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Dimerization of p15RS mediated by a leucine-zipper-like motif is critical for its inhibitory role on Wnt signaling

Xuanzi Fan[1,2#], Juan Zhao[1#], Fangli Ren[1], Yinyin Wang[1], Yarui Feng[1], Lidan Ding[1], Linpeng Zhao[3], Yu Shang[3], Jun Li[4], Jianquan Ni[1], Baoqing Jia[5], Yule Liu[2], Zhijie Chang[1*]

[1]State Key Laboratory of Membrane Biology, School of Medicine, Tsinghua University, Beijing (100084), China; [2]School of Life Sciences, Tsinghua University, Beijing (100084); [3]The Key Laboratory for Cell Proliferation and Regulation Biology of Ministry of Education, Department of Cell Biology, College of Life Sciences, Beijing Normal University, Beijing (100875), China; [4]Institute of Immunology, PLA, The Third Military Medical University, Chongqing (400038), China; [5]Department of General Surgery/Pathology, Chinese PLA General Hospital, Beijing (100853), China.

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#These authors contribute equally to the work.

* To whom correspondence should be addressed:
Zhijie Chang: State Key Laboratory of Membrane Biology, School of Medicine, Tsinghua University, Beijing (100084), China;
zhijiec@tsinghua.edu.cn; Tel. +86 10 62785076; Fax. +86 10 62773624.

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ABSTRACT

We previously demonstrated that p15RS, a newly-discovered tumor suppressor, inhibits Wnt/β-catenin signaling by interrupting the formation of β-catenin•TCF4 complex. However, it remains unclear how p15RS helps exert such an inhibitory effect on Wnt signaling based on its molecular structure. In this study, we reported that dimerization of p15RS is required for its inhibition on the transcription regulation of Wnt-targeted genes. We found that p15RS forms a dimer through a highly-conserved leucine-zipper-like motif in the CCT domain. In particular, residues L248 and L255 were identified responsible for p15RS dimerization, as mutation of these two leucines into prolines disrupted the homodimer formation of p15RS and weakened its suppression of Wnt signaling. Functional studies further confirmed that mutations of p15RS at these residues results in diminishment of its inhibition on cell proliferation and tumor formation. We therefore concluded that dimerization of p15RS governed by the leucine-zipper-like motif is critical for its inhibition of Wnt/β-catenin signaling and tumorigenesis.

Wnt signaling pathway regulates a variety of biological events including cell proliferation and organogenesis (1,2). Based on extensive studies, Wnt signaling pathway has been classified into canonical and non-canonical two categories. The canonical Wnt pathway starts from the binding of Wnt proteins as ligands to receptors Frizzled and LRP5/6, which activates Dishevelled proteins (Dvls) in the cytoplasm. Dvls bridge the recruitment of a destructive complex containing APC-Axin-GSK3β-CK1 to the receptors, allowing cytoplasmic β-catenin proteins free from degradation. The aggregated β-catenin proteins then translocate into the nucleus to interact with T cell-specific factor (TCF)/LEF and therefore initiates the transcription of Wnt-targeted genes (3). Aberrant accumulation of β-catenin in the nucleus promotes the transcription of numerous oncogenes such as *c-MYC* and *CCND1*. As a result, Wnt signaling pathway contributes to tumorigenesis of several cancers, including colon cancer (4), lung cancer (5) and melanoma (6,7) .

p15RS, also known as RPRD1A, is a *p15INK4b*-related gene discovered by a differential display experiment in cells overexpressing p15INK4b (8). Recent studies revealed that p15RS is evolutionarily homologous to yeast Rtt103 and human CREPT (9-11), whereas its function in the regulation of cell proliferation is proved to be opposite to CREPT (9,11,12). p15RS was initially observed to inhibit cell proliferation, as downregulation of p15RS expression resulted in an up-regulation of cyclin D and cyclin E in A375 cells (8). It was also found that p15RS downregulates the mRNA levels of proteins known to promote cell invasion, like cathepsin B and MMP-9 (13). Furthermore, p15RS was identified to participate in the regulation of gene transcription, as it associated with RNA polymerase II (RNAP II) (14,15). p15RS seemed to predominantly interacted with phosphorylated RNAP II, leading to the reduction of CTD S5- and S7-phosphorylation during transcription elongation (16). In particular, we have reported that p15RS attenuates the transcriptional regulation of Wnt/β-catenin signaling. p15RS

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was found to not only block the complex formation of β-catenin•TCF4 (12) but also partially recruit HDAC2 to the TCF4 binding region of targeted promoters to reduce the level of acetylated histone H3 (17).

Protein dimerization is crucial in the functional regulation of proteins such as transcription factors (18). Dimerization of transcription-associated factors facilitates the formation of multiprotein complexes required for transcription initiation by enabling proteins to bind simultaneously to different partners with overlapping binding sites (19). As these multiprotein complexes are dynamic, protein dimerization also acts as a reversible switch to control the off/on status of the information flow during signal transduction (20). Previous studies revealed that one of the most important motifs mediating protein dimerization is the leucine zipper, a parallel coiled-coil domain (21). A typical leucine zipper consists of a series of heptads, a seven-amino-acid motif, with the presence of a leucine residue in each heptad (22). Previous studies observed that the CCT (coiled-coil terminus) domain of p15RS owns a potential to form homodimer (14,15). In this study, we attempt to examine whether the dimerization of p15RS via a leucine-zipper-like motif affects the complex formation of β-catenin•TCF4 and thereafter inhibits Wnt signaling during tumorigenesis.

Results

p15RS forms homodimer via its CCT domain

Previously-resolved crystal structure suggested that the CCT domain of p15RS forms dimers (14,15). To examine whether full-length p15RS dimerizes in *vitro*, we performed a reciprocal immunoprecipitation (IP) experiment, co-expressing Myc-p15RS and Flag-p15RS in HEK293T cells. The results showed that Flag-p15RS and Myc-p15RS interacted with each other, as precipitated by either an anti-Flag (Fig. 1A) or an anti-Myc antibody (Fig. 1B). As p15RS contains a RPR (regulation of nuclear pre-mRNA) domain and a CCT (coiled-coil terminus) domain (Fig. 1C), we questioned the exact domain responsible for p15RS dimerization. For this purpose, different domains of Flag-tagged p15RS were co-expressed with full-length Myc-p15RS in HEK293T cells for an IP assay. The result indicated that the CCT domain (Fig. 1D, fifth lane) interacted with full-length p15RS but the RPR domain (Fig. 1D, fourth lane) lost this ability. Another IP experiment showed that the CCT domain formed a homodimer as well (Fig. 1E, fourth lane). These data suggested that the CCT domain of p15RS is responsible for its dimeric interaction in *vitro*.

To further confirm whether full-length p15RS forms homologous dimer, we performed formaldehyde cross-linking assays in HEK293T cells transfected with Flag-tagged full-length, RPR or CCT domain of p15RS. Western blotting analysis of the cross-linked cells transfected with full-length p15RS demonstrated the presence of an additional band of about 80 kDa, twice the size of a p15RS monomer (about 39 kDa with tag) (Fig. 1F). This result suggested that full-length p15RS dimerizes. We also observed that the CCT domain per se formed homodimers while RPR domain failed to dimerize (Fig. 1G), corresponding to the IP results. Interestingly, we observed two bands around the dimerized position when the CCT domain was expressed (Fig. 1G). We speculated that the higher band

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represented dimers of the CCT domain with the endogenous full-length p15RS and the lower band represented dimers of the homo CCT domain. This result was consistent with the observation from the IP experiments, showing that CCT domain interacted with CCT domain and full length p15RS (Fig. 1D, 1E). Taken together, all these results indicated that full-length p15RS dimerizes due to its CCT domain.

Mutation of the leucine-zipper-like motif impairs p15RS dimerization

To reveal the exact motif responsible for the homologous interaction of p15RS, we analyzed the amino acid sequence in the CCT domain. Interestingly, we identified that from residue 217 to residue 276, the amino acid sequence of p15RS shares a high similarity with leucine zipper motif, based on an alignment analysis of p15RS with typical leucine zipper family proteins including C/EBP, Jun, Fos and GCN4 (Fig. 2A) (23-25). In particular, p15RS was observed to contain 4 typical leucine zipper heptads from residue L245. This finding suggested that p15RS contains a leucine-zipper-like motif in its CCT domain.

As leucine zipper motif is well-recognized to specifically regulate protein dimerization (21), we speculated that it is this leucine-zipper-like motif within p15RS CCT domain that mediate p15RS dimerization. To clarify this, point mutations were introduced to substitute the first two heptadic leucines at residue 248 and residue 255 into prolines ($p15RS^{L248P/L255P}$) or alanines ($p15RS^{L248A/L255A}$) (Fig. 2B) (26,27). An immunostaining experiment showed that both mutants located in the nucleus, similar to the wild type p15RS (Fig. 2C), suggesting that the leucine-zipper-like motif had no effect on protein distribution.

To determine whether this leucine-zipper-like motif regulates p15RS dimerization, we performed an immunoprecipitation co-expressing wild type Flag-p15RS with Myc-tagged mutants (Fig. 2D). Intriguingly, we observed that Flag-p15RS failed to interact with Myc-$p15RS^{L248P/L255P}$ but weakly interacted with Myc-$p15RS^{L248A/L255A}$. Further GST pull-down experiments using purified proteins from either *E. Coli* (Fig. 2E) or mammalian cells (Fig. 2F) showed that $p15RS^{L248P/L255P}$ no longer interacted with wild type p15RS while $p15RS^{L248A/L255A}$ remained weak dimeric interaction in mammalian cells. A crosslinking assay strengthened these findings, showing that $p15RS^{L248P/L255P}$ lost the ability to form dimers (Fig. 2G, P), while $p15RS^{L248A/L255A}$ maintained dimerization ability when it was subjected to a sufficient formaldehyde treatment (Fig. 2G, A), consistent with the eukaryotic GST pull-down results (Fig. 2F). Taken together, these data revealed that mutations of the leucines into prolines at the leucine-zipper-like motif abolishes the dimerization of p15RS.

Homologous interaction of p15RS is critical for its inhibition on Wnt signaling

p15RS was previously reported as an intrinsic inhibitor of Wnt signaling (12). To address whether dimerization contributes to the inhibitory effect of p15RS on Wnt signaling, we examined the transcriptional activity of Wnt-targeted genes using the SuperTop-luciferase reporter. Luciferase assays in both HEK293T (Fig. 3A) and A375 cells (Fig. 3B) showed that expression of $p15RS^{L248P/L255P}$

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failed to inhibit the Wnt1-stimulated luciferase activities, while p15RS$^{L248A/L255A}$, similar to wild type p15RS, remained to suppress Wnt signaling. These results suggested that dimerization of p15RS is crucial for its inhibition on Wnt signaling pathway.

p15RS was proved to interact with β-catenin and TCF4 (12). To address whether the dimeric interaction of p15RS alters its affinity in forming complex with β-catenin or TCF4, we performed IP experiments to examine the association of TCF4, β-catenin and p15RS mutants. The results showed that the interaction of p15RS$^{L248P/L255P}$ with TCF4 was decreased comparing with the affinity of wild type p15RS with TCF4 (Fig. 3C, P vs WT). However, the interaction of p15RS$^{L248P/L255P}$ with β-catenin was dramatically increased comparing with wild type p15RS and p15RS$^{L248A/L255A}$ (Fig. 3D, P). These results suggested that dimerized p15RS (wild type) prefers to interact with TCF4 whereas p15RS monomer (L245P/L255P mutant) tends to associate with β-catenin. Consistently, a GST pull-down assay using proteins purified from eukaryotic cells confirmed that p15RS$^{L248P/L255P}$ interacted with β-catenin with a higher affinity (Fig. 3E). To further address whether dimerization of p15RS affects the complex formation of β-catenin•TCF4, we examined the interaction of β-catenin and TCF4 under over-expression of wild type or mutated p15RS. An IP result showed that the interaction of Flag-β-catenin with HA-TCF4 was recovered when p15RS$^{L248P/L255P}$ was co-expressed (Fig. 3F, P), while wild type p15RS and p15RS$^{L248A/L255A}$ decreased the interaction compared with the positive control (Fig. 3F, WT and A vs EV). This result indicated that wild type p15RS and p15RS$^{L248A/L255A}$ blocked the interaction of β-catenin and TCF4 while p15RS$^{L248P/L255P}$ failed to maintain this blockage. All these data further suggested that dimerization of p15RS participates in interrupting TCF4•β-catenin complex formation.

To investigate whether the dimerization of p15RS is required for its occupancy on promoters of Wnt-targeted genes (12), we performed ChIP assays using primers recognizing TCF4 binding sequence (TBS) within promoters of *CCND1* and *c-MYC*, two typical Wnt down-stream genes. The results showed that unlike wild type p15RS and p15RS$^{L248A/L255A}$, occupancies of p15RS$^{L248P/L255P}$ on the TBS of *CCND1* (Fig. 3G) or *c-MYC* (Fig. 3H) were weakened, suggesting that aberrant dimerization of p15RS impairs its occupancy on the promoters of Wnt targeted genes. Furthermore, we observed that both wild type p15RS and p15RS$^{L248A/L255A}$ significantly inhibited the binding of β-catenin and TCF4 on the TBS, while p15RS$^{L248P/L255P}$ failed to block their occupancy (Fig. 3I). These results suggested that dimerization of p15RS facilitates its inhibition on Wnt downstream genes transcription.

To examine whether β-catenin affects the dimerization of p15RS, we performed a GST pull-down assay using cells overexpressing GST-tagged and Myc-tagged p15RS together with β-catenin (Fig. 3J). The result showed that the presence of β-catenin decreased homo-interaction of p15RS, indicating that β-catenin might disassociate the dimerized p15RS. Further analyses revealed that RPR domain of p15RS exhibited an enhanced interaction with β-catenin comparing to full-length p15RS,

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while CCT domain failed to interact with β-catenin (Fig. S1A). We also confirmed that a region from residue 407 to 533 in β-catenin was required for its interaction with p15RS (Fig. S1B, S1C). Based on these observations, we proposed that β-catenin may compete with p15RS through its high affinity with p15RS monomer and prevent p15RS from inhibiting the transcription of Wnt-targeted genes.

Dimerization is required for p15RS to inhibit tumor cell proliferation and invasion

To address whether dimerization of p15RS affects cell growth through its participation in wnt signal pathway, we examined cell proliferation in metastatic melanoma A375 cells lines stably overexpressing pLVX-GFP-p15RS, pLVX-GFP-p15RS$^{L248P/L255P}$ or pLVX-GFP-p15RS$^{L248A/L255A}$. Right after lentivirus infection, same number of GFP positive cells among groups were seeded (Fig. 4A, top panels). Along cell passages, we were surprised to observe that the amounts of GFP-positive cells overexpressing p15RS or p15RS$^{L248A/L255A}$ decreased dramatically, whereas p15RS$^{L248P/L255P}$ overexpression exerted no inhibitory effect in cell growth, similar with the negative control cell line overexpressing pLVX vector (Fig. 4A, bottom panels). FACS analyses indicated that GFP-positive cells account for 90.56% and 73.37% in the mock and p15RS$^{L248P/L255P}$ groups but decreased to 8.61% and 8.49% in the groups overexpressing p15RS and p15RS$^{L248A/L255A}$ (Fig. 4A, FACS). Consistently, a MTT assay confirmed that p15RS$^{L248P/L255P}$ loses the ability to inhibit cell proliferation (Fig. 4B). Together, these results suggested that the ability of p15RS to inhibit cell proliferation was weakened by aberrant dimerization.

To examine whether dimerization of p15RS influences the colony formation ability of tumor cells, we performed a clone formation experiment in the A375 cells. The result showed that stable overexpression of p15RS or p15RS$^{L248A/L255A}$ inhibited clone formation but p15RS$^{L248P/L255P}$ overexpressing cells formed more colonies (Fig. 4C). A statistics analysis confirmed that the colony numbers of p15RS$^{L248P/L255P}$ cells were significantly increased in comparison with other groups (Fig. 4D). To address whether p15RS dimerization affects the invasion of tumor cells, we performed a wound healing experiment. The data showed that overexpression of p15RS$^{L248P/L255P}$ failed to inhibit cell migration (Fig. 4E, 4F). Taken together, these results suggested that p15RS loses its ability to inhibit tumor cell proliferation and invasion when its dimerization ability is broken.

Dimerization of p15RS plays a crucial role in tumor suppression

To investigate whether the dimeric ability of p15RS is involved in its inhibition on tumor growth, we performed a tumorigenesis assay by injecting A375 overexpressing cell lines into BALB/c nude mice through their armpits (Fig. 5A). The results showed that p15RS suppressed tumor formation comparing with mock cells, while p15RS$^{L248P/L255P}$ overexpressing cells exhibited a similar ability to form tumors in comparison with the mock cells (Fig. 5B). Consistently. We observed that the weight (Fig. 5C) and size (Fig. 5D) of the tumors were dramatically decreased by the overexpression of wild type p15RS, while

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restored, although not completely, by p15RS$^{L248P/L255P}$. All these results suggested that dimerization of p15RS is critical for tumor suppression.

Based on all the results, we proposed that dimerization of p15RS is critical for its down-regulation of Wnt signaling and thus for tumor suppression (Fig. 5E). Dimerization of p15RS facilitates its association with TCF4 and thereafter maintains TCF4 in an inactive status without Wnt signaling. However, when Wnt signaling is activated, nuclear β-catenin binds with p15RS monomer with increased affinity so that it may break down the dimerized p15RS. Due to decreased association between monomer p15RS and TCF4, dimerized p15RS is removed from TCF4 so as to allow β-catenin to associate with TCF4, and to initiate transcription of Wnt-targeted genes.

Discussion

Dimerization is proved to be a key process in the activity regulation of proteins, including enzymes, ion channels, receptors and transcription factors (18). Previous crystal structure analyses demonstrated that the CCT domain of p15RS owns a potential to form homodimer (14,15). However, the function of the homodimerization of full-length p15RS remained unknown. In this study, we revealed that dimerization of p15RS is required for its inhibitory role on Wnt signaling. We used biochemistry experiments to confirm the dimerization of full-length p15RS, similar to the CCT domain. In particular, we identified several leucine residues responsible for the dimeric interaction. We speculated that the the leucine-zipper-like motif within the CCT domain holds two p15RS molecules together to form an increased association interface with TCF4. Since the size of the association interface tends to correlate with the affinity of interaction between two proteins (28), we proposed that the dimerized p15RS has an increased affinity to interact with TCF4. Therefore, dimerized p15RS exhibits a stronger ability to inhibit the transcriptional activity of TCF4 and to maintain Wnt signaling in a quiescent status. Accordingly, blocking the interface by de-dimerization of p15RS appears to impair the association of p15RS with TCF4. Our study has provided compelling evidence for the association of p15RS with TCF4 using the mutants which lost the ability of dimerization. Furthermore, we identified that β-catenin, by interacting with the monomer p15RS, interrupts the dimerization of p15RS, leading to release of p15RS from TCF4. Our model illustrated that β-catenin eventually interacts with TCF4 to activate the transcription of Wnt-targeted genes after dimerized p15RS is broken and released from the promoter (Fig. 5E). This study provided a detailed molecular mechanism of how β-catenin compete with dimerized p15RS during its binding to TCF4 and thus to activate transcription of Wnt targeted genes (12).

Our model is supported by the mutation of the leucine-zipper-like motif where L248 and L255 are critical residues. Indeed, mutation of leucines 248/255 into prolines or alanines impaired the dimeric interaction (Fig. 2D-F). Interestingly, mutation of leucine 248/255 into alanines appeared with equally strong dimerized interaction by the formaldehyde cross-linking experiment (Fig. 2G) and weak interaction by the IP experiment in mammalian cells (Fig. 2D, 2F) while with no

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interaction by the GST pull-down experiment using proteins purified from *E. coli* (Fig. 2E). Functional studies showed that mutation into proline completely lost, but mutation into alanine remained, the ability of p15RS to inhibit cell proliferation and invasion (Fig. 4). These results were also consistent to the luciferase analyses on the transcription activity by the Wnt reporter (Fig. 3A, 3B) and the IP experiments for the effect of p15RS on the β-catenin•TCF4 complex formation (Fig. 3F). Therefore, we concluded that mutation of leucine 248/255 into proline lost the dimerization ability and thereafter impaired the ability to inhibit Wnt signaling. However, we could not exclud the role of other two leucines, which also fit the heptad structure in the leucine-zipper-like motif, on the formation of p15RS dimer.

We have proved our model that dimerized wild type p15RS interacts strongly with TCF4 but the mutated monomer (L248P/L255P) diminishes the interaction with TCF4 (Fig. 3C). It echoes to the strong interaction of β-catenin with monomer p15RS (L248P/L255P) (Fig. 3D, 3E, S2). Furthermore, we observed that dimerized p15RS was dramatically reduced in the presence of β-catenin (Fig. 3J). Consistently, we found that the RPR domain, in a monomer status, interacted with the residues 407-533 of β-catenin with a higher affinity, comparing with full-length p15RS (Fig.S1A). Together with the results of the occupancy of p15RS, TCF4 and β-catenin (Fig. 3G-I), we proposed that β-catenin, after translocated into the nucleus upon Wnt stimulation, breaks down the dimerized p15RS into monomer, to abrogate the ability of p15RS to associate with TCF4. However, since the association of proteins is a dynamic process, we could not exclude the possibility that β-catenin may block the dimer formation by sequestering monomer p15RS. Further analyses are needed to decipher whether β-catenin disrupts the dimerized p15RS or blocks the formation of dimer from monomer p15RS in the nucleus (29,30). Also, our previous studies revealed that p15RS also associated with HDAC2 (17) and its homologues CREPT interacted with HDAC1(10). Whether the dimerization of p15RS is critical for its association with HDAC2 remains to be unveiled.

Previous studies demonstrated that p15RS has malignancy-inhibitory functions. In this study, we confirmed that p15RS suppresses cell proliferation and invasion. We further identified that dimerization of p15RS is critical for its inhibitory role in tumor cell growth and formation. Due to the participation of p15RS in Wnt signaling pathway, we therefore speculated that p15RS inhibits cell proliferation via down regulating Wnt signaling pathway. Since aberrant Wnt signal regulations always cause a wide spectrum of cancers (31), we speculate that p15RS may be a potent tumor suppressor. This study validated that p15RS dimerization is required for p15RS to fulfill its inhibitory effects on tumor cells. We have identified that L248/L255 are critical residues to maintain the dimerization and the inhibition role of p15RS on tumorigenesis. We speculate that loss of p15RS dimerization may act as a key to trigger tumorigenesis. However, it remains unclear whether L248/L255 mutation occurs in human cancers. Our preliminary mutagenesis analyses in cBioPortal for Cancer Genomics based on

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TCGA database showed that most of the single-amino-acid mutations in p15RS in cancer patients are located in the CCT domain, although not L248/L255 (data not shown). Nevertheless, we expect that, along the emergence of techniques like PROTAC (32), facilitating the dimerization of p15RS may become a way to cure cancers.

Experimental procedures

Plasmids and Reagents

Myc-p15RS, Myc-p15RS/RPR, Myc-p15RS/CCT, Wnt1, Flag-β-catenin, HA-TCF4 plasmids were constructed or obtained previously (12,15,17). Flag-p15RS, Flag-p15RS/RPR, Flag-p15RS/CCT, prokaryotic GST-p15RS expressing construct and eukaryotic GST-p15RS were generated by inserting the PCR-amplified fragments into a pCDNA3.1/Flag vector, a pGEX-4T-1 or a pXJ40 vector. Site-directed mutations were generated by two pairs of primers (Sangon Biotech Co. Ltd., Shanghai, China) designed to mutate the target amino acids L248 and L255: L248P/L255P (assigned as $p15RS^{L248P/L255P}$), 5'-AAGGAAGCCCCCGCAGAGAAA-3' and 5'-TTGACAACGGGGAAAATCTGCT-3'; L248A/L255A (assigned as $p15RS^{L248A/L255A}$), 5'-AAGGAAGCCGCTGCAGAGAAA-3' and 5'-TTGACAACGAGCAAAATCTGCT-3'. SuperTop-luciferase reporter was a gift from Dr. Wei Wu (School of Life Science, Tsinghua University, China). 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 and Transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FBS and penicillin (100 units/ml)/streptomycin (100 units/ml). A375 cells were cultured in RPMI Medium 1640 basic (1×) (Gibco) with 10% FBS and penicillin (100 units/ml)/ streptomycin (100 units/ml). All cells were grown at 37°C with 5% CO_2. Serum were purchased from Invitrogen. Stable cell lines were selected by FACS after infected with specific lentivirus. Cells were transfected with plasmids as indicated using Vigofect (Vigorous Inc. Beijing, China) according to the protocols.

Protein extraction and purification

Prokaryotic GST-p15RS was expressed in *E. coli* BL21 for 4 h at 30°C and induced with 0.5 mM isopropyl-I-thio-β-galactopyranoside at 30°C for 4 h. *E. coli* cells were harvested and sonicated. Glutathione S-transferase (GST) fusion proteins were purified by glutathione-Sepharose 4B (GE Healthcare) according to the manufacturer's instructions. Eukaryotic Glutathione S-transferase (GST) fusion proteins were purified by adding glutathione-Sepharose 4B beads into HEK293T cell lysates expressing pXJ40/p15RS, $pXJ40/p15RS^{L248P/L255P}$ and $pXJ40/p15RS^{L248A/L255}$.

Immunoprecipitation Assay (IP), GST pull-down and Western Blotting (WB)

For immunoprecipitation assays, HEK293T cells were plated in 60-mm dishes

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transfected with indicated plasmids. Cells were harvested in RIPA buffer (50 mM Tris-Cl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1% SDS, pH 8.0) together with protease inhibitors after 24-48 h transfection. Whole-cell lysates were incubated with indicated antibodies and protein G/A-Sepharose beads at 4°C overnight. After washed the beads with lysis buffer (50 mM pH7.6 Tris-Cl, 150 mM NaCl, 0.5 M EDTA, 0.05% NP40) for 4 times, precipitates were eluted with 2× SDS-PAGE sample buffer from the beads and analyzed by Western blotting with the indicated antibodies. GST pull-down were performed by adding prokaryotic or eukaryotic purified GST-tagged proteins into cell lysates expressing targeted proteins and then followed IP procedures after incubation.

Cross-linking Assay

Transiently transfected HEK293T cells were cross-linked by 1% formaldehyde for 30 min at 37°C and 0.25 M glycine was added for 5 min at room temperature to stop the cross-linking reaction. Cells were then collected and lysed directly in 2 × SDS-PAGE sample buffer and analyzed by Western blotting with specific antibodies.

Immunofluorescent Analysis

Cells were plated and incubated overnight on glass coverslips placed in 6-well dishes ahead of use. 24 h after transfected with specific plasmids, cells were rinsed with PBS, fixed with 4% paraformaldehyde for 15-20 min at room temperature, and permeated with 0.3% Triton X-100 for 10 min. After blocked with 10% goat serum for 1 h at room temperature, cells were incubated with indicated primary antibodies at 4°C overnight. Secondary antibodies conjugated with FITC (green) were added for 1 h at room temperature to detect bound primary antibodies. DAPI were applied to indicate the nucleus. Stained cells were analyzed by a laser scanning confocal microscopy (Zeiss780) at 40 × oil lens.

Luciferase Assay

HEK293T and A375 cells were plated in 24-well dishes and transfected with the indicated plasmids together with SuperTOP-luciferase reporter plasmid and an internal control pRL-TK vector (from Promega, Madison, WI). 24 h after transfection, the luciferase activity was determined by the Dual-Luciferase Assay System (Vigorous Inc. Beijing, China). The firefly luciferase activity was normalized by Renilla luciferase activity and presented as mean ±S.D. from triplicate experiments.

Chromatin Immunoprecipitation (ChIP) Assay

Cells were cross-linked by 1% formaldehyde at 37°C for 10-15 min and then treated with 125 mM glycine for 5 min. Cells were collected and suspended in 500 µl of ChIP SDS lysis buffer. The mixture was then sonicated for 10-s pulsed with a 30-s interval at 4% output power to yield DNA fragments of 200 to 500 bp in size. After centrifugation, lysates containing protein-DNA complexes were diluted 10-fold and immunoprecipitated with the indicated antibodies. The precipitated complexes were de-associated by 5M NaCl and treated with protein kinase K to remove proteins. DNAs were eluted by phenol:chloroform extraction and analyzed by PCR. The fragment corresponding to the TCF4

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binding site in the *CCND1* promoter was amplified by primers (33): 5'-CACCTCCACCTCACCCCCTAAATCC-3' and 5'-ACTCCCCTGTAGTCCGTGTGACG-TT-3', and that from *c-MYC* promoter was amplified with 5'-TTGCTGGGTTATTTTAA-TCAT-3' and 5'-ACTGTTTGACAAACCG-CATCC-3' (34).

Real Time PCR

Real time PCR was performed to quantify samples from ChIP assays, using a Real-MasterMix (SYBR Green) kit (TIANGEN Biotech, Beijing, China) under the following condition: denature, 95°C, 20 s; annealing, 60°C, 20 s; and extension, 72°C, 30 s. Primers were the same as those used in ChIP assays.

MTT Assay and Wound Healing Assay

For MTT assay, 1×10^3 A375 stable cells were seeded in triplicate in 96-wells. After cultured for indicated times, 20 ul of MTT (5 mg/ml) was added for 4 h. Cells were then dissolved in 150 ul DMSO for 10 min in the dark. OD 490 and 630 were read by Bio-Rad model 680 microplate reader. Data were collected for 7 days. For the wound healing assay, monolayer cells were wounded by a sterile plastic tip. Cell migration was observed by microscopy 24 h later.

Colony Formation Assay

Cells were seeded on 6-well plates at a density of 500 cells/well. After cultured for 2 weeks, cells were fixed with methanol for 10 min and incubated with 0.1% crystal violet (Sigma) for 10 min at room temperature. The number of colonies was counted and presented as mean ± SD.

Tumorigenesis Assay

A375 cells (2×10^5 cells/mouse) were injected subcutaneously into 6-week-old BALB/c nude mice, with control cells and tested cells bilaterally at the armpit of each mouse. When tumors were about 1.5 cm in diameter, mice were sacrificed and tumor tissues were collected, weighted and measured in size. This protocol follows the institutional guidelines and regulations on the animal health and ethics, approved by the animal health and ethics committee in Tsinghua University.

Statistical Analyses

All experiments were repeated at least 3 times. Data were presented as mean +/- standard deviation. Significant differences between groups were determined using a Student's t-test.

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

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FOOTNOTES

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Figure Legends

Fig. 1. The CCT domain of p15RS mediates its dimerization in *vitro*.

(A and B) Homologous interaction of p15RS in *vitro*. Lysates of HEK293T cells transfected with Myc-tagged and Flag-tagged p15RS were immunoprecipitated by anti-Flag antibody (A) or anti-Myc antibody (B) and subjected to Western blotting with indicated antibodies. (C) Graphic representation of p15RS protein structure: RPR domain from amino acid 1 to 135 and CCT domain from amino acid 136 to 312. (D) CCT domain is responsible for the dimerization of p15RS. Flag-tagged full-length p15RS, RPR or CCT domain were co-expressed with Myc-tagged full-length p15RS in HEK293T cells. Cell lysates were incubated with an anti-Flag antibody for the IP assay. (E) The CCT domain of p15RS per se dimerizes. Myc-tagged full-length (Myc-p15RS) or CCT domain of p15RS (Myc-CCT) were co-expressed in HEK293T cells with Flag-tagged CCT domain of p15RS (Flag-CCT). An anti-Myc antibody was used for immunoprecipitation. (F) p15RS forms a homodimer. HEK293T cells transiently over-expressing Flag-p15RS were cross-linked by 1% formaldehyde for indicated times at room temperature 24 h after transfection. An anti-p15RS antibody was used to detect the 39-kDa monomer and the 78-kDa dimer of Myc-p15RS. (G) The CCT domain of p15RS determines dimerization while RPR domain stays as monomer. HEK293T cells transfected with Flag-tagged RPR or CCT domain of p15RS were subjected to cross-linking with 1% formaldehyde for indicated times. The monomers and dimers were revealed by Western blotting using an anti-Flag antibody. Note that dimers of endogenous p15RS with the CCT domain are also marked.

Fig. 2. Dimerization of p15RS depends on the leucine-zipper-like motif within its CCT domain.

(A) A similarity analysis of amino acid sequences of p15RS with typical leucine-zipper-containing proteins by an alignment using Bioedit software. Identical amino acids were back-colored in black while residues sharing similar characteristics were back-colored in gray. * indicates critical leucines in the heptad structure. (B) A schematic diagram of the mutation in the leucine-zipper-like motif of p15RS. $p15RS^{L248P/L255P}$ (referred hereafter to as P): L248 and L255 double-substituted into prolines; $p15RS^{L248A/L255A}$ (referred hereafter to as A) L248 and L255 double-substituted into alanines. Full-length p15RS is referred hereafter to as WT. (C) Mutations failed to affect p15RS localization in the nucleus. MCF-7 cells expressing Flag-p15RS, Flag-$p15RS^{L248P/L255P}$ and Flag- $p15RS^{L248A/L255A}$ were fixed and stained with an anti-Flag antibody followed by an anti-mouse IgG conjugated with FITC. Nuclei were counterstained with DAPI. Cells were observed by a confocal microscope with a 400-fold magnification. (D) $p15RS^{L248P/L255P}$ no longer dimerizes. Myc-tagged full-length p15RS, $p15RS^{L248P/L255P}$ or $p15RS^{L248A/L255A}$ were co-expressed with Flag-tagged p15RS in HEK293T cells. Cell lysates were incubated with an anti-Myc antibody and subjected to Western blotting by an anti-Flag antibody. (E and F) Leucines L248/L255 of p15RS are required for the dimeric interaction *in vivo*. GST pull-down assays were performed with purified prokaryotic (E) or eukaryotic (F) GST-p15RS proteins and Flag-tagged p15RS, $p15RS^{L248P/L255P}$ or Flag-$p15RS^{L248A/L255A}$ proteins expressed in HEK293T cells. Cell lysates were incubated with GST beads and subjected to Western

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blotting by an anti-Flag antibody. (G) p15RS$^{L248P/L255P}$ remains as monomer while p15RS$^{L248A/L255A}$ forms dimer. HEK293T cells transfected with Flag-tagged full-length p15RS, p15RS$^{L248P/L255P}$ or Flag-p15RS$^{L248A/L255A}$ were subjected to cross-linking and detected by Western blotting using an anti-Flag antibody.

Fig. 3. The dimeric ability of p15RS is required for its inhibitory role on Wnt signaling.
(A and B) Dimerization of p15RS participates in the inhibition of Wnt1-stimulated transcriptional activity. Luciferase assays were performed using HEK293T (A) or A375 (B) cells with transient expression of Myc-tagged p15RS (referred hereafter to as WT), p15RS$^{L248P/L255P}$ (referred hereafter to as P) or Flag-p15RS$^{L248A/L255A}$ (referred hereafter to as A) together with a SuperTop-luciferase reporter and pRL-TK (as an internal control). EV indicated empty vector as a control. Wnt1 expression was generated by transfection of a Wnt1 plasmid. Relative luciferase activities were normalized with the internal control. Results are presented from three independent experiments, and data are represented as mean ±S.D. (n = 3). Asterisk indicates a statistically significant difference. *, $p < 0.05$. (C) p15RS$^{L248P/L255P}$ interacts with TCF4 with a decreased affinity. Myc-tagged p15RS, p15RS$^{L248P/L255P}$ or p15RS$^{L248A/L255A}$ were co-expressed with HA-TCF4 in HEK293T cells. Cell lysates were incubated with an anti-Myc antibody and subjected to Western blotting by an anti-HA antibody. Relative binding affinity was represented as fold change based on the level of the HA-TCF4 and Myc-p15RS. (D and E) Decreased dimerization leads to tighter bond between p15RS and β-catenin. Myc-tagged p15RS, p15RS$^{L248P/L255P}$ or Flag-p15RS$^{L248A/L255A}$ were co-expressed with Flag-β-catenin in HEK293T cells. Cell lysates were incubated with an anti-Myc antibody and subjected to Western blotting by an anti-Flag antibody. Cell lysates expressing Flag-β-catenin were incubated with eukaryotic purified GST-tagged p15RS, p15RS$^{L248P/L255P}$ or Flag-p15RS$^{L248A/L255A}$ proteins, together with GST beads, and then subjected to Western blotting by an anti-Flag antibody (E). (F) Diminished dimerization of p15RS enhances the interaction of β-catenin and TCF4. HA-TCF4 and Flag-β-catenin were co-expressed with Myc-tagged p15RS, p15RS$^{L248P/L255P}$ or Flag-p15RS$^{L248A/L255A}$ in HEK293T cells. The cell lysates were subjected to an IP experiment with an anti-Flag antibody and examined by Western blotting using indicated antibodies. (G and H) Dimerization of p15RS is critical for its occupancy on the TBS of Wnt-targeted gene promoters. HEK293T cells were transfected with Myc-tagged p15RS, p15RS$^{L248P/L255P}$ or Flag-p15RS$^{L248A/L255A}$ and cross-linked to perform a ChIP assay using an anti-Myc antibody. The co-immunoprecipitated DNA was examined by PCRs or quantitative PCRs using specific primers for TBS of *CCND* 1 (G) or *c-MYC* (H). (I) Dimerization of p15RS is crucial for its blockage of the TCF4•β-catenin complex to occupy on the TBS of *c-MYC*. HA-TCF4 and Flag-β-catenin were co-expressed with Myc-tagged p15RS, p15RS$^{L248P/L255P}$ or Flag-p15RS$^{L248A/L255A}$ in HEK293T cells. Cells were cross-linked to perform a ChIP assay using an anti-β-catenin antibody. Primers specifically recognizing the TBS of *c-MYC* were used to detect and quantify the precipitated DNAs. (J) β-catenin weakens the dimeric interaction of p15RS. HEK293T cells transfected with GST-p15RS, Myc-p15RS or Flag-β-catenin were precipitated by GST beads

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and the dimerized p15RS was examined by Western blotting using an anti-Myc antibody.

Fig. 4. p15RS dimerization participates in the inhibition of cell proliferation.
(A) $p15RS^{L248P/L255P}$ failed to inhibit cell proliferation. A375 cells were infected with pLVX/GFP-tagged p15RS, $p15RS^{L248P/L255P}$ or $p15RS^{L248A/L255A}$ lentivirus packaged by pLVX plasmid system. The same efficiency of infection was set for overexpression of p15RS and its mutants. Bright field and fluorescence were shown. After cultured for 10 days, GFP-positive cells were counted by FACS. (B) Dimerization of p15RS is important for cell growth inhibition. 1×10^3 A375 cells stably over-expressing GFP-tagged p15RS, $p15RS^{L248P/L255P}$ or $p15RS^{L248A/L255A}$ by pLVX lentivirus were seeded in triplicate on 96-well plates and cultured for indicated time periods. Cell densities were measured at OD 490 nm/630 nm. (C and D) Disabled dimerization loses the ability to inhibit cell clone formation. A375 stable cell lines were seeded on 6-well plates at a density of 1×10^3 cells/well and maintained for two weeks for the colony formation. Cells were fixed with methanol and stained with 0.1% crystal violet (C). Colony numbers were counted from three independent experiments (D). Data are presented as means ± SD. *, $p<0.05$. (E and F) The effect of dimerization on cell migration. Monolayer A375 cells stably over-expressing GFP-tagged p15RS, $p15RS^{L248P/L255P}$ or $p15RS^{L248A/L255A}$ were scratched with sterile pipette tips. Cell migration was observed and photographed by microscopy 24 h later (E). The migration rates were measured by the software Image J (F). Data are presented as means ± SD. **, $p<0.01$.

Fig. 5. Dimerization of p15RS plays a critical role in tumor suppression.
(A, B, C and D) $p15RS^{L248P/L255P}$ exhibits little inhibitory effect on tumor formation. Tumorigenesis assay was performed by injecting 2×10^5 indicated A375 cells into BALB/c nude mice. Each mouse was injected with control cells and testing cells bilaterally into the armpit (A). Tumors were cultured to reach a diameter of about 1.5 cm (B). The weight (C) and size (D) of the tumors were collected and calculated. Data are presented as means ± SD. (E) A proposed model of how dimerization of p15RS participates in Wnt signaling inhibition. Under Wnt quiescence condition (Off), dimerized p15RS associates with TCF4 to suppress its transcription. When Wnt signaling is activated (On), β-catenin is accumulated and translocated into nucleus, where β-catenin disassociates the dimerized p15RS by preferably interacting with monomer p15RS. In such a way, β-catenin competes with dimerized p15RS and then forms a complex with TCF4 to activate transcription.

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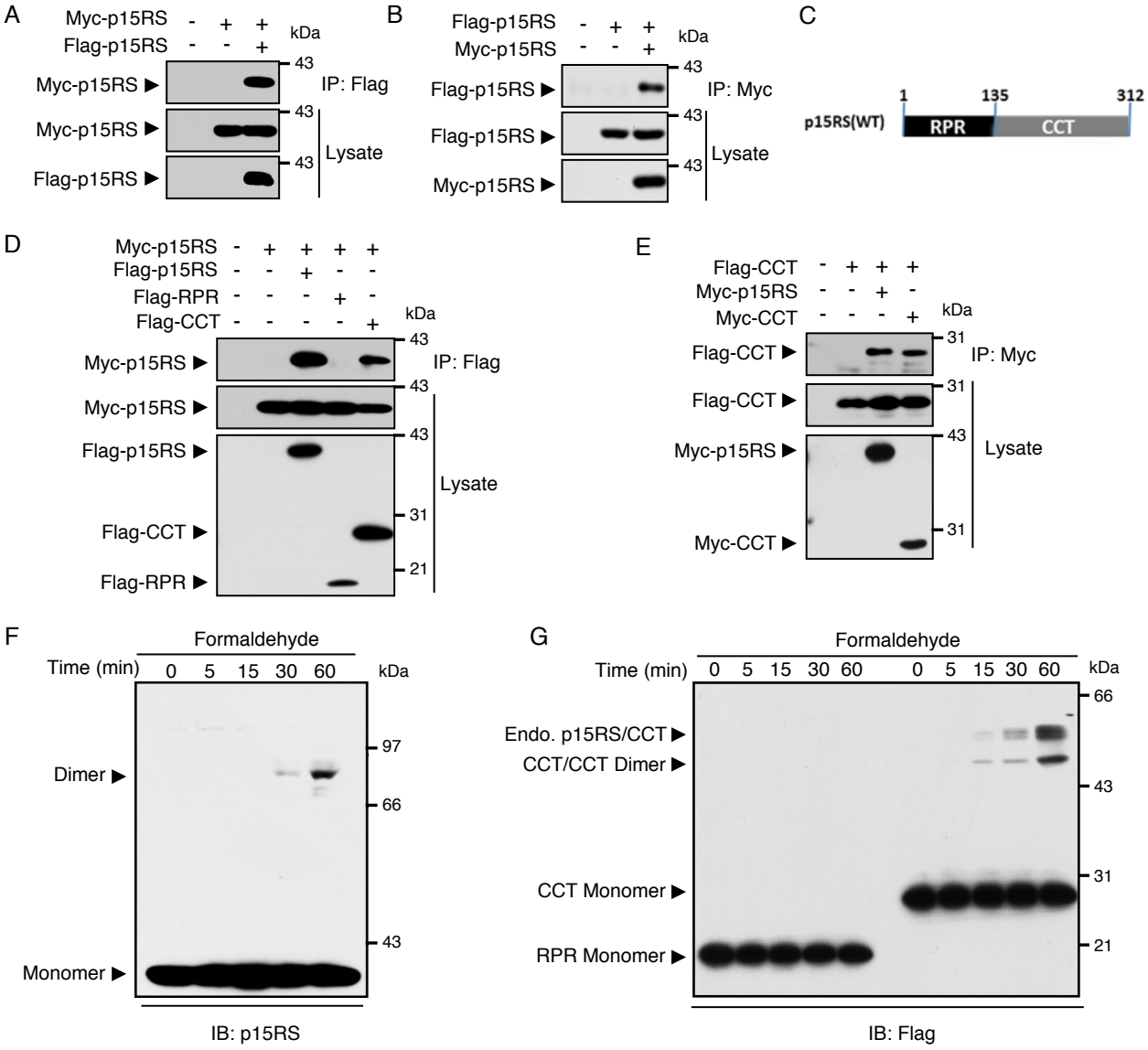
Fig. 1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Flag-p15RS ▶
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Flag-CCT ▶
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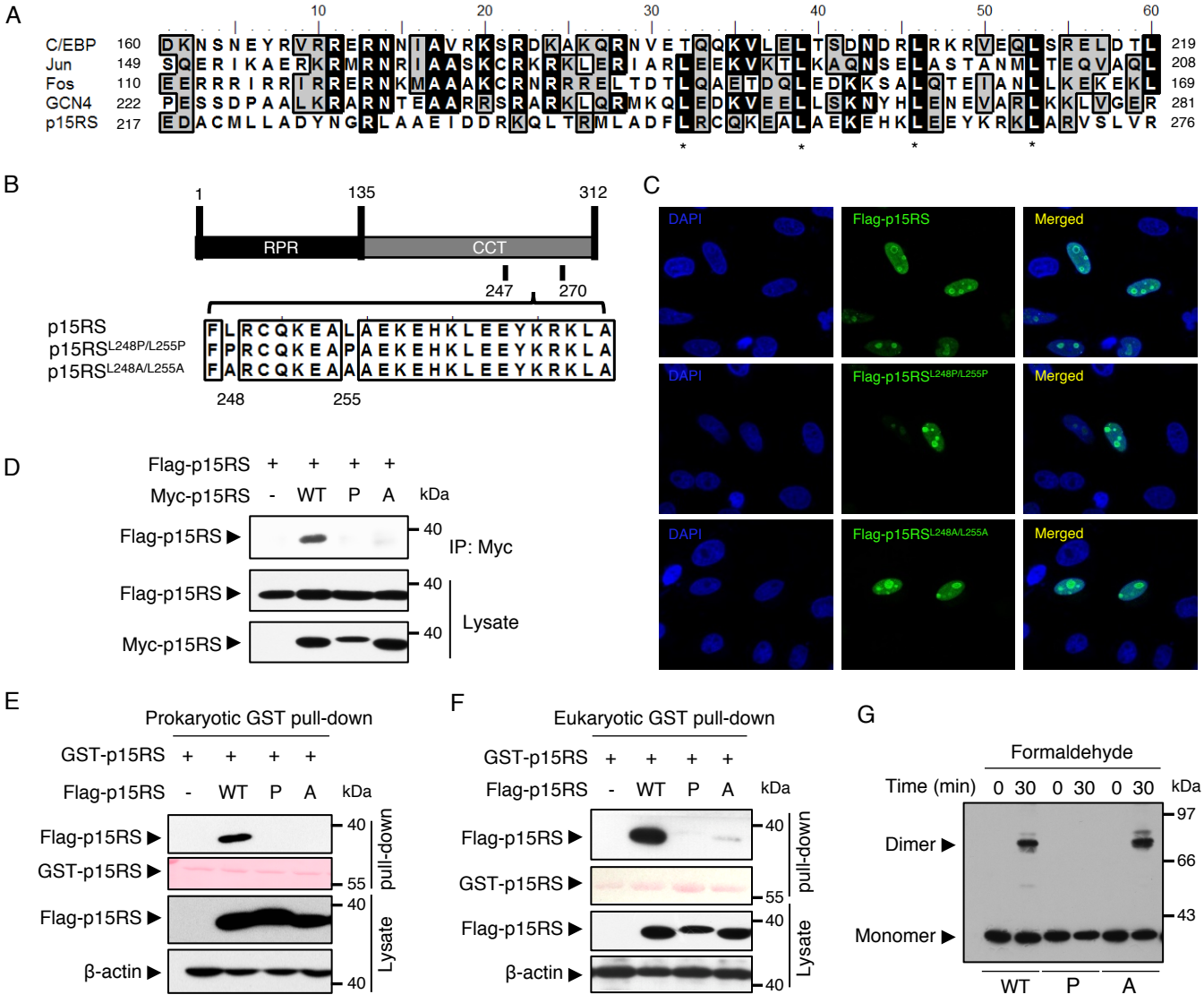
Fig. 2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p15RS 217 EDACMLLADYNGRLAAEIDDRKQLTRMLADFLRCQKEALAEKEHKLEEYKRKLARVSLVR 276
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312
RPR
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Merged
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Myc-p15RS ▶ 40
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GST-p15RS ▶ 55
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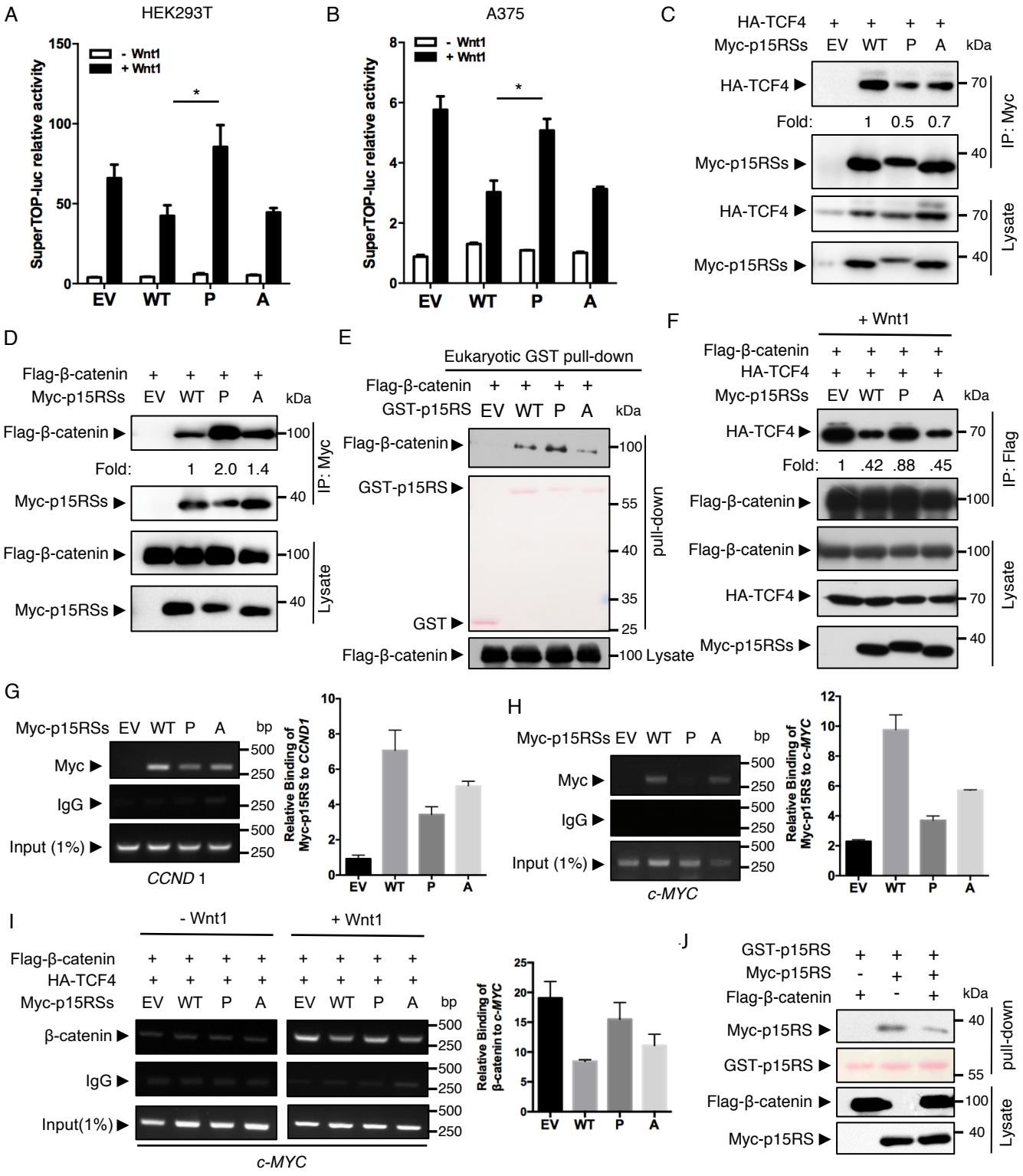
Fig. 3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Flag-β-catenin
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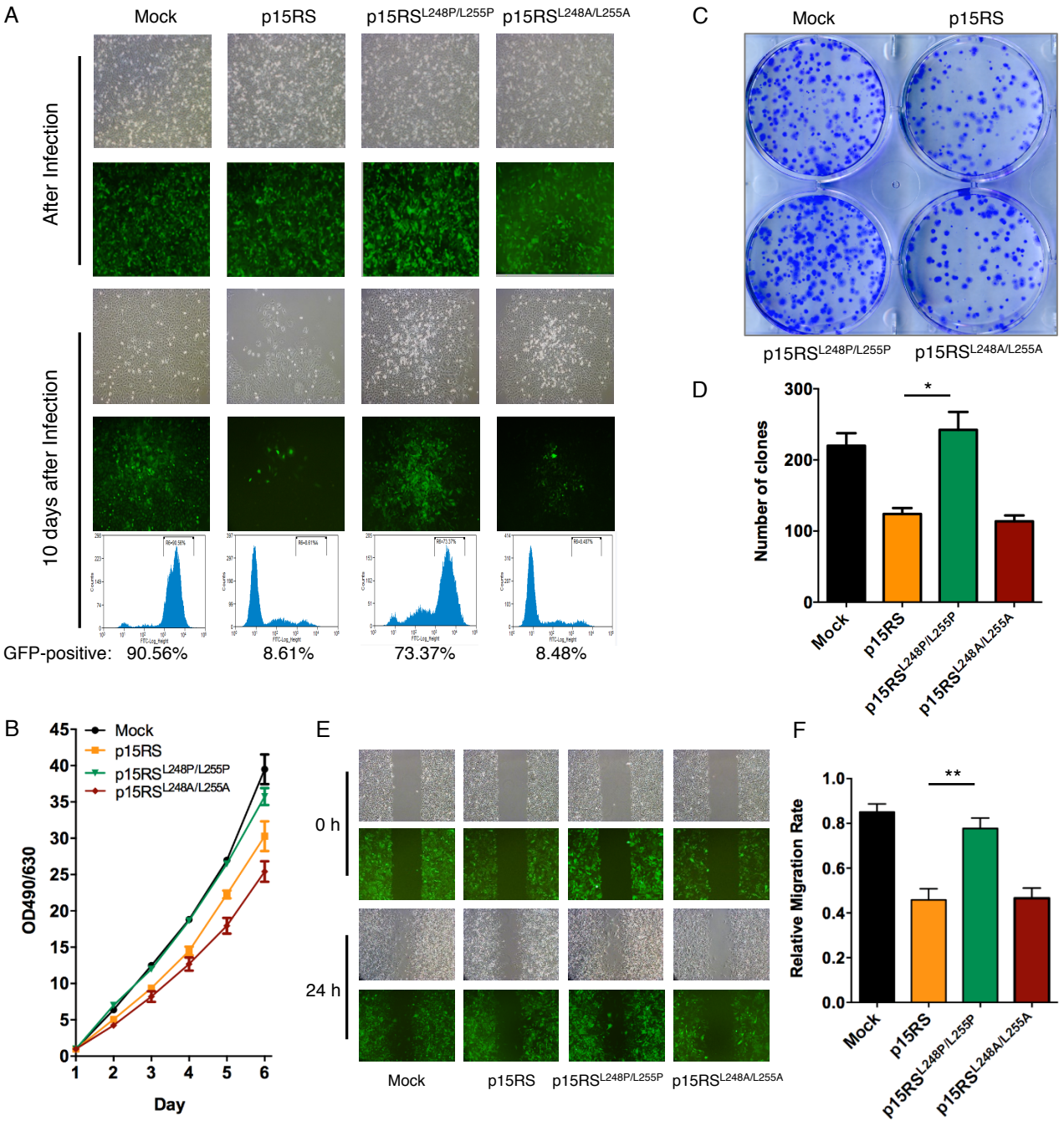
Fig. 4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p15RS
p15RS$^{L248P/L255P}$
p15RS$^{L248A/L255A}$
D
Number of clones
300
200
100
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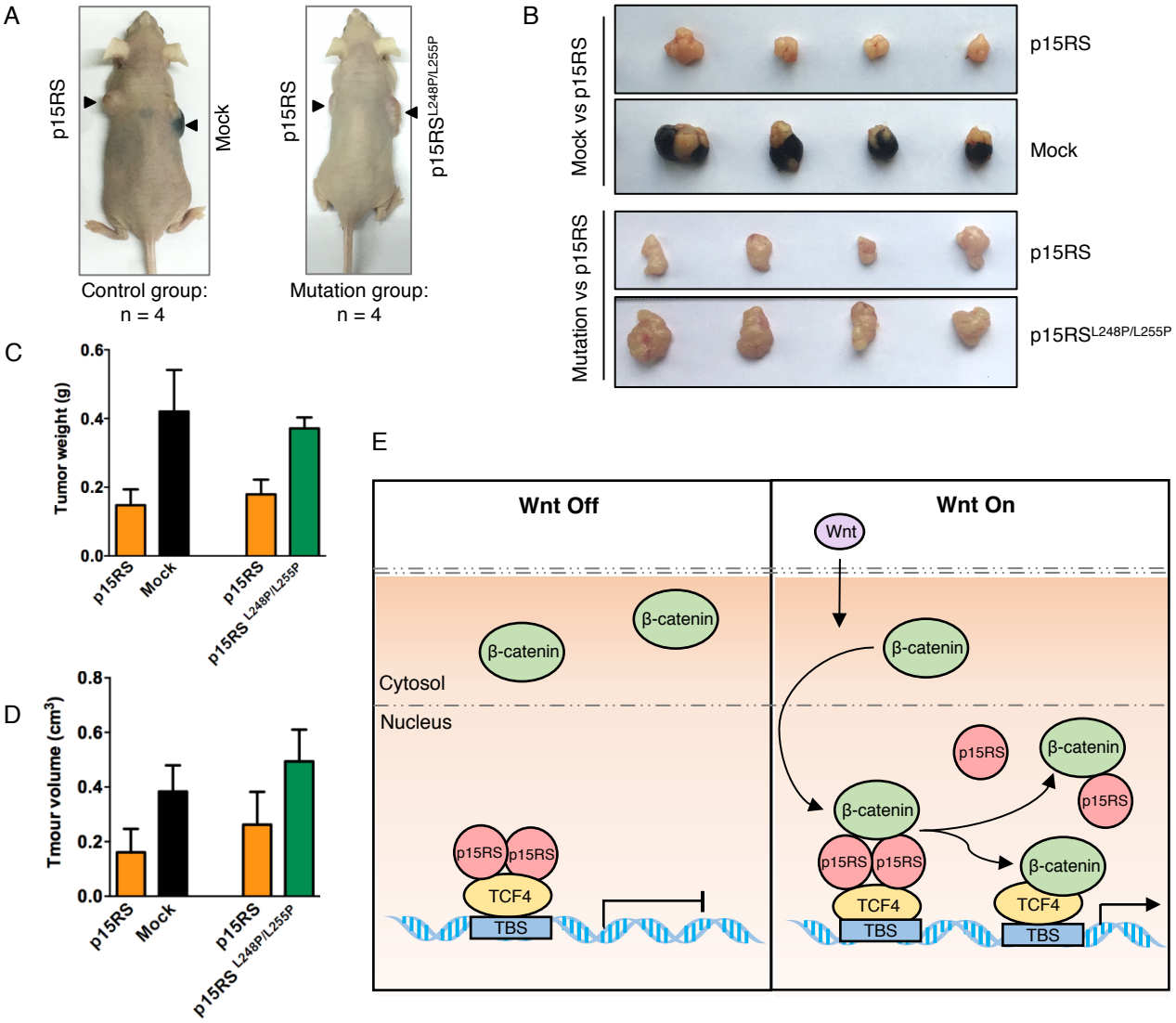
Fig. 5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Control group:
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n = 4
B
Mock vs p15RS
p15RS
Mock
Mutation vs p15RS
p15RS
p15RS L248P/L255P
C
Tumor weight (g)
0.6
0.4
0.2
0.0
p15RS
Mock
p15RS
p15RS L248P/L255P
D
Tmour volume (cm3)
0.8
0.6
0.4
0.2
0.0
p15RS
Mock
p15RS
p15RS L248P/L255P
E
Wnt Off
Wnt On
Wnt
β-catenin
β-catenin
Cytosol
Nucleus
p15RS
p15RS
TCF4
TBS
β-catenin
β-catenin
p15RS
p15RS
p15RS
p15RS
p15RS
β-catenin
TCF4
TBS
β-catenin
TCF4
TBS

Dimerization of p15RS mediated by a leucine-zipper-like motif is critical for its inhibitory role on Wnt signaling

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