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YY1 restrained cell senescence through repressing the transcription of p16

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ABSTRACT

The transcription factor YY1 has been implicated to play a role in cell growth control. In this report, we demonstrate that YY1 was able to suppress NCI-H460 cell senescence through regulating the expression of $p16^{INK4a}$, a cyclin-dependent kinase inhibitor. We also show that YY1 participated in the repression of $p16^{INK4a}$ expression in 293T cells through an epigenetic mechanism involving histone acetylation modification. Specifically, HDAC3 and HDAC4 inhibited the $p16^{INK4a}$ promoter activity. The chromatin immunoprecipitation (ChIP) assays verified that HDAC3 and HDAC4 were recruited to $p16^{INK4a}$ promoter by YY1. Moreover, co-immunoprecipitation assays revealed that these three protein factors formed a complex. Furthermore, knockdown of these factors induced cell enlargement and flattened morphology and significantly increased the SA-β-gal activity, a biochemical marker of cell senescence. Overall, data from this study suggest that YY1, HDAC3 and HDAC4 restrained cell senescence by repressing $p16^{INK4a}$ expression through an epigenetic modification of histones.

1. Introduction

The transcription factor YY1 (Yin Yang 1) is a ubiquitous, highly conserved zinc finger transcription factor [1–3] that activates or represses several different eukaryotic genes, among which are the c-Myc, c-Fos, β-casein, β-actin, interleukin-3 and -5, and IFN-γ genes, as well as some viral promoters [3–7]. In support of the hypothesis that the actions of YY1 are controlled by protein–protein interactions, a wide variety of transcription factors have been shown to associate with YY1, such as Sp1 [8], c-Myc [9] and C/EBP [10]. Subsequently, it was found that the histone deacetylases HDAC1, HDAC2 and HDAC3 could also bind YY1 and repress transcription when targeted to promoters [11–14]. We have recently demonstrated by using ChIP assays that histone deacetylase HDAC4 was recruited by YY1 to the interleukin-5 promoter and repressed the transcription activity of the gene [15]. Many other proteins have been reported to interact with YY1, such as p300, PCAF [13] and CBP [16], which possess the histone acetyltransferase (HAT) activity. It is therefore appealing to speculate that YY1 may activate transcription by the recruitments of HAT proteins. Conceivably, the selective binding of YY1 with either HAT(s) or HDAC(s) determines whether it will mediate the activation or repression of transcription of a given gene.

The role of protein acetylation in the transcriptional control has been extensively studied and is generally considered a major step toward gene expression [17]. Two categories of enzymes with antagonistic activities, i.e., histone acetyltransferases (HATs) and histone deacetylases (HDACs), contribute substantially to the control of gene transcription through modifications of the acetylation state of core histones, and in cases, the transcription factors [18]. In general, histone hyperacetylation correlates with gene activation, whereas histone deacetylation is associated with gene silencing [19].

The $p16^{INK4a}$ (hereafter p16), a tumor suppressor protein, functions as an inhibitor of Cdk4 and Cdk6, the cyclin-dependent kinases that initiate the phosphorylation of the retinoblastoma protein (pRb) [20]. pRb is a corepressor that binds several E2F family transcription factors to silence a host of genes required for cell cycle advancement from G1 to S-phase. Thus, p16 has the capacity to arrest the cell cycle in G1, and one of its physiological roles appears to be to induce irreversible growth arrest characteristic of replicative senescence [21]. Recently, studies by Brachen et al. implicated that the depletion of some polycomb group proteins, such as EZH2, SUZ12 and BMI1, activated p16 transcription and promoted cell senescence [22]. Also, Seligson et al. reported that expression of YY1 correlated with the resistance to immune-mediated apoptosis [23]. Whether YY1, as a polycomb group protein, participates in cell senescence has not been elucidated. In this report, we present experimental data showing that knockdown of YY1 by a specific siRNA vector (YY1i) increased expression of p16 and induced cell senescence. Moreover overexpression of HDAC3 and HDAC4 resulted in the repression of p16 expression. We also provide evidence that HDAC3 and HDAC4 were recruited to p16 promoter by

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YY1, and discuss the possible mechanisms of the functional interactions among YY1, HDAC3 and HDAC4 in p16 transcriptional inhibition and their effects on cell senescence.

2. Materials and methods

2.1. Cell culture, transfection and luciferase reporter assay

NCI-H460 and 293T cells were maintained in IMDM medium supplemented with 10% FBS, 100 mg/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. Transient transfection of 293T cells and NCI-H460 cells was performed using the conventional calcium phosphate–DNA precipitation method and Fu GENE HD transfection reagent (Roche), respectively. Twenty-four hours after the transfection of the expression vectors, or 48 h after transfection of RNAi vectors, cells were harvested for luciferase reporter activity, RT-PCR, Western or ChIP assays. Transfected cells were analyzed for luciferase reporter activity using a Promega dual-luciferase reporter assay system. The *Renilla* luciferase control plasmid pREP7-RLuc was co-transfected in each experiment for normalization. All the results represent the means ± S.D. based on at least three independent experiments.

2.2. Plasmid constructs

The p16 promoter reporter (−869 to +1 bp from the ATG translation initiation site) ligated to the luciferase reporter gene (pGL2 basic, Promega) was provided by Dr. E. Hara (Imperial Cancer Research Fund Laboratories, London, U.K.). Plasmids expressing human HDAC1–6 (fused to the FLAG-epitope) were gifts from Dr. W.C. Greene (Gladstone Institute of Virology and Immunology, San Francisco, CA, USA) and Dr E. Seto (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA). YY1 was kindly supplied by Dr. E. Bonneloy (Regulation de la. Transcription et Maladies Genetiques, CNRS UPR 2228, Universite Rene Descartes, France). The plasmids expressing human HDAC4 (fused to the GFP-epitope) were generously provided by Dr. R. Bassel-Duby (Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA).

2.3. RNA extraction and RT-PCR

Total cellular RNA was extracted from the 293T cells according to the Promega Total RNA Isolation System manual. RNA was re-suspended in RNase-free water and quantitated by spectrophotometry before being reverse transcribed. PCR products were resolved in 2% agarose gel. The p16 primer pairs were: sense 5′-ttcctggacacgctggt-3′ and antisense 5′-caatcggggatgtctgag-3′. The β-actin primer pairs were: sense 5′-tcgtgcgtgacattaaggag-3′ and antisense 5′-atgccagggtacatggtggt-3′. The number of cycles of PCR amplification was 25 for the β-actin, and 30 for the p16 gene.

2.4. Chromatin immunoprecipitation (ChIP)

The protocol for chromatin immunoprecipitation (ChIP) was described previously [24]. Briefly, 1×10^6 cells were treated with 2% formaldehyde in IMDM medium for 10 min at 37 °C to crosslink protein–DNA. To stop the reaction glycine (125 mM final) was added. After washed 4 times with cold PBS, cells were lysed by incubating in nucleus lysis buffer. The lysate was sonicated with a Uibra cell TM-130 sonicator. After centrifugation at 12000 rpm for 10 min, the supernatant was diluted 1:10 in ChIP dilution buffer. The chromatin solution was precleared with 50 μl of protein A-agarose beads (Upstate Biotechnology). The soluble fraction was collected and 5 μg of anti-acetyl-histone H3 (Upstate Biotechnology), anti-acetyl-histone H4 (Upstate Biotechnology), anti-YY1 (Santa Cruz, sc-1703) or anti-Flag (Sigma, F3165) antibodies was added. After immunoprecipitation, the precipitated complexes were collected by adding 60 μl of protein A-agarose beads. After washing, freshly prepared elution buffer (1% SDS, 0.1M $NaHCO_3$) was added, and the crosslinking was reversed at 65 °C for 4 h. DNA was recovered by Proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. For re-ChIP, the immuno-complexes were eluted by adding 100 ml re-ChIP elution buffer (10 mM DTT) after washing for 30 min at room temperature with rotation. The supernatant was diluted 1:20 in ChIP dilution buffer and the antibody against the second protein of interest was added, and following treatment as indicated above. Samples were analyzed by PCR. The sequences of the primers used were: P1 sense 5′-agtttcgctcttgtctcccag-3′, antisense 5′-atggcgaaaccctgtctctac-3′; P2 sense 5′-agacagccgttttacacgcag-3′, antisense 5′-caccgagaaatcgaaatcacc-3′; and P3 sense 5′-taggaaggttgtatcgcggagg-3′, antisense 5′-caaggaaggaggactgggctc-3′ [25]. The locations of P1, P2 and P3 at the p16 promoter are illustrated in Fig. 2F. The number of cycles of PCR amplification was 30 for the input samples, 33 for the RNAi vector transfected samples and 35 for the expression vector transfected samples. All the experiments were repeated three times, and one of the representative results is shown in the text.

2.5. Western blot and co-immunoprecipitation (Co-IP) assays

NCI-H460 and 293T cells were harvested after transfection. 1×10^6 cells were digested and lysed in the lysis buffer for 30 min at 4 °C. Total cell extracts were separated in 12% SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred to a polyvinylidene fluoride membrane. The membrane was incubated with anti-p16 (Santa Cruz, sc-468), anti-Flag, anti-YY1, anti-HDAC3 (Santa Cruz, sc-11417), anti-HDAC4 (Santa Cruz, sc-11418) or anti-β-actin (Sigma, A1978) antibodies, and visualized by using the Chemiluminescent Substrate method with the SuperSignal West Pico kit provided by Pierce Co. β-actin was used as an internal control for normalizing the loading materials.

Co-precipitation was performed in 293T cells. The 293T cells were incubated with DSP for 30 min, and then lysed in Buffer I (50 mM Tris–HCl, pH7.5, 150 mM NaCl, 1% NP40, 0.05% sodium deoxycholate and protease inhibitors). Total cell extracts were precleared with 40 μl Protein A-agarose at 4 °C for 1 h. The supernatant was incubated with the anti-YY1 and anti-GFP antibodies (Upstate, 06-896) with gentle shaking for 1 h at 4 °C followed by the addition of 40 μl of Protein A-agarose and incubated for another 3 h. The beads were re-suspended in 100 μl of 2× loading buffer and boiled for 10 min. The proteins were separated on a 12% SDS-PAGE gel and then transferred to polyvinylidene fluoride membrane for immunoblotting detection with anti-YY1 or anti-GFP antibodies.

2.6. RNA interference (RNAi)

The target sequence for YY1 RNAi was 5′-gagcagaagcaggtgcaga-3′ [26], and that for HDAC4 was 5′-cgtcaacatggctttcacc-3′ [18]. Oligonucleotides that represent small hairpin RNAs (shRNAs) targeting these sequences were designed and cloned into the pSilencer2.0-U6 vector (Ambion) between BamHI and HindIII restriction sites according to the manufacture's instructions. The HDAC3i vector was constructed previously [27].

2.7. Immunofluorescence staining

The treated 293T cells were washed twice in PBS, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 at room temperature and then quenched in ice-cold PBS. After blocking with 5% BSA, collected cells were incubated with rabbit anti-YY1 antibody for 1 h and stained with TRITC-conjugated goat anti-rabbit secondary antibody (Zhongshan, China) for 45 min at 4 °C. Cells were examined under a fluorescence microscope (Nikon, Japan) and the images were collected at ×20 magnification with appropriate filters.

2.8. Senescence-associated galactosidase activity assay

Cells were lysed in reporter lysis buffer after transfection for 7 days. Cell lysates containing equal amounts of protein were diluted in equal volumes of 2× assay buffer

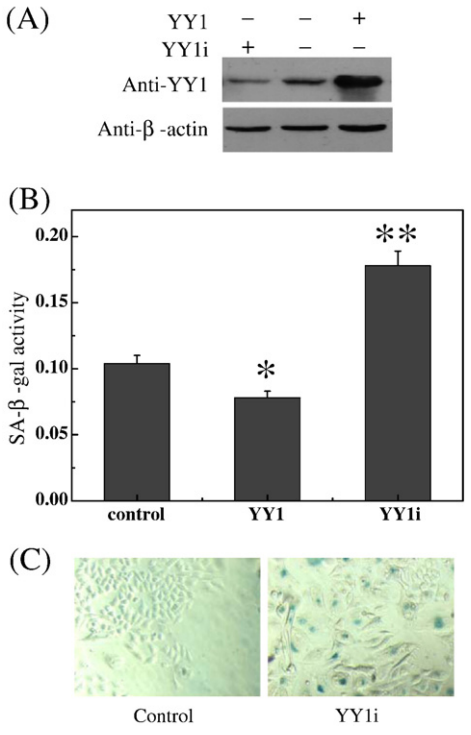

Fig. 1. Knockdown of endogenous YY1 enhanced NCI-H460 cell senescence. (A) Western blotting analysis of the YY1 protein in NCI-H460 cells transfected with YY1i vector or YY1 expression vector. The pcDNA3.1 empty vector and an irrelevant siRNA vector were used as controls. (B) YY1 restrained cell senescence. NCI-H460 cells transfected with YY1 or YY1i vectors were lysed and tested for the SA-β-gal activity, using ONPG as substrate at pH6.0. The controls were the pcDNA3.1 empty vector and an irrelevant siRNA vector. $^{**}P< 0.01$, $^{*}P< 0.05$, ($n=3$). (C) Representative photomicrographs of the SA-β-gal staining at day 7 post YY1i transfection. The irrelevant siRNA vector was used as the control.

containing 1.33 mg/ml *o*-nitrophenyl-D-galactopyranoside (ONPG), 2 mM $MgCl_2$, and 100 μl 2-mercaptoethanol in 200 mM phosphate buffer (pH 6.0), and incubated at 37 °C for 4 h. The absorbance at 420 nm was measured after the addition of an equal volume of 1 M Na_2CO_3. All the results represent the means±S.D. based on at least three independent experiments.

NCI-H460 cells were transfected with the YY1siRNA, HDAC3siRNA or HDAC4-siRNA vectors, or an irrelevant siRNA vector as the control. At day 7 after transfection, cells were processed using a Senescence β-Galactosidase Staining Kit (Cell Signaling Technology). Cells were examined under a fluorescence microscope (Nikon, Japan) and the images were collected at ×10 magnification with appropriate filters. These experiments were repeated three times, and one of the representative results is shown.

2.9. Electrophoretic mobility shift assay (EMSA)

Biotin end-labeled sense and antisense oligonucleotides covering −544/−55 bp of the p16 promoter were: 5′-gaagccatactttccctatgacaccaaacaccccgattcaatttggcagtta-3′ (−544 bp/−493 bp); 5′-gggggagcgcggctgggagcagggaggccggagggcggtgtgggggggcaggt-3′ (−401 bp/−349 bp); 5′-gggaggagcccagtcctccttccttgccaacgctggctctggcgagggctgc-3′ (−348 bp/−297 bp); 5′-gagacccaacctggggcgacttcaggggtgccacattcgctaagtgctcgga-3′ (−275 bp/−224 bp); and 5′-aagaggaggggctggctggtcaccagagggtggggcggaccgagt-3′ (−99 bp/−55 bp). The oligonucleotides were labeled using a biotin 3′-end labeling kit following the manufacturer's instructions (Pierce), respectively, and then annealed to generate the double-stranded probes. EMSA was performed by pre-incubating 5 μg of nucleus extract in 2 μg poly-dI:dC, 2 μl binding buffer on ice for 10 min. Twenty-five fmol biotin-labeled double-stranded probe was added to the mix and incubated on ice for another 15 min. DNA/protein complexes were resolved in a 6% polyacrylamide gel and analyzed according to the EMSA kit instructions (Pierce). Supershift assays were done by adding 1 μg of anti-YY1 antibody after DNA/protein incubation.

3. Results

3.1. RNAi-induced silencing of endogenous YY1 enhanced NCI-H460 cell senescence

To determine whether YY1 affects cell senescence, we assayed the senescence-associated β-galactosidase (SA-β-gal; pH6.0) activity and SA-β-gal staining, a biomarker tightly associated with cell senescence in human cells [28,29]. Senescent human fibroblasts express a β-Gal, an endogenous product that can be detected in single cells by X-Gal at pH 6.0, by forming a local blue precipitate upon cleavage [29]. We first constructed the siRNA vector specific to YY1 (YY1i) to knockdown the endogenous YY1 expression. The inhibitory efficiency of YY1i on endogenous YY1 expression in NCI-H460 cells was verified by Western blotting, and the exogenous expression of the YY1 vector was also measured (Fig. 1A). Next, the transfected NCI-H460 cells were lysed and assayed for the SA-β-gal activity. As shown in Fig. 1B, a 1.5-fold increase in SA-β-gal activity was seen after 7 days of YY1i transfection, while overexpression of YY1 led to a reverse effect. In addition, cells transfected with YY1i exhibited phenotypic changes that resembled those observed in the cells undergoing replicative senescence. These changes include increased SA-β-gal staining, flattened cell morphology, and enlarged cell size (Fig. 1C). Together, these data implicated that YY1 played a role in restraint of cell senescence.

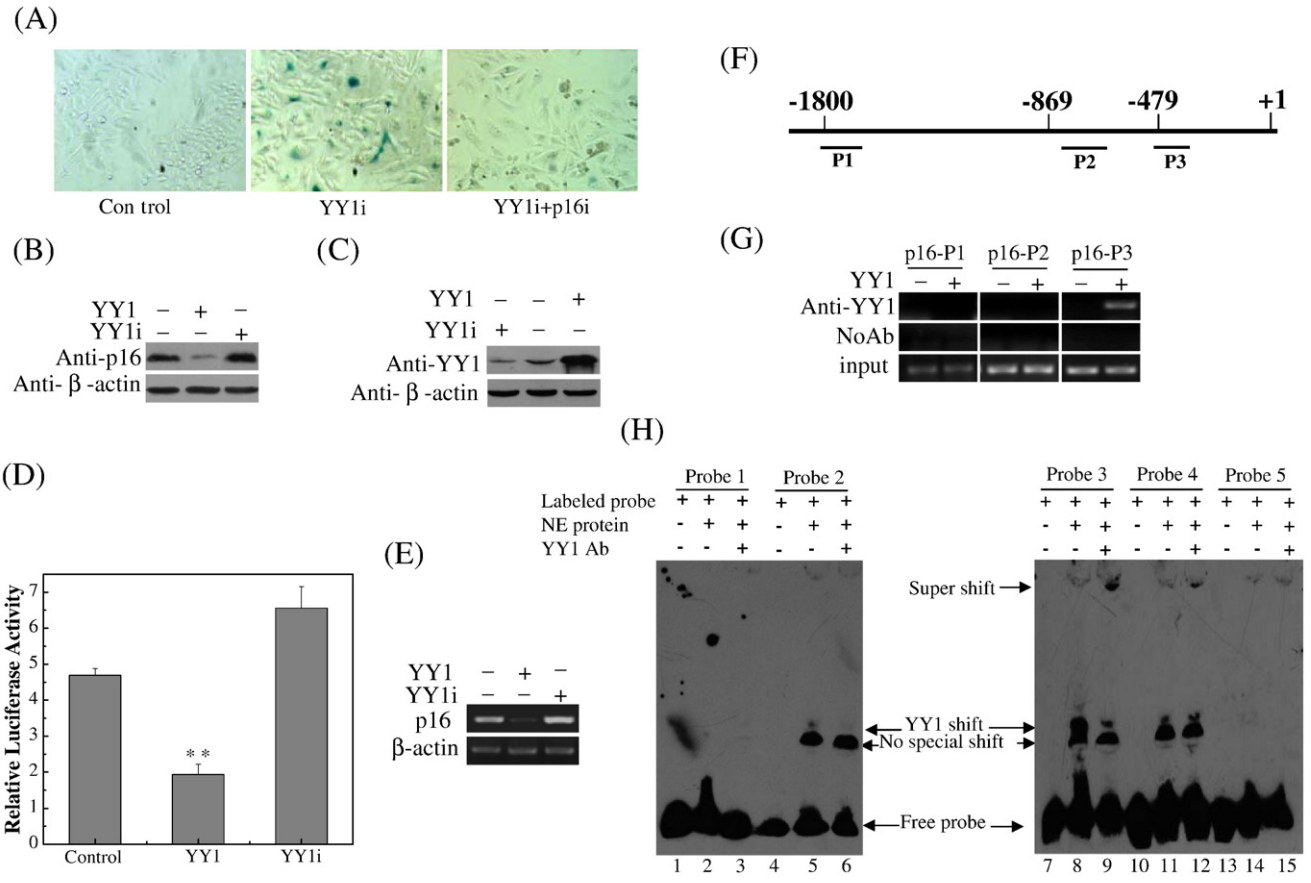

Fig. 2. YY1 repressed p16 expression to restrain cell senescence. (A) YY1 restrained senescence of NCI-H460 cells through p16. Representative photomicrographs of the SA-β-gal staining at day 7 after YY1i, or YY1i plus p16i transfection. An irrelevant siRNA vector was used as the control. (B) YY1 repressed p16 protein expression. Western blots of the p16 protein in NCI-H460 cells transfected with YY1 or YY1i vector. (C) Western blot analysis of the YY1 protein in 293T cells transfected with YY1i vector or YY1 expression vector, pcDNA3.1 empty vector and an irrelevant siRNA vector as the control. (D) YY1 inhibited p16 promoter activity. 293T cells were transiently transfected with YY1 or YY1i vector, and the p16 promoter activity was examined by luciferase reporter assay. The controls were the pcDNA3.1 empty vector and an irrelevant siRNA vector. $^{**}P<0.01$, ($n<3$). (E) YY1 repressed endogenous p16 mRNA. 293T cells were transiently transfected with YY1 or YY1i vector. Total RNA was isolated, reverse transcribed and p16 mRNA was measured by PCR. β-actin was used as an internal control. (F) Diagram of the 5′-flanking region of p16 gene. Lines (P1–3) indicate the three regions of p16 promoter amplified by specific primers in ChIP analysis. (G) Binding of YY1 on p16 promoter. ChIP assays with anti-YY1 antibody in 293T cells transfected with YY1 expression vector. (H) YY1 interacted with p16 promoter. Nuclear extracts from 293T cells were incubated with 5 biotin-labeled oligonucleotides corresponding to region −544 to −55 bp of the p16 promoter. The reactions without nuclear extracts were used as control (lanes 1, 4, 7, 10 and 13). Lanes 2, 5, 8, 11 and 14 were the reaction with nuclear extracts. Anti-YY1 antibody was added for super-shift study (lanes 3, 6, 9, 12 and 15).

3.2. YY1 restrained cell senescence through repressing p16 expression

Since p16 has been implicated to play a pivotal role in cell senescence, we sought to determine whether YY1 induced cell senescence through p16. We used a p16siRNA vector (p16i) to knockdown the endogenous p16 expression [30]. Apparently, compared with the cells transfected with YY1i alone, co-transfection of cells with YY1i and p16i vectors failed to induce cell senescence (Fig. 2A). Western blotting assays demonstrated that the p16 protein expression was decreased upon overexpression of YY1, while it was enhanced by knockdown of the endogenous YY1 in NCI-H460 cells (Fig. 2B). A previous study demonstrated that the HDAC inhibitor butyrate (NaB) was able to increase the expression of p16 in NCI-H460 cells [31]. To look further into the roles of YY1 in regulation of p16 expression, we tested the effect of YY1 on p16 expression in 293T cells. First, we measured the expression of YY1 in 293T cells by using Western blotting (Fig. 2C). We then showed that overexpression of YY1 inhibited the p16 promoter activity, while knockdown of the endogenous YY1 expression by YY1i increased the activity of p16 promoter (Fig. 2D). Also, it can be seen from Fig. 2E that the p16 mRNA level was decreased upon the overexpression of YY1, but increased by the knockdown of the endogenous YY1. Additionally, we designed a series of primer coordinates to the three regions in p16 promoter for ChIP assays (Fig. 2F). P1 locates at the far upstream of p16 promoter (−1800 bp) as a negative control, whereas P2 and P3 locate at the downstream of p16 promoter (−800 bp and −300 bp), representing the important regulatory regions of p16 gene. As can be seen in Fig. 2G, the ChIP data revealed that YY1 was enriched at the p16 promoter (P3) upon the overexpression of YY1. Furthermore, Electrophoretic Mobility Shift Assays (EMSA) was performed to confirm the ChIP results. The EMSA results shown in Fig. 1H revealed that although the nuclear extracts could significantly retard the probes 2, 3 and 4 (lanes 8, 11 and 14), only probe 3 was supershifted by anti-YY1 antibody (lane 9). This demonstrated that YY1 was able interact with the p16 promoter, confirming the ChIP results of YY1 enrichment at the p16 promoter (P3) upon the overexpression of YY1 (Fig. 2G). These results further support the notion that YY1 participates in cell senescence through inhibiting p16 transcription.

3.3. HDAC3 and HDAC4 downregulated p16 expression

In a previous report, we described that the histone acetyltransferase p300 promoted p16 expression [30], implying that HDAC(s) may also be involved in p16 regulation as antagonistic enzyme(s) to HATs. To test this and to determine which HDACs participate in the inhibition of p16 expression, 293T cells were transfected with p16 promoter reporter, together with the expression vectors of HDAC1-6. Of the six HDACs tested, HDAC3 and HDAC4 were found to exhibit much more prominent effects on p16 repression, compared with other HDACs (Fig. 3A). We then used HDAC3i and HDAC4i vectors to knockdown the HDAC3 and HDAC4 expression, and the Western blotting data in Fig. 3B confirmed that the expression of HDAC3 and HDAC4 was decreased by this RNAi protocol. Next, the transfection experiments revealed that the siRNA-mediated silencing of endogenous HDAC3 and HDAC4 activated the p16 promoter activity, while overexpression of HDAC3 and HDAC4 inhibited p16 promoter activity (Fig. 3C). Moreover, the p16 mRNA level was also decreased upon HDAC3 and HDAC4 overexpression, but increased by knockdown of HDAC3 and HDAC4 (Fig. 3D). These results implicated that HDAC3 and HDAC4 inhibited p16 transcription.

3.4. HDAC3 and HDAC4 downregulated p16 to suppress cell senescence

First, the inhibitory efficiency of HDAC3i and HDAC4i on endogenous HDAC3 and HDAC4 expression in NCI-H460 cells was verified by Western blotting (Fig. 4A). And then we showed that, as expected, the p16 protein level was decreased by overexpression of HDAC3 and HDAC4, while it was enhanced by knockdown of these HDACs in NCI-H460 cells (Fig. 4B and C). Also, knockdown of the endogenous HDAC3 and HDAC4 expression by RNAi increased the SA-β-gal activity,

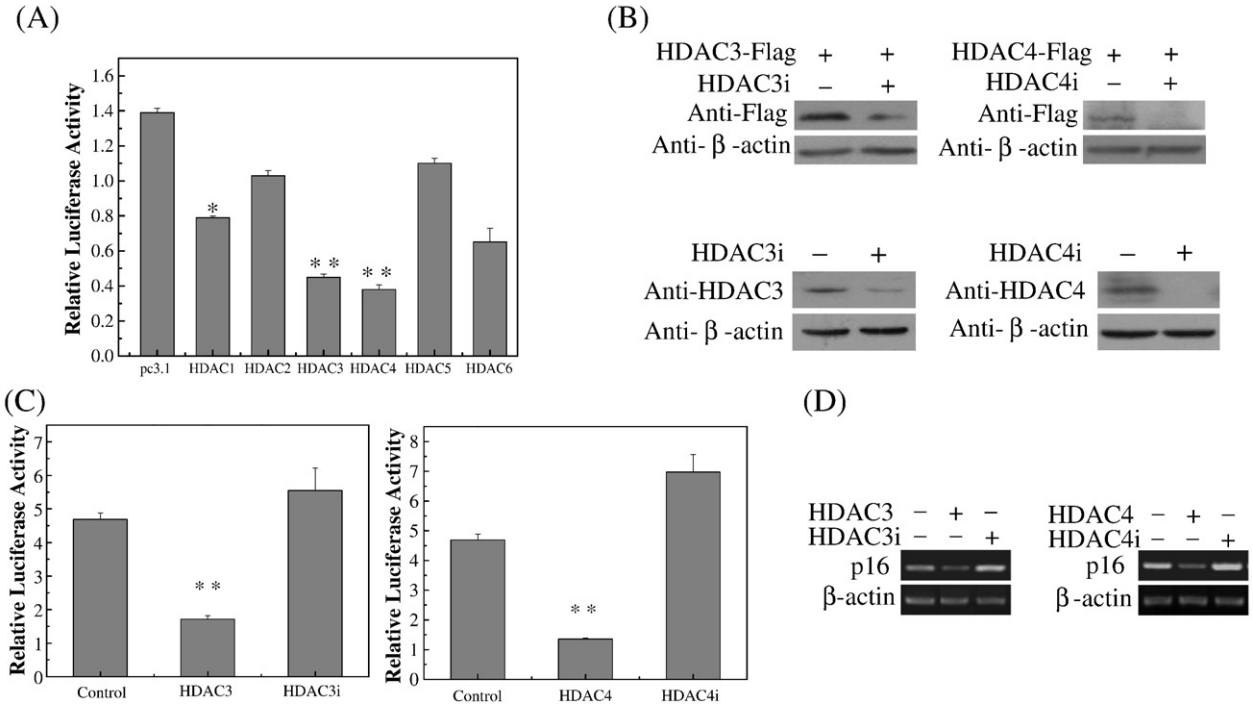

Fig. 3. HDAC3 and HDAC4 downregulated p16 expression. (A) HDACs downregulated the p16 promoter activity. One μg of the p16 reporter vector, together with 1 μg of each HDAC1–6 were co-transfected into 293T cells. Luciferase activity was determined 24 h after transfection and normalized to the *Renilla* activity. The pcDNA3.1 vector was used as control. $^{**}P<0.01$, $^{*}P<0.05$, ($n=3$). (B)Western blots of the HDAC3/4. The inhibitory efficiency of HDAC4i and HDAC3i on HDAC4 and HDAC3 proteins was measured in 293T. (C) HDAC3 and HDAC4 inhibited the p16 promoter activity. 293T cells were transfected with: 1 μg HDAC3 or 5 μg HDAC3i vector (Left), 1 μg HDAC4 or 5 μg HDAC4i vector (Right). The controls were the pcDNA3.1 empty vector and an irrelevant siRNA vector. $^{**}P<0.01$, ($n=3$). (D) Quantitative estimation of p16 mRNA level. Cells were transfected with HDAC3/4 vectors, HDAC3i or HDAC4i vector. Total RNA was isolated, reverse transcribed and p16 mRNA was measured by RT-PCR. β-actin was used as an internal control.

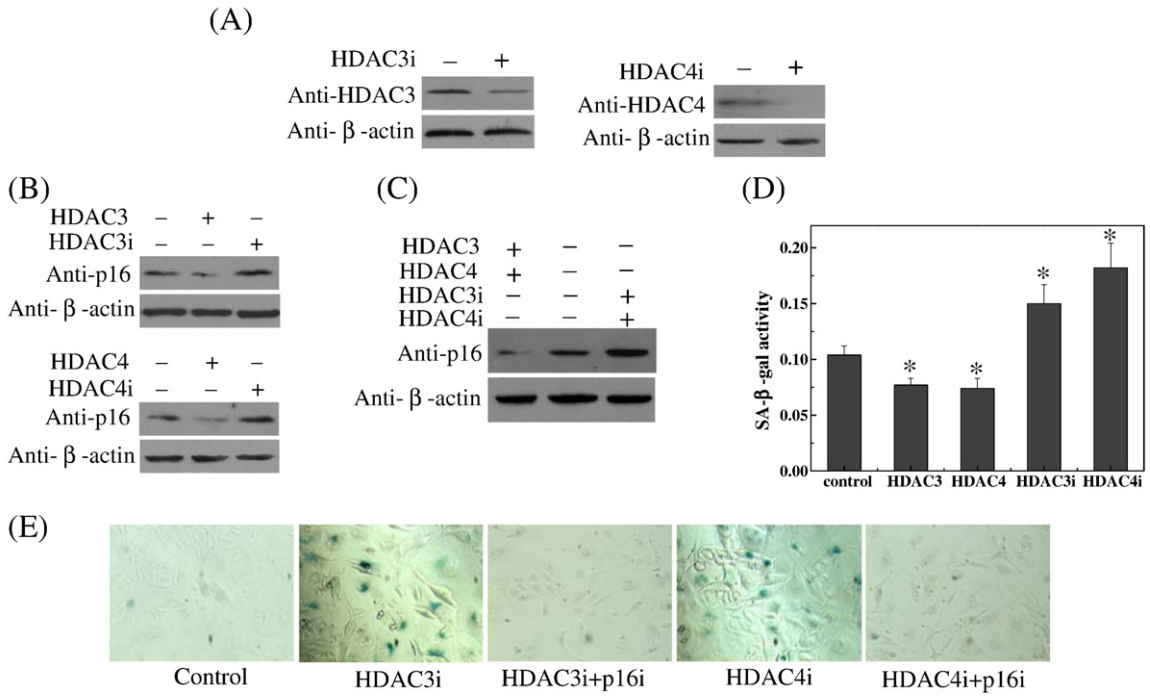

Fig. 4. HDAC3 and HDAC4 repressed p16 expression to affect cell senescence. (A) Western blotting analysis of the HDAC3 and HDAC4 proteins in NCI-H460 cells transfected with HDAC3i and HDAC4i vectors. An irrelevant siRNA vector was used as the control. (B–C) HDAC3/4 inhibited p16 expression. Western blots of the p16 protein in NCI-H460 cells transfected with HDAC3/4, HDAC3i or HDAC4i vector (B), and with HDAC3/HDAC4, or HDAC3i/HDAC4i (C). (D) HDAC3/4 suppressed cell senescence through repressing p16. NCI-H460 cells transfected with HDAC3, HDAC4 or HDAC3i, HDAC4i vectors were lysed and tested for the SA-β-gal activity, using ONPG as substrate at pH 6.0. The control was the pcDNA3.1 empty vector and an irrelevant siRNA vector. *$P<0.05$, ($n=3$). (E) Knockdown of HDAC3 and HDAC4 increased the SA-β-gal activity of NCI-H460 cells. Representative photomicrographs of the SA-β-gal staining at day 7 post transfection (HDAC3i, HDAC4i, or p16i) are presented. An irrelevant siRNA vector was used as the control.

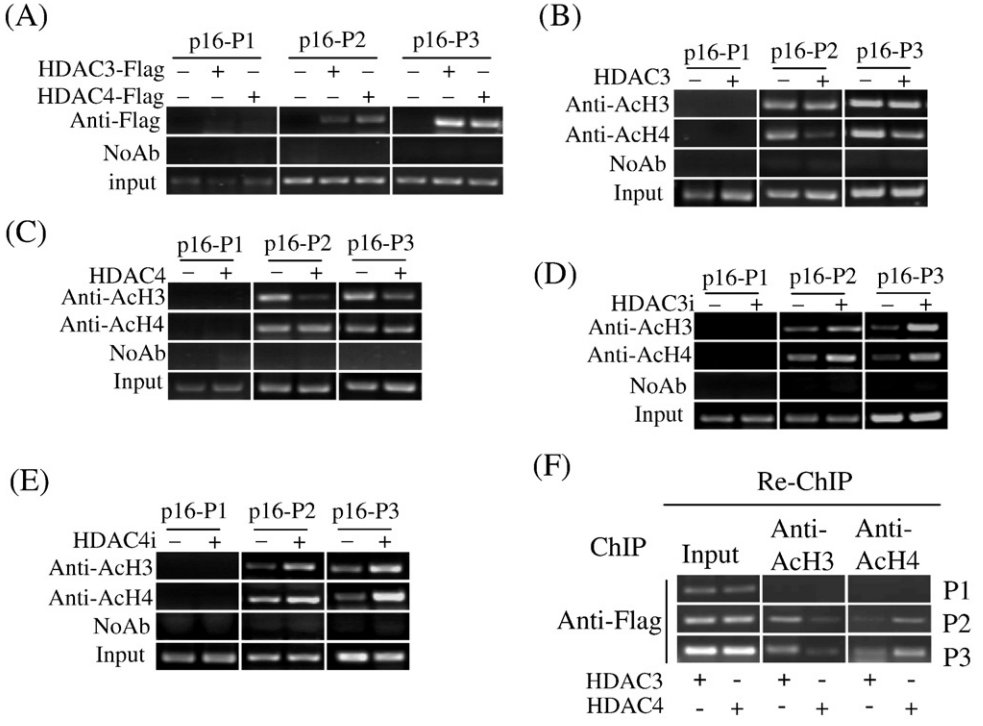

Fig. 5. HDAC3/4 induced histone hypoacetylation at the p16 promoter. (A) ChIP data showing the HDAC3/4 recruitments on p16 promoter. 293T cells were crosslinked with formaldehyde 24 h after transfection with HDAC3-Flag and HDAC4-Flag vectors. DNA was sheared and immunoprecipitated with anti-Flag antibody. PCR products were resolved on a 1.5% agarose gel. Input indicates the DNA prior to immunoprecipitation. (B–E) ChIP assays for the detection of the presence of acetylated histone H3 and H4 on p16 promoter. 293T cells were transfected with HDAC3 (B), HDAC4 (C), HDAC3i (D), or HDAC4i (E) vector. Cells were harvested, DNA was sheared and immunoprecipitated with anti-acetylated histone H3 antibody and anti-acetylated histone H4 antibody. (F) Data of re-ChIP assays. The 293T cells were transfected with HDAC3-Flag or HDAC4-Flag, and the samples were immunoprecipitated with anti-Flag antibody as the first antibody. Then the samples were immunoprecipitated with the second antibody, anti-acetylated histone H3 antibody or anti-acetylated histone H4 antibody.

while overexpression of these HDACs decreased the SA-β-gal activity (Fig. 4D). Furthermore, the cell senescence was also confirmed by using the SA-β-gal staining assay of the cells. As evidenced in Fig. 4E, knockdown of HDAC3 and HDAC4 enhanced the SA-β-gal staining, while co-transfection of cells with HDAC3i/4i and p16i vectors failed to induce cell senescence. These results strongly suggest that HDAC3 and HDAC4 implemented their cell senescence restraint role via inhibition of p16 transcription.

3.5. HDAC3 and HDAC4 were present at the p16 promoter to induce histone hypoacetylation

Our ChIP experiments with anti-Flag antibody revealed that both HDAC3 and HDAC4 were enriched at the downstream regions of p16 promoter (P2 and P3) upon their overexpression (Fig. 5A). Additionally, ChIP assays with anti-acetylated histone H3 and H4 antibodies demonstrated that the acetylation level of histone H4 was reduced by exogenous expression of HDAC3 at P2 and P3 regions, whereas the acetylation level of H3 was not affected (Fig. 5B). Meanwhile, the acetylation level of histone H3 was reduced by exogenous expression of HDAC4 at P2 and P3 regions, while the acetylation of H4 was not affected (Fig. 5C). Contrarily, HDAC3i and HDAC4i enhanced the acetylation levels of both H3 and H4 (Fig. 5D and E). Re-ChIP assays indicated that the acetylation levels of histone H4 and H3 at P2 and P3 regions of p16 promoter, were reduced upon the exogenous expression of HDAC3 and HDAC4, respectively (Fig. 5F). These experimental data clearly indicated that HDAC3 and HDAC4 played an important role in p16 repression via inducing histone deacetylation modification.

3.6. The interactions among YY1, HDAC3 and HDAC4

The results described above implicated that HDAC3 and HDAC4, together with YY1, were able to inhibit p16 gene expression. Next, we sought to investigate whether a physical interaction among these factors exists. First, immunofluorescence microscopy demonstrated that HDAC3 and YY1, as well as HDAC4 and YY1, were co-localized in cell nuclei (Fig. 6A). Then, we investigated the physical interactions among these protein factors by using co-immunoprecipitation (Co-IP) assays. The nuclear extracts from the 293T cells were immunoprecipitated by anti-YY1, anti-HDAC3 and anti-HDAC4 antibodies, and detected in immunoblotting with anti-YY1 and anti-HDAC4 antibodies, respectively (Fig. 6B, upper). Meanwhile, the nuclear extracts from the 293T cells transfected with HDAC3-GFP and HDAC4-GFP expression vectors were immunoprecipitated by anti-GFP and anti-YY1 antibodies, and detected in immunoblotting with respective antibodies (Fig. 6B, lower). The Co-IP results suggested that HDAC3,

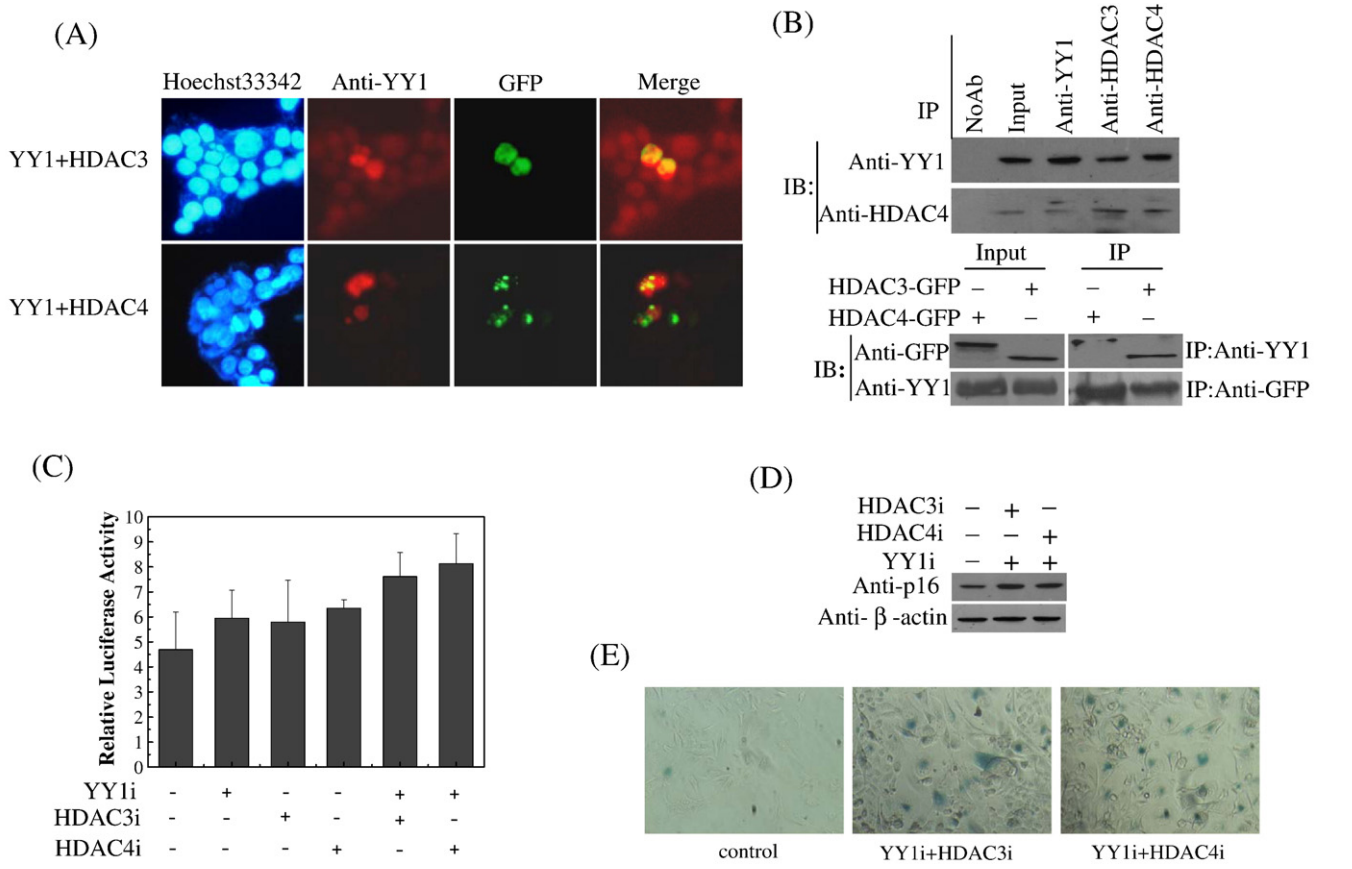

Fig. 6. The interactions among YY1, HDAC3 and HDAC4. (A) Co-localization of YY1 and HDAC3/4. 293T cells were plated onto glass slides and transfected with HDAC3-GFP or HDAC4-GFP plus YY1. Cells were fixed in formaldehyde and stained with anti-YY1 antibody followed by a TRITC secondary antibody, and visualized under a fluorescence microscope. YY1 was immunostained in red and HDAC3/4 in green. The nuclei were counterstained with Hoechst 33342 (blue). The merged images were created with Simple PCI software (Cimaging). (B) Co-IP assays for association of HDAC3/4 with YY1. The cell nuclear extracts were prepared and precipitated with anti-YY1, anti-HDAC3 and anti-HDAC4 antibodies, and detected by using immunoblotting with anti-YY1 and anti-HDAC4 antibodies (upper). The nuclear extracts from the cells transfected with HDAC3-GFP or HDAC4-GFP vectors were precipitated with anti-YY1 and anti-GFP antibodies, and detected by the respective antibodies (lower). Input: proteins prior to immunoprecipitation. (C) Knockdown of YY1 and HDAC3 or HDAC4 increased the activity of p16 promoter. One μg of p16 reporter vector with 5 μg of YY1i and HDAC3i vectors, or with YY1i and HDAC4i, were transfected into 293T cells. The p16 promoter activity was examined by luciferase reporter assay. The control was an irrelevant siRNA vector. (D) Knockdown of YY1 and HDAC3 or HDAC4 increased p16 protein expression. Western blots of the p16 protein in NCI-H460 cells transfected with YY1i and HDAC3i vectors, or with YY1i and HDAC4i vectors. (B) Knockdown of YY1 and HDAC3 or HDAC4 stimulated NCI-H460 cell senescence. Representative photomicrographs of the SA-β-gal staining at day 7 after YY1i and HDAC3i, or YY1i and HDAC4i transfection. An irrelevant siRNA vector was used as the control.

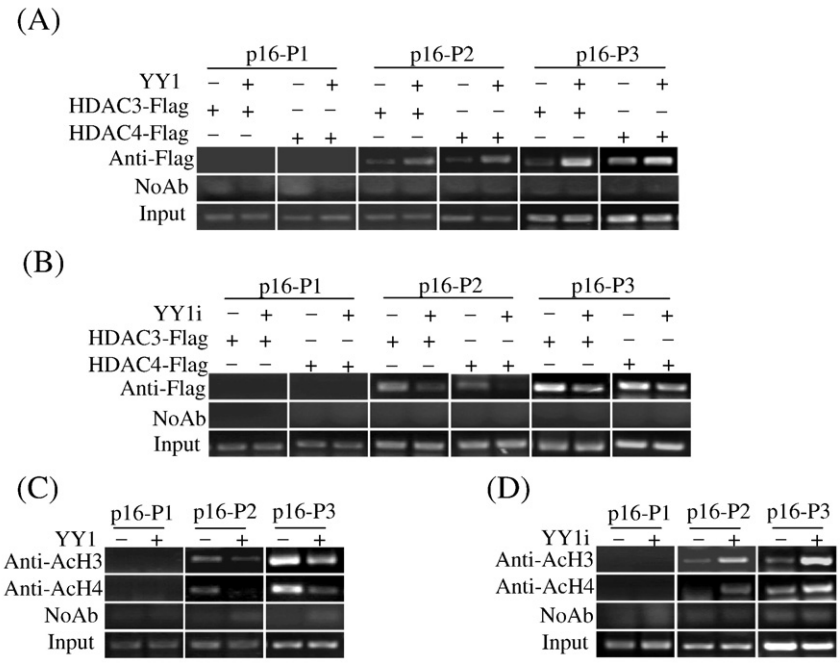

Fig. 7. HDAC3 and HDAC4 were recruited to p16 promoter by YY1. (A–B) YY1 recruited HDAC3/4 to p16 promoter. Cells were transfected with YY1 (A), or with YY1i (B), together with HDAC3-Flag or HDAC4-Flag vector. Samples were immunoprecipitated with anti-Flag antibody, and the precipitated DNA was amplified using PCR. (C–D) ChIP assays for the detection of acetylated histone H3 or H4 on p16 promoter. 293T cells were transfected with YY1 (C) and with YY1i (D). Cells were harvested, and DNA was sheared and immunoprecipitated with anti-acetylated H3 and anti-acetylated H4 antibodies.

HDAC4 and YY1 were presented in the same complex. Also, transfection experiments revealed that the siRNA-mediated silencing of endogenous YY1 and HDAC3 or HDAC4 activated the p16 promoter activity (Fig. 6C). Western blotting assays demonstrated that the p16 protein expression was enhanced by knockdown of the endogenous YY1 and HDAC3 or HDAC4 in NCI-H460 cells (Fig. 6D). Co-transfection of cells with YY1i and HDAC3i or HDAC4i vectors induced cell senescence (Fig. 6E). Thus, data presented above provided evidence that YY1 together with HDAC3 and HDAC4 interacted and worked coordinately to contribute to the repression of p16 expression.

3.7. HDAC3 and HDAC4 were recruited to p16 promoter by YY1

To determine whether HDAC3 and HDAC4 were recruited to p16 promoter by YY1, we examined the binding of HDAC3 and HDAC4 at the p16 promoter regions, and the results showed the binding of HDAC3 and HDAC4 was significantly increased after YY1 overexpression (Fig. 7A), while they were reduced by knockdown of the endogenous YY1 (Fig. 7B). Also, the ChIP data indicated that the acetylation levels of H3 and H4 were decreased by YY1 overexpression (Fig. 7C), whereas they were increased by transfection of YY1i (Fig. 7D), implicating that HDAC3/4 participated in repression of p16 by YY1. Thus, data presented above provide evidence that HDAC3 and HDAC4 were recruited to p16 promoter via YY1.

4. Discussion

The putative role of YY1 in tumorigenesis has been supported by its known interactions with the cell cycle regulators, such as Cyclin D, p53 and c-myc [32]. It would not be surprising to see that deregulated YY1 activity might serve as a central factor causing dysfunctional cell proliferation and increased resistance to cell death. Thus, YY1 may act as an initiator of tumorigenesis and may potentially serve both as a diagnostic and a prognostic tumor marker [32]. Recent evidence showed that inhibition of YY1 by YY1 siRNA sensitized the cells to TRAIL apoptosis concomitantly with DR5 up-regulation [33,34]. Data from Affar et al. not only shed new light on the molecular basis for YY1's roles in developmental and cellular functions, but also provided insight into the general mechanisms controlling eukaryotic cell proliferation, apoptosis, and differentiation [35]. Our results from this study supported this notion by showing that overexpression of YY1 decreased the SA-β-gal activity, while knockdown of the endogenous YY1 expression by RNAi increased both the SA-β-gal activity (Fig. 1B) and the SA-β-gal staining (Fig. 1C). Meanwhile, co-transfection of cells with YY1i and p16i vectors failed to cause cell senescence (Fig. 2A). A previous study by Brachen et al. implicated that the depletion of some polycomb group proteins, such as EZH2, SUZ12 and BMI1, activated p16 transcription and promoted cell senescence [22]. These authors also reported that polycomb proteins participated in p16 transcription regulation and senescence induction by changing the methylation status of lysine 9 of histone H3 at the p16 promoter [22]. In this report, we showed that YY1, a member of polycomb group proteins, restrained cell senescence by regulating p16 expression, through an epigenetic mechanism involving histone acetylation modification. Presumably, the YY1 function described in this report may represent the common feature of the polycomb proteins, similar to those reported by Brachen et al.

YY1 is a multi-functional transcription factor that can act on the genes' promoters, enhancers and initiator elements [36,37]. Here we demonstrate that YY1 functioned as a repressor of the p16 expression. We presented evidence that overexpression of YY1 decreased both the p16 mRNA level (Fig. 2E) and p16 protein production (Fig. 2B), as well as p16 promoter activity (Fig. 2D). These results showed that YY1 played a repression role in p16 expression. It has been suggested that YY1 represses transcription through multiple mechanisms [32]. Most frequently, these mechanisms involve the competition of YY1 with activating factors in overlapping binding sites, thereby decreasing promoter activity and resulting in transcriptional repression. Other hypotheses include the negative regulation of YY1 on neighboring promoter-bound activators [38]. The third model, and the most recent one to emerge, suggests that YY1 recruits a corepressor or complex to a promoter and that this corepressor either negatively affects other

factors that are present, or alters the local chromatin structure to bring about repression [3]. Corepressors that have attracted extensive research interests in the area of chromatin modification include HDAC1, HDAC2, HDAC3 and HDAC4 [11–15]. In this study, we identified that overexpression of YY1 resulted in an enrichment of HDAC3 and HDAC4 on p16 promoter (Fig. 7A), suggesting that YY1 recruited HDAC3 and HDAC4 to p16 promoter. Our experimental evidence also supports the important roles of HDAC activity of HDAC3/4 in p16 expression repression (Fig. 5). It is likely that the inhibitory function of YY1 on p16 gene expression fits the model described by Thomas and Seto [3], which involves the recruitment of corepressors and chromatin modifiers to the gene promoter.

Data presented in this report demonstrate that both HDAC3 and HDAC4 restrained cell senescence via inhibition of p16 gene transcription (Fig. 4D and E). An early study revealed that the histone deacetylase inhibitors Trichostatin A (TSA) and butyrate induced senescent-like phenotype in MECL cells [39], indicating that acetylation of histones participated in cell senescence. However, whether HDACs affect the cell senescence has not been previously described. This report provided the first evidence that HDAC3 and HDAC4 played inhibitory roles in cell senescence through repression of p16 transcription.

To conclude, we demonstrate in this report that the multi-functional transcription factor YY1 is able to restrain cell senescence through inhibition of p16 expression, and this process involves the recruitment of HDAC3 and HDAC4 to the p16 promoter by YY1. Moreover, we provide experimental evidence that YY1, HDAC3 and HDAC4 co-exist in the same complex and work coordinately to contribute to the repression of p16 expression that in turn induces cell senescence.

Acknowledgements

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