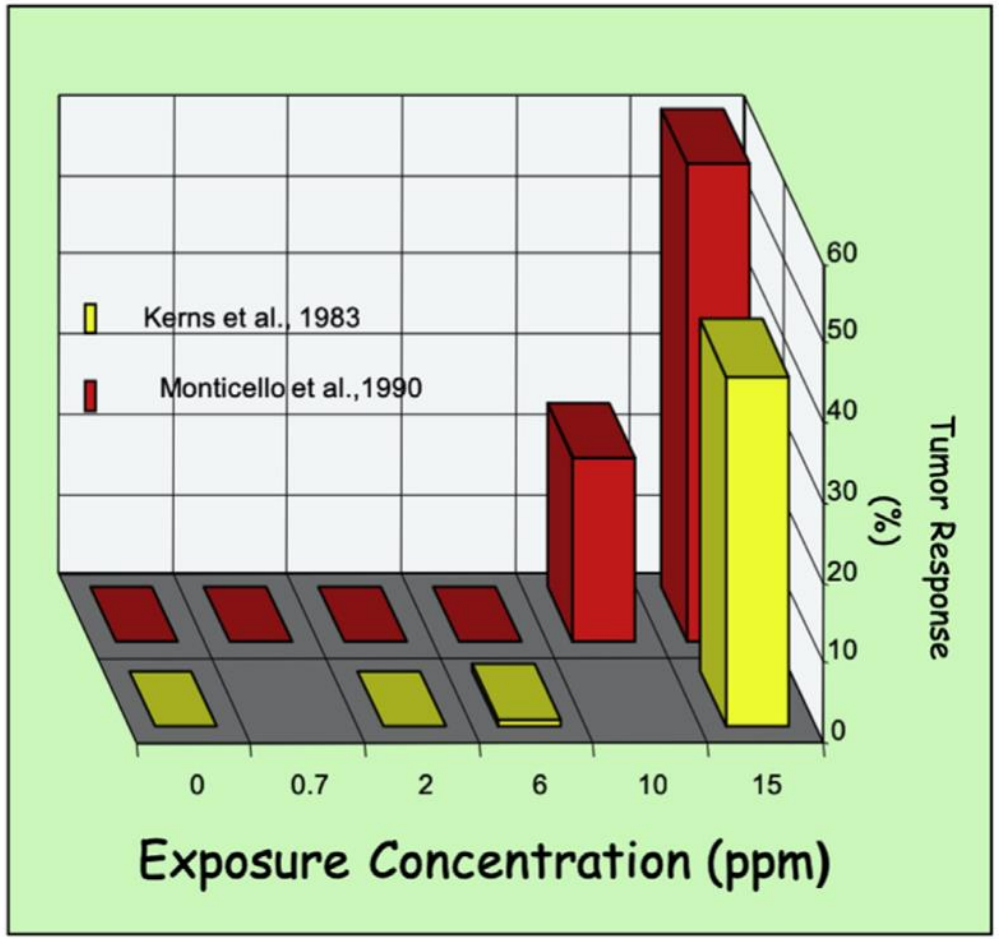
Tox Strategies

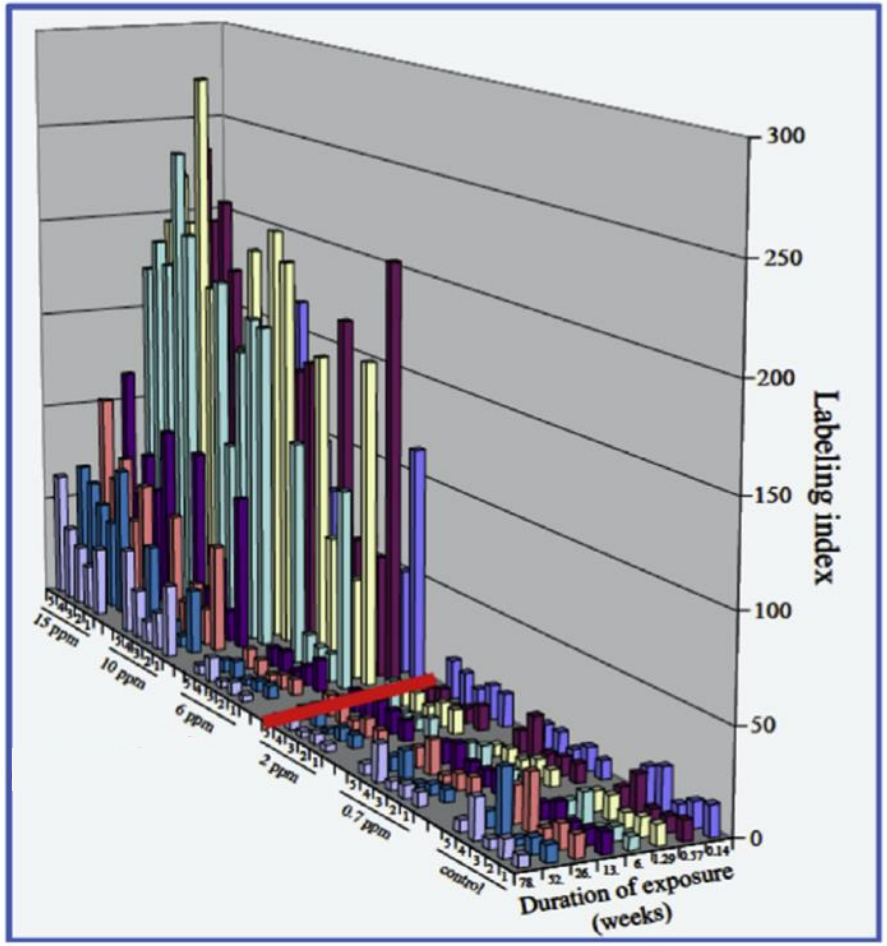
Mechanistic and Pharmacokinetic Considerations to Aid in Determining Causality: New Frontiers for Data Integration

An Updated MOA Analysis for Formaldehyde-Induced Nasal Tumors

Chad Thompson, PhD

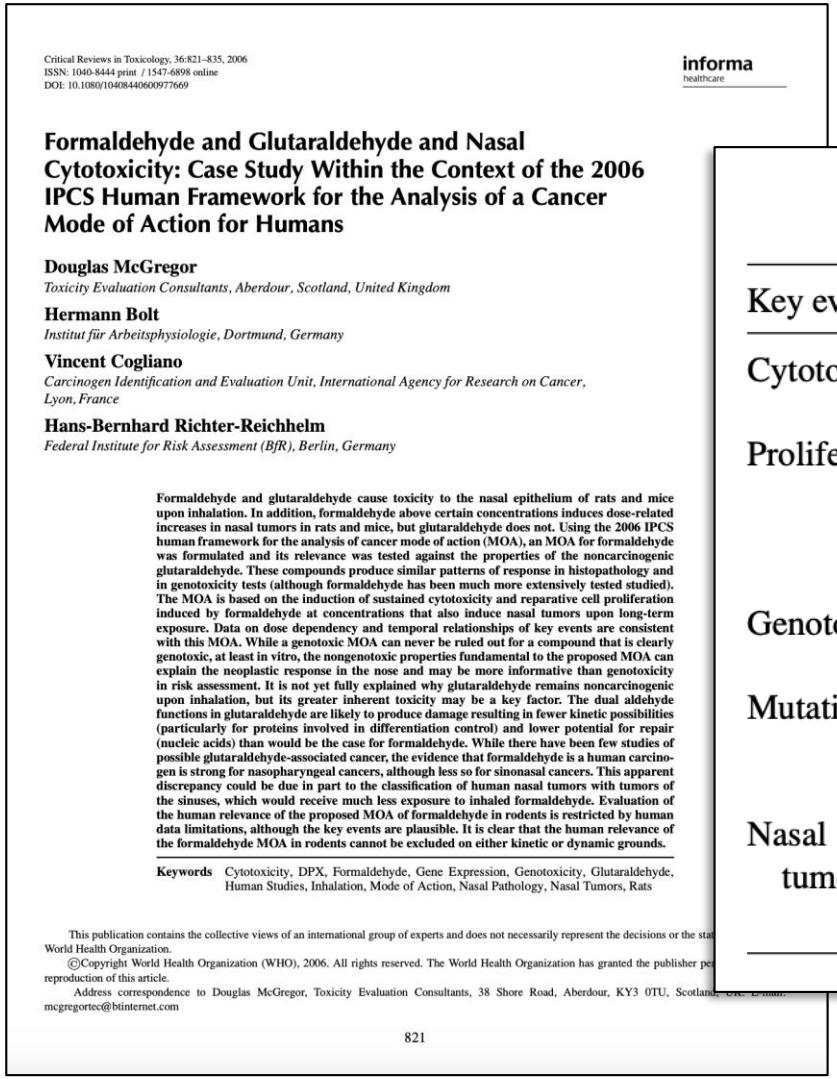
Formaldehyde-Induced Cell Proliferation & Nasal Tumors in Rats





Andersen et al. (2019)

MOA for Formaldehyde-Induced Nasal Tumors



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Formaldehyde and Glutaraldehyde and Nasal Cytotoxicity: Case Study Within the Context of the 2006 IPCS Human Framework for the Analysis of a Cancer Mode of Action for Humans

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Federal Institute for Risk Assessment (BfR), Berlin, Germany

Formaldehyde and glutaraldehyde cause toxicity to the nasal epithelium of rats and mice upon inhalation. In addition, formaldehyde above certain concentrations induces dose-related increases in nasal tumors in rats and mice, but glutaraldehyde does not. Using the 2006 IPCS human framework for the analysis of cancer mode of action (MOA), an MOA for formaldehyde was formulated and its relevance was tested against the properties of the noncarcinogenic glutaraldehyde. These compounds produce similar patterns of response in histopathology and in genotoxicity tests (although formaldehyde has been much more extensively tested studied). The MOA is based on the induction of sustained cytotoxicity and reparative cell proliferation induced by formaldehyde at concentrations that also induce nasal tumors upon long-term exposure. Data on dose dependency and temporal relationships of key events are consistent with this MOA. While a genotoxic MOA can never be ruled out for a compound that is clearly genotoxic, at least in vitro, the nongenotoxic properties fundamental to the proposed MOA can explain the neoplastic response in the nose and may be more informative than genotoxicity in risk assessment. It is not yet fully explained why glutaraldehyde remains noncarcinogenic upon inhalation, but its greater inherent toxicity may be a key factor. The dual aldehyde functions in glutaraldehyde are likely to produce damage resulting in fewer kinetic possibilities (particularly for proteins involved in differentiation control) and lower potential for repair (nucleic acids) than would be the case for formaldehyde. While there have been few studies of possible glutaraldehyde-associated cancer, the evidence that formaldehyde is a human carcinogen is strong for nasopharyngeal cancers, although less so for sinonasal cancers. This apparent discrepancy could be due in part to the classification of human nasal tumors with tumors of the sinuses, which would receive much less exposure to inhaled formaldehyde. Evaluation of the human relevance of the proposed MOA of formaldehyde in rodents is restricted by human data limitations, although the key events are plausible. It is clear that the human relevance of the formaldehyde MOA in rodents cannot be excluded on either kinetic or dynamic grounds.

Keywords Cytotoxicity, DPX, Formaldehyde, Gene Expression, Genotoxicity, Glutaraldehyde, Human Studies, Inhalation, Mode of Action, Nasal Pathology, Nasal Tumors, Rats

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TABLE 3
Formaldehyde concordance table

Key event	Evidence in animals	Evidence in humans
Cytotoxicity	Positive in vivo (target cells)	Plausible
Proliferation	Positive in vivo (target cells)	Plausible (some evidence but confounded by coexposure)
Genotoxicity	DPX (target cells in vivo)	DPX (nontarget cells, ie lymphocytes)
Mutations	Positive in vitro; unconvincing in vivo	Positive (? cells)
Nasal tumors	Positive (mainly anterior lateral meatus)	Positive (nasopharyngeal) ? (sinonasal)

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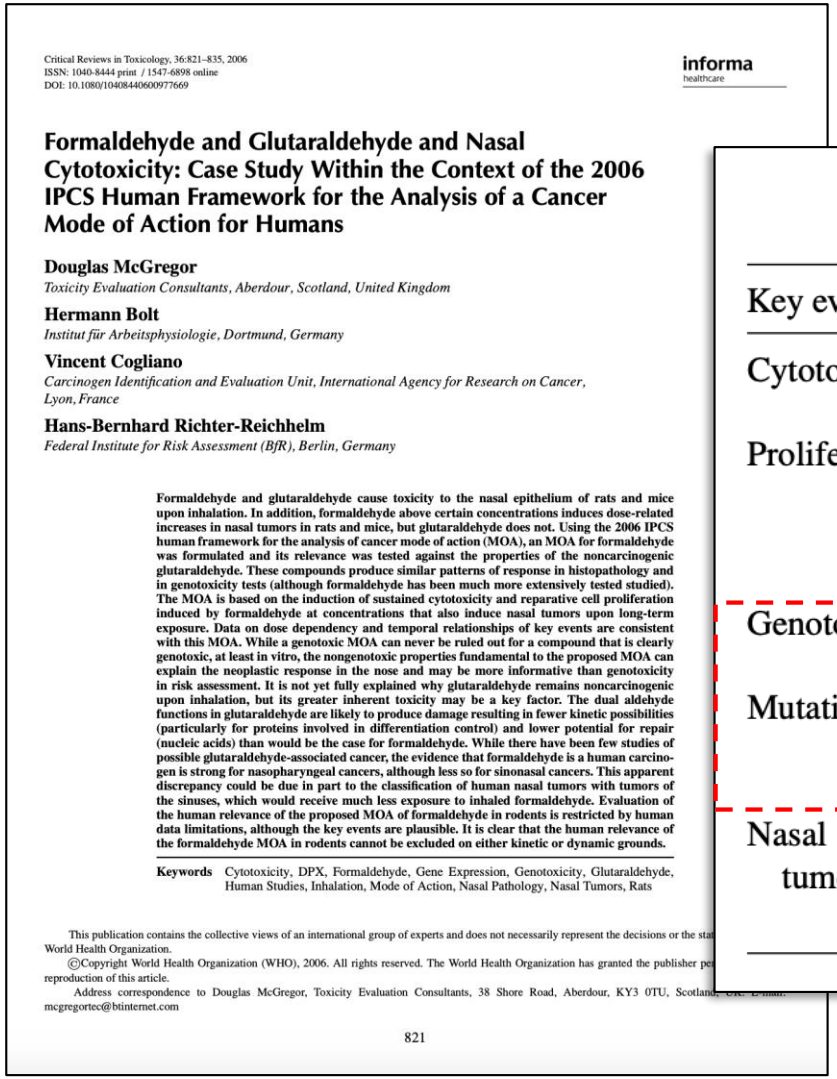
MOA for Formaldehyde-Induced Nasal Tumors



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Uncertainties Regarding the Role of Genotoxicity

- Prolonged exposure to formaldehyde above a critical concentration induces sustained cytotoxicity and cell proliferation. **As a result of genetic changes within this proliferating cell population, neoplasia emerges.**
- Formaldehyde is a genotoxic substance in vitro and forms DNA–protein cross-links (DPX) …**Apart from the abundance of DPX observations in rats, there is little evidence that formaldehyde is mutagenic to mammalian cells in vivo.**
- There is the possibility that mutagenicity could play a role in the development of formaldehyde-induced tumors… **but is generally not genotoxic in standard in vivo assays**...

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- There is the possibility that mutagenicity could play a role in the development of formaldehyde-induced tumors… **but is generally not genotoxic in standard in vivo assays**…

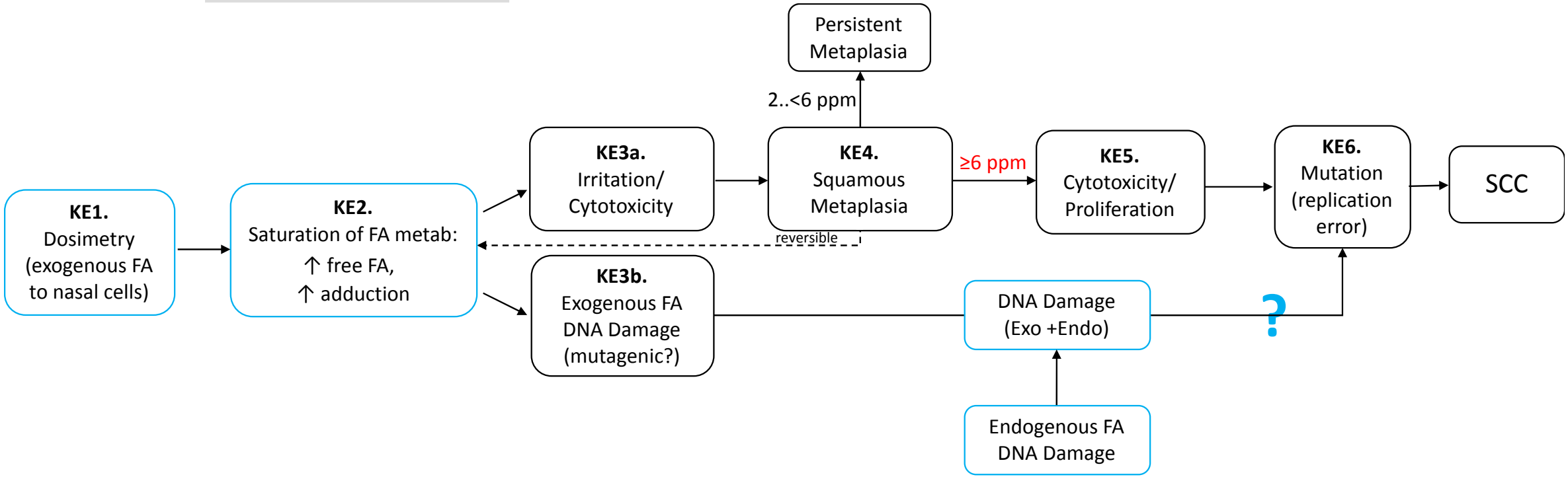
New Data Published After 2006

- Heavy isotope labelling
  - Distinguish endogenous and exogenous adducts
- In vivo genotoxicity assays in nasal cavity
  - Mutant frequency (MF)
  - Micronuclei (MN)
- Mouse models
  - $p53^{+/-}$ exposure study conducted by NTP
  - $Adh5^{-/-}$ mice deficient in FDH

    Measured FA-DNA adducts in $Adh3^{-/-}$
  - $Adh5^{-/-}$/Big Blue mice

    MF data

Updated MOA (2020)



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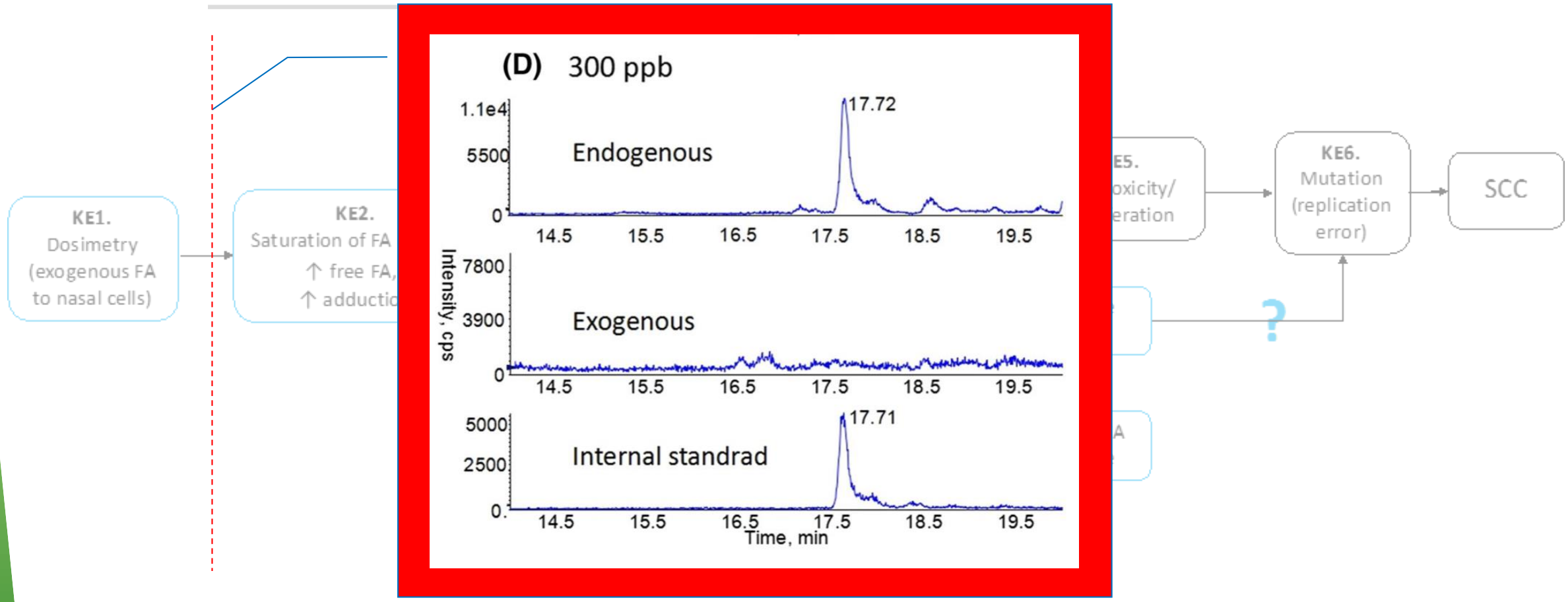
Updated MOA (2020) – Potential Dosimetry Threshold



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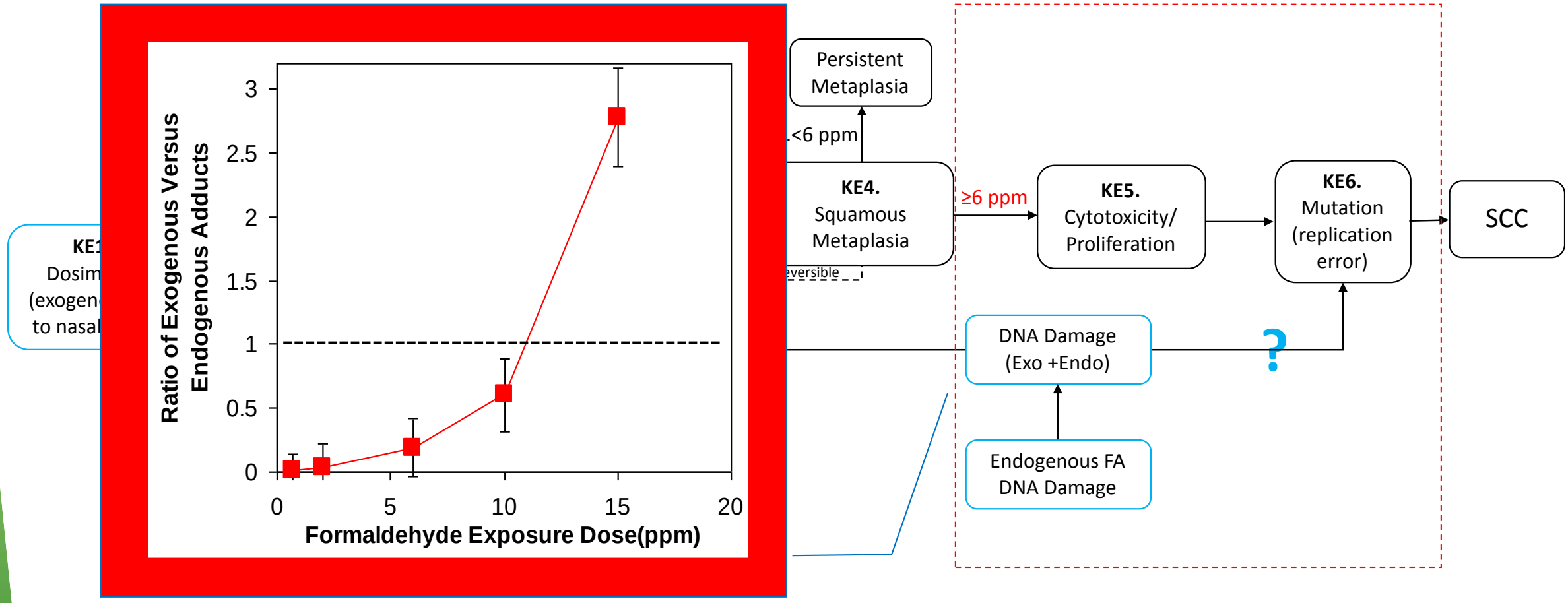
Updated MOA (2020) – Focus of this Talk



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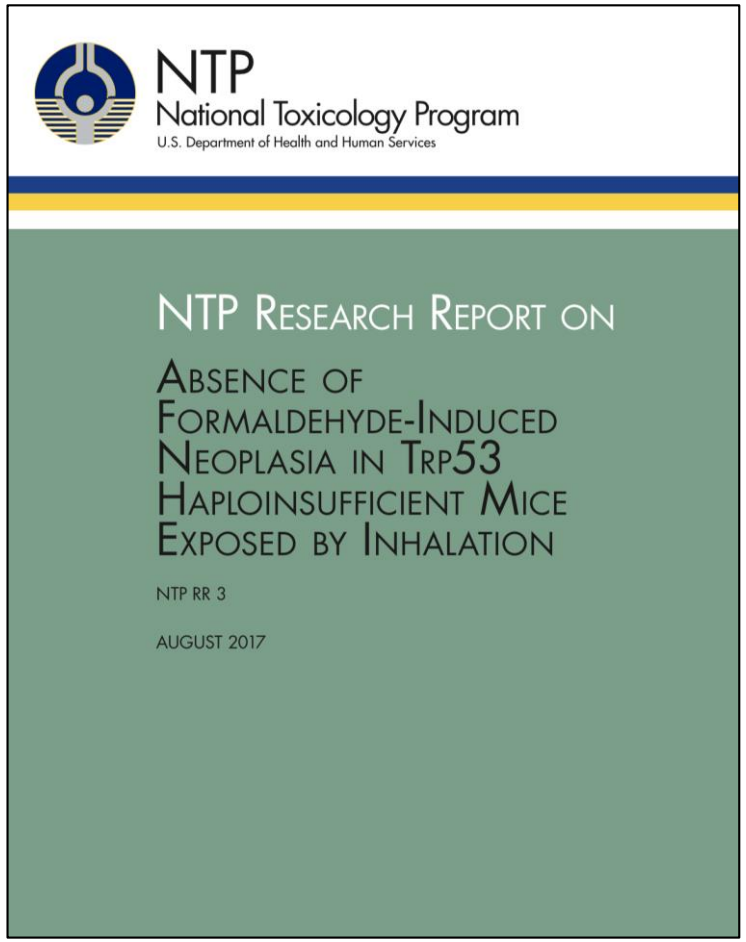
Next Series of Slides

- Discuss 2017 NTP study in $p53^{+/-}$ mice
  - No increase in nasal tumors
- Discuss relevant studies in genetically modified mouse models
  - Loss of formaldehyde detoxification increases DNA adducts
  - Loss of formaldehyde detoxification does no lead to increased mutations
- Discuss more traditional *in vivo* genotoxicity results in rats
  - No increase in mutagenic and clastogenic endpoints in nasal cavity

NTP Study (2017) Inhalation Study in $p53^{+/-}$ Mice



"Considerable evidence shows that formaldehyde-induced mutations in the tumor suppressor gene Trp53 are important in the pathogenesis of nasal cancer ."

"Mutations in Trp53 were identified in formaldehyde-induced nasal SCC in rats, and abnormal Trp53 protein was shown to accumulate in the nasal tissue of formaldehyde-exposed rats."

"We hypothesized that formaldehyde-induced loss of Trp53 would be increased in $Trp53^{+/-}$ mice, resulting in an increased incidence of SCC of the nose and leukemia or lymphohematopoietic cancer, and potentially neoplasms at other sites."

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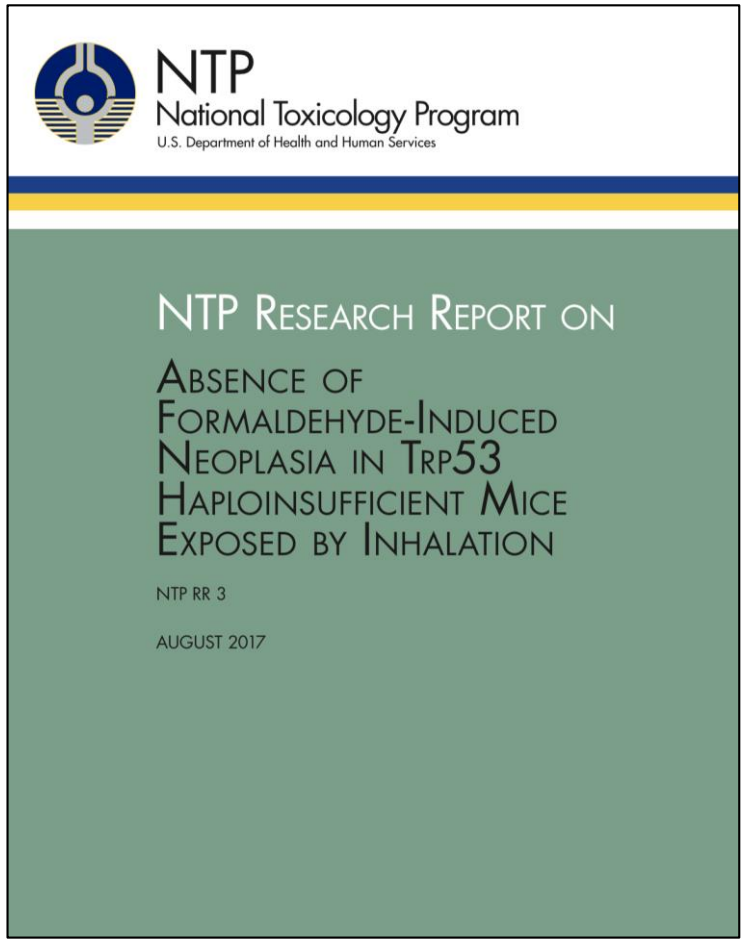
NTP Study (2017) Inhalation Study in $p53^{+/-}$ Mice



Exposures: 0, 7.5, & 15 ppm

6 hr/day, 5d/wk x 8-wk

Held to ~50 weeks old:

- ≅ 32 wk latency

25 mice per group ($p53^{+/-}$)

- Two different strains of $p53^{+/-}$ mice
- These strains are used to test genotoxic carcinogens

Endpoints:

- Histopathology
- Hematology
- Bone marrow smears

*Note: RD_{50} in mice at ~3 ppm

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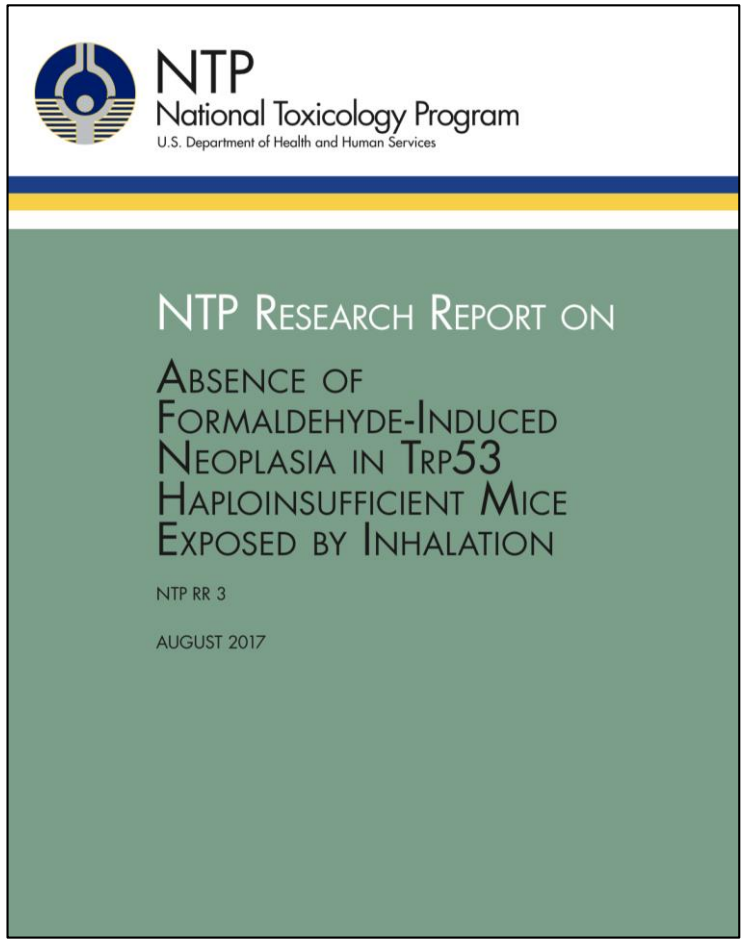
No Increase in Nasal SCC



Endpoint	0 ppm	7.5 ppm	15 ppm
C3B6.129F1-Trp53^{tm1Brd}			
squamous metaplasia	0/21	14/21	22/23
hyperplasia	0/21	0/21	1/23
SCC	none	none	none
B6.129-Trp53^{tm1Brd}			
squamous metaplasia	0/22	13/27	17/26
SCC	none	none	none

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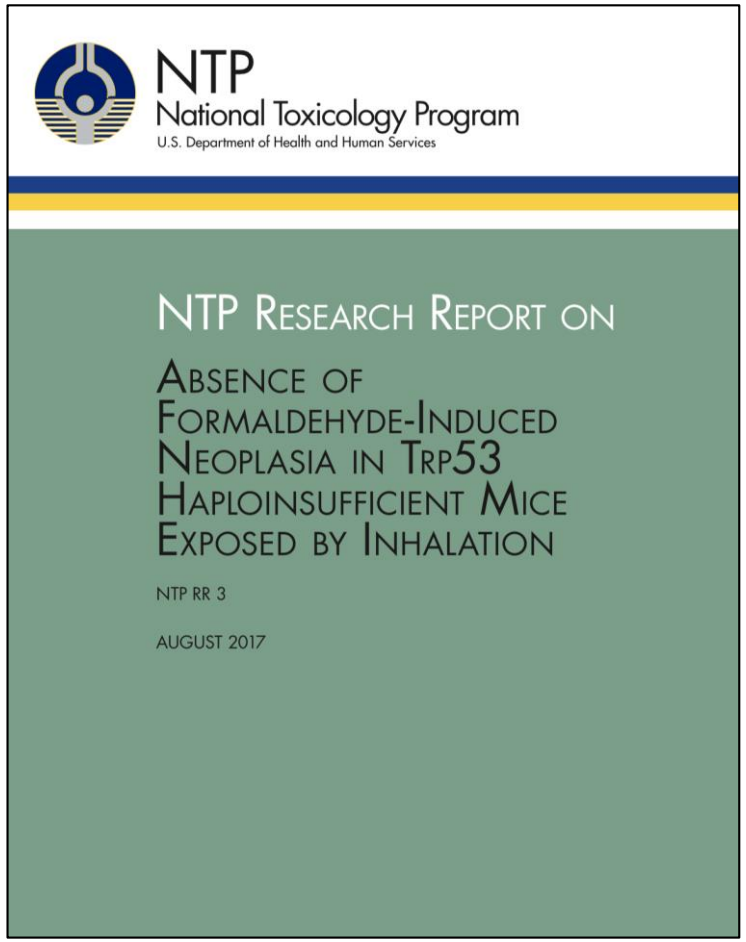
No Increase in Nasal SCC



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squamous metaplasia	0/22	13/27	17/26
SCC	none	none	none

NTP: "The results of this short-term carcinogenicity study do not support a role for *Trp53* in formaldehyde-induced neoplasia. "

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Implications of the NTP (2017) Study

- Squamous metaplasia indicates dosimetry to target tissue and irritation to the nasal mucosa
- Lack of hyperplasia indicates that metaplasia was protective of further damage
- We don't know the exogenous adduct levels in mice exposed to formaldehyde, but based on rat data, adducts were likely present but below endogenous levels
- **Lack of SCC does not support low-dose genotoxicity**

Endpoint	0 ppm	7.5 ppm	15 ppm
C3B6.129F1-Trp53^{tm1Brd}			
squamous metaplasia	0/21	14/21	22/23
hyperplasia	0/21	0/21	1/23
SCC	none	none	none
B6.129-Trp53^{tm1Brd}			
squamous metaplasia	0/22	13/27	17/26
SCC	none	none	none

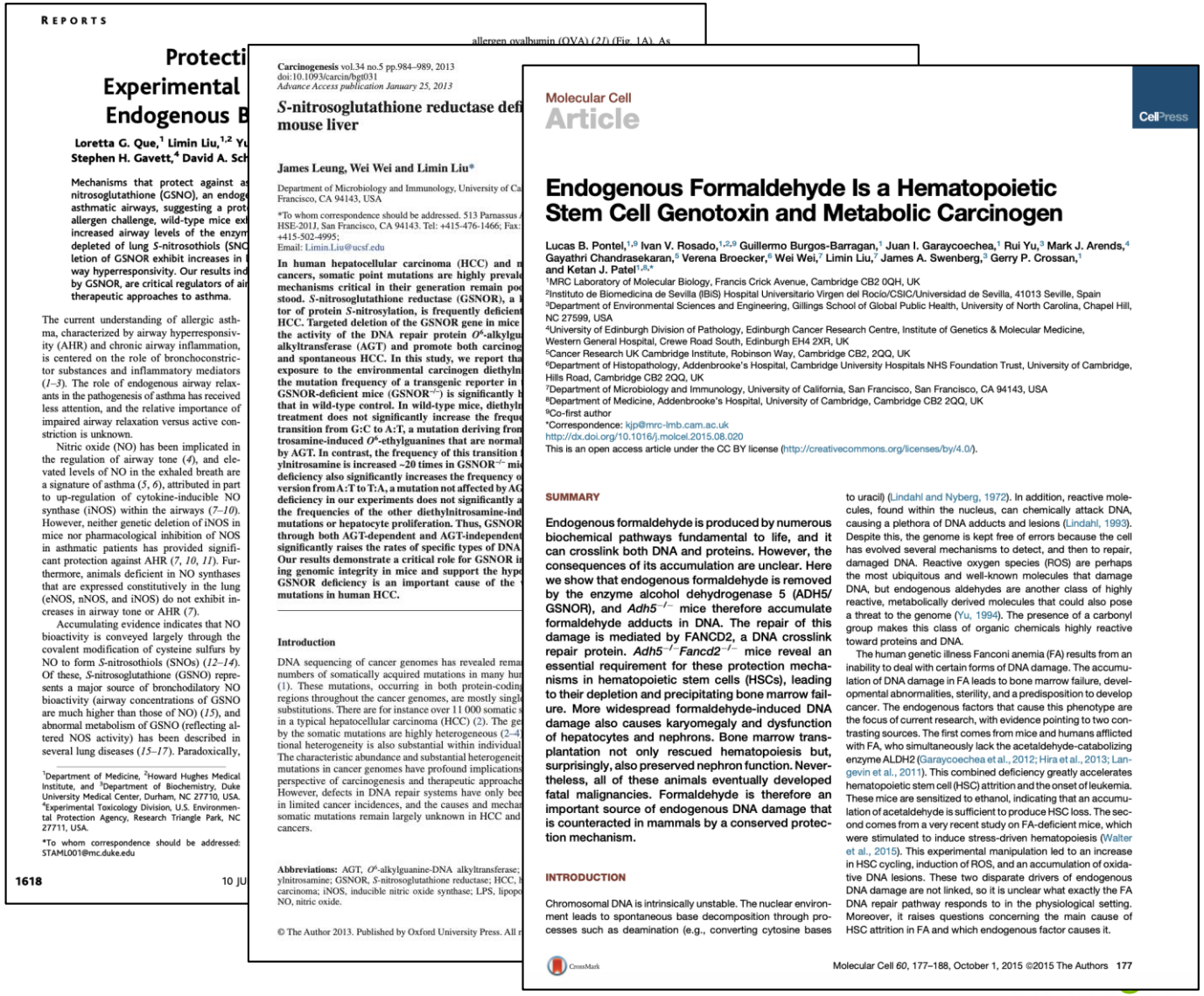
Formaldehyde Dehydrogenase Knockout Models

1. Formaldehyde Metabolism

- Formaldehyde dehydrogenase (FDH)
- Alcohol dehydrogenase (*Adh3*) in rodents
- *Adh5* in humans
  - Oxidizes formaldehyde to formate
  - GSH, NAD+

2. Nitric Oxide (NO) Homeostasis

- GSNO reductase (GSNOR)
  - Reduces GSNO to S-(N-hydroxyamino)glutathione
  - Regulates NO signaling & protein S-nitrosylation
  - GSH, NADH


REPORTS

Protecti
Experimental
Endogenous B

Loretta G. Que,[1] Limin Liu,[1,2] Yu
Stephen H. Gavett,[4] David A. Sch

Mechanisms that protect against as
nitrosoglutathione (GSNO), an endoge
asthmatic airways, suggesting a prot
allergen challenge, wild-type mice ex
increased airway levels of the enzym
depleted of lung S-nitrosothiols (SNO
letion of GSNOR exhibit increases in
way hyperresponsivity. Our results ind
by GSNOR, are critical regulators of air
therapeutic approaches to asthma.

The current understanding of allergic asth-
ma, characterized by airway hyperresponsiv-
ity (AHR) and chronic airway inflammation,
is centered on the role of bronchoconstric-
tor substances and inflammatory mediators
(*1–3*). The role of endogenous airway relax-
ants in the pathogenesis of asthma has received
less attention, and the relative importance of
impaired airway relaxation versus active con-
striction is unknown.

Nitric oxide (NO) has been implicated in
the regulation of airway tone (*4*), and ele-
vated levels of NO in the exhaled breath are
a signature of asthma (*5*, *6*), attributed in part
to up-regulation of cytokine-inducible NO
synthase (iNOS) within the airways (*7–10*).
However, neither genetic deletion of iNOS in
mice nor pharmacological inhibition of NOS
in asthmatic patients has provided signifi-
cant protection against AHR (*7*, *10*, *11*). Fur-
thermore, animals deficient in NO synthases
that are expressed constitutively in the lung
(eNOS, nNOS, and iNOS) do not exhibit in-
creases in airway tone or AHR (*7*).

Accumulating evidence indicates that NO
bioactivity is conveyed largely through the
covalent modification of cysteine sulfurs by
NO to form *S*-nitrosothiols (SNOs) (*12–14*).
Of these, *S*-nitrosoglutathione (GSNO) repre-
sents a major source of bronchodilatory NO
bioactivity (airway concentrations of GSNO
are much higher than those of NO) (*15*), and
abnormal metabolism of GSNO (reflecting al-
tered NOS activity) has been described in
several lung diseases (*15–17*). Paradoxically,

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1618

allergen ovalbumin (OVA) (2*f*) (Fig. 1A). As

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S-nitrosoglutathione reductase defi
mouse liver

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+415-502-4995;
Email: Limin.Liu@ucsf.edu

**In human hepatocellular carcinoma (HCC) and n
cancers, somatic point mutations are highly preval
mechanisms critical in their generation remain poo
stood. *S*-nitrosoglutathione reductase (GSNOR), a
tor of protein *S*-nitrosylation, is frequently deficient
HCC. Targeted deletion of the GSNOR gene in mice
the activity of the DNA repair protein O^6-alkylgu
alkyltransferase (AGT) and promote both carcinog
and spontaneous HCC. In this study, we report tha
exposure to the environmental carcinogen diethyln
the mutation frequency of a transgenic reporter in
GSNOR-deficient mice ($GSNOR^{-/-}$) is significantly h
that in wild-type control. In wild-type mice, diethyln
treatment does not significantly increase the freque
transition from G:C to A:T, a mutation deriving fro
trosamine-induced O^6-ethylguanines that are normal
by AGT. In contrast, the frequency of this transition
ylnitrosamine is increased ~20 times in $GSNOR^{-/-}$ mi
deficiency also significantly increases the frequency o
version from A:T to T:A, a mutation not affected by AG
deficiency in our experiments does not significantly a
the frequencies of the other diethylnitrosamine-ind
mutations or hepatocyte proliferation. Thus, GSNOR
through both AGT-dependent and AGT-independent
significantly raises the rates of specific types of DNA
Our results demonstrate a critical role for GSNOR i
ing genomic integrity in mice and support the hyp
GSNOR deficiency is an important cause of the
mutations in human HCC.**

Introduction

DNA sequencing of cancer genomes has revealed rema
numbers of somatically acquired mutations in many hu
(1). These mutations, occurring in both protein-coding
regions throughout the cancer genomes, are mostly singl
substitutions. There are for instance over 11 000 somatic
in a typical hepatocellular carcinoma (HCC) (2). The ge
by the somatic mutations are highly heterogeneous (2–4
tional heterogeneity is also substantial within individual
The characteristic abundance and substantial heterogeneit
mutations in cancer genomes have profound implications
perspective of carcinogenesis and therapeutic approache
However, defects in DNA repair systems have only bee
in limited cancer incidences, and the causes and mechan
somatic mutations remain largely unknown in HCC and
cancers.

Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase;
ylnitrosamine; GSNOR, *S*-nitrosoglutathione reductase; HCC,
carcinoma; iNOS, inducible nitric oxide synthase; LPS, lipopo
NO, nitric oxide.

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Molecular Cell
Article

CellPress

Endogenous Formaldehyde Is a Hematopoietic Stem Cell Genotoxin and Metabolic Carcinogen

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SUMMARY

Endogenous formaldehyde is produced by numerous biochemical pathways fundamental to life, and it can crosslink both DNA and proteins. However, the consequences of its accumulation are unclear. Here we show that endogenous formaldehyde is removed by the enzyme alcohol dehydrogenase 5 (ADH5/GSNOR), and *Adh5*$^{-/-}$ mice therefore accumulate formaldehyde adducts in DNA. The repair of this damage is mediated by FANCD2, a DNA crosslink repair protein. *Adh5*$^{-/-}$*Fancd2*$^{-/-}$ mice reveal an essential requirement for these protection mechanisms in hematopoietic stem cells (HSCs), leading to their depletion and precipitating bone marrow failure. More widespread formaldehyde-induced DNA damage also causes karyomegaly and dysfunction of hepatocytes and nephrons. Bone marrow transplantation not only rescued hematopoiesis but, surprisingly, also preserved nephron function. Nevertheless, all of these animals eventually developed fatal malignancies. Formaldehyde is therefore an important source of endogenous DNA damage that is counteracted in mammals by a conserved protection mechanism.

INTRODUCTION

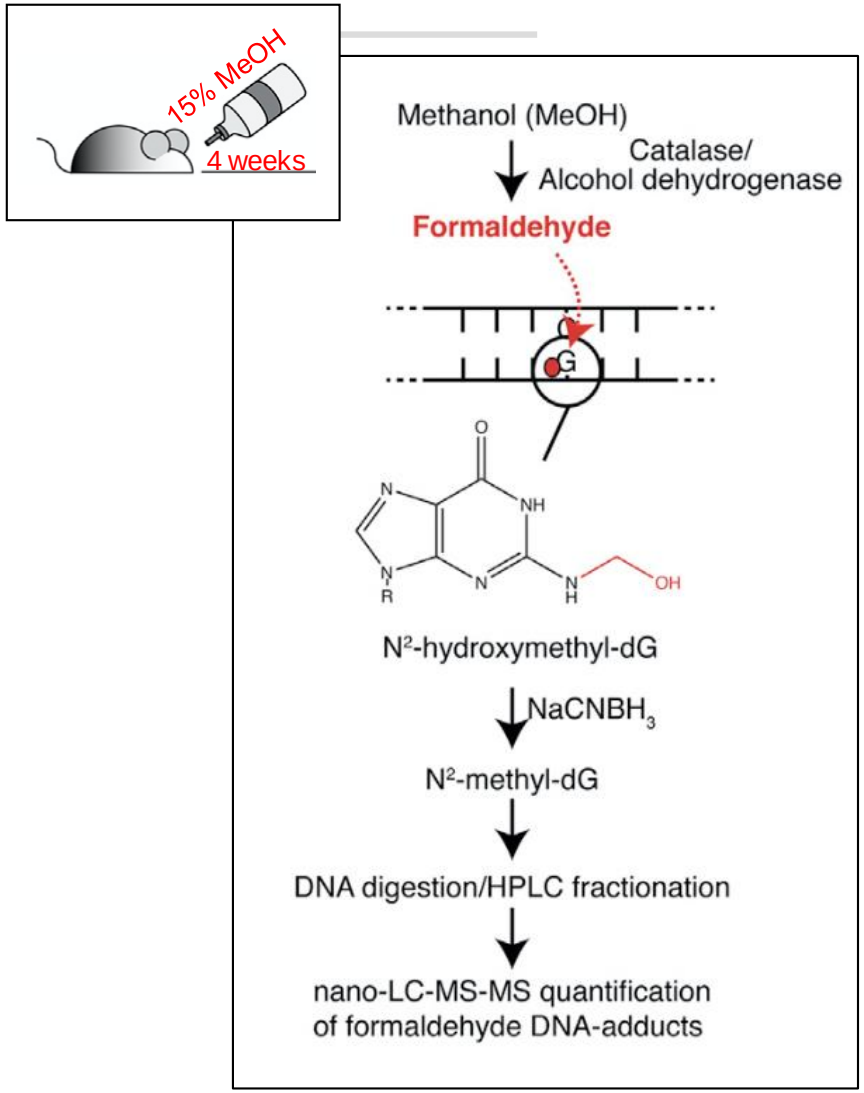
Chromosomal DNA is intrinsically unstable. The nuclear environment leads to spontaneous base decomposition through processes such as deamination (e.g., converting cytosine bases to uracil) (Lindahl and Nyberg, 1972). In addition, reactive molecules, found within the nucleus, can chemically attack DNA, causing a plethora of DNA adducts and lesions (Lindahl, 1993). Despite this, the genome is kept free of errors because the cell has evolved several mechanisms to detect, and then to repair, damaged DNA. Reactive oxygen species (ROS) are perhaps the most ubiquitous and well-known molecules that damage DNA, but endogenous aldehydes are another class of highly reactive, metabolically derived molecules that could also pose a threat to the genome (Yu, 1994). The presence of a carbonyl group makes this class of organic chemicals highly reactive toward proteins and DNA.

The human genetic illness Fanconi anemia (FA) results from an inability to deal with certain forms of DNA damage. The accumulation of DNA damage in FA leads to bone marrow failure, developmental abnormalities, sterility, and a predisposition to develop cancer. The endogenous factors that cause this phenotype are the focus of current research, with evidence pointing to two contrasting sources. The first comes from mice and humans afflicted with FA, who simultaneously lack the acetaldehyde-catabolizing enzyme ALDH2 (Garaycoechea et al., 2012; Hira et al., 2013; Langevin et al., 2011). This combined deficiency greatly accelerates hematopoietic stem cell (HSC) attrition and the onset of leukemia. These mice are sensitized to ethanol, indicating that an accumulation of acetaldehyde is sufficient to produce HSC loss. The second comes from a very recent study on FA-deficient mice, which were stimulated to induce stress-driven hematopoiesis (Walter et al., 2015). This experimental manipulation led to an increase in HSC cycling, induction of ROS, and an accumulation of oxidative DNA lesions. These two disparate drivers of endogenous DNA damage are not linked, so it is unclear what exactly the FA DNA repair pathway responds to in the physiological setting. Moreover, it raises questions concerning the main cause of HSC attrition in FA and which endogenous factor causes it.

CrossMark

Molecular Cell *60*, 177–188, October 1, 2015 ©2015 The Authors 177

ADH5 Protects Against Exogenous FA-DNA Adducts



Pontel et al. (2015)

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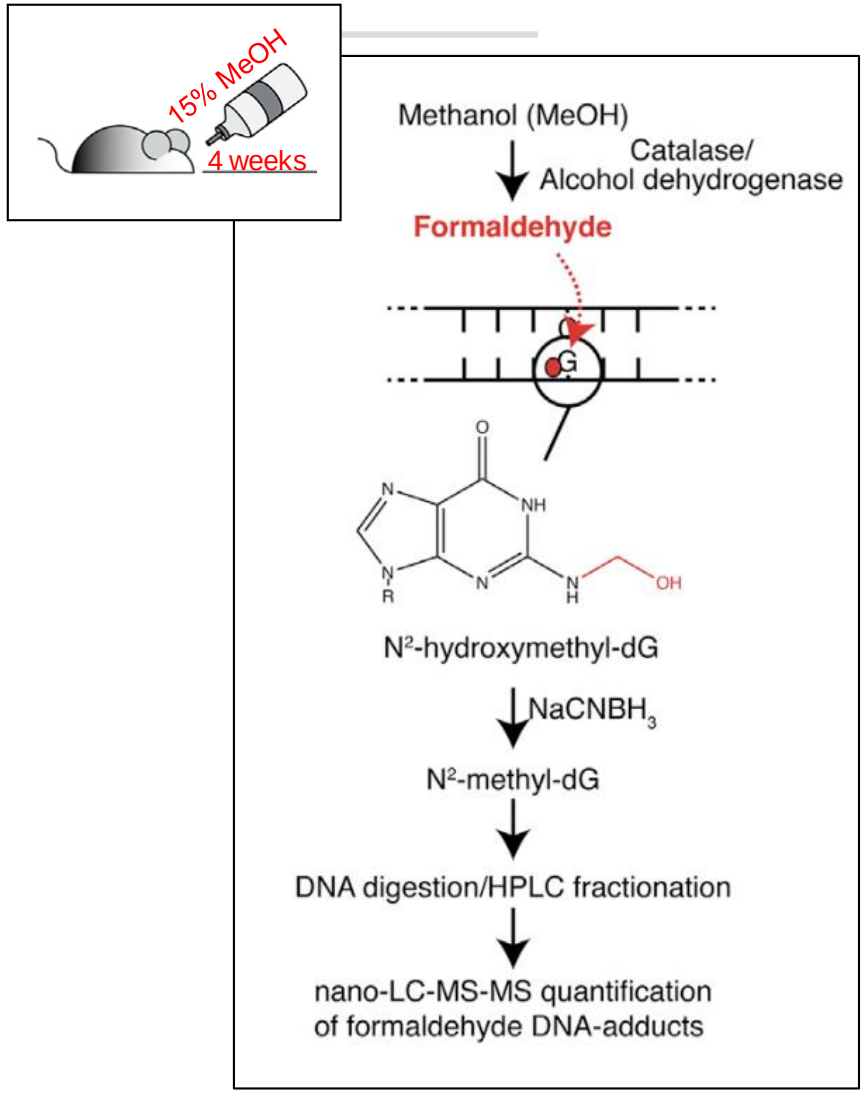
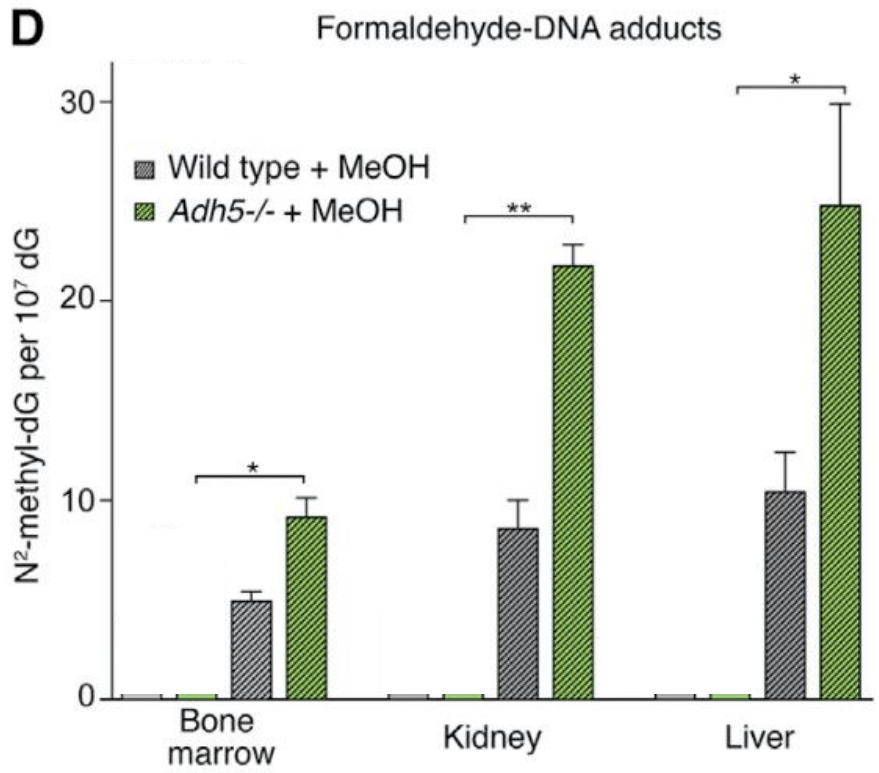
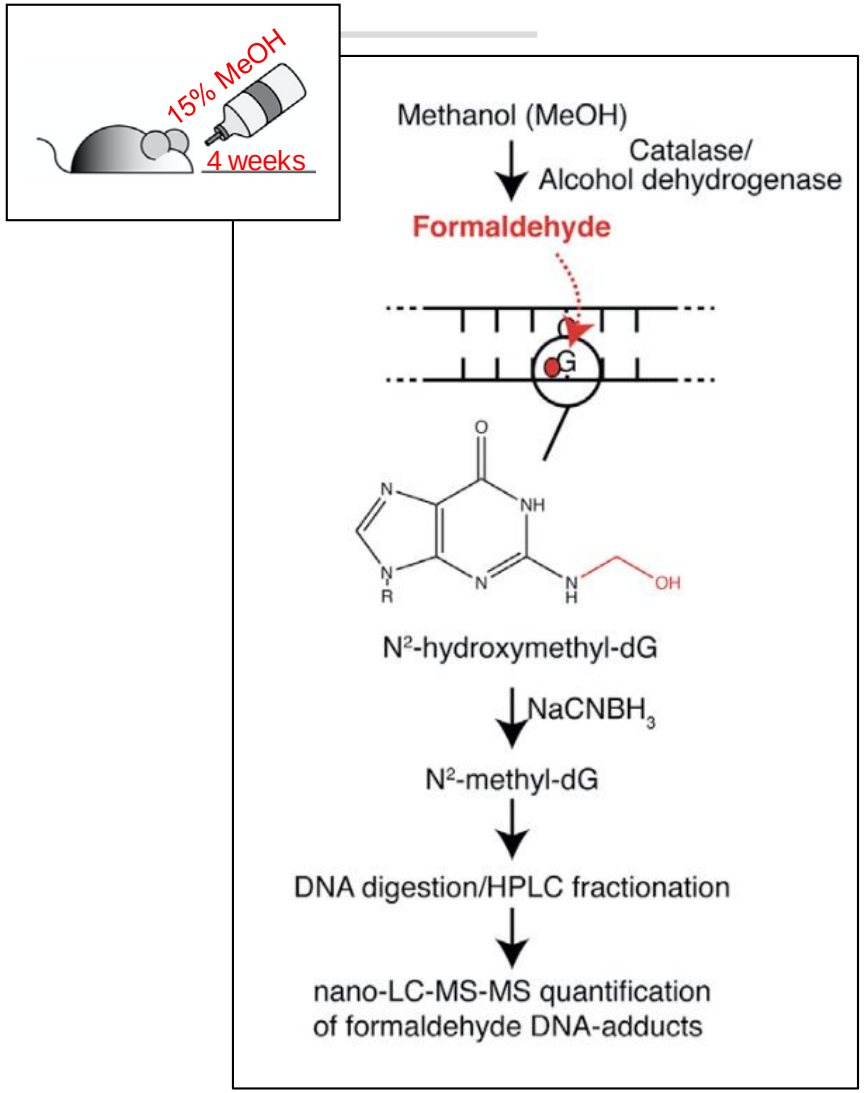
ADH5 Protects Against Exogenous FA-DNA Adducts

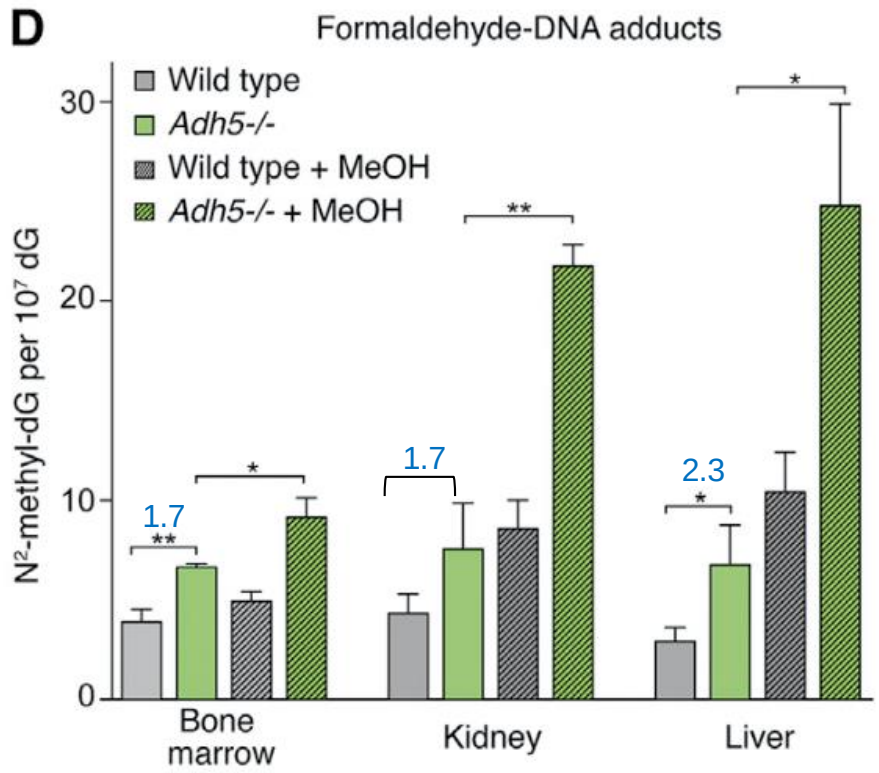




Pontel et al. (2015)

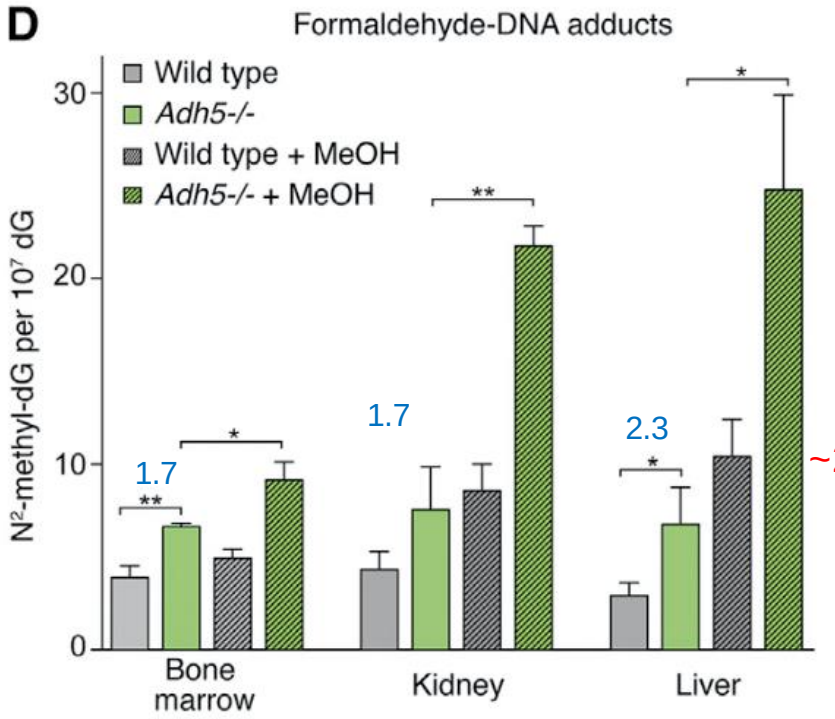
ADH5 Protects Against Exogenous FA-DNA Adducts





Pontel et al. (2015)

FA-DNA Adducts in Adh5$^{-/-}$ Mice Are Comparable to 6-10 ppm Formaldehyde Exposure



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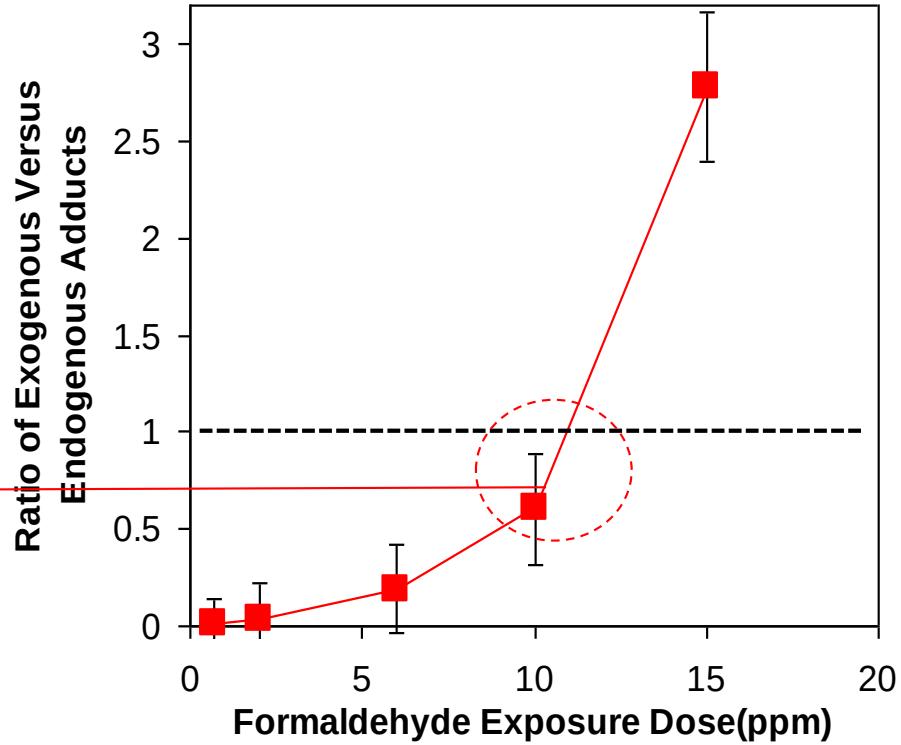
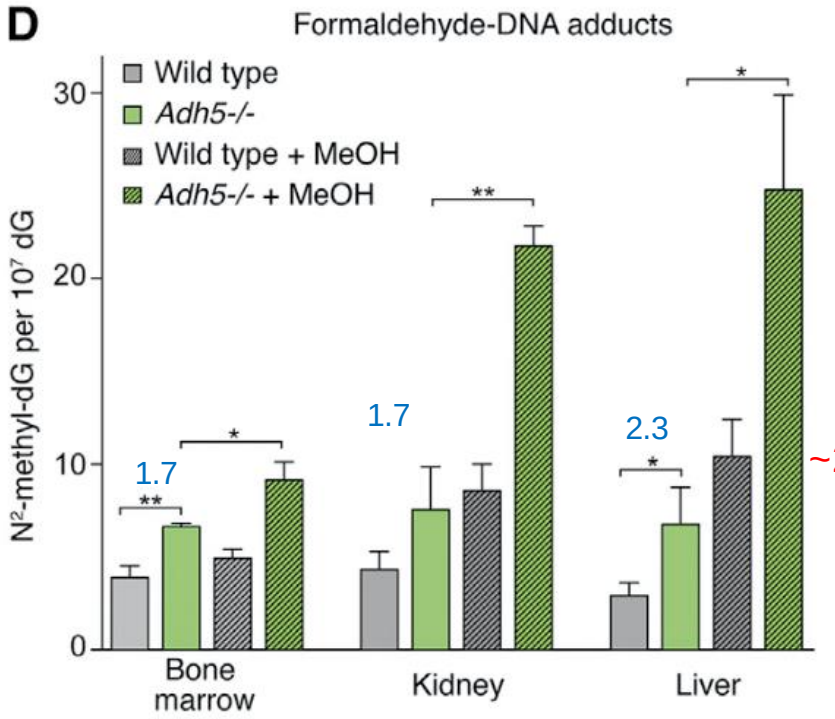


ToxStrategies

FA-DNA Adducts in Adh5$^{-/-}$ Mice Are Comparable to 6-10 ppm Formaldehyde Exposure



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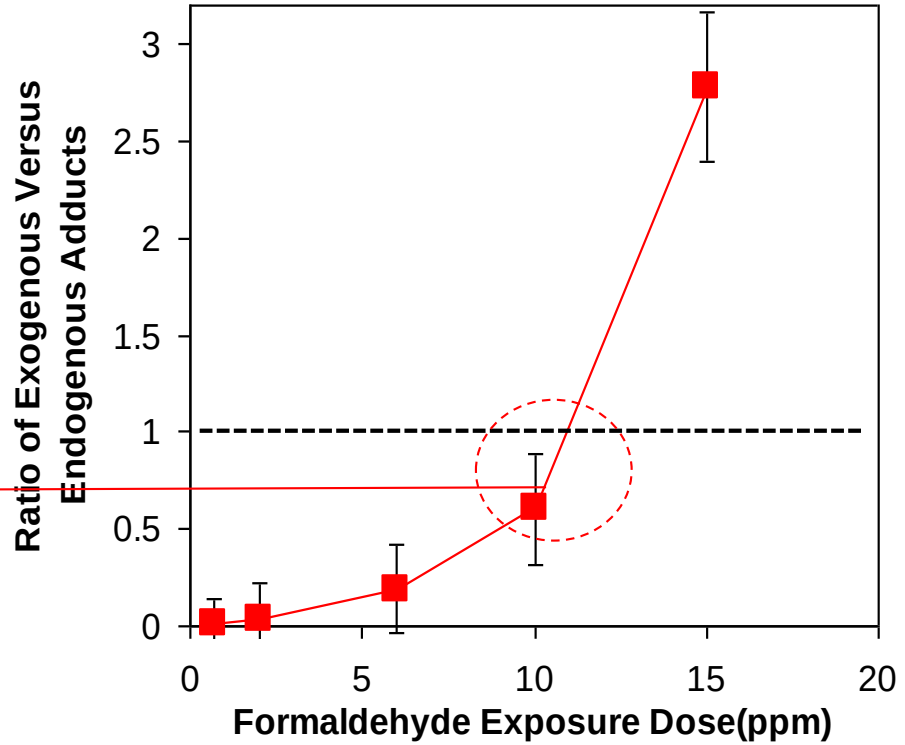
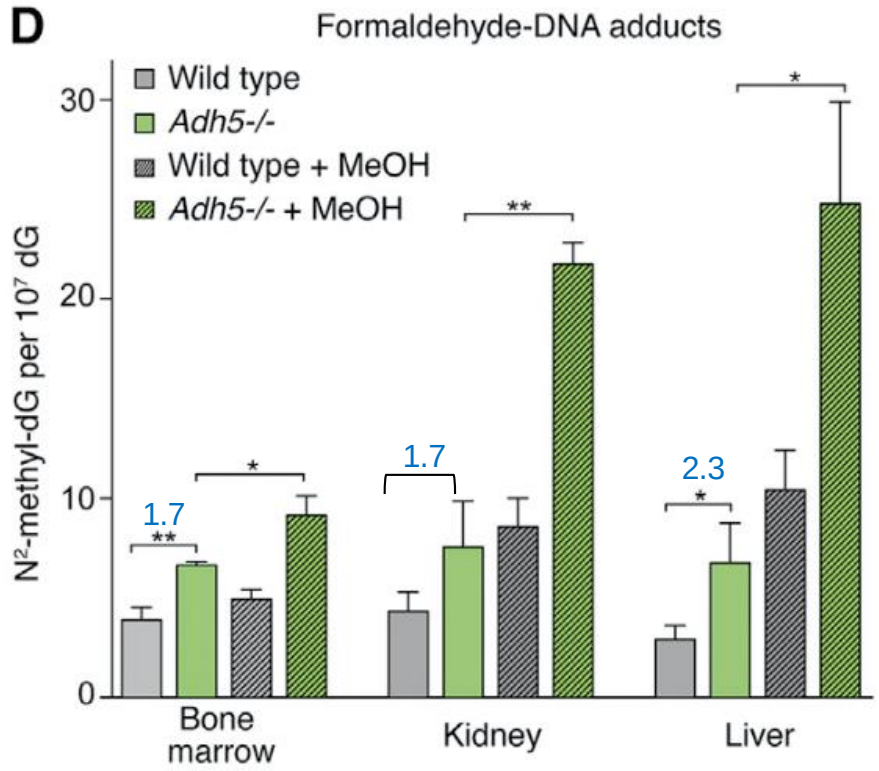


Is there other evidence of genotoxicity?

ToxStrategies

ADH5 Protects Against Exogenous FA-DNA Adducts

- Adh5$^{-/-}$ mice did not exhibit increased γ-H2AX activity in other assays
- Adh5$^{-/-}$ mice did not exhibit increased nuclear aberrations in other assays



Pontel et al. (2015)

ToxStrategies

Adh5$^{-/-}$ Mice Do Not Exhibit Increased Mutant Frequency

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doi:10.1093/carcin/bgt031
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S-nitrosoglutathione reductase deficiency increases mutagenesis from alkylation in mouse liver

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In human hepatocellular carcinoma (HCC) and many other cancers, somatic point mutations are highly prevalent, yet the mechanisms critical in their generation remain poorly understood. *S*-nitrosoglutathione reductase (GSNOR), a key regulator of protein *S*-nitrosylation, is frequently deficient in human HCC. Targeted deletion of the GSNOR gene in mice can reduce the activity of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) and promote both carcinogen-induced and spontaneous HCC. In this study, we report that following exposure to the environmental carcinogen diethylnitrosamine, the mutation frequency of a transgenic reporter in the liver of GSNOR-deficient mice (GSNOR$^{-/-}$) is significantly higher than that in wild-type control. In wild-type mice, diethylnitrosamine treatment does not significantly increase the frequency of the transition from G:C to A:T, a mutation deriving from diethylnitrosamine-induced O^6-ethylguanines that are normally repaired by AGT. In contrast, the frequency of this transition from diethylnitrosamine is increased ~20 times in GSNOR$^{-/-}$ mice. GSNOR deficiency also significantly increases the frequency of the transversion from A:T to T:A, a mutation not affected by AGT. GSNOR deficiency in our experiments does not significantly affect either the frequencies of the other diethylnitrosamine-induced point mutations or hepatocyte proliferation. Thus, GSNOR deficiency, through both AGT-dependent and AGT-independent pathways, significantly raises the rates of specific types of DNA mutations. Our results demonstrate a critical role for GSNOR in maintaining genomic integrity in mice and support the hypothesis that GSNOR deficiency is an important cause of the widespread mutations in human HCC.

Introduction

DNA sequencing of cancer genomes has revealed remarkably large numbers of somatically acquired mutations in many human cancers (1). These mutations, occurring in both protein-coding and other regions throughout the cancer genomes, are mostly single-nucleotide substitutions. There are for instance over 11 000 somatic substitutions in a typical hepatocellular carcinoma (HCC) (2). The genes affected by the somatic mutations are highly heterogeneous (2–4), and mutational heterogeneity is also substantial within individual tumors (1). The characteristic abundance and substantial heterogeneity of somatic mutations in cancer genomes have profound implications in both our perspective of carcinogenesis and therapeutic approaches to cancer. However, defects in DNA repair systems have only been described in limited cancer incidences, and the causes and mechanisms of the somatic mutations remain largely unknown in HCC and many other cancers.

Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; DEN, diethylnitrosamine; GSNOR, *S*-nitrosoglutathione reductase; HCC, hepatocellular carcinoma; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide.

Human HCC, the third leading cause of cancer deaths worldwide, develops mostly in the context of chronic viral hepatitis (5). Inducible nitric oxide synthase (iNOS), a key mediator of innate immune response and inflammation, is often increased both in HCC cells and in the hepatocytes of patients with chronic viral hepatitis and other diseases that predispose to HCC (6–9). Nitric oxide (NO) affects functions of a wide range of proteins—including many important to tumorigenesis—through *S*-nitrosylation, the covalent modification of cysteine thiols (10). *S*-nitrosylation is not only influenced by NOS activities but is also prominently regulated by *S*-nitrosoglutathione reductase (GSNOR), a major denitrosylase (11–13). The human GSNOR gene (*ADH5*) is located at ~4q23, a region in which chromosomal deletion occurs most frequently in HCC (14–17). We showed that the abundance and activity of GSNOR were significantly decreased in cancer samples from ~50% of patients with HCC (18). Interestingly, gene-expression profiling showed that both GSNOR (ADH5) deficiency and iNOS overexpression in the liver are closely associated with *de novo* hepatocarcinogenesis after tumor resection and a poor prognosis in HCC patients (19). Thus, excessive *S*-nitrosylation from GSNOR deficiency and concurrent iNOS overexpression in the liver may contribute critically to human HCC.

Recent studies using a mouse line with targeted deletion of the GSNOR gene demonstrated that *S*-nitrosylation from GSNOR deficiency inactivates the key DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT; also known as O^6-methylguanine-DNA-methyltransferase) and promotes HCC (18,20). O^6-alkylguanines in DNA are produced by alkylating *N*-nitroso compounds, including dialkylnitrosamines, which are widely present in the environment and can also be formed endogenously (21–23). O^6-alkylguanines are mispaired by DNA polymerases to thymine during DNA replication, and the O^6-alkylguanine:T mispairing, through a further round of DNA replication, can result in G:C to A:T mutations (22). Mutagenic O^6-alkylguanines are repaired primarily by AGT (24) although repair of O^6-alkylguanines might be affected by nucleotide excision repair (25). AGT is important for protection against HCC (26–28). We showed that during inflammatory responses following intraperitoneal injection of diethylnitrosamine (DEN) or lipopolysaccharide (LPS), GSNOR deficiency resulted in *S*-nitrosylation, ubiquitination and proteosomal degradation of AGT, leading to its significant reduction in livers of GSNOR$^{-/-}$ mice (18). Consequently, the repair of carcinogenic O^6-ethylguanines in the livers of DEN-challenged GSNOR$^{-/-}$ mice was impaired. GSNOR$^{-/-}$ mice were found to be very susceptible to both spontaneous and DEN-induced HCC. Predisposition to HCC, *S*-nitrosylation and depletion of AGT, and accumulation of O^6-ethylguanines due to GSNOR deficiency were remarkably all abolished by concurrent deletion of the iNOS gene in GSNOR$^{-/-}$iNOS$^{-/-}$ mice, further underscoring the critical role of iNOS-derived *S*-nitrosylation in AGT inactivation and liver carcinogenesis in GSNOR$^{-/-}$ mice (18). Moreover, hepatocyte-specific deletion of GSNOR caused nitrosative inactivation of liver AGT and increased mortality from DEN (20), demonstrating the importance of GSNOR regulation of *S*-nitrosylation in liver parenchymal cells. *S*-nitrosylation may affect DNA repair pathways other than AGT (29), and dialkylnitrosamines can cause a number of mutagenic DNA alkylations in addition to O^6-alkylguanines (30). NO and related reactive nitrogen species at high levels may also damage DNA directly (31). However, it is unknown whether GSNOR deficiency *in vivo* affects the rate or spectrum of DNA mutation.

To examine the impact of GSNOR deficiency on DNA mutations, we employed Big Blue transgenic mice, a well-established system for detecting DNA mutations *in vivo* (32), and measured the rates of both spontaneous and DEN-induced mutations in livers of GSNOR$^{-/-}$ Big Blue double-transgenic mice. We found that after DEN treatment,

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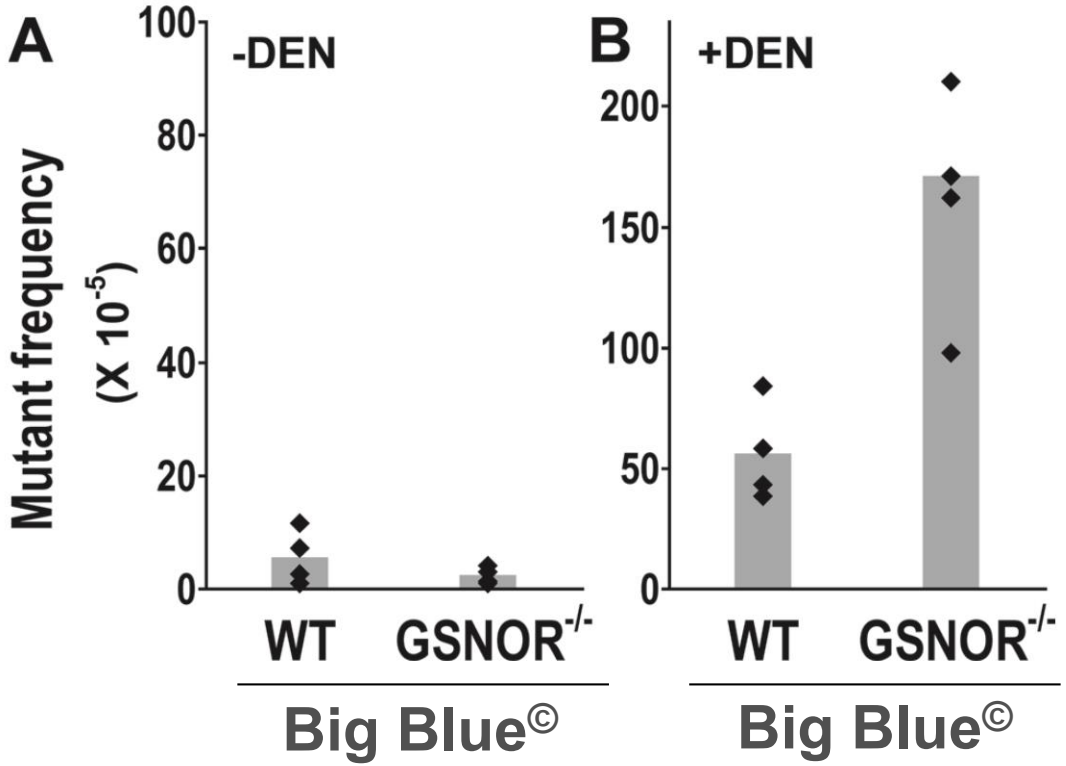
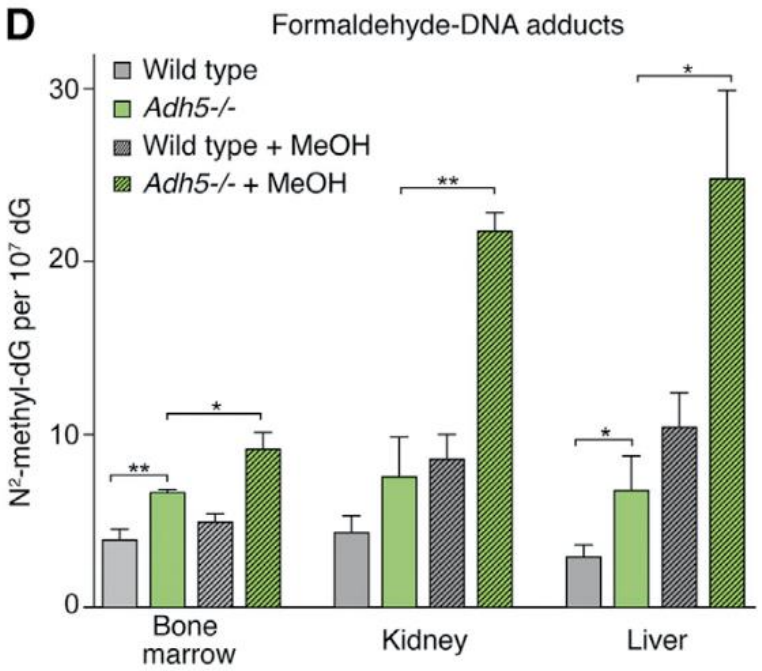
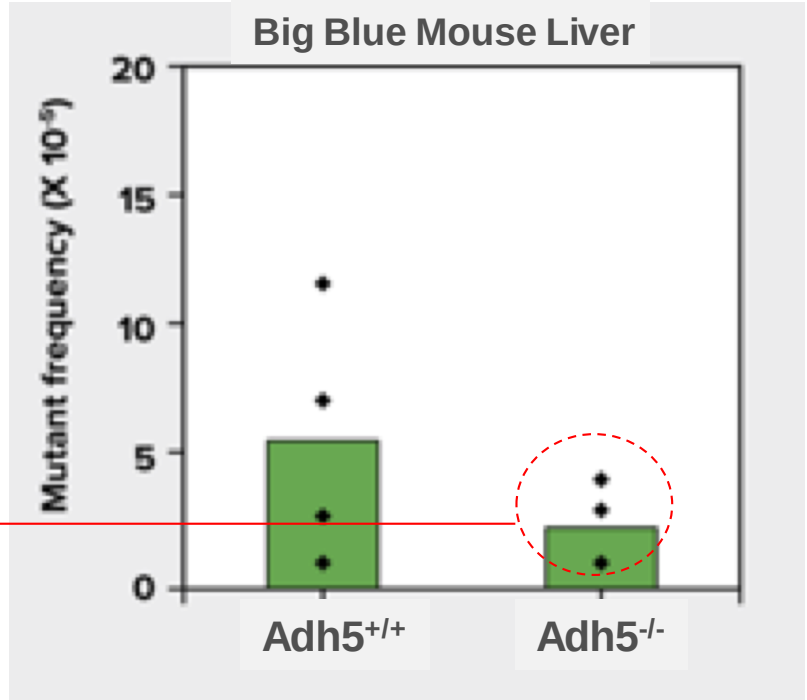


ToxStrategies

Bridging Across Knockout Studies: 2-fold Increases in Adducts do not Increase Mutant Frequency



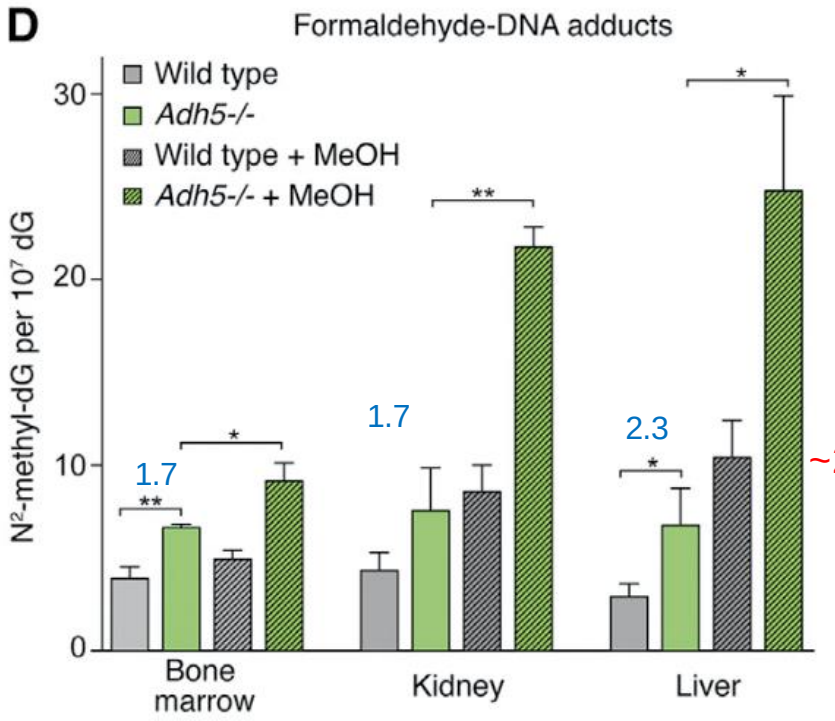
Pontel et al. (2015)



Leung et al. (2013)

ToxStrategies

FA-DNA Adducts in Adh5$^{-/-}$ Mice Are Comparable to 6-10 ppm Formaldehyde Exposure



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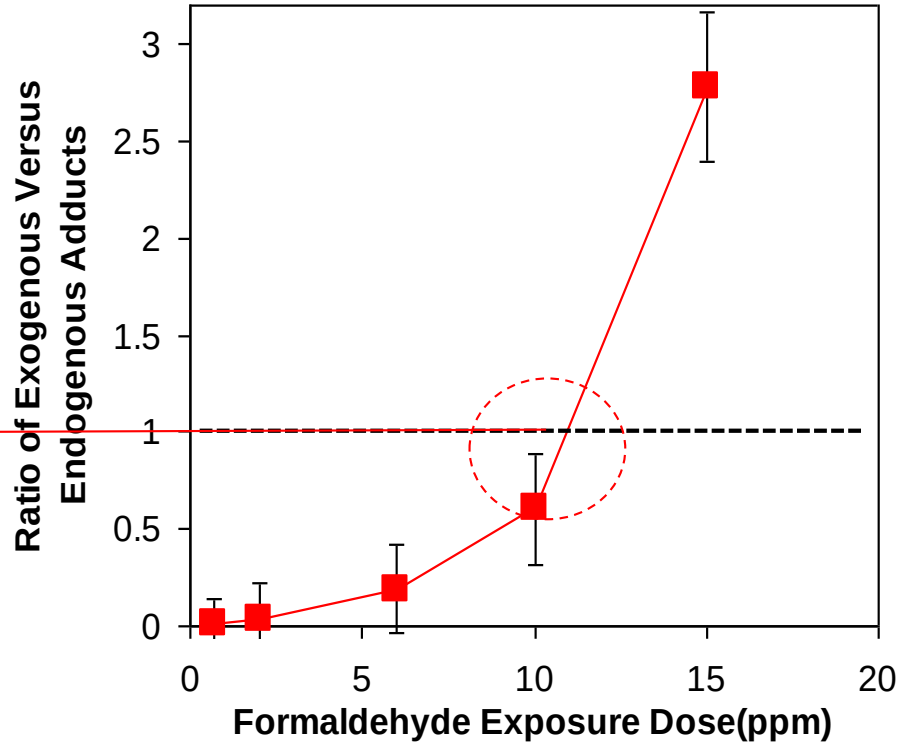
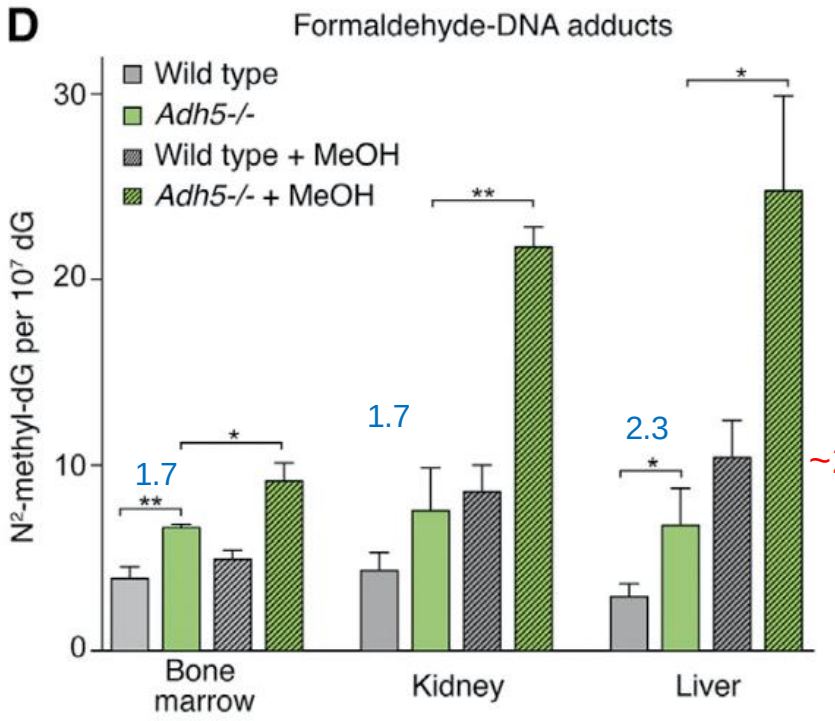


- **Exogenous adduct levels associated with 2x (1:1 ratio) may not be mutagenic (red circle)**

ToxStrategies

FA-DNA Adducts in Adh5$^{-/-}$ Mice Are Comparable to 6-10 ppm Formaldehyde Exposure



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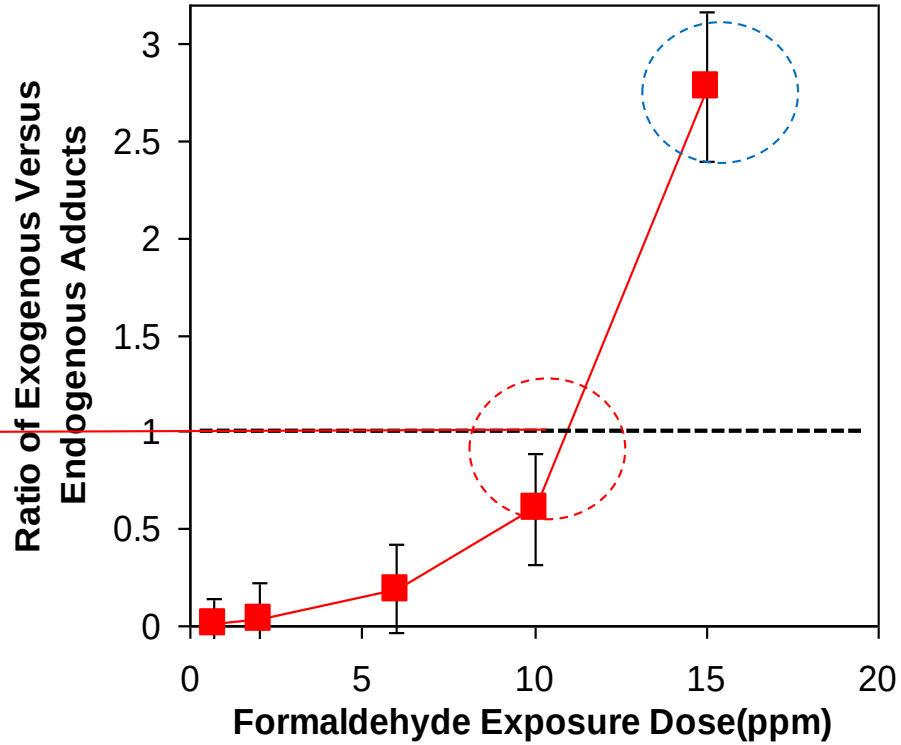

- **Exogenous adducts levels associated with 2x (1:1 ratio) may not be mutagenic (red circle)**
- **What data are available for higher adduct levels (blue circle)?**

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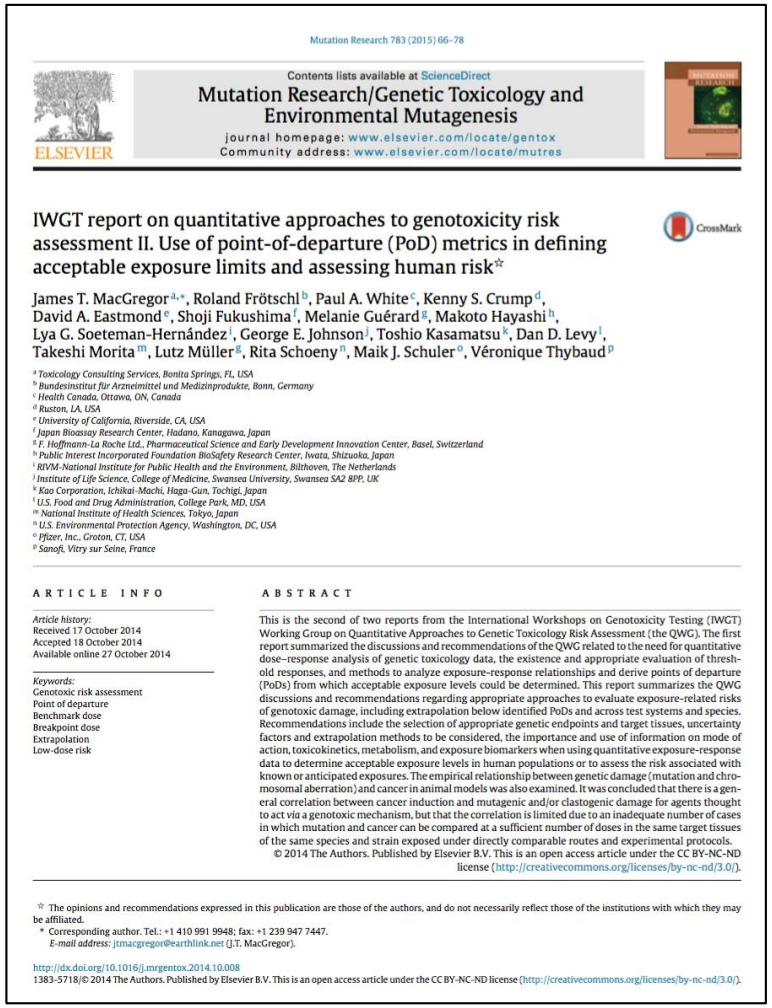
IWGT Recommendations for In Vivo Genotoxicity Assays



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IWGT report on quantitative approaches to genotoxicity risk assessment II. Use of point-of-departure (PoD) metrics in defining acceptable exposure limits and assessing human risk☆

James T. MacGregor[a,*], Roland Frötschl[b], Paul A. White[c], Kenny S. Crump[d], David A. Eastmond[e], Shoji Fukushima[f], Melanie Guérard[g], Makoto Hayashi[h], Lya G. Soeteman-Hernández[i], George E. Johnson[j], Toshio Kasamatsu[k], Dan D. Levy[l], Takeshi Morita[m], Lutz Müller[g], Rita Schoeny[n], Maik J. Schuler[o], Véronique Thybaud[p]

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ABSTRACT

This is the second of two reports from the International Workshops on Genotoxicity Testing (IWGT) Working Group on Quantitative Approaches to Genetic Toxicology Risk Assessment (the QWG). The first report summarized the discussions and recommendations of the QWG related to the need for quantitative dose–response analysis of genetic toxicology data, the existence and appropriate evaluation of threshold responses, and methods to analyze exposure-response relationships and derive points of departure (PoDs) from which acceptable exposure levels could be determined. This report summarizes the QWG discussions and recommendations regarding appropriate approaches to evaluate exposure-related risks of genotoxic damage, including extrapolation below identified PoDs and across test systems and species. Recommendations include the selection of appropriate genetic endpoints and target tissues, uncertainty factors and extrapolation methods to be considered, the importance and use of information on mode of action, toxicokinetics, metabolism, and exposure biomarkers when using quantitative exposure-response data to determine acceptable exposure levels in human populations or to assess the risk associated with known or anticipated exposures. The empirical relationship between genetic damage (mutation and chromosomal aberration) and cancer in animal models was also examined. It was concluded that there is a general correlation between cancer induction and mutagenic and/or clastogenic damage for agents thought to act *via* a genotoxic mechanism, but that the correlation is limited due to an inadequate number of cases in which mutation and cancer can be compared at a sufficient number of doses in the same target tissues of the same species and strain exposed under directly comparable routes and experimental protocols.

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☆ The opinions and recommendations expressed in this publication are those of the authors, and do not necessarily reflect those of the institutions with which they may be affiliated.

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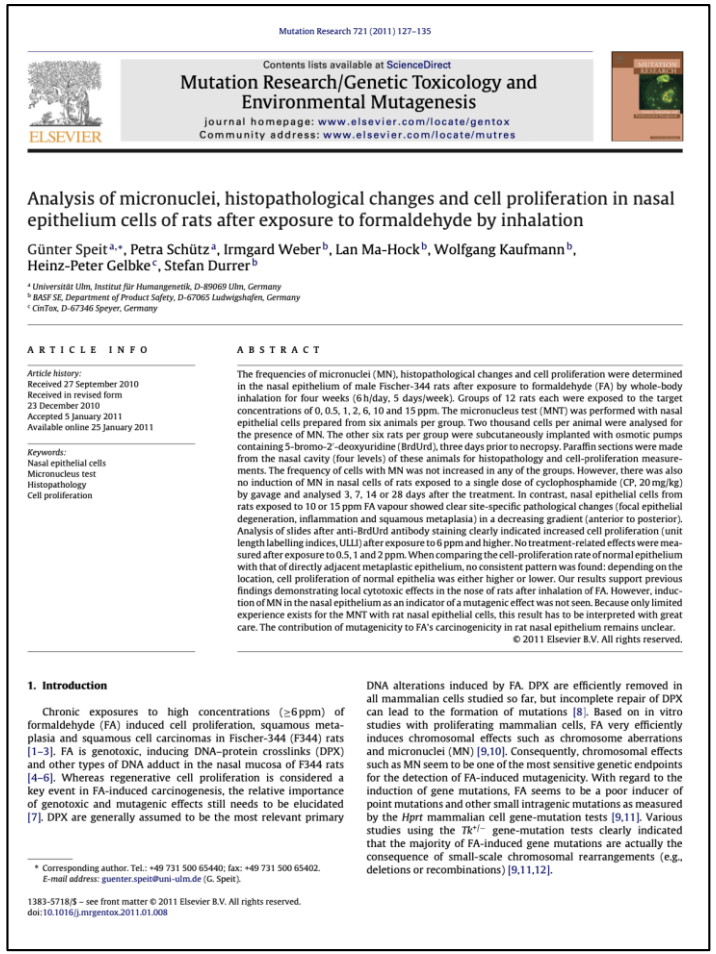
- Ideally conducted in a proliferative tissue
  - Bone marrow (hematopoietic)
  - Colon
  - Young liver
- Ideally at site of carcinogenic action
  - **Nasal epithelium for FA**
- Ideally in tissue with high dosimetry (e.g. site of contact)
  - **Nasal epithelium for FA**

ToxStrategies

Assessment of Micronucleus Formation in Nasal Epithelium



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Analysis of micronuclei, histopathological changes and cell proliferation in nasal epithelium cells of rats after exposure to formaldehyde by inhalation

Günter Speit[a,*], Petra Schütz[a], Irmgard Weber[b], Lan Ma-Hock[b], Wolfgang Kaufmann[b], Heinz-Peter Gelbke[c], Stefan Durrer[b]

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ABSTRACT

The frequencies of micronuclei (MN), histopathological changes and cell proliferation were determined in the nasal epithelium of male Fischer-344 rats after exposure to formaldehyde (FA) by whole-body inhalation for four weeks (6 h/day, 5 days/week). Groups of 12 rats each were exposed to the target concentrations of 0, 0.5, 1, 2, 6, 10 and 15 ppm. The micronucleus test (MNT) was performed with nasal epithelial cells prepared from six animals per group. Two thousand cells per animal were analysed for the presence of MN. The other six rats per group were subcutaneously implanted with osmotic pumps containing 5-bromo-2′-deoxyuridine (BrdUrd), three days prior to necropsy. Paraffin sections were made from the nasal cavity (four levels) of these animals for histopathology and cell-proliferation measurements. The frequency of cells with MN was not increased in any of the groups. However, there was also no induction of MN in nasal cells of rats exposed to a single dose of cyclophosphamide (CP, 20 mg/kg) by gavage and analysed 3, 7, 14 or 28 days after the treatment. In contrast, nasal epithelial cells from rats exposed to 10 or 15 ppm FA vapour showed clear site-specific pathological changes (focal epithelial degeneration, inflammation and squamous metaplasia) in a decreasing gradient (anterior to posterior). Analysis of slides after anti-BrdUrd antibody staining clearly indicated increased cell proliferation (unit length labelling indices, ULLI) after exposure to 6 ppm and higher. No treatment-related effects were measured after exposure to 0.5, 1 and 2 ppm. When comparing the cell-proliferation rate of normal epithelium with that of directly adjacent metaplastic epithelium, no consistent pattern was found: depending on the location, cell proliferation of normal epithelia was either higher or lower. Our results support previous findings demonstrating local cytotoxic effects in the nose of rats after inhalation of FA. However, induction of MN in the nasal epithelium as an indicator of a mutagenic effect was not seen. Because only limited experience exists for the MNT with rat nasal epithelial cells, this result has to be interpreted with great care. The contribution of mutagenicity to FA's carcinogenicity in rat nasal epithelium remains unclear.

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1. Introduction

Chronic exposures to high concentrations (≥6 ppm) of formaldehyde (FA) induced cell proliferation, squamous metaplasia and squamous cell carcinomas in Fischer-344 (F344) rats [1–3]. FA is genotoxic, inducing DNA–protein crosslinks (DPX) and other types of DNA adduct in the nasal mucosa of F344 rats [4–6]. Whereas regenerative cell proliferation is considered a key event in FA-induced carcinogenesis, the relative importance of genotoxic and mutagenic effects still needs to be elucidated [7]. DPX are generally assumed to be the most relevant primary DNA alterations induced by FA. DPX are efficiently removed in all mammalian cells studied so far, but incomplete repair of DPX can lead to the formation of mutations [8]. Based on in vitro studies with proliferating mammalian cells, FA very efficiently induces chromosomal effects such as chromosome aberrations and micronuclei (MN) [9,10]. Consequently, chromosomal effects such as MN seem to be one of the most sensitive genetic endpoints for the detection of FA-induced mutagenicity. With regard to the induction of gene mutations, FA seems to be a poor inducer of point mutations and other small intragenic mutations as measured by the *Hprt* mammalian cell gene-mutation tests [9,11]. Various studies using the *Tk*$^{+/-}$ gene-mutation tests clearly indicated that the majority of FA-induced gene mutations are actually the consequence of small-scale chromosomal rearrangements (e.g., deletions or recombinations) [9,11,12].

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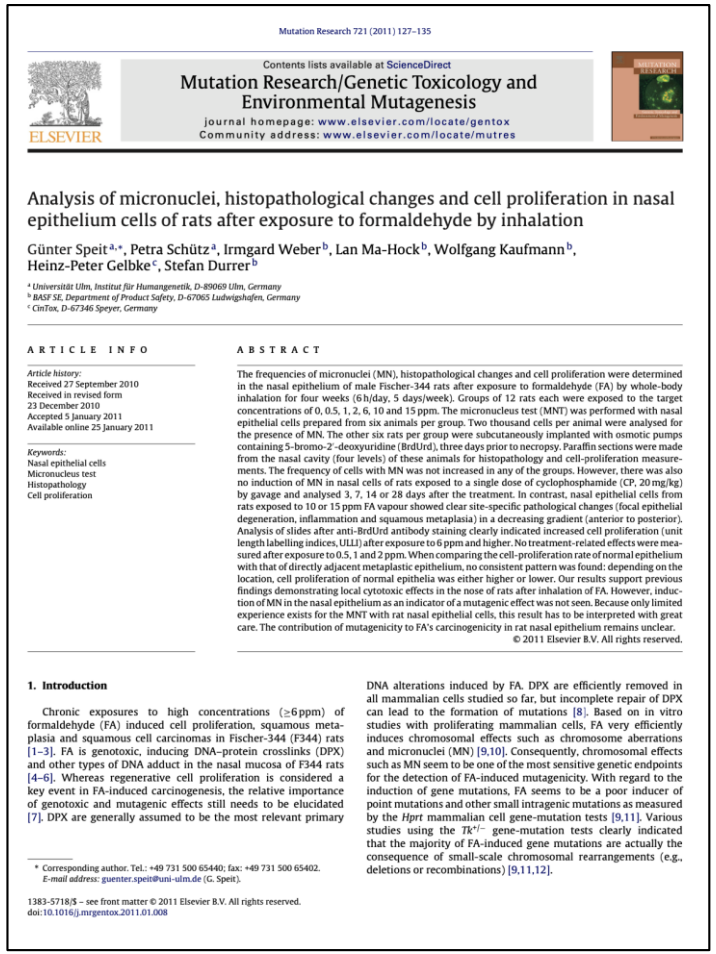
doi:10.1016/j.mrgentox.2011.01.008

- **Exposures: 0, 0.5, 1, 2, 6, 10, 15 ppm**
- **6 hr/day, 5d/wk x 4-wk**
- **Proliferation increased at ≥6 ppm**
- **No increases in nasal cell MN**
- **Limitations**
  - No nasal MN by CP (positive control)
  - More experience needed with nasal MN

ToxStrategies

Assessment of Micronucleus Formation in Nasal Epithelium



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Günter Speit[a,*], Petra Schütz[a], Irmgard Weber[b], Lan Ma-Hock[b], Wolfgang Kaufmann[b], Heinz-Peter Gelbke[c], Stefan Durrer[b]

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1. Introduction

Chronic exposures to high concentrations (≥6 ppm) of formaldehyde (FA) induced cell proliferation, squamous metaplasia and squamous cell carcinomas in Fischer-344 (F344) rats [1–3]. FA is genotoxic, inducing DNA–protein crosslinks (DPX) and other types of DNA adduct in the nasal mucosa of F344 rats [4–6]. Whereas regenerative cell proliferation is considered a key event in FA-induced carcinogenesis, the relative importance of genotoxic and mutagenic effects still needs to be elucidated [7]. DPX are generally assumed to be the most relevant primary DNA alterations induced by FA. DPX are efficiently removed in all mammalian cells studied so far, but incomplete repair of DPX can lead to the formation of mutations [8]. Based on in vitro studies with proliferating mammalian cells, FA very efficiently induces chromosomal effects such as chromosome aberrations and micronuclei (MN) [9,10]. Consequently, chromosomal effects such as MN seem to be one of the most sensitive genetic endpoints for the detection of FA-induced mutagenicity. With regard to the induction of gene mutations, FA seems to be a poor inducer of point mutations and other small intragenic mutations as measured by the *Hprt* mammalian cell gene-mutation tests [9,11]. Various studies using the *Tk*$^{+/-}$ gene-mutation tests clearly indicated that the majority of FA-induced gene mutations are actually the consequence of small-scale chromosomal rearrangements (e.g., deletions or recombinations) [9,11,12].

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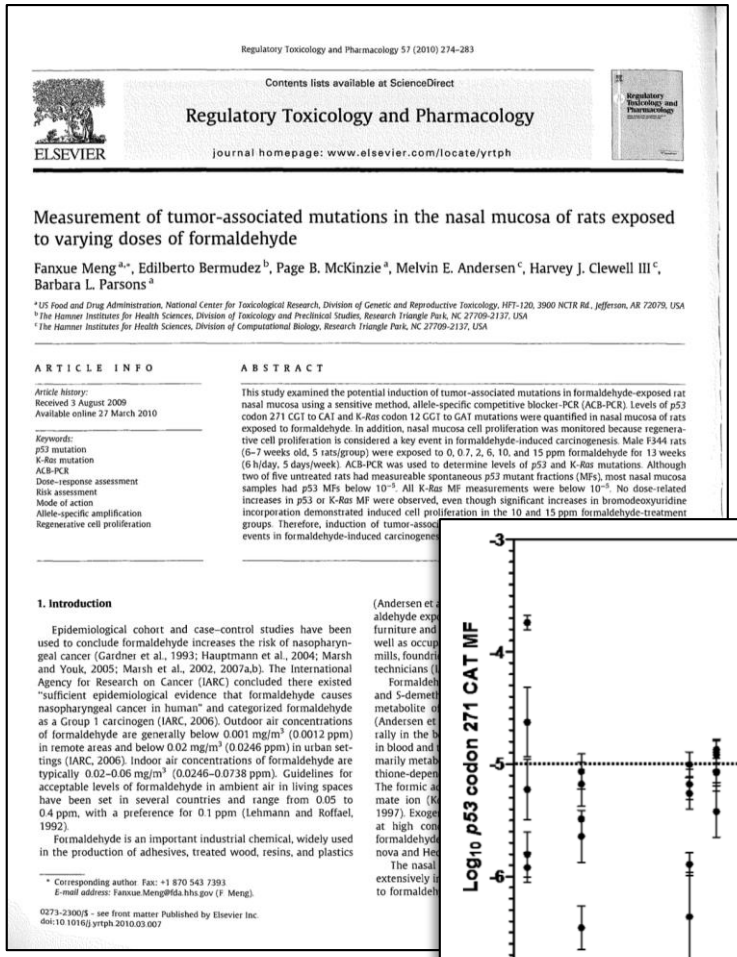
- **Exposures: 0, 0.5, 1, 2, 6, 10, 15 ppm**
- **6 hr/day, 5d/wk x 4-wk**
- **Proliferation increased at ≥6 ppm**
- **No increases in nasal cell MN**
- **Limitations**
  - No nasal MN by CP (positive control)
  - More experience needed with nasal MN

Inability to detect ↑MN in highly proliferative tissue does not support a genotoxic MOA.
Inability to detect ↑MN amongst increased FA-DNA adducts does not support a genotoxic MOA.

ToxStrategies

Assessment of Mutant Frequency in Nasal Epithelium



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Measurement of tumor-associated mutations in the nasal mucosa of rats exposed to varying doses of formaldehyde

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Allele-specific amplification
Regenerative cell proliferation

ABSTRACT

This study examined the potential induction of tumor-associated mutations in formaldehyde-exposed rat nasal mucosa using a sensitive method, allele-specific competitive blocker-PCR (ACB-PCR). Levels of p53 codon 271 CGT to CAT and K-*Ras* codon 12 GGT to GAT mutations were quantified in nasal mucosa of rats exposed to formaldehyde. In addition, nasal mucosa cell proliferation was monitored because regenerative cell proliferation is considered a key event in formaldehyde-induced carcinogenesis. Male F344 rats (6–7 weeks old, 5 rats/group) were exposed to 0, 0.7, 2, 6, 10, and 15 ppm formaldehyde for 13 weeks (6 h/day, 5 days/week). ACB-PCR was used to determine levels of p53 and K-*Ras* mutations. Although two of five untreated rats had measureable spontaneous p53 mutant fractions (MFs), most nasal mucosa samples had p53 MFs below 10^{-5}. All K-*Ras* MF measurements were below 10^{-5}. No dose-related increases in p53 or K-*Ras* MF were observed, even though significant increases in bromodeoxyuridine incorporation demonstrated induced cell proliferation in the 10 and 15 ppm formaldehyde-treatment groups. Therefore, induction of tumor-associ
events in formaldehyde-induced carcinogenes

1. Introduction

Epidemiological cohort and case–control studies have been used to conclude formaldehyde increases the risk of nasopharyngeal cancer (Gardner et al., 1993; Hauptmann et al., 2004; Marsh and Youk, 2005; Marsh et al., 2002, 2007a,b). The International Agency for Research on Cancer (IARC) concluded there existed "sufficient epidemiological evidence that formaldehyde causes nasopharyngeal cancer in human" and categorized formaldehyde as a Group 1 carcinogen (IARC, 2006). Outdoor air concentrations of formaldehyde are generally below 0.001 mg/m^3 (0.0012 ppm) in remote areas and below 0.02 mg/m^3 (0.0246 ppm) in urban settings (IARC, 2006). Indoor air concentrations of formaldehyde are typically 0.02–0.06 mg/m^3 (0.0246–0.0738 ppm). Guidelines for acceptable levels of formaldehyde in ambient air in living spaces have been set in several countries and range from 0.05 to 0.4 ppm, with a preference for 0.1 ppm (Lehmann and Roffael, 1992).

Formaldehyde is an important industrial chemical, widely used in the production of adhesives, treated wood, resins, and plastics

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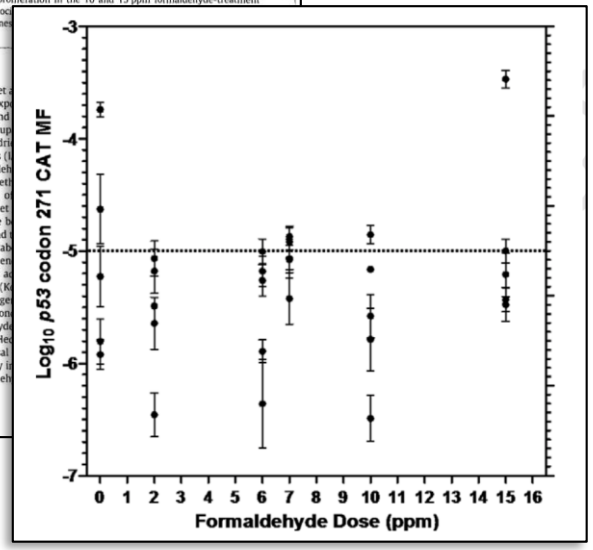

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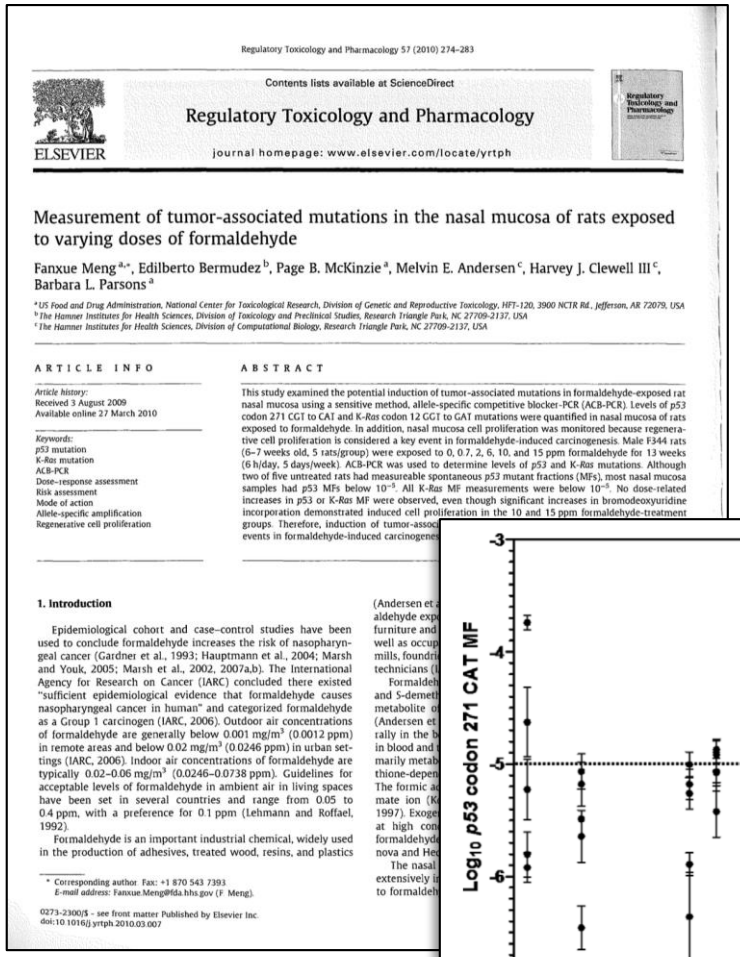

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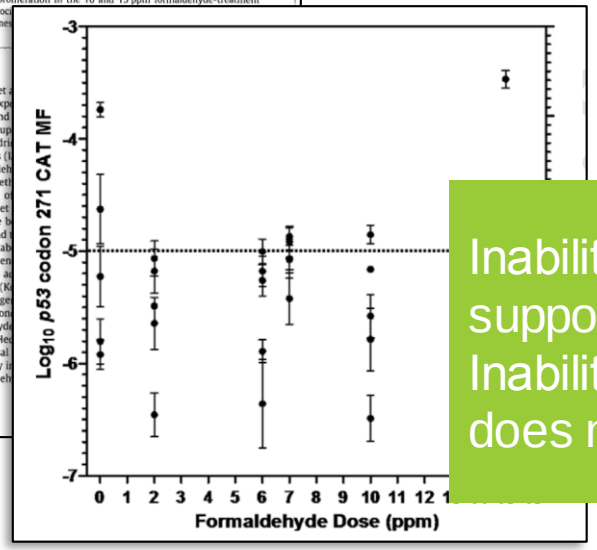

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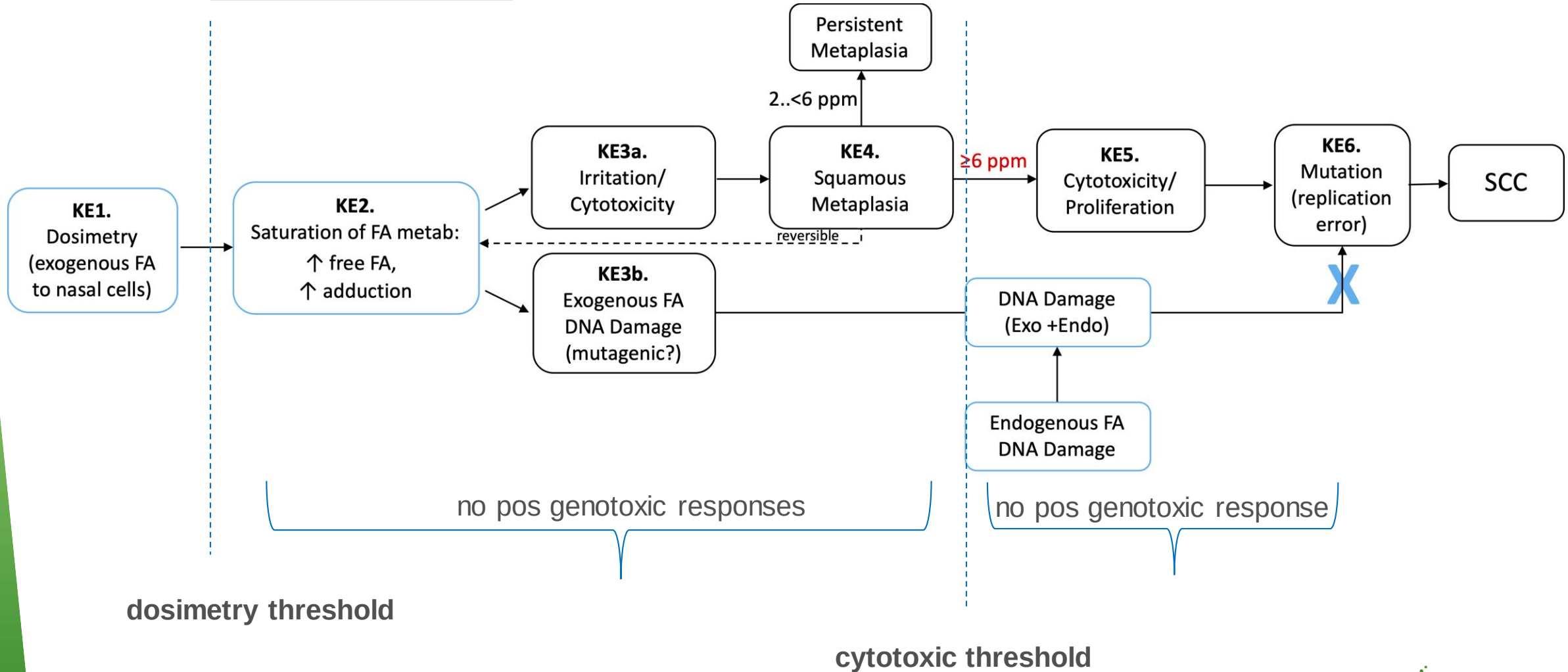
Updated MOA (2020)



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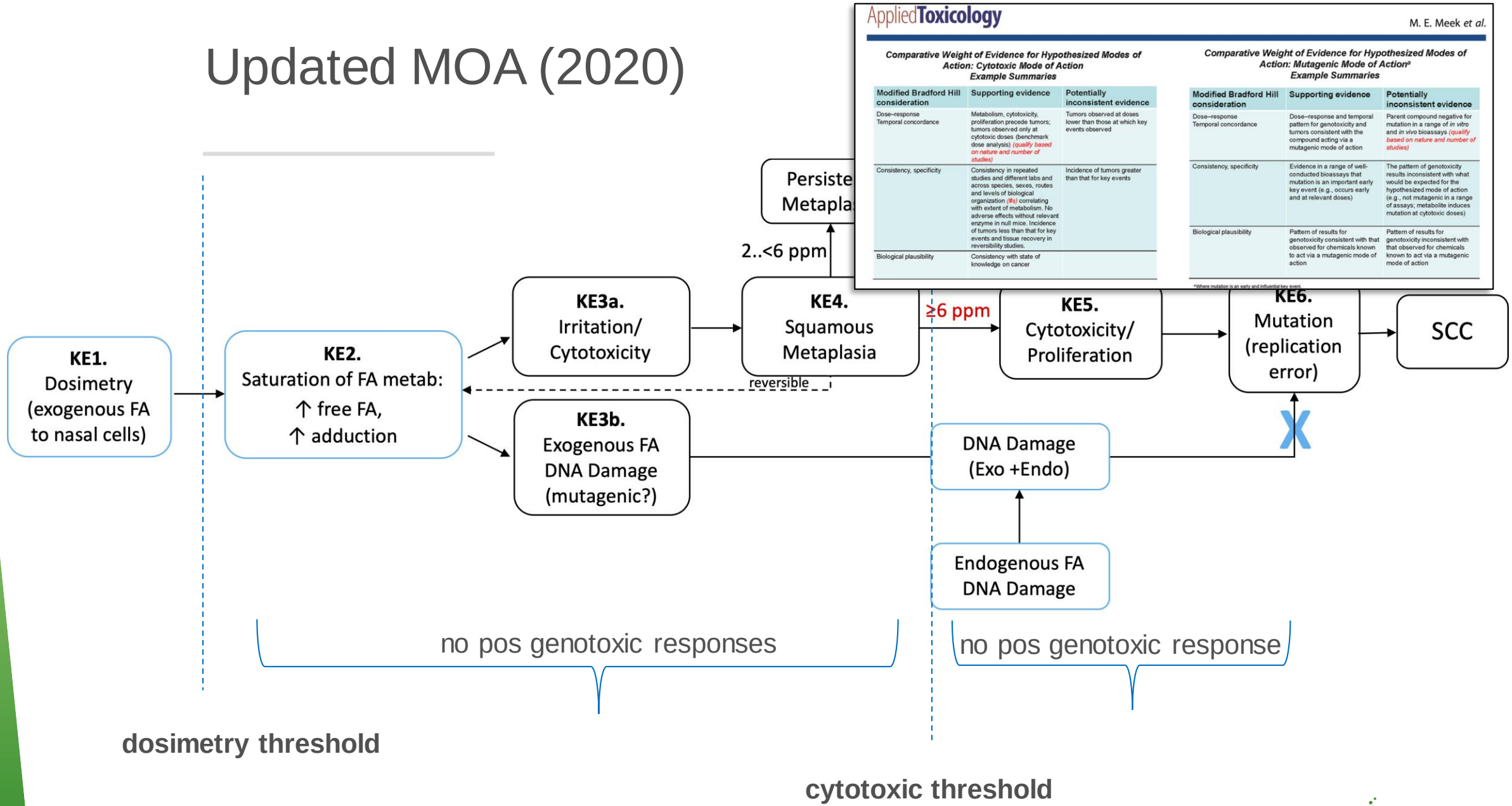

AppliedToxicology M. E. Meek *et al.*

Comparative Weight of Evidence for Hypothesized Modes of Action: Cytotoxic Mode of Action Example Summaries

Modified Bradford Hill consideration	Supporting evidence	Potentially inconsistent evidence
Dose–response Temporal concordance	Metabolism, cytotoxicity, proliferation precede tumors; tumors observed only at cytotoxic doses (benchmark dose analysis) *(qualify based on nature and number of studies)*	Tumors observed at doses lower than those at which key events observed
Consistency, specificity	Consistency in repeated studies and different labs and across species, sexes, routes and levels of biological organization *(Rs)* correlating with extent of metabolism. No adverse effects without relevant enzyme in null mice. Incidence of tumors less than that for key events and tissue recovery in reversibility studies.	Incidence of tumors greater than that for key events
Biological plausibility	Consistency with state of knowledge on cancer	

Comparative Weight of Evidence for Hypothesized Modes of Action: Mutagenic Mode of Action[a] Example Summaries

Modified Bradford Hill consideration	Supporting evidence	Potentially inconsistent evidence
Dose–response Temporal concordance	Dose–response and temporal pattern for genotoxicity and tumors consistent with the compound acting via a mutagenic mode of action	Parent compound negative for mutation in a range of *in vitro* and *in vivo* bioassays *(qualify based on nature and number of studies)*
Consistency, specificity	Evidence in a range of well-conducted bioassays that mutation is an important early key event (e.g., occurs early and at relevant doses)	The pattern of genotoxicity results inconsistent with what would be expected for the hypothesized mode of action (e.g., not mutagenic in a range of assays; metabolite induces mutation at cytotoxic doses)
Biological plausibility	Pattern of results for genotoxicity consistent with that observed for chemicals known to act via a mutagenic mode of action	Pattern of results for genotoxicity inconsistent with that observed for chemicals known to act via a mutagenic mode of action

[a]Where mutation is an early and influential key event.

Summary

- MOA proposed in McGregor et al. (2006) was consistent with a threshold
  - Some uncertainty of the role of genotoxicity
  - Data published after 2006 address these uncertainties
- Increases in exogenous FA-DNA adducts are not detected below 0.3 ppm
- 2-fold increases in FA-DNA adducts does not appear genotoxic
- Several studies published after 2006 have examined the in vivo genotoxicity of formaldehyde in rats up to 15 ppm
  - Negative for clastogenic DNA damage (MN)
  - Negative for mutagenic damage (MF)
- Data published since 2006 further support a threshold MOA

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- Seneca Fitch, ToxStrategies
- Kun Lu, UNC
- Harvey Clewell, Ramboll

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