Supporting Information

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SI Methods

Gene Targeting of CMG2 and TEM8. The CMG2 targeting vector was constructed from 3 CMG2 genomic DNA fragments: the 3.8-kb 5′ homology arm from intron 11; the 1.1-kb loxP arm (with a loxP site at the 5′ end) covering the junctions of intron 11, exon 12, and intron 12; and the 5.4-kb 3′ homology arm from intron 12. Similarly, the TEM8 gene targeting vector was constructed from 3 TEM8 genomic DNA fragments: the 4.1-kb 5′ homology arm from intron 12; the 0.9-kb loxP arm (with a loxP site at the 5′ end) covering the junctions of intron 12, exon 13, and intron 13; and the 2.9-kb 3′ homology arm from intron 13. Genomic DNA fragments were obtained by PCR from the C57BL/6 mouse genome, confirmed by DNA sequencing, and cloned into pPacN, a plasmid from Ozgene containing a PKG-Neo expression cassette flanked by 2 FRT sites and one downstream loxP site, to create CMG2 and TEM8 targeting vectors (Fig. S1*A* and Fig. S2*A*). The ES cell injections, screening, and initial mouse breeding work were performed by Ozgene under contract from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Subsequent genotyping was performed by PCR using mouse tail DNA. The TEM8-targeted mice were made in a 129;C57BL/6 mixed background and CMG2-targeted mice in a pure C57BL/6 background. All studies were carried out in accordance with protocols approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

RT-PCR and Real-Time RT-PCR. Total RNA isolated from various mouse tissues by TRIZOL Reagent (Invitrogen) was subjected to reverse transcription reaction using the SuperScript II Reverse Transcriptase (Invitrogen). The 451-bp TM-containing CMG2 cDNA fragment was amplified from WT tissues using a forward and reverse primer pair, 5′-ggaagagcagtcacgtcgatcagtca-3′ and 5′-gacctccgtagtaggaagcgt-3′. These primers could amplify a 355-bp TM-deleted CMG2 cDNA fragment from $CMG2^{-/-}$ tissues. The 410-bp TM-containing TEM8 cDNA fragment was amplified from WT tissues using a forward and reverse primer pair, 5′-Tggcatgaaagctgcactgcaggtcagcat-3′ and 5′-catattcttgctctggcatcttgactcgtg-3′. These primers amplified a 314-bp TM-deleted TEM8 cDNA fragment from $TEM8^{-/-}$ tissues.

For relative quantification of CMG2 and TEM8 expression levels, real-time RT-PCR analyses were carried out using the Applied Biosystems 7000 sequence detection system and the TaqMan gene expression master mix (Applied Biosystems). Primers for detection of CMG2 (catalog no. Mm01196014_g1), TEM8 (Mm00712952_m1), and β-actin (Mm00607939_s1) were purchased from Applied Biosystems. TEM8 and CMG2 expression levels in tissues are presented as amounts relative to those of β-actin in the same tissues.

Protein Purification. PA, PA-U7, PA-3M, LF, and FP59 were purified as previously described (1–3). EF was purified as described (4).

Schild Plot Analysis. In Schild plot analyses, the apparent affinity values can be obtained from cytotoxicity assays using PA + FP59 in the presence of the nontoxic PA protein, PA-U7, in which the furin cleavage sequence RKKR is changed to the noncleavable sequence PGG (5). Cells precooled to 4 °C were incubated with various concentrations of PA plus FP59 (constant at 1.9 nM) in the presence of different fixed concentrations of PA-U7 for 2 h at 4 °C. The cells were then moved to and incubated at 37 °C for 2 h. Then cells were changed with fresh medium and cultured until 48 h followed by cell viability MTT assay. Addition of various fixed concentrations of PA-U7 shifted the cytotoxicity dose-response curves rightward. We determined the EC_{50}s of PA in the presence of each fixed concentration of PA-U7. The Prism program was used to fit the resulting EC_{50}s to the equation $Y = -\log(X + 10\log K_d) - P$ (where $Y = -\log[EC_{50}]$, $X = [PA\text{-}U7]$, and P is a constant) to determine apparent binding K_d of PA-U7 to $TEM8^{-/-}$ MEFs and $CMG2^{-/-}$ MEFs.

Tissue Extract Preparation. $CMG2^{-/-}$ and WT control mice were injected with or without 100 μg of PA. One hour later, the mice were killed, and serum, liver, and kidney were removed. The organs extracts were obtained as the supernatants of the tissues homogenized in serum-free DMEM, allowing extractions of the mixtures of PA and the putative CMG2 decoys in the local tissues. The livers were homogenized in 2 mL of serum-free DMEM per liver, and kidneys (left and right) in 1 mL of serum-free DMEM.

1. Liu S, Leung HJ, Leppla SH (2007) Characterization of the interaction between anthrax toxin and its cellular receptors. *Cell Microbiol* 9:977–987.
2. Rosovitz MJ, et al. (2003) Alanine scanning mutations in domain 4 of anthrax toxin protective antigen reveal residues important for binding to the cellular receptor and to a neutralizing monoclonal antibody. *J Biol Chem* 278:30936–30944.
3. Gupta PK, Moayeri M, Crown D, Fattah RJ, Leppla SH (2008) Role of N-terminal amino acids in the potency of anthrax lethal factor. *PLoS ONE* 3:e3130.
4. Firoved AM, et al. (2005) *Bacillus anthracis* edema toxin causes extensive tissue lesions and rapid lethality in mice. *Am J Pathol* 167:1309–1320.
5. Liu S, Bugge TH, Leppla SH (2001) Targeting of tumor cells by cell surface urokinase plasminogen activator-dependent anthrax toxin. *J Biol Chem* 276:17976–17984.

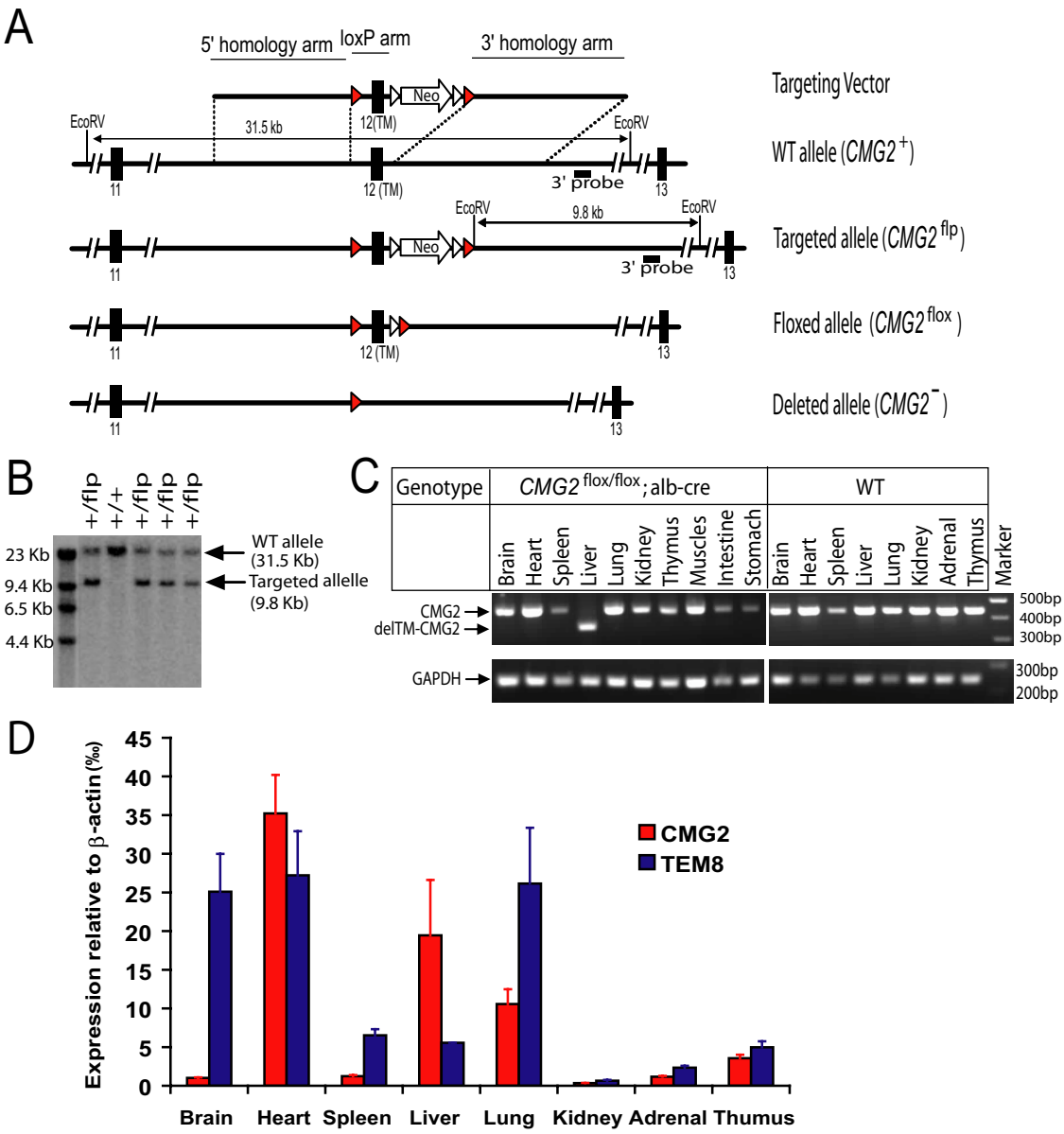

Fig. S1. Targeting of *CMG2* gene. (*A*) Diagram of the WT *CMG2* genomic locus (+), the targeted CMG2 allele (flp), the floxed CMG2 allele (flox), and the CMG2 null allele (−). The white and the red arrowheads indicate FRT and LoxP sites, respectively. (*B*) Representative Southern blot analyses of offspring of *CMG2* targeted mice. Genomic DNA digested with EcoRV was hybridized with a 3′ external probe as shown in (*A*). (*C*) RT-PCR analyses of CMG2 expression in various mouse tissues of WT and liver-specific $CMG2^{-/-}$ mice. Primers flanking the CMG2 TM region were used to amplify a CMG2 cDNA fragment. Note that in the liver-specific $CMG2^{-/-}$ mouse ($CMG2^{flox/flox}$/*Alb-Cre*), the CMG2 TM domain was deleted specifically in the liver. (*D*) Real-time RT-PCR analyses of TEM8 and CMG2 expression in various tissues in WT mice normalized to β-actin. The data are reported as mean of amounts relative to β-actin ± SD.

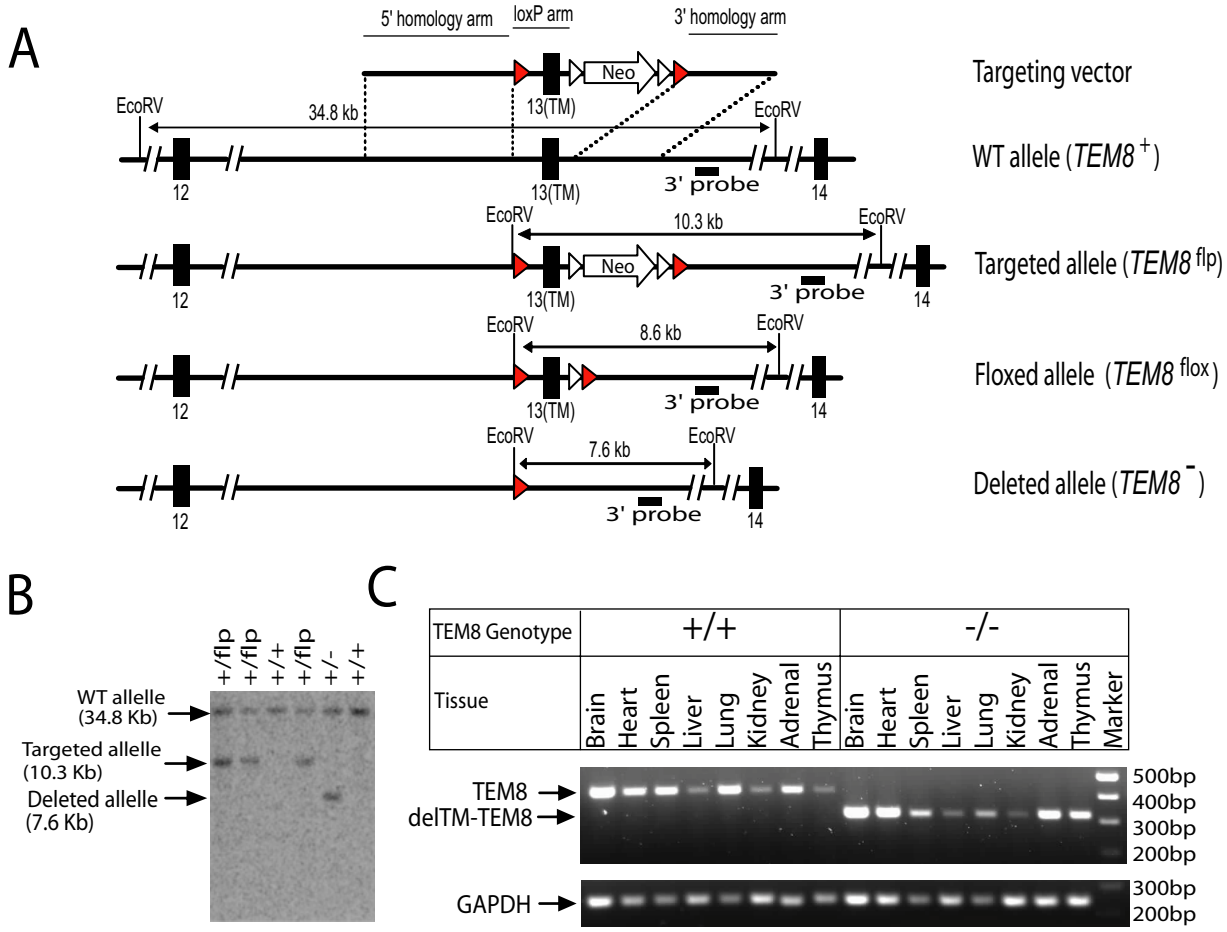

Fig. S2. Targeting of *TEM8* gene. (*A*) Diagram of the WT *TEM8* genomic locus (+), the targeted TEM8 allele (flp), the floxed TEM8 allele (flox), and the TM region deleted TEM8 allele (−). White and red arrowheads indicate FRT and LoxP sites, respectively. (*B*) A representative of Southern blot analyses of the offspring of *TEM8* targeted mice. Mouse tail genomic DNA digested with EcoRV was hybridized with a 3′ external probe as shown in *A*. (*C*) RT-PCR analyses of TEM8 expression in various tissues isolated from WT and $TEM8^{-/-}$ mice. A pair of primers flanking TEM8 TM region were used to show that the TM-deleted TEM8 was still expressed in $TEM8^{-/-}$ mice.

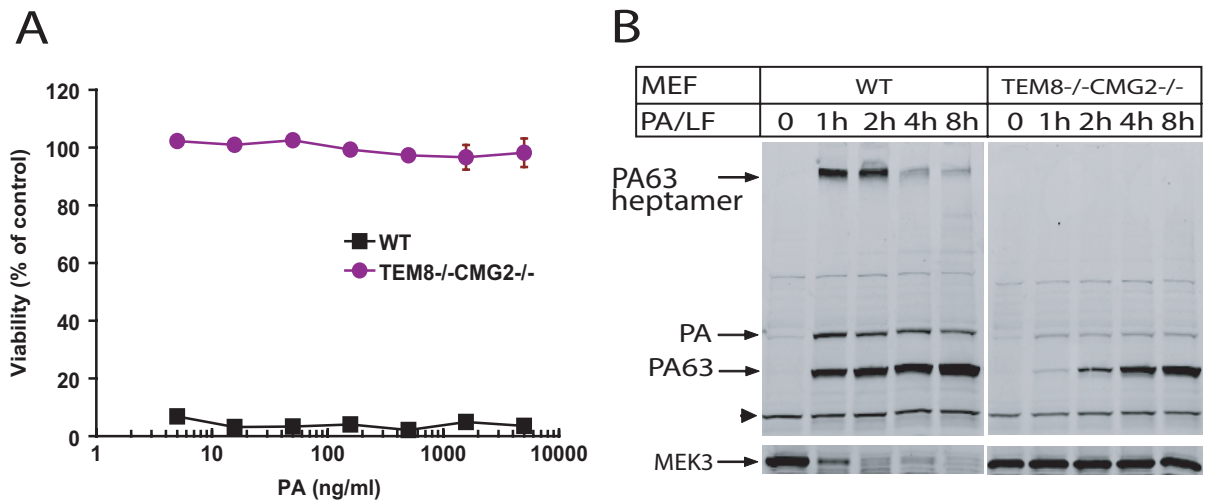

Fig. S3. *TEM8*$^{-/-}$ *CMG2*$^{-/-}$ MEFs are totally resistant to PA in the presence of FP59. (*A*) Cytotoxicity of PA plus FP59 to WT and *TEM8*$^{-/-}$ *CMG2*$^{-/-}$ MEFs. MEFs were treated with various concentrations of PA (0–5000 ng/mL) plus FP59 (1000 ng/mL) for 48 h at 37 °C before assessing cell viability using MTT. Data are reported as mean viability ± SD. (*B*) Binding and processing of PA on WT and *TEM8*$^{-/-}$ *CMG2*$^{-/-}$ MEFs. Cells were incubated with 1 μg/mL PA and 1 μg/mL LF at 37 °C for various time as indicated. Cells were washed, lysed, and cell lysates subjected to Western blotting using either a PA antiserum or a MEK3 antibody. A nonspecific cross-reactive band indicated by the arrow head at the left of the images serves as protein loading controls.

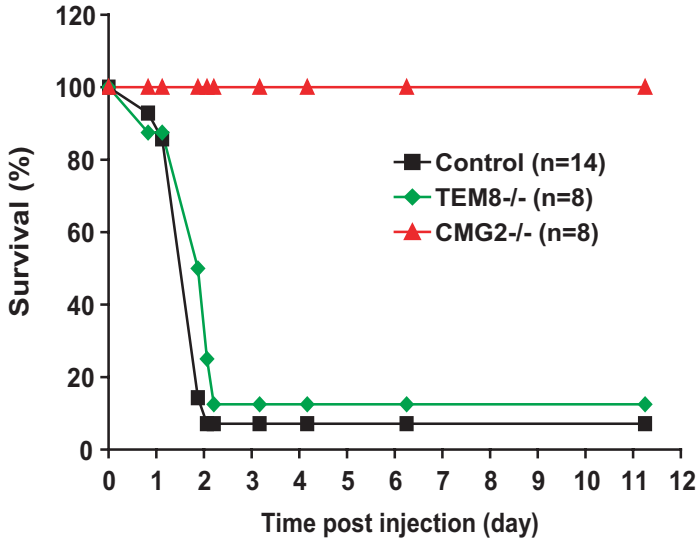

Fig. S4. *CMG2*$^{-/-}$ mice but not *TEM8*$^{-/-}$ mice were completely resistant to one dose of 50-μg ET challenge. *TEM8*$^{-/-}$ and *CMG2*$^{-/-}$ mice and their littermate control (WT, *TEM8*$^{+/-}$, and *CMG2*$^{+/-}$) mice were intravenously treated with ET (50 μg) and monitored for survival.

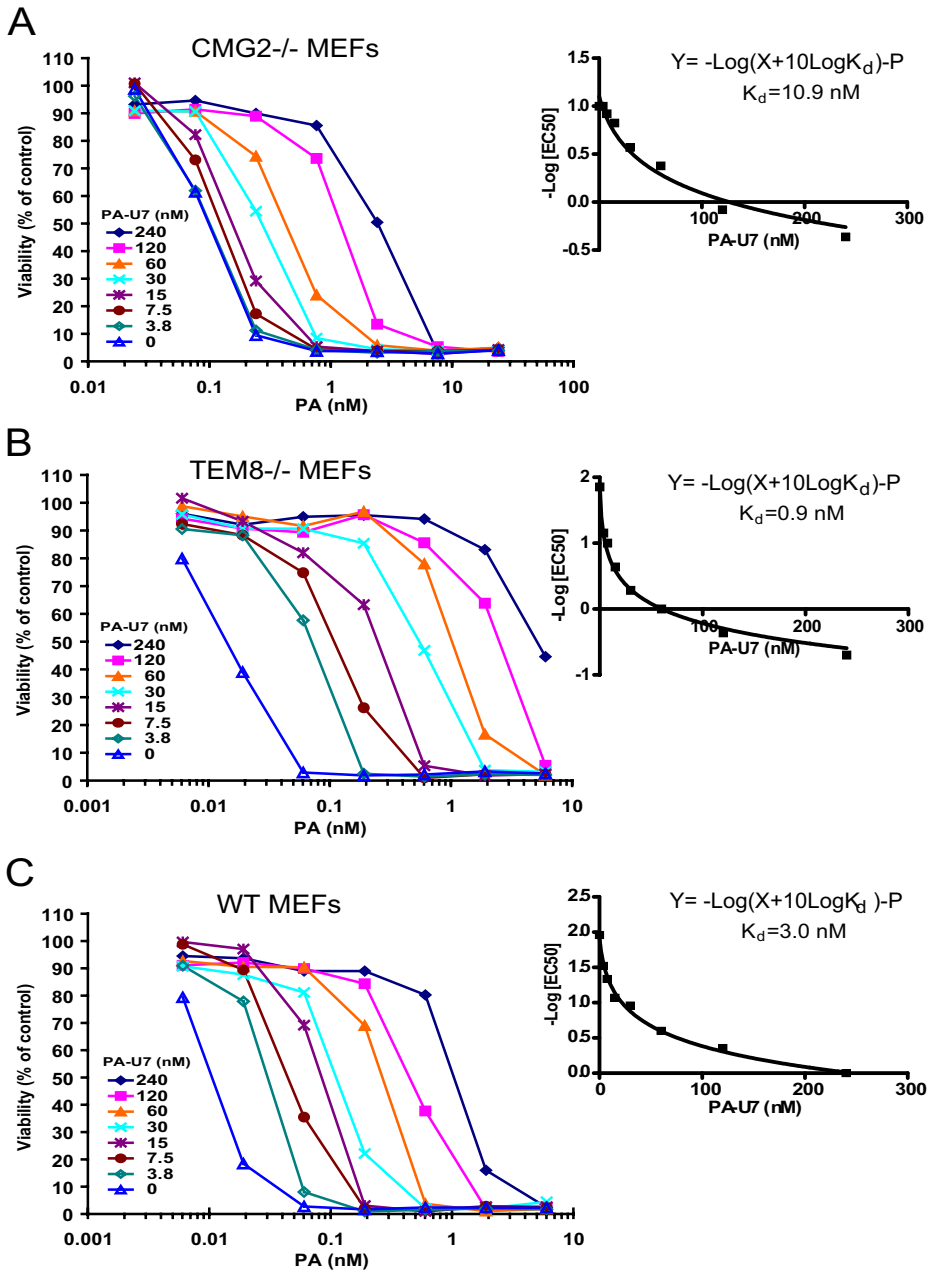

Fig. S5. CMG2 has a higher affinity than TEM8 for PA. (*A*) Evaluation of the apparent binding affinities of PA-U7 to TEM8-expressing MEFs (*CMG2*$^{-/-}$), (*B*) CMG2-expressing MEFs (*TEM8*$^{-/-}$), and (C) WT MEFs expressing both receptors using Schild plot analyses. The TEM8-, CMG2-, and both TEM8- and CMG2-expressing MEFs were incubated with various concentrations of PA plus FP59 (constant at 1.9 nM) and different concentrations of PA-U7 as indicated for 2 h at 4 °C. Then the cells were moved to 37 °C and incubated for 2 h. After that, toxins were removed and the cells were incubated with the toxin-free medium for 48 h, and cell viability was determined by MTT assay. Schild plot calculations were performed as described in *SI Methods*. The inserts shown in *A*–*C* are the regression curves obtained using GraphPad Prism. The data demonstrates that PA has a 11-fold higher binding affinity for CMG2 than for TEM8 (K_d values 0.9 nM vs. 10.9 nM).

Table S1. Genotyping analyses of offspring of TEM8 and CMG2 heterozygous intercrosses reveal that both *TEM8*$^{-/-}$ and *CMG2*$^{-/-}$ mice are viable

	No. of progeny genotyped as			
Genotyping	WT	Heterogeneous	Homozygous	Total
TEM8	69 (24.5%)	141 (50.2%)	71 (25.3%)	281 (100%)
CMG2	61 (28.8%)	98 (46.2%)	53 (25.0%)	212 (100%)

Genotyping was performed on mice at weaning age. In parentheses are the percentage of the mice genotyped. No sex bias was observed in various genotypes.

Table S2. Infertility of female *TEM8*$^{-/-}$ and *CMG2*$^{-/-}$ mice

Breeder					
Female	Male	No. of pairs	No. of pups	Pups/breeder	*P**
TEM8$^{+/-}$	*TEM8*$^{+/-}$	9	376	42	Control
TEM8$^{-/-}$	*TEM8*$^{-/-}$	6	0	0	<0.0000001
TEM8$^{-/-}$	*TEM8*$^{+/-}$	8	0	0	<0.0000001
TEM8$^{+/-}$	*TEM8*$^{-/-}$	4	164	41	0.88
CMG2$^{+/-}$	*CMG2*$^{+/-}$	5	192	38	Control
CMG2$^{-/-}$	*CMG2*$^{-/-}$	8	0	0	<0.00001
CMG2$^{-/-}$	*CMG2*$^{+/-}$	8	0	0	<0.00001
CMG2$^{+/-}$	*CMG2*$^{-/-}$	4	123	31	0.49

The data were obtained during a 5-month breeding period.

*The significance of differences between the tested breeders and the *TEM8* or *CMG2* heterozygous intercrosses was obtained by two-tailed Student's *t* test using Microsoft Excel.

Table S3. Sensitivity of MEFs with various genotypes to PA in the presence of FP59

MEF genotype	EC_{50} of PA from independent experiments, ng/ml	Average EC_{50}	
		ng/ml	Relative to WT
WT	1.8; 2.0; 1.6; 1.7	1.8	1.0
TEM8$^{-/-}$	2.8; 2.3; 2.6; 2.6, 3.2	2.7	1.5
CMG2$^{-/-}$	12; 12; 12; 10; 13; 13	12	6.7
CMG2$^{+/-}$	3.1; 2.5; 2.5; 2.2; 2.6	2.6	1.5

EC_{50}: the concentration of PA needed to kill 50% of cells in the presence of FP59 (100 ng/ml) in cytotoxicity assay as described in *Materials and Methods*.