cassette) and 5 μg of ZFN-encoding mRNAs were co-transfected into 4.5×10^6 of *Oct4*-Venus ESCs (Wistar) at passage 3. As a control experiment, 10 μg of the targeting plasmid without ZFNs was transfected into 4.5×10^6 of *Oct4*-Venus ESCs at passage 3. One out of 46 (2.2%) clones was $p53^{C/C}$, while seven of 46 (15%) clones were $p53^{C/Z}$. A sequence data revealed an 8-bp deletion in the $p53^{C/Z1}$ clone (Figure S2B). This ZFN-induced small deletion was also confirmed by a downward band shift (indicated by asterisks in Supplementary Fig. S2a). As a control experiment, the targeting vector alone was introduced without ZFN. Although 14 geneticin-resistant colonies appeared, they did not achieve homologous recombination (Supplementary Fig. S2a, lane 5). Knockout clones were also produced using a 2nd-step recombination by introducing the 10 μg of targeting plasmid (5′arm-CAG-tdTomato-IRES-Neo-pA-3′arm cassette) and 5 μg of ZFN-encoding mRNAs into 2.5×10^6 cells of a $p53^{+/Cs}$ ESC clone at passage 9 (Fig. 6). Eight red fluorescence (tdTomato)-positive clones were chosen without geneticin selection, and successful homologous recombination was achieved in three of these (38%, $p53^{C/R}$; Supplementary Fig. S2c).

Surveyor nuclease (Cel-I) assay. A ZFN target locus was amplified by PCR (35 cycles: 10 s denaturing at 98°C, 30 s annealing at 62°C and 1 min elongation at 72°C) using primers 1 and 5 (Fig. 6 and Supplementary Table S3). The Cel-I assay was carried out following the manufacturer's protocol (TRANSGENOMIC, Inc.).

ALP staining, immunohistochemistry (IHC) and Annexin V-apoptosis assay. Cells were fixed in 4% paraformaldehyde. ALP staining was performed with the Vector Blue substrate (Vector Labs) according to the manufacturer's instructions. Formalin-fixed and paraffin-embedded slides were stained with hematoxylin and eosin or used for IHC. Antigen retrieval was performed by autoclave in a sodium citrate buffer. The slides were incubated with Sox2 (BioLegend, 1 : 200) or Cleaved Caspase-3 (Cell Signaling, 1/1000) primary antibody at 4°C overnight. The next day, after washing, the samples were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. They were then washed and incubated with 3,3′-diaminobenzidine tetrahydrochloride DAB (Thermo Scientific). An assay for apoptotic ESCs was performed using Annexin V-Cy5 following the manufacturer's protocol (BioVision). Pluripotent ESC colonies were solely harvested and dissociated with Accutase, followed by incubating 1–5 $\times 10^5$ cells with the Annexin V-Cy5 for 5 min in the dark.

Q-PCR analysis. Total RNA was isolated using ISOGEN (Nippongene). cDNA was synthesized with 2 μg of the total RNA using Super Script III RT (Invitrogen) and oligo-dT primer (Invitrogen). cDNAs were used for PCR using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). Optimization of the q-PCR reaction was performed according to the manufacturer's instructions (PE Applied Biosystems, Tokyo, Japan). All quantitations were normalized to an endogenous control *GAPDH*.

Microarray analysis. A one-color microarray-based gene expression analysis system (Agilent Technologies) using SurePrint G3 Rat GE 8 × 60 K Kit containing 30507 probes (26930 genes) was used following the manufacturer's instructions.

EB formation. After ES cells were dissociated into single cells using Accutase, 5×10^5 cells were cultured in PAC medium. After overnight incubation, the EB contained media were separated and cultured in media with or without PAC on a low cell-binding dish (NUNC). After 7 days of incubation, the cell number of EBs was counted after dissociation with Accutase.

Chimera production. In all blastocyst injection experiments, 12 ESCs were injected into E4.5 blastocysts. YPAC or PAC inhibitors were constantly included in media during both microinjection and blastocyst incubation. ESC-injected blastocysts were transferred to E3.5 pseudo-pregnant rats. The contribution of ESCs to the resulting chimeras was determined by the appearance of coat-color or fluorescence.

Karyotype analyses in $p53^{-/-}$ cells. G-band staining was performed in cultured cells from embryos, ESCs, or ESC-derived differentiated cells. Head of E14.0 chimeric embryo was dissociated with Accutase and karyotype analysis was examined in the cells at passage 4. $p53^{C/R2}$ ESCs at five passages after the generation of the gene-targeted null mutation or EB-derived differentiated $p53^{C/R2}$ cells at seven passages were analyzed. EBs were formed at passage 5 and cultured for 2 weeks, followed by two passages to expand the cells. The differentiated state was confirmed by a loss of *Oct4*-Venus expression, as well as by the cell morphology.

Statistical analysis. Results are given as the mean ± SD. Statistical analysis was conducted using Student *t*-tests. $P < 0.05$ was considered significant.

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Author contributions

M.K. designed and performed experiments. M.K. and T.O. wrote the manuscript. T.O. supervised the project.

Additional information

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