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HuR represses Wnt/β-catenin-mediated transcriptional activity by promoting cytoplasmic localization of β-catenin

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ABSTRACT

β-Catenin is the key transcriptional activator of canonical Wnt signaling in the nucleus; thus, nuclear accumulation of β-catenin is a critical step for expressing target genes. β-Catenin accumulates in the nucleus of cancer cells where it activates oncogenic target genes. Hu antigen R (HuR) is a RNA binding protein that regulates multiple post-transcriptional processes including RNA stability. Thus, cytoplasmic HuR protein may be involved in tumorigenesis by stabilizing oncogenic transcripts, but the molecular mechanism remains unclear. Here, we observed that Wnt/β-catenin signaling induced export of the HuR protein, whereas HuR overexpression promoted accumulation of the β-catenin protein in the cytoplasm. Thus, Wnt/β-catenin-mediated transcriptional activity in the nucleus was reduced by overexpressing HuR. These results suggest novel and uncharacterized cytoplasmic β-catenin functions related to HuR-mediated RNA metabolism in cancer cells.

1. Introduction

Wnt signaling controls many physiological processes, including determining cell fate, cell proliferation, and homeostasis [1]. Aberrant activation of the Wnt pathway is linked to developmental defects and various diseases including cancers [2,3]. β-Catenin is a critical transcription factor downstream of the Wnt signaling pathway; thus, it remains at low levels in normal cells by proteolytic regulation in the cytoplasm. Once activated by Wnt, cytoplasmic β-catenin is stabilized and translocates to the nucleus where it activates transcription of oncogenic target genes, such as *cyclin D1*, *c-myc*, and *Axin2* [4–7]. A number of proteins interact with β-catenin, including various RNA binding proteins (RBPs), but how these interactions contribute to β-catenin oncogenic activity is not understood [8].

Hu antigen R (HuR) is a ubiquitously expressed RNA binding protein (RBP) that regulates many post-transcriptional steps, including mRNA alternative splicing, polyadenylation, and mRNA stability and translation [9]. HuR is localized mainly to the nucleus but overexpressed HuR protein in cancer cells is exported to the cytoplasm, which might contribute to RNA-mediated tumorigenesis [10,11]. HuR binds the AU rich elements of target transcripts, including *cyclooxygenase-2* (*COX-2*) mRNA [12,13]. We found previously that β-catenin binds to the 3′ untranslated region of *COX-2* mRNA and interacts with HuR to stabilize *COX-2* mRNA. β-Catenin recruits HuR to *COX-2* mRNA and forms a tertiary ribonucleoprotein (RNP) complex in the cytoplasm of cancer cells, which increases oncogenic COX-2 protein levels [14,15].

In this study, we examined whether the HuR protein affects subcellular localization of β-catenin and measured the influence of HuR on Wnt/β-catenin transcriptional activity. We found that the β-catenin protein was highly abundant in the perinuclear fraction in which HuR was also localized, and that overexpressing HuR induced translocation of β-catenin from the nucleus and the perinucleus into the cytoplasm. The HuR protein decreased β-catenin transcriptional activity significantly in the basal and Wnt activated states. Considering the multiple functions of β-catenin, we propose that perinuclear and cytoplasmic β-catenin has novel and uncharacterized functions, and that HuR is a functionally important regulator of Wnt/β-catenin signaling.

2. Materials and methods

2.1. Cell culture and reagents

Human cervical adenocarcinoma HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). NIH3T3 cells were cultured in DMEM supplemented with 10% fetal calf serum. Human colorectal carcinoma HCT116 cells were cultured in RPMI1640 medium with 10% FBS. Human embryonic kidney 293 (HEK293) cells were cultured in MEM medium with 10% FBS. Actinomycin D (Act D) was added at 5 μg/ml for 90 min. The cells were also treated with 10 mM lithium chloride (LiCl) for 2 h. These

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chemicals were purchased from Sigma (St. Louis, MO, USA). The β-catenin deletion clone (ΔC) was obtained by polymerase chain reaction (PCR) amplification of full-length (FL)-β-catenin. The primers used in this study are shown in Table 1. PCR fragments were cloned into the XbaI/BamHI site of the p3xFlag CMV10 vector. The HuR overexpressing clones pEGFP-HuR and pCI-Flag-HuR were kindly provided by Sung-Gil Chi (Korea University) and Takbum Ohn (Chosun University), respectively.

2.2. Immunocytochemistry

The cells were fixed in 4% paraformaldehyde for 15 min and permeablized with 0.5% Triton X-100. After a 1 hr incubation in blocking buffer with 5% NHS, the cells were incubated with anti-HuR or anti-HA antibodies. Following washes with PBS containing 0.1% Triton X-100, the samples were incubated with secondary anti-mouse Alexa488 or anti-rabbit Cy3 (The Jackson Laboratory, Bar Harbor, ME, USA) and Hoechst 33342. The samples were visualized with a Zeiss microscope.

2.3. Cell fractionation and Western blot analysis

The cellular fractionation method followed that described previously [16]. Briefly, cells were resuspended in hypotonic buffer [10 mM Tris–HCl (pH 7.5), 10 mM NaCl, 1.5 mM $MgCl_2$, and protease inhibitor cocktail] and allowed to swell for 5 min on ice. NP40 (0.5%) was added to the lysates and pelleted at 1000×*g* for 3 min. The supernatant was collected as the cytoplasmic extract (CE). Perinuclear extracts (PNE) were prepared by washing the pellets in buffer containing 1% NP40 and 0.5% sodium deoxycholate. After centrifugation at 1000×*g* for 3 min, the supernatant was stored as the PNE. The nuclear pellets were sonicated in RIPA buffer for 10 min, centrifuged at 4 °C for 10 min, and designated as the nuclear extract (NE). Cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted with monoclonal antibodies that recognize the specific proteins, as indicated. The following antibodies were used for Western blotting: green fluorescent protein (#527131) and β-actin (#6276) antibodies were purchased from Abcam (Cambridge, MA, USA); β-catenin (#610154) antibody was purchased from BD Bioscience (San Diego, CA, USA); HuR (#5261) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. Luciferase reporter assay

The luciferase reporters (TOPFlash and FOPFlash) to monitor Wnt/β-catenin transcriptional activity were described previously [17]. The cells were seeded on 12-well plates and transfected in triplicate using Lipofectamine™ (Invitrogen, Carlsbad, CA, USA) containing the luciferase reporter plasmid (TOPFlash or FOPFlash for TCF/β-catenin; nuclear factor-kappa beta [NF-κB] synthetic reporter) and the pRL-TK plasmid (internal control) in the presence of expression clones. Luciferase activity was measured after 24 h using the GloMax® 20/20 luminometer (Promega, Madison, WI, USA).

Table 1
Sequence of primers used in this work.

Name	F/R	Sequence
Full length β-catenin	For	5′-GCGAGATCTATGGCTACTCAAGCTGATTTG-3′
	Rev	5′-CACAGATCTTTACAGGTCAGTATCAAACCAGGC-3′
ΔC β-catenin	For	5′-GCGAGATCTATGGCTACTCAAGCTGATTTG-3′
	Rev	5′-GTAGGATCCTGGCTTGTCCTCAGACATTCG-3′
Axin-2	For	5′-GCTACGAGGAAGACCCGCAG-3′
	Rev	5′-TAGTGGTGGTGGACATGCTTCG-3′
C-myc	For	5′-AGGCTCCTGGCAAAAGGTCA-3′
	Rev	5′- ACATTCTCCTCGGTGTCCGA-3′
HuR	For	5′-GTGTCGTATGCTCGCCCG-3′
	Rev	5′-TCTGCCTCCGACCGTTTG-3′
GAPDH	For	5′-TGACATCAAGAAGGTGGTGA-3′
	Rev	5′-TCCACCACCCTGTTGCTGTA-3′

3. Results

3.1. Wnt/β-catenin promotes cytoplasmic localization of HuR

We found previously that β-catenin interacts with HuR in the cytoplasm of cancer cells and promotes stability of the oncogenic gene target mRNA [14,15]. As various signaling pathways are involved in β-catenin cytoplasmic export and regulation of RNA stability by HuR [18–21], we tested whether Wnt signaling is relevant to subcellular localization of HuR. Act D was used as the positive control for HuR export to the cytoplasm [19]. The HuR protein accumulated in the cytoplasm when the cells were treated with LiCl, a glycogen synthase kinase-3β inhibitor that activates β-catenin (Fig. 1A). Next, we tested the role of overexpressing β-catenin as a Wnt signaling downstream effector and also found increased cytoplasmic export of HuR in HeLa cells (Fig. 1B). HuR was clearly localized to the cytoplasm in most HA-β-catenin expressing NIH3T3 cells (Fig. 1C). Taken together, we conclude that Wnt/β-catenin signaling promotes cytoplasmic localization of HuR.

3.2. HuR enhances β-catenin cytoplasmic localization from the perinuclear fraction

Next, we asked whether the HuR protein influences subcellular localization of β-catenin. Thus, HEK293 cells were transfected with HuR overexpressing clones, and the cells were fractionated. We obtained three fractions derived from the nucleus (NE), the cytoplasm (CE), and the nuclear-associated perinuclear fraction (PNE). Interestingly, β-catenin was highly abundant in the PNE, but overexpressing HuR led to accumulation of β-catenin in the CE (Fig. 2A and B). Moreover, the HuR protein was found in the PNE and NE, suggesting that co-localization of HuR and β-catenin in the PNE may be important for regulating both proteins (Fig. 2B). These data clearly demonstrate that the HuR protein induced β-catenin accumulation in the cytoplasm, probably from the PN.

3.3. HuR reduces TCF/β-catenin-mediated transcription

Because overexpressing HuR completely blocked nuclear localization of β-catenin, we then tested whether HuR influences β-catenin transcriptional activity. Overexpressing the HuR protein was confirmed by Western blot (Fig. 3A). Luciferase reporters containing wild-type or mutant TCF/β-catenin binding elements, such as TOPFlash (TOP) or FOPFlash (FOP), were utilized. Significantly reduced β-catenin transcriptional activity was observed (Fig. 3B). Specificity of luciferase activity was tested with non-specific NF-κB responsive reporters (Fig. 3C). The Wnt/β-catenin transcriptional target genes, such as *Axin-2* and *C-myc* mRNA, were measured by RT-PCR to test whether HuR regulates β-catenin target gene expression levels (Fig. 3D). A significant reduction in luciferase activity was detected from natural promoters of the *COX-2* and *Cyclin D1* reporters (Fig. 3E and F). These results indicate that HuR represses β-catenin transcriptional activity and significantly reduces its target gene expression.

3.4. Wnt activated or β-catenin stabilized transcription activity is modulated by HuR

Finally, we tested whether Wnt activated transcription is also regulated by the HuR protein. NIH3T3 cells were treated with

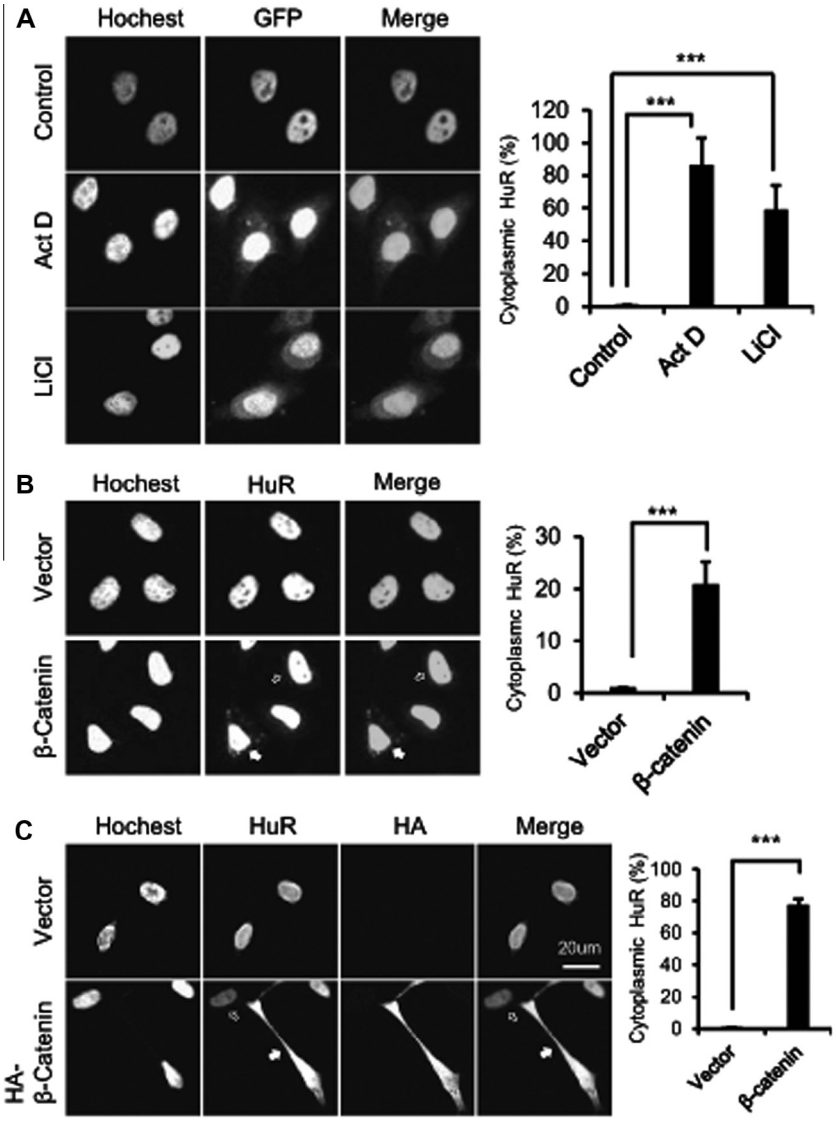

Fig. 1. Cytoplasmic accumulation of Hu antigen R (HuR) following LiCl treatment and β-catenin. (A) HuR immunocytochemistry in HeLa cells. The cells were treated with either actinomycin D (Act D) or LiCl. Cytoplasmic HuR levels in chemically treated cells were compared with those of control cells ($n = 40$, $^{***}p < 0.0001$). (B) Intracellular translocation of HuR upon overexpression of β-catenin. HeLa cells were transfected with β-catenin, and the subcellular locations of HuR were determined by immunocytochemistry. Black arrow indicates a cell with cytoplasmic HuR and the white arrow indicates a cell without cytoplasmic HuR. The percentage of cells with cytoplasmic HuR is shown ($n = 100$, $^{***}p < 0.0001$). (C) Localization of HuR in HA-β-catenin-transfected NIH3T3 cells. The HA antibody was used to detect β-catenin expressing cells. The percentage of cytoplasmic HuR in HA-β-catenin expressing cells (black arrow) were compared with that of control cells (white arrow; $n = 14$, $^{***}p < 0.0001$).

Wnt3a conditioned media, and transcriptional activity was measured with the TOPFlash reporter. Strikingly, Wnt-induced reporter activity decreased significantly by HuR, as did basal TCF/β-catenin transcriptional activity (Fig. 4A). We transfected β-catenin as a FL or as a ΔC to test whether such Wnt activated transcription is mediated by β-catenin. As the β-catenin C-terminal domain is the HuR binding site as well as that for other transcriptional activators [14,22,23], no significant activation of transcription or HuR-mediated repression was observed. In contrast, FLβ-catenin induced β-catenin-mediated reporter activity, which, in turn, was reduced by HuR, confirming specific activation and repression by interactions between β-catenin and HuR (Fig. 4B). The phosphorylation site of the mutated β-catenin protein mimics the Wnt activated state by avoiding proteolysis [2,23,24]. We used HCT116 colorectal carcinoma cells, in which the β-catenin gene was mutated, to stabilize the proteins and test whether HuR modulates such oncogenic β-catenin mutants. TCF/β-catenin-specific transcription activity decreased in a HuR dose-dependent manner (Fig. 4C). Taken together, these results suggest that overexpressed HuR may function as a regulator of Wnt-activated cancer cells.

4. Discussion

In this study, we demonstrated that HuR and β-catenin regulate their cell localization reciprocally; thus, HuR modulates oncogenic transcription of Wnt/β-catenin signaling. As summarized in Fig. 4D, highly abundant β-catenin in the perinuclear compartment was translocated to the nucleus and cytoplasm. Once HuR induced cytoplasmic localization of β-catenin from the perinuclear compartment and/or nucleus, it may reduce β-catenin-mediated transcriptional activity. Considering the well-characterized functional role of nuclear β-catenin during oncogenic transcription, it is counter-intuitive that HuR-induced cytoplasmic localization of β-catenin is relevant to oncogenic transformation. However, our recent findings on the β-catenin and HuR RNP with *COX-2* mRNA reveal a critical role for cytoplasmic β-catenin in oncogenic gene

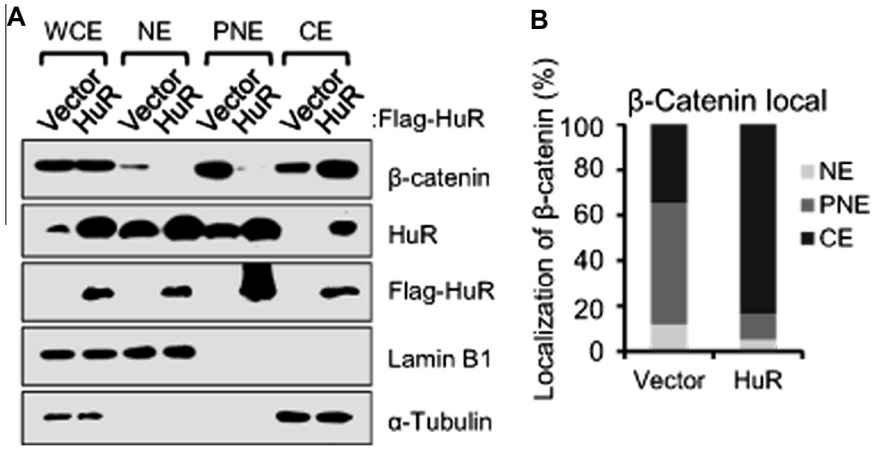

Fig. 2. Enhanced β-catenin cytoplasmic localization by overexpressing Hu antigen R (HuR). (A) Western blot analysis with fractionated HEK293 cells. Nuclear, perinuclear, and cytoplasmic extracts were prepared and designated as NE, PNE, and CE, respectively. Flag-HuR was overexpressed, and the locations of β-catenin and HuR were determined with the indicated antibodies. Anti-α-tubulin and anti-lamin B1 antibodies were used to confirm the subcellular fraction. (B) Quantitation of subcellular β-catenin in (A). Percentages of β-catenin in the NE, PNE, and CE were compared with those in HuR expressing cells.

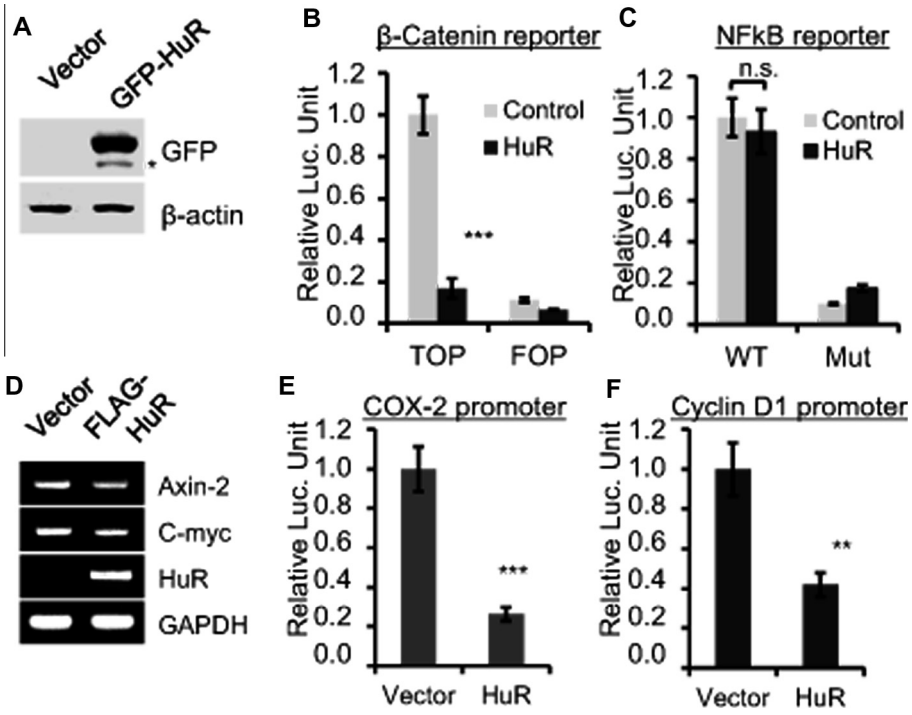

Fig. 3. Repression of TCF/β-catenin-mediated transcription by Hu antigen R (HuR). (A) Western blot analysis of GFP-HuR expression using an anti-GFP antibody in NIH3T3 cells. Anti-β-actin antibody was used as the loading control. Asterisk represents a non-specific band. (B) Luciferase reporter assay with wild-type TCF binding element containing TOPFlash (TOP) or mutant element containing FOPFlash (FOP) reporters. GFP-HuR expression clone was co-transfected as in (A). Firefly luciferase activity was normalized to Renilla luciferase activity, and relative luciferase units are presented as mean ± standard deviation from three independent experiments. (C) Luciferase reporter assay with the will-type nuclear factor-kappa beta (NF-κB) binding element containing the reporter. Results are presented as in (A). (D) Reverse transcription-polymerase chain reaction analysis of target gene expression from Flag-HuR expressed cells. (E) Luciferase reporter assay with the cyclooxygenase (COX)-2 natural promoter reporter. Results are presented as in (A). (F) Luciferase reporter assay with the COX-2 natural promoter reporter. Results are presented as in (A). $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

expression, either by stabilizing RNA or by RNA translation. Interestingly, mobilizing β-catenin and HuR is important for the initiation and progression of tumorigenesis, suggesting an operative role for HuR during Wnt/β-catenin signaling.

Overexpression of HuR and β-catenin is important for tumorigenesis in several cancers [25–34]. In addition, HuR may be an important regulator of Wnt/β-catenin signaling. First, HuR regulates β-catenin protein expression by stabilizing *β-catenin* mRNA and enhancing translation by activating mTOR and increasing HSF1 [34,35]. Second, Wnt receptor *Lrp6* mRNA is a HuR target, so *Lrp6* mRNA is stabilized and its translation is enhanced by HuR. Third, HuR knockdown in stem-cell like WBF344 cells results in a significant decrease in *β-catenin* mRNA levels compared with that in control cells [36]. Thus, HuR must be an important post-transcriptional regulator for the various target mRNAs involved in the Wnt/β-catenin signaling pathway, regardless of the mechanism.

It is assumed that nuclear β-catenin is critical for oncogenic transcription of many target genes. However, our results suggest that a more careful analysis is required to determine the role of

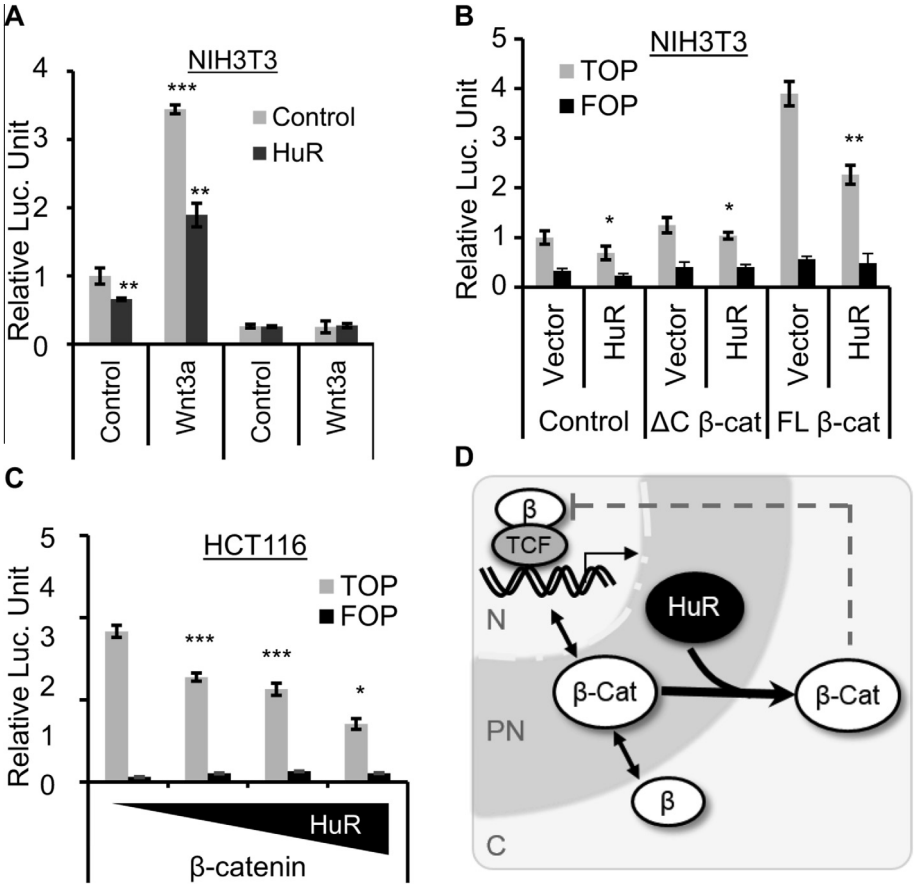

Fig. 4. Modulation of Wnt activation and stabilized β-catenin by Hu antigen R (HuR). (A) Luciferase reporter assay with Wnt3a-activated NIH3T3 cells. TOPFlash (TOP) or FOPFlash (FOP) reporters were co-transfected along with the HuR overexpression clone. Firefly luciferase activity was normalized to Renilla luciferase activity, and relative luciferase units are presented as mean ± standard deviation from three independent experiments. ($^{**}p < 0.001$; $^{***}p < 0.0001$). (B) Luciferase reporter assay with the C-terminal deleted (ΔC) or full-length (FL) β-catenin clones in the presence or absence of the HuR clone. Data are presented as in (A) ($^{*}p < 0.05$; $^{**}p < 0.001$). (C) Luciferase reporter assay in HCT116 cells. An increasing amount of the HuR clone was transfected. Data are presented as in (A) ($^{*}p < 0.05$; $^{***}p < 0.0001$). (D) Model describing the enriched localization of β-catenin in the perinuclear compartment and HuR-induced β-catenin cytoplasmic accumulation. HuR represses β-catenin transcriptional activity by promoting β-catenin export to the cytoplasm. Nuclear (N), perinuclear (PN), and cytoplasmic (C) compartments are indicated. β-Cat or β represent the β-catenin protein.

cytoplasmic β-catenin in tumorigenic events, if any. Moreover, the mechanisms for subcellular translocation of β-catenin are not well understood; thus, how HuR regulates β-catenin translation will be an extremely interesting topic to investigate. Notably, cytoplasmic β-catenin and HuR form RNP with oncogenic transcripts; thus, a mechanistic study on this aberrant RNP will help with understanding RNA-mediated tumorigenesis.

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