HDAC1 Cooperates with C/EBPα in the Inhibition of Liver Proliferation in Old Mice*

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Epigenetic control of liver proliferation involves cooperation between transcription factors and chromatin-remodeling proteins. In this work, we found that the levels of HDAC1 (histone deacetylase 1) are increased in quiescent livers of old mice. The elevation of HDAC1 in liver is mediated by the RNA-binding protein CUGBP1. We found that the age-associated CUGBP1-eIF2 complex binds to the 5′ region of HDAC1 mRNA and increases translation of HDAC1 in the liver. Further analyses showed that CUGBP1 also increases expression of HDAC1 in cultured cells, in the livers of CUGBP1 transgenic mice, and in the livers of mice injected with cyclin D3, which enhances the formation of the CUGBP1-eIF2 complex. In livers of old mice, HDAC1 interacts with the transcription factor C/EBPα and is recruited by this protein to E2F-dependent promoters as a component of high M,C/EBPα-Brm complexes. The recruitment of HDAC1 to c-Myc and FoxM1B promoters leads to deacetylation of histone H3 at Lys-9 on these E2F-dependent promoters. We show that HDAC1 is a critical mediator of growth-inhibitory activity of C/EBPα and that small interfering RNA-mediated inhibition of HDAC1 reduces the ability of C/EBPα to inhibit cell proliferation. In addition, we have found that both elevation of HDAC1 and interaction of C/EBPα with HDAC1 are controlled by cyclin D3-dependent mechanisms. Treatment of old mice with growth hormone, which reduces cyclin D3 levels, leads to the reduction of the CUGBP1-eIF2 complex, normalization of HDAC1 levels, and inhibition of interactions of HDAC1 with C/EBPα-Brm complexes. Thus, our data demonstrate that translational elevation of HDAC1 in livers of old mice is involved in the assembly of high M,C protein-protein complexes that inhibit liver proliferation.

Although the liver is a quiescent tissue, it is able to completely regenerate itself in response to injury and after partial hepatectomy. It has been shown that aging significantly reduces regenerative capacities of the liver and proliferative response after partial hepatectomy (1–3). The first fundamental work was performed by Bucher et al. (1) in 1964 and showed that old animals have a delayed and reduced peak of DNA synthesis. This phenomenon was later confirmed by a number of publications from other groups (2–5). Despite intensive work, very little is known about biochemical pathways by which aging inhibits liver proliferation. Recent studies revealed that expression and activities of two transcription factors, C/EBPα and FoxM1B, are altered in livers of old mice (4–7). The C/EBP family member C/EBPα is expressed at high levels in the liver and is involved in the regulation of liver growth and differentiation (8–10). C/EBPα inhibits liver proliferation in young mice through the interactions with Cdk2 and p21, leading to the inhibition of Cdk2 kinase activity (11–13). In old livers, C/EBPα is recruited to Brm complexes to repress E2F targets through the occupation of E2F-dependent promoters (5, 14). FoxM1B is not detectable in quiescent livers but is elevated after partial hepatectomy and is required for proper liver proliferation (4, 15). Several publications from Costa and co-workers (4, 15) have shown that aged livers fail to elevate FoxM1B after partial hepatectomy, and this failure correlates with the reduced proliferation. We have recently found that the age-associated appearance of the C/EBPα-Brm complex inhibits expression of FoxM1B in livers of old mice after partial hepatectomy (7). Although the elevation of the C/EBPα-Brm complex in old livers is well described (5, 14), the composition of such complex and mechanisms by which this complex represses promoters are not known.

Proliferation of the liver is controlled by a complex cooperation of several networks that determine expression of genes through remodeling of the chromatin structure. One of the proteins that control chromatin structure is HDAC1. HDAC1 is involved in the regulation of many biological processes, including cellular senescence (16, 17). A recent paper has demonstrated that the induction of HDAC1 in melanocytes inhibits cell proliferation and triggers cellular senescence through formation of complexes with Brg1/Brm1 and Rb proteins (17). Despite the established role of HDAC1 in many tissues, very little is known about the role of HDAC1 in the biological processes of the liver. Transgenic mice with liver-specific overexpression of HDAC1 have been generated and have shown that the elevation of HDAC1 leads to down-regulation of p21 (18). It has recently been shown that hepatitis B virus-induced development of hepatocellular carcinoma is mediated by the HBx-dependent repression of estrogen receptor and that this repression involves HDAC1 (19). Aging changes expression of the...
proteins on different levels, including alterations of protein translation. We have previously shown that the RNA-binding protein CUGBP1 regulates translation of certain mRNA in different tissues of mice (20) and that aging enhances translational activity of CUGBP1 by increasing its interactions with eukaryotic initiation translation factor eIF2 (21). It has also been shown that CUGBP1 binds to the 5’ region of C/EBPβ mRNA and increases translation of C/EBPβ in regenerating livers (22, 23) and in livers of old mice (21). Although the translational function of CUGBP1 has been described by many groups, little is known about translational targets of CUGBP1 and their roles in liver biology.

In this work, we have identified an additional translational target of CUGBP1, HDAC1. We found that HDAC1 protein is elevated in old quiescent livers, whereas HDAC1 mRNA is not changed. Searching for the mechanisms of HDAC1 elevation in the livers of old mice, we found that the age-specific CUGBP1-eIF2 complex is associated with HDAC1 mRNA and increases translation of HDAC1. Using tissue culture systems and animal models, we showed that activation of the CUGBP1-eIF2 pathway leads to elevation of HDAC1. Furthermore, we present evidence that, in livers of old mice, HDAC1 is associated with C/EBPα-Brm and is required for the repression of E2F targets. Thus, the studies described in this work have identified a role of translational control in the appearance of the age-specific complex repressor C/EBPα-Brm-HDAC1.

EXPERIMENTAL PROCEDURES

Animals—In this study, we have used young mice (4–6 months) and old mice (20–22 months). Generation of CUGBP1 TR mice was described in our previous work (20). In these studies, we have used 4–6-month-old CUGBP1 TR2 mice. To increase the CUGBP1-eIF2 complex in livers of young mice, cyclin D3 plasmid was injected into 4-month-old mice, as previously described (7, 21). Animal experiments were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine (protocol AN-1439).

Isolation of Protein Extracts—Cytoplasmic and nuclear extracts were isolated as previously described (5, 7, 10). Briefly, livers were homogenized in buffer A containing 20 mM Tris-HCl, pH 7.5, 0.42 M NaCl, 10 mM β-mercaptoethanol, and inhibitors of phosphatases. Nuclei were spun down at 12,000 rpm for 15 min, and supernatant (cytoplasm) was kept in the −80 °C freezer. The pellet (nuclei) was treated with buffer B containing 20 mM Tris-HCl, pH 7.5, 0.42 M NaCl, 10 mM β-mercaptoethanol, 25% sucrose, 5 mM MgCl2, and inhibitors of phosphatases. After a 30-min incubation on ice, nuclei were spun down at 12,000 rpm for 10 min, and supernatant (nuclear extract) was frozen and kept in a −80 °C freezer.

Partial Purification of CUGBP1 from Cytoplasm Using Chromatography on a DEAE Column—Since the RNA binding activity of CUGBP1 is significantly inhibited in cytoplasm by endogenous RNA (22), we have partially purified CUGBP1 from cytoplasm using a DEAE column. The detailed procedure has been described in our previous work (22). Briefly, 1 mg of cytoplasmic proteins were loaded onto a DEAE-Sephadex column and washed from unbound proteins. The bound proteins were eluted with different concentrations of NaCl, including 0.1, 0.2, 0.3, and 0.4 M NaCl. Under these conditions, endogenous RNA remains bound to the column, whereas the majority of proteins are eluted. The location of CUGBP1 within elution fractions was determined by Western blotting and by gel shift-supershift as shown in Fig. 2, C and D. CUGBP1 is usually eluted with 0.3 M NaCl.

Western Blotting and Co-immunoprecipitations—50 μg of proteins were loaded on gradient 8–16% or 4–20% SDS-polyacrylamide gel (Bio-Rad). Proteins were transferred on nitrocellulose membrane, and the membrane was blocked with 10% dry milk on TTBS for 1 h. The membrane was incubated with primary antibodies for 2–4 h, washed, and incubated with secondary antibodies for 1 h. After wash, the signals were detected by detection reagents (Amersham Biosciences). Protein loading was verified by a reprobe of the membranes with β-actin and by Coomassie stain. Antibodies to C/EBPα (A144), CUGBP1 (B1), HDAC1, and eIF2e were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to β-actin were from Sigma True-Blot secondary antibodies and IP beads from Eibioscience were used for Western blotting and co-immunoprecipitation studies.

Gel Shift and UV Cross-link Assays—Gel shift and UV cross-link assays were performed with RNA probes containing C/EBPβ mRNA probe or the 5’ region of HDAC1 mRNA (Fig. 2). UV cross-linking analysis was performed as described in our previous studies (22–24). RNA probes were labeled by [γ-32P]ATP and T4 kinase. Equal amounts of radioactive RNA probes (50–200,000 cpm) were incubated with proteins for 30 min at room temperature and subjected to UV treatment for 5 min at 125 ml. Reaction mixtures were loaded on denaturing SDS-polyacrylamide gel. Proteins were transferred onto the membrane and autoradiographed. The membranes were stained with Coomassie Brilliant Blue to verify protein loading.

Size Exclusion Chromatography and Analyses of Protein-Protein Complexes—This procedure was used for the isolation of CUGBP1-eIF2 and C/EBPα-HDAC1-Brm complexes. CUGBP1-eIF2 complex was separated from free CUGBP1 by size exclusion chromatography of the cytoplasmic extracts on a SEC400 column (DuoFlow; Bio-Rad), as previously described (5–7). The complex was immunoprecipitated from high M, fractions with Abs to CUGBP1 and RNA was isolated from the complex and used for RT-PCR with primers to HDAC1 mRNA. For the isolation of C/EBPα-Brm complex, nuclear extracts from livers of old mice were fractionated on an SEC400 column. Five runs were combined, and C/EBPα was immunoprecipitated from fractions containing the complex. C/EBPα IPs were separated by two-dimensional gel as described (6, 10) and stained with Coomassie Brilliant Blue or transferred on the filters and probed with Abs to HDAC1 and HP1α.

Examination of HDAC1 mRNA—Total RNA was isolated from livers and from cultured cells using TRIzol reagent (Invitrogen). The RNA was reverse transcribed and used for

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2 The abbreviations used are: TR, transgenic; IP, immunoprecipitation; Ab, antibody; ChiP, chromatin immunoprecipitation; HPLC, high pressure liquid chromatography; GH, growth hormone; siRNA, small interfering RNA; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; WT, wild type.
HDAC1 Inhibits Proliferation in Livers of Old Mice

We have previously shown that RNA-binding protein, CUGBP1, activates translation of C/EBPβ mRNA in the liver of old mice (21). The aged livers increase translational activities of CUGBP1 by enhancement of the interaction of CUGBP1 with eIF2 (21). Given correlations between activation of CUGBP1 and induction of protein levels of HDAC1 in livers of old mice, we asked if the CUGBP1-eIF2 complexes might regulate translation of HDAC1. We first determined whether HDAC1 mRNA is associated with the CUGBP1-eIF2 complexes. Cytoplasm of old livers was fractionated by size exclusion chromatography, and the CUGBP1-eIF2 complex was localized within fractions by EMSA (Fig. 1E) and by UV cross-link with C/EBPβ probe (Fig. 1F). The complex was isolated from high Mr fractions by immunoprecipitation with Abs to CUGBP1 as shown in Fig. 1G. RT-PCR with RNA isolated from the complex showed that HDAC1 mRNA was associated with the CUGBP1-eIF2 complex in old livers (Fig. 1G). The association of the CUGBP1-eIF2 complex with HDAC1 mRNA in the aged livers is specific, since phosphoenolpyruvate carboxykinase (PEPCK) serves as a negative control.

Chromatin Immunoprecipitation (ChIP) Assay—The chromatin immunoprecipitation assay was performed using the ChIP-IT kit (Active Motif). The chromatin solutions were isolated from livers of young and old animals, and DNA was sheared by enzymatic digestion according to the instruction manual. Antibodies against C/EBPβ, C/EBPα, E2F4, Brm, HDAC1, Rb (Santa Cruz Biotechnology), acetyl-histone H3-Lys-9 (Cell Signaling), and histone H3-trimethyl Lys-9 (Abcam) were added to each aliquot of precleared chromatin and incubated overnight. Protein G beads were added and incubated for 1.5 h at 4 °C. DNA was isolated and used for PCRs with primers specific for E2F binding sites within the c-Myc and FoxM1B promoters. The sequences of the primers for these promoters are described in our previous studies (5, 7). PCR mixtures were amplified for one cycle of 95 °C for 5 min, annealing temperature for primers (62 °C) for 5 min, and 72 °C for 2 min. Then PCR mixtures were amplified for 34 cycles of 95 °C for 1 min, annealing temperature for 2 min, and 72 °C for 1.5 min. PCR products were separated by 1.5% agarose gel electrophoresis or by 4% PAGE.

RESULTS

Protein Levels of HDAC1 Are Increased in Livers of Aged Mice—Since HDAC1 plays a critical role in cellular senescence (17), we examined expression of HDAC1 in livers of old and young mice. Western blotting analysis showed that protein levels of HDAC1 are 2.5–3-fold higher in livers of old mice (Fig. 1A and B). Consistent with the elevation of protein levels, enzymatic activity of HDAC1 was also increased in old livers (Fig. 1C). Examination of mRNA levels of HDAC1 by RT-PCR, however, showed no significant differences (Fig. 1D), suggesting that HDAC1 is elevated in livers of old mice at the level of translation or protein stability.

Real time PCR with primers specific for HDAC1 mRNA. The sequence of these primers is as follows: forward, 5'-ATTCCCTGGTTTCTATTCGCC CAGA-3'; reverse, 5'-TTAGCAGTTCCAGGATGGCCAAGA-3'. The levels of HDAC1 mRNA were calculated as ratios to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. A summary of two experiments with three animals of each age group is shown. E, purification of the CUGBP1-eIF2 complex by size exclusion chromatography. The CUGBP1-eIF2 complex was separated from free CUGBP1 by size exclusion chromatography. The location of the complex was determined by gel shift assay with the C/EBPβ probe, and the complex was immunoprecipitated with monoclonal Abs to CUGBP1. F, examination of the size exclusion fractions by UV cross-link with C/EBPβ probe. The position of CUGBP1 is shown by an arrow. Positions of molecular weight markers are shown on the right. G, HDAC1 mRNA is associated with the CUGBP1-eIF2 in livers of old mice. RNA was isolated from the CUGBP1-eIF2 complex and examined by RT-PCR with primers specific to HDAC1 mRNA. RT-PCR with primers to phosphoenolpyruvate carboxykinase (PEPCK) served as a negative control.
HDAC1 Inhibits Proliferation in Livers of Old Mice

CUGBP1 binds to the 5′ region of HDAC1 mRNA and increases HDAC1 levels in Hep3B2 cells. A, the nucleotide sequence of the 5′ region of mouse HDAC1 mRNA. GCN repeats (binding site for CUGBP1) are shown by red. AUG codon is shown by blue. B, purified CUGBP1 specifically binds to the 5′ region of HDAC1 mRNA. Bacterially expressed CUGBP1 was incubated with the HDAC1 probe. Cold HDAC1 RNA and AU-rich RNA were added to the binding reactions to examine specificity of the interactions. The bottom image shows a Coomassie stain of the membrane. C, CUGBP1 isolated from mouse liver binds to the HDAC1 mRNA. Bacterially expressed MBP-CUGBP1 protein (lane 1) and DEAE fractions were incubated with the HDAC1 probe, linked to the probe by UV treatment, and separated by gel electrophoresis. V, void volume (unbound to DEAE column). D, purified CUGBP1 (0.3 M NaCl fraction of DEAE) was incubated with the HDAC1 probe and examined by EMSA. Antibodies to CUGBP1 were incorporated in the binding reaction. Positions of CUGBP1, supershift, and free probe are shown on the left. E, highly purified CUGBP1-eIF2 complex binds to the HDAC1 mRNA. UV cross-link was performed with the purified CUGBP1-eIF2 complex (see “Experimental Procedures”) and HDAC1 probe. The position of CUGBP1 is shown. F, ectopic expression of CUGBP1 increases protein levels of HDAC1 in cultured cells. GFP-CUGBP1 was transfected into Hep3B2 cells, and levels of HDAC1 and CUGBP1 were examined by Western blotting. Antibodies to CUGBP1 detect expression of endogenous CUGBP1 and GFP-CUGBP1. Endo-CUGBP1 and HDAC1 probe completely blocks the binding (Fig. 2B). Therefore, we purified CUGBP1 by siRNA from the liver by chromatography on DEAE column and used this protein for further investigations. UV cross-link assay with

bozykinase mRNA used as the control was not observed in these complexes (Fig. 1G).

CUGBP1 Binds to the 5′ Region of HDAC1 mRNA and Increases Translation of HDAC1—We next examined if CUGBP1 interacts with the HDAC1 mRNA and if CUGBP1 increases translation of HDAC1. In the initial experiments, we determined a region of HDAC1 mRNA to which CUGBP1 binds. The 5′ region of the mouse HDAC1 mRNA contained several GCN repeats (Fig. 2A). Previous studies have shown that CUGBP1 binds to GCN repeats and that mutations of GC islands within RNAs abolished interactions of CUGBP1 with RNAs (23–25). Therefore, we synthesized an RNA probe, the sequence of which is shown in Fig. 2A, and examined interactions of this probe with bacterially expressed purified MBP-CUGBP1 and with CUGBP1 purified from proliferating livers using fractionation on a DEAE column as described (22). Initial UV cross-link experiments with bacterially expressed CUGBP1 showed that CUGBP1 binds to the 5′ region of HDAC1 mRNA and that this binding is specific, since the addition of cold HDAC1 probe completely blocks the binding (Fig. 2B). We have previously shown that the DEAE steps of purification significantly increase RNA binding activity of CUGBP1 due to a removal of endogenous RNA, which competes for the interactions with the probe (22). Therefore, we purified CUGBP1 from the liver by chromatography on DEAE column and used this protein for further investigations. UV cross-link assay with
CUGBP1 purified from the liver showed that CUGBP1 (located in the 0.3 M NaCl elution fraction) interacts with the HDAC1 mRNA (Fig. 2C). To confirm the identity of CUGBP1 in the 0.3 M NaCl fraction, we performed EMSA in which antibodies to CUGBP1 were incorporated into the binding reactions. As can be seen in Fig. 2D, antibodies to CUGBP1 supershifted the complex, confirming that CUGBP1 binds to the 5′ region of HDAC1 mRNA. Given the association of the CUGBP1-eIF2 complex with HDAC1 mRNA in the livers of old mice (Fig. 1), we suggested that the CUGBP1-eIF2 complex might increase translation of HDAC1 through the interaction with the 5′ region of HDAC1 mRNA. To examine this suggestion, the biologically active CUGBP1-eIF2 complex was isolated from the cytoplasm of old livers using three steps of HPLC-based chromatography, as described in our previous work (21). Elution fractions from the last step of purification (chromatography on CHT1 column) were examined by a UV cross-link assay with the HDAC1 mRNA probe. The CUGBP1-eIF2 complex consists of three RNA-binding proteins, one of which is CUGBP1 (21). Consistent with these observations, three proteins of the complex interacted with the HDAC1 mRNA (Fig. 2E). To determine if CUGBP1 increases translation of HDAC1, we overexpressed GFP-CUGBP1 in Hep3B2 cells and examined transfection of the control RNA does not change the expression of HDAC1 and CUGBP1. Examination of the HDAC1 mRNA demonstrated that the inhibition of CUGBP1 does not affect levels of HDAC1 mRNA (Fig. 2G, bar graphs). Thus, these studies showed that CUGBP1 binds to the HDAC1 mRNA and that ectopic expression of CUGBP1 increases translation of HDAC1 in cultured hepatocytes, whereas the inhibition of CUGBP1 reduces translation of HDAC1.

The CUGBP1-eIF2 Complex Increases Translation of HDAC1 in the Liver—To examine if the binding of CUGBP1-eIF2 complex to the HDAC1 mRNA increases translation of HDAC1 in the liver, we used two mouse models that up-regulate the CUGBP1-eIF2 complex in young livers: CUGBP1 TR mice and mice injected with cyclin D3, which phosphorylates CUGBP1 and increases amounts of CUGBP1-eIF2 complex (21). To ensure that CUGBP1 TR livers used for these studies contained the CUGBP1-eIF2 complex, we performed co-IP studies and found that CUGBP1 is associated with eIF2α (Fig. 3A). Western blotting with antibodies to CUGBP1 detects both endogenous and GFP-CUGBP1, which allows us to monitor a ratio of transfected and endogenous CUGBP1 (see Fig. 2F). As can be seen in Fig. 2F, CUGBP1 increased expression of HDAC1 up to 2.5–3-fold. This increase is specific, since the levels of β-actin are not changed by CUGBP1. Examination of HDAC1 mRNA in cells transfected with CUGBP1 showed no change, suggesting that CUGBP1-mediated elevation of HDAC1 does not involve activation of transcription or stabilization of the HDAC1 mRNA (Fig. 2F, bar graphs). Since the levels of ectopically expressed GFP-CUGBP1 are above physiological levels of endogenous CUGBP1 (Fig. 2F), we next examined if the inhibition of endogenous CUGBP1 in HepB2 cells will affect expression of HDAC1. The endogenous CUGBP1 was inhibited with siRNA, as previously described (21), and the levels of CUGBP1 and HDAC1 proteins were determined by Western blotting. Transfection of unrelated short RNA was used as the control. Fig. 2G shows that the inhibition of CUGBP1 leads to the significant reduction of HDAC1, whereas expression of HDAC1 protein.

FIGURE 3. The CUGBP1-eIF2 complex increases translation of HDAC1 in the liver. A, the CUGBP1-eIF2 complex is abundant in CUGBP1 TR mice. CUGBP1 was immunoprecipitated from cytoplasms of WT and CUGBP1 TR livers and probed with Abs to eIF2α and CUGBP1. The bottom images (input) show Western blotting of protein extracts used for co-IPs. B, ectopic expression of cyclin D3 in young livers increases amounts of the CUGBP1-eIF2 complex. Cytoplasmic extracts from livers of mice injected with empty vector (control) and cyclin D3 plasmid were fractionated by size exclusion chromatography. Fractions were analyzed by Western blotting with Abs to CUGBP1 and eIF2α. Positions of size exclusion markers are shown on the top. The position of the CUGBP1-eIF2 complex is shown below. The bottom part of each image shows Western blotting of CUGBP1 IPs from each fraction with antibodies to eIF2α. C, examination of HDAC1 levels in livers of CUGBP1 TR mice and in mice of each step of purification (chromatography on SEC400). The CUGBP1-eIF2 complex was isolated from the bottom images below. D, levels of HDAC1 were calculated as ratios to β-actin. The bar graphs represent a summary of three independent experiments. E, levels of HDAC1 mRNA were determined by real time PCR and calculated as ratios to β-actin. Summary of three independent experiments is shown.
time PCR showed no significant differences in the levels of HDAC1 mRNA (Fig. 3E). Since the formation of CUGBP1-eIF2 complex is controlled by cyclin D3-Cdk4 (21), we injected cyclin D3 into 4-month-old mice and examined the CUGBP1-eIF2 complex and levels of HDAC1 protein. Cytoplasmic cyclin D3 into 4-month-old mice and examined the CUGBP1 complex is controlled by cyclin D3-Cdk4 (21), we injected antibodies to HDAC1 showed that the elevation of the increase of protein levels of HDAC1.

Thus, this elevation of protein levels of HDAC1.

HDAC1 Is a Component of the C/EBPα-Brm Complex in Livers of Old Mice—Given established mechanisms of the elevation of HDAC1 in the mouse liver, we next determined biological consequences of this elevation. Livers of old animals have reduced regenerative capacities (1–3), which are associated with the elevation of a complex repressor C/EBPα-Brm (5, 14). Since previous studies have shown that HDAC1 interacts with C/EBPα (26), we examined if the elevation of HDAC1 in livers of old mice might be involved in the repression of genes through interactions with the C/EBPα-Brm complex. We also tested heterochromatin-associated protein 1α (HP1α), because HP1α is associated with both C/EBPα and HDAC1 (27). Several approaches were applied to examine the association of C/EBPα with HDAC1 and HP1α. Co-IP studies revealed that interactions of C/EBPα with HDAC1 and HP1α were increased in livers of old mice (Fig. 4A). To confirm these data, C/EBPα was immunoprecipitated from young and old livers, and enzymatic activity of HDAC1 was determined in HDAC1 IPs. We found that HDAC1 activity is 2.5–3-fold higher in C/EBPα IPs from old livers than in IPs from young livers. The HDAC1 activity is specific, since trichostatin A (TSA) blocks the HDAC1 activity in C/EBPα IPs (Fig. 4B).

We next examined the interactions of HDAC1 and PH1α with C/EBPα using HPLC-based techniques. In these studies, we have also asked if HDAC1 and HP1α might be associated with the C/EBPα-Brm complex, which is abundant in livers of old mice. We have fractionated nuclear extracts of old livers by size exclusion chromatography and examined C/EBPα, HDAC1, and HP1α proteins were determined by Western blotting with antibodies to C/EBPα, HDAC1, and HP1α was performed. Gel filtration fractions and C/EBPα IPs from each fraction were examined by an HDAC1 enzymatic assay with and without the inhibitor of HDAC1, trichostatin A.

HDAC1 Inhibits Proliferation in Livers of Old Mice

FIGURE 4. HDAC1 Is a component of the C/EBPα-Brm complex in old livers. A, the interaction of HDAC1 with C/EBPα is increased in livers of old mice. C/EBPα was immunoprecipitated from young and old livers, and HDAC1, C/EBPα, and HP1α were examined in these IPs. Input, levels of HDAC1 and HP1α proteins were determined by Western blotting with protein extracts used for Co-IP studies. B, C/EBPα-HDAC1 complexes possess deacetylase activity. C/EBPα IPs were examined for the deacetylase activity. Trichostatin A (TSA) was added to reaction mixtures to examine the specificity of the assay. C, HDAC1 activity is associated with the high M, C/EBPα-Brm complex. Nuclear extracts from old livers were fractionated by size exclusion chromatography. Western blotting with Abs to C/EBPα, HDAC1, and HP1α was performed. Gel filtration fractions and C/EBPα IPs from each fraction were examined by an HDAC1 enzymatic assay with and without the inhibitor of HDAC1, trichostatin A.
new data for the role of the translational CUGBP1-eIF2 complex in the elevation of HDAC1, we asked if the reduction of cyclin D3 by growth hormone (GH) will affect the CUGBP1-eIF2 complexes, expression of HDAC1, and interactions of HDAC1 with the C/EBPα-Brm complex. We first examined if GH changed cyclin D3 levels in cytoplasm. Fig. 6A shows that protein levels of cyclin D3 were higher in cytoplasm from livers of old mice compared with levels of cyclin D3 in the cytoplasm of livers of young mice. However, the levels of cyclin D3 were significantly reduced in the cytoplasm of old mice by treatment with GH. Examination of CUGBP1 and eIF2α showed that, consistent with previous reports, protein levels and phosphorylation of CUGBP1 were increased in the cytoplasm of old livers but were reduced by the treatment with GH. The levels of eIF2α did not differ significantly between young and old mice. We next precipitated CUGBP1 from cytoplasmic extracts and examined the association of eIF2α and CRT (components of the age-specific CUGBP1-eIF2 complex (21)) with CUGBP1. Fig. 6B shows that the CUGBP1-eIF2 complexes were abundant in old livers and that treatment of the mice with GH significantly reduced amounts of the CUGBP1-eIF2 complexes. Western blotting of nuclear proteins showed that protein levels of HDAC1 were also reduced in old livers after treatment with GH (Fig. 6C). Thus, these data revealed that elimination of the CUGBP1-eIF2 complex correlates with the reduction HDAC1 in GH-treated mice. We next examined if the association of HDAC1 with C/EBPα-Brm complex was affected by GH. Coimmunoprecipitation studies revealed that the C/EBPα-Brm complex is abundant in livers of old mice and that GH eliminates the complex. This GH-mediated reduction of the C/EBPα-Brm complex also reduced interactions of HDAC1 with C/EBPα (Fig. 6D). Taking together these data, we conclude that HDAC1 interacts with the C/EBPα-Brm complex in livers of old mice. These studies also revealed that GH-cyclin D3 pathway controls formation of the translational CUGBP1-eIF2 complex and interactions of HDAC1 with C/EBPα-Brm complex in the liver. These data are consistent with the hypothesis that the increase of HDAC1-C/EBPα-Brm complexes in old livers is mediated by a reduction of GH and following elevation of cyclin D3.

**HDAC1 Occupies E2F-dependent Promoters in Livers of Old Mice as the Component of C/EBPα Complexes and Inhibits Cell Proliferation**—We next examined if HDAC1 is associated with E2F promoters and if this association requires formation of the C/EBPα-Brm complex. The chromatin immunoprecipitation assay was performed with c-Myc and FoxM1B promoters, since these two genes are critical regulators of liver proliferation and are repressed by the C/EBPα-Brm complex in livers of old mice. We found that E2F4 and Rb were observed on the c-Myc and FoxM1B promoters in young animals, whereas E2F4, Rb, C/EBPα, and HDAC1 were associated with these promoters in old livers (Fig. 6E). GH-mediated elimination of the C/EBPα-Brm-HDAC1 complexes led to the removal of C/EBPα, Rb, and HDAC1 from the promoters. In these studies, Brm was not detectable on the c-Myc and FoxM1 promoters. We suggest that the lack of Brm on the E2F-dependent promoters might be due to masking of the site of interaction with antibodies within the C/EBPα-Brm complex. It is also possible that Brm is required for the assembly of the complex, but it is dissociated from the

**The Growth Hormone-Cyclin D3 Pathway Regulates CUGBP1-eIF2 Complexes and Interactions of HDAC1 with C/EBPα in the Livers of Old Mice**—We have previously found that aging liver increases cyclin D3-Cdk4 in cytoplasm and nuclei and that this accumulation leads to the formation of C/EBPα-Brm and CUGBP1-eIF2 complexes (6, 21). Given our

**FIGURE 5. Detection of HDAC1 and HP1α within the C/EBPα-Brm complex using a two-dimensional Western blot approach.** A, the C/EBPα-Brm complex was separated from free C/EBPα by size exclusion chromatography. Five runs were combined, and the C/EBPα-Brm complex was immunoprecipitated. B, aliquots were loaded on a two-dimensional gel for the Coomassie stain (upper panel) and for Western blotting with Abs to HDAC1 and HP1α. The middle and lower panels show results of Western blotting. The positions of Brm and phosphorylated forms of C/EBPα, Rb, and cyclin D3 are shown by black arrows. The positions of HDAC1 and HP1α are shown by red arrows.

shown) and several additional unknown proteins (Fig. 5A). Western blotting revealed that two of these new proteins interact with antibodies to HDAC1 and with antibodies to HP1α (Fig. 5B). Taken together, these studies revealed that HDAC1 and HP1α are components of the C/EBPα-Brm complex in old livers and that the C/EBPα-Brm-HDAC1 complex possesses deacetylase activity.
complex on the chromatin. Since HDAC1 inhibits transcription of the genes via deacetylation of histone H3, we have examined the occupation of c-Myc and FoxM1B promoters by Lys-9-acetylated histone H3 and by histone H3 trimethylated at Lys-9. The acetylation of histone H3 at Lys-9 is a mark of transcriptionally active regions, whereas the methylation of histone H3 at Lys-9 is associated with transcriptional repression. ChIP assays with specific antibodies showed that, in livers of young mice, acetyl-Lys-9-H3 is bound to the FoxM1B and c-Myc promoters; however, this histone mark is not detectable on these promoters in livers of old mice. On the contrary, trimethyl-Lys-9-H3 is not detectable on these promoters in livers of young mice, but it is present on the promoters in livers of old mice. These patterns of occupation of the FoxM1B and c-Myc promoters are consistent with the hypothesis that the C/EBPα-mediated recruitment of HDAC1 to E2F-dependent promoters represses transcription of these genes. Interestingly, treatments of old mice with GH reverse the patterns of binding to those observed in livers of young mice. Thus, these studies showed that HDAC1 is a component of C/EBPα complexes that occupy E2F-dependent promoters in old livers and that occupation of the E2F-dependent promoters by HDAC1 leads to transcriptional repression of these genes. C/EBPα is one of the key proteins of the C/EBPα-Brm complex that determines age-specific formation of the complex in the liver (5, 7). To examine the role of HDAC1 in growth-inhibitory activity of C/EBPα, the expression of HDAC1 was inhibited in Hep3B2 cells by siRNA (Fig. 7A, top), and C/EBPα was transfected in cells with reduced levels of HDAC1. A colony formation assay showed that the ability of C/EBPα to arrest cell proliferation was significantly reduced in cells with inhibited HDAC1 (Fig. 7, A and B). Note that we obtained around 50% inhibition of the activity of C/EBPα in cells with reduced HDAC1. The remaining activity of C/EBPα might be due to additional pathways of C/EBPα growth arrest, such as inhibition of CdK2 and stabilization of p21 (11, 12), or due to incomplete inhibition of HDAC1 by siRNA.

**DISCUSSION**

**The Translational CUGBP1-eIF2 Complex Elevates HDAC1 in the Liver**—Previous studies have established the role of transcriptional control in the age-associated loss of proliferative capacities of the liver (4, 5, 7, 14). In this work, we have presented evidence for the contribution of translational pathways in the formation of age-specific complexes that inhibit liver proliferation. We have found that HDAC1 protein levels are increased in quiescent old livers by a translational mechanism that involves CUGBP1 and eIF2. These two proteins form a complex that binds to HDAC1 mRNA *in vitro* and is associated with the HDAC1 mRNA in the livers of old mice. We have also found that elevation or inhibition of CUGBP1 in cultured hepatocytes increases or reduces HDAC1 expression correspondingly. The formation of the
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CUGBP1-eIF2 complex depends on the phosphorylation of CUGBP1 at Ser-302, which is mediated by Cdk4/D-type cyclins (21). We have shown that activation of Cdk4 by cyclin D3 in the livers of young mice resulted in elevation of the CUGBP1-eif2 complex and increase of HDAC1. The critical role of CUGBP1 in the elevation of HDAC1 was confirmed by experiments with CUGBP1 TR mice. These observations show a new transla- tional pathway that controls expression of HDAC1 in the liver.

**HDAC1 Induces Epigenetic Alterations in Livers of Old Mice via Interactions with C/EBPα**—HDAC1 belongs to the family of HDAC proteins that play a critical role in the regulation of many biological processes. In this work, we have found that protein levels of HDAC1 are increased in livers of old mice and that this increase leads to the association of HDAC1 with C/EBPα-Brm complexes. HPLC-based examination of total HDAC1 complexes showed that HDAC1 and HDAC1 activity are detectable in fractions containing C/EBPα-Brm complexes and in fractions with lower Mₗ that do not contain C/EBPα (Fig. 4). This pattern of distribution suggests that the elevation of HDAC1 in old livers might affect other biological processes through the association with other transcription factors. We have focused our studies on the interactions of HDAC1 with C/EBPα-Brm complex. Previous investigations revealed that the formation of C/EBPα-Brm complex is tightly controlled by cyclin D3-Cdk4 in tissue culture systems (28) and in the liver (6, 7) and that cyclin D3 is critical for the inhibitory activity of C/EBPα (28). In this study, we have found that HDAC1 is an important component of the C/EBPα-Brm complex and that the complex possesses deacetylase activity. ChIP studies revealed that HDAC1 is located on E2F-dependent promot- ers as a component of complexes containing C/EBPα, Rb, and E2F. It is known that administration of GH in old mice increases liver proliferation after partial hepatectomy (4) and that GH eliminates the C/EBPα-Brm complex in livers of old mice (6, 7). In this work, we have found that the elimination of the C/EBPα-Brm complex by GH-mediated down-regulation of cyclin D3 removes HDAC1 from the E2F-de- pendent promoters. This removal is consistent with the ability of GH to correct proliferation in old livers (4). The HDAC1-dependent repression of the promoters is associated with deacetylation of histone H3 at Lys-9 and with the following methylation of histone H3 at Lys-9. Our ChIP studies revealed that the recruit- ment of HDAC1 by C/EBPα-Brm complex to E2F-dependent promoters leads to deacetylation of histone H3 at Lys-9 and to trimethyla- tion of histone H3 on these promoters. The inhibition of HDAC1 by siRNA revealed that HDAC1...
tion of HDAC1 reduces the ability of C/EBPα to cause growth arrest. Consistent with these observations, treatment of old mice with GH also normalizes expression of HDAC1 (Fig. 6C) and reduces the C/EBPα-Brm-HDAC1 complex. Taken together, these observations revealed that the elevation of HDAC1 in livers of old mice is involved in the inhibition of proliferation.

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