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RhoB is epigenetically regulated in an age- and tissue-specific manner

Young Soo Yoon, Jung Ha Choo, Taekyung Yoo, Keunsoo Kang, Jae Hoon Chung *

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 335 Gwahangno, Yuseoung-gu, Daejeon 305-701, South Korea

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Abstract

RhoB, a member of the Rho family of small GTPases, regulates the organization of the actin cytoskeleton, malignant transformation, and genotoxic stress-induced signaling. In order to characterize epigenetic regulation of *RhoB* transcription during aging, the mRNA levels of *RhoB* were investigated using various mouse tissues of different ages. Bisulfite sequencing revealed that the CpG dinucleotide methylation patterns of the *RhoB* promoter region were not altered in skeletal muscle and lung during aging. ChIP analysis showed that levels of histone H3 and H4 acetylation were reduced in a tissue-specific manner during aging due to direct HDAC1 binding. Histone H3 lysine 9 trimethylation level and deposition of HP1β increased in *RhoB* promoter during aging, whereas histone H3 lysine 4 dimethylation level gradually decreased. It was concluded that mouse *RhoB* transcription is epigenetically regulated in a tissue-specific manner during aging by histone modification, but not by CpG methylation.

Keywords: *RhoB*; Aging; DNA methylation; Histone modification

Aging can be viewed as a progressive decline in body efficiency after an organism has achieved a maximum reproductive competence [1]. Many theories of aging have been proposed to explain the complex nature of the aging process. One particular genetic program theory is the epigenetic theory of aging. It proposes that gradual changes in the epigenetic status of certain genomic loci accumulate as cells duplicate and organisms age [2]. The altered epigenetic status in aged cells or organisms would result in detrimental effects on the integrity of tissue/organ functions.

DNA methylation and histone modifications constitute major means of epigenetic regulations. In the normal mammalian genome, methylation only occurs at cytosines of CpG dinucleotides. CpG islands are CpG-rich areas, usually located in the vicinity of genes, often found near the promoters widely expressed genes. The CpG islands are the sites of almost all the DNA methylation in mammals [3]. A role for DNA methylation in regulation of gene transcription has been suggested by the fact that highly expressed genes tend to be hypomethylated, and silent genes tend to be hypermethylated [4,5]. Histones can undergo multiple covalent modifications, including acetylation, methylation, ubiquitination, and sumoylation. Histone modifications play important roles in recruiting proteins that regulate overall chromatin structure. Therefore, an additional level of gene regulation is accomplished through histone modifications that contribute to numerous biological processes [6].

In this study, mouse *RhoB* mRNA levels were found to gradually decrease in a tissue-specific manner during aging. The status of DNA methylation, histone acetylation, and histone methylation were investigated to determine epigenetic modifications that affect *RhoB* transcription during aging.

Materials and methods

Animals and tissue samples. Newborn C57B1/6J mice were kept in a specific pathogen free (SPF) room during the course of the study (100 weeks). Aged mice that had any detectable abnormalities after dissection, such as tumors, were excluded. Tissues were obtained from 10 autopsied C57Bl/6J mice at the ages of 4, 15, 30, 60, and 100 weeks.

Total RNA isolation and semi-quantitative RT-PCR. Total RNA was isolated using the Trizol reagent (Invitrogen). One microgram of RNA

* Corresponding author. Fax: +82 42 869 2610.
E-mail address: jhc@kaist.ac.kr (J.H. Chung).

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was reversed-transcribed using the ImProm-II™ Reverse Transcription System (Promega). The primers used for RT-PCR were *RhoB*-RTf (5′-ATG GCG GCC ATC CGC AAG AA), *RhoB*-RTr (5′-TCA TAG CAC CTT GCA GCA GTT GAT), β-*actin* RTf (5′-GGT TCC GAT GCC CTG AGG CT), and β-*actin* RTr (5′-CGC AGC TCA GTA ACA GTC CG).

Quantitative real time RT-PCR. Quantitative real time RT-PCR was performed with iQ SYBR green supermix (Bio-rad) using the icycler iQ™ multicolor real-time detection system (Bio-rad). All PCR were carried out for 40 cycles under the standard PCR conditions. The results of quantitative real time RT-PCR were analyzed based on the threshold cycle (C_t) value. This experiment was performed at least three times.

Bisulfite sequencing. Genomic DNA was isolated using a standard procedure and digested with HindIII. One microgram of digested DNA was treated with bisulfite using the EZ DNA Methylation kit (Zymo Research). The primers used for amplification of bisulfite treated DNA were Bis-1f (5′-GGT TGT TTT GGG TAA GTG TTA G), Bis-1r (5′-AAA TTC ACT CTA AAC CTA AAA TAC AAA AAC), Bis-2f (5′-GTT TTT GTA TTT TAG GTT TAG AGT GAA TTT), and Bis-2r (5′-ATA ATC CTC CTA ACC TAC CAT ATC). The PCR products were cloned using the pGEM-T-EASY vector system (Promega) and sequenced.

Chromatin immunoprecipitation (ChIP) assay. Chromatin Immunoprecipitation was performed according to the protocol provided by Upstate Biotechnology (Upstate Biotech). Three hundred milligrams of skeletal muscle, lung, and brain tissues from 4-, 30-, and 100-week-old mice were used for ChIP assay. The nuclei were immunoprecipitated with 5 μL of anti-acetyl-histone H3, anti-acteyl-histone H4, anti-dimethyl histone H3 (Lys4), anti-trimethyl histone H3 (Lys9), anti-HDAC1 (Upstate Biotech), and anti- HP1β (Santa Cruz), respectively. Precipitated DNA and protein complexes were reverse cross-linked and purified through phenol/chloroform extraction. The primers used for the ChIP assay were ChIP-f (5′-ACT CAG AGA GGC TGC GAC GAA) and ChIP-r (5′-GCA GAC GAC GAC AAC CGA ACT).

Results

Age-dependent changes in mouse RhoB mRNA levels

It has been previously reported that the expression of *RhoB* was changed in aged mouse skeletal muscle tissue by DNA microarray analysis [7]. To confirm the change of *RhoB* expression and determine the levels of *RhoB* mRNA in tissues of different ages, *RhoB* mRNA quantification was performed by RT-PCR. The mRNA was isolated from the tissues of 4-, 15-, 30-, 60-, and 100-week-old C57BL/6J mice. *RhoB* mRNA was quantitated to examine basal transcription levels in liver, skeletal muscle, brain, kidney, thymus, spleen, and lung tissues from 4-week-old mice (Fig. 1A). The mRNA levels of *RhoB* in each sample were normalized using the mRNA levels of β-*actin* for quantitative comparisons. Relatively high transcriptional levels were observed in liver, skeletal muscle, kidney, and lung with the highest level in brain. *RhoB* mRNA was negligible in either thymus or spleen. These data suggest that *RhoB* transcription is regulated in a tissue-specific manner.

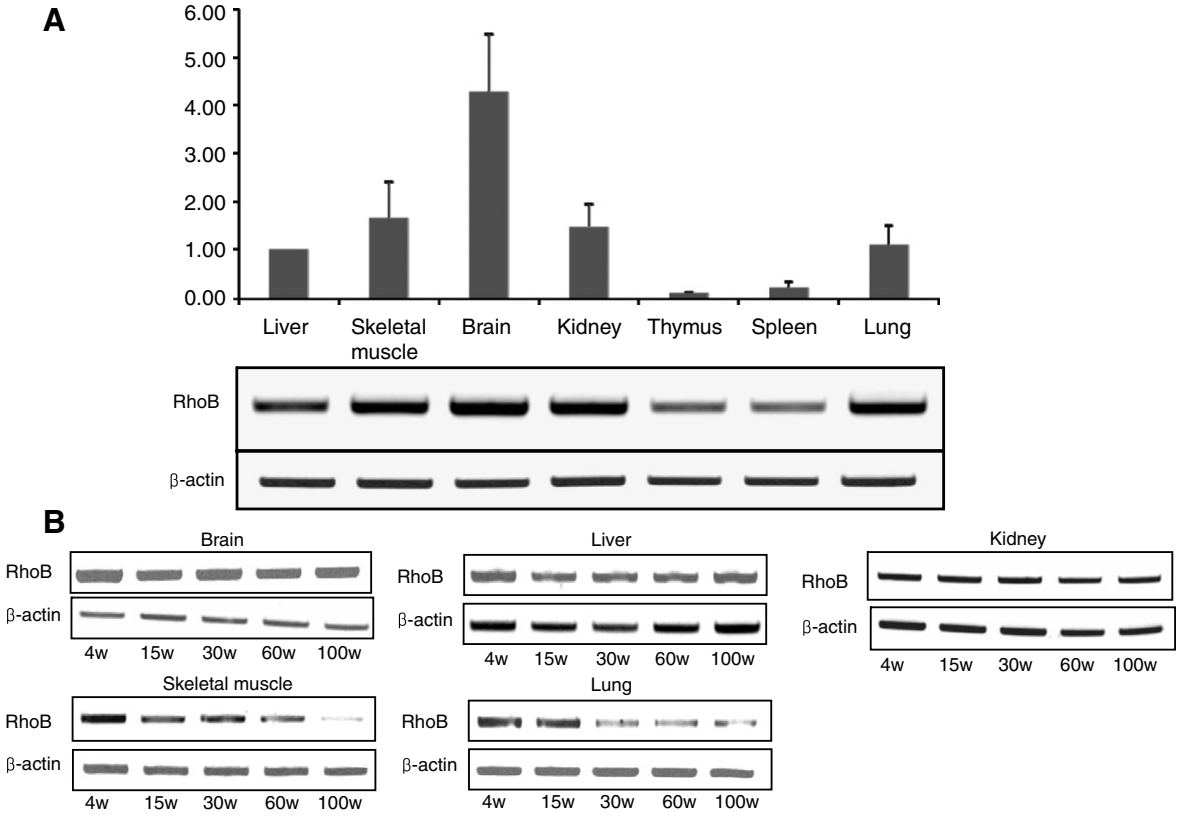

Fig. 1. Age- and tissue-dependent changes in *RhoB* mRNA levels. (A) *RhoB* mRNA levels in several tissues of 4-week-old mice were analyzed by quantitative real time RT-PCR and semi-quantitative RT-PCR. The *RhoB* mRNA levels of each tissue were first normalized with β-*actin* and later compared with the *RhoB* mRNA of the liver tissue. The values in graphs are the average fold differences with standard deviations (SD). (B) *RhoB* mRNA levels in brain, liver, kidney, skeletal muscle, and lung tissue of 4-, 15-, 30-, 60-, and 100-week-old mice were analyzed by semi-quantitative RT-PCR.

The change of *RhoB* mRNA levels during aging was investigated by semi-quantitative RT-PCR in brain, liver, kidney, skeletal muscle, and lung tissues of 4-, 15-, 30-, 60-, and 100-week-old mice (Fig. 1B). These tissues were chosen because *RhoB* mRNA was expressed at high levels in 4-week-old mice. The mRNA levels of *RhoB* gradually decreased in lung and skeletal muscle with age. There were no changes in *RhoB* mRNA levels in the rest of the tissues. Thus, the transcription of *RhoB* appears to be regulated in a tissue-specific manner during the course of aging.

DNA methylation of the RhoB promoter in both skeletal muscle and lung during aging

It was previously reported that DNA methylation of promoter regions is strongly correlated with transcriptional repression [4,8]. To test whether the dramatic changes of *RhoB* mRNA levels in skeletal muscle and lung were associated with changes in DNA methylation of *RhoB* promoter, bisulfite sequencing analysis at the upstream CpG island of *RhoB* was performed (Fig. 2). The *RhoB* promoter contains CpG-rich regions at 3001–3987 bp (GenBank Accession No. X99963), which overlaps the exon [9]. For the cloning purpose, the *RhoB* promoter was divided into two regions, Region 1 (3001–3638) and Region 2 (3512–3987). There are 48 and 65 CpG dinucleotides in these two regions, respectively. In skeletal muscle, the only methylated CpG dinucleotide was found at position 14: the CpG dinucleotides were methylated in 4-, 15-, and 30-week-old mice and unmethylated in 60- and 100-week-old mice; the other CpG dinucleotides did not show significantly altered methylation patterns (Fig. 2A). In lung, all CpG dinucleotides in Region 1 were unmethylated. No changes were observed among all of the age groups (Fig. 2B). Six CpG dinucleotides from 10th to 15th were almost invariably methylated in both skeletal muscle and lung from mice in every age group. Region 2 exhibited unaltered methylation patterns during the course of aging (Fig. 2A and B). These data suggest that changes in *RhoB* transcription are not associated with DNA methylation of the promoter region during the process of aging.

Histone modifications at the RhoB promoter during aging

In order to examine whether histone modifications at the *RhoB* promoter are related to age-dependent changes in *RhoB* transcription levels, a chromatin immunoprecipitation (ChIP) assay was performed using polyclonal antibodies against acetylated H3, acetylated H4, and HDAC1 (Fig. 3). It was previously reported that an inverted CCAAT box in the *RhoB* promoter is crucial for *RhoB* induction in a human non-small cell lung carcinoma cell line [10]. Mouse *RhoB* promoter including two CCAAT boxes was amplified for ChIP assay. In skeletal muscle and lung tissues, where the mRNA levels of *RhoB* decreased during aging, histone H3 (Fig. 3A) and histone H4 (Fig. 3B) acetylation levels of the CCAAT boxes in the *RhoB* promoter also decreased remarkably. In contrast, histone H3 (Fig. 3A) and histone H4 (Fig. 3B) acetylation levels were unaltered in brain, where the mRNA levels of *RhoB* remained constant during the course of aging. These correlations strongly suggest that *RhoB* transcription is regulated by histone H3 and H4 acetylation at the CCAAT box in the *RhoB* promoter.

To explore whether decreased H3 or H4 histone acetylation levels in skeletal muscle and lung tissues from older mice correlate with interactions between HDAC1 and the *RhoB* promoter, ChIP assays were performed using a polyclonal antibody against HDAC1 (Fig. 3C). In skeletal muscle and lung, the level of HDAC1 binding to the CCAAT boxes was altered with age. The HDAC1 was not associated with the CCAAT boxes in young tissues, while the HDAC1 was directly bound to the CCAAT boxes during the process of aging. The HDAC1 binding was not observed in brain tissue from both young and aged mice brains (Fig. 3C). The observation indicates that the age-dependent reduction of *RhoB* mRNA levels is caused by HDAC1 binding, which leads to heterochromatic transition at the *RhoB* promoter.

RhoB transcription is regulated by histone H3 methylation and HP1β deposition

It was reported that histone H3 lysine 4 dimethylation and histone H3 lysine 9 trimethylation are associated with transcriptional activation and repression, respectively [11]. To correlate the reduction of *RhoB* transcription with histone H3 lysine 4 dimethylation and histone H3 lysine 9 trimethylation, ChIP assays were performed using a polyclonal antibody against histone H3 lysine 4 dimethylation and histone H3 lysine 9 trimethylation. In skeletal muscle and lung, histone H3 lysine 4 dimethylation levels gradually decreased during aging. In brain tissue, there is no difference in histone H3 lysine 4 dimethylation levels (Fig. 4A). In contrast to histone H3 lysine 4 dimethylation, histone H3 lysine 9 trimethylation levels gradually increased in skeletal muscle and lung during the course of aging. Histone H3 lysine 9 trimethylation was not detected in either skeletal muscle or lung of 4-week-old mice. However, histone H3 lysine 9 trimethylation levels considerably increased in both aged lung and skeletal muscle. Histone H3 lysine 9 trimethylation was not detected in brain tissue samples from young and old mice (Fig. 4B).

Heterochromatin protein 1 (HP1) was known to bind to N-terminal of histone H3 on trimethylated lysine 9, playing a critical role in heterochromatin formation and epigenetic control of transcription [12,13]. To explore whether HP1β binding correlates with H3 lysine 9 trimethylation in the *RhoB* promoter, ChIP assays were performed using a polyclonal antibody against HP1β (Fig. 4C). In skeletal muscle and lung, HP1β was bound to trimethylated histone H3 lysine 9. The level of HP1β binding increased with age. In contrast, the HP1β binding was not observed in brain tissue from both young and aged mice (Fig. 4C). Histone

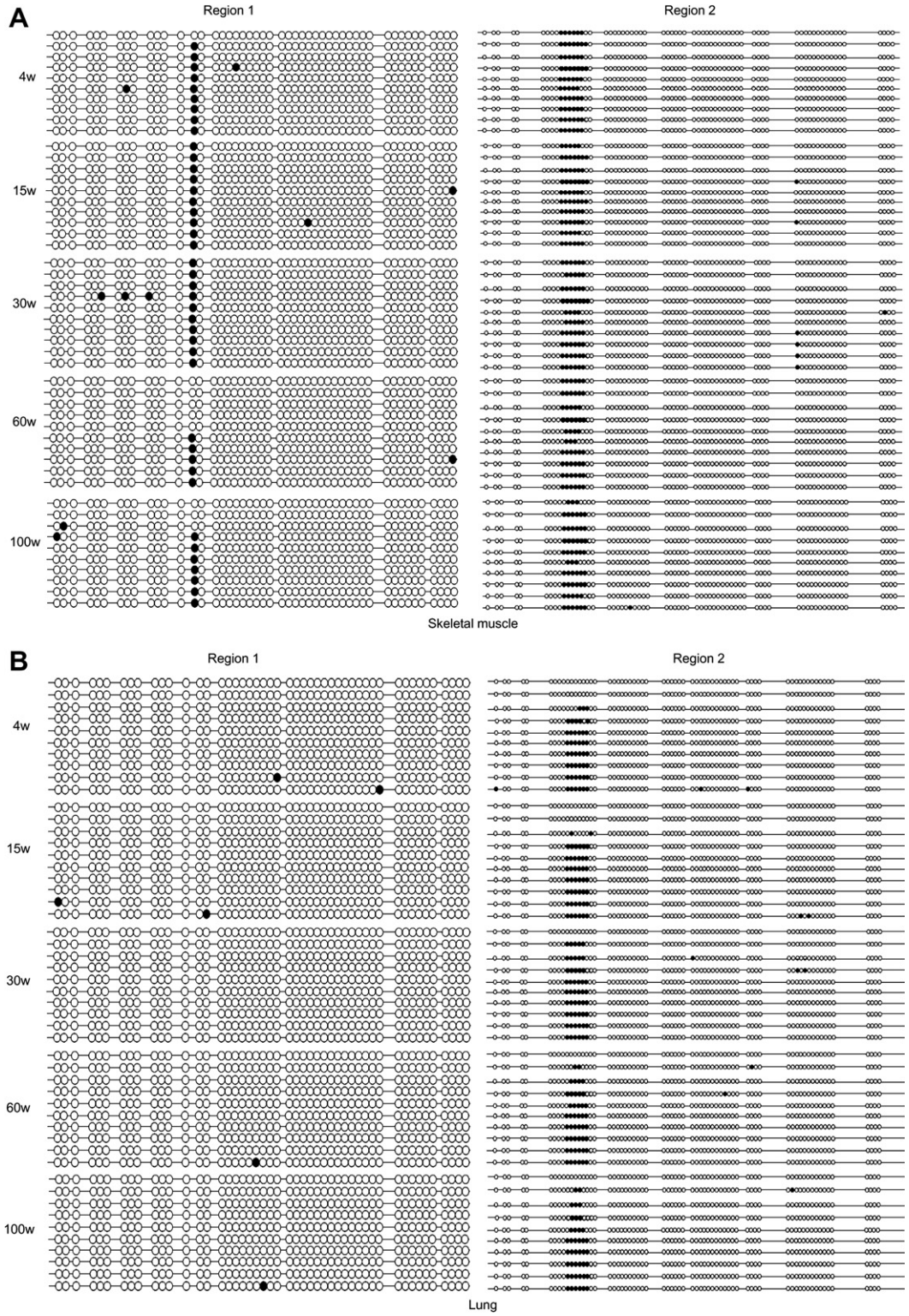

Fig. 2. Methylation status of the mouse *RhoB* promoter. The methylation profiles of the *RhoB* promoter Regions 1 and 2 are shown for skeletal muscle (A) and lung tissue (B) from 4-, 15-, 30-, 60-, and 100-week old mice. Methylated CpGs are indicated by filled circles and unmethylated CpGs are depicted by open circles. Each line represents independent PCR clones.

H3 lysine 9 trimethylation thus appears to play a role in the maintenance of the transcription-repressive chromatin structure of the *RhoB* promoter in aged mice. The HP1β is believed to create and compact chromatin structure that does not permit transcription. These observations indicate that the chromatin structure of the *RhoB* promoter gradually changes to heterochromatic state during aging in a tissue-specific manner.

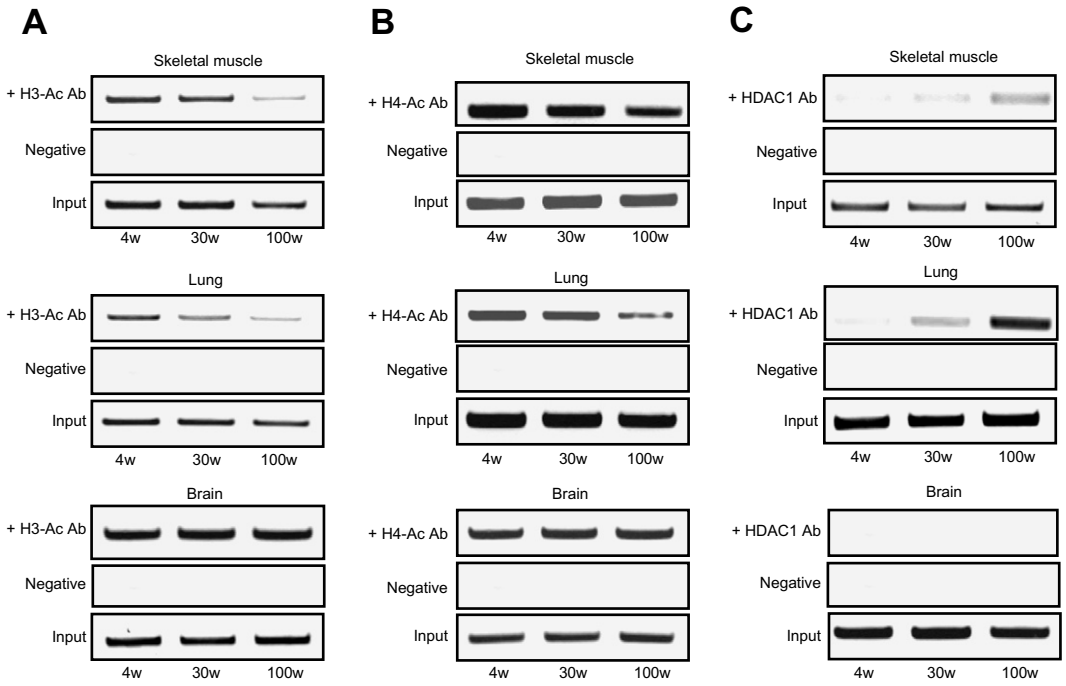

Fig. 3. Age-dependent histone acetylation at the CCAAT box region of the *RhoB* promoter. Changes in histone H3 acetylation levels (A), histone H4 acetylation levels (B), and HDAC1 binding levels (C) in the *RhoB* promoter region are shown. The input and negative control are shown. The immunoprecipitated chromatin was normalized using input levels. Antibodies used: +H3-Ac Ab: anti-acetyl-histone H3; +H4-Ac Ab: anti-acetyl-histone H4; and +HDAC1 Ab: anti-HDAC1.

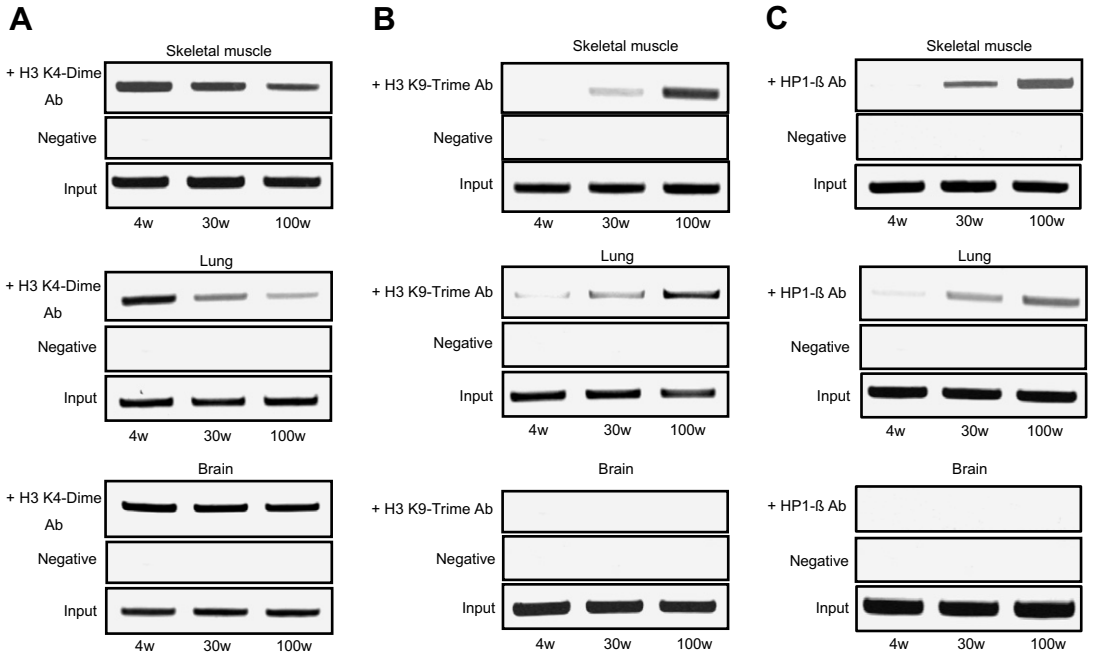

Fig. 4. ChIP analysis of histone H3 lysine 4 dimethylation, histone H3 lysine 9 trimethylation and HP1β binding. Histone H3 lysine 4 dimethylation (A), hisotne H3 lysine 9 trimethylation (B) levels and association of HP1β (C) were determined by ChIP assay. Antibody used: +H3 K4-Dime Ab: anti-dimethyl-histone H3 lysine 4, +H3 K9-Trime Ab: anti-trimethyl-histone H3 lysine 9, and +HP1β Ab: anti-HP1β.

Discussion

The observations reported here revealed the tissue-specific transcription patterns and mechanisms of epigenetic transition of *RhoB* during the aging process. The observed differences in tissue-specific *RhoB* mRNA levels are most likely due to variations in *RhoB* transcriptional activity [14]. Age-dependent *RhoB* transcriptional activity is correlated with histone modifications of *RhoB* promoter. The observations indicate that the chromatin structure of the

RhoB promoter gradually changes to heterochromatic state during aging in a tissue-specific manner. HDAC1 in aged tissue gradually deacetylates the promoter of *RhoB*. Deacetylated lysine residues are then available for subsequent histone methylation and HP1β binding events that establish the long-term silencing of *RhoB* in certain tissues over the course of aging. In mice, a decrease in *RhoB* mRNA levels from aged skeletal muscle and lung tissues raises the possibility that a reduction of *RhoB* mRNA levels explains increased cancer rates with age. It has been reported that *RhoB* is required for apoptosis in cells transformed by DNA-damaging agents [15]. RhoB seems to function as a suppressor or negative modifier in cancer progression [16]. Thus, decreased levels of *RhoB* mRNA during aging may diminish tissue response to either DNA damage or to genotoxic stimuli, predisposing the damaged cells more vulnerable to the progression to a cancerous state. Our results support the idea that a reduction in *RhoB* mRNA from aged mice might increase the occurrence of cancer in a tissue specific manner, as was explained in a human non-small lung carcinoma cell line [10].

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