Supporting Table for Essentiality Call to be Included in Annex 1:

Summary of Supporting Evidence for Essentiality

Event	***Direct Evidence***	***Indirect Evidence***	***Uncertainties or inconsistencies***
DNA alkylation (MIE)	N/A	1. Exposure of mice to diethylnitrosamine (DEN) (which requires metabolism to create alkyl adducts) causes increases in DNA alkylation and mutations (KE2) in liver, but not bone marrow cells (Suzuki et al., 1994; Mientjes et al., 1998). This is consistent with the fact that bone marrow cells are unable to metabolize DEN, but liver cells can, and provides support for the essentiality of alkyl adducts to cause mutations. 2. Dimethylnitrosamine and diethylnitrosamine exposure do not cause increases in bone marrow micronuclei, but do cause increases in mouse spermatid micronuclei, consistent with knowledge of metabolic capacity of these tissues to cause alkyl adducts (Cliet et al. 1993). The data are important because they address the requirement of adducts in the key cell type for the AOP (male germ cells) for DNA alterations to occur.	None.
Insufficient repair (KE1)	**Knock-out of DNA repair leads to increases in mutations (KE2)** Inactivation of MGMT sensitizes cells to alkylation-induced mutagenesis resulting in an increased number of mutations per adduct (Thomas et al. 2013).	**Overexpression in repair leads to decreases in mutations (KE2)** 1. MGMT overexpression protects mgt1 mutant yeast against alkylation-induced mutation (Xiao and Fontanie 1995). 2. Big Blue® mice over-expressing human AGT exhibit greatly reduced O6-methylguanine-mediated lacI	None.

		and K-ras mutations in the thymus following treatment with MNU (Allay et al. 1999) relative to wild type Big Blue® mice.	
Mutations (KE2)	N/A	N/A	N/A

Overall:

Based on the supporting evidence for all KEs and the considerations in Annex 1, the weight of ***evidence for the KEs in the context of the AOP overall*** is considered: **Moderate**

This moderate call is based principally on indirect evidence from several well designed studies on manipulation of DNA alkyl transferase repair enzymes for the MIE and KE1. Specifically, studies have demonstrated that when DNA repair (MGMT) is knocked out (in vitro), DNA mutations (KE2) increase; in contrast, when repair is enhanced in vivo, mutations decline.

To our knowledge it is not possible to block the MIE (other than by enhancing DNA repair). However, studies on alkylating agents that require metabolism demonstrate that tissues that lack the required metabolic enzymes do not have increased DNA alkyl adducts or subsequent increases in mutations.

To our knowledge there is no way to test for the essentiality of mutations in sperm on the AO (heritable mutations).

References:

Allay, E., M. Veigl and S.L. Gerson (1999), "Mice over-expressing human O6 alkylguanine-DNA alkyltransferase selectively reduce O6 methylguanine mediated carcinogenic mutations to threshold levels after N-methyl-N-nitrosourea", *Oncogene*, 18(25): 3783-3787.

Cliet, I., Melcion, C. and A. Cordier (1993) “Lack of predictivity of bone marrow micronucleus test versus testis micronucleus test: comparison with four carcinogens”. *Mutat Res*. 292(2):105-11.

Mientjes, .E.J, Luiten-Schuite, A., van der Wolf, E., Borsboom, Y., Bergmans, A., Berends, F., Lohman, P.H., Baan, R.A., and J.H. van Delft (1998) “DNA adducts, mutant frequencies, and mutation spectra in various organs of lambda lacZ mice exposed to ethylating agents*”, Environ Mol Mutagen*. 31(1):18-31.

Suzuki, T., Hayashi, M and T. Sofuni (1994) “Initial experiences and future directions for transgenic mouse mutation assays”, Mutat Res.307(2):489-94.

Thomas, A.D., G.J. Jenkins, B. Kaina, O.G. Bodger, K.H. Tomaszowski, P.D. Lewis, S.H. Doak, G.E. Johnson (2013), "Influence of DNA repair on nonlinear dose-responses for mutation", *Toxicol. Sci.*, 132(1): 87-95.

Xiao, W. and T. Fontanie (1995), "Expression of the human MGMT O6-methylguanine DNA methyltransferase gene in a yeast alkylation-sensitive mutant: its effects on both exogenous and endogenous DNA alkylation damage", *Mutat. Res*, 336(2): 133-42.

Table II. Summary of the overall weight of evidence for assessment of the AOP based on questions provided in the OECD's Users' Handbook.

1. Support for Biological Plausibility of KERS	**Defining Question**	**High (Strong)**	**Moderate**	**Low (Weak)**
	a) Is there a mechanistic relationship between KE_{up} and KE_{down} consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance.	KER is plausible based on analogy to accepted biological relationships, but scientific understanding is incomplete	Empirical support for association between KEs , but the structural or functional relationship between them is not understood.
MIE => KE1: Alkylation of DNA in male pre-meiotic germ cells leading to insufficient or incorrect repair in male pre-meiotic germ cells	**STRONG.** Broad understanding and extensive evidence that exposure to high enough doses of alkylation agents overwhelms DNA repair machinery leading to the retention of DNA adducts and subsequent mutation upon replication in virtually any species/cell type. The primary DNA repair enzyme (AGT) for alkylated DNA is a suicide enzyme that is well established to become overwhelmed at high enough doses.			
KE1 => KE2: Insufficient or incorrect repair in male pre-meiotic germ cells leading to mutations in male pre-meiotic germ cells	**STRONG.** There is extensive understanding of the various DNA repair systems involved in the removal of DNA adducts, and extensive understanding that the primary enzyme is suicide inactivated causing saturation of repair and mutation at high doses.			
KE2 => AO: Mutations in male pre-meiotic germ cells leading to heritable mutations in offspring	**STRONG.** This is dogma.			
MIE=> KE2: Alkylation of DNA in male pre-meiotic germ cells leading to mutations in male pre-meiotic germ cells	**STRONG.** Alkylating agents are prototypical male germ cell mutagens. These agents, especially those chemicals that preferentially cause O-alkylation in DNA, induce mutations across different species, cell types and tissues. Mechanisms are broadly understood.			
MIE=> AO (Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations in offspring)	**STRONG.** There is extensive understanding that alkylating agents, especially those chemicals that preferentially cause O-alkylation in DNA, induce mutations in the offspring of exposed males. ENU (N-ethyl-N-nitrosourea) is a prototypical agent used to derive offspring with de novo mutations inherited from exposed males. ENU mutagenicity is a standard bench tool for genetic screens in which one can identify new mutations associated with a phenotype of interest.			
2. Support for Essentiality of KEs	Defining Question	High (Strong)	Moderate	Low (Weak)
	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
KE2: (Mutations in male pre-meiotic germ cells)	Essentiality is **MODERATE.** **KE1 - Correct repair of the alkylated DNA (i.e., a block of KE1) will not lead to mutation.** Enhanced DNA repair reduces mutation frequencies (KE1). Reduction in repair increases mutation frequency (KE1). NOTE: Exposure to N-alkylators of DNA (instead of O-alkylation) does not lead to point mutations in somatic or germ cells (specificity for O-alkyl adducts). **Rest of KEs cannot be tested for essentiality (see text for explanation).**			
3. Empirical Support for KERs	Defining Questions	High (Strong)	Moderate	Low (Weak)
	Does empirical evidence support that a change in KE_{up} leads to an appropriate change in KE_{down}? Does KE_{up} occur at lower doses and earlier time points than KE_{down} and is the incidence of KE_{up} > than that for KE_{down}? Inconsistencies?	Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. No or few critical data gaps or conflicting data	Demonstrated dependent change in both events following exposure to a small number of stressors. Some inconsistencies with expected pattern that can be explained by various factors.	Limited or no studies reporting dependent change in both events following exposure to a specific stressor; and/or significant inconsistencies in empirical support across taxa and species that don't align with hypothesized AOP

MIE => KE1: (Alkylation of DNA in male pre-meiotic germ cells leading to insufficient or incorrect repair in male pre-meiotic germ cells)	**MODERATE** Insufficient DNA repair is measured indirectly through persistence of DNA adducts or through the observation of increases in mutation. Extensive evidence in somatic cells to support this KER (in particular for temporal concordance), but not in a format to support evaluating concordance of dose response. There are no apparent inconsistencies. A limited number of studies provide data in support of this in male germ cells.
KE1 => KE2: Insufficient or incorrect repair in male pre-meiotic germ cells leading to mutations in male pre-meiotic germ cells	**MODERATE** Main empirical evidence: synthetic oligonucleotides containing DNA lesions genetically engineered in viral or plasmid genomes and introduced into cells. Sequencing confirmed that replication of alkylated DNA (i.e., unrepaired DNA) causes mutations in addition to revealing the DNA repair pathways and polymerases involved. Thus, there is demonstrated dependent change in the two events in somatic cells. However, no studies to our knowledge have addressed this in germ cells.
KE2 => AO: Mutations in male pre-meiotic germ cells leading to heritable mutations in offspring	**LOW** There are data from only two studies that permit analysis of mutation frequencies in sperm and offspring for the same mutational endpoint. These are consistent with the expected concordance, but more work is necessary.
MIE => KE2: Alkylation of DNA in male pre-meiotic germ cells leading to mutations in male pre-meiotic germ cells	**STRONG** A significant amount of work in somatic cells supports that adducts occur at lower doses than mutations. Extensive numbers of studies have demonstrated that chemicals that alkylate oxygen also cause germ cell mutations. Extrapolation across studies demonstrates concordance in the incidence response and temporal relationships for the two KEs.
MIE => AO: Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations in offspring	**MODERATE** Extensive work has been done on a single chemical: ENU. The studies demonstrate that alkyl adducts occur within hours of exposure in spermatogonia and that this occurs prior to the mutations for tandem repeat DNA sequences and for genes associated with visible phenotypic traits in mice. Moreover, incidence evaluation shows that levels of alkylated DNA are at least one order of magnitude greater than induced inherited mutations at a locus for a similar dose of ENU.
MIE => KE1: Alkylation of DNA in male pre-meiotic germ cells leading to insufficient or incorrect repair in male pre-meiotic germ cells	Inconsistencies / Uncertainties of MIE => KE1 Rationale: The KER is based on indirect measures. However, there are no inconsistencies. The data are based primarily on effects in somatic cells; however, inflection points in the dose-response curve for mutation in germ cells suggest that this consistent with effects in germ cells.
KE1 => KE2: Insufficient or incorrect repair in male pre-meiotic germ cells leading to mutations in male pre-meiotic germ cells	Inconsistencies / Uncertainties of KE1 => KE2 Rationale: No inconsistencies based on the somatic cell literature. However, limited data available in germ cells. Spectrum of mutations in germ cells and somatic cells is consistent in demonstrating what alkyl adducts are least effectively repaired at high doses and what mutations result.
KE2 => AO: Mutations in male pre-meiotic germ cells leading to heritable mutations in offspring	Inconsistencies / Uncertainties of KE2 => KE3 Rationale: Not all mutations are viable for the sperm or the early embryo. Thus, these mutations that occur in sperm will not be seen in the offspring. However, mutations that do not affect spermatogenesis and are viable will be inherited by the offspring. There are very limited data to support this, thus there are uncertainties associated with this KER. This is expected to be an area of extensive future research.