

RB1CC1 activates the *p16* promoter through the interaction with hSNF5

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Abstract. *RB1*-inducible coiled-coil 1 (RB1CC1, also known as FIP200) is involved in dephosphorylation and increase of retinoblastoma tumor suppressor protein (RB1), but the RB1CC1 molecular mechanism in the dephosphorylation of RB1 is not fully understood. We determined that RB1CC1 activates the expression of *p16* (also called INK4a/CDKN2a) through the activation of its promoter, using chromatin immunoprecipitation (ChIP) and *p16* promoter-luciferase reporter assays. In addition, RB1CC1 essentially requires binding with hSNF5 (also known as BAF47/INI1, a chromatin-remodeling factor) to activate the *p16* promoter, in order to enhance the RB1 pathway and acts as a tumor suppressor. Evaluation of the RB1CC1 mechanism of action is expected to provide useful information for clinical practice and future therapeutic strategies in human cancers.

Introduction

INK4a/CDKN2a is generally known as *p16*, a tumor suppressor protein that functions as an inhibitor of CDK4 and CDK6, which initiate phosphorylation of retinoblastoma tumor suppressor protein, RB1. *p16* induces G1 cell cycle arrest, and plays a physiological role in the implementation of irreversible growth arrest (1). Thus, *p16* plays a key role in cellular senescence in human cells, and is important in resistance to oncogenic transformation (1). It is known that *p16* transcription is activated by hSNF5 (2-4) and Ets1/2 (5,6), and is inhibited by Id1 and Bmi-1 (7-9). Genetic mutation and deletion of these *p16* regulators have not been widely reported, while hSNF5 is mutated with loss of functionality in malignant rhabdoid tumor (MRT), a rare and extremely aggressive childhood cancer. Indeed, the functional relation-

ship between *p16* and RB is frequently deregulated in many types of human cancer. However, the mutations and deletions in *p16*, RB and upstream regulators of this pathway are not frequent (10). There could presumably be other factors than the *p16* and RB that regulate the pathway relating to progression or prevention of tumorigenesis.

RB1-inducible coiled-coil 1 [RB1CC1: the symbol referred to here and approved by the Human Genome Organization (HUGO) Gene Nomenclature Committee; also known as FIP200] was identified as an RB1 regulator. In our previous report, we described that RB1CC1 activates the RB1 promoter through the 201 bp upstream GC-rich region (11). We have also reported that RB1CC1 forms a complex with hSNF5 and/or p53, and coordinates with it to activate the *p16* promoter (12).

In the present study, we initially performed an immunohistochemical analysis, and noted that nuclear RB1CC1 expression was significantly correlated with *p16* expression. In addition, the labeling indices of hSNF5 were constantly high (~80%) in all cases of breast cancer, and there was no correlation between hSNF5 and *p16*. We hypothesized that RB1CC1 regulated the quantitative activation of the *p16* promoter. Therefore, we analyzed the mechanism of how RB1CC1 regulated *p16* transcription enhancing its amount. Here, we discovered that RB1CC1 controlled the transcription level of *p16* in the presence of hSNF5, and that RB1CC1 was an upstream regulator of *p16* transcription in many different types of cancer, except for MRT, an extremely rare malignancy.

Materials and methods

Antibodies. Rabbit antisera against RB1CC1 (aa. 25-271 as epitope) were generated as previously reported (13), and the purified IgG fractions were used in the experiments. Anti-*p16* (G175-405) and anti-BAF47 (25) were purchased from BD Bioscience (NJ, USA). Anti-HA (3F10) and anti-Flag (1E6) were from Roche (CT, USA) and Wako (Japan, Osaka), respectively.

Histology and immunohistochemistry. To evaluate the correlation between RB1CC1, *p16* and hSNF5 in human breast cancer

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tissue, surgical specimens from 59 cases were embedded in paraffin after overnight fixation in 10% buffered formalin, and processed for a conventional histopathological diagnosis according to the World Health Organization (WHO) guidelines. Then, serially sliced 4- μ m sections from a specimen of each case were deparaffinized and incubated overnight at 4°C with primary antibodies to RBICC1, p16 and hSNF5 after immersion in 0.3% H₂O₂ and autoclaving at 120°C for 1 min. The sections were rinsed with 1X PBS and incubated with the secondary antibody (Simple Stain MAX-PO; Nichirei, Japan) at room temperature for 1 h. The sections were then stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin.

Microscopic evaluation and statistical analysis of correlation between RBICC1 staining and the labeling index of p16 or hSNF5. The extent of RBICC1 expression was classified into three categories: I, negative stain in both cytoplasm and nuclei; II, positive stain only in cytoplasm and negative in nuclei; III, positive stain in nuclei and/or cytoplasm. For p16 and hSNF5, a nuclear staining reaction that was brownish in color was labeled as positive. The p16 and hSNF5 labeling indices were determined as follows: positive tumor cells/total tumor cells \times 100. One-way factorial ANOVA and multiple comparison tests accompanied by Scheffe's significance were used to evaluate the relationships between RBICC1 grade and p16 labeling index. Spearman's rank correlation coefficient was used to evaluate the correlations between hSNF5 and p16 labeling indices.

Cell culture. HeLa, TTC642, H1299 and HEK293 cells were purchased from either American Type Culture Collection (MD, USA) or Japanese Collection of Research Bioresources (Osaka, Japan), and cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 containing 10 % fetal bovine serum (FBS). All cell culture media were supplemented with penicillin (50 units/ml) and streptomycin (50 mg/ml).

Plasmid DNA and gene transfer. The mammalian expression vector for RBICC1 (RBICC1wt: pcDNA-RBICC1) was generated as reported previously (14). The luciferase plasmid, including the *p16* (*p16* wild-type: p16wt), *RB1*, *p21* or *TP53AIP1* promoter, was constructed as reported previously (12). *p16* mutant plasmids (A, B, C, AB and BC) were generated by a combination of PCR-based manipulations with appropriate external primers at the positions described below and restriction enzyme digestion. Referring to Fig. 2A, the *p16*-mutants A, B, C, AB and BC contained -870 to -624, -624 to -315, -326 to -22, -870 to -315 and -624 to -22, respectively (the initiation ATG was defined as +1). The plasmids of *p16* promoter (-Ebox1, -GT, -SpW, -sGC, -iGC, -Sp1, -Sp2, -Ebox2, -NF κ B, -GATA, -Ets1,GATA, -Ets1, -Ets3, -Ets2,Ets3, -Ets1,Ets3 and -Ets1,Ets2,Ets3) were generated. Referring to Fig. 2B, *p16*mut-Ebox1 (5'-GTACAGGaaATTTTCG-3'), -GT (5'-TTCTCGGaaGGGCTC-3'), -SpW (5'-ACGGGCaiaaaCGGATTT-3'), -sGC (5'-AACGGGaaGGGGCG-3'), -iGC (5'-GCGGGGaaGATTTTC-3'), -Sp1 (5'-CTGGCAG aagGGAGC-3'), -Sp2 (5'-CGGAGGaaGTGTGG-3'), and -Ebox2 (5'-GGGCAGGaaGGGGAGG-3') were mutated from the wild-type *p16* promoter sequence (5'-GTACAGGTATTT

CG3'), (5'-TTCTCGGTGGGGCTC-3'), (5'-ACGGGGCGGGGGCGGATTT-3'), (5'-AACGGGGCGGGGGCG-3'), (5'-GCGGGGGCGGATTTTC-3'), (5'-CTGGCAGGCGGGGAGC-3'), (5'-CGGAGGGCGGTGTGG-3'), and (5'-GGGCAGGTGGGGAGC-3'), respectively. Referring to Fig. 2C, p16mut-NF κ B (5'-CCCCGacGAGAgCAACCT-3'), -GATA (5'-CTGGAA AaATgCCGCGGT-3'), -Ets1 (5'-GCTaCTTaaGGCTGGT-3'), -Ets2 (5'-GCTCTTaaGCCAGCA-3'), and -Ets3 (5'-AGCACCCcAttAAGAAAGA-3') were mutated from the wild-type *p16* promoter sequence, (5'-CCCCGGGGGAGACCCAACCT-3'), (5'-CTGGAAAGATACCGCGGT-3'), (5'-GCTGCTTCCGGCTGGT-3'), (5'-GCTCTTCCGCCAGCA-3'), and (5'-AGCACCGGAGGAAGAAAGA-3'), respectively. *p16*mut-Ets1,GATA, -Ets2,Ets3, -Ets1,Ets3 and -Ets1,Ets2,Ets3 contained (Ets1 and GATA), (Ets2 and Ets3), (Ets1 and Ets3) and (Ets1, Ets2 and Ets3) mutant sequences, respectively. Referring to Fig. 5A, flag-tagged mutants of RBICC1; dCCB and dLZ, were generated by deleting the amino acids of 1078-1363 and 1357-1594 for RBICC1 wild-type, respectively. All plasmid constructs were made by PrimeSTAR™ Mutagenesis Basal Kit (Takara, Japan) according to the manufacturer's instructions, and confirmed by DNA sequencing. Transfection preceded using FuGene HD (Roche) or Lipofectamine 2000 (Invitrogen) according to the supplier's recommendations.

Luciferase-reporter assay. The *p16* wild-type- or mutant-, *RB1*-, *p21*- or *TP53AIP1*- luciferase reporter plasmid was co-transfected using FuGene HD (Roche) with empty vector (pcDNA) and/or RBICC1-expression vector (RBICC1wt or RBICC1mut) into the HeLa cells one day after seeding into 35-mm-well plates at the concentration of 4 \times 10⁴ cells. Twenty-four hours later, luciferase activity was analyzed using a luciferase assay kit (Toyo Inc., Japan) and luminometer (EG&G Berthold Lumat LB9507). β -gal activity was evaluated as an internal control for transfection efficiency using Aurora™ kit (ICN Biomedicals, CA, USA).

Chromatin immunoprecipitation (ChIP) assay. ChIP assay of the endogenous *p16* promoter regions in HeLa or TTC642 cells was done by the ChIP-IT™ control and ChIP-IT™ Express kits (Active Motif, CA, USA) according to the manufacturer's instructions using anti-RBICC1 IgG, anti-hSNF5 IgG and control IgG (Active Motif). The amount of co-immunoprecipitated DNA was evaluated by the quantitative polymerase chain reaction (qPCR). The amplified region of *p16* promoter was from -711 to -529 (the initiation ATG was defined as +1). The sequences of the PCR primers were 5'-AGGAATCCTTTGAACTAGGG-3' and 5'-GGGAAAGTATGGCTTCTTCG-3'. The PCR conditions were multiple cycles of 3 steps (98°C, 10 sec at 58°C, 30 sec at 68°C, 30 sec).

Immunoprecipitation and Western blotting. Before immunoprecipitation, cells were lysed in 1X TNE buffer (20 mM Tris-HCl containing 150 mM NaCl, 5 mM EDTA, 1% NP-40 and 1 mM Na₃VO₄ and a mixture of protease inhibitors). After cleaning lysed material by centrifugation at 15,000 rpm for 10 min, the supernatant was rotary-incubated

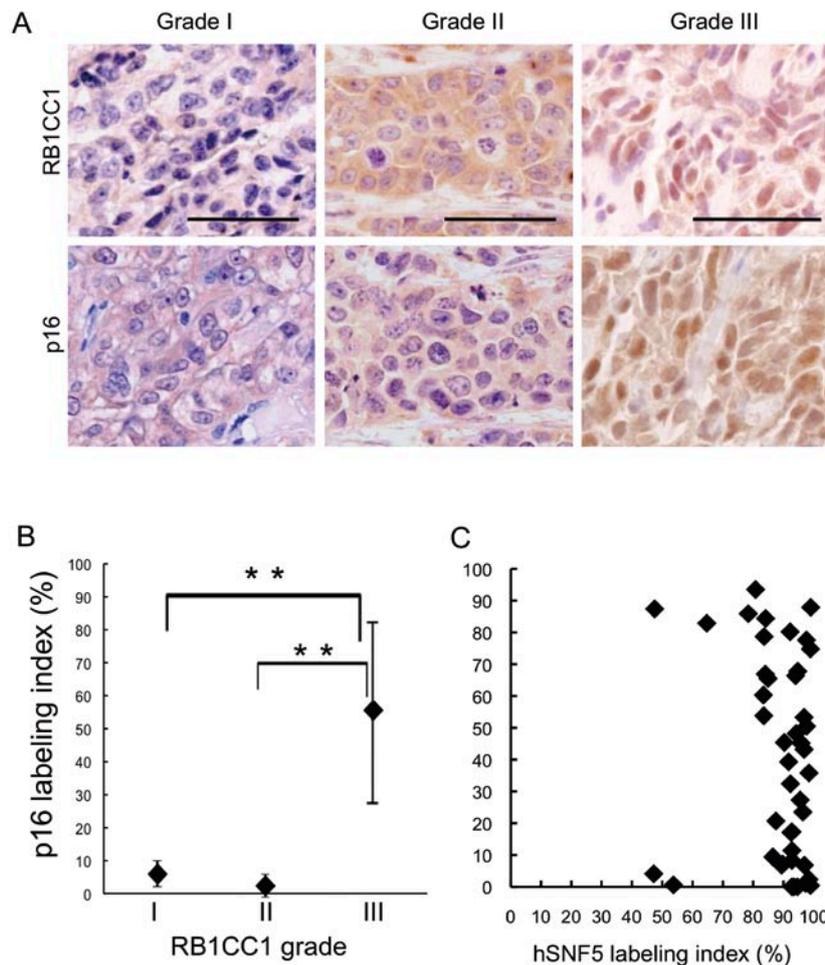


Figure 1. RB1CC1 regulates p16 expression in cancer tissues. (A) Representative cases of immunohistochemical RB1CC1 grades of expression are demonstrated in the upper row, and p16 expressions in each serial section are shown in the lower row. Grade I is defined as negative staining in both cytoplasm and nuclei; II, positive staining only in cytoplasm and negative in nuclei; III, positive staining in nuclei and/or cytoplasm. p16 expression is remarkable in RB1CC1 grade III case. Each scale bar represents 50 μ m. (B) The relationship between the expressional grade of RB1CC1 and p16 labeling index in breast cancer. p16 labeling indices (mean \pm SD) were $5.94\pm 4.11\%$, $2.37\pm 3.44\%$ and $55.60\pm 29.56\%$ in grade I (8 cases), II (6 cases), and III (45 cases) RB1CC1 expression categories, respectively. p16 labeling index is significantly high in the case with nuclear RB1CC1 expression (grade III). One-way factorial ANOVA and multiple comparison tests accompanied by Scheffe's significance were used to evaluate the relationship (** $p < 0.01$). (C) The relationship between hSNF5 and p16 labeling indices in breast cancer. No statistical significance was detected in the relationship by Spearman's rank correlation coefficient.

with anti-HA (Roche) immobilized beads for 2 h at 4°C. The beads were washed five times with 1X TNE buffer and boiled in SDS sample buffer. Proteins resolved by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with the indicated antibodies.

Results

RB1CC1 and p16 expressions correlate in cancer tissues. RB1CC1 and p16 were immunohistochemically analyzed in 59 cases of breast cancers. Nuclear RB1CC1 expression correlated with high p16 expression (Fig. 1A). p16 labeling indices (mean \pm standard deviation) were $5.94\pm 4.11\%$, $2.37\pm 3.44\%$ and $55.60\pm 29.56\%$ in grade I (8 cases), II (6 cases), and III (45 cases) RB1CC1 expression categories, respectively. The p16 labeling index was significantly high in grade III cases of RB1CC1 (One-way factorial ANOVA and multiple comparison tests accompanied by Scheffe's significance, $p < 0.01$; Fig. 1B). These results suggested that RB1CC1 nuclear expression correlated with the p16 expression level.

It has been reported that hSNF5 largely contributes to p16 expression (2-4). To evaluate the contribution of hSNF5 to p16 expression *in vivo*, we immunohistochemically analyzed the labeling indices of hSNF5 and p16 in similar cases of breast cancers. The labeling indices of hSNF5 were $>80\%$ in most of the breast cancer cases, and there was no correlation between hSNF5 and p16 labeling indices (Fig. 1C).

RB1CC1 enhances the activity of p16 promoter. In order to identify the critical region for RB1CC1 to activate the p16 promoter, the luciferase reporter assay was used. Wild-type (p16wt) and the various deletion mutants (A, B, C, AB and BC) of the p16 promoter reporter plasmid were co-transfected into HeLa cells with the RB1CC1-expression vector (RB1CC1) or empty (pcDNA) plasmid (Fig. 2). While the luciferase activity of the p16 mutant A (-870 to -624 bp) was approximately equal to that co-transfected with pcDNA, the promoters, which contained the region of B (-624 to -315 bp) and/or C (-326 to -22 bp), were ~ 3 -5-fold enhanced by RB1CC1. Importantly, the promoter containing both regions (BC, -624 to -22 bp)

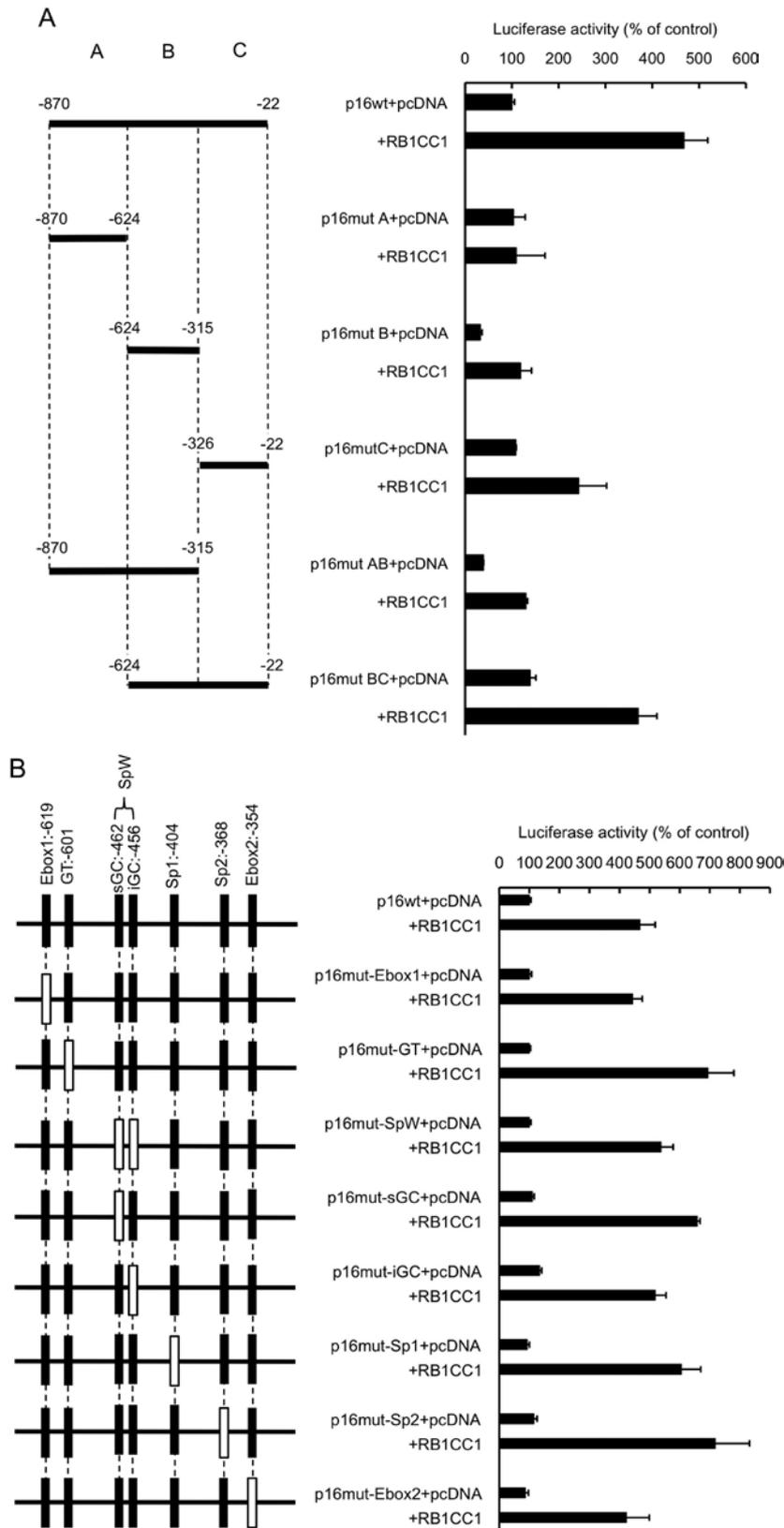


Figure 2. RBICC1 enhances *p16* promoter activity. (A) Schematic representation (left) of p16wt and five kinds of reporter plasmids containing *p16* promoter deletion-mutants (p16mut A, B, C, AB and BC) used in luciferase assays, corresponding to the graph on the right. (B) Schematic representation (left) of *p16wt* and eight kinds of reporter plasmids containing point-mutations of *p16* promoter (p16mut-Ebox1, -GT, -SpW, -sGC, -iGC, -Sp1, -Sp2 and -Ebox2) used in luciferase assays, corresponding to the graph on the right.

was additively stimulated by RBICC1, and reached similar levels to that of the wild-type promoter (p16wt). These results

suggested that RBICC1 required the limited domain (-624 to -22 bp) of the *p16* promoter to activate the promoter in a dose

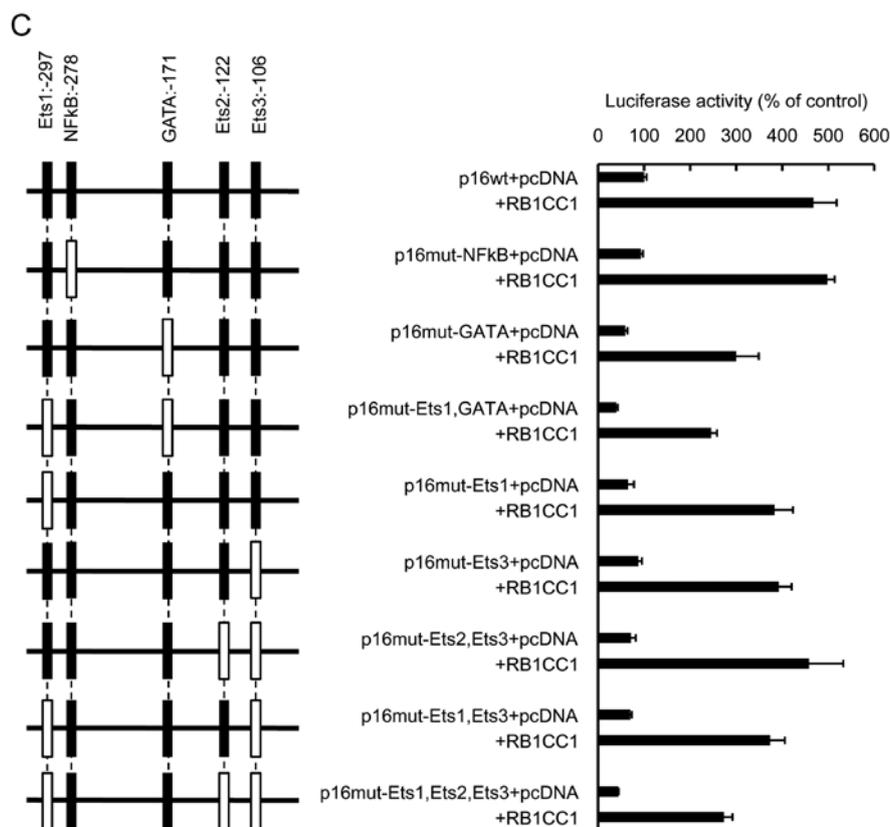


Figure 2. (C) Schematic representation (left) of *p16wt* and eight kinds of reporter plasmids containing point-mutations of *p16* promoter (*p16mut*-NF κ B, -GATA, -Ets1GATA, -Ets1, -Ets3, -Ets2Ets3, -Ets1Ets3 and -Ets1Ets2Ets3) used in luciferase-reporter assays, corresponding to the right graph. The translation start site is determined as +1. All the luciferase values were calculated as the relative fold induction versus *p16wt* co-transfected with pcDNA. The values indicated the means \pm standard errors from quadruplicate experiments in HeLa cells.

(DNA-length) -dependent manner. Additionally, to identify the critical narrow domain of the *p16* promoter activated by RB1CC1, we modified the *p16* wild-type reporter plasmid and screened each activity co-transfected with RB1CC1. We made 8 (p16mut-Ebox1, -GT, -SpW, -sGC, -iGC, -Sp1, -Sp2, -Ebox2) and 8 (p16mut-NF κ B, -GATA, -Ets1GATA, -Ets1, -Ets3, -Ets2Ets3, -Ets1Ets3 and -Ets1Ets2Ets3) kinds of the promoter mutants in the regions of B (-624 to -315 bp) and C (-326 to -22 bp), respectively. The *p16* promoter activity was maintained irrespective of any other mutations (Fig. 2B and C). Altogether, while the critical narrow domain of *p16* promoter was not required for the activation caused by RB1CC1 expression, the region BC (-624 to -22 bp) was required in a DNA-length-dependent manner.

RB1CC1 requires hSNF5 for binding to the *p16* promoter. To examine whether RB1CC1 binds to the *p16* promoter, the ChIP experiments were performed using HeLa and TTC642 (hSNF5^{-/-}) cells. Immunoprecipitated DNA fragments were amplified by PCR using the primer pairs specific to the *p16* promoter (Fig. 3). In HeLa cells, the immunoprecipitates containing a *p16* promoter fragment were significantly enriched by antibodies against RB1CC1 and hSNF5, in contrast to precipitates of normal IgG control. In TTC642 cells, which are genetically null for hSNF5, antibody against RB1CC1 failed to enrich the immunoprecipitates of *p16*

promoter fragments. Therefore, we considered that RB1CC1 binds to the -711 to -529 bp region of the *p16* promoter, and that the binding essentially requires hSNF5 to activate the *p16* promoter. RB1CC1 could not interact with the *p16* promoter in the hSNF5-null cells such as TTC642.

RB1CC1 requires hSNF5 to activate the *p16* promoter. Recently, we have identified that RB1CC1 requires the involvement with hSNF5 and/or p53 to enhance the RB1 pathway (12). To evaluate the requirement of hSNF5 and p53 to activate *p16*, we utilized HeLa, TTC642 (hSNF5^{-/-}) and H1299 (p53^{-/-}) cells. Luciferase plasmid containing *p16* promoter was transfected into these cells together with pcDNA or RB1CC1 (Fig. 4). RB1CC1 could not activate *p16* promoter adequately in TTC642 and H1299 cells, while the activation increased by about 7-fold in HeLa cells. The data suggested that RB1CC1 required the involvement of hSNF5 and p53 to activate the *p16* promoter. Similarly, each luciferase-reporter containing *RB1*, *p21* or *TP53AIP1* was applied into these cells, and we validated the molecular requirement of hSNF5 or p53 for the promoter activation caused by RB1CC1. RB1CC1 could activate neither the *RB1* promoter in TTC642 cells, nor the *p21* or *TP53AIP1* promoters in H1299 cells. These results suggested that RB1CC1 required hSNF5 to activate the *RB1* promoter, and p53 to enhance the promoters of *p21* and *TP53AIP1* (Fig. 4).

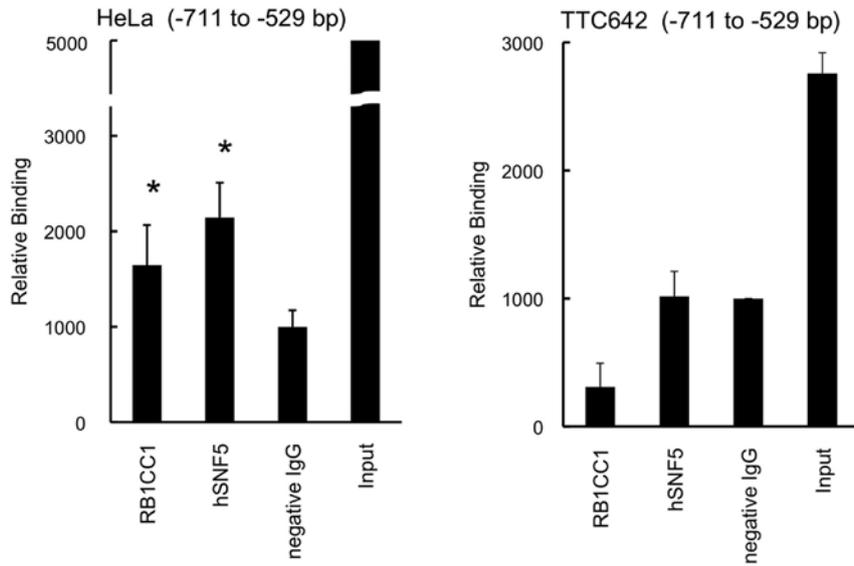


Figure 3. RBICC1 requires hSNF5 for the binding to the *p16* promoter. The ChIP PCR analysis was performed in HeLa and TTC642 (hSNF5^{-/-}) cells using anti-RBICC1 and anti-hSNF5 antibodies. The immunoprecipitated DNA fragments were amplified by PCR using the primer-pairs specific to *p16* promoter (-711 to -529 bp). The values indicated the means ± standard errors from more than quadruplicate experiments. Student's t-test; asterisks (*p<0.05) indicate statistically significant differences with negative IgG.

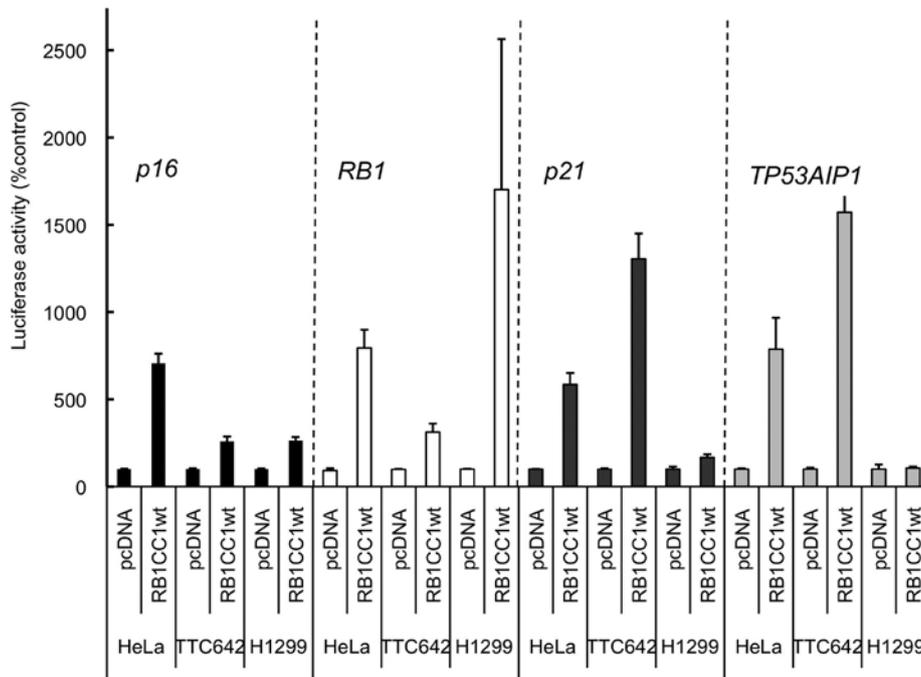


Figure 4. RBICC1 requires the involvement with hSNF5 to activate the *p16* promoter. RBICC1 also requires hSNF5 for *RB1* promoter activation, and needs p53 for the activations of *p21* and *TP53AIP1*. Luciferase plasmid containing *RB1*, *p16*, *p21* or *TP53AIP1* promoter was transfected into HeLa, TTC642 (hSNF5^{-/-}) or H1299 (p53^{-/-}) cells together with RBICC1. Luciferase activity was calculated as the relative fold induction of each versus the value of co-transfection with pcDNA. The values indicate the means ± standard errors from quadruplicate experiments.

hSNF5 binds to C-terminus of RBICC1, which is largely required to activate the *p16* promoter. To confirm that RBICC1 requires hSNF5 to activate the *p16* promoter, the binding between RBICC1 and hSNF5 was evaluated, and it was additionally analyzed whether an RBICC1 mutant that was unable to bind to hSNF5 could activate the *p16* promoter. We generated RBICC1 deletion-mutants (dCCB: aa1078-1363 deleted; and dLZ: aa1357-1594 deleted) (Fig. 5A). HA-tagged

hSNF5 was cotransfected with the plasmids encoding flag-tagged RBICC1 wild-type or mutants in HEK293 cells. In this co-immunoprecipitation assay, hSNF5 could not bind the dLZ mutant, which had lost the C-terminal region of RBICC1, but could bind the dCCB mutant and RBICC1wt (Fig. 3B). In addition, *p16* promoter activity caused by RBICC1wt or the mutants was validated using the luciferase assay. The RBICC1 dLZ mutant that was unable to bind to hSNF5, could

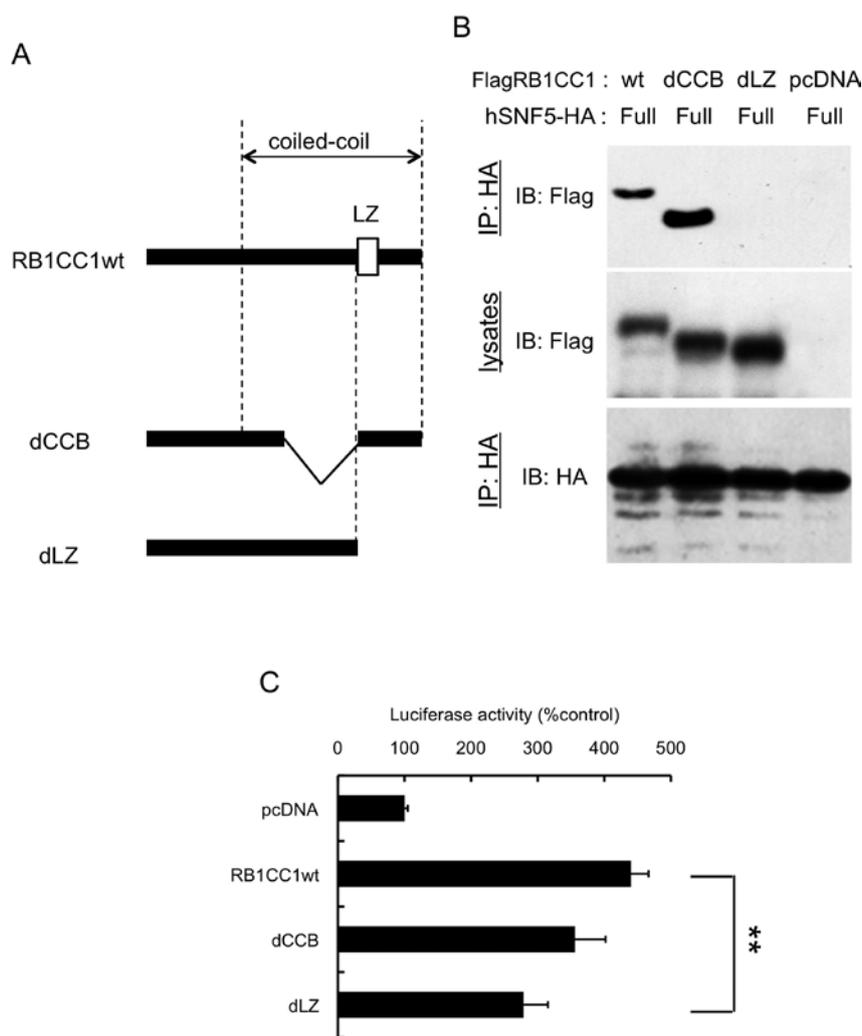


Figure 5. hSNF5 binds to the C-terminus of RB1CC1, which is essentially required for *p16* promoter activation. (A) Schematic representation (left) of RB1CC1wt and two kinds of RB1CC1 deletion-mutants (dCCB; aa1078-1363 deleted and dLZ; aa1357-1594 deleted) used in immunoprecipitation and promoter assay. LZ, leucine zipper. (B) HA-tagged hSNF5 was cotransfected with the plasmids encoding flag-tagged RB1CC1wt or mutants in HEK293 cells. Anti-HA immunoprecipitated lysate and total cell lysate were analyzed by Western blotting. (C) Luciferase plasmid containing *p16* promoter was transfected into HeLa cells together with RB1CC1wt or mutants. Luciferase activity was calculated as the relative fold induction of each versus the value of co-transfection with pcDNA. The values indicated the means \pm standard errors from quadruplicate experiments in HeLa cells. Student's t-test; double-asterisks (** $p < 0.01$) indicate statistically significant differences between RB1CC1wt and the mutants.

inadequately activate the *p16* promoter, while RB1CC1wt or the dCCB mutant activated it adequately. In summary, RB1CC1 could activate the *p16* promoter, depending to a large extent on the interaction with hSNF5.

Discussion

INK4a/CDKN2a/p16 functions as an inhibitor of D-type cyclin-dependent kinases (CDK4 and CDK6) that initiate phosphorylation of RB1, so p16 has the capacity to arrest cells in G1 phase of the cell cycle (1). Transcription of p16 is activated by Ets1/2 (5,6) and hSNF5, a chromatin-remodeling factor (2-4). Reciprocally, BMI-1, a polycomb protein, and Id1, a bHLH protein, are known as negative regulators of p16 (7-9).

RB1CC1 enhances the transcription of RB1 (11), and forms a complex with hSNF5 and/or p53 (12). Nuclear RB1CC1 expression was correlated with a high p16 expression (Fig. 1A-B), so it was suggested that RB1CC1 activated the *p16* promoter. In the present study, hSNF5 was expressed in all of

the breast cancer cases, and there was no correlation between hSNF5 and p16 expressions (Fig. 1C). Following-up on these immunohistochemical results; we analyzed the mechanism of how RB1CC1 controls p16 expression.

To detect the RB1CC1-activated site of the *p16* promoter, we performed the promoter mapping using the luciferase-reporter assay. A limited domain (-624 bp to -22 bp) of the *p16* promoter was required in a dose (DNA-length)-dependent manner for the transcriptional activation caused by RB1CC1 (Fig. 2A). We screened the *p16* promoter from -624 bp to -22 bp, to identify the critical narrow domain of the promoter, but were unable to find it (Fig. 2B-C). These results made us hypothesize that another essential important factor might be a mediator, not in a sequence-specific but in a dose (DNA-length) -dependent manner, when the *p16* transcription was activated by RB1CC1. Previously, we indicated that RB1CC1 interacted with hSNF5 (12) and suggested that RB1CC1 activated the *p16* promoter through the interaction with hSNF5.

To identify the possible cooperation between RBICC1 and hSNF5 in activating the *p16* promoter, we evaluated whether RBICC1 and hSNF5 bound to the promoter at -711 to -529 bp. It was revealed that both molecules bound to this region of the *p16* promoter in HeLa cells, while neither could bind there in hSNF5-null TTC642 cells (Fig. 3). In addition, our preliminary ChIP-sequence data using the next-generation sequencer (Solid, Life Science Technology) suggested that RBICC1 bound to the -621 to -575 bp region of the *p16* promoter (data not shown) in HeLa cells. This region of the *p16* promoter is consistent with the binding region of hSNF5 that has been reported previously (3,4). Together, it is strongly suggested that hSNF5 functions as an essential interacting molecule when RBICC1 binds to the *p16* promoter. hSNF5 is a chromatin-remodeling factor that can activate the promoters without requiring specific binding sequences. Therefore, RBICC1 activated the *p16* promoter not in a sequence-specific, but in a dose (DNA-length)-dependent manner in our luciferase-reporter assay.

RBICC1 binds to hSNF5 (12) as well as p53 (15), so the promoter enhancements caused by RBICC1 were compared among HeLa, TTC642 (hSNF5-null) and H1299 (p53-null) cells, in order to validate the requirement for hSNF5 or p53 when RBICC1 activated *RBI*, *p16*, *p21* and *TP53AIP1*. It was revealed that RBICC1 required p53 as an essential molecule to activate *p21* and *TP53AIP1*, which are known as p53-targeting genes. Considering the *RBI* promoter, RBICC1 required hSNF5 rather than p53. Reciprocally, in order to activate *p16* promoter, RBICC1 required not only hSNF5 but also p53 as the important partners (Fig. 4). These results suggested that RBICC1 might importantly form a large transcriptional complex with hSNF5 as well as p53 for *p16* promoter enhancement.

Finally, we examined the binding between RBICC1 and hSNF5 and the activation of *p16*, because it had been reported that hSNF5 was the upstream transcriptional activator for *p16* (2-4). It was revealed that hSNF5 bound to the C-terminal region of RBICC1, and that an RBICC1 mutant without this region reduced significantly the ability to activate the *p16* promoter (Fig. 5). Together with the finding that the C-terminus of RBICC1 is essential for its nuclear localization (11), it is suggested that RBICC1 can bind to hSNF5 in cell nuclei and is recruited to the *p16* promoter by hSNF5 in order to activate the promoter. hSNF5 is expressed in most cancer cells, while hSNF5 is specifically deleted only in MRT, a childhood rare malignancy. In many different types of cancer, other factors, including RBICC1, may play important roles in *p16* expression, although hSNF5 can recruit them to the promoter region.

In conclusion, the present study revealed that RBICC1 activates the *p16* promoter and enhances *p16* expression, and that hSNF5 is an essential interacting molecule that makes it possible for RBICC1 to play this role. Further studies on RBICC1 are needed in order to help clarify the way forward in research on oncogenesis and senescence. Clinically, the evaluation of RBICC1 status is a possible biomarker for the prognostic indicator in patients with various types of cancer.

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