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Dynamic Association of ORCA with Prereplicative Complex Components Regulates DNA Replication Initiation

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In eukaryotes, initiation of DNA replication requires the assembly of a multiprotein prereplicative complex (pre-RC) at the origins. We recently reported that a WD repeat-containing protein, origin recognition complex (ORC)-associated (ORCA/LRWD1), plays a crucial role in stabilizing ORC to chromatin. Here, we find that ORCA is required for the G_1-to-S-phase transition in human cells. In addition to binding to ORC, ORCA associates with Cdt1 and its inhibitor, geminin. Single-molecule pulldown experiments demonstrate that each molecule of ORCA can bind to one molecule of ORC, one molecule of Cdt1, and two molecules of geminin. Further, ORCA directly interacts with the N terminus of Orc2, and the stability of ORCA is dependent on its association with Orc2. ORCA associates with Orc2 throughout the cell cycle, with Cdt1 during mitosis and G_1, and with geminin in post-G_1 cells. Overexpression of geminin results in the loss of interaction between ORCA and Cdt1, suggesting that increased levels of geminin in post-G_1 cells titrate Cdt1 away from ORCA. We propose that the dynamic association of ORCA with pre-RC components modulates the assembly of its interacting partners on chromatin and facilitates DNA replication initiation.

In eukaryotes, initiation of DNA replication requires the assembly of a prereplicative complex (pre-RC) in late mitosis and G_1, with the sequential loading of the origin recognition complex (ORC), Cdc6, Cdt1, and MCM2-7 onto replication origins (6). The Cdt1-mediated recruitment of the helicase MCM2-7 to chromatin also requires MCM9 (31). At the G_1-S transition, pre-RC is converted to preinitiation complex (pre-IC) when MCM proteins are activated by the cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK). CDK- and DDK-dependent phosphorylation activities, in addition to molecules like MCM10, enable the recruitment of Cdc45 and GINS onto MCM2-7 in order to activate the helicase (16, 37, 40, 55, 60). Upon initiation of DNA replication, the pre-RC is disassembled, and Cdt1 and Cdc6 are released from the origins, thereby preventing rereplication (11).

Several mechanisms ensure that replication occurs "once and only once" during each cell cycle (2, 54). This "licensing" process is well coordinated, and the loss of licensing causes DNA rereplication, genomic instability, and tumorigenesis (2). The regulation of the licensing factors Cdt1 and Cdc6 during the cell cycle is a crucial regulatory mechanism to prevent DNA rereplication (2, 7, 8, 15). In *Saccharomyces cerevisiae*, Cdc6 is degraded, whereas Cdt1 is exported out of the nucleus at the end of G_1 (12, 36, 59). In *Schizosaccharomyces pombe*, both Cdc6 and Cdt1 are subject to proteolytic degradation in post-G_1 cells (17, 19, 21, 22, 43). In mammalian cells, multiple parallel mechanisms operate to prevent Cdc6 and Cdt1 activity in post-G_1 phase: nuclear export of Cdc6 (25), cleavage of Cdc6 (41), SCF^{Skp2}- and Cul4/Ddb1-mediated degradation of Cdt1 (39, 65), and the presence of the Cdt1 inhibitor geminin (34). Geminin is predominantly present in post-G_1 cells and is degraded by APC^{Cdh1} during late mitosis to enable Cdt1 to assemble on chromatin (5, 27, 28, 50, 61). Recent studies have also indicated that geminin promotes the Cdt1-dependent loading of MCM2-7 in G_1, though the molecular nature of this geminin-Cdt1 complex remains to be understood (30). It has been suggested that the stoichiometry of Cdt1 and geminin in cells during different cell cycle stages determines the recruitment of MCM2-7 for licensing or inhibiting pre-RC assembly (9, 30) and that a fine-tuned balance between Cdt1 and geminin is crucial for genomic stability (45). Surprisingly, overexpression of Cdt1 mutants that lack the MCM binding domain can still induce rereplication by derepressing endogenous Cdt1 by titrating the levels of PCNA and cyclin (53). Using quantitative fluorescence microscopy, it has been demonstrated that Cdt1 can associate with geminin and chromatin simultaneously and that these dynamic associations provide spatiotemporal control of licensing (62).

We and others recently reported that origin recognition complex-associated protein (ORCA/LRWD1) is a novel ORC-associated protein that binds to ORC, Cdt1, and geminin (4, 47, 58). ORCA shows cell cycle dynamics similar to those of ORC, with the maximum level during G_1 and progressive decrease as the cells enter S phase. ORCA and ORC colocalize throughout the cell cycle, including in heterochromatic regions. Furthermore, ORCA stabilizes the binding of ORC to chromatin. The dynamic association of ORCA with the pre-RC components and its functional relevance are beginning to be appreciated.

In the present study, we demonstrate that the cellular, as well as chromatin-bound, Orc2 levels are severalfold higher than those of ORCA in human cells. Using single-molecule pulldown (SiMPull) experiments, we demonstrate that one molecule of ORCA is bound to one ORC, one Cdt1, and two geminin molecules, suggesting that an ORCA-independent ORC complex exists in cells.

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We show that ORCA directly interacts with Orc2 and that the binding of Orc2 to ORCA stabilizes cellular ORCA. ORCA associates with Orc1 during G_1, with Orc2 throughout the cell cycle, with Cdt1 during G_1, with phosphorylated Cdt1 during mitosis, and with geminin in post-G_1 cells. Overexpression of geminin results in loss of Cdt1 association with ORCA, suggesting that the increased levels of geminin titrate ORCA away from Cdt1. Based on our results, we propose that at the end of the G_1 phase, when the levels of geminin begin to rise, ORCA binding to Cdt1 is lost, resulting in the disassembly of the pre-RC. Collectively, these results suggest a crucial role for ORCA in pre-RC assembly and replication initiation.

MATERIALS AND METHODS

Cell culture and transfection. HeLa, U2OS, MCF7, and WI38 cells were grown in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS) (HyClone). Lipofectamine 2000 (Invitrogen) and Lipofectamine RNAiMAX (Invitrogen) were used for transient transfections and RNA interference (RNAi), respectively, according to the manufacturers' protocols.

Plasmids and antibodies. Human Orc2, Cdt1, and geminin cDNAs were cloned into the pCGT vector and pEYFP-C1 with the cytomegalovirus (CMV) promoter (Clontech) to generate T7-Orc2, T7-Cdt1, T7-geminin, and yellow fluorescent protein (YFP)-geminin. Truncated mutants were obtained by PCR from Orc2, Cdt1, and geminin cDNAs and were also cloned into the pCGT vector.

The following antibodies were used for immunoprecipitations (IP) and immunoblots, as indicated: ORCA polyclonal antibody (pAb) (2853-2 and 2854-1), Orc1 monoclonal antibody (MAb) (pKS1-40), Orc2 pAb (205), Orc3 pAb (Abcam), Orc4 pAb (Abcam), Orc5 pAb (BD Pharmingen), Orc6 pAb (982), Cdt1 pAb (Yue Xiong Laboratory), geminin pAb (Santa Cruz Biotechnology), tubulin MAb (Sigma-Aldrich), T7 MAb (Novagen), and green fluorescent protein (GFP) MAb (Covance).

Insect cell culture and baculovirus expression. For expression analysis, the full-length human ORCA was first cloned in the pFastBac HT-B (Invitrogen) vector and later was amplified, along with a polyhistidine tag, using pFastBac HT-B-ORCA as the template. Similarly, the open reading frames (ORFs) of human ORC subunits, Cdt1, and geminin were also amplified by PCR (primer sequences will be provided upon request). The PCR product of human ORCA was first cloned in the pFastBac-Dual vector at BssHII and SpeI sites, followed by the cloning of human ORC subunits, Cdt1, or geminin individually in the same backbone, so that each subunit of ORC, Cdt1, or geminin would be expressed along with ORCA from the same virus. Similarly, viruses expressing individual proteins as histidine tags were also generated. DNA sequencing was performed to confirm the constructs, and recombinant baculoviruses were produced according to the manufacturer's protocol (Bac-to-Bac baculovirus expression system; Invitrogen). Hi5/Sf9 insect cells were infected either with baculoviruses expressing ORCA and individual subunits of ORC/Cdt1/geminin or with a combination of different viruses, each at a multiplicity of infection of 5 to 10, and incubated at 28°C for 60 to 72 h. Nuclear extracts were prepared in PK50 buffer (in the presence or absence of 2 mM ATP), and proteins were precipitated by saturation at 45% ammonium sulfate (48). The reconstituted proteins in PK50 buffer were immunoprecipitated using anti-ORCA antibody, followed by immunoblotting using anti-ORC, Cdt1, or geminin antibody.

Immunoprecipitation. Nuclear extracts or whole-cell lysates were incubated with GammaBind G Sepharose resin (Amersham) at 4°C for 45 min for preclearing. Antibodies were added to the supernatant after the removal of the resin, and binding was carried out at 4°C overnight. Resin was then added for 1 h to capture the complex, washed three times in nuclear extraction buffer, and resuspended in Laemmli buffer for immunoblot analysis. If elution of the complex was needed, extracts were incubated with antibody-conjugated resin overnight. The resin was then washed and incubated with appropriate peptides three times at 30°C for 15 min. The eluate was then collected by low-speed centrifugation.

SiMPull. SiMPull experiments were carried out on methoxy polyethylene glycol (mPEG)-passivated quartz slides doped with biotin PEG as described previously (20). Antibodies were immobilized on NeutrAvidin (Thermo)-coated flow chambers either by incubation with biotinylated T7 antibody (Novagen) for 10 min or by incubation with biotinylated anti-rabbit IgG secondary antibodies for 10 min, followed by ORCA antibody for 20 min. RIPA buffer-lysed samples were then incubated in the chamber for 20 min and washed twice with buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml bovine serum albumin [BSA]). Single-molecule data were acquired with a prism-type total internal reflection fluorescence (TIRF) microscope and analyzed using scripts written in Matlab. For ORCA-Orc1 SiMPull analysis, lysates were made from the YFP-Orc1 stable cell line (U2OS cells) where T7-ORCA was transiently transfected. For ORCA-Cdt1 SiMPull analysis, lysates were made from U2OS cells where T7-ORCA and YFP-Cdt1 were transiently transfected. Similarly, for ORCA-geminin SiMPull analysis, lysates were prepared from cells transiently transfected with T7-ORCA and YFP-geminin.

Gel filtration and glycerol gradient sedimentation. Nuclear extracts were subjected to size exclusion chromatography using a Superdex-200 column (GE Inc.) at 0.25 ml/min, and fractions (600 μl each) were collected and precipitated with trichloroacetic acid (TCA). After centrifugation at 14,000 rpm for 5 min, the pellet was washed with acetone twice and resuspended in Tris, pH 8.7, and Laemmli buffer for immunoblots.

For glycerol gradient sedimentation, IP eluate or marker (200 μl) was layered on a 10 to 50% glycerol gradient (buffer: 27 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% NP-40, protease inhibitors). The mixture was centrifuged at 48,000 rpm for 16 h. Fractions (200 μl each) were collected and prepared for immunoblots as described above.

Synchronization. To synchronize cells at the G_1/S boundary, 2 mM thymidine was added. After 24 h, the cells were washed three times with fresh medium, grown for 12 h, and incubated with 2 mM thymidine for an additional 24 h. The cells were then released, and aliquots were taken at 4 h and 8 h for S and G_2 cells. For M and G_1 populations, cells that had been released from the double-thymidine block for 8 h were further treated with 50 ng/ml nocodazole for 14 to 16 h to arrest them in mitosis and were released for 5 to 6 h into G_1 phase.

RESULTS

ORCA is required for G_1/S transition. We previously demonstrated that depletion of ORCA in human primary diploid fibroblasts results in cells accumulating in the G_1 phase of the cell cycle (47). However, it remained to be determined whether ORCA depletion results in a delay in G_1 progression or an arrest in the G_1 phase of the cell cycle. To resolve these possibilities, we serum starved WI38 diploid fibroblasts and then treated the cells with control or ORCA-specific small interfering RNA (siRNA). The cells were then released from arrest and harvested at 12 and 24 h for immunoblot and flow cytometry analyses (Fig. 1Aa). The control and ORCA siRNA-treated cells at 12 h postrelease looked identical. However, at 24 h postrelease, control cells efficiently entered the cell cycle and progressed through S and G_2/M, whereas the ORCA siRNA-treated cells failed to enter S phase, with 67.7% of the cells in G_1 compared to 25.9% of the control cells (Fig. 1Ac). The appearance of a population of ORCA-depleted cells in S phase could indicate either incomplete depletion of ORCA or extremely slow progression through S phase. Further, chromatin fractionation and immunoblot analysis revealed decreased ORC (25.3% of Orc2 on chromatin), as well as MCM (43.3% of MCM3 on chromatin), loading onto chromatin, suggesting that the pre-RC checkpoint had been activated because of defects in loading MCM

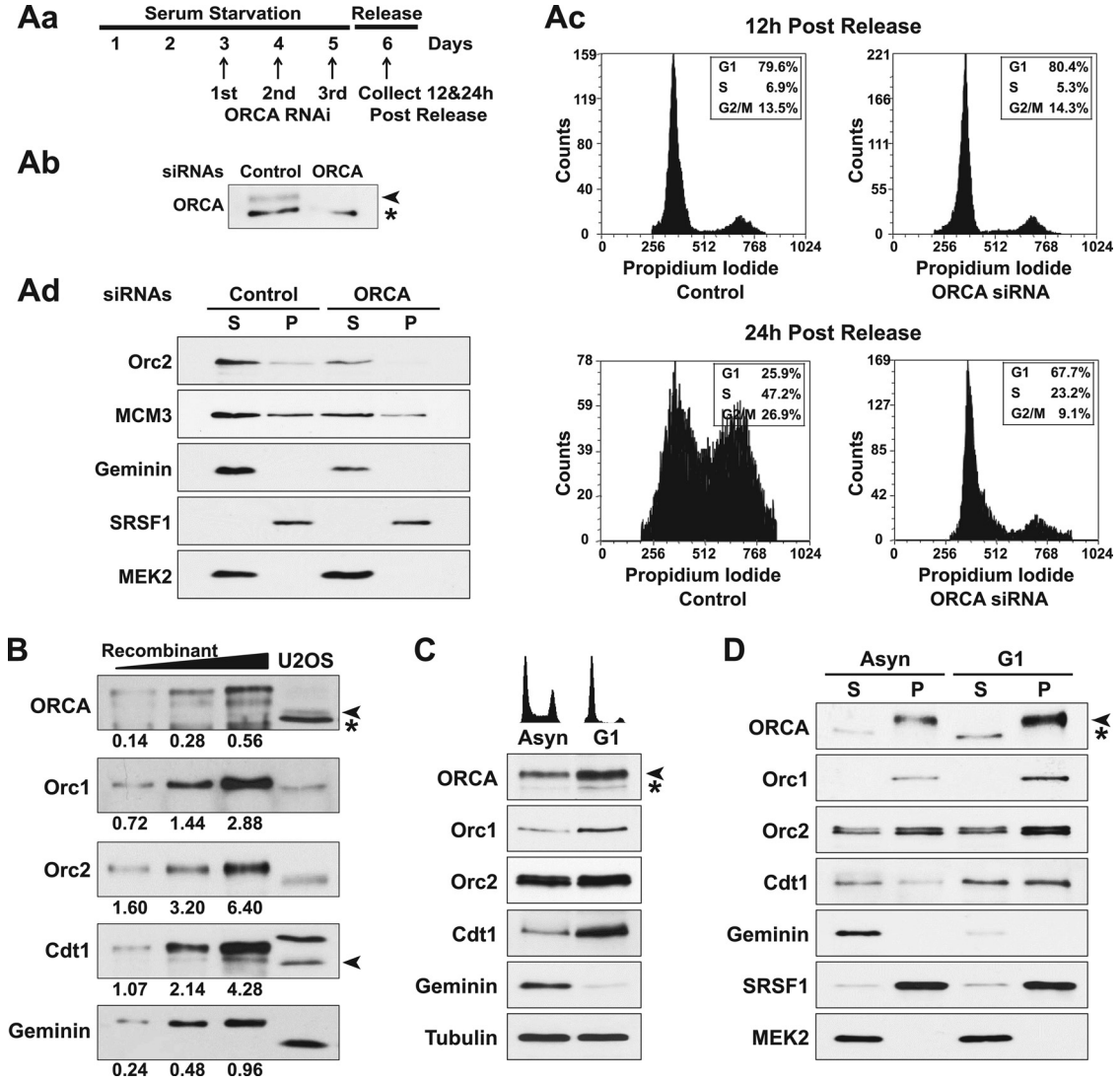


FIG 1 ORCA is required for entry into the cell cycle. (Aa) Scheme of the experiment in WI38 cells. Cells were serum starved for 5 days. ORCA or control siRNA was transfected three times at intervals of 24 h starting day 3. On day 6, the cells were released from arrest for fluorescence-activated cell sorter (FACS) or immunoblot analysis. (Ab) Immunoblot showing efficient knockdown of ORCA. (Ac) FACS analysis at 12 h and 24 h postrelease in control and ORCA siRNA-treated cells. Note the efficient release of cells into the cell cycle in the 24-h FACS profile for control cells but a G_1 arrest in ORCA-depleted cells. (Ad) Chromatin fractionation in control and ORCA siRNA-treated cells and immunoblotting with Orc2, MCM3, and geminin. SRSF1 and MEK2 are shown as loading controls for chromatin (P) and cytosolic (S) fractions, respectively. (B) Relative levels of ORCA, Orc1, Orc2, Cdt1, and geminin in asynchronously growing human U2OS cells. GST-tagged ORCA and His-tagged Orc1, Orc2, Cdt1, and geminin were loaded as indicated (ng) for quantitation. (C) Relative levels of ORCA, Orc1, Orc2, Cdt1, and geminin in the G_1 phase of U2OS cells. Note that ORCA, Orc1, and Cdt1 levels are high during G_1, whereas geminin levels are negligible in G_1. Asyn, asynchronous. (D) Relative abundances of ORCA, Orc1, Orc2, Cdt1, and geminin on chromatin (P) during G_1 in human U2OS cells. The asterisks indicate cross-reacting bands. The arrowheads indicate endogenous ORCA (ORCA immunoblot) and endogenous Cdt1 (Cdt1 immunoblot).

onto chromatin (Fig. 1Ad). Reduced levels of geminin are consistent with cells accumulating in the G_1 phase of the cell cycle. These results suggest that in the absence of ORCA, the transition from G_1 to S phase is affected.

We previously demonstrated that ORCA interacts with ORC, Cdt1, and geminin. In order to test the cellular levels of ORCA protein relative to Orc2, Cdt1, and geminin in human U2OS cells, cell lysates were prepared, and immunoblotting using antibodies against ORCA, ORC, Cdt1, and geminin was conducted. The relative stoichiometry of ORCA and various ORC subunits was normalized using standard curves with purified proteins that were in the linear detection range. Silver staining was used to quantify the purified 6×histidine-tagged recombinant protein that was expressed in SF9 cells and purified on a Talon column or glutathione *S*-transferase (GST) fusion proteins purified from bacterial cells. Based on these calculations, human Orc2 was found to be 10-fold more abundant than ORCA in asynchronously growing human cells (Fig. 1B).

ORCA is highly dynamic throughout the cell cycle, with elevated protein levels during G_1 and subsequent decrease at the G_1/S boundary, and is present predominantly at heterochromatic sites in post-G_1 cells (47). We next examined the relative abundance of ORCA compared to ORC subunits during the G_1 phase of the cell cycle. Cells synchronized at G_1 were used for conducting immu-

noblot analysis to ascertain the levels of ORCA, ORC, Cdt1, and geminin. Consistent with previous studies, ORCA, Orc1, and Cdt1 levels were increased in G_1 compared to Orc2, which was found to be unaltered. Geminin levels were negligible during G_1 (Fig. 1C). To calculate the relative levels of the chromatin-associated pool of ORCA and ORC (asynchronous as well as G_1), we performed chromatin fractionation and quantitated the levels of ORC, ORCA, Cdt1, and geminin (Fig. 1D). Similar to Orc1 and Cdt1, both the total and chromatin-bound levels of ORCA showed increases during the G_1 phase of the cell cycle (around 2-fold increases in G_1 samples compared to asynchronous samples). Orc1 and Orc2 were found to be equimolar on the G_1 chromatin, consistent with a 1:1 ratio of Orc1 to Orc2. Orc1 and Orc2 levels were found to be 3-fold higher than those of ORCA on the G_1 chromatin, suggesting that either multiple ORC proteins associate with ORCA or an ORCA-independent ORC complex exists in G_1 cells. To further address these two possibilities, we evaluated the relative stoichiometry of ORCA bound to ORC, Cdt1, and geminin using single-molecule analysis.

SiMPull analyses of the stoichiometry of ORCA and pre-RC. To further probe the stoichiometry between ORCA and pre-RC components, SiMPull assays were performed (20). SiMPull combines a conventional pulldown assay with single-molecule fluorescence microscopy. Cellular protein complexes are pulled down from cell lysate onto a passivated surface at a low density (0.1 to 0.2 molecules μm^{-2}) and then imaged with single-fluorophore sensitivity. When the proteins are stoichiometrically labeled with fluorophores, such as genetically encoded fluorescent proteins, single-molecule photobleaching analysis can reveal the stoichiometry of the proteins constituting the complex (20, 35). Lysates from cells coexpressing T7-ORCA and YFP-Orc1 (as the representative of ORC) were added to a surface coated with T7 antibody (Fig. 2A). After washing away the unbound proteins, the surfaces were imaged for YFP fluorescence under a single-molecule total internal reflection fluorescence microscope. Figure 2Bb depicts representative single-molecule fluorescence images. The lysate that expressed only YFP-Orc1 served as the control and exhibited 5-fold-reduced binding (Fig. 2Bb and Bc). Photobleaching of individual YFP molecules is a discrete phenomenon, and the number of photobleaching steps provides information about the stoichiometry of the labeled protein (Fig. 2Ab and Ac) (20, 56). Nearly 80% (864 out of 1,075) of pulled-down YFP-Orc1 molecules bleached in a single step (Fig. 2Bd and Be), indicating that each complex has one molecule of Orc1. Similar analysis was performed on lysates expressing T7-ORCA and YFP-Cdt1; ~82% of the YFP-Cdt1 fluorescence spots showed one photobleaching step (Fig. 2C), indicating that each ORCA-Cdt1 complex has one molecule of Cdt1. Next, we examined the stoichiometry of geminin in ORCA-geminin complexes. Biotinylated anti-rabbit IgG secondary antibody was immobilized onto the chamber, followed by ORCA antibody or rabbit IgG (Fig. 2Da). Cell lysates expressing T7-ORCA and YFP-geminin were incubated in the chamber. There was a 5-fold enrichment of YFP spots in the anti-ORCA-coated chamber compared to the control (Fig. 2Db and Dc). Photobleaching analysis demonstrated that 54% of YFP molecules displayed two photobleaching steps, while 43% of the molecules exhibited one-step photobleaching (Fig. 2Dd and De). This is consistent with two geminin molecules per complex, given the ~75% active fraction of YFP (20, 56). Our results demonstrate that each molecule of ORCA can interact with one ORC, one Cdt1, and/or two geminin molecules. A future project would be to use SiMPull technology to study complex assembly and also the stoichiometry of multiple components within the complex. This would entail using multiple fluorescent tags and would require further technical advances.

Cell cycle-dependent association of ORCA with ORC, Cdt1, and geminin. Since ORCA interacts with ORC, Cdt1, and geminin, we examined if they exist in one complex or are present in independent complexes. We fractionated HeLa nuclear extracts on a Superdex-200 gel filtration column and examined the fractions by immunoblotting (Fig. 3A). As reported previously, ORCA and Orc2 cofractionated in the range of >440 kDa (Fig. 3A, fractions 5 and 6), with some present at higher molecular mass (>669 kDa), indicating that ORCA, along with all ORC subunits (predicted mass, 450 kDa), exists in mammalian cells (47). Cdt1 was present in a higher molecular-mass range. In contrast, geminin was enriched only in fractions 4 to 8 in a lower-molecular-mass complex with negligible monomeric form in fractions 12 to 13 (Fig. 3A). These data suggest that distinct complexes may exist in human cells.

Pre-RC components are highly dynamic, with Orc1, Cdc6, and Cdt1 being cell cycle regulated to ensure that replication occurs only once during the cell cycle. The levels of ORCA are elevated during the G_1 phase of the cell cycle and drop as the cells enter S phase. To address the question of whether the existence of ORCA with ORC, Cdt1, and geminin in different subcomplexes reflected its cell cycle-regulated association with each of these components, we performed immunoprecipitation using ORCA antibody during different cell cycle stages (Fig. 3Ba). ORCA associated with Orc2 throughout the cell cycle. ORCA associated with Orc1 predominantly during the G_1 phase of the cell cycle and with less affinity to the phosphorylated form of Orc1 during mitosis. ORCA associated with Cdt1 in G_1 and with geminin from G_1/S to G_2 predominantly and interacted with both Cdt1 and geminin in M phase regardless of the phosphorylation status (Fig. 3Ba). Treatment of extracts with phosphatase demonstrated that Orc1 (data not shown), Cdt1, and geminin (Fig. 3Bb) are phosphorylated during mitosis.

To further evaluate the composition of the ORCA-containing complexes with ORC, Cdt1, and geminin during different stages of the cell cycle, we performed immunoprecipitation of ORCA, followed by peptide elution, and subjected the eluate to 10% to 50% glycerol gradient sedimentation in asynchronous (Fig. 3C) and G_1 (Fig. 3D) extracts and mitotic extracts (Fig. 3E). Immunoblot analysis from asynchronous cells revealed that Orc2 cosedimented with ORCA at a molecular mass of >200 kDa (Fig. 3C). Cdt1, in addition to being a monomer (fraction 9), was also present in the higher molecular-mass range. Geminin was present in fraction 7 in a lower-molecular-mass complex and also in a much larger complex (Fig. 3C, fractions 13 to 15). The glycerol gradient analysis suggests the existence of a large complex composed of ORCA, ORC, Cdt1, and geminin. Immunoprecipitation and sedimentation analysis of G_1 extracts (Fig. 3D) revealed the coexistence of ORCA, Orc2, and Cdt1, but not geminin (in fraction 11). In mitotic extracts, ORCA, Orc2, Cdt1, and geminin were found to cosediment (fraction 10) (Fig. 3E). Similarly, gel filtration analysis of ORCA immunoprecipitates from mitotic extracts further corroborated the existence of the ORCA-ORC-Cdt1-geminin complex (Fig. 3F, fraction 8). To confirm the presence of ORCA-Orc2-Cdt1-geminin during mitosis, we conducted sequential im-

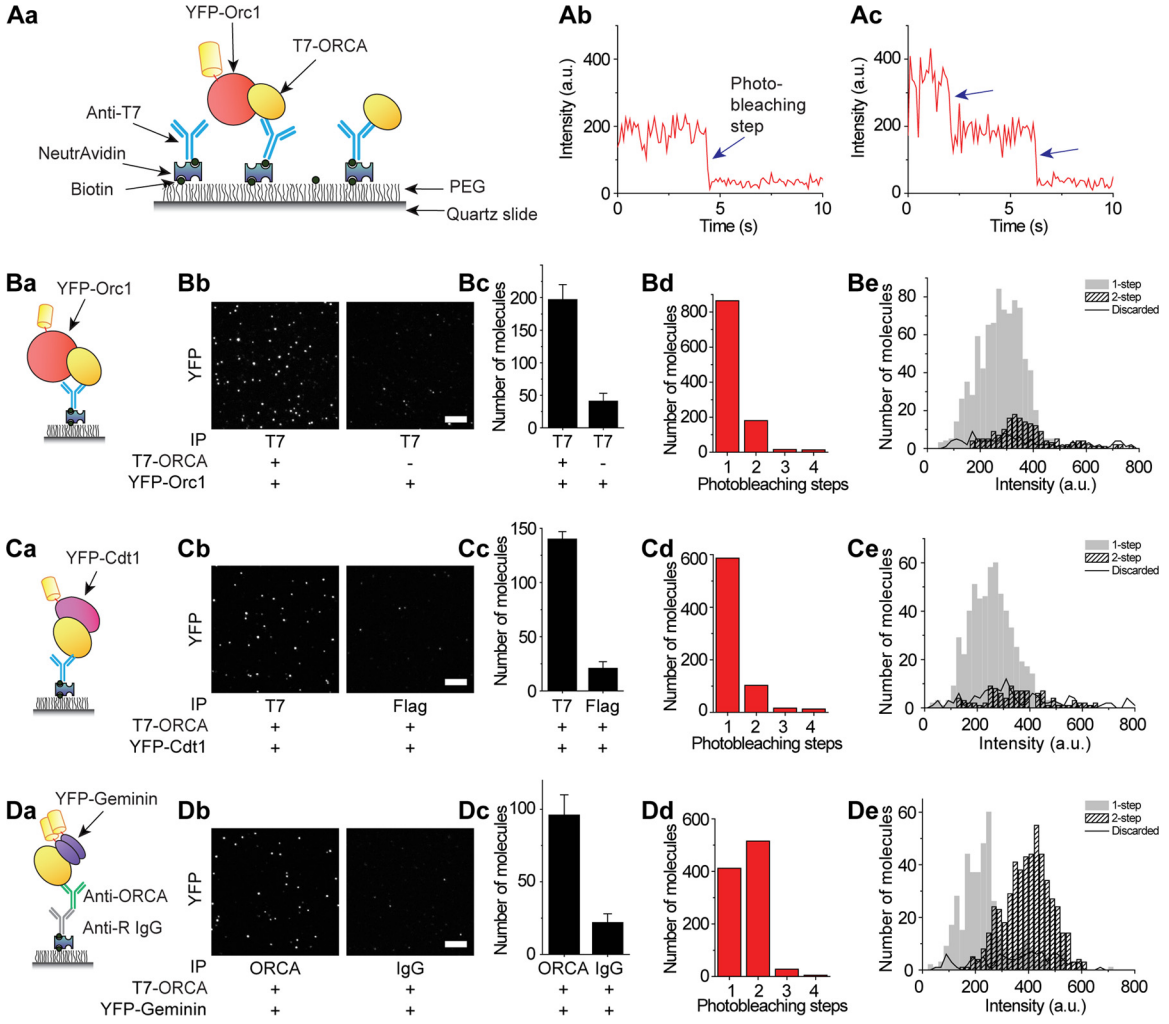
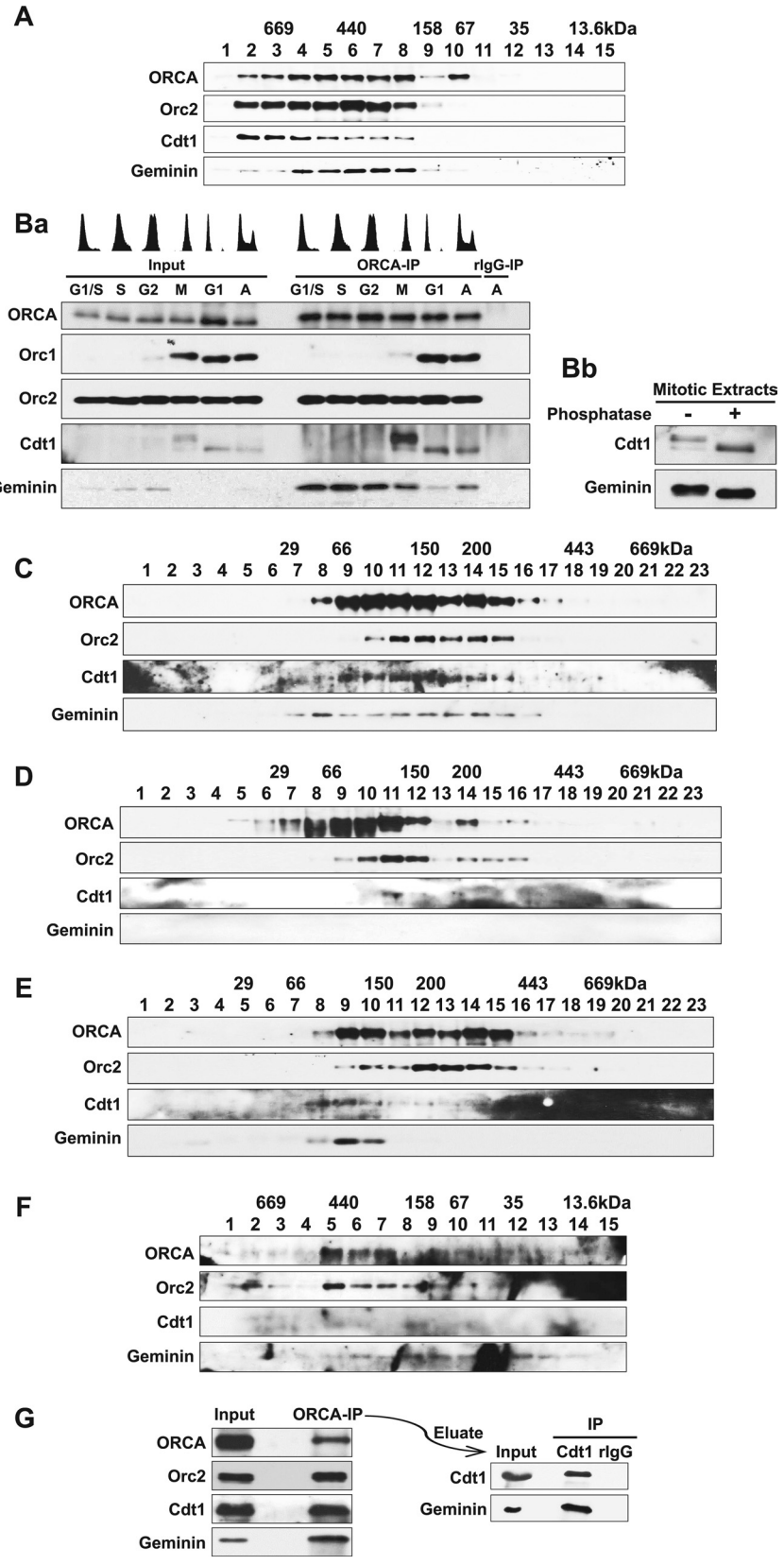


FIG 2 Stoichiometry of ORCA bound to ORC, Cdt1, and geminin using SiMPull analyses. (Aa) Schematic representation of the SiMPull assay. (Ab and Ac) Representative single-molecule fluorescence time trajectories of YFP-geminin molecules that exhibit one-step (Ab) and two-step (Ac) photobleaching. (B) ORCA-Orc1 pulldown. (Ba and Bb) Schematic (Ba) and TIRF (Bb) images of YFP molecules pulled down from U2OS cell lysates expressing T7-ORCA and YFP-Orc1 using biotinylated T7 antibody. The lysate expressing only YFP-Orc1 served as the control. (Bc) Average numbers of YFP fluorescent molecules per imaging area (2,500 μm^2). The error bars indicate standard deviations of the mean values from 20 imaging areas. (Bd) Photobleaching step distribution for YFP-Orc1 bound to T7-ORCA. (Be) Fluorescence intensity distribution of YFP molecules exhibiting 1 and 2 photobleaching steps. Nearly 15% of the molecules could not be unambiguously scored and were discarded; the discarded molecules showed no enrichment of intensity. (Ca to Ce) ORCA-Cdt1 pulldown. Shown are YFP molecules pulled down from U2OS cell lysates expressing T7-ORCA and YFP-Cdt1 using biotinylated T7 antibody. The same lysate incubated with the biotinylated Flag antibody served as the control. (Da to De) ORCA-geminin pulldown. YFP molecules pulled down from U2OS cell lysates expressing T7-ORCA and YFP-geminin using biotinylated anti-rabbit IgG and ORCA antibody were analyzed. The same lysate incubated with the biotinylated anti-rabbit IgG and rabbit IgG served as the control. The fluorescence intensity of molecules bleaching in two steps was nearly twice that of molecules bleaching in a single step.

munoprecipitation. ORCA immunoprecipitates were peptide eluted, and the eluate was subjected to Cdt1 immunoprecipitation. The presence of geminin was evaluated in this assay. Sequential immunoprecipitation clearly revealed the existence of an ORCA complex consisting of Orc2, Cdt1, and geminin (Fig. 3G). Our results suggest the existence of an ORCA-ORC-Cdt1 complex during G_1 and an ORCA-ORC-Cdt1-geminin complex during mitosis.

ORCA associates with Orc2 directly. ORCA was originally identified in an Orc2 immunoprecipitation-mass spectrometry screen (47). ORCA was also found to coimmunoprecipitate with all ORC subunits, Cdt1, and geminin (47). However, whether ORCA displays direct interaction with any of these components remained to be elucidated. To address this possibility, we generated baculoviruses expressing each of the ORC subunits, ORCA, Cdt1, and geminin, for expression in insect cells. Insect cells were either infected with baculoviruses expressing ORCA and individual subunits of ORC/Cdt1/geminin or with a combination of different viruses, each at a multiplicity of infection of 5 to 10, and incubated at 28°C for 60 to 72 h. Whole-cell extracts or nuclear extracts were prepared (see Materials and Methods), and immunoprecipitation was carried out using the ORCA antibody (Fig. 4A). Orc2 bound ORCA directly in whole-cell extracts (Fig. 4Ab), as well as nuclear extracts (data not shown) in this assay. Immunoprecipitation using Orc2 antibody on purified Orc2 and ORCA proteins further confirmed direct interaction between Orc2 and ORCA (Fig. 4B). Orc1 and Orc3 showed weak interactions with ORCA only when nuclear extracts were used (data not shown).


A
669 440 158 67 35 13.6kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
ORCA
Orc2
Cdt1
Geminin
Ba
Input
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G1/S S G2 M G1 A G1/S S G2 M G1 A A
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Several studies have successfully reconstituted the ORC complex *in vitro* using the baculovirus expression system (10, 48, 57). We examined whether ORCA could be efficiently incorporated into the ORC complex *in vitro*. Coinfection of viruses expressing ORCA and ORC subunits followed by immunoprecipitation of Orc2 (Fig. 4C) or ORCA (Fig. 4D) clearly demonstrated that ORCA could be efficiently incorporated into the ORC complex, suggesting that ORCA is an integral component of this multiprotein complex.

To address the functional relevance of ORCA binding to Cdt1 and geminin, we first addressed *in vitro* binding of ORCA to Cdt1 and geminin. Using binary infections in insect cells, we demonstrate that ORCA can associate with Cdt1 (Fig. 4E), as well as geminin (Fig. 4F), *in vitro*.

ORCA utilizes its WD domain to bind to ORC, Cdt1, and geminin. We previously demonstrated that the WD domain of ORCA is required for chromatin association of ORCA and for binding of ORCA to ORC (47). To map the region of ORCA that associates with Cdt1 and geminin, we tagged different domains of ORCA expressing *l*eucine-*r*ich *r*epeat (LRR) (amino acids [aa] 1 to 127), LRR plus linker (aa 1 to 270), WD alone (aa 270 to 647), and WD plus linker (aa 128 to 647) (Fig. 4G) and performed coimmunoprecipitation experiments to determine which domains were sufficient to interact with Cdt1 and geminin. Immunoprecipitation was carried out using T7 antibody, and the interaction of ORCA with Cdt1 and geminin was assessed by immunoblot analysis. Like Orc2 (47), Cdt1, as well as geminin, interacted with the WD domain of ORCA (Fig. 4H and I). Based on bioinformatics predictions, ORCA has 5 discernible WD domains (*S*imple *M*odular *A*rchitecture *R*esearch *T*ool [SMART]). Deletion of even a single WD domain resulted in complete loss of interaction between ORCA and ORC, Cdt1, and geminin (data not shown). These results demonstrate that the entire WD domain of ORCA is critical for its interaction with pre-RC components, as well as for its association with chromatin.

Orc2 is required for stabilization of ORCA. In order to map the region of Orc2 that is critical for binding to ORCA, we generated several truncation mutants of Orc2 with a T7 tag (Fig. 5A). Each of these mutants was transfected into human cells, and immunoprecipitations were conducted using T7 antibody and immunoblotted for ORCA and Orc3 to address which domains of Orc2 are sufficient for interaction with ORCA and Orc3. Our results demonstrate that the N-terminal fragment of Orc2 (aa 1 to 240) binds to ORCA efficiently, whereas the Orc2 fragment (aa 227 to 451) is sufficient to associate with Orc3 (Fig. 5A and B). Further fine mapping of the N-terminal fragment demonstrated that the first 100 aa of Orc2 are sufficient to bind to ORCA (Fig. 5C). We previously demonstrated that ORCA is required for ORC binding to chromatin. We now addressed the impact of Orc2 depletion on ORCA stability and observed that the human cancerous U2OS cells (Fig. 5D), as well as primary diploid fibroblasts (Fig. 5E) lacking Orc2, showed destabilization of cellular ORCA levels, similar to what has been reported for Orc3 (42). No obvious changes were observed for Orc1 levels in cells lacking Orc2. Similarly, depletion of Orc1 did not affect the cellular levels of Orc2 or ORCA (Fig. 5D and E). To address whether expression of the N-terminal half (aa 1 to 240) of Orc2, which showed robust binding to ORCA, can rescue the ORCA destabilization in Orc2-depleted cells, we utilized 2 different siRNAs against Orc2, so that only the endogenous Orc2 was depleted without affecting the exogenously expressed Orc2 truncation mutants (oligonucleotide 2.1 in cells expressing MT3, aa 227 to 577, and oligonucleotide 2.6 in cells expressing MT1, aa 1 to 240) (Fig. 5F). Rescue assays revealed that only the MT1 domain, but not MT3, rescued ORCA degradation in the absence of endogenous Orc2. These results clearly demonstrated that Orc2 is required for the stability of ORCA in mammalian cells, suggesting that ORCA is always in a complex with Orc2.

ORCA binds to distinct domains of geminin and Cdt1. ORCA associates with geminin during post-G_1 and with Cdt1 predominantly in the G_1 phase of the cell cycle. During mitosis, ORCA, along with ORC, associates with both geminin and Cdt1. To address whether a quaternary complex consisting of ORCA-Cdt1-geminin exists in cells during defined periods of the cell cycle, we mapped the interaction sites between ORCA-Cdt1 and ORCA-geminin. Several geminin mutants with a T7 tag (Fig. 6A) were transfected into human cells, and T7 immunoprecipitations, followed by immunoblot analysis with Cdt1, were carried out. Our results are consistent with previously published observations that showed that the central region of geminin is responsible for the Cdt1-geminin association (3, 46). We show that the aa 80 to 160 fragment of geminin (G3) is sufficient to associate with Cdt1 (Fig. 6B). In contrast, ORCA immunoprecipitations from the cell lysates expressing T7-tagged geminin mutants showed that the C terminus of geminin is required for ORCA binding (Fig. 6C). The absence of ORCA association with the fragment aa 80 to 160 of geminin suggested that the binding site of ORCA within geminin is between aa 160 and 209. We made smaller truncation mutations spanning aa 92 to 140, 92 to 160, and 161 to 209, but all these mutants failed to associate with ORCA, possibly due to loss of conformation of the mutant proteins. Based on these mapping analyses, we propose that ORCA binds to the C-terminal end of geminin and Cdt1 interacts with the central region of geminin (Fig. 6B and C), consistent with the possibility that ORCA, Cdt1,

FIG 3 ORCA association with ORC, Cdt1, and geminin is cell cycle regulated. (A) Association of ORCA with ORC, Cdt1, and geminin in human cells. Shown are HeLa nuclear extract fractionated over a Superdex 200 gel filtration column and fractions analyzed for ORCA, Orc2, Cdt1, and geminin by immunoblotting. Molecular mass markers are labeled above the blot. (Ba) ORCA immunoprecipitation during different stages of the cell cycle. ORCA associates with Orc2 throughout the cell cycle. ORCA associates with Orc1 during G_1 but not efficiently with the phosphorylated Orc1 during mitosis. ORCA associates with Cdt1 during G_1, as well as robustly with phosphorylated Cdt1 during mitosis. ORCA associates strongly with geminin from G_1/S to mitosis. (Bb) Immunoblot analysis of whole-cell extract from nocodazole-arrested mitotic extracts treated with phosphatase and untreated. Note that the phosphorylated form of Cdt1 and geminin collapses on phosphatase treatment. (C) Glycerol gradient sedimentation analysis of ORCA complex on material (asynchronous sample) immunoprecipitated using ORCA antibodies. The corresponding molecular mass markers are labeled. Note that ORCA and Orc2 cosediment in fractions 11 to 15, ORCA and Cdt1 cosediment in fractions 11 to 14, and ORCA and geminin cosediment in fractions 8 and 13 to 15. Fractions 13 and 14 contain ORCA, Orc2, Cdt1, and geminin, suggesting the existence of a quaternary complex, as well. (D and E) Glycerol gradient sedimentation analysis of ORCA-containing complex from G_1 extracts (D) and mitotic extracts (E). Note the cosedimentation of ORCA-Orc2-Cdt1 (fraction 11) in G_1 extracts and ORCA-Orc2-Cdt1-geminin (fractions 9 and 10) in mitotic extracts. (F) Gel filtration analysis of ORCA-containing complex during mitosis. (G) Sequential immunoprecipitation of ORCA, followed by Cdt1 from mitotic extracts. Immunoblot analysis of geminin corroborated the presence of ORCA-Orc2-Cdt1-geminin complex in mitotic extracts.

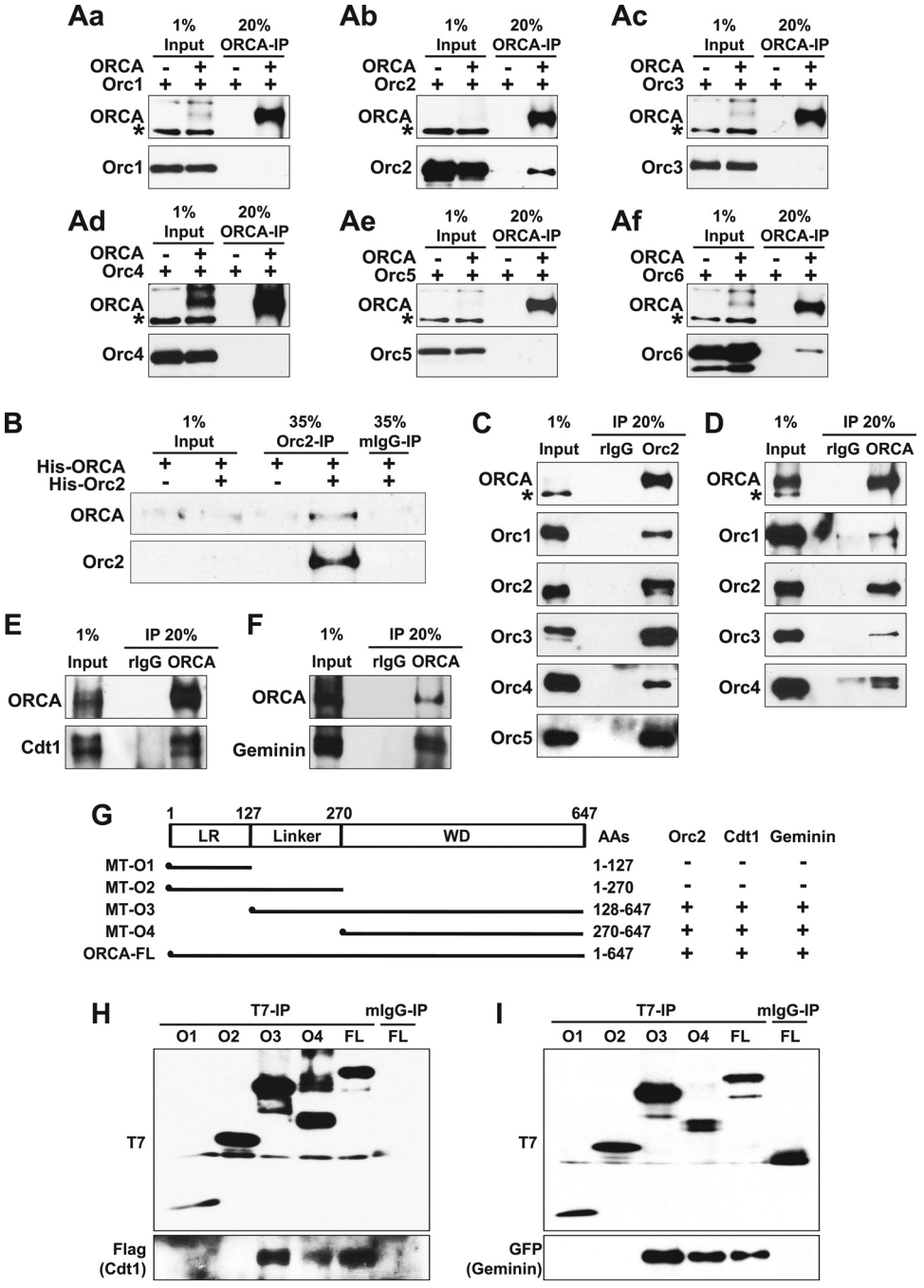

FIG 4 ORCA associates with Orc2 directly and utilizes WD to associate with ORC, Cdt1, and geminin. (Aa to Af) Binary infections using ORCA baculovirus in combination with Orc1 (Aa), Orc2 (Ab), Orc3 (Ac), Orc4 (Ad), Orc5 (Ae), and Orc6 (Af) in insect cells, followed by immunoprecipitation using ORCA antibody from whole-cell extracts. Note the prominent interaction between Orc2 and ORCA. (B) Direct interaction between ORCA and Orc2. His-ORCA and His-Orc2 were purified on Talon columns, and the two proteins were incubated; then, immunoprecipitation was carried out using Orc2 antibody. (C and D) Reconstitution of the ORCA-ORC complex. Coinfection by baculovirus carrying ORCA plus Orc1, Orc2, Orc3, Orc4, or Orc5 in insect cells and immunoprecipitation of whole-cell extracts with Orc2 (C) or ORCA (D) antibody. (E and F) Binary infection using ORCA baculovirus in combination with Cdt1 and geminin in insect cells, followed by immunoprecipitation using ORCA antibody from nuclear extracts. (G) Schematic representation of various truncation mutants containing a T7 epitope tag at the N terminus of ORCA. (H) IP in U2OS cells expressing various T7-ORCA mutants and Flag-Cdt1 using T7 antibody and analysis of Cdt1 by immunoblotting. (I) IP in U2OS cells expressing various T7-ORCA mutants and YFP-geminin using T7 antibody and analysis of geminin by GFP immunoblotting. Note that only ORCA constructs possessing the WD40 domain efficiently interact with Orc2 (47), Cdt1, and geminin (T7-ORCA.128-647, T7-ORCA.270-647, and T7-ORCA.1-647). The asterisks indicate cross-reacting bands.

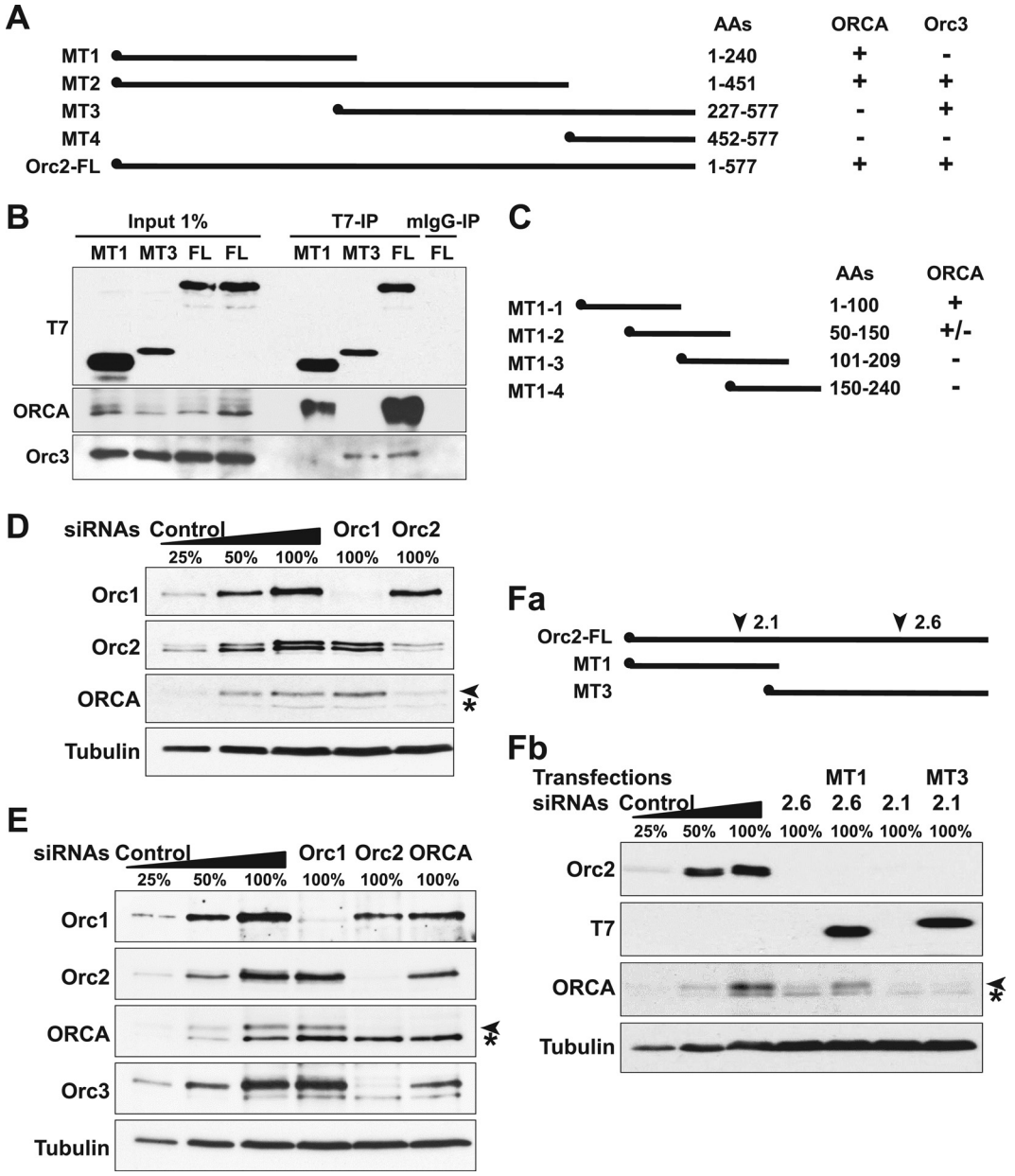

FIG 5 ORCA requires Orc2 for its stability. (A) Schematic representation of various truncation mutants containing a T7 epitope tag at the N terminus of Orc2 (MT1 to MT4). (B) IP using T7 antibody in U2OS cells expressing various mutants of T7-Orc2 and analysis of ORCA and Orc3 by immunoblotting. Note that the aa 1 to 240 fragment (MT1) associates with ORCA and that aa 227 to 577 (MT3), but not aa 452 to 577, binds to Orc3. (C) Schematic representation of smaller truncation mutants of Orc2 within the N-terminal 240 aa (MT1-1 to MT1-4) and their interaction status with ORCA. Amino acids 1 to 100 of Orc2 efficiently show ORCA binding. (D and E) Depletion of Orc2, but not Orc1, results in destabilization of cellular ORCA in human cancerous U2OS and WI38 cells. (Fa and Fb) Transient expression of MT1 (aa 1 to 240) or MT3 (aa 227 to 577) in U2OS cells depleted of endogenous Orc2 and immunoblot analysis using ORCA antibody. Note that the expression of MT1 can rescue the destabilization of ORCA. The asterisks indicate cross-reacting bands. The arrowheads indicate endogenous ORCA.

and geminin coexist simultaneously in one complex. It remains to be determined if the phosphorylation of geminin and Cdt1 facilitates the quaternary complex with ORCA and ORC.

To address whether ORCA can associate with Cdt1 and geminin in a single complex, we further mapped the interaction domain of Cdt1 to ORCA and geminin. T7-tagged Cdt1 mutants (Fig. 6D) were transfected into human cells, and immunoprecipitation was carried out using the T7 antibody. The C-terminal fragment of Cdt1, aa 368 to 546 (MT C5), could interact with ORCA, but not with geminin. However, aa 1 to 368 interacted with ORCA, as well as geminin, suggesting that ORCA can bind to multiple domains within Cdt1 (Fig. 6E and F). While there is at least one distinct domain within Cdt1 that can associate with ORCA but not with geminin, it supports the existence of a quaternary complex within human cells. It has previously been demonstrated that the C-terminal end, con-

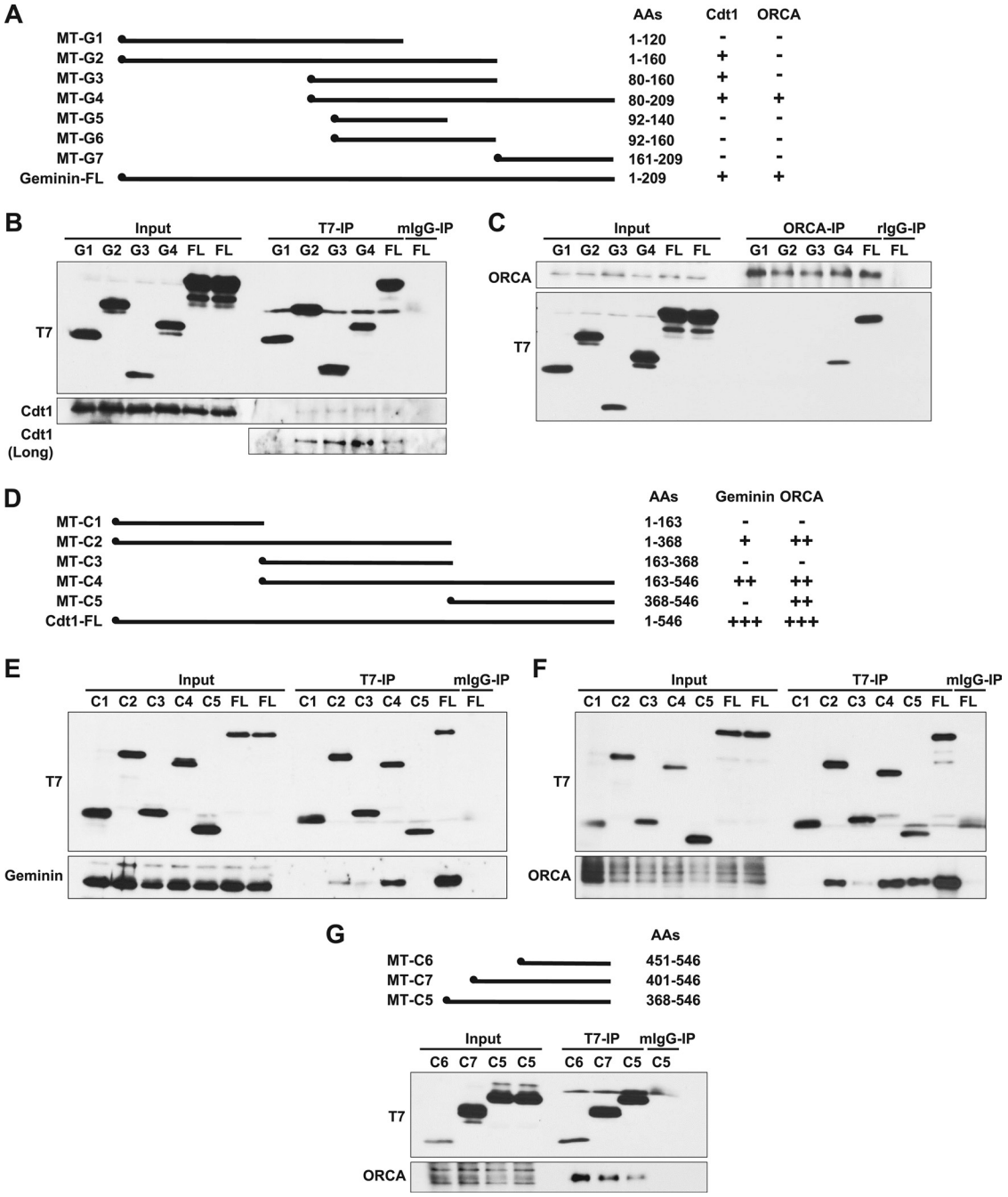

FIG 6 ORCA interacts with the C terminus of geminin and Cdt1. (A) Schematic representation of various truncation mutants containing a T7 epitope tag at the N terminus of geminin (MT-G1 to MT-G7). (B) IP in U2OS cells expressing various T7-geminin mutants using T7 antibody and analysis of Cdt1 by immunoblotting. Note that aa 80 to 160 of geminin is sufficient to bind Cdt1. (C) IP in U2OS cells expressing various T7-geminin mutants using ORCA antibody and analysis of T7-geminin by immunoblotting. Note that ORCA associates with the C-terminal end of geminin. (D) Schematic representation of various truncation mutants containing a T7 epitope tag at the N terminus of Cdt1 (MT-C1 to MT-C5). The plusses indicate the extent of interaction based on the immunoblots shown in panels E and F. (E) IP in U2OS cells expressing various T7-Cdt1 mutants using T7 antibody and analysis of geminin by immunoblotting. (F) IP in U2OS cells expressing various T7-Cdt1 mutants and hemagglutinin (HA)-ORCA using T7 antibody and analysis of ORCA by immunoblotting. Note that the aa 368 to 546 fragment of Cdt1 associates with ORCA but not with geminin. (G) IP in U2OS cells expressing T7-tagged C terminus mutants of Cdt1 and HA-ORCA using T7 antibody and analysis of ORCA by immunoblotting. Note that the aa 451 to 546 fragment of Cdt1 efficiently associates with ORCA.

sisting of the last 377 residues of *Xenopus* Cdt1, is essential for licensing and the extreme C terminus is responsible for MCM binding (14). Similarly, the C-terminal aa 392 to 471 of human Cdt1 have been shown to interact with MCM6 (64). To fine map the C-terminal region of Cdt1 that associates with ORCA, we generated C-terminal mutants, including aa 451 to 546, 401 to 546, and 368 to 546. The fragment aa 451 to 546 of Cdt1 showed robust interaction with ORCA (Fig. 6G).

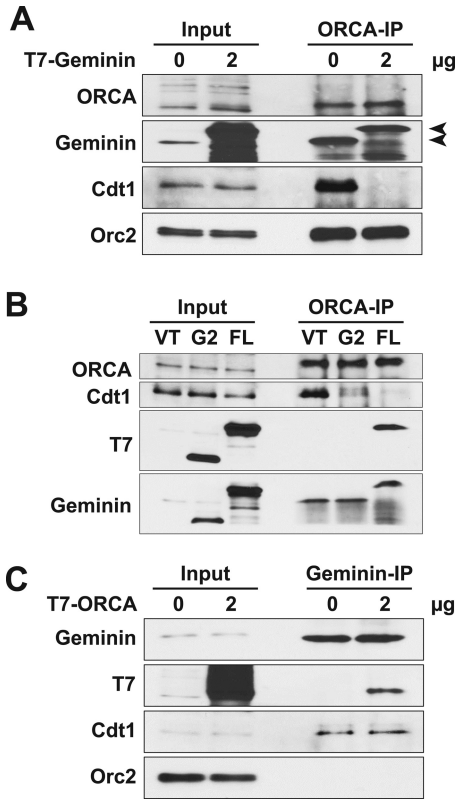

FIG 7 Overexpression of geminin disrupts ORCA-Cdt1 interaction. (A) ORCA immunoprecipitation in U2OS cells transiently overexpressing T7-geminin, followed by ORCA, geminin, Orc2, and Cdt1 immunoblots. Note the loss of ORCA-Cdt1, but not ORCA-Orc2, interaction in cells overexpressing geminin. The arrowheads indicate endogenous and overexpressed geminin. (B) ORCA immunoprecipitation in U2OS cells transiently overexpressing T7 vector (VT), T7-geminin (full-length [FL]), or T7-geminin aa 1 to 160 (G2, which associates only with Cdt1, but not ORCA), followed by geminin, ORCA, Cdt1, and T7 immunoblots. Note that overexpression of full-length geminin, as well as truncated geminin, disrupts ORCA-Cdt1 binding. (C) geminin immunoprecipitation in U2OS cells transiently overexpressing T7-ORCA, followed by geminin, T7, Cdt1, and Orc2 immunoblotting. Note that the overexpression of ORCA does not affect Cdt1-geminin complex formation.

Overexpression of geminin results in the loss of ORCA-Cdt1 interaction. To gain insight into the functional significance of ORCA binding to Cdt1 and geminin, we addressed how changing the expression of geminin affects the interaction between ORCA and Cdt1. In mammalian cells, geminin levels begin to increase at the G_1/S boundary, coincident with a decrease in the levels of ORCA and Cdt1. We therefore asked if the balance between these components is critical for entry into S phase and to prevent relicensing. geminin was overexpressed in human cells, and immunoprecipitation using ORCA antibody was performed. In untransfected cells, ORCA interacted efficiently with endogenous geminin, as well as Cdt1 (Fig. 7A). In cells transfected with 2 μg of geminin, ORCA interacted with endogenous geminin, as well as T7-geminin. Interestingly, in the presence of overexpressed geminin, ORCA failed to show any interaction with Cdt1 (Fig. 7A), suggesting that excess quantities of geminin titrated all the Cdt1 away from ORCA. Alternatively, all of the ORCA was bound to geminin and, as a result, no free ORCA molecules were left behind to interact with Cdt1. To address each of these possibilities, we overexpressed a T7-geminin mutant (aa 1 to 160) that can associate with Cdt1, but not with ORCA. Immunoprecipitation with ORCA in these cells showed that even though ORCA cannot associate with the overexpressed geminin, the binding of ORCA to Cdt1 is lost (Fig. 7B). Overexpression of ORCA did not perturb the Cdt1 binding to geminin (Fig. 7C), indicating that geminin has a higher affinity for Cdt1 than for ORCA. The above-mentioned results indicated that the excess geminin inhibited the ORCA-Cdt1 interaction. It is paradoxical to the fact that during mitosis a quaternary complex consisting of ORCA-ORC-geminin-Cdt1 exists in cells. One possibility could be that the various cell cycle-specific posttranslational modifications on each of these components might influence the complex assembly. For instance, the hyperphosphorylation of Cdt1, as well as geminin, during mitosis may facilitate complex assembly. We propose that ORCA binding to Cdt1 and geminin at the end of G_1 adds another layer of complexity in regulating licensing and preventing rereplication.

DISCUSSION

DNA replication is a highly coordinated process, and its intricate regulation is crucial for maintaining genomic integrity. The involvement of ORCA in DNA replication and chromatin organization is beginning to be appreciated. We demonstrate that ORCA levels peak during the G_1 phase of the cell cycle, with ORC being severalfold in excess in asynchronous, as well as in G_1 phase cells. However, SiMPull analyses demonstrate that ORCA and ORC are stoichiometric and that a single ORCA molecule could associate with a single ORC molecule or one Cdt1 and two geminin molecules. The excess of ORC over ORCA in cells suggests that ORCA-independent ORC complexes exist in cells. It remains to be determined whether the ORCA bound to ORC defines specific origins. Whether ORCA recruits ORC to a specific set of origins, for example, late origins that are enriched in heterochromatic regions, or facilitates loading of ORC at all origins remains to be elucidated. It has previously been demonstrated that in yeast, origin-bound ORC, along with Cdc6, recruits two Cdt1 molecules, which in turn initiates the loading of a double hexamer of the MCM2-7 helicase (51). One possibility is that the ORCA-independent ORC associates with multiple Cdt1 molecules. It is also possible that the scenario in human cells is different from that in yeast, since there is no evidence as yet for an ORCA ortholog in yeast.

ORCA associates with Orc2 directly, and Orc2, in turn, is required for the stability of cellular ORCA. This suggests that ORCA is always in a complex with Orc2 in human cells. Our results demonstrate the existence of an ORCA-ORC-Cdt1 complex during G_1, an ORCA-ORC(2-5)-geminin complex in S phase, and an ORCA-ORC(2-5)-phosphorylated Cdt1-phosphorylated geminin in mitotic cells (Fig. 8). The phosphorylated Orc1 present during mitosis fails to associate with ORCA efficiently, suggesting that the binding of Orc1 to ORCA-ORC(2-5) is an obligatory step to establish the pre-RC. It is interesting that the phosphorylated Cdt1 binds to ORCA very efficiently during mitosis.

Replication licensing is a key mechanism that ensures that DNA replication occurs once and only once in each cell cycle. Loss of licensing control is therefore deleterious to a cell and results in rereplication and genomic instability (2). Cells regulate this by modulating the levels and cellular distribution of prereplicative complex proteins, including Cdt1 and Cdc6, in a cell cycle-dependent manner. Cdt1 interacts with MCM2-7 DNA helicase complex and loads the complex onto chromatin (13, 38, 44). At the

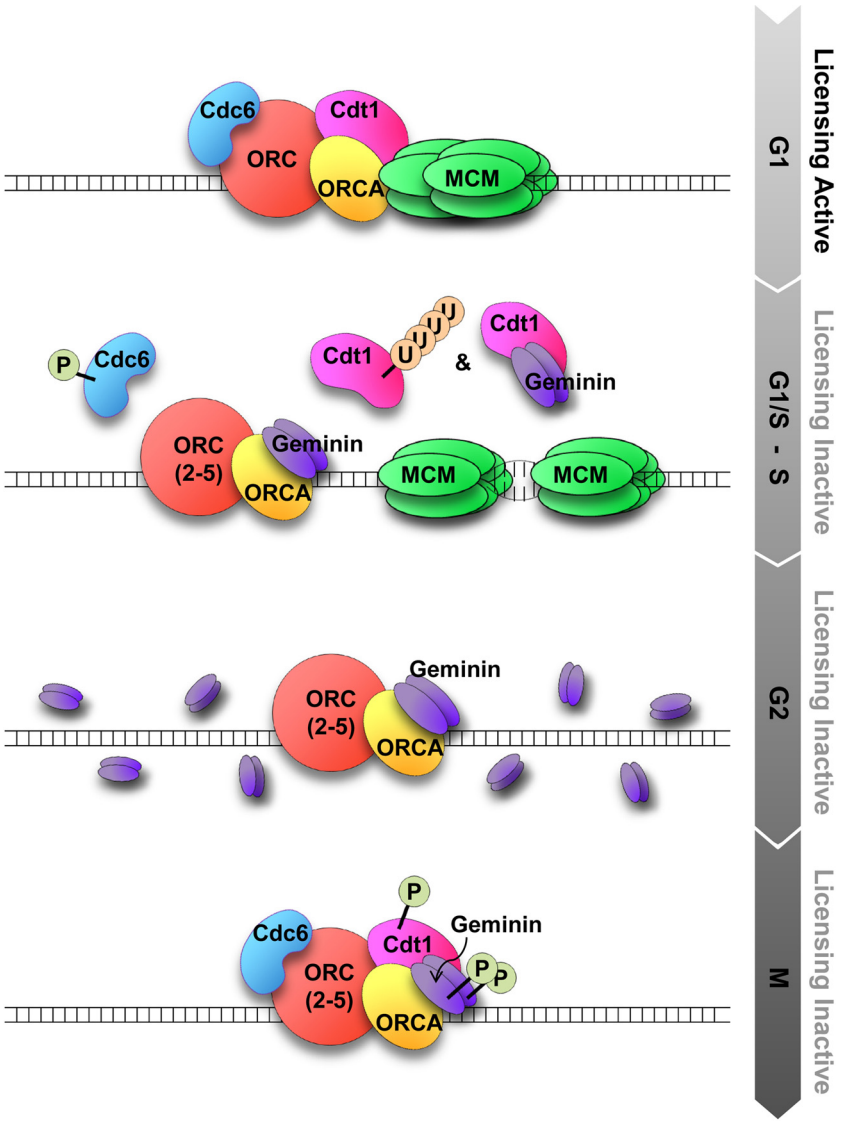

FIG 8 Model depicting ORCA association with the pre-RC components in a cell cycle-regulated manner. In late M/G_1, ORCA associates with ORC and Cdt1. Along with Cdc6, pre-RCs are assembled at origins. ORCA is abundant in the G_1 phase. One prediction would be that ORCA acts as a scaffold and facilitates the assembly of Cdt1 during M and G_1 and that of geminin at the end of G_1 on the chromatin. At the end of G_1, geminin levels begin to rise. The balance in the Cdt1-geminin ratio at this time point favors replication licensing. At the G_1/S boundary, as geminin levels begin to increase significantly and ORCA levels begin to decrease, ORCA-Cdt1 interaction is lost and geminin titrates all the Cdt1 away from ORCA, and hence from the origins. This makes the complex licensing inactive, following which Cdt1 is ubiquitinated and degraded. During G_2, the ORCA-ORC(2-5)-geminin complex prevents licensing. During mitosis, an ORCA-ORC(2-5)-phosphorylated Cdt1-phosphorylated geminin complex exists, and at the end of mitosis, geminin is degraded and Orc1 is dephosphorylated, and thus, a functional pre-RC is assembled.

beginning of S phase, CDK and DDK activate these replication origins (11, 18, 52). Upon initiation of DNA replication, Cdc6 and Cdt1 are released from origins and degraded by proteasomes and/or exported from the nucleus (1, 2, 7, 55). In higher eukaryotes, geminin inhibits Cdt1, thereby preventing the association of MCM2-7 with Cdt1 or preventing the loading of MCMs during the S and G_2 phases of the cell cycle (26, 33, 50, 61). Recent studies suggest that geminin and Cdt1 form a complex that is competent for licensing and that in turn is competent for loading MCM2-7 on chromatin (30). How the Cdt1-geminin complex permits licensing as well as preventing licensing remains to be elucidated. It has been suggested that the stoichiometry of the Cdt1-geminin complex plays a key role in determining these activities (9, 30). Accordingly, it has been suggested that the heterotrimer, where Cdt1 has exposed surfaces that enable loading of MCM, represents a "licensing-permissive" state even in the presence of geminin. The inhibitory complex is heterohexameric, possibly due to an increased local concentration of Cdt1 and geminin (9). While geminin can associate with Cdt1 at a different stoichiometry and dictate the ability to license or prevent it, how this stoichiometry is achieved at the end of the G_1 phase remains to be determined. In *Xenopus* egg extracts, geminin has been shown to associate with chromatin in a Cdt1-dependent manner at the G_1/S boundary when the pre-IC is formed (33). MCM9 has recently

been shown to bind to Cdt1 and to modulate the Cdt1-geminin ratio to enable licensing (32). ORCA associates with Cdt1 primarily during the G_1 phase of the cell cycle, whereas it binds to geminin in post-G_1 cells. Overexpression of geminin results in the loss of ORCA association with Cdt1. However, overexpression of a geminin mutant that can associate with Cdt1 but not ORCA still inhibits the binding of ORCA to Cdt1. These results suggest that at the G_1/S boundary, increased binding of geminin to Cdt1 could decrease the affinity of ORCA for Cdt1. The loss of interaction between ORCA and Cdt1 might be a key step in disassembling the pre-RC at the end of the G_1 phase. In late G_1, as the level of ORCA is reduced and the geminin level begins to rise, more geminin is available to bind to Cdt1, which in turn prevents licensing. A quaternary complex consisting of ORCA-ORC-Cdt1-geminin exists in mitotic cells, where both geminin and Cdt1 are heavily phosphorylated. It is a possibility that the phosphorylation of Cdt1 and geminin (during mitosis) facilitates this complex assembly, whereas the dephosphorylated geminin (at the G_1/S boundary) inhibits ORCA-Cdt1 association. This will be tested in future experiments.

The presence of WD repeats on ORCA, which are required for chromatin association, as well as the requirement for the WD to enable the association with ORC, Cdt1, and geminin, suggests that ORCA is a key chromatin-associated protein that can mediate the assembly with chromatin of proteins that in themselves do not have the ability to associate with chromatin or DNA. Mouse Cdt1 has been reported to be capable of binding DNA in a sequence-, strand-, and conformation-independent manner. This DNA binding domain of Cdt1 overlaps with the geminin binding domain, suggesting that the association of Cdt1 with DNA was inhibited by the presence of geminin (63). However, recent reports have demonstrated that Cdt1 can simultaneously associate with geminin and chromatin *in vivo*, and this in turn facilitates the recruitment of geminin to chromatin (62).

We demonstrate that the C terminus of Cdt1 that has been previously shown to bind MCM2-7 also harbors the ORCA binding domain. Though the C terminus of Cdt1 binds to MCM2-7, the essential N terminus of Cdt1 has been shown to be critical for loading MCM2-7, along with Cdc45 and GINS, which stimulate helicase activity (51). The recent solution structure of the C-terminal domain of Cdt1 (aa 450 to 557 and aa 420 to 557 of mouse Cdt1) by X-ray crystallography and nuclear magnetic resonance (NMR) revealed the presence of a winged-helix domain that could possibly interact with MCM (23, 24). Similarly, NMR studies have revealed the interaction between human Cdt1 (aa 410 to 440) and MCM6 (aa 708 to 821) (29). It is possible that the binding of ORCA to Cdt1 facilitates the loading of MCM2-7, similar to the chromatin remodeler SNF2H, which has been proposed to promote MCM loading via its interaction with Cdt1 (49). Alternatively, ORCA binding to the C terminus of Cdt1 inhibits the interaction of Cdt1 and MCM in G_1 cells. Only when geminin levels begin to rise does the binding of geminin to Cdt1 (resulting in the loss of ORCA-Cdt1 interaction) permit the efficient association of MCM with the exposed domain of Cdt1. This model is supported by previous data showing that the Cdt1-geminin complex and not free Cdt1 is the MCM2-7 loader (30). This active complex is inactivated by the addition of more geminin after origin firing. Our results demonstrate that the binding of ORCA to Cdt1 and geminin is yet another way that ORCA functions in replication initiation and cell cycle entry.

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