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Supplementary Materials for

PRMT8 as a phospholipase regulates Purkinje cell dendritic arborization and motor coordination

Jun-Dal Kim, Kyung-Eui Park, Junji Ishida, Koichiro Kako, Juri Hamada, Shuichi Kani, Miki Takeuchi, Kana Namiki, Hajime Fukui, Shigetomo Fukuhara, Masahiko Hibi, Makoto Kobayashi, Yasunori Kanaho, Yoshitoshi Kasuya, Naoki Mochizuki, Akiyoshi Fukamizu

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(available at advances.sciencemag.org/cgi/content/full/1/11/e1500615/DC1)

Supplemental Materials and Methods

Whole-mount *in situ* hybridization

To make a riboprobe for *prmt8b* cDNA was amplified by reverse-transcription PCR with primers: 5’-CGGGATCCATGGGACTGAGGCACTCATC-3’ and 5’-GCTCTAGATTACCGCATTTTGTAGTCGT-3’ from zebrafish larvae and subcloned to pBluescript II SK+ (Agilent). Digoxygenin-labeled antisense *prmt8b* riboprobe was generated by transcription with T7 RNA polymerase (Promega). Whole-mount *in situ* hybridization was performed as described previously (*47*) except hybridization was at 65°C. For detection, NBT/BCIP (Roche) was used as the substrate for alkaline phosphatase. The NBT/BCIP signals were acquired using an AxioPlan-2 microscope and AxioCam CCD camera (Zeiss).

Generation of *prmt8b* knockout zebrafish

To make knockout zebrafish, we used transcription activator-like effector nucleotide (TALEN) Targeter 2.0 (https://tale-nt.cac.cornell.edu) to design TALEN pair that targets *prmt8b*. The target sequence of TAL-*prmt8b* was 5’-TGGCCAAACTGCTCAACccagaggagatgacaTCTCGAGATTACTATTTCGA-3’ (capital letters were sequences of left [TAL-*prmt8b*-L] and right [TAL-*prmt8b*-R] arms, respectively). The two expression plasmids of the TALEN were constructed by pT3TS-GoldyTALEN (*48*). TALEN mRNAs were synthesized *in vitro* by T3 mMessage mMACHINE kit (Ambion, USA). To induce double strand breaks in the *prmt8b* target

sequence, both 50 pg of TAL-*prmt8b*-L mRNA and TAL-*prmt8b*-R mRNA were injected into 1-2-cell stage *Tg(elavl3:GFP)* embryos. Each injected founder (F0) fish was outcrossed with wild-type fish to obtain F1 progeny from the individual founders. To analyze TALEN induced mutations, genomic DNA from F1 embryos was isolated using lysis buffer (50 mM Tri-HCl (pH 8.0), 100 mM NaCl, 100 mM EDTA, 1% SDS and 0.2 mg/ml proteinase K) and purified by phenol/chloroform extraction. The genotyping PCR primers for prmt8b were 5'-CAGCAGCAGCAAAAACACAAGCAAC-3' and 5'-CCCCAAAACTCCATCCAATTACCTC-3'. The 289 bp (wild-type [WT] allele) or 282 bp (*ncv102* allele) PCR products were confirmed by direct DNA sequence.

Immunofluorescence analysis

For immunofluorescence staining of zebrafish, anti-Vglut1 (1:1,000, purified antibody) and anti-parvalbumin 7 (1:5,000, mouse ascites) antibodies were used, and immunostaining of larvae was performed as described previously (*49*). Alexa Fluor 488 and Alexa Fluor 568 goat anti-rabbit and goat anti-mouse IgG (H+L, Molecular Probes, Invitrogen) were used as secondary antibodies. Fluorescence images were obtained with a Zeiss LSM700 confocal microscope. These fluorescence images were constructed from Z-stack sections by a 3D projection program associated with the microscope.

Morpholino oligonucleotides

Antisense morpholino oligonucleotides (MOs) were synthesized by Gene-Tools (LLC). The sequences were *prmt8*-MO1 (translation-blocking) and *prmt8*-MO2 (splice-blocking) were 5'-ACCGCGATGAGTGCCTCAGTCCCAT-3' and 5'-TGGAGCATTCGATCTACAAGACAAG-3', respectively. The MOs were dissolved in and diluted with water and injected into the yolk of one-cell-stage embryos.

Footprint analysis

For the footprint test, mouse paws were dipped in nontoxic water-based inks (*prmt8*$^{+/+}$ mice in black and *prmt8*$^{-/-}$ mice in red ink). Mice were trained to walk along a 50-cm-long, 10-cm-wide, paper-covered runway (with 10-cm-high walls) into an enclosed box. A fresh sheet of squared paper was placed on the floor of the runway for each run. The footprint patterns were assessed quantitatively by three length measurements including stride, sway and stance. Mean values were used for statistical analysis using Prism 5 (GraphPad Software).

Immunoprecipitation and Western blot

The human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's Medium (DMEM, WAKO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Cell Culture Technology), penicillin/streptomycin (100 units/ml) and maintained at 37°C, 5% CO_2. Expression vectors were transfected using GeneJuice transfection reagent according to the manufacturer's instructions (Novagen). Transfected HEK293T cells were washed in

ice-cold PBS, and resuspended in lysis buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol, and 1x protease inhibitors (Nacalai tesque). After 30 min on ice, lysates were cleared by centrifugation at 13,200 rpm at 4°C for 10 min. The supernatant was treated with anti-HA antibody (12CA5, Roche) and Protein G Sepharose beads (GE Healthcare) at 4°C for 6 h. Immunoprecipitated proteins resolved by 10% SDS-PAGE were transferred to an Immobilon-P membrane (Millipore) before Western blot detection using anti-HA antibody (3F10, Roche Applied Science) at 1:1,000 dilution. Visualization was carried with horseradish peroxidase coupled secondary antibody and developed using Luminata Forte Western HRP substrate (Millipore) and exposed to medical X-ray films (Fuji Film).

In vitro PC lipase assay for thin-layer chromatography, TLC

PC lipase activity was determined by measuring the generation of ^{3}H-labeled choline from [choline-methyl-^{3}H] dipalmitoyl-PC ([^{3}H]-DPPC) as previously described (*50*). For the *in vitro* PC lipase assay of PRMT8, three µl of [^{3}H]-DPPC (specific activity of 30-60 Ci/mmol, Perkin Elmer) were sonicated in 100 µl of PBS buffer using a bath type sonicator, and incubated with the immunoprecipitated wild-type PLD2-HA, K758R mutant (*44*), anti-Flag agarose beads-conjugated wild-type PRMT8-Flag or K107R mutant at 37°C for 1.5 h. The reaction was terminated by the addition of 100 µl of chloroform/methanol (2:1, v/v). The aqueous and organic phases were extracted and dried by centrifugation under vacuum. The reaction products were resuspended in 20 µl of chloroform/methanol/acetic acid/water (23:20:5:1, v/v). [^{3}H]-choline was separated

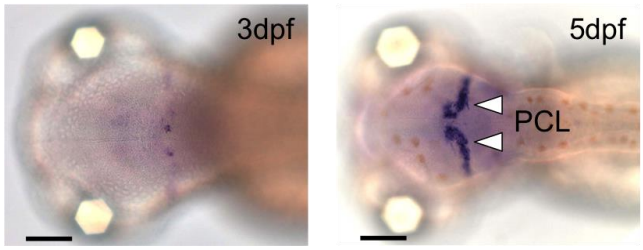
on a silica gel 60 HPTLC plate using chloroform/methanol/acetic acid/water (23:20:5:1, v/v) as the solvent. The plate was sprayed with EN^{3}HANCE (Perkin Elmer) and exposed to Hyperfilm ECL (GE Healthcare) for 1 day at -80°C.

***In vitro* methylation assay**

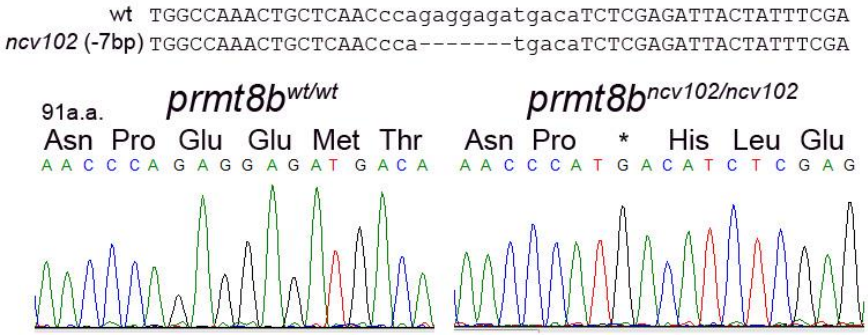
Purified wild-type PRMT8-Flag, K107R, S120A and G121A mutants were added to 2 μg of GST-EWS (RGG3) (*11*), and incubated with 2 μl of *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]SAM) (specific activity of 15.0 Ci/mmol, Perkin Elmer) for 1 h at 37°C in a final volume of 50 μl of PBS. The reaction products were fractionated by SDS-PAGE. Gels stained with CBB staining solution (0.05% CBB, 50% methanol, 10% acetic acid) were incubated for 30 min with Amplify Fluorographic Reagent (Perkin Elmer), dried and exposed to Hyperfilm ECL (GE Healthcare) as -80°C.

fig. S1

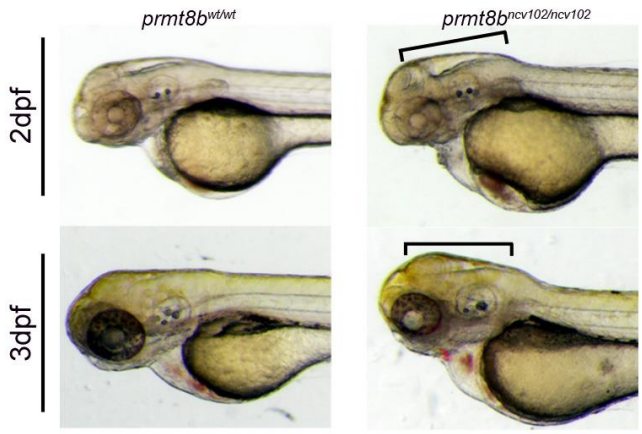
A


3dpf
5dpf
PCL

B


wt TGGCCAAACTGCTCAACccagaggagatgacaTCTCGAGATTACTATTTCGA
ncv102 (-7bp) TGGCCAAACTGCTCAACcca-------tgacaTCTCGAGATTACTATTTCGA
91a.a.
prmt8b wt/wt
prmt8b ncv102/ncv102
Asn Pro Glu Glu Met Thr
Asn Pro * His Leu Glu
A A C C C A G A G G A G A T G A C A
A A C C C A T G A C A T C T C G A G

C


prmt8b wt/wt
prmt8b ncv102/ncv102
2dpf
3dpf

D

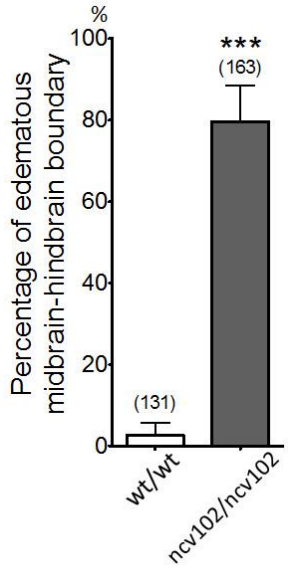

%
100
80
60
40
20
0
Percentage of edematous midbrain-hindbrain boundary
***
(163)
(131)
wt/wt
ncv102/ncv102

fig. S1

E

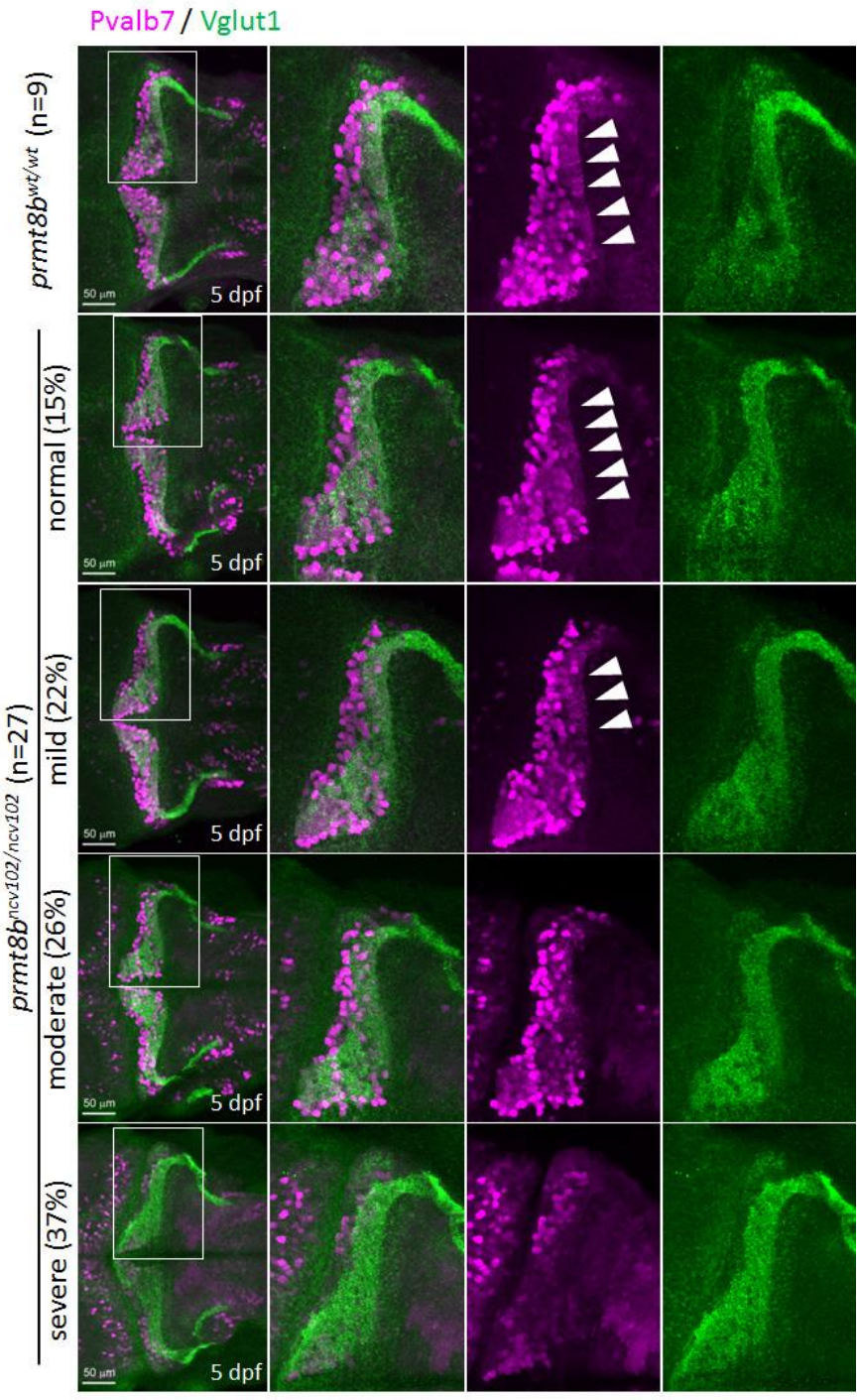

Pvalb7 / Vglut1
prmt8b^wt/wt (n=9)
prmt8b^ncv102/ncv102 (n=27)
normal (15%)
mild (22%)
moderate (26%)
severe (37%)
5 dpf
50 µm

fig. S1

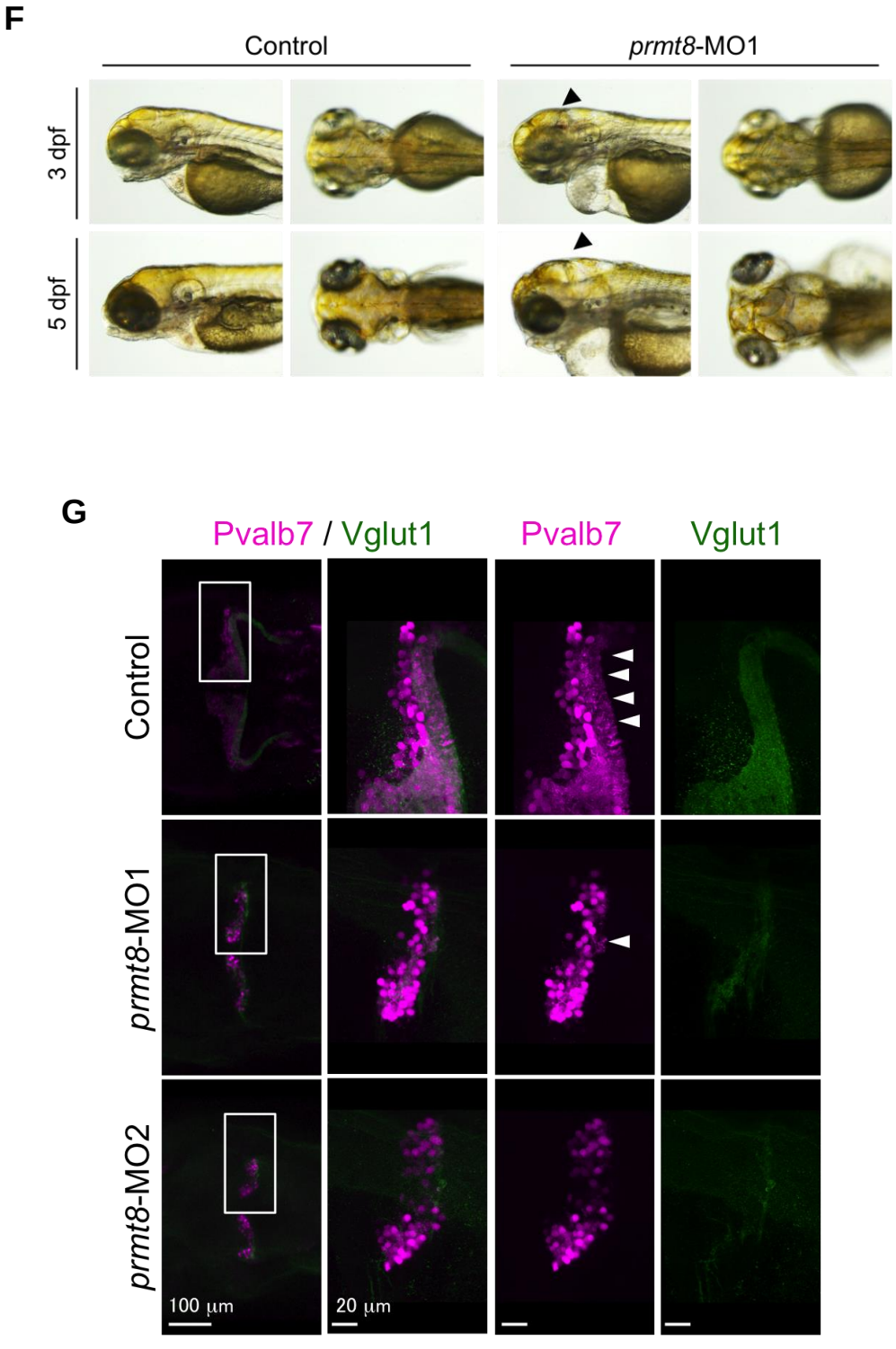

F
Control
prmt8-MO1
3 dpf
5 dpf
G
Pvalb7 / Vglut1
Pvalb7
Vglut1
Control
prmt8-MO1
prmt8-MO2
100 μm
20 μm

fig. S1. Functional analysis of *prmt8* using zebrafish. (**A**) PRMT8 distribution in zebrafish embryos. Expression of the zebrafish *prmt8* mRNA was visualized by whole mount *in situ* hybridization in the early larval stages [3 and 5 days post-fertilization (dpf), dorsal view]. A white arrowhead indicates the location of *prmt8* mRNA in the Purkinje cell layer (PCL) at 5 dpf. Scale bars, 100 μm. (**B**) TALEN-induced *prmt8b* gene (Accession number XM_005164655) mutation at the targeted loci. A deletion of seven base pairs in the *ncv102* allele result in a premature stop codon after Pro (92 a.a.). Upper and lower case letters denote repeat variablediresidues and spacer region for the target of TALEN, respectively. (**C**) Bright field images of anterior region at 2 dpf (top) and 3 dpf (bottom). The *prmt8b*$^{ncv102/ncv102}$ mutant embryos showed morphological changes of the midbrain/hindbrain boundary (bracket). (**D**) Quantitative analysis of the incidence of edematous midbrain/hindbrain boundary at 3 dpf. Total number of embryos analyzed in each group is indicated at the top of the column. (**E**) Immunohistochemical analyses of cerebellum using anti-Parvalbumin7 (Pvalb7) and anti-Vglut1 antibody that detect Purkinje cells (magenta) and granule cells (green), respectively. According to the reduction of dendrite number and extension of Purkinje cells, defects were categorized as indicated at the left. Eighty-five percentage of *prmt8b*$^{ncv102/ncv102}$ mutant larvae exhibited defects of dendrites of Purkinje cells. Boxed areas in left panels are enlarged in right panels. Arrowheads indicate the dendrite arbors of Purkinje cells. Dorsal views, anterior to the left. Scale bars, 50 μm. (**F**) PRMT8 deficiency causes morphological defects in zebrafish. Embryos were injected with 4 ng of *prmt8*-MO1, and brain morphology of embryo was examined at 3 dpf (upper panel) and 5 dpf (lower panels).

Compared to normal morphology of non-injected embryos (Control) (lateral view and dorsal view), *prmt8* morphants showed brain malformations with unclear boundaries between brain subdivisions, especially at the midbrain-hindbrain boundary (arrowheads). (**G**) Morphology of the Purkinje cells. Dorsal view images of the PCL at 5 dpf larval zebrafish that were injected with *prmt8*-MO1 or *prmt8*-MO2. Co-staining with an anti-Pvalb7 and an anti-Vglut1 antibody to specifically label Purkinje cells (magenta) and granule cells (green), respectively. Boxes represent enlarged areas with the Purkinje cell layer. The dendritic arbors of Purkinje cells were indicated by arrowheads. Scale bars, 100 and 20 μm.

fig. S2

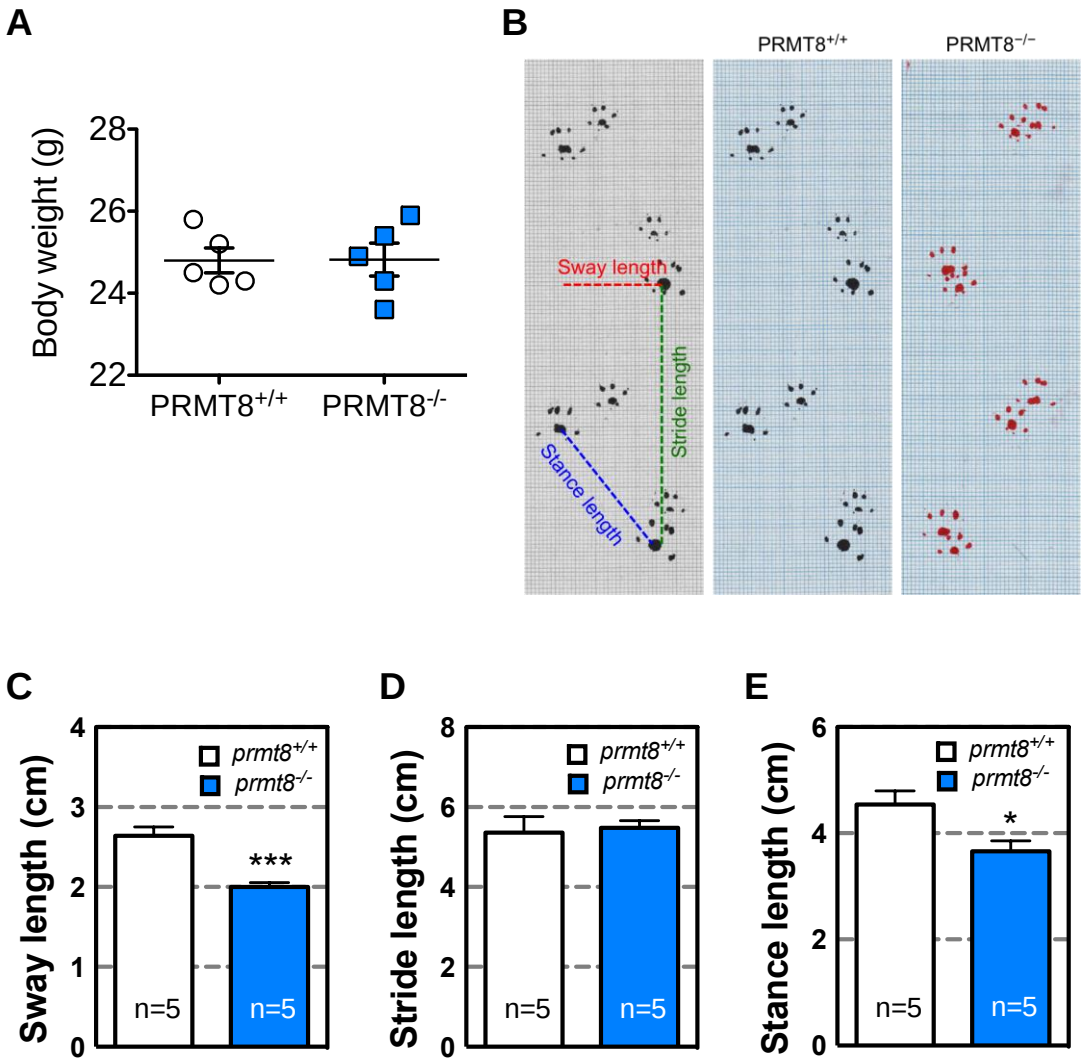


fig. S2. Quantitative analysis of the gait abnormalities in the *prmt8*$^{-/-}$ mice. (**A**) Body weight was measured in 10- to 11-week-old male *prmt8*$^{+/+}$ and *prmt8*$^{-/-}$ mice. (**B**) The parameters measured in the footprint analysis with lines are shown in the left panel. (**C** to **E**) Footprints of *prmt8*$^{+/+}$ mice and *prmt8*$^{-/-}$ mice were evaluated for sway, stride and stance length. Quantification of footprints reveals significantly shorter sway and

stance length in the *prmt8*$^{-/-}$ mice compared with the *prmt8*$^{+/+}$ mice (mean ± SEM, n = 5, *$p < 0.05$, ***$p < 0.0001$). Stride length was not significantly different.

fig. S3

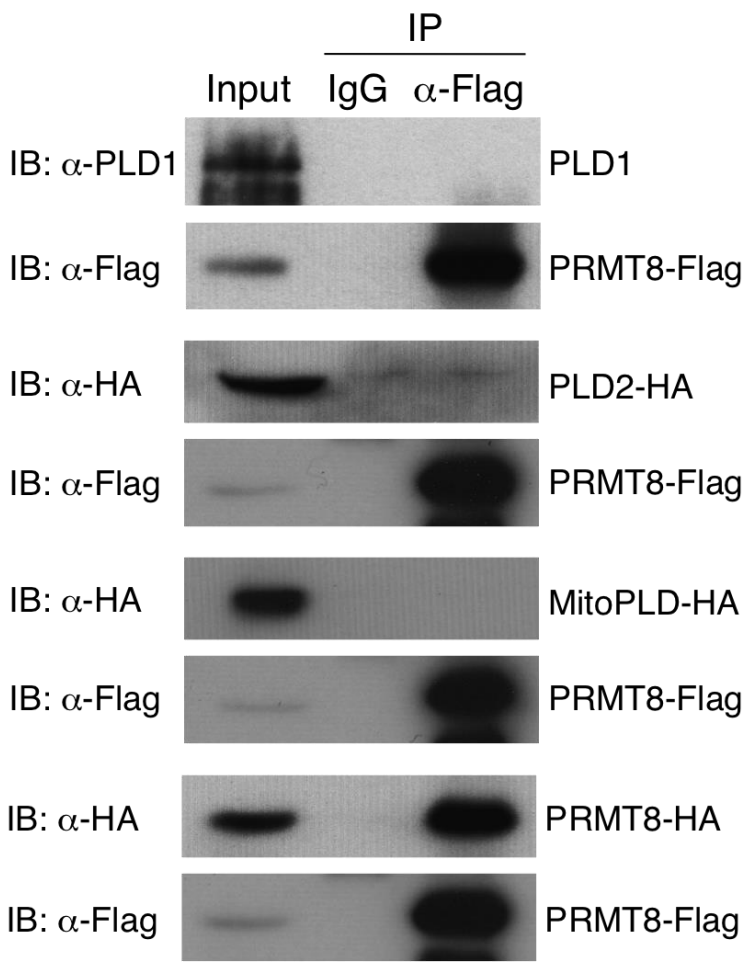


fig. S3. Interaction of PRMT8-Flag and mammalian PLDs. Co-IP analysis of Flag-tagged PRMT8 with PLD1, PLD2-HA or MitoPLD-HA in HEK293T cells. PRMT8-Flag was co-expressed with HA-tagged PLD2 or MitoPLD. PRMT8-Flag protein was precipitated using anti-FLAG antibody and, co-precipitation of PLD1, PLD2-HA or MitoPLD-HA was detected by Western Blot using an anti-PLD1 or anti-HA antibodies.

fig. S4

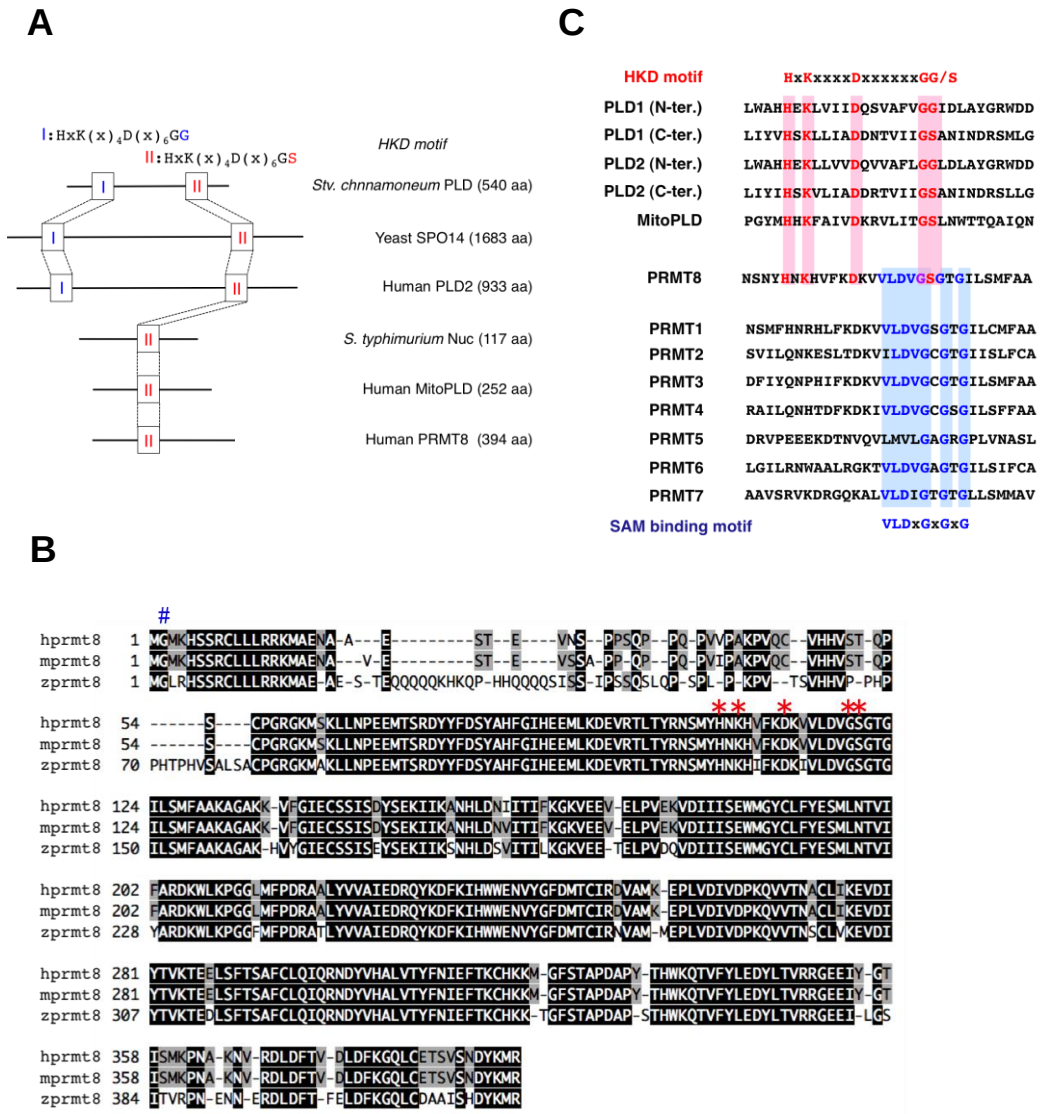


fig. S4. Organizations of the catalytic domains in PLD superfamily. (**A**) PLDs share two conserved amino acid sequences. Regions I and II contain a common conserved amino acid sequence $HxK(x)_4D(x)_6GG/S$ (x, any amino acid), and are referred to as the 1st and 2nd HKD motifs, respectively. (**B**) Human PRMT8 shares sequence homologies

with PRMT8 of other vertebrate species. Black shading represents identical amino acids. Blue # = myristoylation target glycine, Red * = HKD motif. (**C**) Amino acid sequence alignment of regions in the vicinity of HKD motif of PLD isoforms, PRMT family and PRMT8. Amino acids highlighted in red indicate HKD motif. Note that the SAM binding domain (boxed with blue) overlaps with this motif of PRMT8.

fig. S5

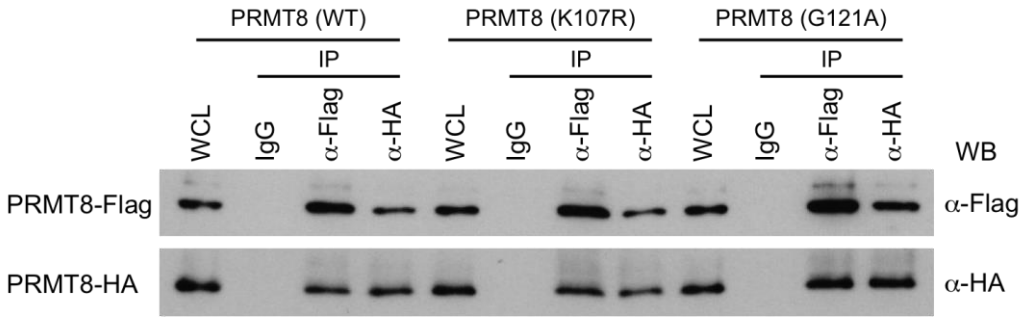


fig. S5. Dimerization of PRMT8-Flag wild-type (WT) and K107R and G121A mutants. HEK293T cells were co-transfected with Flag-tagged PRMT8 wild-type, K107R mutant (phospholipase-inactive), or G121A mutant (methyltransferase-inactive) and HA-tagged PRMT8 wild-type, K107R, or G121A. PRMT8-Flag and PRMT8-HA were immunoprecipitated with anti-Flag and anti-HA antibody, respectively, and the immunoprecipitates were subjected to Western blot analysis with anti-HA or anti-Flag antibody.

fig. S6

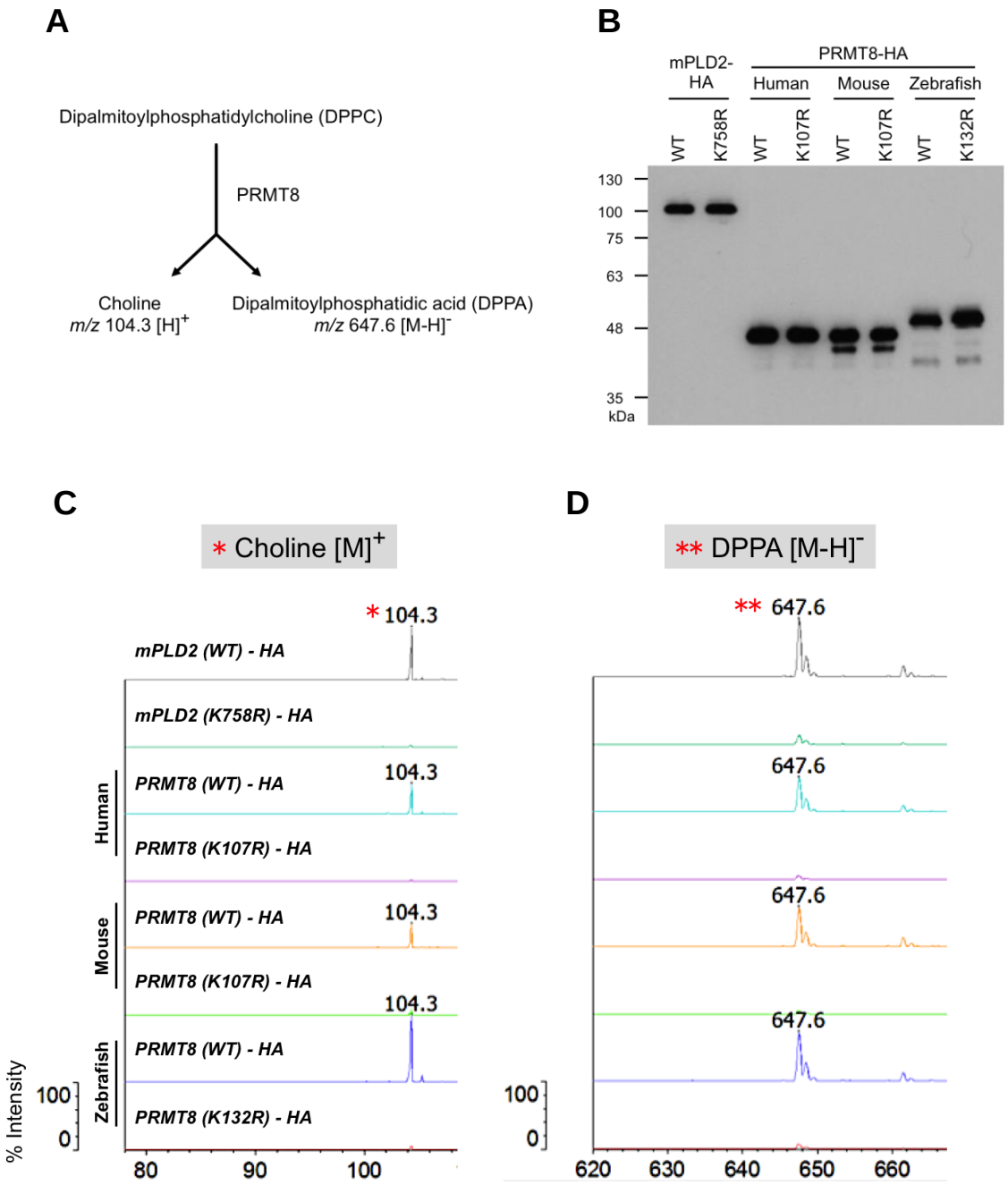


fig. S6. Measuring vertebrate PRMT8 paralog-derived products from PC by

MALDI-QIT-TOF/MS. (**A**) Schematic representation of the hydrolysis of DPPC to choline and DPPA by PRMT8. (**B**) The HA-tagged wild-type PLD2 and the catalytically inactive K758R mutant, and human, mouse and zebrafish wild-type PRMT8 and a lysine to arginine mutants, which were expressed in HEK293T cells. Proteins were purified by immunoprecipitation with anti-HA antibody, followed by immunoblotting, as indicated. (**C** and **D**) MS spectra of reaction products with 9-AA as matrix in positive ion mode and in negative ion mode. The peak of choline (*) and DPPA (**) were indicated at *m/z* 104.3 $[M]^+$ and at *m/z* 647.6 $[M-H]^-$, respectively.

fig. S7

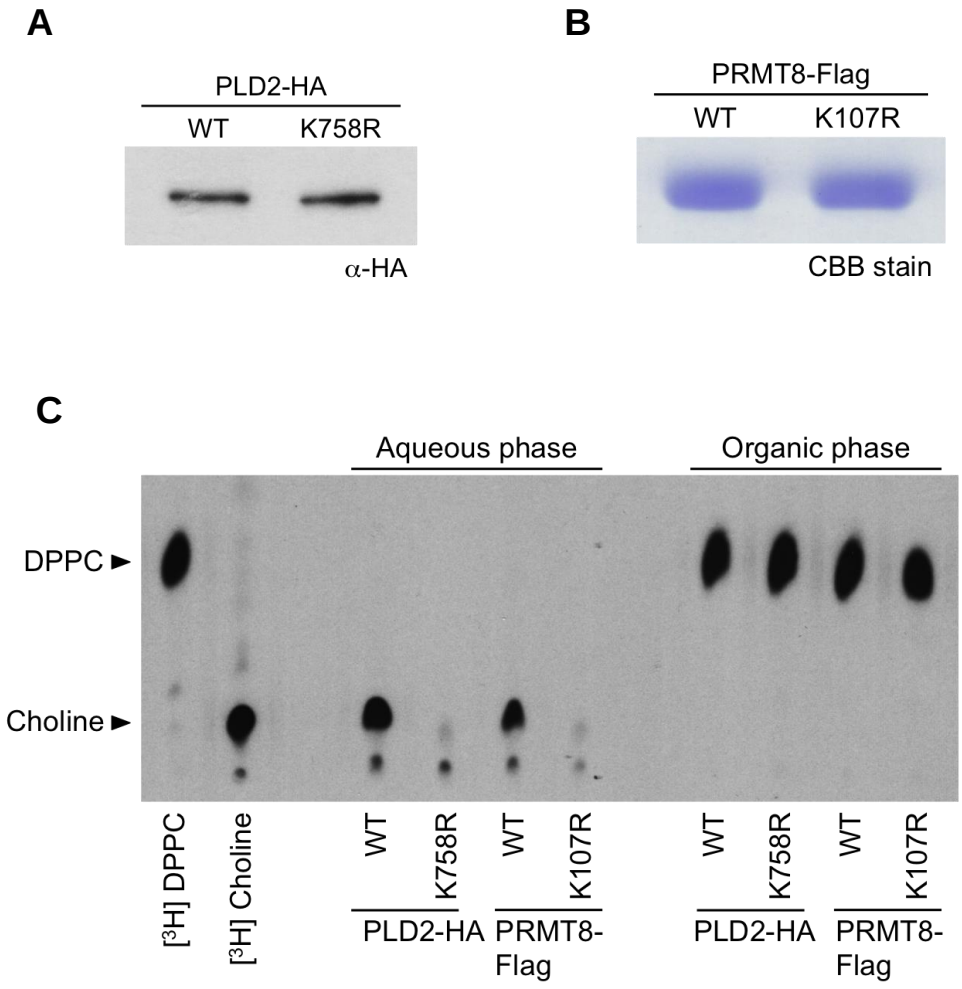


fig. S7. Measuring PRMT8-derived [^{3}H]choline from [^{3}H]DPPC using TLC method. (**A**) HEK293T cells were transfected with plasmids expressing mouse wild-type PLD2-HA or catalytically inactive K758R mutant, as indicated. PLD2-HA

immunoprecipitates were immunoblotted with anti-HA antibody. (**B**) Purified wild-type PRMT8-Flag and the catalytically inactive PRMT8 K107R mutant were visualized by Coomassie blue staining. (**C**) The wild-type PLD2-HA, K758R mutant, wild-type PRMT8-Flag or K107R mutant purified proteins were incubated with ^{3}H-labeled DPPC, and then the water-soluble products were analyzed by TLC. TLC was carried out as described in Supplementary Materials and Methods.

fig. S8

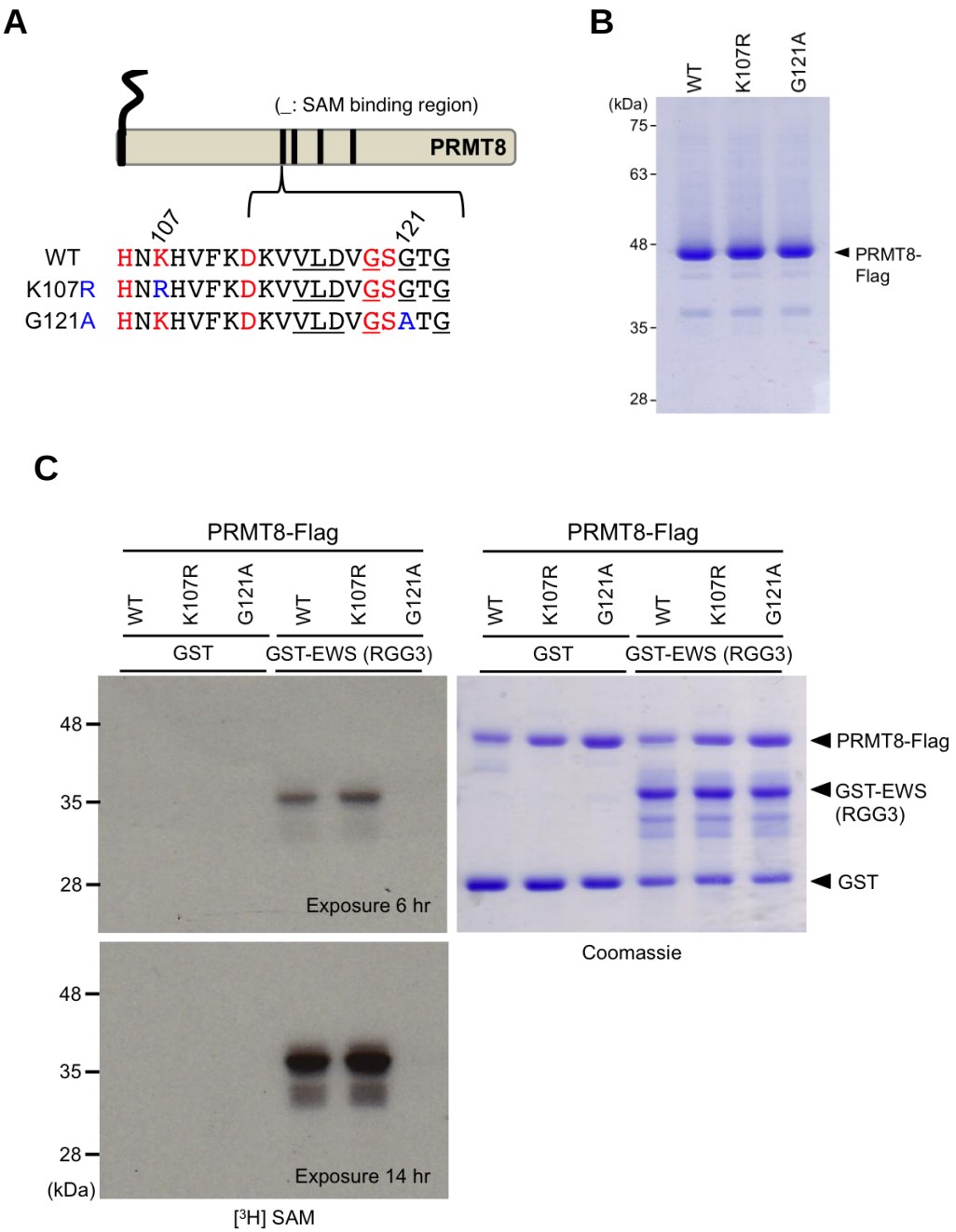

A
(_: SAM binding region)
PRMT8
107
121
WT HNKHVFKDKVVLDVGSGTG
K107R HNRHVFKDKVVLDVGSGTG
G121A HNKHVFKDKVVLDVGSATG
B
WT
K107R
G121A
(kDa)
75
63
48
35
28
PRMT8-Flag
C
PRMT8-Flag
WT
K107R
G121A
GST
GST-EWS (RGG3)
48
35
28
Exposure 6 hr
Exposure 14 hr
(kDa)
[3H] SAM
Coomassie
PRMT8-Flag
GST-EWS (RGG3)
GST

fig. S8

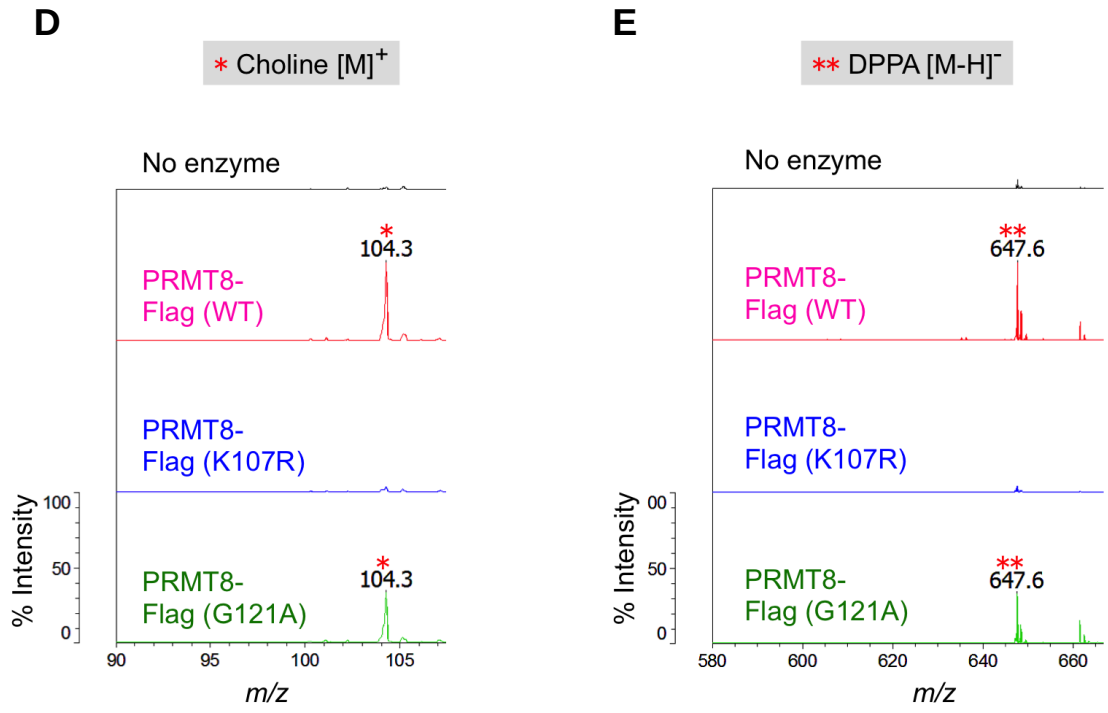


fig. S8. Methyltransferase and PC phospholipase activities of PRMT8. (**A**) Schematic representation of the K107R and the methyltransferase-inactive G121A mutation generated in human PRMT8. (**B**) Purified wild-type PRMT8-Flag, K107R and G121A mutants were visualized by Coomassie blue staining. (**C**) GST-EWS (RGG3) proteins were incubated with wild-type PRMT8-Flag, K107R or G121A mutant in the presence of [^{3}H]SAM. Reaction products were analyzed by autoradiography (Left panels, upper; exposure time 6 h and lower; 14 h) and Coomassie brilliant blue staining (Right panel). (**D** and **E**) MS spectra analyzing choline and DPPA on reaction products

of wild-type PRMT8, K107R or G121A mutants with DPPC. The peaks of choline (*) in positive ion mode and DPPA (**) in negative ion mode were indicated at *m/z* 104.3 $[M]^+$ and at *m/z* 647.6 $[M-H]^-$, respectively.

fig. S9

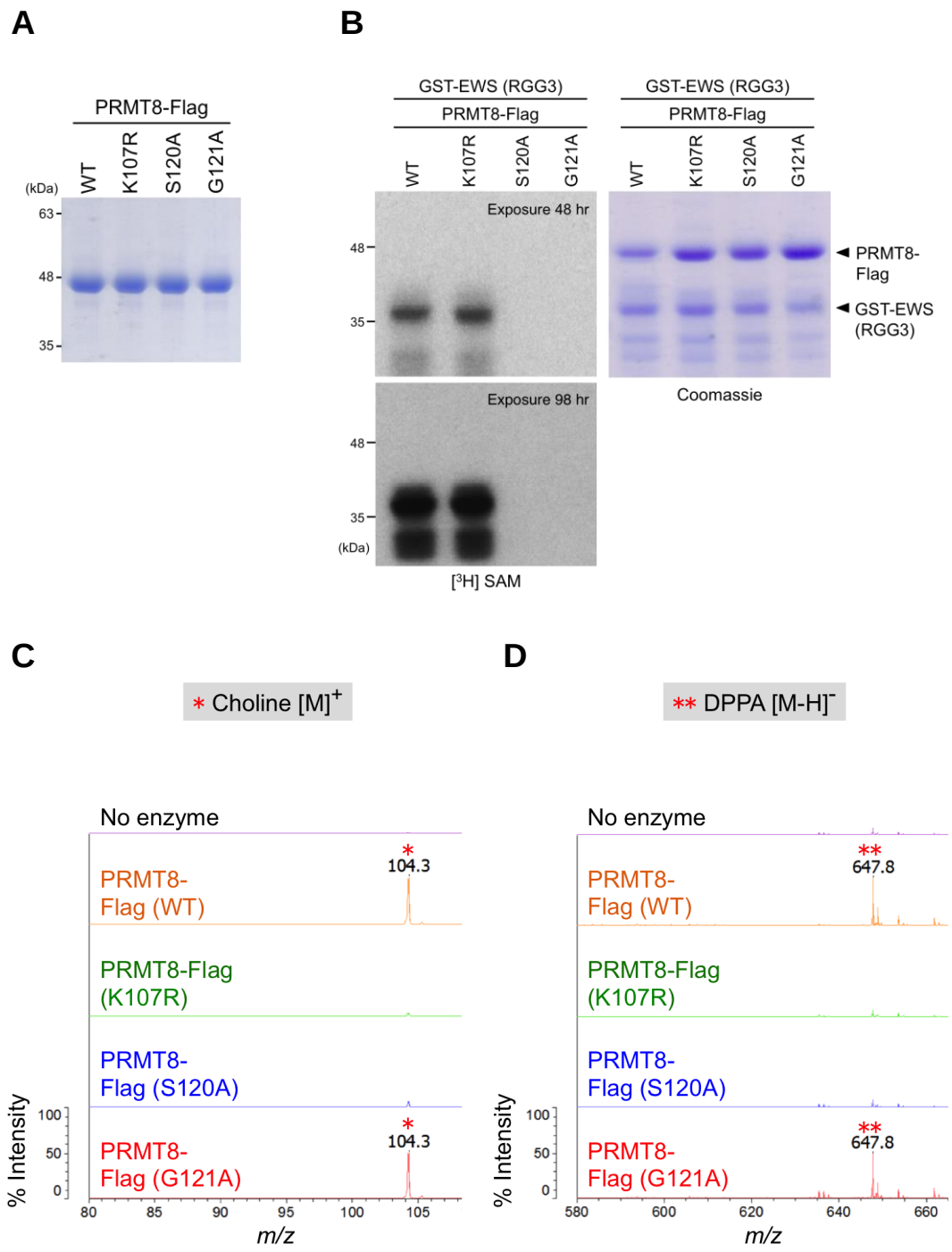


fig. S9. Loss of catalytic activities of PRMT8 by replacing serine 120 to alanine. (**A**) Purified wild-type PRMT8-Flag, K107R, S120A and G121A mutants were visualized

by Coomassie blue staining. (**B**) GST-EWS (RGG3) proteins were incubated with wild-type PRMT8-Flag, K107R, S120A or G121A mutant in the presence of [^{3}H]SAM. Reaction products were analyzed by autoradiography (Left panels, upper; exposure time 48 h and lower; 98 h) and Coomassie brilliant blue staining (Right panel). (**C** and **D**) MS spectra analyzing choline and DPPA on reaction products of wild-type PRMT8, K107R, S120A or G121A mutants with DPPC. The peaks of choline (*) in positive ion mode and DPPA (**) in negative ion mode were indicated at *m/z* 104.3 $[M]^{+}$ and at *m/z* 647.6 $[M-H]^{-}$, respectively.

Table S1

Gene	Detection		Amplicon length (bp)	Accession number
PRMT8	Forward	TTTGCCAGGGACAAGTGGTT	69	NM_201371.2
	Reverse	GGGCAGCTTTGTACGTGGTA		
ChAT	Forward	TGGGTCTCTGAATACTGGCTGA	71	NM_009891
	Reverse	GGGCTAGAGTTGACTGGCAGG		
PLD1	Forward	AGTGCTTCAGACTTGTCCTGGGTT	119	NM_001164056.1
	Reverse	TATGGTAGCGTTTCGAGCTGCTGT		
PLD2	Forward	TTGCGGAAGCACTGTTTCAGTGTG	116	NM_001302475.1
	Reverse	TTGTTCTCCGCTGTTTCTTGCCAC		
GAPDH	Forward	TGTGTCCGTCGTGGATCTGA	150	NM_008084.2
	Reverse	TTGCTGTTGAAGTCGCAGGAG		

PRMT8; protein arginine methyltransferase 8, ChAT; choline acetyltransferase, PLD1; phospholipase D1, PLD2; phospholipase D2, GAPDH; glyceraldehyde 3-phosphate dehydrogenase

Table S1. Primers used for qPCR quantification of mRNA. Primer sequences of the target genes are shown with the expected amplicon length and accession number.

Supplementary movie

Movie S1. Behavior of mice in a novel environment. This movie complements the text. The behavior of 12-week-old homozygous (*prmt8*$^{-/-}$) mouse (right cage) displayed a hyperactive phenotype as compared to wild-type *prmt8*$^{+/+}$ mouse (left cage) in a new cage.