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Hypomorphic mutation in an essential cell-cycle kinase causes growth retardation and impaired spermatogenesis

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***Cdc7* kinase is essential for initiation of DNA replication. *Cdc7*$^{-/-}$ mouse embryonic stem (ES) cells are non-viable but their growth can be rescued by an ectopically expressed transgene (*Cdc7*$^{-/-}$tg). Here we report that, despite the normal growth capability of *Cdc7*$^{-/-}$tg ES cells, the mice with the identical genetic background exhibit growth retardation. Concomitantly, *Cdc7*$^{-/-}$tg embryonic fibroblasts (MEFs) display delayed S phase entry and slow S phase progression. Furthermore, spermatogenesis of *Cdc7*$^{-/-}$tg mice is disrupted prior to pachytene stage of meiotic prophase I. The impairment in spermatogenesis correlates with the extremely low level of Cdc7 protein in testes, and is rescued by introducing an additional allele of transgene, which results in increase of Cdc7 expression. The increased level of Cdc7 also recovers the growth of *Cdc7*$^{-/-}$tg MEFs and mice, indicating that the developmental abnormalities of *Cdc7*$^{-/-}$tg mice are due to insufficiency of Cdc7 protein. Our results indicate the requirement of a critical level of a cell-cycle regulator for mouse development and provide genetic evidence that Cdc7 plays essential roles in meiotic processes in mammals.**

Keywords: *Cdc7* kinase/cellular proliferation/growth retardation/hypomorphic mutation/spermatogenesis

Introduction

Cell-cycle regulators involved in DNA replication or cell division generally play essential roles in cell viability, and their roles in mammalian ontogeny, including in the development of specific tissues and organs, cannot be analyzed through the studies of conventional gene targeting. In particular, effects of reduced expression of these essential regulators on mouse development have not been addressed.

Cdc7, originally discovered in budding yeast, is required for initiation of DNA replication (Hollingsworth and Sclafani, 1990; Sclafani and Jackson, 1994; Stillman, 1996; Dutta and Bell, 1997; Johnston *et al*., 1999; Bell and Dutta, 2002). *Cdc7* homologs have been identified from fission yeast, *Xenopus*, mouse, human and other eukaryotes, suggesting that DNA replication in higher eukaryotes may be regulated by a conserved mechanism involving *Cdc7* (Masai *et al*., 1995; Jiang and Hunter, 1997; Sato *et al*., 1997; Kim *et al*., 1998). In higher eukaryotes, *Cdc7* plays essential roles in cell-cycle progression from late G_1 to S phase in cooperation with *Cdk2–cyclin E*. The kinase activity of Cdc7 is regulated by association with its regulatory subunit, Dbf4/ASK (Johnston and Thomas, 1982a,b; Jackson *et al*., 1993; Brown and Kelly, 1999; Jiang *et al*., 1999; Kumagai *et al*., 1999; Takeda *et al*., 1999). The levels of Dbf4/ASK increase sharply at the G_1/S boundary and peak in S phase, while those of Cdc7 remain constant during the cell cycle (Chapman and Johnston, 1989; Brown and Kelly, 1999; Jiang *et al*., 1999; Kumagai *et al*., 1999; Takeda *et al*., 1999).

Characterization of *cdc7*^{ts} revealed potential roles of *Saccharomyces cerevisiae Cdc7* in meiosis. Sporulation is arrested in *cdc7*^{ts} diploid cells, presumably due to impaired synaptonemal complex formation (Sclafani *et al*., 1988). In adult mice, *Cdc7* transcript and protein are expressed at a high level in testis (Kim *et al*., 1998), suggesting possible involvement of *Cdc7* in germ cell development in mammals. However, the roles of *Cdc7* in mammalian development are unknown.

We previously reported that *Cdc7*-deficient mice die between embryonic day (E) 3.5 and E6.5, indicating that *Cdc7* functions are essential for early embryogenesis and cell proliferation (Kim *et al*., 2002). We also established a conditional *Cdc7*-deficient (mu*Cdc7*$^{-/-}$tg) embryonic stem (ES) cell line lacking both alleles of the *Cdc7* gene in the presence of a single copy of transgene carrying a *loxP*-flanked *Cdc7* cDNA. mu*Cdc7*$^{-/-}$tg ES cells showed morphology and growth properties indistinguishable from those of wild-type ES cells. Upon removal of the transgene by Cre-recombinase, DNA synthesis rapidly ceases within S phase and checkpoint responses are triggered, leading to recombinational repair and eventually to p53-dependent cell death (Kim *et al*., 2002).

In this study, to further investigate the functions of *Cdc7* during mammalian development, we generated mice harboring a genetically modified *Cdc7* allele using mu*Cdc7*$^{-/-}$tg ES cells. Despite the normal growth capability of the mu*Cdc7*$^{-/-}$tg ES cells, the mutant mice (*Cdc7*$^{-/-}$tg mice) exhibited growth retardation. This paralleled with reduced growth rate of *Cdc7*$^{-/-}$tg mouse embryonic fibroblasts (MEFs). Most strikingly, testicular development was severely impaired and spermatogenesis was abrogated in *Cdc7*$^{-/-}$tg mice. We found that *Cdc7*$^{-/-}$tg MEFs and testes expressed Cdc7 at levels considerably lower than those of wild-type counterparts, and showed that all these defects were corrected by introducing an additional allele of transgene *in vivo*, which resulted in increase of Cdc7 expression. Our results demonstrate the

requirement of a critical level of Cdc7 for mammalian development and its crucial roles in spermatogenesis.

Results

Cdc7$^{-/-}$tg mice exhibit growth retardation

To elucidate the functions of *Cdc7* during mammalian development, we attempted to generate mice harboring a genetically modified *Cdc7* allele using mu*Cdc7*$^{-/-}$tg ES cells (Figure 1A–C). Two independent ES cell clones (DKO20 and DKO28) transmitted the mutation into the germline. The *Cdc7*$^{+/-}$tg mice (derived from DKO28 ES cells) were indistinguishable from the wild-type mice in size and appearance. The crosses of *Cdc7*$^{+/-}$tg mice with *Cdc7*$^{+/-}$ mice generated *Cdc7*$^{-/-}$tg mice, but at a considerably reduced frequency; at birth, only 1.6% were *Cdc7*$^{-/-}$tg instead of the expected Mendelian ratio of 12.5% (Table I). Of the *Cdc7*$^{-/-}$tg newborns, <25% (only 0.38% of total offspring) survived to adulthood. Indeed, *Cdc7*$^{-/-}$tg embryos at E18.5 were substantially smaller and thinner than their littermates (Figure 1D), and >75% of *Cdc7*$^{-/-}$tg newborns died within 3 days *post partum*. This indicates that transgene-encoded Cdc7 rescued early embryonic lethality, but that *Cdc7*$^{-/-}$tg mice were susceptible to prenatal or neonatal lethality. *Cdc7*$^{-/-}$tg mice that survived to weaning age had a normal life span but weighed only 50–55% of their normal littermates (Figure 1E). The sizes of the major organs, including heart, liver, kidney, lung,

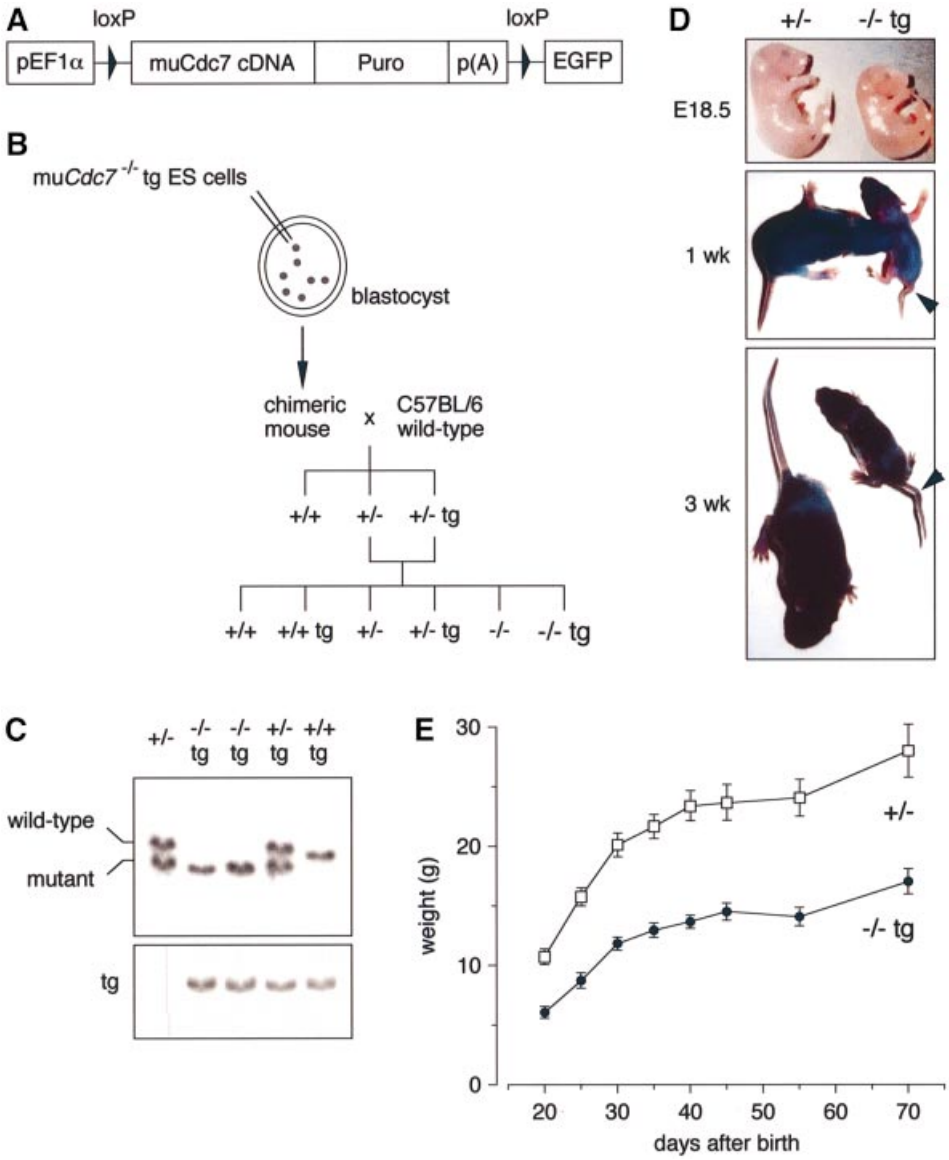

Fig. 1. *Cdc7*$^{-/-}$tg mice exhibit growth retardation. (**A**) The schematic drawing of the transgene used to create conditional *Cdc7*-deficient (*Cdc7*$^{-/-}$tg) mice, consisting of the human elongation factor 1α promoter (pEF1α), *loxP*-flanked murine *Cdc7* cDNA, puromycin resistance cassette (Puro) and polyadenylation signal (pA). (**B**) The strategy for generating *Cdc7*$^{-/-}$tg mice. (**C**) Southern blot hybridization. Tail DNA was digested with *Bgl*II and hybridized with a 3′ flanking probe (Kim *et al*., 2002). The positions of the DNA fragments derived from wild-type and mutant alleles hybridized to the probe are indicated. Tg indicates the band derived from transgene. (**D**) Gross appearance of embryos (E18.5; top), 1- (middle) and 3-week-old (bottom) mice, with *Cdc7*$^{+/-}$ (left) or *Cdc7*$^{-/-}$tg (right) genotype. Note the flexion in the tail of the *Cdc7*$^{-/-}$tg mice (arrowheads). (**E**) Representative growth curve of *Cdc7*$^{+/-}$ (n = 6) and *Cdc7*$^{-/-}$tg (n = 4) male mice. Mice were weighed at the indicated days after birth.

thymus, spleen and brain, of *Cdc7*$^{-/-}$tg mice were proportional to body weight, and histological examination of these organs revealed no discernable defects (data not shown). In contrast, the testes and ovaries were extremely small and exhibited severe developmental defects (see below). In addition, *Cdc7*$^{-/-}$tg mice exhibited a tail flexion anomalies (Figure 1D), as has been observed in *curly-tail* (*ct*) mutant mice (Gruneberg, 1954). The identical phenotypes were observed with DKO20 ES-derived mice.

Cdc7$^{-/-}$*tg MEFs display decreased proliferative capability*

To investigate whether the growth retardation of *Cdc7*$^{-/-}$tg mice reflected a defect in cellular proliferation, we examined the growth properties of *Cdc7*$^{-/-}$tg MEFs. The increase in cell number of *Cdc7*$^{-/-}$tg MEFs was considerably slower than in wild-type or *Cdc7*$^{+/-}$tg MEFs (Figure 2A). FACS analysis of asynchronously growing *Cdc7*$^{-/-}$tg MEFs revealed an increased G_0/G_1 phase

Table I. Genotypes of offspring from crosses between *Cdc7*$^{+/-}$tg and *Cdc7*$^{+/-}$ mice

Stage	Genotype						Total
	+/+	+/+ tg	+/–	+/– tg	–/–	–/– tg	
E13.5	15 (1)	14 (0.93)	27 (1.8)	28 (1.87)	0 (0)	16 (1.07)	100
3 weeks old	354 (1)	332 (0.94)	696 (1.97)	724 (2.04)	0 (0)	8 (0.02)	2114
Expected Medelian ratio	1	1	2	2	1	1	

The numbers and the genotypes of viable embryos (E13.5) and mice (3 weeks old) are given. The values in parentheses indicate the ratio with respect to the number of wild-type (+/+) mice.

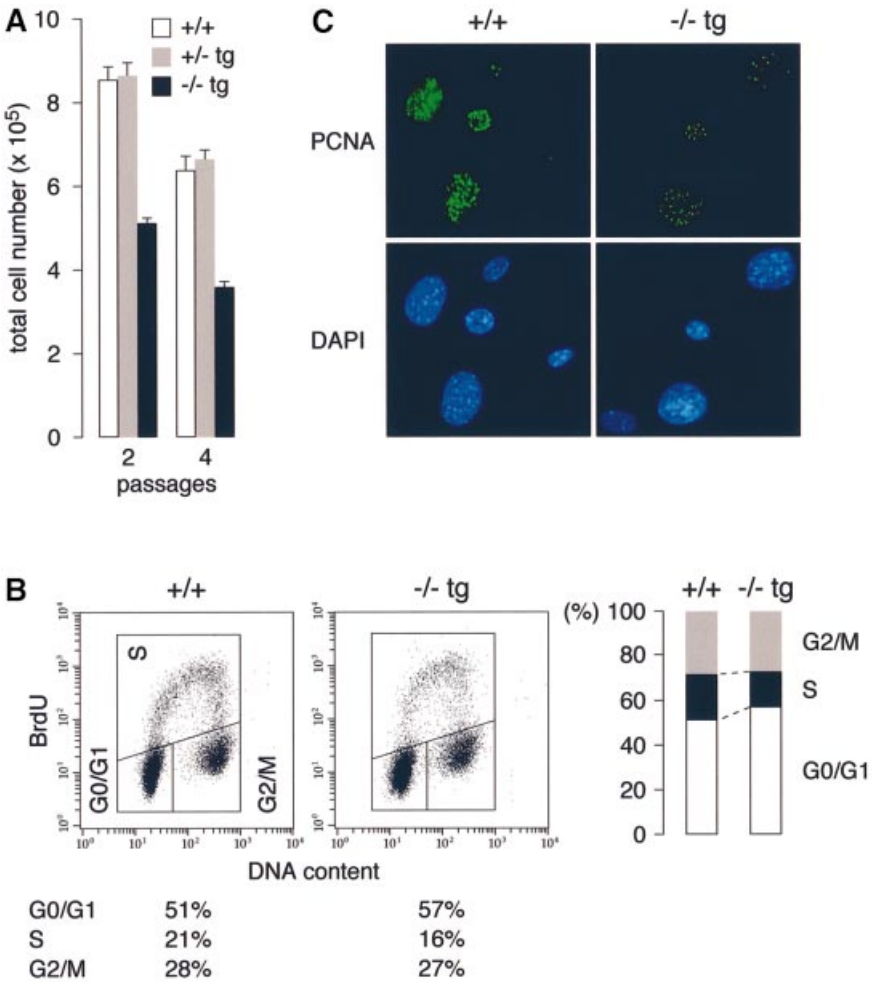

Fig. 2. *Cdc7*$^{-/-}$tg MEFs display decreased proliferative capacity with reduced fraction of S phase cells. (**A**) Passage 2 and 4 MEFs were plated in triplicate at 3×10^5/60 mm dish. Cell numbers, which represent the average of three independent experiments, were determined after 2 days. (**B**) Cell cycle distribution of asynchronously growing MEFs. The decrease in S phase population of *Cdc7*$^{-/-}$tg MEFs was accompanied by a moderate increase in the G_0/G_1 phase population. The data represent three separate experiments with different clones of MEFs. (**C**) Passage 3 MEFs were fixed in methanol/acetone and stained for PCNA. The intensity and numbers of nuclear foci of PCNA staining in the *Cdc7*$^{-/-}$tg MEFs (right) were significantly reduced compared with wild-type MEFs (left).

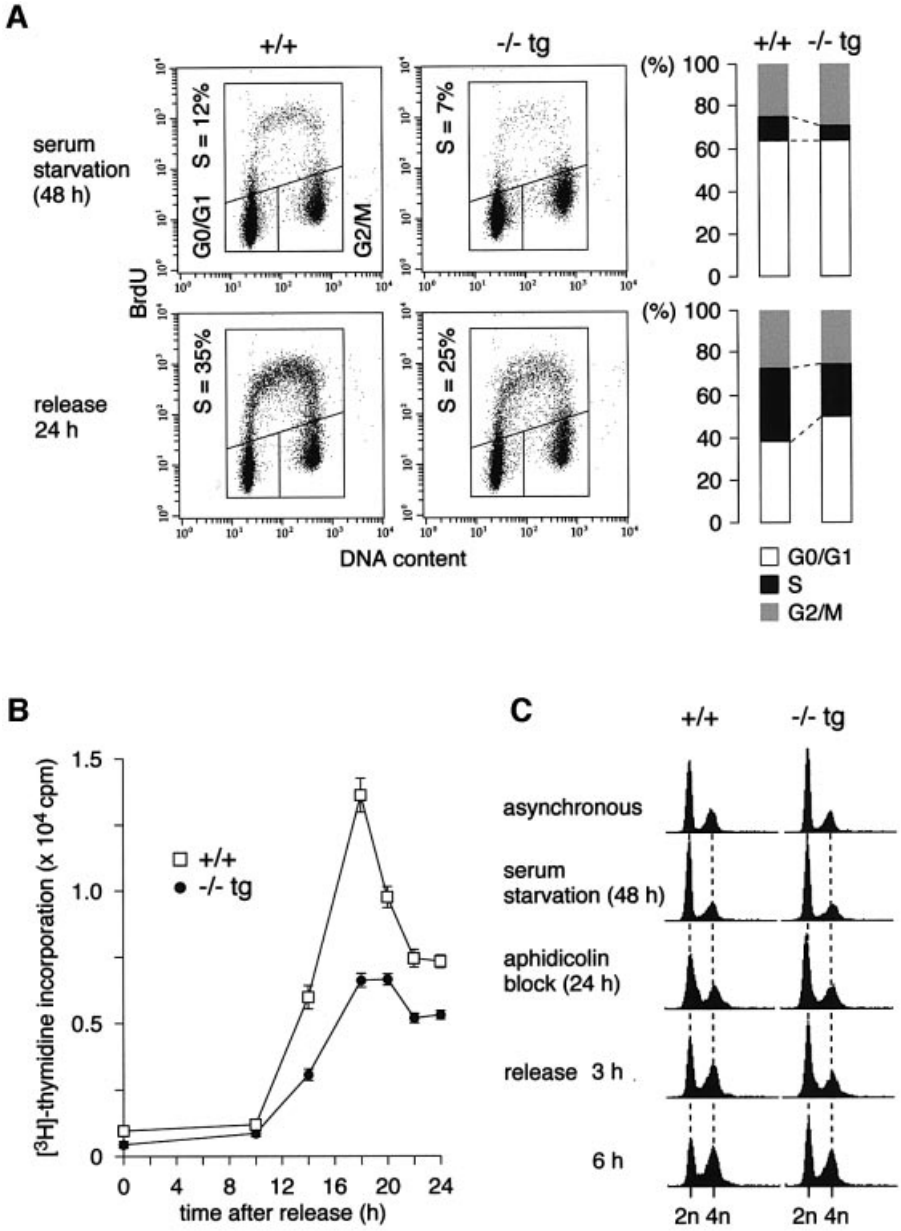

Fig. 3. *Cdc7*$^{-/-}$tg MEFs exhibit delayed S phase entry and slowed S phase progression. (**A**) Cell cycle distribution of *Cdc7*$^{-/-}$tg MEFs following serum starvation and release. The proportion of BrdU-positive cells was lower in the *Cdc7*$^{-/-}$tg MEFs (25%) than in the wild-type MEFs (35%). The gate settings for cells in G_0/G_1, S and G_2/M phases and percentage of cells in S phase are indicated. Consistent data were obtained from two independent experiments. (**B**) Incorporation of [^{3}H]thymidine during 2 h pulses at indicated time points following release from serum starvation. The values represent average of three independent experiments. (**C**) FACS analysis for DNA content. The cells were sequentially arrested in G_0 and early S phase by serum starvation and aphidicolin treatment, respectively. At the time of aphidicolin block, *Cdc7*$^{-/-}$tg MEFs contain more G_0/G_1 cells, and at 3 and 6 h after release from the aphidicolin block, they contain less S phase cells than the wild-type (+/+) MEFs.

population with concomitant decrease in S phase population compared with wild-type MEFs (Figure 2B). [^{3}H]thymidine incorporation in *Cdc7*$^{-/-}$tg MEFs was about half that in the wild-type MEFs (see Figure 9C). To detect actively proliferating cells, we performed immunofluorescence study using anti-proliferating cell nuclear antigen (PCNA) antibody (Figure 2C). While an extensive and strong nuclear PCNA staining was observed in the wild-type MEFs, the intensity and numbers of nuclear foci in the *Cdc7*$^{-/-}$tg MEFs were significantly low. These results indicate the numbers of replication forks and rate of DNA replication are significantly reduced in *Cdc7*$^{-/-}$tg cells, causing slow progression of S phase.

To further investigate the nature of the proliferative characteristics observed in the *Cdc7*$^{-/-}$tg MEFs, we examined cell cycle entry from quiescence. Following serum starvation and release, DNA synthesis of primary MEFs was measured by examining the incorporation of BrdU and [^{3}H]thymidine. FACS analysis demonstrated that, at 24 h after serum addition, the proportion of BrdU-positive cells was lower in the *Cdc7*$^{-/-}$tg MEFs (25%) than in the wild-type (35%) population (Figure 3A). The level of [^{3}H]thymidine incorporation of the *Cdc7*$^{-/-}$tg MEFs was about half that of the wild-type MEFs at most of the time points analyzed (Figure 3B). Delayed S phase entry was also observed in the *Cdc7*$^{-/-}$tg MEFs released from

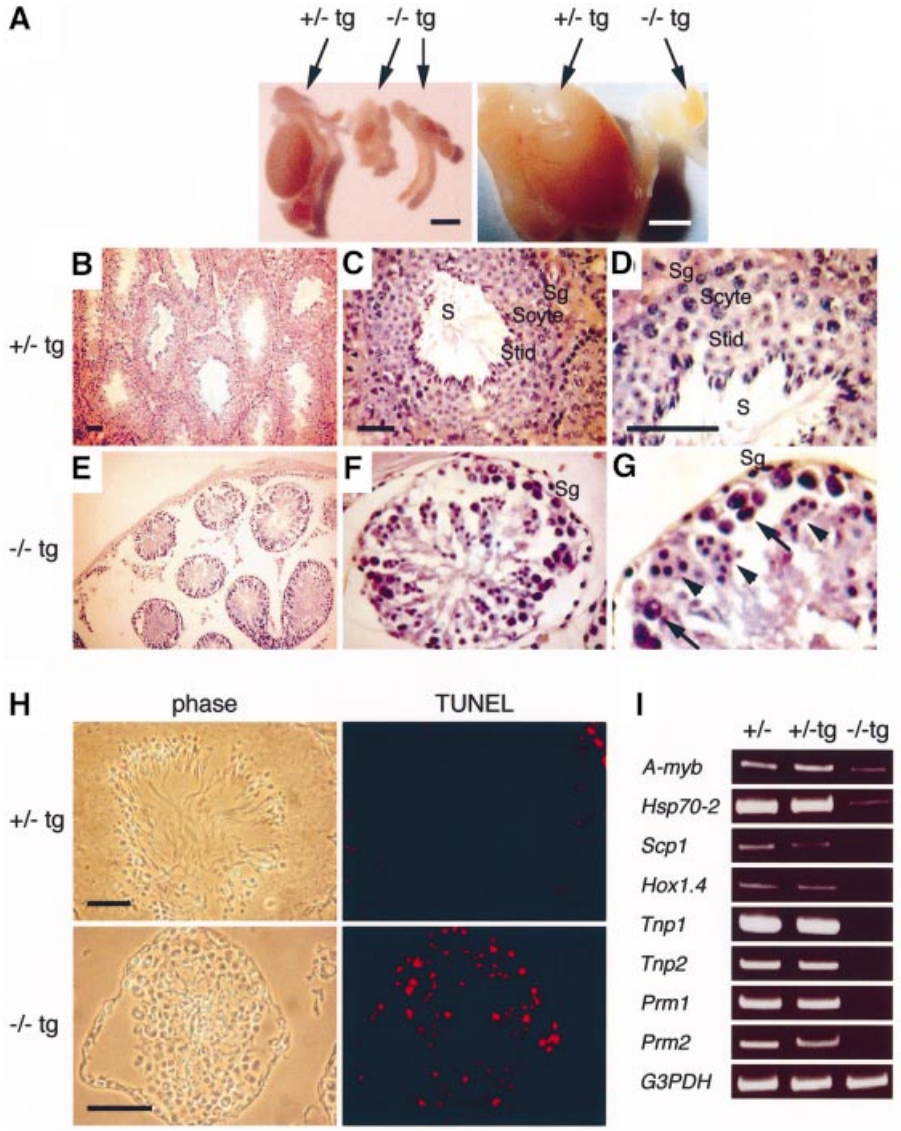

Fig. 4. Spermatogenesis is disrupted in *Cdc7*$^{-/-}$tg mice. (**A**) Gross appearance of testes from *Cdc7*$^{-/-}$tg mice. *Cdc7*$^{-/-}$tg testis is considerably smaller than *Cdc7*$^{+/-}$tg testis. Scale bars, 3 mm. (**B–G**) Histological analysis of *Cdc7*$^{-/-}$tg testes. Staining with hematoxylin and eosin reveals considerably reduced number of spermatogonia (Sg), degenerated spermatocytes (arrows) and accumulation of abnormal multi-nucleated cells (arrowheads) in seminiferous tubules of the *Cdc7*$^{-/-}$tg mice (E–G) in comparison with normal seminiferous tubules observed in *Cdc7*$^{+/-}$tg mice (B–D). Spermatids (Stid) and spermatozoa (S) are completely absent in *Cdc7*$^{-/-}$tg testes. Scyte, spermatocytes. Scale bars, 50 μm. (**H**) TUNEL assay demonstrates a massive increase of apoptotic cells (red fluorescence) in *Cdc7*$^{-/-}$tg testes as compared with the *Cdc7*$^{+/-}$tg testes. Scale bars, 30 μm. (**I**) Expression of developmental markers in testes of *Cdc7*$^{-/-}$tg mice. RT–PCR analysis shows very low level of A-*myb*, *Hsp70-2*, barely detectable level of *Scp1*, and complete absence of *Hox1.4*, *Tpn1*, *Tpn2*, *Prm1* and *Prm2* in *Cdc7*$^{-/-}$tg testes.

aphidicolin block at G_1/S boundary (Figure 3C). The cells were sequentially arrested in G_0 and early S phase by serum starvation and aphidicolin treatment, respectively. Although both *Cdc7*$^{-/-}$tg and wild-type MEFs were arrested with similar DNA content following serum starvation, more G_0/G_1 phase population was detected in *Cdc7*$^{-/-}$tg MEFs (69%) than in the wild-type MEFs (58%) after aphidicolin block. Furthermore, the increase of S phase population after release from the aphidicolin block was slower in *Cdc7*$^{-/-}$tg MEFs; at 3 and 6 h after release, S phase populations of *Cdc7*$^{-/-}$tg MEFs were 7 and 12%, respectively, whereas those of wild-type MEFs were 20 and 27%. Taken together, these results suggest that the decreased proliferative capability of *Cdc7*$^{-/-}$tg MEFs is attributable to delayed S phase entry and slowed S phase progression.

Cdc7$^{-/-}$*tg mice show testicular atrophy*

Male *Cdc7*$^{-/-}$tg mice were infertile. *Cdc7*$^{-/-}$tg testes were extremely small compared with those of the littermates (Figure 4A). Histological analysis of *Cdc7*$^{+/-}$tg testes showed seminiferous tubules containing spermatogonial stem cells at the periphery of the tubule and more differentiated cells towards the lumen, into which the mature spermatozoa are finally released (Figure 4B–D). In contrast, *Cdc7*$^{-/-}$tg testes showed considerably reduced number of seminiferous tubules that contained less spermatogonia and degenerated primary spermatocytes, together with abnormal multi-nucleated cells (Figure 4E–G). Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assays revealed that there were large numbers of apoptotic cells in *Cdc7*$^{-/-}$tg seminiferous tubules (Figure 4H). Remarkably, *Cdc7*$^{-/-}$tg

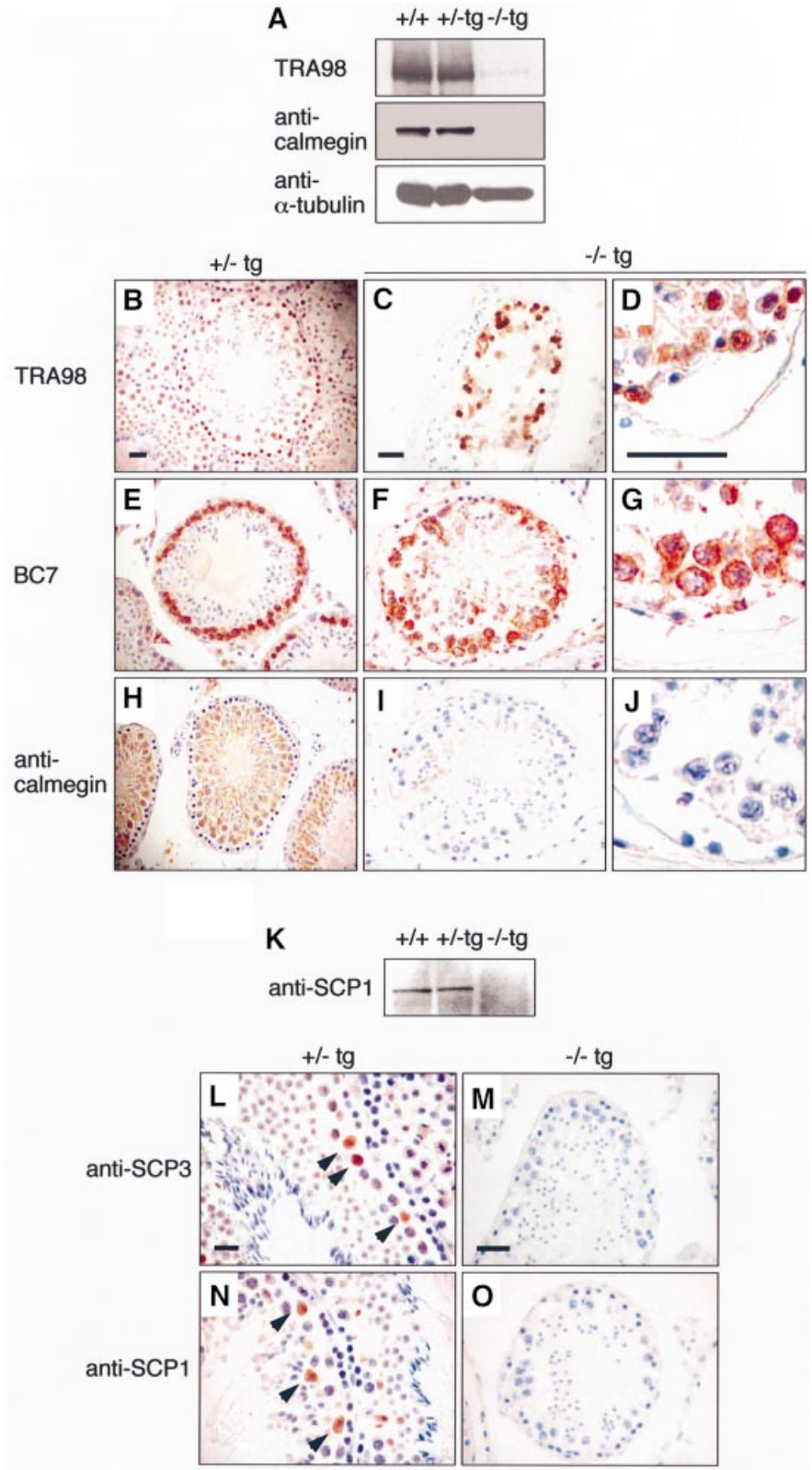

Fig. 5. Spermatogenesis is abrogated prior to pachytene stage in *Cdc7*$^{-/-}$tg male mice. (**A**) Western blot analysis using TRA98 and anti-calmegin antibody. Antigen for TRA98 is expressed at low levels in *Cdc7*$^{-/-}$tg testes, and calmegin is absent in *Cdc7*$^{-/-}$tg testes. (**B–J**) Immunostaining with TRA98 (B–D), BC7 (E–G) and anti-calmegin antibody (H–J). In contrast to the *Cdc7*$^{+/-}$tg testes (B, E and H), in which TRA98-, BC7- and anti-calmegin-positive cells are observed, the *Cdc7*$^{-/-}$tg testes show small number of TRA98-positive (C and D) and BC7-positive (F and G) cells, and no anti-calmegin-positive (I and J) cells. Scale bars, 20 μm. (**K**) Western blot analysis using anti-SCP1 antibody. SCP1 is expressed in the wild-type and *Cdc7*$^{+/-}$tg testes but not in *Cdc7*$^{-/-}$tg testes. (**L–O**) Immunostaining with anti-SCP3 and anti-SCP1 antibodies. In *Cdc7*$^{+/-}$tg testes (L and N), anti-SCP3-positive and anti-SCP1-positive spermatocytes were detected. In contrast, cells positive for either antibody were completely absent in *Cdc7*$^{-/-}$tg testes (M and O). Scale bars, 20 μm. Hematoxylin was used for counter staining.

testes were completely devoid of post-meiotic cells such as spermatids or mature spermatozoa, indicating that spermatogenesis is severely impaired in *Cdc7*$^{-/-}$tg mice. To further define the defect in spermatogenesis, we determined the expression of developmental markers by RT–PCR analysis (Figure 4I). A-*myb* is highly expressed in spermatogonia and spermatocytes at the stages of leptotene to pachytene (Mettus *et al*., 1994; Trauth *et al*., 1994). Expression of *Hsp70-2* is first detected in leptotene and peaks in pachytene spermatocytes (Allen *et al*., 1988; Zakeri *et al*., 1988). The expression of these earliest spermatogenesis markers was markedly decreased in *Cdc7*$^{-/-}$tg testes. *Scp1*, the expression of which is restricted in zygotene to diplotene spermatocytes (Meuwissen *et al*., 1992), was barely detectable in *Cdc7*$^{-/-}$tg testes. Expression of *Hox1.4*, which initiates in pachytene spermatocytes and persists thereafter (Rubin *et al*., 1986), was not detected in *Cdc7*$^{-/-}$tg testes. Likewise, *transition protein-1* (*Tnp1*) and *Tnp2*, as well as *protamine-1* (*Prm1*) and *Prm2*, which sequentially replace histones in post-meiotic spermatids (Kleene *et al*., 1984; Meistrich, 1989; Wolgemuth and Watrin, 1991), were not expressed in *Cdc7*$^{-/-}$tg testes. Together with the histological observations, these results indicate that spermatogenesis in *Cdc7*$^{-/-}$tg male is abrogated in the early stages of meiotic prophase I.

Spermatogenesis is abrogated prior to pachytene stage of meiotic prophase I in Cdc7$^{-/-}$*tg male mice*

To explore more closely the substages of meiosis at which spermatogenesis is disrupted in *Cdc7*$^{-/-}$tg testes, we examined the expression of stage-specific antigens of spermatogenic germ cells. Western blotting analysis using TRA98, an antibody the antigen of which is expressed in all types of spermatogonia, spermatocytes and round spermatids (Tanaka *et al*., 1997), detected a 110 kDa band in testicular extracts of all genotypes (Figure 5A). However, the expression level was markedly decreased in *Cdc7*$^{-/-}$tg testes. Immunostaining with TRA98 also showed the reduced numbers of TRA98-positive cells in *Cdc7*$^{-/-}$tg testes (Figure 5C and D) compared with *Cdc7*$^{+/-}$tg testes (Figure 5B). Immunostaining with BC7 (Koshimizu *et al*., 1993) detected zygotene and early pachytene spermatocytes near the periphery of the *Cdc7*$^{+/-}$tg seminiferous tubules (Figure 5E). In contrast, BC7-positive cells in *Cdc7*$^{-/-}$tg testes were reduced in numbers and were more dispersed within the seminiferous tubule (Figure 5F and G). Calmegin, a 93 kDa protein expressed in cells ranging from early pachytene spermatocytes to elongated spermatids (Watanabe *et al*., 1992, 1994), was detected in wild-type and *Cdc7*$^{+/-}$tg testes, but not in *Cdc7*$^{-/-}$tg testes (Figure 5A). Immunostaining results also confirmed the absence of calmegin in *Cdc7*$^{-/-}$tg testes (Figure 5I and J). These results indicate that spermatogenesis in the *Cdc7*$^{-/-}$tg males was abrogated prior to pachytene stage of meiotic prophase I. Most of the TRA98- and BC7-positive spermatocytes in *Cdc7*$^{-/-}$tg testes were also TUNEL-positive (data not shown), indicating they were undergoing apoptosis.

The absence of pachytene spermatocytes were further confirmed using antibodies against key regulatory proteins for the formation of synaptonemal complex (SC): SCP3/COR1 and SCP1/SYN1. During the zygotene and pachytene stages, SCP3 and SCP1 are localized along the chromosome synapsis (Dobson *et al*., 1994). Western blot analysis revealed that SCP1 was expressed in wild-type and *Cdc7*$^{+/-}$tg testes, whereas its expression was not observed in the *Cdc7*$^{-/-}$tg testis (Figure 5K). Immunostaining also confirmed the expression of SCP3 and SCP1 in *Cdc7*$^{+/-}$tg testes (Figure 5L and N), but not in *Cdc7*$^{-/-}$tg testes (Figure 5M and O). Based on these observations, we conclude that spermatogenesis in *Cdc7*$^{-/-}$tg mice is disrupted prior to pachytene stage of meiotic prophase I, most likely at the early zygotene stage.

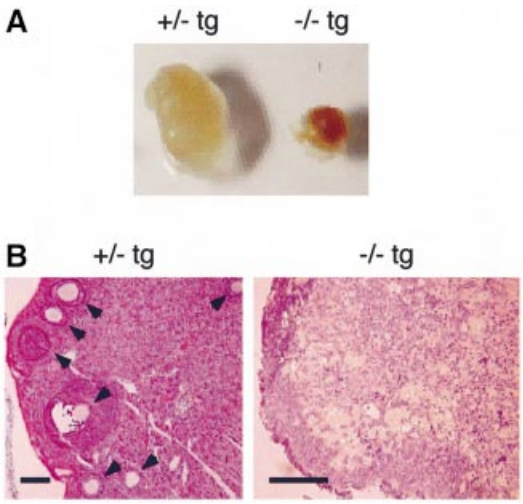

Fig. 6. Abnormal oogenesis in *Cdc7*$^{-/-}$tg females. (**A**) Gross appearance of each ovary from *Cdc7*$^{+/-}$tg and *Cdc7*$^{-/-}$tg females. The ovary from the *Cdc7*$^{-/-}$tg female was considerably smaller than that from *Cdc7*$^{+/-}$tg female. (**B**) Ovaries from adult *Cdc7*$^{+/-}$tg and *Cdc7*$^{-/-}$tg females were stained with hematoxylin and eosin. Arrows indicate the oocytes at various stages of maturation, forming large follicles in *Cdc7*$^{+/-}$tg female (left). No oocytes and follicles are present in *Cdc7*$^{-/-}$tg ovary (right). Scale bars, 100 μm.

Oogenesis is impaired in Cdc7$^{-/-}$*tg female mice*

Cdc7$^{-/-}$tg female mice were infertile. Ovaries of *Cdc7*$^{-/-}$tg females were considerably small and grossly malformed (Figure 6A). Histological analysis showed that the ovaries of *Cdc7*$^{-/-}$tg females contained no oocytes and follicles (Figure 6B). In contrast, the ovaries of *Cdc7*$^{+/-}$tg females contained oocytes at various stages of maturation, forming large follicles.

A limited amount of Cdc7 protein was expressed in Cdc7$^{-/-}$*tg MEFs and testes*

To understand the molecular basis underlying growth defects and testicular hypoplasia in *Cdc7*$^{-/-}$tg mice, we examined the expression of transgene-encoded Cdc7 (Figure 7). Murine Cdc7 protein is expressed in two catalytically active forms of different sizes in ES cells, MEFs and various tissues, resulting from the alternative splicing (Kim *et al*., 1998). Both forms are identical in terms of kinase activity *in vitro* (J.M.Kim and H.Masai, unpublished data). cDNA used to generate mu*Cdc7*$^{-/-}$tg ES cells encodes the shorter form and can perfectly support normal growth of *Cdc7*-deficient ES cells (Kim *et al*., 2002). In mu*Cdc7*$^{-/-}$tg ES cells, the expression level of Cdc7 was ~3- to 5-fold higher than that in the wild-type ES cells. In contrast, the expression level of Cdc7 in

Cdc7$^{-/-}$tg MEFs was five times lower than that in wild-type MEFs (Figure 7A). Thus, the transgene-encoded Cdc7 may not be present at a level sufficient to support normal growth of *Cdc7*$^{-/-}$tg MEFs. The expression of Cdc7 in *Cdc7*$^{-/-}$tg testes was even more severely decreased (Figure 7B). This closely correlates with developmental aberration of *Cdc7*$^{-/-}$tg testes. It would thus appear that decreased Cdc7 expression in testes leads to impaired testicular development and profound defect in spermatogenesis.

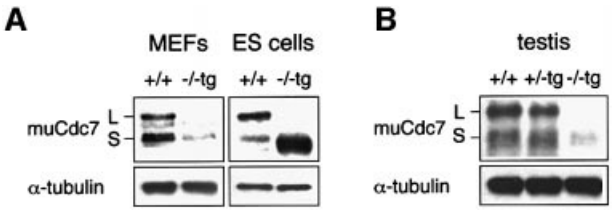

Fig. 7. Diminished expression of Cdc7 in *Cdc7*$^{-/-}$tg MEFs and testes. The levels of Cdc7 protein in MEFs [(**A**), left], ES cells [(A), right] and testes (**B**) were determined by western blot analysis. *Cdc7*$^{-/-}$tg MEFs express Cdc7 protein at levels five times lower than that in wild-type MEFs, whereas the level of Cdc7 in *Cdc7*$^{-/-}$tg ES cells is ~3- to 5-fold higher than that of the wild-type ES cells. The Cdc7 protein level in *Cdc7*$^{-/-}$tg testes is 10 times lower than those in the wild-type and *Cdc7*$^{+/-}$tg testes. α-tubulin is shown as a loading control. L and S denote two alternative spliced forms of murine Cdc7 protein, which are identical in functions. The transgene encodes the form S.

Thymic and peripheral lymphocytes develop normally in Cdc7$^{-/-}$*tg mice*

In contrast to the impaired testicular development, no developmental defect was found in thymi of *Cdc7*$^{-/-}$tg mice. Histological examination of *Cdc7*$^{-/-}$tg thymi showed normal cortex–medulla compartmentalization (data not shown), and there were no significant differences in the CD4 and CD8 levels and distribution of subsets (Figure 8A). Moreover, proliferative response of *Cdc7*$^{-/-}$tg thymocytes was unperturbed; rather, they showed modestly increased [^{3}H]thymidine incorporation in response to stimulation with anti-CD3 plus varying doses of interleukin (IL)-2 (Figure 8B). Consistently, the expression level of transgene-encoded Cdc7 was only modestly decreased in thymi of *Cdc7*$^{-/-}$tg mice (Figure 8C).

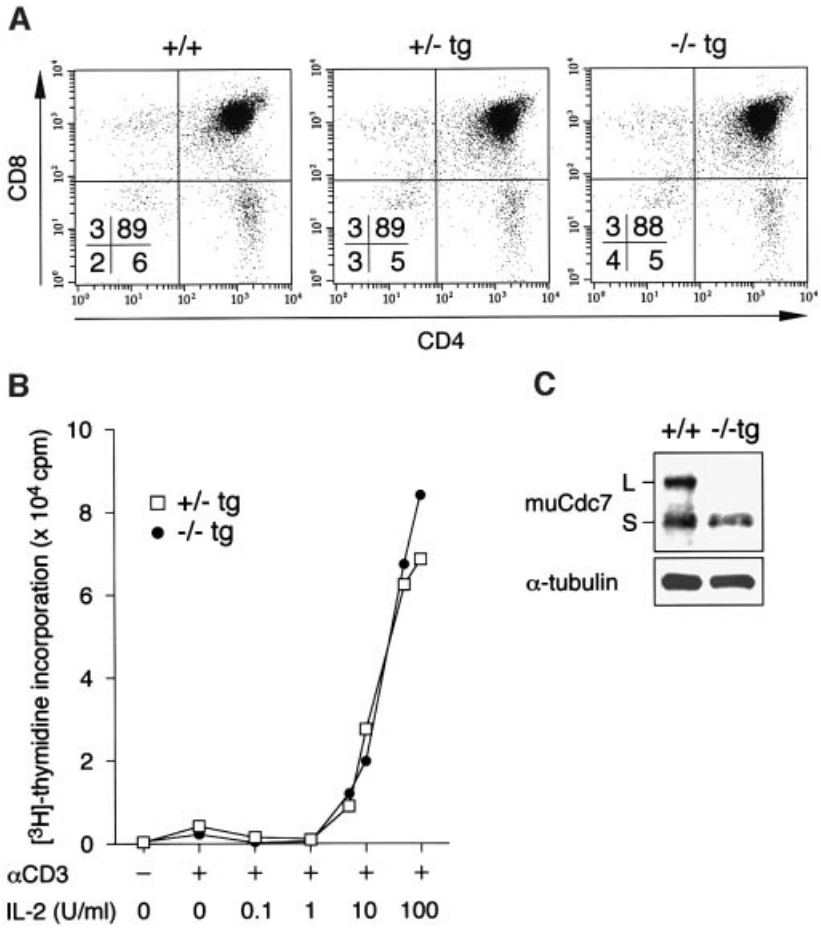

Fig. 8. Thymic development was normal in *Cdc7*$^{-/-}$tg mice. (**A**) Flow-cytometric analysis of thymocytes from *Cdc7*$^{-/-}$tg mice. Expression of CD4 and CD8 in thymocytes of *Cdc7*$^{-/-}$tg mice and their control littermates was analyzed. Proportions of cells in the quadrants are shown. Staining and analysis were performed on at least three animals of each genotype with similar results. (**B**) Thymocytes prepared from *Cdc7*$^{+/-}$tg and *Cdc7*$^{-/-}$tg mice were stimulated using anti-CD3ε antibody and IL-2 of various doses. Mitogenic activation of *Cdc7*$^{-/-}$tg thymocytes is unperturbed. Data shown are averages of three independent experiments. (**C**) The levels of Cdc7 protein in thymi from wild-type (+/+) and *Cdc7*$^{-/-}$tg mice were determined by western blot analysis with anti-murine Cdc7 antibody. *Cdc7*$^{-/-}$tg thymi express Cdc7 protein at the level ~65% that of wild-type thymi. α-tubulin is shown as a loading control. L and S denote two alternative spliced forms of murine Cdc7 protein.

Peripheral CD4 T and B cells of *Cdc7*$^{-/-}$tg mice also expressed amounts of Cdc7 comparable to the wild-type level and retained the proliferative capacity in response to various mitogenic stimulations (data not shown).

Proliferative capacity was recovered in Cdc7$^{-/-}$*tg/tg MEFs*

We next examined whether increase in Cdc7 protein level can rescue the developmental abnormalities of *Cdc7*$^{-/-}$tg mice. To achieve this, we generated *Cdc7*$^{-/-}$tg/tg mice harboring the homozygous *Cdc7* transgene by extensive intercrossing between *Cdc7*$^{+/-}$tg mice. The level of Cdc7 expression in *Cdc7*$^{-/-}$tg/tg MEFs was recovered to approximately two-thirds that of the wild-type MEFs (Figure 9A). Accordingly, the proliferation rate and level of DNA synthesis of *Cdc7*$^{-/-}$tg/tg MEFs were restored (Figure 9B and C). *Cdc7*$^{-/-}$tg/tg MEFs also showed a level of [^{3}H]thymidine incorporation similar to that observed in the wild-type MEFs, following release from serum starvation (Figure 9D). These results are in a sharp contrast to the reduced proliferative activities of *Cdc7*$^{-/-}$tg MEFs, and indicate that increase of Cdc7 expression can indeed restore normal growth in *Cdc7*$^{-/-}$tg MEFs.

Spermatogenesis is restored in Cdc7$^{-/-}$*tg/tg mice*

Cdc7$^{-/-}$tg/tg mice gained normal body weight (Figure 10A). Notably, these mice were fertile and yielded progeny, with significant recovery of Cdc7 protein level in testes (Figure 10B). Histological analysis revealed the presence of considerable numbers of spermatids and mature spermatozoa in *Cdc7*$^{-/-}$tg/tg testes (Figure 10C), indicating that increased Cdc7 expression restored spermatogenesis and rescued infertility, although the size of testes was not completely restored by the presence of an additional copy of the transgene (data not shown).

Discussion

Highly regulated cell-cycle progression is attained by concerted actions of multiple kinases. *Cdc7* plays essential roles in initiation of DNA replication, and its targeted deletion leads to early embryonic lethality, precluding further analyses of its role in mouse development. In this study, we generated and analyzed mice with a hypomorphic allele of *Cdc7* under a *Cdc7*-null background. Our results demonstrate that the levels of Cdc7 are critical for normal mouse development. The mutant mice were growth-retarded and suffered from severe defects in gametogenesis. MEFs derived from *Cdc7* mutant mice showed decreased proliferative properties and exhibited a substantial delay in S phase entry after serum starvation–restimulation. Intriguingly, all these defects were corrected by increasing the expression level of Cdc7 *in vivo*.

Growth defects of Cdc7$^{-/-}$*tg mice*

Cdc7$^{-/-}$tg mice arose at a frequency considerably lower than the expected Mendelian ratio. The growth retardation in *Cdc7*$^{-/-}$tg mice was observed as early as at E13.5, and expected frequencies of viable *Cdc7*$^{-/-}$tg embryos were observed until E17.5 (data not shown), suggesting that most of the missing *Cdc7*$^{-/-}$tg mice died during later stages of embryogenesis or just after birth. One notable abnormality of *Cdc7*$^{-/-}$tg mice was tail flexion anomalies. All of *Cdc7*$^{-/-}$tg newborns that died at or shortly after birth displayed severe tail flexion, whereas the *Cdc7*$^{-/-}$tg mice that reached adulthood suffered from hunched posture but from less severe tail abnormality. Among a number of mutant mice displaying tail flexion anomalies, the *ct* mutant mouse (Gruneberg, 1954) is regarded as an animal model of neural tube defects (NTDs) in humans, of which spina bifida and anencephaly are the most prevalent forms (Embury *et al*., 1979). Since spinal NTD in *ct* mutant mice has been ascribed to decreased cellular proliferation in the

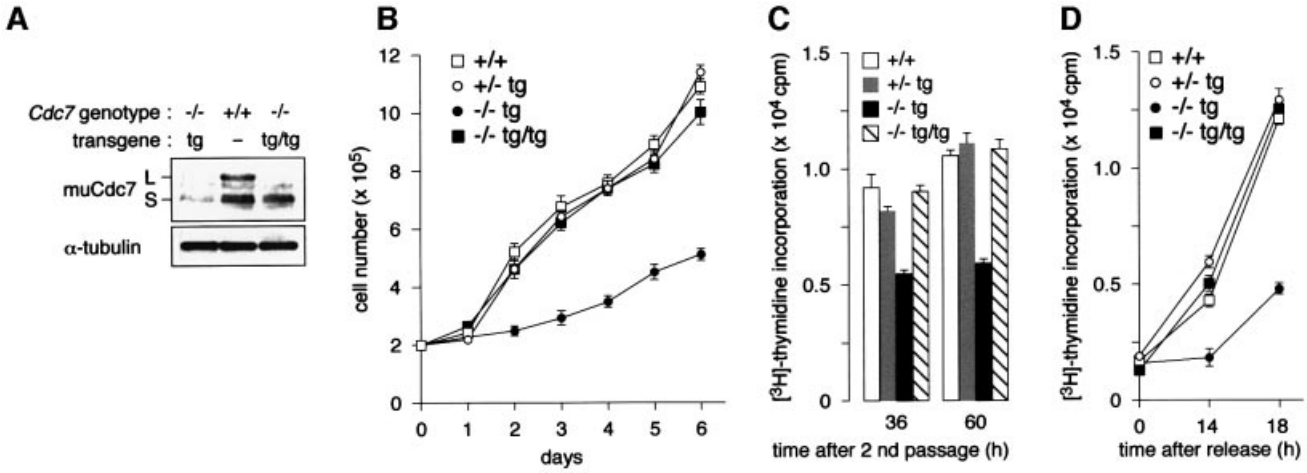

Fig. 9. An additional allele of transgene rescues proliferation defects of *Cdc7*$^{-/-}$tg MEFs. (**A**) Expression of Cdc7 protein in MEFs derived from wild-type (+/+), *Cdc7*$^{-/-}$tg and *Cdc7*$^{-/-}$tg/tg embryos. The level of Cdc7 in *Cdc7*$^{-/-}$tg/tg MEFs is restored close to that in wild-type MEFs. (**B**) MEFs at passage 2 were plated in duplicate at 2×10^5/60 mm plate, and cell numbers were determined daily for 6 days. The cell number at each time point is the mean value of three independent experiments. *Cdc7*$^{-/-}$tg/tg MEFs grow at a rate comparable to that of the wild type MEFs. In (**C**) and (**D**), [^{3}H]thymidine incorporation was measured in asynchronously growing MEFs [passage 2, (C)] or in MEFs released from serum starvation [passage 3, (D)], respectively. The levels of [^{3}H]thymidine incorporation in *Cdc7*$^{-/-}$tg/tg MEFs are restored to those in the control MEFs in both experiments. The values represent averages of three independent experiments.

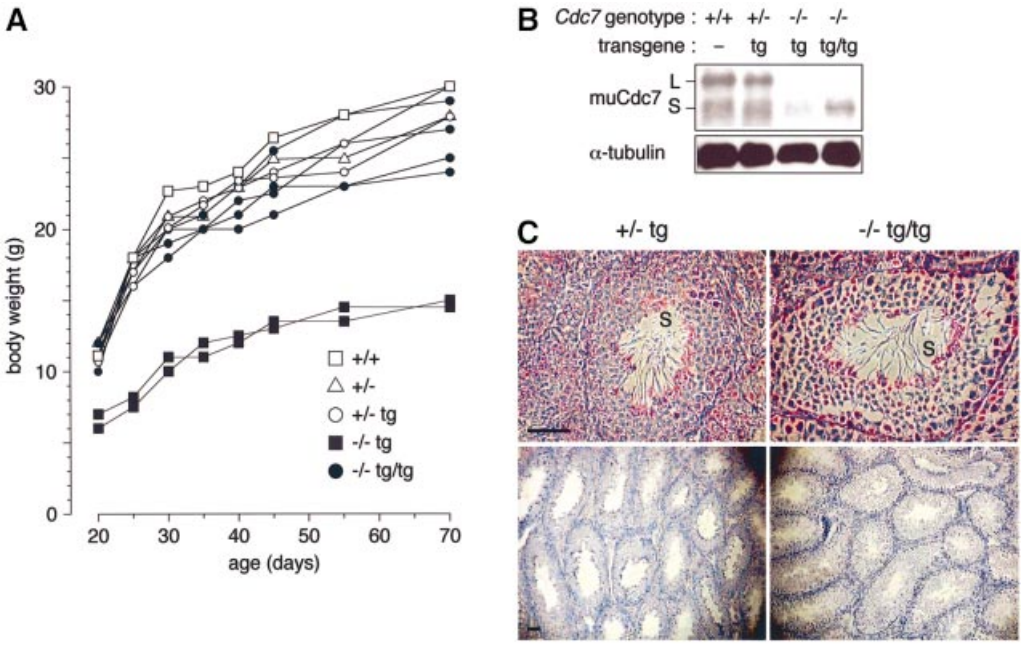

Fig. 10. *Cdc7*$^{-/-}$tg/tg mice exhibit normal body weight and restored spermatogenesis. (**A**) Growth curve of wild-type, *Cdc7*$^{+/-}$, *Cdc7*$^{+/-}$tg, *Cdc7*$^{-/-}$tg and *Cdc7*$^{-/-}$tg/tg mice. Mice were weighed at intervals and plotted against age in days. (**B**) The level of Cdc7 protein in testes from mice with the indicated genotypes. Significant recovery of the Cdc7 protein level was observed in *Cdc7*$^{-/-}$tg/tg testes. L and S denote two alternative spliced forms of murine Cdc7 protein. (**C**) Histological analyses in *Cdc7*$^{+/-}$tg (left) and *Cdc7*$^{-/-}$tg/tg (right) testes. *Cdc7*$^{-/-}$tg/tg testes contained considerable numbers of spermatozoa (S). Scale bars, 50 μm. See Figure 3F for comparison.

hindgut endoderm and notochord (Copp *et al*., 1988a,b), it is conceivable that the suboptimal expression of Cdc7 is detrimental to normal neurulation during embryogenesis. Histological examination of *Cdc7*$^{-/-}$tg adult brains revealed normal morphogenesis, and mutant mice did not display neurological abnormalities such as incomplete eye opening, impaired movements and the leg-clasping reflex (data not shown). These results suggest that no obvious defects are associated with adult neural tissues in *Cdc7*$^{-/-}$tg mice, although there may be some defects in development of neural cells during embryogenesis. The tail flexion anomalies may also be caused by abnormal skeletal development (Connor *et al*., 1997; Ishijima *et al*., 1998; Regnier *et al*., 2002). The longitudinal growth of the skeleton arises from the continuous process of bone formation, in which osteoblast proliferation comprises a critical component. Thus, it is also possible that Cdc7 is involved in the control of osteoblast proliferation in embryos undergoing bone formation, and its insufficiency results in prenatal or neonatal lethality in some *Cdc7*$^{-/-}$tg mice.

Essential roles of Cdc7 in mouse germ cell development

Spermatogenesis in mammals is characterized by a well-defined sequence of mitotic and meiotic divisions that lead to the production of mature spermatozoa (McCarrey, 1993). The mitotic divisions of spermatogonial stem cells lead to the development of type A and type B spermatogonia, the latter of which differentiate into preleptotene spermatocytes. After meiotic S phase, preleptotene spermatocytes enter meiotic prophase I as leptotene spermatocytes (de Rooij and van Dissel-Emiliani, 1997). *Cdc7*$^{-/-}$tg mice showed testicular atrophy with reduced numbers of spermatogonia, degenerated primary spermatocytes, and accumulation of abnormal multi-nucleated cells. Immunohistochemical analyses revealed the presence of arrested, degenerating zygotene spermatocytes in *Cdc7*$^{-/-}$tg testes. These observations raise the intriguing possibility that Cdc7 might be involved in early meiotic prophase I in addition to its expected roles in germ cell proliferation. We recently found that a temperature-sensitive mutation in *hsk1*, the fission yeast homolog of *Cdc7* (Masai *et al*., 1995; Takeda *et al*., 2001), results in arrest of meiotic process at or prior to prophase of meiosis I (K.Ogino and H.Masai, submitted), suggesting that essential roles of *Cdc7* kinase in meiosis may be conserved from yeasts to mammals. Similar testicular atrophy has been also observed in *Cdk4*-null mice (Rane *et al*., 1999; Tsutsui *et al*., 1999). Seminiferous tubules in *Cdk4*$^{-/-}$ mice show reduced numbers of spermatogonia, degeneration of spermatocytes and numerous apoptotic bodies. Three D-type *cyclins* (*cyclin D1*, *D2* and *D3*) are differentially expressed at the various stages throughout spermatogenesis (Ravnik *et al*., 1995; Zhang *et al*., 1999; Beumer *et al*., 2000). Thus, it seems that *Cdk4*, in combination with D-type *cyclins*, is involved in germ cell proliferation and differentiation. In somatic cell-cycle progression, activation of *Cdk4* during early- to mid-G_1 phase is followed by activation of *Cdk2* and *Cdc7* at the G_1/S transition. Therefore, *Cdc7* may promote mitotic cell cycle in spermatogonia downstream of *Cdk4*. *Cdc7* may be also required for meiotic S phase and, possibly, the following meiotic prophase I in mice, as was previously indicated for budding yeast *Cdc7* kinase (Sclafani *et al*., 1988).

Cdc7$^{-/-}$tg females were also infertile and we did not observe any oocytes and follicles in the ovary. In mammals, oocytes undergo meiosis from E12 to E18 in

the fetal ovary, and by 5 days *post partum* all oocytes are arrested in the postpachytene dictyate stage ready for maturation (Hogan *et al.*, 1994). In contrast to male spermatogenesis, where germ cells proliferate continuously and repeat the differentiation cycle throughout their lifetime, oogonia do not proliferate after birth. By analogy with the disrupted spermatogenesis observed in *Cdc7*$^{-/-}$tg males, defects of oogenesis in *Cdc7*$^{-/-}$tg females may manifest before the pachytene stage *in utero*. Therefore, the meiotic arrest and subsequent apoptotic cell death may result in the complete degeneration of germ cells in *Cdc7*$^{-/-}$tg females.

Requirement of a critical level of Cdc7 protein for mammalian development

Diminished expression of Cdc7 in *Cdc7*$^{-/-}$tg testes and MEFs correlated with their developmental and proliferative defects. We also generated another line of *Cdc7*$^{-/-}$tg mice bearing a single copy of transgene by the microinjection method. These mice displayed similar growth retardation and disrupted spermatogenesis with decreased expression of Cdc7. Since the repression of transgene expression is not restricted to testis and is observed in at least two independent strains, this could be more or less general repression of the EF1α promoter in some of the differentiated cells. However, we cannot exclude the possibility that the transgenes of both mice are integrated into one of the loci that undergo silencing in differentiating testes, since overall gene expression profile is profoundly altered during spermatogenesis (Sassone-Corsi, 2002). In contrast, the development of thymic and peripheral lymphocytes of *Cdc7*$^{-/-}$tg mice was largely normal, in which the expression level of Cdc7 was only modestly decreased. The increased level of Cdc7 protein in *Cdc7*$^{-/-}$tg/tg mice, although still below the level of endogenous one, reversed the defects of testes and MEFs. We did not detect any abnormality in Cdc7 heterozygous mice, supporting the notion that the endogenous Cdc7 protein level of wild-type cells may be present in excess. Taken together, it appears that Cdc7 protein must be kept above a certain threshold level to achieve normal mouse development. Since mouse Cdc7 is expressed in two catalytically active forms, it is also conceivable that each isoform has some specific functions, and both are required for normal mouse development.

A strategy for genetic characterization of cell-cycle regulators using hypomorphic mutant mice

Tissue-specific gene targeting can be achieved by excision of a specific DNA segment, which is flanked by *loxP* sites, upon expression of Cre recombinase in a tissue of interest. Although this approach is commonly used, it has been shown that Cre expression in primary MEFs or developing spermatids can cause growth-inhibitory and genotoxic effects (Schmidt *et al.*, 2000; de Alboran *et al.*, 2001; Loonstra *et al.*, 2001; Silver and Livingston, 2001). Such Cre toxicity may confuse interpretation of the phenotypic analysis of the conditional deletion, especially when the gene of interest is involved in cell growth. Our approach utilizes mutant mice that express a given protein only from transgene under the control of a heterologous promoter. By modifying the tissue specificity and the strength of the promoter used for the transgene, one would be able to generate mutant mice with a tissue-specific hypomorphic allele.

In conclusion, our genetic studies point, for the first time, to the critical role for an appropriate protein level of an essential cell-cycle regulator in mouse development. Possible involvement of hypomorphic mutations in cell-cycle-regulatory genes in human diseases remains to be examined. Our study also disclosed the pivotal roles of *Cdc7* in the early stages of spermatogenesis and oogenesis. It will be interesting to examine whether hypomorphic *Cdc7* mutations are associated with individuals suffering from infertility.

Materials and methods

Generation of *Cdc7*$^{-/-}$tg mice

mu*Cdc7*$^{-/-}$tg ES cells were injected into C57BL/6 blastocysts. Chimeric mice were crossed with C57BL/6 females to produce *Cdc7*$^{+/-}$tg mice, which were subsequently crossed with *Cdc7*$^{+/-}$ mice to generate *Cdc7*$^{-/-}$tg mice. *Cdc7*$^{-/-}$tg/tg mice with the homozygous transgene were generated by intercrossing *Cdc7*$^{+/-}$tg mice.

Antibodies

Rabbit polyclonal anti-mouse Cdc7 antibody was described previously (Kim *et al.*, 1998). Polyclonal mouse anti-COR1 (anti-SCP3) antibody that recognizes mouse SCP3 and rabbit anti-SYN1 (anti-SCP1) antibody that recognizes mouse SCP1 were kindly provided by Dr P.B.Moens (York University). Rat monoclonal antibodies TRA98, anti-calmegin (TRA369), and BC7 were generously provided by Dr Y.Nishimune (Osaka University). The antibodies against PCNA and α-tubulin were purchased from Santa Cruz and Sigma, respectively.

Isolation of MEFs

Cdc7$^{+/-}$tg males were mated with *Cdc7*$^{+/-}$ females, and embryos were isolated at E13.5. MEFs were prepared according to standard procedures and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G/streptomycin sulfate.

BrdU labeling of MEFs

Passage 2 MEFs were incubated for 2 h in the presence of 10 μM BrdU. Cells were stained with FITC-conjugated anti-BrdU antibody (PharMingen) and subjected to FACS analysis.

Immunostaining with anti-PCNA antibody

Cells were fixed for 5 min in methanol:acetone solution (1:1) and incubated with anti-PCNA antibody (1:200 dilution) for 20 min at 37°C. Alexa 488-conjugated anti-mouse IgG (Molecular Probes) was used as a secondary antibody.

Synchronization of MEFs

Passage 3 MEFs were incubated in DMEM containing 0.1% FBS for 48 h, and were restimulated in culture medium containing 15% FBS. For [^{3}H]thymidine incorporation, the cells were incubated with 1 μCi of [^{3}H]thymidine for 2 h at each time point after serum release. For BrdU incorporation, cells were pulse-labeled with 100 μM BrdU for 30 min. For aphidicolin block, cells serum-starved for 48 h were subsequently incubated in medium containing 15% FBS and aphidicolin (1 μg/ml) for 24 h. After release from the aphidicolin block, cells were harvested at various times for FACS analysis.

Histological analysis

Tissues were fixed in 10% buffered neutral formalin, dehydrated and embedded in paraffin. Sections (5 μm) from each tissue were stained with hematoxylin–eosin. Apoptotic cells were detected using an *in situ* Cell Death Detection Kit (Roche).

Immunohistochemistry

Testes were fixed in Bouin's solution for 24 h and embedded in paraffin. Sections were incubated with TRA98, anti-calmegin and BC7 at 1:400, and anti-SCP3 and anti-SCP1 antibodies at 1:200. Horseradish peroxidase (HRP)-conjugated secondary antibodies were used at 1:100, and the detection was performed by HRP reaction with DAB as a substrate.

RT–PCR

Total RNA was extracted from adult testes of the wild-type, *Cdc7*$^{+/-}$tg and *Cdc7*$^{-/-}$tg mice using TRIZOL reagent (Gibco-BRL). Single-stranded cDNAs were prepared by reverse transcription using 5 μg of testes RNA. The following sequences were used as the primers: *Hsp70-2*, sense, 5′-AGTCATCACTGTTCCTGCCTA-3′, antisense, 5′-TAGAGCGAGTC-GATCTCTATG-3′; *Tnp1*, sense, 5′-ATGTCGACCAGCCGCAAGCTA-3′, antisense, 5′-CGAATTTCGTCACGATGGCAT-3′; *Tnp2*, sense, 5′-ATGGACACCAAGATGCAGAGC-3′, antisense, 5′-TCACTTGTATC-TTCGCCCTGA-3′; *Prm1*, sense, 5′-ATGGCCAGATACCGATGCTGC-3′, antisense, 5′-CTAGTATTTTTTACACCTTAT-3′; *Prm2*, sense, 5′-ATGGTTCGCTACCGAATGAGG-3′, antisense, 5′-TTAGTGATGGT-GCCTCCTACA-3′; *G3pdh*, sense, 5′-AGTGGAGATTGTTGCCA-TCAACGA-3′, antisense, 5′-TCATACTTGGCAGGTTTCTCCAGG-3′. The sequences of the primers for A-*myb*, *Scp1* and *Hox1.4* were described previously (Tanaka *et al*., 2000).

Western blot analysis

MEFs were lysed in a lysis buffer [50 mM Tris pH 7.6, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptine and 10 μg/ml aprotinin]. Freshly prepared mouse organs were homogenized in a lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 20 μg/ml leupeptine and 20 μg/ml aprotinin). Lysates were cleared by centrifugation and were used for western blot analysis. TRA98 and anti-calmegin rat monoclonal antibodies were diluted 1:1000, and polyclonal rabbit anti-SCP1 antibody was diluted 1:500. The detection was performed by chemiluminescence ECL kit using HRP-conjugated secondary antibodies.

Activation of thymocytes and proliferation assay

Thymocytes (2×10^5) were placed into 96-well plates and were stimulated using anti-CD3ε (200 ng/ml) antibodies and varying doses of IL-2 (0.1–100 U/ml). Cells were pulsed at 48 h with 1 μCi of [^{3}H]thymidine, and harvested 12 h later.

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References

Allen,R.L., O'Brien,D.A. and Eddy,E.M. (1988) A novel hsp70-like protein (P70) is present in mouse spermatogenic cells. *Mol. Cell. Biol.*, **8**, 828–832.

Bell,S.P. and Dutta,A. (2002) DNA replication in eukaryotic cells. *Annu. Rev. Biochem.*, **71**, 333–374.

Beumer,T.L., Roepers-Gajadien,H.L., Gademan,I.S., Kal,H.B. and de Rooij,D.G. (2000) Involvement of the D-type cyclins in germ cell proliferation and differentiation in the mouse. *Biol. Reprod.*, **63**, 1893–1898.

Brown,G.W. and Kelly,T.J. (1999) Cell cycle regulation of Dfp1, an activator of the Hsk1 protein kinase. *Proc. Natl Acad. Sci. USA*, **96**, 8443–8448.

Chapman,J.W. and Johnston,L.H. (1989) The yeast gene, *DBF4*, essential for entry into S phase is cell cycle regulated. *Exp. Cell Res.*, **180**, 419–428.

Connor,F., Bertwistle,D., Mee,P.J., Ross,G.M., Swift,S., Grigorieva,E., Tybulewicz,V.L. and Ashworth,A. (1997) Tumorigenesis and a DNA repair defect in mice with a truncating *Brca2* mutation. *Nat. Genet.*, **17**, 423–430.

Copp,A.J., Brook,F.A. and Roberts,H.J. (1988a) A cell-type-specific abnormality of cell proliferation in mutant (*curly tail*) mouse embryos developing spinal neural tube defects. *Development*, **104**, 285–295.

Copp,A.J., Crolla,J.A. and Brook,F.A. (1988b) Prevention of spinal neural tube defects in the mouse embryo by growth retardation during neurulation. *Development*, **104**, 297–303.

de Alboran,I.M., O'Hagan,R.C., Gartner,F., Malynn,B., Davidson,L., Rickert,R., Rajewsky,K., DePinho,R.A. and Alt,F.W. (2001) Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. *Immunity*, **14**, 45–55.

de Rooij,D.G. and van Dissel-Emiliani,F.M.F. (1997) Regulation of proliferation and differentiation of stem cells in the male germ line. In Potten,C.S. (ed.), *Stem Cells*. Academic Press, San Diego, CA, pp. 283–313.

Dobson,M.J., Pearlman,R.E., Karaiskakis,A., Spyropoulos,B. and Moens,P.B. (1994) Synaptonemal complex proteins: occurrence, epitope mapping and chromosome disjunction. *J. Cell Sci.*, **107**, 2749–2760.

Dutta,A. and Bell,S.P. (1997) Initiation of DNA replication in eukaryotic cells. *Annu. Rev. Cell. Dev. Biol.*, **13**, 293–332.

Embury,S., Seller,M.J., Adinolfi,M. and Polani,P.E. (1979) Neural tube defects in *curly-tail* mice. I. Incidence, expression and similarity to the human condition. *Proc. R. Soc. Lond. B Biol. Sci.*, **206**, 85–94.

Gruneberg,H. (1954) Genetical studies on the skeleton of the mouse: VIII. *curly-tail*. *J. Genet.*, **52**, 52–67.

Hogan,B., Beddington,R., Costantini,F. and Lacy,E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hollingsworth,R.E.,Jr and Sclafani,R.A. (1990) DNA metabolism gene *CDC7* from yeast encodes a serine (threonine) protein kinase. *Proc. Natl Acad. Sci. USA*, **87**, 6272–6276.

Ishijima,J., Yasui,H., Morishima,M. and Shiroishi,T. (1998) Dominant lethality of the mouse skeletal mutation tail-short (*Ts*) is determined by the *Ts* allele from mating partners. *Genomics*, **49**, 341–350.

Jackson,A.L., Pahl,P.M., Harrison,K., Rosamond,J. and Sclafani,R.A. (1993) Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. *Mol. Cell. Biol.*, **13**, 2899–2908.

Jiang,W. and Hunter,T. (1997) Identification and characterization of a human protein kinase related to budding yeast Cdc7p. *Proc. Natl Acad. Sci. USA*, **94**, 14320–14325.

Jiang,W., McDonald,D., Hope,T.J. and Hunter,T. (1999) Mammalian Cdc7–Dbf4 protein kinase complex is essential for initiation of DNA replication. *EMBO J.*, **18**, 5703–5713.

Johnston,L.H. and Thomas,A.P. (1982a) A further two mutants defective in initiation of the S phase in the yeast *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **186**, 445–448.

Johnston,L.H. and Thomas,A.P. (1982b) The isolation of new DNA synthesis mutants in the yeast *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **186**, 439–444.

Johnston,L.H., Masai,H. and Sugino,A. (1999) First the CDKs, now the DDKs. *Trends Cell Biol.*, **9**, 249–252.

Kim,J.M., Sato,N., Yamada,M., Arai,K. and Masai,H. (1998) Growth regulation of the expression of mouse cDNA and gene encoding a serine/threonine kinase related to *Saccharomyces cerevisiae CDC7* essential for G_1/S transition. Structure, chromosomal localization and expression of mouse gene for *S. cerevisiae CDC7*-related kinase. *J. Biol. Chem.*, **273**, 23248–23257.

Kim,J.M., Nakao,K., Nakamura,K., Saito,I., Katsuki,M., Arai,K. and Masai,H. (2002) Inactivation of Cdc7 kinase in mouse ES cells results in S-phase arrest and p53-dependent cell death. *EMBO J.*, **21**, 2168–2179.

Kleene,K.C., Distel,R.J. and Hecht,N.B. (1984) Translational regulation and deadenylation of a protamine mRNA during spermiogenesis in the mouse. *Dev. Biol.*, **105**, 71–79.

Koshimizu,U., Watanabe,D., Sawada,K. and Nishimune,Y. (1993) A novel stage-specific differentiation antigen is expressed on mouse testicular germ cells during early meiotic prophase. *Biol. Reprod.*, **49**, 875–884.

Kumagai,H., Sato,N., Yamada,M., Mahony,D., Seghezzi,W., Lees,E., Arai,K. and Masai,H. (1999) A novel growth- and cell cycle-regulated protein, ASK, activates human Cdc7-related kinase and is essential for G_1/S transition in mammalian cells. *Mol. Cell. Biol.*, **19**, 5083–5095.

Loonstra,A., Vooijs,M., Beverloo,H.B., Allak,B.A., van Drunen,E., Kanaar,R., Berns,A. and Jonkers,J. (2001) Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. *Proc. Natl Acad. Sci. USA*, **98**, 9209–9214.

Masai,H., Miyake,T. and Arai,K. (1995) *hsk1*$^+$, a *Schizosaccharomyces pombe* gene related to *Saccharomyces cerevisiae* CDC7, is required for chromosomal replication. *EMBO J.*, **14**, 3094–3104.

McCarrey,J.R. (1993) Development of the germ cell. In Ewing,L.L. (ed.), *Cell and Molecular Biology of the Testis*. Oxford University Press, Oxford, UK, pp. 58–89.

Meistrich,M.L. (1989) Histone and basic nuclear protein transitions in mammalian spermatogenesis. In Hnilica,L.S., Stein,G.S., Stein,J.L.,

Rauterberg,J. and Jaeger,E. (eds), *Histones and Other Basic Nuclear Proteins*. CRC, Boca Raton, FL, pp. 165–182.

Mettus,R.V., Litvin,J., Wali,A., Toscani,A., Latham,K., Hatton,K. and Reddy,E.P. (1994) Murine A-*myb*: evidence for differential splicing and tissue-specific expression. *Oncogene*, **9**, 3077–3086.

Meuwissen,R.L., Offenberg,H.H., Dietrich,A.J., Riesewijk,A., van Iersel,M. and Heyting,C. (1992) A coiled-coil related protein specific for synapsed regions of meiotic prophase chromosomes. *EMBO J.*, **11**, 5091–5100.

Rane,S.G., Dubus,P., Mettus,R.V., Galbreath,E.J., Boden,G., Reddy,E.P. and Barbacid,M. (1999) Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in β-islet cell hyperplasia. *Nat. Genet.*, **22**, 44–52.

Ravnik,S.E., Rhee,K. and Wolgemuth,D.J. (1995) Distinct patterns of expression of the D-type cyclins during testicular development in the mouse. *Dev. Genet.*, **16**, 171–178.

Regnier,C.H., Masson,R., Kedinger,V., Textoris,J., Stoll,I., Chenard,M.P., Dierich,A., Tomasetto,C. and Rio,M.C. (2002) Impaired neural tube closure, axial skeleton malformations and tracheal ring disruption in TRAF4-deficient mice. *Proc. Natl Acad. Sci. USA*, **99**, 5585–5590.

Rubin,M.R., Toth,L.E., Patel,M.D., D'Eustachio,P. and Nguyen-Huu,M.C. (1986) A mouse homeo box gene is expressed in spermatocytes and embryos. *Science*, **233**, 663–667.

Sassone-Corsi,P. (2002) Unique chromatin remodeling and transcriptional regulation in spermatogenesis. *Science*, **296**, 2176–2178.

Sato,N., Arai,K. and Masai,H. (1997) Human and *Xenopus* cDNAs encoding budding yeast Cdc7-related kinases: *in vitro* phosphorylation of MCM subunits by a putative human homologue of Cdc7. *EMBO J.*, **16**, 4340–4351.

Schmidt,E.E., Taylor,D.S., Prigge,J.R., Barnett,S. and Capecchi,M.R. (2000) Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. *Proc. Natl Acad. Sci. USA*, **97**, 13702–13707.

Sclafani,R.A. and Jackson,A.L. (1994) Cdc7 protein kinase for DNA metabolism comes of age. *Mol. Microbiol.*, **11**, 805–810.

Sclafani,R.A., Patterson,M., Rosamond,J. and Fangman,W.L. (1988) Differential regulation of the yeast *CDC7* gene during mitosis and meiosis. *Mol. Cell. Biol.*, **8**, 293–300.

Silver,D.P. and Livingston,D.M. (2001) Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. *Mol. Cell*, **8**, 233–243.

Stillman,B. (1996) Cell cycle control of DNA replication. *Science*, **274**, 1659–1664.

Takeda,T., Ogino,K., Matsui,E., Cho,M.K., Kumagai,H., Miyake,T., Arai,K. and Masai,H. (1999) A fission yeast gene, *him1*$^{+}$*/dfp1*$^{+}$, encoding a regulatory subunit for Hsk1 kinase, plays essential roles in S-phase initiation as well as in S-phase checkpoint control and recovery from DNA damage. *Mol. Cell. Biol.*, **19**, 5535–5547.

Takeda,T., Ogino,K., Tatebayashi,K., Ikeda,H., Arai,K.-i. and Masai,H. (2001) Regulation of initiation of S phase, replication checkpoint signaling and maintenance of mitotic chromosome structures during S phase by Hsk1 kinase in the fission yeast. *Mol. Biol. Cell.*, **12**, 1257–1274.

Tanaka,H., Pereira,L.A., Nozaki,M., Tsuchida,J., Sawada,K., Mori,H. and Nishimune,Y. (1997) A germ cell-specific nuclear antigen recognized by a monoclonal antibody raised against mouse testicular germ cells. *Int. J. Androl.*, **20**, 361–366.

Tanaka,S.S., Toyooka,Y., Akasu,R., Katoh-Fukui,Y., Nakahara,Y., Suzuki,R., Yokoyama,M. and Noce,T. (2000) The mouse homolog of *Drosophila Vasa* is required for the development of male germ cells. *Genes Dev.*, **14**, 841–853.

Trauth,K., Mutschler,B., Jenkins,N.A., Gilbert,D.J., Copeland,N.G. and Klempnauer,K.H. (1994) Mouse A-*myb* encodes a trans-activator and is expressed in mitotically active cells of the developing central nervous system, adult testis and B lymphocytes. *EMBO J.*, **13**, 5994–6005.

Tsutsui,T., Hesabi,B., Moons,D.S., Pandolfi,P.P., Hansel,K.S., Koff,A. and Kiyokawa,H. (1999) Targeted disruption of *CDK4* delays cell cycle entry with enhanced p27^{Kip1} activity. *Mol. Cell. Biol.*, **19**, 7011–7019.

Watanabe,D., Sawada,K., Koshimizu,U., Kagawa,T. and Nishimune,Y. (1992) Characterization of male meiotic germ cell-specific antigen (Meg 1) by monoclonal antibody TRA 369 in mice. *Mol. Reprod. Dev.*, **33**, 307–312.

Watanabe,D., Yamada,K., Nishina,Y., Tajima,Y., Koshimizu,U., Nagata,A. and Nishimune,Y. (1994) Molecular cloning of a novel Ca^{2+}-binding protein (calmegin) specifically expressed during male meiotic germ cell development. *J. Biol. Chem.*, **269**, 7744–7749.

Wolgemuth,D.J. and Watrin,F. (1991) List of cloned mouse genes with unique expression patterns during spermatogenesis. *Mamm. Genome*, **1**, 283–288.

Zakeri,Z.F., Wolgemuth,D.J. and Hunt,C.R. (1988) Identification and sequence analysis of a new member of the mouse HSP70 gene family and characterization of its unique cellular and developmental pattern of expression in the male germ line. *Mol. Cell. Biol.*, **8**, 2925–2932.

Zhang,Q., Wang,X. and Wolgemuth,D.J. (1999) Developmentally regulated expression of cyclin D3 and its potential *in vivo* interacting proteins during murine gametogenesis. *Endocrinology*, **140**, 2790–2800.

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