Thiopurine-mediated impairment of hematopoietic stem and leukemia cells in *Nudt15*^{R138C} knock-in mice

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Supplementary material and methods

Cells

Ba/F3 cells were maintained by culture in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 20% conditioned medium from the WEHI-3 cell line and incubation in 5% CO2 at 37 °C. WEHI cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% penicillin, 2 mM L-glutamine, and 5×10^{-5} M β-mercaptoethanol. Cells were maintained in our laboratory under condition without contamination with mycoplasma after validating the immunophenotype by fluorescence-activated cell sorting. To generate the conditioned medium, WEHI-cultured medium were centrifuged at 2500 rpm for 15 minutes twice and the supernatant was passed through 0.45 μm PVDF filters. Lenti-X 293T cells were purchased from Takara (Kusatsu, Shiga, Japan) and cultured in DMEM supplemented with 10% FBS.

Vectors and lentiviral transduction

The entire coding sequence of *Nudt15* was amplified from cDNA of BA/F3 cells and cloned to the pCAD lentiviral vector, which is equipped with IRES-GFP[1], to generate the pCAD-Nudt15^{wt} vector. The pCAD-Nudt15^{R138C} vector was generated by PCR from the pCAD-

Nudt15^{wt} vector using the point mutated primers, GGCTCTCTGCTGTCTAAAAGAGCAAG and CAGAAAAGCTGGTCTAAGGGAGGGAA. Lentiviral supernatant was generated in Lenti-X 293T cells as described previously passed through 0.45 μm PVDF filters and concentrated 100 times by ultracentrifugation for 2 hours at 25,000 rpm, 4 °C, using a SW32Ti rotor (Beckman, Brea, CA, USA). Ba/F3 cells were cocultured with pCAD-Nudt15^{wt} or pCAD-Nudt15^{R138C} containing supernatant supplemented with 10% FBS, 20% conditioned medium from the WEHI-3 cell line and 4 μg/mL polybrene. After incubation for 24 hours and extensive wash, transduced BA/F3 cells were cultured in viral-free culture medium for more than 8 days. GFP-positive cells were sorted by FACS Aria (BD Biosciences, San Jose, CA, USA) and used for in-vitro assay.

Quantitative PCR

Total RNA was extracted using Sepasol reagent (Nacalai Tesque, Kyoto, Japan). Complementary RNA was synthesized by PrimeScript^{TM} II reverse transcriptase (Takara). Real-time PCR was performed using LightCycler480 System II (Roche, Basel, Switzerland) and a THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan). The primer sequences are

provided in Supplementary Table 1.

Western blotting

Total tissue lysates were extracted in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P40, 1 mM EDTA, 1 mM sodium vanadate, 50 mM sodium fluoride, 17 mM sodium pyrophosphate, 1 mM PMSF, 1% glycerol and protease inhibitor cocktail (Complete Mini; Roche)]. We used anti-Nudt15 (ab122958; Abcam, Cambridge, UK) and anti-Actin (sc-47778; Santa Cruz Biotechnology, Dallas, TX, USA) as primary antibodies. HRP-conjugated anti-rabbit (NA934v; GE Healthcare, Little Chalfont, UK) or anti-mouse (NA931v; GE Healthcare) were used as secondary antibodies. Protein signals were visualized with ImageQuant LAS 4000mini (GE Healthcare) and the quantification was performed with the ImageQuant TL software (GE Healthcare).

Nudt15 enzyme activity analyses

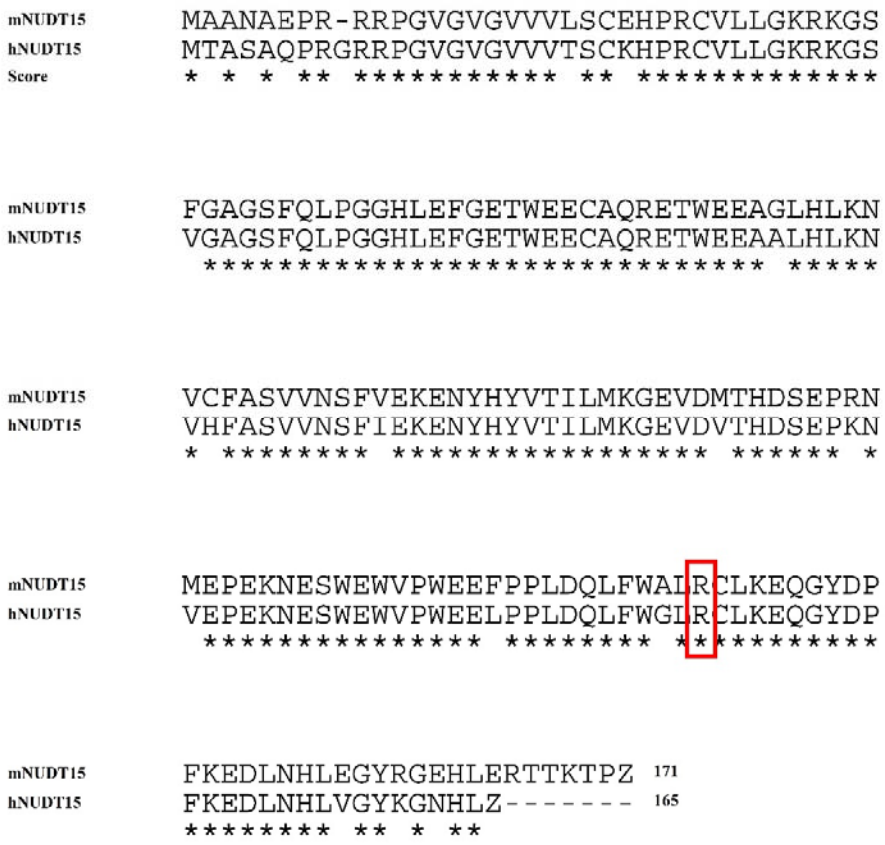
Blood samples from mice were collected using tubes containing ethylenediaminetetraacetic acid (EDTA). After hematocrit measurement, 100 µl of red blood cells (RBCs) were separated from the plasma and buffy coat by centrifugation for 10 minutes at 1600 g, washed

twice with 100 µl of saline, and stored at -80 °C until analysis. For analysis, we added 10 times the volume of distilled water to packed erythrocytes. To a 100 ml mixture of 250 mM Tris-HCl (pH7.5), 100 mM NaCl, 25 mM $MgCl_2$ and 0.0125% Tween20, a substrate containing 50 µl of 5 mM DTT and 50 µl of 0.2 mM 6-thio-guanosine-5'-triphosphate (6-TGTP; Jena Bioscience GmBH, Germany) and 50 µl of diluted RBCs. After incubation for 2 hours at 37 °C and acetonitrile deproteinization, the supernatant was concentrated in nitrogen and dissolved in 200 µl of water. The 6-thio-guanosine-5'-monophosphate (6-TGMP) in the samples was measured by LC-MS/MS according to a previously reported method[2], with some modifications. In brief, the data were acquired on a UPLC system (Agilent 1290 Infinity II; Agilent Technologies, Palo Alto, CA, USA) equipped with a Hypercarb® analytical column (particle size, 5 µm; 2.1 x 100 mm; Thermo Scientific) and guard cartridge (particle size, 5 µm; 2.1 x 10 mm) and coupled to an electrospray ionization quadrupole tandem mass spectrometer (Agilent 6470, Agilent Technologies). Using solvent A, which consisted of 1000 ml of 5 mM ammonium hydrogen carbonate, 1 ml of 50 mM EDTA and 3.5 ml of 28% ammonium hydroxide, and solvent B, which was 100% acetonitrile, the gradient program was set as follows: the first 9 minutes with 7 to 20% solvent B at a flow rate of 0.3 ml/min at 25 °C; an additional 2 minutes holding at 20% solvent B, and another 1

minute with 50% solvent B at a flow rate of 0.6 ml/min. The mass spectrometer was operated in positive mode with a capillary voltage of 3500 V. The nebulizing N_2 gas pressure was 45 psi, and the dry gas flow was 5 l/min at 300 °C. The injection volume was 10 μl. The precursor ion (m/z), product ion (m/z) and collision energies (CE) of 6-TGMP were set at 380, 168 and 33 V, respectively.

Statistical analysis

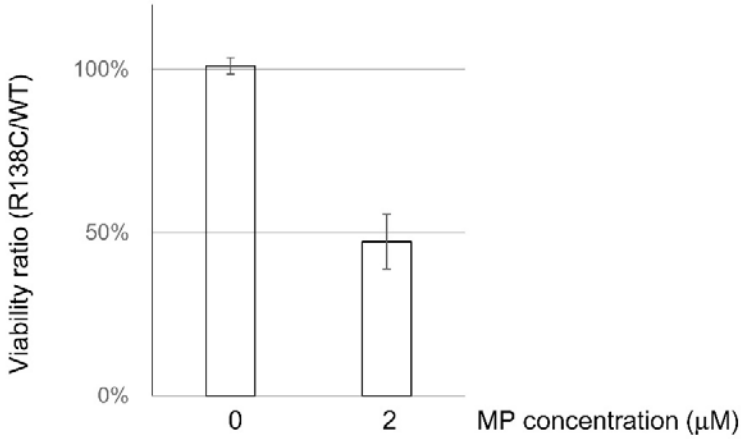
For multiple comparison analyses of toxicity and hematopoiesis among mouse genotypes, we used more than 4 mice to ensure adequate statistic power and adopted ANOVA followed by Tukey's tests. For two-pair comparison, we used more than 3 mice and adopted Student t-test. All analyses were conducted in GraphPad Prism version 6 (GraphPad software, La Jolla, CA, USA). All tests were two-sided, and a p-value <0.05 was considered significant. In survival analysis of MP administration and leukemia model in mice, the non-blinded but random group allocation was performed.



Supplementary Figure 1

Alignment of the protein sequence between murine Nudt15 and human NUDT15.

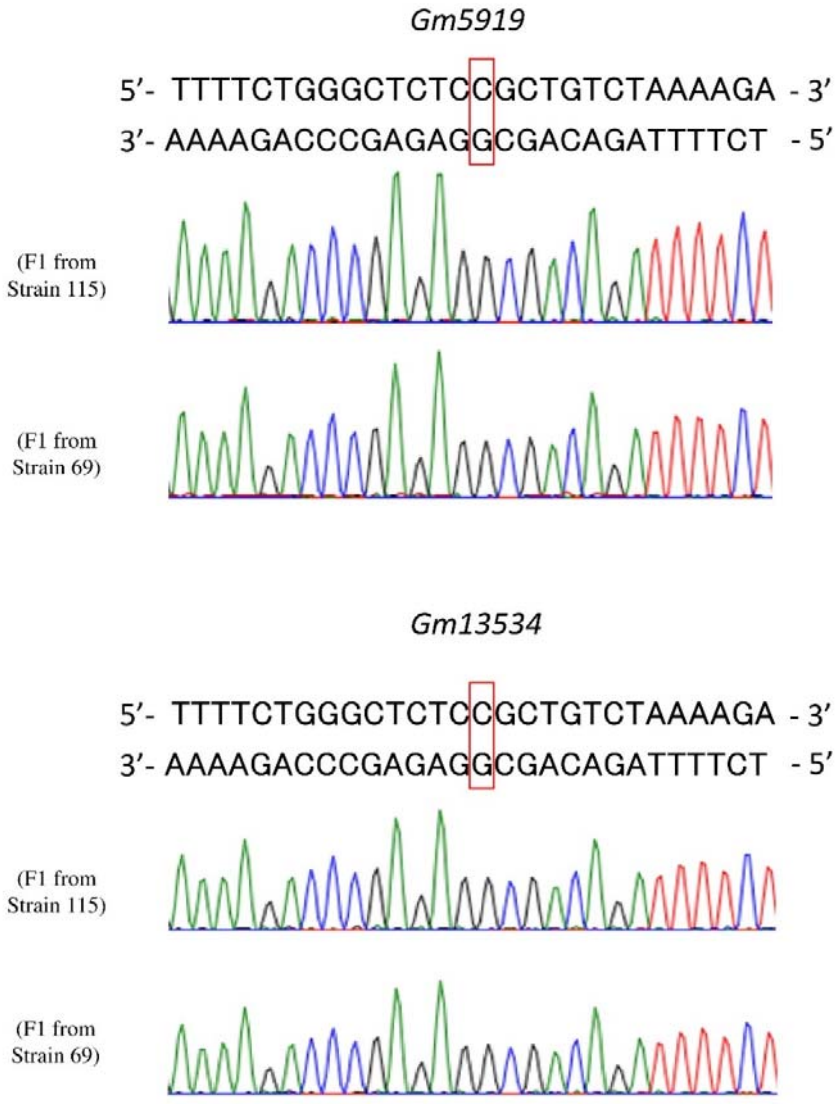
Protein sequence is aligned between murine Nudt15 (mNUDT15) and human NUDT15 (hNUDT15). Asterisks indicate the conserved amino acid. The red box indicates R138C of murine Nudt15 and R139C of human NUDT15.



Supplementary Figure 2

Enhanced susceptibility to MP by overexpression of Nudt15 R138C.

pCAD-Nudt15^{wt} or pCAD-Nudt15^{R138C} transduced Ba/F3 cells were treated with 0 μM or 2 μM MP for 2 days and the number of cells were counted after trypan-blue staining. The Y axis indicates the viability ratio that the number of pCAD-Nudt15^{R138C} transduced cells is divided by the number of pCAD-Nudt15^{wt} transduced cells. Mean and SD are presented (n=3). WT means by wild-type.

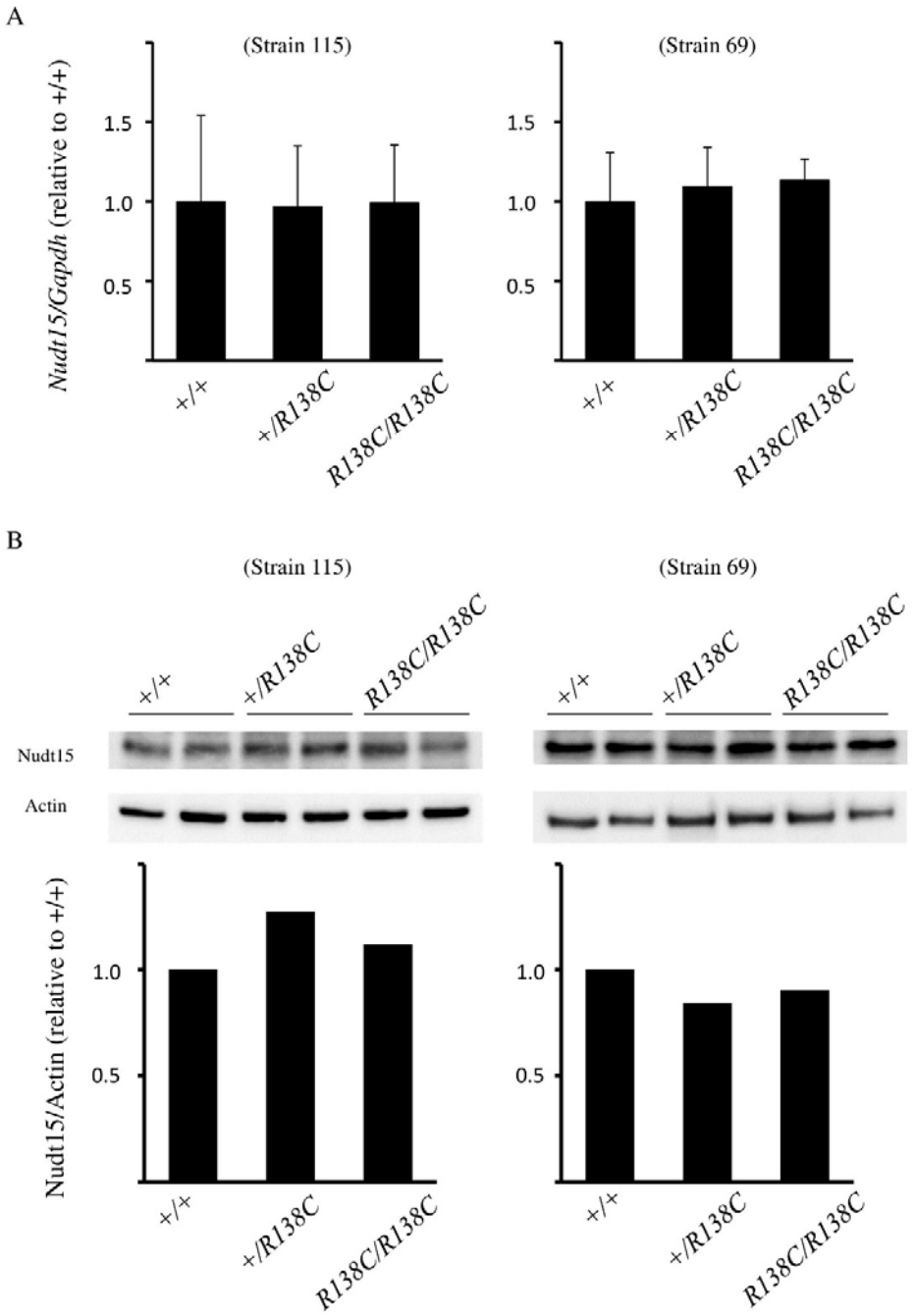


Supplementary Figure 3

Sequence data of pseudogenes Gm5919 and Gm13534 in F1 mice.

No mutations were validated in two pseudogenes which have the same sequence area as the target of sg RNAs. Sequence data in F1 mice were presented. The red boxes indicate the base which can be theoretically converted from C to T by the sgRNAs and the oligoDNA that were

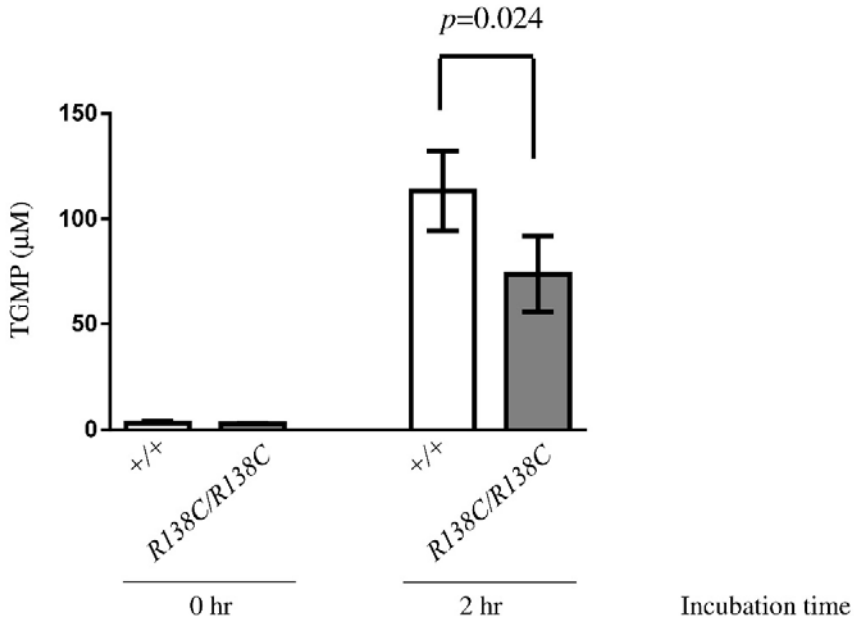
used to establish the *Nudt15*^{R138C} allele.



Supplementary Figure 4

Expression of Nudt15 in mRNA level and protein level in *Nudt15*^{R138C} knock-in mice.

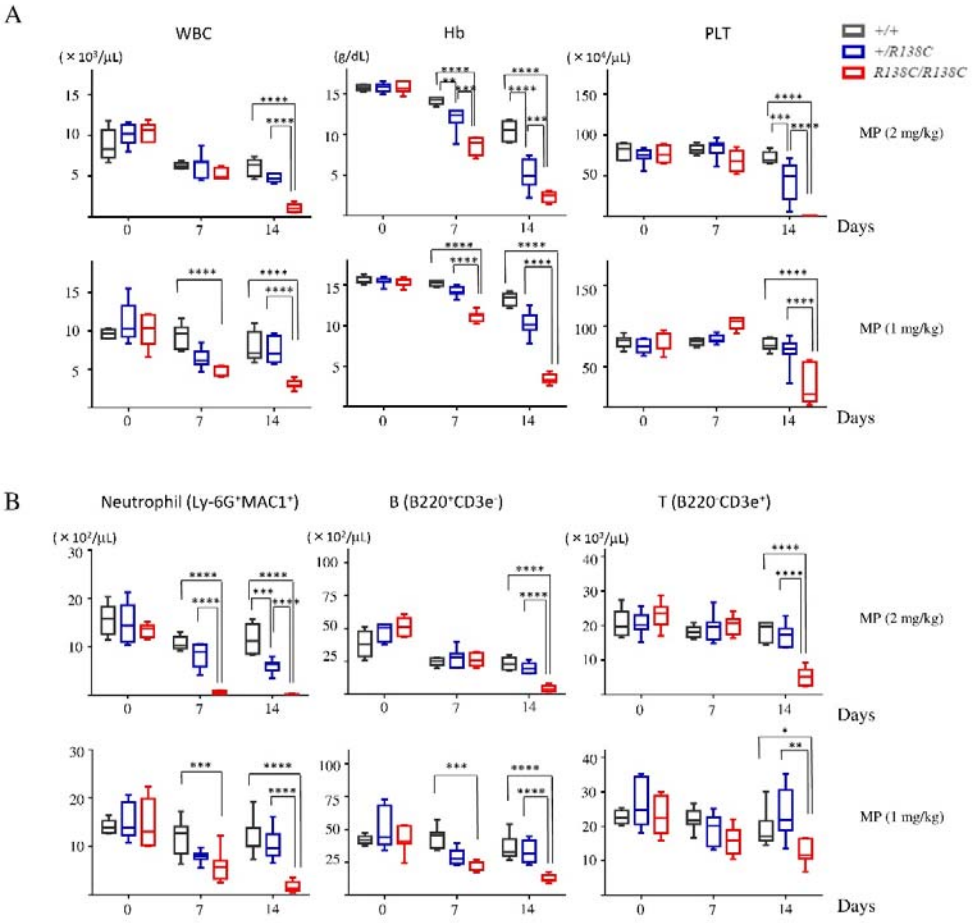
(A) The *Nudt15* transcript level in *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, or *Nudt15*$^{R138C/R138C}$ mice. The data are shown as the relative change in comparison to *Nudt15*$^{+/+}$ mice after normalization to *Gapdh*. The data are presented as mean with standard deviations for 3 independent experiments. (B) The Nudt15 protein level in *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, or *Nudt15*$^{R138C/R138C}$ mice. The upper panels show the raw data of western blotting. The bottom panels show the quantification data of Nudt15 signals after normalization to the Actin signals by ImageQuant TL software. The mean in independently twice experiments is presented.



Supplementary Figure 5

Nudt15 enzyme activity in *Nudt15*^{R138C} knock-in mice.

The data shows the TGMP concentration 0 hr or 2 hr after adding TGTP as a substrate to the lysate of packed red blood cells from *Nudt15*$^{+/+}$ or *Nudt15*$^{R138C/R138C}$ mice in strain 115 (n=4). The *p*-value is 0.024 (Student t-test).

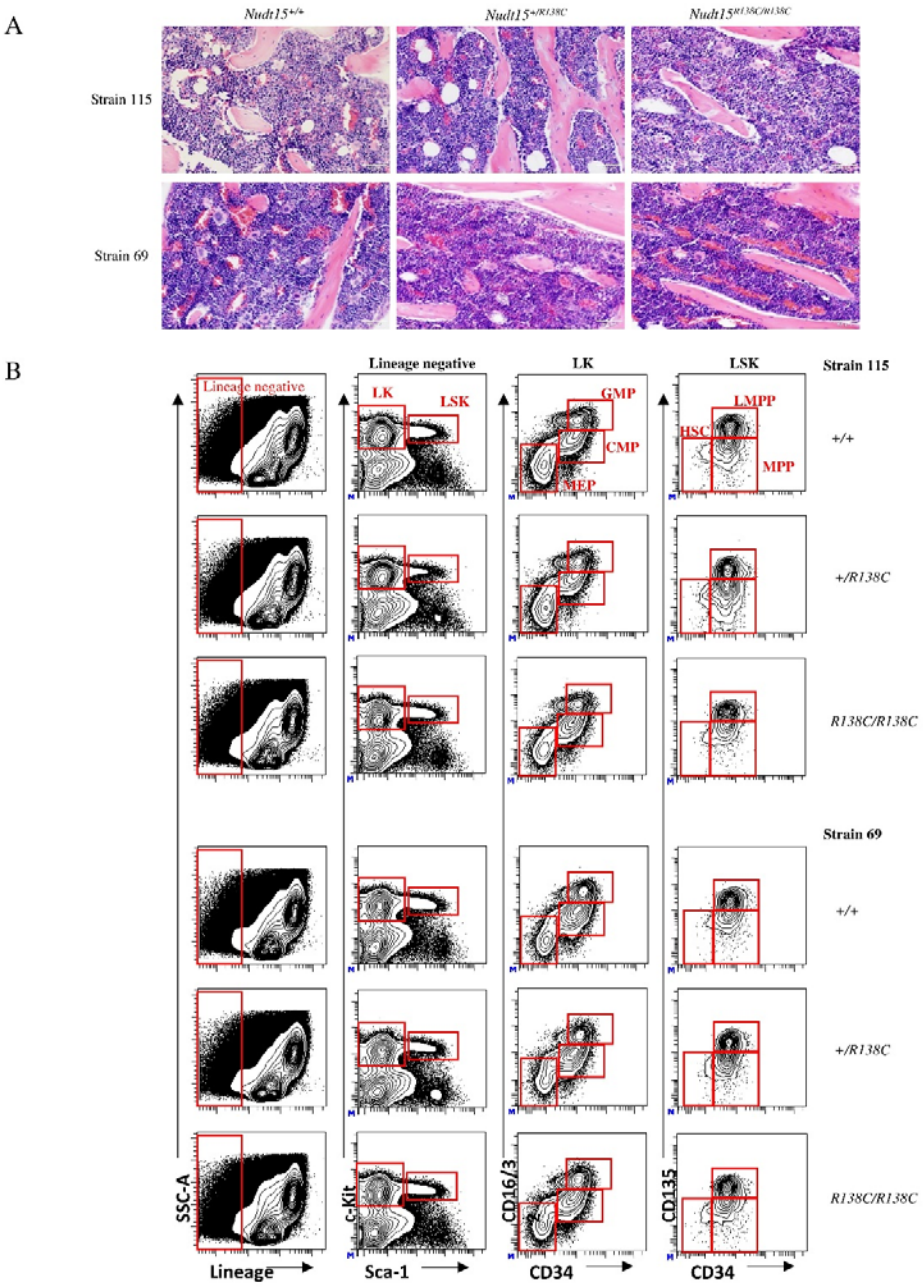


Supplementary Figure 6

Enhanced cytopenia by MP in *Nudt15*$^{+/R138C}$ and *Nudt15*$^{R138C/R138C}$ mice in strain 69.

(A) Peripheral blood cell counts and (B) the number of cells in neutrophils, B lymphocytes and T lymphocytes after oral administration of 1 or 2 mg/kg MP for 7 or 14 days in strain 69. Data are presented in box and whiskers with 10 to 90 percentile. Horizontal lines in boxes indicate the means. 6–8 mice were analyzed in each group. *$p < 0.05$, **$p < 0.01$, ***$p <$

1 0.001, and ****$p < 0.0001$ (ANOVA followed by Tukey's test).

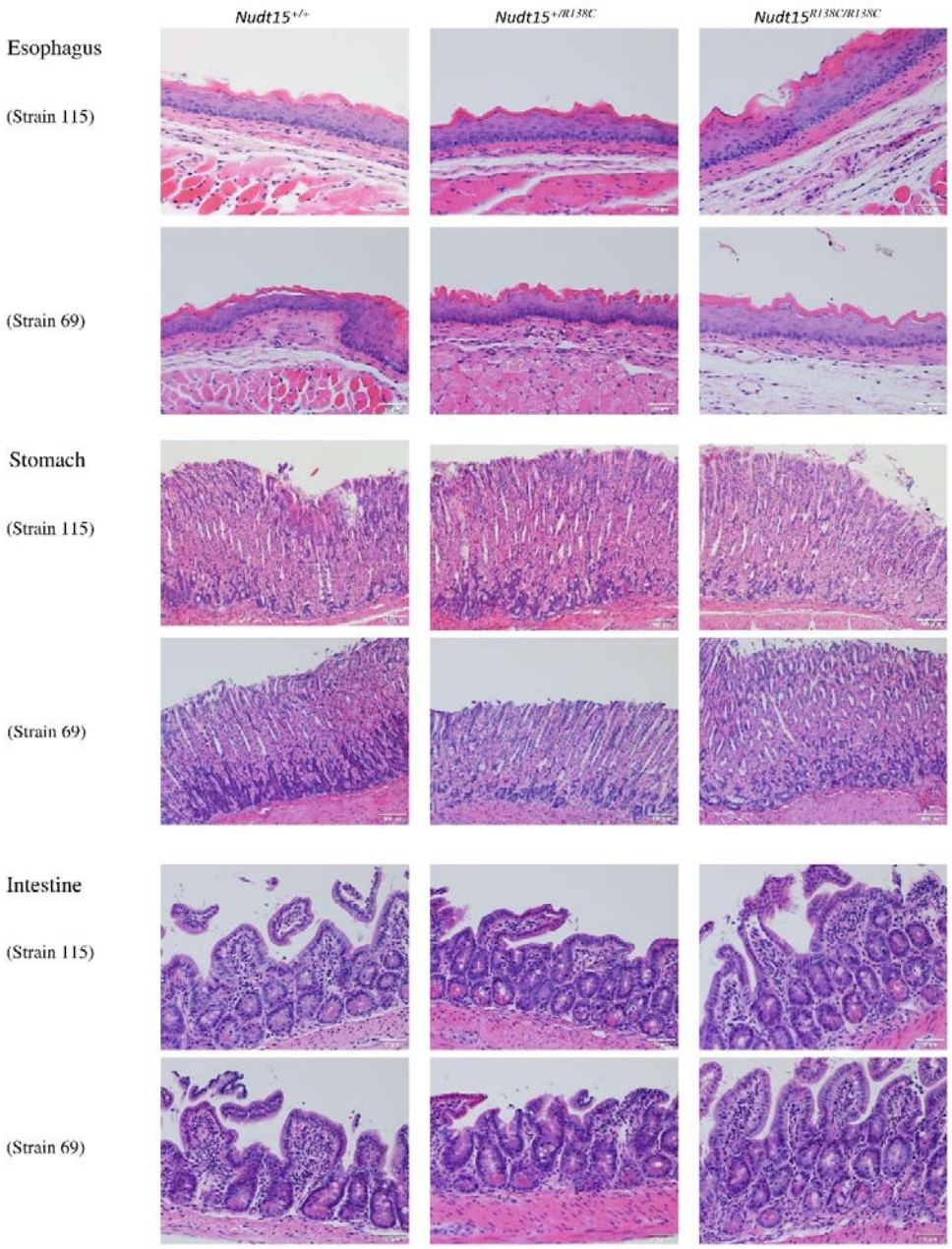


1 **Supplementary Figure 7**

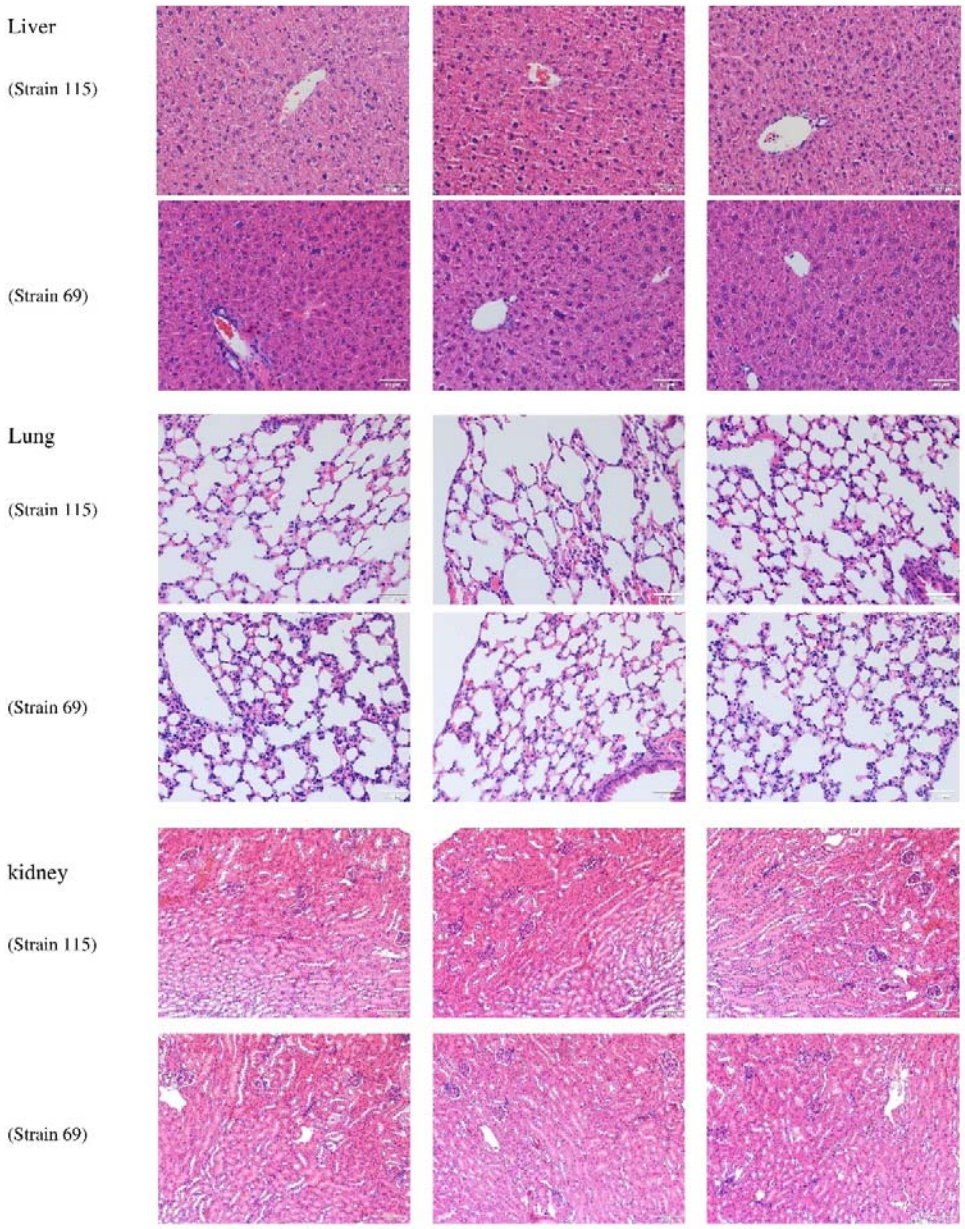
2 **Histological and immunophenotypical BM analyses in steady state.**

3 (A) BM histology from *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, or *Nudt15*$^{R138C/R138C}$ mice in steady state.

HE staining was performed after decalcification. Each *Nudt15* genotype is indicated at the top of panels. Scale bars indicate 50 μm. (B) FACS plots of BM cells from *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, or *Nudt15*$^{R138C/R138C}$ mice in steady state. Each population including LK (Lineage$^-$Sca-1$^-$c-Kit$^+$), LSK (Lineage$^-$Sca-1$^+$c-Kit$^+$), CMP (Lineage$^-$Sca-1$^+$c-Kit$^+$CD16/32^{low}CD34^{low}), GMP (Lineage$^-$Sca-1$^+$c-Kit$^+$CD16/32$^+$CD34$^+$), MEP (Lineage$^-$Sca-1$^+$c-Kit$^+$CD16/32$^-$CD34$^-$), HSCs (CD34$^{-/low}$Flt3$^-$Lineage$^-$Sca-1$^+$c-Kit$^+$), MPP (CD34$^+$Flt3$^-$Lineage$^-$Sca-1$^+$c-Kit$^+$), and LMPP (CD34$^+$Flt3$^+$Lineage$^-$Sca-1$^+$c-Kit$^+$) is indicated and labeled in red squares and red characters. Each *Nudt15* genotype and the strain number are described at the right of panels.



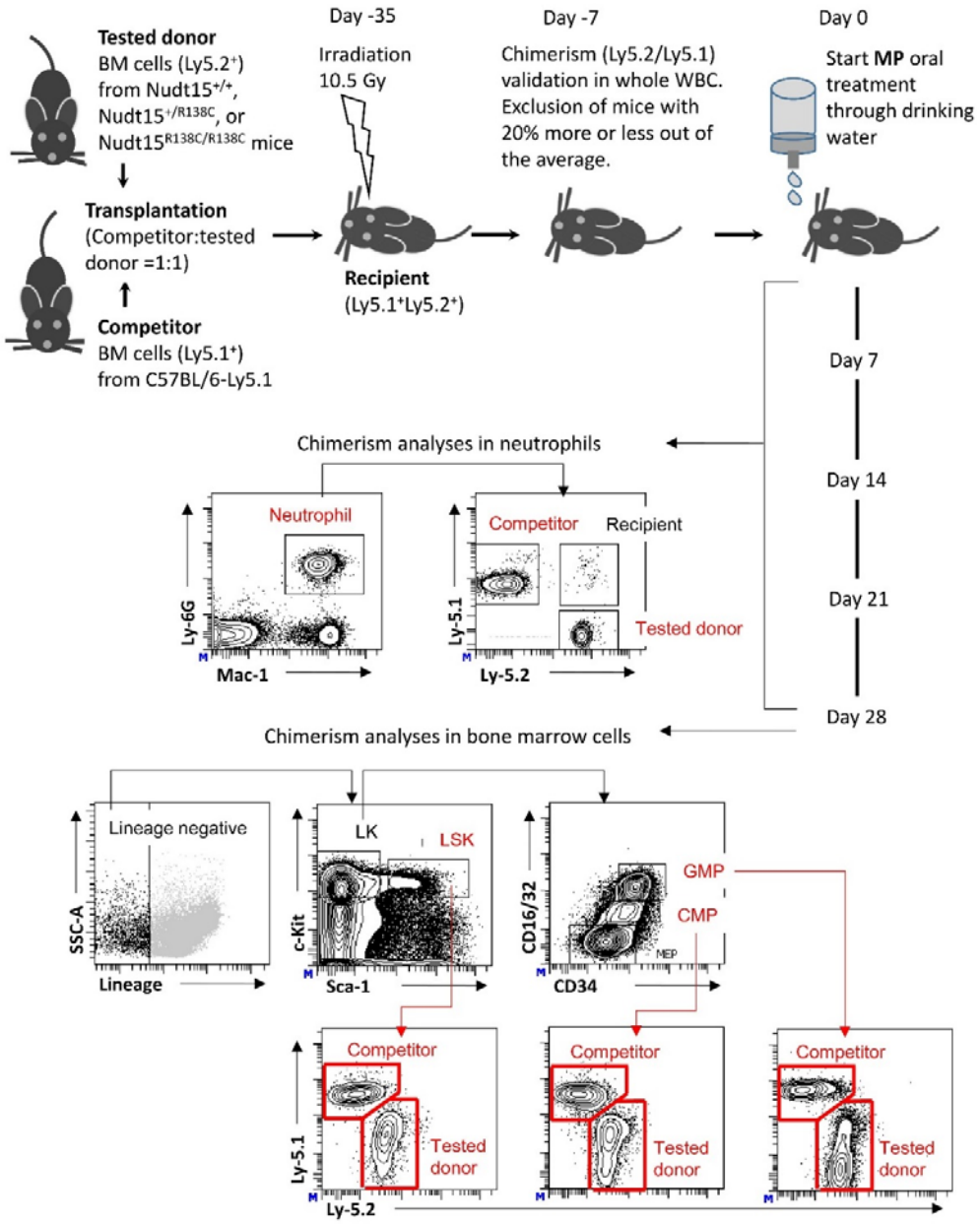
1 **Supplementary Figure 8 (to be continued)**



Supplementary Figure 8

Histology of organs after intraperitoneal injection of 5 mg/kg MP for 5 days.

Histology of organs including esophagus, stomach, intestines, liver, lungs, and kidneys after intraperitoneal injection of 5 mg/kg MP for 5 days are presented. Each *Nudt15* genotype is indicated at the top of panels. Scale bars indicate 50 μm.

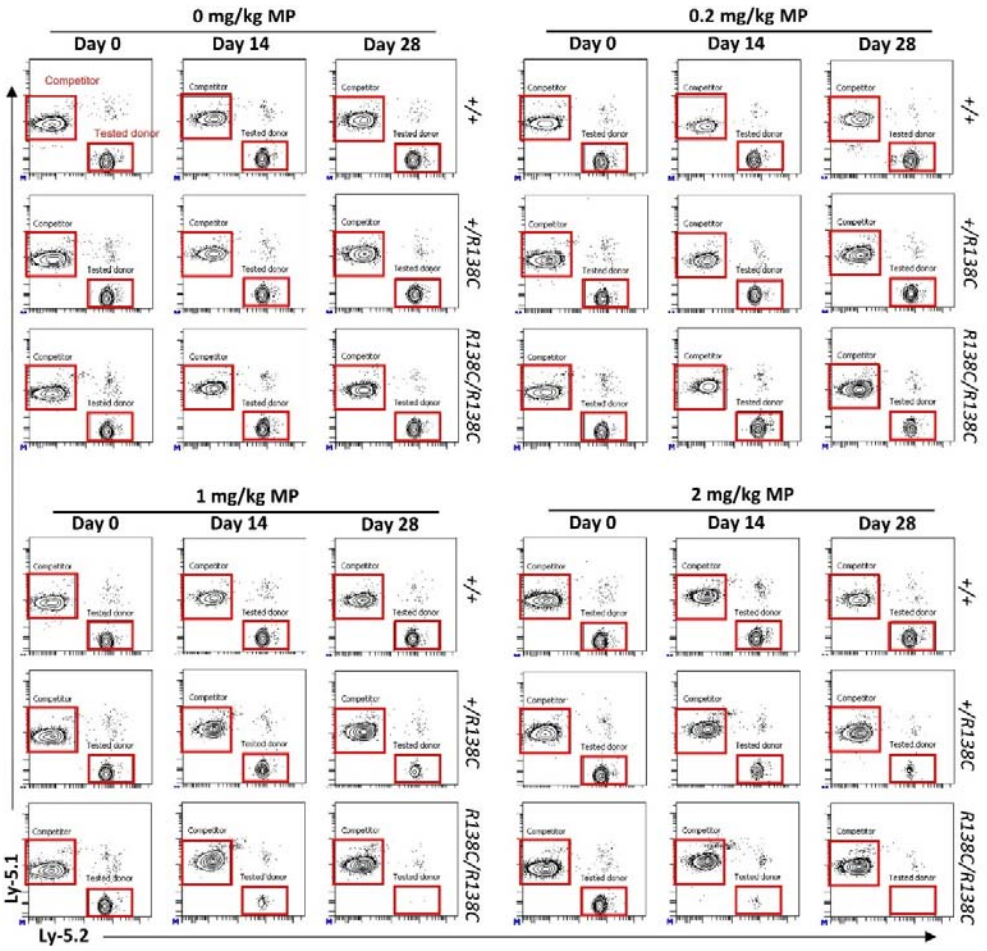


1 **Supplementary Figure 9**

2 **Schematic diagram of a competitive transplantation assay followed by MP treatment.**

3 The method for a competitive transplantation assay followed by MP treatment is

schematically presented. The gating method for chimerism analyses in neutrophils, LSKs, CMPs, and GMPs is delineated.

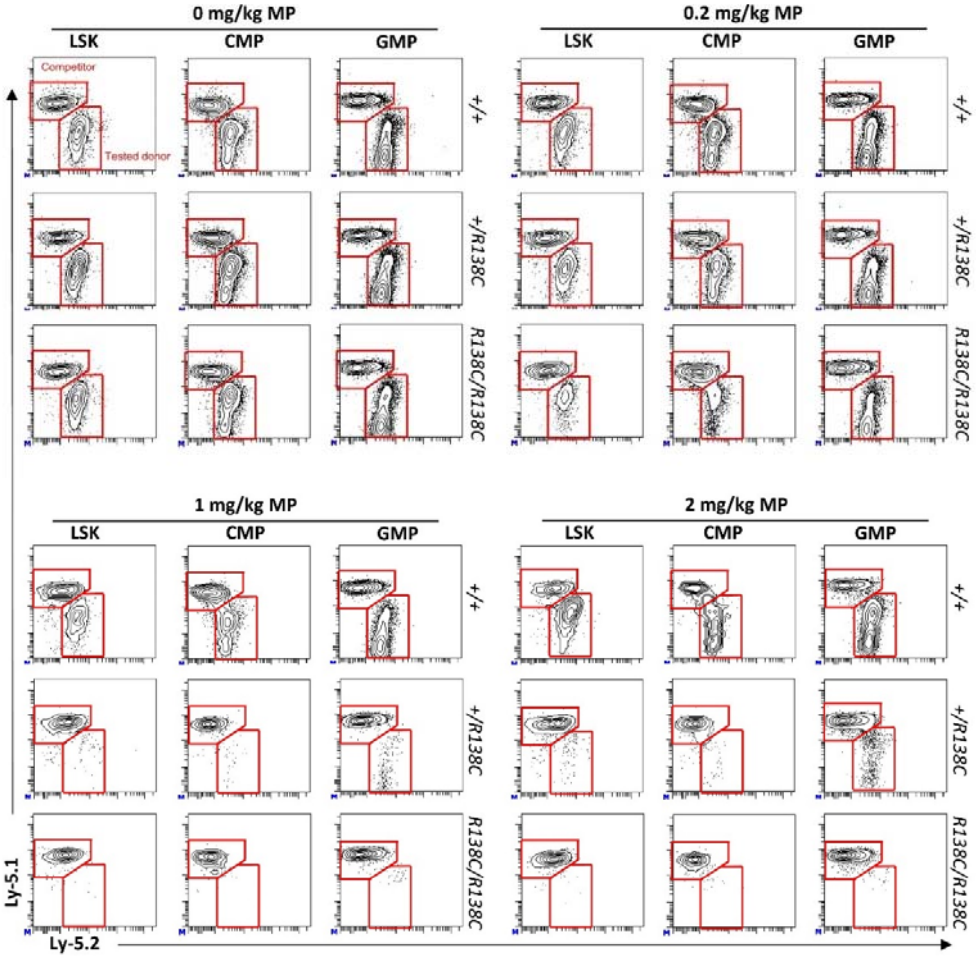


Supplementary Figure 10

Representative FACS plots of neutrophil chimerism.

Representative FACS plots of neutrophil chimerism in a competitive transplantation assay followed by MP treatment are presented. Panels are displayed in grouping each MP dose. The MP dose and the number of treatment days are indicated at the top of panels in each group. Gates for competitor cells ($Ly5.1^{+}$) derived from BM donor cells of wild-type

congenic mice and for tested cells (Ly5.2^{+}) derived from BM donor cells of *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, or *Nudt15*$^{R138C/R138C}$ mice are displayed in the red boxes. Each *Nudt15* genotype is described at the right of panels.



Supplementary Figure 11

Representative FACS plots of chimerism in LSKs, CMPs, or GMPs.

Representative FACS plots of chimerism in HSPC populations including LSKs, CMPs, or GMPs from BM cells in competitive transplantation assay followed by MP treatment are presented. Panels are displayed in grouping each MP dose. The MP dose and each HSPC population name are indicated at the top of panels in each group. Gates for competitor cells

(Ly5.1^{+}) derived from BM donor cells of wild-type congenic mice and for tested cells (Ly5.2^{+}) derived from BM donor cells of *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, or *Nudt15*$^{R138C/R138C}$ mice are displayed in the red boxes. Each *Nudt15* genotype is described at the right of panels.

1 **Supplementary tables**

2 **Supplementary Table 1 Sequence for primers, sgRNA targets and oligoDNAs.**

Targets and OligoDNAs for generating *Nudt15*$^{+/R138C}$	
Strain #69	
target	CTCCGCTGTCTAAAAGAGCAAGG
OligoDNA	GTTGGGAGTGGGTTCCATGGGAAGAATTCCCTCCCT TAGACCAGCTTTTCTGGGCTCTCTGCTGTCTAAAAG AGCAAGGTTATGACCCATTTAAAGAGGACCTGAACC ACCTGGAAGGGT
Strain #115	
target	AACCTTGCTCTTTTAGACAGCGG
OligoDNA	ACCCTTCCAGGTGGTTCAGGTCCTCTTTAAATGGGT CATAACCTTGCTCTTTTAGACAGCAGAGAGCCCAGA AAAGCTGGTCTAAGGGAGGGAATTCTTCCCATGGAA CCCACTCCCAAC
Genotyping primers	
Nudt15 set1	

Nudt15-gFW1	ATGACCTCGAAAGTTCCTGTGTT
Nudt15-gRV1	TGTCAGACAGTTCACAGAAACGA
Nudt15-Sequencing primer1	GGCATCTAGCCTGTAATATAGACAT
Nudt15 set2	
Nudt15-gFW2	TCCTACCAGGACTCTCACCTCATG
Nudt15-gRV2	CAGAGGTAGGTAGGCAGATCTGAG
Nudt15-Sequencing primer2	CCCGGCCTGCAGGTCTATGCCACCAGGACAATTCAG
Gm13534 set	
Gm13534-gFW	TGACTCAGCTATCAGACTGAGCTG
Gm13534-gRV	GAAGCCATCCTTCCTCATACACTCA
Gm13534-Sequencing primer	CCCGGCCTGCAGGTCTATGCCACCAGGACAATTCAG
Gm5919 set	
Gm5919-gFW	TGAGAGAGGTAGCAAGTTCCAGTG

Gm5919-gRV	CCTTACTCTTTTGACTAGTTTTGGGTTG
Gm5919-Sequencing primer	CCCGGCCTGCAGGTCTATGCCACCAGGACAATTCAG
Cas9 set	
Cas9-F	AAAGTGGCACCGAGTCGGTGC
Cas9-R	TTCTTGCTGGGCACCTTGTACTCG
qPCR primers	
Nudt15	
Nudt15-FW	CCGAGGAATATGGAGCCTGAA
Nudt15-RV	AAGCTGGTCTAAGGGAGGGAA
Gapdh	
Gapdh-FW	AGGTCGGTGTGAACGGATTTG
Gapdh-RV	TGTAGACCATGTAGTTGAGGTCA

1

2

Supplementary references

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2. Hofmann U, Heinkele G, Angelberger S, et al. Simultaneous quantification of eleven thiopurine nucleotides by liquid chromatography-tandem mass spectrometry. *Anal Chem*. 2012;84(3):1294-1301.