Nucleosome-directed replication origin licensing independent of a consensus DNA sequence

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ABSTRACT

The numerous enzymes and cofactors involved in eukaryotic DNA replication are conserved from yeast to human, and the budding yeast *Saccharomyces cerevisiae* (S.c.) has been a useful model organism for these studies. However, there is a gap in our knowledge of why replication origins in higher eukaryotes do not use a consensus DNA sequence as found in S.c.. Using in vitro reconstitution and single-molecule visualization, we show here that S.c. origin recognition complex (ORC) stably binds nucleosomes and that ORC-nucleosome complexes have the intrinsic ability to load the replicative helicase MCM double hexamers onto adjacent nucleosome-free DNA regardless of sequence. Furthermore, we find that *Xenopus laevis* nucleosomes can substitute for yeast ones in engaging with ORC. Combined with new analysis of genome-wide ORC binding, our results lead us to propose that the yeast origin recognition machinery contains the cryptic capacity to bind nucleosomes near a nucleosome-free region and license origins, and that this nucleosome-directed origin licensing paradigm generalizes to all eukaryotes.

INTRODUCTION

Complete and accurate genome duplication prior to cell division is a critical process for essentially all living organisms. The basic mechanism for the initiation of DNA replication is shared across domains of life [1,2]. An "initiator" first binds to genomic sites referred to as origins of replication, then recruits the replicative helicase responsible for unwinding parental duplex DNA, creating two complementary templates for DNA polymerases to form two identical copies of daughter chromosomes. In eukaryotes, multiple origins across the genome are licensed for firing once, and only once, per cell cycle and their firing follows a temporally controlled program [3-5]. The eukaryotic initiator is known as the origin recognition complex (ORC), which consists of six conserved subunits, Orc1-6 [6,7]. ORC works in concert with Cdc6 and Cdt1 to coordinate the loading of two Mcm2-7 helicase complexes (MCM) onto the origin DNA [8,9], forming the MCM double hexamer (DH), or "pre-replication complex" (pre-RC). Only a fraction of MCMs loaded on chromatin become activated during the subsequent S phase to produce bidirectional replication forks. The excess of chromatin-associated MCM over those used for replication is referred to as the "MCM paradox" [10].

In the budding yeast *Saccharomyces cerevisiae* (S.c.), origins are located at a set of "replicator" positions known as Autonomously Replicating Sequences (ARS), each containing a 17-bp AT-rich ARS consensus sequence (ACS) element and other less conserved "B elements" [1]. The ARS sequence is AT rich which is not conducive to nucleosome assembly, forming a "nucleosome free region" (NFR). An NFR at an ARS is important for origin function [11], and when nucleosomes are permitted to encroach upon the ARS they are demonstrated to be inhibitory [12-14]. While S.c. has been an instrumental model organism to study eukaryotic replication initiation, the use of a consensus origin DNA sequence is only found in some budding yeasts [15], and therefore a consensus origin sequence is the exception rather than the norm in eukaryotes [15,16]. For example, defined origin elements are not present in the yeast, *Schizosaccharomyces pombe* [17]. The specificity of S.c. ORC for ARS is due to a unique insertion helix (IH) in the Orc4 subunit, which specifically interacts with the ACS sequence [18-21]. S.c. strains with Orc4 IH mutations alter genome-wide firing patterns [19,20] and may enable initiation in NFRs that are different from ARS, such as at transcriptional promotors. It is also worth noting that ARS and its internal ACS and B elements are not strictly required in vitro for S.c. ORC binding or MCM loading [9,22]. These findings, combined with the lack of ACS-like elements in higher eukaryotes, indicate that there exist other chromosomal features that enable the licensing and firing of eukaryotic replication origins [15,23].

One distinct challenge for ORC is the need to navigate through chromosomes prevalently packaged into nucleosomes [24], which influence multiple aspects of eukaryotic replication [25-27]. The nucleosome is often viewed as a barrier to ORC binding and origin activity that must be overcome by certain DNA sequences or chromatin-remodeling enzymes [12-14]. ORC is known to directly interact with nucleosomes by multiple connections [28,29]. Given the continuing uncertainties of the various contacts of ORC subunits to nucleosomes, the interplay between ORC and nucleosomes and its role in origin selection and function remain an area of active research.

In the current study we unexpectedly discovered that budding yeast contain a cryptic nucleosome-dependent mechanism for origin licensing that is independent of an ARS consensus, implying its capability to license origins independent of DNA sequence, and suggesting this process may provide a general mechanism for origin selection that applies to all eukaryotes. On hindsight, this is supported by an extensive ChIP-seq study that determined about one-third of S.c. origins lack a recognizable consensus ACS sequence [11]. Based on the facts that (1) ORC binds nucleosomes [28,29], and (2) pre-RCs are predominantly licensed at nucleosome-free regions (NFRs) [11,12], we hypothesize that

nucleosomes recruit ORC and that this can lead to assembly of the MCM DH onto DNA provided, at a minimum, that there is an adjacent NFR that can accommodate ORC binding and MCM loading (**Figure 1**). We presume that there may be additional requirements for origin activation in vivo at these "non-ARS" sites, such as histone modifications or nucleosome remodelers.

Why would S.c. have developed sequence-specific origins and also utilize a general nucleosome-directed but non-sequence-specific method for origin selection? We presume S.c. evolved ARS sequences, located at intergenic regions, for preferred ORC binding due to its small genome size and therefore urgent need to avoid transcription-replication conflicts, as proposed earlier [20]. To test whether origin licensing in S.c. can occur at random DNA sequence that is adjacent to a nucleosome, we develop herein a single-molecule platform to visualize the dynamic behavior of ORC and MCM on nucleosomal DNA. We find that S.c. ORC stably associates with nucleosomes and loads MCM DHs at adjacent nucleosome-free regions. This finding of ARS sequence-independent yet nucleosome-directed ORC binding and subsequent MCM DH loading reveals that the S.c. system has an inherent sequence-independent origin licensing activity that is akin to that in higher eukaryotes, thereby providing insight and a unifying mechanism for eukaryotic origin selection.

RESULTS

A single-molecule platform to directly visualize eukaryotic origin licensing

To perform single-molecule studies on pre-RC formation, we used the bacteriophage λ genomic DNA containing an engineered S.c. ARS1 sequence [30] placed 14 kb from one end (**Figure 2A & Supplementary Figure 1**). The DNA construct (termed λ_{ARS1}) was biotinylated at both ends. We expressed and purified the S.c. Orc1-6 and Mcm2-7 complexes (referred to as ORC and MCM hereafter) as well as Cdc6 and Cdt1 (**Supplementary Figure 2**). To generate fluorescently labeled ORC for direct visualization, we site-specifically attached a Cy3 fluorophore to the N terminus of the Orc1 subunit via a 12-residue S6 peptide tag. A single λ_{ARS1} DNA molecule was tethered between a pair of streptavidin-coated beads in the microfluidic chamber of a dual-trap optical tweezers instrument combined with multicolor confocal fluorescence microscopy [31,32] (**Figure 2B**). Upon moving the tethered DNA into a channel containing Cy3-ORC (+/- Cdc6) and ATP, we observed ORC binding to DNA in real time. In the presence of Cdc6, ORC displayed short-lived and diffusive binding to non-ARS1 DNA, while remaining stably bound at the ARS1 site (**Figure 2C**). Cdc6 enhances the overall binding of ORC to DNA and is required for its ARS specificity (**Figure 2D & 2E**). These results are consistent with previous single-molecule studies [33-35], thus confirming the normal function of proteins used in this study. The distinctive behavior of ORC at the engineered ARS1 site versus all other DNA sites indicates that the λ_{ARS1} template does not contain a second strong ACS motif, which is corroborated by sequence analysis of the λ genomic DNA (**Supplementary Figure 3**).

ORC mediates MCM DH loading onto both ARS and non-ARS DNA

Next we generated a S.c. strain expressing Mcm2-7 with S6-tagged Mcm3, enabling us to site-specifically label MCM complexes with a fluorophore. We used LD650-labeled MCM, along with unlabeled ORC, Cdc6 and Cdt1, to examine pre-RC formation on λ_{ARS1} DNA (**Figure 3A**). Surprisingly, we observed that the majority (86%) of MCM that stably bound to DNA were at positions distant from ARS1 (**Figure 3B & 3C**). Considering that non-ARS DNA sites vastly outnumber the sole ARS1 site in our template, ORC/MCM may still have a higher affinity to ARSs than to other DNA sequences. We then conducted photobleaching analysis to examine the stoichiometry of MCM bound to DNA, and observed that a significant fraction (36%) of MCMs underwent two-step photobleaching (**Figure 3D & 3E**). Given

114 the estimated labeling efficiency for MCM (~60%), this result suggests that the majority of MCMs on
115 DNA observed in our experiments were double hexamers. The presence of ORC is strictly required for
116 MCM binding to DNA (**Figure 3F**). Notably, we have shown that ORC displays diffusive behavior at non-
117 ARS sites (**Figure 2C & 2E**). Therefore, it appears that MCM stabilizes ORC binding to non-ARS DNA
118 sequences.
119 To obtain further evidence for MCM DH formation, we moved the DNA tether into a separate
120 channel containing a high-salt buffer (0.5 M NaCl) after ORC-mediated MCM binding (**Figure 3G**). We
121 found that a significant portion of the MCM complexes remained associated with DNA upon high-salt
122 wash—either stably residing at the initial position or diffusing along the DNA (**Figure 3H & 3I**), consistent
123 with the behavior of properly loaded MCM DHs [9,34,35]. The rest of the MCMs dissociated from DNA at
124 high salt, likely representing those not topologically encircling the DNA duplex. The fraction of loaded
125 MCMs at the ARS1 site (62%) is much higher than that at non-ARS sites (25%). Therefore, our results
126 confirm that ARS1 represents a preferred position for ORC-mediated MCM loading, but also
127 unambiguously show that this process can still occur on non-ARS sequences. In other words, our data
128 suggest that ARS enhances the likelihood, but is not strictly required, for ORC to license an origin.
129
130 **ORC preferentially binds nucleosomes over nucleosome-free DNA**
131 Next, we set out to examine the behavior of ORC on chromatinized DNA. We reconstituted both
132 S.c. and *Xenopus laevis* (X.l.) histone octamers for comparative studies (**Supplementary Figure 2 &**
133 **Supplementary Figure 4**). In both cases, a unique cysteine residue is placed on the histone H2A, enabling
134 site-specific labeling of the nucleosome. We used the histone chaperone Nap1 for in situ nucleosome
135 assembly [26,36]. By titrating the protein concentrations, we found a condition that yields sparsely
136 populated nucleosomes (usually between 1 and 7) within the tethered λ_{ARS1} DNA, such that most if not
137 all of the nucleosomes are flanked by substantial NFRs (**Figure 4A & Supplementary Figure 5**). When
138 incubating the DNA tether harboring Cy3-labeled S.c. nucleosomes with LD650-ORC and Cdc6, we made
139 the striking observation that ORC predominantly colocalized with the nucleosomes, rather than residing
140 within the long stretches of bare DNA between nucleosomes (**Figure 4B**). Importantly, stable association
141 of ORC with the nucleosome is independent of whether the nucleosome is located at the ARS1 position
142 or non-ARS sites (**Figure 4C**). In fact, most nucleosomes were located at non-ARS positions and yet the
143 vast majority (>90%) had a stably bound ORC using only a low concentration (2 nM) of ORC (**Figure 4D**).
144 Sometimes we observed ORC at the nucleosome-free ARS1 DNA position where it remained stably
145 bound in the presence of Cdc6 (e.g. second kymograph in **Supplementary Figure 6A**), again supporting
146 the expected and normal ORC function. In contrast, when ORC binds to non-ARS nucleosome-free
147 regions of the nucleosomal DNA tether, it displays diffusive behavior and the diffusion was confined
148 between adjacent nucleosomes that are occupied by ORC (**Figure 4B & Supplementary Figure 6**).
149 Comparing **Figure 2E** (ORC behavior on non-nucleosomal DNA) and **Figure 4C** (ORC behavior on
150 nucleosomal DNA), it becomes apparent that the presence of nucleosomes on DNA abrogates the
151 requirement of ARS sequences for stable ORC binding. In other words, ORC has the ability to stably
152 engage with a nucleosome regardless of its adjacent DNA sequence. Interestingly, stable association of
153 ORC with nucleosomes appears to be a conserved phenomenon as we obtained similar results using
154 either S.c. or X.l. histone octamers (**Figures 4C & 4D**). Nucleosome targeting by ORC can conceivably be
155 achieved by either a three-dimensional (3D) search (direct binding from solution) or a one-dimensional
156 (1D) search (sliding along the DNA from a non-nucleosomal site). We observed both modes in our data,

157 with 3D search being the more dominant mode, especially for S.c. nucleosomes (**Supplementary Figure**
158 **6**).
159
160 **ORC mediates MCM loading to nucleosomes**
161 Next we asked whether ORC binding to nucleosomes can lead to MCM helicase loading and pre-
162 RC formation. We used fluorescently labeled MCM complexes (with unlabeled ORC, Cdc6, and Cdt1) to
163 examine their recruitment to nucleosomal DNA and its dependence on ORC. We found that MCM
164 frequently colocalized with nucleosomes that were sparsely distributed across the DNA tether (**Figure**
165 **5A & 5B**). This result was observed for both S.c. and X.l. nucleosomes. MCM binding to the nucleosome
166 requires the presence of ORC, as omitting ORC completely eliminated MCM-nucleosome colocalization
167 (**Figure 5C**). Considering that the free DNA sites vastly outnumber the nucleosome sites in our assay (a
168 few nucleosomes within 48-kbp DNA), the observed frequency of MCM-nucleosome colocalization
169 indicates that nucleosomes are preferred sites for ORC-mediated MCM binding (**Figure 5D**). Again, ARS
170 sequences are not required for MCM-nucleosome colocalization, as these events were mostly observed
171 at non-ARS sites (**Figure 5E**). To examine whether MCM can form double hexamers at the nucleosome,
172 we performed three-color fluorescence experiments using A488-labeled histone octamers and a mixture
173 of Cy3- and LD650-labeled MCMs (**Figure 5F**). Indeed, we detected colocalization of dual-color MCMs
174 (green and red) with nucleosomes (blue), suggesting MCM DH formation at the nucleosome site.
175 To test whether MCM DHs are truly loaded (i.e. topologically encircling DNA), we moved the
176 tethered nucleosomal DNA with bound ORC and MCM into a high-salt buffer channel containing 0.5 M
177 NaCl. As explained earlier, recruited MCMs that do not encircle DNA are expected to dissociate at high
178 salt, whereas loaded MCM DHs are expected to stay on DNA and can slide on it if ORC is dislodged by
179 high salt [8,9]. Notably, we found that, unlike ORC removal from free DNA (ARS1 or non-ARS1) by high salt
180 in the absence of nucleosomes, MCM-ORC complexes appeared more resistant to salt when bound to
181 nucleosomes. Thus we use labeled ORC to examine the ability to dissociate ORC from nucleosomes at
182 0.5 M NaCl. Upon comparing the two types of nucleosomes (S.c. vs. X.l.), we found that about half of S.c.
183 nucleosome-bound ORC was dissociated by 0.5 M NaCl, and about 20% of X.l. nucleosome-bound ORC
184 was removed (**Supplementary Figure 7**). Then we analyzed the behavior of fluorescent MCM (red) bound
185 to S.c. ORC-S.c. fluorescent nucleosomes (blue) upon high-salt wash. The example kymograph in **Figure**
186 **6A** shows multiple MCMs on tethered DNA. Upon high-salt wash, the MCM colocalized with a non-ARS
187 positioned nucleosome underwent sliding on DNA, as did another MCM located at the ARS1 DNA site
188 (without a nucleosome). More kymographs are shown in **Supplementary Figure 8**, including an example
189 of dual-color MCM DH sliding at high salt. Overall we observed ~30% of MCMs formed at ORC-
190 nucleosome sites to diffuse on DNA and another ~30% to remain associated with the nucleosome (**Figure**
191 **6B**). The immobile MCM fraction can be attributed to the strong engagement of ORC with the
192 nucleosome (**Supplementary Figure 7**), which in turn holds MCM next to the nucleosome. The remainder
193 of the MCMs dissociated into solution upon high-salt wash, which presumably represent MCMs that
194 were not fully loaded onto DNA.
195
196 **Genome-wide analysis of ORC/MCM localization**
197 The single-molecule results presented in this study suggest that ORC binding and MCM loading
198 may occur over genomic regions that lack an ARS consensus sequence (ACS) element. In support of this
199 observation, it has been previously shown that only 67% of well documented ARS sites in the S.c. genome
200 contain a recognizable ACS sequence [11]; while the remaining 33% were presumed to contain "novel ACS

sequences". We propose, based on the work presented in the current report, that the novel ACS sequences may have been random DNA sequences adjacent to a nucleosome at an NFR.

To test this hypothesis, we re-analyzed published S.c. Orc1 and Mcm2-7 ChIP-seq data [11,37]. Consistent with previous analysis [11], we identified 295 Orc1 ChIP peaks genome-wide, and most of these sites also bind to Mcm2-7 (**Figure 7A**). Most of the Orc1 peaks (225 out of 295) overlap with annotated origins of replication [38] (**Supplementary Table 1**). Examples of ORC/MCM peaks at known origin sites are shown in **Figure 7B**. We extracted DNA sequences +/- 500 bp flanking the center of the ChIP peaks, scanned each 17-bp window with the ACS position weight matrix (PWM) [39], and recorded all the windows with a PWM score of > 9 (previous studies recommended a cutoff score of 11.9). We found that, even with this less stringent criterion, 145 out of the 295 Orc1 peaks contain no qualified window, 105 with one qualified window, and the remaining 45 with multiple qualified windows. The PWM scores are not correlated with the strength of the ChIP peaks (**Figure 7C**). For example, among the six examples shown in **Figure 7B**, ARS609, ARS523, and ARS416 contain an ACS with high-to-medium scores of 13.2, 14.0, and 10.1, respectively, while the rest contain no window with a score above 9. Yet all of these sites show comparable Orc1 and Mcm2-7 ChIP signals. This unexpected data suggests that ORC/MCM binding to established S.c. origins does not require a highly consensus ACS.

To rule out the possibility that the above conclusion depends on the choice of the cutoff score, we calculated the fraction of origin sequences that contain at least one qualified window using different cutoff values (**Figure 7D**). As expected, fewer qualified sequences were identified with a higher cutoff ACS PWM score. Using the conventional cutoff of 11.9, the ACS consensus is only identified in ~20% of Orc1 ChIP peaks. Among the 225 Orc1 peaks that overlap with annotated origins, we observed a similar number (~20%) (**Figure 7E**). This number only mildly increases to ~25% when we analyzed the subset of Orc1 ChIP peaks that also have high Mcm2-7 ChIP signals (**Supplementary Figure 9A&B**). To test the above conclusion further, we also examined the recent Orc1 ChIP-exo data [40]. Although only 81 Orc1 peaks were identified in this dataset, the probability of finding a consensus ACS motif in these peaks is essentially the same (~20% with a cutoff score of 11.9) (**Supplementary Figure 9C**). As a quality control of our method, we applied the same analysis to sequence-specific transcription factors Abf1 and Reb1, and found that their cognate DNA motifs occur at a much higher frequency near their respective ChIP peaks (~75% and ~55% at recommended cutoff of 8.2 and 8.7, respectively) (**Figure 7F**). Overall, these results suggest that a significant fraction of established replication origins in the yeast genome do not contain a highly consensus ACS.

We next investigated the location of Orc1 ChIP peaks relative to the neighboring nucleosomes. Nucleosome occupancy measured by MNase assay is also shown in **Figure 7B** [41]. For the first three origins with a well-defined ACS, the consensus sequence all locates inside NFRs (indicated by the red lines). Indeed, when we collected all 62 Orc1 peaks with a consensus ACS (score > 11.9), aligned at their ACS locations, and plotted the nearby nucleosome occupancy, we observed strong nucleosome depletion over most of the sites (**Figure 7G**). This is consistent with the previous finding that a consensus ACS engineered into a well-positioned nucleosome leads to nucleosome displacement [42]. For some Orc1 binding sites without a strong ACS (score < 9), the Orc1/Mcm2-7 peaks seem to locate near a nucleosome-NFR junction (**Figure 7B**).

Taken together, these findings are consistent with the model that ORC either binds to consensus ACS elements in the genome—which may lead to NFR formation—or binds to nucleosomes near an NFR, indicating a level of flexibility in yeast origin architecture that has also been reported before [43].

DISCUSSION

Nucleosomes play a major role in directing ORC function in origin licensing

The prevailing model for eukaryotic origin selection takes a DNA-centric view, which argues that ARS—especially the ACS element within ARS—largely dictates where ORC binds in the genome [1]. Nonetheless, in vitro studies demonstrate that these elements are not strictly required for pre-RC formation or replication initiation even for the yeast system where ARSs were initially identified [6,9,22]. For example, the in vitro MCM loading efficiency was similar between wildtype ARS1 and ACS-deleted ARS1, and the specificity of MCM DH loading at ARS1 required addition of competitor DNA [9]. Moreover, the yeast genome contains far more ACS motifs than functional origins [44]. Therefore, the determinants of eukaryotic origin selection must include other chromosomal features, the identity of which are still under study. By imaging ORC's behavior on nucleosomal DNA in real time, our work provides clear evidence that ORC preferentially binds nucleosomes—independent of a nearby ACS motif—over non-nucleosomal DNA, and importantly, that this interaction is functionally relevant to MCM DH loading at nucleosomal sites. These findings support the hypothesis illustrated in **Figure 1** that nucleosomes are the dominant directive of origin function in all cells. This demonstration of ARS-sequence independent, but nucleosome-directed origin licensing, is applicable to all eukaryotes including those where a consensus origin sequence has not been found. In this hypothesis, a nucleosome and an adjacent NFR sufficiently wide to accommodate ORC and an MCM DH are the two main prerequisites for pre-RC formation.

The influence of nucleosomes on origin function has been previously investigated [45], but mainly reported as a secondary mechanism to reinforce the DNA sequence-encoded origin specificity by targeting ORC to ARS instead of other DNA sequences occupied by nucleosomes [26,27]. Here we show that nucleosomes—in and of themselves—represent a primary instructive code for replication origins. Given the highly conserved nature of replication initiation factors, we presume that this principle will generalize to higher eukaryotes, just as the many earlier findings in the S.c. system.

NFR adjacent to a nucleosome enables pre-RC assembly independently of ARS

The second prerequisite for pre-RC formation in our model is the presence of a sufficiently wide NFR that can accommodate the pre-RC complex. It is known that an NFR flanked by regularly positioned nucleosomes is a pronounced feature of S.c. ARS origin sites [11,12]. Our single-molecule data suggest that an MCM DH can be loaded onto DNA lacking an ARS sequence as long as it is adjacent to a nucleosome. Our genomic analyses show that S.c. indeed utilizes many origin sites without a strong ACS element. ARS may be involved in creating some of these NFRs [42], but is not a necessity. Indeed, there exist numerous non-ARS NFRs in yeast chromosomes located at 5' and 3' ends of genes. These NFRs are 80-300 bp long and flanked by well-positioned nucleosomes [46-48]. Importantly, recent studies have shown that "humanizing mutations" of S.c. ORC that abrogate its ability to bind ACS change the genome-wide origin pattern such that initiation occurs at NFRs lacking ACS, such as at promotors [19,20], supporting the notion that some other NFRs that border a nucleosome can in principle be utilized as an origin site.

On the other hand, the requirement for an NFR permissive to helicase loading excludes many genomic regions from becoming origin sites, such as the tightly packaged heterochromatin prevalent in eukaryotic chromosomes. This view explains why replication initiation cannot occur at just any nucleosome. The hierarchical chromatin organization in vivo also influences how the timing of different

289 origins is controlled [17]. There is evidence that origins located within heterochromatin fire later in S phase
290 compared to those in the more open euchromatin [49], perhaps stemming from the time needed for
291 factors such as chromatin remodelers to create wide enough NFRs for ORC binding and MCM DH
292 assembly.
293
294 **Why have some yeast species evolved DNA sequence-specific origins?**
295 Our model (**Figure 1**) infers that there is no fundamental difference in the origin selection
296 mechanism between yeast and higher eukaryotes, but simply that some yeast species (for example, S.c.)
297 have evolved a dependence on ARS sequences, perhaps to increase fitness. Yet S.c. cells contain some
298 origins that simply lack a recognizable ACS for ORC [11]. This evolutionary pressure may be related to the
299 high gene density in organisms such as *S. cerevisiae* with a small genome size. The slight advantage that
300 the ACS element confers on yeast ORC binding to non-nucleosomal DNA, along with the strong binding
301 of ORC to a nucleosome adjacent to an ARS sequence, may serve to place replication origins within
302 intergenic regions to help avoid replication-transcription conflicts and genome instability [20]. As such,
303 ARSs add an additional layer of "security" to prevent spurious origin firing. Nevertheless, our results here
304 show that the S.c. system still retains the nucleosome-directed origin licensing capability that is likely
305 also used by higher eukaryotes. Metazoans, which have much larger genomes, may have evolved other
306 mechanisms to circumvent or tolerate transcriptional interference, and thus did not need to evolve
307 sequence-specific origins [50].
308
309 **A potential explanation for the MCM paradox**
310 Our proposed model that any NFR might enable MCM DH formation mediated by ORC-
311 nucleosome interaction in G1 phase suggests a rather "sloppy" process of origin licensing. It follows that
312 many more sites may be licensed than can be used in S phase for origin firing. While we do not expect
313 replication initiation to take place in every NFR in each cell cycle, because of additional unknown
314 requirements (e.g. histone modifications, MCM requirements to mature to CMG helicases), it is still
315 possible that many NFRs that occur within the genome allow MCM DHs to be assembled but not normally
316 used. Thus, our findings may offer a mechanistic basis for the "MCM paradox" that refers to the excess
317 MCMs distributed in the genome compared to actively used origins [10]. One may question whether the
318 extra MCMs can be those binding nonspecifically to chromatin or are actually in the form of pre-RC MCM
319 DHs. The first cryo-EM study of the MCM DH provides evidence for the latter scenario [51]. In that study,
320 sufficient MCM DHs were obtained by DNase treatment of yeast chromosomal DNA without protein
321 overexpression for high-resolution structural analysis of the MCM DH. Hence, one may infer a natural
322 abundance of MCM DH on normal yeast chromosomes. These unused "dormant" origins may become
323 activated in the event of DNA damage or other cellular stress that limits the ability of replication forks
324 to progress [52].
325 There are further steps in S phase that prompt the maturation of MCM DH to dual CMG
326 replicative helicases [26], some of which may involve elements of ARS sequences that can recruit
327 nucleosome remodeling enzymes. Moreover, epigenetic features of the chromatin, such as
328 posttranslational modifications of histones—which were missing in our current study and thus not
329 required for functional binding of nucleosomes to ORC to facilitate pre-RC formation—may nonetheless
330 contribute to fine-tuning the "nucleosome origin code" by making particular nucleosomes a better or
331 worse binding partner for ORC [53,54].
332

333 In conclusion, our work provides a model for how ORC specifies eukaryotic replication origins in
334 general and an explanation for how an excess of MCM DHs can be loaded onto DNA in G1 phase. Future
335 studies, conducted in diverse eukaryotic systems including human, are needed to fully elucidate the
336 regulatory mechanisms for nucleosome-ORC-MCM interaction and function.

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AUTHOR CONTRIBUTIONS

S.Liu and M.E.O. oversaw the project. M.R.W. prepared the DNA templates. O.Y. prepared the yeast expression constructs for protein production, and purified the factors. M.R.W., O.Y. and S.Li prepared and labeled the replication proteins. S.Li prepared the nucleosome samples and performed the biochemical assays. S.Li and M.R.W. performed the single-molecule experiments. L.B. performed the genomic data analysis. S.Liu, M.E.O., S.Li and L.B. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

356 **MATERIALS AND METHODS**
357 **Protein expression and purification**
358 *S6-ORC*
359 A recombinant strain of *S. cerevisiae* co-expressing the six subunits of ORC, having a 12-aa S6 tag
360 for fluorescent labeling at the N terminus of Orc1, was constructed as follows. We used either an
361 Orc1 gene with an N-terminal 3× Flag (wt ORC) or further modified the Orc1 subunit gene by
362 insertion of DNA encoding the "S6" peptide (GDSLSWLLRLLN) at the N terminus (S6-ORC) [55]. The
363 six subunits of ORC complex were cloned into integration vectors having the galactose inducible
364 Gal1/10 bidirectional promotor for induction by galactose and were cloned and integrated into
365 the genome of strain OY001 (*ade2-1 ura31 his311,15 trp11 leu23,112 can1100 bar1*Δ,
366 *MAT*a *pep4øKANMX6*), a strain constructed from W303 [56]. The order of integration was: (1) genes
367 encoding 3×Flag-Orc1 or S6-3×Flag-Orc1, and Orc3 (both cloned into pRS404/GAL); (2) genes
368 encoding Orc4 and Orc5 (both cloned into pRS405/GAL); (3) the gene encoding Orc2 (cloned into
369 pRS403/GAL); and (4) the gene encoding Orc6 (cloned into pRS402/GAL). The wt and S6-ORC
370 overexpression strains were constructed by integrating the expression plasmids described above
371 in the yeast genome. Both wt ORC and S6-ORC were purified by the same procedure below.
372 One liter of S6-ORC cells were grown under selection at 30 °C in SC glucose, then split into
373 twelve 2-L fluted flasks, each containing 1 L of YP-glycerol media and grown to an optical density
374 at 600 nm (OD_{600}) of 0.4 at 30 °C, arrested with α-factor (50 µg/L) for 2 h, and then induced for 3
375 h upon addition of 20 g/L of galactose. Cells were harvested by centrifugation, resuspended in a
376 minimal volume of 20 mM HEPES pH 7.6, 1.2% polyvinyl pyrrolidone, and protease inhibitors
377 (Sigma-Aldrich #5056489001) and 0.5 mM PMSF, then snap frozen by dripping into liquid
378 nitrogen. Purification of the S6-ORC complex was performed by lysis of 12 L of frozen cells using
379 two SPEX cryogenic grinding mills (6970 EFM). Ground cells were thawed and debris removed by
380 centrifugation (19,000 rpm in an SS34 rotor for 2 h at 4 °C); then the supernatant was applied to
381 a 1-mL anti-Flag M2 affinity column (Sigma) equilibrated in buffer H (50 mM HEPES pH 7.5, 250
382 mM potassium glutamate, 1 mM EDTA, 10% glycerol). Elution was in buffer H containing 0.15
383 mg/mL 3× Flag peptide (EZBiolab). Peak fractions were diluted with 2 volumes of buffer H and
384 loaded onto a 1-mL SP HP column (GE Healthcare) and washed with buffer C (50 mM HEPES pH
385 7.5, 100 mM KOAc). Elution was with an 8-mL linear gradient of 100–600 mM KOAc in 50 mM
386 HEPES pH 7.5. The S6-ORC complex eluted at approximately 400 mM KOAc. Protein
387 concentration of column fractions was determined by Bradford reagent (Bio-Rad), and stored at
388 -80 °C.
389
390 *S6-MCM*
391 Recombinant S.c. strains that co-expressed the six subunits of Mcm2-7 complex having an N-
392 terminal S6-3×Flag tag for fluorescent labeling (S6-MCM), or only a 3×Flag tag on Mcm3 (wt
393 MCM) were constructed as follows. The six subunits of Mcm2-7 were cloned into integration
394 vectors, each having the galactose inducible Gal1/10 bidirectional promotor for induction by
395 galactose. The vectors were then integrated into the genome of strain OY001. Genes encoding
396 Mcm4 and 3×Flag-S6-Mcm3 (or 3×Flag-Mcm3) were cloned into pRS404/GAL (Trp); genes
397 encoding Mcm6 and Mcm7 were cloned into pRS405/GAL (Leu); the gene encoding Mcm2 was
398 cloned into pRS403/GAL (His); and the Mcm5 gene was cloned into pRS406/GAL (Ura). The
399 vectors were integrated in OY001 in the order described above to yield S6-MCM (or wt MCM).

400 S6-MCM cells were grown and induced as described for S6-ORC. Purification of S6-MCM complex
401 was performed by lysis of 12 L of frozen cells with a SPEX cryogenic grinding mill (6970 EFM).
402 Ground cells were thawed and debris removed by centrifugation (12,500 rpm in a SLC-1500 rotor
403 at 4 °C). The clarified extract was applied to a 3-mL anti-Flag M2 affinity column (Sigma)
404 equilibrated in buffer M [50 mM HEPES pH 7.5, 100 mM potassium glutamate, 2 mM DTT, 1 mM
405 EDTA, 10 mM $Mg(OAc)_2$, 0.5 mM ATP, 10% glycerol]. The column was washed with 25 mL buffer
406 M, then eluted with buffer M containing 0.15 mg/mL 3× Flag peptide (EZBiolab). Protein
407 concentration of column fractions was determined by Bradford reagent (Bio-Rad), then
408 aliquoted, snap frozen in liquid N_2 and stored at -80 °C.
409
410 *Cdc6*
411 An *E. coli* optimized gene sequence for expression of S.c. Cdc6 in *E. coli* (a generous gift of Dr.
412 Megan Davey, Western University, Canada) was cloned into pET11 (Novagen). The Cdc6 gene
413 was then subcloned into pGEX-6P-1 (GeneScript) to provide an N-terminal GST tag with a
414 PreScission protease site which we refer to as pGST-Cdc6 PST/P. *E. coli* BL21(DE3) cells were
415 transformed with the pGST-Cdc6 PST/P plasmid and cells were grown in LB + 100 µg/mL ampicillin
416 at 37 °C with shaking until reaching an OD_{600} of 0.49, at which time the culture temperature was
417 quickly reduced to 14 °C by shaking in an ice bath. Then IPTG (1 mM) and 0.2% ethanol were
418 added to induce GST-Cdc6 expression for 24 h at 15 °C. Cells were harvested by low speed
419 centrifugation at 4 °C, and the cell pellet was resuspended in buffer G (50 mM Tris-HCl pH 7.5,
420 1mM EDTA, 10% glycerol) plus 30 µM spermidine and 500 mM NaCl. Cells were lysed by French
421 Press and the lysate was clarified by centrifugation at 14,000 rpm for 1 h at 4 °C. The supernatant
422 was loaded onto a 5-mL glutathione column (GE Healthcare), followed by washing with 20
423 column volumes of buffer G plus 300 mM NaCl. Elution was performed using 25 mL of 20 mM
424 Tris-HCl pH 8.0, 1 mM EDTA, 10% glycerol, 47 mM glutathione and 300 mM NaCl. Fractions of 1
425 mL were collected and analyzed by SDS PAGE. Fractions containing GST-Cdc6 were treated with
426 200 U PreScission protease (ThermoFisher) for 2 h on ice, then were diluted with buffer A (20
427 mM HEPES pH 7.5, 1 mM EDTA, 10% glycerol) to a conductivity of 85 uS/CM and loaded onto an
428 8-mL SP-Sepharose column (Sigma). Cdc6 was eluted in 20-mL steps of buffer A containing either
429 0.2 M, 0.3 M, 0.4 M, 0.5 M NaCl. Fractions were analyzed for Cdc6 by SDS PAGE, and fractions
430 containing Cdc6 were pooled and passed through a 2-mL GST column to remove remaining GST
431 contaminants. Protein concentration was determined by Bradford reagent (Bio-Rad), then
432 aliquoted, snap frozen in liquid N_2 and stored at -80 °C.
433
434 *Cdt1*
435 Cdt1 was cloned into a pET16b vector followed by replacement of the NcoI/NdeI region with
436 insertion of a DNA segment encoding a 3× Flag tag. *E. coli* was transformed with the pFlag-Cdt1-
437 pET plasmid and cells were grown in LB + 100 µg/mL ampicillin at 37 °C with shaking until reaching
438 an OD_{600} of 0.6, at which time the culture temperature was quickly reduced to 15 °C by shaking
439 in an ice bath. Then IPTG (1 mM) was added to induce Cdt1 expression for 10 h at 15 °C. Cells
440 were then harvested by low speed centrifugation at 4 °C, and the cell pellet was resuspended
441 in 50 mL of buffer B (50 mM HEPES pH 7.5, 1 mM EDTA, 2 mM DTT, 2 mM $MgCl_2$, 10% glycerol)
442 plus 800 mM NaCl, and lysed by French Press. Cell lysate was clarified by centrifugation at 14,000
443 rpm for 1 h at 4 °C. The supernatant was treated with 1.5 mL Flag beads (Sigma) for 1 h with

444 gentle agitation, then packed into a 5-mL GE C column equilibrated in buffer B at 4 °C. Cdt1 was
445 eluted with buffer B containing 175 mM NaCl and 0.2 mg/mL 3× Flag peptide. The preparation
446 was then diluted 2-fold with buffer B to reduce conductivity, applied to a 1-mL Heparin agarose
447 column (Sigma), and eluted with a 10-mL linear gradient of 100 mM NaCl to 500 mM NaCl in
448 buffer B. Protein concentration of column fractions was determined by Bradford reagent (Bio-
449 Rad), then aliquoted, snap frozen in liquid N_2 and stored at -80 °C.
450
451 *Nap1*
452 S.c. Nap1 was expressed in *E. coli* from the gene inserted into pGEX-6P-1, a kind gift of Dr. Aaron
453 Johnson (University of Colorado, Denver). The GST-Nap1 was purified essentially as described
454 [57,58], except for passage of the final prep through a GST column (ThermoFisher). Briefly, the pGEX-
455 GST-Nap1 expression plasmid was transformed into *E. coli* BL21 (DE3) cells, and 6 L were grown
456 in LB plus 100 μg/mL ampicillin to an OD_{600} of 0.5 at which time the culture temperature was
457 quickly reduced to 15 °C by shaking in an ice bath. Then IPTG (1 mM) was added to induce Nap1
458 expression for 10 h at 15 °C. Cells were harvested by low speed centrifugation at 4 °C, and the
459 cell pellet was resuspended in 100 mL of PBS (ThermoFisher) plus 500 mM NaCl, 1.5 mM DTT, 1
460 mM EDTA, 30 mM spermidine and 0.5% Triton X-100. Cells were lysed by French Press, and the
461 cell lysate was clarified by centrifugation. The supernatant was loaded onto a 4-mL GST column
462 (GE Healthcare) equilibrated in PBS containing 1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 0.5%
463 Triton X-100, 10% glycerol, and 350 mM NaCl. Elution was with 50 mM Tris-HCl pH 8.0, 1 mM
464 EDTA, 5 mM DTT, 300 mM NaCl, 10% glycerol and 47 mM glutathione. Fractions of 2 mL were
465 collected and analyzed by SDS PAGE for presence of Nap1. Fractions containing Nap1 were
466 pooled and 100 U of PreScission protease (ThermoFisher) was added prior to dialysis overnight
467 against buffer C (20 mM Tris pH 7.5, 1 mM EDTA, 1mM DTT, 150 mM NaCl, 10% glycerol). Twenty
468 eight mL of the dialysate was then loaded onto a 1-mL MonoQ column and eluted with a 20-mL
469 gradient from 150 mM to 1 M NaCl in buffer C. Peak fractions containing Nap1 were pooled and
470 then passed over a GST column to remove any contaminating GST tag and GST-PreScission
471 protease. Protein concentration of column fractions was determined by Bradford reagent (Bio-
472 Rad), then aliquoted, snap frozen in liquid N_2 and stored at -80 °C.
473
474 *Histone octamer*
475 Recombinant *Xenopus laevis* and S.c. histones and their mutants were purified as previously
476 described [59]. Briefly, histones were expressed in BL21 (DE3) cells. Histones were extracted from
477 inclusion bodies under denaturing conditions and purified through Q FF and SP FF columns (GE
478 Healthcare). Histone octamers were then refolded by dialysis and purified by gel filtration using
479 a Superdex 200 10/300 GL column.
480
481 **Fluorescent labeling of proteins**
482 To label S6-ORC and S6-MCM, the protein, Sfp synthase and dye-CoA (dye = Cy3 or LD650) were
483 incubated at a 1:2:5 molar ratio for 1 h at room temperature in the presence of 10 mM $MgCl_2$.
484 Excess dyes and Sfp were removed by a 100-kDa Amicon spin filter. The sample was then buffer-
485 exchanged into 50 mM HEPES pH 7.5, 100 mM KOAc, 250 mM potassium glutamate, 1 mM EDTA,
486 10% glycerol, and 10 mM $MgSO_4$ for S6-ORC; or into 50 mM HEPES KOH pH 7.5, 100 mM KOAc, 2

487 mM DTT, 10 mM $Mg(OAc)_2$, 0.5 mM ATP and 10% glycerol for S6-MCM. The final products were
488 aliquoted, flash frozen, and stored at -80 °C.
489 To label histones, the single-cysteine construct $H2A^{K120C}$ was generated by site-directed
490 mutagenesis. All histones were purified and labeled as previously described [59]. Briefly, they were
491 incubated with Cy3 maleimide (GE Healthcare) or A488 C_5 maleimide (ThermoFisher) at 1:5 molar
492 ratio in a labeling buffer (20 mM Tris-HCl pH 7.0, 7 M Guanidine-HCl, 5 mM EDTA, and 1.25 mM
493 TCEP) for 4 h at room temperature. The labeling reaction was quenched with 80 mM β-
494 mercaptoethanol. Excess dyes were removed by dialysis with a 10-kDa Amicon spin filter. The
495 labeling efficiency varies among batches, ranging from ~50% to >90%.
496
497 **Preparation of DNA template for single molecule experiments**
498 To generate λ_{ARS1} DNA, a 501-bp DNA fragment containing the 185-bp Stillman minimum ARS1
499 sequence [60] (5'-
500 TCGAGAAGCAGGTGGGACAGGTGAACTTTTGGATTGGAACTCGATTTCTGACTGGGTTGGAAGGCAAG
501 AGAGCCCCGAAAGCTTACATTTTATGTTAGCTGGTGGACTGACGCCAGAAAATGTTGGTGATGCGCTTA
502 GATTAAATGGCGTTATTGGTGTTGATGTAAGCGGAGGTGTGGAGACAAATGGTGTAAAAGACTCTAAC
503 AAAATAGCA**AATTTCGTCAAAAATGCT**AAGAAATAGGTTATTACTGAGTAGTATTT**ATTTAAGTATT**GTT
504 TGTGCACTTGCCTGCAGGCCTTT**TGAAAAGCAAGCA**TAAAAG**ATCTAAACATAAAATCT**GTAAAATAAC
505 AAGATGTAAAGATAATGCTAAATCATTTGGCTTTTTGATTGATTGTACAGGAAAATATACATCGCAGGG
506 GGTTGACTTTTACCATTTCACCGCAATGGAATCAAACTTGTTGAAGAGAATGTTCACAGGCGCATACGCT
507 ACAATGACCCGATTCTTG-3'; the 185-bp ARS1 sequence is underlined; bold sequences represent
508 B3, B2, B1 and ACS elements) was amplified from the yeast chromosome (S288C_ChrIV
509 BK0069382: 462,279–462,787) and inserted into λ DNA (Roche, Cat# 11558706910) with XhoI
510 and NheI restriction enzymes (New England BioLabs). The product was then packaged into phage
511 particles using phage extract (MaxPlax, Epicentre). Plaques were generated on LE392 *E. coli*
512 bacterial lawns (Epicentre) and screened for the ARS1 insert. A screened plaque was used as a
513 phage source to purify λ_{ARS1} DNA by lytic growth [61]. The final λ_{ARS1} DNA is 47,822 bp in length and
514 the ARS1 site is located 33,499–33,999 bp from the left end of the the phage genome. To create
515 a terminally biotinylated λ_{ARS1} DNA, the 12-base 5' overhang on each end was filled in with a
516 mixture of unmodified and biotinylated nucleotides by the exonuclease-deficient DNA
517 polymerase I Klenow fragment (New England BioLabs). The reaction was conducted by incubating
518 10 nM λ_{ARS1} DNA, 33 μM each of dGTP/dATP/biotin-11-dUTP/biotin-14-dCTP (Thermo Fisher),
519 and 5 U Klenow in 1× NEB2 buffer at 37 °C for 45 min, followed by heat inactivation at 75 °C for
520 20 min. The DNA was then ethanol precipitated overnight at -20 °C in 2.5× volume cold ethanol
521 and 300 mM NaOAc pH 5.2. Precipitated DNA was recovered by centrifugation at 20,000× g at 4
522 °C for 15 min. After removing the supernatant, the pellet was air-dried, resuspended in TE buffer
523 (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored at 4 °C.
524
525 **Single-molecule experiments**
526 *Data acquisition*
527 Single-molecule experiments were performed at room temperature on a LUMICKS C-Trap
528 instrument as previously described [32]. Laminar-flow-separated channels 1-3 were used to form
529 DNA tethers between two 4.35-μm streptavidin-coated polystyrene beads (Spherotech).
530 Channels 4 and 5 served as protein loading and imaging channels. A488, Cy3, and Cy5/LD650

531 fluorophores were excited by 488 nm, 532 nm and 638 nm laser lines, respectively. Kymographs
532 were generated via confocal line scanning through the center of the two beads.
533
534 *Nucleosome assembly in situ*
535 Optical traps tethering a single DNA were moved to a channel containing 1 nM of fluorescently
536 labeled S.c. or X.l. histone octamers and 2 nM Nap1 in HR buffer [30 mM Tris-OAc pH 7.5, 20 mM
537 $Mg(OAc)_2$, 50 mM KCl, 1 mM DTT, 40 µg /mL BSA], and incubated under a tension below 1 pN
538 until a few fluorescent spots were seen. The tether was then moved to Channel 3 containing 0.5
539 mg/mL salmon sperm DNA (Thermo Fisher) in HR buffer except for MCM/ORC salt stability
540 assays, in which Channel 3 contained a high-salt buffer [50 mM HEPES pH 7.5, 40 µg/mL BSA, 2
541 mM DTT, 10 mM $Mg(OAc)_2$, 500 mM NaCl, 2.5 mM ATP]. A microfluidic flow was turned on for 1
542 min to gently remove free histones and free Nap1.
543
544 *Visualization of ORC*
545 Optical traps tethering a single bare DNA or nucleosomal DNA were moved to a separate channel
546 containing 2 nM Cy3- or LD650-ORC and 5 nM Cdc6 in an imaging buffer containing 25 mM Tris-
547 OAc pH 7.5, 5% glycerol, 40 µg/mL BSA, 3 mM DTT, 2 mM TCEP, 0.1 mM EDTA, 10 mM $Mg(OAc)_2$,
548 50 mM potassium glutamate, and 2.5 mM ATP. The imaging buffer was supplemented with an
549 ATP-regeneration system [60 mg/mL creatine phosphokinase (Sigma) and 20 mM
550 phosphocreatine (Sigma)], a triplet-state quenching cocktail [1 mM cyclooctatetraene (Sigma), 1
551 mM 4-nitrobenzyl alcohol (Sigma) and 1 mM Trolox (Sigma)], as well as an oxygen scavenging
552 system [10 nM protocatechuate-3,4-dioxygenase (Sigma) and 2.5 mM protocatechuic acid
553 (Sigma)]. The kymograph was typically recorded for 10 min.
554
555 *Visualization of MCM*
556 For two-color MCM/nucleosome experiments, a tethered nucleosomal DNA loaded with Cy3 or
557 A488-labeled octamers was moved to a channel containing 10 nM LD650-MCM and 14 nM Cdt1,
558 with or without 2 nM ORC and 5 nM Cdc6, in the imaging buffer described above in the presence
559 of 5 mM ATP. For MCM DH loading experiments, a tethered nucleosomal DNA was moved to a
560 channel containing 10 nM Cy3-MCM, 10 nM LD650-MCM, 4 nM unlabeled ORC, 10 nM Cdc6, 28
561 nM Cdt1, and 5 mM ATP. For MCM/ORC salt stability experiments, the tether was moved to a
562 channel containing the high-salt buffer described above. The oxygen scavenging system was
563 omitted from the imaging buffer for the MCM photobleaching experiments.
564
565 *Data analysis*
566 Single-molecule force and fluorescence data from the .h5 files generated from C-Trap
567 experiments were analyzed using tools in the lumicks.pylake Python library supplemented with
568 other Python modules in a custom GUI Python script titled "C-Trap .h5 File Visualization GUI"
569 (https://harbor.lumicks.com/single-script/c5b103a4-0804-4b06-95d3-20a08d65768f), which
570 was written to extract confocal images and traces.
571
572 **Genomic analysis**
573 We analyzed the following published datasets: SRR034475 and SRR034476 (Orc1 ChIP-seq) [11];
574 SRR1261333 (Mcm2-7 ChIP-seq) [37]; GSE147927 (ChIP-exo data for Orc1, Abf1, and Reb1) [40];

575 GSM2589911 (MNase data) [41]. The ChIP-seq data were aligned to yeast genome (version Scer3)
576 with bowtie2, and peaks were called using MACS2 (threshold: effective p value 0.01). Orc1 and
577 Mcm2-7 ChIP peaks tend to be broader than typical transcription factors, probably due to the
578 large size of the complex. As a result, multiple peaks tend to appear as clusters within a few
579 hundred bps. We wrote a MATLAB algorithm to select a single peak with the largest area
580 underneath within each cluster. We found 295 Orc1 peaks in total (sorted based on the total
581 intensity of the Orc1 ChIP-seq signal in the +/- 1kb region in **Figure 7A**). ARS annotation was
582 downloaded from the Saccharomyces Genome Database. All Orc1 peak locations (peak center
583 +/- 100bp) and the corresponding ARSs are shown in **Supplementary Table 1**.
584 To get the Orc1 motif information, we extracted the genomic sequences within peak
585 center +/- 500bp. Using the PWM in [39], we used a MATLAB program to scan through the sequence
586 and calculated the PWM score for each 17-bp elements on both strands. At each base, PWM
587 score calculated the $\log_2$ of probability of the appearance of a base divided by the probability for
588 that base to appear in the genome. For example, if for Position X, "A" appears 90% of time, while
589 in the genome it is 25%, then the score of Position X is $\log_2(0.9/0.25)$. The final score is the sum
590 of scores for all positions in the element. Abf1 and Reb1 motifs were analyzed the same way,
591 except that we scanned through shorter sequences (peak center +/- 150bp) because the ChIP
592 peaks of these factors are sharper.
593
594 **QUANTIFICATION AND STATISTICAL ANALYSIS**
595 Errors reported in this study represent the standard deviation (SD). *P* values were determined
596 from unpaired two-tailed *t* tests using GraphPad Prism 9 (ns, not significant; *$P < 0.05$; **$P < 0.01$;
597 ***$P < 0.001$; ****$P < 0.0001$).

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746 **FIGURES**
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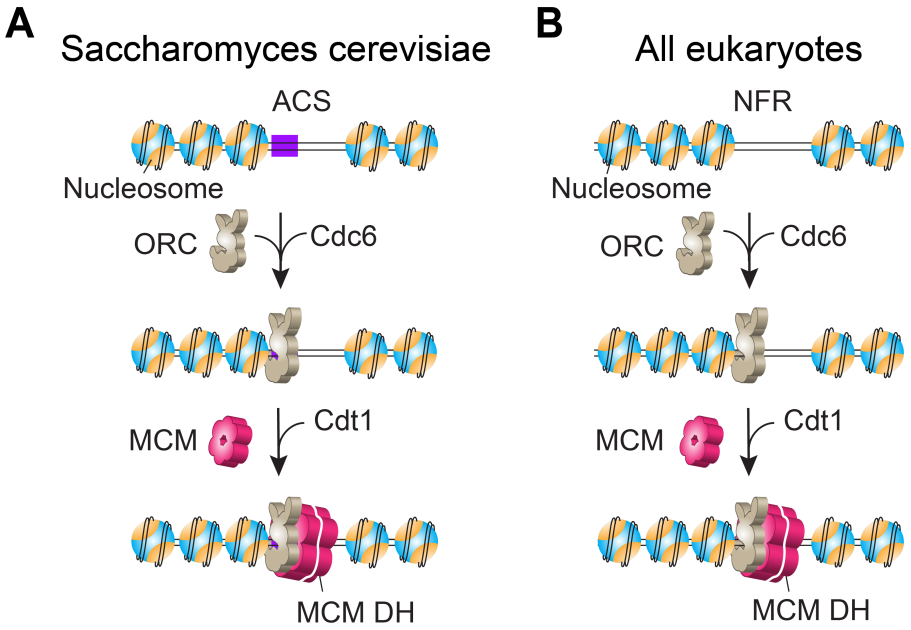

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749 **Figure 1. Model for origin licensing in *Saccharomyces cerevisiae* vs. in most other eukaryotes**
750 (**A**) In a chromatinized genome, ORC searches for nucleosomes and stably associates with them
751 regardless of whether a nearby ARS consensus sequence (ACS) exists. ORC then loads MCM
752 double hexamers in conjunction with Cdc6 and Cdt1 at nucleosomal sites in G1 phase. Due to its
753 small genome, *S. cerevisiae* may have evolved ARS-dependent origins in order to limit ORC
754 binding to specific sequences and thus avoid replication-transcription conflicts [20], which does not
755 generalize to higher eukaryotes. (**B**) Origins in most eukaryotes lack a consensus ACS motif, but
756 still utilize ORC which is known to bind nucleosomes. We demonstrate in this study that S.c. ORC
757 harbors a cryptic ability to bind nucleosomes and direct MCM DH formation within nucleosome-
758 free regions (NFRs) that lack ARS consensus sequences. We propose that this mechanism is the
759 normal process for most eukaryotes, whose origin sites are chiefly defined by the nucleosomal
760 architecture. In this model, ACS confers origin specificity in S.c. by facilitating ORC binding to
761 nucleosomes proximal to a nucleosome-free ARS sequence.

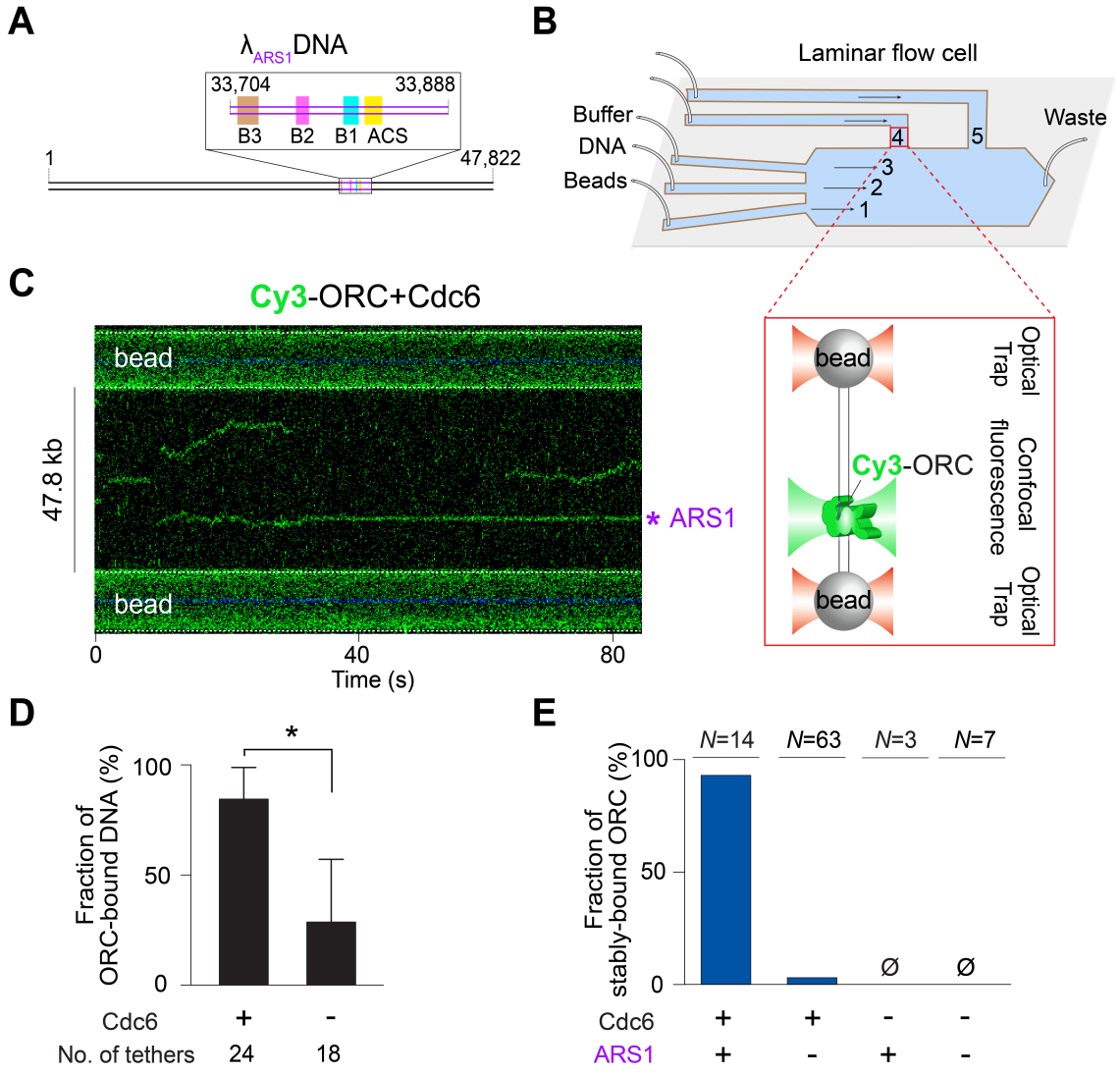

Figure 2. A single-molecule platform to study eukaryotic replication initiation

(**A**) Cartoon of the λ_{ARS1} DNA template. The inserted ARS1 element is illustrated in the inset box. (**B**) Schematic of the single-molecule experimental setup. Channels 1-3 are separated by laminar flow. Beads are optically trapped in channel 1, moved to channel 2 to tether DNA, then moved to channel 3 to characterize the force-extension curve of the tether. Once a correct tether is confirmed, the beads-DNA is moved to channel 4 or 5 containing the proteins. The zoom-in box illustrates the final assembly in the imaging channel (not drawn to scale). (**C**) A representative kymograph showing the behavior of Cy3-labeled ORC on λ_{ARS1} DNA in the presence of Cdc6. The engineered ARS1 position is indicated. (**D**) Fraction of λ_{ARS1} DNA tethers that were observed to have at least one ORC bound in the presence or absence of Cdc6. The protein concentrations used in this experiment are: 2 nM for ORC, and 5 nM for Cdc6. The number of tethers analyzed for each condition is indicated. Error bars represent SD. (**E**) Fraction of ORC molecules that stably reside at the ARS1 site versus non-ARS1 sites in the presence or absence of Cdc6. *N* incidates the number of ORC molecules analyzed for each condition.

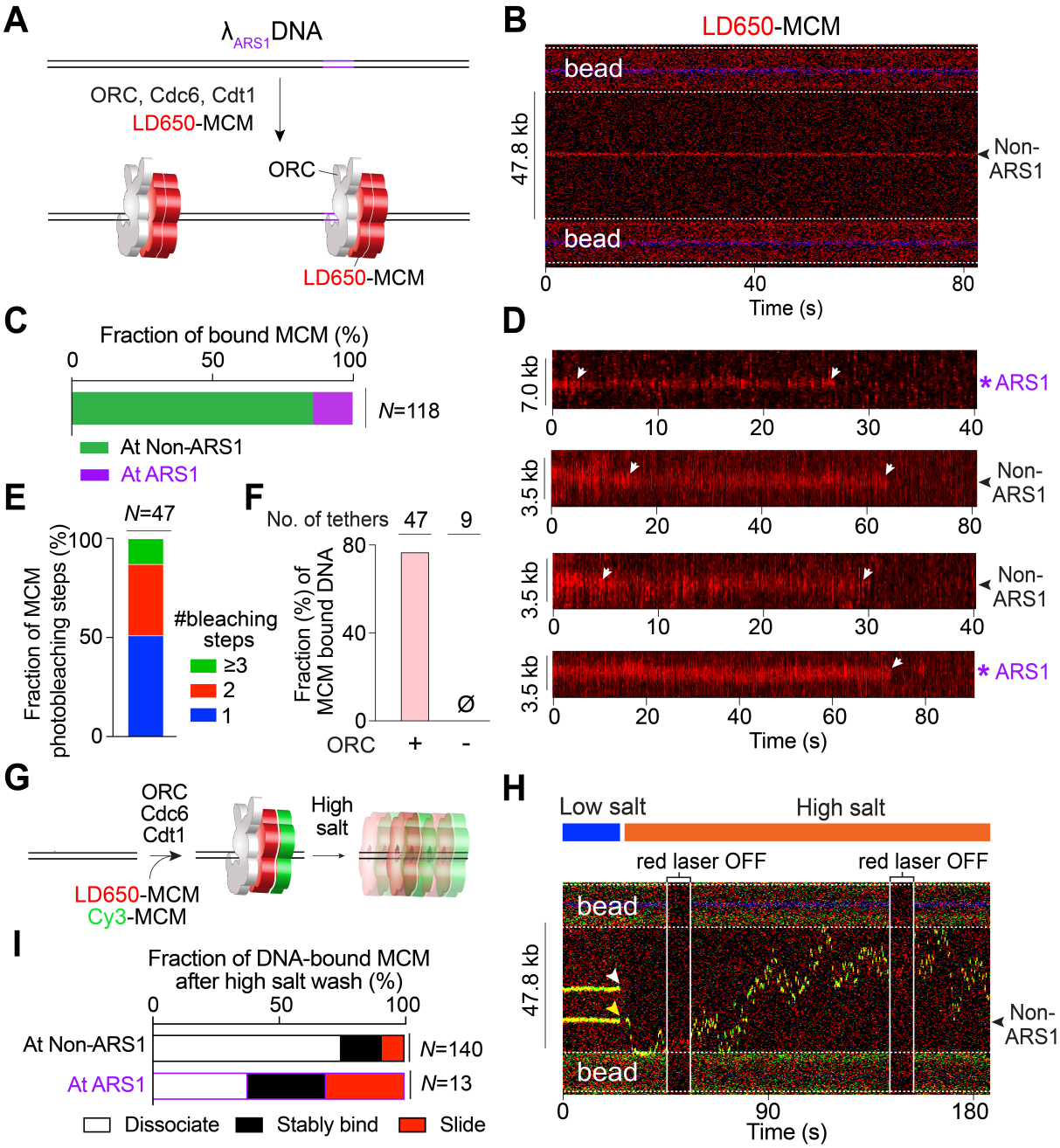

Figure 3. ORC-dependent MCM loading occurs frequently at non-ARS DNA sites

(**A**) Cartoon of the single-molecule pre-RC assembly experiment using λ_{ARS1} DNA, unlabeled ORC, Cdc6, Cdt1, and LD650-labeled MCM (red). (**B**) An example kymograph showing that the MCM fluorescence signal appeared at a non-ARS1 position on DNA. (**C**) Fraction of stably bound MCM complexes that were observed at ARS1 versus non-ARS1 positions. *N* indicates the number of MCM complexes analyzed. (**D**) Example kymographs showing the photobleaching steps (white arrows) of MCM fluorescence at ARS1 and non-ARS1 positions on the DNA tether. (**E**) Distribution of the number of photobleaching steps observed in each MCM fluorescence trajectory. *N*

789 indicates the number of trajectories analyzed. (**F**) Fraction of DNA tethers that were observed to
790 harbor at least one fluorescent MCM complex in the presence or absence of ORC. The protein
791 concentrations used in this experiment are: 10 nM for MCM, 2 nM for ORC, and 5 nM for Cdc6.
792 The number of tethers analyzed for each condition is indicated. (**G**) Cartoon of the high-salt wash
793 experiment to demonstrate MCM loading on DNA using a mixture of LD650-MCM and Cy3-MCM,
794 unlabeled ORC, Cdc6 and Cdt1. (**H**) A representative kymograph showing large-scale mobility of
795 an MCM DH (indicated by the dual-color complex which appeared as yellow) loaded at a non-
796 ARS1 position traversing the entire length of the tethered DNA upon high-salt wash (yellow
797 arrowhead). The other MCM complex dissociated at high salt (white arrowhead). (**I**) Fraction of
798 MCM complexes on nucleosome-free DNA that underwent diffusion without dissociation (red),
799 remained stably bound to the DNA position (black), or dissociated into solution (white) upon
800 high-salt wash. *N* indicates the number of MCM complexes analyzed.

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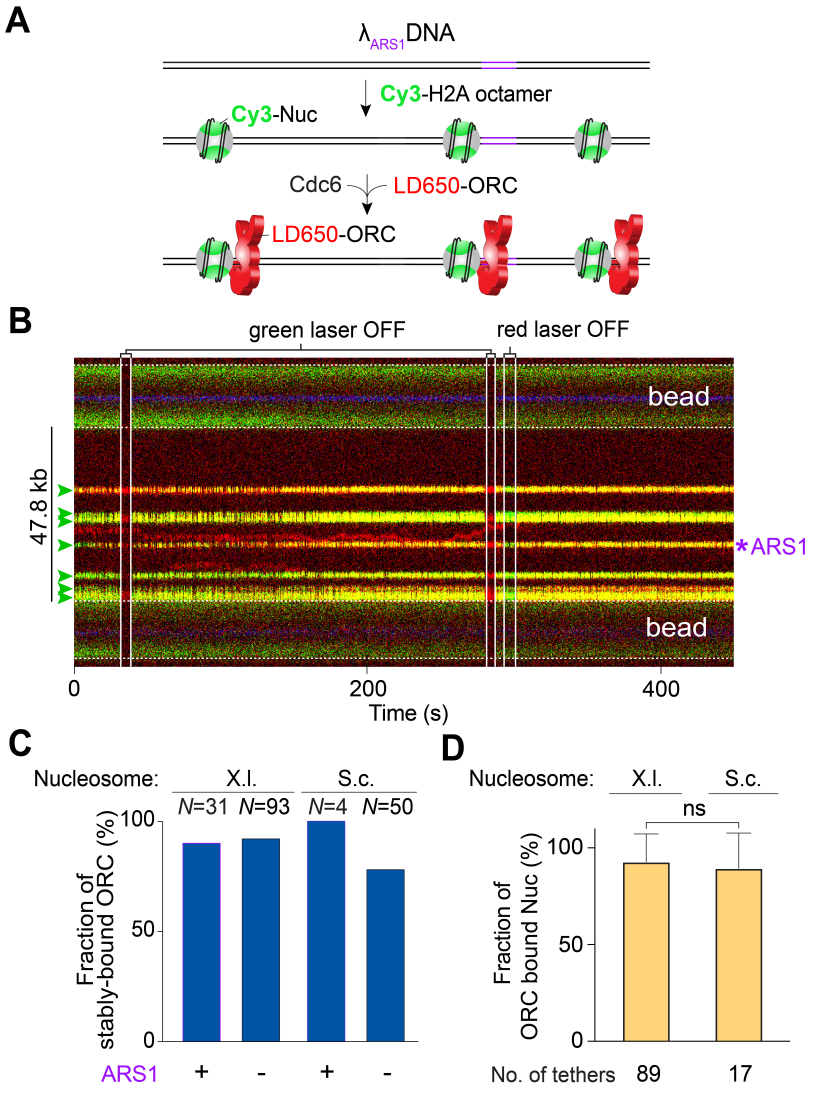

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804 **Figure 4. ORC predominantly binds to nucleosomes over ARS DNA**

805 (**A**) Cartoon of the λ_{ARS1} DNA sparsely loaded with Cy3-labeled S.c. nucleosomes (green) and
806 incubated with LD650-labeled ORC (red) and Cdc6. (**B**) A representative kymograph showing a
807 λ_{ARS1} DNA tether loaded with multiple nucleosomes (positions indicated by green arrowheads),
808 all of which were located at non-ARS1 sites except one. Each nucleosome was observed to be
809 stably bound by ORC (red). The presence of ORC on the nucleosomes is confirmed by turning off
810 the green laser, which showed only the red fluorescence from ORC; alternatively, turning off the
811 red laser showed only the green fluorescence from the nucleosomes. (**C**) Fraction of ORC stably
812 bound to nucleosomes (X.l. or S.c.) located at either the ARS1 site or non-ARS1 sites. *N* indicates
813 the number of ORC molecules analyzed for each condition. (**D**) Fraction of nucleosomes (X.l. or
814 S.c.) within a given DNA tether that were observed to be ORC-bound in the presence of 2 nM
815 ORC and 5 nM Cdc6. The number of tethers analyzed for each condition is indicated. Error bars
816 represent SD.

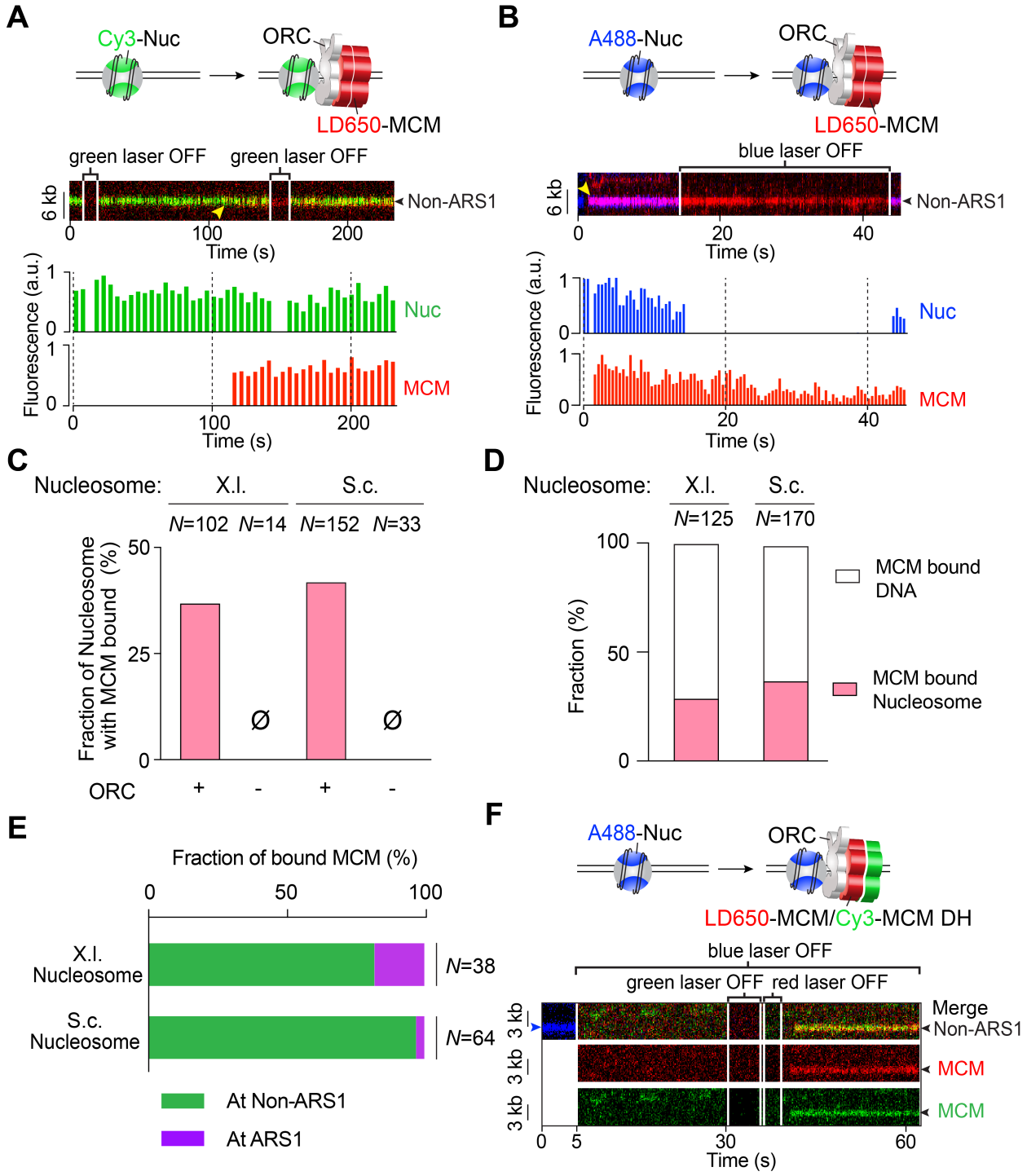

Figure 5. MCMs are recruited by ORC to nucleosomes independently of ARS DNA

(**A**) Cartoon (top), an example kymograph (middle) and the corresponding fluorescence intensities (bottom) of the pre-RC assembly experiment using Cy3-labeled X.l. nucleosomes (green), LD650-labeled MCM (red), unlabeled ORC, Cdc6 and Cdt1. Yellow arrowhead in the kymograph indicates the time when the MCM fluorescence signal appeared at the nucleosomal site. (**B**) Cartoon (top), an example kymograph (middle) and the corresponding fluorescence intensities (bottom) of the pre-RC assembly experiment using A488-labeled S.c. nucleosomes

825 (blue), LD650-labeled MCM (red), unlabeled ORC, Cdc6 and Cdt1. In both examples in **A** and **B**
826 the nucleosomes were at non-ARS1 positions on the DNA. (**C**) Fraction of nucleosomes (X.l. or
827 S.c.) that were observed to have colocalized MCM signals in the presence or absence of ORC. *N*
828 indicates the number of nucleosomes analyzed for each condition. (**D**) Fraction of MCM
829 complexes on a nucleosome-loaded (X.l. or S.c.) tether that colocalized with a nucleosome versus
830 with nucleosome-free DNA. *N* indicates the number of MCM complexes analyzed. (**E**) Fraction of
831 MCM-nucleosome (X.l. or S.c.) colocalization events observed at ARS1 versus non-ARS1 positions.
832 *N* indicates the number of events analyzed. (**F**) Cartoon (top) and an example kymograph
833 (bottom) of the three-color experiments using A488-labeled S.c. nucleosomes (blue), both
834 LD650-labeled MCM (red) and Cy3-labeled MCM (green), unlabeled ORC, Cdc6 and Cdt1. The
835 colocalization of a dual-color MCM with a nucleosome indicates MCM DH recruitment to the
836 nucleosomal site. Individual lasers were occasionally turned off to confirm the fluorescence
837 signals from the other channels.

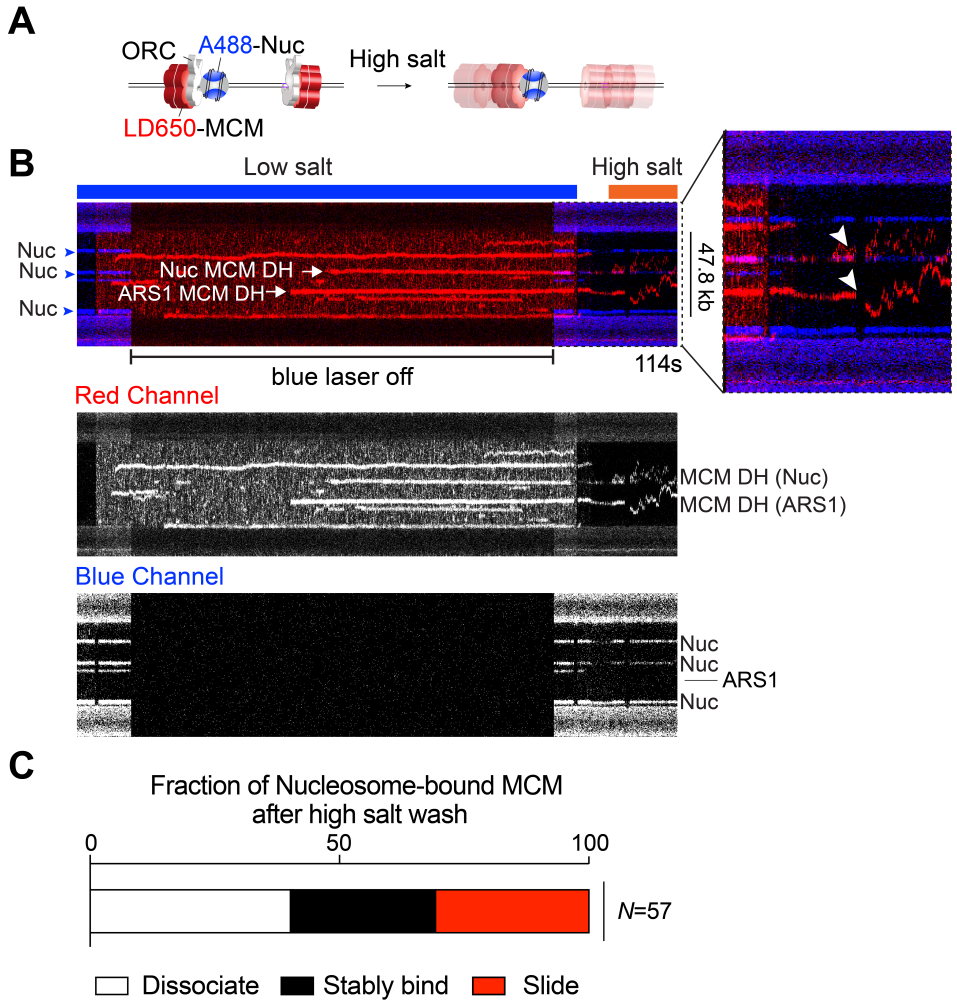

Figure 6. ORC-mediated MCM loading occurs at nucleosomal sites

(**A**) Cartoon illustrating the experimental assay that evaluates MCM loading at nucleosomal sites via high salt wash. (**B**) A representative kymograph showing that MCM complexes (red) formed on a section of bare DNA in the presence of unlabeled ORC, Cdc6 and Cdt1. Some of the MCMs colocalized with a S.c. nucleosome (blue). Upon moving to a high-salt buffer (0.5 M NaCl), a fraction of the MCMs displayed diffusive behavior from both bare DNA sites and nucleosomal sites (white arrowheads in the zoomed-in view) without dissociation, demonstrating their successful loading onto DNA. Blue arrowheads indicate nucleosome positions, all of which were at non-ARS1 sites on the tether shown in this example. MCM and nucleosome fluorescence signals are also separately shown in grey scale at the bottom. (**C**) Fraction of nucleosome-colocalized MCM complexes that underwent diffusion without dissociation (red), remained stably bound to the nucleosome (black), or dissociated into solution (white) upon high-salt wash. *N* indicates the number of MCM complexes analyzed.

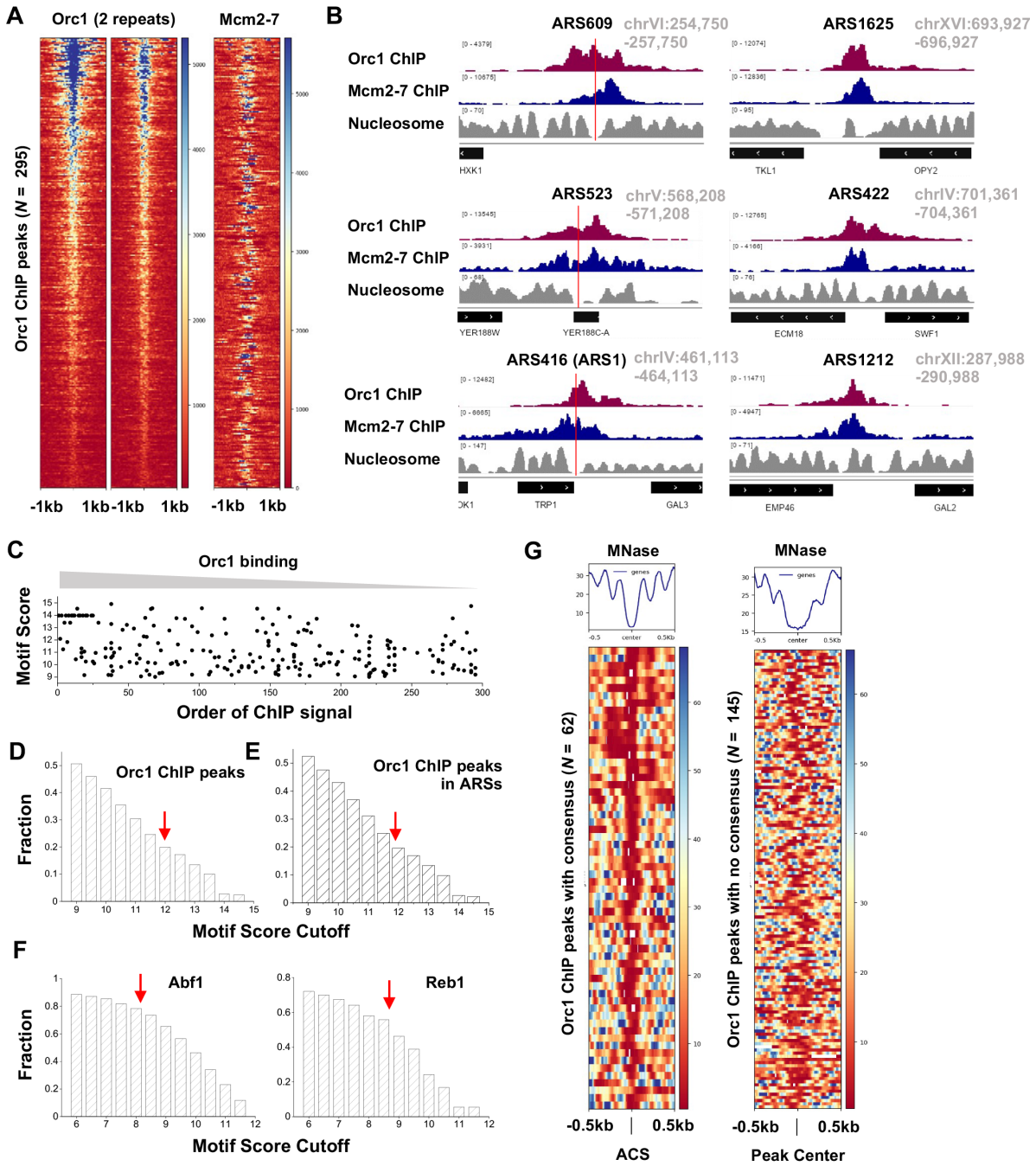

Figure 7. Genome-wide analysis of ORC/MCM localization

(**A**) Heatmap of Orc1 ChIP peaks (*N* = 295) and Mcm2-7 ChIP signal at the corresponding sites. (**B**) Examples of Orc1 and Mcm2-7 ChIP data and nucleosome occupancy at six ARSs. Red vertical lines represent the location of ACS consensuses in these regions. The three ARSs on the right contain no consensus with score above 9. (**C**) ACS motif score vs. the Orc1 ChIP peak strength. The motifs were identified in a 1kb region near Orc1 ChIP peaks (peak center +/- 500 bp). All elements with a PWM score >9 are shown here. For the *x* axis, "1" represents the largest Orc1 ChIP peak, and "295" is the weakest. (**D**) Fraction of sequences underlying all Orc1 peaks that

863 contain motifs above a certain threshold (varying from 9 to 15). The red arrow represents the
864 recommended cutoff of 11.9. (**E**) Same as in **D** except using a subset of Orc1 peaks that overlap
865 with previously annotated ARSs. (**F**) Same as in **D** except for Abf1 and Reb1. The red arrows
866 represent recommended cutoffs for these two factors. (**G**) Heatmap of nucleosome occupancy
867 near Orc1 ChIP peaks. The left panel includes 62 peaks containing ACS consensus (score > 11.9),
868 with each row aligned at the consensus site. The right panel includes 145 peaks with no
869 consensus above 9, and it was aligned at the center of the ChIP peaks.

870 **SUPPLEMENTARY FIGURES**

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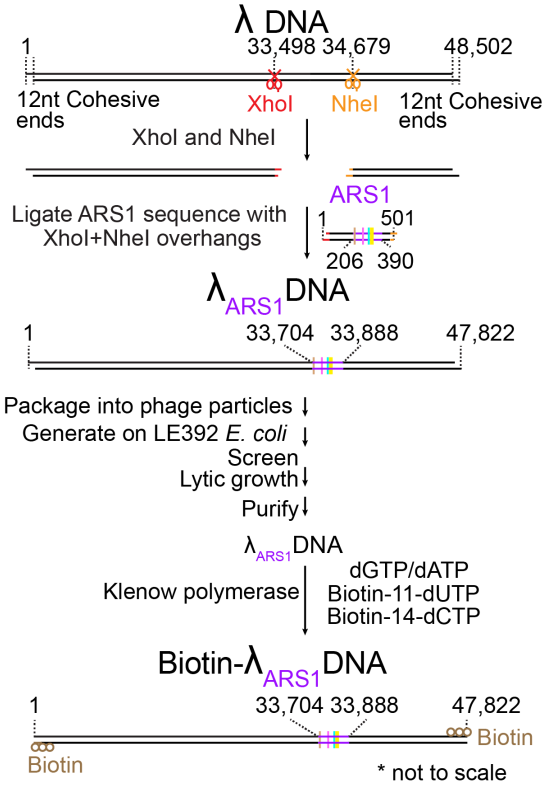

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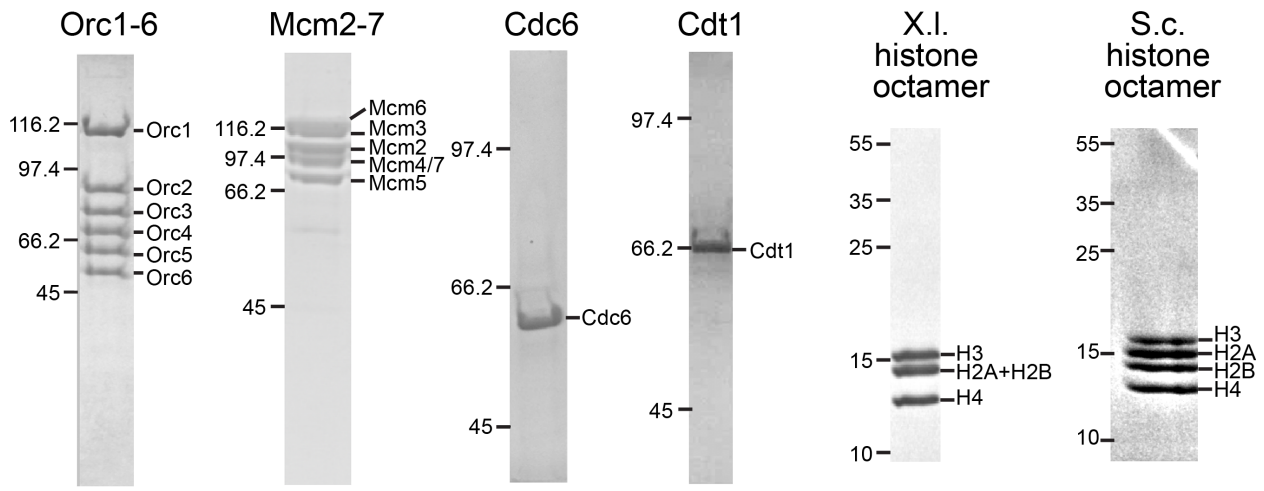
875 **Supplementary Figure 1. Workflow for generating the biotinylated λ_{ARS1} DNA**



Supplementary Figure 2. SDS-PAGE gels showing the proteins used in this work

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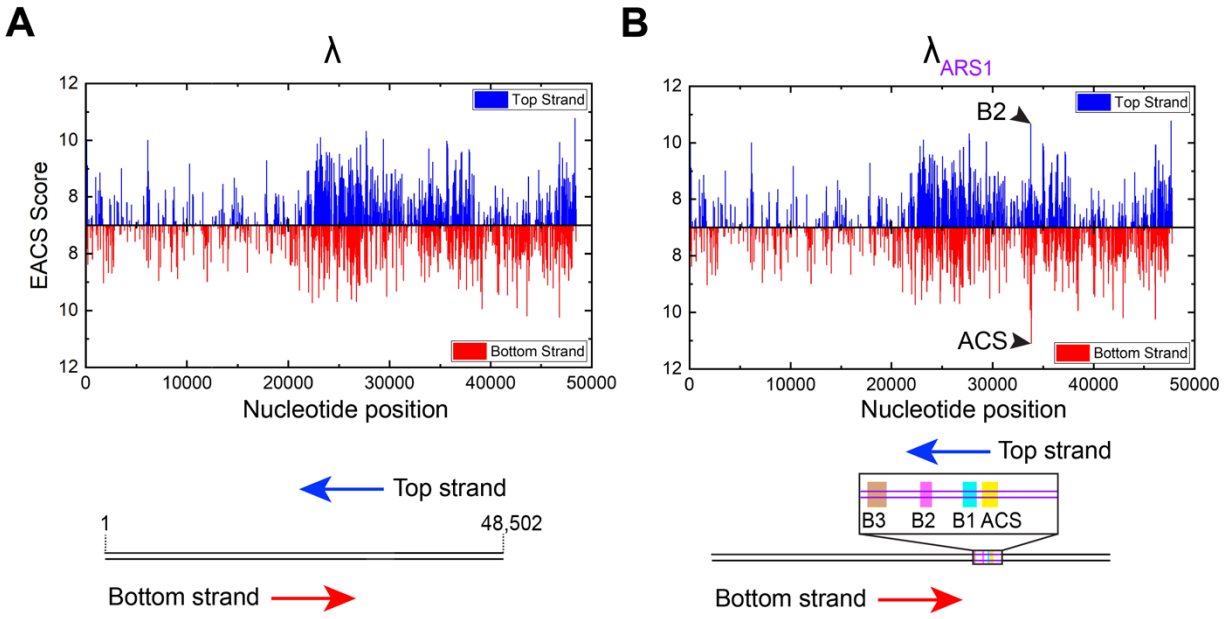

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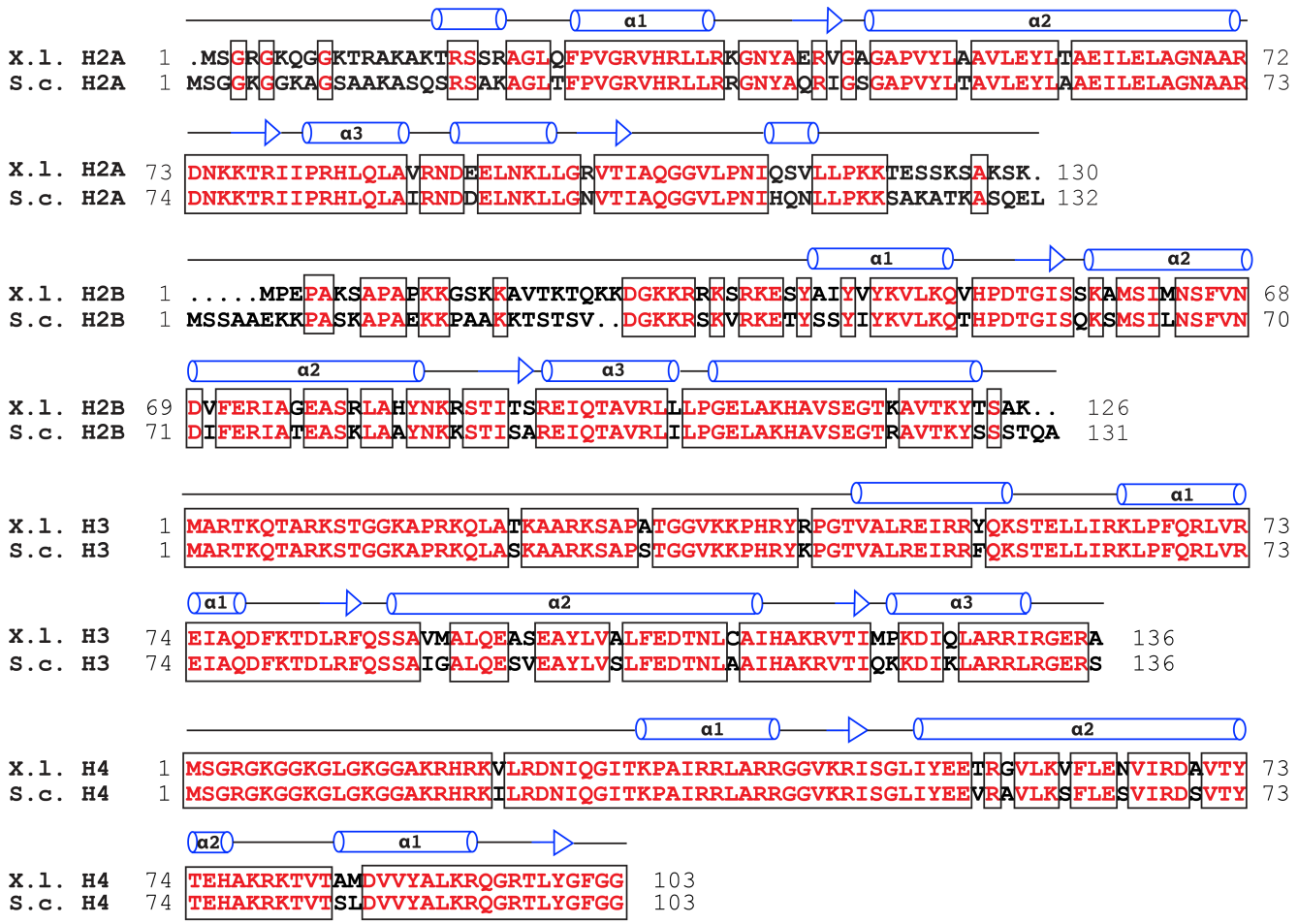
882 **Supplementary Figure 3. Analysis of potential ACS motifs within λ DNA**
883 (**A**) Extended ACS (EACS) scores for the native λ genomic DNA using a 17-bp position weight
884 matrix (PWM) generated from multiple sequence alignment of functional ARS elements
885 identified in *S. cerevisiae* and previously used to score potential ORC binding sites in a given DNA
886 sequence [62,63]. (**B**) EACS scores for the engineered λ_{ARS1} DNA. The highest scores correspond to
887 the ARS1 elements (black arrowheads) inserted in the λ template.



Supplementary Figure 4. Sequence alignment of *Xenopus laevis* and *Saccharomyces cerevisiae* histones. Identical residues are colored in red.

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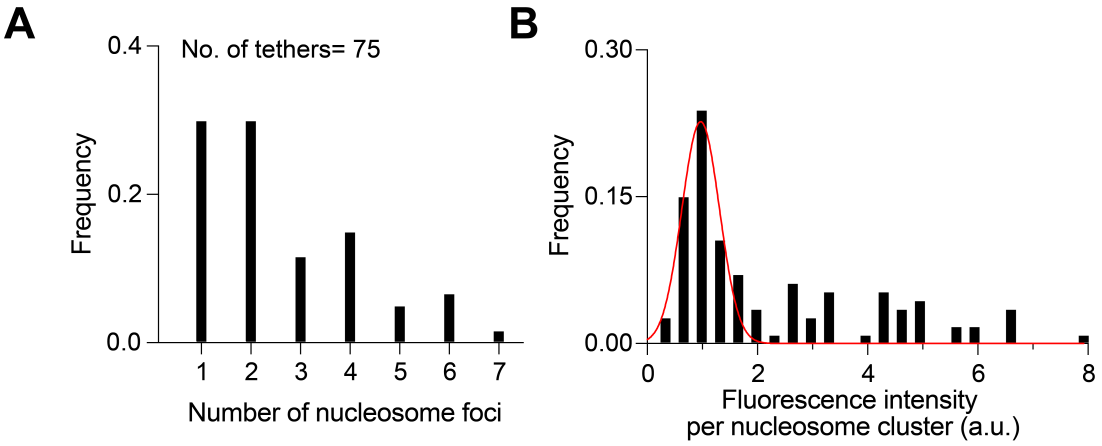

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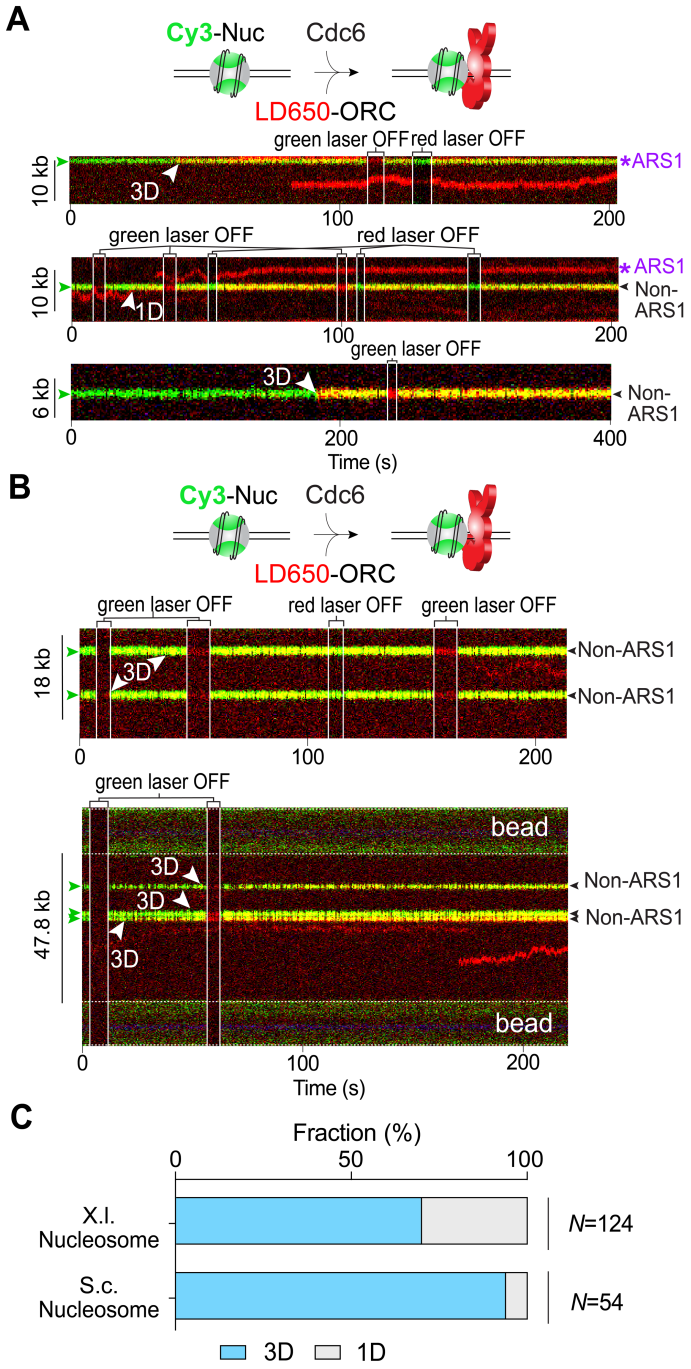
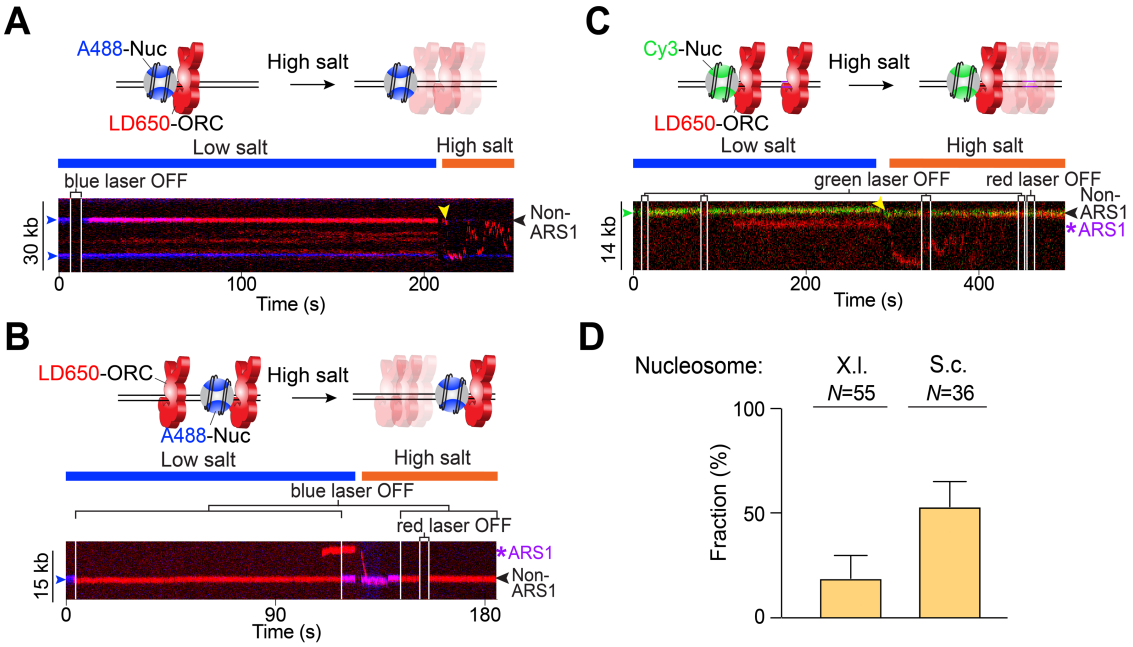
895 **Supplementary Figure 5. Evaluation of nucleosome loading on tethered DNA**
896 (**A**) Distribution of the number of fluorescently labeled nucleosome foci per DNA tether. (**B**) Distribution
897 of the fluorescence intensity per nucleosome cluster. The main peak (red curve) represents the
898 mononucleosome population.

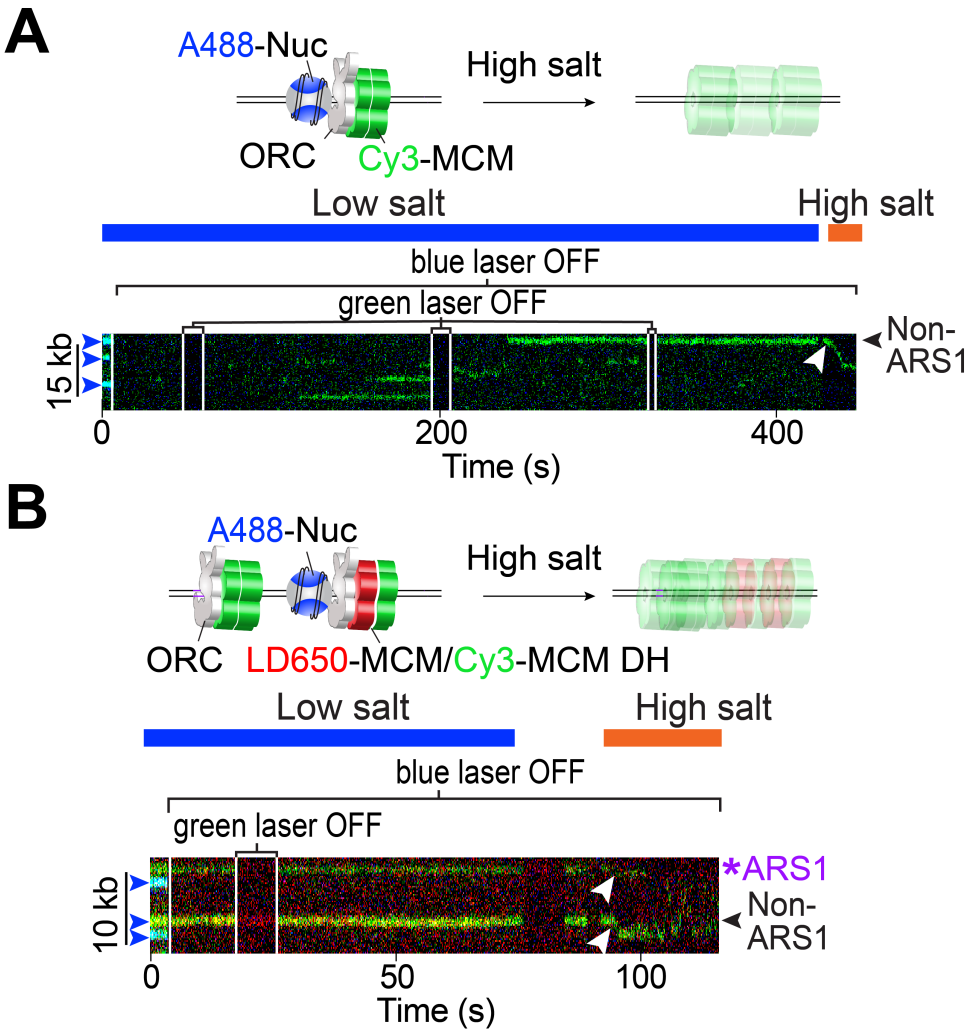


Supplementary Figure 6. Analysis of the search mode used by ORC to target nucleosomes
(**A**) Three example kymographs showing LD650-labeled ORC (red) stably associated with Cy3-labeled X.l. nucleosomes (green). White arrowheads indicate the time when ORC arrived at the nucleosomal site (ARS1 or non-ARS1) via three-dimensional (3D) or one-dimensional (1D) search. Green arrowheads indicate the positions of nucleosomes. (**B**) Two example kymographs showing LD650-labeled ORC (red) binding to Cy3-labeled S.c. nucleosomes (green). (**C**) Fraction of ORC molecules that were observed to

907 target a nucleosome (X.l. or S.c.) by 3D or 1D search. *N* indicates the number of events analyzed for each
908 condition.

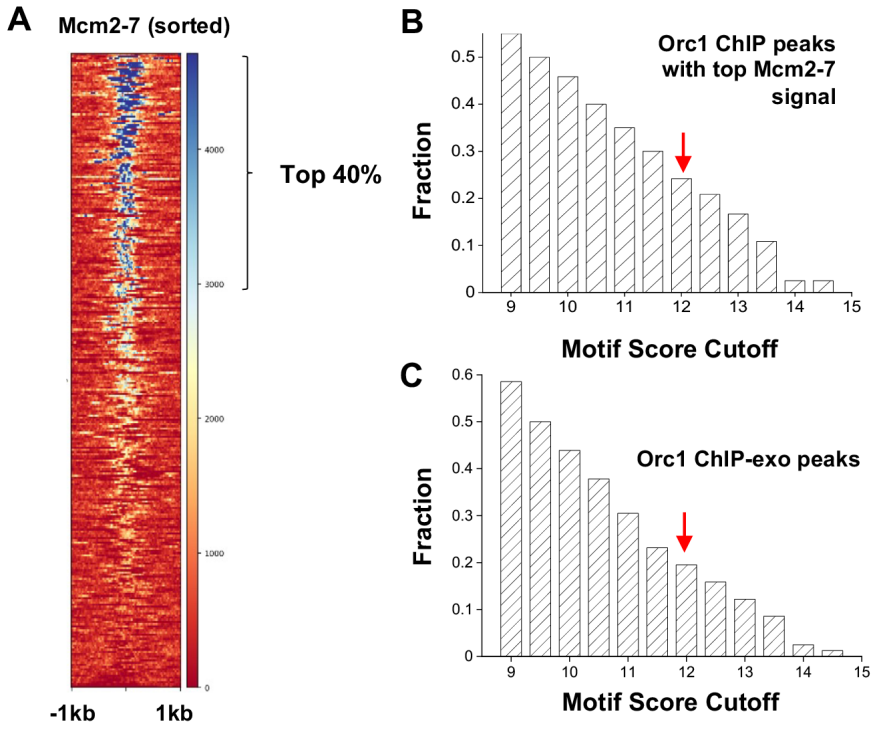


Supplementary Figure 7. The fraction of ORC that remained associated with nucleosomes at high salt (**A**) Cartoon and an example kymograph showing ORC (red) colocalized with a S.c. nucleosome (blue) in a low-salt buffer but departed from the nucleosome and underwent diffusive motions on DNA upon high-salt wash (yellow arrowhead). (**B**) Cartoon and an example kymograph showing an ORC molecule (red) colocalized with a S.c. nucleosome (blue) and remained bound to the nucleosome at high salt. Another ORC bound at the ARS1 DNA position underwent diffusion upon high-salt wash. (**C**) Cartoon and an example kymograph showing an ORC molecule (red) colocalized with a Cy3-labeled X.l. nucleosome (green) and remained bound to it at high salt. Another ORC bound at the ARS1 DNA position underwent diffusion upon high-salt wash (yellow arrowhead). (**D**) Fraction of ORC molecules that were observed to depart from a nucleosomal site (X.l. vs. S.c.) upon high-salt wash. *N* indicates the number of ORC molecules analyzed for each condition. In all experiments, ORC was first incubated with tethered nucleosomal DNA at low salt in the presence of Cdc6; then the whole assembly was moved to another channel containing high salt.



Supplementary Figure 8. Other examples of nucleosome-bound MCM undergoing diffusive motions upon high-salt wash

(**A**) Cartoon and kymograph showing MCM (green) colocalized with a S.c. nucleosome (blue) in the presence of unlabeled ORC, Cdc6 and Cdt1. Upon moving to a high-salt buffer, the MCM diffused away from the nucleosome (white arrowhead) as expected for a loaded MCM DH that encircles DNA. Blue arrowheads indicate the positions of nucleosomes. (**B**) Cartoon and kymograph showing two MCM complexes—one formed on the ARS1 DNA (top) and the other at a non-ARS1 nucleosome (bottom)—both of which displayed diffusive behavior upon high-salt wash (white arrowheads).



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933 **Supplementary Figure 9. Additional genome-wide analysis of ORC/MCM localization**

934 (**A**) Heatmap of sorted Mcm2-7 ChIP signals among the 295 Orc1 ChIP peaks. The top 40% of these sites
935 are included in the analysis in **B**. (**B**) Fraction of sequences that contain motifs above a certain threshold
936 (varying from 9 to 15). These sequences are extracted from the Orc1 peaks with high Mcm2-7 ChIP
937 signals. The red arrow represents the recommended cutoff of 11.9. (**C**) Same as in **B** except that the
938 sequences are extracted from Orc1 ChIP-exo peaks (*N* = 81).