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Cyclin A Is Redundant in Fibroblasts but Essential in Hematopoietic and Embryonic Stem Cells

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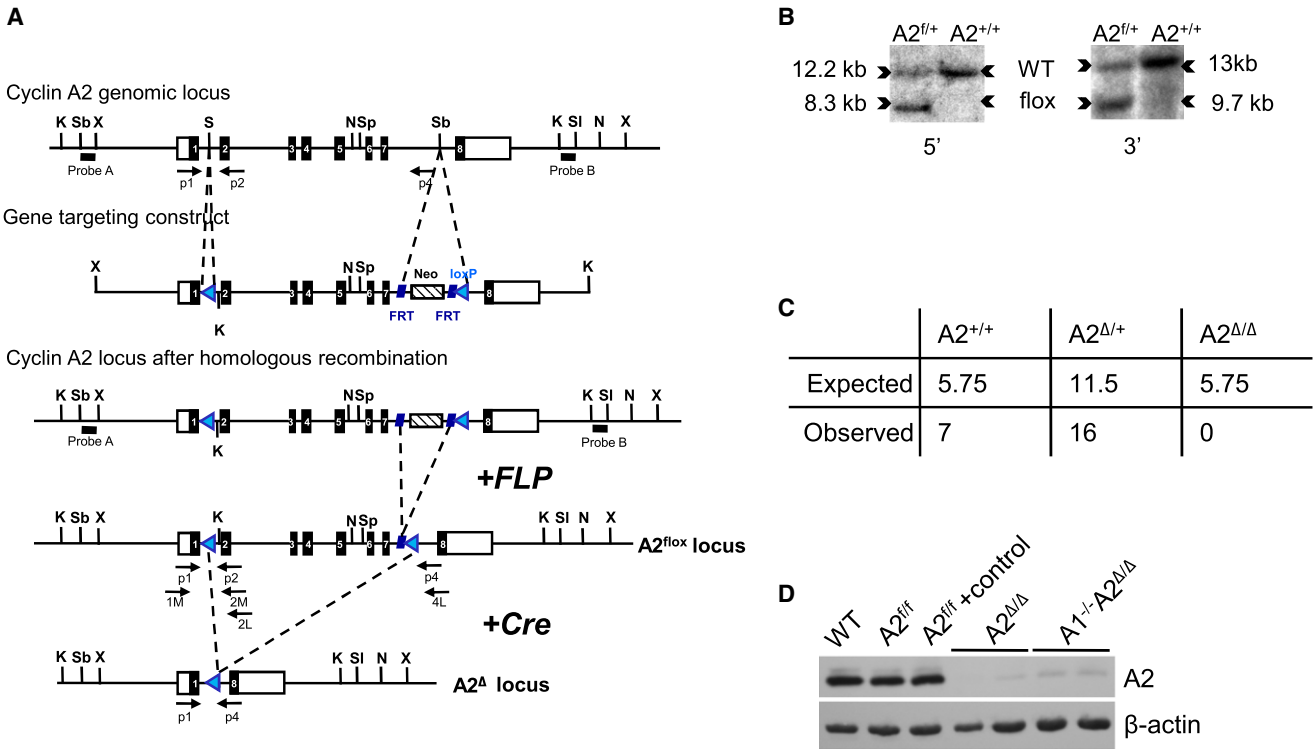
SUMMARY

Cyclins are regulatory subunits of cyclin-dependent kinases. Cyclin A, the first cyclin ever cloned, is thought to be an essential component of the cell-cycle engine. Mammalian cells encode two A-type cyclins, testis-specific cyclin A1 and ubiquitously expressed cyclin A2. Here, we tested the requirement for cyclin A function using conditional knockout mice lacking both A-type cyclins. We found that acute ablation of cyclin A in fibroblasts did not affect cell proliferation, but led to prolonged expression of another cyclin, cyclin E, across the cell cycle. However, combined ablation of all A- and E-type cyclins extinguished cell division. In contrast, cyclin A function was essential for cell-cycle progression of hematopoietic and embryonic stem cells. Expression of cyclin A is particularly high in these compartments, which might render stem cells dependent on cyclin A, whereas in fibroblasts cyclins A and E play redundant roles in cell proliferation.

INTRODUCTION

Replication of genetic material during cell division in Metazoan organisms is thought to be driven by cyclin A. Cyclin A was the first cyclin cloned in any organism (Swenson et al., 1986). It was originally described as a protein with periodic expression pattern in clam embryos (Evans et al., 1983). Subsequently, *cyclin A* genes have been found in all multicellular organisms, including humans (Pines and Hunter, 1990). While only a single *cyclin A* gene is present in the genomes of *C. elegans* and *Drosophila,* mammalian cells express two A-type cyclins, A1 and A2 (Nieduszynski et al., 2002). Cyclin A1 is expressed almost exclusively in the testes and during meiosis in the male germline (Sweeney et al., 1996; Yang et al., 1997), and male knockout mice lacking cyclin A1 are sterile because of an arrest in meiotic prophase at the diplotene stage, just before the first meiotic division (Liu et al., 1998; van der Meer et al., 2004). The second mammalian A-type cyclin, cyclin A2, is ubiquitously expressed in all proliferating cells and is generally considered to be the mammalian S phase cyclin (Hochegger et al., 2008; Pines and Hunter, 1990; Yam et al., 2002). During cell-cycle progression, cyclin A2 is induced at the beginning of the S phase (Erlandsson et al., 2000; Girard et al., 1991). Once induced, cyclin A2 binds and activates its catalytic partners, cyclin-dependent kinases Cdk2 and Cdk1. These cyclin A2-Cdk complexes phosphorylate critical proteins that play role in DNA synthesis, thereby driving S phase progression (Yam et al., 2002). Cyclin A2 is expressed throughout the S and G_2 phases, and is rapidly degraded upon entry of cells into mitosis (Geley et al., 2001; den Elzen and Pines, 2001). Injection of anti-cyclin A2 antibodies or antisense constructs into in vitro-cultured fibroblasts or other cell types blocked DNA synthesis, consistent with the essential function for cyclin A in DNA replication (Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992). In addition, cyclin A2 was postulated to play a role in entry of cells into mitosis (Swenson et al., 1986), and injection of anti-cyclin A2 antibodies into in vitro-cultured fibroblasts, or inhibition of cyclin A2 function by $p21^{Cip1}$ during the G_2 phase, blocked $G_2 \rightarrow M$ phase progression (Furuno et al., 1999; Pagano et al., 1992). An essential function for cyclin A in cell proliferation is supported by the observations that *cyclin A2* knockout mouse embryos died shortly after implantation (Murphy et al., 1997). These studies have led to the current model that the "core" components of the cell-cycle machinery (cyclins A and B) represent absolutely integral elements of the cell-cycle engine (Hochegger et al., 2008; Murphy et al., 1997). In the work described below, we decided to revisit the requirement for cyclin A function in cell proliferation using conditional *cyclin A* knockout mice.



C	$A2^{+/+}$	$A2^{\Delta/+}$	$A2^{\Delta/\Delta}$
Expected	5.75	11.5	5.75
Observed	7	16	0

Figure 1. Generation of *cyclin A2*$^{f/f}$ Mice

(A) *cyclin A2* gene targeting strategy. Coding exons are shown as filled boxes and are numbered. Neo, neomycin phosphotransferase gene. *loxP* and *FRT* sequences are indicated, as light blue triangles and dark blue rectangles, respectively. Restriction enzyme recognition sites: K, KpnI; Sb, SnaBI; X, XbaI; N, NdeI; Sp, SphI; Sl, SalI; S, SmaI. Solid black lines represent Southern blotting probes A and B used to screen for homologous recombination. Arrows denote PCR primers used for genotyping animals (p1, p2, p4,) or for nested PCR (1M, 2M, 2L, 4L). Also shown is the conditional *cyclin A2*^{flox} locus and the deleted *cyclin A2*$^{\Delta}$ allele after Cre-mediated recombination.

(B) Southern blot analysis of genomic DNA extracted from wild-type (WT) and *A2*$^{f/+}$ ESC clones. DNA was digested with KpnI and SphI and hybridized with probe A (5′ end screening) or digested with NdeI and hybridized with probe B (3′ end screening). The sizes of WT and "floxed" alleles are shown.

(C) Expected and observed frequency of E7.5 embryos obtained in *cyclin A2*$^{\Delta/+}$ × *A2*$^{\Delta/+}$ cross.

(D) Western blot analysis of cyclin A2 in wild-type mouse embryo fibroblasts (WT), *A2*$^{f/f}$ cells, *A2*$^{f/f}$ cells transduced with a retrovirus expressing an inactive, point mutant version of Cre ($A2^{f/f}$ +control), *A*$^{f/f}$ cells transduced with Cre-expressing virus ($A2^{\Delta/\Delta}$), or *A1*$^{-/-}$*A2*$^{f/f}$ cells transduced with Cre ($A1^{-/-}A2^{\Delta/\Delta}$).

RESULTS

Generation and Characterization of Conditional Cyclin A2 Knockout Mice

To obtain a conditional *cyclin A2* allele, we inserted *loxP* sites into the first and seventh intron of the murine *cyclin A2* gene (Figure 1A). The gene-targeting construct was introduced into embryonic stem cells (ESCs), and heterozygous *cyclin A2*$^{f/+}$ (*A*^{f} denotes the "floxed" allele) ESCs were obtained through homologous recombination (Figures 1A and 1B). *cyclin A2*$^{f/+}$ ESCs were then injected into mouse blastocysts, and homozygous *cyclin A2*$^{f/f}$ animals were generated via standard procedures (Geng et al., 2003). *cyclin A2*$^{f/f}$ mice were viable and phenotypically normal (data not shown), consistent with our expectation that the "floxed" *cyclin A2* allele is functionally wild-type.

In order to verify that deletion of the "floxed" *cyclin A2* sequences resulted in a functionally null allele, we crossed *cyclin A2*$^{f/f}$ mice with a "deleter" *Meox2-Cre* strain (Tallquist and Soriano, 2000) and generated *cyclin A2*$^{\Delta/+}$ mice (*A2*$^{\Delta}$ denotes the deleted *cyclin A2* allele). We then intercrossed *cyclin A2*$^{\Delta/+}$ heterozygotes, sacrificed pregnant females 7 days post coitum, and genotyped the embryos. No *cyclin A2*$^{\Delta/\Delta}$ embryos were observed (Figure 1C), consistent with the early embryonic lethality associated with a *cyclin A* null phenotype. Hence, deletion of the "floxed" *cyclin A2* sequences converts the conditional *cyclin A2* allele into a functionally null allele.

Analyses of *cyclin A* Null Fibroblasts

We next derived fibroblasts from conditional *cyclin A2* knockout embryos and cultured them in vitro. Transduction of *cyclin A2*$^{f/f}$ cells with Cre-expressing retroviruses led to essentially complete loss of cyclin A2 protein (Figure 1D). Very unexpectedly, we found that an acute ablation of cyclin A2 had no major impact on cell proliferation. Thus, *cyclin A2*$^{\Delta/\Delta}$ cells normally increased cell number during in vitro culture (Figure 2A), and normally re-entered the cell cycle from quiescence (Figure 2B). However, analyses of cell-cycle progression with propidium iodide and anti-BrdU staining revealed that ablation of cyclin A2 increased the fraction of cells in S and G_2/M phases, with concomitant decrease in the G_1 population (Figures 2C and 2D).

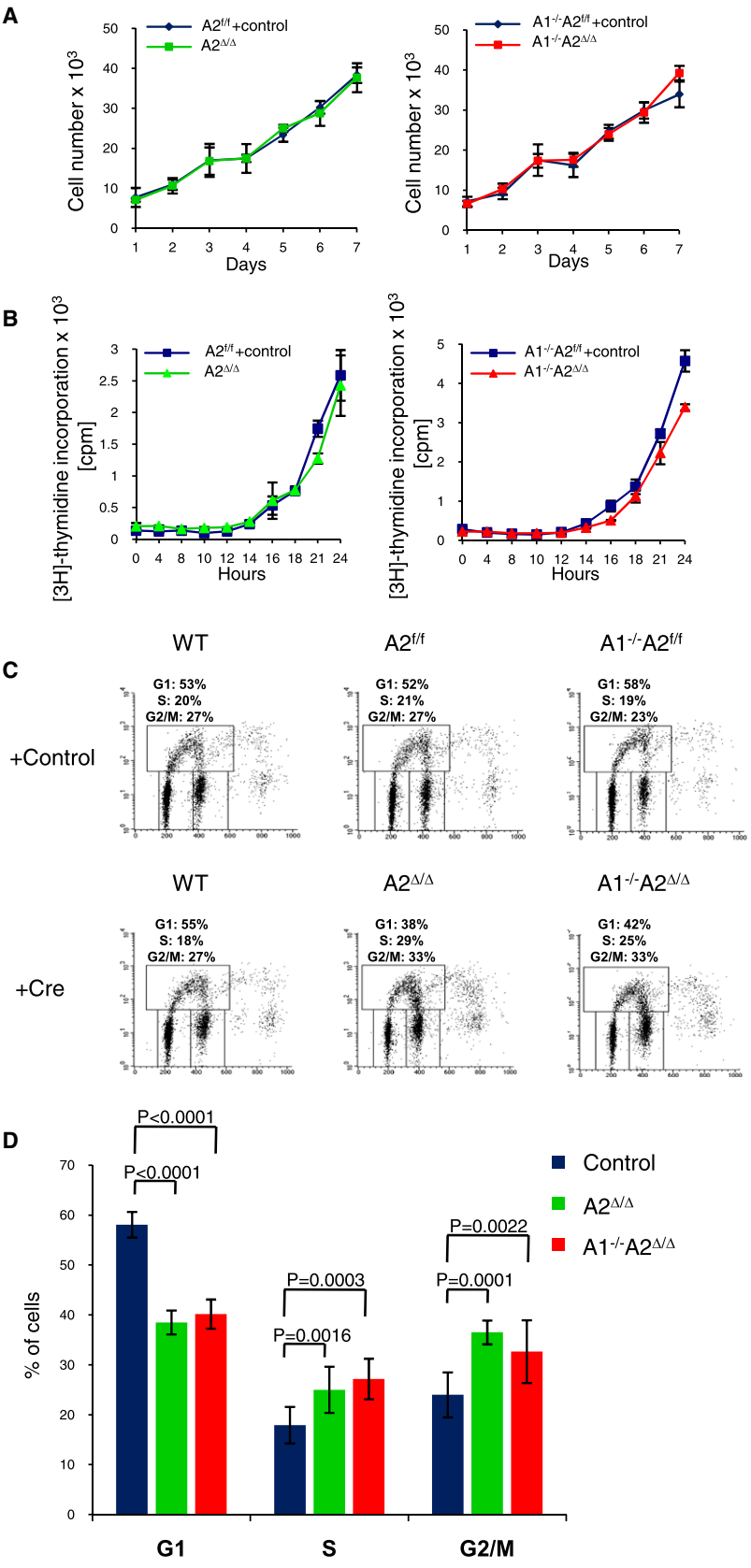


Figure 2. Analyses of *cyclin A2*$^{\Delta/\Delta}$ and *A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ Fibroblasts

(A) In vitro proliferation of fibroblasts transduced with control virus (+control) or with Cre-expressing retrovirus (A2$^{\Delta/\Delta}$ and A1$^{-/-}$A2$^{\Delta/\Delta}$). Equal numbers of cells were plated at the beginning of the experiment. Cells were counted every day for 7 days.

(B) Cell-cycle re-entry analysis. Cells transduced as above were rendered quiescent by serum deprivation and then stimulated to re-enter the cell cycle by addition of serum. Entry into S phase was gauged by measurement of [^{3}H]-thymidine uptake.

(C) Cell-cycle distribution of asynchronously growing mouse embryonic fibroblasts cultured in vitro. Cells were transduced as above, pulsed with bromodeoxyuridine (BrdU) for 1 hr, and then stained with anti-BrdU antibodies and with propidium iodide followed by fluorescence-activated cell sorting (FACS) analysis. The percentages of cells in particular phases of cell cycle are shown.

(D) Histogram representation of the data shown in (C). For each genotype, we analyzed four independent fibroblast cultures, prepared from four different embryos. Shown are mean values ± SD. The significance of differences was analyzed by the t test.

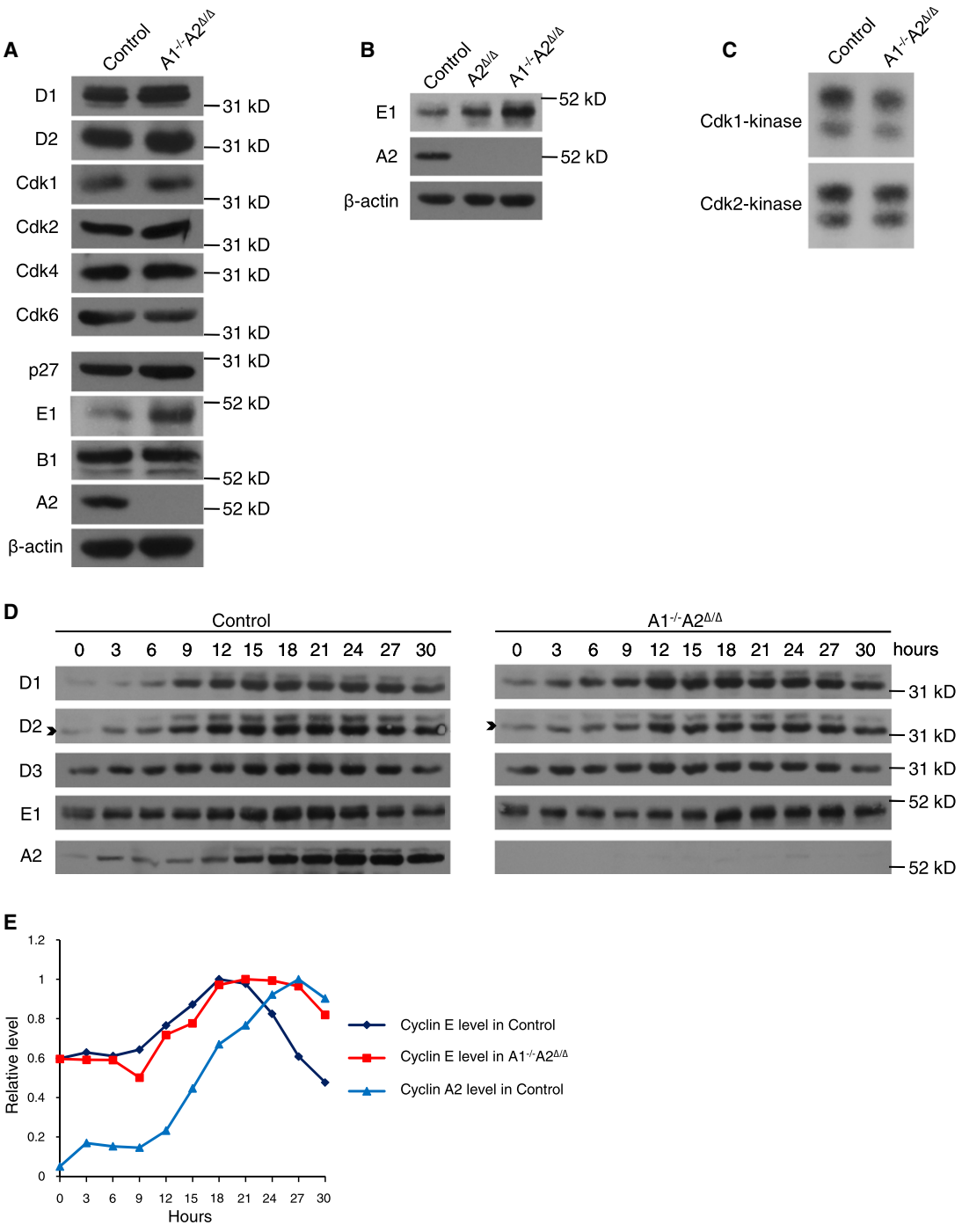


Figure 3. Molecular Analyses of *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ Fibroblasts

(A) The levels of cell-cycle regulators in cyclin A-deficient fibroblasts. Lysates were prepared from *cyclin A1*$^{-/-}$*A2*$^{f/f}$ fibroblasts transduced with control (Control) or Cre-encoding viruses (A1$^{-/-}$A2$^{\Delta/\Delta}$), immunoblotted, and probed with the indicated antibodies.

(B) Similar analysis as in (A) with *A2*$^{\Delta/\Delta}$ and *A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ cells.

(C) Cdk1 and Cdk2 were immunoprecipitated from lysates prepared as above and subjected to in vitro kinase reactions with histone H1 as a substrate.

(D) Levels of cell-cycle regulators during cell-cycle progression. Cells were arrested in G_0 by serum deprivation and stimulated to re-enter the cell cycle by addition of serum. Cells were collected at the indicated time points after serum stimulation and analyzed by western blotting. The arrow indicates a band corresponding to cyclin D2.

(E) Quantification of cyclin E1 and A2 levels at different time-points during re-entry into the cell cycle.

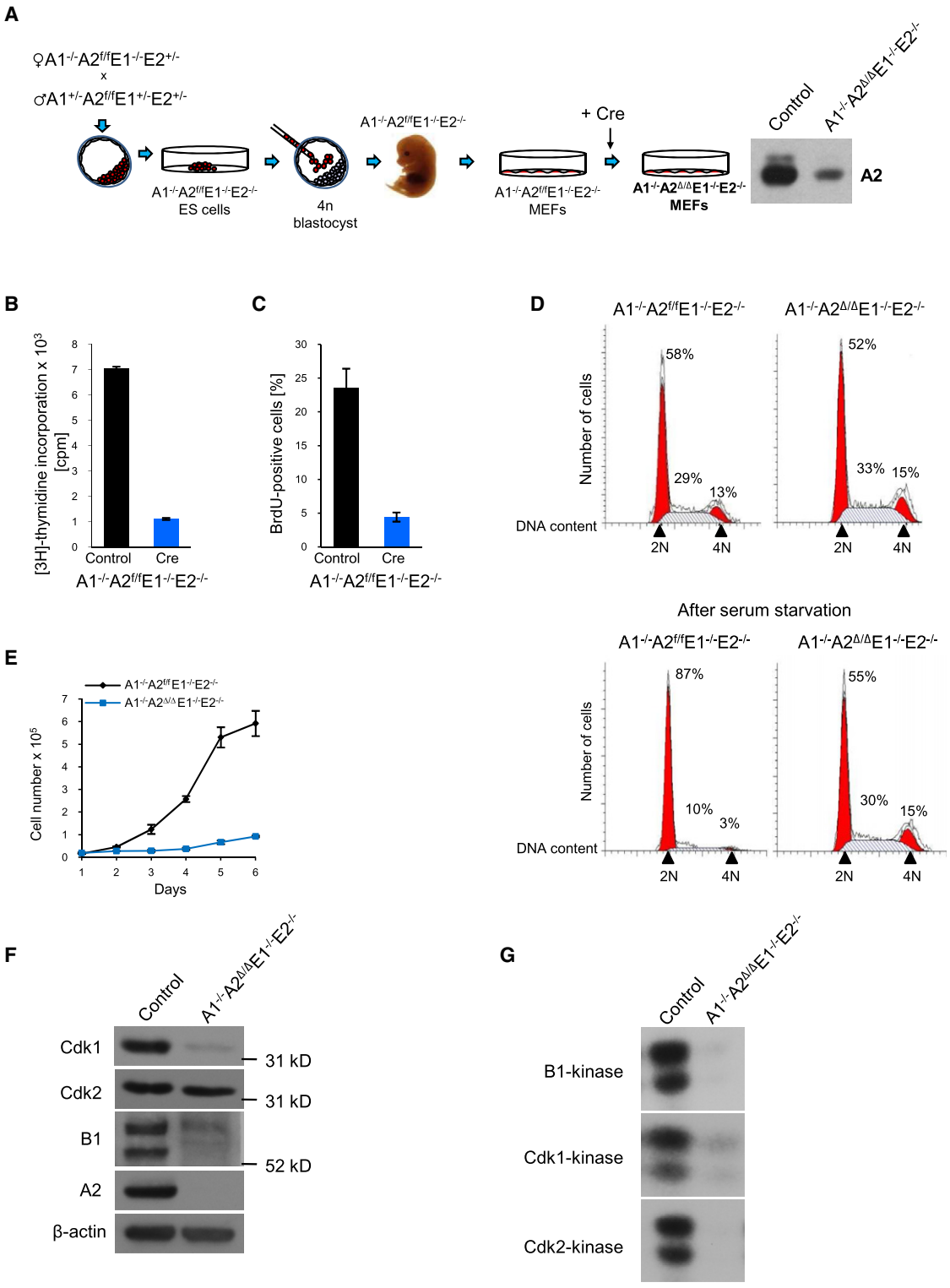


Figure 4. Analyses of Fibroblasts Lacking All A- and E-Type Cyclins

(A) Diagram illustrating generation of quadruple-knockout fibroblasts via tetraploid complementation. The right-hand panel shows western blot analysis of cyclin A2 levels in *cyclin* $A1^{-/-}A2^{f/f}E1^{-/-}E2^{-/-}$ cells transduced with adenovirus encoding empty vector (Control) or Cre ($A1^{-/-}A2^{\Delta/\Delta}E1^{-/-}E2^{-/-}$).

(B and C) Incorporation of [^{3}H]-thymidine (B) and the percentage of BrdU-positive cells (C) in *cyclin* $A1^{-/-}A2^{f/f}E1^{-/-}E2^{-/-}$ fibroblasts transduced as above. Shown are mean values ± SD.

To rule out the possible contribution from cyclin A1, we crossed *cyclin A2*$^{f/f}$ mice with *cyclin A1* null animals (Liu et al., 1998; Ji et al., 2005) and generated *cyclin A1*$^{-/-}$*A2*$^{f/f}$ mice. Fibroblasts were isolated from *cyclin A1*$^{-/-}$*A2*$^{f/f}$ embryos, cultured in vitro, and transduced with Cre-expressing viruses, thereby ablating all cyclin A expression (Figure 1D, and Figure S1A available online). We found that cells lacking all A-type cyclins proliferated normally in culture (Figure 2A) and entered the S phase from G_0 without any delay (Figure 2B). Again, analyses of the cell-cycle profile revealed increased fraction of cells in S and G_2/M phases with concomitant decrease in the G_1 population (Figures 2C and 2D), similar to that seen in *cyclin A2*$^{\Delta/\Delta}$ cells.

To further investigate these cell-cycle alterations, we compared the length of G_1, S, and G_2/M phases in control versus in *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ mouse embryonic fibroblasts (MEFs) using pulse-chase experiments. Specifically, we pulsed in vitro-cultured MEFs with BrdU and followed the progression of BrdU-labeled cells through S, G_2/M, and G_1 phases (see the Supplemental Experimental Procedures). We found that ablation of cyclin A resulted in increased length of the S phase (from approximately 7 hr in control to approximately 8 hr in *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$) and also prolonged the G_2/M phases (from 5 to 6 hr). In contrast, the length of G_1 phase was approximately 1.5 hr shorter upon cyclin A2 ablation. These analyses are consistent with the increased fraction of *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ cells in S and G_2/M phases and decreased percentage in G_1 (Figures 2C and 2D), as well as with overall normal cell division time in *cyclin A* null cells (Figure 2A).

To elucidate the molecular basis of cell division in the absence of A cyclins, we first compared the expression of various cell-cycle proteins between control versus *cyclin A* null (*A1*$^{-/-}$*A2*$^{\Delta/\Delta}$) cells. We found that the levels of cyclins D1, D2, B, Cdk1, Cdk2, Cdk4, Cdk6, and p27^{Kip1}, as well as Cdk2-associated kinase activity, were essentially unchanged in *cyclin A* null fibroblasts, while the activity of Cdk1 kinase was modestly decreased (Figures 3A and 3C). In contrast, we found that cyclin E was markedly upregulated after acute cyclin A shutdown (Figures 3A and 3B).

We also analyzed the expression levels of cyclins and Cdks across cell-cycle progression. In control cells, cyclin E levels peaked in early S phase, and declined thereafter, when most cells were traversing the S phase (Figures 3D and 3E). In *cyclin A* null cells, however, cyclin E assumed a broad cell-cycle expression pattern resembling the combined expression of cyclins E plus A in wild-type cells (Figure 3E). On the other hand, the pattern of cyclin D1, D2, and D3 expression was comparable between control and mutant cells (Figure 3D and data not shown). We therefore hypothesized that cyclin E was responsible for the relatively normal proliferation of *cyclin A* null cells.

To extend these findings, we analyzed phosphorylation status of a panel of cyclin A-Cdk and E-Cdk substrates in cyclin A-deficient cells, using phospho-specific antibodies. We observed essentially unperturbed phosphorylation of these proteins in *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ MEFs (Figure S2A). We also verified normal levels of several E2F target genes in *cyclin A* null cells (Figure S2B), indicating that phosphorylation and concommittant functional inactivation of the retinoblastoma protein and other "pocket" proteins by Cdk-containing complexes proceeded normally in the absence of cyclin A. These findings were consistent with the possibility that cyclin E-Cdk complexes might perform cyclin A-Cdk functions in *cyclin A* null MEFs.

Generation and Analyses of Cells Lacking All A-Type and E-Type Cyclins

To rigorously test this possibility, we decided to combine ablation of A-type and E-type cyclins and to generate cells lacking all these proteins. Of note, mammalian cells express two E-type cyclins, E1 and E2 (Sherr and Roberts, 2004). Ablation of both E cyclins in mice led to embryonic lethality at day 10.75 of gestation because of placental abnormalities (Geng et al., 2003; Parisi et al., 2003). In order to obtain *cyclin E* null fibroblasts, we previously turned to the tetraploid blastocyst complementation rescue method, which essentially provided *cyclin E* null embryos with wild-type (tetraploid) placentas (Geng et al., 2003). Using this method, we bypassed the placental failure and obtained day 13.5 *cyclin E1*$^{-/-}$*E2*$^{-/-}$ embryos, from which in vitro cultures of embryonic fibroblasts were derived. *cyclin E1*$^{-/-}$*E2*$^{-/-}$ fibroblasts proliferated relatively normally during conditions of continuous cell growth, but they were unable to re-enter the cell cycle from quiescence. These *cyclin E* null cells expressed normal levels of cyclin A2, and had normal cyclin A2-associated kinase activity (Geng et al., 2003).

In order to obtain quadruple-knockout cyclin E- and A-deficient embryos and fibroblasts, we again utilized tetraploid blastocyst complementation method. Specifically, we interbred *cyclin A1*$^{-/-}$, *A2*$^{f/f}$, *E1*$^{-/-}$, and *E2*$^{-/-}$ animals and generated compound heterozygotes (Figure 4A). We next intercrossed these animals, sacrificed females at day 3.5 post coitum, and derived ESCs from blastocyst-stage *cyclin A1*$^{-/-}$*A2*$^{f/f}$*E1*$^{-/-}$*E2*$^{-/-}$ embryos. We next injected *cyclin A1*$^{-/-}$*A2*$^{f/f}$*E1*$^{-/-}$*E2*$^{-/-}$ ESCs into tetraploid wild-type blastocysts and implanted them into foster females for further development (Figure 4A). Since the tetraploid cells can contribute only to the extraembryonic tissues (Eggan et al., 2001), in the resulting chimeric embryos the embryo proper was derived entirely from the injected *cyclin A1*$^{-/-}$*A2*$^{f/f}$*E1*$^{-/-}$*E2*$^{-/-}$ ESCs, while the placentas were "wild-type" (tetraploid). We sacrificed embryos at day 13.5 of gestation, derived in vitro cultures of *cyclin A1*$^{-/-}$*A2*$^{f/f}$*E1*$^{-/-}$*E2*$^{-/-}$ fibroblasts, and transduced these cells with viruses expressing Cre recombinase, thereby deleting *cyclin A2* and rendering the cells null for all A- and E-type cyclins (Figures 4A, S1B, and S3). Strikingly, combined ablation of cyclins A and E essentially extinguished

(D) Upper panel: fibroblasts, cultured in medium containing 10% serum, were stained with propidium iodide and analyzed by FACS. Lower panel: cells were placed in serum-free medium for 3 days, stained with propidium iodide, and analyzed by FACS.
(E) In vitro proliferation of fibroblasts. Equal numbers of cells were plated at the beginning of the experiment. Cells were counted every day for 7 days. This experiment was performed with cells immortalized with dominant-negative p53, as cyclin E-deficient cells undergo premature senescence in culture (Geng et al., 2003).
(F) The levels of the indicated proteins in cyclin A- and E-deficient fibroblasts, detected by western blotting.
(G) Cyclin B1, Cdk1, or Cdk2 was immunoprecipitated from protein lysates and used for in vitro kinase reactions with histone H1 as a substrate.

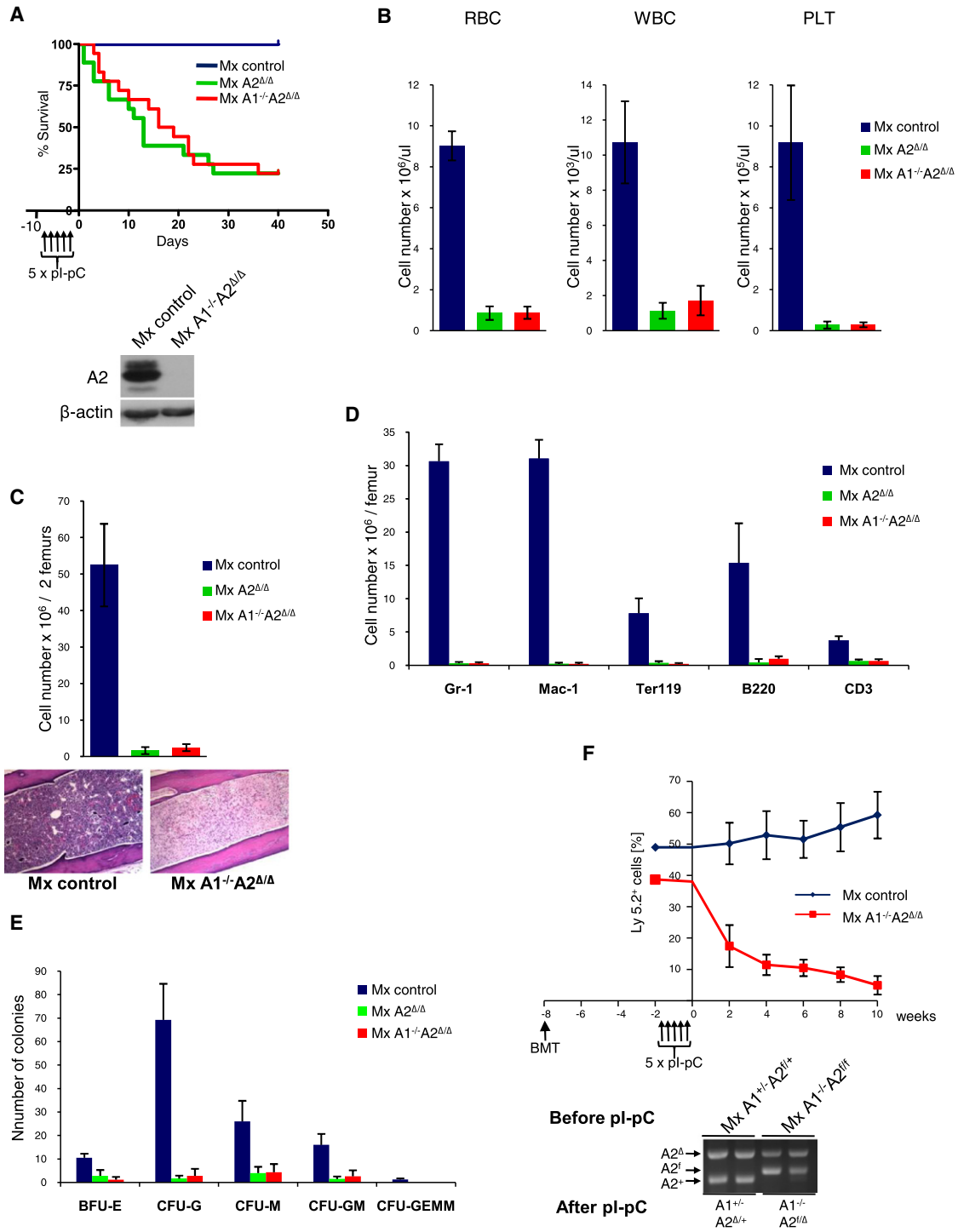


Figure 5. Impact of Cyclin A Ablation on Hematopoiesis

(A) Survival of control *cyclin A1*$^{+/-}$*A2*$^{f/+}$ *MxCre* (Mx Control), *A2*$^{f/f}$ *MxCre* (Mx A2$^{\Delta/\Delta}$), and *A1*$^{-/-}$*A2*$^{f/f}$ *MxCre* (Mx A1$^{-/-}$A2$^{\Delta/\Delta}$) mice after five doses of pI-pC. Each group consisted of 18 mice. Lower panel: deletion of cyclin A2 in bone marrow cells was verified by western blotting 7 days after the last pI-pC injection.

(B) Mean number of red blood cells (RBC), white blood cells (WBC), and platelets (PLT) in the peripheral blood of mice of the indicated genotypes (n = 6 per group) after pI-pC administration.

(C) Mean number of bone marrow cells per two femurs. Lower panel: histological sections of bones with bone marrow were stained with hematoxylin and eosin. Note acellular bone marrow in Mx A1$^{-/-}$A2$^{\Delta/\Delta}$ animals.

(D) Mean number of Gr-1^{+} (granulocyte), Mac-1^{+} (macrophage), Ter119^{+} (erythroid), B220^{+} (B cells), and CD3^{+} (T cells) in the bone marrow. Cells were stained with respective antibodies, followed by FACS.

cell proliferation (Figure 4E) and crippled incorporation of [^{3}H]-thymidine and BrdU (Figures 4B, 4C, and S4), indicating an inability of cells to synthesize DNA. This, together with relatively normal S phase progression of *cyclin E* null (*E1*$^{-/-}$*E2*$^{-/-}$) (Geng et al., 2003; Parisi et al., 2003) and *cyclin A* null (*A1*$^{-/-}$*A2*$^{\Delta/\Delta}$) cells (Figure 2), reveals that either cyclin E or cyclin A must be present to allow DNA synthesis.

Surprisingly, staining of growth-arrested *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ *E1*$^{-/-}$*E2*$^{-/-}$ cells with propidium iodide revealed essentially unchanged percentage of cells with 2N DNA content ("G_1"), cells containing between 2N and 4N DNA ("S"), and cells with 4N DNA content ("G_2/M"), as compared to actively proliferating *cyclin A1*$^{-/-}$*A2*$^{f/f}$ *E1*$^{-/-}$*E2*$^{-/-}$ cells (see Figure 4D, upper panel). The unchanged fraction of cells containing between 2N and 4N DNA, together with crippled thymidine and BrdU incorporation (Figures 4B and 4C), indicates intra-S phase arrest in *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ *E1*$^{-/-}$*E2*$^{-/-}$ cells. Moreover, ablation of cyclin A in quadruple-knockout MEFs led to retention of G_2/M cells with 4N DNA (in the absence of cell division). This indicates that in addition to the S phase block, cyclin A- and E-deficient cells arrest in G_2/M, which is consistent with the proposed function of cyclin A not only in the S phase, but also during $G_2 \rightarrow$ M transition (Swenson et al., 1986; Furuno et al., 1999; Gong et al., 2007; Pagano et al., 1992).

We also performed additional experiments to confirm that *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ *E1*$^{-/-}$*E2*$^{-/-}$ MEFs are "locked" in S and G_2/M phases. Thus, we placed proliferating *cyclin A1*$^{-/-}$*A2*$^{f/f}$*E1*$^{-/-}$ *E2*$^{-/-}$ cells and nonproliferating *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ *E1*$^{-/-}$*E2*$^{-/-}$ cells in serum-free medium, and we analyzed their cell-cycle distribution by propidium iodide staining. As expected, upon serum starvation, proliferating *cyclin A1*$^{-/-}$*A2*$^{f/f}$*E1*$^{-/-}$*E2*$^{-/-}$ cells exited the cell cycle and arrested in G_0/G_1 (2N DNA), with concomitant decrease in S and G_2/M fractions. In contrast, the DNA content of nonproliferating *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ *E1*$^{-/-}$*E2*$^{-/-}$ cells remained unchanged in serum-free medium (Figure 4D, lower panel). These observations further underscore that *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ *E1*$^{-/-}$*E2*$^{-/-}$ cells are "locked" in S and G_2/M phases.

Molecular analyses of *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ *E1*$^{-/-}$*E2*$^{-/-}$ cells revealed that the levels of cyclin B and Cdk1 were greatly decreased upon the loss of all A and E cyclins (Figure 4F). Also, the levels of Cdk1-, Cdk2- and cyclin B-associated kinase were profoundly diminished upon cyclin A/E ablation (Figure 4G). These results are consistent the notion that cyclins A and E represent the redundant activators of Cdk1 and Cdk2. Whereas deletion of either A-type or E-type cyclins did not markedly affect Cdk1/2 catalytic activity (Figure 3C) (Geng et al., 2003), the combined ablation of all A and E cyclins extinguished Cdk1 and Cdk2 kinase and arrested S and G_2/M phase progression.

Requirement for Cyclin A Function in Hematopoietic Stem Cells

Our observations that cyclin A function is dispensable for proliferation of fibroblasts prompted us to study the requirement for cyclin A in other cell types. We decided to focus on bone marrow cells, which contain several different hematopoietic lineages. To avoid ambiguity in interpreting the results, we chose to ablate the expression of all A-type cyclins, by combining our conditional *cyclin A2* knockout strain with *cyclin A1*$^{-/-}$ animals. Thus, we interbred *cyclin A2*$^{f/f}$, *A1*$^{-/-}$, and *MxCre* mice and generated *cyclin A1*$^{-/-}$*A2*$^{f/f}$ *MxCre* animals. The *MxCre* strain expresses interferon-inducible Cre recombinase. Administration of double-stranded RNA polyI-polyC (pI-pC) to *MxCre* mice induces interferon, leading to activation of Cre, and results in a very efficient ablation of the "floxed" sequences in bone marrow cells, including in nearly 100% of hematopoietic stem cells (Kuhn et al., 1995).

Administration of pI-pC to *cyclin A1*$^{-/-}$*A2*$^{f/f}$ *MxCre* mice extinguished cyclin A expression in the bone marrow and resulted in death of the majority of animals within 30 days (Figure 5A). In peripheral blood, we observed substantially decreased numbers of red blood cells and white blood cells, as well as platelets (Figure 5B), pointing to a severe anemia as a most likely cause of death. Analyses of bone marrow revealed greatly reduced numbers of bone marrow cells, with all lineages being equally affected (Figures 5C–5E).

Since in *MxCre* mice the deletion of the "floxed" sequences is not confined to bone marrow, we used bone marrow transplantation assays (Park et al., 2003) to ascertain that the observed phenotype was intrinsic to hematopoietic cells. To this end, we collected bone marrow cells from *cyclin A1*$^{-/-}$*A2*$^{f/f}$ *MxCre* mice (never exposed to pI-pC), mixed them at 1:1 ratio with bone marrow cells derived from wild-type mice, and injected the cells into irradiated wild-type recipients. In this procedure, the injected hematopoietic stem cells reconstitute the bone marrow of recipient animals, which now represents a 1:1 mixture of *cyclin A1*$^{-/-}$*A2*$^{f/f}$ *MxCre* and wild-type cells, while all other tissues of the recipient mouse are wild-type. Importantly, *cyclin A1*$^{-/-}$*A2*$^{f/f}$ *MxCre* hematopoietic cells express a different haplotype of the Ly surface antigen (Ly5.2) than do wild-type cells (Ly5.1), thereby allowing their unequivocal identification (Park et al., 2003). After bone marrow reconstitution, we injected recipient mice with pI-pC, resulting in deletion of cyclin A selectively in Ly.5.2$^+$ bone marrow hematopoietic cells. We found that deletion of cyclin A drastically reduced the numbers of Ly5.2$^+$ blood cells (Figure 5F). At the end of observation period, we sacrificed the mice, collected their bone marrow, and determined that Ly5.2$^+$ cells contributed to only ~5% of bone marrow cells. Flow sorting of these residual Ly5.2$^+$ bone marrow cells

(E) Mean number of myeloid colonies in methylcellulose cultures. Twenty thousand bone marrow cells were isolated from mice (n = 3 per group) 3 days after the last administration of pI-pC (a total of three doses) and plated in duplicate. Colonies were counted after 12 to 14 days.

(F) A total of 2.5 × 10^6 Ly5.2$^+$ bone marrow cells from *A1*$^{+/-}$*A2*$^{f/+}$ *MxCre* (Mx control) or *A1*$^{-/-}$*A2*$^{f/f}$ *MxCre* (Mx A1$^{-/-}$A2$^{\Delta/\Delta}$) mice were adoptively transferred along with 2.5 × 10^6 wild-type (Ly5.1$^+$) bone marrow cells into lethally irradiated (1000 Rad) Ly5.1$^+$ congenic recipients (n = 5 for each group) (BMT). After 6 weeks of engraftment, chimeric mice were treated with five doses of pI-pC, and the percentage of peripheral blood cells expressing Ly5.2 was determined biweekly by flow cytometry (shown are mean values for each time-point). Lower panel: at the end of experiment, genomic DNA was isolated from 5.2$^+$ bone marrow cells and analyzed by PCR for the presence of *A2*$^+$, *A2*^{f}, and *A2*$^\Delta$ alleles.

Error bars represent the SD. In (A) and (F), "time 0" corresponds to the last day of pI-pC injection.

followed by PCR genotyping revealed that they arose from non-deleted *cyclin A1*$^{-/-}$*A2*$^{f/\Delta}$ hematopoietic stem cells (Figure 5F). Altogether, these analyses demonstrated that the requirement for cyclin A function is intrinsic to hematopoietic cells.

To determine at which stage of hematopoiesis cyclin A function becomes first rate limiting, we collected bone marrow cells 7 days after cyclin A ablation and flow sorted hematopoietic stem cells (HSCs), their descendants (lineage-committed common myeloid progenitors, CMP, granulocyte-macrophage progenitors, GMP, and megakaryocyte-erythroid progenitors, MEP), as well as differentiated Mac-1$^+$ (macrophage), Gr-1$^+$ (granulocyte), and Ter119$^+$ (erythroid) cells (Akashi et al., 2000; Morrison et al., 1995). We found that ablation of cyclin A led to a rapid disappearance of hematopoietic stem cells, as well as of lineage-committed HSC descendants, CMP, GMP, and MEP (Figures 6A and 6B). In contrast, the proportions of the more differentiated Gr-1$^+$, Mac-1$^+$, and Ter-119$^+$ cells were unperturbed at this early time point (Figure 6C and not shown). These analyses suggested that the primary defect after cyclin A ablation was at the HSC and early progenitor stage.

To test this possibility further, we flow sorted hematopoietic stem cells from *cyclin A1*$^{-/-}$*A2*$^{f/f}$ mice and transduced them with viruses encoding Cre and GFP. We again sorted GFP-positive HSCs and cultured them—one cell per well—in a cocktail of cytokines that promotes HSC proliferation (Akashi et al., 2000). As a control we used *cyclin A1*$^{+/-}$*A2*$^{f/+}$ HSCs, which were sorted and transduced in an identical fashion. As expected, Cre-transduced *cyclin A1*$^{+/-}$*A2*$^{f/+}$ HSCs proliferated in vitro and formed colonies. PCR of individual colonies revealed that 29/31 of them arose from *cyclin A1*$^{+/-}$*A2*$^{\Delta/+}$ HSCs, confirming that Cre expression led to deletion of the "floxed" *cyclin A2* alleles (Figures 7A and 7B). In contrast, Cre-transduced *cyclin A1*$^{-/-}$*A2*$^{f/f}$ HSCs failed to proliferate and never formed *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ colonies. We did observe a few colonies in Cre-transduced *cyclin A1*$^{-/-}$*A2*$^{f/f}$ HSCs; however, PCR analyses revealed they all arose from the partially recombined *A1*$^{-/-}$*A2*$^{f/\Delta}$ HSCs (Figures 7A and 7B). These results indicate that cyclin A is critically required for proliferation of HSCs.

Requirement for Cyclin A Function in Embryonic Stem Cells

These observations prompted us to analyze the requirement for cyclin A function in another stem cell type, namely embryonic stem cells. To ablate both A cyclins, we interbred *cyclin A1*$^{-/-}$ and *A2*$^{f/f}$ animals, sacrificed pregnant females, collected blastocyst-stage embryos, and derived *cyclin A1*$^{-/-}$*A2*$^{f/f}$ as well as control *cyclin A1*$^{+/-}$*A2*$^{f/+}$ ESCs. We then transduced ESCs with Cre-expressing retrovirus, plated out single cells, and allowed them to form ESC colonies. The genotype of individual colonies was determined by PCR. As expected, expression of Cre in control *cyclin A1*$^{+/-}$*A2*$^{f/+}$ ESCs converted them to *A1*$^{+/-}$*A2*$^{\Delta/+}$ cells, which then proliferated and formed *A1*$^{+/-}$*A2*$^{\Delta/+}$ ESC colonies (78/92 analyzed, Figures 7C and 7D). In contrast, in case of Cre-transduced *cyclin A1*$^{-/-}$*A2*$^{f/f}$ ESCs, we observed 106 ESC colonies derived from cells with one deleted *cyclin A2* allele (*A1*$^{-/-}$*A2*$^{f/\Delta}$), but never detected any *A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ colonies (Figure 7C and 7D). Since it was formally possible that expression levels of Cre in ESCs were sufficient to delete only one but not both "floxed" *cyclin A2* alleles, we transduced *cyclin A1*$^{-/-}$*A2*$^{f/\Delta}$ ESCs with Cre-expressing retrovirus. Out of 135 ESC colonies analyzed, none arose from *cyclin A1*$^{-/-}$*A2*$^{\Delta/\Delta}$ cells (Figure 7C). These results indicate that deletion of both *cyclin A2* alleles is incompatible with ESC proliferation. Hence, as was the case in hematopoietic stem cells, cyclin A is also required for proliferation of embryonic stem cells.

Molecular Analyses of Bone Marrow Cells and Embryonic Stem Cells

Our analyses described above revealed that while in fibroblasts cyclins E and A perform overlapping roles in cell-cycle progression, the proliferation of HSCs and ESCs critically depends on cyclin A. To begin to elucidate the molecular basis of this requirement, we compared the levels of cyclins E and A between hematopoietic bone marrow cells versus fibroblasts.

Our analyses revealed that the levels of cyclin E in bone marrow cells were dramatically lower than in fibroblasts. Even at long exposure times we could barely detect cyclin E signal in bone marrow cells (Figure 7E). In contrast, the amount of cyclin A2 in bone marrow cells was significantly higher than that in fibroblasts (Figure 7E). Of note, in bone marrow samples cyclin A2 migrates at approximately 38 kDa, unlike the 55 kDa isoform seen in other compartments, as reported previously by Welm et al. (2002) (Figures 7E and S5A). This shorter cyclin A2 isoform was reported to lack N-terminal destruction box, but retained Cdk binding and activation, and hence possibly represents hyper-stable cyclin A species (Welm et al., 2002).

We also immunoprecipitated Cdk2 from bone marrow cells and from fibroblasts and probed immunoblots with anti-cyclin E and anti-cyclin A2 antibodies. Again, we observed paucity of cyclin E-Cdk2 complexes in bone marrow cells, while cyclin A-Cdk2 was more abundant in this compartment than in fibroblasts (Figure 7E). The overall levels of Cdk2-kinase activity were comparable between the two compartments, while Cdk1-associated kinase activity was markedly higher in bone marrow cells than in MEFs (Figure 7F). Collectively, these observations suggest that in hematopoietic cells, cyclin A represents the major S phase cyclin, while cyclin E is expressed at very low levels. Consequently, division of these cells critically depends on cyclin A. In contrast, fibroblasts express both E- and A-type cyclins, and each one of these cyclins is sufficient to drive normal cell-cycle progression.

Our analyses of ESCs revealed that the levels of cyclin A2 in these cells were substantially higher than those observed in fibroblasts (Figure 7G). Immunoprecipitation of Cdk1 or Cdk2 followed by immunoblotting with anti-cyclin A2 antibodies indicated that cyclin A2-Cdk1 and A2-Cdk2 complexes are substantially more abundant in ESCs than in fibroblasts (Figures 7G and S5B). We propose that in hematopoietic cells and ESCs, cyclin A-CDK complexes greatly predominate over cyclin E-CDK complexes, and this renders these cells dependent on cyclin A for cell-cycle progression.

DISCUSSION

Cyclin A is considered an essential component of the cell-cycle machinery. This notion is supported by the observations that

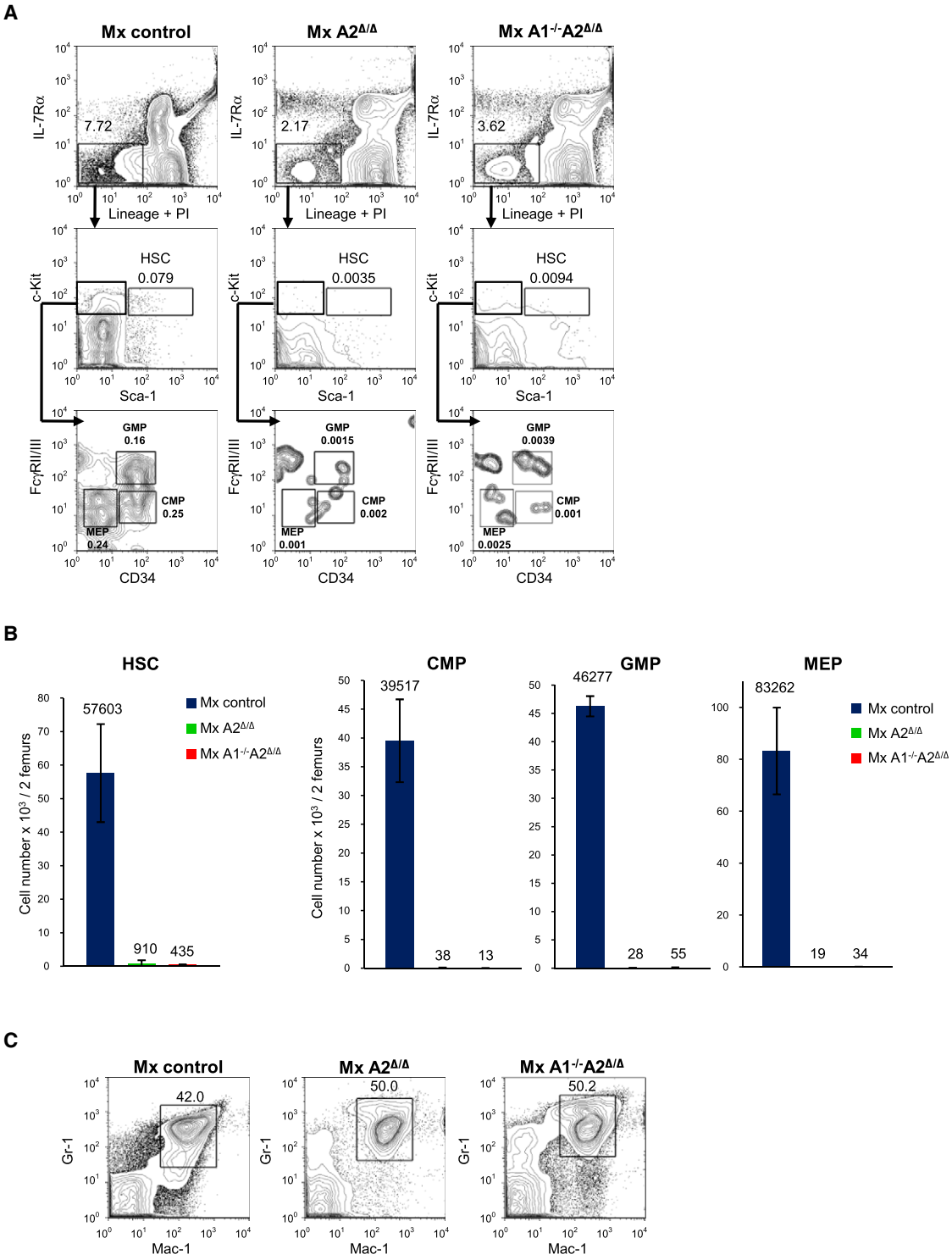


Figure 6. Analyses of Hematopoietic Stem Cells and Progenitors

(A) Cells isolated from bone marrow of pI-pC-treated *cyclin A1*$^{+/-}$*A2*$^{f/+}$ *MxCre* (Mx control), *A2*$^{f/f}$ *MxCre* (Mx A2$^{\Delta/\Delta}$), and *A1*$^{-/-}$*A2*$^{f/f}$ *MxCre* (Mx A1$^{-/-}$A2$^{\Delta/\Delta}$) mice were stained for the presence of hematopoietic stem cells (HSC), common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP). The percentages of particular populations among all bone marrow cells are indicated.

(B) Total number of HSC, CMP, GMP and MEP calculated per two femurs in animals treated as above. Error bars represent the SD.

(C) Bone marrow cells from the same animals as in (B) were stained for the presence of more differentiated Gr-1^{+} and Mac-1^{+} cells.

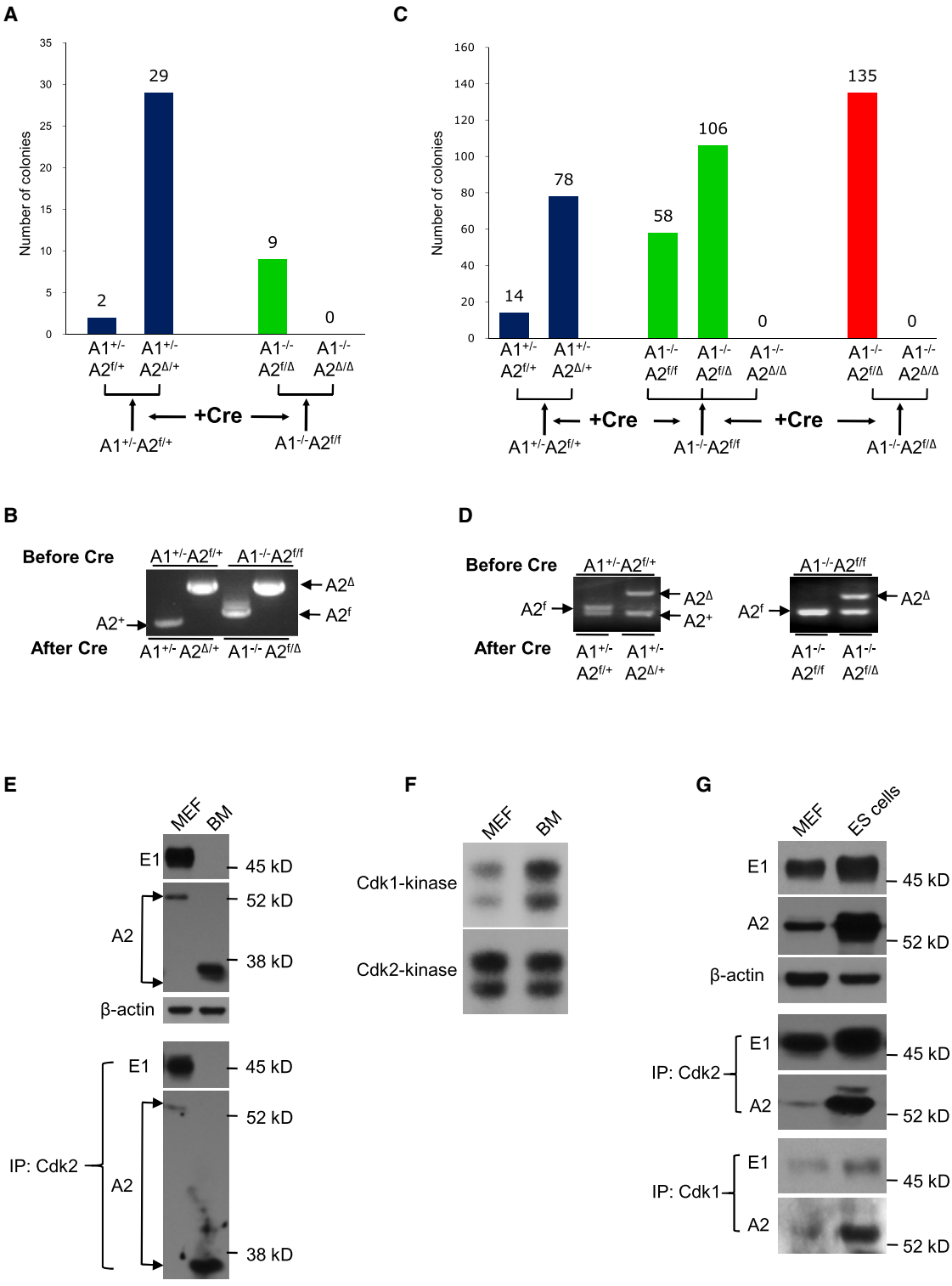


Figure 7. Analyses of Hematopoietic and Embryonic Stem Cells

(A) The numbers of resulting colonies of the indicated genotypes after transduction of in vitro-cultured control *cyclin* $A1^{+/-}A2^{f/+}$ and *cyclin* $A1^{-/-}A2^{f/f}$ HSCs with Cre.

(B) Single-colony PCR of colonies derived from Cre-tranduced HSCs.

(C) The numbers of resulting colonies of the indicated genotypes after transduction of *cyclin* $A1^{+/-}A2^{f/+}$, $A1^{-/-}A2^{f/f}$, and $A1^{-/-}A2^{f/\Delta}$ embryonic stem cells with Cre.

Drosophila embryos arrested their proliferation as soon as maternal cyclin A stores became depleted (Lehner and O'Farrell, 1989; 1990), while *cyclin A2* knockout mice died shortly after implantation (Murphy et al., 1997). In contrast, mice lacking particular G_1 cyclins or Cdks displayed normal proliferation in the overwhelming majority of cell types and presented only narrow, tissue-specific abnormalities (Sherr and Roberts, 2004; Li et al., 2009; Malumbres and Barbacid, 2009). These observations led to the current model that there are essentially two types of cyclin proteins: those that are vital components of the cell-cycle engine (cyclins A and B), and those involved in cell type-specific regulation of the cell cycle (G_1 cyclins) (Hochegger et al., 2008; Murphy et al., 1997).

In this study, we show that the early lethality of *cyclin A* null embryos reflected the requirement for cyclin A function at this particular stage of embryo development, rather than an ubiquitous essential role for cyclin A in cell-cycle machinery. We demonstrate that in fibroblasts—a cell type that has been most extensively studied in the cell-cycle field—either cyclin A or cyclin E is sufficient to drive S phase entry and progression, as well as entry of cells into the M phase. Cyclin A-Cdk complexes are thought to phosphorylate distinct set of proteins than cyclin E-Cdk kinase, and cyclin A-Cdk is believed both to drive S phase progression and to prevent re-replication of genetic material (Yam et al., 2002). However, our analyses of the phosphorylation status of various Cdk1 and Cdk2 substrates in cyclin A-deficient ($A1^{-/-}A2^{\Delta/\Delta}$) and E-deficient ($E1^{-/-}E2^{-/-}$) MEFs revealed essentially unperturbed phosphorylation of these proteins (Figure S2A). Moreover, we found normal levels of E2F targets in cyclin A- and E-deficient cells, indicating normal functional inactivation of the pocket proteins (Figure S2B). Collectively, these analyses indicate that cyclin A- and E-associated kinases can phosphorylate the similar set of proteins in vivo, including proteins involved in centrosome duplication (nucleophosmin), DNA replication (Cdc6), transcription (B-Myb), and cell-cycle progression (pocket proteins).

In contrast to the situation seen in fibroblasts, we found that cyclin A function was essential for proliferation of hematopoietic and embryonic stem cells. We found that in these two cell types, cyclin A was expressed at particularly high levels, while cyclin E was essentially absent in the hematopoietic lineage. We propose that in the two stem cell types studied, cyclin A-Cdk complexes greatly predominate over cyclin E-Cdk complexes, and this renders these cells dependent on cyclin A for cell-cycle progression. The proliferation of several stem cell types was shown to be driven by the Wnt-dependent pathways (Nusse, 2008), and it is possible that these pathways impinge on the S phase machinery by inducing cyclin A. In contrast, mitogenic signals operating in fibroblasts may be capable of upregulating cyclins E and A equally.

An alternative possibility is that cyclin A-Cdk complexes play an essential, nonredundant function in stem cells by phosphorylating stem cell-specific cellular protein(s). Since the identity of these phosphorylation targets is currently unknown, one cannot test this hypothesis by biochemical means. However, this possibility can be verified genetically by creating a knockin mouse strain in which *cyclin A2* coding sequences would be replaced by those of *cyclin E*, and by assessing the ability of knockin cyclin E to drive proliferation of cyclin A-dependent compartments (such as hematopoietic stem cells).

It also remains to be seen whether the function of cyclin A in stem cell compartments is kinase dependent. Currently, the best-documented function of cyclin proteins is their ability to activate cyclin-dependent kinases (Sherr and Roberts, 2004). Hence, it seems likely that the function of cyclin A in vivo is mediated by cyclin A-Cdk1 and A-Cdk2 kinases. However, cyclin D-Cdk4 and D-Cdk6 complexes were shown to play kinase-independent functions during cell-cycle progression (Sherr and Roberts, 2004). Moreover, cyclin E was recently found to play a kinase-independent function in cell-cycle re-entry from quiescence (Geng et al., 2007). Creation of knockin mice expressing kinase-inactive mutant versions of cyclin A would allow one to query the contribution of kinase-dependent and -independent functions of cyclin A to proliferation of cyclin A-dependent compartments.

It is surprising that altered levels of several cell-cycle proteins selectively affected proliferation of stem cells. For example, deficiency in the polycomb family transcriptional repressor Bmi-1 caused increased levels of $p16^{INK4a}$—an inhibitor of cyclin D-Cdk kinase—and led to greatly reduced self-renewal of neural stem cells and hematopoietic stem cells (Molofsky et al., 2003; Park et al., 2003). Consistent with these findings, it was shown that increased $p16^{INK4a}$ levels during aging contribute to age-dependent decline in regenerative capacity of pancreatic islet cells, neural stem cells, and hematopoietic stem cells, while ablation of $p16^{INK4a}$ reversed these changes (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). Moreover, mice lacking all three D-type cyclins died due to an anemia caused by a proliferative failure of embryonic hematopoietic stem cells, while proliferation of the majority of other cell types proceeded unperturbed in the absence of D cyclins (Kozar et al., 2004). Collectively, these observations suggest that wiring of cell-cycle pathways in stem cells may operate in a more rigid and nonredundant fashion than it does in other cell types. This mechanism may allow stem cells to selectively respond to environmental cues by specifically upregulating a particular component of the cell-cycle engine, and to undergo self-renewal, asymmetric division, or cell differentiation.

It remains to be seen whether this strict requirement for particular cell-cycle proteins also operates in cancer stem cells. If it does, a therapy targeting cyclin proteins might be highly selective in shutting off the proliferation of these cells within

(D) Single-colony PCR of colonies derived from ESCs electroporated with Cre.

(E) Upper panel: western blot analysis of wild-type fibroblasts (MEF) and bone marrow cells (BM) probed with the indicated antibodies. Lower panel: Cdk2 was immunoprecipitated (IP), followed by immunoblotting with the indicated antibodies.

(F) Cdk1 or Cdk2 were immunoprecipitated from wild-type MEFs or bone marrow cells (BM) and subjected to in vitro kinase reactions, with histone H1 used as a substrate.

(G) Same analysis as in (E) with fibroblasts and ESCs.

a developing tumor. Cyclin A2-overexpression was shown to endow cells with anchorage-independent growth (Barrett et al., 1995; Guadagno et al., 1993; Yang and Krauss, 1997); therefore, blocking cyclin A2 function may revert an important step in oncogenic transformation. Also, the Myc oncogene has been postulated to signal by upregulating cyclin A (Barrett et al., 1995; Jansen-Durr et al., 1993; Qi et al., 2007), hence Myc-overexpressing human cancers might represent good candidates for anti-cyclin A therapy.

In the opposite setting, transiently stimulating the expression levels of particular cyclins, such as A or D cyclins, might increase the proliferative potential of normal stem cells, thereby augmenting tissue regeneration after an injury or relieving age-related functional changes in the stem cell compartment.

Indeed, overexpression of cyclin A2 in transgenic mice was shown to improve cardiac regeneration after myocardial infarction by augmenting the proliferative capacity of cardiac progenitor cells (Cheng et al., 2007). Therefore, elucidation of the molecular functions of cell-cycle regulators in different stem cell populations may have far reaching implications for treatments of various disease states.

EXPERIMENTAL PROCEDURES

Generation of Conditional *cyclin A2$^{f/f}$* Mice

Generation of *cyclin A2* gene targeting construct, conditional *cyclin A2* knockout ESCs, and mice is described in the Supplemental Experimental Procedures. Genotyping of *cyclin A2* knockout mice was performed with primers p1 (5′-CGC AGC AGA AGC TCA AGA CTC GAC-3′), p2 (5′-TCT ACA TCC TAA TGC AAT GCC TGG-3′), and p4 (5′-CAC TCA CAC ACT TAG TGT CTC TGG-3′) by denaturation of the DNA at 94°C for 3 min, followed by 30 cycles of amplification: 94°C for 1 min, 64°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 7 min. The wild-type *cyclin A2* allele yields a 335 bp product, *cyclin A2^{f}* a 392 bp product, and *cyclin A2$^{\Delta}$* a 580 bp product.

Tetraploid Complementation, Derivation, and Analyses of Fibroblasts

cyclin E1$^{-/-}$E2$^{-/-}$A1$^{-/-}$A2$^{f/f}$ ESCs were derived from blastocyst-stage embryos, cultured, and injected into tetraploid blastocysts as described (Geng et al., 2003). Fibroblasts were derived from embryos at day 13.5 of gestation, cultured, pulsed with BrdU and [^{3}H]-thymidine, and analyzed as described (Geng et al., 2003) (see the Supplemental Experimental Procedures).

For transduction of *cyclin A2$^{f/f}$ or A1$^{-/-}$A2$^{f/f}$* fibroblasts with Cre, we used the hit-and-run system-based retroviruses expressing wild-type Cre, or, as a control, an enzymatically inactive Cre point mutant (Silver and Livingston, 2001). Production of viral supernatants and infection of cells was performed as described (Silver and Livingston, 2001). Fibroblasts were infected twice for 4 hr, with a 24 hr interval between infections, using 10 MOI of virus. For analyses of *cyclin A1$^{-/-}$A2$^{f/f}$E1$^{-/-}$E2$^{-/-}$* fibroblasts, cells were infected twice, as above with 50 MOI of Adeno-Cre (from Vector Development Lab, Baylor College of Medicine) or the same amount of virus carrying an empty vector. Assays were performed after 48–72 hr.

Western Blotting

This was performed as described in the Supplemental Experimental Procedures with antibodies against cyclin A2 (sc-596 from Santa Cruz, or c4710 from Sigma), D2, D3, Cdk2, Cdk6 (all from Santa Cruz), cyclin D1, Cdk4, Cdk1 (all from NeoMarkers), cyclin B1 (Sigma), p27 (BD Bioscience), actin (Chemicon), or cyclin E1 (kindly provided by Dr. B. Clurman). Densitometric analyses of band intensities were performed with ImageJ software (http://rsb.info.nih.gov/ij/index.html).

Hematopoietic Analyses

Eight- to ten-week-old *cyclin A2$^{f/f}$ MxCre*, *A1$^{-/-}$A2$^{f/f}$ MxCre*, or control *A1$^{+/-}$A2$^{f/+}$ MxCre* mice were injected five times, every other day, with 400 μg of polyinosinic-polycytidylic acid (pI-pC, Sigma). Peripheral blood analyses were performed with MASCOT Hemavet 850 counter (CDC Technologies). Staining and flow cytometry of bone marrow cells, as well as methylcellulose cultures, was performed as described (Kozar et al., 2004).

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 7 days after the last pI-pC dose. HSCs and lineage-committed progenitors were stained and analyzed essentially as described (Akashi et al., 2000) (see the Supplemental Experimental Procedures). *cyclin A2* deletion in purified HSCs was performed as described in the Supplemental Experimental Procedures.

Analyses of ESCs

cyclin A1$^{-/-}$A2$^{f/f}$ and control *A1$^{+/-}$A2$^{f/+}$* ESCs were derived from blastocyst-stage embryos as described (Geng et al., 2003). Two hundred thousand ESCs were transduced with hit-and-run retrovirus (Silver and Livingston, 2001), and plated at 5 × 10^3 cells per 10 cm plate with feeders. Colonies were evaluated after 8–12 days.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and five figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00527-3.

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