

Supporting Information

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SI Materials and Methods

Flow Cytometry. Single-cell suspensions of thymocytes and splenocytes were prepared from freshly dissected organs following standard procedures, and were stained with appropriate antibodies in staining buffer (5% BSA and 0.05% NaN₃ in PBS). Cells were washed and analyzed on a FACSCalibur (BD Biosciences) with CellQuest (BD Biosciences) and FlowJo software (BD Biosciences). The antibodies used were as follows: PE-anti-CD8 (Ly-2, BD Pharmingen), FITC-anti-CD4 (L3T4, BD Pharmingen), PE-anti-TCR β (H57-597, BD Pharmingen), and FITC-anti-CD45R/B220 (RA3-6B2, BD Pharmingen).

Cell Culture. CD4⁺ cells were isolated from spleen and cultured as described previously (1, 2).

Lentiviral ZFAT shRNA. The lentivirus-based shRNA vector (pLentiU6+term) was generated from pLenti6/V5-DEST Gateway vector (Invitrogen). The ZFAT [an immune-related transcriptional regulator containing 18 C₂H₂-type zinc-finger domains and one AT-hook] shRNA was subcloned into pLentiU6+term, which was transfected into 293FT cells (Invitrogen), and recombinant lentivirus was collected according to the manufacturer's instructions. The shRNA duplexes used are listed in [Dataset S1](#).

Immunohistochemical Analysis. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (3 μ m) for staining of K_i-67 were treated by sodium citrate buffer (10 mM citric acid, pH 6.0) for 20 min. Sections for staining of cleaved

caspase-3 were treated with 0.3% hydrogen peroxidase in methanol for 30 min and antigen-retrieved by TE buffer (10 mM Tris, 1 mM EDTA, pH8.0) for 20 min. Sections for staining of CD41 were treated with 0.3% hydrogen peroxidase in methanol for 30 min and antigen-retrieved by TE buffer for 10 min. Sections were applied to immunohistochemical analysis using anti-K_i-67 antibody (K_i-67, Thermo Scientific) (3), anticlaved caspase-3 antibody (D175, Cell Signaling Technology) (3, 4), or anti-CD41 antibody (MWReg30, BD Pharmingen) (5). Signals were detected using HISTOFINE simple stain MAX PO (Nichirei) and DAB substrate (Nichirei). Sections were counterstained with hematoxylin and were examined using Biorevo BZ-9000 inverted-phase microscope (Keyence).

ChIP-PCR Assay. ChIP-PCR assays for *Cd41*, *Flk-1*, and *Runx1* were done on E7.5 yolk sacs and kidney as a control tissue. *Kifap3* was used as a hematopoiesis-unrelated control gene. As for immunoprecipitation, 100 μ g of anti-ZFAT monoclonal antibody M16 or Rat IgG (SM14LE, Acris) as a control were used. End-point PCR assays were performed at 35- and 42-cycled PCR. In ChIP-qPCR assay, the total amount of ChIP DNA was normalized by M16-ChIP DNA for *Kifap3* in kidney as 1.0 unit. Amplified regions (about 200-bp length) in the assays for *Cd41*, *Flk-1*, and *Runx1* were selected, based on high DNA-sequence homology with other species in 2-kb upstream region from a transcriptional start site. Primer sets for the assay are listed in [Dataset S1](#).

- Koyanagi M, et al. (2008) ZFAT expression in B and T lymphocytes and identification of ZFAT-regulated genes. *Genomics* 91:451–457.
- Fujimoto T, et al. (2009) ZFAT is an antiapoptotic molecule and critical for cell survival in MOLT-4 cells. *FEBS Lett* 583:568–572.
- Tsunoda T, et al. (2010) Three-dimensionally specific inhibition of DNA repair-related genes by activated KRAS in colon crypt model. *Neoplasia* 12:397–404.
- Ward JM, et al. (2006) Immunohistochemical markers for the rodent immune system. *Toxicol Pathol* 34:616–630.
- Lancrin C, et al. (2009) The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature* 457:892–895.

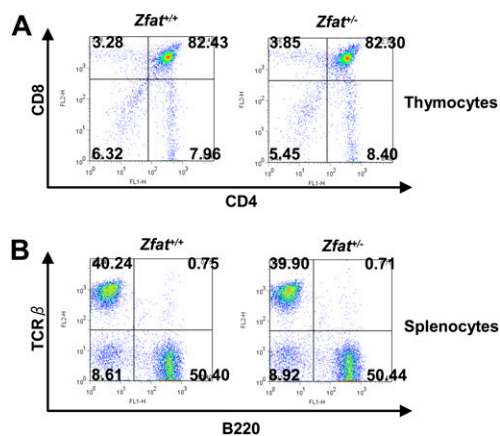


Fig. S1. *Zfat*^{+/-} mice have normal subpopulations of thymocytes and splenocytes. Representative FACS analysis of thymocytes and splenocytes in littermates of *ZFAT*^{+/+} and *ZFAT*^{+/-} mice (male; 7-wk-old). (A) Total thymocytes were gated for live cells and analyzed for CD4 (x axis) and CD8 (y axis) markers. Numbers in quadrants are percentages for each population. (B) Total splenocytes were gated for live cells and analyzed for B220 (x axis) and TCR (y axis) markers. Numbers in quadrants are percentages for each population.

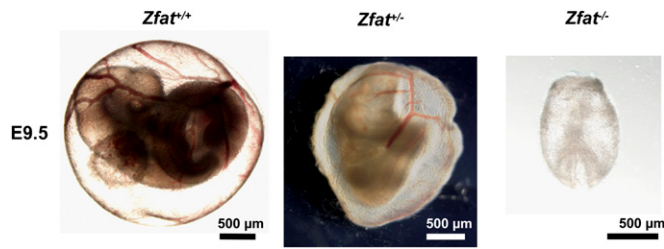


Fig. 52. No obvious abnormality in *Zfat*^{+/-} yolk sacs or embryos at E9.5. Embryos with yolk sacs from *Zfat*^{+/+}, *Zfat*^{+/-}, and *Zfat*^{-/-} mice at E9.5. (Scale bars, 500 µm.)

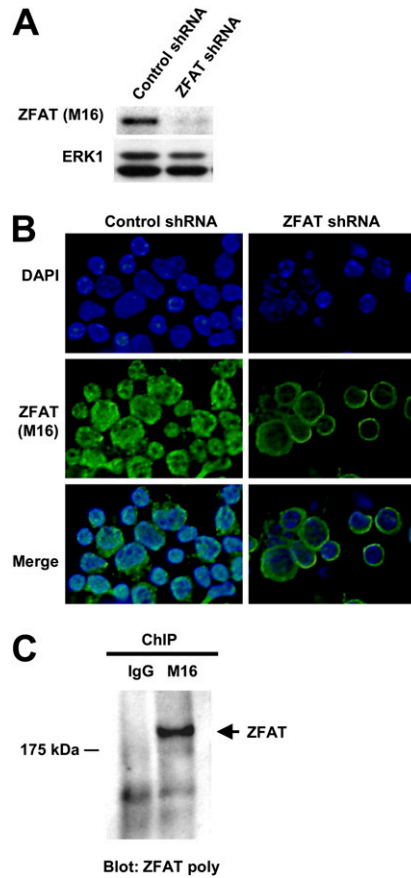


Fig. 53. Properties of M16 anti-ZFAT monoclonal antibody. (A) The M16 antibody recognizes ZFAT protein. Total lysate from mouse CD4⁺ cells was subjected to Western blot analysis. ERK1, loading control. (B) The M16 antibody is useful for immunostaining. Mouse CD4⁺ cells infected with control- or ZFAT-shRNAs were immunostained by the M16 antibody (green) and counterstained by DAPI (blue). (C) The M16 antibody is useful for ChIP. Blot was detected by anti-ZFAT polyclonal antibody (1).

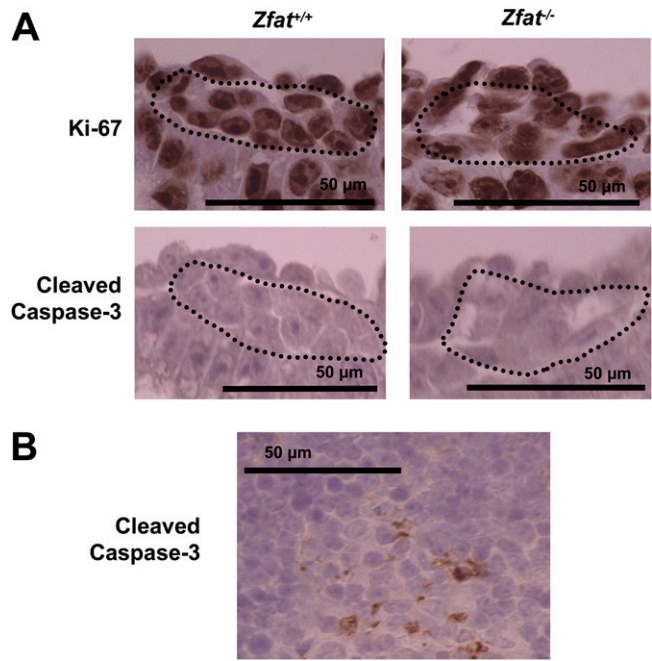


Fig. S4. No obvious difference in expression levels of proliferation- or apoptosis-related markers between *Zfat*^{+/+} and *Zfat*^{-/-} blood islands at E8.0. (A) Signals for *Ki-67* (Upper) and active caspase-3 (Lower) were detected by immunohistochemical staining with each antibody. The region surrounded by the dotted line represents hematopoietic progenitor cells. (Scale bars, 50 μ m.) (B) Active caspase-3 expression in the mouse thymus of the cortico-medullary border as a control signal detected by the immunohistochemical analysis used in A. (Scale bar, 50 μ m.)

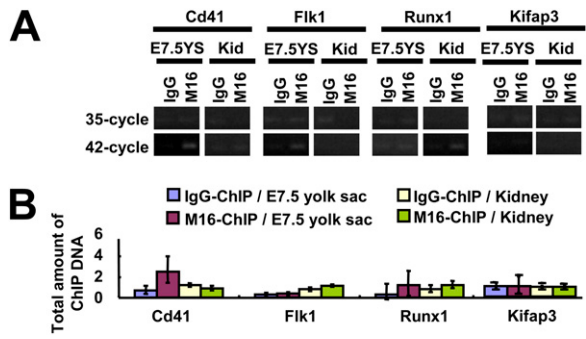


Fig. S5. ChIP-PCR assay for analysis of bindings of ZFAT with the promoter regions for *Cd41*, *Flk-1*, *Runx1*, and *Kifap3* on E7.5 yolk sacs and adult kidney as a control tissue. (A) End-point PCR products at 35- and 42-cycled PCR. YS, yolk sac; Kid, kidney. (B) Quantification of ChIP DNA. Quantities of the ChIP DNA in E7.5 yolk sacs with M16 anti-ZFAT antibody (red bar) and control IgG (blue bar). Quantities of the ChIP DNA in kidney with M16 (green bar) and control IgG (yellow bar). Bar indicates total amount of ChIP DNA normalized by M16-ChIP DNA for *Kifap3* promoter in kidney as 1.0 unit.

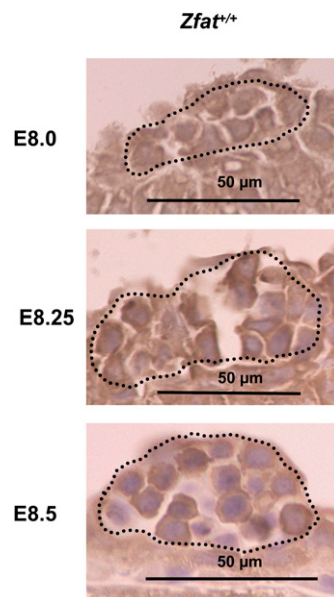


Fig. S6. Expression of CD41 in *Zfat^{+/+}* blood islands. Expression of CD41 at E8.0 (*Top*), E8.25 (*Middle*), and E8.5 (*Bottom*) in *Zfat^{+/+}* blood islands. Expressions were detected by immunohistochemical analysis using CD41 antibody. The region surrounded by the dotted line represents hematopoietic progenitor cells. (Scale bars, 50 μ m.)

Other Supporting Information Files

[Dataset S1 \(DOC\)](#)