Replication licensing — defining the proliferative state?

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The proliferation of eukaryotic cells is a highly regulated process that depends on the precise duplication of chromosomal DNA in each cell cycle. Regulation of the replication licensing system, which promotes the assembly of complexes of proteins termed Mcm2–7 onto replication origins, is responsible for preventing re-replication of DNA in a single cell cycle. Recent work has shown how the licensing system is directly controlled by cyclin-dependent kinases (CDKs). Repression of origin licensing is emerging as a ubiquitous route by which the proliferative capacity of cells is lowered, and Mcm2–Mcm7 proteins show promise as diagnostic markers of early cancer stages. These results have prompted us to propose a functional distinction between the proliferative state and the non-proliferative state (including G0) depending on whether origins are licensed.

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If genetic stability is to be maintained, chromosomal DNA must be precisely duplicated in each cell cycle. In order to achieve this, the DNA polymerases and other DNA processing enzymes working at the replication fork must copy the template DNA with high accuracy. It is also crucial that the replication forks are assembled and disassembled correctly to ensure that all the genome is replicated and that no section of it is replicated more than once. This is a particular problem for eukaryotes, whose very large genomes mean that they must use thousands of REPLICATION ORIGINS (which each initiate a bidirectional pair of replication forks; see Glossary) to duplicate the entire genome in a reasonable period of time. The importance of strict cell-cycle regulation of replication origins is outlined in Box 1.

Here we review recent developments in our understanding of the REPLICATION LICENSING SYSTEM, which normally prevents replication origins from firing (initiating a bidirectional pair of forks) more than once in each cell cycle [1–5]. These recent results suggest that not only is the licensing system the key to ensuring precise chromosome duplication – but that it also plays an important role in determining the proliferative capacity of cells and has potentially important implications for cancer diagnostics.

The replication licensing system

How does the cell know whether or not it has already replicated a section of DNA in S phase? What ensures the strict alternation of S phase and mitosis seen in most cells? Recent work has made it clear that the LICENSING of replication origins, achieved by their loading of mini-chromosome-maintenance 2–7 proteins (Mcm2, 3, 4, 5, 6 and 7), provides crucial information to the cell about whether the DNA has been replicated in the current cell cycle [1–5]. The Mcm2–7 polypeptides form a functional hexameric complex [6] that comprises an important part of the 'pre-replicative complex' (PRE-RC) of replication proteins found at replication origins during G1 phase. On exit from metaphase, the replication licensing system becomes activated, so that each origin becomes loaded with Mcm2–7 (Fig. 1). The replication licensing system ('RLS' in Fig. 1) remains active throughout most of G1 but is inactivated as cells approach S phase. This means that no further Mcm2–7 can be loaded onto origins in S phase, G2 and early mitosis. Only licensed origins containing Mcm2–7 can initiate a pair of replication forks, and, when initiation occurs at an origin, the bound Mcm2–7 is displaced so that the origin cannot fire again (Fig. 1). Mcm2–7 might function as the helicase that unwinds DNA ahead of each replication fork [7], which would explain their displacement from origin DNA when forks are initiated. As a fail-safe mechanism, it is also envisaged that, should an origin be passively replicated by a replication fork emanating from another origin, the Mcm2–7 bound to it would also be displaced.

At least three other proteins are required for origins to load Mcm2–7 and become licensed [4] (Fig. 1b). The origin-recognition complex (ORC) first binds to each replication origin and then recruits two other proteins – Cdc6 (called Cdc18 in the fission yeast *Schizosaccharomyces pombe*) and Cdt1 (also known as RLF-B or *double-parked*) [2–4,8,9]. These proteins in turn load Mcm2–7 complexes and functionally license the origin. A crucial feature is that, although ORC, Cdc6 and Cdt1 are all essential for Mcm2–7 loading, none of them is subsequently

Glossary

CDK: cyclin-dependent kinase, consisting of a small kinase subunit complexed with an activating cyclin subunit. Key activator of cell-cycle transitions.
Licensing: the loading of functional Mcm2–7 hexamers onto replication origins to enable them to support a single round of replication.
Mcm2–7: six related minichromosome maintenance proteins (Mcm2, 3, 4, 5, 6 and 7) found in a hexameric complex.
ORC: the origin-recognition complex, comprising six polypeptides: Orc1, 2, 3, 4, 5 and 6.
Pre-RC: the pre-replicative complex of proteins bound to replication origins during G1 but not G2 of the cell cycle.
Replication licensing system: the set of proteins required to license replication origins.
Replication origin: a site on chromosomal DNA where a bidirectional pair of replication forks initiate.

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Box 1. Ensuring precise chromosome replication.

A small segment of chromosomal DNA replicated from three origins is shown during the different stages of the cell cycle (Fig. I). (a) The DNA is under-replicated as a result of the failure of one of the origins to fire. As sister chromatids are separated during anaphase, the chromosome is likely to be broken near the unreplicated section. (b) The successful duplication of the chromosomal DNA. (c) Over-replication of the chromosomal DNA as a result of one of the origins firing a second time in S phase. The local duplication of DNA in the vicinity of the over-firing origin is likely to represent an irreversible genetic change and might be resolved to form a tandem duplication. The four stages of the cell cycle are shown at the bottom of the figure.

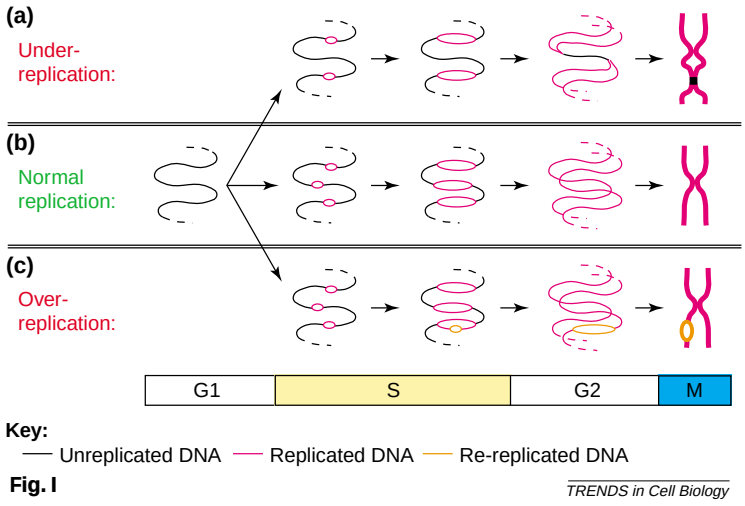

Fig. I

required to maintain the binding of Mcm2–7 to origins [10–13]. Furthermore, it has been shown in *Xenopus* (frog) that, once origin licensing is complete, ORC and Cdc6 (and probably Cdt1 as well) are no longer required for subsequent DNA replication [11,12]. As discussed below, an important consequence is that re-licensing of replicated DNA can be prevented by inhibition or removal of ORC, Cdc6 or Cdt1 once S phase has started, without displacing functional Mcm2–7 at licensed origins.

Overall control of origin licensing by cyclin-dependent kinases

Cyclin-dependent kinases (CDKs) – master regulators of the cell cycle – are activated during late G1, where they eventually induce cells to progress through S phase, G2 and then mitosis; CDK activity is then abolished during late mitosis. Significantly, the licensing system is only active during late mitosis and G1 phase (i.e. when CDK activity is low) (Fig. 1). Experiments performed in *S. pombe* first showed that, if CDK activity was temporarily inhibited in cells at the G2 phase of the cell cycle, the cells would re-replicate their DNA [14,15]. This suggests that reloading of Mcm(2–7) occurs in these CDK-deficient G2 cells. Subsequent work in a range of different organisms has supported this model by showing that CDKs can strongly inhibit the licensing system [16–21]. However, high CDK levels do not displace Mcm2–7 already bound to origins [11,12], thus allowing the normal S phase program to occur in the presence of S-phase-inducing CDKs (Fig. 1). It is possible that different CDKs differ in their ability to inhibit origin licensing, and CDKs normally active in G1 to prepare cells for S phase might be less repressive (see below).

Sometimes cells undergo unusual cell cycles where the daughter cells acquire more DNA than was present in their parent. This can occur as a consequence of developmental signals (such as the increase in chromosome number seen during cardiac myocyte development) or as a consequence of some insult to the cell (such as chemically induced polyploidy). It is useful to separate these unusual cell cycles into two distinct classes, shown schematically in Fig. 2. The first class comprises endoreplication cell cycles (endocycles) [22], where periods of alternating high and low CDK activity still occur. In endocycles, re-licensing of origins occurs when the CDK activity present in G2 or mitotic cells is abolished before cytokinesis has occurred. This G1 re-entry can occur from G2, metaphase or anaphase. The duplicated genomes are held in a single nucleus, except in the case where nuclear division is completed and cytokinesis alone is suppressed, resulting in the formation of a binucleate cell. An example is the endoreplication cycles occurring during *Drosophila* development, where transient interruption of CDK levels after DNA replication leads to cycles of Mcm2–7 reloading and near-complete re-replication of the DNA when CDK levels are restored [23–25]. Because endocycles drive complete S phases, the resultant cells should have DNA contents that are powers of 2 (2, 4, 8, 16, etc.) larger than the DNA content in the starting cell. When mitotic CDK activation is abolished by mutation of the *cdc13* (cyclin B) gene in *S. pombe*, multiple complete rounds of replication are seen and the cellular DNA content approximates this power series [15].

The second way that DNA content might increase is shown in the lower part of Fig. 2. In this case, the cell re-licenses and re-initiates forks at one or more replication origins before S phase has been completed (origin re-firing). The levels of CDK during S phase do not drop, but re-licensing occurs because the licensing components (ORC, Cdc6, Cdt1 or Mcm2–7) cannot respond to the inhibitory CDK signal. Because the re-licensing and re-replication of one particular origin would not be coordinated with any other origin, the resultant cellular DNA content would not represent any simple multiple of the original DNA content. If certain origins were more prone to re-firing, there would be localized amplification of the DNA surrounding them (much as is shown in the bottom panel of Box 1). One of the best-studied examples of this phenomenon in a physiological system occurs within ovarian follicle cells during *Drosophila*

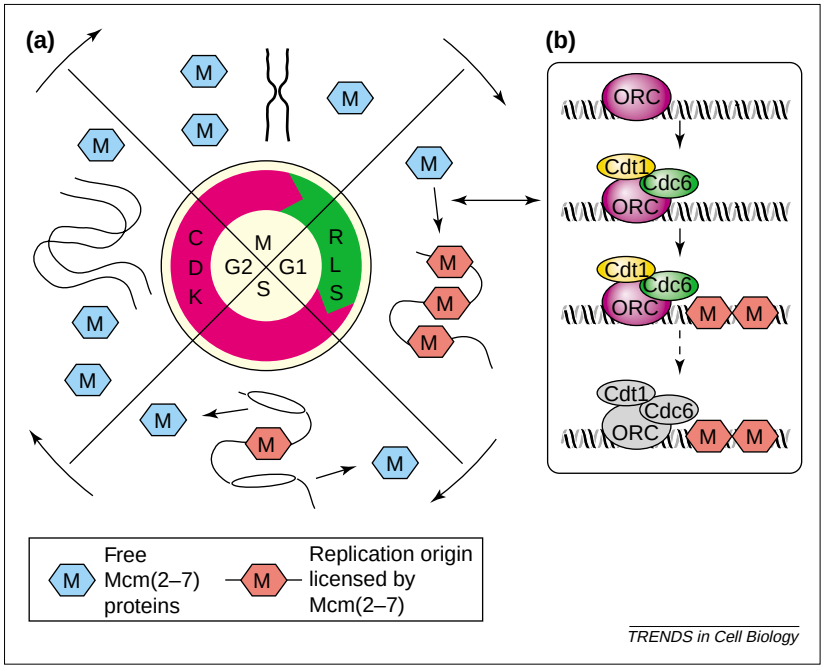

Fig. 1. Replication licensing and cyclin-dependent kinase (CDK) activity through the cell cycle. (a) A small segment of chromosomal DNA is depicted at different stages of the cell cycle, with bound (red hexagons) or unbound (blue hexagons) Mcm2–7 (M). The activity of the replication licensing system (RLS, green) and CDKs (pink) at the different stages are shown in the central circle. (b) Diagram depicting the sequence of events that occurs as each origin binds to Mcm2–7 and becomes licensed [4]. The loading of ORC (purple), Cdt1 (yellow) and Cdc6 (green) and then Mcm2–7 (M, red) is shown. At some time after licensing is complete, ORC, Cdc6 and Cdt1 become inactivated, as indicated by grey shading.

oogenesis, where certain genes are amplified to ensure sufficient production of chorion proteins that make up the eggshell. This occurs by the repeated initiation of replication origins close to the amplified genes in the presence of constant CDK levels [22,26].

CDK targets for preventing re-licensing of origins

Significant progress has recently been made in understanding the mechanisms by which CDKs inhibit replication licensing. Mounting evidence suggests that CDKs are likely to block licensing by a number of partially redundant mechanisms, which is perhaps not surprising given the importance of preventing over-replication of genomic DNA. Indeed, it appears that all four proteins known to be involved in origin licensing (ORC, Cdc6, Cdt1 and Mcm2–7) can each be independently downregulated as a consequence of CDK activity. The redundancy of CDK control over licensing is dramatically demonstrated in a recent paper by Nguyen *et al.* [5]. When CDK inhibition of ORC, Cdc6 and Mcm2–7 was specifically abrogated in the budding yeast *Saccharomyces cerevisiae*, partial re-replication of the genome occurred. This strongly supports the idea that the role of CDKs in preventing re-replication of DNA is primarily mediated by preventing re-licensing of DNA. Abrogation of CDK regulation on individual pairs of these proteins (ORC and Cdc6, ORC and Mcm2–7, or Cdc6 and Mcm2–7) failed to induce significant re-replication of DNA, thus demonstrating the redundancy of the CDK control mechanisms. Because re-replication was only partial when all three of these CDK controls were abolished [5], there might be further pathways preventing re-replication that were still active in these cells. It is also consistent with re-replication being a result of 'origin re-firing' (Fig. 2) rather than arising from CDK oscillation.

Precisely how do CDKs block origin licensing? As discussed in more detail below, each of the four origin proteins (ORC, Cdc6, Cdt1 and Mcm2–7) can be independently regulated by CDKs. Figure 3 summarizes these different mechanisms. This is not a complete list, and further inhibitory pathways might well be discovered. One striking feature shown in Fig. 3 is that, although the four origin proteins are highly conserved throughout the eukaryotic kingdom, the way that they are regulated by CDKs differs significantly. The regulation of these four different proteins by CDKs is discussed in turn.

CDK regulation of ORC

In *S. cerevisiae*, the Orc2 subunit of ORC is phosphorylated by CDKs in late G1, S phase, G2 and mitosis [5]. When the DNA encoding these phosphorylation sites was genetically removed from the *ORC2* gene, cells could undergo partial re-replication under conditions where Cdc6 and Mcm2–7 regulation by CDKs had been abrogated. The phosphorylation of Orc2 during the later stages of the cell cycle appears to be highly conserved throughout eukaryotic evolution and has also been reported in *S. pombe* and *Xenopus*. Phosphorylation of the *S. pombe* Orc2 homolog (Orp2) by CDK has recently been shown to regulate activity of the ORC complex as deletion of CDK phosphorylation sites enhances the re-replication seen when the *S. pombe* Cdc6 homolog (Cdc18) is overexpressed [27]. The situation in metazoans appears somewhat more complex. In *Xenopus*, exposure of chromatin to high CDK levels (such as occur during mitosis) can release ORC from chromatin [11,12], and this appears to play a small but significant role in preventing origin re-licensing [8]. Although ORC appears to bind to DNA with increased affinity in early G1 when CDK activity is low, binding then weakens later in the cell cycle. This decrease in binding affinity of ORC to DNA was not as a result of increases in CDK activity but arose as a consequence of origin licensing ('licensing-dependent origin inactivation'), a process that might help to prevent re-licensing of origins later in the cell cycle [12]. In mammalian cells, there is also evidence that the Orc1 subunit of ORC can be released from DNA during S phase and mitosis, leaving Orc2 still bound [28,29]. This effect is likely to be a result of the high CDK levels present at this stage.

CDK regulation of Cdc6

In yeasts, the abundance of Cdc6 is tightly controlled during the cell-division cycle. *CDC6* transcription peaks during late mitosis and G1 [30–32], with

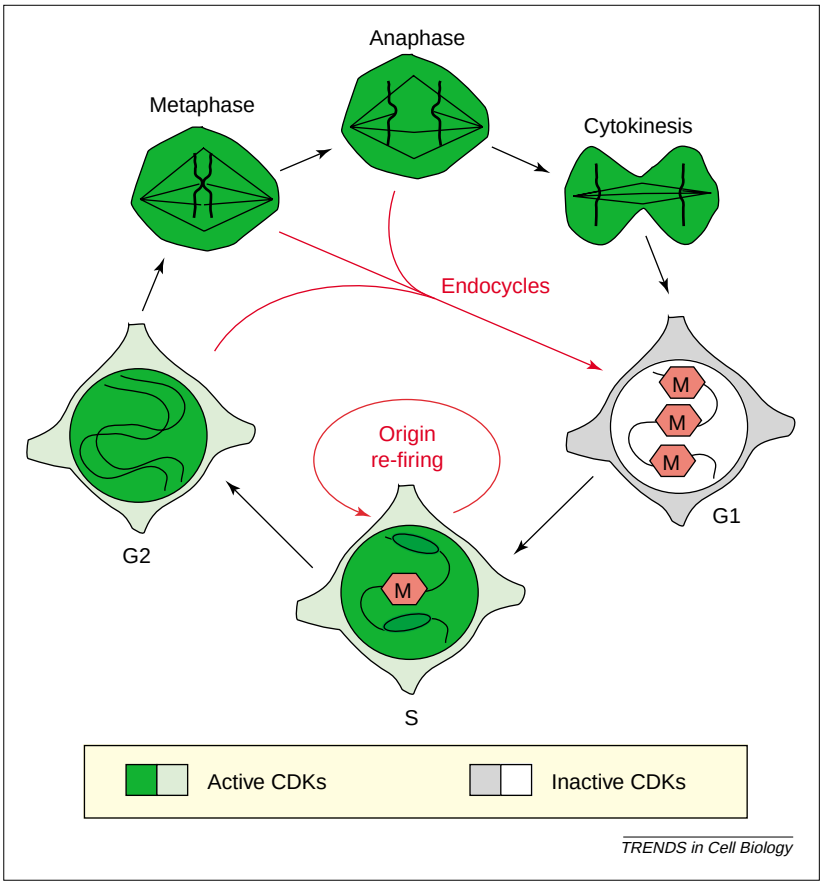

Fig. 2. Different routes by which cellular DNA content can increase. A small segment of chromosomal DNA is depicted at different stages of the cell cycle. Red hexagons show Mcm2–7 bound to origins in G1 and S phases. The outer ring (black arrows) shows the normal sequence of cell-cycle events. The red arrows show routes by which cellular DNA content is increased (endocycles and origin re-firing). Cyclin-dependent kinase (CDK) activity in S, G2 and M phases is denoted by green coloring.

transcription being under the control of the CDK-dependent transcription factor SWI4/Cdc10 [31,33]. Protein levels are also controlled by cell-cycle-specific degradation, probably involving two or more different pathways [34,35] but again controlled by CDK activity [34–36]. To abolish both regulatory mechanisms and to permit DNA re-replication in *S. cerevisiae*, Nguyen *et al.* [5] expressed from a constitutively active promoter a truncated form of Cdc6 that is resistant to cell-cycle-specific degradation.

In vertebrate cells, the regulation of Cdc6 is less well understood. Cdc6 appears to be present throughout the vertebrate cell cycle. Although cell-cycle-dependent degradation of Cdc6 protein is seen, significant amounts of chromatin-bound Cdc6 are observed during G1, S and G2 phases [37,38]. Much of the soluble Cdc6 protein, however, is translocated from the nucleus to the cytoplasm when CDKs are activated in late G1 phase, thus preventing it from further interaction with replication origins [39–41]. One possible explanation for these apparently contradictory results is that the chromatin-bound form of Cdc6 seen in S and G2 phases is not competent to support origin licensing but might play another role in cell-cycle regulation.

CDK regulation of Cdt1

First identified in *S. pombe* as a gene induced by the CDK-dependent transcription factor Cdc10, Cdt1 mRNA and protein levels peak in late mitosis and early G1 [42–44]. In HeLa cells, levels of Cdt1 also peak during G1 [43,45]. A Cdt1 homolog has not yet been identified in *S. cerevisiae*. Therefore, it is possible that re-replication was only partial in *S. cerevisiae* cells containing unregulated ORC, Cdc6 and Mcm2–7 because Cdt1 activity was being inhibited by CDKs [5].

Vertebrate cells are also able to control Cdt1 activity through a specific inhibitor called geminin [8,45,46]; no geminin homolog has yet been identified in yeast. In metaphase-arrested *Xenopus* eggs, geminin inhibition of Cdt1 is the major pathway for prevention of origin licensing [8]. The abundance of geminin, however, has been shown to be indirectly regulated by CDKs. Geminin is specifically degraded during late mitosis by the anaphase-promoting complex (APC/C) that is also responsible for degrading cyclins and which itself is regulated by CDKs [46]. In mammalian cells, geminin levels remain low throughout G1 (when the APC/C is active) and levels rise again during S phase and G2, when the APC/C becomes inactivated and CDK levels rise [45,46]. APC/C regulation has been suggested as a means by which the specialized endocycles of *Drosophila*, mentioned above, are controlled [22].

CDK regulation of Mcm2–7

In *S. cerevisiae*, Mcm2–7 proteins are present in the nucleoplasm only during late mitosis and early G1. Soluble Mcm2–7 proteins are excluded from nuclei at other stages of the cell cycle, although the origin-bound Mcm2–7 hexamer is still seen in S phase nuclei [47–49]. The nuclear exclusion of Mcm2–7 is dependent on the presence of CDK activity. Exclusion can be mediated by the *S. cerevisiae* G1 cyclins (Clns) as well as the B-type cyclins (Clbs) [48], but the B-type cyclins might be more effective [49]. This nuclear exclusion, which formed a major part of the original licensing factor model [1], is an effective way of preventing re-replication of DNA as it separates the license (Mcm2–7) from its substrate (DNA).

There is little evidence, however, that Mcm2–7 activity is regulated by subcellular localization in other organisms. Mcm2–7 proteins have been reported to have a constitutively nuclear localization in *S. pombe*, *Drosophila*, *Xenopus* and a range of mammalian cells. Indeed, there is little evidence that Mcm2–7 proteins are negatively regulated late in the cell cycle of other organisms (e.g. Ref. [19]). However, the possibility of undiscovered modes of Mcm2–7 regulation (such as controlling its assembly into an active hexamer [6]) is still open.

Licensing and the definition of the proliferative state

Only a small proportion of the cells that make up a multicellular organism are likely to be actively

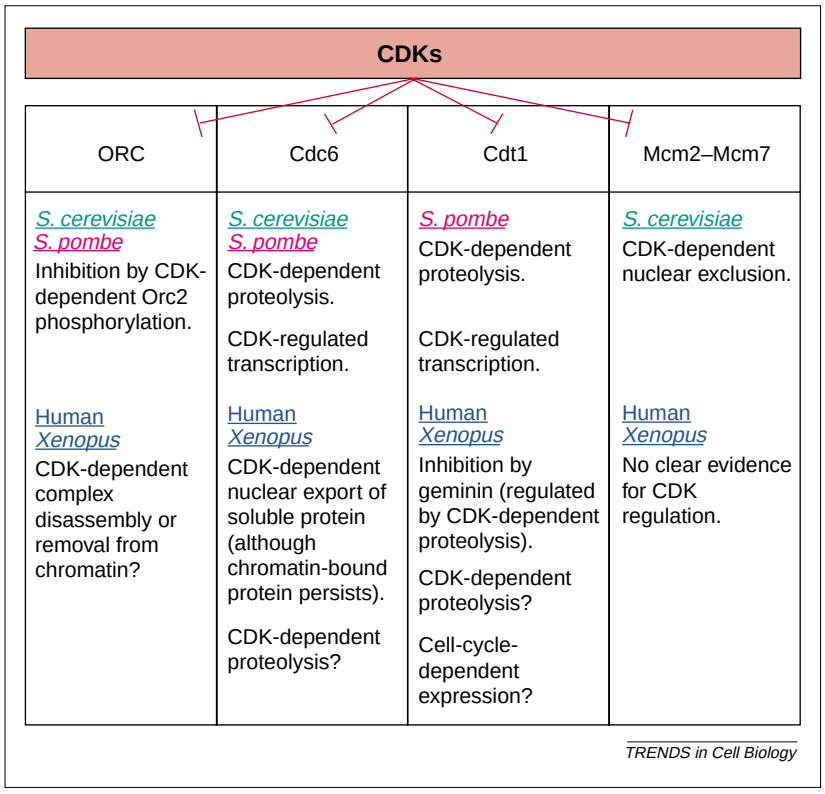

Fig. 3. Pathways for cyclin-dependent kinase (CDK) inhibition of origin licensing. The different mechanisms by which CDKs can inhibit the activity of ORC, Cdc6, Cdt1 and Mcm2–7 in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Xenopus* and human.

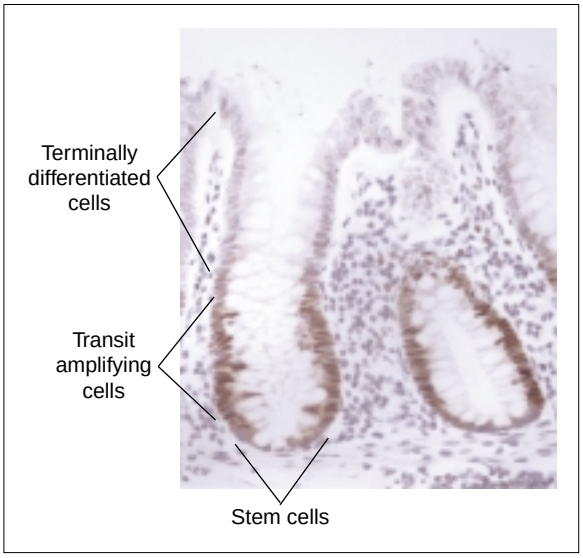

Fig. 4. Mcm2 protein expression in differentiating colonic epithelial cells. Indirect immunoperoxidase staining of colon with an antibody against Mcm2. Anatomical regions containing stem cells, transit amplifying cells and terminally differentiated (non-proliferating) cells are indicated. The transit amplifying cells, which are actively proliferating, stain the most strongly for Mcm2. Reproduced, with permission, from Ref. [56].

engaged in the cell-division cycle at any one time. Some non-proliferating cells will have permanently withdrawn from the cell cycle as a result of terminal differentiation or senescence. Others can be easily stimulated to divide again by appropriate environmental signals such as growth factors; these cells are said to be 'quiescent' or in the G0 phase of the cell cycle. Evidence from a range of different organisms and cell types suggests that both G0 and permanently arrested cells have lost Mcm2–7 proteins and are functionally unlicensed [50–56]. Not only is the Mcm2–7 hexamer removed from DNA as cells pass into G0, but the unbound protein is also lost from the cells. A similar reduction in Cdc6 protein is seen in G0 and permanently arrested cells [53,54,56–58]. ORC levels, however, seem to remain high on progression into quiescence [52,53]. The persistence of ORC in non-proliferating cells is consistent with it having a function in these cells that is independent of DNA replication, such as transcriptional silencing [59].

A recent study of Mcm2–7 proteins in a variety of human tissues by Stoeber *et al.* [56] consolidates these results, and suggests that the removal of the replication license is a common pathway by which proliferation is restrained. An example is shown in Fig. 4 where a section of human colon has been immunostained for Mcm2. The terminally differentiated (non-proliferating) cells at the top of the crypt contain virtually no Mcm2. At the base of the crypts are the stem cells, which, although possessing a high capacity for self-renewal, divide relatively infrequently. These stem cells contain intermediate levels of Mcm2, consistent with them being at different points through the G1–G0 transition. Between the stem cells and the terminally differentiated cells are the transit amplifying cells that are actively proliferating. These proliferating cells stain the most strongly for Mcm2.

The correlation between entry into G0 and terminal differentiation with loss of Mcm2–7 is shown schematically in Fig. 5. During late mitosis and early G1, replication origins become licensed, and they stay licensed throughout the G1 period. From G1, cells have the choice to either enter S phase or withdraw from the cell cycle. Entry into S phase is driven by rising CDK activity, which leads to the initiation of DNA replication and subsequent removal of Mcm2–7. Withdrawal from the cell cycle is accompanied by the gradual loss of Mcm2–7 in the absence of CDK activity or DNA replication. On re-entry into the cell cycle from G0, origins are re-licensed and only then can CDK activity induce entry into S phase [53]. The licensing pathway that is followed during the G0–G1 transition might be regulated differently from the licensing pathway that occurs on exit from mitosis. In particular, many of the pre-RC genes involved in licensing appear to have binding sites for the E2F transcription factor, which is active during late G1 or upon exit from G0 [9,51,57,60–65]. Full E2F activity is dependent on Cdk4/6–cyclin D and Cdk2–cyclin E, suggesting that, at the levels necessary to activate E2F, these particular CDKs might not inhibit licensing (Fig. 5).

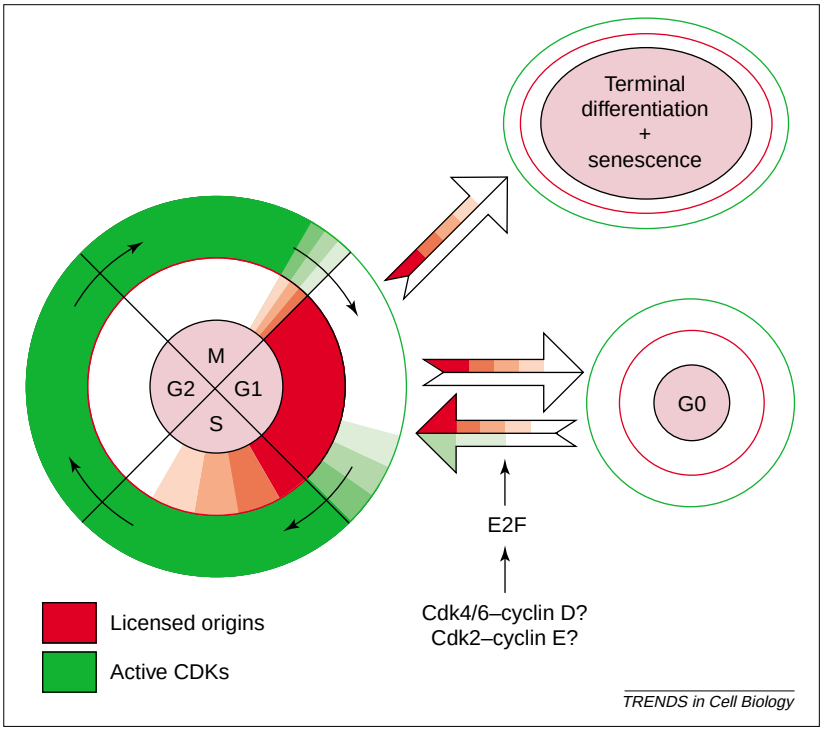

Fig. 5. Entry and exit of cycling cells into quiescence and terminal differentiation. On the left is a cartoon of cells passing through four phases of the cell-division cycle. The presence of licensed origins is shown in red (inner circle), and the presence of active cyclin-dependent kinases (CDKs) is shown in green (outer circle). When cells pass from G1 into G0 or terminally differentiate, origins become unlicensed. When G0 cells are stimulated to re-enter G1, their origins become re-licensed. It is currently unclear whether CDK reactivation of the E2F transcription system is required for this re-licensing to occur.

Detection of Mcm2–7 is therefore a powerful way of assessing the proliferative potential of cells. Because there is currently no formal way of distinguishing G1 from G0 cells, we propose that the description of cells being in G1 should be limited only to cells with licensed origins. The G0 state can then be defined thus: a reversible withdrawal from the cell cycle characterized by unlicensed origins and the absence of CDK activity. We believe that this definition would be useful as it has clear functional significance in distinguishing G0 or differentiated cells that are not proliferating from G1 cells engaged in the cell-division cycle. This distinction, between cells containing Mcm2–7 and cells lacking it, has potential as a diagnostic marker for early cancer stages – that is, cancers with a high proportion of proliferating cells (high growth fraction) should stain strongly for Mcm2–7 [54,56,66,67]. This powerful new approach has already given promising results in the diagnosis of cervical [54], urothelial [66] and bronchial cancers [67].

In their survey of Mcm2–7 levels in different human tissues, Stoeber *et al.* [56] reported that the glandular epithelial cells of the breast showed an interesting exception to the correlation between Mcm2–7 presence and active proliferation. In breast cells from non-pregnant and non-lactating women, a large percentage of glandular epithelial cells (47–65%) expressed Mcm2–7 while only a small percentage (~6%) showed signs of proliferation. During pregnancy, when these cells proliferate rapidly, almost of all them contained Mcm2–7, but expression plunged to <3% during lactation when the glandular epithelial cells undergo differentiation to the secretory state. The persistence of Mcm2–7 in non-proliferating breast cells might be an evolutionary relic from times when women spent most of their fertile years either pregnant or lactating and when it was therefore unnecessary for these cells to be able to withdraw from the G1 state. An important question raised by this finding is whether licensed but slowly proliferating cells such as these have a higher risk of undergoing malignant transformation. Because the lack of origin licensing could be an important mechanism restraining the proliferation of G0 cells, it is possible that failure to downregulate the licensing system (as in these breast cells) might make transition to uncontrolled proliferation significantly easier to achieve. These new observations could therefore have important implications for the pathogenesis of breast cancer and urgently demand further study.

Concluding remarks

The results discussed in this review show that the replication licensing system not only plays a central role in ensuring precise chromosome duplication in each cell cycle, but it also plays an important role in downregulating the proliferative capacity of cells when they withdraw from the cell cycle. This conclusion appears to have important practical implications for cancer diagnosis, which we expect to be consolidated by future work.

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