Bmi1 expression in long-term germ stem cells

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Supplementary Figure Legends

Figure S1. Detection of Bmi1^{High}-derived and PLZF-positive A_{paired} cells in cross sections of seminiferous tubules obtained from *Bmi1$^{creER/+}$/Rosa26$^{Rbw/+}$* mice 7 days after tamoxifen labeling.

The cross sections of seminiferous tubules were stained with anti-PLZF antibody followed by Alexa Fluor 750-labeled secondary antibody. Many PLZF-positive cells (purple) were observed and Bmi1^{High}-derived and PLZF-positive A_{paired} cells were detected. White windows at the right upper corner; magnified pictures of rectangle areas (dotted line). Red arrowheads indicate Bmi1^{High}-derived and PLZF-positive A_{paired} cells. Scale bar = 100μm.

Figure S2. A representative littermates produced by mating B6 mice (female) and *Bmi1$^{creER/+}$/Rosa26$^{Rbw/+}$* mice (male) 10 weeks after tamoxifen administration.

Four fluorescent blue color-stained (indicated by red arrowheads), three fluorescent green color-stained (green arrowheads) and one nonfluorescent (white arrow head) pups are seen.

Figure S3. Tamoxifen does not significantly increase apoptotic germ cells at the dose we used in this study.

(a) B6 mice were injected with tamoxifen and the testes were collected from the mice 12 or 24 hours later. Cross sections of seminiferous tubules from the mice were stained with TUNEL method. A small number of brown-colored nuculeus were detected. Scale bar = 100μm. (b) Percentages of tubules containing TUNEL-positive cells. NS: not

significant.

Figure S4. Comparison of expression of GSC markers in Bmi1^{High}-positive and Bmi1^{Low}-positive cell populations in *Bmi1$^{GFP/+}$* mice without tamoxifen administration.

Male germ cells obtained from *Bmi1$^{GFP/+}$* mice were double-stained with anti-MCAM and c-Kit monoclonal antibodies. (a) SSC/FSC profile of the stained cells. (b) MCAM and c-Kit expression on the Pl gated population of the SSC/FSC profile (a). (c) GFP (Bmi1) expression on the P2 gated population (MCAM-positive and C-Kit-negative cells) of (b). (d) GFP (Bmi1) expression on the P3 gated population (MCAM- and C-Kit-double positive cells) of (b). (e) GFP (Bmi1) expression on the P2+P3+P4 gated populations of (b). (f) Relative gene expression levels in Bmi1^{High}-positive cells and Bmi1^{Low}-positive cells. From the P2+P3+P4 gated populations (e), 1000 GFP^{High} (Bmi1^{High})-positive or GFP^{Low} (Bmi1^{Low})-positive cells were sorted and their mRNA was extracted. cDNA was synthesized from the mRNA and quantitative real-time PCR reactions were performed using various primers such as GFRα1, ID4, Nanos2, Ngn3 and c-Kit. Primers are listed in Table S1. Average of 3 independent experiments. *: $P < 0.05$.

Figure S5. Representative serial cross sections of seminiferous tubes obtained from *Bmi1$^{creER/+}$/Rosa26$^{Rbw/+}$* mice 2 days after tamoxifen labeling.

(a) One A_{single} cell and (b) one A_{paired} cell can be defined by serial cross-sections. Thickness of each cross section was 5 μm, and the sections were cut along long axis.

White windows at the right upper corner; magnified pictures of rectangle areas (dotted line), in which merged images with Hoechst 33342 counter staining (white) are shown. Red arrowheads indicate Bmi1^{High}-derived cells. Scale bar = 100μm. *: Higher magnification of the photographs revealed that small red dots were not cells, but non-specific artifacts.

Figure S6. Increase in the number of patches derived from Bmi1^{High}-positive cells by busulfan induced injury.

(a) Fluorescence image of seminiferous tubes obtained from *Bmi1$^{creER/+}$/Rosa26$^{Rbw/+}$* mice injected with busulfan. *Bmi1$^{creER/+}$/Rosa26$^{Rbw/+}$* mice that had received tamoxifen 2 days before were administered a single dose of busulfan. Twelve weeks later, the testes of the mice were removed and the number of patches was counted. Scale bars = 5 mm in upper panel, 2 mm in middle panel. (b) The injections of busulfan significantly unregulated the number of patches compared with that of untreated mice. $P < 0.01$ by unpaired Student's t-test. w; weeks.

Figure S7. Relationship between "delayed" and "actual" seminiferous stages.

Because of 2-day-delay between tamoxifen injection and analyses of labeled cells, the stage in which original Bmi1^{High}-positive cells exist should be back-calculated from the stage in which Bmi1^{High}-derived cells were observed.

Figure S8. Relationship between GFRα1-positive A_{single} cells and their seminiferous stages.

(a and b) (left) GFRα1-positive (red) cells (right) Hoechst 33342 counter staining

(white) (c) PAS-Hematoxylin staining of continuous specimen to (a). Upper right panel was the magnified images of white square. (d) PAS-Hematoxylin staining of continuous specimen to (b). Lower right panel was the magnified images of white square. Arrow heads: GFRα1-positive A_{single} cells. Roman numbers indicate seminiferous epithelial stages (actual stages). Scale bars = 100 μm

Supplemental movie

Serial images spanning 100 μm of seminiferous tubules in the testis from *Bmi1*$^{creER/+}$/*Rosa26*$^{Rbw/+}$ mice 12 weeks after tamoxifen induction. Hoechst 33342 counter staining is shown in light blue color.

Supplemental Tables

Table S1, List of primers

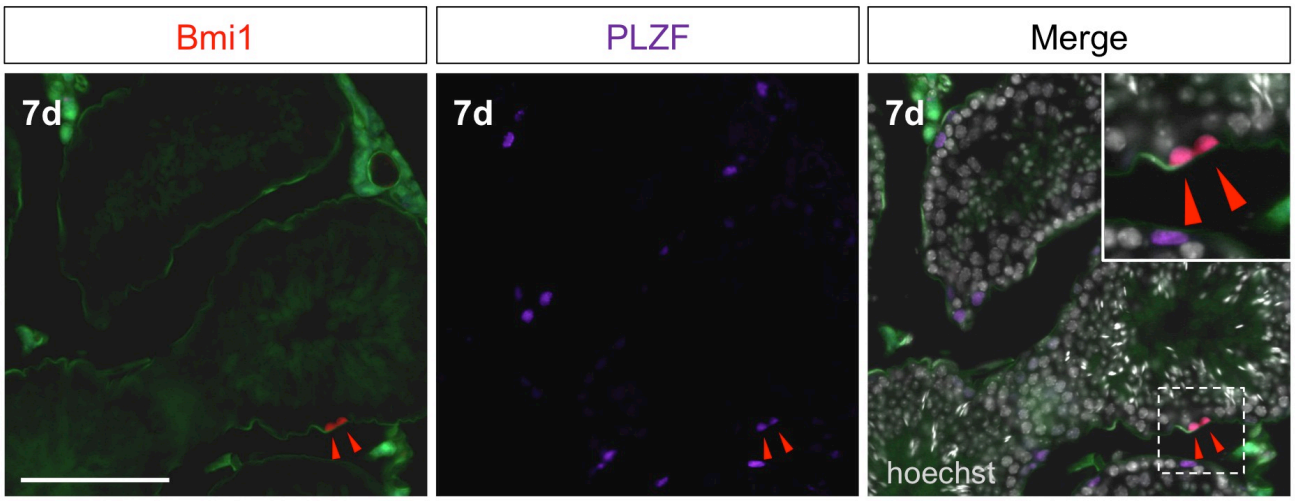
Gene	Forward	Reverse
Gapdh	AAGGGCTCATGACCACAGTC	ACACATTGGGGGTAGGAACA
Bmi1	AAACCAGACCACTCCTGAACA	TCTTCTTCTCTTCATCTCATTTTTGA
Gfrα1	AGAAGCAGTTTCACCCAG	ATCATCACCACCACCATC
ID4	TGAACAAGCAGGGTGACAG	CGGTGGCTTGTTTCTCTTAATTTC
Nanos2	GACCAGGCTCATACACTCAAG	GGAGGGTGTGGGTTGTG
Ngn3	GTCGGGAGAACTAGGATGGC	GGAGCAGTCCCTAGGTATG
c-Kit	ACATCGCCAGAGCCAACG	ATCCACTTTAATTTCGGGTCAA

Table S2 . Summary of stage mismatches

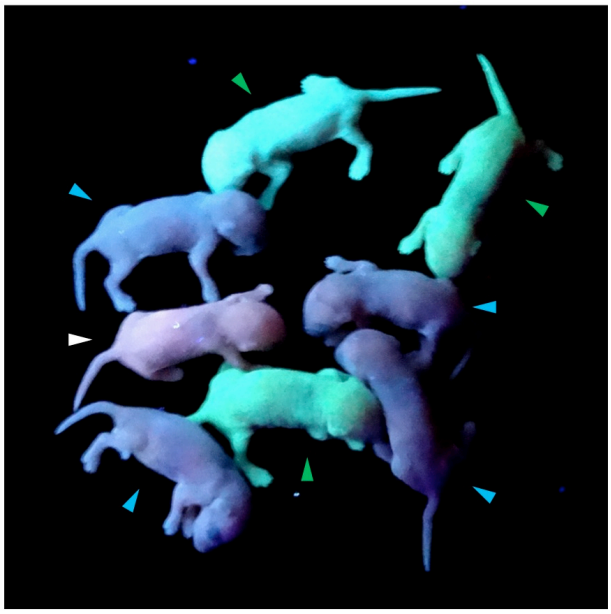
The example number #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
The seminiferous stage of the surrounding areas	XII	XII	XII	XII	XII	I	XII	XII	XII	I	XII	V	VI	V	XII	XII	XII	XII	V
The seminiferous stage of the mismatched areas	IX	IX	VIII	IX	IX	IX	IX	IX	VIII	IX	IX	I	II/III	I	IX	VIII	IX	VIII	I

The summary of the stage mismatches observed in the testes of tamoxifen-injected *Bmi1*$^{creER/+}$*/Rosa26*$^{loxp\text{-}stop\text{-}loxp\text{-}DTA/+}$ mice (Figures 3d and 3e), in which Bmi1^{High}-positive cells were deleted. Seminiferous stages of the surrounding and mismatched areas are shown. Roman numbers indicate seminiferous epithelial stages.

<Figure S1>

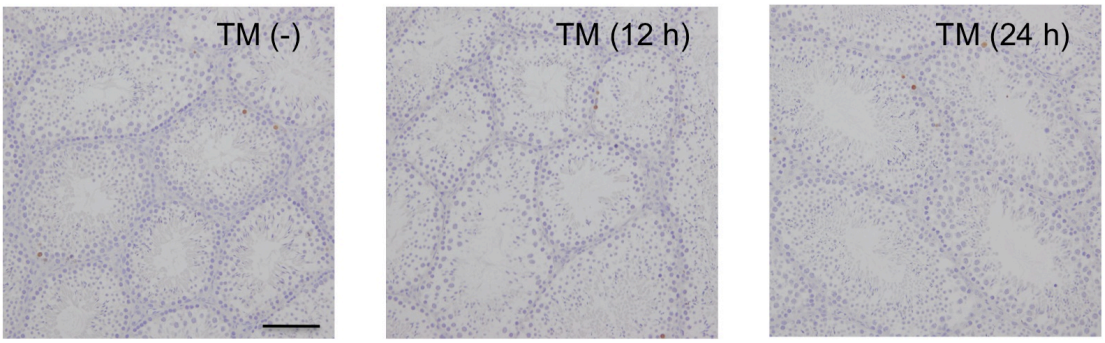


<Figure S2>

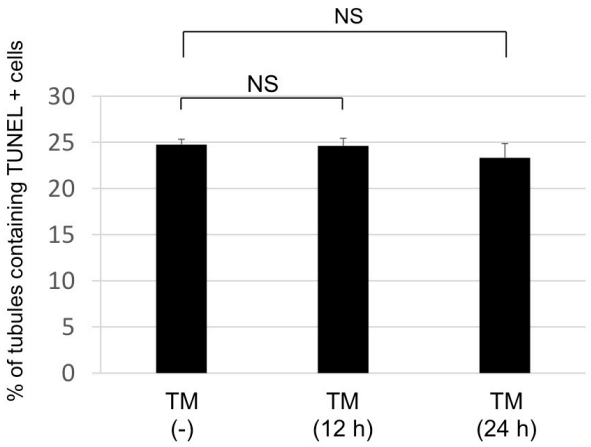


<Figure S3>

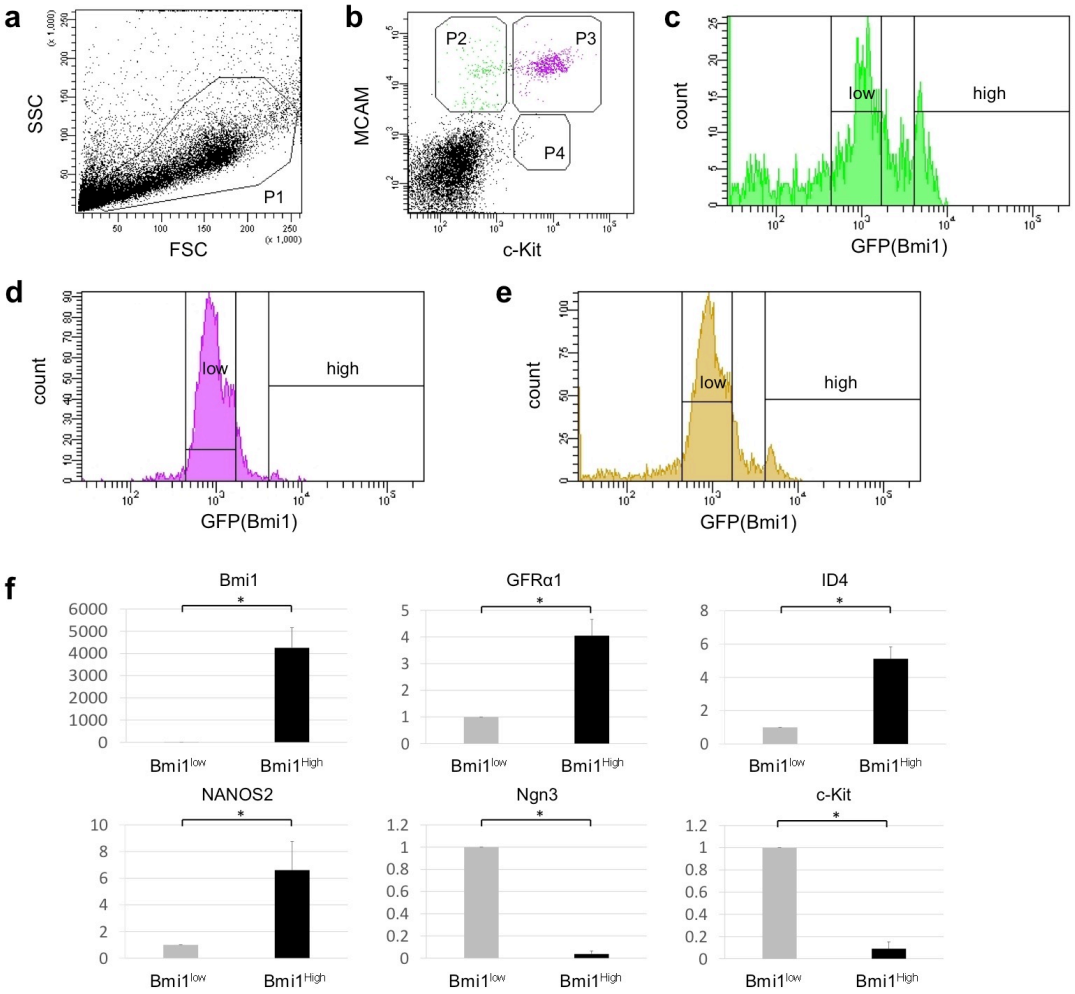
a


TM (-)
TM (12 h)
TM (24 h)

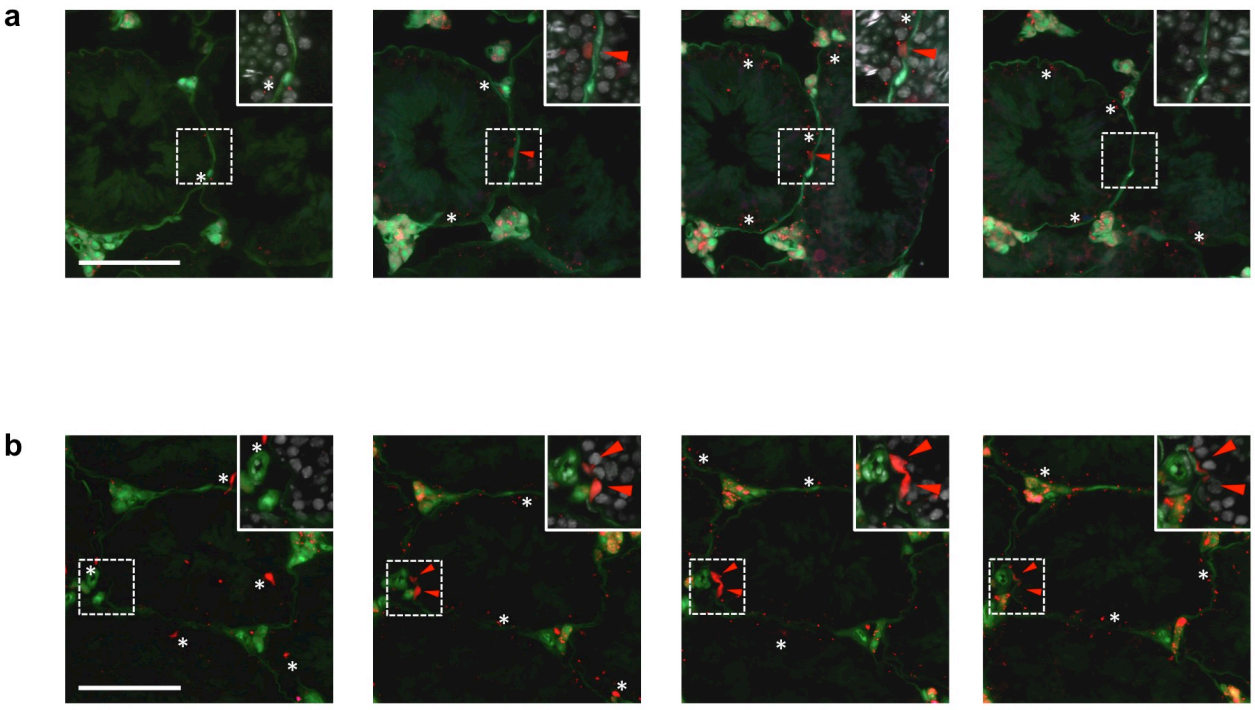
b


NS
NS
% of tubules containing TUNEL + cells
30
25
20
15
10
5
0
TM (-)
TM (12 h)
TM (24 h)

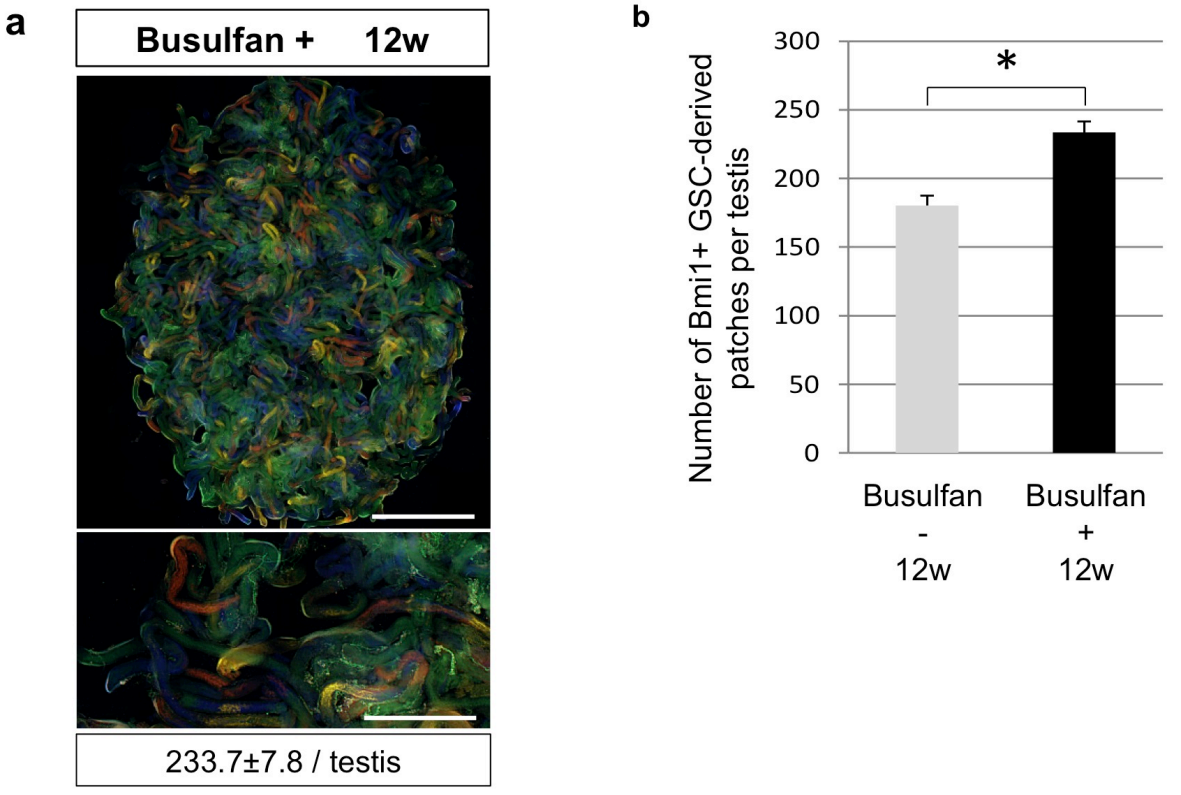
<Figure S4>


a
SSC
FSC
P1
b
MCAM
c-Kit
P2
P3
P4
c
count
GFP(Bmi1)
low
high
d
count
GFP(Bmi1)
low
high
e
count
GFP(Bmi1)
low
high
f
Bmi1
GFRα1
ID4
NANOS2
Ngn3
c-Kit
Bmi1low
Bmi1High

<Figure S5>



<Figure S6>


a
Busulfan + 12w
233.7±7.8 / testis
b
Number of Bmi1+ GSC-derived patches per testis
300
250
200
150
100
50
0
*
Busulfan - 12w
Busulfan + 12w

<Figure S7>

days	1	2	3	4	5	6	7	8	9
delayed stages	Ⅰ	Ⅱ / Ⅲ	Ⅳ	Ⅴ	Ⅵ	Ⅶ	Ⅷ	Ⅸ	Ⅹ	Ⅺ	Ⅻ
actual stages	Ⅺ	Ⅻ	Ⅰ	Ⅱ / Ⅲ	Ⅳ	Ⅴ	Ⅵ	Ⅶ	Ⅷ	Ⅸ	Ⅹ

<Figure S8>