Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice

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We targeted the locus encoding the cyclin-dependent kinase 2 (CDK2) by homologous recombination in mouse embryonic stem (ES) cells. Embryonic fibroblasts lacking CDK2 proliferate normally and become immortal after continuous passage in culture. Elimination of a conditional $Cdk2$ allele in immortal cells does not have a significant effect on proliferation. $Cdk2^{+/−}$ mice are viable and survive for up to two years, indicating that CDK2 is also dispensable for proliferation and survival of most cell types. But CDK2 is essential for completion of prophase I during meiotic cell division. We also show that CDK2 inhibition impedes progression through meiotic prophase I.

CDK2 (refs. 1,2) is thought to be essential in the mammalian cell cycle by driving cells through the G1/S transition (in association with E-type cyclins) and allowing them to progress through the S phase (in association with A-type cyclins). Whereas A-cyclins (A1 and A2) can also associate with the mitotic CDK1 kinase, CDK2 is the only known catalytic partner for E-cyclins (E1 and E2; ref. 3). The precise identity of the physiologically relevant substrates for CDK2 is controversial. Yet, it is well established that CDK2–cyclin E complexes are responsible for completing the inactivation of retinoblastoma (Rb), a step thought to be necessary for initiation of DNA synthesis. Other proposed targets of CDK2–cyclin E include the transcription factor E2F-5 (ref. 5); CDC6, a preinitiation factor essential for generating functional DNA replication origins; p220NPAT, a protein involved in S phase–specific histone gene transcription; nucleaseosmin (NPM/B23) and MPS1, two proteins presumably involved in centrosome duplication, and its own inhibitor, p27 (also called Kip1), which is then targeted for degradation. Loading of CDC45 onto replication origins, a prerequisite for recruitment of DNA polymerase to DNA initiation complexes, also seems to depend on CDK2–cyclin E activity. It is therefore believed that initiation of DNA replication requires a peak of CDK2 activity accomplished by accumulation of cyclin E levels and concomitant disappearance of p27.

Once S phase has started, cyclin E is autophosphorylated by the CDK2–cyclin E complex. Phosphorylated cyclin E is recognized by the F-box protein FBW7 (also called hCDC4 and A Go) and targeted for destruction by the SCF proteasome. Degradation of cyclin E allows CDK2 to bind to its second partner, A-type cyclins. CDK2–cyclin A phosphorylates CDC6 inducing its translocation from the nucleus to the cytoplasm, an event that has been implicated in preventing reinitiation of DNA replication during the S and G2 phases of the cell cycle. CDK2–cyclin A also phosphorylates CDH1, one of the ubiquitin ligase components of the anaphase-promoting complex (APC) required for degradation of cyclin B1. Phosphorylation of CDH1 induces its release from the APC, leading to an increase in cyclin B1 levels required for progression through the G2/M transition. CDK2–cyclin A complexes have also been proposed to have a key role in centrosome duplication.

These observations strongly suggest that CDK2 must be essential for cell proliferation. Moreover, early studies have shown that dominant-negative mutants of CDK2, antibodies against CDK2, cyclin E or cyclin A, and $Cdk2$ antisense RNA block DNA synthesis or cell proliferation. These observations have been recently challenged, however, by the finding that certain cancer cells proliferate despite CDK2 inhibition. To better understand the function of CDK2 in cell proliferation, not only in cultured cells but also in such complex organisms as mice, we targeted the $Cdk2$ locus by homologous recombination in ES cells. Here, we report that ablation of $Cdk2$ in the germ line of mice does not have substantial consequences for embryonic or postnatal development, except in germ cells. Analysis of mouse embryonic fibroblasts (MEFs) indicates that CDK2 is dispensable for cell cycle progression and proliferation but has an unanticipated role in meiotic cell division.

RESULTS
 Generation of mice lacking CDK2

To target the mouse $Cdk2$ locus, we knocked-in two loxP sites flanking coding exons 2 and 3 of $Cdk2$ by homologous recombination in ES cells (Fig. 1). Removal of these sequences by a Cre recombinase eliminates the PSTAIRE cyclin-binding domain. Moreover, splicing from

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exon 1 to exon 4 sequences causes a frameshift in the open reading frame that results in the synthesis of a putative polypeptide of 81 amino acid residues, of which the last 42 are unrelated to CDK2. To select for recombinant ES cells, we inserted a phosphoglycerate kinase promoter (PGK)–neomycin resistance gene (neo) cassette flanked by loxP sites between exons 3 and 4 (Fig. 1). We used ES cells carrying this targeted allele, Cdk2loxneo, to generate Cdk2+/loxneo heterozygous mice and removed the loxP-flanked sequences by crossing Cdk2+/loxneo mice with CMV-cre transgenic mice (Fig. 1). Heterozygous Cdk2+/loxneo mice are normal and fertile. Homozygous Cdk2−/loxneo mice are born from heterozygous parents at the expected mendelian ratio. We confirmed the absence of CDK2 expression in Cdk2−/− mice by western-blot analysis of various tissues (data not shown). Mice lacking CDK2 had no gross anatomical or behavioral abnormalities for up to two years, except for severe atrophy in their gonads (both testes and ovaries), resulting in complete sterility.

**Cdk2−/− MEFs**

Primary MEFs isolated from midgestation Cdk2−/− embryos had no detectable CDK2 expression (Fig. 2a). Immunoprecipitates obtained with antibodies to CDK2 did not phosphorylate histone H1 and Rb, two of the best-known substrates of CDK2 (Fig. 2b). Similarly, immunoprecipitates obtained with antibodies to cyclin E did not phosphorylate these substrates above background levels (Fig. 2b). In contrast, immunoprecipitates obtained with antibodies to cyclin A had substantial H1 kinase activity (Fig. 2b), presumably due to the association of cyclin A with CDK1. Rb was phosphorylated in Cdk2−/− cells. Western-blot analysis with antisera specific for certain CDK-phosphorylated residues showed efficient phosphorylation of Ser795, a substrate of CDK4–cyclin D, and Thr821, a residue thought to be specifically phosphorylated by CDK2–cyclin E (Fig. 2c).

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**Figure 1** Gene-targeting strategy. (a) Schematic representation of Cdk2 alleles used in this study. The mouse Cdk2 locus contains seven exons (boxes) spanning 8 kb. Noncoding sequences (open boxes), nontargeted coding sequences (hatched boxes), targeted exons (filled boxes), loxP sites (filled triangles) and translational initiator (ATG) and terminator (TGA) codons are indicated. The PGK-neo cassette and the PGKT cassette used for selection of homologous recombinant ES clones are indicated by open boxes. Cre, bacteriophage Cre recombinase. Wild-type and mutated Cdk2 alleles are indicated. (b) Southern-blot analysis of recombinant ES cell clones carrying the indicated alleles. The origin of the probes is indicated in a. Sizes of the diagnostic DNA fragments are indicated.

**Figure 2** Properties of Cdk2−/− MEFs. (a) Western-blot analysis of Cdk2+/+, Cdk2−/+ and Cdk2−/− MEFs with antibodies to Cdk2. (b) In vitro CDK2-, cyclin E- and cyclin A-associated kinase activity in extracts of Cdk2−/+ and Cdk2−/− MEFs immunoprecipitated (IP) with antibodies against CDK2, cyclin E and cyclin A using histone H1 and Rb as substrates. A negative control without antibodies (−) is also included. (c) Phosphorylation of Rb residues Ser795 and Thr821 in extracts derived from Cdk2−/+ and Cdk2−/− MEFs using antibodies specific for phosphorylated Ser795-Rb and Thr821-Rb phosphopeptides. (d) Growth curves of primary (P2, P4 and P6) Cdk2+/+ and Cdk2−/− MEFs. (e) Western-blot analysis of Cdk2−/+ and Cdk2−/− primary (P2, P4 and P6) MEFs with antibodies directed against the indicated cell cycle proteins. An antibody against ERK1/2 was used for loading control. (f) S-phase kinetics after restimulation of serum-starved P2 MEFs. (g) Western-blot analysis of Cdk2−/+ and Cdk2−/− immortalized MEFs (P20) with antibodies directed against the indicated cell cycle proteins. An antibody against ERK1/2 was used for loading control.
Thr821 in Cdk2−/− cells is probably phosphorylated by CDK4, a kinase that can phosphorylate Rb, even at sites previously defined as CDK2-specific, when the activity of CDK2 is abolished.

Primary passage 2 (P2) Cdk2−/− MEFs grew well in culture and proliferated with kinetics similar to those of MEFs derived from heterozygous (data not shown) and wild-type embryos (Fig. 2d). At P4, however, Cdk2−/− MEFs had slightly lower proliferation rates, suggesting that they might enter culture crisis earlier than wild-type MEFs. Levels of cyclin E and CDK1 were lower in P4 Cdk2−/− MEFs and expression of cyclin A was almost undetectable (Fig. 2e). By P6, normal and mutant MEFs proliferated poorly, indicating that they had entered crisis (Fig. 2d), and expression of CDK1 and cyclin A was much lower (Fig. 2e). The level of cyclin E was constant in wild-type MEFs but decreased in Cdk2−/− MEFs, probably owing to the absence of CDK2 (Fig. 2e). The expression levels of CDK4, cyclin D1, p27 and p21 (also called Cip1) were similar in Cdk2−/− and Cdk2+/+ MEFs at all passages (Fig. 2e).

Serum-starved P2 Cdk2−/− MEFs entered S phase with the same kinetics as their wild-type counterparts after serum stimulation (Fig. 2f). P4 Cdk2−/− MEFs also entered S phase, but with delayed kinetics (4 h). We observed a parallel delay in the rate of Rb phosphorylation in residues Ser795 and Thr821 in P4 Cdk2 mutant cells (data not shown). Primary Cdk2−/− MEFs formed colonies when plated as single cells, albeit 40% less efficiently than wild-type MEFs. Primary Cdk2−/− MEFs could be transformed either by transfection with Ras and adenoviral E1A oncogenes or by genotoxic insult induced by etoposide, an inhibitor of DNA topoisomerase II, but mutant MEFs were transformed about 30% less efficiently than the corresponding wild-type MEFs. We observed no significant differences in the percentage of cells that underwent G1 arrest (27 ± 11% in wild-type versus 31 ± 7% in Cdk2−/− MEFs) and G2 arrest (62 ± 12% in Cdk2−/− versus 56 ± 4% in Cdk2+/+ MEFs) 24 h after etoposide treatment. In addition, these cells underwent apoptosis with similar rates, 28% of Cdk2−/− and 24% of Cdk2−/− MEFs treated with etoposide, suggesting that loss of CDK2 did not affect DNA-damage checkpoints.
MEFs became immortalized after continuous passage in culture. Whereas all wild-type embryos (n = 8) yielded immortal cell lines when maintained in a 3T3 protocol, only 66% of the mutant Cdk2−/− embryos (n = 9) became immortal. Cdk2−/− cells that did become immortal expressed normal levels of CDK2 and lower levels of cyclin E and cyclin A (Fig. 2g).

To eliminate the possibility that the proliferative properties of MEFs lacking CDK2 might be a consequence of developmental plasticity, we eliminated CDK2 from immortal MEFs containing a conditional Cdk2 allele (Fig. 1). We infected Cdk2lox/− MEFs with a retrovirus expressing Cre recombinase and selected them by single-cell cloning. Three of eight clones tested were Cdk2−/−, and these cells had similar proliferative properties to those of Cdk2+/+ and Cdk2lox/− MEFs, indicating that CDK2 is dispensable for cell proliferation in conditions under which plasticity is unlikely to have a role.

**Defective spermatogenesis in Cdk2−/− mice**

Histological analysis of testes from young Cdk2+/+ and Cdk2−/− male mice showed no detectable differences up to P15, when germ cells had not yet developed beyond tetraploid primary spermatocytes (Fig. 3a,b). At this time, these cells and spermatogonia are the only germ cells present in testes. Defects in Cdk2−/− mice became visible at P20, when germ cells had completed meiosis I to form diploid secondary spermatocytes and some cells were undergoing the second meiotic division to become round spermatids. P20 Cdk2−/− mice had no round spermatids in the seminiferous tubules (Fig. 3c,d), and there was massive apoptosis of spermatocytes (Fig. 3e,f). These observations suggest that CDK2 is essential for completing the first meiotic division in male mice. Notably, spermatogonia continued to proliferate normally in Cdk2−/− mice even at P30, a stage at which the seminiferous tubules are almost completely depleted of germ cells (Fig. 3g,h). Testes of adult (P120) Cdk2−/− mice were atrophic (20% the weight and size of those of wild-type littermates). Histological analysis showed normal levels of spermatogonia, but there were significantly fewer primary spermatocytes in most seminiferous tubes and there were no post-meiotic cells, including round or elongated spermatids and spermatozoa (Fig. 3i,j). Likewise, we observed no spermatozoa in the epididymis of these mutant mice (Fig. 3k,l). The number and distribution of Sertoli and Leydig cells appeared to be normal.

**Cdk2−/− spermatocytes arrest in prophase I**

To determine the stage at which meiosis was altered in Cdk2−/− tests, we examined spermatocytes from P120 mice by immunoconfocal microscopy. Homozygous Cdk2−/− spermatocytes had patterns that resemble leptotene, zygotene and pachytene stages, but we did not observe diplotene or later meiotic stages, suggesting a defect in chromosome pairing during the first meiotic division. To more precisely define the stage at which formation of the synaptonemal complex was affected, we stained Cdk2+/+ and Cdk2−/− squashed spermatocytes with antibodies against STAG3, a marker for the cohesion axis28. STAG3 immunoreactivity in Cdk2−/− pachytene spermatocytes appeared as homogeneous fibers marking the synaptonemal complex (Fig. 4a). In Cdk2−/− pachytene-like spermatocytes, these fibers appeared disorganized and of variable thickness. In addition, antibodies against SYCP3, a marker for the axial element27, showed anomalous distribution of this protein along the fibers and showed the presence of protein aggregates (Fig. 4a). Using chromosome spreading techniques, we observed unsynapsed chromosomes in pachytene-like Cdk2−/− spermatocytes (Fig. 4b). As expected, CDK2 associated with telomeric regions in Cdk2+/+ chromosomes but was absent in those derived from mutant spermatocytes (Fig. 4b). These results suggest that Cdk2−/− spermatocytes have a defect in the formation of the axial element, yielding abundant unpaired chromosomes.

To confirm this hypothesis, we carried out similar experiments using antibodies against SCP1, a marker for the central element of the synaptonemal complex28. SCP1 staining was limited to thick fibers that also stained with antibodies to STAG3 in Cdk2+/+ spermatocytes (Fig. 4b). The absence of SCP1 in a fraction of STAG3-positive fibers indicated that the central element was not efficiently formed in pachytene spermatocytes lacking CDK2. This deficit probably triggers the pachytene checkpoint responsible for the massive apoptosis observed in Cdk2−/− spermatocytes.

**Abnormal Cdk2−/− dictyate oocytes**

Ovaries of E17.5 Cdk2−/− embryos, a time at which most oocytes are at the pachytene stage of meiosis I, had normal morphology and contained approximately the same number of primary oocytes as wild-type embryos (Fig. 5a,b). At P1, a time when most oocytes are at late diplotene (dictyate) stage, Cdk2−/− oocytes had few oocytes (Fig. 5c,d) and more apoptotic cells (Fig. 5e,f). Ovaries of P14 Cdk2−/− mice were devoid of oocytes (data not shown), suggesting that these cells died at the perinatal stage. Ovaries of adult (P120) Cdk2−/− females were considerably atrophic, 15–20% of the size of those of wild-type counterparts. The mutant ovaries showed complete absence of follicles and corpora lutea, and in many cases the ovarian tissue was replaced with cysts lined with a layer of epithelial cells (Fig. 5g,h). The oviduct and uterus of Cdk2−/− mice had a normal appearance (data not shown).
Unlike Cdk2+/- spermatocytes, Cdk2 mutant oocytes developed normally through the leptotene, zygotene and pachytene stages (E14.5–E18.5; data not shown). When oocytes reached the dictyate stage (P1–P2), however, we observed significant differences. At this stage, SYCP3 was no longer present in nucleolar aggregates in Cdk2+/- oocytes but was properly distributed along fibers of fully desynapsed axial elements (Fig. 6a). Moreover, centromeres, which were normally clustered in discrete nuclear locations in wild-type dictyate oocytes, appeared randomly distributed throughout the nucleus of Cdk2+/- oocytes (Fig. 6a). These observations indicate that Cdk2+/- oocytes also had a meiotic defect involving improper distribution of SYCP3, although this occurred at a later stage of prophase I than that of Cdk2+/- spermatocytes.

**DISCUSSION**

Our results illustrate that CDK2 is not essential for mitotic cell division of most, if not all, cell types, contrary to previous thought. Moreover, removal of a conditional Cdk2 allele from growing MEFS does not affect their proliferation. These observations indicate that our results in vivo are probably not due to developmental plasticity. Instead, the dispensability of CDK2 in mitotic cell division is probably due to compensation by another kinase. Phosphorylation of CDK2–cyclin A targets might be compensated by CDK1, a mitotic kinase that also associates with A-type cyclins. If this is the case, however, compensation is not mediated by increased expression of either cyclin A or CDK1. Indeed, CDK2 deficiency results in lower levels of cyclin A and CDK1, in agreement with a role for CDK2 as a positive regulator of CDK1 (ref. 29). The nature of the putative kinase responsible for compensating the activities attributed to CDK2–cyclin E is less obvious. Whereas we detected kinase activity in immunoprecipitates of cyclin E, those of cyclin A did not have kinase activity above background levels, suggesting that CDK2 could be compensated in a cyclin E–independent manner. In the case of Rb phosphorylation, this compensatory effect is probably mediated by cyclin D-dependent kinases CDK4 or CDK6 (refs. 23, 25).

Mice lacking CDK2 cyclin partners have also been generated. Cyclin A1, a protein exclusively expressed in germ cells, is essential for male but not female fertility30. Mice deficient for the widely expressed cyclin A2 die during early embryogenesis, possibly due to loss of CDK1–cyclin A activity31. Ablation of cyclin E (cyclin E1 and E2), only known to interact with CDK2, results in embryonic lethality due to a defect in trophoblast endodermiplication12,33. Moreover, cyclin E–deficient MEFS do not reenter the cycle after serum starvation12. These findings provide genetic evidence suggesting that E-type cyclins may have activities independent of CDK2. In fact, cyclin E mutants unable to bind CDK2 transform rat embryo fibroblasts in combination with H-Ras34. Cdk2+/- MEFS enter DNA synthesis and complete the mitotic cell cycle with similar kinetics as Cdk2+/- MEFS and have normal DNA damage checkpoints. Yet, Cdk2+/- MEFS are not entirely normal. They enter crisis earlier than their wild-type counterparts and immortalize less efficiently. Likewise, they are less susceptible to transformation by oncogenes. Transfection of Cdk2+/- cells with libraries of kinase siRNAs should provide valuable information regarding those enzymes that compensate for CDK2-specific activities. The possibility that the phosphorylation events attributed to CDK2 complexes might not be as critical for cell proliferation as previously thought, though unlikely, cannot be eliminated at this time.

Information linking CDK2 with the meiotic cell cycle is scarce. In *Drosophila*, CDK2 is required for oogenesis, but the specific process in which CDK2 is involved is unknown35. Studies in vertebrate oocytes have implicated CDK2–cyclin E in metaphase II arrest mediated by cytosstatic factor36. In addition, CDK2 has been localized in the telomeric ends of chromosomes from leptotene to diplotene stages of meiosis37. Our results establish that CDK2 is required for completion of prophase I of the meiotic cell cycle. Whereas in male germ cells, CDK2 appears to be essential for synaptonemal complex formation during the pachytene stage, female germ cells progress further to the dictyate stage, a time at which they undergo apoptosis in the absence of CDK2. The differential requirements for this kinase at distinct stages of meiotic prophase I in males and females is not surprising, as there are numerous reports indicating sex-specific regulation of the meiotic cell cycle38.

These observations argue for the existence of specific meiotic substrates for CDK2. CDK2 localizes with MLH1 at crossover sites in midpachytene37, but defects in *Mlh1*-null mice become evident only at metaphase39. Other loci whose inactivation leads to phenotypes similar to that of Cdk2+/- mice include those encoding SP011, a protein involved in DNA double strand breaks40; DMC1, a protein implicated in recombination41; DNA-repair proteins, such as MSH4 (ref. 42) and MSH5 (ref. 43); and SYCP3 (ref. 44). The last is of particular relevance, as CDK2 deficiency results in perturbed distribution of SYCP3 in male and female germ cells. In normal spermatocytes, SYCP3 localizes as aggregates in nucleoli in leptotene. At later stages, SYCP3 becomes distributed along the axial element of the synaptonemal complex15. The absence of CDK2 causes retention of SYCP3 in the nucleoli of Cdk2+/- pachytene spermatocytes and aberrant synaptonemal complex formation. In female germ cells, CDK2 deficiency leads to a precocious disappearance of SYCP3 nucleolar aggregates and abnormal distribution of centromeres at the dictyate stage.
without obvious differences in previous prophase I stages. Thus, CDK2 may promote proper dynamics of SYCP3, either by direct phosphorylation, or by phosphorylating other proteins involved in this process. Because Sycp3+/− females are partially fertile44, however, CDK2 must have additional targets.

CDK2 does not seem to be genetically altered in human tumors, but the expression of some of its direct regulators, such as cyclin E and p27, are frequently altered in human cancers46. These observations, along with the previously assumed role of CDK2 in the cell cycle, has led to considerable efforts to inactivate CDK2 in cancer therapy. Our results predict that highly CDK2-specific inhibitors should have little, if any, effect on the proliferation of somatic cells. But we do not know whether loss of CDK2 activity would affect the proliferation of tumor cells in vivo. Conditional Cdk2lox/lox mice will allow us to test this hypothesis by specifically ablating Cdk2 from experimentally induced tumors. The outcome of such studies should be valuable to predict the clinical utility of CDK2 inhibitors in cancer therapy.

METHODS

Generation of Cdk2+/− mice. We isolated Cdk2 coding sequences from a 129SV1 library (Stratagene) in a single lambda phage containing an insert of 16 kb of genomic DNA. To construct the targeting vector, we subcloned a genomic DNA fragment containing exons 1 to 5 in pBlueScript (Stratagene; Fig. 1). We inserted a PGK-neo cassette derived from pKsloPNPT60 and flanked by loxP sites into the unique SphI site in the third intron of Cdk2 in the same transcriptional orientation. We inserted a third loxP site, subcloned from pHSlox-1 (Novagen), into an Nhel site in the first intron. This strategy introduced novel BamHI and NoI sites in this intron to help genotype the targeted allele. We inserted a PGK-thymidine kinase (TK) cassette from pPNT48 into a unique Xhel site at the 3′ end of the left arm (Fig. 1). We electroporated the targeting vector (20 μg), previously linearized with Sall, into mouse R1 ES cells49 and selected recombinant clones in the presence of G418 and gancyclovir. Southern-blot analysis of 182 G418/Gan6 clones identified 11 homologous recombinants, of which only 3 had undergone recombination 5′ of the Cdk2 exons flanked by loxP sites (Fig. 1b). These clones were aggregated with eight cell–stage CD-1 embryos. Male chimera derived from two of the clones, SO1-87 and SO1-80, transmitted the targeted allele (Cdk2lox/lox) to their offspring. We observed no significant differences between mice derived from these independent clones. We crossed Cdk2+/lox/neo mice with transgenic CMV-cre mice24 to generate Cdk2−/− mice and mated offspring from these crosses with wild-type CD-1 mice to eliminate the CMV-cre transgene. Crosses between these mice generated Cdk2−/− mice. We verified excision of exons 2 and 3 using PCR and Southern-blot analysis of genomic DNA from tail biopsies of Cdk2−/− mice. We verified that splicing from exon 1 to 4 led to a shift of the correct open reading frame using RT–PCR followed by sequencing of the PCR product. All mice. We verified that splicing from exon 1 to 4 led to a shift of the correct open reading frame using RT–PCR followed by sequencing of the PCR product. All

Western-blot, immunoprecipitation and kinase assays. We carried out western-blot analysis, immunoprecipitation and kinase assays as described36. We probed nitrocellulose membranes with antibodies against Cdk2 (M2, Cdk1 (17), Cdk4 (C22), cyclin A (H432), cyclin E (M20), p21 (C19), ERK1 (C16; all from Santa Cruz Biotechnology); cyclin D1 (DCS6; NeoMarkers); p27 (Transduction Laboratories); phosphorylated Rb-S795 (Cell Signaling Technology); and phosphorylated Rb-T821 (Biosource International). For kinase assays, we used 1 μg of mouse Rb protein fragment (amino acids 769–921; 769, Santa Cruz Biotechnology) and histone H1 from calf thymus (Roche) as substrates.

MEF assays. We obtained primary MEFs from E14.5 embryos and cultured them in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For proliferation assays, we plated 5 × 103 cells on six-well plates in duplicate. For colony formation assays, we seeded 5–20 × 103 cells on 10-cm plates in duplicate and cultured them for 2 weeks. We stained the plates with methylene blue and scored the colonies. To analyze S-phase entry, we incubated 108 cells per 10-cm dish in DMEM supplemented with 0.1% FBS for 60 h. We added DMEM supplemented with 10% FBS and then collected cells at the indicated times, stained them with propidium iodide and analyzed by fluorescence-activated cell sorting using a Coulter flow cytometer. We carried out focus formation assays as described35 using 10 μg of pAL8 and pCMV-E1A DNAs. For etoposide treatment, we incubated 106 cells per 10-cm dish with 5 μM etoposide (Sigma-Aldrich) for 24 h. We washed cells with phosphate-buffered saline (PBS) and cultured them for various times in DMEM supplemented with 10% FBS. We carried out immortalization assays following a classical 3T3 protocol.

Histology and immunohistochemistry. We carried out conventional staining with hematoxylin and eosin on 3-μm sections of paraffin-embedded tissues after fixation in formalin for 24 h. We detected germ cells by immunohistochemistry using an antibody to GCNA1 (a gift from G.C. Enders, Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, Kansas, USA). We monitored apoptosis by TUNEL assays (ApoptDETEK, Enzo Life Sciences) and measured spermatogonia proliferation by Ki67 immunostaining using a monoclonal antibody (MIB-1; Dako).

Immunofluorescence analysis of germ cells. To analyze the distribution of synapsin complex components in mouse germ cells, we used a squash procedure that maintains the spatial organization of the cells30 or a spreading technique32. We stained samples with antibodies against human STAG3 (mouse polyclonal antibody m228; ref. 26), human SMCC3 (rabbit polyclonal antibody K987 raised against amino acids 978–1217 expressed in Escherichia coli), rat SYCP3 (rabbit polyclonal antibody K921 and mouse polyclonal antibody N6, both raised against the final 21 C-terminal amino acids), rat SCP1 (rabbit polyclonal antibody K919 raised against the final 23 C-terminal amino acids) and human CDK2 (D-12, Santa Cruz Biotechnology). We obtained antisera to centromere and nucleolus from autoimmune individuals (gifts from G. Roy, Servicio de Immunología, Hospital Ramón y Cajal, Madrid, Spain). Secondary antibodies included Alexa 488-labeled goat antibody to rabbit IgG (Molecular Probes), Cy5-labeled goat antibody to human IgG and Cy3-labeled goat antibody to rabbit IgG (Jackson Immunoresearch Laboratories). For immunofluorescence on squashed mouse germ cells, we diluted primary antibodies 1:50 in PBS with 10% goat serum (GibcoBRL-Life Technologies); we used secondary antibodies (diluted 1:200) in the same conditions. For the spreading procedure, we diluted rabbit polyclonal K921 antibodies to 1:200 and mouse monoclonal D-12 antibody to 1:10. We diluted primary antibodies in PBS containing 10% goat serum; we used secondary antibodies in the same conditions, diluted 1:400.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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