Cdt1 stabilizes an open MCM ring for helicase loading

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ORC, Cdc6 and Cdt1 act together to load hexameric MCM, the motor of the eukaryotic replicative helicase, into double hexamers at replication origins. Here we show that Cdt1 interacts with MCM subunits Mcm2, 4 and 6, which both destabilizes the Mcm2–5 interface and inhibits MCM ATPase activity. Using X-ray crystallography, we show that Cdt1 contains two winged-helix domains in the C-terminal half of the protein and a catalytically inactive dioxygenase-related N-terminal domain, which is important for MCM loading, but not for subsequent replication. We used these structures together with single-particle electron microscopy to generate three-dimensional models of MCM complexes. These show that Cdt1 stabilizes MCM in a left-handed spiral open at the Mcm2–5 gate. We propose that Cdt1 acts as a brace, holding MCM open for DNA entry and bound to ATP until ORC–Cdc6 triggers ATP hydrolysis by MCM, promoting both Cdt1 ejection and MCM ring closure.
The process of eukaryotic DNA replication begins during G1 phase with the loading of the minichromosome maintenance (MCM) heterohexamer comprising Mcm2–7 subunits into head-to-head double hexamers with double-stranded DNA passing through the length of a long central channel. This topological loading requires the Origin Recognition Complex (ORC), Cdc6 and Cdt1 proteins. During S phase, the inactive double hexamer is converted by a set of firing factors into two active CMG (Cdc45-MCM-GINS) replicative helicases in which MCM acts as the ATP-dependent motor for DNA unwinding.

ATP binding and hydrolysis play distinct and crucial roles in MCM loading and ORC, Cdc6 and MCM all contain AAA+ ATPase family members. ATP binding by ORC is required for its stable binding to origin DNA, ATP binding by Cdc6 is required for the formation of a stable ORC–Cdc6 complex at origins and ATP binding by MCM subunits stabilizes the MCM heterohexamer. ATP hydrolysis by ORC and Cdc6 is not required for MCM loading, but ATP hydrolysis by Cdc6 plays an essential role in disassembling incomplete intermediate complexes. MCM loading requires ATP hydrolysis by MCM subunits: mutation of arginine finger residues in several MCM subunits including Mcm2, 3, 5, 6 and 7 all inhibit loading to different extents. Thus, it is important that MCM is bound to ATP before loading.

Previous work has shown that DNA enters the central channel of the MCM hexamer via the Mcm2–5 interface or ‘gate’. How the opening and closing of this gate is regulated to ensure DNA enters only at the correct time is unknown. In budding yeast, Cdt1 forms a stable complex with MCM before loading. Cdt1 is not required for recruitment of MCM to ORC–Cdc6, but does contribute to the stability of MCM subunits in this recruited complex. Cdt1 is released during loading, before double hexamer formation, and its release requires ATP hydrolysis by MCM subunits. Here we use biochemistry and structural approaches to understand how Cdt1 contributes to MCM loading.

Results

Biochemical characterization of Cdt1–MCM interactions. To understand how Cdt1 interacts with MCM, we first set out to determine which MCM subunits interact directly with Cdt1 using individual subunits expressed in E. coli. Figure 1a (arrowheads) shows that both Mcm6, as previously shown, and Mcm2 were co-immunoprecipitated with Cdt1 using an anti-Cdt1 antibody in a Cdt1-dependent manner. We next used glycerol gradients to examine complex formation between Cdt1, Mcm2 and Mcm6 (Supplementary Fig. 1a). Cdt1 formed stable binary complexes with Mcm2 and Mcm6 individually, and a ternary complex with Mcm2 and Mcm6, especially evident from the shift in Cdt1 sedimentation position. We used protein crosslinking with the bifunctional, amino reactive crosslinker bis(sulfo succinimidyl)suberate (BS3), to determine which subunits Cdt1 interacts with in the intact MCM complex. After crosslinking Cdt1–MCM with limiting amounts of BS3, the complex was denatured with SDS and specific subunits were immunoprecipitated under denaturing conditions. Thus, only covalently attached proteins should co-immunoprecipitate under these conditions. We first used complexes in which various MCM subunits were tagged with GFP-FLAG (Supplementary Fig. 1b(i)). Crosslinked proteins were coimmunoprecipitated with anti-FLAG antibody and the presence of Cdt1 in the crosslinked products was examined using anti-Cdt1 antibody (Supplementary Fig. 1b(ii)). Supplementary Fig. 1b(ii) shows that Cdt1 crosslinked strongly to Mcm6 and less strongly to Mcm2 and Mcm4; Cdt1 did not crosslink to Mcm3 or Mcm7 under these conditions. Mcm5 was not examined, because it is unstable when tagged at the carboxy terminus. We next used Cdt1–MCM in which Cdt1 was FLAG-tagged. After denaturing immunoprecipitation with FLAG antibody, four bands were seen above the 180 kDa marker by silver staining, which were not present in the absence of crosslinker, but appeared at low crosslinker concentration (Fig. 1b). We identified the proteins present in these bands by immunoblotting and mass spectrometry. Immunoblotting with Mcm2 or Mcm6 antibodies identified crosslinked complexes containing these proteins (Fig. 1b and Supplementary Fig. 1c), which was confirmed by mass spectrometry (Fig. 1b and Supplementary Table 1). The fastest migrating crosslinked band contained neither Mcm2 nor Mcm6, but was enriched for peptides from Mcm4 by mass spectrometry (Fig. 1b and Supplementary Table 1). From these experiments we conclude that Cdt1 interacts with Mcm6, Mcm2 and, to a weaker extent, Mcm4.

To determine how Cdt1 interacts with Mcm6, its strongest binding partner, we expressed MBP fusions containing different regions of Mcm6 (Fig. 1c(i)) and examined interaction with Cdt1 by amylose–agarose pull down. Figure 1c(ii) shows that Cdt1 did not interact with the amino terminus of Mcm6, but interacted strongly with a fragment containing the entire C-terminal half of Mcm6 and a shorter fragment lacking the AAA+ domain, containing only the C-terminal 228 amino acids of Mcm6. A protein containing all of Mcm6, except for this C-terminal domain, however, was still able to interact with Cdt1 (Supplementary Fig. 1d(i)), although not as well as the full-length protein (Supplementary Fig. 1d(ii)), indicating that Cdt1 interacts with Mcm6 at more than one position, including one in the C terminus and one or within or near the AAA+ domain. This Mcm6AC protein could still form a ternary complex with Mcm2 and Cdt1 (Supplementary Fig. 1e(i)), and a high molecular-weight complex with the full complement of MCM subunits and Cdt1 (Supplementary Fig. 1e(ii)). Cdt1 inhibits the ATPase of the full MCM complex, as well as that of MCM containing Mcm6AC (Supplementary Fig. 1f(i)). Three pairs of MCM subunits, Mcm2–6, Mcm4–7 and Mcm7–3, exhibit ATPase activity on their own. Figure 1d shows that, in addition to the intact MCM complex, Cdt1 also inhibits the ATPase of Mcm2–6 and Mcm4–7 but not Mcm7–3.

We next generated fragments containing the N-terminal (N), middle (M) and C-terminal (C) domains of Cdt1 in various combinations (Fig. 1e(i)), and used these fragments in pull-down experiments to determine which parts of Cdt1 are involved in MCM binding. As shown in Fig. 1e(ii), a fragment containing the 438 N-terminal amino acids of Cdt1 (Cdt1-NM) interacted with neither Mcm2 nor Mcm6, whereas a fragment containing the C-terminal 333 amino acids (Cdt1-MC) interacted with both Mcm2 and 6. Further division of the C terminus of Cdt1 showed that fragments containing amino acid residues 271–496 and 495–640 interacted with both Mcm2 and 6, suggesting there are multiple interactions between Cdt1 and MCM subunits.

We noticed during the course of analysing Cdt1 complexes containing different combinations of MCM subunits by gel filtration that Cdt1 appeared to influence interactions between Mcm2 and 5. An example of this is shown in Supplementary Fig. 1g. A stable tetrameric complex of Mcm6–2–5–3 (the order Mcm6, 2, 5 and 3) was enriched for peptides from Mcm4 by mass spectrometry. Immunoblotting with Mcm2 or Mcm6 antibodies identified crosslinked complexes containing these proteins (Fig. 1b and Supplementary Fig. 1c), which was confirmed by mass spectrometry (Fig. 1b and Supplementary Table 1). The fastest migrating crosslinked band contained neither Mcm2 nor Mcm6, but was enriched for peptides from Mcm4 by mass spectrometry (Fig. 1b and Supplementary Table 1). From these experiments we conclude that Cdt1 interacts with Mcm6, Mcm2 and, to a weaker extent, Mcm4.

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Mcm2 and 6 were retained by Cdt1. We have previously shown that Mcm10, a protein required for helicase activation, also interacts with Mcm2 and 6, but not Mcm3 or 5, and that Cdt1 and Mcm10 compete with each other for MCM binding18. As shown in Fig. 1f, in contrast to Cdt1, Mcm10 pulls down all four subunits of the tetramer, despite not interacting directly with...
Mcm3 or 5. Thus, Cdt1, but not Mcm10, destabilizes the Mcm2–5 interface after binding to Mcm2 and 6.

X-ray crystallography of Cdt1. To understand Cdt1–MCM at an atomic level, we determined crystal structures of two non-overlapping fragments of S. cerevisiae Cdt1 spanning amino acid residues 1–438 and 495–604 (Supplementary Table 2 and Fig. 2). The structure of Cdt1(1–438) was refined using two independent crystal forms to resolutions of 2.7 and 2.1 Å. The fragment comprises a pair of well-defined domains corresponding to the N domain (residues 14–298) and the M domain (residues 299–430) (Fig. 2). The relative orientation between the domains is preserved in all four crystallographically independent copies of the protein chain observed in the crystal structures (Supplementary Fig. 2a), indicating a rigid linkage between the domains. The M domain belongs to the winged helix domain (WHD) family and is predictably similar to the geminin-binding M domain of mouse Cdt1 (Supplementary Fig. 2b). Perusal of the Dali structure comparison server19 revealed a striking similarity between the Cdt1 N domain and Fe(II)/α-ketoglutarate-dependent dioxygenase superfamily of enzymes, such as AlkB and other DNA/RNA dealkylators20, and the Jumonji C histone demethylase21 (Supplementary Fig. 2c). However, the key His and Asp residues involved in chelation of the Fe(II) ion co-factor in the active sites of these enzymes are not conserved in the Cdt1 N domain (Supplementary Fig. 2c). Moreover, an α-helical hairpin (26/27), which extends the dioxygenase fold, occludes the degenerate active site of the N domain (Supplementary Fig. 2c). These observations indicate that the dioxygenase structural fold was repurposed for a non-catalytic function. The structure of Cdt1(495–604) revealed the C domain (Fig. 2), which is highly similar to the analogous WHD domain previously characterized in mouse Cdt1 (refs 22,23) (Supplementary Fig. 2b).

The N terminus of Cdt1 contributes to MCM loading. To examine the role of the dioxygenase-like N domain, we expressed and purified wild-type Cdt1, Cdt1MC (lacking the N domain), the hexameric MCM complex without Cdt1 and the heptameric, co-expressed Cdt1–MCM complex (Supplementary Fig. 3a). Cdt1–MCM complexes were reconstituted by incubation of MCM with an excess of Cdt1 or Cdt1MC before use. Figure 3a shows that MCM is recruited to DNA in the presence of ATP after high-salt wash similarly in the co-expressed Cdt1–MCM complex and in the Cdt1–MCM complex reconstituted from independently expressed MCM and Cdt1 (lanes 1 and 3). MCM loading onto DNA—defined by the generation of Mcm2–7 double hexamers bound to DNA after high-salt extraction of ORC and Cdc6 (refs 2,24,25)—occurred with both the co-expressed and reconstituted complexes, although it was routinely more efficient with the co-expressed complex (Lanes 2 and 4). The reconstituted complex of Cdt1–MCM recruited MCM, as well as the complex containing wild-type Cdt1 (lane 5); however, loading was severely reduced with the truncated Cdt1MC (compare lanes 4 and 6). This is consistent with previous results from Takara and Bell26, who showed reduced MCM loading by Cdt1 lacking its N terminus. Takara and Bell26 further observed that MCM loaded by this truncated Cdt1 was less efficient in replication assays using S phase extracts than MCM loaded by full-length Cdt1 and this was confirmed by our experiments (Supplementary Fig. 3b). This defect could be due to the reduced MCM loading or could reflect some additional role for the N domain of Cdt1 in replication, which might be interesting, given its homology to dioxygenases. To address this we manipulated our loading reactions, so the truncated Cdt1 loaded the same amount of MCM as the full-length protein (Fig. 3b). Figure 3c shows that MCM loaded by the truncated Cdt1 is equally stable to high-salt extraction as the MCM loaded by wild-type Cdt1. If the N domain of Cdt1 had a role in replication beyond MCM loading, these MCM complexes should show reduced replication in extracts. However, as shown in Fig. 3d, Dbf4-dependent kinase (DDK)-dependent replication from these loaded MCM complexes was as efficient as from the complexes loaded with full-length Cdt1 (Fig. 3d, compare lanes 2 and 4). Therefore, the N domain of Cdt1 plays an important role in MCM loading and plays no role in replication downstream of MCM loading. The fact that this domain is essential in vivo may be due to this defect in loading.

EM of Cdt1–MCM. To understand better the function of Cdt1 in MCM loading, we used single-particle electron microscopy (EM) to characterize the MCM complex, either in isolation or bound to Cdt1 (Fig. 4). As judged from end-on view two-dimensional (2D) class averages of negatively stained particles, apo MCM appears to exist as a mixture of open and closed rings (Fig. 4a and Supplementary Fig. 4—as previously observed in ref. 27). When imaged in the presence of ATPγS, however, open MCM rings were not detected, suggesting that interaction with a slowly hydrolysable ATP analogue promotes ring closure (Fig. 4a and Supplementary Fig. 5, in agreement with refs 10,28). It is not formally possible, however, to distinguish closed rings from tilted views of open rings in 2D, especially when using low-resolution negative stain EM. To confirm that ATPγS-treated MCM particles were bona fide closed rings, we have reconstructed a three-dimensional (3D) structure, generated from all particles contributing to high-quality 2D averages, irrespective of their configuration. In these conditions, MCM appears to form a planar ring, with a topologically closed N-terminal tier and a slightly notched AAA+ tier (structure solved to 23.6 Å resolution, Fig. 4a and Supplementary Fig. 5). We then repeated the EM analysis using the Cdt1–MCM complex. In both the apo and the ATPγS-bound form, the heptameric complex appears to contain open MCM rings only, suggesting that Cdt1 has a role in stabilizing MCM in its open configuration (structures solved to 20.4 and 18.0 Å resolution, respectively, Fig. 4a and Supplementary Figs 6 and 7). Three-dimensional reconstruction of the Cdt1-bound MCM shows essentially the same structure in the apo and in the ATPγS-bound form, with a lock-washer ring configuration containing a discontinuity that spans both the N-terminal and the AAA+ tier of MCM (Fig. 4a and Supplementary Figs 6 and 7). An analogous topologically open form of MCM had been previously observed for the Drosophila melanogaster27 and Encephalitozoon cuniculi29 MCM, which were observed to form a left-handed spiral configuration. This was determined by tilt validation analysis and docking of the markedly chiral structure of the N-terminal MCM domain of an archaeal orthologue, available at the time29. In the Drosophila
structure, N-terminal MBP tags were used to establish that the discontinuity in the MCM complex exists between Mcm5 and Mcm2 subunits\textsuperscript{27,29}. Two atomic structures of yeast MCM have since become available (from the inactive double-hexameric MCM and the active Cdc45-MCM-GINS, CMG helicase) and we have used the latter in an atomic docking exercise (fitting the MCM N-terminal tier as a rigid body)\textsuperscript{30,31}. Recognition of the characteristic propeller shape of the A domain of MCM immediately establishes the handedness of the Cdt1–MCM map (Supplementary Fig. 8 and Supplementary Movie 1), indicating that when Cdt1 associated MCM forms a left-handed spiral configuration similar to prior observations on the isolated MCM complex\textsuperscript{29}. Docking of the MCM N-terminal domain (extracted from either the MCM double hexamer or the CMG helicase) into either the apo or the ATP\textsuperscript{G} bound Cdt1–MCM EM map assigns the discontinuity at the Mcm2–5 interface, in agreement with previously published data\textsuperscript{3,10,28,29}, as well as with biochemical data presented here (Fig. 4b and Supplementary Fig. 8). To optimize the atomic docking and accurately model the MCM discontinuity, the N-terminal domain of MCM was separated into two distinct subcomplexes (Mcm5–3–7 and Mcm2–4–6), which were used for rigid body fitting (Fig. 4c and Supplementary Fig. 9). Superposition of full-length MCM subunits, followed by local adjustment of the isolated AAA\textsuperscript{+}WH domains of Mcm5–3–7 and Mcm2–4–6 were used to complete the assignment of MCM domains and model the Mcm2–5 discontinuity in the AAA\textsuperscript{+} tier (Fig. 4d and Supplementary Fig. 9). The marked spiral structure of the Cdt1-associated MCM causes a gross misconfiguration of the Mcm2–5 active site (Fig. 4d and Supplementary Fig. 9), whose reconstitution would require planarization and tightening of the MCM ring. The resulting molecular model for the open MCM agrees with recently published cryo-EM data\textsuperscript{32}. Once MCM subunits were assigned in the Cdt1–MCM map, unoccupied density could be detected, intimately contacting the Mcm6 and Mcm2 subunits, and radially projecting from Mcm2 (Fig. 4b). We assign this density to Cdt1. By docking the crystal structure of NM Cdt1 into the Cdt1 density (Fig. 4b and Supplementary Fig. 9), an MCM interaction can be modelled, whereby the middle domain of Mcm2 is sandwiched between the A-domain and AAA\textsuperscript{+} domain of Mcm2 and extends to contact the A-domain of Mcm6, following the outer perimeter of the MCM N-terminal domain (Fig. 4c–e). Conversely, the N domain of Cdt1 projects away from the MCM ring and does not appear to be involved in any contact with the helicase motor (Fig. 4c,d and Supplementary Movie 1). The domain interaction studies presented in Fig. 1e support this

**Figure 3 | The N domain is important for MCM loading.** (a) Mcm2–7 recruitment (− high-salt wash, HSW) and loading (+ HSW) on DNA analysed by SDS–PAGE and silver staining. Loading of co-expressed Cdt1–MCM complexes (lane 2) is more efficient than loading with reconstituted complexes of Mcm2–7 and Cdt1 WT (lane 4). Loading is dramatically reduced with Cdt1MC (lane 6). Recruitment of Mcm2–7 is not as dramatically affected (lanes 1, 3, 5). (b) Mcm2–7 loading on DNA analysed by SDS–PAGE and silver staining. Loaded Mcm2–7 is stable in high-salt washes up to 2.5 M NaCl, both when loaded with Cdt1 WT or Cdt1MC. (c) Mcm2–7 loading on DNA analysed by SDS–PAGE and silver staining (left), and (d) alkaline agarose gel (right) of replication products after in vitro replication assays using S-phase whole-cell extract. When adjusting levels of Mcm2–7 loading (left) for Cdt1 WT (lane 1) and Cdt1MC (lane 2)-loaded hexamers, DDK-dependent replication products and efficiency is comparable (right).
assignment. Notably, the connectivity between N domain and M
domain observed in our 2.1 Å crystal structure differs drastically
from the atomic model based on a 7 Å resolution cryo-EM
structure of MCM—Cdt1 (EMD-6671), in that N domain (which
appears pseudo two-fold symmetric at 7 Å resolution) is
rotated with respect to M domain32 (Supplementary Fig. 10).
Although the C-terminal WHD of Cdt1 could be accommodated
in our EM structure, interacting with the WHD of Mcm6, we
have not modelled this region using our negative stain data, due
to the limited resolution. Our CTD-Cdt1 crystal structure can
however be docked into the 7 Å resolution cryo-EM structure of
MCM-Cdt1, indicating that the C-terminal WHD of Cdt1
interdigitates between the A domains of Mcm4 and Mcm6, in
agreement with two recently published atomic models32,33.

Figure 4 | Negative-stain electron microscopy of yeast MCM in the absence and presence of Cdt1. (a) Two-dimensional averages of free and Cdt1-
associated MCM, in the apo and ATPγS-bound state (central panel). A red arrowhead points the MCM gate. ATPγS binding causes gate closure in the free
MCM but not in the Cdt1-bound state. A red arrowhead points the MCM gate. Three-dimensional reconstruction of a topologically closed, ATPγS-bound MCM
(left panel) and an open ATPγS-bound Cdt1—MCM (right panel). (b) Atomic docking of the N-terminal MCM is used to identify the register of MCM proteins.
Unoccupied density on the side of the MCM ring is assigned to Cdt1. (c) The N-terminal domain of Cdt1 (green) is not involved in MCM interactions. The
middle domain of Cdt1 contacts the A domains of Mcm2 and Mcm6. (d) Cdt1 binding stabilizes a left-handed spiral configuration of MCM. In this state, the
Mcm2—S ATPase interface is grossly misaligned. The middle domain of Cdt1 is sandwiched between the Mcm2 N domain and AAA + domain. (e) Detail of the
Mcm2—Cdt1 M domain interaction. The M domain of Cdt1 (light blue) is sandwiched in between the A and AAA + domains of Mcm2 (pink).
summary, Cdt1 makes extensive contacts with the outer perimeter of MCM, notably being sandwiched between the A and AAA+ domains of Mcm2. Comparative analysis of the nucleotide-bound Cdt1–MCM form with the post-catalytic MCM double hexamer (corresponding to the loaded form) highlights a gross reconfiguration of the AAA+ tier, which would occur upon transitioning from an open lock-washer to the loaded MCM configuration. This is best visualized using the centre of mass of A and AAA+ domains of the six Mcm subunits (Fig. 5). The ATPase tier globally rotates clockwise, whereas the N-terminal domain of Mcm5 moves counterclockwise to seal the Mcm2–5 ATPase site globally rotates clockwise, whereas the N-terminal and AAA+ domains of the six Mcm subunits (Fig. 5). The ATPase tier globally rotates clockwise, whereas the N-terminal domain of Mcm5 moves counterclockwise to seal the Mcm2–5 ATPase site.

Discussion

We propose that Cdt1 stabilizes a conformation of the MCM ring in which the Mcm2–5 gate is held open to allow duplex DNA passage required for helicase loading. In this state, the Mcm2–5 opening is much wider than in the ORC–Cdc6–Cdt1–MCM complex, suggesting that ORC plays a role in closing, not in opening the MCM structure. We further propose that, by holding MCM in this conformation, ATP hydrolysis is inhibited, which helps ensure MCM-Cdt1 is bound to ATP for subsequent loading (Fig. 5). Previous work in a variety of systems has led to the idea that loading of ring-shaped multi-subunit protein complexes around duplex DNA can occur via two mechanisms: ring making, in which individual protomers assemble around DNA to form a ring, and ring breaking, in which a pre-formed ring is transiently destabilized at one inter-protomer interface by a loading factor before loading. Our results suggest that MCM loading has destabilized at one inter-protomer interface by a loading factor.

Figure 5 | A mechanism for ATPase-mediated Cdt1 ejection during MCM loading. Cdt1 binds to the side of Mcm2 and Mcm6, interacting both with N-terminal and AAA+ ATPase elements. Cdt1 stabilizes a topologically open left-handed spiral configuration of MCM, with a discontinuity between the Mcm5 and Mcm2 subunits, disrupting a functionally essential AAA+ ATPase site. DNA recruitment mediated by ORC and Cdc6 (OCCM formation) planarizes the MCM ring, reconstituting the Mcm5–2 ATPase site, which becomes competent for ATP hydrolysis. Conversion to a post-catalytic, loaded MCM involves the clockwise rotation of the AAA+ ATPase tier. This conformational change probably disrupts a Cdt1 docking site on Mcm2, promoting ATPase-dependent Cdt1 ejection.

MCM must hydrolyse ATP during loading. We propose that the inhibition of ATPase activity by Cdt1 plays an important role in keeping MCM in an appropriately ATP-bound form required for subsequent loading. Our Cdt1–MCM structure, in which MCM subunits are in a left-handed spiral and the Mcm2–5 active site is disrupted, helps explain how Cdt1 association inhibits the MCM ATPase activity. We note that multiple MCM assemblies have been characterized by crystallography and electron microscopy with left handed AAA+ spiral, which have been described as inactive forms of the ATPase. We suggest that Cdt1 acts more like an open-ring stabilizer than a canonical ring breaker. Moreover, once MCM is recruited to ORC–Cdc6 at origins, Cdt1 prevents dissociation of Mcm3,5,7 closure) and concomitant Cdt1 ejection, en route to MCM

Something must occur during loading which counteracts this inhibition of ATPase by Cdt1 and allows ATP hydrolysis by MCM. We propose that this involves the transition from the open spiral with misaligned Mcm2–5 ATPase elements to a planar structure where Mcm2 and Mcm5 are properly configured for hydrolysis. We suggest that it is the binding of Cdt1–MCM to ORC–Cdc6 that promotes this transition. This is supported by the cryo-EM structure of the ATP7S stabilized ORC–Cdc6–Cdt1–MCM loading intermediate in which the MCM subunits are in a planar configuration, containing a more aligned, yet still misconfigured, Mcm2–5 ATPase centre. Cdt1 ejection, en route to MCM.

This is consistent with the finding that MCM subunit mutants defective in ATP hydrolysis do not eject Cdt1 efficiently and is also consistent with recent single molecule experiments showing concomitant release of Cdt1 and MCM ring closure. Multiple ATPase centres including 2–5, 5–3 and 3–7 are required for MCM loading, which is unexpected given that Cdt1 contacts only the Mcm2, 4 and 6 subunits. Comparative analysis of the pre-catalytic Cdt1–MCM complex and the post-
catalytic MCM double hexamer suggests a mechanism for this process. Transition from an open (Cdt1–MCM) to a locked MCM ring (as observed in the MCM double hexamer) involves the clockwise rotation of the AAA + ATPase tier with respect to the N-terminal domain of MCM and this rotation probably disrupts a Mcm2–Cdt1 interaction point, which appears to be the main Cdt1 docking site in our structure (Fig. 5 and Supplementary Movie 2). Combined, our biochemical and structural data explain why Cdt1 keeps the MCM ring open and the ATPase inactive, before ORC-directed DNA association, which is concomitant with a major reconfiguration of the ATPase tier of MCM and Cdt1 ejection. Previous work has shown that Mcm3 binding promotes ATP hydrolysis by ORC–Cdc6 (ref. 9); we propose there is a mutual activation of the MCM ATPase by ORC–Cdc6 binding as well. The N domain of Cdt1 is a dioxygenase, which has been catalytically inactivated during evolution. In metazoan, this N domain is much shorter and shows little or no sequence similarity to the yeast protein, and thus may be structurally unrelated to the yeast N domain. The N domain of the yeast protein plays an important role in MCM loading, but makes little or no contact with MCM in our structure. It is possible that this domain transiently contacts ORC–Cdc6, DNA or MCM in the early stages of helicase loading.

Methods
Reagents. All yeast strains are described in Supplementary Table 3, all plasmids in Supplementary Table 4 and all oligonucleotides in Supplementary Table 5.

Glycerol gradients. Fifteen micrograms of Mcm2, 6 and Cdt1 were loaded onto 5 ml centrifuge tubes (Beckman) containing 15–35% glycerol gradient of buffer A (25 mM Hepes, pH 7.6, 1 mM EDTA, 0.05% NP-40, 10% glycerol and 0.1 M potassium glutamate). Gradients were centrifuged at 42,000 r.p.m. for 16 h (SW55 Ti rotor (Beckman)). Twenty-seven aliquots of 175 μl were taken manually. Of these aliquots, 20 μl were loaded onto SDS–PAGE gel and visualized by silver staining.

Pull downs. Purified Mcm2–7 subunits (0.5 μM) were individually incubated at room temperature for 60 min with purified Cdt1 (0.5 μM) or a buffer control in 100 μl of buffer A/2 mM 2-mercaptoethanol. Reactions were subjected to immuno-precipitation for 2 h at 4°C with ~5 μg of polyclonal α-Cdt1 antibody pre-bound to protein A sepharose. After two washes with 0.5 ml binding buffer each, beads were resuspended in SDS sample buffer and samples analysed by SDS–PAGE and Coomassie staining.

Mcm6 MBP fragments and Cdt1 proteins were dialysed against buffer A for 2 h at 4°C. Each pull-down reaction contained 10 μg of each protein. Once mixed, proteins were incubated for 60 min at 4°C in a rotating wheel. Ten microlitres of slurry amylase-agarose beads (NEB) prewashed with buffer A was used to pull down Mbp-Mcm6 fragments after an incubation of 30 min at 4°C. Minus Cdt1 and Mbp were used as positive and negative controls, respectively. Beads were collected and washed twice with buffer A. Fifty per cent of the final volume was loaded onto SDS–PAGE gel and proteins were stained with Coomassie stains.

Cdt1-His-SUMO fragments together with Mcm2 and 6 were dialysed against buffer B (25 mM Hepes-KOH pH 7.6/0.02% NP-40/10% glycerol/0.1 M K glutamate) for 2 h at 4°C. Ten micrograms of each dialysed protein was used per pull-down reaction. Once mixed, proteins were incubated for 60 min at 4°C in a rotating wheel. Five microlitres of slurry Magnetic beads (Sigma) prewashed with buffer B was used to pull down Cdt1-His-SUMO fragment, after an incubation of 30 min at 4°C. His-SUMO tag alone was used as a negative control. Beads were collected and washed with buffer B twice. Fifty per cent of the final volume was loaded onto SDS–PAGE gel and proteins were stained with Coomassie stain.

Cdt1 FLAG together with Mcm6 fl and AC were dialysed against buffer B for 2 h at 4°C. Ten micrograms of each dialysed protein was used per reaction. Once mixed, proteins were incubated for 60 min at 4°C in a rotating wheel. Five microlitres of slurry FLAG beads (Sigma) prewashed with buffer B was used to pull down Cdt1 FLAG after an incubation of 30 min at 4°C. Beads were collected and washed twice with buffer B. FLAG peptide (Sigma) was added to a final concentration of 0.5 M and the mixture was further incubated for 20 min at room temperature. Supernatant was collected and loaded onto SDS–PAGE gel and bound proteins were visualized by Coomassie stain.

ATPase assays. Ten micrograms of each Mcm subunit was used to form the following dimers: 3/7, 4/7 and 2/6. These pairwise combinations were dialysed against buffer A for 2 h at 4 °C in 200 μl of buffer A/2 mM 2-mercaptoethanol. Reactions were subjected to immuno-precipitation for 2 h at 4°C with ~5 μg of polyclonal α-Mcm antibody pre-bound to protein A sepharose. Twenty micrograms of each Mcm were quantified and used for the ATPase assays, together with recombinant Cdt1 at 3:1 molar ratio (Cdt1:MCM).

ATPase assays. DNA replication assays were carried out essentially as described. ARS1-containing plasmids (pBluescript KS (+) ARS1 WT) were radiolabelled and biotinylated using the Biotin-16-Propargylamine DNA Labeling Kit (Vector Laboratories) according to manufacturer’s instructions and immobilized on streptavidin M-280 magnetic DNA beads. As biotinylation efficiency was estimated to be ~10–20%, 1 μg of DNA was coupled per 5 μl slurry bead. Immobilized DNA was incubated with Mcm2–7–Cdt1 complexes, ORC and Cdc6 in the presence of ATP at 37 °C for 20 min. DNA beads were then washed twice either with low salt wash buffer (25 mM Hepes-KOH pH 7.6, 300 mM K-acetate, 0.02% NP-40, 5 mM Mg(OAc)2, 10% glycerol) or a buffer control in the presence of ATP at 37 °C for 20 min. DNA beads were then treated with MNase at 30°C for 10 min and the supernatant was analysed by SDS–PAGE. For reconstituted Cdt1–MCM complexes, Mcm2–7 was incubated with a fivefold molar excess of Cdt1 WT or Mcm3 binding promotes ATP hydrolysis by ORC–Cdc6 (ref. 9); we propose there is a mutual activation of the MCM ATPase by ORC–Cdc6 binding as well. The N domain of Cdt1 is a dioxygenase, which has been catalytically inactivated during evolution. In metazoan, this N domain is much shorter and shows little or no sequence similarity to the yeast protein, and thus may be structurally unrelated to the yeast N domain. The N domain of the yeast protein plays an important role in MCM loading, but makes little or no contact with MCM in our structure. It is possible that this domain transiently contacts ORC–Cdc6, DNA or MCM in the early stages of helicase loading.

Methods
Reagents. All yeast strains are described in Supplementary Table 3, all plasmids in Supplementary Table 4 and all oligonucleotides in Supplementary Table 5.

Glycerol gradients. Fifteen micrograms of Mcm2, 6 and Cdt1 were loaded onto 5 ml centrifuge tubes (Beckman) containing 15–35% glycerol gradient of buffer A (25 mM Hepes, pH 7.6, 1 mM EDTA, 0.05% NP-40, 10% glycerol and 0.1 M potassium glutamate). Gradients were centrifuged at 42,000 r.p.m. for 16 h (SW55 Ti rotor (Beckman)). Twenty-seven aliquots of 175 μl were taken manually. Of these aliquots, 20 μl were loaded onto SDS–PAGE gel and visualized by silver staining.

Pull downs. Purified Mcm2–7 subunits (0.5 μM) were individually incubated at room temperature for 60 min with purified Cdt1 (0.5 μM) or a buffer control in 100 μl of buffer A/2 mM 2-mercaptoethanol. Reactions were subjected to immuno-precipitation for 2 h at 4°C with ~5 μg of polyclonal α-Cdt1 antibody pre-bound to protein A sepharose. After two washes with 0.5 ml binding buffer each, beads were resuspended in SDS sample buffer and samples analysed by SDS–PAGE and Coomassie staining.

Mcm6 MBP fragments and Cdt1 proteins were dialysed against buffer A for 2 h at 4°C. Each pull-down reaction contained 10 μg of each protein. Once mixed, proteins were incubated for 60 min at 4°C in a rotating wheel. Ten microlitres of slurry amylase-agarose beads (NEB) prewashed with buffer A was used to pull down Mbp-Mcm6 fragments after an incubation of 30 min at 4°C. Minus Cdt1 and Mbp were used as positive and negative controls, respectively. Beads were collected and washed twice with buffer A. Fifty per cent of the final volume was loaded onto SDS–PAGE gel and proteins were stained with Coomassie stain.

Cdt1-His-SUMO fragments together with Mcm2 and 6 were dialysed against buffer B (25 mM Hepes-KOH pH 7.6/0.02% NP-40/10% glycerol/0.1 M K glutamate) for 2 h at 4°C. Ten micrograms of each dialysed protein was used per pull-down reaction. Once mixed, proteins were incubated for 60 min at 4°C in a rotating wheel. Five microlitres of slurry beads Magnetic beads (Sigma) prewashed with buffer B was used to pull down Cdt1-His-SUMO fragment, after an incubation of 30 min at 4°C. His-SUMO tag alone was used as a negative control. Beads were collected and washed with buffer B twice. Fifty per cent of the final volume was loaded onto SDS–PAGE gel and proteins were stained by Coomassie stain.

Cdt1 FLAG together with Mcm6 fl and AC were dialysed against buffer B for 2 h at 4°C. Ten micrograms of each dialysed protein was used per reaction. Once mixed, proteins were incubated for 60 min at 4°C in a rotating wheel. Five microlitres of slurry FLAG beads (Sigma) prewashed with buffer B was used to pull down Cdt1 FLAG after an incubation of 30 min at 4°C. Beads were collected and washed twice with buffer B. FLAG peptide (Sigma) was added to a final concentration of 0.5 M and the mixture was further incubated for 20 min at room temperature. Supernatant was collected and loaded onto SDS–PAGE gel and bound proteins were visualized by Coomassie stain.

Equilibrated in a reaction containing 5 × 105 bacteria (125 μg/ml Hepes-KOH pH 7.6, 50 mM Mg(OAc)2, 0.1% NP-40, 212.5 mM K-glutamate, 25% glycerol), 1 mM DTT, 5 mM ATP, 1 mM spermine (Sigma-Aldrich) and distilled water. The
reaction was supplemented with purified DDK and incubated at 25 °C with agitation for 15 min. The supernatant of the reaction was removed and beads were resuspended in a reaction mix containing 20 mM Hepes-KOH pH 7.6, 160 mM MgCl2, 1 mM DTT, 5 mM ATP, 100 μM dATP/dCTP/dGTP/dTTP (Invitrogen) and 200 μM CTP/UTP (Invitrogen), 40 mM creatine phosphate (Calbiochem) and 10 μg creatine phosphokinase (Calbiochem). YK03 S-phase extract (750 μg) was added last. The reaction was then incubated at 25 °C with agitation for 20 min.

Replication reactions were quenched by addition of 20 mM EDTA and beads were washed with TE buffer, before resuspending in 5 mM EDTA and then adding NaOH and sucrose to 50 mM and 1% v/v, respectively. Samples were incubated at room temperature for 30 min before products were separated through 1% alkaline agarose gels in 30 mM NaOH, 2 mM EDTA for 16 h at 25 V. Gels were fixed with 5% cold TCA, dried onto chromatography paper (Whatman) and autoradiographed with Amersham Hyperfilm-MP (GE Healthcare).

**X-ray crystallography.** *Saccharomyces cerevisiae* Cdt1 fragments spanning residues 1–438 (N domain and M domain) or 495–604 (CTD) were expressed in E. coli as fusions to hexahistidine-SUMO either in native or selenomethionine (SeMet) derivatized form. The recombinant proteins were isolated from bacterial extracts by batch absorption to NiNTA agarose (Qiagen). Following digestion with SUMO protease and ion exchange chromatography, untagged proteins were polished by size-exclusion chromatography through a Superdex 200 16/600 column equilibrated in 200 mM NaCl, 25 mM Tris-HCl pH 7.4. Crystals were grown at 18 °C in hanging drops made by mixing 1 μl protein (10 mg ml−1) and 1 μl reservoir solution.

Cdt1(1–488; NM) was crystallized in two forms. Crystals of form 1 were obtained using SeMet containing protein and reservoir solution containing 30% glycerol (v/v), 20% polyethylene glycol-4000 (w/v), 0.1 M isopropanol (v/v), 0.1 M Tris-HCl pH 8.5. A data set were collected to a resolution of 0.82 Å at a wavelength of 0.9798 Å on the beamline 104 of the Diamond Light Source (Oxfordshire, UK) and processed using XDS41 and Aimless42 via the Xia2 automatic pipeline43. The phases were determined by single-wavelength anomalous dispersion using phasix.autosol software44, which identified 22 Se sites resulting in a figure of merit of 0.281 and 1.8 M ammonium sulphate, 20% glycerol (v/v), 0.1 M MES-NaOH, pH 6.5. X-ray crystallography

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Author contributions

J.F., K.K., M.E.D., D.R. and J.F.X.D. designed the biochemical experiments in Figs 1 and 3, and Supplementary Information, and J.F., K.K., D.R. and M.E.D. performed these experiments. J.F., V.E.P. and C.D. designed and performed crystallization studies, and J.H., L.R. and A.C. designed and performed EM. experiments. J.F.X.D. coordinated writing of the manuscript with other authors.

Additional information

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