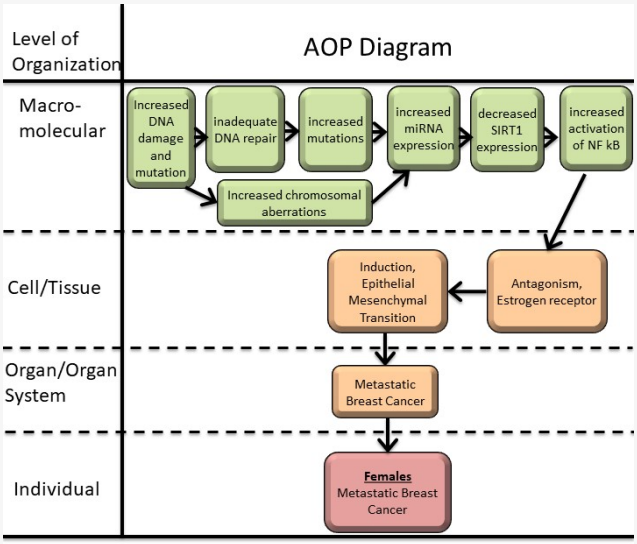
AOP ID and Title:

AOP 443: DNA damage and mutations leading to Metastatic Breast Cancer
Short Title: DNA damage and metastatic breast cancer

Graphical Representation



Authors

Dr Usha S Adiga MD PhD, Professor,Department of Biochemistry,KS Hegde Medical Academy,Nitte Deemed to be University,Mangalore,Karnataka,India

Mrs Sriprajna Mayur MSc (PhD),KS Hegde Medical Academy,Nitte Deemed to be University, Mangalore, Karnataka,India

Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.103	Included in OECD Work Plan

Abstract

This adverse outcome pathway details the effect of alcohol as a stressor in metastatic breast cancer. Aim of this AOP is intended to detail the linkage between alcohol and miRNA- SIRT-1 axis induced metastatic breast cancer which represents a knowledge gap as there are not many references available. Consecutive KEs identified are as follows.

Acetaldehyde, which is a metabolite of alcohol is considered a major mutagen which has been determined to induce genotoxic effects on DNA resulting in increased DNA damage. Inadequate DNA crosslink repair mechanisms leads to accumulation of damaged DNA resulting in impaired DNA synthesis leading to mutations and increased miRNA expression ; leads to disruption of SIRT-1 signalling . This step is followed by increased acetylation and activity of NFkB ; loss of estrogen receptor functions ; molecular alterations of epithelial cells ; gain of mesenchymal cell features ; eventuating in increased invasion and migration of breast cancer cells resulting in Metastatic breast cancer .

Background

Alcoholic beverages are classified by the International Agency for Research on Cancer(IARC) as Group 1 carcinogens. Studies have reported alcohol consumption to be a risk factor for breast cancer in women(Room R et al, 2005). A woman drinking an average of two units of alcohol per day has an 8% higher risk of developing breast cancer than a woman who drinks an average of one unit of alcohol per day[2]. Alcohol is metabolized by alcohol dehydrogenase to acetaldehyde which is a mutagen. Various theories have been proposed which explain the mutagenicity of alcohol. Among them, the most relevant one for carcinoma of the breast has been proposed by Purohita et al, suggesting an alcohol-induced inactivation of the tumor suppressor gene BRCA1 and increased estrogen Responsiveness in breast tissues(Purohit V et al, 2005). Boffetta and Hashibe list plausible mechanisms of breast cancer as a result of the genotoxic effect of acetaldehyde-induced increased estrogen concentration(Boffetta P et al 2006). It has also been found that alcohol stimulates the epithelial-mesenchymal transition (EMT), because of which there is distant metastasis (Forsyth C. B. et al 2010). However, this mechanism needs to be elucidated in detail.

MicroRNAs (miRNAs) are non-coding, single-stranded RNA molecules that regulate target gene expression via post-transcriptional modifications [Mohr A. M& Mott J. L 2015 and Lai E. C. 2002). Several studies indicated the promising role of miRNA in the diagnosis and outcome prediction in several cancers (Mirzaei H et al 2018 and Liu, S. Y et al 2017). miRNA-21 is upregulated and promotes metastasis in several cancers (Kunita, A et al 2018 and Liu Z et al 2015). A Chinese study by Kunita et al proved that plasma levels of miRNA were up-regulated in large B-cell lymphoma patients (Kunita, A et al 2018). A study by Wang et al also proved that plasma levels of miR were upregulated in large B-cell lymphoma patients in China (Chen et al 2014). Although miR-21 was indicated to play a crucial role in the metastasis of lung cancer, ovarian cancer, and head and neck cancer through several signaling pathways, the molecular mechanism of how miR-21 regulates the EMT process in breast cancer is not clear (Liu S. Y et al, Lopez-Santillan et al 2018, Panagal M. et al 2018, Zhou, B.et al 2018, Brabletz T et al and Ye, X. et al 2017).There are a number of miRNAs which regulate SIRT 1 expression. The epithelial-mesenchymal transition (EMT) is a process that which epithelial cells lose their cell polarity and cell adhesion ability, which will lead to cancer metastasis (Vaziri H et al 2001 and Luo, J et al 2001). Epithelial cells exhibit the property of regular cell-cell contacts, adhesion to the surrounding cellular fabric, preventing the detachment of individual cells. Whereas mesenchymal cells do not form intercellular contacts.

Sirtuins are nicotinamide adenine dinucleotide (NAD+)–dependent deacetylases that function as intracellular regulators of transcriptional activity (Blander G & Guarente L 2004 and Roth M & Chen W 2014). It plays important roles in cell survival, signal transduction, and cell apoptosis by deacetylating key cell signaling molecules and apoptotic related proteins, such as NF-kB, p53, Ku70, and HIFs (Zhao, W et al 2008 and Chen W & Bhatia R 2013). Various studies have inconclusive reports on the role of SIRT1 in cancer, because of its opposite effects as both a tumor activator or suppressor in various human cancers, including breast cancer. Deng et al found that the expression of SIRT1 was lower in prostate cancer, bladder cancer, ovarian cancer, and glioblastoma when compared with normal tissues (Han, L et al 2013).On the contrary, it was found that, in leukemia and lung cancer, SIRT1 was significantly higher(Riggio M et al 2012 and Lee M S et al 2015).

This can be explained as follows: SIRT1-mediated deacetylation suppresses the functions of several tumor suppressors including p53, p73, and HIC1, it has been suggested that SIRT1 has a promoting function in tumor development and progression [Pinton G et al 2016, Pillai VB et al 2014, Wan G et al 2017 and Hwang B et al 2014]. In contrast, SIRT1 may have a suppressive activity in tumor cell growth by suppressing NF-κB, a transcription factor playing a central role in the regulation of the innate and adaptive immune responses and carcinogenesis, the dysregulation of which leads to the onset of tumorigenesis and tumor malignancy(Yuan J et al 2009, Wang R H et al 2008, Chen L F et al 2004 and Greten F R & Karin M 2004). Here, we aim to further explore the role of the SIRT1-NF kB signaling pathway in tumorigenesis of the breast as well as its associated mechanisms.

The nuclear factor-κB (NF- κB)/REL family of transcription factors is comprised of a RELA/p65,c-REL, RELB, p105/NF- κB1 and p100/NF- κB2 (Van Laere S J et al 2007). The p105 and p100 proteins can be processed by proteolytic cleavage into p50 and p52, respectively. Activation of the NF-κB signaling pathway leads to the induction of target genes that can inhibit apoptosis, interaction with cell cycle regulation, cell invasion, contribute to tumorigenesis and metastatic invasion (Shostak K & Chariot 2011). Activation NF-κB in breast cancer is loss of Estrogen Receptor (ER) expression and Human Epidermal Growth Factor Receptor 2 (HER-2) overexpressed via epidermal growth factor receptor (EGFR) and Mitogen-Activated Protein Kinase (MAPK) pathway (Ali S & Coombes R C 2002). Indeed, the binding of epidermal growth factor (EGF) to its receptor (EGFR) also ultimately activates NF-κB and most likely contributes to the enhanced activity of this transcription factor in ER-negative breast cancer cells (Kalkhoven E et al 1996).

Loss of ER function has been associated with constitutive NFkB activity and hyperactive MAPK, because of constitutive secretion of cytokine and growth factors, which ultimately culminates in aggressive, metastatic, hormone-resistant cancers (Merkhofer E C et al 2010). Activation of the progesterone receptor can lead to inhibition of NF-κB driven gene expression (Sethi G et al 2008) reducing its DNA binding and transcriptional activity. HER-2 activates NF-κB through the canonical pathway which surprisingly, involves IKKα (Ito, T et al 2010). Activation of NF-κB promotes the survival of tumor cells. Several gene products that negatively regulate apoptosis in tumor cells are controlled by NF-κB activation (Lee J et al 2010). Estrogen plays an important role in breast cancer initiation and progression. Breast cancer over time acquires different mutations and the proportion of estrogen receptor-negative cells in tumors increases. This transformation confers aggressive biological characteristics to breast cancer such as rapid growth, poor differentiation, and poor response to hormone therapy. NF-κB pathway plays important role in this pathway (Lee J et al 2010).

Expression of SIRT1 is controlled at multiple levels by transcriptional, post-transcriptional, and post-translational mechanisms under physiological and pathological conditions. Emerging evidence indicates that miRs are important regulators of SIRT1 expression (Lovis P et al 2008, Ortega F J et al 2010, Zovoilis A et al 2011, Yamakuchi M et al 2008 and Mullany L E et al 2017). Studies have shown that miR-34a directly binds to the 3′ untranslated region (UTR) of SIRT1 mRNA and reduces its expression (Ortega F J et al 2010).

Study findings support the hypothesis that alcohol consumption is able to influence miRNA expression. Considerable evidence from rodent and human studies demonstrates that disruption of the hepatic SIRT1 signaling by ethanol plays a central role in the development of AFLD (Yin H et al 2014, Li M et al 2014).Ethanol down-regulates SIRT1 in hepatic cells and in the animal livers. The ethanol-mediated disruption of SIRT1 signaling leads to excess fat accumulation and inflammatory responses in the liver of animals and humans. Treatment with resveratrol, a known SIRT1 agonist, can alleviate liver steatosis . Accumulating evidence demonstrates that ethanol-mediated SIRT1 inhibition leads to the development of AFLD largely through disruption of a signaling network mediated by various transcriptional regulators and co-regulators, including nuclear transcription factor-κB (NF-κB)(Yin H et al 2014, Li M et al 2014).

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1669	Increased, DNA damage and mutation	Increased, DNA damage and mutation
2	KE	155	Inadequate DNA repair	Inadequate DNA repair
3	KE	185	Increase, Mutations	Increase, Mutations
	KE	1554	Increase Chromosomal Aberrations	Increase chromosomal aberrations
4	KE	1980	Increased microRNA expression	Increase,miRNA levels
5	KE	1981	Decreased SIRT1 expression	Decrease,SIRT1(sirtuin 1) levels
6	KE	1172	Increased activation, Nuclear factor kappa B (NF-kB)	Increased activation, Nuclear factor kappa B (NF-kB)
7	KE	112	Antagonism, Estrogen receptor	Antagonism, Estrogen receptor
8	KE	1457	Induction, Epithelial Mesenchymal Transition	EMT
9	AO	1982	metastatic breast cancer	Metastasis, Breast Cancer

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increased, DNA damage and mutation	adjacent	Inadequate DNA repair	High	High
Inadequate DNA repair	adjacent	Increase, Mutations	High	High
Increase, Mutations	adjacent	Increased microRNA expression	Moderate	Moderate
Increased microRNA expression	adjacent	Decreased SIRT1 expression	Moderate	Moderate
Decreased SIRT1 expression	adjacent	Increased activation, Nuclear factor kappa B (NF-kB)	Moderate	Moderate
Increased activation, Nuclear factor kappa B (NF-kB)	adjacent	Antagonism, Estrogen receptor	Moderate	Moderate
Antagonism, Estrogen receptor	adjacent	Induction, Epithelial Mesenchymal Transition	High	High
Induction, Epithelial Mesenchymal Transition	adjacent	metastatic breast cancer	High	High
Increased, DNA damage and mutation	non-adjacent	Increase Chromosomal Aberrations	High	High
Increase Chromosomal Aberrations	non-adjacent	Increased microRNA expression	High	High

Stressors

Name	Evidence
Ethyl alcohol	High

Overall Assessment of the AOP

Increased DNA damage and mutations [Evidence: high]: DNA damage refers to any modification in the physical and/or chemical structure of DNA resulting in an altered DNA molecule that is different from the original DNA molecule with regard to its physical, chemical, and/or structural properties".External factors to the cell such as environmental or potentially aggressive factors produced by the normal cell metabolism can damage the DNA. The effects caused by the action of endogenous factors may be more serious and/or more extensive than the effect of most of the exogenous DNA damaging factors. Evidence suggested that prolonged alcohol intake is positively associated with an increased risk of cancer. It can cause changes in the sequence of genomic DNA, which may act as a tumor promoter as well. Alcohol consumption can result in the generation of DNA-damaging molecules such as reactive oxygen species (ROS), lipid peroxidation products, and acetaldehyde. Strand breaks and oxidative base damage in DNA can be produced by hydroxyl radicals which are both mutagenic and cytotoxic. Alcohol is a known inducer of microsomal oxidizing system, which includes a specific ethanol-inducible form of cytochrome **P450,** referred to as CYP2EL (Lieber C 1992). This effect on the enzyme system has been associated with liver pathology induced by alcohol (Morimoto M et al 1993, French S et al 1993, Nanji A et al 1994, Albano E et al 1996). Again the damaging effects of high levels of CYP2E1 may be mediated by the generation of ROS (Cederbaum 1989, Reinke L et al 1990, Ishii H et al 1989). ROS that is highly reactive, include the oxygen radicals superoxide anion and hydroxyl radicals and can react with lipids, proteins, and DNA and thereby damage them (Knecht K et al 1990). It has been confirmed in vivo experiments that hydroxyethyl radical formation takes place after ethanol exposure (Albano E et al 1996, Moore D et al 1995, Clot, P et al 1996, Thurman R 1973). Chronic exposure to ethanol also results in increased production of H_2O_2, (Kukieka E et al 1992, Kukielka, E., & Cederbaum, A. I. 1994) which can react with metal ions (such as iron in the Fenton reaction); thus resulting in the production of the highly reactive hydroxyl radicals. DNA is very sensitive to the attack by the hydroxyl radical. A sensitive assay for hydroxyl radical formation from CYP2E1 uses DNA damage (strand breakage) as an endpoint (Breen AP, Murphy JA 1995). Apart from this, more than twenty different types of DNA base damage with diverse biological properties are produced by hydroxyl radical (Moriya M 1993).8-hydroxy-2'-deoxyguanosine, is one such DNA lesion brought about by oxidative stress. **This is** mutagenic, due to the tendency of DNA polymerases to misincorporate deoxyadenosine residues opposite this oxidized base (Song B 1996).

Inadequate DNA cross-link repair mechanisms [Evidence:high]:

As a result of DNA damage, DNA repair activities change. A variety of genotoxic agents, such as N-nitrosodimethylamine, aflatoxin B1, and 2-acetylaminofluorene induce the protein, O6-Alkylguanine-DNA alkyltransferase (ATase), are responsible for the repair of DNA alkylation damage in rats (O'Connor, 1989; Chinnasamy et al.,1996). Grombacher and Kaina (1996) reported an increased human ATase mRNA expression by alkylating agents like N-methyl-N'-nitro-N-nitrosoguanidine and methyl methanesulphonate and by ionizing radiation via the induction of the ATase promoter. ATase mRNA expression was increased in response to treatment with 2-acetylaminofluorene in rat liver (Potter et al., 1991; Chinnasamy et al., 1996). In another study, it was demonstrated that ATase gene induction is p53 gene-dependent: ATase activity was induced in mouse tissues following γ-irradiation in p53 wild-type mice, but not in p53 null animals (Rafferty et al., 1996). Alkylating agents and X-rays also induce DNA glycosylase, alkylpurine-DNA-N-glycosylase (APNG) (Lefebvre et al., 1993; Mitra and Kaina, 1993).

Increased mutations [Evidence: moderate]: Inadequate repair causes damaged DNA to be retained and used as a template during DNA replication. Incorrect nucleotides may be inserted during the replication of damaged DNA, and these nucleotides become 'fixed' in the cell after replication. The mutation propagates to more cells as a result of further replication. Non-homologous end joining (NHEJ) is one of the repair methods employed in human somatic cells to repair DNA double-strand breaks (DSBs). (Petrini et al., 1997; Mao et al., 2008). However, this mechanism is prone to errors and may result in mutations during the DNA repair process. (Little, 2000). As it does not use a homologous template to repair the DSB, NHEJ is considered error-prone. Many proteins work together in the NHEJ pathway to bridge the DSB gap by overlapping single-strand termini that are typically less than 10 nucleotides long. (Anderson, 1993; Getts & Stamato, 1994; Rathmell & Chu, 1994). Errors are introduced during this process, which can result in mutations like insertions, deletions, inversions, or translocations.

Increased micro RNA expression [Evidence: moderate]: DNA damage-responsive transcription factors, such as NF-kB, E2F, and Myc, are also involved in miRNA transcription regulation. The p53 protein also functions as a transcriptional repressor by binding to miRNA promoters and preventing the recruitment of transcriptional activators. For example, p53 prevents the TATA-binding protein from binding to the TAATA site in the promoter of the miR-17-92 cluster gene, suppressing transcription. Under hypoxic conditions, the miR-17-92 cluster is suppressed by a p53-dependent mechanism, making cells more susceptible to hypoxia-induced death (Yan et al.,2009).

Decreased SIRT1 expression [Evidence: moderate]: There are several signaling pathways that establish the role of increased miRNA expression in downregulating the SIRT 1 gene few of which are listed as follows; Butyrate has been demonstrated to cause apoptosis and reduce carcinogenesis in a variety of cancers (Tailor et al.,2014; Rahmani et al.,2002). Although butyrate has been shown to suppress SIRT1 gene expression in various cancers, this has yet to be proven in hepatocellular carcinoma (HCC) (Iglesias et al., 2007). In HCC, miR-22 was found to be downregulated, and its low levels aided carcinogenesis (Zhang et al.,2010). The Huh7 cells' in vitro proliferation was decreased by miR-22 expression, which activated apoptosis. In Huh7 cells, on the other hand, SIRT1 expression was high, which enhanced the expression of antioxidants such as superoxide dismutase (SOD), allowing cell growth to continue (Chen et al.,2012). Butyrate upregulated miR-22 in Huh7 cells, which binds directly to the 3′UTR region of SIRT1 and suppresses its expression.Notch3–SIRT1–LSD1–SOX2 Signaling Pathway **in** metastasis (Wang et al.,2016; Wu et al .,2017).MiR-486 inhibits HCC invasion and tumorigenicity by directly targeting and suppressing SIRT1 expression. This reduced the tumorigenic and chemo-resistant features of LCSCs and inhibited HCC invasion and tumorigenicity (Yan et al.,2019).

Increased activity of NF kB [Evidence: moderate]: SIRT1 deacetylates NFkB. In the context of NFkB, all of the evidence so far points to its signaling being inhibited after SIRT1 deacetylation (Morris, 2012). According to Yeung et al, SIRT1 can directly interact with and deacetylate the RelA/p65 component of the NF-B complex (Yeung et al.,2004). NF-B can be activated by cytokines (TNF-, IL-1), growth factors (EGF), bacterial and viral products (lipopolysaccharide (LPS), dsRNA), UV and ionizing radiation, reactive oxygen species (ROS), DNA damage, and oncogenic stress from inside the cells. Almost all stimuli eventually activate a large cytoplasmic protein complex called the inhibitor of B (IB) kinase (IKK) complex via a so-called "canonical pathway." The exact composition of this complex is unknown, however, it has three fundamental components: IKK1/IKK, IKK2/IKK, and NEMO/IKK. IB is phosphorylated by the activated IKK complex, which marks it for destruction by the -transducin repeat-containing protein (-TrCP)-dependent E3 ubiquitin ligase-mediated proteasomal degradation pathway (Liu et al., 2012;Li et al., 2002). As a result, unbound NF-B dimers can go from the cytoplasm to the nucleus, bind to DNA, and control gene transcription.

Antagonism of estrogen receptor [Evidence: moderate]: Activation NF-kB in breast cancer leads to loss of Estrogen Receptor (ER) expression and Human Epidermal Growth Factor Receptor 2 (HER-2) overexpressed via epidermal growth factor receptor (EGFR) and Mitogen-Activated Protein Kinase (MAPK) pathway (Laere et al.,2007). Indeed, the binding of epidermal growth factor (EGF) to its receptor (EGFR) activates NF-B, which most likely contributes to this transcription factor's increased activity in ER-negative breast cancer cells (Shostak et al.,2011). Because of the constitutive production of cytokines and growth factors, loss of ER function has been linked to constitutive NF-kB activity and hyperactive MAPK, resulting in aggressive, metastatic, hormone-resistant malignancies (Ali et al., 2002). Activation of the progesterone receptor can reduce DNA binding and transcriptional activity by inhibiting NF-B-driven gene expression (Kalkhoven et al., 1996). HER-2 stimulates NF-B via the conventional route, which includes IKK (Merkhofer et al., 2010).

Epithelial-mesenchymal transition cell [Evidence: high]: Estrogen/ERa signaling maintains an epithelial phenotype and suppresses EMT.ERa signaling promotes proliferation and epithelial differentiation and opposes EMT. Various studies support this finding (Eeckhoute et al.,2007, Kouros-Mehr et al.,2008, Nakshatri et al., 2009, Taylor et al.,2010). ER-a negative was related to activation of genes implicated in Wnt, Sonic Hedgehog, and TGF-b signaling, indicating epithelial-mesenchymal transition (EMT)(Wik et al.,2013).

Metastatic breast cancer [Evidence: high]: The “epithelial-mesenchymal transition” (EMT), a key developmental regulatory program, has been reported to play critical and intricate roles in promoting tumor invasion and metastasis in epithelium-derived carcinomas in recent years. Some of the cells undergoing EMT have the characteristics of cancer stem cells (CSCs), which are linked to cancer malignancy (Shibue & Weinberg, 2017; Shihori Tanabe, 2015a, 2015b; Tanabe, Aoyagi, Yokozaki, & Sasaki, 2015). Cancer metastasis and cancer therapeutic resistance are linked to the EMT phenomenon (Smith & Bhowmick, 2016; Tanabe, 2013). EMT causes the cell to escape from the basement membrane and metastasize by increasing the production of enzymes that break down extracellular matrix components and decreasing adherence to the basement membrane (Smith & Bhowmick, 2016).

Overall Assessment:

overall assessment of the AOP was based on the biological domain of the applicability, the essentiality of all KEs, Biological plausibility of each KER, Empirical support for each KER, and Quantitative weight of evidence considerations optional.

	MIE 1669	KE 155	KE 185	KE1980	KE1981	KE 1172
Sex/Life stage /Taxa	Female/Reproductive/Human,human cell line,mice,rat	Female/Reproductive/Rat/rat cel lines/mouse	Female/Reproductive/Mice,yeast,hman cel line	Female/Reproductive/canine,mouse,human cell line	Female/Reproductive/human,human cell ine	Female/Reproductive/human,hum cell ine,mice
Essentiality of KEs	Direct Evidence	Direct Evidence	Direct Evidence	Direct Evidence	Direct Evidence	Direct Evidence
Empirical Support of KER	High for MIE1669 to KE155	High for KE 155 to KE 185	Moderate for KE 185 to KE1980	Moderate for KE1980 to KE1981	Moderate for KE1981 to KE 1172	Moderate for KE 1172 to KE 112
Biological plausibiity of KER	High for MIE1669 to KE155	High for KE 155 to KE 185	Moderate for KE 185 to KE1980	Moderate for KE1980 to KE1981	Moderate for KE1981 to KE 1172	Moderate for KE 1172 to KE 112
Quantitative assessment	PCR-RFLP OHdG – ELISA &RT- PCR, Western Blot MAPK assay, Immunoprecipitation, Western immunoblotting 8.	Quantification of ATase activity – BSA method APNG assay, OXOG glycosylase activity assay, Western immunoblotting, Immunohistochemical detection of ATase.	Acetaldehyde assay, Extract preparation and Western blotting, N^2- Ethyl dGuo quantitation	Western blotting,clonal survival assay,FACs	qRT-PCR,Western blotting,Luciferase reporter assay Micro-array	qRT-PCR,immunohistochemistry
References	Chen CH et al 2011,	Panida Navasumrit et al, 2001),Kotova N et al, 2013,Garaycoechea JI et al, 2012	Abraham J et al 2011,Garaycoechea JI et al, 2018,Voordeckers K et al, 2020	van Jaarsveld MT et al 2014, Abdelfattah, N et al, 2018,Liu Z et al, 2017,Zhang X et al,2011 Wan G et al, 2013,Bulkowska M et al, 2017	Shen ZL et al 2016,Guo S et al 2020,Bae HJ et al 2014,Zhou J et al 2017,Fu H et al 2018,,Lian B et al 2018,Guan Y et al 2017,Yang X et al 2014,Jiang G et al 2016,Luo J et al 2017,Tian Z et al 2016,Yan X et al 2019,Zhang S et al 2016	McGlynn LM et al 2014,Paul Pfluger et al 2008,Yeung F et 2004

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
human	Homo sapiens	Moderate	NCBI
mice	Mus sp.	Moderate	NCBI
rat	Rattus norvegicus	Moderate	NCBI
canine heartworm nematode	Dirofilaria immitis	Moderate	NCBI
yeast	Saccharomyces cerevisiae	Moderate	NCBI

Sex Applicability

Sex	Evidence
Female	High

Sex:The AOP is appicable to women.However study suggests that the relative risk of breast cancer in men is comparable to that in women for alcohol intakes below 60 g per day. It continues to increase at high consumption levels not usually studied in women (Guénel P et al 2004).

Life stage: There are no research articles which highlight the role alcohol in a particular life stage.In addition, age-related differences in response to alcohol exposure are neither uniform nor linear. The data available is insufficient which direct the construction of a catalog of “appropriate” tests or to define all the factors which influence nonlinear effects (Squeglia LM et al 2014).

Taxonomic:The evidences for the key events of this AOP are available in various species ike rat,mice and humans.

Essentiality of the Key Events

Direct evidence is available for all the suggested key events. However the strength of weight of evidence varies from moderate to high. however, some inconsistencies are also available. majority of the experimental evidence is available in rats, mice, canine and human cell lines. only a few studies are available on human subjects.

- Human normal hepatocytes (HL-7702) were subjected to escalating doses of N,N-dimethylformamide for 24 hours (C. Wang et al., 2016). At all concentrations, a concentration-dependent increase in ROS was detected; the rise was statistically significant when compared to control (6.4, 16, 40, 100 mM). Until the highest two concentrations (40 and 100 mM), no significant rise in 8-oxodG was seen, indicating

inadequate repair at these dosages. Excision repair genes (XRCC2 and XRCC3) were considerably up-regulated at 6.4 and 16 mM, well below the doses that significantly produced 8-oxodG, indicating that adequate DNA repair was possible at these low concentrations. These findings show that repair is competent at low concentrations (removing 8-oxodG quickly), but that repair is swamped (i.e., insufficient) at larger doses, where 8-oxodG greatly increases. AS52 Chinese hamster ovary cells (wild type and OGG1-overexpressing) were exposed to varying doses of ultraviolet A (UVA) radiation (Dahle et al., 2008). Formamidopyrimidine glycosylase (Fpg)-sensitive sites were quantified using alkaline elution after increasing repair times (0, 1, 2, 3, 4 h) following 100 kJ/m^2 UVA irradiation. OGG1-overexpressing AS52 cells (OGG1+): Fpg-sensitive sites reduced to 71% within half an hour and down to background levels at 4h.Wild type AS52 cells: at 4h, 70% of the Fpg-sensitive sites remained, indicating accumulation of oxidative lesions. Mutations in the *Gpt* gene was quantified in both wild type and OGG1+ cells by sequencing after 13-15 days following 400 kJ/m^2 UVA irradiation.G:C→T:A mutations in UVA-irradiated OGG1+ cells were completely eliminated (thus, repair was sufficient when repair overexpressed).G:C→T:A mutation frequency in wild type cells increased from 1.8 mutants/million cells to 3.8 mutants/million cells following irradiation – indicating incorrect repair or lack of repair of accumulated 8-oxo-dG.

- There is evidence from knock-out/knock-down studies indicating there is a strong link between DNA repair adequacy and the frequency of mutations. Defects in proteins involved in DNA repair resulted in altered mutation frequencies in all of the instances studied when compared to wild-type cases. In cell lines deficient in LIG4 (Smith et al., 2003) and Ku80 (Feldmann et al., 2000), there were significant decline in the frequency and accuracy of DNA repair; 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). There was more spontaneous DNA damage in Nibrin-deficient mouse cells than in wild-type controls, implying insufficient DNA repair. In vivo mutation frequencies were also observed to be higher in Nibrin-deficient mice than in wild-type mice using the corresponding Nibrin-deficient and wild-type mice (Wessendorf et al., 2014). Furthermore, depending on the XPC status of cancer patients, mutation densities in certain genomic areas were influenced differentially. In XPC-wild-type patients, mutation frequencies were higher at DHS promoters and 100 bp upstream of TSS than in cancer patients missing functional XPC (Perera et al., 2016). Finally,it was found that radiation exposure caused four times more mutations in WKT1 cells with lower repair capacity than in TK6 cells with normal repair capacity in a research (Amundson and Chen, 1996).
- There are findings that strongly link the different elements of DNA damage and repair events to the expression of miRNA. Zhang and coworkers examined genome-wide mature miRNA expression in Atm+/+ and Atm-/- littermate mouse embryonic fibroblasts to see how miRNAs are regulated in the DNA damage response (MEFs)(Zhang et al.,2011). MEFs were given neocarzinostatin (NCS), a radiomimetic medication that causes DSBs (Ziv et al., 2006). Mouse miRNA microarray analysis was used to determine miRNA expression profile in each sample, which was done at several time points (0–24 hr). As many as 71 distinct miRNAs were found to be considerably (2-fold) upregulated in the NCS-treated Atm+/+ MEFs, but not in the corresponding Atm-/- MEFs, implying that DNA damage stress causes broad-spectrum changes in miRNA expression. According to Wan et al., regulatory RNA-binding proteins in the Drosha and Dicer complexes, such as DDX5 and KSRP, drive posttranscriptional processing of primary and precursor miRNAs after DNA damage. The findings show that nuclear export of pre-miRNAs is increased in an ATM-dependent manner after DNA damage. The ATM-activated AKT kinase phosphorylates Nup153, a main component of the nucleopore, resulting in enhanced interaction between Nup153 and Exportin-5 (XPO5) and increased nuclear export of pre-miRNAs. These findings demonstrate that DNA damage signalling is important for miRNA transport and maturation. In agreement with previous reports showing that ATM-activated p53 and KSRP promote miRNA expression (Suzuki et al., 2009; Zhang et al., 2011), the study found 61 p53-dependent miRNAs and 29 KSRP-dependent miRNAs among the ATM-induced miRNAs.
- In Jiang et al's study, the cellular function and molecular mechanism of miR2045p in hepatocellular cancer were investigated (HCC)(Jiang et al.,2016). Shen et al showed that downregulation of miR-199b is associated with distant metastasis in colorectal cancer via activation of SIRT1 and inhibition of CREB/KISS1 signalling(Shen et al., 2016). A study by Tian et al found that MicroRNA-133b targets Sirt1 and suppresses hepatocellular carcinoma cell progression(Tian et al., 2016). In liver cancer, Yan et al discovered that MicroRNA 486 5p acts as a tumour suppressor of proliferation and cancer stem-like cell characteristics by targeting Sirt1(Yan et al.,2019). Zhang et al reported that MicroRNA-22 functions as a tumor suppressor by targeting SIRT1 in renal cell carcinoma (Zhang et al., 2016).
- According to Lu et al, SIRT1 inhibited the growth of gastric cancer through inhibiting the activation of STAT3 and NF-B (Lu et al.,2014). The goal was to look at SIRT1's regulatory effects on gastric cancer (GC) cells (AGS and MKN-45) as well as the links between SIRT1 and STAT3 and NF-B activation in GC cells. The SIRT1 activator (resveratrol RSV) was discovered to contribute to the repression of viability and increase of senescence, which was reversed by SIRT1 inhibitor (nicotinamide NA) and SIRT1 depletion using the CCK-8 and SA-β-gal assays, respectively. SIRT1 activation (RSV supplement) reduced not only STAT3 activation, including STAT3 mRNA level, c-myc mRNA level, phosphorylated STAT3 (pSTAT3) proteins, and acetylizad STAT3 (acSTAT3) proteins, but also pNF-B p65 and acNF-B p65 suppression. The effects of RSV were reversed by NA. Furthermore, when STAT3 or NF-B were knocked down, neither RSV nor NA could affect cellular survival or senescence in MKN-45 cells. Overall, the outcomes of the study revealed that SIRT1 activation could cause GC in vitro to lose viability and senescence. Furthermore, our findings demonstrated that SIRT1 inhibited proliferation in GC cells and was related to deacetylation-mediated suppression of STAT3 and NF-B protein activation. The levels of SIRT1 protein expression in non-small-cell lung cancer (NSCLC) cell lines were examined in a study by Yeung et al.,2004. In comparison to immortalised epithelial human lung NL-20 cells, NSCLC cells exhibit significant quantities of SIRT1 protein, as reported by other researchers (Luo et al, 2001; Vaziri et al, 2001). Pharmacological modulators of Sirtuin activity were employed to see if NF-kB transcription was regulated by Sirtuins (Landry et al, 2000; Bedalov et al, 2001; Howitz et al, 2003). Transient luciferase reporter experiments revealed that cells pretreated with resveratrol had very minimal NF-kB transcription following the presence of TNFa. TNFa-induced NF-kB activity was boosted when cells were pretreated with the Sirtuin inhibitors nicotinamide or splitomicin. NF-kB transcription was also potentiated in cells treated with trichostatin A (TSA), an HDAC class I and class II inhibitor, as expected.
- In specific subclasses of human breast cancer cells and tumour tissue specimens, an enhanced level of activated NF-kB is found, primarily in erbB2-overexpressing ER-negative breast cancer (Biswas et al 2000;2003). Singh et al explored a variety of methods to inhibit NF-kB activation in ER-negative breast cancer cells and looked at the effects on cell proliferation, apoptosis, and tumour growth in xenografts(Singh et al.,2007). In a prospective cohort study, Sampepajung et al used immunohistochemistry (IHC) to examine NF-B expression and intrinsic subtypes of breast cancer tissue and found a significant correlation between negative ER and overexpression of NF-B (p 0.05), with overexpression of NF-B being higher in negative ER (77.3 percent) compared to positive ER (47.4 percent)(Sampepajung et al., 2021). Laere et al suggested that activation of NF-kB in inflammatory breast cancer (IBC) is associated with loss of estrogen receptor (ER) expression, indicating potential crosstalk between NF-kB and ER(Laere et al.,2007). Differential Sensitivity of ER α and ERβ Cells to the NF-kB Inhibitor Go6976 was tested. A differential sensitivity to Go6976 by ER α and ERβ breast cancer cells was observed (Holloway et al.,2004). The ER α cells were more sensitive and less viable after treatment with this NF-kB inhibitor. The IC50 (50% killing) by Go6976 was 1 mM for Era of MDA-MB435 and MDA-MB231 breast cancer cells, whereas it was greater than 10 mM for ERa of MCF-7 and T47D or the normal mammary epithelial H16N cells. At 10 mM Go6976, about 80% of the ERa cells were killed, whereas only 15–30% of ERa and normal H16N cells were sensitive to this compound. The relative resistance of the H16N normal human mammary cells indicates a possible high therapeutic index of Go6976 against ERa cancer cells.
- Endogenous ER silencing causes EMT in ER-positive breast cancer cells. ER-positive MCF-7 cells were infected with ER shRNA lentiviral particles and stable clones were selected with puromycin (optimal dose of 0.8 g/mL) to knock down ER gene expression (Zheng et al.,2014). When the number of cell passages was increased following infection, the expression of ER was gradually knocked down. Saleh et al. hypothesise that loss of oestrogen receptor function, which causes endocrine resistance in breast cancer, also causes trans-differentiation from an epithelial to a mesenchymal phenotype, which causes enhanced aggressiveness and metastatic tendency(Saleh et al., 2011).
- EMT is the most crucial step in initiating metastasis, including metastasis to lymph nodes, because tumour cell movement is a pre-requisite for the metastatic process (Da et al., 2017). Multiple signalling pathways cause cancer cells to lose their cell-to-cell connections and cellular polarity during EMT, increasing their motility and invasive ness (Huang et al., 2017). MMPs cause E-cadherin to be cleaved, which increases tumour cell motility and invasion (Pradella et al., 2017). Chen et al investigated the potential function of MDM2 in ovarian cancer SKOV3 cells' EMT and metastasis(Chen et al.,2015). TGFbeta and Twist induce EMT by upregulating the expression of EMT markers such Snail, Vimentin, N-cadherin, and ABC transporters like ABCA3, ABCC1, ABCC3, and ABCC10 (Saxena et al., 2011).In the treatment with about 0.3, 3, 30 mM of doxorubicin, human mammary epithelial cells (HMLE) stably expressing Twist, FOXC2 or Snail demonstrate increased cell viability compared to control HMLE, dose-dependently (Saxena et al., 2011).

Weight of Evidence Summary

Increased, DNA damage and mutation leads to Inadequate DNA repair

DNA base excision repair (BER) and, to a lesser extent, nucleotide excision repair (NER) are used to repair oxidative DNA damage. Previous research has found thresholded dose-response curves in oxidative DNA damage and attributed these findings to a lack of repair capability at the curve's inflection point (Gagne et al., 2012; Seager et al., 2012). Following chemical exposures, in vivo, a rise and buildup of oxidative DNA lesions was seen despite the activation of BER, suggesting poor repair of oxidative DNA lesions beyond a certain level(Ma et al., 2008).

Empirical Evidence (include consideration of temporal concordance) has been documented in several studies as follows;

Compound class	Species	Study type	Dose	KER findings	Reference
N,N-dimethylformamide	Homosapiens hepatocyte cell line	In vitro Experimental	mM	Increased DNA damage leads to decreased DNA cross link repair mechanisms	Wang et al.,2016
UV radiation	Cricetulus griseus(Chinese hamster)	-do-	kJ/m2	-do-	Dahle et al.,2008
X-rays	Human leukemia cell line	-do-	Gy/min	-do-	Li et al.,2013
X rays	Mice	In vivo Experimental	Gy/min	-do-	Li et al.,2013
Aniline	Rat	-do-	Kg/day	-do-	Ma et al.,2008

Inadequate DNA repair leads to Increase, Mutations

There will be no increase in mutation frequency if DNA repair is capable of appropriately and efficiently repairing DNA lesions caused by a genotoxic stressor.

For alkylated DNA, for example, efficient AGT removal will result in no increases in mutation frequency. However, once AGT reaches a certain dose, it becomes saturated and can no longer effectively remove alkyl adducts. Mutation occurs when O-alkyl adducts are replicated. The evidence that unrepaired O-alkylated DNA replication induces mutations in somatic cells is vast and has been evaluated. (Basu and Essigmann 1990; Shrivastav et al. 2010).

Empirical Evidence (include consideration of temporal concordance) has been documented in several studies as follows;

Compound class	Species	Study type	Dose	KER findings	Reference
				inadequate DNA	

UV radiation	Chinese hamster	In vitro	kJ/m2	Inadequate DNA repair leads to increased mutations	Dahle et al.,2008
	Mice	In vivo		-do-	Klungland et al., 1999
X ray	human	In vitro	Gy	-do-(dose-incidence)	Mcmohan et al., 2016

Increase, Mutations leads to Increase,miRNA levels

Evidences suggest that transcription pathway for miRNAs is regulated in the DNA damage response (DDR).Inadequate repair and mutations increase miRNA expression.DNA damage-responsive transcription factors, such as NF-kB, E2F, and Myc, are also involved in miRNA transcription regulation.The p53 protein also functions as a transcriptional repressor by binding to miRNA promoters and preventing the recruitment of transcriptional activators.The empirical and dose response evidence for increased mutations inducing miRNA expression has been documented as follows;

Compound class	Species	Study type	Dose	KER findings	Reference
Neocarzinostatin	Mouse Fibroblast	In vitro	Ng/ml	Increased mutation leads to increased microRNA expression	Ziv et al.,2006
Neocarzinostatin	Mouse Fibroblast	In vitro	Ng/ml	-do-	Zhang et al.,2011
Cisplatin and IR	Human mammary epithelial cells	In vitro	mM and Gy	-do-	Jaarsveld et al., 2014

Increase,miRNA levels leads to Decrease,SIRT1(sirtuin 1) leves

There are several pathways which suggest suppression of SIRT1 expression when miRNA is elevated.SIRT1 was downregulated at the mRNA and protein levels when miR-138 expression was increased. MiR-138 binds to the SIRT1 gene's 3′UTR unique complimentary site and inhibits SIRT1 expression directly, preventing HCC proliferation, migration, and invasion (Luo et al.,2017).When compared to the normal hepatic cell line L02, SIRT1 is overexpressed, while miR-138 levels are lowered in HepG2, SMMC7721, Bel7404, and HCCM3 .

The evidence for this fact has been listed as follows;

Compound class	Species	Study type	KER findings	Reference
	Human HCC Cell lines	In vitro	Increased miRNA leads to Reduced SIRT1	Jiang et al.,2016; Luo et al.,2017; Tian et al., 2016; Yan et al.,2019; Bae et al.,2014;Zhou et al.,2017
	Human CRC cell lines	In vitro	-do-	Shen et al., 2016;Lian et al.,2018
	Human RCC Cell lines	In vitro	-do-	Zhang et al., 2016;Fu et al.,2018
Astragalus Polysachcharide	Prostate cancer cell lines	In vitro	-do-	Guo et al.,2020;Yang et al.,2014
	Lung cancer cell lines	In vitro	-do-	Guan et al.,2017

Decrease,SIRT1(sirtuin 1) leves leads to Increase activation, Nuclear factor kappa B (NF-kB)

SIRT1 suppresses NF-B signalling either directly by deacetylating the RelA/p65 subunit or indirectly by triggering repressive transcriptional complexes, which frequently involve heterochromatin formation at NF-B promoter regions. SIRT1 expression and signalling are both inhibited by NF-B.

Zhang et al. found that overexpressing RelA/p65 protein increased SIRT1 expression at both the transcriptional and protein levels (36 h treatment), whereas knocking down RelA/p65 expression decreased TNF-induced SIRT1 expression (8 h treatment)(Zhang et al.,2010). They also discovered that the RelA/p65 protein may bind to the SIRT1 promoter's NF-B motifs. These findings suggest that NF-B may promote SIRT1 expression. Given that SIRT1 induction appeared to occur much later than NF-B activation, it appears that this action could represent a feedback response limiting inflammation and eventually generating endotoxin tolerance.

Evidences supporting this key event is as follows;

Compound class	Species	Study type	KER findings	Reference
nicotinamide	Human gastric cancer cell lines	In vitro	Decreased, SIRT1 leads to increased NF kB activity	*Lu* et al.,2014
nicotinamide or splitomicin	non-small-cell lung cancer (NSCLC) cell lines	In vitro	Decreased, SIRT1 leads to increased NF kB activity	Yeung et al.,2004; Luo et al, 2001; Vaziri et al, 2001

Increase activation, Nuclear factor kappa B (NF-kB) leads to Antagonism, Estrogen receptor

NF-kB activation in breast cancer has been extensively documented in oestrogen receptor negative (ER) breast tumours and ER breast cancer cell lines, implying a significant inhibitory interaction between both signalling pathways (Biswas et al, 2000, 2001, 2004; Zhou et al, 2005). A rise in both NF-kB DNA-binding activity (Nakshatri et al, 1997) and expression of NF-kB target genes such IL8 coincides with a transition from oestrogen dependence to oestrogen independence in breast cancer, indicating inhibitory cross-talk. The fact that some breast tumours that are resistant to the tumoricidal action of anti-estrogens become sensitised to apoptosis and show a drop in NF-kB activity after treatment with oestrogen supports the inverse relationship between ER and NF-kB activity.

-This shows that oestrogen's proapoptotic actions in these tumours are mediated via NF-kB suppression.

Both in vivo and in vitro studies support the finding;

Compound class	Species	Study type	Dose	KER findings	Reference
Bortezomib	Breast cancer cell lines	In vitro		Increased activity of NF kB, leads to Reduced Estrogen receptor expression	Singh et al.,2017; Holloway et l.,2004
	Human Breast tissue	In vivo		-do-	Biswas et al 2000;2003
	Human Breast tissue	In vivo		-do-	Sampepajung et al., 2021; Laere et al.,2007; Indra et al.,2021;

Antagonism, Estrogen receptor leads to EMT

E2/ERa signalling, in part through transcriptional activation of luminal/epithelial-related transcription factors, promotes the development of mammary epithelia along a luminal/epithelial lineage. GATA3 and ERa both promote each other (Eeckhoute et al.,2007). In normal breast epithelia, GATA3 is needed for luminal differentiation(Kouros-Mehr et al.,2008) and GATA3 and ERa control many of the same genes (Wilson et al.,2008). In mice, forcing GATA3 expression in mesenchymal breast cancer cells produces mesenchymal–epithelial transition (MET), a reversible mechanism analogous to EMT, and prevents tumour metastasis (Yan et al.,2010). Another ERa-interacting transcription factor, FOXA1, is essential for luminal lineage in mammary epithelia and stimulates ductal development in mice (Bernardo et al.,2010). FOXA1 enhances ERa gene expression by increasing the accessibility of estrogen-response regions for ERa binding (Nakshatri et al., 2009). In breast cancer cells, on the other hand, E2 appears to increase FOXA1 expression. Importantly, ERa, FOXA1, and GATA3 are all positive breast cancer prognostic factors(Nakshatri et al.,2009).

Ye et al. investigated the impact of ERa overexpression in ERa-negative breast cancer cell lines (MDA-MB-468, MDA-MB-231) or ERa knockdown in ERa-positive cell lines (MCF-7, T47D) on Slug and Snail expression and phenotypes in ERa-positive cell lines (MCF-7, T47D)(Ye et al., 2010). Slug is repressed, E-cadherin is increased, and cells develop as adherent colonies with less invasiveness when ERa is forced to get expressed. ERa knockdown, on the other hand, causes an increase in Slug expression, a decrease in E-cadherin, and spindle-shaped invasive cells.

Wik et al used integrated molecular profiling to examine Endometrial cancer samples from a primary investigation cohort and three independent validation cohorts (Wik et al.,2013). Patient survival was closely linked to ER-a immunohistochemical staining and receptor gene (ESR1) mRNA expression. In the study cohort, ER-a negative was related with activation of genes implicated in Wnt, Sonic Hedgehog, and TGF-b signalling, indicating epithelial–mesenchymal transition (EMT)

EMT leads to Metastasis, Breast Cancer

The “epithelial–mesenchymal transition” (EMT), a key developmental regulatory program, has been reported to play critical and intricate roles in promoting tumor invasion and metastasis in epithelium-derived carcinomas.

EMT is marked by a decrease in E-cadherin and β- -catenin expression and an increase in vimentin, fibronectin, and N-cadherin expression (Irani et al., 2018). EMT is a master mechanism in cancer cells that allows them to lose their epithelial characteristics and gain mesenchymal-like qualities. EMT is the most crucial step in initiating metastasis, including metastasis to lymph nodes, because tumour cell movement is a pre-requisite for the metastatic process (Da et al., 2017). Multiple signalling pathways cause cancer cells to lose their cell-to-cell connections and cellular polarity during EMT, increasing their motility and invasive ness (Huang et al., 2017). MMPs cause E-cadherin to be cleaved, which increases tumour cell motility and invasion (Pradella et al., 2017).

Quantitative Consideration

The techniques used for quantifying KE's were reliable with repeatability and reproducibility. Assays were fit for the purpose.

	MIE 1669	KE 155	KE 185	KE1980	KE1981	KE 1172	KE 112	KE1457	AO1982
Human	PCR-RFLP 8-OHdG – ELISA & MDA (Chen CH et al 2011)	-	Acetaldehyde assay, Extract preparation and Western blotting, N^2- Ethyl dGuo quantitation Abraham J et al 2011	-	-	qRT-PCR,immunohistochemistry (McGlynn LM et al 2014)	qRT-PCR, immunohistochemistry (Sampepajung E et al 2021, Van Laere SJ et al 2007,)	IHC,micro array,qPCR, SNP array(Wik E et al 2013)	Liang et al., 2013;Liu et al., 2016;Zhang et al.,2015; Chen et al., 2015;Yue et al.,2019;Wang et al., 2018;Yu et al.,2017
Human Tissues	-	-	-	-	qRT-PCR,Western blotting,Luciferase reporter assay H2,H4,H7,H8,H9 Micro-array (Shen ZL et al 2016)	-	-	-	-
Human Cell lines	RT- PCR, Western Blot MAPK assay, Immunoprecipitation, Western immunoblotting Thymidine uptake ECL-SDS PAGE, RIA Adduct removal measurements, DNA isolation, TLC, LCMS Acetaldehyde estimation, DNA adducts – LC-ESI-MS/ MS-SRM, Western blotting	-	-	Western blotting,clonal survival assay,FACs(van Jaarsveld MT et al 2014)	Micro-array, qRT-PCR,Western blotting,Luciferase reporter assay (Guo S et al 2020, Bae HJ et al 2014, Zhou J et al 2017, Fu H et al 2018, Lian B et al 2018 Guan Y et al 2017 Yang X et al 2014)	qRT-PCR,,Luciferase reporter assay Cell based HDAC assay(Yeung F et al 2004)	qPCR, western blotting, immunoprecipitation, immunofluorescent microscopy, Luciferase reporter assay EMSA, IHC,Cell viability assay (Singh S et al 2007, Holloway JN et al 2004, Biswas DK et al 2000, Song RX et al 2005, Scherbakov AM et al 2005, Scherbakov AM et al 2009)	qRT-PCR,cell viability assay, Western blotting,EdU incorporation assay(Bouris P et al 2015, Liu Y et al 2015, Al Saleh S et al 2011, Zeng Q et al 2014, Ye Y et al 2010, Lin, HY et al 2018)	qRT-PCR,,Luciferase reporter assay ,immunoblotting,immunoprecipitation,cell invasion assay,cell migration assay, bioluminesence imaging,wound healing assay,Wound scratch & Transwell assay, Microarray,Immunofluorescence, Immunohistochemistry, Gujral et al.,2014;Cui et al.,2013;Shiota et al.,2012;Gao et al.,2018;Chen et al.,2017;Liu et al.,2020;Casas et al.,2011;Jackstadt et al.,2013;Kong et al.,2016;Zhang et al.,2014;Huang et al.,2014

	Western blotting, enzymatic assay, LC-ESI-MS/ MS-SRM DNA oxidative damage by ELISA, Immunofluorescence, cell culture, 8-OHdG – ELISA & Ph2Aλ–focformation assays, P53 luciferase assays, qPCR, Western Blotting (Elise A. Triano et al 2003, Etique.Nicolas etiqu et al 2004, Izevbigie EB et al 2002, Przylipiak A et al 1996, Singletary KW et al 2001, Singletary KW et al 2004, Abraham J et al 2011, Zhao M et al 2017, Jessy Abraham J et al 2011)								
Rat	Free radical assay GC-MS-SIM (Hackney JF et al 1992, McDermott EW et al 1992)	Quantification of ATase activity – BSA method APNG assay, OXOG glycosylase activity assay, Western immunoblotting, Immunohistochemical detection of ATase. (Kotova N et al, 2013)	-	Free radicCyQuant cell Proliferation assay (Abdelfattah, N. et al 2018)	-	-	-	-	-
Rat Cell lines	-	Flow cytometric micronucleus assay, Cell cycle analysis, Replication fork elongation assay, Cytotoxicity assay, Recombination assay, (Panida Navasumrit et al, 2001)	-	-	-	-	-	-	-
Mice	Comet assay, ROS generation assay. (Lei Guo et al 2008)	FISH karyotyping, Invivo point mutation assay, Whole genome sequencing of HSC clones. (Garaycoechea JI et al, 2012)	In vivo point mutation assay Garaycoechea JI et al, 2018	Free radicCyQuant cell Proliferation assay (Abdelfattah, N. et al 2018) RNA sequence analysis,Immunostaining,immunoblotting,Flowcytometry,COMET assay,qRT PCR(Liu Z et al 2017) Microarray (Zhang X et al 2011) qRT PCR,RIP assay,Immunogold EM(Wan G et al 2013)	qRT-PCR,Western blotting,Luciferase reporter assay,ELISA,cell culture Bai XZ et al 2018	qRT-PCR,Southern and northern blotting, reporter gene assay(Paul T et al 2008)	EMSA,Autoradiography,Immunofluorescent microscopy, Westernblotting (Biswas DK et al 2001)	-	Chen et al.,2017; Gumireddy et al.,2009; Yu et al., 2016; Sarkar et al.,2015
Canine	-	-	-	micro array(Bulkowska M et al 2017)	-	-	-	-	-
Yeast	-	-	Fluctuation assay Voordeckers K et al, 2020	-	-	-	-	-	-

Considerations for Potential Applications of the AOP (optional)

Intended uses of this AOP:

- Helpful for risk assessors, in assessing the risk of alcohol on metastatic breast cancer
- If the causal relationship is established between key events, it may be useful drug targets
- An alternative model to animal model based test methods

References

44.Non-Technical Summary Archived 24 July 2006 at the Wayback Machine. UK Committee on Carcinogenicity of Chemicals in Food Consumer Products and the Environment (COC)

Abdelfattah, N., Rajamanickam, S., Panneerdoss, S., Timilsina, S., Yadav, P., Onyeagucha, B. C., ... & Rao, M. K. (2018). MiR-584-5p potentiates vincristine and radiation response by inducing spindle defects and DNA damage in medulloblastoma. Nature communications, 9(1), 1-19.

Abraham, J., Balbo, S., Crabb, D., & Brooks, P. J. (2011). Alcohol metabolism in human cells causes DNA damage and activates the fanconi anemia–breast cancer susceptibility (FA-BRCA) DNA damage response network. Alcoholism: Clinical and Experimental Research, 35(12), 2113-2120.

Al Saleh, S., Al Mulla, F., & Luqmani, Y. A. (2011). Estrogen receptor silencing induces epithelial to mesenchymal transition in human breast cancer cells. PloS one, 6(6), e20610

Albano, E., Clot, P., Morimoto, M., Tomasi, A., Ingelman-Sundberg, M., & French, S. W. (1996). Role of cytochrome P4502E1-dependent formation of hydroxyethyl free radical in the development of liver damage in rats intragastrically fed with ethanol. Hepatology, 23(1), 155-163.

Ali, S., & Coombes, R. C. (2002). Endocrine-responsive breast cancer and strategies for combating resistance. Nature Reviews Cancer, 2(2), 101-112.

Amundson, S. A., & Chen, D. J. (1996). Ionizing radiation-induced mutation of human cells with different DNA repair capacities. Advances in Space Research, 18(1-2), 119-126.

Anderson, C. W. (1993). DNA damage and the DNA-activated protein kinase. Trends in biochemical sciences, 18(11), 433-437

Bae, H. J., Noh, J. H., Kim, J. K., Eun, J. W., Jung, K. H., Kim, M. G., ... & Nam, S. W. (2014). MicroRNA-29c functions as a tumor suppressor by direct targeting oncogenic SIRT1 in hepatocellular carcinoma. Oncogene, 33(20), 2557-2567.

Bai, X. Z., Zhang, J. L., Liu, Y., Zhang, W., Li, X. Q., Wang, K. J., ... & Hu, D. H. (2018). MicroRNA-138 aggravates inflammatory responses of macrophages by targeting SIRT1 and regulating the NF-κB and AKT pathways. Cellular Physiology and Biochemistry, 49(2), 489-500.

Basu, A. K., & Essigmann, J. M. (1990). Site-specifically alkylated oligodeoxynucleotides: probes for mutagenesis, DNA repair and the structural effects of DNA damage. Mutation Research/Fundamental and

Molecular Mechanisms of Mutagenesis, 233(1-2), 189-201.

Bedalov, A., Gatbonton, T., Irvine, W. P., Gottschling, D. E., & Simon, J. A. (2001). Identification of a small molecule inhibitor of Sir2p. Proceedings of the National Academy of Sciences, 98(26), 15113-15118.

Bernardo, G. M., Lozada, K. L., Miedler, J. D., Harburg, G., Hewitt, S. C., Mosley, J. D., ... & Keri, R. A. (2010). FOXA1 is an essential determinant of ERα expression and mammary ductal morphogenesis. Development, 137(12), 2045-2054.

Biswas, D. K., Cruz, A. P., Gansberger, E., & Pardee, A. B. (2000). Epidermal growth factor-induced nuclear factor κB activation: a major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. Proceedings of the National Academy of Sciences, 97(15), 8542-8547

Biswas, D. K., Dai, S. C., Cruz, A., Weiser, B., Graner, E., & Pardee, A. B. (2001). The nuclear factor kappa B (NF-κB): a potential therapeutic target for estrogen receptor negative breast cancers. Proceedings of the National Academy of Sciences, 98(18), 10386-10391.

Biswas, D. K., Martin, K. J., McAlister, C., Cruz, A. P., Graner, E., Dai, S. C., & Pardee, A. B. (2003). Apoptosis caused by chemotherapeutic inhibition of nuclear factor-κB activation. Cancer research, 63(2), 290-295

Biswas, D. K., Martin, K. J., McAlister, C., Cruz, A. P., Graner, E., Dai, S. C., & Pardee, A. B. (2003). Apoptosis caused by chemotherapeutic inhibition of nuclear factor-κB activation. Cancer research, 63(2), 290-295.

Biswas, D. K., Shi, Q., Baily, S., Strickland, I., Ghosh, S., Pardee, A. B., & Iglehart, J. D. (2004). NF-κB activation in human breast cancer specimens and its role in cell proliferation and apoptosis. Proceedings of the National Academy of Sciences, 101(27), 10137-10142.

Biswas, D. K., Singh, S., Shi, Q., Pardee, A. B., & Iglehart, J. D. (2005). Crossroads of estrogen receptor and NF-κB signaling. Science's STKE, 2005(288), pe27-pe27.

Blander, G., & Guarente, L. (2004). The Sir2 family of protein deacetylases. Annual review of biochemistry, 73(1), 417-435..

Boffetta, P., & Hashibe, M. (2006). Alcohol and cancer. The lancet oncology, 7(2), 149-156.

Bouris, P., Skandalis, S. S., Piperigkou, Z., Afratis, N., Karamanou, K., Aletras, A. J., ... & Karamanos, N. K. (2015). Estrogen receptor alpha mediates epithelial to mesenchymal transition, expression of specific matrix effectors and functional properties of breast cancer cells. Matrix Biology, 43, 42-60.

Brabletz, T., Kalluri, R., Nieto, M. A., & Weinberg, R. A. (2018). EMT in cancer. Nature Reviews Cancer, 18(2), 128-134.

Breen, A. P., & Murphy, J. A. (1995). Reactions of oxyl radicals with DNA. Free radical biology and medicine, 18(6), 1033-1077.

Bulkowska, M., Rybicka, A., Senses, K. M., Ulewicz, K., Witt, K., Szymanska, J., ... & Krol, M. (2017). MicroRNA expression patterns in canine mammary cancer show significant differences between metastatic and non-metastatic tumours. BMC cancer, 17(1), 1-17.

Casas E, Kim J, Bendesky A, Ohno-Machado L, Wolfe CJ, Yang J.(2011) Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis. Cancer Res. 1;71(1):245-54

Cederbaum, A. I. (1989). Introduction: role of lipid peroxidation and oxidative stress in alcohol toxicity. Free Radical Biology and Medicine, 7(5), 537-539.

Chen L, Mai W, Chen M, Hu J, Zhuo Z, Lei X, Deng L, Liu J, Yao N, Huang M, Peng Y, Ye W, Zhang D.(2017) Arenobufagin inhibits prostate cancer epithelial-mesenchymal transition and metastasis by down-regulating β-catenin. Pharmacol Res. 123:130-142

Chen, C. H., Pan, C. H., Chen, C. C., & Huang, M. C. (2011). Increased oxidative DNA damage in patients with alcohol dependence and its correlation with alcohol withdrawal severity. Alcoholism: Clinical and Experimental Research, 35(2), 338-344.

Chen, H. C., Jeng, Y. M., Yuan, R. H., Hsu, H. C., & Chen, Y. L. (2012). SIRT1 promotes tumorigenesis and resistance to chemotherapy in hepatocellular carcinoma and its expression predicts poor prognosis. Annals of surgical oncology, 19(6), 2011-2019

Chen, L. F., & Greene, W. C. (2004). Shaping the nuclear action of NF-κB. Nature reviews Molecular cell biology, 5(5), 392-401.

Chen, S. P., Liu, B. X., Xu, J., Pei, X. F., Liao, Y. J., Yuan, F., & Zheng, F. (2015). MiR-449a suppresses the epithelial-mesenchymal transition and metastasis of hepatocellular carcinoma by multiple targets. BMC cancer, 15(1), 1-13.

Chen, W., & Bhatia, R. (2013). Roles of SIRT1 in leukemogenesis. Current opinion in hematology, 20(4).

Chen, W., Wang, H., Chen, H., Liu, S., Lu, H., Kong, D., ... & Lu, Z. (2014). Clinical significance and detection of micro RNA-21 in serum of patients with diffuse large B-cell lymphoma in C hinese population. European journal of haematology, 92(5), 407-412.

Chinnasamy, N., Rafferty, J. A., Margison, G. P., O'CONNOR, P. J., & Elder, R. H. (1997). Induction of O 6-alkylguanine-DNA-alkyltransferase in the hepatocytes of rats following treatment with 2-acetylaminofluorene. DNA and cell biology, 16(4), 493-500.

Clot, P. A. O. L. O., Albano, E. M. A. N. U. E. L. E., Eliasson, E. R. I. K., Tabone, M. A. R. C. O., Arico, S. A. R. I. N. O., Israel, Y., ... & Ingelman-Sundberg, M. (1996). Cytochrome P4502E1 hydroxyethyl radical adducts as the major antigen in autoantibody formation among alcoholics. Gastroenterology, 111(1), 206-216.

Cui B, Zhang S, Chen L, Yu J, Widhopf GF 2nd, Fecteau JF, Rassenti LZ, Kipps TJ. (2013)Targeting ROR1 inhibits epithelial-mesenchymal transition and metastasis. Cancer Res. 73(12):3649-60.

Da, C., Wu, K., Yue, C., Bai, P., Wang, R., Wang, G., ... & Hou, P. (2017). N-cadherin promotes thyroid tumorigenesis through modulating major signaling pathways. Oncotarget, 8(5), 8131.

Dahle, J., Brunborg, G., Svendsrud, D. H., Stokke, T., & Kvam, E. (2008). Overexpression of human OGG1 in mammalian cells decreases ultraviolet A induced mutagenesis. Cancer letters, 267(1), 18-25.

Eeckhoute, J., Keeton, E. K., Lupien, M., Krum, S. A., Carroll, J. S., & Brown, M. (2007). Positive cross-regulatory loop ties GATA-3 to estrogen receptor α expression in breast cancer. Cancer research, 67(13), 6477-6483.

Etique, N., Chardard, D., Chesnel, A., Merlin, J. L., Flament, S., & Grillier-Vuissoz, I. (2004). Ethanol stimulates proliferation, ERα and aromatase expression in MCF-7 human breast cancer cells. International journal of molecular medicine, 13(1), 149-155.

Feldmann, E., Schmiemann, V., Goedecke, W., Reichenberger, S., & Pfeiffer, P. (2000). DNA double-strand break repair in cell-free extracts from Ku80-deficient cells: implications for Ku serving as an alignment factor in non-homologous DNA end joining. Nucleic acids research, 28(13), 2585-2596.

Forsyth, C. B., Tang, Y., Shaikh, M., Zhang, L., & Keshavarzian, A. (2010). Alcohol stimulates activation of Snail, epidermal growth factor receptor signaling, and biomarkers of epithelial–mesenchymal transition in colon and breast cancer cells. Alcoholism: Clinical and Experimental Research, 34(1), 19-31.

French, S. W., Wong, K., Jui, L., Albano, E., Hagbjork, A. L., & Ingelman-Sundberg, M. (1993). Effect of ethanol on cytochrome P450 2E1 (CYP2E1), lipid peroxidation, and serum protein adduct formation in relation to liver pathology pathogenesis. Experimental and molecular pathology, 58(1), 61-75.

Fu, H., Song, W., Chen, X., Guo, T., Duan, B., Wang, X., ... & Zhang, C. (2018). MiRNA-200a induce cell apoptosis in renal cell carcinoma by directly targeting SIRT1. Molecular and cellular biochemistry, 437(1), 143-152.

Gagné, J. P., Rouleau, M., & Poirier, G. G. (2012). PARP-1 activation—bringing the pieces together. Science, 336(6082), 678-679.

Gao J, Yang Y, Qiu R, Zhang K, Teng X, Liu R, Wang Y. (2018) Proteomic analysis of the OGT interactome: novel links to epithelial-mesenchymal transition and metastasis of cervical cancer. Carcinogenesis. 39(10):1222-1234

Garaycoechea, J. I., Crossan, G. P., Langevin, F., Daly, M., Arends, M. J., & Patel, K. J. (2012). Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. Nature, 489(7417), 571-575.

Garaycoechea, J. I., Crossan, G. P., Langevin, F., Mulderrig, L., Louzada, S., Yang, F., ... & Patel, K. J. (2018). Alcohol and endogenous aldehydes damage chromosomes and mutate stem cells. Nature, 553(7687), 171-177.

Gocke, E., & Müller, L. (2009). In vivo studies in the mouse to define a threshold for the genotoxicity of EMS and ENU. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 678(2), 101-107.

Greten, F. R., & Karin, M. (2004). The IKK/NF-κB activation pathway—a target for prevention and treatment of cancer. Cancer letters, 206(2), 193-199.

GROMBACHER, T., & KAINA, B. (1996). Isolation and analysis of inducibility of the rat N-methylpurine-DNA glycosylase promoter. DNA and cell biology, 15(7), 581-588

Guan, Y., Rao, Z., & Chen, C. (2018). miR-30a suppresses lung cancer progression by targeting SIRT1. Oncotarget, 9(4), 4924.

Gujral TS, Chan M, Peshkin L, Sorger PK, Kirschner MW, MacBeath G. (2014) A noncanonical Frizzled2 pathway regulates epithelial-mesenchymal transition and metastasis. Cell. 159(4):844-56

Guo, S., Ma, B., Jiang, X., Li, X., & Jia, Y. (2020). Astragalus polysaccharides inhibits tumorigenesis and lipid metabolism through miR-138-5p/SIRT1/SREBP1 pathway in prostate cancer. Frontiers in Pharmacology, 11, 598

Guo, S., Ma, B., Jiang, X., Li, X., & Jia, Y. (2020). Astragalus polysaccharides inhibits tumorigenesis and lipid metabolism through miR-138-5p/SIRT1/SREBP1 pathway in prostate cancer. Frontiers in Pharmacology, 11, 598.

Hackney, J. F., Engelman, R. W., & Good, R. A. (1992). Ethanol calories do not enhance breast cancer in isocalorically fed C3H/Ou mice.

Han, L., Liang, X. H., Chen, L. X., Bao, S. M., & Yan, Z. Q. (2013). SIRT1 is highly expressed in brain metastasis tissues of non-small cell lung cancer (NSCLC) and in positive regulation of NSCLC cell migration. International journal of clinical and experimental pathology, 6(11), 2357.

Holloway, J. N., Murthy, S., & El-Ashry, D. (2004). A cytoplasmic substrate of mitogen-activated protein kinase is responsible for estrogen receptor-α down-regulation in breast cancer cells: the role of nuclear factor-κB. Molecular Endocrinology, 18(6), 1396-1410.

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., ... & Sinclair, D. A. (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature, 425(6954), 191-196.

Huang Y, Zhao M, Xu H, Wang K, Fu Z, Jiang Y, Yao Z. (2014) RASAL2 down-regulation in ovarian cancer promotes epithelial-mesenchymal transition and metastasis. Oncotarget. 5(16):6734-45

Huang, R., & Zong, X. (2017). Aberrant cancer metabolism in epithelial–mesenchymal transition and cancer metastasis: Mechanisms in cancer progression. Critical reviews in oncology/hematology, 115, 13-22

Huang, R., & Zong, X. (2017). Aberrant cancer metabolism in epithelial–mesenchymal transition and cancer metastasis: Mechanisms in cancer progression. Critical reviews in oncology/hematology, 115, 13-22.

Hwang, B. J., Madabushi, A., Jin, J., Lin, S. Y. S., & Lu, A. L. (2014). Histone/protein deacetylase SIRT1 is an anticancer therapeutic target. American journal of cancer research, 4(3), 211.

Irani, S., & Dehghan, A. (2018). The expression and functional significance of vascular endothelial-cadherin, CD44, and vimentin in oral squamous cell carcinoma. Journal of International Society of Preventive & Community Dentistry, 8(2), 110.

Ishii H, Thurman R, Ingelman-Sundberg M, Cederbaum A, Fernandez-Checa J, Kato S, Yokoyama H, Tsukamoto H. (1996). Oxidative stress in alcoholic liver injury. Alcohol Clin Exp Res 20:162A-l67A.

Ito, T., Yagi, S., & Yamakuchi, M. (2010). MicroRNA-34a regulation of endothelial senescence. Biochemical and biophysical research communications, 398(4), 735-740.

Jackstadt R, Röh S, Neumann J, Jung P, Hoffmann R, Horst D, Berens C, Bornkamm GW, Kirchner T, Menssen A, Hermeking H. (2013)AP4 is a mediator of epithelial-mesenchymal transition and metastasis in colorectal cancer. J Exp Med. 210(7):1331-50.

Jiang, G., Wen, L., Zheng, H., Jian, Z., & Deng, W. (2016). miR-204-5p targeting SIRT1 regulates hepatocellular carcinoma progression. Cell biochemistry and function, 34(7), 505-510

Kalkhoven, E., Wissink, S., van der Saag, P. T., & van der Burg, B. (1996). Negative Interaction between the RelA (p65) Subunit of NF-κB and the Progesterone Receptor (∗). Journal of Biological Chemistry, 271(11), 6217-6224.

Klungland, A., Rosewell, I., Hollenbach, S., Larsen, E., Daly, G., Epe, B., ... & Barnes, D. E. (1999). Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. Proceedings of the National Academy of Sciences, 96(23), 13300-13305.

Knecht, K. T., Bradford, B. U., Mason, R. P., & Thurman, R. G. (1990). In vivo formation of a free radical metabolite of ethanol. Molecular Pharmacology, 38(1), 26-30.

Kong J, Sun W, Li C, Wan L, Wang S, Wu Y, Xu E, Zhang H, Lai M. (2016)Long non-coding RNA LINC01133 inhibits epithelial-mesenchymal transition and metastasis in colorectal cancer by interacting with SRSF6. Cancer Lett. 380(2):476-484

Kotova, N., Vare, D., Schultz, N., Gradecka Meesters, D., Stępnik, M., Grawé, J., ... & Jenssen, D. (2013). Genotoxicity of alcohol is linked to DNA replication-associated damage and homologous recombination repair. Carcinogenesis, 34(2), 325-330.

Kouros-Mehr, H., Kim, J. W., Bechis, S. K., & Werb, Z. (2008). GATA-3 and the regulation of the mammary luminal cell fate. Current opinion in cell biology, 20(2), 164-170.

KUKIEŁKA, E., & CEDERBAUM, A. I. (1992). The effect of chronic ethanol consumption on NADH-and NADPH-dependent generation of reactive oxygen intermediates by isolated rat liver nuclei. Alcohol and Alcoholism, 27(3), 233-239.

Kukielka, E., & Cederbaum, A. I. (1994). DNA strand cleavage as a sensitive assay for the production of hydroxyl radicals by microsomes: role of cytochrome P4502E1 in the increased activity after ethanol treatment. Biochemical Journal, 302(3), 773-779.

Kunita, A., Morita, S., Irisa, T. U., Goto, A., Niki, T., Takai, D., ... & Fukayama, M. (2018). MicroRNA-21 in cancer-associated fibroblasts supports lung adenocarcinoma progression. Scientific reports, 8(1), 1-14.

Lai, E. C. (2002). Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. Nature genetics, 30(4), 363-364.

Landry, J., Slama, J. T., & Sternglanz, R. (2000). Role of NAD+ in the deacetylase activity of the SIR2-like proteins. Biochemical and biophysical research communications, 278(3), 685-690.

Lee, J., Padhye, A., Sharma, A., Song, G., Miao, J., Mo, Y. Y., ... & Kemper, J. K. (2010). A pathway involving farnesoid X receptor and small heterodimer partner positively regulates hepatic sirtuin 1 levels via microRNA-34a inhibition. Journal of Biological Chemistry, 285(17), 12604-12611.

Lee, M. S., Jeong, M. H., Lee, H. W., Han, H. J., Ko, A., Hewitt, S. M., ... & Song, J. (2015). PI3K/AKT activation induces PTEN ubiquitination and destabilization accelerating tumourigenesis. Nature communications, 6(1), 1-14.

LEFEBVRE, P., ZAK, P., & LAVAL, F. (1993). Induction of O6-methylguanine-DNA-methyltransferase and N3-methyladenine-DNA-glycosylase in human cells exposed to DNA-damaging agents. DNA and cell biology, 12(3), 233-241.

Lei Guo (2008). Basic and clinical pharmacology and toxicology.103:222-227.

Li, M., Lu, Y., Hu, Y., Zhai, X., Xu, W., Jing, H., ... & Yao, J. (2014). Salvianolic acid B protects against acute ethanol-induced liver injury through SIRT1-mediated deacetylation of p53 in rats. Toxicology Letters, 228(2), 67-74.

Li, Q., & Verma, I. M. (2002). NF-κB regulation in the immune system. Nature reviews immunology, 2(10), 725-734.

Li, Y. S., Song, M. F., Kasai, H., & Kawai, K. (2013). Generation and threshold level of 8-OHdG as oxidative DNA damage elicited by low dose ionizing radiation. Genes and Environment.

Lian, B., Yang, D., Liu, Y., Shi, G., Li, J., Yan, X., ... & Zhang, R. (2018). miR-128 targets the SIRT1/ROS/DR5 pathway to sensitize colorectal cancer to TRAIL-induced apoptosis. Cellular Physiology and Biochemistry, 49(6), 2151-2162.

Lieber, C. S. (1992). Metabolism of ethanol. In Medical and nutritional complications of alcoholism (pp. 1-35). Springer, Boston, MA.

Lin, H. Y., Liang, Y. K., Dou, X. W., Chen, C. F., Wei, X. L., Zeng, D., ... & Zhang, G. J. (2018). Notch3 inhibits epithelial–mesenchymal transition in breast cancer via a novel mechanism, upregulation of GATA-3 expression. Oncogenesis, 7(8), 1-15

Little, J. B. (2000). Radiation carcinogenesis. Carcinogenesis, 21(3), 397-404.

Liu M, Xiao Y, Tang W, Li J, Hong L, Dai W, Zhang W, Peng Y, Wu X, Wang J, Chen Y, Bai Y, Lin J, Yang Q, Wang Y, Lin Z, Liu S, Xiong J, Wang J, Xiang L. (2020) HOXD9 promote epithelial-mesenchymal transition and metastasis in colorectal carcinoma. Cancer Med. 9(11):3932-3943

Liu, F., Xia, Y., Parker, A. S., & Verma, I. M. (2012). IKK biology. Immunological reviews, 246(1), 239-253.

Liu, S. Y., Li, X. Y., Chen, W. Q., Hu, H., Luo, B., Shi, Y. X., ... & Lu, Z. X. (2017). Demethylation of the MIR145 promoter suppresses migration and invasion in breast cancer. Oncotarget, 8(37), 61731.

Liu, Y., Liu, R., Fu, P., Du, F., Hong, Y., Yao, M., ... & Zheng, S. (2015). N1-Guanyl-1, 7-diaminoheptane sensitizes estrogen receptor negative breast cancer cells to doxorubicin by preventing epithelial-mesenchymal transition through inhibition of eukaryotic translation initiation factor 5A2 activation. Cellular Physiology and Biochemistry, 36(6), 2494-2503

Liu, Z., Jin, Z. Y., Liu, C. H., Xie, F., Lin, X. S., & Huang, Q. (2015). MicroRNA-21 regulates biological behavior by inducing EMT in human cholangiocarcinoma. International journal of clinical and experimental pathology, 8(5), 4684.

Liu, Z., Zhang, C., Khodadadi-Jamayran, A., Dang, L., Han, X., Kim, K., ... & Zhao, R. (2017). Canonical microRNAs enable differentiation, protect against DNA damage, and promote cholesterol biosynthesis in neural stem cells. Stem cells and development, 26(3), 177-188

Lopez-Santillan, M., Larrabeiti-Etxebarria, A., Arzuaga-Mendez, J., Lopez-Lopez, E., & Garcia-Orad, A. (2018). Circulating miRNAs as biomarkers in diffuse large B-cell lymphoma: a systematic review. Oncotarget, 9(32), 22850.

Lovis, P., Roggli, E., Laybutt, D. R., Gattesco, S., Yang, J. Y., Widmann, C., ... & Regazzi, R. (2008). Alterations in microRNA expression contribute to fatty acid–induced pancreatic β-cell dysfunction. Diabetes, 57(10), 2728-2736.

Lu, J., Zhang, L., Chen, X., Lu, Q., Yang, Y., Liu, J., & Ma, X. (2014). SIRT1 counteracted the activation of STAT3 and NF-κB to repress the gastric cancer growth. International journal of clinical and experimental medicine, 7(12), 5050.

Luo, J., Chen, P., Xie, W., & Wu, F. (2017). MicroRNA-138 inhibits cell proliferation in hepatocellular carcinoma by targeting Sirt1. Oncology reports, 38(2), 1067-1074.

Luo, J., Nikolaev, A. Y., Imai, S. I., Chen, D., Su, F., Shiloh, A., ... & Gu, W. (2001). Negative control of p53 by Sir2α promotes cell survival under stress. Cell, 107(2), 137-148.

Ma, H., Wang, J., Abdel-Rahman, S. Z., Boor, P. J., & Khan, M. F. (2008). Oxidative DNA damage and its repair in rat spleen following subchronic exposure to aniline. Toxicology and applied pharmacology, 233(2), 247-253.

McDermott, E. W., O'Dwyer, P. J., & O'Higgins, N. J. (1992). Dietary alcohol intake does not increase the incidence of experimentally induced mammary carcinoma. European journal of surgical oncology, 18(3), 251-254.

McGlynn, L. M., Zino, S., MacDonald, A. I., Curle, J., Reilly, J. E., Mohammed, Z. M., ... & Shiels, P. G. (2014). SIRT2: tumour suppressor or tumour promoter in operable breast cancer?. European Journal of Cancer, 50(2), 290-301.

McMahon, S. J., Schuemann, J., Paganetti, H., & Prise, K. M. (2016). Mechanistic modelling of DNA repair and cellular survival following radiation-induced DNA damage. Scientific reports, 6(1), 1-14.

Merkhofer, E. C., Cogswell, P., & Baldwin, A. S. (2010). Her2 activates NF-κB and induces invasion through the canonical pathway involving IKKα. Oncogene, 29(8), 1238-1248.

Mirzaei, H., Masoudifar, A., Sahebkar, A., Zare, N., Sadri Nahand, J., Rashidi, B., ... & Jaafari, M. R. (2018). MicroRNA: A novel target of curcumin in cancer therapy. Journal of Cellular Physiology, 233(4), 3004-3015.

Mitra, S., & Kaina, B. (1993). Regulation of repair of alkylation damage in mammalian genomes. Progress in nucleic acid research and molecular biology, 44, 109-142.

Mohr, A. M., & Mott, J. L. (2015, February). Overview of microRNA biology. In Seminars in liver disease (Vol. 35, No. 01, pp. 003-011). Thieme Medical Publishers.

Moore, D. R., Reinke, L. A., & McCAY, P. B. (1995). Metabolism of ethanol to 1-hydroxyethyl radicals in vivo: detection with intravenous administration of alpha-(4-pyridyl-1-oxide)-Nt-butylnitrone. Molecular pharmacology, 47(6), 1224-1230.

Morimoto, M., Hagbjörk, A. L., Nanji, A. A., Ingelman-Sundberg, M., Lindros, K. O., Fu, P. C., ... & French, S. W. (1993). Role of cytochrome P4502E1 in alcoholic liver disease pathogenesis. Alcohol, 10(6), 459-464.

Moriya, M. (1993). Single-stranded shuttle phagemid for mutagenesis studies in mammalian cells: 8-oxoguanine in DNA induces targeted GC--> TA transversions in simian kidney cells. Proceedings of the National Academy of Sciences, 90(3), 1122-1126

Mullany, L. E., Herrick, J. S., Wolff, R. K., Stevens, J. R., & Slattery, M. L. (2017). Alterations in microRNA expression associated with alcohol consumption in rectal cancer subjects. Cancer Causes & Control, 28(6), 545-555.

Nakshatri, H., & Badve, S. (2009). FOXA1 in breast cancer. Expert reviews in molecular medicine, 11.

Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet Jr, R. J., & Sledge Jr, G. W. (1997). Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. Molecular and cellular biology, 17(7), 3629-3639

Nanji, A. A., Zhao, S., Sadrzadeh, S. H., Dannenberg, A. J., Tahan, S. R., & Waxman, D. J. (1994). Markedly enhanced cytochrome P450 2E1 induction and lipid peroxidation is associated with severe liver injury in fish oil—ethanol-fed rats. Alcoholism: Clinical and Experimental Research, 18(5), 1280-1285.

Navasumrit, P., Margison, G. P., & O'Connor, P. J. (2001). Ethanol modulates rat hepatic DNA repair functions. Alcohol and Alcoholism, 36(5), 369-376

O'Connor, P. J. (1989). Towards a role for promutagenic lesions in carcinogenesis. In DNA repair mechanisms and their biological implications in mammalian cells (pp. 61-71). Springer, Boston, MA.

Ortega, F. J., Moreno-Navarrete, J. M., Pardo, G., Sabater, M., Hummel, M., Ferrer, A., ... & Fernandez-Real, J. M. (2010). MiRNA expression profile of human subcutaneous adipose and during adipocyte differentiation. PloS one, 5(2), e9022.

Panagal, M., SR, S. K., Gopinathe, V., Sivakumare, P., & Sekar, D. (2018). MicroRNA21 and the various types of myeloid leukemia. Cancer Gene Therapy, 25(7), 161-166.

Perera, D., Poulos, R. C., Shah, A., Beck, D., Pimanda, J. E., & Wong, J. W. (2016). Differential DNA repair underlies mutation hotspots at active promoters in cancer genomes. Nature, 532(7598), 259-263.

Petrini, J. H., Bressan, D. A., & Yao, M. S. (1997, June). TheRAD52epistasis group in mammalian double strand break repair. In Seminars in immunology (Vol. 9, No. 3, pp. 181-188). Academic Press.

Pfluger, P. T., Herranz, D., Velasco-Miguel, S., Serrano, M., & Tschöp, M. H. (2008). Sirt1 protects against high-fat diet-induced metabolic damage. Proceedings of the national academy of sciences, 105(28), 9793-9798

Pillai, V. B., Sundaresan, N. R., & Gupta, M. P. (2014). Regulation of Akt signaling by sirtuins: its implication in cardiac hypertrophy and aging. Circulation research, 114(2), 368-378.

Pinton, G., Zonca, S., Manente, A. G., Cavaletto, M., Borroni, E., Daga, A., ... & Moro, L. (2016). SIRT1 at the crossroads of AKT1 and ERβ in malignant pleural mesothelioma cells. Oncotarget, 7(12), 14366.

Potter, P. M., Rafferty, J. A., Cawkwell, L., Wilkinson, M. C., Cooper, D. P., O'Connor, P. J., & Margison, G. P. (1991). Isolation and cDNA cloning of a rat; O 6-alkyllguanine-DNA-alkyltransferase gene, molecelar analysis of expression in rat liver. Carcinogenesis, 12(4), 727-733.

Pradella, D., Naro, C., Sette, C., & Ghigna, C. (2017). EMT and stemness: flexible processes tuned by alternative splicing in development and cancer progression. Molecular cancer, 16(1), 1-19.

Przylipiak, A., Rabe, T., Hafner, J., Przylipiak, M., & Runnebaum, B. (1996). Influence of ethanol on in vitro growth of human mammary carcinoma cell line MCF-7. Archives of gynecology and obstetrics, 258(3), 137-140.

Purohit, V., Khalsa, J., & Serrano, J. (2005). Mechanisms of alcohol-associated cancers: introduction and summary of the symposium. Alcohol, 35(3), 155-160.

Rada-Iglesias, A., Enroth, S., Ameur, A., Koch, C. M., Clelland, G. K., Respuela-Alonso, P., ... & Wadelius, C. (2007). Butyrate mediates decrease of histone acetylation centered on transcription start sites and down-regulation of associated genes. Genome research, 17(6), 708-719.

Rafferty, J. A., Clarke, A. R., Sellappan, D., Koref, M. S., Frayling, I. M., & Margison, G. P. (1996). Induction of murine O6-alkylguanine-DNA-alkyltransferase in response to ionising radiation is p53 gene dose dependent. Oncogene, 12(3), 693-697.

Rahmani, M., Dai, Y., & Grant, S. (2002). The histone deacetylase inhibitor sodium butyrate interacts synergistically with phorbol myristate acetate (PMA) to induce mitochondrial damage and apoptosis in human myeloid leukemia cells through a tumor necrosis factor-α-mediated process. Experimental cell research, 277(1), 31-47.

Rathmell, W. K., & Chu, G. (1994). Involvement of the Ku autoantigen in the cellular response to DNA double-strand breaks. Proceedings of the National Academy of Sciences, 91(16), 7623-7627.

Reinke, L. A., Rau, J. M., & McCay, P. B. (1990). Possible roles of free radicals in alcoholic tissue damage. Free radical research communications, 9(3-6), 205-211.

Riggio, M., Polo, M. L., Blaustein, M., Colman-Lerner, A., Lüthy, I., Lanari, C., & Novaro, V. (2012). PI3K/AKT pathway regulates phosphorylation of steroid receptors, hormone independence and tumor differentiation in breast cancer. Carcinogenesis, 33(3), 509-518.

Room, R., Babor, T., & Rehm, J. (2005). Alcohol and public health. The lancet, 365(9458), 519-530.

Roth, M., & Chen, W. (2014). Sorting out functions of sirtuins in cancer. Oncogene, 33(13), 1609-1620.

Sampepajung, E., Hamdani, W., Sampepajung, D., & Prihantono, P. (2021). Overexpression of NF-kB as a predictor of neoadjuvant chemotherapy response in breast cancer. Breast Disease, (Preprint), 1-9

Saxena, M., Stephens, M. A., Pathak, H., & Rangarajan, A. (2011). Transcription factors that mediate epithelial–mesenchymal transition lead to multidrug resistance by upregulating ABC transporters. Cell death & disease, 2(7), e179-e179.

Scherbakov, A. M., Lobanova, Y. S., Shatskaya, V. A., & Krasil'nikov, M. A. (2009). The breast cancer cells response to chronic hypoxia involves the opposite regulation of NF-kB and estrogen receptor signaling. Steroids, 74(6), 535-542

Seager, A. L., Shah, U. K., Mikhail, J. M., Nelson, B. C., Marquis, B. J., Doak, S. H., ... & Jenkins, G. J. (2012). Pro-oxidant induced DNA damage in human lymphoblastoid cells: homeostatic mechanisms of genotoxic tolerance. Toxicological Sciences, 128(2), 387-397.

Sethi, G., Sung, B., & Aggarwal, B. B. (2008). Nuclear factor-κB activation: from bench to bedside. Experimental biology and medicine, 233(1), 21-31.

Shen, Z. L., Wang, B., Jiang, K. W., Ye, C. X., Cheng, C., Yan, Y. C., ... & Wang, S. (2016). Downregulation of miR-199b is associated with distant metastasis in colorectal cancer via activation of SIRT1 and inhibition of CREB/KISS1 signaling. Oncotarget, 7(23), 35092

Shibue, T., & Weinberg, R. A. (2017). EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. Nature reviews Clinical oncology, 14(10), 611-629.

Shiota, M., Zardan, A., Takeuchi, A., Kumano, M., Beraldi, E., Naito, S., ... & Gleave, M. E. (2012). Clusterin mediates TGF-β–induced epithelial–mesenchymal transition and metastasis via Twist1 in prostate cancer cells. Cancer research, 72(20), 5261-5272.

Shostak, K., & Chariot, A. (2011). NF-κB, stem cells and breast cancer: the links get stronger. Breast Cancer Research, 13(4), 1-7

Shrivastav, N., Li, D., & Essigmann, J. M. (2010). Chemical biology of mutagenesis and DNA repair: cellular responses to DNA alkylation. Carcinogenesis, 31(1), 59-70.

Singh, S., Shi, Q., Bailey, S. T., Palczewski, M. J., Pardee, A. B., Iglehart, J. D., & Biswas, D. K. (2007). Nuclear factor-κB activation: a molecular therapeutic target for estrogen receptor–negative and epidermal growth factor receptor family receptor–positive human breast cancer. Molecular cancer therapeutics, 6(7), 1973-1982.

Singletary, K. W., Barnes, S. L., & van Breemen, R. B. (2004). Ethanol inhibits benzo [a] pyrene-DNA adduct removal and increases 8-oxo-deoxyguanosine formation in human mammary epithelial cells. Cancer letters, 203(2), 139-144.

Singletary, K. W., Frey, R. S., & Yan, W. (2001). Effect of ethanol on proliferation and estrogen receptor-α expression in human breast cancer cells. Cancer letters, 165(2), 131-137.

Smith, B. N., & Bhowmick, N. A. (2016). Role of EMT in metastasis and therapy resistance. Journal of clinical medicine, 5(2), 17.

Smith, J., Riballo, E., Kysela, B., Baldeyron, C., Manolis, K., Masson, C., ... & Jeggo, P. (2003). Impact of DNA ligase IV on the fidelity of end joining in human cells. Nucleic acids research, 31(8), 2157-2167.

Song, B. J., & Cederbaum, A. I. (1996). Ethanol-inducible cytochrome P450 (CYP2E1): biochemistry, molecular biology and clinical relevance: 1996 update. Alcoholism: Clinical and Experimental Research, 20, 138a-146a.

Song, R. D., Zhang, Z., Mor, G., & Santen, R. J. (2005). Down-regulation of Bcl-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER+ breast cancer cells. Apoptosis, 10(3), 667-678.

Suzuki, H. I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., & Miyazono, K. (2009). Modulation of microRNA processing by p53. Nature, 460(7254), 529-533.

Tailor, D., Hahm, E. R., Kale, R. K., Singh, S. V., & Singh, R. P. (2014). Sodium butyrate induces DRP1-mediated mitochondrial fusion and apoptosis in human colorectal cancer cells. Mitochondrion, 16, 55-64.

Tanabe, S. (2013). Perspectives of gene combinations in phenotype presentation. World journal of stem cells, 5(3), 61.

Tanabe, S., Aoyagi, K., Yokozaki, H., & Sasaki, H. (2015). Regulated genes in mesenchymal stem cells and gastric cancer. World journal of stem cells, 7(1), 208.

Taylor, M. A., Parvani, J. G., & Schiemann, W. P. (2010). The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-β in normal and malignant mammary epithelial cells. Journal of mammary gland biology and neoplasia, 15(2), 169-190.

Thurman, R. G. (1973). Induction of hepatic microsomal NADPH-dependent production of hydrogen peroxide by chronic prior treatment with ethanol. Mol. Pharmacol, 9, 670-675.

Tian, Z., Jiang, H., Liu, Y., Huang, Y., Xiong, X., Wu, H., & Dai, X. (2016). MicroRNA-133b inhibits hepatocellular carcinoma cell progression by targeting Sirt1. Experimental cell research, 343(2), 135-147

Triano, E. A., Slusher, L. B., Atkins, T. A., Beneski, J. T., Gestl, S. A., Zolfaghari, R., ... & Weisz, J. (2003). Class I alcohol dehydrogenase is highly expressed in normal human mammary epithelium but not in invasive breast cancer: implications for breast carcinogenesis. Cancer research, 63(12), 3092-3100.

van Jaarsveld MT, Wouters MD, Boersma AW, Smid M, van Ijcken WF, Mathijssen RH, Hoeijmakers JH, Martens JW, van Laere S, Wiemer EA, Pothof J. (2014) .DNA damage responsive microRNAs misexpressed in human cancer modulate therapy sensitivity. Mol Oncol. 8(3), 458-68

Van Laere, S. J., Van der Auwera, I., Van den Eynden, G. G., Van Dam, P., Van Marck, E. A., Vermeulen, P. B., & Dirix, L. Y. (2007). NF-κB activation in inflammatory breast cancer is associated with oestrogen receptor downregulation, secondary to EGFR and/or ErbB2 overexpression and MAPK hyperactivation. British journal of cancer, 97(5), 659-669

Vaziri, H., Dessain, S. K., Eaton, E. N., Imai, S. I., Frye, R. A., Pandita, T. K., ... & Weinberg, R. A. (2001). hSIR2SIRT1 functions as an NAD-dependent p53 deacetylase. Cell, 107(2), 149-159.

Voordeckers, K., Colding, C., Grasso, L., Pardo, B., Hoes, L., Kominek, J., ... & Verstrepen, K. J. (2020). Ethanol exposure increases mutation rate through error-prone polymerases. Nature communications, 11(1), 1-16

Wan, G., Tian, L., Yu, Y., Li, F., Wang, X., Li, C., ... & Cao, F. (2017). Overexpression of Pofut1 and activated Notch1 may be associated with poor prognosis in breast cancer. Biochemical and biophysical research communications, 491(1), 104-111.

Wan, G., Zhang, X., Langley, R. R., Liu, Y., Hu, X., Han, C., ... & Lu, X. (2013). DNA-damage-induced nuclear export of precursor microRNAs is regulated by the ATM-AKT pathway. Cell reports, 3(6), 2100-2112.

Wang, C., Yang, J., Lu, D., Fan, Y., Zhao, M., & Li, Z. (2016). Oxidative stress-related DNA damage and homologous recombination repairing induced by N, N-dimethylformamide. Journal of Applied Toxicology, 36(7), 936-945.

Wang, R. H., Sengupta, K., Li, C., Kim, H. S., Cao, L., Xiao, C., ... & Deng, C. X. (2008). Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. Cancer cell, 14(4), 312-323.

Wang, R., Li, C., Qiao, P., Xue, Y., Zheng, X., Chen, H., ... & Ba, X. (2018). OGG1-initiated base excision repair exacerbates oxidative stress-induced parthanatos. Cell death & disease, 9(6), 1-15.

Wang, R., Sun, Q., Wang, P., Liu, M., Xiong, S., Luo, J., ... & Cheng, B. (2016). Notch and Wnt/β-catenin signaling pathway play important roles in activating liver cancer stem cells. Oncotarget, 7(5), 5754.

Wessendorf, P., Vijg, J., Nussenzweig, A., & Digweed, M. (2014). Deficiency of the DNA repair protein nibrin increases the basal but not the radiation induced mutation frequency in vivo. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 769, 11-16.

Wik, E., Ræder, M. B., Krakstad, C., Trovik, J., Birkeland, E., Hoivik, E. A., ... & Salvesen, H. B. (2013). Lack of estrogen receptor-α is associated with epithelial–mesenchymal transition and PI3K alterations in endometrial carcinoma. Clinical Cancer Research, 19(5), 1094-1105.

Wilson, B. J., & Giguère, V. (2008). Meta-analysis of human cancer microarrays reveals GATA3 is integral to the estrogen receptor alpha pathway. Molecular cancer, 7(1), 1-8.

Wu, C. X., Xu, A., Zhang, C. C., Olson, P., Chen, L., Lee, T. K., ... & Wang, X. Q. (2017). Notch inhibitor PF-03084014 inhibits hepatocellular carcinoma growth and metastasis via suppression of cancer stemness due to reduced activation of Notch1–Stat3. Molecular cancer therapeutics, 16(8), 1531-1543

Yamakuchi, M., Ferlito, M., & Lowenstein, C. J. (2008). miR-34a repression of SIRT1 regulates apoptosis. Proceedings of the National Academy of Sciences, 105(36), 13421-13426.

Yan, H. L., Xue, G., Mei, Q., Wang, Y. Z., Ding, F. X., Liu, M. F., ... & Sun, S. H. (2009). Repression of the miR-17-92 cluster by p53 has an important function in hypoxia-induced apoptosis. The EMBO journal, 28(18), 2719-2732

Yan, W., Cao, Q. J., Arenas, R. B., Bentley, B., & Shao, R. (2010). GATA3 inhibits breast cancer metastasis through the reversal of epithelial-mesenchymal transition. Journal of Biological Chemistry, 285(18), 14042-14051.

Yan, X., Liu, X., Wang, Z., Cheng, Q., Ji, G., Yang, H., ... & Pei, X. (2019). MicroRNA4865p functions as a tumor suppressor of proliferation and cancer stemlike cell properties by targeting Sirt1 in liver cancer. Oncology reports, 41(3), 1938-1948.

Yao, H., Li, P., Venters, B. J., Zheng, S., Thompson, P. R., Pugh, B. F., & Wang, Y. (2008). Histone Arg modifications and p53 regulate the expression of OKL38, a mediator of apoptosis. Journal of Biological Chemistry, 283(29), 20060-20068.

Ye, X., Brabletz, T., Kang, Y., Longmore, G. D., Nieto, M. A., Stanger, B. Z., ... & Weinberg, R. A. (2017). Upholding a role for EMT in breast cancer metastasis. Nature, 547(7661), E1-E3.

Ye, Y., Xiao, Y., Wang, W., Yearsley, K., Gao, J. X., Shetuni, B., & Barsky, S. H. (2010). ERα signaling through slug regulates E-cadherin and EMT. Oncogene, 29(10), 1451-1462

Yeung, F., Hoberg, J. E., Ramsey, C. S., Keller, M. D., Jones, D. R., Frye, R. A., & Mayo, M. W. (2004). Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. The EMBO journal, 23(12), 2369-2380

Yin, H., Hu, M., Liang, X., Ajmo, J. M., Li, X., Bataller, R., ... & You, M. (2014). Deletion of SIRT1 from hepatocytes in mice disrupts lipin-1 signaling and aggravates alcoholic fatty liver. Gastroenterology, 146(3), 801-811.

Yuan, J., Minter-Dykhouse, K., & Lou, Z. (2009). A c-Myc–SIRT1 feedback loop regulates cell growth and transformation. Journal of Cell Biology, 185(2), 203-211.

Zeng, Q., Zhang, P., Wu, Z., Xue, P., Lu, D., Ye, Z., ... & Yan, X. (2014). Quantitative proteomics reveals ER-α involvement in CD146-induced epithelial-mesenchymal transition in breast cancer cells. Journal of proteomics, 103, 153-169.

Zhang JP, Zeng C, Xu L, Gong J, Fang JH, Zhuang SM. (2014) MicroRNA-148a suppresses the epithelial-mesenchymal transition and metastasis of hepatoma cells by targeting Met/Snail signaling. Oncogene. 33(31):4069-76.

Zhang, H. N., Li, L., Gao, P., Chen, H. Z., Zhang, R., Wei, Y. S., ... & Liang, C. C. (2010). Involvement of the p65/RelA subunit of NF-κB in TNF-α-induced SIRT1 expression in vascular smooth muscle cells. Biochemical and biophysical research communications, 397(3), 569-575.

Zhang, J., Yang, Y., Yang, T., Liu, Y., Li, A., Fu, S., ... & Zhou, W. (2010). microRNA-22, downregulated in hepatocellular carcinoma and correlated with prognosis, suppresses cell proliferation and tumourigenicity. British journal of cancer, 103(8), 1215-1220.

Zhang, X., Wan, G., Berger, F. G., He, X., & Lu, X. (2011). The ATM kinase induces microRNA biogenesis in the DNA damage response. Molecular cell, 41(4), 371-383.

Zhao, M., Howard, E. W., Guo, Z., Parris, A. B., & Yang, X. (2017). p53 pathway determines the cellular response to alcohol-induced DNA damage in MCF-7 breast cancer cells. PLoS One, 12(4), e0175121.

Zhao, W., Kruse, J. P., Tang, Y., Jung, S. Y., Qin, J., & Gu, W. (2008). Negative regulation of the deacetylase SIRT1 by DBC1. Nature, 451(7178), 587-590.

Zhou, B., Wang, D., Sun, G., Mei, F., Cui, Y., & Xu, H. (2018). Effect of miR-21 on apoptosis in lung cancer cell through inhibiting the PI3K/Akt/NF-κB signaling pathway in vitro and in vivo. Cellular Physiology and Biochemistry, 46(3), 999-1008.

Zhou, J., Zhou, W., Kong, F., Xiao, X., Kuang, H., & Zhu, Y. (2017). microRNA34a overexpression inhibits cell migration and invasion via regulating SIRT1 in hepatocellular carcinoma Corrigendum in/10.3892/ol. 2019.11048. Oncology letters, 14(6), 6950-6954.

Zhou, Y., Eppenberger-Castori, S., Eppenberger, U., & Benz, C. C. (2005). The NFkB pathway and endocrine-resistant breast cancer. Endocrine Related Cancer, 12(1), S37.

Zovoilis, A., Agbemenyah, H. Y., Agis-Balboa, R. C., Stilling, R. M., Edbauer, D., Rao, P., ... & Fischer, A. (2011). microRNA-34c is a novel target to treat dementias. The EMBO journal, 30(20), 4299-4308.

Appendix 1

List of MIEs in this AOP

Event: 1669: Increased, DNA damage and mutation

Short Name: Increased, DNA damage and mutation

Key Event Component

Process	Object	Action
DNA damage response, detection of DNA damage	site of DNA damage	increased
mutation		increased
chromosome breakage	chromosome	increased
chromosomal instability	chromosome	increased
abnormal DNA repair	DNA repair complex	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:303 - Frustrated phagocytosis-induced lung cancer	KeyEvent
Aop:409 - Frustrated phagocytosis leads to malignant mesothelioma	KeyEvent
Aop:416 - Aryl hydrocarbon receptor activation leading to lung cancer through IL-6 toxicity pathway	KeyEvent
Aop:417 - Aryl hydrocarbon receptor activation leading to lung cancer through AHR-ARNT toxicity pathway	KeyEvent
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	MolecularInitiatingEvent
Aop:451 - Interaction with lung resident cell membrane components leads to lung cancer	KeyEvent

Stressors

Name
Reactive oxygen species

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals		NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	

Sex Applicability

Sex	Evidence
Unspecific	

The DNA damages and mutations can occur in mammals, male or female, and is generally measured in adults.

Key Event Description

DNA damages are alteration of the DNA backbone including abasic site, single or double strand breaks or inter-strand crosslinks. These damages could be recognized and repaired by specialized enzymes. However, if damages persist, mutation in the DNA sequences can occur. Unlike DNA damages, DNA mutations when both strands are modified cannot be repaired and are heritable. Mutations affect the genotype and could affect phenotype.

Different mechanisms are implicated in DNA damage such as oxidative burst, DNA repair dysfunction or centrosome amplification and chromosome instability [1].

How it is Measured or Detected

DNA damages could be measured using different assays, such as micronucleus formation (OECD n°487) [2], comet assay with different protocols for the detection of double and single-strand breaks, DNA-DNA and DNA-protein crosslinks, adduct and oxidized nucleotides (OECD n°489) [3, 4] and γH2AX for the analysis of DNA strand breaks [5].

DNA mutation could be analyzed with Ames test or *via* the analysis of frequencies of mutations (OECD n°471) [6].

References

1. Zhang Y. Cell toxicity mechanism and biomarker. 2018;7 1:34; doi: 10.1186/s40169-018-0212-7.
2. Kato T, Totsuka Y, Ishino K, Matsumoto Y, Tada Y, Nakae D, et al. Genotoxicity of multi-walled carbon nanotubes in both in vitro and in vivo assay systems. Nanotoxicology. 2013;7 4:452-61; doi: 10.3109/17435390.2012.674571.
3. Pacurari M, Yin XJ, Zhao J, Ding M, Leonard SS, Schwegler-Berry D, et al. Raw single-wall carbon nanotubes induce oxidative stress and activate MAPKs, AP-1, NF-kappaB, and Akt in normal and malignant human mesothelial cells. 2008;116 9:1211-7; doi: 10.1289/ehp.10924.
4. Hiraku Y, Guo F, Ma N, Yamada T, Wang S, Kawanishi S, et al. Multi-walled carbon nanotube induces nitrative DNA damage in human lung epithelial cells via HMGB1-RAGE interaction and Toll-like receptor 9 activation. Particle and fibre toxicology. 2016;13:16; doi: 10.1186/s12989-016-0127-7.
5. Catalan J, Siivola KM, Nymark P, Lindberg H, Suhonen S, Jarventaus H, et al. In vitro and in vivo genotoxic effects of straight versus tangled multi-walled carbon nanotubes. Nanotoxicology. 2016;10 6:794-806; doi: 10.3109/17435390.2015.1132345.
6. Fukai E, Sato H, Watanabe M, Nakae D, Totsuka Y. Establishment of an in vivo simulating co-culture assay platform for genotoxicity of multi-walled carbon nanotubes. Cancer science. 2018; doi: 10.1111/cas.13534.

List of Key Events in the AOP

Event: 155: Inadequate DNA repair

Short Name: Inadequate DNA repair

Key Event Component

Process	Object	Action

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 - 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
rat	Rattus norvegicus	Moderate	NCBI
Syrian golden hamster	Mesocricetus auratus	Moderate	NCBI
Homo sapiens	Homo sapiens	High	NCBI
cow	Bos taurus	Low	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 (FM-HCR)	Nagel et al., 2014	Measures the ability of human cells to repair plasma reporters, which contain different types and amounts of DNA damage; Used to measure repair processes including HR, NHEJ, BER, NER, MMR, and MGMT	HR, NHEJ, BER, NER, MMR, or MGMT	N/A
Alkaline Unwinding Assay with Time Course	Nacci et al. 1991	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 (No. 489)
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
γ-H2AX Foci Staining with Time Course	Mariotti et al. 2013 Penninckx et al. 2021	Histone H2AX is phosphorylated in the presence of DNA strand breaks, the rate of its disappearance over time is used as a measure of DNA repair	DSBs	N/A
Alkaline Elution Assay with Time Course	Larsen et al. 1982	DNA with strand breaks elute faster than DNA without, plotted against time intervals to determine the rate at which strand breaks repair	SSBs	N/A
53BP1 foci Detection with Time Course	Penninckx et al. 2021	53BP1 is recruited to the site of DNA damage, the rate at which its level decreases over time is used to measure DNA repair	DSBs	N/A

References

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Event: 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 - 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
Assorted Gene Loci Mutation Assays	Tindall et al., 1989; Kruger et al., 2015	After exposure to a chemical/mutagen, mutations can be measured by the ability of exposed cells to form colonies in the presence of specific compounds that would normally inhibit colony growth; Usually only cells -/- for the gene of interest are able to form colonies	N/A
TK Mutation Assay	Yamamoto et al. 2017; Liber et al., 1982; Lloyd and Kidd, 2012	After exposure to a chemical/mutagen, mutations are detected at the thymidine kinase (TK) loci of L5178Y wild-type mouse lymphoma TK (+/-) cells by measuring resistance to 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., 2017	Using this PCR technique, single base pair substitution mutations within oncogenes or tumour suppressor genes can be detected by selectively amplifying specific point mutations within an allele and selectively blocking amplification of the wild-type allele	N/A
Transgenic Rodent Mutation Assay	OECD 2013; Lambert 2005; Lambert 2009	This *in vivo* test detects gene mutations using transgenic rodents that possess transgenes and reporter genes; After *in vivo* exposure to a chemical/mutagen, the transgenes are analyzed by transfecting bacteria with the reporter gene and examining the resulting phenotype	Yes (No. 488)
Conditionally inducible transgenic mouse models	Parsons 2018 (Review)	Inducible mutations linked to fluorescent tags are introduced into transgenic mice; Upon exposure of the transgenic mice to an inducing agent, the presence and functional assessment of the mutations can be easily ascertained due to expression of the linked fluorescent tags	N/A
Error-Corrected Next Generation Sequencing (NGS)	Salk 2018 (Review)	This technique detects rare subclonal mutations within a pool of heterogeneous DNA samples through the application of new error-correction strategies to NGS; At present, few laboratories in the world are capable of doing this, but commercial services are becoming available (e.g., Duplex sequencing at TwinStrand BioSciences)	N/A

References

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Event: 1554: Increase Chromosomal Aberrations

Short Name: Increase chromosomal aberrations

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Biological Context

Level of Biological Organization
Molecular

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

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

Key Event Description

The term "structural chromosomal aberrations" refers to chromosome damage caused by breaks in the DNA that can result in the deletion, addition, or rearrangement of chromosomal segments. According to whether one or both chromatids are affected, chromosomal aberrations can be classified into two main groups: chromatid-type and chromosome-type. Additionally, they can be divided into rejoined and non-rejoined aberrations. Translocations, insertions, dicentrics, and rings are examples of rejoined aberrations, whereas acentric fragments and breaks are examples of unrejoined aberrations (Savage, 1976). Some of these abnormalities, like reciprocal translocations, are long-lasting and can last for many years (Tucker and Preston, 1996). Others, such as dicentrics and acentric fragments, are unstable and weaken with each cell division due to cell death (Boei et al., 1996). After cell division, these activities might still be visible, and the DNA is irreversibly damaged. The occurrence of chromosomal abnormalities is linked to cancer development and cell death (Mitelman, 1982).

A missing, excess, or asymmetrical part of chromosomal DNA is referred to as a chromosomal aberration (CA). There are various double-strand break (DSB) repair mechanisms that could be responsible for these DNA modifications in the chromosome structure (Obe et al., 2002).

The four basic categories of CAs are inversions, translocations, duplications, and deletions. When a section of a chromosome's genetic material is destroyed, deletions take place. When a chromosome's end portion is cut, terminal deletions result.

When a chromosome splits into two different places and wrongly rejoins, leaving the middle portion out, interstitial deletions result. Duplications occur when excess genetic material is added to or rearranged; they can take the forms of transpositions, tandem duplications, reverse duplications, and misplaced duplications (Griffiths et al., 2000). A segment of one chromosome is transferred to a non-homologous chromosome in translocations (Bunting and Nussenzweig, 2013). A reciprocal translocation occurs when regions of two non-homologous chromosomes are switched. When an inversion occurs, the DNA sequence is effectively reversed because both ends of the chromosome split and are ligated at the opposite ends.

The copy number variant is a fifth type of CA that can exist in the genome (CNV). CNVs are deletions or duplications that can range in size from 50 base pairs (Arlt et al., 2012; Arlt et al., 2014; Liu et al., 2013) up into the megabase pair range and may make up more than 10% of the human genome (Shlien et al., 2009; Zhang et al., 2016; Hastings et al., 2009). (Arlt et al., 2012; Wilson et al., 2015; Arlt et al., 2014; Zhang et al., 2016). According to Wilson et al. (2015), CNV regions are particularly abundant in large active transcription units and genes, and they are especially problematic when they result in the duplication of oncogenes or the loss of tumor suppressor genes (Liu et al., 2013; Curtis et al., 2012).

Recurrent and non-recurrent CNVs are two different types. Non-allelic homologous recombination (NAHR), a recombination process that occurs during meiosis, is hypothesised to be the cause of recurrent CNVs (Arlt et al., 2012; Hastings et al., 2009). These germline CNVs, also known as recurrent CNVs, may be inherited and are hence prevalent in various people (Shlien et al., 2009; Liu et al., 2013). It is thought that non-recurrent CNVs are created in mitotic cells during the replication process. It has been proposed that replication-related stress, particularly stalled replication forks, triggers microhomology-mediated processes to break the replication stall, which frequently leads to duplications or deletions, despite the fact that the mechanism is not well understood.

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).

Depending on whether the aberration affects the chromatid or the chromosome, CAs can be categorized. Chromosome-type aberrations (CSAs) are chromosome breakage and chromatid swaps; ring chromosomes, marker chromosomes, and dicentric chromosomes are examples of chromatid-type aberrations (CTAs) (Bonassi et al., 2008; Hagmar et al., 2004). Micronuclei (MN; small nucleus-like structures that contain a chromosome or a fragment of a chromosome that was lost during mitosis) and nucleoplasmic bridges (NPBs; physical linkages between the two nuclei) are visible in binucleated cells when cells are halted at the cytokinesis step (El-Zein et al., 2014). The DNA sequence can be examined to evaluate other CAs, as it is for identifying copy number variants (CNVs) (Liu et al., 2013).

Essentiality of the key event

Chromosomal aberrations, such as mutations, deletions, and translocations, are indicative of genetic damage, which can result from exposure to genotoxic agents. This key event represents a mechanistic step that contributes to the overall progression of the pathway, helping to bridge the gap between the initial exposure and the manifestation of adverse effects.

By showcasing experimental evidence that supports the occurrence of increased chromosomal aberrations in response to the MIE, the AOP gains scientific credibility and biological plausibility. Studies demonstrating the genotoxic effects of certain substances provide empirical support for the connectivity of events within the pathway. For example, genotoxicity assays that detect structural changes in chromosomes can serve as evidence of chromosomal aberrations (e.g., Ames test, in vitro micronucleus assay).

Furthermore, the presence of increased chromosomal aberrations is indicative of potential genetic harm, which aligns with the adverse outcome. This insight aids in risk assessment and regulatory decision-making, as the occurrence of genotoxicity informs the evaluation of the potential health risks associated with exposure to certain agents.

Fischer et al., in their mRNA expression profiles showed that the tumor subtypes of neuroblastoma had significantly more segmental genomic imbalances, indicating that a combination of expression profiling (miRNAs and mRNAs) with analysis of DNA copy number alterations, will lead to improved prognostication of this often fatal tumor subtype (Fischer et al., 2010)

How it is Measured or Detected

Assay	References	Description
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
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.
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)
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
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.
High-throughput biomarker assays (indirect measures to confirm clastogenicity)	Bryce et al. 2014, 2016, 2018 Khoury et al., 2013, Khoury et al., 2016) Hendriks et al., 2012, 2016; Wink et al., 2014	Multiplexed biomarkers can be measured by flow cytometry are used to discern clastogenic and aneugenic mechanisms for MN induction. Flow cytometry-based quantification of γH2AX foci and p53 protein expression (Bryce et al., 2016). Prediscreen Assay– In-Cell Western-based quantification of γH2AX Green fluorescent protein reporter assay to detect the activation of stress signaling pathways, including DNA damage signaling including a reporter porter that is associated with DNA double strand breaks.
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
		DNA can be stained using fluorescent dyes and

High content imaging	Shahane et al., 2016	micronuclei can be scored high-throughput microscopy image analysis.
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, 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
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
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

References

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Fischer, M., Bauer, T., Oberthür, A., Hero, B., Theissen, J., Ehrich, M., ... & Berthold, F. (2010). Integrated genomic profiling identifies two distinct molecular subtypes with divergent outcome in neuroblastoma with loss of chromosome 11q. *Oncogene*, *29*(6), 865-875.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Event: 1980: Increased microRNA expression

Short Name: Increase,miRNA levels

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Stressors

Name
Ethyl alcohol

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mice	Mus sp.	Moderate	NCBI
human and other cells in culture	human and other cells in culture	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Female	Moderate

Regulation of miRNA expression by DNA replication,damage and repair responses,transcription and translation has been proved in animals like mice,canine and cell line experiments.

Key Event Description

Biological state

The elevation of microRNA (miRNA) levels as a consequence of mutations and chromosomal aberrations is a multifaceted outcome stemming from the intricate regulatory dynamics of gene expression. These genetic alterations can trigger a cascade of events that influence miRNA expression. Mutations and aberrations in regulatory regions can lead to increased transcription of miRNA genes, augmenting the production of precursor miRNAs. Moreover, copy number changes resulting from chromosomal aberrations, such as gene amplification, can amplify the output of miRNA genes, ultimately boosting mature miRNA levels. Disruptions in genes responsible for miRNA processing can perturb the biogenesis pathway, leading to the accumulation of precursor miRNAs and subsequent rise in mature miRNA abundance. In parallel, altered regulatory interactions and epigenetic modifications brought about by genetic changes can free miRNA genes from their constraints, promoting enhanced expression. Additionally, miRNA-mediated feedback loops, influenced by mutations, can indirectly influence miRNA levels. This complex interplay underscores how genetic alterations can reshape the miRNA landscape, potentially influencing downstream gene expression patterns and contributing to diverse cellular outcomes and disease processes.

Genome integrity must be maintained for the proper functioning and survival of an organism. There has been an efficient and rapid response developed by the eukaryotic cells to DNA damage to overcome the harmful effects. As soon as the DNA damage or replication arrest is detected, the activation of cell cycle checkpoint and stopping the progress of the cell cycle thus providing time for the cell to repair the DNA damage. The response to DNA damage also leads to transcriptional regulation, activation of DNA repair, and, in severe cases, initiation of apoptosis (Harper, J.W., and Elledge, S.J. , 2007). Expression of miRNAs may be regulated by the DNA damage response. A study reported that that micro RNA expression is a a partially ATM / ATR-independent manner(Pothof, J. et al , 2009). Subsequent studies have shown that the tumor suppressor p53 promotes PrimeRNA processing via RNA helicase p68 (Suzuki, H.I et al, 2009).

Han et al evaluated miRNA expression pattern in a DNA damage regulatory protein, DDX1 in controls, as well in DDX1-knockdown U2OS cells with the help of reverse transcription quantitative-PCR (qRT-PCR) and human miRNA array (Han C et al, 2014). The study noticed a significant reduction in the expression levels of a subset of miRNAs -200 family such as miR-200a, miR-200b, miR-200c, miR-141 and miR-429 (cut-off >2-fold).d miR-429). The ovarian cancer genomics study revealed a 8-miRNA signature that defines the mesenchymal subtype of serous ovarian cancer (Yang Y, et al, 2011). Among the eight miRNAs, miR-200a, miR-29c, miR-141 and miR-101 were significantly dependent on DDX1, suggesting that DDX1 may play a role in ovarian tumor progression. Nuclear run-on assays were performed to determine whether DDX1 regulates the miRNA expression at transcriptional or post-transcriptional levels, No notable differences were seen in the transcription of pri-miR-200s from the two miR-200 gene clusters (miR-200a/200b/429 and miR-200c/141) in the control and DDX1-silenced cells . However, in the DDX1-knockdown U2OS cells, the levels of mature DDX1-dependent miRNAs, but not control miR-21, were significantly decreased. Due to the potential inhibition of miRNA processing activity, primary transcripts of the DDX1-dependent miRNAs were accumulated. Conversely, these DDX1-dependent miRNAs were up-regulated in the DDX1- overexpressing cells. The above findings suggested that expression of specific miRNAs was promoted by DDX1 at the post-transcriptional level.

Biological compartments:

Cellular, nucleus, cytoplasm and mitochondria

General role in biology:

MicroRNAs (miRNAs) are endogenous non-coding RNAs that contain approximately 22 nucleotides. They function as major regulators of various biological processes, and their dysregulation is associated with many diseases, including cancer.

Cells trigger a specific cellular responses to preserve the integrity of the genome. The DNA damage response (DDR) is one among them along with several distinct DNA repair pathways.Normal cells need to repair DNA damage through various repair mechanisms or induce apoptosis and cell cycle arrest if repair is not possible [Jackson SP and Bartek J, 2009]. Genomic instability and mutagenesis are brought about by the disruption of repair mechanisms.DNA damage response (DDR) determines the fate of the cell and controls microRNAs expression. This will in turn regulate important components of the DNA repair machinery. Various reports suggest the key role of miRNA in the regulation of the DDR [d'Adda di Fagagna F, 2014 and WeiW et al 2012].The DDR and DNA damage are known regulators of miRNA expression [Sharma V et al 2013 and Chowdhury D et al 2013]. Several studies have shown that the cellular sensitivity to chemotherapeutic drugs is affected by DDR- miRNA network.[van Jaarsveld MT et al 2014].

A bidirectional relationship between miRNAs and the DDR has been suggested by studies. The DDR is a known regulator of miRNA expression at both transcriptional and post-transcriptional levels, and miRNA-mediated gene silencing has been shown to modulate the activity of the DDR [d'Adda di Fagagna F, 2014 ; WeiW et al 2012 and Han C et al 2012]. A unique set of miRNAs as well as a common core miRNA signature are activated depending on DNA damage type and level, suggesting that miRNAs regulate the DDR by mechanisms based on the type and/or the intensity of DNA damage [Han C et al 2012]. miRNAs expression may be regulated by transcription factors either binding directly to miRNA promoters and modulating their transcriptional activity, or by modifying the expression of miRNA processing machinery components.

Studies have widely explored the TP53-mediated transcriptional pathways regulating miRNA expression following DNA damage. miRNA-34a-c is induced by DNA damage and oncogenic stress, is one of the transcriptional target of the tumor suppressor TP53 [Hermeking H et al 2012]. TP53 directly binds to the promoter of miRNA-34 and activates transcription. Micro RNA-34 has been reported to repress the mRNA transcripts of several genes involved in the regulation of cell cycle, cell proliferation and survival, such as BCL2, CCND1 CCNE2, MYC, CDK4, CDK6 and SIRT1 [Hermeking H et al 2012]. Activation of miRNA-34a promotes TP53-mediated apoptosis, cell cycle arrest or senescence [Hermeking H et al 2012]. miRNA-34a may target SIRT1, form a positive feedback loop of the acetylation of TP53, expression of its transcriptional targets, regulating cell cycle and apoptosis [Hermeking H et al 2012]. The alternative pathway involving p38 MAPK signaling also induces miR-34c [Cannell IG et al 2010]. Inhibition of miRNA 34 prevents the DNA damage induced cell cycle arrest and results in an increased DNA synthesis [Cannell IG et al 2010].

DNA damage promotes the TP53-dependent upregulation of miRNA-192, miRNA-194 and miRNA-215. The genomic region surrounding the miRNA-194/miRNA-215 cluster contains a putative TP53-binding element, indicating that these miRNAs are transcriptionally activated by TP53 [Hermeking H et al 2012]. The expression of miRNA-192 and miRNA-215 induces cell cycle arrest and targets several transcripts involved in cell cycle checkpoints [Georges SA et al 2008].

MYC and E2F, are the two other transcription factors involved in DNA damage- induced cell cycle checkpoints, that regulate the expression of several miRNAs. Both factors induce transcription of the miRNA-17-92 cluster that forms a feedback loop by inhibiting E2F expression [Aguda BD et al 2008]. E2F transcription factors are repressed by several other miRNAs, including miRNA-106a-92 and miRNA-106b-25 cluster members, miRNA-210, miRNA-128, miRNA-34 and miRNA-20 [Wan G et al 2011].

DNA damage upregulates several miRNAs, including miRNA-16-1, miRNA-143 and miRNA-145. [Suzuki HI et al 2009]. Most TP53 mutations found in cancers are located in a domain required for miRNA processing and transcriptional activity [Suzuki HI et al 2009]. Thus, loss of TP53 functions in miRNAs transcription and processing might contribute to cancer progression. Considering that some miRNAs are reduced after DNA damage in an ATM-dependent manner, ATM could be also involved in inhibitory pathways that downregulate miRNA expression [Wang Y et al 2013]. These findings support the existence of a critical link between the DDR and miRNA processing pathway.

In the DNA damage response, post-transcriptional processing of miRNAs is also regulated. It was reported that DNA damage led to increased levels of some pre-miRNAs and mature miRNAs without significant changes of levels of their primary transcripts, suggesting posttranscriptional mechanisms could contribute to the induction of certain miRNAs under DNA damage stress [Zhang X, et al 2011]. There appears to be functional connections between DNA damage response and miRNA processing and maturation.

Micro RNA - 18a, miR-100, miR-101, miR-181, and miR-421, have been implicated as novel regulators to control the protein level of ATM (Majid S et al 2010). BRCA1, a critical tumor suppressor, BRCA1, is also recruited to DNA damage lesions, where it facilitates DNA repair. The level of BRCA1 is regulated by miR-182, miR-146a, and 146b-5p (Matsui M et al 2013).

The tumor suppressor p53 has a central role in the activation of genes in multiple pathways, including cell cycle regulation, tumor suppression, and apoptosis. Micro RNA-125b and miR-504 have been identified as negative regulators of p53 in several types of human cells (Kreis S et al 2008 and Wang J et al 2012).

The available evidence suggests that DNA damage signaling participates in miRNA biogenesis by regulating both transcriptional and post-transcriptional mechanisms. Further studies can through light on the correlation between DNA damaging signaling and miRNA processing. The majority of the studies have examined the miRNA regulation in response to DNA damage and have focused on events that occur in the nucleus. It is important to extend the investigations in understanding the contribution of cytoplasmic regulation of miRNA biogenesis following DNA damage. It is very interesting to determine whether DNA damage signals can modulate the turnover, stabilization, modification, and degradation of miRNAs.

How it is Measured or Detected

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human cell line	Western blotting,clonal survival assay,FACs(van Jaarsveld MT et al 2014)	Yes	Strong	Yes	Yes	Yes
Mice	Free radicCyQuant cell Proliferation assay (Abdelfattah, N. et al 2018)	Yes	Strong	Yes	Yes	Yes
	RNA sequence analysis,Immuno staining,immunoblotting,Flowcytometry,COMET assay,qRT PCR(Liu Z et al 2017)	Yes	Strong	Yes	Yes	Yes
	Microarray (Zhang X et al 2011)	Yes	Strong	Yes	Yes	Yes
	qRT PCR,RIP assay,Immunogold EM(Wan G et al 2013)	Yes	Strong	Yes	Yes	Yes
Canine	micro array(Bulkowska M et al 2017)	Yes	Strong	Yes	Yes	Yes

References

Abdelfattah, N., Rajamanickam, S., Panneerdoss, S., Timilsina, S., Yadav, P., Onyeagucha, B. C., ... & Rao, M. K. (2018). MiR-584-5p potentiates vincristine and radiation response by inducing spindle defects and DNA damage in medulloblastoma. *Nature communications*, *9*(1), 1-19.

Aguda, B. D., Kim, Y., Piper-Hunter, M. G., Friedman, A., & Marsh, C. B. (2008). MicroRNA regulation of a cancer network: consequences of the feedback loops involving miR-17-92, E2F, and Myc. *Proceedings of the National Academy of Sciences*, *105*(50), 19678-19683.

Bulkowska, M., Rybicka, A., Senses, K. M., Ulewicz, K., Witt, K., Szymanska, J., ... & Krol, M. (2017). MicroRNA expression patterns in canine mammary cancer show significant differences between metastatic and non-metastatic tumours. *BMC cancer*, *17*(1), 1-17.

Cannell, I. G., Kong, Y. W., Johnston, S. J., Chen, M. L., Collins, H. M., Dobbyn, H. C., ... & Bushell, M. (2010). p38 MAPK/MK2-mediated induction of miR-34c following DNA damage prevents Myc-dependent DNA replication. *Proceedings of the National Academy of Sciences*, *107*(12), 5375-5380.

Chowdhury, D., Choi, Y. E., & Brault, M. E. (2013). Charity begins at home: non-coding RNA functions in DNA repair. *Nature reviews Molecular cell biology*, *14*(3), 181-189.

di Fagagna, F. D. A. (2014). A direct role for small non-coding RNAs in DNA damage response. *Trends in cell biology*, *24*(3), 171-178.

Georges, S. A., Biery, M. C., Kim, S. Y., Schelter, J. M., Guo, J., Chang, A. N., ... & Chau, B. N. (2008). Coordinated regulation of cell cycle transcripts by p53-Inducible microRNAs, miR-192 and miR-215. *Cancer research*, *68*(24), 10105-10112.

Han, C., Liu, Y., Wan, G., Choi, H. J., Zhao, L., Ivan, C., ... & Lu, X. (2014). The RNA-binding protein DDX1 promotes primary microRNA maturation and inhibits ovarian tumor progression. *Cell reports*, *8*(5), 1447-1460.

Han, C., Wan, G., Langley, R. R., Zhang, X., & Lu, X. (2012). Crosstalk between the DNA damage response pathway and microRNAs. *Cellular and molecular life sciences*, *69*(17), 2895-2906.

Harper, J. W., & Elledge, S. J. (2007). The DNA damage response: ten years after. *Molecular cell*, *28*(5), 739-745.

Hermeking, H. (2012). MicroRNAs in the p53 network: micromanagement of tumour suppression. *Nature reviews cancer*, *12*(9), 613-626.

Jackson SP, Bartek J. (2009). The DNA-damage response in human biology and disease. Nature, 461,1071-8

Kreis, S., Philippidou, D., Margue, C., & Behrmann, I. (2008). IL-24: a classic cytokine and/or a potential cure for cancer?. *Journal of cellular and molecular medicine*, *12*(6a), 2505-2510.

Liu, Z., Zhang, C., Khodadadi-Jamayran, A., Dang, L., Han, X., Kim, K., ... & Zhao, R. (2017). Canonical microRNAs enable differentiation, protect against DNA damage, and promote cholesterol biosynthesis in neural stem cells. *Stem cells and development*, *26*(3), 177-188.

Majid, S., Dar, A. A., Saini, S., Yamamura, S., Hirata, H., Tanaka, Y., ... & Dahiya, R. (2010). MicroRNA-205–directed transcriptional activation of tumor suppressor genes in prostate cancer. *Cancer*, *116*(24), 5637-5649.

Matsui, M., Chu, Y., Zhang, H., Gagnon, K. T., Shaikh, S., Kuchimanchi, S., ... & Janowski

B. A. (2013). Promoter RNA links transcriptional regulation of inflammatory pathway
Pothof, J., Verkaik, N. S., Van Ijcken, W., Wiemer, E. A., Ta, V. T., Van Der Horst, G. T., ... & Persengiev, S. P. (2009). MicroRNA-mediated gene silencing modulates the UV-induced DNA-damage response. *The EMBO journal*, *28*(14), 2090-2099.
Sharma, V., & Misteli, T. (2013). Non-coding RNAs in DNA damage and repair. *FEBS letters*, *587*(13), 1832-1839.
Suzuki, H. I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., & Miyazono, K. (2009). Modulation of microRNA processing by p53. *Nature*, *460*(7254), 529-533.
Suzuki, H. I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., & Miyazono, K. (2009). Modulation of microRNA processing by p53. *Nature*, *460*(7254), 529-533.
van Jaarsveld, M. T., Wouters, M. D., Boersma, A. W., Smid, M., van IJcken, W. F., Mathijssen, R. H., ... & Pothof, J. (2014). DNA damage responsive microRNAs misexpressed in human cancer modulate therapy sensitivity. *Molecular oncology*, *8*(3), 458-468.
van Jaarsveld MT, Wouters MD, Boersma AW, Smid M, van Ijcken WF, Mathijssen RH, Hoeijmakers JH, Martens JW, van Laere S, Wiemer EA, Pothof J. (2014) .DNA damage responsive microRNAs misexpressed in human cancer modulate therapy sensitivity. Mol Oncol. 8(3), 458-68.
Wan, G., Mathur, R., Hu, X., Zhang, X., & Lu, X. (2011). miRNA response to DNA damage. *Trends in biochemical sciences*, *36*(9), 478-484.
Wan, G., Zhang, X., Langley, R. R., Liu, Y., Hu, X., Han, C., ... & Lu, X. (2013). DNA-damage-induced nuclear export of precursor microRNAs is regulated by the ATM-AKT pathway. *Cell reports*, *3*(6), 2100-2112.
Wang, J., & Li, L. C. (2012). Small RNA and its application in andrology and urology. *Translational andrology and urology*, *1*(1), 33.
Wang, Y., & Taniguchi, T. (2013). MicroRNAs and DNA damage response: implications for cancer therapy. *Cell cycle*, *12*(1), 32-42.
Wei, W., Ba, Z., Gao, M., Wu, Y., Ma, Y., Amiard, S., ... & Qi, Y. (2012). A role for small RNAs in DNA double-strand break repair. *Cell*, *149*(1), 101-112.
Yang, Y., Ahn, Y. H., Gibbons, D. L., Zang, Y., Lin, W., Thilaganathan, N., ... & Kurie, J. M. (2011). The Notch ligand Jagged2 promotes lung adenocarcinoma metastasis through a miR-200–dependent pathway in mice. *The Journal of clinical investigation*, *121*(4), 1373-1385.
Zhang, X., Wan, G., Berger, F. G., He, X., & Lu, X. (2011). The ATM kinase induces microRNA biogenesis in the DNA damage response. *Molecular cell*, *41*(4), 371-383.
Zhang, X., Wan, G., Berger, F. G., He, X., & Lu, X. (2011). The ATM kinase induces microRNA biogenesis in the DNA damage response. *Molecular cell*, *41*(4), 371-383.

Event: 1981: Decreased SIRT1 expression

Short Name: Decrease,SIRT1(sirtuin 1) levels

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Stressors

Name
Ethyl alcohol

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	Moderate	NCBI
mice	Mus sp.	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Female	Moderate

Decreased SIRT1 expression is known to be highly conserved throughout evolution and is present from humans to invertebrates.

Key Event Description

Biological state:

Mammalian SIRTs include seven proteins (SIRT1-7) with deacetylase activity belonging to the class III histone deacetylase family. SIRTs share homology with the yeast deacetylase Sir2, and have different sequences and lengths in both their N- and C-terminal domains (Carafa, V. et al 2012). Expressed from bacteria to humans (Vaquero, A. 2009), SIRTs target histone and non-histone proteins.

Localization of SIRTs is restricted to mitochondria, cytoplasm and nucleus The location of SIRT1, SIRT6, and SIRT7 is predominantly in the nucleus, while SIRT2 in the cytosol, and SIRT3, SIRT4, and SIRT5 are in the mitochondria. Depending on their role in regulating different pathways, SIRTs relocalize under different conditions such as cell cycle phase, tissue type, developmental stage, stress condition, and metabolic status which has been documented in the literature. (McGuinness, D. et al 2011). As per Mitchishita et al, SIRT1, SIRT2, and SIRT7 are often found in both the nucleus and cytoplasm (Michishita, E et al 2005).

Cellular pathways like DNA repair, transcriptional regulation, metabolism, aging, and senescence are modulated by Sirtuins. This has created sufficient interest with Sirtuins as target in cancer research as the above mentioned functions are involved in initiation and progression of cancer. Evidence have suggested the association of SIRTs with metabolism-associated TFs, MYC and hypoxia inducible factor-1 (HIF-1), in terms of energy metabolic reprogramming.(Zwaans, B. M. et al 2014)

The biological effect of SIRTs in cancer is either tumor suppression or tumor promoter (oncogenes) action by altering the cell proliferation, differentiation, and death which in turn depends on cell context and experimental conditions. These two totally opposite function of SIRTs on cancer cell is remains a highly debated and controversial topic. Whether SIRTs act as tumor suppressors or promoters depends on (i) their The different expression levels of SIRTS in tumors and its effects on cell cycle, cell growth, death, their action on specific proto-oncogene and onco-suppressor proteins will determine SIRTs role as tumor suppressor or tumor promoters (Deng, C. X. 2009).

Sirtuin Reactions

The NAD+-dependent deacetylation is well known enzymatic reaction catalyzed by SIRTs. Deacetylation reaction begins with amide cleavage from NAD+ with the formation of nicotinamide and an intermediate of reaction, O-ADP-ribose. This intermediate formed is necessary for the deacetylation process by which SIRTs catalyze the transfer of one acetyl group from a lysine to O-ADP-ribose moiety to form *O*-acetyl-ADP-

ribose and the deacetylated lysine product. This reaction requires a mole equivalent of NAD+ per acetyl group removed and is controlled by the cellular [NAD]/[NADH] ratio (Sauve, A. A. 2010 and Shi, Y. et al 2013).

Among the SIRTs family, only SIRT1, SIRT2, and SIRT3 possess a robust deacetylase activity even though SIRT enzymes are primarily known as protein deacetylases. SIRT4, SIRT5, SIRT6, and SIRT7) exhibit a weak or no detectable deacetylation activity at all.Through these reactions, SIRTs are able to regulate several key cellular processes (Jiang, H., et al 2013 and Zhang, S. et al 2017).

Biological compartments:

Regulation of gene expression takes place in the cell, subcellular site being nucleus.

General role in biology:

Silent Inflammation Regulator 2 (SIR2) proteins belong to the family of histone deacetylases (HDACs) that catalyze deacetylation of both histone and non- histone lysine residues.

Mammalian sirtuins (SIRT1-7) are involved in diverse biological processes including energy metabolism, lifespan and health span regulation (Longo VD et al 2006). Mammalian sirtuins possess will bring about an array of biological functions through its enzymatic activity such as histone deacetylase, mono-ADP-ribosyltransferase, desuccinylase, demalonylase, demyristoylase, and depalmitoylase activity (Michan S et al 2007). SIRT1 located in the nucleus play an important role in genomic stability, telomere maintenance, and cell survival (Chen J et al 2011 and, Haigis MC et al 2006).

Among the 7 SIRTs, SIRT1 is the largest in terms of total DNA and amino acid sequence studied sirtuin [Fang, Y. and M.B. Nicholl 2011]. SIRT1, a class 3 histone deacetylase, is implicated in the modulation of apoptosis, senescence, proliferation, and aging. It's actions arebrought about by cellular nicotinamide adenosine dinucleotide (NAD+) which acts as a cofactor for deacetylation reactivity. The liberated nicotinamide from NAD+, generates a novel metabolite o-acetyl-ADP-ribose . SIRT1 can mediate the actions at translational level. Various mechanisms have been proposed to be involved in dysregulation of SIRT1 in cancer cells [Yao, C., et al.2016]. In human breast, lung and prostate cancers SIRT1 is significantly elevated . It plays a role in tumorigenesis by anti-apoptotic activity through oncogene and epigenetic regulator action.[Saunders, L. and E. Verdin 2007]. SIRT1 deacetylates pro-apoptotic proteins such as p53 and promotes cell survival under genotoxic and oxidative stresses [Kojima, K., et al 2010]. It's critical role in multiple aspects of resistance to anti-cancer drugs is also well documented [Duan, K., et al 2015]. Therefore, SIRT1 overexpression is associated with the subsequent higher level of tumor cell proliferation, invasion, and migration [Wang, X., et al 2016].

SIRT1 expression is increased in human colon cancer, acute myeloid leukemia, and some skin can- cers (Bradbury, C. A et al 2005, Hida, Y. et al 2007, Huffman, D. M. et al 2007 and Stunkel, W.2007). SIRT1 , by interacting with and inhibiting p53 may act as tumor promoter (van Leeuwen, I., and Lain, S. 2009). Repression of tumor suppression protein expression and DNA repair protein ,are other roles of SIRT1 in cancer cells. In colon cancer , SIRT1 limits β-catenin signaling while in breast cancer it interacts with BRCA1 signaling . However it has been observed that SIRT1 expression is decreased in ovarian cancer, glioblastoma, and bladder carcinoma (Deng, C. X. 2009). In these cancers , SIRT1 might serve as a tumor suppressor by blocking oncogenic pathways. Thus SIRT1 can serve as a tumor promoter or tumor suppressor, depending on the oncogenic pathways specific to particular tumors.

In hepatocellular carcinoma , SIRT1 was overexpressed in HCC cells and tissues, and significantly promoted the migration and invasion ability of HCC cells by inducing the epithelial and mesenchymal transition[Hao C et al 2014]. This in vivo study also supported the oncogenic functions of SIRT1 in enhancing metastasis[Hao C et al 2014]. Bae et al [Bae HJ et al 2014] found that knockdown of SIRT1 inhibited cell growth by transcriptional deregulation of cell cycle proteins, leading to hypophosphorylation of pRb, which inactivated E2F/ DP1 target gene transcription, and thereby caused the G1/S cell cycle arrest. In addition, miR29c was identified as a suppressor of SIRT1 by comprehensive miRNA profiling and ectopic miR29c expression recapitulated SIRT1 knockdown effects in HCC cells [Bae HJ et al 2014]. To contradict the above findings, Zhang et al [Zhang ZY et al 2015] reported that SIRT1 has anticarcinogenic effects in HCC via the AMPK mammalian target of rapamycin (mTOR) pathway. They evaluated the relationship between p53 mutations and activation of SIRT1 in 252 patients with hepatitis B virus positive HCC and found that activated SIRT1 was associated with a longer recurrence free survival in HCC tissues harbouring mutant p53. He reported that inhibition of SIRT1 increased cell growth, bearing mutated p53, by suppressing AMPK activity and enhancing mTOR activity.The conflicting results from different published data indicated that SIRT1 is multifunctional gene and its biological features are left unsolved.

These above evidence indicates the involvement of SIRTs in regulating three important tumor processes: epithelial-to-mesenchymal transition (EMT), invasion, and metastasis. Many SIRTs are responsible for cellular metabolic reprogramming and drug resistance by inactivating cell death pathways and promoting uncontrolled proliferation. These observations are for the future development of novel tailored SIRT-based cancer therapies.

Wang et al showed that SIRT1 expression was increased in several cancer cell lines, and is generally associated with poor prognosis and overall survival (Wang, C., et al 2017). Vaziri et al reported that SIRT1 interacted with P53, triggering its deacetylation in Lys382 residue, and determined a block of all P53-dependent pathways, leading to uncontrolled cell cycle and inactivation of the apoptotic process (Vaziri, H., et al 2011).

SIRT1 has a function in metastasis and invasiveness in several cancers that has been reported in several studies. Among them ,the deacetylation of many proteins involved in tumor suppressor processes or DNA damage repair, and the inactivation of specific pathways support the role of SIRT1 as a tumor promoter. The role of SIRT1 in the initiation, promotion, and progression of several malignant tumors including prostate cancer (Jung-Hynes, B. et al 2009), breast cancer (Jin, X., et al 2018), lung cancer (Han, L. et al 2013) and gastric cancer (Han, L. et al 2013) are well documented. Wilking el al showed in his in vitro experiments that the inhibition of SIRT1 by treatment with small molecule SIRT1 inhibitors determines a significant decrease in cell growth, proliferation and viability (Wilking, M. J., et al 2014).

How it is Measured or Detected

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human tissues	qRT-PCR,Western blotting,Luciferase reporter assay H2,H4,H7,H8,H9 Micro-array (Shen ZL et al 2016)	yes	Strong	Yes	Yes	Yes
Human cell lines	Micro-array, qRT-PCR,Western blotting,Luciferase reporter assay (Guo S et al 2020, Bae HJ et al 2014, Zhou J et al 2017, Fu H et al 2018, Lian B et al 2018 Guan Y et al 2017 Yang X et al 2014)	yes	Strong	Yes	Yes	Yes
Mouse	qRT-PCR,Western blotting,Luciferase reporter assay,ELISA,cell culture Bai XZ et al 2018	yes	Moderate	Yes	Yes	Yes

References

Bae, H. J., Noh, J. H., Kim, J. K., Eun, J. W., Jung, K. H., Kim, M. G., ... & Nam, S. W. (2014). MicroRNA-29c functions as a tumor suppressor by direct targeting oncogenic SIRT1 in hepatocellular carcinoma. *Oncogene*, *33*(20), 2557-2567.

Bae, H. J., Noh, J. H., Kim, J. K., Eun, J. W., Jung, K. H., Kim, M. G., ... & Nam, S. W. (2014). MicroRNA-29c functions as a tumor suppressor by direct targeting oncogenic SIRT1 in hepatocellular carcinoma. *Oncogene*, *33*(20), 2557-2567.

Bai, X. Z., Zhang, J. J., Liu, Y., Zhang, W., Li, X. Q., Wang, K. J., ... & Hu, R. H. (2018). MicroRNA-138 aggravates

Bai, X. Z., Zhang, J. L., Liu, T., Zhang, W., Li, X. Q., Wang, K. J., ... & Hu, D. H. (2018). MicroRNA-138 aggravates inflammatory responses of macrophages by targeting SIRT1 and regulating the NF-κB and AKT pathways. *Cellular Physiology and Biochemistry*, *49*(2), 489-500.

Bradbury, C. A., Khanim, F. L., Hayden, R., Bunce, C. M., White, D. A., Drayson, M. T., ... & Turner, B. M. (2005). Histone deacetylases in acute myeloid leukaemia show a distinctive pattern of expression that changes selectively in response to deacetylase inhibitors. *Leukemia*, *19*(10), 1751-1759.

Carafa, V., Nebbioso, A., & Altucci, L. (2012). Sirtuins and disease: the road ahead. *Frontiers in pharmacology*, *3*, 4.

Chen, J., Zhang, B., Wong, N., Lo, A. W., To, K. F., Chan, A. W., ... & Ko, B. C. (2011). Sirtuin 1 is upregulated in a subset of hepatocellular carcinomas where it is essential for telomere maintenance and tumor cell growth. *Cancer research*, *71*(12), 4138-4149..

Deng, C. X. (2009). SIRT1, is it a tumor promoter or tumor suppressor?. *International journal of biological sciences*, *5*(2), 147.

Duan, K., Ge, Y. C., Zhang, X. P., Wu, S. Y., Feng, J. S., Chen, S. L., ... & Fu, C. H. (2015). miR-34a inhibits cell proliferation in prostate cancer by downregulation of SIRT1 expression. *Oncology letters*, *10*(5), 3223-3227.

Fang, Y., & Nicholl, M. B. (2011). Sirtuin 1 in malignant transformation: friend or foe?. *Cancer letters*, *306*(1), 10-14.

Fu, H., Song, W., Chen, X., Guo, T., Duan, B., Wang, X., ... & Zhang, C. (2018). MiRNA-200a induce cell apoptosis in renal cell carcinoma by directly targeting SIRT1. *Molecular and cellular biochemistry*, *437*(1), 143-152.

Guan, Y., Rao, Z., & Chen, C. (2018). miR-30a suppresses lung cancer progression by targeting SIRT1. *Oncotarget*, *9*(4), 4924.

Guo, S., Ma, B., Jiang, X., Li, X., & Jia, Y. (2020). Astragalus polysaccharides inhibits tumorigenesis and lipid metabolism through miR-138-5p/SIRT1/SREBP1 pathway in prostate cancer. *Frontiers in Pharmacology*, *11*, 598.

Haigis, M. C., & Guarente, L. P. (2006). Mammalian sirtuins—emerging roles in physiology, aging, and calorie restriction. *Genes & development*, *20*(21), 2913-2921.

Han, L., Liang, X. H., Chen, L. X., Bao, S. M., & Yan, Z. Q. (2013). SIRT1 is highly expressed in brain metastasis tissues of non-small cell lung cancer (NSCLC) and in positive regulation of NSCLC cell migration. *International journal of clinical and experimental pathology*, *6*(11), 2357.

Hao, C., Zhu, P. X., Yang, X., Han, Z. P., Jiang, J. H., Zong, C., ... & Wei, L. X. (2014). Overexpression of SIRT1 promotes metastasis through epithelial-mesenchymal transition in hepatocellular carcinoma. *BMC cancer*, *14*(1), 1-10.

Hida, Y., Kubo, Y., Murao, K., & Arase, S. (2007). Strong expression of a longevity-related protein, SIRT1, in Bowen's disease. *Archives of dermatological research*, *299*(2), 103-106.

Huffman, D. M., Grizzle, W. E., Bamman, M. M., Kim, J. S., Eltoum, I. A., Elgavish, A., & Nagy, T. R. (2007). SIRT1 is significantly elevated in mouse and human prostate cancer. *Cancer research*, *67*(14), 6612-6618.

Jiang, G., Wen, L., Zheng, H., Jian, Z., & Deng, W. (2016). miR-204-5p targeting SIRT1 regulates hepatocellular carcinoma progression. *Cell biochemistry and function*, *34*(7), 505-510.

Jiang, H., Khan, S., Wang, Y., Charron, G., He, B., Sebastian, C., ... & Lin, H. (2013). SIRT6 regulates TNF-α secretion through hydrolysis of long-chain fatty acyl lysine. *Nature*, *496*(7443), 110-113.

Jin, X., Wei, Y., Xu, F., Zhao, M., Dai, K., Shen, R., ... & Zhang, N. (2018). SIRT1 promotes formation of breast cancer through modulating Akt activity. *Journal of Cancer*, *9*(11), 2012.

Jung-Hynes, B., Nihal, M., Zhong, W., & Ahmad, N. (2009). Role of sirtuin histone deacetylase SIRT1 in prostate cancer: a target for prostate cancer management via its inhibition?. *Journal of Biological Chemistry*, *284*(6), 3823-3832..

Kojima, K., Fujita, Y., Nozawa, Y., Deguchi, T., & Ito, M. (2010). MiR-34a attenuates paclitaxel-resistance of hormone-refractory prostate cancer PC3 cells through direct and indirect mechanisms. *The Prostate*, *70*(14), 1501-1512.

Lian, B., Yang, D., Liu, Y., Shi, G., Li, J., Yan, X., ... & Zhang, R. (2018). miR-128 targets the SIRT1/ROS/DR5 pathway to sensitize colorectal cancer to TRAIL-induced apoptosis. *Cellular Physiology and Biochemistry*, *49*(6), 2151-2162.

Longo, V. D., & Kennedy, B. K. (2006). Sirtuins in aging and age-related disease. *Cell*, *126*(2), 257-268.

Luo, J., Chen, P., Xie, W., & Wu, F. (2017). MicroRNA-138 inhibits cell proliferation in hepatocellular carcinoma by targeting Sirt1. *Oncology reports*, *38*(2), 1067-1074.

McGuinness, D., McGuinness, D. H., McCaul, J. A., & Shiels, P. G. (2011). Sirtuins, bioageing, and cancer. *Journal of aging research*, *2011*.

Michan, S., & Sinclair, D. (2007). Sirtuins in mammals: insights into their biological function. *Biochemical Journal*, *404*(1), 1-13.

Michishita, E., Park, J. Y., Burneskis, J. M., Barrett, J. C., & Horikawa, I. (2005). Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Molecular biology of the cell*, *16*(10), 4623-4635.

Saunders, L. R., & Verdin, E. (2007). Sirtuins: critical regulators at the crossroads between cancer and aging. *Oncogene*, *26*(37), 5489-5504.

Sauve, A. A. (2010). Sirtuin chemical mechanisms. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1804*(8), 1591-1603..

Shen, Z. L., Wang, B., Jiang, K. W., Ye, C. X., Cheng, C., Yan, Y. C., ... & Wang, S. (2016). Downregulation of miR-199b is associated with distant metastasis in colorectal cancer via activation of SIRT1 and inhibition of CREB/KISS1 signaling. *Oncotarget*, *7*(23), 35092.

Shi, Y., Zhou, Y., Wang, S., & Zhang, Y. (2013). Sirtuin deacetylation mechanism and catalytic role of the dynamic cofactor binding loop. *The journal of physical chemistry letters*, *4*(3), 491-495.

Shuang, T., Wang, M., Zhou, Y., & Shi, C. (2015). Over-expression of Sirt1 contributes to chemoresistance and indicates poor prognosis in serous epithelial ovarian cancer (EOC). *Medical oncology*, *32*(12), 1-7.

Stünkel, W., Peh, B. K., Tan, Y. C., Nayagam, V. M., Wang, X., Salto-Tellez, M., ... & Wood, J. (2007). Function of the SIRT1 protein deacetylase in cancer. *Biotechnology Journal: Healthcare Nutrition Technology*, *2*(11), 1360-1368.

Tian, Z., Jiang, H., Liu, Y., Huang, Y., Xiong, X., Wu, H., & Dai, X. (2016). MicroRNA-133b inhibits hepatocellular carcinoma cell progression by targeting Sirt1. *Experimental cell research*, *343*(2), 135-147.

van Leeuwen, I., & Lain, S. (2009). Sirtuins and p53. *Advances in cancer research*, *102*, 171-195.

Vaquero, A. (2009). The conserved role of sirtuins in chromatin regulation. *International Journal of Developmental Biology*, *53*(2-3), 303-322.

Vaziri, H., Dessain, S. K., Eaton, E. N., Imai, S. I., Frye, R. A., Pandita, T. K., ... & Weinberg, R. A. (2001). hSIR2SIRT1 functions as an NAD-dependent p53 deacetylase. *Cell*, *107*(2), 149-159.

Wang, C., Yang, W., Dong, F., Guo, Y., Tan, J., Ruan, S., & Huang, T. (2017). The prognostic role of Sirt1 expression in solid malignancies: a meta-analysis. *Oncotarget*, *8*(39), 66343.

Wang, X., Yang, B., & Ma, B. (2016). The UCA1/miR-204/Sirt1 axis modulates docetaxel sensitivity of prostate cancer cells. *Cancer chemotherapy and pharmacology*, *78*(5), 1025-1031.

Wilking, M. J., Singh, C., Nihal, M., Zhong, W., & Ahmad, N. (2014). SIRT1 deacetylase is overexpressed in human melanoma and its small molecule inhibition imparts anti-proliferative response via p53 activation. *Archives of biochemistry and biophysics*, *563*, 94-100.

Yan, X., Liu, X., Wang, Z., Cheng, Q., Ji, G., Yang, H., ... & Pei, X. (2019). MicroRNA4865p functions as a tumor suppressor of proliferation and cancer stemlike cell properties by targeting Sirt1 in liver cancer. *Oncology reports*, *41*(3), 1938-1948.

Yang, X., Yang, Y., Gan, R., Zhao, L., Li, W., Zhou, H., ... & Meng, Q. H. (2014). Down-regulation of mir-221 and mir-222 restrain prostate cancer cell proliferation and migration that is partly mediated by activation of SIRT1. *PloS one*, *9*(6), e98833.

Yao, C., Liu, J., Wu, X., Tai, Z., Gao, Y., Zhu, Q., ... & Gao, S. (2016). Reducible self-assembling cationic polypeptide-based micelles mediate co-delivery of doxorubicin and microRNA-34a for androgen-independent prostate cancer therapy. *Journal of Controlled Release*, *232*, 203-214.

Zhang S, Zhang D, Yi C, Wang Y, Wang H, Wang J. (2016). MicroRNA-22 functions as a tumor suppressor by targeting SIRT1 in renal cell carcinoma. Oncol Rep. 35(1), 559-67.

Zhang, Z. Y., Hong, D., Nam, S. H., Kim, J. M., Park, Y. H., Jon, J. W., ... & Kim, S. J. (2015). SIRT1 regulates oncogenesis via a mutant p53-dependent pathway in hepatocellular carcinoma. *Journal of hepatology*, *62*(1), 121-130.

Zhang, S., Huang, S., Deng, C., Cao, Y., Yang, J., Chen, G., ... & Zou, X. (2017). Co-ordinated overexpression of SIRT1 and STAT3 is associated with poor survival outcome in gastric cancer patients. *Oncotarget*, *8*(12), 18848.

Zhou, J., Zhou, W., Kong, F., Xiao, X., Kuang, H., & Zhu, Y. (2017). microRNA34a overexpression inhibits cell migration and invasion via regulating SIRT1 in hepatocellular carcinoma Corrigendum in/10.3892/ol. 2019.11048. *Oncology letters*, *14*(6), 6950-6954.

Zwaans, B. M., & Lombard, D. B. (2014). Interplay between sirtuins, MYC and hypoxia-inducible factor in cancer-associated metabolic reprogramming. *Disease models & mechanisms*, *7*(9), 1023-1032.

Event: 1172: Increased activation, Nuclear factor kappa B (NF-kB)

Short Name: Increased activation, Nuclear factor kappa B (NF-kB)

Key Event Component

Process	Object	Action
regulation of I-kappaB kinase/NF-kappaB signaling		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:382 - Angiotensin II type 1 receptor (AT1R) agonism leading to lung fibrosis	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:319 - Binding to ACE2 leading to lung fibrosis	KeyEvent
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Stressors

Name
Reactive oxygen species

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
epithelial cell

Organ term

Organ term
tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Moderate

Sex Applicability

Sex	Evidence
Mixed	Not Specified

The ROS directly influences NF-κB signalling, resulting in differential production of cytokines and chemokines (McKay and Cidlowski, 1999). NIn accordance with the OECD AOP Handbook, the pathway begins with increased levels of reactive oxygen species (ROS), serving as the Molecular Initiating Event (MIE), which subsequently triggers the Activation of the NF-κB Signaling Pathway . This activation, in turn, directly influences the expression of genes involved in the Differential Production of Cytokines and Chemokines , culminating in the regulation of Pro-Inflammatory Responses Transcriptionally Mediated by NF-κB (. The resultant exaggerated and dysregulated pro-inflammatory response contributes to chronic inflammation and tissue damage, representing the Adverse Outcome (AO). This sequence of events is underpinned by the works of McKay and Cidlowski (1999) and aligns with the guidelines set forth in the OECD AOP Handbook.F-κB regulates pro-inflammatory responses that are transcriptionally mediated by NF-κB.

Key Event Description

The NF-kB pathway consists of a series of events where the transcription factors of the NF-kB family play a key role. The proinflammatory cytokine (IL-1beta) can be activated by NF-kB , including Reactive Oxygen Species produced by NADPH oxidase (NOX). Upon pathway activation, the IKK complex will be phosphorylated, which in turn phosphorylates IkBa. There, this transcription factor can express pro-inflammatory and pro-fibrotic genes. This can be achieved by ROS, IKK enhancer or nuclear translocation enhancer.

How it is Measured or Detected

NF-kB transcriptional activity: Beta lactamase reporter gene assay (Miller et al. 2010). NF-kB transcription: Lentiviral NF-kB GFP reporter with flow cytometry (Moujalled et al. 2012)

NF-κB translocation: RelA-GFP reporter assay (Wink et al 2017)

IκBa phosphorylation: Western blotting (Miller et al. 2010)

NF-κB p65 (Total/Phospho) ELISA

ELISA for IL-6, IL-8, and Cox

References

McKay LI, Cidlowski JA. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. Endocr Rev. 1999 Aug;20(4):435-59.

Miller SC, Huang R, Sakamuru S, Shukla SJ, Attene-Ramos MS, Shinn P, Van Leer D, Leister W, Austin CP, Xia M. Identification of known drugs that act as inhibitors of NF-kappaB signaling and their mechanism of action. Biochem Pharmacol. 2010 May 1;79(9):1272-80.

Moujalled DM, Cook WD, Lluis JM, Khan NR, Ahmed AU, Callus BA, Vaux DL. In mouse embryonic fibroblasts, neither caspase-8 nor cellular FLICE-inhibitory protein (FLIP) is necessary for TNF to activate NF-κB, but caspase-8 is required for TNF to cause cell death, and induction of FLIP by NF-κB is required to prevent it. Cell Death Differ. 2012 May;19(5):808-15.

Wink S, Hiemstra S, Herpers B, van de Water B. High-content imaging-based BAC-GFP toxicity pathway reporters to assess chemical adversity liabilities. Arch Toxicol. 2017 Mar;91(3):1367-1383.

Event: 112: Antagonism, Estrogen receptor

Short Name: Antagonism, Estrogen receptor

Key Event Component

Process	Object	Action
estrogen receptor activity	estrogen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:30 - Estrogen receptor antagonism leading to reproductive dysfunction	MolecularInitiatingEvent
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
hepatocyte

Domain of Applicability

Taxonomic applicability: Steroid receptors, including ER are thought to have evolved in the chordate lineage (Baker 1997, 2003; Thornton 2001). An ER ortholog has been isolated from a mollusk species, but no ER orthologs have been detected in arthropods or nematodes (Thornton et al. 2003). Broadly speaking, most vertebrates can be expected to have functional ERs, while most invertebrates do not, although there may be exceptions within the mollusk lineage and evolutionarily-related organisms.

Key Event Description

Site of action: The site of action for the molecular initiating event is the liver (hepatocytes).

Responses at the macromolecular level: Estrogen receptor antagonists have been shown to interact with the ligand binding domain of ERs. However, those interactions occur at different contact sites than those of estrogen agonists, leading to a different conformation in the transactivation domain (Brzozowski et al. 1997; Katzenellenbogen 1996).

Characterization of chemical properties: Two broad categories of ER antagonists have been described. Type I, like tamoxifen act as mixed agonists and antagonists. Type II, like ICI164384 are pure antagonists (Katzenellenbogen 1996). Due to their potential utility for treating estrogen-dependent breast cancers and other estrogen-related disease states as well as concerns regarding endocrine disruption, there is an extensive body of literature on the identification and design of chemical structures that act as ER antagonists (e.g., (Brooks et al. 1987; Brooks and Skafar 2004; Lloyd et al. 2006; Sodero et al. 2012; Vedani et al. 2012; Wang et al. 2006).

How it is Measured or Detected

- The BG1luc estrogen receptor transactivation test method for identifying estrogen receptor agonists and antagonists (OECD Test Guideline 457) has been validated by the National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods (NICEATM) and Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as an appropriate assay for detecting ER antagonism. (OECD, 2012b).
- Other human ER-based transactivation assays that have been used to detect ERα antagonism include the T47D-Kbluc assay (Wilson et al. 2004); ERα CALUX assay (van der Burg et al. 2010); MELN assay (Witters et al. 2010); and the yeast estrogen screen (YES; (De Boever et al. 2001)). Each of these assays have undergone some level of validation.
- In aquatic ecotoxicology, vitellogenin synthesis in primary fish liver cells and liver slices has also been used to screen for anti-estrogenic activity (e.g., (Bickley et al. 2009; Navas and Segner 2006; Schmieder et al. 2000; Schmieder et al. 2004; Sun et al. 2010). Although these approaches have generally not been subject to as much formal validation as human ER-based transactivation assays, in the case of fish-specific AOPs linked to this key event, these measures of anti-estrogenicity may be more directly relevant to predicting other key events in the pathway.

References

- Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753-758.
- Katzenellenbogen B. 1996. Estrogen receptors: Bioactivities and interactions with cell signaling pathways. Biology of Reproduction 54:287-293.
- Brooks SC, Wappler NL, Corombos JD, Doherty LM, Horwitz JP. 1987. Estrogen structure-fuction relationships. Berlin:Walter de Gruyter & Co., 443-466.
- Brooks SC, Skafar DF. 2004. From ligand structure to biological activity: Modified estratrienes and their estrogenic and antiestrogenic effects in mcf-7 cells. Steroids 69:401-418.
- Lloyd DG, Smith HM, O'Sullivan T, Knox AS, Zisterer DM, Meegan MJ. 2006. Antiestrogenically active 2-benzyl-1,1-diarylbut-2-enes: Synthesis, structure-activity relationships and molecular modeling study for flexible estrogen receptor antagonists. Medicinal chemistry 2:147-168.
- Sodero AC, Romeiro NC, da Cunha EF, de Oliveira Magalhaaes U, de Alencastro RB, Rodrigues CR, et al. 2012. Application of 4d-qsar studies to a series of raloxifene analogs and design of potential selective estrogen receptor modulators. Molecules 17:7415-7439.
- Vedani A, Dobler M, Smiesko M. 2012. Virtualtoxlab - a platform for estimating the toxic potential of drugs, chemicals and natural products. Toxicology and applied pharmacology 261:142-153.
- Wang CY, Ai N, Arora S, Erenrich E, Nagarajan K, Zauhar R, et al. 2006. Identification of previously unrecognized antiestrogenic chemicals using a novel virtual screening approach. Chemical research in toxicology 19:1595-1601.
- Denny JS, Tapper MA, Schmieder PK, Hornung MW, Jensen KM, Ankley GT, et al. 2005. Comparison of relative binding affinities of endocrine active compounds to fathead minnow and rainbow trout estrogen receptors. Environmental toxicology and chemistry / SETAC 24:2948-2953.
- Lee HK, Kim TS, Kim CY, Kang IH, Kim MG, Jung KK, et al. 2012. Evaluation of in vitro screening system for estrogenicity: Comparison of stably transfected human estrogen receptor-alpha transcriptional activation (oecd tg455) assay and estrogen receptor (er) binding assay. The Journal of toxicological sciences 37:431-437.
- Rider CV, Hartig PC, Cardon MC, Lambright CR, Bobseine KL, Guillette LJ, Jr., et al. 2010. Differences in sensitivity but not selectivity of xenoestrogen binding to alligator versus human estrogen receptor alpha. Environmental toxicology and chemistry / SETAC 29:2064-2071.
- OECD. 2012b. Test no. 457: Bg1luc estrogen receptor transactivation test method for identifying estrogen receptor agonists and antagonists:OECD Publishing.
- Wilson VS, Bobseine K, Gray LE, Jr. 2004. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. Toxicological sciences : an official journal of the Society of Toxicology 81:69-77.
- van der Burg B, Winter R, Weimer M, Berckmans P, Suzuki G, Gijsbers L, et al. 2010. Optimization and prevalidation of the in vitro eralpha calux method to test estrogenic and antiestrogenic activity of compounds. Reproductive toxicology 30:73-80.
- Witters H, Freyberger A, Smits K, Vangenechten C, Lofink W, Weimer M, et al. 2010. The assessment of estrogenic or anti-estrogenic activity of chemicals by the human stably transfected estrogen sensitive mcf7 cell line: Results of test performance and transferability. Reproductive toxicology 30:60-72.
- De Boever P, Demare W, Vanderperren E, Cooreman K, Bossier P, Verstraete W. 2001. Optimization of a yeast estrogen screen and its applicability to study the release of estrogenic isoflavones from a soygerm powder. Environmental health perspectives 109:691-697.
- Bickley LK, Lange A, Winter MJ, Tyler CR. 2009. Evaluation of a carp primary hepatocyte culture system for screening chemicals for oestrogenic activity. Aquatic toxicology 94:195-203.
- Navas JM, Segner H. 2006. Vitellogenin synthesis in primary cultures of fish liver cells as endpoint for in vitro screening of the (anti)estrogenic activity of chemical substances. Aquatic toxicology 80:1-22.
- Schmieder P, Tapper M, Linnum A, Denny J, Kolanczyk R, Johnson R. 2000. Optimization of a precision-cut trout liver tissue slice assay as a screen for vitellogenin induction: Comparison of slice incubation techniques. Aquatic toxicology 49:251-268.
- Schmieder PK, Tapper MA, Denny JS, Kolanczyk RC, Sheedy BR, Henry TR, et al. 2004. Use of trout liver slices to enhance mechanistic interpretation of estrogen receptor binding for cost-effective prioritization of chemicals within large inventories. Environmental science & technology 38:6333-6342.
- Sun L, Wen L, Shao X, Qian H, Jin Y, Liu W, et al. 2010. Screening of chemicals with anti-estrogenic activity using in vitro and in vivo vitellogenin induction responses in zebrafish (danio rerio). Chemosphere 78:793-799.
- Baker ME. 1997. Steroid receptor phylogeny and vertebrate origins. Molecular and cellular endocrinology 135:101-107.

- Baker ME. 2003. Evolution of adrenal and sex steroid action in vertebrates: A ligand-based mechanism for complexity. BioEssays : news and reviews in molecular, cellular and developmental biology 25:396-400.
- Thornton JW. 2001. Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. Proceedings of the National Academy of Sciences of the United States of America 98:5671-5676.
- Thornton JW, Need E, Crews D. 2003. Resurrecting the ancestral steroid receptor: Ancient origin of estrogen signaling. Science 301:1714-1717.

Event: 1457: Induction, Epithelial Mesenchymal Transition

Short Name: EMT

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:241 - Latent Transforming Growth Factor beta1 activation leads to pulmonary fibrosis	KeyEvent
Aop:206 - Peroxisome proliferator-activated receptors γ inactivation leading to lung fibrosis	KeyEvent
Aop:280 - α-diketone-induced bronchiolitis obliterans	KeyEvent
Aop:347 - Toll-like receptor 4 activation and peroxisome proliferator-activated receptor gamma inactivation leading to pulmonary fibrosis	KeyEvent
Aop:414 - Aryl hydrocarbon receptor activation leading to lung fibrosis through TGF-β dependent fibrosis toxicity pathway	KeyEvent
Aop:415 - Aryl hydrocarbon receptor activation leading to lung fibrosis through IL-6 toxicity pathway	KeyEvent
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
humans	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

Regulation of miRNA expression by DNA replication,damage and repair responses,transcription and translation has been proved in animals like mice,canine and cell line experiments.

Key Event Description

Process:transition of epithelial cells to mesenchymal Object: epithelial cells

Action:increased

Process:transition of epithelial cells to mesenchymal Object: epithelial cells

Action:increased

Biological state

An epithelial-mesenchymal transition (EMT) is a biologic process in which epithelial cells are polarized, interact through their basal surface with basement membrane, and undergo biochemical changes to assume a mesenchymal cell phenotype.

This phenotypic transformation has various characters such as enhanced migratory capacity, high invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components (Kalluri, R., and Neilson, E.G. 2003). The completion of an EMT is signalled by the degradation of the underlying basement membrane and the formation of a mesenchymal cell that can migrate away from the epithelial layer in which it originated.

EMT has a number of distinct molecular processes like activation of transcription factors, expression of specific cell surface proteins, reorganization and expression of cytoskeletal proteins, production of ECM-degrading enzymes, and changes in the expression of specific microRNAs. These factors are used as biomarkers to demonstrate the passage of a cell through an EMT.

Biological compartment

Cellular

Role in General Biology:

Excessive proliferation of epithelial cells and angiogenesis mark the initiation and early growth of primary epithelial cancers. (Hanahan, D., and Weinberg, R.A. 2000). The subsequent acquisition of invasiveness, initially manifest by invasion through the basement membrane, is thought to herald the onset of the last stages of the multi-step process that leads eventually to metastatic dissemination, with life-threatening consequences. There has been an intense research going on in the genetic controls and biochemical mechanisms underlying the acquisition of the invasive phenotype and the subsequent systemic spread of the cancer cell. Activation of an EMT program has been proposed as the critical mechanism for the acquisition of malignant phenotypes by epithelial cancer cells (Thiery, J.P. 2002).

Pre-clinical experiments such as mice models and cell culture experiments has demonstrated that carcinoma cells can acquire a mesenchymal phenotype and express mesenchymal markers such as α-SMA, FSP1, vimentin, and desmin (Yang, J., and Weinberg, R.A. 2008). These cells are seen at the invasive front of primary tumors and are considered to be the cells that eventually enter into subsequent steps of the invasion-metastasis cascade, i.e., intravasation, transport through the circulation, extravasation, formation of micro metastases, and ultimately colonization (the growth of small colonies into macroscopic metastases) (Thiery, J.P. 2002, Fidler, I.J., and Poste, G. 2008, Brabletz, T., et al. 2001).

An apparent paradox comes from the observation that the EMT-derived migratory cancer cells typically establish secondary colonies at distant sites that resemble, at the histopathological level, the primary tumor from which they arose; accordingly, they no longer exhibit the mesenchymal phenotypes ascribed to metastasizing carcinoma cells. Reconciling this behaviour with the proposed role of EMT as a facilitator of metastatic dissemination requires the additional notion that metastasizing cancer cells must shed their mesenchymal phenotype via a MET during the course of secondary tumor formation (Zeisberg, M et al 2005). The tendency of disseminated cancer cells to undergo EMT likely reflects the local microenvironments that they encounter after extravasation into the parenchyma of a distant organ, quite possibly the absence of the heterotypic signals they experienced in the primary tumor that were responsible for inducing the EMT in the first place (Thiery, J.P. 2002, Jechlinger, M et al 2002, Bissell, M.J et al 2002). These evidences indicate that induction of an EMT is likely to be a centrally important mechanism for the progression of carcinomas to a metastatic stage and implicates MET during the subsequent colonization process. However, many steps of this mechanistic model still require direct experimental validation. It remains unclear at present whether these phenomena and molecular mechanisms relate to and explain the metastatic dissemination of non-epithelial cancer cells.

The entire spectrum of signaling agents that contribute to EMTs of carcinoma cells remains unclear. One theory suggests that the genetic and epigenetic alterations undergone by cancer cells during the course of primary tumor formation render them especially responsive to EMT-inducing heterotypic signals originating in the tumor-associated stroma. Oncogenes induce senescence, and recent studies suggest that cancer cell EMTs may also play a role in preventing senescence induced by oncogenes, thereby facilitating subsequent aggressive dissemination (Smit, M.A., and Peeper, D.S. 2008, Ansieau, S., et al. 2008, Weinberg, R.A. 2008). In the case of many carcinomas, EMT-inducing signals emanating from the tumor-associated stroma, notably HGF, EGF, PDGF, and TGF-β, appear to be responsible for the induction or functional activation in cancer cells of a series of EMT-inducing transcription factors, notably Snail, Slug, zinc finger E-box binding homeobox 1 (ZEB1), Twist, Goosecoid, and FOXC2 (Thiery, J.P. 2002, Jechlinger, M et al 2002, Shi, Y., and Massague, J. 2003, Niessen, K., et al. 2008, Medici, D et al 2008, Kokudo, T., et al. 2008). Once expressed and activated, each of these transcription factors can act pleiotropically to choreograph the complex EMT program, more often than not with the help of other members of this cohort of transcription factors. The actual implementation by these cells of their EMT program depends on a series of intracellular signaling networks involving, among other signal- transducing proteins, ERK, MAPK, PI3K, Akt, Smads, RhoB, β-catenin, lymphoid enhancer binding factor (LEF), Ras, and c-Fos as well as cell surface proteins such as β4 integrins, α5β1 integrin, and αVβ6 integrin (Tse, J.C., and Kalluri, R. 2007). Activation of EMT programs is also facilitated by the disruption of cell-cell adherens junctions and the cell-ECM adhesions mediated by integrins (Yang, J., and Weinberg, R.A. 2008, Weinberg, R.A. 2008, Gupta, P.B et al 2005, Yang, J et al 2006, Mani, S.A., et al. 2007, Mani, S.A., et al. 2008, Hartwell, K.A., et al. 2006, Taki, M et al 2006)..

How it is Measured or Detected

Loss of E-cadherin and cell polarity is considered to be a fundamental event in epithelial-mesenchymal transition. The simultaneous expression of epithelial (e.g. E-cadherin) and mesenchymal markers (e.g. N-cadherin and vimentin) within the airway epithelium are indicative for ongoing transition (Borthwick et al. 2009, 2010).

	Method/ measurement referenc	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human cell line	qRT-PCR,cell viability assay, Western blotting,EdU incorporation assay	+	Strong	Yes	Yes	Yes
Human	IHC,micro array,qPCR, SNP array	+	Moderate	Yes	Yes	Yes

References

Borthwick, L. A., Parker, S. M., Brougham, K. A., Johnson, G. E., Gorowiec, M. R., Ward, C., … Fisher, A. J. (2009). Epithelial to mesenchymal transition (EMT) and airway remodelling after human lung transplantation. *Thorax*, *64*(9), 770–777. https://doi.org/10.1136/thx.2008.104133

Borthwick, L. A., McIlroy, E. I., Gorowiec, M. R., Brodlie, M., Johnson, G. E., Ward, C., … Fisher, A. J. (2010). Inflammation and epithelial to mesenchymal transition in lung transplant recipients: Role in dysregulated epithelial wound repair. *American Journal of Transplantation*, *10*(3), 498–509. https://doi.org/10.1111/j.1600-6143.2009.02953.x

Al Saleh, S., Al Mulla, F., & Luqmani, Y. A. (2011). Estrogen receptor silencing induces epithelial to mesenchymal transition in human breast cancer cells. PloS one, 6(6), e20610.

Bissell, M. J., Radisky, D. C., Rizki, A., Weaver, V. M., & Petersen, O. W. (2002). The organizing principle: microenvironmental influences in the normal and malignant breast. Differentiation, 70(9-10), 537-546.

Bouris, P., Skandalis, S. S., Piperigkou, Z., Afratis, N., Karamanou, K., Aletras, A. J., ... & Karamanos, N. K. (2015). Estrogen receptor alpha mediates epithelial to mesenchymal transition, expression of specific matrix effectors and functional properties of breast cancer cells. Matrix Biology, 43, 42-60.

Brabletz, T., Jung, A., Reu, S., Porzner, M., Hlubek, F., Kunz-Schughart, L. A., ... & Kirchner, T. (2001). Variable β-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proceedings of the National Academy of Sciences, 98(18), 10356-10361.

Brabletz, T., Jung, A., Reu, S., Porzner, M., Hlubek, F., Kunz-Schughart, L. A., ... & Kirchner, T. (2001). Variable β-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proceedings of the National Academy of Sciences, 98(18), 10356-10361.

idler, I. J., & Poste, G. (2008). The “seed and soil” hypothesis revisited. The lancet oncology, 9(8), 808.

Gupta, P. B., Mani, S., Yang, J., Hartwell, K., & Weinberg, R. A. (2005, January). The evolving portrait of cancer metastasis. In Cold Spring Harbor symposia on quantitative biology (Vol. 70, pp. 291-297). Cold Spring Harbor Laboratory Press.

Hanahan, D., and Weinberg, R.A. (2000). The hall- marks of cancer. Cell. 100:57–70.

Hartwell, K. A., Muir, B., Reinhardt, F., Carpenter, A. E., Sgroi, D. C., & Weinberg, R. A. (2006). The Spemann organizer gene, Goosecoid, promotes tumor metastasis. Proceedings of the National Academy of Sciences, 103(50), 18969-18974.

Jechlinger, M., Grünert, S., & Beug, H. (2002). Mechanisms in epithelial plasticity and metastasis: insights from 3D cultures and expression profiling. Journal of mammary gland biology and neoplasia, 7(4), 415-432.

Kalluri, R., & Neilson, E. G. (2003). Epithelial-mesenchymal transition and its implications for fibrosis. The Journal of clinical investigation, 112(12), 1776-1784.

Kokudo, T., Suzuki, Y., Yoshimatsu, Y., Yamazaki, T., Watabe, T., & Miyazono, K. (2008). Snail is required for TGFβ-induced endothelial-mesenchymal transition of embryonic stem cell-derived endothelial cells. Journal of cell science, 121(20), 3317-3324.
Lin, H. Y., Liang, Y. K., Dou, X. W., Chen, C. F., Wei, X. L., Zeng, D., ... & Zhang, G. J. (2018). Notch3 inhibits epithelial–mesenchymal transition in breast cancer via a novel mechanism, upregulation of GATA-3 expression. Oncogenesis, 7(8), 1-15.

Liu, Y., Liu, R., Fu, P., Du, F., Hong, Y., Yao, M., ... & Zheng, S. (2015). N1-Guanyl-1, 7-diaminoheptane sensitizes estrogen receptor negative breast cancer cells to doxorubicin by preventing epithelial-mesenchymal transition through inhibition of eukaryotic translation initiation factor 5A2 activation. Cellular Physiology and Biochemistry, 36(6), 2494-2503.

Mani, S. A., Yang, J., Brooks, M., Schwaninger, G., Zhou, A., Miura, N., ... & Weinberg, R. A. (2007). Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. Proceedings of the National Academy of Sciences, 104(24), 10069-10074.

Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., ... & Weinberg, R. A. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell, 133(4), 704-715.

Medici, D., Hay, E. D., & Olsen, B. R. (2008). Snail and Slug promote epithelial-mesenchymal transition through β-catenin–T-cell factor-4-dependent expression of transforming growth factor-β3. Molecular biology of the cell, 19(11), 4875-4887.

Niessen, K., Fu, Y., Chang, L., Hoodless, P. A., McFadden, D., & Karsan, A. (2008). Slug is a direct Notch target required for initiation of cardiac cushion cellularization. The Journal of cell biology, 182(2), 315-325.

Shi, Y., & Massagué, J. (2003). Mechanisms of TGF-β signaling from cell membrane to the nucleus. cell, 113(6), 685-700.

Smit, M. A., & Peeper, D. S. (2008). Deregulating EMT and senescence: double impact by a single twist. Cancer cell, 14(1), 5-7.

Taki, M., Verschueren, K., Yokoyama, K., Nagayama, M., & Kamata, N. (2006). Involvement of Ets-1 transcription factor in inducing matrix metalloproteinase-2 expression by epithelial-mesenchymal transition in human squamous carcinoma cells. International journal of oncology, 28(2), 487-496.

Thiery, J. P. (2002). Epithelial–mesenchymal transitions in tumour progression. Nature reviews cancer, 2(6), 442-454.

Tse, J. C., & Kalluri, R. (2007). Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment. Journal of cellular biochemistry, 101(4), 816-829.

Weinberg, R. A. (2008). Twisted epithelial–mesenchymal transition blocks senescence. Nature cell biology, 10(9), 1021-1023.

Wik, E., Ræder, M. B., Krakstad, C., Trovik, J., Birkeland, E., Hoivik, E. A., ... & Salvesen, H. B. (2013). Lack of estrogen receptor-α is associated with epithelial–mesenchymal transition and PI3K alterations in endometrial carcinoma. Clinical Cancer Research, 19(5), 1094-1105.

Yang, J., & Weinberg, R. A. (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Developmental cell, 14(6), 818-829.

Yang, J., Mani, S. A., & Weinberg, R. A. (2006). Exploring a new twist on tumor metastasis. Cancer research, 66(9), 4549-4552.

Ye, Y., Xiao, Y., Wang, W., Yearsley, K., Gao, J. X., Shetuni, B., & Barsky, S. H. (2010). ERα signaling through slug regulates E-cadherin and EMT. Oncogene, 29(10), 1451-1462.

Zeisberg, M., Shah, A. A., & Kalluri, R. (2005). Bone morphogenic protein-7 induces mesenchymal to epithelial transition in adult renal fibroblasts and facilitates regeneration of injured kidney. Journal of Biological Chemistry, 280(9), 8094-8100.

Zeng, Q., Zhang, P., Wu, Z., Xue, P., Lu, D., Ye, Z., ... & Yan, X. (2014). Quantitative proteomics reveals ER-α involvement in CD146-induced epithelial-mesenchymal transition in breast cancer cells. Journal of proteomics, 103, 153-169.

- Zeng, Q., Zhang, P., Wu, Z., Xue, P., Lu, D., Ye, Z., ... & Yan, X. (2014). Quantitative proteomics reveals ER-α involvement in CD146-induced epithelial-mesenchymal transition in breast cancer cells. *Journal of proteomics, 103*, 153-169.

List of Adverse Outcomes in this AOP

Event: 1982: metastatic breast cancer

Short Name: Metastasis, Breast Cancer

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	AdverseOutcome

Stressors

Name
Ethyl alcohol

Biological Context

Level of Biological Organization
Organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	High

Sex Applicability

Sex	Evidence
Mixed	High

Increased metastasis of cancerous cells is known to be highly conserved throughout evolution and is present from humans to invertebrates.

Key Event Description

Processs: metastasis of cancer cells Object:metastasis Process:Increased

Biological state:

Dissemination of the cancer cells from one organ to another which is not directly connected to the primary site is called metastasis. It has a crucial role in the prognosis of cancer patients. In the initial stage of metastasis, cancer cells detach from the primary tumor and disseminate in the tissue. Subsequently cancer cells enter the vascular or lymphatic channels (23-25). The establishment of micro-metastasis mainly depends on the survival of the circulating tumor cells (CTCs) inside the lymphatic or blood channels. Extravasation of cancer cells through the vessel wall takes place resulting in the proliferation of cancer cells in the secondary site. Various signalling pathways are involved in each of the above mentioned process. Few theories have been proposed to explain the mechanism of metastasis. The “organ selection concept” theory suggests that the growth factors establish a successful metastasis in the metastatic site (26,27) whereas the “adhesion theory” proposes the tissue specific adhesion molecules are expressed on endothelial cells of recipient organs which will anchor the migrating cancer cells, providing the a pre-metastatic niche. The role of chemokine receptor has been explained in “chemo-attraction theory” while Paget reported the theory of “seed” for metastatic tumor cells and of “soil” for the secondary site. As per this concept the organ distribution is determined by the site and histopathological type of the primary tumor. The recent understanding suggested, pre-metastatic niche has been indicated to explain metastasis. It is interesting to note that prior to co-localization, the primary tumor induces the micro environment of secondary site by CTCs.

Subsequently, a metastatic niche is generated to support disseminated tumor cells (DTCs) and localize them to develop a metastasis. The most recent theory describes a bidirectional relationship between the primary and secondary sites. According to this theory, the surviving cancer cells in the metastatic tumor can return to the primary site to promote the primary tumor progression (28,29). Efficient and direct blood flow can explain the probability of metastasis to the specific organs like hepatic metastasis in patients with colon cancer which receive direct blood flow from the primary site .Vascular permeability is also the other factor which significantly promotes extravasation at the metastatic site. However at present, understanding of molecular mechanisms of metastasis remains incomplete.

Biological compartment

Organs,Cellular

Role in general biology

Epithelial- mesenchymal transition (EMT) and its reverse mesenchymal-epithelial transition (MET) are characteristics of cellular plasticity during embryogenesis and tumor metastasis (30). There has been decreased expression of E-cadherin and β-catenin and elevated expression levels of vimentin, fibronectin and N-cadherin in EMT (31). In cancers, EMT is a major process by which cancer cells lose their epithelial characteristics

to acquire mesenchymal-like properties. Tumor cell migration is a pre-requisite for the metastatic process in which, EMT is the most critical step to initiate metastasis including metastasis to lymph nodes (32). During EMT, cancer cells lose their cell-to-cell junctions and cellular polarity via multiple signaling pathways which increase the motilities and invasive phenotype of them (33). Cleavage of E-cadherin mediated by the MMPs increases the tumor cell motility and invasion . Apart from this ,EMT has a key role in drug resistance. This is supported by the finding that high levels of vimentin was found in adriamycin and vinblastine resistant breast cancer cell lines (34). EMT promotes CSCs motility, cancer cell invasion, tumor metastasis and recurrence and drug resistance. Expression of stem cell like markers and formation of tumor spheres by CSCs are enhanced by EMT process. CSCs acquire mesenchymal features by undergoing EMT phenomenon. By acquiring mesenchymal features, CSCs become resistant to anti-cancer therapies; hence, they can survive and cause cancer recurrence. In addition to this ,CSCs invade to the adjacent stromal tissues, enter the vascular channels, and finally reach the distant organs. In the target organs, CSCs cause MET phenomenon which results in the acquisition of epithelial characteristics. MET phenomenon also increases the cell-to-cell attachment, cancer cells proliferation and differentiation to form metastatic lesions (35). Altogether , EMT induces CSC properties and metastatic activities. On the other hand, EMT and CSCs collaborate in invasion capacity hence targeting the EMT/CSC phenotype can be a therapeutic approach for the treatment of metastasis and tumor recurrence (36).

EMT programs are regulated by a network of signal- ling pathways that involve components such as growth factors (transforming growth factor-β [TGF-β], epider- mal growth factor [EGF]) and their associated signalling proteins (Wnt, Notch, Hedgehog, nuclear-factor kappa B [NF-κB], extracellular signal-regulated kinase [ERK], and phosphatidylinositol 3-kinase [PI3K]/Akt) in response to stresses involved in tumorigenesis, including hypoxia, oncogenic or metabolic stress, inflammation, and physical constraints [37-41].

These signals activate EMT-inducing transcription factors, including Snail/Slug, ZEB1/δEF1, ZEB2/SIP1, Twist1/ 2, and E12/E47 [42-44]. EMT-inducing transcription factors regulate the expression of proteins involved in cell polarity, cell-cell contact, cytoskeletal structural maintenance, and extracellular matrix (ECM) degradation, and they sup- press key epithelial genes. Loss of E-cadherin is considered a hallmark of EMT; these EMT-inducing transcription factors bind to E-box elements in the E-cadherin gene promoter to repress its transcription. Of particular note, Snail is an early marker of EMT that is involved in the initial cell-migratory phenotype, and it occasionally induces other factors .

During EMT, epithelial cells reorganize cytoskeleton and resolve cell–cell junctions, which are accompanied with switching off the expression of epithelial markers and turning on mesenchymal genes. Although changes in epithelial and mesenchymal markers during EMT can vary significantly in different biologic contexts, a network of transcription factors, including TWIST1/2, SNAIL1/2, ZEB1/2, and FOXC2, are consistently required to orchestrate the EMT program (45). The expression of these transcription factors is associated with poor prognosis and distant metastasis in various human cancers has been documented in various studies. (46). Besides its role in promoting tumor cell invasion, EMT is shown to confer tumor cells with resistance to apoptosis and anoikis (47), thus allowing cell survival in the blood stream after intravasation. EMT could also facilitate tumor cells' escape from the senescence program, especially through TWIST1 and ZEB1 (48,49). Furthermore, EMT has been shown to cancer cells with cancer stem cell (CSC)–like features, which further aid tumor dormancy and chemo resistance (50,51).Tumor samples or experimental tumor xenograft models have provided convincing evidence for the activation of EMT in various primary epithelial tumors in various studies. . Interestingly, more recent studies reveal a dynamic requirement of EMT in tumor metastasis: activation of EMT promotes local tumor invasion, intravasation, and extravasation of the systemic circulation, whereas reversion of EMT is essential to establish macrometatasis in distant organs (52,53).

How it is Measured or Detected

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Cell line,humans,Human cell line studies	qRT-PCR,,Luciferase reporter assay ,immunoblotting,immunoprecipitation,cell invasion assay,cell migration assay, bioluminesence imaging,wound healing assay,Wound scratch & Transwell assay, Microarray,Immunofluorescence, Immunohistochemistry,	+	Strong	Yes	Yes	Yes

Regulatory Significance of the AO

The Adverse Outcome Pathway (AOP) holds substantial regulatory significance as a structured framework for understanding and predicting the biological sequence of events leading from DNA damage to a metastatic breast cancer. By elucidating the causal relationships between key events along the pathway, AOP offer a comprehensive understanding of toxicological mechanisms and provide a basis for informed decision-making in risk assessment and regulatory decision-making. AOPs facilitate the integration of diverse scientific data, enabling regulators to evaluate the potential impact of chemical exposures on human health and the environment. These pathways empower the development of targeted testing strategies, alternative methods, and safer chemical design, ultimately enhancing the efficiency and accuracy of risk assessment and regulatory policies.

References

Anastas JN, Moon RT. (2013) WNT signalling pathways as therapeutic targets in cancer. Nat Rev Cancer. 13(1):11–26.

Ansieau S, Bastid J, Doreau A, Morel A-P, Bouchet BP, Thomas C, et al. (2008) Induction of EMT by TWIST proteins as a collateral effect of tumor- promoting inactivation of premature senescence. Cancer Cell. 14: 79–89.

Da C, Wu K, Yue C, Bai P, Wang R, Wang G, et al. (2017) N-cadherin promotes thyroid tumorigenesis through modulating major signaling pathways. Oncotarget. 8:8131-8142.

Huang R, Zong X. (2017) Aberrant cancer metabolism in epithelialmesenchymal transition and cancer metastasis: Mechanisms in cancer progression. Crit Rev Oncol Hematol.115:1322.

Irani S, Dehghan A. (2018) The expression and functional significance of vascular endothelial-cadherin, CD44, and vimentin in oral squamous cell carcinoma. J Int Soc Prev Community Dent. 8:408-417.

Ishiwata T.(2016) Cancer stem cells and epithelial-mesenchymal transition: Novel therapeutic targets for cancer. Pathol Int.66:601-608.

Jolly MK, Tripathi SC, Jia D, Mooney SM, Celiktas M, Hanash SM, et al. (2016) Stability of the hybrid epithelial/mesenchymal phenotype. Oncotarget.7:27067-27084.

Lamouille S, Xu J, Derynck R.(2014) Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 15(3):178–96.

Liu S, Ye D, Guo W, Yu W, He Y, Hu J, et al. (2015) G9a is essential for EMT-mediated metastasis and maintenance of cancer stem cell-like characters in head and neck squamous cell carcinoma. Oncotarget . 6:6887-6901.

Peinado H, Olmeda D, Cano A.(2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer. 7(6):415–28.

Pickup M, Novitskiy S, Moses HL. (2013)The roles of TGFbeta in the tumour microenvironment. Nat Rev Cancer. 13(11):788–99.

Puisieux A, Brabletz T, Caramel J. (2014) Oncogenic roles of EMT-inducing transcription factors. Nat Cell Biol. 16(6):488–94.

S_x0019_anchez-Tillo_x0019_ E, Liu Y, de Barrios O, Siles L, Fanlo L, Cuatrecasas M, et al. (2012) EMT-activating transcription factors in cancer: beyond EMT and tumor invasiveness. Cell Mol Life Sci.69:3429–56.

Pradella D, Naro C, Sette C, Ghigna C. (2017) EMT and stemness: flexible processes tuned by alternative splicing in development and cancer progression. Mol

cancer.16:8.

Sommers CL, Heckford SE, Skerker JM, Worland P, Torri JA, Thompson EW, et al. (1992) Loss of epithelial markers and acquisition of vimentin expression in adriamycin- and vinblastine-resistant human breast cancer cell lines. Cancer Res. 52:5190-5197.

Thiery JP, Acloque H, Huang RYJ, Nieto MA.(2009) Epithelial-mesenchymal transitions in development and disease. Cell 139:871–90.

Wang Y, Shi J, Chai K, Ying X, Zhou BP.(2013) The Role of Snail in EMT and Tumorigenesis. Curr Cancer Drug Targets. 13(9):963–72.

Zavadil J, Bottinger EP. TGF-beta and epithelial-to-mesenchymal transitions. Oncogene. 2005;24(37):5764–74.

Browne G, Sayan AE, Tulchinsky E.(2010) ZEB proteins link cell motility with cell cycle control and cell survival in cancer. Cell Cycle.9:886–91.

Casas E, Kim J, Bendesky A, Ohno-Machado L, Wolfe CJ, Yang J.(2011) Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis. Cancer Res. 1;71(1):245-54.

Chen L, Mai W, Chen M, Hu J, Zhuo Z, Lei X, Deng L, Liu J, Yao N, Huang M, Peng Y, Ye W, Zhang D.(2017) Arenobufagin inhibits prostate cancer epithelial-mesenchymal transition and metastasis by down-regulating β-catenin. Pharmacol Res. 123:130-142.

Chen Y, Wang DD, Wu YP, Su D, Zhou TY, Gai RH, Fu YY, Zheng L, He QJ, Zhu H, Yang B.(2017) MDM2 promotes epithelial-mesenchymal transition and metastasis of ovarian cancer SKOV3 cells. Br J Cancer. 117(8):1192-1201.

Chen, Sp., Liu, Bx., Xu, J. *et al.* (2015). MiR-449a suppresses the epithelial-mesenchymal transition and metastasis of hepatocellular carcinoma by multiple targets. *BMC Cancer.* **15,** 706

Cui B, Zhang S, Chen L, Yu J, Widhopf GF 2nd, Fecteau JF, Rassenti LZ, Kipps TJ. (2013)Targeting ROR1 inhibits epithelial-mesenchymal transition and metastasis. Cancer Res. 73(12):3649-60.

De Craene B, Berx G. (2013) Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer.13:97–110.

De Craene B, Berx G. (2013)Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer. 13(2):97–110. doi:10.1038/nrc3447.

Derksen PWB, Liu X, Saridin F, van der Gulden H, Zevenhoven J, Evers B, et al. (2006)Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. Cancer Cell.10:437–49.

Gao J, Yang Y, Qiu R, Zhang K, Teng X, Liu R, Wang Y. (2018) Proteomic analysis of the OGT interactome: novel links to epithelial-mesenchymal transition and metastasis of cervical cancer. Carcinogenesis. 39(10):1222-1234.

Gujral TS, Chan M, Peshkin L, Sorger PK, Kirschner MW, MacBeath G. (2014) A noncanonical Frizzled2 pathway regulates epithelial-mesenchymal transition and metastasis. Cell. 159(4):844-56.

Gumireddy K, Li A, Gimotty PA, Klein-Szanto AJ, Showe LC, Katsaros D, Coukos G, Zhang L, Huang Q. (2009) KLF17 is a negative regulator of epithelial-mesenchymal transition and metastasis in breast cancer. Nat Cell Biol. 11(11):1297-304.

Huang Y, Zhao M, Xu H, Wang K, Fu Z, Jiang Y, Yao Z. (2014) RASAL2 down-regulation in ovarian cancer promotes epithelial-mesenchymal transition and metastasis. Oncotarget. 5(16):6734-45.

Irani S, Moshref M, Lotfi A. (2004) Metastasis of a gastric adenocarcinoma to the mandible:A case report. Oral Oncol extra.40:85-87.

Irani S. (2017) Metastasis to the Jawbones: A review of 453 cases. J Int Soc Prev Community Dent.7:71-81.

Irani S, Bidari –Zerehpoush F, Sabeti S. (2016) Prevalence of pathological entities in neck masses: A study of 1208 consecutive cases. Avicenna J Dent Res.8:e25614.

Irani S. (2016) Distant metastasis from oral cancer: A review and molecular biologic aspects. J Int Soc Prev Community Dent.6:265-271.

Irani S. (2011) Metastasis to head and neck area: a 16-year retrospective study. Am J Otolaryngol.32:24-27.

Irani S. (2016) Metastasis to the oral soft tissues: A review of 412 cases. J Int Soc Prev Community Dent.6:393-401.

Irani S.(2016) Pre-cancerous lesions in the oral and maxillofacial region: A literature review with special focus on etopathogenesis. Iran j pathol.11:303-322.

Jackstadt R, Röh S, Neumann J, Jung P, Hoffmann R, Horst D, Berens C, Bornkamm GW, Kirchner T, Menssen A, Hermeking H. (2013)AP4 is a mediator of epithelial-mesenchymal transition and metastasis in colorectal cancer. J Exp Med. 210(7):1331-50.

Kong J, Sun W, Li C, Wan L, Wang S, Wu Y, Xu E, Zhang H, Lai M. (2016)Long non-coding RNA LINC01133 inhibits epithelial-mesenchymal transition and metastasis in colorectal cancer by interacting with SRSF6. Cancer Lett. 380(2):476-484.

Liang YJ, Wang QY, Zhou CX, Yin QQ, He M, Yu XT, Cao DX, Chen GQ, He JR, Zhao Q. (2013)MiR-124 targets Slug to regulate epithelial-mesenchymal transition and metastasis of breast cancer. Carcinogenesis.34(3):713-22.

Liu M, Xiao Y, Tang W, Li J, Hong L, Dai W, Zhang W, Peng Y, Wu X, Wang J, Chen Y, Bai Y, Lin J, Yang Q, Wang Y, Lin Z, Liu S, Xiong J, Wang J, Xiang L. (2020) HOXD9 promote epithelial-mesenchymal transition and metastasis in colorectal carcinoma. Cancer Med. 9(11):3932-3943.

Liu Y, Wang G, Yang Y, Mei Z, Liang Z, Cui A, Wu T, Liu CY, Cui L. (2016) Increased TEAD4 expression and nuclear localization in colorectal cancer promote epithelial-mesenchymal transition and metastasis in a YAP-independent manner. Oncogene. 35(21):2789-800.

Mani SA, Guo W, Liao M-J, Eaton EN, Ayyanan A, Zhou AY, et al. (2008)The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell.133:704–15.

Morel A-P, Lievre M, Thomas C, Hinkal G, Ansieau S, Puisieux A. Gener- ation of breast cancer stem cells through epithelial-mesenchymal transi- tion. PLoS ONE 2008;3:e2888.

Sarkar TR, Battula VL, Werden SJ, Vijay GV, Ramirez-Peña EQ, Taube JH, Chang JT, Miura N, Porter W, Sphyris N, Andreeff M, Mani SA. (2015) GD3 synthase regulates epithelial-mesenchymal transition and metastasis in breast cancer. Oncogene. 34(23):2958-67.

Shiota M, Zardan A, Takeuchi A, Kumano M, Beraldi E, Naito S, Zoubeidi A, Gleave ME. (2012) Clusterin mediates TGF-β-induced epithelial-mesenchymal transition and metastasis via Twist1 in prostate cancer cells. Cancer Res. 72(20):5261-72.

Tsai JH, Donaher JL, Murphy DA, Chau S, Yang J. (2012) Spatiotemporal regu- lation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. Cancer Cell.22:725–36.

Wang L, Tong X, Zhou Z, Wang S, Lei Z, Zhang T, Liu Z, Zeng Y, Li C, Zhao J, Su Z, Zhang C, Liu X, Xu G, Zhang HT. (2018) Circular RNA hsa_circ_0008305 (circPTK2) inhibits TGF-β-induced epithelial-mesenchymal transition and metastasis by controlling TIF1γ in non-small cell lung cancer. Mol Cancer. 17(1):140.

Yu CP, Yu S, Shi L, Wang S, Li ZX, Wang YH, Sun CJ, Liang J. (2017) FoxM1 promotes epithelial-mesenchymal transition of hepatocellular carcinoma by targeting Snai1. Mol Med Rep. 16(4):5181-5188.

Yu J, Lei R, Zhuang X, Li X, Li G, Lev S, Segura MF, Zhang X, Hu G. (2016) MicroRNA-182 targets SMAD7 to potentiate TGFβ-induced epithelial-mesenchymal transition and metastasis of cancer cells. Nat Commun. 7:13884.

Yue, B., Song, C., Yang, L. *et al.* (2019) METTL3-mediated N6-methyladenosine modification is critical for epithelial-mesenchymal transition and metastasis of gastric cancer. *Mol Cancer.* **18,** 142.

Zhang JP, Zeng C, Xu L, Gong J, Fang JH, Zhuang SM. (2014) MicroRNA-148a suppresses the epithelial-mesenchymal transition and metastasis of hepatoma cells by targeting Met/Snail signaling. Oncogene. 33(31):4069-76.

Zhang W, Shi X, Peng Y, Wu M, Zhang P, Xie R, Wu Y, Yan Q, Liu S, Wang J. (2015) HIF-1α Promotes Epithelial-Mesenchymal Transition and Metastasis through Direct Regulation of ZEB1 in Colorectal Cancer. PLoS One. 10(6):e0129603.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

[Relationship: 2608: Increased, DNA damage and mutation leads to Inadequate DNA repair]

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Female	High

In any eukaryotic or prokaryotic cell, oxidative DNA damage can develop and overwhelm the cell's repair processes. This KER has been seen in mammalian cells, yeast, and bacteria, among other places.

Key Event Relationship Description

Upstream event: Increased, DNA damage and mutation

Downstream event: DNA repair mechanism, Reduced

The Key Event Relationship (KER) described involves a cascade of events related to DNA integrity and repair. The upstream event entails "Increased DNA damage and mutation," wherein exposure to various genotoxic agents leads to the accumulation of DNA lesions and mutations. These genetic alterations can arise from factors like chemical exposure, radiation, or other external agents.

The downstream event in this KER is the "Reduced DNA repair mechanism." As a response to increased DNA damage and mutations, the cellular machinery responsible for DNA repair mechanisms becomes compromised or less effective. The cell's ability to identify and rectify DNA lesions and mutations is hindered, potentially due to the overwhelming load of damage or the inefficiency of repair pathways.

Together, this KER illustrates a cause-and-effect relationship wherein heightened DNA damage and mutations contribute to a reduction in the cell's DNA repair mechanisms. This sequence of events highlights the delicate balance between damage induction and repair processes within the cell, emphasizing the importance of understanding these interactions for maintaining genomic stability and preventing the accumulation of detrimental mutations.

Evidence Supporting this KER

- DNA damage leading to inadequate repair mechanisms :

- As a result of DNA damage, DNA repair activities change. A variety of genotoxic agents, such as N-nitrosodimethylamine, aflatoxin B1, and 2-acetylaminofluorene induce the protein, O6-Alkylguanine-DNA alkyltransferase (ATase), which is responsible for repair of DNA alkylation damage in rats (O'Connor, 1989; Chinnasamy et al.,1996). Grombacher and Kaina (1996) reported an increased human ATase mRNA expression by alkylating agents like N-methyl-N'-nitro-N-nitrosoguanidine and methyl methanesulphonate and by ionizing radiation via the induction of the ATase promoter. ATase mRNA expression was increased in response to treatment with 2-acetylaminofluorene in rat liver (Potter et al., 1991; Chinnasamy et al., 1996). In another study, it was demonstrated that ATase gene induction is p53 gene-dependent: ATase activity was induced in mouse tissues following γ-irradiation in p53 wild type mice, but not in p53 null animals (Rafferty et al., 1996).
- Alkylating agents and X-rays also induce DNA glycosylase, alkylpurine-DNA-N-glycosylase (APNG) (Lefebvre et al., 1993; Mitra and Kaina, 1993).
- As a consequence of these and other observations, there is considerable interest in investigating DNA repair modulation as a possible risk factor in carcinogenesis.

- Due to low levels of reactive oxygen species (ROS) and other free radicals generated by endogenous redox reactions, oxidative DNA lesions are present in the cell at steady state.
- The most important oxidative DNA lesions include 7, 8-dihydro-8oxo-deoxyGuanine (8-oxo-dG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPydG), and thymidine glycol (Tg).
- Under homeostatic settings, cells can control the amount of free radicals in the environment and quickly repair oxidised DNA bases with basal repair mechanisms, preventing irreparable damage (Swenberg et al., 2011). Oxidative DNA lesions are mainly repaired by base excision repair (BER) initiated by DNA glycosylases such as oxoguanine glycosylase 1 (OGG1), endonuclease III homologue 1 (NTH1), and Nei-like DNA glycosylases (NEIL 1/2), which detect and remove damaged bases.
- Endonucleases or lyases cleave abasic sites, resulting in transitory single-strand breaks (SSB) that can be repaired in either short-patch or long-patch fashion. To a lesser extent, nucleotide excision repair (NER) is involved in repairing oxidised bases. (Shafirovich et al., 2016).
- Increased levels of free radicals or exposure to oxidising agents can increase the number of oxidative DNA lesions and overload repair mechanisms, lowering repair quality. If the repair mechanisms are weakened, oxidative lesions might build up (insufficient repair), resulting in erroneous base pairing during replication or incomplete repair (indicated by accumulation of repair intermediates) (Markkanen et al., 2017).

Biological Plausibility

BER and, to a lesser extent, NER are used to repair oxidative DNA damage. Previous research has found thresholded dose-response curves in oxidative DNA damage and attributed these findings to a lack of repair capability at the curve's inflection point (Gagne et al., 2012; Seager et al., 2012). Following chemical exposures, in vivo, a rise and buildup of oxidative DNA lesions was seen despite the activation of BER, suggesting poor repair of oxidative DNA lesions beyond a certain level(Ma et al., 2008).

OGG1 and NTH1, the glycosylases that initiate the BER of 8-oxo-dG and thymine glycol (Tg) lesions, respectively, are bifunctional, containing both glycosylase and lyase activities. By cleaving the glycosidic link, the glycosylase eliminates the oxidised guanine and creates an apurinic site.The lyase then cleaves the phosphodiester bond 5' to the AP site; a transient SSB is created for further processing in BER (Delaney et al., 2012). Abasic sites created by OGG1 and other glycosylases are also processed by apuric/apyrimidinic endonucleases (APE1) to create the 5' nick (Allgayer et al., 2016).

Previous research has shown that an imbalance in any of the BER's several phases might result in an accumulation of repair intermediates and failed repair. Given that OGG1 is slower than other glycosylases in releasing its catalytic product, a disproportionate rise in oxidative DNA lesions compared to the quantity of accessible OGG1 is highly likely to result in an imbalance between lesions and the BER initiating step (Brenerman et al., 2014). As a result, oxidative lesions would begin to accumulate. Furthermore, overexpression of OGG1 and NTH1 has been linked to the accumulation of SSB, suggesting that the unbalanced lyase activity causes an excess of SSB intermediates(Yang et al., 2004; Yoshikawa et al., 2015; Wang et al., 2018).

Increases in oxidative lesions may result in more lesions and repair intermediates being produced in close proximity. Previous research on mammalian cell extracts has shown that when oxidative damages occur in parallel or opposite each other, repair effectiveness is reduced.OGG1 showed reduced binding to 8-oxo-dG near an AP site incision. Furthermore, the OGG1-8-oxo-dG complex has been observed to hinder the repair of neighbouring AP site incision, delaying the completion of BER; It's been claimed that this interaction between BER enzymes causes a buildup of oxidative lesions and repair intermediates(Pearson et al., 2004; Budworth et al., 2005; Bellon et al., 2009; Yoshikawa et al., 2015; Sharma et al., 2016).

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

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

Empirical Evidence

In vitro studies demonstrating dose and temporal concordance, or essentiality

  - Human normal hepatocytes (HL-7702) were subjected to escalating doses of N,N-dimethylformamide for 24 hours (C. Wang et al., 2016)
  - At all concentrations, a concentration-dependent increase in ROS was detected; the rise was statistically significant when compared to control (6.4, 16, 40, 100 mM)
  - Until the highest two concentrations (40 and 100 mM), no significant rise in 8-oxodG was seen, indicating inadequate repair at these dosages.
  - Excision repair genes (XRCC2 and XRCC3) were considerably up-regulated at 6.4 and 16 mM, well below the doses that significantly produced 8-oxodG, indicating that adequate DNA repair was possible at these low concentrations.
  - These findings show that repair is competent at low concentrations (removing 8-oxodG quickly), but that repair is swamped (i.e., insufficient) at larger doses, where 8-oxodG greatly increases.
- AS52 Chinese hamster ovary cells (wild type and OGG1-overexpressing) were exposed to varying doses of ultraviolet A (UVA) radiation (Dahle et al., 2008)
  - Formamidopyrimidine glycosylase (Fpg)-sensitive sites were quantified using alkaline elution after increasing repair times (0, 1, 2, 3, 4 h) following 100 kJ/m^2 UVA irradiation
  - OGG1-overexpressing AS52 cells (OGG1+): Fpg-sensitive sites reduced to 71% within half an hour and down to background levels at 4h
  - Wild type AS52 cells: at 4h, 70% of the Fpg-sensitive sites remained, indicating accumulation of oxidative lesions

- Mutations in the *Gpt* gene was quantified in both wild type and OGG1+ cells by sequencing after 13-15 days following 400 kJ/m^2 UVA irradiation
  - G:C→T:A mutations in UVA-irradiated OGG1+ cells were completely eliminated (thus, repair was sufficient when repair overexpressed).
  - G:C→T:A mutation frequency in wild type cells increased from 1.8 mutants/million cells to 3.8 mutants/million cells following irradiation – indicating incorrect repair or lack of repair of accumulated 8-oxo-dG.
  - The importance of 8-oxo-dG production in oxidative DNA damage-induced G to T transversion mutations is further demonstrated by the above result.

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

In vivo studies demonstrating dose concordance

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

- For 30 days, male Sprague-Dawley rats were fed 0.5 mmol aniline/kg/day. Spleen tissues were used to acquire genomic DNA, nuclear extracts, and mitochondrial extracts (Ma et al., 2008).
  - 8-OHdG was measured on digested genomic DNA using an enzyme-linked immunosorbent assay (ELISA). The number of lesions in aniline-fed rats was 2.8 times higher than in control rats.
  - OGG1 activity was measured in both nuclear and mitochondrial extracts of aniline-treated rats, with a 1.32-fold and 1.15-fold increase in enzyme activity (both significant; p0.05) seen in the corresponding extracts.
  - Western blotting was used to assess the OGG1 enzyme content in the extracts; the increase in OGG1 content in aniline-treated rats was consistent with the OGG1 activity assay.
  - The amount of 8-OHdG rose despite an increase in OGG1 enzyme concentration and activity.

  - These findings show that because 8-oxodG adducts are rapidly eliminated, repair is sufficient at low doses. 8-oxo-dG begins to significantly increase at larger concentrations, indicating that repair is being overwhelmed (i.e., insufficient).

Uncertainties and Inconsistencies

Despite the fact that OGG1's dual activity as a glycosylase and lyase has been widely acknowledged and proved experimentally, investigations suggest that apurinic endonuclease 1 is primarily responsible for the cleavage of phosphodiester link 5' to the lesion (APE1) (Allgayer et al., 2016; R. Wang et al., 2018). In rare circumstances, APE1 may be the primary driver of BER intermediate buildup. According to some research, OGG1 is involved in the repair of non-transcribed strands but isn't essential for transcription-coupled 8-oxo-dG repair.; Le Page et al. reported efficient repair of 8-oxo-dG in the transcribed sequence in *Ogg1* knockout mouse cells (Le Page et al., 2000). Furthermore, the repair of 8-oxo-dG is influenced by the sequences surrounding it; the location of the lesions may have a negative impact on repair effectiveness. (Pastoriza-Gallego et al., 2007). We note that the study by Allgayer et al. was investigating the fate and effect of 8-oxo-dG during transcription; repair mechanism may vary by situation and availability of repair enzymes at the time.

Quantitative Understanding of the Linkage

The specific relationship between oxidative DNA lesions and when repair is regarded insufficient has yet to be determined; this relationship will most likely differ between cell types and tissues, making it difficult to define. There are computational models of 8-oxo-dG repair kinetics.

To explore the rate of clearance of BER repair intermediates, Sokhansanj and Wilson III [2004] used a quantitative model of BER and a literature estimate for the rate of generation of endogenous 8-oxo-dG (Sokhansanj and Wilson III, 2004).

- The OGG1, AP lyases, polymerases, and ligases activities were incorporated in the BER model, which used Michaelis-Menten enzyme kinetics.
- The model assumed that endogenous oxidative lesions formed at a rate of 500 8-oxo-dG/day.
  - Based on the information above, it was calculated that after a sudden increase in 8-oxo-dG to 20,000 8-oxo-dG/cell, the total amount of repair intermediates would revert to baseline in 4000 seconds (less than 1 hour)
  - This model also assumed that OGG1 was available in excess
- The glycosylase reaction kinetics of OGG1 (a bifunctional glycosylase/lyase) were reported to increase when APE1 (AP site endonuclease) was present. It's thought to be because the two enzymes work together.
- When OGG1 kinetics were reduced by tenfold, 8-oxo-dG increased tenfold, while no other repair intermediates increased.
- Quantitative understanding is represented as below;
-

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Rat	Quantification of ATase activity – BSA method APNG assay, OXOG glycosylase activity assay, Western immunoblotting, Immunohistochemical detection of ATase (Kotova et al.,2013)	Yes	Strong	Yes	Yes	Yes
Rat cell line	Flow cytometric micronucleus assay, Cell cycle analysis, Replication fork elongation assay, Cytotoxicity assay, Recombination assay, (Panida et al.,2001)	Yes	Strong	Yes	Yes	Yes
mouse	FISH karyotyping, Invivo point mutation assay, Whole genome sequencing of HSC clones (Garayacoechea et al.,2012)	Yes	Strong	Yes	Yes	Yes

Response-response relationship

Linear increase in DNA damage was noted following exposure to the stressor.

Time-scale

Changes were noted within 24 hours of treatment with the stressor, however after withdrawl of the stressor, persisted for 3-4 weeks.

The acute ethanol dose significantly inhibited O^6-alkylguanine-DNA alkyltransferase (ATase) activity by 21–32% throughout the 24-h post-treatment period and this was confirmed by immunohistochemical detection of the ATase protein in hepatic nuclei. Twelve hours after the ethanol treatment, the activities of the DNA glycosylases, alkylpurine-DNA-*N*-glycosylase (APNG) and 8-oxoguanine-DNA glycosylase (OXOG glycosylase) were each increased by ~44%. In contrast, when given chronically via the liquid diet, ethanol initially had no effect on ATase activity, but after 4 weeks ATase activity was increased by 40%. Following ethanol withdrawal, ATase activity remained elevated for at least 12 h, but, by 24 h, the activity had fallen to the uninduced control level. DNA glycosylase activities were again affected differently. After 1 week of dietary ethanol exposure, there was no effect on APNG activity but it was inhibited by 19% at 4 weeks. OXOG glycosylase activity, on the other hand, was increased by 53% after 1 week, but decreased by 40% after 4 weeks.

Known modulating factors

DNA repair mechanism depends on the cell type,age of the cell and extra cellular environment.

Known Feedforward/Feedback loops influencing this KER

Not found to the best of our knowledge.

References

Allgayer, J., Kitsera, N., Bartelt, S., Epe, B., & Khobta, A. (2016). Widespread transcriptional gene inactivation initiated by a repair intermediate of 8-oxoguanine. *Nucleic acids research*, *44*(15), 7267-7280.

Bellon, S., Shikazono, N., Cunniffe, S., Lomax, M., & O'Neill, P. (2009). Processing of thymine glycol in a clustered DNA damage site: mutagenic or cytotoxic. *Nucleic acids research*, *37*(13), 4430-4440.

Brenerman, B. M., Illuzzi, J. L., & Wilson III, D. M. (2014). Base excision repair capacity in informing healthspan. *Carcinogenesis*, *35*(12), 2643-2652.

Budworth, H., Matthewman, G., O'Neill, P., & Dianov, G. L. (2005). Repair of tandem base lesions in DNA by human cell extracts generates persisting single-strand breaks. *Journal of molecular biology*, *351*(5), 1020-1029.

Çağlayan, M., & Wilson, S. H. (2015). Oxidant and environmental toxicant-induced effects compromise DNA ligation during base excision DNA repair. *DNA repair*, *35*, 85-89.

Chinnasamy, N., Rafferty, J. A., Margison, G. P., O'CONNOR, P. J., & Elder, R. H. (1997). Induction of O 6-alkylguanine-DNA-alkyltransferase in the hepatocytes of rats following treatment with 2-acetylaminofluorene. *DNA and cell biology*, *16*(4), 493-500.

Dahle, J., Brunborg, G., Svendsrud, D. H., Stokke, T., & Kvam, E. (2008). Overexpression of human OGG1 in mammalian cells decreases ultraviolet A induced mutagenesis. *Cancer letters*, *267*(1), 18-25.

Delaney, S., Jarem, D. A., Volle, C. B., & Yennie, C. J. (2012). Chemical and biological consequences of oxidatively damaged guanine in DNA. *Free radical research*, *46*(4), 420-441.

Freudenthal, B. D., Beard, W. A., & Wilson, S. H. (2013). DNA polymerase minor groove interactions modulate mutagenic bypass of a templating 8-oxoguanine lesion. *Nucleic acids research*, *41*(3), 1848-1858.

Gagné, J. P., Rouleau, M., & Poirier, G. G. (2012). PARP-1 activation—bringing the pieces

together. *Science*, *336*(6082), 678-679.

Garaycoechea, J. I., Crossan, G. P., Langevin, F., Daly, M., Arends, M. J., & Patel, K. J. (2012). Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. *Nature*, *489*(7417), 571-575.

Gehrke, T. H., Lischke, U., Gasteiger, K. L., Schneider, S., Arnold, S., Müller, H. C., ... & Carell, T. (2013). Unexpected non-Hoogsteen–based mutagenicity mechanism of FaPy-DNA lesions. *Nature chemical biology*, *9*(7), 455-461.

Greenberg, M. M. (2012). The formamidopyrimidines: purine lesions formed in competition with 8-oxopurines from oxidative stress. *Accounts of chemical research*, *45*(4), 588-597.

GROMBACHER, T., & KAINA, B. (1996). Isolation and analysis of inducibility of the rat N-methylpurine-DNA glycosylase promoter. *DNA and cell biology*, *15*(7), 581-588.

Hashimoto, K., Tominaga, Y., Nakabeppu, Y., & Moriya, M. (2004). Futile short-patch DNA base excision repair of adenine: 8-oxoguanine mispair. *Nucleic acids research*, *32*(19), 5928-5934.

Kotova, N., Vare, D., Schultz, N., Gradecka Meesters, D., Stępnik, M., Grawé, J., ... & Jenssen, D. (2013). Genotoxicity of alcohol is linked to DNA replication-associated damage and homologous recombination repair. *Carcinogenesis*, *34*(2), 325-330.

Le Page, F., Klungland, A., Barnes, D. E., Sarasin, A., & Boiteux, S. (2000). Transcription coupled repair of 8-oxoguanine in murine cells: the ogg1 protein is required for repair in nontranscribed sequences but not in transcribed sequences. *Proceedings of the National Academy of Sciences*, *97*(15), 8397-8402.

LEFEBVRE, P., ZAK, P., & LAVAL, F. (1993). Induction of O6-methylguanine-DNA-methyltransferase and N3-methyladenine-DNA-glycosylase in human cells exposed to DNA-damaging agents. *DNA and cell biology*, *12*(3), 233-241.

Li, Y. S., Song, M. F., Kasai, H., & Kawai, K. (2013). Generation and threshold level of 8-OHdG as oxidative DNA damage elicited by low dose ionizing radiation. *Genes and Environment*.

Ma, H., Wang, J., Abdel-Rahman, S. Z., Boor, P. J., & Khan, M. F. (2008). Oxidative DNA damage and its repair in rat spleen following subchronic exposure to aniline. *Toxicology and applied pharmacology*, *233*(2), 247-253.

Maddukuri, L., Ketkar, A., Eddy, S., Zafar, M. K., & Eoff, R. L. (2014). The Werner syndrome protein limits the error-prone 8-oxo-dG lesion bypass activity of human DNA polymerase kappa. *Nucleic acids research*, *42*(19), 12027-12040.

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

Mitra, S., & Kaina, B. (1993). Regulation of repair of alkylation damage in mammalian genomes. *Progress in nucleic acid research and molecular biology*, *44*, 109-142.

O'Connor, P. J. (1989). Towards a role for promutagenic lesions in carcinogenesis. In *DNA repair mechanisms and their biological implications in mammalian cells* (pp. 61-71). Springer, Boston, MA.

Navasumrit, P., Margison, G. P., & O'Connor, P. J. (2001). Ethanol modulates rat hepatic DNA repair functions. *Alcohol and Alcoholism*, *36*(5), 369-376.

Pearson, C. G., Shikazono, N., Thacker, J., & O'Neill, P. (2004). Enhanced mutagenic potential of 8-oxo-7, 8-dihydroguanine when present within a clustered DNA damage site. *Nucleic acids research*, *32*(1), 263-270.

Potter, P. M., Rafferty, J. A., Cawkwell, L., Wilkinson, M. C., Cooper, D. P., O'Connor, P. J., & Margison, G. P. (1991). Isolation and cDNA cloning of a rat; O 6-alkyllguanine-DNA-alkyltransferase gene, molecelar analysis of expression in rat liver. *Carcinogenesis*, *12*(4), 727-733.

Rafferty, J. A., Clarke, A. R., Sellappan, D., Koref, M. S., Frayling, I. M., & Margison, G. P. (1996). Induction of murine O6-alkylguanine-DNA-alkyltransferase in response to ionising radiation is p53 gene dose dependent. *Oncogene*, *12*(3), 693-697.

Seager, A. L., Shah, U. K., Mikhail, J. M., Nelson, B. C., Marquis, B. J., Doak, S. H., ... & Jenkins, G. J. (2012). Pro-oxidant induced DNA damage in human lymphoblastoid cells: homeostatic mechanisms of genotoxic tolerance. *Toxicological Sciences*, *128*(2), 387-397.

Shafirovich, V., Kropachev, K., Anderson, T., Liu, Z., Kolbanovskiy, M., Martin, B. D., ... & Geacintov, N. E. (2016). Base and nucleotide excision repair of oxidatively generated guanine lesions in DNA. *Journal of Biological Chemistry*, *291*(10), 5309-5319.

Shah, A., Gray, K., Figg, N., Finigan, A., Starks, L., & Bennett, M. (2018). Defective base excision repair of oxidative DNA damage in vascular smooth muscle cells promotes atherosclerosis. *Circulation*, *138*(14), 1446-1462.

Sharma, V., Collins, L. B., Chen, T. H., Herr, N., Takeda, S., Sun, W., ... & Nakamura, J. (2016). Oxidative stress at low levels can induce clustered DNA lesions leading to NHEJ mediated mutations. *Oncotarget*, *7*(18), 25377.

Sokhansanj, B. A., & Wilson III, D. M. (2004). Oxidative DNA damage background estimated by a system model of base excision repair. *Free Radical Biology and Medicine*, *37*(3), 422-427.

Swenberg, J. A., Lu, K., Moeller, B. C., Gao, L., Upton, P. B., Nakamura, J., & Starr, T. B. (2011). Endogenous versus exogenous DNA adducts: their role in carcinogenesis, epidemiology, and risk assessment. *Toxicological sciences*, *120*(suppl_1), S130-S145.

Taggart, D. J., Fredrickson, S. W., Gadkari, V. V., & Suo, Z. (2014). Mutagenic potential of 8-oxo-7, 8-dihydro-2′-deoxyguanosine bypass catalyzed by human Y-family DNA polymerases. *Chemical research in toxicology*, *27*(5), 931-940.

Wang, C., Yang, J., Lu, D., Fan, Y., Zhao, M., & Li, Z. (2016). Oxidative stress-related DNA damage and homologous recombination repairing induced by N, N-dimethylformamide. *Journal of Applied Toxicology*, *36*(7), 936-945.

Wang, R., Li, C., Qiao, P., Xue, Y., Zheng, X., Chen, H., ... & Ba, X. (2018). OGG1-initiated base excision repair exacerbates oxidative stress-induced parthanatos. *Cell death & disease*, *9*(6), 1-15.

Yang, N., Galick, H., & Wallace, S. S. (2004). Attempted base excision repair of ionizing radiation damage in human lymphoblastoid cells produces lethal and mutagenic double strand breaks. *DNA repair*, *3*(10), 1323-1334.

Yoshikawa, Y., Yamasaki, A., Takatori, K., Suzuki, M., Kobayashi, J., Takao, M., & Zhang-Akiyama, Q. M. (2015). Excess processing of oxidative damaged bases causes hypersensitivity to oxidative stress and low dose rate irradiation. *Free radical research*, *49*(10), 1239-1248.

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		
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

The described Key Event Relationship (KER) outlines a sequence of events related to DNA repair and its consequences. The upstream event is characterized by "Inadequate DNA repair," indicating that the cellular mechanisms responsible for repairing DNA damage are compromised or insufficient. This could result from various factors, such as genetic mutations, environmental exposures, or other cellular processes.

The downstream event in this KER is an "Increase in Mutations." As a consequence of inadequate DNA repair, the accumulation of unrepaired or incorrectly repaired DNA damage can lead to an elevated rate of mutations in the genome. These mutations can involve changes in the DNA sequence, structure, or arrangement, which may have various implications for cellular function, including potential disruptions to normal processes and pathways.

This KER highlights the critical role of DNA repair mechanisms in maintaining genomic stability and preventing the buildup of mutations that can contribute to various biological outcomes, including disease development and other adverse effects.

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 (Perera et al., 2016).

Time-scale

Inadequate Repair of DSB

Two studies were used to provide data regarding the time scale of DNA repair and the appearance of mutations. In a study using plants, DNA damage was evident immediately following radiation with 30 Gy of radiation; 50% of repairs were complete by 51.7 minutes, 80% by 4 hours, and repair was completed by 24 hours post-irradiation. Although no mutational analysis was performed during the period of repair, irradiated plants were found to have increased mutations when they were examined 2 - 3 weeks later (Ptácek et al., 2001). Both DNA repair and mutation frequency were examined at the same time in a study comparing simple and complex ligation of linearized plasmids. In this study, repaired plasmids were first detected between 6 - 12 hours for simple ligation events and between 12 - 24 hours for more complex ligation events; this first period was when the most error-free rejoining occurred in both cases. After this initial period of repair until its completion at 48 hr, repair became increasingly more erroneous such that mutations were found in more than half of the repaired plasmids at 48 hr regardless of the type of required ligation (Smith et al., 2001).

Known modulating factors

Not identified.

Known Feedforward/Feedback loops influencing this KER

Not identified.

References

Albertini, R.J. et al. (1997), "Radiation Quality Affects the Efficiency of Induction and the Molecular Spectrum of HPRT Mutations in Human T Cells", 148(5 Suppl):S76-86.

Amundson, S.A. & D.J. Chen (1996), "Ionizing Radiation-Induced Mutation of Human Cells With Different DNA Repair Capacities.", Adv. Space Res. 18(1-2):119-126.

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

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

Basu, A.K. and J.M. Essigmann (1990), "Site-specific alkylated oligodeoxynucleotides: Probes for mutagenesis, DNA repair and the structure effects of DNA damage", *Mutation Research*, 233: 189-201.

Beranek, D.T. (1990), "Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents", *Mutation Research*, 231(1): 11-30.

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

Bhowmick, R., S. Minocherhomji & I.D. Hickson (2016), "RAD52 Facilitates Mitotic DNA Synthesis Following Replication Stress", Mol. Cell., 64(6):1117-1126.

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

Deem, A. et al. (2011), "Break-Induced Replication Is Highly Inaccurate", PLoS Biol., 9(2):e1000594, doi: 10.1371/journal.pbio.1000594.

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

Douglas, G.R., J. Jiao, J.D. Gingerich, J.A. Gossen and L.M. Soper (1995), "Temporal and molecular characteristics of mutations induced by ethylnitrosourea in germ cells isolated from seminiferous tubules and in spermatozoa of lacZ transgenic mice", *Proc. Natl. Acad. Sci. USA*, 92(16): 7485-7489.

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

Ellison, K.S., E. Dogliotti, T.D. Connors, A.K. Basu and J.M. Essigmann (1989), "Site-specific mutagenesis by O6-alkyguanines located in the chromosomes of mammalian cells: Influence of the mammalian O6-alkylguanine-DNA alkyltransferase", *Proc. Natl. Acad. Sci. USA*, 86: 8620-8624.

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

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

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

Gocke, E. and L. Muller (2009), "In vivo studies in the mouse to define a threhold for the genotoxicity of EMS and ENU", *Mutat. Res*., 678, 101-107.

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

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

Kaina, B., M. Christmann, S. Naumann and W.P. Roos (2007), "MGMT: Key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents", *DNA Repair*, 6: 1079–1099.

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

Kramara, J., B. Osia & A. Malkova (2018), "Break-Induced Replication: The Where, The Why, and The How", Trends Genet. 34(7):518-531, doi: 10.1016/j.tig.2018.04.002.

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

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

Little, J.B. (2000), "Radiation carcinogenesis.", Carcinogenesis 21(3):397-404 doi:10.1093/carcin/21.3.397.

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

Mao Z, Bozzella M, Seluanov A, Gorbunova V. 2008. DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells. Cell Cycle. 7(18):2902–2906. doi:10.4161/cc.7.18.6679.

Matuo Y, Izumi Y, Furusawa Y, Shimizu K. 2018. Mutat Res Fund Mol Mech Mutagen Biological e ff ects of carbon ion beams with various LETs on budding yeast Saccharomyces cerevisiae. Mutat Res Fund Mol Mech Mutagen. 810(November 2017):45–51. doi:10.1016/j.mrfmmm.2017.10.003.

Mcmahon SJ, Schuemann J, Paganetti H, Prise KM. 2016. Mechanistic Modelling of DNA Repair and Cellular Survival Following Radiation-Induced DNA Damage. Nat Publ Gr.(April):1–14. doi:10.1038/srep33290.

Minocherhomji, S. et al. (2015), "Replication stress activates DNA repair synthesis in mitosis", Nature, 528(7581):286-290.

Minowa, O., Arai, T., Hirano, M., Monden, Y., Nakai, S., Fukuda, M., Itoh, M., Takano, H., Hippou, Y., Aburatani, H., Masumura, K., Nohmi, T., Nishimura, S., Noda, T. (2000), Mmh/Ogg1 gene inactivation results in accumulation of 8-hydroxyguanine in mice, Proc Natl Acad Sci USA, 97:4156-4161.

Muller, L., E. Gocke, T. Lave and T. Pfister (2009), "Ethyl methanesulfonate toxicity in Viracept – A comprehensive human risk assessment based on threshold data for genotoxicity", *Toxicology Letters*, 190: 317-329.

Nagashima, H. et al. (2018), "Induction of somatic mutations by low-dose X-rays : the challenge in recognizing radiation-induced events.", J. Radiat. Res., Na 59(October 2017):11–17. doi:10.1093/jrr/rrx053.

NRC (1990), "Health Effects of Exposure to Low Levels of Ionizing Radiation", (BEIR V).

O'Brien, J.M., A. Williams, J. Gingerich, G.R. Douglas, F. Marchetti and C.L. Yauk CL. (2013), "No evidence for transgenerational genomic instability in the F1 or F2 descendants of Muta™Mouse males exposed to N-ethyl-N-nitrosourea", *Mutat. Res*., 741-742:11-7

O'Brien, J.M., M. Walker, A. Sivathayalan, G.R. Douglas, C.L. Yauk and F. Marchetti (2015), "Sublinear response in lacZ mutant frequency of Muta™ Mouse spermatogonial stem cells after low dose subchronic exposure to N-ethyl-N-nitrosourea", Environ. Mol. Mutagen., 56(4): 347-55.

Pegg, A.E., (2011), "Multifaceted roles of alkyltransferase and related proteins in DNA repair, DNA damage, resistance to chemotherapy, and research tools", *Chem. Res. Toxicol*., 24(5): 618-639.

Perera, D. et al. (2016), "Differential DNA repair underlies mutation hotspots at active promoters in cancer genomes.", Nature 532, 259-263.

Petrini, J.H.J., D.A. Bressan & M.S. Yao (1997), "The RAD52 epistasis group in mammalian double strand break repair.", Semin Immunol. 9(3):181–188. doi:10.1006/smim.1997.0067

Philippin, G., J. Cadet, D. Gasparutto, G. Mazon, R.P. Fuchs (2014), "Ethylene oxide and propylene oxide derived N7-alkylguanine adducts are bypassed accurately in vivo", *DNA Repair (Amst)*, 22:133-6.

Pouget, J.P. & S.J. Mather (2001), "General aspects of the cellular response to low- and high-LET radiation.", Eur. J. Nucl. Med. 28(4):541–561. doi:10.1007/s002590100484

Preston, R. et al. (2013), "Uncertainties in estimating health risks associated with exposure to ionising radiation", Journal of Radiological Protection, Vol.33/3, IOP Publishing, Bristol, https://doi.org/10.1088/0952-4746/33/3/573.

Ptácek, O. et al. (2001), "Induction and repair of DNA damage as measured by the Comet assay and the yield of somatic mutations in gamma-irradiated tobacco seedlings.", Mutat Res. 491(1-2):17–23

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

Pzoniak, A., L. Muller, M. Salgo, J.K. Jone, P. Larson and D. Tweats (2009), "Elevated ethyl methansulfonate in nelfinavir mesylate (Viracept, Roche): overview", *Aids Research and Therapy*, 6: 18.

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

Rodriguez, G.P., Song, J.B., Crouse, G.F. (2013), In Vivo Bypass of 8-oxodG, PLoS Genetics, 9:e1003682.

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

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

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

Sassa, A., Kamoshita, N., Kanemaru, Y., Honma, M., Yasui, M. (2015), Xeroderma Pigmentosum Group A Suppresses Mutagenesis Caused by Clustered Oxidative DNA Adducts in the Human Genome, PLoS One, 10:e0142218.

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

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

Shrivastav, N., D. Li and J.M. Essignmann (2010), "Chemical biology of mutagenesis and DNA repair: cellular response to DNA alkylation", *Carcinogenesis*, 31(1): 59-70.

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

Singer, B., F. Chavez, M.F. Goodman, J.M. Essigman and M.K. Dosanjh (1989), "Effect of 3' flanking neighbors on kinetics of pairing of dCTP or dTTP opposite O6-methylguanine in a defined primed oligonucleotide when Escherichia coli DNA polymerase I is used", *Proc. Natl. Acad. Sci. USA*, 86(21): 8271-8274.

Sishc-Brock J. & A.J. Davis (2017), "The role of the core non-homologous end joining factors in carcinogenesis and cancer.", Cancers (Basel). 9(7). doi:10.3390/cancers9070081.

Smith, J. et al. (2001), "The influence of DNA double-strand break structure on end-joining in human cells.", Nucleic Acids Res. 29(23):4783–4792

Smith, J. et al. (2003), "Impact of DNA ligase IV on the ® delity of end joining in human cells.", Nucleic Acids Res., 31(8):2157-67. doi:10.1093/nar/gkg317

Tan, X., Grollman, A., Shibutani, S. (1999), Comparison of the mutagenic properties of 8-oxo-7,8-dihydro-2'-deoxyadenosine and 8-oxo-7,8-dihydro-2'-deoxyguanosine DNA lesions in mammalian cells, Carcinogenesis, 20:2287-2292.

Thomas, A.D., G.J. Jenkins, B. Kaina, O.G. Bodger, K.H. Tomaszowski, P.D. Lewis, S.H. Doak and G.E. Johnson (2013), "Influence of DNA repair on nonlinear dose-responses for mutation", *Toxicol. Sci.*, 132(1): 87-95.

van Delft, J.H. and R.A. Baan (1995), "Germ cell mutagenesis in lambda lacZ transgenic mice treated with ethylnitrosourea; comparison with specific-locus test", *Mutagenesis*, 10(3): 209-214.

Waters, C.A. et al. (2014), "The fidelity of the ligation step determines how ends are resolved during nonhomologous end joining.", Nat Commun. 5:1–11. doi:10.1038/ncomms5286.

Wessendorf P. et al. (2014), "Mutation Research / Fundamental and Molecular Mechanisms of Mutagenesis Deficiency of the DNA repair protein nibrin increases the basal but not the radiation induced mutation frequency in vivo.", Mutat. Res. - Fundam. Mol. Mech. Mutagen. 769:11–16. doi:10.1016/j.mrfmmm.2014.07.001.

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

Relationship: 2609: Increase, Mutations leads to Increase,miRNA levels

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
mice	Mus sp.	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified

Sex Applicability

Sex	Evidence
Female	High

Not specific through any particular life stage or gender.

Key Event Relationship Description

Upstream event: increased, mutations

Downstream event: increased miRNA

The Key Event Relationship (KER) depicted involves a chain of events associated with genetic changes and molecular responses. The upstream event is characterized by "Increase in Mutations," indicating a heightened occurrence of changes in the DNA sequence, structure, or arrangement. These mutations can arise from various factors, such as environmental exposures, errors during DNA replication, or DNA repair deficiencies.

The downstream event in this KER is the "Increase in miRNA levels." As a consequence of increased mutations, there is an elevation in the levels of microRNAs (miRNAs), which are small non-coding RNA molecules that play a role in regulating gene expression. The alterations in DNA resulting from mutations can influence the expression of miRNAs, leading to changes in their abundance.

This KER suggests a potential link between genetic changes and miRNA regulation. It highlights the intricate molecular interactions within the cell, where mutations in the genome can impact miRNA expression patterns. Understanding this relationship contributes to a broader understanding of how genetic alterations can influence gene expression and cellular responses.

Evidence Supporting this KER

Evidences suggest that transcription pathway for miRNAs is regulated in the DNA damage response (DDR). The tumour suppressor p53 is a well-known transcription factor that is activated in response to DNA damage and causes cell growth arrest, promotes apoptosis, inhibits angiogenesis, and mediates DNA repair (Meek et al., 2009). Global miRNA expression investigations identified the miRNA components of p53 transcriptional pathways and demonstrated that a cohort of miRNAs are up-regulated in a p53-dependent manner following DNA damage. The miR-34 family (miR-34a, miR-34b/c) was the first transcriptional target of p53 to be discovered. p53 directly transactivates miR-15a/16-1, miR-29, miR-107, miR-145, miR-192, miR-194, miR-215, and miR-605 in addition to the miR-34 family.

Biological Plausibility

Various transcription factors regulate miRNA expression.The p53 protein also functions as a transcriptional repressor by binding to miRNA promoters and preventing the recruitment of transcriptional activators.For example, p53 prevents the TATA-binding protein from binding to the TAATA site in the promoter of the miR-17-92 cluster gene, suppressing transcription. Under hypoxic conditions, the miR-17-92 cluster is suppressed by a p53-dependent mechanism, making cells more susceptible to hypoxia-induced death (Yan et al.,2009). Cells can amplify the p53 signal by fine-tuning the p53 signalling pathway and the miRNA network, which improves cell sensitivity to external signals.

Other DNA damage-responsive transcription factors, such as NF-kB, E2F, and Myc, are also involved in miRNA transcription regulation. Both E2F and Myc promote miR-17-92 cluster transcription, which reduces E2F and Myc expression, establishing an autoregulatory negative feedback loop. Furthermore, Myc-induced miRNAs have an impact on cell proliferation and cell fate in Myc-mediated cells (Kim et al.,2010). Myc-induced miR-20a, for example, targets cdkn1a, a gene that encodes a negative regulator of cell-cycle progression, while Myc-induced miR-221 and miR-222 target the CDKN1b and CDKN1c genes, respectively, that trigger cell-cycle arrest. Little is currently known about how miRNA gene expression is transcriptionally regulated due to a lack of basic information about miRNA gene structure. Global prediction and verification of promoter regions of miRNA genes would allow us to further explore the functional interaction of transcriptional machinery and epigenetic miRNA regulation. p53 regulates miRNA maturation not only during transcription but also during processing of initial miRNA transcripts, resulting in crosstalk between the p53 network and the DDR's miRNA biogenesis machinery. Many p53-regulated miRNAs target proteins in the DDR, such as cell cycle progression and apoptosis, to affect the DDR.

Empirical Evidence

There are findings that strongly link the different elements of DNA damage and repair events to the expression of miRNA.

- Zhang and coworkers examined genome-wide mature miRNA expression in Atm+/+ and Atm-/- littermate mouse embryonic fibroblasts to see how miRNAs are regulated in the DNA damage response (MEFs)(Zhang et al.,2011).
- MEFs were given neocarzinostatin (NCS), a radiomimetic medication that causes DSBs (Ziv et al., 2006). Mouse miRNA microarray analysis was used to determine miRNA expression profile in each sample, which was done at several time points (0–24 hr). As many as 71 distinct miRNAs were found to be considerably (2-fold) upregulated in the NCS-treated Atm+/+ MEFs, but not in the corresponding Atm-/- MEFs, implying that DNA damage stress causes broad-spectrum changes in miRNA expression.
- The functioning of the Atm gene is required for the induction of these miRNAs. The DNA damage induction of these miRNAs was entirely eliminated when ATM was knocked out of the Atm-/- MEFs, implying that ATM is a critical regulator of KSRP activity in miRNA synthesis.
- To see if DNA damage enhances the transcription of these miRNAs, researchers used quantitative reverse-transcriptase PCR (RT-PCR) using primer sets built particularly for pri-miRNAs to look at the expression levels of primary miRNA transcripts (pri-mRNAs) in both Atm+/+ and Atm-/- MEFs. Regardless of ATM status, these pri-miRNAs did not show any significant induction or reduction (50 percent change) in transcription levels during the DNA damage response.
- The study used a pair of human fibroblast cell lines with proficient (GM0637) or deficient (GM9607) ATM to assess the levels of six representative mature miRNAs that were randomly selected out of the pool for both ATM- and KSRP-induced miRNAs to confirm that DNA damage-mediated miRNA induction in MEFs was not species specific.

- Following NCS treatment, the levels of these miRNAs increased dramatically in ATM-proficient cells but not in ATM-deficient cells, matching the findings from MEFs. DNA damage was not induced by the control miR-218, which is not regulated by ATM or KSRP.
- After DNA damage, -KSRP was necessary for miRNA induction. When KSRP was knocked out, the induction of these miRNAs was significantly reduced, demonstrating a functional connection between KSRP and ATM in miRNA synthesis.

- According to Wan et al., regulatory RNA-binding proteins in the Drosha and Dicer complexes, such as DDX5 and KSRP, drive posttranscriptional processing of primary and precursor miRNAs after DNA damage. The findings show that nuclear export of pre-miRNAs is increased in an ATM-dependent manner after DNA damage. The ATM-activated AKT kinase phosphorylates Nup153, a main component of the nucleopore, resulting in enhanced interaction between Nup153 and Exportin-5 (XPO5) and increased nuclear export of pre-miRNAs. These findings demonstrate that DNA damage signalling is important for miRNA transport and maturation.

- To test the DNA-damage induction of miRNAs in human cells, researchers looked at mature miRNA expression in human fibroblast GM0637 cells treated with the radiomimetic drug neocarzinostatin (NCS) in the presence or absence of the ATM inhibitor KU55933.

- In agreement with previous reports showing that ATM-activated p53 and KSRP promote miRNA expression (Suzuki et al., 2009; Zhang et al., 2011), the study found 61 p53-dependent miRNAs and 29 KSRP-dependent miRNAs among the ATM-induced miRNAs.
- The study also examined the levels of different forms of miRNAs (pri- miRNAs, pre-miRNAs, and mature miRNAs) selected from the ATM-induced miRNAs, including KSRP-dependent miRNAs (let-7a, 15a, 15b, 16, 125b, 21, 27b, 98, and 199a), p53- dependent miRNAs (34a), and KSRP/p53-independent miRNAs (181a, 382, and 338).
- As a control, miR-218, which is unaffected by DNA damage, was also included in the examination. With the exception of miR-34a, which is known to be transactivated by p53, there were no significant increases in expression of primary transcripts for these miRNAs following DNA damage. -These results suggest that DNA damage may promote posttranscriptional maturation of the miRNAs.

- Neither KSRP nor p53 were required for the expression of miR-181a, miR-382, or miR-338. Stable knockdown of KSRP or p53 could not prevent their induction following DNA damage, but knockdown of ATM did, indicating that the increased miRNAs in the DDR are accounted for by another ATM-dependent mechanism. The miR-34a and miR-21 controls were reliant on p53 and KSRP, respectively.

- These findings imply that DNA damage may increase miRNA maturation after transcription. Neither KSRP nor p53 were required for the expression of miR-181a, miR-382, or miR-338. Stable knockdown of KSRP or p53 could not prevent their induction following DNA damage, but knockdown of ATM did, indicating that the increased miRNAs in the DDR are accounted for by another ATM-dependent mechanism. As previously observed (He et al., 2007; Trabucchi et al., 2009), the controls miR-34a and miR-21 were reliant on p53 and KSRP, respectively.

- A group of miRNAs is induced in an ATM-dependent way after DNA damage. Prior to NCS (500 ng/ml) treatment, human fibroblast GM0637 (ATM-proficient) cells were pretreated with ATM inhibitor KU55933 (10 mM) or DMSO. For microarray analysis, cells were taken 4 hours after NCS treatment.

- There was an increase in miRNA levels as well as a drop in miRNA levels.
- The miRNA expression profile from GM0637 cells treated with DMSO or ATM inhibitor was used to identify ATM-dependent (ATM-IN/Ctrl 0.67) and ATM-independent (ATM-IN/Ctrl > 0.67) miRNAs.
- Human Mammary Epithelial progenitor Cells (HMEpC) and Human Small Airway Epithelial progenitor Cells (HSAEpC) were cultivated. Cells were cultivated to population doubling 5 before being aliquoted and frozen in Promocell culture media, which contained 10% DMSO and 5% Human Serum Albumin (HSA, Octalbine). All tests used cells cultivated from a freshly thawed vial and expanded until population doubling was reached. Up to 16 population doublings, the HMEpC/HSAEpC maintained their proliferation characteristics (Jaarsveld et al,.2014)

- As previously stated, miRNA RT-qPCR expression analysis was performed on 8 normal breast tissue samples and 84 breast tumour tissue samples utilising microfluidic cards (A&B TLDA arrays, Applied Biosystems) (Van der Auwera et al., 2010).
- MiRNA microarray analysis was carried out on i.untreated and cisplatin/IR treated HMEpC and HSAEpC cells (4 replicates for each condition), (ii) 18 lung tumour tissue tissue samples and 14 adjacent 'normal' lung tissue samples (representing NSCLC subtypes), and (iii) 52 breast cancer cell lines (Riaz et al., 2013) and 12 lung cancer cell lines. RNA Bee was used to isolate total —RNA (Bio- Connect, the Netherlands). One mg of RNA was hybridised with an Exiqon LNATM-based probeset (versions 10 and 7 for cell lines and lung tissue, respectively), and spotted in duplicate on Nexterion E slides (Pothof et al., 2009).

- The severity of DNA damage determines the outcome of DDR signalling, i.e. repair and survival or apoptosis/senescence. Since there is evidence in the literature that all these branches within the DDR can be defective in cancer, conditions were established to identify miRNAs that are regulated upon DNA damage in general, independent of biological outcome.
- Two genotoxic therapeutic agents (cisplatin and IR) were used for which a dose was determined that allows for cellular recovery after DNA damage and a dose that induces primarily cell death or senescence.
- To define the conditions for recovery clonal survival assay was used, which determines the capacity of individual cells to recover and form colonies after DNA damage.
- 0.25 mM cisplatin or 1.5 Gy IR were chosen, both resulting in a 50% reduction of HMEpC colony formation , thus 50% of all initially damaged cells can still grow out into a colony within 10 days. --As expected, these conditions activate the DDR and induce cell cycle arrest
- Following that, a higher dose at which the cells suffer apoptosis following cisplatin treatment was determined using an MTT assay. HMEpC viability was reduced by 50% after 48 hours of treatment with 15 mM cisplatin, which was accompanied by lower cellular PARP1 levels, an apoptosis marker. It's worth noting that at this concentration, all cells will eventually die. In contrast to cisplatin, a high dose of IR caused cellular senescence rather than apoptosis, which has no effect on cellular viability as assessed by the MTT experiment.
- HMEpCs were treated with low and high doses of cisplatin and IR to characterise the miRNA response to DNA damage. Total RNA was isolated 6 hours, 12 hours, and 24 hours after the commencement of treatment for each genotoxic treatment and dose based on miRNA kinetics after DNA damage treatment (Pothof et al., 2009; Zhang et al., 2011). MiRNA profiling was carried out on 725 human miRNAs utilising miRNA arrays with Locked Nucleic Acid-based capture probes. Before normalisation, the reproducibility between biological replicates (n 14 4) was examined, revealing that the correlation between replicates was higher than that between non-replicates. After normalisation, condition-specific regulation of miRNAs was shown to be dependent on the kind of genotoxic stress and dose, as well as miRNAs that showed oppo-site regulation after IR and cisplatin treatment.
- General miRNA responders to DNA damage, i.e. significantly regulated miRNAs across all genotoxic conditions per time point were focused.Several general DDR miRNAs were identified and most were regulated at 6 h and 12 h after treatment , which is in agreement with published miRNA expression kinetics after DNA damage (Pothof et al., 2009;). In conclusion, the study could identify several miRNAs that can be characterized as general responders to genotoxic cancer treatments in HMEpCs.

Uncertainties and Inconsistencies

In response to stressors like as ionising radiation, miRNAs are differently regulated. When exposed to IR, miRNA expression is frequently disrupted. Some miRNAs are induced by IR, while others are suppressed, a decision that is likely based on the target genes implicated. This figure summarises the miRNAs mentioned in this review whose expression changes in response to IR. Lists of miRNAs whose induction or repression has been detected are on the left and right, respectively. MiRNAs are in the middle, and both induction and repression have been found in many cell types. The centre contains the biggest group of miRNAs, demonstrating how diverse the miRNA profile can be from one cell type to the next. Bold miRNAs play a role in several parts of the DDR.

Induced miRNAs were reported by some studies (Cha et al.,2009;Chaudhary et al 201; Chaudhary et al 2012; Chaudhary et al 2013;Kwon et al.,2013;Mueller et al.,2013;Shin et al.,2009;Sokolov et al.,2012;Wagner et al.,2010),whereas repressed miRNAs are observed in some(Cha et al., 2009,Chaudhari et al.,2010).Both induction and repression of some miRNA were seen in different cell types and results are inconclusive (Cha et al.,2009;Chaudhary et al 201; Chaudhary et al 2012; Chaudhary et al 2013;Kwon et al.,2013;Mueller et al.,2013;Shin et al.,2009;Sokolov et al.,2012;Wagner et al.,2010; Kraemer et al., 2011; Moskwa et al.,2011;Niemoeller et al.,2011;Sokolov et al., 2012;Wagner et al.,2010).This inconsistency could be due to different doses of stressor.

DNA damage response influences miRNA expression, at the same time miRNA can also influence DDR, cell cycle etc.The miR-34 family produces a cell-cycle arrest in the G1 phase and slows cell-cycle progression by targeting multiple cell cycle regulators when ectopically produced, implying tumor-suppressing potential. The miR-34 family, for example, specifically targets and inhibits cyclin-dependent kinase 4 (CDK4), CDK6, E2F3, Myc, and NMYC (Chang et al.,2007,He et al., 2007).

MiRNA expression can be influenced by DNA damage and mutation, but miRNA can also regulate DNA damage response and cell cycle.By suppressing the transcripts of numerous genes that govern cell-cycle checkpoints or metabolism, these p53-induced miRNAs contribute to cell-cycle arrest (Su et al.,2010;Georges et al.,2008;Hermeking et al.,2012;Klein et al., 2010; Liu et al.,2011; Suh et al.,2011). Wip1 phosphatase, a master inhibitor in the DDR that inhibits the activation and stability of p53, is targeted and repressed by miR-16 and miR-29, resulting in p53 induction (Ugalde et al.,2011; Zhang et al.,2010). Cellcycle arrest is induced by ectopic expression of miR-192/215, which targets a number of genes that regulate the G1/S and G2/M checkpoints (Bulavin et al.,2004).

The oncogene c-Myc is directly targeted by miR-145, implying that p53 suppresses cMyc activities through regulating miRNA expression (Sachdeva et al.,2009, Suh et al.,2011). p53-induced miRNAs, interestingly, influence p53 activity in a positive feedback loop (Han et al.,2012, Hermeking et al.,2012). SIRT1 acetylation and activation are increased when miR-34 inhibits it (Yamakuchi et al., 2008). Mdm2 expression is directly inhibited by miR-192, miR-194, miR-215, and MiR-605, while Wip1 is inhibited by miR-29, resulting in higher p53 levels and activity. (Braun et al.,2008, Pichiorri et al.,2010, Xiao et al., 2011).

Quantitative Understanding of the Linkage

The below table gives the evidence for DNA damage responses influencing the expression of miRNA as well as miRNA expression influencing DNA damage response.

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human cell line	Western blotting,clonal survival assay, FACs(Jaarsveld et al., 2014)	YEs	Strong	Yes	Yes	Yes
Mice	Free radic CyQuant cell Proliferation assay (Abdelfattah et	Yes	Strong	Yes	Yes	Yes

	al.,2018)					
	RNA sequence analysis, Immuno staining, immunoblotting, Flowcytometry, COMET assay, qRT PCR(Liu et al., 2017)	Yes	Strong	Yes	Yes	Yes
	Microarray (Zhang et al.,2011)	Yes	Strong	Yes	Yes	Yes
	qRT-PCR, RIP assay, Immunogold EM(Wan et al.,2013)	Yes				
Canine	micro array(Bulkowska et al., 2017)	Yes	Strong	Yes	Yes	Yes

Response-response relationship

Activity of pri-miR-218, pri-miR-16-1, pri-miR-21, and pri- miR-199a had significantly increased binding with Drosha (2.5- to 3.2-fold) after DNA damage (Zhang et al.,2011)

Time-scale

It has been noted that, within hours of DNA damage,miRNA expression were induced(Wan et al.,2013).

Known modulating factors

miRNA expression profiles are influenced by a variety of DNA damaging stressors. Pothof et al. were the first to notice differences in miRNA expression in cell-cycle checkpoints and DNA repair in UV-treated cells (Pothof et al., 2009). Other DNA damaging agents, such as cisplatin, doxorubicin, IR, and NCS, were used to examine miRNA expression profiles in cells (Galluzzi et al., 2010, Saleh et al.,2011;Suzuki et al.,2009). Different levels of DNA damage appear to activate different groups of miRNAs, implying that miRNAs regulate the DDR through a mechanism that is dependent on the type and severity of the DNA damage.

Known Feedforward/Feedback loops influencing this KER

Not specific ones available.

References

Abdelfattah, N., Rajamanickam, S., Panneerdoss, S., Timilsina, S., Yadav, P., Onyeagucha, B. C., ... & Rao, M. K. (2018). MiR-584-5p potentiates vincristine and radiation response by inducing spindle defects and DNA damage in medulloblastoma. *Nature communications*, *9*(1), 1-19.

Braun, C. J., Zhang, X., Savelyeva, I., Wolff, S., Moll, U. M., Schepeler, T., ... & Dobbelstein, M. (2008). p53-Responsive micrornas 192 and 215 are capable of inducing cell cycle arrest. *Cancer research*, *68*(24), 10094-10104.

Bulkowska, M., Rybicka, A., Senses, K. M., Ulewicz, K., Witt, K., Szymanska, J., ... & Krol, M. (2017). MicroRNA expression patterns in canine mammary cancer show significant differences between metastatic and non-metastatic tumours. *BMC cancer*, *17*(1), 1-17.

Bulavin, D. V., Phillips, C., Nannenga, B., Timofeev, O., Donehower, L. A., Anderson, C. W., ... & Fornace, A. J. (2004). Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK–mediated activation of the p16 Ink4a-p19 Arf pathway. *Nature genetics*, *36*(4), 343-350.

Cha, H. J., Shin, S., Yoo, H., Lee, E. M., Bae, S., Yang, K. H., ... & An, S. (2009). Identification of ionizing radiation-responsive microRNAs in the IM9 human B lymphoblastic cell line. *International journal of oncology*, *34*(6), 1661-1668.

Chaudhry, M. A., & Omaruddin, R. A. (2012). Differential regulation of microRNA expression in irradiated and bystander cells. *Molecular Biology*, *46*(4), 569-578.

Chaudhry, M. A., Omaruddin, R. A., Brumbaugh, C. D., Tariq, M. A., & Pourmand, N. (2013). Identification of radiation-induced microRNA transcriptome by next-generation massively parallel sequencing. *Journal of radiation research*, *54*(5), 808-822..

Chaudhry, M. A., Omaruddin, R. A., Kreger, B., De Toledo, S. M., & Azzam, E. I. (2012). Micro RNA responses to chronic or acute exposures to low dose ionizing radiation. *Molecular biology reports*, *39*(7), 7549-7558.

Chaudhry, M. A., Sachdeva, H., & Omaruddin, R. A. (2010). Radiation-induced micro-RNA modulation in glioblastoma cells differing in DNA-repair pathways. *DNA and cell biology*, *29*(9), 553-561..

Chang, T. C., Wentzel, E. A., Kent, O. A., Ramachandran, K., Mullendore, M., Lee, K. H., ... & Mendell, J. T. (2007). Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Molecular cell*, *26*(5), 745-752.

Galluzzi, L., Morselli, E., Vitale, I., Kepp, O., Senovilla, L., Criollo, A., ... & Kroemer, G. (2010). miR-181a and miR-630 regulate cisplatin-induced cancer cell death. *Cancer research*, *70*(5), 1793-1803.

Georges, S. A., Biery, M. C., Kim, S. Y., Schelter, J. M., Guo, J., Chang, A. N., ... & Chau, B. N. (2008). Coordinated regulation of cell cycle transcripts by p53-Inducible microRNAs, miR-192 and miR-215. *Cancer research*, *68*(24), 10105-10112.

Han, C., Wan, G., Langley, R. R., Zhang, X., & Lu, X. (2012). Crosstalk between the DNA damage response pathway and microRNAs. *Cellular and molecular life sciences*, *69*(17), 2895-2906.

Han, C., Liu, Y., Wan, G., Choi, H. J., Zhao, L., Ivan, C., ... & Lu, X. (2014). The RNA-binding protein DDX1 promotes primary microRNA maturation and inhibits ovarian tumor progression. *Cell reports*, *8*(5), 1447-1460.

Hermeking, H. (2012). MicroRNAs in the p53 network: micromanagement of tumour suppression. *Nature reviews cancer*, *12*(9), 613-626.

He, L., He, X., Lim, L. P., De Stanchina, E., Xuan, Z., Liang, Y., ... & Hannon, G. J. (2007). A microRNA component of the p53 tumour suppressor network. *Nature*, *447*(7148), 1130-1134.

Kim, J. W., Mori, S., & Nevins, J. R. (2010). Myc-induced microRNAs integrate Myc-mediated cell proliferation and cell fate. *Cancer research*, *70*(12), 4820-4828.

Klein, U., Lia, M., Crespo, M., Siegel, R., Shen, Q., Mo, T., ... & Dalla-Favera, R. (2010). The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. *Cancer cell*, *17*(1), 28-40.

Kraemer, A., Anastasov, N., Angermeier, M., Winkler, K., Atkinson, M. J., & Moertl, S. (2011). MicroRNA-mediated processes are essential for the cellular radiation response. *Radiation research*, *176*(5), 575-586.

Kwon, J. E., Kim, B. Y., Kwak, S. Y., Bae, I. H., & Han, Y. H. (2013). Ionizing radiation-inducible microRNA miR-193a-3p induces apoptosis by directly targeting Mcl-1. *Apoptosis*, *18*(7), 896-909.

Liu, M., Lang, N., Chen, X., Tang, Q., Liu, S., Huang, J., ... & Bi, F. (2011). miR-185 targets RhoA and Cdc42 expression and inhibits the proliferation potential of human colorectal cells. *Cancer letters*, *301*(2), 151-160.

Liu, Z., Zhang, C., Khodadadi-Jamayran, A., Dang, L., Han, X., Kim, K., ... & Zhao, R. (2017). Canonical microRNAs enable differentiation, protect against DNA damage, and promote cholesterol biosynthesis in neural stem cells. *Stem cells and development*, *26*(3), 177-188.

Meek, D. W. (2009). Tumour suppression by p53: a role for the DNA damage response?. *Nature Reviews Cancer*, *9*(10), 714-723.

Moskwa, P., Buffa, F. M., Pan, Y., Panchakshari, R., Gottipati, P., Muschel, R. J., ... & Chowdhury, D. (2011). miR-182-mediated downregulation of BRCA1 impacts DNA repair and sensitivity to PARP inhibitors. *Molecular cell*, *41*(2), 210-220.

Mueller, A. C., Sun, D., & Dutta, A. (2013). The miR-99 family regulates the DNA damage response through its target SNF2H. *Oncogene*, *32*(9), 1164-1172.

Niemoeller, O. M., Niyazi, M., Corradini, S., Zehentmayr, F., Li, M., Lauber, K., & Belka, C. (2011). MicroRNA expression profiles in human cancer cells after ionizing radiation. *Radiation oncology*, *6*(1), 1-5.

Pichiorri, F., Suh, S. S., Rocci, A., De Luca, L., Taccioli, C., Santhanam, R., ... & Croce, C. M. (2010). Downregulation of p53-inducible microRNAs 192, 194, and 215 impairs the p53/MDM2 autoregulatory loop in multiple myeloma development. *Cancer cell*, *18*(4), 367-381.

Pothof, J., Verkaik, N. S., Van Ijcken, W., Wiemer, E. A., Ta, V. T., Van Der Horst, G. T., ... & Persengiev, S. P. (2009). MicroRNA-mediated gene silencing modulates the UV-induced DNA-damage response. *The EMBO journal*, *28*(14), 2090-2099.

Riaz, M., van Jaarsveld, M. T., Hollestelle, A., Prager-van der Smissen, W. J., Heine, A. A., Boersma, A. W., ... & Martens, J. W. (2013). miRNA expression profiling of 51 human breast cancer cell lines reveals subtype and driver mutation-specific miRNAs. *Breast cancer research*, *15*(2), 1-17.

Shin, S., Cha, H. J., Lee, E. M., Lee, S. J., Seo, S. K., Jin, H. O., ... & An, S. (2009). Alteration of miRNA profiles by ionizing radiation in A549 human non-small cell lung cancer cells. *International journal of oncology*, *35*(1), 81-86.

Sokolov, M. V., Panyutin, I. V., & Neumann, R. D. (2012). Unraveling the global microRNAome responses to ionizing radiation in human embryonic stem cells. *PloS one*, *7*(2), e31028.

Su, X., Chakravarti, D., Cho, M. S., Liu, L., Gi, Y. J., Lin, Y. L., ... & Flores, E. R. (2010). TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs. *Nature*, *467*(7318), 986-990.

Suh, S. O., Chen, Y., Zaman, M. S., Hirata, H., Yamamura, S., Shahryari, V., ... & Dahiya, R. (2011). MicroRNA-145 is regulated by DNA methylation and p53 gene mutation in prostate cancer. *Carcinogenesis*, *32*(5), 772-778.

Sachdeva, M., Zhu, S., Wu, F., Wu, H., Walia, V., Kumar, S., ... & Mo, Y. Y. (2009). p53 represses c-Myc through induction of the tumor suppressor miR-145. *Proceedings of the National Academy of Sciences*, *106*(9), 3207-3212.

Suh, S. O., Chen, Y., Zaman, M. S., Hirata, H., Yamamura, S., Shahryari, V., ... & Dahiya, R. (2011). MicroRNA-145 is regulated by DNA methylation and p53 gene mutation in prostate cancer. *Carcinogenesis*, *32*(5), 772-778.

Saleh, A. D., Savage, J. E., Cao, L., Soule, B. P., Ly, D., DeGraff, W., ... & Simone, N. L. (2011). Cellular stress induced alterations in microRNA let-7a and let-7b expression are dependent on p53. *PloS one*, *6*(10), e24429.

Suzuki, H. I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., & Miyazono, K. (2009). Modulation of microRNA processing by p53. *Nature*, *460*(7254), 529-533.

Ugalde, A. P., Ramsay, A. J., De La Rosa, J., Varela, I., Mariño, G., Cadiñanos, J., ... & López-Otín, C. (2011). Aging and chronic DNA damage response activate a regulatory pathway involving miR-29 and p53. *The EMBO journal*, *30*(11), 2219-2232.

van Jaarsveld, M. T., Wouters, M. D., Boersma, A. W., Smid, M., van IJcken, W. F., Mathijssen, R. H., ... & Pothof, J. (2014). DNA damage responsive microRNAs misexpressed in human cancer modulate therapy sensitivity. *Molecular oncology*, *8*(3), 458-468.

Van der Auwera, I., Limame, R., Van Dam, P., Vermeulen, P. B., Dirix, L. Y., & Van Laere, S. J. (2010). Integrated miRNA and mRNA expression profiling of the inflammatory breast cancer subtype. *British journal of cancer*, *103*(4), 532-541.

Wan, G., Zhang, X., Langley, R. R., Liu, Y., Hu, X., Han, C., ... & Lu, X. (2013). DNA-damage-induced nuclear export of precursor microRNAs is regulated by the ATM-AKT pathway. *Cell reports*, *3*(6), 2100-2112.

Wagner-Ecker, M., Schwager, C., Wirkner, U., Abdollahi, A., & Huber, P. E. (2010). MicroRNA expression after ionizing radiation in human endothelial cells. *Radiation oncology*, *5*(1), 1-10.

Xiao, J., Lin, H., Luo, X., Luo, X., & Wang, Z. (2011). miR-605 joins p53 network to form a p53: miR-605: Mdm2 positive feedback loop in response to stress. *The EMBO journal*, *30*(3), 524-532.

Yamakuchi, M., Ferlito, M., & Lowenstein, C. J. (2008). miR-34a repression of SIRT1 regulates apoptosis. *Proceedings of the National Academy of Sciences*, *105*(36), 13421-13426.

Yan, H. L., Xue, G., Mei, Q., Wang, Y. Z., Ding, F. X., Liu, M. F., ... & Sun, S. H. (2009). Repression of the miR-17-92 cluster by p53 has an important function in hypoxia-induced apoptosis. *The EMBO journal*, *28*(18), 2719-2732.

Zhang, X., Wan, G., Mlotshwa, S., Vance, V., Berger, F. G., Chen, H., & Lu, X. (2010). Oncogenic Wip1 phosphatase is inhibited by miR-16 in the DNA damage signaling pathway. *Cancer research*, *70*(18), 7176-7186.

Zhang, X., Wan, G., Berger, F. G., He, X., & Lu, X. (2011). The ATM kinase induces microRNA biogenesis in the DNA damage response. *Molecular cell*, *41*(4), 371-383.

Relationship: 2610: Increase,miRNA levels leads to Decrease,SIRT1(sirtuin 1) levels

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
human and other cells in culture	human and other cells in culture	High	NCBI
mice	Mus sp.	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Female	High

The key event relationship was observed in humans,animals irrespective of gender and life stage specificity.

Key Event Relationship Description

Upstream event: Increased, miRNA

Downstream event: SIRT1, Reduced

The described Key Event Relationship (KER) outlines a sequence of events involving microRNA (miRNA) regulation and its downstream consequences. The upstream event is characterized by "Increased miRNA," indicating an elevation in the levels of miRNA molecules within the cell. miRNAs are small non-coding RNA molecules that play a role in post-transcriptional gene regulation by targeting messenger RNAs (mRNAs) for degradation or translational repression.

The downstream event in this KER is "SIRT1 Reduced," which suggests a reduction in the levels or activity of the protein SIRT1. SIRT1 is a member of the sirtuin family of proteins involved in various cellular processes, including DNA repair, metabolism, and stress response. The miRNAs, as part of their regulatory function, can target and inhibit the expression of genes, including SIRT1, leading to a decrease in its abundance or function.

This KER underscores the intricate regulatory mechanisms within cells, where miRNAs can modulate the expression of target genes and consequently influence cellular processes. Understanding these interactions contributes to a comprehensive grasp of how molecular events are interconnected and how changes in miRNA levels can impact downstream protein expression and cellular responses.

Evidence Supporting this KER

Biological Plausibility

There are several signaling pathways that establishing the role of increased miRNA expression in downregulating SIRT 1 gene few of which are listed as follows;

Butyrate–miR-22–SIRT1

Butyrate, a short-chain fatty acid, is produced by the intestinal microbiome via anaerobic fermentation and is subsequently absorbed by the hepatocytes (Besten et al., 2013). Butyrate has been demonstrated to cause apoptosis and reduce carcinogenesis in a variety of cancers (Tailor et al.,2014; Rahmani et al.,2002). Although butyrate has been shown to suppress SIRT1 gene expression in various cancers, this has yet to be proven in hepatocellular carcinoma (HCC) (Iglesias et al., 2007). In HCC, miR-22 was found to be downregulated, and its low levels aided carcinogenesis (Zhang et al.,2010). The Huh7 cells' in vitro proliferation was decreased by miR-22 expression, which activated apoptosis. In Huh7 cells, on the other hand, SIRT1 expression was high, which enhanced the expression of antioxidants such superoxide dismutase (SOD), allowing cell growth to continue (Chen et al.,2012). Butyrate upregulated miR-22 in Huh7 cells, which binds directly to the 3′UTR region of SIRT1 and suppresses its expression; this decreased SOD function and increased ROS generation, increasing caspase 3 and cytochrome c activity, and encouraging apoptosis (Pant et al .,2017).Furthermore, by downregulating SIRT1, miR22 increased PTEN and gsk-3 expression and downregulated β catenin and p-akt expression and thus may promote apoptosis and decrease HCC proliferation (Pant et al .,2017).

Notch3–SIRT1–LSD1–SOX2 Signaling Pathway

Lysine demethylase 1 (LSD1) is an epigenetic regulator responsible for demethylating various histones and controls the pluripotency of stem cells (Adamo et al.,2011; Whyte et al., 2012; Thambyrajah et al., 2016).

In comparison to normal hepatic parenchyma, HCC cells overexpress LSD1. Furthermore, LSD1 is highly expressed in LCSCs, where it regulates SOX2 gene transcription, promotes self-renewal and carcinogenesis, and is linked to a poor patient prognosis (Liu et al.,2018). LSD1 demethylated the SOX2 promoter, increasing its expression and improving LCSC stemness in a similar way to SIRT1 via DNMT3A. Acetylation suppresses LSD1's enzymatic activity and promotes its breakdown via UPP. SIRT1 enhanced the stability of LSD1 by deacetylating it . Notch signalling is essential for cell survival and proliferation (Bouras et al., 2008). Notch receptors are overexpressed in most HCCs, and their ligand expression has been linked to aggressive tumour characteristics (Tschaharganeh et al., 2013). The Wnt/-catenin pathway was activated by Notch, which enhanced HCC growth and metastasis (Wang et al.,2016; Wu et al .,2017). Notch signalling has also been demonstrated to increase CSC self-renewal. SIRT1 expression was enhanced by Notch3 signalling, which also promoted LDS1 deacetylation and activated LSD1 which consequently promotes LCSC self-renewal. The Notch3- dependent pathway was crucial for LCSC self-renewal and in vivo tumor dissemination.

MiR-133b is a tumour suppressor that has been found to be significantly decreased in a variety of malignancies (Hu et al.,2010). When compared to paired neighbouring normal tissue, miR-133b expression was shown to be lower in the majority of HCC samples (El-Halawany et al.,2015). Furthermore, overexpression of miR-133b in HepG2 cells inhibited HCC cell growth and invasion while promoting apoptosis (Tian et al.,2016). In nude mice with orthotopic HepG2 cell tumours, increase of miR-133b also reduced tumour growth. In human HCC cells, miR-133b targets SIRT1 and has an adverse relationship with it. Increased miR-133b expression significantly reduced SIRT1 mRNA and protein expression. Overall, miR-133b appears to have an anti-cancer effect in HCC cells through suppressing SIRT1 expression.

In Huh7 and HepG2 cell lines, inhibiting the SIRT1–SREBP pathway lowered proliferation and DNA synthesis, reduced lipid anabolism, and repressed tumorigenesis (Zhang et al.,2014).

MiR-486 inhibits HCC invasion and tumorigenicity by directly targeting and suppressing SIRT1 expression. This reduced the tumorigenic and chemo-resistant features of LCSCs and inhibited HCC invasion and tumorigenicity (Yan et al.,2019).

The miR-29 family inhibits tumour growth by targeting Mcl-1 and Bcl-2. MiR-29c inhibits hepatocytic SIRT1 and so has tumor-suppressing properties. Ectopic miR-29c expression suppressed cell growth by lowering SIRT1 expression. In hepatocytes, miR-29c directly targets and inhibits SIRT1 mRNA translation (Bae et al., 2014).

SIRT1 was downregulated at the mRNA and protein levels when miR-138 expression was increased. MiR-138 binds to the SIRT1 gene's 3′UTR unique complimentary site and inhibits SIRT1 expression directly, preventing HCC proliferation, migration, and invasion (Luo et al.,2017).When compared to the normal hepatic cell line L02, SIRT1 is overexpressed, while miR-138 levels are lowered in HepG2, SMMC7721, Bel7404, and HCCM3

Empirical Evidence

- In Jiang et al's study, the cellular function and molecular mechanism of miR2045p in hepatocellular cancer were investigated (HCC)(Jiang et al.,2016). Real-time reverse transcription polymerase chain reaction was used to analyze at SIRT1 mRNA and miR2045p. Western blotting was used to determine SIRT1 protein levels. To confirm colony formation, a cell proliferation assay was used. The invasion experiment was carried out using a

transwell technique.

-Overexpression of miR2045p in human HCC cell lines (BEL7405 and QGY7701) resulted in cell death, increased apoptosis, and increased drug sensitivity. SIRT1 levels were inversely associated to miR2045p levels and were overexpressed in human HCC tissues. These findings suggest that miR204 5p and SIRT1 may play a role in the progression of HCC.

-Introducing miR20405p into BEL7405 and QGY7701 cells lowered SIRT1 mRNA and protein expression. These findings imply that in HCC cells, SIRT1 is a direct target of miR2045.

-It was also found that SIRT1 was negatively related to miR-204-5p expression in HCC tissues.

- Using real-time PCR and western blot assays, Luo et al discovered that miR-138 expression was low while sirtuin type 1 (Sirt1) mRNA expression was high in hepatocellular carcinoma tissues and cell lines, and that miR-138 functions were achieved by targeting Sirt1 using luciferase reporter gene vector and RNA immunoprecipitation assays(Luo et al .,2017).

-Using CCK-8 and BrdU tests, overexpression of miR-138 reduced Sirt1 expression and hindered cell growth.

-Forced production of Sirt1 in cells could partially reverse the inhibitory impact of miR-138. The study findings demonstrated that miR-138 plays a critical role in the regulation of hepatocellular carcinoma cell development via the miR-138/Sirt1 axis, and that miR-138 could be an important future target for hepatocellular carcinoma clinical therapy.

- Shen et al showed that downregulation of miR-199b is associated with distant metastasis in colorectal cancer via activation of SIRT1 and inhibition of CREB/KISS1 signalling(Shen et al., 2016).
- MiR-199b expression levels in six CRC cell lines in comparison to NCM460, a normal colorectal cell line. qRT-PCR detection of relative miR-199b expression after transfection with miR-199b mimics and its negative control (NC).After overexpression of miR-199b, the invasive capacity of SW480 and SW620 cells was examined using the Transwell test.The migratory ability of SW620 and SW480 cells was assessed using a wound healing test. Quantification was done by measuring the wound's smallest clearance distance.After overexpression of miR-199b, Western blot examination revealed the expression levels of invasion-related molecules MMP2 and MMP9, the epithelial-mesenchymal transition (EMT) marker E-cadherin, and Vimentin.The expression of miR-199b was shown to be adversely linked with SIRT1 in CRC specimens in the study. The effects of SIRT1 knockdown on biological behaviour were similar to those of miR-199b overexpression.Furthermore, Human Tumor Metastasis PCR Array revealed that KISS1 was one of SIRT1's downstream targets. SIRT1 silencing increased the acetylation of the transcription factor CREB, which increased KISS1 expression. The latter was activated further by attaching to the KISS1 promoter, which resulted in transcription.

-According to the findings, miR-199b modulates the SIRT1/CREB/KISS1 signalling pathway and could be used as a prognostic marker or a novel treatment target for CRC patients.

- A study by Tian et al found that MicroRNA-133b targets Sirt1 and suppresses hepatocellular carcinoma cell progression(Tian et al., 2016).qRT-PCR was used to examine miR-133b expression levels in 37 cases of primary hepatoma carcinoma tissues and their surrounding normal equivalents.Sirt1 is a direct target of miR-133b, and its expression in HCC is inversely linked with that of miR-133b.After transfecting the miR-133b expression plasmid into HepG2 and SMMC7721 cells, Sirt1 expression was elevated, indicating that the effect of miR-133b on HCC cells is dependent on Sirt1 repression. In comparison to the control group of HCC cells pretreated with miR-133b overexpression vector, transfection of the Sirt1 overexpression vector restored Sirt1 protein levels.Restoring Sirt1 partially reverses the effect of up-regulation of miR-113b on HCC cell proliferation, invasion, and apoptosis in vitro.
- In liver cancer, Yan et al discovered that MicroRNA 486 5p acts as a tumour suppressor of proliferation and cancer stem-like cell characteristics by targeting Sirt1(Yan et al.,2019).

-The qPCR analysis revealed that miR 486 decreased Sirt1 expression in HepG2 and PLC cells. Transfection of a miR 486 inhibitor, on the other hand, enhanced Sirt1 mRNA levels.

-The study focused primarily on Sirt1 as a miR 486 target gene. A luciferase assay in 293T cells was used to further investigate the connection between miR 486 and Sirt1 3'UTR. The Dual Luciferase Assay System was used to detect luciferase activity in cells co transfected with miR 486 mimics or NC using the pGL3-Sirt1-3'UTR vector or pGL3 basic vector. Sirt1-3'UTR reporter firefly luciferase activity was considerably reduced by co-transfection with miR 486 mimics, but not by the pGL3 reporter. Western blot analysis revealed that in the miR 486 overexpression group, Sirt1 was downregulated. Furthermore, IHC labelling revealed that Sirt1 expression was downregulated in tumour tissues produced from miR 486 overexpression cells.

- Zhang et al reported that MicroRNA-22 functions as a tumor suppressor by targeting SIRT1 in renal cell carcinoma (Zhang et al., 2016).

  -Real-time quantitative RT-PCR was used to determine the miR-22 expression pattern (qRT-PCR).

-Quantitative real-time PCR results revealed that miR-22 was considerably downregulated in RCC samples compared to non-cancerous tissues, and this was related with tumour stage and lymph node metastasis.In vitro, forced overexpression of miR-22 decreased proliferation, migration, and invasion, as well as promoted cell death, and suppressed tumour growth in vivo, according to a functional investigation.In addition, a luciferase reporter test showed SIRT1 as a direct target of miR-22.

-MiR-22 overexpression suppressed epithelial-mesenchymal transition via activating p53 and its downstream targets p21 and PUMA, as well as the apoptosis markers CASP3 and PARP (EMT). These findings revealed that miR-22 acted as a tumour suppressor in RCC and inhibited RCC growth and metastasis by directly targeting SIRT1, implying an unique therapeutic role for RCC treatment.

- Astragalus Polysaccharides(APS) inhibits Tumorigenesis and Lipid Metabolism Through the miR-138-5p/SIRT1/SREBP1 Pathway in Prostate Cancer (Guo et al.,2020).APS was discovered to suppress the proliferation and invasion of PCa cells in vitro and in vivo in a dose- and time-dependent manner in the current investigation.Under APS exposure, SIRT1 expression was significantly decreased, according to microarray results.

-SIRT1 knockdown enhances AMPK/SREBP1 signalling and its related target genes considerably.

- Bae et al reported that MicroRNA-29c functions as a tumor suppressor by direct targeting oncogenic SIRT1 in hepatocellular carcinoma(Bae et al., 2014).

  -In this study, SIRT1 expression in a subset of HCCs was performed.

-The study found that overexpression of SIRT1 increased HCC cell proliferation by inactivating p21Cip1, p27Kip1, and p15INK4B, as well as activating CDK2, CDK6, cyclin D3, and cyclin D1.

-In addition, miRNA expression profiling was used to explore for deregulated miRNAs in HCC, and five miRNAs targeting SIRT1 were shown to be highly downregulated in the disease.

-The liver cancer cell lines SNU-182, HepG2, and Hep3B grew slower when SIRT1 was knocked off.

-miR-29c inhibits SIRT1 mRNA translation in the liver, acting as a tumour suppressor. In the rescue experiment, SIRT1-expressing plasmid (pcDNA3.1- SIRT1-His) or Mock (empty vector, pcDNA3.1-His) were introduced into SNU-182 cell lines in the presence or absence of ectopic miR-29c expression. MiR-29c mimic (miR-29c) and 30 UTR-negative SIRT1 expression plasmids were transfected into cells (pcDNA3.1-SIRT1-His).

-After 48 h of transfection, endogenous SIRT1, not ectopically expressed SIRT1 (SIRT1-His), was suppressed by ectopic miR-29c transfection as found in Western blot analysis .

-Anti-growth effect of ectopic miR-29c was attenuated by co-transfection of SIRT1-expressing plasmid

- In a study by Zhou et al,the miR-34a overexpression vector or scramble control was transfected into human Hep3B and Huh7 cell lines(Zhou et al 2017).

-To determine the impact of miR-34a expression on HCC cell invasion and migration, researchers performed Transwell assays, Matrigel, and wound healing experiments.

-Using quantitative reverse transcription polymerase chain reaction, the expression of miR-34a and the mRNA expression of other related proteins was discovered, and protein levels were determined using western blot analysis.

-MiR-34a expression was considerably downregulated in Hep3B and Huh7 cells compared to the control, however this was reversed by transfection with exogenous miR-34a.

-Overexpression of miR-34a increased the expression of sirtuin 1 while decreasing the amount of acetylate-p53.

- Study by Fu et al concluded that MiRNA-200a induce cell apoptosis in renal cell carcinoma by directly targeting SIRT1(Fu et al.,2018).The expression of miR-200a was found to be considerably lower in renal cell carcinoma (RCC) specimens and RCC cell lines in the study. In RCC cell lines, restoring miR-200a decreased cell growth, halted cell cycle progression, and accelerated cell death.

-Sirtuin 1 (SIRT1) was identified as one of the downregulated proteins after miR-200a overexpression in 786-O cells using qRT-PCR array technique. SIRT1 was confirmed as a direct target of miR-200a after a second assay using a luciferase reporter system. Furthermore, knocking down SIRT1 with siRNA could partially mimic the effects of miR-200a overexpression.

-Overexpression of truncated SIRT1 (without an endogenous 30 -UTR) on 786-O cells, on the other hand, may rescue the effect of miR-200a overexpression, suggesting that the SIRT1 30 -UTR is preferentially targeted by miR-200a.

-These findings add to the growing body of data for the miR-200a's tumor-suppressive effect in RCC, as well as establishing a novel regulatory mechanism that could play a role in SIRT1 overexpression in RCC.

- Study by Lian et al performed Quantitative real-time PCR analysis to detect the expression of microRNA-128 (miR-128) in tissues from patients with CRC and CRC cell lines. The effect of miR-128 on Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), an anti-tumor medication, caused cytotoxicity against CRC cell lines was assessed using MTT assays (Lian et al.,2018).

-Flow cytometry was used to detect the distribution of death receptor 5 (DR5) and the formation of reactive oxygen species (ROS).Western blot, flow cytometry, and luciferase reporter assays were used to look into the mechanism and pathway of miR-128-induced apoptosis in TRAIL-treated CRC cells.

-MiR-128 expression was found to be downregulated in CRC tumour tissues as well as CRC cell lines in vitro. MiR-128 overexpression made CRC cells more susceptible to TRAIL-induced cytotoxicity through triggering apoptosis. MiR-128 directly targeted sirtuin 1 (SIRT1) in CRC cells, according to bioinformatics, western blot analysis, and luciferase reporter tests. Overexpression of miR128 reduced SIRT1 expression, which increased ROS generation in TRAIL-treated CRC cells.

This increase of ROS subsequently induced DR5 expression, and thus increased TRAIL-induced apoptosis in CRC cells.

- A study by Guan et al confirmed SIRT1 as a direct target of miR-30a by measuring SIRT1 expression in lung cancer cells following overexpression or knockdown of miR-30a and using a luciferase assay (Guan et al.,2017).

-In vitro and in vivo, miR-30a decreased lung cancer cell proliferation, invasion, and increased apoptosis through inhibiting SIRT1.This research discovered a new regulatory axis in which miR-30a and SIRT1 control lung cancer cell proliferation, invasion, and apoptosis, as well as lung carcinogenesis. After overexpression or knockdown of miR-30a, two human lung cancer cell lines (A549 and H1975) were employed to confirm the direct link between miR-30a and SIRT1.Cellular levels of miR-30a were greatly elevated in A549 and H1975 cells transfected with miR-30a mimics and dramatically decreased in cells transfected with miR-30a inhibitor, as expected.As a result, overexpression of miR-30a dramatically decreased SIRT1 protein expression in A549 and H1975 cells, whereas the miR-30a inhibitor considerably boosted SIRT1 protein levels in lung cancer cells. The studies were repeated, and the expression of SIRT1 mRNA after transfection was evaluated to see how much miR-30a influenced SIRT1 expression. The level of SIRT1 mRNA was unaffected by overexpression or knockdown of miR-30a.

- In cells co-transfected with luciferase reporter plasmid and miR-30a mimics, luciferase activity was drastically reduced. Then, to delete the expected miR-30a binding site, point mutations were induced into the SIRT1 3′-UTR binding region. The overexpression or knockdown of miR30a has no effect on this mutant luciferase reporter. This finding revealed that the binding sites play a significant role in miR-30a-SIRT1 mRNA interaction.

- Yang et al suggested Down-Regulation of miR-221 and miR-222 Restrain Prostate Cancer Cell Proliferation and Migration That Is Partly Mediated by Activation of SIRT1(Yang et al.,2014).

- When compared to LNCap cells, PC-3 cells had higher levels of miR-221 and miR-222 expression. The proliferation and migration rates of PC-3 cells dropped after miR-221 or miR-222 expression was inhibited, but the apoptosis rate rose. Furthermore, after cells were transfected with miR-221 or miR-222 inhibitors, SIRT1 protein was up-regulated. In comparison to the controls, cells transfected with siSIRT1 demonstrated greater migration and a lower rate of apoptosis, but no significant influence on cell proliferation. Although there was a negative connection between miR-221 and SIRT1, no direct target relationship was discovered. These findings show that miR-221 and miR-222 are abundant in PC-3 cells. In prostate cancer cells, inhibiting these proteins reduces cell proliferation and migration while increasing apoptosis. Upregulation of SIRT1 may be responsible for these effects.

Uncertainties and Inconsistencies

Not specific.

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human tissues	qRT-PCR,Western blotting,Luciferase reporter assay Micro-array	Yes	Strong	Yes	Yes	Yes
Human cell lines	Micro-array, qRT-PCR,Western blotting, Luciferase reporter assay Micro-array	Yes	Strong	Yes	Yes	Yes
Mouse(A1)	qRT-PCR,Western blotting,Luciferase reporter assay,ELISA,cell culture	Yes	Moderate	Yes	Yes	Yes

Response-response relationship

No specific pattern of response response relationship was observed.

Time-scale

In study by Jiang et al, it was shown that miR-204-5p targeting SIRT1 regulates hepatocellular carcinoma progression. The results were noted within 48 hours during the experiment (Jiang et al., 2016).

Known modulating factors

SIRT1 has been found to have a number of endogenous and external regulators. SIRT1 activity is naturally inhibited by the protein encoded by deleted in breast cancer 1 (DBC1). DBC1 forms a tumour suppressor complex with SIRT1, but knocking out DBC1 increases SIRT1 activity, promoting tumorigenesis. The small molecule resveratrol was the first to be discovered to regulate SIRT1 activity and extend life span. After the effect of resveratrol upon SIRT1 was characterized, high throughput screening was used to find other small molecule activators of SIRT.

Known Feedforward/Feedback loops influencing this KER

p53-miR-34a–SIRT1 Signaling Pathway

0404 is a DNA-damaging substance that has no cytotoxic effects on human hepatocytes that aren't malignant. In an in vivo HepG2 HCC model, 0404 caused apoptosis and inhibited proliferation. P53 WT HepG2 cells, on the other hand, were more susceptible to 0404 than p53 mutant Huh7 cell lines (Xia et al.,2017). P53 influences the expression of several miRs. As a result, a large number of miRs target the 3′UTR region of the p53 mRNA. As a result, p53 and miRs could establish a feedback loop (Zhang et al., 2015). The miR-34 family has been identified as the most common p53-induced miRs and is commonly suppressed in diverse malignancies (Xiao et al.,2014, Lou et al.,2015). In HCC cells, miR-34a increased p53 transcription and acetylation while also inducing apoptosis. 0404 enhanced p53 and miR-34a expression, elevated acetylated p53, and downregulated SIRT1 protein expression in HepG2 but not Huh7 cell lines, inhibiting HCC growth (Xiao et al.,2014).

In HCC, the lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is highly expressed, promoting development and invasion. MALAT1 stimulates the formation of HCC CSCs by activating the mechanistic target of rapamycin (mTOR) signalling pathway (Malakar et al.,2017;Yuan et al 2016). MiR-204, in contrast to MALAT1, promotes apoptosis by activating p53 and suppressing Bcl-2, an anti-apoptotic protein (Ryan et al.,2012). Cancer stemness and EMT were also suppressed by miR-204, which increased chemosensitivity (Ryan et al.,2012 ;Sacconi et al.,2012). MALAT1 expression, on the other hand, was negatively linked with miR-204 levels. MALAT1 binds to miR-204 and inhibits its expression by binding directly to it (Hou et al., 2017). SIRT1 appears to play a key role in the interaction between MALAT1 and miR-204. SIRT1 is recognised to play a role in HCC EMT, migration, and invasion. MiR-204 specifically targets SIRT1 and silences it (Hou et al.,2017).

However, because SIRT1 and MALAT1 bind to the same miR-204 region, MALAT1 may compete with SIRT1 for miR204 binding, reducing miR-204-induced SIRT1 suppression. Overall, MALAT1 inhibited miR-204 activity, resulting in an elevation in SIRT1, which encouraged HCC migration and invasion (Hou et al., 2017). MALAT1 inhibition reduced the aggressiveness of HCC, making it a possible therapeutic target (Hou et al., 2017).

References

Adamo, A., Barrero, M. J., & Izpisua Belmote, J. C. (2011). LSD1 and pluripotency: a new player in the network. *Cell Cycle*, *10*(19), 3215-3216.

Bae, H. J., Noh, J. H., Kim, J. K., Eun, J. W., Jung, K. H., Kim, M. G., ... & Nam, S. W. (2014). MicroRNA-29c functions as a tumor suppressor by direct targeting oncogenic SIRT1 in hepatocellular carcinoma. *Oncogene*, *33*(20), 2557-2567.

Bai, X. Z., Zhang, J. L., Liu, Y., Zhang, W., Li, X. Q., Wang, K. J., ... & Hu, D. H. (2018). MicroRNA-138 aggravates inflammatory responses of macrophages by targeting SIRT1 and regulating the NF-κB and AKT pathways. *Cellular Physiology and Biochemistry*, *49*(2), 489-500.

den Besten, G. K. van Eunen AK Groen K. Venema DJ Reijngoud, and Bakker BM 2013. *The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. j. Lipid Res*, *54*, 2325-2340.

Bouras, T., Pal, B., Vaillant, F., Harburg, G., Asselin-Labat, M. L., Oakes, S. R., ... & Visvader, J. E. (2008). Notch signaling regulates mammary stem cell function and luminal cell-fate commitment. *Cell stem cell*, *3*(4), 429-441.

Chen, H. C., Jeng, Y. M., Yuan, R. H., Hsu, H. C., & Chen, Y. L. (2012). SIRT1 promotes tumorigenesis and resistance to chemotherapy in hepatocellular carcinoma and its expression predicts poor prognosis. *Annals of surgical oncology*, *19*(6), 2011-2019.

El-Halawany, M. S., Ismail, H. M., Zeeneldin, A. A., Elfiky, A., Tantawy, M., Kobaisi, M. H., ... & Abdel Wahab, A. H. A. (2015). Investigating the pretreatment miRNA expression patterns of advanced hepatocellular carcinoma patients in association with response to TACE treatment. *BioMed research international*, *2015*.

Fu, H., Song, W., Chen, X., Guo, T., Duan, B., Wang, X., ... & Zhang, C. (2018). MiRNA-200a induce cell apoptosis in renal cell carcinoma by directly targeting SIRT1. *Molecular and cellular biochemistry*, *437*(1), 143-152.

Guo, S., Ma, B., Jiang, X., Li, X., & Jia, Y. (2020). Astragalus polysaccharides inhibits tumorigenesis and lipid metabolism through miR-138-5p/SIRT1/SREBP1 pathway in prostate cancer. *Frontiers in Pharmacology*, *11*, 598.

Guan, Y., Rao, Z., & Chen, C. (2018). miR-30a suppresses lung cancer progression by targeting SIRT1. *Oncotarget*, *9*(4), 4924.

Hou, Z., Xu, X., Zhou, L., Fu, X., Tao, S., Zhou, J., ... & Liu, S. (2017). The long non-coding RNA MALAT1 promotes the migration and invasion of hepatocellular carcinoma by sponging miR-204 and releasing SIRT1. *Tumor Biology*, *39*(7), 1010428317718135.

Hu, G., Chen, D., Li, X., Yang, K., Wang, H., & Wu, W. (2010). miR-133b regulates the MET proto-oncogene and inhibits the growth of colorectal cancer cells in vitro and in vivo. *Cancer biology & therapy*, *10*(2), 190-197.

Jiang, G., Wen, L., Zheng, H., Jian, Z., & Deng, W. (2016). miR-204-5p targeting SIRT1 regulates hepatocellular carcinoma progression. *Cell biochemistry and function*, *34*(7), 505-510.

Lian, B., Yang, D., Liu, Y., Shi, G., Li, J., Yan, X., ... & Zhang, R. (2018). miR-128 targets the SIRT1/ROS/DR5 pathway to sensitize colorectal cancer to TRAIL-induced apoptosis. *Cellular Physiology and Biochemistry*, *49*(6), 2151-2162.

Liu, C., Liu, L., Chen, X., Cheng, J., Zhang, H., Zhang, C., ... & Qian, C. (2018). LSD1 stimulates cancer-Associated fibroblasts to drive Notch3-Dependent self-Renewal of liver cancer stem–like cells. *Cancer research*, *78*(4), 938-949.

Lou, G., Liu, Y., Wu, S., Xue, J., Yang, F., Fu, H., ... & Chen, Z. (2015). The p53/miR-34a/SIRT1 positive feedback loop in quercetin-induced apoptosis. *Cellular Physiology and Biochemistry*, *35*(6), 2192-2202.

Luo, J., Chen, P., Xie, W., & Wu, F. (2017). MicroRNA-138 inhibits cell proliferation in hepatocellular carcinoma by targeting Sirt1. *Oncology reports*, *38*(2), 1067-1074.

Malakar, P., Shilo, A., Mogilevsky, A., Stein, I., Pikarsky, E., Nevo, Y., ... & Karni, R. (2017). Long noncoding RNA MALAT1 promotes hepatocellular carcinoma development by SRSF1 upregulation and mTOR activation. *Cancer research*, *77*(5), 1155-1167.

Pant, K., Yadav, A. K., Gupta, P., Islam, R., Saraya, A., & Venugopal, S. K. (2017). Butyrate induces ROS-mediated apoptosis by modulating miR-22/SIRT-1 pathway in hepatic cancer cells. *Redox Biology*, *12*, 340-349.

Rada-Iglesias, A., Enroth, S., Ameur, A., Koch, C. M., Clelland, G. K., Respuela-Alonso, P., ... & Wadelius, C. (2007). Butyrate mediates decrease of histone acetylation centered on transcription start sites and down-regulation of associated genes. *Genome research*, *17*(6), 708-719.

Rahmani, M., Dai, Y., & Grant, S. (2002). The histone deacetylase inhibitor sodium butyrate interacts synergistically with phorbol myristate acetate (PMA) to induce mitochondrial damage and apoptosis in human myeloid leukemia cells through a tumor necrosis factor-α-mediated process. *Experimental cell research*, *277*(1), 31-47.

Ryan, J., Tivnan, A., Fay, J., Bryan, K., Meehan, M., Creevey, L., ... & Stallings, R. L. (2012). MicroRNA-204 increases sensitivity of neuroblastoma cells to cisplatin and is associated with a favourable clinical outcome. *British journal of cancer*, *107*(6), 967-976.

Sacconi, A., Biagioni, F., Canu, V., Mori, F., Di Benedetto, A., Lorenzon, L., ... & Blandino, G. (2012). miR-204 targets Bcl-2 expression and enhances responsiveness of gastric cancer. *Cell death & disease*, *3*(11), e423-e423.

Shen, Z. L., Wang, B., Jiang, K. W., Ye, C. X., Cheng, C., Yan, Y. C., ... & Wang, S. (2016). Downregulation of miR-199b is associated with distant metastasis in colorectal cancer via activation of SIRT1 and inhibition of CREB/KISS1 signaling. *Oncotarget*, *7*(23), 35092.

Tailor, D., Hahm, E. R., Kale, R. K., Singh, S. V., & Singh, R. P. (2014). Sodium butyrate induces DRP1-mediated mitochondrial fusion and apoptosis in human colorectal cancer cells. *Mitochondrion*, *16*, 55-64.

Tian, Z., Jiang, H., Liu, Y., Huang, Y., Xiong, X., Wu, H., & Dai, X. (2016). MicroRNA-133b inhibits hepatocellular carcinoma cell progression by targeting Sirt1. *Experimental cell research*, *343*(2), 135-147.

Thambyrajah, R., Mazan, M., Patel, R., Moignard, V., Stefanska, M., Marinopoulou, E., ... & Lacaud, G. (2016). GFI1 proteins orchestrate the emergence of haematopoietic stem cells through recruitment of LSD1. *Nature cell biology*, *18*(1), 21-32.

Tschaharganeh, D. F., Chen, X., Latzko, P., Malz, M., Gaida, M. M., Felix, K., ... & Breuhahn, K. (2013). Yes-associated protein up-regulates Jagged-1 and activates the Notch pathway in human hepatocellular carcinoma. *Gastroenterology*, *144*(7), 1530-1542.

Wang, R., Sun, Q., Wang, P., Liu, M., Xiong, S., Luo, J., ... & Cheng, B. (2016). Notch and Wnt/β-catenin signaling pathway play important roles in activating liver cancer stem cells. *Oncotarget*, *7*(5), 5754.

Whyte, W. A., Bilodeau, S., Orlando, D. A., Hoke, H. A., Frampton, G. M., Foster, C. T., ... & Young, R. A. (2012). Enhancer decommissioning by LSD1 during embryonic stem cell differentiation. *Nature*, *482*(7384), 221-225.

Wu, C. X., Xu, A., Zhang, C. C., Olson, P., Chen, L., Lee, T. K., ... & Wang, X. Q. (2017). Notch inhibitor PF-03084014 inhibits hepatocellular carcinoma growth and metastasis via suppression of cancer stemness due to reduced activation of Notch1–Stat3. *Molecular cancer therapeutics*, *16*(8), 1531-1543.

Xiao, Z., Li, C. H., Chan, S. L., Xu, F., Feng, L., Wang, Y., ... & Chen, Y. (2014). A small-molecule modulator of the tumor-suppressor miR34a inhibits the growth of hepatocellular carcinoma. *Cancer research*, *74*(21), 6236-6247.

Lou, G., Liu, Y., Wu, S., Xue, J., Yang, F., Fu, H., ... & Chen, Z. (2015). The p53/miR-34a/SIRT1 positive feedback loop in quercetin-induced apoptosis. *Cellular Physiology and Biochemistry*, *35*(6), 2192-2202.

Yan, X., Liu, X., Wang, Z., Cheng, Q., Ji, G., Yang, H., ... & Pei, X. (2019). MicroRNA4865p functions as a tumor suppressor of proliferation and cancer stemlike cell properties by targeting Sirt1 in liver cancer. *Oncology reports*, *41*(3), 1938-1948.

Yang, X., Yang, Y., Gan, R., Zhao, L., Li, W., Zhou, H., ... & Meng, Q. H. (2014). Down-regulation of mir-221 and mir-222 restrain prostate cancer cell proliferation and migration that is partly mediated by activation of SIRT1. *PloS one*, *9*(6), e98833.

Yuan, P., Cao, W., Zang, Q., Li, G., Guo, X., & Fan, J. (2016). The HIF-2α-MALAT1-miR-216b axis regulates multi-drug resistance of hepatocellular carcinoma cells via modulating autophagy. *Biochemical and biophysical research communications*, *478*(3), 1067-1073.

Zhang, H., Feng, Z., Huang, R., Xia, Z., Xiang, G., & Zhang, J. (2014). MicroRNA-449 suppresses proliferation of hepatoma cell lines through blockade lipid metabolic pathway related to SIRT1. *International journal of oncology*, *45*(5), 2143-2152.

Zhang, J., Yang, Y., Yang, T., Liu, Y., Li, A., Fu, S., ... & Zhou, W. (2010). microRNA-22, downregulated in hepatocellular carcinoma and correlated with prognosis, suppresses cell proliferation and tumourigenicity. *British journal of cancer*, *103*(8), 1215-1220.

Zhang, S., Zhang, D., Yi, C., Wang, Y., Wang, H., & Wang, J. (2016). MicroRNA-22 functions as a tumor suppressor by targeting SIRT1 in renal cell carcinoma. *Oncology reports*, *35*(1), 559-567.

Zhang, Y., Dai, J., Deng, H., Wan, H., Liu, M., Wang, J., ... & Tang, H. (2015). miR-1228 promotes the proliferation and metastasis of hepatoma cells through a p53 forward feedback loop. *British journal of cancer*, *112*(2), 365-374.

Zhou, J., Zhou, W., Kong, F., Xiao, X., Kuang, H., & Zhu, Y. (2017). microRNA34a overexpression inhibits cell migration and invasion via regulating SIRT1 in hepatocellular carcinoma Corrigendum in/10.3892/ol.2019.11048. *Oncology letters*, *14*(6), 6950-6954.

Relationship: 2611: Decrease,SIRT1(sirtuin 1) levels leads to Increased activation, Nuclear factor kappa B (NF-kB)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
human	Homo sapiens	Moderate	NCBI
mice	Mus sp.	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified

Sex Applicability

Sex	Evidence
Female	High

The KER has been noted in human and animal cell lines irrespevtive of gender or any specific life stage.

Key Event Relationship Description

Upstream event: Decreased, SIRT1

Downstream event: NF kB activity, Increased

The described Key Event Relationship (KER) delineates a sequence of events involving the regulatory impact of SIRT1 and its downstream effects. The upstream event is characterized by "Decreased SIRT1," indicating a reduction in the levels or activity of the protein SIRT1. SIRT1 is a member of the sirtuin family of proteins that plays a role in various cellular processes, including gene expression regulation and stress response.

The downstream event in this KER is an "Increased NF-κB activity," signifying an elevation in the activity of the nuclear factor kappa B (NF-κB) signaling pathway. SIRT1 has been recognized as a modulator of NF-κB activity. Decreased SIRT1 levels can lead to enhanced NF-κB activity, potentially due to the loss of SIRT1-mediated deacetylation and inhibition of NF-κB transcriptional activity.

This KER underscores the intricate interplay between proteins and signaling pathways within the cell, where changes in the levels of one protein, like SIRT1, can impact downstream signaling and cellular responses. The reduction in

SIRT1 levels can contribute to heightened NF-κB activity, which in turn may influence various cellular processes, including inflammation, immune responses, and stress-related pathways.

Evidence Supporting this KER

SIRT1 deacetylates NFkB. In the context of NFkB, all of the evidence so far points to its signalling being inhibited after SIRT1 deacetylation (Morris, 2012). SIRT1 or SIRT1 activation by resveratrol and other polyphenols, in fact, has been found to reduce inflammatory response by deacetylating and inhibiting NFkB in both in vitro and in vivo investigations. The essential significance of NFkB in many cellular processes implicated in inflammation, ageing, cancer, and other diseases makes these findings particularly intriguing.

Biological Plausibility

-The acetylation of many lysines on NFkB has been identified, which leads to its activation (Kiernan et al., 2003). A novel class of deacetylases known as Sirtuins has heightened interest in modulating NFkB activity. The activation of sirtuins actually inhibits NFkB.

- According to Yeung et al, SIRT1 can directly interact with and deacetylate the RelA/p65 component of the NF-B complex (Yeung et al.,2004). Deacetylation of Lys310 decreased the transactivation ability of the RelA/p65 subunit and, as a result, lowered the transcription of NF-B-dependent genes. Furthermore, deacetylation of Lys310 in the RelA/p65 protein exposed it to methylation at Lys314 and Lys315, resulting in increased ubiquitination and destruction of the protein (Yang et al.,2010). SIRT1 inhibition of NF-B signalling has been demonstrated in a number of recent studies, and activation of SIRT1 could ameliorate a variety of NF-B-driven inflammatory and metabolic illnesses (Salminen et al.,2008; Yu et al.,2010; Yao et al.,2012; Xie et al.,2013).

SIRT1 suppresses NF-B signalling either directly by deacetylating the RelA/p65 subunit or indirectly by triggering repressive transcriptional complexes, which frequently involve heterochromatin formation at NF-B promoter regions. SIRT1 expression and signalling are both inhibited by NF-B.

Zhang et al. found that overexpressing RelA/p65 protein increased SIRT1 expression at both the transcriptional and protein levels (36 h treatment), whereas knocking down RelA/p65 expression decreased TNF-induced SIRT1 expression (8 h treatment)(Zhang et al.,2010). They also discovered that the RelA/p65 protein may bind to the SIRT1 promoter's NF-B motifs. These findings suggest that NF-B may promote SIRT1 expression. Given that SIRT1 induction appeared to occur much later than NF-B activation, it appears that this action could represent a feedback response limiting inflammation and eventually generating endotoxin tolerance.

Empirical Evidence

- According to Lu et al, SIRT1 inhibited the growth of gastric cancer through inhibiting the activation of STAT3 and NF-B (Lu et al.,2014). The goal was to look at SIRT1's regulatory effects on gastric cancer (GC) cells (AGS and MKN-45) as well as the links between SIRT1 and STAT3 and NF-B activation in GC cells. The SIRT1 activator (resveratrol RSV) was discovered to contribute to the repression of viability and increase of senescence, which was reversed by SIRT1 inhibitor (nicotinamide NA) and SIRT1 depletion using the CCK-8 and SA-β-gal assays, respectively. SIRT1 activation (RSV supplement) reduced not only STAT3 activation, including STAT3 mRNA level, c-myc mRNA level, phosphorylated STAT3 (pSTAT3) proteins, and acetylizad STAT3 (acSTAT3) proteins, but also pNF-B p65 and acNF-B p65 suppression. The effects of RSV were reversed by NA.
- Furthermore, when STAT3 or NF-B were knocked down, neither RSV nor NA could affect cellular survival or senescence in MKN-45 cells. Overall, the outcomes of the study revealed that SIRT1 activation could cause GC in vitro to lose viability and senescence. Furthermore, our findings demonstrated that SIRT1 inhibited proliferation in GC cells and was related with deacetylation-mediated suppression of STAT3 and NF-B protein activation.
- The levels of SIRT1 protein expression in non-small-cell lung cancer (NSCLC) cell lines were examined in a study by Yeung et al.,2004. In comparison to immortalised epithelial human lung NL-20 cells, NSCLC cells exhibit significant quantities of SIRT1 protein, as reported by other researchers (Luo et al, 2001; Vaziri et al, 2001).
- Pharmacological modulators of Sirtuin activity were employed to see if NF-kB transcription was regulated by Sirtuins (Landry et al, 2000; Bedalov et al, 2001; Howitz et al, 2003).

Transient luciferase reporter experiments revealed that cells pretreated with resveratrol had very minimal NF-kB transcription following the presence of TNFa. TNFa-induced NF-kB activity was boosted when cells were pretreated with the Sirtuin inhibitors nicotinamide or splitomicin. NF-kB transcription was also potentiated in cells treated with trichostatin A (TSA), an HDAC class I and class II inhibitor, as expected.

Uncertainties and Inconsistencies

- SIRT1 can inhibit NF-κB signaling directly or indirectly, in turn the NF-κB system suppresses SIRT1-mediated functions by inhibiting the downstream targets of SIRT1. Given that SIRT1 and NF-κB signaling have antagonistic characteristics, these pathways control many of the physiologically relevant metabolic and inflammatory switches required for the maintenance of cellular and organismal homeostasis.
- PGC-1 is a downstream target of the SIRT/AMPK signalling cascade that promotes oxidative metabolism by promoting mitochondrial biogenesis (Fernandez et al.,2011). In cardiac cells, Alvarez-Guardia et al. found that the RelA/p65 member of the NF-B complex was constitutively linked to the PGC-1 protein. They also discovered that activating NF-B after TNF exposure boosted the association between the RelA/p65 and PGC-1 proteins, resulting in an increase in glucose oxidation (Alvarez et al., 2010). These findings show that deacetylation of PGC-1 promotes mitochondrial oxidative respiration, whereas activation of NF-B signalling inhibits SIRT1/PGC-1 communication and activates aerobic glycolysis. This shift is known as the Warburg effect, which can be seen in cancer cells but also in ageing (Salminen et al., 2010). Overexpression of PGC-1, on the other hand, decreased the transcriptional activity of NF-B by lowering the phosphorylation of the transactivating RelA/p65 component(Eisele et al.,2013)

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human cell line	qRT-PCR,,Luciferase reporter assay Cell based HDAC assay(Luo et al.,2001)	Yes	Strong	Yes	Yes	Yes
Humans	qRT-PCR,immunohistochemistry (McGlynn et al.,2014)	Yes	Strong	Yes	Yes	Yes
Mouse	qRT-PCR,Southern and northern blotting, reporter gene assay(Paul et al.,2008)	Yes	Low	Yes	Yes	Yes

Response-response relationship

- Studies have been done on pancreatic cancer cells, Joudah et colleagues investigated the processes and correlations between SIRT1 and NF-B activation .The results showed that a 1 μM SIRT1 aptamer might limit NF-B activation by increasing SIRT1 protein activity(Joudah et al.,2021). According to the findings of SIRT1 aptamer mechanisms, it is possible that SIRT1 aptamer will be used in the treatment of pancreatic cancer in the future.

-To explore the mechanism of SIRT1 aptamer in cell lines, SIRT1 activity was measured in parallel on Aspc-1, BxPc-3, and Capan-2 cell lines under the same conditions. SIRT1 activity was measured in BxPc-3 cell lines using SIRT1 aptamer at 0.25, 0.5, and 1M. Then, using 100M resveratrol (SIRT1 activator control), 100M suramin, and nicotinamide(SIRT1 inhibitor control), assess its activity .

-The results revealed that using SIRT1 aptamer at 1M boosted SIRT1 activity in Capan-2 cells when compared to high concentrations of 100M resveratrol, 100M Suramin, and 100M Nicotinamide.

-The activation of SIRT1 in the Aspc-1 cell line when treated with SIRT1 at 1μM was higher than that of 100μM resveratrol, Suramin, and Nicotinamide.

-the effect of SIRT1 aptamer on NF-kB activation was determined in nuclear extracts of BxPC-3, Capan-2, and AsPC-1 cell lines using an ELISA-based test to measure the capacity of NF-kB p65 subunit for DNA-binding.

-At 1 μM, adding a SIRT1 aptamer caused biphasic alterations in NF-kB. At 8 hours, NF-kB binding activity in Bx-PC-3, Capan-2, and AsPC-1 cell lines was reduced by 150 percent, 130 percent, and 130 percent, respectively, compared to control 100 percent. In Bx-PC-3,Capan-2, and AsPC-1 cell lines, the decline was 180 percent, 145 percent, and 140 percent of the control 100 percent, $P<0.005$ at 16 hours respectively.

Time-scale

The events connected by this KER occur within hours.

Known modulating factors

- NF-B can be activated by cytokines (TNF-, IL-1), growth factors (EGF), bacterial and viral products (lipopolysaccharide (LPS), dsRNA), UV and ionising radiation, reactive oxygen species (ROS), DNA damage, and oncogenic stress from inside the cells. Almost all stimuli eventually activate a large cytoplasmic protein complex called the inhibitor of B (IB) kinase (IKK) complex via a so-called "canonical pathway." The exact composition of this complex is unknown, however it has three fundamental components: IKK1/IKK, IKK2/IKK, and NEMO/IKK. IB is phosphorylated by the activated IKK complex, which marks it for destruction by the -transducin repeat-containing protein (-TrCP)-dependent E3 ubiquitin ligase-mediated proteasomal degradation pathway (Liu et al., 2012;Li et al., 2002). As a result, unbound NF-B dimers can go from the cytoplasm to the nucleus, bind to DNA, and control gene transcription.
- SIRT6 is a nuclear sirtuin that regulates the acetylation status and transcriptional activity of HIF1 and NFkB. SIRT6 deacetylates histone 3 lysine 8 (H3K9) at HIF1 target gene promoters and so acts as a corepressor of HIF1 transcriptional activity. SIRT6 modulation of glucose flow appears to be crucial, as SIRT6 deficiency results in fatal hypoglycemia (Zhong et al., 2010). SIRT6 inhibits NFkB function through a mechanism that is strikingly similar. SIRT6 also deacetylates H3K9 on the promoters of specific NFkB target genes, reducing NFkB's accessibility to these promoters (Kawahara et al., 2009). SIRT6 has a compensating impact in SIRT1 deficient animals, attenuating the enhanced NFkB activity due to an elevated acetylation state (Schug et al., 2010). Finally, although having different methods, both SIRT1 and SIRT6 are negative regulators of NFkB activity.
- SIRT2 has been demonstrated to deacetylate the cytoplasmic lysine 310 (K310) of NFkB subunit p65 (Rothgiesser et al., 2010). SIRT2 suppresses NFkB activation and transcription of NFkB target genes in response to TNF stimulation in this way (Rothgiesser et al., 2010). After TNF exposure, SIRT2 silenced cells show higher NFkB activity and a reduced probability of cell death (Rothgiesser et al., 2010). As a result, SIRT2 in the cytosol and SIRT1 in the nucleus can both deacetylate NFkB.

Known Feedforward/Feedback loops influencing this KER

- SIRT1 and AMPK have a close interaction in the control of energy metabolism and inflammation as they can promote each other's activity (Ruderman et al., 2010). SIRT1 stimulates AMPK by deacetylating LKB1, which then activates AMPK (Lan et al., 2008), whereas AMPK promotes the synthesis of cellular NAD+, which is necessary for SIRT1 activity (Canto et al.,2009). SIRT1 and AMPK have many similar activities in the control of energy metabolism as a result of this positive feedback.
- AMPK appears to be an efficient inhibitor of NF-B signalling and inflammatory reactions, according to new research. This topic was recent discussed in depth (Salminen et al.,2011). In a nutshell, AMPK inhibits RelA/p65 by

activating SIRT1. PGC-1 is also phosphorylated by AMPK, which increases its activation (Canto et al.,2009). As a result, PGC-1 can block RelA/p65-mediated NF-B signalling.

- The transcription factor FoxO3a, which is involved in metabolic and immunological homeostasis, was activated by AMPK (Eijkelenboom et al.,2013). Overexpression of FoxO3a decreased NF-B activation in cultured cells, such as after TNF treatment, by suppressing nuclear translocation of the RelA/p65 component. The inhibition of NF-B signalling by FoxO3a was corroborated in a study (Lee et al.,2008) who found that overexpression of FoxO3a caused the production of B-Ras1, an inhibitor of NF-B activation. However, FoxO3a has recently been discovered to activate the NF-B system via BCL10, which is expressed in B lymphocytes (Li et al., 2012).

References

Alvarez-Guardia, D., Palomer, X., Coll, T., Davidson, M. M., Chan, T. O., Feldman, A. M., ... & Vázquez-Carrera, M. (2010). The p65 subunit of NF-κB binds to PGC-1α, linking inflammation and metabolic disturbances in cardiac cells. *Cardiovascular research*, *87*(3), 449-458.

Bedalov, A., Gatbonton, T., Irvine, W. P., Gottschling, D. E., & Simon, J. A. (2001). Identification of a small molecule inhibitor of Sir2p. *Proceedings of the National Academy of Sciences*, *98*(26), 15113-15118.

Cantó, C., Gerhart-Hines, Z., Feige, J. N., Lagouge, M., Noriega, L., Milne, J. C., ... & Auwerx, J. (2009). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature*, *458*(7241), 1056-1060.

Cantó, C., & Auwerx, J. (2009). PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Current opinion in lipidology*, *20*(2), 98.

Eijkelenboom, A., & Burgering, B. M. (2013). FOXOs: signalling integrators for homeostasis maintenance. *Nature reviews Molecular cell biology*, *14*(2), 83-97.

Fernandez-Marcos, P. J., & Auwerx, J. (2011). Regulation of PGC-1α, a nodal regulator of mitochondrial biogenesis. *The American journal of clinical nutrition*, *93*(4), 884S-890S.

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., ... & Sinclair, D. A. (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature*, *425*(6954), 191-196.

Kawahara, T. L., Michishita, E., Adler, A. S., Damian, M., Berber, E., Lin, M., ... & Chua, K. F. (2009). SIRT6 links histone H3 lysine 9 deacetylation to NF-κB-dependent gene expression and organismal life span. *Cell*, *136*(1), 62-74.

Kiernan, R., Brès, V., Ng, R. W., Coudart, M. P., El Messaoudi, S., Sardet, C., ... & Benkirane, M. (2003). Post-activation turn-off of NF-κB-dependent transcription is regulated by acetylation of p65. *Journal of Biological Chemistry*, *278*(4), 2758-2766.

Lan, F., Cacicedo, J. M., Ruderman, N., & Ido, Y. (2008). SIRT1 modulation of the acetylation status, cytosolic localization, and activity of LKB1: possible role in AMP-activated protein kinase activation. *Journal of Biological Chemistry*, *283*(41), 27628-27635.

Landry, J., Slama, J. T., & Sternglanz, R. (2000). Role of NAD+ in the deacetylase activity of the SIR2-like proteins. *Biochemical and biophysical research communications*, *278*(3), 685-690.

Lee, H. Y., Youn, S. W., Kim, J. Y., Park, K. W., Hwang, C. I., Park, W. Y., ... & Kim, H. S. (2008). FOXO3a turns the tumor necrosis factor receptor signaling towards apoptosis through reciprocal regulation of c-Jun N-terminal kinase and NF-κB. *Arteriosclerosis, thrombosis, and vascular biology*, *28*(1), 112-120.

Li, Z., Zhang, H., Chen, Y., Fan, L., & Fang, J. (2012). Forkhead transcription factor FOXO3a protein activates nuclear factor κB through B-cell lymphoma/leukemia 10 (BCL10) protein and promotes tumor cell survival in serum deprivation. *Journal of Biological Chemistry*, *287*(21), 17737-17745.

Liu, F., Xia, Y., Parker, A. S., & Verma, I. M. (2012). IKK biology. *Immunological reviews*, *246*(1), 239-253.

Li, Q., & Verma, I. M. (2002). NF-κB regulation in the immune system. *Nature reviews immunology*, *2*(10), 725-734.

Lu, J., Zhang, L., Chen, X., Lu, Q., Yang, Y., Liu, J., & Ma, X. (2014). SIRT1 counteracted the activation of STAT3 and NF-κB to repress the gastric cancer growth. *International journal of clinical and experimental medicine*, *7*(12), 5050.

Luo, J., Nikolaev, A. Y., Imai, S. I., Chen, D., Su, F., Shiloh, A., ... & Gu, W. (2001). Negative control of p53 by Sir2α promotes cell survival under stress. *Cell*, *107*(2), 137-148.

Joudah, M. S., Arif, I. S., & Al-Sudani, B. T. (2021). Crosstalk Between Sirt1 Activators And Nf-Kb Axis As A Therapeutic Target To Reduce Pancreatic Cancer. *Systematic Reviews in Pharmacy*, *12*(3), 207-212.

McGlynn, L. M., Zino, S., MacDonald, A. I., Curle, J., Reilly, J. E., Mohammed, Z. M., ... & Shiels, P. G. (2014). SIRT2: tumour suppressor or tumour promoter in operable breast cancer?. *European Journal of Cancer*, *50*(2), 290-301.

Yang, L., Wu, D., Wang, X., & Cederbaum, A. I. (2012). Cytochrome P4502E1, oxidative stress, JNK, and autophagy in acute alcohol-induced fatty liver. *Free Radical Biology and Medicine*, *53*(5), 1170-1180.

Pfluger, P. T., Herranz, D., Velasco-Miguel, S., Serrano, M., & Tschöp, M. H. (2008). Sirt1 protects against high-fat diet-induced metabolic damage. *Proceedings of the national academy of sciences*, *105*(28), 9793-9798.

Rothgiesser, K. M., Erener, S., Waibel, S., Lüscher, B., & Hottiger, M. O. (2010). SIRT2 regulates NF-κB-dependent gene expression through deacetylation of p65 Lys310. *Journal of cell science*, *123*(24), 4251-4258.

Ruderman, N. B., Xu, X. J., Nelson, L., Cacicedo, J. M., Saha, A. K., Lan, F., & Ido, Y. (2010). AMPK and SIRT1: a long-standing partnership?. *American Journal of Physiology-Endocrinology and Metabolism*.

Salminen, A., & Kaarniranta, K. (2010). Glycolysis links p53 function with NF-κB signaling: Impact on cancer and aging process. *Journal of cellular physiology*, *224*(1), 1-6.

Eisele, P. S., Salatino, S., Sobek, J., Hottiger, M. O., & Handschin, C. (2013). The peroxisome proliferator-activated receptor γ coactivator 1α/β (PGC-1) coactivators repress the transcriptional activity of NF-κB in skeletal muscle cells. *Journal of Biological Chemistry*, *288*(4), 2246-2260.

Salminen, A., Kauppinen, A., Suuronen, T., & Kaarniranta, K. (2008). SIRT1 longevity factor suppresses NF-κB-driven immune responses: regulation of aging via NF-κB acetylation?. *Bioessays*, *30*(10), 939-942.

Salminen, A., Hyttinen, J. M., & Kaarniranta, K. (2011). AMP-activated protein kinase inhibits NF-κB signaling and inflammation: impact on healthspan and lifespan. *Journal of molecular medicine*, *89*(7), 667-676.

Schug, T. T., Xu, Q., Gao, H., Peres-da-Silva, A., Draper, D. W., Fessler, M. B., ... & Li, X. (2010). Myeloid deletion of SIRT1 induces inflammatory signaling in response to environmental stress. *Molecular and cellular biology*, *30*(19), 4712-4721.

Vaziri, H., Dessain, S. K., Eaton, E. N., Imai, S. I., Frye, R. A., Pandita, T. K., ... & Weinberg, R. A. (2001). hSIR2SIRT1 functions as an NAD-dependent p53 deacetylase. *Cell*, *107*(2), 149-159.

Xie, J., Zhang, X., & Zhang, L. (2013). Negative regulation of inflammation by SIRT1. *Pharmacological Research*, *67*(1), 60-67.

Yao, H., & Rahman, I. (2012). Perspectives on translational and therapeutic aspects of SIRT1 in inflammaging and senescence. *Biochemical pharmacology*, *84*(10), 1332-1339.

Yang, X. D., Tajkhorshid, E., & Chen, L. F. (2010). Functional interplay between acetylation and methylation of the RelA subunit of NF-κB. *Molecular and cellular biology*, *30*(9), 2170-2180.

Yeung, F., Hoberg, J. E., Ramsey, C. S., Keller, M. D., Jones, D. R., Frye, R. A., & Mayo, M. W. (2004). Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. *The EMBO journal*, *23*(12), 2369-2380.

Yu, J., & Auwerx, J. (2010). Protein deacetylation by SIRT1: an emerging key post-translational modification in metabolic regulation. *Pharmacological research*, *62*(1), 35-41.

Zhang, H. N., Li, L., Gao, P., Chen, H. Z., Zhang, R., Wei, Y. S., ... & Liang, C. C. (2010). Involvement of the p65/RelA subunit of NF-κB in TNF-α-induced SIRT1 expression in vascular smooth muscle cells. *Biochemical and biophysical research communications*, *397*(3), 569-575.

Zhong, L., D'Urso, A., Toiber, D., Sebastian, C., Henry, R. E., Vadysirisack, D. D., ... & Mostoslavsky, R. (2010). The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1α. *Cell*, *140*(2), 280-293.

Relationship: 2612: Increased activation, Nuclear factor kappa B (NF-kB) leads to Antagonism, Estrogen receptor

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mice	Mus sp.	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Moderate

Sex Applicability

Sex	Evidence
Female	High

KER has been observed in humans and animals irrespective of the gender and life stage.

Key Event Relationship Description

Upstream event: Increased, NF kB activity

Downstream event: Estrogen receptor, Reduced

The described Key Event Relationship (KER) outlines a sequence of events involving the activation of the nuclear factor kappa B (NF-κB) pathway and its impact on the estrogen receptor. The upstream event is marked by "Increased NF-κB activity," signifying an elevation in the activation of the NF-κB signaling pathway. NF-κB is a transcription factor that plays a crucial role in various cellular processes, including inflammation, immune response, and cell survival.

The downstream event in this KER is "Reduced Estrogen receptor," indicating a decrease in the levels or activity of the estrogen receptor. The NF-κB pathway has been linked to the modulation of estrogen receptor function. Increased NF-κB activity can lead to the downregulation or interference of estrogen receptor signaling, which can affect cellular responses to estrogen and its related pathways.

This KER emphasizes the intricate cross-talk between signaling pathways and transcriptional regulation within cells. The heightened NF-κB activity can exert downstream effects on the estrogen receptor, potentially impacting hormone-related processes and cellular functions. Understanding these interactions contributes to a broader comprehension of how cellular pathways influence each other and how changes in one pathway can lead to consequences in another.

Evidence Supporting this KER

Activation NF-κB in breast cancer leads to loss of Estrogen Receptor (ER) expression and Human Epidermal Growth Fac- tor Receptor 2 (HER-2) overexpressed via epidermal growth factor receptor (EGFR) and Mitogen Activated Protein Kinase (MAPK) pathway (Laere et al.,2007). Indeed, the binding of epidermal growth factor (EGF) to its receptor (EGFR) activates NF-B, which most likely contributes to this transcription factor's increased activity in ER negative breast cancer cells (Shostak et al.,2011). Because of constitutive production of cytokines and growth factors, loss of ER function has been linked to constitutive NF-kB activity and hyperactive MAPK, resulting in aggressive, metastatic, hormone-resistant malignancies (Ali et al., 2002). Activation of the progesterone receptor can reduce DNA binding and transcriptional activity by inhibiting NF-B-driven gene expression (Kalkhoven et al., 1996). HER-2 stimulates NF-B via the conventional route, which includes IKK (Merkhofer et al., 2010).

Biological Plausibility

-NF-kB activation in breast cancer has been extensively documented in oestrogen receptor negative (ER) breast tumours and ER breast cancer cell lines, implying a significant inhibitory interaction between both signalling pathways (Biswas et al, 2000, 2001, 2004; Zhou et al, 2005). A rise in both NF-kB DNA-binding activity (Nakshatri et al, 1997) and expression of NF-kB target genes such IL8 coincides with a transition from oestrogen dependence to oestrogen independence in breast cancer, indicating inhibitory cross-talk. The fact that some breast tumours that are resistant to the tumoricidal action of anti-estrogens become sensitised to apoptosis and show a drop in NF-kB activity after treatment with oestrogen supports the inverse relationship between ER and NF-kB activity.

-This shows that oestrogen's proapoptotic actions in these tumours are mediated via NF-kB suppression.

Empirical Evidence

- In specific subclasses of human breast cancer cells and tumour tissue specimens, an enhanced level of activated NF-kB is found, primarily in erbB2-overexpressing ER-negative breast cancer (Biswas et al 2000;2003).

- Singh et al explored a variety of methods to inhibit NF-kB activation in ER-negative breast cancer cells and looked at the effects on cell proliferation, apoptosis, and tumour growth in xenografts(Singh et al.,2007). Several cell lines were utilised as representative cultured cell models for subclasses of human breast cancer, including ER negative and erbB2 positive (SKBr3 and MDA-MB453), ER negative and erbB1 positive (MDA-MB231), and ER positive and erbB1/erbB2 negative (MCF-7). IKK, the primary kinase in NF-nB activation, was disabled using a conditional dominant-negative gene construct and small-molecule inhibitors. Bortezomib, a proteasome inhibitor, was used to prevent NF-kB activation.

-The study compared the results of NF-kB activation patterns in ER-negative and erbB2-positive SKBr3 and MDA-MB453 cell lines as representative functional systems of this subclass of human breast cancer to the ER-positive and erbB2-negative MCF-7 cell line. Growing SKBr3 cells in deficient (minimum) media followed by supplementation with the particular mitogenic growth factor HRG (+HRG) allowed the degree of activated NF-kB to be experimentally controlled. This therapy increased IKK activity in as little as 15 minutes, while also increasing NF-nB DNA-binding activity and NF-nB–driven reporter gene expression. These findings imply that HRG-initiated signalling in erbB2-positive cells is mediated by IKK-induced NF-kB activation. MDA-MB453, a second erbB2-positive and ER-negative breast cancer cell line, showed similar results.

- In a prospective cohort study, Sampepajung et al used immunohistochemistry (IHC) to examine NF-B expression and intrinsic subtypes of breast cancer tissue and found a significant correlation between negative ER and overexpression of NF-B (p 0.05), with overexpression of NF-B being higher in negative ER (77.3 percent) compared to positive ER (47.4 percent)(Sampepajung et al., 2021)

- Laere et al suggested that activation of NF-kB in inflammatory breast cancer (IBC) is associated with loss of estrogen receptor (ER) expression, indicating a potential crosstalk between NF-kB and ER(Laere et al.,2007).

In this study, the activation of NF-kB in IBC and non-IBC cells was investigated in relation to ER and EGFR expression, ErbB2 expression, and MAPK hyperactivation.The expression of eight NF-kB target genes was associated with the expression of a qRT–PCR-based ER signature in tumours with and without transcriptionally active NF-kB. Hierarchical clustering was performed using a combined ER/NF-kB signature. MAPK hyperactivation was associated to tumour phenotype, ER and EGFR overexpression, and/or ErbB2 overexpression, according to a recently reported MAPK signature.

In breast tumours without transcriptionally active NF-kB, the expression of most ER-modulated genes was much higher. Furthermore, the expression of most ER-modulated genes was highly anticorrelated with that of most NF-kB target genes, demonstrating that ER and NF-kB activity are inversely related.

-The activation of the transcription factors of ER and NF-kB are inversely linked.

- Indra et al employed the prospective cohort approach to investigate 62 samples in an observational analysis.The positive and negative expression of NF-B, ER, and HER2 overexpression were among the data used in this investigation(Indra et al.,2021). The cases were separated into two groups: those who responded to neoadjuvant chemotherapy and those who did not. Negative NF-B expression (82.5%), positive HER2 status (85.7%), and negative ER status (85.7%) were all associated with a larger percentage of responding individuals (71.9 percent).

-NF-B expression, ER status, and HER2 all had a substantial relationship with the response to anthracycline-based neoadjuvant chemotherapy for locally advanced breast cancer, with NF-B expression having the strongest link.

-A higher percentage of responding participants (71.9 percent) had a negative ER status, indicating that ER expression and chemotherapy response have a substantial relationship (p 0.05).

- This finding is consistent with alanalysis by Osako et al, of 103 individuals with locally advanced KPD. Neoadjuvant chemotherapy with anthracyclines and taxanes was given to the patients. The pCR chemotherapy results were significantly correlated with negative ER and PR expression results (Osako et al.,2012).

The expression of NF-B, HER2, and ER status has a strong association with chemotherapy response, according to these findings. Multivariate analysis of the specific association between NF-B expression and chemotherapeutic response revealed that NF-B expression and HER2 status were both related with chemotherapy response , whereas ER status had no such relationship.

- Sarkar et al assessed NF-B expression in breast cancer tissue and fibroadenoma tissue as test samples and controls, respectively. The Western Blot Technique was used to measure the p65 protein from the NF-kB superfamily of transcription factors. Immunohistochemistry was used to determine the levels of ER, PR, and HER-2/neu(Sarkar et al., 2013).Large tumour size (5 cm), high grade tumours, negative ER, negative PR, and positive HER-2/neu are all related with -NF-B/p65.

-NF-B activation was shown to be more common in ER-negative tumours (81.8%) than in ER-positive cancers (38.5%), a statistically significant difference.

-NF-B expression is linked to ER negative status and is also linked to a higher NPI value, which indicates a poor prognosis.

- NF-kB activity is elevated in hormone-independent and ER-negative breast tumors , and hyperactiva- tion of MAPK leads to enhanced NF-kB activity through induction of autocrine factors such as HB- EGF (Norris et l.,1999;Pearson et l.,2001;McCarthy et l.,1995;Troppamair et l.,1998). NF-kB activity is elevated in MCF-7 breast cancer cells with elevated MAPK activity(Holloway et l.,2004).

-NF-kB activity is about 5-fold higher than parental MCF-7 in all of our model cell lines. This elevated NF-kB activity is attributable to hyperactivation of MAPK because NF-kB activity is returned to normal levels (basal levels in co-MCF7 cells) by dnERKs 1 and 2 .

Uncertainties and Inconsistencies

No specific uncertainties and inconsistencies reported to the best of our knowledge.

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of	Assay fit for	Repeatability/ reproducibility	Direct measure

			evidence	purpose		
Human cell line	qPCR, western blotting, immunoprecipitation, immunofluorescent microscopy, Luciferase reporter assay EMSA, IHC,Cell viability assay	Yes	Strong	Yes	Yes	Yes
Humans	qRT-PCR, immunohistochemistry	Yes	Strong	Yes	Yes	Yes
Mouse	EMSA,Autoradiography Immunofluorescent microscopy, Westernblotting	Yes	Strong	Yes	Yes	Yes

Response-response relationship

Differential Sensitivity of ER α and ERβ Cells to the NF-kB Inhibitor Go6976. A differential sensitivity to Go6976 by ER α and ERβ breast cancer cells was observed (Holloway et al.,2004). The ER α cells were more sensitive and less viable after treatment with this NF-kB inhibitor. The IC50 (50% killing) by Go6976 was 1 mM for Era of MDA-MB435 and MDA-MB231 breast cancer cells, whereas it was greater than 10 mM for ERa of MCF-7 and T47D or the normal mammary epithelial H16N cells . At 10 mM Go6976, about 80% of the ERa cells were killed, whereas only 15–30% of ERa and normal H16N cells were sensitive to this compound. The relative resistance of the H16N normal human mammary cells indicates a possible high therapeutic index of Go6976 against ERa cancer cells.

This observation is consistent with the previously observed role of NF-kB as an antiapoptotic agent. FACS analysis demonstrated accumulation of sub-G1 population (67%) in Go6976- treated (48 h at 1 mM) ERa vs. only 10–15% in ERa cells, indicating enhanced apoptotic cell death preferentially of ERa cells caused by this low molecular weight compound.

Time-scale

Key events connected by this KER occur within hours of exposure.

Known modulating factors

Estradiol has been shown to decrease transcriptional activity and expression of NF-kB in a variety of experimental models (Biswas et al., 2005;Lobanova et al.,2007). Estrogen treatment of MCF-7 or MCF-7/H cells resulted in a significant suppression of NF-kB activity in both cell lines, according to research. The antiestrogen tamoxifen boosted NF-kB activity in the cells, indicating that ER plays a key role in NF-kB down-regulation in both parent and hypoxia-tolerant cells.

-MCF-7/T2H cells were discovered to have a partial tolerance to acute cobalt chloride-induced hypoxia while maintaining their estrogen-independent phenotype. In contrast to the MCF-7/H subline, MCF-7/T2H cells had a non-affected baseline NF-kB level, indicating that estrogens are responsible for NF-kB downregulation (Scherbakov et al., 2009).

Known Feedforward/Feedback loops influencing this KER

- Multiple pathways are implicated in the crosstalk between NF-KB and ER. Through many mechanisms, including collaboration with FOXA1 to strengthen latent ER-binding sites and trigger translation of their synergistic genes, NF-KB directly interacts with the DNA-binding activity of ER (Franco et al. 2015). Furthermore, NF-KB affects ER via interacting with its ER co-activator or co-repressor, which changes ER transcriptional activity (Park et al. 2005). Similar to ER, NF-KB has been reported to have a role as a downstream effector for the growth factor pathway, which is recognized to be involved in both ligand-dependent and non-ligand-dependent ER activation, leading to resistance to a wide range of anti- oestrogen drugs (Zhou et al. 2005a, Sas et al. 2012, Frasor et al. 2015).

-NF-KB is also involved in the anti-apoptotic pathway and immune surveillance systems, both of which have been linked to endocrine resistance (Hu et al. 2015; Lim et al. 2016). Furthermore, NF-KB inhibition of ER activity has been observed. The zinc finger repressor B-lymphocyte-induced maturation protein (BLIMP1), which can bind to the ER promoter area and restrict ER transcription, is triggered by the NFB subunit RelB. (Wang et al. 2009). Increasing data suggests that NF-KB plays an important role in the complexities of the endocrine resistance environment in breast cancer.

-NF-KB and ERS1 mutations in breast cancer patients who are resistant to endocrine therapy

TNF needs NF-KB and FOXA1 to change the breast cancer cell transcriptome by modulating latent ER-binding sites.

References

Ali, S., & Coombes, R. C. (2002). Endocrine-responsive breast cancer and strategies for combating resistance. *Nature Reviews Cancer*, *2*(2), 101-112.

Kalkhoven, E., Wissink, S., van der Saag, P. T., & van der Burg, B. (1996). Negative Interaction between the RelA (p65) Subunit of NF-κB and the Progesterone Receptor (∗). *Journal of Biological Chemistry*, *271*(11), 6217-6224.

Allred, D. C., & Mohsin, S. K. (2000). Biological features of premalignant disease in the human breast. *Journal of mammary gland biology and neoplasia*, *5*(4), 351-364.

Biswas, D. K., Shi, Q., Baily, S., Strickland, I., Ghosh, S., Pardee, A. B., & Iglehart, J. D. (2004). NF-κB activation in human breast cancer specimens and its role in cell proliferation and apoptosis. *Proceedings of the National Academy of Sciences*, *101*(27), 10137-10142.

Biswas, D. K., Martin, K. J., McAlister, C., Cruz, A. P., Graner, E., Dai, S. C., & Pardee, A. B. (2003). Apoptosis caused by chemotherapeutic inhibition of nuclear factor-κB activation. *Cancer research*, *63*(2), 290-295.

Biswas, D. K., Cruz, A. P., Gansberger, E., & Pardee, A. B. (2000). Epidermal growth factor-induced nuclear factor κB activation: a major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. *Proceedings of the National Academy of Sciences*, *97*(15), 8542-8547.

Biswas, D. K., Dai, S. C., Cruz, A., Weiser, B., Graner, E., & Pardee, A. B. (2001). The nuclear factor kappa B (NF-κB): a potential therapeutic target for estrogen receptor negative breast cancers. *Proceedings of the National Academy of Sciences*, *98*(18), 10386-10391.

Biswas, D. K., Singh, S., Shi, Q., Pardee, A. B., & Iglehart, J. D. (2005). Crossroads of estrogen receptor and NF-κB signaling. *Science's STKE*, *2005*(288), pe27-pe27.

Franco, H. L., Nagari, A., & Kraus, W. L. (2015). TNFα signaling exposes latent estrogen receptor binding sites to alter the breast cancer cell transcriptome. *Molecular cell*, *58*(1), 21-34.

Frasor, J., El-Shennawy, L., Stender, J. D., & Kastrati, I. (2015). NFκB affects estrogen receptor expression and activity in breast cancer through multiple mechanisms. *Molecular and cellular endocrinology*, *418*, 235-239..

Holloway, J. N., Murthy, S., & El-Ashry, D. (2004). A cytoplasmic substrate of mitogen-activated protein kinase is responsible for estrogen receptor-α down-regulation in breast cancer cells: the role of nuclear factor-κB. *Molecular Endocrinology*, *18*(6), 1396-1410.

Hu, R., Warri, A., Jin, L., Zwart, A., Riggins, R. B., Fang, H. B., & Clarke, R. (2015). NF-κB signaling is required for XBP1 (unspliced and spliced)-mediated effects on antiestrogen responsiveness and cell fate decisions in breast cancer. *Molecular and cellular biology*, *35*(2), 379-390.

Manginstar, C., Islam, A. A., Sampepajung, D., Hamdani, W., Bukhari, A., Syamsu, S. A., ... & Faruk, M. (2021). The relationship between NFKB, HER2, ER expression and anthracycline-based neoadjuvan chemotherapy response in local advanced stadium breast cancer: A cohort study in Eastern Indonesia. *Annals of Medicine and Surgery*, *63*, 102164.

Lim, S. O., Li, C. W., Xia, W., Cha, J. H., Chan, L. C., Wu, Y., ... & Hung, M. C. (2016). Deubiquitination and stabilization of PD-L1 by CSN5. *Cancer cell*, *30*(6), 925-939.

Lobanova, Y. S., Scherbakov, A. M., Shatskaya, V. A., & Krasil'nikov, M. A. (2007). Mechanism of estrogen-induced apoptosis in breast cancer cells: role of the NF-κB signaling pathway. *Biochemistry (moscow)*, *72*(3), 320-327.

McCarthy, S. A., Samuels, M. L., Pritchard, C. A., Abraham, J. A., & McMahon, M. (1995). Rapid induction of heparin-binding epidermal growth factor/diphtheria toxin receptor expression by Raf and Ras oncogenes. *Genes & development*, *9*(16), 1953-1964.

Merkhofer, E. C., Cogswell, P., & Baldwin, A. S. (2010). Her2 activates NF-κB and induces invasion through the canonical pathway involving IKKα. *Oncogene*, *29*(8), 1238-1248.

Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet Jr, R. J., & Sledge Jr, G. W. (1997). Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Molecular and cellular biology*, *17*(7), 3629-3639.

Norris, J. L., & Baldwin, A. S. (1999). Oncogenic Ras enhances NF-κB transcriptional activity through Raf-dependent and Raf-independent mitogen-activated protein kinase signaling pathways. *Journal of Biological Chemistry*, *274*(20), 13841-13846.

Osako, T., Nishimura, R., Okumura, Y., Toyozumi, Y., & Arima, N. (2012). Predictive significance of the proportion of ER-positive or PgR-positive tumor cells in response to neoadjuvant chemotherapy for operable HER2-negative breast cancer. *Experimental and therapeutic medicine*, *3*(1), 66-71.

Park, K. J., Krishnan, V., O'Malley, B. W., Yamamoto, Y., & Gaynor, R. B. (2005). Formation of an IKKα-dependent transcription complex is required for estrogen receptor-mediated gene activation. *Molecular cell*, *18*(1), 71-82.

Pearson, G., English, J. M., White, M. A., & Cobb, M. H. (2001). ERK5 and ERK2 cooperate to regulate NF-κB and cell transformation. *Journal of Biological Chemistry*, *276*(11), 7927-7931.

Sampepajung, E., Hamdani, W., Sampepajung, D., & Prihantono, P. (2021). Overexpression of NF-kB as a predictor of neoadjuvant chemotherapy response in breast cancer. *Breast Disease*, (Preprint), 1-9.

Sarkar, D. K., Jana, D., Patil, P. S., Chaudhari, K. S., Chattopadhyay, B. K., Chikkala, B. R., ... & Chowdhary, P. (2013). Role of NF-κB as a prognostic marker in breast cancer: a pilot study in Indian patients. *Indian journal of surgical oncology*, *4*(3), 242-247.

Sas, L., Lardon, F., Vermeulen, P. B., Hauspy, J., Van Dam, P., Pauwels, P., ... & Van Laere, S. J. (2012). The interaction between ER and NFκB in resistance to endocrine therapy. *Breast Cancer Research*, *14*(4), 1-14.

Scherbakov, A. M., Lobanova, Y. S., Shatskaya, V. A., & Krasil'nikov, M. A. (2009). The breast cancer cells response to chronic hypoxia involves the opposite regulation of NF-kB and estrogen receptor signaling. *Steroids*, *74*(6), 535-542.

Shostak, K., & Chariot, A. (2011). NF-κB, stem cells and breast cancer: the links get stronger. *Breast Cancer Research*, *13*(4), 1-7.

Singh, S., Shi, Q., Bailey, S. T., Palczewski, M. J., Pardee, A. B., Iglehart, J. D., & Biswas, D. K. (2007). Nuclear factor-κB activation: a molecular therapeutic target for estrogen receptor–negative and epidermal growth factor receptor family receptor–positive human breast cancer. *Molecular cancer therapeutics*, *6*(7), 1973-1982.

Song, R. D., Zhang, Z., Mor, G., & Santen, R. J. (2005). Down-regulation of Bcl-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER+ breast cancer cells. *Apoptosis*, *10*(3), 667-678.

Troppmair, J., Hartkamp, J., & Rapp, U. R. (1998). Activation of NF-κB by oncogenic Raf in HEK 293 cells occurs through autocrine recruitment of the stress kinase cascade. *Oncogene*, *17*(6), 685-690.

Van Laere, S. J., Van der Auwera, I., Van den Eynden, G. G., Van Dam, P., Van Marck, E. A., Vermeulen, P. B., & Dirix, L. Y. (2007). NF-κB activation in inflammatory breast cancer is associated with oestrogen receptor downregulation, secondary to EGFR and/or ErbB2 overexpression and MAPK hyperactivation. *British journal of cancer*, *97*(5), 659-669.

Wang, X., & Belguise, K. (2009). O' Neill, CF, Sanchez-Morgan, N. *Romagnoli, M., Eddy, SF, Mineva, ND, Yu, Z., Min, C., Trinkaus-Randall, V. et al*, 3832-3844.

Zhou, Y., Eppenberger-Castori, S., Eppenberger, U., & Benz, C. C. (2005). The NFkB pathway and endocrine-resistant breast cancer. *Endocrine Related Cancer*, *12*(1), S37.

Relationship: 2613: Antagonism, Estrogen receptor leads to EMT

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified

Sex Applicability

Sex	Evidence
Female	Moderate

Humans and animals with no specific gender or life stage specificity.

Key Event Relationship Description

Upstream event: Decreased, Estrogen receptor activity

Downstream event: EMT, Increased

The outlined Key Event Relationship (KER) elucidates a sequence of events involving the modulation of estrogen receptor activity and its influence on Epithelial-Mesenchymal Transition (EMT). The upstream event is characterized by "Decreased Estrogen receptor activity," indicating a reduction in the functioning or expression of the estrogen receptor. Estrogen receptors are crucial for mediating the effects of estrogen hormone signaling.

The downstream event in this KER is "Increased EMT," denoting an elevation in the occurrence of Epithelial-Mesenchymal Transition. EMT is a cellular process where epithelial cells lose their characteristics and adopt a more mesenchymal phenotype, which can lead to increased cell motility and invasiveness. A decrease in estrogen receptor activity has been associated with promoting EMT in certain contexts.

This KER underscores the intricate relationships between molecular pathways and cellular behaviors. The reduced activity of estrogen receptors can impact cellular responses, potentially contributing to the initiation of EMT. Understanding these connections sheds light on how changes in receptor activity can influence fundamental cellular processes and cellular plasticity.

Evidence Supporting this KER

Estrogen/ERa signaling maintains an epithelial phenotype and suppresses EMT.ERa signaling promotes proliferation and epithelial differentiation and opposes EMT. ERa activated by E2 inhibits TGF-b signaling and cytokine signaling through Smad and NF-kB, respectively, both of which promote EMT. EMT-related transcription factors and microRNAs are likewise suppressed by ERa signalling. This anti-EMT stance is thought to be a major component in luminal A breast cancer's low spreading potential and excellent prognosis. ERa signalling, on the other hand, promotes the proliferation and survival of ERa-positive breast cancer cells by increasing cell cycle and anti-apoptotic gene expression. Furthermore, because GATA3 is a marker for luminal progenitor cell development and both GATA3 and FOXA1 are cofactors that affect ERa signalling and activity, ERa signalling interacts with luminal-related transcription factors GATA3 and FOXA1 to promote an epithelial phenotype. These elements work together to enhance cell–cell adhesion, basolateral polarity, and low motility in epithelial tissues.

Biological Plausibility

E2/ERa signalling, in part through transcriptional activation of luminal/epithelial-related transcription factors, promotes the development of mammary epithelia along a luminal/epithelial lineage. GATA3 and ERa both promote each other (Eeckhoute et al.,2007). In normal breast epithelia, GATA3 is needed for luminal differentiation(Kouros-Mehr et al.,2008) and GATA3 and ERa control many of the same genes (Wilson et al.,2008). In mice, forcing GATA3 expression in mesenchymal breast cancer cells produces mesenchymal–epithelial transition (MET), a reversible mechanism analogous to EMT, and prevents tumour metastasis (Yan et al.,2010). Another ERa-interacting transcription factor, FOXA1, is essential for luminal lineage in mammary epithelia and stimulates ductal development in mice (Bernardo et al.,2010). FOXA1 enhances ERa gene expression by increasing the accessibility of estrogen-response regions for ERa binding (Nakshatri et al., 2009). In breast cancer cells, on the other hand, E2 appears to increase FOXA1 expression. Importantly, ERa, FOXA1, and GATA3 are all positive breast cancer prognostic factors(Nakshatri et al.,2009).

ERa signalling enhances primary lesion formation (and therefore is mitogenic), but it can control the EMT process (and thus is anti-metastatic) up to a point. Signaling pathways that lead to EMT are antagonised by E2/ERa signalling. TGF-b, for example, has been demonstrated to generate EMT in human mammary epithelial cells, and overexpression of the EMT-inducing protein Snail boosted TGF-b signalling and invasiveness while decreasing adhesion and ERa expression in MCF-7 cells (Taylor et al.,2010). TGF-b has an anti-estrogen impact on MCF-7 cells. Smad2/3 and the Smad-selective E3 ubiquitin ligase Smurf create a ternary complex with ERa, which enhances the proteosomal degradation of Smad proteins, according to Ito et al (Ito et al.,2010).

Empirical Evidence

- Ye et al. investigated the impact of ERa overexpression in ERa-negative breast cancer cell lines (MDA-MB-468, MDA-MB-231) or ERa knockdown in ERa-positive cell lines (MCF-7, T47D) on Slug and Snail expression and phenotypes in ERa-positive cell lines (MCF-7, T47D)(Ye et al., 2010). Slug is repressed, E-cadherin is increased, and cells develop as adherent colonies with less invasiveness when ERa is forced to get expressed. ERa knockdown, on the other hand, causes an increase in Slug expression, a decrease in E-cadherin, and spindle-shaped invasive cells.

-In luminal-type breast tumours, E2 signalling modulates the activity of ERa and associated cofactors GATA3 and FOXA1 to promote an epithelial phenotype and repress EMT and pro-metastatic progression. In vitro, direct regulatory linkages have been shown, such as increased invasion and beginning of EMT in MCF-7 cells after overexpression of RELB or Snail due to suppression of ERa and E-cadherin, and in vivo results support these findings.

-In breast cancer patients, high levels of ERa, GATA3, and FOXA1 expression are linked to a better prognosis. Invasive basal-like and claudin-low breast cancer subtypes, on the other hand, are associated with high levels of RELB and Snail expression (as well as no/low expression of ERa, GATA3, and FOXA1).

- Wik et al used integrated molecular profiling to examine Endometrial cancer samples from a primary investigation cohort and three independent validation cohorts (Wik et al.,2013). Patient survival was closely linked to ER-a immunohistochemical staining and receptor gene (ESR1) mRNA expression. In the study cohort, ER-a negative was related with activation of genes implicated in Wnt, Sonic Hedgehog, and TGF-b signalling, indicating epithelial–mesenchymal transition (EMT).

-In conclusion, the absence of ER-a in endometrial cancer is linked to EMT and a shorter survival time.

- Bouris et al used specialised shRNA lentiviral particles to create stably transfected MCF-7 cells by knocking down the ER gene (identified as MCF-7/SP10 + cells) and compared them to control cells (MCF-7/c). In MCF-7 cells, ER suppression triggered cellular phenotypic changes as well as significant changes in gene and protein expression of many markers associated with epithelial to mesenchymal transition (EMT). These cells, in particular, showed increased cell proliferation, migration, and invasion(Bouris et al.,2015).

- N1-Guanyl-1,7-Diaminoheptane Sensitizes Estrogen Receptor Negative Breast Cancer Cells to Doxorubicin by Preventing Epithelial-Mesenchymal Transition by Inhibiting Eukaryotic Translation Initiation Factor 5A2 Activation (Liu et al.,2015)

- Saleh et al. hypothesise that loss of oestrogen receptor function, which causes endocrine resistance in breast cancer, also causes trans-differentiation from an epithelial to a mesenchymal phenotype, which causes enhanced aggressiveness and metastatic tendency(Saleh et al., 2011).

-siRNA-mediated oestrogen receptor silencing in MCF7 breast cancer cells resulted in estrogen/tamoxifen resistant cells (pII) with altered morphology, increased motility with cytoskeleton rearrangement and switch from keratin/actin to vimentin, and ability to invade simulated extracellular matrix components.

-Invasion assay of MCF7, E2, pII and MDA231 cells through simulated ECM protein components was carried out.Cells were plated into the upper chambers of the cell invasion plate and incubated for 48 h prior to measurement of the fluorescence intensity of the invading cells in the bottom chambers. MCF7 and E2 cells were considered non- invasive (,20 FU/m), whereas pII and MDA231 cells progressively invaded the BME (100 FU/m).

- Quantitative proteomics demonstrates ER participation in CD146-induced epithelial-mesenchymal transition in breast cancer cells, according to Zheng et al. They used a three-step Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) method to examine whole cell protein profiles of MCF-7 cells that had undergone EMT as CD146 expression increased from moderate to high levels (Zheng et al.,2014).

-In total, 2293 proteins were identified in this investigation, with 103 showing changes in protein abundance that linked with CD146 expression levels, demonstrating substantial morphological and biochemical changes associated with EMT.

-According to the Ingenuity Pathway Analysis (IPA), oestrogen receptor (ER) was the transcription regulator that was most strongly suppressed during CD146-induced EMT. In addition, functional experiments demonstrated that in cells undergoing CD146-induced EMT, ER expression was suppressed, but re-expression of ER eliminated their migratory and invasive characteristics. Finally, we discovered that ER- exerted its effects on CD146-induced EMT via inhibiting Slug, a major EMT transcriptional component.

- By immunohistochemistry, Ye et al found a high direct link between ERa and E-cadherin expression in human breast tumours, showing that ERa signalling may regulate E-cadherin and influence EMT and tumour growth(Ye et al.,2010).They looked at the impacts of ERa signalling in ERa-transfected ERa-negative breast carcinoma cell lines MDA-MB-468 and MDA-MB-231, as well as the effects of ERa knockdown in naturally expressing ERa-positive lines MCF-7 and T47D, to test this theory and the mechanisms behind it.17b-estradiol (E2) decreased slug and increased E-cadherin in ERa-negative lines when ERa was overexpressed.

-In Matrigel, clones that showed the most of these alterations developed in clusters and were less invasive.Slug rose, E-cadherin reduced, cells became spindly, and Matrigel invasion increased when ERa was knocked down in ERa-positive lines. Slug expression was reduced by ERa signalling in two ways: directly, by repression of slug transcription via the formation of a corepressor complex containing ligand-activated ERa, HDAC inhibitor (HDAC1), and nuclear receptor corepressor (N-CoR) that bound the slug promoter in three half-site oestrogen response elements (EREs); and indirectly, by phosphorylation and inactivation of GSK-3b via phosphoinositide (Akt). Inactivation of GSK-3b suppressed slug expression while increasing E-cadherin. There was a substantial inverse connection between slug and ERa and E-cadherin immunoreactivity in human breast cancer cases. The data suggest that E-cadherin and EMT are regulated by ERa signalling through slug.

Uncertainties and Inconsistencies

No specific Uncertainties and Inconsistencies noted to the best of our knowledge.

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of	Assay fit for	Repeatability/ reproducibility	Direct measure

			evidence	purpose		
Human cell line	qRT-PCR, cell viability assay, Western blotting, EdU incorporation assay (C1,c2,C3,C5,c6,c8)	Yes	Strong	Yes	Yes	Yes
Human	IHC,microarray,qPCR, SNP array(H)	Yes	Moderate	Yes	Yes	Yes

Response-response relationship

- Endogenous ER silencing causes EMT in ER-positive breast cancer cells.

ER-positive MCF-7 cells were infected with ER shRNA lentiviral particles and stable clones were selected with puromycin (optimal dose of 0.8 g/mL) to knockdown ER gene expression (Zheng et al.,2014).

-When the number of cell passages was increased following infection, the expression of ER was gradually knocked down.

-ER gene expression was decreased by roughly 25% four passages after infection compared to control lentiviral particles transfected cells (MCF-7/c cells). The ER gene expression was lowered even more in the following passage (passage 5 post-infection) (by around 50% compared to MCF-7/c cells). In passage 7, a significant reduction in ER gene expression (about 75–80%) was seen, along with a distinct transition of cells from an epithelial to a mesenchymal phenotype.

- When MCF-7 cells reach confluency, they develop as closely packed colonies that produce sheet-like monolayer structures. Stable clones from stage 7 post-infection, on the other hand, grew as more elongated individual cells rather than tight clusters, with a spindle-like shape. For stable clones with a distinct mesenchymal character, a very substantial down-regulation of ER gene expression (above 99 percent) was found from passage 10 and beyond. MCF-7/SP10+ was given to these cells to emphasise the stable transfection (S) and passage 10 or more (P10+). The substantial down-regulation of ERa was confirmed by immunofluorescence and Western blot analysis of the same stable clones (MCF-7/ SP10 + cells).

Time-scale

Downstream key event occurs within hours of the occurrence of the upstream key event.

Known modulating factors

Tumour characteristics and heterogeneity, biological changes of tumour progression and interacting molecules, all of which can influence the degree of hormone responsiveness in a particular individual with hormone receptor-positive breast cancer.

Known Feedforward/Feedback loops influencing this KER

EMT is inhibited by ERa, and microRNAs either promote or inhibit EMT . These findings raise the question of whether microRNAs have a role in the control of EMT by targeting ERa mRNA. The large (>4000 nt) 30 untranslated region (30-UTR) of human ERa mRNA, as well as results that particular microRNAs are differentially expressed between ERa-positive and ERa-negative breast tumours , suggest the possibility of microRNA-mediated control of human ERa mRNA(Adams et al.,2008).

Pro-metastatic/anti-proliferative (miR-206), pro-metastatic/pro-proliferative (miR-221/222), and anti-proliferative/anti-metastatic (miR-221/223) ERa-targeting microRNAs (miR-130a, miR-145). MiR-17/92 appears to be prometastatic, although it is implicated in several feedback loops, which could make miR-17/92's expression and effects on proliferation extremely reliant on the microenvironment as well as the genetic and epigenetic background.

Accurate identification of micro-RNAs that contribute significantly to a particular pathway (such as EMT) within breast cancers in situ is one hurdle. MicroRNAs have hundreds of potential targets, and in vivo studies will be needed to identify physiologically important targets in the context of breast cancer, as well as to develop effective treatments for breast cancer that involve manipulating microRNA expression levels and identifying off-target effects. (Adams et al.,2007;Zhao et al.,2008;Leva et al.,2010;Stinson et al.,2011;Acunzo et al.,2011;Castellano et al.,2009).

References

Acunzo, M., Visone, R., Romano, G., Veronese, A., Lovat, F., Palmieri, D., ... & Croce, C. M. (2012). miR-130a targets MET and induces TRAIL-sensitivity in NSCLC by downregulating miR-221 and 222. *Oncogene*, *31*(5), 634-642.

Adams, B. D., Guttilla, I. K., & White, B. A. (2008, November). Involvement of microRNAs in breast cancer. In *Seminars in reproductive medicine* (Vol. 26, No. 06, pp. 522-536). © Thieme Medical Publishers.

Adams, B. D., Furneaux, H., & White, B. A. (2007). The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-α (ERα) and represses ERα messenger RNA and protein expression in breast cancer cell lines. *Molecular endocrinology*, *21*(5), 1132-1147.

Al Saleh, S., Al Mulla, F., & Luqmani, Y. A. (2011). Estrogen receptor silencing induces epithelial to mesenchymal transition in human breast cancer cells. *PloS one*, *6*(6), e20610.

Bernardo, G. M., Lozada, K. L., Miedler, J. D., Harburg, G., Hewitt, S. C., Mosley, J. D., ... & Keri, R. A. (2010). FOXA1 is an essential determinant of ERα expression and mammary ductal morphogenesis. *Development*, *137*(12), 2045-2054.

Bouris, P., Skandalis, S. S., Piperigkou, Z., Afratis, N., Karamanou, K., Aletras, A. J., ... & Karamanos, N. K. (2015). Estrogen receptor alpha mediates epithelial to mesenchymal transition, expression of specific matrix effectors and functional properties of breast cancer cells. *Matrix Biology*, *43*, 42-60.

Castellano, L., Giamas, G., Jacob, J., Coombes, R. C., Lucchesi, W., Thiruchelvam, P., ... & Stebbing, J. (2009). The estrogen receptor-α-induced microRNA signature regulates itself and its transcriptional response. *Proceedings of the National Academy of Sciences*, *106*(37), 15732-15737.

Di Leva, G., Gasparini, P., Piovan, C., Ngankeu, A., Garofalo, M., Taccioli, C., ... & Croce, C. M. (2010). MicroRNA cluster 221-222 and estrogen receptor α interactions in breast cancer. *JNCI: Journal of the National Cancer Institute*, *102*(10), 706-721.

Eeckhoute, J., Keeton, E. K., Lupien, M., Krum, S. A., Carroll, J. S., & Brown, M. (2007). Positive cross-regulatory loop ties GATA-3 to estrogen receptor α expression in breast cancer. *Cancer research*, *67*(13), 6477-6483.

Ito, I., Hanyu, A., Wayama, M., Goto, N., Katsuno, Y., Kawasaki, S., ... & Yanagisawa, J. (2010). Estrogen inhibits transforming growth factor β signaling by promoting Smad2/3 degradation. *Journal of biological chemistry*, *285*(19), 14747-14755.

Kouros-Mehr, H., Kim, J. W., Bechis, S. K., & Werb, Z. (2008). GATA-3 and the regulation of the mammary luminal cell fate. *Current opinion in cell biology*, *20*(2), 164-170.

Lin, H. Y., Liang, Y. K., Dou, X. W., Chen, C. F., Wei, X. L., Zeng, D., ... & Zhang, G. J. (2018). Notch3 inhibits epithelial–mesenchymal transition in breast cancer via a novel mechanism, upregulation of GATA-3 expression. *Oncogenesis*, *7*(8), 1-15.

Liu, Y., Liu, R., Fu, P., Du, F., Hong, Y., Yao, M., ... & Zheng, S. (2015). N1-Guanyl-1, 7-diaminoheptane sensitizes estrogen receptor negative breast cancer cells to doxorubicin by preventing epithelial-mesenchymal transition through inhibition of eukaryotic translation initiation factor 5A2 activation. *Cellular Physiology and Biochemistry*, *36*(6), 2494-2503.

Nakshatri, H., & Badve, S. (2009). FOXA1 in breast cancer. *Expert reviews in molecular medicine*, *11*.

Stinson, S., Lackner, M. R., Adai, A. T., Yu, N., Kim, H. J., O'Brien, C., ... & Dornan, D. (2011). TRPS1 targeting by miR-221/222 promotes the epithelial-to-mesenchymal transition in breast cancer. *Science signaling*, *4*(177), ra41-ra41.

Taylor, M. A., Parvani, J. G., & Schiemann, W. P. (2010). The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-β in normal and malignant mammary epithelial cells. *Journal of mammary gland biology and neoplasia*, *15*(2), 169-190.

Wilson, B. J., & Giguère, V. (2008). Meta-analysis of human cancer microarrays reveals GATA3 is integral to the estrogen receptor alpha pathway. *Molecular cancer*, *7*(1), 1-8.

Wik, E., Ræder, M. B., Krakstad, C., Trovik, J., Birkeland, E., Hoivik, E. A., ... & Salvesen, H. B. (2013). Lack of estrogen receptor-α is associated with epithelial–mesenchymal transition and PI3K alterations in endometrial carcinoma. *Clinical Cancer Research*, *19*(5), 1094-1105.

Yan, W., Cao, Q. J., Arenas, R. B., Bentley, B., & Shao, R. (2010). GATA3 inhibits breast cancer metastasis through the reversal of epithelial-mesenchymal transition. *Journal of Biological Chemistry*, *285*(18), 14042-14051.

Ye, Y., Xiao, Y., Wang, W., Yearsley, K., Gao, J. X., Shetuni, B., & Barsky, S. H. (2010). ERα signaling through slug regulates E-cadherin and EMT. *Oncogene*, *29*(10), 1451-1462.

Zeng, Q., Zhang, P., Wu, Z., Xue, P., Lu, D., Ye, Z., ... & Yan, X. (2014). Quantitative proteomics reveals ER-α involvement in CD146-induced epithelial-mesenchymal transition in breast cancer cells. *Journal of proteomics*, *103*, 153-169.

Zhao, J. J., Lin, J., Yang, H., Kong, W., He, L., Ma, X., ... & Cheng, J. Q. (2008). MicroRNA-221/222 negatively regulates estrogen receptorα and is associated with tamoxifen resistance in breast cancer. *Journal of Biological Chemistry*, *283*(45), 31079-31087.

Relationship: 2614: EMT leads to Metastasis, Breast Cancer

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Female	High

EMT induces cancer invasion, metastasis (*Homo sapiens*)(P. Zhang et al., 2015).

EMT is related to cancer drug resistance in MCF-7 human breast cancer cells (*Homo sapiens*)(B. Du & Shim, 2016).

Key Event Relationship Description

Upstream event: Increased, EMT

Downstream event: Metastasis

The described Key Event Relationship (KER) outlines a consequential sequence of events pertaining to cellular transitions and their impact on metastasis. The upstream event is marked by "Increased Epithelial-Mesenchymal Transition (EMT)," signifying an elevation in the occurrence of EMT—a process where epithelial cells transition into a mesenchymal phenotype with increased motility and invasiveness.

The downstream event in this KER is "Metastasis," indicating the spread of cancer cells from the primary tumor site to distant locations in the body. EMT has been recognized as a critical step in the metastatic cascade, as it can equip cancer cells with the traits necessary for invading surrounding tissues, entering the bloodstream, and establishing secondary tumors at distant sites.

This KER underscores the pivotal role of EMT in driving the metastatic potential of cancer cells. The transition from an epithelial to mesenchymal state enhances the ability of cancer cells to navigate through tissues and disseminate to distant locations, contributing to the aggressive nature of metastatic disease. Understanding these relationships is crucial for developing strategies to inhibit metastasis and improve cancer treatment outcomes.

Evidence Supporting this KER

The “epithelial–mesenchymal transition” (EMT), a key developmental regulatory program, has been reported to play critical and intricate roles in promoting tumor invasion and metastasis in epithelium-derived carcinomas in recent years. The EMT program allows stationary and polarized epithelial cells, which are connected laterally via several types of junctions and normally interact with the basement membrane via their basal surfaces to maintain apical–basal polarity, to undergo multiple biochemical changes that enable them to disrupt cell–cell adherence, lose apical–basal polarity, dramatically remodel the cytoskeleton, and acquire mesenchymal characteristics such as enhanced migratory capacity, invasiveness, elevated resistance to apoptosis and greatly increased production of ECM components. (Boyer et al., 1993).Some of the cells undergoing EMT have the characteristics of cancer stem cells (CSCs), which are linked to cancer malignancy (Shibue & Weinberg, 2017; Shihori Tanabe, 2015a, 2015b; Tanabe, Aoyagi, Yokozaki, & Sasaki, 2015).Cancer metastasis and cancer therapeutic resistance are linked to the EMT phenomenon (Smith & Bhowmick, 2016; Tanabe, 2013). EMT causes the cell to escape from the basement membrane and metastasize by increasing the production of enzymes that breakdown extracellular matrix components and decreasing adherence to the basement membrane (Smith & Bhowmick, 2016). Therapy resistance is linked to morphological alterations seen during EMT (Smith & Bhowmick, 2016).

Biological Plausibility

Biological Plausibility

EMT is marked by a decrease in E-cadherin and β- -catenin translocation into the nucleus and an increase in vimentin, fibronectin, and N-cadherin expression (Irani et al., 2018,Tanabe et al., 2016). EMT is a master mechanism in cancer cells that allows them to lose their epithelial characteristics and gain mesenchymal-like qualities. EMT is the most crucial step in initiating metastasis, including metastasis to lymph nodes, because tumour cell movement is a pre-requisite for the metastatic process (Da et al., 2017). Multiple signalling pathways cause cancer cells to lose their cell-to-cell connections and cellular polarity during EMT, increasing their motility and invasive ness (Huang et al., 2017). MMPs cause E-cadherin to be cleaved, which increases tumour cell motility and invasion (Pradella et al., 2017).

Invasiveness and medication resistance are linked to the morphological and physiological changes associated with EMT (Shibue & Weinberg, 2017). In initial tumours, EMT-activated carcinoma cells penetrate the surrounding stroma (Shibue & Weinberg, 2017). EMT-activated carcinoma cells interact with the extracellular matrix protein to activate focal adhesion kinase and extracellular signal-related kinase, followed by TGFbeta and canonical and/or noncanonical Wnt pathways to develop cancer stem cell (CSC) traits, which contribute to drug resistance (Shibue & Weinberg, 2017).

Drug efflux and cell proliferation are slowed by EMT-associated downregulation of several apoptotic signalling pathways, resulting in general resistance of carcinoma cells to anti-cancer drugs (Shibue & Weinberg, 2017).Snail, an EMT-related transcription factor, promotes the production of the AXL receptor tyrosine kinase, which allows cancer cells to survive by activating AXL signalling when its ligand, growth arrest-specific protein 6 (GAS6), binds to it (Shibue & Weinberg, 2017).

EMT-activated cells are resistant to the deadly effects of cytotoxic T cells, which include increased expression of programmed cell death 1 ligand (PD-L1), which binds to the inhibitory immune-checkpoint receptor programmed cell death protein 1 (PD-1) on the cell surface of cytotoxic T cells(Shibue & Weinberg, 2017).

The reversing process of EMT, which names as a mesenchymal-epithelial transition (MET), maybe one of the candidates for the anti-cancer therapy, where the plasticity of the cell phenotype is of importance and under investigation (Shibue & Weinberg, 2017).

Empirical Evidence

Incidence concordance

- By inhibiting PUMA (also known as BBC3, encoding Bcl-2-binding component 3) and conferring resistance to p53-mediated apoptosis of hematopoietic progenitors, Slug/Snai2, a ces-1-related zinc finger transcription factor gene, confers resistance to p53-mediated apoptosis of hematopoietic progenitors (Inukai et al., 1999; Shibue & Weinberg, 2017; W.-S. Wu et al., 2005).TGFbeta-1 induced EMT results in the acquisition of cancer stem cell (CSC) like properties by inducing the expression of multiple members of the ATP-binding cassette (ABC) transporter family, which results in doxorubicin resistance (Saxena et al.,2011; Shibue & Weinberg, 2017). (Pirozzi et al., 2011; Shibue & Weinberg, 2017).Cancer metastasis and resistance to dendritic cell-mediated immunotherapy are promoted by snail-driven EMT (Kudo-Saito, Shirako, Takeuchi, & Kawakami, 2009).EMT induced by the zinc finger E-box-binding homeobox (ZEB1) relieves miR-200-mediated repression of programmed cell death 1 ligand (PD-L1) expression, a major inhibitory ligand for the programmed cell death protein (PD-1) immune-checkpoint protein on CD8+ cytotoxic T lymphocytes (CTL), resulting in immunosuppression and metastasis of CD8+ T cells (Chen et al., 2014).
- Wnt signalling is important for embryonic development, and genetic abnormalities in this network have been linked to colorectal cancer(Gujral et al.,2014). The Wnt receptor Frizzled2 (Fzd2) and its ligands Wnt5a/b are enhanced in metastatic liver, lung, colon, and breast cancer cell lines and in high-grade malignancies, and their expression correlates with epithelial-mesenchymal transition markers (EMT)(They created an anti-Fzd2 antibody that decreases tumour growth and metastasis in xenografts by reducing cell migration and invasion(**Support for essentiality**). Patients with malignancies that exhibit high levels of Fzd2 and Wnt5a/b may benefit from blocking this pathway, according to the researchers.
- In breast, colon, liver, and 186 lung cancer cell lines, researchers discovered a link between Fzd2 and its ligands Wnt5a/b and mesenchymal markers.
  - Fzd2 mRNA expression is significantly increased in late stages (stages III and IV) of primary liver and lung cancers compared with normal tissue
- -Fzd2 regulated cell migration.
- - Fzd2 signaling regulates EMT program
- -expression of Fzd2 in Huh7 cells decreased levels of the epithelial markers E-cadherin and Occludin and increased levels of the mesenchymal markers Foxc1 and Vimentin.
  - Exposing cells to an inhibitor of Wnt secretion (C59) decreased Stat3 transcriptional activity in FOCUS cells 2- to 4-fold, whereas overexpressing Fzd2 in Huh7 cells increased Stat3 activity 2-fold.
- Cui et al discovered a link between metastasis and the expression of ROR1, a type I receptor tyrosine kinase–like orphan receptor that is expressed throughout embryogenesis and by a variety of malignancies but not by normal postpartum tissues(Cui et al.,2013). ROR1 expression has been linked to the epithelial–mesenchymal transition (EMT), which occurs during embryogenesis and cancer metastasis, according to their findings. Breast adenocarcinomas with high ROR1 expression were more likely to have gene expression profiles consistent with EMT and had greater rates of recurrence and metastasis than those with low ROR1 expression. Suppressing ROR1 expression in metastasis-prone breast cancer cell lines MDA-MB-231, HS-578T, or BT549 decreased expression of proteins associated with EMT (e.g., vimentin, SNAIL-1/2, and ZEB1), increased expression of E-cadherin, epithelial cytokeratins (e.g., CK-19), and tight junction proteins (e.g., ZO-1), and impaired their migration/invasion capacity in vitro.(**Support for essentiality)**
- -Conversely, transfection of MCF-7 cells to express ROR1 reduced expression of E-cadherin and CK-19, but enhanced the expression of SNAIL-1/2 and vimentin. Treatment of MDA-MB-231 with a monoclonal antibody specific for ROR1 induced downmodulation of vimentin and inhibited cancer cell migration and invasion in vitro and tumor metastasis in vivo.
  - ROR1 associates with metastatic cancer phenotypes
  - ROR1 is associated with early metastatic relapse in breast adenocarcinoma
  - Silencing ROR1 inhibits orthotopic lung metastasis
  - Silencing ROR1 inhibits experimental lung and bone metastasis
  - An anti-ROR1 antibody inhibits cancer metastasis(**Support for essentiality**)
- At 37°C, monoclonal antibodies (mAb) specific for ROR1's extracellular domain were created, and one (D10) was chosen to elicit fast downmodulation of surface ROR1 . ROR1 internalisation was observed in MDA-MB-231 cells treated with D10, as determined by confocal microscopy . As measured by flow cytometry with a separate mAb specific for a unique, non–cross-blocking epitope of ROR1, this resulted in a considerable reduction in ROR1 . D10 treatment of MDA-MB-231 reduced cytoplasmic vimentin expression , which was bound to ROR1 in coimmunoprecipitation studies . In vitro, D10 treatment significantly reduced the ability of MDA-MB-231 to migrate and invade . D10 may also be able to stop other ROR1 cancer cell lines from migrating or invading.
- Chen et al investigated the potential function of MDM2 in ovarian cancer SKOV3 cells' EMT and metastasis(Chen et al.,2015).
- Wound-healing and transwell tests were used to mimic MDM2's regulatory effects on cell motility. By displaying the expression levels of epithelial marker E-cadherin as well as critical components of the Smad pathway, the

impacts on EMT transition and Smad pathway were explored. The connection of MDM2 expression levels with the stages of 104 ovarian cancer patients was explored using an immunohistochemical assay to assess the clinical relevance of their findings.

- Results show that MDM2 plays a significant role in driving EMT and motility in ovarian SKOV3 cells by promoting the activation of the TGF-b-Smad pathway, which leads to increased snail/slug transcription and a decrease of E-cadherin levels.
- Such induction of EMT is sustained in either E3 ligase-depleted MDM2 or E3 ligase inhibitor HLI-373-treated cells, but is reduced by MDM2 N-terminal deletion, as evidenced by Nutlin-3a, the N-terminal targeting agent's inhibitory effects on EMT. MDM2 expression levels are substantially correlated with ovarian cancer stages, and increased MDM2 expression in combination with TGFB is associated with a bad prognosis and predicts a high risk of ovarian cancer patients.
- This research suggests that MDM2 activates the Smad pathway to promote EMT in ovarian cancer metastasis, and that targeting MDM2's N-terminal can reprogram EMT and limit cancer cell mobility.
- HOXD9, a Hox family member, is involved in cancer growth and metastasis. But, its regulation mechanism at the molecular level particularly in colo rectal cancer (CRC), is mostly unknown.Liu and colleagues used immunofluorescence, immunohistochemistry (IHC), and western blot to examine the levels of HOXD9 protein expression. Colony formation and EdU in-corporation, CCK-8, wound scratch and transwell invasion assays, and animal models were used to determine the in vivo and in vitro roles of HOXD9 in CRC. In CRC, HOXD9 expression was higher than in matched healthy tissues (Liu et al.,2020). High HOXD9 expression has been linked to advanced stages of cancer, tumour differentiation, lymph node metastasis, and other serious invasions, as well as a poor prognosis, according to the American Joint Committee on Cancer (AJCC). In CRC cells, HOXD9 promoted proliferation, motility, and EMT processes in vitro. TGF-1 also stimulated the expression of HOXD9, which was dosage dependent, and HOXD9 downregulation suppressed TGF-1-induced EMT. Through orthotopic implantation, HOXD9 promoted the invasiveness and metastasis of CRC cells in vivo.The ectopic expression of HOXD9 promoted the invasion metastasis in cells of the colorectal tumor by induction of EMT in vitro and vivo.
- Twist1, Snail1, Snail2, ZEB1, and ZEB2 are among a group of transcription factors that have been demonstrated to promote tumour spread by inducing epithelial mesenchymal transition (EMT). However, it is unknown whether these transcription factors activate the EMT program separately or in concert. Twist1 requires direct induction of Snail2 to induce EMT, according to the study by Casas et al. Twist1's capacity to decrease E-cadherin transcription is totally blocked when Snail2 is knocked off. Twist1 induces Snail2 transcription by binding to an evolutionarily conserved E-box on the proximal promoter. Twist1-induced cell invasion and distant metastasis in mice require Snail2 induction. Twist1 and Snail2 expression in human breast cancers are significantly linked.Results of the study by Casas et al show that Twist1 needs to induce Snail2 to suppress the epithelial branch of the EMT program and that Twist1 and Snail2 act together to promote EMT and tumor metastasis in human mammary epithelial cell lines (Casas et al.,2012).
- The basic helix-loop-helix transcription factor AP4/TFAP4/AP-4 is encoded by a c-MYC target gene and is up-regulated in colorectal cancer (CRC) and a variety of other tumour types at the same time as c-MYC. A combination of microarray, genome-wide chromatin immunoprecipitation, next-generation sequencing, and bioinformatic studies were used to characterise AP4 DNA binding and mRNA expression across the genome. Hundreds of AP4 target genes were identified as activated and repressed as a result. SNAIL, E-cadherin/CDH1, OCLN, VIM, FN1, and the Claudins 1, 4, and 7 were among the AP4 target genes, which included markers of stemness (LGR5 and CD44) and epithelial–mesenchymal transition (EMT) such as SNAIL, E-cadherin/CDH1, OCLN, VIM, and FN1. As a result, AP4 activation promoted EMT and increased CRC cell motility and invasion. Down-regulation of AP4 hindered migration and invasion by causing mesenchymal–epithelial transition (Jackstadt et al.,2013).
- EMT, migration, and invasion produced by ectopic expression of c-MYC also needed AP4 induction. Lung metastasis in mice was reduced when AP4 was inhibited in CRC cells. Increased AP4 expression was linked to liver metastases and poor patient survival in primary CRC. These findings point to AP4 as a novel EMT regulator that plays a role in CRC and maybe other carcinomas' metastatic processes.

Uncertainties and Inconsistencies

Whenever cell phenotype plasticity is crucial and under investigation, the reverse of EMT, known as the mesenchymal-epithelial transition (MET), may be one of the prospects for anti-cancer therapy (Shibue & Weinberg, 2017).

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Cell line,humans,Human cell line studies	qRT-PCR,,Luciferase reporter assay ,immunoblotting,immunoprecipitation,cell invasion assay,cell migration assay, bioluminesence imaging,wound healing assay,Wound scratch & Transwell assay, Microarray,Immunofluorescence, Immunohistochemistry	Yes	Strong	Yes	Yes	Yes

Response-response relationship

TGFbeta and Twist induce EMT by upregulating the expression of EMT markers such Snail, Vimentin, N-cadherin, and ABC transporters like ABCA3, ABCC1, ABCC3, and ABCC10 (Saxena et al., 2011).In the treatment with about 0.3, 3, 30 mM of doxorubicin, human mammary epithelial cells (HMLE) stably expressing Twist, FOXC2 or Snail demonstrate increased cell viability compared to control HMLE, dose-dependently (Saxena et al., 2011).

Time-scale

When Twist/FOXC2/Snail overexpressed HMLE is treated with doxorubicin for 48 hours, cell viability increases compared to control HMLE (Saxena et al., 2011).When Twist or Zeb1 were inhibited with small interference RNA (siRNA), cell viability was reduced relative to control MDAMB231 cells treated with doxorubicin for 48 hours (Saxena et al., 2011).

Known modulating factors

In EMT-activated cells, ABC transporters linked to drug resistance are overexpressed (Saxena et al., 2011). In EMT-activated cells, the expression of PD-L1, which binds to PD-1 on cytotoxic T cells, is upregulated, inhibiting cancer immunity and increasing resistance to cancer therapy (Shibue & Weinberg, 2017).

Known Feedforward/Feedback loops influencing this KER

- Understanding the association between EMT and cancer malignancy necessitates further research into the EMT-cancer stem cells (CSC) relationship. Non-CSCs in cancer can spontaneously undergo EMT and dedifferentiate into new CSCs, resulting in tumorigenic potential renewal (Marjanovic, Weinberg, & Chaffer, 2013; Shibue & Weinberg, 2017).The plastic CSC theory demonstrates bidirectional conversions between non-CSCs and CSCs, which could help EMT-activated cells acquire cancer malignancy (Marjanovic et al., 2013).

- Long non-coding RNAs (lncRNAs) play crucial roles in many biological and pathological processes, including tumor metastasis. Kong et al reported a novel lncRNA, LINC01133 that was downregulated by TGF- β, which could inhibit epithelial–mesenchymal transition (EMT) and metastasis in colorectal cancer (CRC) cells (Kong et al.,2016). SRSF6, an alternative splicing factor that interacts directly with LINC01133, was found to enhance EMT and metastasis in CRC cells even when LINC01133 was not present. The study also found that the EMT process in CRC cells was regulated by LINC01133 in the presence of SRSF6. In vivo, the ability of LINC01133 to prevent metastasis was confirmed. Furthermore, clinical data revealed that LINC01133 expression was favourably correlated with E-cadherin and negatively correlated with Vimentin, and that low LIINC01133 expression in tumours was associated with poor CRC survival. These findings show that LINC01133, by directly binding to SRSF6 as a target mimic and inhibiting EMT and metastasis, could be used as a predictive biomarker and an effective target for anti-metastasis therapy in CRC.
- MiR-148a inhibited Met expression directly by binding to its 30-UTR, according to Zhang et al's findings. Furthermore, reintroducing miR-148a reduced the nuclear accumulation of Snail, a transcription factor that promotes EMT, by inhibiting Met's downstream signalling, such as activating phosphorylation of AKT-Ser473 and inhibitory phosphorylation of GSK-3b-Ser9 (Zhang et al.,2015). MiR-148a, when combined, may suppress hepatoma cell EMT and metastasis by adversely regulating Met/Snail signalling.

References

- BOYER, B., & THIERY, J. P. (1993). Epithelium-mesenchyme interconversion as example of epithelial plasticity. *Apmis*, *101*(1-6), 257-268.

Chen, S. P., Liu, B. X., Xu, J., Pei, X. F., Liao, Y. J., Yuan, F., & Zheng, F. (2015). MiR-449a suppresses the epithelial-mesenchymal transition and metastasis of hepatocellular carcinoma by multiple targets. *BMC cancer*, *15*(1), 1-13.

- Cui, B., Zhang, S., Chen, L., Yu, J., Widhopf, G. F., Fecteau, J. F., ... & Kipps, T. J. (2013). Targeting ROR1 inhibits epithelial–mesenchymal transition and metastasis. *Cancer research*, *73*(12), 3649-3660.

Chen, Y., Wang, D. D., Wu, Y. P., Su, D., Zhou, T. Y., Gai, R. H., ... & Yang, B. (2017). MDM2 promotes epithelial–mesenchymal transition and metastasis of ovarian cancer SKOV3 cells. *British journal of cancer*, *117*(8), 1192-1201..

- Casas, E., Kim, J., Bendesky, A., Ohno-Machado, L., Wolfe, C. J., & Yang, J. (2011). Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis. *Cancer research*, *71*(1), 245-254.

- Chen, L., Mai, W., Chen, M., Hu, J., Zhuo, Z., Lei, X., ... & Zhang, D. (2017). Arenobufagin inhibits prostate cancer epithelial-mesenchymal transition and metastasis by down-regulating β-catenin. *Pharmacological research*, *123*, 130-142.

Chen, L., Gibbons, D. L., Goswami, S., Cortez, M. A., Ahn, Y. H., Byers, L. A., ... & Qin, F. X. F. (2014). Metastasis is regulated via microRNA-200/ZEB1 axis control of tumour cell PD-L1 expression and intratumoral immunosuppression. *Nature communications*, *5*(1), 1-12.

Da, C., Wu, K., Yue, C., Bai, P., Wang, R., Wang, G., ... & Hou, P. (2017). N-cadherin promotes thyroid tumorigenesis through modulating major signaling pathways. *Oncotarget*, *8*(5), 8131.

Du, B., & Shim, J. S. (2016). Targeting epithelial–mesenchymal transition (EMT) to overcome drug resistance in cancer. *Molecules*, *21*(7), 965.

- Gao, J., Yang, Y., Qiu, R., Zhang, K., Teng, X., Liu, R., & Wang, Y. (2018). Proteomic analysis of the OGT interactome: novel links to epithelial–mesenchymal transition and metastasis of cervical cancer. *Carcinogenesis*, *39*(10), 1222-1234.

- Gumireddy, K., Li, A., Gimotty, P. A., Klein-Szanto, A. J., Showe, L. C., Katsaros, D., ... & Huang, Q. (2009). KLF17 is a negative regulator of epithelial–mesenchymal transition and metastasis in breast cancer. *Nature cell biology*, *11*(11), 1297-1304.
- Gujral, T. S., Chan, M., Peshkin, L., Sorger, P. K., Kirschner, M. W., & MacBeath, G. (2014). A noncanonical Frizzled2 pathway regulates epithelial-mesenchymal transition and metastasis. *Cell*, *159*(4), 844-856.

Huang, Y., Zhao, M., Xu, H., Wang, K., Fu, Z., Jiang, Y., & Yao, Z. (2014). RASAL2 down-regulation in ovarian cancer promotes epithelial-mesenchymal transition and metastasis. *Oncotarget*, *5*(16), 6734.

- Huang, R., & Zong, X. (2017). Aberrant cancer metabolism in epithelial–mesenchymal transition and cancer metastasis: Mechanisms in cancer progression. *Critical reviews in oncology/hematology*, *115*, 13-22.

- Inukai, T., Inoue, A., Kurosawa, H., Goi, K., Shinjyo, T., Ozawa, K., ... & Look, A. T. (1999). SLUG, a ces-1-related zinc finger transcription factor gene with antiapoptotic activity, is a downstream target of the E2A-HLF

oncoprotein. *Molecular cell*, *4*(3), 343-352.
- Irani, S., & Dehghan, A. (2018). The expression and functional significance of vascular endothelial-cadherin, CD44, and vimentin in oral squamous cell carcinoma. *Journal of International Society of Preventive & Community Dentistry*, *8*(2), 110.
- Jackstadt, R., Röh, S., Neumann, J., Jung, P., Hoffmann, R., Horst, D., ... & Hermeking, H. (2013). AP4 is a mediator of epithelial–mesenchymal transition and metastasis in colorectal cancer. *Journal of Experimental Medicine*, *210*(7), 1331-1350.
- Kong, J., Sun, W., Li, C., Wan, L., Wang, S., Wu, Y., ... & Lai, M. (2016). Long non-coding RNA LINC01133 inhibits epithelial–mesenchymal transition and metastasis in colorectal cancer by interacting with SRSF6. *Cancer letters*, *380*(2), 476-484.
- Kudo-Saito, C., Shirako, H., Takeuchi, T., & Kawakami, Y. (2009). Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. *Cancer cell*, *15*(3), 195-206.
- Liang, Y. J., Wang, Q. Y., Zhou, C. X., Yin, Q. Q., He, M., Yu, X. T., ... & Zhao, Q. (2013). MiR-124 targets Slug to regulate epithelial–mesenchymal transition and metastasis of breast cancer. *Carcinogenesis*, *34*(3), 713-722.
- Liu, Y., Wang, G., Yang, Y., Mei, Z., Liang, Z., Cui, A., ... & Cui, L. (2016). Increased TEAD4 expression and nuclear localization in colorectal cancer promote epithelial–mesenchymal transition and metastasis in a YAP-independent manner. *Oncogene*, *35*(21), 2789-2800.

Liu, M., Xiao, Y., Tang, W., Li, J., Hong, L., Dai, W., ... & Xiang, L. (2020). HOXD9 promote epithelial-mesenchymal transition and metastasis in colorectal carcinoma. *Cancer medicine*, *9*(11), 3932-3943.

Marjanovic, N. D., Weinberg, R. A., & Chaffer, C. L. (2013). Cell plasticity and heterogeneity in cancer. *Clinical chemistry*, *59*(1), 168-179.

- Pirozzi, G., Tirino, V., Camerlingo, R., Franco, R., La Rocca, A., Liguori, E., ... & Rocco, G. (2011). Epithelial to mesenchymal transition by TGFβ-1 induction increases stemness characteristics in primary non small cell lung cancer cell line. *PloS one*, *6*(6), e21548.

- Pradella, D., Naro, C., Sette, C., & Ghigna, C. (2017). EMT and stemness: flexible processes tuned by alternative splicing in development and cancer progression. *Molecular cancer*, *16*(1), 1-19.
- Sarkar, T. R., Battula, V. L., Werden, S. J., Vijay, G. V., Ramirez-Peña, E. Q., Taube, J. H., ... & Mani, S. A. (2015). GD3 synthase regulates epithelial–mesenchymal transition and metastasis in breast cancer. *Oncogene*, *34*(23), 2958-2967.
- Saxena, M., Stephens, M. A., Pathak, H., & Rangarajan, A. (2011). Transcription factors that mediate epithelial–mesenchymal transition lead to multidrug resistance by upregulating ABC transporters. *Cell death & disease*, *2*(7), e179-e179.
- Shibue, T., & Weinberg, R. A. (2017). EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. *Nature reviews Clinical oncology*, *14*(10), 611-629.
- Shiota, M., Zardan, A., Takeuchi, A., Kumano, M., Beraldi, E., Naito, S., ... & Gleave, M. E. (2012). Clusterin mediates TGF-β–induced epithelial–mesenchymal transition and metastasis via Twist1 in prostate cancer cells. *Cancer research*, *72*(20), 5261-5272.
- Smith, B. N., & Bhowmick, N. A. (2016). Role of EMT in metastasis and therapy resistance. *Journal of clinical medicine*, *5*(2), 17.
- Tanabe, S. (2013). Perspectives of gene combinations in phenotype presentation. *World journal of stem cells*, *5*(3), 61.
- Tanabe, S. (2015). Origin of cells and network information. *World journal of stem cells*, *7*(3), 535.
- Tanabe, S. (2015). Signaling involved in stem cell reprogramming and differentiation. *World journal of stem cells*, *7*(7), 992.
- Tanabe, S., Aoyagi, K., Yokozaki, H., & Sasaki, H. (2016). Regulation of CTNNB1 signaling in gastric cancer and stem cells. *World journal of gastrointestinal oncology*, *8*(8), 592–598.
- Tanabe, S., Aoyagi, K., Yokozaki, H., & Sasaki, H. (2015). Regulated genes in mesenchymal stem cells and gastric cancer. *World journal of stem cells*, *7*(1), 208.
- Wang, L., Tong, X., Zhou, Z., Wang, S., Lei, Z., Zhang, T., ... & Zhang, H. T. (2018). Circular RNA hsa_circ_0008305 (circPTK2) inhibits TGF-β-induced epithelial-mesenchymal transition and metastasis by controlling TIF1γ in non-small cell lung cancer. *Molecular cancer*, *17*(1), 1-18.
- Wu, W. S., Heinrichs, S., Xu, D., Garrison, S. P., Zambetti, G. P., Adams, J. M., & Look, A. T. (2005). Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing puma. *Cell*, *123*(4), 641-653.
- Yu, C. P., Yu, S., Shi, L., Wang, S., Li, Z. X., Wang, Y. H., ... & Liang, J. (2017). FoxM1 promotes epithelial-mesenchymal transition of hepatocellular carcinoma by targeting Snai1. *Molecular medicine reports*, *16*(4), 5181-5188.
- Yu, J., Lei, R., Zhuang, X., Li, X., Li, G., Lev, S., ... & Hu, G. (2016). MicroRNA-182 targets SMAD7 to potentiate TGFβ-induced epithelial-mesenchymal transition and metastasis of cancer cells. *Nature communications*, *7*(1), 1-12.
- Yue, B., Song, C., Yang, L., Cui, R., Cheng, X., Zhang, Z., & Zhao, G. (2019). METTL3-mediated N6-methyladenosine modification is critical for epithelial-mesenchymal transition and metastasis of gastric cancer. *Molecular cancer*, *18*(1), 1-15.
- Zhang, P., Sun, Y., & Ma, L. (2015). ZEB1: at the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. *Cell cycle*, *14*(4), 481-487.
- Zhang, J. P., Zeng, C., Xu, L., Gong, J., Fang, J. H., & Zhuang, S. M. (2014). MicroRNA-148a suppresses the epithelial–mesenchymal transition and metastasis of hepatoma cells by targeting Met/Snail signaling. *Oncogene*, *33*(31), 4069-4076.
- Zhang, W., Shi, X., Peng, Y., Wu, M., Zhang, P., Xie, R., ... & Wang, J. (2015). HIF-1α promotes epithelial-mesenchymal transition and metastasis through direct regulation of ZEB1 in colorectal cancer. *PloS one*, *10*(6), e0129603.

List of Non Adjacent Key Event Relationships

Relationship: 2728: Increased, DNA damage and mutation leads to Increase chromosomal aberrations

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	non-adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mice	Mus sp.	High	NCBI
human	Homo sapiens	High	NCBI
human and other cells in culture	human and other cells in culture	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

Not specific,

Key Event Relationship Description

Increased DNA damage leads to increased chromosomal aberrations

The presented relationship outlines a direct correlation between two genetic events. The upstream event, "Increased DNA damage," signifies an augmentation in the occurrence of genetic lesions and alterations within the DNA molecule. This damage can result from various sources, such as exposure to radiation, chemicals, or errors during DNA replication.

The downstream event in this relationship is "increased chromosomal aberrations," which signifies a rise in the number or frequency of structural abnormalities in chromosomes. Chromosomal aberrations can encompass various changes, including deletions, insertions, translocations, or inversions of genetic material within chromosomes.

This relationship underscores the close connection between genetic lesions and chromosomal abnormalities. Increased DNA damage can directly contribute to an elevated occurrence of chromosomal aberrations, as the integrity of DNA is essential for maintaining the proper structure of chromosomes. Understanding this relationship is crucial in the context of genomic stability and its implications for various biological outcomes, including genetic disorders and cancer development.

Evidence Supporting this KER

DNA double-strand breaks (DSB) are the crucial lesions underlying the formation of CA [M.A Bender et al.,1974; G.Obe et al., 2002]. Chromosomes are uninemic; each chromatid contains one continuous DNA molecule. Consequently, an unrepaired DSB, appears at mitosis as a terminal deletion (or an incomplete exchange), leading to loss of genetic material and eventually cell death or loss of heterozygosity in diploid cells. On the other hand, misrepaired DSB generate intra- or inter-chromosomal exchanges which may or may not be lethal, depending on the exact form they take. Concluding a controversy that lasted a number of years ([K.H Chandwick et al.,1981] and references therein), there is now a general agreement that the dose–response curve for the induction of DSB is linear over several orders of magnitude [K Rothkamm et al., 2003]

Biological Plausibility

DNA damage and unrepaired or insuffifficiently repaired DNA double-strand breaks as well as telomere shortening contribute to the formation of structural chromosomal

aberrations (CAs). Non-specifific CAs have been used in the monitoring of individuals

exposed to potential carcinogenic chemicals and radiation. The frequency of CAs in

peripheral blood lymphocytes (PBLs) has been associated with cancer risk and the

association has also been found in incident cancer patients. CAs include chromosome

type aberrations (CSAs) and chromatid-type aberrations (CTAs) and their sum CAtot.

Structural CAs may be specifific, such as translocations and inversions, or non-specifific, such as chromatid breaks, fragmented or missing parts of chromosomes, and fusions

resulting in dicentric and ring chromosomes (Bignold, 2009).

The former are often recurrent and they are currently analyzed by molecular cytogenetic methods while the latter are scored by classical cytogenetic techniques, which are able to recognize chromosome-type aberrations (CSAs) and chromatid-type aberrations (CTAs) according to morphological changes (Hagmar et al., 2004). CTAs are formed due to insufficiently repaired double-strand breaks (DS00Bs) during the late S or G2 phase of the

cell cycle (Natarajan and Palitti, 2008; Bignold, 2009; Durante et al., 2013), whereas CSAs are the result of direct DNA damage due to radiation, chemical mutagens, or shortening of telomeres during the G0/G1 phase (Albertini et al., 20; Jones et al., 2012). Non-specifific CAs have been used in the monitoring of populations occupationally exposed to potential carcinogenic chemicals and radiation and an increased frequency of CAs in peripheral blood lymphocytes (PBLs) has been associated with cancer risk and the association has also been found in incident cancer patients (Rossner et al., 2005; Vodicka et al., 2010; Vodenkova et al., 2015).

Unrepaired or insuffiffifficiently repaired DSBs, as well as telomerase dysfunction, represent the mechanistic bases for the formation of structural CAs (Natarajan and Palitti, 2008;

Bignold, 2009; Durante et al., 2013; Vodicka et al., 2018; Srinivas et al., 2020). However, even other types of DNA repair pathways may contribute to CA formation as these are

found in inherited syndromes manifesting DNA repair gene mutations (Rahman, 2014).

Empirical Evidence

A study by Solange et al evaluated the effects of exposure to formaldehyde (FA) in human peripheral blood lymphocytes, a group of laboratory workers exposed occupationally to FA and control subjects were tested for chromosomal aberrations (CAs) and DNA damage (comet assay)(Solange et al 2015). The level of exposure to FA in the workplace air was evaluated. The association between genotoxicity biomarkers and polymorphic genes of xenobiotic - metabolising and DNA repair enzymes were also assessed.

All cytogenetic parameters evaluated—total-CA, CSAs, CTAs, gaps and aneuploidies—were significantly elevated in anatomy pathology professionals exposed to FA (mean 0.38 ppm) compared with control subjects. FA-exposed individuals showed an increase of 91% in total-CAs frequency compared with controls. Mean frequencies of both CAs types, CSAs and CTAs were also significantly higher in exposed workers . Although there is a paucity of studies assessing CAs in FA occupationally exposed subjects, our findings are in agreement with most of the published literature. (He.J.L *et al.* 1998)found higher frequencies of CAs in PBLs of 13 anatomy students exposed to FA (mean level 2.37 ppm) during a 12-week anatomy class. Similarly, in a recent study involving FA-exposed personnel working in pathology departments ($n = 21$; mean level 0.72 ppm), total-CA and CTAs were significantly elevated

compared with controls (Jakab et al., 2010). A significant increase in CAs frequencies was also observed in industrial workers (kitaeva et al.,1996).

Multiaberrant cells frequency was significantly higher (4-fold) in FA-exposed workers than in control individuals, whereas aberrant cells frequency was significantly increased by 1.7-fold in the exposed group(Solange et al., 2015).

Uncertainties and Inconsistencies

In contrast, no significant differences were found in CAs frequencies between individuals working in different laboratories of a Cancer Research Institute, including an anatomical pathology laboratory (Pala M et al.,2008)

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Mice	Chromosomal abberation assay, Genotoxicity assessment assay, (Evgenii Plotnikov., et al 2016) CT8 Assay (Francesco Marchett., et al 2015)	+	Strong	Yes	Yes	Yes
Human	Micronuclease (CBMN) Assay, Comet assay, (Qiang Liu., et al 2009.) CAs analysis, Comet assay, PCR-RFLP (S. Costa et al., 2015)	+	Strong	Yes	Yes	Yes
Human Cell lines	Polyploid assay, Sister chromatid exchange test, V79/HPRT mutation assay, Cell transformation assay, Tumorigenicity test, ,spore rec assay (Hirohisa Tsuda et al., 1993)	+	Strong	Yes	Yes	Yes

Response-response relationship

Multiaberrant cells frequency was significantly higher (4-fold) in formaldehyde-exposed workers than in control individuals, whereas aberrant cells frequency was significantly increased by 1.7-fold in the exposed group(Solange et al., 2015).

Time-scale

It is generally accepted that exchanges formed in the G1-phase originate from the interaction of two spatially distinct radiogenic damaged sites (DSB) [Heck et al., 2008], which runs counter to the once-popular concept encompassed by so-called “one-hit models” for the formation of translocations, dicentrics and other exchanges [Pala M et al.,2008].

Known Feedforward/Feedback loops influencing this KER

Not known.

References

Albertini, R. J., Anderson, D., Douglas, G. R., Hagmar, L., Hemminki, K., Merlo, F., ... & Aitio, A. (2000). IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. *Mutation*

Research/Reviews in Mutation Research, *463*(2), 111-172.

Bignold, L. P. (2009). Mechanisms of clastogen-induced chromosomal aberrations: A critical review and description of a model based on failures of tethering of DNA strand ends to strand-breaking enzymes. *Mutation Research/Reviews in Mutation Research*, *681*(2-3), 271-298.

Costa, S., Carvalho, S., Costa, C., Coelho, P., Silva, S., Santos, L. S., ... & Teixeira, J. P. (2015). Increased levels of chromosomal aberrations and DNA damage in a group of workers exposed to formaldehyde. *Mutagenesis*, *30*(4), 463-473.

Durante, M., Bedford, J. S., Chen, D. J., Conrad, S., Cornforth, M. N., Natarajan, A. T., ... & Obe, G. (2013). From DNA damage to chromosome aberrations: joining the break. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *756*(1-2), 5-13.

G. Obe, P. Pfeiffer, J.R.K. Savage, C. Johannes, W. Goedecke, P. Jeppsen, A.T. Natarajan,W.Martinez-Lopez, G.A. Folle,M.E. (2002) Drets,Chromosomal aberrations: formation, identifification, and distribution, Mutat. Res. 504.3–16.

Hagmar, L., Strömberg, U., Tinnerberg, H., & Mikoczy, Z. (2004). Epidemiological evaluation of cytogenetic biomarkers as potential surrogate end-points for cancer. *IARC scientific publications*, (157), 207-215.

He, J. L., Jin, L. F., & Jin, H. Y. (1998). Detection of cytogenetic effects in peripheral lymphocytes of students exposed to formaldehyde with cytokinesis-blocked micronucleus assay. *Biomedical and environmental sciences: BES*, *11*(1), 87-92.

Heck, H. D. A., & Casanova, M. (2004). The implausibility of leukemia induction by formaldehyde: a critical review of the biological evidence on distant-site toxicity. *Regulatory Toxicology and Pharmacology*, *40*(2), 92-106.

Jakab, M. G., Klupp, T., Besenyei, K., Biró, A., Major, J., & Tompa, A. (2010). Formaldehyde-induced chromosomal aberrations and apoptosis in peripheral blood lymphocytes of personnel working in pathology departments. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *698*(1-2), 11-17.

Jones, C. H., Pepper, C., & Baird, D. M. (2012). Telomere dysfunction and its role in haematological cancer. *British journal of haematology*, *156*(5), 573-587.

K.H. Chadwick, H.P. Leenhouts, The Molecular Theory of Radiation Biology, Springer-Verlag, Berlin, (1981).

Kitaeva, L. V., Mikheeva, E. A., Shelomova, L. F., & Pla, S. (1996). Genotoxic effect of formaldehyde in somatic human cells in vivo. *Genetika*, *32*(9), 1287-1290.

K. Rothkamm, M. Löbrich, (2003).Evidence for a lack of DNAdouble-strand break repair in human cells exposed to very low X-ray doses, Proc. Natl. Acad. Sci. U. S. A. 100. 5057–5062.

Liu, Q., Cao, J., Li, K. Q., Miao, X. H., Li, G., Fan, F. Y., & Zhao, Y. C. (2009). Chromosomal aberrations and DNA damage in human populations exposed to the processing of electronics waste. *Environmental science and pollution research*, *16*(3), 329-338.

ALL. A. Bender, H.G. Griggs, J.S. Bedford,(1974). Mechanisms of chromosomal aberration production. III: chemicals and ionizing radiation, Mutat. Res. 23.197–212.

Marchetti, F., Bishop, J., Gingerich, J., & Wyrobek, A. J. (2015). Meiotic interstrand DNA damage escapes paternal repair and causes chromosomal aberrations in the zygote by maternal misrepair. *Scientific reports*, *5*(1), 1-7.

Natarajan, A. T., & Palitti, F. (2008). DNA repair and chromosomal alterations. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *657*(1), 3-7.

Pala, M., Ugolini, D., Ceppi, M., Rizzo, F., Maiorana, L., Bolognesi, C., ... & Vecchio, D. (2008). Occupational exposure to formaldehyde and biological monitoring of Research Institute workers. *Cancer detection and prevention*, *32*(2), 121-126.

Plotnikov, E., Silnikov, V., Gapeyev, A., & Plotnikov, V. (2016). Investigation of DNA-damage and chromosomal aberrations in blood cells under the influence of new silver-based antiviral complex. *Advanced Pharmaceutical Bulletin*, *6*(1), 71.

Rahman, N. (2014). Realizing the promise of cancer predisposition genes. *Nature*, *505*(7483), 302-308.

Rossner, P., Boffetta, P., Ceppi, M., Bonassi, S., Smerhovsky, Z., Landa, K., ... & Šrám, R. J. (2005). Chromosomal aberrations in lymphocytes of healthy subjects and risk of cancer. *Environmental health perspectives*, *113*(5), 517-520.

Solange Costa, Sandra Carvalho, Carla Costa, Patrícia Coelho, Susana Silva, Luís S. Santos, Jorge F. Gaspar, Beatriz Porto, Blanca Laffon, João P. Teixeira, Increased levels of chromosomal aberrations and DNA damage in a group of workers exposed to formaldehyde, *Mutagenesis*, Volume 30, Issue 4, July 2015, Pages 463–473,

Srinivas, N., Rachakonda, S., & Kumar, R. (2020). Telomeres and telomere length: a general overview. *Cancers*, *12*(3), 558.

Tsuda, H., Shimlzu, C. S., Taketomi, M. K., Hasegawa, M. M., Hamada, A., Kawata, K. M., & Inui, N. (1993). Acrylamide; induction of DNA damage, chromosomal aberrations and cell transformation without gene mutations. *Mutagenesis*, *8*(1), 23-29.

Vodicka, P., Musak, L., Vodickova, L., Vodenkova, S., Catalano, C., Kroupa, M., ... & Hemminki, K. (2018). Genetic variation of acquired structural chromosomal aberrations. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *836*, 13-21.

Vodicka, P., Polivkova, Z., Sytarova, S., Demova, H., Kucerova, M., Vodickova, L., ... & Hemminki, K. (2010). Chromosomal damage in peripheral blood lymphocytes of newly diagnosed cancer patients and healthy controls. *Carcinogenesis*, *31*(7), 1238-1241.

Vodenkova, S., Polivkova, Z., Musak, L., Smerhovsky, Z., Zoubkova, H., Sytarova, S., ... & Vodicka, P. (2015). Structural chromosomal aberrations as potential risk markers in incident cancer patients. *Mutagenesis*, *30*(4), 557-563.

Relationship: 2729: Increase chromosomal aberrations leads to Increase,miRNA levels

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	non-adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	High

Not specific through any particular life stage or gender

Key Event Relationship Description

KER :Increased, chromosomal aberration leads to microRNA expression, increased

Upstream event: increased, chromosomal aberration

Downstream event: increased miRNA expression

The depicted Key Event Relationship (KER) outlines a sequence of events involving genetic alterations and their potential impact on microRNA (miRNA) regulation. The upstream event, "Increased chromosomal aberration," suggests an elevation in the occurrence of structural abnormalities within chromosomes. These aberrations can encompass various changes, including deletions, duplications, inversions, or translocations of genetic material.

The downstream event in this KER is "increased miRNA expression," signifying a rise in the levels of microRNA molecules within the cell. Genetic alterations, such as chromosomal aberrations, can influence the expression of miRNAs, leading to changes in their abundance.

This KER underscores the potential interplay between genetic changes and miRNA regulation. Genetic alterations can influence miRNA expression patterns, potentially impacting downstream gene expression and cellular responses. Understanding these relationships contributes to a broader understanding of how genomic changes can influence post-transcriptional gene regulation and cellular processes.

Evidence Supporting this KER

The first report linking a chromosomal breakpoint with the genomic location of miRNAs was published a couple of decades ago (Gauwerky et al.,1989). A masked t(8;17) translocation resulted in a high activation of the MYC OG: *MYC* from chromosome 8 was truncated at the end of the first exon (which is noncoding), and the coding region joined the regulatory elements of a gene located on chromosome 17, called *BCL3* (B cell leukemia/lymphoma 3). Despite extensive genomic search, *BCL3* remained an elusive entity until the identification of the human miRNAs. Fifteen years after the initial discovery, the *miR-142* gene was found to be located 50 nt from the t(8;17) break involving chromosome 17 and *MYC,* meaning that the regulatory elements of this miRNA are likely involved in the overexpression of MYC (Calin, G.A., et al. 2002). The clinical consequences were dramatic for the patient, leading to aggressive acute prolymphocytic leukemia (Gauwerky et al.,1989). Apart from the involvement in the t(8;17) breakpoint of B cell acute leukemia, *miR-142-3p* and *miR-142-5p* are also within the 17q23 minimal amplicon described in breast cancer (Barlund, M., et al. 2000) and near the FRA17B site, a target for HPV16 integration in cervical tumors (Calin, G.A., et al. 2004).

Biological Plausibility

Chromosomal translocations alter PCG loci through two main mechanisms (Russo, G., et al. 1988). The first is the juxtaposition of promoter/enhancer elements from one gene to the intact coding region of another gene, while the second is the recombination of the coding regions of two different genes. The former is more frequently found in B and T cell lymphomas and leukemias and the latter in human myeloid leukemias and soft-tissue sarcomas. The translocations that alter miRNA loci can be classified by analogy with these mechanisms . At least five different situations can be postulated, the last three of which have yet to be identified in human cancers: (a) juxtaposition of promoter/enhancer elements from miRNA genes to a PCG ORF with overexpression of the protein [e.g., t(8;17)(q24;q22)]; (b) disruption of the region of interaction between the target PCG and the interactor miRNA with the disruption of the repression and the overexpression of the protein (e.g., 12q15 translocations involving HMGA2 gene); (c) juxtaposition of promoter/enhancer elements from PCG to a miRNA gene with overexpression of the noncoding gene; (d) juxtaposition of promoter/enhancer elements from miRNA to another miRNA gene with overexpression of the noncoding gene (termed “promoter swapping”); and (e) miRNA gene–to–miRNA gene fusion with the consequent production of a “new” cluster of coexpressed or independently expressed miRNAs.

Empirical Evidence

miRNA genes are located near breakpoint regions. *miR-180* is only 1 kb from the *MN1* gene involved in a t(4;22) chromosomal translocation in meningioma that inactivates *MN1* and possibly the miRNA gene located in the same position. Also, in a patient with precursor B cell acute lymphoblastic leukemia, an insertion of *miR-125b-1* into a rearranged immunoglobulin heavy-chain locus was described, possibly as an early step in leukemogenesis (Sonoki, T et al., 2009-2010). Chromosomal translocations fusing the BCL-6 OG to the regulatory elements of *miR-28* or to the lipoma preferred partner were described in primary central nervous system lymphomas and may be associated with aberrant somatic hypermutation or defective class switch recombination (Schwindt, H., et al. 2006).

Uncertainties and Inconsistencies

The contribution of microRNAs (miR) to the pathogenesis of mantle cell lymphoma (MCL) is not well known. The expression of 86 mature miRs mapped to frequently altered genomic regions in MCL in CD5+ /CD5 normal B cells, reactive lymph nodes, and purified tumor cells of 17 leukemic MCL, 12 nodal MCL, and 8MCL cell lines were investigated. Genomic alterations of the tumors were studied by single nucleotide polymorphism arrays and comparative genomic hybridization. Leukemic and nodal tumors showed a high number of differentially expressed miRs compared with purified normal B cells, but only some of them were commonly deregulated in both tumor types. An unsupervised analysis of miR expression profile in purified leukemic MCL cells revealed two clusters of tumors characterized by different mutational status of the immunoglobulin genes, proliferation signature, and number of genomic alterations. The expression of most miRs was not related to copy number changes in their respective chromosomal loci. Only the levels of miRs included in the miR-17-92 cluster were significantly related to genetic alterations at 13q31. Moreover, overexpression of miR-17-5p/miR-20a from this cluster was associated with high MYC mRNA levels in tumors with a more aggressive behavior. In conclusion, the miR expression pattern of MCL is deregulated in comparison with normal lymphoid cells and distinguishes two subgroups of tumors with different biological features.

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human	Microarray, CGH analysis (dehan et al., 2007)	+	Strong	Yes	Yes	Yes
Human cell line and blood samples	Northern blotting (Calin et al., 2004)	+	Strong	Yes	Yes	Yes
	Cytogenetic techniques (Lionetti M et al., 2009 ; Min DJ et al., 2013 ;Huang JJ et al., 2012 ; Pichiorri F, et al., 2011; Roccaro AM et al., 2009; Gao X et al., 2009 ; Corthals SL et al.,2010 ; Pichiorri F et al., 2008 ; Yang RF et al., 2010; Rio-Machin A et al., 2013 ; Gutiérrez NC et al., 2010 ; Kuehl WM et al.,	+	Strong	Yes	Yes	Yes

	2012 ; Pichiorri F et al., 2010 ; Gatt ME et al., 2011 ; Zhang Y-K et al., 2011 ;Misiewicz-Krzeminska I et al., 2013;Wong KY et al., 2011 ;Chim CS et al., 2010)					

Response-response relationship

Detailed investigation of the 13q14.3 deletions showed that both members of an miRNA cluster, *miR-15a* and *miR-16-1*, are deleted or downregulated in approximately 68% of CLL cases as compared with healthy donors (Calin, G.A., et al. 2002). Furthermore, a rare mutation lowering the expression of these genes was identified in two CLL patients including one from a family with individuals having CLL and breast cancer, and was found to be associated with the loss of the normal allele in the leukemic cells (Calin, G.A., et al. 2005). It was shown that the levels of both *miR-15* and *miR-16* inversely correlate with the BCL-2 protein expression and that BCL-2 repression by these miRNAs induces apoptosis in leukemia cells (Cimmino, A., et al. 2005).

Levels of *miR-16* were decreased in NZB lymphoid tissue, and exogenous *miR-16* delivered to an NZB malignant B-1 cell line resulted in cell cycle alterations and increased apoptosis. Linkage of the *miR-15a/miR-16-1* complex to the development of CLL in this spontaneous mouse model suggests that the altered expression of these genes is the molecular lesion in CLL (Raveche, E.S., et al. 2007).

The only miRNA found to be overexpressed in any type of solid tumor analyzed (breast, colon, lung, prostate, stomach, and endocrine pancreas tumors, glioblastomas, and uterine leiomyomas) is *miR-21* (Volinia, S., et al. 2006, Ciafre, S.A., et al. 2005; . Krichevsky et al.,2003; Wang, T., et al. 2007). This gene is located in the 3′UTR of the vacuole membrane protein 1 (*VMP1*) gene at chromosome 17q23.2, a region frequently found amplified in neuroblastomas and breast, colon, and lung cancers. Knockdown of *miR-21* in glioblastoma cell lines induces a caspase-mediated apoptosis, further supporting the oncogenic role of this miRNA (Chan, et al.,2005).

Time-scale

Studies performed in solid cancer cell lines showed that *miR-16* negatively regulated cellular growth and cell cycle progression. *miR-16*–downregulated transcripts were enriched with genes whose silencing by small interfering RNAs causes an accumulation of cells in G0/G1. Simultaneous silencing of these genes was more effective at blocking cell cycle progression than was disruption of the individual genes. Thus, *miR-16* coordinately regulates targets that may act in concert to control cell cycle progression (Linsley, P.S., et al. 2007)

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
UV rays,cisplatin, doxorubicin, IR	Impaired DNA repair	Altered miRNA expression	Pothof et al., 2009,Galluzzi et al., 2010, Saleh et.al.,2011;Suzuki et al.,2009

Known Feedforward/Feedback loops influencing this KER

Not mentioned.

References

Bärlund, M., Monni, O., Kononen, J., Cornelison, R., Torhorst, J., Sauter, G., ... & Kallioniemi, A. (2000). Multiple genes at 17q23 undergo amplification and overexpression in breast cancer. *Cancer research*, *60*(19), 5340-5344.

Calin, G. A., Dumitru, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., ... & Croce, C. M. (2002). Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the national academy of sciences*, *99*(24), 15524-15529.

Calin, G. A., Sevignani, C., Dumitru, C. D., Hyslop, T., Noch, E., Yendamuri, S., ... & Croce, C. M. (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proceedings of the National Academy of Sciences*, *101*(9), 2999-3004.

Calin, G. A., Ferracin, M., Cimmino, A., Di Leva, G., Shimizu, M., Wojcik, S. E., ... & Croce, C. M. (2005). A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *New England Journal of Medicine*, *353*(17), 1793-1801.

Calin, G. A., & Croce, C. M. (2006). MicroRNAs and chromosomal abnormalities in cancer cells. *Oncogene*, *25*(46), 6202-6210.

Chan, J. A., Krichevsky, A. M., & Kosik, K. S. (2005). MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer research*, *65*(14), 6029-6033.

Chim, C. S., Wong, K. Y., Qi, Y., Loong, F., Lam, W. L., Wong, L. G., ... & Liang, R. (2010). Epigenetic inactivation of the miR-34a in hematological malignancies. *Carcinogenesis*, *31*(4), 745-750.

Ciafre, S. A., Galardi, S., Mangiola, A., Ferracin, M., Liu, C. G., Sabatino, G., ... & Farace, M. G. (2005). Extensive modulation of a set of microRNAs in primary glioblastoma. *Biochemical and biophysical research communications*, *334*(4), 1351-1358.

Cimmino, A., Calin, G. A., Fabbri, M., Iorio, M. V., Ferracin, M., Shimizu, M., ... & Croce, C. M. (2005). miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proceedings of the National Academy of Sciences*, *102*(39), 13944-13949.

Corthals, S. L., Jongen-Lavrencic, M., de Knegt, Y., Peeters, J. K., Beverloo, H. B., Lokhorst, H. M., & Sonneveld, P. (2010). Micro-RNA-15a and micro-RNA-16 expression and chromosome 13 deletions in multiple myeloma. *Leukemia research*, *34*(5), 677-681.

Dehan, E., Ben-Dor, A., Liao, W., Lipson, D., Frimer, H., Rienstein, S., ... & Kaminski, N. (2007). Chromosomal aberrations and gene expression profiles in non-small cell lung cancer. *Lung cancer*, *56*(2), 175-184.

Galluzzi, L., Morselli, E., Vitale, I., Kepp, O., Senovilla, L., Criollo, A., ... & Kroemer, G. (2010). miR-181a and miR-630 regulate cisplatin-induced cancer cell death. *Cancer research*, *70*(5), 1793-1803.

Gao, X., Zhang, R., Qu, X., Zhao, M., Zhang, S., Wu, H., ... & Chen, L. (2012). MiR-15a, miR-16-1 and miR-17-92 cluster expression are linked to poor prognosis in multiple myeloma. *Leukemia research*, *36*(12), 1505-1509.

Gatt, M. E., Zhao, J. J., Ebert, M. S., Zhang, Y., Chu, Z., Mani, M., ... & Carrasco, D. R. (2010). MicroRNAs 15a/16-1 function as tumor suppressor genes in multiple myeloma. *Blood*, 61-65.

Gauwerky, C. E., Huebner, K., Isobe, M., Nowell, P. C., & Croce, C. M. (1989). Activation of MYC in a masked t (8; 17) translocation results in an aggressive B-cell leukemia. *Proceedings of the National Academy of Sciences*, *86*(22), 8867-8871.

Gutiérrez, N. C., Sarasquete, M. E., Misiewicz-Krzeminska, I., Delgado, M., De Las Rivas, J., Ticona, F. V., ... & San Miguel, J. F. (2010). Deregulation of microRNA expression in the different genetic subtypes of multiple myeloma and correlation with gene expression profiling. *Leukemia*, *24*(3), 629-637.

Huang, J. J., Yu, J., Li, J. Y., Liu, Y. T., & Zhong, R. Q. (2012). Circulating microRNA expression is associated with genetic subtype and survival of multiple myeloma. *Medical oncology*, *29*(4), 2402-2408.

Krichevsky, A. M., King, K. S., Donahue, C. P., Khrapko, K., & Kosik, K. S. (2003). A microRNA array reveals extensive regulation of microRNAs during brain development. *Rna*, *9*(10), 1274-1281.

Kuehl, W. M., & Bergsagel, P. L. (2012). Molecular pathogenesis of multiple myeloma and its premalignant precursor. *The Journal of clinical investigation*, *122*(10), 3456-3463.

Linsley, P. S., Schelter, J., Burchard, J., Kibukawa, M., Martin, M. M., Bartz, S. R., ... & Lim, L. (2007). Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. *Molecular and cellular biology*, *27*(6), 2240-2252.

Lionetti, M., Biasiolo, M., Agnelli, L., Todoerti, K., Mosca, L., Fabris, S., ... & Neri, A. (2009). Identification of microRNA expression patterns and definition of a microRNA/mRNA regulatory network in distinct molecular groups of multiple myeloma. *Blood, The Journal of the American Society of Hematology*, *114*(25), e20-e26.

Min, D. J., Ezponda, T., Kim, M. K., Will, C. M., Martinez-Garcia, E., Popovic, R., ... & Licht, J. D. (2013). MMSET stimulates myeloma cell growth through microRNA-mediated modulation of c-MYC. *Leukemia*, *27*(3), 686-694.

Misiewicz-Krzeminska, I., Sarasquete, M. E., Quwaider, D., Krzeminski, P., Ticona, F. V., Paíno, T., ... & Gutiérrez, N. C. (2013). Restoration of microRNA-214 expression reduces growth of myeloma cells through positive regulation of P53 and inhibition of DNA replication. *haematologica*, *98*(4), 640.

Pichiorri, F., Suh, S. S., Ladetto, M., Kuehl, M., Palumbo, T., Drandi, D., ... & Croce, C. M. (2008). MicroRNAs regulate critical genes associated with multiple myeloma pathogenesis. *Proceedings of the National Academy of Sciences*, *105*(35), 12885-12890.

Pichiorri, F., Suh, S. S., Rocci, A., De Luca, L., Taccioli, C., Santhanam, R., ... & Croce, C. M. (2010). Downregulation of p53-inducible microRNAs 192, 194, and 215 impairs the p53/MDM2 autoregulatory loop in multiple myeloma development. *Cancer cell*, *18*(4), 367-381.

Pichiorri, F., De Luca, L., & Aqeilan, R. I. (2011). MicroRNAs: new players in multiple myeloma. *Frontiers in genetics*, *2*, 22.

Pothof, J., Verkaik, N. S., Van Ijcken, W., Wiemer, E. A., Ta, V. T., Van Der Horst, G. T., ... & Persengiev, S. P. (2009). MicroRNA-mediated gene silencing modulates the UV-induced DNA-damage response. *The EMBO journal*, *28*(14), 2090-2099.

Raveche, E. S., Salerno, E., Scaglione, B. J., Manohar, V., Abbasi, F., Lin, Y. C., ... & Marti, G. E. (2007). Abnormal microRNA-16 locus with synteny to human 13q14 linked to CLL in NZB mice. *Blood, The Journal of the American Society of Hematology*, *109*(12), 5079-5086.

Rio-Machin, A., Ferreira, B. I., Henry, T., Gómez-López, G., Agirre, X., Alvarez, S., ... & Cigudosa, J. C. (2013). Downregulation of specific miRNAs in hyperdiploid multiple myeloma mimics the oncogenic effect of IgH translocations occurring in the non-hyperdiploid subtype. *Leukemia*, *27*(4), 925-931.

Roccaro, A. M., Sacco, A., Thompson, B., Leleu, X., Azab, A. K., Azab, F., ... & Ghobrial, I. M. (2009). MicroRNAs 15a and 16 regulate tumor proliferation in multiple myeloma. *Blood, The Journal of the American Society of Hematology*, *113*(26), 6669-6680.

Russo, G., Isobe, M., Pegoraro, L., Finan, J., Nowell, P. C., & Croce, C. M. (1988). Molecular analysis of at (7; 14)(g35; g32) chromosome translocation in a T cell leukemia of a patient with ataxia telangiectasia. *Cell*, *53*(1), 137-144.

Saleh, A. D., Savage, J. E., Cao, L., Soule, B. P., Ly, D., DeGraff, W., ... & Simone, N. L. (2011). Cellular stress induced alterations in microRNA let-7a and let-7b expression are dependent on p53. *PloS one*, *6*(10), e24429.

Schwindt, H., Akasaka, T., Zühlke-Jenisch, R., Hans, V., Schaller, C., Klapper, W., ... & Deckert, M. (2006). Chromosomal translocations fusing the BCL6 gene to different partner loci are recurrent in primary central nervous system lymphoma and may be associated with aberrant somatic hypermutation or defective class switch recombination. *Journal of Neuropathology & Experimental Neurology*, *65*(8), 776-782.

Sonoki, T., Iwanaga, E., Mitsuya, H., & Asou, N. (2005). Insertion of microRNA-125b-1, a human homologue of lin-4, into a rearranged immunoglobulin heavy chain gene locus in a patient with precursor B-cell acute lymphoblastic leukemia. *Leukemia*, *19*(11), 2009-2010.

Suzuki, H. I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., & Miyazono, K. (2009). Modulation of microRNA processing by p53. *Nature*, *460*(7254), 529-533.

Volinia, S., Calin, G. A., Liu, C. G., Ambs, S., Cimmino, A., Petrocca, F., ... & Croce, C. M. (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. *Proceedings of the National Academy of Sciences*, *103*(7), 2257-2261.

Wang, T., Zhang, X., Obijuru, L., Laser, J., Aris, V., Lee, P., ... & Wei, J. J. (2007). A micro-RNA signature associated with race, tumor size, and target gene activity in human uterine leiomyomas. *Genes, Chromosomes and Cancer*, *46*(4), 336-347.

Wong, K. Y., So, C. C., Loong, F., Chung, L. P., Lam, W. W. L., Liang, R., ... & Chim, C. S. (2011). Epigenetic inactivation of the miR-124-1 in haematological malignancies. *PloS one*, *6*(4), e19027.

Yang, R. F., Chen, L. J., Li, J. Y., Li, C. M., Xu, J. R., Wu, Y. J., & Lu, H. (2010). microRNA-21 and microRNA-30b expression in multiple myeloma. *Zhonghua xue ye xue za zhi= Zhonghua Xueyexue Zazhi*, *31*(1), 38-41.

Zhang, X., Wan, G., Berger, F. G., He, X., & Lu, X. (2011). The ATM kinase induces microRNA biogenesis in the DNA damage response. *Molecular cell*, *41*(4), 371-383.