FEBS Letters 586 (2012) 2803–2812

journal homepage: www.FEBSLetters.org

Review

Control over DNA replication in time and space

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ARTICLE INFO

Article history:
Received 13 July 2012
Revised 18 July 2012
Accepted 18 July 2012
Available online 25 July 2012

Edited by Miguel De la Rosa, Felix Wieland and Wilhelm Just

Keywords:
DNA replication
Licensing
Origin
S phase
Replisome

ABSTRACT

DNA replication is precisely regulated in time and space, thereby safeguarding genomic integrity. In eukaryotes, replication initiates from multiple sites along the genome, termed origins of replication, and propagates bidirectionally. Dynamic origin bound complexes dictate where and when replication should initiate. During late mitosis and G1 phase, putative origins are recognized and become "licensed" through the assembly of pre-replicative complexes (pre-RCs) that include the MCM2–7 helicases. Subsequently, at the G1/S phase transition, a fraction of pre-RCs are activated giving rise to the establishment of replication forks. Origin location is influenced by chromatin and nuclear organization and origin selection exhibits stochastic features. The regulatory mechanisms that govern these cell cycle events rely on the periodic fluctuation of cyclin dependent kinase (CDK) activity through the cell cycle.

1. Introduction

Precise spatiotemporal regulation of DNA replication is crucial for the maintenance of genomic integrity. DNA replication in eukaryotic cells initiates from hundreds of sites along the genome, called origins of replication. In mammals, 30,000 to 50,000 origins are activated in each cell cycle. Origin firing, which refers to the unwinding of double stranded DNA at origins of replication, results in the formation of two replication forks at each origin that move in opposite directions. Dynamic multi-protein complexes bind onto origins throughout the cell cycle and govern where and when replisomes catalyzing DNA synthesis will assemble.

In eukaryotic cells, initial DNA unwinding at origins and progressive helicase activity at replication forks require the hetero-hexameric MCM2–7 complex, which forms the core of the replicative helicase. The loading and activation of MCM2–7 complexes are strictly regulated to ensure accurate and complete replication of the genome. These processes are executed in a stepwise manner (Fig. 1). A key initial step is the binding of the origin recognition complex (ORC) onto replication origins. This complex marks all potential origins providing spatial control of origin positioning and is required for the chromatin loading of downstream replication factors. In late mitosis and during G1 phase, ORC is recognized by loading factors that recruit MCM2–7 complexes to origins, forming the pre-Replicative Complex (pre-RC). Following MCM2–7 loading, origins are licensed for a new round of DNA replication. Nevertheless, replication initiation does not occur until the G1/S phase transition. From the G1/S transition and throughout S-phase, initiation factors are recruited to a fraction of licensed origins forming the pre-Initiation Complex (pre-IC). They will activate the MCM2–7 helicases and recruit the replication machinery, contributing to DNA unwinding, formation of Replisome Progression Complexes and migration of replication forks bi-directionally away from the origin. As forks coming in from opposite directions converge, replisomes are disassembled and MCM2–7 proteins released from chromatin. From S-phase onset and throughout S and G2 phases, MCM2–7 helicases cannot re-associate with origins. Licensing will only take place again following passage through mitosis, ensuring that each origin is replicated only once in each cell cycle.

In this review, we outline the key events that take place at origins of DNA replication through the cell cycle of eukaryotic cells. We discuss the regulatory mechanisms that control origin activation in time and space and ensure that all genomic regions are replicated exactly once within the same cell cycle. Finally, we consider the spatiotemporal coordination of DNA replication within the cell nucleus. Aiming to outline key concepts in a concise manner, references to primary work on which concepts are based may be incomplete. For an extensive presentation of the primary literature on each of these topics the reader is referred to a number of comprehensive recent reviews [1–10].

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0014-5793
http://dx.doi.org/10.1016/j.febslet.2012.07.042

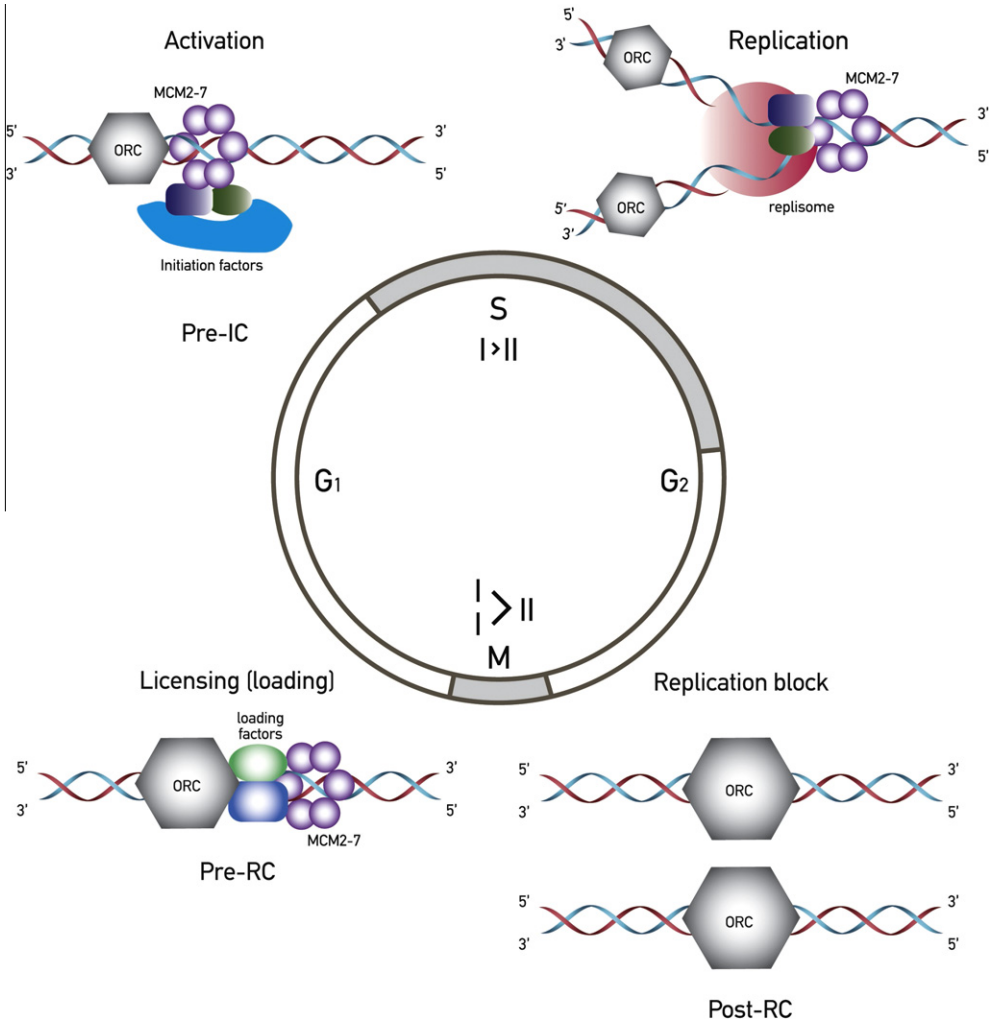

Fig. 1. Assembly of multi-protein complexes onto replication origins determines DNA replication events. Eukaryotic DNA replication starts from multiple sites along the genome, called replication origins. The origin recognition complex (ORC) directly recognizes and binds onto origins marking them throughout the cell cycle. In late mitosis and during G1 phase, the MCM2–7 helicase ring assembles onto origins via loading factors, forming the pre-replicative complex (pre-RC), thereby licensing chromatin for a new round of replication. Following the G1 to S phase transition, several initiation factors are recruited to origins converting the pre-RC to a pre-initiation complex (pre-IC). Subsequently, replication factors are loaded to the pre-IC leading to the assembly of the replisome and initiation of DNA synthesis. MCM proteins move away from origins with the replication fork and de novo licensing is globally inhibited after S-phase onset. Only ORC subunits remain bound to origins forming the post-replicative complex (post-RC).

2. Dynamic origin bound complexes dictate when and where replication should initiate

The initiation of DNA replication is tightly controlled to ensure that each genomic region is replicated fully and only once during each cell cycle. This process is mediated by the ordered transitions of origin bound complexes and is divided into two discrete steps. The first step, referred to as "origin licensing", takes place from late mitosis and through G1 phase and involves the formation of a multiprotein complex, the pre-replicative complex (Pre-RC), on origins of replication. During the second step, which occurs following the G1-S transition, the pre-RC becomes activated leading to the formation of the replisome and the firing of origins (reviewed in [3,6,7]). Key factors and mechanisms are highly conserved across eukaryotes and mechanistic insight has largely benefited from biochemical studies in Xenopus extracts and a combination of genetic and biochemical studies in budding yeast. We will present current knowledge based on the budding yeast paradigm (Fig. 2, left panel) and discuss specific aspects of the process as it emerges for higher eukaryotes (Fig. 2, right panel).

2.1. Licensing DNA for a new round of replication: formation of Pre-replicative complexes during G1

Pre-RC formation occurs in G1 phase in a stepwise manner that results in the loading of MCM2–7 helicases onto origins of DNA replication, but in an inactive state. The MCM proteins were initially identified during genetic screens for mutants that affected plasmid maintenance in yeast. MCM2 to MCM7 are six sequence-related AAA + type ATPases/helicases, which form a hetero-hexameric ring. The ring is believed to encircle DNA and functional ATPase sites generated between adjacent subunits, as in related helicases [11].

The first step of licensing involves the binding of the Origin Recognition Complex (ORC) to the origin in an ATP-bound state. ORC consists of six subunits (ORC1–ORC6) and its binding to DNA is required for the recruitment of the Cdc6 ATPase and Cdt1. Both Cdc6 and Cdt1 are necessary for the subsequent association of the MCM2–7 helicases onto origin DNA. In budding yeast, Cdt1 and MCM2–7 form a stable complex and are recruited concomitantly onto origin DNA. Interactions of Cdt1 with Orc6 are important for

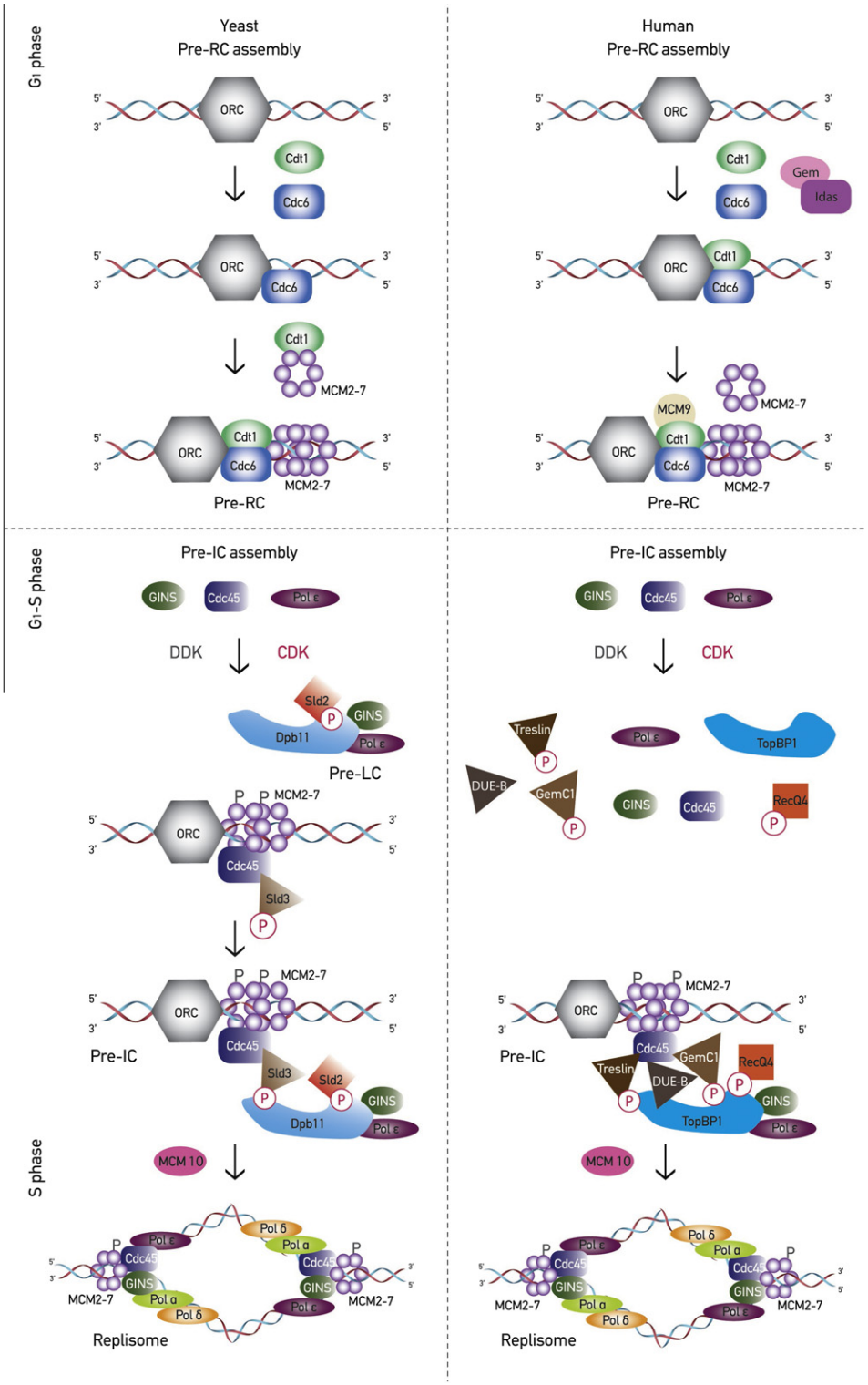

Fig. 2. DNA replication licensing and initiation steps in budding yeast and human cells. Licensing of origins constitutes a conserved process in all eukaryotes that occurs in late mitosis and during G1 phase. It involves the ordered assembly of pre-replicative complexes (pre-RCs) comprising the origin recognition complex (ORC), Cdt1, Cdc6 and MCM2–7 helicases. In higher eukaryotes, licensing is negatively regulated by Geminin. At the G1 to S phase transition, the concerted actions of cyclin-dependent kinases (CDK) and Dbf4-dependent kinase (DDK) activate the pre-RCs converting them into pre-initiation complexes (pre-ICs), through the recruitment of additional initiation factors. In both yeast and human, DDK phosphorylates predominantly the MCM2–7 helicases. In budding yeast, Sld2 and Sld3 constitute the major CDK targets which interact with Dpb11 and lead to the recruitment of Cdc45 and GINS onto pre-RCs. In higher eukaryotes, putative functional orthologues of Sld2 (RecQ4), Sld3 (Treslin and possibly GemC1 and DUE-B) and Dpb11 (TopBP1) have been characterized. Although the manner by which pre-ICs are formed may differ among species, in both yeast and higher eukaryotes these reactions enable the assembly of an active Cdc45/MCM2–7/GINS (CMG) helicase. Additional replication factors are recruited to the CMGs, leading to the formation of two sister replisomes at each origin, which proceed bidirectionally along the DNA. The proposed steps are based primarily on studies in budding yeast and analogies drown for human cells based on finding in higher eukaryotes. Please refer to the text for a detailed description.

this recruitment step [12]. ATP hydrolysis by Cdc6 stimulates the stable loading of the MCM2–7 complex onto chromatin, which is accompanied by the dissociation of Cdt1 and Cdc6 [13]. Moreover, ATP hydrolysis by the ORC complex is necessary for the reiterative loading of MCM2–7 complexes to origins. In vitro reconstitution of pre-RC formation using purified budding yeast proteins coupled to electron microscopy revealed that MCM2–7 proteins are loaded as double hexamers at origins of replication [14,15]. The two hexamers are arranged in a head-to-head orientation and are connected via their N-terminal rings. Moreover, the MCM2–7 double hexamers encircle double stranded DNA and are able to slide along it [14,15]. The presence of two Cdt1 binding sites on ORC is consistent with cooperative loading of two MCM2–7 hexamers delivered by two Cdt1 molecules [12]. Taken together, these data suggest that licensing involves conformational changes at origin bound complexes which result in loading of two closed MCM2–7 rings around double stranded DNA, facing in opposite orientations, but catalytically inactive.

The mechanism of origin licensing appears conserved in higher eukaryotes [6,7] (Fig. 2, right panel). For example, a recent study provided evidence for the loading of MCM2–7 double hexamers onto sperm chromatin in Xenopus egg extracts [16]. Additional levels of control may however exist. The G1 phase lasts several hours in most mammalian cell types. Both ORC subunits and Cdt1 have been shown to bind dynamically onto chromatin throughout G1 in mammalian cells [17,18] while MCM proteins bind stably [19], suggesting that MCM loading takes place throughout G1. Reiterative licensing through G1 may permit additional control points [20]. Origin selection and decisions on the timing of origin firing within a given chromatin region are both established during the G1 phase in mammalian cells, and reiterative licensing following these decision points may be important to ensure that multiple licensed origins exist in each genomic region. In metazoa, licensing is also negatively regulated by Geminin, a small coiled-coil protein which binds onto Cdt1 and inhibits MCM loading [21,22]. Transitions between active and inactive Cdt1-Geminin complexes have been suggested to regulate timely licensing [23]. An additional protein with homology to the Geminin coiled-coil was also recently identified and named Idas [24]. Idas, which is present in vertebrates, is essential for correct execution of the cell cycle and was shown to associate with Geminin and inhibit Cdt1-Geminin interactions, suggesting the possibility of an additional layer of regulation in higher eukaryotes [24]. MCM2–7 loading to chromatin was also shown to require another MCM-like protein, MCM9, which positively regulates licensing by binding to Cdt1 and preventing its association with Geminin [25]. MCM9 was however recently shown to function in a complex with MCM8 during homologous recombination [26–28] and additional studies will be required to clarify its function during licensing.

2.2. Initiating DNA replication: activation of Pre-RCs and replisome assembly

The activation of the MCM2–7 helicase occurs following the G1-S transition in situ at origins. The inactive double MCM2–7 hexamer which is loaded onto double stranded DNA during licensing must be converted to an active processive helicase. Recent studies indicate that the replicative helicase translocates along single stranded DNA moving 3′ to 5′ on the leading strand template [29]. Therefore DNA replication initiation would require local unwinding of the double helix and extrusion of the lagging strand template possibly through a transient opening in the MCM2–7 ring. It may also entail dissociation of the double head-to-head hexamer into opposite moving helicases. These events must be carefully controlled in time and space. The action of two kinases, Cyclin Dependent Kinase (CDK) and Dbf4-Dependent Kinase (DDK), triggers a cascade of interactions among different auxiliary factors that results in the recruitment of Cdc45 and GINS and the formation of a transient pre-initiation complex (Pre-IC), which is necessary for the activation of the MCM2–7 complex and the loading of replication factors to form the replisome.

Both Cdc45 and GINS interact with MCM2–7 proteins and are required for initiation as well as for the progression of DNA replication forks. The recruitment of Cdc45 at an origin of replication depends on its temporal program of activation, which implies that Cdc45 may serve as an indicator of origin firing [30,31]. GINS comprises a heterotetrameric complex that consists of four small proteins (Psf1, Psf2, Psf3 and Sld5) initially identified in budding yeast and Xenopus egg extracts [32,33]. GINS is required for the maintenance of a stable interaction between Cdc45 and the MCM2–7 complex [34]. Moreover, GINS interacts with polymerase α and triggers its polymerase activity [35].

In eukaryotic cells, the MCM2–7 proteins constitute the helicase "catalytic core", although they have weak unwinding activity in vitro [36]. Several lines of evidence indicate that the helicase activity of MCM2–7 complex gets induced upon its interaction with Cdc45 and GINS, which leads to the formation of the CMG complex (Cdc45/MCM2–7/GINS). These three factors were co-isolated at sites of a circular DNA plasmid in Xenopus egg extracts where fork progression was paused using a streptavidin–biotin complex [37]. The components of the CMG complex were co-purified from Drosophila embryo extracts. This complex was shown to exhibit ATP-dependent helicase activity in vitro, since it could displace a 40 nt oligonucleotide annealed to a single-stranded plasmid [38]. Biochemical experiments using reconstituted Drosophila MCM2–7 and CMG complexes provided direct evidence for the activation step of MCM2–7 complex following its association with Cdc45 and GINS. Parallel comparison of helicase activities of the two complexes revealed that the CMG complex exhibits a higher rate of ATP hydrolysis and improved DNA affinity relative to the MCM2–7 complex [39]. The mechanism through which Cdc45 and GINS activate the MCM2–7 complex was explored in a structural study that used single particle electron microscopy [40]. It was shown that the MCM2–7 complex exists in two different conformations, a spiral lock-washer state and a planar gapped-ring state with a gap between the MCM2 and MCM5 subunits. On the contrary, the CMG complex adopts only a closed conformation, which implies that CDC45 and GINS may activate the MCM2–7 helicase complex by bridging the gap between the MCM2 and MCM5 subunits [40].

The recruitment of Cdc45 and GINS to origins as well as the formation of the CMG complex has been extensively studied in budding yeast (Fig. 2, left panel). This process requires CDK activity and depends on several auxiliary factors including Sld2, Sld3, Dpb11 and polymerase ε, which contribute to the assembly of a pre-initiation complex (Pre-IC) [1,5]. The majority of these factors are not incorporated into the replication fork. Dpb11 has two pairs of tandem BRCT repeats, via which it binds to phospho-proteins [41]. The C-terminal pair of BRCT repeats binds to phosphorylated Sld2 and the N-terminal pair binds to phosphorylated Sld3. Sld2 and Sld3 constitute the principal targets of CDK activity during the initiation of DNA replication [42,43]. These two factors were identified in a screen for mutants synthetically lethal with Dpb11 [44,45]. Sld3 has been shown to form a complex with Cdc45 [45]. Following the onset of DNA replication, Sld3 does not migrate with the replication fork but it dissociates from origins [46]. A recent study revealed that Sld3 competes with GINS for binding to MCM2–7 and Cdc45 [47]. In this regard, Sld3 may deliver Cdc45 to origins. Upon entry into S phase, GINS may induce the displacement of Sld3 from Cdc45 and MCM2–7, enabling the assembly of the CMG complex [47].

How do these factors associate with Pre-RCs? According to the current model describing the assembly of the pre-IC in budding

yeast, Sld3 interacts with Cdc45 and both proteins are recruited to early firing origins in G1 and to late firing origins during S phase, in a mutually dependent way [45]. The binding of Dpb11 to phosphorylated Sld2 triggers the assembly of a transient pre-loading complex (Pre-LC), composed of Dpb11, Sld2, polymerase ε and GINS [1,48]. At the same time, following phosphorylation by CDK, Sld3 associates with Dpb11. In this model, Dpb11 functions as a scaffold, bridging phosphorylated Sld2 and phosphorylated Sld3, which results in the delivery of GINS to origins. In addition to Dpb11, DNA polymerase ε appears to be essential for the assembly of the Pre-LC, since it is required for the binding of GINS to the Sld2–Dpb11 complex. Taken together, CDK activity promotes the formation of the pre-LC, through which GINS is recruited to pre-RCs. Upon initiation of DNA replication, Sld2, Sld3 and Dpb11 do not participate in the replisome, whereas Cdc45, GINS and MCM2–7 assemble into an active helicase complex [1,48]. An additional evolutionarily conserved factor, MCM10, participates in the conversion of the pre-RC to a replisome and is required for the recruitment of DNA polymerase α to origins and activation of the CMG complex [49–51].

The exact process by which pre-RCs are converted to pre-ICs in higher eukaryotes is not yet fully clarified (Fig. 2, right panel), mainly due to the difficulty in retrieving orthologues of key replication proteins such as Sld2 and Sld3. Dpb11 orthologues containing BRCT repeats have been identified in metazoans (TopBP1/Cut5/Mus101) [52]. The vertebrate orthologue of budding yeast Sld2 is proposed to be the RecQ4 DNA helicase [53,54]. It has been shown that RecQ4 is necessary for the initiation of DNA replication and is able to interact with TopBP1 via its N-terminus which shows sequence homology to Sld2 [53,54]. However, unlike Sld2, RecQ4 binding to TopBP1 is not mediated by CDK phosphorylation, implying that RecQ4 may not constitute a major CDK target [53]. Furthermore, RecQ4 appears not to be required for CMG assembly in Xenopus [53,54]. Interestingly, human RecQ4 has been reported to interact directly with MCM10 as well as with the CMG complex. The interaction between MCM10 and RecQ4 is regulated by CDK-mediated phosphorylation and likely triggers RecQ4 helicase activity [55]. Recently, three different proteins, Treslin/Ticrr (TopBP1-interacting, replication-stimulating protein/ TopBP1-interacting, checkpoint and replication regulator) [56,57], GemC1 (Geminin coiled-coil-containing potein 1) [58] and DUE-B (DNA unwinding element) [59], were characterized as putative functional orthologues of Sld3 in vertebrates. All of these proteins interact with TopBP1 and are required for the association of Cdc45 with chromatin. However, only Treslin's interaction with TopBP1 is positively regulated by CDK phosphorylation and Treslin exhibits sequence homology to Sld3, making it likely that is constitutes the Sld3 orthologue [60,61]. The function of GemC1 and DUE-B at origin bound complexes remains to be elucidated. It is intriguing that GemC1 possesses a coiled-coil similar to Geminin's coiled-coil [58] and its closest paralogue is Idas [24].

In addition to CDK, a second kinase, DDK is required for the initiation of eukaryotic DNA replication across evolution. DDK is comprised of the Cdc7 serine/threonine kinase in association with the regulatory subunit Dbf4. DDK gets activated at the onset of S phase, since Dbf4 is absent in G1 and accumulates in S phase [7]. Both in vitro and in vivo studies indicate that the MCM2–7 complex is the major target of DDK [5]. Instead of acting as a global modulator, DDK appears to act locally at individual origins, which is consistent with its activity being limiting in cells [62–64]. In line with this observation, Cdc7 is recruited to origins through Dbf4 interactions with several pre-RC components, including MCM2, MCM4, ORC2 and ORC3 [65–67]. Moreover, Cdc7 has been shown to preferentially phosphorylate MCM2–7 complexes that are stably bound to chromatin and promote their association with Cdc45 [66,68,69]. DDK has been shown to act prior to CDK for activating origins of replication in Xenopus [70,71] and more recently in budding yeast [72].

The N-terminal regions of MCM2, MCM4 and MCM6 constitute the prime phosphorylation substrates of DDK [5]. The functional significance of MCM2–7 phosphorylation mediated by DDK has been predominantly established by analyzing the N-terminal region of budding yeast MCM4 subunit [66,73,74]. This region contains a "DDK docking domain" through which Cdc7-Dbf4 is recruited and phosphorylates adjacent phosphoacceptor sites [66]. A similar "DDK docking site" has been found to exist in the N-terminus region of budding yeast MCM2 [75]. Moreover, the hyperphosphorylated form of MCM4 is considerably enriched in the Cdc45–MCM2–7 complex, which implies that the MCM2–7 phosphorylation is likely to be required for the stable association of MCM2–7 with Cdc45. Interestingly, the exact sequence of the N-terminal region of MCM4 appears not to be important for its phosphorylation by Cdc7, since it can be substituted by the corresponding N-terminus of MCM2 [66]. In a more recent study, it was reported that phosphorylation of MCM4 by Cdc7 suppresses an inhibitory activity of the N-terminal region of MCM4 subunit [74]. The exact mechanism through which the phosphorylation of MCM2–7 complex by DDK activates the MCM2–7 helicases remains elusive. It is very likely that this modification generates a conformational change in MCM2–7 complex, enabling it to function as a helicase. This possibility is supported by the characterization of a specific mutation in MCM5 subunit in budding yeast, the *mcm5-bob1,* which can bypass the requirement for Cdc7-Dbf4 in initiation of DNA replication [76,77]. Studies of archaeal MCM complexes propose that *mcm5-bob1* mutant may mimic a conformational change in the MCM2–7 complex that is normally induced by Cdc7-Dbf4 phosphorylation [78]. This conformational change may facilitate association with other replication factors such as Cdc45, local unwinding of double stranded DNA or dissociation of the double head-to-head MCM2–7 hexamer into single hexamers able to move away with replication forks.

In summary, although the proteins and the detailed mechanisms involved in the assembly of Pre-IC and the firing of origins differ in metazoans from unicellular eukaryotic organisms, both systems exhibit conserved features. In all eukaryotes, initiation of DNA replication requires a protein (Dpb11/Cut5/Rad4/TopBP1) that serves as a scaffold for the recruitment of auxiliary factors (Sld2 and Sld3/Treslin/Tcirr) that mediate the loading of Cdc45 and GINS to MCM2–7 complex at origins. The interaction between the scaffold and the auxiliary factors is stimulated by CDK activity, thereby ensuring that this process is triggered after the completion of G1 phase. Moreover, activation of Pre-RCs requires the DDK mediated phosphorylation of MCM2–7 complex, which contributes to the assembly of a progressive CMG complex on origins, in all species.

3. Cyclin dependent kinases govern transitions of origin bound complexes, ensuring once per cell cycle replication

It is vital for cells to be able to distinguish which parts of their genome have been replicated and which not at every point in time and everywhere along the genome, in order to prevent reinitiation events from occurring during the same cell cycle. It is now well established that CDKs globally orchestrate transitions at origin bound complexes regulating licensing and initiation events to ensure that each origin is fired only once per cell cycle. The CDK cycle is intricately linked to cell cycle specific proteolysis through the Anaphase Promoting Complex/Cyclosome (APC/C) and co-operates with DDK to bring about timely DNA replication (Fig. 3).

CDKs play a pivotal role in driving progression through the cell cycle in all eukaryotic cells. CDK activity oscillates during the cell

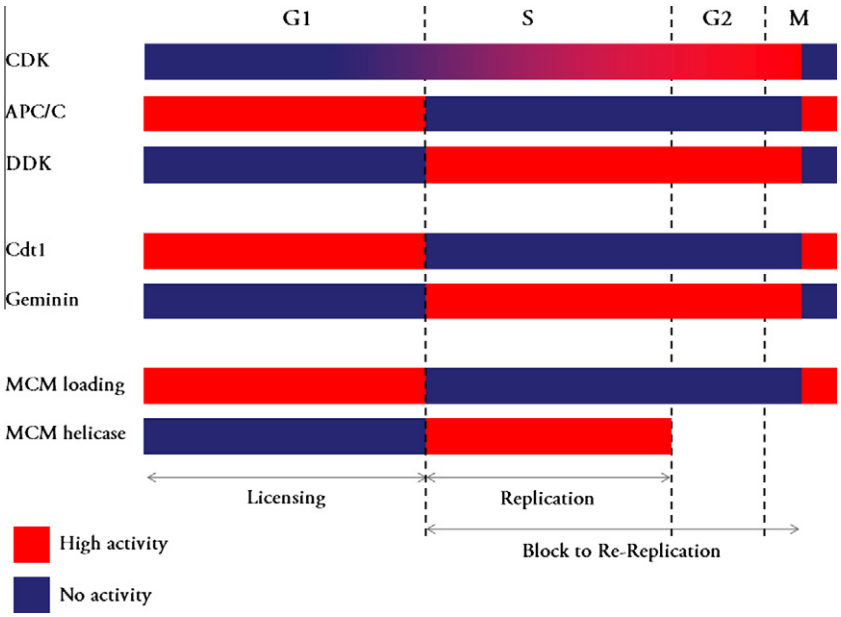

Fig. 3. Once per cell cycle replication in higher eukaryotes is regulated by cyclin-dependent kinases (CDK), assisted by the Dbf4-dependent kinase (DDK) and the anaphase promoting complex/cyclosome (APC/C). CDK activity is low in G1 phase (blue) permitting DNA replication licensing (MCM loading). As CDK levels rise at the G1/S transition, origin firing is initiated (MCM helicase activation) and licensing is globally inhibited (block to rereplication), until CDK levels drop again at the end of mitosis. Cdt1 and Geminin are crucial regulators of licensing in metazoa. The replication factor Cdt1 is essential for loading MCM2–7 complexes onto replication origins and is inhibited by Geminin. Cdt1 accumulates specifically during G1 phase while Geminin is absent during G1 and accumulates in S and G2.

cycle, dictating the pace of cell cycle transitions. The activity of CDKs depends on their binding to members of the cyclin protein family. While several different cyclin-CDK complexes are formed during the cell cycle, each with its own substrates, it appears that it is the total level of available CDK activity, rather than the presence of specific CDK complexes, which dictates substrate choice and governs cell cycle transitions [79,80]. Total CDK activity is low during the G1 phase, due to the low levels of cyclins and the presence of CDK inhibitors (Fig. 3). As G1 phase progresses, cyclin levels increase, promoting a gradual increase in the catalytic activity of CDKs. DNA licensing can take place only from the end of mitosis to the end of G1 phase, when CDK activity is low. During this period, CDK levels are not sufficient to promote pre-RC activation, and thus initiation of DNA replication is prevented. At the G1/S transition, CDK activity rises past the threshold for S-phase onset, inducing firing of origins and promoting initiation of DNA replication. CDK levels over the G1/S transition threshold prevent further Pre-RC assembly. Pre-RC assembly and activation are therefore restricted to different phases of the cell cycle. CDK activity continues to rise during S and G2 phases and a sharp rise in CDK activity at the G2/M transition marks entry into mitosis. Pre-RCs can reassemble only after cells exit mitosis, when CDK activity drops again to reset the cell cycle. This mechanism ensures that no initiation of DNA replication will occur before cell division is completed.

High levels of CDK activity during S, G2 and M phases inhibit origin licensing by targeting multiple Pre-RC components. This ensures that MCM2–7 loading to origins occurs only in a strict time window from late mitosis to late G1 phase, when DNA replication cannot initiate. Different organisms have developed a wide range of mechanisms for regulating Pre-RC formation and multiple overlapping mechanisms operate in every cell, ensuring that rereplication is prevented at every origin. In budding yeast, CDKs block the Cdt1/MCM2–7 complex by triggering its export to the cytoplasm [81], while phosphorylation of Cdc6 results in its SCF mediated proteolysis [82–84]. Moreover, CDK phosphorylates ORC2 and ORC6 subunits. ORC phosphorylation inhibits MCM2–7 loading to origins by preventing the interaction between Cdt1 and ORC6 [12].

In metazoans, inhibition of Cdt1 activity constitutes a fundamental regulatory mechanism through which relicensing is blocked (Fig. 3). Cdt1 accumulates in G1 phase, while upon entry into S phase, human Cdt1 is rapidly targeted for degradation by the independent action of two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4-Cdt2 [85,86]. The Cdt1 interaction with Skp2 is dependent on the prior phosphorylation of Cdt1 by CDK2 [87,88] and is believed to globally regulate Cdt1 levels in the nucleus during both S phase and G2 [85]. The targeting of Cdt1 for ubiquitination dependent proteolysis by Cul4-Ddb1-Cdt2 requires Cdt1 recruitment to chromatin during S phase through its interaction with Proliferation Cell Nuclear Antigen (PCNA) [85,89]. It is therefore linked to ongoing replication. To ensure tight control over licensing, metazoans have implemented an additional mechanism for the negative regulation of Cdt1, Geminin [21]. Geminin binds tightly to Cdt1, thereby blocking its ability to load MCM2–7 onto chromatin [22]. Geminin is expressed during S and G2 phases and is degraded by the anaphase promoting complex/cyclosome (APC/C) at the metaphase to anaphase transition [21] (Fig. 3). Downregulation of Geminin during late mitosis and G1, when APC/C is active, releases Cdt1 allowing its participation in the licensing process exclusively during this period. Thus, the combined action of E3 ubiquitin ligases and Geminin ensures that Cdt1 is not active during S and G2 phases, resulting in the prevention of origin relicensing. Ectopic expression of Cdt1 or downregulation of Geminin leads to rereplication in human cells, underlining the importance of a tight control over Cdt1 for maintaining genome stability. Apart from Cdt1 downregulation, several other components of the Pre-RC are directly inhibited by CDK-mediated phosphorylation. Upon entry into S phase, the major fraction of Cdc6 gets phosphorylated by CDK and is excluded from the nucleus, while a small fraction of the protein remains bound to chromatin throughout S phase [90–96]. Finally, after the onset of S phase, ORC activity is reduced predominantly due to the phosphorylation of the ORC1 subunit, which reduces its affinity for chromatin [97] and/or promotes its degradation [98]. Furthermore, ORC1 phosphorylation by CDKs permits its export to the cytoplasm [99].

In addition to inhibiting relicensing of replication origins outside of G1 phase, CDKs positively regulate initiation of DNA replication by activating pre-RCs at the G1/S phase transition. CDKs promote the assembly of pre-ICs and the loading of Cdc45 and GINS to origins, which are necessary for origin firing. In budding yeast, phosphorylation of Sld2 and Sld3 generates binding sites for Dpb11 (see also Section 2.2 above). Interestingly, it has been shown that, although only Thr84 mediates Sld2 binding to Dpb11, prior phosphorylation of other sites is also required. It seems likely that CDK-mediated phosphorylation of Sld2 causes conformational changes that facilitate the exposure of Thr84 to CDKs. The existence of such a mechanism may set a high threshold for CDK activity to trigger initiation of DNA replication [100]. This, in turn, ensures that there is enough time for Pre-RC components to get phosphorylated and be inhibited before firing of origins. In metazoans, phosphorylation of Treslin/Ticrr by CDKs promotes its binding to TopBP1, which is required for Cdc45 loading to origins [56].

Thus, the periodic fluctuation in CDK activity dictates proper progression through cell cycle events. CDK activity both induces initiation of replication at the G1/S transition and inhibits any reinitiation events from taking place by blocking Pre-RC formation. Furthermore, CDKs act by multiple mechanisms to block pre-RC assembly, in all eukaryotic systems. Given the importance of inhibiting relicensing and thus rereplication of DNA within the same cell cycle, this multilayer regulatory mechanism provides the cells with overlapping, partly redundant control points to safeguard genomic integrity.

4. Replication is spatially organized within the cell nucleus

We have so far considered the ordered transitions of multi-protein complexes which regulate replication initiation at a given origin of replication. Hundreds of thousands of putative origin sites however exist in higher eukaryotic genomes and thousands of these will be selected and will fire at different times during S phase in each cell. Origin selection and timing of origin activation is influenced by DNA sequence, chromatin structure, transcription and nuclear positioning and is coordinated with other origin sites along the genome. It also exhibits a high degree of stochasticity.

Origin specification differs considerably across evolution and DNA sequence influences origin location to a different extent in different organisms. Budding yeast origins constitute sequence-specific sites referred to as autonomously replicating sequences (ARSs). These sites share a highly conserved 12 bp consensus sequence (ARS consensus sequence, ACS), which is required for the recruitment of the ORC [101,102]. A second sequence adjacent to the ACS is also necessary for origin activity [103]. In contrast to budding yeast, the location of replication origins in other eukaryotes appears less well determined. In fission yeast, origins are located predominantly in intergenic regions that are rich in A and T residues [104]. This feature can be attributed to the fission yeast ORC4 subunit, which contains AT-hook motifs that bind to short AT stretches [105]. Similar to fission yeast, no specific sequence elements have been revealed for metazoan origins. In Drosophila and Xenopus early embryos, any genomic region can serve as an origin without any sequence characteristics required [106,107], while in human and mouse, initiation of DNA replication has been shown to correlate well with unmethylated CpGs islands often present at promoter regions, and genomic regions that are rich in actively transcribed genes [108–110].

Chromatin structure and modifications affect origin selection and origin activation. Several studies suggest that nucleosome positioning at replication origins significantly inhibits the association of ORC with chromatin. Indeed, in budding yeast, ORC localizes in nucleosome free genomic regions [111,112], while in metazoans, AT-rich intergenic regions, which are preferred sites for ORC binding, exhibit lower nucleosomal occupancy [113]. Furthermore, ORC associates predominantly with open chromatin structures. In line with this notion, acetylation of histone tails has a positive impact on Pre-RC assembly. The histone deacetylase (HDAC) of budding yeast, Sir2, prevents MCM association with some origins by inducing unfavorable positioning of nucleosomes [114]. In addition, acetylation of H3 and H4 histones facilitates DNA replication of a plasmid [115]. In human cells, the histone acetyltransferase (HAT) Hbo1, has been shown to interact with Cdt1 and to be required for licensing of replication origins [116,117]. Recently, Orc1 was shown to bind specifically to histone H4 dimethylated at lysine 20 [118] suggesting that histone methylation may directly affect origin selection. Chromatin structure also appears to affect the timing of replication, with euchromatic, transcriptionally active regions replicating early in S phase while heterochromatic regions replicate late. Direct interactions of replication proteins with chromatin components may also affect the timing of DNA replication: in fission yeast, Swi6, an orthologue of heterochromatin protein 1 (HP1), was shown to recruit DDK to heterochromatic loci, allowing origin firing in early S phase [119]. Interestingly, HP1 has also been shown to interact with ORC in Drosophila, Xenopus and human cells [6]. It seems likely that chromatin structure regulates DNA replication by controlling the accessibility of origin DNA to both licensing and replication factors. However, while an open chromatin structure in actively transcribed genomic regions positively correlates with origin activity, ongoing transcription of a given gene suppresses initiation within the transcription unit [120], possibly through displacing pre-RC complexes. Transcription therefore both renders chromatin accessible to replication factors at a given locus and restricts origin usage to non-transcribed segments of the locus.

Nuclear positioning is also intricately linked with origin specification, replication timing and S phase progression. Eukaryotic chromosomes occupy distinct territories within the cell nucleus and the subnuclear topology of a given chromosomal region affects its replication [9]. Chromosomal territories are established early in the G1 phase, and their establishment is causally linked with the decision of when a given chromosomal domain will be replicated during S phase (Timing Decision Point) [9]. DNA replication itself is also highly spatially organized within the nucleus. Ongoing DNA replication can be visualized within the cell nucleus as discrete focal sites called ‘‘replication foci’’. It is estimated that each focus contains 10–100 active origins which fire synchronously in clusters. Live-cell imaging of mammalian cells has shown that these replication foci exhibit distinct spatial distribution as the cell progresses through S phase. Upon entry into S phase, numerous small foci are distributed throughout the cell nucleus. In middle S phase, replication foci become larger and are concentrated around the nucleoli and at the nuclear periphery. Finally, in late S phase, replication foci appear even larger and fewer. The organization of DNA replication into discrete replication foci offers several advantages. Nuclear sub-compartmentalization of DNA replication could contribute to the sequestration of replication factors that may be limiting for this process into replication foci [4]. Moreover, clustering of origins can provide the cells with the possibility of regulating the initiation events at two levels: within a cluster as well as among different clusters. For example, when replication forks are stalled due to DNA damage, neighboring dormant origins within the same cluster get activated ensuring complete DNA duplication. At the same time, checkpoint activation suppresses or delays the initiation of replication at unreplicated late-firing clusters [2].

Monitoring the dynamics of the assembly and disassembly of DNA replication factories by live-cell imaging has provided useful

information about how DNA synthesis progresses in the cell nucleus. These studies revealed that new replication factories are formed adjacent to the ones that recently disassembled [121,122]. These observations have led to the proposal of the "domino model" according to which the completion of replication of a specific genomic region triggers the initiation of replication of a neighboring region. A possible explanation for this model is that DNA replication induces local de-condensation and relaxation of chromatin making it more accessible to replication factors [4].

Though the decision of which origin will fire and when is affected by many features including sequence, chromatin structure and nuclear topology, it also entails a high degree of stochasticity [104,123]. Of the many putative origins along the genome, only a subset will get selected to fire in each cell in a population, apparently at random. Different cells in a population will therefore activate a different set of origins at every cell division. Replication timing is also affected by stochastic origin activation, with efficient origins firing on average earlier in S phase than inefficient ones [123]. Stochastic origin selection, though a source of uncertainty, provides a clear benefit: many putative origins exist, which can be called upon when needed [104]. For example, under conditions of DNA replication stress, when active forks stall, the presence of ready-to-fire dormant origins is crucial for maintaining genome integrity [2].

5. Perspectives

Findings in the last decade have allowed detailed insight into the processes which take place at replication origins to ensure that the genome is accurately copied at every cell division. Recent methodological advances, including the establishment of cell-free systems in yeast able to reproduce the licensing and initiation steps in vitro [15,72], are expected to permit a comprehensive description of the molecular interactions leading to origin firing. In parallel, genome-wide analyses combined with modeling and in silico analysis are beginning to offer a view of the progression of replication across complete genomes (see for example [124]). Several key questions however remain. How is replication organized within the cell nucleus? How is activation of origins at different sites coordinated to ensure complete replication under different conditions and how does it respond to external and internal signaling cues? How is replication linked to transcription and the establishment of cell fate during development? And how is DNA replication linked to other nuclear processes such as DNA repair? DNA replication must be closely linked with DNA damage responses (DDR) to ensure genome stability and the factors discussed in this review are likely to link the two processes. For example, Cdt1 is recruited to sites of damage [125] and specifically degraded in damaged cells [126] and has been suggested to link DNA licensing to the DDR. Methods enabling us to study DNA replication at the single cell level within the context of the living cell will hopefully permit a more complete understanding of controls acting over DNA replication in time and space.

Aberrations in the controls acting over DNA replication in time and space are linked to disease (reviewed in [6],[10]). Both under- and over-replication lead to DNA replication stress, genomic instability and predispose for cancer [127,128]. For example, a hypomorphic mutation in MCM4 leads to breast cancer in the mouse [129], while Cdt1 ectopic expression predisposes for cancer [130] and is an early event during carcinogenesis, which is linked to aneuploidy in cancer specimens [128,131,132]. Cancer cells appear to respond differently to licensing system aberrations in comparison to normal cells, suggesting that drugs targeting licensing factors may provide cancer-cell specific treatments [127,128]. In addition to links with cancer, aberrations in licensing have been linked to growth retardation. Inherited mutations in pre-replication complex components lead to primodial dwarfism syndromes [133–135] suggesting that correct licensing is essential for developmentally regulated growth. Understanding how the spatio-temporal control of DNA replication is brought about in different cell types through the use of animal models will hopefully allow insight into both the maintenance of genome integrity and how this is linked to growth and development.

Acknowledgments

Work in the ZL lab is supported by the Association for International Cancer Research and the European Research Council.

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