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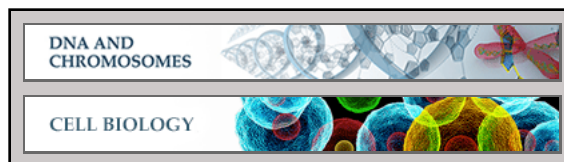
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The Involvement of Acidic Nucleoplasmic DNA-binding Protein (And-1) in the Regulation of Prereplicative Complex (pre-RC) Assembly in Human Cells*

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Background: The assembly of a replicative helicase MCM2–7 onto replication origins is essential for initiation of DNA replication.
Results: And-1 facilitates assembly of MCM2–7 onto replication origins.
Conclusion: And-1 is proposed to be a critical regulator of prereplicative complex assembly in human cells.
Significance: These studies reveal a novel role for human And-1 in the licensing of replication origins.

DNA replication in all eukaryotes starts with the process of loading the replicative helicase MCM2–7 onto chromatin during late mitosis of the cell cycle. MCM2–7 is a key component of the prereplicative complex (pre-RC), which is loaded onto chromatin by the concerted action of origin recognition complex, Cdc6, and Cdt1. Here, we demonstrate that And-1 is assembled onto chromatin in late mitosis and early G_1 phase before the assembly of pre-RC in human cells. And-1 forms complexes with MCM2–7 to facilitate the assembly of MCM2–7 onto chromatin at replication origins in late mitosis and G_1 phase. We also present data to show that depletion of And-1 significantly reduces the interaction between Cdt1 and MCM7 in G_1 phase cells. Thus, human And-1 facilitates loading of the MCM2–7 helicase onto chromatin during the assembly of pre-RC.

DNA replication is regulated by sequential, interactive mechanisms involving many types of proteins acting in concert to ensure that the genome is accurately replicated only once per cell cycle. Initiation of DNA replication in all eukaryotes occurs at replication origins via a process requiring the assembly of a prereplicative complex (pre-RC)[3] in late mitosis and G_1 phase

of cell cycle (1, 2). Pre-RC assembly is a cell cycle-regulated process that starts with the assembly of a six-subunit origin recognition complex (ORC) at replication origins. ORC then promotes the recruitment of two important replication factors, Cdt1 and Cdc6, onto origins in mitosis. Following assembly of Cdc6 and Cdt1 at replication origins, the minichromosome maintenance (MCM) complex is recruited onto origins by the concerted action of ORC, Cdc6, and Cdt1 during late mitosis through G_1 phase. The MCM2–7 complex consists of six proteins that are structurally related and highly conserved in eukaryotes (3). MCM2–7 is the replicative helicase, which plays a critical role in both initiation and elongation by unwinding DNA double helix during DNA replication (3–5).

Cdt1 and Cdc6 are critical for the recruitment of MCM2–7 onto chromatin. Cdt1 interacts with MCM2–7, and this interaction is important for Cdt1 to recruit MCM2–7 onto origins (6). Cdc6 promotes the assembly of MCM2–7 onto origins by enhancing the interaction between Cdt1 and MCM2–7 via interactions with both proteins (7). Following pre-RC assembly at replication origins during the M to G_1 phase transition (termed "origin licensing"), the initiation of DNA replication is triggered by the cooperative action of two protein kinases, Cdk2-CcnE and Cdc7-Dbf4. They are required to recruit Cdc45 onto replication origins and to stimulate initiation by phosphorylating MCM2 (1, 8, 9).

Although the mechanisms governing DNA replication are conserved from yeast to human, exceptions have been reported (10). For example, the histone H4-specific acetylase HBO1 appears to acetylate histone H4 at replication origins to promote the loading of MCM2–7 onto replication origins in human cells (11, 12), but an HBO1 homolog does not exist in yeast (13, 14). Thus, human cells clearly contain mechanisms that regulate the loading of MCM2–7 onto replication origins

[3] The abbreviations used are: pre-RC, prereplicative complex; And-1, acidic nucleoplasmic DNA-binding protein 1; MCM2–7, minichromosome main-

tenance protein 2–7; Cdt1, Cdc10-dependent transcript 1; Cdc6, cell division cycle 6; ORC, origin recognition complex; CldU, chlorodeoxyuridine; IdU, iododeoxyuridine.

but are absent from yeast cells. Results reported here reveal that And-1 is yet another example.

And-1 is an acidic nucleoplasmic DNA-binding protein containing an amino-terminal WD40 domain and a carboxyl-terminal high mobility group motif (15). The WD40 domain functions to mediate protein-protein interactions, whereas high mobility group motif has DNA-binding activity, and it usually facilitates the formation of nucleoprotein complexes on the chromatin by modulating DNA structure (16–18). Ctf4, the And-1 ortholog in *Saccharomyces cerevisiae*, was originally identified in a genetic screen for mutants affecting chromosome transmission fidelity (19), and subsequent studies indicated that Ctf4 regulates telomere replication and sister chromatid cohesion (20–23). The And-1 homolog in *Schizosaccharomyces pombe*, *mcl1*, is essential for viability, maintenance of genome integrity, DNA damage repair, and regulation of telomere replication (22, 23). We previously reported that And-1 is required for loading DNA polymerase α p180 onto chromatin during S phase in *Xenopus* egg extracts and is required for the maintenance of the stability of DNA polymerase α p180 in human cells (15). Other studies further indicate that And-1 is involved in the formation of the Cdc45-MCM2–7-GINS (Go, Ichi, Nii, and San) complex, stimulates the activities of DNA polymerases α and ϵ, and couples MCM2–7 to DNA polymerase alpha (24–28). More recently, we demonstrated that And-1 interacts with and maintains the stability of the histone acetyltransferase, Gcn5 (29, 30). Thus, And-1 is an important protein that plays multiple roles in the regulation of chromatin function and DNA replication.

Using *Xenopus* egg extracts, we have found that And-1 is loaded onto chromatin in S phase after MCM2–7 chromatin assembly (15). Surprisingly, we and others (24, 30) also have observed that human And-1 relocalizes onto chromatin in late mitosis, suggesting that human cells have a distinct mechanism governing the association of And-1 with chromatin. In an effort to elucidate the role of And-1 during this period of the mammalian cell cycle, we considered the possibility that human And-1 is recruited onto chromatin during late mitosis in order to play a role in regulating pre-RC assembly.

In this study, we investigate the biological function of And-1 during late mitosis and G_1 phase in human cells. We find that And-1 forms complex with MCM2–7 and down-regulation of And-1 with siRNA significantly suppresses loading of MCM2–7 onto chromatin in late mitosis and G_1 phase. Furthermore, we find that human And-1 interacts with Cdt1, and depletion of And-1 suppresses the interaction between Cdt1 and MCM7 in G_1 phase. Thus, our data suggest that human And-1 is an important regulator governing the assembly of MCM2–7 at replication origins during origin licensing in human cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Synchronization, and FACS Analyses—U2OS, 293T, and HCT116 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37 °C in 5% CO_2 supply. HCT116 cells expressing FLAG-And-1 or FLAG alone were constructed by infecting cells with retrovirus expressing FLAG-And-1 or FLAG, followed by single col-

ony selection. Cell cycle synchronization was achieved by treating cells with 40 ng/ml nocodazole in complete medium for 16 h, mitotically arrested cells were harvested by mitotic shake off, followed by washing in PBS, and released into nocodazole-free complete medium. To synchronize cells in G_1 phase, L-mimosine (final concentration, 300 μM) was used to treat cells for 24 h. Flow cytometry analysis (FACS) was performed as described previously (31).

siRNA Transfection—siRNA transfections were performed with 100 nM siRNA oligonucleotide duplexes using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instructions. Cells were harvested for analysis 36–48 h after transfection. siRNA oligonucleotides against And-1 were made to the following target sequences (sense): And-1–1, 5′-TGACTCGCTTCACTACAAA-3′; And-1–2, 5′-GATGG-TCAAGAAGGCAGCA-3′; And-1–3, 5′-GGTGTAGGTAAC-AGGACAT-3′; and control Gl2, 5′-AACGTACGCG-GAATACTTCGA-3′.

Antibodies—Anti-MCM2 (3619), anti-H3K9Ac (9671), anti-H4K5Ac (9672), and H4K12Ac (2591) were from Cell Signaling. Anti-α-tubulin (T6199), anti-FLAG-M2 (A2220), anti GAPDH (G9545) and anti-HA (H9658) were from Sigma. Anti-Gcn5 (sc-20698 and sc-130374), anti-β-actin, anti-PCNA (SC-56), anti-Cdc6 (sc-8342), anti-Mcm7 (sc-9966), anti-Cdt1 (sc-28262), anti-polymerase α (sc-5921), anti-cyclin B1 (sc-752), and anti-Mcm6 (sc9843) were from Santa Cruz Biotechnology. Anti-Orc2 (559266). Anti-PAN-MCM (559541) was from BD Biosciences Pharmingen. Anti-And-1 was produced as described previously (30).

Plasmids—FLAG-And-1 and GST-And-1 (full-length and various fragments) were constructed as described previously (15).

Immunoblotting, Immunofluorescence, and Chromatin Binding Assay—To make total protein extracts, cells were lysed in radioimmune precipitation assay buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0), followed by sonication. Immunofluorescence assays were described previously (30). Chromatin fractionation assays were performed as described previously (32).

Immunoprecipitation—Cells were lysed in lysis buffer (25 mM HEPES-KOH at pH 7.6, 150 mM KAc, 5 mM $MgCl_2$, 1 mM Na_2 EGTA, 10% glycerol, 0.1% Nonidet P-40, protease inhibitor, supplemented with 300 μg/ml ethidium bromide and 15 kunitz units of DNase I) for 20 min on ice, followed by sonication. After centrifugation, the resulting supernatants were mixed with anti-FLAG beads for 2 h. Beads were then washed three times with lysis buffer. Associated proteins were eluted by incubating beads with SDS loading buffer for immunoblotting.

In-Gel Trypsin Digestion and Mass Spectrometry—FLAG-And-1 immunoprecipitates were prepared as described in immunoprecipitation. Gel bands were excised and digested as described previously (33). Concentrated peptides from each band were injected via an autosampler (6 μl) and loaded onto a Symmetry C18 trap column (5 μm, 300 μm, inner diameter $\times$ 23 mm, Waters) for 10 min at a flow rate of 10 μl/min, 100% A. The sample was subsequently separated by a C18 reverse-phase column (Michrom Bioresources) at a flow rate of 300 nl/min using an Eksigent nano-hplc system (Dublin, CA). The mobile

phases consisted of water with 0.1% formic acid (A) and 90% acetonitrile (B). A 65 min linear gradient from 5 to 60% B was employed. Eluted peptides were introduced into the mass spectrometer via Michrom Bioresources CaptiveSpray. The spray voltage was set at 1.4 kV, and the heated capillary was set at 200 °C. The LTQ-Orbitrap-XL (Thermo Fisher Scientific) was operated in data-dependent mode with dynamic exclusion in which one cycle of experiments consisted of a full-MS in the Orbitrap ($300-2000$ m/z) survey scan in profile mode, with a resolution of 30,000, and five subsequent MS/MS scans in the LTQ of the most intense peaks in centroid mode using collision-induced dissociation with the collision gas (helium) and normalized collision energy value set at 35%.

Each file was searched for protein identification using the sequest algorithm in the Bioworks Browser software (version 3.3.1, Thermo Fisher Scientific) against the Uniprot database indexed for human species and for fully tryptic peptides, two missed cleavages, and potential modification of oxidized methionine (15.9949 Da). DTA generation parameters were Peptide Tolerance of 50 ppm and Fragment Ion Tolerance of 1 Da. Search result files were loaded into ProteoIQ software (NuSep, Bogart, GA) and filtered based on the following: XCorr > 1.9, four spectra per peptide, two unique peptides per protein, 0.98 peptide probability, and 0.95 protein probability. Protein fold change values were calculated by using the ratio of scan counts for each sample *versus* its paired control per protein. Protein annotations were acquired using the Keyword and GO terms in the Uniprot Protein Knowledgebase.

Molecular Combing—HCT116 cells transfected with siRNAs were labeled for 20min with 100 μM iododeoxyuridine (IdU), washed in PBS, and then labeled for another 20 min with 100 μM chlorodeoxyuridine (CldU). Cells were then collected by trypsinization, and DNA was gently extracted for molecular combing as described previously (34). DNA was combed on silanized surfaces (Microsurfaces, Inc.) and denatured in 1 N NaOH and probed with the following primary antibodies: mouse anti-BrdU (IdU-specific; clone BD44, Becton Dickinson), rat anti-BrdU (CldU-specific; clone BU-75, AbD Serotec) and mouse anti-ssDNA (clone 16-19, Millipore). The following secondary antibodies were used: anti-mouse Alexa Fluor 594, anti-rat Alexa Fluor 488, and anti-mouse Alexa Fluor 647, respectively (Invitrogen). Images were acquired on an epifluorescence microscope (BD Pathway) using Attovision software. Signals were measured using NIH ImageJ software with custom-made modifications. The images of DNA fibers were made by aligning selected DNA fibers containing IdU and CldU tracks using Photoshop. Fork velocity data sets were evaluated for statistical significance using non-parametric Kruskal-Wallis test.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were carried out with chromatin immunoprecipitation kit (EZ-ChIP, Millipore) according to the manufacturer's instruction manual. Briefly, cells ($\sim$1–2 $\times$ 10^7 cells) were cross-linked with 1% formaldehyde for 10 min and quenched with glycine (final concentration, 0.125 M). Cells were then lysed in 1 ml of cell lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.1). Chromatin samples were sonicated to an average size of 500 bp. The chromatin samples were diluted 10-fold in dilution buffer

(0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) and precleared with protein G-agarose beads, followed by incubation with 2 μg of antibody. Each immunoprecipitation was incubated for an additional 1 h with protein G-agarose beads. Precipitates were washed with 1 ml of low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, and then twice with TE (Tris, EDTA) buffer. DNA was then eluted in elution buffer (0.1 M NaHCO$_3$ and 1% SDS) and incubated at 65 °C overnight with NaCl (final concentration, 0.2 M) to reverse the formaldehyde cross-links. DNA was purified using the Qiaquick PCR purification kit. DNA samples were analyzed by quantitative real-time PCR using primers for DNA replication origins and surrounding regions as described (35).

RESULTS

Identification of MCM2–7 as And-1-associated Proteins—To further characterize the role of And-1 in DNA replication, we set out to identify new And-1-associated proteins. To this end, we developed HCT116 cell lines stably expressing FLAG alone or FLAG-And-1 fusion proteins at a level similar to that of the endogenous And-1 (data not shown). We then prepared whole cell extracts from each cell line and subjected the extracts to affinity purification using anti-FLAG beads. To rule out the possibility that the protein interactions were mediated by chromatin, we included ethidium bromide and DNase I in the lysis buffer to destroy chromatin structure. The purified proteins were then resolved by gel electrophoresis, and the resolved proteins were subjected to in-gel digestion followed by mass spectrometry analyses and database searching for protein identification. Through these analyses, we identified proteins that were present more frequently in FLAG-And-1 complexes than in FLAG control complexes. The And-1-associated DNA replication proteins identified in this experiment are listed in Fig. 1, *A* and *B*. They include Cdc45, DNA polymerase α (p180), DNA primase subunits 1 and 2 (PRIM1/2), DNA polymerase epsilon catalytic subunit A, claspin, and MCM proteins 2, 3, 4, 5, 6, and 7 (Fig. 1, *A* and *B*). All of these proteins are known to be involved in the initiation and/or elongation processes of DNA replication. Because we were particularly interested in the associations of And-1 with MCM2–7, we performed co-immunoprecipitation assays to confirm these interactions. As shown in Fig. 1*C*, we successfully detected DNA polymerase α, MCM2, MCM6, and MCM7 from the FLAG-And-1 but not FLAG immunoprecipitates (Fig. 1*C*). Thus, our data indicate that And-1 interacts with MCM2–7, suggesting a possible role of And-1 in the regulation of MCM2–7 function.

Human And-1 Proteins Are Loaded onto Chromatin in Late Mitosis—It is known that the total cellular levels of And-1 do not change throughout the cell cycle, but the chromatin-associated And-1 is cell cycle-regulated (15, 24, 28). We and others recently found that unlike *Xenopus*, And-1 that is loaded onto chromatin at the onset of S phase, human And-1 is loaded onto chromatin in late mitosis (24, 30), suggesting an important role of human And-1 in regulating chromatin function during late mitosis through G$_1$ phase. The fact that And-1 interacts with MCM2–7 proteins prompted us to investigate whether human And-1 regulates the association of MCM2–7 with chromatin.
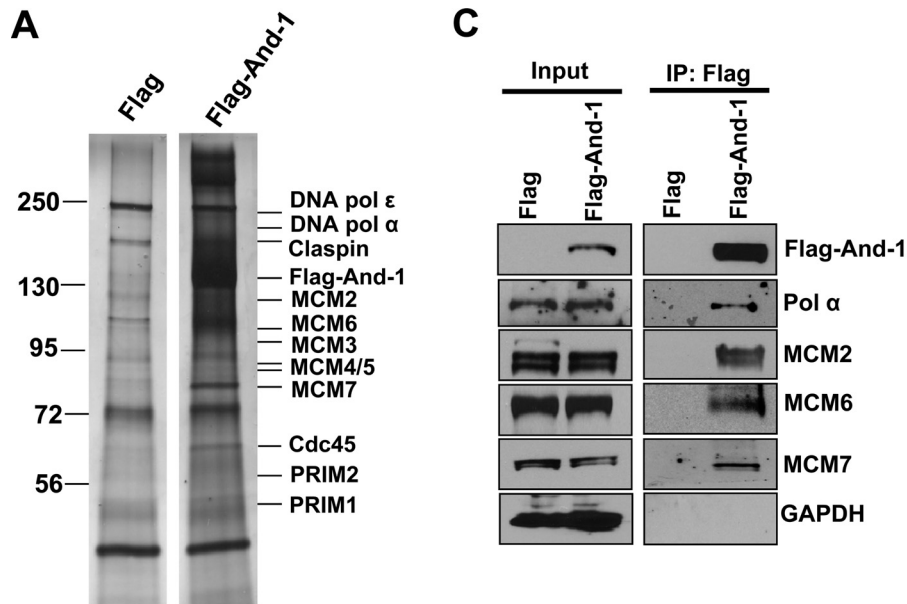
FIGURE 1. **Identification of MCM2–7 as And-1-associated proteins.** *A,* purification of And-1-associated proteins. Cell extracts from HCT116 cells stably expressing FLAG-And-1 proteins or FLAG alone were subjected to immunoprecipitation using anti-FLAG beads. FLAG immunoprecipitates (*IP*) were resolved on SDS-PAGE followed by silver staining. Proteins that are presented more in purified FLAG-And-1 complexes than in control FLAG complexes by mass spectrometry were indicated. *B,* identification of And-1-associated proteins that are involved in DNA replication by mass spectrometry. *C,* And-1 interacts with MCM2–7 proteins. Co-immunoprecipitation assays were performed using HCT116 cell lines expressing FLAG-And-1 or FLAG alone. FLAG immunoprecipitates were resolved on SDS-page gel and immunoblotted for the indicated proteins.

To explore this possibility, we first compared the associations of And-1 or MCM7 with chromatin in HCT116 cells at different stages of the cell cycle by immunofluorescence. Both And-1 and MCM7 proteins were dispersed throughout the nucleus during interphase (Fig. 2*A*). However, at prometaphase, metaphase and anaphase, both proteins dissociated from chromatin, as revealed by the lack of And-1 and MCM7 in the DAPI-stained area (Fig. 2*A*). At telophase, both And-1 and MCM7 reassociated with chromatin (Fig. 2*A*). Interestingly, we noticed in some telophase cells that MCM7 was still segregated from the chromosomes, whereas And-1 had already acquired exclusive association with chromosomes (Fig. 2*A*). Because we did not observe association of MCM2–7 with chromosomes in the

absence of chromosome bound And-1 (data not shown), our results suggest that localization of And-1 onto chromosomes precedes that of MCM2–7 during late mitosis.

A visualization of the localization of And-1 on the chromatin under microscopy does not definitively indicate an actual association of And-1 with chromatin. To examine the chromatin association of human And-1 in more detail, we analyzed the association of And-1 with chromatin in cells that had been released from mitotic arrest by nocodazole. These cells were harvested at various times after release for chromatin fractionation assays. The DNA content of each fraction was determined by FACS. As shown in Fig. 2*B,* cells released from the nocodazole block progressed throughout mitosis and entered into G_1
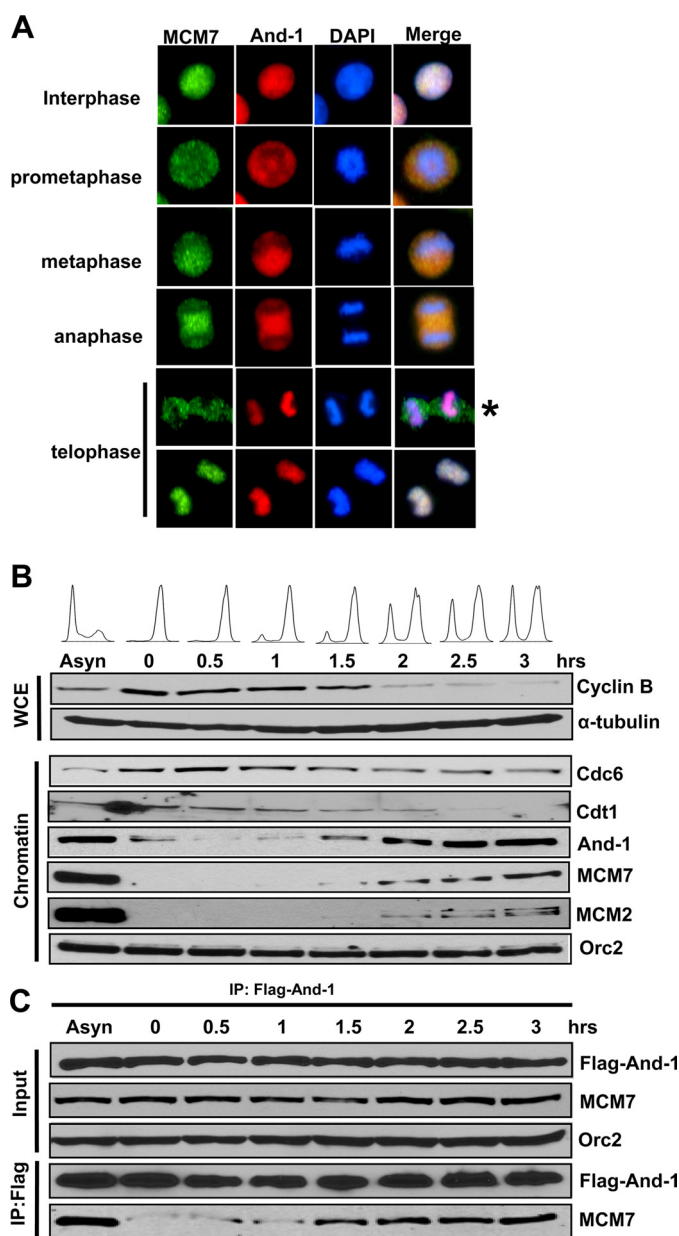
FIGURE 2. **Human And-1 associates with chromatin at telophase.** *A*, And-1 localizes to chromatin at telophase prior to MCM7. Asynchronized HCT116 cells were immunostained for the indicated proteins. Nuclei were stained with DAPI. An *asterisk* indicates cells in which And-1 but not MCM7 was relocalized onto chromatin. *B*, And-1 associates with chromatin in late mitosis. HCT116 cells were synchronized in mitosis by treatment with nocodazole (40 ng/ml) for 16 h. Mitotically arrested cells were harvested by mitotic shaking and then released to nocodazole-free medium. Cells were harvested at indicated time points postrelease. Harvested cells were either processed for FACS analyses or chromatin fractionation assays followed by immunoblot for the indicated proteins. *Asyn*, asynchronous cells. *C*, the interaction between And-1 and MCM7 is increased in late mitosis. HCT116 cells expressing FLAG-And-1 were synchronized at mitosis and then released as in *B*. Cells were harvested at various point points and then processed in lysis buffer containing ethidium bromide (300 μg/ml) and DNase I (15 kunitz units). The extract was used for FLAG-And-1 IPs, which were resolved in SDS-PAGE and then immunoblotted for the indicated proteins. *WCE*, whole cell extract. *Asyn*, asynchronous cells.

phase at ~1.5 h post-release, as indicated by FACS data and cyclin B protein levels. Orc2 was consistently bound to chromatin at the same protein levels in all time points, whereas Cdc6 and Cdt1 accumulated on chromatin soon after cells were

released from mitosis arrest. Following Cdc6 and Cdt1, MCM2–7 reassociated with chromatin at 1.5 h after release. Unlike *Xenopus* egg extracts (15), we could detect small amounts of chromatin-associated And-1 protein in metaphase cells or in cells released immediately from the nocodazole block (0–1 h). Chromatin-associated And-1 rapidly increased at approximately the same time as MCM2–7 (Fig. 2*B*). We infer from these data that human And-1 binds to chromatin during mitosis prior to the assembly of the pre-RCs.

Given that human And-1 forms complexes with MCM2–7 and exhibits a chromatin association pattern similar to that of MCM2–7, we next investigated whether the interactions between And-1 and MCM2–7 are cell cycle-dependent. To this end, we examined the interactions between And-1 and MCM7 by co-immunoprecipitation assays in cells that were arrested and then released into nocodazole-free medium for various lengths of time. Weak interaction between And-1 and MCM7 was detected in cells immediately released from nocodazole arrest (Fig. 2*C*). Strikingly, there was a rapid increase in the interaction between And-1 and MCM7 at 1.5 h after nocodazole release, and this strong interaction was maintained at later time points (Fig. 2*C*). The increased interaction between And-1 and MCM7 occurred at the time when MCM2–7 began to associate with chromatin (Fig. 2*B*), suggesting that And-1 may trigger the association of MCM2–7 with chromatin via protein-protein interactions.

And-1 Is Required for Association of MCM2–7 with Chromatin—To determine whether or not And-1 regulates binding of MCM2–7 with chromatin, we examined the association of MCM2–7 with chromatin in cells treated either with control siRNA (siGl2) or three independent And-1 siRNAs, each of which produced >90% knockdown of And-1 proteins (Fig. 3*A* and siAnd-1–3 not shown). Although it did not affect the total levels of MCM2–7, And-1 down-regulation resulted in the reduction of chromatin-associated MCM2–7, but it had no detectable effect on the association of Orc2 with chromatin (Fig. 3*A* and siAnd-1–3 not shown).

Previous studies have shown that association of human MCM2–7 with chromatin is cell cycle-dependent, with a stronger association at G_1/S phase than at late S/G_2 phase (5, 36). Given that And-1 depletion causes an S/G_2 phase accumulation in human cells (15), it is likely that the a reduction of chromatin-associated MCM2–7 could be an indirect effect of cell cycle arrest associated with And-1 depletion. To rule out this possibility, we synchronized And-1-depleted cells in G_1 phase using L-mimosine, a plant amino acid that reversibly arrests cells in G_1 phase by increasing p27 levels (Fig. 3*B*) (37, 38). FACS analyses indicated that L-mimosine arrested siRNA-transfected cells in G_1 phase regardless whether they were treated with siAnd-1 or siGl2 (Fig. 3*C*). In the absence of And-1, the chromatin associations of MCM2/6/7 were significantly reduced, whereas chromatin associated Cdc6 and Cdt1 were not affected (Fig. 3*D*). To verify that the reduced chromatin associations of MCM2–7 in the siAnd-1-treated cells was due specifically to an And-1 deficiency, we performed rescue experiments. Chromatin association of MCM7 was restored by expressing wild-type And-1 in cells in which endogenous And-1 was depleted using siAnd-1–3 targeting 3′-UTR of And-1 mRNA (see Fig. 7*D*).
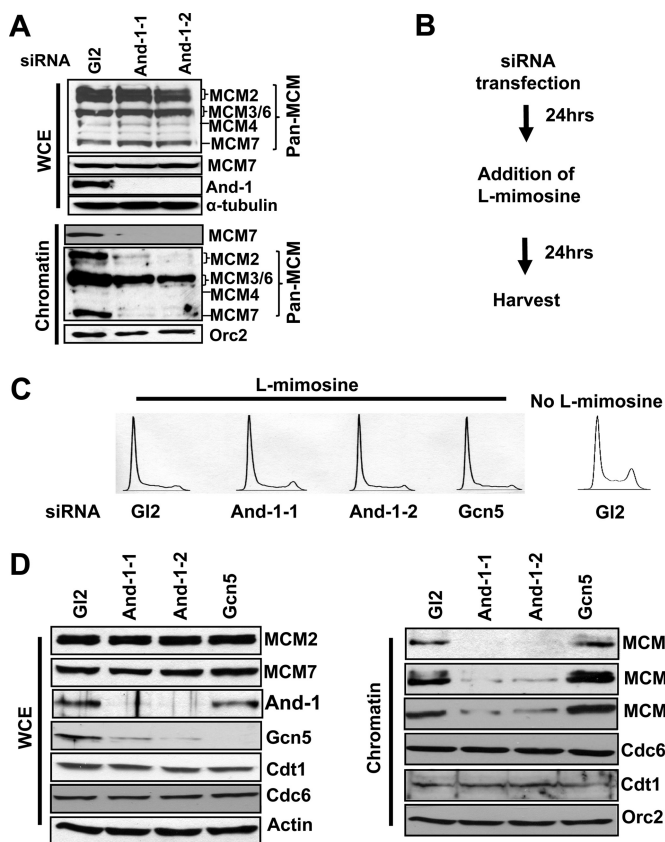
FIGURE 3. **And-1 is required for the association of MCM2–7 with chromatin in G_1 phase.** *A,* depletion of And-1 reduces the chromatin association of MCM2–7 proteins. HCT116 cells were transfected with two independent siRNA targeting And-1. Cells were harvested for chromatin fractionation assays 48 h post-siRNA transfection. Chromatin fractionations were then resolved on SDS-PAGE and immunoblotted for the indicated proteins. *B,* schematic of cell preparation for experiments performed in *C* and *D. C,* FACS analyses of cells treated as in *B*. Note that L-mimosine treatments arrest siRNA-transfected cells in G_1 phase. *D,* cells treated as in *B* were used for chromatin fractionation analyses. *WCE* (whole cell extractions) or chromatin fractions were resolved on SDS-PAGE and immunoblotted for the indicated proteins.

FIGURE 4. **And-1 is required for the assembly of MCM7 onto chromatin in late mitosis.** 24 h after siRNA transfection, HCT116 cells were synchronized at mitosis by nocodazole treatment for 16 h. Synchronized cells were then released into nocodazole-free medium and harvested at the indicated time points. Harvested cells were either processed for FACS analyses (*A*) or chromatin fractionation assays (*B*). Whole cell extracts (*WCE*) or chromatin fractionations were resolved in SDS-PAGE and immunoblotted for the indicated proteins. *Asyn,* asynchronous cells.

In previous studies, we showed that And-1 is required for maintaining the stability of histone acetyltransferase Gcn5 and thereby maintaining acetylation of histone H3 at K9 and K56 (29, 30). Because histone modification (H4 acetylation) is required for loading the MCM2–7 helicase onto replication origins (11, 39), we next investigated whether defects in MCM2–7 loading that resulted from And-1 depletion are due to the reduction of Gcn5. As shown in Fig. 3*D*, depletion of Gcn5 had no effect on either the total cellular levels or the amount of chromatin associated MCM2/6/7, indicating that Gcn5 did not mediate the effect of And-1 on the association of MCM2–7 with chromatin.

And-1 Is Required for Efficient Assembly of MCM2–7 Proteins onto Chromatin in Late Mitosis and Early G_1 Phase—In the absence of And-1, chromatin-associated MCM2–7 are significantly reduced in G_1 phase, suggesting that And-1 is required either for maintaining the association of MCM2–7 with chromatin during G_1 phase or for promoting the assembly of MCM2–7 onto chromatin during late mitosis. Given that And-1 is loaded onto chromatin before MCM2–7 and the interaction between And-1 and MCM2–7 is increased at late
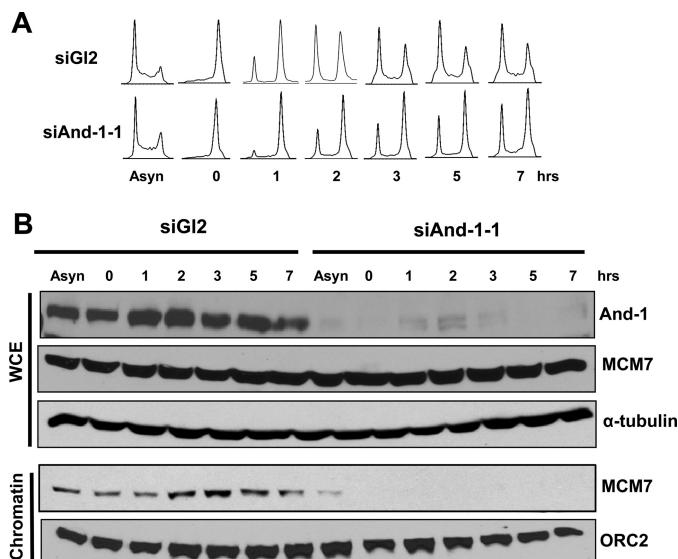
mitosis when MCM2–7 are loaded onto chromatin, we hypothesized that And-1 is required for loading MCM2–7 onto chromatin in late mitosis. To test this possibility, we examined the association of MCM7 with chromatin in And-1 down-regulated cells that were synchronized in late mitosis and G_1 phase. Specifically, U2OS cells transfected with siRNAs were arrested in metaphase using nocodazole and then released into nocodazole-free medium to allow them to resume mitosis. The cells were harvested at various times after release for analyses. Interestingly, FACS analyses indicated that siAnd-1-treated cells progressed through mitosis and G_1 phase at a much slower rate than the siGl2-treated cells (Fig. 4*A*). In siGl2-treated cells, the association of MCM7 with chromatin accumulated at two hours after nocodazole release when cells began to enter G_1 phase and slightly decreased at 7-h post release when cells progressed into S phase. However, the chromatin-associated MCM7 was reduced significantly in And-1-depleted cells up to 7 h after release when ~25% of the cells had entered G_1 phase (Fig. 4*B*). These data strongly suggest that And-1 is essential for the assembly of MCM2–7 onto chromatin in late mitosis.

And-1 Regulates the Assembly of MCM Proteins at Replication Origins—The fact that And-1 is required for the assembly of MCM proteins onto chromatin in G_1 phase suggested that And-1 plays an important role in the loading of MCM proteins onto replication origins. To explore this possibility, we investigated the assembly of MCM7 proteins onto two well characterized replication origins in the human genome, the BG40.9 origin located in the β-globin locus and the LB2 origin located in the lamin B2 locus (35). Using ChIP assays with cells arrested in G_1 phase by L-mimosine, we found that the association of MCM7 with these two replication origins was reduced significantly in the cells treated with siAnd-1 compared with cells treated with the siGl2 control (Fig. 5). These results reveal that
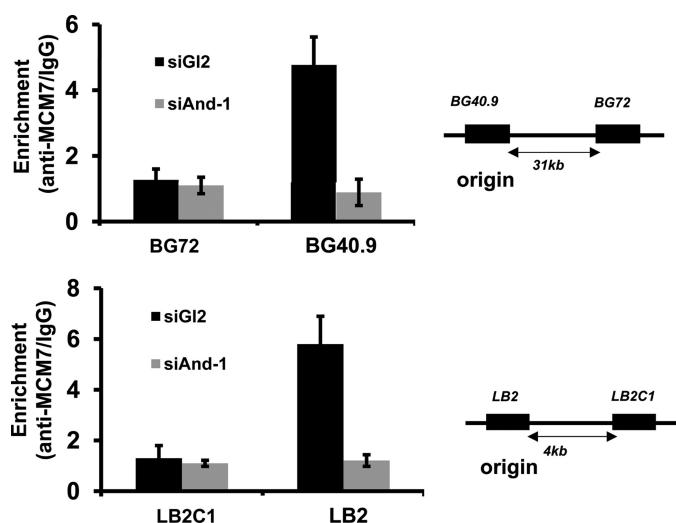
FIGURE 5. **Depletion of And-1 reduces the association of MCM7 with replication origins.** Cells treated as in Fig. 3*B* were harvested for ChIP assays. MCM7 protein levels at replication origins BG40.9 or LB2 as well as their flanking region BG72 or LB2C1 were shown. IgG was used as the measurement of nonspecific chromatin precipitation. Quantitative PCR was used to ascertain the MCM7 levels at indicated chromatin regions. The *y* axis represents the relative enrichment of the MCM7 proteins compared with the IgG control (after normalization with input precipitations). Data are represented as the mean + S.D. from three independent experiments.

And-1 is required for the assembly of MCM2–7 onto replication origins in G_1 phase.

Depletion of And-1 Reduces Both the Velocity and Density of Replication Forks—To further characterize the role of And-1 in DNA replication, we examined the progression of replication forks (fork velocity) and the number of forks per length of DNA (fork density) in siGl2- and siAnd-1-treated cells using molecular combing (34, 40, 41). Cells transfected with either siGl2 or siAnd-1 were labeled with 100 μM IdU for 20 min and then 100 μM CldU for 20 min before being harvested at 36 h after transfection. DNA fibers were prepared for molecular combing (Fig. 6*A*). Newly synthesized DNA (red fluorescence) migrated away from the replication origin and was subsequently elongated (green fluorescence) (Fig. 6, *A* and *B*). The lengths of both red and green tracks in the DNA fibers isolated from the cells treated with siAnd-1 were shorter than those in siGl2-treated cells (Fig. 6*B*), revealing that the velocity of DNA replication forks in And-1 down-regulated cells was slower relative to control cells. The replication fork velocity in siGl2-treated cells was ~1.5 kb/min, in agreement with a previous report (Fig. 6*C*) (42), whereas fork velocity in cells treated with siAnd-1 was ~0.4 kb/min. These data suggest that And-1 is critical for the progression of replication forks.

We next measured the density of replication forks (total number of forks divided by total length of DNA examined) in proliferating cells. Fork density in cells treated with siGl2 was 3.19 forks/megabase (Fig. 6*D*), similar to a previous report by Guilbaud *et al.* (43), whereas the fork density in cells depleted of And-1 by either siAnd-1–1 or siAnd-1–2 was 2.61 forks/megabase and 2.78 forks/megabase, respectively (Fig. 6*D*). At 36 h after siAnd-1 transfection, we could not detect any DNA damage (data not shown). Thus, the reduced fork density is unlikely due to the fork collapse associated with DNA damage, instead,
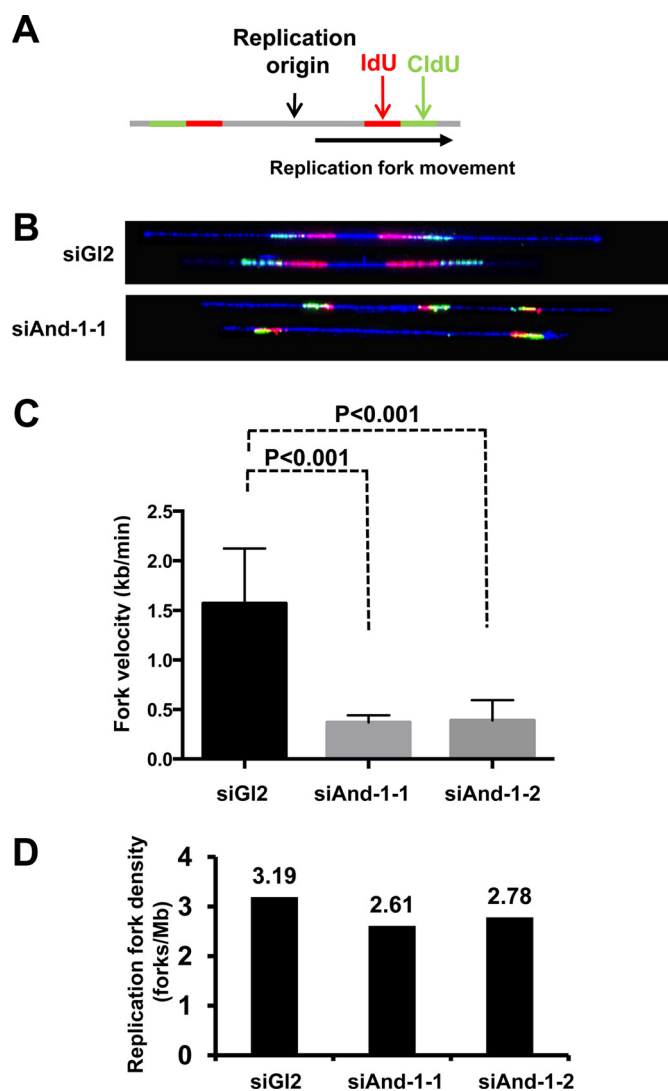

FIGURE 6. **Depletion of And-1 reduces the speed of replication fork movement and the global fork density.** *A*, schematic of cell preparation for single-molecule analysis. HCT116 cells tansfected with control or And-1 siRNA were labeled with 100 μM IdU for 20 min and then 100 μM CldU for 20 min before being harvested 36 h after siRNA transfection. *Red*, IdU; *Green*, CldU. *B*, the individual fork velocity decreases after depletion of And-1 as reflected by the length of IdU- and CldU-labeled DNA fibers. *C*, depletion of And-1 reduces fork velocity. Means were shown with S.D. for each data set. At least 200 replication forks were counted. *D*, the global fork density is reduced after And-1 depletion. The global replication fork density was determined as the ratio of the total number of IdU and CldU foci to the total length of DNA examined, corrected for contamination by non-replicating G_1 or G_2/M cells. 300–400 megabase of DNA length were examined.

it is more likely that loss of And-1 resulted in inefficient origin utilization.

And-1 Facilitates the Interaction between MCM7 and Cdt1—Given that Cdt1 interacts with MCM2–7 and that this interaction is essential for recruiting MCM2–7 to replication origins (6), we next asked whether And-1 could regulate MCM2–7 by affecting their interaction with Cdt1. To test this possibility, we first examined the interaction between And-1 and Cdt1 by expressing FLAG-tagged wild-type And-1 and a set of FLAG-tagged And-1 truncation mutants in U2OS cells and examining the interactions of And-1 and its mutants with endogenous Cdt1 (Fig. 7*A*). We found that FLAG-And-1 co-precipitated with both Cdt1 and MCM6/7 and that the interactions were
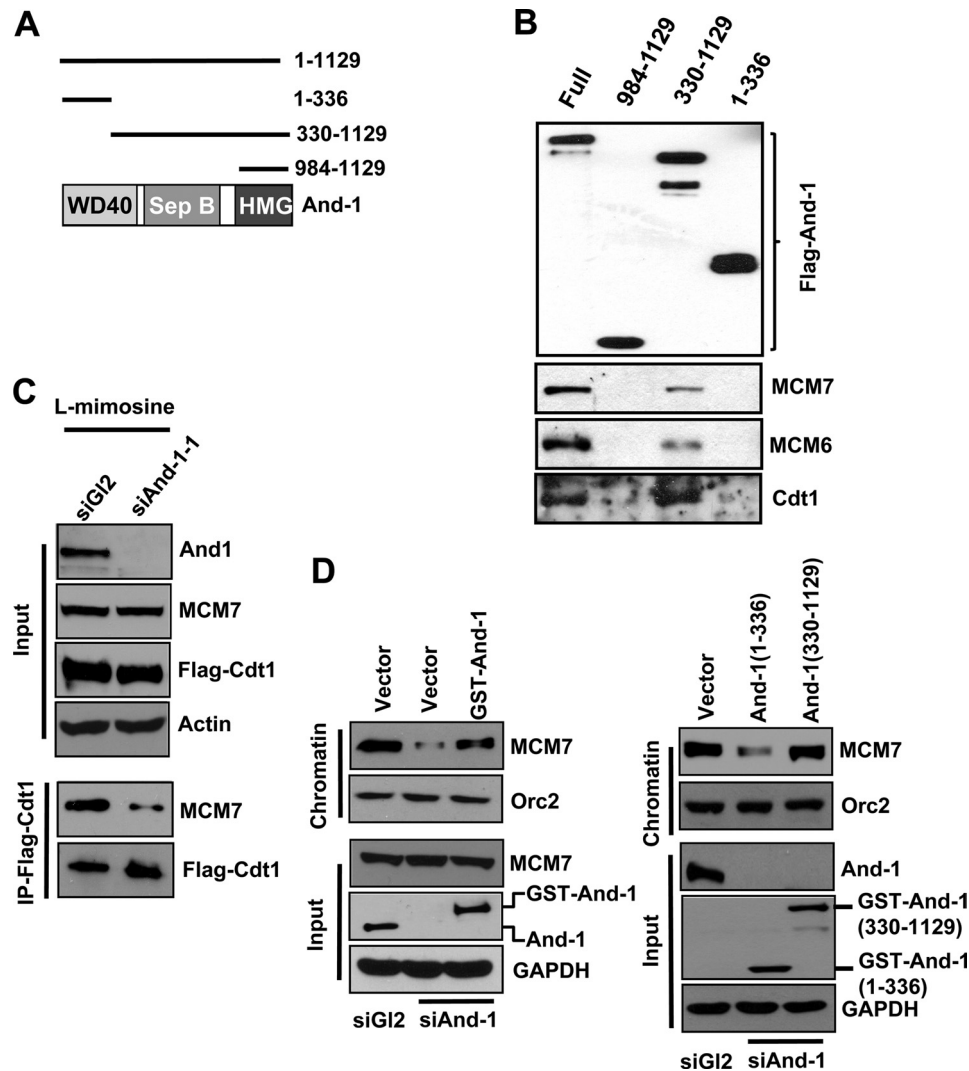
FIGURE 7. **Loss of And-1 reduces the interaction between MCM7 and Cdt1.** *A*, schematic of the And-1 protein domains and deletion mutants used for protein interactions. *B*, the interactions between And-1 and MCM6/7 or Cdt1. U2OS cells transfected with plasmids expressing the indicated proteins were harvested and immunoprecipitated for FLAG-And-1. FLAG-And-1 immunoprecipitates were then resolved on SDS-PAGE and immunoblotted for the indicated proteins. *C*, U2OS cells expressing FLAG-Cdt1 were transfected with the indicated siRNAs. Twenty-four hours post-siRNA transfection, the cells were treated with L-mimosine. Cells were harvested 48 h after siRNA transfection and processed for FLAG-Cdt1 IP, which was subjected to SDS-PAGE and immunoblotting analyses for the indicated proteins. *D*, HCT116 cells transfected with indicated plasmids and siRNAs, followed by treatment with L-mimosine for 24 h after siRNA transfection. Cells were then harvested 48 h after siRNA transfection for chromatin fractionations as in Fig. 3*A*. *HMG*, high mobility group.

detected in full-length And-1 and mutant And-1(330–1129), but not in And-1(1–336) or And-1(984–1129) (Fig. 7*B*), suggesting that the SepB domain of And-1 is critical for the association of And-1 with MCM6/7 and Cdt1.

The finding that And-1 interacts with both MCM7 and Cdt1 suggested that And-1 may facilitate the interaction between Cdt1 and MCM7. To test this possibility, we examined the interaction between Cdt1 and MCM7 in cells transfected with siAnd-1 and then synchronized in G_1 phase using L-mimosine. Although And-1 depletion had no effect on the total cellular levels of Cdt1 and MCM7, And-1 depletion reduced significantly the amount of MCM7 associated with Cdt1 (Fig. 7*C*). These results indicate that And-1 mediates the interaction between Cdt1 and MCM7.

Because And-1 interacts with MCM2–7, we next asked whether the interaction between them is required for the association of MCM2–7 with chromatin. To test this possibility, we

depleted And-1 using siAnd-1–3, which targets the 3′-UTR of And-1 mRNA in cells expressing wild-type GST-And-1, GST-And-1(1–336), or GST-And-1(330–1129). And-1 depletion consistently resulted in a reduction of chromatin associated MCM7, which was recovered by expression of wild-type GST-And-1, GST-And-1(330–1129) but not GST-And-(1–336), which does not interact with MCM7 (Fig. 7*D*). Thus, the interaction between And-1 and MCM7 is required for the association of MCM7 with chromatin.

And-1 Is Not Required for HBO1-mediated Histone Acetylation at Replication Origins—A recent study showed that HBO1, a histone H4-specific acetylase, is involved in the loading of MCM2–7 onto replication origins by promoting histone H4 acetylation at the origins (11). We therefore investigated whether And-1 regulates the loading of MCM2–7 onto chromatins by a mechanism involving HBO1-mediated histone H4 acetylation. First, chromatin fractionation assays were carried
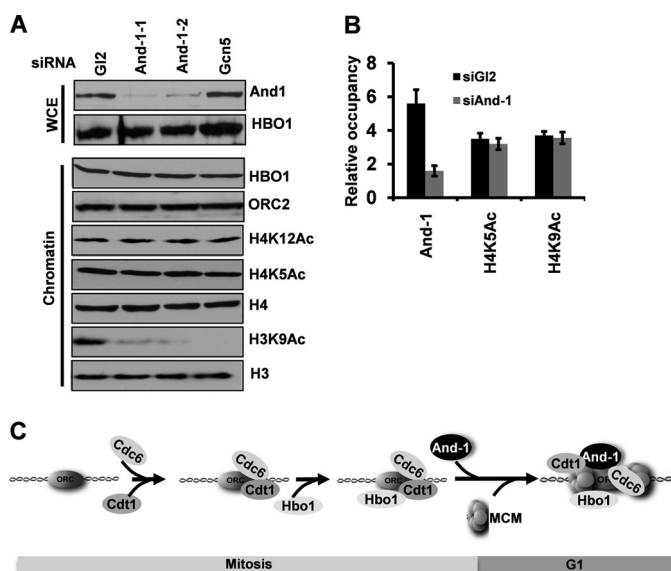
FIGURE 8. **Depletion of And-1 does not affect HBO1-mediated histone H4 acetylation.** *A*, HCT116 cells treated as in Fig. 3*B* were harvested for chromatin fractionation assays. Whole cell extract (*WCE*) or chromatin fractions were resolved on SDS-PAGE and immunoblotted for the indicated proteins. *B*, HCT116 cells treated as in Fig. 3*B* were harvested for ChIP assays. And-1 and H4 acetylation levels at replication origins were indicated. The quantification of quantitative PCR was as described in Fig. 5. Data are represented as the mean + S.D. from three independent experiments. *C*, a proposed model for the role of human And-1 in the regulation of chromatin loading of MCM2–7. Human And-1 is loaded onto chromatin in late mitosis after the assembly of ORC, Cdc6, and Cdt1 at origins. And-1 then facilitates the assembly of MCM2–7 onto chromatin in a manner independent on HBO1.

out on U2OS cells that had been transfected by a control siRNA, And-1 siRNA, or Gcn5 siRNA and then synchronized in G$_1$ phase using L-mimosine. Consistent with our previous observation (30), depletion of And-1 or Gcn5 significantly reduced the acetylation of H3K9 (Fig. 8*A*). However, depletion of either And-1 or Gcn5 did not affect the total cellular level of HBO1 protein levels, the amount of chromatin-associated HBO1, or the acetylation of histone H4 at K5 and K12 (Fig. 8*A*).

Although And-1 depletion did not affect the total amount of acetylated histone H4 on chromatin, And-1 depletion might affect acetylation of histone H4 specifically at replication origins, thereby causing a reduction in the amount of MCM2–7 loaded onto replication origins. To test this possibility, we performed ChIP assays to compare the levels of acetylated histone H4 at the replication origin LB2 in cells treated with siGl2 or siAnd-1. siAnd-1 transfection remarkably reduced the amount of origin-associated And-1, but it had no effect on the level of histone H4 acetylation at replication origins (Fig. 8*B*). These results collectively demonstrate that And-1 facilitates the loading of MCM2–7 onto chromatin by an HBO1-independent pathway.

DISCUSSION

DNA replication in eukaryotes begins with the process of loading the replicative helicase MCM2–7 onto chromatin in late mitosis and G$_1$ phase of the cell cycle. Here, we have analyzed the function of human And-1 in the assembly of MCM2–7 at replication origins during late mitosis through G$_1$ phase. Significantly, we find that human And-1 forms a com-

plex with MCM2–7 during late mitosis and is required for loading of MCM2–7 onto chromatin for pre-RC assembly (Fig. 8*C*).

The assembly of MCM2–7 onto replication origins occurs from late mitosis to G$_1$ phase. In parallel to chromatin loading of MCM2–7, human And-1 proteins are also recruited onto chromatin in late mitosis (Fig. 2) (24, 30). These results are different from the observations obtained in studies using *Xenopus* egg extracts, in which And-1 is loaded onto chromatin at early S phase after the assembly of pre-RC (15). This discrepancy might result from the fundamental differences between *Xenopus* eggs and human somatic cells, or from differences between assays using *Xenopus* egg extracts and assays involving cultured human cells. Although conservative mechanisms governing pre-RC assembly have been seen among the different species from yeast to human (1, 10), additional mechanisms to control pre-RC assembly have been identified in metazoans. For instance, geminin prevents the pre-RC assembly by inhibiting Cdt1 in metazoans, but there is no functional equivalent of geminin in yeast cells (44). The loading of MCM2–7 onto chromatin is promoted by HBO1-mediated acetylation of histone H4, whereas HBO1 does not appear to exist in yeasts (11, 13, 14). Although depletion of MCM8 inhibits DNA replication without affecting pre-RC assembly in *Xenopus* egg extracts (45). However, human MCM8 appears to be essential for pre-RC assembly by regulating the recruitment of Cdc6 to chromatin (46). These examples reveal that species-specific differences exist in origin licensing. Our studies suggest that And-1 is yet another example. Human And-1 facilitates the assembly of pre-RCs in human cells, although it does not appear to serve this functional in frog eggs.

The results presented here suggest And-1 regulates the chromatin assembly of MCM2–7 via facilitating the interaction between Cdt1 and MCM2–7. Given that And-1 regulates histone H3 acetylation by maintaining the stability of Gcn5 (29, 30), it is possible that And-1 may affect MCM2–7 chromatin loading indirectly via Gcn5. However, our data indicate that this is not the case because Gcn5 depletion had no effect on the chromatin loading of MCM2–7 (Fig. 3). Moreover, human And-1 did not regulate the chromatin assembly of MCM2–7 via HBO1-mediated histone H4 acetylation at replication origins (Fig. 8, *A* and *B*). Therefore, we suggest that human And-1 regulates DNA replication during transition from mitosis to G$_1$ phase by regulating loading of MCM2–7 onto replication origins. And-1 promotes the assembly of MCM2–7 onto chromatin by facilitating the interaction between Cdt1 and MCM2–7 via a HBO-1-independent pathway (Fig. 8*C*).

Human And-1 is an evolutionarily conserved protein with homologs in most eukaryotes. Interestingly, human And-1 but not yeast Ctf4 has a C-terminal high mobility group domain, a key domain to regulate assembly of nucleoprotein complexes on chromatin (16, 17). This structural difference may explain why human And-1 has different functions from yeast Ctf4. For instance, we recently reported that depletion of And-1 leads to the degradation of histone acetyltransferase Gcn5 in human cells but not in yeast cells (30). However, *Xenopus* And-1 does have a high mobility group domain, indicating that the different function of And-1 in human cells and *Xenopus* egg extracts is not due to the structure difference. Nevertheless, our study has

elucidated a novel mechanism governing the chromatin loading of MCM2–7 in human cells. In the future, it will be important to determine how And-1 regulates the interactions between Cdt1 and MCM2–7.

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