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Mechanisms and regulation of DNA replication initiation in eukaryotes

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Abstract

Cellular DNA replication is initiated through the action of multiprotein complexes that recognize replication start sites in the chromosome (termed origins) and facilitate duplex DNA melting within these regions. In a given cell cycle, initiation occurs only once per origin and each round of replication is tightly coupled to cell division. To avoid aberrant origin firing and re-replication, eukaryotes tightly regulate two events in the initiation process: loading of the replicative helicase, MCM2-7, onto chromatin by the Origin Recognition Complex (ORC), and subsequent activation of the helicase by incorporation into a complex known as the CMG. Recent work has begun to reveal the details of an orchestrated and sequential exchange of initiation factors on DNA that give rise to a replication-competent complex, the replisome. Here we review the molecular mechanisms that underpin eukaryotic DNA replication initiation – from selecting replication start sites to replicative helicase loading and activation – and describe how these events are often distinctly regulated across different eukaryotic model organisms.

Keywords

DNA replication; ORC; Cdc6; initiator; Cdt1; MCM2-7; CMG; helicase

Introduction

The success of biological organisms depends on the faithful transmission of genetic information from parent to progeny. All life-forms store their genetic content in the form of nucleic acids, and the replication and dissemination of this information forms the fundamental basis of inheritance. In cells, the process of replication involves two primary tasks: (1) the separation of duplex DNA into two single-stranded templates and (2) semi-conservative replication of each strand. These events are coupled with cell division to produce progeny with essentially identical copies of the parent's genetic information.

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Through all cellular lineages, a conserved division of labor has been applied to the process of DNA replication, such that separable tasks (e.g., start site selection, duplex unwinding, DNA synthesis) are allocated to different, albeit sometimes overlapping, factors. Although this basic framework is conserved throughout the bacterial, archaeal, and eukaryote kingdoms, there has been significant evolutionary diversification of the molecules that complete each task, to the point where it is now clear that aspects of the replicative machinery emerged twice, independently, during cellular evolution (Edgell and Doolittle, 1997, Leipe et al., 1999). Replication initiation in eukaryl species has become particularly elaborated by disparate forms of regulation to meet the specific demands of multicellularity, development and large genome size.

Replication is started by a trans-acting “initiator” factor that directs, in both space and time, loading of the replicative machinery onto particular genomic loci known as origins. In general, the number of origins scales with genome size, thereby ensuring that chromosome duplication can be carried out on a physiologically manageable timescale (Gilbert, 2004). Bacteria, as well as certain archaea, frequently initiate replication with a single chromosomal start site (Costa et al., 2013, Wu et al., 2014b). Conversely, some archaeal chromosomes possess multiple origins (Wu et al., 2014b), as do all eukaryotic genomes (the 12 Mbp *S. cerevisiae* genome contains around 400 origins and the 3 Gbp human genome ~30,000–50,000 origins (Leonard and Mechali, 2013)). The large number of origins and the need to coordinate initiation across these sites represents a fundamental challenge to DNA replication in eukaryotes; other factors, such as the use of multiple linear chromosomes, as opposed to a single circular chromosome, add additional replicative complexity. Replication is particularly problematic in multicellular eukaryotes, where the process of development can alter replication timing and frequency, and in the context of cellular differentiation, which changes the chromatin landscape and requires the coordinated transmission of epigenetic marks. Despite these and other challenges, more than 15 trillion repetitive rounds of DNA replication and cell division are successfully executed on the developmental path of a fertilized human embryo to the adult human body (Bianconi et al., 2013). Although our understanding of the replicative process is far from complete, we are beginning to understand how eukaryotes utilize a variety of sequential and redundant regulatory mechanisms to achieve this biological feat.

The eukaryotic replisome is built from the regulated and stepwise assembly of multiple intermediary replication factor complexes. In *S. cerevisiae*, 42 individual proteins are sufficient to fully reconstitute DNA replication *in vitro*, and since many of these proteins function within large macromolecular assemblies, fewer than fifteen pre-assembled replication factors are required (Yeeles et al., 2015). In short, replication initiation entails four steps (Figure 1): (1) demarcation of start sites by the Origin Recognition Complex (ORC) and the Cdc6 helicase-loader; (2) reiterative loading of an inactive form of the replicative helicase, MCM2-7, by ORC•Cdc6 and the Cdt1 chaperone to form the pre-replication complex (pre-RC); (3) helicase activation by the formation of the Cdc45•MCM2-7•GINS (CMG) complex (the pre-initiation complex, pre-IC); and (4) generation of a bidirectional replication fork that depends on prior origin melting by the MCM2-7 complex and on the tethering of DNA polymerases and additional accessory factors to the replicative helicase. Here we review the molecular mechanisms underpinning

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eukaryotic replication initiation, from origin specification to helicase activation. This review, although focused on eukaryotic mechanisms, will, as needed, reference studies of the archaeal system to fill critical gaps in knowledge. Given the extensive number of publications in the field, we apologize to those colleagues whose work is not referenced due to space limitations.

Origins of replication

Origins of replication are chromosomal regions that recruit replication initiators for facilitating assembly of the replication machinery (Francois Jacob, 1963). The defining features of eukaryotic origins are complicated and continuously evolving; for more thorough coverage we refer the reader to a number of excellent reviews on the topic (MacAlpine and Bell, 2005, Leonard and Mechali, 2013, Creager et al., 2015). Here we briefly discuss the most salient features of origins in *S. cerevisiae*, *S. pombe*, and metazoans, with a particular focus on details pertinent to later topics of discussion.

S. cerevisiae origins of replication

S. cerevisiae origins of replication were initially identified as chromosomal regions capable of conferring replicative properties to exogenous plasmids (Stinchcomb et al., 1979). These AT-rich, autonomous replication sequences (ARSs) function as replication start sites (Huberman et al., 1987, Brewer and Fangman, 1987, Huberman et al., 1988) and serve to recruit the eukaryotic initiator, ORC (Bell and Stillman, 1992). The ARS contains a number of essential elements, the most important of which is the eleven-basepair ‘A element’, which constitutes the ARS consensus sequence (ACS) and represents the primary site of initiator binding (Marahrens and Stillman, 1992, Rao et al., 1994, Theis and Newlon, 1994, Rao and Stillman, 1995). Notably, amongst the different model eukaryotic systems used for studying replication, only *S. cerevisiae* appears to utilize a specific consensus sequence (Figure 2).

Although there are over 12,000 ACS-like sequences in the yeast genome, only about 400 facilitate replication initiation (Wyrick et al., 2001, Nieduszynski et al., 2006, Xu et al., 2006). This low usage of possible *S. cerevisiae* origins (< 5%) derives in part from an additional level of origin specification that is imposed by the local chromatin structure. *S. cerevisiae* origins, like those of other eukaryotes, are maintained as nucleosome-free regions (NFRs) (Eaton et al., 2010, Berbenetz et al., 2010). However, surrounding an ARS, ORC-dependent nucleosome phasing directly affects the efficiency of origin usage (Thoma et al., 1984, Simpson, 1990, Lipford and Bell, 2001, Berbenetz et al., 2010), and the eviction of ORC results in nucleosome encroachment into ARS regions (Thoma et al., 1984, Eaton et al., 2010). Mechanistically, NFRs allow ORC access to DNA, while phased nucleosomes provide additional favorable sites for binding ORC (Muller et al., 2010, Hizume et al., 2013). Thus, *S. cerevisiae* origins are defined by ORC binding to both an ACS and specific chromatin features (Leonard and Mechali, 2013, Hoggard et al., 2013).

S. pombe origins of replication

Genome-wide studies demonstrate that the *S. pombe* genome contains around 400 origin sequences that are generally nucleosome-free (Givens et al., 2012, Xu et al., 2012), AT-rich,

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and around 1 kilobase long (Segurado et al., 2003, Dai et al., 2005, Heichinger et al., 2006). These findings are consistent with biochemical studies showing that origin usage in fission yeast depends on clustered stretches of adenine and thymine (Clyne and Kelly, 1995, Kim et al., 2001, Okuno et al., 1999, Dai et al., 2005). Origin selection in *S. pombe* is facilitated by a species-specific insertion in the Orc4 subunit of ORC that encodes a DNA-binding element that specifically recognizes the minor groove of AT-rich sequences (Figure 2) (Chuang and Kelly, 1999, Moon et al., 1999).

Metazoan origins of replication

Unlike budding yeast ORC, which shows a degree of sequence-specificity, metazoan ORC binds DNA promiscuously (Vashee et al., 2003, Remus et al., 2004). This behavior is consistent with the observation that metazoan replication initiates from diverse sequences (Mechali and Kearsey, 1984, Heinzel et al., 1991, Hyrien and Mechali, 1993). Despite origin sequence variability, the genome-wide analysis of replication start sites has revealed some common patterns in metazoan origins. As with budding and fission yeast, metazoan ORC binds to NFRs in the genome (MacAlpine et al., 2010, Karnani et al., 2010, Eaton et al., 2011), which in turn favorably contributes to assembly of the replication machinery (Lubelsky et al., 2011). Interestingly, G-rich sequences and CpG islands are highly enriched in metazoan origins (Delgado et al., 1998, Cadoret et al., 2008, Prioleau, 2009, Sequeira-Mendes et al., 2009) and have been proposed to serve two purposes: NFR maintenance (Huppert and Balasubramanian, 2007, Wong and Huppert, 2009, Fenouil et al., 2012) and the favoring of G-quadraplex formation (Cayrou et al., 2011, Cayrou et al., 2012, Valton et al., 2014, Cayrou et al., 2015). Preliminary analyses suggest that ORC may preferentially associate with G-rich elements (Zellner et al., 2007, Hoshina et al., 2013), indicating that a conserved structural feature in DNA, rather than a specific consensus sequence, may aid ORC binding in metazoans. As in *S. cerevisiae*, metazoan origin selection by ORC is further fine-tuned by direct interactions with nucleosomes and chromatin-associated factors. For example, the N-terminal Bromo-Adjacent Homology (BAH) domain in Orc1 directly interacts with histones to direct origin usage (Figure 2) (Noguchi et al., 2006, Muller et al., 2010, Kuo et al., 2012).

Although ORC can be targeted to specific genomic loci, origins cannot be strictly defined by the position of ORC binding on chromatin. Indeed, once loaded onto DNA by ORC, the MCM2-7 helicase (which eventually nucleates replisome assembly following duplex melting) appears to lack positional restraints and is free to either diffuse away from ORC (Remus et al., 2009, Evrin et al., 2009) or be forcibly displaced by other chromatin-localized cellular processes (such as the transcriptional machinery) (Ritzi et al., 1998, Edwards et al., 2002, Powell et al., 2015, Gros et al., 2015). Thus, origins in eukaryotes must be defined flexibly, as the site of initiator binding does not always reflect the site of replication initiation.

Overall, both yeast and metazoa contain conserved sequence elements at origins that are known or proposed to guide ORC binding. In yeast, these elements are encoded within the DNA primary sequence and in metazoa potentially by a propensity to form distinctive secondary structures. Chromatin context plays an additional critical, but poorly understood

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role in origin usage in all eukaryotes. Understanding how these *cis*- and *trans*-acting origin elements interface is an important and active area of future research.

The Origin Recognition Complex (ORC)

Eukaryotic origins direct the recruitment of the Origin Recognition Complex (ORC), a conserved heterohexameric protein assembly identified for its ability to specifically recognize the double-stranded form of the yeast ACS (Bell and Stillman, 1992). Upon recruitment to chromosomal replication start sites, ORC binds an additional factor, Cdc6, as a necessary prerequisite to helicase loading. Despite its centrality to ORC function, the mechanism of DNA binding by the initiator has long remained ambiguous. However, structural studies from archaea and eukaryotes have revealed a conserved mechanism for the association of ORC with DNA that informs not only our understanding of how the ORC•Cdc6 initiator stably binds replication start sites, but also how ORC mediates downstream helicase loading events (Dueber et al., 2007, Gaudier et al., 2007, Sun et al., 2013, Bleichert et al., 2015).

The Origin Recognition Complex and Cdc6

ORC was first identified by fractionation of ARS-binding proteins in budding yeast (Bell and Stillman, 1992). Although many other ARS-binding factors had been identified previously (Jazwinski and Edelman, 1982, Sweder et al., 1988, Diffley and Stillman, 1988, Buchman et al., 1988, Shore et al., 1987), ORC proved uniquely able to bind the ACS (Diffley and Cocker, 1992, Marahrens and Stillman, 1992, Li and Herskowitz, 1993), and temperature sensitive mutants exhibited cell-cycle arrest at a stage consistent with a role in the early aspects of DNA replication (Bell et al., 1993, Foss et al., 1993, Micklem et al., 1993). Following the discovery of ORC in budding yeast, the broad eukaryotic conservation of ORC was demonstrated with the identification of orthologs in *S. pombe* (Muzi-Falconi and Kelly, 1995, Grallert and Nurse, 1996), *D. melanogaster* (Gossen et al., 1995, Landis et al., 1997), *X. laevis* (Carpenter et al., 1996, Romanowski et al., 1996b), and humans (Gavin et al., 1995). The mechanism of ORC function has now been investigated across multiple model organisms, revealing that the core components, subunit organization, and function of ORC are broadly conserved (although not universally, particularly in protozoa (El-Sayed et al., 2005)). Despite this conservation, there do exist certain species-specific alterations that appear to have generated notable functional differences.

S. cerevisiae ORC is a roughly 400 kDa assembly composed of six proteins (Orc1-6) named in descending order of molecular weight (Bell et al., 1993). Orc1-5 exhibit relatively good conservation across species and are members of the ATPases associated with diverse cellular activities (AAA+) superfamily of proteins; Orc1-5 also contain a C-terminal winged-helix (WH) domain (Loo et al., 1995, Bell et al., 1995, Muzi-Falconi and Kelly, 1995, Liu et al., 2000). Overall, the Orc1-5 AAA+ and WH domains, which account for a bit over half the total mass of ORC, display an average of ~45–50% similarity and 25–30% identity between yeast and human orthologs (Tugal et al., 1998, Speck et al., 2005). A majority of AAA+ family members bind and hydrolyze ATP through the formation of a composite, bipartite active site that is generated between neighboring AAA+ protomers upon subunit

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oligomerization (Guenther et al., 1997, Lenzen et al., 1998, Putnam et al., 2001). AAA+ proteins, like the broad P-loop family of NTPases to which they belong (Iyer et al., 2004), contain so-called 'Walker A' and 'Walker B' signature sequence motifs that contribute to nucleotide binding and hydrolysis, respectively. A third motif, generally critical to AAA+ ATPase function, is the arginine finger, which is presented in *trans* to the neighboring subunit to reconstitute an active ATPase site (Neuwald et al., 1999, Zhang et al., 2000). While many of the structural elements important for ATP binding are conserved in Orc1, Orc4, and Orc5, only Orc1 has been found to possess ATPase activity (Klemm et al., 1997, Makise et al., 2003, Ranjan and Gossen, 2006, Siddiqui and Stillman, 2007, Bleichert et al., 2015); Orc2 and Orc3 retain degenerate AAA+ scaffolds that lack functional active site motifs altogether (Speck et al., 2005, Clarey et al., 2006, Bleichert et al., 2015). Intriguingly, although many AAA+ family members function as toroidal hexameric assemblies (reviewed in (Hanson and Whiteheart, 2005)), ORC retains only five proteins with AAA+ domains. Orc6 lacks a AAA+ domain (Chesnokov et al., 2003, Balasov et al., 2007, Bleichert et al., 2013) and its primary sequence is only weakly conserved between yeast and human, making it the least conserved ORC subunit (Dhar and Dutta, 2000). Nonetheless, certain elements of Orc6 are conserved across species, including an N-terminal TFIIB-like domain and a short conserved region at the extreme C-terminus of the protein (Liu et al., 2011, Bleichert et al., 2013).

Cdc6 forms a complex with ORC at origins and is required for initiator function. Cdc6 was first identified in *S. cerevisiae* mutant screens (Hartwell, 1976) and was later found to have a role in replication initiation (Palmer et al., 1990), with functional requirements prior to S-phase (Kelly et al., 1993, Hogan and Koshland, 1992, Zwerschke et al., 1994). A genetic interaction between ORC and Cdc6 suggested a coordinated activity for these factors (Liang et al., 1995), with supporting data demonstrating an ORC-dependent recruitment of Cdc6 to origins that depends on a direct interaction between the proteins (Santocanale and Diffley, 1996, Leatherwood et al., 1996, Grallert and Nurse, 1996, Coleman et al., 1996, Cocker et al., 1996, Liang et al., 1995, Kong et al., 2003). Importantly, analysis of the Cdc6 primary sequence reveals conserved nucleotide binding and hydrolysis motifs (Lisziewicz et al., 1988, Zhou et al., 1989, Zwerschke et al., 1994), as well as close homology to ORC's AAA + subunits, particularly Orc1 (Bell et al., 1995, Quintana et al., 1997). Cdc6 associates with chromatin-bound ORC in an ATP-dependent fashion (Perkins and Diffley, 1998, Kneissl et al., 2003, Evrin et al., 2013, Kang et al., 2014, Coster et al., 2014, Ticau et al., 2015), an interaction that activates Cdc6's ATPase activity and is consistent with the reconstitution of canonical AAA+ interactions (Randell et al., 2006, Speck and Stillman, 2007). Thus, Cdc6 recruitment provides a sixth AAA+ subunit to the initiator complex overall.

Structure of ORC and ORC•Cdc6

Orc/Cdc6 homologs have been identified in all eukaryotic and archaeal species analyzed (Aves et al., 2012). In many archaea, the initiation factors are genetically streamlined such that certain species possess only a single Orc/Cdc6 gene (Barry and Bell, 2006) and the relative simplicity of the archaeal system has been exploited to help understand the structure and function of eukaryotic Orc homologs. A conserved three-domain architecture is observed for Orc and Cdc6 homologs, with two domains at the N-terminus forming the

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central AAA+ module and a third at the C-terminus comprising a loosely-tethered WH domain (Figure 3A) (Liu et al., 2000, Singleton et al., 2004, Gaudier et al., 2007, Dueber et al., 2007, Bleichert et al., 2015). The structure of archaeal Orc in complex with DNA has shown that both the WH and AAA+ domains contact DNA (Gaudier et al., 2007, Dueber et al., 2007), and biochemical studies have demonstrated that contacts mediated by the AAA+ domain contribute to origin specificity (Dueber et al., 2011). Surprisingly, structures of Orc/DNA complexes have not revealed evidence for the formation of an ATPase-competent Orc dimer (Gaudier et al., 2007, Dueber et al., 2007), despite the existence of a conserved arginine finger in these proteins that would otherwise suggest that oligomerization might occur. The mechanism that promotes formation of a catalytically active ATPase in the archaeal system is unclear.

S. cerevisiae Cdc6 forms a stable complex with ORC in the presence of DNA and ATP (Wang et al., 1999, Mizushima et al., 2000, Seki and Diffley, 2000, Speck et al., 2005, Randell et al., 2006). By using a non-hydrolyzable ATP analog, a stable Cdc6•ORC complex can be trapped in the absence of DNA (Speck et al., 2005). Low-resolution 3D electron microscopy reconstructions of eukaryotic ORC have revealed an elongated, crescent-shaped particle that, in the presence of Cdc6 and ATPγS, transforms into a closed ring with a large central cavity (Speck et al., 2005, Clarey et al., 2006, Clarey et al., 2008, Sun et al., 2012). Structural investigation of *D. melanogaster* ORC has demonstrated a subunit order of Orc1→Orc4→Orc5→Orc3→Orc2 around the ORC ring (Bleichert et al., 2015), with a physical gap between the terminal Orc subunits that accommodates Cdc6 and is consistent with the direct interaction observed between Cdc6 and Orc1 (Sun et al., 2012, Sun et al., 2013, Wang et al., 1999). The C-terminus of Orc6 has been found to tether this subunit to a conserved domain insertion within Orc3 (Bleichert et al., 2013, Bleichert et al., 2015); *S. cerevisiae* Orc6 also binds Orc2 (Chen et al., 2008, Sun et al., 2012), possibly through a region that is specific to fungal homologs (Bleichert et al., 2013). The adjoining nature of Orc1 and Orc4 within the ternary complex is consistent with the known formation of a joint ATPase site between the two subunits (Klemm et al., 1997, Chesnokov et al., 2001, Bowers et al., 2004, Giordano-Coltart et al., 2005).

The ORC•Cdc6 assembly represents the functional initiator complex at origins. Interestingly, ORC itself exhibits differing levels of stability across species, suggesting that, in certain cases, ORC subcomplexes may be sequentially recruited. Indeed, unlike *D. melanogaster* and *S. cerevisiae* ORC, which form stable heterohexamers (Bell and Stillman, 1992, Chesnokov et al., 1999), human and *X. laevis* ORC appear to have alternative core subcomplexes. Human Orc1 and Orc6 loosely associate with an Orc2-5 core (Dhar et al., 2001, Vashee et al., 2001, Siddiqui and Stillman, 2007), whereas in *X. laevis,* the Orc6 subunit is labile (Gillespie et al., 2001). The weak interactions of certain Orc subunits likely play an important role in regulation. For example, vertebrate Orc1 is selectively released from chromatin after initiation (Rowles et al., 1999, Natale et al., 2000, Li et al., 2004), which helps to prevent re-initiation and provides a means to alter origin usage in a developmental- or differentiation-dependent fashion (Li and DePamphilis, 2002). The ability of either Orc1 or Orc6 to dissociate from ORC without disrupting the remaining core complex is consistent with the terminal position of Orc1 within the core ring and the peripheral binding site of Orc6; one exception to this trend occurs in *S. pombe*, whereby an

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ORC pentamer can be purified that lacks Orc4, an internal component of the ORC ring (Moon et al., 1999, Kong and DePamphilis, 2001). Whether S. pombe ORC retains additional stabilizing elements that can compensate for the absence of Orc4 in such instances is not known.

The recent atomic-resolution structure of *D. melanogaster* ORC (Bleichert et al., 2015) has helped clarify both our understanding of ORC organization and also mechanistic models for origin engagement and helicase loading. ORC adopts a two-tiered, notched ring architecture in which the WH domains of Orc1-5 sit atop a layer of AAA+ subunits (Figure 3B). Interestingly, the arrangement of AAA+ and WH domain contacts is domain swapped in ORC, such that the WH domain of Orc1 rests on the AAA+ region of Orc4, the WH domain of Orc4 sits on the AAA+ region of Orc5, and so forth. The open-ended, pentameric AAA+ core of ORC in principle provides an opportunity for the formation of four bipartite AAA+ interfaces. Within the *D. melanogaster* ORC structure, however, only one of the observed subunit interfaces evinces a typical AAA+ active-site configuration (Orc4/Orc5). Two others (Orc5/3 and Orc3/2) approximate the correct protomer alignment but fail to reconstitute a canonical active site; in the case of Orc3/2, the two subunits lack the amino acids necessary to bind ATP, as predicted. Of the remaining possible AAA+ interactions, Orc1 was unexpectedly found to be rotated more than 90 degrees out-of-plane from Orc4, rendering this catalytic center inoperative (Figure 3B). This finding was surprising, as Orc1 alone is capable of both binding and hydrolyzing ATP, using an arginine finger donated by Orc4 (Klemm et al., 1997, Chesnokov et al., 2001). Given that the *D. melanogaster* Orc1 conformation seen crystallographically is also seen in 3D electron microscopy reconstructions of ORC (Bleichert et al., 2013, Bleichert et al., 2015), this observation suggests that metazoan ORC can transition between at least two conformations, an autoinhibited and active conformation. In the future, it will be important to understand how the cell regulates the equilibria of ORC between these states, as well as whether this conformational transition is preserved in other ORC homologs.

A two-state model for ORC origin recognition

Despite the fundamental role of ORC in origin selection and recognition, the mechanism by which ORC associates with DNA has remained highly enigmatic. The presence of numerous interspecies peculiarities, such as divergent origin features, species-specific DNA-binding elements, and the effect of chromatin-bound *trans*-acting factors, have all challenged our understanding of how ORC is recruited to and stably binds origins. However, an analysis of ORC behavior across species suggests that in all cases, the origin-binding properties of ORC can be interpreted within a model containing at least two states: a transient ORC recruitment event that is mediated through diverse and sometimes species-specific interactions, and a second, mechanistically conserved step that positions ORC for productive origin engagement and that leads to stable Cdc6 association. We will first discuss the conserved mechanism by which ORC stably associates with origins and then detail the interactions that facilitate ORC recruitment.

Structural studies of origin-bound archaeal Orc have revealed a coordinated role for both the AAA+ and WH domains in DNA binding (Gaudier et al., 2007, Dueber et al., 2007). The

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WH domains show extensive contacts with origin DNA by a canonical helix-turn-helix (HTH) and β-hairpin wing interface, and a near compete loss of DNA binding by archaeal Orc is observed upon mutation or deletion of this region (Singleton et al., 2004, Dueber et al., 2011). The WH-DNA interaction positions the AAA+ domain in an orientation where a characteristic α-helical insertion within the initiator/helicase loader subgroup of AAA+ proteins, the initiator specific motif (ISM), contacts DNA. Given the different subunit compositions and oligomeric states observed between archaeal (monomer) and eukaryotic initiators (heterohexamer) it was initially unclear to what extent the mechanism of archaeal origin binding would be conserved. However, superposing the structure of DNA-bound archaeal Orc onto the *D. melanogaster* ORC crystal structure reveals nearly perfect co-axial positioning of the DNA within the ORC central channel (Bleichert et al., 2015). Notably, the eukaryotic ISMs and the wings of the WH domains each form a contiguous DNA-binding surface that lines the ORC central channel, and are thus positioned to engage DNA in a manner similar to that of archaeal Orc (although not identically, as the HTH motif of ORC's WH domains are buried between inter-subunit contacts in the complex (Bleichert et al., 2015)). This modeling also accounts for an unresolved region of density in an early 3D electron microscopy reconstruction of an ORC•Cdc6•DNA complex (Sun et al., 2012). Overall, these data indicate that archaeal and eukaryotic ORC engage DNA by a generally conserved mechanism.

The insights gleaned from the available structural studies suggest a conserved mechanism for stable origin association by eukaryotic ORC. In this model, duplex DNA is loaded laterally into the ORC central channel through the discontinuity between Orc1 and Orc2 (Speck et al., 2005). This gap is then blocked off by the subsequent recruitment of Cdc6, generating a stable, closed-ring conformation that encircles origin DNA (Bleichert et al., 2015). Domain swapping between the WH domain of Orc2 and the AAA+ region of Cdc6 (and between the WH domain of Cdc6 and the AAA+ element of Orc1) are predicted to form, stabilizing the complex (Bleichert et al., 2015). The ability of the ORC•Cdc6 complex to encircle DNA would be predicted to underpin ORC's observed persistence at origins (Speck et al., 2005, Duzdevich et al., 2015) and is consistent with the observation that both yeast and metazoan Cdc6 can stabilize ORC on DNA (Harvey and Newport, 2003, Houchens et al., 2008).

Interestingly, an additional DNA-binding element has been identified between the N-terminal BAH and AAA+ domain of *S. cerevisiae* Orc1 that is essential for ARS binding (Kawakami et al., 2015). While the related residues are largely conserved in metazoan Orc1, they are positioned outside of the ORC central pore and thus it is unclear how this interaction is coordinated with the encirclement mechanism described here, or whether it contributes only to the initial recruitment of ORC to DNA.

Diverse mechanisms for origin recruitment of ORC

Although the encirclement model accounts for stable origin binding, eukaryotic ORC has been observed to interact with DNA in a mode that exhibits fast kinetics (Harvey and Newport, 2003, Remus et al., 2004, Houchens et al., 2008, Duzdevich et al., 2015) and, in *S. cerevisiae*, allows for a one-dimensional linear search for *bona fide* origin sites (Duzdevich

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et al., 2015). This initial recruitment of ORC to DNA likely functions as an important intermediate on the path towards stable origin binding. Interestingly, substantial mechanistic plasticity appears to have been introduced to the recruitment step, co-evolving in certain cases with species-specific features.

In *S. pombe* ORC, the preference for AT-rich origins correlates with a unique domain insertion in Orc4 comprising nine AT-hook motifs (Chuang and Kelly, 1999, Moon et al., 1999), a DNA-binding element that facilitates interactions with the minor groove of AT-rich DNA sequences (Aravind and Landsman, 1998). Notably, the AT-hook motif is absent in *S. cerevisiae* and metazoan Orc4. Unlike *S. cerevisiae* ORC, which utilizes all AAA+ domain-containing Orc subunits for DNA binding (Lee and Bell, 1997), *S. pombe* Orc4 is necessary and sufficient for initial origin engagement (Chuang and Kelly, 1999, Moon et al., 1999, Kong and DePamphilis, 2001, Gaczynska et al., 2004), and the AT-hook motifs of Orc4 are additionally required for viability (Chuang et al., 2002). Interestingly, *S. pombe* ORC shows a biphasic mechanism of DNA binding, with an initial, salt-sensitive DNA binding event that precedes the formation of a salt-stable form, a state that in turn can be further stabilized by the addition of Cdc6 (Houchens et al., 2008). These findings suggest that *S. pombe* ORC is recruited to chromosomes by the Orc4 AT-hook motif, which then leads to stable DNA association through a mechanism that likely involves the encirclement of duplex DNA.

Analogous to *S. pombe* Orc4, metazoan Orc6 has been shown to have a distinct DNA binding activity. Despite the absence of an ATPase or WH domain, exclusion of Orc6 from *D. melanogaster* ORC results in the loss of ATP-dependent DNA binding, the same effect observed for Orc1 Walker A (ATP binding) and Walker B (ATP hydrolysis) mutants (Chesnokov et al., 2001). Analysis of the *D. melanogaster* and human Orc6 N-terminus reveals structural homology with the DNA-binding domain of transcription factor TFIIB (Chesnokov et al., 2003, Balasov et al., 2007), and mutation of the Orc6 TFIIB domain abolishes DNA binding (Liu et al., 2011). Although the Orc6 TFIIB domain was initially considered unique to metazoans, subsequent sequence analysis indicates that the domain is conserved in fungal Orc6, but lacks specific DNA-binding elements (Bleichert et al., 2013), a finding consistent with *S. cerevisiae* Orc6 being dispensable for origin recognition *in vitro* (Lee and Bell, 1997, Chen et al., 2007). Given the available data, it seems likely that metazoan ORC utilizes Orc6 to loosely tether the complex to DNA in a functionally analogous manner as *S. pombe* Orc4, and that this action aids with the initial ORC recruitment event that precedes stable origin association. This model begs the question, however, of how ORC is recruited to chromosomes in species where Orc6 is a labile subunit (Dhar et al., 2001, Vashee et al., 2001, Gillespie et al., 2001). One possible answer is that chromatin-bound Orc6 may function as a recruitment platform for the core Orc1-5 subunits.

In addition to “hard-wired” DNA binding domains, eukaryotic ORC can also bind a plethora of chromatin-associated factors that provide additional means for recruiting and regulating ORC’s association with origins (Chakraborty et al., 2011). Early studies recognized that ORC played dual roles in replication and transcriptional regulation (Bell et al., 1993, Loo et al., 1995, Palacios DeBeer et al., 2003, Leatherwood and Vas, 2003), and a number of transcriptional regulators have been shown to bind ORC, including HP1, E2F, HMGA1a and Sir1 (Triolo and Sternglanz, 1996, Pak et al., 1997, Fox et al., 1997, Gardner et al., 1999,

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Royzman et al., 1999, Bosco et al., 2001, Prasanth et al., 2004, Thomae et al., 2008, Prasanth et al., 2010). Although chromatin accessibility and transcriptional programs are clearly regulated in an ORC-dependent manner (Bell et al., 1993, Foss et al., 1993, Micklem et al., 1993, Loo et al., 1995, Fox et al., 1995, Pak et al., 1997, Huang et al., 1998, Bose et al., 2004, Chesnokov, 2007, Shor et al., 2009), whether chromatin bound transcriptional regulators also direct ORC origin usage is less clear. Certain cases have been investigated in some detail; for example, ORC can be targeted to chromatin-bound HMGA1a, where it directs assembly of replication complexes (Thomae et al., 2008), while genome-wide analysis of metazoan origins reveals ORC association with HP1 sites (Cayrou et al., 2011). Thus, transcriptional regulators represent an additional means of tethering ORC to chromosomes and likely affect ORC function in replication.

Eukaryotic ORC is also directly recruited to histones by the N-terminal Bromo-Adjacent Homology (BAH) domain in Orc1 (Zhang et al., 2002, Noguchi et al., 2006, Muller et al., 2010). This element selectively binds H4K20me2, an interaction that directs replication licensing (Kuo et al., 2012). Other histone modifications also have been found to direct origin usage (such as H4K20me1, H3K4me2/3, and H4 acetylation), but how these effect ORC positioning or downstream licensing reactions remains unclear (Rice et al., 2002, Vogelauer et al., 2002, Aggarwal and Calvi, 2004, Knott et al., 2009, Tardat et al., 2010, Miotto and Struhl, 2010, Costas et al., 2011, Eaton et al., 2011, Liu et al., 2012b). A factor known as ‘ORCA’ (for Origin Recognition Complex Associated) additionally has been reported to directly recruit human ORC to chromosomes, as well as to bind to other initiation factors that promote replication licensing and S-phase progression (Shen et al., 2010, Shen et al., 2012). Conversely, the assembly of the replication machinery at telomeres has been reported to be controlled by an interaction between ORC and the TRF2 subunit of the shelterin complex (Atanasiu et al., 2006, Tatsumi et al., 2008, Deng et al., 2009, Higa et al., 2016).

Collectively, multiple lines of data demonstrate that localizing ORC to DNA prior to productive origin engagement (i.e., Cdc6-dependent DNA encircling) is a critical step in replication initiation, and that many diverse recruitment mechanisms are sufficient to demarcate a replication start site. Consistent with this idea is the demonstration that a fusion between ORC and the Gal4 DNA binding domain can result in the initiation of DNA replication on a plasmid containing a tandem array of Gal4 binding sites (Takeda et al., 2005). In light of the many mechanisms that can facilitate ORC recruitment, an important future direction will be to understand how different recruitment pathways cooperate or antagonize each other in specifying sites of replisome assembly.

The Minichromosome Maintenance (MCM) Complex

Following the active designation of an origin by ORC, the complex next facilitates initiation at these sites by loading the heterohexameric MCM2-7 helicase onto DNA. Studies into MCM2-7 function have revealed a surprisingly complex enzyme that appears capable of harnessing ATP to promote both the melting of double-stranded DNA and translocation of single-stranded DNA substrates. Although precise mechanisms have yet to be elaborated, a

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picture of how specific MCM2-7 elements couple ATP turnover to DNA remodeling and movement is beginning to emerge.

Identification of the MCM2-7 complex

MCM proteins were originally identified in genetic screens that aimed to uncover factors required for replication and cell cycle progression in yeast (Maine et al., 1984, Yan et al., 1991, Chen et al., 1992, Hennessy et al., 1990, Moir et al., 1982). Homologs were subsequently identified in *D. melanogaster* (Treisman et al., 1995), *X. laevis* (Madine et al., 1995) and in humans (Todorov et al., 1995), and like yeast MCMs, were shown to have an essential role in DNA replication. Investigation into MCM function converged with studies by Laskey and Blow, who identified a replication licensing factor (RLF) that restricted replication to once per cell cycle by nuclear exclusion until after nuclear envelope breakdown (Blow and Laskey, 1988, Blow, 1993). Indeed, MCM proteins, like the RLF, showed redistribution from the cytosol to nucleus upon completion of mitosis (Hennessy et al., 1990), and immunodepletion of MCM proteins inhibited replication (Kubota et al., 1995, Madine et al., 1995). Importantly, a purified RLF fraction was found to resolve into two separate factors, RLF-M and RLF-B, with RLF-M containing multiple proteins that cross-reacted with MCM antibodies (Chong et al., 1995).

Purification of RLF-M, as well as co-immunoprecipitation studies, revealed that MCM proteins reside within large multimeric assemblies with other MCM2-7 family members (Kubota et al., 1995, Madine et al., 1995, Chong et al., 1995, Burkhart et al., 1995, Kimura et al., 1995, Musahl et al., 1995, Romanowski et al., 1996a, Chong et al., 1996). The predominant assembly *in vivo* is an MCM2-7 heterohexamer, although low levels of subassemblies have been reported to exist that may represent intermediates (Su et al., 1996, Ishimi, 1997, Adachi et al., 1997, Thommes et al., 1997, Lee and Hurwitz, 2000, Prokhorova and Blow, 2000). As with Orc1-5, MCM proteins are predicated upon a conserved AAA+ ATPase element (Koonin, 1993, Iyer et al., 2004). However, unlike Orc1-5, which show differing levels of active site conservation, the six subunits in the MCM2-7 complex each contain the complete set of catalytic residues expected to be necessary for supporting ATP hydrolysis.

MCM architecture

The basic architecture of an MCM protein is conserved across archaea and eukaryotes. MCMs contain a central AAA+ ATPase fold flanked by conserved N- and C-terminal domains (termed ‘NTD’ and ‘CTD,’ respectively). Structural analyses of MCMs have revealed three conserved subdomains within the NTD, allowing subdivision of this element into the NTD-A, -B, and -C regions (Figure 4A) (Fletcher et al., 2003, Liu et al., 2008, Brewster et al., 2008, Bae et al., 2009, Fu et al., 2014, Li et al., 2015). NTD-C, the most highly conserved NTD subdomain, possesses an oligonucleotide/oligosaccharide-binding (OB) fold. Within an MCM hexamer, interactions between adjacent NTD-C elements serve as the primary interface for subunit assembly, with this interaction alone sufficient to facilitate hexamerization of the NTD (Fletcher et al., 2003, Kasiviswanathan et al., 2004). Although NTD-A is the least conserved N-terminal subdomain, it forms a helical bundle with similarity to helix-turn-helix type DNA binding proteins (Costa et al., 2008). The NTD-

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B element comprises a Zn-finger motif that, with the exception of MCM3, is universally conserved in MCM2-7 (Li et al., 2015), and that facilitates higher order organization of single NTD hexamers into a conserved, head-to-head double hexamer (Figure 4B) (Kelman et al., 1999, Shechter et al., 2000, Chong et al., 2000, Fletcher et al., 2005, Remus et al., 2009, Evrin et al., 2009, Costa et al., 2014, Li et al., 2015).

The AAA+ domain is the most highly conserved region across MCM homologs. The MCM AAA+ fold contains three unique insertions relative to prototypical AAA+ proteins, and falls within the pre-sensor II (PSII) insert clade of the ATPase superfamily (Iyer et al., 2004, Erzberger and Berger, 2006). One such insertion is distinctive in that it remodels a portion of the AAA+ cassette to orient a catalytic amino acid known as the sensor II motif in *trans* with the active site of an adjacent protomer (Moreau et al., 2007, Bae et al., 2009), rather than in *cis*, which serves as the more usual arrangement in AAA+ oligomers. By comparison, the other two insertions are found in many other AAA+ subgroups and consist of two β–hairpin insertions termed the pre-sensor I (PSI)-insert and the helix 2 (H2)-insert. Within the context of the hexamer, these two β-hairpins are positioned within the central channel and are integral to helicase mechanism (Figure 5). With regard to the MCM catalytic centers, a recent high-resolution cryo-electron microscopy structure of a full-length *S. cerevisiae* MCM2-7 double hexamer reveals significant structural variability between the six radially-arranged ATPase sites, with a catalytically competent conformation but unequal nucleotide occupancy observed for four of the sites (Li et al., 2015). This variability is consistent with the unequal ATPase activity observed for the six MCM2-7 active sites (Bochman et al., 2008) and with the non-equivalent function of these sites across the different stages of helicase recruitment, loading, and activation (Ilves et al., 2010, Coster et al., 2014, Kang et al., 2014).

Insofar as the MCM CTD, solution structures have shown this region to adopt a canonical winged-helix (WH) domain (Figure 4A) (Wei et al., 2010, Liu et al., 2012a, Wiedemann et al., 2015), which based on the weak or absent CTD density in structures of many full-length MCM proteins, appears flexibly tethered to the AAA+ core. Although the position of this domain with respect to the hexamer was at first unclear, recent structural and biochemical work demonstrates that the WH domain sits distal to the NTD (Li et al., 2015, Wiedemann et al., 2015, Yuan et al., 2016). The *S. solfataricus* MCM WH domain can bind single-stranded DNA weakly (Pucci et al., 2007); however, what role this activity plays *in vivo*, and whether the WH domains of the eukaryotic MCMs can also bind DNA, are open questions. The majority of data reported thus far suggests that, at least in archaea, the MCM WH domain functions to allosterically modulate the ATP hydrolysis rate of the AAA+ domain (Jenkinson and Chong, 2006, Barry et al., 2007, Wiedemann et al., 2015). In addition, this region can serve as a protein/protein interaction site, with the WH domains of eukaryotic MCM3 and MCM6 having been shown to engage Cdc6 and Cdt1, respectively (You and Masai, 2008, Liu et al., 2012a, Frigola et al., 2013). Interestingly, recent structural analyses of the activated yeast helicase (the CMG complex) have revealed that the Mcm5 and Mcm6 WH domains partially occlude the Mcm2-7 central channel, thus positioning them to perhaps function in the translocation process (Yuan et al., 2016). Despite this observation, the precise role of the MCM WH domains remains to be determined.

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Although the ATPase activity of MCM proteins localizes exclusively to the core AAA+ domain, hexamer formation is facilitated by both the AAA+ and NTD elements in the order MCM5→MCM3→MCM7→MCM4→MCM6→MCM2 (Schwacha and Bell, 2001, Crevel et al., 2001, Davey et al., 2003, Costa et al., 2011). A loop within each NTD-A OB-fold, termed the allosteric communication loop (ACL), contacts the AAA+ domain of its adjacent protomer, further stabilizing the hexamer (Miller et al., 2014, Li et al., 2015). Collectively, these interactions facilitate the formation of a particle consisting of two stacked rings (a AAA+ tier and an NTD tier). In single and double MCM2-7 hexamers, the central channel is sufficiently large enough to accommodate double-stranded DNA (Figure 4B) (Remus et al., 2009, Evrin et al., 2009, Costa et al., 2011, Lyubimov et al., 2012, Costa et al., 2014, Li et al., 2015); later, in an unknown series of events, the helicase undergoes an isomerization reaction that appears coupled to the extrusion of one of the two strands through a natural discontinuity between the MCM2/5 subunits (Bochman and Schwacha, 2008, Costa et al., 2011, Bruck and Kaplan, 2015b), permitting translocation along single-stranded DNA (Ishimi, 1997, Kelman et al., 1999, Shechter et al., 2000, Ilves et al., 2010, Fu et al., 2011).

MCM mechanism in origin melting and processive unwinding

The MCM2-7 complex matures through a variety of intermediates before being incorporated into the active helicase present at replication forks. Within the MCM2-7 lifecycle, two stable complexes are observed: an origin-bound double hexamer (Evrin et al., 2009, Remus et al., 2009) and a replication fork-associated single hexamer (Fu et al., 2011, Duzdevich et al., 2015). Interestingly, the double hexamer serves not only as a platform for recruiting helicase-activating factors but also is likely responsible for the initial origin melting event (Sun et al., 2014, Li et al., 2015, Bochman and Schwacha, 2015). Thus, the MCM complex must utilize the ATP-hydrolysis-driven repositioning of its DNA-binding elements to carry out two very distinct tasks, origin opening as a double hexamer and processive DNA unwinding as a single hexamer. Although the MCM2-7 DNA-binding elements are relatively well defined, it is currently unclear whether the two functionalities of the helicase require an overlapping or mutually exclusive set of protein interactions with DNA.

The MCM2-7 hexamer has multiple tiers of DNA-binding elements that are positioned to engage DNA passing through the central pore (Figure 5). The NTD-B Zn-fingers are situated at one end of the hexamer, forming a skirt of DNA-binding elements that run parallel to and extend the central channel. This domain is required for double hexamer formation, and mutation or deletion of the archaeal NTD-B reduces DNA binding and results in loss of helicase activity (Poplawski et al., 2001, Pucci et al., 2004, Kasiviswanathan et al., 2004). The Zn-finger motifs of eukaryotic MCM2-7 also facilitate double-hexamer formation (Li et al., 2015) and represent a putative DNA-binding region, but the role of this domain in helicase activity has not been investigated directly. The Zn-finger motif of *S. cerevisiae* Mcm2 and Mcm5 are required for cellular proliferation, suggesting an essential and conserved function for this region (Yan et al., 1991, Dalton and Hopwood, 1997).

As one moves through the central MCM2-7 pore, from the NTD to the CTD, the next DNA-binding elements encountered are the β–hairpin insertions within the OB-fold (NTD-C). The

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NTD-C β–hairpin constricts the central channel (Fletcher et al., 2003, Li et al., 2015) and, in archaea, is electropositive in nature. Mutation of basic residues within this loop and on the adjacent, pore-lining surface of the OB-fold dramatically reduce both DNA binding and helicase activity in archaeal MCM (Fletcher et al., 2003, Pucci et al., 2004, McGeoch et al., 2005, Fletcher et al., 2008). The NTD-C β-hairpin is highly variable in length and composition within the eukaryotic MCM2-7 family, and while a majority are enriched in basic residues, this conservation is not universal. Nevertheless, the NTD-C region appears critical for eukaryotic MCM function, as *S. cerevisiae* strains harboring mutations within this β–hairpin of Mcm5 show an increased rate of minichromosome loss (Leon et al., 2008). This effect can be repressed by the addition of multiple ARS sequences, which suggests that origin recruitment, but not helicase activity, is defective in this particular mutant.

In addition to the NTD-C β–hairpin, the crystal structure of an archaeal MCM NTD bound to single-stranded DNA has revealed a second DNA-binding region within the NTD-C, the MCM-single-stranded-DNA binding motif (MSSB) (Froelich et al., 2014). The MSSB is formed primarily by two positively charged residues that extend from the OB-fold and, interestingly, bind single-stranded DNA in an orientation perpendicular to the long axis of the central channel. The MSSB DNA-binding residues are conserved among three consecutive subunits of the eukaryotic MCM2-7 complex (MCM4, MCM6, and MCM7), but not the other subunits. It has been suggested that the MSSB plays a role in DNA melting, working against a DNA pumping action of the ATPase domains to induce topological strain that encourages strand separation (Froelich et al., 2014). Thus, the MSSB may have a role in the early steps of initiation, consistent with the defects observed for *S. cerevisiae* MSSB mutants in *in vitro* loading reactions (Froelich et al., 2014); whether or how this motif contributes to helicase function during elongation is unknown.

The AAA+ PSI β–hairpin insertion constitutes the most C-terminal DNA-binding element. This pore-lining loop shows the highest level of conservation of the various DNA binding elements discussed thus far, and contains an invariant lysine that projects towards the central pore. The PSI β-hairpin has been compared to the translocation β–hairpin of SF3 helicases (e.g., the papillomavirus E1 protein and the SV40 Large T-antigen), which in the context of a hexamer forms a vertically aligned, right-handed staircase that tracks the DNA backbone with a conserved lysine (Enemark and Joshua-Tor, 2006, Enemark and Joshua-Tor, 2008). Consistent with a critical role in helicase function, mutation of the PSI lysine in archaeal MCM weakens DNA binding and ATP hydrolysis, and fully abrogates helicase activity (McGeoch et al., 2005); mutation of this residue within the context of the *D. melanogaster* CMG similarly abolishes helicase function (Petojevic et al., 2015). Interestingly, genetic analysis of the *S. cerevisiae* PSI β–hairpin lysines has highlighted non-equivalent functions for this residue in the six Mcm2-7 homologs, with only the mutation of this residue in Mcm3 abolishing an ability to complement deletion strains (Lam et al., 2013, Ramey and Sclafani, 2014).

How are the activities of the MCM2-7 DNA-binding elements functionally coordinated with ATP turnover to achieve the different functionalities observed for the helicase? At least two other loops seem to facilitate communication between the AAA+ domain and the DNA-binding elements lining the central channel. One is a motif known as the H2-insert, which

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sits in the MCM AAA+ fold and forms an extended loop that junctions the ATPase and NTD tiers, creating a pore-lining feature at the interface (Brewster et al., 2008, Bae et al., 2009, Li et al., 2015). Notably, analysis of the current data suggests that ATP-dependent repositioning of the H2-insert may switch the helicase between an origin melting conformation to one that facilitates processive unwinding. Despite a low level of sequence conservation, the length of the H2-insert is fully conserved in archaea and all MCM2-7 homologs and is sufficiently long to directly contact the MSSB. Interestingly, the H2-insert is rich in charged amino acids and alignment of the six yeast Mcm proteins reveals two fully conserved acidic residues that, in the context of the double hexamer, are positioned to shield the MSSB from binding DNA (Figure 5). Consistent with this proposal, the H2-insert dramatically affects DNA binding in the archaeal MCM complex, with removal of the loop enhancing MCM affinity for both double and single-stranded DNA (Jenkinson and Chong, 2006). The position of the H2-insert is thought to be modulated in an ATP-dependent fashion, such that the presence of ATP results in a more buried state for this loop (Jenkinson and Chong, 2006). In conclusion, the available data suggest a critical role for the H2-insert in transitioning the helicase between different functional states.

In addition to H2-insert dependent crosstalk between MCM subunits, the ACL of NTD-C projects upward from the OB-fold and toward the AAA+ domain of an adjacent protomer, where it is sandwiched between the H2-insert and PSI β–hairpin (Sakakibara et al., 2008, Barry et al., 2009, Li et al., 2015). Like the other functional motifs within the NTD, the ACL is absolutely required for helicase activity. However, the ACL does not affect DNA binding activity, but instead modulates the ATPase activity of the AAA+ domain; mutation of ACL residues or deletion of the region results in markedly reduced ATPase activity and inhibition of duplex unwinding (Sakakibara et al., 2008, Barry et al., 2009). Like the H2-insert, the ACL position with respect to the AAA+ domain is altered under different nucleotide-bound states (Barry et al., 2009).

Overall, the DNA-binding elements within the MCM NTD appear to play a critical function in both origin melting and unwinding. Although functional data point to an ATP-controlled connection between the positional status of specific DNA binding elements within the MCM2-7 ring and their ability to grasp or release substrate, direct observation of these elements in either a DNA melting or translocation mode is currently lacking. Recent electron microscopy models of the activated eukaryotic MCM2-7 helicase in the context of the CMG have revealed that there exists conformational coupling between the NTD and CTD tier, suggestive of coordinated action between these regions during processive unwinding (Yuan et al., 2016, Abid Ali et al., 2016). NTD-A also exhibits conformational dynamics and has been seen to rotate markedly away from the hexamer axis, a movement that may be coordinated through direct binding of DNA to the NTD-A (Fletcher et al., 2003, Chen et al., 2005, Hoang et al., 2007, Liu et al., 2008, Costa et al., 2008). As additional substrate-bound structures are solved for the archaeal and eukaryotic MCM homologs, exciting insights into its DNA remodeling and motor mechanisms are certain to emerge.

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Loading of the replicative helicase

The defining step in eukaryotic replication licensing is loading of MCM2-7 onto duplex DNA into a stable head-to-head double hexamer (Figure 1) (Remus et al., 2009, Evrin et al., 2009, Gambus et al., 2011). To attain this state, single MCM2-7 hexamers are sequentially loaded by an interaction with ORC•Cdc6 that is chaperoned by the Cdc10-dependent transcript 1 (Cdt1) protein. Structural characterization of multiple intermediates, together with the ability to reconstitute and study loading *in vitro*, are revealing how the stepwise and carefully orchestrated exchange of pre-RC factors at origins underlies this complex reaction (Figure 6).

Mechanism of helicase recruitment to origins

Maturation of the pre-RC occurs in a sequential fashion during the G1 phase of the cell cycle (Romanowski et al., 1996b, Gillespie et al., 2001, Tsuyama et al., 2005, Remus et al., 2009, Evrin et al., 2009, Tsakraklides and Bell, 2010, Ticau et al., 2015). Demarcation of replication start sites by ORC represents the inaugurating event, and permits the recruitment of and stable association with Cdc6 (Liang et al., 1995, Cocker et al., 1996, Coleman et al., 1996, Donovan et al., 1997, Seki and Diffley, 2000, Tsuyama et al., 2005). In an *in vitro* setting, Cdc6 regulates the fidelity of origin selection by two distinct mechanisms. First, Cdc6 helps to restrict the initiator from acting at illegitimate origins by sequestering the free initiator (thereby lowering the effective concentration of the initiator to increase origin specificity) (Duzdevich et al., 2015), and by triggering the dissociation of ORC from non-origin DNA (Mizushima et al., 2000). Second, Cdc6 ATPase activity is enhanced when bound to non-ARS sequences, which triggers the dissociation of Cdc6 from the initiator (Speck et al., 2005, Speck and Stillman, 2007). Ultimately, the secure association between Cdc6 and ORC at origins primes the initiator for helicase recruitment and loading (Rowles et al., 1996, Donovan et al., 1997, Perkins and Diffley, 1998, Feng et al., 2000, Tsuyama et al., 2005); this same complex will later facilitate the release of the double hexamer for subsequent activation at the onset of S phase (Chang et al., 2015).

The MCM2-7 helicase must first be recruited to origins to initiate the loading reaction. Notably, the helicase is sensitive to solution conditions and shows substantial conformational heterogeneity (Gomez-Llorente et al., 2005, Bochman and Schwacha, 2008, Costa et al., 2011), a feature that is pertinent for understanding the mechanism of association with the origin-bound initiator. Biochemical and structural analyses of the MCM2-7 complex has revealed that, in the absence of ATP, archaeal, fungal, and metazoan complexes can adopt a cracked ring architecture that bears a discontinuity, or ‘gate,’ between MCM2 and MCM5 (Bochman and Schwacha, 2008, Costa et al., 2011, Lyubimov et al., 2012, Boskovic et al., 2016, Samson et al., 2016). Nucleotide binding constricts the ring and favors the formation of MCM2/5 interactions across the ring break (Samel et al., 2014), but does not appear to irreversibly close the gate in single MCM2-7 hexamers (Costa et al., 2011, Lyubimov et al., 2012). Thus, MCM2-7 may not require active ring-opening prior to loading, but instead may simply be stably aligned around DNA by origin-bound ORC•Cdc6.

In addition to the ORC•Cdc6 helicase-loader complex, Cdt1 is also required for helicase loading in yeast and metazoa (Maiorano et al., 2000, Nishitani et al., 2000, Whittaker et al.,

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2000, Claycomb et al., 2002, Devault et al., 2002). However, the role of this factor has been enigmatic. Although Cdt1 was initially thought to be required for helicase recruitment to ORC (Asano et al., 2007, Chen et al., 2007, Chen and Bell, 2011), recent data suggest that MCM2-7 is recruited by direct interactions with the initiator (Fernandez-Cid et al., 2013, Frigola et al., 2013). With the exception of Orc6, all initiation factors discussed thus far possess a central AAA+ ATPase unit; Cdt1 represents an additional non-AAA+ protein involved in the process. Cdt1 was first identified in *S. pombe*, and through an ability to induce re-replication in the absence of cell division, was recognized as an essential and potent initiator of replication (Hofmann and Beach, 1994, Nishitani et al., 2000). Cdt1 is universally conserved in metazoa but is inconsistently found in other eukaryotic supergroups (Tada et al., 1999, Maiorano et al., 2000, Whittaker et al., 2000, Aves et al., 2012). The weak conservation of Cdt1 across species aggravated initial attempts to identify an *S. cerevisiae* homolog, but eventually the TAH11 (Topoisomerase-A hypersensitive 11) protein was identified as the budding yeast counterpart (Tanaka and Diffley, 2002, Devault et al., 2002). TAH11 was first isolated in genetic screens for genes that showed synthetic lethality with a mutant topoisomerase I allele (Fiorani and Bjornsti, 2000), and proteomic studies in human cells have suggested there exists an interaction between Cdt1 and both topoisomerase I and topoisomerase IIa (Sugimoto et al., 2008). Archaea also contain a Cdt1-related protein, termed WhiP; however, this factor appears to uniquely function in the Orc-independent assembly of pre-RCs (Robinson and Bell, 2007, Samson et al., 2013).

Interestingly, Cdt1 has markedly diverged between *S. cerevisiae* and metazoa, and functional analyses have suggested that there exist differing mechanisms for Cdt1-dependent action across species. All Cdt1 homologs are built from a conserved C-terminal pair of WH domains (Lee et al., 2004, Khayrutdinov et al., 2009). By contrast, the N-terminal region of Cdt1 is not conserved, and while the N-terminal domain of metazoa and *S. pombe* Cdt1 has a basic pI (human Cdt1 pI = 10.5) that is predicted to be unstructured, the *S. cerevisiae* N-terminus has an acidic pI (pI = 5.1) and possesses sufficient secondary structure to likely constitute an ordered domain (modeling using Phyre2 (Kelley et al., 2015) suggests that this region corresponds to a catalytically defunct oxygenase fold). Interestingly, an *S. cerevisiae* Cdt1 construct lacking the non-conserved N-terminal domain fails to load the helicase into an activation-competent conformation (Takara and Bell, 2011, Fernandez-Cid et al., 2013), whereas the N-terminus of metazoa Cdt1 is dispensable for the loading reaction (Ferenbach et al., 2005).

The factors with which Cdt1 stably associates also evince species-specific differences. In *S. cerevisiae*, Cdt1 and Mcm2-7 form a tight complex and exhibit interdependent nuclear import (Tanaka and Diffley, 2002, Kawasaki et al., 2006, Remus et al., 2009, Takara and Bell, 2011); Cdt1 similarly helps maintain the structural integrity of the Mcm2-7 complex (Wu et al., 2012, Frigola et al., 2013). Conversely, *Xenopus* Cdt1 and MCM2-7 are biochemically separable initiation factors, with immunodepletion of MCM3 from *Xenopus* egg extracts removing MCM2-7 but having no effect on Cdt1 (Maiorano et al., 2004) (it is worth noting that mouse Cdt1 has been reported to form a complex with MCM2-7 *in vitro* (You and Masai, 2008), but the stability of this assembly has not been investigated). Multiple explanations could account for the differences in behavior between *S. cerevisiae* and *Xenopus* Cdt1; for example, differences in the relative affinities of Cdt1 for the MCM2-7

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complex could reflect an alteration to the sequential assembly of the pre-RC factors in metazoa, such that Cdt1 associates first with ORC•Cdc6 rather than with the helicase (Maiorano et al., 2000, Waga and Zembutsu, 2006). Finally, metazoa contain an additional Cdt1-binding protein, Geminin, which negatively regulates licensing by inhibiting the Cdt1-dependent loading of the helicase into a stable double hexamer (Wohlschlegel et al., 2000, Edwards et al., 2002, Yanagi et al., 2002, Wu et al., 2014a).

Despite the differences in Cdt1 structure and its preferred binding partners between budding yeast and metazoans, multiple studies have identified MCM6 as the primary Cdt1 interaction site on the helicase hexamer (Yanagi et al., 2002, Ferenbach et al., 2005, Zhang et al., 2010, Wei et al., 2010, Liu et al., 2012a, Wu et al., 2012) (mouse Cdt1 also interacts with MCM2 (You and Masai, 2008)). Cdt1/MCM6 contacts are facilitated by the C-terminal WH domain of each protein (Ferenbach et al., 2005, Teer and Dutta, 2008, Khayrutdinov et al., 2009, Jee et al., 2010, Wei et al., 2010, Takara and Bell, 2011), with structural studies of the human proteins revealing that a helical extension which projects from the Cdt1 WH region engages the helix-turn-helix motif of the MCM6 C-terminus (You and Masai, 2008, Liu et al., 2012a). Mutagenesis of residues in the interaction surface abolishes helicase loading and DNA replication in budding yeast, suggesting that the MCM6-Cdt1 mechanism of binding is widely conserved (Liu et al., 2012a). Despite this contact, *in vitro* binding studies have suggested that there exist additional undefined sites of interaction between Cdt1 and MCM2-7 (Khayrutdinov et al., 2009, Fernandez-Cid et al., 2013).

S. cerevisiae Cdt1 has also been reported to bind Orc6 (Semple et al., 2006, Asano et al., 2007, Chen et al., 2007, Chen and Bell, 2011). This interaction was initially proposed to facilitate helicase recruitment by helping to tether MCM2-7 to the ORC•Cdc6 complex (it is unclear whether metazoan Cdt1 and Orc6 interact (Yanagi et al., 2002)); however, other work has indicated that *S. cerevisiae* Mcm2-7 contains elements that directly engage ORC•Cdc6 and that are wholly sufficient for recruitment (Fernandez-Cid et al., 2013, Frigola et al., 2013). Cdt1's participation in modulating MCM/ORC•Cdc6 interactions is still under debate, with one study noting that budding yeast Cdt1 relieves an Mcm6-dependent autoinhibitory mechanism that prevents Cdt1-independent recruitment (i.e., deletion of an Mcm6 autoinhibitory domain results in helicase recruitment in the absence of Cdt1) (Fernandez-Cid et al., 2013), and another showing that Cdt1 is fully dispensable for helicase recruitment (here only an interaction between Cdc6 and the extreme C-terminus of Mcm3 appears necessary for recruitment (Frigola et al., 2013). Consistent with the proposed role of the eukaryotic MCM3 C-terminus, recent studies in archaea have found that the MCM C-terminal WH domain directly binds Orc to facilitate recruitment of the helicase to replication origins (Samson et al., 2016).

Together, the available data suggest that Cdt1 may be dispensable for helicase recruitment, and that this step instead predominantly relies on interactions between ORC•Cdc6-bound origins and MCM2-7. What then might be the significance of Cdt1 and its interaction with Orc6? Beyond a potential ability to relieve MCM6-dependent autoinhibition (Fernandez-Cid et al., 2013), *S. cerevisiae* Cdt1 stabilizes Mcm2-7 at origins in an Orc6-dependent manner (Chen et al., 2007), such that in the absence of Cdt1, topologically linked Mcm2-7 hexamers fail to load (Chen and Bell, 2011). Notably, an N-terminal deletion construct of budding

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yeast Cdt1 permits loading of double hexamers, but in a state that is incapable of being subsequently activated (Takara and Bell, 2011). Thus, Cdt1 is required for events downstream of recruitment, which appear to include the formation of an MCM2-7 double hexamer intermediate that is competent for replication. Overall, the molecular details underlying the role of Cdt1, and in particular its Orc6 association, are in need of further study.

Loading the double hexamer

Ultimately, the initiator-dependent recruitment of MCM2-7 to origins leads to the formation of a stable chromatin-bound MCM2-7 complex that, unlike ORC•Cdc6, is resistant to high-salt extraction (Donovan et al., 1997, Edwards et al., 2002, Bowers et al., 2004). Notably, the MCM2-7 loading reaction has been fully reconstituted *in vitro* with purified proteins from *S. cerevisiae* (Remus et al., 2009, Evrin et al., 2009), which has in turn allowed the use of single molecule and structural techniques to understand both the nature of the salt-stable complex and the events leading to its formation. The details of the loading reaction discussed below are derived exclusively from studies with *S. cerevisiae* initiation factors; given some of the species-specific differences observed to date, it will be important to reconstitute the loading reaction *in vitro* with other organisms for comparison.

Following the recruitment of Cdt1•Mcm2-7 to ORC•Cdc6-bound origins, Mcm2-7 is deposited onto DNA while both Cdc6 and Cdt1 are ejected from the pre-RC in an ATP hydrolysis-dependent fashion. Cdc6 release occurs prior to Cdt1 both for single- and double-hexamer loading (Remus et al., 2009, Evrin et al., 2009, Tsakraklides and Bell, 2010, Kang et al., 2014, Ticau et al., 2015) (Figure 6). Singly-loaded Mcm2-7 hexamers result in the transient formation of a meta-stable ORC•Cdc6•Cdt1•Mcm2-7 (OCCM) intermediate, which can be stabilized by the presence of a non-hydrolyzable ATP analog. This property has been exploited to permit imaging of the complex by 3D cryo-electron microscopy (Sun et al., 2013). Within the OCCM, Cdt1 interfaces with the N-terminal tier of Mcm2, Mcm6, and Mcm5. The Mcm3 C-terminus resides proximal to Cdc6, consistent with the observed interaction between these regions *in vitro* and the proposed role of this interaction in helicase recruitment (Sun et al., 2013, Frigola et al., 2013). Cdt1 and Orc6 make no visible contacts in the OCCM structure; indeed the N-terminal TFIIB domain of Orc6 does not appear ordered. Notably, the Cdt1•Mcm2-7 heptamer is oriented such that the MCM C-terminal domains (the region containing the AAA+ and WH domains) abut the ORC WH domains (Bleichert et al., 2015), leaving the MCM NTD accessible. Although analysis of the loading reaction in bulk has indicated that singly-loaded Mcm2-7 hexamers are salt-sensitive (Remus et al., 2009, Evrin et al., 2009, Kang et al., 2014), single molecule analysis of the reaction reveals that nearly 50% of the singly-loaded helicases are salt-stable, suggesting that at this stage Mcm2-7 ring closure has occurred (Ticau et al., 2015). This discrepancy between bulk and single-molecule studies currently lacks explanation, although may arise from the relatively high protein concentrations used in the bulk assays and/or by their limited kinetic sensitivity.

After the first loading event, Cdc6 re-associates with the ORC•Mcm2-7 complex, forming a short-lived ORC•Cdc6•Mcm2-7 (OCM) intermediate that is competent for recruitment of a

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second Cdt1•Mcm2-7 heptamer (Fernandez-Cid et al., 2013, Ticau et al., 2015). At present, the mechanics by which a second hexamer is recruited are unclear. It has been proposed that loading of the second hexamer requires equivalent interactions as the first, with the Mcm3 C-terminus being required for both loading steps (Frigola et al., 2013). However, if loading the second helicase into a double hexamer requires an interaction with ORC•Cdc6, this mechanism must involve some fairly complicated acrobatics, mainly because the first loaded Mcm2-7 complex should still be present to block the MCM-binding surface on ORC, and because the second Mcm2-7 complex must be placed at a location more than ~100 Å from ORC's initial binding site on DNA, and in an inverted orientation from the first helicase. This spatial problem could be solved if two ORCs were used for the loading of two helicases with opposing polarity; however, single-molecule analysis has demonstrated that a single, stably bound ORC is sufficient to load a double hexamer (Ticau et al., 2015). Because of these steric complications, an alternative mechanism has been proposed wherein the first, loaded hexamer templates loading of the second through a distinct mechanism that does not require direct ORC•Cdc6 interactions (Ticau et al., 2015). Consistent with this idea, the kinetics of loading the second hexamer are slower than the first (Fernandez-Cid et al., 2013), as is the release of Cdc6 and Cdt1 (Ticau et al., 2015), suggesting different reaction intermediates are accessed.

While the model described above does not directly account for the demonstrated role of the budding yeast Mcm3 WH domain in the loading of both the first and second Mcm2-7 hexamers (Frigola et al., 2013), previous work has revealed that deletion of the Mcm3 C-terminus results in a dramatic loss of Mcm2-7 ATPase activity (Sun et al., 2014). Since proper ATPase function by the Mcm2-7 hexamer is critical at all stages of the loading reaction in *S. cerevisiae*, this loss of activity in the Mcm3 WH domain mutant could account for the observed defect in loading the second hexamer. Accordingly, Mcm2-7 complexes with individual active site mutations also show defective recruitment (particularly Mcm2 Walker A and Mcm6 arginine finger mutants), Cdt1 release, and dramatically reduced levels of salt-stable double hexamers (Kang et al., 2014, Coster et al., 2014). Although a marginal level of double-hexamer formation can be achieved with particular MCM ATPase mutants, these loaded complexes are generally deficient for downstream activation events and cannot drive DNA replication *in vitro* (Kang et al., 2014). Collectively, the present data indicate that ATP binding and hydrolysis by *S. cerevisiae* Mcm2-7 is required from the early steps of helicase recruitment to the concluding activation events. These results are difficult to rationalize with the observation that *Xenopus* MCM6 and MCM7 Walker A mutants are loaded normally (Ying and Gautier, 2005), but this finding may again reflect species-specific differences.

Although double-hexamer loading is critically dependent on the ATPase activity of Mcm2-7, ATP hydrolysis by Cdc6 and ORC perform additional roles that are consistent with a requirement for their ATPase activity *in vivo* (Zwerschke et al., 1994, Weinreich et al., 1999, Wang et al., 1999, Schepers and Diffley, 2001, Takahashi et al., 2002, Bowers et al., 2004). ATP hydrolysis by Orc1 is dispensable for the loading reaction (Evrin et al., 2013, Fernandez-Cid et al., 2013, Coster et al., 2014), but resets the initiation machinery to allow reiterative loading of MCM double hexamers at origins (Bowers et al., 2004). By comparison, Cdc6 has been reported to function as a quality control factor, whose ATPase

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activity ensures that defective or incomplete Mcm2-7 complexes are released from origins (Frigola et al., 2013, Kang et al., 2014, Coster et al., 2014). Another major function of Cdc6 ATPase activity appears to be in the final step of pre-RC formation, where it is responsible for releasing successfully loaded double hexamers from ORC, an event that enables the downstream activation of the helicase (Chang et al., 2015). Loss of Cdc6 ATPase activity also results in inefficient Cdt1 release (Randell et al., 2006), although ATP turnover by Mcm2-7 appears primarily responsible for Cdt1 ejection (Kang et al., 2014, Coster et al., 2014). Overall, Cdc6 functions as a linchpin factor critical for multiple events in the loading process.

Helicase activation

Although a double MCM2-7 hexamer is loaded during pre-RC formation, at replication forks this structure is dissolved to yield two single MCM2-7 hexamers that move in opposite directions (Yardimci et al., 2010, Ticau et al., 2015). On their own, single MCM2-7 hexamers possess little or no helicase activity (Ishimi, 1997, Lee et al., 2000, Kaplan et al., 2003, You et al., 2003, Ilves et al., 2010). Initially, this observation was difficult to rationalize with the strong evidence that pointed towards a role for the complex as the replicative helicase, but it is now appreciated that MCM2-7 is incorporated into a larger Cdc45•MCM2-7•GINS (CMG) complex that possesses robust helicase activity. Like pre-RC assembly, double hexamer dissolution and CMG formation occur through a highly orchestrated and interconnected series of events that provide yet another means for regulating the DNA replication initiation reaction.

Identification of the Cdc45•MCM2-7•GINS (CMG) complex

The MCM2-7 genes are essential proteins in DNA replication (Moir et al., 1982, Maine et al., 1984, Gibson et al., 1990, Dalton and Whitbread, 1995) and their phylogenetic lineage to helicases led to early suggestions that these factors might function as nucleic acid motor proteins (Koonin, 1993). Together with work on archaeal MCMs, which have been found to possess robust helicase activity (Chong et al., 2000, Kelman et al., 1999, Shechter et al., 2000), these data indicated that the MCM2-7 complex likely served as the replicative helicase in eukaryotes. Interestingly, initial *in vitro* studies were unable to detect helicase activity from the eukaryotic MCM2-7 complex, although an MCM subcomplex composed of subunits $(4,6,7)_2$ showed weak helicase activity (Ishimi, 1997, Lee et al., 2000, Kaplan et al., 2003, You et al., 2003). These data suggested that the MCM2-7 assembly might play a more limited role in replication, such as origin melting. However, the persistence of MCM2-7 at replication forks (Aparicio et al., 1997), coupled with the observed S-phase arrest after MCM2-7 inactivation (Labib et al., 2000, Pacek and Walter, 2004, Shechter et al., 2004), provided irrefutable evidence for the role of the complex in elongation (Todorov et al., 1995, Krude et al., 1996). Nevertheless, the demonstration of helicase activation remained elusive.

Eventually, conditions were identified that recovered *in vitro* helicase activity for budding yeast Mcm2-7 (Bochman and Schwacha, 2008). Roughly contemporaneously, several lines of analysis began to reveal that the helicase also existed within a larger macromolecular assembly (Masuda et al., 2003, Calzada et al., 2005, Pacek et al., 2006, Gambus et al., 2006,

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Moyer et al., 2006). Three parallel studies – two using an unbiased assessment of Cdc45 and GINS interacting factors and a third using a candidate-based approach to determine the composition of the helicase stalled at replication forks – have now demonstrated that MCM2-7 forms a stable complex with Cdc45 and GINS (Pacek et al., 2006, Gambus et al., 2006, Moyer et al., 2006). Cdc45 and GINS are essential replication fork components (Moir et al., 1982, Hopwood and Dalton, 1996, Dalton and Hopwood, 1997, Zou et al., 1997, Kamimura et al., 1998, Kanemaki et al., 2003, Masuda et al., 2003), which mimic the chromatin association pattern of MCM subunits (Takayama et al., 2003, Kubota et al., 2003). Each MCM2-7 binds a single copy of Cdc45 and one GINS tetramer to form a stable, eleven-protein complex termed the CMG (for Cdc45•MCM2-7•GINS). The CMG has been shown to possess robust helicase activity (Moyer et al., 2006, Ilves et al., 2010), and bioinformatics analyses have indicated that a full set of CMG factors are likely conserved from archaea to eukaryotes (Makarova et al., 2012), with recent work demonstrating that, as observed for the eukaryotic accessory factors, archaea's Cdc45 and GINS homologs activate MCM *in vitro* and *in vivo* (Xu et al., 2016).

Regulating helicase activation

Appropriate recruitment of the factors necessary for CMG formation constitutes a highly-regulated step towards replisome formation. Consistent with the Cdc6-dependent release of the MCM2-7 double hexamer from ORC (Chang et al., 2015), helicase activation steps no longer require the function of either ORC or Cdc6 (Hua and Newport, 1998, Rowles et al., 1999, Jares and Blow, 2000, Walter, 2000, Yeeles et al., 2015). The newly formed MCM2-7 double hexamer serves itself as a binding platform and target for the Dbf4-dependent kinase (DDK) that, in collaboration with S-phase cyclin-dependent kinase (S-CDK), constitute the minimal set of kinases needed to regulate the recruitment of Cdc45 and GINS. Together, the activity of these two kinases coordinate the assembly of a pre-IC that is poised for activation and formation of a bidirectional replication fork (Figure 6) (Aladjem, 2007).

DDK is formed by the direct association of the Cdc7 kinase with Dbf4, a protein co-factor that relieves Cdc7 autoinhibition (Kitada et al., 1992, Jackson et al., 1993, Dowell et al., 1994) and that is regulated in a cell-cycle dependent fashion (Cheng et al., 1999, Oshiro et al., 1999, Nougarede et al., 2000). DDK phosphorylates multiple MCM subunits (Lei et al., 1997, Weinreich and Stillman, 1999, Masai et al., 2006, Montagnoli et al., 2006, Cho et al., 2006, Sheu and Stillman, 2006, Tsuji et al., 2006) and is required for initiating DNA replication (Chapman and Johnston, 1989, Hollingsworth and Sclafani, 1990, Jiang et al., 1999a). Like DDK, S-CDK is required for replication (Broek et al., 1991, Hayles et al., 1994) and exists in an inactive conformation by engaging Sic1, a repressive protein that restricts S-CDK activity into late G1/S phase (Mendenhall, 1993, Donovan et al., 1994, Schwob et al., 1994, Knapp et al., 1996, Schneider et al., 1996). Redundant regulation of S-CDK occurs by the cell-cycle dependent expression of activating cyclins (Abreu et al., 2013). It is currently unclear whether there is a conserved sequential order for DDK and CDK activity. In a *X. laevis* egg extract system, DDK activity is independent of CDK, and exposing the pre-RC first to CDK fully ablates DNA replication initiation (Jares and Blow, 2000, Walter, 2000), suggesting that DDK acts first. Such a consensus has not been found with *S. cerevisiae* proteins, where the sequential action of DDK and CDK depends on the

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experimental setup (Nougarede et al., 2000, Heller et al., 2011) and has been found to be inconsequential in a fully *in vitro* reconstituted DNA replication system (Yeeles et al., 2015).

DDK is recruited to Mcm2-7 through interactions with Mcm4 and Mcm2 (Sheu and Stillman, 2010, Ramer et al., 2013) and targets specific MCM subunits within the context of a double, but not a single, MCM2-7 hexamer (Francis et al., 2009, Evrin et al., 2014, Sun et al., 2014, Kang et al., 2014, Costa et al., 2014). Notably, the MCM subunits responsible for recruiting DDK are non-adjacent within the context of a single hexamer; however, the rotational offset within the double hexamer results in the adjacent placement of MCM2 and MCM4 from separate hexamers (Costa et al., 2014, Sun et al., 2014, Li et al., 2015), thus forming a composite DDK-interaction interface. In addition, MCM2, MCM4, and MCM6, which are all phosphorylated by DDK (Lei et al., 1997, Masai et al., 2006, Tsuji et al., 2006, Sheu and Stillman, 2006, Cho et al., 2006, Bruck and Kaplan, 2009, Randell et al., 2010), reside in close proximity within the double hexamer and the interface between the two hexamers is somewhat splayed apart at this position, creating a gap that may provide DDK access to the N-terminal serine/threonine rich domains (NSD) of its MCM targets (Sheu and Stillman, 2010, Sun et al., 2014, Li et al., 2015). Collectively, the available data suggest that the DDK-dependent activation of MCM2-7 is temporally regulated by selective recruitment of the kinase to and action upon a double hexameric MCM2-7, a structure that uniquely positions the relevant MCM subunits in close proximity (Sun et al., 2014). In addition to DDK, budding yeast Mcm2-7 has been shown to be phosphorylated by CDK and Mec1 kinases, which sensitize Mcm2-7 to DDK activity (Devault et al., 2008, Randell et al., 2010); however, *in vitro* DDK alone is required for activation of *S. cerevisiae* Mcm2-7 (Yeeles et al., 2015).

In *S. cerevisiae*, the DDK-dependent phosphorylation of Mcm2-7 is important for facilitating the Sld3 and Sld7-chaperoned recruitment of Cdc45 onto the double hexamer (Masai et al., 2006, Yabuuchi et al., 2006, Sheu and Stillman, 2006, Tanaka et al., 2011a). In humans, two proteins known as Treslin and MTBP (for MDM2 binding protein) have been proposed to represent functional homologs of budding yeast Sld3 and Sld7, respectively (Matsuno et al., 2006, Sanchez-Pulido et al., 2010, Sansam et al., 2010, Kumagai et al., 2010, Bruck and Kaplan, 2015c, Boos et al., 2013). *In vitro,* Cdc45 recruitment depends on the prior association of Sld3 and Sld7 with the DDK-phosphorylated N-termini of Mcm2, Mcm4, and Mcm6 (Deegan et al., 2016, Fang et al., 2016); however, *in vivo* Sld3/7 and Cdc45 form a stable complex and may be co-recruited to double hexamers (Kamimura et al., 2001, Tanaka et al., 2011a, Tanaka et al., 2011b). Notably, in the absence of DDK, no additional initiation factors are recruited to Mcm2-7 double hexamers. Conversely, in the absence of CDK activity, Cdc45 as well as Sld3 and Sld7 are still efficiently recruited (Heller et al., 2011, Yeeles et al., 2015). Thus, recruitment of Cdc45 appears to rely exclusively on DDK.

How does DDK-dependent MCM2-7 phosphorylation facilitate Cdc45 recruitment? Although we lack a complete mechanistic picture for DDK-dependent activation, three distinct MCM modifications have been identified that result in DDK bypass, suggesting there exists a complex role for DDK that likely involves a concerted change within multiple MCM subunits. First, hyperphosphorylation of the *S. cerevisiae* Mcm4 N-terminus relieves

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an autoinhibitory function that this region imposes on the helicase (indeed, deletion of the Mcm4 N-terminus bypasses the DDK requirement) (Sheu and Stillman, 2006, Sheu and Stillman, 2010). Although a mechanism entailing a steric block to the Cdc45 binding site or the adoption of a different non-permissive orientation would satisfy this observation, within the atomic structure of the *S. cerevisiae* Mcm2-7 double hexamer the Mcm4 N-terminus is disordered, suggesting that autoinhibition is relieved by an alternative mechanism (Li et al., 2015). Second, phosphorylation of Mcm4 and Mcm6 generates a phosphopeptide binding site for recruiting Sld3 (phosphomimetic mutants of these MCM subunits can bypass the requirement for DDK) (Deegan et al., 2016). Finally, a mutation within budding yeast Mcm5, known as the *bob1* allele, has been found to bypass DDK-activated initiation (Hardy et al., 1997).

In addition to DDK-dependent recruitment of Cdc45, Mcm2-7 activation requires an association with GINS and, for origin firing, with Replication Protein A (RPA), MCM10, Polα, Polε, and CTF4 (van Deursen et al., 2012, Yeeles et al., 2015). Notably, CDK's action on the growing, chromatin-associated replication assembly, as well as on replicative proteins yet to be integrated into the replisome, results in the recruitment of the remaining initiation factors that are needed for origin firing. In this swarm of activity, CDK targets Cdc45, Sld2, Sld3, and Sld7 (Masumoto et al., 2002, Zegerman and Diffley, 2007, Tanaka et al., 2007, Muramatsu et al., 2010, Heller et al., 2011, Yeeles et al., 2015), and, together with a protein known as Dpb11, facilitates recruitment of a multiprotein assembly composed of GINS, Sld2, and Polε to the growing complex (Araki et al., 1995, Kamimura et al., 1998, Kamimura et al., 2001, Takayama et al., 2003, Muramatsu et al., 2010, Tanaka et al., 2013). Thus, DDK and CDK drive formation of an active helicase by phosphorylating key assembly factors that allow for the sequential recruitment of Cdc45 and GINS. For its part, MCM10 is recruited by interactions with the CMG (Homesley et al., 2000, Wohlschlegel et al., 2002, Douglas and Diffley, 2016) and further appears to help stabilize the replisome once formed (Gregan et al., 2003, Ricke and Bielinsky, 2004). Polε is likewise recruited directly to the CMG by an interaction with GINS (Araki et al., 1995, Muramatsu et al., 2010), whereas Polα is physically coupled to the helicase by CTF4 (Villa et al., 2016, Simon et al., 2014).

Many questions remain for the events that transition the growing MCM2-7 complex into a competent bi-directional replication fork. For example, it is currently unclear at what point and how origin melting occurs, or how an MCM2-7 double hexamer, which encircles duplex DNA, transitions into two uncoupled CMG particles that encircle single-stranded DNA (Yardimci et al., 2010, Ticau et al., 2015). Interestingly, recent data suggests that the process of origin melting and strand extrusion may be interdependent and temporally intertwined with CMG assembly. Indeed, *S. cerevisiae* Sld2, Sld3, and Dpb11, which chaperone GINS into a complex with the helicase, compete with GINS for Mcm2-7 binding, a function that is relieved in the presence of single-stranded DNA (Bruck et al., 2011, Bruck and Kaplan, 2011, Bruck and Kaplan, 2014, Bruck and Kaplan, 2015c, Dhingra et al., 2015) (the metazoan Sld2 and Sld3 equivalents also bind single-stranded DNA (Sangrithi et al., 2005, Ohlenschlager et al., 2012, Bruck and Kaplan, 2015c)). Thus, GINS binding may not only be anticipated by origin melting and the generation of single-stranded DNA, but by strand extrusion as well, as the separated origin strands may be contained within the Mcm2-7 double hexamer for a period of time (Sun et al., 2014, Li et al., 2015). The topological

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transformations to DNA in the context of the maturing CMG complex are likely aided in by the phosphorylation of Mcm2 by DDK (Lei et al., 1997, Bruck and Kaplan, 2009), an event that weakens the Mcm2/5 gate and promotes ring opening (Bruck and Kaplan, 2015a, Bruck and Kaplan, 2015b). Consistent with this idea, origin melting and strand extrusion have been reported to occur concomitantly with DDK-dependent activation but prior to complete CMG assembly (Bruck and Kaplan, 2015a). Interestingly, recent data suggests that the single-stranded DNA binding activity of budding yeast Mcm10 is required for initiation and that it may help stabilize the origin melting reaction (Perez-Arnaiz and Kaplan, 2016).

Despite the available insights, the mechanisms that transition a loaded double hexamer into an active, bi-directional replication fork remain shrouded in mystery. An important future direction will be to understand the timing of different events in the lifecycle of the helicase, such as when double-hexamer dissociation and replisome factor recruitment occurs (such as MCM10, Polα and RPA), as compared to origin melting and strand extrusion. The physical mechanisms that coordinate CMG structural changes with origin remodelling likewise remain ill-defined, and require structural studies of different intermediates that, at present, have not been stably isolated.

Mechanism of CMG function

The cracked ring architecture of the MCM2-7 complex is an asset when it comes to loading the helicase around double-stranded DNA, but would seem to be a potential liability in terms of processive DNA unwinding functions (Bochman and Schwacha, 2008, Ilves et al., 2010, Petojevic et al., 2015). Although nucleotide binding by MCM2-7 helps to overcome a natural tendency of the helicase to splay open (Samel et al., 2014), cracked-ring conformations still readily form (Costa et al., 2011, Lyubimov et al., 2012), providing a physical basis for the weak *in vitro* helicase activity of MCM2-7 (Bochman and Schwacha, 2008, Ilves et al., 2010). Incorporation of MCM2-7 into the CMG counteracts the functional deficiencies of the MCM2-7 complex for DNA unwinding. 3D electron microscopic reconstructions of both the *Drosophila* and budding yeast CMG have revealed that GINS and Cdc45 seal off the MCM2/MCM5 discontinuity and reduce the conformational dynamics of the MCM2/5 gate, likely as a means to favor productive ATPase interface contacts (Figure 7A-B) (Costa et al., 2011, Costa et al., 2014, Abid Ali et al., 2016, Yuan et al., 2016). Thus, instead of being directly involved in translocation *per se*, it would seem that GINS and Cdc45 help shift the structural disposition of the MCM2-7 AAA+ domains into productive conformations. Consistent with this notion, incorporation of *Drosophila* MCM2-7 into the CMG increases the v_{max} of ATP hydrolysis by over 300-fold and results in a 10-fold higher affinity for DNA, resulting in a dramatic increase in helicase activity (Ilves et al., 2010). Interestingly, in addition to participating in the control of MCM2-7 ring status, both GINS (Boskovic et al., 2007, Ilves et al., 2010) and Cdc45 (Krastanova et al., 2012, Bruck and Kaplan, 2013, Szambowska et al., 2014) have been shown to bind DNA. Cdc45, which is a catalytically-defunct homolog of the RecJ exonuclease (Sanchez-Pulido and Ponting, 2011, Simon et al., 2016), also appears to assist with capture of the leading strand during transient CMG gate opening (Petojevic et al., 2015), thereby possibly playing a protective role during replication fork stalling (Bruck and Kaplan, 2013).

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Structural analysis of the CMG reveals the presence of two distinct conformers that are, as observed for the MCM2-7 helicase core, differentiated by the relative positioning of the gating subunits (Yuan et al., 2016, Abid Ali et al., 2016). As the MCM NTD is stabilized by extensive contacts with GINS and Cdc45, changes within the AAA+ ATPase ring appear primarily responsible for the alterations observed between the two conformational states (Costa et al., 2011, Costa et al., 2014, Abid Ali et al., 2016, Yuan et al., 2016). Altering the position of the MCM2/5 gate appears to propagate structural changes around the MCM2-7 ring that result in significant translational and rotational rearrangement of MCM6 and, to a lesser extent, MCM4. These changes in turn are coupled to a transition from a constricted conformer state, where the MCM2-7 NTD and CTD tiers are co-planar, to a relaxed, tilted form, which generates an asymmetric expansion of the gap between the NTD and CTD tiers on one side of the ring (Figure 7C). It has been suggested that switching between these conformations could elicit a pumpjack motion, with the CTD tier rocking with respect to a stable NTD tier during DNA translocation (Yuan et al., 2016), and that these dynamics could be used to drive a linear, as opposed to rotational, mechanism of DNA translocation (Yuan et al., 2016, Abid Ali et al., 2016). Higher-resolution structures of distinct translocation intermediates, coupled with single-molecule measurements of ring dynamics and displacement, will be needed to test these ideas.

An interesting point of discussion in the field has been whether the ATPase regions of MCMs serve as single or double-stranded DNA translocases. Based on a distant phylogenetic kinship to RuvB, a known double-strand translocase, MCM2-7 was initially proposed to move along duplex DNA, with the NTD-associated GINS/Cdc45 accessory subunits serving as a ploughshare to separate the two strands (Takahashi et al., 2005). However, subsequent biochemical studies of the *Drosophila* CMG have shown that the complex requires a fork for unwinding (Ilves et al., 2010), consistent with both archaeal MCM and metazoan MCM4,6,7 functioning as single-stranded DNA translocases on model substrates (Ishimi, 1997, Kelman et al., 1999, Shechter et al., 2000, Moyer et al., 2006, Kang et al., 2012). Along these lines, studies in archaea have reported that an MCM construct lacking the NTD tier (and that has no associated GINS/Cdc45) still possesses helicase activity (Barry et al., 2007). Moreover, replisomes have been challenged with leading and lagging strand roadblocks in an *X. laevis* extract system, with only leading strand roadblocks proving capable of stalling fork progression (MCM2-7 is a $3'\rightarrow5'$ helicase that tracks on the leading strand) (Fu et al., 2011). Consistent with this observation, recent analysis of the pattern of forked DNA crosslinking to the CMG reveals that the leading strand shows robust crosslinking to all MCM2-7 subunits, as well as Cdc45, while lagging strand crosslinks are markedly fewer and those that occur are all on the exterior surface of MCM2-7 (Petojevic et al., 2015). Collectively, these data strongly indicate that the CMG functions as a single-stranded DNA translocase.

Structural analysis of the apo and substrate-bound CMG states also support a single-strand tracking model. In particular, upon binding of the CMG to a forked duplex substrate, nucleic acid density can be visualized within the central channel whose dimensions appear sufficient to accommodate single but not double-stranded DNA (Abid Ali et al., 2016). This finding is consistent with a previous structural analysis of the CMG bound to duplex DNA containing a single $3'$-tail, which showed that the duplex region of a DNA fork is positioned outside the

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central channel in an orientation consistent with the known 3′→5′ polarity of DNA engagement (Costa et al., 2014). Interestingly, the high-resolution structure of the *S. cerevisiae* CMG reveals that the WH domains of Mcm5 and Mcm6, which are disordered within the context of the double hexamer (Li et al., 2015), become ordered and take up a position within the central pore of the CMG that constricts the AAA+ pore to a point where it is too small to accommodate double-stranded DNA (Yuan et al., 2016). Overall, these lines of evidence largely corroborate the view that the CMG tracks along single-stranded templates, albeit with a potential capacity to switch to a double-stranded DNA mode of translocation, as might occur during replication termination, when forks converge (Dewar et al., 2015) (parenthetically, there is evidence that the *E. coli* DnaB helicase can undergo a transition between single and double-stranded translocation modes (Kaplan, 2000, Kaplan and O'Donnell, 2002)). Understanding the role of the WH domains awaits determination of substrate-bound CMG structures in which the disposition of the nucleic acid substrate and the WH domains are fully defined.

Overall, while a variety of mechanistic models exist to account for hexameric helicase activity, the so-called steric exclusion model appears most compatible with the observed data to date for the CMG. The steric exclusion model posits that translocation occurs along single-stranded DNA with the non-template strand being excluded from the central channel. One assumption of this framework has been that the lagging strand is functionally passive, implying that once displaced, it is inconsequential to helicase function. However, emerging lines of evidence are starting to suggest that the lagging strand may wrap around the external surface of the CMG and support helicase activity (Figure 7D). Consistent with this notion, DNA interactions with the external surface of archaeal MCM is integral to helicase function (Rothenberg et al., 2007, Costa et al., 2008, Graham et al., 2011, Graham et al., 2016). DNA binding to the MCM external surface appears at least partially facilitated by the NTD-A region, which can undergo large structural rearrangements to expose a DNA-binding site (Fletcher et al., 2003, Chen et al., 2005, Costa et al., 2008, Miller et al., 2014). Whether the eukaryotic MCM NTD-A also facilitates DNA binding is unclear; however, the MCM AAA + domain contains a conserved, surface-exposed β–hairpin that has been reported to directly engage DNA (Brewster et al., 2010, Graham et al., 2011) and, in the context of the eukaryotic CMG, is integral to helicase function (Petojevic et al., 2015). Thus, the CMG may function by a mechanism of single-stranded DNA translocation that results in both steric exclusion and wrapping of lagging strand DNA (Graham et al., 2011).

Regulatory mechanisms for preventing re-initiation

Origin licensing and firing are the most tightly regulated events in the process of DNA replication initiation. Multiple interdependent and redundant mechanisms ensure that origins can be marked and utilized only within a certain window of the cell cycle. While the previous discussion of helicase activation has demonstrated the role of CDK and DDK in positively regulating initiation, a plethora of mechanisms exist to negatively regulate replication licensing and prevent re-replication as well. This section will discuss some general principles for preventing re-replication; although regulatory approaches show substantial evolutionary diversification and expansion, certain common themes do exist. For a more thorough discussion of the regulation of DNA replication initiation in eukaryotes, we

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point the reader to a number of dedicated reviews on the topic (see (Arias and Walter, 2007, Hook et al., 2007, Masai et al., 2010, Araki, 2010, Siddiqui et al., 2013)).

Initiator and helicase regulation

By temporally separating helicase loading from activation, S-CDK can simultaneously activate the helicase while also inhibiting helicase loading factors to prevent origin re-firing. A major means by which CDK protects against re-licensing in S-phase is to regulate initiator interactions and their association with chromatin. Although budding yeast ORC remains stably bound throughout the cell cycle (Diffley et al., 1994, Liang and Stillman, 1997, Fujita et al., 1998), vertebrate Orc1 is the target of CDK-dependent and CDK-independent mechanisms that remove it from chromatin (Figure 8) (Rowles et al., 1999, Findeisen et al., 1999, Natale et al., 2000, Li and DePamphilis, 2002, Li et al., 2004, Kreitz et al., 2001, Sun et al., 2002). In both yeast and metazoans, initiation factors are targeted for proteolysis in a CDK-dependent fashion, with budding yeast exclusively targeting Cdc6 (Piatti et al., 1995, Drury et al., 1997, Perkins et al., 2001, Mimura et al., 2004) and metazoans targeting both Orc1 and Cdc6 (Mendez et al., 2002, Tatsumi et al., 2003, Ohta et al., 2003, Lidonnici et al., 2004, Kalfalah et al., 2015). In *S. pombe*, Orc2 is also targeted by CDK to prevent re-licensing, but the mechanism utilized in this instance is unclear (Vas et al., 2001, Wuarin et al., 2002). In *S. cerevisiae*, CDK-dependent control is further extended to regulate Cdc6 transcription and nuclear localization (Moll et al., 1991, Honey and Futcher, 2007); metazoan Cdc6 likewise undergoes CDK-dependent nuclear export to limit replication (Saha et al., 1998, Jiang et al., 1999b, Petersen et al., 1999). Interestingly, budding yeast Orc6 and Cdc6 stably associate with CDK during S-phase and this interaction sterically inhibits interactions necessary for pre-RC assembly, such as with Cdt1 (Mimura et al., 2004, Wilmes et al., 2004, Chen and Bell, 2011). In metazoans, Cdt1 has not yet been reported to interact with Orc6.

In addition to regulating ORC and Cdc6, in *S. cerevisiae* CDK targets Mcm2-7 to prevent new, productive interactions with chromatin-bound ORC•Cdc6 complexes. This action occurs through the CDK-dependent nuclear exclusion and export of Mcm2-7 (Labib et al., 1999, Nguyen et al., 2001, Tanaka and Diffley, 2002, Liku et al., 2005). There is currently no evidence that CDK targets metazoan MCM2-7 directly, although phosphorylation of the initiator reduces helicase association with origins (Findeisen et al., 1999). Interestingly, a new, CDK-independent MCM regulatory mechanism has recently been identified in budding yeast that involves the SUMOylation of all members of the Mcm2-7 hexamer in G1. The SUMO modification in turn, through phosphatase recruitment, prevents phosphorylation-dependent helicase activation and thus negatively regulates initiation (Wei and Zhao, 2016). How the SUMO pathway integrates with the other mechanisms that regulate initiation is currently unclear.

Cdt1: a master regulatory nexus

Cdt1 plays an essential part in the loading of MCM2-7 onto DNA as a stable double hexamer. As such, Cdt1 turns out to be a common regulatory point. *S. cerevisiae* Cdt1 activity is restricted by the CDK-dependent inhibition of its interaction with Orc6 (Chen and Bell, 2011) and by nuclear export of Cdt1 in G1 (Tanaka and Diffley, 2002). Conversely,

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metazoan and *S. pombe* Cdt1 protein levels are regulated in a cell-cycle dependent fashion such that Cdt1 is actively degraded upon S-phase entry (Figure 8) (Wohlschlegel et al., 2000, Nishitani et al., 2000, Gopalakrishnan et al., 2001, Nishitani et al., 2001, Zhong et al., 2003, Nishitani et al., 2004). Degradation of Cdt1 is restricted to S-phase by an interaction with chromatin-bound PCNA, which serves as a platform for Cdt1 recognition and ubiquitination by the Cullin-RING ligase 4 (CRL4) ubiquitin ligase (Arias and Walter, 2005, Arias and Walter, 2006, Senga et al., 2006, Hu and Xiong, 2006, Guarino et al., 2011). A conserved PCNA-interacting protein (PIP) degron within the Cdt1 N-terminus facilitates the interaction with chromatin-bound PCNA and is required for degradation (Senga et al., 2006, Havens and Walter, 2009, Havens and Walter, 2011). Similarly, the CDK-dependent phosphorylation of Cdt1 leads to its recognition and ubiquitination by the SCF^{Skp2} E3 ubiquitin ligase, providing an additional mechanism to limit Cdt1 protein levels and prevent re-replication (Li et al., 2003, Liu et al., 2004, Sugimoto et al., 2004, Thomer et al., 2004, Kondo et al., 2004, Nishitani et al., 2006). Notably, Cdt1 ubiquitination can be reversed through the function of the ubiquitin hydrolase USP37, whose activity stabilizes Cdt1 and promotes helicase loading (Hernandez-Perez et al., 2016).

Metazoan Cdt1 is also uniquely regulated by binding to a partner protein, Geminin. Geminin is a coiled-coil protein (Saxena et al., 2004, Lee et al., 2004) that was initially identified in *Xenopus* egg extract screens for proteins destabilized in mitosis (McGarry and Kirschner, 1998). This study, along with many others, revealed that Geminin, a nuclear protein whose levels become elevated during S-phase, targets and restricts Cdt1 activity to G1, thus preventing Cdt1-induced re-replication (Quinn et al., 2001, Tada et al., 2001, Mihaylov et al., 2002, Cook et al., 2004, Yoshida et al., 2005, Lutzmann et al., 2006, Kerns et al., 2007). Geminin binds directly to Cdt1 (Wohlschlegel et al., 2000, Lutzmann et al., 2006, De Marco et al., 2009), and this interaction has been shown to inhibit Cdt1 binding to mouse MCM6 (Yanagi et al., 2002). Recent work demonstrates that Geminin binding to Cdt1 inhibits ORC-dependent helicase loading but not recruitment (Wu et al., 2014a), a finding consistent with studies in budding yeast showing Cdt1-independent recruitment of the helicase to the origin-bound initiator (Frigola et al., 2013, Fernandez-Cid et al., 2013).

Interestingly, Geminin serves two seemingly dichotomous functions. Although clearly an inhibitor of licensing, Geminin is also required for replication initiation through its ability to stabilize Cdt1 levels by protecting the protein from degradation (Ballabeni et al., 2004, Narasimhachar and Coue, 2009). Thus, metazoans regulate Geminin protein levels and cellular localization to liberate Cdt1 from Geminin and promote pre-RC assembly in G1 (McGarry and Kirschner, 1998, Tsunematsu et al., 2013, Dimaki et al., 2013). In addition, the stoichiometry of the Cdt1•Geminin complex has been suggested to regulate Geminin's activity, such that a lower order Cdt1:Geminin complex (1:2) is permissive to pre-RC assembly whereas a higher order (2:4) complex is not (De Marco et al., 2009). How this stoichiometry is regulated in the context of the other regulatory mechanisms governing Geminin and Cdt1 protein levels and cellular localization is not known.

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Concluding remarks

At this point, many of the major events and intermediates that facilitate pre-RC and pre-IC assembly have been defined. However, between these stable intermediates there exist multiple, undefined dynamic and transient protein interactions, modifications, and exchanges that represent critical steps towards building a replisome. The questions that remain are both broad and specific: how does ORC balance sequence preference and *trans*-acting chromatin contextual cues when selecting origins? What is the role of Cdt1 in MCM2-7 loading and, given the minimal conservation of this protein, is Cdt1 mechanism conserved across eukarya? Does MCM2-7 melt origins and unwind duplex DNA (as part of the CMG) using an overlapping or mutually exclusive set of protein-nucleic acid interactions, and how is this functional switch regulated? What type of molecular dance must take place to transition the pre-RC product, the double-stranded DNA-bound MCM2-7 double hexamer, into the CMG, and then again into a bi-directional replication fork defined by two single helicases bound to single-stranded DNA? It will be critical to address these and other questions in multiple model eukaryotic organisms to begin to understand what level of conservation can be expected for such a complex process. Future work that aims to resolve these and other questions will undoubtedly reveal exciting new mechanisms that underlie the highly orchestrated and regulated process of replication initiation in eukarya.

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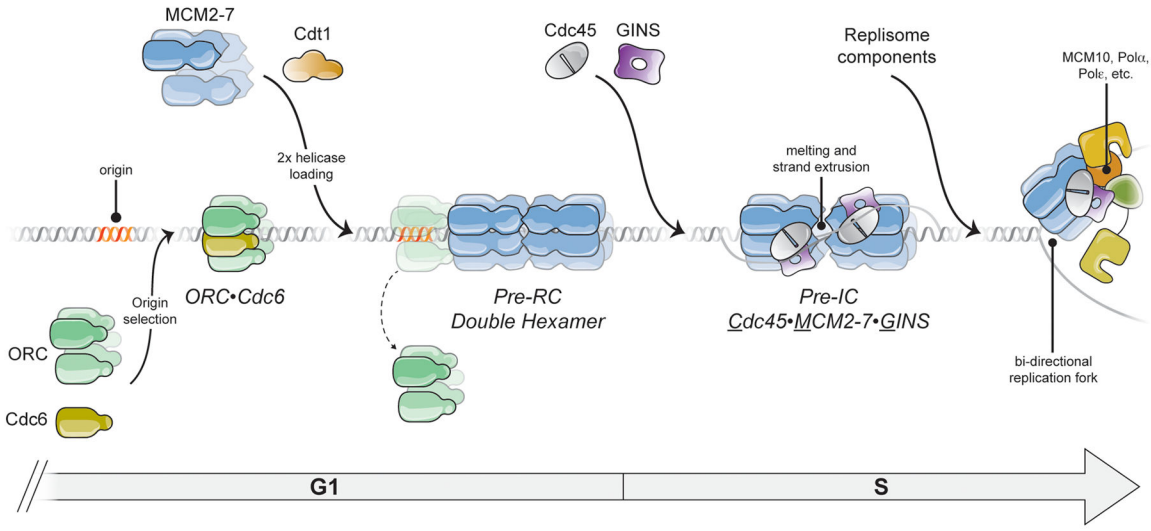

Figure 1.

Mechanistic outline of DNA replication initiation in eukarya. During the G1 phase of the cell cycle, an origin-bound ORC•Cdc6 complex together with Cdt1 facilitates the sequential recruitment and loading of two MCM2-7 complexes into a stable double hexamer that encircles duplex DNA (pre-RC). At the onset of S phase, the helicase is activated, leading to origin unwinding. The recruitment of other initiation factors (Cdc45 and GINS, the pre-IC) and double-hexamer dissolution activate the helicase to drive fork progression as a single-stranded DNA-bound Cdc45•MCM2-7•GINS (CMG) complex. A color version of this figure is available online.

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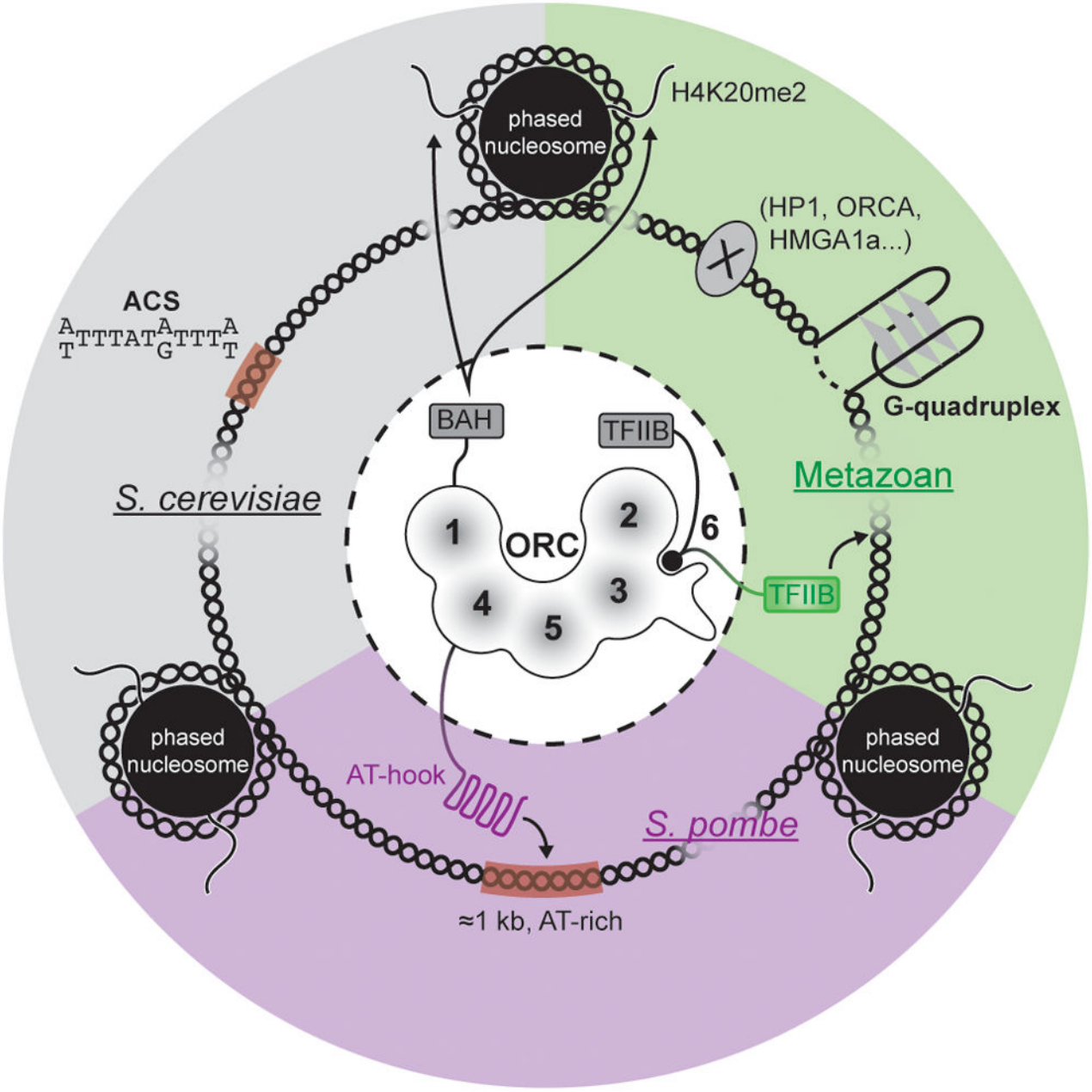

Figure 2.

Molecular details of eukaryotic origins and mechanisms of ORC binding. (GREY) *S. cerevisiae* origins are distinctive among eukaryotes for conforming to a consensus sequence, the ACS. *Sc*ORC can bind the ACS directly and specifically, although interactions between the Orc1-BAH domain and nucleosomes can also modulate ORC origin selection. (PURPLE) Although they do not possess a strict consensus sequence, *S. pombe* origins are AT-rich. *Sp*ORC specifically binds such sites using a domain insertion unique to SpOrc4 that encodes a DNA-binding AT-hook motif. (GREEN) Metazoan ORC can be targeted to chromosomes through a variety of mechanisms, including the Orc1 BAH domain, the DNA-binding TFIIB domain of Orc6, and through interactions with chromatin-associated factors.

A majority of metazoan origins are also predicted to contain G-quadruplex secondary structure elements, but how this feature affects ORC binding is currently unclear. A color version of this figure is available online.

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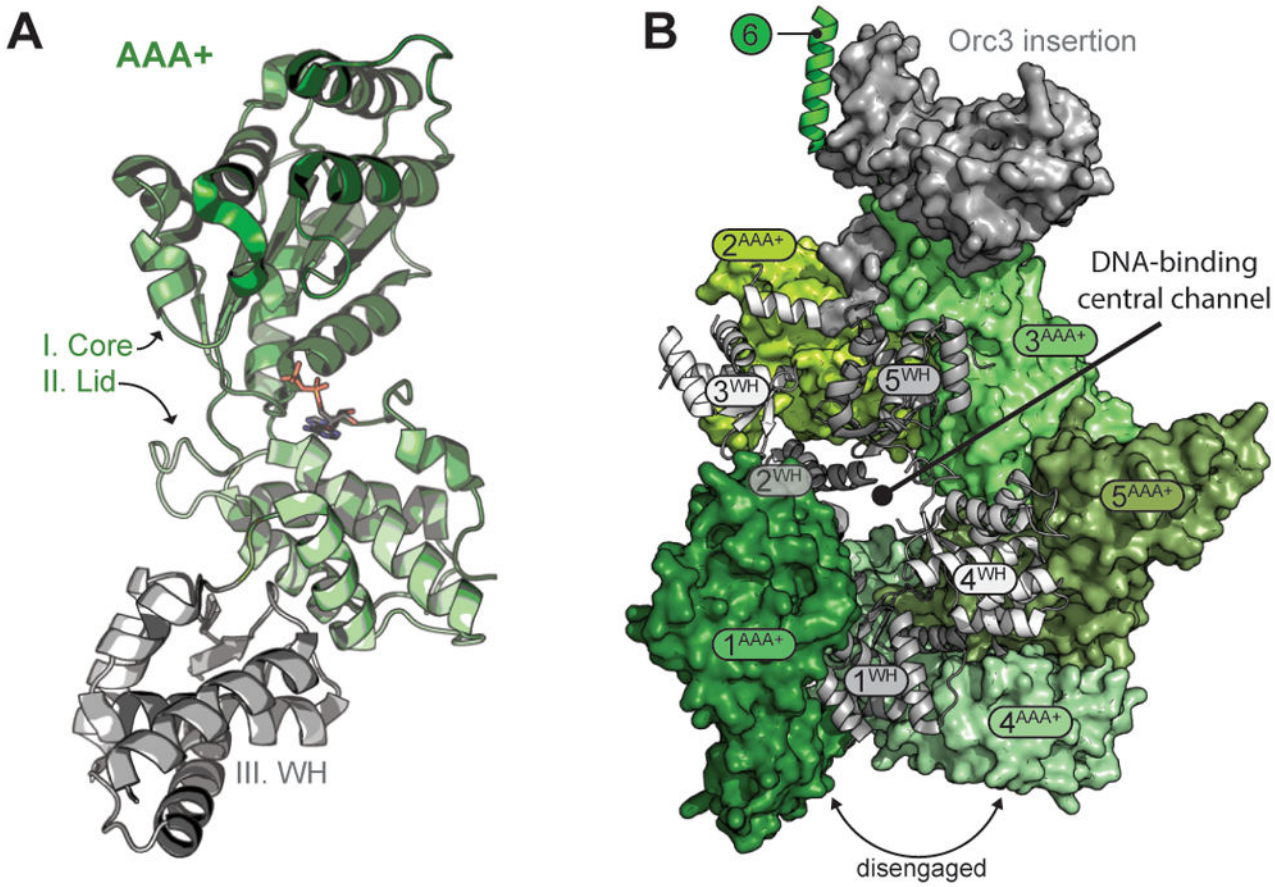

Figure 3.

ORC architecture. A) Cdc6/Orc homologs are characterized by three domains, two of which form the AAA+ module (green) and a third that encodes a winged-helix (WH) domain (grey). Bound nucleotide is shown as sticks (PDB = 1FNN). B) The *D. melanogaster* ORC heterohexamer is a crescent shaped molecule with the AAA+ (green surface) and WH (grey cartoon) domains forming a domain-swapped arrangement. Orc6 is bound by a domain insertion in the AAA+ domain of Orc3. Although the Orc1/Orc4 active site is required for activity, in the *D. melanogaster* structure Orc1 is disengaged from Orc4 and positioned above the plane of the AAA+ ring (PDB = 4XGC). A color version of this figure is available online.

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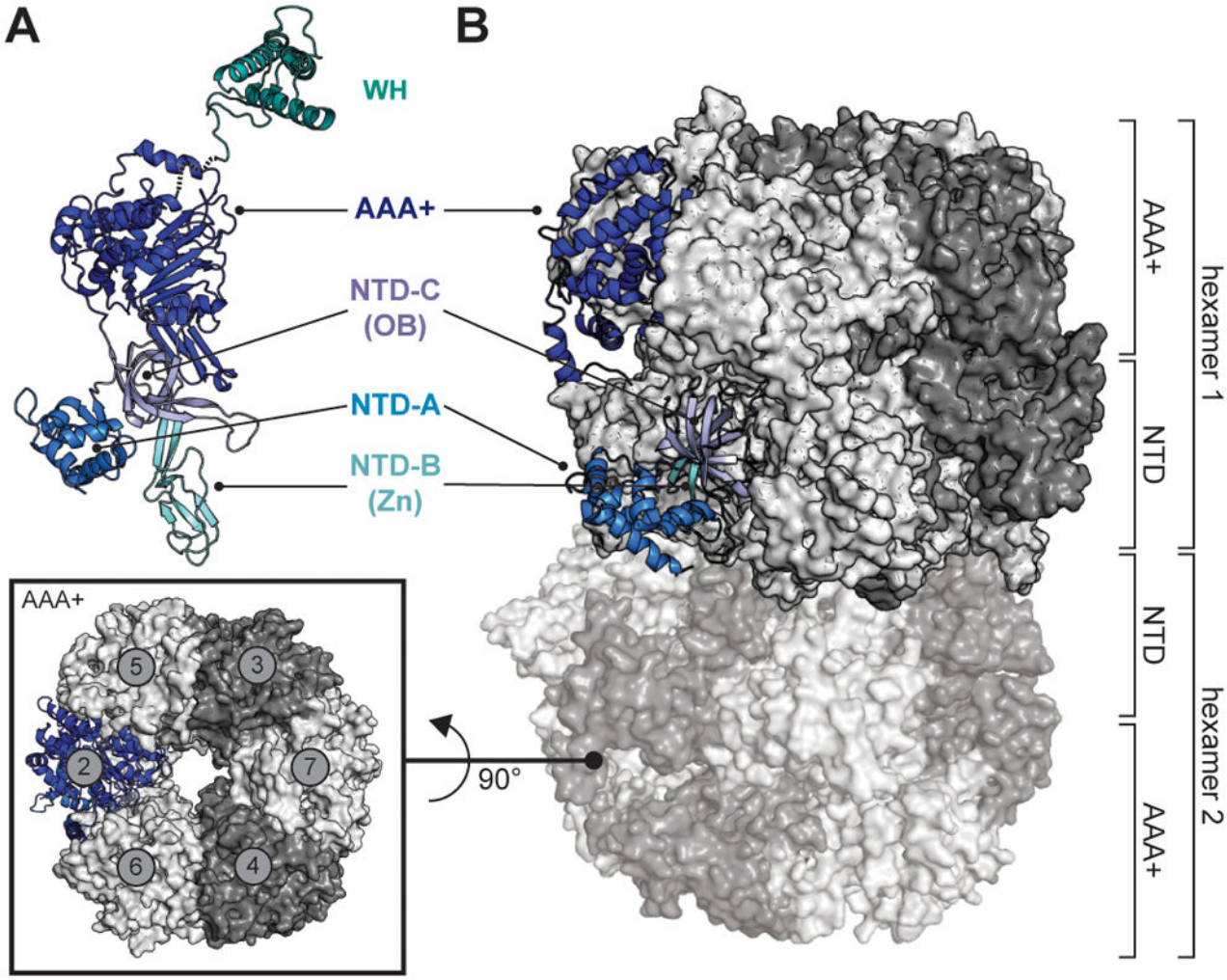

Figure 4.

MCM architecture. A) MCM homologs are characterized by three domains: NTD, AAA+, and CTD. The NTD can be subdivided into NTD-A (a small helical bundle), NTD-B (Zn-finger), and NTD-C (OB fold). The CTD forms a WH domain (for AAA+ and NTD, PDB = 3F9V; for WH domain, PDB = 2KLQ). B) Two physiologically relevant MCM oligomers have been observed, a hexamer that is formed by lateral interactions between the AAA+ and NTD domains of adjacent protomers, and a double hexamer that is formed by interactions between the NTD-B Zn-finger domains of two MCM2-7 rings. The double hexamer structure from S. cerevisiae Mcm2-7 is shown (Li et al., 2015), with one hexamer faded compared to the other. The inset shows a top-down view through the central cavity and the radial arrangement of eukaryotic MCM2-7 subunits (the double hexamer was built from two copies of PDB = 3JA8 fit to the EM density map EMD-6338 (Li et al., 2015)). A color version of this figure is available online.

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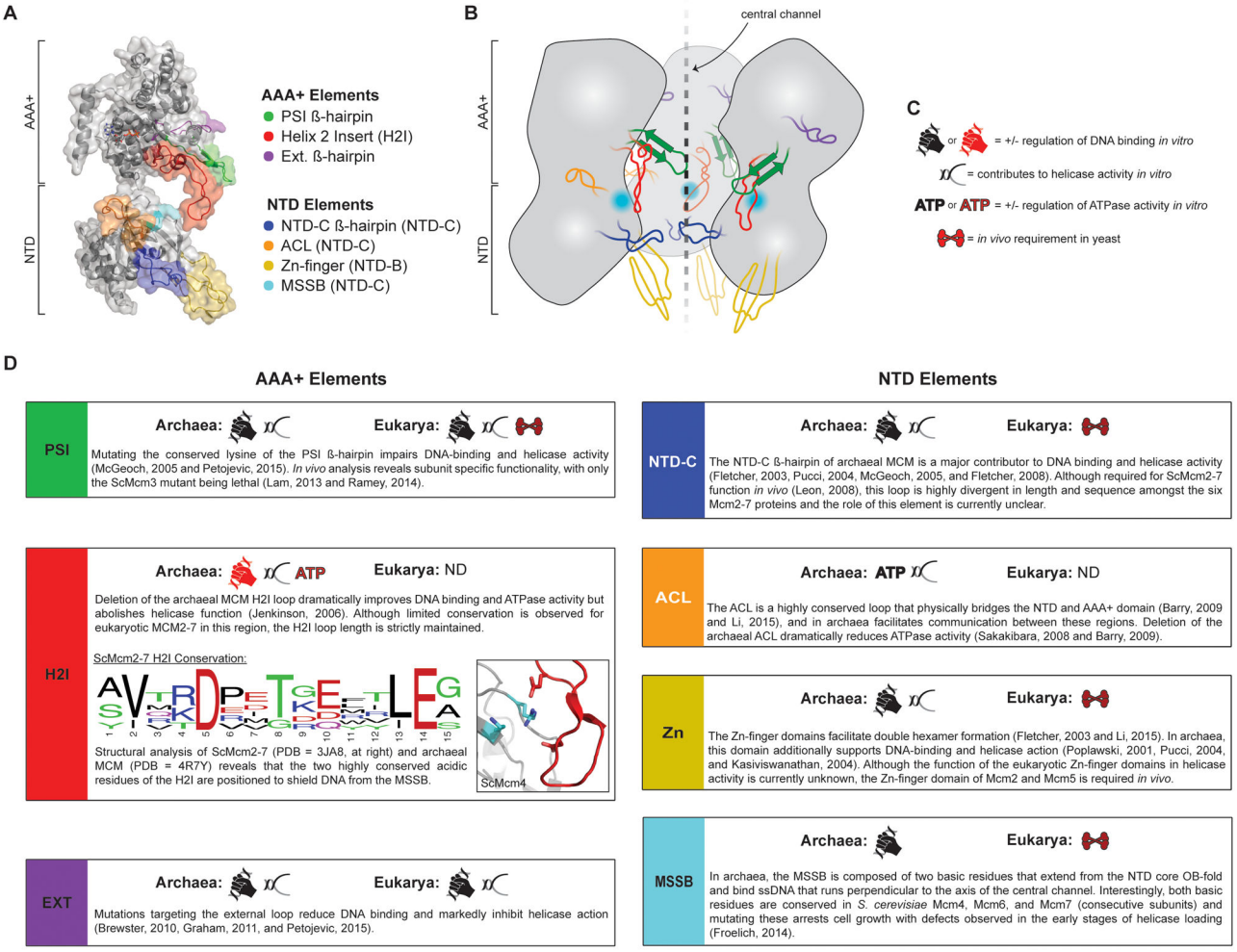

Figure 5.

Functional elements of MCM helicases. A) Each MCM monomer contains multiple functional elements, including DNA-binding/sensing motifs, regions that modulate ATPase activity, and loops that communicate between the NTD and AAA+ domain (PDB = 3JA8, chain 2). B) In the context of a hexamer, the MCM functional elements (excepting the external β-hairpin) line a central cavity through which DNA translocates (modeled after PDB = 3JA8, chains 4, 6, and 7). C) Symbol key. D) Detailed functional description for each MCM element known to contribute to activity. A color version of this figure is available online.

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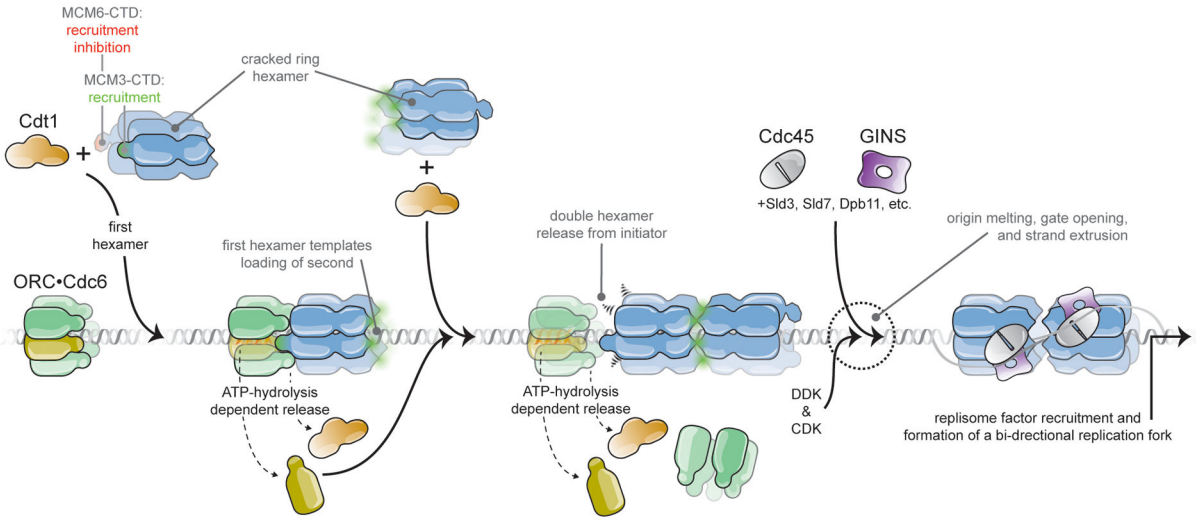

Figure 6.

MCM2-7 complex loading and maturation into the CMG. Two sequential rounds of helicase recruitment and loading at origins is required for building an MCM2-7 double-hexamer. For both hexamers, DNA is threaded into the central channel through a discontinuity between MCM2 and MCM5. The first hexamer is recruited through direct interactions with the initiator (MCM3-Cdc6) and may require Cdt1 for overcoming an MCM6-mediated autoinhibited state of the helicase. After the first hexamer loads, both Cdc6 and Cdt1 are released. Rebinding of Cdc6 to ORC primes the system for recruiting and loading a second hexamer in the opposite direction as the first, an event that has been proposed to be controlled by ORC•Cdc6, but templated by the first MCM2-7 hexamer. Cdt1 and Cdc6 recruitment and ejection are required for both loading events. Phosphorylation of the double hexamer and other initiation factors by CDK and DDK facilitate origin melting, GINS and Cdc45 recruitment/assembly, DNA strand extrusion, and activation of the helicase for DNA unwinding. A color version of this figure is available online.

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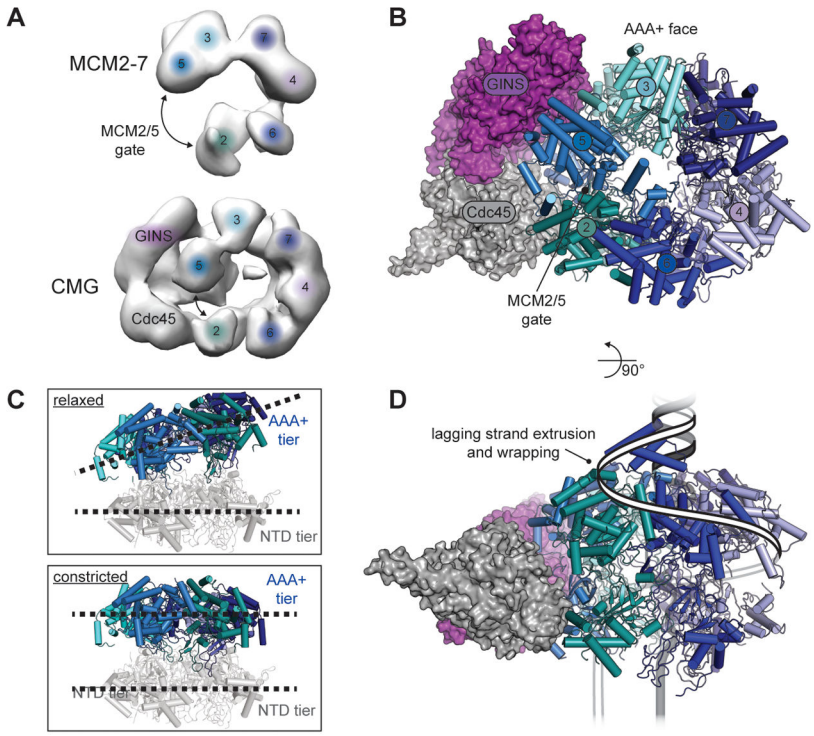

Figure 7.

CMG helicase organization and dynamics. (A) MCM2-7 adopts a spiral, cracked-ring architecture with a discontinuity between MCM2 and MCM5. Upon incorporation into the CMG, Cdc45 and GINS convert the helicase into a planar form and seal off the MCM2/5 gate (EM density maps = EMD-1835 and EMD-1833 for MCM2-7 and CMG, respectively). (B) A view of the CMG from the AAA+ face illustrating the overall architecture of the complex (PDB = 3JC5 (Yuan et al., 2016)). (C) At least two conformational states exist for the CMG, a constricted state in which the AAA+ and NTD rings are coplanar (bottom panel), and a relaxed state where one end of the AAA+ tier lifts up from NTD tier (top panel). These conformations appear coupled to alterations in the relative disposition of the gating subunits, MCM2 and MCM5 (constricted and relaxed conformer PDB codes = 3JC5 and 3JC7, respectively). (D) The activated CMG helicase is thought to translocate along single-stranded DNA, unwinding downstream template through a combined steric exclusion and DNA wrapping mechanism. A color version of this figure is available online.

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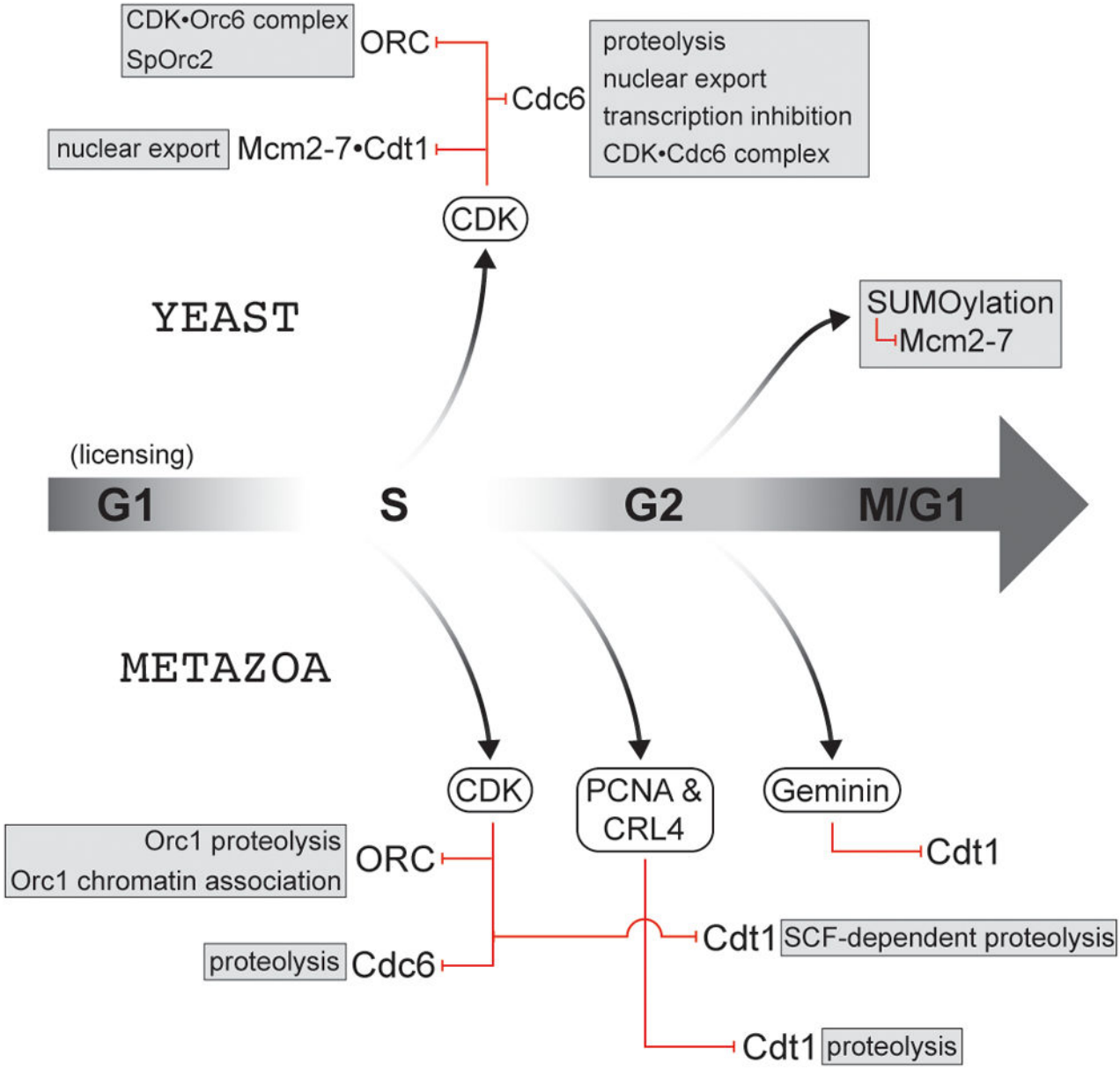

Figure 8.

Mechanisms to prevent re-replication. Multiple, redundant mechanisms are utilized to prevent re-licensing of origins after S-phase has initiated. Whereas yeast seem to exclusively utilize CDK-dependent mechanisms to prevent re-licensing, metazoans also employ CDK-independent pathways for negatively regulating Cdt1 activity. A color version of this figure is available online.