

Published in final edited form as:

Nat Rev Mol Cell Biol. 2005 June ; 6(6): 476–486. doi:10.1038/nrm1663.

## Preventing re-replication of chromosomal DNA

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### Preface

To ensure its faithful duplication, chromosomal DNA must be precisely duplicated in each cell cycle, with no sections left unreplicated and no sections replicated more than once. Eukaryotic cells achieve this by dividing replication into two non-overlapping phases. During late mitosis and G1, replication origins are 'licensed' for replication by loading the Mcm2-7 proteins to form a pre-replicative complex (pre-RC). Mcm2-7 proteins are then essential for initiating and elongating replication forks during S phase. Recent data have provided biochemical and structural insight into the process of replication licensing, and the mechanisms that regulate it during the cell cycle.

### Introduction

In order for the cell division cycle to produce two daughter cells that inherit a perfect copy of the genetic material originally present in the mother cell, it must accomplish two difficult tasks: the chromosomal DNA must first be precisely duplicated, with no errors, deletions or duplications, and then the two copies must be precisely segregated to the two daughter cells. The accuracy of these events is particularly crucial to multicellular organisms, where any changes to the genome could potentially give rise to cancers which threaten the life of the entire organism. This review will concentrate on the first of these linked problems: how eukaryotic cells ensure that their chromosomal DNA is precisely duplicated during S phase of the cell cycle.

A large number of replication origins (typically from  $\sim 10^3 - 10^5$ ) (see BOX 1) are used by eukaryotes to ensure that the entire genome is fully replicated. But these origins must be regulated very strictly. How does the cell know whether or not it has already replicated a section of DNA in S phase? Eukaryotes have solved this problem by providing a marker to distinguish replicated from unreplicated DNA. The fact that replicated DNA differs from unreplicated DNA was first suggested by the classic cell fusion experiments of Rao and Johnson<sup>1</sup>. In hybrids of G1 and G2 cells, the unreplicated G1 nucleus passes directly into S-phase, whilst the DNA of the G2 nucleus (which has already been replicated) does not replicate again until after the hybrid cell has passed through mitosis. The G2 nucleus is therefore refractory to undergoing further replication. Subsequent work using cell-free extracts of *Xenopus* eggs refined this idea and suggested a model whereby replication origins were "licensed" for replication during late mitosis and G1, but the licence was removed as the DNA was replicated<sup>2</sup>. Dividing the process of DNA replication into two non-overlapping phases (one phase permissive for the licensing of DNA replication and a second phase that is permissive for the initiation of replication but not for licensing) can potentially explain how cells ensure the precise duplication of chromosomal DNA in a single cell cycle.

Detailed experimental support for the licensing model has now been obtained, which shows that it comprises four essential features<sup>3,4</sup>. First, replication origins are licensed by stably

binding complexes of the mini-chromosome-maintenance 2-7 proteins (Mcm2, 3, 4, 5, 6 and 7). Mcm2-7 proteins form an essential component of the “pre-replicative complex” (pre-RC) of proteins found at replication origins during G1 phase. Second, the binding of Mcm2-7 proteins to origin DNA is essential for the origin to be able to initiate a pair of replication forks. Third, the licensing of origins and the loading of Mcm2-7 onto DNA is restricted to late mitosis and G1 of the cell cycle. Fourth, Mcm2-7 proteins are displaced from origins as DNA replication is initiated, probably by travelling ahead of the replication fork. The licence is therefore never associated with replicated DNA.

These features can explain why DNA replicates only once in each cell cycle (Fig 1). Recent results also indicate that the presence or absence of licensed origins has even deeper implications for cell biology, as it might play a role in determining the proliferative capacity of cells<sup>3</sup>.

## Understanding the function of Mcm2-7

The Mcm2-7 complex consists of 6 closely related proteins that are highly conserved throughout the eukaryotic kingdom (forming the MCM/P1 family). Each protein has a relatively highly conserved C-terminus which shows sequence homology to hexameric DNA helicases and contains an ATPase motif. A range of complexes have been described containing different combinations of the Mcm2-7 proteins. A purified hexamer containing Mcm4, 6 and 7 has been shown to have limited 3' to 5' DNA helicase activity *in vitro*<sup>5</sup>. However, only the heterohexamer that contains all 6 Mcm2-7 proteins can support licensing in *Xenopus* egg extract<sup>6</sup>. *In vitro*, all 6 proteins are required for maximum ATPase activity, and the ATPase motifs of all 6 proteins are required for viability in *S. cerevisiae*<sup>7</sup>. Chromatin immunoprecipitation has revealed that Mcm proteins are associated with replication forks as they elongate along chromosomal DNA<sup>8</sup>, and inhibition of Mcm2-7 function during S phase causes a rapid cessation of DNA synthesis<sup>9-11</sup>, indicating that Mcm2-7 function is required for fork progression as well as initiation. These observations all suggest that Mcm2-7 functions as a helicase that unwinds DNA ahead of the replication fork. The Mcm2-7 helicase is therefore likely to be displaced from replicated DNA by virtue of its moving ahead of the replication fork. The displacement of the replication licence from replicated DNA is one of the major features (point 4 above) of the licensing factor model.

The archaea possess proteins with high sequence homology to eukaryotic MCM/P1 proteins. The single MCM protein from *Methanobacterium thermoautotrophicum* (*MmMCM*) forms dodecamers that display DNA helicase activity *in vitro*<sup>12-14</sup>. A crystal structure of the N-terminus of the *MmMCM* has recently been reported<sup>15</sup>. This reveals a double hexameric structure, with a large positively charged channel running through the centre that is wide enough to surround double-stranded DNA (FIG. 2a-c). The C-terminal helicase domain that is missing from the crystal structure could potentially form an extra ring facing away from the dimer interface. This conclusion is supported by electron microscopy of the full-length *MmMCM* which shows a bilobed hexameric structure with a large central channel<sup>16</sup>. Modelling indicated that the N-terminal domain fits well into the central part of the structure, whereas the helicase-containing C-terminus could form the extra lobe (Fig 2d).

This structure has interesting implications for the function of the eukaryotic Mcm2-7 proteins and their role in replication licensing. As can be seen from FIG. 1, it is crucial that the binding of Mcm2-7 to DNA is both stable and regulated. Mcm2-7 complexes that are loaded onto DNA in late mitosis must remain associated through G1 and into S phase. It is also crucial that once the licensing system is turned off in late G1, there is no illegitimate association of Mcm2-7 with DNA. These problems can be overcome if the loading of Mcm2-7 onto DNA represents the Mcm2-7 hexamer encircling the DNA. This would lead to

a stable association between Mcm2–7 and the DNA, which could be tightly controlled by regulating the activity of the loader which would be required to open up the hexamer and clamp it around DNA. The *MmMCM* structure therefore provides an explanation for two of the fundamental features (points 1 and 3 above) of the licensing model — the stability and temporal control of Mcm2–7 protein binding.

The *MmMCM* structure is highly congruent with the helicase domain of the SV40 large T antigen, the crystal structure of which has recently been determined<sup>17</sup>. T antigen is the replicative helicase for SV40 DNA. Like *MmMCM*, T antigen forms a bilobed double hexamer with a large central channel. Interestingly, T antigen contains “exit channels” through which single-stranded DNA might be extruded as the helicase progresses, and features consistent with this can be seen in *MmMCM*<sup>16</sup>. Li *et al* propose a mechanism by which T antigen unwinds DNA as it is replicated (FIG. 3). T antigen encircles both strands of template DNA prior to initiation, which for Mcm2-7 would correspond to the licensed but uninitiated state. On initiation, one of the single-strands is extruded through the side channels, whilst the other single strand exits the helicase at the hexamer-hexamer interface. This model is consistent with previous models based on the extruded ‘rabbit ears’ of single-stranded DNA that had been observed when T antigen interacts with origin DNA *in vitro*<sup>18</sup>.

### The ‘Mcm Paradox’

There are some observations, however, that do not fit neatly into a model of Mcm2-7 functioning as a simple replication fork helicase. First, whereas one might have thought that only one double hexamer needs to be loaded onto each replication origin, in fact the number in eukaryotes seems much higher, with 10 – 40 Mcm2-7 hexamers present at each origin<sup>19-22</sup>. The loading of Mcm2-7 onto chromatin depends on the Origin Recognition Complex (ORC; see below), which is thought to bind specifically to replication origins. ORC recruits two further proteins – Cdc6 and Cdt1 – which together facilitate the loading of Mcm2-7. However, studies in HeLa cells and *Xenopus* extracts indicate that Mcm2-7 is also loaded onto sites on the chromosome that are distant from ORC<sup>22-26</sup>. ATP hydrolysis by *S. cerevisiae* ORC is specifically required for loading the subsequent Mcm2-7 complexes after the first ones have been loaded onto DNA<sup>27</sup>, which provides further evidence that multiple copies of Mcm2-7 are loaded onto each origin. The distribution of Mcm2-7 on chromatin therefore does not match the expected distribution of a simple replicative helicase, which should be loaded at replication origins and at only two copies per origin. A second discrepancy is that although some Mcm2-7 colocalizes with replication forks<sup>8</sup>, the bulk of the chromatin-bound protein is not associated with sites of DNA synthesis<sup>28-30</sup>.

There are a number of possible explanations for this paradoxical distribution of Mcm2–7 on chromosomes. One possibility is that Mcm2-7 does not act as a typical DNA helicase, but instead acts as a fixed pump that forces DNA into immobilized replication forks where DNA synthesis occurs<sup>31</sup>. Because the DNA will be twisted as it is pumped, this will provide the energy to unwind the DNA ahead of the DNA polymerases, obviating the need for a helicase at the fork. However, for this to happen, the DNA between the Mcm2-7 pump and the DNA polymerases must be somehow protected from topoisomerase-mediated relaxation, which would dissipate the stored-up energy and allow the DNA to rewind.

An alternative possibility is that although a small fraction of Mcm2–7 is required to licence and initiate the replication of DNA, the bulk of the Mcm2-7 is not involved in DNA synthesis, either having some completely different function<sup>32,33</sup> or only being required for some contingency. Consistent with the idea that most of the Mcm2–7 is not normally involved in DNA synthesis is the observation that near-maximal replication rates can be achieved in *Xenopus* with only 1-2 heterohexamers of Mcm2-7 per replication

origin<sup>21,22,34</sup>. Although *Xenopus* Cdc6 is required for the loading of Mcm2-7 onto DNA, its affinity for replication origins decreases once the first Mcm2-7 hexamers have been loaded onto each origin<sup>34</sup>. This plausibly ensures that under conditions where the extent of Mcm2-7 loading is limited, each origin gets the minimum allocation for it to support initiation. But what is the function of the excess Mcm2-7 hexamers? One proposal is that the excess Mcm2-7 might activate intra-S phase checkpoints. Human Mcm7 interacts with ATRIP, a protein that binds and activates the ATR kinase which is involved in checkpoint signalling when DNA replication is disturbed<sup>35</sup>. When Mcm7 levels were slightly reduced so that replication rates were not significantly reduced, checkpoint signalling pathways were also disrupted<sup>35</sup>.

The presence of excess Mcm2-7 at replication origins might explain a puzzling feature of eukaryotic replication origins, which in many circumstances form diffuse initiation sites that are not precisely specified by DNA sequence<sup>36</sup>. If the Mcm2-7 helicase has a similar mode of action to SV40 T antigen, then the site of assembly of a back-to-back Mcm2-7 dimer on DNA could potentially specify a replication origin. It is possible that the 10–40 Mcm2-7 hexamers loaded onto each origin could form a number of back-to-back dimers, each of which could provide a different site at which initiation could occur. This might provide redundant origins for use if various components of the replication machinery were to fail.

### The mechanism of licensing and Mcm2-7 protein loading

The loading of Mcm2-7 onto DNA that occurs as an origin is licensed is likely to involve the opening of the Mcm2-7 ring to allow it to encircle the DNA. Other protein components of the pre-RC are required to load Mcm2-7 onto chromatin. One of the first events is the binding of ORC to DNA (FIG. 4). ORC in turn recruits two further proteins – Cdc6 and Cdt1 – which are all required for the loading of Mcm2-7 and the functional licensing of the origin<sup>3,4,37</sup>. This process has been reconstituted on *Xenopus* sperm chromatin using purified ORC, Cdc6, Cdt1 and Mcm2-7, in addition to a chromatin remodelling protein, nucleoplasmin, which is required for the initial binding of ORC to DNA<sup>38</sup>.

Little is known about this reaction, but the available evidence indicates that ORC, Cdc6 and Cdt1 act together as a clamp loader to open up the Mcm2-7 ring and load it around DNA<sup>37</sup>. Alternatively, the ORC, Cdc6 and Cdt1 molecules might facilitate the assembly of the Mcm2-7 hexamer from the many subcomplexes of Mcm proteins that have been described. Consistent with these models, ORC, Cdc6 and Cdt1 are only required for the loading of Mcm2-7 onto DNA (licensing), but are not required for the continued association of Mcm2-7 on DNA once this has occurred<sup>39-42</sup>. Both ORC and Cdc6 are ATPases, and the licensing reaction is dependent on hydrolysable ATP<sup>38</sup>. ATP hydrolysis could plausibly provide the energy to break open the hexameric ring of Mcm2-7 to allow it to reform around DNA. Cdt1 can directly bind components of the Mcm2-7 hexamer<sup>43-45</sup>, and so the role of Cdt1 might be to recruit Mcm2-7 to the DNA. Cdc6 must already be bound to chromatin before Cdt1 can join the complex in an active form, consistent with Cdt1 having a role in recruiting Mcm2-7 to a clamp-loader that comprises ORC and Cdc6<sup>46</sup>. Geminin, a major inhibitor of the licensing system in metazoans (see below) binds tightly to Cdt1<sup>47,48</sup>, potentially blocking its ability to bind Mcm2-7<sup>49</sup>. As both ORC and the Mcm2-7 hexamer are asymmetric complexes, it is possible that the orientation of ORC on DNA determines the orientation that Mcm2-7 is loaded onto DNA. If, as discussed above, initiation requires two back-to-back Mcm2-7 complexes, it might then be necessary to have two ORC-Cdc6-Cdt1 complexes in different orientations on the DNA to achieve this (FIG. 4d). Alternatively, Mcm2-7 might be loaded as preformed double-hexamers.

The components of the pre-RC may also have functions other than supporting origin licensing. Several lines of evidence indicate that ORC has an important role in generating transcriptionally silent chromatin in a process that is independent of its role in DNA replication<sup>50</sup>. Several reports also suggest that Cdc6 is involved in the activation of checkpoint pathways within S phase or in the progression from G2 into mitosis<sup>34,51,52</sup>.

## Regulation of the Licensing System

To prevent the possibility of replicated origins becoming re-licensed during S phase, it is important that the ability to license new replication origins is down-regulated before entry into S phase. Because ORC, Cdc6 and Cdt1 are required for the loading of Mcm2-7 onto DNA, but are not required for the continued association of Mcm2-7 on DNA, down-regulation of their activity at the end of G1 is an effective way of preventing the re-licensing of replicated DNA.

### Regulation of licensing in yeast

Early work in fission yeast (*Schizosaccharomyces pombe*) showed that continued activity of cyclin-dependent kinases (CDKs; see BOX 2) in S phase and G2 is necessary to prevent re-replication of DNA. Down-regulation of either Cdk1<sup>53</sup> or the mitotic cyclin Cdc13<sup>54</sup> in G2 permitted extensive re-replication of DNA in a single cell cycle. This implied that re-loading of Mcm2-7 occurs in G2 cells if CDK activity is ablated. Similar experiments in budding yeast (*Saccharomyces cerevisiae*) showed that pre-RC assembly (origin licensing) is inhibited by Cdk1 activity<sup>55</sup>. This suggested an intellectually satisfying model where low CDK level at the beginning of the cell cycle (late mitosis and early G1) permit origin licensing whilst rising CDK levels at the end of G1 both prevent further licensing and at the same time promote the initiation of replication<sup>56</sup>.

In yeasts, CDKs appear to have multiple redundant effects on preventing re-licensing of replicated DNA, targeting all of the components of the licensing system<sup>3,4</sup>. One of the major substrates of CDK regulation in *S. cerevisiae* is Cdc6 (or the *S. pombe* homologue Cdc18), which is targeted for degradation following phosphorylation by CDKs at the G1-S phase transition<sup>57-59</sup>. Cdc6/Cdc18 levels are also regulated at the transcriptional level to give maximum expression in late mitosis and G1<sup>3,4</sup>. In addition, the ability of ORC to support re-replication is directly inhibited by CDK phosphorylation<sup>60,61</sup>. In both fission and budding yeast, CDKs are directly recruited to ORC bound to origin DNA, and this helps maintain ORC in an inactive state during S phase and G2<sup>62,63</sup>. CDKs can also bind directly to Cdc6, which may also contribute to the prevention of re-replication<sup>64</sup>.

In *S. pombe*, Cdt1 levels peak in late mitosis and early G1 and may be controlled by CDK-dependent transcription and proteolysis in a manner similar to Cdc6/Cdc18. This is likely to be important in preventing re-replication of DNA, since overexpression of Cdt1 enhances re-replication induced by overexpression of *SpCdc18/Cdc6*<sup>65</sup>. In *S. cerevisiae*, CDKs promote the nuclear export of Cdt1 and Mcm2-7 during S phase, G2 and early mitosis, thus preventing them from gaining access to chromosomal DNA<sup>66,67</sup>. Experiments in *S. cerevisiae* showed that in order for significant re-replication to occur, all these different CDK-dependent controls must be inactivated. Partial over-replication was only seen when unphosphorylatable mutants of *orc2* and *orc6* were combined with non-degradable Cdc6 and constitutively nuclear Mcm2-7 and Cdt1<sup>60</sup>. These experiments suggest that in yeasts, direct inhibition of components of the licensing system by CDKs prevents the relicensing of replicated DNA.



## Regulation of licensing in metazoans

There is evidence that CDK levels are also important for regulating the licensing system in metazoans<sup>4</sup>. For example, fluctuations of cyclin E levels are required to drive endoreduplication cycles in *Drosophila*<sup>68,69</sup>. However, the role of CDKs in inhibiting the licensing system in metazoans is less clear-cut than it is in yeasts (Fig 5B), and it seems likely that CDK levels only indirectly affect the activity of the licensing system. ORC from mammalian somatic cells is a labile complex. The Orc1 subunit is loosely associated with the Orc2, 3, 4, 5 subcomplex and is targeted for degradation in S phase by an SCF dependent polyubiquitination reaction that may depend on CDK activity<sup>70-72</sup>. Alternatively, in some cell lines, Orc1 appears to be stable in S phase but is mono-ubiquitinated and/or phosphorylated as S phase progresses<sup>73,74</sup>. It has been suggested that this Orc1 cycle contributes to the regulation of the licensing system, but experimental evidence for this is still lacking. Unlike the situation in yeast, metazoan Cdc6 appears to be bound to chromatin throughout much of the cell cycle, including G<sub>2</sub><sup>75,76</sup>. Excess Cdc6 is exported out of the nucleus during S phase as a consequence of CDK activity, but there is no evidence that this plays a part in preventing re-replication of DNA<sup>77-80</sup>. Only in mitosis is there clear evidence in metazoans of a direct inhibitory effect of high CDK levels on the licensing system<sup>47,81</sup>.

Instead, the major route by which metazoans prevent licensing during S phase and G<sub>2</sub> is by downregulation of Cdt1 activity. This is brought about both by degradation of the Cdt1 protein and by activation of a Cdt1 inhibitory protein called geminin. Cdt1 is degraded at the end of G<sub>1</sub> and early S phase (Fig. 5b) in a process that depends on the activity of SCF-class ubiquitin ligases<sup>48,82-86</sup>. In human cells, a Skp2-containing SCF interacts with Cdt1, dependent on prior phosphorylation of Cdt1 by Cdk2 or Cdk<sup>485,87-89</sup>. CDK-dependent degradation of Cdt1 has also been observed in *Xenopus*<sup>90</sup>. There are, however, Skp2-independent pathways that can still degrade Cdt1, because mutants of Cdt1 that are not phosphorylated by CDK and do not interact with Skp2 are still degraded normally at the onset of S phase (ref<sup>91</sup> and D. Takeda and A. Dutta, unpublished results). In *Caenorhabditis elegans*, a related CUL-4 ubiquitin ligase is required to downregulate Cdt1 levels at the end of G<sub>1</sub><sup>86</sup>. Loss of CUL-4 leads to Cdt1-dependent re-licensing and replication<sup>86</sup>. Similarly, overexpression of Cdt1 in human, *Drosophila* or *Xenopus* led to significant re-replication, an effect that was also enhanced by co-expression of Cdc6<sup>79,90-94</sup>. In *Xenopus*, simultaneous loss of geminin and stabilisation of Cdt1 is sufficient to cause extensive re-licensing and re-replication<sup>92</sup>.

In addition to being degraded during S phase, Cdt1 is stabilised during G<sub>2</sub> and M by binding to geminin, which appears to protect Cdt1 from ubiquitin-mediated degradation<sup>81</sup>. This stabilisation by geminin allows Cdt1 levels to build up in an inactive form, ready for its function in late mitosis. If only geminin-bound Cdt1 is protected from degradation, this ensures that the quantity of Cdt1 does not exceed the amount that can be inhibited by geminin.

Geminin inhibits Cdt1 by binding to it and provides the major inhibitor of licensing during S phase and G<sub>2</sub><sup>47,48,84,95</sup>. Geminin activity is down-regulated in late mitosis, either through proteolysis<sup>95</sup> or by posttranslational inactivation such as CDK-dependent ubiquitination<sup>84,96</sup>, and this allows activation of the licensing system as cells exit from metaphase. At the end of G<sub>1</sub>, geminin is then stabilised and/or re-activated, thus preventing any further licensing. The importance of geminin in preventing re-replication is demonstrated by the observation that the loss of geminin in *Drosophila* or human tissue culture cells leads to extensive re-replication of DNA<sup>83,97-99</sup>. Geminin has been reported to be a regulator of transcription factors, and so misregulation of transcription could contribute to the re-replication<sup>100,101</sup>. However, overexpression of Cdt1 can by itself

stimulate re-replication, suggesting that Cdt1 is the relevant target of geminin for the prevention of re-replication<sup>79,90-94</sup>. Taken together, these results suggest that in most metazoan cells the major feature preventing the re-licensing of replicated DNA is the absence of Cdt1 activity, which can occur despite any potential CDK-dependent inhibitory phosphorylation of ORC, Cdc6 and Mcm2-7 proteins.

Several structural studies have recently illuminated how geminin might inhibit Cdt1 function. Geminin forms an elongated dimer through the interaction of its coiled-coil domains<sup>49,102-104</sup>. The crystal structure of the central region of Cdt1 complexed with a fragment of geminin has been solved<sup>49</sup> (Fig 6). One molecule of Cdt1 interacts asymmetrically with the geminin dimer forming contacts between highly conserved amino acids in helix H1 and surface residues on the coiled-coil domain of both subunits of geminin. A second unstructured loop immediately N-terminal to the coiled-coil domain of geminin makes a second contact with Cdt1 that is essential for the inhibition of DNA replication. A structure-function study carried out with full length Cdt1 indicated that the N-terminal 160 residues of Cdt1 that were not present in the co-crystal also interact with the N-terminal unstructured loop of geminin<sup>103</sup>. In addition, an electron microscopic study indicated that geminin dimers may associate with each other to form tetramers<sup>105</sup>. These studies indicate that the geminin-Cdt1 interaction might interfere with Cdt1-Mcm2-7 interaction by steric hindrance and thus interfere with the loading of Mcm2-7.

## Checkpoint pathways and re-replication

When the licensing system is artificially misregulated to allow cells to inappropriately re-license their DNA, re-replication of DNA is observed<sup>60,79,92,97-99</sup>. This indicates that replication licensing is the major system preventing re-replication of DNA in a single cell cycle. However, when re-replication occurs, various checkpoint pathways are activated. Checkpoint kinases – comprising the upstream ATM and ATR kinases and the Chk1 and Chk2 downstream kinases – are typically activated when DNA is damaged or if problems are detected during progression through the cell cycle. Although the precise mechanism is not understood, it seems likely that checkpoint activation in response to misregulation of the licensing system is a consequence of some structural problem occurring during re-replication (such as a fork running into the back of another fork), rather than re-licensing itself<sup>106</sup>.

Overexpression of Cdt1 or depletion of geminin (which induces re-replication) activates ATM/ATR-Chk1/Chk2 dependent checkpoint pathways that lead to an arrest of the cell-cycle and/or apoptosis<sup>79,92,97-99</sup>. Cells overexpressing Cdt1 and Cdc6 utilize p53 as the major effector downstream from the ATM/ATR checkpoint kinases, while cells depleted of geminin utilize the checkpoint kinase Chk1 as the major effector. The reason for this differential use of effectors is unclear but could suggest a role of either Cdc6 or of geminin in determining how the checkpoint kinases are directed to their substrates<sup>34</sup>. Consistent with this idea, Cdc6 has been reported to be involved in checkpoint activation independent of its role in licensing<sup>34,51,52,99</sup>. The activation of p53, of course, leads to apoptosis in addition to the arrest of the cell-cycle. The cells also undergo apoptosis (but in a p53-independent reaction) if the Chk1-dependent G2 arrest seen following geminin depletion is over-ridden. Thus, metazoans are spared the ill consequences of re-replication by at least two back-up pathways: checkpoint induced cell-cycle inhibition and/or apoptosis.

In addition to being degraded during S phase, Cdt1 is also degraded in response to DNA damaging agents. Treatment of human or *Drosophila* tissue culture cells with ionising radiation led to an almost complete loss of Cdt1 protein<sup>107</sup>. This Cdt1 degradation occurred in cells during G1 and depended on the CUL4 ubiquitin ligase, but not on ATR or ATM-

dependent checkpoint pathways. Human tissue culture cells treated with UV also undergo Cdt1 degradation<sup>108</sup>. In contrast to the findings with ionising radiation, the sensitivity of UV-induced Cdt1 degradation to various checkpoint kinase inhibitors suggested that the ATR kinase activity is involved. These results suggest that downregulation of the licensing system is an additional route by which cells can prevent DNA replication in the face of DNA damage.

## Co-ordination of Licensing and Proliferative Potential

When cells withdraw from the cell cycle, either temporarily into G0 (quiescence), or permanently as a consequence of terminal differentiation or senescence, Cdc6, Cdt1 and Mcm2-7 are down-regulated and degraded, leaving only ORC at the origins (FIG. 7; reviewed in Ref. 3). At first sight it seems surprising that unreplicated DNA becomes unlicensed on exit from the cell cycle. One possibility is that this provides a barrier to prevent non-proliferating cells from re-entering the cell division cycle. Indeed, an early change that is seen in pre-malignant lesions is the presence of Mcm2-7 in cells which do not normally express them<sup>109</sup>. As the loss of the licensed state represents a profound change to the replicative potential of the cell as it exits from G1 into G0, it provides an attractive functional definition of exit from the proliferative state<sup>3,110</sup>.

The transcription of *CDC6*, *CDT1* and *MCM2-7* all appear to be responsive to the E2F family of transcription factors which are activated on exit from G0<sup>111-113</sup>. E2F-driven transcription is normally repressed by members of the Rb family, which is in turn repressed by CDK-dependent phosphorylation (primarily by cyclin E-Cdk2 and cyclin D-Cdk4/6). Cyclin E knockout embryos (cyclin E1<sup>-/-</sup> cyclin E2<sup>-/-</sup>) show virtually normal proliferation and developmental capacity. However, they fail to make trophoblast giant cells and megakaryocytes, both cell types that need to endoreduplicate to attain a >2N DNA content<sup>114,115</sup>. Mouse embryo fibroblasts (MEFs) derived from the cyclin E knockout embryos were unable to re-enter S phase after being driven into G0 by serum starvation or contact inhibition. In particular, these quiescent cyclin E knockout cells failed to load Mcm2 onto chromatin despite the normal loading of ORC and Cdc6<sup>114,115</sup>. These studies indicate that whereas cyclin E is dispensable for licensing to occur after exit from normal mitosis, it is essential for licensing at other times – either on exit from G0 or during endoreduplication cycles. Consistent with this idea, work in *Drosophila* has also shown a requirement for cyclin E in Mcm2-7 loading during endoreduplication cycles<sup>68,69,116</sup>.

The picture is further complicated by the observation that Cdk2 knockout mice – which lack the only known kinase partner of cyclin E – are viable, and produce MEFs that can exit normally from serum starvation<sup>117,118</sup>. Although the precise role of CDKs in activating the licensing system is unclear, it is possible that during normal cell cycle progress, a range of CDKs (including cyclin B-Cdk1<sup>96</sup>) play redundant roles in activating the licensing system. On exit from G0 fewer CDKs may be available – whilst cyclin A-Cdk1 may be able to replace cyclin E-Cdk2 in Cdk2 knockout cells, cyclin A-Cdk2 may not be able to do so in cyclin E knockout cells<sup>115</sup>.

What is the essential role of cyclin E in licensing during exit from G0? One possibility is that cyclin E is required for the synthesis of Cdt1 (ORC and Cdc6 levels were normal in the cyclin E<sup>-/-</sup> MEFs). Another possibility is that cyclin E is directly or indirectly required for the activation of one of the pre-RC components when cells exit from G0. Consistent with this suggestion, an *in vitro* system that supports the replication of nuclei isolated from cells exiting from G0 shows a requirement for cyclin E in loading Mcm2-7 proteins onto chromatin<sup>119</sup>. One step that might require CDK activity is the inactivation of geminin. On exit from metaphase in *Xenopus* egg extracts, geminin is inactivated as a consequence of



CDK-dependent ubiquitination<sup>96</sup>. An analogous reaction requiring cyclin E for the inactivation of geminin could be postulated on exit from G<sub>0</sub>. However, geminin expression is also low in quiescent cells<sup>120,121</sup>, and this explanation would require geminin levels to rise prior to the completion of licensing when cells re-enter the cell cycle (FIG. 7), a point that has so far not been studied.

As licensing must be complete before cells enter S phase, it is plausible that cells possess a 'licensing checkpoint' that blocks entry into S phase until licensing is complete. In support of this idea, primary cells that over-expressed constitutively active geminin arrested in a G<sub>1</sub>-like state with hypophosphorylated Rb and showing no evidence of attempts to initiate DNA replication<sup>122,123</sup>. By contrast, a variety of cancer-derived cell lines progressed into an unsuccessful S phase and ultimately underwent apoptosis. The nature of the 'licensing checkpoint' seen in primary cells is currently unclear, but may provide a useful route by which to induce cancer-specific cell-killing.

## Conclusions and Perspectives

Over the past few years, the basic mechanisms that ensure once-per-cycle replication of chromosomal DNA have been elucidated. Although the regulation seems to differ between yeasts and metazoans, in both groups it centres upon restricting the rebinding of Mcm2-7 to replicated DNA, thus conforming to the original licensing factor hypothesis. In yeasts, the re-licensing of replicated DNA is prevented by high CDK activity which represses different pre-RC components late in the cell cycle. In contrast, metazoans prevent the re-licensing of replicated DNA largely by inhibiting Cdt1 activity, which in turn may largely be due to the presence of active geminin. With the understanding of these basic pathways in place, the time is now ripe to address a number of further questions. It will be of great interest to understand the biochemical events occurring when Mcm2-7 are loaded onto DNA. Are Mcm2-7 loaded as hexamers or as double hexamers? Are there any features in the DNA sequence or chromatin that direct ORC to specific sites on the chromosomes? What is the relationship between the sites where ORC is bound, the sites where Mcm2-7 is bound, and the sites where replication forks actually initiate? The answers to these questions may also shed light on the 'Mcm paradox': why there is such an excess of Mcm2-7 over replication origins. It will also be of great interest to understand how the licensing system is regulated when cells enter and exit quiescence. What roles do CDKs play in reactivating the licensing system on exit from quiescence? Are there checkpoint or feedback systems monitoring the success of this process? Is the unlicensed state of quiescent cells important for preventing illegitimate re-entry into the cell cycle? Do cancer cells acquire errors in these checkpoint and feedback pathways, and do such errors lead to genomic instability? Answering these and other questions will help to integrate our knowledge of cell cycle control with the biophysical events occurring during the process of DNA replication and with the genomic instability that is the hallmark of many cancers.

## Supplementary Material

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## Acknowledgments

Thanks to Margret Michalski-Blow for assembling the Glossary. This work was supported by Cancer Research UK grant C303/A3135 and NIH grants CA60499 and CA89406.

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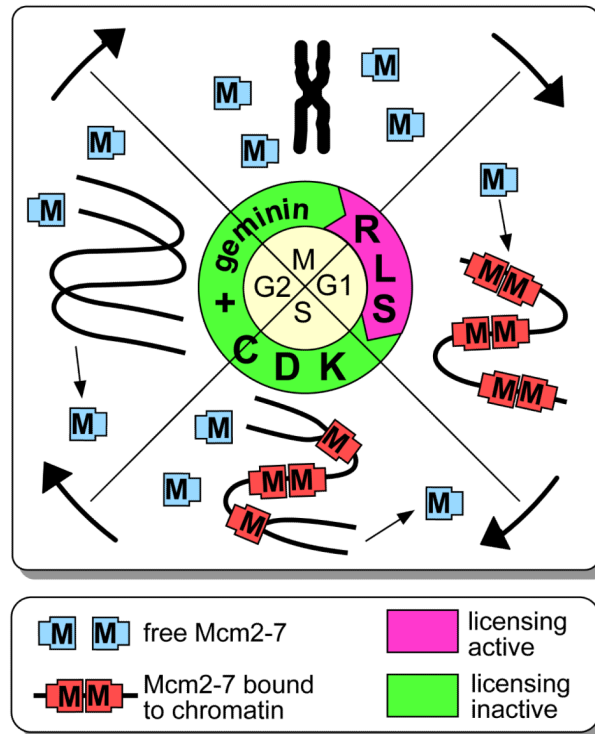
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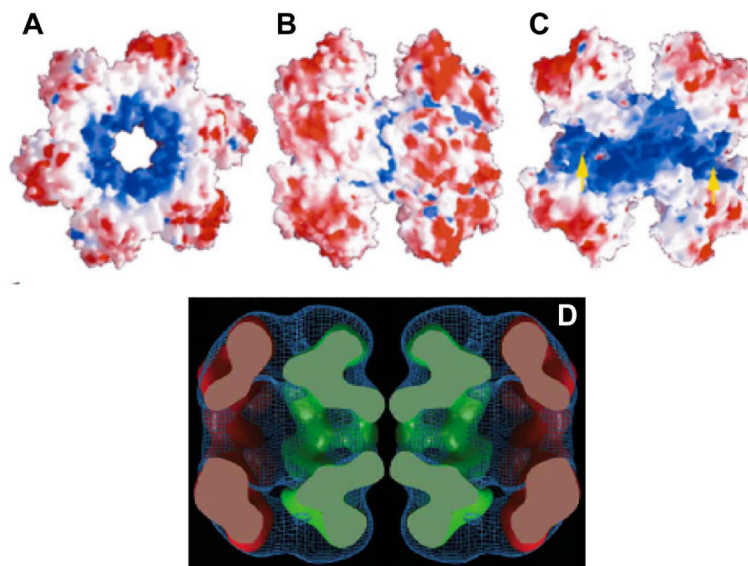
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**Figure 1.**

Regulated loading and unloading of Mcm2-7 during the cell cycle.

A small segment of chromosomal DNA that encompasses 3 replication origins is shown. At the end of mitosis (M), the replication licensing system (RLS) is activated, which causes Mcm2-7 (the asymmetric rectangles marked 'M') to be loaded onto potential replication origins (origin licensing). The licensing system is turned off at the end of G1 by inhibition by CDKs and/or geminin. During S phase, the Mcm2-7 complexes are displaced from replicated DNA by moving ahead of the replication fork, and are removed from DNA at fork termination. In this way, replicated DNA cannot undergo further initiation events until passage through mitosis.

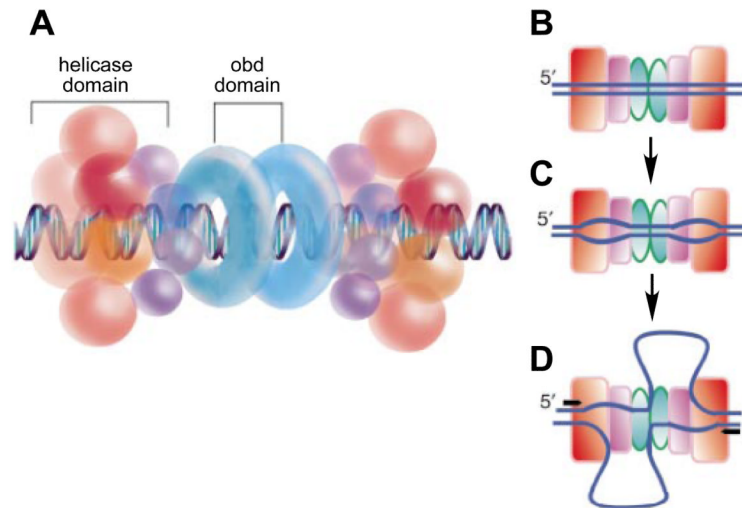


**Figure 2.**

Crystal structure of the N terminus of the *Mm*MCM.

**A - C.** Different views of the N terminus of the *Mm*MCM dodecamer, highlighting the central channel that runs through it. Positive charges are shaded in blue, negative charges in red. **A**, End view. **B**, Side view. **C**, Same view as B, but with the two front-most monomers removed to reveal the central channel. Yellow arrows show the side channels passing between exterior and interior. Reproduced from reference 15. **D** A side view of the electron density of full-length *Mm*MCM, aligned to approximately correspond to the side view of the crystal structure shown in B. In green, crystal structure of the N terminus has been fitted into the electron density. In red is fitted a hexameric model of the core AAA+ domain of RuvB, which has significant homology to the C-terminus of the Mcm2-7 proteins. Reproduced from reference 16.

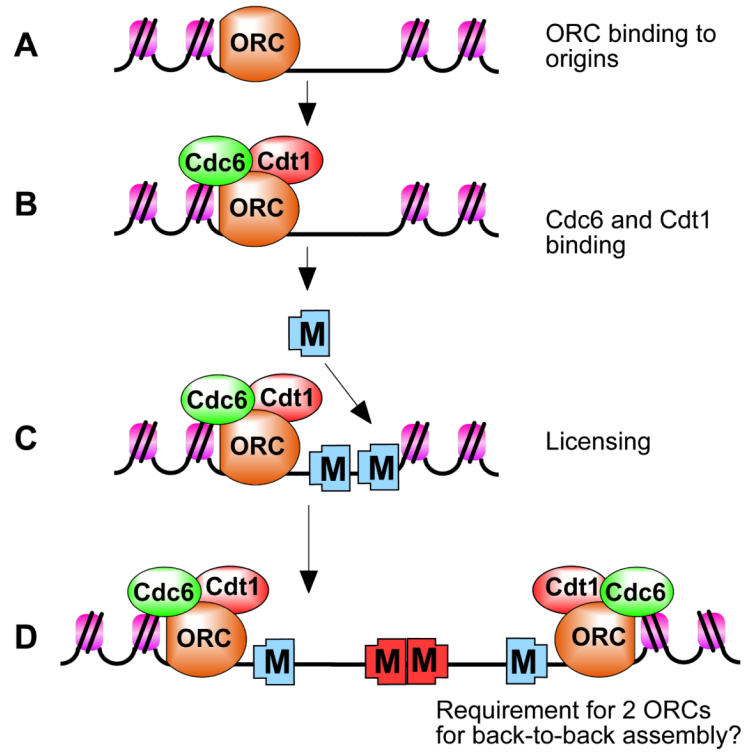




**Figure 3.**

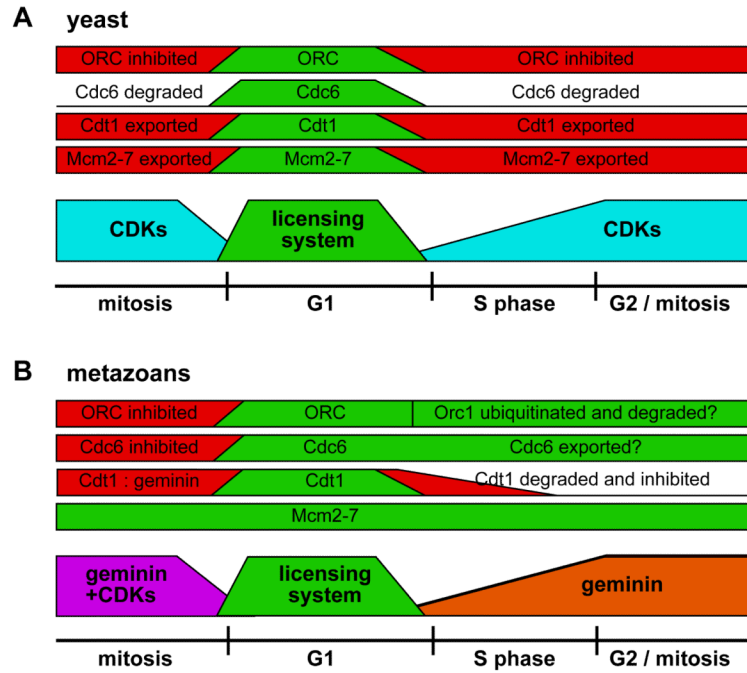
Model for SV40 T antigen function at replication initiation.

Model for the function of the SV40 T antigen based on the crystal structure of the helicase domain. **A**, A double hexamer of the SV40 T antigen, comprising a bilobed helicase domain and an origin binding domain (obd), is shown encircling origin DNA. **B**, **C**, **D**, Model for the possible extrusion of unwound DNA through a side channel of the helicase domain to unwind the two forks bidirectionally. **A**, T antigen binds and encircles origin DNA. **B**, Origin DNA is unwound, possibly by the two T antigen hexamers rotating relative to one another. **C**, A conformational change to form the fully initiated complex may involve extrusion of one of the single strands through an exit channels in the helicase domain, with the other single strand being extruded at the hexamer-hexamer interface. Reproduced from reference17.



**Figure 4.**

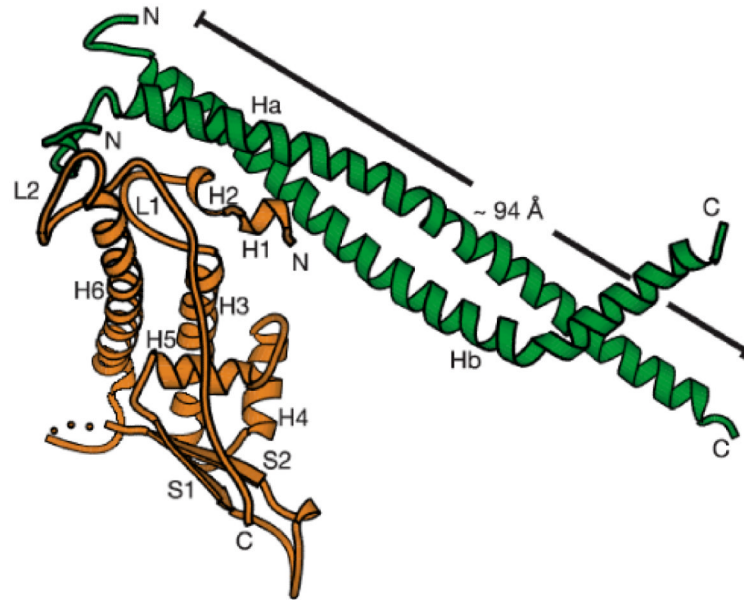
Stepwise assembly of pre-replicative complex (pre-RC) proteins during origin licensing. **A**, ORC is first recruited to the replication origin. **B**, ORC recruits Cdc6 and Cdt1. **C**, ORC, Cdc6 and Cdt1 act together to load multiple Mcm2-7 hexamers onto the origin, which licenses the DNA for replication. **D**, Initiation-competent complexes are probably formed by the back-to-back assembly of two Mcm2-7 complexes. Since ORC is asymmetrical, this might require a second ORC molecule in the opposite orientation to load Mcm2-7 in the opposite orientation.



**Figure 5.**

Cell cycle regulation of the licensing system in yeasts and metazoans.

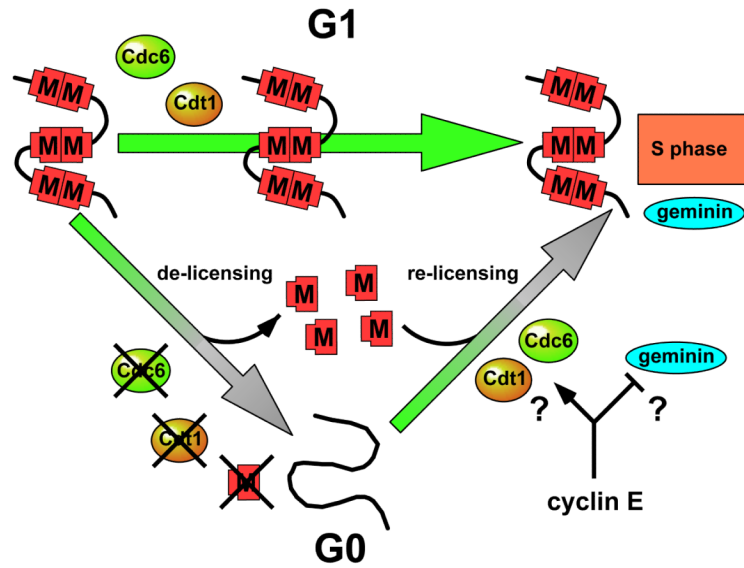
The activity of components of the licensing system during the cell cycle of yeasts (**A**) and metazoans (**B**) is shown. In the lower part of each figure, the licensing system is shown active (green) only in G1. In yeasts, licensing is inhibited at other times by CDKs (blue), whilst in metazoans, licensing is inhibited in S phase and G2 by geminin (orange) and in mitosis by a combination of geminin and CDKs (purple). Above this, the activity of different pre-RC components (ORC, Cdc6, Cdt1 and Mcm2-7) is shown: green for active, red for inhibited.



**Figure 6.**

Structure of a geminin : Cdt1 complex.

Structure of a complex between the central regions of geminin (tGeminin, in green) and Cdt1 (tCdt1, in orange). The N-terminal ten residues are structured in the tGeminin monomer that binds to tCdt1 but are disordered in the other tGeminin monomer, indicating that this region might undergo induced folding after binding to tCdt1. Comparison with the structure of geminin on its own<sup>103</sup> suggests that the kink at the C terminal end of geminin may be a distortion due to a packing interaction. Reproduced from reference<sup>49</sup>.



**Figure 7.** The possible role of cyclin E in promoting re-licensing of DNA on exit from G0. Mcm2-7 (red shapes ‘M’) are shown on a small segment of chromosomal DNA in a cell either passing directly through G1 (top arrow) or passing into G0 and then into S phase (lower arrows). During passage through G1, Mcm2-7 loaded onto DNA in late mitosis remains stably bound to DNA. Geminin is activated in late G1, just prior to entry into S phase. From G1, cells also have the option of entering quiescence (the G0 state). On entry into G0 from G1, the DNA is de-licensed and Mcm2-7, Cdc6 and Cdt1 are degraded. This might provide a barrier that prevents the inappropriate proliferation of these cells. On exit from G0 into S phase, quiescent cells must relicense their DNA, and cyclin E is required for this re-licensing. Cyclin E might be required to drive the re-synthesis or activation of Mcm2-7, Cdc6 or Cdt1, or cyclin E might be required to temporarily suppress geminin activity, which is also resynthesized prior to entry into S phase.