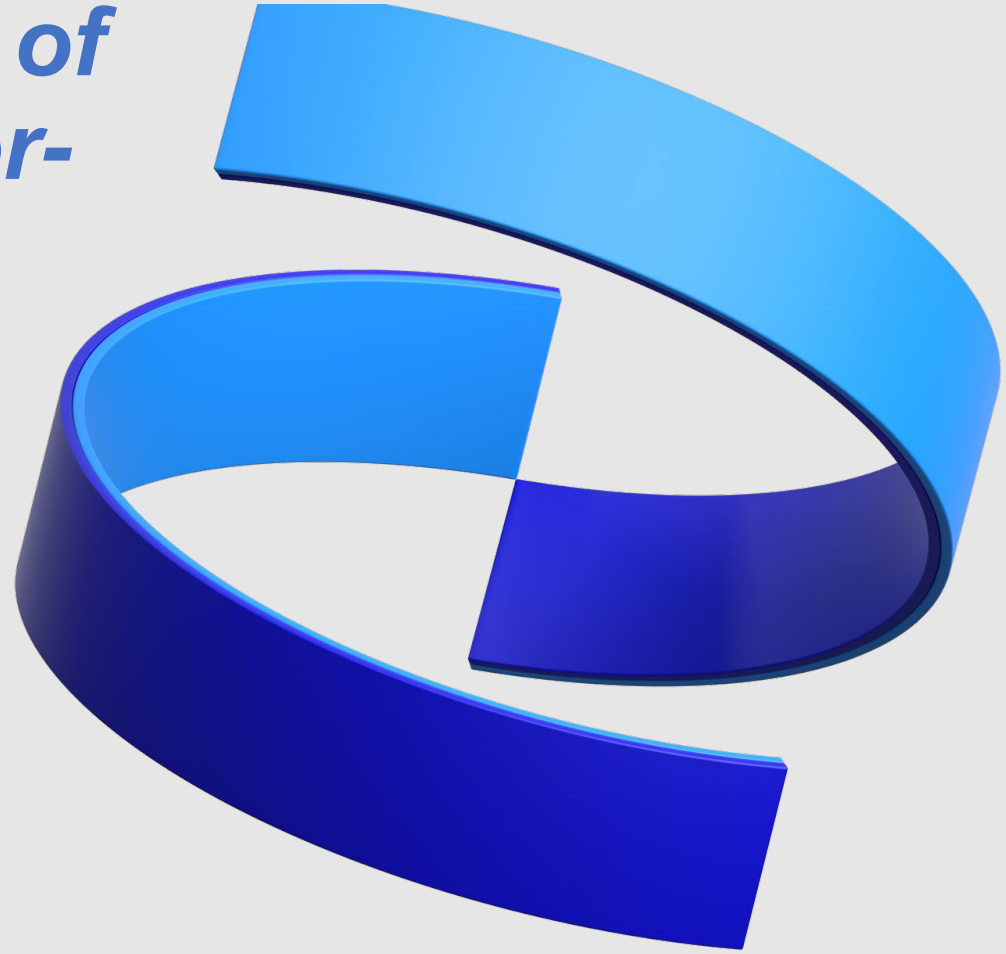
In vivo genotoxicity assessment of N-nitrosodiethylamine with Error-Corrected Next-Generation Sequencing



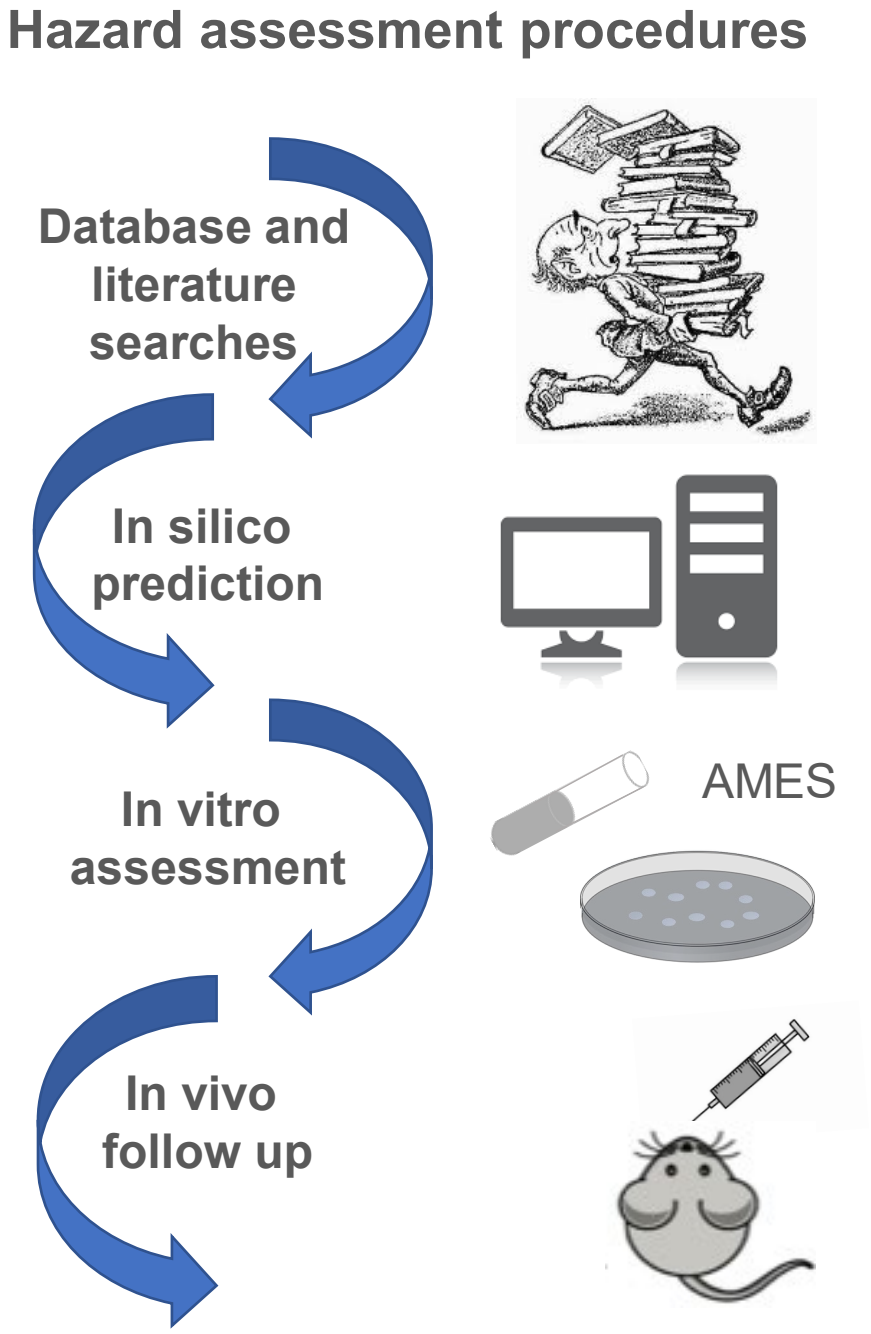
Shaofei Zhang

May 5th, 2023

ICH M7: Impurities Classification

1. Known mutagenic carcinogens (Compound-specific acceptable limit)
2. Known mutagens with unknown carcinogenic potential (Appropriate TTC)
3. Alerting structure, unrelated to the structure of the drug substance; no mutagenicity data (Appropriate TTC or AMES)
4. Alerting structure, same alert in drug substance or compounds related to the drug substance (Non-mutagenic impurity)
5. No structural alerts, or alerting structure with sufficient data to demonstrate lack of mutagenicity or carcinogenicity (Non-mutagenic impurity)

https://database.ich.org/sites/default/files/M7_R1_Guideline.pdf



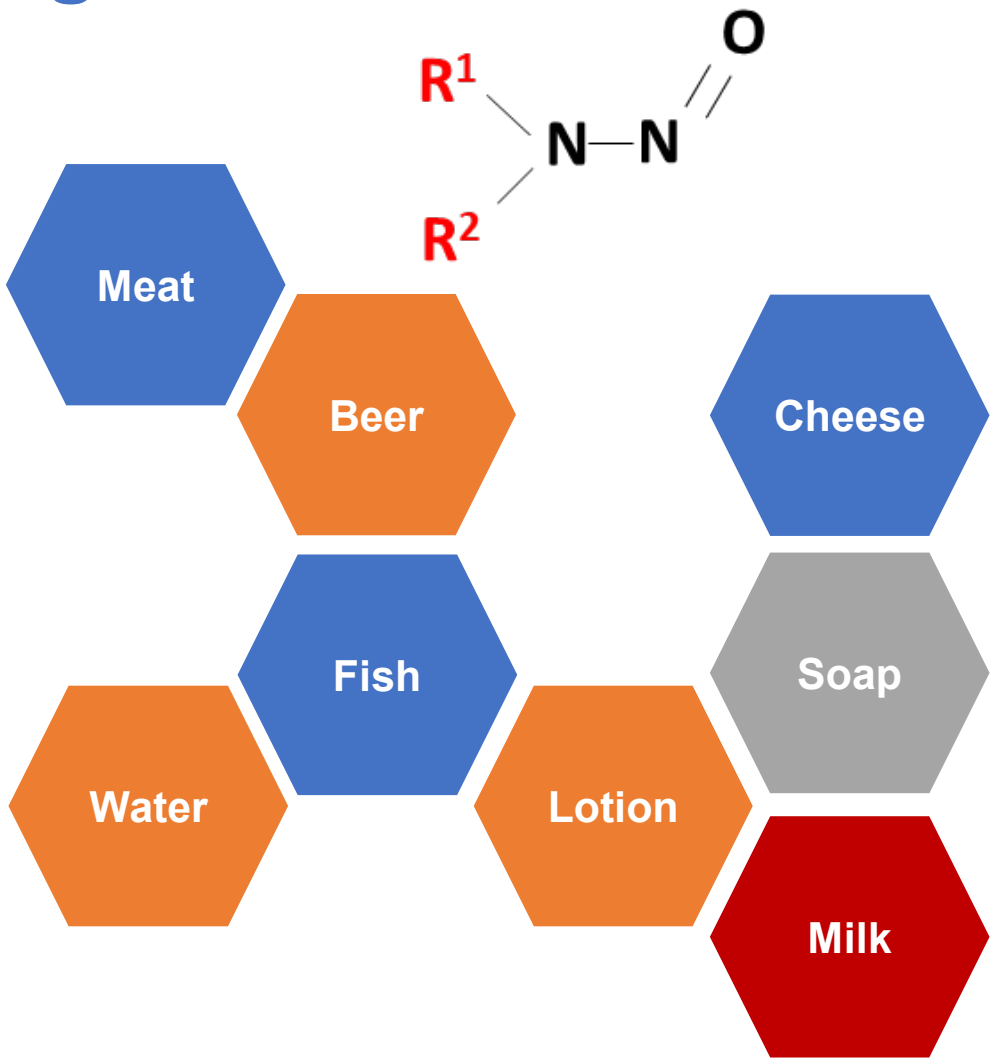
Nitrosamines are classified as ‘Cohort of Concern (CoC)’ in the ICH M7 guideline

Nitrosamines have been found in foods, beverages, cosmetics, tobacco and packing materials.

Detection of N-nitrosodimethylamine (NDMA) in sartans in July 2018.

~18% the compounds were non-carcinogenic out of 228 N-nitrosamines tested

Considerable overlap of toxicological potency with non-N-nitrosamines not part of the CoC category



J. Med. Chem. 2022, 65, 15584−15607

Acceptable Intakes for N-Nitrosamine in Active Pharmaceutical Ingredients

Threshold of Toxicologic Concern (TTC) – is not justified for Cohort of Concern (CoC).

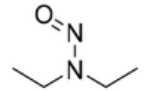
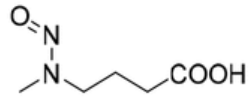
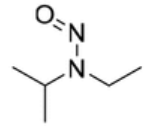
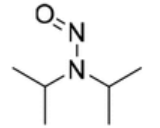
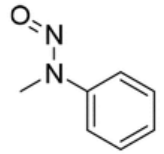
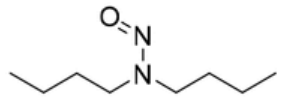
Acceptable intake (AI): daily human exposure to a N-nitrosamine that approximates a 1:100,000 cancer risk after 70 years of exposure.

Compound-specific AI limit is calculated by linear extrapolation of rodent carcinogenicity (TD50)

The DNA repair enzyme can restore DNA integrity and result in a nonlinear dose response at low level of exposure

Need a methodology to calculate PDE based on *in vivo* genotoxicity data

Maximal Daily Acceptable Intake for some *N*-Nitrosamines found in drug products

N-nitrosamine	Structure	Daily (ng/day)[a,b] AI
NDMA		96.0
NDEA		26.5
NMBA		96.0[c]
NIPEA		26.5[d]
NDIPA		26.5[d]
NMPA		26.5[d,e] 34.3[f]
NDBA		26.5[d]

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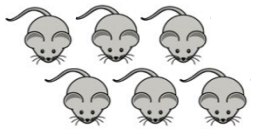
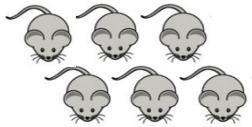
Study design

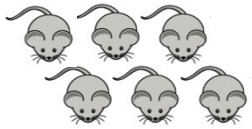
Vehicle Control



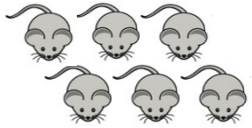
Positive Control
(ENU 20 mg/kg/day for 6 days)



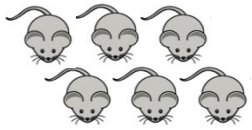
Test Article
NDEA



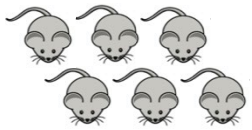
(0.001mg/kg/day)



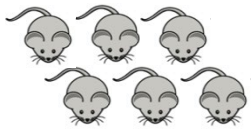
(0.01mg/kg/day)



(0.1mg/kg/day)



(1 mg/kg/day)

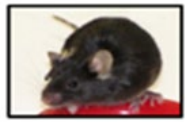


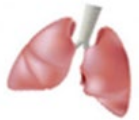
(3 mg/kg/day)

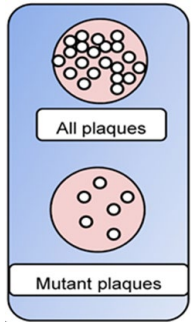
28-day of daily treatment

plus 3-day expression

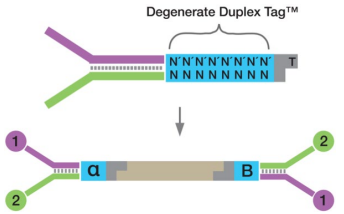
TGR assay
Big Blue

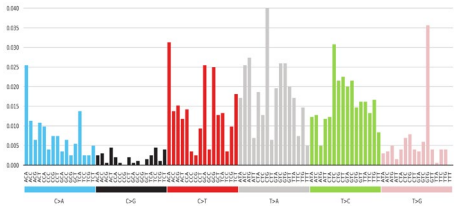






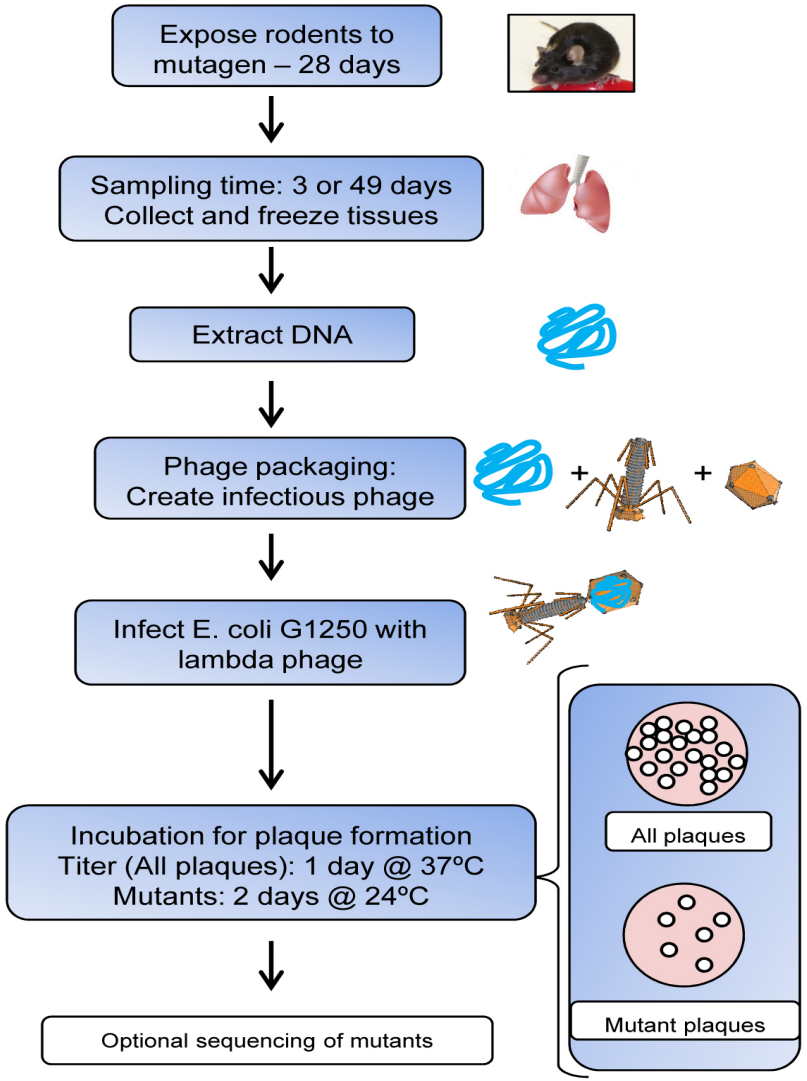
Duplex Sequencing





All Procedures performed on the animals in these studies were in accordance with regulations and established guidelines and were reviewed and approved by an Institutional Animal Care and Use Committee.

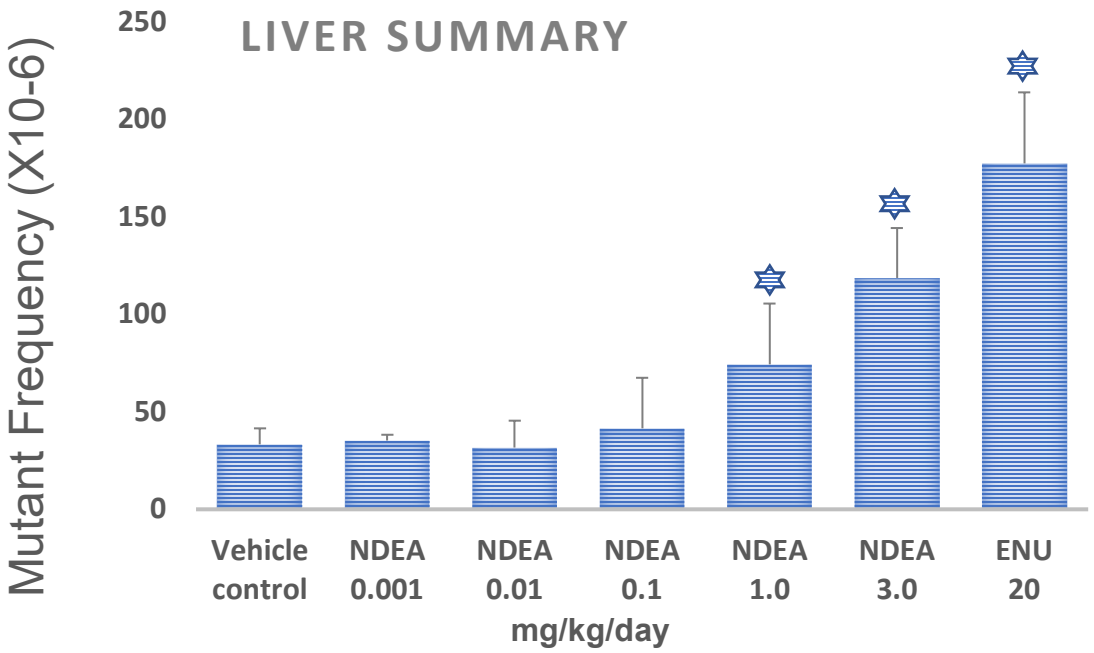
Big Blue® Assay: Overview



TGR: Current gold standard to assess mutations *in vivo*

- ***cII* gene** – Controls lytic/lysogenic cycle of bacteriophage
- **Lysis (lytic cycle)** – Replication of bacteriophage inside host cell resulting in lysis of cell and form plaques on bacterial lawn
- **Lysogeny (lysogenic cycle)** – Bacteriophage DNA integrates into bacteria's genome or as circular replicon in cytoplasm with no lysis
- Wild-type and *cII* mutants form plaques at 37ºC
- Only *cII* mutants form plaques at 24ºC

NDEA was positive for inducing genotoxicity in the rat liver in this study



Group No.	Treatment	Dose level mg/kg/day	Animals/ group	Mutant Frequency ± Standard Deviation (x 10^{-6})
1	VC	0	5	33.1±8.2
2	NDEA	0.001	5	35.0±3.1
3	NDEA	0.01	5	31.5±13.7
4	NDEA	0.1	5	41.3±26.0
5	NDEA	1	5	74.0±31.4
6	NDEA	3	5	118.3±25.6
7	ENU	20	5	177.0±36.4

- Dose-related increase in the mutant frequency induced by NDEA treatment
- Vehicle control and two lowest dose levels have similar mutant frequency
- Top two dose levels show statistically significant difference relative to the vehicle control
- The MF induced by treatment of the top two doses is also out of 95% control limit

Limitations of the Transgenic rodent assay

1

Stand alone assay

Not conductive to integrating into other toxicology studies

2

Transgenic animals

The transgenic animals required for Big Blue assays are expensive and have very limited availabilities

3

Indirect measurement

Relies on a phenotypic measure of mutagenesis

4

Artificial reporter gene

Limited to evaluation of a gene playing no role in carcinogenesis

5

Clonal expansion

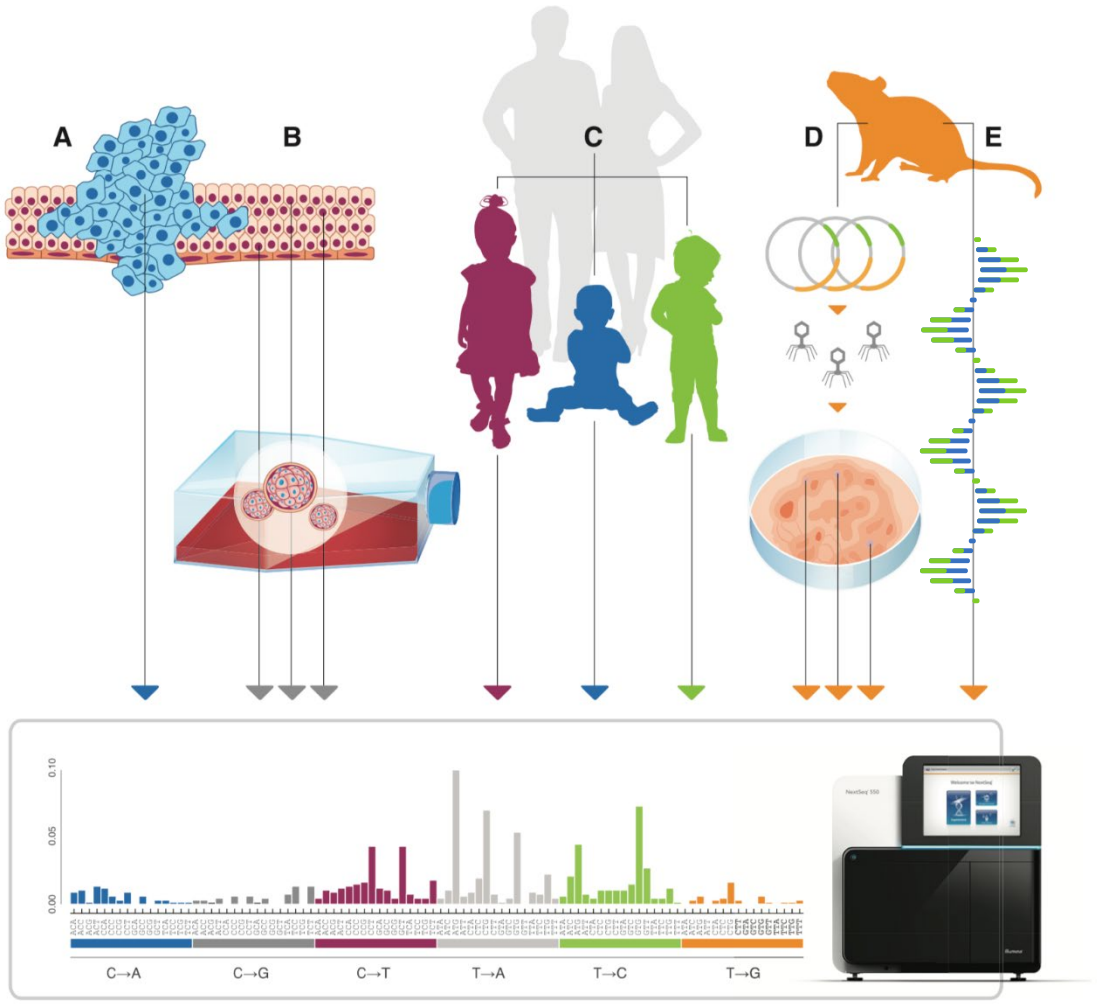
Subject to artificially high mutation frequencies by clonal expansion

6

No mutational spectrum

Does not provide information on types of mutation induced

Power to directly examine the somatic genome with Duplex Sequencing



- *Any* species can be used *in vivo* or *in vitro*. No need for transgenic organisms.
- *Any* tissue or source of double-stranded DNA can be used. No restrictions on cell type.
- *Any* genetic locus can be interrogated. No limitation to artificial reporters.
- Data is unbiased: Clonal amplification is inherently detected and discounted.
- Data is simple, relevant and statistically robust.

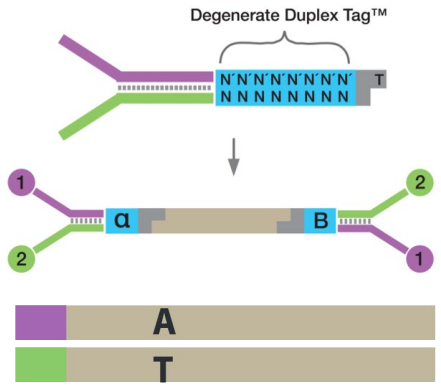
Mutation frequency

Mutational spectrum

Trinucleotide signature

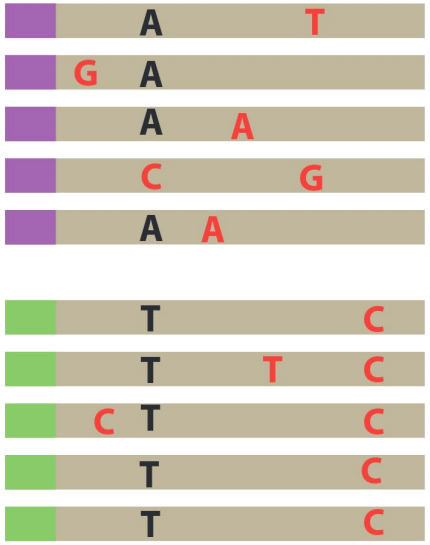
Principle of Duplex Sequencing™ Technology

Starting DNA molecule (truth)



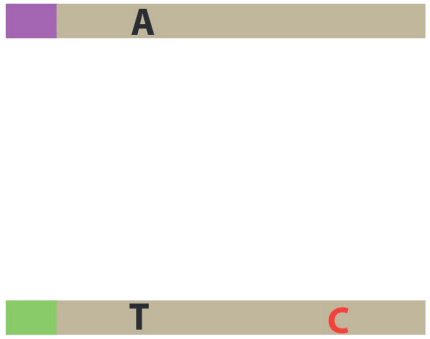
PCR amplify both strand.

Group amplicons by unique tag sequence and strand



Remove intra-strand errors in RED

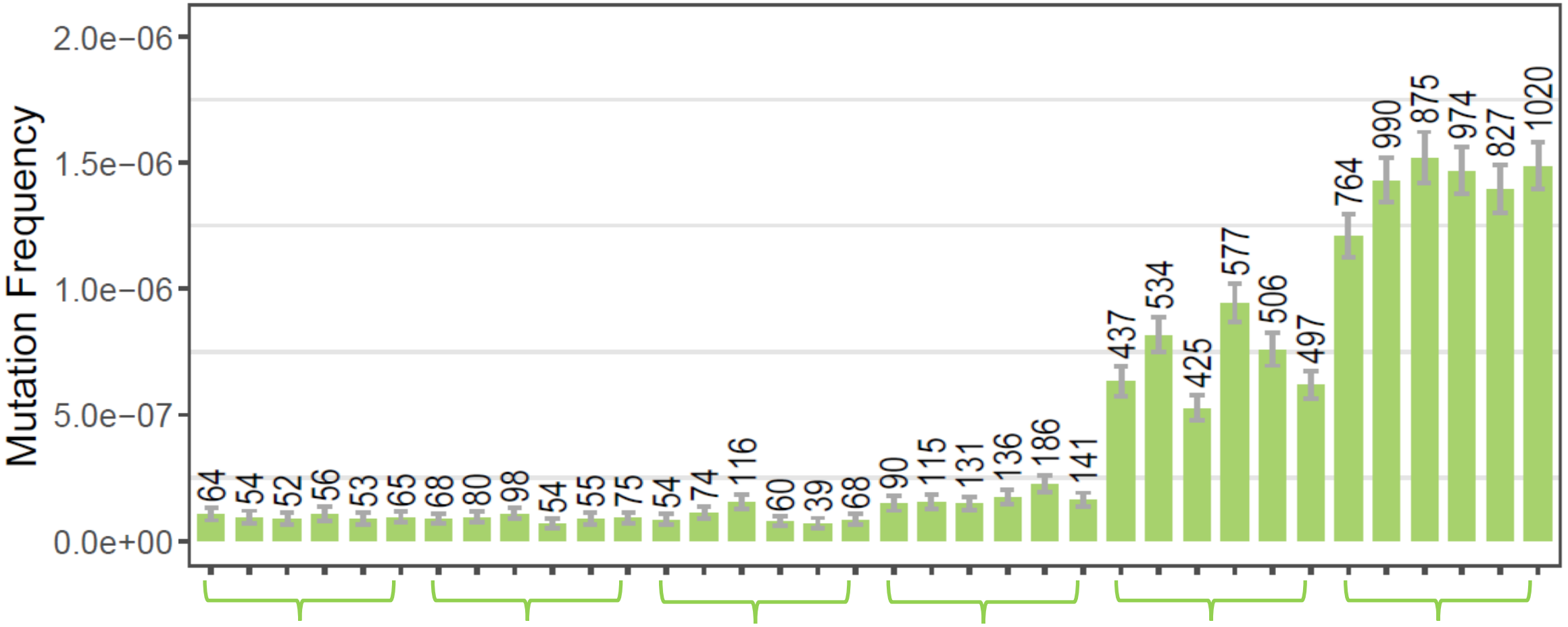
Strand level errors remain



Remove errors in only one strand

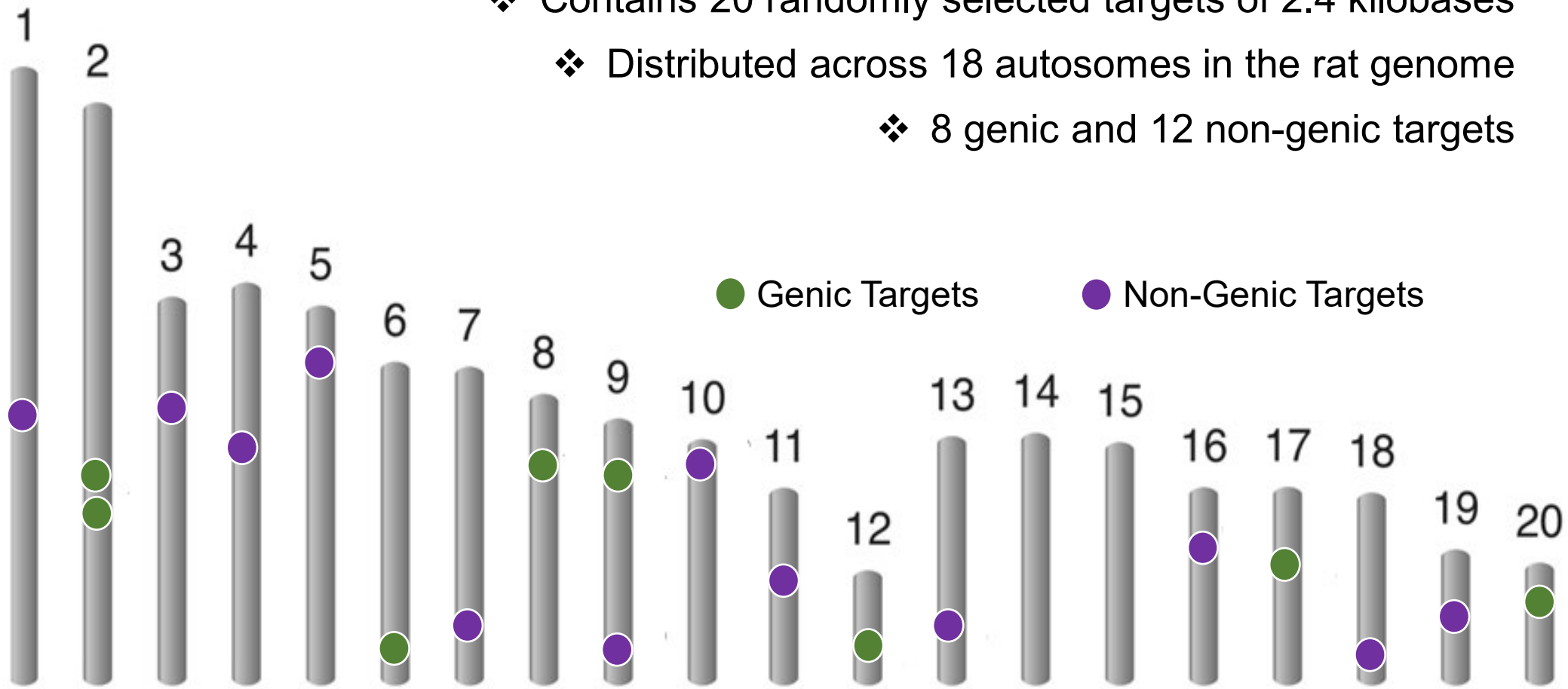
True mutants remain

Dose-dependent increases in the mutations induced by NDEA treatment in this study

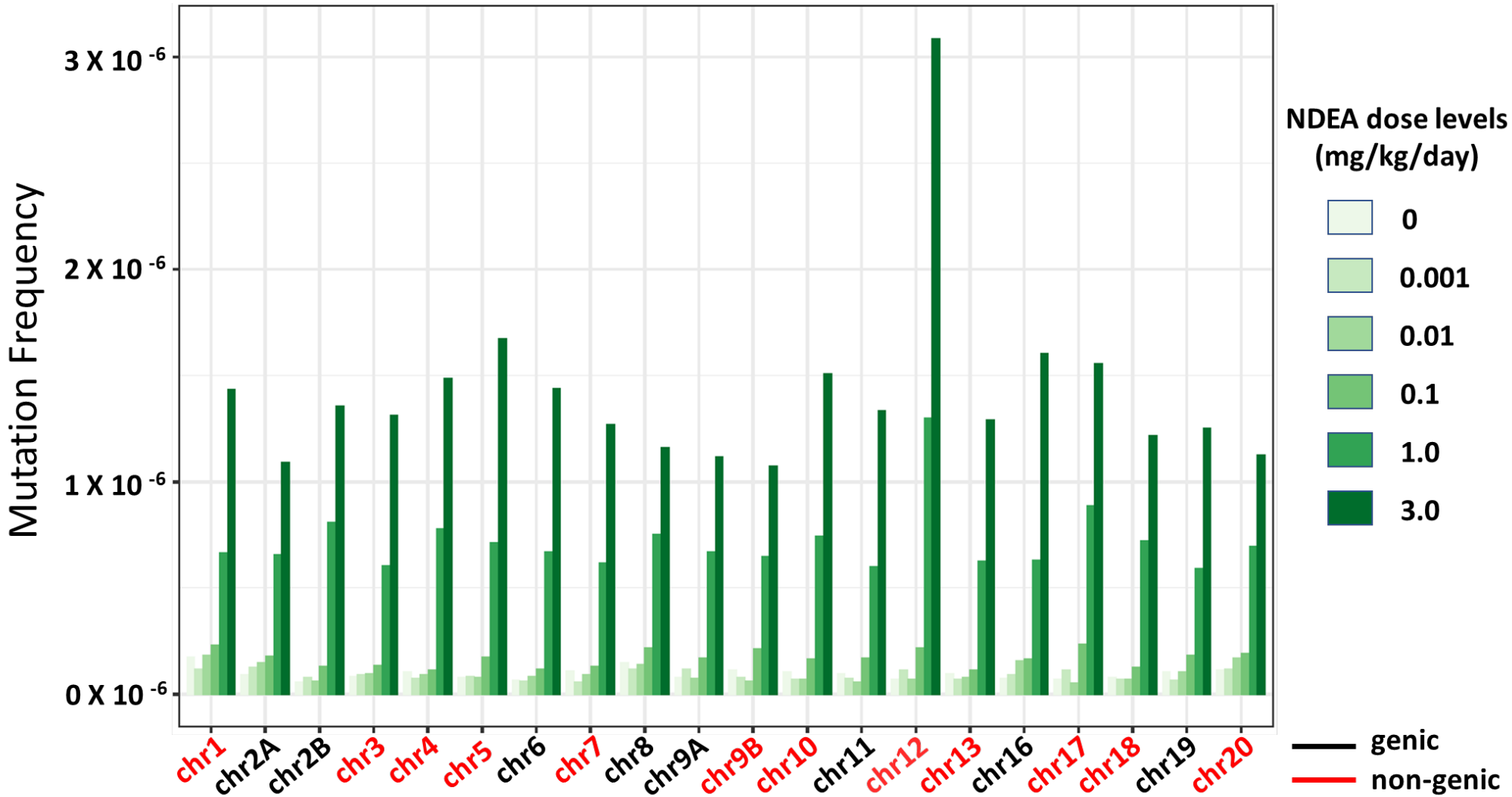


Panel Design: Rat Genotoxicity Panel

- Contains 20 randomly selected targets of 2.4 kilobases
  - Distributed across 18 autosomes in the rat genome
    - 8 genic and 12 non-genic targets



NDEA induced mutation frequency varies at different genomic locations



BMD analysis using in vivo mutagenicity and carcinogenicity dose–response data

Endpoint	BMDL[a] (mg/kg/day)	BMDU[b] (mg/kg/day)
cII MF	0.1	1.0
DuplexSeq MF	0.04	0.09
liver cell tumors [c]	0.022	0.046

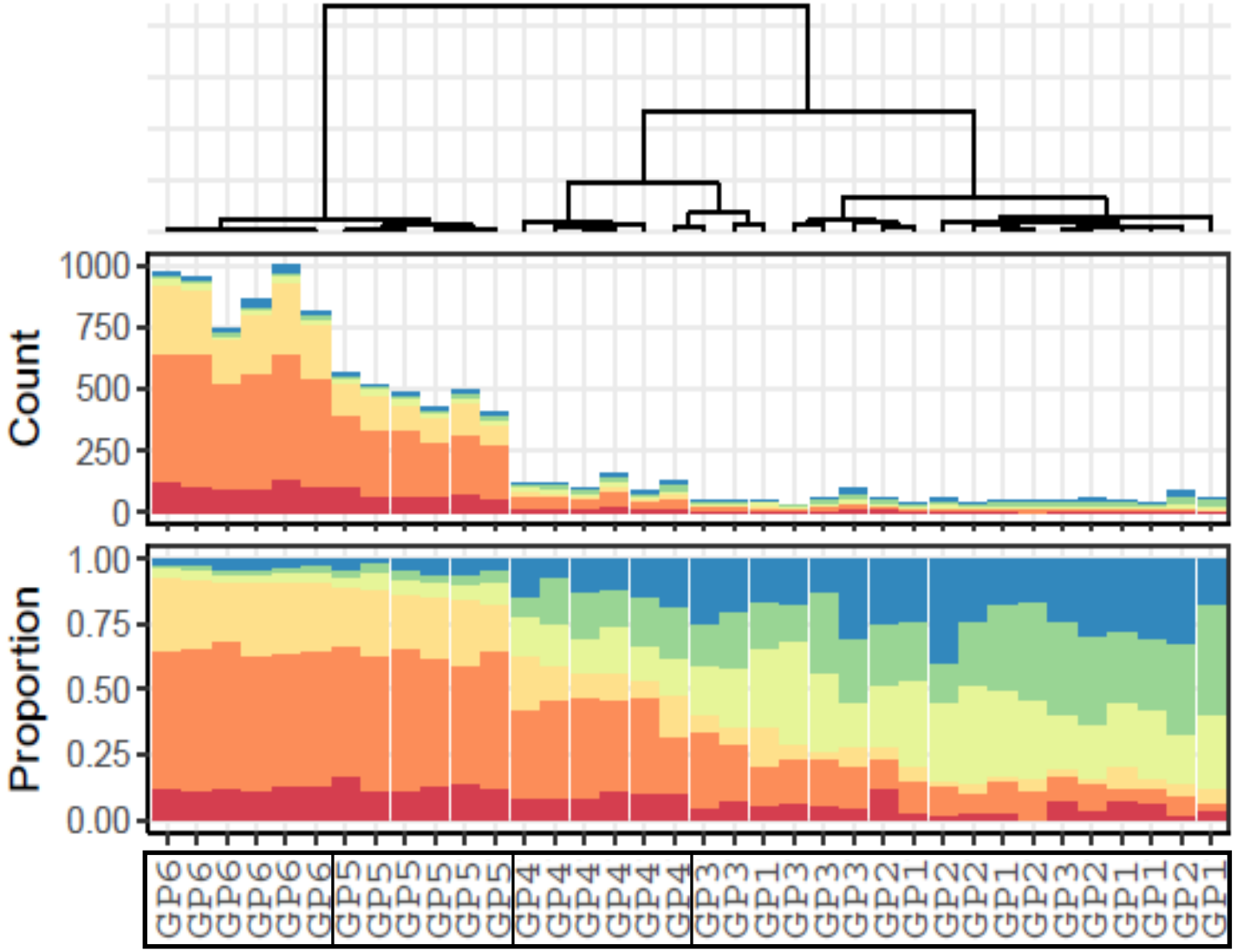
MF – Mutation Frequency

a. Benchmark dose response (50% CES) at the lower 95th confidence interval

b. Benchmark dose response (50% CES) at the upper 95th confidence interval

c. Increased incidence of liver cell tumors in Colworth rats by Johnson et al. *Environ Mol Mutagen. 2021 Jun;62(5):293-305*

Dose-dependent changes in the simple base substitution spectra



Substitution Types:

- C>A
- C>G
- C>T
- T>A
- T>C
- T>G

Group1: Vehicle Control

Group2: 0.001 mg/kg/day

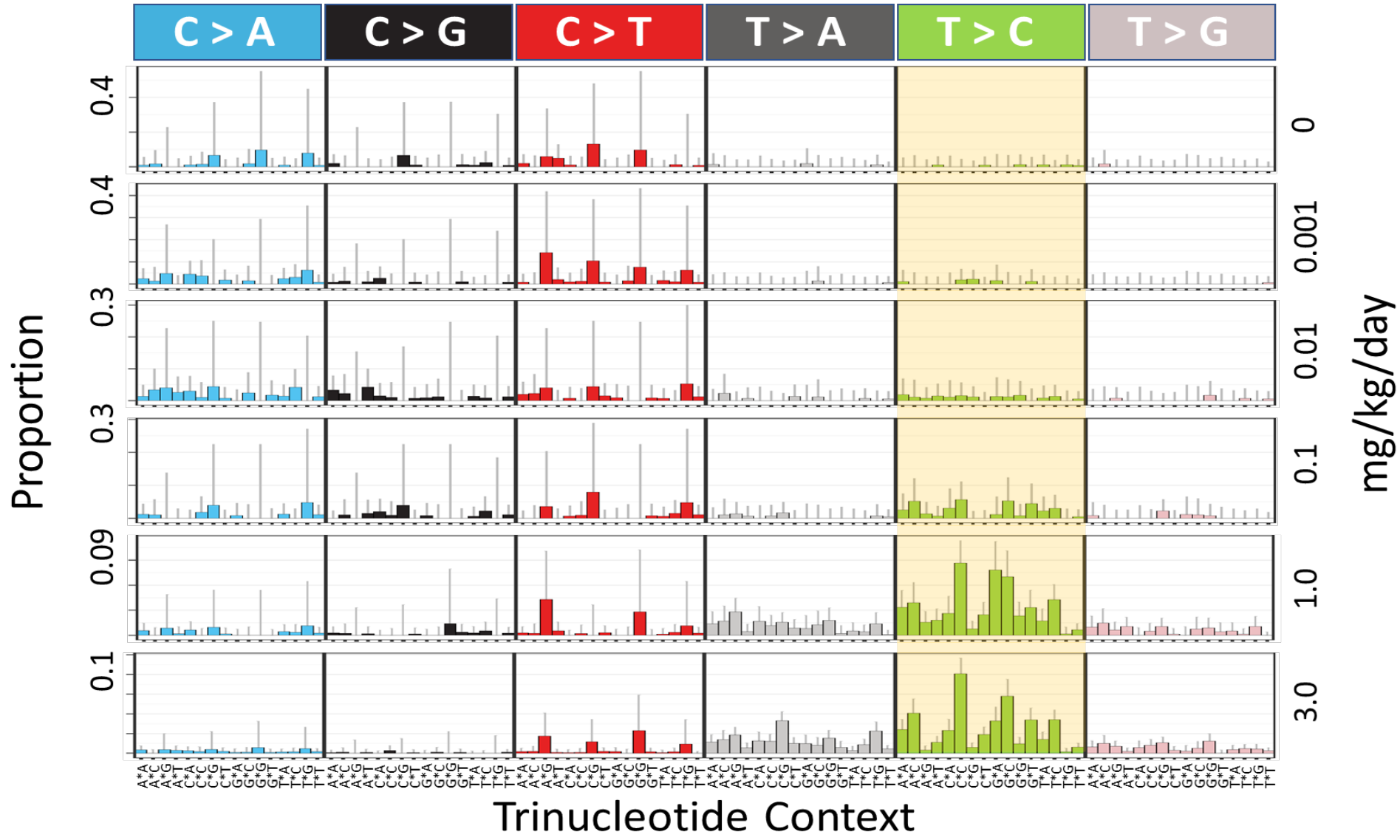
Group3: 0.01 mg/kg/day

Group4: 0.1 mg/kg/day

Group5: 1 mg/kg/day

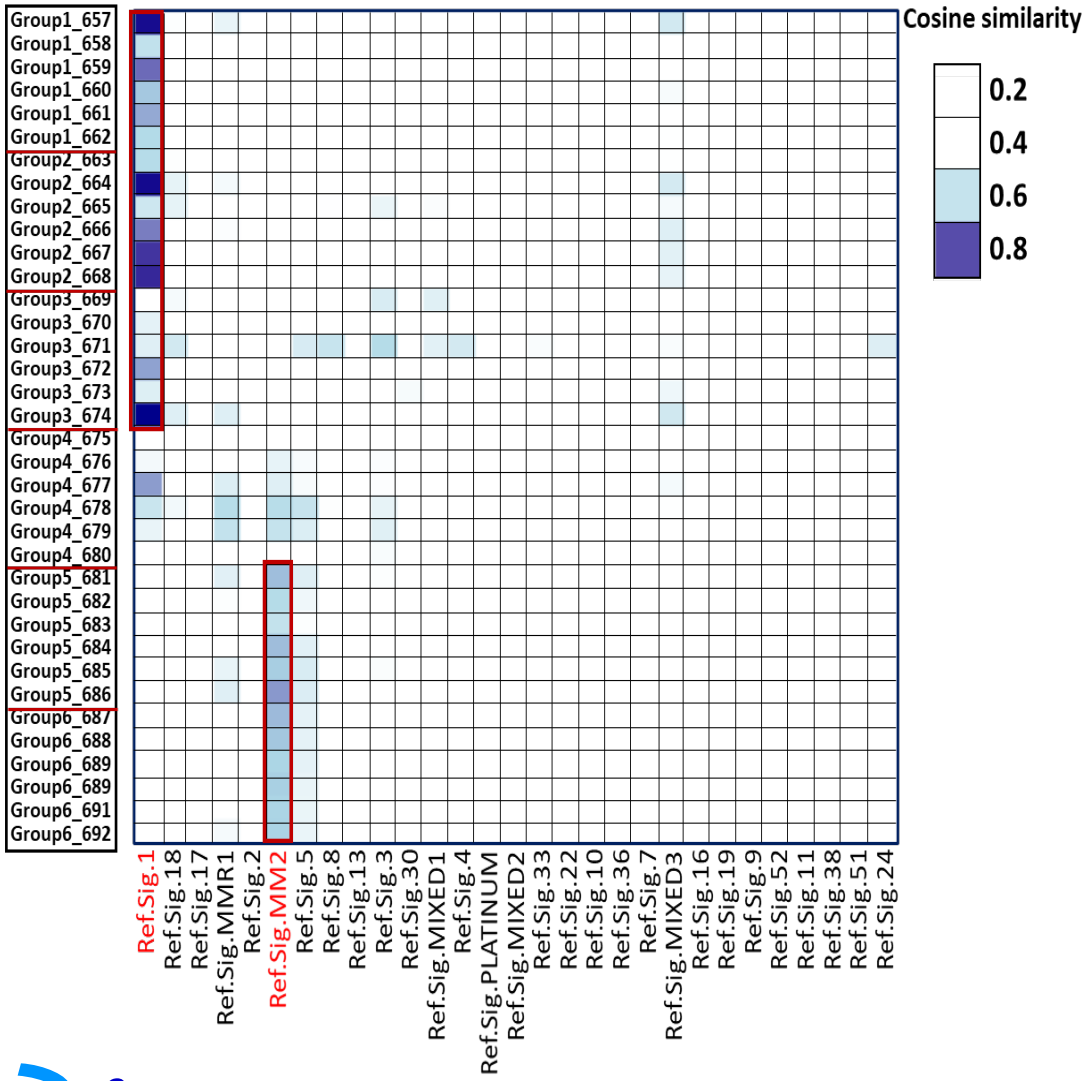
Group6: 3 mg/kg/day

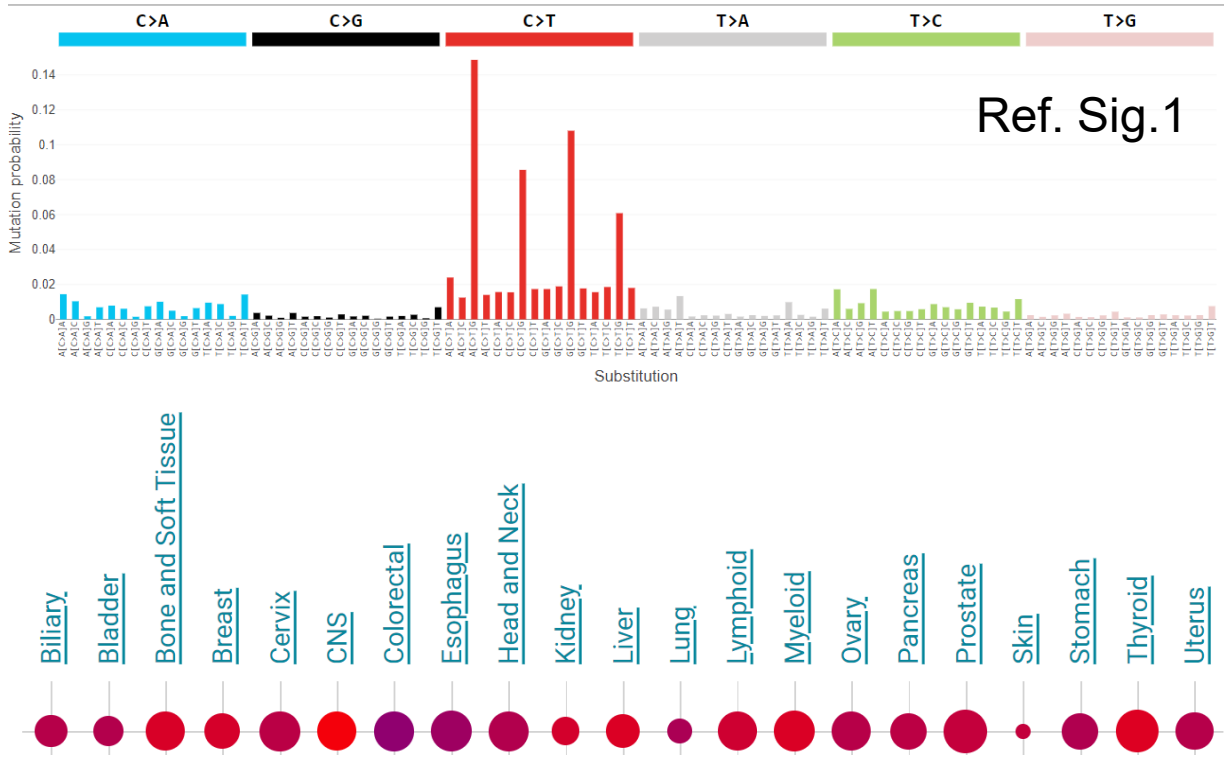
Dose-dependent changes in the trinucleotide spectra



Comparation of trinucleotide spectra with the cancer signatures

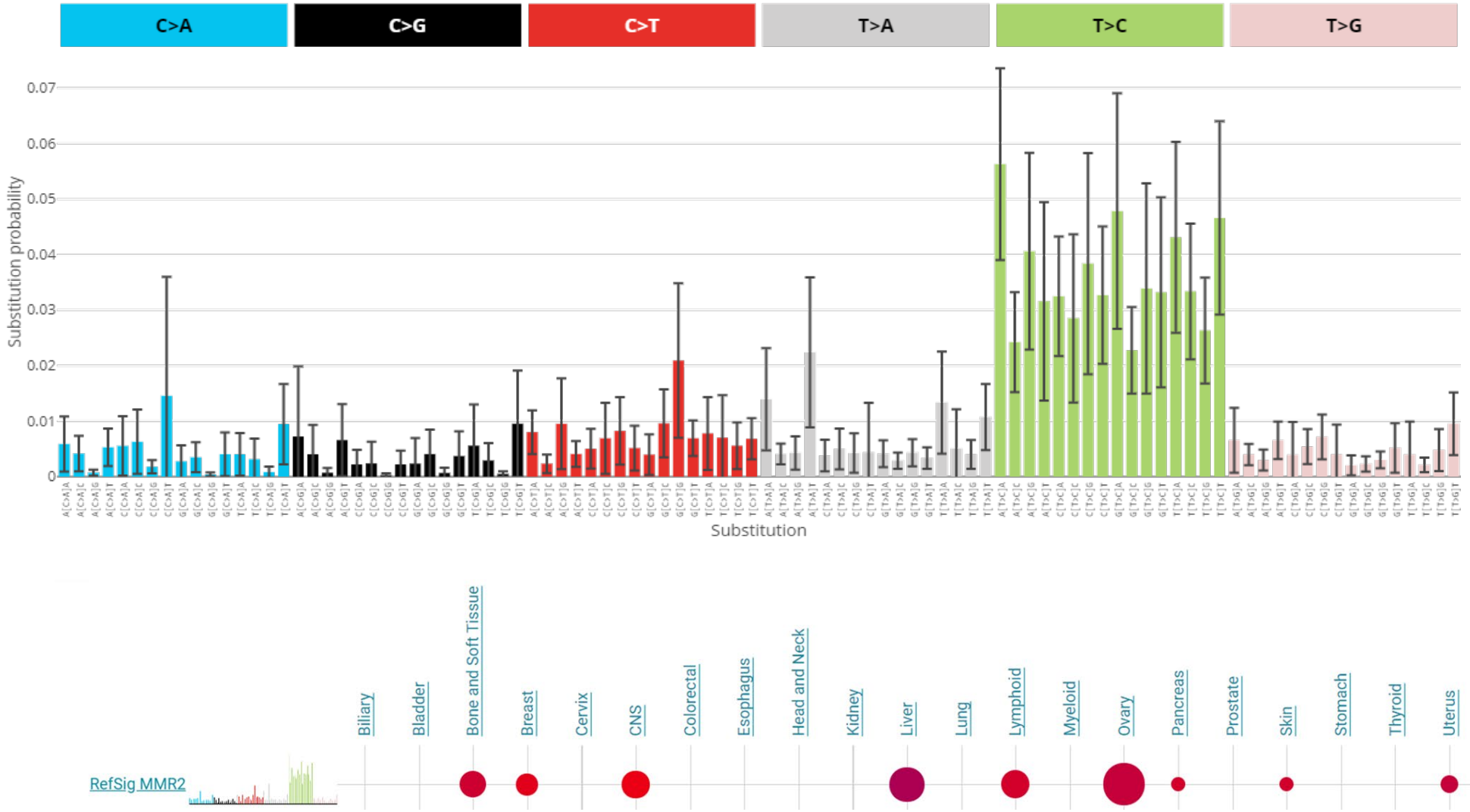
- SIGNAL database: 3,107 whole genome sequenced (WGS) primary cancers of 21 organs



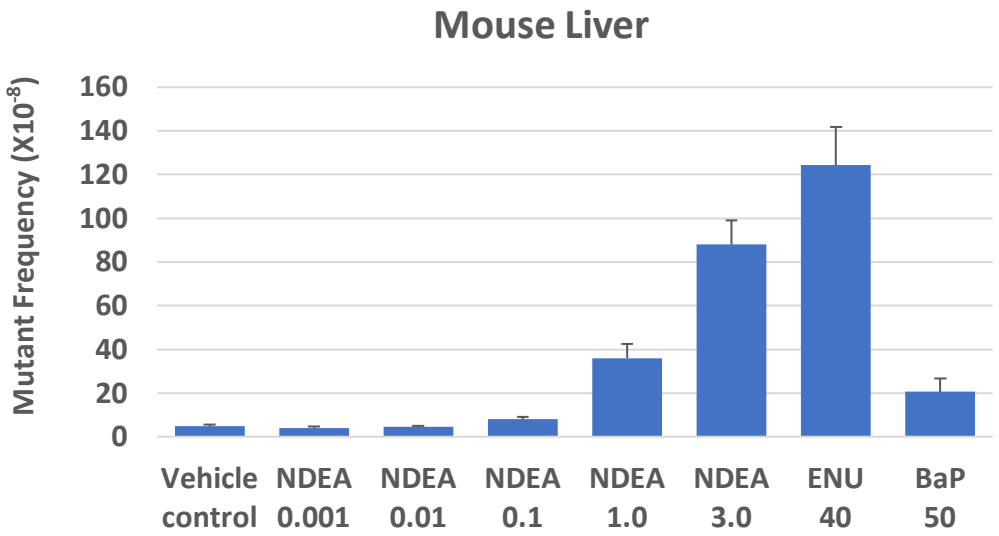


RefSig 1 is the result of spontaneous deamination of 5-methylcytosine.

Deficiency in Mismatch repair (MMR)2 signature is frequently found in liver cancer



Dose-dependent increases in the MF in mice liver

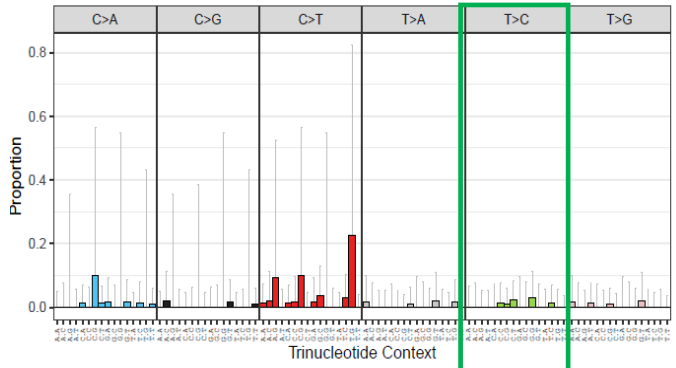


Group No.	Treatment	Dose level mg/kg/day	Animals/group	Mutant Frequency ± Standard Dviation (x 10^{-6})
1	VC	0	6	4.8±0.9
2	NDEA	0.001	6	4.1±0.7
3	NDEA	0.01	6	4.6±0.4
4	NDEA	0.1	6	8.1±1.1
5	NDEA	1	6	36±6.5
6	NDEA	3	6	87.9±11.2
7	ENU	40	6	124.2±17.6
8	BaP	50	6	20.7±6.0

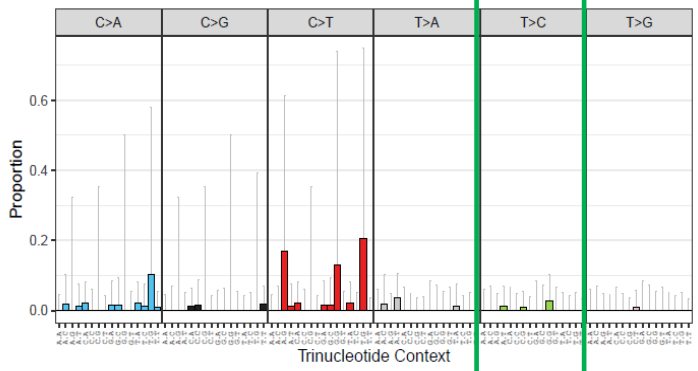
- Dose-related increase in the mutant frequency in mouse liver
- The positive control and two top dose groups show statistically significant difference relative to the vehicle control
- The third highest dose level group almost doubles the MF compared to vehicle control group

The characterized T>C mutation shows dose-related increase in mice

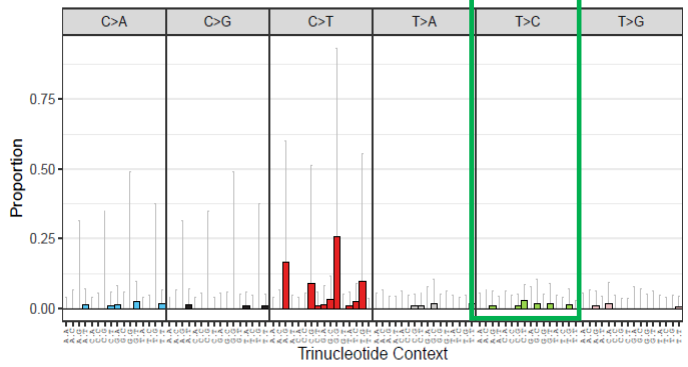
Vehicle Control



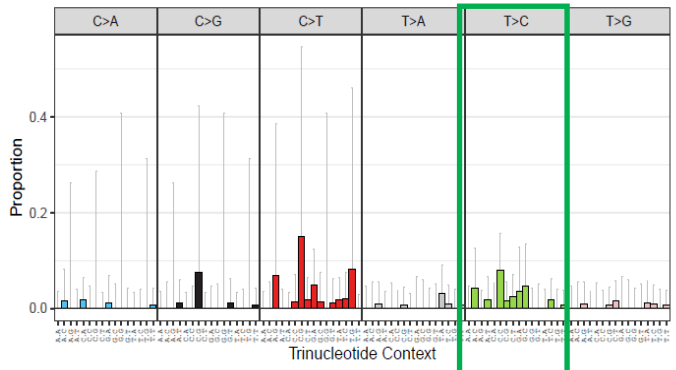
NDEA 0.001 mg/kg/day



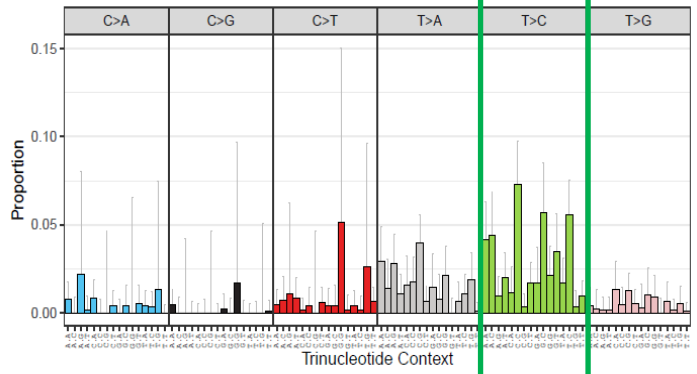
NDEA 0.01 mg/kg/day



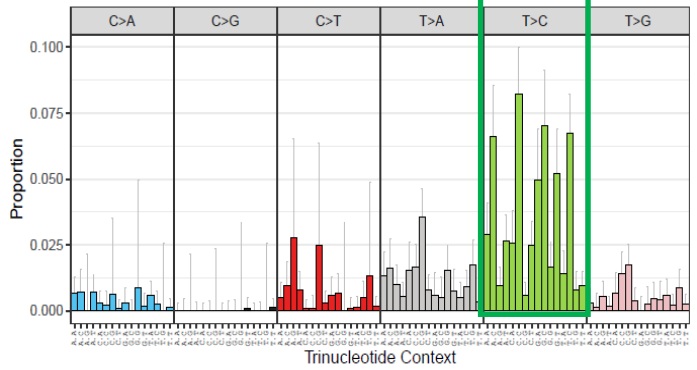
NDEA 0.1 mg/kg/day



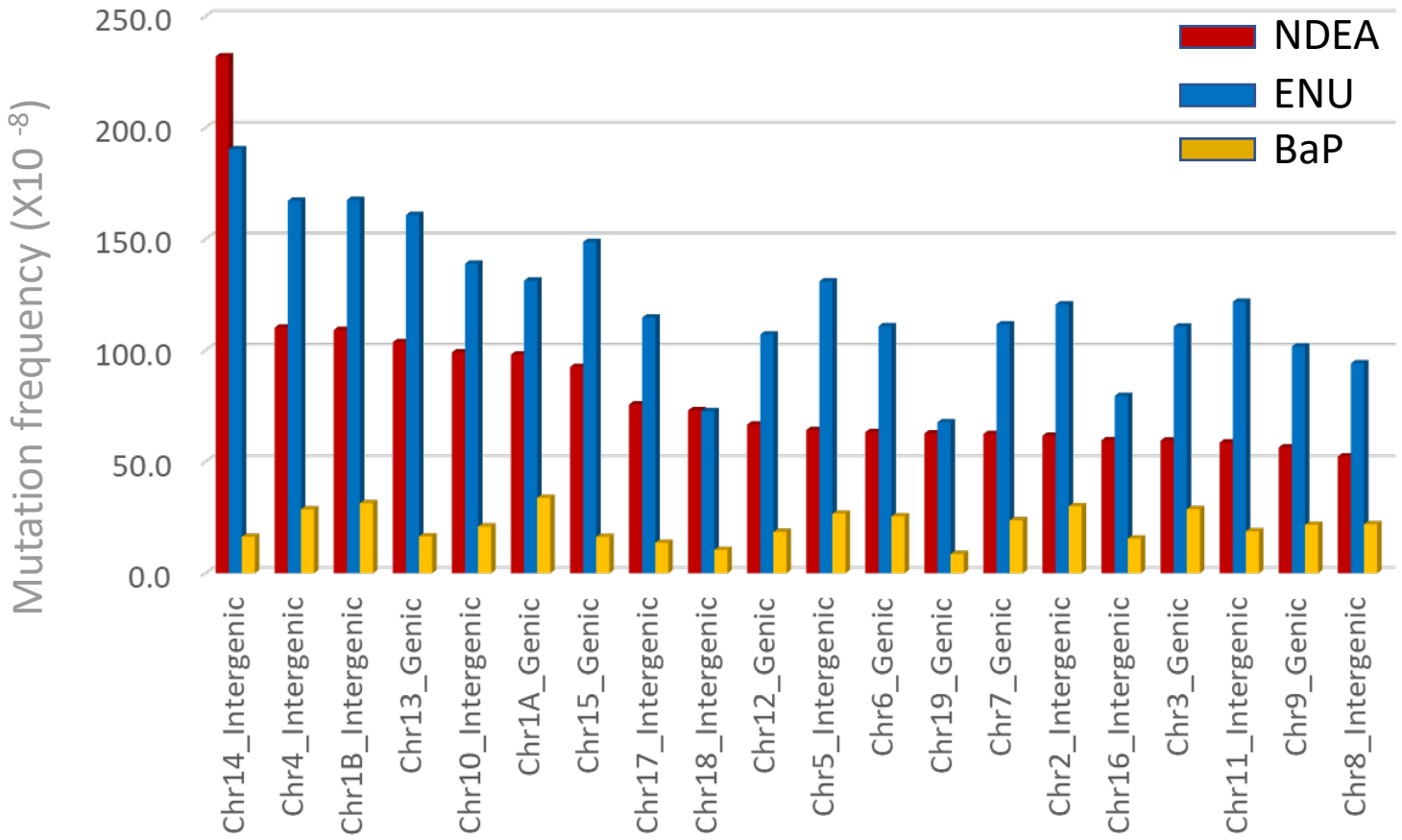
NDEA 1.0 mg/kg/day



NDEA 3.0 mg/kg/day



Mutation frequency varies by treatments and genomic locations



Chromosomes	NDEA	ENU	BaP
Chr14_Intergenic	232.0	190.3	16.4
Chr4_Intergenic	110.3	167.2	28.7
Chr1B_Intergenic	109.2	167.6	31.4
Chr13_Genic	103.7	160.8	16.5
Chr10_Intergenic	99.1	138.9	20.9
Chr1A_Genic	98.1	131.3	33.9
Chr15_Genic	92.6	148.7	16.3
Chr17_Intergenic	75.7	114.8	13.7
Chr18_Intergenic	73.1	72.8	10.4
Chr12_Genic	66.7	107.2	18.5
Chr5_Intergenic	64.2	131.0	26.7
Chr6_Genic	63.3	110.9	25.5
Chr19_Genic	62.7	67.8	8.6
Chr7_Genic	62.5	111.7	23.7
Chr2_Intergenic	61.7	120.7	30.1
Chr16_Intergenic	59.7	79.6	15.6
Chr3_Genic	59.6	110.7	28.8
Chr11_Intergenic	58.7	121.8	18.8
Chr9_Genic	56.5	101.8	21.7
Chr8_Intergenic	52.4	94.2	22.0

Conclusions

- Treatment of NDEA led to increase in MF and change of mutational signatures in a dose-related manner, both in rats and in mice, in this study
- The results of Duplex Sequencing show good correlation with traditional TGR assay
- DS shows slightly higher sensitivity than TGR assay, with the mutagenicity BMD results comparable with carcinogenicity studies
- Both Duplex Sequencing and TGR assays confirmed that a threshold for genotoxic effect level could be observed for NDEA
- Duplex Sequencing provides additional mechanistic information underlying the mutagenic process

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