FAD24 Acts in Concert with Histone Acetyltransferase HBO1 to Promote Adipogenesis by Controlling DNA Replication*

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Preadipocytes differentiate into adipocytes through approximately two rounds of mitosis, referred to as mitotic clonal expansion (MCE), but the events early in the differentiation process are not fully understood. Previously, we identified and characterized a novel gene, fad24 (factor for adipocyte differentiation 24), induced to express at the early stages of adipocyte differentiation. Although fad24 clearly has crucial roles in adipogenesis, its precise functions remain unknown. Here we show that the knockdown of fad24 by RNAi in 3T3-L1 preadipocytes repressed MCE. Moreover, FAD24 interacts with HBO1, a histone acetyltransferase and positive regulator of DNA replication initiation. The knockdown of hbo1 repressed MCE and adipogenesis, indicating that FAD24 acts in concert with HBO1 to promote adipogenesis by controlling DNA replication. Regarding the molecular mechanisms behind the regulation of DNA replication by fad24, we revealed that FAD24 co-localizes with HBO1 to chromatin during late mitosis, which is when the pre-replication initiation complex is assembled. Furthermore, chromatin immunoprecipitation experiments indicated that FAD24 localizes to origins of DNA replication with HBO1. When fad24 expression was inhibited during adipocyte differentiation, the recruitment of HBO1 to origins of DNA replication was reduced. Thus, FAD24 controls DNA replication by recruiting HBO1 to origins of DNA replication and is required for MCE during adipocyte differentiation.

Adipose tissue is important not only for energy homeostasis, but also for secreting adipocytokines such as adiponectin, leptin, resistin, and tumor necrosis factor-α (1–4). Obesity, an excess of adipose tissue, is a major risk factor for diabetes, hypertension, hyperlipidemia, and also arteriosclerosis (5). The development of obesity is thought to be mainly dependent on the expansion of adipocytes both in size and in number (6, 7). Because the proliferation and subsequent differentiation of preadipocytes could increase the number of adipocytes, further insight into the molecular basis of these two processes is required.

When growth-arrested 3T3-L1 preadipocytes, fibroblastic cells often used to study the physiology of adipocytes, are treated with inducers of differentiation, they undergo approximately two rounds of mitosis, referred to as mitotic clonal expansion (MCE), and then differentiate into mature adipocytes (8–11). MCE appears to be necessary for optimal differentiation, and DNA replication and changes in chromatin structure during MCE might facilitate the transcription of genes leading to the terminal differentiation (9, 10). It is clear that the transcription factor CCAAT/enhancer-binding protein (C/EBP)β and the cyclin-dependent kinase inhibitor p27 play crucial roles in MCE (12–14), but this process is not fully understood.

It is known that the mid- and late stages of adipocyte differentiation are regulated by three classes of transcription factors, namely peroxisome proliferator-activated receptor γ (PPARγ), C/EBPs, and sterol regulatory element-binding protein-1 (SREBP-1) (15, 16). Moreover, it is becoming clear that adipogenesis is regulated by an elaborate network of transcription factors and cell-cycle regulators (17). However, the earliest step in the differentiation process remains largely unknown, although others have recently reported regulators that are important during this period (18–21).

Previously, we isolated 102 genes induced to express at the beginning of the differentiation of 3T3-L1 cells, using the PCR-subtraction-cloning method (22, 23). Of the genes, those for regulator of G protein signaling 2 and TCL/TC10β promote adipogenesis (24, 25). Moreover, 3 of the 102 genes were identified as novel, factor for adipocyte differentiation (fad) 24, fad104, and fad158 (26–28). Gain-of-function and/or loss-of-function experiments indicated that these gene products are positive regulators of adipogenesis (26–28), but the molecular functions of these factors have not been investigated in detail.

In this study, we show that MCE was inhibited when fad24 expression was knocked down in adipocyte differentiation. It

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2 The abbreviations used are: MCE, mitotic clonal expansion; BrdU, bromodeoxyuridine; C/EBP, CCAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; DAPI, 4′,6-diamidino-2′-phenylindole dihydrochloride; EGFP, enhanced green fluorescence protein; fabp4, fatty acid-binding protein 4; fad, factor for adipocyte differentiation; HBO1, histone acetyltransferase binding to ORC1; ING, inhibitor of growth; MCM, minichromosome maintenance; Noc, nucleolar complex-associated protein; ORC, origin recognition complex; ORF, open reading frame; PPARγ, peroxisome proliferator-activated receptor γ; pre-RC, pre-replicative complex; RNAi, RNA interference; shRNA, short hairpin RNA; SREBP-1, sterol regulatory element-binding protein 1; WCE, whole cell extracts; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate.
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was also revealed that FAD24 interacts with histone acetyltransferase binding to ORC1 (HBO1), a positive regulator of DNA replication initiation (29–33). The knockdown of hbo1 impaired the ability of 3T3-L1 cells to differentiate into mature adipocytes by inhibiting MCE. Moreover, immunofluorescence and chromatin immunoprecipitation (ChIP) experiments indicated that FAD24 contributes to the regulation of DNA replication through the recruitment of HBO1 to origins of DNA replication. The functional relationship between FAD24 and HBO1 in DNA replication is discussed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Differentiation, and Cell Counting**—Mouse 3T3-L1 preadipocyte cells were cultured and induced to differentiate as previously described (26). HeLa cells were also cultured as described (26). For counting during adipocyte differentiation, the cells were trypsinized in 24-well plates at each time point after induction and collected by centrifugation. Then, an aliquot was subjected to cell counting using a hemocytometer.

**RNAi**—The shRNA expression plasmids for fad24 and the negative control plasmid were used as described (26). The three target regions (1) 564–584 bp; (2) 941–961 bp; (3) 1679–1699 bp) were selected according to Qiagen siRNA online design tool for the RNAi experiment with hbo1. A 19-base shRNA-coding fragment with a 5′-TTCAAGAGA-3′ loop was subcloned into the ApaI/EcoRI site of pSilencer 1.0-U6 (Ambion). Transfection of the shRNA expression plasmids into 3T3-L1 cells was performed as previously described (26).

**RNA Isolation, Q-PCR, and Northern Blot Analyses**—Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer’s instructions. The reverse transcription and Q-PCR were performed as previously described (26). The pre-designed primers and probe sets for fad24, hbo1, cyclin E1, pparγ, cebpα, srebp-1, fabp4, and 18 S rRNA were obtained from Applied Biosystems. Northern blot analyses were also performed as described (26).

**BrdU Labeling, Immunofluorescence, and FACS Analyses**—For BrdU labeling and immunofluorescence microscopy, 3T3-L1 cells were plated on coverslips and induced with differentiation cocktails. At 16 h after induction, cells were labeled for 2 h with 30 µg/ml BrdU (BD Biosciences). Coverslips were fixed in 70% ethanol for 30 min and incubated in 100% methanol for 10 min at room temperature. The fixed cells on coverslips were then treated for 30 min with 1.5 M HCl, blocked with 0.5% Tween 20 in PBS for 5 min, incubated with FITC-anti-BrdU monoclonal antibody (BD Biosciences) for 30 min at room temperature, and incubated with 7-aminooctanoyl D (BD Biosciences) for 5 min at room temperature. At each step described above, the cells were washed with PBS three times after treatment. For FACS analyses, BrdU-labeled 3T3-L1 cells were treated with a BrdU flow kit (BD Biosciences) as directed by the supplier. These cells were analyzed with FACScan (Becton Dickinson).

**Plasmid Construction**—For the construction of the 3×FLAG-tagged FAD24 expression plasmid or the EGFP-fused FAD24 expression plasmid, the mouse FAD24 open reading frame (ORF) was subcloned into the EcoRI/EcoRV site of pCMV10 (Sigma) or the EcoRI/BamHI site of pEGFP-C1 (BD Biosciences Clontech). The ORFs of mouse ING5 and rat HBO1 were amplified by RT-PCR using 3T3-L1 RNA and rat liver RNA, respectively, and subcloned into the EcoRV site of pBluescript KS (Stratagene). To generate the Myc-tagged ING5 expression plasmid and the Myc-tagged HBO1 expression plasmid, the ING5 ORF and the HBO1 ORF were subcloned into the EcoRI/BglII site and Sall/NotI site of pCMV-Myc (BD Biosciences Clontech), respectively. The sequences were determined with an ABI PRISM 310 (Applied Biosystems).

**Western Blot Analyses and Antibodies**—Proteins were resolved using SDS/PAGE, transferred to the polyvinylidene difluoride membrane, and probed using primary antibodies and secondary antibodies conjugated with alkaline phosphatase. Specific proteins were detected with BCIP/NBT phosphatase substrate (KPL). The antibody against mouse FAD24 was raised in rabbits using peptide 8 (residues 426–446 of FAD24) conjugated with KLH. The following antibodies were obtained commercially: anti-β-actin (AC-15, Sigma), anti-HBO1 (N-18, Santa Cruz Biotechnology), anti-ORC4 (ab9641, Abcam), anti-MCM2 (N-19, Santa Cruz Biotechnology), anti-CDK6 (180.2, Santa Cruz Biotechnology), anti-FLAG M2 (SIGMA), and anti-c-Myc (BD Biosciences Clontech).

**Immunoprecipitation Assay**—The 3× FLAG-tagged FAD24 expression plasmid or empty plasmid was introduced into HeLa cells by the calcium phosphate co-precipitation method (34). The cells were harvested 48 h after transfection, and...
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FIGURE 2. Effect of the knockdown of fad24 on MCE. A, Q-PCR analyses of the expression of fad24. Total RNA was obtained from 3T3-L1 cells transfected with plasmids expressing shRNA for fad24 (shfad24: white bar) or with plasmids expressing scrambled shRNA as a control (Control: black bar) at 12 h after induction. The expression level of fad24 was determined by Q-PCR and normalized with 18 S rRNA expression. B, effect of shRNA for fad24 on cell proliferation during MCE. Day 0 post-confluent shfad24-treated cells (gray line) or control cells (black line) were induced to differentiate into adipocytes. Cell numbers were determined at different time points after induction. C, effect of shRNA for fad24 on the incorporation of BrdU during MCE. The shfad24-treated cells or control cells were labeled with BrdU at 16 h after induction and stained with the FITC-conjugated anti-BrdU monoclonal antibody and 7-amino-actinomycin D (DNA). The fluorescence of BrdU (green) and DNA (red) was detected with a fluorescence microscope. The cells labeled with BrdU in three microscopic view fields were counted for each experiment, and the results from three experiments were averaged. Each column represents means ± S.D. (n = 3). The asterisk indicates significant difference when compared with the value for control cells (p < 0.05). D, for FACS analyses, shfad24-treated cells (white bar) or control cells (black bar) were labeled with BrdU at 16 h after induction, washed, and stained with the BrdU flow kit. The percentages of cells in S phase were determined by flow cytometry. E, effect of shRNA for fad24 on the expression of cyclin E1. Total RNA obtained from shfad24-treated cells (white bar) or control cells (black bar) at different time points after induction was subjected to Q-PCR for cyclin E1. The expression levels were normalized with 18 S rRNA expression. Each value in A, B, D, and E represents the mean ± S.D. (n = 3). The asterisks in B, D, and E indicate significant differences when compared with the values for control cells (p < 0.01).

RESULTS

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lysates were prepared with TNE buffer (10 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 0.15 M NaCl, and 1 mM EDTA) containing the protease inhibitor mixture. These lysates were incubated with the anti-FLAG M2 antibody or the anti-HBO1 antibody overnight at 4 °C. The mixtures were incubated with Protein G-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. For control experiments, normal mouse IgG or normal goat IgG (Santa Cruz Biotechnology) was used. After extensive washing, the bound proteins were separated by SDS/PAGE and detected by Western blotting.

Subcellular Localization of FAD24 and HBO1—HeLa cells were plated onto coverslips 1 day before transfection. The cells were transfected with the EGFP-fused FAD24 expression plasmid and/or the Myc-tagged HBO1 expression plasmid by the calcium phosphate co-precipitation method, and fixed with 3% paraformaldehyde, 0.1 mM CaCl₂, and 0.1 mM MgCl₂ for 20 min at room temperature. The coverslips were incubated with anti-c-Myc or anti-C23 (MS-3, Santa Cruz Biotechnology) monoclonal antibody in PBS overnight at 4 °C. After five washes with PBS, the secondary antibody labeled with TRITC (Sigma-Aldrich) was incubated for 30 min at room temperature. After five more washes with PBS, EGFP and TRITC signals were detected with a fluorescence microscope or a confocal laser scanning microscope.

ChIP Assay—Approximately 1.0 × 10⁷ cells were incubated with 1% formaldehyde for 10 min at room temperature. Cross-linking was terminated by the addition of the stop solution (10 mM Tris-HCl, pH 8.0, and 2 mM glycine). The cells were washed with PBS and then scraped into 1 ml of cold lysis buffer (25 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, and 150 mM NaCl) containing the protease inhibitor mixture. These cell lysates were sonicated 10 times for 10 s each time to generate DNA fragments that ranged in size from 200 to 1000 bp. The sheared chromatin-lysed extracts were incubated with either 2 μg of the indicated antibody or 2 μg of normal IgG overnight at 4 °C. Each extract-antibody mixtures were incubated for an additional 1 h with protein G-Sepharose beads. The immunoprecipitates were washed according to the conventional method (35), suspended in the extraction buffer (TE pH 8.0, and 1% SDS), and incubated overnight at 65 °C and for 2 h at 37 °C with 100 μg of proteinase K to reverse proteins/DNA cross-links. Finally, these samples were processed for DNA purification by the phenol-chloroform extraction method and ethanol precipitation. PCRs were performed in 20 μl with 1:1000 to 1:4000 of input DNA and 1:10 of the immunoprecipitates as described either using the primers LB2 and LB2C2 (35), or using the primers amplicon 15 and amplicon 19 (36). The number of PCR cycles yielding products within the linear range was determined by using 2-fold serial dilutions of the input DNA. PCR products were separated on an 8% polyacrylamide gel and stained with ethidium bromide.

RESULTS

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When only one inducer was added to the medium. The results showed that dexamethasone, insulin, and 3-isobutyl-1-methylxantine induce the fad24 expression (Fig. 1A). Next, when only one inducer was omitted from the medium, the expression level of fad24 was not elevated only in the condition without insulin (Fig. 1B). Therefore, it seems that the expression of fad24 was mainly dependent on insulin.

The possibility was considered that fad24 is involved in MCE, because the expression of fad24 was rapidly induced early in the differentiation of adipocytes (26) and the induction was mainly dependent on insulin which is essential for MCE (Fig. 1). To characterize the functional roles of fad24 in MCE, we introduced a plasmid expressing short hairpin RNA for fad24 (shfad24) into 3T3-L1 cells and confirmed a decrease of fad24 expression in the transfected cells compared with control cells (Fig. 2A). These cells were treated with inducers, and cell numbers were counted during adipocyte differentiation (Fig. 2B). The numbers of shfad24-treated cells were lower than control values 1–6 days after induction. The calculation of the blockage percentage of replication in each point in shfad24-treated cells showed the 19–40% reduction, indicating that MCE was inhibited by the knockdown of fad24 expression.

Next, DNA synthesis was evaluated using BrdU labeling to verify whether it was blocked by the knockdown of fad24 expression. Sixteen hours after the induction of differentiation, shfad24-treated cells and control cells were pulse-labeled for 2 h with BrdU, and immunostained with the anti-BrdU antibody. As shown in Fig. 2C, less BrdU was incorporated into shfad24-treated cells than the control cells (Fig. 2C). FACS analyses showed that the percentage of cells in S

largely unknown (26). For differentiation of 3T3-L1 preadipocytes into adipocytes, four inducers are added to the medium. Therefore, to clarify the agents required for the elevated expression of fad24, the expression level of fad24 was determined

FIGURE 3. Interaction of FAD24 with histone acetyltransferase HBO1. A, interaction of FAD24 with DNA replication initiation factors. HeLa cells were transfected with the 3× FLAG-tagged FAD24 expression plasmid or empty plasmid, and WCEs were prepared. Immunoprecipitation (IP) experiments were performed with the anti-FLAG antibody. Normal mouse IgG was used as a negative control. Immunoprecipitates (lanes 2, 3, 5, and 6) and 5% of input (lanes 1 and 4) were resolved by SDS/PAGE (8% gel) and detected by Western blotting with antibodies against FLAG and each protein. The asterisk indicates nonspecific bands against IgG heavy chain. B, interaction of FAD24 with HBO1 by reverse IP. IP were performed with the anti-HBO1 antibody using the WCE in A. Immunoprecipitates (lanes 2 and 3) and 5% of input (lane 1) were resolved by SDS/PAGE (8% gel) and detected by Western blotting with antibodies against FLAG and HBO1. C, interaction of FAD24 with ING5, a subunit of the HBO1-ING5 complex. The Myc-tagged ING5 expression plasmid was co-transfected into HeLa cells with the 3× FLAG-tagged FAD24 expression plasmid or empty plasmid, and WCEs were prepared. IP was performed with the anti-FLAG antibody. Immunoprecipitates (lanes 2, 3, 5, and 6) and 5% of input (lanes 1 and 4) were resolved by SDS/PAGE (10% gel) and detected by Western blotting with antibodies against epitope tags for FLAG and Myc. Experiments in A, B, and C were conducted three times, and similar results were obtained in each experiment. D, fractionation scheme. HeLa cells were separated into soluble cytoplasmic (S2), soluble nucleoplasmic (S3), and chromatin-enriched (P3) fractions as described previously (42). E, HeLa cells transfected with the 3× FLAG-tagged FAD24 expression plasmid were fractionated as outlined in D. Each fraction prepared from the equivalent numbers of cells was resolved by SDS/PAGE (8% gel) and detected by Western blotting with antibodies against FLAG and HBO1. This experiment was conducted two times, and the reproducibility was confirmed.
that the knockdown of *fad24* expression impairs MCE through the inhibition of G₁-S transition.

**FAD24 Interacts with Histone Acetyltransferase HBO1, a Component of the Pre-replicative Complex (Pre-RC)**—Our previous report suggested *fad24* to be a mammalian ortholog of nucleolar complex-associated protein 3 (Noc3p), a yeast protein required for DNA replication initiation (26, 37). It is possible that *fad24* plays important roles in the regulation of DNA replication initiation, because the knockdown of *fad24* expression blocked G₁-S transition and MCE (Fig. 2). DNA replication initiation requires the assembly of a pre-RC, including origin recognition complex (ORC) 1–6, CDC6, CDT1, HBO1, and minichromosome maintenance complex (MCM) 2–7 at origins of DNA replication (33, 38–43). Therefore, we examined whether FAD24 interacts with components of the pre-RC. Whole cell extracts (WCE) were prepared from HeLa cells transfected with the 3xFLAG-tagged FAD24 expression plasmids or empty plasmids, and immunoprecipitated with the anti-FLAG antibody. The resulting immunoprecipitates were assayed by immunoblotting with antibodies against CDC6, HBO1, MCM2, and ORC4. HBO1 and also ORC4, but not CDC6 and MCM2, were immunoprecipitated only in the WCE expressing 3xFLAG-tagged FAD24 (Fig. 3A), although these proteins were not immunoprecipitated when normal mouse IgG was used as a negative control. In the reciprocal experiments, 3xFLAG-tagged FAD24 was co-immunoprecipitated with HBO1 (Fig. 3B). These results demonstrate that FAD24 interacts with HBO1 and also ORC4.

Recently, HBO1 complexes including inhibitor of growth (ING) 4 or ING5 were purified, and it was suggested that the HBO1-ING5 complex plays essential roles in DNA replication (44). To examine whether FAD24 interacts with ING5, we transfected the Myc-tagged ING5 expression plasmids into HeLa cells with the 3xFLAG-tagged FAD24 expression plasmids or the empty plasmids. The WCE were prepared and immunoprecipitated with the anti-FLAG antibody. Myc-tagged ING5 was immunoprecipitated only in the WCE expressing 3x FLAG-tagged FAD24, although this protein was not immunoprecipitated when normal mouse IgG was used as a negative control (Fig. 3C). This result suggests that FAD24 interacts with ING5.

Next, we examined whether FAD24 associates with chromatin by conducting a chromatin-binding assay using HeLa cells transfected with the 3x FLAG-tagged FAD24 expression plasmids (45) (Fig. 3, D and E). The 3x FLAG-tagged FAD24 was mainly detected in the nucleoplasmic (S3) and chromatin-bound (P3) fractions, whereas a large amount of HBO1 was...
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detected in the P3 fraction as reported (33). These results strongly indicate that some FAD24 interacts with HBO1 on the chromatin.

Hbo1 Is Required for MCE and Adipogenesis—Histone acetyltransferases are known to play important roles in changes of chromatin structure, followed by various nuclear processes including transcription, DNA repair, and DNA replication (46, 47). Because it is also becoming clear that histone acetylation is important for adipogenesis (48–50), we focused on the roles of hbo1 in adipogenesis.

First, we determined the expression of hbo1 during the differentiation of 3T3-L1 cells by conducting Northern blot analyses. In contrast with the transient induction of fad24 early in the differentiation process, the expression of hbo1 was detectable in the cells before induction, decreased until 6 h after induction, and increased again 12 h after induction (Fig. 4A).

Next, we examined the effect on adipogenesis of hbo1 knockdown by RNA interference (RNAi). The level of hbo1 expression in 3T3-L1 cells transfected with the shhbo1 expression plasmids declined compared with that in control cells (Fig. 4B). These cells were treated with adipogenic inducers. At 8 days post-induction, the cells were fixed and stained with Oil red O (Fig. 4C), and the amounts of triacylglycerols were determined (Fig. 4D). The number of Oil red O-stained cells and the accumulation of triacylglycerols were decreased in shhbo1-treated cells. Moreover, the expression profiles of adipogenic marker genes were determined by real-time quantitative RT-PCR (Q-PCR). Expression levels of pparγ, c/ebpα, and fatty acid-binding protein 4 (fabp4) were reduced in shhbo1 cells (Fig. 4E). These results strongly indicate that the knockdown of hbo1 expression inhibits adipogenesis. Interestingly, the expression of srebp-1 in shhbo1 cells was unchanged when compared with that in control cells, as well as in the case of fad24 knockdown (26).

As described in Fig. 2, fad24 is an important regulator of MCE. Therefore, we examined whether the knockdown of hbo1 expression affects MCE. The numbers of shhbo1 cells were smaller than those of control cells 1–4 days after induction (Fig. 4F). Thus, MCE was inhibited by the knockdown of hbo1 expression.

FAD24 Partially Co-localized with HBO1 in the Nucleus during Interphase—Previously, we reported the localization of FAD24 to the nucleolus and nuclear speckles using an enhanced green fluorescence protein (EGFP)-fused N-terminal-deleted form of FAD24 (amino acids 76–807) (26). In this report, we determined the localization of the entire FAD24 by transiently transfected an EGFP-fused full-length FAD24 into HeLa cells. The transfected cells were immunostained with the antibody against C23, a marker of the nucleolus. The signals were detected with a fluorescence microscope. EGFP-fused FAD24 localized to the nucleolus and the nucleoplasm in a granular pattern (Fig. 5A). However, these granules are not identical to the nuclear speckles, because GFP does not colocalize with SC35, which identifies nuclear speckles. These granules are also different from PML bodies, which are the major spotted components in the nucleus (data not shown). The same results were obtained when using the FAD24 expression plasmid having a smaller 3× FLAG tag at the N-terminal, and also using the

![FIGURE 5. Intracellular localization of FAD24 and HBO1 during interphase.](image)

![FIGURE 6. Intracellular localization of FAD24 and HBO1 during mitosis.](image)

FAD24-EGFP expression plasmid in which EGFP was joined to the C-terminal portion of FAD24 (data not shown). These results indicate that FAD24 concentrates in the nucleolus and also in the unidentified granules in the nucleoplasm.

Next, to further characterize the molecular functions of FAD24 and HBO1, HeLa cells were transfected with the EGFP-fused FAD24 expression plasmid along with the Myc-tagged HBO1 expression plasmid and immunostained with the antibody against the Myc epitope tag. The distribution of EGFP-fused FAD24 overlapped with the staining pattern of Myc-tagged HBO1 in the nucleolus and the nucleoplasm in a granular pattern (Fig. 5B), indicating that FAD24 partially colocalizes with HBO1 in the nucleus during interphase.
The localization of HBO1 to origins of DNA replication—The above results raised the possibility that FAD24 is involved in the regulation of DNA replication. To determine whether FAD24 localizes to mammalian origins of DNA replication, we performed ChIP analyses using HeLa cells transfected with the 3× FLAG-tagged FAD24 expression plasmid or empty plasmids. The immunoprecipitated DNA was amplified by PCR with primer pairs against the Lamin B2 locus (LB2), an origin of DNA replication, or an outside region (LB2C2) as a negative control (35). The signal against LB2 was strong relative to the signal against LB2C2 only in HeLa cells transfected with the 3× FLAG-tagged FAD24 expression plasmid, although neither signal was detected when normal mouse IgG was used (Fig. 7A). This indicates that 3× FLAG-tagged FAD24 localizes to a human origin of DNA replication. Similar experiments were performed with the antibody against HBO1 and showed that HBO1 also localizes to LB2 (Fig. 7B). Moreover, the ChIP analyses using 3T3-L1 cells revealed that 3× FLAG-tagged FAD24 localized to a mouse origin of DNA replication with HBO1 (Fig. 7C and D) (36). These results suggest that FAD24 might directly regulate DNA replication with HBO1.

The Knockdown of FAD24 Expression Inhibits the Localization of HBO1 to Origins of DNA Replication during MCE—To gain insights into the roles of FAD24 and HBO1 in the regulation of DNA replication during MCE, we first determined protein levels of FAD24 and HBO1 at the early stages of adipocyte differentiation by conducting Western blot analyses with antibodies against FAD24 or HBO1. The level of FAD24 was slightly elevated 8 h after induction and declined until 24 h. In contrast, the level of HBO1 declined until 8 h and increased again 12 h after induction (Fig. 8A).
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Next, we performed ChIP analyses using 3T3-L1 cells before induction (0 h) and at 12 h after induction. The immunoprecipitated DNA was amplified by PCR with primer pairs against a mouse origin of DNA replication (ORI), or an outside region (non-ORI) as a negative control. The signal against ORI was strongly detected relative to the signal against non-ORI at 12 h after induction, but not 0 h, although neither signal was detected when normal goat IgG was used (Fig. 8B). This indicates that HBO1 localizes to origins of DNA replication early in the differentiation program.

These results raised the possibility that the induction of FAD24 expression and the localization of HBO1 to origins of DNA replication are required for the recruitment of HBO1 to ORI during MCE. Then, ChIP analyses were performed using 3T3-L1 cells transfected with the shfad24 expression plasmid or control plasmid. The signal against ORI was decreased in shfad24-treated cells, indicating that the localization of HBO1 to ORI was inhibited when fad24 expression was knocked down (Fig. 8C). Overall, these results suggest that FAD24 is required for the recruitment of HBO1 to origins of DNA replication at the early stages of adipocyte differentiation.

FIGURE 8. Effect of the knockdown of fad24 on the localization of HBO1 to origins of DNA replication during MCE. A, Western blot analyses of FAD24 and HBO1 during adipocyte differentiation. WCE from 3T3-L1 cells at different time points after induction were prepared, resolved by SDS/PAGE (8% gel), and detected by Western blotting with antibodies against FAD24, HBO1, and β-actin as a loading control. This experiment was conducted two times, and the reproducibility was confirmed. B, localization of HBO1 to origins of DNA replication during the early stages of adipocyte differentiation. A ChIP assay was performed on 3T3-L1 cells at 0 h and 12 h after induction using the anti-HBO1 antibody (lanes 2 and 5). Normal goat IgG was used as a negative control (lanes 3 and 6). Input DNA (1:1000) and immunoprecipitated DNA were amplified by PCR using primer sets against ORI or non-ORI. PCR products were resolved on an 8% polyacrylamide gel and stained with ethidium bromide. The intensities were determined with a fluoroimager. This experiment was conducted three times, and ChIP efficiency against ORI was calculated as a percentage of immunoprecipitated material (right panel). Each value represents the mean ± S.D. (n = 3). The asterisk indicates significant difference when compared with the value for 3T3-L1 cells at 0 h (p < 0.05). C, effect of shRNA for fad24 on the localization of HBO1 to origins of DNA replication at the early stages of adipocyte differentiation. A ChIP assay was performed on 3T3-L1 cells transfected with the shfad24 expression plasmid or the control plasmid at 12 h after induction using the anti-HBO1 antibody (lanes 2 and 5). Normal goat IgG was used as a negative control (lanes 3 and 6). Input DNA (1:2000) and immunoprecipitated DNA were amplified by PCR using primer sets against ORI or non-ORI. Results are shown as in Fig. 8B. Each value represents the mean ± S.D. (n = 3). The asterisk indicates significant difference when compared with the value for control cells (p < 0.05).

DISCUSSION

In this study, we revealed that fad24 is required for MCE, an early and essential step in the differentiation program. Moreover, BrdU labeling experiments indicated that the inhibition of MCE by the knockdown of fad24 expression is followed by a blocking of G1-S transition. Other groups reported that C/EBPβ and p27 are important regulators of MCE (12–14). The functional disruption of C/EBPβ and the inhibition of p27 degradation blocked G1-S transition followed by the inhibition of MCE. Therefore, the regulation of G1-S transition might be a key step in MCE.

It was revealed that FAD24 interacts with HBO1, a component of the pre-RC. The functional analysis of hbo1 using RNAi revealed that hbo1 as well as fad24 is required for MCE and adipogenesis. Therefore, FAD24 may act in concert with HBO1 to stimulate adipogenesis by controlling DNA replication. Moreover, we revealed that FAD24 interacts with ING5, a component of the HBO1-ING5 complex that plays positive roles in the regulation of DNA replication. However, it remains unclear whether FAD24 interacts with the HBO1-ING5 complex. Because the expression of ing5 was transiently induced at the early stages of adipocyte differentiation (data not shown), the further analysis of the link between FAD24 and the HBO1-ING5 complex may provide useful information for the biological and biochemical roles of FAD24 in adipocyte differentiation.

During mitosis, FAD24 constantly localized to chromatin. It was reported that ORC1, a subunit of ORC, localizes to chromatin throughout mitosis (51). ORC is thought to function as the landing pad for the recruitment of other components of the pre-RC, so FAD24 may act as a cofactor for the establishment and maintenance of the pre-RC. In contrast, HBO1 mainly localized to chromatin only during telophase in which pre-RC assembly takes place. FAD24 might regulate the localization of HBO1 to chromatin during telophase, because the knockdown of fad24 expression impaired the localization of HBO1 to origins of DNA replication.

This is the first report that FAD24 and HBO1 colocalize to mammalian origins of DNA replication. Moreover, the knock-
down of fad24 expression inhibited the localization of HBO1 to origins of DNA replication at 12 h after induction when DNA replication initiates. These results strongly indicated that FAD24 acts as a cofactor for the recruitment of pre-RC including HBO1 to origins of DNA replication. In fact, Zhang et al. (37) reported that Noc3p, a yeast ortholog of FAD24, plays a direct role in the initiation of DNA replication by interacting with MCM and ORC, as a cofactor for the recruitment of Cdc6p and MCM. These findings support our speculation that FAD24 functions as an important regulator of pre-RC assembly.

Studying how FAD24 regulates pre-RC assembly and DNA replication with HBO1 is very interesting. One may speculate that FAD24 localizes to origins of DNA replication together with ORC, and in turn recruits HBO1 when the replication begins. Then, the histone acetylation by recruited HBO1 could change the conformation of chromatin and enable pre-RC to assemble. The impaired recruitment of HBO1 by the knockdown of fad24 expression might block pre-RC assembly and DNA replication, because DNA replication requires the precise formation of the pre-RC. In fact, the inhibition of HBO1 blocked the binding of MCM to chromatin and DNA replication in mammalian cells and in the well-characterized cell-free system of Xenopus eggs (33, 44). Moreover, recent findings identified a critical function for the transcription factor c-Myc in DNA replication through non-transcriptional control (52). This regulatory mechanism for DNA replication might involve c-Myc-dependent modifications to chromatin such as histone acetylation. Therefore, further functional analyses of FAD24 should help to uncover the connection between mammalian DNA replication and histone acetylation.

Our previous report indicated that an N-terminal deleted form of FAD24 localized to the nucleolus and nuclear speckles during interphase (26), but the present study revealed that full-length FAD24 localized to the nucleolus and the nucleoplasm in a granular pattern. This N-terminal region includes the nuclear localization signal, suggesting its importance for the localization of FAD24. It was reported that Noc3p localizes to the nucleolus and nucleoplasm and is required for ribosome biogenesis (53). FAD24 might be involved in ribosome biogenesis with HBO1, because the link between DNA replication and ribosome biogenesis is thought to be important for cell proliferation control (54).

In summary, our findings indicate that FAD24 acts in concert with HBO1 to promote adipogenesis by controlling DNA replication. The regulation of DNA replication by FAD24 involves the recruitment of HBO1 to origins of replication during the early stages of adipocyte differentiation. A change in the conformation of chromatin during MCE is thought to be required for optimal differentiation. Therefore, FAD24 might function as a key regulator of the adipocyte-specific chromatin structure accompanied by DNA replication during MCE with HBO1. Further characterization of FAD24 including the search for proteins interacting with FAD24 and analyses of histone modifications changed by the overexpression or the knockdown of fad24 should help us to understand the roles of MCE and the signaling pathway early on in the adipocyte differentiation process.

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