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Pulling the Trigger to Fire Origins of DNA Replication

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1. Introduction

DNA replication is a fundamental aspect of cell biology. The process is essential for chromosome doubling and segregation during cell division. Additionally, the DNA replication program can be manipulated to allow a reduction in ploidy as occurs during meiosis or an increase in ploidy as observed in endo-cycles during some developmental processes [1]. The importance of the integrity of the chromosome duplication process is inherently obvious. In somatic cells failure to replicate prevents cell division or leads to a catastrophic reductional division and cell death. Less drastic defects in DNA replication can appear as problems leading to gene amplification, chromosome breaks or chromosome missegregation [2]. These can manifest as birth defects or increased susceptibility to cancer [3]. The integrity of the DNA replication process is ensured partly by DNA repair mechanisms and checkpoint controls. However, the primary mechanism that safeguards the DNA replication process is the complex and multi-step process that leads to the assembly and activation of an active replication complex at chromosomal origins of DNA replication.

The assembly and activation of DNA replication complexes on eukaryotic chromosomes is critically dependent upon two cell cycle regulated protein kinase complexes; Cyclin Dependent Kinase (CDK) and Dbf4 Dependent Kinase (DDK). These protein kinases phosphorylate multiple protein substrates that play roles in assembling a replisome through promoting specific protein-protein interactions that recruit essential components to the complex and stabilize the assembled complex. Additionally, CDK and DDK play roles in the activation of the DNA replication complex and its helicase activity [4].

This chapter will review the key regulatory roles played by CDK and DDK activity in promoting timely assembly of DNA replication complexes. The focus of the article will be on the budding yeast *Saccharomyces cerevisiae* where the assembly and activation of origins of DNA replication has been extensively studied. However, the yeast system will be compared and

contrasted with other eukaryotes in order to emphasize universal features of the process and highlight unique characteristics of DNA replication in different organisms and cell types.

2. Origins of replication: Where it all starts

DNA replication is a fundamental aspect of cellular proliferation. Bacterial cells with relatively small chromosomes initiate DNA replication from a single well-defined site on each chromosome referred to as *oriC* [5]. Eukaryotic chromosomes can be from 10 to 1000 times larger than bacterial chromosomes. In order to completely replicate so much chromosomal DNA within a timely fashion that will allow proliferation, eukaryotic cells employ multiple sites on each chromosome that act as origins for the initiation of DNA replication. These sites are referred to as origins of DNA replication (ORIs). In most metazoans ORIs are poorly defined in the sense that they lack a specific consensus DNA sequence but appear to localize to large regions of a chromosome and are defined by the structure of the chromatin and modification state of the histones and chromatin proteins rather than by specific DNA sequences [6-8]. Indeed, even in the single celled fission yeast *Schizosaccharomyces pombe* DNA replication initiates from relatively broad chromosomal regions [9, 10]. The budding yeast and particularly *Saccharomyces cerevisiae* differs from other eukaryotes in this regard. Autonomously Replicating Sequences (ARS) were first identified in *S. cerevisiae* chromosomal DNA in 1979 [11]. When incorporated into plasmid DNA an ARS sequence allowed for efficient replication and maintenance of the extrachromosomal plasmid. Characterization of ARSs revealed specific DNA sequence elements that act as ORIs reviewed by [12]. These sequences are about 100 – 150 basepairs in length and are composed of elements referred to as A, B1, B2, other sequence elements referred to as B3 and C are sometimes present [13]. The A module harbors an AT-rich 11 basepair ARS Consensus Sequence (ACS). Together the A and B1 element contribute to the formation of a binding site for Origin Recognition Complex (ORC) proteins [14], discussed in the next section. The B2 sequence module contains a double stranded DNA unwinding element (DUE). This sequence is where unwinding of the double helical DNA initiates to create a replication bubble [15, 16]. The B3 element acts as a binding site for the transcription factor Abf1 and excludes nucleosome occupancy of the origin sites [17]. The C element has transcription factor binding sites that may stimulate the utilization of some ORIs but are not essential for ORI function [12, 18].

Although there are specific sequence determinants for *S. cerevisiae* origins of replication, even in this yeast not all ORIs are equal. Significant heterogeneity exists among ORIs in the frequency with which they are activated and utilized [19]. Indeed, there are some origin sequences in the *S. cerevisiae* genome that are not utilized and appear to be dormant [20]. In addition to the frequency of activation there is a distinct temporal order to ORI activation with a subset of origins being activated at early times in S-phase and others being activated later in S-phase [21, 22]. DNA combing studies with *S. cerevisiae* have revealed that at the single molecule level origin activation is highly stochastic with different sets of ORIs being activated in each cell cycle [19, 23]. Indeed while there are approximately 700 potential ORIs in the *S. cerevisiae* nuclear genome only about 200 are activated in any given S-phase. Recent genome-

wide studies investigating origin activation combined with mathematical modeling have suggested that replication timing can be explained by a stochastic mechanism [24-27]. The basis for the differential frequency of ORI activation and temporal regulation has been argued to be due to a limited availability of some essential activators [28-31]. In the case of *S. cerevisiae* over expression of Dbf4, the activating subunit of the Dbf4 dependent kinase (DDK) along with the Cdk substrates Sld2, Sld3 and their binding partner Dbp11 allow early activation of late firing ORIs [28]. Since Dbf4, Sld2, Sld3 do not remain associated with the replication complex once it has been activated, it has been proposed that once an origin fires, the limiting subunits are released from the complex and can then interact with another ORI and trigger its activation. In this scenario ORIs with the highest affinity for the rate limiting factors will have the highest probability of being activated and will have a high probability of being activated at early times in S-phase. ORIs with a lower affinity for the rate limiting factors will fire after those factors have been released from other ORIs. Hence a temporal order of ORI activation can be created. These models propose that the rate limiting activators of DNA replication have a higher affinity for some ORIs than others [28]. This differential affinity may be due to structural aspects of the chromatin in which the ORI is embedded as well as modification of the chromatin proteins by acetylation, methylation, and potentially other post-translational events [32-34]. Further, there is evidence that ORI usage can be influenced by the presence of nearby transcriptional units [35-37].

3. Assembly of the pre-RC: Orc marks the spot

The model of specific chromosomal locations acting as sequence specific sites for binding of protein complexes to initiate DNA replication is conserved across organisms from eukaryotes to prokaryotes and archaea. However, as already described there is no conservation of DNA sequences that act as ORIs across organisms. Indeed, even in *S. cerevisiae*, which has well defined ORIs the sequence of the origins of replication are rather degenerate with only the core ACS being well conserved. In other eukaryotic organisms ORIs display little similarity beyond being rich in AT sequences. Although the DNA sequences that act as sites for initiation of DNA replication are not conserved among eukaryotes the protein complex that binds to ORIs, the Origin Recognition Complex (ORC) is well conserved across eukaryotes and archaea [38-40]. The conserved ORC complex is a hetero-hexamer composed of six subunits Orc1 to Orc6. This complex binds directly to the chromosomal DNA. The *S. cerevisiae* Orc1-6 proteins bind as a hetero-hexamer to the ORI sequence constitutively throughout the cell cycle with Orc1, Orc2, Orc4, and Orc5 making direct contact with the A and B1 sequence ORI DNA sequence [41-43]. In contrast metazoans and even the fission yeast *S. pombe* display regulated binding of the ORC complex to the chromosomal ORI sites. In particular the Orc1 subunit dissociates from the chromatin in G2-phase and re-associates with the complex in G1 [31, 44]. In *D. melanogaster* and human cells Orc1 is subject to degradation by the Anaphase Promoting Complex (APC) in G2-phase [44-48]. As Orc1-6 is required for DNA replication initiated at ORIs, the regulated binding of Orc in metazoans provides an additional layer of regulation that may be used to control the initiation of DNA replication.

The Orc1-6 proteins act as a marker of chromosomal ORI sites and a platform for the assembly of replication complexes. Orc1-6 does not perform this function in an entirely static fashion. Rather successful initiation of DNA replication requires that the Orc1-6 be capable of binding and hydrolyzing ATP, reviewed by [49]. The Orc1 and Orc5 subunits possess nucleotide-binding motifs, Orc1 has conserved Walker A and Walker B motifs and Orc5 has a Walker A motif and a questionable Walker B sequence [50]. Both Orc1 and Orc5 can bind DNA but only Orc1 displays ATPase activity and while mutations that inactivate the Orc1 Walker A sequence cause defects in DNA replication, mutations to the Orc5 Walker A sequence do not [50-52]. In yeast this activity is essential to allow Orc1-6 to bind specifically to chromosomal ORI DNA and to load other replication complex components on to the ORI [43, 50]. Site-specific binding of Orc1-6 to ORI DNA requires the ability to bind ATP; however ATP hydrolysis is not required, suggesting that ATP binding modulates Orc1 structure and its ability to complex with both DNA and other Orc subunits [50]. In contrast ATP hydrolysis is strictly required for the loading of other replication complex proteins and the formation of a functional DNA replication complex [50-52].

DNA replication is essential for developmental processes as well as for somatic cell proliferation. It is frequently the case that the cell cycle is altered or modified from the canonical form it takes in mature cells to achieve specific developmental aims. Orc1-6 is essential for DNA replication in many developmental contexts. Mutations in human Orc1 and Orc4 proteins are responsible for Meier-Gorlin syndrome, a developmental disorder characterized by primary dwarfism, microcephaly, developmental abnormalities of ear and patella [53, 54]. Additionally, Orc3 is essential for neuronal development and maturation [55]. However, there is some diversity in the regulation of Orc1-6 during developmental. For example endo-reduplication in *D. melanogaster* does not require Orc1 [56, 57]. The developmental regulation of Orc binding to chromatin may be influenced by changes in chromatin modification that occur during development since changes in chromatin acetylation have been associated with and shown to regulate the transition to endo-reduplication and the redistribution of Orc proteins during development [58]. And, while Orc1-6 and DNA replication is essential for premeiotic DNA replication, the requirements for these proteins and the mechanism by which they are organized to promote the initiation may differ between mitotic and meiotic S-phases [9].

4. Assembly of the pre-RC: Enter the helicase

The chromatin bound Orc1-6 acts as a nucleation site for the construction of a replication complex (RC). This begins with the assembly of a pre-Replicative Complex (pre-RC). The pre-RC is the multi-protein complex assembled on to ORIs in G1-phase prior to the initiation of DNA replication in S-phase. The base of the pre-RC is the chromatin bound Orc1-6, which acts as a landing pad for the assembly of a series of other protein factors required to assemble a replication fork and initiate bidirectional DNA synthesis. A key requirement for processive DNA synthesis is a dsDNA helicase that can unwind the chromosomal DNA. The Orc1-6 itself has no helicase activity but is essential for recruitment of the replicative helicase to origins of DNA replication. The replicative helicase in *S. cerevisiae* is the minichromosome maintenance

complex (Mcm2-7). The Mcm complex is a hetero-hexamers composed of the subunits Mcm2 – Mcm7 [59-61]. The Mcm subunits interact with each other in a 1:1 ratio to form a ring-like structure that initially binds by wrapping around the DNA such that the double helix passes through the rings central channel. Extensive investigation using biochemical characterization and mutagenesis studies have revealed that the Mcm ring structure has a subunit assembly with the order Mcm5 – Mcm3 – Mcm7 – Mcm4 – Mcm6 – Mcm2 [62]. Sub-complexes of the full Mcm2-7 ring can exist in vivo and in vitro and indeed a trimer composed of Mcm4 – Mcm6 – Mcm7 has ATPase activity and can unwind duplex DNA in vitro [63, 64]. Multiple potential ATPase active sites are formed by interactions between the Mcm subunits: however, only the ATPase activity catalyzed by sites formed by Mcm3 – Mcm7 and Mcm7 – Mcm4 are essential for the helicase activity of the Mcm2-7 holo-complex [64, 65].

In G1 phase of the cell cycle the Mcm2-7 complex is recruited and loaded on to Orc1-6 bound ORI sequences. The helicase is loaded on to the B2 sequence element as a pair of hexamers arranged on the DNA in a head – to – head orientation [66, 67]. The helicase initially assembles on to the DNA as an open complex with a central channel; the ring can be closed around the DNA helix by an ATP dependent conformational change (Figure 1). This involves ATP binding to the Mcm2 – Mcm5 subunits and acting as a “switch” that closes the open gate around the duplex DNA [68].

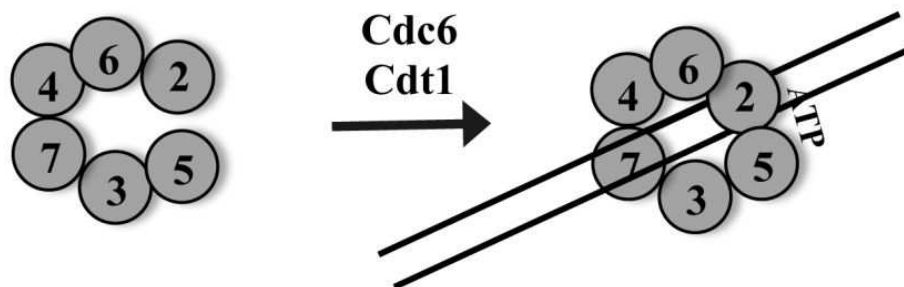


Figure 1. The Mcm2-7 hexamer assembles as an open complex that can be closed through ATP binding. The Mcm2-7 subunits can assemble with each other and in the presence of ATP the complex can assume a ring conformation. In vivo the hexamer is loaded on to Orc1-6 bound ORI duplex DNA. This loading is dependent upon the loading factors Cdc6 and Cdt1. The hexamer can be closed loosely around the duplex through binding to ATP.

Loading Mcm2-7 on to the Orc1-6 bound ORI DNA is accomplished through the combined action of the ATPase activity inherent to the chromatin bound Orc1-6 complex and interaction with the AAA⁺ ATPase loading factor Cdc6. An additional protein required for loading of the Mcm complex is Cdt1, which was first identified in *S. pombe*, but subsequently functional homologs were discovered in *S. cerevisiae*, *X. laevis*, *D. melanogaster*, and mammalian cells [69-73]. The carboxyl-terminus of Cdt1 binds to the Mcm2 and Mcm6 subunits and these contacts are essential for recruitment of the functional Mcm2-7 helicase to Orc1-6 bound origins of DNA replication [74]. ATP hydrolysis catalyzed by both Orc1-6 and the Orc bound Cdc6 stimulate the recruitment of multiple Cdt1-Mcm2-7 complexes [75]. This allows two hexameric Mcm2-7 rings to bind the ORI in a head-to-head orientation, with the dsDNA running through a central channel in the complex [67, 76]. The double hexamers can slide on the duplex DNA

creating the potential to load multimers of double hexamer structures at a single ORI. This may explain why the number of double hexamers loaded on to the DNA can greatly exceed the number of origins that are activated in the subsequent S-phase [77]. Following loading of the Mcm2-7 complexes Cdt1, and Cdc6 are released and do not remain at the ORI as the replication complex continues to assemble [78].

Association of the Mcm2-7 complex with Orc1-6 is a tightly regulated process. In *S. pombe*, Cdt1 mRNA accumulates in the G1 and early S-phase of the cell cycle and in both *S. pombe* and mammalian cells the abundance of the Cdt1 protein is regulated through its destruction by the ubiquitin-proteasome system [71, 73]. In contrast the abundance of Cdt1 protein in *S. cerevisiae* does not fluctuate throughout the cell cycle [69, 79]. In metazoans Cdt1 binding to Mcm2-7 and recruitment to Orc1-6 is negatively regulated by the protein geminin [80]. No protein with a similar function to geminin has been identified in yeast; however, recruitment of *S. cerevisiae* Cdt1-Mcm2-7 complexes to Orc1-6 are negatively regulated by phosphorylation of Orc subunits by Cyclin Dependent Kinase (Cdk) activity [81]. This is an important mechanism to ensure that ORIs are loaded and licensed only once in each cell cycle. Additionally, the gene encoding the loader *CDC6* is transcriptionally regulated such that the mRNA accumulates exclusively during G1 and early S-phase [82]. The Cdc6 protein itself accumulates only in late G1 and early S-phase and is targeted for degradation outside of G1-phase by the Skip1-Cdc53-F box protein (SCF) mediated ubiquitin-proteasome complex [83]. The rigorous regulation applied to Cdc6 and Cdt1 ensures that the Orc1-6 complexes can only be loaded with the replicative DNA helicase machinery in G1 and early S-phase. This is essential to avoid the possibility of origin re-licensing during a cell cycle, which could lead to over replication of some segments of the genome, unscheduled changes in ploidy, the formation of structures that could be at risk for damage, and inappropriate recombination leading to chromosome damage and instability [2, 84].

5. Activating the pre-RC: DDK and CDK usher in the replication complex

Loading the Mcm2-7 helicase complex on to an Orc1-6 bound ORI creates a pre-RC, which licenses the origin and provides the potential for it to be activated or “fired” in S-phase. However, activation of the Mcm2-7 complex and unwinding of the DNA depends upon the further ordered addition of the protein factors Sld3, Cdc45, Sld2, Dpb11, the GINS complex (composed of Psf1, Psf2, Psf3, and Sld5), Mcm10, the replicative DNA polymerases Pol ϵ , Pol δ , and Pol α -primase, along with numerous accessory factors. The addition of these factors to the ORI bound Orc1-6 – Mcm2-7 is dependent upon the activity of two protein kinases DDK and CDK.

DDK (Dbf4 Dependant Kinase) is composed of a catalytic subunit, Cdc7 and an activating subunit, Dbf4 [4]. DDK is essential for the initiation of DNA replication and loss of function mutations in either subunit are lethal resulting in a G1 – S-phase arrest characterized by “dumbbell” morphology in *S. cerevisiae* [85, 86]. DDK is an acidophilic protein kinase [87]. It phosphorylates serine/threonine residues and displays a preference for phosphorylating

serine or threonine residues that are followed by an acidic aspartic acid or glutamic acid residue [88-90]. Additionally, DDK will phosphorylate serine or threonine residues that precede a serine or threonine that has been phosphorylated by another kinase. This is the case with the DDK substrate protein Mer2 where phosphorylation of a serine residue by Cdk1 acts as a priming event to allow phosphorylation by DDK [88, 91]. In *S. cerevisiae* the catalytic subunit Cdc7 does not fluctuate in abundance through the cell cycle; however the kinase activity associated with the protein significantly increases in late G1 and S-phase [92]. The kinase activity associated with Cdc7 is regulated primarily through the interaction of Cdc7 with its positively acting regulatory subunit Dbf4. While the abundance of Cdc7 is relatively constant through the cell cycle, Dbf4 displays a striking accumulation in late G1 and early S-phase and rapidly disappears following the completion of DNA replication [93]. The accumulation of Dbf4 in late G1 and S-phase is accounted for in part by transcriptional regulation; the gene is expressed exclusively in late G1 and S-phase [85], and by regulated destruction of Dbf4 by the ubiquitin-proteasome system [94]. Binding of Dbf4 to Cdc7 leads to a conformational shift in the structure of the inert Cdc7 monomer, that stabilizes the active state of the enzyme [95]. Dbf4 displays localization to ORIs [96]. This localization is driven by sequence motifs in Dbf4 that bind specifically to Orc2, Orc3, and to Mcm4 [97, 98]. Contacts with Mcm4 are particularly critical to achieve recruitment of DDK to the pre-RC. Thus, while Cdc7 possesses the catalytic kinase activity, Dbf4 is required to activate the enzyme and target its kinase activity to the appropriate substrates.

The second protein kinase required for conversion of the pre-RC into an active DNA replication complex is CDK. The enzyme is composed of a catalytic subunit Cdk1 (formerly known as Cdc28 in *S. cerevisiae*) that can be activated by association with a cyclin. Like Cdc7, the monomeric Cdk1 has little associated kinase activity [99]. Also similar to Cdc7 the abundance of Cdk1 does not vary appreciably through the cell cycle; however its associated kinase activity fluctuates from very low levels in early G1 to peak levels occurring in M-phase [100, 101]. Binding to an activating cyclin subunit triggers a conformational change in Cdk1 that reveals the active site and promotes the enzymes protein kinase activity [102]. *S. cerevisiae* expresses 9 Cdk1 activating cyclins that promote Cdk1 kinase activity in different phases of the cell cycle. Cln1, Cln2, and Cln3 are required for budding and events in G1 phase, Cln1 and Cln2 accumulate in late G1 and early S-phase while Cln3 is expressed throughout the cell cycle. Clb1, Clb2, Clb3, and Clb4 accumulate in G2 and M-phases, and promote events in G2 and mitosis [103]. Clb5-Cdk1, and Clb6-Cdk1 are the predominant Cdk complexes that promote the initiation of DNA replication during a normal cell cycle in *S. cerevisiae*. *CLB5* and *CLB6* are transcriptionally regulated such that their mRNAs accumulate in late G1 and S-phase. The Clb5 and Clb6 proteins begin to accumulate in late G1-phase [104-106]. Clb6 is targeted for destruction by the SCF and degraded early in S-phase whereas Clb5 persists into G2-phase [107]. Owing to its destruction early in S-phase Clb6-Cdk1 influences only early firing ORIs whereas Clb5-Cdk1 can regulate both early and later firing ORIs [107, 108]. Among the cyclin subunits Clb5 and Clb6 are the most effective at triggering ORI activation and henceforth I will refer to them as S-Cdk. Their effectiveness in activating DNA replication is in part due to the timing of their accumulation; however, even if other cyclins are expressed in late G1 and early S-phase they cannot activate DNA replication as effectively as S-Cdk [109-112]. Both Clb5

and Clb6 have a hydrophobic patch on their surfaces with an MRAIL sequence motif that allows them to interact with target proteins that have Arg-x-Leu or Lys-x-Leu sequences [111, 113, 114]. Whereas DDK physically interacts with the Mcm2-7 complex and this interaction is essential for conversion of a pre-RC to an active replicative complex, there is no evidence that Cdk must bind to the pre-RC in order to drive its conversion to an active complex. Clb5 can bind to Orc6 and does so following the initiation of DNA replication but this is a mechanism to prevent re-licensing and reactivation of ORIs rather than to promote their initial activation in S-phase [115].

6. Activating the licensed origins: All aboard the helicase train

The first additional components to interact with the loaded and licensed pre-RC are Sld3, its partner Sld7 and Cdc45 [116-118]. These factors associate with early firing ORIs and bind to the Mcm2-7 complex in G1 phase. Sld3 was originally identified in a genetic screen designed to isolate mutations that were synthetically lethal in an *S. cerevisiae* strain that harbored a temperature sensitive mutant allele of the DNA polymerase ϵ binding protein *DPB11* [119]. *CDC45* was discovered through its genetic interactions with *MCM5* and *MCM7* mutants [120]. Mutations in either *CDC45* or *SLD3* that cause loss of function prevent DNA replication and are thus lethal [116, 118]. Chromatin immunoprecipitation and in vitro reconstitution experiments indicate that the binding of Sld3 and Cdc45 to ORIs in G1-phase is relatively weak [121, 122]. DDK activity and binding of DDK to the pre-RC is required for the stable recruitment of Sld3 and Cdc45 both in vitro [121], and in vivo [116, 123, 124]. In addition, Sld3 and Cdc45 are required for each others interaction with the ORI bound Mcm2-7 complex.

Association of Cdc45, Sld3 and its partner Sld7 with ORIs is dependent upon DDK [29, 121]. Neither Sld3-Sld7 nor Cdc45 are directly phosphorylated by DDK rather Mcm2, Mcm4 and potentially Mcm6 are the critical S-phase substrates for DDK [89, 98, 125]. Indeed, modification of the structural architecture of the Mcm2-7 complex is likely the critical function for DDK in the activation of DNA replication since a mutation of Mcm5 that changes proline 83 to leucine alters the structure of the Mcm2-7 complex and allows cells lacking DDK to survive and replicate their DNA [122, 126, 127]. Additionally, DDK binds to the Mcm2-7 complex through interactions with a docking domain in Mcm4 and mutations in the Mcm can bypass the requirement for DDK [98, 125]. The initial interaction of DDK with the Mcm2-7 complex is dependent upon prior phosphorylation of at least Mcm4 and Mcm6 by yet to be identified protein kinases [89, 90].

The binding of Cdc45, Sld3 and Sld7 is a pre-requisite for the further assembly and conversion of the pre-RC to an active replication complex (RC). Following the loading of these factors Cdk activity is required. Accumulating S-Cdks interact with both Sld2 and Sld3 through RxL motifs in the substrate proteins [113-115, 128]. This leads to phosphorylation of Sld2 and Sld3 at multiple sites [129, 130]. The multi-site phosphorylation of Sld2 leads to a conformational change in the protein that allows the additional phosphorylation of threonine 84, which does not reside within a canonical Cdk recognition motif [131]. Phosphorylation of T⁸⁴ allows Sld2

to interact with Dpb11 a protein originally identified based upon its interactions with the replicative DNA polymerase, Pol ϵ [132]. Dpb11 has BRCT repeat domains at both its amino-terminal and carboxyl-terminal regions [133]. These sequence motifs function as phosphopeptide binding domains [134] allowing the phosphorylated Sld2 to bind the carboxyl-terminal BRCT phosphopeptide binding domain of Dpb11 [119, 129, 130]. Similarly phosphorylation of Sld3 allows Sld3 to bind the amino-terminal BRCT repeat of Dpb11 thus recruiting the Sld2-Sld3-Dpb11 complex to the Mcm2-7 complex and origin of replication [129, 130]. Dpb11 binds Pol ϵ , the leading strand replicative DNA polymerase in *S. cerevisiae* [132]. The interaction of Dpb11 with DNA Pol ϵ is not Cdk dependent but binding to phosphorylated Sld2 and Sld3 allows recruitment of the entire complex to the licensed ORI [135].

Although Sld2 and Sld3 are not the only components of the replication complex that can be phosphorylated by Cdk1 they are the critical substrates since phosphomimetic mutations in Sld2 and fusion of Sld3 with Dpb11 can bypass the need for Cdk1 activity to initiate DNA synthesis [129, 130].

The binding of Sld2 and Sld3 to the pre-RC allows the recruitment of GINS to the Mcm2-7 hexamer. GINS is a protein complex composed of Psf1, Psf2, Psf3 and Sld5 and is named after the number based names of its components Go, Ichi, Ni, San (Japanese for 5, 1, 2, 3). Sld5 was identified in a genetic screen for mutants that displayed synthetic lethality when combined with a thermo-sensitive *dpb11* allele [116]. Subsequent investigations revealed partners of Sld5 (Psf1, Psf2, Psf3) that formed a complex required for initiation and DNA strand elongation during DNA replication [136]. GINS associates with Cdc45 at the ORI and its recruitment leads to stable engagement of Cdc45 with the Mcm2-7 complex. In vitro Cdc45 and GINS strongly stimulate the ATPase and DNA unwinding activity of Mcm2-7 complex [137]. There is evidence that Cdc45 makes specific contacts with Mcm2 while GINS binds to Mcm5, when GINS and Cdc45 bind one another this tightly closes the Mcm2-7 rings "gate" with DNA trapped within the central channel of the Mcm ring structure reviewed by [59]. There is no evidence that Cdk phosphorylates either Cdc45 or GINS or regulates their activity, the primary role played by the Cdk appears to be in promoting their recruitment to the chromatin bound Mcm2-7 complex. The binding of the additional components including GINS results in conversion of the pre-RC into the CMG (Cdc45/Mcm2-7/GINS) complex, this is also referred to as the pre-initiation complex (pre-IC) [138]. While Sld2, Sld3 and Sld7 are released from the complex following stable engagement of Cdc45 and GINS, both of the latter factors remain associated with the Mcm2-7 and are required for elongation of the nascent DNA strands following the initiation of DNA synthesis [136, 139].

Mcm10 is an additional factor required for assembly of a functional replisome and conversion of the pre-IC to an RC. Mcm10 was originally identified in a screen similar to that used for the identification of other *S. cerevisiae* MCM genes [140, 141]. Homologs of *MCM10* can be found from yeast to humans [142, 143]. Mcm10 is an abundant chromatin bound protein that interacts with all six subunits of the Mcm2-7 complex and localizes to origins of DNA replication [141, 142, 144]. Mcm10 has a critical role in conversion of the pre-RC to an active RC as it makes contacts with DNA Pol α and the CMG complex components [145-147]. It is certain that Mcm10 plays a role in stabilizing the Mcm2-7 complex with DNA Pol α [148]; however its precise role

in the initial recruitment of DNA polymerases or their accessory factors to the replisome is not entirely clear.

The accumulation and action of DDK and CDK set in motion the assembly and conversion of the pre-RC to an activated RC. The use of two independent kinases to achieve this goal allows tight regulation over the assembly and activation process. Since both kinases are required to activate and “fire” the ORI it seems that there are in fact two triggers that can be pulled independently. For the initiation of DNA replication to take place both triggers must be pulled with the correct timing.

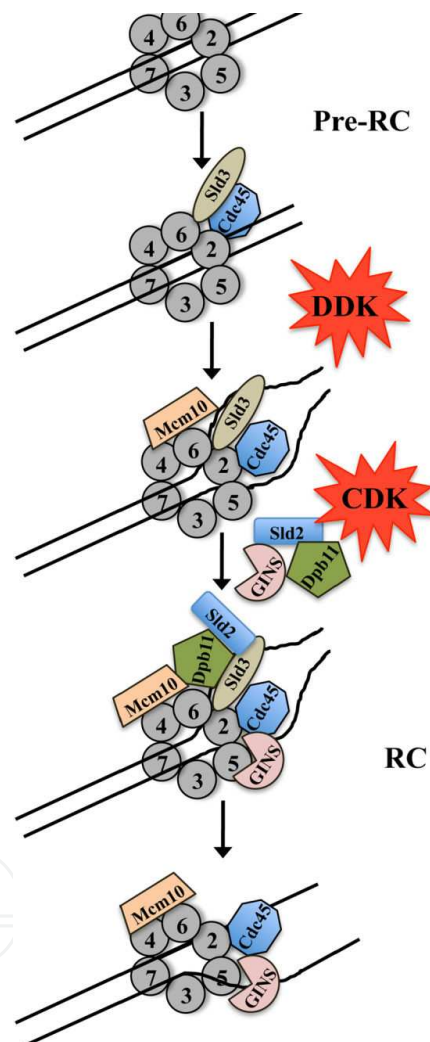


Figure 2. DDK and CDK promote assembly and activation of replication complexes at chromosomal origins of DNA replication. Sld3 and Cdc45 associate loosely to the ORI bound Mcm2-7 hexamer in G1-phase. Phosphorylation of the Mcm subunits by DDK promote tight binding by Cdc45 and Sld3, Mcm10 may associate with the complex at this time and plays an important role in unwinding of the ORI DNA duplex. CDK phosphorylation of Sld3, and Sld2 recruit Sld2, Dpb11, Pole and GINS to the Mcm2-7 complex. GINS binding increases the helicase activity of the Mcm2-7 hexamer allowing unwinding of duplex DNA. The association of GINS also marks a transition when Mcm2-7 binding to duplex DNA changes to binding such that a single strand is retained in the central channel, while the other strand is moved to the external surface of the complex.

7. The business end: Polymerases at the origin

The final critical steps of origin firing are the recruitment of the replicative polymerases, unwinding of the dsDNA and initiation of DNA synthesis. While all cells encode multiple different DNA polymerases the enzymes with the most well characterized roles in nuclear chromosomal DNA replication are DNA Pol ϵ , DNA Pol δ , and DNA Pol α – primase. DNA Pol ϵ acts as the leading strand DNA polymerase for nuclear DNA replication in *S. cerevisiae* [149]. Through its interaction with Dpb11 it is recruited to the pre-RC complex following Cdk1 mediated phosphorylation of Sld2, and Sld3. DNA Pol δ is the major lagging strand DNA polymerase in *S. cerevisiae* [150]. Although DNA Pol δ plays a key role in nuclear DNA replication it is currently unclear how this enzyme is recruited to the nascent RC. DNA Pol α -primase is essential for the initiation of DNA replication as primase synthesizes RNA primers that Pol α extends with short DNA oligonucleotides on the unwound ORI DNA providing primers for DNA Pol ϵ and DNA Pol δ . [151, 152]. Mcm10 binds DNA Pol α and this DNA polymerase may be initially recruited to the Mcm2-7 complex through these interactions. The primase polypeptide forms a complex with the carboxyl-terminus of Pol α allowing the two to be incorporated into the growing replisome simultaneously [153]. Following or perhaps concurrent with recruitment of the replicative DNA polymerases there is a reorganization of the complex as it undergoes conversion from a pre-IC to RC. During this process Dpb11, Sld2 and Sld3 are ejected from the complex while Pol ϵ remains bound. Within the RC, DNA Pol ϵ makes contacts with Mrc1 that help to retain it within the complex [154]. It is currently unclear how Mrc1 is recruited to the complex upon conversion to a nascent RC or whether unwinding of the ORI DNA is required. Pol α makes contacts initially with Mcm10 and once incorporated into the RC, it makes further contacts with Ctf4 a component of Replication Factor C (RFC), these contacts help stabilize the binding of Pol α to the complex [155, 156]. During the remodeling of the pre-RC into an activated RC several accessory proteins: Replication Factor C (RFC), Proliferating Cell Nuclear Antigen (PCNA), and Replication Protein A (RPA) are added to the complex. The mechanism that leads to recruitment of these accessory proteins has not been determined. It may be that they simply recognize and bind to the protein-DNA structure formed by the initial unwinding of the ORI DNA. Owing to its ssDNA binding capability RPA associates with the RC once unwinding of the ORI DNA is underway; here it assists in stabilizing the nascent replication bubble and provides access for the replicative DNA polymerases [157]. All three subunits of DNA Pol δ make contact with PCNA and these interactions are essential for processive lagging strand DNA synthesis [158]. These factors influence the processivity and integrity of DNA synthesis.

Unwinding the ORI DNA to provide ssDNA as template for the DNA polymerases and to construct bidirectional replication forks is accomplished by the activated Mcm2-7 hexamer in concert with associated proteins Cdc45, GINS, Mcm10 and the replicative DNA polymerases. In vitro the Mcm2-7 hexamer unwinds DNA by tracking along a single strand while displacing the other strand [65, 159]. Achieving this end requires that the dsDNA initially bound be melted and locally unwound allowing release of one strand to the outside surface of the complex and retaining the other within the central channel of the hexamer. Although the molecular details

of this process remain unclear some of the current models to explain ORI unwinding by Mcm2-7 have been recently reviewed in detail [59].

Sld2, Sld3, and Mcm10 all display some ability to bind ssDNA and it has been speculated that they might participate in the initially melting of the dsDNA, allowing the Mcm2-7 rings to undergo conformational change such that they close around one of strands of the melted duplex. Mcm10 may be a real candidate for this role based upon its stable incorporation into the RC and ability to bind ssDNA [160]. Determining the precise mechanism and timing of ORI DNA unwinding will await higher resolution structural and biochemical analysis.

8. Who's on first? Ordered action of DDK and CDK in the activation of ORIs

The assembly of a preRC and its conversion first to an RC and then an active replication fork is a multistep process that requires the activity of both DDK and CDK. Multiple investigations have been performed to determine the order in which DDK and CDK act at the ORIs to trigger their activation. Genetic studies with *S. cerevisiae* have suggested that DDK cannot complete its function without prior S-Cdk activation implying either that Cdk must act before DDK or that DDK performs a multiple functions at the pre-RC and that some of them require Cdk activity for completion [161]. In *X. laevis* egg extracts DDK can complete its essential function in the absence of Cdk activity, however Cdk cannot perform its vital function in the absence of DDK [162, 163]. Recent investigations using an *S. cerevisiae* in vitro DNA replication system suggest that assembly and activation of origins of replication require that DDK act before Cdk but that completion of DDKs essential functions require Cdk activity [90, 121]. The apparent conflict in these results may reflect differences between DNA replication control in somatic cells and eggs. Additionally, some of the differences may stem from the limitations inherent to both genetic and in vitro biochemical experimental systems. Redundant systems and limits to the speed with which activities can be activated and inactivated in vivo place limits on genetic approaches to understanding the specific requirements for DDK and CDK. While in vitro it may be difficult to accurately recapitulate the in vivo environment. For example, Cdk activity increases during G1-phase in a graded fashion both in total kinase activity and kinase specificity. Relatively low levels of Cdk activity are sufficient to activate DNA replication and elevated levels of Cdk activity that accumulate in S, G2, and M-phases prevent licensing and activation of origins by promoting destruction of Cdc6, nuclear export of Mcm2-7 components and by binding to Orc6 and excluding recruitment of Mcm to ORIs [164, 165]. It is possible that low levels of Cdk activity are required prior to DDK initiating its function. Indeed phosphorylation of Mcm4 and Mcm6 is a prerequisite for DDK binding to the pre-RC and further inducing activation. It has been proposed that phosphorylation of Mcm4 by G1-Cdk activity may be required to allow DDK to bind to the Mcm2-7 complex [89].

9. Conclusion

DNA replication is a fundamental aspect of cellular proliferation and development. Many aspects of this process are well conserved not only within the domain of eukaryotes but also across bacteria and archaea. The multi-step assembly and activation of origins of DNA replication is more complicated and more rigorously regulated in eukaryotes than it is in either prokaryotes or archaea. This complexity stems in part from the size of the eukaryotic genomes that necessitates multiple origins of replication on each chromosome. Additionally, multiple layers of regulation act as a safeguard that ensures each origin of DNA replication is activated only once in each cell cycle. This is crucial to prevent over replication, amplification of chromosomal segments and chromosome instability.

The initiation of DNA replication in *S. cerevisiae* has served as an exceptional model owing to the genetic and biochemical accessibility of this organism. Our current understanding of the steps leading to the initiation of DNA replication in *S. cerevisiae* can be summarized as follows. Orc1-6 bound ORI sequences act as a binding site for Cdc6, which in conjunction with Cdt1 recruits Mcm2-7 hexamers to the ORI. DDK is recruited to this structure by virtue of the affinity of Dbf4 for docking domains in Mcm4. DDK phosphorylates the Mcm2-7 helicase, promoting the recruitment of Sld3 and Cdc45. Next, S-CDK-dependent phosphorylation of Sld2 and Sld3 leads to their binding Dpb11 and recruitment of the complex, along with GINS and Pol ϵ to the pre-RC thus forming a CMG complex. These proteins then serve to both recruit Mcm10 and fully activate the Mcm2-7 helicase, which uses ATP hydrolysis to melt the origin DNA. Pol α -primase and Pol δ can then be loaded on to the ssDNA at the unwound ORI, leading to the formation of a complete replisome with accessory proteins such as PCNA, Mrc1, RFC, RPA, and topoisomerase. The helicase activity of the Mcm2-7 hexamers then drives bidirectional dsDNA unwinding and replication fork movement along the chromosome allowing the synthesis of new DNA.

Initiating DNA replication is a serious event for a cell. The chromosomal DNA is rarely more at risk of damage than when it is being unwound and copied. During this processes single stranded DNA is revealed and the fork structures with the potential for breakage and recombination are formed. The requirement for two protein kinases, DDK and CDK, that perform non-redundant functions in the assembly and activation of replication complexes suggests that there are in fact two triggers that must be pulled to fire the origin. The requirement for two different kinases that are independently regulated and that each have distinct substrate specificity allows the initiation of DNA replication to be regulated with exquisite sensitivity. Perhaps rather than considering these two kinases as triggers they should really be thought of as a double failsafe mechanism where each trigger must be pulled with the appropriate timing to allow DNA replication to proceed.

Despite our general understanding of this process many aspects of its molecular basis remain to be elucidated. How are Sld3 and Cdc45 initially recruited to the pre-RC? How does the Mcm2-7 helicase melt ORI DNA and what is the mechanism by which it is converted to a machine that directionally tracks along and unwinds dsDNA? Does DDK travel with the Mcm2-7 complex along the DNA? How are DNA Pol δ and the accessory proteins RFC, and

PCNA recruited to the replication fork? It is likely that a combination of genetic analysis, biochemistry and high-resolution structure analysis will be required to answer these questions.

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