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

Yuanliang Zhai, Philip Y. K. Yung, Lin Huo and Chun Liang*

Section of Biochemistry and Cell Biology, Division of Life Science, and Center for Cancer Research, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

*Author for correspondence (bccliang@ust.hk)

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

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 (Difflrey, 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, 1996). 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 described Cdc14p as the physiological phosphatase that dephosphorylates initiation proteins to promote pre-RC formation and DNA replication (Norton and Diffl ey, 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). Nevertheless, 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 initiation proteins previously tested (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, 1996). 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 described Cdc14p as the physiological phosphatase that dephosphorylates initiation proteins to promote pre-RC formation and DNA replication (Norton and Diffl ey, 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). Nevertheless, the essential role of Cdc14p in replication licensing remains controversial.

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-termination 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) (Norton and Diffl ey, 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 (Norton and Diffl ey, 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 Cdc14p protein. 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 Cdc14p protein.

![Fig. 1. Cdc14p is required for DNA replication in the mitotic rereplication system.](image-url)
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 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-PP1 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
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 Cib–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; Visinini 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 overloaded 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 undetectable (for orc2-6A) or less severe (for orc6-4A and mcms-5A) phenotypes. (It is possible that some cryptic CDK phosphorylation sites are still present in the orc6-4A and mcms-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 Cdc14ΔC283S 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–Cdc14ΔC283S, 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–Cdc14ΔC283S (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 Cdc14ΔC283S, whereas the non-phosphorylatable Orc2-6A or Orc6-4A did not interact with Cdc14p or Cdc14ΔC283S (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 Cdc14ΔC283S was detected by co-immunoprecipitation assays, with a stronger interaction between Cdc6p and Cdc14ΔC283S, when both Cdc6p and Cdc14p or Cdc14ΔC283S 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-cdc14C283S) 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-cdc14C283S expression. Cell extracts were prepared and immunoprecipitated 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–cdc14C283S were immunoblotted for the association of HA–Cdc14 or HA–Cdc14C283S. (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–cdc14C283S (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 yeast mouse strains 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 Cdc14C283S 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–Cdc14C283S (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 Cdc14C283S (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–Cdc14C283S (GST–Cdc14CS) (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.

**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 Cdc14C283S 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 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.
Disruption of phospho-regulation of pre-RC components bypasses the requirement for Cdc14p in DNA replication

In light of the known mechanisms of anti-replication (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, 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 APlCdh1, 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
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 Cdc14p 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 Ctb2p 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, Ctb2p–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 nucleus. 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 Clb2p 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 pHL112 (Hallen et al., 2000) by inserting the corresponding open reading frames (ORFs) between the NotI and XhoI sites to replace ACM1. All wild-type ORF inserts were generated by PCR amplification with KOD polymerase (Toyobo, Osaka, Japan) using W303-1a genomic template. The plasmid pRS416–GAL1–3FLAG–myc was derived from RS416–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 by replacing ACM1 with the ubi-DHFRts–myc degron from pKL187 (a gift from Karim Labib, Paterson Institute for Cancer Research, Manchester, UK). Plasmid pRS403–GAL1–sic1ΔNT was generated by inserting the EcoRI–EcoRV fragment (containing GAL–sic1ΔNT) from pLD1 (Notton and Dillay, 2000) into pRS403 at the Smal and EcoRI sites. Plasmids pJB283–cdc6Δnt, containing cdc6Δnt lacking the N-
Rereplication with pulsed expression of GAL-cdc14-td

GAL-cdc14-td protein was generated as described above. Cdc14 was coexpressed with Cdc6 to induce Cdc14-td expression. After, the cells were collected and suspended in YPD (10 μg/ml) or 10 μM nocodazole for 1 hour at 30°C to arrest cells in the G2-M phase. The cells were then released into fresh YPD medium. After 1 hour, nocodazole (10 μg/ml) was added to block cells in M phase. Subsequently, cells were incubated at 37°C to arrest cells in the G2-M phase. Afterwards, cells were incubated at 37°C for 1 hour, after which cells were released into YPD plus nocodazole (25 μg/ml). Cells were collected at various time points, during which extra nocodazole (25 μg/ml) was added to the culture every hour.

In vitro and in vivo phosphatase assays

Recombinant GST-Cdc14 and GST-Cdc14ΔC350 were purified to near homogeneity (supplementary material Fig. S8) using columns packed with glutathione (GSH)–agarose (Sigma). Rosetta 2 (DE3) Escherichia coli cells (Novagen) harboring GST–Cdc14 or GST–Cdc14ΔC350 fusion constructs were grown to OD600 = 0.5–0.8 at 37°C before induction with 0.1 mM IPTG for 16 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 NaHPO4, 3.6 mM K2HPO4), 10% glycerol, 200 mM NaCl, 1% Triton X-100 and 1 mM DTT, and lysed by sonication. The GST columns bound with GST fusion proteins were washed with buffer W (pH 9.0, containing 1 X PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.8 mM K2HPO4), 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 Cdc6p) was isolated using spheroplasted (see chromatin-binding assay) mitotic cdc14-1 cells synthesized 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 mitotic cdc14-1 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 YL1076 isolated from YPD plus nocodazole. Phosphatase assays were performed essentially as previously described (Jaspersen and Morgan, 2000), with minor modifications. Briefly, Cdc14 and Orc6 were resolved by 7.5% and 10% SDS-PAGE, respectively, and elongation factors, 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 MnCl2 and 0.1 mg/ml BSA) before purified GST–Cdc14, GST–Cdc14ΔC350 and α-phosphatase (NEB) were added in a 50 μl reaction volume. Protein phosphatase inhibitors (2 mM ZnSO4, 50 μM NaF, 1 mM Na2VO4 and 2 mM Na3P04) 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 Cdc14ΔC350. The cells were arrested at 0°C and washed with 3.5 M NaCl, 1% Triton X-100 and 1 mM DTT, and then resuspended in YPD containing Cdc14p, Orc6p and Cdc6p. Cells were collected at various time points, during which extra nocodazole (25 μg/ml) was added to the culture every hour. Rereplication with pulsed expression of GAL-cdc14-td

GAL-cdc14-td protein was generated as described above. Cdc14 was coexpressed with Cdc6 to induce Cdc14-td expression. After, the cells were collected and suspended in YPD (10 μg/ml) or 10 μM nocodazole for 1 hour at 37°C to arrest cells in the G2-M phase. The cells were then released into fresh YPD medium. After 1 hour, nocodazole (10 μg/ml) was added to block cells in M phase. Subsequently, cells were incubated at 37°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 to YPG plus nocodazole (10 μg/ml) for 1 hour of GAL-sic1ΔNT induction, followed by a change in medium to YPD plus nocodazole (25 μg/ml) in which extra nocodazole (25 μg/ml) 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 OD600 = 0.5 in YPR and arrested in G1 phase with α-factor (5 μg/ml). The culture was then released into fresh YPR. 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 37°C for 1 and 3 hours, respectively. Orc6p, Mcm3p and Cdc6p were resolved by 7.5% and 10% SDS-PAGE, respectively, and then transferred to nitrocellulose (NC) membrane. Phosphatase activity was detected using sc-Mcm3p (anti-scMcm3 #18). Immunoprecipitates were then isolated with protein-G beads, before incubation with antibodies [sc6317, sc12045 and sc9071 (Santa Cruz)]. Cdc6p was detected with α-Mcm3 (anti-scMcm3 #18). Immunoprecipitates were then isolated with protein-G beads, before incubation with antibodies [sc6317, sc12045 and sc9071 (Santa Cruz)]. Cdc6p was detected with α-Mcm3 (anti-scMcm3 #18). Immunoprecipitates were then isolated with protein-G beads, before incubation with antibodies [sc6317, sc12045 and sc9071 (Santa Cruz)]. Cdc6p was detected with α-Mcm3 (anti-scMcm3 #18). Immunoprecipitates were then isolated with protein-G beads, before incubation with antibodies [sc6317, sc12045 and sc9071 (Santa Cruz)]. Cdc6p was detected with α-Mcm3 (anti-scMcm3 #18). Immunoprecipitates were then isolated with protein-G beads, before incubation with antibodies [sc6317, sc12045 and sc9071 (Santa Cruz)]. Cdc6p was detected with α-Mcm3 (anti-scMcm3 #18). Immunoprecipitates were then isolated with protein-G beads, before incubation with antibodies [sc6317, sc12045 and sc9071 (Santa Cruz)]. Cdc6p was detected with α-Mcm3 (anti-scMcm3 #18). Immunoprecipitates were then isolated with protein-G beads, before incubation with antibodies [sc6317, sc12045 and sc9071 (Santa Cruz)]. Cdc6p was detected with α-Mcm3 (anti-scMcm3 #18). Immunoprecipitates were then isolated with protein-G beads, before incubation with antibodies [sc6317, sc12045 and sc9071 (Santa Cruz)]. Cdc6p was detected with α-Mcm3 (anti-scMcm3 #18). Immunoprecipitates were then isolated with protein-G beads, before incubation with antibodies [sc6317, sc12045 and sc9071 (Santa Cruz)]. Cdc6p was detected with α-Mcm3 (anti-scMcm3 #18). Immunoprecipitates were then isolated with protein-G beads, before incubation with antibodies [sc6317, sc12045 and sc9071 (Santa Cruz)]. Cdc6p was detected with α-Mcm3 (anti-scMcm3 #18). Immunoprecipitates were then isolated with protein-G beads, before incubation with antibodies [sc6317, sc12045 and sc9071 (Santa Cruz)]. Cdc6p was detected with α-Mcm3 (anti-scMcm3 #18).


Fig. S2.
Fig. S3.
Fig. S4.
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Fig. S5.
Fig. S5. continued

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For **WT GAL-sic1 Δ NT /38°C**:

R/Noc/38°C → G/Noc/38°C

For **cdc14-3 GAL-sic1 Δ NT /38°C**:

R/Noc/38°C → G/Noc/38°C
Fig. S6.
Table S1. Yeast strains used in this study

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YW303-1a  This study

YL1039  MAT a CDC14 pRS416-GAL1-3FLAG-CDC6

YW303-1a  This study

YL1083  MAT a CDC14 pRS416-GAL1-3FLAG-CDT1

YW303-1a  This study

YL1093  MAT a cdc14-3 MCM4-EGFP-KanMX4 ura3-1:: GAL-sic1ΔNT-URA3 his3::GAL-sic1ΔNT-HIS3

YW303-1a  This study

YL985  MAT a MCM4-EGFP-KanMX4 ura3-1:: GAL-sic1ΔNT-URA3

YW303-1a  This study

YIL17  MAT a cdc15-2, ura3-1:: GAL-sic1ΔNT-URA3

YW303-1a  (Noton and Diffley, 2000)

A1311  MAT a sic1Δ::HIS3 ade2-1 trp1-1 can1-100 GAL psi+
PDS1-HA-LEU2::pds1  3xGAL-CDC14::URA3

YW303-1a  (Visintin et al., 1998)

YL1026  MAT a CDC14 pRS416-td

YW303-1a  This study

YJL3248  MAT a orc2-6A orc6-4A MCM7-2NLS cdc20:: MET3-HA3-CDC20-TRP1 ura3:: GAL-cdc6ΔNT-URA3 bar1:: LEU2 trp1-289 leu2 ade2 ade3

A364a  (Nguyen et al., 2001)

YL1063  MAT a cdc14-3 orc2-6A orc6-4A MCM7-2NLS CDC20

YW303-1a  This study

ura3:: GAL-cdc6ΔNT-URA3 bar1:: LEU2 trp1-289 ade2 ade3

YL1118  MAT a cdc14-3 ORC2 orc6-4A MCM7-2NLS cdc20:: MET3-HA3-CDC20-TRP1 ura3:: GAL-cdc6ΔNT-URA3 trp1-289 ade2 ade3

YW303-1a  This study

AH109  MAT a trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ
gal80ΔLYS2::GAL1UAS-GALITATA-HIS3GAL2UAS-
GAL2TATA-ADE2 URA3::MEL1UAS-MELITATA-lacZ

MEL1

YW303-1a  Clontech

YL1084  MAT a sic1Δ::HIS3

YW303-1a  This study

YL1085  MAT a cdc14-1 sic1Δ::HIS3

YW303-1a  This study

YL1086  MAT a cdc14-3 sic1Δ::HIS3

YW303-1a  This study

YL1117  MAT a cdc14-td ura3:: GAL-sic1ΔNT-URA3 his3::GAL-
sic1ΔNT-HIS3

YW303-1a  This study

3218  MAT a cdc28-as1 MCM4-TEV-PrA-7His::Sphis5+

YW303-1a  (Devault et al., 2008)

YL1023  MAT a cdc28-as1 MCM4-EGFP-KanMX4

YW303-1a  This study

YL1024  MAT a cdc28-as1 cdc14-3 MCM4-EGFP-KanMX4

YW303-1a  This study

YL1030  MAT a MCM4-EGFP-KanMX4 MCM7-2NLS-klTRP ura3-1:: GAL-sic1ΔNT-URA3

YW303-1a  This study

YL1031  MAT a cdc14-3 MCM4-EGFP-KanMX4 MCM7-2NLS-
klTRP ura3-1:: GAL-sic1ΔNT-URA3 his3::GAL-sic1ΔNT-
HIS3

YL1059 MAT a CDC14 MCM4-EGFP-KanMX4 MCM7-2NLS-klTRP leu2::pJJ283-cdc6ΔNT::LEU2 ura3-1:: GAL-sic1ΔNT-URA3 W303-1a This study

YL1098 MAT a cdc14-3 MCM4-EGFP-KanMX4 MCM7-2NLS-klTRP leu2::pJJ283-cdc6ΔNT::LEU2 ura3-1:: GAL-sic1ΔNT-URA3 his3::GAL-sic1ΔNT-HIS3 W303-1a This study

YL1028 MAT a MCM4-EGFP ura::GAL-CDC14-URA3 W303-1a This study

YL1029 MAT a MCM4-EGFP sic1Δ::HIS3 ura3::GAL-CDC14-URA3 W303-1a This study