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Cabin1 Represses MEF2 Transcriptional Activity by Association with a Methyltransferase, SUV39H1*

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Hyonchol Jang[‡], Doo-Eun Choi[‡], Hyungsoo Kim[‡], Eun-Jung Cho[§], and Hong-Duk Youn[‡1]

From the [‡]Department of Biochemistry and Molecular Biology, Cancer Research Institute, Interdisciplinary Program in Genetic Engineering, Seoul National University College of Medicine, Seoul 110-799 and [§]College of Pharmacy, Sungkyunkwan University, Suwon 440-746, Republic of Korea

Myocyte enhancer factor 2 (MEF2) plays pivotal roles in various biological processes, and its transcriptional activity is regulated by histone acetylation/deacetylation enzymes in a calcium-dependent fashion. A calcineurin-binding protein 1 (Cabin1) has been shown to participate in repression of MEF2 by recruiting mSin3 and its associated histone deacetylases. Here, we report that histone methylation also takes a part in Cabin1-mediated repression of MEF2. Immunoprecipitate of Cabin1 complex can methylate histone H3 by association with SUV39H1. SUV39H1 increased Cabin1-mediated repression of MEF2 transcriptional activity in MEF2-targeting promoters. The SUV39H1 was revealed to bind to the 501–900-amino acid region of Cabin1, which was distinct from its histone deacetylase-recruiting domain. In addition, the Gal4-Cabin1-(501–900) alone repressed a constitutively active Gal4-tk-promoter, indicating that Cabin1 recruits SUV39H1 and represses transcriptional activity. Finally, both SUV39H1 and Cabin1 were shown to bind on the MEF2 target promoter in a calcium-dependent manner. Thus, Cabin1 recruits chromatin-modifying enzymes, both histone deacetylases and a histone methyltransferase, to repress MEF2 transcriptional activity.

Myocyte enhancer factor 2 (MEF2)[2] family proteins were originally identified on the basis of their role in muscle differentiation and consist of four members encoded by different genes: *MEF2A*, *MEF2B*, *MEF2C*, and *MEF2D* (1–6). All MEF2 proteins contain a highly conserved 57-amino acid MADS box motif, which is named after the first four members of the family to be identified: MCM1, Agamous, Deficiens, and serum-response factor, at the extreme N terminus (1–6). This MADS box motif serves as a minimal DNA binding domain, which requires an adjacent 29-amino acid extension, referred to as the MEF2 domain, for high affinity DNA binding and dimerization (1–6).

MEF2 proteins play important roles in morphogenesis and myogenesis of skeletal, cardiac, and smooth muscle cells (1–6). In lymphocyte development, expression of the Nur77 gene, an immediate-early gene involved in apoptosis of autoreactive T cells in response to T cell receptor signaling, is critically regulated by MEF2 (7). In neuronal cells, MEF2 is implicated in neuronal activity-dependent cell survival, and recently MEF2 has been shown to regulate excitatory synapse number and postsynaptic differentiation of dendrites (8–10).

Eukaryotic gene expression is controlled by proteins that recruit the basal transcription machinery or alter chromatin structure (11–14). Chromatin structure can be altered by ATP-dependent nucleosome remodeling or covalent modifications of histones (11–14). Covalent modifications of N-terminal tails of histones include acetylation/deacetylation and methylation/demethylation (11–14). In general, chromatin in the neighborhood of transcriptionally active gene is enriched in acetylated histones, and the enzymes responsible for both acetylation and deacetylation are often recruited to sites where gene expression is to be activated or repressed, respectively (11–14). Histone lysine methylation has proven to "mark" chromatin for downstream events that result in the "activation" or "repression" of specific genes and large chromosomal regions (11–14). Among the histone methyltransferases involved in the repression process, the best characterized is the human homologue of *Drosophila melanogaster* Su(var)3–9, SUV39H1 (15). SUV39H1 has been shown to generate a binding site for the heterochromatin-associated protein HP1 through trimethylation of lysine 9 of histone H3 (16–18).

As with many DNA binding transcriptional factors, MEF2 alters target gene expression by recruiting specific chromatin-modifying activities to promoter regions (6). In the absence of transactivating stimuli, MEF2 represses target genes by association with histone deacetylases (HDACs). MEF2 recruits HDAC1/HDAC2 via Cabin1-mSin3 complex or via MEF2-interacting transcriptional repressor and directly recruits HDAC4/HDAC5 (19–21). In the presence of transactivating stimuli, the association with HDACs is disrupted and MEF2 recruits a calcineurin-activated transcription factor, NF-AT, and a histone acetyltransferase, p300 (19). However, the detailed mechanism of the regulation of MEF2 is not fully elucidated.

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[1] To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Cancer Research Institute, Interdisciplinary Program in Genetic Engineering, Seoul National University College of Medicine, 28 Yongon-dong, Chongro-gu, Seoul 110-799, Republic of Korea. Tel.: 82-2-3668-7455; Fax: 82-2-3668-7431; E-mail: hdyoun@snu.ac.kr.

[2] The abbreviations used are: MEF2, myocyte enhancer factor-2; Cabin1, calcineurin-binding protein-1; SUV39H1, human homologue of *D. melanogaster* Su(var)3–9; HDAC, histone deacetylase; H3-K9, lysine 9 of histone H3; HEK, human embryonic kidney; GST, glutathione *S*-transferase; GFP, green fluorescent protein.

Cabin1, which was initially identified as a calcineurin-binding and -inhibitory protein, is widely expressed in a variety of tissues (22, 23). The open reading frame of human Cabin1 encodes 2,220 amino acids. A yeast two-hybrid system with a C-terminal 502-amino acid fragment of Cabin1 as bait, MEF2B was initially found to bind Cabin1 via the MADS/MEF2 box (7). MEF2 is normally sequestered by Cabin1 in a transcriptionally inactive state. In this state, Cabin1 recruits mSin3 and its associated HDAC1 and HDAC2 to mediate transcriptional repression. Cabin1 also inhibits the association between MEF2 and a coactivator, p300. An increase in intracellular calcium concentration induces the dissociation of MEF2 from Cabin1 as a result of competitive binding of activated calmodulin to Cabin1. The release of Cabin1 from MEF2 results in release of HDACs, and it also provides the binding sites for p300 (7, 19). Interestingly, Cabin1-mediated transcriptional repression of MEF2D cannot be fully rescued by HDAC inhibitors (19), suggesting that additional chromatin modifications may occur. In the present study, we investigated the possibility that histone methylation contributes to the transcriptional repressive potential of Cabin1 in addition to the chromatin modifications already identified.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Myc-Cabin1 (FL), Myc-Cabin1-(320–2220), Myc-Cabin1-(1–1800), Myc-Cabin1-(1–900), Myc-Cabin1-(1–315), Gal4-Cabin1 (FL), pGL-Nur77-(−307 to −242)-luc, Myc-MEF2D, and pOF-MEF2-luc were described previously (7, 19). Gal4-tk-luc was described previously (24). Cabin1-(366–500), Cabin1-(321–700), Cabin1-(501–900), Cabin1-(501–700), and Cabin1-(701–900) were generated by subcloning of EcoR1-BamH1 PCR products from full-length Cabin1 into pcDNA3.1/myc-His(-) B (Invitrogen). Gal4-Cabin1-(501–900) was generated by subcloning of EcoR1-BamH1 PCR products into pcDNA3.1/Ga14-myc-His(-) B, which was generated by inserting the Gal4 DNA binding domain into the HindIII site of pcDNA3.1/myc-His(-) B (Invitrogen). FLAG-Cabin1 (FL), FLAG-Cabin1-(1–2116), and FLAG-Cabin1-(Δ501–900/FL) were generated by subcloning of PCR products from full-length Cabin1 into pcDNA5/FRT/DEST-FLAG (Invitrogen). cDNA of SUV39H1 was generated by PCR amplification from 293 human embryonic kidney (HEK293) cells and was cloned into pcDNA3.0-FLAG, which was generated by inserting a FLAG tag in the XbaI site of pcDNA3.0 (Invitrogen). A bacterial expression construct for SUV39H1 was generated by subcloning of BamH1-SalI PCR fragment into pGEX-4T-1 (Amersham Biosciences). cDNA of histone H3 was generated by PCR from HEK293 cells and was cloned into pRSET B (Invitrogen). Glutathione *S*-transferase (GST) or His fusion proteins were expressed in the *Escherichia coli* strain BL21 (DE3), and the proteins were isolated according to the manufacturer's instructions.

Antibodies for anti-Cabin1 and anti-MEF2D were described previously (7, 25). Anti-SUV39H1 (MG44) and anti-trimethyl-histone H3(K9) antibodies were purchased from Upstate, anti-p300 (N-15) polyclonal antibody from Santa Cruz Biotechnology, anti-acetylhistone H3(K9) antibody from Cell Signaling, anti-Myc (9E10) and anti-hemagglutinin (16B12) monoclonal antibodies from Covance, and anti-FLAG (M2) monoclonal antibody from Sigma.

Transfection, Immunoprecipitation, and Reporter Gene Assay—DO11.10 T hybridoma cells were cultured at 37 °C and 5% CO_2 in RPMI medium 1640 supplemented with 1 mM glutamine, 10% fetal bovine serum, and antibiotics. DO11.10 cells were transfected using electroporation (250 V, 975 μF). HEK293 cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics and then transfected using the calcium phosphate coprecipitation methods or Lipofectamine reagent (Invitrogen).

For coimmunoprecipitation assays, transfected cells were harvested 36 h post-transfection, washed in phosphate-buffered saline, and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitors). Cell lysates were immunoprecipitated with suitable antibodies along with Protein-A/G beads (Santa Cruz). The immunoprecipitates were washed in lysis buffer, denatured in sodium dodecyl sulfate (SDS) loading buffer, and analyzed by Western blotting.

For the reporter gene assays, cells were transfected with suitable promoter-luciferase reporter plasmids and expression vectors. Cells were harvested 30 h after transfection. Luciferase activities were measured with a Sirius luminometer (Berthold Detection Systems).

Histone Methyltransferase Assay—Nuclear extracts from 3×10^8 DO11.10 cells were immunoprecipitated by anti-Cabin1 rabbit polyclonal antibody or normal rabbit IgG antibody. Immunoprecipitates were divided into 1:2, and each immunoprecipitate was incubated for 90 min at 37 °C in 40 μl of appropriate buffer (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) containing 2.5 μg of bacterially purified His-tagged histone H3 as the substrate and 1 μCi of ^{3}H-labeled *S*-adenosylmethionine as the methyl donor. The reaction was stopped by the addition of 10 μl of SDS loading buffer, and samples were resolved by 15% SDS-PAGE followed by gel drying and autoradiography.

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation assays were carried out as described previously (25). Briefly, DO11.10 cells (4×10^7/lane) were treated with phorbol 12-myristate 13-acetate (40 nM) and ionomycin (1 μM) for 3 h. Formaldehyde-treated nuclear lysates were subjected to immunoprecipitation with various antibodies. DNA fragments present in the immunoprecipitates were quantified by real-time quantitative PCR using SYBR® Green fluorescence on the iQ5 Real-time PCR Detection System (Bio-Rad). Results were normalized by total input DNA using the $2^{-\Delta\Delta CT}$ calculation method (26). The position and sequence of primers used to amplify chromatin immunoprecipitation-enriched DNA spanning the MEF2-responsive elements in the Nur77 promoter were −521 to −498 from the translation start site, 5′-AGGACAGACTGGGAAAGGGACAAA-3′, and −254 to −232, 5′-AGGGAGCGCGGATTGTTTGAT-3′. As a control, the DNA fragments present in the immunoprecipitates were amplified with primers that specifically recognize the coding region of the Nur77, +434 to +456, 5′-GGGTAGGGGGTTGTCTGAGCATG-3′, and +641 to +662, 5′-CCTTCAGCAACCCCCAGGTCTT-3′.

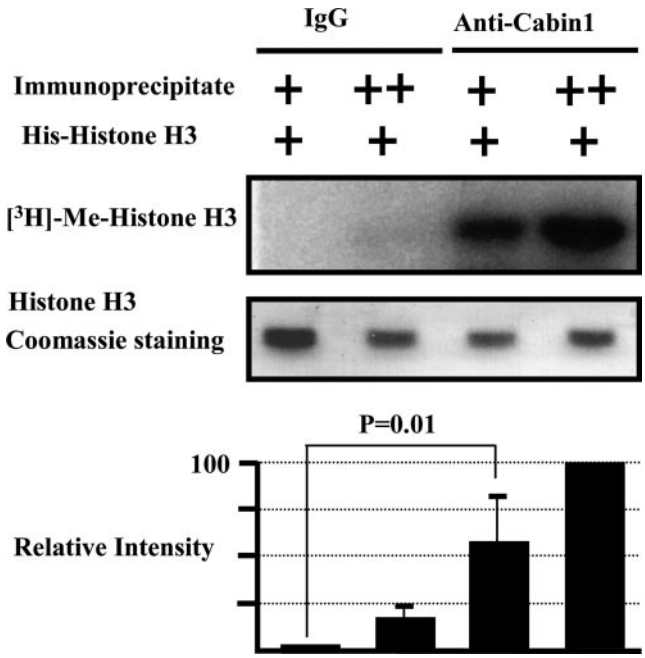

FIGURE 1. **Cabin1 recruits an active histone methyltransferase.** Nuclear extracts from DO.11.10 cells were immunoprecipitated with either normal rabbit IgG or anti-Cabin1 antibody. Precipitates were incubated with purified His-histone H3 in the presence of [^{3}H]*S*-adenosylmethionine. Detailed information about histone methyltransferase assays was described under "Experimental Procedures." The *bar graph* represents relative intensity of the blots. The values represent means ± S.E. ($n = 3$).

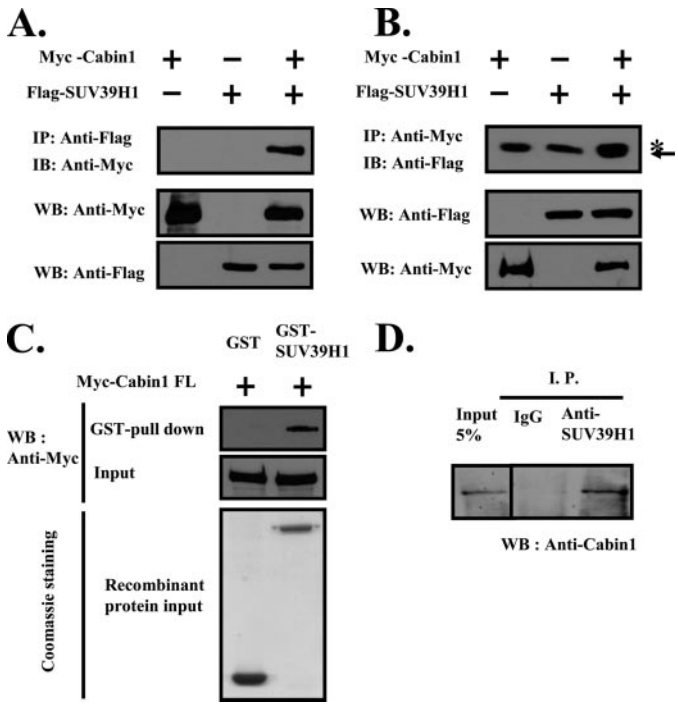

FIGURE 2. **SUV39H1 is associated with Cabin1 *in vivo* and *in vitro*.** *A* and *B*, whole HEK293 cell lysates containing either Myc-Cabin1 alone, FLAG-SUV39H1 alone, or both were immunoprecipitated with either anti-FLAG antibody or anti-Myc antibody. Immunoprecipitates were then analyzed by immunoblotting with either anti-Myc antibody or anti-FLAG antibody. The *arrow* indicates precipitated FLAG-SUV39H1, and the asterisk (*) indicates immunoglobulin heavy chains (IgG). *C*, whole HEK293 cell lysates containing Myc-Cabin1 were incubated with GST or GST-SUV39H1 and then affinity-precipitated with glutathione-Sepharose beads. Affinity precipitates were analyzed by immunoblotting with an anti-Myc antibody. *D*, nuclear extracts from DO11.10 cells were immunoprecipitated with normal IgG or anti-SUV39H1 antibody. Immunoprecipitates were then analyzed by immunoblotting with an anti-Cabin1 antibody. All figures are representative of at least three separate experiments.

Confocal Microscopy—DO11.10 cells were transfected with FLAG-SUV39H1 and green fluorescent protein (GFP), or GFP-Cabin1, or both FLAG-SUV39H1 and GFP-Cabin1. After 30 h of transfection, cells were fixed with 4% (w/v) paraformaldehyde and permeabilized with 0.5% Triton X-100. The cells were then stained with anti-FLAG antibody and Cy3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch). The cells were suspended in 50% (v/v) glycerol containing 4′,6-diamidino-2-phenylindole solutions and loaded on the slide glass and covered with cover glass. Immunofluorescence was observed under a Zeiss LSM 510 laser scanning microscope.

Statistics—Data are presented as means ± S.D., and p value was calculated using Student's t test calculator (www.physics.csbsju.edu/stats/t test.html). A value of $p < 0.05$ was considered statistically significant. All data presented are representative of at least three separate experiments.

RESULTS

Cabin1 Recruits an Active Histone Methyltransferase, SUV39H1—To investigate the role of histone methylation on the transcriptional repression of MEF2 by Cabin1, we analyzed whether Cabin1 can recruit any active histone methyltransferase. To initially test this hypothesis, we immunoprecipitated endogenous Cabin1 complex from DO11.10 cell nuclear extracts and assayed *in vitro* histone methyltransferase activity with recombinant histone H3 as the substrate. The immunoprecipitated Cabin1 complex specifically had histone H3 methyltransferase activity in a dose-dependent manner (Fig. 1).

In mammals, one of the best characterized histone H3 methyltransferases that has repressive potential is SUV39H1 (15). Therefore, we investigated whether Cabin1 associated with SUV39H1. Mammalian HEK293 cells were cotransfected with expression vectors for FLAG-tagged SUV39H1 and for Myc-tagged Cabin1. Cabin1 specifically interacted with SUV39H1, as observed by coimmunoprecipitation using either anti-FLAG antibody or anti-Myc antibody, respectively (Fig. 2, *A* and *B*). Bacterially purified GST-SUV39H1 could also pull down transfected Myc-tagged Cabin1, whereas GST alone could not (Fig. 2*C*). To ascertain the physiological association of SUV39H1 and Cabin1, we performed immunoprecipitation assay using the endogenous proteins in DO11.10 cells. Anti-SUV39H1 antibody, but not preimmune serum (IgG), could specifically immunoprecipitate Cabin1 (Fig. 2*D*).

Next, we examined whether SUV39H1 and Cabin1 could colocalize in cells. When both GFP-tagged Cabin1 and FLAG-tagged SUV39H1 were cotransfected in DO11.10 cells, a significant portion was found to colocalize in nuclei. Whereas GFP was diffusely distributed in both nuclei and cytosol, GFP and SUV39H1 rarely colocalized within nuclei (Fig. 3).

SUV39H1 Increases Cabin1-mediated Repression of MEF2 Transcriptional Activity—To determine the functional consequence of the association between Cabin1 and SUV39H1, we analyzed the role of SUV39H1 in MEF2-targeting promoters. Transcriptional activity of MEF2 was measured by luciferase

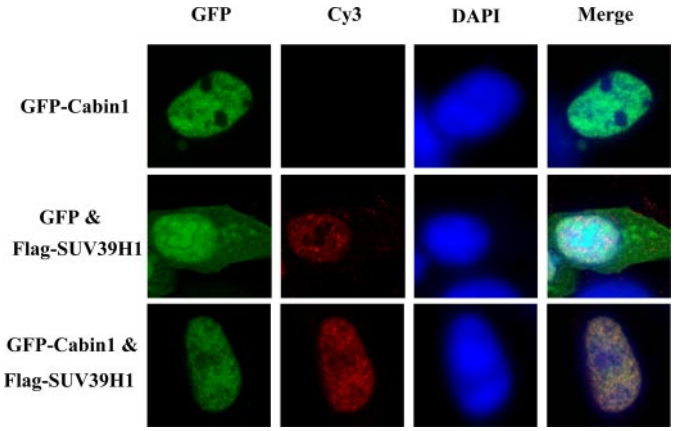

FIGURE 3. **SUV39H1 and Cabin1 colocalize in nucleus.** DO11.10 cells were transfected with expression vectors for GFP-Cabin1 only, or FLAG-SUV39H1 and GFP, or both GFP-Cabin1 and FLAG-SUV39H1. Cells were stained as described under "Experimental Procedures," and immunofluorescence was observed under a Zeiss LSM 510 laser scanning microscope.

reporter gene under the control of two tandem MEF2 binding sites (pOF-MEF2-luc) or under the control of two MEF2 binding sites within the minimal Nur77 promoter region (pGL-Nur77-Luc) in DO11.10 cells or HEK293 cells. Cotransfection of MEF2D with luciferase reporter gene enhanced the reporter gene significantly, and this enhanced activity was repressed by additional cotransfection of Cabin1 (FL). Moreover, additional cotransfection of SUV39H1 further inhibited MEF2D-driven activation of the reporter gene whereas Cabin1-(1–2116), which lacks the MEF2 binding domain, did not inhibit MEF2D-driven activation of the MEF2 reporter gene. In addition, SUV39H1 did not synergize with Cabin1-(1–2116) in repression of the MEF2 reporter gene (Fig. 4). These results suggest that SUV39H1 represses MEF2 transcriptional activity via Cabin1.

The 501–900-Amino Acid Region of Cabin1 Binds to SUV39H1—To further analyze the role of SUV39H1 in Cabin1-mediated repression of MEF2 transcriptional activity, we first determined the SUV39H1 binding domain within Cabin1. Various truncation mutants of Cabin1 were overexpressed in HEK293 cells by transient transfection or labeled with [^{35}S]methionine by *in vitro* transcription and translation. Bacterially purified GST-SUV39H1 was mixed with cell lysates containing Myc-Cabin1 deletion mutants or *in vitro* translational products of [^{35}S]Cabin1 deletion mutants followed by GST affinity precipitation of SUV39H1 using glutathione-Sepharose beads. The interaction between Cabin1 deletion mutant and SUV39H1 was detected by immunoblotting with anti-Myc antibody or autoradiography (Fig. 5, *A* and *B*). The N-terminal 900 amino acids of Cabin1 were initially revealed to bind to SUV39H1 (Fig. 5, *A* and *D*). Cabin1-(501–900), Cabin1-(501–700), and Cabin1-(701–900) were recruited by SUV39H1 (Fig. 5, *B* and *D*). However, neither Cabin1-(1–315) nor Cabin1-(366–500) was recruited by GST-SUV39H1, suggesting Cabin1-(501–900) was responsible for the interaction between Cabin1 and SUV39H1 (Fig. 5, *B* and *D*). Pull-down experiments using Cabin1 (FL) and Cabin1-(Δ501–900/FL), which lacks the 501–900-amino acid region of Cabin1, showed that GST-SUV39H1 could recruit Cabin1 (FL) but not Cabin1-(Δ501–900/FL) (Fig. 5, *C* and *D*). Thus, SUV39H1 bound to Cabin1

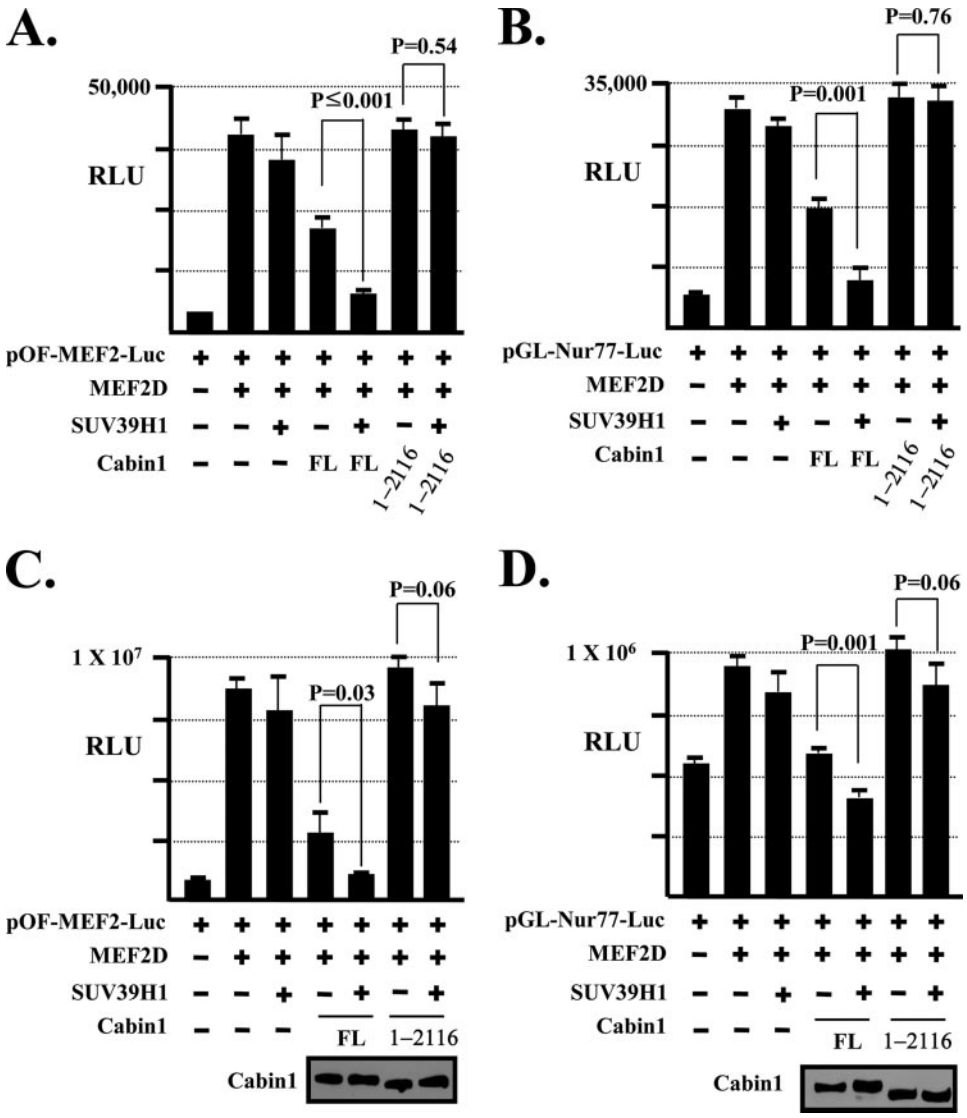

FIGURE 4. **SUV39H1 increases Cabin1-mediated repression of MEF2D transcriptional activity.** DO11.10 cells (*A* and *B*) or HEK293 cells (*C* and *D*) were transfected with the indicated luciferase reporter gene along with MEF2D, SUV39H1, and Cabin1 (FL) or Cabin1-(1–2116). Luciferase activity was measured 30 h post-transfection with a luminometer. The values represent relative luminescent unit means ± S.E. ($n \geq 3$).

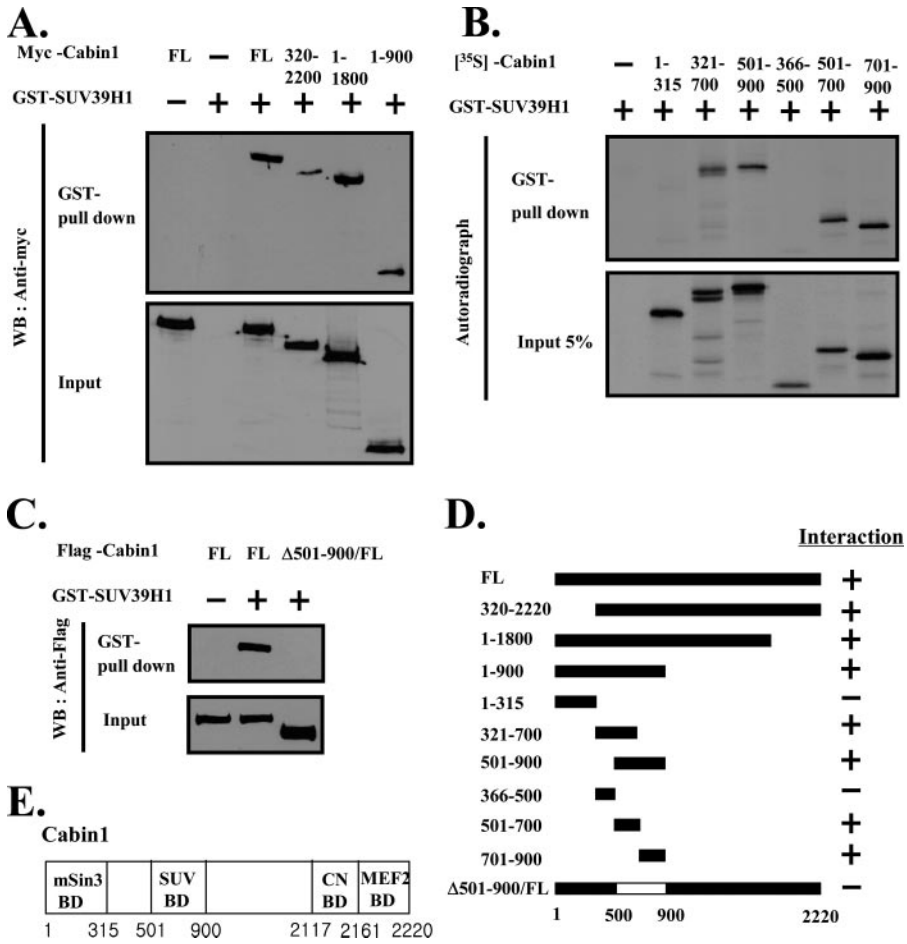

FIGURE 5. **The 501–900-amino acid region of Cabin1 interacts with SUV39H1.** Pulldown assays were performed in the presence of glutathione-Sepharose beads using GST-SUV39H1 and whole cell lysates from transiently transfected HEK293 cells (*A* and *C*) or *in vitro* transcription/translation products (*B*) with the indicated mammalian expression vectors for various deletion mutants of Cabin1. All figures are representative of at least three separate experiments. *D*, diagram of the full-length Cabin1 and its deletion mutants used in this study. *E*, various protein-interacting domains in Cabin1. The *numbers* represent the amino acid sequences of human Cabin1. *mSin3BD*, mSin3 binding domain; *SUVBD*, SUV39H1 binding domain; *CNBD*, calcineurin binding domain; *MEF2BD*, MEF2 binding domain. The figure is not to scale.

at a different site from the MEF2 binding domain (MEF2BD) at the C-terminal of Cabin1 (Fig. 5*E*). This result suggested that Cabin1 can simultaneously bind to both SUV39H1 and MEF2 via its 501–900 amino acids and C-terminal MEF2BD, respectively.

The 501–900-Amino Acid Region of Cabin1 Alone Represses a Constitutively Active Promoter—The 501–900-amino acid region of Cabin1, which was identified as a SUV39H1-interacting domain, is distinct from mSin3 and its associated HDAC-interacting domain, mSin3BD (Fig. 5*E*) (19). Because we found that Cabin1 represses MEF2 transcriptional activity by recruiting SUV39H1, we tested whether the 501–900-amino acid region could have repressive ability when recruited to the vicinity of a constitutively active promoter. We thus expressed a fusion protein between the Gal4 DNA binding domain and the 501–900-amino acid region of Cabin1 and determined the ability of the resultant fusion protein to repress a constitutively active reporter gene placed in close proximity to multimerized Gal4 binding sites. This fusion protein indeed repressed the reporter gene activity in a concentration-dependent manner (Fig. 6, *A* and *B*). It has been reported that a fusion protein between the Gal4 DNA binding domain and full-length Cabin1 or the N-terminal 315 amino acids of Cabin1 represses a constitutively active reporter gene placed in close proximity to multimerized Gal4 binding sites, presumably via mSin3 and its associated HDACs (19). The C-terminal fragment Cabin1-(2007–2220), which cannot recruit mSin3 and HDACs, had no repressive effect on the reporter gene activity (19). Moreover, SUV39H1 did not increase the Cabin1-(Δ501–900/FL)-mediated repression of MEF2 transcriptional activity (Fig. 6*C*). Thus, the repressive potential of Cabin1-(501–900) is probably due to the recruitment of SUV39H1 through Cabin1-(501–900).

Both SUV39H1 and Cabin1 Bind on the MEF2 Target Promoter in a Calcium-dependent Manner—To address the physiological association of SUV39H1 on MEF2-regulated promoters, we analyzed the Nur77 promoter, which has been known to be regulated by MEF2D in a calcium-dependent manner (19). This promoter contains two MEF2 binding sites (19). Chromatin immunoprecipitation analysis of DO11.10 cells revealed that Cabin1 and SUV39H1 bound on the Nur77 promoter in the absence of a calcium signal, but in the presence of a calcium signal both Cabin1 and SUV39H1 were released from the promoter (Fig. 7, *A* and *B*) whereas MEF2D bound on the Nur77 promoter both in the absence and presence of a calcium signal and p300 bound on the Nur77 promoter only in the presence of a calcium signal (Fig. 7, *A* and *B*). Moreover, in the absence of a calcium signal trimethylation of lysine 9 at histone H3 (H3-K9) was detected, and this trimethylation decreased in the presence of a calcium signal. On the contrary, acetylation of H3-K9 increased in the presence of a calcium signal (Fig. 7, *A* and *B*). These findings demonstrate that SUV39H1 is recruited to MEF2 target promoters by association with Cabin1-MEF2 complexes. Calcium signals abrogate the interaction between Cabin1 and MEF2. Consequently, SUV39H1 is also released from MEF2.

DISCUSSION

In this study, we have revealed previously unrecognized interaction between Cabin1 and a histone methyltransferase. Histones are methylated on either lysine or arginine residues. Lysine side chains are mono-, di-, or trimethylated,

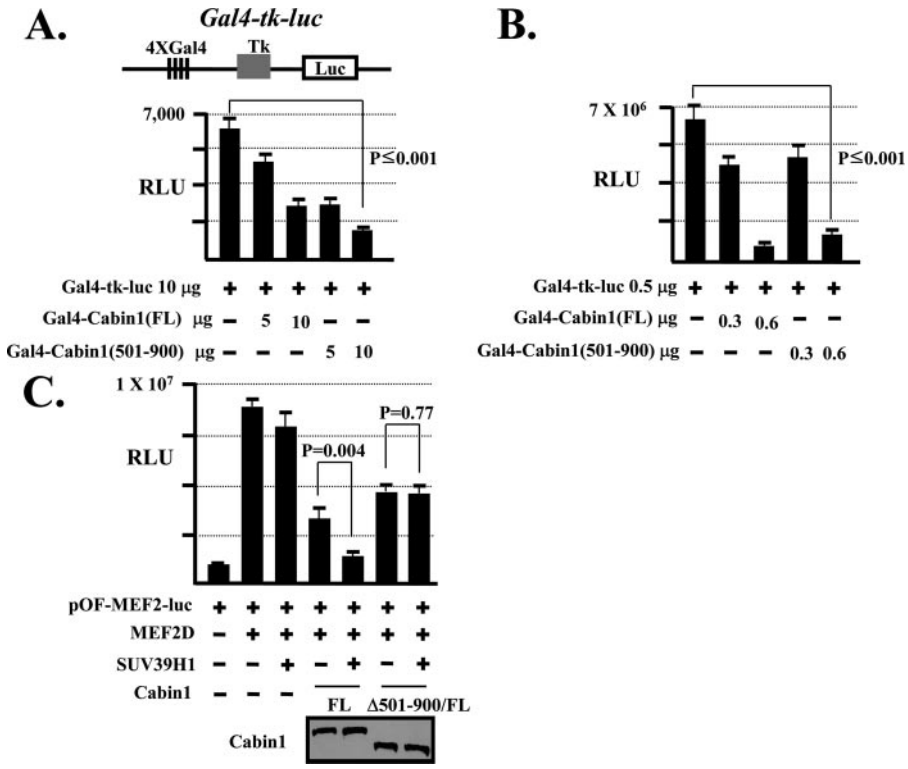

FIGURE 6. **The 501–900-amino acid region of Cabin1 has transcriptional repression activity.** DO11.10 cells (*A*) or HEK293 cells (*B*) were transfected with Gal4-tk luciferase reporter gene and increasing amounts of Gal4-Cabin1 (FL) or GAL4-Cabin1-(501–900). *C*, HEK293 cells were transfected with pOF-MEF2 luciferase reporter gene along with MEF2D, SUV39H1, and Cabin1 (FL) or Cabin1-(Δ501–900/FL). Luciferase activity was measured 30 h post-transfection with a luminometer. The values represent relative luminescent unit means ± S.E. ($n = 3$).

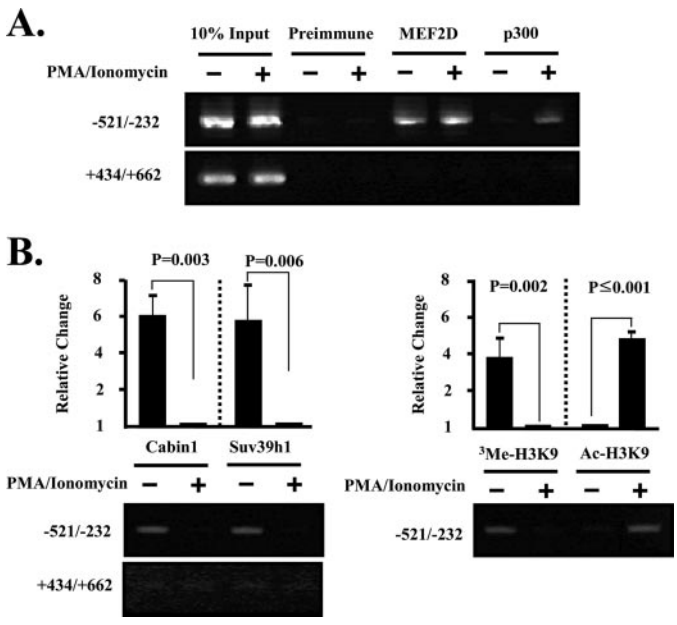

FIGURE 7. **Both SUV39H1 and Cabin1 bind on the MEF2 target promoter in a calcium-dependent manner.** Chromatin immunoprecipitation analysis was performed on DO11.10 cells with or without treatment of phorbol 12-myristate 13-acetate and ionomycin. *A*, DNA fragments present in the immunoprecipitates were amplified with primers that specifically recognize a fragment of the Nur77 promoter (−521 to −232 from the translation start site). As a negative control, primers that recognize the coding region of the Nur77 (+434 to +662) were used. PCR products were separated on 2% agarose gels and stained with ethidium bromide. *B*, enrichment of the Nur77 promoter in the described antibody immunoprecipitates was analyzed by semiquantitative PCR using ethidium bromide staining and real-time quantitative PCR using SYBR® Green fluorescence. The values represent relative change means ± S.E. ($n \geq 3$).

whereas arginine side chains are monomethylated or (symmetrically or asymmetrically) dimethylated (14, 27, 28). The methylation of arginine residues has only been linked to active transcription because this modification is solely found on chromatin when genes are actively transcribed (14, 29). The methylation of lysine residues can signal either activation or repression, depending on the particular lysine residue that is methylated (30). Even within the same lysine residue, the biological consequence of methylation can differ depending on the methylation state (31, 32). Among the lysine residues, the lysine 9 of histone H3 (H3-K9) is the best characterized as a methylation site linked with repression. Among methyltransferases that are responsible for the methylation of the H3-K9, SUV39H1 and G9a are the major H3-K9 methyltransferases in higher eukaryotes; SUV39H1 is the major H3-K9 methyltransferase that is responsible for trimethylation of H3-K9, whereas G9a is the major H3-K9 methyltransferase that is responsible for mono- and dimethylation of H3-K9 in euchromatin (33–36). Thus, the best candidates for the methyltransferase that associates with Cabin1 and represses MEF2 transcriptional activity are SUV39H1 and G9a. We first tested whether SUV39H1 is associated with Cabin1 and found that indeed SUV39H1 bound Cabin1 *in vitro* and *in vivo* (Fig. 2). We thus focused on the effect of SUV39H1 on the function of Cabin1. Later we found that G9a is also associated with Cabin1 (data not shown). It will be interesting to further investigate the relationship between G9a and Cabin1.

The SUV39H1, mSin3, and MEF2 binding domains in Cabin1 are all distinct (Fig. 5*E*). Thus, it is possible that Cabin1, MEF2, mSin3 and its associated HDACs, and SUV39H1 make up a complex. The previously known mechanism by which calcium regulates MEF2D in this promoter is through dissociation of calcium-sensitive MEF2 co-repressors (Cabin1/HDACs, HDAC4/5) and the association with the calcineurin-activated transcription factor NF-AT and the coactivator p300 (7, 19). From the findings in this study and previous results, the Cabin1-mediated repression mechanism of MEF2 can be depicted like this: Cabin1 is recruited to promoters through its interactions with DNA sequence-specific-binding protein MEF2. The association of Cabin1 with mSin3 and its associated HDACs and histone methyltransferase SUV39H1 provides an efficient mechanism for modifying nucleosomal histone tails. HDACs deacetylate the H3-K9, and then SUV39H1 trimethyl-

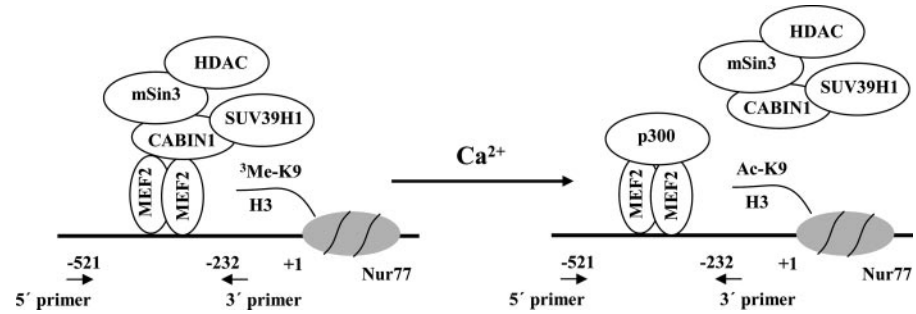

FIGURE 8. **Role of the chromatin-modifying activities in the transcriptional co-repressor function of Cabin1.** Cabin1 is recruited to promoters through its interactions with DNA sequence-specific-binding protein MEF2. The association of Cabin1 with mSin3 and its associated HDACs and a histone methyltransferase SUV39H1 provides an efficient mechanism for modifying nucleosomal histone tails. HDACs deacetylate the H3-K9, and then SUV39H1 trimethylates this site, maintaining chromatin in repressive states. Increase of the intracellular calcium concentration abrogates the interaction between Cabin1 and MEF2. Consequently, HDACs and SUV39H1 are also released from the MEF2 target promoters. The dissociation of Cabin1 from MEF2 also provides a binding site for histone acetyltransferase p300. Acetylation of histone H3 by p300 makes the chromatin suitable for transcription.

ates this site, maintaining chromatin in repressive states. Increase of the intracellular calcium concentration abrogates the interaction between Cabin1 and MEF2. Consequently, HDACs and SUV39H1 are also released from MEF2 target promoters. The dissociation of Cabin1 from MEF2 also provides a binding site for histone acetyltransferase p300. Acetylation of histone H3 by p300 makes the chromatin suitable for transcription (Fig. 8).

Because SUV39H1 trimethylates H3-K9 and histone acetylation can occur only on the unmethylated lysine, this trimethylated lysine should be demethylated before acetylation. Until recently, histone methylation was thought to be an irreversible process. However, now it is revealed that not only histone phosphorylation and acetylation but also histone methylation are reversible processes. Recently, histone demethylases such as lysine-specific demethylase 1 and JmjC domain-containing proteins JHDM1, JHDM2A, and JMJD2A have been reported (37–40). Among them, JMJD2A reverses trimethylated H3-K9 to di- but not mono- or unmethylated products (40), and JHDM2A demethylates mono- and dimethyl H3-K9 (39). So, it may be possible that JMJD2A and JHDM2A cooperate in demethylation of trimethyl H3-K9 to unmethylated H3-K9. In this regard, JMJD2A and JHDM2A may be also required in calcium-dependent activation of MEF2 transcriptional activity.

It appears that different degrees of methylation are associated with distinct chromatin regions or transcriptional states. Trimethylation of H3-K9 is mainly associated with pericentromeric heterochromatin and transcriptional repression, whereas dimethylation of H3-K9 appears to occur on repressed genes in euchromatin (33–36). However, repression of euchromatic genes by SUV39H1 has also been identified. For example, in retinoblastoma-mediated transcriptional repression of E2F, retinoblastoma is recruited to promoters through its interaction with the DNA sequence-specific-binding protein E2F. Retinoblastoma associates with HDACs and SUV39H1 to repress E2F target promoters (41–44). Furthermore, class II HDACs are known to bind MEF2 and repress its transcriptional activity by recruiting class I HDACs and SUV39H1 (21, 45). These mechanisms are quite parallel to the Cabin1-mediated repression mechanism proposed in Fig. 8.

In this study, we found the involvement of SUV39H1, a H3-K9 methyltransferase, in Cabin1-mediated repression of MEF2 transcription factor. Histone methylation is critical for maintaining the chromatin as "activated" or "inactivated" state. Thus, the findings that SUV39H1 associates with Cabin1 and determines the H3-K9 states in MEF2-driven promoters disclose that Cabin1 serves as a co-repressor of MEF2 transcription factor. Moreover, because Cabin1 is a huge protein and has a potential for recruiting other types of proteins involved in transcriptional repression, it will be interesting to further investigate the different types of Cabin1-mediated repression of MEF2 transcription factor.

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