Molecular Mechanisms of Aflatoxin-mediated Carcinogenesis: Implications for Hepatocellular Carcinogenesis

R. Stephen Lloyd
Oregon Health & Science U.
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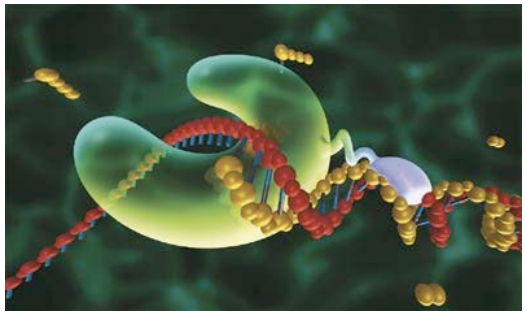
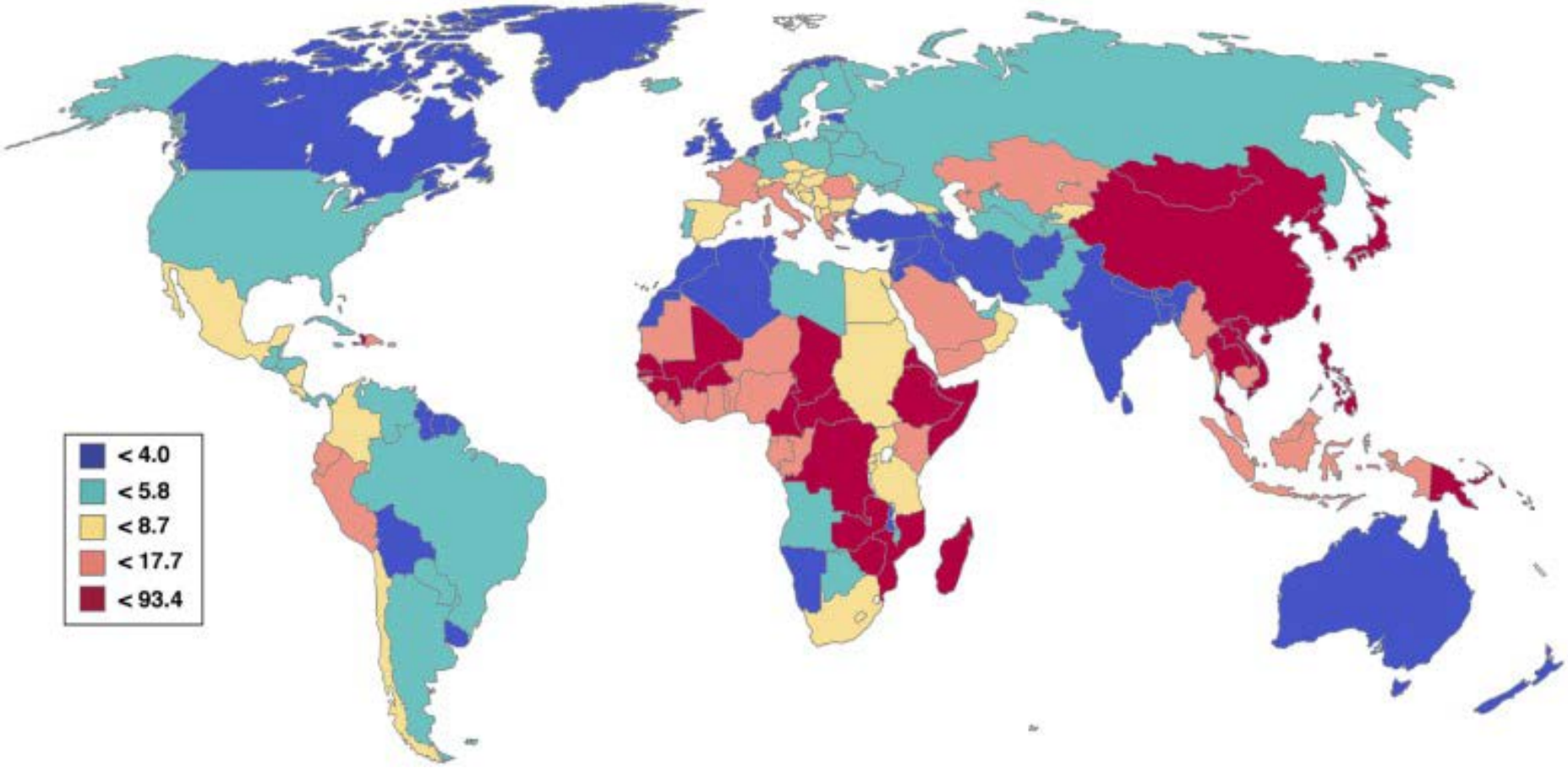
NIH Video Conference



The PROBLEM:
Global incidence of hepatocellular carcinomas (HCC)

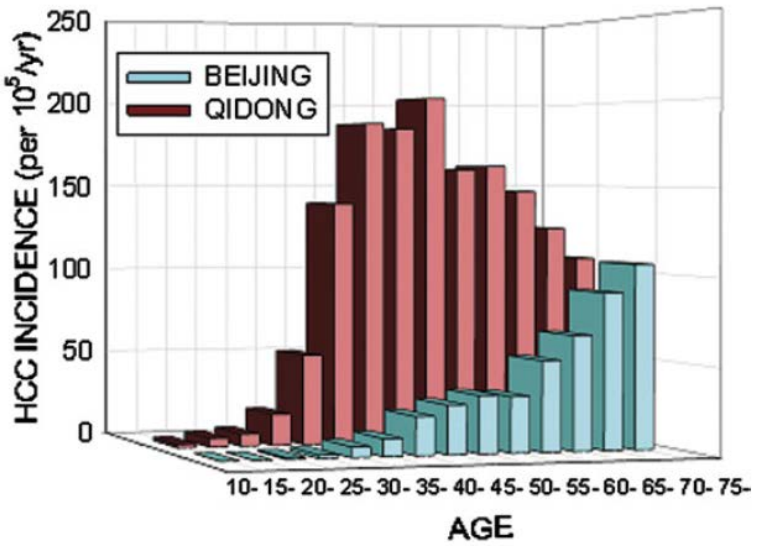


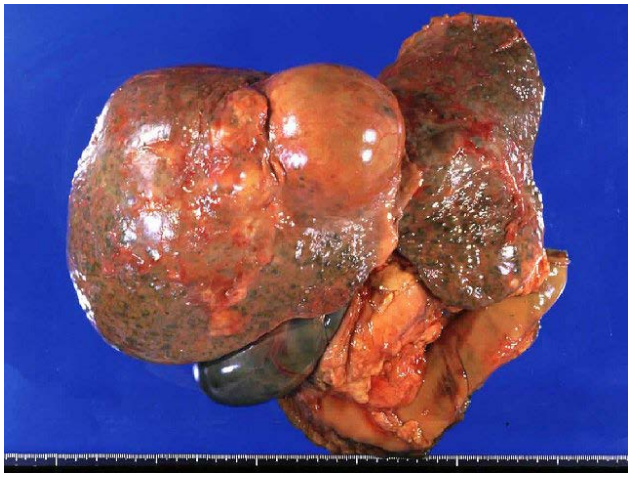
Regional variations in the mortality rates of HCC categorized by age-adjusted mortality rates. The rates are reported per 100,000 persons.

H.B. El–Serag & K.L. Rudolph
Gastroenterology 2007;132:2557–2576

HCC in the People's Republic of China

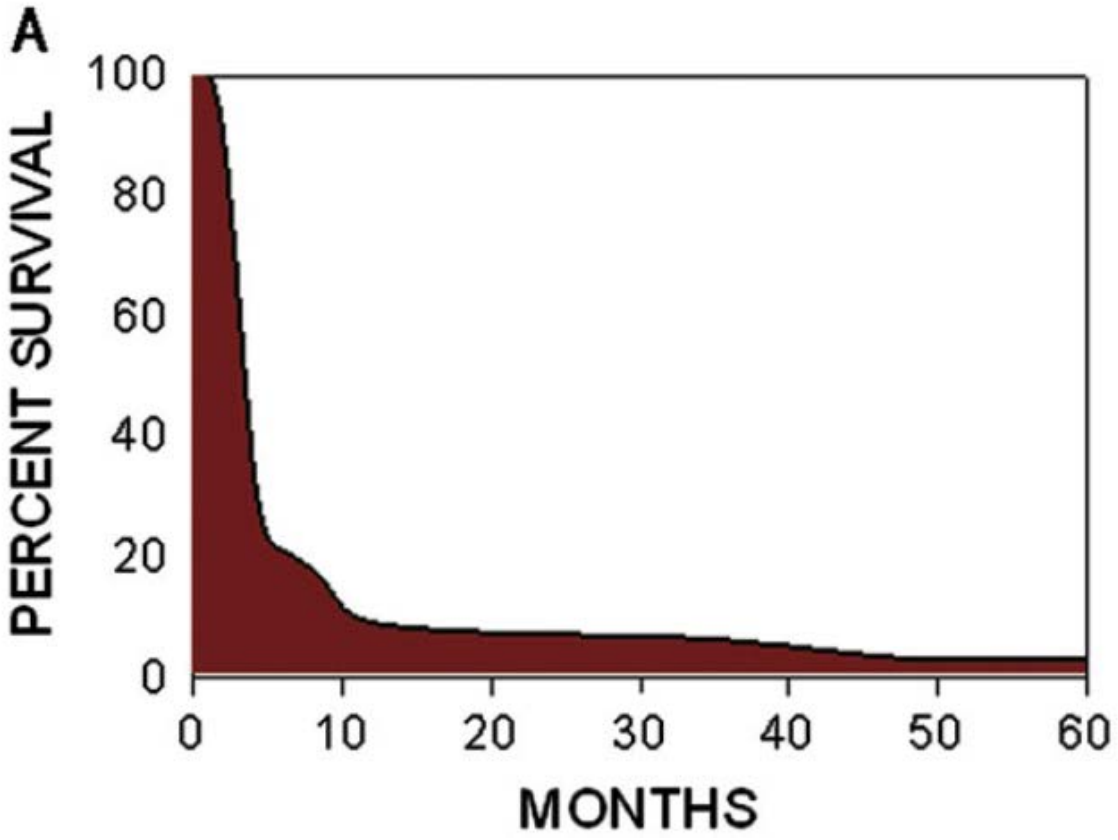
- ~700,000 new cases/yr
- ~300,000 deaths/yr
- Males 5x more likely to develop HCCs
- Age of onset: ~20 yr; peaks 40-49 (male), 50-59 (female)
- Bimodal age distribution in specific regions





Kensler et al., Toxicol. Sci. 2011, 120, S28-S48

Early onset of HCC with poor prognosis



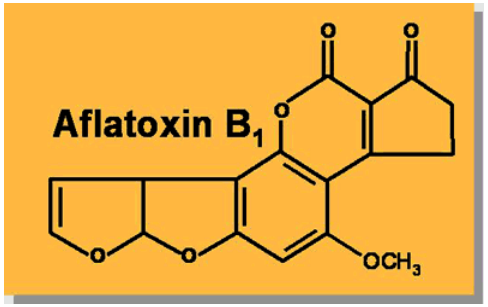
Progressive understanding of the molecular mechanisms underlying geographically-enhanced HCC

- Clue #1:

  1950s-1960s outbreaks of “turkey X” disease – massive poultry deaths(~100,000) from consumption of peanut meal contaminated with *Aspergillus flavus*

- Clue #2:

  Aspergillus flavus produces a toxic substance: aflatoxin



Progressive understanding of the molecular mechanisms underlying geographically-enhanced HCC (continued)

- Clue #3:

Contamination of corn and peanuts

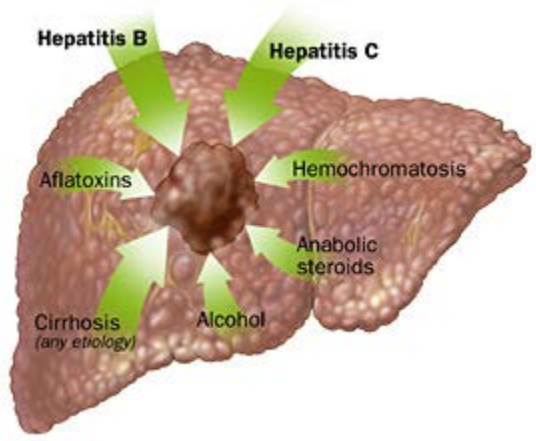
with *Aspergillus flavus* is widespread

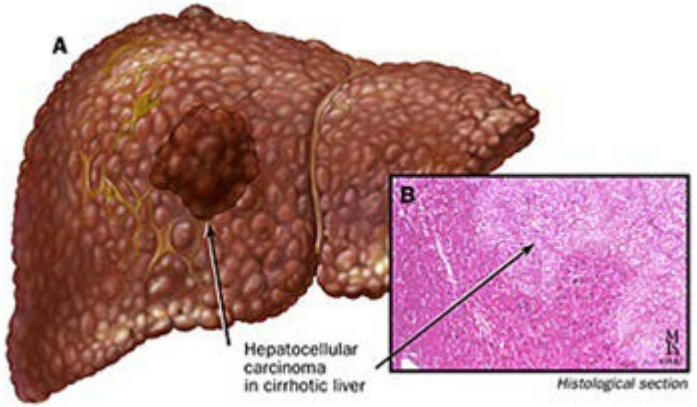
Progressive understanding of the molecular mechanisms underlying geographically-enhanced HCC (continued)

- Clue #4:

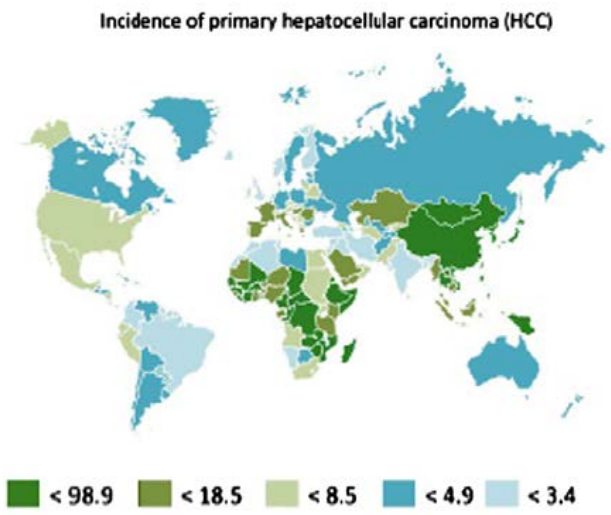
  World-wide epidemiology studies link HCC with aflatoxin (AFB_1) exposures (as determined by AFB_1-HSA adducts in blood, AFB_1 metabolites in urine, and AFB_1 DNA adducts)

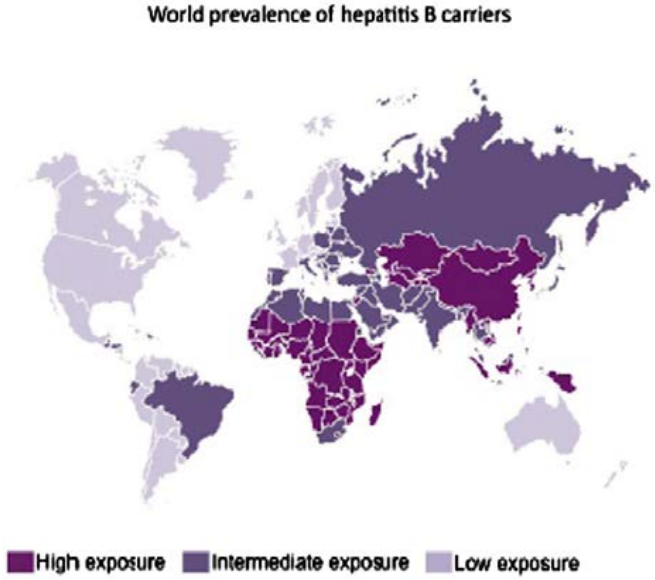
  World-wide epidemiology studies link HCC with chronic infections of hepatitis B and C

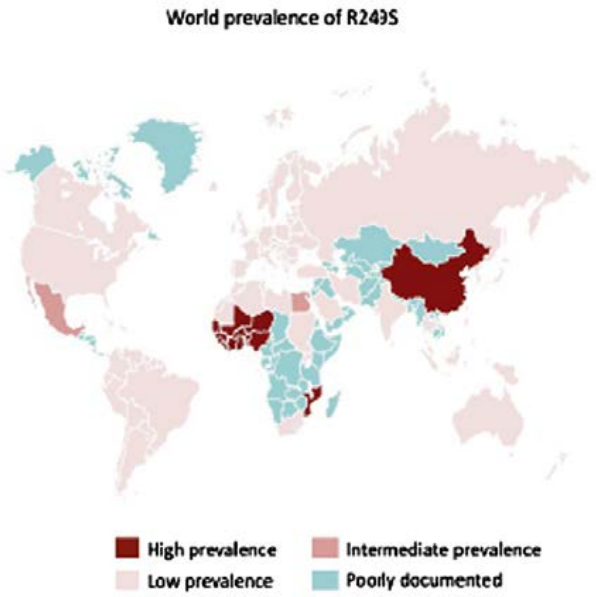




Geographic distribution of HCC and aflatoxin exposure/hepatitis carriers

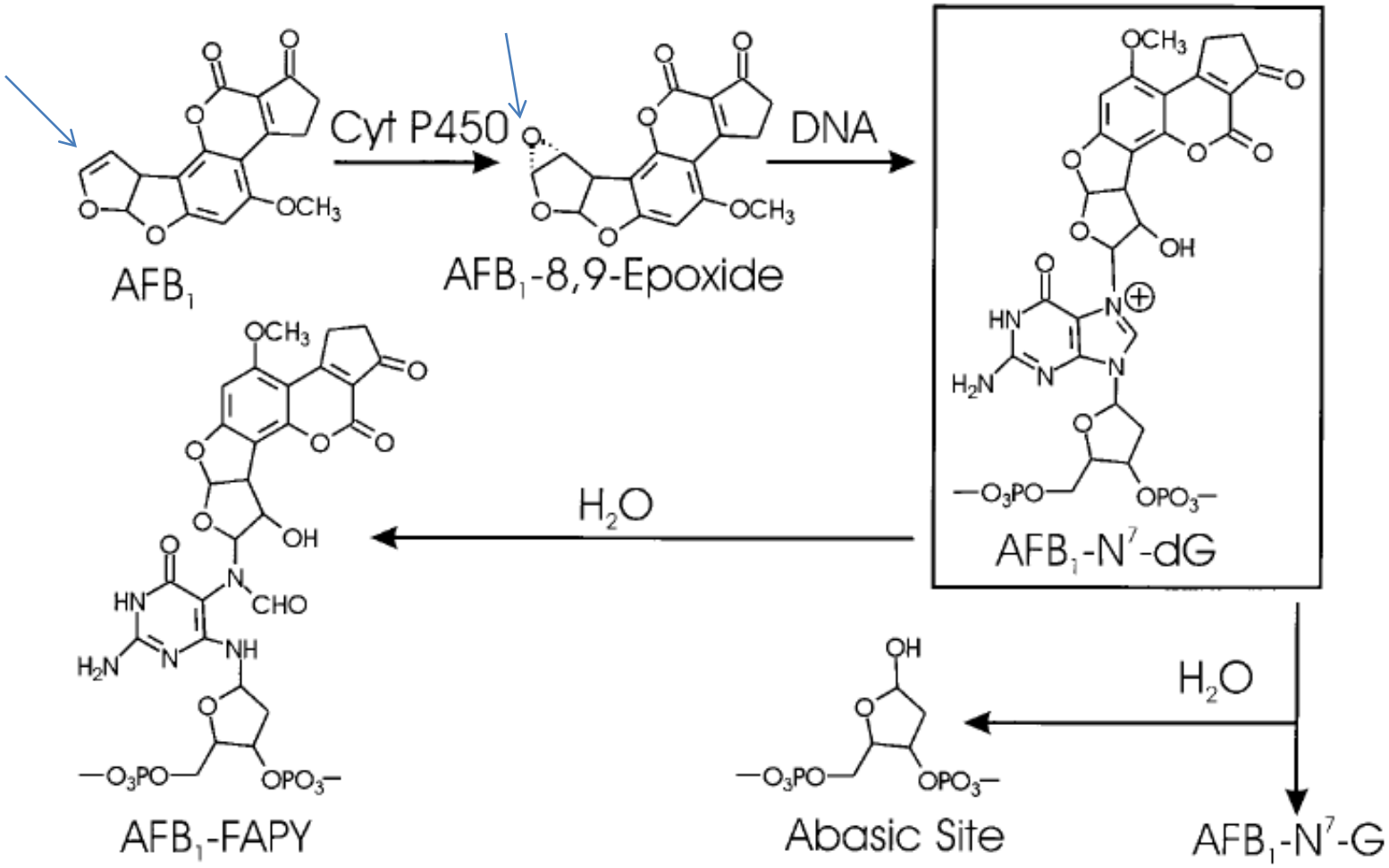






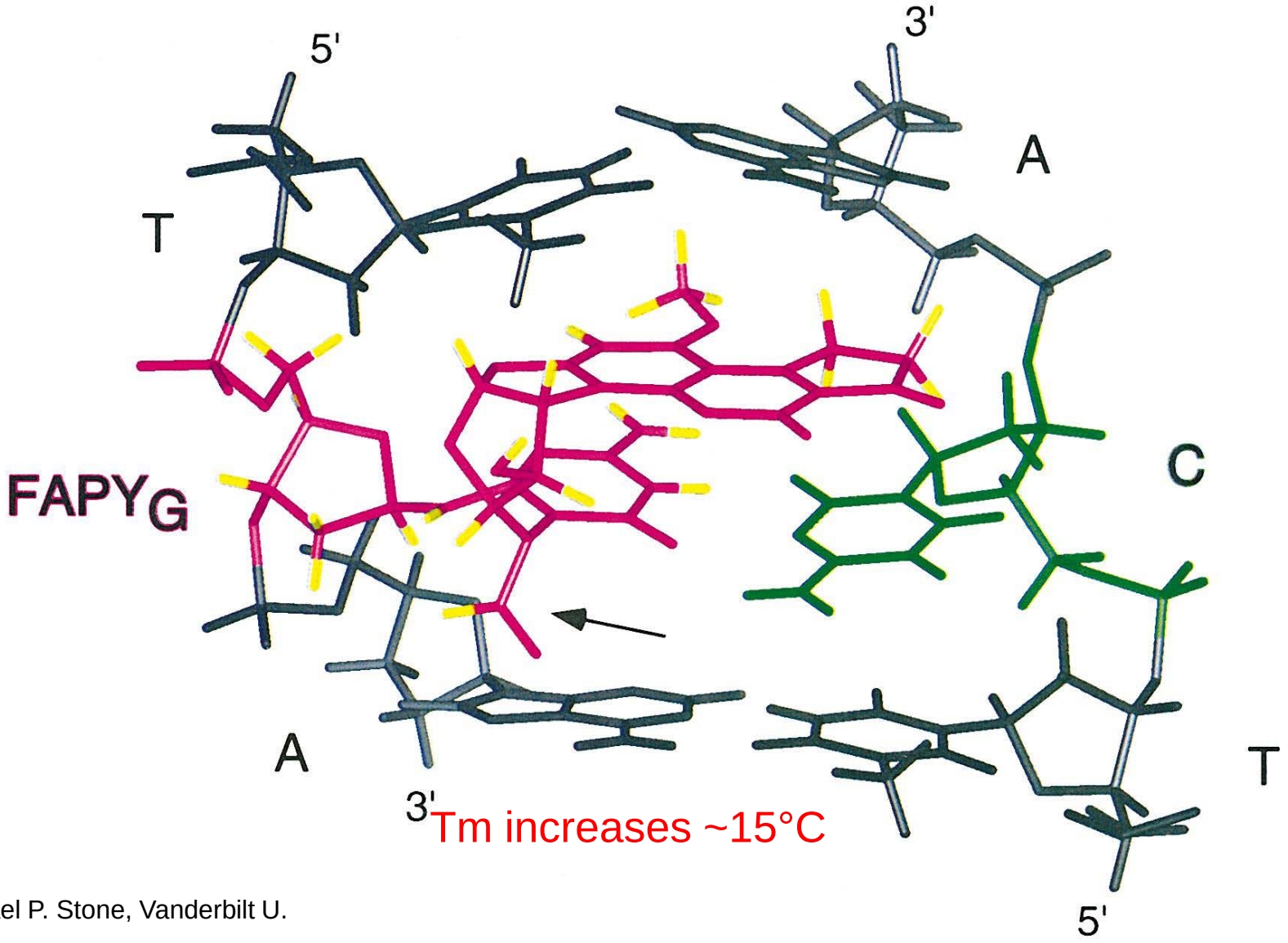
Gouas, et al, 2009

Metabolic activation of AFB_1 to form DNA adducts



Alekseyev et al., Carcinogenesis 2004, 25 (6), p 1045-1051

AFB_1 adducts intercalate within and stabilize duplex DNA



Michael P. Stone, Vanderbilt U.

Insights into the mutagenic potential of AFB_1

- G to T mutations at the third position of codon 249 of the p53 gene is found in >50% of HCC cases examined from high AFB_1 contaminated areas
- In *E.coli*, AFB_1- N7-dG induces G to T mutations (4%)
- In *E.coli*, AFB_1-FAPY induces G to T mutations (32%)
- AFB_1-FAPY-dG is a more effective replication block than AFB_1-N7-dG in *E. coli*
- Both AFB_1 DNA adducts are repaired by nucleotide excision repair in *E. coli*

Goals of our investigation

- Determine the genetic consequences of replication past both the cationic N7-AFB_1-dG and AFB_1-FAPY-dG adducts in primate cells
- Identify the DNA polymerases that may account for error-free and error-prone replication past these adducts
- Explore alternative DNA repair mechanisms that could influence the mutagenic outcomes

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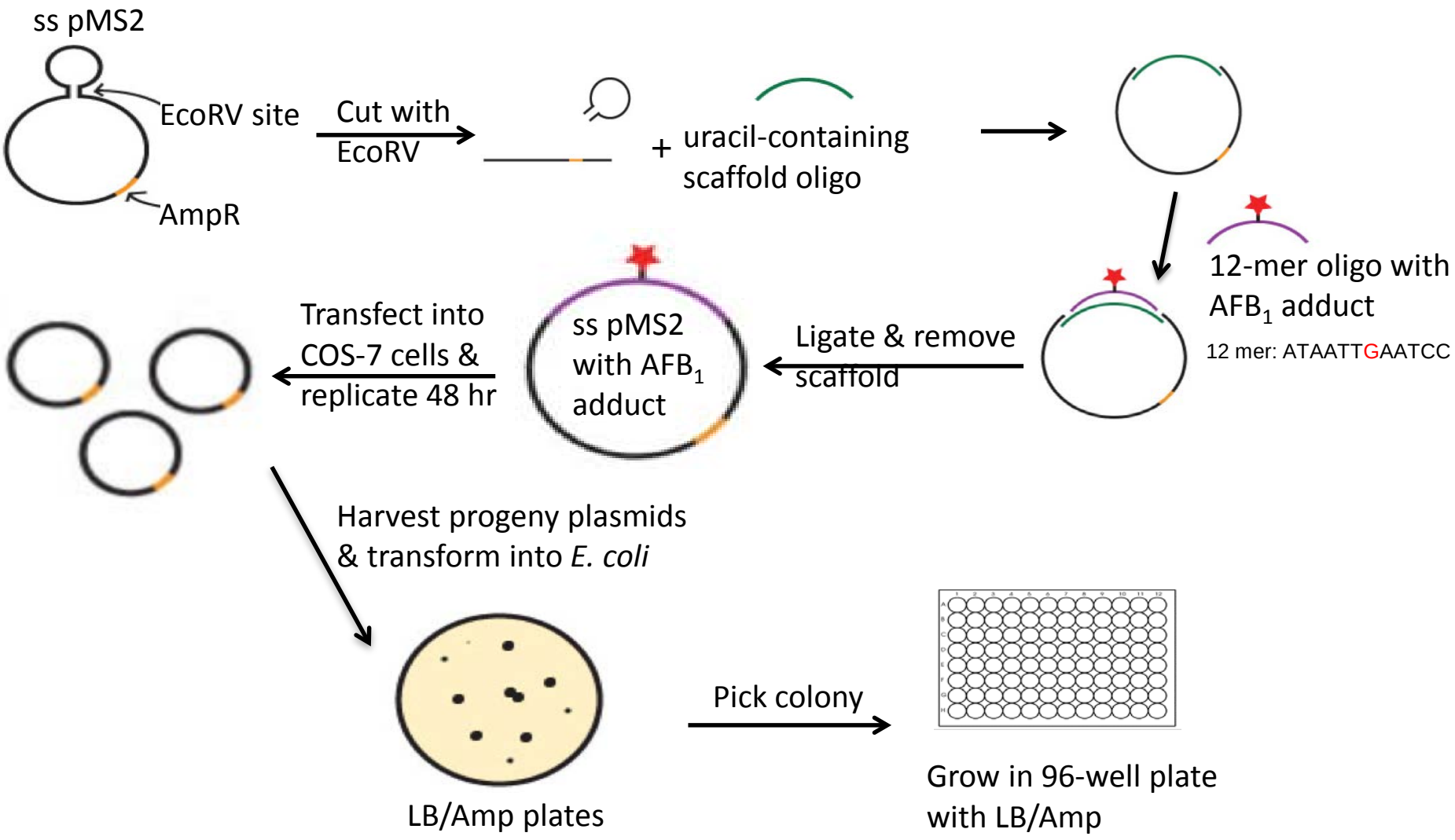
Genetic consequences of replicating single-stranded DNAs containing either the cationic N7-AFB1-dG or AFB_1-FAPY-dG adducts in primate cells

Experimental rationale criteria:

1. Need DNAs of known sequence containing site-specific DNA adducts: Synthetic DNA synthesis – MP Stone, VU
2. Utilize a replication strategy that measures only the consequences of replication bypass of the adduct, not a combined effect of repair and replication: Use of single-stranded DNA shuttle vector that allows replication in primate cells but prevents repair; the resulting double-stranded DNAs can be analyzed in *E. coli* for mutations
3. Design a procedure that measures the consequence of replication in progeny DNAs: Use differential DNA hybridization and sequencing to screen for mutations

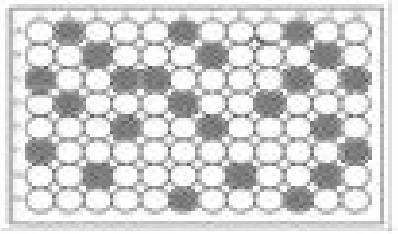
Site-specific mutagenesis assay

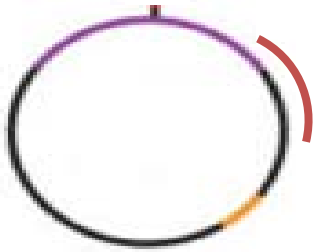


Lin et al, 2014 a,b *Carcinogenesis; J. Biol. Chem*.

Analyses by differential DNA hybridization

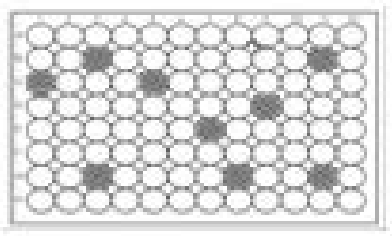
Hybridize with bridge probe to ensure the presence of insert sequences

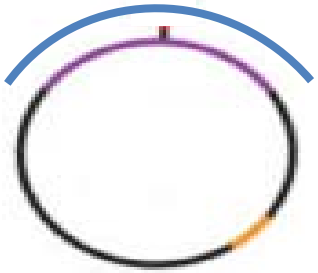




Bridge probe

Lyse cells & cross-link DNA on two membranes





NM,
G-> T,
G-> A or
Del probe

Hybridize with NM, G to T, G to A, & deletion probes

Lin et al, 2014 a,b *Carcinogenesis; J. Biol. Chem*.

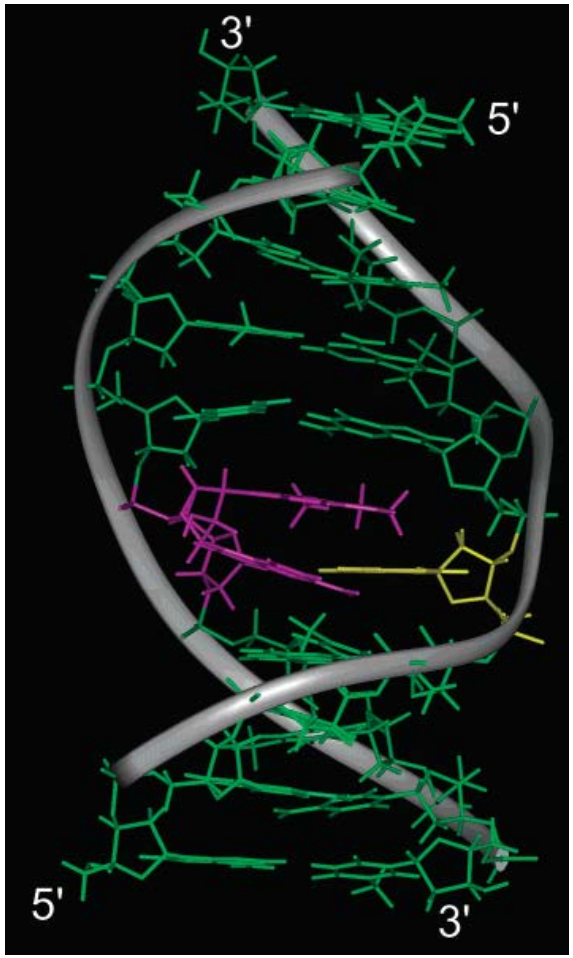
Both AFB_1- FAPY & AFB_1-N7-dG adducts induce G to T mutations in primate cells

DNA modification	Colonies scored	Mutated	Single base substitutions			Deletions	Other position substitution	Frequency of mutation (%)
			G->T	G->A	G->C			
Nondamage	189	0	0	0	0	0	0	0
AFB_1-N7-Gua	216	98	80 (81.6%)	12 (12.2%)	1 (1%)	3 (3.1%)	2 (2%)	45.4
AFB_1-FAPY	203	197	170 (86.3%)	16 (8.1%)	5 (2.5%)	5 (2.5%)	1 (0.5%)	97

The high frequencies of G to T mutations are highly consistent with mutation data observed in patients with early onset HCC

Lin et al, 2014 a,b *Carcinogenesis; J. Biol. Chem*.

Solution structure of AFB_1-FAPY-dG mismatched with dA



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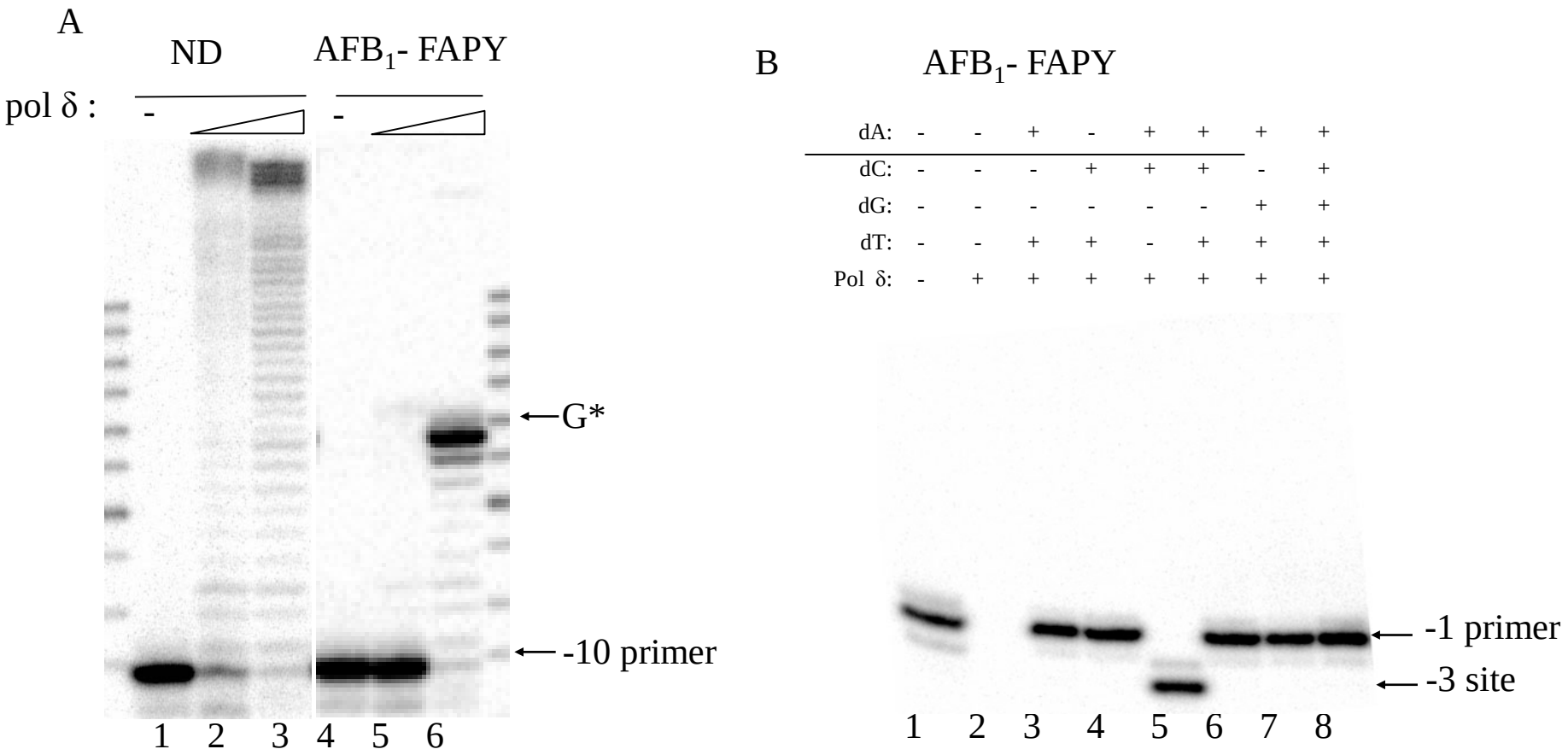
DNA Replication Bypass of AFB_1-FAPY-dG

Collaboration with Dr. Peter Burgers, Washington University, St. Louis

AFB_1-FAPY-dG blocks replicative pol δ

```
5′-ATTATGCAGCGATAGAATAATTGAATCCATCGCTGGTACCGACTCG-3′
                                  3′-GACCATGGCTGAGC-5′ (-10 primer)
                          3′-TTAGGTAGCGACCATGGC-5′      (-1 primer)
```



Lin et al, 2014 *Carcinogenesis*

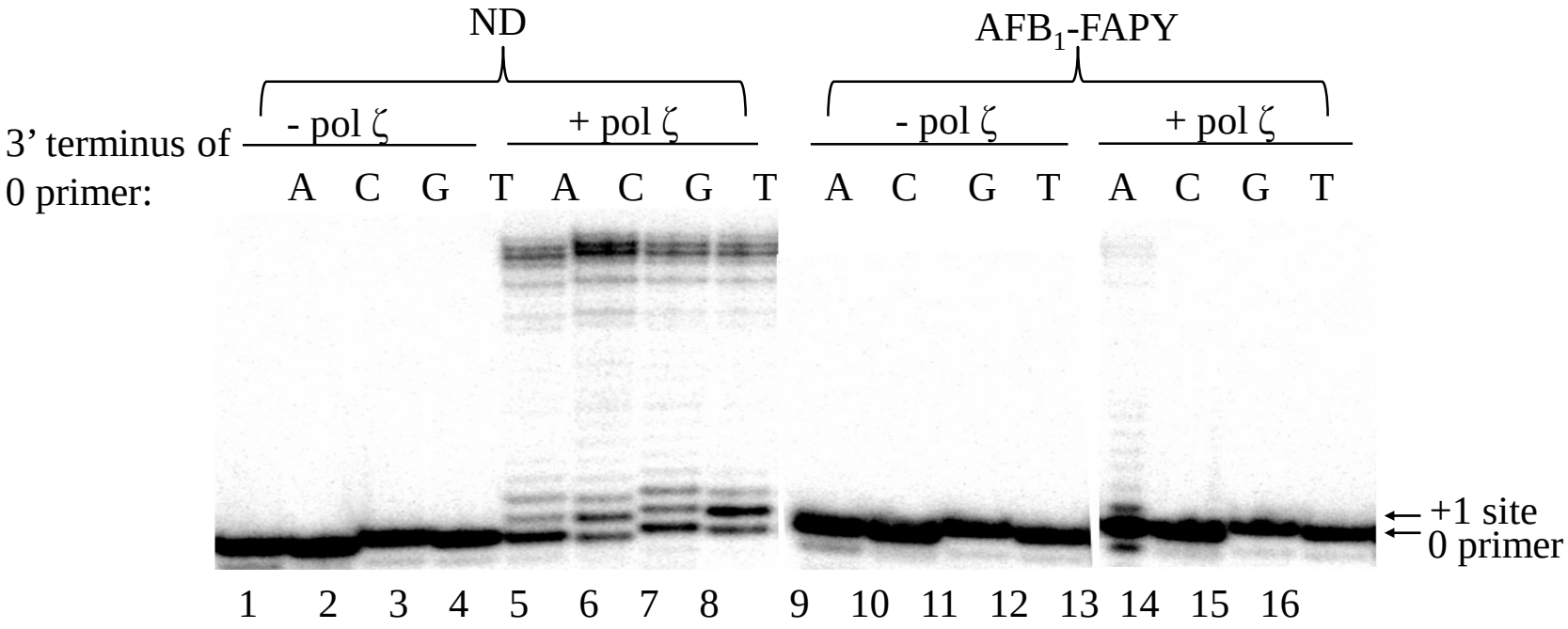
Table 1 | **Mammalian DNA polymerases**

DNA polymerase	Catalytic subunit (gene, size of protein and protein domain structure* in humans)	Function	Family‡
Pol α	*POLA1* (166 kDa)	DNA replication priming	B
Pol δ	*POLD1* (124 kDa)	DNA replication, NER and MMR	B
Pol ε	*POLE* (262 kDa)	DNA replication, NER and MMR	B
Pol γ	*POLG* (140 kDa)	Mitochondrial DNA replication and repair	A
Pol β	*POLB* (38 kDa)	BER and meiotic recombination	X
Pol λ	*POLL* (63 kDa)	V(D)J recombination; possibly end joining and BER	X
Pol μ	*POLM* (55 kDa)	V(D)J recombination; possibly end joining	X
TDT	*DNTT* (58 kDa)	Immunoglobulin diversity at junctions of coding regions	X
Pol ζ	*REV3L* (353 kDa)	TLS and mutagenesis	B
REV1	*REV1* (138 kDa)	TLS and mutagenesis, anchor for several DNA polymerases	Y
Pol η	*POLH* (78 kDa)	Bypass of UV radiation-induced DNA adducts, especially CPDs	Y
Pol ι	*POLI* (80 kDa)	Backup enzyme for bypass of UV radiation-induced DNA adducts and BER	Y
Pol κ	*POLK* (99 kDa)	Bypass of bulky adducts, backup enzyme for NER	Y
Pol θ	*POLQ* (290 kDa)	Defence against ionizing radiation-induced DNA damage	A
Pol ν	*POLN* (100 kDa)	ICL repair or testis-specific function?	A

Lange et al, 2011

Pol ζ-mediated bypass of AFB_1-FAPY-dG

```
5′-ATTATGCAGCGATAGAATAATTGAATCCATCGCTGGTACCGACTCG-3′
                          3′-TTAGGTAGCGACCATGGC-5′(-1 primer)
                         3′-NTTAGGTAGCGACCATGG-5′ (0 primer)
```



Lin et al, 2014 *Carcinogenesis*

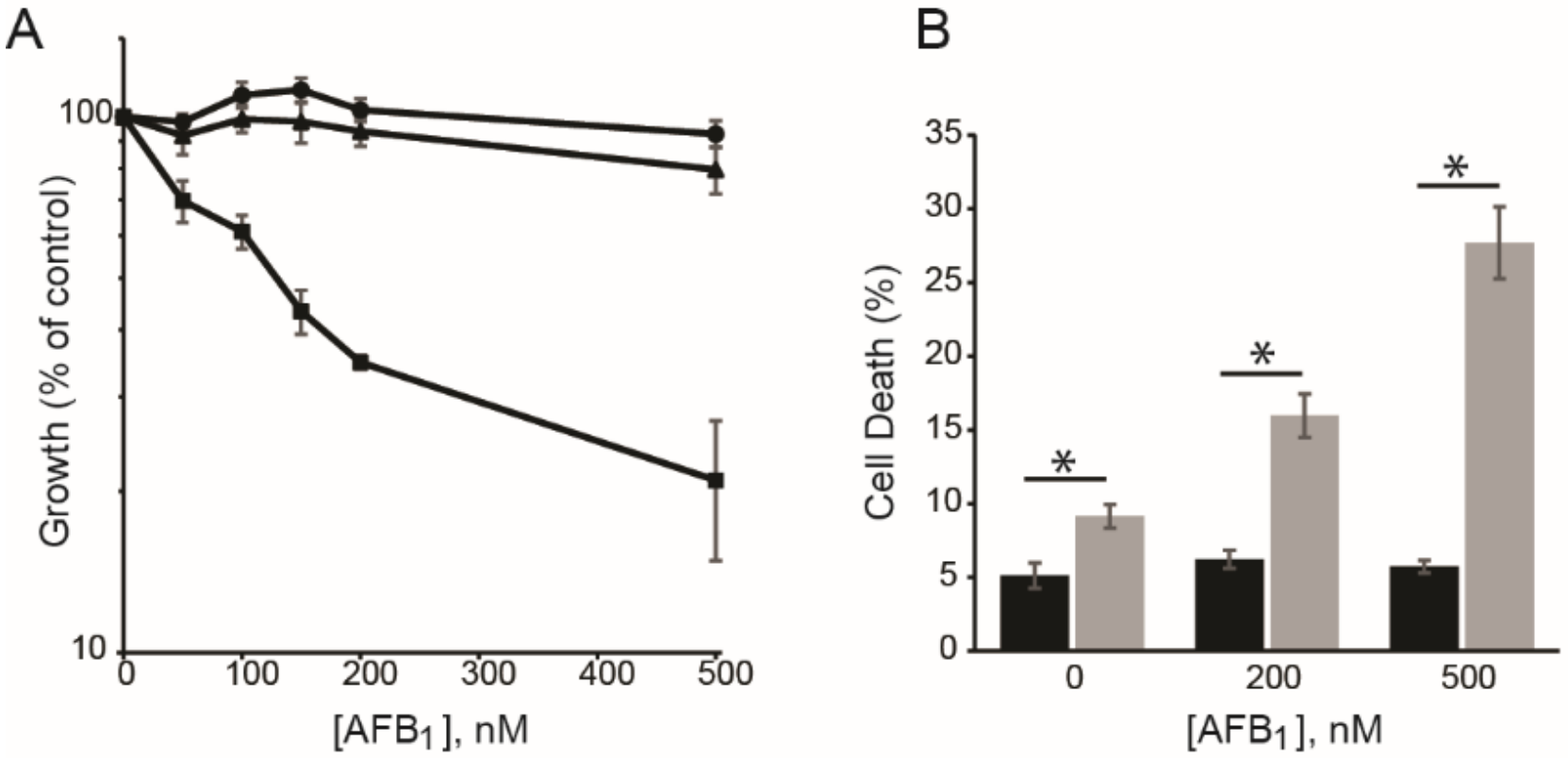
Biological consequences of polymerase ζ-deficiency following aflatoxin exposure

- No other TLS polymerase was able to efficiently bypass AFB_1-Fapy-dG adducts; although polymerase κ very low efficiency and fidelity

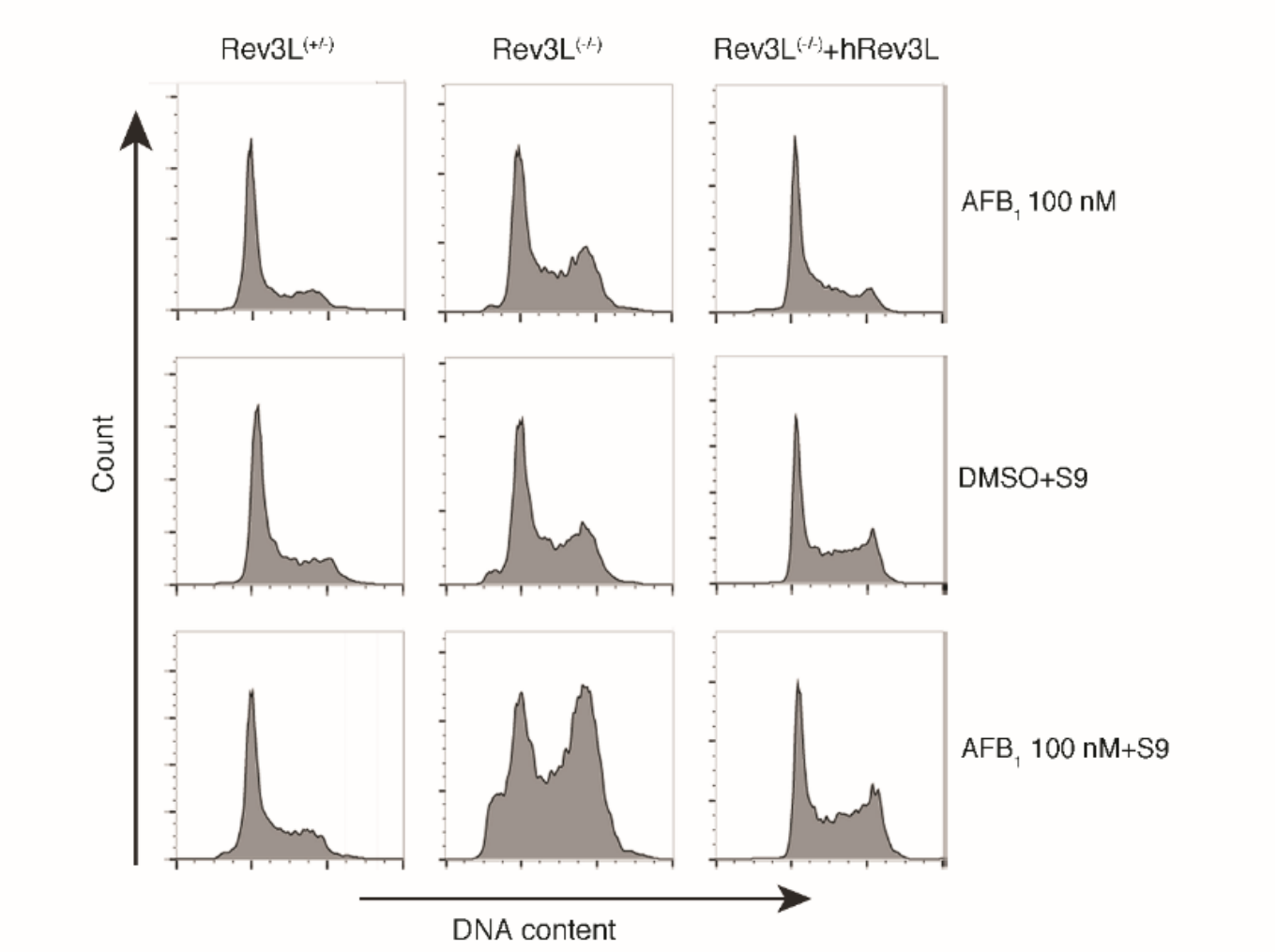
These data suggested that if polymerase ζ was primarily responsible for TLS, then polymerase ζ-deficient cells, would manifest a strong biological phenotype following aflatoxin exposure

Initiated collaboration with Dr. Rick Wood, MD Anderson Cancer Institute, Smithville to obtain polymerase ζ-deficient cells (*Rev3L*$^{-/-}$)

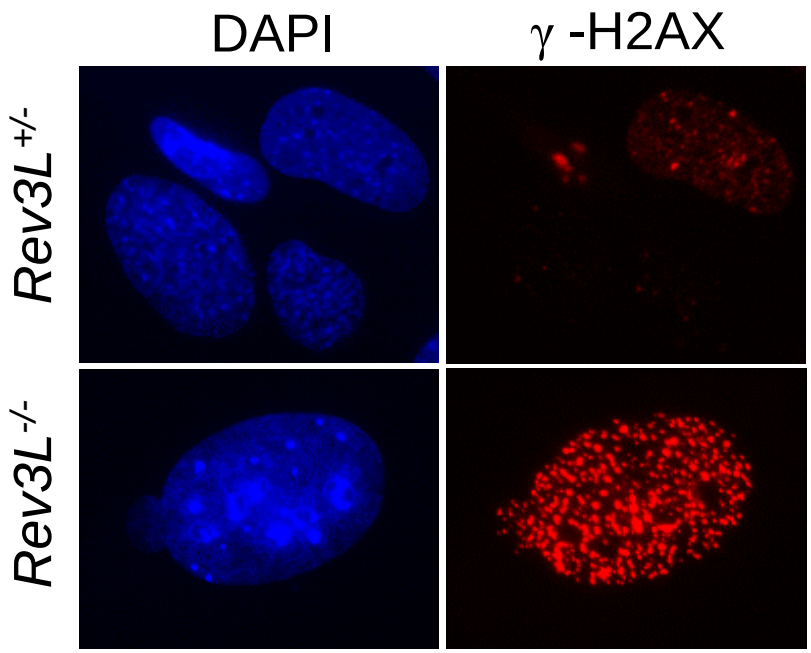
Polymerase ζ-deficient cells are sensitive to the cytotoxic effects of aflatoxin

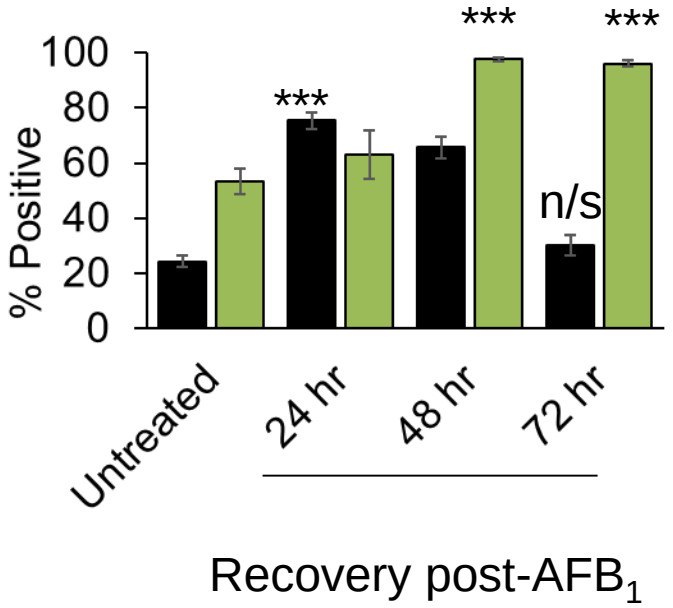


Polymerase ζ-deficient cells arrest in G2 following aflatoxin exposures

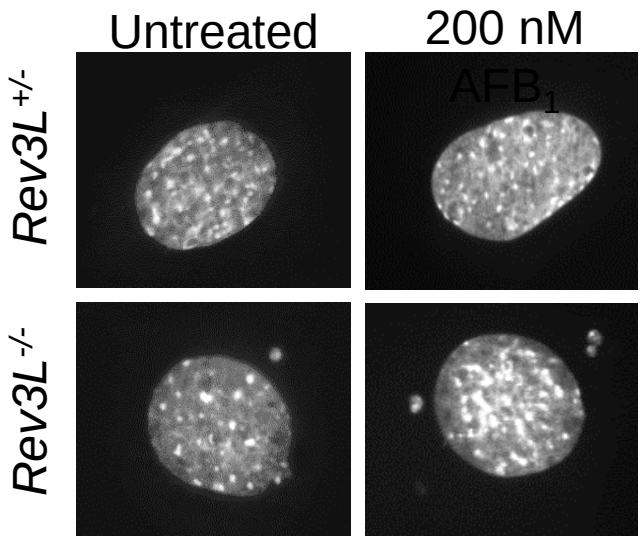


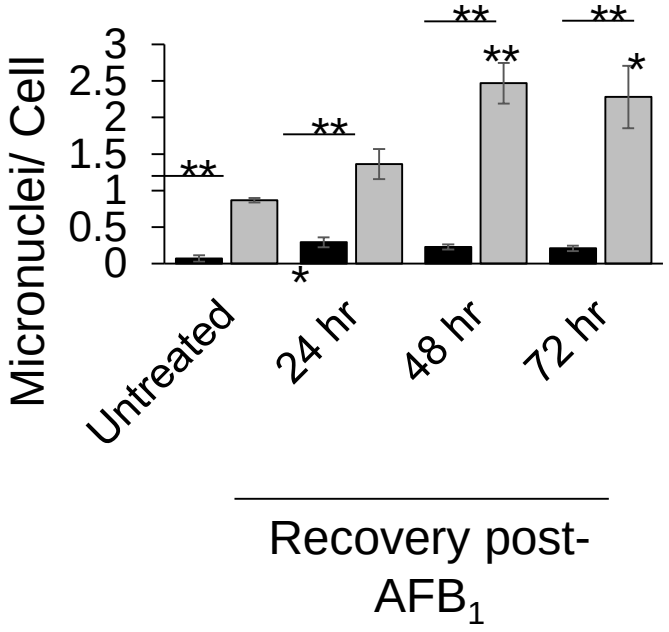
In polymerase ζ-deficient cells, aflatoxin adducts manifest as double-stranded breaks: γH2AX foci



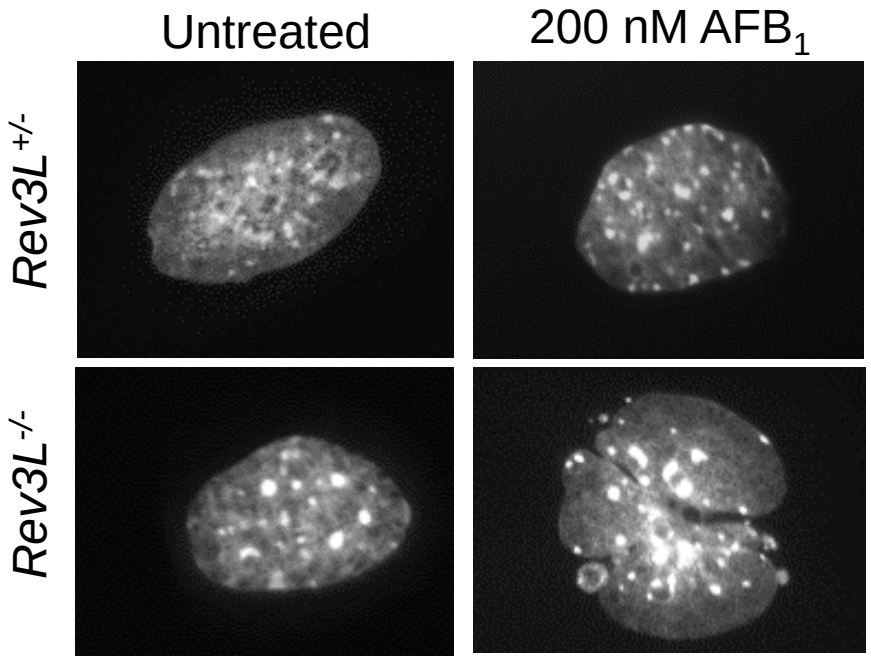


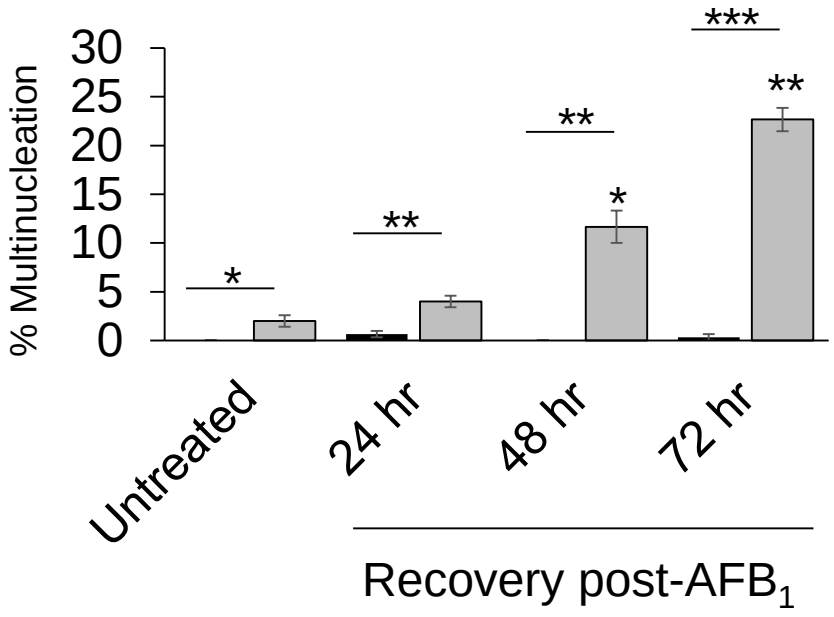
In polymerase ζ-deficient cells, aflatoxin adducts manifest by an increase in micronuclei



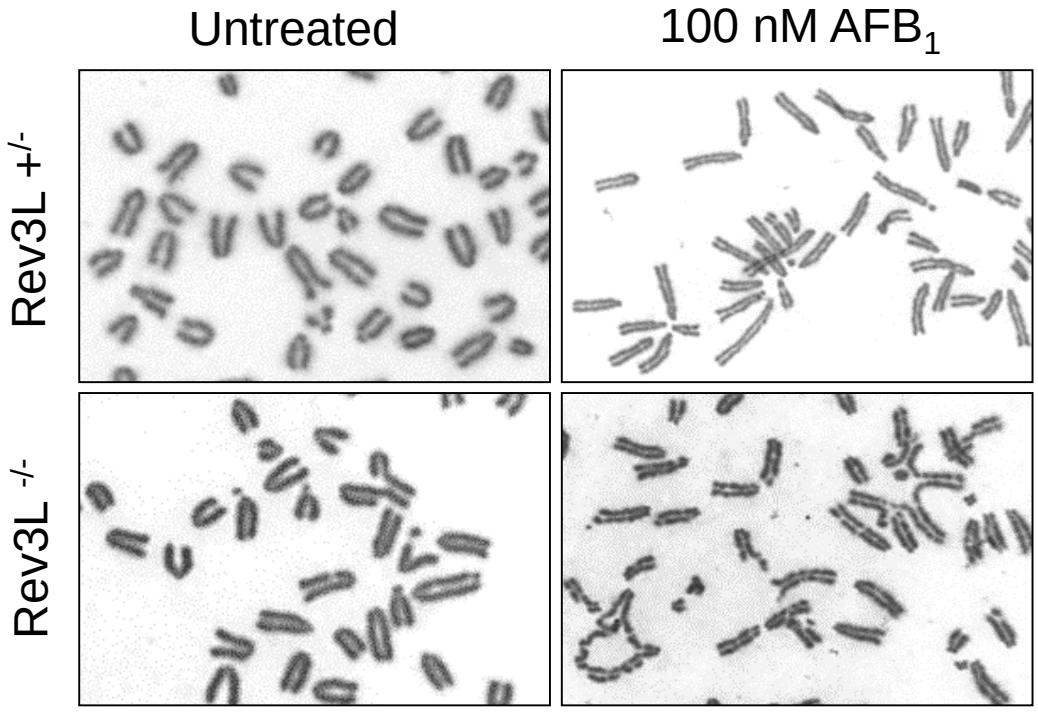


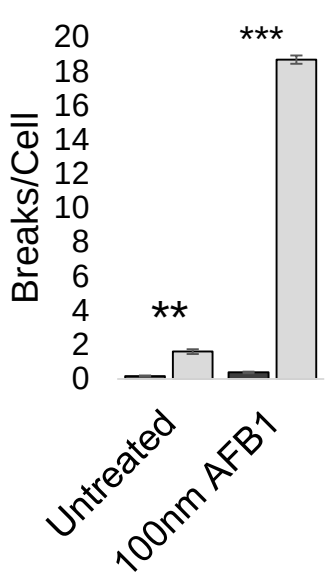
In polymerase ζ-deficient cells, aflatoxin adducts manifest as an increase in multinucleated cells

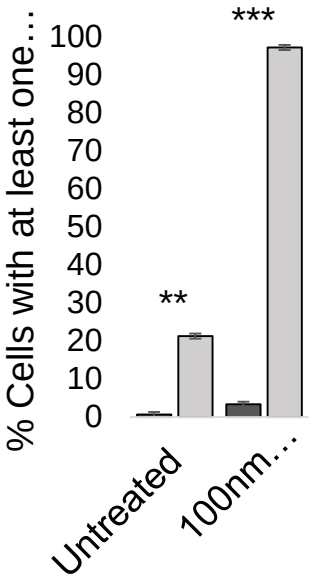




In polymerase ζ-deficient cells, aflatoxin adducts manifest as double-stranded breaks: chromosome breaks & radials

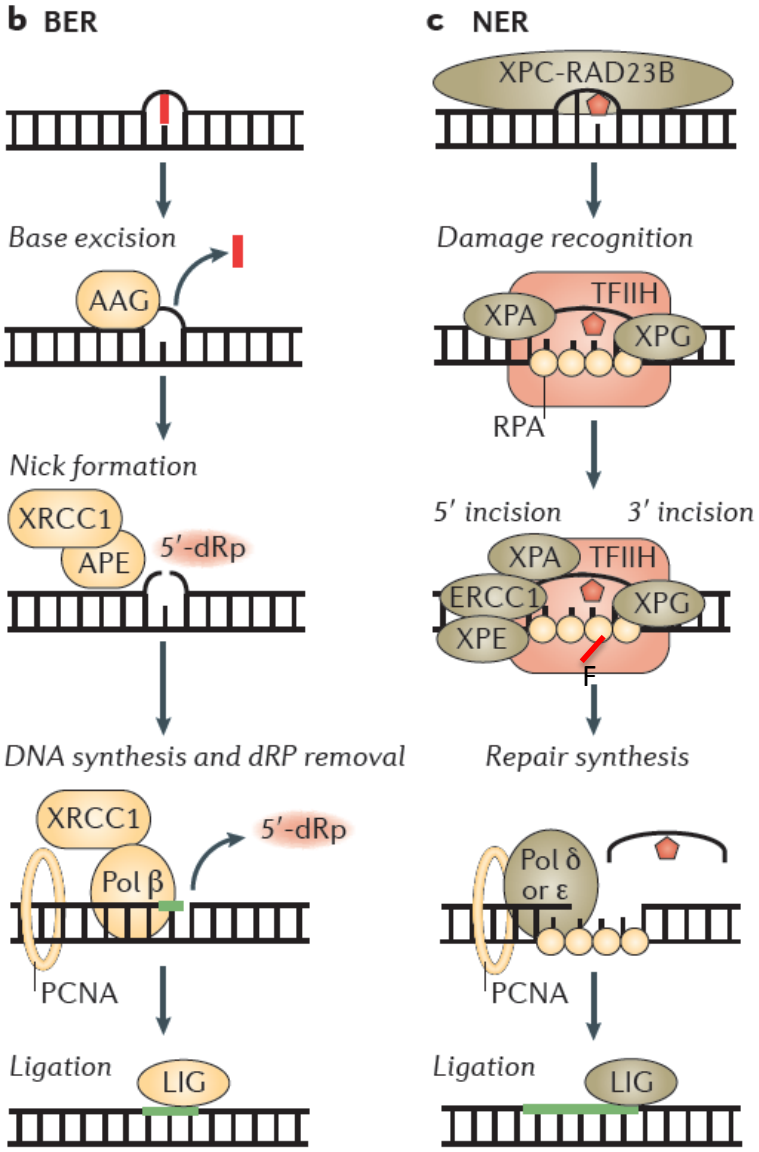






DNA Repair Pathways for Aflatoxin Adducts: Base vs. nucleotide excision repair

E. coli FPG: conflicting data



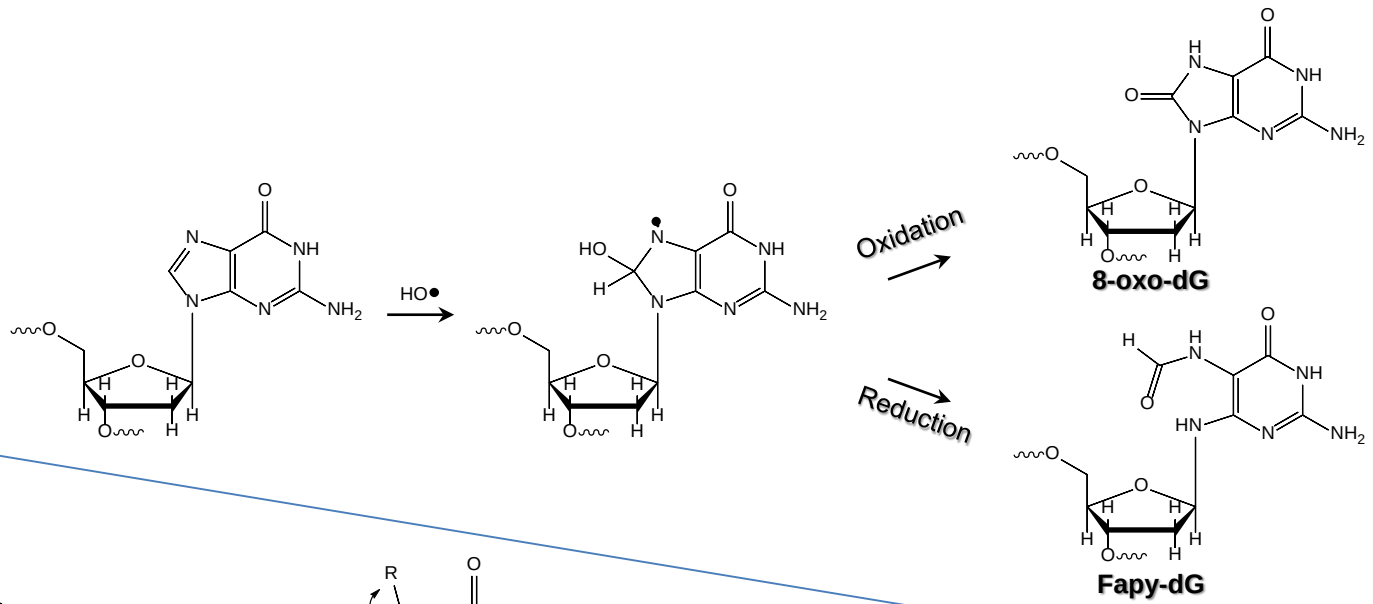
E. coli
UVR ABC excision nuclease – Excellent removal

Human and monkey
Efficient removal of subset of adducts

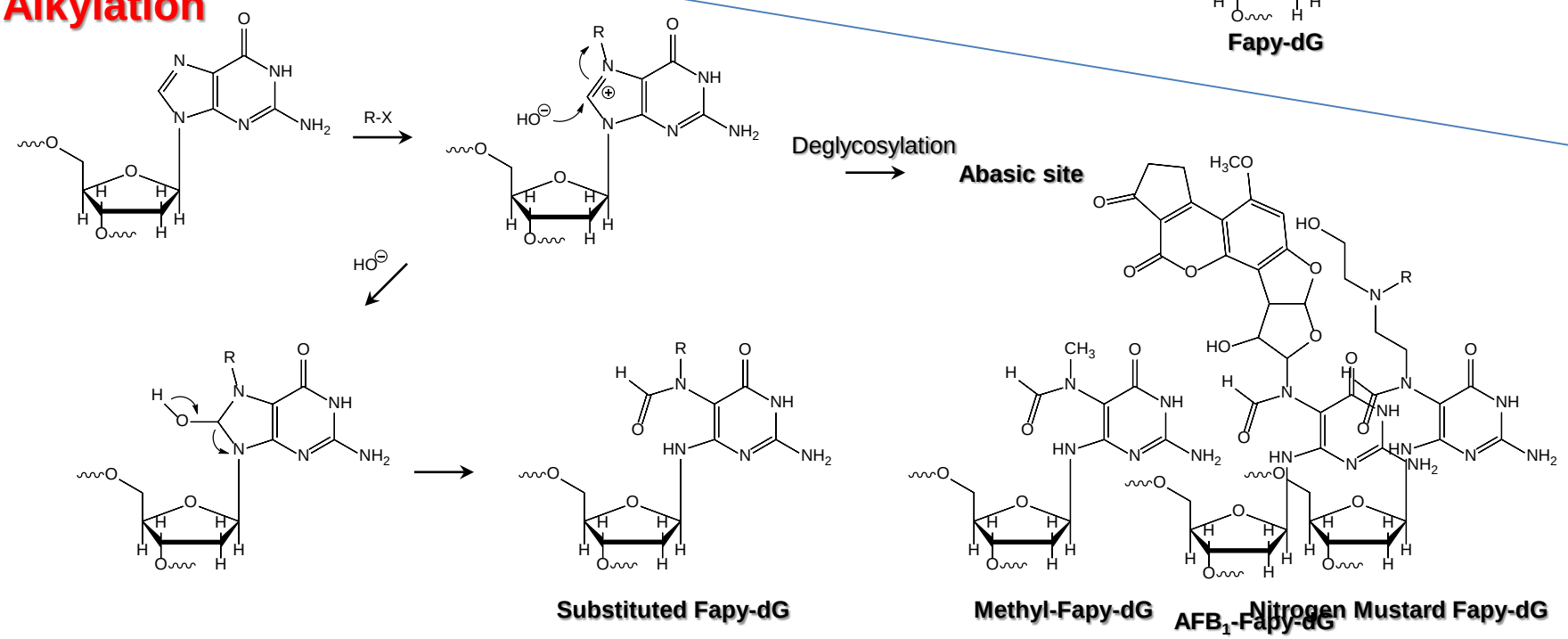
Fu, et al 2012

Common

Oxidation



Alkylation

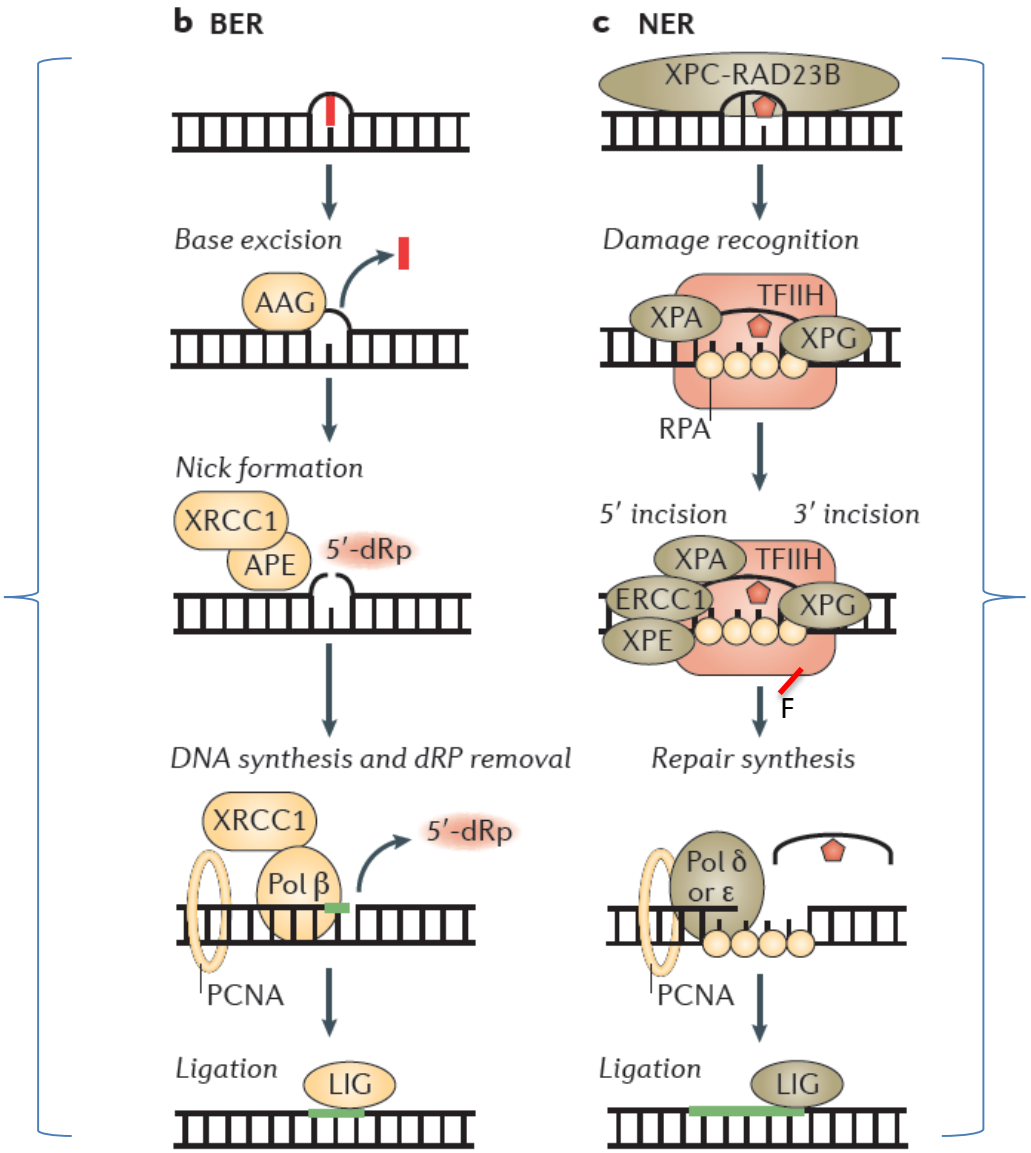


Greenberg, M. M. *Acc. Chem. Res.* **2012**, *45*, 588-597

Gates, K.S.; Nooner, T; Dutta, S. *Chem. Res. Toxicol.* **2004**, *17*, 839-856

DNA Repair Pathways for Aflatoxin Adducts:
Base vs. nucleotide excision repair

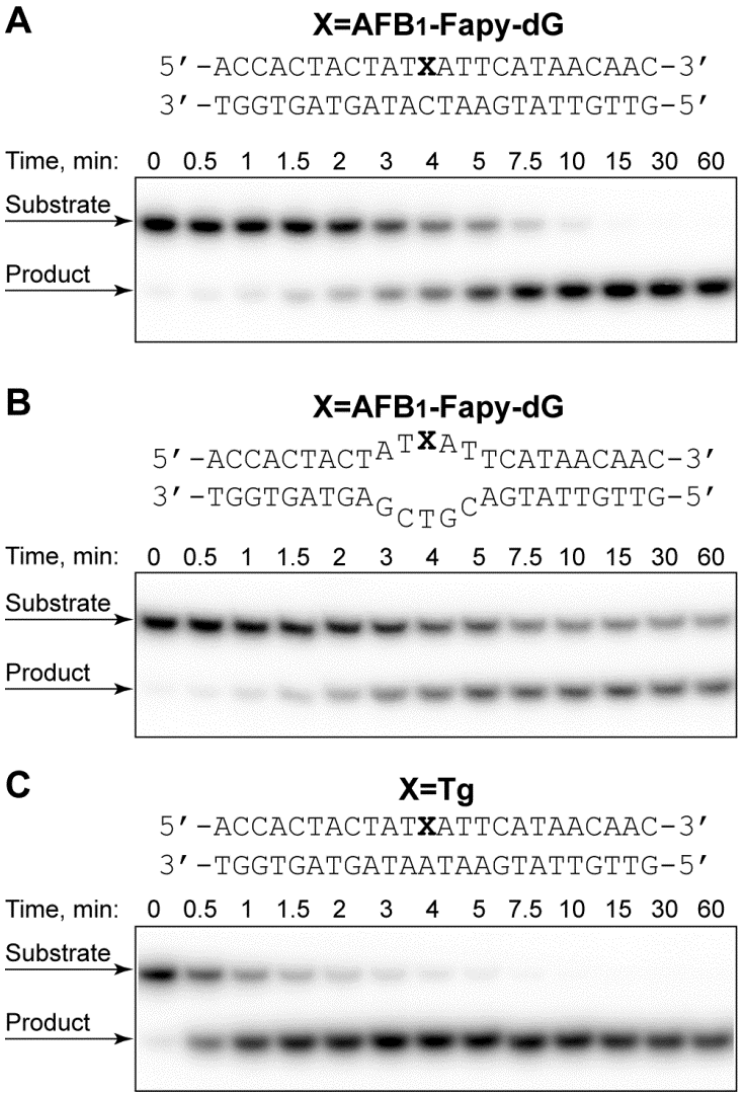
BER assumed not functional for aflatoxin adduct repair

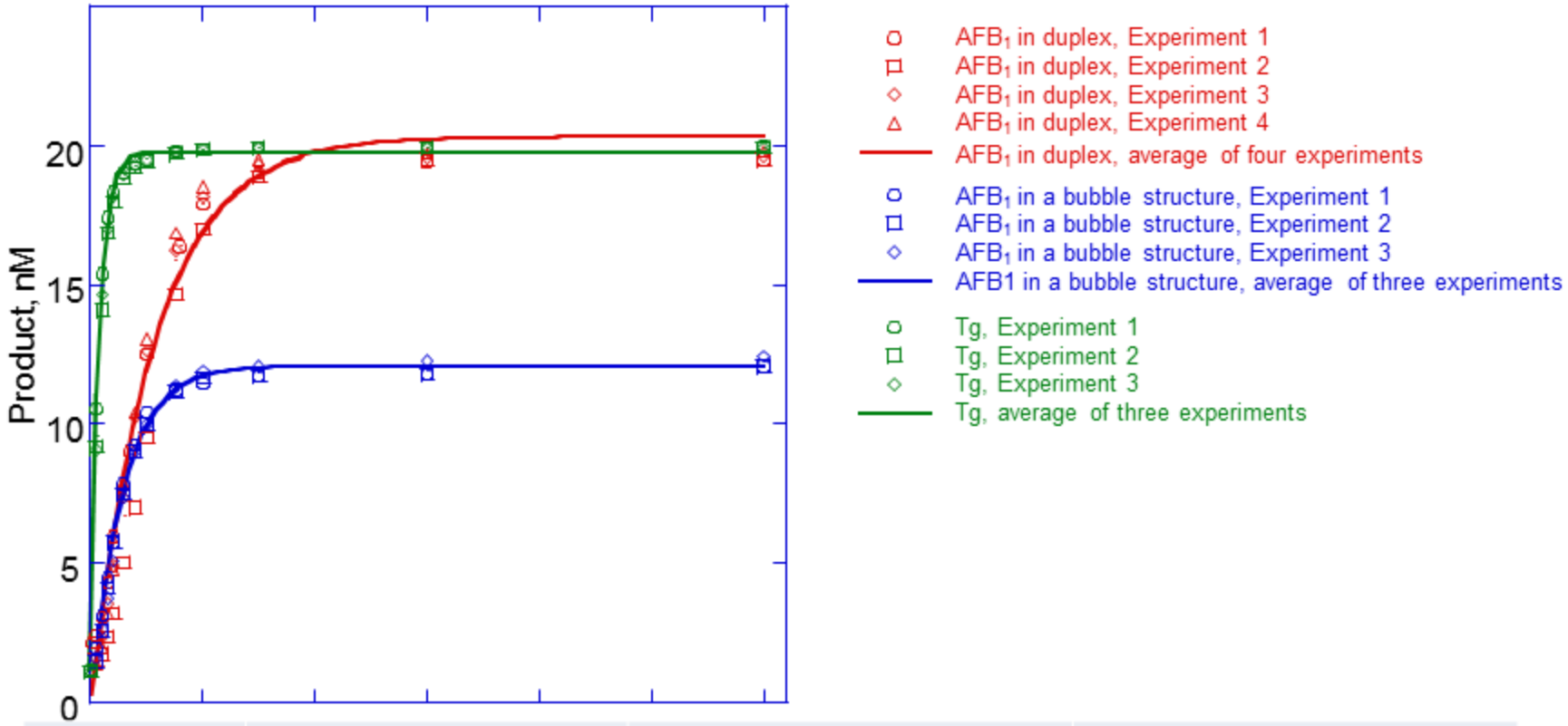


NER capable of removing a some aflatoxin adducts

Fu, et al 2012

NEIL1-catalyzed incision of DNAs containing an AFB_1-Fapy-dG





	AFB_1 in duplex		AFB_1 in a bubble structure		Thymine glycol	
	k_{obs}	Extrapolated maximal product	k_{obs}	Extrapolated maximal product	k_{obs}	Extrapolated maximal product
Experiment 1	0.194	19.9	0.349	11.9	1.497	19.7
Experiment 2	0.131	20.5	0.319	12.1	1.247	19.7
Experiment 3	0.178	20.4	0.288	12.5	1.291	19.7
Experiment 4	0.181	20.6				
AVE	0.171	20.4	0.319	12.2	1.345	19.7
SD	0.028	0.3	0.031	0.3	0.133	0

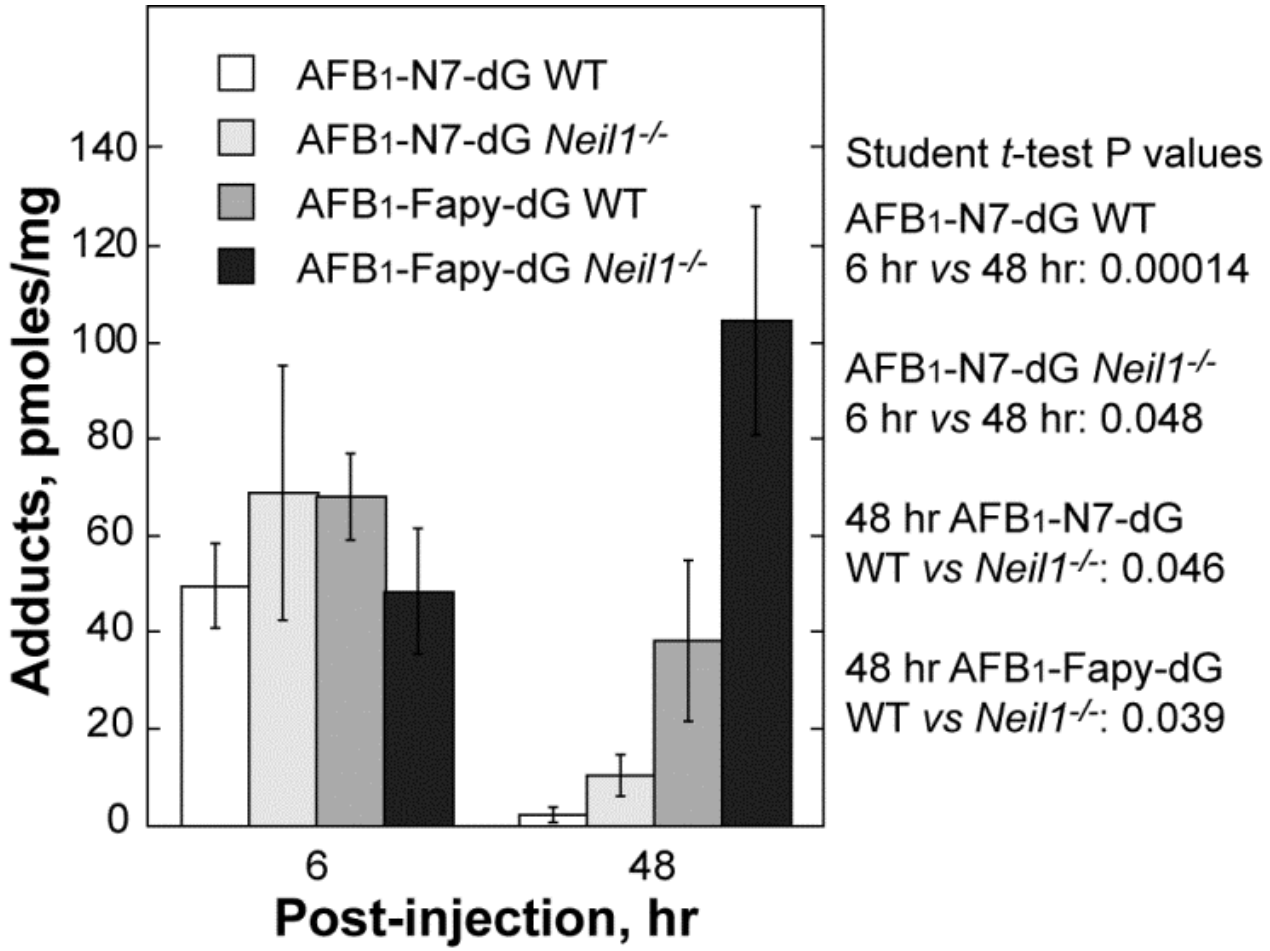
Is NEIL1-initiated base excision repair active in AFB_1 adduct repair *in vivo*?

- Collaboration with Drs. John Essigmann, MIT and Dr. John Groopman, Johns Hopkins U.

Hypothesis: if NEIL1-initiated BER is a significant contributor to the repair of AFB_1-Fapy-dG adducts (in addition to NER), then the persistence of these adducts should be greater in *Neil1*$^{-/-}$ mice vs WT mice

Experimental design: expose newborn (<6 day-old) WT and *Neil1*$^{-/-}$ mice to i.p. 3.5 mg/kg AFB_1 in DMSO & DMSO control; harvest livers at 6 and 48 hr post injection, harvest DNA, analyze for AFB_1 adducts by mass spectrometry

Formation and persistence of AFB_1 DNA adducts in WT and *Neil1*$^{-/-}$ mice: effect of deficient base excision repair



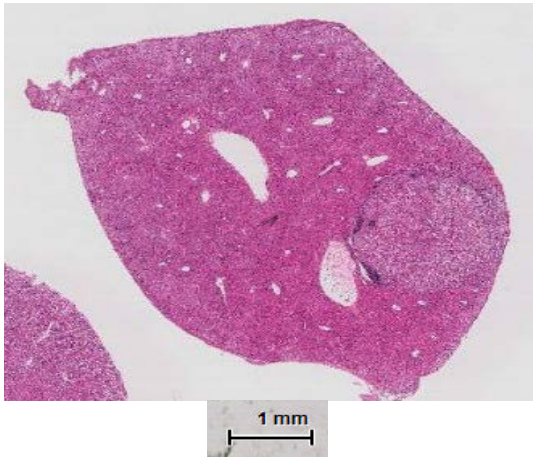
Is there a role for NEIL1 in limiting aflatoxin-induced carcinogenesis?

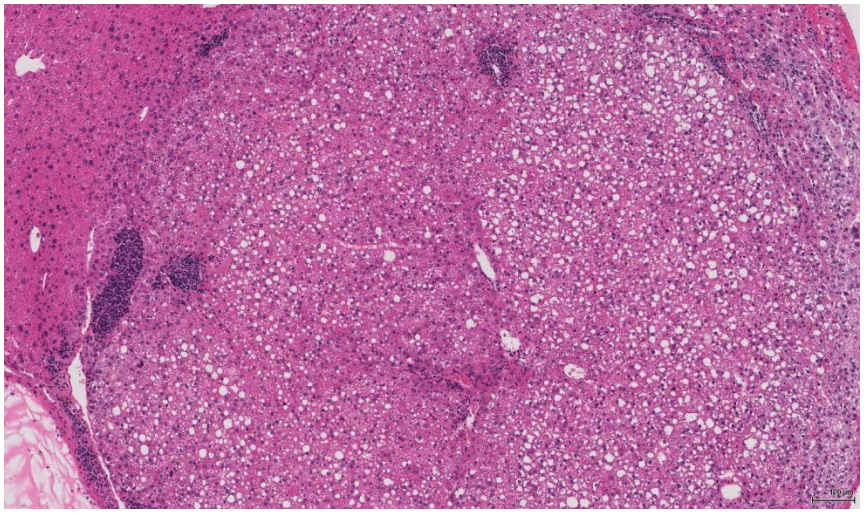
- Given that AFB_1 Fapy adducts are good substrates for NEIL1 incision
- Given that AFB1-Fapy-dG adducts differentially accumulate in *Neil1*$^{-/-}$ mice

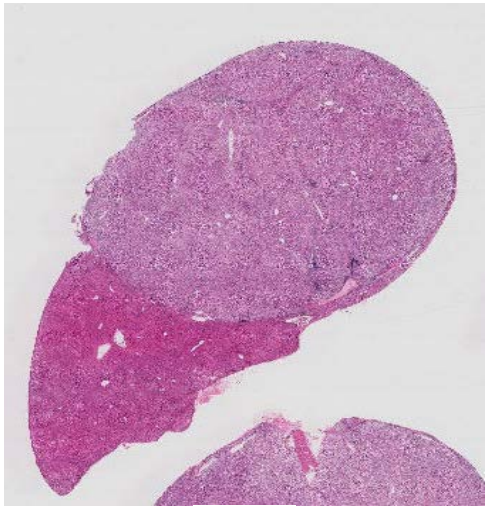
Hypothesis: *Neil1*$^{-/-}$ mice would be more susceptible to AFB_1 carcinogenesis vs WT

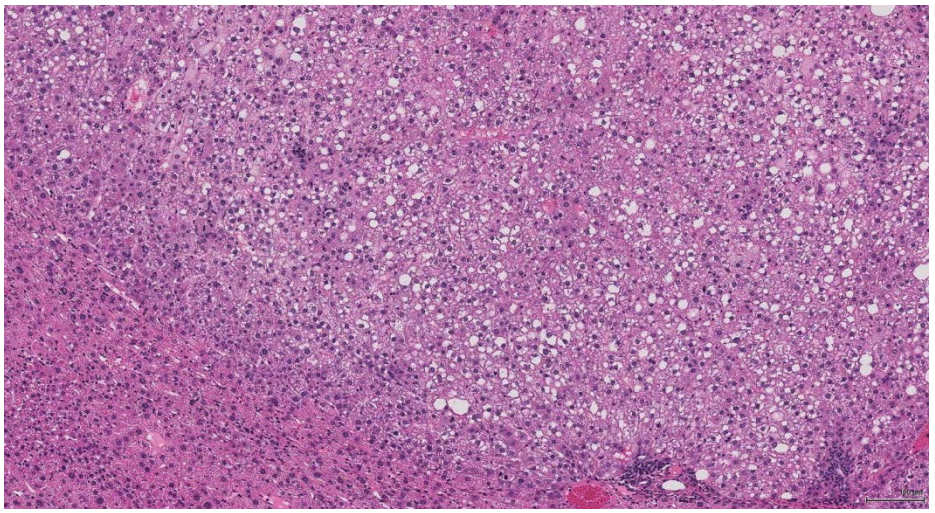
Experimental Design: <6 day old C57Bl6/J ± *Neil1* (~40 mice per group) given a single IP injection of DMSO, 1.0 or 7.5 mg/kg AFB_1; followed for ~15 months

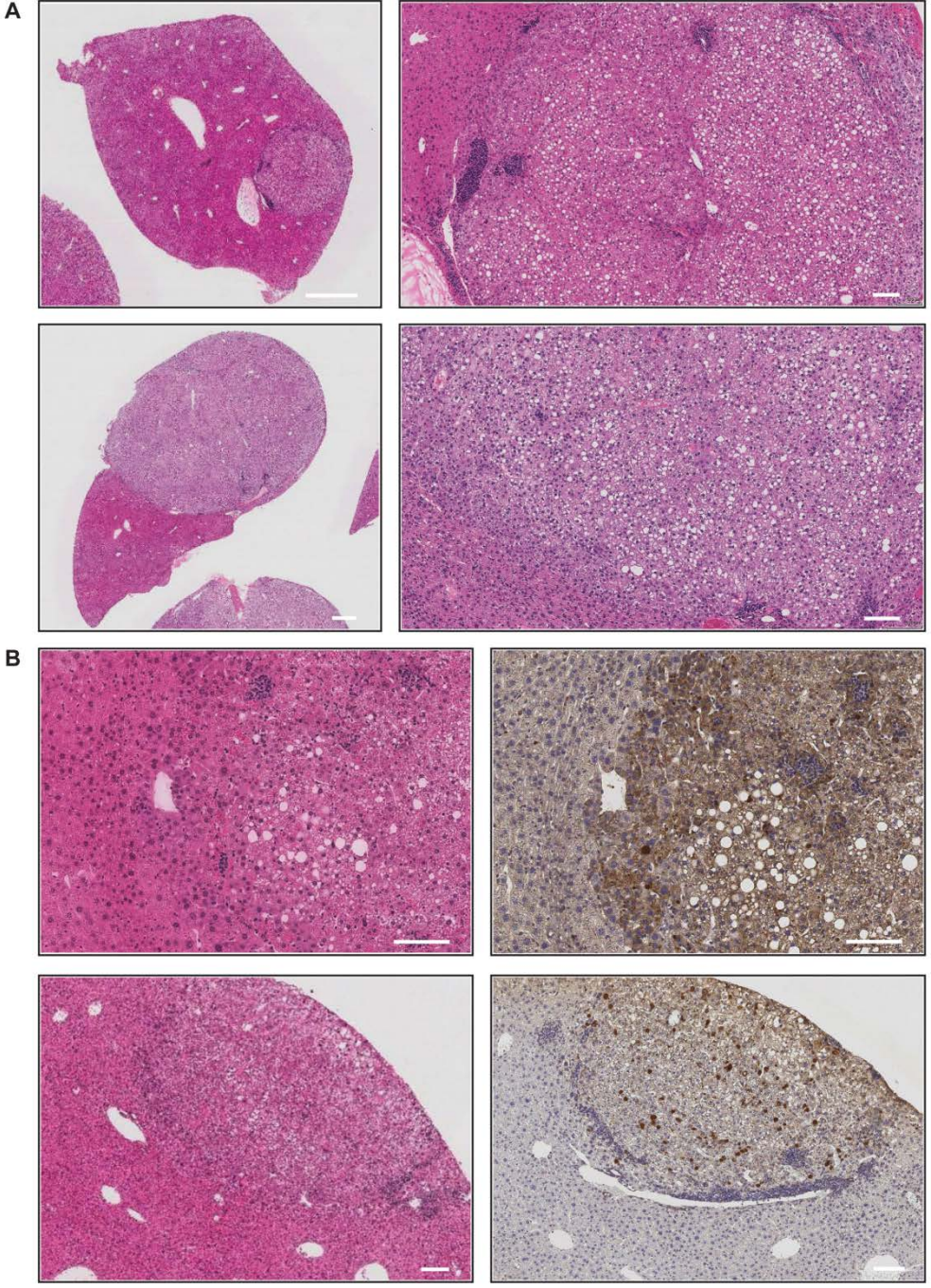
Histopathology of *Neil1*$^{-/-}$ Liver Tumors



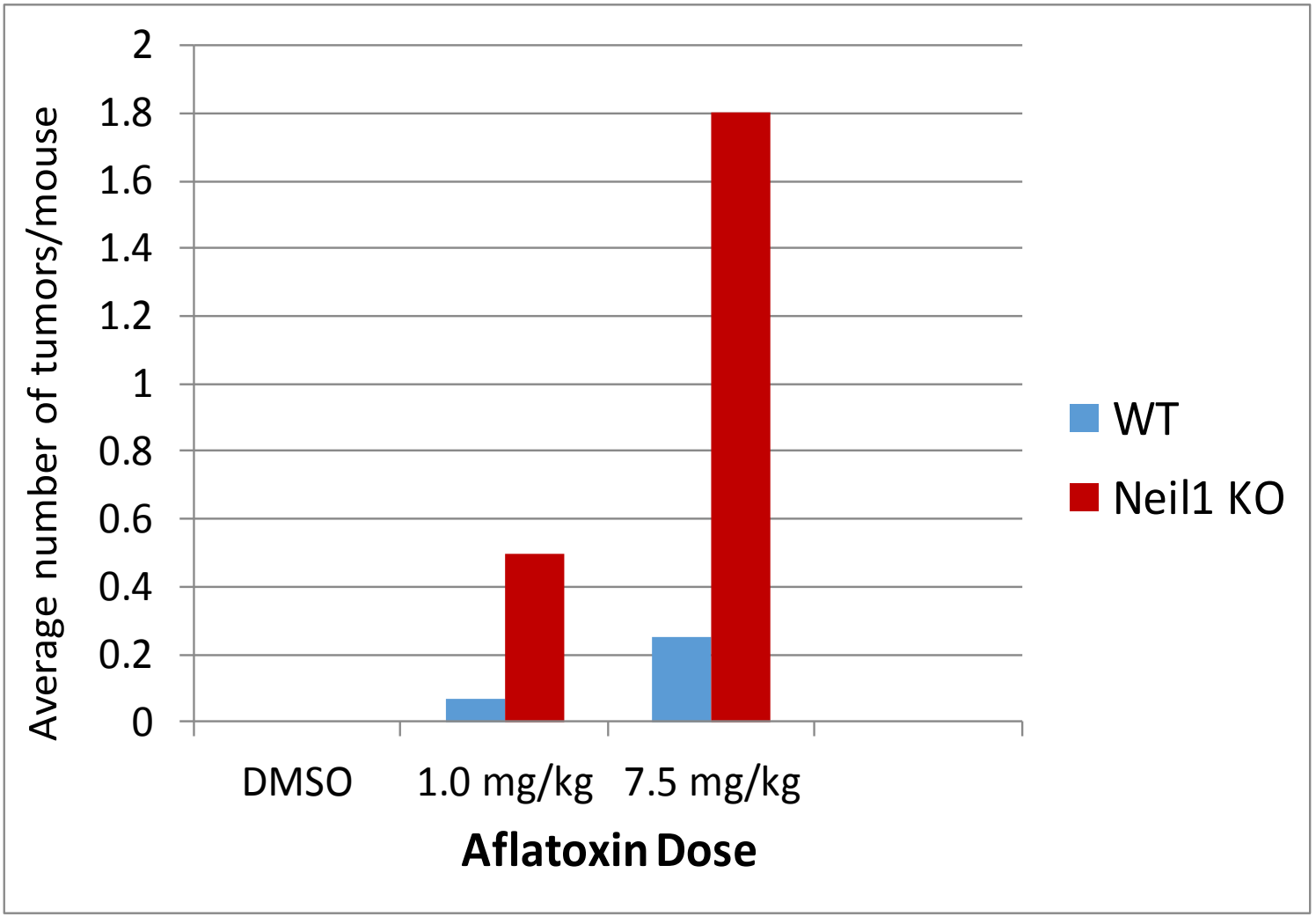




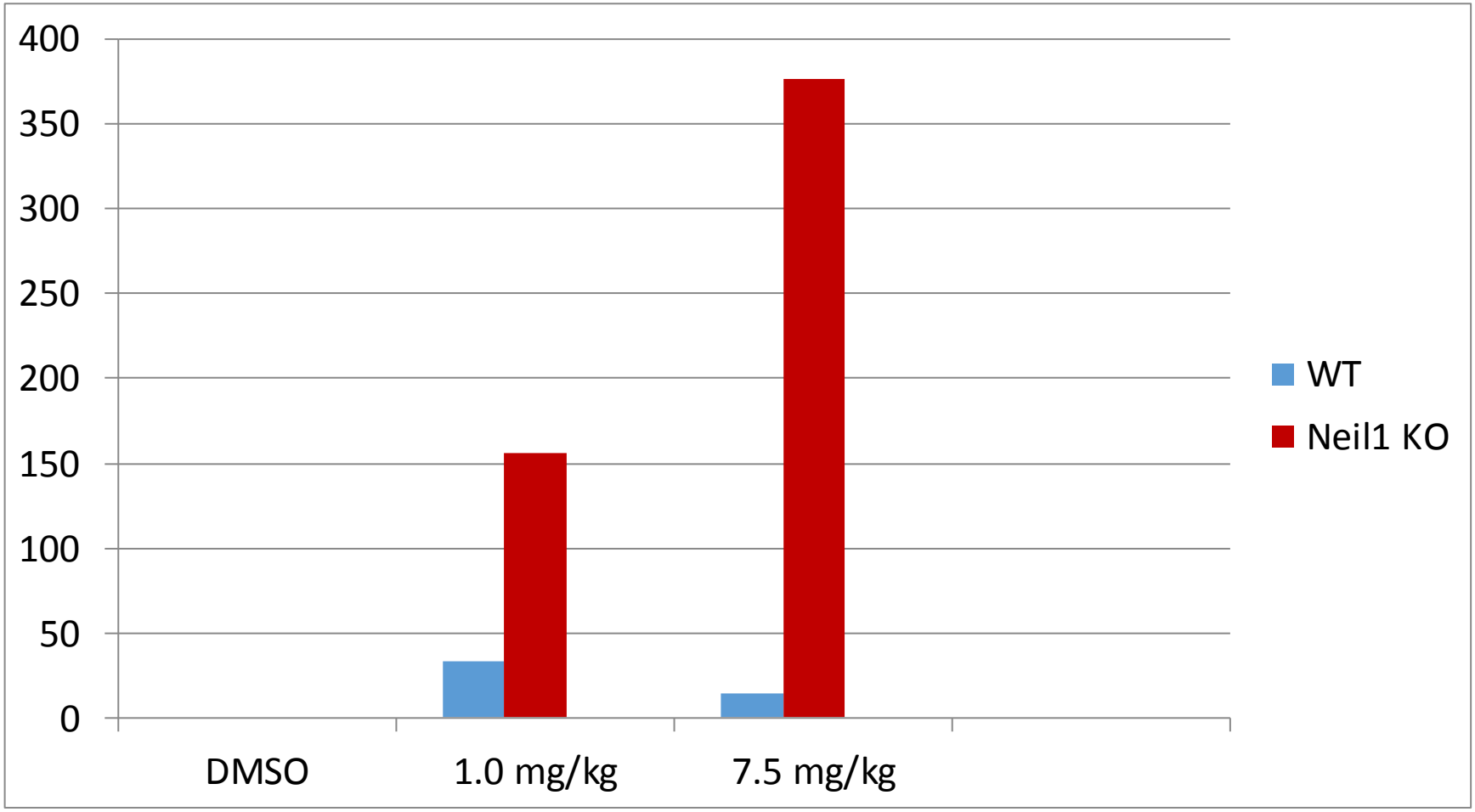



A
B

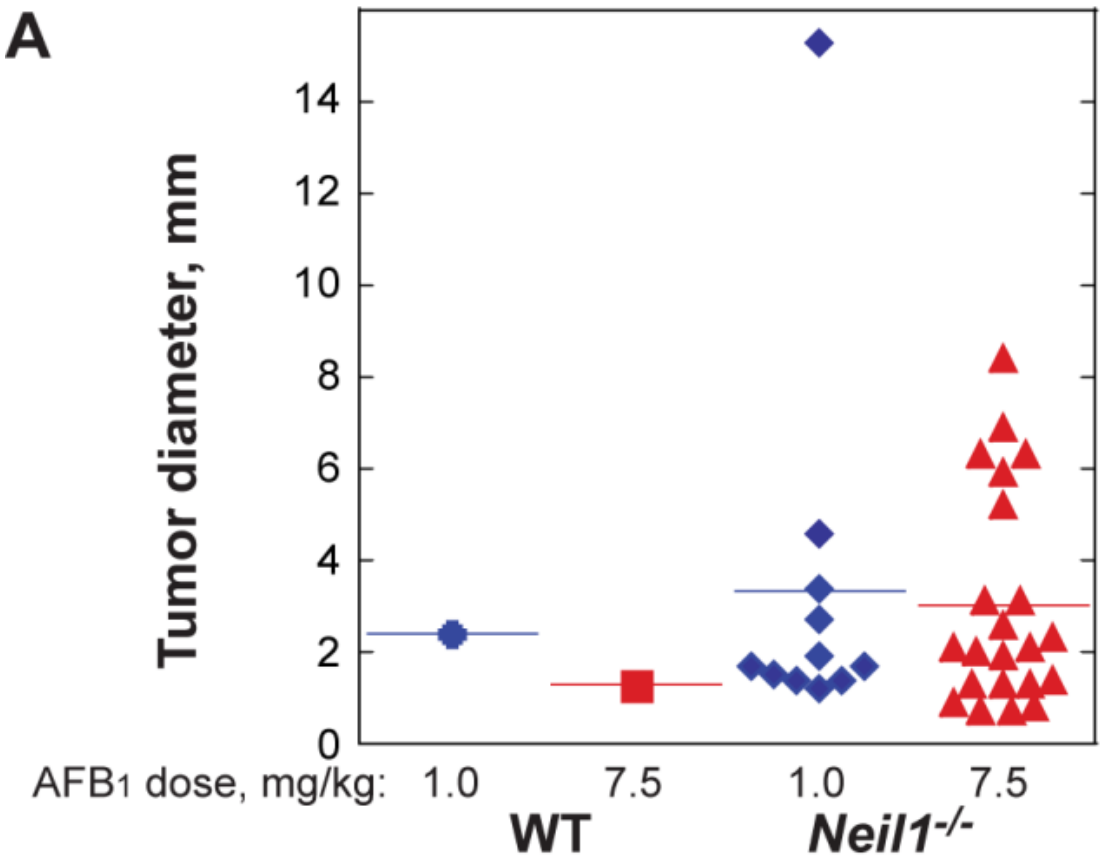
Average Number of Tumors/Mouse



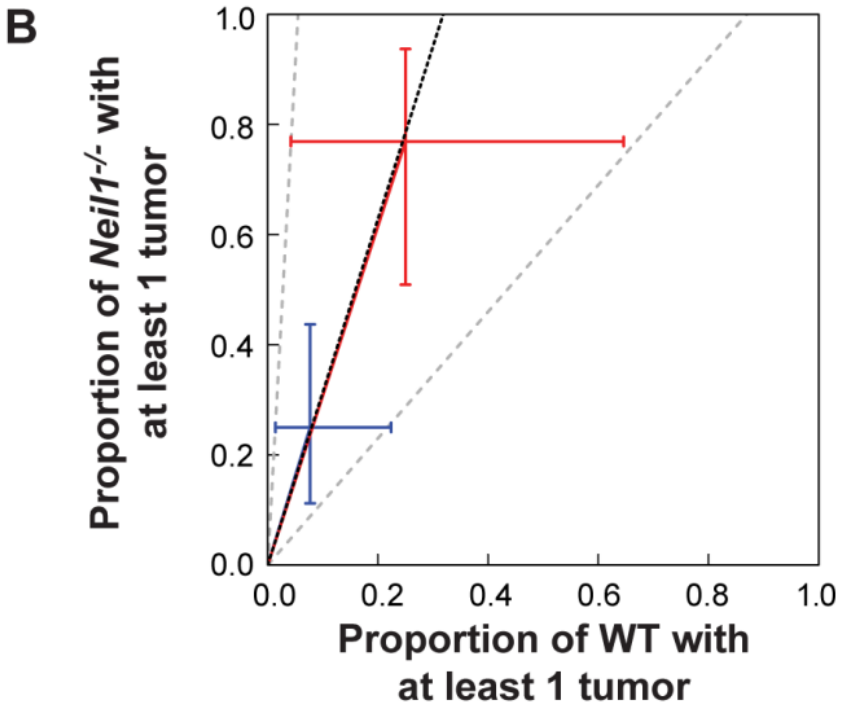
Average tumor size mm^3/mouse



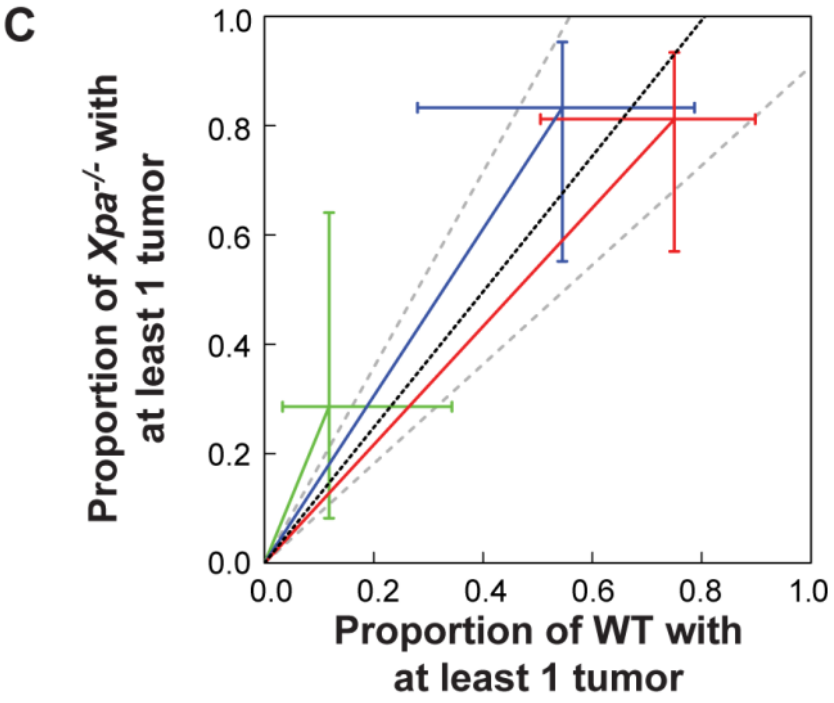
Individual tumor data: *Neil1*$^{-/-}$ vs WT



Relative importance of BER- vs NER-initiated repair of AFB_1 adducts in HCCs



Risk increase from *Neil1* deficiency:
3.4



Risk increase from XPA deficiency:
1.2

Human Health Implications

- Mouse carcinogenesis data suggest that deficiencies in NEIL1 lead to increased susceptibility to aflatoxin-induced liver cancers
- At least 2 of the 4 known human polymorphic variants of *NEIL1* produce glycosylase-deficient enzymes
- Data suggest a potential increased disease susceptibility for individuals carrying inactivating SNPs

Human polymorphic variants of *NEIL1*

Residue #	Frequency*	Activity
S82C	1.1 %	Wild type
G83D	1.1 %	No glycosylase
R136C	1.1 %	No glycosylase
I182M	0.5%	Reduced glycosylase
D252N	2.4%	Wild type

** NCBI SNP database*

Relevance to human health

Could polymorphic variants in *NEIL1* within the human population in China, SE Asia, Africa affect genetic susceptibility to the development of early onset HCCs arising from aflatoxin exposure?

Propose that DNA sequencing of the NEIL1 gene from DNAs isolated from tumors of early onset HCC could be a key to susceptibility

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