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Competing interests statement

The authors declare no competing financial interests.

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INNOVATION

Control of DNA replication and its potential clinical exploitation

Michael A. Gonzalez, Kiku-e K. Tachibana, Ronald A. Laskey and Nicholas Coleman

Abstract | Multiple conserved mechanisms limit DNA replication to once per cell cycle.

One vital level of control focuses on the loading of the heterohexameric ring of minichromosome maintenance proteins (MCMs) onto chromatin in the hierarchical assembly of the pre-replication complex at origins of replication. An essential role in proliferation for MCMs and their regulators makes them potentially important biomarkers for routine clinical use in cancer detection and prognosis.

Most cells in the human body are not cycling and exist in a state of quiescence (the G0 phase). A minority of actively cycling cells are located at specific sites in tissues such as epithelia (for example, in the cervix, colon

and skin) and bone marrow. Exogenous growth factors stimulate cell-cycle entry from G0 into the first gap phase (G1). During late G1, cells are committed to DNA replication (which occurs in S phase), which is followed by a second gap phase (G2) and segregation of the replicated chromatin between the two daughter cells in mitosis (M). Cell-cycle progression is driven by changes in cyclin–cyclin-dependent kinase (CDK) pairs^{1,2}. Cyclin D–CDK4, cyclin D–CDK6 and cyclin E–CDK2 regulate G1. S phase is triggered by cyclin A–CDK2, and cyclin B–CDK1 regulates progression through G2 and entry into mitosis.

To ensure the completion of each phase before entry into the next, surveillance mechanisms or cell-cycle checkpoints monitor the processes. If errors or defects are detected,

progression is reversibly halted by CDK inhibitors. The Ink4 family of CDK inhibitor proteins (**INK4A** (p16), **INK4B** (p15), **INK4C** (p18) and **INK4D** (p19)) induce a G1 phase arrest by inhibiting CDK4 and CDK6, whereas the Cip/Kip family (**WAF1** (p21, CIP1), **KIP1** (p27) and **KIP2** (p57)) inhibit CDK2.

During transformation to the malignant phenotype, control of the cell cycle is disrupted. The cancer cell typically acquires somatic mutations in genes that regulate the cell cycle (for example, cyclins, CDKs and CDK inhibitors)². Progress through the cycle might be stimulated by overexpression of cyclins (for example, amplification of the cyclin D1 gene (**CCND1**) or locus translocation of 11q13 is seen in a wide range of epithelial malignancies), enhanced CDK activity (for example, CDK4 on 12q13 is

amplified in sarcomas and gliomas), or inactivation of the CDK inhibitors (for example, **CDKN2A** (encoding INK4A) on 9p21 is frequently hypermethylated, mutated or deleted in many carcinomas)¹. Cell-cycle checkpoints might also be defective, allowing genomic instability and progression towards uncontrolled cellular proliferation.

Control of DNA replication

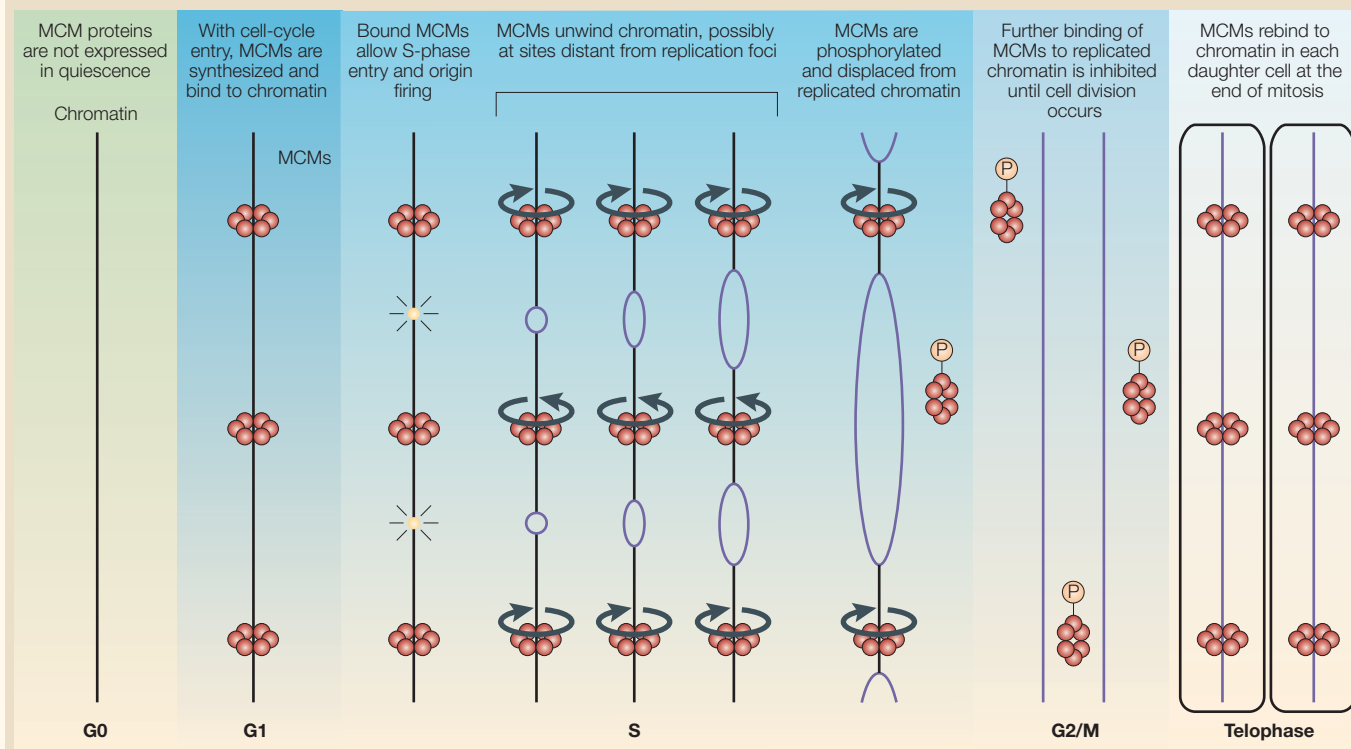
DNA replication is a highly conserved mechanism that requires the presence of the heterohexameric complex of minichromosome maintenance proteins (MCMs) 2–7 — so called because mutants in yeast are defective in maintaining extrachromosomal elements (minichromosomes) — on chromatin³ (BOX 1). The MCMs display weak helicase activity and form a ring structure with a central pore

that could accommodate double-stranded DNA. It has been suggested that MCMs function as replicative helicases. Before and during S phase, MCMs are distributed on unreplicated DNA, and they do not preferentially localize to sites of DNA replication in animal cells. These and other observations have led to the hypothesis that MCMs might function as rotary molecular pumps that are tethered to the nuclear matrix in S phase⁴.

As DNA replication proceeds, MCMs are phosphorylated by high CDK activity, and/or the DBF4–CDC7 kinase (DBF4-dependent kinase, DDK), and are displaced from chromatin leaving replicated DNA free of MCMs. The displaced MCMs remain soluble in the nucleus throughout S phase, G2 and early mitosis, before re-binding to chromatin in late mitosis and G1 in preparation for

Box 1 | Control of DNA replication in human cells

It is essential that genome replication produces precisely two copies. Small unreplicated regions will result in deletions and breaks at mitosis, whereas local over-replication would result in gene amplification and possibly oncogene amplification. The coupling of DNA replication to cell-cycle progression is complicated by the fact that eukaryotic genomes are large and discontinuous. Initiation from thousands of replication origins on each chromosome makes efficient duplication possible⁵⁵. An average S phase lasts approximately 6–8 hours in proliferating human primary fibroblasts in tissue culture, although the exact duration of DNA replication varies according to cell type^{56,57}. In addition to the spatial distribution of replication origins, there is temporal variation in origin firing, with some origins firing ‘early’ during S phase and others firing ‘late’⁵⁸. Euchromatin that harbours transcriptionally active genes is replicated early, whereas heterochromatic regions tend to be replicated late. The spatio-temporal regulation of replication sites has not been found to differ between normal and immortalized or transformed human cells⁵⁹. The cyclical binding of minichromosome maintenance proteins (MCMs) to chromatin limits DNA replication to once per cell cycle. MCMs might function as rotary molecular pumps that are tethered to the nuclear matrix in S phase. In this model, ATP-dependent rotation of the tethered MCM ring relative to the linear axis of DNA propels DNA through immobilized sites of replication, rotating and unwinding the DNA as it does so⁴. Although this model can reconcile the distribution of MCMs with their probable function as helicases, key features remain to be tested. Purple DNA strands represent replicated chromatin.



“Multiple mechanisms cooperate to ensure that each region of DNA is replicated precisely once between consecutive divisions.”

another round of replication⁵. Notably, daughter cells that have committed themselves to exit the cell cycle and enter quiescence downregulate MCMs, which are no longer required in quiescence⁶.

Multiple mechanisms cooperate to ensure that each region of DNA is replicated precisely once between consecutive divisions. The MCMs conform exactly to predictions of

the original licensing factor model^{7,8}, which proposed that an essential positive activator of replication was bound to DNA before replication and was inactivated by passage of the replication fork. MCMs would therefore ‘license’ only one round of replication at a time.

As MCMs are present in the nucleus throughout the cell cycle, their cyclical chromatin binding is strictly regulated by recruitment factors and CDK activity³ (FIG. 1). The origin recognition complex (ORC), which is bound to chromatin throughout the cell cycle (with the exception of ORC1, which is degraded during S phase⁹), forms a landing pad for the MCM loading factors CDC6 and CDT1 to form the pre-replication complex (Pre-RC). An increase in cyclin A–CDK2 levels and the attendant activity of DDK results in origin firing and the start of S phase.

Following S phase initiation, CDC6 and CDT1 are phosphorylated and displaced from chromatin. CDC6 is exported to the cytoplasm, whereas CDT1 is targeted for degradation in the nucleus. In addition, CDT1 is specifically inhibited by **geminin**, which is expressed in S phase, G2 and early mitosis and prevents illegitimate origin firing during these cell-cycle phases as well as the development of genomic instability^{10–12} (BOX 2).

MCMs as cancer biomarkers

MCMs have recently emerged as useful biomarkers of cell cycle ‘state’; that is, whether a cell is capable of proliferating, rather than being quiescent or senescent. Expression of all six MCMs is seen throughout all phases of the cell cycle and is downregulated following exit from the cell cycle into quiescence, differentiation or senescence^{6,13,14}. This is

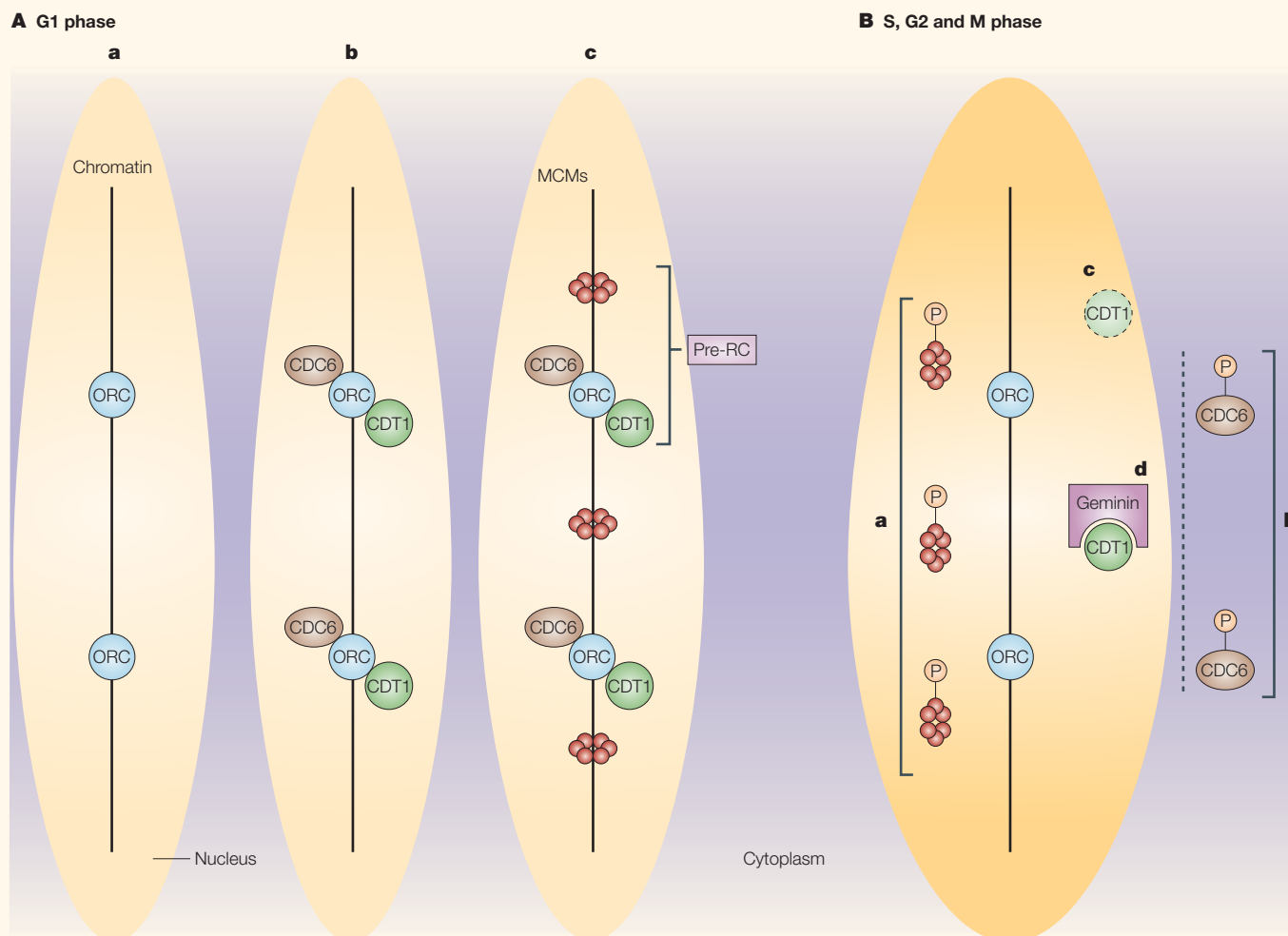


Figure 1 | Factors regulating the binding of minichromosome maintenance proteins (MCMs) to chromatin. A | G1 phase. The multisubunit protein complex ORC (origin recognition complex) constitutively binds to origins of replication (a). Following cell-cycle entry from quiescence, ORC functions as a landing pad for the replication factors CDC6 and CDT1 (b), which in turn recruit the hexameric MCMs (MCM2–7) to chromatin (c). Assembly of this complex of proteins, known as the pre-replication complex (Pre-RC), allows the initiation of S-phase. **B | S, G2 and M phase.** Following S phase entry, the Pre-RC is disassembled. High cyclin A–CDK2 activity results in the phosphorylation and displacement of MCMs (a) and CDC6 from chromatin. CDC6 is exported to the cytoplasm (b). CDT1 undergoes proteolysis (c) and geminin binds to any CDT1 that has escaped degradation (d). P, phosphorylated.

evident on immunohistochemical examination of normal stratified epithelium, for example the ectocervix, where MCMs are confined to the basal proliferative compartment and are absent in the terminally differentiated superficial keratinocytes^{15–18} (FIG. 2).

By contrast, in pre-invasive cervical lesions the proliferative compartment progressively expands with increasing histological grade, and this is paralleled by the appearance of MCM-positive cells at the surface of the epithelium (>90% in high-grade squamous

intraepithelial lesions (HSILs), approximately 40% in low-grade squamous intraepithelial lesions (LSIL)). Dysregulated expression of MCMs is also characteristic of dysplasia and malignancy in a range of other sites, including colorectal^{19,20}, oesophageal^{21–23}, ovarian²⁴, laryngeal²⁵, urothelial²⁶ and vulval²⁷ epithelium.

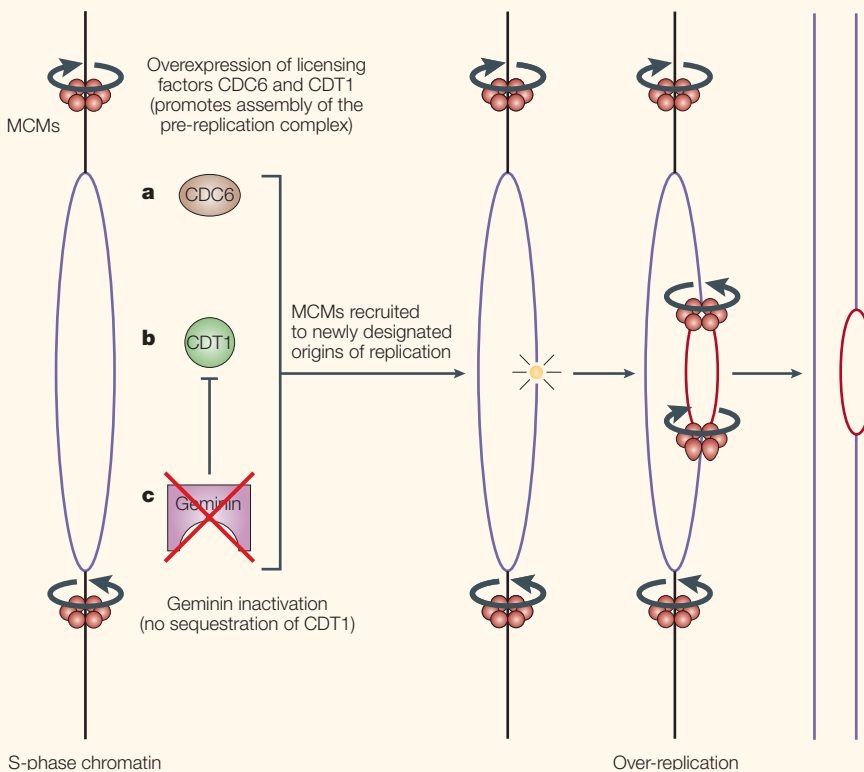
The ability of MCMs to distinguish cycling cells from quiescent cells has prompted a potential clinical application in cancer screening approaches that rely on the detection of malignant or pre-malignant cells exfoliated from surface epithelia, such as in cervical screening¹⁶. Existing biomarkers of cell-cycle entry include Ki67 and proliferating cell nuclear antigen (PCNA). These biomarkers are not as sensitive as MCMs in detecting cells that are in cycle and therefore are unlikely to be as effective in this clinical setting²⁸. MCMs are expressed throughout G1 phase, whereas Ki67 might not be expressed until late G1 phase²⁹. Despite its widespread use to infer proliferative capacity, the function of this extremely large nuclear protein (~395 kDa) remains unknown and there is evidence that it might be involved in other non-cell-cycle-related cellular processes, such as ribosomal biosynthesis³⁰. Ki67 expression in cycling cells might also be downregulated owing to nutritional deprivation³¹. Likewise, PCNA is a less useful marker of cell-cycle entry than MCMs. PCNA is an auxiliary factor for DNA polymerase- δ , and its nuclear expression is strong and maximal during S phase, with weak staining observed in G1, G2 and mitotic phases³². This results in a qualitative variation in immunostaining, particularly when antigenicity is also affected by technical factors such as fixation time³³. PCNA also has an important role in DNA repair, which reduces its value as a specific cell-cycle marker³⁴.

Immunostaining for MCMs in routine cervical smears could potentially improve the current method of **cervical cancer** screening by cytological assessment using the Papanicolaou (Pap) stain. This stain is composed of Harris's haematoxylin, to assess nuclear morphology, and three cytoplasmic dyes (orange G, eosin Y and light green) to determine the degree of keratinocyte differentiation, and it is subjective and error-prone as an isolated test¹⁷.

Furthermore, identification of neoplastic cells using antibodies against MCMs might also prove to be important in population screening for a range of organ sites where exfoliated cells can be obtained for cytological analysis, such as from the genitorurinary and aerodigestive tracts^{19,21,24,25}. MCMs might be detected *in situ* using high-throughput

Box 2 | The roles of MCMs and their regulators in genomic instability

A surplus of minichromosome maintenance proteins (MCMs) is present throughout the cell cycle, therefore overexpressing the limited loading factors could induce inappropriate MCM binding to chromatin and might affect DNA replication. Overexpressing CDC6 (a) and CDT1 (b) in transformed, p53-deficient (but not p53 wild-type) cells results in re-replication of DNA, presumably by recruiting MCMs to chromatin and permitting firing of newly replicated origins⁶⁰. In addition, overexpression of RIS2, the mouse homologue of CDT1, in mouse primary fibroblasts allows faster entry into S phase following release from G0 and subcutaneous injection of such cells causes tumour formation in nude mice⁶¹. CDT1 overexpression has also been observed in a number of human tumour cell lines⁶¹. Analogously, geminin has recently been identified as having an important role in genomic stability in cells by inhibiting the untimely assembly of MCMs onto chromatin and thereby preventing re-replication of DNA (c). Functional knockdown of geminin by small interfering RNA leads to MCMs remaining chromatin-bound during an extended S phase, in which both normal human primary and malignant cells acquire an aneuploid DNA content^{62,63}. Dysregulation of CDC6 or the balance between CDT1 and geminin might therefore affect the state of MCM binding to chromatin, which might trigger illegitimate origin firing and genomic instability. CDC6 and CDT1 are therefore potentially important oncogenes, which like MCMs and geminin are under E2F transcriptional regulation^{64,65}. Evidence also indicates that geminin is a potential tumour suppressor, particularly as overexpression of non-degradable geminin in the HCT116 colorectal carcinoma cell line suppresses tumorigenesis *in vivo*⁶⁶. However, a screen for mutation of the geminin gene or loss of its phase-specific expression in breast carcinoma tissue samples and cell lines showed no abnormalities¹¹. As DNA replication licensing is a crucial requirement for proliferation of cancer cells, proteins that regulate this essential cellular process might be indispensable in tumours. Such proteins might then be expected to function as accurate markers of the cell cycle in neoplastic as well as normal cells. Purple DNA strands represent replicated chromatin; red DNA strands represent re-replicated chromatin.



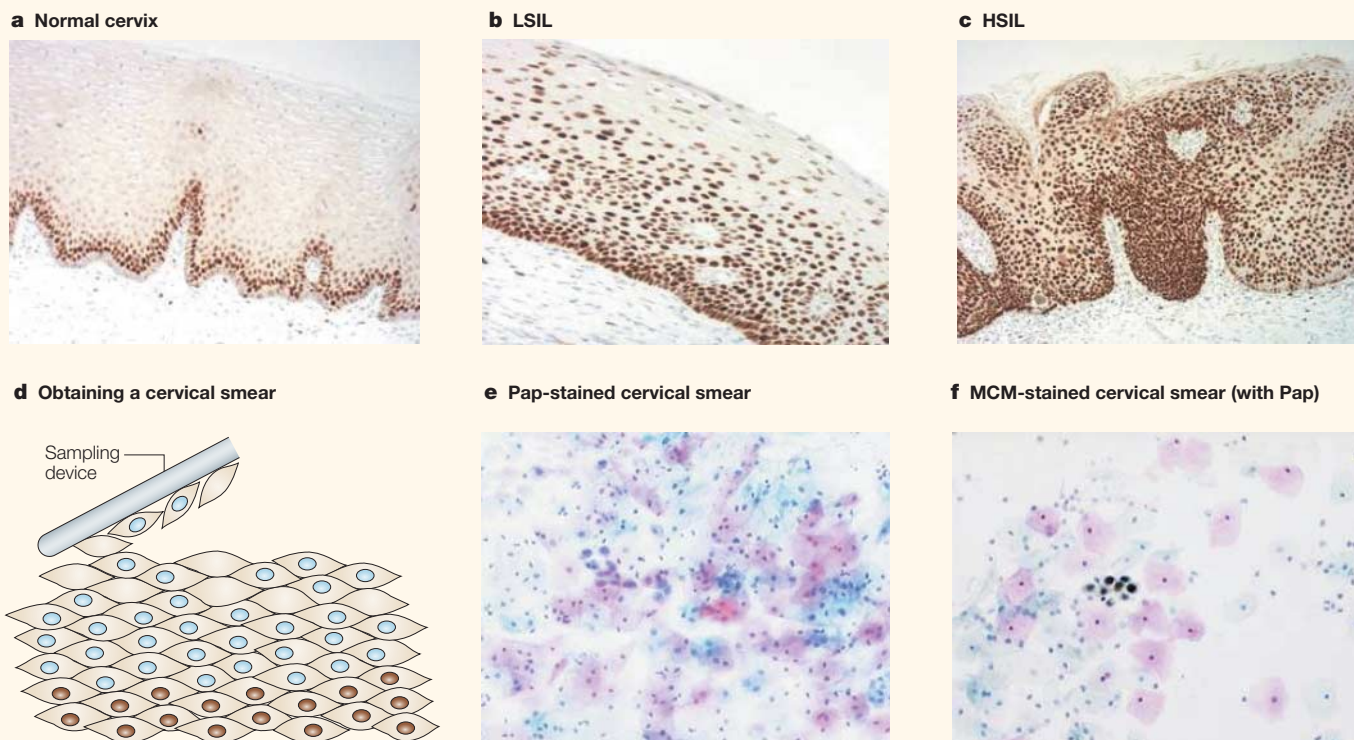


Figure 2 | Minichromosome maintenance proteins (MCMs) detect cells in cycle in normal and pre-malignant cervical tissue. a | MCM immunostaining of normal cervical epithelium identifies cycling cells in the basal proliferative compartment (brown). **b** | In low-grade squamous intraepithelial lesions (LSIL) there is expansion of the proliferative layer with appearance of cycling cells in more superficial epithelial layers. **c** | In high-grade squamous intraepithelial lesions (HSIL) the majority of the epithelium consists of cycling cells as detected by MCMs. **d** | Surface cervical epithelial cells obtained by conventional sampling in a normal routine smear are MCM negative (blue); cells with brown nuclei are MCM positive. By contrast, 90% are positive in HSIL and approximately 40% in LSIL. **e** | Abnormal cells (present in the centre of the field) might be difficult to detect using a routine Papanicolaou (Pap) stain. **f** | Detection of abnormal cells in a cervical smear is facilitated by staining for MCMs in an immunoenhanced Pap smear.

microscopy or in lysed specimens using a liquid-phase immunoassay^{26,35}.

In this context, the MCM loading factor CDC6, which is also only present in cycling cells, has previously been proposed to be a promising diagnostic marker^{16,36}. MCMs, however, are more abundant, stable and immunogenic than CDC6, and their cellular localization during the cell cycle is uniform. CDC6 shuttles between the nucleus and cytoplasm (FIG. 1), whereas MCMs are confined to the nucleus (except following nuclear breakdown in mitosis), which facilitates assessment of immunostained cytological or tissue specimens.

MCMs/geminin in cancer prognosis

Two different technical approaches have identified MCMs as powerful predictors of clinical outcome in several epithelial malignancies. Immunohistochemical studies using MCMs as candidate markers have described a potential role for the analysis of MCM expression (thereby indicating the frequency of cell-cycle entry by tumour cells) in predicting survival in patients with **breast cancer**³⁷, **non-small cell lung cancer**^{38,39}, **brain tumours**^{40,41}, **prostate**

cancer⁴², **oesophageal cancer**⁴³, **renal cell cancer**⁴⁴, **bladder cancer**⁴⁵ and **oral squamous cell carcinoma**⁴⁶. In selecting treatment for cervical cancer, the MCM labelling index might also prove to be of value as a predictive marker of tumours that respond to radiation therapy⁴⁷.

In addition, expression microarray analysis has independently identified the genes that encode MCMs as being upregulated at the mRNA level in malignancy on a large-scale meta-analysis⁴⁸ and as being associated with an adverse clinical outcome^{49–53}. Genes that encode MCMs are present in ‘poor’ prognostic signatures for a

range of malignancies, including breast cancer^{49–51}, medulloblastoma⁵² and mantle cell lymphoma⁵³. No specific MCM subunit has been shown to be specifically upregulated in isolation. **MCM2** (REF. 53), **MCM4** (REF. 51), **MCM5** (REF. 52) and **MCM6** (REFS 49,50) have all been reported to be overexpressed in carcinomas with a clinically aggressive phenotype. The functional significance of increased expression of individual MCMs or combinations of MCMs should be determined.

Geminin, an inhibitor of pre-replication complex assembly, has also been shown to convey strong, independent prognostic value as a marker of cell-cycle progression (through S, G2 and mitosis), both immunohistochemically¹² and on expression array analysis⁵¹. Immunohistochemical assessment of geminin expression might complement information obtained from assessment of MCM labelling of any given tissue sample (frozen or paraffin-embedded) by identifying the proportion of cells that have progressed past G1 phase and are therefore actively proliferating but have not exited mitosis (an indication of cell-cycle ‘rate’).

“The ability of MCMs to distinguish cycling cells from quiescent cells has prompted a potential clinical application in cancer screening approaches...”

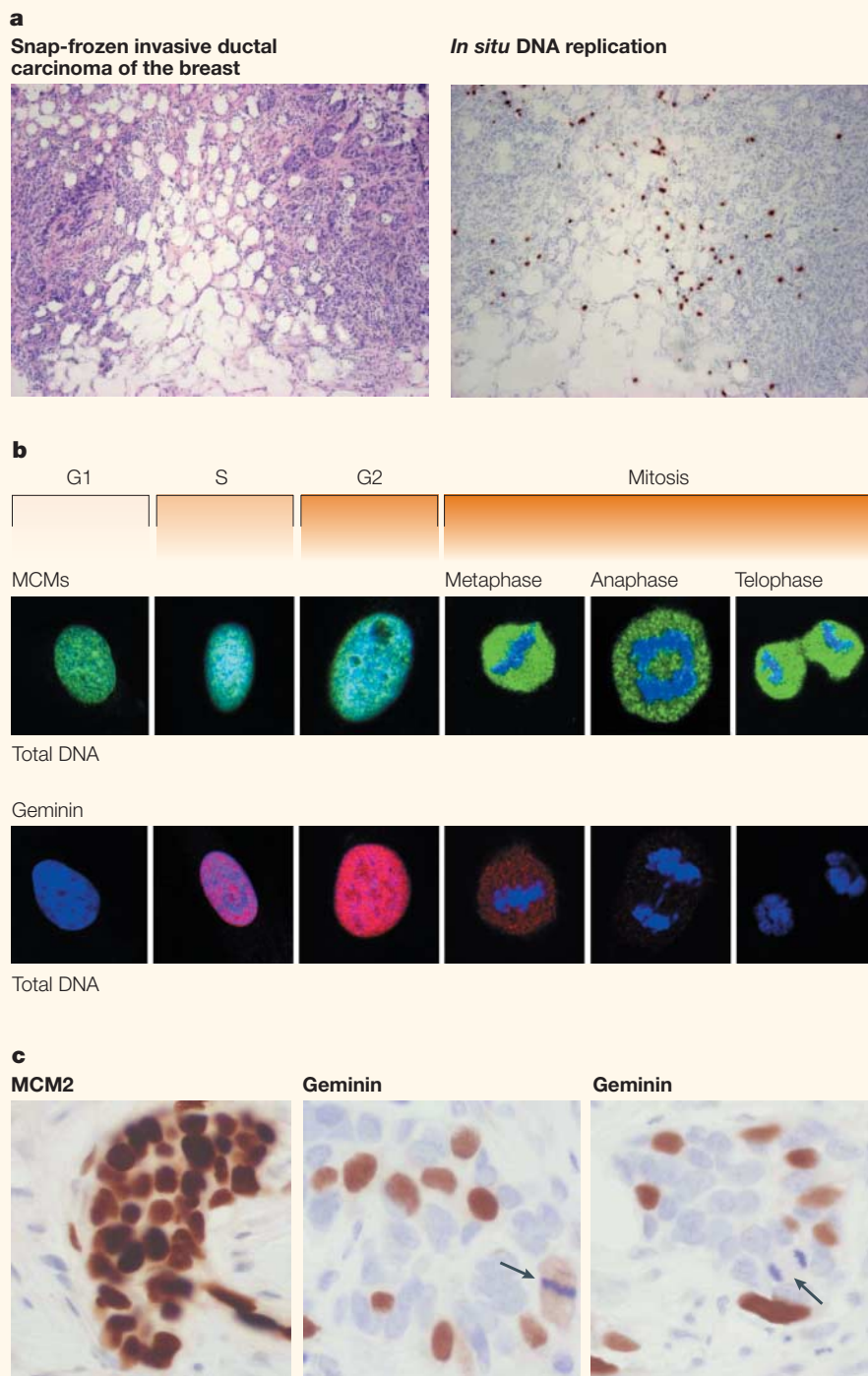


Figure 3 | Novel methods for histological assessment of cell-cycle state and rate of cycling.
a | *In situ* DNA replication in frozen section. S phase cells can be identified in tissue by their ability to incorporate labelled nucleotides (producing brown staining). However, the technique requires carefully processed frozen sections and may therefore not be suitable for high-throughput applications in the routine histopathology laboratory⁵⁴. **b** | Cell-cycle expression of MCMs and geminin in U2OS cells. Minichromosome maintenance proteins (MCMs (green); MCM5 shown here) are present during all phases of the cell cycle, in contrast to geminin (red), which is specifically expressed during S phase, G2 and early mitosis. Geminin is degraded at the metaphase–anaphase transition by the anaphase promoting complex^{9–11}. Blue, total DNA; turquoise, merged MCMs/total DNA; pink, merged geminin/total DNA. **c** | MCMs and geminin as cell-cycle biomarkers in routinely processed formalin-fixed paraffin-embedded tissue. MCM2 immunohistochemical labelling identifies all cycling cells in an invasive ductal carcinoma of the breast of no special type (formalin-fixed, paraffin-embedded tissue). Geminin labelling detects cells in S phase, G2 and early mitosis with degradation at the metaphase–anaphase transition (arrows show a geminin-positive invasive breast cancer cell in metaphase and geminin-negative cell in telophase).

The applicability of such methods to archival paraffin-embedded specimens provides substantial practical advantages over other approaches to estimate cell-cycle kinetics in tissue, such as assessment of the S phase fraction by *in situ* DNA replication⁵⁴, which requires carefully processed frozen sections (FIG. 3).

Differences between the MCM and geminin labelling indices can be accounted for by cells that are progressing through G1 phase or that have arrested in this phase. In any given histological section, such cells might represent a highly significant proportion of the total cell population, as G1 is the longest and most variable phase of the cell cycle. Examination of normal, premenopausal breast epithelium, for example, has revealed that, unlike other normal tissues, a high proportion of cells express MCMs in ducts and lobules, but levels of Ki67 or geminin expression are low and equivalent to one another^{12,14,37}. This indicates early cell-cycle entry of mammary epithelial cells in G1 phase, with limited progress through to S phase, G2 and mitosis. If repeated growth stimuli promote cell-cycle progression in these cells, the risk of conversion to breast carcinoma might increase. The relative frequency of MCM expression in normal breast epithelium is highly variable, so it will be interesting to determine whether a high MCM labelling index in normal breast or benign breast disease poses a higher cancer risk.

Conclusion

The control of DNA replication is a rapidly advancing research field that is providing multiple opportunities for translational applications. The replication licensing proteins have emerged as important biomarkers in cancer pathology, with the potential to improve diagnosis, prognosis and assessment of treatment response for a range of common tumour types. These proteins also provide attractive drug targets, and the design of new antitumour therapies to target positive regulators of DNA replication, such as Pre-RC proteins, or to mimic the replication inhibitory function of geminin is a rich area for future exploitation.

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Competing interests statement

The authors declare **competing financial interests**. See web version for details.

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