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p15RS/RPRD1A (p15INK4b-related Sequence/Regulation of Nuclear Pre-mRNA Domain-containing Protein 1A) Interacts with HDAC2 in Inhibition of the Wnt/β-Catenin Signaling Pathway*

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Background: p15RS/RPRD1A inhibits Wnt/β-catenin signaling by recruiting HDAC2.
Results: p15RS interacts with HDAC2 and enhances the occupancy of HDAC2 to promoters of Wnt-targeted genes, keeping histone3 in a deacetylated state.
Conclusion: p15RS cooperates with HADC2 in the inhibition of Wnt/β-catenin signaling.
Significance: p15RS/HDAC2 is a novel co-repressor in the regulation of Wnt/β-catenin signaling.

We previously reported that p15RS (p15INK4b-related sequence), a regulation of nuclear pre-mRNA domain containing protein, inhibited Wnt signaling by interrupting the formation of the β-catenin·TCF4 complex. However, how p15RS functions as an intrinsic repressor to repress transcription remains unclear. In this study, we show that p15RS, through a specific interaction with HDAC2 (histone deacetylase 2), a deacetylase that regulates gene transcription, maintains histone H3 in a deacetylated state in the promoter region of Wnt-targeted genes where β-catenin·TCF4 is bound. We observed that histone deacetylase inhibitors impair the ability of p15RS in inhibiting Wnt/β-catenin signaling. Depletion of HDAC2 markedly disabled p15RS inhibition of Wnt/β-catenin-mediated transcription. Interestingly, overexpression of p15RS decreases the level of acetylated histone H3 in the c-*MYC* promoter. Finally, we demonstrate that p15RS significantly enhances the association of HDAC2 and TCF4 and enhances the occupancy of HDAC2 to DNA, resulting in the deacetylation of histone H3 and the failure of β-catenin interaction. We propose that p15RS acts as an intrinsic transcriptional repressor for Wnt/β-catenin-mediated gene transcription at least partially through recruiting HDAC2 to occupy the promoter and maintaining deacetylated histone H3.

The Wnt/β-catenin signaling pathway, commonly referred to as the canonical Wnt cascade, has been unveiled as a highly conserved pathway from fly to diverse vertebrates, including frog, mouse, and human (1, 2). Canonical Wnt signal transduction eventually activates the TCF/LEF[2] transcription factors (2, 3), which are modulated by different complexes formed with either co-activators or co-repressors in the promoters of a group of genes, including *c-MYC* and *CCND1* (4). Upon activation of canonical Wnt signaling, TCF/LEF forms an active transcriptional complex with β-catenin, a major component in the signaling pathway, which is dephosphorylated and accumulates and translocates into the nucleus (4–6). In contrast, in the absence of Wnt signaling, TCF/LEF forms a complex with co-repressors, including Groucho and histone deacetylases (HDACs) (7, 8), resulting in inhibition of gene transcription. In particular, TCF/LEF was reported to act as a repressor by associating with HDAC1 and HDAC2, leading to the hypo-acetylation of *LEF1* target genes from a hyper-acetylated status (9).

HDACs are a family of enzymes that catalyze the removal of acetyl groups from lysine residues in histone and non-histone proteins involved in a variety of biological events, including cell proliferation, apoptosis, and differentiation (10, 11). In mammals, the HDAC family contains 18 members, categorized into four classes, including class I (HDAC-1, -2, -3, and -8), class II (HDAC-4, -5, -6, -7, -9, and -10), class III (sirtuins), and class IV (HDAC-11) (12, 13). Among these HDAC proteins, the function of HDAC1/2 in the regulation of gene transcription has been widely studied (14, 15). HDAC1/2 *per se* is unable to bind to DNA but is recruited by transcription factors, including SP1/

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[2] The abbreviations used are: TCF/LEF, T-cell factor/lymphoid enhancer factor; HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid; TSA, trichostatin A; RPR, regulation of nuclear pre-mRNA; TRITC, tetramethylrhodamine isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBS, TCF4-binding sequence.

SP3 (16), and the tumor suppressors p53 (17) and BRCA1 (18). Recently, HDAC1/2 was reported to regulate the Wnt/β-catenin signaling pathway (19), seemingly through competing with β-catenin in the interaction with TCF4. The biological role of HDAC1/2 in regulating Wnt signaling was attributed to allowing oligodendrocytes to differentiate on an appropriate schedule (8). HDAC1 also functions as a component of the Groucho·TLE repressor complex, which maintains Wnt signaling in a quiescent state (4, 20). Interestingly, another report proposed that the activation of TCF/LEF target genes by β-catenin occurred by a two-step process. The first step involves HDAC1 dissociation from LEF-1, and as a consequence, the target gene promoter is inactive but poised for activation. Once HDAC1-dependent repression is relieved, β-catenin then binds LEF-1 at the promoter and activates downstream target genes of Wnt signaling (21). However, how HDAC2 specifically functions on Wnt signaling, differentially from HDAC1, remains unclear.

p15RS, a *p15INK4b*-related gene, also named *RPRD1A*, was identified by a differential display experiment from cells overexpressing p15INK4b (22). p15RS contains a RPR domain and evolutionarily is homologous to yeast Rtt103 and human CREPT (23–25). Inhibition of p15RS expression by an antisense DNA led to increased expression of cyclin D and cyclin E in A375 cells (22). Therefore, p15RS was considered as an inhibitor for cell proliferation (26). Other reports demonstrated that p15RS might function through an association with RNA polymerase II during gene transcription (25, 27), similar to its yeast homolog Rtt103 (28). Our group recently found that p15RS played an important role in attenuating Wnt/β-catenin signaling by disrupting the β-catenin/TCF4 interaction (29) and proposed a model where p15RS interacts with TCF4 to block the interaction of β-catenin with TCF4. Here, we demonstrate that p15RS specifically interacts with HDAC2, not HDAC1 or -3, and reduces the level of acetylated histone H3 in the TCF4 binding region of the c-*MYC* promoter. We reveal that p15RS·HDAC2 is a novel co-repressor complex binding to TCF4.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—HEK293T, HEK293 and MCF-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units/ml)/streptomycin (100 units/ml). All cells were grown at 37 °C with 5% CO_2. Media and serum were purchased from Invitrogen. Cells were transfected with plasmids as indicated using Vigofect (Vigorous Inc. Beijing, China), according to the manufacturer's instruction. To generate the stable cell line, MCF-7 and HEK293 cells were infected by lentivirus, which was produced by HEK293T cells transiently transfected with vectors of pLVX and pLL3.7 carrying different cDNAs. GFP-positive cells were selected by FACS.

Plasmids and RNA Interference—pLVX-p15RS plasmid was constructed by inserting a PCR-amplified fragment into pLVX vector. FLAG-β-catenin plasmid was kindly provided by Dr. Xi He, Harvard Medical School. HA-TCF4 and pTOP/FOP-Luc were kindly provided by Dr. Hans Clevers, Hubrecht Institute. HA-β-catenin, FLAG-HDAC1, FLAG-HADC2, and FLAG-HDAC3 were kindly provided by Dr. Wei Wu, Tsinghua University. pLVX and pLL3.7 vectors were provided by Dr. Jing Cheng, Tsinghua University. For generation of an shRNA against p15RS, we synthesized two single strand DNA fragments, 5′-GATCCACCAAACAGGAAGCTTACTTTCAAGAGAAGTAAGCTTCCTGTTTGGTTTA-3′ and 5′-AGCTTAAACCAAACAGGAAGCTTACTTCTCTTGAAAGTAAGCTTCCTGTTTGGTG-3′, to target the human p15RS gene (target is underlined) and used a nonspecific shRNA, 5′-TACAAGACCTAAGTGCACTGTTCAAGAGACAGTGCACTTAGGTCTTGTTTTTTTC-3′ and 5′-TCGAGAAAAAAACAAGACCTAAGTGCACTGTCTCTTGAACAGTGCACTTAGGTCTTGTA-3′, as a control and inserted them into HpaI-XhoI sites in the pLL3.7 vector.

Reagents and Antibodies—Trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) were purchased from Sigma. Antibody against β-actin was purchased from Sigma. Anti-Myc (9E10), anti-p15RS, anti-HDAC1, anti-HDAC2, anti-HADC3, anti-β-catenin, and anti-HA (F-7) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against TCF4, Ac-H3, and c-MYC were purchased from Cell Signaling Technology. Fluorescent secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) were purchased from Jackson ImmunoResearch.

Luciferase Assays—Luciferase assays were performed in 24-well plates using HEK293T and MCF-7 cells transfected with the Super-TOPFlash-Luc reporter plasmid along with an internal control (pRL-TK vector from Promega, Madison, WI). 24–36 h after transfection, reporter activity was determined by the Dual-Luciferase Assay System (Vigorous Inc. Beijing, China). Firefly luciferase activity was normalized against *Renilla* luciferase activity and presented as a mean ± S.D.

Co-immunoprecipitation and Western Blot—For co-immunoprecipitation assays, HEK293T cells were plated in a 60-mm dish transfected with the indicated plasmids. 24–48 h after transfection, cells were lysed in 800 μl of cell lysis buffer (80 mmol/liter KCl, 10 mmol/liter Na_2HPO_4, 1 mmol/liter EDTA (pH 8.0), 0.5% Nonidet P-40, 10% glycerol, 1 mmol/liter DTT, 0.1 mmol/liter Na_3VO_4, 1 mmol/liter phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). 600 μl of whole-cell lysates were incubated with 2 μg of the indicated antibody and 30 μl of protein G/A-Sepharose beads at 4 °C overnight. Beads were washed four times with cell lysis buffer, and precipitates were eluted with 2× SDS-PAGE sample buffer and analyzed by Western blotting with the indicated antibodies.

Immunofluorescent Analysis—Cells were plated on glass coverslips in 6-well dishes, incubated overnight at 37 °C, and then transfected with the indicated plasmids. 24 h after transfection, cells were rinsed with PBS three times, fixed with 4% paraformaldehyde in PBS for 15–20 min at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were blocked with 10% goat serum for 1 h at room temperature. The primary antibodies, diluted in PBS with 3% bovine serum albumin, were incubated overnight at 4 °C, and bound antibodies were detected with secondary antibodies conjugated with FITC (green) or TRITC (red) for 1 h at room temperature. Finally, cells were stained with DAPI. Stained cells were ana-

lyzed with a laser scanning confocal microscopy (OLYMPUS FV10i-Oil).

Cross-linking Experiment—Transiently transfected HEK293T cells were washed twice with PBS and then cross-linked for 30 min at 37 °C with 1% formaldehyde. The reaction was stopped by the addition of 0.25 M glycine for 5 min at room temperature. After cross-linking, cells were boiled directly in SDS-PAGE sample buffer and analyzed by Western blotting with mAbs against the Myc, FLAG, or HA epitope.

Chromatin Immunoprecipitation (ChIP) Assay—A modified protocol from Upstate Biotechnology was used. Briefly, cells were fixed at 37 °C for 10 min with 1% formaldehyde for cross-linking. The cells were resuspended in 300 μl of ChIP lysis buffer and mixed at 4 °C and then sonicated for 30 s at level 2 (Ultrasonic Processor, Sonics) to yield DNA fragments that were 100–500 bp in size. Eluted DNA was recovered with QIAquick columns (Qiagen, Germany) and used as templates for PCR amplifications. The input control was from the supernatant before precipitation. The fragment corresponding to the TCF4-binding site in the c-*MYC* promoter was amplified by PCR with primers 5′-TTGCTGGGTTATTTTAATCAT-3′ and 5′ACTGTTTGACAAACCGCATCC-3′. Real time PCR was used to quantify the precipitated DNA fragments.

Real Time Reverse Transcription (RT)-PCR—Total RNA was extracted using TRIzol reagent (Invitrogen). Reverse transcription was done using a Quant script RT kit (TIANGEN Biotech, Beijing, China). Real time PCR was performed using a Real-MasterMix (SYBR Green) kit (TIANGEN Biotech, Beijing, China) using the following conditions: denature, 95 °C, 20 s; annealing, 58 °C, 20 s; and extension, 68 °C, 30 s. Primers used for the human c-*MYC* gene were 5′-TGGTCGCCCTCCTATGTTG-3′ and 5′-CCGGGTCGCAGATGAAACTC-3′. Primers used for the human *CCND1* gene were 5′-CCGAGAAGCTGTGCATTACAC-3′ and 5′-AGGTTCCACTTGAGCTTGTTCAC-3′. Primers used for the human *GAPDH* gene were 5′-AGACCACAGTCCATGCCATC-3′ and 5′-TTGCCCACAGCTTGGCAG-3′. Primers used for real time PCR analyses for the human *c-MYC* gene were as follows: region A, 5′-TGCCTCTATCATTCCTCCCT-3′ and 5′-TCCACACCGAGAACGCAC-3′; region B, 5′-CATATCGCCTGTGTGAGCCA-3′ and 5′-TAAAAGGATCAGGGCGGTT-3; region C, 5′-CCTCAAATTGGACTTTGGGCA-3′ and 5′-AGGATTGAAATTCTGTGTAACTGCT-3′; region D, 5′-TTTCTTCCCCCTCCCAACCA-3′ and 5′-GCCCCCAGACCCATTTCAAC-3′. Primers used for real time PCR analyses for the human *CCND1* gene were as follows: region A, 5′-GGTACCTGGTGGAAAAGCGGGT-3′ and 5′-AAGCTTCACCTTCACGCTTTCCTC-3′; region B, 5′-GAGAAGCCAGTGCCAGGG-3′ and 5′-CAATAAAAGAGCAAAGATGGC-3′; region C,5′-GGCTCACGCTTACCTCAAC-3′ and 5′-GGAAACATGCCGGTTACAT-3′; region D, 5′-TGTGGGAAGTTCAGCAGCAT-3′ and 5′-TTGTCTCGCGTGGTTGG-3′.

Cell Proliferation—Cell proliferation was measured through an MTT assay. 293 cells were transfected with indicated plasmids in addition to Wnt3a conditioned medium, which was produced from a Wnt3a stable cell line. Cells were seeded into a 96-well plate at a density of 1000 cells/well. When cells were cultured for the indicated times, MTT was added. Cells were incubated for another 4 h before measurement. The MTT-produced formazan was dissolved in 150 μl of dimethyl sulfoxide, and the absorbance at 530 nm was measured with a reference filter of 630 nm using a spectrophotometer (model 680, Bio-Rad). Three independent experiments were performed.

RESULTS

HDACs Contribute to the Inhibitory Function of p15RS—HDACs bind to the TCF/LEF transcription factor to repress the Wnt target gene expression in the absence of Wnt ligands (19). Because p15RS functions as an intrinsic inhibitor of Wnt signaling, we attempted to address whether HDAC proteins might contribute to the inhibitory effect of p15RS. For this purpose, we examined the effect of TSA and SAHA, two HDAC inhibitors (30), on the inhibitory role of p15RS in Wnt-3a-induced transcriptional activation. A luciferase reporter assay using TOP/FOP-Flash-Luc, which responds to canonical Wnt signaling, demonstrated that overexpression of p15RS significantly inhibited the luciferase activity in control cells in the presence of solvent DMSO but had no significant effect when the HEK293T cells (Fig. 1*A*) and MCF-7 cells (Fig. 1*B*) were treated with TSA and SAHA.

To study the physiological role of HDAC inhibitors on p15RS, we examined the expression of *CCND1* and *c-MYC*, two Wnt3a-targeted genes. The results showed that p15RS decreased the expression of *CCND1* (Fig. 1*C*) significantly in the presence of DMSO but has no significant effect in the presence of TSA or SAHA. Similarly, overexpression of p15RS significantly decreased the expression of c-*MYC,* and both TSA and SAHA weakened this effect (Fig. 1*D*). Taken together, these data suggest that HDAC inhibitors counteract the inhibitory ability of p15RS on Wnt-3a-stimulated gene expression.

HDAC2, but Not HDAC1 or HDAC3, Interacts with p15RS in Mammalian Cells—The fact that HDAC inhibitors TSA and SAHA impaired the role of p15RS in the inhibition of Wnt signaling implied that p15RS might function through HDACs. To identify individual HDACs, we examined whether HDAC1, -2, and -3, the class I HDACs, localize to the nucleus and associate with p15RS, which is also localized to the nucleus (29). An immunoprecipitation experiment showed that Myc-p15RS interacts with FLAG-HDAC2 but not FLAG-HDAC1 or FLAG-HDAC3 in HEK293T cells (Fig. 2*A*). These results therefore identify that p15RS specifically interacts with HDAC2. Because p15RS consists of RPR and CCT domains (Fig. 2*B*), we questioned how p15RS interacts with HDAC2. To map the region of p15RS responsible for the interaction with HDAC2, we performed an immunoprecipitation assay using different domains of p15RS. The results show that both the RPR and the CCT domains showed decreased binding abilities with FLAG-HDAC2, and seemingly, the CCT domain had a lower binding affinity (Fig. 2*C*). To further examine the interaction of endogenous HDAC2 with p15RS under physiological conditions, we performed an immunoprecipitation experiment using an antibody against HDAC1, -2, or -3 in HEK293T cells where p15RS and HDAC1, -2, or -3 are simultaneously expressed. The results showed that the endogenous p15RS protein was precipitated down by an anti-HDAC2 antibody but not by antibodies against

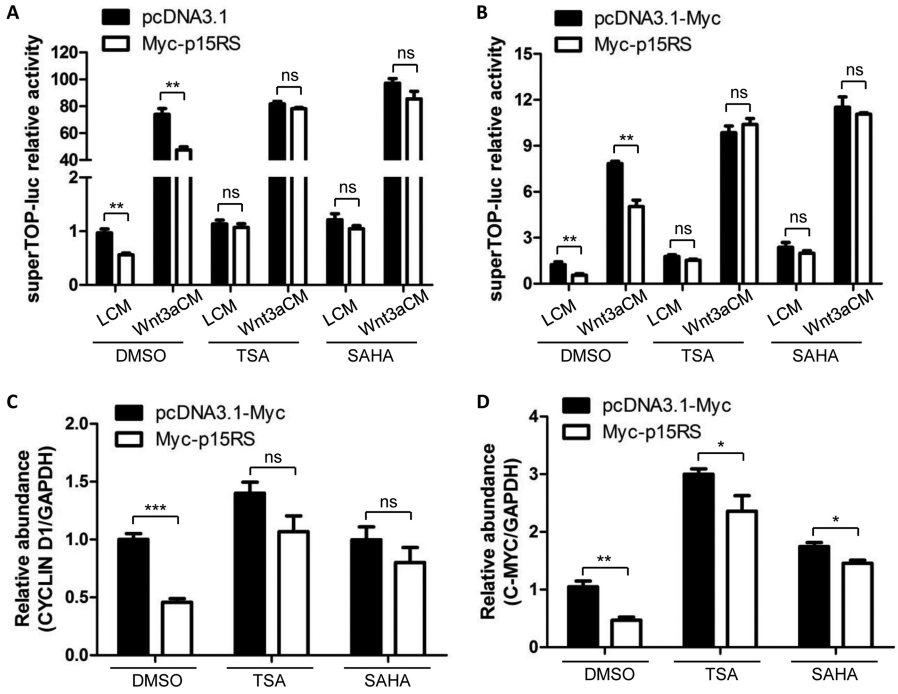


FIGURE 1. **HDACs contribute to the inhibitory function of p15RS.** *A* and *B,* TSA and SAHA impaired the inhibitory effect of p15RS on Wnt signaling. TOP/FOP-Flash-Luc (0.1 μg) and pRT-TK (0.05 μg) were co-transfected into HEK293T cells (*A*) and MCF-7 cells (*B*) with Myc-p15RS (0.05 μg). Cells were treated with TSA (10 ng/ml) for 12 h in combination with Wnt3a conditioned medium (*Wnt3aCM*) treatment for 8 h. The solvent DMSO was used as a control. *C* and *D,* TSA and SAHA blocked the inhibitory effect of p15RS on Wnt target gene expression. mRNA levels of cyclin D1 (*C*) and c-MYC (*D*) were examined by RT-PCR analysis in HEK293T cells with or without overexpression of p15RS and treatment with TSA (10 ng/ml) or SAHA (10 ng/ml) for 12 h. Experiments were repeated in triplicate, and data are represented as the mean ± S.D. ($n = 3$). *Asterisk* indicates a statistically significant difference (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *ns* indicates no statistical difference.

HDAC1 or HDAC3 (Fig. 2*D*). These results firmly confirm that p15RS specifically interacts with HDAC2 in mammalian cells.

As p15RS was reported to associate with TCF4 in the nucleus (29), we next examined whether HDAC2, TCF4, and p15RS form a complex in mammalian cells. An immunoprecipitation experiment demonstrated that FLAG-HDAC2 precipitated down both HA-TCF4 and Myc-p15RS simultaneously (Fig. 2*E*). Consistently, another immunoprecipitation experiment using endogenous proteins showed that an antibody against HDAC2 (Fig. 2*F*) or p15RS (Fig. 2*G*) precipitated down TCF4, and an antibody against TCF4 simultaneously precipitated down p15RS and HDAC2 (Fig. 2*H*). All these results suggest that p15RS, HDAC2, and TCF4 form a complex in mammalian cells.

We next examined whether p15RS and HDAC2 co-localize in intact cells. An immunostaining experiment showed that both p15RS and HDAC2 localize to the nucleus, and p15RS co-localized with HDAC2 (Fig. 2*I*). In line with this observation, we further demonstrated that both p15RS and HDAC2 co-localized with TCF4 in the nucleus, which is consistent with previous observations (Fig. 2*I*) (19, 29).

We next observed whether the three proteins could form a triplex complex. To clarify this, we co-transfected HEK293T cells with vectors encoding differentially tagged p15RS, HDAC2, and TCF4. Interestingly, formaldehyde treatment of cells that co-express Myc-p15RS (~38 kDa), FLAG-HDAC2 (~65 kDa), and HA-TCF4 (~62 kDa) revealed the presence of an additional band of ~170 kDa (Fig. 2*J*), corresponding to the expected size of a heterotrimer formed by these three molecules. We also co-expressed Myc-p15RS (~38 kDa), FLAG-HDAC1 (~65 kDa), and HA-TCF4 (~62 kDa) for the same experiment with formaldehyde cross-linking. The results showed that p15RS, HDAC1, and TCF4 failed to form a triplex complex at ~170 kDa (Fig. 2*K*). Taken together, our results show that p15RS, HDAC2, and TCF4 build a heterotrimer in living cells.

HDAC2 Is Involved in the p15RS-conducted Inhibition of Wnt/β-Catenin—To determine whether the inhibitory role of p15RS on Wnt-3a-mediated transcriptional activity is specifically dependent upon HDAC2, we depleted HDAC1, HDAC2, or HDAC3 using siRNAs and examined TOP/FOP-Flash-Luc reporter activity stimulated by Wnt3a conditioned medium in the presence of overexpressed p15RS in HEK293T cells. The results showed that depletion of HDAC2 disabled or impaired the role of p15RS on the inhibition of luciferase activity under basal conditions or with Wnt stimulation, whereas depletion of either HDAC1 or HDAC3 had no effect on p15RS inhibition of Wnt signaling (Fig. 3*A*), suggesting that the inhibitory role of p15RS on Wnt signaling specifically depends on HDAC2. Meanwhile, when we depleted p15RS, we observed increased activation of Wnt signaling (Fig. 3*B*), which is consistent with our previous findings (29). Intriguingly we found that depletion of p15RS retained a similar effect on reporter activity in control and HDAC1 and -3 depletion cells, whereas depletion of p15RS did not further facilitate Wnt signaling when HDAC2 was depleted (Fig. 3*B*). There results suggest that p15RS inhibits Wnt signaling through HDAC2 rather than the other HDACs. A Western blot result showed the efficiency of knockdown of the HDACs and p15RS in these cells (Fig. 3, *C* and *D*). Taken

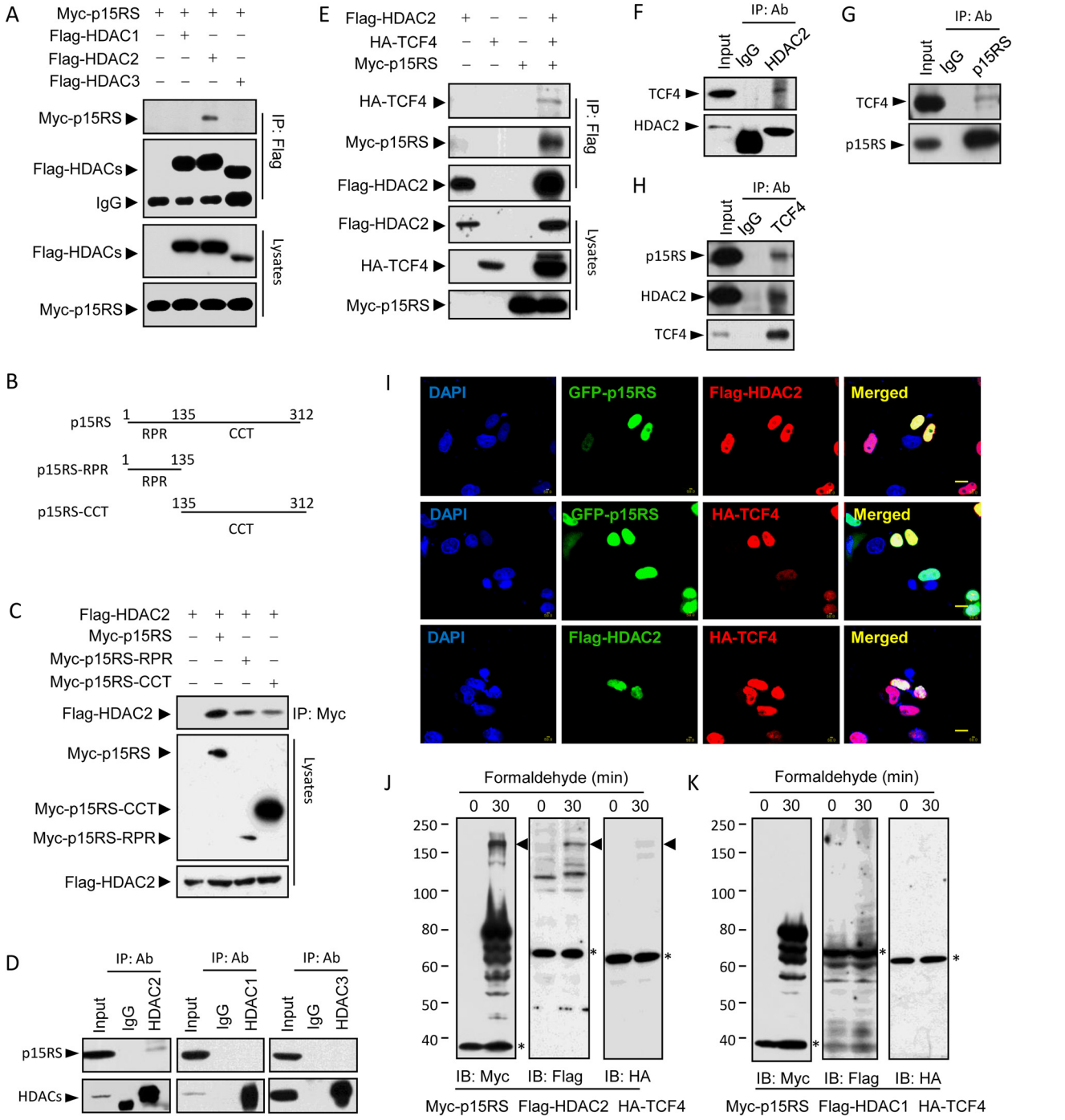


FIGURE 2. **p15RS specifically interacts with HDAC2.** *A,* p15RS interacts with HDAC2 but not with HDAC1 or HDAC3. HEK293T cells co-transfected with Myc-p15RS (4 μg) and FLAG-HDACs (4 μg) were harvested for an immunoprecipitation (*IP*) experiment using an anti-FLAG antibody. Myc-p15RS and FLAG-HDACs were revealed by an anti-Myc antibody and an anti-FLAG antibody in the Western blot analyses. *IB,* immunoblot. *B,* schematic illustration of p15RS and its domains. The RPR domain is an RNA polymerase II CTD-interacting domain, and the CCT domain is assigned as a coiled-coil terminus. *C,* both the RPR and the CCT domains interact weakly with HDAC2. The full-length and each of the two domains of p15RS were co-expressed with FLAG-HDAC2. Immunoprecipitation was performed using an anti-Myc antibody (*Ab*). Precipitants were detected with an anti-FLAG antibody. *D,* endogenous p15RS interacts with HDAC2. Nuclear extracts from HEK293T cells were immunoprecipitated with an anti-HDAC1, anti-HDAC2, or anti-HDAC3 antibody. The immunoprecipitated complex was revealed using an anti-p15RS antibody. *E,* p15RS forms a complex with HDAC2 and TCF4. HEK293T cells were co-transfected with the indicated plasmids. Antibodies used for IP and Western blotting are shown. *F* and *G,* endogenous TCF4 interacts with HDAC2 and p15RS. Nuclear extracts from HEK293T cells were immunoprecipitated with an anti-HDAC2 (*F*) or an anti-p15RS (*G*) antibody. The immunoprecipitates were analyzed by Western blotting. *H,* endogenous p15RS forms a complex with HDAC2 and TCF4. Antibodies used for IP and Western blotting are shown. *I,* p15RS, HDAC2, and TCF4 co-localize in the nucleus. MCF-7 cells expressing GFP-p15RS, FLAG-HDAC2, and HA-TCF4 were stained with an anti-FLAG or anti-HA antibody. Images were viewed with a confocal microscope. Co-localization of the proteins is shown as *yellow*. *Scale bar,* 10 μm. *J,* p15RS, HDAC2, and TCF4 form a heterotriplex. Transiently transfected HEK293T cells expressing Myc-p15RS, FLAG-HDAC2, and HA-TCF4 proteins were cross-linked for 30 min at 37 °C with 1% formaldehyde 24 h after transfection. Specific mAbs were used for the detection of the ~38-kDa monomer of Myc-p25RS and the ~65- and ~62-kDa monomers of FLAG-HDAC2 and HA-TCF4, respectively (*). Heterotrimer of ~170 kDa is indicated by the *dark arrowheads*. *K,* p15RS, HDAC1, and TCF4 failed to form a heterotriplex. The assay was performed according to *J* except with the expression of FLAG-HDAC1 instead of FLAG-HDAC2. Molecular masses are indicated in kDa.

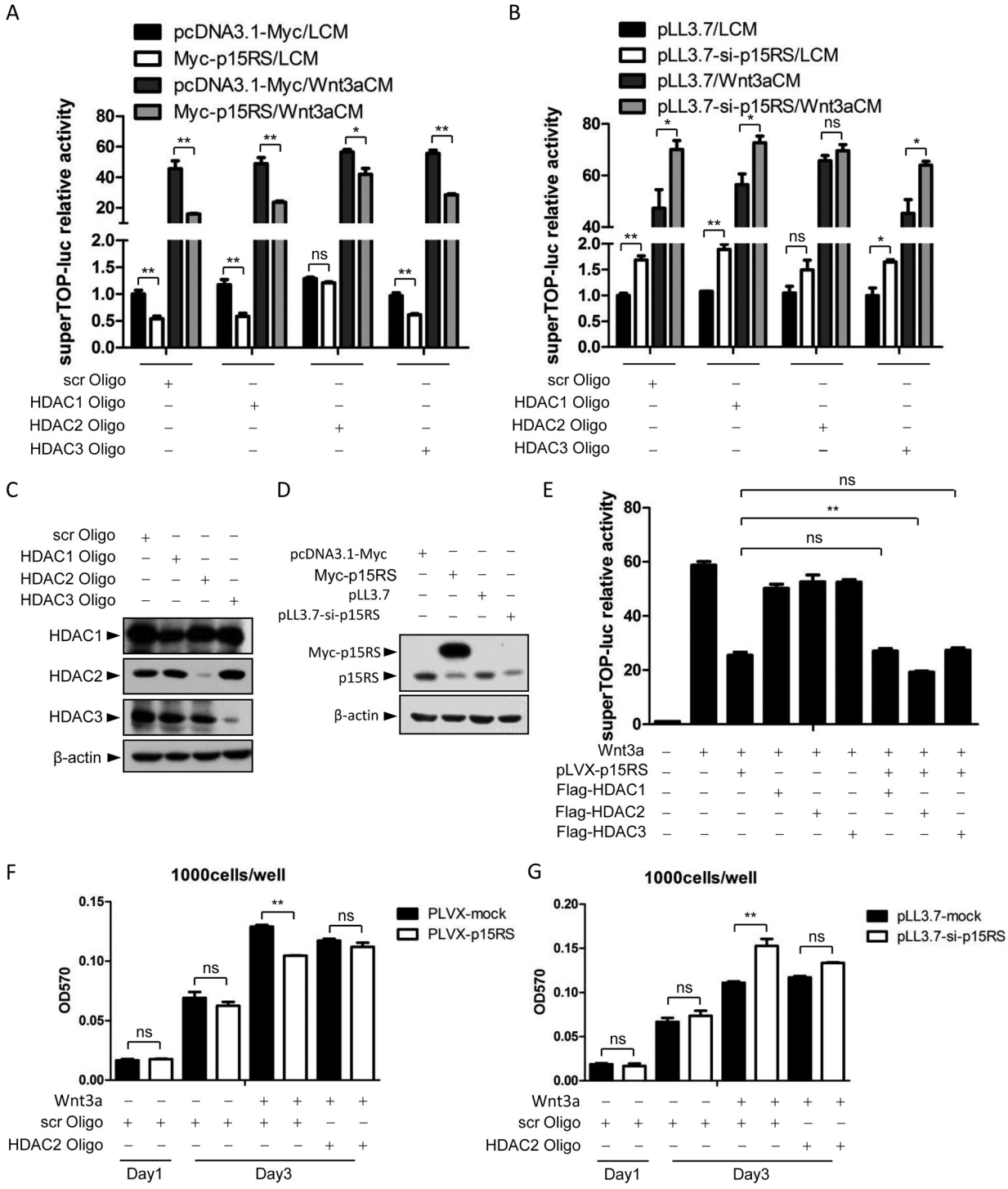


FIGURE 3. **HDAC2 is involved in the p15RS-mediated inhibition of Wnt/β-catenin signaling.** *A* and *B,* overexpression of p15RS results in lower inhibitory function when HDAC2 was depleted. HEK293T cells were transfected with an siRNA against HDAC1, HDAC2, or HDAC3, TOP/FOP-Flash-Luc (0.1 μg), and pRT-TK (0.05 μg) in the presence of Myc-p15RS (0.05 μg) (*A*) or pLL3.7-si-p15RS (*B*). Cells were treated with Wnt3a conditioned medium (*Wnt3aCM*) or control medium for 8 h. The luciferase activity was examined as in Fig. 1. *C* and *D,* protein levels of HDACs and p15RS were detected. Western blot shows the effect of the siRNA in the regulation of HDACs expression. A nonspecific siRNA was used as a control (*C*). The effect of an siRNA against p15RS is shown in *D*. *E,* HDAC2 cooperates with p15RS to repress Wnt signaling. Super-TOP-luciferase (0.1 μg), FLAG-HDACs (0.1 μg), and Myc-p15RS were co-transfected into HEK293T cells for a luciferase assay. Experiments were repeated in triplicate, and the data represent the mean ± S.D. ($n = 3$). *Asterisk* indicates a statistically significant difference (*, $p < 0.05$; **, $p < 0.01$). *ns* indicates no statistical difference. *F,* overexpression of p15RS has lower inhibitory effect on cell growth under the depletion of HDAC2. Cell proliferation experiments were performed using 293 cells stably overexpressing p15RS, stimulated with or without Wnt3a. 293 cells transfected with a siRNAs against HDAC2 or a nonspecific siRNA were seeded into 96-well plate at a density of 1000 cells/well. OD_{570} was measured to assess cell numbers. A *t* test was performed to detect statistical difference between the two groups (**, $p < 0.01$). *G,* depletion of p15RS failed to promote cell growth when HDAC2 was knocked down. Depletion of p15RS in 293 cells was examined for its effect on cell growth under Wnt3a stimulation. 293 cells transfected with a siRNAs against HDAC2 or a nonspecific siRNA were seeded into 96-well plate at a density of 1000 cells/well. Results shown are representative of three independent experiments. A *t* test was performed to detect statistical difference between the two groups (**, $p < 0.01$).

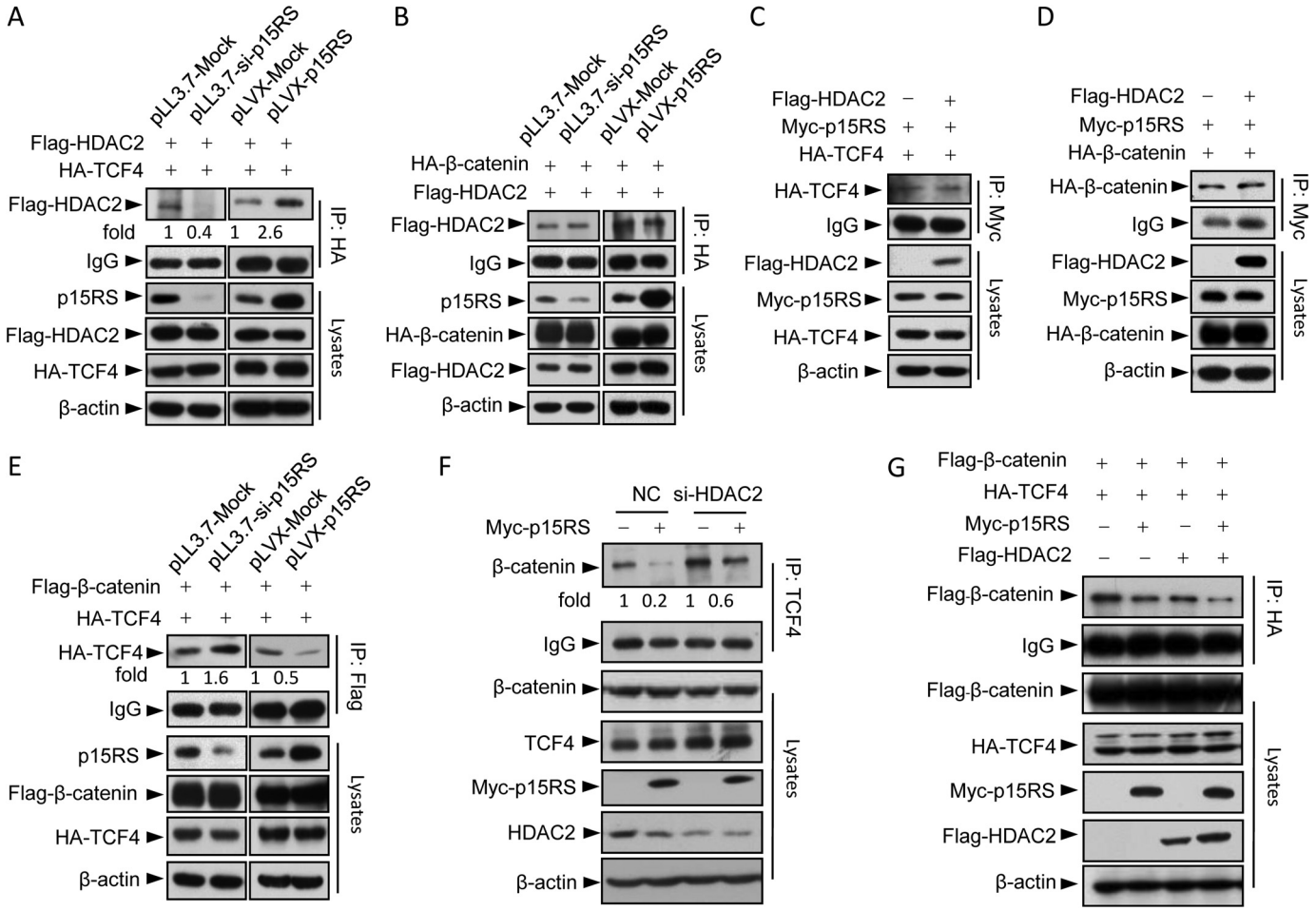


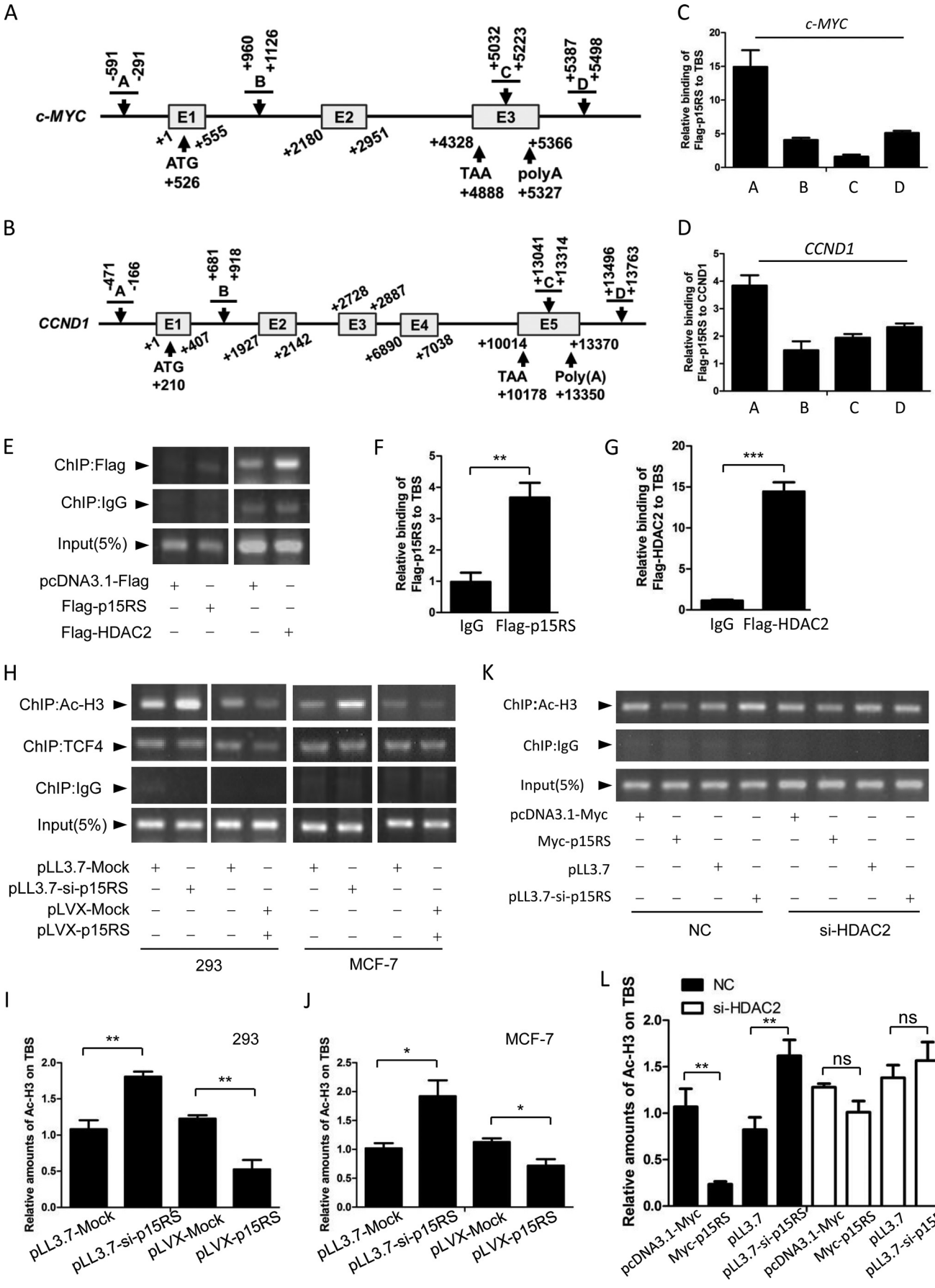
FIGURE 4. **p15RS enhances the interaction of HDAC2 and TCF4.** *A,* p15RS promotes the TCF4·HDAC2 complex formation. 293 cells stably expressing p15RS (*pLVX-p15RS*) and an shRNA against p15RS (*pLL3.7-si-p15RS*) by lentivirus were co-transfected with FLAG-HDAC2 (2 μg) and HA-TCF4 (3 μg) for IP using an anti-HA antibody. FLAG-HDAC2 associated with HA-TCF4 was revealed by immunoblotting using an anti-FLAG antibody. *B,* β-catenin-HDAC2 complex is not affected when p15RS was overexpressed or depleted. The HA-β-catenin and FLAG-HDAC2 complexes were revealed using 293 stable cell lines co-transfected with FLAG-HDAC2 (2 μg) and HA-β-catenin (2 μg). *C* and *D,* HDAC2 fails to affect the interaction of p15RS with TCF4 or β-catenin. HEK293T cells were co-transfected with Myc-p15RS and HA-TCF4 (3 μg) (*C*) or HA-β-catenin (2 μg) (*D*) with or without FLAG-HDAC2 (2 μg). IP was performed using an anti-Myc antibody. *E,* p15RS interfered with the interaction of β-catenin and TCF4. 293 cells co-transfecting with FLAG-β-catenin (1 μg) and HA-TCF4 (3 μg) were harvested for IP using an anti-FLAG antibody. HA-TCF4 was revealed by an anti-HA antibody (*upper panel*). *F,* depletion of HDAC2 recovered the β-catenin/TCF4 interaction, which is disrupted by p15RS. HEK293T cells were transfected with an siRNA against HDAC2. Myc-p15RS was expressed as indicated. IP was performed using anti-TCF4 antibody. *G,* p15RS and HDAC2 coordinately inhibit β-catenin/TCF4 interaction. HEK293T cells were transfected with the indicated plasmids. The cells were harvested for IP by using an anti-HA antibody.

together, these results indicate that p15RS inhibits the transcription of Wnt targeted genes through recruiting HDAC2.

The specific role of HDAC2 on p15RS in the inhibition of Wnt signaling was further demonstrated in an overexpression experiment. A luciferase reporter result showed that HDAC1, HDAC2, or HDAC3 did not repress Wnt-3a-induced TOP/FOP-Flash-Luc reporter transcription when transfected alone (Fig. 3*E, 4th* to *6th lanes*), which might be due to a high level of endogenous HDAC proteins in a cell. This result is similar to that of a previous report, where overexpressing HDAC1 alone did not repress Wnt-1-induced LEF-1 transcription (9). However, co-expression of HDAC2 with p15RS had a synergistic effect on the inhibition of the luciferase activity (Fig. 3*E*, compare *3rd, 5th,* and *8th lanes*) but co-expression of either HDAC1 or HDAC3 showed no further inhibitory effect on the luciferase activity (Fig. 3*E*, *7th* and *9th lanes*). These results suggest that HDAC2 contributes to the inhibition of Wnt signaling conducted by p15RS.

To examine whether the role of p15RS on cell growth is affected by HDAC2, we sought to deplete HDAC2 in 293 cells where p15RS was stably overexpressed or depleted. A cell proliferation experiment showed that overexpression of p15RS inhibited cell proliferation upon Wnt3a stimulation (Fig. 3*F*, *5th* and *6th lanes*), but the inhibition was weakened when HDAC2 was depleted (Fig. 3*F*, *7th* and *8th lanes*). In contrast, p15RS depletion cells grew faster than mock cells under Wnt3a treatment (Fig. 3*G*, *5th* and *6th lanes*), whereas depletion of p15RS showed no further effect on cell proliferation when HDAC2 was depleted (Fig. 3*G*, *7th* and *8th lanes*). These results indicate that the negative regulation of cell proliferation by p15RS under Wnt3a stimulation is partly dependent on HDAC2.

p15RS Enhances HDAC2/TCF4 Interaction—To reveal how HDAC2 participates in the inhibition of Wnt signaling conducted by p15RS, we examined whether p15RS could influence the association of HDAC2 with TCF4 or HDAC2 could affect



the association of p15RS with TCF4 in the nucleus. For this purpose, we generated different cell lines to stably overexpress (using vector pLVX) or deplete (using vector pLL3.7-si) p15RS in 293 cells that constitutively express moderate levels of p15RS (data not shown). An immunoprecipitation experiment indicated that the interaction of FLAG-HDAC2 and HA-TCF4 was decreased in the p15RS depletion cells, whereas it was enhanced in the p15RS overexpression cells (Fig. 4*A*). Because β-catenin interacts with HDAC2 (8), we questioned whether p15RS influences the β-catenin·HDAC2 complex. Interestingly, we observed that the interaction of HA-β-catenin and FLAG-HDAC2 was not affected in either the p15RS overexpression or p15RS depletion cell line (Fig. 4*B*). Furthermore, we questioned whether HDAC2 affects the interaction of p15RS and TCF4. A reciprocal immunoprecipitation experiment demonstrated that Myc-p15RS and HA-TCF4 were equally precipitated in the absence or presence of FLAG-HDAC2 in HEK293T cells (Fig. 4*C*), suggesting that HDAC2 failed to affect the interaction of TCF4 and p15RS. The interaction of HA-β-catenin and Myc-p15RS was also not affected in HDAC2 overexpression cells (Fig. 4*D*).

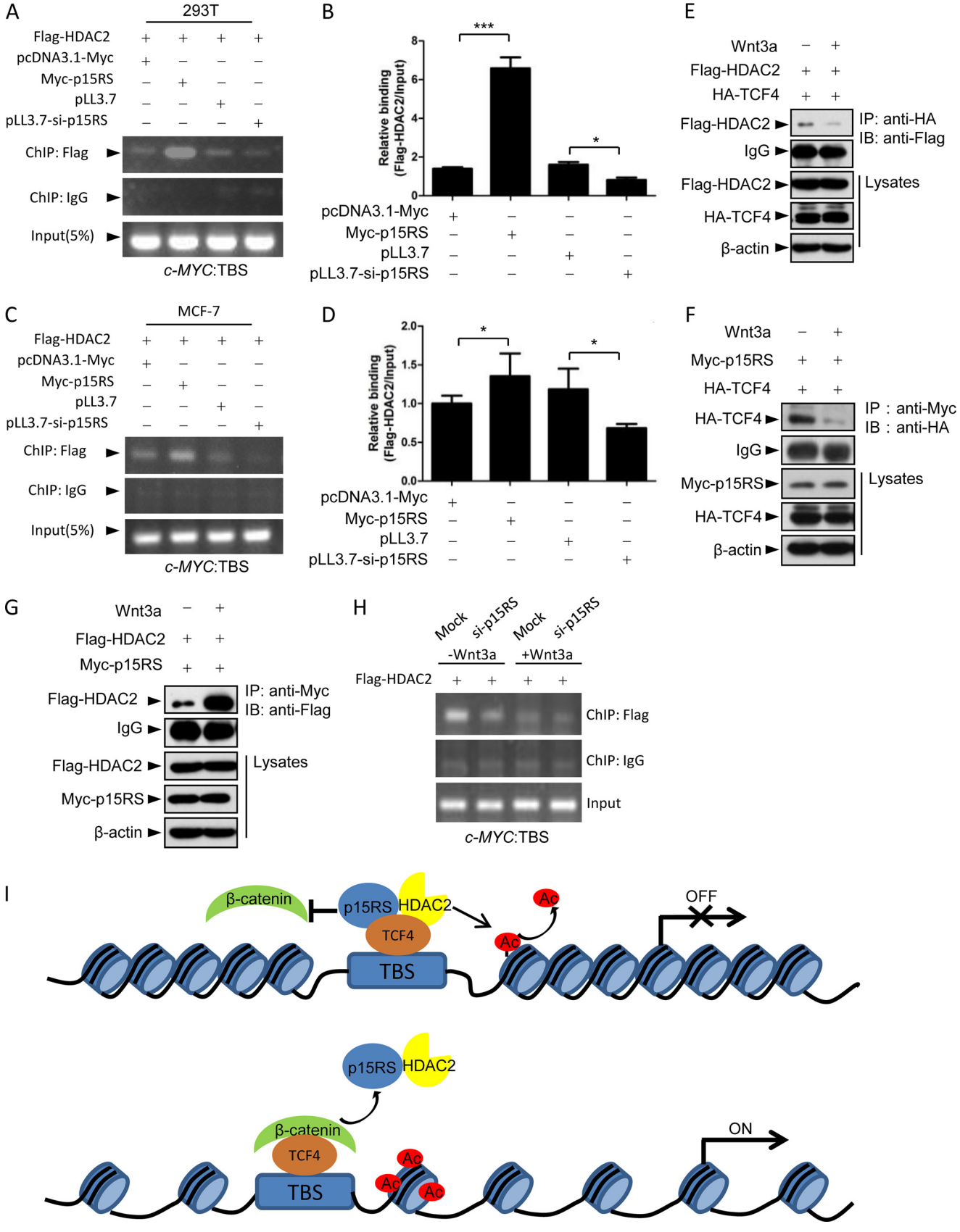
We previously demonstrated that p15RS interfered with the formation of the β-catenin·TCF4 complex (30). Although we confirmed that overexpression of p15RS decreased the interaction of β-catenin with TCF4 and the amount of complex (Fig. 4*E*), we questioned whether HDAC2 is required for p15RS to block the β-catenin and TCF4 interaction. An immunoprecipitation experiment showed that expression of Myc-p15RS impaired the interaction of β-catenin and TCF4 strongly in control cells, but the inhibition was weakened when HDAC2 was depleted (Fig. 4*F*). To address whether p15RS and HDAC2 coordinately regulate the formation of the β-catenin and the TCF4 complex, we examined the interaction of β-catenin and TCF4 under co-expression of p15RS and HDAC2. To this end, FLAG-β-catenin and HA-TCF4 were co-expressed in HEK293T cells with overexpression of Myc-p15RS or FLAG-HDAC2. Immunoprecipitation results showed that the interaction of FLAG-β-catenin with HA-TCF4 was further decreased when Myc-p15RS and FLAG-HDAC2 were co-expressed compared with the case where Myc-p15RS or FLAG-HDAC2 was expressed separately (Fig. 4*G*). These results indicate that HDAC2 contributes to the function of p15RS in interfering with the formation of the β-catenin·TCF4 complex.

p15RS Decreases the Level of Ac-H3 in the c-MYC Promoter by Recruiting HADC2—As p15RS enhanced the interaction of HDAC2 with TCF4, we questioned whether p15RS maintains the association of HDAC2 to the promoters of Wnt target genes. A chromatin immunoprecipitation (ChIP) experiment using different pairs of primers along the c-*MYC* (Fig. 5*A*) and *CCND1* genes (Fig. 5*B*) revealed that p15RS localized mostly to the promoter region of these two genes (marked *A*) (Fig. 5, *C* and *D*). Further ChIP experiments using a TCF4-binding sequence (TBS) (31) revealed that both p15RS and HDAC2 localized to the c-*MYC* promoter (Fig. 5, *E*–*G*), suggesting that both p15RS and HDAC2 are involved in regulating c-MYC expression. To address whether the co-localization of p15RS and HDAC2 functions on the maintenance of deacetylated histones, we examined the level of acetylated histone 3 (Ac-H3), a representative histone acetylated in activated gene promoters, in the promoter of the c-*MYC* gene in the 293 and MCF-7 cell lines. A ChIP analysis showed that the level of Ac-H3 bound to the TBS of the c-*MYC* promoter was markedly increased when p15RS was depleted (Fig. 5*H*, *top panel,* compare *2nd* with *1st column*), whereas the bound TCF4 level remained unchanged (Fig. 5*H*, *2nd image lane*). Conversely, overexpression of p15RS decreased the level of Ac-H3 significantly (Fig. 5*H, top panel,* compare *4th* to *3rd column*). Quantitative analyses for the amounts of ChIPed DNA by real time PCR further confirmed the increased or decreased change in acetylated H3 by depletion or overexpression of p15RS (Fig. 5, *I* and *J*). These results suggest that p15RS blocks the acetylation or promotes the deacetylation of H3 in the c-*MYC* promoter. To address whether p15RS affects the Ac-H3 level through HDAC2 in the deacetylation of H3, we examined the Ac-H3 level in the c-*MYC* promoter in 293 cells where HDAC2 was depleted. The results showed that p15RS failed to reduce the Ac-H3 level when HDAC2 was depleted (Fig. 5, *K* and *L*), suggesting that p15RS maintains the deacetylation of H3 through HDAC2.

p15RS Enhances the Occupancy of HDAC2 to the c-MYC Promoter—To address whether the interaction of p15RS and HDAC2 affects the association of HDAC2 to the promoters of Wnt target genes, we investigated the occupancy of HDAC2 to DNA. A ChIP analysis indicated that overexpression of p15RS resulted in significantly enhanced, although depletion of p15RS led to decreased, HDAC2-DNA binding to the TBS of the c-*MYC* promoter compared with that in control HEK293T cells (Fig. 6*A*). An RT-PCR result further confirmed the role of

FIGURE 5. **p15RS decreases the level of acetylated histone H3 (Ac-H3) on the c-*MYC* promoter.** *A* and *B,* graphic representation of the c-*MYC* (*A*) and *CCND1* (*B*) genomic structures. Fragments detected by PCR are shown as *A*–*D*. *C* and *D,* p15RS mostly localizes to the promoter region of the c-*MYC* (*C*) or *CCND1* (*D*) genes. HEK293T cells were transfected with FLAG-p15RS and then harvested for ChIP assay using an anti-FLAG antibody. DNA fragments from ChIP assay were detected using RT-PCR with primer pairs indicated as *A*–*D*. The relative binding of FLAG-p15RS on the different regions were normalized with the respective input. *E,* both p15RS and HDAC2 localize to the TCF4 binding region (TBS) in the c-*MYC* promoter. HEK293T cells were transfected with FLAG-p15RS or FLAG-HDAC2 and then harvested for the ChIP assay using an anti-FLAG antibody. PCR bands indicating the presence of p15RS and HDAC2 on the TBS were normalized with the respective input. *F* and *G,* relative amounts of FLAG-p15RS (*F*) or FLAG-HDAC2 (*G*) on the TBS in the c-*MYC* promoter. The amounts of ChIPed DNA from *E* were quantitated by real time PCR. *H,* p15RS decreases the level of Ac-H3 on the TBS. Stable 293 and MCF-7 cell lines which constitutively express p15RS, or si-p15RS, were harvested for ChIP assay using an anti-Ac-H3 antibody and an anti-TCF4 antibody, respectively. The PCR bands from the Ac-H3 precipitated complex on the TBS were normalized with the respective input. *I* and *J,* relative amounts of Ac-H3 on the TBS of HEK293T (*I*) and MCF-7 (*J*) cells. Real time PCR was used to quantify the amounts of ChIPed DNA from *H. K,* p15RS-mediated maintenance of deacetylated H3 depends on HDAC2. HEK293T cells were transfected with an siRNA against HDAC2 (*si-HDAC2*). Cells were transfected with Myc-p15RS or si-p15RS. ChIP was performed using an anti-Ac-H3 antibody. *NC,* negative control. PCR bands were quantified and normalized with the respective input. *L,* relative amounts of Ac-H3 on the TBS region. The graph is a quantification of the ChIPed DNA from (*K*) examined by real time PCR. *Asterisk* indicates a statistically significant difference (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

p15RS on the occupancy of HDAC2 to promoters (Fig. 6*B*). Similar results were obtained in MCF-7 cells (Fig. 6, *C* and *D*). Taken together, these results suggest that p15RS enhances the interaction of HDAC2 with TCF4 and the occupancy of HDAC2 in the c-*Myc* promoter to repress the gene transcription by maintaining deacetylated H3.



To unveil the mechanism of the inhibitory role of p15RS and HDAC2 on the transcription of Wnt target genes, we investigated whether the interaction of p15RS or HDAC2 with TCF4 is dependent upon Wnt signaling. IP results showed that the interaction of p15RS (Fig. 6*E*) or HDAC2 (Fig. 6*F*) with TCF4 decreased when cells were treated with Wnt3a. However, another IP experiment demonstrated that the interaction of p15RS with HDAC2 was increased under Wnt3a stimulation (Fig. 6*G*). These results suggest that both p15RS and HDAC2 are removed from TCF4 and form a complex free from binding with TCF4 when Wnt signaling is activated. Further ChIP analyses indicated that HDAC2 bound to the TBS of the c-*MYC* promoter in the static condition but that the binding of HDAC2 to the promoter was decreased when p15RS was depleted (Fig. 6*H*, *1st* and *2nd lanes*). Consistently, the binding of HDAC2 was significantly decreased after Wnt3a treatment (Fig. 6*H*, compare *3rd* with *1st* and *4th* with *2nd lanes*). All the results suggest that both p15RS and HDAC2 collectively bind to the promoter region of Wnt target genes, and their binding can be released by Wnt signaling.

DISCUSSION

We have demonstrated that p15RS requires deacetylation activity to repress gene transcription. Interestingly, we identified that p15RS interacts with HDAC2 but not HDAC1 or HDAC3. Our results provide evidence for a model that p15RS and HDAC2 coordinately inhibit TCF4-initiated transcription. Previously, we proposed that p15RS blocked the interaction of β-catenin with TCF4 (29). In this study, we observed that p15RS not only interacted with TCF4 but also with HDAC2. We revealed that the interaction of p15RS with HDAC2 results in decreased acetylation of histone H3. We propose that p15RS maintains HDAC2 to occupy the promoter and keeps H3 in a deacetylated state. In such a way, β-catenin fails to associate with TCF4, and gene transcription is repressed. Therefore, the role of p15RS in the inhibition of gene transcription in the absence of Wnt stimulation is by simultaneously blocking the interaction of β-catenin with TCF4 and maintaining H3 in a deacetylated (or hypo-acetylated) state, which is less accessible to transcriptional activators or the basal transcription machinery (Fig. 6*E*, *top*) (8, 9). However, when Wnt signaling is activated, β-catenin translocates into the nucleus to associate with p15RS, removing both p15RS and HDAC2 from binding to TCF4. This ultimately results in the formation of the β-catenin and TCF4 complex at the promoters of Wnt target genes (Fig. 6*E*, *bottom*).

Several groups have proposed that TCF/LEF might interact with HDACs, probably in an indirect manner, for instance, via the recently described NuRD complex containing HDAC and ATPase (32, 33). In this study, we found that TCF4 interacts with HDAC2 and p15RS, indicating that p15RS and HDAC2 are likely a novel co-repressor complex involved in the inhibition of the Wnt/β-catenin signaling pathway. Our findings echo the observation that different TCF/LEF family members may interact with different co-repressors, including two exclusive complexes, the Groucho complex with human TCF1, *Drosophila melanogaster* dTCF, and *Xenopus laevis* TCF-3 (XTCF-3) (34), and the NuRD complex with mTCF3 and mTCF4 (33). Our study supports the notion that LEF/TCF associates with different complexes in different cell types or under different physiological conditions. The interaction of TCF4 with HDAC2 and p15RS might represent a condition that a cell requests for this complex to repress the transcription of a specific gene.

We have found that p15RS, coordinating with HDAC2, decreases the transcriptional activity of Wnt/β-catenin signaling by interfering with the β-catenin·TCF4 complex formation. However, how p15RS and HDAC2 interfere with the formation of the β-catenin·TCF4 complex remains to be elucidated. It was reported that β-catenin is modified by acetylation and deacetylation. The acetylation of β-catenin occurs at Lys-49 by CBP (35) and at Lys-345 by p300 (36), leading to an increased binding affinity with TCF4 and an up-regulated transcriptional activity of Wnt/β-catenin signaling. Instead, deacetylation of β-catenin results in decreased binding affinity with TCF4. Therefore, we envisioned that p15RS might interfere with the formation of the β-catenin and TCF4 complex by recruiting HDAC2 to deacetylate β-catenin and thus decrease the binding affinity of β-catenin for TCF4. Indeed, we observed decreased levels of acetylated β-catenin when p15RS was overexpressed (data not shown). Therefore, we consider that the inhibitory role of p15RS on Wnt target genes could be through recruiting HDAC2, which maintains both β-catenin and histone H3 in a deacetylated state.

Previous studies reported that HDAC1 could also inhibit Wnt target genes. However, our study provided evidence that HDAC1 failed to interact with p15RS and showed no effect on p15RS-mediated inhibition (Fig. 3). We considered that HDAC1 might function independently of p15RS and may recruit a different regulator. For instance, NFATc2 was reported to directly interact with HDAC1 and suppresses CDK4 transcription (37). Therefore, HDAC1 and HDAC2 may reciprocally recruit independent factors for the inhibition of gene transcription. Indeed, NFATc2 was reported not to interact with HDAC2 (37), similar to the case we describe here

FIGURE 6. **p15RS enhances the occupancy of HDAC2 to the c-*MYC* promoter.** HEK293T (*A* and *B*) and MCF-7 (*C* and *D*) cells were co-transfected with FLAG-HDAC2, Myc-p15RS, or si-p15RS. Cells were harvested for ChIP using an anti-FLAG antibody. Results from PCR (*A* and *C*) and quantitative PCR (*B* and *D*) using primers targeting the TBS are presented. *E,* Wnt3a decreases the interaction of HDAC2 and TCF4. FLAG-HDAC2 and HA-TCF4 were co-expressed in HEK293T cells together with or without Wnt3a. IP experiments were performed using an anti-HA antibody. *F,* Wnt3a released the interaction of p15RS and TCF4. Myc-p15RS and HA-TCF4 were co-expressed in HEK293T cells together with or without Wnt3a. The IP experiments were performed using an anti-Myc antibody. *G,* Wnt3a enhanced the interaction of p15RS and HDAC2. Myc-p15RS and FLAG-HDAC2 were co-expressed in HEK293T cells together with or without Wnt3a. IP experiments were performed using an anti-Myc antibody. *H,* depletion of p15RS attenuated the binding of HDAC2 to TBS. 293 cells were transfected with FLAG-HDAC2 for 24 h with or without Wnt3a treatment. ChIP assay was performed using an anti-FLAG antibody. The ChIPed DNA was examined by PCRs using primers for the TBS of the c-*MYC* gene. *I,* model demonstrating the inhibitory role of p15RS and HDAC2 on the canonical Wnt signaling pathway. In the quiescent state, p15RS maintains the interaction of HDAC2 with TCF4 on the promoters of Wnt-target genes and keeps histone proteins in a deacetylation state (*upper panel*). When Wnt signaling is activated, β-catenin translocates into the nucleus where it removes p15RS and HDAC2 from TCF4, and then initiates gene transcription.

where p15RS does not interact with HDAC1. Because differential roles for HDAC1 and HDAC2 have been demonstrated (38), our study provides a case where p15RS specifically recruits HDAC2, but not HDAC1 or HDAC3, for the inhibition of Wnt signaling.

The role of p15RS in gene transcription seems quite different from its yeast homolog Rtt103 and the human/mouse homolog CREPT or RPRD1B. Although yeast *Rtt103* was demonstrated to be a gene that favors cell growth (as the double deletion of Rtt103 is synthetic lethal) (39) and human/mouse CREPT promotes tumor cell proliferation (24, 40), p15RS was reported to inhibit cell growth as demonstrated by both Liu *et al.* (22) and Chang and co-workers (29). Interestingly, although yeast Rtt103 binds only to the 3′-end of genes to regulate transcriptional termination by a mechanism called the *Torpedo* model, our results indicated that p15RS preferably binds to the promoter regions of the c-*MYC* and *CCDN1* genes as shown here (Fig. 5, *C* and *D*) and in our previous report (29). These results suggest that mammalian p15RS functions differently from its yeast homolog Rtt103. Intriguingly, the role of p15RS in the regulation of gene transcription also differs from that of CREPT as we observed that CREPT binds to both the promoter and termination region of the *CCND1* gene (24) and promotes Wnt signaling (40). However, the differential roles of p15RS and CREPT in regulating gene transcription were not elucidated by partial structural analyses (23, 25, 27). Deciphering the molecular mechanisms of this new family of RPR-containing proteins will provide new insight for understanding the fine regulation of gene transcription.

In summary, our findings extend our knowledge on the molecular mechanisms underlying the negative regulation of Wnt signaling by p15RS. In parallel to its function in interfering with the interaction of β-catenin and TCF4, p15RS exerts its inhibitory effect by recruiting HDAC2 to keep chromatin in a repressive state. These mechanisms of p15RS in repressing Wnt targeted gene transcription may work jointly or independently, possibly depending on the cell type and differentiation state.

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REFERENCES

1. Siegfried, E., Chou, T. B., and Perrimon, N. (1992) wingless signaling acts through zeste-white 3, the *Drosophila* homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. *Cell* **71,** 1167–1179
2. Behrens, J., von Kries, J. P., Kühl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996) Functional interaction of β-catenin with the transcription factor LEF-1. *Nature* **382,** 638–642
3. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destrée, O., and Clevers, H. (1996) XTcf-3 transcription factor mediates β-catenin-induced axis formation in *Xenopus* embryos. *Cell* **86,** 391–399
4. Clevers, H., and Nusse, R. (2012) Wnt/β-catenin signaling and disease. *Cell* **149,** 1192–1205
5. Cadigan, K. M., and Waterman, M. L. (2012) TCF/LEFs and Wnt signaling in the nucleus. *Cold Spring Harb. Perspect. Biol.* **4,** a007906
6. Anastas, J. N., and Moon, R. T. (2013) WNT signalling pathways as therapeutic targets in cancer. *Nat. Rev. Cancer* **13,** 11–26
7. Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destrée, O., and Clevers, H. (1998) The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395,** 608–612
8. Ye, F., Chen, Y., Hoang, T., Montgomery, R. L., Zhao, X. H., Bu, H., Hu, T., Taketo, M. M., van Es, J. H., Clevers, H., Hsieh, J., Bassel-Duby, R., Olson, E. N., and Lu, Q. R. (2009) HDAC1 and HDAC2 regulate oligodendrocyte differentiation by disrupting the β-catenin-TCF interaction. *Nat. Neurosci.* **12,** 829–838
9. Billin, A. N., Thirlwell, H., and Ayer, D. E. (2000) β-Catenin-histone deacetylase interactions regulate the transition of LEF1 from a transcriptional repressor to an activator. *Mol. Cell. Biol.* **20,** 6882–6890
10. Reichert, N., Choukrallah, M. A., and Matthias, P. (2012) Multiple roles of class I HDACs in proliferation, differentiation, and development. *Cell. Mol. Life Sci.* **69,** 2173–2187
11. Glozak, M. A., Sengupta, N., Zhang, X., and Seto, E. (2005) Acetylation and deacetylation of non-histone proteins. *Gene* **363,** 15–23
12. Bieliauskas, A. V., and Pflum, M. K. (2008) Isoform-selective histone deacetylase inhibitors. *Chem. Soc. Rev.* **37,** 1402–1413
13. Beumer, J. H., and Tawbi, H. (2010) Role of histone deacetylases and their inhibitors in cancer biology and treatment. *Curr. Clin. Pharmacol.* **5,** 196–208
14. Aghdassi, A., Sendler, M., Guenther, A., Mayerle, J., Behn, C. O., Heidecke, C. D., Friess, H., Büchler, M., Evert, M., Lerch, M. M., and Weiss, F. U. (2012) Recruitment of histone deacetylases HDAC1 and HDAC2 by the transcriptional repressor ZEB1 downregulates E-cadherin expression in pancreatic cancer. *Gut* **61,** 439–448
15. Harms, K. L., and Chen, X. (2007) Histone deacetylase 2 modulates p53 transcriptional activities through regulation of p53-DNA binding activity. *Cancer Res.* **67,** 3145–3152
16. Doetzlhofer, A., Rotheneder, H., Lagger, G., Koranda, M., Kurtev, V., Brosch, G., Wintersberger, E., and Seiser, C. (1999) Histone deacetylase 1 can repress transcription by binding to Sp1. *Mol. Cell. Biol.* **19,** 5504–5511
17. Juan, L. J., Shia, W. J., Chen, M. H., Yang, W. M., Seto, E., Lin, Y. S., and Wu, C. W. (2000) Histone deacetylases specifically down-regulate p53-dependent gene activation. *J. Biol. Chem.* **275,** 20436–20443
18. Yarden, R. I., and Brody, L. C. (1999) BRCA1 interacts with components of the histone deacetylase complex. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 4983–4988
19. Li, H., and Richardson, W. D. (2009) Genetics meets epigenetics: HDACs and Wnt signaling in myelin development and regeneration. *Nat. Neurosci.* **12,** 815–817
20. Winkler, C. J., Ponce, A., and Courey, A. J. (2010) Groucho-mediated repression may result from a histone deacetylase-dependent increase in nucleosome density. *PloS one* **5,** e10166
21. Toh, Y., Kuninaka, S., Endo, K., Oshiro, T., Ikeda, Y., Nakashima, H., Baba, H., Kohnoe, S., Okamura, T., Nicolson, G. L., and Sugimachi, K. (2000) Molecular analysis of a candidate metastasis-associated gene, MTA1: possible interaction with histone deacetylase 1. *J. Exp. Clin. Cancer Res.* **19,** 105–111
22. Liu, J., Liu, H., Zhang, X., Gao, P., Wang, J., and Hu, Z. (2002) Identification and characterization of P15RS, a novel P15(INK4b) related gene on G_1/S progression. *Biochem. Biophys. Res. Commun.* **299,** 880–885
23. Ni, Z., Olsen, J. B., Guo, X., Zhong, G., Ruan, E. D., Marcon, E., Young, P., Guo, H., Li, J., Moffat, J., Emili, A., and Greenblatt, J. F. (2011) Control of the RNA polymerase II phosphorylation state in promoter regions by CTD interaction domain-containing proteins RPRD1A and RPRD1B. *Transcription* **2,** 237–242
24. Lu, D., Wu, Y., Wang, Y., Ren, F., Wang, D., Su, F., Zhang, Y., Yang, X., Jin, G., Hao, X., He, D., Zhai, Y., Irwin, D. M., Hu, J., Sung, J. J., *et al.* (2012) CREPT accelerates tumorigenesis by regulating the transcription of cell-cycle-related genes. *Cancer Cell* **21,** 92–104
25. Mei, K., Jin, Z., Ren, F., Wang, Y., Chang, Z., and Wang, X. (2014) Structural basis for the recognition of RNA polymerase II C-terminal domain by CREPT and p15RS. *Sci. China Life Sci.* **57,** 97–106
26. Zhang, X., Cao, Q., Liu, X., Liu, S., Wang, J., Sun, S., Wang, O., Tian, Z., Liu, H., Kuang, J., and Zhang, W. (2012) Cellular and molecular evidence for malignancy-inhibitory functions of p15RS. *Cell Cycle* **11,** 1988–1998

27. Ni, Z., Xu, C., Guo, X., Hunter, G. O., Kuznetsova, O. V., Tempel, W., Marcon, E., Zhong, G., Guo, H., Kuo, W. H., Li, J., Young, P., Olsen, J. B., Wan, C., Loppnau, P., *et al.* (2014) RPRD1A and RPRD1B are human RNA polymerase II C-terminal domain scaffolds for Ser5 dephosphorylation. *Nat. Struct. Mol. Biol.* **21,** 686–695
28. Lunde, B. M., Reichow, S. L., Kim, M., Suh, H., Leeper, T. C., Yang, F., Mutschler, H., Buratowski, S., Meinhart, A., and Varani, G. (2010) Cooperative interaction of transcription termination factors with the RNA polymerase II C-terminal domain. *Nat. Struct. Mol. Biol.* **17,** 1195–1201
29. Wu, Y., Zhang, Y., Zhang, H., Yang, X., Wang, Y., Ren, F., Liu, H., Zhai, Y., Jia, B., Yu, J., and Chang, Z. (2010) p15RS attenuates Wnt/β-catenin signaling by disrupting β-catenin.TCF4 Interaction. *J. Biol. Chem.* **285,** 34621–34631
30. Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R., and Pavletich, N. P. (1999) Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* **401,** 188–193
31. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) Identification of c-MYC as a target of the APC pathway. *Science* **281,** 1509–1512
32. Sakai, H., Urano, T., Ookata, K., Kim, M. H., Hirai, Y., Saito, M., Nojima, Y., and Ishikawa, F. (2002) MBD3 and HDAC1, two components of the NuRD complex, are localized at Aurora-A-positive centrosomes in M phase. *J. Biol. Chem.* **277,** 48714–48723
33. Xue, Y., Wong, J., Moreno, G. T., Young, M. K., Côté, J., and Wang, W. (1998) NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol. Cell* **2,** 851–861
34. Jennings, B. H., and Ish-Horowicz, D. (2008) The Groucho/TLE/Grg family of transcriptional co-repressors. *Genome Biol.* **9,** 205
35. Wolf, D., Rodova, M., Miska, E. A., Calvet, J. P., and Kouzarides, T. (2002) Acetylation of β-catenin by CREB-binding protein (CBP). *J. Biol. Chem.* **277,** 25562–25567
36. Lévy, L., Wei, Y., Labalette, C., Wu, Y., Renard, C. A., Buendia, M. A., and Neuveut, C. (2004) Acetylation of β-catenin by p300 regulates β-catenin-Tcf4 interaction. *Mol. Cell. Biol.* **24,** 3404–3414
37. Baksh, S., Widlund, H. R., Frazer-Abel, A. A., Du, J., Fosmire, S., Fisher, D. E., DeCaprio, J. A., Modiano, J. F., and Burakoff, S. J. (2002) NFATc2-mediated repression of cyclin-dependent kinase 4 expression. *Mol. Cell* **10,** 1071–1081
38. Dovey, O. M., Foster, C. T., and Cowley, S. M. (2010) Histone deacetylase 1 (HDAC1), but not HDAC2, controls embryonic stem cell differentiation. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 8242–8247
39. Kim, M., Krogan, N. J., Vasiljeva, L., Rando, O. J., Nedea, E., Greenblatt, J. F., and Buratowski, S. (2004) The yeast Rat1 exonuclease promotes transcription termination by RNA polymerase II. *Nature* **432,** 517–522
40. Zhang, Y., Liu, C., Duan, X., Ren, F., Li, S., Jin, Z., Wang, Y., Feng, Y., Liu, Z., and Chang, Z. (2014) CREPT/RPRD1B, a recently identified novel protein highly expressed in tumors, enhances the β-catenin·TCF4 transcriptional activity in response to Wnt signaling. *J. Biol. Chem.* **289,** 22589–22599

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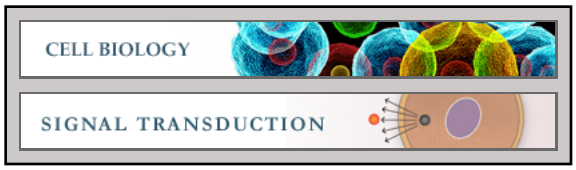
p15RS/RPRD1A (p15INK4b-related Sequence/Regulation of Nuclear Pre-mRNA Domain-containing Protein 1A) Interacts with HDAC2 in Inhibition of the Wnt/β -Catenin Signaling Pathway



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