Interaction between HMGA1a and the origin recognition complex creates site-specific replication origins

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In all eukaryotic cells, origins of DNA replication are characterized by the binding of the origin recognition complex (ORC). How ORC is positioned to sites where replication initiation is unknown, because metazoan ORC binds DNA without apparent sequence specificity. Thus, additional factors might be involved in ORC positioning. Our experiments indicate that a family member of the high-mobility group proteins, HMGA1a, can specifically target ORC to DNA. Coimmunoprecipitations and imaging studies demonstrate that HMGA1a interacts with different ORC subunits in vitro and in vivo. This interaction occurs mainly in AT-rich heterochromatic regions to which HMGA1a localizes. Fusion proteins of HMGA1a and the DNA-binding domain of the viral factor EBNA1 or the prokaryotic tetracycline repressor, TetR, can recruit ORC to cognate operator sites forming functional origins of DNA replication. When HMGA1a is targeted to plasmid DNA, the prereplicative complex is assembled during G1 and the amount of ORC correlates with the local concentration of HMGA1a. Nascent-strand abundance assays demonstrate that DNA replication initiates at or near HMGA1a-rich sites. Our experiments indicate that chromatin proteins can target ORC to DNA, suggesting they might specify origins of DNA replication in metazoan cells.

Eukaryotic cells duplicate their genomes with remarkable precision and in a timely coordinated fashion. The process of DNA replication initiates at multiple origins of replication, which are recognized by the heterohexameric origin recognition complex (ORC), consisting of Orc1–6 (1). Human ORC is a dynamic cell cycle-regulated complex and can be regarded as an interactive platform for the assembly of the prereplicative complex (preRC), consisting of Orc1–6, Cdt1, Cdc6, and the MCM2–7 complex (1). The assembly of preRCs licenses origins for replication initiation. The human ORC subunits Orc2–5 form a stable core complex, whereas the association of the largest subunit Orc1 is cell-cycle-regulated (1). Biochemical studies indicate that the smallest subunit Orc6 is only loosely attached, and the existence of a hexameric holocomplex has only recently been postulated in human cells (2). ORC and most other proteins involved in initiation of DNA replication are conserved among eukaryotes, but specification of origins in mammalian cells remains elusive and is controversially discussed (3, 4).

How metazoan ORC recognizes origins is unknown, because ORC does not bind to DNA sequence specifically (3–5). The positioning of ORC at origins might be determined by veiled DNA sequence motifs, local chromatin structures, or accessory targeting factors such as AIF-C, Trf2, Ku80, or EBNA1, which can specify sites of ORC binding (6–10), and recently a direct role of Myc in replication initiation has been suggested (11). In Schizosaccharomyces pombe (Sp), origins contain large stretches of AT-rich sequences, and SpORC is recruited to these origins via the SpOrc4 subunit. It contains a unique N-terminal extension with nine AT-hook motifs, which mediate origin binding (12). In metazoan cells, AT-hook motifs are a hallmark of the HMGA family of high-mobility group (HMG) proteins. One member, HMGA1a, binds with high specificity to the minor groove of AT tracks and induces conformational changes (13). It is known as a structural nonhistone chromatin constituent that competes and antagonizes histone H1-mediated repression of genes (14), thus contributing to cell proliferation (15–17). However, no direct role in origin definition or DNA replication has been ascribed to HMGA proteins.

In this study, we describe a functional interaction between HMGA1a and ORC. Targeting HMGA1a to specific sites on plasmid DNA recruits ORC and generates artificial origins of DNA replication. Replication initiates at or in close vicinity of HMGA1a-rich sites, and preRCs form at these loci during the G1 phase of the cell cycle. We demonstrate that HMGA1a and ORC directly interact in vivo and in vitro. An HMGA1a variant with mutated AT-hook motifs and competition experiments with Hoechst 33342 both indicate that this interaction occurs mainly in AT-rich chromatin domains. Our data suggest that genuine chromatin proteins might contribute to the specification of chromosomal origins of DNA replication and their recognition by ORC at the molecular level.

Results

HMGA1a Supports Replication When Targeted to Plasmid DNA. Studying mammalian origins of DNA replication in the context of chromosomal DNA is difficult because of the scarcity of tractable model systems. Therefore, we concentrated on plasmid models, which can be addressed genetically and biochemically. We took advantage of the latent origin of Epstein–Barr virus (EBV), oriP. It mediates extrachromosomal replication of EBV genomes and has a modular bipartite structure. The family of repeats (FR) binds the EBV-encoded protein EBNA1 (Fig. L4) and tethers oriP to chromatin mediating replication-independent nuclear retention and long-term plasmid stability. The dyad symmetry element (DS) acts as a bona fide eukaryotic replication origin.


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and HMGA1a:EBNA1-DBD confers replication competence of oriP plasmids (20, 21) and EBV genomes (22). Thus, we asked whether HMGA1a could recruit ORC to DNA and thereby contribute to the molecular definition of a replication origin. Transient replication assays with an oriP reporter were performed in two HEK293 cell lines, which express EBNA1 or HMGA1a:EBNA1-DBD (Fig. 1A). Two to three weeks after transfection and selection, low-molecular-weight DNA was isolated and digested with DpnI to frequently cleave nonreplicated plasmid DNA, which had retained the dam methylation pattern acquired in the prokaryotic host. *Escherichia coli* cells were transformed with 500 ng of DNA, and ampicillin-resistant colonies were determined. Similar colony numbers were obtained with DNA from cells expressing EBNA1 or HMGA1a:EBNA1-DBD (Fig. 1A). Parental HEK293 cells or HEK293 cells expressing the DNA-binding domain of EBNA1, only, did not give rise to colonies (data not shown), confirming the function of HMGA1a in this system (20). ChIP experiments in synchronized HEK293 derivatives (supporting information (SI) Fig. 5) clearly indicated that HMGA1a:EBNA1-DBD mediates DNA replication of oriP in an ORC-dependent manner in synchrony with the cell cycle as expected (18).

**HMGA1a and ORC Associate.** The replication functions of EBNA1 depend on the interaction between this viral protein and ORC (7, 9). To assess whether HMGA1a:EBNA1-DBD interacted with ORC, we generated a HEK293 cell line with a Strep-tagged version of HMGA1a:EBNA1-DBD. Pull-down experiments indicated that it coprecipitated the four ORC subunits Orc1, 2, 4, and 6 (Fig. 1B, lane 6), suggesting that HMGA1a interacts with the entire ORC holocomplex. Albeit the interaction between HMGA1a:DBD-Strep and different ORC subunits was weak, it depended on HMGA1a, because the DNA-binding domain of EBNA1 alone, EBNA1-DBD, did not precipitate ORC subunits (data not shown). Conversely, an Orc2-specific antibody coprecipitated HMGA1a:EBNA1 (Fig. 1B, lane 8). Endogenous HMGA1a interacts with the Orc6 component of ORC (Fig. 1C Left and SI Fig. 6), as shown by coimmunoprecipitation with an HMGA1a-specific antibody but not an isotype control. Pull-down experiments with recombinant HMGA1a and Orc6 proteins indicate this interaction is direct and independent of DNA (Fig. 1C Right). Despite the relatively weak coprecipitation of ORC and HMGA1a:EBNA1-DBD, transient replication (Fig. 1A) and ChIP experiments (SI Fig. 5) provided clear evidence that the HMGA1a fusion could mediate specific binding of ORC, generating a functional origin of DNA replication.

**HMGA1a and ORC Interact in AT-Rich Heterochromatin in Living Cells.** To explore the character of the HMGA1a-ORC interaction in living cells, we used bimolecular fluorescent complementation (BiFC), which visualizes protein–protein interactions (23). HMGA1a was fused to the C-terminal fragment of YFP and YFP’s N-terminal part to different ORC subunits. We observed fluorescence complementation in living and fixed cells in the combination of HMGA1a and Orc1 and HMGA1a and Orc6 (Fig. 2A and SI Fig. 7) but not with other ORC subunits (SI Table 1). Seventy percent of Orc1:HMGA1a BiFC-positive cells and 73.9% of Orc6:HMGA1a BiFC-positive cells displayed interactions at the nuclear periphery and perinucleolar regions (Fig. 2A and B; for detailed numbers and intensity profiles, see SI Fig. 8). In living cells, the BiFC efficiencies between HMGA1a and Orc1 or Orc6 were 32.5% and 84.8%, respectively (Fig. 2A). Orc1 is cell cycle-regulated and becomes unstable after entry into S phase, which might explain the lower efficiency of the Orc1:HMGA1a BiFC complex.

Our coimmunoprecipitation experiments indicated that HMGA1a interacts with ORC. To validate whether fluorescence complementation between Orc components and HMGA1a origin (18). Deleting DS results in a replication-defective oriP mutant, indicating that EBNA1, when bound to FR, does not contribute to oriP replication (19). The transactivation domain of EBNA1 can be functionally substituted by HMGA1a (Fig. 1A).
(Figs. 1 B and C and 2A) is representative for this interaction, we investigated the localization of the core component Orc2 in relation to an HMGA1a-eGFP fusion in HepG2 cells. Consistent with our BiFC assays (Fig. 2 A), a significant colocalization of HMGA1a-eGFP with endogenous Orc2 was observed in perinucleolar regions (Fig. 2B; SI Fig. 9A). We also asked whether HMGA1a might direct the localization of ORC via HMGA1a's AT-hook domains, which mediate its preference for AT-rich regions. We used an HMGA1a mutant, HMGA1a(R3xG), in which glycines replaced the arginines (R28G, R69G, and R86G) of the AT-hook consensus motifs (GRP). This HMGA1a mutant shows a diffuse nuclear localization pattern (16). HeG2 cells expressing Orc6 and HMGA1a(R3xG)-BiFC proteins (93.6%) showed fluorescence complementation, and most Orc6/HMGA1a complexes were now found in nucleoli (Fig. 2 A; SI Fig. 8), indicating that HMGA1a can alter the localization of Orc6. All BiFC cells analyzed displayed a healthy morphology, and the diameter of the nucleoli of transfected cells was similar to those of untransfected cells (SI Fig. 8C).

HMGA1a preferentially binds through its AT-hooks to the minor groove of AT-rich sequences and can be displaced by several dyes binding to DNA (13). In living cells, Hoechst 33342 competed with the prevalent binding of HMGA1a/Orc6 complexes to AT-rich domains in a time-dependent manner (Fig. 2C a and b). The intensity profiles of the BiFC complexes were unaltered when the cells were incubated with Hoechst 33342 for 1 min, but HMGA1a/Orc6 relocated to the nucleoli after 10 min (SI Fig. 9 B and C). The prominent nucleolar localization of the HMGA1a/Orc6 complex after competition with this dye is most likely mediated by interactions of Orc6 with components of the ribosome biogenesis pathway (24) (M. Rohrmoser and A.W.T., unpublished data). Cotransferring the heterochromatin protein HP1α-mRFP revealed that HMGA1a/Orc6 partially colocalizes with HP1α (Fig. 2C and SI Fig. 9D for intensity profiles). This observation further indicates that the interactions of HMGA1a and ORC-subunits occur mainly in AT-rich heterochromatic domains but not in the entire heterochromatin. In summary, our data suggested that HMGA1a's AT-hook domains mediate the preferred heterochromatin positioning of the ORC/HMGA1a complex, but the integrity of this complex is independent of HMGA1a's subnuclear localization.

It is likely that nuclear factors other than HMGA1a can also determine ORC localization and specify replication origins, because immunofluorescence experiments with Orc2 and HMGA1a-eGFP clearly showed Orc2 localization beyond HMGA1a rich domains (25, 26). These results suggested that a certain molecular ratio between HMGA1a and ORC proteins might determine ORC localization to AT-rich heterochromatin regions. To analyze the targeting functions of HMGA1a, we first monitored the localization of ORC via BiFC between Orc6/Orc4 and Orc6/Orc5. The diffuse nuclear distribution is characteristic for interphase nuclei in Orc6/Orc4 and Orc6/Orc5 BiFC-

Fig. 2. BiFC analysis and colocalization of HMGA1a and Orc2. Fluorescence images of HepG2 cells expressing the indicated proteins were acquired 24 h after DNA transfection. (A) Analysis of interaction of HMGA1a with Orc1 and Orc6. HMGA1a fused to the N-terminal domain of YFP was cotransfected with Orc1 (I) and Orc6 (II) linked to the C-terminal fragment of YFP. (II) An HMGA1a variant with mutated AT-hooks (R3xG) was cotransfected with the Orc6-BiFC construct. To calculate BiFC efficiencies, cells were cotransfected with BiFC vectors and an mRFP-expression vector. For example, for Orc1, 32.5% of all cotransfected cells (n = 151) showed BiFC and 70% of BiFC-positive cells showed the depicted pattern. For intensity profiles, see SI Figs. 8 and 9. (B) Colocalization of Orc2 and HMGA1a-eGFP was visualized in fixed HepG2 cells by immunofluorescence using an Orc2-specific antibody. HMGA1a-eGFP and Orc2 colocalize especially in perinucleolar regions (arrows). (C) HMGA1a and Orc2 BiFC experiments in the presence of 5 μg/ml Hoechst 33342 incubated for 1 (a) and 10 min (b). (Scale bars: 10 μm.) (c) Signal complexes of HMGA1a/Orc6 BiFC- and HP1α-mRFP partially overlap. Orc6 and HMGA1a-BiFC plasmids were cotransfected with an HP1α-mRFP expression plasmid (see also SI Fig. 9D). To increase contrast, the yellow BiFC signal was changed to green. (D) Orc6/Orc6 and Orc5/Orc6 BiFC signals altered after overexpression of HMGA1a-mRFP. Orc4/Orc6 (a) and Orc5/Orc6 were cotransfected into HepG2 cells. Cotransfection of the indicated BiFC plasmids (a' and b') and HMGA1a-mRFP (a' and b') resulted in a significant colocalization of ORC (a' and b'). In A–C, Left shows interference contrast figures of the transfected cells. (Scale bars: 10 μm.)
experiments (Fig. 2D a and b) (26). Altering the molecular ratio between ORC after overexpression of HMGAla-mRFP caused relocation of the diffuse BiFC patterns of Orc4/Orc6 and Orc5/Orc6 resulting in colocalization with HMGAla-mRFP (Fig. 2D a’’’ and b’’’). It thus appeared that HMGAla could target ORC to AT-rich chromatin regions. Moreover, and consistent with our observations in Fig. 1C, the BiFC experiments confirmed Orc6 as an integral part of the human origin recognition complex in living cells.

HMGAla-Dependent Replication of Extrachromosomal Plasmids. The HMGAla/ORC interaction could also point to nonreplicative functions of ORC, such as heterochromatin formation (27), but the potential role of HMGAla in the definition of replication origins is of immediate interest. HMGAla-specific and ChIP grade antibodies are not available for analysis of endogenous HMGAla at chromosomal origins. Therefore, we asked whether HMGAla might be sufficient to target ORC to DNA creating a functional origin of DNA replication in a plasmid model system. HMGAla was fused to the single-chain tetracycline repressor scTetR to form the chimeric scTetR:HMGAla gene. scTetR:HMGAla and, as a control, the scTetR gene alone were stably integrated in HEK293/EBNA1+ cells (Fig. 3A) (28). Both HEK293 cell lines, EBNA1+/scTetR+ and EBNA1+ /scTetR:HMGAla+ were transfected with three reporter plasmids: a wild-type oriP control plasmid (oriP) or two test plasmids with four tetO sites (FRtet4tetO32) or 32 tetO sites (FRtetO32) to create clusters of binding sites with different density for scTetR:HMGAla (Fig. 3B; SI Table 2). In all tet reporter plasmids, EBNA1-binding sites within FR confer nuclear retention of plasmid DNA molecules only, but no DNA replication. Both FRtet4tetO plasmids did not replicate in EBNA1+/scTetR+ cells as expected, indicating that the tetracycline-repressor scTetR does not confer a functional replicator/initiator interaction (Fig. 3C). Similarly, an oriP-mutant with a deleted DS element (FRtetO2) was replication-deficient in 293/EBNA1+ cells (SI Fig. 10B). Under selection the FRtetO tetO32-plasmids stably replicated in EBNA1+/scTetR:HMGAla+ HEK293 cells similar to oriP (Fig. 3C) according to the once-per-cell cycle rule as assessed in Melsen-Stahl experiments (data not shown). Under nonselective conditions, both tetO plasmids were relatively unstable. Doxycyclin, which prevents binding of scTetR:HMGAla to tetO motifs (data not shown), further diminished their copy numbers in contrast to oriP (Fig. 3C).

Pull-down and imaging experiments (Figs. 1C and 2) suggested an interaction between HMGAla and ORC subunits. To assess this observation for scTetR:HMGAla, we performed coimmunoprecipitations with nuclear extracts of scTetR:HMGA1a, and an appropriately matched Ig control. The association of Orc3 at or near the oriP was compared by quantitative ChIP was performed. Drug-selected EBNA1+/scTetR:HMGAla+HEK293 cells carrying the four different tetO plasmids or the oriP control plasmid (Fig. 4A) were cross-linked, and the fragmented chromatin was subjected to immunoprecipitations using a human Orc3-specific antibody and an appropriately matched Ig control. The association of Orc3 at or near the tetO motifs was compared by quantitative PCR to that at distal reference sites (see map in Fig. 4C). As expected, oriP showed reproducible enrichment of Orc3 at the tetO proximal PCR fragment in relation to two distal control sites (Fig. 4C). Similar results were obtained with an Orc2-specific antibody (data not shown). The abundance of ORC components correlated directly with the number of tetO motifs and led to a higher replication efficacy of the test plasmids.

Repli-
indicates site-specific ORC binding. For each experiment, 500-cells (green bars) and scTetR:HMGA1a expressing scTetR:HMGA1a. Nascent DNA was purified from parental HCT116 C/two (FRwttetO8), four (FRwttetO16), and eight (FRwttetO32) arrays of tetO4-motifs. Efficient targeting of HMGA1a Specifies Replication Origins. Multiple dependent plasmids and recruit ORC to the origin of DNA replication in a sequence-independent manner (Fig. 4.D). The resulting plasmid pEPI-tetO20 was introduced in parental HCT116 cells and a derivative expressing scTetR:HMGA1a. Replication start sites were determined by nascent strand analysis after G418 selection. In parental HCT116 cells, pEPI-tetO20 did not show any locus-specific preference of nascent strand plasmid DNA (green bars, Fig. 4.D), as expected (31), pEPI-tetO20 exhibited a 5-fold abundance of nascent-strand DNAs near the integrated tetO sites relative to reference sites in cells expressing scTetR:HMGA1a, only (compare red and green bars, Fig. 4.D). These data indicated that high local concentrations of HMGA1a not only recruit ORC in a site-specific manner but also form a dominant origin in a replication system with otherwise multiple, apparently sequence-independent initiation sites.

Discussion

HMGA1a fused to the DNA-binding and dimerization domains of EBNA1 or TetR targets ORC to the cognate operator sites generating functional origins of DNA replication. ORC binding and replication competence did not rely on the plasmid background or an oriP-like configuration of tetO sites, but because an array of tetO sites integrated into the pEPI-vector generated a dominant and site-specific origin (Fig. 4.D). This study is not the first example that origins can be specified on plasmid DNAs (32, 33). However, a direct interaction between ORC and a chromatin constituent like HMGA1a in vivo, and a possible contribution to origin formation has not been described before.

Our in vivo and in vitro data indicate a direct interaction between HMGA1a and ORC subunits, corroborating HMGA1a’s interaction with the ORC holocomplex. Coimmunoprecipitation experiments with tagged and endogenous proteins point to a transient interaction between HMGA1a and Orc6 (Figs. 1 and 2). Orc6 is biochemically less tightly associated with other ORC subunits but is essential for ORC DNA binding in Drosophila (34). A higher local concentration of HMGA1a at a given site might stabilize this interaction leading to a more efficient recruitment of ORC and thus increased replication efficiency (Fig. 4). Assuming that HMGA1a is also involved in cellular DNA replication, only a subset of chromosomal AT-sites might become competent for HMGA1a-mediated ORC binding and DNA replication when HMGA1a is present at high local density. Our BiFC experiments indicate that these sites are preferentially located in AT-rich heterochromatin domains.

HMGA1a is a multifunctional chromatin protein. Our data suggest now that it plays a role in recruiting ORC to AT-rich heterochromatin domains. All members of the HMGA protein family have been described as architectural transcription factors, and several studies link HMGA proteins to cell proliferation. Overexpression of HMGA1a can transcriptionally up-regulate cell cycle and growth regulators, i.e., cyclin A, p38 MAPK or N-myc (17). In addition, steady-state protein levels of HMGA1a are elevated in cancer cells with high proliferative potential (16) but decreased in differentiated cells. In contrast, deletion of one Hmga allele results in a pygmy phenotype in mice (15), and the expression of antisense RNA or a dominant-negative HMGA1a variant diminishes proliferation of tumor cells (17). Our own results suggest an additional nontranscriptional function of
HMGA1a in proliferation control, on the basis of chromosomal DNA replication.

A connection between ORC and heterochromatin is well documented and seems to be conserved throughout evolution. For example, ScORC is essential for silencing the HMR-loci (35, 36), and mutations in the Orc2 subunit of Drosophila reduce this ability to form heterochromatin (37). Therefore, Leatherwood and Vas hypothesized that heterochromatin might require additional ORC to replicate these tightly condensed regions (38). In analogy to the S. pombe Orc4 subunit, our data suggest a cofactor model in which HMGA1a recruits ORC to certain sites in heterochromatin to form functional origins of DNA replication in metazoan cell DNA. Further experiments shall reveal whether HMGA1a is instrumental in replicating chromosomal origins.

Materials and Methods

DNA Transfection, Plasmid Rescue, and Southern Blotting. Plasmid DNAs were transfected with Polyfect into HEK293 cells and derivatives, which were selected with 100 μg/ml hygromycin, 250 μg/ml puromycin, or 200 μg/ml neomycin. Five hundred nanograms of low-molecular-weight DNA was digested with DpnI and electroporated into E. coli DH10B, which were selected with ampicillin. For Southern blotting, 6 μg of DNA was separated, transferred onto nylon membranes (Amersham), and probed with a radiolabeled prokaryotic probe. Signals were quantified with the Imager FLA-5100 (Fuji).

ChIP and Real-Time PCR Analysis. ChIP experiments were performed as described (9). (1) (10) were isolated, cross-linked with formaldehyde for 10 min at 37°C, washed, and lysed by adding Na-laurylsarcosine (2% final concentration). After washing, the chromatin was resuspended in 2 ml of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA) and sonicated. Five hundred micrograms of nucleoprotein was immunoprecipitated with 10 μg of polyclonal (Orc2, Orc3, and Mcm7) or monoclonal (EBNA1) antibodies in 50 mM Tris, 150 mM NaCl, 0.5 mM EDTA, and 0.5% Nonidet P-40 (NET). Coprecipitated DNA was isolated and purified, and quantitative real-time PCR was performed as described (9). Primer pairs are listed in Table S1.

Inmunofluorescence and Live-Cell Microscopy. Fluorescence complementation (23) was performed with a Leica TCS-SP2/AOBS instrument. 22-26 h after transfection of 1 μg of the indicated expression plasmids. For immunolocalizations, cells were fixed in 2% formaldehyde/PBS, washed, and permeabilized. Antibodies to detect HA- and Flag-tags were used as described in SI Text; DNA was visualized with 5 μg/ml Hoechst 33342.

Commmunprecipitation Assays and Western Blot. Chromatin-bound proteins were isolated by high salt extraction from 2.5 × 106 cells and incubated with 5–10 μl of antibodies coupled to protein A or G Sepharose. Bound proteins were eluted, separated on SDS/PAGE, blotted, and detected with the indicated antibodies.

Nascent-Strand Analysis. Nascent DNA of HCT116 cells transfected with pePltetO2β was analyzed as described (39).

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*Numbers in parentheses denote the size of the plasmids in kbp.

†Destabilized d2GFP
Table 3. Oligos used in this work for PCR analysis

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SI Text

Material and Methods

Cell lines. The HEK293 cell line is derived from primary human embryonal kidney cells transformed with human adenovirus type 5 DNA (1). 293'D is a clonal derivative established in our laboratory. A clonal HEK293 EBNA1-expressing cell line was established by stable transfection of 293'D cells with two plasmids, which express EBNA1 (p2816) and neomycin phosphotransferase (p2727), respectively, and selection with 200 µg/ml G418. scTetR (p3248) and scTetR:HMGA1a (p3265) expressing HEK293 cell clones were generated similarly with plasmids encoding hygromycin and puromycin resistance and selection with hygromycin (100 µg/ml) and puromycin (250 ng/ml), respectively. A HEK293 cell clone, which expresses both EBNA1 and scTetR:HMGA1a was established on the basis of HEK293 EBNA1 cells. This cell line was cotransfected with expression plasmids encoding scTetR:HMGA1a (p3265.12) and puromycin resistance (p3223.9). The expression of EBNA1, HMGA1a:EBNA1, scTetR, or scTetR:HMGA1a proteins in the clonal derivatives of HEK293 cells were confirmed by Western blot analysis with the monoclonal antibody 1H4 directed against EBNA1 or a polyclonal rabbit antisera directed against TetR B protein (2). All HEK293 cells were maintained in RPMI1640 medium with 10% FCS, 100 units of penicillin per ml, and 100 µg of streptomycin per ml at 37°C in a 5% CO₂ atmosphere. HCT116 was cultivated in McCoy’s medium, C2C12, and HepG2 cells, a human hepatoma-derived cell line (3), were maintained in Dulbecco-modified medium. SF9 cells were cultivated in Grace’s Insect Medium (Gibco/Invitrogen) supplemented with 10% FCS.

Plasmids. Plasmids carrying oriP, hybrid oriP replicons, or oriP-like replicons are summarized in SI Table 2 along with their key features. All plasmid DNAs were prepared with Jetstar 2.0 columns (Genomed). pEPI is a kind gift from H. J. Lipps (Witten/Herdecke) (4). pEPI-tetO²⁰ is based on pEPI and was generated by oligomerization of oligonucleotides containing tetO motifs. Genetic modification of the DS element was done with synthetic oligonucleotides at the appropriate locations by conventional cloning techniques. DNA sequencing confirmed the sequence composition of the critical regions. The plasmids encoding EBNA1, scTetR and
scTetR:HMGA1a express their transgenes from the immediate-early human cytomegalovirus promoter. The expression plasmid encoding scTetR pWHE120 (sB+B)/scTA2(2) is published; scTetR:HMGA1a (p3265.12) is based on pWHE120 (sB+B)/scTA2 in which its triple F domain was replaced by the entire coding sequence of the human HMGA1a cDNA. For BiFC cDNAs were subcloned into pBiFC-YC155 and pBiFC-YN155 expression vectors (5), which were kindly provided by Tom Kerppola. These vectors allow the expression of HA-tag-YC or Flag-tag-YN fusion proteins. BiFC plasmids encoded HMGA1a, HMGA1aR3xG (R3xG)(6), and Orc1, -2, -5, and -6. All plasmid DNA sequences and cloning procedures are available upon request. All plasmid DNA sequences are available upon request.

**DNA transfections.** DNA transfections into HEK293 cells and derivatives were performed with Polyfect (Qiagen). Cells were seeded at about 50% confluence into six-well cluster plates one day before transfection. For transfection, cells in six well cluster plates were incubated in 1 ml OptiMEM medium (Invitrogen) for 1h and incubated with plasmid DNA embedded in lipid micelles (1 µg DNA, 5 µl Polyfect per well, 100 µl of RPMI1640 without supplements; preincubation for 15min at room temperature) overnight. After one to two days, the cells were transferred onto 13 cm cell culture dishes and cultivated in the presence of 100 µg/ml hygromycin, 250 ng/ml puromycin or 200 µg/ml G418.

**Plasmid rescue, Southern blotting, and quantification.** To determine the number of copies in transfected HEK293 cells low molecular weight DNA was isolated (7) and digested with DpnI to cleave unreplicated plasmid DNAs, which retain the dam methylation pattern that the plasmids had acquired during propagation in *Escherichia coli*. Five hundred nanograms of DNA was introduced into the *E. coli* DH10B strain by electroporation (1,800 V, 25 µF, 100 Ohm, 1-mm gap cuvettes, Genepulser, Bio-Rad). Transformants were selected on agar plates containing 100 µg/ml ampicillin. Several colonies were picked and the DNA composition of the rescued plasmid was confirmed with restriction enzyme analysis. For Southern blotting, 6 µg of isolated low molecular weight DNA was separated on an 0.8% TAE gel, transferred onto a nylon membrane (Amersham Pharmacia) and detected with a radiolabeled probe. Signal strengths were quantified with the Imager FLA-5100 (Fuji).
**Chromatin-immunoprecipitation assay and real-time PCR analysis.** For chromatin immunoprecipitation experiments, nuclei were prepared as described (8). For each sample $1 \times 10^7$ cells were harvested, washed with PBS and resuspended in 250 µl hypotonic buffer A [10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM MgCl$_2$, 0.34M sucrose, 10% glycerol, 1 mM DTT, protease inhibitor mix Complete (Roche)]. Cells were lysed by adding 0.04% Triton X-100 and incubated for 10 min on ice. Samples were centrifuged (4 min, 1,300 g, 4°C) to separate soluble cytosolic and nucleosolic proteins from chromatin. Nuclei were washed at a concentration of $1 \times 10^8$ nuclei/ml in ice-cold buffer A supplemented with 200 mM NaCl. After centrifugation (1,300 g, 5 min, 4°C) nuclei were carefully resuspended in 1 ml buffer A. Nine milliliters prewarmed buffer A supplemented with 1.1% formaldehyde were added and the nuclei cross-linked for 10 min at 37°C. Fixed nuclei were washed twice with PBS/0.5% Nonidet P-40, resolved in 2.7 ml LSB (10 mM Hepes pH7.9, 10 mM KCl, 1.5 mM MgCl$_2$) and lysed by adding 300 µl 20% Sarkosyl. The chromatin was transferred onto a 40 ml sucrose cushion (LSB plus 100 mM sucrose) and centrifuged (10min, 4°C, 4000g). Supernatant was removed and the chromatin was resuspended in 2 ml TE and sonicated (Branson sonifier 250-D, 35% amplitude, 2min in 1sec intervals). For partial DNA digests, 2 mM CaCl$_2$ and 8 units MNase (Roche) were added and incubated for 10 min at 37°C. The reaction was stopped by adding 5 mM EGTA. For each immunoprecipitation, 500 µg of the nucleoprotein was adjusted with 1/10 volume of 11xNET (final concentration: 50 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40). 10 µg of polyclonal rabbit antibodies directed against Orc2, Orc3 and Mcm7 proteins or 50 µl hybridoma supernatant of the monoclonal EBNA1-specific antibody 1H4 were added. The immunoprecipitation and purification of coprecipitated DNA was performed as described (9). Real-time PCR analysis was performed according to the manufacturer’s instructions using the same parameters and the primer pairs as described (9). The primer pairs used are given in Tab. 1.

**Immunofluorescence and live cell microscopy.** Fluorescence complementation in living cells was inspected 24-28 h after cotransfection of 1 µg of the indicated expression vectors. A Leica TCS-SP2/AOBS microscope and the 514 nm laser line of an argon laser were used for detection. Interference contrast was used to control viability and integrity of cells. For DNA staining in living cells, Hoechst dye 33342 was added at 5 µg/ml for one and ten minutes. After a brief wash...
step in culture medium, cells were inspected immediately within the next 10 min. For parallel immunolocalizations, cells grown on cover slips were washed in PBS, fixed in 2% formaldehyde/PBS for 15 min, washed again and permeabilized in ice cold 0.5% Triton/PBS for 5 min. Expression of YC-fusion proteins was controlled using monoclonal rat-antibodies directed against HA-tag (1:50); expression of YN-fusion proteins was controlled using mouse-antibodies directed against Flag-tags (1:500; Sigma). Secondary antibodies were anti-rat-Cy5 (1:100; Dianova) and anti-mouse-Tx-red (1:100, Dianova). Antibodies were diluted in PBS (pH 7.4) and incubated subsequently for 45 min in a humidified chamber at room temperature. To stain DNA, 10 µl of Hoechst dye/PBS (5 µg/ml) were added and incubated for further 10 min. After two final washing steps, cover slips were mounted in Mowiol.

HepG2 cells were grown on glass cover slips and cotransfected with the 1 µg of the indicated expression vectors using HD Fugene (Roche) according to the manufacturer’s instructions. C2C12 mouse myoblast cells stably expressing HMGA1a-eGFP were generated by retroviral transfection and grown in DMEM containing 4.5 g/liter glucose (Gibco/Invitrogen), supplemented with 1% L-glutamine and 10% FCS. For immunofluorescence of endogenous Orc2, a rabbit polyclonal antibody (pAB205) was used (10). Immunofluorescence images were analyzed with a Leica TCS-SP2/AOBS microscope using a HCX PI APO lbd.Bl. 63 × 1.4 oil immersion lens. Images were analyzed in the sequential scan mode using the 405 nm diode for detection of Hoechst dye, the 514 nm laser line of the argon laser for YFP, the 561 nm DPSS-Laser for Tx-red and a HeNe-Laser at 633 nm for Cy5. GFP was analyzed using the 488 nm laser line of an argon laser.

BiFC efficiencies were determined by counting of transfected cells under a fluorescence microscope. Cotransfection of an mRFP expression plasmid was used as transfection marker. Intensity profiles were measured using optical sections and the ‘Profile’ tool of the Leica software. Relative intensities were calculated using the formula $1/(MI/I_{dx})$ with $MI = \text{mean intensity}$ and $I_{dx} = \text{intensity measured at distance } x$.

**Pulldown experiments with recombinant HMGA1a and Orc6.** For the expression of recombinant Orc6, Sf9-insect cells were infected at an MOI = 5 with a baculovirus encoding C-
terminal His$^{(6)}$-tagged Orc6 (kindly provided by R. Knippers). 60 h after infection, cells were lysed by the addition of ice-cold lysis buffer (PBS, 2 mM MgCl$_2$, 0.1% Nonidet P-40, 10% glycerol) and NaCl was added to a final concentration of 500 mM. After centrifugation, the supernatant containing Orc6-His$^{(6)}$ was subjected to a Ni NTA-affinity purification. Orc6 containing fractions were pooled and dialyzed against pulldown buffer (125 mM NaCl, 5% Glycerol, 10 mM Hepes pH 7.9, 1.5 mM MgCl$_2$). HMGA1a was cloned into pET3c vector (Novagen) and expressed as an N-terminal hemagglutinin-Strep-Tag II fusion protein in *E. coli Rosetta*(DE3) pLysS. After IPTG induction for 4h at 37°C, cells were harvested and HA-Strep-HMGA1a was acid extracted like described (11). To remove nucleic acid from the proteins, 1 M NaCl washing steps were included during purification. For pulldown experiments 10 µg recombinant HA-Strep-Tag-II-HMGA1a and Orc6-His$^{(6)}$ were rotated with 20 µl Strep-Tactin-Sepharose for 1 h at 4°C. After repeated washes with pulldown buffer the retained proteins were eluted by the addition of 10 mM desthiobiotin (dissolved in pulldown buffer; IBA Bio TAGnology). Eluates were analyzed by Western blotting. As a control experiment, Orc6-His$^{(6)}$ was incubated with Strep-Tactin-Sepharose in the absence of HA-Strep-Tag-II-HMGA1a to rule out unspecific binding of Orc6- His$^{(6)}$ to the affinity matrix.

**Coimmunoprecipitation assays and Western blot analysis.** Protein extracts were prepared as follows: after swelling cells in hypotonic buffer (10 mM KCl, 340 mM Sucrose, 10 mM Hepes pH 7.9, 1.5 mM MgCl$_2$, 10% glycerol), nuclei were prepared either by mechanical disruption in a Wheaton tight 7.5 ml douncer or by the addition of Triton X- 100 to a final concentration of 0.06%; Chromatin proteins were extracted for 1h with extraction buffer (450 mM NaCl, 10 mM Hepes pH 7.9, 1.5 mM MgCl$_2$). After centrifugation at 200,000g for 1h the extract was dialyzed against 125 mM NaCl, 10 mM Hepes, pH 7.9, 1.5 mM MgCl$_2$, until NaCl concentration reached 150 mM (all buffers contained freshly added 1 mM ATP, 1 mM DTT, complete Protease inhibitor EDTA free, Roche). 5-10 µg antibody was used per immunoprecipitation of extracts from 2.5 × 10$^7$ cells. Immunocomplexes were precipitated using protein G- or protein A Sepharose. Bound proteins were eluted either with 5% N-Laurylsarcosine or Laemmli sample buffer, separated on SDS/PAGE, and blotted with the respective antibodies. For HMGA1a immunoprecipitations a rabbit polyclonal antibody was purchased from Santa Cruz.
**Nascent-strand analysis (12).** HCT116 cells were transfected at a density of $5 \times 10^5$ cell per well in six-well dishes with 2 $\mu$g of pEPI-tetO$^{20}$ plasmid DNA using Lipofectamine according to the manufacturer’s instructions. Cells were selected for 3 weeks with 200 $\mu$g/ml G418. Up to $8 \times 10^6$ cells were directly loaded onto an alkaline low melting point 1.2% agarose gel (running gel buffer: 50 mM NaOH, 1 mM EDTA). After 15 min of cell lysis DNA was separated at 45V at 4°C for 24 h. Single-strand DNA was excised in a range of 700 to 1100bp and purified (Macherey & Nagel 2 in1 Nucleospin Extract Kit II) following the manufacturer’s instructions. Nascent strands were quantified by real time PCR using pEPI-tetO$^{20}$ dilutions as a standard. Nascent strand abundance was calculated relative to the control region spanning the HSV polyA signal, which was arbitrarily set to one.


Supplementary Figure 1: HMGA1a:EBNA1 fusion supports oriP-mediated replication in an ORC-dependent manner.

An oriP plasmid (p2832) was transfected into HMGA1a:EBNA1-DBD expressing HEK293 cells, which were selected for two weeks. Cells were synchronized with mimosine (0.5mM) or nocodazole (40ng/ml) at G1/S or in mitosis, respectively. Immunoprecipitations with Orc2-, EBNA1- or Mcm7-specific antibodies were performed with 500µg formaldehyde cross-linked nucleoprotein and coprecipitated plasmid DNA was quantified by real-time PCR analysis using the DS-specific primer pair sc5 (left panel) (1). No enrichment was detected at a reference site located in the puromycin resistance gene (right panel). The histogram shows the results of cells synchronized in G1/S (black bars) and M phase (white bars). The heights of the columns indicate the relative enrichment (mean values and standard deviation of three independent experiments) on a logarithmic scale expressed as the difference between PCR values obtained with the indicated specific antibodies versus controls obtained with pre-immune serum or an isotype-matched antibody.

Supplementary Figure 6: sctetR:HMGA1a and ORC coprecipitate
To scrutinize the interaction between HMGA1a and ORC, nuclear extracts from HEK293 cells either expressing scTetR:HMGA1a or scTetR were immunoprecipitated with antibodies against human Orc2 (lanes 1-3) or Orc6 (lanes 4-6, 9, 10). Western blot analysis was performed with antibodies specific for Orc1 (top), TetR (middle), and Orc6 (bottom). scTetR:HMGA1a migrates at the size of 68 kDa, scTetR at the expected size of 52 kDa comigrating with the heavy chain of the precipitating antibody (*). Lanes 7, 8, 11 and 12 show a Western blot of nuclear extracts from 5x10^4 cells.
Supplementary Figure 2

Supplementary Figure 2. Top panel: Formation of the bFosYC-bJunYN Bimolecular Fluorescent Complex as described by Hu and Kerppola\(^1\) as a positive control. Bottom panels: For immunolocalization in HepG2 cells transiently transfected with the indicated BiFC plasmids. YN-plasmids express a FLAG-tagged fusion proteins, the corresponding YC-plasmids express an HA-tagged fusion proteins for detection of both bimolecular fluorescence (left row) and immunofluorescence (middle rows). DNA is stained with DAPI (right row). Only doubly transfected cells show bimolecular fluorescence complementation. The bars indicate 10µm.

Supplementary Figure 3

A

B

non-transfected  A1a / Orc1  A1a / Orc6  A1a-R3xG / Orc6

nuclear diameter (μm)
Supplementary Figure 4

A

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Relative intensity profiles:

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Distance in μm
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Relative intensity profile:

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Distance in μm
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Supplementary Figure 5: Control experiments in HEK293 cells expressing only scTetR or EBNA1

(A) Design of different vector constructs: The wild-type oriP plasmid consists of the 'family of repeats' (FR) and the replicator element, the dyad symmetry element (DS). FR is an array of 20 high affinity EBNA1 binding sites (black circles); The DS element encompasses two pairs of EBNA1 binding sites (black circles). The plasmid FR<sup>wt</sup>/DS<sup>Δ</sup> lacks DS. Four tet-operator sites (tetO<sup>4</sup>, red circles) replace DS in the plasmid FR<sup>wt</sup>/tetO<sup>4</sup>. (B) The plasmids FR<sup>wt</sup>/tetO<sup>4</sup>, FR<sup>wt</sup>/DS<sup>Δ</sup>, and oriP were transfected into HEK293 cells expressing either scTetR:HMGA1a or EBNA1. Low molecular weight DNA was prepared after two to three weeks of selection and digested with DpnI and HindIII. The radioactive probe used in the Southern blot hybridizations recognized a specific DNA fragment of 3.5 kbp (black arrow head). A background signal appears at 2.5kbp (open arrow head). The FR<sup>wt</sup>/DS<sup>Δ</sup> plasmid was not maintained in both cell lines suggesting that it does not replicate because it lacks a functional origin. As expected in EBNA1<sup>+</sup> cells, only the oriP plasmid replicated and was retained. It carries the two essential cis-acting nuclear maintenance and replicator elements, which are recognized by EBNA1 (right). In the absence of EBNA1 in scTetR:HMGA1a cells (left), the plasmid FR<sup>wt</sup>/tetO<sup>4</sup> was presumably not extrachromosomally retained during selection.