Supplementary Materials

T cell depletion and Treg cell isolation

Whole bone marrow cells (WBMCs) from WT C57/BL6 mice were subjected to T cell depletion using Thy 1.2 (CD90.2) microbeads (Miltenyi Biotech, Auburn, CA). Splenocytes and cells from lymph nodes were enriched for T cells using magnetic microbeads (MACS, Pan-T cell isolation kit, Miltenyi Biotech, Auburn, CA) or for Treg cells using $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotech, Auburn, CA). Treg cell fraction ($CD4^+CD25^+$) and Teff cell fraction ($CD4^+CD25^-$) were used for BMT.

CFSE labeling

In vitro proliferation assays were conducted by labeling $CD4^+CD25^-$ (Teffs, $2.5X10^6$ cells/ml) with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE)/ml (Invitrogen, Grand Island, NY) for 10 minutes and quenched by ice cold PBS. Cells were then co-cultured with $CD4^+CD25^+$ cells freshly isolated from either WT, *Fanca*$^{-/-}$, or *Fancd2*$^{-/-}$ mice at a ratio of 2:1 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) for 5 days followed by flow cytometry analysis for CFSE retention.

Histopathology

Histopathological specimens from liver, lung, skin and small intestine were obtained on days 14 after transplantation, fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA) and embedded into paraffin blocks. Tissue sections were stained with hematoxylin and eosin and examined at ×400 (Skin & Liver) or ×200 (Lung & Small intestine) by microscope respectively.

Assessment of GVHD

The degree of GVHD was assessed using a cumulative scoring system that measures changes in 5 clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity (maximum index 10).[31] Mice were monitored daily, and those with GVHD clinical scores greater than or equal to 6 were culled and the date of death recorded as the next day in accordance with institutional animal ethics guidelines.

Assessment of serum cytokine level

Cytokines (TNF-α, IFN-γ and IL-6) from serum samples were determined by using the cytometric beads array kit (CBA, BD Biosciences Pharmingen, San Diego, CA). All assays were performed according to manufacturer's protocol.

SA-β-galactosidase (SA-β-gal) staining

SA-β-galactosidase (SA-β-gal) staining in Treg cells before or after *p65* deletion was conducted following the manufacturer's instruction (Cell Signaling, Boston, MA).

Preparation of cell extracts, Immunoblotting

To prepare whole cell extracts, cells were washed with ice-cold PBS, and resuspended in ice-cold lysis buffer containing 50mM Tris-HCL (pH 7.4), 0.1% NP40, and 1M NaCl supplemented with protease and phosphatase inhibitors (10μg/ml aprotinin, 25μg/ml leupeptin, 10μg/ml pepstatin A, 2mM PMSF, 0.1M NaP_2O_4, 25mM NaF and 2mM sodium orthovandate) for 30 min on ice. Cell lysates were resolved on SDS-PAGE, and immunoblots were analyzed with antibodies for the active form of p16 (Abcam, Cambridge, MA), or β-actin (Sigma-Aldrich, St Louis, MO). Each lane contains protein

from ~50,000 cells. Signals were visualized by incubation with anti-mouse or anti-rabbit secondary antibodies followed by ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ).

RNA Isolation, Reverse Transcriptase (RT)-PCR

Total RNA from H-$2^{b+}CD4^{+}$ cells isolated from recipients transplanted with Treg cells from WT, *Fanca*$^{-/-}$ or *Fancd2*$^{-/-}$ mice was prepared with RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's procedure. Reverse transcription was performed with random hexamers and Superscript II RT (Invitrogen, Grand Island, NY) and was carried out at 42 °C for 60 min and stopped at 95 °C for 5 min. First-strand cDNA was used for real-time PCR using primers listed in Table 1. Samples were normalized to the level of *GAPDH* mRNA, and the relative expression levels were determined by the standard curve method.

Statistical analysis

Paired or unpaired student's *t-test* was used for two-group comparisons, and one-way ANOVA for more than two-group comparisons. Survival data were plotted by the Kaplan-Meier curve method and analyzed by the log-rank test. Values of $p<0.05$ were considered statistically significant. Results are presented as mean ± SD. * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$.

Supplementary figures

Fig S1

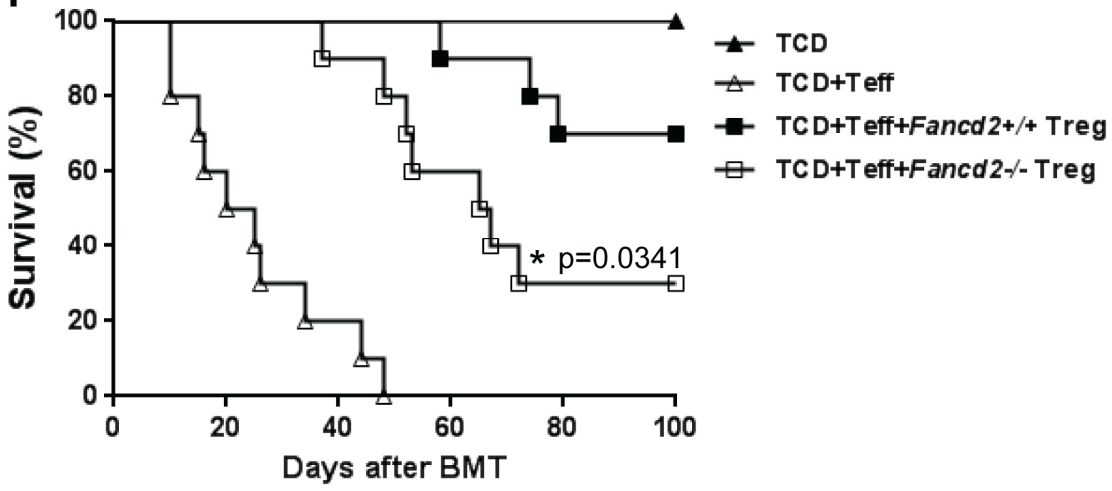

Fig S1. FA Treg cells are less suppressive in preventing GVHD *in vivo*. Lethally irradiated Balb/c recipients were transplanted with 5×10^6 T cell depleted bone marrow cells (TCD) from WT C57Bl/6 animals alone, or WT TCD plus sorted WT Teff cells ($CD4^+CD25^-$, 5×10^5) with or without equal numbers of sorted $CD4^+CD25^+$ Treg cells from either WT C57BL/6 or *Fancd2*$^{-/-}$ mice. Survival of the recipients was monitored, and analyzed by the Kaplan-Meier curve method. Each group includes 7-10 mice.

Fig S2

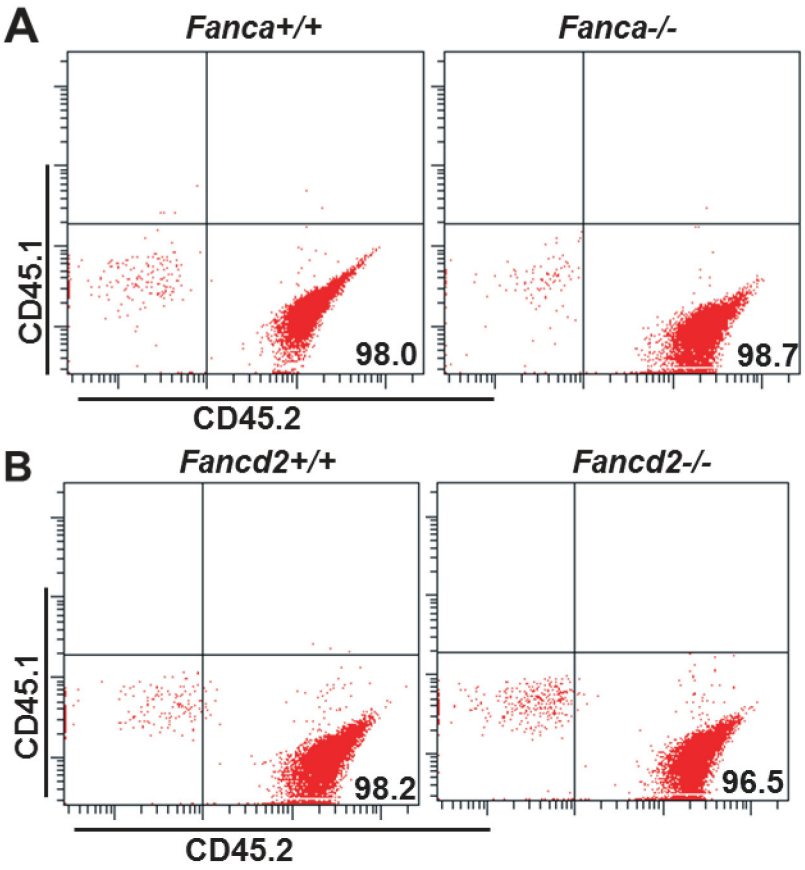

Fig S2. Generation of WT or FA chimera. 5×10^6 whole bone marrow cells (WBMCs) plus 5×10^6 splenocytes from WT C57BL/6 mice (B6, H-2^{b+}, CD45.2^+) or 10×10^6 WBMCs plus 5×10^6 splenocytes from *Fanca*$^{-/-}$ (A) or *Fancd2*$^{-/-}$ (B) mice (C57BL/6: B6, H-2^{b+}, CD45.2^+) were injected to lethally irradiated Boy J recipients (C57BL/6: B6, H-2^{b+}, CD45.1^+). Donor derived chimera (CD45.2^+) were assessed by Flow Cytometric analysis at 4 month after BMT.

Fig S3

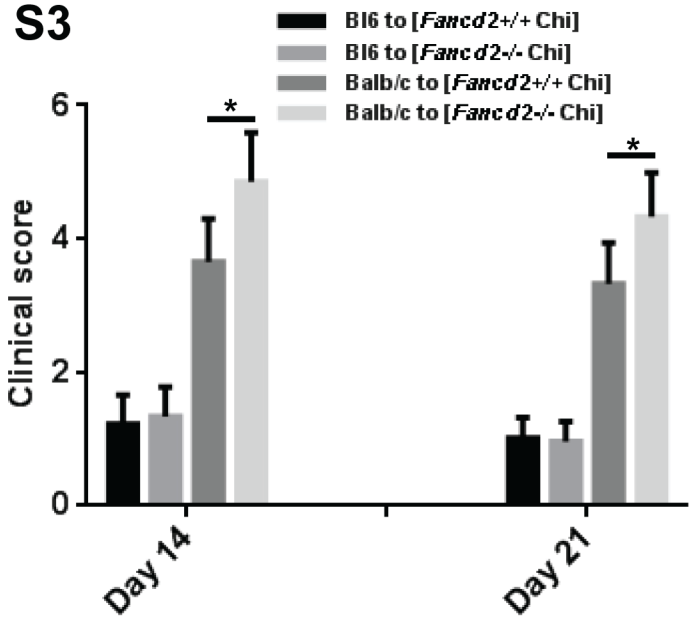

Fig S3. FA chimeras exhibit increased GVHD-inducing potential. The mice described in Fig S1 with greater than 95% donor-derived chimera were subjected to 2nd BMT by 9 Gy irradiation and injecting 5×10^6 BM cells along with 2×10^6 T cells isolated from either B6 (syngeneic; H-2^{b+}, CD45.2^+) or Balb/c (allogeneic; H-2^{d+}, CD45.2^+) mice. GVHD clinical scores were determined as a measure of GVHD severity on days 14 and 21 after allogeneic BMT. Data are presented as means plus or minus SD of two independent experiments (n=7-10 per group).

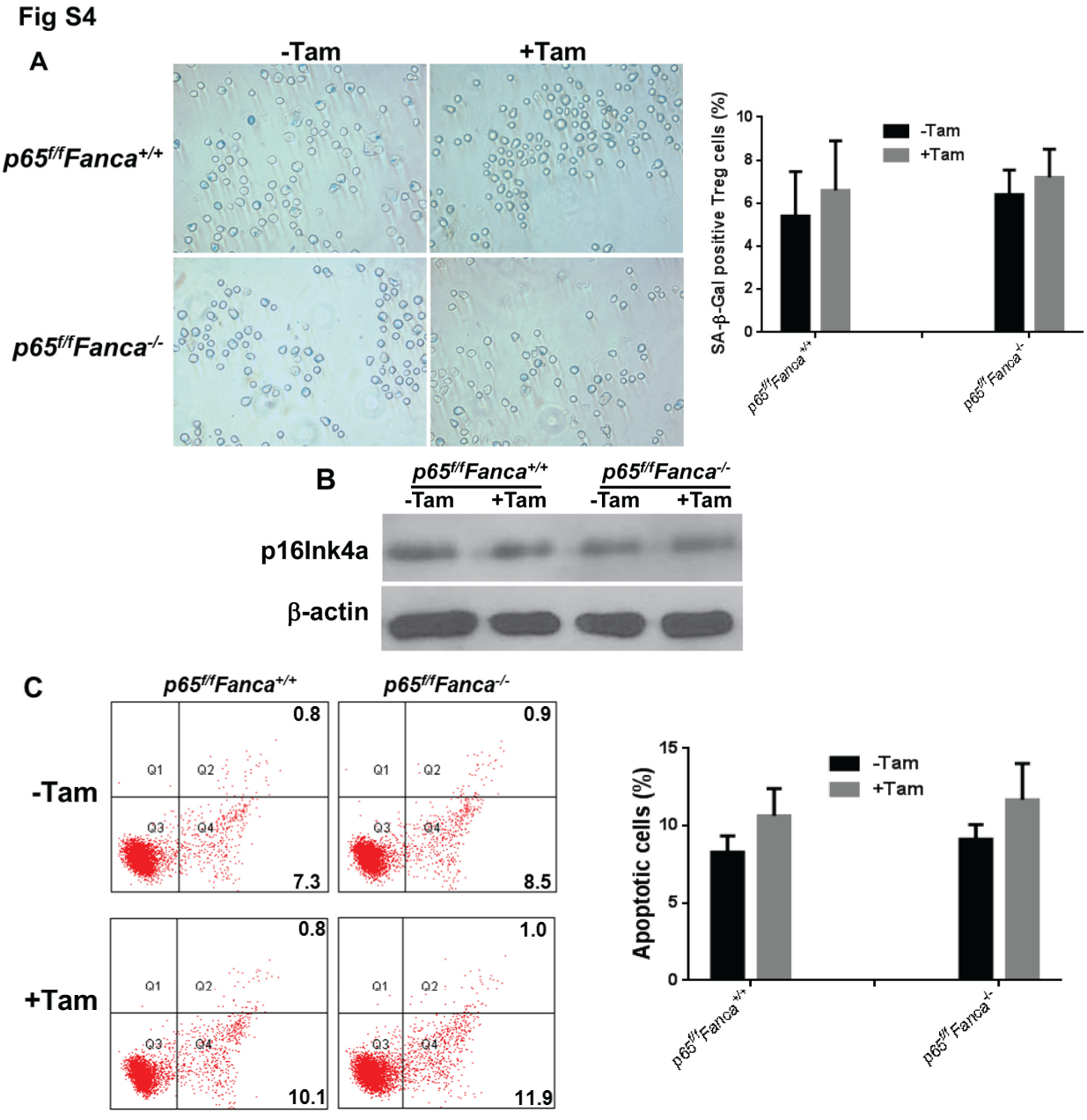

Fig S4. *p65* gene deletion does not alter cellular senescence or apoptosis in Tregs. (A) Loss of *p65* does not increase SA-β-gal-positive Treg cells. $CD4^+CD25^+$ Treg cells isolated from indicated genotype of mice were subjected to SA-β-galactosidase (SA-β-gal) staining. Representative images (Left) and quantification (Right) are shown. (B) *p65* gene deletion does not alter p16Ink4a level. Cells described in (A) were subjected to

immunoblotting against p16ink4a or β-actin. (C) *p65* gene deletion does not increase apoptosis in Tregs. Cells described in (A) were subjected to apoptosis analysis. Representative images (Left) and quantification (Right) were shown. Data are presented as means plus or minus SD of two independent experiments (n=3 per group).