DNA replication licensing in somatic and germ cells

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Summary

The DNA replication (or origin) licensing system ensures precise duplication of the genome in each cell cycle and is a powerful regulator of cell proliferation in metazoan. Studies in yeast, Drosophila melanogaster and Xenopus laevis have characterised the molecular machinery that constitutes the licensing system, but it remains to be determined how this important evolutionary conserved pathway is regulated in Homo sapiens. We have investigated regulation of the origin licensing factors Cdc6, Cdt1, Mmc2 and Geminin in human somatic and germ cells. Cdc6 and Cdt1 play an essential role in DNA replication initiation by loading the Mcm2-7 complex, which is required for unwinding the DNA helix, onto chromosomal origins. Geminin is a repressor of origin licensing that blocks Mcm2-7 loading onto origins. Our studies demonstrate that Cdc6, Cdt1 and Mmc2 play a central role in coordinating growth during the proliferation-differentiation switch in somatic self-renewing systems and that Cdc6 expression is rate-limiting for acquisition of replication competence in primary oocyte. In striking contrast, we show that proliferation control during male gametogenesis is not linked to Cdc6 or Mmc2, but appears to be coordinated by the negative regulator Geminin with Cdt1 becoming rate-limiting in late prophase. Our data demonstrate a striking sexual dimorphism in the mechanisms repressing origin licensing and preventing untimely DNA synthesis during meiosis I, implicating a pivotal role for Geminin in maintaining integrity of the male germline genome.

Key words: Ki67, Cdc6, Cdt1, MCM, Geminin, DNA replication licensing, Oogenesis, Spermatogenesis, Gametogenesis

Introduction

Initiation of chromosomal replication depends on sequential assembly of pre-replicative complexes (pre-RCs) at replication origins during late mitosis and early G1 phase of the mitotic cell cycle (Mendez and Stillman, 2000; Dimitrova et al., 2002). Assembly of pre-RCs renders origins competent or ‘licensed’ for DNA replication during S phase (Blow and Hodgson, 2002). The initial step in origin licensing is binding of the origin recognition complex (ORC) to chromatin (DePamphilis, 2003). ORC functions as a landing platform for Cdc6 and Cdt1 which in turn load the minichromosome maintenance complex (Mcm2-7) onto chromatin (Lei and Tye, 2001). At the G1/S transition, firing of the pre-RC by cyclin-dependent kinases (CDK) and the Cdc7/ASK kinase (Wuarin and Nurse, 1996; Masai and Arai, 2002) triggers a conformational change in the origin licensing complex resulting in recruitment of Cdc45, DNA polymerase alpha, replication protein A and elongation factors. During this process, referred to as origin melting, the DNA helix is unwound by the minichromosome maintenance (MCM) complex (Labib and Diffley, 2001) and replication is initiated by the primase activity of DNA polymerase alpha (Bell and Dutta, 2002). Replication initiation is tightly coupled to removal of the license and thus prevention of re-licensing after origin firing. This step is critical as origins must fire once and only once per cell cycle to ensure genomic stability. Mammalian cells have adopted several strategies for the prevention of origin re-licensing. These include elevated CDK activity during S, G2 and M phases resulting in inactivation and/or removal of replication licensing factors, changes in gene expression and/or cell-cycle regulated ubiquitin-mediated proteolysis of licensing factors, and expression of a repressor of origin licensing known as Geminin (Nishitani and Lygerou, 2002). Geminin was identified in two independent screening procedures using Xenopus as a vertebrate model; firstly in a biochemical screen for mitotically degraded proteins (McGarry and Kirschner, 1998), and secondly in an expression cloning screen for early patterning factors that specify neural cell fate (Kroll et al., 1998). Geminin is not evolutionarily conserved and is found only in higher eukaryotes (Bell and Dutta, 2002). Studies in a variety of metazoan cell types and cellular extracts have firmly established that Geminin inhibits origin licensing via its interaction with Cdt1 thereby blocking MCM loading onto chromatin (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000; Tada et al., 2001; Quinn et al., 2001; Mihaylov et al., 2002; Shreeram et al., 2002). In human cells, following ubiquitination by the anaphase-promoting complex and degradation at the metaphase to anaphase transition, Geminin is absent during G1 until origin licensing is complete, and
accumulates during S, G2 and M phases (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000). Intriguingly, Geminin may represent a bifunctional molecule given its early neuronal specification role in Xenopus (Kroll et al., 1998) and Drosophila (Quinn et al., 2001), an activity mapped to an N-terminal domain (Kroll et al., 1998) distinct from the C-terminal coiled-coil domain linked to regulation of chromosomal replication (McGarry and Kirschner, 1998; Thepaut et al., 2002).

Importantly, initiation of chromosomal replication also represents a final and critical step in growth control downstream of complex networks of cell signalling pathways that have evolved to specify when and where cells divide in multicellular organisms (Stoeber et al., 2001). Over the last decade studies in yeast and Xenopus have identified some of the molecular mechanisms that control this critical decision point in cell proliferation, but it remains unclear how origin licensing is regulated in human tissue development and maintenance. The major role of cell division in adult life is to maintain the number of differentiated functional cells, replacing cells lost through death or injury. Tissues in the adult with the most rapid turnover are referred to as self-renewing systems and include skin, gut, testis and the haemopoietic system. These tissues share similar hierarchies of cellular development from stem cells to terminally differentiated mature cells via transit amplifying cells (Myster and Duronio, 2000; Watt and Hogan, 2000). We have previously shown that the origin licensing pathway plays a critical role in coordinating growth in human tissues; specifically in self-renewing tissues the transition from progenitor cells to the non-mitotic, terminally differentiated phenotype is coupled to repression of origin licensing through downregulation of the MCM helicase and its chromatin loading factor Cdc6 (Stoeber et al., 2001; Williams et al., 1998). Thus differentiation and DNA replication licensing appear to be mutually exclusive processes in keeping with the concept of antagonism between cellular circuits controlling proliferation and differentiation (Olson, 1992; Olson and Spiegelman, 1999). In this context two intriguing questions arise; first, is the physical connection of differentiation-promoting and replication-inhibiting domains within Geminin involved in triggering the proliferation-differentiation switch in self-renewing systems? Secondly, does Geminin play a role in preventing untimely DNA synthesis during other physiological processes?

To address these important questions for tissue development and maintenance, we first investigated regulation of origin licensing factors including Geminin in cycling human cells using a novel non-chemical synchronisation methodology (Thornton et al., 2002; Helmstetter et al., 2003). Extension of our findings in cultured cells to expression profiling of Cdc6, Cdt1, Mcm2 and Geminin in human gut epithelium and testis has revealed fundamental differences in the control of origin licensing between self-renewing systems of somatic and germ cell type. To investigate further whether Geminin blocks untimely DNA synthesis during germ cell development in higher vertebrates, we compared origin licensing in male and female human germ cells. Our findings show that human primary spermatocytes and primary oocytes prevent untimely DNA synthesis during meiosis I (MI) through repression of origin licensing by very different mechanisms. This intriguing sexual dimorphism may contribute to the striking difference in error rates during meiosis in the male and female germ lines.

Materials and Methods

Tissue specimens

Formalin-fixed, paraffin-embedded archival human colon, testis, and ovary tissue blocks from diagnostic biopsy or resection specimens were retrieved from the archives of the Department of Histopathology, University College London, UK. Ethical approval was obtained from the Joint UCL/UCLH Committees on the Ethics of Human Research. Haematoxylin/Eosin (H and E) stained and immunostained tissue sections were examined by two experienced pathologists.

Cell culture and synchronisation

MOLT-4 human leukemic lymphoblasts (ATCC CRL-1582; Rockville, MD, USA) were cultured at 37°C in CO2-independent Leibovitz (L-15) medium supplemented with 2 g/l dextrose, 50 units/ml penicillin G, 50 µg/ml streptomycin sulphate and 10% FCS (all from Invitrogen, Paisley, UK). Newborn (early G1 phase) cells were collected by membrane elution and followed during synchronous growth as described previously (Helmstetter et al., 2003; Thornton et al., 2002). Samples were removed at specific time points for determination of cell concentrations, cell sizes, DNA and protein content. Cell density and size were determined using a Coulter Z2 Particle Count and Size Analyzer (Beckman-Coulter, High Wycombe, UK).

Asynchronous HL-60 cells (ECACC 8501143, Salisbury, Wiltshire, UK) were maintained between 1-5x10⁶ cells/ml at 37°C and 5% CO₂ in RPMI 1640 medium supplemented with 2 mM glutamine and 10% FCS. HeLa S3 cells (ECACC 87110901) were cultured at 37°C and 5% CO₂ in DMEM supplemented with 10% FCS and synchronised in S phase by blocking with thymidine (2.5 mM) for 25 hours and releasing for 2 hours prior to harvesting.

Rabbit polyclonal antibody generation

pET14b-Geminin (Wohlschlegel et al., 2000) was expressed in E. coli BL21(DE3) and His₆-tagged Geminin protein purified by Ni-NTA metal affinity chromatography following the manufacturer’s instructions (Qiagen, Crawley, UK). Recombinant Geminin was further purified using a (FPLC) Hi-load Q Sepharose 16/10 column in NaPi buffer, and eluted with increasing concentrations of NaCl. Rabbits were injected with recombinant Geminin (125 µg) and received three boost injections following a standard immunisation protocol (Eurogentec, Seraing, Belgium). Sera were affinity-purified on a CNBr column against 10 mg of recombinant Geminin, eluted with 0.1 M glycine pH 2.5, and dialysed into PBS/1% BSA/0.1% sodium azide. An equal volume of sterile glycerol was added and antibodies designated G94 and G95 were stored at –20°C. Antibody purification was quality controlled by SDS-PAGE and ELISA. Specificity of antibodies was demonstrated by immunoblotting, by quenching all immunohistochemical staining after preincubating antibodies with recombinant Geminin for 1 hour at room temperature, and by flow cytometric analyses of asynchronous MOLT-4 cells after preincubation with either antibody G95 and recombinant Geminin (1:10 ratio), or G95 alone. Rabbit polyclonal antibodies were raised against a C-terminal fragment (amino acid residues 238-546) of bacterially expressed human Cdt1 as previously described (Wohlschlegel et al., 2000).

Flow cytometric analysis of DNA, Ki67 and Geminin content

For flow cytometric analysis of DNA content, cells were fixed in 80% ethanol and stained with propidium iodide as described previously (Helmstetter et al., 2003). For determination of Ki67 and Geminin content, cells were fixed and permeabilised with non-ionic detergent as described previously (Gong et al., 1995). After centrifugation, cell
pellets were resuspended in Dulbecco’s phosphate-buffered saline (D-PBS) and either 10 µl of FITC-conjugated Ki67 mAb (clone B56) or matched non-specific staining control (FITC-conjugated IgG; Pharmingen™, Oxford, UK), or 0.1 µg of anti-Geminin antibody G95, or G95 plus recombinant Geminin (1:10 ratio). After a 2-hour incubation at 4°C, cells were washed with D-PBS/1% BSA, concentrated via centrifugation and stained with 50 µg/ml propidium iodide and 20 µg/ml RNase A in PBS. Analyses of light scatter properties and DNA/protein content were performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Cell doublets were excluded by gating on a dot plot of the width of DNA fluorescence intensity (Erlanson and Lane, 1999). In most samples 10⁶ cells were examined and data stored/analysed using CellQuest™ software (BD Biosciences), Cyclyched (V.1.0.0.1) and WinMDI (V.2.7).

Preparation of protein extracts and immunoblotting
MOLT-4 and HL-60 total cell lysates (2×10⁸ cells) were prepared in sample buffer (3% SDS, 100 mM DTT, 60 mM Tris pH 6.8, 0.01% Bromphenol Blue, 10% glycerol) as described previously (Harlow and Lane, 1999). Lysates from asynchronous Jurkat and HeLa cells were obtained commercially (BD Biosciences, Oxford, UK) and 12.5 µg lystate was loaded per well. Lysates were separated on 10% SDS-polyacrylamide gels (Invitrogen) and immunoblotted as described previously (Stoeber et al., 2001) using anti-Geminin antibodies G94 and G95 (and corresponding preimmune sera), anti-Mcm5 mAb (Stoeber et al., 2002) and antibodies obtained from commercial sources against the following antigens: Cdc6 (NeoMarkers, Fremont, CA, USA; clone DCS-180.2), Mcm2 (clone 46) and PCNA (clone 24) (BD Transduction Laboratories™, Lexington, KY, USA), Mcm7 (NeoMarkers; clone 47DC141), Cyclin A (Santa Cruz; CA, USA; clone C-19) and Actin (Sigma Aldrich, Gillingham, UK; clone AC-15). Western blots of human tissue lysates were obtained from Geno Technology, Inc (St Louis, MO, USA) and probed with G94, G95 and anti-Cdc6 antibody.

Immunoprecipitation
Total lysates from HeLa cells (500 µg) harvested 2 hours after release from thymidine block were prepared in RIPA buffer containing 1 mM 1,4-dithiothreitol (DTT) and 1 tablet of Complete Mini, EDTA-free protease inhibitor cocktail per 10 ml buffer (Roche Diagnostics GmbH, Mannheim, Germany). Geminin protein was immunoprecipitated with anti-Geminin antibody G94 (1.5 µg) essentially as described previously (Harlow and Lane, 1999) with final washes in lysis buffer A (140 mM NaCl, 10 mM Tris pH 8.0, 0.5% NP-40) before resuspension in Laemmli buffer. Proteins were separated by SDS-PAGE, and immunoblots were probed with anti-Geminin antibody G95.

(q)RT-PCR analysis of geminin splice variants
For conventional RT-PCR using primers spanning the coding region of geminin, 300 ng of total RNA isolated with RNeasy Mini Kit (Qiagen) was reverse transcribed for 1 hour at 42°C in a reaction mix containing 10 mM dNTPs, 0.5 µl RNasin (Promega, Southampton, UK), 80 pmol of oligo(dT) primers, AMV RT buffer and 0.6 µl AMV reverse transcriptase (both Promega). After purification with Qiaprep PCR Purification Kit (Qiagen), 4 µl cDNA was amplified for 35 cycles (95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute) in 50 µl reactions containing Pfu polymerase buffer, 1 µl Pfu polymerase (each from Stratagene, Amsterdam, the Netherlands), 10 mM dNTPs and 0.2 µg of each geminin-specific primer. Geminin primers were designed within the first and last exons (exon 7) as follows [for: 5′ AGC AGG GCT TTG CAG AG 3′; rev: 5′ AAG TCA TGG CTG ACA ACT CAG A 3′]. For real-time RT-PCR analyses of geminin isoforms, RNA from human tissues was obtained from BD Biosciences and 2 µg was reverse transcribed as described previously (Lobenhofer et al., 2002) for 10 minutes at 25°C, 30 minutes at 48°C and 5 minutes at 95°C in a reaction mix containing 1× RT buffer (Applied Biosystems, Warrington, UK), 5.5 mM MgCl₂, 500 µM of each dNTP, 2.5 µM random hexamers, 0.4 units/µl RNase inhibitor and 1.25 U/µl MultiScribe™ reverse transcriptase (Applied Biosystems). Undiluted cDNA was amplified in duplicate or triplicate PCR reactions containing either (1) Geminin splice-variant-specific primers and SYBR Green PCR mastermix (Quantitect™ SYBR® Green PCR kit, Qiagen, La Jolla, CA, USA) or (2) 18s rRNA dual-labelled probes and TaqMan® Universal PCR Master Mix (each from Applied Biosystems). All reactions were performed on an ABI 7700 Sequence Detector (Applied Biosystems) following the manufacturers’ recommendations with thermal cycling parameters of 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Primers for four Geminin splice variants designated GemA [for: 5′ CTG TGG CCT TTT CGG AGT T 3′; rev: 5′ GGG TGG AGA CGC TCA ATC C 3′], GemB [for: 5′ GGG CCT CGG GGA CAA 3′; rev: 5′ TTA TGT AGA TGG TGA AGC ACA GA 3′], GemD [for: 5′ AGT TAG CAG GGC TTT ACT GA 3′; rev: 5′ AGA AGA CTA CGA TCA TCC CCA C 3′] and GemE [for: 5′ CGT CTG CTG GAT TGG GAC 3′; rev: 5′ AAA GCC CTG CTA ACT CCG C 3′] were designed using GenBank and Primer Express software (V.1.0; Applied Biosystems). Primer pairs for GemB, GemD and GemE are specific as one or both primers were designed within unique exons. Using Sequence Detection Software (Applied Biosystems), the threshold cycle (Ct) for each amplification reaction was determined. A variation of n cycles between reactions or samples represents a 2ⁿ-fold difference in message levels.

Immunohistochemistry
Anti-Mcm2 mAb (clone 46) was obtained from BD Transduction Laboratories™ (Lexington, KY, USA), anti-human Ki67 mAb (clone MIB-1) from DAKO (Glostrup, Denmark), and anti-Cdc6 mAb (clone DCS-180) from Neomarkers. Three µm sections of formalin-fixed, paraffin-embedded tissues were cut onto DAKO Techmate™ 52024 slides, dewaxed in xylene and rehydrated through an alcohol series to water. For antigen retrieval, tissues were pressure-cooked for 2-3 minutes (Ki67, Cdc6, Cdt1, Mcm2) or microwaved for 20 minutes in 0.1 M citrate buffer pH 6.0 (Geminin). For Ki67, Mcm2 and Geminin detection, automatic immunostaining was performed on a DAKO TechMate™ 500 as described previously (Dogan et al., 2000); Cdt1 immunostaining was performed manually. After blocking endogenous peroxidase activity, slides were incubated with primary antibodies for 1 hour at room temperature using the following concentrations: (a) ovary: Ki67 (1/50), Cdt1 (1/6000), Mcm2 (1/4000), G94 (1/4000), G95 (1/2000); (b) testis: Ki67 (1/50), Cdt1 (1/7500), Mcm2 (1/4000), G94 (1/4000), G95 (1/1500); (c) colon: Ki67 (1/50), Cdt1 (1/9000), Mcm2 (1/2000), G94 (1/4000), G95 (1/2000). Antigen-bound primary Ki67, Cdt1, Mcm2 and Geminin antibodies were detected with ChemMate™ EnVision™ Detection Kit (DAKO). For staining of Cdc6, slides were covered with 50 µl of Cdc6 antibody (1/50) for 4 hours at 37°C. To detect antigen-bound Cdc6 antibody, a labelled streptavidin-biotin visualisation system was utilised (ChemMate™ Detection Kit K5001, DAKO). Primary antibodies were omitted in negative controls and, in addition, appropriate tissue sections were used as positive and negative controls. To detect in situ expression of Geminin in ethanol-fixed MOLT-4 cells, staining was performed as described above with G95 (1/2000) and detected with the labelled streptavidin-biotin visualisation system (ChemMate™ Detection Kit K5001, DAKO).

Protein expression profile analysis
Slides were examined and images captured with an Olympus BX51.
microscope/CCD camera setup using ANAlysis software (SIS, Münster, Germany). Captured images were printed and cells expressing the protein of interest counted. In colon, epithelial cells in the basal compartment (BC: lower third of colonic crypts) and luminal compartment (LC, upper two thirds of colonic crypts) were evaluated separately. In testis, spermatogonia (SG), primary spermatocytes (SC), early spermatids (EST) and late spermatids (LST) were examined separately. Different stages of spermatogenesis were identified according to established morphological criteria (Trainer, 1997). In ovary, primary oocytes located within primordial follicles of the ovarian cortex were investigated. To assess interobserver variability, 10% of all cases were recounted by an independent investigator with high agreement.

Results
Protein levels of origin licensing factors oscillate during the human mitotic cell cycle
To study the regulation of Geminin in human cultured cells and tissues, we raised rabbit polyclonal antibodies against full-length human Geminin (Fig. 1A). In immunoblots of total cell lysates from asynchronously proliferating Jurkat, HeLa, MOLT-4 and HL-60 cells, affinity-purified anti-Geminin antibodies G94 and G95 detected a single protein of the reported molecular mass for Geminin (~33 kDa) (McCarty and Kirschner, 1998; Wohlschlegel et al., 2000). Both antibodies also recognised recombinant Geminin corresponding to the reported size of human Geminin. Antibodies G94 and G95 detected a single ~33 kDa protein in total cell lysates from asynchronous Jurkat, HeLa, MOLT-4 and HL-60 cells, and recognised nanogram quantities of recombinant Geminin (~35 kDa) whereas preimmune (PI) sera did not.

Immunoprecipitation (IP) of HeLa S phase cell lysates with antibody G94 followed by immunoblotting (IB) with G95 shows the 33 kDa protein in the immunoprecipitate whereas immunoprecipitation with preimmune (PI) sera did not. (D) Bivariate flow cytometry of asynchronous MOLT-4 cells with antibody G95 alone, or G95 plus recombinant Geminin. Note that cells in G1 (2C) are Geminin-negative (depicted as area within the trapezoidal region) whereas cells in S or G2/M are positive (area outside the trapezoidal region). Preincubation of antibody G95 with recombinant Geminin blocked detection of this cell cycle specific expression.

Statistical analysis
Colon and testis data were analysed using identical statistical methods. For each of these sites, five subjects each provided 100 crypts (colon) or tubules (testes) for data analysis. Each of the five markers was used to stain 20 crypts/tubules from each subject. For each crypt/tubule we formed a summary statistic (Altman, 1991), the proportion of cells stained positive by the marker used for the crypt/tubule. The labelling index (percentage of positive cells) was estimated for each marker using a linear mixed model, a form of analysis of variance that accounts for the repeated measures within subject (Laird and Ware, 1982). For ovary, five subjects each provided five histology sections for data analysis. Each marker was used to stain one section from each subject. In each section, all primary oocytes were assessed for protein expression by calculating the proportion of cells stained positive. A minimum of 10 primary oocytes were evaluated per case for each protein (overall between 120 and 164 primary oocytes were examined for protein expression). The labelling index was estimated for each marker by averaging these proportions across the subjects. Labelling indices for different pairs of markers, or for the same marker in different compartments, were compared by calculating ratios of the indices for each subject, and averaging these across the subjects. Intervals that did not contain the value 1 indicated that the two indices were considered different at the 5% level of statistical significance. Analysis was carried out using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA) and S-PLUS 6 (MathSoft Inc., Seattle, WA, USA).

To study expression of origin licensing factors including Geminin during synchronous growth of proliferating MOLT-4 human lymphoblastic lymphocytes shows that Geminin is absent in cells with a 2C (or G1 phase) DNA content and increases during S/G2 in keeping with previous reports (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000). Preincubation of antibody G95 with recombinant Geminin blocked detection of this cell-cycle-specific expression (Fig. 1D). Taken together, these data show that antibodies G94 and G95 are specific for a cell cycle regulated protein in human cell lysates with a molecular mass corresponding to the reported size of human Geminin.

To study expression of origin licensing factors including Geminin during synchronous growth of proliferating human cells, we exploited membrane elution (Fig. 2A), a novel non-chemical synchronisation methodology (Thornton et al., 2002; Helmstetter et al., 2003). Newborn (early G1 phase) MOLT-4 cells isolated by membrane elution progressed through a full cycle of synchronous growth as demonstrated by FACS analysis of the DNA content (Fig. 2B). In total cell lysates polypeptide (Fig. 1C). Bivariate flow cytometric analyses of DNA and Geminin content in asynchronously proliferating MOLT-4 human lymphoblastic lymphocytes shows that Geminin is absent in cells with a 2C (or G1 phase) DNA content and increases during S/G2 in keeping with previous reports (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000). Preincubation of antibody G95 with recombinant Geminin blocked detection of this cell cycle specific expression.

Fig. 1. Generation and characterisation of anti-Geminin antibodies. (A) Coomassie staining of purified bacterially expressed His6-Geminin. (B) Immunoblots of recombinant Geminin and human cell lysates with affinity-purified anti-Geminin antibodies G94 and G95. Antibodies G94 and G95 detected a single ~33 kDa protein in total cell lysates from asynchronous Jurkat, HeLa, MOLT-4 and HL-60 cells, and recognised nanogram quantities of recombinant Geminin (~35 kDa) whereas preimmune (PI) sera did not. (C) Immunoprecipitation (IP) of HeLa S phase cell lysates with antibody G94 followed by immunoblotting (IB) with G95 shows the 33 kDa protein in the immunoprecipitate whereas immunoprecipitation with preimmune (PI) sera did not. (D) Bivariate flow cytometry of asynchronous MOLT-4 cells with antibody G95 alone, or G95 plus recombinant Geminin. Note that cells in G1 (2C) are Geminin-negative (depicted as area within the trapezoidal region) whereas cells in S or G2/M are positive (area outside the trapezoidal region). Preincubation of antibody G95 with recombinant Geminin blocked detection of this cell cycle specific expression.
Repression of origin licensing in human tissues

Repression of origin licensing in human tissues prepared from synchronous batch cultures (Fig. 2C), Cdc6 oscillates during the cell cycle with levels increasing during S phase before diminishing in late G2/M. Cdt1 expression peaks in early G1 with protein levels declining during S phase progression. Protein levels of the DNA helicase subunits Mcm2, Mcm5 and Mcm7 increase linearly and in parallel in early G1 up to a maximum in S phase before decreasing during G2/M. Oscillation of MCM protein levels was also observed in a second successive cycle of synchronous MOLT-4 growth (data not shown), suggesting that these changes in protein levels may have been previously obscured by metabolic perturbation through use of chemical synchronisation agents. As shown in asynchronous MOLT-4 cells, by flow cytometry (Fig. 1D), Geminin is absent during the permissive window for pre-RC assembly in G1 with levels becoming detectable at the G1/S transition and increasing linearly during S/G2 before mitotic degradation. The specificity of the anti-Geminin antibodies and the cell cycle periodicity of Geminin expression is further supported by in situ analysis of MOLT-4 cells showing expression restricted to late S/G2 phase but not in G1 phase (Fig. 2D). The periodicity in Geminin expression is similar to that seen for Cyclin A and is consistent with findings in HeLa cells (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000). Protein levels of proliferating cell nuclear antigen (PCNA) and the cytoskeletal filamentous protein Actin were essentially unchanging during synchronous progression through the cell cycle. Moreover, flow cytometric analysis showed that the fraction of cells expressing the standard proliferation marker Ki67 during synchronous growth (85-95%) remained constant and was similar to the proportion of Ki67-positive cells in asynchronous culture.

Origin licensing factors are downregulated during the proliferation-differentiation switch in colon but not testis

To investigate regulation of the origin licensing pathway in human somatic and germ cell self-renewing tissues, we analysed the protein expression profiles of Ki67, Cdc6, Cdt1, Mcm2 and Geminin in colon and testis. Labelling indices (percentage of positive cells) for each protein are described in Table 1, and representative immunostained tissue sections are

![Fig. 2. Expression profiles of origin licensing factors during the mitotic cycle of human synchronous cells. (A) Schematic of membrane elution methodology. Asynchronously proliferating MOLT-4 cells are immobilised on surfaces such that cell division results in release of one daughter cell into the effluent while the other daughter cell remains surface-bound. Newborn (early G1 phase) cells are continuously released in the effluent and grow synchronously without evidence of disturbance. (B) FACS DNA profiles of synchronously proliferating MOLT-4 cells at 3-hour intervals. (C) (Top) Cell-cycle-phase distributions as determined by DNA profiles in B. Immunoblots of origin licensing factors and control proteins in total cell lysates from equivalent numbers of synchronously proliferating MOLT-4 cells at the indicated times. (D) In situ staining of Geminin (G95) in asynchronous, early G1-phase and S/G2 phase MOLT-4 cells. Synchronous cells were obtained using the membrane elution method.](Image)

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<td></td>
<td>BC</td>
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<tr>
<td>Ki67</td>
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<td>Mcm2</td>
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<td>Cdt1</td>
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Values are mean of the compartment percentage of cells stained positive (s.d.). BC, basal compartment; LC, luminal compartment; SG, spermatogonia; SC, primary spermatocytes; EST, early spermatids; LST, late spermatids.
shown in Figs 3 and 4. In colonic crypts (Fig. 3), expression of Ki67 and Mcm2 is found in a high proportion of stem-transit progenitor cells within the basal compartment (BC; 82% and 88%, respectively). A significantly lower percentage of these cells express Geminin [28% (with antibody G94); 33% (G95)] and Cdt1 (44%). Low level expression (1.6%) of Cdc6 is detected in crypt cells within the BC. Geminin expression is restricted to a subset of cells in the BC. Cdc6 expression is restricted to basal proliferating cells. Note the absence of origin licensing factors at the base of the crypts (putative stem cell compartment).

In contrast to the basal proliferative compartment, the Ki67 labelling index is significantly lower in the luminal compartment (82% in the BC against 12% in the LC) of
Repression of origin licensing in human tissues

colonic crypts. In the latter compartment, which contains differentiating goblet and enteroendocrine cells, the drop in Ki67 labelling index corresponds to loss of proliferative capacity as cells migrate to the surface. Triggering of the proliferation-differentiation switch also coincides with downregulation of Cdc6 (1.6% in the BC compared with 0.1% in the LC) and Cdt1 (44% in the BC against 13% in the LC). Similarly, Geminin is significantly downregulated as cells migrate from the proliferative to the differentiated compartment [28% (G94)/33% (G95) in the BC compared to 3.1% (G94)/3.7% (G95) in the LC]. Importantly, expression of Mcm2 persists at relatively high levels (40%) in early differentiation with downregulation occurring only as cells terminally differentiate. Notably, these origin factors are absent in cells at the base of colonic crypts (fourth position or below), the putative stem cell compartment (Potten, 1986). This suggests that colonic stem cells, which have a high capacity for self-renewal but divide infrequently, reside in an out-of-cycle, non-licensed quiescent state (S. R. Kingsbury, M.L., T.F., E. C. Obermann, unpublished observation).

In testis, the migration of cells into the differentiated LC, which contains germ cells in meiotic and post-meiotic phases, is also linked to downregulation of Ki67 as cells lose their ability to initiate DNA synthesis. This is reflected in the significant decrease in the Ki67 labelling index from 24% for spermatogonia to 0.6% in spermatocytes. However, in marked contrast to early differentiating colonocytes, in which

downregulation of origin licensing factors occurs in parallel with Ki67, early differentiation in testis (i.e. maturation of spermatogonia into primary spermatocytes) is associated with continued presence of these replicative factors (Fig. 5). Primary spermatocytes show strikingly high levels of Cdc6, Cdt1, Mcm2 and Geminin in contrast to the marked downregulation seen in the differentiating LC of colon [Cdc6: 42% against 0.1%; Cdt1: 33% against 13%; Mcm2: 99% against 40%; Geminin: 91% (G94)/95% (G95) against 3.1% (G94)/3.7% (G95)]. Cdt1 expression levels fall in late prophase

**Fig. 5.** The proliferation-differentiation switch in colon and loss of DNA replication competence are coupled to downregulation of Ki67, Cdc6, Cdt1 and Geminin. In testis, the proliferation-differentiation switch and loss of DNA replication competence are also coupled to downregulation of Ki67. However, in contrast to somatic cells, early stages of germ cell differentiation and entry into meiosis are associated with high levels of Cdc6, Cdt1, Mcm2 and Geminin. Late stages of differentiation in somatic and germ cell self-renewing systems are linked to downregulation of Cdc6, Cdt1, Mcm2 and Geminin. Note that in contrast to Ki67, Cdc6, Cdt1 and Geminin, downregulation of Mcm2 is coupled to the final stages of the differentiation programme. BC, basal compartment; LC, luminal compartment; TDC, terminally different compartment; SG, spermatogonia; SC, primary spermatocytes; EST, early spermatids and late spermatids (LST)

**Fig. 6.** Ki67, Cdc6, Cdt1, Mcm2 and Geminin protein expression in human ovary (original magnification ×120). The schematic drawing of adult ovary (cross section; top left) shows that the predominant follicles are primordial follicles (white arrow) composed of primary oocytes arrested in the prophase of MI and surrounded by flattened follicular cells (black thin arrow). Primordial follicles mature into Graafian follicles following a period of follicular growth. Follicular growth involves proliferation of follicular cells and increase in size of oocytes to form primary follicles (white arrowhead). Primary oocytes do not express Ki67 contrasting with expression in follicular cells of primary follicles. Virtually all primary oocytes express Cdt1 and Mcm2 proteins. Geminin is found in a proportion of proliferating follicular cells, but is not expressed in primary oocytes. Cdc6 is detected in proliferating follicular cells of primary and secondary follicles (inset; arrowhead) but not in primary oocytes.
Quantitative RT-PCR was used to study these and two bp), which was not found within the smaller variant (GemE).

(designated GemA) contained a sequence within exon 1 (132 stable (liver) or permanent (heart) tissues, the two alternatively renewing tissues such as testis, colon and bone marrow than in
geminin message levels were generally more abundant in self-primers spanning the geminin locus gave two distinct bands spermatogenesis. Conventional RT-PCR analyses using germ cell-specific transcripts of geminin are expressed during unique mechanisms of gene expression in testis (Goldberg, the male germline genome.

implicate Geminin as a key factor in maintaining stability of DNA synthesis during meiosis I is repressed into spermatids, there is downregulation of Cdc6, Mcm2 and Geminin.

Untimely DNA synthesis during meiosis I is repressed via different mechanisms in the male and female germline

Like primary spermatocytes, primary oocytes (within the primordial follicles of the ovary) are arrested in prophase of MI. Represented immunostained tissue sections of human ovary are illustrated in Fig. 6. A comparison of labelling indices for Cdc6, Cdt1, Mcm2, Geminin and Ki67 in primary spermatocytes and primary oocytes is described in Fig. 7. Neither primary spermatocytes nor primary oocytes express Ki67. In contrast, the male and female germ cells each express significant levels of Cdt1 (spermatocytes: 33%, oocytes: 97%) and Mcm2 (spermatocytes: 98.9%, oocytes: 99.6%). Notably, although Cdc6 and Geminin are present at high levels in primary spermatocytes (42% and 95%, respectively), these origin licensing factors are absent in primary oocytes (Fig. 7). These data demonstrate a striking sexual dimorphism in DNA replication licensing of male and female germ cells, and implicate Geminin as a key factor in maintaining stability of the male germline genome.

Given high Geminin levels in primary spermatocytes and unique mechanisms of gene expression in testis (Goldberg, 1996; Walker et al., 1999), we sought to investigate whether germ cell-specific transcripts of geminin are expressed during spermatogenesis. Conventional RT-PCR analyses using primers spanning the geminin locus gave two distinct bands when total RNA from a panel of cell lines (Fig. 8A) and human tissues (Fig. 8B) (including testis) was investigated. Although geminin message levels were generally more abundant in self-renewing tissues such as testis, colon and bone marrow than in stable (liver) or permanent (heart) tissues, the two alternatively spliced forms were not equally expressed in different tissues. Sequence analysis of the isoforms revealed that the larger form (designated GemA) contained a sequence within exon 1 (132 bp), which was not found within the smaller variant (GemE).

Quantitative RT-PCR was used to study these and two additional variants supported by cDNA clones within AceView (http://www.ncbi.nlm.nih.gov) in RNA from human testis, ovary, colon and cervix (Fig. 8C). Analyses revealed that each variant was present in each tissue with geminin message levels generally higher in testis (data not shown). Message levels of the endogenous control gene (18s rRNA) were virtually identical for all tissues (inset, Fig. 8C). Significantly, the GemE splice variant was considerably more abundant (∼8-fold) in testis than in ovary, colon or cervix. GemE represents a mRNA that could encode 258 amino acids if translated. Analysis of additional amino acids at the N terminus suggests that the predicted protein could possess a zinc finger motif which may facilitate protein-protein or protein-DNA interactions (Sujatha and Chatterji, 1991). Interestingly, the GemE isoform also has cell cycle periodicity in MOLT-4 cells during synchronous growth with a peak in expression at G1/S (data not shown).

At the protein level, immunoblotting of human testis and ovary lysates with anti-Geminin antibodies G94 and G95 detected the 33 kDa form of Geminin in testes but not ovary (Fig. 8D). These data are in keeping with our immunohistochemical findings (Figs 4 and 6). Interestingly, in addition to the 33 kDa form, the Geminin-specific antibodies also detected a polypeptide with a molecular mass of ∼20 kDa in testis lysates. Preincubation of the antibodies with recombinant Geminin blocks this interaction (Fig. 8D), suggesting that the additional polypeptide detected may constitute a testis-specific Geminin isoform. This band could of course also be a result of non-specific proteolytic cleavage after tissue lysis or merely a protein with a shared epitope. However, it is noteworthy that similar proteolytic cleavage after lysis of other tissues was not observed and that detection of the 20 kDa polypeptide with two independent polyclonal antibodies was blocked after preincubation with recombinant Geminin. As is the case for Geminin, a single protein with a molecular mass of ∼62 kDa consistent with the reported electrophoretic mobility of human Cdc6 was detected with an anti-Cdc6 antibody in lysates from testis but not ovary (Fig. 8E), again confirming our immunohistochemical finding that Cdc6 is absent in primary oocytes (Fig. 6). Taken together, our data suggest alternative transcripts of geminin may be involved in germ cell-specific functions. Future detailed analyses at RNA and protein level are required to characterise these isoforms.

**Discussion**

Although the general scheme of cellular hierarchies is similar for self-renewing systems, differences are observed between those of somatic and germ cell type. In somatic self-renewing systems such as skin and gut, cell production occurs in the basal proliferative compartment through mitotic cell divisions with migration and differentiation of post-mitotic cells into the luminal compartment (Watt and Hogan, 2000; Potten, 1997). In testis, a more complex cellular hierarchy is found. Germ cell development occurs in successive mitotic, meiotic and post-meiotic phases with germ cells moving from the basal compartment to the lumen of seminiferous tubules as cells engage their differentiation programme (spermatogenesis) (Potten, 1997). The basic features of meiosis, two cell divisions with no intervening DNA replication, result in halving of the chromosomes complement. Importantly, it is during the long prophase of meiotic division I (MI) that genetic recombination
Repression of origin licensing in human tissues occurs, increasing the genetic variability of the organism (Champion and Hawley, 2002). Following engagement of the differentiation programme and migration of cells into the luminal compartment of colon there is abrupt downregulation of Ki67, Cdc6, Cdt1, Mcm2 and Geminin expression. Downregulation of Cdc6, Cdt1 and MCM proteins provides a powerful downstream mechanism to suppress DNA synthesis in this differentiated luminal compartment (Stoeber et al., 2001; Stoeber et al., 1998; Yan et al., 1998). The protein expression profile of Geminin and its downregulation during the proliferation-differentiation switch in this somatic self-renewing tissue is consistent with Geminin’s role as an inhibitor of origin licensing in cycling cells (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000). We detected Geminin in a subpopulation of colonic progenitor transit cells, in slightly fewer cells than Ki67, which is present throughout all phases of the mitotic cell cycle (Brown and Gatter, 2002), and consistent with the S-G2-M phase labelling index observed in cultured human cells (Wharton et al., 2004). Interestingly, increased expression of Geminin during the proliferation-differentiation switch when cells migrate from the proliferative to luminal compartments was not observed, suggesting that Geminin does not function as an inducer or regulator of differentiation in somatic self-renewing systems. Onset of differentiation and migration of germ cells into the luminal compartment of testis is also associated with loss of cell proliferation as indicated by downregulation of Ki67. However, in striking contrast with colon, the halt to DNA synthesis is not coupled to downregulation of DNA replication licensing factors (Fig. 5). Indeed Cdc6, Cdt1 and Mcm2 protein levels are high in primary spermatocytes, germ cells in prophase of MI, despite the fact that suppression of chromosomal replication during this stage is critical for production of a haploid genome (Champion and Hawley, 2002). In keeping with our findings, a screen of human tissues has shown that Cdt1 mRNA is most highly expressed in testis and thymus (Nishitani et al., 2001) and, notably, studies in yeast and p53–/– human cells have demonstrated that high levels of Cdc6 and Cdt1 can trigger re-replication events (Gopalakrishnan et al., 2001; Vaziri et al., 2003). Such events during meiosis would be catastrophic, leading to genetic instability with formation of abnormal gametes and embryos. The unexpected high levels of origin licensing factors in primary spermatocytes raises the question of alternative functions for these replicative factors including roles in genetic recombination, DNA repair or coordination of reduction divisions. Importantly, in this study we have found that Geminin is present at very high levels in primary spermatocytes, suggesting that this negative regulator plays a pivotal role in repressing origin licensing during MI, thus ensuring integrity of the male germline genome. Interestingly Cdt1 becomes rate-limiting in late prophase, suggesting a dual mechanism for repressing origin licensing...
during this critical stage of male gametogenesis. High levels of Geminin during the proliferation-differentiation switch is also consistent with a putative coordinating role in germ cell differentiation. Support for Geminin as a coordinator of proliferation and differentiation comes from two recent studies which provide compelling evidence that the products of genes regulating development (hox, polycomb and six) also control cell proliferation by directly inhibiting the interaction of Geminin with Cdt1 (Del Bene et al., 2004; Luo et al., 2004). Completion of the differentiation programme, however, with transition of spermatocytes into spermatids and spermatozoa (spermiogenesis) is associated with downregulation of Cdc6, Mcm2 and Geminin as observed in colonic. Thus the terminally differentiated phenotype of end stage cells in both germ line and somatic self-renewing systems is characterised by repression of origin licensing through downregulation of Cdc6, Cdt1 and MCM helicase proteins, a powerful downstream mechanism for suppressing proliferative capacity.

The presence of high Geminin levels in primary spermatocytes suggests protein expression and/or prolonged half-life in these cells, which are locked in prophase of MI. At transcription level, testis is unique in that many germ cell-specific transcripts are produced during spermiogenesis (Goldberg, 1996). Our detection of a geminin mRNA isoform significantly more abundant in testis than in its female germline counterpart or in somatic tissues, suggests that GeminE may indeed have testis-specific functions during spermiogenesis. Notably it has been suggested that deletions within the 5’UTR, as is the case for GemE when compared with the larger isoform, could be associated with increased mRNA expression or transcriptional modulatory function (Yu et al., 2001). At the protein level, a prolonged half-life of Geminin protein may occur through testis-specific mechanisms that modulate anaphase promoting complex-targeted proteolysis during meiosis. This has been observed for Cyclin B1, a target for the anaphase promoting complex that is incompletely degraded at the end of MI in Xenopus and that is essential for suppression of DNA synthesis after Cdc2 inactivation at MI exit (Iwabuchi et al., 2000; Iwabuchi et al., 2002; Stern, 2003).

Cell cycle kinetics in the human germline are remarkably different. Oogonia begin meiosis during foetal development but arrest part-way through prophase of MI to form primary oocytes, and do not complete the first division until ovulation. The second meiotic division (MII) is completed only if the egg is fertilised. Thus oogenesis may last for several decades. In contrast, male meiosis begins at puberty and is a continuous process with spermatocytes progressing through prophase I and through the second reduction division in around 22 days (Wolgemuth et al., 2002). Our data and those of Nishitani et al. (Nishitani et al., 2001) suggest that all factors required for origin licensing are present within tests. This implicates Geminin as an inhibitor of origin licensing and thus a suppressor of DNA replication in primary spermatocytes during the prolonged prophase of MI. An important question is whether a similar mechanism operates during female meiosis. Our studies have revealed a very different mechanism by which primary oocytes suppress chromosomal replication (Fig. 7). Although primary oocytes express the MCM helicase proteins and Cdt1, the second essential loading factor Cdc6 is absent. Interestingly, Geminin, which in contrast to primary spermatocytes would not be required to suppress origin licensing in the absence of Cdc6, is also absent in primary oocytes. A similar molecular mechanism for acquisition of DNA replication competence during oocyte maturation has been reported for Xenopus, in that competence to replicate in the unfertilised egg is conferred by Cdc6 during meio tic maturation (Lemaître et al., 2002; Whitmire et al., 2002). Our finding that Geminin is absent in human primary oocytes is also in keeping with the reported absence of Geminin in stage VI (arrested) Xenopus oocytes with the synthesis of Geminin being induced during oocyte maturation (McGarry, 2002). Thus Cdc6 is most probably the crucial regulator in humans, which ensures that eggs do not engage in untimely DNA replication during the prolonged prophase of MI which (might last several years). It is noteworthy that Cdc6 is rate-limiting for acquisition of DNA replication competence not only in female germ cells, but also in somatic cells re-entering the cell division cycle from quiescence (Stoeber et al., 1998; Lea et al., 2003).

Sexual dimorphism has been identified at many levels in relation to meiosis and the production of haploid gametes. Mammalian males and females use different strategies, transit meiosis with different levels of success, and exit with different end products. Importantly, the quality control mechanisms that ensure genetic integrity of gametes during meiosis also show a striking sexual dimorphism (Hunt and Hassold, 2002). It has been shown that the pachytene and spindle assembly checkpoints are missing or less stringent in mammalian oogenesis than in spermatogenesis (Wolgemuth et al., 2002; Voet et al., 2003), and that these differences might account for the high error rates observed in oocytes. Direct studies of human gametes show chromosomal abnormalities in up to 20% of oocytes but only 3-4% of sperm (Martin and Rademaker, 1991). It is possible that the alternative strategies for repression of origin licensing observed by us in this study of human gametogenesis could contribute to the striking difference in error rates during meiosis in the male and female germlines.

Here we demonstrate that suppression of proliferation in terminally differentiated cells of self-renewing tissues (spermatocytes and surface colonocytes) is achieved through downregulation of the Cdc6, Cdt1 and MCM origin licensing factors. Differentiated hepatocytes of adult liver, a stable tissue, and mature adult neurons and myocardocytes, the latter examples of permanent tissues (Leblond, 1963), also suppress origin licensing through Cdc6 and MCM downregulation (Stoeber et al., 2001). Primary oocytes, and also quiescent T cells (Lea et al., 2003), mimic somatic cells with induction of Cdc6 rate-limiting for acquisition of replication competence. Cdc6, the loading factor for the MCM helicase, therefore functions as the ‘master regulator’ of cell proliferation control in these tissues. In contrast to somatic and female germ cells, repression of origin licensing in primary spermatocytes appears to depend on inhibition of origin licensing by Geminin with Cdt1 only becoming rate-limiting in late prophase. Taken together our studies have revealed a remarkable heterogeneity in the regulation of origin licensing in somatic and germ cell tissues.

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