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Single-Molecule Visualization of MCM2-7 DNA Loading: Seeing Is Believing

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The first event in the initiation of eukaryotic DNA replication is the recruitment of the MCM2-7 ATPase, the core of the replicative DNA helicase, to origins. Ticau et al. use single-molecule imaging to reveal how ORC, Cdc6, and Cdt1 cooperate to load MCM2-7 onto DNA, enabling bidirectional replication.

Eukaryotic cells copy their vast genomes by initiating DNA replication from thousands of origins of replication. To insure that replication initiates at every origin precisely once per cell cycle, cells divide the process of initiation into two stages. In G1 phase, two copies of the MCM2-7 ATPase are loaded onto origin DNA to form a "pre-replication complex" (pre-RC) (Figure 1). This process requires three "licensing" factors: a hexameric AAA+ ATPase called ORC (*o*rigin *r*ecognition *c*omplex), another AAA+ ATPase called Cdc6, and Cdt1. When it is first loaded, MCM2–7 encircles double-stranded DNA and is inactive as a DNA helicase. In S phase, the two *M*CM2–7 complexes associate with the helicase co-factors, *C*dc45 and *G*INS, forming two active CMG helicases that encircle single-stranded DNA, unwind the origin, and nucleate the assembly of two replisomes that travel away from the origin, copying DNA as they go. Importantly, once cells enter S phase, multiple mechanisms prevent de novo MCM2–7 loading onto origins. As a result, each origin fires only once per cell cycle. In this issue, Ticau et al. (2015) use single-molecule imaging to reveal how yeast MCM2–7 double hexamers are loaded at replication origins (Figure 1).

Recent studies showed that ORC, Cdc6, Cdt1, and MCM2–7 are necessary and sufficient for pre-RC assembly (Remus et al., 2009); revealed various MCM2–7 loading intermediates (Fernández-Cid et al., 2013; Sun et al., 2013; Sun et al., 2014); and determined how MCM2–7 subunits interact via their N termini within the so-called double hexamer (Costa et al., 2014; Sun et al., 2014). However, the most fundamental question—how an origin containing a single ORC-binding site supports the head-to-head loading of two MCM2–7 molecules—remains unanswered: Are the two MCM2–7 hexamers loaded simultaneously or one at a time? Are the two MCMs loaded via the same or different mechanisms? Does one DNA-bound ORC complex load both MCM2–7 hexamers, or is there participation by a second ORC bound at a cryptic site? Of the helicase-loading intermediates captured in recent structural studies, which complexes are on pathway? Other questions refer to the exact roles of Cdc6 and Cdt1 and the order in which they arrive and depart from the origin during licensing.

To answer these questions, Ticau et al. established a single-molecule loading assay with recombinant yeast proteins. A fluorescently labeled DNA containing the yeast origin of replication was immobilized on a coverslip and imaged via total internal reflection fluorescence microscopy. One or two fluorescently labeled licensing factors (for example, MCM2-7 and Cdc6, or MCM2-7 and Cdt1) and ATP were added to the flow cell, and protein binding and unbinding on DNA was monitored in real time by co-localizing the fluorescent signals from the nucleic acid and the protein of interest. This assay determined the arrival and departure times of proteins relative to each other and identified short-lived intermediates not detected in ensemble or structural approaches. Photobleaching experiments established the stoichiometry of bound factors.

Monitoring the binding of fluorescently labeled MCM2–7 hexamers to DNA revealed that MCM2–7 is recruited one hexamer at a time, providing definitive support for previous models (Fernández-Cid et al., 2013; Sun et al., 2013, 2014). The authors then examined the relative timing of Cdc6 and Cdt1 recruitment to replication origins. MCM2–7 and Cdt1 form a hetero-heptameric complex in solution (Kawasaki et al., 2006) while DNA-bound ORC forms a complex with Cdc6 (Sun et al., 2012). The single-molecule approach showed that Cdc6 binds to ORC before MCM2-7•Cdt1 arrives at an origin, indicating that Cdc6 primes the origin recognition complex to recruit the first MCM2–7 hexamer (Figure 1). Moreover, after MCM2-7•Cdt1 binding, Cdc6 is always released before Cdt1. Interestingly, Cdc6/Cdt1 departure times are significantly longer after loading of the second MCM2–7 ring compared to the first MCM2–7 ring, suggesting that the two hexamers are recruited differently. In addition, the kinetics of Cdc6/Cdt1 departure suggests that several processes occur between the arrival of MCM2–7 and the release of Cdc6/Cdt1. The number and identity of these steps is unclear and should be investigated in future studies.

Finally, Ticau et al. examined ORC dynamics during pre-RC assembly. By simultaneously monitoring fluorescently labeled ORC and MCM2–7, they discovered that a single ORC complex remains bound to the origin during the arrival of

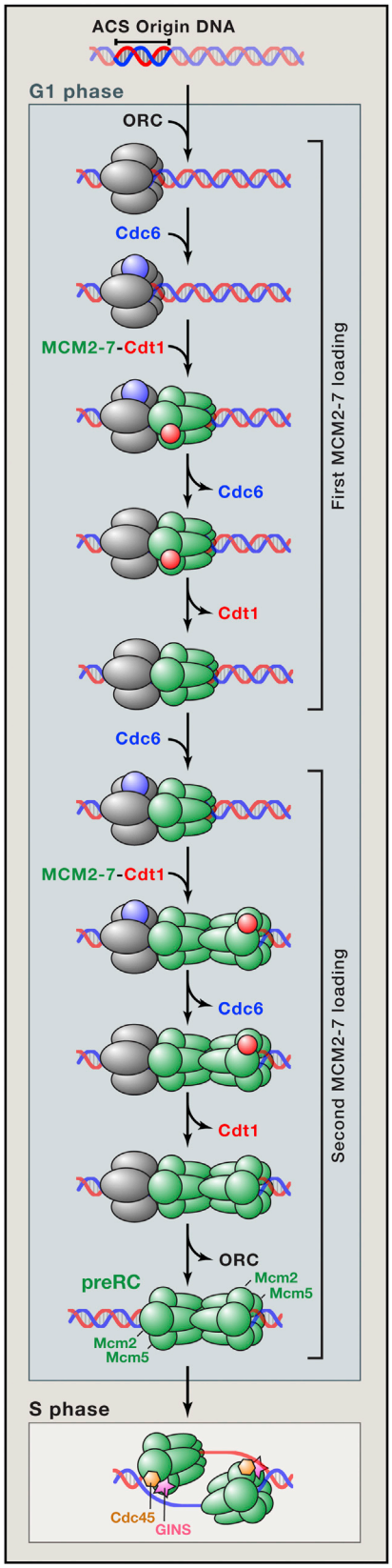

Figure 1. Loading Stages of the Eukaryotic Replicative Helicase

During G1, the eukaryotic replicative helicase is loaded as a preRC complex at the replication origin (ACS in yeast). Each preRC consists of two MCM2–7 hexamers encircling dsDNA. Establishing the necessary head-to-head association of the hexamers involves cooperation between the origin recognition complex (ORC) and licensing factors Cdc6 and Cdt1. At the beginning of the S phase, the preRC is activated and associates with Cdc45 and GINS to form two CMG complexes that travel on ssDNA away from the origin. Ticau et al. dissected the stages of preRC loading.

both MCM2–7 hexamers, ruling out any models that require two ORCs to form a preRC (Figure 1). Importantly, ORC interacts with the C-terminal domains of MCM2–7 during loading (Sun et al., 2013), yet in the final pre-RC, the two MCM2–7 rings interact via their N termini. Therefore, ORC cannot recruit the first and second MCM2–7 hexamers by the same mechanism, in agreement with the longer departure time of Cdc6/Cdt1 after the second MCM2–7 arrival. As MCM2–7 complexes do not interact in solution, the first loaded MCM2–7 complex must adopt a conformation that is competent for interaction with the second MCM2–7 through their N termini. Notably, Cdc6 binding (presumably to ORC) precedes loading of the second MCM2–7 complex, suggesting that it is required for this event. It will be interesting to understand how Cdc6 performs this function, given its distal location relative to the second MCM2–7 loading event (Figure 1). Ticau et al. also revealed that ORC dissociates from the origin soon after loading of the second MCM2–7, disfavoring mechanisms in which one DNA-bound ORC assembles several pre-RCs.

The work by Ticau et al. is complemented by a study in *Molecular Cell* (Duzdevich et al., 2015), which explores other facets of pre-RC assembly, as well as downstream events of origin activation. This work shows that Cdc6 reduces the affinity of soluble ORC for DNA, effectively insuring that ORC normally binds DNA before Cdc6. Inducing pre-RC activation with yeast S-phase extract reveals that activation of the two MCM2–7 complexes in the pre-RC is temporally and thus probably mechanistically coupled. The study also confirms models featuring actively replicating forks containing a single copy of the MCM2–7 ATPase. Finally, experiments by Duzdevich and colleagues support the findings of Ticau et al. that one and the same ORC complex directs the loading of both MCM2–7 hexamers comprising the preRC.

The work by Ticau et al. illustrates the power of simultaneously labeling pairs of proteins and watching them assemble into a multi-protein complex. The work provides the most definitive roadmap to date of the complex process underlying pre-RC assembly and identifies which intermediates should be pursued in structural studies. Now that origin unwinding and replisome assembly have also been reconstituted with purified components (Yeeles et al., 2015), we can expect the full power of single-molecule analysis to be applied to understanding the dynamics of these processes.

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