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Efficiency and equity in origin licensing to ensure complete DNA replication

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Abstract

The cell division cycle must be strictly regulated during both development and adult maintenance, and efficient and well-controlled DNA replication is a key event in the cell cycle. DNA replication origins are prepared in G1 phase of the cell cycle in a process known as origin licensing which is essential for DNA replication initiation in the subsequent S phase. Appropriate origin licensing includes: (1) Licensing enough origins at adequate origin licensing speed to complete licensing before G1 phase ends; (2) Licensing origins such that they are well-distributed on all chromosomes. Both aspects of licensing are critical for replication efficiency and accuracy. In this minireview, we will discuss recent advances in defining how origin licensing speed and distribution are critical to ensure DNA replication completion and genome stability.

Introduction

Animal development begins from a single cell, and the number of cells in a human adult can be more than 30 trillion. Thousands of divisions occur during the development of each individual. Cell proliferation must be strictly regulated during development and homeostasis, and uncontrolled cell proliferation can lead to abnormal development or diseases such as cancer. For each cell cycle in somatic cells, the genome is replicated once and only once to produce two exact copies of the chromosomes.

DNA replication in mammalian cells initiates at many thousands of discrete sites called replication origins [1]. Before the initiation of DNA replication, cells load heterohexameric minichromosome maintenance (MCM) complexes onto origin DNA; this process is called origin “licensing.” Origin licensing starts from the binding of the heterohexameric Origin Recognition Complex (ORC) to double-stranded DNA, and ORC in turn recruits the CDT1 and CDC6 proteins. These three components then load MCM complexes onto DNA to form double hexamers of two MCM complexes, and the loaded MCM complexes encircle duplex DNA (Figure 1A)[2]. Origin licensing is unidirectional and confined to G1 phase, and the loaded MCM complexes are very stably associated with DNA in G1 [3]. During S phase, the loaded MCM complexes form the core of the replicative helicase, and MCM complexes

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in different genomic regions are activated at different times in S phase. Activated helicases lead the replication forks, unwinding DNA for synthesis, and are then unloaded as DNA replication completes (Figure 1B).

Properly-controlled origin licensing is critical for efficient and complete genome duplication. Reduced origin licensing can lead to under-replication, DNA damage, and even chromosome breaks [4–7]. Successful genome duplication is required for cell proliferation and demands extraordinary precision and accuracy [8]. Inadequate origin licensing can cause disease; for example, hypomorphic mutations in *ORC1, ORC4, ORC6, CDT1, CDC6, MCM4, MCM5* and *MCM7* are strongly associated with Meier-Gorlin syndrome, a form of human primordial dwarfism characterized by reduced proliferation [9–12]. Moreover, low MCM expression causes replication stress and limits fetal erythropoiesis and hematopoietic stem cell differentiation during mouse development [5]. The biochemistry of origin licensing is already well-defined primarily due to work with purified *Saccharomyces cerevisiae* (budding yeast), *Drosophilia melanogaster* (fruit fly), and *Xenopus laevis* (frog) proteins [13–17], but how cells achieve appropriate origin licensing in time and in space during G1 is still not well understood. In this review, we highlight research suggesting that origin licensing speed and the distribution of licensed origins have a profound impact on the efficiency and accuracy of DNA replication.

Licensing the appropriate number of origins

In theory, a single replication origin for each chromosome could completely duplicate a genome. However, due to the huge size of the human genome, thousands of origins are necessary to fully duplicate human chromosomes in a reasonable amount of time. A paucity of licensed origins prevents efficient replication and can induce replication stress, genome instability and malignant transformation [6].

Even though cells license thousands of origins, only around 5%– 10% of licensed origins are normally used, with the majority remaining dormant. During S phase, replication forks can stall at sites of DNA damage, repetitive DNA, collisions with RNA Polymerase, or other barriers [18]. Dormant origins will initiate at nearby stalled replication forks that emanated from primary origins to ensure complete replication [19]. The risk of incomplete replication is higher when cells lack dormant origins [20, 21]. The existence of dormant origins allows flexibility in origin usage to match local conditions. It is still unclear however, if dormant origins and primary origins that will initiate are licensed differently.

Reduced origin licensing is also called under-licensing. During S phase, fewer licensed origins means fewer initiation events, thus extending the time required for full genome duplication. An under-licensed chromosome also has a higher risk for under-replication because there are not enough licensed dormant origins to rescue replication near forks that stall [5, 20]. This challenge is even greater for common fragile sites (CFS) which are more sensitive to reduced licensing and show increased breakage because they are inherently prone to under-licensing already from the effects of local transcription [22–25]. Under-licensing is closely related to abnormal development and disease [9]; A hypomorphic MCM4 mutation results in a genetic syndrome of growth retardation and natural killer cell

deficiency [26]. The patients' fibroblasts also exhibited genomic instability that was rescued by the expression of WT-MCM4 [26]. A Meier-Gorlin syndrome variant in MCM7 interferes with MCM complex formation and impacts S phase efficiency [27]. Meier-Gorlin mutations in *orc6* impair ORC complex formation, according to work with Drosophila proteins [28]. The majority of Meier-Gorlin syndrome alleles are predicted to have similar effects on replication efficiency and thus, cell proliferation [11, 27, 29–31].

Origin licensing speed

To ensure precise and complete chromosome replication, origin licensing can begin as early as telophase [32] and is stopped at S phase entry. The licensing system is turned off during S phase and G2, ensuring that no replication origins can fire more than once in a single cell cycle. If licensing were to occur on replicated DNA, then that DNA could be re-replicated, and re-replication is a potent driver of genome instability [33–35]. Therefore all MCM loading factors are inactivated or degraded at the beginning of S phase, leaving no opportunity to license new origins once cells have entered S phase. G1 phase is the only opportunity for origin licensing, and it is critical for cells to license enough origins during G1 phase [36].

G1 length varies widely among different cell types. For example, specialized developmental and immune cell cycles have minimal G1 lengths of mere minutes [37, 38], and G1 in cultured somatic cells is often greater than 12 hours [39]. To avoid under-licensing, short G1 cells must license origins faster than long G1 cells. The number of licensed origins influences how effectively S phase cells can accommodate both endogenous and exogenous sources of replication stress and also the timing of replication within S phase. To lower the risk of genome instability, appropriate origin licensing speed is necessary to archive enough licensed origins before S phase begins. If MCM loading speed is not coordinated with G1 length, cells with short G1 phases will experience under-licensing and genome instability (Figure 2). For example, overexpression of the well-known oncogene *CCNE1* shortens G1, and causes significant under-licensing, DNA double-stranded breaks, and chromosome rearrangements both in cultured cells and human cancers [40–45]. *CCNE1* overexpression does not affect licensing speed, so the shortened G1 results in under-licensing [41]. If cells could increase licensing speed in a short G1, then the risk of under-replication would be lower in S phase, but what controls licensing speed is still unknown.

In a prior study, we derived a method to measure MCM loading rates in asynchronous populations of pluripotent vs differentiated human cells [41]. We found a faster origin licensing speed in stem cells with short G1 phases compared to differentiated cells with longer G1 phases. During the differentiation of stem cells, licensing speed also became slower. Since induced pluripotent stem cells also license origins fast, presumably reprogramming increases licensing speed; the mechanism of licensing acceleration is still unclear however. We also demonstrated that faster origin licensing helps protect stem cell pluripotency, and it is a hallmark of pluripotency. These findings indicated that licensing speed is both developmentally regulated and itself can influence early development. However, the underlying mechanism of how loading speed regulates pluripotency it is still

unknown, but it is possible that artificially increasing MCM loading speed could improve reprogramming to pluripotency.

There are still large knowledge gaps about how MCM loading speed is established for different cell types and how cells coordinate MCM loading amount and speed within G1. Multiple proteins are required normal origin licensing, and differences in their concentrations or activities likely contribute to different origin licensing speeds. There are several possibilities for how MCM loading speed can accelerate: (1) Higher concentrations of MCM loading factors may result in faster MCM loading. For example, we found that CDT1 levels are higher in G1 phase of fast-loading pluripotent cells than G1 of slower-loading differentiated cells. On the other hand, CDT1 may not be rate-limiting for licensing speed in all cell types. For example, overproducing CDT1 in differentiated epithelial cells (RPE1-hTert) had no effect on MCM loading rate, but increasing the amount of CDC6 in G1 through a stabilizing mutation [46] accelerated MCM loading in epithelial cells [41]. These observations indicate that the MCM loading rate is likely set by whatever loading factor is rate limiting in a given cell type.

Either MCM loading factors themselves or accessory factors may contribute to licensing speed indirectly. For example, the RIF1 phosphatase supports efficient origin licensing by protecting the ORC1 subunit of ORC from degradation in G1 [47]; (2) MCM loading factors may be more dynamic and efficient in fast-loading cells. Unlike MCM, ORC, CDC6 and CDT1 are released after a licensing reaction is complete and can then load other MCM complexes [48]. If the loading factors recycle faster, then the MCM loading speed will be faster. Mechanisms to alter *in vivo* activity of loading factors have not yet been elucidated, so this possibility is still in the realm of speculation. (3) The chromatin environment might affect MCM loading speed. We recently measured MCM loading dynamics in G1 phase and found that in early G1 phase, MCM loading is faster in euchromatin than in heterochromatin, but in middle and late G1 heterochromatin loading more closely matches that of euchromatin [49]. These findings indicate that licensing speed is closely related to chromatin environments. In support of this idea, differentiated cells with slow origin licensing have more compacted heterochromatin than stem cells which have fast origin licensing [41, 50]. Chromatin remodeling proteins (SNF2H, GRWD1, SAF-A) [51–53], and histone acetylation enzyme (HBO1) [54, 55] have also been shown to promote the origin licensing, and their concentrations or activities could affect licensing speed. (4) PCNA-K164 ubiquitination may also promote origin licensing by an unknown mechanism, and changes in the levels of this modification could therefore impact licensing speed [56]. The combination of all of the MCM loading factors and their accessory factor concentrations and activities will determine the maximum licensing speed in a given cell type. We postulate that normal cells coordinate licensing speed with the length of G1 by adjusting rate limiting activities. When this coordination is disrupted by mutation or environmental perturbation, cells risk under-licensing and subsequent under-replication.

Origin licensing distribution

Metazoans origins are not defined by DNA sequence, but are instead defined by features of local chromatin environment that influence ORC binding and subsequent origin licensing [57, 58].

Different approaches have been developed to characterize human origins, including optical methods and sequencing methods [59–61]. Chromatin environments vary widely as a result of differences in chromosome compaction and histone modification states [62–64]. For example, the acetylation of histone H4 (H4AC) is generally linked to more open and active chromatin, and the trimethylation of histone H3 at lysines 9 and 27 (H3K9me3 and H3K27me3) are common markers of more compacted and inactive chromatin [63, 65, 66]. In addition, multiple studies have documented chromatin-dependent differences in origin licensing and/or origin initiation. For example, chromatin immunoprecipitation followed by sequencing (ChIP-seq) mapped ORC and MCM binding sites in fruit fly cells and human cells, and this study correlated high ORC-DNA binding with transcription start sites and other open chromatin regions [67]. Analysis of ORC/MCM density at different sites found ORC and MCM density are low within actively transcribed gene bodies, but enriched at active gene promoters [68]. Both of these studies in metazoan cells agreed with previous work in budding yeast that demonstrated preferential ORC binding (and MCM loading) in nucleosome-depleted DNA [69, 70]. A recent study discovered that the histone variant H2A.Z promotes histone H4 lysine 20 dimethylation which, in turn, recruits ORC for MCM loading [71]. These and similar studies have documented genomic locations where origin licensing is most abundant or efficient, and those locations are frequently associated with open euchromatin.

Because it is critical to ensure enough primary and dormant origins for complete genome duplication, origin licensing should not be overly concentrated in accessible chromatin at the expense of less accessible chromatin. Despite substantial amounts of compacted and less accessible heterochromatin, *all* DNA must be licensed to be fully replicated. High total amounts of loaded MCM, but inappropriately distributed MCM complexes along chromatin creates a risk of under-replication if the distribution leaves sparsely licensed genomic regions (Figure 3). However, very little is known about how MCM complexes are sufficiently distributed before S phase starts, particularly at less preferred origins or in compacted chromatin.

Dukaj and Rhind examined the effects of reducing MCM levels in budding yeast. Limiting total MCM resulted in differential loading at yeast origins, and origins with intrinsically low ORC affinity were more prone to reduced MCM loading compared to origins with high ORC affinity [72]. This finding highlights the intrinsic differences among potential origins for efficient origin licensing. Two recent studies by Fox and colleagues described molecular mechanisms to reduce disparities in MCM loading levels among different yeast origins. In the first study, they demonstrated that a histone deacetylase localizes to origins that are intrinsically preferred by ORC. Histone deacetylation at those sites suppresses excess MCM loading to allow more equitable loading at the less-preferred sites [73]. In the second study, a sequence-specific DNA binding protein enhances ORC recruitment to origins that are

otherwise intrinsically less favored [74]. Thus, origin licensing distribution can be adjusted by suppressing excess licensing at “strong” origins and enhancing licensing at “weak” origins. It remains to be determined if similar mechanisms operate in metazoans where ORC binding is sequence-independent whereas budding yeast ORC has some sequence specificity [58, 75]. In support of mechanisms in mammalian cells for enhancing licensing in heterochromatin, a heterochromatin binding protein, ORCA/LRWD1, binds to H3K9me3 (a heterochromatin mark) and recruits ORC [76]. A dedicated mechanism to recruit ORC to heterochromatin regions is expected to specifically enhance licensing in regions that are inherently less accessible, and our analysis of licensing dynamics in heterochromatin supports this idea [49].

Until very recently, it was not known if origin licensing activity occurs at all origins throughout G1 phase or if there is a temporal order to licensing itself. It is well-known that origin initiation within S phase follows a cell type-specific stereotypical timing pattern [77, 78]. Moreover, the amount of total MCM loading at different sites correlates with early vs late replication, underscoring inherent differences between potential origins. We recently found that origins in euchromatin are licensed relatively earlier than origins in heterochromatin within G1 phase, and that euchromatin loading rates slow during G1, but heterochromatin loading rates are more constant. ORCA-mediated ORC recruitment specifically to heterochromatin sustains heterochromatin loading in late G1 to promote equitable licensing between euchromatin and heterochromatin by the end of G1 [49]. It is unclear if the preference for fast euchromatin loading in early G1 is due to general accessibility or to specific interactions between loading factors and euchromatin marks. Moreover, the dynamics of origin licensing at sites that are likely to be primary origins compared to sites more likely to be dormant origins are also unknown. Predicting primary vs dormant origins prior to S phase is not a trivial challenge because the sites of primary origins can vary among individual cells and from one cell cycle to the next [79, 80].

Origin licensing distribution may not be simply a consequence of where ORC binds and loads MCM complexes. There are suggestions that once loaded, MCM complexes can slide away from their original loading site in G1 phase. For example, analysis of MCM and ORC localization in cultured fruit fly cells found that many MCM complexes were not near ORC complexes, and that this separation depends on cyclin E/CDK2. This dependence suggested redistribution of MCM localization after cyclin E/CDK2 activation in late G1 phase or early S phase [81]. It was not yet appreciated at the time of this study that ORC can release from DNA after MCM loading, so it remains to be determined if loaded MCM complexes moved away from ORC or if ORC departed from the loaded MCM complexes. This sliding behavior in G1 phase has thus far only been observed *in vitro*. It is still an open question whether a loaded MCM can translocate past nucleosomes either by an active chromatin remodeling mechanism, perhaps with an associated remodeling enzyme, or by passive sliding in G1 phase. In addition, MCM complexes loaded in the transcribed regions of genes can be displaced, either by sliding or by active removal upon encounters with RNA polymerase [40, 82]. This transcription-dependent mechanism explains how licensed origins are generally depleted from active gene bodies [68].

Origin licensing checkpoint

Because origin licensing is the most important step to prepare for DNA replication, cells may have a means to prolong G1 phase while licensing is still low; such a mechanism is referred to as a “licensing checkpoint”. S phase entry delay was indeed reported in several non-transformed cell types when origin licensing was artificially inhibited by various methods such as depleting MCM2-7, CDC6, CDT1, or ORC subunits by RNAi or by overproducing a licensing inhibitor [83–86]. It remains unclear how this checkpoint operates and whether it is the number or the distribution of licensed origins that signals for S phase delay or by what mechanism that signal would be generated. What is known thus far is that CDK2 activity in licensing-deficient cells is delayed by a variety of molecular markers, and the delay is only observed in p53-proficient cell types [85, 86].

Future directions

An important unanswered question is what mechanisms ensure adequate MCM loading distribution? How does licensing overcome differences in accessibility in different chromatin regions. Also is there a defined order of licensing at the level of individual origins or chromatin domains or in broad chromatin accessibility categories? We suggest that the last genomic regions to be licensed in G1 may also be the most vulnerable to under-replication since they may receive less MCM or less well-distributed MCM. Demonstrating differential loading dynamics between euchromatin and heterochromatin is a first step [49], but exploring licensing dynamics at the resolution of origins will likely require technological advances for single cell analyses. Furthermore, it is still unclear if G1 length determines the speed of origin licensing or if the speed of licensing determines G1 length. Changes in G1 length control are common in cancer-derived cells lines, and a hallmark of cancer is genome instability; such instability could be partly driven by poor licensing coordination. Defining the mechanism of G1 length and MCM loading speed coordination could therefore inspire new routes to developing cancer therapies and diagnostics.

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Perspectives

- Origin licensing is the most important step for DNA replication preparation. Aberrant origin licensing can cause genome instability and is tightly linked to a variety of diseases such as developmental disorders and cancer. Thus, understanding the regulation of origin licensing will help understand cell cycle regulation and can inspire the development of novel disease therapies.
- Cells must license enough origins within the time allotted for G1 phase to ensure complete replication in S phase. Origin licensing speed is different between cell types and depends on the concentration and activity of licensing proteins as well as chromatin environments.
- Well-distributed origin licensing in G1 phase protects against under-replication in vulnerable genomic regions. Human origin licensing distribution is affected by chromatin environments, but much remains to be learned about licensing distribution mechanisms.

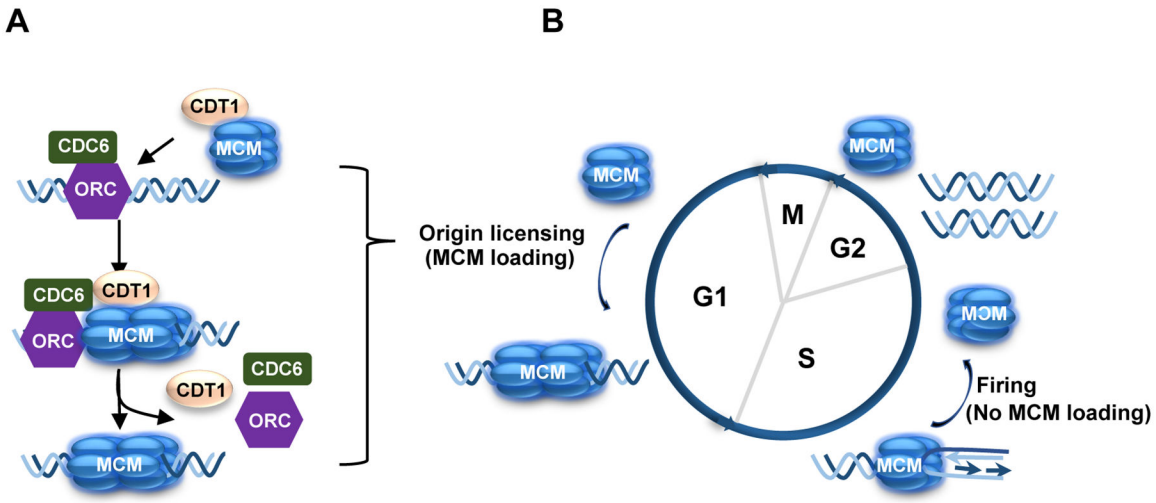

Fig 1. Origin licensing in the cell cycle

(A) Diagram of the sequence of events that occur as each origin is licensed. ORC selects the sites and bind to DNA, CDC6 and CDT1 are recruited, then direct the loading of MCM. (B) Origin licensing starts in late mitosis and continues throughout G1 phase. The loaded MCM complexes become the core of active DNA helicases and unwind DNA and then are unloaded once replication is finished in S phase. After S phase begins, no new origin licensing is allowed.

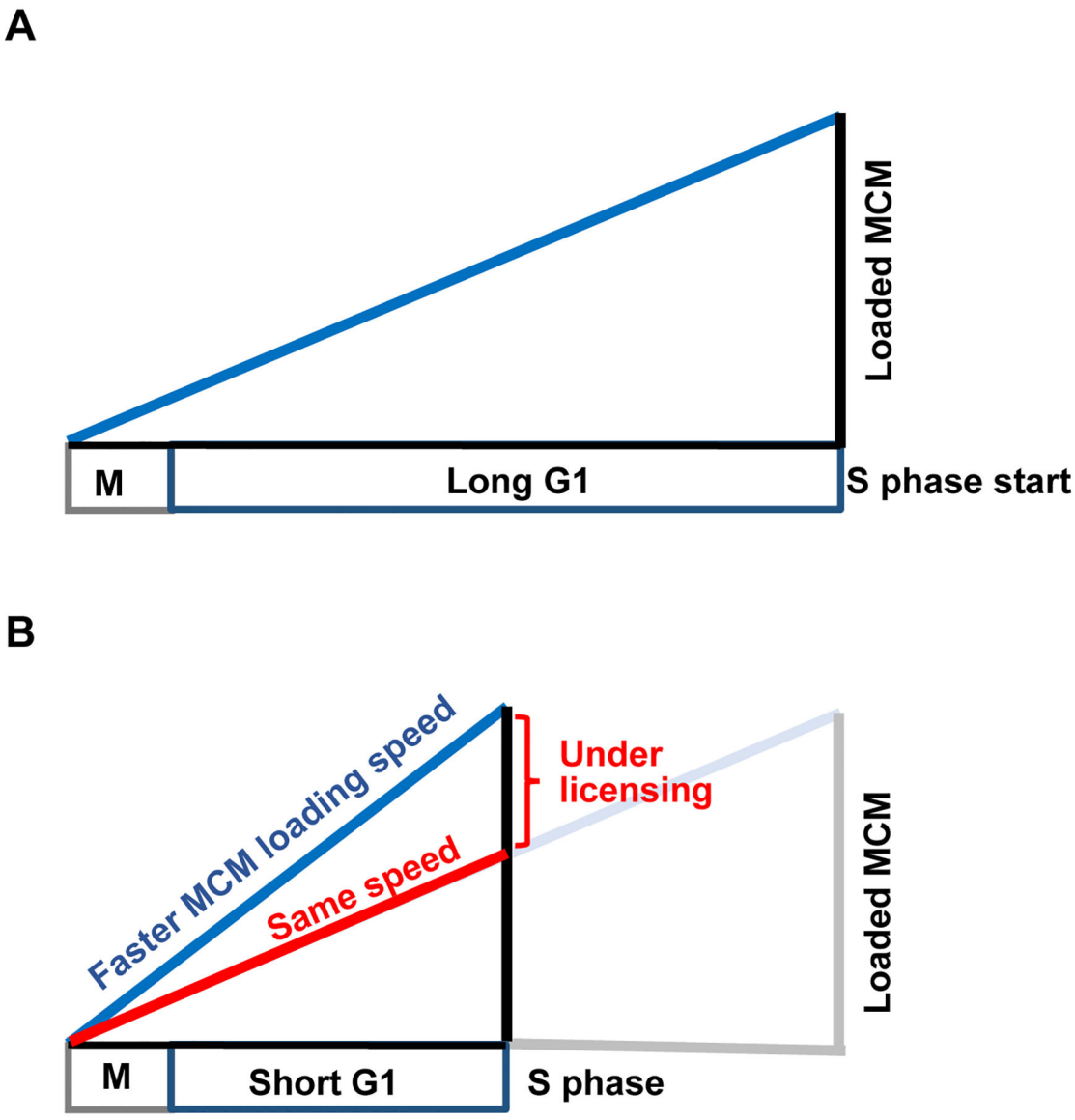

Fig 2. Origin licensing speed

(A) Origin licensing in normal cells. Origin licensing is unidirectional in G1 phase, the loaded MCM complexes won't be unloaded until S phase. (B) Origin licensing in cells with short G1 length. If cells license origins with the same speed as in cells with long G1 phases (indicated with red line), cells will experience under-licensing at S phase entry. If cells license in a short G1, they will avoid under-licensing (indicated with blue line).

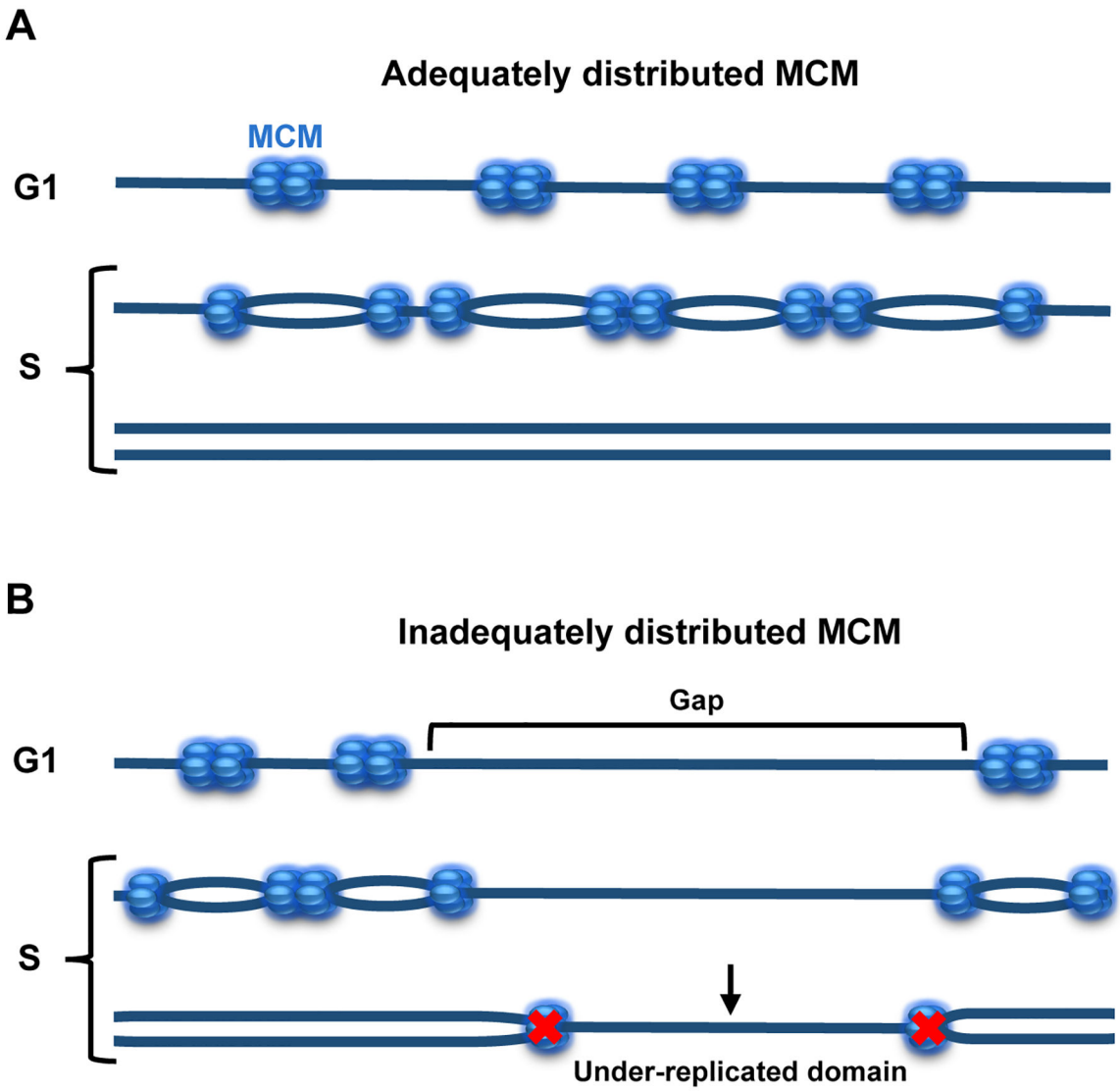

Fig 3. Origin licensing distribution

(A) Adequate distribution of licensed origins (loaded MCM complexes). The DNA replication is successful in the subsequent S phase. (B) Inappropriate MCM loading distribution elevates the risk of under-replication in S phase.