Cdc14p resets the competency of replication licensing by dephosphorylating multiple initiation proteins during mitotic exit in budding yeast

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Summary

In eukaryotes, replication licensing is achieved through sequential loading of several replication-initiation proteins onto replication origins to form pre-replicative complexes (pre-RCs), and 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. However, whether the removal of the inhibitory phosphorylations on the initiation proteins is essential and the identity of the acting phosphatase(s) remain unknown. Here, we show that cell division cycle protein 14 (Cdc14p) dephosphorylates replication-initiation proteins Orc2p, Orc6p, Cdc6p and Mcm3p to restore their competence for pre-RC assembly in the budding yeast *Saccharomyces cerevisiae*. Cells without functional Cdc14p fail to dephosphorylate initiation proteins and to form pre-RCs – even when CDK activities are suppressed – and cannot replicate DNA in mitotic rereplication systems, whereas pulsed ectopic expression of Cdc14p in mitotic cells results in efficient pre-RC assembly and DNA rereplication. Furthermore, Cdc14p becomes dispensable for DNA rereplication in mitotic cells with combined non-phosphorylatable and/or phosphorylation-insensitive alleles of the initiation proteins. These data unravel the essential role of Cdc14p in replication licensing, beyond its established functions in mitotic exit, providing new insight into the intricate regulation of DNA replication through the interplay of CDKs and the Cdc14p phosphatase.

Key words: Cdc14p phosphatase, DNA replication, Replication licensing, Replication-initiation proteins, Cell cycle, Budding yeast

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Introduction

Faithful inheritance of the genetic material in eukaryotic cells requires DNA replication to be precisely controlled so that it occurs exactly once per cell cycle. The key to this regulation lies within the initiation of DNA replication. The prerequisite of replication initiation is pre-replicative complex (pre-RC) assembly by stepwise loading of multiple replication-initiation proteins onto replication origins during the M to G1 transition; this process is referred to as replication licensing (Bell and Dutta, 2002). The cycle of pre-RC assembly and disassembly is governed by the oscillating rhythm of activities of cyclin-dependent kinases (CDKs). During the G1 to S transition in the budding yeast *Saccharomyces cerevisiae*, replication origins are activated by CDK-dependent phosphorylation of replication-initiation proteins, including Orc2p, Orc6p, Cdc6p, Mcm3p and Sld2p, and some other proteins. Whereas phosphorylation of some initiation proteins, such as Sld2p and Sld3p, is required for origin activation, phosphorylation of other initiation proteins is inhibitory for pre-RC formation. For example, phosphorylation leads to degradation of Cdc6p, exclusion of the Mcm2-7 complex from the nucleus and origin recognition complex (ORC) proteins inactivation (Diffley, 2004). Hence, the loss of competence of pre-RC reassembly by inhibitory phosphorylation of some of the initiation proteins in the process of origin activation constitutes the major block to genome reduplication within the same cell cycle (Arias and Walter, 2007).

During mitotic exit, CDK activities are reduced to low levels because of the degradation of cyclins and the accumulation of the CDK inhibitor Sic1p (Stegmeier and Amon, 2004). Meanwhile, Orc2p and Orc6p become dephosphorylated, Cdc6p is synthesized de novo, and the Mcm2-7 complex is imported into the nucleus and then loaded onto replication origins, forming, with other initiation proteins, pre-RCs (Diffley, 2004). Whereas the CDK-mediated anti-rereplication mechanisms have been studied in considerable detail, whether dephosphorylation of initiation proteins is necessary for origin resetting and the identity of the acting phosphatase(s) that might control replication licensing remain largely unknown.

The temporal profile of pre-RC assembly coincides with the functional window of Cdc14p (Bell and Dutta, 2002) – a master phosphatase that orchestrates various events essential for mitotic exit (Stegmeier and Amon, 2004; Sullivan and Morgan, 2007) – in the cell cycle. Cdc14p is sequestered in the nucleolus for most of the cell cycle, and is only released and activated sequentially through the action of the Cdc fourteen early release (FEAR) and mitotic exit network (MEN) pathways upon anaphase onset to counteract CDK-dependent phosphorylations of mitotic substrates (Stegmeier and Amon, 2004). However, it is not known whether Cdc14p is also responsible for dephosphorylating replication-initiation proteins for pre-RC formation during mitotic exit.

A link between Cdc14p and DNA replication initiation has been implicated by a series of genetic data. Similar to all known initiation mutants tested (Loo et al., 1995; Zou et al., 1997; Tye, 1999; Zhang et al., 2002), the plasmid-loss phenotype of *cdc14-1* cells was suppressed by adding multiple copies of an autonomously

replicating sequence (ARS) to the plasmid (Hogan and Koshland, 1992). Furthermore, overexpression of the initiation protein Orc6p caused synthetic dosage lethality in *cdc14-1* cells (Kroll et al., 1996), and synthetic lethality was observed between *cdc14-1* and *cdc6-1*, *orc2-1* or *orc5-1* (Loo et al., 1995; Hardy, 1996; Yuste-Rojas and Cross, 2000). In addition, Orc6p was found to be dephosphorylated by recombinant Cdc14p in vitro, although the corresponding in vivo role was not favored (Bloom and Cross, 2007). In spite of these indirect clues, it has not been determined whether Cdc14p has a direct role in the initiation of DNA replication. By contrast, a previous study has discounted Cdc14p as the physiological phosphatase that dephosphorylates initiation proteins to promote pre-RC formation and DNA replication (Noton and Diffley, 2000). A recent study has reported genome-wide under-replication in *cdc14-1* cells, which was attributed to dosage insufficiency of some replication proteins; however, the possible function of Cdc14p in promoting replication licensing by dephosphorylating pre-RC components was not addressed (Dulev et al., 2009). Therefore, the essential role of Cdc14p in replication licensing remains controversial.

Here, we present genetic and biochemical evidence to show the essential role of Cdc14p in dephosphorylating the initiation proteins Orc2p, Orc6p, Cdc6p and Mcm3p to reset the competency of replication licensing during mitotic exit in budding yeast.

Results

Cdc14p is required for DNA replication in mitotic rereplication systems

In an effort to identify factors that are involved in or regulate DNA replication initiation, we have previously carried out a phenotypic screen with yeast cells that were randomly mutagenized (Cheng et al., 2010; Ma et al., 2010; Wang et al., 2010). From this screen, we isolated several new hypomorphic alleles of *cdc14* mutants that, like *cdc14-1* (Hogan and Koshland, 1992) and all known replication-initiation mutants previously tested (Loo et al., 1995; Zou et al., 1997; Tye, 1999; Zhang et al., 2002), have high rates of plasmid loss that can be rescued by adding multiple copies of an ARS to the plasmid (Ma et al., 2010). These results and the previous genetic data described above, prompted us to re-examine the role of Cdc14p in DNA replication licensing.

Because of the parallel temporal profiles of mitotic exit and pre-RC assembly, which occur upon anaphase onset in budding yeast, it is difficult to use loss-of-function approaches to examine the function of Cdc14p in replication licensing without affecting mitotic exit. To circumvent the requirement for Cdc14p in mitotic exit, we employed a mitotic rereplication system based on pulsed expression of an N-terminally truncated, relatively stable form of Sic1p (Sic1ΔNT) (Noton and Diffley, 2000). Sic1ΔNT can inhibit mitotic CDK activities and, hence, drive pre-RC assembly when overexpressed from a galactose-inducible promoter (Dahmann et al., 1995). Resumption of CDK activities through subsequent repression of the *GAL* promoter activates replication origins and causes rereplication when cells are still blocked in mitosis by nocodazole. It is noteworthy that a previous study using *cdc14-1* cells based on this system failed to detect the requirement for Cdc14p in DNA replication (Noton and Diffley, 2000). We suspect that this is due to the leakiness of the *cdc14-1* allele and insufficient inactivation (1 hour at 37°C) of the Cdc14-1 protein. In fact, we found that the *cdc14-3* allele is much more temperature sensitive than *cdc14-1*, as *cdc14-3* cells did not survive even at 30°C, whereas *cdc14-1* had moderate growth (supplementary material Fig. S1), consistent with a recent publication (Miller et al., 2009). Therefore, we tested the requirement for Cdc14p in mitotic DNA rereplication using *cdc14-3* cells. Indeed, fluorescence-activated cell sorting (FACS) analysis revealed a failure of rereplication when *cdc14-3* cells were incubated at 38°C for 2 or 3 hours to heat inactivate the Cdc14-3 protein before Sic1ΔNT induction (Fig. 1C,D). The severity of rereplication defects correlated with the extent of Cdc14-3 protein inactivation, because the ability of *cdc14-3* cells to replicate DNA was progressively lost with increased time of heat inactivation from 0 to 3 hours at 38°C (Fig. 1A–D).

To confirm the role of Cdc14p in replication, we constructed a temperature-inducible degron allele of *CDC14* (*cdc14-td*) in the mitotic rereplication strain. The *cdc14-td* allele consists of a temperature-sensitive degron fused at the N terminus of the

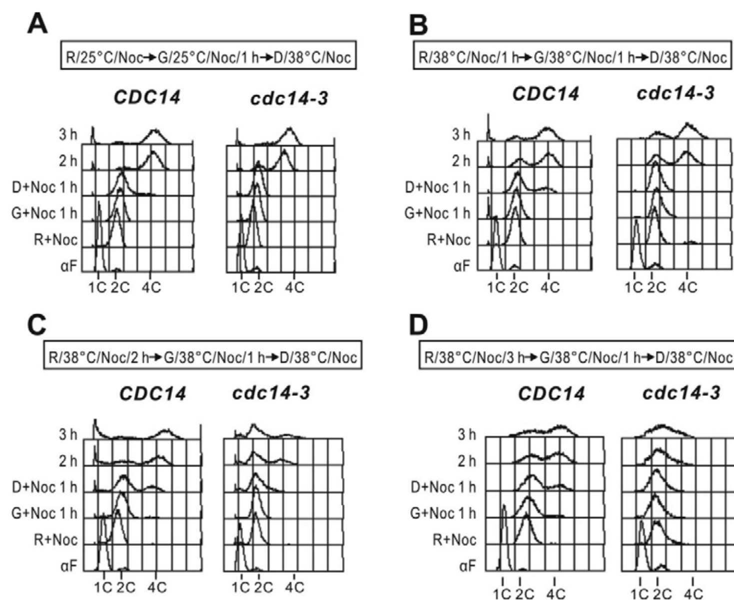

Fig. 1. Cdc14p is required for DNA replication in the mitotic rereplication system. Strains YL985 (*CDC14 GAL-sic1ΔNT*) and YL1093 (*cdc14-3 GAL-sic1ΔNT*) were synchronized in M phase in YPR plus nocodazole (R+Noc) at 25°C. After heat inactivation at 38°C for 1–3 hours (**B–D**), or without heat inactivation (**A**), cells were changed into YPG plus nocodazole (G+Noc) to induce Sic1ΔNT expression for 1 hour and then into YPD plus nocodazole (D+Noc) for the rest of the time points to repress Sic1ΔNT expression at 38°C and thus allow DNA rereplication. Samples were processed for FACS analysis to examine rereplication. αF, α-factor.

endogenous *CDC14*. Upon heat inactivation, the entire fusion protein can be efficiently degraded through the ubiquitin-mediated proteolysis pathway (Dohmen et al., 1994; Sanchez-Diaz et al., 2004). The Cdc14-td protein was completely degraded upon heat inactivation for 3 hours (supplementary material Fig. S2E). The rereplication assays based on *cdc14-td* showed similar results to those based on *cdc14-3* (supplementary material Fig. S2A–D). Note that a shorter heat-inactivation time (1 hour) for both *cdc14-3* (Fig. 2B) and *cdc14-td* (supplementary material Fig. S2B) strains led to subtle or mild rereplication defects; this might explain the results from *cdc14-1* cells with 1 hour of heat inactivation in a previous report (Noton and Diffley, 2000).

To further confirm the role of Cdc14p in DNA replication, we used a mitotic rereplication system based on transient chemical inhibition of an analogue-sensitive (*as*) allele of *CDC28* (*cdc28-as1*) (Bishop et al., 2000). The PP1 analog II, 1-NM-PP1, acts as a specific, potent and reversible inhibitor of the mutant Cdc28-as1 protein. Transient treatment of *cdc28-as1* mitotic cells with 1-NM-PP1 efficiently drove origin licensing and firing, leading to DNA reduplication in both wild-type and *cdc14-3* cells at the permissive temperature of 25°C (supplementary material Fig. S3A). By contrast, incubation of *cdc14-3* cells at 38°C for 2 hours before the 1-NM-PP_1 treatment totally abolished rereplication (supplementary material Fig. S3C), just as it did in the Sic1ΔNT-based rereplication system described above. Also similar to the results with the Sic1ΔNT system, incubation of *cdc14-3* cells for 1 hour was insufficient to produce obvious replication defects in the *cdc28-as1* system (supplementary material Fig. S3B).

Because the full release and activation of Cdc14p from the nucleolus during mitotic exit requires the MEN signaling cascade (Stegmeier and Amon, 2004), we also tested whether the MEN component Cdc15p is required for mitotic rereplication. Incubation of *cdc15-2* cells for 1 hour at 38°C before Sic1ΔNT induction resulted in mild rereplication defects (supplementary material Fig. S4B), and heat inactivation for 2 hours caused significant rereplication defects (supplementary material Fig. S4C). Taken together, the data from *cdc14-3* cells in two different rereplication systems and from *cdc14-td* and *cdc15-2* cells in a single

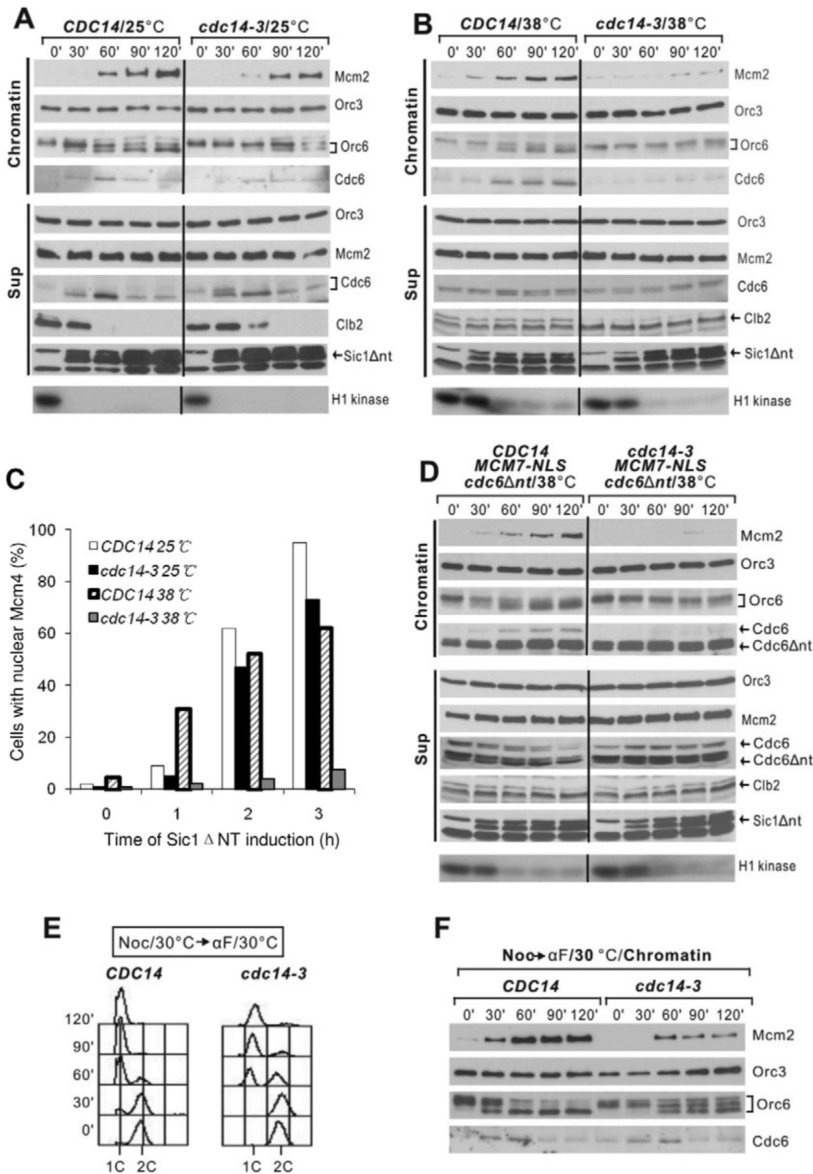

Fig. 2. Cdc14p is essential for pre-RC assembly in both the mitotic rereplication system and the normal cell cycle. (**A**,**B**) Strains YL985 (*CDC14 GAL-sic1ΔNT*) and YL1093 (*cdc14-3 GAL-sic1ΔN*) were synchronized in M phase in YPR plus nocodazole at 25°C. After 2 hours of heat inactivation at 38°C (B; 0′), galactose was added to the medium to induce Sic1ΔNT expression, and α-factor was added to suppress Cln–CDK activity. The control cells without heat inactivation were kept at 25°C throughout the experiment (A). Cells from both 25°C and 38°C cultures were harvested for chromatin-binding assays along the course (30′–120′) of Sic1ΔNT induction. Mitotic CDK activities were determined by kinase assays with histone H1 as the substrate and precipitates by $p13^{Suc1}$ agarose beads from whole-cell extracts as the source of the CDKs. (**C**) Percentages of *CDC14* wild-type and *cdc14-3* cells with nuclear localization of Mcm4-EGFP upon Sic1ΔNT expression monitored by fluorescence microscopy (see supplementary material Fig. S5 for fluorescence images). Cell samples were collected as described in A and B, except that α-factor was not used during the mitotic arrest. (**D**) Chromatin-binding assays were performed to detect pre-RC assembly in strains YL1059 (*CDC14 MCM7-NLS cdc6ΔNT GAL-sic1ΔNT*) and YL1098 (*cdc14-3 MCM7-NLS cdc6ΔNT GAL-sic1ΔNT*) in the same experiments as described in A and B. (**E**,**F**) *CDC14* and *cdc14-3* cells were synchronized in M phase in YPD plus nocodazole at 25°C, and the cultures were then incubated at 30°C for 30 minutes. Afterwards, the cultures were released into fresh YPD plus α-factor at 30°C. Samples were collected at 30 minute intervals starting at the point of release, and were processed for FACS analysis (E) and chromatin-binding assays (F).

rereplication system demonstrate that Cdc14p is required for DNA replication.

Cdc14p is essential for pre-RC assembly in the mitotic replication system and in the normal cell cycle

We reasoned that the faulty DNA replication in *cdc14-3* cells probably resulted from pre-RC assembly deficiency when the Cdc14-3 protein was inactivated. To test this possibility, we used chromatin-binding assays to examine the efficiency of pre-RC formation after Sic1ΔNT overexpression in mitotic cells. Because mini-chromosome maintenance (MCM) proteins bind to the chromosomes only at ARS sequences when pre-RCs are formed in wild-type cells, a reduction in or absence of chromatin association of MCM proteins in *cdc14-3* cells compared with wild-type cells under the same treatment indicates pre-RC assembly defects in the mutant. Our data show that dephosphorylation of Orc6p and chromatin association of Cdc6p and Mcm2p were partially defective at the permissive temperature of 25°C (Fig. 2A) and totally faulty at 38°C (Fig. 2B) in *cdc14-3* cells, indicating a reduction in and failure of, respectively, pre-RC formation. Such defects were not due to inefficient CDK inactivation in *cdc14-3* mutant cells, because Sic1ΔNT was overexpressed to similar levels and CDK activity was reduced to equally low levels in *CDC14* wild-type and *cdc14-3* mutant cells (Fig. 2B).

Because Clb–CDK phosphorylation of Mcm3p drives nuclear export of the MCM complex into the cytoplasm to prevent rereplication (Nguyen et al., 2000; Liku et al., 2005), we examined the efficiency of nuclear import of the MCM complex when Sic1ΔNT was overexpressed in mitotic cells and tested the requirement for Cdc14p in this process. Inactivation of the Cdc14-3 protein at 38°C largely abolished the nuclear localization of Mcm4–EGFP, and partial defects were observed even at 25°C (Fig. 2C; supplementary material Fig. S5). These results suggest that Cdc14p is required for the nuclear import of the MCM complex.

It is known that Cdc14p also dephosphorylates the transcription factor Swi5p, which, in turn, promotes de novo synthesis of Cdc6p (Piatti et al., 1995; Visintin et al., 1998). Thus, the chromatin-loading failure of MCM proteins, which might be present in the nucleus at levels below the detection limit of fluorescence microscopy, might be due to an indirect effect of Cdc6p insufficiency in *cdc14-3* cells. However, we ruled out this possibility by showing that Mcm2p was still absent from the chromatin at 38°C in *cdc14-3* cells expressing stable Cdc6Δnt and Mcm7–NLS, which targets MCM to the nucleus (Fig. 2D), and that these cells failed to reduplicate their genome after pulsed expression of Sic1ΔNT (supplementary material Fig. S6). These results reinforce the idea that dephosphorylation of ORC, Cdc6p and Mcm3p by Cdc14p and, hence, antagonization of rereplication blockades, are essential for replication licensing.

To demonstrate the role of Cdc14p in replication licensing in the normal cell cycle, we examined pre-RC formation during the M to G1 transition at 30°C without using the mitotic rereplication systems. Under these semi-permissive conditions, the residual function of the Cdc14-3 protein was able to promote complete mitotic exit when *cdc14-3* cells were released from M phase into G1 phase (Fig. 2E). However, pre-RC assembly was obviously suppressed; Orc6p was only partially dephosphorylated and the chromatin association of Mcm2p was significantly reduced compared with wild-type cells (Fig. 2F). Together, our data indicate that Cdc14p is required for pre-RC assembly in the normal cell cycle as well as in the mitotic rereplication systems, and that CDK inactivation alone, without dephosphorylation of initiation proteins promoted by Cdc14p, is not sufficient to reset the competency of replication licensing.

The pre-RC components Orc2p, Orc6p, Cdc6p and Mcm3p are substrates of the Cdc14p phosphatase

Given that Orc2p, Orc6p, Cdc6p and Mcm3p are targets of CDKs (Ubersax et al., 2003) and that Cdc14p prefers to dephosphorylate many CDK substrates (Stegmeier and Amon, 2004; Sullivan and Morgan, 2007), we suspected that these initiation proteins are also substrates of Cdc14p. Because overexpression of Orc6p causes synthetic dosage lethality in *cdc14-1* cells (Kroll et al., 1996), presumably by overloading the weakened Cdc14-1 phosphatase with one of its substrates, we employed the same genetic assay to test whether similar phenotypic enhancement occurs when other initiation proteins are overexpressed. Indeed, overexpression of Orc2p, Orc6p, Cdc6p and Mcm3p individually resulted in severe growth defects in *cdc14-1* but not wild-type *CDC14* cells (Fig. 3A,B). By contrast, overexpression of the corresponding non-phosphorylatable forms, in which all serine or threonine residues in the conserved CDK phosphorylation sites are replaced with alanine, resulted in undectable (for *orc2-6A*) or less severe (for *orc6-4A* and *mcm3-5A*) phenotypes. (It is possible that some cryptic CDK phosphorylation sites are still present in the *orc6-4A* and *mcm3-5A* mutant genes.) Cdt1p, an MCM loader without any predicted or known CDK phosphorylation motifs, had no observable effect on *cdc14-1* cells (Fig. 3A). The genetic aggravation of the *cdc14-1* growth defects by a high dosage of one of the initiation proteins that can be phosphorylated by CDKs indicates a potential enzyme–substrate relationship between Cdc14p and the initiation proteins Orc2p, Orc6p, Cdc6p and Mcm3p.

We then determined whether Cdc14p can bind to the phosphorylated initiation proteins in vivo. The phosphatase-dead mutant Cdc14^{C283S} has previously been used as a substrate trap to capture the complexes formed between Cdc14p and its cognate phospho-substrates (Hall et al., 2008). We found that Orc2p, Orc6p and Mcm3p could be co-immunoprecipitated with FLAG–Cdc14^{C283S}, but not wild-type FLAG–Cdc14p, expressed from the *GAL1* promoter to override the sequestration of Cdc14p by its inhibitors in mitotic cells (Fig. 3C). By contrast, non-phosphorylatable forms of Orc2p (Orc2-6A) and Orc6p (Orc6-4A), which are functional in DNA replication, failed to interact with either FLAG–Cdc14p or FLAG–Cdc14^{C283S} (supplementary material Fig. S7). Furthermore, yeast two-hybrid analysis showed that, of the six ORC subunits, only Orc2p and Orc6p formed weak interactions with Cdc14p and stronger interactions with Cdc14^{C283S}, whereas the non-phosphorylatable Orc2-6A or Orc6-4A did not interact with Cdc14p or Cdc14^{C283S} (Fig. 3E,F). Together, these results suggest that the interactions between Cdc14p and Orc2p, Orc6p and Mcm3p are transient and require intact CDK phosphorylation sites, consistent with an enzyme–substrate relationship between Cdc14p and the initiation proteins. In addition, physical interaction of Cdc6p with either Cdc14p or Cdc14^{C283S} was detected by co-immunoprecipitation assays, with a stronger interaction between Cdc6p and Cdc14^{C283S}, when both Cdc6p and Cdc14p or Cdc14^{C283S} were ectopically expressed in mitotic cells (Fig. 3D).

Next, we asked whether Cdc14p can dephosphorylate the initiation proteins in vivo. Cdc14p was ectopically expressed from

the *GAL1* promoter in mitotic cells to allow accumulation of active Cdc14p in both the nucleus and cytoplasm (Pereira et al., 2002). Using overexpression of Glc7p (a protein phosphatase 1 catalytic subunit) and Sit4p (a protein phosphatase 2A-related phosphatase) as specificity controls, a previous study demonstrated the specific dephosphorylation of the Cdc14p substrates by the overexpressed

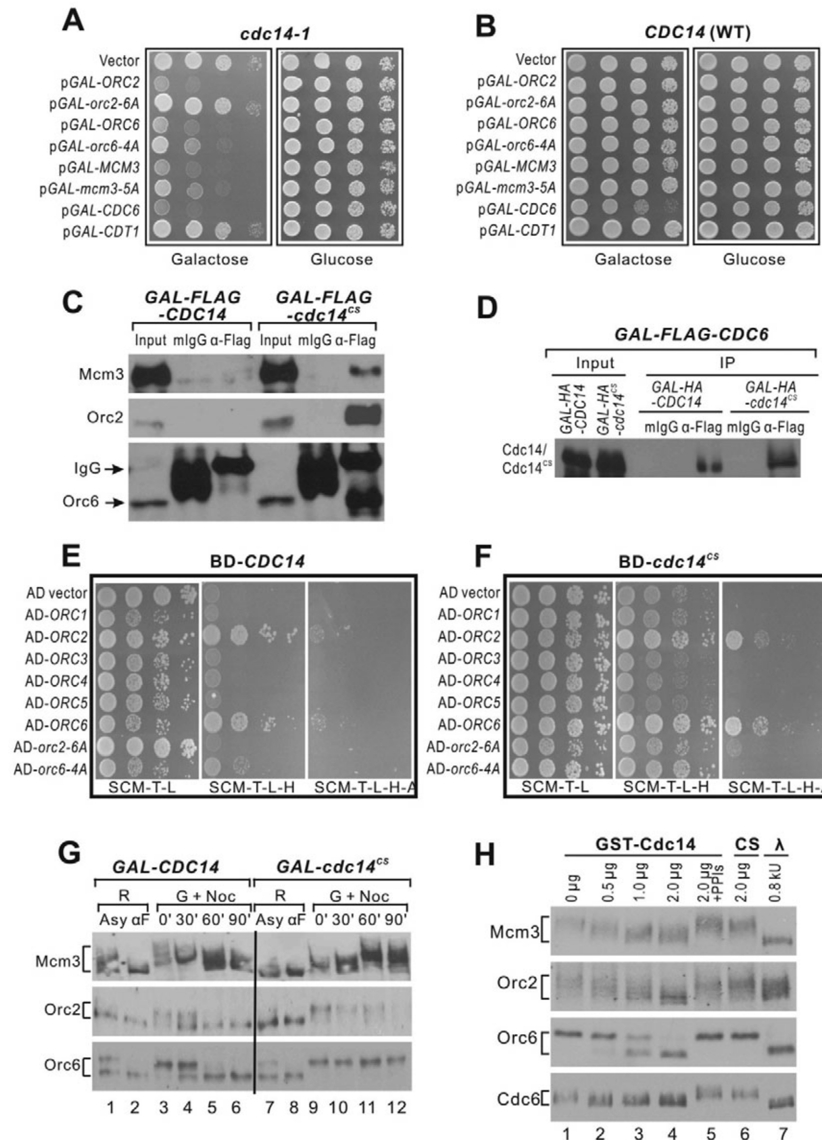

Fig. 3. The pre-RC components Orc2p, Orc6p, Cdc6p and Mcm3p are substrates of the Cdc14p phosphatase. (**A**,**B**) Synthetic dosage lethality between *cdc14-1* and the overexpressed initiation proteins. Tenfold serial dilutions of *cdc14-1* cells (A) or the control wild-type (WT) W303-1a cells (B) harboring the indicated plasmids were spotted onto SC-Ura/galactose and SC-Ura/glucose media plates and incubated at 25°C for 3 days. (**C**,**D**) Physical interactions between Cdc14p and the initiation proteins detected by co-immunoprecipitation. (C) Cells from strains YL944 (*cdc26Δ GAL-FLAG-CDC14*) and YL946 (*cdc26Δ GAL-FLAG-cdc14^{C283S}*) were cultured in YPR at 37°C for 3 hours to obtain metaphase synchrony resulting from *CDC26* deletion. Galactose was then added to induce *GAL-FLAG-CDC14* or *GAL-FLAG-cdc14^{C283S}* expression. Cell extracts were prepared and immunoprecipited with the anti-FLAG antibody or the control mouse IgG (mIgG), and the associated initiation proteins were detected by immunoblotting. (D) Anti-FLAG immunoprecipitates from mitotic cells co-overexpressing FLAG–Cdc6 with either HA–Cdc14 or HA–cdc14^{C283S} were immunoblotted for the association of HA–Cdc14 or HA–Cdc14^{C283S}. (**E**,**F**) Physical interactions between Cdc14p and the initiation proteins detected by yeast two-hybrid analysis. Growth of the yeast two-hybrid host AH109 cells transformed with DNA-binding domain (BD)-*CDC14* (E) or BD-*cdc14^{C283S}* (F), together with the activation domain (AD) vector or individual AD-fusion plasmids indicated on the left of the panels, was tested by spotting tenfold serial dilutions of the cells on different synthetic complete medium (SCM) drop-out plates as indicated. T, tryptophan; L, leucine; H, histidine; A, adenine. Orc2-6A and Orc6-4A are serine and threonine to alanine substitution mutants in all of the predicted CDK phosphorylation sites. (**G**) In vivo Cdc14p phosphatase assays. Whole-cell extracts from M-phase cells overexpressing either Cdc14p or Cdc14^{C283S} were prepared and immunoblotted to detect in vivo dephosphorylation of the initiation proteins. Samples from asynchronous (Asy) and G1 cells synchronized by α-factor (αF) were used to mark the relative positions of the phosphorylated and dephosphorylated forms of the proteins. (**H**) In vitro Cdc14p phosphatase assays. Chromatin (as the source of ORC) and immunoprecipitates (as the source of Mcm3p and Cdc6p) prepared from M-phase cells synchronized using nocodazole were incubated with the indicated amounts of purified GST–Cdc14 or phosphatase-dead GST–Cdc14^{C283S} (CS) (supplementary material Fig. S8), and dephosphorylation of the substrates was monitored by immunoblotting after SDS-PAGE. λ-phosphatase was used as a positive control. PPIs, protein phosphatase inhibitors.

Cdc14p in vivo (Visintin et al., 1998). Our results show that the phosphorylated forms of Orc2p, Orc6p and Mcm3p were progressively converted into dephosphorylated, faster-migrating forms in step with the expression of Cdc14p (Fig. 3G, lanes 4–6) but not Cdc14^{C283S} (Fig. 3G, lanes 10–12). Cdc6p was not examined in the same experiment because it is highly unstable. However, the de novo synthesized Cdc6p was kept in the dephosphorylated form when Cdc14p was activated (Fig. 2A) or ectopically expressed (Fig. 4A). These data suggest that Cdc14p can specifically promote the dephosphorylation of Orc2p, Orc6p and Mcm3p, and probably also Cdc6p, in vivo.

We then used a well-established in vitro Cdc14p phosphatase assay (Visintin et al., 1998; Jaspersen and Morgan, 2000; Pereira et al., 2002; Bloom and Cross, 2007; Hall et al., 2008; Clemente-Blanco et al., 2009) to test whether Cdc14p is able to dephosphorylate the initiation proteins in vitro. Orc2p, Orc6p, Cdc6p and Mcm3p isolated from mitotic cells were efficiently dephosphorylated in vitro when they were incubated with recombinant GST-tagged Cdc14 (GST–Cdc14) (Fig. 3H, lanes 2–4). The amount of GST–Cdc14 and the assay conditions were similar to those previously used to demonstrate the specific dephosphorylation of known substrates by Cdc14p (Jaspersen and Morgan, 2000; Bloom and Cross, 2007). As negative controls, the proteins incubated with GST–Cdc14 plus phosphatase inhibitors (Fig. 3H, lane 5) and with the phosphatase-dead mutant GST–Cdc14^{C283S} (GST–Cdc14^{CS}) (Fig. 3H, lane 6) remained phosphorylated. These results show that Cdc14p can dephosphorylate Orc2p, Orc6p, Cdc6p and Mcm3p in vitro as well as in vivo.

Taken together, the data from analysis of synthetic dosage lethality and physical interactions, as well as the phosphatase assays, strongly suggest that phosphorylated pre-RC components Orc2p, Orc6p, Cdc6p and Mcm3p are physiological substrates of the Cdc14p phosphatase.

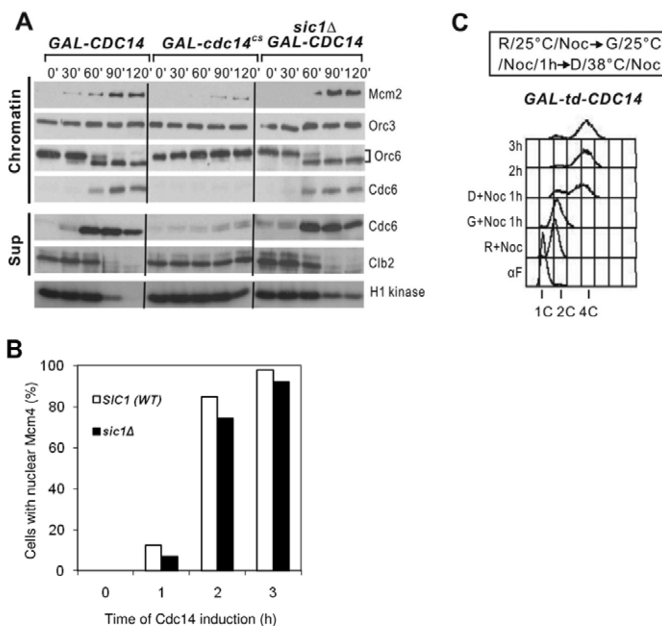

Fig. 4. Ectopic expression of Cdc14p drives efficient MCM nuclear localization and pre-RC assembly, and pulsed expression of the Cdc14-td protein induces DNA rereplication. (**A**) Strains HCY115 (*GAL–CDC14*), HCY116 (*GAL–cdc14^{C283S}*) and A1311 (*GAL–CDC14 sic1Δ*) were induced in YPRG plus nocodazole to express Cdc14p or Cdc14^{C283S}. Cells were harvested along the course of induction and processed for chromatin-binding and H1 kinase assays. (**B**) Cdc14p was induced in strains YL1028 (*MCM4-EGFP GAL-CDC14*) and YL1029 (*sic1Δ MCM4-EGFP GAL-CDC14*) in M phase in YPRG plus nocodazole. Cells were harvested at the indicated time points and processed for fluorescence microscopy to monitor the nuclear localization of Mcm4–EGFP (see supplementary material Fig. S9 for fluorescence images). (**C**) FACS analysis of *GAL–cdc14-td* cells undergoing mitotic rereplication after pulsed expression (i.e. induction and then degradation) of the Cdc14-td protein. D+Noc, YPD plus nocodazole; G+Noc, YPG plus nocodazole; R+Noc, YPR plus nocodazole; αF, α-factor.

Ectopic expression of Cdc14p drives MCM nuclear localization and pre-RC assembly, and pulsed expression of the Cdc14-td protein induces rereplication in mitotic cells

We then asked, instead of inactivating CDKs by Sic1ΔNT overexpression or by chemical inhibition of Cdc28-as1, whether ectopic expression of Cdc14p alone in mitotic cells is sufficient to drive pre-RC assembly. Indeed, along the time course of Cdc14p ectopic expression, Orc6p was efficiently dephosphorylated, both Cdc6p and Mcm2p were progressively loaded onto the chromatin, CDK activity was lowered following Clb2 degradation (Fig. 4A), and Mcm4–EGFP efficiently accumulated in the nucleus (Fig. 4B; supplementary material Fig. S9). Importantly, the observed ORC dephosphorylation and MCM nuclear import and chromatin association were Sic1p independent (Fig. 4A,B), excluding the possibility that these events were the indirect results of CDK inactivation due to Sic1p accumulation when Cdc14p was overexpressed (Visintin et al., 1998). In the negative control, Orc6p remained phosphorylated and MCM loading was almost undetectable when the mutant Cdc14^{C283S} was ectopically expressed (Fig. 4A), consistent with Cdc14p phosphatase activity being essential for pre-RC assembly.

Interestingly, dephosphorylation of Orc6p and loading of Cdc6p and Mcm2p onto chromatin were in step with Cdc14p overexpression but ahead of CDK inactivation (Fig. 4A), suggesting that Cdc14p can dephosphorylate these initiation proteins and promote pre-RC assembly even in the presence of high CDK activity (see the 60 minute time point in Fig. 4A). By contrast, a time lag was required for pre-RC assembly after the CDK activity dropped to an undetectable level in the *GAL–sic1ΔNT*-based system (Fig. 2A). It is conceivable that this time lag is needed for the release and activation of Cdc14p after CDK inactivation by Sic1ΔNT overexpression. Taken together, these data reinforce the idea that Cdc14p is the factor that directly resets the competence of replication licensing.

To determine whether the pre-RC that formed upon ectopic expression of Cdc14p is functional, we used *GAL–cdc14-td* instead of *GAL–CDC14* cells. First, galactose was used to induce Cdc14-td expression to promote pre-RC assembly in M-phase cells at 25°C. Cdc14-td expression was then suppressed in glucose medium, and the Cdc14-td protein was degraded at 38°C to allow CDK reactivation and origin firing when the cells were kept in M phase. Indeed, the mitotic genome was efficiently reduplicated (Fig. 4C). These data indicate that the Cdc14-td protein can promote the assembly of pre-RCs that are functional for DNA replication. The results from the Cdc14p and Cdc14-td ectopic expression experiments further demonstrate that Cdc14p not only antagonizes CDK activity but also dephosphorylates initiation proteins to reprogram the initiation of DNA replication.

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Disruption of phospho-regulation of pre-RC components bypasses the requirement for Cdc14p in DNA replication

In light of the known mechanisms of anti-rereplication (Nguyen et al., 2001; Arias and Walter, 2007), the requirement for Cdc14p in pre-RC assembly and DNA replication (Figs 1 and 2), and the enzyme–substrate relationship between Cdc14p and the initiation proteins (Fig. 3), we reasoned that Orc2p, Orc6p, Cdc6p and Mcm3p might represent the major set of Cdc14p substrates whose dephosphorylation during mitotic exit is essential for replication licensing. If this is true, combined non-phosphorylatable and/or phosphorylation-insensitive mutant alleles of these initiation proteins should bypass the function of Cdc14p in pre-RC assembly. In fact, this idea was supported by a previous study that demonstrated that pre-RC assembly, origin firing and partial mitotic genome reduplication occurred upon ectopic expression of Cdc6ΔNT in a quadruple mutant strain (*orc2-6A orc6-4A MCM7–NLS GAL–cdc6ΔNT*) in which the CDK consensus phosphorylation motifs in Orc2p and Orc6p were mutated to non-phosphorylatable forms, the MCM complex was constitutively targeted to the nucleus and Cdc6p was expressed in a stabilized form (Nguyen et al., 2001). In such a system, high mitotic CDK activity remained unchanged and the nucleus was undivided during the course of rereplication, suggesting that Cdc14p was not released from the nucleolus and thus did not mediate the observed rereplication. To rigorously rule out the involvement of Cdc14p in this deregulated rereplication system, we compared DNA rereplication in *CDC14* wild-type and *cdc14-3* mutant cells in the quadruple mutant background upon Cdc6ΔNT ectopic expression after Cdc14-3 protein inactivation. Just as previously reported (Nguyen et al., 2001), efficient DNA rereplication occurred in *CDC14* wild-type cells, although the mitotic genome was not completely reduplicated (Fig. 5, right panel). We found that DNA rereplication also occurred in *cdc14-3* cells as efficiently as in *CDC14* wild-type cells (Fig. 5, middle panel). By contrast, intact *ORC2* completely suppressed DNA reduplication in *cdc14-3 ORC2 orc6-4A MCM7–NLS GAL–cdc6ΔNT* cells, that is, there was no increase in the DNA content beyond 2C (Fig. 5, left panel). These results strongly suggest that,

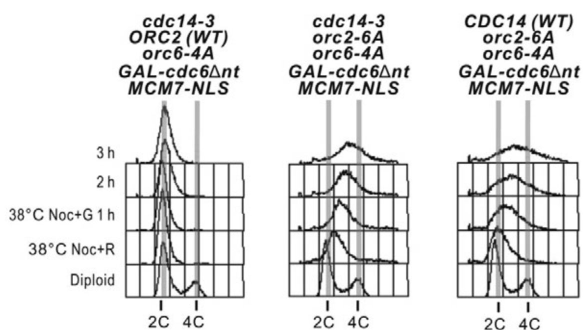

Fig. 5. Mutants with combined non-phosphorylatable and/or phosphorylation-insensitive alleles of the initiation proteins do not require Cdc14p for DNA rereplication. Strains YL1063 (*cdc14-3 orc2-6A orc6-4A MCM7-NLS GAL-cdc6Δnt*), YL1118 (*cdc14-3 ORC2 orc6-4A MCM7-NLS GAL-cdc6Δnt*) and YJL3248 (*orc2-6A orc6-4A MCM7-NLS GAL-cdc6Δnt*) were arrested in M phase in YPR plus nocodazole (Noc+R) at 25°C and then shifted to 38°C for 2 hours to inactivate the Cdc14-3 protein. Galactose was then added to cultures (Noc+G) to induce Cdc6Δnt expression, and rereplication was examined by FACS analysis. A diploid strain was used to mark the positions of the DNA content of 2C and 4C.

of all initiation proteins, Orc2p, Orc6p, Cdc6p and Mcm3p represent the major set of Cdc14p substrates whose dephosphorylation is each necessary for pre-RC assembly and DNA replication.

Discussion

Eukaryotic cells use elaborate intrinsic mechanisms to block DNA rereplication and ensure faithful inheritance of the genetic material (Arias and Walter, 2007). It is, therefore, important to remove these blocks before cells undergo another cell cycle. Otherwise, existence of the obstacles to DNA replication from the previous cell cycle might cause genome under-replication and chromosome instability. Although how the CDK-mediated phosphorylations of initiation proteins inhibit genome rereplication has been elucidated in considerable detail, the necessity of and mechanisms involved in removing these blocks to allow replication licensing for the next cell cycle are not well understood, nor is the identity of the phosphatase that counteracts the inhibitory phosphorylations known. In particular, despite the genetic evidence from previous studies, the role of Cdc14p in DNA replication remains controversial. The identification of Cdc14p as the responsible phosphatase in this study answered these questions; we demonstrate that dephosphorylation of Orc2p, Orc6p, Cdc6p and Mcm3p by Cdc14p is essential for origin resetting and pre-RC assembly during mitotic exit.

Identification of multiple pre-RC components as a new set of Cdc14p substrates extends the physiological significance of this phosphatase outside the scope of mitotic exit. Putting the data from this study and from others together, we propose that Cdc14p provides a favorable environment for resetting the competency of replication licensing during mitotic exit by dephosphorylating multiple initiation proteins and other factors whose dephosphorylated forms can promote the expression and/or stabilization of the initiation proteins (see diagrams in Fig. 6). During mitotic exit, Cdc14p dephosphorylates Swi5p (Visintin et al., 1998), triggering nuclear localization of Swi5p, which, in turn, induces the expression of both Sic1p and Cdc6p (Piatti et al., 1995; Visintin et al., 1998). Cdc14p also stabilizes the newly synthesized Sic1p and Cdc6p by keeping them in dephosphorylated forms that cannot be recognized by the Skp1-Cul1-F-box-protein (SCF) ubiquitination system (Verma, 1997; Perkins et al., 2001). Dephosphorylation of Cdh1p by Cdc14p activates APC^{Cdh1}, which, in turn, mediates Clb2p degradation to free Cdc6p to bind chromatin for pre-RC assembly (Visintin et al., 1998; Jaspersen et al., 1999; Mimura et al., 2004). Clb2p degradation and Sic1p accumulation also lead to CDK inactivation (Stegmeier and Amon, 2004). Dephosphorylation of Orc2p and Orc6p by Cdc14p restores the overall ability of the ORC to bind ATP (Makise et al., 2009) and other initiation proteins, and nuclear import of the MCM complex is promoted when Mcm3p is dephosphorylated by Cdc14p. As such, pre-RCs can be assembled stepwise onto replication origins for replication initiation in the next S phase.

Our discovery of the requirement for Cdc14p in resetting replication licensing is reminiscent of previous findings concerning Cdc14p-mediated Cdh1p activation in mitotic exit (Amon, 2008), in that both processes were once perceived as default or passive dephosphorylation phenomena when CDKs are inactivated, without the need for Cdc14p. With the use of the *cdc14-1* allele, it was once suggested that CDK inactivation alone, but not the Cdc14p phosphatase, was the sole driving force for origin resetting during mitotic exit (Noton and Diffley, 2000). Remarkably, overexpression

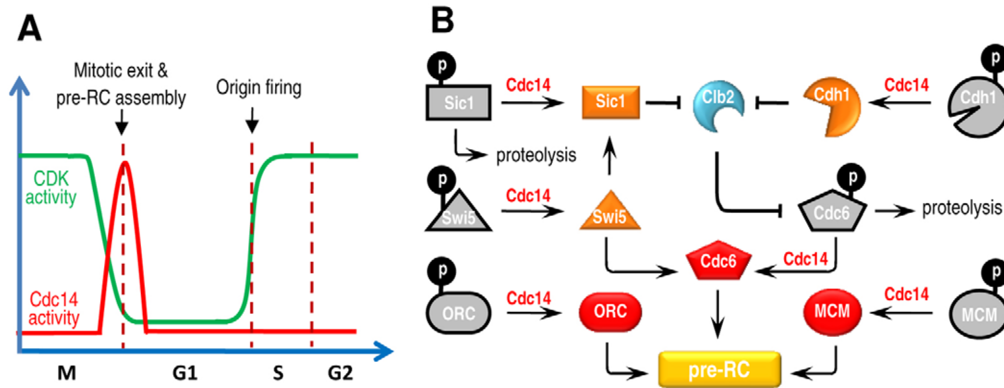

Fig. 6. Model depicting the essential role of Cdc14p in pre-RC assembly during mitotic exit. (**A**) Schematic diagram illustrating the change in CDK and Cdc14p phosphatase activities in the cell cycle. (**B**) Diagram of Cdc14p substrates related to pre-RC assembly. Phosphorylated (p), inactive Cdc14p substrates are shaded gray, whereas their dephosphorylated, active forms are shaded red (substrates identified in this study) or orange (previously identified substrates). See Discussion for details.

of Sic1p can rescue cell viability of *cdc14-1* at 37°C (Jaspersen et al., 1998; Archambault et al., 2003; Machin et al., 2006), but cannot suppress the temperature sensitivity of *cdc14-3* cells even at 30°C (Lengronne and Schwob, 2002), suggesting that *cdc14-3* is a much tighter allele than *cdc14-1*. Consistent with this, *cdc14-1*, but not *cdc14-3*, cells can grow at 30°C (supplementary material Fig. S1) (Miller et al., 2009). We thus suspect that the leakiness of the *cdc14-1* allele and insufficient length (1 hour) of heat inactivation might allow the residual phosphatase activity of the Cdc14-1 protein to promote pre-RC assembly at some replication origins; this was sufficient for DNA replication in the *GAL–sic1ΔNT* rereplication system. By contrast, we observed near-total defects in DNA rereplication (Fig. 1C,D) and pre-RC assembly (Fig. 2B) in the more temperature-sensitive *cdc14-3* and *cdc14-td* alleles with a longer inactivation time (2–3 hours). Moreover, the rereplication defects of *cdc14-3* cells were similar in two different mitotic rereplication systems.

It has previously been reported that Orc6p can be directly dephosphorylated by recombinant Cdc14p in vitro; Orc6p was not considered to be a physiological substrate of Cdc14p in vivo (Bloom and Cross, 2007). Our in vitro and in vivo phosphatase assays, together with the physical interaction and genetic data, demonstrate the role of Cdc14p in dephosphorylating the pre-RC components. Moreover, inactivation of the MEN component Cdc15-2p, which is required for Cdc14p release and activation, also leads to failure of rereplication, consistent with the role of Cdc14p in replication licensing. In addition, pre-RC assembly induced by ectopic expression of Cdc14p and DNA rereplication resulting from transient expression of the Cdc14-td protein further demonstrate that Cdc14p is the acting phosphatase that resets replication licensing during mitotic exit.

Upon *GAL–sic1ΔNT* induction, and thus CDK inactivation, at least some Cdc14p should be released from the nucleolus to perform its function to reset origin licensing in the mitotic rereplication system. As a consequence of Cdc14p activation, active APC^{Cdh1} promotes Clb2p degradation and Orc6p is converted to the dephosphorylated form in *CDC14* wild-type cells. However, by monitoring Cdc14–EGFP fluorescence, we did not observe obvious Cdc14-EGFP release from the nucleolus upon *GAL–sic1ΔNT* induction (data not shown), just as reported in similar experiments (Bloom and Cross, 2007). During normal mitotic exit, Clb2p–CDK phosphorylates Net1p, releasing Cdc14p from sequestration (Azzam et al., 2004). In the mitotic rereplication system, however, CDK inactivation by *GAL–sic1ΔNT* induction might maintain the nucleolar protein Net1p in the hypophosphorylated form, thereby constraining a large portion of Cdc14p in the nucleolus. Under such conditions, therefore, it is difficult to detect by regular fluorescent microscopy the small amount of Cdc14p released, and a more sensitive and quantitative fluorescent measurement might be required to trace Cdc14p release (Lu and Cross, 2009). Nonetheless, we indirectly detected Cdc14p activity by observing Clb2p degradation upon *GAL–sic1ΔNT* induction (Fig. 2A), because Cdc14p is required for Clb2 degradation brought about by ectopic CDK inactivation (Amon, 2008). It will be interesting to determine whether partial Cdc14p release in the mitotic rereplication systems is regulated by an as yet uncharacterized Net1p-independent pathway.

Although CDK-dependent mechanisms that block genome reduplication are diverse in eukaryotes (Arias and Walter, 2007), our findings using budding yeast underscore the importance of identifying phosphatases or other CDK-antagonizing activities that extinguish the inhibitory phosphorylations of initiation proteins for replication licensing in other organisms. It is quite possible that the essential role of Cdc14p in pre-RC assembly is conserved throughout Eukarya. Given 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 promises to be an important protein to study in both normal and cancer cells. Furthermore, it will be interesting to examine whether direct dephosphorylation of initiation proteins, and, hence, pre-RC assembly, under high overall cellular CDK activity is permitted at some Cdc14p-concentrated genomic loci for DNA amplification in specialized normal tissues as well as in tumorigenesis.

Materials and Methods

Plasmid construction

All pRS416-*GAL1-3FLAG*-based plasmids were derived from pHLP112 (Hall et al., 2008) by inserting the corresponding open reading frames (ORFs) between the *Not*I and *Xho*I sites to replace *ACM1*. All wild-type ORF inserts were generated by PCR amplification with KOD polymerase (Toyobo, Osaka, Japan) using W303-1a genomic DNA as the template. *cdc14*^{C283S}, containing a C283S mutation in the Cdc14p catalytic domain, was made using site-directed mutagenesis. Both *orc2-6A* and *orc6-4A* ORFs were amplified using the genomic DNA of YJL1737 (*MATa orc2-6A orc6-4A leu2 ura3-52 trp1-289 ade2 ade3 bar1::LEU2*) (Nguyen et al., 2001) as the template. The plasmid pRS416-*GAL1-3FLAG-mcm3-5A* was derived from pRS416-*GAL1-3FLAG-MCM3* by introducing mutations that change serine and threonine to alanine in the CDK consensus phosphorylation sites at residues 761, 765, 781, 786 and 845. Plasmid pRS416-*GAL1-cdc14-td* was derived from RS416-*GAL1-3FLAG-CDC14* by replacing *3FLAG* with the *ubi-DHFR*^{ts}*-myc* degron from pKL187 (a gift from Karim Labib, Paterson Institute for Cancer Research, Manchester, UK). Plasmid pRS403-*GAL-sic1ΔNT* was generated by inserting the *Eco*RI-*Eco*RV fragment (containing *GAL-sic1ΔNT*) from pLD1 (Noton and Diffley, 2000) into pRS403 at the *Sma*I and *Eco*RI sites. Plasmid pJJ283-*cdc6Δnt*, containing *cdc6Δnt* lacking the N-

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terminal 46 residues, was cloned into the *Sma*I site of pJJ283. pFA6a-2NLS-klTRP was derived from pFA6a-3HA-klTRP (Janke et al., 2004) by replacing 3HA with 2NLS [two tandem copies of the simian virus 40 (SV40) nuclear localization sequence (NLS)] amplified from the genomic DNA of YJL3248 (Nguyen et al., 2001). Full-length *CDC14* and *cdc14*^{C283S} were cloned into pGEX-5X-3 vector (GE Healthcare) at the *Bam*HI and *Xho*I sites to obtain GST fusion constructs. All constructs were confirmed by DNA sequencing.

Strain construction

PCR-based approaches (Longtine et al., 1998; Janke et al., 2004) were used to generate the *cdc26Δ*, *MCM4–EGFP* and *GAL–3HA–CDC6* modifications in the relevant background strains. pLD1 linearized at the *Apa*I site was integrated at the *ura3* locus to generate the *GAL–sic1ΔNT* strains. Additional copies of *GAL–sic1ΔNT* were integrated at the *his3* locus in the *cdc14-3* strains and the corresponding wild-type strains using *Nhe*I-linearized pRS403-*GAL-sic1ΔNT* to obtain the wild-type and *cdc14-3* strains with near-equal levels of Sic1ΔNT expression upon induction. The *cdc14-3 cdc28-as1* double-mutant strain was obtained by crossing 3128 (*MATa cdc28-as1 MCM4-TEV-PrA-7His::Sphis5+*) (Devault et al., 2008) with *cdc14-3 MCM4-EGFP* (*MAT*α). The *cdc14-td* mutant strain was generated by fusing a td cassette to the N terminus of *CDC14* at its genomic locus in strain YLD12. To obtain YL1059 (*MCM7-2NLS-klTRP cdc6Δnt*) and YL1098 (*cdc14-3 MCM7-2NLS-klTRP cdc6Δnt*), two tandem copies of the SV40 NLS were first fused to the C terminus of *MCM7* in YL985 and YL1093 using the one-step PCR-based tagging strategy with pFA6a-2NLS-klTRP as the template, generating YL1030 and YL1031, which were further integrated with the *Cla*I-linearized pJJ283-*cdc6Δnt* at the *leu2* locus through homologous recombination. To obtain YL1063 (*cdc14-3 orc2-6A orc6-4A MCM7-2NLS GAL-cdc6Δnt-UAR3*), *cdc14-3* (*MAT*α) was first crossed with YJL1737 (*orc2-6A orc6-4A*) to obtain the *cdc14-3 orc2-6A orc6-4A* (*MAT*α) triple-mutant strain, which was then crossed with YJL3248 (*orc2-6A orc6-4A MCM7-2NLS GAL-cdc6Δnt-UAR3 MET3-CDC20*) to generate YL1063, whose genotype was validated by DNA sequencing, growth phenotypes and immunoblotting. For yeast strains used in this study see supplementary material Table S1.

Growth media, cell-cycle synchronization procedures and FACS analysis

Cells were grown in YP medium (1% yeast extract and 2% peptone) with different carbon sources: YP medium plus 2% dextrose (YPD), 2% raffinose (YPR) or 2% galactose (YPG). For some of the experiments, galactose (final 0.5%) was added to YPR (YPRG) for induction of *GAL*-promoter-driven gene expression.

To arrest cells in G1 phase, log-phase yeast cultures were treated with α-factor (5 μg/ml) at 25°C for ~3 hours, during which α-factor was replenished every 90 minutes. To block cells in M phase, cultures pre-synchronized in G1 phase were released into fresh medium, and nocodazole (10–15 μg/ml) was added for 60 minutes starting from the point of bud emergence (~1 hour after release from α-factor). For co-immunoprecipitation assays, log-phase cultures with *cdc26Δ* background were arrested at metaphase by incubation at 38ºC for 4 hours. FACS analysis was performed essentially as previously described (Liang and Stillman, 1997), except that gating was applied to record cells with a DNA content of 1C and above in the FACS analysis shown in Fig. 5.

Mitotic rereplication assays

Rereplication induced by *GAL–sic1ΔNT* transient expression

Cells were grown to an optical density at 600 nm (OD_{600}) of 0.5 in YPR and then pre-synchronized with α-factor (5 μg/ml) in G1 phase for 3 hours at 25°C. The culture was then released into fresh YPR, allowing progression through S phase. After ~1 hour, nocodazole (10 μg/ml) was added to the culture for 30 to 60 minutes to block cells in M phase. Afterwards, aliquots of the culture were shifted to 38°C for 1, 2 and 3 hours, and an aliquot of cells was kept at 25°C as the control. After heat inactivation, cells were changed into YPG plus nocodazole (10 μg/ml) for 1 hour of *GAL–sic1ΔNT* induction, followed by a change in medium to YPD plus nocodazole (10 μg/ml) (dextrose was used to suppress *GAL–sic1ΔNT* expression). Cell samples were collected at various time points, during which extra nocodazole (2.5 μg/ml) was added to the culture every hour to maintain mitotic block. Pre-warmed media were used for the heat-inactivation procedures.

To measure pre-RC assembly by chromatin-binding assays in the mitotic rereplication system, instead of changing cells into YPG, galactose (final 2%) was added to YPR to induce *GAL–sic1ΔNT* expression. Meanwhile, α-factor (5 μg/ml) was added every half hour to suppress Cln–CDK activity during the course of sample collection.

Rereplication with transient treatment of 1-NM-PP1

cdc28-as1 cells were grown to OD_{600}=0.5 in YPD and arrested in G1 phase with α-factor. The culture was then released into fresh YPD. After ~1 hour, nocodazole (10 μg/ml) was added to block cells in M phase. Afterwards, the culture was split into three aliquots; one was kept at 25°C as the control, and the other two were shifted to 38°C for 1 or 2 hours. Then, 1-NM-PP1 (final 5 μM) (Calbiochem) was added into the culture for 1 hour, after which cells were changed into pre-warmed YPD plus nocodazole (15 μg/ml). Samples were collected at various time points, during which extra nocodazole (2.5 μg/ml) was added to the culture every hour.

Rereplication with pulsed expression of *GAL–cdc14-td*

YL1026 cells harboring pRS416-GAL1-cdc14-td were grown to OD_{600}=0.5 in synthetic complete medium without uracil and with raffinose (SC-Ura/raffinose medium), and arrested in G1 phase with α-factor at 25°C. The culture was then released into fresh SC-Ura/raffinose medium. After ~1 hour, nocodazole (10 μg/ml) was added to block cells in M phase, followed by addition of galactose (2%) to induce *GAL–cdc14-td* expression. Afterwards, the cells were changed into YPD plus nocodazole (12.5 μg/ml) at 38°C to repress the expression of and degrade the Cdc14-td protein. FACS samples were collected at various time points, during which extra nocodazole (2.5 μg/ml) was added to the culture every hour.

In vitro and in vivo phosphatase assays

Recombinant GST–Cdc14 and GST–Cdc14^{C283S} were purified to near homogeneity (supplementary material Fig. S8) using columns packed with glutathione (GSH)–agarose (Sigma). Rosseta 2 (DE3) *Escherichia coli* cells (Novagen) harboring GST–*CDC14* or GST–*cdc14*^{C283S} fusion constructs were grown to OD_{600}=0.5–0.8 at 37°C before induction with 0.1 mM IPTG at 16°C for 12 hours. Harvested cell pellets were resuspended in buffer L, pH 9.0, containing 2× PBS (280 mM NaCl, 5.4 mM KCl, 20.2 mM Na_2HPO_4, 3.6 mM KH_2PO_4), 10% glycerol, 200 mM NaCl, 1% Triton X-100 and 10 mM DTT, and lysed by sonication. The GSH columns bound with GST fusion proteins were washed with buffer W, pH 9.0, containing 1× PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4), 10% glycerol, 350 mM NaCl, 1% Triton X-100 and 10 mM DTT, four times before elution in buffer E, pH 9.0 (Tris-HCl pH 9.0, 10% glycerol, 500 mM NaCl, 1% Triton X-100, 10 mM DTT and 50 mM reduced glutathione). Eluate was dialyzed against buffer D (25 mM Hepes-NaOH pH 7.4, 10% glycerol, 500 mM NaCl, 0.1% Triton X-100 and 1 mM DTT) and then adjusted to 50% glycerol for storage at –80°C before use. Chromatin (as the source of the phosphorylated forms of Orc2p and Orc6p) was isolated using spheroplasted (see chromatin-binding assay) mitotic *cdc14-3* cells synchronized by incubation at 38°C for 3 hours. The phosphorylated form of Mcm3p was obtained by immunoprecipitation using α-Mcm3 (anti-scMcm3 #18; a gift from Bruce Stillman, Cold Spring Harbor Laboratory, New York) from extracts of spheroplasts from mitotic *cdc14-3* cells. The phosphorylated form of hemagglutinin (HA)-tagged Cdc6p (HA–Cdc6p) was obtained by immunoprecipitation using α-HA (12CA5; Roche) from whole-cell extracts overexpressing HA–Cdc6p from strain YL746 arrested in M phase with YPG plus nocodazole. Phosphatase assays were performed essentially as previously described (Jaspersen and Morgan, 2000), with minor modifications. Briefly, isolated chromatin containing Orc2p and Orc6p, and the immunoprecipitates of Mcm3p and Cdc6p were separately equilibrated in phosphatase buffer (25 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 2 mM $MnCl_2$ and 0.1 mg/ml BSA) before purified GST–Cdc14, GST–Cdc14^{C283S} or λ-phosphatase (NEB) were added in a 50 μl reaction volume. Protein phosphatase inhibitors (2 mM $ZnSO_4$, 50 μM NaF, 1 mM Na_3VO_4 and 2 mM $Na_4P_2O_7$) were added before GST–Cdc14 in the negative control. After incubation at 30°C for 30 minutes, chromatin and immunoprecipitates were separately pelleted and boiled in 2× Laemmli sample buffer for SDS-PAGE analysis described below.

For in vivo phosphatase assays, strains HCY115 and HCY116 were first cultured in YPR and then arrested in M phase by nocodazole before galactose (0.5%) was added to induce ectopic expression of Cdc14p or Cdc14^{C283S}. Cells were harvested at 30 minute intervals from the point of induction and onwards, and their whole-cell extracts were analyzed by immunoblotting.

To observe the mobility shift of pre-RC components from the in vivo phosphatase assays, samples were resolved by 7.5% SDS-PAGE supplemented with 25 μM Phos-tag acrylamide (Kinoshita et al., 2009) (NARD Institute) in a 10 cm path-length midi-gel system (CBS Scientific). For the samples from the in vitro phosphatase assays, Orc6p and Cdc6p were resolved by 7.5% and 10%, respectively, SDS-PAGE supplemented with 5 μM Phos-tag in the midi-gel system, and Orc2p and Mcm3p were resolved by 7.5% SDS-PAGE with 7.5 μM Phos-tag and 5% SDS-PAGE with 3.5 μM Phos-tag, respectively, in a 22 cm path-length electrophoresis system (CBS Scientific).

Co-immunoprecipitation assay and immunoblotting

Yeast whole-cell extracts were prepared by bead beating in immunoprecipitation lysis buffer [20 mM Tris HCl pH 7.8, 20 mM $MgCl_2$, 2 mM EDTA, 1 mM EGTA, 5% glycerol, 0.1% Triton X-100, 125 mM $(NH_4)_2SO_4$], together with freshly added 1× protease inhibitor cocktails (Roche), 2 μg/ml pepstatin A and 1 mM PMSF. Extracts (5–10 mg/ml) treated with 500 U/ml DNase I (Sigma) were precleared with protein-G beads (Invitrogen) before incubation with antibodies [α-HA (12CA5; Roche), α-FLAG (M2; Sigma), α-Orc3 (SB3; a gift from Bruce Stillman) and α-Mcm3 (anti-scMcm3 #18)]. Immunoprecipitates were then isolated with protein-G beads, washed four times in a total of 1–2 hours and boiled in 2× Laemmli sample buffer. Protein samples were then subjected to 10% SDS-PAGE. When needed, the immunoprecipitation lysis buffer was supplemented with phosphatase inhibitors, including 10 mM NaF, 2 mM $Na_4P_2O_7$ and 0.1 mM $NaVO_3$. For immunoblotting, Orc2p, Orc3p, Orc6p, Mcm2p and Mcm3p were detected with monoclonal antibodies SB67, SB3, SB49, anti-Mcm2#28 and anti-Mcm3#18 (gifts from Bruce Stillman) at dilutions of 1:1000, 1:30,000, 1:2000, 1:20,000 and 1:2000, respectively. Cdc6p, Cdc14p and Clb2p were detected with sc6317, sc12045 and sc9071 (Santa Cruz

Biotechology), respectively, at 1:500. GST fusion proteins were detected with mouse anti-GST (#13-6700 Zymed, Invitrogen) at 1 μg/ml.

Chromatin-binding assay

Chromatin-binding assays were performed as previously described (Liang and Stillman, 1997), with modifications. Collected yeast cells were incubated at room temperature for 10 minutes in 1 ml of prespheroplasting buffer [100 mM PIPES (pH 9.4), 10 mM DTT] containing 1 mM PMSF and 2 μg/ml pepstatin A, followed by incubation in 1 ml of spheroplasting buffer [50 mM KH_2PO_4/K_2HPO_4 (pH 7.5), 0.6 M sorbitol, 10 mM DTT] containing 1 mM PMSF, 2 μg/ml pepstatin A and 10 μl of 5 mg/ml recombinant lyticase (purified from *E. coli* using an expression construct from Susan Gasser, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland) at 37°C for 5–10 minutes (20 μl of lyticase were used for cells with heat inactivation at 38°C) with mixing every 2–3 minutes, until the OD_{600} of a 1:100 dilution of the cell suspension (in 0.2% Triton X-100) dropped to 5–10% of the value before digestion. Sphereoplasts were pelleted at 1700 *g* for 2 minutes in a microcentrifuge at 4°C, and washed with 0.5 ml of ice-chilled sphereoplasts wash buffer [100 mM KCl, 50 mM HEPES-KOH (pH 7.5), 2.5 mM $MgCl_2$ and 0.4 M sorbitol] containing 1 mM PMSF and 2 μg/ml pepstatin A. The spheroplast pellet was then lysed by vigorous mixing in 400 μl of extraction buffer (EXB) [100 mM KCl, 50 mM HEPES-KOH (pH 7.5), 2.5 mM $MgCl_2$, 50 mM NaF, 5 mM $Na_4P_2O_7$, 0.1 mM $NaVO_3$ and 0.5% Triton X-100] containing 1 mM PMSF, 2 μg/ml pepstatin A and 1× protease inhibitor cocktail (Roche). The lysate was incubated on ice for 10 minutes with vigorous mixing every 3–4 minutes. The lysate was spun twice at 2390 *g* for 1 minute to remove most of the unlysed cells. An equal volume of 30% sucrose (~350 μl) was added to the bottom of the lysate, and the tube was centrifuged at 20,800 *g* for 10 minutes at 4°C. The pellet (chromatin fraction) was washed twice with 100 μl of EXB, resuspended in 40 μl of EXB, supplemented with 50 μl of 2× Laemmli sample buffer, and then boiled for 3 minutes prior to SDS-PAGE and immunoblotting.

Histone H1 kinase assay

Whole-cell extracts were prepared by bead beating with lysis buffer [25 mM Tris-HCl pH 7.5, 10 mM NaF, 5 mM EDTA, 1 mM EGTA, 0.1 % NP-40, 100 mM NaCl, 1 mM DTT, 5% glycerol and protease inhibitors added just before use (1 mM PMSF, 2 μg/ml pepstatin A and 1× protease inhibitor cocktail; Roche)]. To pull down the Cdc28p-associated kinase from the extracts, 10 μl of 50% slurry of $p13^{Suc1}$-agarose beads (Millipore), mixed with 12.5 μl of 50% slurry of Sephadex G-50 to reduce bead loss during the pull-down and kinase assays, were added to each extract containing 250 μg of total proteins. After incubation at 4°C for 2 hours, the beads were washed four times with lysis buffer and twice with H1 kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT). Half of the beads were then incubated at 25°C for 10 minutes before 20 μl of kinase assay mixture (0.5 μCi [γ-^{32}P]ATP, 10 μg Histone H1, 500 μM ATP in 1× kinase buffer) were added. The mixture was incubated at 25°C for 10 minutes. The reaction was stopped by adding 20 μl of 2× Laemmli sample buffer, followed by boiling at 95°C for 4 minutes. Samples were resolved by 12.5% SDS-PAGE and subjected to autoradiography.

Fluorescence microscopy of Mcm4–EGFP

Living cells were pelleted and resuspended in 100% ethanol at –20°C for 3 minutes. Cells were then washed once with 0.5 ml of ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 43 mM Na_2HPO_4 and 14 mM KH_2PO_4 at pH 7.4), resuspended in 20 μl of PBS containing 0.1 μg/ml DAPI (Sigma) and placed on ice for 2 minutes. Samples (2 μl each) were spotted on glass slides coated with a 4% agarose pad and observed under a fluorescence microscope within 10 minutes. Photographs of cells were taken and processed using a Nikon TE2000 E inverted fluorescence microscope with a Nikon plan Apo 63× oil NA 1.4 DIC and appropriate filter sets, a digital camera (SPOT RT1200) and Metamorph 6.2 software (Molecular Devices, Sunnyvale, CA).

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