or *KRAS* may be prevented or reversed using microRNAs (miRNAs) that are linked with p53 and KRAS regulation. Two miRNAs were examined: miR-223-3p and miR-34a. The connection between miR-223-3p, p53 and tumourigenesis was explored in a study using lung squamous cell carcinoma tumours, NSCLC cell lines with mutant p53, and mouse xenograft models using nude mice inoculated with primary human lung squamous cell carcinoma tumour fragments. In general, miR-223-3p expression was found to be significantly decreased in tumours of human origin and in successful mouse xenografts.

When miR-223-3p expression was examined in relation to *TP53* mutational status, miR-223-3p expression was significantly lower in the tumours with mutant p53 relative to those with wild-type p53. Confirming this reciprocity, silencing the mutant *TP53* in NSCLC cell lines using short interfering RNA (siRNA) significantly increased miR-223-3p expression. Similarly, transfection of NSCLC cells with a vector to overexpress the mutant p53 resulted in decreased miR-223-3p expression. This led to experiments involving the *in vivo* treatment of the xenograft tumours with a miR-223-3p agonist. In comparison to non-agonist treated tumours, treatment with the miR-223-3p agonist resulted in not only increased expression of miR-223-3p, but also significant decreases in tumour weight, tumour volume and p53 expression (Luo et al. 2019).

Comparable results were found in a study using a mouse model with Cre-inducible heterozygous mutations in both *KRAS* and *TP53*. Intratracheal delivery of Cre via an adenovirus directly to the lungs resulted in significant tumour growth in the lungs after several weeks. When the mice were treated with a lentivirus carrying miR-34a at the same time as the Cre-adenovirus, there were significantly fewer tumours found in the treated animals, and the lungs of the treated animals were significantly smaller than the tumour-burdened, inflamed lungs of the untreated group. Furthermore, treatment with miR-34a 10 weeks after delivery of the Cre-adenovirus resulted in tumour regression by 4 weeks post-treatment, with tumour numbers and sizes decreasing significantly to near baseline levels in treated mice relative to the untreated controls (Kasinski and Slack 2012).

Tumour regression has also been achieved using the EGFR inhibitor EGF816. Multiple rodent xenograft models, with tumours derived from several cell lines with different *EGFR* mutations, were examined after administration of EGF816. Relative to rodents treated with vehicle, those treated with the inhibitor showed a reduction in tumour growth over 14, 18 or 21 days of treatment. In most cases, there was a dose-dependent increase in tumour regression, such that tumours were smaller in animals given higher doses of the inhibitor (Jia et al. 2016).

In contrast, the process of tumourigenesis may be expediated by addition of a carcinogen. Exposure of *Gprc5a* knock-out mice to a known carcinogen, NNK, resulted in a faster rate of lung tumourigenesis and more somatic mutations. Mice treated with NNK for 2 months showed increased tissue abnormalities within 1 month of treatment and detectable tumours by 3 months. At 6 months post-treatment, all NNK-exposed mice were presenting with lung adenocarcinomas, and the tumour burden significantly increased from 6 to 7 months post-treatment. In comparison, saline-treated controls had very few tissue abnormalities present at 7 months post-treatment, and did not develop adenocarcinomas until 16 months post-treatment. The NNK-treated animals also showed an increased somatic mutation burden at 5 - 7 months post-treatment relative to saline-treated controls at 16+ months post-treatment (Fujimoto et al. 2017).

Uncertainties and Inconsistencies

Uncertainties and inconsistencies in this KER are as follows:

1. Tumours often have many different mutations present, some at such low levels that they are very difficult to detect. This is an issue, as these low-level mutants may still play a significant role in tumour growth, relapse and resistance to therapy. There has been some improvement in detecting these mutations with new technologies such as consensus sequencing-based error-correction approaches (Salk et al. 2018).
2. Opposing results were found for two studies examining *TP53* mutations in lung tumours from New Mexico uranium miners. In an earlier study by Vahakangas (1992), lung tumours were examined from 19 underground miners exposed to an average of 111 WLM of radon. Seven of the tumours harboured a *TP53* mutation, but none of the mutations were found to be G to T transversions in the coding strand of *TP53*. In contrast, a study by Taylor (1994) that examined *TP53* mutations in lung tumours of 29 New Mexico uranium miners exposed to an average of 1,382 WLM of radiation found that 16 of the *TP53* mutations were G to T transversions at codon 249. An *in vitro* study using normal human bronchial epithelial cells irradiated with alpha particles equivalent to 1,460 WLM (4 Gy) was also performed, mimicking the above studies. The resulting irradiated cells exhibited malignant characteristics such as distinct morphology, a high rate of mitosis, and an extended lifespan. The mutational analysis, however, was in agreement with the results from Vahakangas(1992) , as there were no G to T transversions found at codon 249 and codon 250 of *TP53* (Hussain et al. 1997).

Quantitative Understanding of the Linkage

Quantitative understanding of the relationship between mutation frequency and lung cancer incidence is not well-defined. Although it is well known that mutations are linked with cancer incidence and that some mutations are more common or specific to certain types of cancer, it is difficult to precisely predict cancer incidence from the somatic mutation frequency. A review paper by Saini (2018) discusses mutation loads in healthy and cancerous cells and methods of measuring these mutations. Interestingly, pre-cancerous, healthy cells are thought to be responsible for generating the majority of somatic mutations found in tumours (Tomasetti et al. 2013).

Mutation frequencies for healthy and cancerous cells are summarized in the table below.

Reference	Summary
Milholland et al., 2017	Observation of somatic mutation rates in healthy human & mouse cells observed: human cells: $2.8x10^{-7}$ mutations per base pair and $2.66x10^{-9}$ mutations per base pair per mitosis. Mouse cells: 4.4×10^{-7} mutations per base pair and 8.1×10^{-9} mutations per base pair per mitosis.
Vogelstein, 2004	Tumor mutation rates are thought to be similar to mutation rates in healthy human cells of a similar number of generations. Observation of 1 mutation per megabase pair.
Saini, 2018	Somatic mutations in cancerous cells, 100 to 10^6 mutations per genome.
Alexandrov, 2013	Somatic mutations in cancerous cells, 0.001 to > 400 mutations per megabase pair. Higher mutation frequencies in cancers that are linked to environmental causes (e.g. lung cancer).
Danesi, 2003	Clinical detection of lung cancer observed 10-20 genetic events.

Response-response relationship

Studies assessing the nature of the relationship between mutation frequencies and cancer incidence directly are difficult to locate. There are, however, separate studies that assess the relationship between radiation exposure and mutation frequencies, and the relationship between radiation exposure and lung cancer incidence. More research is required to directly assess the response-response relationship between mutations and lung cancer.

Mutation frequencies were found to increase in a positive, dose-dependent manner with increasing gamma-ray radiation doses between 0 Gy and 6 Gy in Chinese hamster embryonal lung fibroblasts (Canova et al. 2002) and normal human bronchial epithelial cells (Suzuki and Hei 1996). Similarly, fibroblasts exposed to a number of different ions of varying LETs were found to have a positive, dose-dependent relationship between oncogenic transformations and radiation doses ranging from 0 - 1 Gy (Miller et al. 1995). This positive, dose-dependent relationship was also found between the incidence of lung cancer in rats and their cumulative radon exposure between 25 and 3000 WLM (Monchaux et al. 1994). (According to a conversion factor from Jostes (Jostes 1996), 25 WLM is equivalent to 0.02 - 0.25 Gy, and 3000 WLM is equivalent to 2.4 - 30 Gy.) Furthermore, two epidemiological studies examining lung cancer in radon-exposed uranium miners found a positive, linear relationship between the relative risk of lung cancer and the cumulative radon exposure (Lubin et al. 1995; Ramkissoon et al. 2018).

Time-scale

It is difficult to pinpoint exact time scales in terms of how long it takes for lung cancer to develop after mutations are accumulated. Differing experimental or biological conditions may modify the time scale between these events, making it challenging to predict exactly when tumours will develop. Another level to this challenge is the difficulty in pinpointing exactly when mutations occur after exposure to a stressor. Synthesis of results from various studies highlights this variety in time scales between stressor exposure, mutation induction and tumourigenesis.

Studies examining the time scale between mutations and lung cancer generally agree that tumourigenesis occurs at least weeks or months after the induction of mutations. In cells whose nuclei were precisely irradiated with 1 - 8 alpha particles, mutations were evident 2 weeks after irradiation (Hei et al. 1997). Oncogenic transformations, however, were not evident until 7 weeks after irradiation (Miller et al. 1999). Likewise, xenografts using human bronchial epithelial cells that were transformed into tumour cells by irradiation resulted in detectable tumours in Nu/Nu mice within 13 weeks of injection; the tumours grew to diameters of 0.6 - 0.7 cm by 6 months post-injection (Hei et al. 1994). In *Gprc5a* knock-out mice, there were tissue abnormalities present in approximately 10% of mice at 10-11 months of age, but spontaneous tumours did not develop until at least 20 months of age. Exposure of these mice to known tobacco carcinogen NNK from 2 - 4 months of age resulted in a faster rate of tumourigenesis, with tissue abnormalities present in roughly 65% of the population by 1 month post-exposure (5 months of age), and adenocarcinomas in approximately 15% of the population by 3 months post-exposure (7 months of age). At 6 months post-exposure (10 months of age), 100% of the population presented with adenocarcinomas; one month later, there was a significant increase in the tumour burden. Furthermore, somatic mutation burdens in NNK-treated mice between the ages of 9 and 11 months were higher relative to untreated mice of at least 20 months of age (Fujimoto et al. 2017). Moreover, epidemiological analysis of radon-exposed uranium miners found that the relative risk of lung cancer was amplified with increasing years of radon exposure (Lubin et al. 1995).

Cre-inducible transgenic mouse models of lung cancer are likewise useful for highlighting that mutations precede lung tumourigenesis. In the presence of Cre-induced mutant *K-Ras4b* expression, focal hyperplasia lesions were present within 7 - 14 days of expression induction, and tumours were present by 2 months post-induction. In animals with an additional constitutive mutation, tumours were present within 1 month of mutant *K-Ras4b* expression (Fisher et al. 2001). Likewise, clinically detectable lung cancer was present in the lungs of transgenic mice with Cre-inducible *KRAS* and *TP53* mutations within 15 to 37 weeks of the mutations being expressed, depending on the dose of Cre-carrying adenovirus used (Kasinski and Slack 2012).

Known modulating factors

There are known modulating factors that affect the relationship between mutations and lung cancer. Overall, increasing age is correlated with more mutations (Tomasetti et al. 2013), and a higher incidence of cancer has been documented in those exposed to radiation at a younger age (Bijwaard et al. 2001). A direct relationship has also been established between the degree of tissue damage in the respiratory structures and the consumption of cigarettes (Auerbach et al. 1957). Furthermore, mutations linked to lung cancer are more common in specific groups of people. *EGFR* mutations have been found more frequently in non-smokers (Lim et al. 2009; Sanders and Albitar 2010; Paik et al. 2012; Cortot et al. 2014), adenocarcinoma patients (Lim et al. 2009; Sanders and Albitar 2010), and females (Lim et al. 2009; Cortot et al. 2014). In general, *KRAS* mutations are more common in smokers (Paik et al. 2012; Cortot et al. 2014); however, the *KRAS* G12D transition variant is more common in non-smokers, while the G12V transversion variant is more common in smokers (Paik et al. 2012). Patients with stage I NSCLC also tend to have more frequent mutations in *KRAS* compared to patients at a higher stage (Cortot et al. 2014). Although *TP53* mutations are not associated with smoking status overall, G to T transversions were found to be more common in smokers (Cortot et al. 2014).

Known Feedforward/Feedback loops influencing this KER

Not identified.

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Relationship: 1985: Increase, Chromosomal aberrations leads to Increase, lung cancer

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deposition of energy leading to lung cancer	non-adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The domain of applicability applies to mammals such as mice, rats, hamsters and humans.

Key Event Relationship Description

Chromosomal aberrations (CAs) are described as irregularities in chromosome structure due to segments of the chromosome that have been lost, gained, or rearranged. This can lead to two categories of chromosomal exchanges: balanced, which do not impact the overall frame of chromosome structure, and unbalanced, which refers to CAs that do alter the frame of chromosome structure (Genetic Alliance 2010) . Specific categories of CAs include chromosome-type aberrations (CSAs) such as chromosome-type breaks, ring chromosomes, marker chromosomes, and dicentric aberrations; chromatid-type aberrations (CTAs) such as chromatid breaks and chromatid exchanges (Hagmar et al. 2004; Bonassi et al. 2008); micronuclei (MN); nucleoplasmic bridges (NPBs); and copy number variants (CNVs). When CAs affect genes related to tumourigenesis or their regulatory regions (Shlien and Malkin 2009; Liu et al. 2013), this may lead to an abnormal accumulation of malignant cells and ultimately may result in cancer. Lung cancer in particular may occur if these tumourigenesis-related CAs (which are more often unbalanced than balanced in lung cancer (Mitelman et al. 1997) occur in cells of the lung.

Evidence Supporting this KER

Biological Plausibility

Biological Plausibility

The biological rationale linking CAs with lung cancer is strongly supported. There are many epidemiological studies that provide evidence of a link between increasing CAs and cancer incidence. Several published reports spanning over 22,000 study subjects across multiple European countries have examined the association between the presence of CAs in cultured blood lymphocytes and the incidence of cancer. In every cohort examined, the presence of CAs was predictive of cancer risk (Bonassi et al. 2000; Hagmar et al. 2004; Norppa et al. 2006; Boffetta et al. 2007; Bonassi et al. 2008). Although CSAs and CTAs both had predictive value, CSAs were considered to be slightly more indicative of cancer risk (Norppa et al. 2006). Similarly, studies examining chromosomes in lymphocytes from lung cancer patients found significant increases in CTAs, CSAs, and overall CAs relative to lymphocytes from healthy controls. Furthermore, the CAs were shown to be significant predictors of lung cancer risk (Vodenkova et al. 2015). Analysis of MN and NPB levels within binucleated cells also found that these CAs were significantly increased in lung cancer patients relative to healthy controls (Lloyd et al. 2013; El-zein et al. 2014; El-zein et al. 2017), with very similar results for geographically-separated test and validation cohorts (El-zein et al. 2014).

Exposure to radiation has also been epidemiologically linked to the relationship between CAs and cancer. Studies of radon-exposed uranium miners have revealed evidence of an association between exposure to radon gas and an increased incidence of lung cancer (Roscoe et al. 1989; Tirmarchel et al. 1993; Smerhovsky et al. 2001; Smerhovsky et al. 2002; Vacquier et al. 2008; Walsh et al. 2010). Analysis of CAs in the blood lymphocytes of miners from the Czech Republic found that miners with higher levels of CAs had a significantly elevated risk of cancer (Smerhovsky et al. 2001; Smerhovsky et al. 2002). The results from these studies were likely not due to smoking status of the miners, as a study examining a cohort of 516 white, never-smoker American uranium miners found that the mortality rates from lung cancer were higher in the miners than in the general non-smoking population (Roscoe et al. 1989).

Beyond epidemiology, there are also many genetic and molecular studies that provide strong evidence of a relationship between CAs and cancer. A subset of these studies have investigated copy number variants (CNVs). Examination of CNVs and known cancer genes in a large population revealed that CNVs often overlap with cancer genes and thus have the potential to amplify carcinogenesis (Shlien and Malkin 2009; Ohshima et al. 2017)Moreover, using only CNV genetic information from a database, Zhang et al (2016) were able to categorize 3,480 samples into their respective cancer type based solely on the CNVs of the samples. This was accomplished by developing a panel of 19 discriminating genes that could predict cancer type with a high level of accuracy using only the CNV number. Interestingly, many of these discriminating genes have known associations with cancer or processes known to be important in cancer development (Zhang et al. 2016). Furthermore, cancer-prone individuals tend to have more CNV instability, which has been attributed to inherently less efficient DNA repair mechanisms (Shlien and Malkin 2009). In their 2013 review, Liu et al provided lists of cancer-related genes typically amplified by CNVs (*ERBB2, EGFR, MYC, PIK3CA, IGF1R, FGFR1/2, KRAS, CDK4, CCDN1, MDM2, MET,* and *CDK6*) and deleted by CNVs (*RB1, PTEN, CDKN2A/B, ARID1A, MAPSK4, NF1, SMAD4, BRCA1/2, MSH2/6, DCC,* and *CDH1*). There is also evidence associating CNVs to lung cancer specifically. Analysis of primary NSCLC samples revealed 27 chromosomal regions where CNVs were present in at least one third of the samples (Wrage et al. 2009). Furthermore, medically-relevant CNVs were found in 60% of lung cancer patients, encompassing genes such as *TP53, BAP1, STK11, BRCA2, CDKN2A,* and *RB1* (Mukherjee et al. 2016).

Likewise, lung cancer-specific studies have been performed to identify chromosomes most often affected by CNV gains and losses. Analysis of DNA from primary human lung tumours and early-passage primary cell lines established from human tumours revealed that gains were most frequently found at chromosomes 3q, 5p, 7p, and 8q, while losses were most frequent at chromosome 3p (Balsara et al. 1997). Separation of CNV analyses into squamous cell carcinoma and lung adenocarcinoma groups demonstrated differences between the two lung cancers (Petersen et al. 1997; Bjorkqvist et al. 1998; Feder et al. 1998; Massion et al. 2002). In general, CNV changes were present in 84% of squamous cell carcinoma samples, but only in 68% of adenocarcinomas (Bjorkqvist et al. 1998). In squamous cell carcinoma, the most frequent gain was found at chromosome 3q (Bjorkqvist et al. 1998), specifically at 3q26 (Massion et al. 2002); other common CNV gains were found at chromosomes 8q, 5p and 7p (Bjorkqvist et al. 1998). Losses in 3p were also more common in squamous cell carcinoma than adenocarcinoma (Feder et al. 1998). In adenocarcinoma, the most common documented CNV was a gain at chromosome 7p (Feder et al. 1998). Gains in squamous cell carcinoma were often found in genes *GLUT2, THRB, PIK3CA* and *BCL6*, and losses in *FHIT, EG9F2* and *CACNAID*. Interestingly, CNV gains affecting *PIK3CA* were correlated with increased activity of PKB in squamous cell carcinoma (Massion et al. 2002). A review by Knuutila et al (1999) summarizes DNA copy number losses found in 73 human tumour types, with results separated by chromosome number.

Loss of heterozygosity is also a common occurrence in cancer. Preneoplastic lesions from seven NSCLC tumours were histologically categorized and genetically analyzed. Consistent with above studies that revealed CNV losses at chromosome 3p, loss of heterozygosity was also common at the 3p locus. Percentages of 3p loss of heterozygosity increased from hyperplastic lesions (76%) to dysplastic lesions (86%) to carcinoma *in situ* (100%). Overall, cumulative loss of heterozygosity was nearly doubled in carcinoma *in situ* and invasive carcinoma lesions relative to preneoplastic hyperplasia and dysplasia lesions (Hung et al. 1995).

Other studies have revealed a link between gene rearrangements and cancer. Truncated tumour suppressor genes *TP53, BRCA1* and *BRAC2* have been reported in prostate cancer (Mao et al. 2011). In lung cancer, the gene *ALK* has been observed to undergo rearrangements, often in the form of gene fusions with *EML4* (Sanders and Albitar 2010; Sasaki et al. 2010). These *ALK* rearrangements often result in increased activity of *ALK*, higher activation of PI3K-AKT pathways, and ultimately an increased risk of tumourigenesis (Sanders and Albitar 2010). Another common example of a gene fusion is the Philadelphia chromosome, which is formed by a translocation between chromosome 9 and 22 and results in the fusion of *BCR* and *ABL* genes. The resulting *BCR/ABL* gene fusion product was found to be the cause of chronic myelogenous leukemia (reviewed by (Trask 2002). This fusion may be induced by stressors such as ionizing radiation; exposure of human leukemic promyelocytic cells (HL-60) to 5 Gy of gamma-ray radiation resulted in homologous *BCR* and *ABL* genes in closer proximity to each other and to the centre of the nucleus (Bartova et al. 2000).

Several rearrangements have also been significantly associated with lung cancer. A balanced translocation at chromosome 19 that results in overexpression of Notch3 in lung epithelial cells has been identified in a number of NSCLC lung cancer cell lines and tumours. This is significant, as Notch3 is not normally expressed in the cells of the lungs (Dang et al. 2000). In fact, transgenic mice engineered to overexpress Notch3 in the lung epithelium died at birth. Analysis of these embryos at embryonic day 18.5 revealed tissue abnormalities in locations where Notch3 mRNA was found, which suggests that overexpression of Notch3 in the lungs may play a role in lung tumourigenesis (Dang et al. 2003). Significant associations have been found between rearrangements in chromosome Xp and higher NSCLC tumour stage, as well as rearrangements in 17p and lower NSCLC tumour stage; 3p and 6q rearrangements were linked with better NSCLC survival (Feder et al. 1998).

CAs that affect pathways controlling cellular growth and apoptosis may promote the development of cancer. In some cases, CAs may alter the activity of proto-oncogenes or tumour suppressor genes (Mitelman et al. 1997; Albertson et al. 2003). Proto-oncogene regulation may be modified such that its gene product is overexpressed; alternatively, the product of the proto-oncogene itself may be affected, producing an abnormally-functioning protein. CAs that affect tumour suppressor genes may inactivate its expression; deletion of the chromosome housing the tumour suppressor gene(s) or unbalanced structural rearrangements may also prevent the expression of tumour suppressor genes (Mitelman et al. 1997). If these alterations enhance cell growth and/or inhibit apoptosis, the cell may become excessively proliferative and unresponsive to external environmental signals (Albertson et al. 2003). There are several pathways that could conceivably be pushed towards malignant transformation by the formation of CAs, including signalling pathways AKT-PI3K-mTOR and RAS-REF-MEK. If a CA occurs within gene(s) related to either of these pathways such that the activity is augmented, this may contribute to the development of a tumour (Sanders and Albitar 2010).

Other factors that may also contribute to increasing the CAs in a tumour include aberrant centromeres and telomerase deficiencies. In some cases, centromeres may become abnormally large due to aberrant amplification. These large centromeres may no longer separate the chromosomes appropriately during cell division, increasing the CA burden in the resulting daughter cells. In telomerase-deficient tumour cells that are proliferating but not being monitored closely, the telomeres may become abnormally short. This becomes an issue for cells that continue dividing because the chromosomes may become damaged during cell division, resulting in chromosomal fusions and breakages. Ultimately, this would also increase the CAs in the daughter cells (Albertson et al. 2003).

Ionizing radiation may also play a role in carcinogenesis. A series of studies focussed on irradiating human papillomavirus (HPV18)-immortalized human bronchial epithelial cells and transplanting the cells into nude mice. The transplantation of these irradiated cells resulted in tumour induction, an effect that was not found when unirradiated cells were transplanted into the mice (Hei et al. 1994). From these tumours, 6 different tumour cell lines were established and analyzed for cytogenetics. All of the lines were found to have CAs, and all harboured losses in genetic information (Weaver et al. 1997). Establishment of further tumour cell lines and their subsequent genetic analysis confirmed that there were CAs, especially in the form of deletions, that were common among the different tumour cell lines (Weaver et al. 2000).

Whether the CA is spontaneous or inherited may also be an important factor in the development of cancer. Non-clonal CAs, which are acquired spontaneously, promote genetic instability and are thought to confer a growth advantage. Ionizing radiation and carcinogens are two stressors that are thought to push the cell towards production of non-clonal CAs, which dominate during the pre-crisis stage of tumour development. After the tumour cells have passed the crisis stage and become immortal, clonal CAs (which are stable, inherited and recurrent in the cell population) dominate the CA landscape of the tumour. Clonal CAs are thought to confer a survival advantage to the cells. Overall, it is suggested that the shifting of equilibrium between non-clonal and clonal CAs is key in the initiation and progression of cancers (Heng, Stevens, et al. 2006; Heng, Bremer, et al. 2006). Interestingly, non-clonal CAs are affected by genotype. In both mouse embryonic stem cells and cultured lymphocytes that were lacking ATM, the spontaneous frequency of non-clonal CAs were significantly increased relative to wild-type cells; the same pattern was also observed in p53-/- cells from a human lung cancer cell line and an ovarian carcinoma cell line (Heng, Stevens, et al. 2006).

Empirical Evidence

There is moderate empirical evidence supporting the relationship between the incidence of CAs and the development of lung cancer in the presence of ionizing radiation. The evidence presented below is summarized in table 11, here (click link). Radon gas exposure in particular is linked to this process, and there are several published reviews that provide evidence for associations between radon exposure and the appearance of CAs, and radon exposure and the incidence of lung cancer (Jostes 1996; Al-Zoughool and Krewski 2009; Robertson et al. 2013). Genetic abnormalities found in lung cancer that result in genomic instability are also discussed by Massion (2003). Overall, however, there is little empirical evidence available supporting a dose and incidence concordance, some empirical evidence supporting a temporal concordance, and little empirical evidence supporting essentiality for this KER.

Dose and Incidence Concordance

There is a lack of empirical evidence to show dose and incidence concordance between CAs and lung cancer, particularly in the field of ionizing radiation. As described above, numerous studies are available that provide strong evidence linking CAs to cancer incidence, radiation exposure to CA frequency, and radiation exposure to cancer incidence. However, there is a lack of studies that assess whether increasing doses of a stressor, such as ionizing radiation, translate into dose-dependent changes in CA frequencies and dose-dependent changes in cancer incidences. Attempts were thus made to locate studies using similar radiological and biological conditions that assessed CA frequency and/or cancer incidence in response to a stressor.

Evidence from several epidemiological studies suggests a dose/incidence concordance between the appearance of CAs and lung cancer incidence upon radiation exposure. In humans, this association has been studied in different cohorts of uranium miners that were occupationally exposed to radon in the 1900s. (It is important to note that radon exposure was reported as working level months (WLM). One WLM is calculated based on 170 hours of exposure to one working level (WL), where 1 WL refers to the equivalent of 1.3 x10^5 MeV of alpha particle energy in 1 L of air. Damage from 1 WLM is thought to be equivalent to 0.8 - 10.0 mGy (Jostes 1996); this corresponds to 100 - 1250 WLM/Gy.) One of these uranium miner studies examined the relationship between radon exposure, CAs and lung cancer. In a cohort consisting of 225 radon-exposed miners from the Czech Republic, 1,323 cytogenetic assays were performed and 20 cases of respiratory and intrathoracic organ cancers were recorded. Over the course of their employment, mine workers were estimated to be exposed to 1.7 - 662.3 WLM, with approximately one-third of miners exposed to doses above 80 WLM. There were significant associations found between the radiation dose and both the percentage of aberrant cells and frequency of chromatid breaks. Furthermore, an increased risk of lung cancer was revealed in subjects with high frequencies of CAs. Radon exposure was also found to be a significant predictor of lung cancer incidence (Smerhovsky et al. 2002). In addition, other studies examining uranium miners from different countries found a significant association between the cumulative radon exposure and the risk of lung cancer (Tirmarchel et al. 1993; Vacquier et al. 2008; Walsh et al. 2010).

Evidence from *in vivo* and *in vitro* studies has also revealed similar associations. In a study examining mouse bronchial epithelial cells for CAs, 1 Gy of X-ray radiation was found to induce a significant increase in the percentage of binucleated cells with MN relative to unirradiated controls (Werner et al. 2017). Similarly, rats irradiated with 1 Gy of thoracic X-rays between 1 and 15 weeks of age were found to develop significantly more lung tumours than unirradiated controls (Yamada et al. 2017). Several studies using lung and non-lung cell lines have also shown that a dose-dependent increase in CAs occurs with increasing radiation doses of X-rays between 0 and 5 Gy (Yamada et al. 2002) and alpha particles between 0 and 2.23 Gy (Nagasawa et al. 1990; Deshpande et al. 1996; Yamada et al. 2002; Stevens et al. 2014). Coinciding with these results, oncogenic transformations were found in non-lung cell lines irradiated with similar radiation doses; specifically, dose-dependent increases in oncogenic transformations were evident between 0 and 2.5 Gy of both X-rays (Robertson et al. 1983) and alpha particles (Robertson et al. 1983; Miller et al. 1996). Relative to X-ray exposed cells, those exposed to alpha particles had more MN accumulated per Gy (Yamada et al. 2002) and more oncogenic transformations (Robertson et al. 1983). Likewise, *in vivo* studies

demonstrated a dose-dependent increase in MN in lung fibroblasts isolated from Wistar rats exposed to 0 - 11.3 Gy of gamma-ray radiation (Brooks et al. 1995), Wistar rats exposed to 0 - 323 WLM of radon, and Syrian hamsters exposed to 0 - 278 WLM of radon (Khan et al. 1995). When the incidence of lung carcinomas was examined in Sprague-Dawley rats exposed to radon and radon progeny at exposure levels similar to uranium miners, there was a dose-dependent increase in lung carcinomas between 25 and 3000 WLM (Monchaux et al. 1994).

In further support of this dose-dependency between CAs and lung cancer, analyses of lung tissue with varying levels of tumourigenesis exhibited corresponding accumulations of CAs. In a *Kras*^{LA2} mouse model of lung cancer, tumours collected when the mice were 6 months of age were categorized according to size. Genomic instability in the form of CNVs significantly increased with increasing tumour size; this was especially true in chromosome 6, which houses the *KRAS* gene (To et al. 2011). Similarly, analysis of human lung tissue categorized according to the level of damage (normal epithelium, hyperplasia, metaplasia, dysplasia, carcinoma *in situ* or invasive carcinoma) found that the loss of heterozygosity was increased with higher levels of tissue damage (Thibervile et al. 1995; Wistuba et al. 1999). These findings were especially pronounced in the chromosome 3p region (Wistuba et al. 1999), specifically at 3p21-22 (Thibervile et al. 1995). Interestingly, a review by Zabarovsky (2002) suggests that there may be multiple tumour suppressor genes at chromosome 3p, which are thought to play an important role in carcinogenesis (Zabarovsky et al. 2002). Loss of heterozygosity was also found commonly at 9p21-22 and 5q21 (Thibervile et al. 1995).

Temporal Concordance

There is some empirical evidence of temporal concordance between CA frequency and lung cancer incidence after exposure to ionizing radiation. With respect to the time of irradiation, CAs have been shown to occur prior to lung tumourigenesis. Results from a number of different studies found that an increased CA burden was evident within hours or days of irradiation (Nagasawa et al. 1990; Khan et al. 1995; Deshpande et al. 1996; Yamada et al. 2002; Stevens et al. 2014; Werner et al. 2017). The development of cancer, however, was a longer process. *In vitro* oncogenic transformations were documented weeks after irradiation (Robertson et al. 1983; Miller et al. 1996), while *in vivo* lung tumours were not detected until months or years after the radiation exposure (Tirmarchel et al. 1993; Yamada et al. 2017). In terms of examining increased CAs and cancer incidence directly, injection of a CA-carrying agent into mice was shown to induce cancer within 21 - 31 days of the injection (Pear et al. 1998; Kuramochi et al. 2001).

Essentiality

There are few studies available that demonstrate the essentiality of CAs for the induction of lung cancer. However, two agonist-type studies were found that supported the relationship between CAs and cancer, though they were not specific to the lung. In the first study, addition of a known pulmonary carcinogen to cultures of peripheral blood lymphocytes from both lung cancer patients and healthy controls resulted in significantly increased MN, NPBs and nuclear buds relative to the respective untreated cultures (Lloyd et al. 2013). The second study demonstrated a clear relationship between the *BCR/ABL* translocation and chronic myelogenous leukemia. In this study, BALB/c mice that were lethally irradiated received a bone marrow transplant containing retroviruses carrying the *BCR/ABL* translocation. Within 21 - 31 days of the transplant, all of the infected mice were found to have a disease equivalent to the human chronic myelogenous leukemia (Pear et al. 1998).

A further study manipulated *TSCL1* dynamics in a xenograft mouse model. The human lung cancer cell line, A549, harbours a loss of heterozygosity at chromosome 11, which results in highly reduced levels of *TSCL1*. Upon injection of these cells into BALB/c mice, tumours were detectable at the injection sites by 3 weeks post-injection. In an effort to correct this defect, mini genes were engineered to carry a full-length *TSCL1* gene and transfected into A549 cells which were then injected into mice. Although tumours still developed, they were fewer in number and slower growing (Kuramochi et al. 2001). Thus correction of one CA may have a measurable effect on cancer progression.

Uncertainties and Inconsistencies

Uncertainties and inconsistencies in this KER are as follows:

1. CNVs are often difficult to detect in cancer cells, even with current advances in next generation sequencing. This is due to the sheer number of CNVs that could possibly be present within one tumour; the unknown ratio of cancer cells and healthy cells within a tumour sample; the unknown ploidy of tumours; and the possible presence of multiple clones in one tumour, including possible low-number subclones that may be difficult to detect (Liu et al. 2013).
2. In some studies, smoking does not affect the CA-cancer relationship (Bonassi et al. 2000; Bonassi et al. 2008; El-zein et al. 2014; Vodenkova et al. 2015; El-zein et al. 2017), but it does have a significant effect in other studies (Paik et al. 2012; Lloyd et al. 2013; Minina et al. 2017).
3. In a study examining MN in lung fibroblasts isolated from Wistar rats and Syrian hamsters exposed to radon, Syrian hamsters were found to have a significantly increased rate of MN per 1000 bincleated cells per Gy relative to rats. According to the literature however, Wistar rats have a higher documented sensitivity to radon-induced lung cancer than Syrian hamsters (Khan et al. 1995).

Quantitative Understanding of the Linkage

In terms of quantitatively linking the frequency of CAs with the incidence of cancer in order to form predictions, there are few studies that directly link these two events. Estimates suggest that the accumulation of 10 - 20 genetic abnormalities is required for detectable lung cancer (Danesi et al. 2003). Along a similar line of reasoning, normal cells that have been converted to tumourigenesis are thought to harbour an average loss of heterozygosity of at least 25 - 30%; it is common, however, for there to be allele losses of greater than 75% in tumour cells (Vogelstein and Kinzler 2004). Although our current overall quantitative understanding of this KER hints that it may be possible to predict CAs and lung cancer risk for known radiation exposures, more research is required to further confirm and refine the direct quantitative understanding between a radiation-based stressor, CA rates, and cancer incidence.

Below are two tables that provide examples of the quantitative understanding that currently exists between CA frequency and lung cancer, often described in terms of a radiation stressor. The first highlights predictions of CA frequency rates, while the second provides examples that highlight cancer predictions.

Reference	Summary
Brooks, 1995	Irradiating lung fibroblasts from wistar rats in the dose (D) range 0 - 11.3 Gy resulted in a postive increase in estimated CA rate (y) (of the form y = a + bD): 4 hours (a,b := 0.02 ± 0.03, (2.38 ± 0.44)x10^{-2}), 67 hours (a,b:= 0.01 ± 0.06, (1.01 ± 0.10)x10^{-2}).
Khan, 1995	Lung fibroblasts from Wistar rats and Syrian hamsters were arradiated with Radon with equivalent doses (D) of 0-323 WLM (Wistar) and 0-278 WLM (Syrian). The estimated CA response (y) (of the form y = a + bD) were found to be: Wistar (a,b := 15.5 ± 14.4, 0.53 ± 0.06), Syrian (a,b := 38.3 ± 15.1, 0.80 ± 0.08).
Girard et al., 2000	NSCLC and SCLC cell lines undergo allelic loss: NSCLC - 22 ± 8 loci, SCLC - 17 ± 4.
Yamada, 2002	Rat alveolar epithelial cell line irradiated with X-rays or alpha particles in dose ranges 0 - 5 Gy (X-rays) and 0 - 2 Gy (alpha particles). Observation of 6.7 % increase in MN / Gy (X-rays) and 28.5 % in MN / Gy (alpha particles).
Stevens, 2014	V79-4 cells irradiated to alpha particles in the dose (D) range 0-2.23 Gy resulted in positive CA rate (y) (of the form y = a + bD) were found to be: Acute/High dose rate (a,b := 0.633 ± 0.2, 0.0208 ± 0.0068), Syrian (a,b := 0.523 ± 0.18, 0.0103 ± 0.0051).

Reference	Summary
Timarche, 1993	Study of French Uranium miners exposed to Radon in the dose (D) range of 0 - 300 WLM resulted in a calculated lung cancer risk (y) (of the form y = a + bD) based on a 0.6% per exposure to 1 WLM: (a,b) := 1.68, 0.0058.
Walsh, 2010	Study of German of miners exposed to Radon in the dose (D) range of 0 - 1500 WLM resulted in a calculated lung cancer risk of 1.1% per WLM (radon exposure rate: 2.7 WL).
Miller, 1995	C3H10T1/2 cells exposed to alpha particles with a dose of 0 - 1 Gy. Resulted in a calculated cancer risk of 22.7 ± 2.0 transformants per 10^4 surviving cells per Gy.

Response-response relationship

There is evidence of a response-response relationship between radiation exposure and CAs in cells of the lung, and between radiation exposure and the risk of lung cancer in radon-exposed miners. In two different studies using lung fibroblasts isolated from irradiated rodents, there was a positive, linear, dose-dependent relationship found between the radiation dose and the number of MN (Brooks et al. 1995; Khan et al. 1995). A number of *in vitro* studies also confirmed the presence of a positive, linear dose-dependent relationship between the number of radiation-induced CAs and the radiation dose (Nagasawa et al. 1990; Yamada et al. 2002; Stevens et al. 2014). In studies examining mortality from lung cancer in radon-exposed uranium miners from France and Germany, there was a positive linear relationship between the radon exposure and risk of lung cancer mortality (Tirmarchel et al. 1993; Walsh et al. 2010). This relationship was found to be exponentially modified by the age at median exposure, the time since median exposure, and the radon exposure rate (Walsh et al. 2010). Furthermore, oncogenic transformations in C3H10T1/2 cells irradiated with alpha particles were found to increase in a positive, linear dose-dependent fashion (Miller et al. 1996).

Time-scale

There is evidence suggesting that time-related predictions can be made for CA incidence and the development of lung cancer after exposure to ionizing radiation. CAs have been demonstrated to occur within hours of irradiation and persist for days afterwards. In mouse bronchial epithelial cells, 1 Gy of X-ray radiation induced a significant increase in the percentage of binucleated cells with MN by 24 hours post-irradiation. These levels remained significantly elevated at 48 hours and 72 hours post-irradiation, though there was a time-dependent decrease in the percentage of cells with CAs. By 7 days post-irradiation, these levels were no longer significantly different from controls (Werner et al. 2017). In a similar study, lung fibroblasts were isolated and cultured from Wistar rats, Syrian hamsters and Chinese hamsters after exposure to 323, 278 and 496 WLM of radon, respectively, at 0.2, 15, and 30 days post-exposure. In all species, MN levels were highest at 0.2 days post-irradiation, and decreased over 30 days. The MN levels in the irradiated fibroblasts, however, remained significantly elevated at all time points relative to unirradiated control cells (Khan et al. 1995). Other in vitro studies have shown the presence of CAs within 13 - 82 hours post-irradiation (Nagasawa et al. 1990; Deshpande et al. 1996; Yamada et al. 2002; Stevens et al. 2014). It was noted in one study that the number or sister chromatid exchanges per cell were significantly higher than non-irradiated control cells at 72 hr post-irradiation, but these levels did not change appreciably at 74, 76, 78 or 82 hours post-irradiation (Deshpande et al. 1996).

In comparison to the time between radiation exposure and CA detection, there is a much longer gap between radiation exposure and the incidence of lung cancer. Oncogenic transformations in fibroblasts irradiated with alpha particles or X-rays were present 4 - 8 weeks after radiation exposure (Robertson et al. 1983; Miller et al. 1996). *In vivo* irradiation of 1 week-, 5 week- and 15 week-old rats by 1 Gy of thoracic X-rays was found to induce lung tumours months to years after the radiation treatment, with the highest risk for lung tumours found in rats that died between 600 and 900 days of age (Yamada et al. 2017). Similarly, French uranium miners exposed to radon and radon progeny for a minimum of two years were diagnosed at least 10 years after the first radon exposure (Tirmarchel et al. 1993).

Furthermore, direct injection of a CA into mice has also been shown to result in cancer several weeks after the CA administration. Injection of tumourigenic A549 cells that harbour a loss of heterozygosity at chromosome 11 resulted in tumour growth 3 weeks after injection (Kuramochi et al. 2001). Similarly, administration of the *BCR/ABL* translocation resulted in the mouse equivalent of chronic myelogenous leukemia by 21 - 31 days post-injection (Pear et al. 1998).

Known modulating factors

Some studies have documented modulating factors that affect CAs in lung cancer, including age, ethnicity (Lloyd et al. 2013), smoking (Feder et al. 1998; Paik et al. 2012; Lloyd et al. 2013; Minina et al. 2017), sex (Feder et al. 1998), and genotype (Kim et al. 2012; Minina et al. 2017). In NSCLC patients, *ALK* and *EML4* rearrangements have reportedly been influenced by confounding variables such as age (Shaw et al. 2009; Wong et al. 2009; Sasaki et al. 2010), sex (Shaw et al. 2009), and smoking history (Koivunen et al. 2008; Shaw et al. 2009; Wong et al. 2009; Sasaki et al. 2010).

Known Feedforward/Feedback loops influencing this KER

Not identified.

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