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Duplex Sequencing Studies to Characterize Chemical Mutagenic Mechanisms in Mouse Somatic Tissues and Germ Cells

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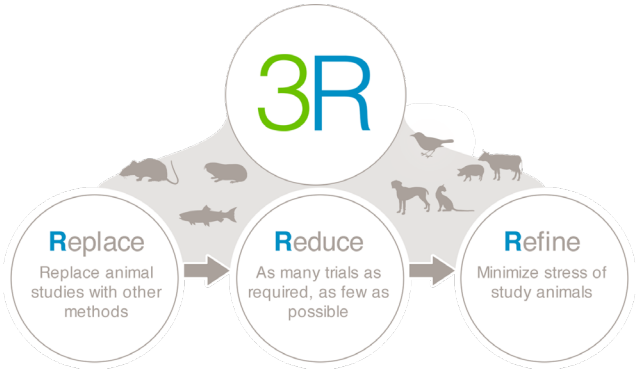
YOUR HEALTH AND SAFETY... OUR PRIORITY.

Major Challenges and Needs in Mutagenicity Testing

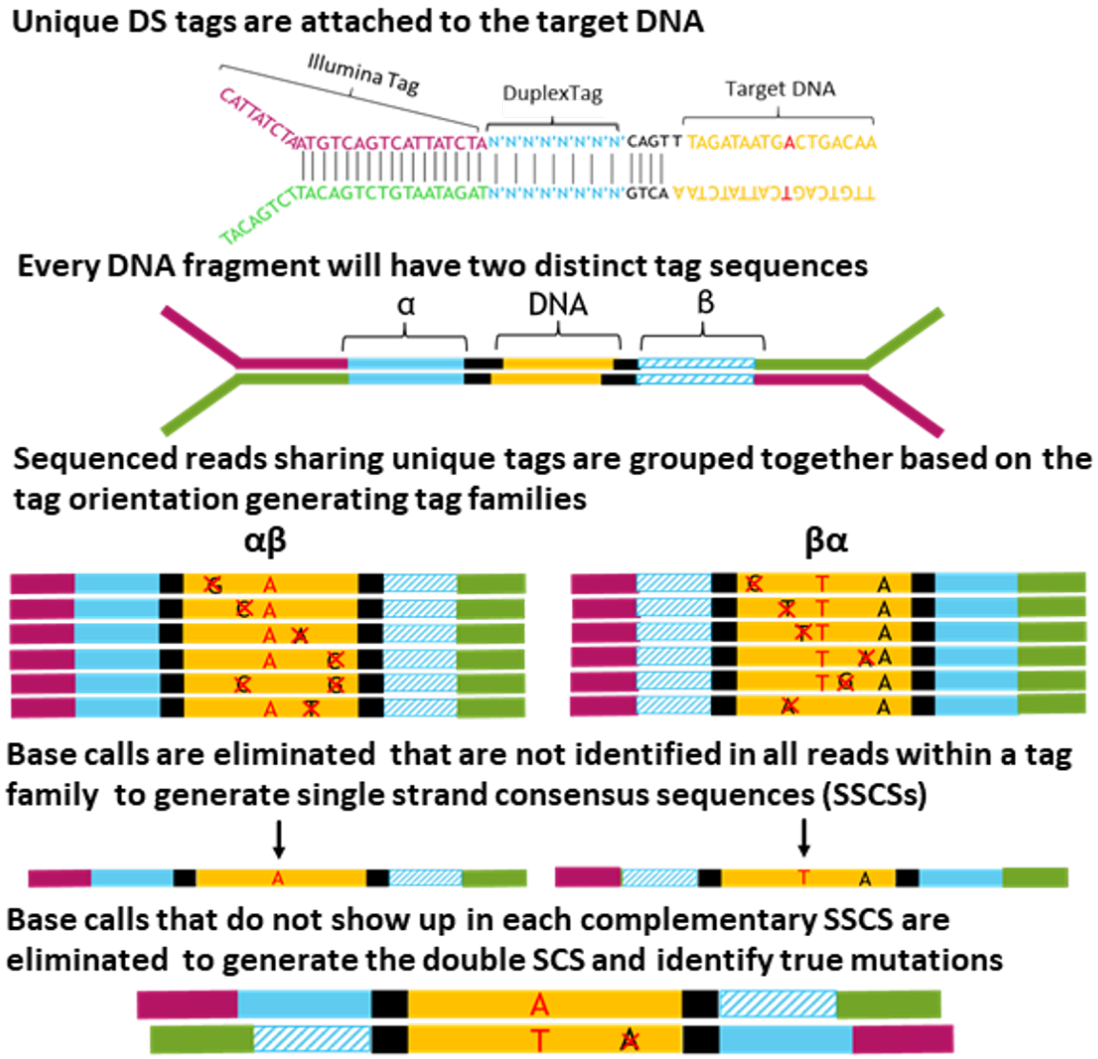
- Current *In vivo* and *in vitro* mutagenicity tests have <u>limitations</u>
  - Use specific rodents and cell lines (cannot be integrated into other tests)
  - Limited to bacterial reporter genes or single mammalian genes
  - Assessing mutations that cause a phenotypic change
  - Characterization of mutation spectrum requires extensive further work

- Need improved mutagenicity assays for a modernized toxicity testing paradigm



- Integrated
- Comprehensive
- Mechanistic
- Human-relevant
- Efficient

In vivo Mutagenicity Studies with Duplex Sequencing (DS)



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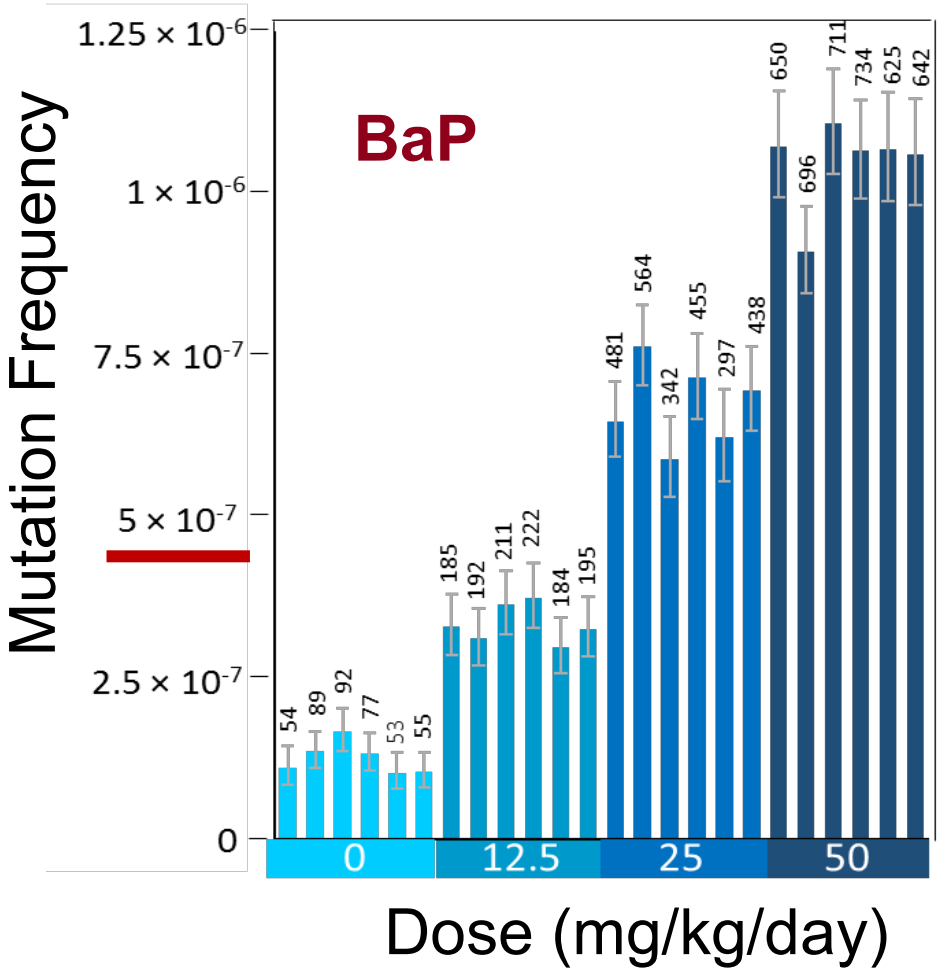
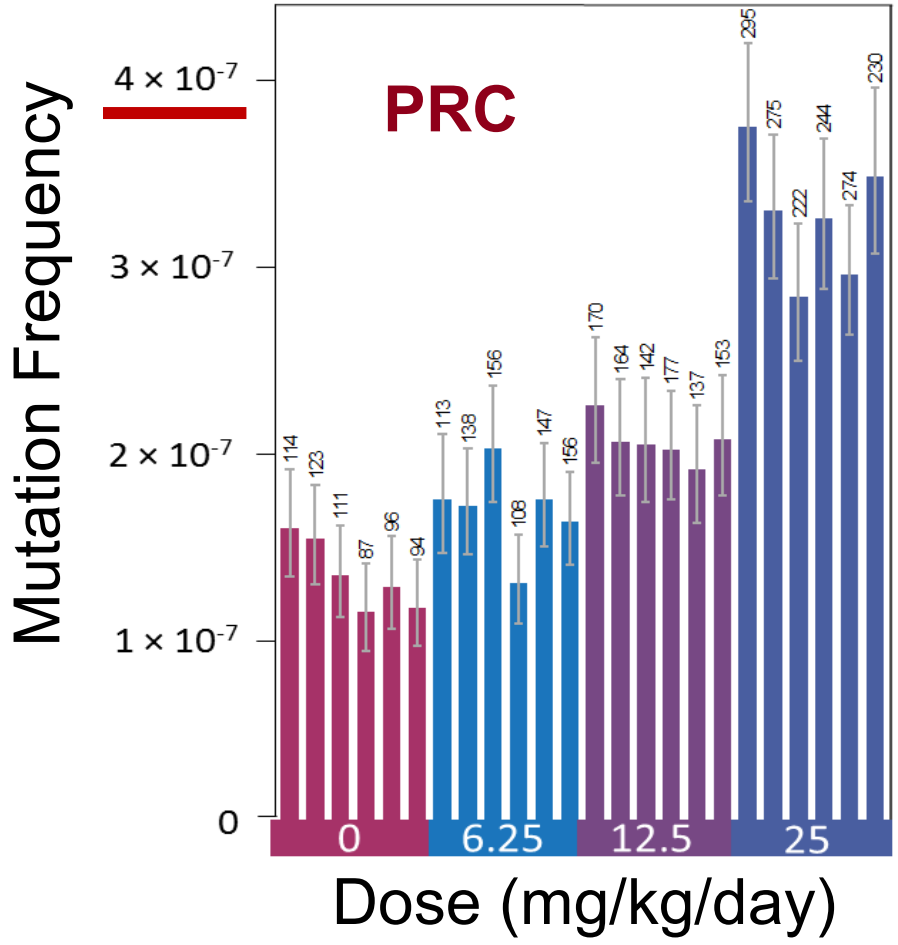
Mouse Mutagenesis Panel

- Study design
- Cross-laboratory concordance
- Sensitivity
- Mechanistic analyses
- Tissue specificity
- Concordance with plaque-based TGR assay

Dose-Dependent Increases in Mutations in Bone Marrow Following Exposures to BaP or PRC





Detection of Mutations in Somatic Tissues by DS

- DS performs well versus the "gold standard" *lacZ* assay in bone marrow
  - High cross-laboratory concordance in library prep and sequencing
  - High detection sensitivity and low sample-to-sample variability
- DS yields novel insights into genomic features that influence mutation induction and potential mechanisms that underlie cancer development
  - Intergenic loci have higher spontaneous and chemically-induced mutations
  - The mutation signature of BaP matched the signatures associated with tobacco-induced lung cancers
  - The mutation signature of PRC was associated with defective mismatch repair signatures
- Preliminary power analyses show that three animals per group are sufficient to detect a 1.5-fold increase above background with a >80% power

Detection of Mutations in Germ Cells by DS: Study Design

Gavage 28 days → → → **28 day sampling time**

N-ethyl-N-nitrosourea (ENU)
Procarbazine (PRC)
Benzo(a)pyrene (BaP)

MutaMouse

Control
(n = 6)

Low
(n = 6)

Medium
(n = 6)

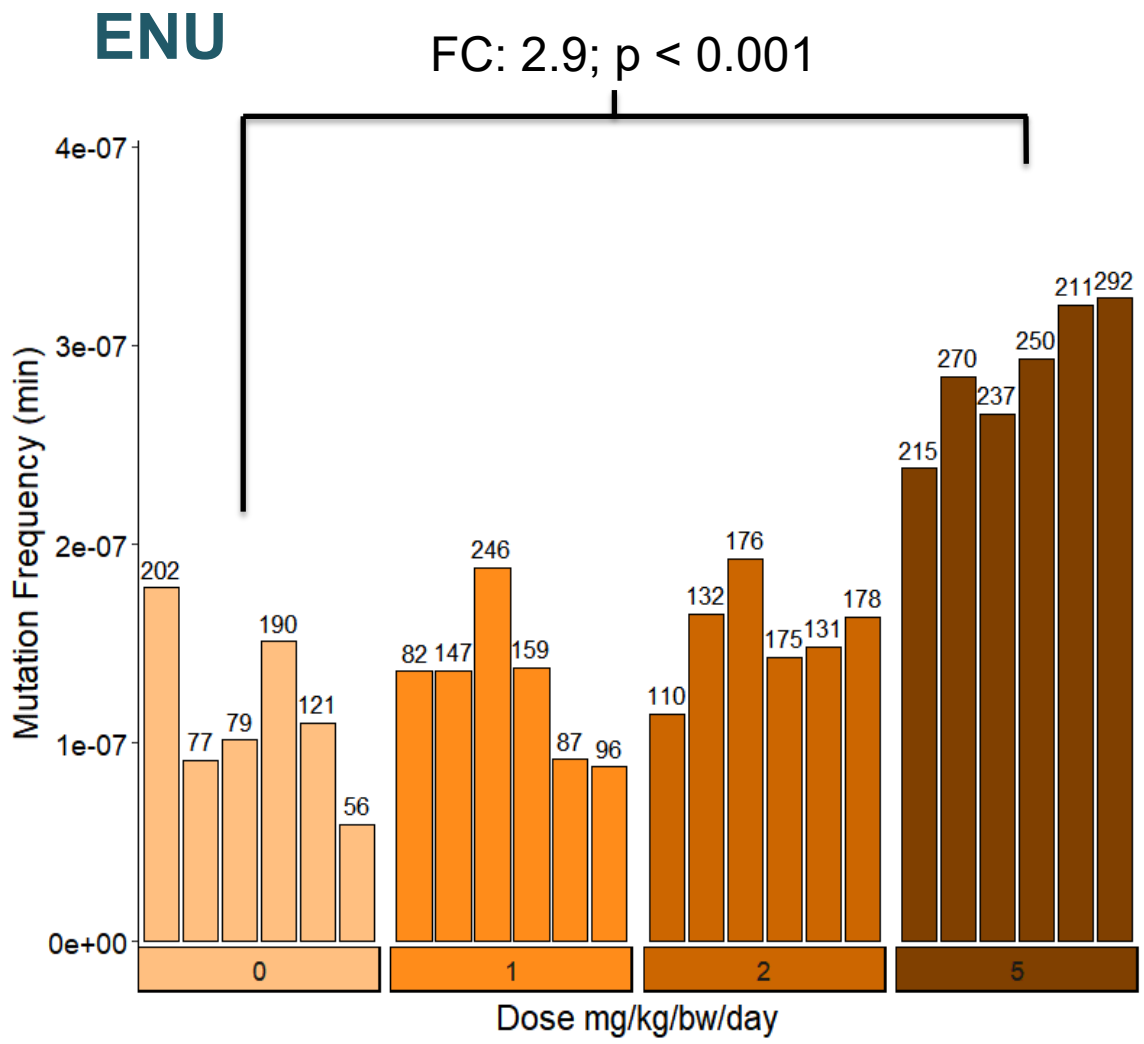
High
(n = 6)

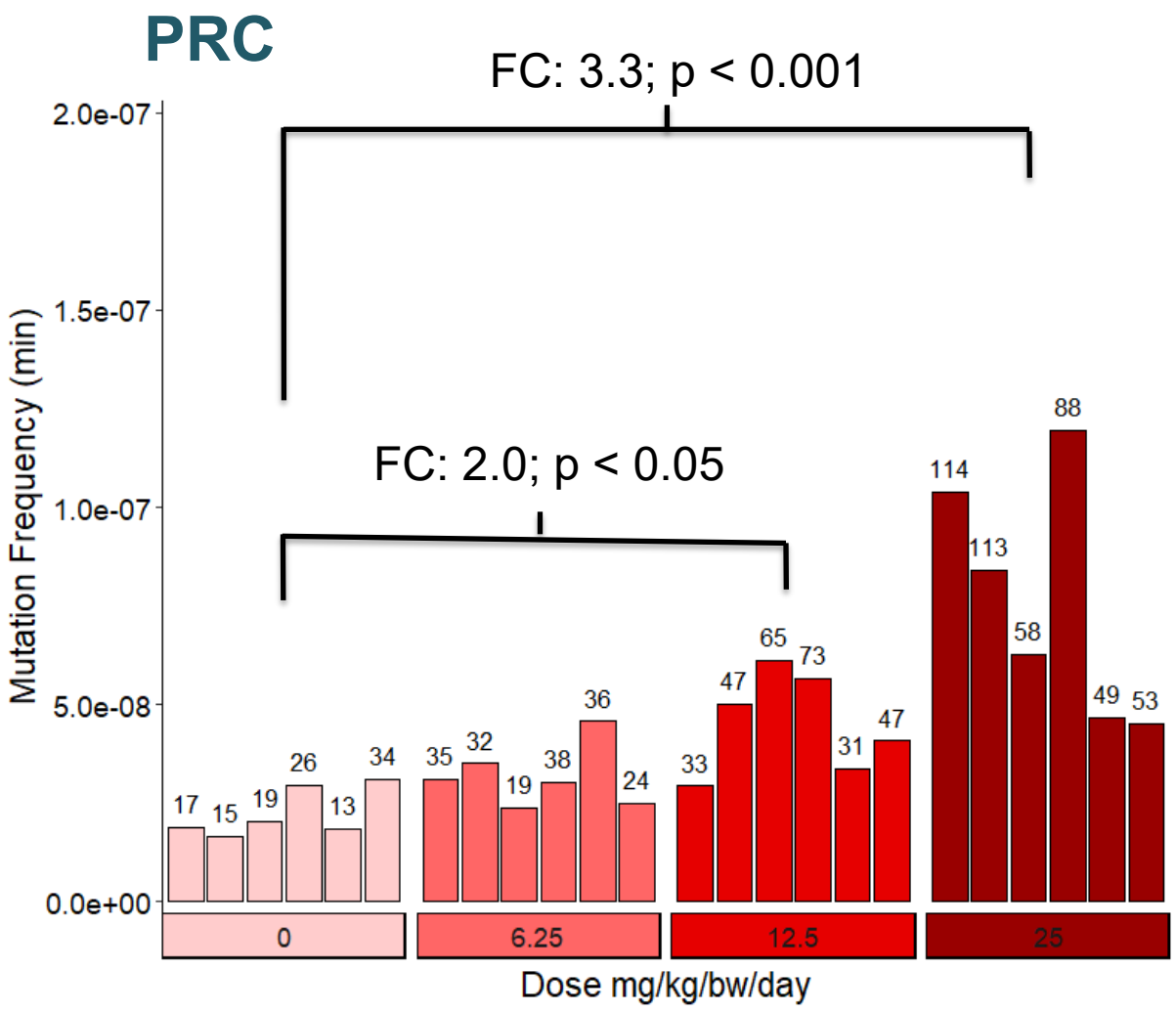
Seminiferous Tubules

DNA isolation – Qiagen DNeasy Kit
↓
Duplex Sequencing (DS)

DNA isolation – Phenol Chloroform
↓
***lacZ* assay**

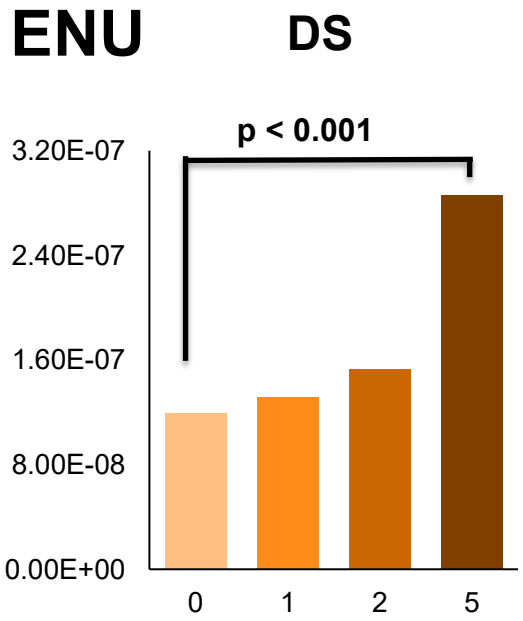
DS Shows Significant Increases in Mutations in Germ Cells Following Exposures to ENU or PRC

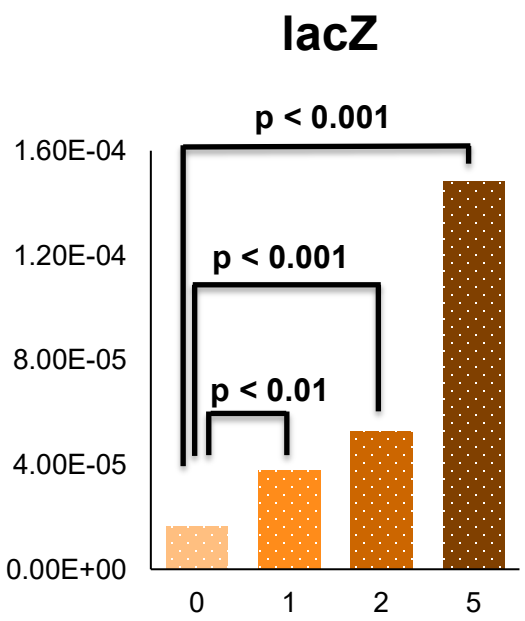




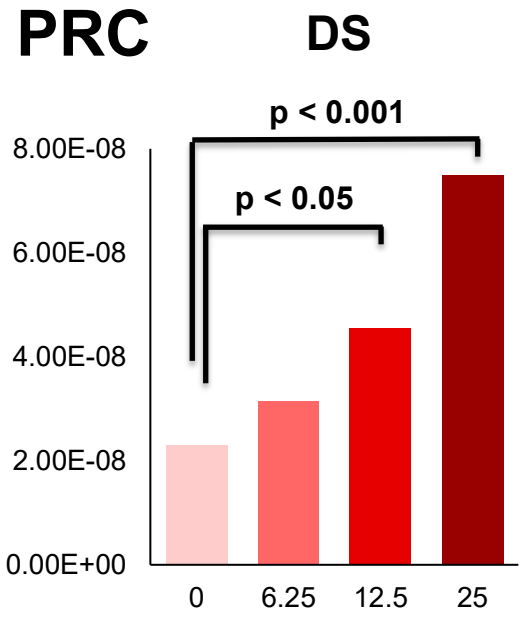
DS Results are Concordant with the *lacZ* Assay

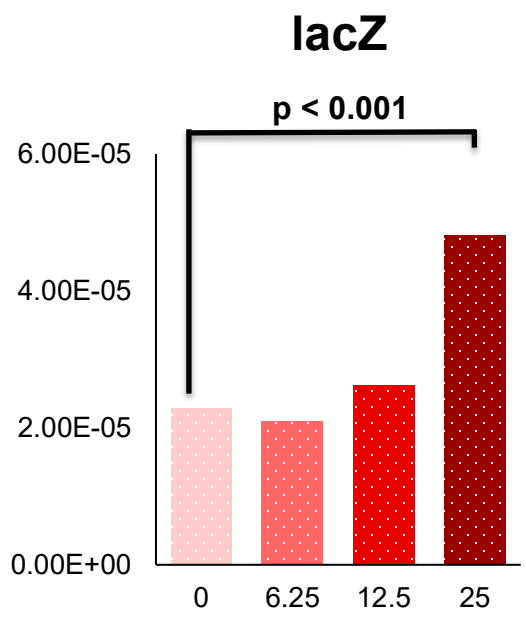
ENU

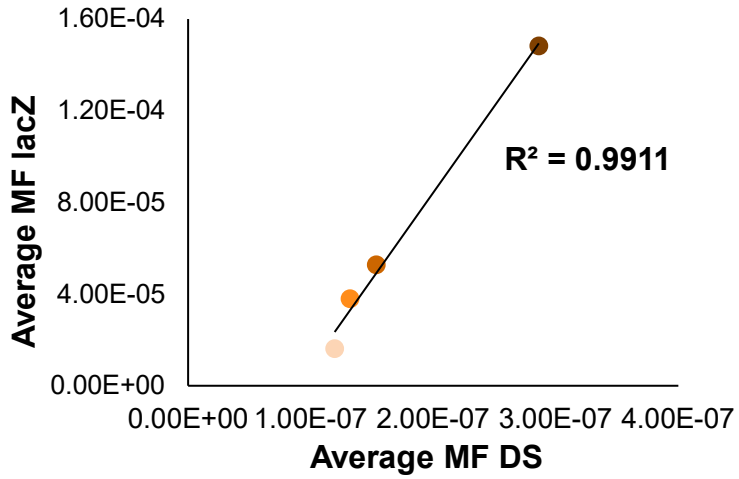


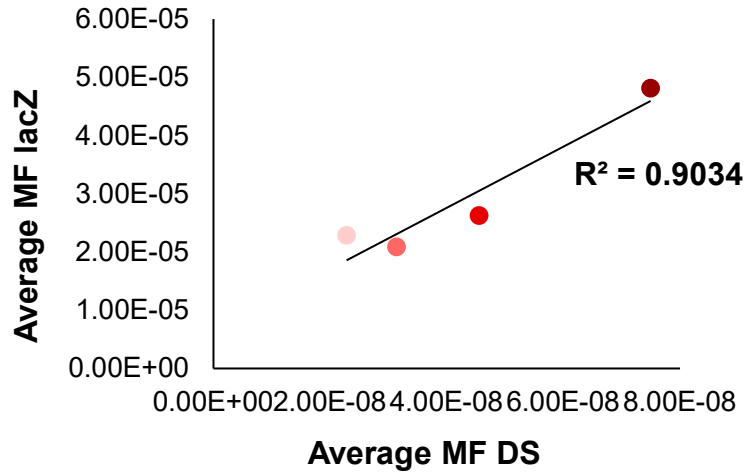


PRC



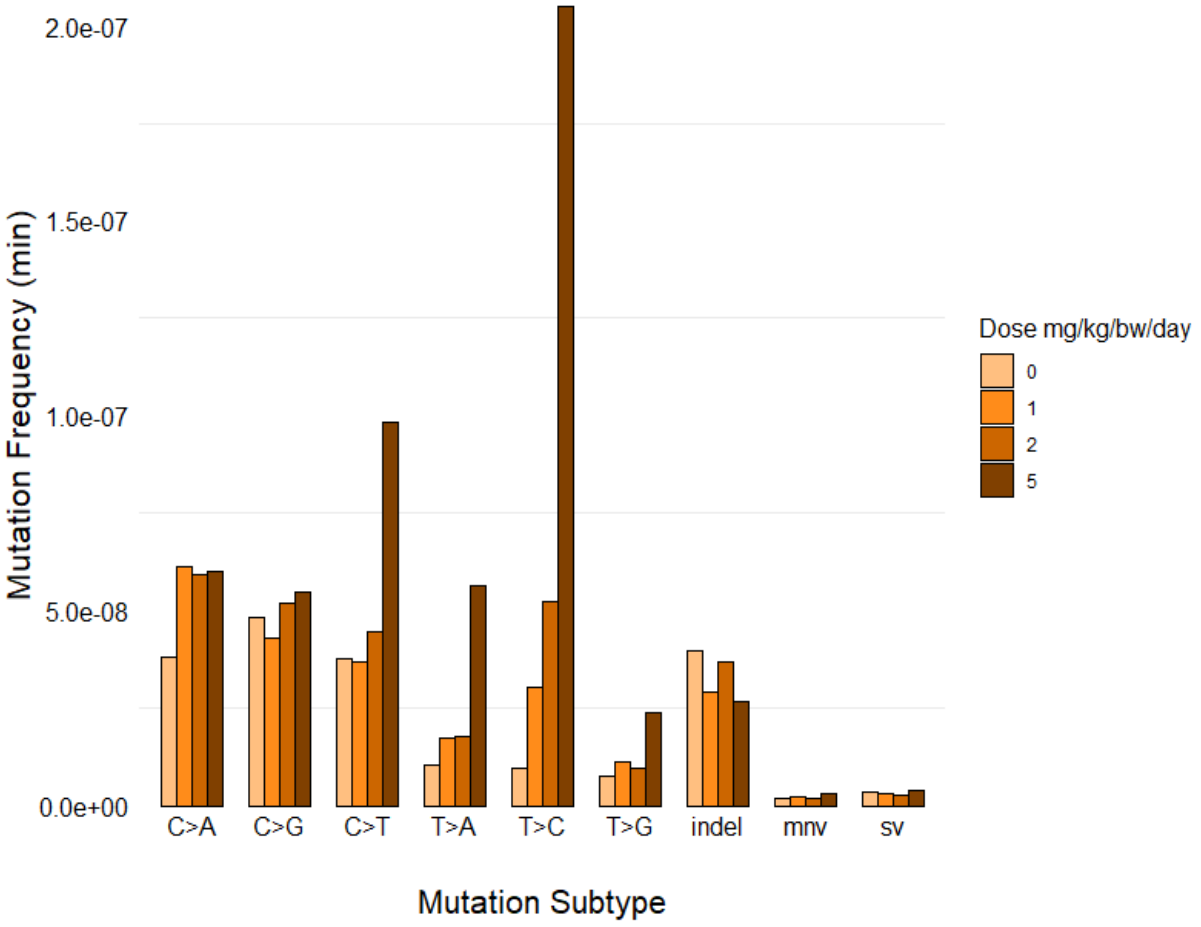




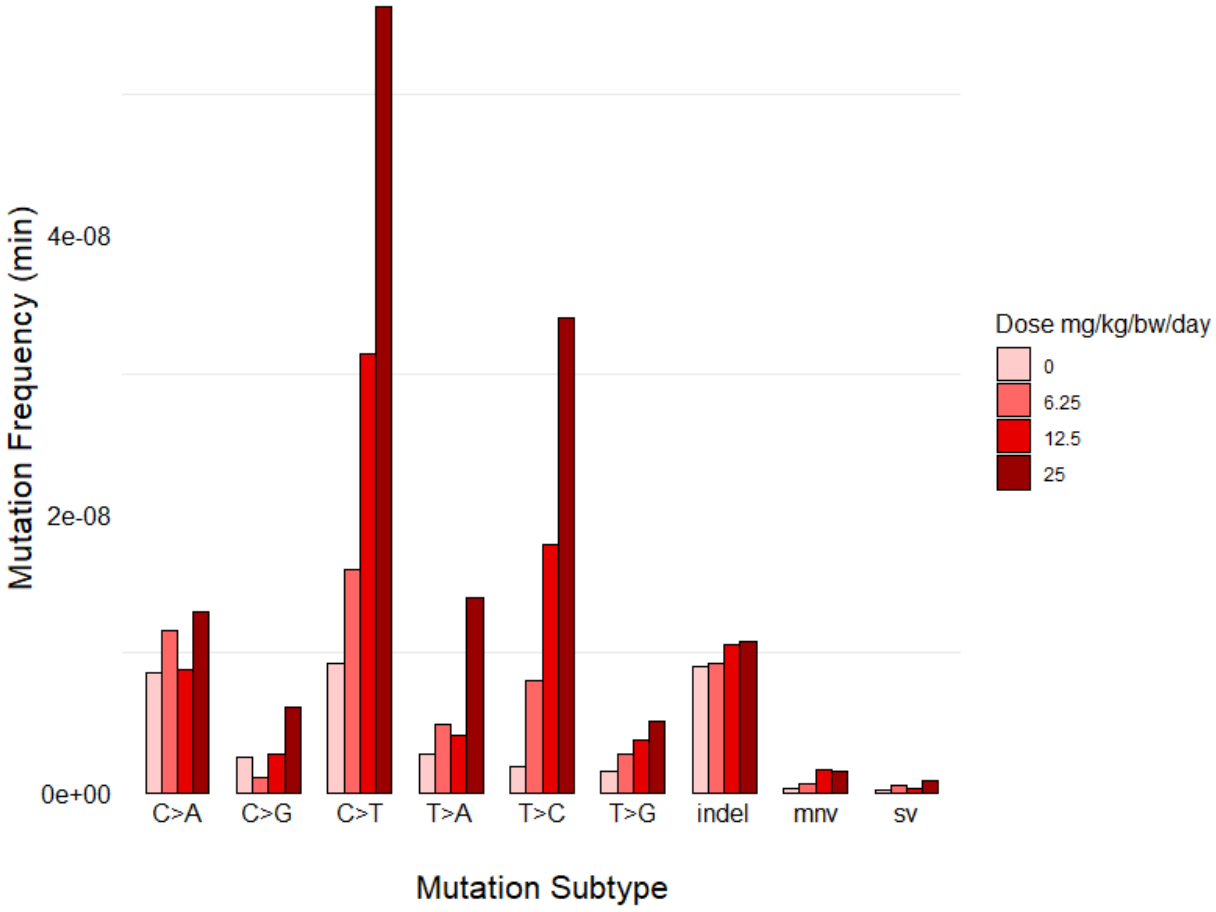


DS Shows High Levels of Indels in Controls and Similarities in the Spectra of ENU and PRC

ENU

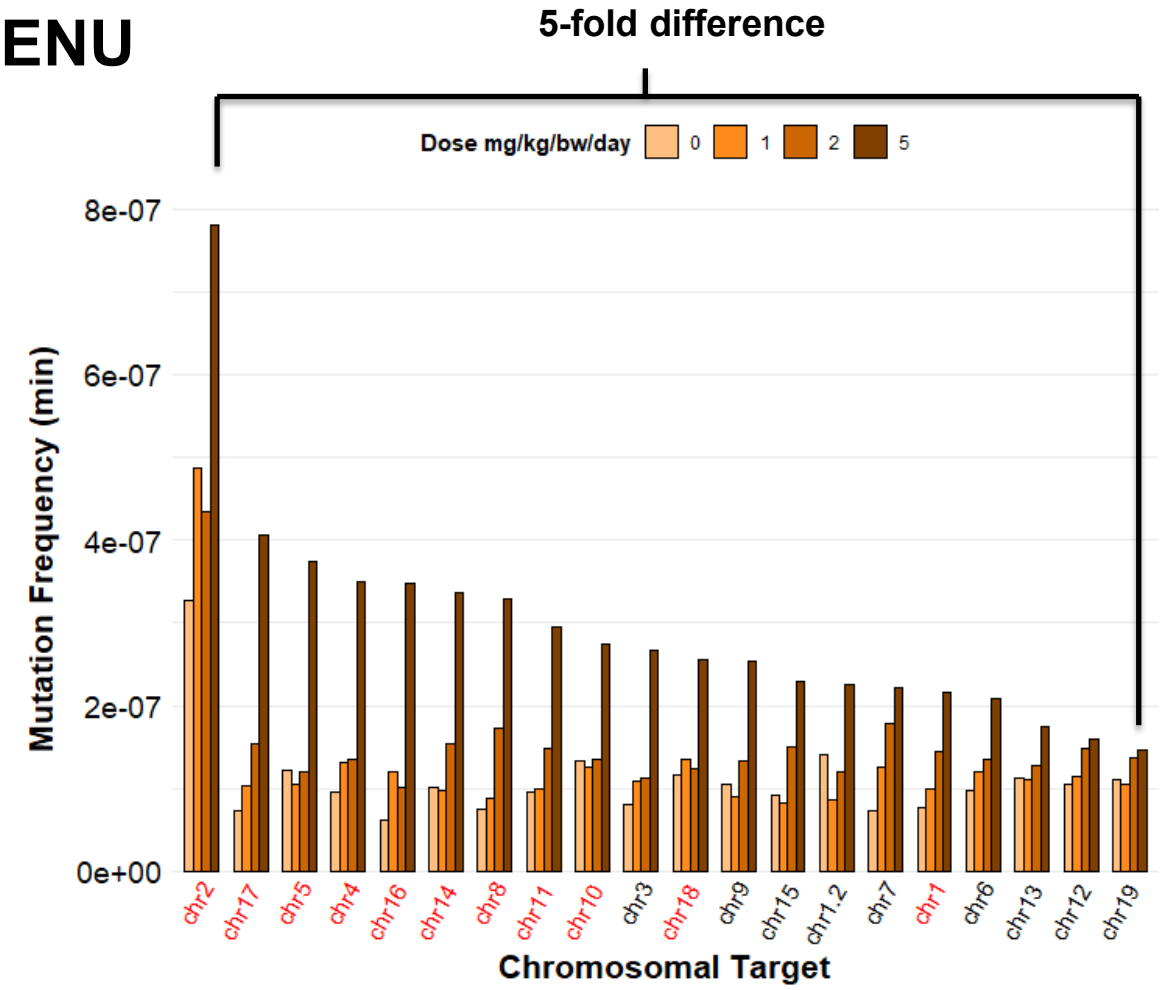


PRC



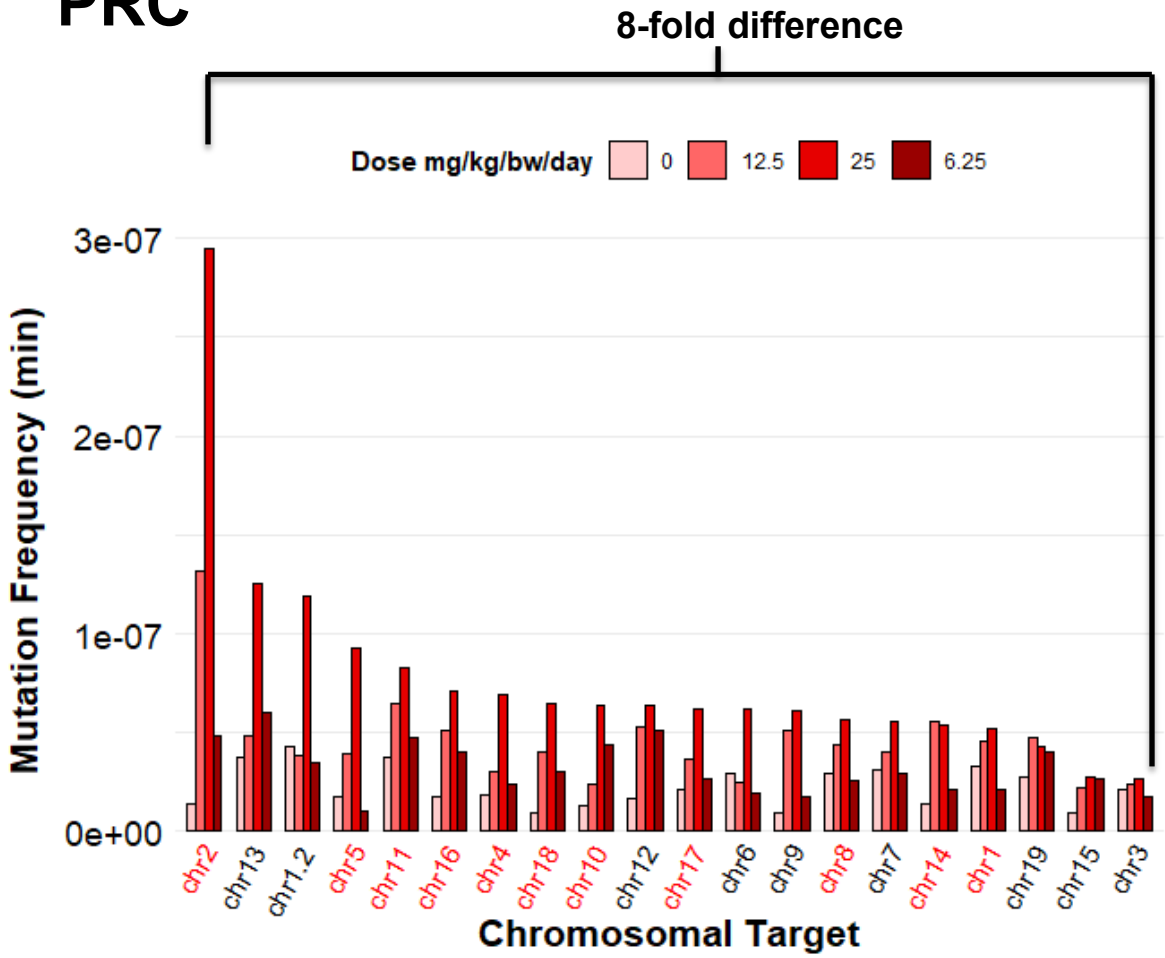
Beyond lacZ: as for Bone Marrow, DS shows Intra- & Inter-genic Difference in Mutation Frequencies in Germ Cells

ENU



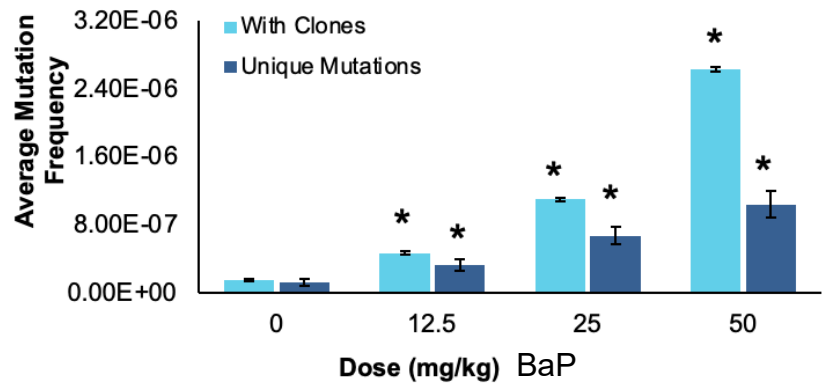
Intergenic Genic

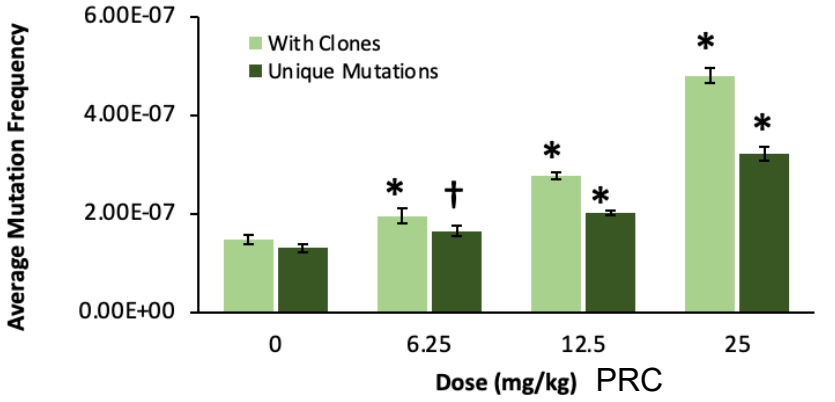
PRC



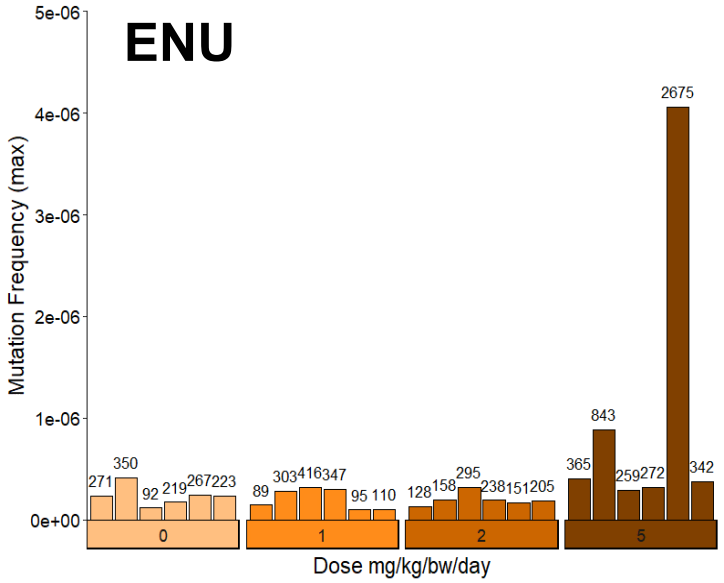
Clonally Expanded Mutations Affect Germ Cell Results More than in Bone Marrow

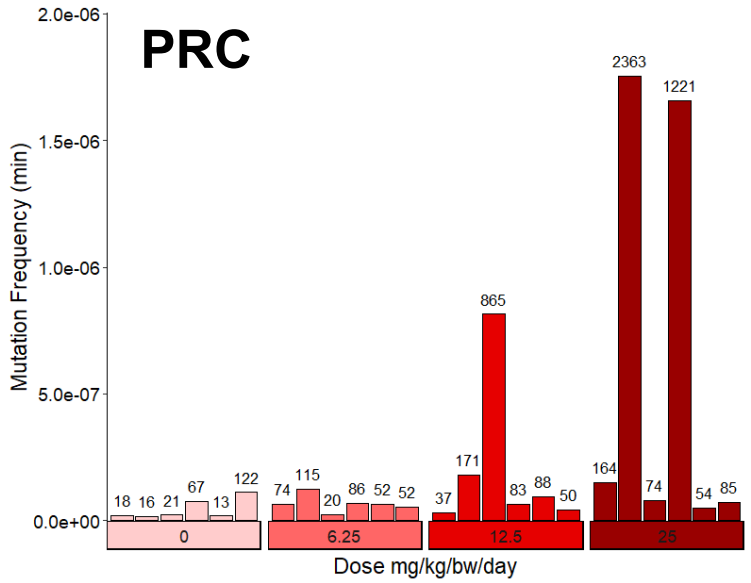
Bone Marrow





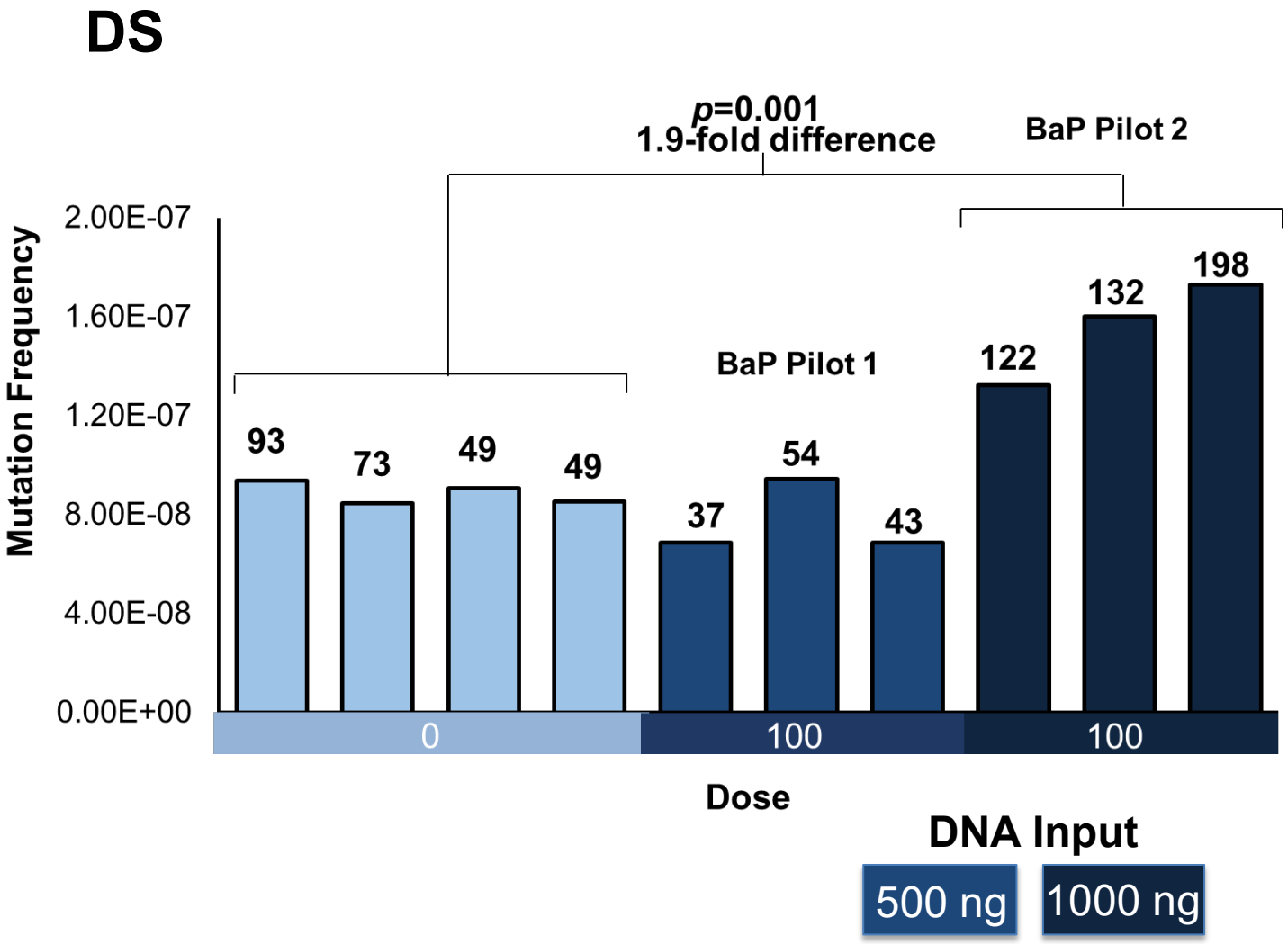
Germ Cells

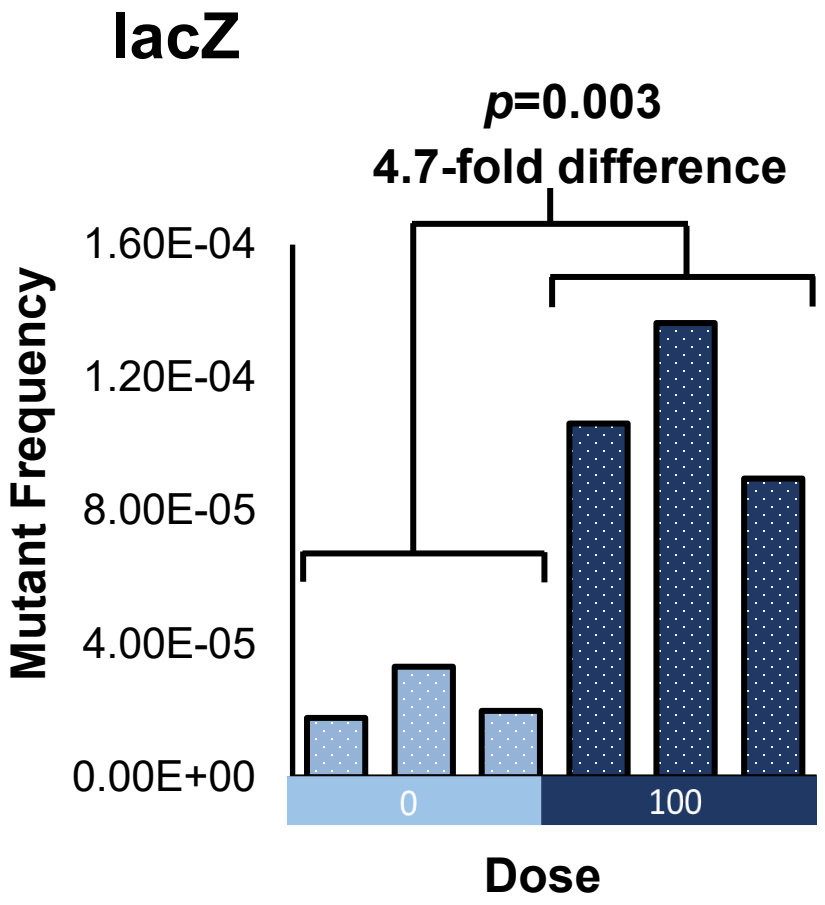




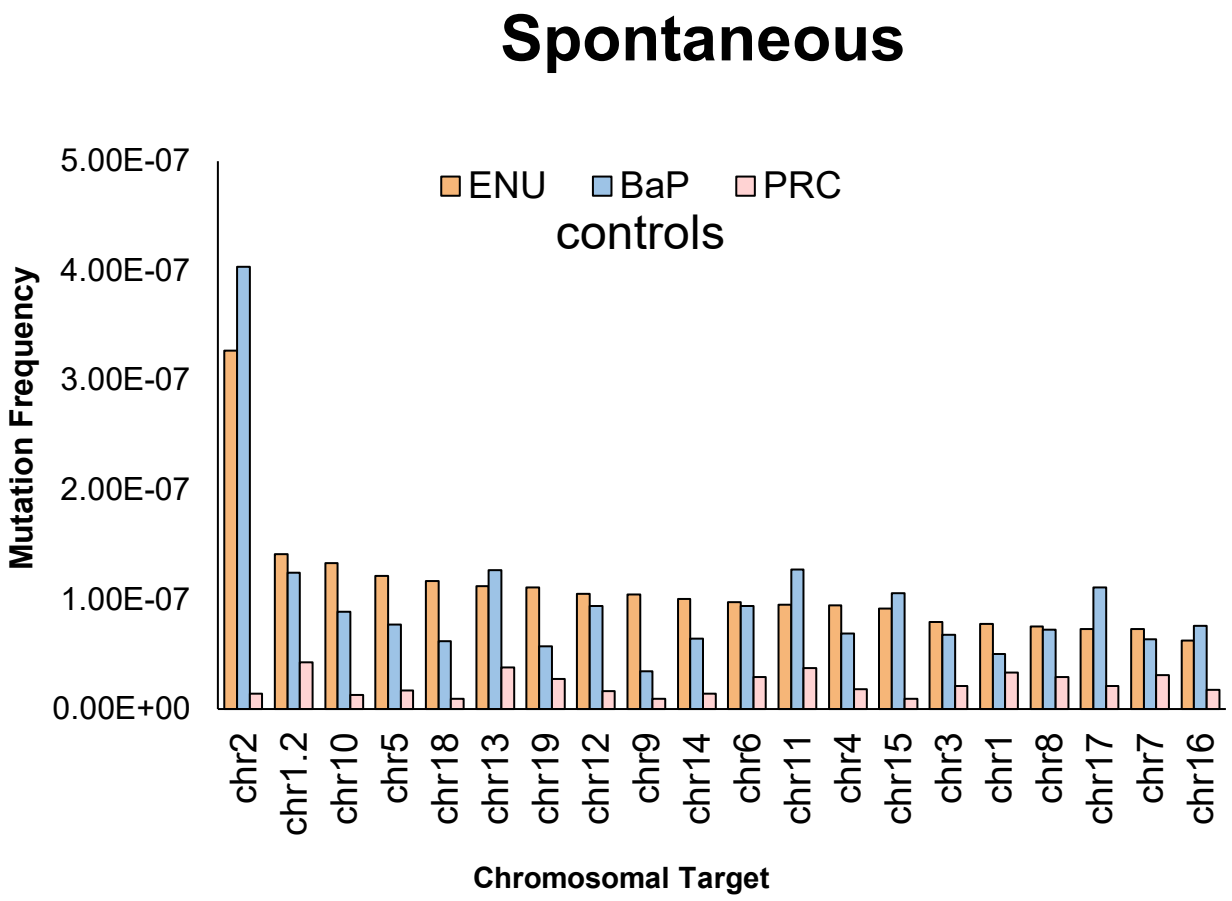
- Mutations in a few stem cells affecting a large portion of developing germ cells

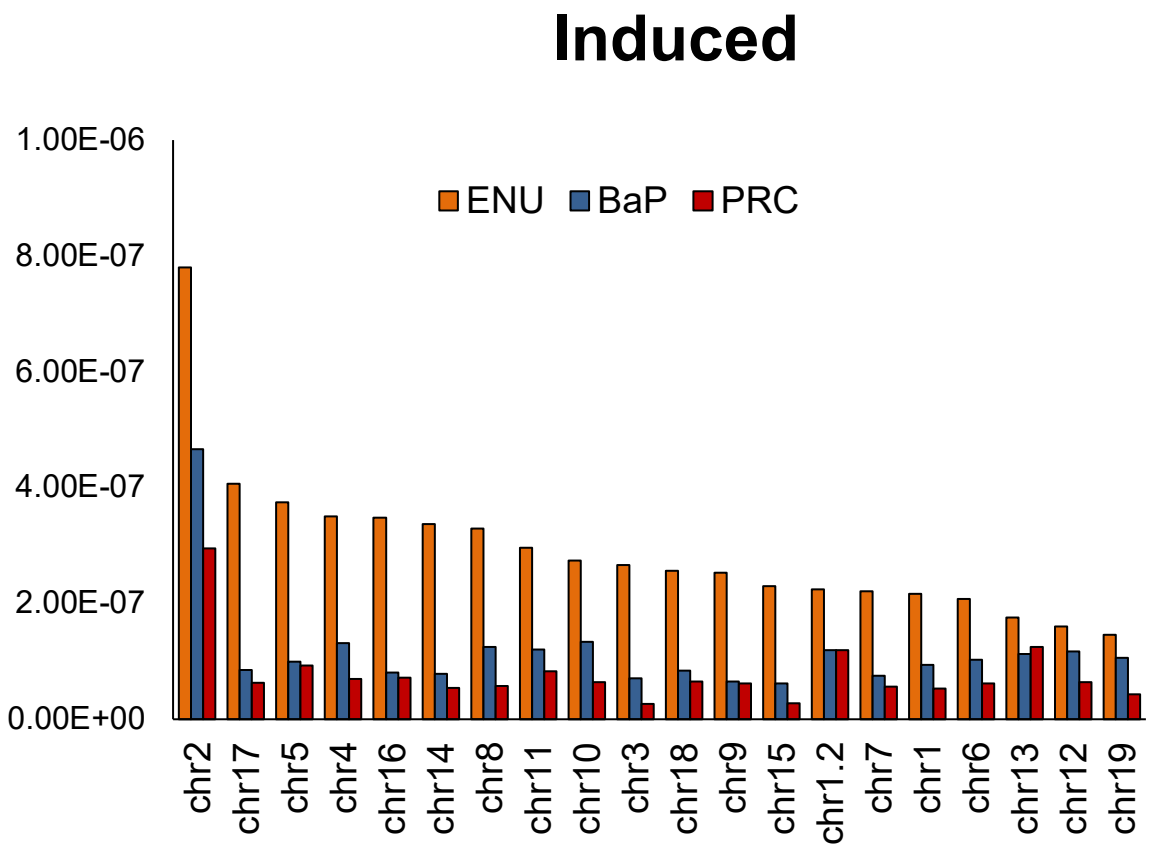
Higher DNA Input is Necessary to Detects an Increase in Mutation Frequencies with BaP



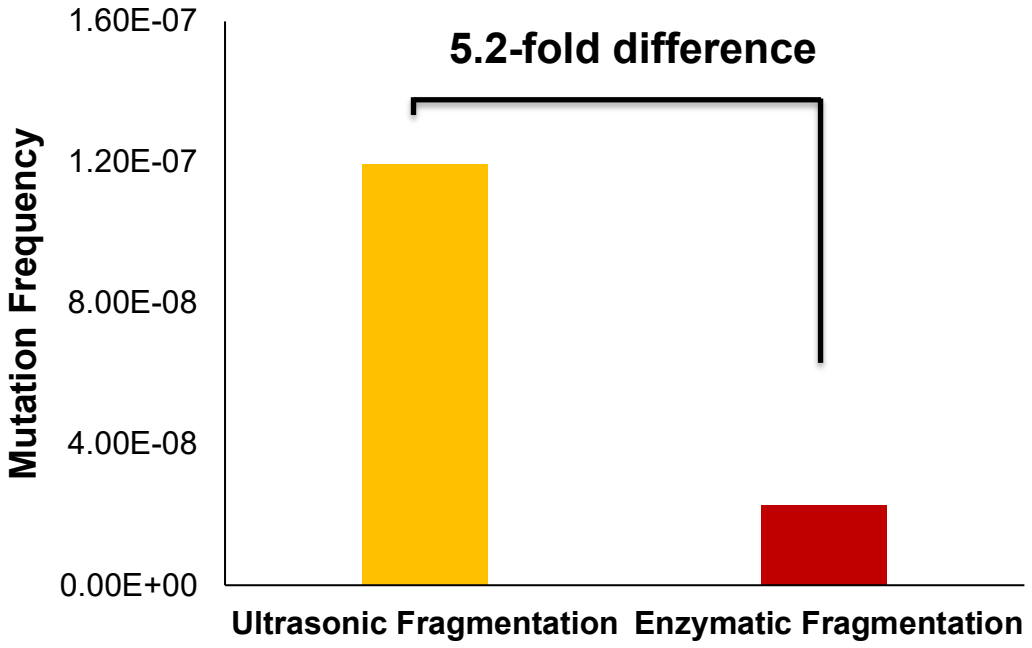


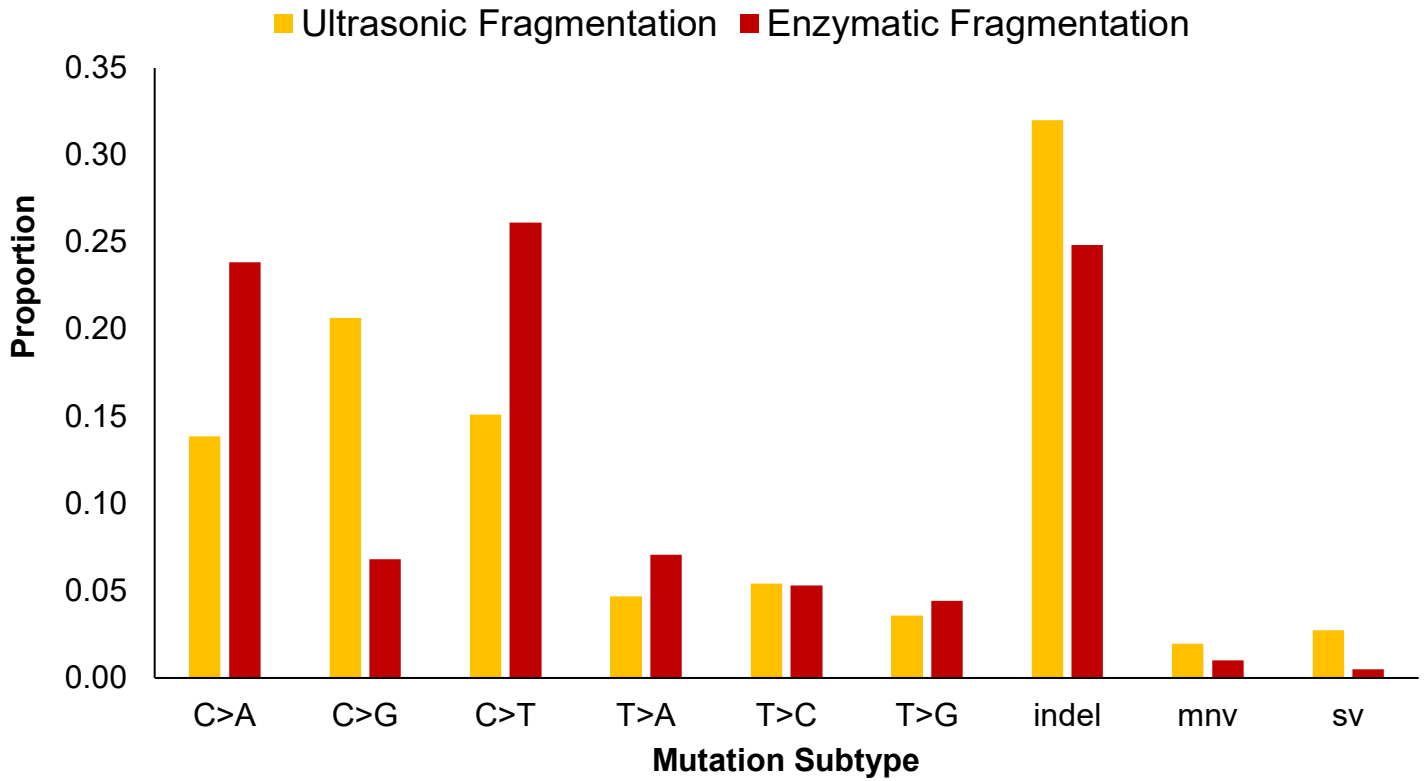
Target on Chr 2 in Germ Cells Shows High Susceptibility to Spontaneous and Induced Mutations





Fragmentation Method Impacts Background Mutation Frequency and Spectrum



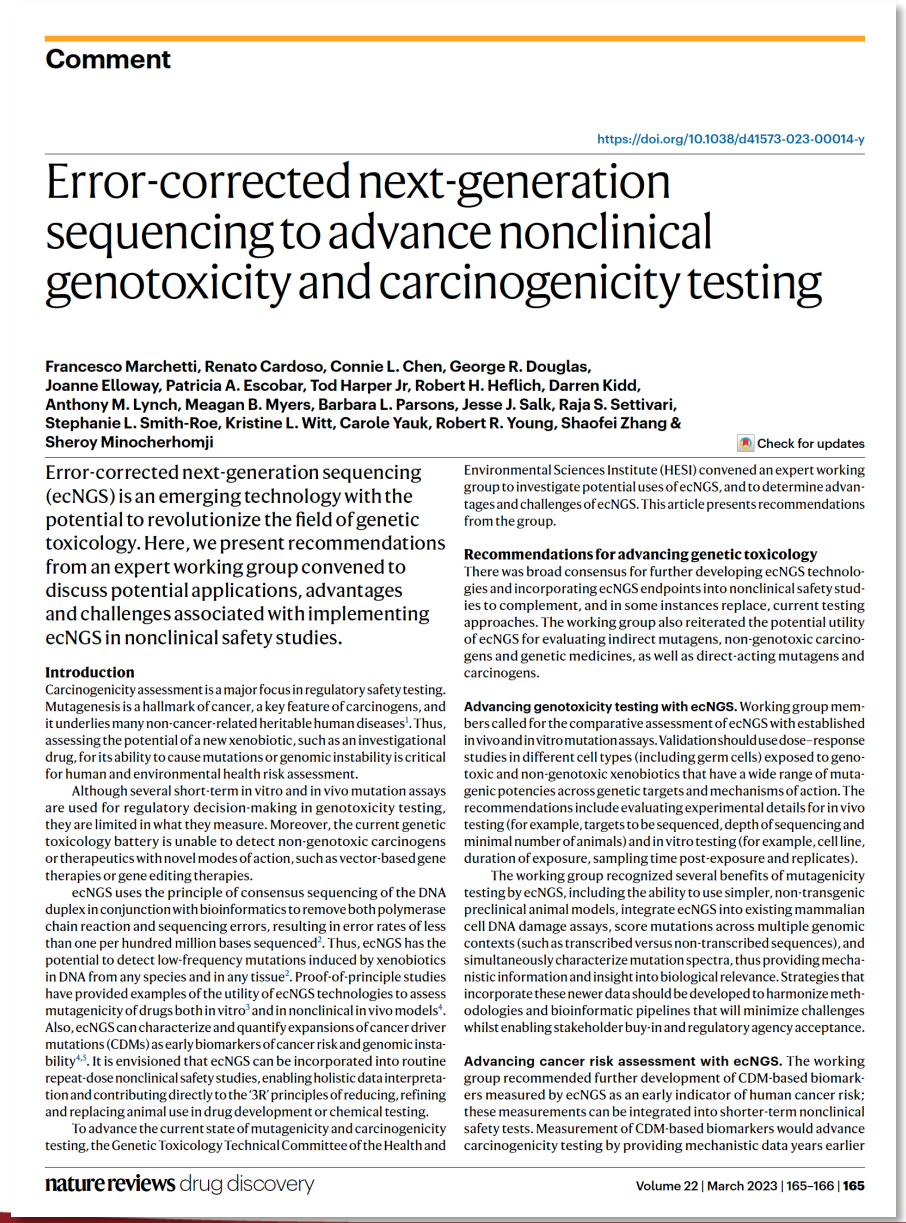


Detection of Mutations in Germ Cells by DS

- DS is effective at detecting chemically-induced mutations in male germ cells
  - Significant increase in mutation frequencies observed for ENU, PRC and BaP
  - Mutation spectra are consistent with known mutagenic mechanisms
  - Good correlation *with* lacZ assay results was observed
  - Enzymatic fragmentation affected background mutation frequencies and spectrum

- These initial studies highlights a few germ cell-specific characteristics
  - Larger impact of clonally expanded mutations on mutation frequencies
  - Higher DNA input may be required to yield a significant response
  - Target on chr 2 shows much higher susceptibility to chemical induced mutagenesis than any other target in germ cells but not in bone marrow
  - Indels are appear to be more common in germ cells than bone marrow

A Roadmap for Regulatory Uptake



Comment

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Error-corrected next-generation sequencing to advance nonclinical genotoxicity and carcinogenicity testing

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Check for updates

Error-corrected next-generation sequencing (ecNGS) is an emerging technology with the potential to revolutionize the field of genetic toxicology. Here, we present recommendations from an expert working group convened to discuss potential applications, advantages and challenges associated with implementing ecNGS in nonclinical safety studies.

Introduction

Carcinogenicity assessment is a major focus in regulatory safety testing. Mutagenesis is a hallmark of cancer, a key feature of carcinogens, and it underlies many non-cancer-related heritable human diseases[1]. Thus, assessing the potential of a new xenobiotic, such as an investigational drug, for its ability to cause mutations or genomic instability is critical for human and environmental health risk assessment.

Although several short-term in vitro and in vivo mutation assays are used for regulatory decision-making in genotoxicity testing, they are limited in what they measure. Moreover, the current genetic toxicology battery is unable to detect non-genotoxic carcinogens or therapeutics with novel modes of action, such as vector-based gene therapies or gene editing therapies.

ecNGS uses the principle of consensus sequencing of the DNA duplex in conjunction with bioinformatics to remove both polymerase chain reaction and sequencing errors, resulting in error rates of less than one per hundred million bases sequenced[2]. Thus, ecNGS has the potential to detect low-frequency mutations induced by xenobiotics in DNA from any species and in any tissue[2]. Proof-of-principle studies have provided examples of the utility of ecNGS technologies to assess mutagenicity of drugs both in vitro[3] and in nonclinical in vivo models[4]. Also, ecNGS can characterize and quantify expansions of cancer driver mutations (CDMs) as early biomarkers of cancer risk and genomic instability[4,5]. It is envisioned that ecNGS can be incorporated into routine repeat-dose nonclinical safety studies, enabling holistic data interpretation and contributing directly to the '3R' principles of reducing, refining and replacing animal use in drug development or chemical testing.

To advance the current state of mutagenicity and carcinogenicity testing, the Genetic Toxicology Technical Committee of the Health and Environmental Sciences Institute (HESI) convened an expert working group to investigate potential uses of ecNGS, and to determine advantages and challenges of ecNGS. This article presents recommendations from the group.

Recommendations for advancing genetic toxicology

There was broad consensus for further developing ecNGS technologies and incorporating ecNGS endpoints into nonclinical safety studies to complement, and in some instances replace, current testing approaches. The working group also reiterated the potential utility of ecNGS for evaluating indirect mutagens, non-genotoxic carcinogens and genetic medicines, as well as direct-acting mutagens and carcinogens.

Advancing genotoxicity testing with ecNGS. Working group members called for the comparative assessment of ecNGS with established in vivo and in vitro mutation assays. Validation should use dose–response studies in different cell types (including germ cells) exposed to genotoxic and non-genotoxic xenobiotics that have a wide range of mutagenic potencies across genetic targets and mechanisms of action. The recommendations include evaluating experimental details for in vivo testing (for example, targets to be sequenced, depth of sequencing and minimal number of animals) and in vitro testing (for example, cell line, duration of exposure, sampling time post-exposure and replicates).

The working group recognized several benefits of mutagenicity testing by ecNGS, including the ability to use simpler, non-transgenic preclinical animal models, integrate ecNGS into existing mammalian cell DNA damage assays, score mutations across multiple genomic contexts (such as transcribed versus non-transcribed sequences), and simultaneously characterize mutation spectra, thus providing mechanistic information and insight into biological relevance. Strategies that incorporate these newer data should be developed to harmonize methodologies and bioinformatic pipelines that will minimize challenges whilst enabling stakeholder buy-in and regulatory agency acceptance.

Advancing cancer risk assessment with ecNGS. The working group recommended further development of CDM-based biomarkers measured by ecNGS as an early indicator of human cancer risk; these measurements can be integrated into shorter-term nonclinical safety tests. Measurement of CDM-based biomarkers would advance carcinogenicity testing by providing mechanistic data years earlier

❖ Regulatory adoption of ecNGS - goals

1. Complement and/or eventually replace the in vivo TGR mutation tests
2. Integrate mutagenicity assessment within standard in vivo toxicity testing
3. Complement and/or eventually replace components of the current in vitro genotoxicity battery
4. Develop ecNGS technologies for characterizing off-target effects of gene editing
5. Develop ecNGS assays to quantify the extent of carcinogenic clonal expansion
6. Develop tools for biomonitoring of human populations following occupational and environmental exposures, as well as subjects in clinical trials

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