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Cyclin E Ablation in the Mouse

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Summary

E type cyclins (E1 and E2) are believed to drive cell entry into the S phase. It is widely assumed that the two E type cyclins are critically required for proliferation of all cell types. Here, we demonstrate that E type cyclins are largely dispensable for mouse development. However, endoreplication of trophoblast giant cells and megakaryocytes is severely impaired in the absence of cyclin E. Cyclin E-deficient cells proliferate actively under conditions of continuous cell cycling but are unable to reenter the cell cycle from the quiescent G_0 state. Molecular analyses revealed that cells lacking cyclin E fail to normally incorporate MCM proteins into DNA replication origins during $G_0 \rightarrow S$ progression. We also found that cyclin E-deficient cells are relatively resistant to oncogenic transformation. These findings define a molecular function for E type cyclins in cell cycle reentry and reveal a differential requirement for cyclin E in normal versus oncogenic proliferation.

Introduction

Cyclins are key components of the core cell cycle machinery. These proteins constitute regulatory subunits that bind, activate, and provide substrate specificity for their associated cyclin-dependent kinases (CDKs). Two classes of cyclins operate during the G_1 phase in mammalian cells: D type cyclins (D1, D2, and D3) and E type cyclins (E1 and E2) (Sherr and Roberts, 1999). The expression of D type cyclins is controlled by the extracellular mitogens. Once induced, D type cyclins associate with CDK4 and CDK6, and phosphorylate the retinoblastoma protein, pRB, and pRB-related proteins p107 and p130 (Adams, 2001).

In contrast to growth factor-inducible D type cyclins, the expression of E type cyclins is controlled by an autonomous mechanism and peaks sharply at the G_1/S border (Dulic et al., 1992; Koff et al., 1992). The two E type cyclins, cyclin E1 (formerly called cyclin E) and the recently described cyclin E2, show significant amino acid similarity (75% within the cyclin box, 47% throughout the entire coding sequence) and are coexpressed in virtually all proliferating cells (Geng et al., 2001; Gudas et al., 1999; Lauper et al., 1998; Zariwala et al., 1998). E type cyclins associate with CDK2, CDK3, and possibly with other CDKs (Geisen and Moroy, 2002; Koff et al., 1992; Lauper et al., 1998; Zariwala et al., 1998). E type cyclins are believed to complete phosphorylation of pRB, initiated by the action of cyclin D-CDK complexes (Harbour et al., 1999; Lundberg and Weinberg, 1998). However, E type cyclins were shown to be critically required also for proliferation of pRB-negative cells, while ectopic overexpression of cyclin E in pRB-negative cells accelerates their cell cycle (Kelly et al., 1998; Ohtsubo et al., 1995). These observations reveal that E type cyclins control cell cycle progression also through pRB-independent targets. Among putative cyclin E-CDK substrates are proteins involved in the initiation of DNA replication (Arata et al., 2000), proteins governing centrosome duplication (nucleophosmin) (Hinchcliffe et al., 1999; Okuda et al., 2000), histone biosynthesis ($p220^{NPAT}$) (Ma et al., 2000; Zhao et al., 2000), and cell cycle progression ($p27^{Kip1}$, CDC25) (Hoffmann et al., 1994; Sheaff et al., 1997). Thus, it is currently assumed that cyclin D-CDK and cyclin E-CDK enzymes have distinct functions in regulating G_1 progression, with cyclin D coupling extracellular signals to the cell cycle machinery via pRB phosphorylation, while cyclin E controls the initiation of DNA replication, centrosome duplication, and possibly other functions. Importantly, it is widely believed that E type cyclins are absolutely required for cell cycle progression of all cell types. The work presented below challenges this notion and reveals that our understanding of the cell cycle machinery needs to be reevaluated.

Results

Generation of Cyclin E1- and Cyclin E2-Deficient Mice

In order to rigorously test the requirement for E type cyclins in development and in oncogenesis, we generated mice lacking cyclin E1 or cyclin E2, using gene targeting in embryonal stem cells. In our strategy for cyclin E1 targeting, we deleted coding exons 2–11. In case of cyclin E2 targeting, we deleted coding exons 1–8 (Figure 1A). Homozygous cyclin $E1^{-/-}$ and $E2^{-/-}$ mice were generated using standard methods, and the absence of the deleted exons was verified by Southern blotting (Figures 1B and 1C).

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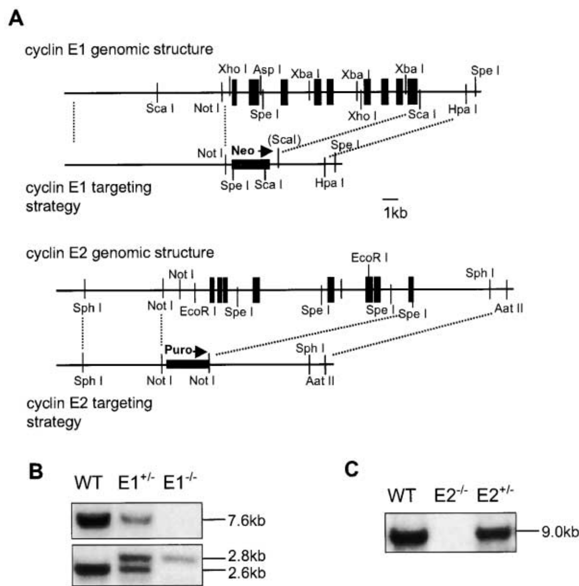

Figure 1. Targeted Inactivation of the Cyclin E1 and E2 Genes

(A) Top: *cyclin E1* gene targeting strategy. Bottom: *cyclin E2* gene targeting strategy. The coding exons are shown as filled boxes. Neo, the neomycin phosphotransferase gene; Puro, the puromycin resistance gene; arrows show transcriptional orientation. The restriction map of the cyclin E2 locus was provided by Drs. B. Amati and A. Beck.

(B) Southern blot analysis of genomic DNA extracted from wild-type (WT), cyclin $E1^{+/-}$, and cyclin $E1^{-/-}$ mice, digested with ScaI and SpeI, and hybridized with murine cyclin E1 cDNA probe containing exons 4–12 and the 3′ untranslated region. Note the absence of the 7.6 and 2.6 kb exon-containing bands and the appearance of a novel band of 2.8 kb in cyclin $E1^{-/-}$ DNA.

(C) Southern blot analysis of genomic DNA extracted from WT, cyclin $E2^{+/-}$, and cyclin $E2^{-/-}$ mice, digested with EcoRI, and hybridized with murine cyclin E2 cDNA probe containing exons 1–6. Note the absence of the exon-containing 9.0 kb band in cyclin $E2^{-/-}$ DNA.

Requirement for Cyclin E in Male Spermatogenesis

Cyclin $E1^{-/-}$ mice were born with the expected Mendelian ratio and appeared indistinguishable from the wild-type littermates. Detailed histopathological analyses revealed normal morphogenesis in all tissues examined. These cyclin E1-deficient animals displayed normal life spans and appeared normal throughout their entire lives.

Also, mice lacking cyclin E2 were born at the expected frequencies and were initially indistinguishable from control littermates. However, we found that cyclin E2-deficient males displayed reduced fertility, with approximately 50% of males being sterile. Our analyses revealed that cyclin $E2^{-/-}$ males displayed reduced testicular size (Figure 2B) and greatly reduced sperm counts (Figure 2C), as compared with wild-type littermates. Microscopic analyses demonstrated a significant testicular atrophy in cyclin $E2^{-/-}$ mice (Figure 2A). Testes of the mutant males displayed thinned walls of the seminiferous tubules with great reduction in the number of spermatogenetic cells. We also observed frequent abnormal meiotic figures within the spermatocyte layers (Figure 2A) and the presence of multinuclear giant cells within the seminiferous epithelium. These observations reveal that cyclin E2 is required for normal development of male germ cells and suggest a role for cyclin E2 in the meiosis that takes place in this compartment. Importantly, cyclin E2-deficient females develop normally and are fully fertile. Moreover, all other compartments analyzed develop normally in cyclin $E2^{-/-}$ mice (data not shown), and mutant animals display normal life spans.

To further probe the requirement for E type cyclins in development, we crossed cyclin E1- and E2-deficient mice, and we generated mice expressing only a single cyclin E allele, namely cyclin $E1^{-/-}E2^{+/-}$ and cyclin $E1^{+/-}E2^{-/-}$ animals. We found that cyclin $E1^{-/-}E2^{+/-}$ mice (like cyclin $E1^{-/-}$ animals) were indistinguishable from control mice and displayed normal morphogenesis in all tissues. On the other hand, cyclin $E1^{+/-}E2^{-/-}$ animals resembled cyclin $E2^{-/-}$ mice and displayed testicular hypoplasia and male infertility, which was more pronounced than that of mice lacking only cyclin E2 (data not shown). Collectively, these phenotypic analyses suggested to us that the two E type cyclins perform overlapping functions in the cell cycle progression and that a single cyclin E allele is sufficient to afford normal proliferation and development in mice. An alternative explanation would be that the proliferation of the developing tissues does not require E type cyclins. However, this notion would be incompatible with our current understanding of the cell cycle progression.

Requirement for E Type Cyclins in the Endoreplication of Trophoblast Giant Cells

We further intercrossed cyclin E-deficient animals in order to generate cyclin $E1^{-/-}E2^{-/-}$ mice. No double mutant mice were born live, prompting us to collect embryos at different stages of development. We found the expected fractions of live cyclin $E1^{-/-}E2^{-/-}$ embryos until day 10.25 of gestation (Figure 3A). At embryonic day (E)10.75, approximately 50% of cyclin $E1^{-/-}E2^{-/-}$ conceptuses were alive, and these mutant embryos appeared growth retarded, as compared with wild-type littermates (Figure 3B). No live cyclin $E1^{-/-}E2^{-/-}$embryos were observed after E11.5. Surprisingly, histopathological examination of the mutant embryos revealed that—despite some growth retardation—these cyclin E-deficient embryos displayed normal morphogenesis and no signs of pathological lesions. Also, analyses of yolk sacs revealed normal blood vessel formation and the presence of blood islands filled with numerous blood cell precursors (data not shown). In contrast, examination of other extraembryonal tissues revealed a profound abnormality within the mutant placentas.

Specifically, we found that the layer of trophoblast giant cells, usually very prominent in wild-type placentas due to the giant size of trophoblast cell nuclei, was virtually missing in mutant tissues. Instead, cyclin $E1^{-/-}E2^{-/-}$ placentas contained a layer of greatly underdeveloped "small" trophoblast cells with much smaller nuclei than the corresponding layer in wild-type animals (Figure 3C).

Trophoblast giant cells represent a very unique population that normally undergoes repeated rounds of DNA synthesis without intervening mitoses (endoreplication), which leads to formation of giant nuclei containing up to 1000 N of DNA (Cross, 2000; Zybina and Zybina, 1996). The greatly diminished size of trophoblast nuclei

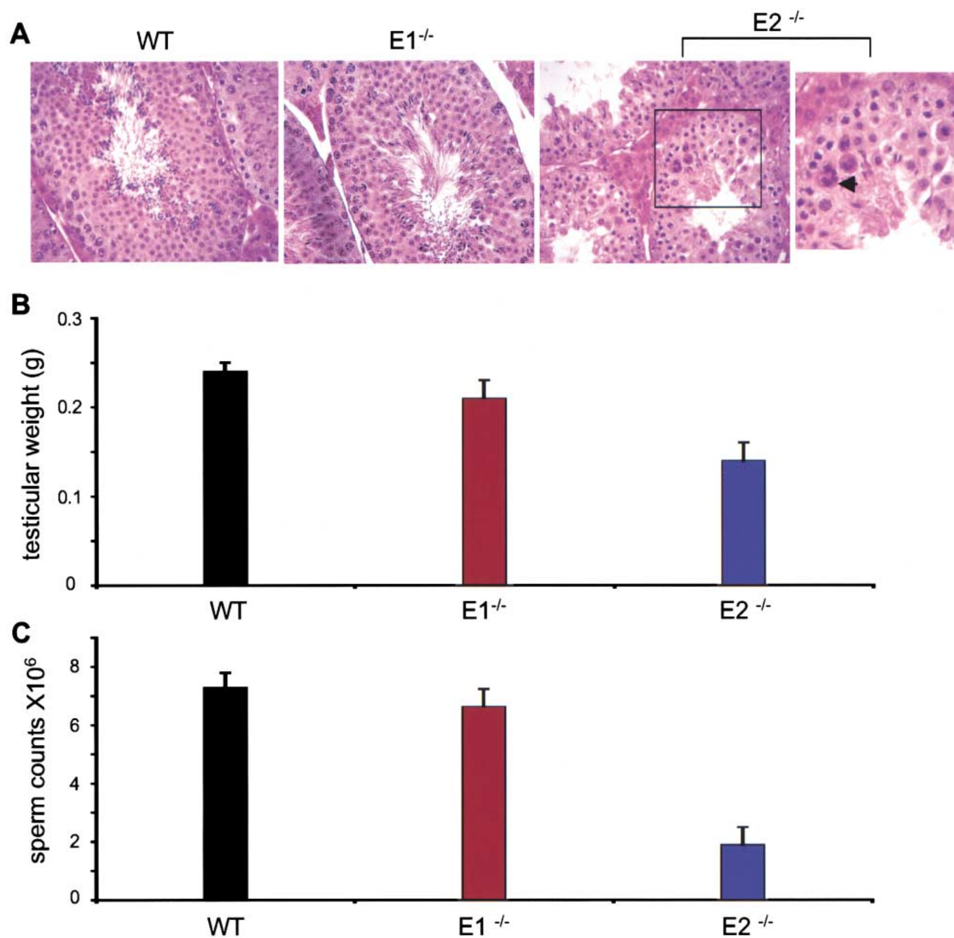

Figure 2. Testicular Abnormalities in Cyclin E-Deficient Mice

(A) Histologic sections of testes from 2- to 3-month-old wild-type (WT), cyclin E1$^{-/-}$, and cyclin E2$^{-/-}$ males, stained with hematoxylin and eosin. Note the paucity of cells in the seminiferous tubules of cyclin E2$^{-/-}$ animals and the presence of aberrant meiotic figures (arrow). Original magnification 20×. The right panel shows higher magnification of the boxed region.

(B) The mean testicular weight in 2- to 3-month-old WT (n = 10), cyclin E1$^{-/-}$ (n = 12), and cyclin E2$^{-/-}$ (n = 12) males. Error bars denote standard errors.

(C) Sperm counts in same experimental groups as in (B).

in cyclin E1$^{-/-}$E2$^{-/-}$ conceptuses suggested to us that the mutant trophoblast cells might fail to undergo normal endoreplicative cycles. To verify this hypothesis, we quantitated the DNA content in trophoblast giant cells of wild-type mice and in the corresponding trophoblast cells of cyclin E1$^{-/-}$E2$^{-/-}$ animals by Feulgen staining. Diploid cells of the maternal decidua were used to standardize our measurements. As expected, in wild-type placentas, trophoblast giant cells contained up to 1000 N of DNA, with the majority of cells containing over 100 N of DNA. In contrast, the DNA content of cyclin E1$^{-/-}$E2$^{-/-}$ trophoblast cells was greatly reduced, with virtually all cells containing less than 100 N DNA (Figure 3D). These findings suggest that E type cyclins are required for repeated rounds of endoreplication in trophoblast giant cells, and consequently, in the absence of E type cyclins, the trophoblast cells fail to undergo normal endoreplicative cycles. Given the critical central function of trophoblast giant cells in placental physiology, including invasion into the maternal tissues, formation of the blood vessels in the labyrinth layer, promotion of maternal blood flow, and production of angiogenic factors and hormones that influence placental function (Cross, 2000; Cross et al., 2002), we hypothesized that the placental dysfunction due to trophoblast cell defect might be the cause of lethality in cyclin E1$^{-/-}$E2$^{-/-}$ embryos.

E Type Cyclins Are Dispensable for the Development of the Embryo Proper

To verify this hypothesis, we turned to the tetraploid complementation rescue method (Eggan et al., 2001; Tanaka et al., 1997). In this technique, two-cell-stage embryos are collected from pregnant wild-type females and fused by a pulse of an electric current, yielding one-cell tetraploid embryos. These embryos are then kept in vitro for 2 days and develop into tetraploid blastocysts. Mutant ES cells are then injected into tetraploid blastocysts and the chimeric embryos are implanted into foster females for further development. Importantly, tetraploid blastocysts cannot contribute to the embryo proper but can form normal placentas and other extraembryonic

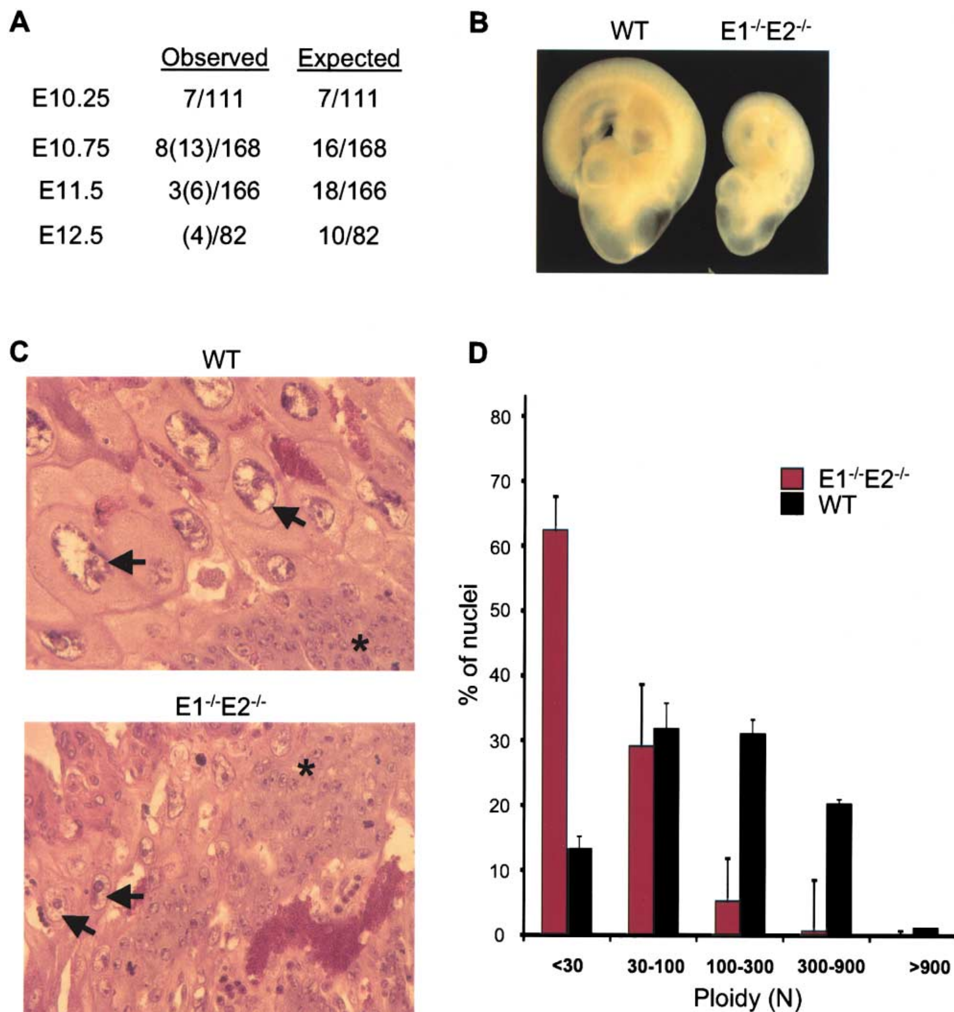

Figure 3. Phenotype of Cyclin $E1^{-/-}E2^{-/-}$ Embryos

(A) Survival of $E1^{-/-}E2^{-/-}$ embryos at various stages of development. Shown are the ratios between the numbers of observed live $E1^{-/-}E2^{-/-}$ embryos and the total numbers of embryos analyzed. Numbers in parentheses denote the dead $E1^{-/-}E2^{-/-}$ embryos observed at each time point. In the right-hand column, the expected frequency of mutant embryos is shown.

(B) Appearance of viable WT and $E1^{-/-}E2^{-/-}$ embryos at day 10.75 of gestation.

(C) Histologic sections of placentas derived from WT and $E1^{-/-}E2^{-/-}$ embryos at day 10.5 of gestation. Trophoblast giant cell nuclei are marked by arrows. Asterisks mark the decidual layer of the placentas (containing diploid cells). Original magnification 40×.

(D) Ploidy distribution of trophoblast giant cells in wild-type placentas and the ploidy of trophoblast cells in the corresponding layer of $E1^{-/-}E2^{-/-}$ placentas.

tissues (Tanaka et al., 1997). Hence, all cells in the developing embryo are derived from the injected mutant ES cells, while the extraembryonic tissues are formed by cells deriving from wild-type (tetraploid) blastocysts. We reasoned that if the observed lethality in cyclin $E1^{-/-}E2^{-/-}$ embryos is indeed caused by the placental failure, we should be able to rescue this lethality by tetraploid complementation.

First, we mated cyclin $E1^{+/-}E2^{+/-}$ males and females, collected blastocysts at E3.5, and derived several independent cyclin $E1^{-/-}E2^{-/-}$ embryonal stem (ES) cell lines using conventional methods (Hogan et al., 1986). The appearance and the proliferation rate of these cyclin E-deficient ES cells were very similar to those of control wild-type ES cells (data not shown). Next, we injected cyclin $E1^{-/-}E2^{-/-}$ ES cells into tetraploid blastocysts, and we analyzed the development of the chimeric embryos. We found that this procedure fully rescued the embryonal lethality, and we recovered viable cyclin $E1^{-/-}E2^{-/-}$ embryos at all points of development and at postnatal day (P)1 (Figures 4A and 4B). Since even wild-type embryos produced by tetraploid complementation often die at birth (Eggan et al., 2001), we were not able to study cyclin $E1^{-/-}E2^{-/-}$ mice during postnatal life.

The observed rescue of the embryonal lethality by the tetraploid complementation method points to the placental abnormality as a cause of death in cyclin $E1^{-/-}E2^{-/-}$ animals. Moreover, these analyses reveal that the E type cyclins are not required for embryo development (with the exception of cardiovascular system, see below).

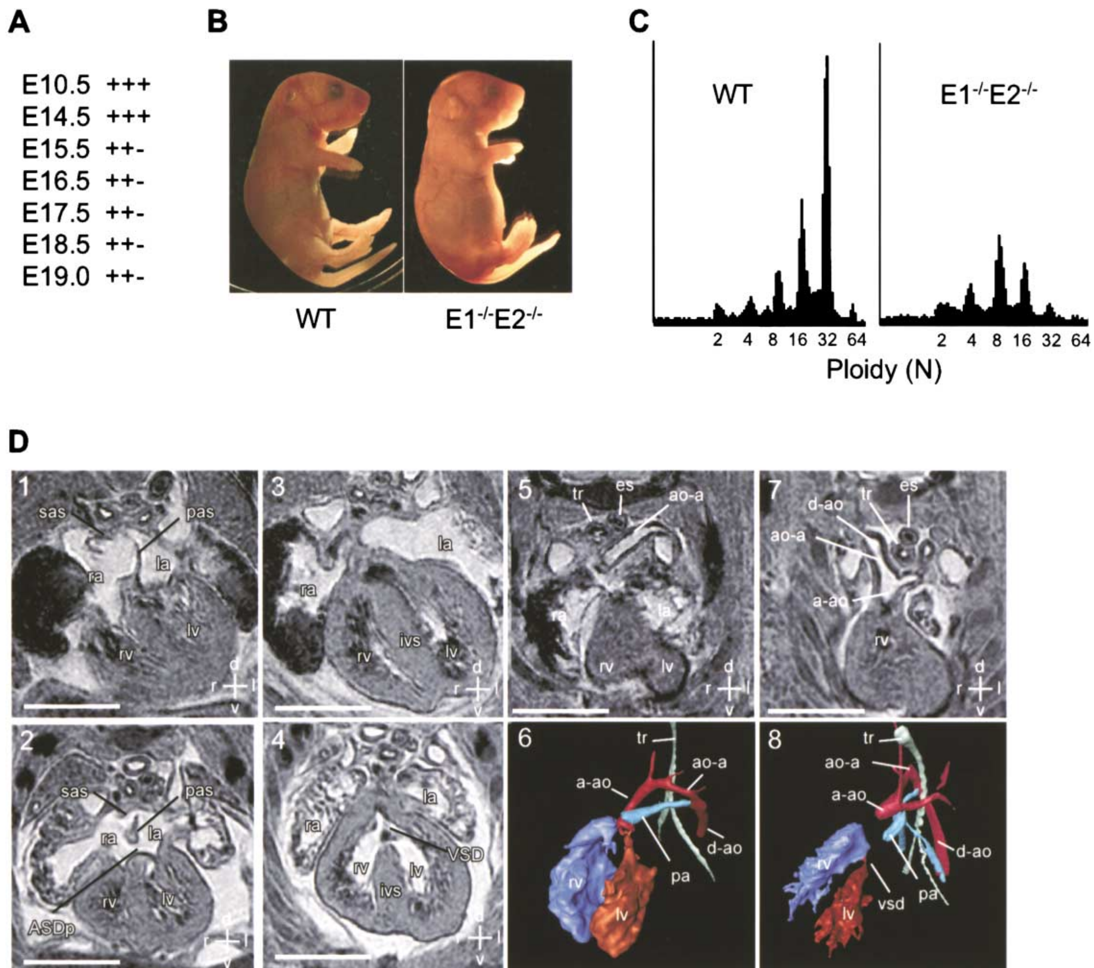

Figure 4. Rescue of Cyclin E1$^{-/-}$E2$^{-/-}$ Lethality by Tetraploid Blastocyst Complementation

(A) Survival of the rescued embryos at various stages of development. +++ indicates normal development; ++− indicates that a fraction of embryos were found dead at this stage.

(B) Normal appearance of rescued E1$^{-/-}$E2$^{-/-}$ animals at postnatal day (P)1. A wild-type (WT) neonate is shown for comparison.

(C) Ploidy distribution of WT and cyclin E1$^{-/-}$E2$^{-/-}$ megakaryocytes cultured in vitro for 3 days in the presence of thrombopoietin. Peaks representing each ploidy class are labeled.

(D) Cardiovascular abnormalities in cyclin E1$^{-/-}$E2$^{-/-}$ embryos. Magnetic resonance imaging of cardiac malformations in E1$^{-/-}$E2$^{-/-}$ embryos. Scale bars = 1 mm; axes: d, dorsal; v, ventral; r, right; l, left. All images are transverse sections from day 15.5 embryos. Panels 1, 3, and 5 are images from a wild-type embryo. Abbreviations: sas, secondary atrial septum; pas, primary atrial septum; ra, la, right and left atria, respectively; rv, lv, right and left ventricles, respectively; ivs, interventricular septum; a-ao, ascending aorta; d-ao, descending aorta; ao-a, aortic arch; pa, pulmonary artery; tr, trachea; and es, esophagus. Panel 6 is a 3D reconstruction (left oblique view) of normal cardiac anatomy; note the aortic arch on the left of the trachea. Panels 2, 4, and 7 are images from a cyclin E-deficient embryo, and panel 8 is a 3D reconstruction of mutant cardiac anatomy. These show a primum atrial septal defect (ASDp, panel 2), a ventricular septal defect (VSD, panel 4), and a right-sided aortic arch that arises from the right ventricle (panels 7 and 8), i.e., double-outlet right ventricle.

Cardiovascular Abnormalities in Cyclin E1$^{-/-}$E2$^{-/-}$ Embryos

While we detected live cyclin E1$^{-/-}$E2$^{-/-}$ embryos until P1, we found that a fraction (approximately half) of mutant embryos died between E15.5 and E18.5 (Figure 4A). To determine the cause of this lethality, we analyzed cyclin E-deficient embryos by serial sectioning and by magnetic resonance imaging (Schneider et al., 2003). These analyses revealed multiple cardiac abnormalities in cyclin E1$^{-/-}$E2$^{-/-}$ conceptuses. Specifically, mutant embryos displayed ventricular septal defect (VSD) combined with primum atrial septal defect (ASD), leading to the appearance of common atrioventricular canal in mutant hearts (Figure 4D, panels 2 and 4). Moreover, in cyclin E-deficient hearts, both the aorta and the pulmonary artery arose incorrectly from the right ventricle, giving rise to the so-called double-outlet right ventricle (Figure 4D, panels 7 and 8). This indicates that E type cyclins are required for the normal development of atrioventricular endocardial cushions and associated structures (Webb et al., 1998). Lastly, we detected multiple abnormalities in the patterning of aortic arches (Figure 4D, panels 7 and 8). Similar defects were reported in mutant animals with abnormal neural crest migration and patterning (Kirby and Waldo, 1995) and suggest that E type cyclins may play a role in this process. Importantly, very extensive histopathological analyses involving serial sectioning of the entire rescued cyclin

$E1^{-/-}E2^{-/-}$ embryos revealed normal morphogenesis in all other compartments. These observations indicate that the E type cyclins are largely dispensable for normal development.

Requirement for E Type Cyclins in Endoreplication of Megakaryocytes

In addition to trophoblast giant cells, the platelet precursors, megakaryocytes, are another cell type that normally undergoes endoreplicative cycles. The availability of rescued cyclin E-deficient embryos allowed us to address the requirement for E type cyclins in endoreplication in this compartment. To this end, we isolated megakaryocytes from E14.5 wild-type and cyclin E-deficient livers, cultured them in vitro in the presence of thrombopoietin to stimulate endoreplicative cycles, and determined the DNA content. As expected, wild-type megakaryocytes displayed polyploid DNA content, with the majority of cells containing 32 N of DNA. In contrast, DNA content was clearly reduced in cyclin $E1^{-/-}E2^{-/-}$ megakaryocytes (Figure 4C), revealing impaired endoreplication in the absence of E type cyclins. Together with our earlier studies of trophoblast giant cells, these analyses reveal that E type cyclins are required for endoreplication of diverse mammalian cellular compartments.

Continuous Cell Cycling in the Absence of E Type Cyclins

We next derived fibroblasts (MEFs) from rescued cyclin $E1^{-/-}E2^{-/-}$ embryos, and we used them for cell cycle and molecular studies. Analyses of in vitro cultured early-passage cyclin $E1^{-/-}E2^{-/-}$ MEFs revealed that these cells actively proliferated in vitro, with a higher fraction of mutant cells residing in the G_1 phase (Figure 5B). We also determined that the rate of the S phase progression (incorporation of $[^3H]$thymidine per 10^3 of S phase nuclei per unit of time) was very similar in wild-type and cyclin $E1^{-/-}E2^{-/-}$ cells (data not shown).

Detailed analyses of the in vitro proliferation rates revealed that the proliferation of cyclin E-deficient cells was modestly impaired compared with wild-type controls. We found that after 7 days of in vitro culture, cyclin E-deficient cells on average increased cell number 30-fold, i.e., 71.3% of the increase seen in wild-type controls (Figure 5C). These findings are consistent with apparently normal proliferation of cells within cyclin $E1^{-/-}E2^{-/-}$ embryos. Since during repeated in vitro passaging cyclin E-deficient cells underwent replicative crisis earlier (passage four) compared to wild-type cells (P7), all experiments described in this paper used cells freshly explanted from embryos (P0) or at P1 and P2.

E type cyclins were postulated to play a key role in the phosphorylation of the retinoblastoma protein (Harbour et al., 1999; Lundberg and Weinberg, 1998), the induction of cyclin A (Zerfass-Thome et al., 1997), and in the degradation of the cell cycle inhibitor $p27^{Kip1}$ (Sheaff et al., 1997). Importantly, absence of any of these events is predicted to arrest cell cycling. Hence, proliferation of cyclin $E1^{-/-}E2^{-/-}$ cells is very surprising, and it is hard to reconcile with our current understanding of cell cycle progression.

We started our molecular analyses by comparing the phosphorylation of the retinoblastoma protein in cyclin E-deficient embryos and MEFs versus their wild-type counterparts, using Western blotting. We found that the appearance of phosphorylated species of pRB was similar in wild-type and mutant samples (Figure 5A), revealing that at least at this level of resolution, E type cyclins are not required for normal pRB phosphorylation. More-

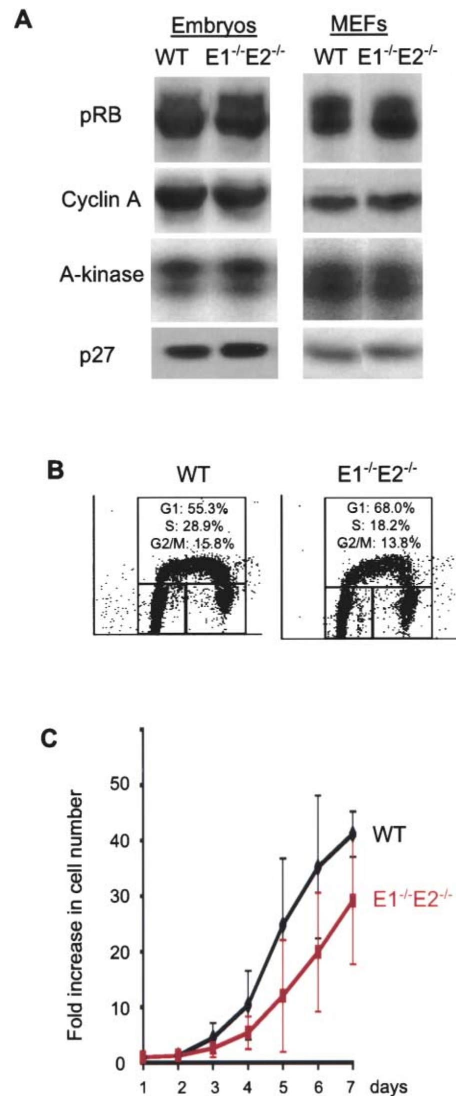

Figure 5. Analyses of Cyclin E-Deficient Embryos and In Vitro Cultured Mouse Embryonal Fibroblasts

(A) Western blot analyses of the retinoblastoma protein, pRB, cyclin A, and $p27^{Kip1}$ in wild-type (WT) and $E1^{-/-}E2^{-/-}$ embryos (left) and MEFs. The activity of cyclin A-associated kinase was also determined using histone H1 as a substrate.

(B) Cell cycle distribution of asynchronously growing mouse embryo fibroblasts cultured in vitro. Cells were pulsed with bromodeoxyuridine (BrdU) for 1 hr and then stained with anti-BrdU antibodies and with propidium iodide followed by FACS analysis. The percentages of cells in particular phases of cell cycle are shown.

(C) In vitro proliferation of wild-type and cyclin E-deficient fibroblasts. Equal amounts of cells were plated at the beginning of the experiment. Cell numbers were determined every day for total of 7 days. Values shown are means of three independent experiments utilizing cells derived from four $E1^{-/-}E2^{-/-}$ and four control embryos. Error bars denote standard deviation.

over, we found that the levels of cyclin A, as well as the levels of cyclin A-associated kinase, were similar in wild-type and mutant cells (Figure 5A). Hence, the activity of cyclin E is not required for the induction of cyclin A. Based on our additional analyses (see below), we propose that in cyclin $E1^{-/-}E2^{-/-}$ cells, the phosphorylation of pRB is carried out by cyclin A. Moreover, we found that the levels of $p27^{Kip1}$ did not differ between wild-type and mutant cells and embryos (Figure 5A), again revealing that the levels of this inhibitor can be held in check without any contribution from E type cyclins.

In addition to its well-documented cell cycle functions, E type cyclins were postulated to play a key role in duplication of centrosomes, initiation of DNA replication, and histone biosynthesis (see Introduction). However, staining of cyclin $E1^{-/-}E2^{-/-}$ MEFs with the centrosomal marker γ tubulin revealed normal appearance of this organelle in mutant cells (data not shown). Moreover, relatively normal cell cycling of mutant cells in vitro and normal development of cyclin E-deficient embryos implies that both DNA and histones can be synthesized normally in the absence of cyclin E activity. Collectively, these results indicate that contrary to the widely held view, E type cyclins are largely dispensable for continuous cell cycling.

Critical Requirement for E Cyclins in Cell Cycle Reentry

Next, we analyzed the ability of cyclin $E1^{-/-}E2^{-/-}$ cells to reenter the cell cycle from the quiescent G_0 state. To this end, we arrested cells in G_0 phase by serum deprivation, stimulated them to reenter the cell cycle by serum readdition, and monitored their entry into S phase by measuring thymidine incorporation. As expected, wild-type cells synchronously entered the S phase starting at 12 hr after serum readdition. In contrast, quiescent cyclin $E1^{-/-}E2^{-/-}$ cells were unable to normally reenter the S phase (Figure 6A). In an alternative approach, cells were rendered quiescent by contact inhibition and were then triggered to reenter the cell cycle by replating under low-density conditions. Again, cells lacking cyclin E were unable to enter the S phase (data not shown). We concluded that in the absence of E type cyclins, mutant cells are unable to reenter the cell cycle from the quiescent G_0 state.

In order to understand the molecular basis of this requirement for E type cyclins in cell cycle reentry, we first focused on the phosphorylation of the retinoblastoma protein. We compared the pRB phosphorylation at time zero and at 18 hr poststimulation, a time where wild-type cells were at the peak of the S phase progression (Figure 6A). Surprisingly, we found that pRB was clearly phosphorylated in cyclin $E1^{-/-}E2^{-/-}$ cells (Figure 6B). Analyses of time course of pRB phosphorylation following serum stimulation revealed that in wild-type cells, hyperphosphorylated pRB species started to appear at 12 hr poststimulation, concomitant with appearance of cyclin E-associated kinase (Figure 6C). In cyclin $E1^{-/-}E2^{-/-}$ cells, pRB phosphorylation was delayed by 2–3 hr, and it coincided with the appearance of cyclin A-associated kinase (Figure 6C and data not shown). These analyses suggest that in mutant cells, cyclin A is responsible for bringing about pRB phosphorylation. We noted, however, that the overall extent of pRB phosphorylation was slightly lower in mutant cells (Figures 6B and 6C).

Phosphorylation and functional inactivation of pRB is expected to liberate E2F transcription factors, leading to the induction of E2F target genes (Dyson, 1998). Hence, we asked whether these E2F targets are normally induced in the absence of cyclin E. Our analyses revealed that several known E2F targets, such as CDC6, CDC45, and cyclin A, are induced normally in cyclin $E1^{-/-}E2^{-/-}$ cells (Figure 6D and data not shown). Collectively, these observations essentially rule out the absence of pRB phosphorylation as the cause of the failure of S phase entry in $E1^{-/-}E2^{-/-}$ cells.

Cyclin E and MCM Loading into DNA Replication Origins

Recent work of Coverley et al. (2002) revealed that in a cell-free in vitro reconstitution system, cyclin E triggers loading of the MCM proteins into DNA replication origins during $G_0 \rightarrow S$ progression. These findings, together with our observations that cyclin $E1^{-/-}E2^{-/-}$ cells failed to normally enter the S phase from the G_0 state, suggested to us that mutant cells may be unable to incorporate MCM into their DNA replication origins. To test this possibility, we stimulated wild-type and mutant cells to reenter the cell cycle, harvested cells at various time points, and prepared protein lysates. We then separated the proteins into chromatin bound and chromatin unbound free fractions and examined the presence of MCM2 in these fractions by Western blotting. As expected (Madine et al., 2000; Stoeber et al., 1998), in wild-type cells, MCM2 was not bound to DNA in the G_0 and in early G_1 cells, but it became associated with chromatin approximately 12 hr after serum readdition (Figure 7A), coinciding with the appearance of cyclin E kinase activity (data not shown). In contrast, MCM2 failed to associate with the DNA in cyclin $E1^{-/-}E2^{-/-}$ cells through the entire observation period (Figure 7A). Importantly, we verified that the levels of unbound, free MCM2 were very similar in wild-type and cyclin $E1^{-/-}E2^{-/-}$ cells (Figure 7A, bottom). Hence, cyclin E-deficient cells contain normal levels of MCM2 but fail to induce its loading into DNA replication origins during cell cycle reentry.

These findings prompted us to analyze loading of various other components of the prereplicative complexes onto DNA replication origins during $G_0 \rightarrow S$ phase progression of wild-type and mutant cells. As expected (Madine et al., 2000; Stoeber et al., 1998), we found that in wild-type cells, the ORC2 protein remained associated with DNA replication origins at all points during cell cycle reentry. CDC6 was loaded onto DNA at approximately 12 hr, followed by DNA loading of the MCM at 12–15 hr (Figure 7B). In cyclin E-deficient cells, the ORC2 protein was also constitutively associated with DNA, and CDC6 was loaded at a similar time as in the wild-type cells. However, as we observed before, mutant cells were deficient in MCM loading (Figure 7B). These analyses reveal that the assembly of the prereplicative complexes is blocked in cyclin $E1^{-/-}E2^{-/-}$ cells at the MCM loading stage.

Relatively normal proliferation of continuously cycling cyclin $E1^{-/-}E2^{-/-}$ cells, documented above, prompted

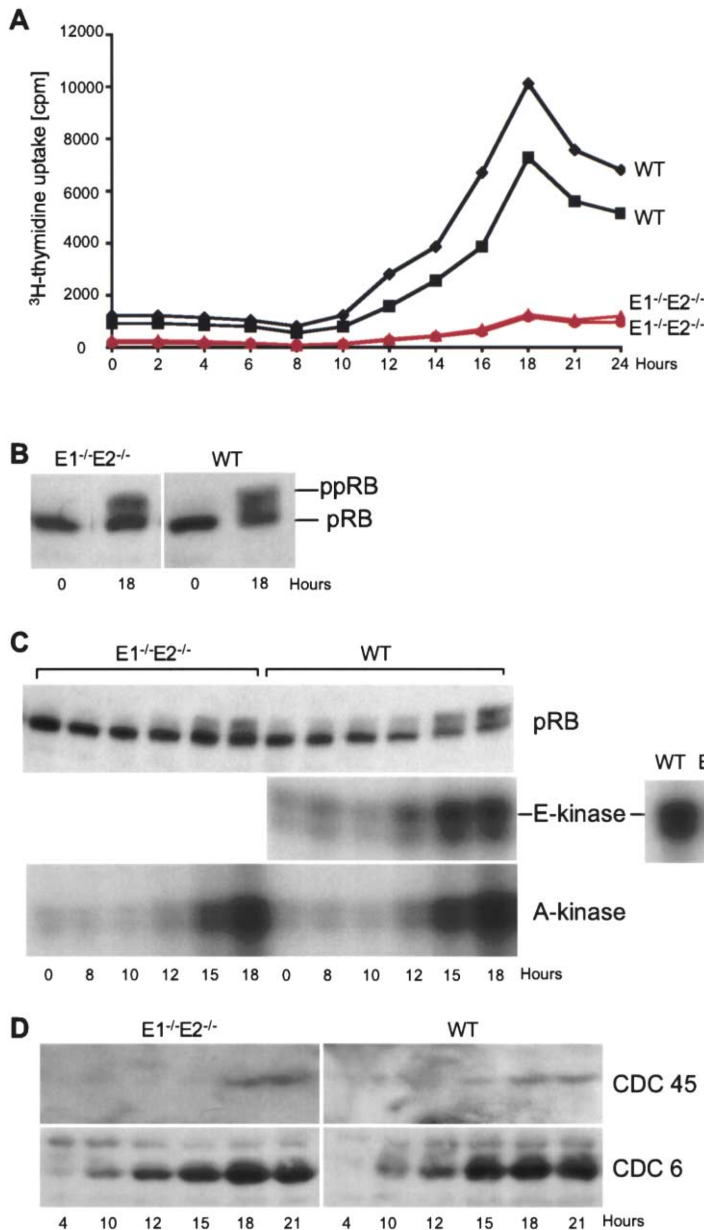

Figure 6. Impaired Cell Cycle Reentry in Cyclin E-Deficient Cells

Wild-type and E1$^{-/-}$E2$^{-/-}$ mouse embryonal fibroblasts (MEFs) were rendered quiescent by serum deprivation and then were restimulated to reenter the cell cycle by serum readdition.

(A) Entry into the S phase was gauged by measuring [^{3}H] thymidine uptake.

(B) Phosphorylation status of the retinoblastoma protein (pRB) was determined by Western blotting at time zero and 18 hr after the stimulation. ppRB denotes hyperphosphorylated pRB.

(C) pRB phosphorylation status at different time points following serum stimulation was determined by Western blotting (top). Cyclin E- and cyclin A-associated kinase activities were determined in the same samples using histone H1 as a substrate (middle and bottom). Right-hand panel shows control immunoprecipitation with anti-cyclin E antibody using extracts from continuously growing wild-type (WT) and E1$^{-/-}$E2$^{-/-}$ MEFs. Cyclin E-associated kinase activity was determined as above.

(D) The levels of known E2F targets, CDC45, and CDC6, were determined by Western blotting.

us to analyze the chromatin association of MCM in these cells. Our analyses revealed that in contrast to cells reentering the cell cycle from the quiescent G_0 state, continuously cycling E1$^{-/-}$E2$^{-/-}$ cells displayed normal loading of MCM2, as well as of ORC2 and CDC6 (Figure 7C). Collectively, these results strongly suggest that cyclin E activity is critically required for loading of MCM into prereplication complexes during $G_0 \rightarrow S$ phase progression, but not in continuously cycling cells, as postulated by Coverley et al. (2002). Consequently, in the absence of E type cyclins, mutant cells fail to assemble normal prereplication complexes during $G_0 \rightarrow S$ progression, explaining an inability of mutant cells to reenter the cell cycle. We note here that a similar critical role for cyclin E in MCM loading was postulated to operate in endoreplication (see Discussion).

Resistance of Cyclin E-Deficient Cells to Oncogenic Transformation

Lastly, we asked whether the absence of E type cyclins alters the response of E1$^{-/-}$E2$^{-/-}$ cells to various oncogenic stimuli. First, we infected wild-type and cyclin E1$^{-/-}$E2$^{-/-}$ MEFs with retroviruses encoding the c-*Myc* oncogene, selected the infected cells, and plated them at low densities. While wild-type cells formed numerous colonies following Myc expression, cyclin E1$^{-/-}$E2$^{-/-}$ cells failed to do so (Figure 8). Importantly, we verified that the levels of the ectopically expressed Myc were very similar in wild-type and mutant cells (data not shown).

Next, we infected cells with retroviruses encoding oncogene pairs: *H-Ras* plus c-*Myc*, or with *H-Ras* plus dominant-negative (DN) p53, or *H-Ras* plus *E1A*, and

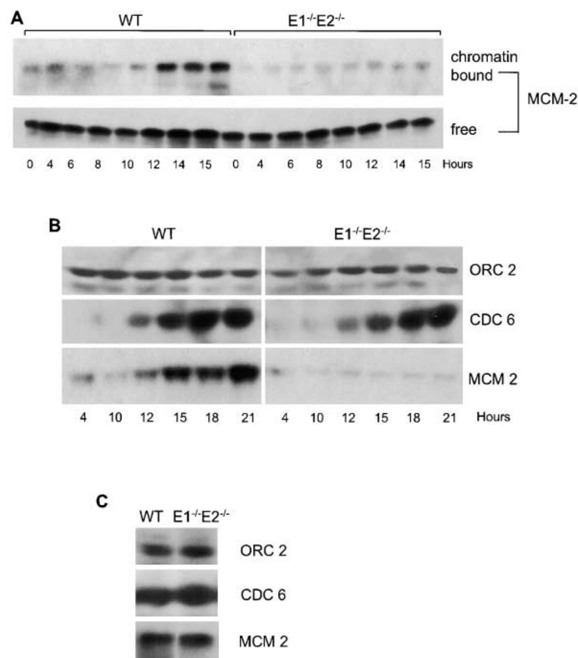

Figure 7. Defective MCM Loading in Cyclin E-Deficient Cells

(A and B) Wild-type (WT) and cyclin E1$^{-/-}$E2$^{-/-}$ mouse embryonal fibroblasts (MEFs) were rendered quiescent by serum deprivation and then were restimulated to reenter the cell cycle by serum readdition. (A) Protein extracts were separated into chromatin bound and chromatin unbound free fractions, and the levels of MCM2 in these fractions were determined by Western blotting. (B) The levels of ORC2, CDC6, and MCM2 in the chromatin bound fraction were determined by Western blotting.

(C) The levels of ORC2, CDC6, and MCM2 in the chromatin bound fraction of continuously proliferating MEFs.

scored the ability of these oncogenes to transform cyclin E-deficient MEFs using focus-forming assay. In each case, equal expression of the introduced oncogenes was verified in wild-type versus mutant cells by Western blotting (data not shown). We found that cyclin E1$^{-/-}$E2$^{-/-}$ monolayers failed to form foci following introduction of oncogenic Ras plus Myc or Ras plus DN p53 (Figure 8). In the case of *H-Ras* plus *E1A* oncogenes, we observed the presence of some putative foci in cyclin E1$^{-/-}$E2$^{-/-}$ monolayers. However, the number of these foci was reduced approximately 10- to 12-fold, as compared wild-type cells, and cyclin E-deficient foci displayed very flattened appearance (Figure 8). We concluded that mutant cells display greatly reduced susceptibility to oncogenic transformation.

Discussion

In this study, we tested the requirement for cyclin E in mouse development by genetic means. Contrary to the prevailing view, we found that cyclin E is largely dispensable for normal mouse embryogenesis. Thus, while cyclin E was shown to be critically required for the development of *Drosophila melanogaster* (Knoblich et al., 1994) and *Caenorhabditis elegans* (Fay and Han, 2000), this is clearly not the case in the mammalian embryos. On the other hand, we found that cyclin E is essential for normal development of mammalian placentas. Similar results were independently obtained by T. Parisi et al. (submitted). Intriguingly, recent reports indicate that mice lacking other components of the cell cycle machinery, namely the retinoblastoma protein (Wu et al., 2003), as well as the E2F partner DP1 (Kohn et al., 2003), also die due to placental abnormalities, revealing the specific requirement for these proteins in extraembryonal development.

It is widely assumed that E type cyclins are critically required for normal proliferation of virtually all mammalian cell types. E type cyclins were postulated to contribute to pRB inactivation (Harbour et al., 1999; Lundberg and Weinberg, 1998), control centrosome duplication (Hinchcliffe et al., 1999; Okuda et al., 2000), trigger the initiation of DNA replication (Arata et al., 2000; Krude et al., 1997), control histone biosynthesis via phosphorylation of $p220^{NPAT}$ (Ma et al., 2000; Zhao et al., 2000), activate E2F-5 (Morris et al., 2000), and bind and activate CDC25 phosphatase (Hoffmann et al., 1994). In several instances, the relevant function was uniquely ascribed to cyclin E, but not to other cyclins. However, our observations that nearly normal mouse development takes place in the absence of E type cyclins indicate that our current understanding of the cell cycle machinery needs to be reevaluated.

Our analyses of in vitro cultured cyclin E-deficient cells revealed that E type cyclins are not essential for continuous cell cycling but are critically required for cell cycle reentry. Since most cells of the developing embryo are in an actively proliferating state (Kaufman and Bard, 1999), these observations likely explain normal embryonal development seen in cyclin E1$^{-/-}$E2$^{-/-}$ animals. Active proliferation of cyclin E-deficient cells indicates that all functions specifically ascribed to E type cyclins can be carried out by other proteins. For instance, our analyses indicate that cyclin A mediates phosphorylation of pRB in E1$^{-/-}$E2$^{-/-}$ fibroblasts, and this phosphorylation occurs at a time when cyclin A normally phosphorylates pRB in wild-type cells.

E Type Cyclins and MCM Loading

During continuous cell cycling, binding of MCM to the replication origins occurs immediately after the exit from mitosis, i.e., in the absence of any cyclin E-CDK activity (Mendez and Stillman, 2000). As the cells traverse the G_1 and enter the S phase, cyclin E-CDK activity is believed to be responsible for the origin firing by phosphorylating as yet unidentified targets (Krude et al., 1997; Stoeber et al., 1998). Cyclin E action is thought to allow loading of CDC45 onto replication origins, which results in origin melting and recruitment of polymerase α (Arata et al., 2000; Zou and Stillman, 2000). Our demonstration that cyclin E1$^{-/-}$E2$^{-/-}$ cells proliferate in vivo and in vitro suggests that in continuously growing cells, other critical steps in origin firing are likely carried out by cyclin A or by other proteins.

In contrast to active continuous cell cycling, we found that cyclin E-deficient cells were unable to normally reenter the cell cycle from the quiescent G_0 state. Importantly, these cyclin E1$^{-/-}$E2$^{-/-}$ cells failed to enter S

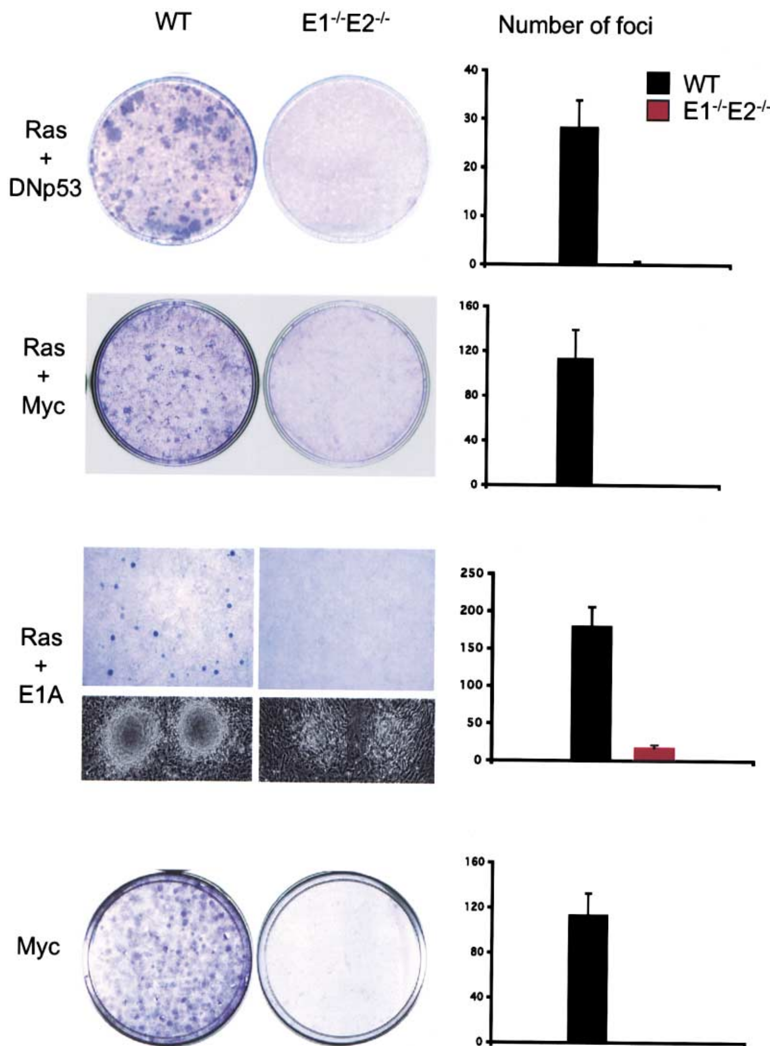

Figure 8. Resistance of Cyclin E-Deficient Cells to Oncogenic Transformation

Appearance of crystal violet-stained plates (left) and the mean number of foci or colonies seen in wild-type and cyclin E1$^{-/-}$E2$^{-/-}$ MEFs infected with retroviruses encoding indicated oncogenes. Error bars denote SD.

In the case of v-Ha-Ras plus E1A infection, low magnification of the crystal violet-stained monolayers and higher magnification of unstained foci (bottom) is shown.

phase despite normal induction of cyclin A and cyclin A-associated kinase activity and nearly normal phosphorylation of the retinoblastoma protein. Thus, cyclin E plays a specific, critical function in $G_0 \rightarrow S$ progression, and this function cannot be carried out by other cyclins. Our molecular analyses revealed that in the absence of E type cyclins, mutant cells fail to normally load MCM proteins onto their DNA replication origins.

Unlike in continuously cycling cells, in quiescent G_0 state, MCM and CDC6 are displaced from chromatin and must be reloaded during cell cycle reentry (Madine et al., 2000; Stoeber et al., 1998). Coverley et al. (2002) showed that in cell-free in vitro reconstitution systems, cyclin E activity is critically required for loading of MCM onto replication origins during $G_0 \rightarrow S$ phase transition. Importantly, the work of Coverley et al. (2002) reveals that cyclin E must act before cyclin A to allow normal DNA replication in this setting, and addition of cyclin A to in vitro reactions prior to cyclin E completely blocked MCM loading and subsequent S phase entry. Hence, Coverley et al. (2002) postulate that during normal $G_0 \rightarrow S$ phase progression, a window of opportunity for MCM loading exists, which is opened by cyclin E action and closed as soon as cyclin A appears. We believe that in our E1$^{-/-}$E2$^{-/-}$ cells, this window of opportunity for MCM loading fails to open, due to the absence of E type cyclins, explaining an inability of mutant cells to reenter the cell cycle. These observations, together with normal MCM loading in continuously cycling E1$^{-/-}$E2$^{-/-}$ cells, reveal that the molecular basis of the S phase entry differs, depending on whether cells emerge from the M or G_0 phase.

E Type Cyclins and Endoreplication

In addition to $G_0 \rightarrow S$ transition of mammalian cells, cyclin E was postulated to cause loading of MCM proteins onto DNA replication origins also in another very different setting, namely during endoreplicative cycles of *Drosophila melanogaster* salivary glands. Thus, Su and O'Farrell (1998) observed that MCM2 becomes chromatin bound in less than 8 min after inducible activation of cyclin E in endoreplicating salivary glands and that this relocalization of MCM2 precedes cyclin E-induced S phase entry. Based on these observations, Su and O'Farrell (1998) postulated that in normal endoreplicative cycles, activation of cyclin E triggers MCM relocalization to DNA replication origins. It is very tempting to speculate that both the inability of cyclin E1$^{-/-}$E2$^{-/-}$

cells to reenter the cell cycle and the endoreplicative defect seen in $E1^{-/-}E2^{-/-}$ mice are caused by the same molecular defect, namely defective binding of MCM to replication origins in the absence of cyclin E.

Regardless of the exact molecular mechanism of the endoreplicative defect seen in cyclin E-deficient mice, our results reveal that cyclin E is required for endoreplication in mammalian cells and that this requirement operates in both trophoblast giant cells and in megakaryocytes. Importantly, cyclin E was previously postulated to play key role in endoreplication occurring in *Drosophila* (Duronio and O'Farrell, 1995; Knoblich et al., 1994; Sauer et al., 1995), revealing that this function for cyclin E is conserved during evolution.

E Type Cyclins and Oncogenic Transformation

Our analyses revealed that cyclin E-deficient cells are resistant to transformation by various oncogene pairs. This observation may seem unexpected. However, recent work of Zou et al. (2002) showed that cells lacking CDK4 are also oncogene resistant, but proliferate normally under nononcogenic conditions. The resistance of $CDK4^{-/-}$ cells to oncogenic transformation has been traced to the increased levels of the cell cycle inhibitor $p21^{Cip1}$ in these cells (Zou et al., 2002). Importantly, the levels of $p21^{Cip1}$ and of other cell cycle inhibitors remain unperturbed in cyclin E-deficient cells expressing activated oncogenes, as compared to oncogene-expressing wild-type cells (data not shown). However, we believe that the resistance of cyclin E-deficient and $CDK4^{-/-}$ cells to transformation can be explained by the same mechanistic principle. Thus, we propose that there is a critical threshold for cyclin-associated kinase activities that is required for oncogenic cell cycling. This threshold can be compromised either by decreasing the activity of kinases (by upregulating $p21^{Cip1}$, in the case of $CDK4^{-/-}$ cells) or by eliminating the activators of kinases, cyclins (in the case of $E1^{-/-}E2^{-/-}$ cells).

Amplification of the *cyclin E* gene, located on chromosome 19q12, and overexpression of cyclin E protein are seen very frequently in human malignancies (Donnellan and Chetty, 1999). In the case of breast cancers, elevated levels of cyclin E strongly correlate with poor clinical outcome (Porter et al., 1997). Thus, elucidation of the molecular functions for cyclin E in normal proliferation may help to understand how these functions are subverted in human neoplastic disease.

Experimental Procedures

Generation of Cyclin E1- and E2-Deficient Mice

The cyclin E1 gene-targeting construct was assembled by removing a 10 kb NotI-ScaI fragment of the murine cyclin E1 gene (containing coding exons 2–11) and replacing it with the neomycin phosphotransferase (*neo*) gene. The construct was electroporated into embryonal stem (ES) cells and clones were selected as described (Sicinski et al., 1995). Ten out of 198 ES cell clones analyzed underwent homologous recombination at the cyclin E1 locus. Two recombinant ES cell clones were used to generate cyclin E1-deficient animals.

Cyclin E2 gene-targeting vector was generously provided by Drs. Bruno Amati and Andreas Beck. The construct was modified by replacing a 14kb NotI-SpeI fragment of the *cyclin E2* gene (encoding coding exons 1–8) with the puromycin resistance gene. Such modified gene targeting construct was electroporated into cyclin $E1^{+/-}$ ES cells. Four out of 320 ES cell clones analyzed underwent homologous recombination at the cyclin E2 locus. The recombinant ES cells were then used to generate cyclin E2-deficient and cyclin $E1^{+/-}E2^{+/-}$ animals, using standard procedures (Sicinski et al., 1995).

Histologic Analyses

Organs or embryos were fixed in Bouin's fixative (Sigma) and embedded in paraffin. Histologic sections were stained with hematoxylin and eosin. Sperm counts were performed as described (Searle and Beechey, 1974). The number of sperm cells was expressed as the number of cells per both epididymi.

For quantification of DNA ploidy in trophoblast giant cells, placentas were collected from E10.5 wild-type and mutant embryos, fixed in 4% paraformaldehyde and embedded in paraffin. 5 μm thick sections were cut and stained with Feulgen stain. Four wild-type and four cyclin $E1^{-/-}E2^{-/-}$ placentas were analyzed. Images were quantified using Scion Image software (Scion Corporation, Frederick MD). The ploidy of trophoblast giant cells was expressed relative to diploid decidual cells.

Derivation of Embryonal Stem Cells and Tetraploid Blastocyst Complementation

Cyclin $E1^{-/-}E2^{-/-}$ ES cells were derived from E3.5 blastocysts essentially as described (Hogan et al., 1986). Tetraploid blastocyst complementation was performed as described (Eggan et al., 2001). Briefly, two-cell embryos were collected from B6D2F1 mice and fused with two electrical pulses of 90 V applied for 30 μs to produce one-cell tetraploid embryos. Embryos were then incubated in KSOM media (Specialty Media) for 2 days at 37°C and developed into tetraploid blastocysts. ES cells were injected into tetraploid blastocysts using piezo micromanipulator (Primetech, Japan), and chimeric embryos were implanted into pseudopregnant recipient females for further development.

Isolation, In Vitro Culture, and Analyses of Megakaryocytes

Cells were isolated from E14.5 livers and cultured for 3 days in DMEM 10% fetal bovine serum (Sigma) supplemented with 1% tissue-culture supernatant from a murine c-Mpl ligand-producing cell line (kindly provided by Dr. R. Shivdasani). Megakaryocytes were stained with FITC-conjugated anti-CD41 antibody (PharMingen) together with propidium iodide, and DNA content among CD41-positive cells was determined by flow cytometry.

Magnetic Resonance Imaging

Paraformaldehyde-fixed embryos were embedded in 1% agarose containing 2 mM gadolinium-diethylenetriamine pentaacetic anhydride (Gd-DTPA) and imaged using an 11.7 Tesla vertical magnet interfaced to a Bruker Avance spectrometer as described (Schneider et al., 2003). 3D datasets were analyzed and surface reconstructions generated using Amira 3.0 (TGS Europe).

Western Blotting and Chromatin Binding Analyses

Blots were probed with antibodies against pRB (Pharmingen), cyclin A (Santa Cruz), $p27^{Kip1}$ (Santa Cruz), MCM2 (Pharmingen), CDC6 (Santa Cruz), and ORC2 (kindly provided by Dr. A. Dutta). As secondary antibodies, peroxidase-conjugated IgG (Jackson ImmunoResearch) was used, followed by enhanced chemiluminescence detection (Amersham Pharmacia).

For kinase assays, 100 μg of proteins was incubated with anti-cyclin E antibody or with anti-cyclin A antibody conjugated to agarose beads (Santa Cruz). Immunoprecipitations and kinase reactions were performed as described (Geng et al., 1999).

Separation of proteins into chromatin bound and free fractions was performed using standard methods (Arata et al., 2000; Madine et al., 2000). Briefly, cells were incubated for 15–20 min on ice in 0.1% Nonident P-40-containing CSK buffer (10 mM PIPES [pH 7.0], 100 mM NaCl, 3 mM $MgCl_2$, and 300 mM sucrose). Insoluble fractions were collected by centrifugation at 5000 rpm for 5 min at 4°C and added to SDS sample buffer prior to SDS-PAGE. In each sample analyzed, proteins deriving from 10^6 nuclei were used for Western blot analyses.

Cell Proliferation Analyses and Oncogenic Transformation Assays

Cells were grown in 10% fetal bovine serum in DMEM. For cell cycle reentry, cells were starved in serum-free DMEM for 48–72 hr before being restimulated with 10% fetal bovine serum for indicated times. Alternatively, cells were kept at full confluence for 3 days, released from quiescence by being replated at low density (20%–30% confluent density), and harvested at indicated time points. For growth-curve assays, cells were plated at 3×10^4 cells per well in 12-well plates. At indicated times, cells were quantified.

Oncogenic transformation assays on cell monolayers were performed on freshly explanted (passage zero) MEFs as described (Zou et al., 2002) using the following retroviruses: LXSN vector for coexpression of DNp53 (GSE56) plus H-Ras^{Val12} (kindly provided by Dr. A. Gudkov), pLPC-E1A-ires-H-ras^{Val12} (kindly provided by Dr. S. Lowe), pBabe-puro-H-Ras^{Val12}, and pBabe-hygro-c-Myc. Immortalization with Myc was performed using pBabe-hygro-c-Myc retrovirus as described (Bouchard et al., 1999). Foci and colonies were stained with crystal violet and quantified after 2–3 weeks.

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