Supporting Information

Semerad *et al*. 10.1073/pnas.0808866106

SI Materials and Methods

Mice. E2A −/− and E2A^{GFP} mice were described previously (1, 2). Unless otherwise stated in the text, we used 8- to 12-week-old mice in all studies. Mice were housed in a specific pathogen-free environment at the University of California, San Diego. Animal care and experimental procedures were approved by the Animal Subjects Committee, University of California, San Diego.

Peripheral Blood and Bone Marrow Analysis. Blood was obtained by retro-orbital venous plexus sampling in polypropylene tubes containing EDTA. Bone marrow was harvested by flushing both femurs and tibias with α-MEM containing 10% FBS or by crushing and passing through a 70-μm filter.

Flow Cytometry. Hematopoietic stem cell (HSC) and hematopoietic progenitors were analyzed and sorted with a FACSAria instrument (Becton Dickinson). All antibodies were purchased from eBioscience, unless otherwise indicated. For HSC analysis and sorting, adult bone marrow cells were stained with fluorescein isothiocyanate (FITC)-CD34 (RAM34) (PharMingen), phycoerythrin (PE)-Flt-3 (A2F10), allophycocyanin (APC)- or AlexaFluor 750 (AF750)-c-kit (2B8), phycoerythrin-Cy5.5 (PE-Cy5.5)-Sca-1 (D7), and APC- or Biotin-conjugated lineage antibodies including: CD3 (145–2C11), CD4 (RM4–5), CD8 (53–6.7), B220 (RA3–6B2), CD19 (MB19–1), Gr-1 (RB6–8C5), Mac-1 (M1/70), NK1.1 (PK136), and Ter-119, followed by avidin-APC-Cy7 when applicable. LSK cells were sorted as Lin$^-$/c-kit$^+$/Sca-1$^+$. Alternatively, cells were stained with FITC-CD34, PE-Flt3, PE-Cy5-IL7Rα, PECy5.5-CD19 and -CD11c, PECy7-B220 and -NK1.1, APC-FcγR, AF680-Sca-1 (I.L.W. Laboratory, Stanford University School of Medicine), AF750-c-kit, Pacific Blue (PacBlue)-Mac-1 (I.L.W. Laboratory), -Ter119 (I.L.W. Laboratory), -CD4 (I.L.W. Laboratory), and -CD8 (I.L.W. Laboratory), Pacific Orange (PacO)-Gr-1 (I.L.W. Laboratory), and biotin-CD150 followed by streptavidin-Qdot-605 (Invitrogen). CLPs were analyzed by using PE-IL-7Rα, APC- or AF750-c-kit, PE-Cy5.5-Sca-1, and APC- or Biotin-conjugated lineage antibodies followed by avidin-APC-Cy7 when applicable. CLPs were identified as Lin$^-$/IL-7Rα^+/ckit^{int}/Sca-1^{int}. Myeloid progenitors were analyzed using APC-conjugated lineage antibodies, AF750-c-kit, PE-Cy5.5-Sca-1, phycoerythrin-Cy7 (PE-Cy7)-CD16/32 (93), and Biotin-CD34 followed by avidin-PE. The erythromyeloid subpopulations were identified as Lin$^-$/Sca-1$^-$/IL-7Rα^-/c-kit$^+$/CD34$^+$/FcγR^{low} for CMPs, as Lin$^-$/Sca-1$^-$/IL-7Rα^-/c-kit$^+$/CD34$^+$/FcγR^{high} for GMPs, and as Lin$^-$/Sca-1$^-$/IL-7Rα^-/c-kit$^+$/CD34$^-$/FcγR^{low} for MEPs. Alternatively, to further subdivide the Lin-ckit+Sca-1- compartment, cells were stained with FITC-CD34, PE-CD41, PECy5-IL7Ra, -Mac-1, -B220, -CD4, and -CD8, PECy5.5-CD19 and -CD11c, PECy7-Ter119, APC-FcγR, AF680 Sca-1, AF750 c-kit, PacBlue-CD105, PacO-Gr-1, and biotin-CD150 with streptavidin Qdot-605. Data were analyzed with FlowJo software (Treestar).

Bone Marrow Homing Assay. Bone marrow was harvested from 8–10 week old wild type (CD45.2$^+$) and E2A −/− (CD45.2$^+$) mice as described (n = 6 mice for each genotype). The cells were pooled and subjected to ammonium chloride mediated RBC lysis. The samples were depleted of Lin$^+$ cells by staining with biotin-conjugated lineage antibodies (CD3, CD4, CD8, B220, CD19, Gr-1, Mac-1, NK1.1, and Ter-119), incubation with anti-biotin microbeads (Miltenyi Biotech), and isolation of Lin$^-$ cells by using AutoMACS (Miltenyi Biotech). A portion of the Lin$^-$ fraction was analyzed by flow cytometry to determine the input percentages of the LSK, LSKFlk2$^-$, and LSKFlk2$^+$ cell subsets in the wild-type and E2A −/− samples. The Lin$^-$ cells were resuspended and 500,000 cells injected intravenously into lethally γ-irradiated (single dose of 1,000 cGy 24 h before transplantation) CD45.1 congenic C57/B6 mice at 10 weeks of age (n = 5 recipients per genotype); 18 h posttransplantation, bone marrow was harvested from the recipient mice as described above and analyzed by flow cytometry for the presence of CD45.2$^+$LSK, CD45.2$^+$LSKFlk2$^-$, and CD45.2$^+$LSKFlk2$^+$ populations.

CFU-G Colony Assay. A total of 5×10^4 bone marrow mononuclear cells were plated in 2.5 mL of methylcellulose media (MethoCult 3231; Stem Cell Technologies), supplemented with G-CSF at 100 ng/mL (Peprotech) for CFU-G cultures. Standard morphologic criteria were used to score colonies containing at least 50 cells on day 7 for CFU-G.

1. Bain G, *et al.* (1997) Both E12 and E47 allow commitment to the B cell lineage. *Immunity* 6:145–154.

2. Zhuang Y, Jackson A, Pan L, Shen K, Dai M (2004) Regulation of E2A gene expression in B-lymphocyte development. *Mol Immunol* 40:1165–1177.

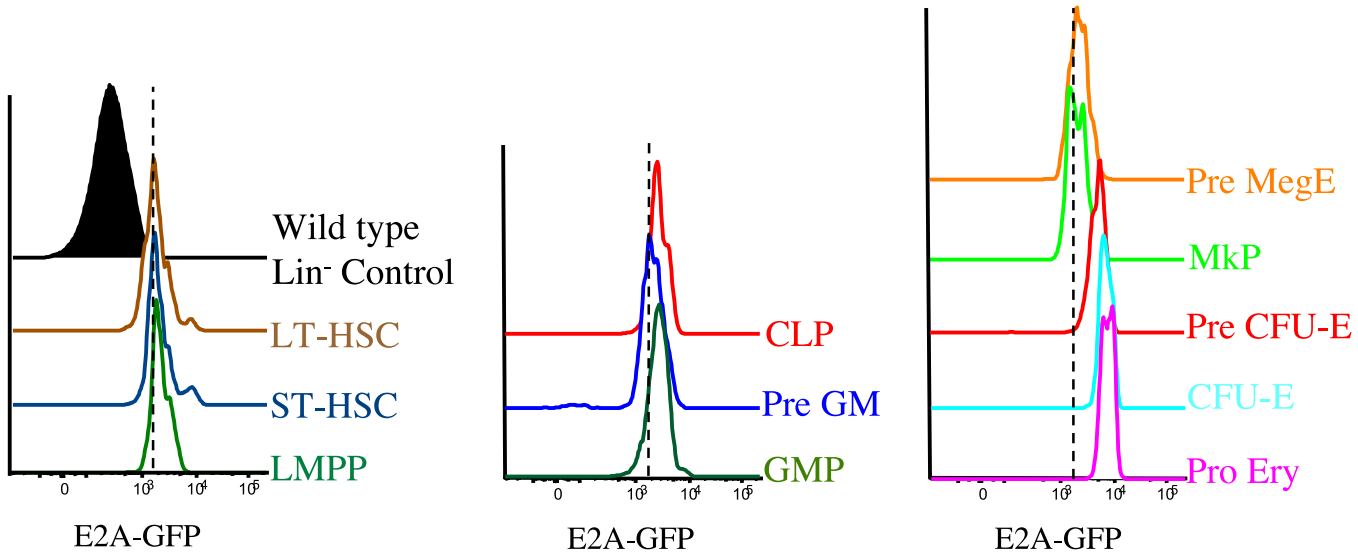

Fig. S1. Expression of E2A in adult mouse bone marrow (BM) HSCs and hematopoietic progenitors. The BM HSCs and indicated hematopoietic progenitor populations from wild-type and E2A$^{GFP/GFP}$ mice were analyzed and gated as described. The fluorescence intensity of GFP in each progenitor population is specified by histograms. The open colored histograms represent GFP fluorescence from E2A$^{GFP/GFP}$ HSCs and hematopoietic progenitor cells. As a control, the solid black histogram represents background GFP fluorescence of wild-type Lin^{-} cells. The dotted line in each plot corresponds to the level of GFP fluorescence for the LT-HSCs.

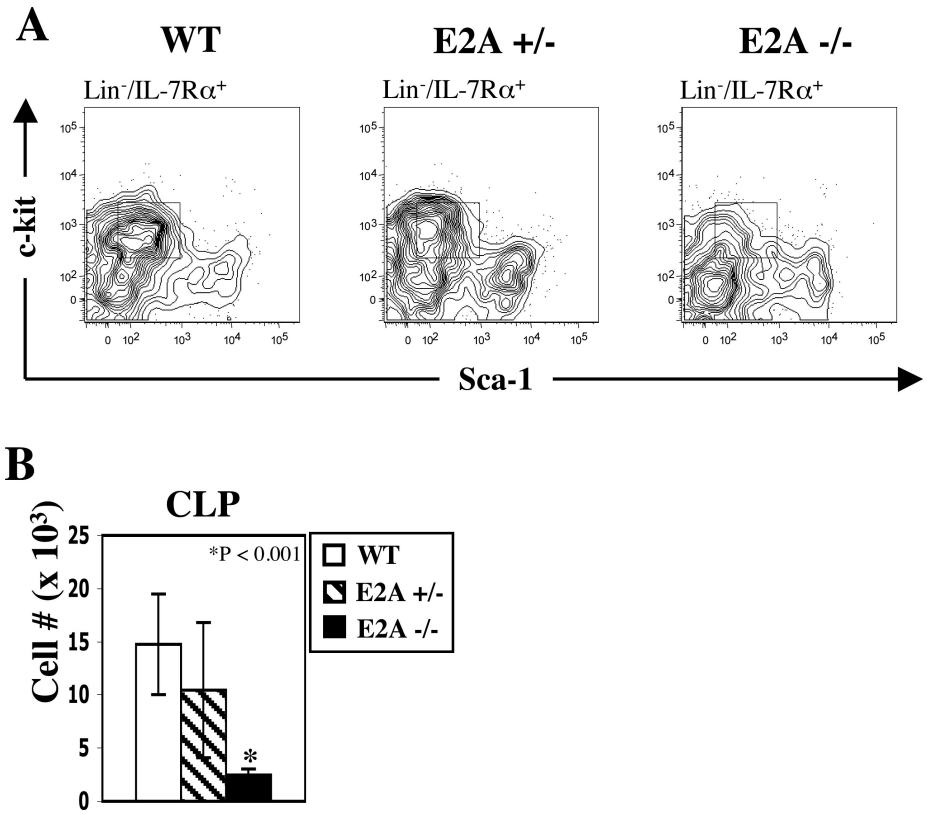

Fig. S2. Effect of E2A deletion on the number of common lymphoid progenitors in adult mouse BM. Total BM cells from wild-type, E2A +/−, and E2A −/− mice were harvested and prepared for analysis by flow cytometry as described. (*A*) Shown are representative staining profiles for CLPs. (*B*) Reduced number of CLPs in the BM of E2A −/− mice. Shown are the absolute numbers of CLPs in the BM of wild-type, E2A +/−, and E2A −/− mice ($n = 8$). Data represent the mean ± SD. Statistical significance determined by 2-sided Student's *t* test, compared with wild-type.

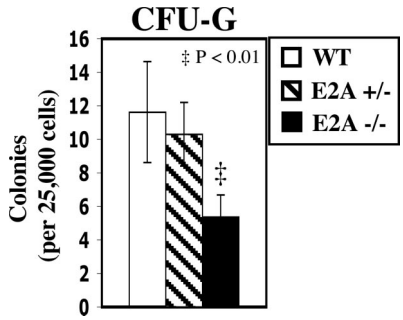

Fig. S3. Reduced number of CFU-G colonies in the BM of E2A −/− mice. Total BM cells from wild-type, E2A +/−, and E2A −/− mice ($n = 4$) were plated in methylcellulose-containing media supplemented with G-CSF. Shown is the number of colonies per 25,000 total BM cells. Standard morphologic criteria were used to score colonies containing >50 cells. Statistical significance determined by 2-sided Student's *t* test, compared with wild-type.

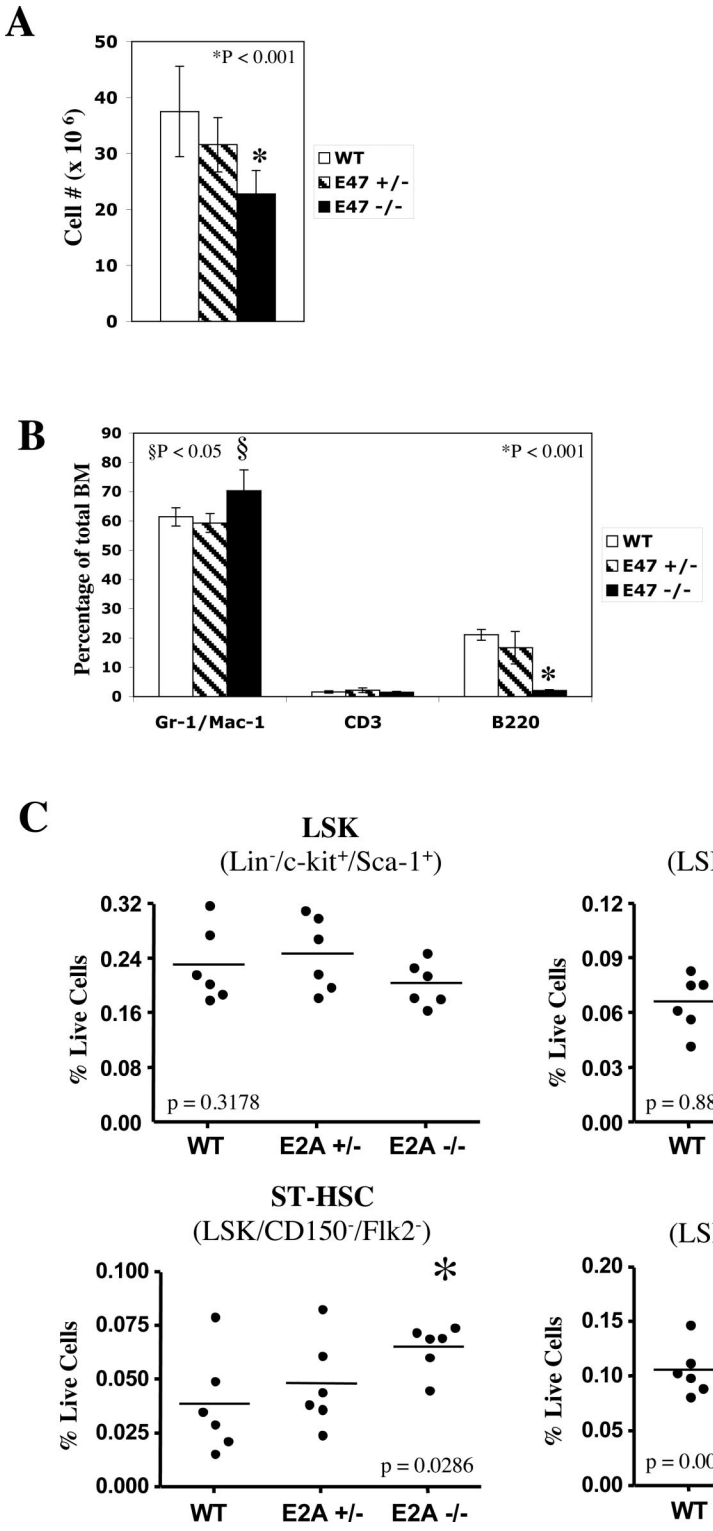

Fig. S4. Effects of E2A deletion on bone marrow mononuclear cell number and the frequency of HSCs in adult mouse BM. (*A*) Decreased BM cellularity in E2A −/− mice. Shown are the absolute numbers of bone marrow mononuclear cells from the hind limbs of 8–9 week old wild-type, E2A +/−, and E2A −/− mice (n = 8). (*B*) Altered distribution of lineage positive cell subsets in the BM of E2A −/− mice. Shown are the percentages of Gr-1$^+$/Mac-1$^+$, CD3$^+$, and B220$^+$ cell subsets in the bone marrow of wild-type, E2A +/−, and E2A −/− mice as determined by flow cytometry (n = 8). (*C*) Frequencies of the LSK (Lin$^-$/c-kit$^+$/Sca-1$^+$), LT-HSC (LSK/CD150$^+$/Flk2$^-$), ST-HSC (LSK/CD150$^-$/Flk2$^-$), and MPP (LSK/CD150$^+$/Flk2$^+$) cell subsets in the BM of wild-type, E2A +/−, and E2A −/− mice (n = 6). Statistical significance determined by unpaired t test, 2-tailed, between E2A −/− and wild-type.

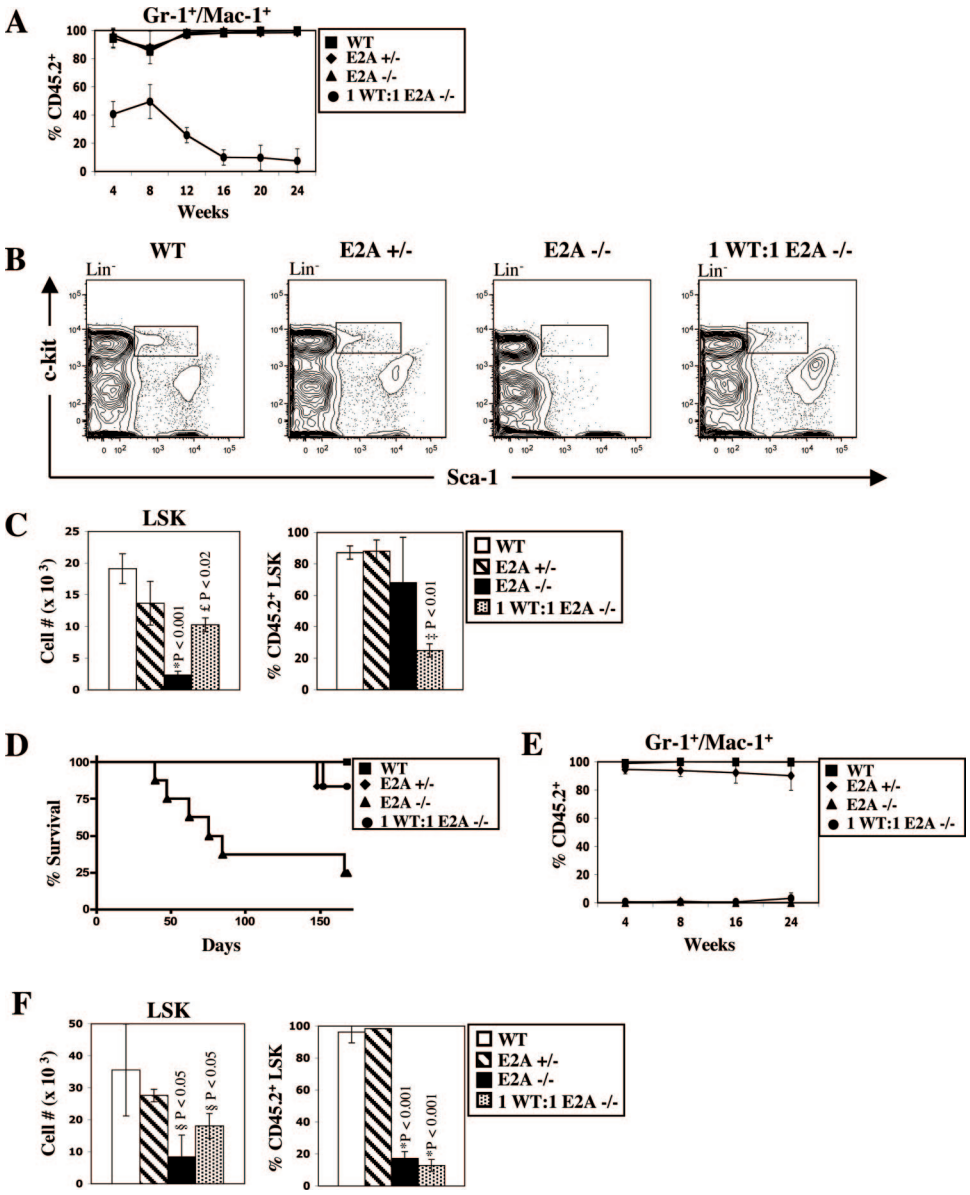

Fig. S5. E2A-deficient HSCs are defective in their long-term and serial repopulating ability. A competitive reconstitution assay by using FACS-purified LSK cells from wild-type and E2A −/− mice was used to assess the repopulating ability of E2A −/− HSCs. (*A*) Peripheral blood was analyzed monthly posttransplant to assess the contribution of wild-type, E2A +/−, or E2A −/− (CD45.2$^+$) HSCs to the myeloid lineage (Gr-1$^+$/Mac-1$^+$ cells) (n = 4 recipients per group). (*B*) Representative staining profiles of LSK cells from the BM of primary LSK cell transplant recipient mice at 24-weeks posttransplant. (*C*) Reduced numbers of LSK cells in the BM of primary E2A −/− LSK cell recipient mice 24-weeks posttransplant. (*Left*) Absolute numbers of LSK cells found in the BM of the primary transplants; (*Right*) and the percentage of the LSK cells that are donor or E2A −/− derived (CD45.2$^+$) (n = 4 recipients per group). (*D*) Survival curve for recipients of secondary BM transplants. Lethally irradiated CD45.1$^+$ recipient mice were transplanted with 2 × 10^6 whole BM cells isolated from the primary LSK cell transplant recipients described above (n = 8 recipients per group). (*E*) Peripheral blood was analyzed monthly posttransplant to assess the contribution of donor or E2A −/− (CD45.2$^+$) BM cells to the myeloid lineage (Gr-1$^+$/Mac-1$^+$ cells) in the secondary recipients (n = 2–8 recipients per group). (*F Left*) Absolute numbers of LSK cells found in the BM of the secondary transplant recipients; (*Right*) and the percentage of the LSK cells that are donor or E2A −/− derived (CD45.2$^+$) (n = 2–8 recipients per group). Data represent the mean ± SD. Statistical significance determined by 2-sided Student's t test, compared with wild type.

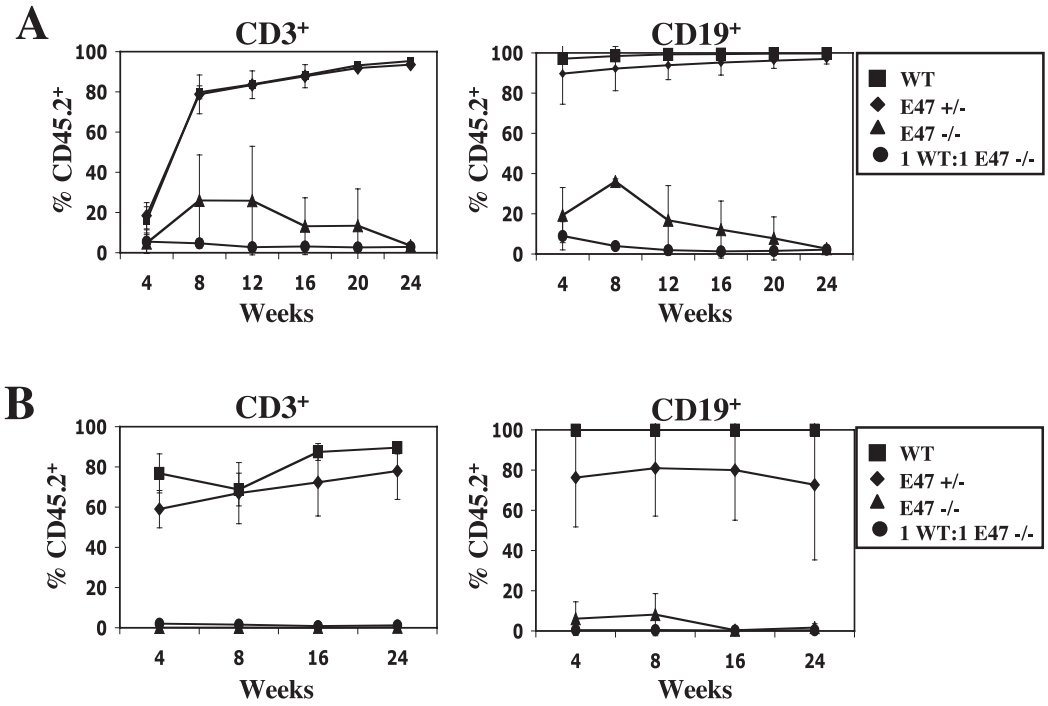

Fig. S6. Defective repopulation of the T- and B-lymphoid lineages by E2A-deficient HSCs. A competitive reconstitution assay by using FACS-purified LSK cells from wild-typeand E2A −/− mice was used to assess the repopulating ability of E2A −/− HSCs. Peripheral blood was analyzed monthly posttransplant to assess the contribution of wild-type, E2A +/−, or E2A −/− (CD45.2$^+$) HSCs to the T- and B-lymphoid lineages primary LSK cell transplants (n = 4 recipients per group) (*A*); and secondary BM transplants (n = 2–8 recipients per group) (*B*).

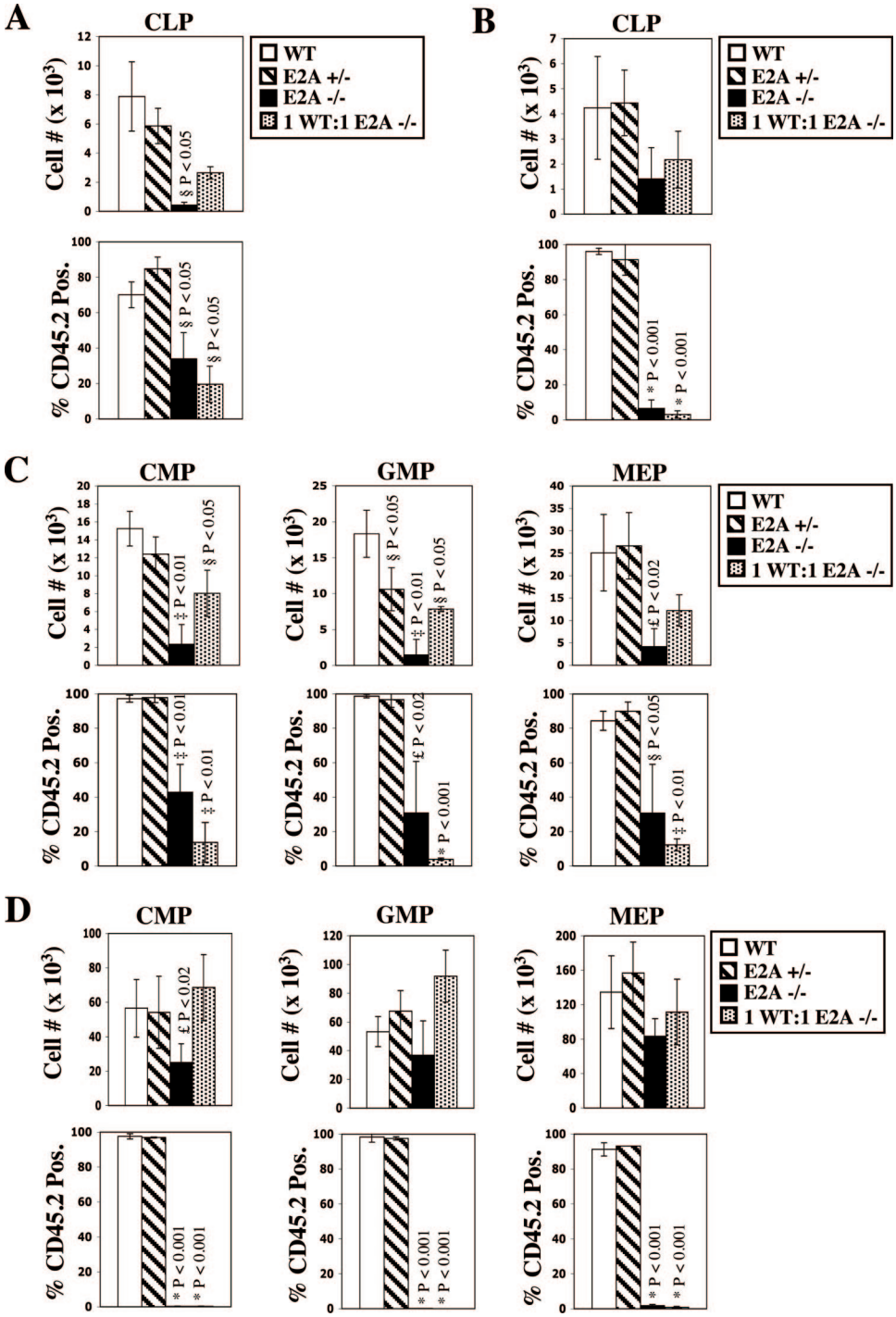

Fig. S7. Defective repopulation of hematopoietic progenitors by E2A-deficient HSCs. BM from primary transplant recipients receiving FACS-purified LSK cells and secondary transplant recipients receiving whole BM isolated from the primary LSK cell transplants was analyzed 24-weeks posttransplant for the presence of wild-type or E2A −/− derived lymphoid and erythromyeloid hematopoietic progenitors. (*A* and *B*) Absolute numbers (*Upper*) of CLPs and the percentage (*Lower*) of CLPs that are donor or E2A −/− (CD45.2$^+$) derived in the BM of the primary LSK cell transplant recipients (*A*) and the secondary transplant recipients (*B*) 24-weeks posttransplant. (*C* and *D*) Absolute numbers (*Upper*) of CMPs, GMPs, and MEPs and the percentage (*Lower*) of each that are donor or E2A −/− (CD45.2$^+$) derived in the BM of the primary LSK cell transplant recipients (*C*) and the secondary transplant recipients (*D*) 24-weeks posttransplant.

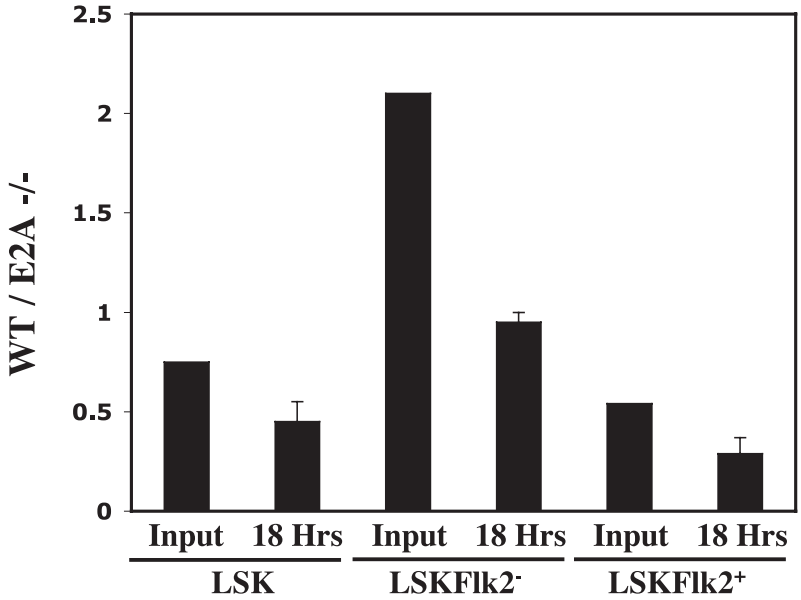

Fig. S8. Normal homing capacity of E2A-deficient HSCs. Lin^- BM cells from wild-type and E2A −/− mice were isolated and transplanted into lethally irradiated recipient mice as described; 18 h posttransplantation, bone marrow was harvested from the recipient mice ($n = 6$) as described and analyzed for the presence of $CD45.2^+LSK$, $CD45.2^+LSKFlk2^-$, and $CD45.2^+LSKFlk2^+$ cells by flow cytometry. To assess the level of homing to the bone marrow by each HSC subset, the ratio of wild-type to E2A −/− (wild-type/E2A −/−) was determined for each HSC subset within the Lin^- preparations injected and compared with the wild-type to E2A −/− ratio determined 18 h posttransplant ($n = 5$ recipients per genotype).

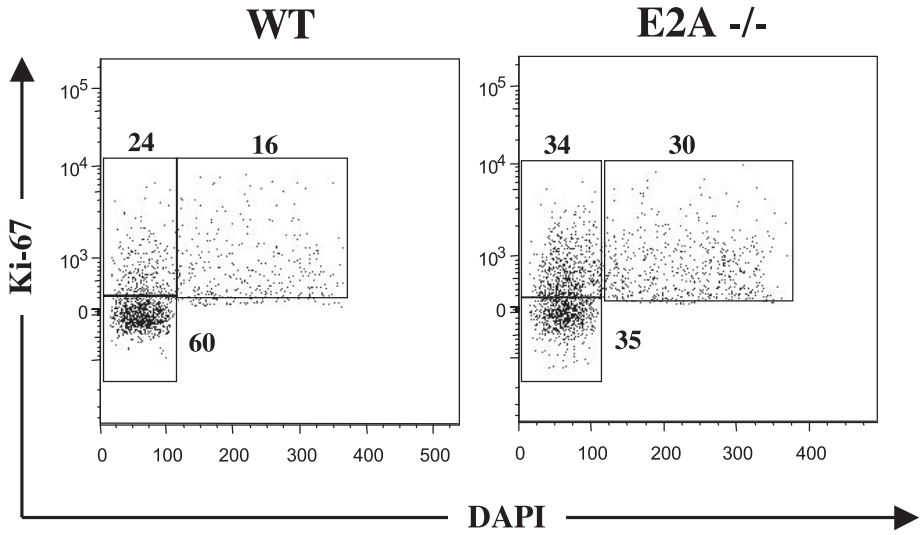

Fig. S9. Representative FACS profile of cell cycle analysis by staining of LSKFlk2$^-$ HSCs with Ki-67 and DAPI. Cell cycle distribution was analyzed by Ki-67 and DAPI staining of LSKFlk2$^-$ and LSKFlk2$^+$ HSC subsets of wild-type and E2A −/− mice. Shown is a representative FACS profile of the LSKFlk2$^-$ HSC subset.

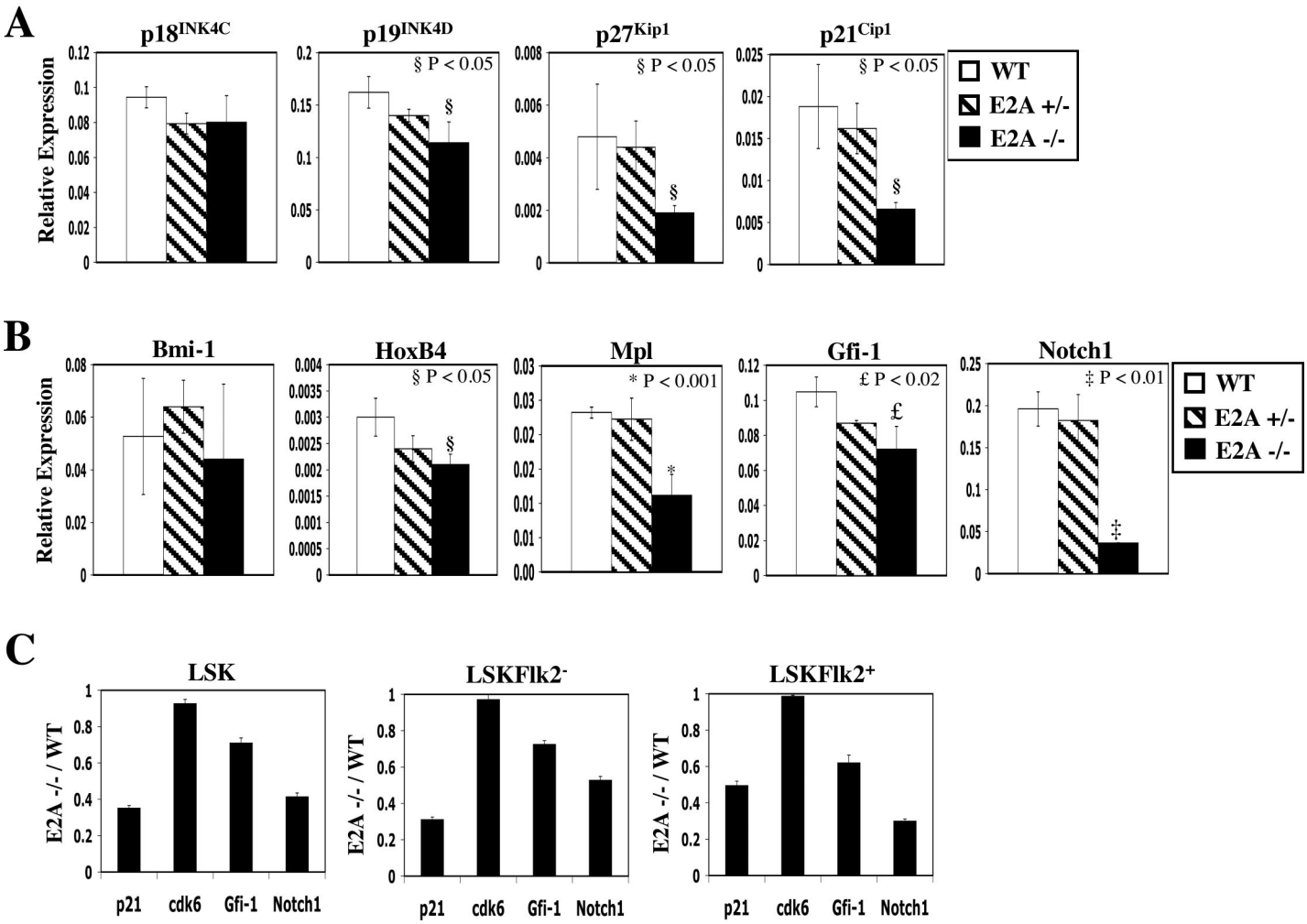

Fig. S10. Quantitative PCR expression analysis of known regulators of cell cycle progression in E2A-deficient LSK Cells. (*A*) Quantitative PCR expression analysis of $p18^{INK4C}$, $p19^{INK4D}$, $p27^{Kip1}$, and $p21^{Cip1}$ in LSK cells sorted from wild-type and E2A −/− mice. Shown is the relative amount of target mRNA compared with β-actin mRNA. (*B*) Quantitative PCR expression analysis of Bmi-1, HoxB4, Mpl, Gfi-1, and Notch1 in LSK cells sorted from wild-type and E2A −/− mice. Shown is the relative amount of target mRNA compared with β-actin mRNA. Data represent the mean ± SD. Statistical significance determined by 2-sided Student's *t* test, compared with wild-type. (*C*) Quantitative PCR expression analysis of $p21^{Cip1}$, cdk6, Gfi-1, and Notch1 in LSK, LSKFlk2-, and LSKFlk2+ cell subsets FACS-sorted from wild-type and E2A −/− mice. Transcript levels are shown as a ratio of E2A −/− transcript levels compared with wild-type (E2A −/−/wild-type) normalized by β-actin mRNA expression.