postulated\(^1\) and we have used a re-replication assay to identify Cdt1 (ref. 14) as one such factor. Cdt1 cooperates with Cdc18 to promote DNA replication, interacts with Cdc18, is located in the nucleus, and its concentration peaks as cells finish mitosis and proceed to S phase. Both Cdc18 and Cdt1 are required to load the MCM protein Cdc21 onto chromatin at the end of mitosis and this is necessary to initiate DNA replication. Genes related to Cdt1 have been found in Metazoa and plants (A. Whitaker, I. Roysman and T. Orr-Weaver, personal communication), suggesting that the cooperation of Cdc6/Cdc18 with Cdt1 to load MCM proteins onto chromatin may be a generally conserved feature of DNA licensing in eukaryotes.

Cdc6/18 has a central role in regulating S-phase onset in a number of eukaryotes\(^1\). We employed a re-replication assay in fission yeast to identify new factors that cooperate with Cdc18 to induce DNA synthesis. Increased expression of Cdc18 induces fission yeast cells to undergo continued DNA synthesis in the absence of mitosis, leading to re-replication\(^2\). When Cdc18 was expressed in cells at increasing levels (low, L; medium, M; and high, H), a corresponding increase in the extent of re-replication was observed (Fig. 1a, b). There was hardly any re-replication in strain L, whereas an average DNA content of 6C and 16C was observed in strains M and H, respectively. We reasoned that co-expression of a factor that cooperates with Cdc18 might induce the low-expressing strain L to re-replicate more effectively. The fission yeast Cdt1 protein is a good candidate for a Cdc18 partner as it is under the same transcriptional regulation as Cdc18 (ref. 14) and, like Cdc18 (ref. 11), is required both for S-phase onset and for the DNA replication checkpoint\(^4\). Overexpression of Cdc18 did not promote re-replication on its own (data not shown). However, co-expression of Cdt1 in strain L generated cells with an average DNA content of 16C, similar to the level of re-replication observed in strain H and significantly higher than the level seen in the medium-expressing strain M (Fig. 1c, d). We saw no enhancement of DNA synthesis when Cdc30 (SpOrc1; refs 16, 17), Hsk1 (ref. 18; a CDC7 homologue) or Cdc22 (ref. 19; the large subunit of ribonucleotide reductase) were co-expressed with Cdc18 (data not shown). We conclude that Cdt1 expression enhances the effect of Cdc18 to promote continuing DNA synthesis, indicating that Cdc18 and Cdt1 cooperate to induce DNA replication.

To determine whether Cdt1 and Cdc18 can physically interact, Cdt1 was tagged with glutathione S-transferase (GST–Cdt1) and Cdc18 with the myc epitope (myc–Cdc18), and both fusion proteins were co-expressed in fission yeast. As shown in Fig. 2a, precipitation of GST–Cdt1 (and not the control GST protein) specifically co-precipitated myc–Cdc18 (compare lanes 3 and 4, lower panel). The reciprocal experiment is shown in Fig. 2b; immunoprecipitation of myc–Cdc18 specifically co-precipitates GST–Cdt1. The association between Cdt1 and Cdc18 was confirmed using cells expressing untagged Cdt1 and Cdc18 (Fig. 2c); immunoprecipitation with anti-Cdt1 antibodies specifically brings down Cdc18 (lane 4). To map the domain of Cdc18 required for Cdt1 interaction, the amino-terminal and carboxy-terminal regions of Cdc18 were tagged with the haemagogulin (HA) epitope and independently expressed together with either GST–Cdt1 or GST alone as a control. As shown in Fig. 2d, the C terminus but not the N terminus of Cdc18 is specifically co-precipitated with GST–Cdt1 (lane 6). We therefore conclude that Cdc18 can form in vivo complexes with Cdt1, primarily through its C terminus. We have previously shown that it is the C terminus of Cdc18 which induces re-replication and activates the DNA replication checkpoint, whereas the N terminus associates with the Cdc2 protein kinase\(^20\).

The re-replication induced by the C terminus of Cdc18 is also postulated\(^3\) and we have used a re-replication assay to identify Cdt1 (ref. 14) as one such factor. Cdt1 cooperates with Cdc18 to promote DNA replication, interacts with Cdc18, is located in the nucleus, and its concentration peaks as cells finish mitosis and proceed to S phase. Both Cdc18 and Cdt1 are required to load the MCM protein Cdc21 onto chromatin at the end of mitosis and this is necessary to initiate DNA replication. Genes related to Cdt1 have been found in Metazoa and plants (A. Whitaker, I. Roysman and T. Orr-Weaver, personal communication), suggesting that the cooperation of Cdc6/Cdc18 with Cdt1 to load MCM proteins onto chromatin may be a generally conserved feature of DNA licensing in eukaryotes.

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The Cdt1 protein is required to license DNA for replication in fission yeast

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To maintain genome stability in eukaryotic cells, DNA is licensed for replication only after the cell has completed mitosis, ensuring that DNA synthesis (S phase) occurs only once every cell cycle\(^1\). This licensing control is thought to require the protein Cdc6 (Cdc18 in fission yeast) as a mediator for association of minichromosome maintenance (MCM) proteins with chromatin\(^2\). The control is overridden in fission yeast by overexpressing Cdc18 (ref. 11) which leads to continued DNA synthesis in the absence of mitosis\(^2\). Other factors acting in this control have been
behaves similarly, we investigated the levels and subcellular localization of Cdt1 through the cell cycle. We found that Cdt1 protein levels fluctuated as cells progressed through a synchronous cell cycle, peaking at the end of mitosis, in parallel with Cdc18 protein levels (Fig. 2f). Both Cdc18 and Cdt1 localized to the nuclei of binucleate cells that have just completed mitosis and are in G1 or early S phase (Fig. 2g). Either no, or very weak, nuclear staining was observed in septated cells which were further on in S phase, or in G2 cells. These observations were confirmed using synchronous cultures (data not shown). Cdt1 and Cdc18 are therefore present in the cell nucleus after mitosis when DNA licensing takes place.

DNA licensing is believed to require chromatin association of the MCM proteins2±5, and in budding yeast and *Xenopus* this association depends on the Cdc6 protein6±10, the homologue of Cdc18 in these organisms. To confirm MCM protein association with chromatin in fission yeast, and establish the requirement for Cdc18 and

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**Figure 1** Cdt1 enhances the ability of Cdc18 to induce continuing DNA synthesis. a, b, Three strains were constructed which express Cdc18, under the inducible nmt promoter, at different levels (L, low; M, medium; H, high). The promoter was derepressed and cells expressing both Cdc18 and Cdt1 were assessed by Western blotting (b). Cdc2 protein levels are shown as loading control.

c, d, Strain L was transformed with plasmid pREP4±Cdt1, in which Cdt1 is under the nmt promoter, and expression of both Cdc18 and Cdt1 was induced. As controls, strains M and H transformed with vector alone were used. DNA content was measured by FACS (c) and levels of both Cdc18 and Cdt1 were assessed by Western blotting (d). Cdt1 overexpression led to a small increase in Cdc18 protein levels, perhaps due to stabilization of the Cdc18 protein in repressing cells. The band marked with an asterisk in d is a non-specific band that serves as loading control.

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**Figure 2** Cdt1 can form a complex with Cdc18, and accumulates in the cell nucleus in G1. a, Cell extracts were prepared from cells co-expressing myc±Cdc18 (in pRMH41) and GST (lanes 1 and 3) or GST±Cdt1 (lanes 2 and 4) (both in pREP4) and the GST proteins precipitated with glutathione beads. Cell extracts (lanes 1 and 2) and precipitates (lanes 3 and 4) were subjected to Western blotting with anti-Cdt1 (upper panel) or anti-Cdc18 antibodies (lower panel). 5 µg cell extract and the precipitate from 25 µg extract, and 1 µg extract and the precipitate from 125 µg extract, were loaded for the Cdt1 and Cdc18 blots, respectively. b, Myc±Cdc18 in the cell extract used in a, lane 2, was precipitated with anti-myc (lane 1) or anti-HA antibodies (lane 2), and the precipitates blotted with anti-Cdt1 (upper panel) or anti-Cdc18 antibodies (lower panel). Precipitates from 125 µg and 25 µg of extract were loaded for the Cdt1 blot and the Cdc18 blot (lower panel), respectively. c, Untagged Cdc18 and Cdt1 were co-expressed using pREP41±Cdc18 and pREP4±Cdt1. Immunoprecipitation was performed using anti-Cdc18 (lane 2) or anti-Cdt1 antibodies (lane 4) or pre-immune serum for Cdc18 (lane 3) or Cdt1 (lane 5). 25 µg of total cell extract (lane 1) and precipitates from 125 µg extract (lanes 2±5) were western blotted using anti-Cdc18 antibodies. d, N terminus of Cdc18 (residues 1±141 in pH441) tagged with HA was co-expressed with GST±Cdt1 (lanes 1 and 5) or GST alone (lanes 3 and 7), both in pREP4X. Similarly, the C terminus of Cdc18 (residues 150 to end in pH441) tagged with HA was co-expressed with GST±Cdt1 (lanes 2 and 6) or GST alone (lanes 4 and 8). 2.5 µg cell extract (lanes 1±4) and precipitates from 250 µg extract using glutathione beads (lanes 5±8) or BSA beads as control (lane 9) were western blotted with anti-HA antibodies. e, Cdc18-C (in pREP81) was expressed alone (upper panel) or with Cdt1 (in pREP42) (lower panel) and the DNA content measured after 20 h expression. f, cdc25-22 cells were synchronized at the G2±M transition by incubating for 4 h at the non-permissive temperature and released into a synchronous cell cycle. Samples were taken every 20 min and the levels of Cdc18 (top), Cdt1 (middle) and α-tubulin (bottom, loading control) were assessed by Western blotting. For cent septation is indicated at the bottom. Initiation of DNA replication immediately precedes the peak of septation. g, Cells expressing Cdc18±myc (upper panel) or Cdt1±myc (lower panel), both under their endogenous promoters, were fixed with methanol and stained with anti-myc antibody (left), propidium iodide (middle) or visualized by Nomarski optics (right). Only binucleate cells show strong nuclear staining for either protein.
Cdt1 for this association, we raised antibodies against Cdc21 (ref. 22; an MCM4 homologue) and developed a fractionation protocol (Fig. 3a) which enriches for chromatin-associated components. In the total cell extract and detergent-soluble protein fractions, Cdc21 protein levels remained constant through the cell cycle. However, the appearance of Cdc21 in the chromatin-enriched preparation was periodic through the cell cycle. When synchronized cells completed mitosis, Cdc21 became associated with chromatin and this association persisted through G1 into S phase (Fig. 3b, left bottom panel, Fig. 3c). It decreased to a low level during G2 and reappeared after the second mitosis. Sp Orc1 protein levels in total, soluble and chromatin fractions were constant and served as loading controls. A significant fraction of the chromatin-associated Cdc21 migrated slower than the detergent-soluble protein (Fig. 3d, lanes 1 and 2). This was due to phosphorylation of the chromatin-associated Cdc21 (Fig. 3d, lanes 3 and 4). We used a strain in which Cdc18 was under the control of the thrime-repressible low strength nmt promoter to test the effect of Cdc18 depletion on Cdc21 chromatin association. Cells depleted of Cdc18 at the G2–M transition completed mitosis normally but failed to initiate DNA replication (data not shown). In the absence of Cdc18, there was no increase in Cdc21 chromatin association (Fig. 3e) or phosphorylation (data not shown) as cells completed mitosis and entered G1. This establishes that Cdc18 is required for the chromatin association and phosphorylation of Cdc21 at the G1–S transition. Cdt1 was also found to be present in the chromatin-enriched fraction during the G1 and S phases of the cell cycle, but in contrast to Cdc21 there was no requirement for Cdc18 to bring about this association (Fig. 3e).

We next investigated the effects of Cdt1 depletion on DNA replication and MCM association with chromatin. To find out whether Cdt1 is primarily required for early or late S phase, cells in which Cdt1 is under the control of the nmt promoter were arrested with hydroxyurea at the beginning of S phase. Thiamine

Figure 3 Cdc21 associates with chromatin as cells prepare for S phase in a Cdc18-dependent manner. A, Flowchart of chromatin association assay. B, cdc25-22HA-Orc1 cells were blocked at the G2–M transition and released into a synchronous cell cycle. Samples collected every 15 min were fractionated as shown in a. The total cell lysate (T), Triton extractable (E) and chromatin–associated fractions (C) were analysed by western blotting using anti-Cdc21 (left) or anti-HA antibodies (right). A higher exposure is shown for Cdc21 (C). We estimate that around 20% of the total Cdc21 is found in the chromatin-enriched fraction in G1. Per cent seapted cells are indicated at the bottom. FACs analysis (c) shows when initiation of DNA replication occurs. D, The Triton extractable (lane 1) and chromatin-associated fraction (lanes 2–4) from the 60 min time point of b were western blotted for Cdc21 without prior treatment (lane 2) or after incubation with calf intestinal alkaline phosphatase in the absence (lane 3) or presence (lane 4) of phosphatase inhibitors. E, cdc25-22 cells expressing Cdc18 under the control of the thrime-repressible nmt promoter as their only functional copy were blocked at G2–M for 4 h at 36 °C in the absence (−T) or presence (+T) of thiamine and then released to 25 °C (always − or +T). Samples were taken every 25 min and fractionated as in a. Total cell lysate and chromatin-associated fractions were western blotted using anti-Cdc21, anti-Cdc18, and anti-Cdc18 antibodies as shown. A higher exposure is shown for the chromatin-associated fraction of Cdc21. As loading controls, an α-tubulin western blot is shown for the total cell lysate, and a Coomassie-stained gel of the chromatin-associated fractions.

Figure 4 Cdt1 depletion blocks the initiation of DNA replication by inhibiting the association of Cdc21 with chromatin. A, Cells expressing Cdt1 under the control of the thrime-repressible nmt promoter as their only functional copy (nmt-cdt1) were arrested at early S phase with hydroxyurea (HU), in the absence or presence of thiamine (−T, +T). After 4 h, HU was washed out and cells were released, always −T or +T. A western blot with anti-Cdt1 antibodies is shown in a. Coomassie staining serves as loading control. Cdt1 levels decrease after 4 h in HU even in the absence of thiamine, indicating that Cdt1 is unstable in HU blocked cells. DNA content at the indicated times (h) is shown in b. C, cdc25-22 mnt-cdt1 cells were arrested with HU for 6 h at 25 °C without or with thiamine (−T or +T). After removal of the HU, cells were shifted to 36 °C for 3 h to arrest them at the G2–M transition and then released into a synchronous cell cycle, always in the presence or absence of thiamine. Samples were collected every 25 min and processed as in Fig. 3a. The total cell lysate and the chromatin-associated fraction were western blotted and visualized with anti-Cdc21, anti-Cdc18 and anti-Cdt1 antibodies as indicated while an α-tubulin western blot and Coomassie staining serve as loading controls for the total lysate and chromatin-extractable fractions, respectively.
was added half the culture at the same time as the hydroxyurea to repress Cdt1 expression, and a western blot showed that Cdt1 levels dropped by over 90% within two hours (Fig. 4a). After washing off the hydroxyurea, both the cells expressing Cdt1 (Fig. 4b, left panels) and the Cdt1-depleted cells (Fig. 4b, right panels) completed DNA replication within one hour. Thus, Cdt1 depletion does not prevent the later stages of DNA replication. However, cells lacking Cdt1 were unable to undergo a second round of DNA replication and accumulated in G1. We conclude that Cdt1 is required for the initial stages of DNA replication. Cells lacking Cdt1 subsequently proceeded inappropriately into mitosis, generating a ‘cut’ phenotype (data not shown), verifying that Cdt1 is required for the checkpoint control inhibits mitosis when S phase has not taken place1,4. To determine whether Cdt1 depleted cells were defective in the association of Cdc21 with chromatin, cells were released from a hydroxyurea block, synchronized at the G2/M transition, and samples taken as cells completed mitosis and progressed to S phase. Like Cdc18 depleted cells, Cdt1 depleted cells were defective in both the chromatin association and phosphorylation of Cdc21 (Fig. 4c). In contrast, Cdc18 could still associate with chromatin in the absence of Cdt1, although possibly at a reduced level. We conclude that Cdt1 is required for the association of Cdc21 with chromatin at or before S phase.

We have identified Cdt1 as being required to license DNA for replication after exit from mitosis. It can associate with Cdc18 in cells, it accumulates within the nucleus, associates with chromatin and is expressed periodically during the cell cycle, peaking from mitotic exit to the onset of S phase. Elevated Cdt1 expression promotes the ability of Cdc18 to bring about continued DNA synthesis, and the absence of either Cdt1 or Cdc18 blocks the association of Cdc21 (MCM4) with chromatin and prevents the initiation of DNA replication. We propose that Cdt1 acts cooperatively with Cdc18 during the G1 phase of the cell cycle, bringing about the construction of pre-initiation complexes on replication origins by loading MCM proteins onto chromatin associated with the origin recognition complex (ORC). Genes related to Cdt1 have been found in Drosophila, human, mouse, zebrafish, Caenorhabditis elegans and Arabidopsis, although not yet in budding yeast, and mutant analysis of the Cdt1 related gene in Schizosaccharomyces pombe is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis. EMBO J. 13, 425–434 (1994).


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