Supplemental Data

Cyclin A – Redundant in Fibroblasts, Essential in Hematopoietic and Embryonal Stem Cells

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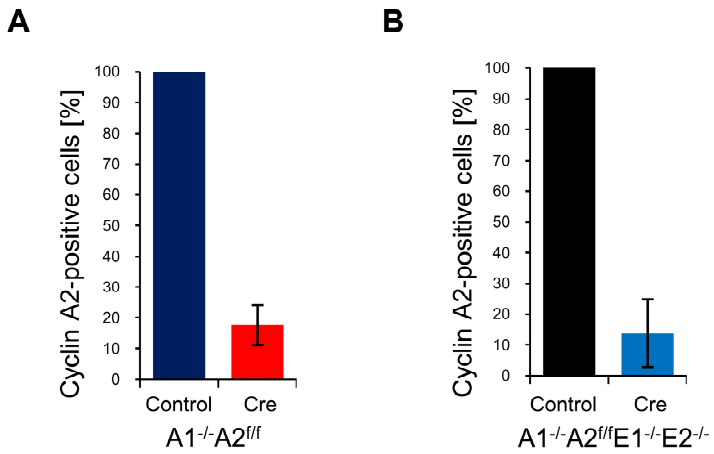

Figure S1. Estimation of the Efficiency of Cyclin A2 Deletion in Cultured Cells Using Immunostaining

(A) Cyclin $A1^{-/-}A2^{f/f}$ mouse embryonic fibroblasts were transduced with Cre-expressing virus (Cre), or with enzymatically inactive Cre point-mutant (Control) and were plated on cover slips. Subsequently, cells were fixed in 4% paraformaldehyde and stained with anti-cyclin A2 antibody (sc-596, Santa Cruz). As a secondary antibody, Alexa Fluor goat anti-rabbit (Invitrogen) was used. Cell nuclei were stained with DAPI (Vectashield, Vector Laboratories). We first determined the fraction of cyclin A2-positive cells in control cultures, and this was set at 100%. The fraction of cyclin A2-positive cells in Cre-transduced cultures

was then determined and presented as the percentage of control values. Shown are mean values ±SD.

(B) Similar analysis as above for cyclin $A1^{-/-}A2^{f/f}E1^{-/-}E2^{-/-}$ cells.

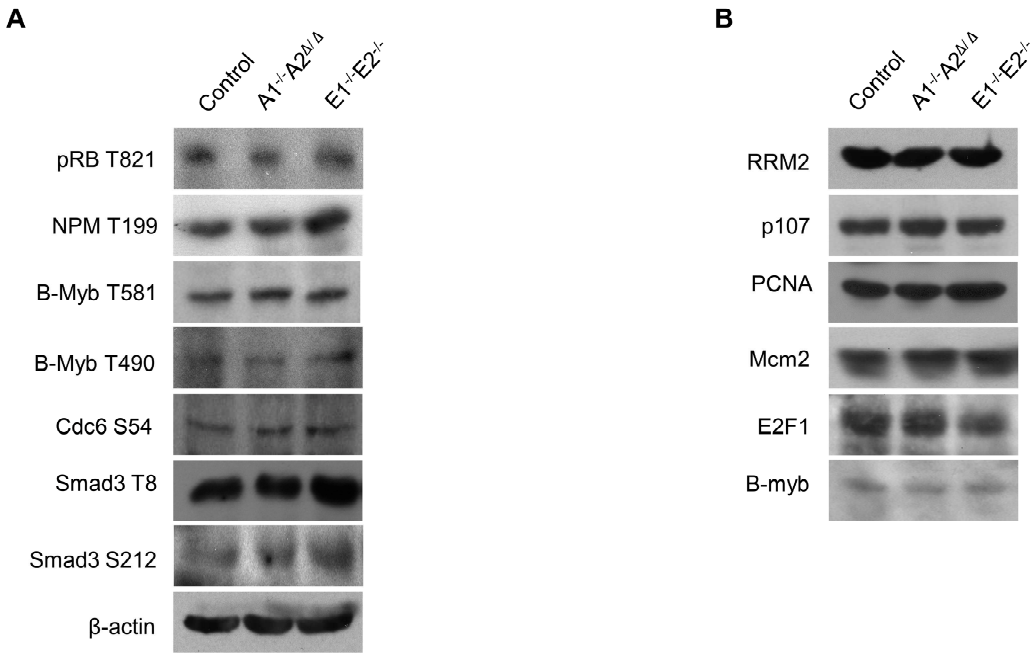

Figure S2. Analyses of Cyclin A- and E-deficient Mouse Embryo Fibroblasts (MEF)

(A) Lysates were prepared from cyclin $A1^{-/-}A2^{f/f}$ fibroblasts transduced with Cre-encoding viruses ($A1^{-/-}A2^{\Delta/\Delta}$), or from cyclin $E1^{-/-}E2^{-/-}$ MEFs (Geng et al., 2003), immunoblotted and probed with the indicated phospho-specific antibodies against: retinoblastoma protein, pRB T821 (Sigma-Aldrich). This residue was shown to be phosphorylated by cyclin A-Cdk2 and cyclin E-Cdk2 (Zarkowska and Mittnacht, 1997). Nucleophosmin, NPM T199 (Abcam). This residue is thought to represent cyclin E-Cdk2 substrate, but it can be phosphorylated *in vitro* by cyclin A-Cdk2 (Tokuyama et al., 2001). B-Myb T581 and T490 (both from Santa Cruz). These residues of B-Myb represent substrates of cyclin A-Cdk2 (Saville and Watson, 1998; Bartsch et al., 1999). Cdc6 S54 (Santa Cruz). This site was shown to be phosphorylated by cyclin A-Cdk2 (Petersen et al., 1999) or by E-Cdk2 (Mailand and Diffley, 2005). Smad3 T8 and S212 (kindly provided by Dr. Fang Liu). These sites were shown to be collaboratively phosphorylated by Cdk2- and Cdk4-containing complexes (Matsuura et al., 2004).

(B) Lysates were probed with antibodies against the following E2F targets: Ribonucleotide Reductase M2 (RRM2, AbD Serotec), p107, PCNA, E2F1, B-Myb (all from Santa Cruz), Mcm2 (BD Biosciences).

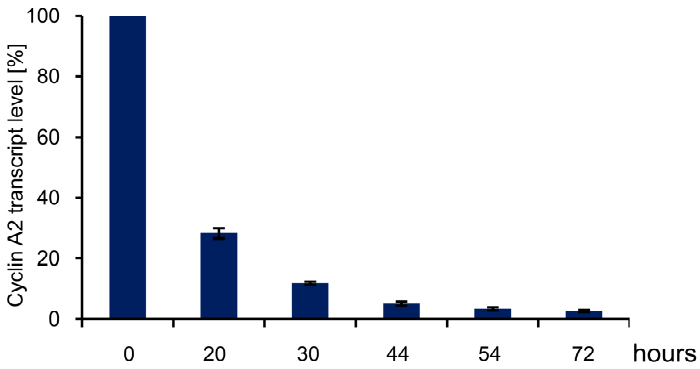

Figure S3. Analyses of Cyclin A2 Transcript Levels at Different Time-Points after Transduction with Cre

Cyclin $A1^{-/-}A2^{f/f}E1^{-/-}E2^{-/-}$ cells were transduced with Cre-expressing viruses as described in the Experimental Procedures. At different time-points after the transduction, RNA was isolated, and the levels of cyclin A2 transcript were determined by reverse transcription - real time PCR (see Supplemental Experimental Procedures for details). The levels observed before the infection (time 0) were set at 100%. Shown are mean values $\pm$SD.

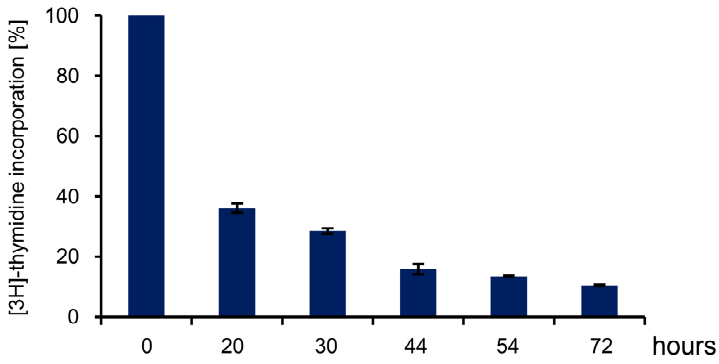

Figure S4. Analyses of Thymidine Incorporation at Different Time-Points after Transduction with Cre

Cyclin $A1^{-/-}A2^{f/f}E1^{-/-}E2^{-/-}$ MEFs were transduced with Cre-expressing viruses as described in the Experimental Procedures. At different time-points after the transduction, cells were fed with [^{3}H]-thymidine for 1 hr, and incorporation of [^{3}H]-thymidine was determined. The incorporation observed before the infection (time 0) was set at 100%. Shown are mean values $\pm$SD.

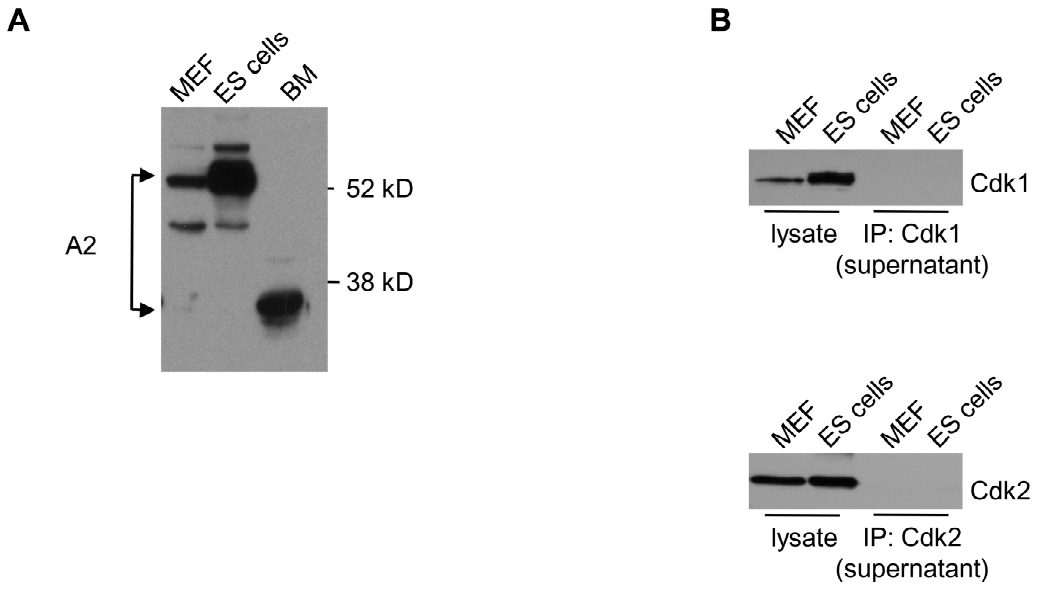

Figure S5. Analyses of Bone Marrow, MEFs and ES cells

(A) Western blot analysis of *in vitro* cultured fibroblasts (MEF), ES cells and bone marrow cells (BM) probed anti-cyclin A2 antibody (sc-596 from Santa Cruz) that is different from the one used in Figure 7E (c4710, from Sigma). Both antibodies detect a shorter,approximately 38 kDa species in bone marrow, as reported by Welm et al. (2002).

(B) These blots represent control lanes for the Cdk1 and Cdk2 immunoprecipitations shown in Figure 7G. Shown here are the levels of Cdk1 and Cdk2 in the lysate before (lysate), and after the immunoprecipitation (supernatant). Note that the immunoprecipitations of Cdk1 and Cdk2 were essentially complete, as no detectable Cdk1 or Cdk2 protein was detected in the supernatant.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of Conditional Cyclin $A2^{f/f}$ Mice

The cyclin A genomic fragment was subcloned from clone #RP22-513P3 derived from RPCI-22 mouse 129s6/Sv BAC library (BACPAC Resources Center, Children's Hospital Oakland Research Institute). We assembled the gene-targeting construct by inserting 5' *loxP* site into the SmaI site within the first intron, and the 3' *loxP* site together with neomycin resistance (NEO) cassette flanked by two FRT recombination sites into the SnaBI site in the seventh intron of the cyclin A2 locus (main text, Figure 1A). The gene-targeting construct was electroporated into embryonal stem (ES) cells and cells were selected with neomycin. Homologous recombination was verified by Southern blotting using probes A (SnaBI –XbaI 552 bp fragment) and B (KpnI-SalI 186 bp) (Figure 1A). 14/480 of ES cell clones underwent homologous recombination. Two recombinant ES cell clones were electroporated with a plasmid expressing FLP-e recombinase, leading to the excision of the NEO cassette (Figure 1A) giving rise to cyclin $A2^{f/+}$ ES. These cyclin $A2^{f/+}$ ES cells were then injected into C57BL/6 mouse blastocysts and cyclin $A2^{f/f}$ mice were obtained using standard methods (Geng et al., 2003). For some analyses, cyclin $A2^{f/f}$ mice were bred with cyclin $A1^{-/-}$ (Liu et al., 1998), $E1^{-/-}$, $E2^{-/-}$ (Geng et al., 2003), MxCre (Kuhn et al., 1995) or Meox2-Cre animals which express Cre in epiblast-derived tissues (Tallquist and Soriano, 2000) (the latter two strains were purchased from the Jackson Laboratory).

Derivation of Mouse Embryonic Fibroblast Cultures

Mouse embryonic fibroblasts (MEFs) were isolated as follows. Mice were mated and the day when a vaginal plug was detected was designated as day 0.5 *post coitum*. Embryos were sacrificed by decapitation at day 13.5 *post coitum* and the internal organs were removed. The remaining tissues were minced in 0.25% trypsin-EDTA and further digested with trypsin at 37^{o}C for 45 minutes. After

digestion, the tissue was resuspended by pipetting in DMEM supplemented with 10% fetal bovine serum and plated out in T75 flasks. Once the cells reached confluence, they were trypsinized in 0.25% trypsin-EDTA, and passaged by 1:4 splitting (this was designated passage 1). Subsequent passages were also performed by 1:4 splitting, and cells were cultured in DMEM plus 10% fetal bovine serum. For most of experiments, cells from passage 1 to 2 were used for infections and analyses.

Pulse-Chase Experiments

S phase cells were labeled by addition of bromodeoxyuridine (BrdU, 75 uM) to the culture medium for 2 hours. Subsequently, cells were returned to BrdU-free medium containing DMEM plus 10% fetal bovine serum. Cells were harvested at 0, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 22, and 25 hours after the end of BrdU labeling, and stained with propidium iodide. We gated BrdU-positive cells and analyzed their DNA content by flow cytometry. The length of the S phase was defined as a time period during which BrdU-positive cells exit the S-phase (i.e. the time of disappearance of $BrdU^+$ cells with DNA content between 2N and 4N DNA). The length of G_2/M was defined as a time period between the end of BrdU labeling (time 0) and a time when BrdU positive cells start accumulating in G_1 phase (2N DNA content). The length of G_1 was defined as a time period between appearance of BrdU-positive G_1 cells (2N) and a time when BrdU-positive cells start entering the next S phase (>2N DNA content).

Western Blotting

Cell pellets were lysed on ice for 15 minutes in ELB buffer (0.1% NP-40, 160 mM NaCl, 50 mM HEPES pH 7.4, 5mM EDTA pH 7.5) supplemented with protease inhibitors cocktail (Complete, Roche Diagnostics). Cell debris was cleared by centrifugation at 4^oC, 12,000 rpm for 10 minutes, and protein concentration was measured in the supernatant using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Inc.) according to manufacturer instructions. 50-100 micrograms of

proteins per sample were resolved using SDS-PAGE, and transferred to Immobilon-P membrane (Millipore).

Reverse Transcription – Real Time PCR

MEFs were transduced with Cre-expressing viruses as described in the Experimental Procedures in the main text. The end of the first infection was considered the beginning of Cre expression. Cells were harvested at 20-72 hours after the end of the first infection. Total RNA was isolated from cells using TRIzol reagent (Invitrogen) and purified with RNeasy Mini Kit (Qiagen). cDNAs was synthesized using reverse transcriptase (First Strand cDNA Synthesis Kit, GE Healthcare) according to manufacturer's instructions. 6 nanograms of cDNA was amplified in the PCR Mix solution (SybrGreen, Applied Biosystems) using cyclin A2-specific primers for exon 1 (5'-ATGTCAACCCCGAAAAACTG-3') and exon 2 (5'-GCAGTGACATGCTCATCGTT). Only undeleted cyclin A2 locus can give rise to a transcript spanning exons 1 and 2 (Figure 1A). As an internal standard, GAPDH primers were used: forward (5'-AAAATGGTGAAGGTCGGTGT -3'), reverse (5'-AATCTCCACTTTGCCACTGC-3'). Real-time PCR was performed in the 7300 RT-PCR system (Applied Biosystems), using the default thermocycler program for both genes: 10 minutes of pre-incubation at 95℃ followed by 40 cycles con sisting of 15 seconds at 95℃ and one minute at 60℃.

Analyses of Hematopoietic Stem Cells and Progenitors

Bone marrow cells were collected from cyclin $A1^{-/-}A2^{f/f}$ MxCre, $A2^{f/f}$ MxCre and control $A1^{+/-}A2^{f/+}$ MxCre mice seven days after the last pI-pC dose. Hematopoietic stem cells (HSC) were stained as $IL7R\alpha^{-}Lin^{-}Sca\text{-}1^{hi}c\text{-}Kit^{hi}$, common myeloid progenitors (CMP) as $IL7R\alpha^{-}Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}CD34^{+}Fc\gamma RII/III^{lo}$, granulocyte / macrophage progenitors (GMP) as $IL7R\alpha^{-}Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}CD34^{+}Fc\gamma RII/III^{hi}$, and megakaryocyte/erythrocyte progenitors (MEP) as $IL7R\alpha^{-}Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}CD34^{-}Fc\gamma RII/III^{lo}$, essentially as described (Akashi et al., 2000). Briefly, HSC staining was performed using APC-conjugated anti-c-Kit

(2B8), FITC conjugated anti-Sca-1 (D7), biotinylated anti- IL7Rα (A7R34) (eBioscience, San Diego, CA) and PE-Cy5-conjugated rat antibodies specific for lineage markers: CD4 (RM4-5), CD8 (5H10), B220 (6B2), Gr-1 (8C5), and CD19 (6D5) (Caltag, Burlingame, CA), followed by avidin-PE (eBioscience, San Diego, CA). For myeloid progenitors, bone marrow cells were stained with FITC conjugated anti-CD34 (RAM34) monoclonal antibodies, PE-conjugated anti-FcγRII/III (93), APC-conjugated anti-c-Kit (2B8), biotinylated anti-Sca-1 (D7), and PE-Cy5-conjugated rat antibodies specific for IL7Rα (A7R34) and the lineage markers described above, followed by avidin-APC-Cy7 (eBioscience). For mature myeloid and erythoroid cell analysis, bone marrow cells were stained with FITC-conjugated Mac-1, PE-conjugated Ter-119 and APC-conjugated Gr-1(BD Biosciences, San Jose, CA). All cell populations were analyzed using a multi color Moflo flow-cytometer (Dako, Denmark). All flow cytometry data were analyzed with FlowJo software (Treestar, Ashland, OR)

Cyclin A2 deletion in purified HSC was performed using MSCV-Cre -ires-EGFP vector as described previously (Iwasaki et al., 2005). Briefly, the virus supernatant was obtained from the cultures of 293T cells co-transfected with the target retrovirus vector, gag-pol– and vesicular stomatitis virus G (VSV-G)–expression plasmids using an ExGen 500 (Fermentas). HSC purified from cyclin $A1^{-/-}A2^{f/f}$ or $A1^{+/-}A2^{f/+}$ mice by FACS, as above, were cultured for 48 hr in recombinant fibronectin fragment–coated culture dish (RetroNectin dish; Takara, Tokyo, Japan) with 1 ml of the virus supernatant containing SCF (100 ng/ml), LIF (20 ng/ml), sIL-6 R (100ng/ml) and IL-6 (20ng/ml). At the completion of transduction, GFP-positive HSC were purified by FACS, single HSC were sorted into Terasaki 60-well plates containing Iscove's Modified Dulbecco's Medium (Invitrogen) supplemented with 20% fetal calf serum (Stem Cell Technology) plus SCF (20 ng/ml), IL-3 (20 ng/ml), GM-CSF (10 ng/ml), G-CSF (10 ng/ml), Epo (2 unit/ml), and Tpo (10 ng/ml) (R&D Systems, Minneapolis, MN). Colonies were enumerated under an inverted microscope for 14 days. All cultures were incubated at 37℃ in a humidified chamber under 5% C O_2. Single colony PCR to

genotype cyclin A2 locus was performed using nested PCR; the first round of PCR was with primers
1M (5′- GGA ACG TGC GTG GAC CCG CG -3′), 2L (5′-CAC GAA GAA TAC TTG CTT TAT GTC-3′) and 4L (5′- GCC GGC AGT CTT TCA CTC AC -3′) , followed by second PCR with primers p1 (5′- CGC AGC AGA AGC TCA AGA CTC GAC -3′), 2M (5'-CCT TCC CTG AAG TAG TAA TCT G-3') and p4 (5′- CAC TCA CAC ACT TAG TGT CTC TGG-3′). PCR conditions were as described for cyclin A2 locus in Experimental Procedures in the main text.

Bone Marrow Reconstitution

Bone marrow reconstitution assays were performed as described (Sicinska et al., 2006). Animals were injected 5 times with 400µg of pI-pC, six weeks after receiving bone marrow.

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