

# Cyclin A1 protein shows haplo-insufficiency for normal fertility in male mice

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## Abstract

In higher eukaryotes, the cyclins constitute a family of proteins involved in progression through the cell cycle. The cyclin A1 gene (*Ccna1*) is expressed during meiosis and is required for spermatogenesis. Targeted disruption of the *Ccna1* gene with a *LacZ* reporter gene has allowed us to study the expression pattern of this gene in more detail. We have confirmed expression in mouse pre-meiotic spermatocytes and also detected expression in the accessory olfactory bulb, hippocampus and amygdala of the adult brain. We have also found that the amount of cyclin A1 protein influences the fertility of male mice and its action is modulated by genetic background. On an outbred genetic background (129S6/SvEv × MF1), *Ccna1*<sup>tm1Col</sup> −/− animals are sterile due to spermatogenic arrest prior to the first meiotic division while *Ccna1*<sup>tm1Col</sup> +/− mice show reduced sperm production and fertility. This is even more pronounced on an inbred genetic background (129S6/SvEv) where *Ccna1*<sup>tm1Col</sup> +/− male mice are sterile due to a severe reduction in the total number of sperm.

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## Introduction

The cyclins form a large protein family involved in the regulation of the eukaryotic cell cycle. Cyclin binding is a key event required for activation of cyclin-dependent protein kinases (CDKs), which regulate progression between phases of the cell cycle. There are four types of cyclins: A, B, D and E. D- and E-type cyclins are involved in the passage through G1 and entry into the S-phase. B-type cyclins are necessary for mitosis or meiosis. Evidence from the point of cell cycle arrest in ablation experiments indicate that A-type cyclins act at two points in the cell cycle, at the beginning of the S-phase and during mitosis. Two A-type cyclins (A1 and A2) have been identified in *Xenopus* (Howe *et al.* 1995), mice (Sweeney *et al.* 1996, Ravnik & Wolgemuth 1999) and humans (Henglein *et al.* 1994, Yang *et al.* 1997).

Cyclin A1 differs from the other cyclins in that its expression pattern is predominantly restricted to the germ line. Its precise pattern of expression has been investigated in male mice and compared with that of cyclin A2. While cyclin A2 is exclusively expressed during mitotic proliferation prior to the meiotic cycle and during the pre-meiotic S-phase, cyclin A1 expression is restricted to

meiotic cells. Specifically, cyclin A1 mRNA and protein are first detected in late pachytene spermatocytes with the amount of protein rising to a maximum during diplotene (Ravnik & Wolgemuth 1999). As with the role of cyclin A2/CDK during mitosis, the role of cyclin A1/CDK during meiosis remains obscure. However, cyclin A1 is essential for the completion of male meiosis as mice homozygous for a null mutation of cyclin A1 are sterile due to the complete arrest of spermatogenesis in the latter stages of meiotic prophase, while females appear to be phenotypically normal and fertile (Liu *et al.* 1998). It has been proposed that the role of cyclin A1/CDK may include the phosphorylation of Cdc25 phosphatases which are required for the activation of the cyclin B/CDK1 complex (or M-phase promoting factor) essential for the G2/M transition (Liu *et al.* 2000).

In humans, there are data that suggest a role for cyclin A1 in haematopoiesis and more definitely in the development of myeloid leukaemia. Very low levels of cyclin A1 are detected in normal haematopoietic tissues, and relatively high levels have been detected in several leukaemic cell lines and in the peripheral blood cells of

patients with certain haematological malignancies (Kramer *et al.* 1998). Transgenic mice over-expressing cyclin A1 in the myeloid lineage exhibit abnormal myelopoiesis and can develop acute myeloid leukaemia (Liao *et al.* 2001). Although the role of cyclin A1 in the development of acute myeloid leukaemia is unknown, there is some evidence to suggest that the kinase activity of cyclin A1/CDK2 may enhance the activity of the B-MYB transcription factor which is essential for the G1/S transition in leukaemic cells (Muller-Tidow *et al.* 2001). Human cyclin A1/CDK2 can phosphorylate other important cell cycle regulators such as E2F- and Rb- related proteins (Yang *et al.* 1999).

We have described here the generation of transgenic mice in (designated *Ccna1*<sup>tm1Col</sup>) which the cyclin A1 gene (*Ccna1*) has been disrupted and tagged with a *LacZ* reporter gene. We have confirmed previous reports that homozygous mutant males are sterile due to a failure to complete spermatogenesis (Liu *et al.* 1998). We have also made the new observation that *Ccna1*<sup>tm1Col</sup> +/- male mice show reduced fertility because of a reduction in sperm numbers and that the severity of this phenotype is strain dependent. These data suggested that cyclin A1 is acting as a dose-dependent regulator of the successful production of haploid cells and its activity is modulated by genetic background. If applicable to humans, cyclin A1 levels and/or activity may contribute to idiopathic cases of oligospermia (reduced sperm numbers) in infertile men.

## Materials and Methods

### Generation of *Ccna1*<sup>tm1Col</sup> mice

CCB ES cells (129S6Sv/Ev) were grown on primary embryonic fibroblast feeder cells in Dulbecco's modified Eagle's medium/F12 (Gibco, Invitrogen Life Technologies, Paisley, Scotland) supplemented with 20% fetal calf serum (LabTech International, Lewes, East Sussex, UK), 1 mM L-glutamine (Sigma, Poole, Dorset, UK), 10<sup>-4</sup> M β-mercaptoethanol (Sigma) and 100 units/ml each of penicillin and streptomycin (Sigma). ES cells (2 × 10<sup>7</sup>) were electroporated with 0.5 μg of the linearised targeting vector at 250 V and 960 μF capacitance. ES cell clones were selected in 250 μg/ml G418 (Sigma) and screened by Southern analysis for targeting events. Two independently targeted ES cell clones were used to generate chimaeric mice by injection into C57BL/6 blastocysts. Animal experiments were performed in accordance with UK legal requirements. Male chimaeras were bred with MF1 (outbred strain) or 129S6/SvEv (inbred strain) females and heterozygous offspring identified by Southern analysis and/or PCR. Primers B158 (tgctcaagtcagatctccactcc) and B159 (ttatattctcccacactccacc) were used to amplify a 1 kb fragment from the intact *Ccna1* gene. Primers (gaagaactcgtaaggcgatagaa and ggggggagagcggtattcggctat) were used to amplify a 765 bp fragment from the neomycin phosphotransferase (*neo*) gene as a marker for the targeted allele. Reaction

conditions were 95 °C for 5 min followed by 40 cycles of 93 °C for 30 s, 60 °C for 1 min and 70 °C for 1 min. A null mutation at the *Ccna1* locus was confirmed by both RT-PCR and western analysis. The RT-PCR analysis was performed using primers RTF (atgcatcgccagagctccaagagtggagtc) and RTR (cctctgcatactccgttacgtaacacat) which span intron II of the *Ccna1* gene and amplify a 0.5 kb fragment. The reaction conditions were first-strand cDNA synthesis: 50 °C for 30 min, 94 °C for 2 min; second-strand cDNA synthesis and PCR amplification: 10 cycles of 94 °C for 20 s, 65 °C for 30 s and 68 °C for 30 s followed by 25 cycles of 94 °C for 20 s, 65 °C for 30 s and 68 °C for 30 s with an extension of 5 s per cycle and a final cycle of 68 °C for 7 min. Western analysis used homogenates prepared from decapsulated testes and an anti-cyclin A1 antibody (Sweeney *et al.* 1996) or an anti-cyclin B2 antibody (Brandeis *et al.* 1998) or an anti-CDK2 antibody (sc162 from Santa Cruz Biotechnology, Sant Cruz, CA, USA).

### Histological analysis

Intact tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and assessed for β-galactosidase expression by overnight incubation in staining solution (0.1M PBS, 2mM MgCl<sub>2</sub>, 5mM potassium ferricyanide, 5mM potassium ferrocyanide and X-Gal at 1 mg/ml). After staining, tissues were post-fixed in 4% paraformaldehyde, embedded in paraffin wax and sectioned. The brain was fixed in 4% paraformaldehyde for 15 min and then set in 3% agarose and slices were cut at 2 mm intervals. The slices were fixed in 4% paraformaldehyde for a further 15 min and then washed, stained and fixed as before. Tissues were embedded in paraffin wax and 7 μm sections cut and stained with haematoxylin and eosin. For staging the seminiferous cycle, Bouin-fixed, historesin-embedded adult testes were sectioned at 5 μm and counter-stained with methylene blue. Spermatogenic stages were defined by morphological appearance as described by Russell *et al.* (1990). For cell counts, a complete cross-section (as opposed to a longitudinal section) of a stage VII tubule was selected and photographed. When necessary, several photographs were taken to cover the whole tubule. All pachytene and haploid cells on the photographs were counted. The sections were cut at 5 μm and stained with methylene blue to allow good visualisation of nuclei. Only cells with complete nuclear membranes on the photographs were counted. The cells of interest were counted by the appearance of the corresponding nuclei. Since the diameter of pachytene cells and step 7 spermatids are approximately 12 μm and 5 μm respectively sections as thin as 5 μm rarely result in overlapping of cells, which might lead to a misrepresentation in cell counting.

### Analysis of reproductive capability

Eight- to nine-week-old male mice were housed in individual cages and set up with 6- to 8-week-old MF1

females. Females were checked for the presence of a copulatory plug every morning. Any plugged female was removed and replaced. Plugged females were killed at 10 days post coitum (dpc) and examined for the number of embryos. For each male being tested, at least one littermate with a different genotype was also tested. This regime was carried out for 5 weeks. For sperm counts, the vas deferens was dissected from one side and its length measured. A 1 ml syringe fitted with a 34 gauge blunt ended needle was used to flush 0.5 ml of water through the vas deferens into a 1.5 ml Eppendorf tube. After a brief vortex, 10  $\mu$ l of the suspension was loaded onto a haemocytometer and the sperm were counted. The frequencies of mating and pregnancy in the male fertility test were analysed using the chi-square test of significance. A two-tailed Mann–Whitney test (InStat GraphPad Software, San Diego, CA, USA) was used to compare the results obtained in the analysis of testes weight and sperm recovery.

## Results

### **Male chimaeras showed an unusually low rate of germ-line transmission of the ES cell genome**

A standard gene-targeting strategy was used to disrupt the *Ccna1* gene (Fig. 1A). The targeting vector contained a *LacZ* gene fused immediately after the initiator methionine of the cyclin A1 protein. The *LacZ* gene was expressed from the endogenous cyclin A1 promoter, which provides a convenient method of visualising *Ccna1* gene expression. The targeted allele had all the protein coding exons of the *Ccna1* gene deleted. Germ-line transmission of the *Ccna1* null mutation was achieved from only two out of 34 male chimaeras generated from two independently targeted ES clones. This frequency of transmission (6%) was much lower than usually observed in our laboratory where 76% of male chimaeras normally transmit the ES cell genome ( $n = 58$  chimaeras). In addition, neither of the two germ-line chimaeras showed complete ES cell colonisation of the germ line as judged by transmission of agouti coat colour to offspring. Of the 34 chimaeras, 12 (35%) failed to sire any offspring compared with a failure rate of around 7% ( $n = 58$  chimaeras) for other gene-targeting experiments. The *Ccna1* null mutation was established on an outbred (129S6/SvEv  $\times$  MF1) and an inbred (129S6/SvEv) genetic background by breeding from the two germ-line chimaeras. Offspring from outbred *Ccna1* heterozygote crosses gave the expected Mendelian ratio of genotypes and viable homozygous mutant animals (Fig. 1B). The null mutation was confirmed by both RT-PCR and Western blot analysis with no mRNA or protein detected in homozygous mutant animals (Fig. 1C and D). The western blot indicated that approximately half the amount of protein was present in the testes of heterozygous mice (Fig. 1D).

### ***Ccna1*<sup>tm1Col</sup> homozygous mutant males have small testes and are azoospermic**

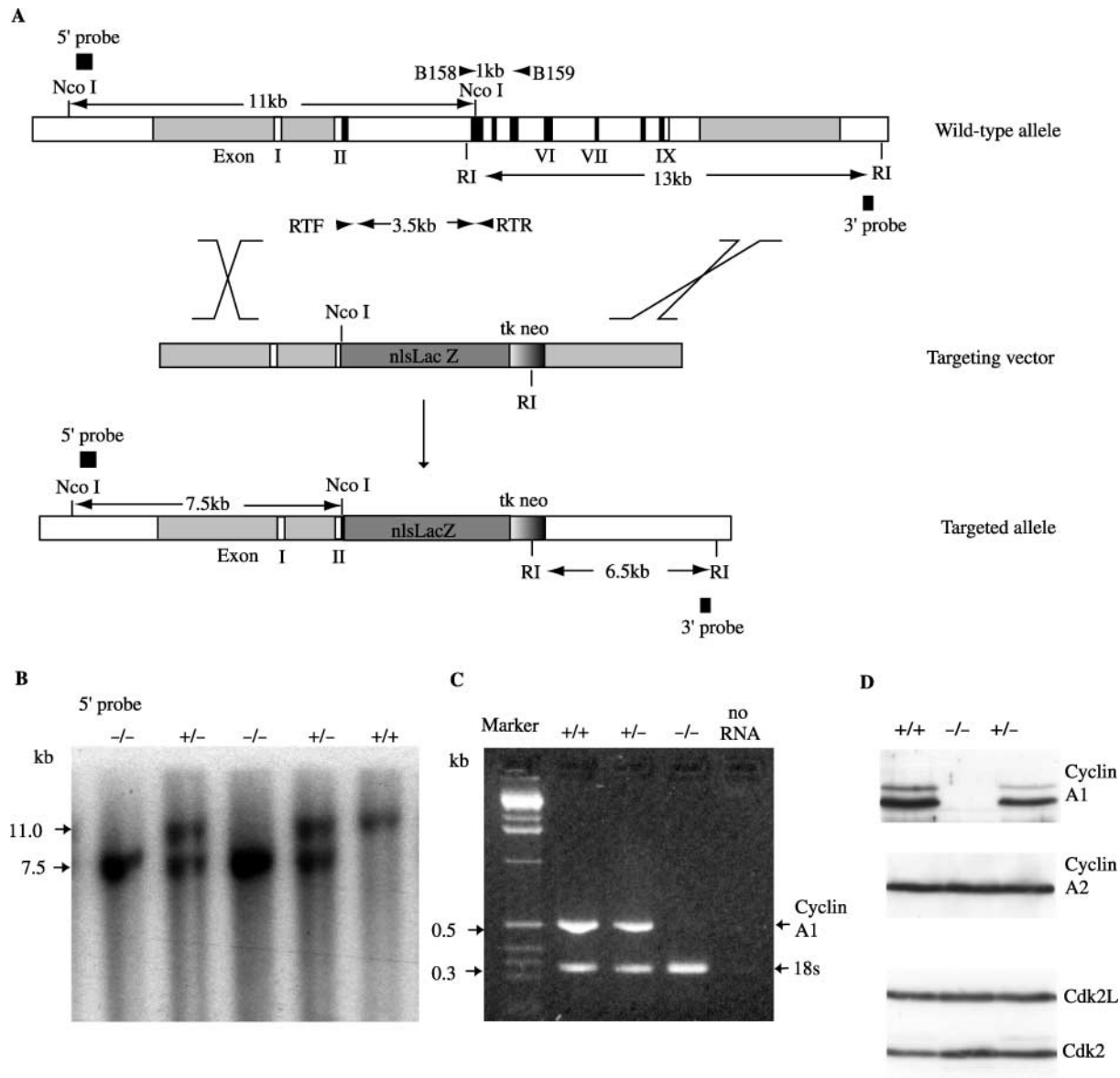
The combined weight of both testes from *Ccna1*<sup>tm1Col</sup>  $-/-$  mice was around 50% of the weight from age-matched *Ccna1*<sup>tm1Col</sup>  $+/+$  animals. On the outbred genetic background (MF1  $\times$  129S6/SvEv), pairs of testes from *Ccna1*<sup>tm1Col</sup>  $+/+$  mice had a weight of  $0.23 \pm 0.02$  g ( $n = 5$  animals), *Ccna1*<sup>tm1Col</sup>  $+/-$  mice had a paired testes weight of  $0.20 \pm 0.01$  g ( $n = 9$  animals) and *Ccna1*<sup>tm1Col</sup>  $-/-$  mutants had a paired testes weight of  $0.12 \pm 0.002$  g ( $n = 6$  animals). There was no significant difference in the body weights of male mice at 20 weeks of age ( $35 \pm 1.2$  g ( $n = 5$  animals) for *Ccna1*<sup>tm1Col</sup>  $+/+$  mice and  $36 \pm 0.98$  g ( $n = 6$  animals) for *Ccna1*<sup>tm1Col</sup>  $-/-$  mice).

Post-meiotic cell types (spermatids and spermatozoa) were absent from *Ccna1*<sup>tm1Col</sup>  $-/-$  testes but the spermatogonial population appeared normal (Fig. 2A and C compared with Fig. 2B and D). Degenerating cells with condensed and fragmented nuclear material identified by intense haematoxylin staining were found in the adluminal compartment of seminiferous tubules. Occasionally, large multi-nucleated cells were also present (Fig. 2D); these are often observed when spermatogenesis is disrupted (Knudson *et al.* 1995, Nantel *et al.* 1996). All cell types from the spermatogenic lineage were present in testes from *Ccna1*<sup>tm1Col</sup>  $+/-$  mice. Testes from *Ccna1*<sup>tm1Col</sup>  $-/-$  mice stained for  $\beta$ -galactosidase activity predominantly in degenerating spermatocytes (Fig. 2F). In testes from *Ccna1*<sup>tm1Col</sup>  $+/-$  mice, faint staining was seen in spermatocytes with stronger staining in round spermatids (Fig. 2E). Spermatozoa showed little or no staining and no staining was observed in ovaries or eggs (not shown). The testes of control wild-type mice also showed no staining.

The lumen of the vas deferens and epididymis from wild-type mice (Fig. 2G and I respectively) were packed with spermatozoa. In contrast, spermatozoa were absent from the vas deferens (Fig. 2H) and epididymis (Fig. 2J) from *Ccna1*<sup>tm1Col</sup>  $-/-$  mice. The lumen of the epididymis from *Ccna1*<sup>tm1Col</sup>  $-/-$  mice contained many degenerating spermatocytes and some giant multi-nucleated cells.

### **Cyclin A1 is haplo-insufficient for normal male fertility due to reduced sperm numbers**

Only one out of five *Ccna1*<sup>tm1Col</sup>  $+/-$  breeding pairs on the outbred genetic background (MF1  $\times$  129S6/SvEv) produced offspring and no pups were ever obtained from heterozygous matings on the inbred genetic background (129S6/SvEv). Preliminary breeding experiments showed that *Ccna1*<sup>tm1Col</sup>  $+/-$  females were fertile so the fertility of males was assessed in more detail. No differences were observed in the ability of male mice of any genotype to plug *Ccna1*<sup>tm1Col</sup>  $+/+$  females (Fig. 3A). However, there was a significant difference in the pregnancy rate between each genotype. Around 90% of females that were plugged

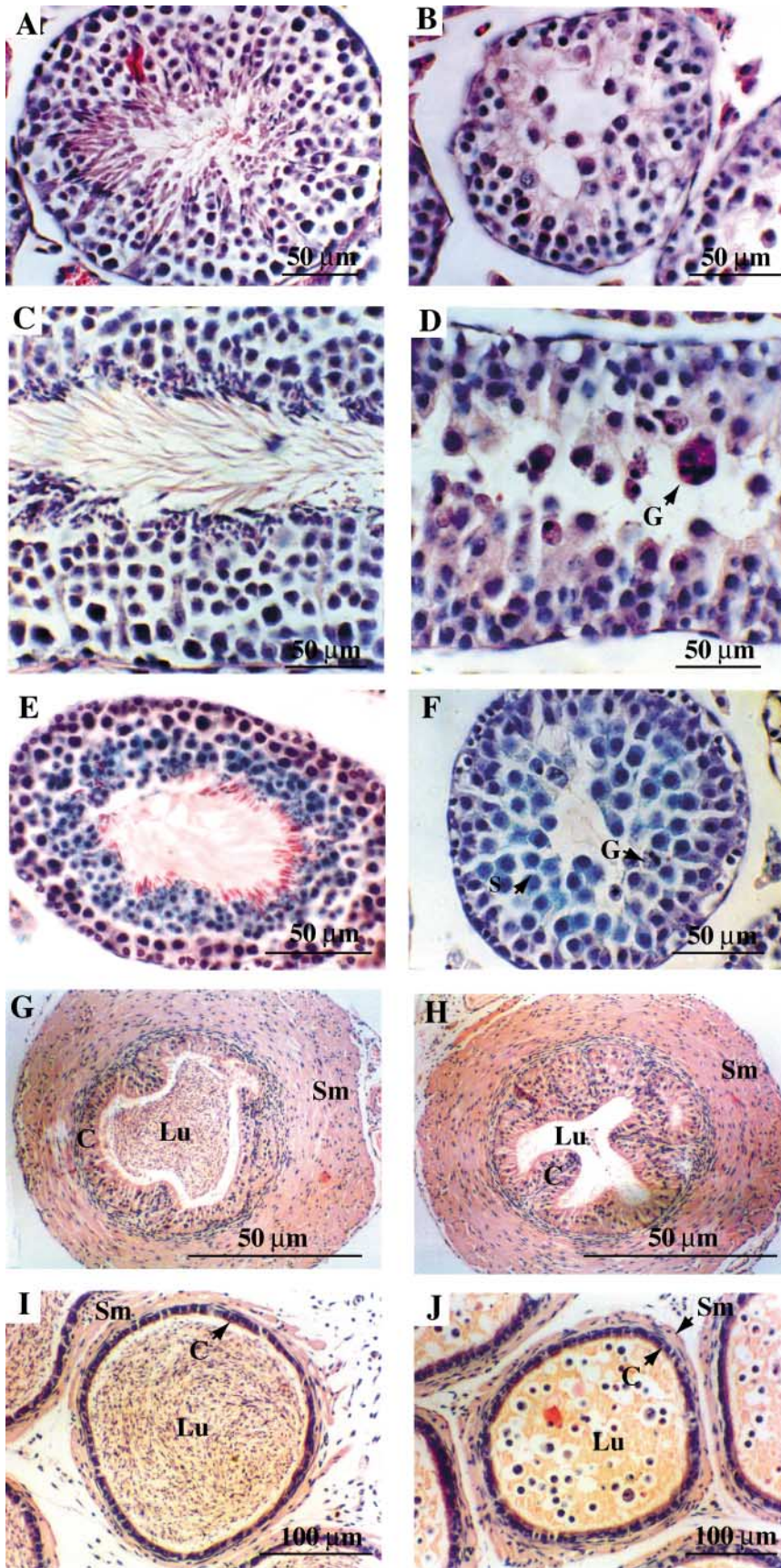


**Figure 1** Generation of *Ccna1*<sup>tm1Col</sup> mice. (A) Targeted disruption of the murine cyclin A1 gene (*Ccna1*): the targeting vector contains 5 kb of 5' homology including the *Ccna1* promoter and 3.5 kb of 3' homology (homology shown in light grey). The *LacZ* reporter gene (dark grey) encodes a  $\beta$ -galactosidase tagged with a nuclear localisation signal sequence (nls) and is driven by the *Ccna1* promoter. The *neo* selection cassette is under the control of the thymidine kinase (*tk*) promoter (gradation to black). Homologous recombination results in loss of all coding exons II to IX (coding region shown in black). RI, *EcoRI* restriction site. B158, B159, RTF, RTR indicate primer locations. (B) Southern analysis of *Ccna1*<sup>tm1Col</sup> mice generated from heterozygote intercrosses: genomic DNA was digested with *NcoI* and hybridised with a 5' probe external to the targeting vector. (C) Detection of *Ccna1* expression in the testes of *Ccna1*<sup>tm1Col</sup> mice: RT-PCR was performed with primers RTF and RTR spanning intron II of the *Ccna1* gene to amplify a product of 0.5 kb from cyclin A1 cDNA. As a positive control an 18S rRNA primer pair which amplifies a 0.3 kb product was used. (D) Western blot analysis of testes lysates: anti-cyclin A1 antibodies detected two cyclin A1 polypeptides with relative molecular masses of 54 kDa and 57 kDa in the testes of both *Ccna1*<sup>tm1Col</sup> +/+ and +/-, but not -/- mice. The signal from the *Ccna1*<sup>tm1Col</sup> +/- sample was approximately half as strong as that from the *Ccna1*<sup>tm1Col</sup> +/+ sample. The anti-cyclin B2 and anti-Cdk2 antibodies acted as positive controls for loading differences.

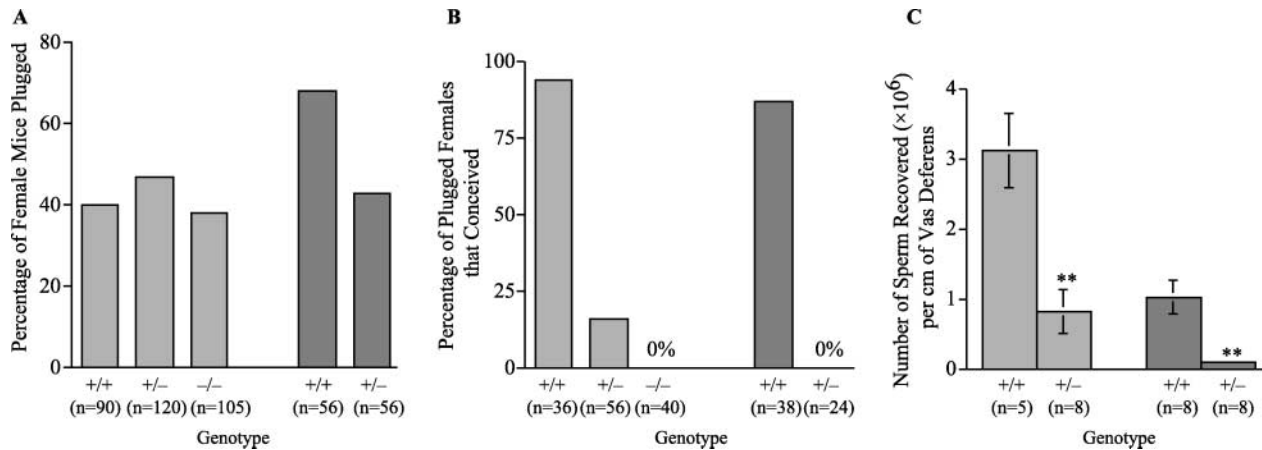
by wild-type males became pregnant irrespective of whether the males were outbred or inbred (Fig. 3B). The conception rate was reduced to 16% for heterozygous males of outbred genetic background while inbred heterozygous males gave no pregnancies from 24 copulatory plugs (Fig. 3B). Thus, *Ccna1*<sup>tm1Col</sup> -/- mice could not

be obtained on an inbred genetic background because of the infertility of the *Ccna1*<sup>tm1Col</sup> +/- males on this genetic background. As expected from the absence of sperm, outbred *Ccna1*<sup>tm1Col</sup> -/- males also failed to conceive.

To establish the reason for the reduced fertility of *Ccna1*<sup>tm1Col</sup> +/- mice, the number of sperm recovered



**Figure 2** Histology of the male reproductive system. Seminiferous tubules from *Ccna1*<sup>tm1Col</sup> -/- mice (B, D and F) lack post-meiotic cell types and are characterised by degenerating cells with condensed and fragmented nuclear material and giant multi-nucleated cells (G). The spermatogenic lineage is intact in seminiferous tubules from *Ccna1*<sup>tm1Col</sup> +/+ and +/- mice (A, C and E). For *Ccna1*<sup>tm1Col</sup> testes stained for  $\beta$ -galactosidase activity (E and F), a small amount of staining was seen in some spermatocytes with much stronger staining in round spermatids in +/- testes (E) while the blue stain was predominantly seen in spermatocytes (S) in -/- testes (F). The lumen (Lu) of the vas deferens (G) and epididymis (I) from *Ccna1*<sup>tm1Col</sup> +/+ mice contained spermatozoa, which were absent from the vas deferens (H) and epididymis (J) of *Ccna1*<sup>tm1Col</sup> -/- mice. Sm, smooth muscle; C, cuboidal epithelia. All sections were cut at 7  $\mu$ m and stained with haematoxylin and eosin.



**Figure 3** Fertility analysis of *Ccna1*<sup>tm1Col</sup> mice. (A) Frequency of copulatory plug. Seven males of each genotype were housed with females over a 4-week period and copulatory plugs recorded. The total number of females to which each male had access is given in parentheses. (B) Frequency of conception. The number of females with a copulatory plug (in parentheses) that became pregnant was recorded. (C) Recovery of sperm from the vas deferens. Sperm were flushed from the vas deferens of *Ccna1*<sup>tm1Col</sup> +/+ and +/- males. No sperm were recovered from homozygous -/- outbred animals. The number of males analysed is shown in parentheses. \*\**P* < 0.001 (two-tailed Mann–Whitney). For all three panels, outbred mice are shown in light grey, inbred mice in dark grey.

from the vas deferens was measured (Fig. 3C). On both the outbred and inbred genetic backgrounds, significantly less sperm were recovered from *Ccna1*<sup>tm1Col</sup> +/- mice than from *Ccna1*<sup>tm1Col</sup> +/+ mice (*P* < 0.001, two-tailed Mann–Whitney). Significantly fewer (*P* = 0.03, two-tailed Mann–Whitney) sperm were also recovered from inbred (129S6/SvEv) *Ccna1*<sup>tm1Col</sup> +/+ males compared with the outbred *Ccna1*<sup>tm1Col</sup> +/+ males, consistent with the reduced fecundity of inbred strains of mice in general (Fig. 3C). No sperm were isolated from any *Ccna1*<sup>tm1Col</sup> -/- mice (*n* = 6).

To determine the origin of the reduced sperm numbers in the *Ccna1*<sup>tm1Col</sup> +/- mice, pachytene spermatocytes and haploid spermatids were quantitated in testes sections from mice on the outbred and inbred genetic backgrounds (Fig. 4). No significant differences were found in the number of pachytene cells between *Ccna1*<sup>tm1Col</sup> +/- and *Ccna1*<sup>tm1Col</sup> +/+ mice of the same genetic background. In contrast, the number of haploid spermatids was significantly lower in *Ccna1*<sup>tm1Col</sup> +/- than in *Ccna1*<sup>tm1Col</sup> +/+, indicating a greater failure of some cells to complete meiosis (Table 1). More cells failed to complete meiosis on the inbred background (Table 1).

### Cyclin A1 is also expressed in the brain

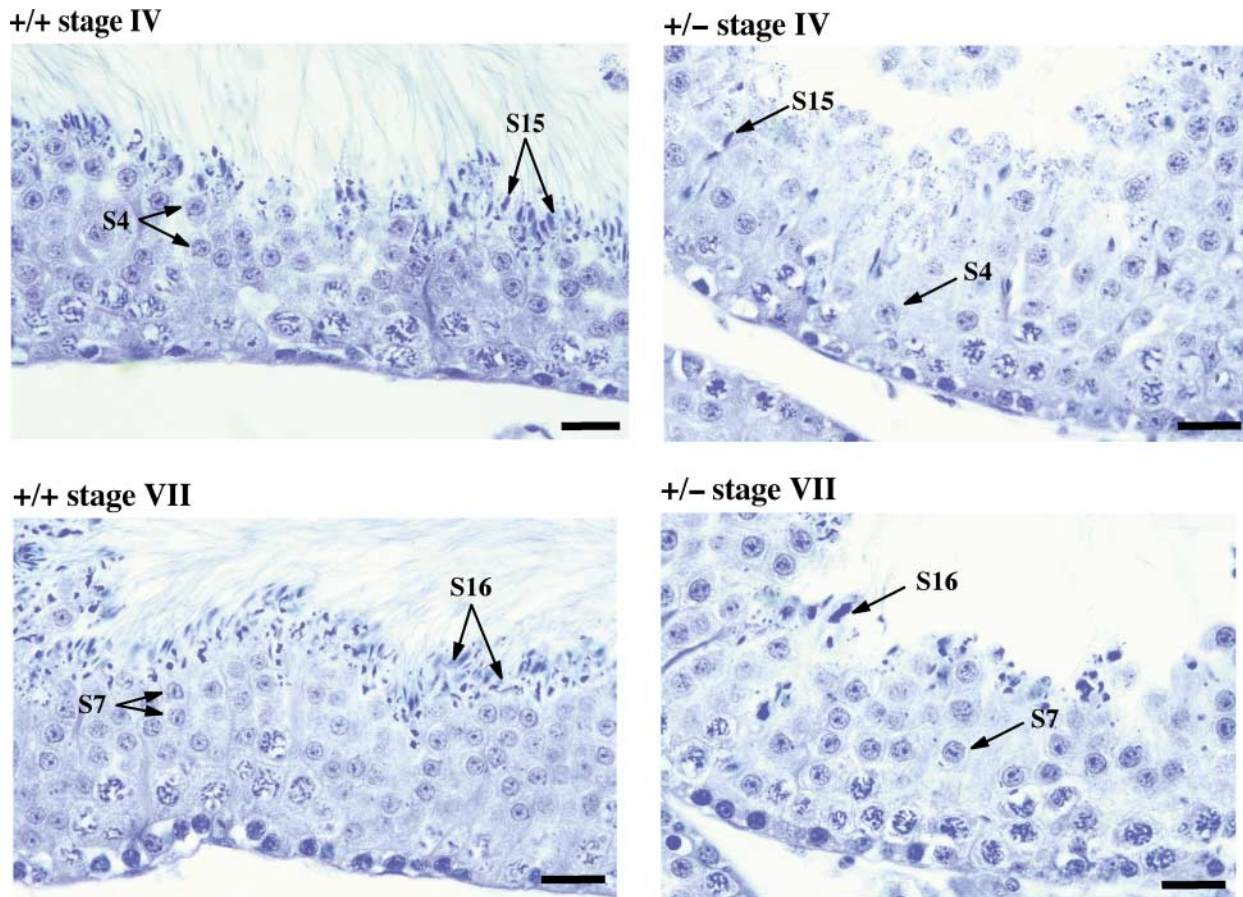
To determine whether the cyclin A1 gene is transcribed exclusively in the testes, other tissues were examined for  $\beta$ -galactosidase activity. Staining was observed in brain slices from both *Ccna1*<sup>tm1Col</sup> +/- and -/- but not in +/+ controls (Fig. 5A). Similar staining patterns were observed in mice derived from both targeted ES clones and no obvious difference in the size of these staining regions was apparent between +/- and -/- animals. Intense staining was found in the accessory olfactory bulb,

the dentate gyrus, the Ammon's horn of the hippocampus and in the amygdala. Less intense staining was seen in the thalamus, hypothalamus and some cell layers of the cerebellum (not shown).  $\beta$ -Galactosidase activity was not observed in any other tissues examined (lung, heart, spleen, liver, kidney and ovaries) or in post-implantation embryos at 10.5 dpc (*n* = 13) and 12.5 dpc (*n* = 12) (data not shown). To confirm that the *LacZ* expression pattern reflected *Ccna1* expression, the expression of endogenous cyclin A1 in the brain was examined by RT-PCR. Cyclin A1 mRNA was detected in the brain of *Ccna1*<sup>tm1Col</sup> +/+ and *Ccna1*<sup>tm1Col</sup> +/- mice but not in the brain of *Ccna1*<sup>tm1Col</sup> -/- mutant animals (Fig. 5B).

### Discussion

The disruption of spermatogenesis in *Ccna1*<sup>tm1Col</sup> -/- mice was consistent with that reported by Liu *et al.* (1998) and confirms that cyclin A1 is essential for meiosis in male germ cells. Liu *et al.* (1998), however, did not describe a phenotype in heterozygous mice. The data presented here show that *Ccna1*<sup>tm1Col</sup> +/- males with an outbred genetic background (MF1  $\times$  129S6/SvEv) are sub-fertile and those with an inbred genetic background (129S6/SvEv) are sterile. On both these genetic backgrounds, significantly fewer sperm were recovered from the reproductive tract of *Ccna1*<sup>tm1Col</sup> +/- animals compared with *Ccna1*<sup>tm1Col</sup> +/+, suggesting that a low sperm count is responsible for this sub-fertility. It has long been recognised that reduced sperm numbers (oligozoospermia) can severely compromise normal fertility in humans.

Western analysis showed that *Ccna1*<sup>tm1Col</sup> +/- mice have around half the cyclin A1 protein compared with normal mice. Cyclin A1 haplo-insufficiency reduces the number of cells that make the meiotic transition from



**Figure 4** Histological evaluation of meiosis in heterozygous mouse testes. Comparison of seminiferous tubules from *Ccna1<sup>tm1Col</sup> +/+* mice (left-hand panels) and *+/-* mice (right-hand panels) on an inbred genetic background (129S6/SvEv). At both the morphological stages shown (stages IV and VII of spermatogenesis) the number of haploid spermatids and of spermatozoa (with tails in the lumen) was significantly reduced in heterozygotes. Roman numerals correspond to particular stages of the spermatogenic cycle as defined by Russell *et al.* (1990). S4/7/15/16, step 4/7/12/15/16 spermatids. Scale bars represent 20  $\mu$ m.

spermatocyte to haploid spermatid (Table 1). This may be caused by an altered transit time through meiosis and increased loss of spermatocytes. It is unlikely to be caused by selective loss of spermatids carrying the mutated *Ccna1<sup>tm1Col</sup>* allele since functional sperm with this allele are produced by outbred *Ccna1<sup>tm1Col</sup> +/-* mice and the availability of cyclin A1 protein to each spermatocyte will be similar via protein movement between cytoplasmic bridges (Braun *et al.* 1989). Interestingly,

haplo-insufficiency has also been found for the mitotic checkpoint protein MAD2, suggesting that the cell cycle may be particularly sensitive to changes in the amount of regulatory proteins (Michel *et al.* 2001).

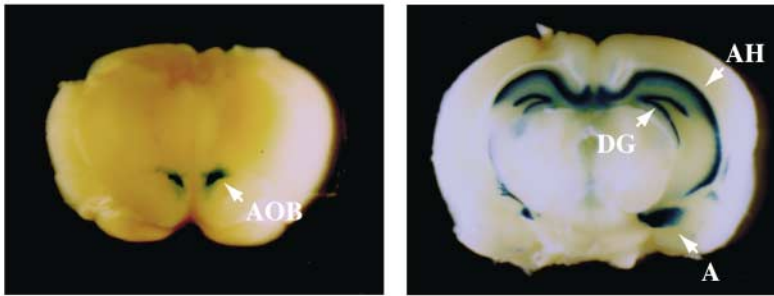
As heterozygous inbred males are sterile, it is perhaps surprising that male chimaeras (generated from 129S6/SvEv ES cells) transmitted the targeted allele. It is noteworthy that the rate of germ-line transmission was considerably lower than for other gene-targeted mice that we

**Table 1** Comparison of pachytene and haploid cell numbers in *Ccna1<sup>tm1Col</sup> +/+* and *+/-* mice. Cells were counted in cross sections of seminiferous tubules. Values are given as mean  $\pm$  S.E.M. per tubule cross section, with number of tubules examined in parentheses. Analyses were performed using testes from four outbred and two inbred mice of each genotype.

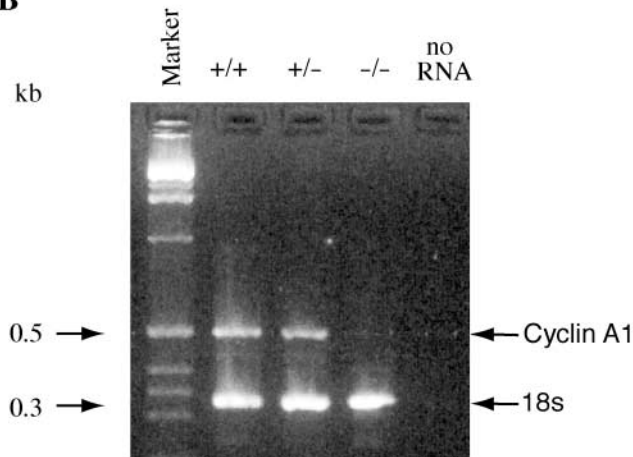
Genotype	Strain	No. of pachytene cells/tubule cross section	No. of haploid cells/tubule cross section
+/+	Outbred (129S6/SvEv $\times$ MF1)	64 $\pm$ 3.7 (16)	153 $\pm$ 10.4 (16)
+/-		65 $\pm$ 3.9 (16)	122 $\pm$ 7.8 (16)*
+/+	Inbred (129S6/SvEv)	84 $\pm$ 8.1 (8)	207 $\pm$ 26.9 (8)
+/-		74 $\pm$ 6.8 (8)	85 $\pm$ 3.9 (8)**

\* $P = 0.024$ , \*\* $P = 0.0005$ , statistically significant differences between *Ccna1<sup>tm1Col</sup> +/+* and *+/-* mice are indicated (two-tailed, Student's *t*-test).

A



B



**Figure 5** (A) Detection of cyclin A1 expression in the brain. Brain from a *Ccna1<sup>tm1Col</sup> -/-* adult male bisected through the cerebral hemispheres in the coronal plane and stained for  $\beta$ -galactosidase activity. AOB, accessory olfactory bulb; AH, Ammon's horn (hippocampus); DG, dentate gyrus (hippocampus); A, amygdala. (B) Detection of cyclin A1 expression in the forebrain by RT-PCR. RT-PCR was carried out with 100 ng total RNA prepared from the forebrain of adult mice. Two specific primer pairs were used: RTF and RTR, which span intron II of the cyclin A1 gene and amplify a PCR product of 0.5 kb from cyclin A1 cDNA. As an internal control an 18S rRNA primer/competimer mix (Ambion, Huntingdon, Cambridge, UK) was used, these primers amplify a 0.3 kb product from 18S cDNA.

have generated and many of the male chimaeras failed to produce any offspring. Partial germ-line transmission was only obtained from two chimaeras and pups were obtained from both ES cell (12956/SvEv)-derived sperm and host blastocyst-derived sperm (C57Bl/6). It is likely that the ES cell-derived sperm required the presence of C57Bl/6 sperm to achieve a total sperm count above the threshold required for fertility. Interestingly, the *Ccna1* targeted allele generated by Liu *et al.* (1998) was transmitted through female chimaeras.

The  $\beta$ -galactosidase staining pattern in the testes was consistent with the cyclin A1 promoter initiating expression of the *LacZ* reporter gene in the appropriate developmentally regulated manner. Expression was initiated in spermatocytes but also continued in haploid spermatids where the cyclin A1 protein is not usually found (Sweeney *et al.* 1996). This is probably due to the persistence of  $\beta$ -galactosidase mRNA and/or protein beyond the meiotic divisions consistent with our observations in transgenic mice expressing a cyclin A1-promoted *LacZ* transgene (J S-T, unpublished observations).

The  $\beta$ -galactosidase staining in the brain suggested that cyclin A1 expression is not restricted to germ cells. The presence of cyclin A1 transcripts in the brain was confirmed by RT-PCR. Expression of cyclin A1 in the brain has not been reported in the mouse but was detected at low levels in humans (Yang *et al.* 1997) and a number of

human expressed sequence tags for cyclin A1 have been found in the brain. The role that cyclin A1 might have in the brain is not clear as *Ccna1<sup>tm1Col</sup> -/-* mutants exhibited no obvious abnormal behaviour. Future tests may reveal whether an absence of cyclin A1 protein in key brain regions has any measurable effect on odourant identification, behaviour or memory.

In conclusion, we have shown that homozygous normal levels of cyclin A1 are essential for normal fertility in male mice and that the severity of haplo-insufficiency is strain dependent. Cyclin A1 has a similar pattern of expression in mice and humans and it is likely to have the same essential role during spermatogenesis in both species. It would be interesting to investigate human cases of idiopathic oligozoospermia and azoospermia for mutations in the *CCNA1* gene.

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