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Cdk2 Knockout Mice Are Viable

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Summary

Background: Cyclin-dependent kinases (Cdks) and their cyclin regulatory subunits control cell growth and division. Cdk2/cyclin E complexes are thought to be required because they phosphorylate the retinoblastoma protein and drive cells through the G1/S transition into the S phase of the cell cycle. In addition, Cdk2 associates with cyclin A, which itself is essential for cell proliferation during early embryonic development.
Results: In order to study the functions of Cdk2 in vivo, we generated *Cdk2* knockout mice. Surprisingly, these mice are viable, and therefore *Cdk2* is not an essential gene in the mouse. However, *Cdk2* is required for germ cell development; both male and female $Cdk2^{-/-}$ mice are sterile. Immunoprecipitates of cyclin E1 complexes from $Cdk2^{-/-}$ spleen extracts displayed no activity toward histone H1. Cyclin A2 complexes were active in primary mouse embryonic fibroblasts (MEFs), embryo extracts and in spleen extracts from young animals. In contrast, there was little cyclin A2 kinase activity in immortalized MEFs and spleen extracts from adult animals. $Cdk2^{-/-}$ MEFs proliferate but enter delayed into S phase. Ectopic expression of Cdk2 in $Cdk2^{-/-}$ MEFs rescued the delayed entry into S phase.
Conclusions: Although *Cdk2* is not an essential gene in the mouse, it is required for germ cell development and meiosis. Loss of Cdk2 affects the timing of S phase, suggesting that Cdk2 is involved in regulating progression through the mitotic cell cycle.

Introduction

Cyclin-dependent kinases (Cdks) are master regulators of eukaryotic cell proliferation (for review see [1]). Cdks are activated by binding to cyclin regulatory subunits. More than ten different Cdks and cyclins have been identified. These are expressed in different combinations during specific phases of the cell cycle. The D-type cyclin-dependent kinases Cdk4 and Cdk6, whose expression is modulated by growth-stimulatory signals, function in early to middle G1 phase. They are followed by the activation of Cdk2 in complex with cyclin E during late G1. Cdk2/cyclin A complexes function in S phase, whereas Cdc2/cyclin B and A complexes promote the G2/M transition. In general, regulation of the Cdk activity requires the interplay among the phosphorylation by activating kinases (CAK, for review see [2]), their association with the cyclins, and the Cdk inhibitors (Cki). There are two Cki families: the Ink4 family ($p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$), whose members bind only to Cdk4 and Cdk6, and the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$), whose members bind all Cdks (for review see [3]). In addition, all Cdk/cyclin/Cki complexes are affected by transcriptional regulation, subcellular localization, and ubiquitin-mediated degradation, all of which influence the progression through the cell cycle. Although we possess a wealth of knowledge about the individual components of this regulatory network, in vivo investigations of the cell cycle regulators remain necessary if we are to fully understand their function and identify potential overlapping pathways.

Cell cycle regulatory proteins were thought to be essential for mouse viability because of their crucial roles in cell division. However, in vivo targeted disruption of mouse genes encoding Cdk4 [4, 5], cyclin D1 or D2 [6–8], cyclin B2 [9], cyclin A1 [10], members of the Cip/Kip family [11–15], or members of the Ink4 family [16–19] have not resulted in lethality. Developmental defects occur in some of the mouse models, indicating that these regulatory proteins are important but not essential for viability. In contrast, cyclin A2 [20] and B1 [9] inactivation causes embryonic lethality; demonstrating that these cyclins mediate essential functions in the development of the mouse.

Using transformed cell lines and purified proteins, researchers have been able to study Cdk2 extensively in the last decade. From these experiments, it was suggested that Cdk2 orchestrates several cell cycle events (for review see [1]) such as G1/S transition, progression in S phase through initiation and maintenance of DNA replication, exit from S phase, progression into G2, and maybe entry into mitosis [21]. The importance of Cdk2 in the regulation of the cell cycle has been demonstrated by the expression of a dominant-negative Cdk2 mutant (D145N) that induces a G1 block in human osteosarcoma cell lines [22]. This block is rescued by coexpression of wild-type Cdk2 or cyclin D1. Furthermore, overexpression of the Cdk2 inhibitor $p27^{Kip1}$ or microinjection of antibodies against Cdk2, cyclin E, or cyclin A causes cell cycle arrest [23, 24] and blocks initiation of DNA synthesis in mammalian cells [23, 25]. The most established function of Cdk2 is related to progression from G1 to the S phase, which requires the sequential phosphorylation of the retinoblastoma protein (Rb) by Cdk4/Cdk6 and Cdk2 [26–29]. In its hypophosphorylated state, Rb sequesters the E2F family of transcription factors [28]. After phosphorylation of Rb by Cdk4/6 and Cdk2, E2F proteins are released and promote transcriptional activation of genes required for entry into S phase. In addition, Cdk2 mediates its functions through phosphorylation of many substrates, including targets that trigger the firing of replication origins [30–35], regulate centrosome duplication [36, 37], p53 [38], and the Cdk

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inhibitor $p27^{Kip1}$ [39–41]. Phosphorylation of p27 on threonine 187 leads to the degradation of p27 and allows full activation of Cdk2/cyclin E complexes. Although these data suggest essential functions for Cdk2 in cell cycle progression, it is unclear whether Cdk2 is essential in vivo.

Recently, it has been reported that human colon cancer cell lines proliferate normally after depletion of Cdk2 [42], indicating that Cdk2 is not required for cell cycle progression in immortalized human cell lines. The closest homolog of Cdk2 is Cdk3 [43]. Like *Cdc2* and *Cdk2*, *Cdk3* can complement *cdc28* temperature-sensitive mutations in budding yeast [43–46]. Expression of a dominant-negative mutant of human Cdk3 blocks cells in G1 phase [22], and Cdk3/cyclin E complexes can promote S phase entry in quiescent cells as efficiently as can Cdk2/cyclin E complexes [47]. Nevertheless, a non-sense mutation in the *Cdk3* gene has been reported from most *Mus musculus* strains commonly used in the laboratory [48], and this mutation prevents expression of the Cdk3 protein in the mouse. Therefore, Cdk3 cannot compensate for the loss of Cdk2 in the mouse.

We generated *Cdk2* knockout mice with a targeted disruption of the kinase domain to determine Cdk2 functions in the context of a living animal. Here we describe the analysis of phenotypes of *Cdk2* null mice and embryonic fibroblasts (MEFs) derived from them. $Cdk2^{-/-}$ mice were unexpectedly viable and developed normally, but they were sterile and slightly smaller. Primary $Cdk2^{-/-}$ MEFs were able to proliferate but displayed a delay in entry into S phase. Cyclin E1 complexes did not display kinase activity toward histone H1 in $Cdk2^{-/-}$ spleen extracts from adult animals. Cyclin A2 complexes were active in primary MEF, embryo, and spleen extracts from young animals, but there was little activity in immortalized MEF or spleen extracts from adult animals. Our results suggest that Cdk2 is essential for meiosis and involved in maintaining normal proliferation in primary MEFs.

Results

Generation of Mice Lacking *Cdk2*

To establish the in vivo role of Cdk2 in control of cell proliferation, we used gene-targeting techniques to generate *Cdk2*-deficient mice. We functionally inactivated the *Cdk2* gene in ES cells by homologous recombination. In our targeting vector, we replaced exons 4 and 5, encoding the core kinase domain of Cdk2, with a PGK-neomycin cassette to render Cdk2 nonfunctional (Figure 1A). The complete *Cdk2* locus (12.6 kb) was retrieved from a bacterial artificial chromosome [49, 50]. This retrieval occurred by recombination between the extremities of the C*dk2* locus and the retrieval vector, containing two short *Cdk2* homology arms (70 base oligonucleotides), in the context of *E. coli* deficient for the recBCD nuclease. Exons 4 and 5 were replaced by a neomycin cassette according to the same strategy. After electroporation and double selection with G418 and gancyclovir, two independent embryonic stem (ES) cell lines that had the *Cdk2* locus correctly targeted were identified. We used these lines to generate chimeric animals. Male chimeras were mated with wild-type C57BL/6 females, and their offspring were analyzed for *Cdk2* recombination via PCR and Southern blotting (Figure 1B).

After successfully targeting the *Cdk2* locus, we aimed to verify the absence of *Cdk2* mRNA and protein in $Cdk2^{-/-}$ mice. We analyzed *Cdk2* transcripts from thymus by Northern blots. The insertion of the neomycin cassette prevented the expression of wild-type transcripts in thymus of $Cdk2^{-/-}$ animals (Figure 1C, left panel); nevertheless, we cannot exclude the possibility that the neomycin cassette might affect the expression of neighboring genes of the *Cdk2* locus. We also performed RT-PCR on RNA from mouse embryonic fibroblasts (MEFs). The full ORF of the *Cdk2* cDNA was amplified from wild-type but not $Cdk2^{-/-}$ MEFs (Figure 1C, right panel). We observed the same result when we used a reverse primer anchored in the deleted exon 4. We also investigated the expression of *Cdc2* by Northern blot and by RT-PCR. We found a similar level of expression in tissues from animals of both genotypes, suggesting that *Cdc2* RNA expression was not affected by inactivation of *Cdk2*. Western blot analysis corroborated that the Cdk2 protein was not expressed in tissues obtained from $Cdk2^{-/-}$ mice (see Figure 3A). Nevertheless, a small *Cdk2* transcript, encompassing exon 1 to exon 3, was detectable by RT-RCR in wild-type and knockout animals (Figure 1C, right panel) although we were unable to detect any truncated Cdk2 protein (data not shown). Our data suggest that $Cdk2^{-/-}$ mice do not express Cdk2 or its closest homolog, Cdk3, the two cyclin-dependent kinases implicated in the control of the G1/S transition and S phase of the cell cycle.

Phenotypic Analysis of *Cdk2* Knockout Mice

To assess the role of *Cdk2* in normal development, we intercrossed $Cdk2^{+/-}$ mice. In the mixed strain background (129S1/SvImJ × C57BL/6), *Cdk2*-deficient mice were born and viable (Figure 1D); however, the frequency was slightly below the Mendelian ratio. From a total of 234 animals, with a normal male:female ratio (54:46), we observed 45 homozygote $Cdk2^{-/-}$ mice (19.2%) compared to 78 wild-type (33.3%) and 111 heterozygote (47.5%) siblings ($\chi^2 = 9.92$, p value < 0.01). Inactivation of *Cdk2* may cause prenatal lethality at low penetrance, although at birth homozygous *Cdk2* null animals did not differ in any obvious way from their littermates. In spite of the widespread expression of *Cdk2* during embryogenesis (data not shown), its loss did not affect fetal development in the mixed background 129S1/SvImJ × C57BL/6. Survival of $Cdk2^{-/-}$ mice was not reduced; however, after weaning they were slightly smaller than wild-type littermates (Figure 1D, left panel and data not shown). Nevertheless, $Cdk2^{-/-}$ animals developed normally, and an anatomical and histological examination of the mutant mice revealed no obvious malformations (data not shown), with the noticeable exception of the gonads (Figure 1D, right panel). In fact, breedings of $Cdk2^{-/-}$ males and females led to vaginal plugs, suggesting a normal mating behavior, but pregnancies did not occur. The same observations were made for matings between wild-type and homozygote mutant mice, indicating that both $Cdk2^{-/-}$ males and females are sterile. Indeed, a severe atrophy of the gonads was observed even before sexual maturity and became more severe in adult animals (ovaries are shown in Figure 1D; see also Figure 2).

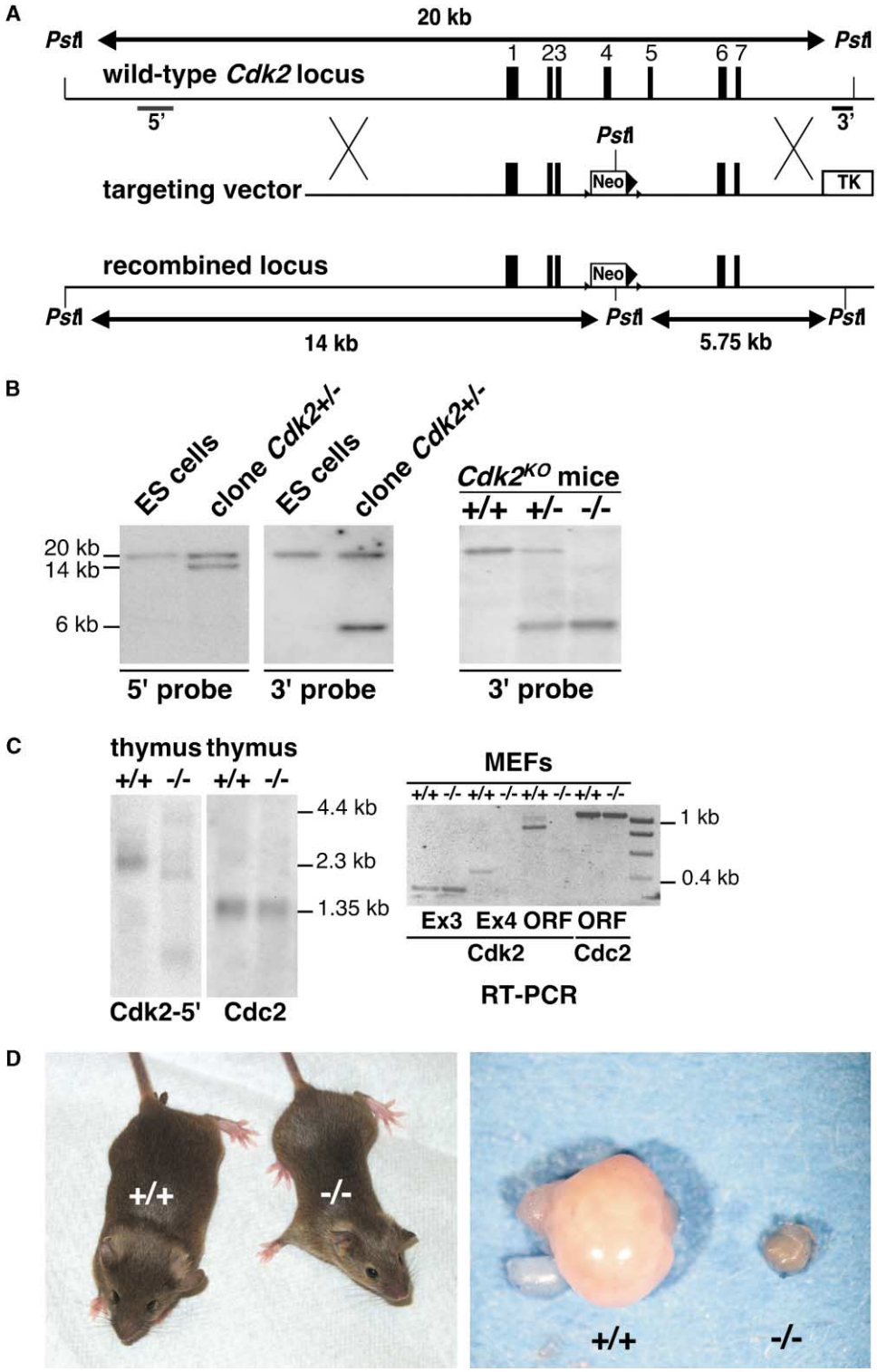

Figure 1. Generation of *Cdk2*$^{-/-}$ Mice and *Cdk2* Expression Analysis

The *Cdk2* gene was inactivated in ES cells via the targeting construct shown in (A). This strategy introduces a neomycin cassette in place of exons 4 and 5. The PGK-TK was inserted at the 3′ end of the targeting vector. 5′ and 3′ probes, located outside of the targeting vector, were used for Southern blot analysis with a PstI digest. Southern blot analysis of recombined ES clones and offspring mice from a chimera is shown in (B). Northern blots ([C], left panel) were performed on RNA from wild-type or *Cdk2*$^{-/-}$ thymus with a Cdk2-5′ probe (partial *Cdk2* cDNA encoded by exon 1 to exon 3) and Cdc2 probe (full-length ORF). RT-PCR analyses ([C], right panel) were performed from RNA of MEFs with *Cdk2*- or *Cdc2*-specific oligonucleotides (*Cdk2* amplification: exon 1 to exon 3, exon 1 to exon 4, or full ORF; *Cdc2* amplification: full-length ORF). *Cdk2*$^{-/-}$ mice are viable and develop normally, although adult animals appear to be slightly smaller than their littermates ([D], left panel). Ovaries from wild-type and *Cdk2*$^{-/-}$ animals are shown in (D) (right panel).

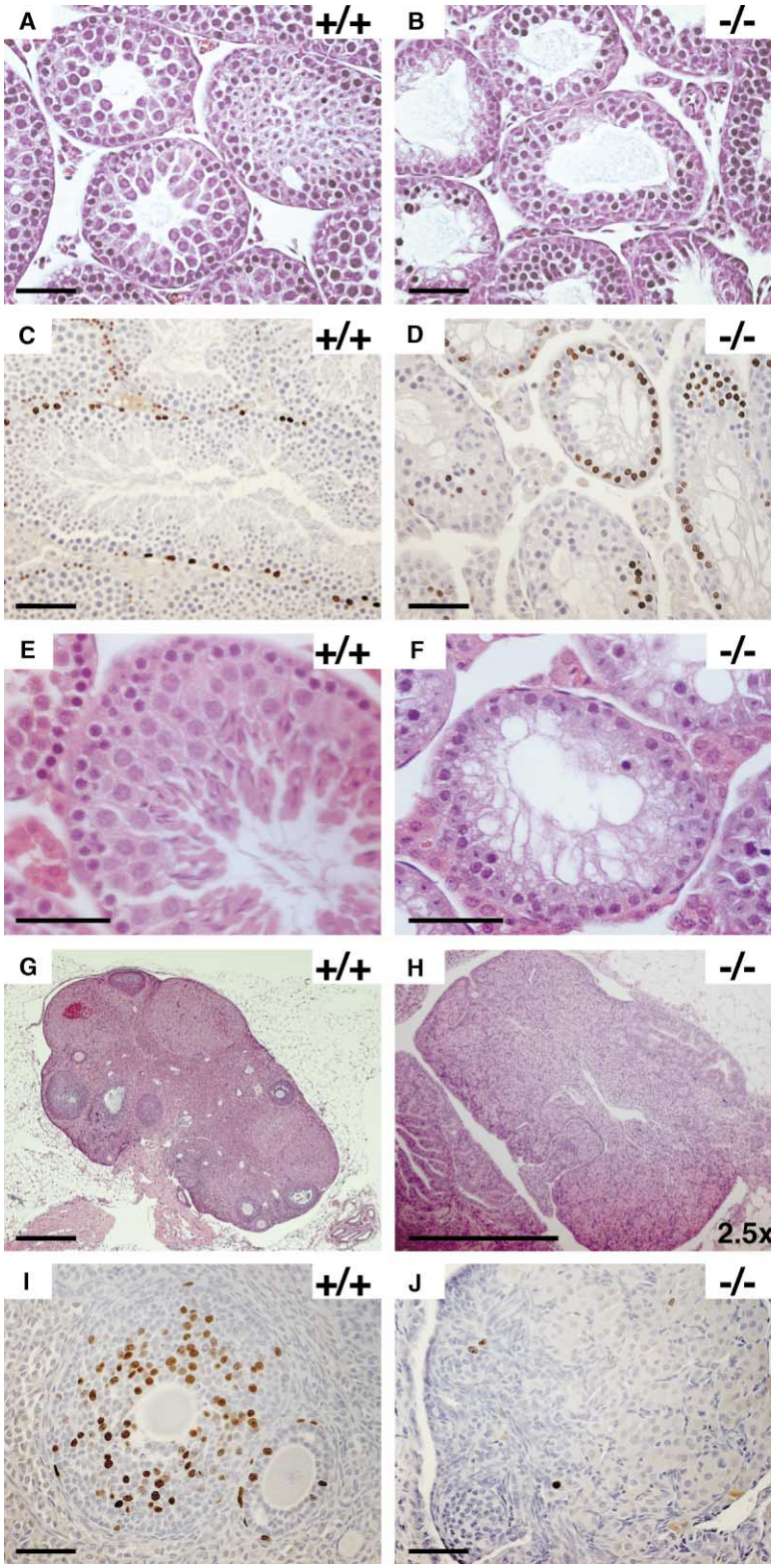

Figure 2. Abnormal Germ Cell Development in *Cdk2*$^{-/-}$ Mice

Histological sections of gonads were stained with hematoxylin and eosin. The males were 3 weeks old (A and B) or 3 months old (C–F), and the females were 3 months old (G–J). Tissues were fixed in 4% paraformaldehyde for 24 hr prior to being processed and embedded in paraffin wax. Wild-type seminiferous tubules with normal germ cell differentiation are shown in (A, C, and E). *Cdk2*$^{-/-}$ seminiferous tubules are smaller than the wild-type and show defective germ cell development (B, D, and F). Post-meiotic cells (late spermatocytes, spermatids, and spermatozoa) are absent. In vivo BrdU staining is shown in (C) and (D). Wild-type ovaries with follicles are shown in (G) and (I). *Cdk2*$^{-/-}$ atrophic ovaries display a lack of follicle development (H and J). In vivo BrdU staining is shown in (I) and (J). The scale bar represents 50 μm for (A–F) and (I–J) and 500 μm for (G) and (H).

Defective Differentiation of Male and Female Germ Cells

To determine the causes of the sterility in *Cdk2*$^{-/-}$ males, we performed a histological analysis in sexually immature and mature male animals (Figures 2A–2F). Male germ cell development begins at the periphery of the seminiferous tubule where spermatogonial stem cells reside. These mitotic self-renewal cells differentiate progressively to spermatocytes, where meiosis occurs, and to spermatids that colonize the more-internal tubular

layers. Ultimately, spermatids yield mature spermatozoa that can be visualized in the lumina of the seminiferous tubule ([51] and references therein). We observed that the seminiferous tubules underwent an overall size reduction resulting from a large depletion of germ cells (Figures 2A/C/E versus 2B/D/F). Spermatozoa were completely absent in the lumina, as in epididymis, consistent with the sterility of these *Cdk2*$^{-/-}$ animals. The spermatogonia were present, and it seemed that Sertoli cells were not affected by the inactivation of *Cdk2*. In accordance with these observations, the inactivation of *Cdk2* induced a defect in the differentiation of male germ cells in young animals. This phenotype was already observed in three-week-old males (Figure 2A versus 2B), showing that the first wave of germ line differentiation did not take place and therefore that germ cells did not enter meiosis in *Cdk2* null males. The proliferation as measured by in vivo BrdU incorporation was similar in wild-type and *Cdk2*$^{-/-}$ testis (Figures 2C and 2D), corroborating that germ cells are affected after spermatogonia proliferation when meiosis occurs.

Using the same method, we analyzed ovaries to determine if oogenesis took place in *Cdk2*$^{-/-}$ females. Female germ cells proliferate through mitotic cell cycles before they enter meiosis during embryonic development and arrest in prophase of meiosis I. These oocytes remain arrested until puberty, when a pool of oocytes is recruited to grow and complete the first meiotic division, which is followed by a second meiotic round, when fertilization takes place [52]. We analyzed histological sections of ovaries from three-month-old *Cdk2*$^{-/-}$ and wild-type females (Figures 2G–2J). In wild-type ovaries, we observed a normal development of oocytes and different stages of follicles (Figures 2G and 2I). In *Cdk2*$^{-/-}$ ovaries, we did not observe any primordial follicles, and apparently the oocytes did not develop in these atrophic ovaries (Figure 2H). BrdU staining was only detected in wild-type (Figure 2I) and not in *Cdk2*$^{-/-}$ ovaries (Figure 2J). The absence of primordial follicle differentiation in *Cdk2* null animals suggests a block during embryonic development prior to oocyte differentiation. An atrophy of the uterus was also observed, possibly as a result of the absence of ovary development, and may be linked to a hormonal deficit (data not shown).

Lack of Cyclin E1 Activity in *Cdk2*$^{-/-}$ Spleen Extracts

In order to determine the impact of *Cdk2* inactivation and to seek an explanation for the unexpected viability of the *Cdk2*$^{-/-}$ mice, we analyzed the expression of several cell cycle regulators in a range of adult tissues (Figure 3). We performed Western blots by using antibodies against Cdks, cyclins, or Ckis. The Cdk2 protein was expressed at high levels in thymus and spleen and at low levels in kidney and lung but was not detectable in brain and liver (Figure 3A, top panel). In contrast, Cdk2 was not expressed in all tissues from *Cdk2*$^{-/-}$ mice (Figure 3A). The expression of Cdks (Cdk4, Cdk6, and Cdc2) was similar in wild-type and *Cdk2*$^{-/-}$ tissues, with the exception of lower levels of Cdk6 in kidney from *Cdk2*$^{-/-}$ mice (Figure 3A, lanes 7 and 8). Cyclin E1 was expressed at high levels in brain and at lower levels in thymus from wild-type and *Cdk2*$^{-/-}$ extracts, whereas cyclin A2 was detectable in liver and kidney and moderately in lung from both types of extracts. Neither cyclin E1 and nor cyclin A2 exhibited any difference in its expression pattern as a result of the loss of Cdk2 (Figure 3A). Similarly, p27, an inhibitor of Cdk2/cyclin E complexes, was expressed at high levels in all tissues except kidney in both genotypes. The p27 protein has to be phosphorylated by Cdk2 on threonine 187 to be degraded, thus allowing full activity of Cdk2/cyclin E complexes [39–41]. We used a phospho-specific antibody (see Supplemental Experimental Procedures available with this article online) to detect the T187-phosphorylated form of p27 and observed an equivalent level of phosphorylation of p27 in wild-type and *Cdk2*$^{-/-}$ tissues (Figure 3A, bottom panel). This is in agreement with our findings that the levels of p27 were similar in both types of tissues and indicated that other kinases can phosphorylate p27^{T187}.

Protein levels may not solely reflect the activity of Cdk/cyclin complexes. Therefore, we performed immunoprecipitations from spleen extracts, a tissue showing a high expression of Cdk2 (Figure 3A, lane 3), and analyzed the kinase activity (Figure 3B) and the binding partners of Cdk2 (Figure 3C). We analyzed the activity of Cdks by using in vitro kinase assays and the substrate histone H1. Immunoprecipitation of Cdk2 yielded high Cdk2 activity from wild-type but not from *Cdk2*$^{-/-}$ spleen lysates (Figure 3B, lane 1 versus lane 2) from adult animals. Similarly, the kinase activity associated with cyclins E1 and A2 was detectable in wild-type but not in *Cdk2*$^{-/-}$ spleen extracts from adult mice (Figure 3B, lane 3 versus lane 4, lane 5 versus lane 6, and lane 13 versus lane 14). The absence of cyclin E1 and A2 activity suggested that Cdk2 is the main kinase that binds cyclins E1 and A2, at least in spleen tissue from adult mice. However, cyclin A2 activity was detected in *Cdk2*$^{-/-}$ embryo extracts (11.5 days post-coitum [dpc]), spleen extracts from young animals (Figure 3B, lanes 10 and 12), and in primary *Cdk2*$^{-/-}$ MEFs (see Figure 4D). Therefore, cyclin A2 activity is present in embryonic tissues, corroborating its being essential for embryonic development [20]. We observed a similar level of Cdc2 (Figure 3B, lanes 7 and 8) and cyclin B1 (data not shown) activity in both wild-type and *Cdk2*$^{-/-}$ spleen extracts, indicating that the Cdc2 activity was unchanged in the absence of Cdk2 activity. After immunoprecipitation with Cdk2, cyclin E1, and cyclin A2 antibodies, as well as immunoblotting for Cdk2, we detected the Cdk2 protein only in wild-type samples (Figure 3C and data not shown). These results are in agreement with our observations of the kinase activities. The Cdc2 protein was detectable after Cdc2 immunoprecipitation in wild-type and *Cdk2*$^{-/-}$ spleen (Figure 3C), confirming that Cdc2 protein levels and activity were similar in wild-type and *Cdk2*$^{-/-}$ spleen extracts. Cdc2 was immunoprecipitated by cyclin A antibodies in wild-type spleen extracts from young and adult animals. In *Cdk2*$^{-/-}$ adult spleen extract, the amount of Cdc2 bound to cyclin A was clearly reduced (Figure 3C, right panel).

These results indicated that the in vivo expression of all the cell cycle regulators tested was not affected by the loss of Cdk2. Moreover, the absence of Cdk2 activity

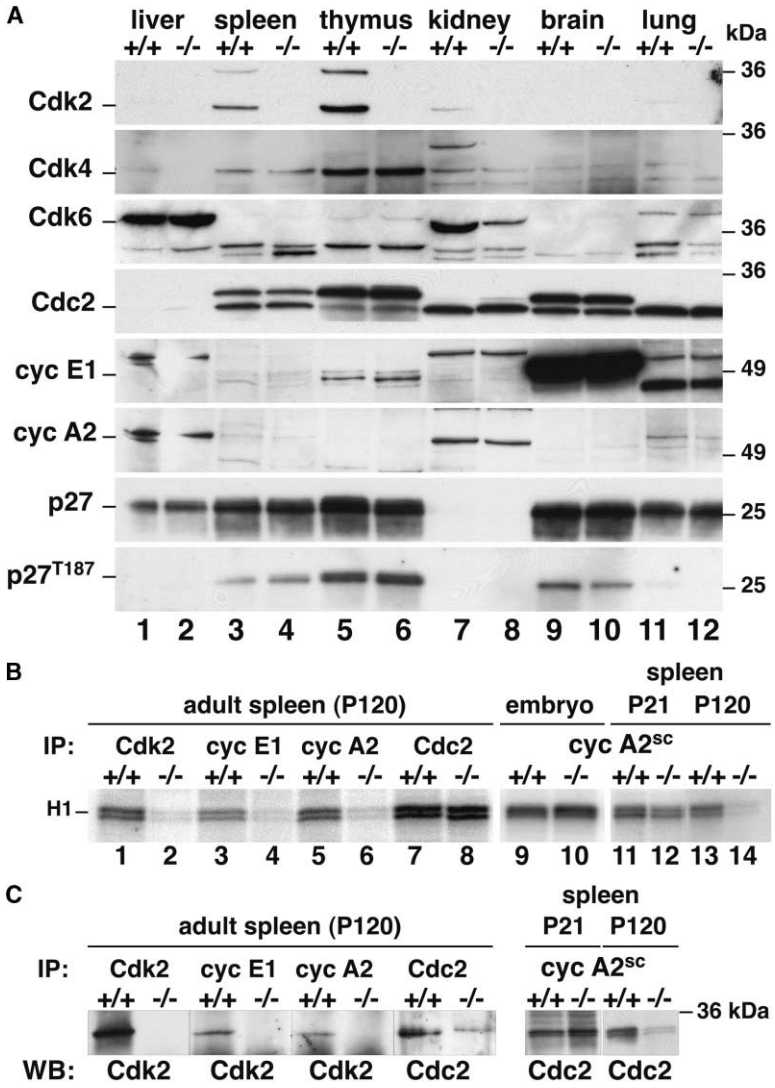

Figure 3. Expression of Cell Cycle Regulators in *Cdk2*$^{-/-}$ Tissues

Western blot analysis of Cdks, cyclin E1, and cyclin A2 as well as p27 expression were performed with 20 μg protein lysates from various adult tissues of wild-type or *Cdk2*$^{-/-}$ mice (A). Cdk2/Cdc2-associated histone H1-kinase activity was measured in wild-type or *Cdk2*$^{-/-}$ spleen extracts from young and adult animals or embryo extracts (11.5 dpc) after immunoprecipitation with indicated antibodies and as described in the Supplemental Experimental Procedures (B). Immunoprecipitations of Cdk2, cyclin E1, cyclin A2, cyclin A2^{sc} (Santa Cruz H-432), and Cdc2, as well as subsequent Western blot analysis with antibodies against Cdk2 and Cdc2 are shown in (C).

did not affect p27^{T187} phosphorylation, which occurs in the G1/S phases, or the Cdc2 activation, which usually happens in the G2/M phases of the cell cycle. Nevertheless, cyclin A2 activity was present in extracts from embryos but not from adult spleen. Therefore, we aimed to investigate the transitions between the cell cycle phases in more detail, and we addressed this question in vitro by using mouse embryonic fibroblasts derived from wild-type and *Cdk2*$^{-/-}$ embryos.

Proliferation of *Cdk2*$^{-/-}$ Mouse Embryonic Fibroblasts

We generated fibroblasts from *Cdk2*$^{-/-}$ mouse embryos (13.5 dpc) and from wild-type littermate embryos to study the proliferation of *Cdk2*$^{-/-}$ MEFs. Cultures of primary *Cdk2*$^{-/-}$ MEFs were morphologically indistinguishable from those of wild-type MEFs. We plated 10,000 cells from both genotypes in 24-well plates and counted them daily without passaging them. During the first 4 days of culture, both *Cdk2*$^{-/-}$ and wild-type MEFs displayed similar patterns of proliferation. From day 4 to 6, wild-type MEFs started to proliferate more quickly than *Cdk2*$^{-/-}$ MEFs (Figure 4A). The number of cells remained constant for *Cdk2*$^{-/-}$ MEFs (entered plateau phase) one day earlier than for wild-type MEFs, indicating that contact inhibition in *Cdk2*$^{-/-}$ MEFs than in wild-type MEFs might occur at lower cell densities. This result suggests that embryonic fibroblasts continue to proliferate in the absence of Cdk2.

Cell Cycle Kinetics of Unsynchronized Primary Fibroblasts

In order to gain insight into the mechanisms underlying the proliferation in *Cdk2*$^{-/-}$ fibroblasts, we studied the cell cycle kinetics of primary unsynchronized MEFs 2 days after seeding (Figure 4B). We measured the proportion of cells in S phase by pulse labeling cells with 100 μM BrdU 1 hr before harvesting the cells. In proliferating populations of *Cdk2*$^{-/-}$ MEFs, 28% of cells were in S phase of the cell cycle, as indicated by BrdU incorporation, whereas in wild-type MEFs 25% were in S phase (Figure 4B). The percentage of cells in both G1 and G2/M was also similar in both genotypes. Therefore, the same number of cells were proliferating, corroborating that primary MEFs lacking Cdk2 proliferate without major defect.

To further characterize the proliferation of wild-type

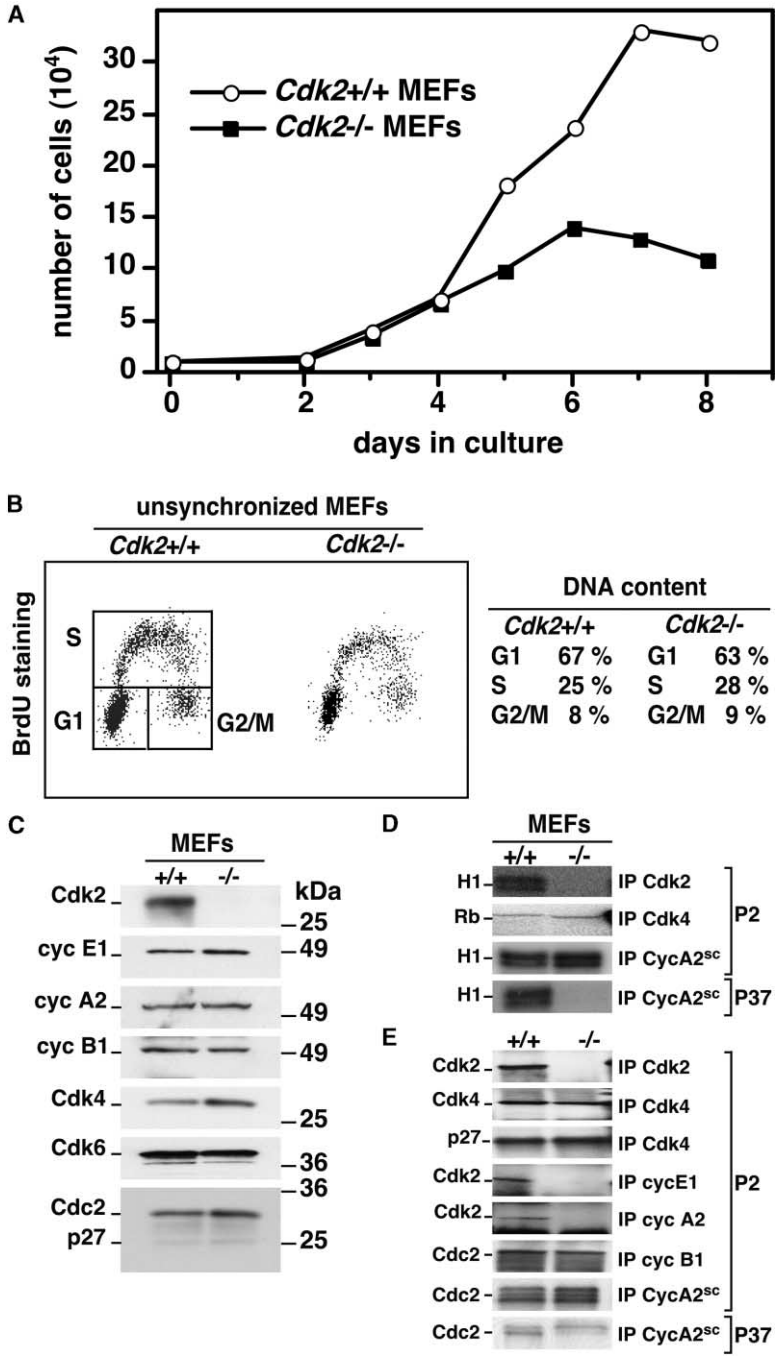

Figure 4. Proliferation Analysis in Mouse Embryonic Fibroblasts

Growth curves of wild-type and *Cdk2*$^{-/-}$ MEFs at early passage are shown in (A). Ten thousand cells were seeded in each well and counted every day. The number of cells at each time point represents the average of triplicate wells. The data shown represent the average of nine *Cdk2*$^{-/-}$ MEF clones from three different litters. Cell cycle analysis of unsynchronized MEFs by flow cytometry is shown in (B). Cells were pulse labeled with BrdU for 1 hr at day 2 after being seeded and were stained with propidium iodide. Data represent averages of two experiments. Western blot analysis of the expression of cell cycle proteins in unsynchronized MEFs is shown in (C). Cdk-associated kinase activity in unsynchronized MEFs is shown in (D). Cdk2-, Cdk4-, or cyclin A-associated protein complexes were immunoprecipitated from cell lysates, and activity toward histone H1 or Rb was measured in vitro as described in the Supplemental Experimental Procedures. MEF lysates were immunoprecipitated (E) with antibodies against Cdk2 (top panel), Cdk4 (second and third panels from top), cyclin E1 (fourth panel from top), cyclin A2 (first, second, and fourth panels from bottom), and cyclin B1 (third panel from bottom), and immunoblotted with antibodies against Cdk2 (first, fourth, and fifth panels from top), Cdc2 (bottom three panels), Cdk4 (second panel from top), or p27 (third panel from top).

and *Cdk2*$^{-/-}$ MEFs, we analyzed the expression of cell cycle regulatory proteins (Figure 4C) and their kinase activities (Figure 4D). We used primary wild-type and *Cdk2*$^{-/-}$ MEF extracts to perform Western blots by using specific antibodies against Cdks, cyclins, and p27 (Figure 4C). Protein levels of Cdc2, Cdk4, Cdk6, p27, cyclin A2, cyclin E1, and cyclin B1 were similar in primary *Cdk2*$^{-/-}$ MEFs and wild-type MEFs.

To measure the kinase activity of Cdks, we performed immunoprecipitations, followed by kinase assays with the substrates histone H1 or Rb (Figure 4D). Cdk2 activity was detected in primary wild-type MEFs (Figure 4D, top panel) but not in *Cdk2*$^{-/-}$ MEFs, corroborating our results from spleen extracts (see Figure 3B) and embryo extracts (data not shown). Cyclin A2 activity was detected in primary *Cdk2*$^{-/-}$ MEFs (Figure 4D, second panel from bottom) but not in immortalized *Cdk2*$^{-/-}$ MEFs (Figure 4D, bottom panel). The kinase activity of Cdk4 toward Rb was similar in both *Cdk2*$^{-/-}$ and wild-type MEFs (Figure 4D, second panel from top). These results suggested that *Cdk2*$^{-/-}$ MEFs are able to proliferate without increased Cdk4 activity.

In addition, we investigated the composition of the Cdk/cyclin/Cki complexes in lysates prepared from wild-type and *Cdk2*$^{-/-}$ MEFs to determine the impact of the absence of Cdk2. We performed immunoprecipita-

tions with lysates from both genotypes and antibodies against Cdk2, cyclin E1, cyclin A2, cyclin B1, and Cdk4, then performed Western blots (Figure 4E). Cdk2 was immunoprecipitated with Cdk2 antibodies in wild-type but not in *Cdk2*$^{-/-}$ MEFs (Figure 4E, top panel). We obtained the same result when we used immunoprecipitates of cyclin E1 or A2 (Figure 4E, fourth and fifth panel from top). Cdc2 was bound to cyclin A2 in primary and immortalized MEFs (Figure 4E, bottom two panels). Immunoprecipitation with antibodies against Cdk4 pulled down similar amounts of Cdk4 protein from wild-type and *Cdk2*$^{-/-}$ MEFs (Figure 4E, second panel from top). In addition, we pulled down similar amounts of p27 in the Cdk4 immunoprecipitates for each genotype (Figure 4E, third panel from top). This indicated that in the absence of Cdk2, the same amount of p27 is bound to Cdk4. Finally, similar levels of Cdc2 were observed in both wild-type and *Cdk2*$^{-/-}$ MEFs after immunoprecipitation with antibodies against cyclin B1 (Figure 4E, third panel from bottom).

Taking these results together, we found that wild-type and *Cdk2*$^{-/-}$ MEFs express similar levels of cell cycle regulators. The loss of Cdk2 affected neither the amount of p27 bound to Cdk4 nor the formation of cyclin A2/Cdc2 and cyclin B1/Cdc2 complexes.

Loss of Cdk2 Affects Timing of S Phase Entry

Unsynchronized cells do not reveal the timing of cell cycle progression; therefore, we synchronized our primary MEFs by serum starvation to study the impact of loss of Cdk2 on the G1/S transition. MEFs were plated at high densities (3–4 $\times$ 10^6 cells/100 mm-dish), starved for 72 hr by culturing in medium supplemented with 0.1% FBS, and then released by the addition of 10% FBS. Cells were harvested at the indicated time points after serum stimulation. In addition, cells from both *Cdk2*$^{-/-}$ and wild-type MEFs were pulse labeled with BrdU for 1 hr and then subjected to flow cytometry (Figures 5A and 5B). Wild-type MEFs started to enter S phase by 12 hr, whereas *Cdk2*$^{-/-}$ cells started to enter into S phase between 14 and 15 hr (Figure 5B, left panel). By 18 hr, a good percentage of wild-type MEFs were in S phase, whereas *Cdk2*$^{-/-}$ MEFs required approximately 4 hr more. These results suggest that the absence of Cdk2 delayed the G1/S transition by at least 4 hr or affected the total number of cells entering S phase. We confirmed this observation by examining the BrdU incorporation during the 12–15 hr timeframe in 1 hr increments after serum stimulation (data not shown). In order to investigate if the S phase delay is due to the loss of Cdk2, we reintroduced Cdk2-HA into *Cdk2*$^{-/-}$ MEFs by retroviral infection (see Supplemental Experimental Procedures) and synchronized the cells as described above. Expression of Cdk2-HA resulted in approximately twice the number of cells entering S phase as did so in *Cdk2*$^{-/-}$ MEFs (Figure 5B, right panel), indicating that Cdk2 is involved in regulating the timing of S phase entry.

We performed kinase assays by using histone H1 as a substrate, after immunoprecipitation with antibodies against Cdk2, cyclin A2, or cyclin B1. Cdk2 kinase activity toward histone H1 was detected after 18 hr and increased up to the 24 hr time point (Figure 5C, top panel, lanes 1–7). The increase of Cdk2 activity coincided with the entry into S phase (see Figures 5A and 5B). Cdk2 activity was not detected in *Cdk2*$^{-/-}$ MEFs (Figure 5C, top panel, lanes 8–14). The cyclin A2 activity followed the same pattern as Cdk2 in the wild-type MEFs (Figure 5C, middle panel, lanes 1–7), but there was little cyclin A2 activity in the *Cdk2*$^{-/-}$ MEFs (Figure 5C, middle panel, lanes 8–14). However, after 34 hr (data not shown), cyclin A2 kinase activity was increased to levels similar to those shown in Figure 4D (second panel from bottom). The cyclin B1/Cdc2-associated kinase activity followed a pattern similar to that of the Cdk2 activity in wild-type MEFs (Figure 5C, bottom panel, lanes 1–7). In *Cdk2*$^{-/-}$ MEFs, the cyclin B1/Cdc2 activity was slightly lower than in wild-type MEFs (Figure 5C, bottom panel, lanes 8–14), which is most likely a result of the S phase delay. In addition, as compared to wild-type MEFs, the *Cdk2*$^{-/-}$ MEFs were spontaneously immortalized in a 3T3 protocol after a delay of approximately ten passages (data not shown). Similar results have been recently reported for *Cdk2*$^{-/-}$ MEFs [53] and for *cyclin E1*$^{-/-}$*cyclin E2*$^{-/-}$ MEFs [54].

From our in vitro studies on primary MEFs, we demonstrated that the loss of Cdk2 has little effect on proliferation but that cells are delayed in their entry into S phase. This phenotype was rescued by ectopic expression of Cdk2 in the *Cdk2*$^{-/-}$ MEFs. Further investigations will help to link the in vitro phenotype to the in vivo defects of germ cell development.

Discussion

The goal of the present study was to determine the functions of Cdk2 in vivo. We have generated mice lacking *Cdk2* and found that they are viable. Loss of Cdk2 caused sterility and slightly decreased body weight in adult mice. Moreover, embryonic fibroblasts generated from *Cdk2*$^{-/-}$ embryos proliferated almost normally but displayed a delay in S phase entry. The phenotype of our *Cdk2*$^{-/-}$ mice is very similar to the one described when exons 2 and 3 are conditionally removed with the Cre/loxP system [53].

Mice lacking *Cdk2* (in a *Cdk3*$^{-/-}$ background) developed normally, indicating that Cdk2 is not required for embryonic development in the mouse. Similarly, mice lacking important cell cycle regulators (Cdks/cyclins/Ckis) are viable (see Introduction and, for review, see [55]). This indicates that other mechanisms can compensate for the key cell cycle regulators, including both Cdk2 and Cdk3. However, all of these knockout mice exhibit various phenotypes in adult mice, demonstrating that the ablated gene is required in specific tissues.

Cdk2 and Cdk4 share common features, such as that they phosphorylate Rb and they bind to p27^{Kip1}. We have shown in the present study that p27 is phosphorylated on threonine 187, which is known to be a site for Cdk2, in wild-type and *Cdk2*$^{-/-}$ tissues (Figure 3A). Therefore, kinases other than Cdk2 are able to phosphorylate p27 on threonine 187. The most likely candidate is Cdc2 because it displays substrate specificity similar to that of Cdk2 in vitro, although other kinases cannot be excluded at this time. This phosphorylation leads to the

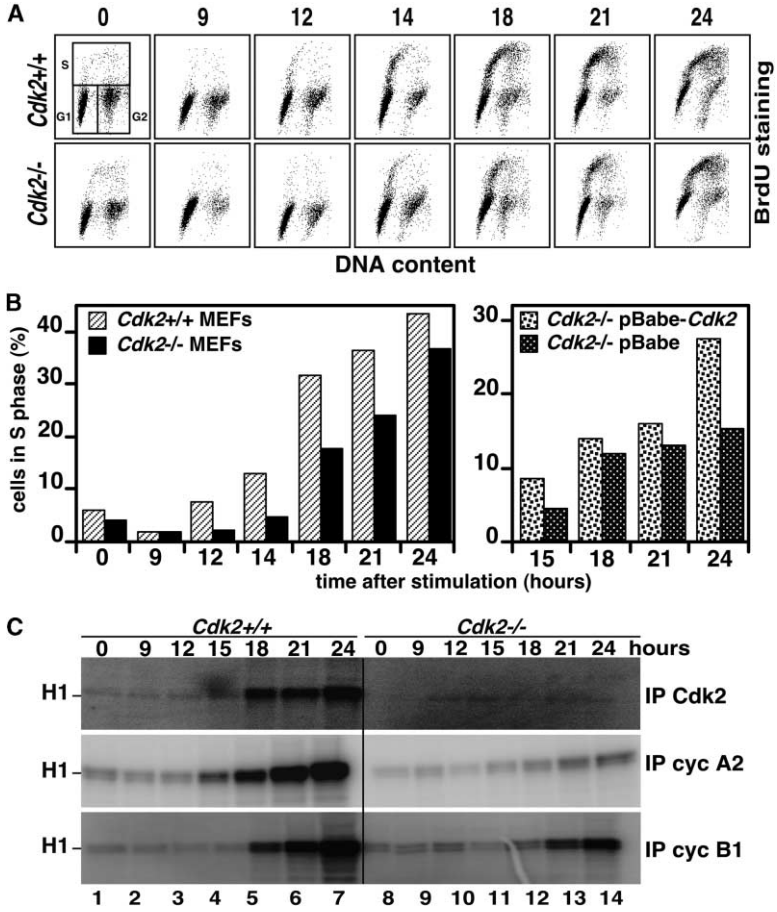

Figure 5. Delayed Entry into the S Phase of *Cdk2*$^{-/-}$ MEFs after Serum Starvation

MEFs were harvested from wild-type and *Cdk2*$^{-/-}$ embryos at 13.5 dpc and cultured in the presence of 10% FBS for two passages. We then induced quiescence by culturing cells in 0.1% FBS for 72 hr (time zero) and subsequently stimulated cells with medium containing 10% serum. Cells were pulse labeled with 100 μM BrdU for 1 hr and harvested at the times indicated after stimulation. Cells were stained with anti-BrdU antibodies and propidium iodide and subjected to flow cytometry (A). The X axis represents the DNA content, and the Y axis represents the amount of BrdU incorporated into newly synthesized DNA. The upper left panel shows the gate settings for cells in G1, S, and G2/M phases of the cell cycle. The percentage of cells in S phase from each time point was calculated and plotted (B) from data shown in (A). *Cdk2*$^{-/-}$ MEFs display a delay in S phase entry at 12 hr after stimulation ([B], left panel). Cdk2-HA was reintroduced by retroviral transfer into *Cdk2*$^{-/-}$ MEFs, and the experiment shown in (A) was repeated ([B], right panel). Cdk2-, cyclin A2-, and cyclin B1-associated kinase activity is shown in (C). Cdk2, cyclin A2, and cyclin B1 complexes were immunoprecipitated from 200 μg cell lysates, and kinase activity toward histone H1 was measured in vitro as described in the Supplemental Experimental Procedures.

degradation of p27 [39–41]. In agreement with this result, we did not observe elevated levels of p27 in *Cdk2*$^{-/-}$ tissues or MEFs (Figures 3A and 4C).

The delay in S phase entry in *Cdk2*$^{-/-}$ MEFs is similar to that observed in *Cdk4* null MEFs [5] and suggests that Cdk2 activity is required for proper timing of S phase entry. One function of Cdk4 is to sequester p27, and it thus prevents inhibition of Cdk2 activity by p27. In *Cdk4*$^{-/-}$ MEFs, more p27 is bound to Cdk2, thus lowering its activity [5]. The delay in S phase entry in *Cdk4*$^{-/-}$ MEFs is most likely due to Cdk2 inhibition because the delay was almost completely rescued by removal of p27 in *Cdk4*$^{-/-}$*p27*$^{-/-}$ MEFs [5]. Therefore, Cdk2 is one of the determining factors for timing of S phase entry, which is consistent with our results (see Figure 5).

Both Cdk4/6 and Cdk2 phosphorylate Rb, which activates the transcriptional activity of E2F by releasing it from sequestration [26, 28, 29]. One possible explanation for the progression of the cell cycle in the absence of either Cdk2 or Cdk4 would be that one could compensate for the other if it were lost. It has been shown that Cdk4 was able to phosphorylate Rb on Cdk2-specific sites in certain colon cancer cells when depleted of Cdk2 [42]. Inhibition of Cdk2 activity by overexpression of p27, expression of a dominant-negative mutant of Cdk2 (D145N), or depletion of Cdk2 through antisense oligonucleotides did not cause cell cycle arrest in specific colon cancer cell lines [42], indicating that there is no stringent requirement for Rb to be phosphorylated by Cdk2. On the other hand, the expression of dominant-negative mutants of Cdk2 and Cdk3 caused a G1 block in human U2OS osteosarcoma cells and other cell lines [22] and inhibited mitogen-induced cell proliferation of oligodendrocyte progenitors [56]. These observations are in agreement with the notion that Cdk2 is dispensable for cell proliferation but might be required in specific cell lines or tissues, as we reported for germ line development. In addition, the Cdk2^{D145N} phenotype is rescued by cyclin D1 and, with less efficiency, by both cyclins A and E [22]. Therefore, the mechanisms by which Cdk2^{D145N} arrests cell lines might include more than suppressing the endogenous Cdk2 kinase activity.

One of the main reasons that *Cdk2*$^{-/-}$ mice are viable could be related to the cyclin A2 activity. Cyclin A2 binds to Cdk2, and Cdc2 and is an essential gene in the mouse [20]. In the absence of Cdk2, cyclin A2 is still able to bind to Cdc2 and therefore fulfill its essential missions. In primary *Cdk2*$^{-/-}$ MEFs, cyclin A2 is active (see Figure 4D and [53]), but upon ablation of Cdk2 in the immortalized human C33A cell line, there is no detectable cyclin A activity [42]. Our results indicate that although there is cyclin A2 activity in primary MEFs, there is very little in immortalized MEFs (Figure 4D). Furthermore, we detected cyclin A2 activity in embryo and spleen extracts from young animals, but there was no cyclin A2 activity in spleen extracts from adult animals. These results sug-

gest that cyclin A2 activity is required during embryonic development but might not be essential in adult mice.

Another explanation of why the progression of the cell cycle is not impaired in the absence of Cdk2 could be related to cyclin E. Binding of cyclin E to Cdk2 in late G1 is thought to drive the G1/S transition. Moreover, Cdk2 is thought to be the only Cdk that binds cyclin E. In the present study, we observed that cyclin E1-associated kinase activities are decreased in *Cdk2*$^{-/-}$ spleen extracts compared to wild-type spleen extracts (Figure 3B). It has been reported that cyclin E mutants that cannot bind Cdk2 and are still able to transform primary rat embryonic fibroblasts in cooperation with Ha-Ras [57]. Cyclin E might have an essential noncatalytic function to allow progression through the cell cycle. Our results are in agreement with this hypothesis because the progression of the cell cycle is maintained in the absence of measurable cyclin E1-associated kinase activity. In addition, a recent report demonstrated that *cyclin E1*$^{-/-}$*cyclin E2*$^{-/-}$ mice [54] display a more severe phenotype than our *Cdk2*$^{-/-}$ mice. On the other hand, cyclin E2 is required for normal spermatogenesis, indicating the importance of Cdk2/cyclin E2 in meiosis. Meiosis is more severely affected in *Cdk2*$^{-/-}$ than in *cyclin E2*$^{-/-}$ animals, suggesting that Cdk2 functions in meiosis extend further than those of cyclin E2. Targeted inactivation of cyclin E genes during gametogenesis will be interesting to investigate if cyclin E functions are directly dependent on Cdk2. Interestingly, though, mice expressing only a single cyclin E allele have no defect in female gametogenesis [54], whereas *Cdk2*$^{-/-}$ females are severely affected (see Figure 2).

Conclusions

The results presented in this report indicate that loss of Cdk2 leads to almost no proliferation defects in vivo or in vitro. Cdk2 is clearly essential in meiosis and affects the timing of S phase entry in MEFs. It is a surprise that Cdk2 is dispensable in the mitotic cell cycle, indicating either that phosphorylation of Cdk2 substrates is not essential or that there are other kinases that can phosphorylate the essential Cdk2 substrates. It will be important to study the impact of Cdk2 inactivation on tumor progression and cancer therapy in vivo.

Supplemental Data

Supplemental Experimental Procedures are available with this article online at http://www.current-biology.com/cgi/content/full/13/20/1775/DC1/.

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