Chapter 9
The Origin Recognition Complex in the Initiation of DNA Replication

Timothy Hoggard and Catherine A. Fox

Abstract The origin recognition complex (ORC) is the six-subunit heteromeric protein complex that binds directly to the positions on eukaryotic chromosomes where the protein-DNA complex required for the activation of DNA replication origins assembles. This protein-DNA complex, named the MCM complex, contains the replicative helicases and performs the actual unwinding of the parental DNA duplex (a.k.a. origin activation or firing) at the origin. While there is no evidence that ORC is required for origin unwinding directly, it is nevertheless essential for origin activation because of its key role in the MCM complex loading reaction. ORC loads the MCM complex onto chromosomal DNA during G1-phase, thus "licensing" origins for activation in the subsequent S-phase. To perform this role, ORC directly contacts double-stranded DNA, positioning the substrate onto which the MCM complex must load as well as providing a protein interaction surface to guide MCM complex loading. ORC binds and hydrolyzes ATP, and uses these steps to alter its conformation. These conformational changes in turn alter ORC's ability to interact with both DNA and partner proteins to achieve a complete MCM complex loading cycle. Other chapters in this volume focus on the steps of the MCM complex loading reaction (a.k.a. pre-RC formation; origin licensing). While a discussion of the ORC's role in replication initiation naturally requires consideration of this reaction, this chapter emphasizes the current understanding of the structural and functional features of ORC that must impinge on MCM complex loading, and perhaps other as yet undiscovered roles for ORC in DNA replication.

Keywords ORC • Origins • pre-RC • Cell cycle • MCM

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A Brief History

The ORC was identified in *Saccharomyces cerevisiae* (herein referred to as *Sc*ORC) as a stable, six-subunit heteromer that binds to an essential element within budding yeast origin DNA [1]. This element includes a conserved sequence called the ACS, for *A*RS *c*onsensus *s*equence, because it can be found in all chromosomal fragments that can provide for autonomous replication of bacterial plasmids in budding yeast [2, 3]. The ACS is required for yeast origin function in both plasmid and native chromosomal contexts [4–8]. Thus by analogy to the prokaryotic cellular initiator dnaA binding to the *E. coli* chromosomal origin, oriC, and several viral initiators binding to their cognate sites, there was strong reason to hypothesize that the ACS was a prime target for the binding of the eukaryotic initiator. The importance of having a defined, functional sequence linked to origins cannot be overemphasized—in no other eukaryote were origins defined at a level to be useful for this type of direct biochemistry and this fact remains true. The identification of *Sc*ORC as a protein complex that protected the ACS, by a DNaseI footprinting assay, and required it for DNA binding therefore provided compelling evidence that it was, or was strongly linked to, the eukaryotic initiator that functioned directly at the DNA replication origins of the cellular chromosomes of eukaryotes. *Sc*ORC was also intriguing because it bound ATP, indicating another similarity to previously characterized bacterial and viral initiators (reviewed in [9]). Following *Sc*ORC's biochemical discovery, temperature-sensitive mutant alleles of two *Sc*ORC genes, *ScORC2* and *ScORC5*, were isolated in forward genetic screens, allowing for their rapid cloning [10–12]. *ScORC6* was identified and cloned using a one-hybrid protein-DNA interaction assay, and the remaining yeast ORC genes were cloned using degenerate primers designed from protein sequence [13, 14]. Orthologs of each of the six *Sc*ORC genes have since been identified in many other organisms, and sequence and structural information about ORC subunits from archeal and eukaryotic model organisms, as described in the following sections, extend the analogy between ORC and other initiators of DNA replication to the structural level (discussed in several reviews [15–17]).

Primary and Secondary Structural Features of ORC Subunits

The ORC is a complex comprised of six distinct subunits. To understand how these individual subunits interact to form a functional ORC, this section will first consider what is known about some of the key primary and secondary features of the subunits. Five of the six *Sc*ORC subunits, Orc1-5 contain a region with substantial sequence similarities to the defining domain of the AAA+ family of ATP-binding and hydrolysis proteins and these subunits will be discussed together [16, 18]. Orc6, which has a distinct structure, will be discussed separately.

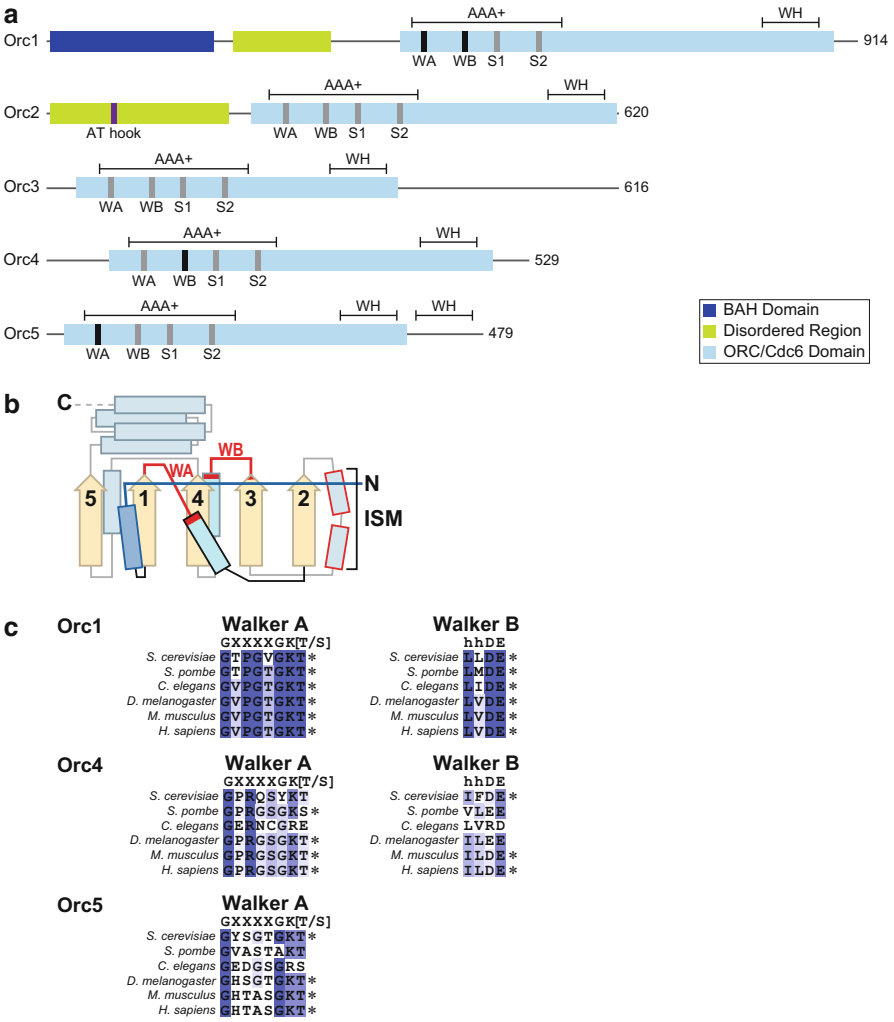

Fig. 9.1 (**a**) Schematics of primary structure of *S. cerevisiae* Orc1-5 subunits adapted from [16]. Perfects matches to Walker A (WA) and B (WB) motifs are colored in *black*. Deviations from consensus motif signatures of the AAA+ domain are colored in *grey*. (**b**) Schematic of the AAA+ secondary fold for the Initiator clade adapted from [19]. WA and WB motif positions indicated in *red*, and the Initiator Specific Motif (ISM) (two similarly sized alpha helices following beta strand 2) is outlined in *red*. (**c**) Alignments of the WA and WB motifs of Orc1, Orc4, and Orc5

The primary structure of the five AAA+ *Sc*ORC subunits is diagrammed in Fig. 9.1a. Each subunit is named based on its relative molecular weight, so Orc1 is the largest subunit and Orc6 is the smallest subunit of ORC. This size differential is generally conserved, though in metazoans Orc3 is similar in size or slightly larger than Orc2, and metazoan Orc6 is considerably smaller than *Sc*Orc6. Orc1-5, and

particularly Orc1, share sequence similarity with Cdc6, a seventh AAA+ protein that joins with ORC in G1-phase to form the active AAA+ oligomeric core bound to origin DNA. It is this ORC-Cdc6-DNA complex that functions to load the MCM complex [18].

The AAA+ Domain

Because the AAA+ domains of Orc1-5 subunits and Cdc6 together form the active MCM complex loading machine, a discussion of this domain in general and its variation within individual Orc subunits is warranted. AAA+ (*A*TPases *A*ssociated with diverse cellular *A*ctivities) proteins define a superfamily of proteins that share features of ATP binding and hydrolysis that allow them to participate in a vast array of cellular processes [18]. Both primary and secondary structural characteristics define this domain [19]. Key primary characteristics include the Walker A and Walker B motifs, associated with ATP binding and hydrolysis, respectively (Fig. 9.1). The key secondary characteristic is the core sheet of parallel beta strands arranged in a 51432 topology (Fig. 9.1b). While Orc1-5 share secondary and primary features with the AAA+ family, there is variation among these subunits with respect to the catalytic core motifs within the Walker A and Walker B boxes both within and between species [18] (Fig. 9.1c). In budding yeast, only two subunits, *Sc*Orc1 and *Sc*Orc5, contain perfect matches to the Walker A signature (GXXXXGK[T/S]), consistent with the experimental finding that only the Orc1 and Orc5 subunits of *Sc*ORC bind ATP [20]. In metazoans, a perfect match to the Walker A signature can also be found in Orc4. Metazoan Orc1, Orc4, and Orc5 each bind ATP (Figs. 9.1c and 9.2) [21, 22].

While *Sc*Orc1-5 each contain a recognizable AAA+ domain, and both *Sc*Orc1 and *Sc*Orc5 can bind ATP, only *Sc*Orc1 contains a perfect match to the Walker B signature, hydrophobic-hydrophobic-DE (hhDE). Consistent with this observation, Orc1 is the subunit within *Sc*ORC that hydrolyzes ATP and in fact is the major ATPase of ORC in all eukaryotes examined. Interestingly, Orc2 contains a highly conserved sequence at the predicted position of the Walker A motif, though it does not match the canonical Walker A motif, suggesting that it performs an important but as yet undefined function of ORC [18]. Orc3 is the most diverged of the Orc1-5 subunits [16, 18]. In summary, experimental work, including a recently solved crystal structure of *Drosophila melanogaster* ORC (*Dm*ORC), and sequence conservation provide strong evidence that the AAA+ domains of the Orc1-5 subunits drive the assembly of a functional complex [23]. The fundamental role of ATP binding and hydrolysis by ORC that is essential to MCM complex loading onto chromosomal DNA is performed by the Orc1 subunit.

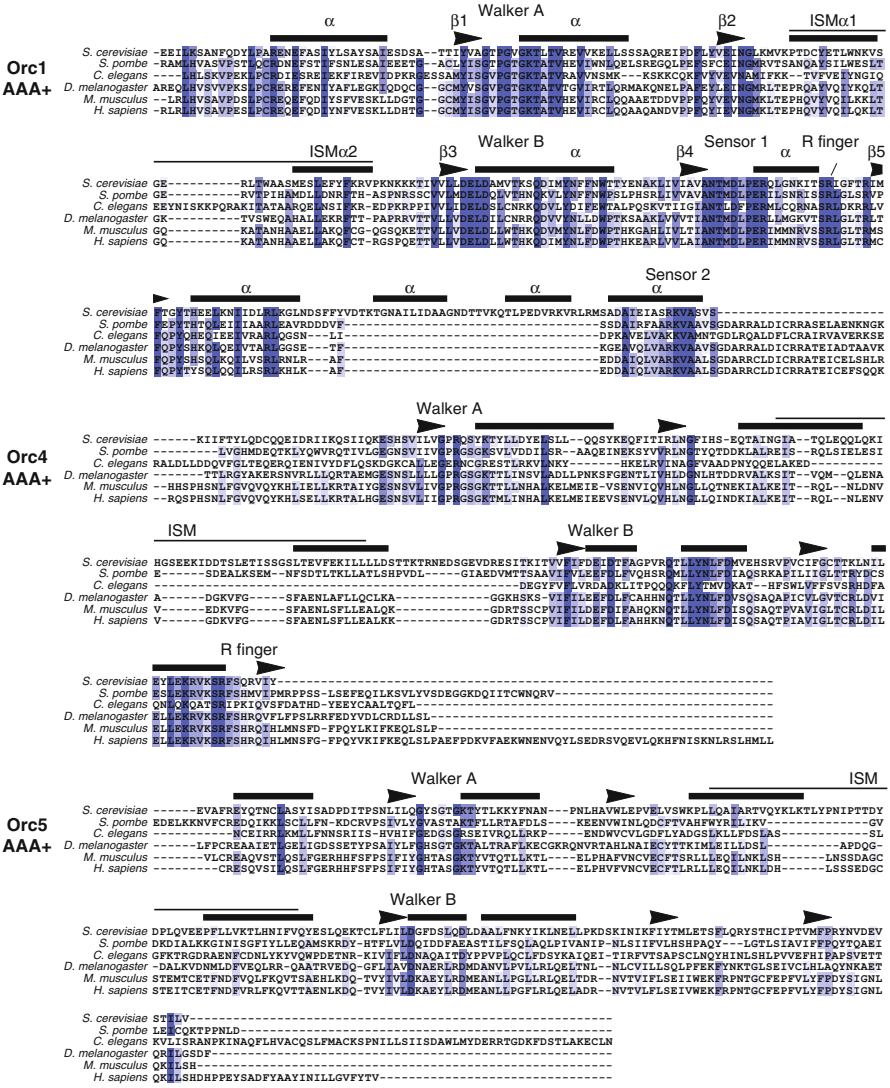

Fig. 9.2 Alignments of the AAA+ domains of Orc1, Orc4, and Orc5: Positions of relative amino acids are indicated. For Orc1 the beta strands are numbered to aid in comparison to Fig. 9.1b

Initiator Specific Motif (ISM) and Winged-Helix (WH) Domain

Fundamental to ORC's catalytic role in loading the MCM complex is its ability to directly contact double-stranded DNA. By binding DNA, ORC serves to position it for MCM complex loading. Two lines of evidence reveal that these ORC-DNA contacts, which do not necessarily play a role in the selection of *specific* chromosomal sites by ORC (see below), are likely performed by two motifs present in the Orc1-5

subunits. One motif is the ISM that is part of the AAA+ fold in these subunits (Fig. 9.1b). Specifically, the Orc1-5 subunits are members of the DnaA/Cdc6/ORC Initiator clade within the AAA+ superfamily whose defining feature is the presence of an extra helical insertion, resulting in two similarly sized alpha helices after strand 2 [9, 10, 19] (Fig. 9.1b). In dnaA, the bacterial initiator, the ISM forms a wedge structure that helps push the subunits of the dnaA homo-oligomer into a spiral that binds single-stranded DNA (ssDNA) and thus contributes to origin DNA melting [9, 24, 25]. In an archeal Orc1 (*S. solfataricus*) the ISM forms a different, more parallel arrangement that contacts double-stranded DNA [26]. It has been proposed that the eukaryotic Orc1-5 subunits contain similar ISMs that adopt a conformation in which each subunit ISM interfaces with the duplex DNA (dsDNA) substrate [9, 26, 27]. This conformation may be adapted for the role of ORC/Cdc6 in stabilizing both the DNA substrate and the MCM helicase for loading onto double-stranded DNA [28]. Notably, the recently solved crystal structure of *Dm*ORC, which will be discussed more in sections that follow, and modeling using the *S. solfataricus* Orc1-1 structure, reveals a central channel through the *Dm*ORC that forms from the ISMs of Orc2-5 and the β-hairpin wings of the WH domains of Orc1 and Orc3-5 that can fully accommodate dsDNA [23].

It is worth noting here that in vitro and in the absence of ATP, *Sc*ORC can bind single-stranded DNA (ssDNA) and does so in a distinct conformation, indicating that there are forms of *Sc*ORC that favor ssDNA binding [29]. However, no biological role for this interaction has been demonstrated and current models do not predict that such an activity is necessary for the MCM complex loading reaction [30–32].

The second motif likely involved in catalytically relevant DNA contacts (i.e., regulation of ATPase activity; regulation of MCM complex loading) is the winged-helix (WH) DNA binding domain. The Orc1-5 subunits contain regions predicted to form winged-helix (WH) DNA binding domains C-terminal to their AAA+ domains (Fig. 9.1a). The WH domains, predicted based on a secondary structural signature, can be found in multiple Orc orthologs, including archeal Orc1/Cdc6 orthologs. For archeal Orc1s, the WH domains participate in extensive interactions with DNA and contribute to sequence-specific binding, although an unexpectedly small number of protein side-chain-DNA-base contacts suggest that context or deformability plays a larger role in origin specificity than sequence [26, 27, 33]. Regardless, these observations led to the obvious hypothesis that the WH domains of eukaryotic ORC subunits contact DNA in a similar manner to archeal Orc1 that forms a lobster claw-like structure, with the ISM and WH domains forming the two halves of the claw that bind DNA. However, the WH domains of eukaryotic ORCs have not been studied in great detail, perhaps because they do not appear to contribute substantially to ORC-origin specificity across species. Interestingly, while the isolated WH domains of human Orc1 (*Hs*Orc1) bind DNA, the WH domain of *Sc*Orc1 does not but instead forms oligomers in solution [34]. It is posited that the WH domain of *Sc*Orc1 may have evolved instead for protein-protein interactions important to the mechanism of transcriptional silencing. After the genome duplication in budding yeast, the *SIR3* gene, an *ORC1* paralog, evolved for dedicated transcriptional silencing, and indeed the Sir3 WH domain is important for both Sir3 oligomerization and silencing in

yeast [34, 35]. Nevertheless, these observations indicate that a WH domain of an ORC subunit can function in mediating protein-protein interactions. The recent crystal structure of *Dm*ORC reveals that the WH domains of the Orc1-5 subunits are integral to the stability of the complex because they mediate extensive contacts with the AAA+ domains on adjacent subunits [23]. In fact these contacts rely on the canonical DNA recognition helices of the WH domains interacting extensively with the AAA+ domains and thus made unavailable for DNA binding.

Other DNA Binding Domains in ORC

The ORC-DNA contacts mediated by the ISM and WH domains discussed above are likely conserved and critical for the catalytic role of ORC in loading the MCM complex onto DNA. However, ORC is also important for selecting specific positions on chromosomes to serve as MCM complex loading sites. It is likely that for most eukaryotic ORCs, with the possible exception of budding yeast, that these ORC-DNA contacts are quite distinct from the contacts discussed above that are intimately connected to ORC's mechanism in MCM complex loading. In addition, it appears that these types of contacts are not well conserved. For example, *S. pombe* Orc4 (*Sp*Orc4) contains a distinct N-terminal extension with nine AT-hook motifs important for ORC-origin binding and replication function in vivo, but the binding mediated by the AT-hook appears to be separable from tighter ORC-DNA interactions needed for ORC's catalytic function [36, 37]. *Sc*ORC is the only eukaryotic ORC that shows sequence-specific DNA binding to origins in vitro and this binding, but not nonspecific DNA binding, can regulate the Orc1 ATPase activity [20]. Thus for *Sc*ORC, there appears to exist a connection or overlap between ORC-DNA interactions critical for ORC's selection of chromosomal origins and ORC-DNA interactions with functional roles in MCM complex loading. However even in budding yeast, recent in vitro studies indicate that the specific sequence of yeast origin DNA, that is the conserved ORC binding site, is not intrinsically essential to ORC's ability to establish functional replication origins [38, 39]. Regardless the mechanism by which particular ORC-DNA interactions function "catalytically" in the MCM complex loading reaction and their relationship to ORC-origin interactions required primarily for localizing ORC to appropriate chromosomal regions for this reaction to occur remain open questions. These questions have been raised to a new level of interest by the crystal structure of *Dm*ORC that suggests a stable form of the ATP-bound complex exists in an auto-inhibited form that prevents ORC from engaging with the double-stranded DNA in a catalytically useful way [23]. In particular, perhaps ORC-DNA, or ORC-chromatin contacts important for origin selection by ORC help relieve this auto-inhibited form of ORC such that it can encircle dsDNA.

For metazoans, the most prominent models for origin selection by ORC invoke specific interactions between certain types of chromatin structures and ORC, as described below and later in this review. Thus the prominent model is that origin

selection mechanisms in metazoan cells do not involve substantial and direct sequence-specific contacts between ORC and origin DNA. However, a recent study of *Hs*ORC-DNA binding challenges an overly rigid take on this model [40]. In particular, *Hs*ORC, via discrete region of the Orc1 protein N-terminal to the AAA+ domain, shows in vitro binding preference for G-quadruplex (G4)-preferable G-rich ssDNA or RNA. Notably this observation is not the first example of ORC-origin interactions involving nucleic acids other than dsDNA [41–44]. However, what is particularly significant about these biochemical observations is that G4-preferable elements are common features in human DNA replication origins [45–47]. In summary there appear to be multiple acceptable mechanisms for localizing ORC to chromosomal regions in a way that will allow for catalytically relevant ORC-DNA contacts to form subsequently that direct the MCM complex loading reaction. It is possible that these differing selection mechanisms influence ORC dynamics with the DNA in a manner that is relevant to the efficiency of MCM complex loading, as discussed later.

Orc1 Bromo Adjacent Homology (BAH) Domain

Many observations suggest that metazoan ORC relies on specific contacts with chromatin to select and bind to the chromosomal positions that will serve as MCM complex loading sites. The largest subunit of Orc1 contains a BAH domain, a protein module found in many nuclear proteins that likely functions, at least in part, through direct interactions with nucleosomes [48–51]. This domain is defined primarily at the level of secondary structure. While it can be found in most Orc1 orthologs, from yeast to humans, there is limited conservation between yeast and metazoan Orc1BAH domains at the sequence level (Fig. 9.3). Nevertheless accumulating evidence supports a role for the Orc1BAH domain in ORC-chromatin interactions in both metazoans and yeast.

In human cells the Orc1BAH domain contributes to re-binding of Orc1 to chromosomes in G1-phase and the replication of a plasmid that depends on the Epstein-Barr virus oriP [52]. The metazoan Orc1BAH domain binds a histone H3 N-terminal peptide dimethylated on lysine 20 (H3K20me2) [51] (Fig. 9.3). Mutations that cause defects in this interaction reduce ORC binding to an origin in vivo and cause growth defects in a zebrafish model that mimic Meier-Gorlin Syndrome (MGS), a form of human dwarfism associated with defects in several proteins that function in pre-RC assembly [53]. These data provide a direct link between ORC and specific chromatin modifications relevant to ORC-origin binding. More recent studies demonstrate that the MGS-associated substitution in the Orc1BAH domain, R105Q, reduces an Orc1BAH-nucleosome interaction in vitro [54]. All together, these data provide evidence that a specific metazoan Orc1-BAH-nucleosome interaction is important for ORC-chromatin binding and ORC's replication function in metazoans.

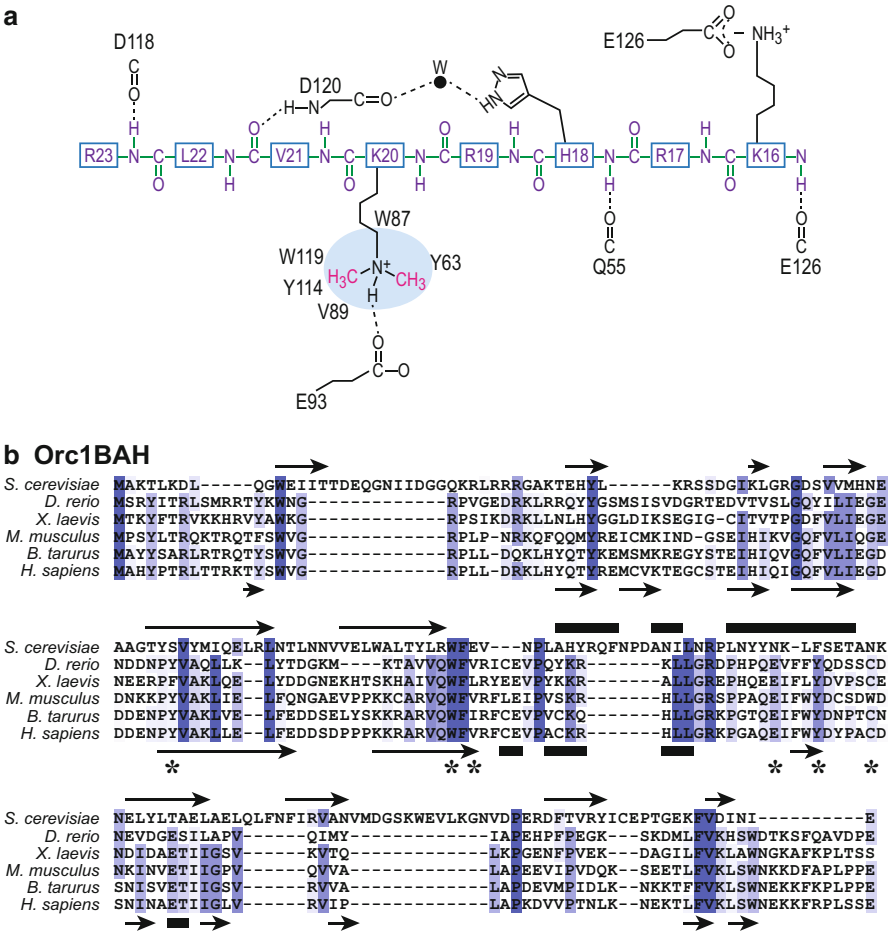

Fig. 9.3 (**a**) Schematic of the H3K20me2 peptide interactions with the Orc1BAH domain adapted from [51]. (**b**) Alignments of the Orc1BAH domains from the indicated species, *asterisks* refer to key residues involved in forming the hydrophobic cage that binds K20me2 on histone H3 peptide

The paradigm for *Sc*ORC-origin binding is that interactions between ORC and specific sequences within yeast origin DNA are key determinants. While this model is generally correct and contributes predictive power with respect to identifying yeast origins, accumulating evidence reveals that ORC-chromatin interactions likely play a positive role in yeast ORC-origin binding just as they do in metazoans [55, 56]. While the yeast Orc1BAH domain does not bind H3K20me2, it does bind chromatin [50]. Given recent structural studies of the similar Sir3BAH domain bound to nucleosomes, it is likely that the yeast Orc1BAH domain binds nucleosomes directly [48]. In addition, while a yeast mutant lacking the Orc1BAH domain (i.e., *ORC-bahΔ*) shows no growth defects, ORC association with chromatin is reduced, and multiple individual origins show substantially reduced ORC binding

and defects in activation in vivo [14, 56]. Thus the *Sc*Orc1BAH domain is important for ORC-origin binding, but ORC-origin binding capability must exist in excess of what is required for yeast cell viability. Consistent with this interpretation, viable mutations in genes encoding proteins that participate in pre-RC assembly are synthetically lethal when combined with an *orc1bahΔ* mutation [56]. However, no specific *Sc*Orc1BAH-histone interaction has been reported, and *Sc*ORC lacking an Orc1BAH domain can still bind origins in chromatin in vitro, though this result may indicate that the appropriate *Sc*Orc1BAH-dependent nucleosome configuration or modification is currently missing from the in vitro system and/or that other regions of *Sc*ORC may contact chromatin to facilitate origin binding [56–58]. While the specifics of the molecular interactions may differ, these observations provide evidence that the Orc1BAH domain is a conserved chromatin-binding module from yeast to humans important for ORC-origin binding.

The Orc6 Subunit

Orc6 is the only subunit of ORC that is not an AAA+ protein, and thus it must use distinct mechanisms to incorporate into ORC. While yeast Orc6 co-purifies as a stoichiometric component of *Sc*ORC, it is not essential for *Sc*ORC-origin DNA binding [1, 59]. However, experiments using a conditional yeast Orc6 mutant provide strong evidence that Orc6 is required in ORC for stable MCM complex loading and DNA replication [60]. This function may relate to the ability of Orc6 to bind to Cdt1, an MCM chaperone needed for stable loading of the MCM complex onto DNA in the presence of ATP, though data are conflicting with respect to the precise role of this interaction [60, 61]. While in yeast Orc6 is the only subunit dispensable for *Sc*ORC-origin binding, metazoan Orc6 *is* required for ORC-origin binding as well as replication [62].

In comparison to the AAA+-domain-containing ORC subunits, Orc6 is less conserved between yeast and metazoans [16]. However, a recent study presents revised Orc6 sequence alignments to reveal greater similarity in Orc6 domain structure between yeast and metazoans than previously thought [63]. In addition, experiments show that yeast and metazoan Orc6 actually use a similar mechanism for binding to ORC [41].

Orc6 is conserved among metazoan species and contains three distinct domains. Two of these domains are similar to regions in Transcription Initiation Factor II B (TFIIB, domains A and B, Fig. 9.4a) [62]. The third region of similarity occurs in an otherwise novel C-terminal domain (Orc6 CTD). Importantly, similarity to all three of these regions can also be located within *Sc*Orc6, once a large insertion between the TFIIB domains is correctly accounted for [41]. The region of highest conservation within the Orc6 CTD contains a missense substitution in human Orc6 (Y232S) associated with MGS. The corresponding substitution in *Dm*Orc6 (Y225S) reduces Orc6 binding to Orc1-5 in vitro and MCM loading in vivo [63]. This metazoan Orc6-(Orc1-5) interaction is mediated by an interface between a highly

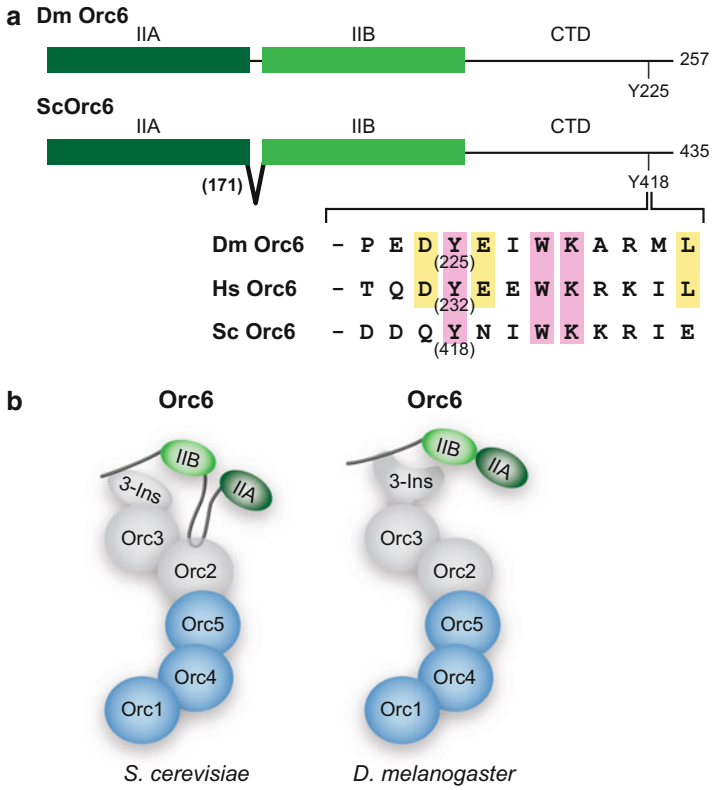

Fig. 9.4 (**a**) Schematics of the primary structure for *Dm*Orc6 and *Sc*Orc6 adapted from [63]. Region of the Orc6 CTDs that contain the "MGS" implicated Y residue and contact Orc3 are highlighted for *Homo sapiens*, *Drosophila melanogaster*, and *Saccharomyces cerevisiae*. (**b**) Cartoon models depicting the basic subunit organization of *Sc*ORC and *Dm*ORC as adapted from [63]. These models are based on the analyses of EM structures as described in the text [63]; a recent crystal structure of *Dm*ORC revised the order of the subunits from Orc1-Orc4-Orc5-Orc2-Orc3 in the AAA+ core to Orc1-Orc4-Orc5-Orc3-Orc2, and shows that ATPγS bound *Dm*ORC forms a ring with an enclosed central channel that could accommodate dsDNA [23]

conserved C-terminal region of Orc6 and a distinct region of Orc3 (Fig. 9.4b). These results align well with data that show that human Orc6 and Orc3 interact [64, 65]. While these data are consistent with the human Orc6 MGS-mutant's (Y232S)) effects on DNA replication causing MGS-associated phenotypes, it must be noted that Orc6, including the Orc6CTD (and other subunits of ORC), has additional non-replicative functions in metazoans that may also contribute to MGS phenotypes [66–74].

*Sc*Orc6's interaction with the Orc1-5 AAA+ core complex appears to involve both yeast-specific features and metazoan-like features. Independent studies provide evidence that *Sc*Orc6 interacts with *Sc*Orc2 or *Sc*Orc3 to incorporate into ORC. In particular the C-terminal region of *Sc*Orc6 binds ORC through an

interaction with *Sc*Orc3, but *Sc*Orc6 also binds *Sc*Orc2 under conditions where it cannot bind *Sc*Orc3 [60, 75]. A *Sc*Orc6 mutant carrying the MGS substitution (Y418S) abolishes an Orc6-Orc3 interaction in vitro, but not *Sc*Orc6's interaction with the Orc1-5 AAA+ core [63]. Together these studies suggest that *Sc*Orc6 contains at least two independent interfaces that help it incorporate into *Sc*ORC, a conserved Orc6 CTD that binds *Sc*Orc3 and a yeast-specific region that binds *Sc*Orc2 (Fig. 9.4b). Nevertheless, these studies identify strong similarities between Orc6 and its structure, both alone and in the context of ORC, between yeast and metazoans. Interestingly, the yeast-specific insertion plays an important functional role in yeast ORC, as it contains cyclin-dependent kinase (CDK) sites and an S-cyclin binding motif, both of which help regulate ORC's activities in yeast [76, 77]. While the core structure and mechanical functions of ORC are probably highly conserved from yeast to metazoans, strategies to regulate its activity appear to vary considerably (e.g., [64]).

Architecture and Conformational States of the ORC

As is true for many AAA+ proteins, the Orc1-5 subunits acquire their functionality by coming together in an oligomeric complex, ,with the ATP-binding pockets of individual subunits participating in the subunit-subunit interfaces [19]. The arrangement of the ATP-binding pockets at subunit interfaces plays a role in ATPase activity of AAA+ oligomeric complexes [78]. In particular, X-ray crystal structures of AAA+ oligomers suggest an arginine of one subunit, referred to as an "arginine finger," interacts with the ATP-binding pocket of the neighboring subunit forming interactions with the bound ATP, promoting ATP hydrolysis [78]. *Sc*Orc1 and *Sc*Orc4 follow this paradigm, with the R267 residue of *Sc*Orc4 being essential for ATP hydrolysis by *Sc*Orc1 in ORC [79]. Interestingly, while both EM structures of *Sc*ORC and *Dm*ORC and the recent crystal structure of *Dm*ORC reveal that Orc1 and Orc4 are adjacent subunits, consistent with these experimental findings (Fig. 9.4b), the recent crystal structure of *Dm*ORC indicates that the arginine finger of *Dm*Orc4 is 40 angstroms away from nucleotide binding cleft of *Dm*Orc1. This and additional observations strongly suggest that *Dm*Orc1 must undergo a large conformational shift so that *Dm*Orc4 can trigger its ATPase activity [23]. Such changes could be achieved in part by ORC binding to DNA and/or other proteins that participate in the MCM complex loading reaction, including Cdc6 and MCM itself. In addition the *Dm*ORC crystal structure suggests that ATP binding and hydrolysis by *Dm*Orc4, regulated by an arginine finger from the neighboring subunit of *Dm*Orc5, may execute important *Dm*ORC functions in some organisms, consistent with metazoan Orc4s containing both consensus Walker A and B motifs. Because *Sc*Orc4 lacks a Walker A motif, whatever this role of metazoan Orc4 may be, it is not essential in budding yeast and therefore likely more critical for regulation than core mechanism.

Substantial efforts to define the structures of *Sc*ORC and *Dm*ORC by electron microscopy (EM), and more recently an X-ray crystal structure of *Dm*ORC, have generated images of ORC that provide clues about how it may function in the MCM complex loading reaction [18, 23, 28, 63, 75, 80, 81]. Single-particle reconstructions of negative-stained EM complexes in which the heterologous Maltose Binding Protein is fused to individual Orc subunits allowed the subunit organization within *Sc*ORC and *Dm*ORC to be determined with impressive accuracy, though the recent crystal structure of *Dm*ORC reveals that the subunit arrangement of Orc2-Orc3 is actually Orc3-Orc2 [23, 63, 81] (Fig. 9.4b). Regardless, all together these studies indicate that both the subunit organization and basic shape of *Sc*ORC and *Dm*ORC are similar, as predicted based on the fundamental role ORC plays at DNA replication origins (Fig. 9.4b) [63].

For *Dm*ORC, high levels of ATPγS were required to provide the level of resolution necessary to locate the subunits in the EM images and make a useful comparison to *Sc*ORC, suggesting that the ATP-bound *Dm*ORC forms a more stable and homogenous conformation. Based on EM analysis the ORCs from both organisms form a two-lobed structure in which roughly one half contains the subunits with ATP-binding and/or hydrolysis capabilities in the following order: Orc1-Orc4-Orc5, and the other half contains the remaining subunits in the following order: (Orc2-Orc3)-Orc6 [63] (Fig. 9.4b). The crystal structure of *Dm*ORC shows that ATP-bound ORC reveals a ring structure with a clear central channel for duplex DNA and, as mentioned above, revises the placement of Orc2 and Orc3 within ORC1-5 to Orc1-Orc4-Orc5-Orc3-Orc2 [23]. Interestingly, this structure provides no entry point for duplex DNA, suggesting metazoan ORC must undergo conformational changes even in the ATP-bound state to allow for DNA binding. There are two distinct tiers to *Dm*ORC, with one being formed by both canonical and noncanonical interactions between adjacent AAA+ domains and the other being formed by WH domain interactions between the AAA+ domains of *neighboring* subunits. These interactions lead to the WH domains forming a "collar" that is offset from the AAA+ ring. It is the flexible linker regions between the AAA+ and WH domains on most subunits that allow for these interactions to occur in this way. While there is as yet no reported crystal structure for *Sc*ORC, and potentially important differences may exist between *Dm*ORC and *Sc*ORC, the overall similarity in shape and organization derived from completely independent EM studies is substantial and comforting. In addition, all of these data are consistent with previous examinations of ORC organization as well as subunit-subunit interactions in these and other systems, including human ORC [59, 64, 65, 82, 83].

EM structural studies to address possible conformational changes in ORC have been performed with *Sc*ORC and include examining the effect of relevant *Sc*ORC partners, including Cdc6 and origin DNA and, most recently, a partially assembled putative intermediate in the MCM loading reaction, the OCCM (for ORC-Cdc6-Cdt1-MCM) on ORC structure [18, 28, 75, 81]. In each of these situations ORC's structure is determined in the presence of ATPγS to stabilize intermediates. The ORC-dependent MCM loading machine consists of ORC and Cdc6 together on origin DNA, such that the loading complex contains six related AAA+ protein

subunits and Orc6. In yeast, ORCs remain bound to most origins throughout the cell cycle, and Cdc6 joins ORC only in G1-phase when the MCM complex is loaded onto chromosomal DNA [84, 85]. In vitro, Cdc6 preferentially binds ORC when ORC is bound to origin DNA, and upon doing so creates an extended ORC-Cdc6 DNaseI footprint that includes sequences adjacent to the ORC binding site but not required for ORC binding per se [18]. In fact this extended footprint contains an origin element required for MCM complex loading and looks similar to the extended pre-RC (G1-phase) footprint initially defined at yeast origins by in vivo footprinting [85–87]. Together these observations suggest that distinguishing features of the in vivo G1-footprint result from the ORC-Cdc6 complex participating in MCM complex loading, rather than the loaded MCM complex itself. The formation of this ORC-Cdc6-origin DNA complex, including the diagnostic extended DNaseI footprint, requires ATP and a *functional* ORC binding site, that is defined as an ORC site that can support origin function in vivo and not merely ORC binding in vitro [18]. ORC-1A is an ORC containing a defective Orc1 subunit (a K485A substitution in Orc1). ORC-1A is lethal in vivo and does not support formation of the diagnostic ORC-Cdc6-origin DNA complex in vitro, providing evidence that this distinctive ternary complex is functionally relevant to the essential MCM complex loading reaction [18, 20].

A single-particle cryo-EM-derived structure of the *Sc*ORC-origin DNA and *Sc*ORC-Cdc6-origin DNA complexes in the presence of ATPγS indicates that conformational changes in ORC accompany these assemblies [75]. First, ORC binding to origin DNA stabilizes the ORC structure considerably and induces a rotation of the Orc1-Orc4-Orc5 region relative to the rest of ORC. Second, upon Cdc6 binding, additional conformational changes in ORC are observed, including a shift in the Orc1 N-terminal domain, which contains the nucleosome-binding BAH module. This shift may accommodate new Cdc6-Orc1 contacts. Orc6 is also rearranged in the complex upon binding Cdc6 such that it juts out of the central portion of the ring's surface and comes into contact with Orc1, which is supported by additional Orc1-Orc6 interaction data. This change is proposed to position Orc6 to help recruit the MCM complex via direct contacts with MCM-bound Cdt1. Recent studies with purified proteins suggest that this interaction is not intrinsically essential for recruitment of MCM to ORC-Cdc6-DNA, though both Orc6 and Cdt1 are required for a complete reaction that culminates in stable loading of the MCM complex [60, 61]. These major conformational changes to Orc1 and Orc6 within *Sc*ORC observed from the EM studies are consistent with earlier limited protease digestion patterns of ORC compared to ORC-Cdc6 [88]. The bottom line is that *Sc*ORC conformations are altered considerably by nucleotide binding, origin DNA, and the key protein partner Cdc6.

More recently, *Sc*ORC structure by cryo-EM has been examined in a trapped and putative intermediate in the MCM complex loading reaction on origin DNA termed the OCCM for ORC-Cdc6-Cdt1-MCM [28]. The formation of this intermediate is accompanied by additional changes in the conformation of ORC-Cdc6 in which the relatively flat complex changes to a more dome-like shape, with the concave face directed toward the Cdt1-MCM complex. While the initial interpretation of this

structure was that the AAA+ domains reach toward the MCM subunits within the Cdt1-MCM complex, the recent model based on the crystal structure of *Dm*ORC suggests that the WH collar on ORC contacts the AAA+ ring on the MCM [23]. Based on the EM structural analysis of the OCCM, the movement of *Sc*Orc6 from the middle of the *Sc*ORC-Cdc6 complex seems to be essential to allow for the binding of the Cdt1-MCM complex and to help promote additional transitions in *Sc*Orc3 and *Sc*Cdc6. Interestingly, one outcome of these transitions is that Orc4 and Orc1 come into closer contact, consistent with the notion that these conformational changes promote Orc4's ability to stimulate ATP hydrolysis by Orc1, a reaction essential for efficient MCM complex loading in vitro as well as cell viability [79]. Together with the crystal structure of *Dm*ORC that shows *Dm*Orc4 too far away from *Dm*Orc1 to stimulate Orc1 ATPase, these data suggest that ORC's catalytically important ATPase activity is highly regulated by steps in the MCM complex loading reaction. Another conformational change is that the ORC-Cdc6 complex forms a right-handed spiral structure within the OCCM that has a 34 angstrom helical rise from bottom (Orc3) to top (Cdc6), matching the helical pitch of B-form dsDNA. Thus, an attractive hypothesis is that these matching structures allow ORC-Cdc6 to hold the DNA substrate for loading of the MCM complex [28].

As an AAA+ initiator protein, the observed conformational changes in ORC in response to ATP binding and interactions with DNA and other proteins would be predicted to drive the mechanical work that culminates in the loading of a replication-competent MCM complex onto DNA. The challenge, of course, is to link these conformational states to discrete biochemical steps in the MCM loading reaction, and currently a strong opportunity for meeting this challenge exists in the yeast system where the MCM complex loading reaction, and most recently the entire origin reaction, from *Sc*ORC binding to origin activation, can be reconstituted with recombinant proteins [61, 89–92]. While it makes sense that the structural changes described to date cause the changes in protein-protein and protein-DNA interaction affinities necessary to drive the G1-phase MCM complex loading cycle, precisely how ORC's conformational states fit into this scheme remains an area of intense research and debate [93, 94]. The recent reports about both *Sc*ORC in the OCCM and the high resolution crystal structure of *Dm*ORC described above raise the possibility that the MCM complex loading step(s) promote ORC-Cdc6 architectural changes that modulate both the ORC and Cdc6 ATPases. Interestingly, recent studies show that the ATP hydrolysis by subunits of MCM complex substrate itself is required for MCM complex loading [89, 90]. Thus, while there may be an intrinsic rate of ATP hydrolysis by ATP-bound ORC-Cdc6 on DNA that could conceivably function in proofreading (i.e., dissolution of any incomplete MCM complexes that may form) as proposed, perhaps another and not mutually exclusive possibility is that a fully loaded MCM complex (i.e., double hexamer of MCMs) signals its "maturation" to its ORC-Cdc6 loading complex and promotes ORC-Cdc6 ATPase activity and release from DNA [61]. In this model, a loaded MCM complex is made competent for S-phase activation (i.e., now released from ORC-Cdc6 and free to move away for the "loading site" on double-stranded DNA) and, at the same time, ORC and Cdc6 are "reset" such that

they can now participate in another independent MCM complex loading reaction at the same or another origin [79]. The end result would be a cycle that could continue to load MCM complexes onto chromosomes through G1-phase as long as Cdt1-MCM complexes were available. For a cycle to work efficiently and to load multiple MCM complexes, a "mature" MCM complex would have to move out of the way to allow another loading event. This movement on double-stranded DNA is not hard to envision given the demonstrated passive sliding of the MCM complex on naked DNA in vitro and the dynamic behavior of nucleosomes observed near origins [30, 32, 95]. The bottom line based on several lines of accumulating evidence, in terms of ORC, is that ORC must *bind* and *release* its origin DNA substrate to establish one complete MCM complex loading reaction. Mechanisms that facilitate re-binding of released ORC would promote multiple MCM complex loading reactions in a given G1-phase. The recent models based on the crystal structure of *Dm*ORC raise the possibility that origin binding dynamics could be regulated by the rate that ATP-bound *Dm*ORC is able to alter its conformation to access double-stranded DNA (i.e., the "on" reaction) [23]. Specific interactions between ORC subunits and chromatin structures neighboring origins are suggested as one possible mechanism for achieving such a conformational alteration.

Roles for ATP Binding and Hydrolysis by ORC

The specific roles for ATP binding and hydrolysis events catalyzed by ORC have been studied extensively in yeast using genetic tools, purified proteins and extracts. In one basic approach, a defined mutant form of a *Sc*ORC subunit is generated. The mutant subunit is then expressed in vivo as the sole source of that subunit and/or as a component of an overproduced ORC (i.e., where all subunits are overproduced to make an excess of the desired mutant ORC complex). In addition, the mutant subunit is reconstituted with the remaining *Sc*ORC subunits in an expression system (Baculovirus infected Sf9 cells or a yeast strain optimized for protein expression and purification) to facilitate purification of a defined mutant form of *Sc*ORC. The biochemical functions of the mutant *Sc*ORC are then compared to that of a wild-type *Sc*ORC using a variety of in vitro assays. Similar in vitro expression approaches can be used to study metazoan ORCs in vitro as well, as mentioned below.

Because only Orc1 and Orc5 were shown to bind ATP within *Sc*ORC, an early study addressed their ATP-binding functions in the context of ORC by generating mutant versions of each of these subunits in which their Walker A motifs were altered to abolish their ATP-binding function [20]. Two distinct mutant forms of *Sc*ORC can be assembled that either cannot bind ATP at the Orc1 subunit (*Sc*ORC-1A) or cannot bind ATP at the Orc5 subunit (*Sc*ORC-5A). *Sc*ORC-1A but not *Sc*ORC-5A cannot bind a yeast origin in vitro, indicating that ATP binding by ORC via the Orc1 subunit but not the Orc5 subunit is essential for specific *Sc*ORC-origin binding. In addition an *orc1-A* mutant allele fails to complement an *orc1Δ* mutation in vivo, while an *orc5-A* mutant allele complements an *orc5Δ* mutation.

Interestingly, the biochemical role of ATP binding by *Sc*Orc5 remains unknown, even though ATP binding by this subunit is conserved. A clue comes from a genetic screen to identify mutant alleles that enhance gross chromosomal rearrangements (GCRs) in yeast that isolated *orc5-70,* an allele that encodes a G42R substitution in *Sc*Orc5's Walker A motif [96]. While *orc5-70* causes temperature-sensitive growth defects, it causes no detectable defects in origin activation per se, in contrast to the temperature-sensitive *orc5-1* allele [11, 84, 97]. However, an increase in origin number per chromosome exacerbates the elevated GCR caused by *orc5-70*, suggesting that whatever defect in *Sc*ORC this allele causes is related in some way to *Sc*ORC behavior at origins.

To examine the role of ATP hydrolysis by *Sc*ORC, ,which occurs through the *Sc*Orc1 subunit, a similar approach was taken. One type of allele was identified in *ORC1* through a dominant negative overexpression screen that assessed the effect of mutations in the region encoding the Orc1 Walker B motif predicted to be important for ATP hydrolysis [98]. Two alleles were isolated that affect the same amino acid, *orc1-d1*(D569Y) and *orc1-d2*(D569F). The goal of this screen was to isolate a "clean" defective version of *Sc*ORC in which Orc1 ATPase was nonfunctional but ATP binding was unaffected. While this goal was not achieved—an *Sc*ORC-d1 mutant complex has a 10-fold reduction in the Km for ATP and a 16-fold defect in kcat for ATPase activity—the analyses of these mutants provide important insights into ORC function. Interestingly the *orc1-d* alleles support viability and complement an *orc1Δ* mutation, indicating that a substantial reduction in intrinsic ATPase activity of *Sc*ORC is tolerated in vivo. Consistent with this observation, under saturating levels of ATP, a mutant *Sc*ORC-d1 binds yeast origin DNA indistinguishably from wild-type *Sc*ORC. Thus the fundamental role for ORC in origin binding appears unperturbed in vivo, indicating that MCM complexes must be loaded onto origin DNA by *Sc*ORC-d mutants. However, overproduction of an orc1-d subunit *together with overproduction of each of the other Orc subunits* produces a dominant negative growth phenotype. Thus an excess of ORC-d1 complexes is dominant negative over wild-type ORC. (Overexpression of *orcd-1* alone is *not* dominant negative because an ORC-d1 complex can provide enough ATPase activity to support viability—only when excess ORC-d1 is made, such that it accumulates in the nucleus is a dominant negative effect observed.) However, this dominant negative effect of ORC-d1 can be bypassed by overexpressing CDC6. The interpretation is that ORC-d1, essentially acting as an excess of ORC in the ATP-bound state, is titrating limiting levels of Cdc6 away from any ORC (wild type or ORC-d1) bound to origins. Cdc6 has the highest affinity for the ORC-DNA-ATP complex [18]. Thus this observation is consistent with a model whereby ORC's ATPase is required to release ORC-Cdc6 from DNA and possibly each other, thus recycling the proteins for further rounds of MCM complex loading [79, 99].

While the Cdc6 overexpression rescue of the dominant negative effect of *Sc*ORC-d1 makes sense, an unanswered question is why *Sc*ORC-d1 or *Sc*ORCd2, when expressed at normal levels and as the sole source of ORC, provides for normal levels of cell growth. At least two different models may explain this observation. The first is that, in the context of Cdc6, origin DNA, and MCM-Cdt1, the

ORC-d1 (or -d2)-Cdc6 complex has no substantial defect in whatever level of ATPase activity is required by this complex to participate in a normal MCM complex loading reaction. Related to this point, it must be acknowledged that the requirement and roles of ATP hydrolysis by the ORC-Cdc6 complex for MCM complex loading remain incompletely understood. Indeed, recent reports show that, in a purified system, the Cdc6-ATPase is not required for loading a replication-competent MCM complex in vitro, even though the Cdc6-ATPase is required for viability *and* MCM complex loading in a crude extract [89, 90, 100]. Nevertheless, this first model posits that, in the biologically relevant context, ORC-d1 or ORC-d2 has little to no defect in ORC ATPase activity despite showing weak intrinsic ATPase activities as isolated complexes in vitro. A second possibility is that, while MCM complex loading at origins can still occur in cells in which ORC-d1 or ORC-d2 is the only source of ORC, the MCM loading cycle may be substantially less efficient. However, perhaps in yeast cells, as in mammalian cells, levels of loaded MCM complexes (i.e., numbers of *potential* origins) are in excess of what is needed to complete an unchallenged S-phase [101–103].

A second mutant of version ORC defective in ATPase activity is called ORC-4R to signify that the gene encoding the Orc4 subunit contains a missense mutation in the codon for arginine 267 (i.e., *orc4-R267A*, *orc4-R267K*, *orc4-R267E*), the arginine "finger" discussed above needed for Orc1's ATPase activity [79]. In contrast to the *orc1-d* alleles, *orc4-R* alleles do not support yeast viability. In addition, in vitro ORC-4R complexes show no ORC ATPase activity but can bind ATP similarly to wild-type ORC, indicating Orc1's ability to bind ATP remains intact. As predicted by this result, ORC-4R binds origin DNA similarly to WT ORC both in vivo and in vitro. However, ORC-4R reduces MCM complex loading efficiency in an in vitro MCM complex loading extract in which wild-type ORC is depleted and replaced with recombinant ORC-4R. Interestingly, in vitro, MCM complex loading, as measured by salt-resistant origin DNA-associated MCM, is not abolished by ORC4-R, only reduced. These and other data lead to a model that posits that ATP hydrolysis by ORC is required for multiple rounds of MCM complex loading reactions onto DNA in vitro, but is not essential for loading per se. These data also support a model in which the ORC ATPase activity functions to recycle the ORC-Cdc6 loading machine and, perhaps, also release the loaded MCM complex for "activation competence" in S-phase, as discussed above. With respect to these ideas, it would be useful to know the cell cycle arrest point of orc-4R mutants, and whether ORC and/or MCM are associated with origins at this arrest stage. ORC4-R may remain "stuck" at origins, or it may leave, but load inadequate levels of MCM complexes to support replication. Suppressors of orc-4R lethality might also be informative, though not necessarily trivial (or even possible) to obtain.

In terms of ATP binding and ATPase activity, metazoan ORC, as represented by studies of *Dm*ORC, shows strong similarities to *Sc*ORC even though *Dm*ORC DNA binding shows considerably lower preference for any particular DNA sequence [21, 104, 105]. In particular, both tight DNA binding and ATPase activity of ORC depend on *Dm*Orc1 [21]. Furthermore, chromatin binding by ORC as well as DNA replication in a cell-free extract depends on an intact Orc1, i.e., an Orc1 containing wild

type and functional Walker A and Walker B motifs. Analogous defects in Orc4 or Orc5 have only minimal effects. In substantial contrast to *Sc*ORC, however, *Dm*Orc6 is essential for DNA and chromatin binding by ORC. In general, however, there are strong functional similarities between *Dm*ORC and *Sc*ORC in terms of ATP binding and hydrolysis.

Studies of human ORC (*Hs*ORC),), however, suggest that, while mechanistic aspects of Orc1-ATP binding and hydrolysis are conserved once ORC is bound to an origin and presumably participating in the MCM complex loading reaction, differences in terms of regulation of *Hs*ORC are apparent [22, 64, 65]. As with *Sc*ORC and *Dm*ORC, Orc1 is required to support ATP hydrolysis of *Hs*ORC. However, in contrast to *Dm*ORC, recombinant *Hs*ORC requires the Walker A motifs of Orc1, Orc4, and Orc5 to associate with chromatin and support replication in a cell-free extract derived from *Xenopus laevis* [22]. A partial explanation for these somewhat unexpected observations may be provided by the subsequent studies of recombinant *Hs*ORC that show its assembly into a stable complex requires ATP binding by the Orc4 and Orc5 subunits and follows an ordered pathway [64, 65]. In particular, *Hs*Orc4 binds to a stable subcomplex of *Hs*Orc2-3-5 only in the presence of ATP, and intact Walker A motifs in Orc4 and Orc5 are necessary. Moreover, Orc1 association with an Orc2-5 subcomplex also requires ATP, though Orc1 does not interact with Orc4 on its own, regardless of ATP. Thus a model is proposed whereby ATP binding by the Orc4 and Orc5 subunits controls an ordered assembly of *Hs*ORC, with Orc1 joining the complex last in G1-phase to allow ORC to participate in the MCM complex loading reaction. This assembly process may proceed in reverse during S-phase, after an origin is activated, and be important for the S-phase mediated degradation of Orc1 in human cells and the inactivation of ORC activity until the following cell division is complete and the cells re-enter G1-phase [106, 107].

Regulation of ORC Activity

The eukaryotic cell division cycle demands a precise coordination between chromosome duplication in S-phase and chromosome segregation in M-phase. In particular, every chromosome has to be duplicated completely and only once during a normal S-phase or chromosome breakage and aneuploidy can result after M-phase. In terms of DNA replication origins, this rule means that any given region of a chromosome, including a region containing a potential origin, is replicated only once during a given cell cycle, regardless of whether the origin-containing region is replicated from the functional activation of that origin or a replication fork emanating from a neighboring origin. In either situation, the origin must be inactivated and made incapable of firing until cell division is complete and the new daughter cells enter their own S-phases. To achieve this goal, multiple mechanisms exist that inhibit the MCM complex loading reaction during S-phase and promote it only during G1-phase. Thus, the proteins required for the MCM complex loading reaction, including ORC, Cdc6, Cdt1, and MCM itself, are prime targets for these inhibitory

mechanisms. While the specific mechanisms vary between organisms, and the pathways emphasized for inhibition vary as well, the ultimate result is the same—the MCM complex loading reaction is strictly confined to G1-phase. A complete discussion of this topic is beyond this chapter's scope, but ORC activity, because of its central role in the MCM complex loading reaction, is one relevant target and mechanisms for its regulation, some of which are mentioned above, are briefly summarized here.

As mentioned above, *Sc*ORC binds origins throughout most of the cell cycle, though recently a class of origins was discovered that lacks robust ORC association in G2-phase [108–110]. Nevertheless, *Sc*ORC does not appear to be regulated in general or substantially at the level of ORC-origin binding during the cell cycle, at least compared to metazoans. Instead, as cells enter S-phase, *Sc*ORC is phosphorylated by S-phase cyclin-dependent kinases (CDKs) on the Orc2 and Orc6 subunits [76]. In addition, one S-phase cyclin, Clb5, binds directly to an RXL motif in Orc6 [111]. While phosphorylation does not appear to reduce ORC-origin binding, it inhibits *Sc*ORC's function in the MCM complex loading reaction [61, 77]. Thus while S-phase CDKs activate targets needed for origin activation during S-phase, they simultaneously phosphorylate and inhibit targets that function in the MCM complex loading reaction.

Evidence from studies of *Dm*ORC suggests both similarities and differences in terms of ORC regulation by CDKs. In particular *Dm*ORC is also a target CDK-dependent phosphorylation, though the target subunits are Orc1 and Orc2. In vitro studies show that *Dm*ORC's ATPase activity but not ATP-binding affinity is reduced by this phosphorylation. Moreover, ATP-dependent ORC-DNA binding is also inhibited, suggesting a mechanism different from that used by *Sc*ORC. Finally, as mentioned above, *Hs*ORC activity in MCM complex loading is regulated by the degradation of Orc1 during S-phase [106]. Thus the assembly of a complete *Hs*ORC functional for MCM complex loading is confined to G1-phase by this mechanism and possibly ATP-dependent ordered assembly of the remainder of the complex as well [64, 65].

Selecting Chromosomal Origins

While ORC binding is not sufficient to establish an origin, it is obviously an essential step. The mechanisms that recruit ORC to specific chromosomal regions and their relationship to the catalytic role of ORC in MCM complex loading remain incompletely understood. For example, while it is reasonable to think that key aspects of the ISM and WH domain contacts with origin DNA have fundamental catalytic roles and therefore are similar at all origins, the mechanisms responsible for localizing ORC to a chromosomal region where it has the opportunity to make such contacts in the first place appear to vary considerably between organisms and even between origins within a single cell. This variability creates substantial challenges to understanding precisely how ORC selects origins across species, and how

these selection mechanisms might affect origin activity. However, this variability may also create opportunities for achieving cell-type-specific modulation of ORC function as well as increasing our basic understanding of the relationships between chromatin structure and origin activity.

Because *Sc*ORC shows binding specificity for a conserved sequence element found within yeast origins, origin selection is relatively well understood in *S. cerevisiae*. Indeed, an ORC binding site can be identified in most yeast origins [3]. However, even in *S. cerevisiae*, an ORC binding site is not sufficient to identify a yeast origin. For example, a tight ORC binding site in vitro does not necessarily predict an efficient origin in vivo [57, 112]. In fact, a large number of budding yeast origins actually have surprisingly weak ORC binding sites such that the intrinsic strength of the ORC-DNA interaction at these origins is insufficient to explain the strength of ORC-origin binding in vivo [57]. In addition, a number of highly efficient ORC binding sites can be found in the yeast genome that do not function as origins or even bind ORC in vivo [57]. Thus, while ORC binding site specificity certainly aids in mechanistic studies of yeast origins, there is as yet no clear understanding of ORC-DNA contacts critical for ORC's catalytic roles versus contacts used "simply" to localize or stabilize ORC to a particular chromosomal region. In budding yeast this issue is challenging because the same DNA element that localizes ORC to origins appears to have catalytic functions as well.

Moreover, accumulating evidence in budding yeast suggests that, as in metazoans, ORC accessory factors, in the form of specific chromatin structures and/or other DNA binding proteins, may have direct and positive roles in promoting selective *Sc*ORC-origin binding. In fact, the best predictor of a functional *Sc*ORC binding site in vivo is not the closest sequence match to a consensus ORC site but rather an ORC binding site contained within a defined nucleosome configuration in which the predicted ORC site is relatively free of nucleosomes [55]. As mentioned above, the *Sc*Orc1BAH domain, a putative nucleosome-binding module, is needed for wild-type levels of *Sc*ORC-origin binding at most yeast origins, and origins that are particularly dependent on the Orc1BAH domain for *Sc*ORC binding are enriched for a distinctive local nucleosome configuration that places two nucleosomes closer to the Orc1 subunit side of ORC [56]. Recently, another distinct Orc1BAH-independent group of yeast origins have been identified that likely rely on the presence of additional proteins, including chromatin, to either properly remodel otherwise weak ORC sites or to provide for direct ORC-protein interactions [57]. While the specific ORC-accessory interactions that promote ORC-origin binding at these yeast origins have not been defined, a recent study demonstrates a physical interaction between an evolutionarily conserved transcription factor, a member of the forkhead transcription factor family conserved from yeast to metazoans, named Fkh1 and *Sc*ORC [113]. In addition, forkhead protein binding sites are enriched near many *S. cerevisiae* DNA replication origins and contribute to their normal early activation during S-phase [113, 114]. Therefore, while sequence specificity of *Sc*ORC-double-stranded origin DNA interactions still makes it exceptional among eukaryotes, accumulating evidence makes it clear that, as for ORCs in other organisms, accessory factors, including nucleosomes, play a role in promoting functional ORC-origin binding.

In metazoans, including human cells and excluding the rapidly dividing cells of early embryos, the guiding paradigm for origin selection by ORC is that ORC-origin binding specificity is achieved by ORC accessory factors, including specific modification states of chromatin that directly bind regions of ORC as discussed above (e.g., K3K20me2 binding by Orc1BAH). Consistent with this model, ORC interacting proteins that have a key role in recruiting *Hs*ORC to origins have been identified. HMGA1a, a high-mobility group chromatin-associated protein, interacts directly with *Hs*ORC and, when tethered to a heterologous DNA binding domain, can recruit ORC to a heterologous DNA region and establish a functional origin [115]. Thus the HMGA1a-ORC contacts govern the specificity of ORC-origin binding, but establishing the functional MCM complex loading site has little, if any, requirement for specific ORC-DNA contacts. The interaction between HMGA1a and ORC is robust within AT-rich heterochromatin, consistent with a proposal that such ORC-protein interactions may be particularly critical in difficult-to-replicate heterochromatin, an abundant component of metazoan genomes [116]. ORCA/LRWD1 (ORC associated/leucine rich repeats and WD containing 1) is a more recently described ORC accessory factor with similar roles to HMGA1a in recruiting ORC to DNA [117, 118]. Again, the most abundant co-localization between ORCA and ORC occurs within heterochromatic regions. ORCA also interacts with other regulators of MCM complex loading such as Cdt1. Depletion experiments in embryonic stem cells and human primary cells show that ORCA is required for normal levels of chromatin-associated MCM. A recent review discusses many other ORC accessory factors that have been described in metazoans, several of which appear to function after the ORC-origin binding step and facilitate the MCM complex loading reaction [119]. What is clear from these studies is that metazoan ORC relies on a number of accessory factors to perform its role in establishing origins in complex genomes. The relatively stringent requirement for a specific sequence for ORC-origin binding in the small compact genome of budding yeast may have evolved to insure origins would not be disrupted by or interfere with gene transcription, though even in budding yeast robust gene transcription makes some origins more sensitive to defects in MCM complex loading components compared to others [120]. Regardless, in larger genomes with substantial gene-free regions, there is an increased opportunity for ORC-origin interactions that may have relieved some of the evolutionary pressure for sequence-specific binding by ORC. Despite differences between ORC-origin binding in budding yeast and metazoans, it is worth noting that ORC binding regions in chromatin occur within chromatin structures that appear, at least in terms of local nucleosome organization, similar to the structure described for yeast origins [55, 121–123].

An important question is whether these varied ORC-origin selection mechanisms have any impact on ORC's catalytic role in loading MCM complexes onto chromosomal DNA and/or other steps relevant to origin activation. Interestingly, independent studies in *S. pombe* and *S. cerevisiae* indicate a link between ORC-origin binding dynamics and origin activation time during S-phase, even though no functional role for ORC in origin activation during S-phase is known or proposed [57, 110, 124]. The multiple origins that replicate eukaryotic chromosomes are not acti-

vated simultaneously during S-phase, but rather individual origins each activate with a distinct probability and at a characteristic point during S-phase, with some origins activating soon after S-phase begins and others activating later after much of the genome has been duplicated. In *S. pombe*, origins that bind to ORC early after M-phase, presumably because they have a higher affinity for ORC, also show earlier MCM recruitment *and* are activated earlier in the subsequent S-phase compared to origins that bind ORC later [124]. One interpretation is that an origin with high affinity for ORC increases the probability of a successful MCM complex loading event occurring, thus allowing for multiple MCM complexes to be loaded at such an origin in a given G1-phase. Because the MCM complex is the target for limiting S-phase activators, origins that have received more MCM complexes during G1-phase can more effectively compete for these limiting activators and hence activate soon after S-phase begins [125, 126]. In *S. cerevisiae*, a recent study provides a complimentary correlative observation [110, 127, 128]. In particular, while budding yeast origins are thought to be bound by *Sc*ORC throughout the cell cycle, a recent study used a more sensitive measure for ORC binding in vivo than used in prior genome-wide studies to identify a subset of yeast origins that fail to associate with ORC immediately following the completion of replication in G2-phase. The origins that show this delay in ORC binding are enriched for origins that activate relatively late during S-phase. Of course, other models that do not invoke reiterative loading of MCM complexes are reasonable. For example, whatever chromatin structure promotes early ORC binding to an origin may also promote access of that origin to limiting S-phase factors, and the correlation between ORC binding and activation time may merely reflect a "shared response" to the same chromatin cues. However, some experimental evidence argues against this interpretation of the correlative data [124].

Another series of studies focused on *Sc*ORC-origin binding interactions may seem to contradict these findings at first glance, but on closer consideration all of these data may be reconciled [57, 112]. In these studies, yeast origins that bind ORC tightly because they possess an ORC DNA binding site with a high-intrinsic affinity for *Sc*ORC, as measured in vitro, are enriched for later activating origins, a result that is the opposite of that described for *Sp*ORC above. However, it is important to recall that *Sp*ORC selects origins via an AT-hook on the Orc4 subunit acting essentially as an ORC-origin tether. Therefore, the *Sp*ORC catalytic DNA contacts, presumably mediated by the ISM and WH domains conserved in all ORCs, are distinct from the contacts *Sp*ORC uses to select an origin site (more accurately thought of as selecting an MCM complex loading site). Based on the role of accessory ORC factors in ORC-origin binding in metazoans as discussed above, it seems likely that a similar division of labor occurs for metazoan ORC, except that the ORC-origin tethering may be achieved through ORC-protein or ORC-chromatin contacts as opposed to an AT-hook-DNA interaction. In contrast to these situations, *Sc*ORC, at least at these high-affinity DNA-dependent origins, appears to rely on the same DNA element for both origin selection and for ORC's catalytic role in MCM complex loading. Thus for *Sc*ORC an intrinsically tight ORC binding site may inhibit the kind of ORC-DNA dynamics required for efficient MCM complex loading (i.e., efficient

binding *and* release). In support of this interpretation, budding yeast origins that also bind *Sc*ORC tightly in vivo but instead show weak intrinsic ORC-origin DNA interactions are enriched for origins that are activated early and efficiently in S-phase. These origins are referred to as "chromatin-dependent" because they use factor(s) extrinsic to the ORC binding site, such as ORC-chromatin contacts, to "tether" ORC to the origin. Thus at these origins *Sc*ORC is positioned to have a dynamic relationship with the DNA that controls its catalytic function, similarly to how the *Sp*Orc4 AT-hook positions *Sp*ORC. The bottom line is that ORC may require a dynamic interaction with its catalytically relevant DNA contacts (i.e., it must bind and release and rebind efficiently) to either maximize the rate of an MCM complex loading cycle in G1-phase, as suggested by biochemical data or, alternatively, "release" a loaded MCM complex for activation during S-phase [99]. An ORC-origin tethering mechanism distinct from catalytically important ORC-DNA interactions may facilitate such dynamics.

While the mechanisms that control origin activation timing are mechanistically interesting and relevant to genome stability and cell differentiation in their own right, several studies now provide evidence that the *number* of MCM complexes loaded onto a chromosome directly reflects the number of *potential* origins on that chromosome, and these potential origins are essential for chromosomal stability under conditions of replicative stress, including the levels of stress encountered during the typical lifespan of a multicellular organism [101–103, 129–133]. Thus specific ORC-origin selection mechanisms may facilitate distinct ORC-DNA dynamics that in turn establish the rate for an MCM complex loading cycle within a particular chromosomal region. An efficient cycle would help establish the "excess" number of potential origins, in the form of loaded and competent MCM complexes, which appears so critical to the stable inheritance of eukaryotic genomes.

References

1. Bell SP, Stillman B. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature. 1992;357(6374):128–34.
2. Stinchcomb DT, Struhl K, Davis RW. Isolation and characterisation of a yeast chromosomal replicator. Nature. 1979;282(5734):39–43.
3. Siow CC, Nieduszynska SR, Muller CA, Nieduszynski CA. OriDB, the DNA replication origin database updated and extended. Nucleic Acids Res. 2012;40(Database issue):D682–6. Pubmed Central PMCID: 3245157.
4. Deshpande AM, Newlon CS. The ARS consensus sequence is required for chromosomal origin function in Saccharomyces cerevisiae. Mol Cell Biol. 1992;12(10):4305–13.
5. Newlon CS, Theis JF. The structure and function of yeast ARS elements. Curr Opin Genet Dev. 1993;3(5):752–8.
6. Marahrens Y, Stillman B. A yeast chromosomal origin of DNA replication defined by multiple functional elements. Science (New York, NY). 1992;255(5046):817–23.
7. Marahrens Y, Stillman B. Replicator dominance in a eukaryotic chromosome. EMBO J. 1994;13(14):3395–400.
8. Brewer BJ, Fangman WL. The localization of replication origins on ARS plasmids in S. cerevisiae. Cell. 1987;51(3):463–71.

9. Duderstadt KE, Berger JM. A structural framework for replication origin opening by AAA+ initiation factors. Curr Opin Struct Biol. 2013;23(1):144–53. Pubmed Central PMCID: 3572333.
10. Foss M, McNally FJ, Laurenson P, Rine J. Origin recognition complex (ORC) in transcriptional silencing and DNA replication in S. cerevisiae. Science (New York, NY). 1993;262(5141):1838–44.
11. Loo S, Fox CA, Rine J, Kobayashi R, Stillman B, Bell S. The origin recognition complex in silencing, cell cycle progression, and DNA replication. Mol Biol Cell. 1995;6(6):741–56.
12. Micklem G, Rowley A, Harwood J, Nasmyth K, Diffley JF. Yeast origin recognition complex is involved in DNA replication and transcriptional silencing. Nature. 1993;366(6450):87–9.
13. Li JJ, Herskowitz I. Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. Science (New York, NY). 1993;262(5141):1870–4.
14. Bell SP, Mitchell J, Leber J, Kobayashi R, Stillman B. The multidomain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. Cell. 1995;83(4):563–8.
15. Bell SP. The origin recognition complex: from simple origins to complex functions. Genes Dev. 2002;16(6):659–72.
16. Duncker BP, Chesnokov IN, McConkey BJ. The origin recognition complex protein family. Genome Biol. 2009;10(3):214. Pubmed Central PMCID: 2690993.
17. Li H, Stillman B. The origin recognition complex: a biochemical and structural view. Subcell Biochem. 2012;62:37–58. Pubmed Central PMCID: 3779782.
18. Speck C, Chen Z, Li H, Stillman B. ATPase-dependent cooperative binding of ORC and Cdc6 to origin DNA. Nat Struct Mol Biol. 2005;12(11):965–71.
19. Iyer LM, Leipe DD, Koonin EV, Aravind L. Evolutionary history and higher order classification of AAA+ ATPases. J Struct Biol. 2004;146(1-2):11–31.
20. Klemm RD, Austin RJ, Bell SP. Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. Cell. 1997;88(4):493–502.
21. Chesnokov I, Remus D, Botchan M. Functional analysis of mutant and wild-type Drosophila origin recognition complex. Proc Natl Acad Sci U S A. 2001;98(21):11997–2002. Pubmed Central PMCID: 59756.
22. Giordano-Coltart J, Ying CY, Gautier J, Hurwitz J. Studies of the properties of human origin recognition complex and its Walker A motif mutants. Proc Natl Acad Sci U S A. 2005;102(1):69–74. Pubmed Central PMCID: 544074.
23. Bleichert F, Botchan MR, Berger JM. Crystal structure of the eukaryotic origin recognition complex. Nature. 2015;519(7543):321–6. Pubmed Central PMCID: 4368505.
24. Duderstadt KE, Chuang K, Berger JM. DNA stretching by bacterial initiators promotes replication origin opening. Nature. 2011;478(7368):209–13. Pubmed Central PMCID: 3192921.
25. Erzberger JP, Mott ML, Berger JM. Structural basis for ATP-dependent DnaA assembly and replication-origin remodeling. Nat Struct Mol Biol. 2006;13(8):676–83.
26. Dueber EL, Corn JE, Bell SD, Berger JM. Replication origin recognition and deformation by a heterodimeric archaeal Orc1 complex. Science (New York NY). 2007;317(5842):1210–3.
27. Gaudier M, Schuwirth BS, Westcott SL, Wigley DB. Structural basis of DNA replication origin recognition by an ORC protein. Science (New York NY). 2007;317(5842):1213–6.
28. Sun J, Evrin C, Samel SA, Fernandez-Cid A, Riera A, Kawakami H, et al. Cryo-EM structure of a helicase loading intermediate containing ORC-Cdc6-Cdt1-MCM2-7 bound to DNA. Nat Struct Mol Biol. 2013;20(8):944–51. Pubmed Central PMCID: 3735830.
29. Lee DG, Makhov AM, Klemm RD, Griffith JD, Bell SP. Regulation of origin recognition complex conformation and ATPase activity: differential effects of single-stranded and double-stranded DNA binding. EMBO J. 2000;19(17):4774–82.
30. Remus D, Beuron F, Tolun G, Griffith JD, Morris EP, Diffley JF. Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. Cell. 2009;139(4):719–30.
31. Remus D, Diffley JF. Eukaryotic DNA replication control: lock and load, then fire. Curr Opin Cell Biol. 2009;21(6):771–7.

32. Evrin C, Clarke P, Zech J, Lurz R, Sun J, Uhle S, et al. A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. Proc Natl Acad Sci U S A. 2009;106(48):20240–5.
33. Wigley DB. ORC proteins: marking the start. Curr Opin Struct Biol. 2009;19(1):72–8.
34. Oppikofer M, Kueng S, Keusch JJ, Hassler M, Ladurner AG, Gut H, et al. Dimerization of Sir3 via its C-terminal winged helix domain is essential for yeast heterochromatin formation. EMBO J. 2013;32(3):437–49. Pubmed Central PMCID: 3567499.
35. Hickman MA, Rusche LN. Transcriptional silencing functions of the yeast protein Orc1/Sir3 subfunctionalized after gene duplication. Proc Natl Acad Sci U S A. 2010;107(45):19384–9. Pubmed Central PMCID: 2984160.
36. Houchens CR, Lu W, Chuang RY, Frattini MG, Fuller A, Simancek P, et al. Multiple mechanisms contribute to Schizosaccharomyces pombe origin recognition complex-DNA interactions. J Biol Chem. 2008;283(44):30216–24. Pubmed Central PMCID: 2573090.
37. Chuang RY, Chretien L, Dai J, Kelly TJ. Purification and characterization of the Schizosaccharomyces pombe origin recognition complex: interaction with origin DNA and Cdc18 protein. J Biol Chem. 2002;277(19):16920–7.
38. On KF, Beuron F, Frith D, Snijders AP, Morris EP, Diffley JF. Prereplicative complexes assembled in vitro support origin-dependent and independent DNA replication. EMBO J. 2014;33(6):605–20. Pubmed Central PMCID: 3989654.
39. Gros J, Devbhandari S, Remus D. Origin plasticity during budding yeast DNA replication in vitro. EMBO J. 2014;33(6):621–36. Pubmed Central PMCID: 3989655.
40. Hoshina S, Yura K, Teranishi H, Kiyasu N, Tominaga A, Kadoma H, et al. Human origin recognition complex binds preferentially to G-quadruplex-preferable RNA and single-stranded DNA. J Biol Chem. 2013;288(42):30161–71. Pubmed Central PMCID: 3798484.
41. Norseen J, Thomae A, Sridharan V, Aiyar A, Schepers A, Lieberman PM. RNA-dependent recruitment of the origin recognition complex. EMBO J. 2008;27(22):3024–35. Pubmed Central PMCID: 2585170.
42. Mohammad MM, Donti TR, Sebastian Yakisich J, Smith AG, Kapler GM. Tetrahymena ORC contains a ribosomal RNA fragment that participates in rDNA origin recognition. EMBO J. 2007;26(24):5048–60. Pubmed Central PMCID: 2140106.
43. Norseen J, Johnson FB, Lieberman PM. Role for G-quadruplex RNA binding by Epstein-Barr virus nuclear antigen 1 in DNA replication and metaphase chromosome attachment. J Virol. 2009;83(20):10336–46. Pubmed Central PMCID: 2753104.
44. Deng Z, Norseen J, Wiedmer A, Riethman H, Lieberman PM. TERRA RNA binding to TRF2 facilitates heterochromatin formation and ORC recruitment at telomeres. Mol Cell. 2009;35(4):403–13. Pubmed Central PMCID: 2749977.
45. Besnard E, Babled A, Lapasset L, Milhavet O, Parrinello H, Dantec C, et al. Unraveling cell type-specific and reprogrammable human replication origin signatures associated with G-quadruplex consensus motifs. Nat Struct Mol Biol. 2012;19(8):837–44.
46. Cayrou C, Coulombe P, Puy A, Rialle S, Kaplan N, Segal E, et al. New insights into replication origin characteristics in metazoans. Cell Cycle. 2012;11(4):658–67. Pubmed Central PMCID: 3318102.
47. Cayrou C, Coulombe P, Vigneron A, Stanojcic S, Ganier O, Peiffer I, et al. Genome-scale analysis of metazoan replication origins reveals their organization in specific but flexible sites defined by conserved features. Genome Res. 2011;21(9):1438–49. Pubmed Central PMCID: 3166829.
48. Armache KJ, Garlick JD, Canzio D, Narlikar GJ, Kingston RE. Structural basis of silencing: Sir3 BAH domain in complex with a nucleosome at 3.0 A resolution. Science (New York, NY). 2011;334(6058):977–82.
49. Callebaut I, Courvalin JC, Mornon JP. The BAH (bromo-adjacent homology) domain: a link between DNA methylation, replication and transcriptional regulation. FEBS Lett. 1999;446(1):189–93.
50. Onishi M, Liou GG, Buchberger JR, Walz T, Moazed D. Role of the conserved Sir3-BAH domain in nucleosome binding and silent chromatin assembly. Mol Cell. 2007;28(6):1015–28.

51. Kuo AJ, Song J, Cheung P, Ishibe-Murakami S, Yamazoe S, Chen JK, et al. The BAH domain of ORC1 links H4K20me2 to DNA replication licensing and Meier-Gorlin syndrome. Nature. 2012;484(7392):115–9.
52. Noguchi K, Vassilev A, Ghosh S, Yates JL, DePamphilis ML. The BAH domain facilitates the ability of human Orc1 protein to activate replication origins in vivo. EMBO J. 2006;25(22):5372–82.
53. Bicknell LS, Walker S, Klingseisen A, Stiff T, Leitch A, Kerzendorfer C, et al. Mutations in ORC1, encoding the largest subunit of the origin recognition complex, cause microcephalic primordial dwarfism resembling Meier-Gorlin syndrome. Nat Genet. 2011;43(4):350–5.
54. Zhang W, Sankaran S, Gozani O, Song J. A Meier-Gorlin Syndrome mutation impairs the ORC1-nucleosome association. ACS Chem Biol. 2015;10:1176–80.
55. Eaton ML, Galani K, Kang S, Bell SP, MacAlpine DM. Conserved nucleosome positioning defines replication origins. Genes Dev. 2010;24(8):748–53. Pubmed Central PMCID: 2854390.
56. Muller P, Park S, Shor E, Huebert DJ, Warren CL, Ansari AZ, et al. The conserved bromo-adjacent homology domain of yeast Orc1 functions in the selection of DNA replication origins within chromatin. Genes Dev. 2010;24(13):1418–33.
57. Hoggard T, Shor E, Muller CA, Nieduszynski CA, Fox CA. A link between ORC-origin binding mechanisms and origin activation time revealed in budding yeast. PLoS Genet. 2013;9(9), e1003798. Pubmed Central PMCID: 3772097.
58. Hizume K, Yagura M, Araki H. Concerted interaction between origin recognition complex (ORC), nucleosomes and replication origin DNA ensures stable ORC-origin binding. Genes Cells. 2013;18(9):764–79.
59. Lee DG, Bell SP. Architecture of the yeast origin recognition complex bound to origins of DNA replication. Mol Cell Biol. 1997;17(12):7159–68.
60. Chen S, de Vries MA, Bell SP. Orc6 is required for dynamic recruitment of Cdt1 during repeated Mcm2-7 loading. Genes Dev. 2007;21(22):2897–907.
61. Frigola J, Remus D, Mehanna A, Diffley JF. ATPase-dependent quality control of DNA replication origin licensing. Nature. 2013;495(7441):339–43.
62. Liu S, Balasov M, Wang H, Wu L, Chesnokov IN, Liu Y. Structural analysis of human Orc6 protein reveals a homology with transcription factor TFIIB. Proc Natl Acad Sci U S A. 2011;108(18):7373–8. Pubmed Central PMCID: 3088625.
63. Bleichert F, Balasov M, Chesnokov I, Nogales E, Botchan MR, Berger JM. A Meier-Gorlin syndrome mutation in a conserved C-terminal helix of Orc6 impedes origin recognition complex formation. Elife. 2013;2, e00882. Pubmed Central PMCID: 3791464.
64. Siddiqui K, Stillman B. ATP-dependent assembly of the human origin recognition complex. J Biol Chem. 2007;282(44):32370–83.
65. Ranjan A, Gossen M. A structural role for ATP in the formation and stability of the human origin recognition complex. Proc Natl Acad Sci U S A. 2006;103(13):4864–9. Pubmed Central PMCID: 1458761.
66. Hossain M, Stillman B. Meier-Gorlin syndrome mutations disrupt an Orc1 CDK inhibitory domain and cause centrosome reduplication. Genes Dev. 2012;26(16):1797–810. Pubmed Central PMCID: 3426759.
67. Stiff T, Alagoz M, Alcantara D, Outwin E, Brunner HG, Bongers EM, et al. Deficiency in origin licensing proteins impairs cilia formation: implications for the aetiology of Meier-Gorlin syndrome. PLoS Genet. 2013;9(3), e1003360. Pubmed Central PMCID: 3597520.
68. Prasanth SG, Prasanth KV, Siddiqui K, Spector DL, Stillman B. Human Orc2 localizes to centrosomes, centromeres and heterochromatin during chromosome inheritance. EMBO J. 2004;23(13):2651–63. Pubmed Central PMCID: 449767.
69. Prasanth SG, Prasanth KV, Stillman B. Orc6 involved in DNA replication, chromosome segregation, and cytokinesis. Science (New York NY). 2002;297(5583):1026–31.
70. Hemerly AS, Prasanth SG, Siddiqui K, Stillman B. Orc1 controls centriole and centrosome copy number in human cells. Science (New York, NY). 2009;323(5915):789–93.
71. Chesnokov IN, Chesnokova ON, Botchan M. A cytokinetic function of Drosophila ORC6 protein resides in a domain distinct from its replication activity. Proc Natl Acad Sci U S A. 2003;100(16):9150–5. Pubmed Central PMCID: 170887.

72. Balasov M, Huijbregts RP, Chesnokov I. Functional analysis of an Orc6 mutant in Drosophila. Proc Natl Acad Sci U S A. 2009;106(26):10672–7. Pubmed Central PMCID: 2705598.
73. Akhmetova K, Balasov M, Huijbregts RP, Chesnokov I. Functional insight into the role of Orc6 in septin complex filament formation in Drosophila. Mol Biol Cell. 2015;26(1):15–28. Pubmed Central PMCID: 4279225.
74. Bernal JA, Venkitaraman AR. A vertebrate N-end rule degron reveals that Orc6 is required in mitosis for daughter cell abscission. J Cell Biol. 2011;192(6):969–78. Pubmed Central PMCID: 3063140.
75. Sun J, Kawakami H, Zech J, Speck C, Stillman B, Li H. Cdc6-induced conformational changes in ORC bound to origin DNA revealed by cryo-electron microscopy. Structure. 2012;20(3):534–44. Pubmed Central PMCID: 3299985.
76. Nguyen VQ, Co C, Li JJ. Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature. 2001;411(6841):1068–73.
77. Chen S, Bell SP. CDK prevents Mcm2-7 helicase loading by inhibiting Cdt1 interaction with Orc6. Genes Dev. 2011;25(4):363–72. Pubmed Central PMCID: 3042159.
78. Ogura T, Whiteheart SW, Wilkinson AJ. Conserved arginine residues implicated in ATP hydrolysis, nucleotide-sensing, and inter-subunit interactions in AAA and AAA+ ATPases. J Struct Biol. 2004;146(1-2):106–12.
79. Bowers JL, Randell JC, Chen S, Bell SP. ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. Mol Cell. 2004;16(6):967–78.
80. Clarey MG, Erzberger JP, Grob P, Leschziner AE, Berger JM, Nogales E, et al. Nucleotide-dependent conformational changes in the DnaA-like core of the origin recognition complex. Nat Struct Mol Biol. 2006;13(8):684–90.
81. Chen Z, Speck C, Wendel P, Tang C, Stillman B, Li H. The architecture of the DNA replication origin recognition complex in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A. 2008;105(30):10326–31.
82. Chastain 2nd PD, Bowers JL, Lee DG, Bell SP, Griffith JD. Mapping subunit location on the Saccharomyces cerevisiae origin recognition complex free and bound to DNA using a novel nanoscale biopointer. J Biol Chem. 2004;279(35):36354–62.
83. Matsuda K, Makise M, Sueyasu Y, Takehara M, Asano T, Mizushima T. Yeast two-hybrid analysis of the origin recognition complex of Saccharomyces cerevisiae: interaction between subunits and identification of binding proteins. FEMS Yeast Res. 2007;7(8):1263–9.
84. Liang C, Weinreich M, Stillman B. ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. Cell. 1995;81(5):667–76.
85. Cocker JH, Piatti S, Santocanale C, Nasmyth K, Diffley JF. An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. Nature. 1996;379(6561):180–2.
86. Santocanale C, Diffley JF. ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in Saccharomyces cerevisiae. EMBO J. 1996;15(23):6671–9.
87. Wilmes GM, Bell SP. The B2 element of the Saccharomyces cerevisiae ARS1 origin of replication requires specific sequences to facilitate pre-RC formation. Proc Natl Acad Sci U S A. 2002;99(1):101–6.
88. Mizushima T, Takahashi N, Stillman B. Cdc6p modulates the structure and DNA binding activity of the origin recognition complex in vitro. Genes Dev. 2000;14(13):1631–41.
89. Coster G, Frigola J, Beuron F, Morris EP, Diffley JF. Origin licensing requires ATP binding and hydrolysis by the MCM replicative helicase. Mol Cell. 2014;55(5):666–77. Pubmed Central PMCID: 4157578.
90. Kang S, Warner MD, Bell SP. Multiple functions for Mcm2-7 ATPase motifs during replication initiation. Mol Cell. 2014;55(5):655–65. Pubmed Central PMCID: 4156824.
91. Heller RC, Kang S, Lam WM, Chen S, Chan CS, Bell SP. Eukaryotic origin-dependent DNA replication in vitro reveals sequential action of DDK and S-CDK kinases. Cell. 2011;146(1):80–91.
92. Yeeles JT, Deegan TD, Janska A, Early A, Diffley JF. Regulated eukaryotic DNA replication origin firing with purified proteins. Nature. 2015;519(7544):431–5.

93. Samson RY, Bell SD. MCM loading--an open-and-shut case? Mol Cell. 2013;50(4):457–8.
94. Yardimci H, Walter JC. Prereplication-complex formation: a molecular double take? Nat Struct Mol Biol. 2014;21(1):20–5.
95. Dion MF, Kaplan T, Kim M, Buratowski S, Friedman N, Rando OJ. Dynamics of replication-independent histone turnover in budding yeast. Science (New York NY). 2007;315(5817):1405–8.
96. Huang D, Koshland D. Chromosome integrity in Saccharomyces cerevisiae: the interplay of DNA replication initiation factors, elongation factors, and origins. Genes Dev. 2003;17(14):1741–54. Pubmed Central PMCID: 196182.
97. Fox CA, Loo S, Dillin A, Rine J. The origin recognition complex has essential functions in transcriptional silencing and chromosomal replication. Genes Dev. 1995;9(8):911–24.
98. Klemm RD, Bell SP. ATP bound to the origin recognition complex is important for preRC formation. Proc Natl Acad Sci U S A. 2001;98(15):8361–7.
99. Tsakraklides V, Bell SP. Dynamics of pre-replicative complex assembly. J Biol Chem. 2010;285(13):9437–43. Pubmed Central PMCID: 2843193.
100. Randell JC, Bowers JL, Rodriguez HK, Bell SP. Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. Mol Cell. 2006;21(1):29–39.
101. Ge XQ, Jackson DA, Blow JJ. Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress. Genes Dev. 2007;21(24):3331–41.
102. Ibarra A, Schwob E, Mendez J. Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. Proc Natl Acad Sci U S A. 2008;105(26):8956–61.
103. Kawabata T, Luebben SW, Yamaguchi S, Ilves I, Matise I, Buske T, et al. Stalled fork rescue via dormant replication origins in unchallenged S phase promotes proper chromosome segregation and tumor suppression. Mol Cell. 2011;41(5):543–53.
104. Remus D, Beall EL, Botchan MR. DNA topology, not DNA sequence, is a critical determinant for Drosophila ORC-DNA binding. EMBO J. 2004;23(4):897–907.
105. Austin RJ, Orr-Weaver TL, Bell SP. Drosophila ORC specifically binds to ACE3, an origin of DNA replication control element. Genes Dev. 1999;13(20):2639–49.
106. Mendez J, Zou-Yang XH, Kim SY, Hidaka M, Tansey WP, Stillman B. Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol Cell. 2002;9(3):481–91.
107. Ohta S, Tatsumi Y, Fujita M, Tsurimoto T, Obuse C. The ORC1 cycle in human cells: II. Dynamic changes in the human ORC complex during the cell cycle. J Biol Chem. 2003;278(42):41535–40.
108. Diffley JF, Cocker JH, Dowell SJ, Rowley A. Two steps in the assembly of complexes at yeast replication origins in vivo. Cell. 1994;78(2):303–16.
109. Aparicio OM, Weinstein DM, Bell SP. Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell. 1997;91(1):59–69.
110. Belsky JA, MacAlpine HK, Lubelsky Y, Hartemink AJ, MacAlpine DM. Genome-wide chromatin footprinting reveals changes in replication origin architecture induced by pre-RC assembly. Genes Dev. 2015;29(2):212–24. Pubmed Central PMCID: 4298139.
111. Wilmes GM, Archambault V, Austin RJ, Jacobson MD, Bell SP, Cross FR. Interaction of the S-phase cyclin Clb5 with an "RXL" docking sequence in the initiator protein Orc6 provides an origin-localized replication control switch. Genes Dev. 2004;18(9):981–91.
112. Palacios DeBeer MA, Muller U, Fox CA. Differential DNA affinity specifies roles for the origin recognition complex in budding yeast heterochromatin. Genes Dev. 2003;17(15):1817–22.
113. Knott SR, Peace JM, Ostrow AZ, Gan Y, Rex AE, Viggiani CJ, et al. Forkhead transcription factors establish origin timing and long-range clustering in S. cerevisiae. Cell. 2012;148(1-2):99–111. Pubmed Central PMCID: 3266545.
114. Looke M, Kristjuhan K, Varv S, Kristjuhan A. Chromatin-dependent and -independent regulation of DNA replication origin activation in budding yeast. EMBO Rep. 2013;14(2):191–8. Pubmed Central PMCID: 3596130.

115. Thomae AW, Pich D, Brocher J, Spindler MP, Berens C, Hock R, et al. Interaction between HMGA1a and the origin recognition complex creates site-specific replication origins. Proc Natl Acad Sci U S A. 2008;105(5):1692–7. Pubmed Central PMCID: 2234206.
116. Leatherwood J, Vas A. Connecting ORC and heterochromatin: why? Cell Cycle. 2003;2(6):573–5.
117. Shen Z, Sathyan KM, Geng Y, Zheng R, Chakraborty A, Freeman B, et al. A WD-repeat protein stabilizes ORC binding to chromatin. Mol Cell. 2010;40(1):99–111.
118. Shen Z, Chakraborty A, Jain A, Giri S, Ha T, Prasanth KV, et al. Dynamic association of ORCA with prereplicative complex components regulates DNA replication initiation. Mol Cell Biol. 2012;32(15):3107–20. Pubmed Central PMCID: 3434513.
119. Shen Z, Prasanth SG. Emerging players in the initiation of eukaryotic DNA replication. Cell Div. 2012;7(1):22. Pubmed Central PMCID: 3520825.
120. Nieduszynski CA, Blow JJ, Donaldson AD. The requirement of yeast replication origins for pre-replication complex proteins is modulated by transcription. Nucleic Acids Res. 2005;33(8):2410–20. Pubmed Central PMCID: 1087785.
121. Eaton ML, Prinz JA, MacAlpine HK, Tretyakov G, Kharchenko PV, MacAlpine DM. Chromatin signatures of the Drosophila replication program. Genome Res. 2010;21(2): 164–74.
122. Lubelsky Y, Sasaki T, Kuipers MA, Lucas I, Le Beau MM, Carignon S, et al. Pre-replication complex proteins assemble at regions of low nucleosome occupancy within the Chinese hamster dihydrofolate reductase initiation zone. Nucleic Acids Res. 2011;39(8):3141–55. Pubmed Central PMCID: 3082903.
123. Papior P, Arteaga-Salas JM, Gunther T, Grundhoff A, Schepers A. Open chromatin structures regulate the efficiencies of pre-RC formation and replication initiation in Epstein-Barr virus. J Cell Biol. 2012;198(4):509–28. Pubmed Central PMCID: 3514025.
124. Wu PY, Nurse P. Establishing the program of origin firing during S phase in fission Yeast. Cell. 2009;136(5):852–64.
125. Mantiero D, Mackenzie A, Donaldson A, Zegerman P. Limiting replication initiation factors execute the temporal programme of origin firing in budding yeast. EMBO J. 2011;30(23):4805–14. Pubmed Central PMCID: 3243606.
126. Tanaka S, Nakato R, Katou Y, Shirahige K, Araki H. Origin association of Sld3, Sld7, and Cdc45 proteins is a key step for determination of origin-firing timing. Curr Biol. 2011;21(24):2055–63.
127. Rhind N, Yang SC, Bechhoefer J. Reconciling stochastic origin firing with defined replication timing. Chromosome Res. 2010;18(1):35–43. Pubmed Central PMCID: 2862975.
128. Yang SC, Rhind N, Bechhoefer J. Modeling genome-wide replication kinetics reveals a mechanism for regulation of replication timing. Mol Syst Biol. 2010;6:404. Pubmed Central PMCID: 2950085.
129. Ryba T, Hiratani I, Sasaki T, Battaglia D, Kulik M, Zhang J, et al. Replication timing: a fingerprint for cell identity and pluripotency. PLoS Comput Biol. 2011;7(10), e1002225. Pubmed Central PMCID: 3197641.
130. Debatisse M, Le Tallec B, Letessier A, Dutrillaux B, Brison O. Common fragile sites: mechanisms of instability revisited. Trends Genet. 2012;28(1):22–32.
131. Watanabe Y, Maekawa M. Spatiotemporal regulation of DNA replication in the human genome and its association with genomic instability and disease. Curr Med Chem. 2010;17(3):222–33.
132. Smith L, Plug A, Thayer M. Delayed replication timing leads to delayed mitotic chromosome condensation and chromosomal instability of chromosome translocations. Proc Natl Acad Sci U S A. 2001;98(23):13300–5. Pubmed Central PMCID: 60865.
133. Woodward AM, Gohler T, Luciani MG, Oehlmann M, Ge X, Gartner A, et al. Excess Mcm2-7 license dormant origins of replication that can be used under conditions of replicative stress. J Cell Biol. 2006;173(5):673–83.