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# Cell Cycle Control of DNA Replication by Phosphorylation and Dephosphorylation of Replication-Initiation Proteins in Budding Yeast

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## 1. Introduction

Eukaryotic DNA replication initiates in a two-step fashion (Diffley, 2004), and both steps are tightly regulated by phosphorylation and dephosphorylation of replication-initiation proteins. First, a multi-protein complex known as pre-RC (pre-replicative complex) is assembled on replication origins from late M to early G1 phase; this process is referred to as replication licensing. Subsequently, origin activation (firing) occurs in accordance to the rise of S-CDK (cyclin dependent kinase) and DDK (Dbf4p dependent kinase) activities during which replication forks are established and DNA synthesis begins.

### 1.1 Components of replication licensing

Pre-RC is assembled onto replication origins in a stepwise manner, and it includes the following components:

#### 1.1.1 Replicators and replication origins

Replicators and replication origins in budding yeast were together identified as some conserved DNA sequences, autonomously replicating sequences (ARSs). There are about 400 ARSs distributed throughout the yeast genome to promote the association of initiation proteins and the unwinding of DNA double helixes (Raghuraman et al., 2001). Despite the conservation of the initiation proteins in eukaryotes, the origin sequences are highly divergent, and poorly characterized in many organisms (Machida et al., 2005; Sclafani & Holzen, 2007)

#### 1.1.2 ORC (Origin recognition complex)

Origin recognition complex (ORC) can specifically recognize and associate with ARSs directly in an ATP dependent manner throughout the cell cycle, serving as a landing pad for other factors to be assembled. ORC utilizes the energy from ATP hydrolysis to drive pre-RC assembly (Bowers et al., 2004; Randell et al., 2006). Although the six subunits of ORC (Orc1-6p) form a tight complex *in vivo*, only Orc1-5p are required for origin recognition and DNA binding (Stillman, 2005). Nevertheless, Orc6p plays an essential role in pre-RC assembly

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through interacting with Cdt1p (Cdc10p-dependent transcript) to promote multiple rounds of MCM (Minichromosome maintenance) loading onto replication origins (Chen et al., 2007).

ORC is functionally related to DnaA, the initiator protein in bacteria. Structural analysis showed that DnaA displays a helical conformation that, when bound to the replication origin, induces superhelical tension which causes localized DNA melting to facilitate helicase loading (Erzberger et al., 2006). Despite detailed biochemical analysis, the exact mechanism of ORC as the initiator awaits further investigations. However, some studies highlighted its role in manipulating local chromatin environment (Lipford & Bell, 2001; Kan et al., 2008; Espinosa et al., 2010).

### 1.1.3 Cdc6p (Cell division cycle)

The budding yeast Cdc6p was identified as a multicopy suppressor of the *orc5-1* ts mutant (Liang et al., 1995). It interacts with ORC, and is required for loading the hexameric helicase MCM (Minichromosome maintenance) proteins onto chromatin (Cocker et al., 1996; Donovan et al., 1997; Tanaka et al., 1997). Analysis with EM micrograph reconstruction showed that the ORC-Cdc6p complex displayed a ring-shaped structure, which possesses six AAA+ ATPase domains (five from ORC and one from Cdc6p) (Speck et al., 2005). *In vitro* studies suggested that the ATPase activities from both initiation proteins are needed for robust MCM loading (Seki & Diffley, 2000). Importantly, ATP hydrolysis by Cdc6p destabilizes the ORC-Cdc6p complex, and because ARS-specific DNA binding to the complex inhibits the Cdc6p ATPase activity (Speck & Stillman, 2007), it is thus proposed that the ATPase domain of Cdc6p confers specificity on the site of pre-RC assembly.

### 1.1.4 Cdt1p (Cdc10p-dependent transcript)

In contrast to other initiation proteins which were first identified in budding yeast, the helicase loader component Cdt1p was first isolated from fission yeast (Nishitani et al., 2000), *Xenopus* (Maiorano et al., 2000) and *Drosophila* (Whittaker et al., 2000) as a protein involved in DNA replication. Based on homology search, and despite low level of sequence homology, the budding yeast Cdt1p was identified as the gene *TAH11* (Topo-A hypersensitive) (Tanaka & Diffley, 2002), previously isolated by virtue of its genetic interaction with a topoisomerase I mutant. Cdt1p in budding yeast, like the homologs in higher eukaryotes, is essential for loading MCM onto replication origins, acting as a bridge between Mcm2-7p and ORC. On the one hand, Cdt1p interacts with the MCM complex specifically through the Mcm6p subunit (Wei et al., 2010); on the other hand, Cdt1p associates with ORC via transient contact with Orc6p, resulting in the incorporation of Mcm2-7p complex into pre-RC (Chen et al., 2007). Unlike higher eukaryotes and fission yeast where Cdt1p is regulated by degradation, ScCdt1p is stable throughout the cell cycle but is regulated by nuclear import and export (Tanaka & Diffley, 2002).

### 1.1.5 MCM (Minichromosome maintenance) proteins

MCM genes were first isolated in a genetic screen from mutants defective in maintaining the stability of ARS-based plasmids (Maine et al., 1984). The six subunits are highly related to one another in sequence, but each MCM protein is essential for cell growth. MCM proteins migrate with the replication fork (Aparicio et al., 1997) and function as the putative replicative helicase. *In vitro* studies strongly suggest that Mcm2-7 proteins act as DNA helicase (Bochman & Schwacha, 2009). The Mcm 4/6/7 complex purified from Hela cells

displayed weak DNA helicase activity. Similar studies in mouse and fission yeast also identified a DNA helicase activity dependent on the Mcm4/6/7 complex. Surprisingly, although all six Mcm proteins function at the replication fork in budding yeast, the intact Mcm2-7p complex showed no helicase activity *in vitro* (Takahashi et al., 2005). A recent study with biochemical reconstitution of *Drosophila* CMG (Cdc45p, MCM and GINS) complex suggested that the MCM complex is loaded in an inactive form as a pre-RC component (Ilves et al., 2010), and it displays robust ATPase and helicase activity only when it is associated with Cdc45p and GINS. Apart from the association with Cdc45p and GINS, modifications on MCM also play an important role in the helicase activation.

In order to perform its helicase activity, the Mcm2-7p complex needs the action of both DDK and CDKs to phosphorylate some MCM subunits as well as other initiation proteins (Labib, 2010). The N-terminal tails of Mcm2/Mcm4/Mcm6 appears to be the major substrates of DDK for DNA replication, among which Mcm4p phosphorylation by DDK is particularly important. N-terminal serine/threonine-rich domain (NSD) of Mcm4p contains multiple motifs that are targets for DDK, and phosphorylations of these motifs by DDK relieve the inhibitory effect of Mcm4-NSD on DNA replication (Sheu & Stillman, 2006; 2010).

At the heart of the regulation of replication initiation is the loading of the MCM helicase onto origins and its subsequent activation to unwind the double helix. The loading process is well organized spatially and temporally. Oscillation of CDK activity during cell cycle restricts this process only within late anaphase and G1 phase at low CDK activity (Diffley, 2004). At the M-to-G1 transition, the MCM complex is allowed to be imported into the nucleus. Only after the helicase loaders Cdc6p and Cdt1p associate with ORC can the MCM complex be loaded onto chromatin to form pre-RC (Cocker et al., 1996; Donovan et al., 1997; Tanaka & Diffley, 2002). Recent studies using reconstituted yeast pre-RC and EM microscopy demonstrated that MCM is loaded onto replication origins as a double-hexamer, connected in a head-to-head configuration via their N-terminal tails (Evrin et al., 2009; Remus et al., 2009).

### 1.1.6 Noc3p (Nucleolar-associated complex)

Noc3p was isolated as a multicopy suppressor of the *mcm5-1* ts mutant (Zhang et al., 2002). Noc3p mutants display classical ARS-number suppressible plasmid loss phenotype. Noc3p binds to ARS DNA and interacts with ORC and MCM proteins. And inactivation of Noc3p results in failure of pre-RC assembly and G1/S transition (Zhang et al., 2002). Because both ORC and Noc3p bind to the chromatin throughout the cell cycle and depletion of Noc3p only affect the binding of initiation proteins downstream of ORC onto the chromatin, ORC and Noc3p represent ARS-bound scaffold proteins that mark the early step of pre-RC assembly. Besides, since both ORC and Noc3p have been implicated in ribosome biogenesis (Milkereit et al., 2001; Du and Stillman, 2002), they might be important regulator coordinating both cell growth and division (Jorgensen & Tyers, 2004). A study in budding yeast also shows that overexpression of Noc3p caused severe growth defects in *orc* and *cdc6* mutants, just as overexpression of some MCM subunits and some other replication-initiation proteins did (Honey and Futcher, 2007), supporting the role of Noc3p in replication initiation together with ORC and MCM proteins. Noc3p interacts with Cdt1p, Mcm6p and four of the six subunits of ORC that are closely connected with one another (Wu, R., J. Yeung and CL, unpublished data), apparently making a snug fit into the main body of the ORC architecture. We have also isolated separation-of-function mutants in *NOC3* (Wu, R. and CL, unpublished data), indicating that the Noc3p's functions in DNA replication and ribosome biogenesis are separable.

Consistent with the role of Noc3p as an initiation protein, *fad24* (Factor for adipocyte differentiation, the human homolog of Noc3p), is found to regulate differentiation (Tominaga et al., 2004) and is essential for DNA replication in human (Johmura et al., 2008a) and mouse cells (Johmura et al., 2008b). Importantly, *fad24* interacts with HBO1 (Histone acetyltransferase-binding to ORC), which is an Orc1p interacting protein in human cells required for acetylating origin-proximal histone H4 to promote replication licensing (Iizuka & Stillman, 1999). If these interactions are conserved in yeast, it is tempting to speculate that Noc3p might also recruit histone acetyltransferase (HAT) to ARSs. Gcn5p, a recently identified HAT with important role in replication licensing (Espinosa et al., 2010), would be an attractive candidate.

Data from a fission yeast study are also consistent with Noc3p being required for replication initiation, as Noc3p inactivation (probably incompletely) caused delay in S phase entry, slow progression through S phase, and cell cycle arrest in late S and/or G2/M phases, although the authors interpreted the data in favor of Noc3p's role in cell division instead of replication (Houchens et al., 2008). These phenotypes are typical of the majority of the published ORC and MCM ts mutants in both budding and fission yeasts, as partial initiation of DNA replication by the residual mutant proteins even under the restrictive conditions for cell growth allows the cells to replicate most, but not all of the DNA, resulting in late S and/or G2/M arrest. Consistent with this interpretation, these mutants would not arrest in the first G2/M phase if the cells were released into the cell cycle after the mutant proteins were inactivated in early S phase or G2/M phase instead of G1 phase (Zhang et al., 2002; Gibson et al., 2006). Therefore, the apparent cell cycle arrest in G2/M phases is the result of incomplete DNA replication, not because of a cell division defect *per se*.

## 1.2 A model of replication licensing

Summing up the current data, a model of pre-RC assembly is proposed as shown in Fig. 1. ORC and Noc3p bind to ARS elements throughout the cell cycle, and they act as a scaffold to recruit other initiators. During mitotic exit, *de novo* synthesized and stabilized Cdc6p binds with ORC/Noc3p and together they form a ring complex on ARS. At the same time MCM and Cdt1p are imported into the nucleus in an inter-dependent manner. And through the concerted action of Cdc6p and Cdt1p, the MCM helicase is loaded as a double hexamer onto the chromatin to license the replication origin. This complex composed of ORC, Noc3p, Cdc6p, Cdt1p, MCM and possibly other unidentified proteins, is referred to as the pre-RC, which is assembled only at low CDK activity upon mitotic exit to early G1 of the next cell cycle. Worth mentioning, all of the known pre-RC components are conserved from yeasts to humans despite that some of them are regulated differently in different organisms.

## 1.3 Origin activation (Origin firing)

Origin firing refers to the activation of licensed replication origins by the concerted activities of CDK and DDK during which helicase activation is coupled to the recruitment of DNA polymerases.

### 1.3.1 GINS (Go, Ichi, Nii, and San; five, one, two, and three in Japanese)

The GINS complex is composed of Sld5p, Psf1p, -2p and -3p discovered by several groups (Kanemaki et al., 2003; Kubota et al., 2003; Takayama et al., 2003). A functional proteomic



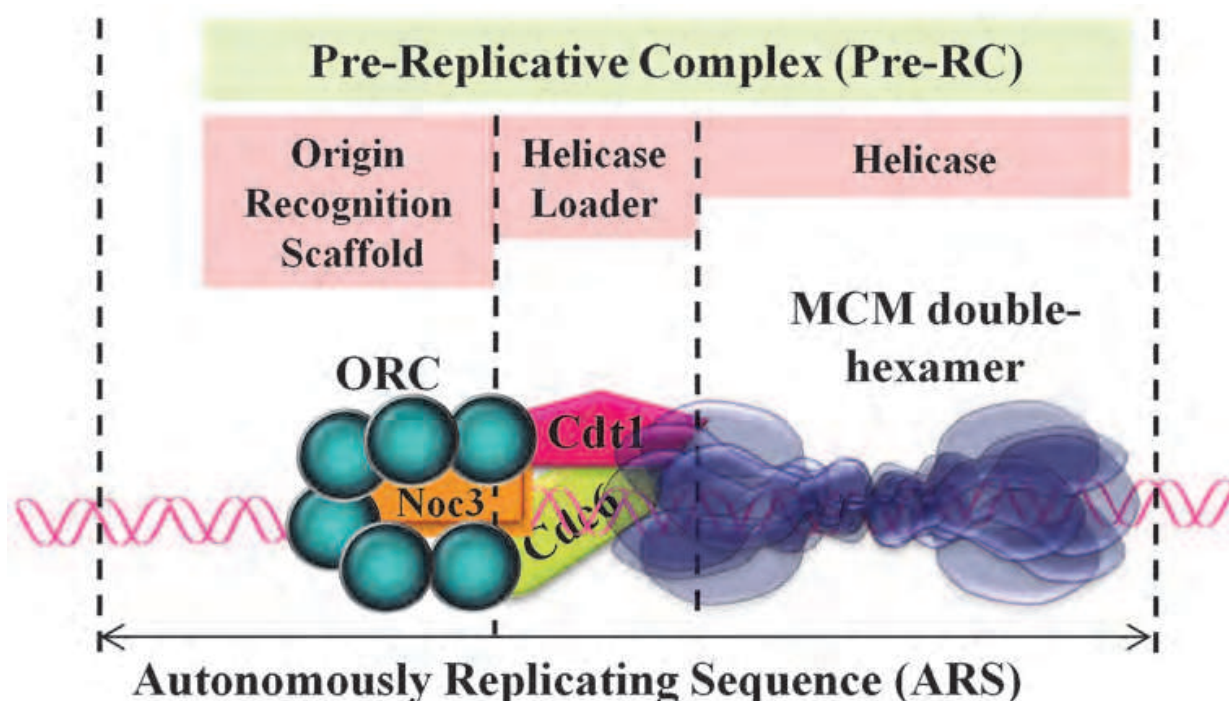


Fig. 1. The pre-replication complex in budding yeast.

The pre-RC is assembled step-wise on ARS during late M to early G1 phase of the cell cycle. It is composed of several groups of proteins including the origin recognition scaffold (ORC and Noc3p), the helicase loaders (Cdc6p and Cdt1p) and the helicase (the MCM complex). See text for details.

screen in budding yeast identified the GINS components that, upon depletion, would affect DNA replication (Kanemaki et al., 2003). *PSF1* and *PSF2* (Partner of Sld Five) were identified as multicopy suppressors of the *sdl5-12* ts mutant, and *PSF3* was identified as a multicopy suppressor of the *psf1-1* ts mutant (Takayama et al., 2003). These proteins interact with one another and are essential for DNA replication in budding yeast. By sequence homology search, they were identified and purified as a ring-like complex from *Xenopus* egg extracts (Kubota et al., 2003). Crystallography analysis reveals that the human GINS complex adopts a trapezium-like structure (Boskovic et al., 2007), rather than the ring-like shape counterpart in *Xenopus* as observed on EM micrograph (Kubota et al., 2003). GINS is only recruited to origins upon S-phase onset, and it travels along with the replication fork as replication proceeds (Kanemaki et al., 2003).

### 1.3.2 Cdc45p

*CDC45* was initially characterized as one of a group of genes, together with *MCM4*, *5*, and *7* that display extensive genetic interactions with one another (Hennessy et al., 1991). Cdc45p forms a complex with Sld3p, and together they are recruited in an inter-dependent manner to licensed origins throughout S-phase (Kamimura et al., 2001). Notably, early-firing origins are marked with Sld3p-Cdc45p even in G1 phase (Kamimura et al., 2001). And because Cdc45p origin binding profile coincides with the activation timing of origins, Cdc45p is considered as a marker for active origins (Aparicio et al., 1997; Zou & Stillman, 1998; Kamimura et al., 2001). In addition, stable recruitment of Cdc45p to pre-RC requires DDK phosphorylation of the Mcm4p N-terminal tail (Sheu & Stillman, 2006).

### 1.3.3 Sld2p, Sld3p and Dpb11p

*DPB11* (DNA polymerase *B*) was first identified as a multicopy suppressor of the *ts* alleles *dpb2-1* and *pol12-1* (Araki et al., 1995), which encode two different subunits of the DNA polymerase  $\epsilon$  in budding yeast. Identified by the same genetic screen strategy, *SLD2*, *SLD3* and other *SLD* genes (including *SLD5* in the GINS complex) were found to suppress the *dpb11-1 ts* allele when they are carried on high copy plasmids (Kamimura et al., 1998).

Dpb11p contains two pairs of tandem BRCT (BRCA1 C-terminal) domains, which are sites known as phosphopeptide binding modules. Dpb11p is proposed to be a bridging factor to promote DNA replication. Both Sld2p and -3p are substrates of S-CDK, showing an S-phase specific phosphorylation profile, and they only bind Dpb11p in the phosphorylated forms (Tanaka et al., 2007; Zegerman & Diffley, 2007). Such interactions are elegantly partitioned over Dpb11p. Notably, the N-terminal proximal BRCT repeats of Dpb11p are required for Sld3p binding while those located in the C-terminal region interact with Sld2p. Mutagenesis study and synthetic phospho-peptide mapping revealed that phosphorylation at T84 on Sld2p, and that T600 and S622 in Sld3p are responsible for mediating Dpb11p binding (Zegerman & Diffley, 2007). By a series of rescue experiments and genetic screens, it was found that the lethality of cells carrying non-phosphorylatable Sld3p (i.e. Sld3p-600A, 609A, 622A) could be rescued when the mutant Sld3p was fused with the N-terminal BRCT repeats-truncated Dpb11p (referred to as SD-fusion) (Zegerman & Diffley, 2007); while cells with non-phosphorylatable Sld2p could be rescued by a Sld2p phospho-mimic mutant (either Sld2-T84D or Sld2-11D) (Tanaka et al., 2007; Zegerman & Diffley, 2007). Moreover, ectopic expression of Sld2-T84D in an SD-fusion genetic background led to DNA replication independent of S-CDK. Remarkably, when these CDK-bypass mutants were combined with the *bob1* allele (an *MCM5* allele that suppresses *cdc7 $\Delta$*  lethality and bypasses the requirement of DDK in replication) (Tanaka et al., 2007; Zegerman & Diffley, 2007) or the *mcm4 $\Delta$ 74-174* allele (a DDK-bypass allele of *MCM4* lacking a self-inhibitory domain) (Sheu & Stillman, 2010), DNA replication still occurred even when the yeast cells were arrested in G1 phase lacking both CDK and DDK activities. Taking together, Sld2p, Sld3p and Mcm4p represent the minimal targets of the S-phase kinases in promoting DNA replication.

### 1.3.4 Mcm10p, Ctf4p and DNA polymerase $\alpha$

Budding yeast Mcm10p is a recruitment factor that targets polymerase  $\alpha$  to the licensed origins in G1 phase and the replication forks during S-phase (Ricke & Bielsky, 2004; Lee et al., 2010). It binds to origins in G1 cells in an MCM-dependent manner and co-migrates with the fork as part of the replisome progression complex (RPC) (Gambus et al., 2006). Study in *Xenopus* suggested that Mcm10p channels its function in part through interacting with a protein known as And1 (Ctf4p in budding yeast), which is important for efficient DNA synthesis and acts as a bridging factor between Mcm10p and Pol  $\alpha$  (Zhu et al., 2007). The budding yeast Ctf4p was initially isolated as a mutant defective in chromosome segregation (Spencer et al., 1990). It travels with the replication fork and is known to participate in sister chromatids cohesion (Hanna et al., 2001; Gambus et al., 2006). Consistent with the *Xenopus* ortholog, Ctf4p was also identified as a RPC component, and it interacts with Mcm10p for stable chromatin association during S-phase (Tanaka et al., 2009). Besides, it helps to stabilize both Mcm10p and Pol  $\alpha$  (Gambus et al., 2009; Wang et al., 2010). And cells without Ctf4p display a delay onset and slow progression of S-phase (Wang et al., 2010).

1.4 Current model of origin activation

Both CDK and DDK activities are required for origin firing, and genetic evidence suggested that CDKs work in advanced of DDK to promote DNA replication. As illustrated in Fig. 2, binding of Sld3p and Cdc45p to pre-RC in G1 cells marks early-firing origins (Kamimura et al., 2001). Upon S-phase onset, rising S-CDK activity phosphorylates both Sld2p and Sld3p, creating binding sites for the BRCT repeats in Dpb11p (Tanaka et al., 2007; Zegerman & Diffley, 2007). The Sld2p-Dpb11p interaction nucleates the formation of an unstable complex known as pre-LC (*pre-loading complex*), which contains the Pol  $\epsilon$  subunit Dpb2p, Mcm10p and GINS, in addition to Sld2p and Dpb11p (Muramatsu et al., 2010). Since phospho-Sld3p binds directly to Dpb11p and both Mcm10p and Sld3p interact with pre-RC components, this transient complex might be formed at the vicinity of licensed origins during S-phase. At the same time, DDK phosphorylates the N-terminal tail of Mcm4p at pre-RC to alleviate its auto-inhibitory activity (Sheu & Stillman, 2010) and to help stabilize Cdc45p binding to pre-RC (Sheu & Stillman, 2006). Stable association of Cdc45p is also facilitated by origin-bound Mcm10p (Sawyer et al., 2004). Since Mcm10p is a pre-LC component, origin-targeting of Pol  $\alpha$  is coupled to the rise of S-CDK activity. In summary, GINS and Cdc45p are recruited for CMG complex assembly and helicase activation, and both Pol  $\alpha$  and Pol  $\epsilon$  are targeted to licensed origins by the joint actions of the S-phase kinases upon origin activation.

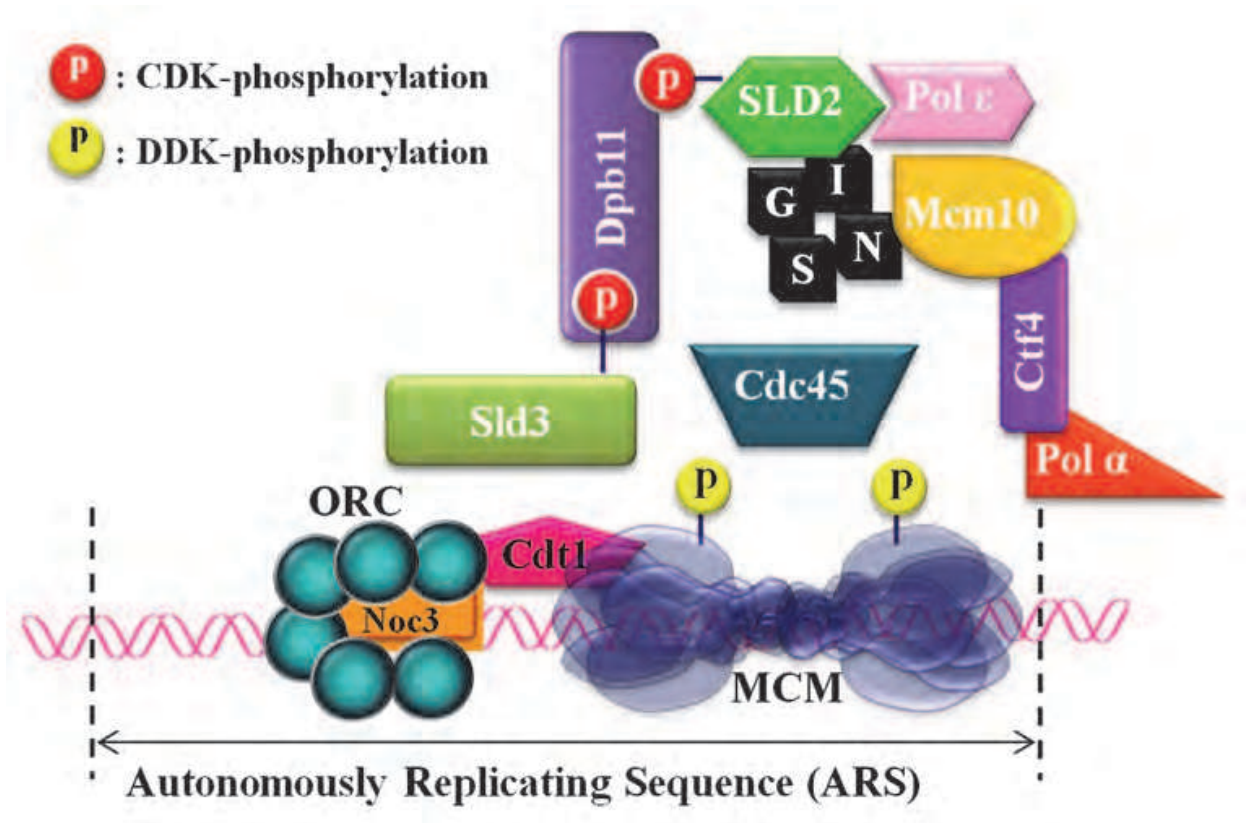


Fig. 2. The current model of origin activation in budding yeast. At the beginning of S-phase, the concerted actions of CDK and DDK trigger origin-recruitment of both Cdc45p and GINS which bind MCM to form the active helicase. At the same time, polymerase  $\epsilon$  and  $\alpha$  are recruited for DNA synthesis. See text for details. Yellow and red circles represent DDK and CDK phosphorylation respectively.



## 2. CDK-dependent anti-rereplication mechanisms

Uncontrolled overreplication of the genome within the same cell cycle is detrimental to cell survival. Hence organisms possess multiple redundant mechanisms to prevent over-replication (Diffley, 2004). As shown in Fig. 3, central to this regulation lies at inhibitory CDK phosphorylations on Orc2p, Orc6p, Cdc6p and Mcm3p, which render these pre-RC components fail to relicense replication origins within the same cell cycle.

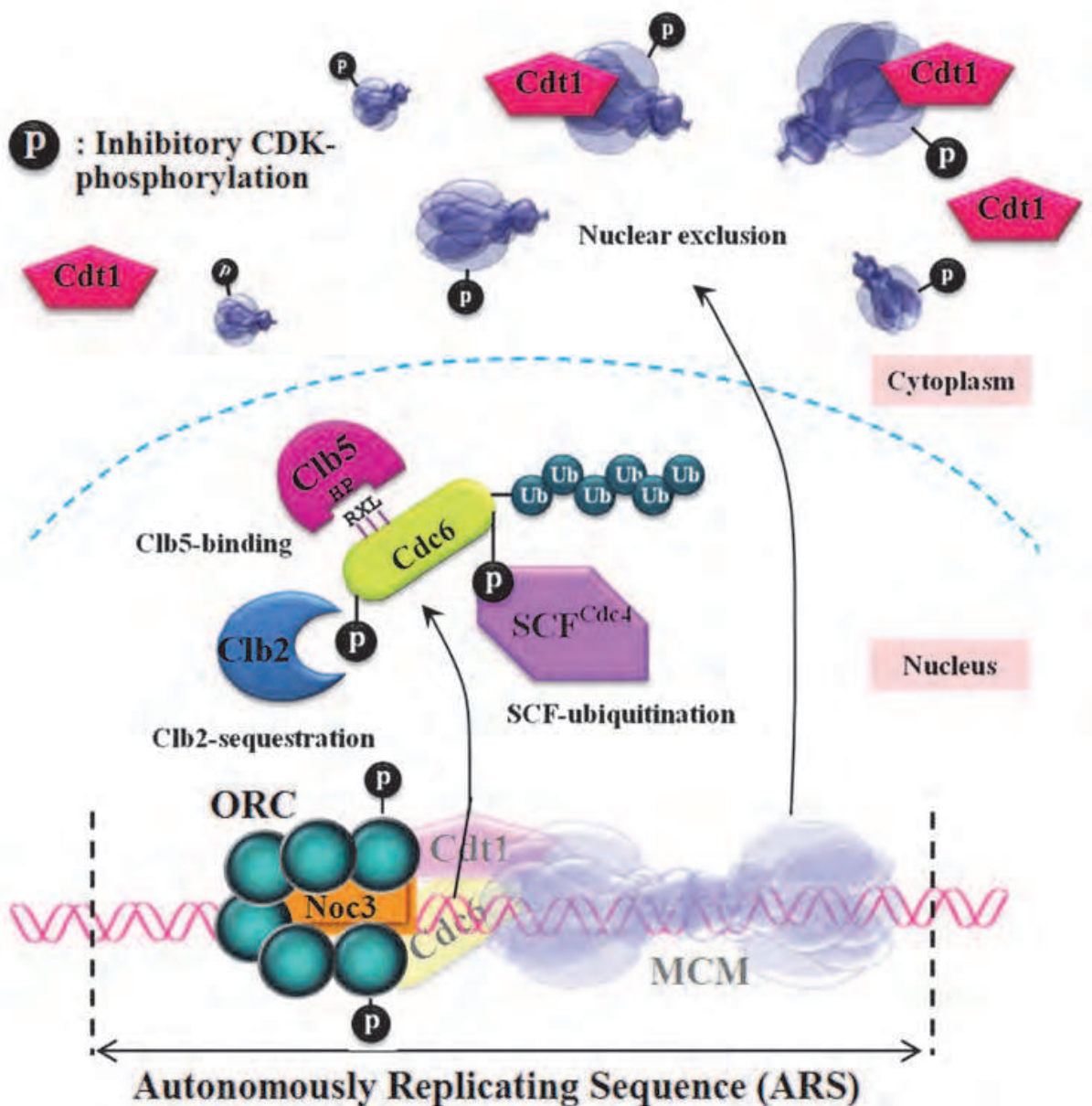


Fig. 3. Anti-rereplication mechanisms in budding yeast.

Anti-rereplication mechanisms in budding yeast targets multiple pre-RC components to render them unable to relicense replication origins within the same cell cycle. All of these mechanisms require CDK-phosphorylations. HP, hydrophobic patch. See text for details

## 2.1 Orc2p

Orc2p is a subunit of the hexameric ORC complex that binds replication origins throughout the cell cycle. Orc2p has six full CDK phosphorylation sites, and it is phosphorylated by Clb-CDK from S-phase until mitotic exit. *In vitro* studies suggest that phosphorylated Orc2p inhibits the ATP binding activity of Orc5p (Makise et al., 2009), and this might affect the overall activity of ORC to promote pre-RC assembly.

## 2.2 Orc6p

Orc6p shares the same phosphorylation profile as Orc2p during the cell cycle. In addition to the four full CDK consensus sites, Orc6p is found to be phosphorylated by CDK at a sub-optimal TP site at amino acid residue 114. Orc6p phosphorylation by CDK blocks the interaction between Cdt1p and Orc6p, thus largely suppressing the loading of Mcm2-7p complex onto replication origins. Furthermore, S-CDKs also bind to the RXL motif of Orc6p to produce a steric hindrance for MCM loading (Chen & Bell, 2011).

## 2.3 Cdc6p

Cdc6p is part of the MCM loader required for pre-RC assembly. From late M to early G1 phase, *de novo* synthesized Cdc6p binds to ORC and activates the ATPase activity of ORC to recruit the Mcm2-7p helicase onto replication origins (Randell et al., 2006; Chen et al., 2007). Cdc6p is a very unstable protein whose protein level is regulated by CDK phosphorylation and ubiquitin mediated proteolysis (Perkins et al., 2001). There are eight CDK consensus sites on Cdc6p; those at the N-terminal region are phosphorylated by Cln-CDK and targeted for robust SCF<sup>Cdc4p</sup>-mediated degradation during the G1/S transition (Drury et al., 1997). In G2/M cells, Cdc6p is phosphorylated by Clb-CDK and degraded through the SCF<sup>Cdc4p</sup> pathway in a much slower rate. And a moderate rate of Cdc6p proteolysis was also observed in alpha-factor blocked cells independent of CDK phosphorylation and the SCF complex (Drury et al., 2000). Although phosphorylation of the N-terminal CDK sites of Cdc6p does not form an SCF phospho-degron in G2/M phase, it creates a strong affinity site for Clb2p-binding that would dislodge Cdc6p from chromatin (Mimura et al., 2004). As such, Cdc6p is incompetent for pre-RC assembly whenever CDK activity persists.

## 2.4 Mcm3p

Mcm3p is a component of the hexameric MCM helicase that is loaded onto chromatin in the last step of pre-RC assembly. Subcellular localization of the MCM complex is cell cycle regulated (Labib et al., 1999). MCM is targeted to the nucleus from late M phase to G1 phase and largely exported to the cytoplasm from S-phase onwards until mitotic exit. Central to this regulation is the phosphorylation status of Mcm3p, which possesses five conserved CDK sites spanning over a NLS-NES (nuclear localization signal-nuclear exit signal) module near the N-terminus. The entire MCM complex contains a single bipartite NLS, which is distributed on two distinct MCM proteins Mcm2p and Mcm3p. Therefore, it is necessary for all six MCM subunits to be assembled into a whole complex in order to generate a functional NLS for nuclear import (Liku et al., 2005). On the other hand, there is an NES in Mcm3p adjacent to the NLS sequence. CDK phosphorylation of Mcm3p is required for nuclear export of the MCM complex, which probably downregulates the NLS activity and upregulates the NES activity (Liku et al., 2005). Therefore, nuclear export of the Mcm2-7p complex also provides a significant contribution to replication control (Nguyen et al., 2001; Liku et al., 2005).

Persistence of high CDK activity from late G1 to M phase ensures that activation of each origin occurs no more than once per cell cycle. The mechanisms that CDKs target multiple pre-RC components for inhibitory phosphorylations contribute to multiple overlaying strategies in cells to antagonize inappropriate DNA rereplication (Blow & Dutta, 2005). In a mutant strain (Nguyen et al., 2001) where all CDK sites of Orc2p and Orc6p were mutated to non-phosphorylatable alanine residues and the MCM complex was constitutively targeted into the nucleus, genome rereplication occurred upon overexpression of stabilized Cdc6p. This indicates that CDK inhibitions on ORC, Cdc6p and Mcm3p act in a redundant manner and each of these mechanisms is mostly sufficient to block the re-assembly of pre-RC during S-phase.

Although the strategies against rereplication vary in metazoans (Arias & Walter, 2005), most of them target pre-RC components. In mammalian cells, Orc1p is degraded by the ubiquitin-proteasome pathway during S-phase via the SCF<sup>Skp2p</sup> E3 ligase (Méndez et al., 2002). Besides, CDK phosphorylation on Orc1p inhibits its chromatin association during mitosis (Li & DePamphilis, 2002). Another initiation protein, the mammalian Cdt1p is targeted by multiple pathways and kept inactive during S and G2-phase. Cdt1p is targeted for degradation by the Cullin-based E3 ligase, Cul4p-Ddb1p<sup>Cdt2p</sup>. Because the degradation depends on the Cdt1p-PCNA interaction (Arias & Walter, 2006), this limits the timing of proteolysis only upon origin firing. Besides, Cdt1p is phosphorylated by CDK in S and G2 phases, which creates a phospho-degron recognized by the SCF<sup>Skp2p</sup> complex and is targeted for degradation (Takeda et al., 2005). Finally a protein known as Geminin was discovered as a binding inhibitor of Cdt1p. Geminin forms a complex with Cdt1p and renders it unable to load the MCM complex onto chromatin, thereby inhibiting pre-RC assembly (Wohlschlegel et al., 2000). Furthermore, a study suggested that Geminin also targets HBO1, which is a histone modifier required to acetylate origin-proximal H4 to promote MCM loading (Iizuka & Stillman, 1999). Geminin, when complexed with Cdt1p, acts as a potent inhibitor against the HBO1 acetyltransferase activity and hence prevents replication licensing (Miotto & Struhl, 2010).

### **3. The quest for a phosphatase to counteract anti-rereplication mechanisms and reset licensing competence during mitotic exit – studies on Cdc14p**

As discussed above, eukaryotic cells utilize elaborate intrinsic mechanisms to prevent DNA rereplication and ensure faithful inheritance of the genetic material. It is of equal importance to remove the blocks by dephosphorylating pre-RC proteins before cells undergo another cell cycle. Otherwise, existence of obstacles to DNA replication from the previous cell cycle may cause genome under-replication and chromosome instability. Cdc14p, a master phosphatase that promotes mitotic exit, fulfills the requirements of such a key regulator of DNA replication.

#### **3.1 Linkage between Cdc14p and DNA replication initiation**

Linkage between Cdc14p and DNA replication initiation has been implicated in a series of genetic data. Similar to all known initiation mutants reported, the plasmid loss of *cdc14-1* cells could be suppressed by adding multiple copies of an ARS to the plasmid (Hogan & Koshland, 1992; Ma et al., 2010). Overexpression of the initiation protein Orc6p caused synthetic dosage lethality in *cdc14-1* cells (Kroll et al., 1996), and synthetic lethality was observed when *cdc14-1* was combined with *cdc6-1*, *orc2-1* or *orc5-1* (Loo et al., 1995; Kroll et al., 1996; Yuste-Rojas & Cross, 2000). Orc6p was found to be dephosphorylated by

recombinant Cdc14p *in vitro* (Bloom & Cross, 2007). A recent study reported that genome-wide under-replication was observed in *cdc14-1* cells (Dulev et al., 2009). Recently, we show that Cdc14p, a dual-specificity phosphatase essential for mitotic exit, is responsible for resetting the competency of replication licensing by dephosphorylating multiple pre-RC proteins (Zhai et al., 2010).

### 3.2 Regulation of Cdc14p activation

Cdc14p is sequestered in the nucleolus for most part of the cell cycle, and it is only released and activated upon anaphase onset sequentially through the FEAR (Cdc fourteen early release) and MEN (mitotic exit network) pathways to counteract several CDK-dependent phosphorylations of mitotic substrates (Stegmeier & Amon, 2004; Sullivan & Morgan, 2007). As such, the functional window of Cdc14p coincides with the temporal profile of pre-RC assembly in the cell cycle.

During mitotic exit, a series of coordinated cellular events occur from anaphase onset to early G1 of the next cell cycle during which CDK activity is inhibited in a graded manner (Sullivan & Morgan, 2007). Cdc14p is the phosphatase responsible for driving these cell cycle events. Cdc14p released by FEAR at early anaphase mainly contributes to promoting onset of sister chromatids segregation, stabilization of anaphase spindle, establishment of spindle midzone, nucleolar segregation and MEN activation (Amon, 2008). The release of Cdc14p by the FEAR pathway is transient and restricted within the nucleus, and Cdc14p will be resequenced to the nucleolus if MEN pathway is defective (Rock & Amon, 2009). The MEN pathway, a Ras-like GTPase signaling cascade, results in complete activation of Cdc14p with the phosphatase located throughout the cell (Stegmeier & Amon, 2004). The main role of Cdc14p activated by the MEN pathway is to trigger CDK inactivation and drive mitotic exit (Amon, 2008). The FEAR and MEN pathways help to ensure a complete segregation of all chromosomes before the onset of mitotic exit and cytokinesis.

The important substrates of the MEN-activated Cdc14p include Cdh1p and Swi5p (Switching deficient) (Visintin et al., 1998). Cdh1p is the late mitotic co-activator of the APC E3 ligase, and it is inactive when phosphorylated by CDK. Dephosphorylation of Cdh1p by Cdc14p activates the APC<sup>Cdh1p</sup> activity which in turn targets mitotic cyclins for destruction. Parallel to this pathway, dephosphorylation of the transcription factor Swi5p triggers its nuclear import to activate *CDC6* and *SIC1* transcription. As a result, high level of APC activity and Sic1p accumulation lead to CDK inhibition.

### 3.3 The role of Cdc14p in resetting replication licensing

In a recent study, our group provided genetic and biochemical evidence to demonstrate the essential role of Cdc14p in dephosphorylating the initiation proteins Orc2p, Orc6p, Cdc6p and Mcm3p and resetting the competency of replication licensing during mitotic exit in budding yeast (Zhai et al., 2010).

In an effort to identify novel factors that are involved in or regulate DNA replication initiation, our lab has carried out a yeast phenotypic screen with randomly mutagenized yeast cells and have obtained several new hypomorphic alleles of *cdc14* mutants as well as mutants in other genes (Ma et al., 2010). These new *cdc14* mutants, like the previous *cdc14-1* (Hogan & Koshland, 1992) and all known replication-initiation mutants reported, have high rates of plasmid loss that can be suppressed by the presence of multiple copies of an ARS on the plasmid (Zou et al., 1997; Tye, 1999; Zhang et al., 2002; Ma et al., 2010). These results and



the previous genetic data described above prompted us to further examine the role of Cdc14p in DNA replication licensing.

Because of the parallel temporal profiles of pre-RC assembly and mitotic exit, it is difficult to perform loss-of-function experiments to examine the function of Cdc14p in replication licensing during the M/G1 transition. To circumvent the requirement of Cdc14p for mitotic exit, a mitotic rereplication system based on pulsed expression of an N-terminally truncated, relatively stable form of Sic1p (Sic1 $\Delta$ NT) was employed (Noton & Diffley, 2000). Sic1 $\Delta$ NT can inhibit mitotic CDK activity and hence drive pre-RC assembly when overexpressed from a galactose-inducible promoter (Dahmann et al., 1995). Resumption of CDK activity through subsequent repression of the GAL promoter and turnover of Sic1 $\Delta$ NT activates replication origins and causes rereplication when cells are still blocked in mitosis. By using this mitotic replication system, we showed that Cdc14p is essential for DNA replication by promoting pre-RC assembly.

Orc2p, Orc6p, Cdc6p and Mcm3p are known targets of CDKs (Ubersax et al., 2003). We showed that Cdc14p physically interacts with and dephosphorylates Orc2p, Orc6p, Cdc6p and Mcm3p *in vivo* and *in vitro*. Consistent with this, overexpression of Orc2p, Orc6p, Cdc6p or Mcm3p, but not the corresponding non-phosphorylatable (S/T to A) forms, resulted in severe growth defects in *cdc14-1* cells, probably by overloading the weakened Cdc14-1 phosphatase with one of its substrates. Inactivation of the Cdc14-3 protein also largely inhibited ORC dephosphorylation, nuclear localization of Mcm4-EGFP, and chromatin association of Cdc6p and MCM proteins.

To demonstrate that Orc2p, Orc6p, Cdc6p and Mcm3p are the major set of Cdc14p substrates whose dephosphorylations during mitotic exit promote replication licensing, we tested if combined non-phosphorylatable and/or phosphorylation-insensitive mutant alleles of these initiation proteins could bypass the function of Cdc14p in pre-RC assembly. A previous study showed that pre-RC assembly, origin firing and partial mitotic genome reduplication occurred upon ectopic expression of Cdc6 $\Delta$ NT in a quadruple mutant strain (*orc2-6A orc6-4A MCM7-NLS GAL-cdc6 $\Delta$ NT*) where the CDK consensus motifs in Orc2p and Orc6p were mutated to non-phosphorylatable forms, the MCM complex was constitutively nuclear-targeted and Cdc6p was expressed in a stabilized form (Nguyen et al., 2001). We compared DNA rereplication in *CDC14* wild-type and *cdc14-3* mutant cells in the quadruple (*orc2-6A orc6-4A MCM7-NLS GAL-cdc6 $\Delta$ NT*) mutant background upon Cdc6 $\Delta$ NT ectopic expression after Cdc14-3 inactivation in cells blocked in mitosis. Efficient DNA rereplication occurred in both *CDC14* and *cdc14-3* cells even when the Cdc14-3 protein was inactivated. These results strongly suggest that, of all initiation proteins, Orc2p, Orc6p, Cdc6p and Mcm3p represent the major set of Cdc14p substrates whose dephosphorylation is necessary for pre-RC assembly and DNA replication.

#### 4. Summary and discussion

In eukaryotes, replication licensing is achieved through sequential loading of several replication-initiation proteins onto replication origins to form pre-replicative complexes (pre-RCs). On the other hand, unscheduled replication licensing is prevented by cyclin-dependent kinases (CDKs) through inhibitory phosphorylations of multiple initiation proteins. It is known that CDK inactivation during mitotic exit promotes pre-RC formation for the next cell cycle. We have recently shown in budding yeast that Cdc14p dephosphorylates Orc2p, Orc6p, Cdc6p and Mcm3p to restore their competence for pre-RC

assembly (Zhai et al., 2010). Cells without functional Cdc14p failed to dephosphorylate the initiation proteins and to form pre-RCs even when CDK activities are inhibited, and they could not replicate DNA in the mitotic rereplication systems. On the other hand, pulsed ectopic expression of Cdc14p in mitotic cells resulted in efficient pre-RC assembly and DNA rereplication. Furthermore, Cdc14p becomes dispensable for DNA rereplication in mitotic cells with combined non-phosphorylatable / phosphorylation-insensitive alleles of the initiation proteins. These results unravel the essential role of Cdc14p in replication licensing besides its established functions for mitotic exit, providing new insight into the intricate regulation of DNA replication by the interplay between CDKs and the Cdc14p phosphatase.

4.1 Proposed model of Cdc14p-driven origin licensing

Identification of multiple pre-RC components as a new set of Cdc14p substrates extends the physiological roles of this phosphatase outside the scope of mitotic exit. Based on the available data, we propose that Cdc14p provides a favorable environment for resetting the competency of replication licensing during mitotic exit by dephosphorylating multiple initiation proteins as well as other factors that promote the expression and/or stabilization of the initiation proteins (see diagrams in Fig. 4). During mitotic exit, Cdc14p dephosphorylates Swi5p, triggering nuclear localization of Swi5p which in turn induces the expression of Sic1p and Cdc6p (Piatti et al., 1995; Visintin et al., 1998). Cdc14p also stabilizes Sic1p and Cdc6p by keeping them in the dephosphorylated forms which are recognized by the SCF ubiquitination system (Verma et al., 1997; Perkins et al., 2001). Dephosphorylation of Cdh1p by Cdc14p activates APC<sup>Cdh1p</sup> which in turn mediates Clb2p degradation to free Cdc6p for pre-RC assembly (Visintin et al., 1998; Jaspersen et al., 1999; Mimura et al., 2004). Clb2p degradation and Sic1p accumulation also contribute to CDK inactivation (Stegmeier & Amon, 2004). Dephosphorylation of ORC by Cdc14p restores the ability of ORC to bind ATP (Makise et al., 2009) and other initiation proteins, and nuclear import of MCM complex is permitted when Mcm3p is dephosphorylated by Cdc14p. Altogether, these events promote pre-RC assembly in a step-wise manner onto replication origins for replication initiation.

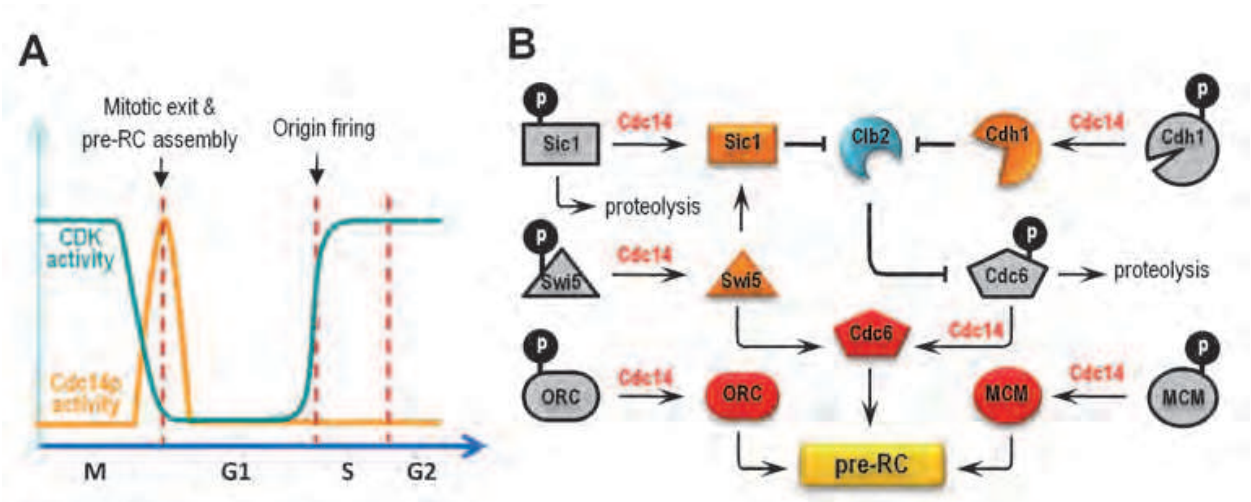


Fig. 4. Model depicting the essential role of Cdc14p in pre-RC assembly during mitotic exit. (A) Schematic diagram illustrating the change of CDK activity and the Cdc14p phosphatase activity in the cell cycle. (B) Diagram of Cdc14p substrates related to pre-RC assembly.

Phosphorylated, inactive Cdc14p substrates are shaded gray, while their dephosphorylated, active forms are highlighted in red (substrates identified by us in Zhai et al., 2010) or orange (previously identified substrates). Modified from Zhai et al., 2010. See text for details.

#### 4.2 Coordinating replication licensing with other mitotic events by Cdc14p

Given the broad spectrum of Cdc14p targets and the sophisticated biphasic control of Cdc14p activation, Cdc14p may help to constitute a framework of a spatiotemporal program to coordinate different phosphorylation-regulated mitotic events. For example, it has been recently demonstrated that the histone acetyltransferase Gcn5p may facilitate replication licensing by inducing local decondensation at ARS-proximal regions (Espinosa et al., 2010). It might be worth testing whether the histone modifier Gcn5p and/or its origin-targeting factor are subject directly or indirectly to Cdc14p control. Genome-wide screening of Cdc14p substrates will likely shed light on this and other issues related to the coordination between replication licensing and mitotic exit.

#### 4.3 Conserved origin-resetting mechanism in other higher eukaryotes?

Although CDK-dependent mechanisms against genome reduplication are diverse in eukaryotes (Arias & Walter, 2007), our findings in budding yeast underscore the importance of looking for phosphatases or other CDK-antagonizing activities that extinguish the inhibitory phosphorylations on initiation proteins for replication licensing in other organisms. There are two homologs of Cdc14p in humans known as Cdc14A and Cdc14B (Amon, 2008). However, their roles in mitotic exit remain controversial. Besides, it was suggested that the protein phosphatase-1 (PP1) might be the responsible phosphatase for driving mitotic exit (Wu et al., 2009). Hence it remains unknown whether or not Cdc14p homologs in higher eukaryotes also control replication licensing.

If the function of Cdc14p is conserved in human cells, the dual roles of Cdc14p in mitosis and DNA replication, and the intimate association of cancer with replication licensing (Shima et al., 2007) and the cell cycle (Hook et al., 2007), Cdc14p may promise to be an important protein to study in both normal and cancer cells.

#### 4.4 Implications in DNA amplification

DNA amplification represents the most extreme case of re-replication in which only specific genomic loci undergo repeated origin firing while replication of the rest of the genome is inhibited by anti-rereplication mechanisms (Claycomb & Orr-Weaver, 2005). DNA amplification provides extra DNA templates to boost gene expression in response to developmental needs and in tumorigenesis. If Cdc14p or a functional analog exists in higher eukaryotes, targeting such phosphatase to amplification loci would contribute to establishing a restricted zone of dephosphorylating activity for pre-RC assembly. If this is true, one could imagine that such phosphatase should only selectively act against inhibitory phosphorylations of pre-RC proteins, but not affect origin activation by CDK.

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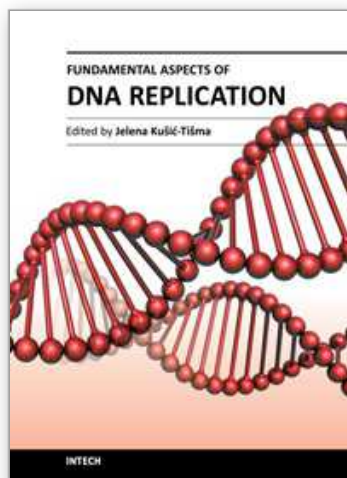
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## **Fundamental Aspects of DNA Replication**

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DNA replication, the process of copying one double stranded DNA molecule to produce two identical copies, is at the heart of cell proliferation. This book highlights new insights into the replication process in eukaryotes, from the assembly of pre-replication complex and features of DNA replication origins, through polymerization mechanisms, to propagation of epigenetic states. It also covers cell cycle control of replication initiation and includes the latest on mechanisms of replication in prokaryotes. The association between genome replication and transcription is also addressed. We hope that readers will find this book interesting, helpful and inspiring.

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