p15RS attenuates Wnt/β-catenin signaling by disrupting β-catenin/TCF4 interaction

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Running Title: p15RS inhibits Wnt signaling

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The formation of a β-catenin/TCF4 complex in the nucleus of cells is well known as a prerequisite for the transcription of Wnt target genes. Although many co-factors have been identified to regulate the activity of the β-catenin/TCF4 complex, it remains unclear how the complex association is negatively regulated. In this study, we report that p15RS, a negative regulator of the cell cycle, blocks β-catenin/TCF4 complex formation and inhibits Wnt signaling. We observed that p15RS interact with β-catenin and TCF4. Interestingly, while the interaction of p15RS with β-catenin is increased, its interaction with TCF4 is decreased upon Wnt1 stimulation. Moreover, over-expression of p15RS reduces the interaction of β-catenin with TCF4, while the depletion of p15RS enhances their interaction. We further demonstrate that over-expression of p15RS suppresses canonical Wnt signaling and results in retarded cell growth, while the depletion of p15RS shows an enhanced effect on Wnt signaling. We analyzed that inhibition of Wnt signaling by p15RS leads to decreased expression of Cyclin D1 and c-Myc, two Wnt targeted genes critical for cell growth. Our data suggest that p15RS inhibits Wnt signaling by interrupting β-catenin/TCF4 complex formation and that Wnt signaling initiates downstream gene expression by removing p15RS from promoters.

Keywords: p15RS; β-catenin; TCF4; Wnt signaling; Cyclin D1

Introduction

The Wnt signaling pathway, an evolutionarily conserved signal transduction cascade, is involved in the regulation of various biological events, including body axis formation, cell proliferation, and organogenesis in many organisms and is important for homeostatic self-renewal in several adult tissues. Disorders in Wnt

signaling cause human degenerative diseases as well as cancers (1-4).

Wnt signaling is initiated by the binding of Wnt proteins to their receptors, Frizzled and LRP5/6. After Wnt protein binding, Wnt signaling branches into three distinct pathways, a canonical Wnt-β-catenin pathway and two non-canonical pathways (Wnt-Ca^{2+}, and Wnt-JNK), via the cytoplasmic phosphoprotein Dishevelled (Dvl) (2,3,5).

In the canonical Wnt pathway, β-catenin protein accumulates upon Wnt stimulation. In the absence of a Wnt signal, β-catenin forms a complex with adenomatous polyposis coli (APC) and Axin, and is phosphorylated by casein kinase Iα (CKIα) and glycogen synthase kinase 3β (GSK3β), and is subsequently degraded by the β-TrCP mediated ubiquitin/proteasome pathway (6,7). When Wnt proteins bind to the receptors Frizzled and LRP5/6, Dishevelled (Dvl) becomes hyper-phosphorylated by CKIε, resulting in the assembly of an LRP5/6-Axin-FRAT complex that leads to the degradation of Axin (8-10). As a consequence, β-catenin dissociates from the APC-Axin-GSK3β complex. Alternatively, Wnt may activate Dvl, which in turn inhibits GSK3β activity (8). In both scenarios, β-catenin escapes from degradation and accumulates in the nucleus, where it displaces the Groucho/histone deacetylases (HDACs) transcription inhibitors from the T cell-specific factor (TCF)/lymphoid enhancer-binding factor 1 (LEF-1) and recruit histone acetylase CBP/p300 to activate gene expression (11). Nuclear β-catenin also interacts with the legless family docking proteins (BCL9 and BCL9L), which coordinate with PYGO family co-activators (PYGO1 and PYGO2), to regulate the transcription of Wnt target genes including *cyclinD1, MYC, FGF20, DKK1* and *WISP1* (12-16). Although it has been well-established that the formation of the nuclear β-catenin/TCF complex plays a pivotal role in the activation of Wnt target genes, the fine details of the mechanisms of transcriptional activation and regulation are still under investigation (17,18).

p15RS, a novel p15INK4b-related gene involved in G1/S progression, was identified by a differential display experiment from cells over-expressing p15INK4b (19). Anti-sense p15RS was shown to up-regulate the expression of *Cyclin D1* in MLIK6 cells. Therefore, p15RS was regarded as a negative regulator in cell cycle control in G1 phase by down regulating Cyclin D1 expression (19). Since *Cyclin D1* is a direct target gene that is transactivated by the β-catenin/TCF4/LEF-1 complex through a LEF-1 binding site in its promoter (20), we speculated that p15RS might be involved in regulating Wnt/β-catenin signaling. In the present study, we report that p15RS blocks the interaction of β-catenin and TCF4, and negatively regulates the canonical Wnt/β-catenin signaling pathway.

Materials and Methods
Plasmids and Reagents- pcDNA3.1/Myc-p15RS and different p15RS deletion mutant plasmids were generated by inserting the PCR-amplified fragments into a pcDNA3.1/Myc vector. Constructs expressing Flag-β-catenin, Flag-β-catenin*, Flag-Dvl2, and Wnt1 as well as the pGL3/LEF-1-Luc vector were 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 deletions were kindly provided

by Dr. Yeguang Chen, Tsinghua University. HA-TCF4 deletions were kindly provided by Dr. Chengwen Wu, National Yang-Ming University. Flag-Chibby was kindly provided by Dr. Randall T. Moon, Northwestern University, Chicago. A plasmid with p15RS siRNA was synthesized using two single-strand DNA fragments: 5'-GATCC ACCAAACAGGAAGCTTACTTTCAAGAG AAGTAAGCTTCCTGTTTGGTTTA-3' and 5'-AGCTTAAACCAAACAGGAAGCTTAC TTCTCTTGAAAGTAAGCTTCCTGTTTGG TG-3' [The underlined sequence is the siRNA target of the human p15RS gene (nt 154-174)], inserted between the BamHI and HindIII sites of pSilencer 4.1/CMV vector (a gift from Dr. Xun Shen, Chinese Academy of Science, Beijing.). A p15RSs siRNA resistant mutant was generated by mutagenesis at nucleotides C162T, G165A, 168GA without affecting the amino acid sequence. Anti-β-Actin (AC-15) and anti-Flag (M2) were from Sigma. Anti-Myc (9E10), anti-β-catenin, anti-TCF4, anti-p15RS and anti-HA (F-7) were purchased from Santa Cruz. Fluorescent secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) were purchased from Jackson ImmunoResearch Laboratories.

Cell Culture- HEK293T, MCF-7, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. SW480 cells were kept in RPMI1640 medium supplemented with 10% FBS. All the cells were kept at 37°C in a 5% CO_2-containing atmosphere. Media and serum were purchased from Gibco. For the generation of stable cell lines, MCF-7 cells were plated at a density of 5 x 10^4 cells/cm^2 the day before transfection. After 24 h, and every 48 h thereafter for 2 weeks, media was

replaced with fresh media containing 1 mg/ml of G418 (Sigma). Resistant clones were maintained in media containing 400 μg/ml of G418. MCF-7 cells at a density of 1,500 cells/well in 96-well plate were used for the cell proliferation experiments.

Co-immunoprecipitations- HEK293T cells were plated in 60 mm dishes the day before transfection. 5 μg of pcDNA3.1/Myc-p15RS and the indicated plasmids were transfected. If necessary, pcDNA3.1/Myc or HA empty vector was added to ensure that an equal amount of plasmid was transfected into each dish. Cells were lysed 24-36 h after transfection in 600 μl cell lysis buffer (50 mM Tris·Cl, 150 mM NaCl, 50 mM NaF, 0.5% NP-40, pH 7.5) with protease inhibitors to prepare whole-cell lysates. Lysates were mixed with the appropriate antibodies and incubated at 4°C for 2 h, followed by the addition of protein G-agarose beads to pellet the immune complexes. Immunoprecipitants and 5% of lysates were analyzed by immunoblotting for the indicated proteins.

For cytoplasmic and nuclear protein extraction, cell pellets were first re-suspended in 400 μl ice-cold Buffer I (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 25 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitors, pH 8.0) and incubated on ice for 15 min. NP-40 (10%) was then added to a final concentration of 1%, and after a 10-second vortex the samples were centrifuged at top speed for 2-3 min. The supernatants were collected as the cytoplasmic fraction. The nuclear pellets were re-suspended in 200 μl ice-cold Buffer II (20 mM HEPES, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, 25

mM NaF, 1 mM Na$_3$VO$_4$, 1 mM PMSF, and protease inhibitors, pH 8.0) and rotated vigorously at 4°C for 30 min. Samples were centrifuged at 4°C at top speed for 10 min, with the supernatant being the nuclear fraction. Lysate, 200 μl, was mixed with the indicated antibodies and incubated at 4°C for 2 h, followed by the addition of protein G-agarose beads to pellet the immune complexes. The immunoprecipitants and 5% of the lysates were analyzed by immunoblotting for the indicated proteins.

Luciferase Assay- HEK293T or MCF-7 cells were plated in 24-well plates the day before transfection. 0.2 μg of reporter plasmid TOP/FOP-Flash-Luc or pGL3/LEF-1-luc together with 5 ng of the internal control plasmid pRL-TK were transfected per well. 0.4 μg of plasmids for the expression of p15RS, β-catenin, β-catenin*, Wnt1 or empty vector were co-transfected as indicated per well. 24-36 hours after transfection, reporter gene activity was assayed using the Dual Luciferase Assay System (E1910; Promega).

Chromatin Immunoprecipitation (ChIP) Assay- A modified protocol from Upstate Biotechnology (http://www.upstate.com) was used. Briefly, cells were fixed at 37°C for 10 min with 1% formaldehyde for cross-linking. The cells were re-suspended in 300 μl ChIP lysis buffer and mixed at 4°C and then sonicated for 30 sec at level 2 (Ultrasonic Processor, Sonics) to yield DNA fragments that were 100 to 500 bp in size. Eluted DNA was recovered with QIAquick columns (QIAGEN) and used as templates for PCR amplifications. The input control was from the supernatant before precipitation. Primers used

for the *Cyclin D1* promoter were: 5'-CACCTCCACCTCACCCCCTAAATCC-3' and 5'-ACTCCCCTGTAGTCCGTGT GACGTT-3'. A 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 Quantscript RT Kit (TIANGEN Biotech, Beijing, China). Real-time PCR was performed using a RealMasterMix (SYBR Green) kit (TIANGEN Biotech, Beijing, China) at the condition: denature, 95°C, 20 sec; annealing, 58°C, 20 sec; extension, 68°C, 30 sec. Primers used for the human *c-Myc* gene were: 5'-TGGTCGCCCTCCTATGTT G-3' and 5'-CCGGGTCGCAGATGAAACT C-3'. Primers used for the human *Cyclin D1* gene were: 5'-CCGAGAAGCTGTGCATCTA CAC-3' and 5'-AGGTTCCACTTGAGCTT GTTCAC-3'. Primers used for the human *p15RS* gene were: 5'-GCTCTGTATGGTGA TAAG-3' and 5'-TCTTGTAATGCTCTAAC G-3'. Primers used for the human *β-Actin* gene were: 5'-GCAAGCAGGAGTATGACG AG-3' and 5'-CAAATAAAGCCATGCCAA TC-3'.

Immunofluorescence Staining and Microscopy- HeLa cells were cultured in 6-well plates with 8 x 10^4 cells per well (Corning Incorporated, Corning, NY). Cells were transfected with the indicated plasmids and fixed after 36 h transfection with 4% paraformaldehyde for 20 min and perforated with 0.3% Triton X-100 for 10 min. The cells were blocked with 10% FBS for 50 min followed by an incubation with the c-Myc antibody at 4°C overnight. Cells were incubated with the secondary antibodies

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conjugated with fluorescein isothiocyanate (green) or goat anti-mouse IgG/TRITC antibody (Jackson Research Laboratories) for 1 h and counterstained with DAPI for 10 min. The stained cells were visualized using a confocal laser scanning microscope (OLYMPUS BX61) with the co-localization of two proteins indicated by a merged image.

Results
p15RS interacts with β-catenin or TCF4 in mammalian cells

To address whether p15RS is involved in the Wnt/β-catenin signaling pathway, we performed an immunoprecipitation experiment to examine whether p15RS forms a complex with β-catenin or TCF4. We co-expressed Myc-p15RS and Flag-β-catenin or HA-TCF4 in HEK293T cells, and immunoprecipitated the cell lysates with an anti-Myc or an anti-HA antibody. A Western blotting of the precipitates with an anti-Flag antibody indicated that Myc-p15RS was co-immunoprecipitated with Flag-β-catenin (Fig. 1A, top panel, lane 4) or HA-TCF4 (Fig. 1A, second panel, lane 5). As a control, we demonstrated that Flag-β-catenin and HA-TCF4 formed a complex under the same condition (Fig. 1A, top panel, lane 6). To confirm that the interaction of p15RS with β-catenin occurs in the nucleus, a mutant of β-catenin (β-catenin*) that has constitutive activity and is localized to the nucleus with TCF4 was used for the immunoprecipitation experiments. The results indicated that p15RS interacts with β-catenin* (Fig. 1B, top panel, lane 4), but not with Dvl2 (Fig. 1B, top panel, lane 5), a key regulator of Wnt signaling which is localized to the cytoplasm, indicating that p15RS interacts with either β-catenin or TCF4 in the nucleus of mammalian cells. To

determine whether p15RS physically binds to β-catenin and TCF4, we purified the GST-p15RS protein and performed a GST pull down experiment. The results showed that either β-catenin or TCF4 was pulled down with GST-p15RS, but not with GST (Fig. 1C, top panel, lane 4 and the second panel, lane 5). These data indicate that p15RS interacts with β-catenin and TCF4 both in vivo and in vitro.

We further examined the interaction of endogenous p15RS with β-catenin or TCF4. Co-immunoprecipitation experiments using nuclear extracts showed that the endogenous p15RS protein was precipitated down by an anti-TCF4 antibody in both HeLa and MCF-7 cells, two cell lines with quiescent Wnt signaling, but not in SW480 cells, which exhibits constitutive β-catenin accumulation in the nucleus due to a loss-of-function mutation in APC (21,22) (Fig. 1D, top panel). At the same time, an anti-β-catenin antibody precipitated down p15RS in the SW480 cells and HeLa cells but not in the MCF-7 cells (Fig. 1D, the second panel). These results firmly confirm that p15RS specifically interacts with β-catenin or TCF4 under physiological conditions.

We next examined whether p15RS and β-catenin or TCF4 co-localize in intact cells. Immunostaining showed that p15RS localizes to the nucleus (Fig. 1E, green), consistent with observations from a previous study (23). Since β-catenin translocates into the nucleus upon Wnt stimulation, we treated cells with Wnt1 and observed the location of p15RS and β-catenin. The results indicate that p15RS co-localized with β-catenin in the nucleus upon Wnt1 stimulation (Fig. 1E, merged). In line with this observation, we further demonstrated that p15RS co-localized with TCF4 in the nucleus, but not with Dvl2 which

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is in the cytoplasm (Fig. 1F). These results confirm that p15RS interacts with β-catenin or TCF4, but not with Dvl2.

The RPR domain of p15RS is required for the interaction with β-catenin or TCF4

p15RS has a RPR domain, which is involved in the regulation of nuclear pre-mRNA (24,25), and CCT domain (Fig. 2A). To determine which domain is involved in the interaction of p15RS with β-catenin, we co-expressed Flag-tagged β-catenin and deletion mutants of p15RS in HEK293T cells. Immunoprecipitation experiment results showed that the RPR domain (Fig. 2B, top panel, lane 3) but not the CCT domain (Fig. 2B, top panel, lane 4) interacted with β-catenin. Using the same deletions, we demonstrated that the RPR domain also interacted with TCF4 (Fig. 2C). These experiments suggest that the RPR domain contributes to the interaction with both β-catenin and TCF4.

Reciprocally, we attempted to determine which domain of β-catenin is critical for the interaction with p15RS. For this purpose, an immunoprecipitation experiment was performed by using different deletions of β-catenin. The results showed that the mutants C4 (543-781), N3 (1-407) and N4 (1-265) failed to interact with p15RS while the other deletion mutants retain a strong interaction (Fig. 2D), suggesting that the aa407–533 region of β-catenin, which covers the armadillo repeats 6–8, a critical region for LEF/TCF binding (26,27), was essential for the interaction with p15RS. Furthermore, we mapped the domain of TCF4 for the interaction with p15RS and found that the N-terminus (amino acids 1–200) of TCF4, which is the binding site for β-catenin (28,29),

accounted for the binding to p15RS (Fig. 2E). All of these data indicate that TCF4 and β-catenin bind to the RPR domain of p15RS, and that p15RS associated with β-catenin at its TCF4 binding site while p15RS associated with TCF4 at its β-catenin binding site, implying a possibility that p15RS competes with β-catenin to bind to TCF4.

p15RS significantly inhibits the canonical Wnt/β-catenin signaling pathway

To examine the role of p15RS on Wnt signaling, we determined whether p15RS has an effect on Wnt-mediated transcriptional activity as measured by the luciferase reporter TOP/FOP-Flash-Luc, a reporter responding to canonical Wnt signaling. The results showed that Wnt1 stimulated reporter activity 3-5 fold but that over-expression of Myc-p15RS inhibited luciferase activity in both HEK293T and MCF-7 cells (Fig. 3A). These data suggest that p15RS inhibits Wnt1-induced transcriptional activity.

To determine whether the inhibitory role of p15RS on Wnt-mediated transcriptional activity is dependent upon β-catenin, we co-expressed p15RS with β-catenin. Results from a luciferase experiment with TOP/FOP-Flash-Luc reporter showed that p15RS reduced β-catenin mediated luciferase activity dramatically in both HEK293T and MCF-7 cells (Fig. 3B). We observed a similar inhibitory effect of p15RS on a LEF-1/Luc reporter (Supplementary Fig. S1A, S1B). These results suggest that the over-expression of p15RS inhibits the transcriptional activity of β-catenin/TCF4.

Since the RPR domain of p15RS is critical for the interaction with both β-catenin and TCF4 (Fig. 2), we speculated that the RPR domain might be responsible for the inhibitory

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role of p15RS on Wnt-mediated transcriptional activity. A luciferase reporter experiment result showed that the expression of the RPR domain reduced Wnt1 mediated luciferase activity significantly in HEK293T cells, but the expression of the CCT domain failed to do so (Fig. 3C). These results suggest that the RPR domain of p15RS is critical for the inhibition of the transcriptional activity of β-catenin/TCF4.

To examine whether endogenous p15RS plays a role in Wnt signaling, we generated an siRNA to deplete the expression of p15RS in MCF-7 cells. A Western analysis showed that the p15RS siRNA reduced the expression of p15RS, but had no effect on endogenous β-catenin or TCF4 protein levels (Fig. 3D). Using the TOP/FOP-Flash-Luc reporter, we observed that Wnt1-stimulated luciferase activity was dramatically enhanced in three clones of cells with depletion of p15RS (Fig. 3E). In line with this result, we further observed that the depletion of p15RS significantly enhanced β-catenin mediated transcriptional activity (Fig. 3F). We obtained similar results with the LEF-1/Luc reporter (Supplementary Fig. S1C, S1D). To demonstrate the specificity of the siRNA targeting p15RS, we generated an siRNA resistant mutant of p15RS with three nucleotides changed for a rescue experiment. The data showed that the siRNA blocked the effect of wild type p15RS on Wnt signaling but had no effect on the inhibitory role of the siRNA resistant mutant of p15RS, indicating a specific role of the siRNA that targeted p15RS. Taken together, these data indicate that p15RS is a negative regulator of Wnt signaling.

p15RS inhibits cell growth and Wnt targeted

gene expression

To examine the role of p15RS on cell growth, we performed a cell proliferation experiment by stably over-expressing or depleting p15RS in MCF-7 cells. The results of these experiments showed that the over-expression of p15RS inhibited (Fig. 4A) but the depletion of p15RS enhanced (Fig. 4B) cell proliferation upon Wnt1 stimulation. At the same time, we stably over-expressed or depleted p15RS in SW480 cells and found that over-expression of p15RS inhibited but depletion of p15RS enhanced cell proliferation (Supplementary Fig. S3, A-D). All of the cell proliferation experiments indicate that p15RS inhibits Wnt-stimulated cell proliferation. Consistent with the cell growth experiment, we observed that over-expression of p15RS inhibited, and depletion of p15RS enhanced, the expression of *Cyclin D1* and *c-Myc*, two Wnt1-targeted genes, upon Wnt1 stimulation, both at the mRNA (Fig. 4C) and protein levels (Fig. 4D). These data suggest that p15RS inhibits cell growth by blocking Wnt-targeted gene expression.

p15RS blocks the β-catenin/TCF4 complex formation

To unveil the mechanism of the inhibitory role of p15RS on the transcription of Wnt targeted genes, we investigated whether the interaction of p15RS with β-catenin or TCF4 is dependent upon Wnt signaling. Our data demonstrated that the interaction of p15RS with β-catenin was increased with Wnt1 stimulation (Fig. 5A), while the interaction of p15RS with TCF4 was decreased when Wnt1 was added (Fig. 5B). Since β-catenin and TCF4 form a complex upon Wnt stimulation, we examined whether p15RS affects the

formation of the β-catenin/TCF4 complex. An immunoprecipitation experiment indicated that the interaction of β-catenin and TCF4 was enhanced in p15RS depleted cells while it was decreased in p15RS over-expressing cells (Fig. 5C). Since we have observed that the RPR domain of p15RS is critical for the interaction of p15RS with both β-catenin and TCF4 (see Fig. 2), we questioned whether the RPR domain of p15RS affects the formation of the β-catenin/TCF4 complex. Our immunoprecipitation results showed that expression of the RPR domain disrupted, to an extent similar to full length p15RS, the association of the β-catenin/TCF4 complex, while the expression of the CCT domain had no effect on the interaction of β-catenin with TCF4 (Fig. 5D). These data suggest that the RPR domain is required for p15RS to block the interaction of β-catenin and TCF4. Taken together with our observation that p15RS, via its RPR domain, interacts with β-catenin at its TCF4 binding domain and with TCF4 at its β-catenin binding site (Fig. 2), we propose that p15RS competes with TCF4 for an association with β-catenin.

To investigate whether p15RS blocks β-catenin/TCF4 complex formation on the promoters of Wnt-target genes, we performed a ChIP assay using a TCF4 binding sequence from a native *Cyclin D1* promoter. The data showed that the association of β-catenin with the promoter sequence was suppressed in p15RS over-expressing cell lines, but was increased in p15RS depleted cell lines (Fig. 5E, upper panel). Under the same conditions, we observed that the binding of TCF4 to the promoter sequence remained unchanged in either p15RS over-expressing or depleted cells (Fig. 5E, lower panel). In line with these observations, we found that the association of

p15RS to the promoter sequence was decreased upon Wnt1 stimulation (Fig. 5F), suggesting that p15RS is released from the TCF4 binding sequence of the promoter when Wnt signaling is activated.

TCF4 transactivates gene expression by forming a transcriptional complex, in which TCF4 provides the DNA-binding moiety and β-catenin contributes a transactivation ability (28,29). To further evaluate whether p15RS-mediated suppression of TCF4 activity is due to the inhibition of TCF4 transcription factor complex formation, we fused the VP16 transactivation domain with TCF4 and performed a TOP/FOP-Flash-Luc reporter assay. The results showed that p15RS inhibited β-catenin-mediated but not VP16-TCF4-mediated transactivation (Fig. 5G), suggesting that p15RS impairs the transactivation of β-catenin while having no effect on TCF4 DNA binding affinity. These data support the results from the ChIP experiments as shown in Fig. 5E. Consistent with these observations, we further found that p15RS interacted with LEF-1, a factor homologous to TCF4, and impaired the interaction of β-catenin and LEF-1 (Supplementary Fig. S2B and S2C). As a negative control we demonstrated that p15RS did not interact with Chibby, another protein that interacts with β-catenin (Supplementary Fig. S2A). Taken together, all these data suggest that p15RS inhibits Wnt-targeted gene transcription by blocking the formation of complex of β-catenin and TCF4.

Discussion

Canonical Wnt signaling regulates gene expression in the nucleus via the activation of the TCF4/LEF-1 complex by β-catenin, which is translocated from the cytoplasm into the

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nucleus. β-catenin has been well documented to form a complex with TCF4/LEF-1, which then recruits the transcription factors Brg1 and CBP to initiate Wnt-targeted gene expression. Many factors have been identified that interact with the β-catenin/TCF4/LEF-1 complex which includes a group of factors, including TATA binding protein (30), Pontin52 (31), Bcl-9/Legless and Pygopus (15,32), that function as co-activators to enhance transcriptional activity, while a second group of factors, including ICAT (33), Sox9 (34), Chibby (35), NLK (Nemo-like kinase) (36), NARF (37) and APC (adenomatous polyposis coli) (38), inhibit the activity of the β-catenin/TCF4/LEF-1 complex. The mechanisms of action of the negative regulators of the β-catenin/TCF4/LEF-1 complex are diverse. For example, NLK (Nemo-like kinase) phosphorylates TCF4 to mediate the dissociation of the β-catenin/TCF4/LEF-1 complex from DNA (36). NARF, a Nemo-like kinase (NLK)-associated ring finger protein mediates the ubiquitination and degradation of TCF4/LEF-1 (37). ICAT (inhibitor of catenin) inhibits the interaction of β-catenin with TCF4 (33). Chibby, a nuclear β-catenin-associated antagonist of Wnt/Wingless pathway, competes with LEF-1 for binding with β-catenin (35). APC enhances the ability of CtBP to inhibit the transcriptional activity of the β-catenin/TCF4/LEF-1 complexes (38). Sox9 interacts with β-catenin to promote the phosphorylation and translocation of β-catenin from the nucleus (34). In this study, we identified p15RS as a new nuclear modulator of the Wnt signaling pathway. We showed that p15RS interacts with β-catenin or TCF4, but not with Dvl2. Intriguingly, the

interactions of p15RS with β-catenin and TCF4 differ substantially upon Wnt signaling. In the presence of Wnt1, we observed that the interaction of p15RS with β-catenin increased but that the interaction of p15RS with TCF4 decreased. At the same time, the presence of p15RS inhibits the interaction of β-catenin with TCF4. These results indicate that p15RS inhibits Wnt signaling via the inhibition of the formation of the β-catenin/TCF4 complex. Since p15RS is constitutively located in the nucleus, we propose that p15RS is an intrinsic factor that inhibits TCF4-mediated transcription when cells are in a quiescence condition (without Wnt stimulation). When the canonical Wnt signaling pathway is activated, β-catenin accumulates in the nucleus and associates with p15RS resulting in the disruption of the interaction of p15RS with TCF4 (removing p15RS), thereafter, the β-catenin/TCF4 complex maintains active binding with promoters of target genes to initiate gene transcription (Fig. 6). This model explains how the over-expression of p15RS results in the inhibition of Wnt signaling and the decreased interaction of β-catenin with TCF4.

A previous report showed that the knockdown of p15RS in MLIK6 cells up-regulates the expression of *Cyclin D1* (19), indicating that p15RS inhibits *Cyclin D1* expression. Since *Cyclin D1* is a direct target gene transactivated by the β-catenin/LEF-1 complex through a LEF-1 binding site in its promoter (20), we speculated that p15RS may negatively regulate *Cyclin D1* expression via the β-catenin/LEF-1 complex. In this study, we provide several lines of evidence to show that p15RS functions as a negative regulator of the canonical Wnt signaling pathway. First, we observed that the over-expression of

p15RS markedly suppresses Wnt–induced transcriptional activity (Fig. 3A, 3B). Next, depletion of endogenous p15RS expression, using an siRNA, results in increased Wnt-induced transcriptional activity (Fig. 3E-G). Importantly, we found that p15RS inhibits the Wnt signaling dependent expression of *Cyclin D1* and *c-Myc* (Fig. 4). In agreement with an inhibitory role on the expression of Wnt-induced genes (*Cyclin D1* and *c-Myc*), we further observed that the over-expression of p15RS inhibits cell proliferation and depletion of p15RS enhances cell proliferation. All of these results consistently suggest that p15RS is an intrinsic factor that inhibits Wnt signaling.

The canonical Wnt signaling has been linked to several tumors (2,4). In this study, we found that p15RS negatively regulates Wnt signaling and thereafter inhibits the expression of Cyclin D1 and c-Myc, two genes critical for cell proliferation. Our study implies that p15RS may be a tumor suppressor. Indeed, our data showed that overexpression of p15RS retards, while depletion of p15RS accelerates, cell growth (see Fig. 4 and Supplementary Fig. S3). This result is in consistency with the notion that p15RS functions as a negative regulator in G1 phase in cell cycle control (19). We speculate that p15RS may play a role in tumorigenesis.

We revealed that a mechanism by which p15RS inhibits Wnt signaling is via the interruption of the association of the β-catenin/TCF4 complex. It remains unclear how p15RS functions in the complex disassociation. Our data demonstrate that p15RS forms a complex with TCF4 in the absence of Wnt signaling. When β-catenin accumulates upon Wnt signaling, β-catenin interacts with p15RS. This interaction results

in the decreased interaction of p15RS with TCF4 and an increase in the interaction of β-catenin with TCF4. Interestingly, we observed that the RPR domain is critical for the interaction of p15RS with both β-catenin or TCF4, since the deletion mutant of p15RS that lacks the RPR domain failed to interact with β-catenin or TCF4. We propose that the RPR domain of p15RS is critical for the interaction of β-catenin and TCF4. Since the RPR domain interacts with β-catenin at its TCF4 binding site and with TCF4 at its β-catenin binding site, we suggest that the RPR domain provides the mechanism for the competition of p15RS between β-catenin and TCF4. This competition results in an exclusive association of p15RS with β-catenin or with TCF4. Our data also indicate that p15RS blocks the binding of the β-catenin/TCF4 complex to the *Cyclin D1* gene promoter. The p15RS interruption of the association between β-catenin and TCF4 protein (Fig. 5D) does not affect the binding of TCF4 to the DNA as demonstrated by the VP16-TCF4 fusion protein (Fig. 5G). Therefore we speculate that p15RS blocks the transcriptional activity of TCF4 without affecting the binding of TCF4 to DNA. Since the RPR domain has only been previously reported to function in mRNA processing (24,25), our study provide new evidence for the functions of this domain in interrupting the formation of a complex between β-catenin and TCF4.

Another question to be addressed is whether p15RS, β-catenin and TCF4 form a complex. Our data indicates that p15RS interacts with either β-catenin or TCF4, however we did not find evidence for a complex of p15RS/β-catenin/TCF4. Based on the fact that the over-expression of p15RS

diminished the formation of the β-catenin/TCF4 complex, we concluded that p15RS does not form a stable complex with β-catenin/TCF4. This suggestion fits our model where p15RS competes withTCF4 for binding with β-catenin and decreases the amount of the β-catenin/TCF4 complex. Intriguingly, in the SW480 cell line, where Wnt signaling is constitutive active, we observed an interaction of p15RS with β-catenin but could not observe an interaction of p15Rs with TCF4, in contrast to Wnt signaling quiescent cells (such as HeLa and MCF-7), where we observed an interaction of p15RS with TCF4. Interestingly the interaction of p15RS with β-catenin in the Wnt signaling quiescent cells is very weak (HeLa) or undetectable (MCF-7). In line with the data from the over-expression of p15RS

on the interaction of β-catenin or TCF4, we propose that p15RS blocks the interaction of β-catenin with TCF4 and that a stable p15RS/β-catenin/TCF4 complex is not formed. Indeed, we failed to demonstrate the existence of a p15RS/β-catenin/TCF4 complex by IP plus re-IP experiments (data not shown).

In conclusion, we identified p15RS as a new nuclear negative regulator of the Wnt signaling pathway. This discovery extends our understanding of regulation of Wnt signaling by negative regulators. Given the well-established roles of Wnt signaling in various diseases, including cancers and degenerative diseases, we expect that our findings on the functional interaction of p15RS with TCF4 and β-catenin will provide useful information for the development of effective therapies against these disorders.

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Figure Legends

Figure 1. Interactions of p15RS with β-catenin or TCF4
(A) p15RS interacts with β-catenin or TCF4 in HEK293T cells. Cells were co-transfected with the indicated plasmids. Immunoprecipitation was performed using an anti-Myc or anti-HA antibody and the precipitants were detected using an anti-Flag or anti-HA antibody. (B) p15RS interacts with β-catenin*, but not with Dvl2. HEK293T cells were co-transfected with the indicated plasmids. Antibodies used for immunoprecipitation and Western blotting are shown. (C) p15RS interacts with β-catenin or TCF4 in vitro. A GST–pull-down assay was performed with purified GST or GST-p15RS protein and Flag-β-catenin or HA-TCF4 protein expressed in HEK293T cells. (D) Endogenous p15RS interacts with β-catenin or TCF4. Nuclear extracts from SW480, HeLa and MCF-7 cells were immunoprecipitated with an anti-β-catenin and an anti-TCF4 antibody. The precipitants were visualized with an anti-p15RS antibody. (E) p15RS co-localizes with β-catenin in the nucleus upon Wnt stimulation. HeLa cells expressing Myc-p15RS and Flag-β-catenin were treated with or without Wnt1 (transfection of 60 ng of Wnt1 plasmid) and the cells were stained with an anti-Myc (green) and M2 (red) antibody. The images were viewed with a confocal microscope. The co-localization of Myc-p15RS and Flag-β-catenin in the nucleus is shown as a yellow color. (F) p15RS co-localizes with TCF4 in the nucleus. HeLa cells expressing Myc-p15RS with HA-TCF4 or Flag-Dvl2 (as a negative control) were stained and viewed with a confocal microscope. *Scale bar*, 10 μm.

Figure 2. Mapping the p15RS domains interacting with β-catenin or TCF4
(A) Schematic illustration of p15RS and its domains. RPR, regulation of nuclear pre-mRNA (or CID, CTD-Interacting Domain), and CCT, coiled-coil terminus, are presented. (B) The RPR but

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not the CCT domain interacts with β-catenin. Full-length and two domains of p15RS were co-expressed with Flag-β-catenin. Immunprecipitation was performed using an anti-Myc antibody. Precipitants were detected with an anti-Flag antibody. (C) The RPR but not the CCT domain interacts with TCF4. Full-length and two domains of p15RS were co-expressed with HA-TCF4. Immunprecipitation was performed using an anti-Myc antibody. Precipitants were detected with an anti-HA antibody. (D) p15RS associates with the armadillo repeats 6–8 of β-catenin. HEK293T cells co-transfected with Myc-p15RS and HA-β-catenin deletion mutants. The lysate were subjected to immunoprecipitation using an anti-HA antibody. Precipitants were detected with an anti-Myc antibody. Different deletions of β-catenin are shown in the top panel. (E) p15RS interacts with the N-terminal of TCF4. HEK293T cells co-transfected with Myc-p15RS and HA-TCF4 deletion mutants. Lysates were subjected to immunoprecipitation using an anti-HA antibody. Precipitants were detected with an anti-Myc antibody. Different deletions of TCF4 are shown in the top panel. CTNNB1 is the β-catenin binding domain and HMG is the DNA-binding domain.

Figure 3. p15RS inhibits the canonical Wnt/β-catenin signaling pathway
(A) p15RS inhibits Wnt1-stimulated transcriptional activity. Luciferase assays were performed using HEK293T cells with transient expression of Myc-p15RS or in MCF-7 cells with stable expression of Myc-p15RS, transfected with a TOP/FOP-Luc Flash reporter and pRL-TK (as an internal control). Wnt1 expression was generated by the transfection of 10 ng of a Wnt1 plasmid. Relative luciferase activities were normalized with the internal control. Results are presented from three independent experiments. (B) p15RS inhibits β-catenin-mediated transcriptional activity. The same cells were used for transfection of β-catenin. (C) The RPR domain inhibits Wnt1-mediated transcriptional activity. (D) p15RS siRNA effectively depletes the expression of endogenous p15RS. A siRNA targeting p15RS was selected to generate three stable cell lines in MCF-7 cells. The endogenous p15RS, β-catenin and TCF4 protein levels were examined by Western blot analysis. (E) Depletion of p15RS enhances Wnt-stimulated transcriptional activity. Luciferase assays were performed using the stable siRNA-p15RS MCF-7 cells transfected with the indicated vectors along with a TOP/FOP-Luc Flash reporter and pRL-TK (internal control). (F) p15RS siRNA increases β-catenin-mediated transcriptional activity. The experiment was done similar to that in (E) except that β-catenin was co-expressed. (G) p15RS siRNA had no effect on the inhibitory role of siRNA-resistant p15RS in Wnt1-mediated transcriptional activity. A siRNA-resistant p15RS was used to show the specificity of the siRNA targeting to p15RS in a lucieferase experiment.

Figure 4. p15RS inhibits cell growth and Wnt targeted gene expression
(A) Over-expression of p15RS inhibits cell growth. Cell proliferation experiments were performed using MCF-7 cells stably over-expressing p15RS, that were stimulated with or without Wnt1 (transfection of 200 ng of Wnt1 plasmid). The seeding densities of cells were 1500 cells/well in a 96-well plate. OD570 was measured to assess cell numbers. A T-test was performed to detect statistical difference between the two groups with ** indicating $p < 0.01$. (B)

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Depletion of p15RS by siRNA promotes cell growth. Stable depletion of p15RS in MCF-7 cells was examined for its affect on cell growth under the stimulation of Wnt1 (transfection of 10 ng of Wnt1 plasmid). Results shown are a representative of three independent experiments. A T-test was performed between the two groups with ** indicating p<0.01. (C) p15RS inhibits Wnt-targeted gene expression. mRNA levels of Wnt-targeted genes, *Cyclin D1* and *c-Myc*, were examined by real-time RT-PCR in MCF-7 cells with stable over-expression or depletion of p15RS. Individual clones (1#, 2#, C2) and a pool of these cells (indicated as P) were used. (D) A Western blot for the protein levels of Wnt-target genes. Protein levels of c-Myc and Cyclin D1 were examined by a Western blot for the cells used in (C).

Figure 5. p15RS blocks β-catenin/TCF4 complex formation

(A) The interaction of p15RS and β-catenin is enhanced by Wnt stimulation. HEK293T cells expressing Myc-p15RS and Flag-β-catenin with or without Wnt1 (transfection of 200 ng of Wnt1 plasmid) were immunoprecipitated with an anti-Myc antibody, and the precipitants were detected using an anti-Flag antibody. (B) The interaction of p15RS with TCF4 is decreased upon Wnt stimulation. Myc-p15RS and HA-TCF4 were expressed in HEK293T cell with or without Wnt1 (transfection of 200 ng of Wnt1 plasmid). Cell lysates were immunoprecipitated with an anti-Myc antibody, and the precipitants were detected using an anti-HA antibody. (C) p15RS disrupts the β-catenin/TCF4 association upon Wnt1 stimulation. The complex of β-catenin and TCF4 was examined by an immunoprecipitation experiment using the M2 antibody (for Flag-β-catenin) and an anti-HA antibody (for Western blotting to HA-TCF4) in MCF-7 cells stably over-expressed (Myc-p15RS) or depleted (siRNA) for p15RS. (D) The RPR domain of p15RS is responsible for the inhibition of the β-catenin/TCF4 association. A co-immunoprecipitation experiment was done in HEK293T cells co-transfected with Wnt1, Flag-β-catenin and HA-TCF4 as well as the wild type (WT) or indicated p15RS domains (RPR or CCT). (E) p15RS impairs β-catenin/TCF4 binding to the *Cyclin D1* promoter upon Wnt stimulation. ChIP assays were performed in the indicated cell lines transfected with HA-TCF4 and Flag-β-catenin. PCR was performed for both the precipitated complexes and the lysates (as inputs). The upper panels indicate the association of β-catenin/TCF4 with the promoter. The lower panels present total TCF4 binding to the promoter. (F) The association of p15RS on the promoter sequence is decreased upon Wnt1 stimulation. A ChIP assay was performed in HEK293T cells expressing Myc-p15RS with or without Wnt1 (transfection of 200 ng of Wnt1 plasmid). An antibody against Myc was used to precipitate the promoter sequence. A result of real-time PCR of the ChIP experiment is shown on right panel. (G) p15RS did not affect VP16-TCF4-mediated transactivation. Luciferase assays were performed using HEK293T cells with transient co-expression of Myc-p15RS with β-catenin or VP16-TCF4, and transfected with a TOP/FOP-Luc Flash reporter and pRL-TK (as an internal control). Relative luciferase activities were normalized to the internal control. Results are presented from three independent experiments.

Figure 6. A model to demonstrate the inhibitory role of p15RS on the canonical Wnt signaling pathway

In the quiescent state (without Wnt), p15RS interacts with TCF4 on the promoters of Wnt-target genes and inhibits transcription. When Wnt signaling is activated, β-catenin translocates into the nucleus to interact with p15RS thereafter removing p15RS from its association with TCF4, allowing β-catenin/TCF4 binding to the promoter to initiate gene transcription.
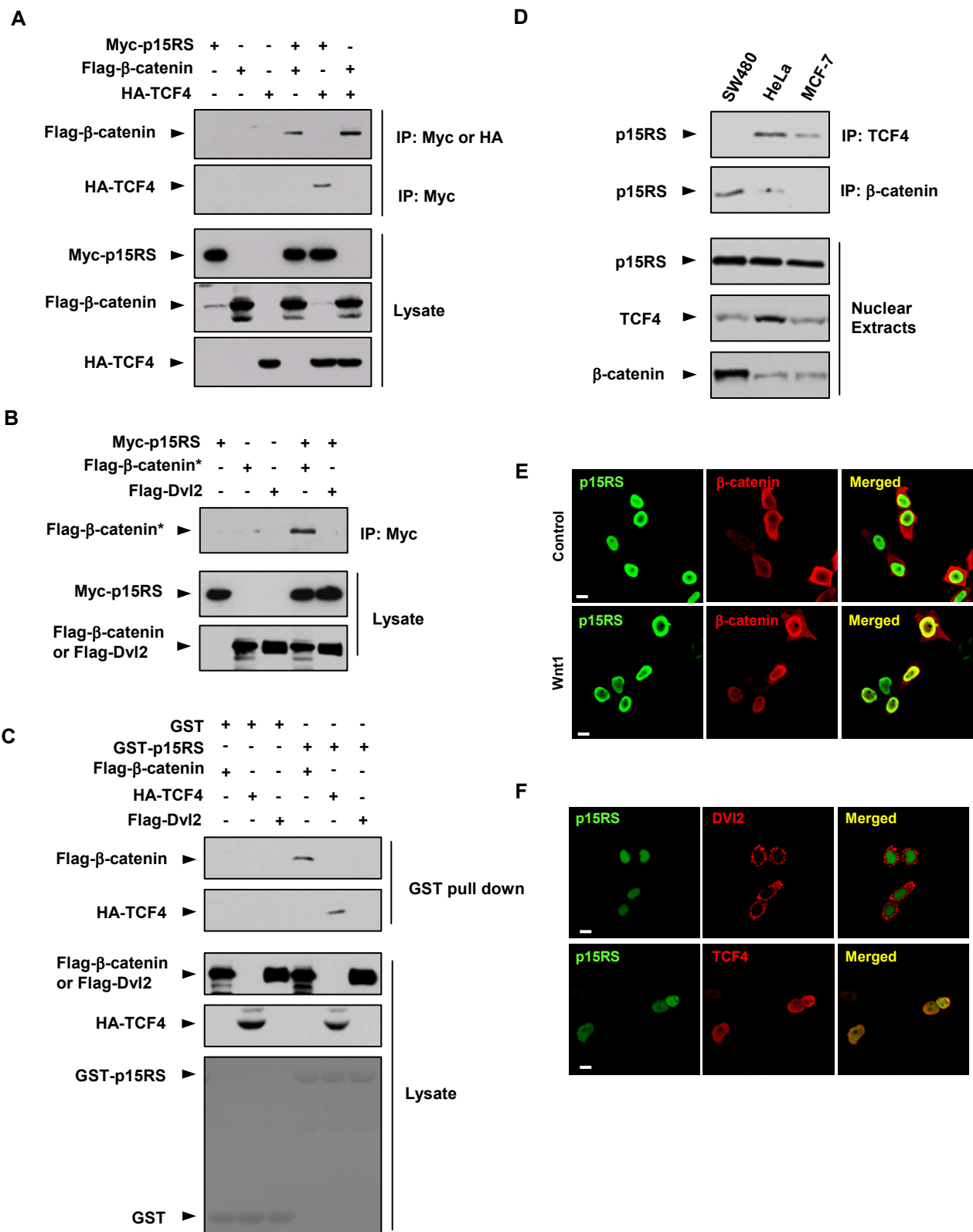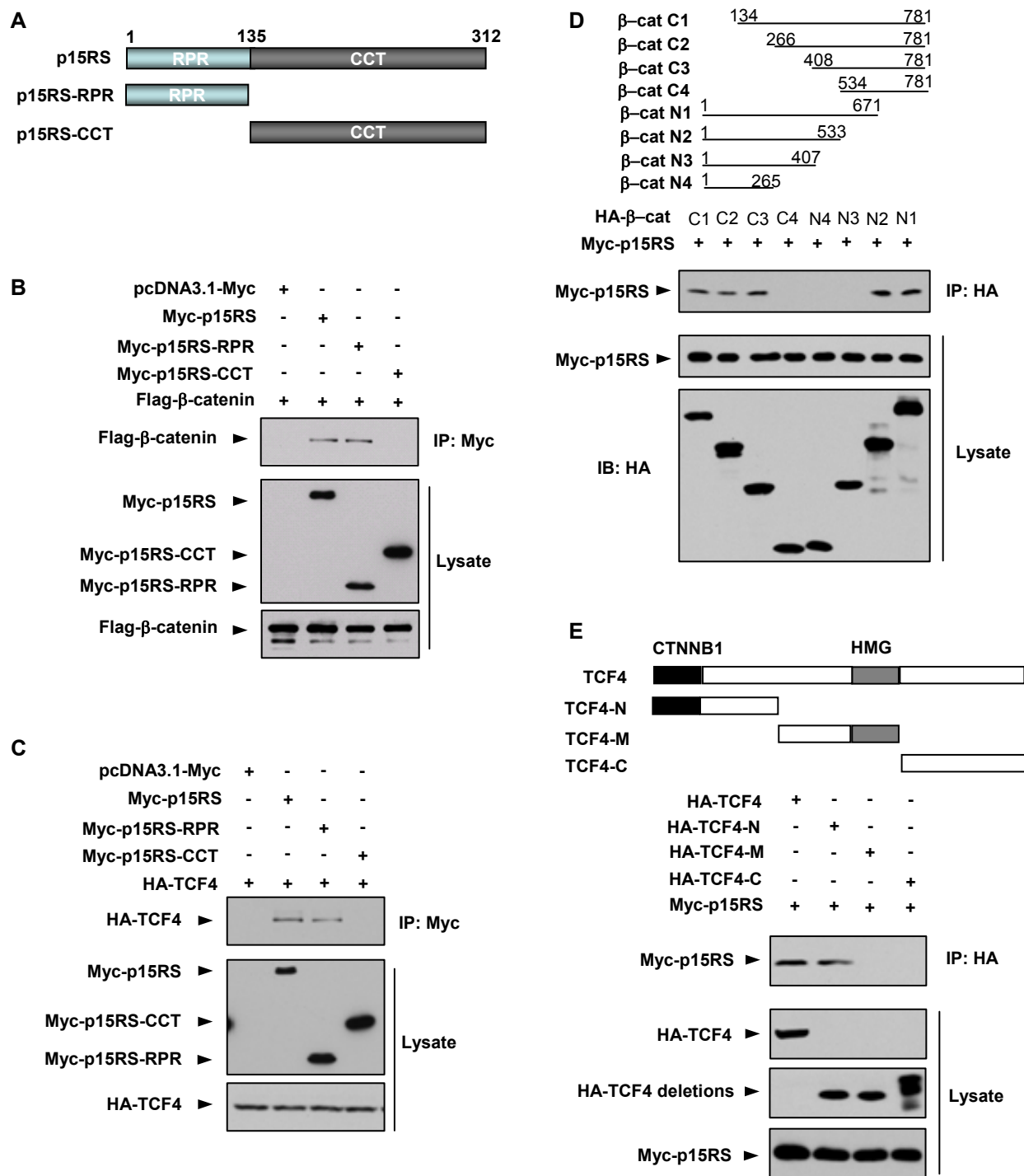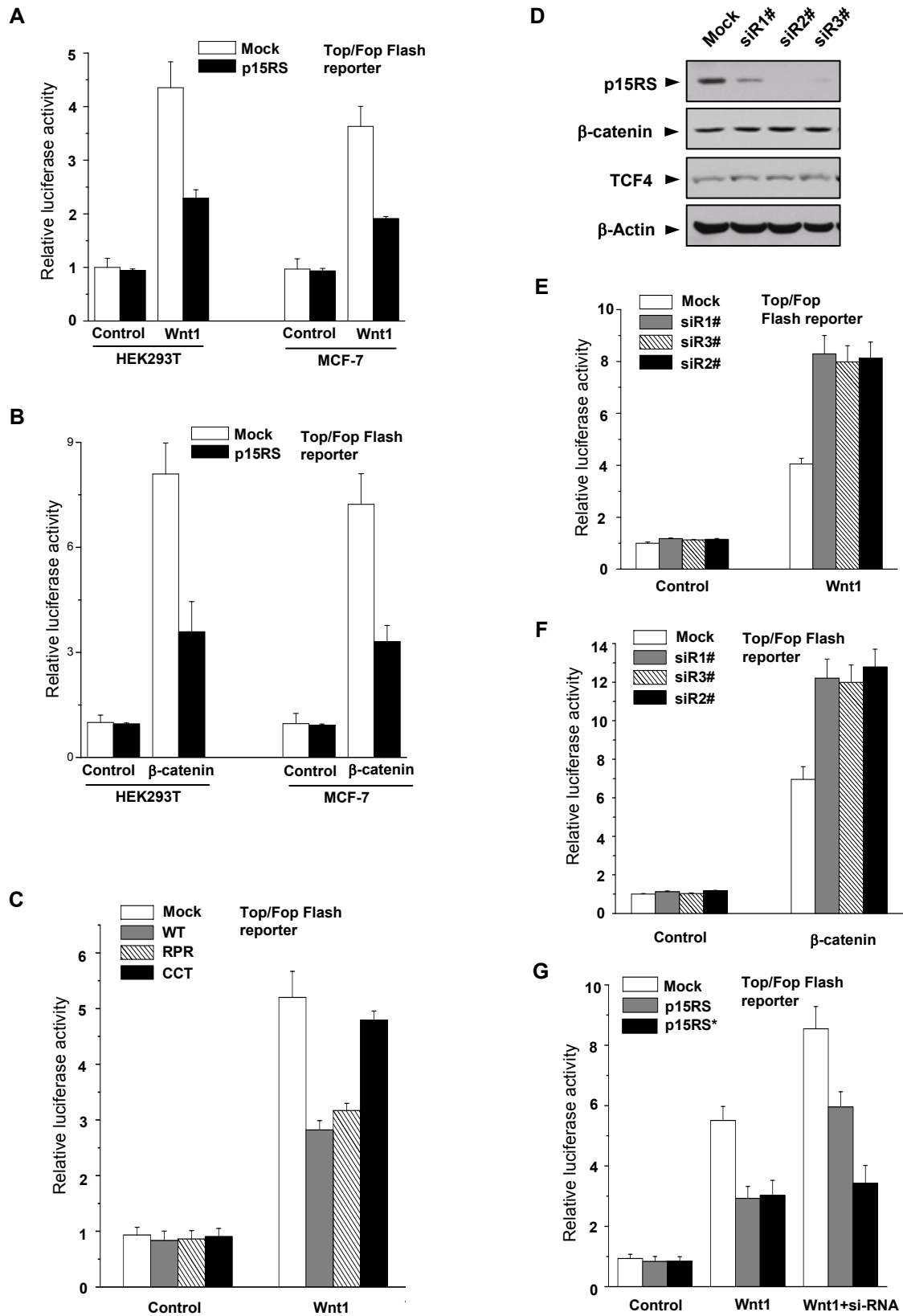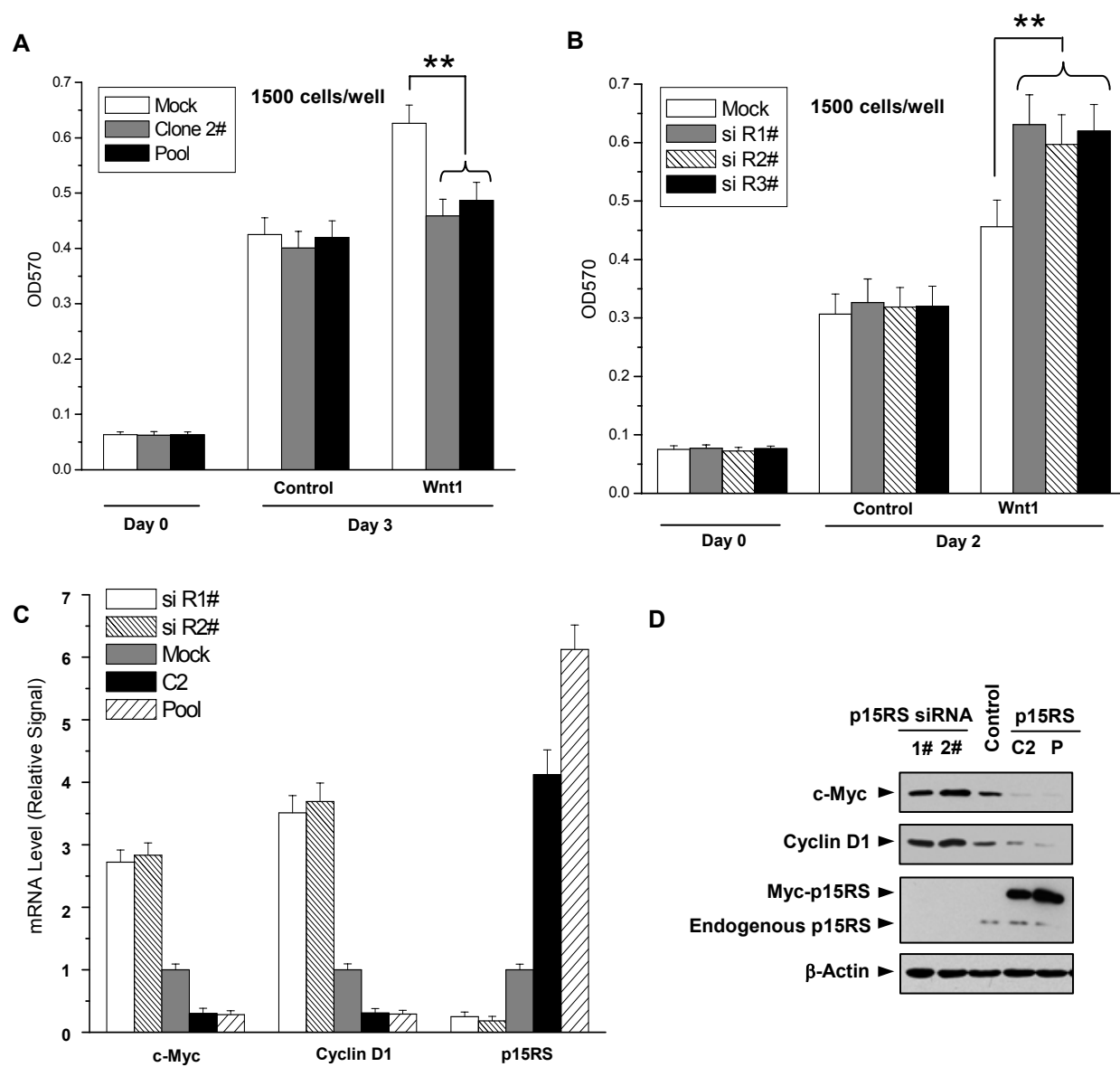
Fig. 1

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