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 3MF1), CcnA1 tm1Col / - animals are sterile due to spermatogenic arrest prior to the first meiotic division while CcnA1 tm1Col / + mice show reduced sperm production and fertility. This is even more pronounced on an inbred genetic background (129S6/SvEv) where CcnA1 tm1Col / - male mice are sterile due to a severe reduction in the total number of sperm.

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 indicates 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 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 leukaemia 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 Ccn1tm1Col) which the cyclin A1 gene (Ccn1) 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 Ccn1tm1Col +/− 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 Ccn1tm1Col mice

CCB ES (129S6/NawEv) 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), 10−5 M β- mercaptoethanol (Sigma) and 100 units/ml each of penicillin and streptomycin (Sigma). ES cells (2 × 106) were electroporated with 0.5 μg of the linearised targeting vector at 250V 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/NawEv (inbred strain) females and heterozygous offspring identified by Southern analysis and/or PCR. Primers B138 (gtgcaagtctgctcccttcc) and B139 (tattatctcccacaaccc) were used to amplify a 1 kb fragment from the intact Ccn1 gene. Primers (gaagaactcgtcaaggcgatagaa and gggtggagagcgtattcggctat) 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 Ccn1 locus was confirmed by both RT-PCR and western analysis. The RT-PCR analysis was performed using primers RTF (atgcatcggcaggtccaaagtg-gagtc) and RTR (ccctgtacactctgctcttacatcacat) which span intron II of the Ccn1 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 MgCl2, 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, histoestin-embedded adult testes were sectioned at 5 μm and counter-stained with thionine green. Spermatogenetic 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 μl 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 Ccn1 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 Ccn1 gene expression. The targeted allele had all the protein coding exons of the Ccn1 gene deleted. Germ-line transmission of the Ccn1 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 Ccn1 null mutation was established on an outbred (129S6/SvEv X MF1) and an inbred (129S6/SvEv) genetic background by breeding from the two germ-line chimaeras. Offspring from outbred Ccn1 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).

Ccn1<sup>tm1Col</sup> homozygous mutant males have small testes and are azoospermic

The combined weight of both testes from Ccn1<sup>tm1Col</sup> –/– mice was around 50% of the weight from age-matched Ccn1<sup>tm1Col</sup> +/+ animals. On the outbred genetic background (MF1 X 129S6/SvEv), pairs of testes from Ccn1<sup>tm1Col</sup> +/+ mice had a weight of 0.23 ± 0.02 g (n = 5 animals), Ccn1<sup>tm1Col</sup> +/- mice had a paired testes weight of 0.20 ± 0.01 g (n = 9 animals) and Ccn1<sup>tm1Col</sup> –/– mutants had a paired testes weight of 0.12 ± 0.002 g (n = 6 animals). There was no significant difference in the body weights of male mice at 20 weeks of age (35 ± 1.2 g (n = 5 animals) for Ccn1<sup>tm1Col</sup> +/- mice and 36 ± 0.98 g (n = 6 animals) for Ccn1<sup>tm1Col</sup> –/– mice).

Post-meiotic cell types (spermatids and spermatozoa) were absent from Ccn1<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 Ccn1<sup>tm1Col</sup> +/- mice. Testes from Ccn1<sup>tm1Col</sup> –/– mice stained for β-galactosidase activity predominantly in degenerating spermatocytes (Fig. 2F). In testes from Ccn1<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 Ccn1<sup>tm1Col</sup> –/– mice. The lumen of the epididymis from Ccn1<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 Ccn1<sup>tm1Col</sup> +/- breeding pairs on the outbred genetic background (MF1 X 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 Ccn1<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 Ccn1<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
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 tm1Col /+ mice could not be obtained on an inbred genetic background because of the infertility of the Ccna1 tm1Col +/- males on this genetic background. As expected from the absence of sperm, outbred Ccna1 tm1Col +/- males also failed to conceive.

To establish the reason for the reduced fertility of Ccna1 tm1Col +/- mice, the number of sperm recovered...
Figure 2 Histology of the male reproductive system. Seminiferous tubules from

$\text{Ccna1}^{tm1Col -/-}$ mice (B, D and F) lack post-meiotic cell types and are characterised by
degenerating cells with condensed and fragment nuclear material and giant multi-
nucleated cells (G). The spermatogenic lineage is intact in seminiferous tubules from

$\text{Ccna1}^{tm1Col +/+}$ and $+/-$ mice (A, C and E). For $\text{Ccna1}^{tm1Col}$ testes stained for $\beta$-galactosi-
dase 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 predo-
mantly seen in spermatocytes (S) in $-/-$ testis (F). The lumen (Lu) of the vas deferens (G) and epididymis (I) from $\text{Ccna1}^{tm1Col}$

$+/-$ mice contained spermatozoa, which were absent from the vas deferens (H) and epididymis (I) of $\text{Ccna1}^{tm1Col} -/-$ mice. Sm, smooth muscle; C, cuboidal epithelia. All sections were cut at 7 $\mu$m and stained with

haematoxylin and eosin.
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 β-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). β-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 × 129S6/SvEv) are subfertile 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
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 $\text{Ccna1} \text{tm1Col}$ allele since functional sperm with this allele are produced by outbred $\text{Ccna1} \text{tm1Col}^{+/+}$ 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 $\text{Ccna1} \text{tm1Col}^{+/+}$ and $+/-$ mice. Cells were counted in cross sections of seminiferous tubules. Values are given as mean ± S.E.M. per tubule cross section, with number of tubules examined in parentheses. Analyses were performed using tests from four outbred and two inbred mice of each genotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Strain</th>
<th>No. of pachytene cells/tubule cross section</th>
<th>No. of haploid cells/tubule cross section</th>
</tr>
</thead>
<tbody>
<tr>
<td>$+/-$</td>
<td>Outbred (129S6/SvEv x MF1)</td>
<td>$64 \pm 3.7$ (16)</td>
<td>$153 \pm 10.4$ (16)</td>
</tr>
<tr>
<td>$+/-$</td>
<td>65 ± 3.9 (16)</td>
<td>122 ± 7.8 (16)**</td>
<td></td>
</tr>
<tr>
<td>$+/-$</td>
<td>Inbred (129S6/SvEv)</td>
<td>$84 \pm 8.1$ (8)</td>
<td>$207 \pm 26.9$ (8)</td>
</tr>
<tr>
<td>$+/-$</td>
<td>74 ± 6.8 (8)</td>
<td>85 ± 3.9 (8)**</td>
<td></td>
</tr>
</tbody>
</table>

* $P = 0.024$, ** $P = 0.0005$, statistically significant differences between $\text{Ccna1} \text{tm1Col}^{+/+}$ and $+/-$ mice are indicated (two-tailed, Student’s t-test).
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 (129SvJ/C57BL6)-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 \( \text{Ccna1}^{\text{tm1Col}2/2} \) 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 \( \text{LacZ} \) 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 \( \text{LacZ} \) 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 \( \text{Ccna1}^{\text{tm1Col}2/2} \) 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 \( \text{CCNA1} \) gene.

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References

normally and are fertile whereas cyclin B1-null mice die in utero. PNAS 95 4344–4349.


