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Temporal association of ORCA/LRWD1 to late-firing origins during G1 dictates heterochromatin replication and organization

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ABSTRACT

DNA replication requires the recruitment of a pre-replication complex facilitated by Origin Recognition Complex (ORC) onto the chromatin during G1 phase of the cell cycle. The ORC-associated protein (ORCA/LRWD1) stabilizes ORC on chromatin. Here, we evaluated the genome-wide distribution of ORCA using ChIP-seq during specific time points of G1. ORCA binding sites on the G1 chromatin are dynamic and temporally regulated. ORCA association to specific genomic sites decreases as the cells progressed towards S-phase. The majority of the ORCA-bound sites represent replication origins that also associate with the repressive chromatin marks H3K9me3 and methylated-CpGs, consistent with ORCA-bound origins initiating DNA replication late in S-phase. Further, ORCA directly associates with the repressive marks and interacts with the enzymes that catalyze these marks. Regions that associate with both ORCA and H3K9me3, exhibit diminished H3K9 methylation in ORCA-depleted cells, suggesting a role for ORCA in recruiting the H3K9me3 mark at certain genomic loci. Similarly, DNA methylation is altered at ORCA-occupied sites in cells lacking ORCA. Furthermore, repressive chromatin marks influence ORCA's binding on chromatin. We propose that ORCA coordinates with the histone and DNA methylation machinery to establish a repressive chromatin environment at a subset of origins, which primes them for late replication.

INTRODUCTION

Eukaryotic cell cycle progression is a highly orchestrated process in which DNA replication, chromatin organization, and transcription need to be precisely coordinated. Each cell needs to replicate the entire genome once and only once per cell cycle, and this process is regulated both temporally and spatially. In eukaryotic cells, DNA replication initiates from multiple distinct sites on each chromosome, which are called replication origins (1,2). The initiation of DNA replication requires the step-wise assembly of pre-replication complex (pre-RC) onto origins during G1 (3). In eukaryotic cells, this process starts with the loading of a six-subunit complex, Origin Recognition Complex (ORC) at origins during G1 phase (3,4). ORC loading results in the sequential recruitment of Cdc6, Cdt1 and the DNA helicase MCM2-7 complex. After the loading of MCM2-7, the origin becomes 'licensed' and will fire during S phase (3). The MCM–Cdc45–GINS (CMG complex) helicase complex is subsequently activated by cell cycle kinases to start replication (5,6).

In *Saccharomyces cerevisiae*, origins are defined by a consensus sequence that is required for ORC binding (7). In *Schizosaccharomyces pombe*, origins are AT rich but do not appear to have a consensus sequence (8). In metazoans, however, no consensus sequence at replication origins has been identified and it remains to be determined as to how origins are selected and how ORC is recruited to origins. Multiple lines of evidence suggest that in metazoan cells, the selection of an origin and recruitment of pre-RC complex is facilitated by different pre-RC interacting proteins with different DNA binding preferences. For example, human Myc protein has been shown to interact with ORC and regulate

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the licensing of lamin B2 origin (9). The *Drosophila* insulator protein Su(Hw) has been shown to facilitate the loading of pre-RC proteins to origins at repressive chromatin regions (10). Factors including HMGA1 (11) or specific histone modifications (12,13) have been implicated in facilitating ORC binding to chromatin. Recently, RepID protein was shown to bind to a subset of origins and regulate replication (14). We have previously identified ORCA as an ORC-associated protein that can stabilize ORC on chromatin (15). However, whether this happens at origins or at heterochromatin remains to be resolved.

Several studies have been conducted to map replication origins genome-wide in different cell types (16). These studies were done by isolating and sequencing 'replication bubbles' (17,18), RNA-primed nascent DNA strand (19,20) or ORC binding sites (21–23). It is clear from these studies that different cell types use different sets of origins and not all the origins that are licensed are fired during S phase. Genome-wide analysis showed that replication origins are enriched for CpG islands (24) and correlate with local transcription status (19,21). Several histone marks, including H3K79me2 and H4K20me, have also been suggested to play a role in the licensing process (25–27). Different origins also fire at different times during S phase and it is generally believed that the temporal firing of origins is affected by local chromatin structure as well as the availability of initiation factors (28,29). Origins at open chromatin regions tend to fire early while those at repressive chromatic regions tend to fire late (12).

A lot of effort has been made to understand how specific origins are selected and how ORC is recruited in metazoan cells. In human cells, ORC binds to chromatin non-specifically and therefore it has been extremely challenging to identify ORC-bound regions. Barring a couple of recent studies showing that preRC complexes are at nucleosome-depleted regions in Drosophila (30) and in mammalian cells Orc1 and Orc2 bind predominantly to open chromatin (21,23), the factors that dictate ORC loading remain to be determined. It has therefore remained a challenge in the field to determine precisely the underlying factor(s) that provide specificity for origin location, and to understand if these factors stimulate origin activation. It was therefore imperative to determine the distribution of factors like ORCA that we have shown to facilitate the chromatin association of ORC.

ORCA localizes to heterochromatin structures and associates with repressive histone marks (15,31,32). We recently demonstrated that ORCA's role in heterochromatin organization during S phase is independent of its role in replication initiation/licensing during G1 (33). Additionally, we have also shown that ORCA is required for ORC loading and replication licensing (34). However, it is not clear if the stabilization of ORC by ORCA occurs at replication origins and/or if ORCA associates with replication origins. Since previous attempts to map ORC-binding sites were carried out in asynchronous cells, it was unclear if ORC binds to specific sites in G1, when licensing occurs. To dissect out ORCA's function in replication initiation and to better understand the step-wise licensing process in G1, we have mapped ORCA binding sites genome-wide by ChIP-seq studies. By carrying our ORCA ChIP-seq in cells synchronized at different stages of G1 phase, we demonstrate that the loading of ORCA at replication origins is likely to be a dynamic process. Our previous studies did not address if ORCA coats repressive chromatin structures or if it binds to distinct sites. We find that ORCA binding sites are enriched for repressive marks and ORCA shows strong co-localization with a subset of origins that are also enriched for repressive marks, including H3K9me3 and methyl-CpG sites. ORCA bound regions predominantly replicate during late S phase. ORCA directly interacts with these repressive marks and these marks in turn are also required for ORCA's association with chromatin. Our results suggest that ORCA associates with late-replicating origins, coordinates with the machinery that establishes repressive chromatin environment, helps in the maintenance of heterochromatin, and dictates replication timing.

MATERIALS AND METHODS

Chromatin immunoprecipitation (ChIP) and ChIP-seq

Detailed ChIP protocol is described in supplementary material and methods.

Construction of ChIP-Seq libraries and sequencing on the HiSeq2500 was carried out at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign (UIUC). The libraries were constructed with the Kapa Hyper Prep Kit from Kapa Biosystems (Kapa Biosystems, MA, USA). Briefly, DNAs (10 ng) were blunt-ended, 3′-end A-tailed and ligated to indexed adaptors with 6nt barcodes. Adaptored DNA were amplified by PCR to selectively enrich for those fragments that have adapters on both ends. Amplification was carried out for 10 cycles with the Kapa HiFi polymerase (Kapa Biosystems, Woburn, MA, USA) to reduce the likeliness of multiple identical reads due to preferential amplification. The final libraries were run on Agilent bioanalyzer DNA high-sensitivity chip (Agilent, Santa Clara, CA, USA) to determine the average fragment size and to confirm the presence of DNA of the expected size range. They were also quantitated by qPCR on a BioRad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc. CA, USA) prior to pooling and sequencing.

The ChIP-Seq libraries were pooled in equimolar concentration based on the qPCR concentration and sequenced on four lanes on an Illumina HiSeq2500 using HiSeq SBS sequencing kits version 4. The raw .bcl files were converted into demultiplexed compressed fastq files using the bcl2fastq 1.8.2 conversion software (Illumina). Independently, ChIP-Seq for the 1.5 and 3 h time points was performed in duplicates with two independent biological replicates using the HiSeq Rapid choice method.

Nascent strand sequencing

Replication origins were mapped using nascent strand sequencing (19,35). In short, the nascent strand samples were prepared by collecting λ exonuclease resistant, 0.5–2.5 kb fragments of single stranded DNA from asynchronous U2OS cell populations. High molecular weight genomic DNA was prepared in parallel from the same population of cells, sonicated and sequenced. All samples were subject to

massively parallel sequencing using standard Illumina protocols (36).

Following sequencing, Genomatix (https://www.genomatix.de/) was used to call peaks against appropriate genomic DNA or input controls. Either the MACS algorithm or the SICER algorithm was used. SICER was used to call peaks for the nascent strand sequencing with the following parameters: redundancy threshold = 2, window size = 200, fragment size = 150, gap size = 600, FDR = 0.01, *P*-value = 200. MACS was used to call peaks for the ORCA ChIP-Seq with the following parameters: Tag size = 20, *q*-value = 0.01, bandwidth = 300, lower and upper limit to mold for (model) = 10–30, redundancy threshold = auto. Bed file peaks identified by SICER or MACS were visualized along with the tiled original data using the Integrative Genome Viewer (https://www.broadinstitute.org/igv/).

BED file comparisons

A custom script (available upon request) was used to create a list of sequences from a reference file (input 1) that are or are not found within a user-defined distance of sequences in the comparator file (input 2). The BedIntersect script outputs the peaks from the reference file that overlap within 2 kb of the comparator, while the BedSubtract script outputs the peaks that are found in the reference and not in the comparator. Series of intersections and subtractions were performed to identify the extent of colocalization among ORCA, methylated CpGs, histone modifications and replication origins. Whole-genome co-localization was visualized and quantified using ColoWeb (37) and verified with Genomatix.

Methylated DNA immunoprecipitation (MeDIP) and MeDIP-seq

Detailed MeDIP protocol is described in supplementary material and methods

For MeDIP-seq, sonicated genomic DNA (6 μg) was used to construct libraries using the Kapa Hyper Prep Kit (Kapa Biosystems, MA) prior to performing MeDIP. Briefly, 6 μg of sonicated DNA were blunt-ended, 3′-end A-tailed and ligated to indexed adaptors with 6nt barcodes. Adaptored DNA was then subjected to MeDIP as described above. Five nanogram of pull down DNA were amplified by PCR to selectively enrich for those fragments that have adapters on both ends. Amplification was carried out for 12 cycles with the Kapa HiFi polymerase (Kapa Biosystems, Woburn, MA, USA) to reduce the likeliness of multiple identical reads due to preferential amplification. The final libraries were run on Agilent bioanalyzer DNA high-sensitivity chip (Agilent, Santa Clara, CA, USA) to determine the average fragment size and to confirm the presence of DNA of the expected size range. They were also quantitated by qPCR on a BioRad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc., CA, USA) prior to pooling and sequencing.

The MeDIP libraries were pooled in equimolar concentration based on the qPCR concentration and sequenced on two lanes on an Illumina HiSeq2500 using HiSeq SBS sequencing kits version 4. The raw .bcl files were converted into demultiplexed compressed fastq files using the bcl2fastq 1.8.2 conversion software (Illumina).

MeDIP-seq data analysis

The MeDIP-seq sequencing reads were aligned to the GRCh37/hg19_noMask version of the Human genome using Bowtie 0.12.7 (38). The single-end MeDIP-seq sequencing reads were aligned with the following Bowtie parameters: -n 2 -l 20 -M 1 –tryhard. MeDIP-seq peaks were called using Model-based Analysis of ChIP-Seq (MACS) with default parameters (39). Input sequencing libraries were used to control for sequencing specific biases.

Data access

All sequencing data have been submitted to NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/). The accession numbers are: H3K9me3 ChIP-seq (GSE68129); ORCA ChIP-seq and MeDIP-seq in U2OS cells (GSE81165); U2OS nascent strand-seq (GSE80391).

RESULTS

Binding of ORCA on chromatin is dynamically regulated during G1

To understand how ORCA regulates heterochromatin replication and/or organization, we evaluated the genome-wide distribution of ORCA using chromatin immunoprecipitation (ChIP) in U2OS cell line stably expressing HA-ORCA (Supplementary Figure S1A). We performed ChIP in cells synchronized at G1 phase, a stage when ORC loading and the licensing process are established (Figure 1). We found that ORCA showed dynamic binding pattern at several genomic loci at different stages of G1. Previous work has shown that the largest ORC subunit, Orc1 protein shows spatio-temporal dynamics during G1 (40). Since the protein levels of ORCA are highest during G1, we conducted ORCA ChIP at different points in G1. Cells were synchronized at prometaphase by nocodazole, and the mitotic cells were collected by shake-off. The cells were released from the arrest and collected for ChIP at various time points within G1 [1.5 h (early G1), 3 h (mid G1) and 5 h (late G1)]. The arrest during mitosis and the synchronous release was monitored by flow cytometry, immunoblot analyses of G1 and S phase markers (Supplementary Figure S1B) and immunofluorescence to evaluate BrdU incorporation (not shown). ChIP was performed using HA antibody followed by deep sequencing at early, mid, and late G1 phase. Our ChIP-seq result showed that both the binding pattern and the number of ORCA binding sites changed as cells progress through G1 phase. We obtained ∼10 000 peaks at early and mid G1 and only 1347 peaks at late G1 (Figure 1A). This change in the number of ORCA binding sites during G1 is consistent with the fact that ORCA protein level peaks at G1 and drops at the G1/S boundary (15). We also found that about 55% of the peaks at early and mid G1 are not found at late G1, whereas most of the peaks at late G1 are also present in the previous two time points (Figure 1A and B). A subset of regions is bound by ORCA throughout G1,

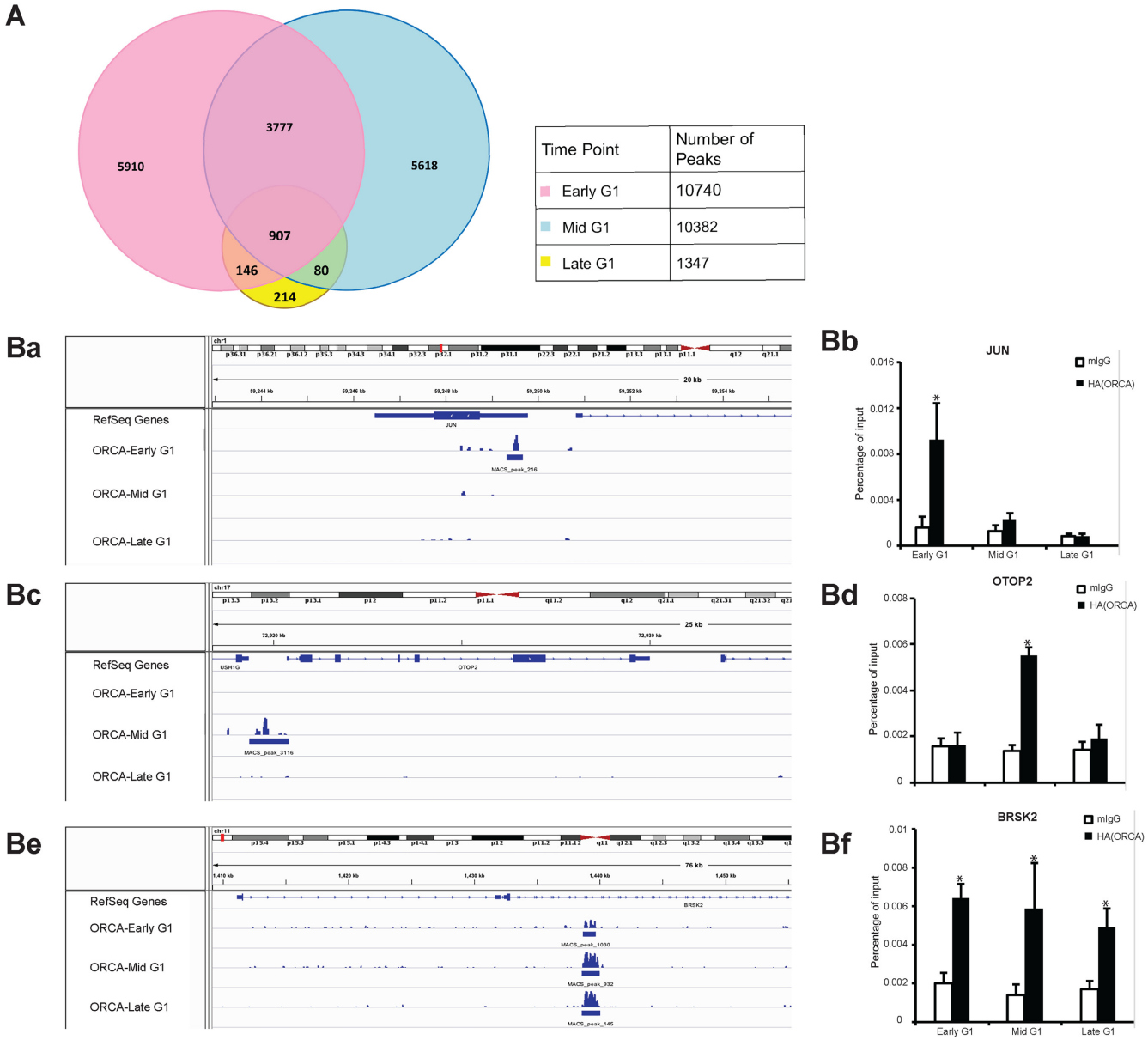

Figure 1. Dynamic binding of ORCA on chromatin during G1 phase. (**A**) Venn diagram summarizing the number of ORCA peaks at each time point during G1 phase and their overlaps. (**B**) Representative screenshots of regions with different ORCA binding pattern throughout G1 visualized with Integrated Genome Viewer (Ba, Bc, Be) and corresponding ChIP-qPCR validation of ORCA binding pattern during G1 (Bb, Bd, Bf). The horizontal bars below the peaks represent the called-peaks. ChIP was performed in the U2OS cell line stably expressing HA-ORCA. Error bars represent S.D., $n = 3$, $^*P < 0.05$.

which contribute to most of the ORCA binding regions we have identified at the late G1 time point (Figure 1A). Representative snapshots of regions with different ORCA binding pattern throughout G1 are shown (Figure 1Ba, Bc, Be). Binding pattern of ORCA for c-Fos locus as a negative control is shown in Supplementary Figure S1C. These results indicate that the binding of ORCA onto chromatin during G1 phase is a temporally regulated process and is dynamic. The temporal binding of ORCA at specific regions was also validated by ChIP-qPCR (Figure 1Bb, Bd, Bf). Similar temporal ORCA binding on chromatin was also observed by ChIP-qPCR in wild-type U2OS cells using ORCA antibody (Supplementary Figure S1Da and Db).

ORCA binding regions contained ∼32–34% intergenic regions, with the other areas either genic or partial (partly contained in genic regions) (Supplementary Figure S1E). Since the ChIP-Seq analysis is limited to single copy regions and does not include repetitive loci, which comprise a large fraction (>50%) of the human genome, the actual abundance of ORCA binding sites intergenic regions is likely to be higher. Remarkably however, ORCA peaks are significantly enriched in promoter regions (Supplementary Figure S1E), consistent with their colocalization with replication origins (see below).

ORCA binding regions strongly co-localize with replication origins

We have previously shown that ORCA interacts with the ORC complex and stabilizes ORC on the chromatin (15). Further, loss of ORCA in primary fibroblasts resulted in cells arresting in G1 with reduced MCM loading, consistent with a role for ORCA in replication origin licensing. In addition to its role in replication initiation, ORCA also regulates heterochromatin organization in a way that is independent of its role in replication initiation (33). To address how ORCA might regulate replication initiation, we asked if the ORCA binding sites during G1 represent replication origins. We mapped the replication initiation sites in U2OS cells by nascent strand abundance analysis (35). Our analysis showed that there is strong co-localization between ORCA-binding sites and replication initiation sites. At all three time points tested, most of the ORCA binding sites (65–85%) were found near (within 2 kb) replication origins (Figure 2A and B). However, these ORCA binding sites represent only 11.5% of the total mapped origins. ORCA binding sites, however, did not show strong co-localization with a random generated genome sequence of the same size distribution and GC content as the origin sequences (Figure 2B), suggesting that the majority of ORCA binding sites identified in our ChIP-seq analysis indeed represent replication origins. We observed that at each time point, ORCA specifies a different subset of origins, indicating that ORCA associates with distinct origins during G1 in a dynamic manner. In fact, at early and mid G1, more than 50% of the origins bound by ORCA is not found at late G1, while a subset of origins are bound by ORCA throughout G1 (Figure 2C and D). In late G1 phase, most of the origins bound by ORCA are present in the two earlier time points and very few origins are bound by ORCA only at late G1 (Figure 2C). These results indicate that the specification of replication origins by ORCA/ORC during G1 may be more dynamic than previously thought. Once an origin is specified by ORCA, it may enable the licensing process and once the licensing process is finished, ORCA may dissociate from its initial loading site.

ORCA binding regions are enriched for H3K9me3 and methyl-CpG marks

We previously demonstrated that ORCA localizes at repressive chromatin structures and regulates heterochromatin organization (15,33). We mapped the genome-wide distribution of repressive marks including H3K9me3 and DNA methylation in human U2OS cells. By aligning the data obtained from the ORCA ChIP-seq with that of H3K9me3 ChIP-seq, we found that there is strong co-localization between ORCA-binding regions and H3K9me3-containing regions (Figure 3Aa and c, Supplementary Figure S2A). Interestingly, the percentage of ORCA peaks that co-localize with H3K9me3 increases slightly as cells progress through G1 (from 45% to 65%, Figure 3Ab) consistent with our cell biological data that ORCA levels decrease at the end of G1 but whatever remains localizes to heterochromatic structures.

To address ORCA's localization in relation to DNA methylation, we carried out methylated DNA immunoprecipitation (MeDIP) with an antibody that specifically recognizes methyl-cytosine. We performed MeDIP followed by deep sequencing in U2OS cells to determine the genome wide localization of methyl-CpG sites. We aligned the MeDIP-seq result with our ORCA ChIP-seq results. Similar to H3K9me3, we also found strong co-localization between ORCA-binding regions and methyl-CpG sites, and the percentage of ORCA peaks that co-localize with methyl-CpG sites also increases slightly as cells progress through G1 (from 49% to 61%, Figure 3Ba and Bb).

ChIP-Seq was performed in two biological replicates for the 1.5 and 3 h time points. In both sets of biological replicates we observed high concordance between the duplicates collected at the same time but low concordance between the two time points. Both biological replicates demonstrated a highly significant colocalization with replication origins and H3K9me3 when compared to randomized controls. These data support our conclusion that ORCA associates with replication origins and H3K9 methylated regions and that ORCA binding undergoes temporal relocation during G1.

ORCA regulates H3K9me3 and methyl-CpG marks at its binding sites

We have previously shown that the loss of ORCA causes dramatic reduction of H3K9me3 at 18% of the H3K9me3 peaks (33). However, we were unable to pinpoint why only specific H3K9me3 sites were altered. To address if ORCA regulates H3K9me3 at its binding sites, we aligned the H3K9me3-containing ORCA peaks with the 18% H3K9me3 peaks (ORCA-dependent H3K9me3 peaks) that showed dramatic reduction upon ORCA depletion. Our results showed that most of the H3K9me3-containing ORCA-binding sites (∼80%) showed H3K9me3 reduction upon ORCA depletion (Figure 3C, Supplementary Figure S2B), indicating that ORCA binds to H3K9me3-containing regions and regulates the local repressive marks. Two snapshots showing the loss of H3K9me3 upon ORCA-depletion occurring at the regions showing co-localization of ORCA and H3K9me3 are provided (Supplementary Figure S2B). Adjacent regions enriched for H3K9me3 (without ORCA binding) remain unaffected upon the loss of ORCA (Supplementary Figure S2B). However, what dictates ORCA-binding to certain repressive sites and not others remains to be determined. Future studies will determine the potential involvement of other factors, chromatin modifications, or DNA methylation in the differential binding of ORCA to repressive marks.

Previous work demonstrated that ORCA binds to nucleosomes most efficiently in the presence of methylated DNA (31). We therefore evaluated if ORCA also regulates DNA methylation status at its binding sites. We carried out MeDIP followed by qPCR in control and ORCA-depleted cells. Our MeDIP-qPCR results showed a significant decrease in DNA methylation at several ORCA-binding sites (the ones that are also enriched for H3K9me3) upon ORCA depletion (Figure 3D). Interestingly, we observed about a 50% reduction in DNA methylation level near the transcription start sites of ribosomal RNA (Figure 3D). The DNA methylation status at the rDNA locus is known to control

Figure 2. ORCA associates with replication origins. (**A**) Genome-wide co-localization of ORCA binding sites as a function of the distance from replication origin sites. (**B**) Percentage of ORCA binding sites that co-localize with replication origins at each time point of G1 phase. (**C**) Venn diagram summarizing the number of ORCA-associated origins at each time point during G1 phase and their overlaps. (**D**) Representative screenshots showing different ORCA-origin co-localization visualized with Integrated Genome Viewer.

rRNA transcription (41). We therefore determined if the methylation change caused by ORCA depletion changed the rRNA transcription status. Our transcription analysis by qPCR showed an increase in 45S pre-rRNA level in ORCA-depleted cells (Supplementary Figure S2C), indicating that the loss of DNA methylation at rDNA locus by ORCA depletion also causes a change in rRNA transcription. Previously, it has been shown in mouse cells that loss of ORCA leads to increased major satellite repeat transcription (42). Here, it appears that ORCA also regulates transcription from certain repeat regions in human cells.

ORCA-bound origins are enriched for H3K9me3 and methyl-CpG marks

Our analysis showed that most of the ORCA-binding regions co-localize with replication origins. However, there appear to be a large number of origins that are not bound by ORCA. This is similar to what has been reported previously for Orc1 binding to human genome (21). We therefore determined the chromatin feature of a subset of distinct origins that are occupied by ORCA. Replication origins are generally enriched at euchromatic regions (12). Further, our

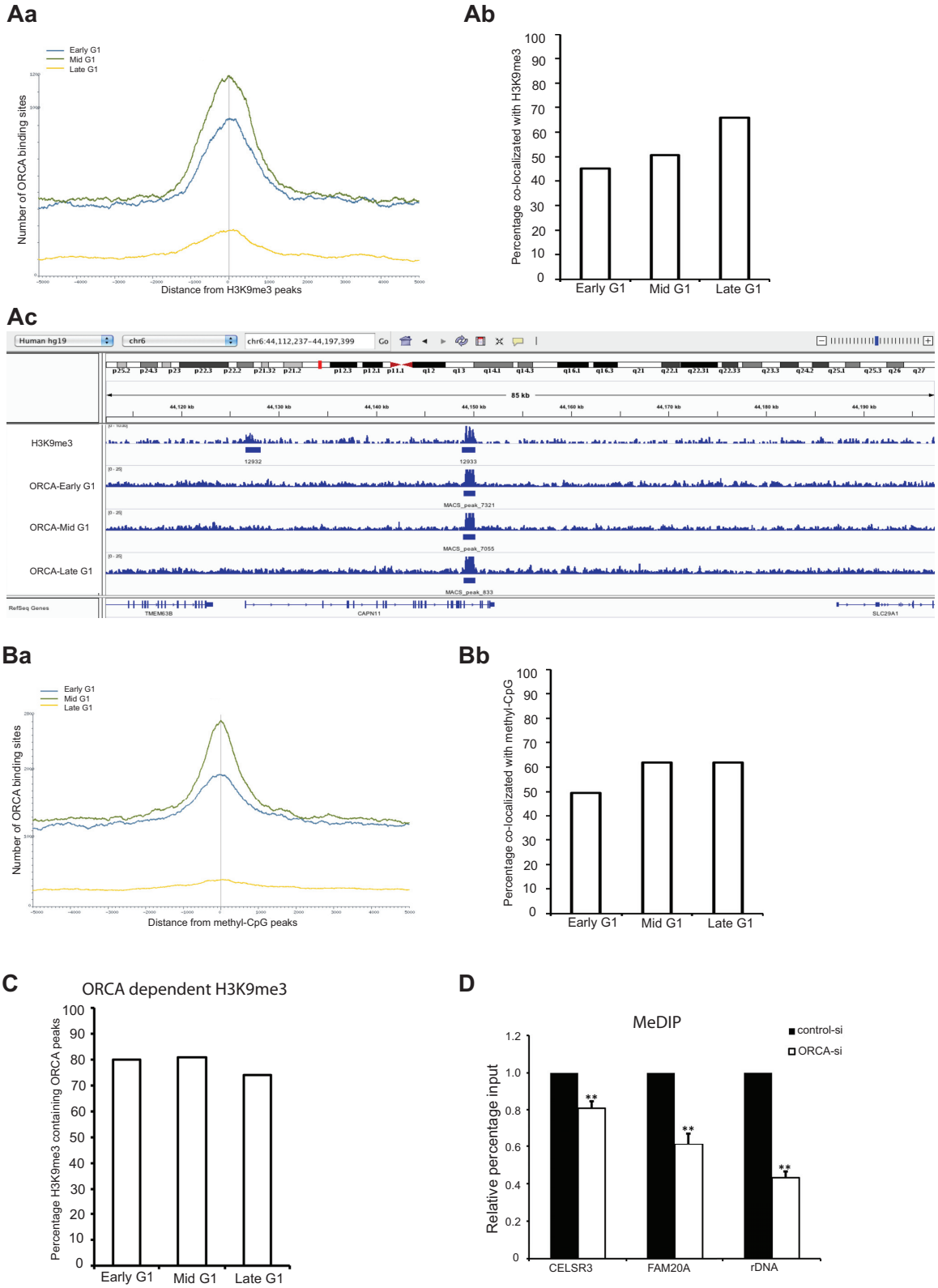

Figure 3. ORCA binding regions are enriched for H3K9me3 and methylated CpG. (**A**) Co-localization of ORCA with H3K9me3. (Aa) Genome-wide co-localization of ORCA binding sites as a function of the distance from H3K9me3 sites. (Ab) Percentage of ORCA binding sites that co-localized with H3K9me3 at each time point of G1 phase. (Ac) Representative screenshots of ORCA and H3K9me3 co-localization visualized with Integrated Genome Viewer. (**B**) Co-localization of ORCA with methyl-CpG sites. (Ba) Genome-wide co-localization of ORCA binding sites as a function of the distance from methyl-CpG sites. (Bb) Percentage of ORCA binding sites that co-localized with methyl-CpG sites at each time point of G1 phase. (**C**) Percentage of H3K9me3 containing ORCA peaks that showed highly significant H3K9me3 reduction upon ORCA depletion. (**D**) MeDIP-qPCR at several ORCA binding regions in control and ORCA-depleted cells. Error bars represent S.D., $n = 3$, $^{**}P < 0.01$.

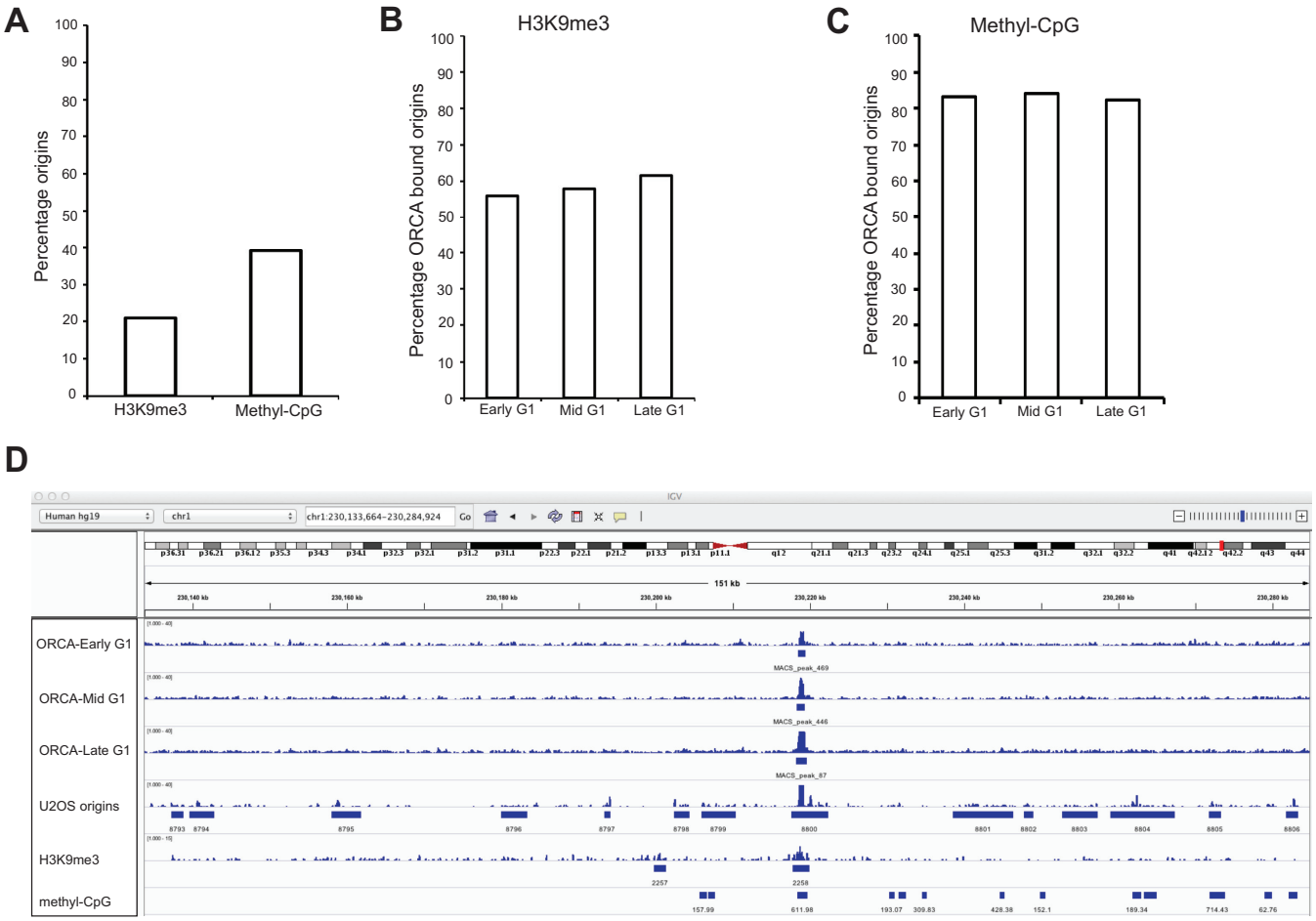

Figure 4. ORCA-associated replication origins are enriched for H3K9me3 and methylated CpG. (**A**) Percentage of total origins that co-localize with H3K9me3 and methyl-CpG sites. (**B**) Percentage of ORCA-associated origins that co-localize with H3K9me3. (**C**) Percentage of ORCA-associated origins that co-localize with methyl-CpG sites. (**D**) Representative screenshots showing one ORCA-associated origin that co-localizes with H3K9me3 as well as methyl-CpG site visualized by Integrated Genome Viewer. Note the adjacent origins that do not co-localize with H3K9me3 and methyl-CpG site are not occupied by ORCA.

analysis also showed that replication initiation events do not show very strong co-localization with H3K9me3 and methylated CpG sites (Figure 4A). In fact, among the total replication initiation sites, only a small subset is found near H3K9me3 (20%) and 40% of the origins are found near methyl-CpG site (Figure 4A). However, ORCA-occupied origins showed strong co-localization with H3K9me3 and methyl-CpG sites (Figure 4B and C). At all the three time points in G1, a large portion of the ORCA-occupied origins are found near H3K9me3 and methyl-CpG sites (Figure 4B and C). A snapshot of an ORCA-bound origin enriched for repressive environment is shown (Figure 4D). These results suggest that ORCA may specify a subset of replication origins with repressive chromatin marks, and facilitate the loading of other pre-RC components at these origins.

Our observation that ORCA-bound regions is enriched for repressive chromatin marks indicates that these regions may be late-replicating regions. Using the recently published U2OS replication timing profile (43), we stratified the genome into regions that replicate during early, early-mid, mid-late and late S phase. Our analysis showed that regions that are bound by ORCA replicated predominantly during late S phase (Supplementary Figure S2D). The numbers of binding regions that intersect with late replicating regions were higher during early G1 (1.5 and 3 hours into G1) but the enrichment was also evident at the 5 hours time point, albeit with fewer peaks overall (73.0% of ORCA 1.5 h; 70.7% of ORCA 3 h and 67.1% of ORCA 5 h, found in late replicating regions). This observation is in line with the notion that repressive regions generally replicate late during S phase. Our analysis showed that H3K9me3 containing ORCA peaks primarily colocalize with origins (Supplementary Figure S2E). This is consistent with the fact that ORCA-bound origins are late-firing origins.

ORCA interacts with methylated DNA sequence *in vitro* and associates with DNA methyltransferases

Our sequencing analyses showed that a large number of ORCA peaks co-localize with H3K9me3 and methyl-CpG peak *in vivo*. Others and we have previously shown that ORCA could interact with both H3K9 methyltransferases and methylated H3K9 peptide *in vitro* (33,42). We now addressed if ORCA can bind methylated DNA as well. To ad-

dress this, we performed electrophoretic mobility shift assay (EMSA) using bacterially purified His-SUMO-ORCA protein. To test whether ORCA interacts with methylated DNA directly, we used a double-stranded DNA probe with 12 CpG sites, with the cytosine being either un-modified or methylated. When we incubated the methylated probe with His-SUMO-ORCA but not His-SUMO, we observed a dramatic shift in mobility, indicating a strong interaction between ORCA and the methylated DNA probe (Figure 5A). Incubation of an un-methylated probe with ORCA, however, did not show an obvious shift, indicating ORCA specifically recognizes and interacts with methylated DNA. We then asked if ORCA only recognizes methyl-cytosine when the CpG sites on both strands are methylated. To address this, we generated DNA substrates that are unmethylated, hemi-methylated (one strand) or methylated (both the strands) and performed EMSA experiment with purified ORCA protein. The result showed that ORCA interacts with both the hemi-methylated and fully methylated probe but not with the un-methylated probe (Figure 5B), suggesting that ORCA may be recognizing the methyl-group on methyl-cytosine. We also performed a similar experiment with another pre-RC protein, Orc1, and did not observe any binding to methylated or unmethylated DNA substrate (Supplementary Figure S3A).

We found that ORCA binds to repressive histone marks and also associates with the enzymes (HKMTs) that catalyze these marks (33). Since we found that ORCA preferentially binds to methylated sequences, we examined if ORCA also interacts with the DNA methylation machinery, DNA methyltransferases (DNMTs). Mammalian cells have three active DNA methyltransferases: DNMT1, DNMT3a and DNMT3b (44). To address whether ORCA interacts with DNA methyltransferases, we performed co-immunoprecipitation (co-IP) experiments. By performing co-IP using T7 antibody in U2OS cells expressing Myc-DNMT1 with or without T7-ORCA, we found that ORCA interacts with DNMT1 (Figure 5C). In a U2OS cell line stably expressing HA-ORCA, we performed IP using HA antibody and the result revealed robust interaction between HA-ORCA and endogenous DNMT3a (Figure 5D). Similarly, endogenous ORCA was found to interact with Myc-DNMT3b (Figure 5E). Reciprocal co-IPs confirmed these interactions (Supplementary Figure S3B–D). To rule out the possibility that DNA or RNA mediated these interactions, we have also performed co-IP experiments in the presence of Ethidium Bromide (EtBr) or RNase A. Interactions between DNMT1, DNMT3a, DNMTb and ORCA were still detected under these conditions (Supplementary Figure S3E–I). These results demonstrate that ORCA associates with methylated DNA and also with the enzymatic machinery catalyzing these marks.

Repressive marks are required for ORCA binding to chromatin

We have observed that ORCA binding regions are enriched for repressive marks including H3K9me3 and methylated CpG sequence *in vivo*. We observe that ORCA directly interacts with both methylated H3K9 peptides and methylated DNA sequences *in vitro*. We reasoned that these repressive marks could also be influencing ORCA recruitment onto chromatin. To test this hypothesis, we depleted H3K9 methyltransferases SUV39H1 and SUV39H2 by siRNA (Supplementary Figure S3J). SUV39H1 and H2 are responsible for establishing H3K9me3 and depletion of these two enzymes leads to a significant loss of H3K9me3 mark (Supplementary Figure S3J and S3K). It has been shown that H3K9me3 and DNA methylation are dependent on each other (45), and we found that depletion of SUV39H1 and H2 also led to a reduction of DNA methylation level at several loci (Supplementary Figure S3L). We then carried out ORCA ChIP in SUV39-depleted cells and found that ORCA binding at several genomic loci is significantly reduced upon SUV39 depletion (Figure 5F), indicating that these repressive chromatin marks are required for ORCA binding at these regions. The total protein level of ORCA did not change significantly in SUV39-depleted cells (Supplementary Figure S3J). Thus, ORCA could be recruited to its binding sites by directly recognizing repressive chromatin marks and hence recruit other factors to its binding sites for its replication and/or heterochromatin functions.

DISCUSSION

ORCA is an ORC-associated protein that plays important roles in replication initiation as well as heterochromatin organization (15,31–34). We report that ORCA shows a dynamic binding pattern on chromatin during G1 phase and that ORCA associates with a distinct set of origins that are enriched for repressive marks, including H3K9me3 and methylated CpG sequence and these origins are predominantly late-replicating origins. We also show that ORCA can directly interact with these repressive marks and binds to the enzymes catalyzing these marks. Finally, the binding of ORCA to specific chromatin sites is required for maintaining the repressive marks at these sites suggesting the existence of a feedback loop to maintain heterochromatin structure.

The loading of ORC onto replication origin marks the initial step of the licensing process. ORC loading triggers the sequential loading of Cdc6, Cdt1, and the hexamer helicase MCM2-7 (3). However, few studies have been done to determine how the whole licensing process is coordinated during the length of G1 and whether all the origins are licensed in a synchronized manner. This is the first study investigating the genome-wide distribution of a preRC or a preRC-associated protein during G1 in synchronized mammalian cells. Previous work reported the 'sedimentation-coefficient shifted chromatin' mapping of Orc1 binding sites in human cells and found that Orc1 binding sites represent a subset of origins. These origins represent active replication origins, and were associated with open chromatin (21). However, the chances of mapping the Orc1 to heterochromatin were diminished because of their experimental design. Our results show that the association of ORCA onto origins during G1 is temporally regulated and highly dynamic. About half of the origins occupied by ORCA at early G1 are no longer bound by ORCA at mid G1 and only a small subset of origins is bound by ORCA throughout G1. This is consistent with our previous observation that ORCA protein level peaks at G1 phase and decreases as cells move towards S

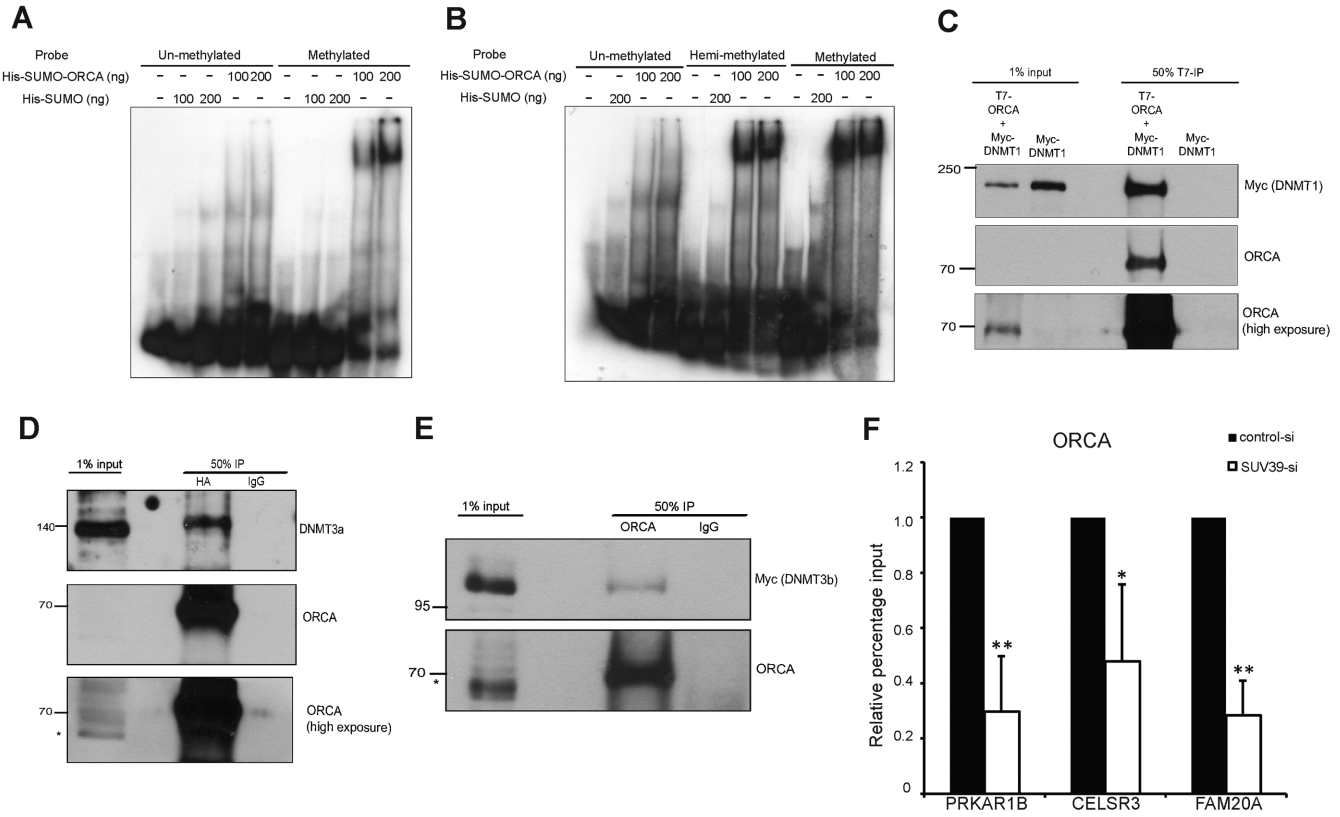

Figure 5. ORCA associates with methylated DNA sequence *in vitro* and interacts with DNA methyltransferases. (**A**) Electrophoretic mobility shift assay (EMSA) showing purified His-SUMO-ORCA protein preferentially interacts with methylated DNA substrate *in vitro*. His-SUMO is used as negative control. (**B**) EMSA showing purified His-SUMO-ORCA protein interacts with both hemi-methylated and fully methylated DNA substrate *in vitro*. (**C**) Immunoprecipitation (IP) in U2OS cells transiently expressing T7-ORCA and Myc-DNMT1 using T7 antibody. Results were analyzed by ORCA and Myc immunoblot. (**D**) IP in U2OS cell line stably expressing HA-ORCA using HA antibody. Results were analyzed by ORCA and DNMT3a immunoblot. (**E**) IP in U2OS cells transiently expressing Myc-DNMT3b using ORCA antibody. Results were analyzed by ORCA and Myc immunoblot. * denotes cross-reacting bands. (F) ORCA ChIP-qPCR in control and SUV39H1 and SUV39H2-depleted cells. Error bars represent S.D., $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$.

phase. These results indicate that the loading of preRC at origin during G1 may be a dynamic process and it is possible that once the licensing process is finished, some of the factors that facilitate the initial loading of preRC components are no longer bound to origins. *In vitro* studies have shown that in *Xenopus laevis* egg extracts, once the MCM complex is loaded, ORC is no longer required for MCM to initiate replication (46). Our results here show that *in vivo*, some of the pre-RC proteins or accessory factors may also dissociate from origins once MCM is loaded.

Metazoan replication origins lack consensus sequences and how origins are selected and licensed during G1 phase remains unknown. It has been proposed that origin selection is determined by the local chromatin structure or by the pre-RC interacting proteins that bind origins. Several studies mapped replication origins in metazoan cells using different methods (19,20,22). Although discrepancies exist, these studies generally agree on the fact that replication initiation events are enriched at open chromatin regions. Nevertheless, heterochromatin regions also need to be replicated and 'licensed' during G1 phase. ORCA could be a protein that can bind to origins with repressive chromatin marks and dictate the binding of other pre-RC proteins. We have previously demonstrated that in human cells ORC exists in excess to ORCA (34). Thus, it is likely that ORCA facilitates the loading of ORC to a subset of late-replicating origins that are enriched for repressive marks whereas other factors, possibly origin binding proteins or specific chromatin marks, may facilitate ORC loading onto origins at other chromatin regions. However our attempts to map Orc1 and Orc2 in human U2OS cells failed and therefore we do not have a conclusive answer on the binding dynamics of ORC. This is similar to what others have reported about lack of significant enrichment over background when attempting MCM and ORC ChIP (47). Further, how ORC binding is affected in the absence of ORCA remains to be answered.

Many factors and epigenetic mechanisms dictate the timing and efficiency of origin firing during S-phase. Spatial organization of origins within nuclear territories is an important determining factor for the origin timing. Early origins are thought to be more efficient with respect to the timing and efficiency of firing, whereas late origins are less efficient (48,49). It is believed that the local concentration of the initiation factors dictates the temporal firing of origins. However, why the initiation factors are recruited to some origins and not to others are unclear. Chromosomal context and chromatin architecture are key elements that determine the replication origin timing and firing (50). Early fir-

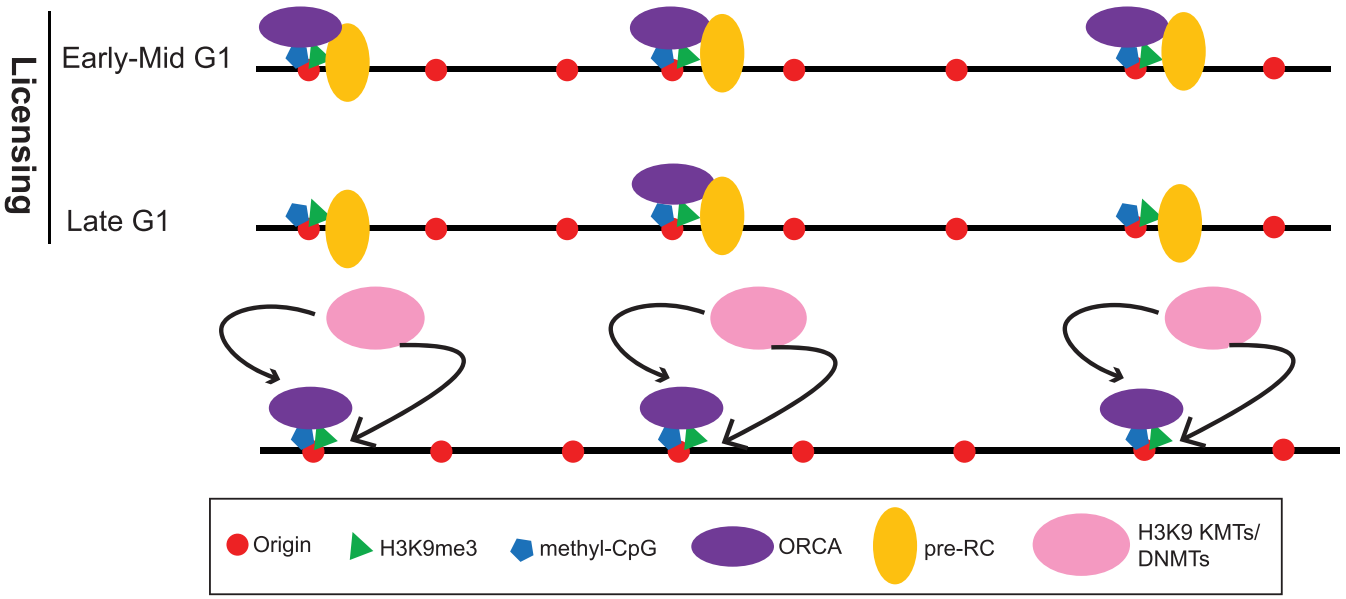

Figure 6. Model depicting ORCA's role in licensing as well as heterochromatin organization. Model showing how ORCA functions during the licensing process (top) and heterochromatin organization (bottom).

ing of origins is the default state. The repressive chromatin environment that flanks the origin causes an origin to fire late (10,51). Early replicating origins are enriched for euchromatic histone modifications including H3K4me1/2/3, H3K9ac, H3K1ac, H3K36me3 and H3K27ac), whereas late replication is marked by repressive chromatin modifications including H3 and H4 hypoacetylation, H3K9me1/3 and H3K27me3 (1,12,52).

Our data demonstrates that ORCA associates with a distinct class of origins that are enriched for repressive marks and primarily replicate late during S phase. The connections between H3K9me and late-firing replication origins has recently been reported in fission yeast as well (53). Our recent study using eight different cell lines, including cancer and non-cancer cells, has shown that the shared replication origins preferentially localize to unmethylated CpGs and with euchromatic marks including H3K4me3 and H3K9ac, whereas, the cell-type specific origins preferentially localize to heterochromatin marks such as H3K9me3 (54). We determined that shared origins replicated throughout S-phase, but the unique origins mostly replicated during late S phase (54). Indeed, we found that the ORCA-bound sites represent unique origins, consistent with ORCA-bound origins representing late-firing origins. We have previously shown that loss of ORCA causes changes in replication timing of a few tested origins. Further, fewer cells were found to exhibit the late S-phase pattern in the absence of ORCA (33). These observations suggest that the association of ORCA with a subset of origins enriched for the repressive marks during G1 phase may also dictate its replication timing during S-phase. Whether the ORCA-bound origins are still functional origins in the absence of ORCA is an interesting question. We hope that the genome-wide replication profiling experiments would address this aspect in the future.

Our ChIP-seq result showed that ORCA co-localizes with repressive marks including H3K9me3 and methyl-CpG sites, and that ORCA associates directly with methylated H3K9 peptides (33) as well as methylated DNA sequence. We propose that ORCA is recruited onto chromatin by directly recognizing and interacting with the repressive marks. In support of this hypothesis, we showed that ORCA binding to certain regions is reduced upon SUV39 depletion, which reduces H3K9me3 as well as other repressive marks on the chromatin. Notably, depletion of SUV39 causes a global change in chromatin structure. Thus, it is also possible that the reduced ORCA binding that we observe upon SUV39-depletion is due to a change in the global chromatin structure. Nevertheless, our results show that the loading of ORCA onto chromatin requires proper chromatin architecture. Interestingly, not only does ORCA bind to the repressive marks, it also appears to regulate these marks at its binding sites. We find that the loss of ORCA causes reduced H3K9me3 as well as DNA methylation level at its binding sites. There is a lot of evidence highlighting the crosstalk between H3K9 methylation and DNA methylation (45). Thus, it is not clear whether the reduction in these two marks upon ORCA depletion is a consequence of one affecting the other. However, ORCA interacts with the enzymes that catalyze these marks, including H3K9 lysine methyltransferases and DNA methyltransferase. Based on our data, we propose a feedback loop between ORCA and repressive chromatin marks (Figure 6). The repressive marks at heterochromatin regions are required for initial ORCA loading. ORCA is then able to recruit the histone and DNA methyltransferases to its binding sites and facilitate the spreading of these repressive marks. During G1, ORCA binds to origins enriched for repressive marks and this in turn may dictate heterochromatin replication in the following S phase.

ACCESSION NUMBERS

GEO GSE86129, GSE81165, GSE80391.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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