Fig. S4 Watanabe et al

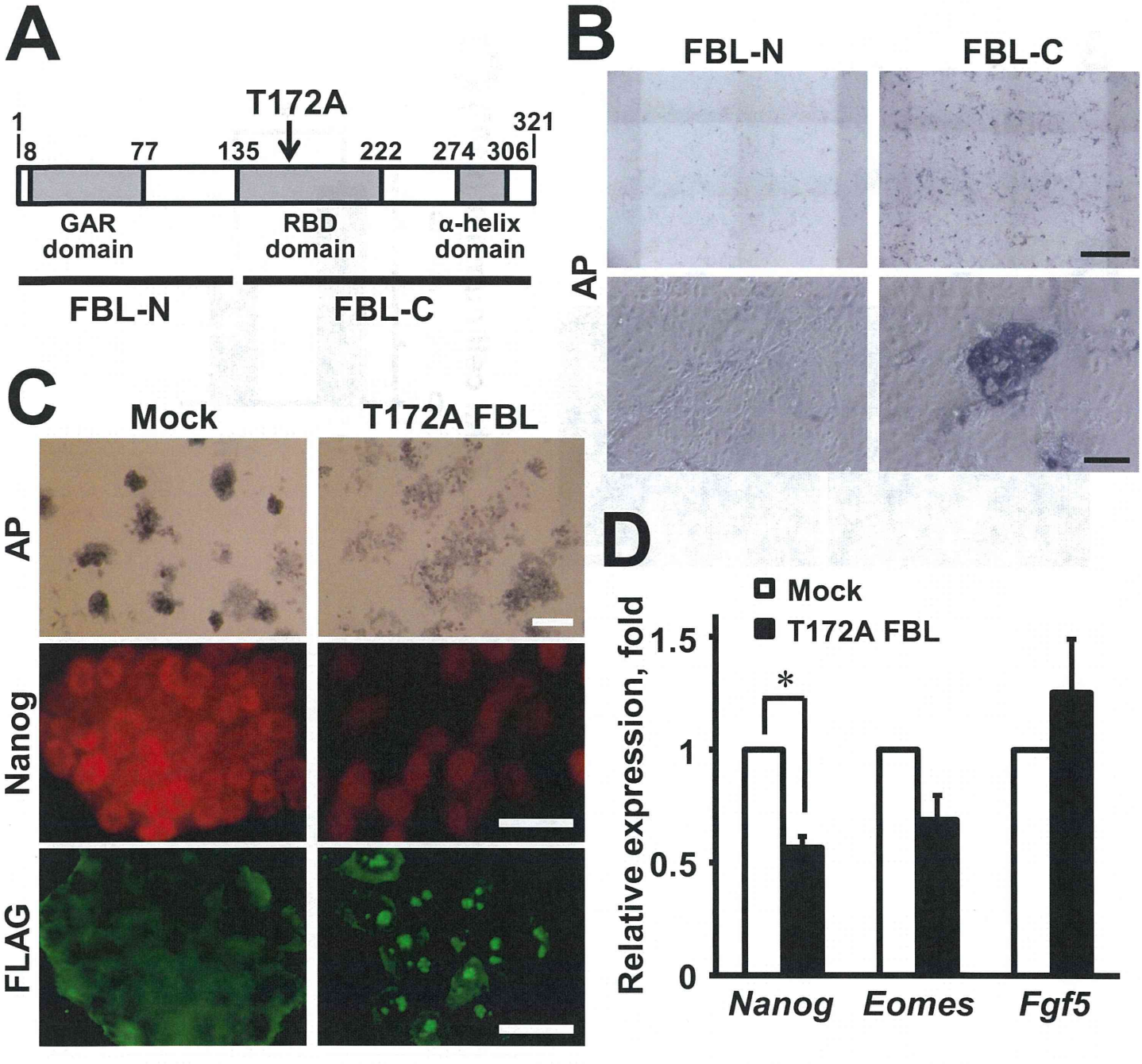


Fig. S5 Watanabe et al

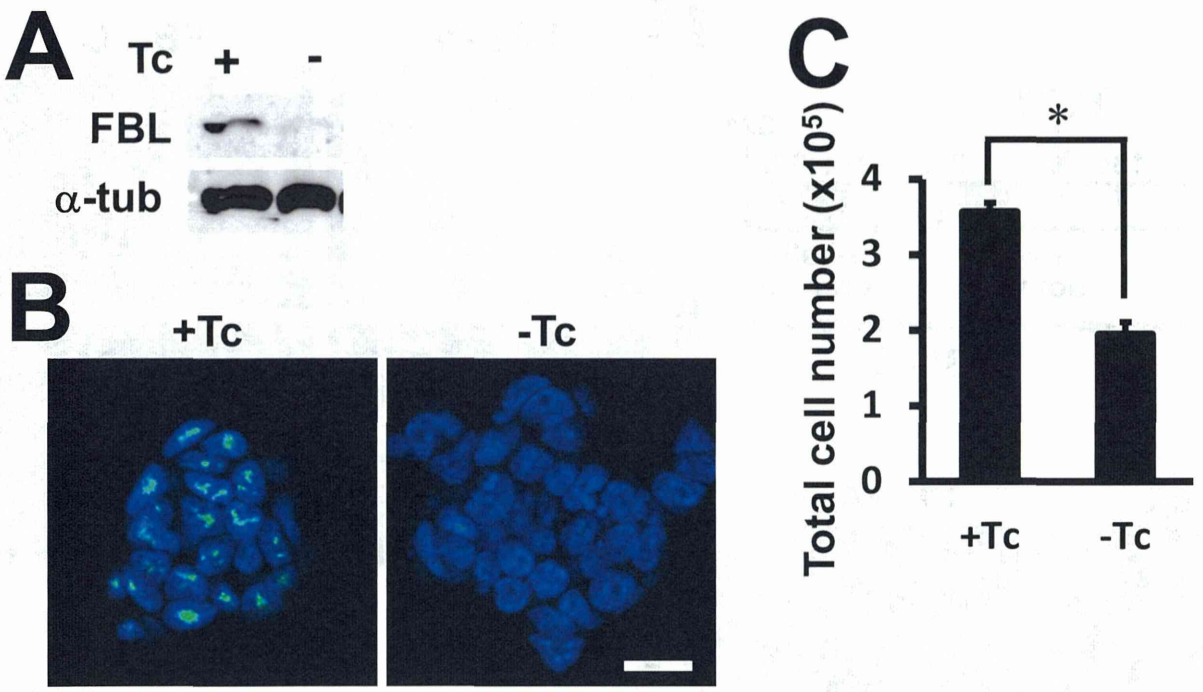


Fig. S6 Watanabe et al

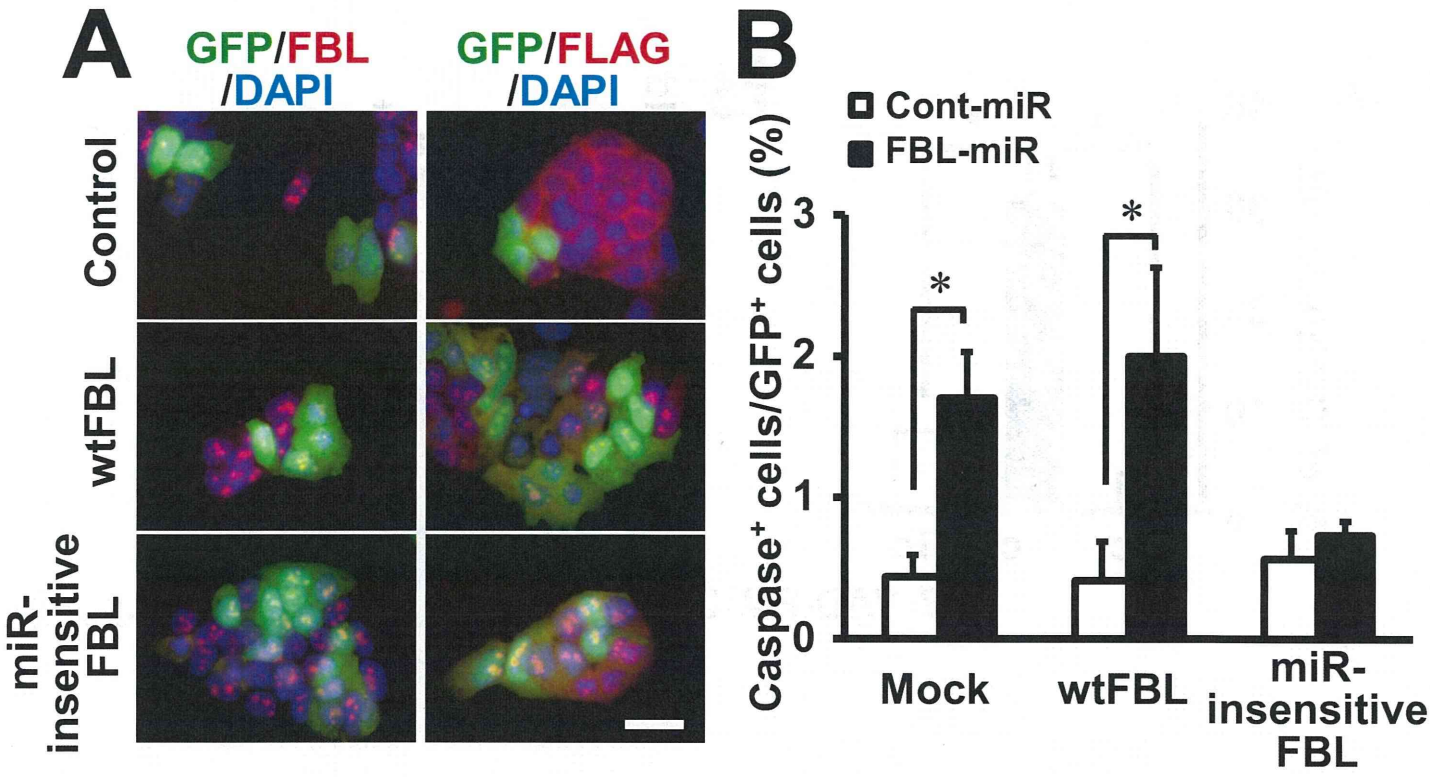


Fig. S7 Watanabe et al

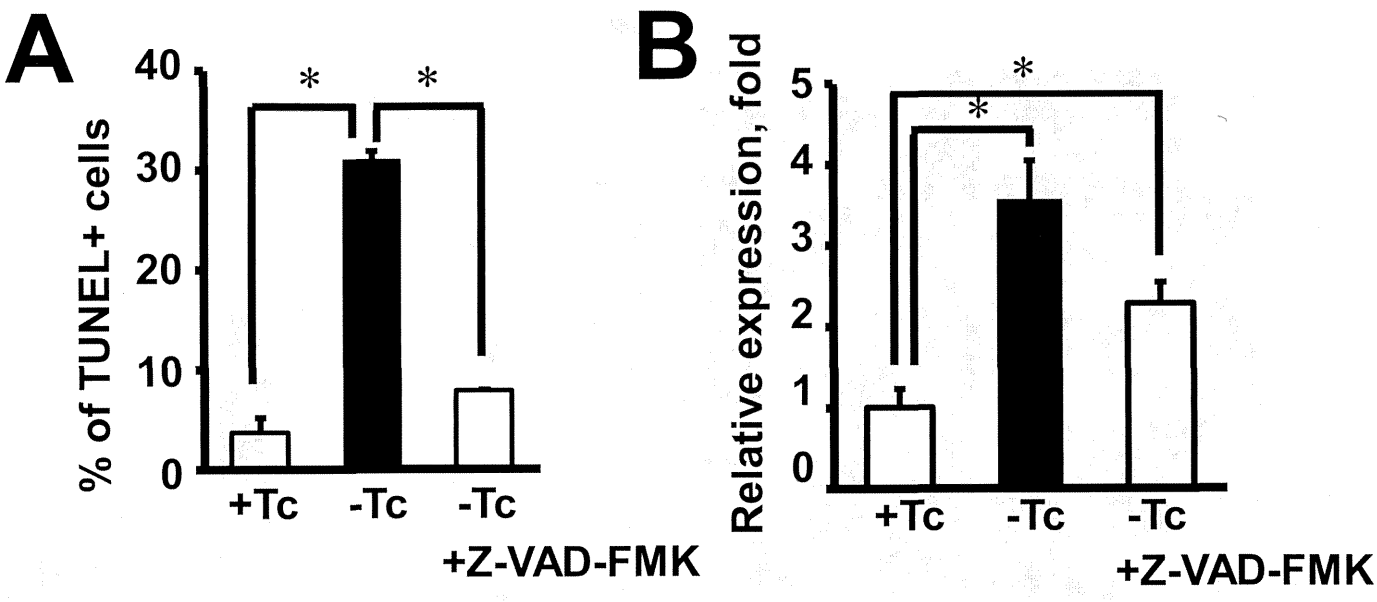


Fig. S8 Watanabe et al

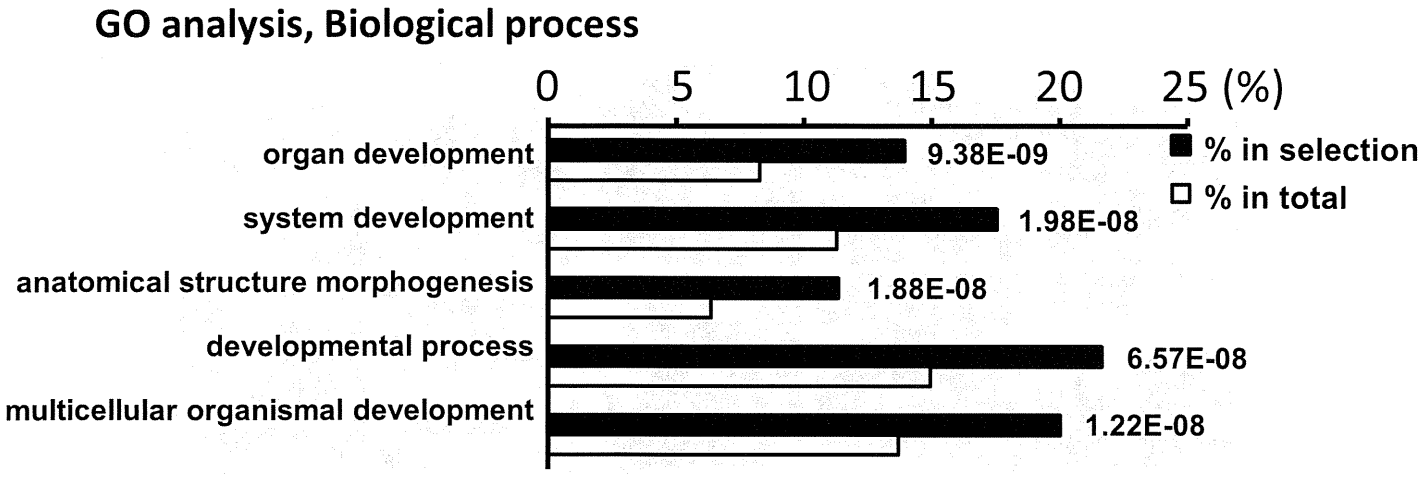


Fig. S9 Watanabe et al

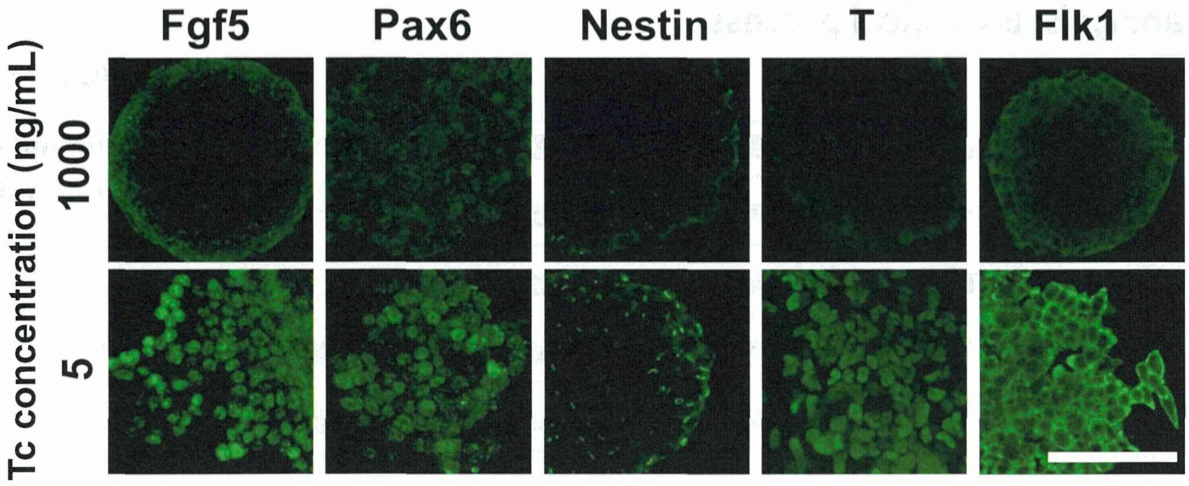


Fig. S10 Watanabe et al

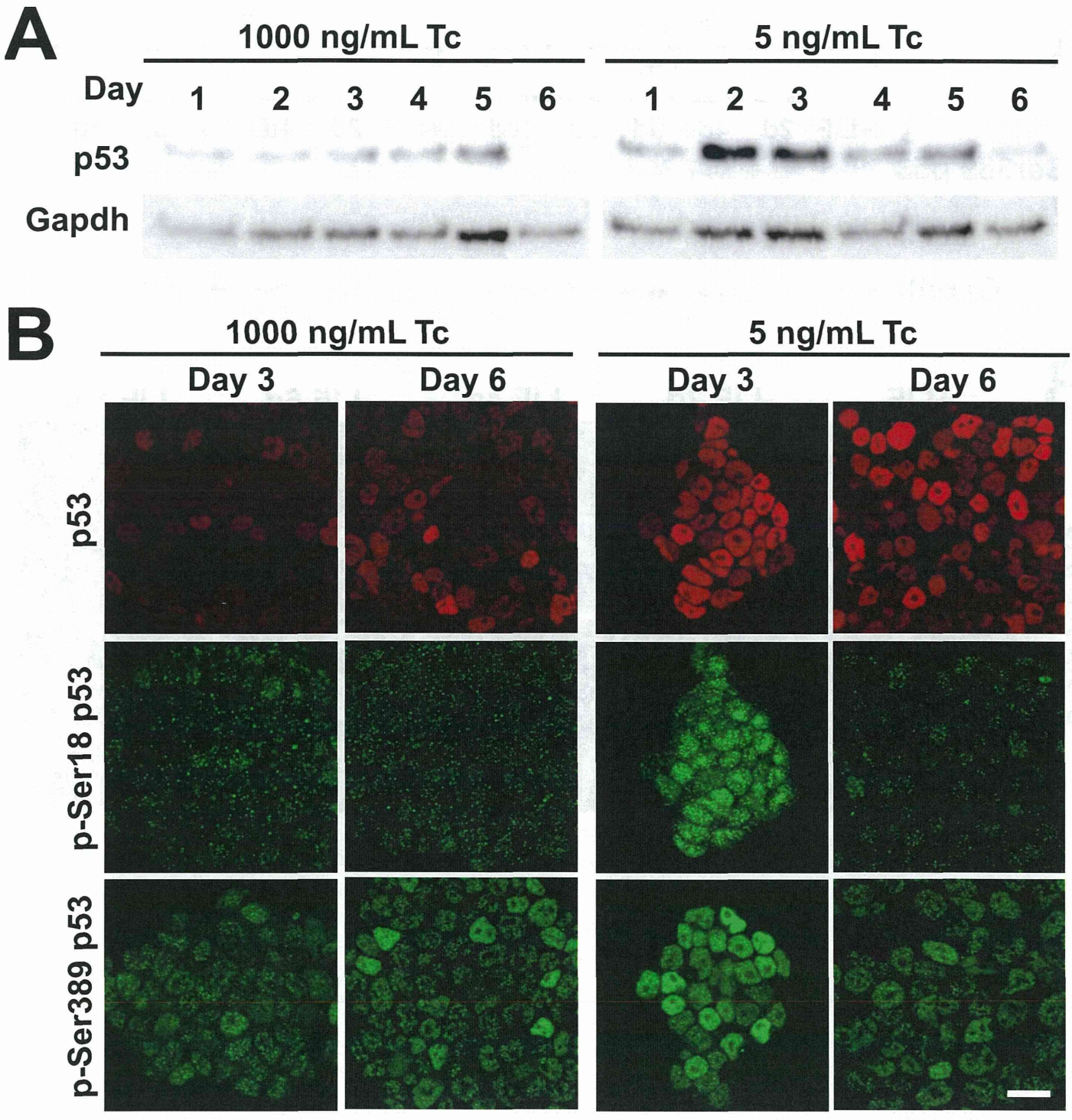
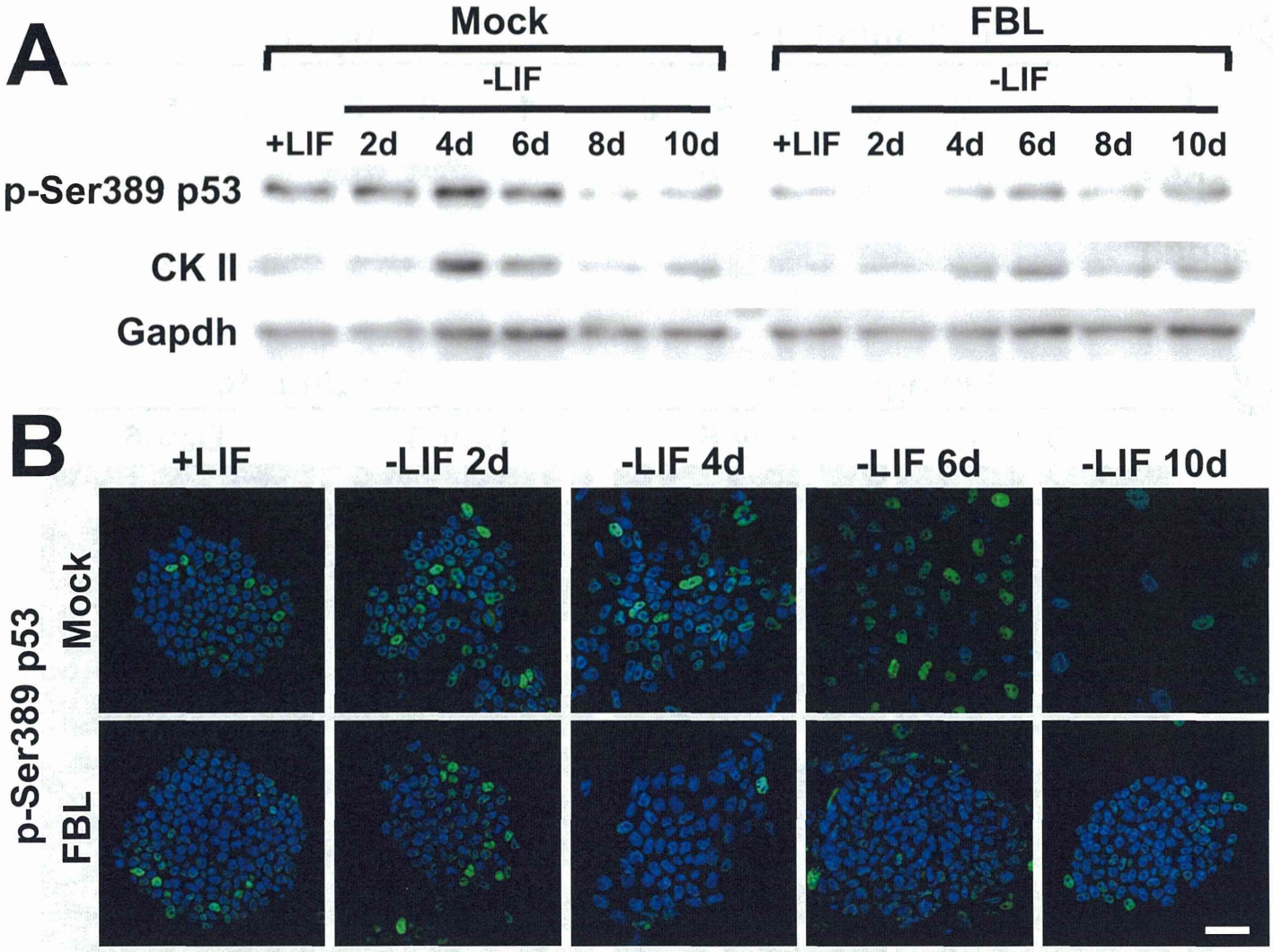


Fig. S11 Watanabe et al



Supplementary Figure legends

Figure S1. Cells stably expressing FBL maintain their large nucleolar morphology even in the absence of LIF. ES cells stably expressing FBL or the control vector were cultured for 10 days with or without LIF, and analyzed by immunofluorescence staining of nucleolar markers. In the control cells, another nucleolar protein, nucleolin, was continuously expressed whereas FBL was reduced after 10 days culture without LIF. Scale bar: 5 μm.

Figure S2. Overexpression of FBL in ES cells does not affect pluripotent ES cells cultured in the presence of LIF. (A) Morphology and the expression of Venus protein of Tc-off-regulated FBL-expressing ES cells. ES cells were cultured for 3 days with or without Tc. (B) Western blot analysis of FLAG-FBL expression in ES cells. (C) Total number of ES cells cultured with or without Tc for 3 days. (D) Expression of Nanog and Oct4 in ES cells cultured with or without Tc for 3 days.

Figure S3. Overexpression of c-Myc and FBL in MEFs and ES cells. (A) Morphology of MEFs infected with FBL or c-Myc retrovirus. Pictures were taken 1 week after

infection. (B) RT-PCR analysis of (A). (C, D) qPCR analysis of endogenous *FBL* and *c-Myc* in (A). (E, F) Overexpression of c-Myc does not rescue FBL-knockdown-induced apoptosis. (E) ES cells were infected with Sendai virus expressing c-Myc and Kusabira orange, passaged on the next day, and cultured for 2 more days without Tc. TUNEL-positive cells (Green) were still observed in c-Myc expressing cells (Red). Scale bar: 100 μm. (F) Quantification of TUNEL-positive cells in c-Myc virus-expressing cells or control non-infected cells. Overexpression of c-Myc did not decrease the number of TUNEL-positive cells in FBL-knockdown ES cells. $*p < 0.01$ in (C).

Figure S4. The functional domain is localized in the C-terminal half of FBL. (A) Schematic of the FBL structure. FBL has 3 domains, the GAR domain, RBD, and the α-helix domain. The C-terminal half of FBL containing the RBD and α-helix domains is the methyltransferase domain. (B) Alkaline phosphatase staining of ES cells. ES cells stably expressing FBL-C maintained alkaline phosphatase activity even after 10 days of culture without LIF. (C) A point mutation introduced into FBL (T172A) decreased alkaline phosphatase activity and Nanog expression in the presence of LIF. (D) qRT-PCR analysis of ES cells stably expressing FBL T172A. Decreased expression of

Nanog in ES cells stably expressing the T172A FBL mutant. *$p < 0.01$ in (D). Scale bars: (B, upper) 2 mm; (B, lower) 300 μm; (C, upper) 200 μm; (C, middle and lower) 30 μm.

Figure S5. A second miRNA for FBL also repressed FBL expression and proliferation of ES cells. (A) Western blot analysis of FBL expression in ES cells. FBL expression were significantly decreased when the ES cells were cultured without Tc. (B) Immunofluorescence analysis of FBL expression. Upon withdrawal of Tc, the FBL expression was notably decreased. The cells were immunostained after 2 days of culture in the absence of Tc. (C) Proliferation of ES cells after knock down of FBL. Total cell number was measured after 3 days of culture with or without Tc. The total cell number significantly decreased after knock down of FBL. *$p < 0.01$ in (C). Scale bar: 20 μm.

Figure S6. The expression of miRNA-insensitive FBL rescues ES cells from apoptotic cell death. ES cells stably expressing wild type FBL, miRNA-insensitive FBL, or the control plasmid were transiently transfected with a FBL-miRNA expression vector, pcDNA6.2-GW/EmGFP-FBL-miR, and the apoptosis was analyzed 48h post transfection. GFP fluorescence indicates the transfected cells with this FBL-knockdown

vector. (A) Immunofluorescence analysis of ES cells expressing FLAG-tagged wild type and miRNA-insensitive FBL with a combination of GFP-FBL-miR. The ES cells were fixed and stained with indicated antibodies. In wild type FBL-expressing ES cells, the expression of both endogenous and exogenous FBL were decreased to almost undetectable levels in GFP-FBL-miR transfected cells. In contrast, in miRNA-insensitive FBL-expressing cells, exogenous FLAG-tagged FBL was still expressed in GFP-positive cells. (B) Quantification of active caspase-positive cells. In miRNA-insensitive FBL-expressing cells, the number of apoptotic cells was decreased to a level that was comparable to that of control cells. $*p < 0.05$ in (B). Scale bar: 50 μm.

Figure S7. Altered pre-RNA processing does not occur as a consequence of apoptosis. (A, B) Knock down of FBL leads to an accumulation of 45S pre-rRNA independently from apoptosis. FBL-knock down ES cells were cultured for 2 days with or without Tc. Z-VAD-FMK was added from the beginning of culture. (A) Quantification of TUNEL-positive cells. Z-VAD-FMK treatment significantly inhibited the induction of apoptosis in the absence of Tc. (B) qRT-PCR analysis of the amount of 45S rRNA. Induction of FBL-knock down leads to an accumulation of 45S pre-rRNA. The

accumulation of 45S pre-rRNA was still evident in the presence of Z-VAD-FMK. *$p <$ 0.05 in (A, B).

Figure S8. Enriched gene ontology (GO) categories having significant numbers of genes differentially expressed in FBL-knockdown cells compared with the control-knockdown cells. White bars indicate percentage of genes in each GO category. Black bars indicate percentage of genes in each GO category that were differentially expressed more than 2-fold. Gene ontology analysis suggests the up-regulation of development-associated genes in FBL-knock down conditions. Numbers on the graph indicate *p*-values.

Figure S9. Immunofluorescence analysis of ES cells under reduced FBL expression with antibodies against differentiation markers. After 6 days of culture, the expression levels of differentiation marker proteins increased under FBL-reduced conditions (5 ng/mL Tc). Scale bar: 100 μm.

Figure S10. p53 was transiently up-regulated after partial knock down of FBL. (A) Western blot analysis of p53 expression in ES cells. p53 expression was increased from

day 2 to day 3 of culture only under FBL-reduced conditions (5 ng/mL Tc). (B) Immunofluorescence analysis of p53 and its active forms. The levels of p53 and its phosphorylated forms, p-Ser18 p53 and p-Ser389 p53, were increased on day 3 under FBL-reduced conditions. Scale bar: 20 μm.

Figure S11. Suppression of p53 activation by FBL during spontaneous differentiation of ES cells. (A) Western blot analysis of p53 p-Ser389 and CK II. The level of the active form of p53, p53 p-Ser389, was significantly increased from day 4 to day 6 in control cells but not in ES cells stably expressing FBL. (B) Immunofluorescence staining of the activated form of p53 in ES cells. Scale bar: 50 μm.

Table I. Primers used for qPCR analyis

Gene	forward	reverse
Nanog	5'-ctccattctgaacctgagctataa-3'	5'-attgctagtcttcaaccactg-3'
Oct4	5'-tctttccaccaggcccccggctc-3'	5'-tgcgggcggacatggggagatcc-3'
Sox2	5'-ccagcgcccgcatgtataac-3'	5'-cgggctgttcttctggttgc-3'
Rex1	5'-aaaatgaatgaacaaatgaagaaaa-3'	5'-ttgcctcgtcttgctttagg-3'
Stella	5'-aggctcgaaggaaatgagtttg-3'	5'-tcctaattcttcccgattttcg-3'
Pecam1	5'-gtcatggccatggtcgagta-3'	5'-ctcctcggcgatcttgctgaa-3'
Eomes	5'-tggatgaggcaggagatttc-3'	5'-gttggtatttgtgcagagact-3'
Gata4	5'-ctcgatatgtttgatgacttct-3'	5'-cgttttctggtttgaatccc-3'
Brachyury	5'-taccccagcccctatgctca-3'	5'-ggcactccgaggctagacca-3'
Flk1	5'-cacctggcactctccaccttc-3'	5'-gatttcatcccactaccgaaag-3'
Nestin	5'-ctgcaggccactgaaaagtt-3'	5'-ttccaggatctgagcgatct-3'
Pax6	5'-agtgtcagttcccgtccaag-3'	5'-gtgcttctaaccgccatttc-3'
Fgf5	5'-aaagtcaatggctcccacgaa-3'	5'-ggcacttgcatggagttttcc-3'
FBL	5'-tttccattaaggccaactgc-3'	5'-cagttcttcaccttggg-3'
*FBL(*endo)	5' -gatgcagcaggagaacatga-3'	5' -aagccacacgtgcaacagta-3'
cMyc(endo)	5' -tgacctaactcgaggaggagctggaatc-3'	5' -aagtttgaggcagttaaaattatggctgaa-3'
45S rRNA	5' -ctcctctctcgcgctctct-3'	5' -gagcgaccaaaggaaccata-3'
Gapdh	5'-cacattgggggtaggaacac-3'	5'-acccagaagactgtggatgg-3'

Supplementary Materials and Methods

Antibodies

The following antibodies were used in this study: FBL (Abcam, ab4566, 1/500 for immunofluorescence staining [IF] and Western blot [WB]), Nanog (ReproCell, RCAB0001P, 1/100 for IF, 1/500 for WB), Oct4 (Santa Cruz, sc-9081, 1/100 for IF, 1/500 for WB), SSEA1 (Kyowa Medex, TM13, 1/100 for IF), p53 (Cell Signaling, 2524S, 1/100 for IF and WB), p53 p-Ser389 (Abcam, ab33889, 1/100 for IF and WB), p53 p-Ser18 (Abcam, ab1431, 1/100 for IF and WB), NF200 (Sigma-Aldrich, N4142, 1/500 for IF), Tuj1 (Convance, MMS-435P, 1/500 for IF), FGF5 (Santa Cruz, sc-7914, 1/100 for IF), Pax6 (Abcam, ab5790, 1/200 for IF), T (R&D system, AF2085, 1/200 for IF), Flk1 (Santa Cruz, sc-6251, 1/100 for IF), CK II (Abcam, ab76025, 1/200 for WB), FLAG (Affinity BioReagents, PA1-984A, 1/200 for IF, 1/500 for WB; Sigma-Aldrich, F3165, 1/2000 for WB), α-tubulin (Sigma-Aldrich, T9026, 1/3000 for WB), and Gapdh (Santa Cruz, sc-20357, 1/300 for WB). Alexa 488- or Alexa 594-conjugated anti-mouse and anti-rabbit IgG secondary antibodies (Molecular Probes) were used for the staining. For SSAE1 antibody, Alexa 488-conjugated anti-mouse IgM secondary antibody (Molecular Probes) was used.

Apoptosis detection

Apoptotic cells were detected on the basis of DNA fragmentation and caspase activity. DNA fragmentation was detected by using the terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling method (DeadEnd™ Fluorometric TUNEL System, Promega) according to the manufacturer's instructions. Briefly, fixed and permeabilized cells were reacted with terminal deoxynucleotidyl transferase using fluorescent nucleotides at 37°C for 1 h. The reaction was terminated by adding 2× saline sodium citrate. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

Active caspase was detected by using the Sulforhodamine Multi-Caspase Activity Kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions. Briefly, cells were incubated with 1× SR-VAD-FMK diluted with culture medium at 37°C for 1 h. The reaction was terminated by adding wash buffer. Cell nuclei were stained with Hoechst dye.

Metabolic labeling and analysis of rRNA processing

ES cells were seeded at a density of 4×10^4 per well in gelatin-coated 12-well plates

and cultured for 2 days in the presence or absence of Tc. To measure rRNA synthesis, the cells were incubated in medium supplemented with [^{3}H]uridine (5 μCi/mL) for 30 min, and then cultured in medium without [^{3}H]uridine for 2 h. Pulse-chase labeling experiments were carried out using [methyl-^{3}H]methionine. Cells were pre-incubated for 15 min in methionine-free medium, incubated for 30 min in medium containing [methyl-^{3}H]methionine (50 μCi/mL), and chased in nonradioactive medium containing 2 mM non-labeled methionine for 15, 30, 45, or 60 min. RNA from the same number of cells was analyzed. Total RNA was purified with ISOGEN, separated in a 1% agarose gel, and transferred to a nylon membrane (GE Healthcare, Hybond N+). Autoradiograph images were scanned with the STORM 830 phosphorimager (GE Healthcare) and quantified by using the Image Quant Solutions software (ver. 1.2, GE Healthcare).

DNA microarray

Total RNA was prepared from FBL-knock down (-Tc) and control (+Tc) ES cells after culturing for 2 days. An Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent) was used to synthesize fluorescently labeled cRNA targets for microarray analysis. Labeled cRNA targets were hybridized on a Whole Mouse Genome array (G4122F, 4×44K, Agilent) according to the manufacturer's protocol. The arrays were

scanned with a G2505C Microarray Scanner System (Agilent). Microarray analysis was performed in duplicate. Data were averaged and analyzed using the GeneSprings GX software (version11.5) and Ingenuity pathway analysis software (Tomy Digital Biology). The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE GSE51139 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51139).

Quantitative real-time reverse transcription-PCR

Total RNA was prepared with Isogen (Nippon Gene) following the manufacturer's recommendations, and cDNAs were synthesized using a Super Script II reverse transcriptase (Life Technologies). Real-time reverse transcription-PCR was performed with a Chromo 4 Real-Time PCR Detector (Bio-Rad) system using the Thunderbird SYBR qPCR Mix (TOYOBO), according to the manufacturer's instructions. All experiments were performed in triplicate, and the data were normalized to the expression level of the *Gapdh* or *TBP* mRNA. Primer sets used in this study are listed in Supplementary Table I.

Induction of neuronal differentiation

Differentiation of ES cells was performed on the basis of a previously described method (1, 2). Briefly, embryoid bodies formed by suspension culture for 5 days were treated with trypsin-EDTA. The dissociated cells were seeded on poly-L-lysine/laminin/fibronectin-coated plates and further cultured for 4 days (1). The concentration of Tc was decreased from 1 μg/mL to 5 ng/mL from day 3 under partial FBL knockdown conditions. At day 9, cells were fixed and stained. In monolayer culture, ES cells were dissociated, seeded on poly-L-lysine/laminin/fibronectin-coated plates, and cultured for 7 days in N2B27 medium (2). After immunostaining, $Tuj1^+$- or $NF200^+$-positive cells with neurites (over 54.5 μm, 50 pixels at ×10) were determined on an ArrayScan (Thermo Fisher Scientific).

Reprogramming of MEFs into induced pluripotent stem cells

For retrovirus production, Plat-E cells were seeded the day before transfection. On the next day, Plat-E cells were transfected with each retroviral vector using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's recommendations. On the next day, the medium was replaced. MEFs were seeded at a density of 5×10^4 cells per well on gelatin-coated 12-well plates. At 48 h post transfection, the virus-containing supernatant was collected by centrifugation at 1,000