Cdt1p, through its interaction with Mcm6p, is required for the formation, nuclear accumulation and chromatin loading of the MCM complex

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Summary

Regulation of DNA replication initiation is essential for the faithful inheritance of genetic information. Replication initiation is a multi-step process involving many factors including ORC, Cdt1p, Mcm2-7p and other proteins that bind to replication origins to form a pre-replicative complex (pre-RC). As a prerequisite for pre-RC assembly, Cdt1p and the Mcm2-7p heterohexameric complex accumulate in the nucleus in G1 phase in an interdependent manner in budding yeast. However, the nature of this interdependence is not clear, nor is it known whether Cdt1p is required for the assembly of the MCM complex. In this study, we provide the first evidence that Cdt1p, through its interaction with Mcm6p with the C-terminal regions of the two proteins, is crucial for the formation of the MCM complex in both the cytoplasm and nucleoplasm. We demonstrate that disruption of the interaction between Cdt1p and Mcm6p prevents the formation of the MCM complex, excludes Mcm2-7p from the nucleus, and inhibits pre-RC assembly and DNA replication. Our findings suggest a function for Cdt1p in promoting the assembly of the MCM complex and maintaining its integrity by interacting with Mcm6p.

Key words: DNA replication, Pre-replicative complex, Cdt1p–Mcm6p interaction, MCM complex formation, Saccharomyces cerevisiae

Introduction

DNA replication is one of the most fundamental cellular processes. To maintain the integrity of the genetic information as it is passed from one generation to the next, the initiation of DNA replication must be stringently controlled to ensure that genome duplication occurs precisely once per cell cycle. Replication initiation is a multi-step process, including replication origin licensing and activation. During origin licensing at the M-to-G1 transition, pre-RCs are formed by the stepwise assembly of Cdc6p, Cdt1p and the minichromosome maintenance (MCM) complex onto the platform formed by the origin recognition complex (ORC) and other proteins at replication origins (Bell and Dutta, 2002; Méndez and Stillman, 2003; Labib, 2010). Subsequently, pre-initiation complexes (pre-ICs) are formed by the loading of other replication-initiation proteins onto replication origins. Activation of replication initiation is then achieved by the actions of cyclin-dependent kinases (CDKs) and the Dbf4p-dependent Cdc7p kinase (DDK), which phosphorylate several pre-RC and pre-IC components.

Cdt1p was first identified as a licensing factor in Xenopus (Maiorano et al., 2000). Along with Cdc6p and other licensing factors, Cdt1p is required for pre-RC formation but not DNA replication elongation (Devault et al., 2002; Tanaka and Diffley, 2002). Unlike other proteins involved in DNA replication, the primary sequences of Cdt1p homologues have a low degree of conservation among diverse species; for example, there is only 11% identity between yeast and human CDT1 proteins. In addition, the regulation of Cdt1 varies significantly among eukaryotes. It is regulated by ubiquitin-mediated proteolysis in fission yeast (Nishitani et al., 2004) and other model organisms (Higa et al., 2003; Arias and Walter, 2005); by binding of the inhibitor Geminin in Xenopus (Wohlschlegel et al., 2000) and mammalian cells (Yanagi et al., 2002); and by nuclear export in budding yeast (Tanaka and Diffley, 2002).

In budding yeast, Mcm2-7p and Cdt1p are imported into the nucleus during the M-to-G1 transition when CDK activity is low, whereas their export to the cytoplasm in late G1 and S phases is promoted by high CDK activity (Labib et al., 1999; Liku et al., 2005; Nguyen et al., 2000). Cdt1p, which does not possess a nuclear localization signal (NLS), requires Mcm2-7p to enter the nucleus (Tanaka and Diffley, 2002). Furthermore, nuclear accumulation of Mcm2-7p requires the formation of a complex containing all six MCM subunits and Cdt1p (Pasios and Forsburg, 1999; Labib et al., 2001; Tanaka and Diffley, 2002). However, the reason why the nuclear localization of the Mcm2-7p complex, in which Mcm2p and Mcm3p have NLSs, depends on Cdt1p is still unknown.

In this study, we discovered that Cdt1p is essential for the formation of the MCM complex in both the cytoplasm and nucleoplasm before pre-RC formation. We found that disruption of the interaction between Cdt1p and Mcm6p prevents the assembly of the MCM complex, abolishes nuclear retention of Mcm2-7p, and inhibits pre-RC formation and DNA replication. These findings suggest a function for Cdt1p in facilitating the assembly and maintaining the integrity of the hexameric MCM complex by interacting with Mcm6p.
Results

Depletion of either Mcm6p or Cdt1p in M phase prevents MCM complex formation

Cdt1p and the MCM proteins are exported separately from the nucleus: Cdt1p is exported to the cytoplasm before replication initiation (Tanaka and Diffley, 2002), whereas Mcm2-7p is exported, possibly as individual subunits, during DNA replication (Nguyen et al., 2000) (see the Discussion). Cdt1p and Mcm2-7p associate to form an MCM–Cdt1p complex in the cytoplasm in M phase (Tanaka and Diffley, 2002). Previous studies showed that depletion of Cdt1p or any one of the MCM subunits abolishes the nuclear import of the MCM complex during the M-to-G1 transition (Labib et al., 2001; Tanaka and Diffley, 2002). Because not every MCM subunit contains a nuclear localization signal and hence Mcm2-7p needs to be imported into the nucleus as a heterohexameric complex, we speculated that the failure of Mcm2-7p nuclear importation when Cdt1p or an MCM subunit is depleted might result from the failure of the MCM subunits to form a complex. To test this hypothesis, we used co-immunoprecipitation to examine the interactions between different MCM subunits in yeast extracts prepared from mcm6-td (td, temperature-inducible degron) and cdt1-td cells depleted of the Mcm6-td and Cdt1-td protein, respectively.

Wild-type control, mcm6-td and cdt1-td cells were synchronized in M phase with the microtubule-depolymerising drug nocodazole. The Mcm6-td or Cdt1-td protein was then synchronized in M phase with the microtubule-depolymerising maintained at the permissive temperature of 25 ˚C without induction, which facilitates td protein degradation. Cells immunoprecipitated with Mcm2p using the extracts from immunoprecipitation experiments using Cdt1p was not precipitated by the control mouse IgG (Fig. 1A). These results indicate that Mcm2p, Mcm3p and Cdt1p can specifically interact with each other and that Cdt1p can be precipitated by Mcm2p at the permissive temperature of 25 ˚C when Cdt1p or Cdt1-td was present (Fig. 1B, lanes 1–9), by contrast, neither Cdt1p nor Mcm3p was co-immunoprecipitated with Mcm2p using the extract from mcm6-td cells at 37 ˚C when the Mcm6-td protein was degraded (Fig. 1A, lanes 10–12). As controls for the specificity of the co-immunoprecipitation, Mcm2p, Mcm3p or Cdt1p was not precipitated by the control mouse IgG (Fig. 1A). These results indicate that Mcm2p, Mcm3p and Cdt1p can no longer be in a complex without Mcm6p. Similarly, co-immunoprecipitation experiments using cdt1-td cells showed that Mcm3p, Mcm4–GFP, Mcm6–HA and Cdt1p or Cdt1-td were co-immunoprecipitated with Mcm2p using the extracts from CDT1 wild-type cells at both 25 ˚C and 37 ˚C and from cdt1-td cells at 25 ˚C when Cdt1p or Cdt1-td was present (Fig. 1B, lanes 1–9), but not from cdt1-td cells at 37 ˚C when the Cdt1-td protein was depleted (Fig. 1B, lanes 10–12). These results suggest that depletion of Cdt1p prevents Mcm2-7p complex formation.

To corroborate the anti-Mcm2 co-immunoprecipitation results, we performed anti-Mcm3, anti-Mcm4–GFP and anti-Mcm6–HA co-immunoprecipitation experiments, separately, under similar conditions as those for Fig. 1B. The results showed that Mcm2p, Mcm3p, Mcm4–GFP, Mcm6–HA and Cdt1p or Cdt1-td were all co-immunoprecipitated by each of the relevant antibodies using extracts from CDT1 wild-type cells at both 25 ˚C and 37 ˚C and from cdt1-td cells at 25 ˚C, but not from cdt1-td cells at 37 ˚C (supplementary material Fig. S1A–C). These results support the conclusion from the anti-Mcm2 co-immunoprecipitation experiments that Cdt1p is required for the formation of the MCM complex.

To confirm that Mcm2-7p cannot assemble into a complex after Cdt1-td depletion, we examined the co-sedimentation of different MCM subunits by sucrose gradient centrifugation. Extracts from wild-type and Cdt1-td-depleted M phase cells were separated on a 20–50% sucrose gradient, and fractions were immunoblotted for the different MCM subunits (Fig. 1C; see Coomassie-stained gels in supplementary material Fig. S1D). Mcm2p, Mcm3p, Mcm4–GFP and Mcm6–HA from wild-type cells co-migrated as both monomeric (∼140 kDa) and hexameric (∼540 kDa) forms, whereas they only appeared in the fractions of the monomers in Cdt1-td-depleted extract. Together with the co-immunoprecipitation data, these results demonstrate that Cdt1p is required for the formation of the Mcm2-7p complex in M phase before the MCM complex enters the nucleus, providing an explanation for the failure of the MCM complex to enter the nucleus in the absence of Cdt1p.

Depletion of Cdt1p in G1 phase abolishes the formation and nuclear retention of non-chromatin-bound Mcm2-7p

To investigate the possible role of Cdt1p in maintaining the integrity of the MCM complex in G1 phase when most of MCM proteins are in the complex and normally locate in the nucleus, we examined the localization of Mcm4–GFP in G1 phase cdt1-td cells depleted of the Cdt1-td protein. When the cells synchronized in G1 phase by the yeast mating pheromone α-factor at 25 ˚C were shifted to 37 ˚C to deplete the Cdt1-td protein, most Mcm4–GFP was excluded from the nucleus, whereas most Mcm4–GFP remained in the nucleus in wild-type cells (supplementary material Fig. S2A,B). However, there remained a low level of GFP signal in the nucleus even after 4 hours of Cdt1-td depletion (supplementary material Fig. S2A). We speculated that this might result from the stabilization of the chromatin-bound MCM complex as a component of the pre-RC. To test this possibility, we examined the chromatin association of Mcm2p in cells depleted of Cdt1p as above. The chromatin-bound Mcm2p in cdt1-td cells at 37 ˚C, although decreased to some extent compared with levels in wild-type cells, appeared constant during the time course of Cdt1-td depletion (supplementary material Fig. S2C). These results suggest that depletion of Cdt1p results in nuclear export of most Mcm2-7p, but does not remove the chromatin-associated MCM proteins once pre-RCs have formed. Consistent with this finding, flow cytometry confirmed that depletion of Cdt1p in G1 phase did not result in any observable defect in cell cycle progression after the cells were released into fresh medium (supplementary material Fig. S2D), indicating that the MCM proteins stably bound on chromatin after Cdt1-td depletion were functional for DNA replication.

To more clearly demonstrate the role of Cdt1p in the nuclear retention of the non-chromatin-bound MCM proteins in G1 cells, we needed to prevent MCM proteins from binding to chromatin in G1 phase before Cdt1-td protein depletion. To do this, we first synchronized cells in G1 phase and then released them from the G1 block while shutting off CDC6 expression under the control of the MET3 promoter to prevent pre-RC formation in the next G1 phase (Labib et al., 1999; Nguyen et al., 2000). These G1 cells were then shifted to 37 ˚C to deplete the Cdt1-td protein (see diagram in Fig. 2A). Fluorescence microscopy showed that without Cdt1p, the nuclear Mcm4–GFP signal decreased dramatically compared with that in the CDT1 wild-type control cells and was reduced to a non-detectable level after 120 minutes.
of Cdt1-td depletion (Fig. 2B,C). Quantification of the Mcm4–GFP nuclear localization indicated that wild-type and cdt1-td cells at 25 °C showed a similar level of the nuclear accumulated Mcm4–GFP (Fig. 2C). By contrast, few cdt1-td cells contained nuclear Mcm4–GFP after 2 hours of Cdt1-td depletion at 25 °C, whereas wild-type cells accumulated nuclear Mcm4–GFP normally (Fig. 2C). Flow cytometry confirmed that the cells remained arrested in G1 phase throughout the experiment (supplementary material Fig. S4). The chromatin-bound and soluble proteins from the cells at each time point were also immunoblotted to confirm that there was no pre-RC (i.e. absence of Mcm2 protein) without Cdc6p, and that the decrease of the nuclear Mcm4–GFP signal during the time course of Cdt1-td depletion was not due to any notable change in the total Mcm4–GFP protein level (Fig. 2D). Together, these results reveal that depletion of Cdt1p abolishes the nuclear retention of non-chromatin-bound MCM proteins.

Because depletion of Cdt1p prevents the formation of the MCM complex in M phase cells (Fig. 1), we reasoned that the loss of nuclear retention of non-chromatin-bound MCM proteins in Cdt1-td-deleted G1 cells probably also resulted from disassembly of the MCM complex. To validate this hypothesis, we performed co-immunoprecipitation with extracts from G1 cells depleted of the Cdt1-td protein and without pre-RC formation as described above. The results showed that neither Mcm3p nor Mcm4–GFP could be co-immunoprecipitated with Mcm2p in G1-phase cell extracts without Cdt1p (Fig. 2E, lanes 10–12), but they were co-immunoprecipitated with Mcm2p in the control G1 extracts in the presence of Cdt1p or Cdt1-td (Fig. 2E, lanes 1–9). Together, these data indicate that Cdt1p is required for the nuclear retention of non-chromatin-bound MCM2-7p in G1 cells by maintaining the integrity of the MCM complex. These findings might have implications in the nuclear export of MCM proteins in S phase (see the Discussion).

Cdt1p and Mcm6p interact through their C-terminal domains

As a component of pre-RC, Cdt1p is known to interact with Mcm6p physically in metazoans (Teer and Dutta, 2008; Wei et al., 2010; Zhang et al., 2010). To further investigate the role of Cdt1p in maintaining the MCM complex integrity in budding yeast, we focused on the interaction between Cdt1p and Mcm6p. Based on the mapping of interaction domains of human CDT1 and MCM6 (Zhang et al., 2010), we truncated Cdt1p and Mcm6p, each into a large N-terminal and a small C-terminal fragment designated Cdt1N (amino acids 1–421), Cdt1C (422–604), Mcm6N (1–914) and Mcm6C (915–1017), respectively (see diagram in Fig. 3A). We performed yeast two-hybrid assays and found that Cdt1p and Cdt1C interacted with both Mcm6p and Mcm6p. Based on the mapping of interaction domains of human CDT1 and MCM6 (Zhang et al., 2010), we truncated Cdt1p and Mcm6p, each into a large N-terminal and a small C-terminal fragment designated Cdt1N (amino acids 1–421), Cdt1C (422–604), Mcm6N (1–914) and Mcm6C (915–1017), respectively (see diagram in Fig. 3A). We performed yeast two-hybrid assays and found that Cdt1p and Cdt1C interacted with both Mcm6p and Mcm6p, whereas Cdt1N and Mcm6N had no interaction either with each other, or with Mcm6p or Cdt1p (Fig. 3B). Immunoblotting showed that the hybrid proteins used in this study were expressed at similar levels in the host cells (supplementary material Fig. S3). These data suggest that Cdt1p and Mcm6p interact through the small C-terminal domain present in each of the two proteins.

To confirm the interactions detected by the yeast two-hybrid analysis, we performed co-immunoprecipitation to examine
interactions of the FLAG-tagged Cdt1p, Cdt1N and Cdt1C with Mcm6–HA and the interactions of FLAG-tagged Mcm6p, Mcm6N and Mcm6C with Cdt1-HA in yeast extracts. The results showed that Mcm6–HA was co-immunoprecipitated with Cdt1–FLAG (Fig. 3C, lane 7) and Cdt1C–FLAG (Fig. 3C, lane 11), but not with Cdt1N–FLAG (Fig. 3C, lane 9). Likewise, Cdt1–HA was co-immunoprecipitated with Mcm6–FLAG and Mcm6C–FLAG, but not with Mcm6N–FLAG (Fig. 3D). Together, the results from the yeast two-hybrid and co-immunoprecipitation assays strongly suggest that Cdt1p and Mcm6p interact with each other through their C-terminal domains.

Our yeast two-hybrid analysis (Fig. 3B) also confirmed the previously reported results using the same assay (Asano et al., 2007) and the GST-pull down assay (Chen et al., 2007) that Cdt1p interacts with Orc6p, and that Mcm6p interacts with Mcm2p, as reported for the homologous proteins in mammalian cells (Kneissl et al., 2003; Yu et al., 2004). Our results further showed that the N-terminal domain of Cdt1p interacts with Orc6p, and that the N-terminal domain of Mcm6p interacted with Mcm2p (Fig. 3B).

To identify the interacting amino acid residues in the C-terminal domains of Cdt1p and Mcm6p, we introduced point mutations in the respective C-terminal domain for yeast two-hybrid analysis (Fig. 3B). The Cdt1p-binding, C-terminal domain of human Mcm6p contains several acidic residues. Our previous study showed that mutations at these and other residues in the C-terminal domain of human MCM6 (Glu757Ala, Glu763Ala and Leu766Ala) significantly reduced the affinity between Cdt1p and Mcm6p (Wei et al., 2010). Sequence alignment showed that three acidic amino acids, Glu945, Asp947 and Glu953, in the C-terminal domain of Mcm6p are conserved from yeast to human (supplementary material Fig. S5A). In addition, Leu951 and Tyr954, corresponding to the Glu763 and Leu766 in human MCM6, are conserved in most species (supplementary material Fig. S5A). Because these residues are on the interacting interface of the human Cdt1p and Mcm6p (Wei et al., 2010), we reasoned that these five residues are likely to be important for the interaction between Cdt1p and Mcm6p. We substituted these residues with alanine in Mcm6p and Mcm6C to construct the Mcm6-5A (full length) and Mcm6C-5A (C-terminal domain) mutants, respectively. Yeast two-hybrid analysis showed that

Fig. 2. Cdt1p is essential for the nuclear retention and integrity of non-chromatin-bound MCM complex. (A) Outline of the experiments to examine the localization and chromatin association of Mcm4–GFP and the integrity of the MCM complex. YL1211 (cdt1-td MET-CDC6) and YL1213 (CDT1 MET-CDC6) cells were cultured in medium lacking methionine, and α-factor was added to synchronize cells in G1 phase at 25°C (–Met +α-F, 25°C). Cells were then released into fresh methionine-containing medium (+Met –α-F, 25°C) grown until buds emerged, and α-factor was then added to block cells in the next G1 phase (+Met +α-F, 25°C). Cells were then shifted to 37°C for 2 hours (+Met +α-F, 37°C) and harvested for analysis. (B) Fluorescence microscopic analysis was performed with living cells in the final G1 phase at 25°C and at 37°C with 30 minute intervals. Scale bar: 10 μm. (C) The proportion of cells with nuclear Mcm4–GFP shown in Fig. 2B was quantified (n=300). (D) WCEs and chromatin fraction (Chr.) prepared from the cells were immunoblotted for Mcm2p, Orc3p, Cdc6p, Cdt1-td, Cdt1p and Mcm4–GFP. (E) WCEs were immunoprecipitated with anti-Mcm2 antibody or the control mouse IgG (Ctrl IgG), and WCEs and the immunoprecipitates were immunoblotted for Cdt1-td, Cdt1p, Mcm4–GFP, Mcm3p and Mcm2p. A dash between two lane numbers indicates a blank lane.
Mcm6-5A and Mcm6C-5A could no longer interact with either Cdt1p or Cdt1C, whereas Mcm6-5A still reserved the interaction with Mcm2p (Fig. 3B). We also determined that Mcm6p fused to the activation domain (AD–Mcm6p), but not AD–Mcm6-5A, expressed from the yeast two-hybrid plasmids that were used for the interaction study in Fig. 3B supported the growth of the GAL-MCM6 cells in which the expression of the endogenous MCM6 was repressed by glucose (supplementary material Fig. S5C), indicating that AD–Mcm6p, but not AD–Mcm6-5A, is biologically functional. These results suggest that some, if not all of the mutated residues in Mcm6p are crucial for the Cdt1–Mcm6p interaction, and that the C-terminal domain of Mcm6p is essential for cell proliferation through its interaction with Cdt1p.

Given that the Cdt1p-interacting surface of Mcm6p contains acidic amino acids, we speculated the residues on the Mcm6p-interacting surface of Cdt1p might contain basic amino acids. We aligned the C-terminal sequences of Cdt1p from different species and identified five highly conserved basic amino acids, Arg486, Arg488, Arg490, Arg501 and Lys512 (supplementary material Fig. S5B). We changed all of the five amino acids to alanine to construct the Cdt1-5A (full length) and Cdt1C-5A (C-terminal domain) constructs. We then tested the interactions between Cdt1-5A and Mcm6p by yeast two-hybrid analysis (Fig. 3C)

These results suggest that the Cdt1p-interacting surface of Mcm6p contains acidic amino acids, and the five conserved basic amino acids in Cdt1p are essential for its interaction with Mcm6p.
domain) mutants. As predicted, Cdt1-5A and Cdt1C-5A no longer interacted with Mcm6p or Mcm6C, but Cdt1-5A still interacted with Orc6p in the two-hybrid assay (Fig. 3B). Furthermore, BD-Cdt1p (Cdt1p fused to the DNA binding domain), but not BD–Cdt1-5A, supported the growth of the GAL–CDT1 cells when the expression of the endogenous CDT1 was repressed (supplementary material Fig. S5C), indicating that BD–Cdt1-5A is no longer functional, unlike BD-Cdt1p. These results suggest that the conserved basic residues in the C-terminal domain of Cdt1p are crucial for the Cdt1p–Mcm6p interaction and essential for Cdt1p function. Taken together, our two-hybrid and co-immunoprecipitation results strongly suggest that the interaction between the small C-terminal domains of Cdt1p and Mcm6p, at least partly through charge–charge interactions, is a part of the bridge that brings ORC and the MCM complex together to form the pre-RC (see diagram in Fig. 3E).

Overexpression of the Mcm6p-interacting domain of Cdt1p impairs cell growth, MCM nuclear localization and initiation of DNA replication

Because the interaction between the C-terminal domains of Mcm6p and Cdt1p is crucial for MCM complex assembly, overexpression of these domains might exert negative dominant effects on cell proliferation. BD-Cdt1p is functional (supplementary material Fig. S5C), therefore we overexpressed different BD-fusion proteins in wild-type yeast cells to examine cell growth. The growth of the cells expressing BD–Cdt1C, but not BD–Cdt1p or BD–Cdt1N, was severely defective (Fig. 4A). However, overexpression of the BD–Cdt1C-5A mutant, which does not interact with Mcm6p as described above, did not show any effect on cell growth (Fig. 4A). These results suggest that the overexpressed BD–Cdt1C hindered cell growth through its interaction with the endogenous Mcm6p.

If overexpression of BD–Cdt1C interferes with the interaction between the endogenous Cdt1p and Mcm6p, and Cdt1p is essential for the formation as well as chromatin loading of the MCM complex, overexpression of BD–Cdt1C should result in mislocalization of the MCM complex. To investigate this possibility, we examined the localization of Mcm4–GFP in cells expressing BD–Cdt1C in G1 phase. By comparing the average Mcm4–GFP signal per unit area in the nucleus and in the whole cell (see the Materials and Methods), we found that cells overexpressing BD–Cdt1C, but not BD–Cdt1 or BD–Cdt1C-5A, showed a reduced relative intensity in the nuclear Mcm4–GFP

Fig. 4. Overexpression of the C-terminal region of Cdt1p impairs MCM nuclear accumulation, initiation of DNA replication and cell growth. (A) Tenfold serial dilutions of YL1216 (Mcm4–GFP) cells containing the empty BD vector or the plasmid expressing BD–Cdt1, BD–Cdt1N, BD–Cdt1C or BD–Cdt1C-5A were plated on a SCM–Trp plate (selective for the BD vector) and incubated for 3 days at 23°C. (B) Microscopic images of G1-phase-synchronized YL1216 (Mcm4–GFP) cells containing the empty BD vector or the plasmid expressing BD–Cdt1, BD–Cdt1C or BD–Cdt1C-5A. Scale bar: 10 μm. Insets represent the single cells in the white boxes. (C) Quantification of the average fluorescence signal intensity of the nucleus normalized to the average fluorescence signal intensity of the whole cell (n=30). The error bars represent s.d. *P<0.001. (D) Quantitative plasmid loss rates were measured for the cells containing the empty BD vector or the plasmid expressing BD–Cdt1C or BD–Cdt1C-5A plus either p1ARS or p8ARSs grown in SCM-Trp medium at 25°C for 10–11 generations. Plasmid loss rates are represented as the means of percentage loss per generation ± s.d. of three separate experiments.
signal (Fig. 4B,C). These results suggest that disruption of nuclear localization of Mcm2-7p is part of the mechanism by which overexpression of BD–Cdt1C impairs cell growth.

To determine whether overexpression of BD–Cdt1C impairs replication initiation, the loss rates of a pair of tester plasmids, p1ARS and p8ARSs (Zhang et al., 2002), in the cells were measured. p1ARS contains one replication origin ARS1, and p8ARSs carries seven additional copies of H4-ARS inserted into p1ARS. It has been well documented that all known replication initiation mutants have a higher rate of p1ARS loss compared with p8ARSs (Hogan and Koshland, 1992; Loo et al., 1995; Zhang et al., 2002; Cheng et al., 2010; Ma et al., 2010; Wang et al., 2010; Zhai et al., 2010). In control cells containing the empty BD vector, the loss rates of p1ARS and p8ARSs were 1.2% and 0.5% per generation, respectively (Fig. 4D), as expected for wild-type cells. In the cells expressing BD–Cdt1C, the loss rate of p1ARS was as high as 15.3% per generation, whereas the loss rate of p8ARSs was significantly reduced to 3.0% (Fig. 4D), indicating that the high p1ARS loss rate was due to defective replication initiation in BD–Cdt1C-expressing cells. Plasmid loss rates of the cells expressing the mutant BD–Cdt1C-5A were similar to those of the vector control cells (Fig. 4D). Together, data in Fig. 4 suggest that the overexpressed BD–Cdt1C impaired cell growth by titrating Mcm6p, thus interfering with the interaction between the endogenous Cdt1p and Mcm6p and impairing nuclear accumulation of the MCM complex and replication initiation.

Disruption of the Cdt1p–Mcm6p interaction by mutation of Cdt1-5A or Mcm6-5A impairs MCM complex formation and nuclear localization, pre-RC assembly and DNA replication

To further probe the physiological significance of the Cdt1p–Mcm6p interaction, we examined the consequences of disrupting the interaction using Cdt1-5A or Mcm6-5A mutants. Because BD–Cdt1p and AD–Mcm6p are functional (supplementary material Fig. S5C), we expressed BD–Cdt1p and BD–Cdt1-5A in cdt1-td cells, and AD–Mcm6p and AD–Mcm6-5A in mcm6-td cells to investigate the effects of the mutations in Cdt1-5A and Mcm6-5A on MCM complex formation and nuclear localization, pre-RC formation and DNA replication.

We first performed co-immunoprecipitation between Mcm2p and other MCM subunits using yeast extracts to determine whether the Cdt1-5A or Mcm6-5A mutant could support MCM complex formation when the corresponding Cdt1-td or Mcm6-td protein is degraded. cdt1-td cells containing the empty BD vector or the plasmid expressing BD–Cdt1p or BD–Cdt1-5A were synchronized in M phase using nocodazole and then shifted to 37°C to degrade the Cdt1-td protein. Mcm2p, Mcm3p and Mcm4–GFP were co-immunoprecipitated with extracts from cells expressing wild-type BD–Cdt1p but not BD–Cdt1-5A mutant or the cells containing the empty vector (Fig. 5A). These results suggest that the Cdt1-5A mutant cannot support the formation of the Mcm2-7p complex. Similarly, using mcm6-td cells containing the empty AD vector or the plasmid expressing AD–Mcm6p or AD–Mcm6-5A, we found that the AD–Mcm6-5A mutant was unable to support MCM complex formation (Fig. 5B). These data strongly suggest that the Cdt1p–Mcm6p interaction through the C-terminal regions of the two proteins is required for the assembly and integrity of the Mcm2-7p complex.

We reasoned that when the BD–Cdt1-5A or AD–Mcm6-5A mutant failed to support MCM complex formation, MCM nuclear accumulation should also be abolished. To verify this, cells from the same strains used in Fig. 5A,B were blocked in M phase using nocodazole, shifted to 37°C to degrade the Cdt1-td or Mcm6-td protein, respectively, and then released into G1 phase in fresh medium containing α-factor at 37°C to examine localization of Mcm4–GFP. G1-phase cells at 25°C and M-phase cells at 37°C were used as the positive and negative controls, respectively, for MCM nuclear localization. The results showed that the BD–Cdt1-5A mutant did not support Mcm4–GFP nuclear accumulation in G1 phase at 37°C when the Cdt1-td protein was depleted. However, Mcm4–GFP localized normally in cells expressing wild-type BD–Cdt1p at both 25°C and 37°C and those expressing BD–Cdt1-5A at 25°C (Fig. 5C). Similarly, the Mcm6-5A mutant failed to allow nuclear accumulation of Mcm4–GFP when the Mcm6-td protein was depleted (Fig. 5C).

We further investigated the effects of the mutations in Cdt1-5A on pre-RC formation during the M-to-G1 transition. cdt1-td cells expressing BD–Cdt1p or BD–Cdt1-5A were synchronized in M phase, shifted to 37°C, and then released into G1 phase in medium containing α-factor at 37°C. Chromatin binding assays were performed to examine Mcm2p and other proteins on chromatin to evaluate pre-RC formation. The results showed that the BD–Cdt1-5A mutant did not support pre-RC formation because Mcm2p was absent from chromatin when cells passed through M phase into G1 phase, whereas in cells expressing the wild-type BD–Cdt1p, Mcm2p was loaded onto chromatin 30 minutes after release from the M-phase block (Fig. 5D,E). These results indicate that the interaction between Cdt1p and Mcm6p is essential for pre-RC formation.

To investigate the effects of the Cdt1-5A mutations on DNA replication, we studied the cell cycle profile of cdt1-td cells expressing BD–Cdt1p or BD–Cdt1-5A. Cells were induced to degrade the Cdt1-td protein in M phase and then released into α-factor-containing medium at 37°C. The synchronized G1 cells were then released into fresh medium at 37°C, and the DNA content of the cells in different time points was analyzed by flow cytometry. As Fig. 5F shows, when the Cdt1-td protein was depleted, the cells expressing the wild-type BD–Cdt1p replicated normally, whereas the cells expressing the BD–Cdt1-5A mutant could not duplicate their DNA, even though cell budding occurred normally. These results indicate that BD–Cdt1-5A does not support DNA replication. Together, data shown in Figs 4 and 5 indicate that disruption of the Cdt1p–Mcm6p interaction by the mutations in Cdt1-5A impairs MCM complex formation and nuclear localization, pre-RC assembly and cell viability.

Discussion

In our effort to better understand the physiological roles of Cdt1p in pre-RC formation and the assembly mechanism of the MCM complex, we provide the first evidence that Cdt1p, through its interaction with Mcm6p with a small C-terminal region of each of the two proteins, is crucial for both the assembly and integrity of the non-chromatin-bound MCM complex. Once this interaction is disrupted, by depleting Cdt1p or Mcm6p, expressing the Mcm6p-interacting region of Cdt1p, or mutating the interacting interface between Cdt1p and Mcm6p, the MCM complex disassembles and loses its ability to accumulate in the nucleus and to form the pre-RC. Furthermore, we provide a molecular explanation as to why Cdt1p is required for the nuclear import of MCM proteins that contain NLSs.
The Cdt1p–Mcm6p interaction is crucial for MCM complex formation in vivo

Several models have been proposed for the assembly of the MCM complex. Studies from *E. coli* to metazoans suggest that the architecture of replicative helicases, including Mcm2-7p, is a ring-shaped hexamer. In addition, depletion of any subunit of Mcm2-7p results in the mislocalization of the MCM complex (Pasion and Forsburg, 1999; Labib et al., 2001). These and other results suggest that Mcm2-7p function together as a heterohexamer (Davey et al., 2003; Evrin et al., 2009; Remus et al., 2009). However, it is not clear how Mcm2-7p assembles to form a hexameric complex, or whether Cdt1p plays a role in this process.

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**Fig. 5.** Mutations in Mcm6-5A and Cdt1-5A abolish Mcm2-7p nuclear accumulation, MCM complex integrity, pre-RC formation and DNA replication. (A) The Cdt1-td protein was depleted in M-phase-synchronized YL1215 (cdt1-td) cells containing the empty BD vector or the plasmid expressing BD-Cdt1 or BD-Cdt1-5A. WCEs were immunoprecipitated with anti-Mcm2p antibody or the control mouse IgG (Ctrl. IgG), and WCEs and immunoprecipitates were immunoblotted with anti-Myc antibody to detect the BD–Cdt1p and BD–Cdt1-5A fusion proteins. Mcm2p, Mcm3p and Mcm4–GFP were also immunoblotted. (B) Mcm6-td was depleted in M-phase-synchronized YL1224 (mcm6-td) cells containing the empty AD vector or plasmid expressing AD–Mcm6 or AD–Mcm6-5A. WCEs were immunoprecipitated with anti-Mcm2p antibody or the control mouse IgG (Ctrl. IgG), and WCEs and immunoprecipitates were immunoblotted with anti-HA antibody to detect the AD–Mcm6p and AD–Mcm6-5A fusion proteins. Mcm2p, Mcm3p and Mcm4–GFP were also immunoblotted. (C) YL1224 (mcm6-td) and YL1215 (cdt1-td) cells containing the empty vector or the plasmid expressing Mcm6p, Cdt1p, Mcm6-5A or Cdt1-5A, were synchronized by α-factor (α-F.) before being released into fresh medium containing nocodazole (Noc.). Cells blocked in M phase were shifted to 37°C to degrade the Mcm6-td or Cdt1-td protein, respectively, and then released to fresh medium at 37°C containing α-factor to synchronize cells in G1 phase. The cells in G1 phase at 25°C (α-F., 25°C) and those synchronized in M (Noc., 37°C) or G1 (α-F., 37°C) phase at 37°C were taken for analysis. The proportion of the cells with nuclear Mcm4–GFP was quantified (n=300). (D) YL1208 (cdt1-td) cells containing the plasmid expressing Cdt1p or Cdt1-5A were synchronized in M phase and were then shifted to 37°C for 1.5 hours before being released into fresh medium containing α-factor at 37°C for two hours. Samples were taken at 30 minute intervals. The chromatin fraction (Chr.) and (WCEs) of the cell samples were immunoblotted for Mcm2p, Orc3p, Cdt1p and Cdt1-5A. (E) Flow cytometry analysis performed for the cells from D. % Bud., percentage of budded cells. (F) YL1208 (cdt1-td) cells containing the plasmid expressing Cdt1p or Cdt1-5A were synchronized in M phase before being shifted to 37°C for 1.5 hours to deplete the Cdt1-td protein. Afterwards, cells were released into fresh medium containing α-factor at 37°C for 2 hours and then released into fresh medium at 37°C. Flow cytometry was performed for the cell samples taken at the indicated time points.
It was reported that approximately equimolar Cdt1p was readily co-purified with the Mcm2-7p complex from yeast cell extracts (Kawasaki et al., 2006; Remus et al., 2009; Tsakraklides and Bell, 2010). It was also shown that, without co-overexpression of Cdt1p, most of the overexpressed MCM subunits in yeast cell extracts formed sub-complexes other than the Mcm2-7p hexamer (Tsakraklides and Bell, 2010). These observations are consistent with our results that non-chromatin-bound MCM proteins cannot form a heterohexameric complex after Cdt1p depletion in vivo. However, a fraction of the overexpressed Mcm2-7p subunits could be purified as hexamers from yeast extracts without Cdt1p co-overexpression in G1 phase cells (Evrin et al., 2009; Tsakraklides and Bell, 2010). The MCM complex purified this way might originate from the chromatin-bound MCM proteins, because we found that depletion of Cdt1p does not affect the integrity of the chromatin-bound MCM complex in G1 phase. In an in vitro system, six separately purified recombinant MCM subunits at 17.6 mM each could form a hexamer without Cdt1p (Davey et al., 2003). However, it is noteworthy that the concentrations of most MCM subunits in the cell are around 1 mM or lower (Ghaemmaghami et al., 2003). It is possible that the in vitro environment with relatively high concentrations of MCM proteins, low ion strength and lack of competition from other proteins facilitates the intermolecular interactions and thus MCM complex formation. In some other reports, the MCM complex was purified from insect cells co-overexpressing Mcm2-7p (Schwacha and Bell, 2001; Bochman and Schwacha, 2007). However, these reports also showed a significant increase of the molecular weight of the purified MCM complex (Schwacha and Bell, 2001), suggesting that the MCM complex is associated with other proteins, possibly including Cdt1p from insect cells.

By contrast, it is reported that Mcm4p–Mcm6p–Mcm7p and Mcm3p–Mcm5p form separated sub-complexes in vitro (You et al., 1999). Our results from yeast two-hybrid assay support these observations in budding yeast, because MCM subunits that are not neighbors in the hexamer also had interactions (data not shown), which is consistent with the data in human and murine MCM (Kneissl et al., 2003; Yu et al., 2004). However, the results from the co-immunoprecipitation and sucrose gradient centrifugation assay in this study did not show the clear existence of sub-complexes. There at least two possible reasons for the absence of sub-complexes in our experiments. First, the salt concentration of our lysate buffer is relatively high (200 mM K-Glutamate) which might break the sub-complexes. Second, the resolution of gradient ultra-centrifugation might not be high enough to differentiate the monomers and sub-complexes. Because it has been shown that the functional MCM complex is the heterohexamer (Gambus et al., 2006; Moyer et al., 2006), we did not further investigate the role of Cdt1p in sub-complex formation.

In this study, we used three different methods, namely cdt1-td protein depletion, overexpression of the Mcm6p-interacting domain of Cdt1p, and mutation of the interacting surface of Mcm6p and Cdt1p, to disrupt the Cdt1p–Mcm6p interaction, and observed similar phenotypes: disassembly and failed nuclear accumulation of the MCM complex, defective pre-RC formation and DNA replication. A recent study showed in budding yeast that Cdt1p C-terminal mutations, which include some of the mutation sites in Cdt1-5A cause a lethal phenotype (Jee et al., 2010), consistent with our results that the Mcm6-5A and Cdt1-5A mutations disrupt the Cdt1p–Mcm6p interaction. These results demonstrate that the interaction between Cdt1p and Mcm6p is crucial for the assembly and integrity of the Mcm2-7p complex in vivo.

The C-terminal region of Cdt1p is conserved in eukaryotes

Despite the fact that Cdt1p is not highly conserved in eukaryotes with only 10–12% protein sequence identity among the homologues, we noted high sequence similarity in the C-terminal region of Cdt1p from different species (supplementary material Fig. S5B). Consistently with the possibility that this conserved domain might perform some essential functions, we demonstrate that, like its homologues in other eukaryotes, the C-terminal domain of yeast Cdt1p interacts with Mcm6p. Our findings also suggest that Cdt1p, as a stabilizer to maintain the integrity of the MCM complex, can be another safeguard to prevent re-replication by ensuring the timely disassembly and nuclear export of the excess MCM complex, which is not loaded onto pre-RCs. Our study also raised a question as to whether the function of Cdt1p in maintaining the Mcm2-7p integrity is conserved in other eukaryotes.

A model for the role of Cdt1p in ensuring timely MCM nuclear export

The timing of MCM complex nuclear export has been extensively studied. Mcm2-7p that is not chromatin bound is excluded from the nucleus at the G1-to-S transition (Labib et al., 1999; Nguyen et al., 2000) (see model in supplementary material Fig. S7A). However, the MCM complexes on the replication forks are exported to the cytoplasm during DNA synthesis (Labib et al., 1999; Nguyen et al., 2000) (see model in supplementary material Fig. S7A). Similarly to the nuclear export of the excess Mcm2-7p in late G1 phase, nuclear exclusion of Cdt1p occurs before activation of Cdc7p-Dbf4p (DDK) (Labib et al., 1999; Nguyen et al., 2000; Tanaka and Diffley, 2002). The coincidence of the timing of nuclear export of Cdt1p and Mcm2-7p at the G1-to-S transition suggests that they are exported together as an MCM–Cdt1p complex during this time window.

It was suggested that, of the six MCM subunits, only Mcm3p contains an NES as a part of the mechanism that regulates the localization of the MCM complex (Liku et al., 2005). However, by carefully analyzing the protein sequences of the six MCM subunits using the eukaryotic linear motif server (Puntervoll et al., 2003), we found that all MCM subunits contained putative NES (supplementary material Fig. S6). Interestingly, most of these NESs, except that in Mcm3p, are located at or near the ATPase motifs or regions that are homologous to the so called ‘A-domain’ of the Sulfolobus solfataricus MCM protein (Brewster et al., 2008) (supplementary material Fig. S6). Because MCM subunits form the MCM complex by interacting with one another through their ATPase motifs (Davey et al., 2003), and the A-domains might also localize to the interface of the MCM subunits, which is suggested by the crystal structure of the Sulfolobus solfataricus MCM protein (Brewster et al., 2008), the NESs in the budding yeast MCM proteins, except that in Mcm3p, might be blocked in the MCM complex.

Based on our findings, we propose a model for the regulation of Cdt1p and MCM complex localization (supplementary material Fig. S7). The NESs on the MCM subunits, except that in Mcm3p, are protected from being recognized by binding with other MCM subunits (supplementary material Fig. S7B). At the
G1-to-S transition, CDK activates the NES on Mcm3p to export the non-chromatin-bound MCM–Cdt1p complex to the cytoplasm (supplementary material Fig. S7A). The MCM complex on chromatin dissociates from replication forks during S-phase progression, and disintegrates because there is no Cdt1p in the nucleus to maintain its integrity (supplementary material Fig. S7A). Dissolution of the Mcm2-7p complex causes the exposure of the NESs and nuclear exportation of all MCM subunits (supplementary material Fig. S7A). In the cytoplasm, the six MCM subunits and Cdt1p assemble to form a Cdt1–MCM complex, which is still excluded from the nucleus by the NES on Mcm3p until late mitosis when the CDK activity is low (supplementary material Fig. S7A). This model, which is mostly supported by data presented here and from others, explains why depletion of any one subunit of the Cdt1p–Mcm2-7p complex abolishes the nuclear importation and retention of other subunits, and why the nuclear localization of the Mcm2-7p complex, in which Mcm2p and Mcm3p have NLSs, depends on Cdt1p, thus providing new insight into the regulation of the Cdt1p–MCM complex in DNA replication.

Materials and Methods

Strains, plasmids and antibodies

The strains and plasmids used in this study are listed in supplementary material Tables S1 and S2, respectively. Anti-Orc3p, anti-Mcm2p, anti-Mcm3p and anti-Cdt1p antibody were kindly gifts from Bruce Stillman (Cold Spring Harbor Laboratory, NY, US) and John Diffley, (London Research Institute, London, UK). Anti-HA, anti–FLAG, anti-Mcm4p and anti–GFP antibodies were purchased from Roche, Sigma-Aldrich and Santa Cruz Biotechnology, respectively.

Yeast two-hybrid assay

Matchmaker system III from Clontech was used for the yeast two-hybrid assay, conducted as previously described (Kan et al., 2008; Lau et al., 2011; Wang et al., 2010).

Cell cycle synchronization and flow cytometry

Cell cycle block and release with f-factor or nocardazole was carried out as described previously for the cdt1-td cells (Tanaka and Diffley, 2002) or mcm6-td cells (Lahib et al., 2001). Doxycycline (20 μg/mI) was added to cdt1-td cells before shifting the cells to 37°C. Flow cytometry was performed as previously described (Zhang et al., 2002), 1 × 10^6 cells were collected and fixed with 70% ethanol at 4°C for at least 1 hour. Cells were resuspended with 1 ml of 50 mM sodium citrate containing 0.25 mg/ml RNaseA. After incubation at 50°C for 1 hour, cells were further treated with 1 mg/ml Proteinase K for 1 hour at 50°C. Finally, cells were stained with 2 μg/ml propidium iodide. Fluorescence of each sample was measured on FACSort (Becton Dickinson).

Chromatin binding assay

The chromatin binding of Mcm4–GFP to examine the cell cycle patterns of chromatin-associated proteins was performed as previously described (Liang and Stillman, 1997; Zhang et al., 2002) with minor modifications. The spheroplasts were prepared by digesting the cell wall with lyticase. The spheroplasts were lysed in extraction buffer containing 0.1% Triton X-100. The lysates were centrifuged at 500 g for 10 minutes to eliminate most of unlysed cells. The clarified lysates were underlaid with a 30% sucrose cushion and centrifuged at 21,500 g for 30 minutes and then immunoprecipitated with specific antibodies or the control mouse IgG overnight at 4°C. The immunoprecipitates were then collected with Protein-G beads for 2 hours at 4°C. The immunoprecipitates were washed five times with pre-chilled Buffer K-200 (Evrin et al., 2009) before elution with SDS sample buffer at 95°C.

Co-immunoprecipitation assay

100 ml of yeast culture at OD600 = 2 (~2 × 10^7 cells/ml) were harvested for one immunoprecipitation. Yeast whole cell extracts were prepared by bead beating in Buffer K-200 (25 mM Hepes, pH 7.5, 5 mM magnesium acetate, 1 mM EDTA, 1 mM EGTA, 200 mM K-glutamate, 0.5% Triton X-100, 10% glycerol, 1 × protease inhibitor). Extracts were diluted to 10 mg/ml, and pre-cleared by centrifugation at 21,500 g for 30 minutes and then immunoprecipitated with specific antibodies or the control mouse IgG overnight at 4°C. The immunoprecipitates were then collected with Protein-G beads for 2 hours at 4°C. The immunoprecipitates were washed five times with pre-chilled Buffer K-200 (Evrin et al., 2009) before elution with SDS sample buffer at 95°C.

Sucrose gradient centrifugation

50 ml of yeast culture at OD600 = 2 (~2 × 10^7 cells/ml) were harvested for each sample. Yeast whole cell extracts were prepared by bead beating in Buffer GF (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 × protease inhibitor). Extracts containing 1 mg of total protein together with molecular size makers (0.5 U alkaline phosphatase and 0.5 U β-galactosidase) were applied on the top of a 2ml 20–50% sucrose gradient in Buffer GF with protease inhibitors. The sucrose gradient was centrifuged at 55,000 r.p.m. for 14 hours at 4°C in a TLS-55 rotor. Twenty-six fractions were collected from the top of the gradient after centrifugation. The fractions containing alkaline phosphatase and β-galactosidase were detected by activity assay (Chen et al., 2010).

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