Mechanism and timing of Mcm2–7 ring closure during DNA replication origin licensing

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The opening and closing of two ring-shaped Mcm2–7 DNA helicases is necessary to license eukaryotic origins of replication, although the mechanisms controlling these events are unclear. The origin-recognition complex (ORC), Cdc6 and Cdt1 facilitate this process by establishing a topological link between each Mcm2–7 hexamer and origin DNA. Using colocalization single-molecule spectroscopy and single-molecule Förster resonance energy transfer (FRET), we monitored ring opening and closing of Saccharomyces cerevisiae Mcm2–7 during origin licensing. The two Mcm2–7 rings were open during initial DNA association and closed sequentially, concomitant with the release of their associated Cdt1. We observed that ATP hydrolysis by Mcm2–7 was coupled to ring closure and Cdt1 release, and failure to load the first Mcm2–7 prevented recruitment of the second Mcm2–7. Our findings identify key mechanisms controlling the Mcm2–7 DNA-entry gate during origin licensing, and reveal that the two Mcm2–7 complexes are loaded via a coordinated series of events with implications for bidirectional replication initiation and quality control.

During eukaryotic DNA replication, origins of replication are licensed when two copies of the ring-shaped, heterohexameric Mcm2–7 helicase topologically encircle origin DNA1. This linkage is established when the Mcm2-Mcm5 gate (the interface between Mcm2 and Mcm5) is opened to allow DNA to enter the central channel of the helicase and then closed to prevent DNA release2,3. The two Mcm2–7 complexes are loaded sequentially. One Mcm2–7, in complex with Cdt1, is initially recruited to origin DNA bound by the ORC and Cdc6 (refs. 4–7). This intermediate rapidly releases Cdc6 and then Cdt1 (refs. 6,8). A second Cdc6 and the Cdt1–Mcm2–7 complex then associate with the ORC and the first Mcm2–7 (refs. 8,9), and subsequently Cdc6, Cdt1 and ORC are released8. The net result is a head-to-head Mcm2–7 double hexamer that encircles the origin DNA and is poised for bidirectional initiation4,10.

ATP binding and hydrolysis are critical for helicase loading. ATP binding is required for the initial DNA association of the three helicase-loading proteins and Mcm2–7 (refs. 11,12). ATP hydrolysis is required for the process to move beyond this initial association and for Mcm2–7 loading to be completed8,13,14. ORC, Cdc6 and Mcm2–7 all bind and hydrolyze ATP. Although not required for helicase loading, ORC ATP hydrolysis is required for the repetition of this event15. Cdc6 ATP hydrolysis also is not required for helicase loading13,14,16; however, it is required for a quality control mechanism that releases incompletely loaded Mcm2–7 from DNA13,14,17. Mcm2–7 ATP hydrolysis by at least a subset of the six Mcm2–7 ATPase motifs is required for helicase loading13,14, but it remains unclear which event(s) depends on the action of these ATPases.

Although previous studies revealed both the order of protein associations during helicase loading and their regulation18, the timing and mechanism of the key event of Mcm2–7 ring opening and closing have remained unclear. ATP binding at the Mcm2–Mcm5 interface is proposed to close the Mcm2–7 ring3, and this idea is supported by findings from electron microscopy studies of ATPγS-bound Mcm2–7 (ref. 9). In contrast, structural studies have shown that Mcm2–7 is in an open state in the presence of ATP19,20. The status of the Mcm2–Mcm5 gate in the initially recruited Cdt1–Mcm2–7 complex is unknown. The sequence similarity and structural similarity of ORC–Cdc6 to sliding clamp loaders have led to a hypothesis that binding to ORC and Cdc6 opens the Mcm2–7 ring5, but this remains to be tested.

Using a single-molecule FRET-based approach, we examined the timing and mechanism of Mcm2–7 ring opening and closing, and their relationship to other events of origin licensing. We found that Mcm2–7 is in an open state upon initial binding, and that this state is independent of Cdt1 binding. Mcm2–7 ring closure occurs independently for each Mcm2–7 at a time that is concomitant with Cdt1 release. In addition, we found that ATP hydrolysis by Mcm5–Mcm3 is required for ring closure and Cdt1 release. When these events were prevented, recruitment of the second Mcm2–7 ring was inhibited. Our findings provide important insights into the mechanism of helicase loading and reveal attributes of this event that favor double-hexamer formation and quality control.

**RESULTS**

**An assay for Mcm2-Mcm5 gate status**

Using the closed Mcm2–7 ring structure as a reference21, we attached donor and acceptor fluorophores to Mcm2 and Mcm5 at positions where FRET should increase in the closed state (Fig. 1a and Supplementary Fig. 1a). This fluorescent variant (Mcm2–7FRET)

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functioned at near-wild-type levels in bulk helicase-loading assays with purified proteins (Supplementary Fig. 1b–d). To measure Mcm2–7 DNA association and changes in apparent FRET efficiency (\(E_{\text{FRET}}\)) during helicase loading, we incubated surface-attached fluorescent origin DNA with purified Mcm2–725FRET, ORC, Cdc6 and Cdt1 (ref. 8). Colocalization of the protein- and DNA-associated fluorophores was indicative of DNA binding. By using alternating excitation of the acceptor and donor fluorophores (Fig. 1b and Supplementary Figs. 2 and 3a), we were able to monitor Mcm2–7 binding to individual DNAs and calculate \(E_{\text{FRET}}\) for bound Mcm2–725FRET. Long-lived sequential increases in Mcm2–7-associated fluorescence revealed the first and second Mcm2–7 binding events (Fig. 1b and Supplementary Fig. 3a). We focused on events in which simultaneous increases in both acceptor-excited and total donor-excited fluorescence (Fig. 1b) indicated an Mcm2–725FRET with both donor and acceptor fluorophores was binding. After initial binding, Mcm2–725FRET showed relatively high donor emission and weak acceptor emission (Fig. 1b; \(\sim 850\) s), resulting in a low \(E_{\text{FRET}}\) value. Long-lived Mcm2–725FRET molecules subsequently displayed decreased donor emission and increased acceptor emission, indicative of increased \(E_{\text{FRET}}\) (Fig. 1b; \(\sim 880\) s).

Analysis of a large number of Mcm2–725FRET helicase-loading trajectories revealed evidence for two major types of DNA–Mcm2–7 complexes with distinct \(E_{\text{FRET}}\) values (Fig. 1c). Early after DNA binding (<15 s), Mcm2–725FRET was predominantly in a state where \(E_{\text{FRET}} \sim 0.18\) (Fig. 1c, Supplementary Fig. 3b and Supplementary Table 1). At intermediate times (15–75 s), we observed a mixture of states with \(E_{\text{FRET}} \sim 0.18\) and \(E_{\text{FRET}} \sim 0.36\). At longer times (>75 s), \(E_{\text{FRET}}\) values were almost all \(\sim 0.36\). We observed a similar set of distributions for binding of a second Mcm2–725FRET, except that an intermediate \(E_{\text{FRET}}\) value of \(\sim 0.28\) was seen at early time points (Supplementary Fig. 3c and Supplementary Table 2). This intermediate value suggests that the first Mcm2–7 remains in the state where \(E_{\text{FRET}} \sim 0.36\), whereas the second Mcm2–7 is initially in the state where \(E_{\text{FRET}} \sim 0.18\). These distributions are consistent with a transition of both the first and the second Mcm2–7 complexes from an open Mcm2–Mcm5 gate (\(E_{\text{FRET}} \sim 0.18\)) to a closed Mcm2–Mcm5 gate (\(E_{\text{FRET}} \sim 0.36\)). We observed a similar transition between discrete low and high \(E_{\text{FRET}}\) states during helicase loading for an alternative Mcm2–7 construct in which Mcm2 was fluorescently labeled at a different location (Supplementary Fig. 3d). This finding is in agreement with the idea that the increase in \(E_{\text{FRET}}\) is caused by a conformational change that decreases the distance between Mcm2 and Mcm5.

Mcm2–725FRET–DNA complexes with time-averaged \(E_{\text{FRET}}\) values greater than 0.25 correlated with long-lived (>100 s) DNA associations (Fig. 1d), which supports the idea that the higher \(E_{\text{FRET}}\) state represents a closed Mcm2–Mcm5 gate. Consistent with higher \(E_{\text{FRET}}\) values being caused by changes in an individual Mcm2–7–hexamer, we observed elevated \(E_{\text{FRET}}\) values only when the donor and the acceptor were on the same Mcm2–7 (Supplementary Fig. 2a). Furthermore, once molecules reached \(E_{\text{FRET}} \sim 0.36\), no persistent excursions at or below \(E_{\text{FRET}}\) values of 0.18 were observed (>5 s; \(N = 5/57\)), consistent with a stably closed Mcm2–7 ring \(\bar{4}\). These findings, combined with subsequent observations, led us to conclude that \(E_{\text{FRET}}\) values of \(-0.18\) and \(-0.36\) for individual Mcm2–725FRET complexes indicate the presence of the open and closed conformations of the Mcm2–Mcm5 gate, respectively.

Cdt1 release is concomitant with Mcm2–7 ring closure

Because Cdc6 and Cdt1 sequentially dissociate from DNA after facilitating the initial Mcm2–7 binding \(\bar{8}\), we asked whether either of these events is correlated with Mcm2–Mcm5 gate closure. For the first Mcm2–7 association, we compared the time of gate closure, defined as the point at which \(E_{\text{FRET}}\) reached \(-0.36\), with previously

![Figure 1](https://example.com/figure1.png)

**Figure 1** Mcm2–7 hexamers associate with DNA while in an open-gate conformation and subsequently close. (a) Schematic of DNA-bound Mcm2–725FRET in the open-gate and closed-gate states. Mcm2 and Mcm5 were labeled with acceptor (A; red circles) and donor (D; green circles) fluorophores, respectively. Mcm2–Mcm5 gate closure increased the proximity of the fluorophores and the FRET efficiency (\(E_{\text{FRET}}\)). Fluorescence excitation (ex) and emission (em) wavelengths are indicated. Acceptor-excited (i) and donor-excited (ii) emission records are shown together with calculated donor-excited total emission (iii) and \(E_{\text{FRET}}\) (iv). Black arrows show the initial association of long-lived Mcm2–725FRET. Black horizontal bars indicate low-\(E_{\text{FRET}}\) intervals observed at the beginning of these associations. AU, arbitrary units. (c) Histograms of \(E_{\text{FRET}}\) values recorded during the indicated time intervals after association of the first Mcm2–725FRET with origin DNA. Fits to the sum (dashed cyan curves) of two Gaussians (red curves) yielded the fit parameters reported in Supplementary Table 1. For accurate \(E_{\text{FRET}}\) determination, we analyzed only the first Mcm2–7 associations that retained donor and acceptor fluorophores for >150 s and that lacked a second Mcm2–7 association within 150 s of DNA association. The same molecules were used for each histogram, and data from 255–(0–15 s), 1,020 (15–75 s) and 1,320 (75–150 s) total time points were plotted. (d) Histogram of single Mcm2–7 dwell times. Mcm2–7 associations with a time-averaged \(E_{\text{FRET}} < 0.25\) (\(N = 91\) molecules) are plotted in red, and those with a time-averaged \(E_{\text{FRET}} > 0.25\) (\(N = 59\) molecules) are in blue. Time-averaged \(E_{\text{FRET}}\) was calculated over the duration of the association or until the association of a second Mcm2–7. For 29 molecules with average \(E_{\text{FRET}} > 0.25\), the measured dwell times were truncated by the end of the recording.
Figure 2  Mcm2–7 gate closure correlates with the release of Cdt1 on DNA but not free in solution. (a) The fraction of dye-labeled Cdc6 (N = 96 molecules) or Cdt1 (N = 72 molecules) bound at various times after the first Mcm2–7 association (ref. 8) (marked along the left-hand vertical axis) and the fraction of Mcm2–725FRET molecules showing gate closure (Mcm2–7 FRET; N = 96 molecules) at various times (marked along the right-hand vertical axis). The 95% confidence interval (CI) for the time of Cdt1 release is indicated by the gray solid lines. The inset shows the mean release times and Mcm2–7 gate-closure time (±95% CI). Gate-closure times were based on the first time point at which the E_{\text{FRET}} value increased by 0.15 over the average of five previous time points and was sustained for at least five time points. (b) The same as in a, but for events after association with the second Mcm2–725FRET. The closed-gate fraction (N = 47 molecules) is shown on the right-hand vertical axis. The release of Cdc6 (N = 70 molecules) and Cdt1 (N = 74 molecules; 95% CI indicated by gray solid lines) can be assessed with the left-hand vertical axis (from ref. 8). Gate-closure times were determined as in a, except that the E_{\text{FRET}} increase threshold was 0.1. (c) Histograms of E_{\text{FRET}} values of tethered Mcm2–725FRET in either the absence (N = 32 molecules, 2,014 total time points) or the presence (N = 60 molecules, 3,712 total time points) of Cdt1, fit with the sum (cyan dashed line) of two (with Cdt1) or three (no Cdt1) Gaussians (red curves), which yielded the fit parameters reported in Supplementary Table 3. The presence of Cdt1 moderately increased the percentage of Mcm2–7 in the open (E_{\text{FRET}} ~ 0.16) state (88% versus 80%). In both cases, the majority of the remaining data fit to a second state with E_{\text{FRET}} ~ 0.28, which is likely to represent a partially closed Mcm2–Mcm5 gate. Insets show schemes of attachment. Mcm2–725FRET was tethered to the slide surface either directly through the Mcm5 subunit (no Cdt1; Mcm2–725FRETbiotin) or indirectly through biotinylated Cdt1 tethered to the surface (with Cdt1).
**Figure 3** Closure of the second Mcm2–7 ring correlates with ORC release. (a) Schematic of Mcm2–725quench labeling in the open-gate and closed-gate states. Mcm2 and Mcm5 were labeled with donor fluorophore (D; green circles) and quencher (BHQ-2; black circles), respectively. Closure of the Mcm2–Mcm5 gate decreases the distance between the fluorophore and quencher, and thus decreases donor emission. (b) Two example traces showing sequential association of two Mcm2–725quench molecules with individual origin DNA molecules. The black arrows show the initial association of Mcm2–725FRET and loaded Mcm2–725FRET. Although the absolute values were different from those seen in the single-molecule experiments owing to incomplete protein labeling (only doubly labeled proteins were assessed in the single-molecule setting), we observed higher FRET values for loaded Mcm2–725FRET (0.129 ± 0.004) than for free Cdt1–Mcm2–725FRET (0.076 ± 0.002). These findings agree with the results of previous low-resolution structural studies showing that free Mcm2–7 has an open Mcm2–Mcm5 gate. We conclude that Cdt1 is not required to prevent Mcm2–7 ring closure, but Cdt1-bound Mcm2–7 may more strongly favor the open state.

**Oncogene release occurs after closure of both Mcm2–7 rings**

To simultaneously monitor the status of the Mcm2–Mcm5 gate and release of fluorescently labeled proteins from individual DNA templates in three-color experiments, we labeled Mcm2 and Mcm5 with a fluorophore and a quencher, respectively (Mcm2–725quench; Fig. 3a). Bulk assays showed that Mcm2–725quench retained ~50% of wild-type helicase-loading activity (Supplementary Fig. 1c,d). Consistent with our Mcm2–725FRET studies, this complex showed high fluorescence upon initial DNA binding (open gate) and reduced fluorescence thereafter (closed gate; Fig. 3b and Supplementary Fig. 4d). Experiments in which we combined Mcm2–725quench with differentially labeled Mcm2–7 (Mcm2–725FRET) showed that the Mcm2–Mcm5 gate of the first Mcm2–7 did not reopen once closed, including during loading of the second Mcm2–7 (Fig. 3c).

As with Mcm2–725FRET labeled Cdc6 and ORC were compatible with Mcm2–725quench, but fluorescently labeled Cdt1 inhibited helicase loading when combined with this form of Mcm2–7. In agreement with previous results showing that Cdc6 is released before Cdt1, labeled Cdc6 was always released before Mcm2–725quench gate closure (Fig. 3d; 62/62 events). When we combined labeled ORC with Mcm2–725quench, we noted a connection between ORC release and closing of the second Mcm2–7 ring. In the majority of events (47/54), the single ORC involved in helicase loading was released at a time within the range of experimental error for gate closure of the second Mcm2–7 ring (Fig. 3e). In the remaining events, ORC was retained on the DNA after the second ring closure. Interestingly, the average time until the second Mcm2–7 ring closure was much longer than the previously determined average time until the establishment of Mcm2–7–Mcm2–7 double-hexamer interactions (as measured by FRET between the N termini of the first and second Mcm2–7 hexamers; Supplementary Fig. 4e). Thus, an open second Mcm2–7 ring engages in initial double-hexamer interactions with a closed first Mcm2–7 ring (Fig. 3c), which raises the possibility that the closed first Mcm2–7 ring could act as a template to facilitate closing of the second Mcm2–7 ring. Taken together, our data strongly suggest that ORC release occurs only after the formation of the Mcm2–7 double hexamer and closure of both
Mcm2–7 rings; such a mechanism would ensure that ORC is retained until the completion of origin licensing.

**Mcm2–7 ATP hydrolysis is required for Cdt1 release and ring closure**

To further investigate the mechanism of Mcm2–Mcm5 gate closure, we asked whether the ATPase activity of Mcm2–7, Cdc6 or ORC controls this event. Given the temporal connection between gate closure and Cdt1 release, we focused on a mutation in the Mcm5-ATPase active site (mcm5-R549A; Supplementary Fig. 5a) that leads to defects in Cdt1 release and Mcm2–7 loading13,14. We incorporated this mutant into Mcm2–725FRET (Mcm2–725FRET-5RA) and monitored the status of the Mcm2–Mcm5 gate. Strikingly, Mcm2–725FRET-5RA remained in an open-gate state (FRET ~ 0.18) and monitored the status of the Mcm2–Mcm5 gate. This allowed us to perform a high-salt wash that removed incompletely loaded Mcm2–7 (32/32 events)12,23.

**DISCUSSION**

Our results support our initial conclusion that Mcm2–725FRET FRET values of ~0.18 and ~0.36 represent the open and closed states of the Mcm2–Mcm5 gate, respectively. The Mcm2–7 ring was at FRET ~ 0.18 before and immediately after DNA binding, in agreement with the idea of an open Mcm2–7 ring allowing DNA access to the central channel. Similarly, all DNA-associated Mcm2–725FRET-5RA molecules were in the open (FRET ~ 0.18) state and were released by a high-salt wash that removed incompletely loaded Mcm2–7 (32/32 events)12,23. In agreement with this conclusion, recent high-resolution cryo-EM structural studies of Mcm2–7 and Cdt1–Mcm2–7 showed that both complexes are in an open-ring conformation24. The Mcm2–7 ATPase, but not the ORC or Cdc6 ATPases, is required for helicase loading13,14. Consistent with the theory that the higher FRET state reflects a loaded, closed-ring Mcm2–7, the Mcm5-Mcm3 ATPase mutant—but not mutations in ORC or Cdc6 ATPases—prevented the formation of this state. Future studies will be required to determine whether other Mcm2–7 ATPase mutants have the same effect. Finally, attainment of FRET ~ 0.36 occurred independently for each Mcm2–7 complex, in agreement with evidence that the hexamers are loaded one at a time6–9. Although structural studies of the loaded double hexamer suggest a completely closed Mcm2–7 ring25, our findings do not exclude the possibility that the closed state we observed in
Cdc6 is followed by the closure of the second Mcm2–7 ring, which charged Mcm2–7 central channel and Cdt1 binding (Fig. 5). Events are causally linked (Fig. 5). Mcm5-Mcm3 ATPase mutant, support a model in which these events of the Mcm2-Mcm5 gate, and the inhibition of both events by the Mcm2–7 ATPases) (iv), and this closure event is associated with the release of Cdc6 before Mcm2–7 ring opening (Figs. 1 and 3), and ATP hydrolysis by ORC or Cdc6 is not required for ring closure (Fig. 4b). This does not eliminate other possible roles for ORC–Cdc6, including stimulation of Mcm2–7 ATP hydrolysis or alteration of the Mcm2–7 conformation to facilitate ring closure.

The ordered release of Cdc6 and Cdt1 and the connection of the latter event to ring closure create a window of time for Mcm2–7 loading quality control. Cdc6 ATP hydrolysis is connected to the release of nonproductive Mcm2–7 complexes. Because the Mcm2–7 ring is open throughout Cdc6-DNA association (Fig. 3d), this quality control mechanism would not require reopening of the Mcm2–7 ring. In addition, the ordered closure of rings would allow the first and second Mcm2–7 complexes to be assessed separately. Although the mechanism of this release is unclear, one simple hypothesis is that an ATP-dependent release of Cdc6 before Mcm2–7 ring closure leads to the simultaneous release of open, nonproductive Mcm2–7 complexes.

Our findings indicate that loading of the two Mcm2–7 complexes associated with origin licensing is the result of a single coordinated event rather than two independent Mcm2–7 loading events. Both the lack of a second Mcm2–7 association for the Mcm2–7 5RA mutant and the finding that gate closure in the first Mcm2–7 always preceded DNA association with the second Mcm2–7 (Fig. 1b and Supplementary Fig. 2a; 47/47 events) strongly suggest that recruitment of the second Mcm2–7 cannot begin until the first loading event is completed. This is inconsistent with models that suggest that two ORC molecules independently recruit and load one Mcm2–7. The connection between ORC release and closure of the second but not the first Mcm2–7 ring (Fig. 3e) also supports the idea of a coordinated mechanism. Importantly, these properties would ensure that single Mcm2–7 loading events occurred only as the first step in the formation of an Mcm2–7 double hexamer.

The combination of fully reconstituted biochemical assays and detailed structural models of key replication intermediate has provided important insights into the events of eukaryotic replication initiation. Single-molecule studies complement these approaches by revealing reaction kinetics that are difficult to assay in asynchronous bulk reactions, by identifying intermediates that are too short-lived or dynamic to be analyzed structurally, and by monitoring changes in protein conformation in real time. Our findings show how the combination of single-molecule colocalization and single-molecule FRET can be used to elucidate the complex and coordinated protein dynamics of helicase-loading events. More important, our findings reveal features of origin licensing that can reduce the number of incomplete or incorrect events, and thus improve genome stability.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
S.T. performed all experiments with feedback from J.G., L.J.F. and S.P.B., except for the ensemble FRET studies, which were performed by K.C. I.R.C. prepared essential reagents. S.T., L.J.F. and J.G. analyzed the data. S.P.B. wrote the paper with input from all other authors. S.P.B. and J.G. directed the project.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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ARTICLES
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1. Yardimci, H. & Walter, J.C. Prereplication-complex formation: a molecular double take? Nat. Struct. Mol. Biol. 21, 20–25 (2014).
2. Samel, S.A. et al. A unique DNA entry gate serves for regulated loading of the eukaryotic replicative helicase MCM2-7 onto DNA. Genes Dev. 28, 1653–1666 (2014).
3. Bochman, M.L. & Schwacha, A. The Mcm2-7 complex has in vitro helicase activity. Mol. Cell 31, 287–293 (2008).
4. Remus, D. et al. Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. Cell 139, 719–730 (2009).
5. Aparicio, O.M., Weinstein, D.M. & Bell, S.P. Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell 91, 59–69 (1997).
6. Fernández-Cid, A. et al. An ORC/Cdc6/MCM2-7 complex is formed in a multistep reaction to serve as a platform for MCM double-hexamer assembly. Mol. Cell 50, 577–588 (2013).
7. Sun, J. et al. Cryo-EM structure of a helicase loading intermediate containing ORC-Cdc6-Cdt1-MCM2-7 bound to DNA. Nat. Struct. Mol. Biol. 20, 944–951 (2013).
8. Ticau, S., Friedman, L.J., Ivica, N.A., Gelles, J. & Bell, S.P. Single-molecule studies of origin licensing reveal mechanisms ensuring bidirectional leading strand loading. Cell 161, 513–525 (2015).
9. Sun, J. et al. Structural and mechanistic insights into Mcm2-7 double-hexamer assembly and function. Genes Dev. 28, 2291–2303 (2014).
10. Ervin, C. et al. A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. Proc. Natl. Acad. Sci. USA 106, 20240–20245 (2009).
11. Bell, S.P. & Stillman, B. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature 357, 128–134 (1992).
12. Randell, J.C.W., Bowers, J.L., Rodríguez, H.K. & Bell, S.P. Sequential ATP hydrolysis by Cdc6 and Orc directs loading of the Mcm2-7 helicase. Mol. Cell 21, 29–39 (2006).
13. Kang, S., Warner, M.D. & Bell, S.P. Multiple functions for Mcm2-7 ATPase motifs during replication initiation. Mol. Cell 55, 655–665 (2014).
14. Corder, G., Frigola, J., Beuron, F., Morris, E.P. & Diffley, J.F. Origin licensing requires ATP binding and hydrolysis by the MCM replicative helicase. Mol. Cell 55, 666–677 (2014).
15. Bowers, J.L., Randell, J.C.W., Chen, S. & Bell, S.P. ATP hydrolysis by ORC catalyzes reiterative MCM2-7 assembly at a defined origin of replication. Mol. Cell 16, 967–978 (2004).
16. Chang, F. et al. Cdc6 ATPase activity disengages Cdc6 from the pre-replicative complex to promote DNA replication. Elife 4, 1–42 (2015).
17. Frigola, J., Remus, D., Mehanna, A. & Diffley, J.F. ATPase-dependent quality control of DNA replication origin licensing. Nature 495, 339–343 (2013).
18. Deegan, T.D. & Diffley, J.F. MCM: one ring to rule them all. Curr. Opin. Struct. Biol. 37, 145–151 (2016).
19. Costa, A. et al. The structural basis for MCM2-7 helicase activation by GINS and Cdc45. Nat. Struct. Mol. Biol. 18, 471–477 (2011).
20. Lyubimov, A.Y., Costa, A., Bleichert, F., Botchan, M.R. & Berger, J.M. ATP-dependent conformational dynamics underlie the functional asymmetry of the replicative helicase from a minimalist eukaryote. Proc. Natl. Acad. Sci. USA 109, 11999–12004 (2012).
21. Li, N. et al. Structure of the eukaryotic MCM complex at 3.8 Å. Nature 524, 186–191 (2015).
22. Chepeva, A. & Diffley, J.F. Mutational analysis of conserved sequence motifs in the budding yeast Cdc6 protein. J. Mol. Biol. 308, 597–608 (2001).
23. Donovan, S., Harwood, J., Druy, L.S. & Diffley, J.F. Cdc6p-dependent loading of MCM proteins onto pre-replicative chromatin in budding yeast. Proc. Natl. Acad. Sci. USA 94, 5611–5616 (1997).
24. Zhai, Y. et al. Open-looped structure of the Cdt1–MCM2-7 complex as a precursor of the MCM double hexamer. Nat. Struct. Mol. Biol. http://dx.doi.org/10.1038/nstmb.3374 (2016).
25. Bowers, J.L., Rabbani, T.P., Deegan, T.D., Janska, A., Early A. & Diffley, J.F. Regulated eukaryotic DNA replication origin firing with purified proteins. Nature 519, 431–435 (2015).
ONLINE METHODS

Protein expression and purification strains. Cdc6SORT49 (pET-GSS-Cdc6), Cdt1SORT49, Mcm2–7SNAP (yST166) and ORCSORT49 (yST163) were purified as described previously. To monitor the Mcm2–Mcm3 gate, we constructed strains that expressed Cdt1–Mcm2–725FRET, by introducing an Asc1 site after amino acid 721 of Mcm2 and amino acid 591 of Mcm5 (ref. 2). A SNAP-tag (Mcm5, NEB) or CLIP-tag (Mcm2, NEB) was inserted with ten amino acid linkers (GSGGGGSGGS) at each junction. For purification of Mcm2–725FRET, Mcm2–721CLIP and Mcm5–591SNAP were expressed in conjunction with the remaining wild-type Mcm2–7 subunits and Cdt1 (yST229) or in the absence of Cdt1 (yST266, to make Mcm2–725FRET-biotin) and labeled with CLIP-Surface 647(NEB) and SNAP-Surface 549 (NEB) as described below. To monitor gate closure by quenching (Mcm2–725quench) or to create an alternate Mcm2–Mcm5 gate (Mcm2–725FRET), we coexpressed Mcm5SORT (Mcm2 with LPETGG NH2-CHHHHHHHHHHHLPETGGG followed by the remainder of the protein) on Cdt1–Mcm2–7 from a strain that expressed Mcm5–R549A on Cdt1 release by purifying Cdt1–Mcm2–7 from a strain that expressed Mcm5–R549A, Mcm4SNAP, Cdt1SORT and the remaining wild-type Mcm subunits (yST291). Mcm4SNAP was labeled with SNAP-JF646 (a gift from Luke Lavis, Janelia Research Campus), and Cdt1SORT was coupled to the peptide NH2-GGGHHHHHHHHHHHC-COOH coupled to maleimide-Dy549, and we coupled Mcm5–591SNAP to SNAP-BHQ-2 to form Mcm2–725quench (see below) or to SNAP-Surface 649 to form Mcm2–725FRET, Mcm2–725FRET-mcm5RA. We monitored the effect of Mcm5–R549A on Cdt1 release by purifying Cdt1–Mcm2–7 from a strain that expressed Mcm5–R549A, Mcm4SNAP, Cdt1SORT and the remaining wild-type Mcm subunits (yST291). Mcm4SNAP was labeled with SNAP-JF646 (a gift from Luke Lavis, Janelia Research Campus), and Cdt1SORT was coupled to the peptide NH2-GGGHHHHHHHHHHHC-COOH coupled to maleimide-Dy549 to make Cdt1SORT49–Mcm2–7SNAP-mcm5RA. We monitored the effect of Mcm5–R549A on Cdc6 release by purifying Cdt1–Mcm2–7 from a strain that expressed Mcm5–R549A, Mcm4SNAP, Cdt1 and the remaining wild-type Mcm subunits (yST299) to make Cdt1–Mcm2–7SNAP-mcm5RA.

Purification and fluorescent labeling of Cdt1–Mcm2–7. S. cerevisiae (W303 background) strains yST229, yST220, yST299 or yST291 were grown to OD600 = 1.2 at 30 °C to make Cdt1–Mcm2–74SNAP-mcm5RA. For SNAP- or CLIP-tagged Cdt1–Mcm2–7, when the proteins were coupled to fluorophore(s), we applied the reaction to a Superdex 200 10/300 gel filtration column equilibrated in buffer A with 0.1 mM EDTA, 0.1 mM EGTA and 0.3 M KGluc. Peak fractions containing Cdt1–Mcm2–7 were pooled, aliquoted and stored at −80 °C.

For SORT-tagged Cdt1–Mcm2–7, after dye coupling, the reaction was applied to a Superdex 200 10/300 gel filtration column equilibrated in buffer A with 0.1 mM EDTA, 0.1 mM EGTA, 0.3 M KGluc and 10 mM imidazole. Peak fractions containing peptide-coupled Cdt1–Mcm2–7 were pooled and incubated with 0.5 ml of Complete His-Tag Purification Resin (Roche) pre-equilibrated in buffer A with 0.1 mM EDTA, 0.1 mM EGTA, 0.3 M KGluc, 10 mM imidazole. Peptide-coupled Cdt1–Mcm2–7 was eluted using buffer A with 0.1 mM EDTA, 0.1 mM EGTA, 0.3 M KGluc and 0.5 M imidazole. Peak fractions were pooled, aliquoted, and stored at −80 °C.

Special note on handling of fluorescent dyes: light sources on all chromatography apparatuses (AKTA FPLC, HPLC) were turned off during preparative runs. Fractions containing fluorescently labeled peptides and proteins were determined during previous analytical runs.

Fluorescent labeling of peptides for Sortase coupling, as well as purification of the Sortase A pentamutant enzyme and Ulp1, was performed as reported previously.

Percent labeling of Mcm2–725FRET. To determine the labeling efficiency of the SNAP- and CLIP-tag labeling approaches in the Mcm2–725FRET context, we purified and labeled Mcm2–725FRET with SNAP-Surface 549 and CLIP-Surface 647 on the Mcm5 and Mcm2 subunits, respectively. We imaged a standard reaction containing 0.25 nM ORC, 1 nM Cdc6 and 2.5 nM Cdt1–Mcm2–725FRET using the described protocol and monitored the colocalization of Mcm2–725FRET fluorescence by DNA fluorescence (to ensure that we were monitoring fully assembled complexes). Each colocalization was scored as exhibiting both D and A fluorescence for only D or only A. By assuming that the labeling reactions of the SNAP and CLIP tags in Mcm2–725FRET were independent, we calculated from the observed D and A colocalization frequencies that SNAP labeling efficiency was ~74%, and CLIP labeling efficiency was ~81%, yielding ~60% of Mcm2–725FRET complexes with both D and A fluorophores.

Synthesis of SNAP-549-Biotin and SNAP-BHQ2. Commercially available compounds were used without further purification. Reaction yields were not optimized. Reversed-phase high-performance liquid chromatography (HPLC) was done on an Agilent LC-MS Single Quad System 1200 Series (analytical) and an Agilent 1100 Preparative-scale Purification System (semi-preparative). Analytical HPLC was done on a Waters Atlantis T3 C18 column (2.1 × 150 mm, 5-µm particle size) at a flow rate of 0.5 ml/min with a binary gradient from phase A (0.1 M triethyl ammonium bicarbonate (TEAB) or 0.1% trifluoroacetic acid (TFA) in water) to phase B (acetonitrile) and monitored by absorbance at 280 nm. Semi-preparative HPLC was done on a Y VyDAC 218TP series C18 polymeric reversed-phase column (22 × 250 mm, 10-µm particle size) at a flow rate of 20 ml/min. Mass spectra were recorded by electrospray ionization (ESI) on an Agilent 6120 Quadrupole LC-MS, an Agilent 6210 Time-Of-Flight (TOF) or a Thermo Scientific QExactive system. We prepared SNAP-BHQ2 (BG-BHQ2; Supplementary Fig. 6a) by reacting the building block BG-NH2 (New England BioLabs) with commercially available BHQ-2 succinimidyl ester (LGC Bioseach) as described23. BHQ-2 succinimidyl ester (2.5 mg, 4.1 µmol) was dissolved in anhydrous DMF (1.0 mL). BG-NH2 (1.1 mg, 4.1 µmol) and triethylamine (0.56 µL, 4.1 µmol) were added, and the reaction was added to a final concentration of 5 mM in buffer A with 0.3 M KGluc. This was mixed with 100 nmol of peptide carrying a Sort-tag and labeled with Dy549-maleimide (Dynoms), dissolved in 200 µL of buffer A with 0.3 M KGluc (the sequences of the peptides used are described below). The reaction was incubated at room temperature for 15 min and then quenched with 20 mM EDTA. The net result of the sortase reaction is coupling of the fluorescently labeled (or biotinylated) peptide to the N terminus of Cdt1 with the sequence NH2-CIHGGGHGHHHHHHHLPETGGG followed by the remainder of the protein or to the C terminus of Mcm2 with the sequence LPETGGHHHHHHHHHHC-OOH.

For SNAP or CLIP-tagged Cdt1–Mcm2–7, when the proteins were coupled to fluorophore(s), we applied the reaction to a Superdex 200 10/300 gel filtration column equilibrated in buffer A with 0.1 mM EDTA, 0.1 mM EGTA and 0.3 M KGluc. Peak fractions containing Cdt1–Mcm2–7 were pooled, aliquoted and stored at −80 °C.

For SORT-tagged Cdt1–Mcm2–7, after dye coupling, the reaction was applied to a Superdex 200 10/300 gel filtration column equilibrated in buffer A with 0.1 mM EDTA, 0.1 mM EGTA, 0.3 M KGluc and 10 mM imidazole. Peak fractions containing peptide-coupled Cdt1–Mcm2–7 were pooled and incubated with 0.5 ml of Complete His-Tag Purification Resin (Roche) pre-equilibrated in buffer A with 0.1 mM EDTA, 0.1 mM EGTA, 0.3 M KGluc, 10 mM imidazole for 1 h with rotation at 4 °C. The flow-through was discarded, and the resin was washed with 5 ml of buffer A with 0.1 mM EDTA, 0.1 mM EGTA, 0.3 M KGluc and 10 mM imidazole. Peptide-coupled Cdt1–Mcm2–7 was eluted using buffer A with 0.1 mM EDTA, 0.1 mM EGTA, 0.3 M KGluc and 0.5 M imidazole. Peak fractions were pooled, aliquoted, and stored at −80 °C.

Special note on handling of fluorescent dyes: light sources on all chromatography apparatuses (AKTA FPLC, HPLC) were turned off during preparative runs. Fractions containing fluorescently labeled peptides and proteins were determined during previous analytical runs.

Fluorescent labeling of peptides for Sortase coupling, as well as purification of the Sortase A pentamutant enzyme and Ulp1, was performed as reported previously8.
mixture was incubated at room temperature with stirring overnight. The solvent was removed under vacuum, and the product was purified by reversed-phase HPLC using a 0.1 M TEAB-acetonitrile gradient (yield, 21%).

**Single-molecule data analysis.** Analysis of the CoSMoS data sets was similar to that described in ref. 34. Specifically, we typically followed these four steps:\(^3\)

1. Define the spatial relationship between the two images created at different excitation and emission wavelengths from the single field of view by the dual-view optical system (‘mating’).
2. Correct the data set for stage drift that occurred during the experiment (‘drift correction’).
3. Image the label on origin DNA to identify the locations of single DNA molecules on the surface, and (4) integrate fluorescence emission from small regions centered at the pre-defined locations of coupled DNA locations in each acquired image to obtain plots of fluorescence intensity versus time. We used custom image-processing software (https://github.com/gelles-brandeis/CoSMoS_Analysis) implemented in MATLAB (The Mathworks, Natick, MA) to carry out these steps. Confidence intervals for kinetic data were determined by bootstrapping.

Both dual-imaging optics and chromatic aberrations result in spatial displacement between fluorescent spot images of colocized species that are labeled with dyes of different colors. Accurate colocalization of the differentially labeled species therefore requires the use of a mapping procedure. For each pair of colors, we acquired a list of several hundred reference spot pairs, using a sample containing a surface-tethered oligonucleotide that was labeled with Alexa Fluor 488, Cy3 and Cy5. We mapped the coordinates of a fluorescent spot to the equivalent location at a different color by using a transformation with fit parameters based on just the 15 nearest reference spots.\(^3\) Drift correction and spot detection were done as described in ref. 35. Fluorescence emission from labeled complexes was integrated over a 0.37-µm² area centered at each drift-corrected origin-DNA location, yielding for each DNA molecule a separate intensity time course for each color of fluorescent label being observed.

**FRET and quenching data analysis.** Images containing spots that were analyzed to produce a FRET time course were first mapped and drift-corrected (see above). By alternating between their laser excitation wavelengths, we monitored the colocalization of donor-labeled and acceptor-labeled Mcm2–7 hexamers with the origin-DNA molecule. To determine the time until formation of the state with \(E_{\text{FRET}} \approx 0.36\), we noted the earliest time point at which the \(E_{\text{FRET}}\) values increased by 0.15 for the first Mcm2–7 or 0.1 for the second Mcm2–7. Only Mcm2–7 molecules that were labeled with both fluorophores were used for analysis of the first Mcm2–7. For analysis of the second Mcm2–7, both the first and the second Mcm2–7 molecules had to be labeled with both fluorophores.

To calculate apparent FRET efficiencies, we first subjected the baseline for each fluorescence intensity trace to a low-pass filter. That smoothed baseline was then subtracted from the starting trace, resulting in a fluorescence time record with a zero mean baseline.\(^2\) (Fig. 1b). The apparent FRET efficiency was calculated as follows:

\[
E_{\text{FRET}} = \frac{I_{\text{Accept}}}{I_{\text{Accept}} + I_{\text{Donor}}}
\]

where \(I_{\text{Accept}}\) and \(I_{\text{Donor}}\) are the acceptor and donor emission intensities observed during donor excitation, respectively. No gamma correction was applied because no systematic change in \(I_{\text{Accept}} + I_{\text{Donor}}\) was observed upon changes in \(E_{\text{FRET}}\) (Fig. 1b and Supplementary Fig. 2a) or upon acceptor photobleaching.

\(E_{\text{quench}}\) was calculated from baseline-corrected data (of single Mcm2–7 molecules) as described in Supplementary Figure 4d.

**Measuring FRET values for soluble Mcm2–7.** To generate sufficient loaded Mcm2–7, we carried out a large-scale helicase-loading reaction with 20 pmol of bead-attached origin DNA, 80 pmol of Mcm2–7, 20 pmol of ORC, and 40 pmol of Cdc6 (ref. 13). After a 20-min incubation, the DNA beads were washed with a high-salt buffer (to remove incompletely loaded protein), and loaded Mcm2–7 was released from beads by DNase I treatment as previously described.\(^1\) The released, loaded Mcm2–7 was a similar concentration of unloaded solution Cdt1–Mcm2–7 was placed in a cuvette and excited at 549 nm. Fluorescence emission was detected from 560–690 nm, and the peak values
of donor (574 nm) and acceptor (670 nm) emissions were used to determine $E_{\text{FRET}}$ values. Reported uncertainties are the s.d. of four separate experiments.

**Statistical analysis.** Confidence intervals were determined by bootstrapping with either 1,000 samples (Figs. 2 and 4 and Supplementary Figs. 4 and 5) or 250 samples (Supplementary Tables 1–3).

**Code availability.** Links to code are included in this section, in the legend of Supplementary Figure 3 legend and in the caption of Supplementary Table 1.

**Data availability.** The data that support the findings of this study are available from the corresponding authors upon reasonable request.

28. Chen, I., Dorr, B.M. & Liu, D.R. A general strategy for the evolution of bond-forming enzymes using yeast display. *Proc. Natl. Acad. Sci. USA* **108**, 11399–11404 (2011).

29. Kindermann, M., Sielaff, I. & Johnsson, K. Synthesis and characterization of bifunctional probes for the specific labeling of fusion proteins. *Bioorg. Med. Chem. Lett.* **14**, 2725–2728 (2004).

30. Smith, B.A. *et al.* Three-color single molecule imaging shows WASP detachment from Arp2/3 complex triggers actin filament branch formation. *eLife* **2**, e01008 (2013).

31. Friedman, L.J. & Gelles, J. Mechanism of transcription initiation at an activator-dependent promoter defined by single-molecule observation. *Cell* **148**, 679–689 (2012).

32. Friedman, L.J., Chung, J. & Gelles, J. Viewing dynamic assembly of molecular complexes by multi-wavelength single-molecule fluorescence. *Biophys. J.* **91**, 1023–1031 (2006).

33. Crawford, D.J., Hoskins, A.A., Friedman, L.J., Gelles, J. & Moore, M.J. Single-molecule colocalization FRET evidence that spliceosome activation precedes stable approach of 5′ splice site and branch site. *Proc. Natl. Acad. Sci. USA* **110**, 6783–6788 (2013).

34. Hoskins, A.A. *et al.* Ordered and dynamic assembly of single spliceosomes. *Science* **331**, 1289–1295 (2011).

35. Friedman, L.J. & Gelles, J. Multi-wavelength single-molecule fluorescence analysis of transcription mechanisms. *Methods* **86**, 27–36 (2015).