SUPPLEMENTARY INFORMATION

An improved Red/ET recombineering system and mouse ES cell culture conditions for the generation of targeted mutant mice

Katsuyoshi Kumagai,1,* Masakatsu Takanashi,2 Shin-ichiro Ohno,2 Masahiko Kuroda,2 and Katsuko Sudo1,*

1Pre-clinical Research Center, University-related Facilities, Tokyo Medical University, Tokyo, Japan
2Department of Pathology, Tokyo Medical University, Tokyo, Japan
* Address all correspondence to Katsuko Sudo, Ph.D., or Katsuyoshi Kumagai, Ph.D.
Animal Research Center, University-related Facilities, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan
Tel.: 81-3-3351-6141
Fax: 81-3-3226-7030
e-mail: ksudo@tokyo-med.ac.jp (Katsuko Sudo), kumagai@tokyo-med.ac.jp (Katsuyoshi Kumagai)
Supplementary Figure S1. Establishment of germline-competent ES cell lines derived from C57BL/6J mice

Morphology and characterization of the ES-like cells derived from C57BL/6J mice. (A) Blastocysts were plated onto 96-well tissue culture plates with a feeder layer using KSR medium. The inner cell mass (ICM) was isolated from the fertilized eggs on day 5 (black arrowhead). ES-like colonies were isolated after dissociation of the ICM, and the cells reached 70%-80% confluence on day 14. (B) Chromosome number analysis demonstrated a karyotype of 40 XY. Expression of alkaline phosphatase (ALP) (C), SSEA-1 (D) and Oct-4 (E) was detected by immunocytochemistry (representative
images obtained from line #2 are shown). (F) Chimeric mice derived from the C57BL/6J ES cell line. F0-chimeric mice, including 100% F0-chimeric mice (yellow arrowhead) and F0 mice with less than 100% chimerism. (G) Analysis of germline transmission by coat color analysis after natural mating between F0-chimeric mice and ICR mice. F1 mice with an agouti coat color (blue arrowhead) were generated from the 100% F0-chimeric mice, confirming germline transmission. In contrast, no F1 mice with an agouti coat color were generated from the F0 mice with less than 100% chimerism. Scale bars represent 200 μm.
SUPPLEMENTARY METHODS

Construction of the Wapl gene cKO targeting vector

A schematic diagram of the experimental procedure is shown, followed by a step-by-step description of the method.

**BAC preparation**

**Day 0**

The C57BL/6J BAC clone RP23_478G5 containing the Wapl gene, which was obtained from Invitrogen Life Technologies Japan (Tokyo, Japan), was cultured on a Lysogeny Broth (LB) plate containing 15 μg/mL of chloramphenicol (cm) (LB + cm plate) at 37 °C overnight.
**Gene retrieval**

**Day 1**

Two colonies growing on the LB plate were cultured in 1 mL of LB + cm (15 μg/mL) medium with shaking at 37 °C overnight.

**Day 2**

1) LB medium (1.4mL) in a microfuge tube was inoculated with 30 μL of the fresh starter culture and incubated for 2 hours at 37 °C with shaking at 1,000 rpm.
2) In an eppendorf tube, 100 ng of pRed/ET plasmid (GENE BRIDGES, Heidelberg, Germany) was mixed with 18 μL of freshly prepared cells.
3) The mixture was transferred to a 1-mm electroporation cuvette, and electroporated at 1,800 V.
4) After electroporation, 1 mL of fresh LB medium was added, and the sample was cultured for 70 min at 30 °C with shaking at 1,000 rpm, and then incubated on an LB plate containing 15 μg/mL of cm and 3 μg/mL of tetracycline (tet) (LB + cm/tet plate) at 30 °C overnight.

**Day 3**

Eight colonies growing on the LB + cm/tet plate were cultured in 1 mL LB + cm (15 μg/mL)/tet (3 μg/mL) medium with shaking at 30 °C overnight.

**Day 4**

1) LB medium (1.4mL) in a microfuge tube was inoculated with 30 μL of the fresh
starter culture and incubated for 2 hours at 30 °C with shaking at 1,000 rpm.

2) Cells were cultured in medium with 50 μL of 10% L-arabinose (Sigma) for 45 min at 37 °C with shaking at 1,000 rpm.

3) In an eppendorf tube, 40 ng of the gene retrieval vector for the exon 3 (E3) and exon 4 (E4) region of the WapI gene was mixed with 18 μL of freshly prepared cells.

4) The mixture was transferred to a 1-mm electroporation cuvette, and electroporated at 1,800 V.

5) After electroporation, 1 mL of fresh LB medium was added, and the sample was cultured for 5 hours at 37 °C with shaking at 1,000 rpm, and then incubated on an LB plate containing 100 μg/mL of ampicillin (amp) (LB + amp plate) at 37 °C overnight.

**Day 5**

1) Transformants were selected by static culturing in 1 mL of LB + amp (100 μg/mL) medium at 30 °C overnight.

2) Positive clones containing the regions indicated in red were identified by PCR analysis (using primer pairs PCR #5 and #6 shown in Supplementary Table S1).

**Insertion of the loxP-neo-loxP-cassette into a plasmid**

**Day 6**

1) LB medium (1.4 mL) in a microfuge tube was inoculated with 30 μL of fresh starter culture and incubated for 2 hours at 37 °C with shaking at 1,000 rpm.

2) In an eppendorf tube, 100 ng of the pRed/ET plasmid was mixed with 18 μL of freshly prepared cells.

3) The mixture was transferred to a 1-mm electroporation cuvette, and electroporated at
1,800 V.

4) After electroporation, 1 mL of fresh LB medium was added, and the sample was cultured for 70 min at 30 °C with shaking at 1,000 rpm, and then incubated on an LB plate containing 100 μg/mL of amp and 3 μg/mL of tet (LB + amp/tet plate) at 30 °C overnight.

**Day 7**

Two colonies growing on the LB + amp/tet plate were cultured with shaking in 1 mL of LB + amp (100 μg/mL)/tet (3 μg/mL) medium at 30 °C overnight.

**Day 8**

1) LB medium (1.4 mL) in a microfuge tube was inoculated with 30 μL of fresh starter culture for 2 hours at 30 °C with shaking at 1,000 rpm.

2) Cells were cultured in medium containing 50 μL of 10% L-arabinose for 45 min at 37 °C with shaking at 1,000 rpm.

3) In an eppendorf tube, 313 ng of the loxP-neo-loxP-cassette that was amplified by PCR (primer pairs shown in Supplementary Table S2) was mixed with 18 μL of freshly prepared cells.

4) The mixture was transferred to a 1-mm electroporation cuvette, and electroporated at 1,800 V.

5) After electroporation, 1 mL of fresh LB medium was added, and the sample was cultured for 5 hours at 37 °C with shaking at 1,000 rpm, and then incubated on an LB plate containing 100 μg/mL of amp and 50 μg/mL of kanamycin (kan) (LB + amp/kan plate) at 37 °C overnight.
Day 9

1) Transformants were selected by culturing in 1 mL LB + amp (100 μg/mL)/kan (50 μg/mL) medium with shaking at 37 °C overnight.

2) Positive clones containing the regions indicated in green were identified by PCR analysis (using primer pairs PCR #7 and #8 shown in Supplementary Table S2).

Deletion of the kanamycin/neomycin selection maker by Cre expression

Day 10

1) LB medium (1.4 mL) in a microfuge tube was inoculated with 30 μL of fresh starter culture, and incubated for 2 hours at 37 °C with shaking at 1,000 rpm.

2) In an eppendorf tube, 87 ng of the 706-Cre plasmid (GENEBRIDGES) was mixed with 18 μL of freshly prepared cells.

3) The mixture was transferred to a 1-mm electroporation cuvette, and electroporated at 1,800 V.

4) After electroporation, 1 mL of fresh LB medium was added, and the sample was cultured for 70 min at 30 °C with shaking at 1,000 rpm, and then incubated on an LB plate containing 100 μg/mL of amp, 50 μg/mL of kan, and 3 μg/mL of tet (LB + amp/kan/tet plate) at 30 °C overnight.

Day 11

Transformants were selected for recombineering of the loxP site by their expression of Cre recombinase, by culturing in 1 mL of LB + amp (100μg/mL) medium with shaking at 30 °C for 2 hours followed by 37 °C overnight.
Day 12

Recombineer of the loxP site was identified by PCR analysis (primer pairs are shown in Supplementary Table S2).

**Insertion of the frt-neo-frt-loxP-cassette onto a plasmid**

1) LB medium (1.4 mL) in a microfuge tube was inoculated with 30 μL of fresh starter culture for 2 hours at 37 °C with shaking at 1,000 rpm.

2) In an eppendorf tube, 100 ng of the pRed/ET plasmid was mixed with 18 μL of freshly prepared cells.

3) The mixture was transferred to a 1-mm electroporation cuvette, and electroporated at 1,800 V.

4) After electroporation, 1 mL of fresh LB medium was added, cultured for 70 min at 30°C with shaking at 1,000 rpm, and then incubated on an LB plate containing 100 μg/mL of amp and 3 μg/mL of tet (LB + amp/tet plate) at 30 °C overnight.

Day 13

Two colonies growing on the LB + amp/tet plate were cultured with shaking in 1 mL LB + amp (100 μg/mL)/tet (3 μg/mL) medium at 30 °C overnight.

Day 14

1) LB medium (1.4 mL) was inoculated with 30 μL of fresh starter culture and incubated for 2 hours at 30 °C with shaking at 1,000 rpm.

2) Cells were cultured in medium containing 50 μL of 10% L-arabinose for 45 min at
37 °C with shaking at 1,000 rpm.

3) In an eppendorf tube, 300 ng of the frt-neo-frt-loxP-cassette that was amplified by PCR (primer pairs shown in Supplementary Table S2) was mixed with 18 μL of freshly prepared cells.

4) The mixture was transferred to a 1-mm electroporation cuvette, and electroporated at 1,800 V.

5) After electroporation, 1 mL of fresh LB medium was added, and the sample was cultured for 4 hours at 37 °C with shaking at 1,000 rpm, and then incubated on an LB plate containing 100 μg/mL of amp and 50 μg/mL of kan (LB + amp/kan plate) at 37 °C overnight.

**Day 15**

1) Transformants were selected by culturing in 1 mL of LB + amp (100 μg/mL)/kan (50 μg/mL) medium with shaking at 37 °C overnight.

2) Positive clones containing the regions indicated in blue were identified by PCR analysis (using primer pairs #9 and #10, shown in Supplementary Table S2).

3) The sequence of the 5’ loxP site of the PCR-positive clones was confirmed using the loxP_seq_1 primer and that of the 3’ loxP site was confirmed using the fgl_R2 primer (Supplementary Table S2).
### Supplementary Table S1

| Target gene | Experiment | Primer name | Sequence (5' to 3') | Product size |
|-------------|------------|-------------|---------------------|--------------|
| **Wapl**   | Amplification of the 5' homology arm of the retrieval vector | Retrieve_long_F4 | AAAACCGCGCAGCGATACA CTGCTCATGCTA | 422 bp |
|            |            | Retrieve_long_R4 | AAAACCGCGGCAGAACTA AGACTGCGACAGGA | |
|            | Amplification of the 3' homology arm of the retrieval vector | Retrieve_short_F1 | AAAACCGGCAGTTGTG GTGTGATGTGATG | 424 bp |
|            |            | Retrieve_short_R1 | AAAAAGACTGCTATCTA ATAAGGAAATTCT | |
|            | Confirmation of amplification of the gene retrieval vectors | ret_F1 | ATGGAACTGATGATGGG AGC | 977 bp |
|            |            | ret_R4 | TTTCCCAGTCACGACGTTG T | |
|            | PCR 1      | ret_F1 | ATGGAACTGATGATGGG AGC | 530 bp |
|            |            | ret_R7 | GTACCAGACCTGCCAGAG GAA | |
|            | PCR 2      | ret_F5 | GTCCCCATCAAAATATGATTG AAT | 588 bp |
|            |            | ret_R4 | TTTCCCAGTCACGACGTTG T | |
| **Pgr**    | Amplification of the 5' homology arm of the retrieval vector | Retrieve_long_F7 | AAAACCGCGCGCCAAATA CCACCTCTGTAGC | 422 bp |
|            |            | Retrieve_long_R7 | AAAACCGCGCAACCAATTA GCATAAGATGAAC | |
|            | Amplification of the 3' homology arm of the retrieval vector | Retrieve_short_F7 | AAAACCGCGCGCAGCA TAACCTAAACCTAG | 424 bp |
|            |            | Retrieve_short_R7 | AAAACCGCGCGCAATTTA TTGTATGCTTTA | |
|            | Confirmation of amplification of the retrieval vector | ret_F1 | ATGGAACTGATGATGGG AGC | 977 bp |
|            |            | ret_R4 | TTTCCCAGTCACGACGTTG T | |
|            | PCR 3      | ret_F1 | ATGGAACTGATGATGGG AGC | 539 bp |
|            |            | ret_R8 | AGGGAGAAGGAAATCCAA GGA | |
|            | PCR 4      | ret_F8 | GAGGAAACACGCACAGCTGA TTC | 524 bp |
|            |            | ret_R4 | TTTCCCAGTCACGACGTTG T | |
### Supplementary Table S2

**Primers for generating the conditional *Wap1* gene targeting vector in this study**

| Experiment | Primer name | Sequence (5' to 3') | Product size |
|------------|-------------|---------------------|--------------|
| Amplification of the loxP-Neo-loxP-cassette | loxP_Neo_F1 | ACAGTAGGAGAGTCTAATGTGTAA ACTTTTTGGTTTTTATTCTAGAT TAATTAACCCCTACTAAAGGCGC | 1,737 bp |
| | loxP_Neo_R1 | TGTCTTCAGACACTCAGAGAGG GCCAGACATCTGTGTTACAGATGTT TGAAATACGACTCATACTGAGGCT | |
| Confirmation of insertion of the loxP-Neo-loxP-cassette into the BAC | PCR 5 | gb_F2 | CTCTTCAGATGGCATAAGCAA | 379 bp |
| | | gb_R2 | TAGGGGAGGAGTAGAAGGTC | |
| | PCR 6 | gb_F3 | TACTAGAAGAGATCGCAGTG | 325 bp |
| | | gb_R2 | TAGGGGAGGAGTAGAAGGTC | |
| Confirmation of the excision of Neo from the BAC | PCR 5 | gb_F2 | CTCTTCAGATGGCATAAGCAA | 379 bp |
| | | gb_R2 | TAGGGGAGGAGTAGAAGGTC | |
| Confirmation of the presence of the 5' loxP site | | loxP_R1 | ATAACTTCGTATAATGTACGATAT | 195 bp |
| Amplification of the Frt-Neo-Frt-loxP-cassette | fneol_F1 | TGTCTAAATATGGCATTAAATA ATTTTCTGGCGGAGAGATGTC TGAAATACGACTCATACTGAGGCT | 1,777 bp |
| | fneol_R1 | GAGGGCATCAGATCAGATTTAAAA CAGTGTGAGGCCAGACATCAGTGTGGA CGTATAACGACTCATACTGAGGCT | |
| Confirmation of insertion of the Frt-Neo-Frt-loxP-cassette into the BAC | PCR 7 | fgl_F1 | GCCCCCAAACGGGTAAGTA | 356 bp |
| | | fgl_R1 | CGCCCTTGGTAGGGGTAAAT | |
| Confirmation of the existence of the 3' loxP site | PCR 8 | fgl_F2 | CTCCCCGTGACATGTCGGA | 404 bp |
| | | fgl_R2 | AGACTAGTGCACATGTCGCAAG | |
| 5' loxP sequence | | loxP_seq_1 | TTGGTGTCCAGGGGACGGA | - |
| 3' loxP sequence | | fgl_R2 | AGACTAGTGCACATGTCGCAAG | - |
## Germline transmission analysis of B6JES cell lines

| Cell lines | 0%-49% | 50%-99% | 100% | No. of F0 chimeric mice | No. of weaned | No. of GLT\(^b\) in |
|------------|--------|---------|------|-------------------------|--------------|-----------------|
| B6JES-#1   | 0      | 0       | 4    | 0                       | 0            | 3/3             |
| B6JES-#2   | 0      | 1       | 5    | 0                       | 0            | 0/1             |
| B6JES-#3   | 0      | 1       | 4    | 0                       | 0            | 0/1             |
| B6JES-#4   | 0      | 0       | 6    | 0                       | 0            | 0/1             |

\(^a\)100% F0-chimeric male mice

\(^b\)GLT, germline transmission
| Genes | No. of picked up ES cells | No. of homologous recombinations in ES cells (%)<sup>a</sup> | No. of F0-chimeric mice | No. of mice with a high contribution (> 70% chimerism) of ES cells (%)<sup>b</sup> | No. of GLT<sup>d</sup> in F0-chimeric mice (%)<sup>c</sup> |
|-------|--------------------------|---------------------------------|------------------------|-------------------------------------------------|--------------------------|
| A     | 256                      | 14 (5.5)                        | 9                      | 6 (66.7)                                         | 5 (55.6)                 |
| B     | 256                      | 8 (3.1)                         | 10                     | 5 (50.0)                                         | 3 (30.0)                 |
| C     | 435                      | 4 (0.9)                         | 7                      | 3 (42.9)                                         | 2 (28.6)                 |
| D     | 544                      | 3 (0.6)                         | 12                     | 5 (41.7)                                         | 2 (16.6)                 |

<sup>a</sup> No. of ES cells showing homologous recombination/no. of ES cells picked up  
<sup>b</sup> No. of mice showing a high contribution (> 70% chimerism) of ES cells/total no. of F0-chimeric  
<sup>c</sup> No. of F0 chimeric mice showing GLT/total no. of F0 chimeric mice  
<sup>d</sup> GLT, germline transmission