SUPPORTING INFORMATION

The DNA repair enzyme MUTYH potentiates cytotoxicity of the alkylating agent MNNG by interacting with abasic sites

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Running Title: *MUTYH potentiates MNNG cytotoxicity via abasic sites*

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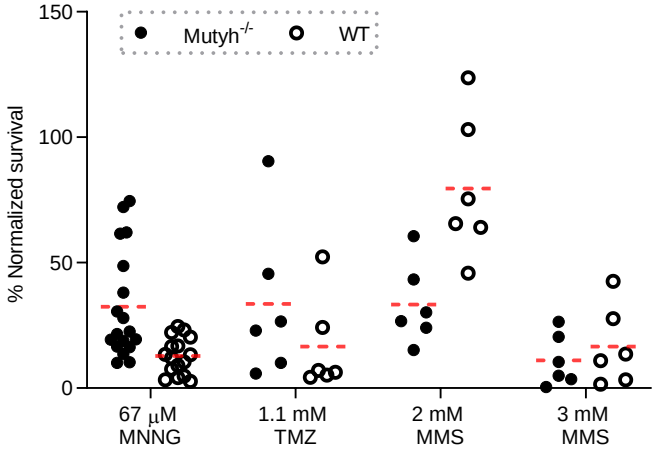


Figure S1. Cytotoxicity of MNNG, MMS and TMZ in *Mutyh*-/- versus WT MEFs. *Mutyh*-/- MEFs are more sensitive to 2 mM MMS (p=0.006) but less sensitive to MNNG versus WT MEFs (p=0.007). Survival to 3 mM MMS and 1.1 mM TMZ was not significantly different. Summary of 7 day survival to alkylating agents from parallel assays, normalized to untreated cells.

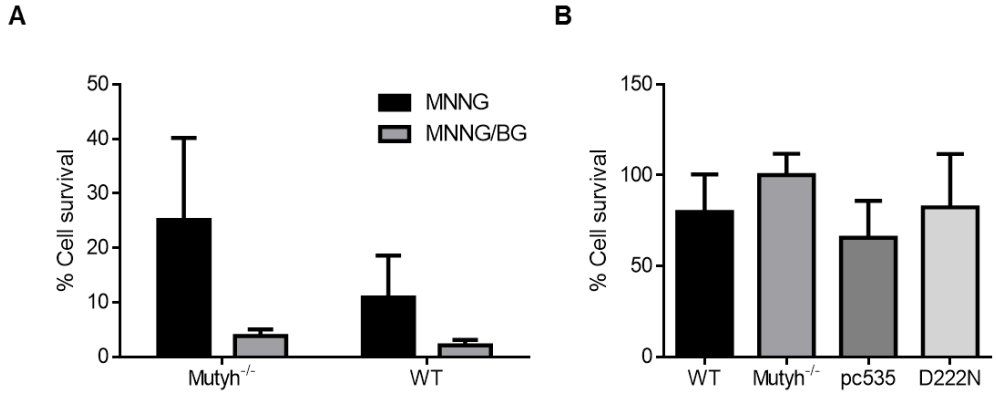


Figure S2. Effect of MGMT inhibitor O^6-benzylguanine (BG) pre-treatment on survival of MNNG treated MEFs. (A) Effect of 150 µM O^6-benzylguanine (BG) pre-treatment on survival to 33 µM MNNG in wild-type versus *Mutyh*$^{-/-}$ MEFs. Summary from five separate experiments, normalized to untreated cells. Two-way ANOVA interaction (genotype x BG treatment) was not significant (p=0.12). (B) Relative survival of MEFs treated with 20 µM O^6-benzylguanine and 33 µM MNNG, normalized to survival with MNNG alone. Differences in survival between WT *Mutyh*$^{-/-}$ MEFs (p=0.45) were not significant; Differences in survival between stably-transfected *Mutyh*$^{-/-}$ MEFs expressing wild-type (pc535) and catalytically-inactive (D222N) MUTYH isoforms (p=0.66) were also not significant. Error bars represent the SD calculated from at least five biological replicates.

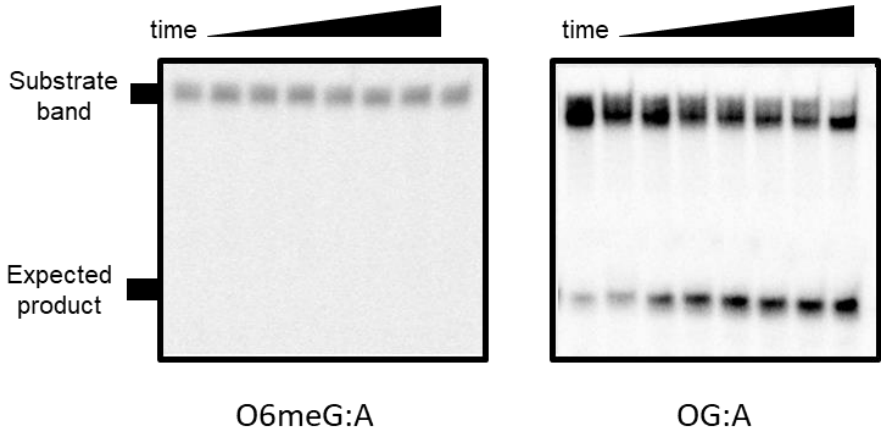


Figure S3. Mutyh glycosylase assay on O^{6}MeG:A versus OG:A-containing duplexes. In vitro glycosylase assay with mouse Mutyh acting on a 30-bp dsDNA duplex containing a centrally located O^{6}meG:A mismatch under single turnover conditions and a separate OG:A glycosylase assay using the same enzyme preparation. Storage phosphor autoradiogram of two separate PAGE gels with O^{6}meG:A (20 nM DNA, 100 nM Mutyh) and OG:A (20 nM DNA, 10 nM Mutyh). Lanes 1-8 represent time points at 20 seconds, 40 seconds, and 1, 3, 5, 10, 20, 40 minutes. Reactions were conducted at 37 °C for 30 min and quenched by the addition of NaOH to a final concentration of 0.2 M. Mixtures were heated to 90 °C for 5 min then analyzed by denaturing PAGE. Representative of three separate trials.

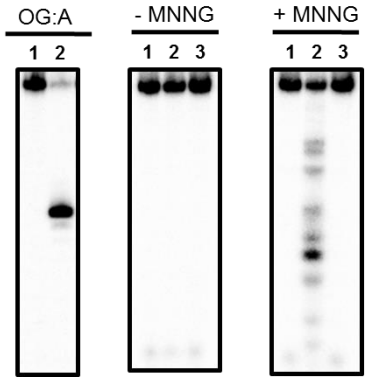
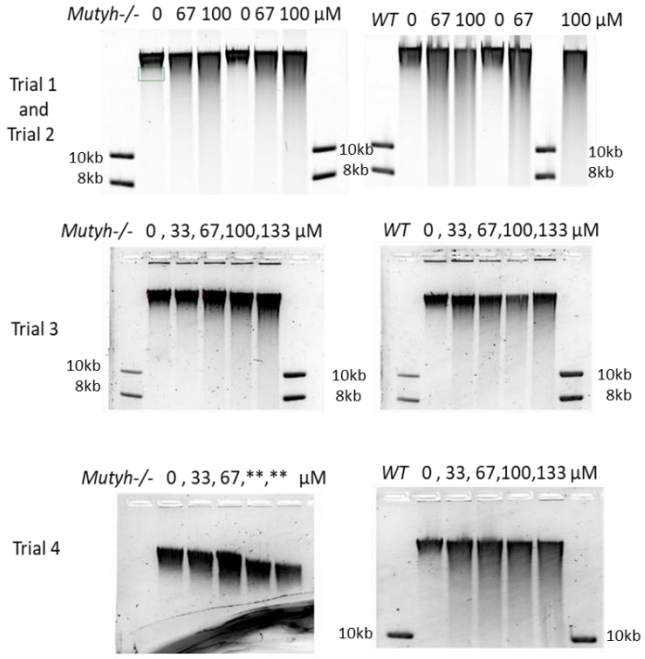


Figure S4. Glycosylase assay on MNNG-treated DNA duplexes. In vitro glycosylase assays with AAG and Mutyh acting on MNNG-treated 30-bp dsDNA. Storage phosphor autoradiograms of PAGE gel under single turnover conditions (20 nM DNA, 100 nM Mutyh) for Mutyh acting on OG:A (left gel) where 1) no enzyme, negative control, and 2) with Mutyh. For the middle and right gel, 1) No enzyme control, 2) reaction with AAG, and 3) reaction with Mutyh. Reactions were conducted at 37 °C for 30 min and quenched by the addition of NaOH to a final concentration of 0.2 M. Mixtures were heated to 90 °C for 5 min then analyzed by denaturing PAGE. Representative of three separate trials.

Figure S5



(Trial 5 shown in main text)

Quantification of % Tail (lower region) density from five trials

	MNNG concentration				
Mutyh-/- MEFs	0	33 uM	67 uM	100 uM	133 uM
Trial 1	15	*	36	48	*
Trial 2	9	*	36	47	*
Trial 3	16	21	28	34	35
Trial 4	20	36	41	**	**
Trial 5	11	16	18	28	35
Average	14	24	32	39	35
std dev	4.3	10.4	9.0	9.7	0.1
	MNNG concentration				
WT MEFs	0	33 uM	67 uM	100 uM	133 uM
Trial 1	26	*	55	55	*
Trial 2	24	*	52	50	*
Trial 3	18	40	46	42	46
Trial 4	17	43	54	53	52
Trial 5	10	31	39	47	48
average	19	38	49	49	47
std dev	6.4	5.9	6.6	5.4	1.6

*data not collected **gel damage prevented quantification

Figure S5. Alkaline gel electrophoresis of WT vs. *Mutyh*$^{-/-}$ MEF genomic DNA. Alkaline gel electrophoresis visualizing DNA strand breaks induced by MNNG [ref. 44]. Genomic DNA was extracted 40 minutes after the indicated MNNG treatment, quantified, incubated in an alkaline loading buffer and run on a 0.8% agarose gel overnight at pH 12.4 as detailed in the Experimental Procedures. Percent of total sample band density in the lower region ('tail') versus the upper band using ImageQuant software from 2 biological replicates (5 gels). WT cells had significantly more DNA in the lower region at 67 µM versus *Mutyh*$^{-/-}$ cells (t-test, $p < 0.01$), indicative of increased strand breaks in WT versus *Mutyh*$^{-/-}$ MEFs. Trial 4 *Mutyh*$^{-/-}$ gel was damaged in the lower region and the molecular weight markers are not visible.

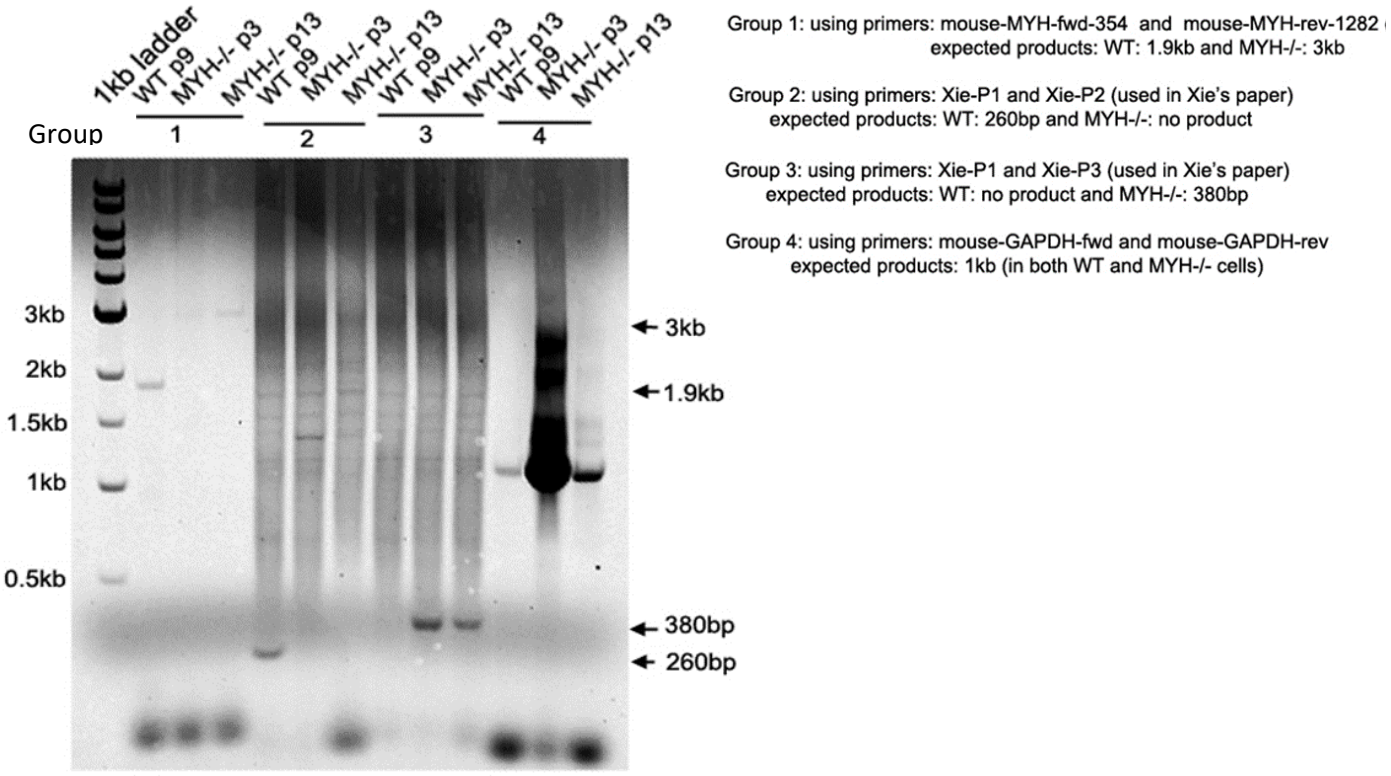


Figure S6. Validation of *Mutyh*$^{-/-}$ MEF genomic knockout by PCR. DNA from WT and *Mutyh*$^{-/-}$ MEF cell lines was extracted using DNAzol. Group 1 shows the expected WT product (1.9kb) and *Mutyh*$^{-/-}$ shows presence of an insert (~3kb) using independently designed primers flanking the putative genomic insertion site (mouse-MYH-fwd-354 5'-CTATTATACTCGATGGATGC-3' and mouse-MYH-rev-1282 5'-CCAGACTGTATACTTGATACG-3') . Group 2 and 3 lanes are validation using primers from Xie, et al. 2004 (ref 90; Xie-P1 5'-CAAGTGCTGGGATCAAAGGTG-3', Xie-P2 5'-GCTCCTTCTTGTAGCCGACG-3', and Xie-P3 5'-TCCTCGTGCTTTACGGTATCG). The products confirm the presence of the inactivating insert in *Mutyh*$^{-/-}$ MEFs and the absence in WT MEF DNA.

Table S1. Quantitation of methyl base adducts released by DNA glycosylases

Enzyme	1mA (pmol)		3mA (pmol)		7mA (pmol)		7mG (pmol)		O^6mG (pmol)	
	Absolute	Relative to HCl	Absolute	Relative to HCl	Absolute	Relative to HCl	Absolute	Relative to HCl	Absolute	Relative to HCl
(5N HCl)	0.22 ± 0.09	1.00	60 ± 6.2	1.00	6.9 ± 0.66	1.00	64 ± 18	1.00	12 ± 1.2	1.00
No enzyme	0.05 ± 0.086	0.22	11 ± 4.3	0.18	0.00	0.00	5.9 ± 1.9	0.09	9.5 ± 1.2	0.80
hAAG	0.00	0.00	27 ± 0.094	0.45	0.00	0.00	29 ± 1.6	0.45	7.3 ± 2.6	0.61
Mutyh	0.00	0.00	5.0 ± 0.98	0.08	0.16 ± 0.27	0.02	4.0 ± 0.73	0.06	3.8 ±0.37	0.31
D207N Mutyh	0.00	0.00	5.7 ± 1.8	0.10	0.00	0.00	5.0 ± 0.89	0.08	9.9 ± 7.1	0.83

Reactions were performed at 37 °C for 1 hour and contained 5 μM enzyme, 10 μg MMNG-treated calf-thymus DNA, 50 mM HEPES pH 7.5, 100 mM KCl, 10 mM DTT, 2 mM EDTA, and 0.1 mg/mL BSA.

Supplemental Materials and Methods

Reagents. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was purchased from Pfaltz & Bauer. O^6-benzylguanine (BG), O-(tetrahydro-2H-pyran-2-yl)-hydroxylamine (OTX), sodium borohydride ($NaBH_4$) and sodium cyanoborohydride ($NaCNBH_3$) were purchased from Sigma-Aldrich. pEGFP-C1 4.7 kb plasmid DNA was purchased from Clontech. Carboxy-H2DCFDA (DCF) probe was purchased from Invitrogen (catalog # C-400). 6-FAM labeled DNA oligonucleotides were purchased from Eurofins Genomics, while unlabeled DNA oligonucleotides were purchased from Integrated DNA Technologies. Methyl base standards were purchased from various vendors: 7-methyladenine (7mA, Sigma-Aldrich), 1-methyladenine (1mA, Acros Organics), 3-methyladenine (3mA, Sigma-Aldrich), O^6-methylguanine (O^6mG, Sigma-Aldrich), and 7-methylguanine (7mG, Santa Cruz Biotech). Calf thymus deoxyribonucleic acid, sodium salt was purchased from Millipore Sigma.

Glycosylase Assay. Glycosylase cleavage assays were performed as described previously [ref. 30] using the DNA substrate duplexes described in main text Methods, where **Y** = A, **X** = T or OG, and the Y-containing strand was ^{32}P-5'-end labeled. Mutyh enzymes were purified as described in the main text Methods and human AAG was commercially available (New England Biolabs). The reaction buffer contained 20 mM Tris-HCl pH 7.6, 10 mM EDTA, 100 μg/mL bovine serum albumin, and 30 mM NaCl. The AAG reaction buffer contained 20 mM Tris-HCl, 0.1% Triton® X-100, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 mM KCl, pH 8.8. Reactions were conducted at 37 °C for 30 min or one hour and quenched by the addition of NaOH to a final concentration of 0.2 M, and mixtures were heated to 90 °C for 5 min then analyzed by denaturing PAGE as described above.