

The RNA-binding Protein RNPC1 Stabilizes the mRNA Encoding the RNA-binding Protein HuR and Cooperates with HuR to Suppress Cell Proliferation*

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Background: The mechanism by which HuR expression is controlled is poorly understood.

Results: HuR mRNA is stabilized by RNPC1. Furthermore, HuR, by repressing c-Myc expression, facilitates RNPC1-induced growth suppression.

Conclusion: HuR is a target of RNPC1 and a mediator of RNPC1-induced growth suppression.

Significance: Our results reveal a novel regulation of HuR by RNPC1 via mRNA stability.

The RNA-binding protein HuR, a member of the embryonic lethal abnormal vision/Hu protein family, plays a critical role in many cellular processes, including cell proliferation, angiogenesis, and inflammatory response. Despite significant progresses in understanding how HuR functions, the mechanism by which HuR expression is controlled is still poorly understood. Here, we showed that RNA-binding protein RNPC1 post-transcriptionally regulates HuR expression via mRNA stability. Specifically, we showed that overexpression of RNPC1 increases, whereas knockdown or knock-out of RNPC1 decreases, the level of HuR transcript and protein. Moreover, we showed that RNPC1, but not mutant RNPC1 deficient in RNA binding, stabilizes HuR transcript via binding to its 3'-untranslated region. Furthermore, to determine the biological significance of RNPC1-enhanced HuR expression, we showed that HuR, by repressing c-Myc expression, facilitates RNPC1-mediated growth suppression. Together, we have uncovered a novel mechanism by which HuR is regulated by RNPC1 via mRNA stability and HuR is a mediator of RNPC1-induced growth suppression.

Hu antigen R (HuR)² is a member of the embryonic lethal abnormal vision family of RNA-binding proteins, which can associate with mRNAs containing AU- and U-rich element in their 3'-untranslated regions (3'-UTRs) (1). In response to stress signals, such as UV irradiation and polyamine depletion, HuR is translocated from nucleus to cytosol wherein HuR binds to numerous target mRNAs and then regulates their stability and/or translation (2–6). These mRNAs encode proto-oncogenes (e.g. c-Fos and c-Myc) (7–9), tumor suppressor (e.g. p53) (10), cell cycle regulators (e.g. Cyclins A, B1, D1, and E) (11–13), cyclin-dependent kinase (cdk) inhibitors (e.g. p21 and p27) (2, 14), and growth factors (e.g. vascular endothelial growth factor) (15). By regulating these targets, HuR has been impli-

cated in various biological progresses, including cell proliferation, immune and stress response, differentiation, and carcinogenesis.

Given the functional importance of HuR, studies have been carried out to elucidate the underlying mechanisms by which HuR is regulated. For example, in response to various stimuli, HuR translocates from nucleus to cytosol where it regulates the stability and/or translation of its targets. The translocation of HuR is tightly controlled by several kinases, including Cdk1, Chk2, p38, PKC α , and PKC δ (4, 16–19). In addition, HuR can be cleaved by caspases-3 and -7 at aspartate 226, and the cleaved HuR product is able to promote apoptotic cell death independent of its RNA-binding activity (7, 20). Recently, HuR was found to negatively regulate its own expression by promoting alternative polyadenylation site usage (21). Furthermore, HuR is found to be regulated by several microRNAs, including miR-519, miR-16, and miR-125b, via protein translation (22–24). Nevertheless, very little is known whether HuR can be regulated by other post-transcriptional mechanisms, such as mRNA stability.

The RNA-binding protein RNPC1, also called RBM38, is expressed as two isoforms, RNPC1a with 239 amino acids and RNPC1b with 121 amino acids (25). RNPC1 belongs to the RNA recognition motif (RRM)-containing RNA-binding protein family, which also includes HuR and nucleolin. Like HuR, RNPC1 is able to bind to transcripts containing AU- or U-rich elements and regulate their stability or translation. Studies from our laboratory and others show that RNPC1 can stabilize p21 transcripts by binding to its 3'-UTR and, subsequently, increase p21 expression (25–27). In addition, we also showed that RNPC1 is able to modulate the RNA-binding activity of, and cooperate with, HuR to regulate p21 mRNA stability via physical interaction (28). Interestingly, unlike their collaborative regulation of p21 mRNA stability, RNPC1 and HuR are found to have opposing effects on p53 translation (10, 29). Although it is unclear how RNPC1 and HuR differentially regulate their common targets, these data suggest that the function of these two proteins is intimately linked. In the current study, we have investigated the role of RNPC1 in regulating HuR expression. Specifically, we found that overexpression of RNPC1 increases, whereas knockdown or knock-out of RNPC1

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² The abbreviations used are: HuR, Hu antigen R; UTR, untranslated region; REMSA, RNA electrophoretic mobility shift assay; MEF, mouse embryonic fibroblast.

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decreases, the level of HuR transcript and protein. Moreover, we showed that RNPC1 stabilizes HuR mRNA via binding to its 3'-UTR. Furthermore, we found that HuR facilitates RNPC1-mediated growth suppression by repressing *c-Myc* expression. Together, these data suggest a novel regulation of HuR by RNPC1 via mRNA stability.

EXPERIMENTAL PROCEDURES

Plasmids—pGEX vectors expressing GST-tagged wild-type RNPC1a or RNP1- or RNP2-deletion RNPC1a mutants (Δ RNP1 or Δ RNP2) were used for producing recombinant protein as previously described (28). pcDNA3 vectors expressing HA-tagged RNPC1a, Δ RNP1, and Δ RNP2 were generated as previously described (28). pcDNA4/TO vectors expressing RNPC1a, HA-RNPC1a, and HA-RNPC1b were generated as previously described (25). All lentivirus vectors (pLKO.1-puro) expressing shRNA of interest were purchased from Sigma. The targeting sequences are 5'-CGC TGA GTA CTT CGA AAT GTC-3' for control luciferase shRNA, 5'-CAG AAG GAC ACC ACG TTC A-3' for RNPC1 shRNA, 5'-CGA GCT CAG AGG TGA TCA AAG-3' for HuR shRNA, and 5'-TGA GAC AGA TCA GCA ACA A-3' for *c-Myc* shRNA.

Cell Culture—RKO, MCF7, HCT116, *p53*^{-/-}HCT116, and H1299 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) as previously described (25). Primary mouse embryonic fibroblasts (MEFs) were generated as previously reported (29). Briefly, MEFs were cultured in DMEM supplemented with 10% fetal bovine serum, 55 μ M β -mercaptoethanol, and 1 \times MEM non-essential amino acid solution (Cellgro).

Tet-on-inducible System and Cell Line Generation—The inducible cell lines in this study were generated by using a Tet-on-inducible system. In this system, two plasmids were used. The first one is called pcDNA6/TR, which encodes the Tet repressor under the control of the human CMV promoter. The second one is called pcDNA4/TO, a plasmid for inducible expression of a target gene, with two tetracycline operator sites in the CMV promoter. In the absence of tetracycline, the Tet repressor binds to the Tet repressor sequence in the promoter of the inducible expression vector and inhibits gene expression. In the presence of tetracycline, the Tet repressor binds to tetracycline with high affinity and thus is unable to induce gene expression.

RKO, MCF7, HCT116, *p53*^{-/-}HCT116, and H1299 cells that can inducibly express HA-tagged RNPC1a or RNPC1b were generated as previously described (30). Briefly, to generate stable cell lines that can inducibly express HA-tagged RNPC1a or RNPC1b, pcDNA4 vector containing HA-tagged RNPC1a or RNPC1b was transfected into parental cells expressing a TetR (pcDNA6). The cells were selected with Zeocin and confirmed by Western blot analysis. To induce HA-tagged RNPC1a or RNPC1b expression, doxycycline (0.5 μ g/ml), a tetracycline analog, was added to the medium for various times.

RNA Interference—Scrambled siRNA (GGC CGA UUG UCA AAU AAU U), siRNAs against RNPC1a (5'-CAC CUU GAU CCA GCG GAC UUA-3'), or HuR (5'-GGG AUA AAG UAG CAG GAC A-3') were purchased from Dharmacon (Chicago, IL). For siRNA transfection, siLentFectTM Lipid Reagent (Bio-

Rad) was used according to the user's manual. For lentiviral production and transduction, a lentivirus vector (10 μ g) expressing shRNA of interest, along with packaging plasmids, pRSV-REV (5 μ g), pMDL g/p RRE (5 μ g), and VSVG (5 μ g), was cotransfected into 293T cells (1×10^7) by using ExpressFectTM transfection reagent (Denville Scientific) according to the user's manual. After 48 h, the supernatant containing shRNA-expressing lentivirus was filtered and concentrated by ultracentrifugation (25,000 rpm, 4 °C, 2 h). The concentrated lentiviral particles were then used to transduce cells, followed by puromycin selection (1 μ g/ml) for 3–4 days.

Western Blot Analysis—Whole cell lysates were prepared by using 2 \times SDS sample buffer. Cell lysates were separated in 8–12% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the indicated antibodies, followed by ECL detection. The antibodies used in this study were: anti-HuR (H-280, Santa Cruz Biotechnology), anti-p21 (purified rabbit polyclonal), anti-*c-Myc* (C-33, Santa Cruz Biotechnology), anti-RNPC1 (purified rabbit polyclonal), anti-actin (Sigma), and anti-HA (HA.11, Covance).

RNA Isolation, RT-PCR, and Quantitative PCR—Total RNA was isolated using TRIzol reagent (Invitrogen) according to the user's manual. cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) according to users' manual. Quantitative PCR was performed in 20- μ l reactions using 2 \times qPCR SYBR Green Mix (ABgene, Epsom, UK) with 5 μ M primers. Reactions were run on a Real-time PCR system (Eppendorf Mastercycler ep realplex, Eppendorf, Germany) using a three-step cycling program: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 68 °C for 30 s. A melting curve (57–95 °C) was generated at the end of each run to verify the specificity. The primers for RNPC1, HuR, p21, and GAPDH were used as previously described (25, 28).

RNA Immunoprecipitation—RNA immunoprecipitation was carried out as previously described (25, 31). Briefly, cells (1×10^7) were lysed with 1 ml of lysis buffer (10 mM HEPES, pH 7.0, 100 mM KCl, 5 mM MgCl₂, 0.5% Nonidet P-40, 1 mM DTT) supplemented with RiboLock ribonuclease inhibitor (Fermentas) for 15 min, and cell lysates were collected by centrifugation (14,000 rpm, 4 °C, 10 min). The RNA-protein immunocomplexes were formed by incubating 400 μ l of cell lysates with 4 μ g of anti-HA or isotype control IgG at 4 °C for 4 h and brought down by 25 μ l of protein A/G bead (50% slurry). RT-PCR analysis was carried out to examine the RNA-protein interaction.

Probe Labeling and REMSA—All probes were labeled by *in vitro* transcription using a PCR template containing the T7 promoter and various regions of HuR 3'-UTR. Briefly, 500 ng of PCR product was incubated with 50 μ Ci of [α -³²P]rUTP, 0.5 mM each of rNTP (A, G, and C), 20 units of T7 RNA polymerase (Ambion) in 20 μ l of reaction at 37 °C for 1 h, followed by DNase I (1 unit) treatment for 10 min. The reaction mixture was purified by using a Sephadex G-50 column to remove unlabeled free nucleotide, and the radioactivity of probes was measured by using a scintillation counter. RNA electrophoretic mobility shift assay (REMSA) was carried out with a modified protocol as previously described (2, 10, 32, 33). Briefly, 250 nM of recombinant protein, 100 μ g/ml yeast tRNA, and 50,000

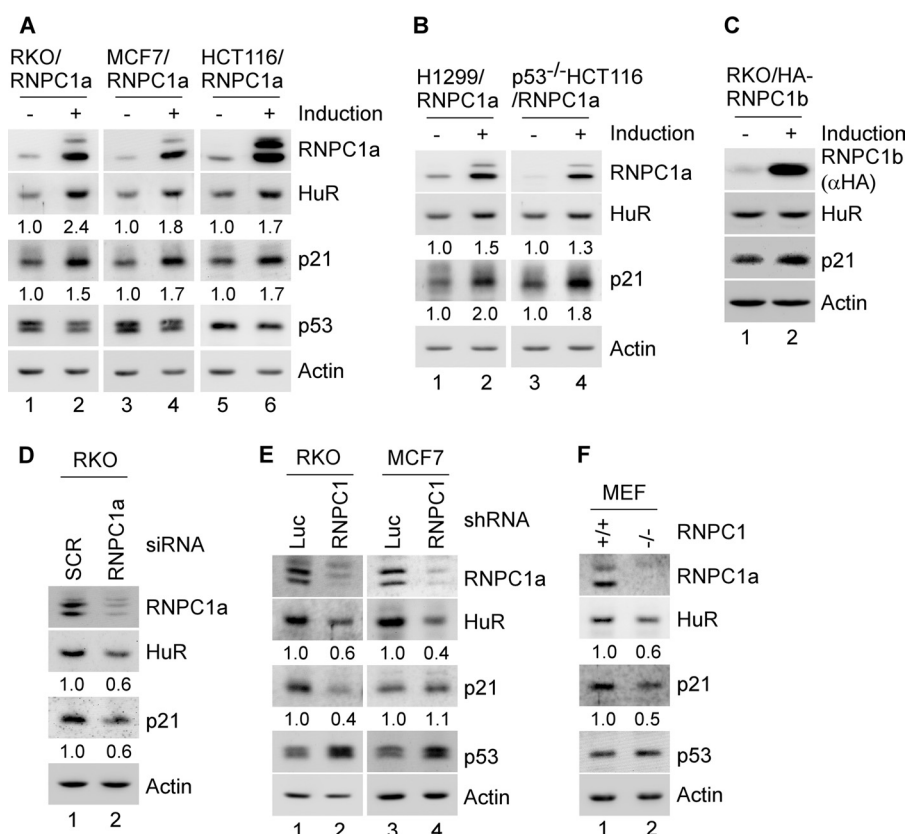


FIGURE 1. HuR expression is enhanced by RNPC1a. *A*, the level of HuR is increased by RNPC1a. RKO, MCF7, and HCT116 cells were un-induced or induced to express RNPC1a for 48 h, and the level of RNPC1a, p21, p53, HuR, and actin was determined by Western blot analysis. The level of HuR or p21 was normalized to that of actin and arbitrarily set at 1.0 in control cells. The relative -fold change of each protein level is shown below each lane. The data are representative of three independent experiments. *B*, RNPC1a increases HuR expression independent of p53. The experiment was performed as described in *A* except that H1299 and p53^{-/-} HCT116 cells were used. The basal level of HuR or p21 was arbitrarily set at 1.0, and relative -fold change of each protein level is shown below each lane. The data are representative of three independent experiments. *C*, RNPC1b has no effect on HuR expression. The level of RNPC1b, HuR, p21, and actin was measured in RKO cells with or without RNPC1b induction for 24 h. The data are representative of three independent experiments. *D*, knockdown of RNPC1a leads to decreased expression of HuR. The level of RNPC1a, p21, HuR, and actin was determined by Western blot analysis in RKO cells transiently transfected with 40 nM of scrambled siRNA (SCR) or siRNA against RNPC1a for 48 h. The basal level of HuR or p21 was arbitrarily set at 1.0, and relative -fold change of each protein level is shown below each lane. The data are representative of three independent experiments. *E*, RKO and MCF7 cells were transfected with a lentivirus expressing a control luciferase (Luc) shRNA or RNPC1 shRNA, selected by puromycin for 3 days. Cell lysates were collected, and the level of RNPC1a, HuR, p21, p53, and actin was determined by Western blot analysis. The basal level of HuR or p21 was arbitrarily set at 1.0, and -fold change is shown below each lane. The data are representative of three independent experiments. *F*, knock-out of RNPC1 leads to increased expression of HuR in mouse embryonic fibroblasts (MEFs). The level of RNPC1a, HuR, p21, p53, and actin in wild-type and RNPC1^{-/-} MEFs was measured by Western blot analysis. The basal level of HuR or p21 was arbitrarily set at 1.0, and relative -fold change of each protein level is shown below each lane. The data are representative of three independent experiments.

CPM of ³²P-labeled RNA probe (≈ 1 nM) were mixed in 20 μ l of reaction buffer (10 mM Tris-Cl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, 1 mM DTT) at 25 °C for 20 min. To remove the unbound region of RNA probe, RNA-protein complexes were digested by adding 100 units of RNase T1 at 37 °C for 15 min and then separated in 6% of native PAGE gel. RNA-protein complexes were visualized by autoradiography. For the supershift assay, 2 μ g of anti-HA antibody was preincubated with HA-tagged proteins for 30 min on ice prior to incubation with RNA probe.

Colony Formation Assay—RKO or H1299 cells (1500 per well) were seeded in each well of a 6-well plate and cultured in the absence or presence of RNPC1a along with or without HuR and/or c-Myc knockdown for 14 days. Cells were then fixed with methanol/glacial acetic acid (7:1, v/v) for 20 min and stained with 0.1% of crystal violet for 6 h. The percent density of the colony was quantified by densitometry.

Statistical Analysis—Statistical significance was determined by two-tailed Student's *t* test. *p* < 0.05 was considered as significant.

RESULTS

HuR Expression Is Enhanced by RNPC1—RKO, HCT116, and MCF7 cells that can express RNPC1a were generated by using a Tet-on-inducible system as describe in the “Experimental Procedures.” To determine whether HuR expression is regulated by RNPC1, cells were treated with or without 0.5 μ g/ml tetracycline to induce or un-induce RNPC1 expression for 48 h, followed by Western blot analysis. We showed that the level of RNPC1a was detectable upon induction (Fig. 1A, RNPC1 panel). Interestingly, the level of HuR was markedly increased upon RNPC1a induction (Fig. 1A, compare lanes 1, 3, and 5 with lanes 2, 4, and 6, respectively). Consistent with previous reports, the level of p21 was increased, whereas p53 was decreased, by RNPC1a (Fig. 1A, p53 and p21 panels) (25, 26, 28, 29). Similarly, we showed that RNPC1a was able to enhance HuR expression in p53-null H1299 and p53^{-/-} HCT116 cells, accompanied with an increased expression of p21 (Fig. 1B, compare lanes 1 and 3 with lanes 2 and 4, respectively). By

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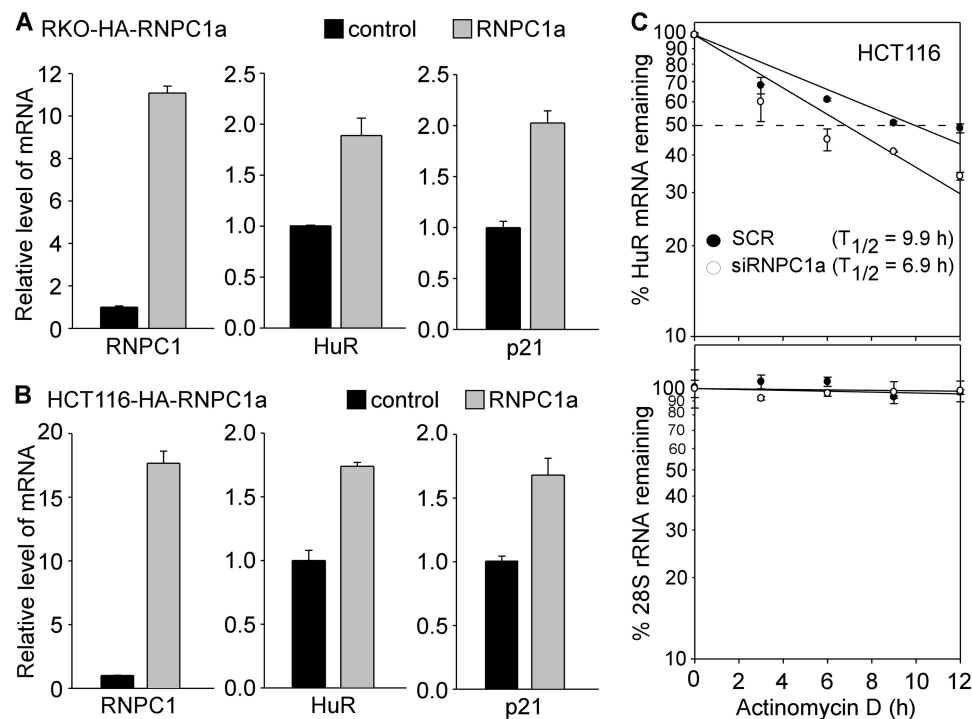


FIGURE 2. HuR mRNA stability is regulated by RNPC1. *A* and *B*, the level of HuR transcript is up-regulated by RNPC1. Total RNAs were isolated, and quantitative RT-PCR was performed in triplicates to measure the level of RNPC1, HuR, and p21 transcripts. RKO (*A*) or HCT116 (*B*) cells were un-induced (control) or induced (RNPC1a) to express RNPC1a for 36 h. The level of RNPC1, HuR, and p21 transcript was normalized to that of the GAPDH transcript. The relative -fold change is the ratio of the transcript level in RNPC1-expressing cells versus that in control cells. The error bars indicate standard deviation. *C*, knockdown of RNPC1 destabilizes HuR transcript. (*upper panel*) and 28S rRNA (*lower panel*) was measured by quantitative RT-PCR in HCT116 cells transiently transfected with 40 nM of scrambled siRNA or siRNA against RNPC1a for 3 days, followed by treatment with actinomycin D (5 μ g/ml) for various times. The relative half-life of the HuR transcript was calculated from triplicate samples and is presented as mean \pm S.D.

contrast, RNPC1b had little, if any, effect on HuR expression (Fig. 1C). Because RNPC1b does not affect HuR expression, this study focused on RNPC1a. To simplify, RNPC1 and RNPC1a are interchangeably used here.

Next, to determine whether HuR is regulated by endogenous RNPC1, scrambled siRNA or siRNA against RNPC1 was transiently transfected into RKO cells. As shown in Fig. 1D, the level of endogenous RNPC1 was decreased by RNPC1 siRNA, but not scrambled siRNA, concomitantly with decreased expression of p21 as expected (Fig. 1D, compare lane 1 with 2). Importantly, the level of HuR was greatly decreased upon RNPC1 knockdown (Fig. 1D, compare lane 1 with 2), consistent with the data that ectopic expression of RNPC1 increases expression of HuR (Fig. 1, *A* and *B*). Likewise, knockdown of RNPC1 by shRNA was able to decrease HuR expression in RKO and MCF7 cells, along with decreased expression of p21 and increased expression of p53 (Fig. 1E, compare lanes 1 and 3 with lanes 2 and 4, respectively). We note that increased expression of p53 leads to elevated transcription of the p21 (*CDKN1A*) gene as evidenced by increased levels of p21 pre-mRNA (29). However, due to the decreased mRNA stability upon knockdown of RNPC1, the level of mature p21 mRNA is decreased, resulting in decreased levels of p21 protein (25, 29). Furthermore, we showed that the level of HuR was markedly decreased in primary *RNPC1*^{-/-} MEFs as compared with that in wild-type MEFs (Fig. 1F, compare lane 1 with 2). Together, these data suggest that RNPC1 enhances HuR expression.

HuR mRNA Stability Is Regulated by RNPC1—RNA-binding proteins are known to regulate gene expression via post-trans-

criptional mechanisms, including mRNA stability. To explore how RNPC1 regulates HuR expression, quantitative RT-PCR was performed to measure the level of HuR transcript in RKO and HCT116 cells that can inducibly express RNPC1. We showed that, upon induction, the level of RNPC1 transcript was increased ~10- 15-fold in RKO and HCT116 cells (Fig. 2, *A* and *B*, *left panels*). Interestingly, following RNPC1 induction, the level of HuR transcript was increased 1.7- and 1.9-fold in RKO and HCT116 cells, respectively (Fig. 2, *A* and *B*, *middle panels*). As a control, p21 transcript was increased 1.5- 2.0-fold by RNPC1 (Fig. 2, *A* and *B*, *right panels*). These results suggest that RNPC1 may regulate the half-life of HuR transcript. To address this, HCT116 cells were transiently transfected with scrambled siRNA or siRNA against RNPC1 for 3 days, followed by treatment of actinomycin D to inhibit *de novo* RNA synthesis for various times, and the level of HuR mRNA was measured by quantitative RT-PCR. We showed that the half-life of HuR mRNA was decreased from ~9.9 h in control cells to ~6.9 h in RNPC1-knockdown cells (Fig. 2C, *upper panel*). As a control, the half-life of 28S rRNA was not altered by RNPC1 knockdown (Fig. 2C, *lower panel*). Together, our data indicate that RNPC1 stabilizes HuR transcript.

RNPC1 Directly Binds to the AU-rich Element in HuR 3'-UTR—To further explore how RNPC1 regulates HuR mRNA stability, we sought to determine whether RNPC1 associates with the HuR transcript. Thus, an RNA immunoprecipitation assay followed by RT-PCR was performed using HCT116 cells that can inducibly express HA-tagged RNPC1. We found that HuR mRNA was present in RNPC1, but not control IgG, immu-

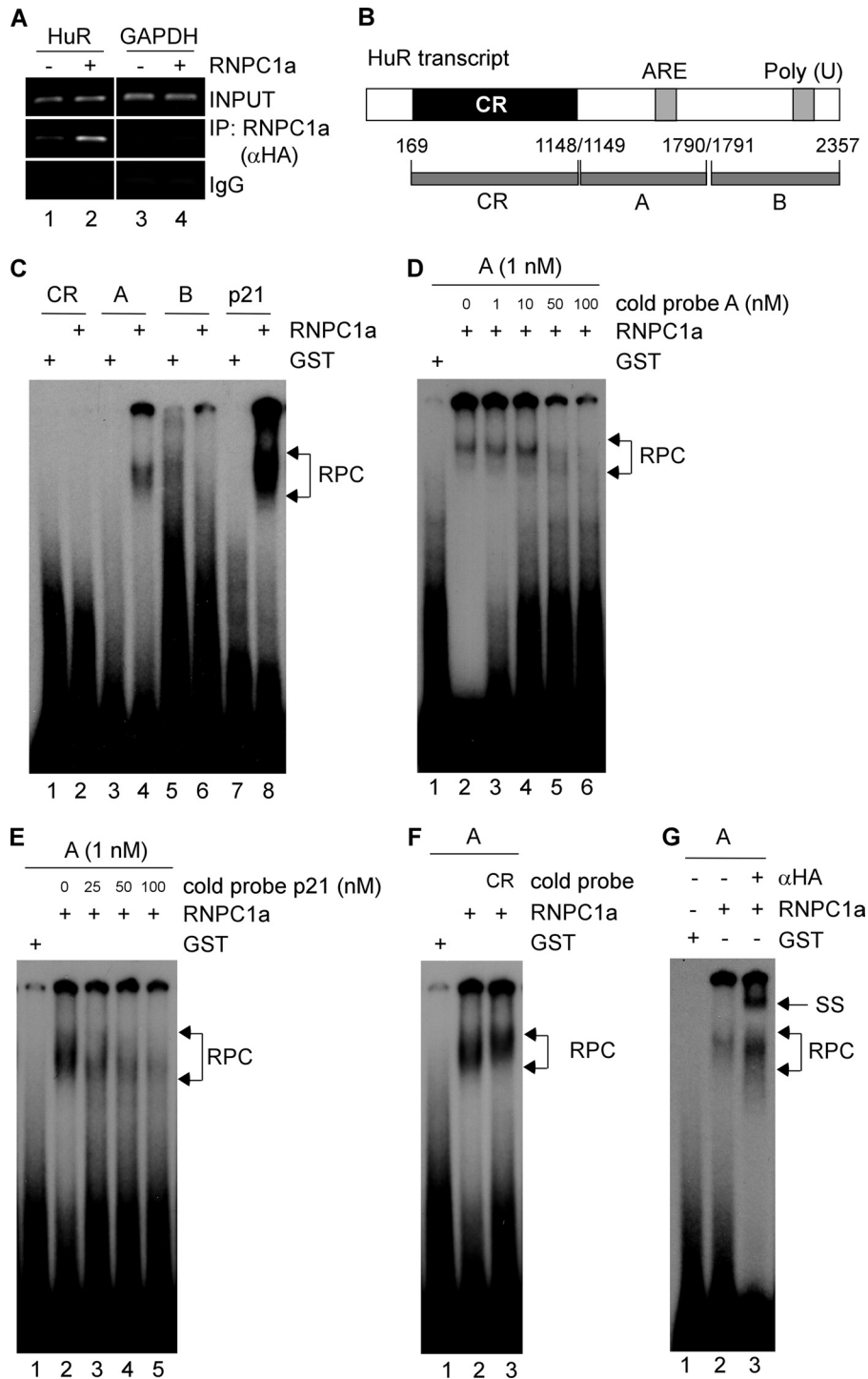


FIGURE 3. RNPC1 directly binds to the 3'-UTR of HuR transcript. *A*, RNPC1 interacts with HuR transcript *in vivo*. RKO cells were un-induced or induced to express HA-tagged RNPC1 for 24 h. Cell lysates were collected and immunoprecipitated with HA antibody or control IgG, followed by RT-PCR analysis to determine the level of the HuR and GAPDH transcripts in control IgG and RNPC1 immunocomplexes. *B*, schematic presentation of HuR transcript and the location of probes. AU-rich element (ARE) and poly(U) regions are shown in the shaded box. *C*, RNPC1 directly binds to the 3'-UTR of HuR transcript. REMSA was performed by mixing a ³²P-labeled RNA probe (CR, A, B, or p21 probe) with recombinant GST or GST-fused HA-RNPC1. The bracket indicates RNA-protein complexes. *D* and *E*, REMSA assay was performed by adding various amounts of unlabeled probe A (*D*) or p21 probe (*E*) in the reaction mixture containing radiolabeled probe A (1 nM). *F*, REMSA assay was performed by adding an excess amount of unlabeled probe CR (100 nM) in the reaction mixture containing radiolabeled probe A (1 nM). *G*, supershift assay was performed by adding 2 μg of anti-HA in the reaction to "supershift" the RNPC1-probe A complex.

nocomplexes (Fig. 3*A*, left panel, compare lane 1 with 2). As a control, RNPC1 was unable to associate with GAPDH mRNA (Fig. 3*A*, right panel). Next, REMSA was performed to map the binding site of RNPC1 in the HuR transcript with three HuR

RNA probes: probe CR, which contains the entire coding region, probe A, which contains the 5' portion of the 3'-UTR, and probe B, which contains the 3' portion of the 3'-UTR (Fig. 3*B*). A p21 probe, which is derived from p21-3'-UTR and

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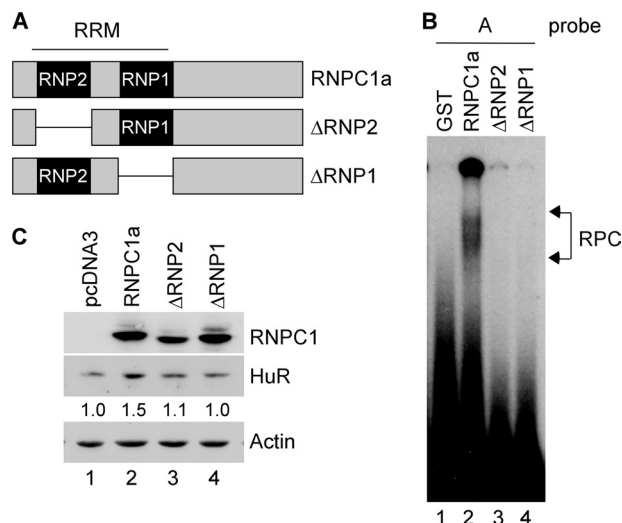


FIGURE 4. The RNA-binding domain in RNPC1 is required for regulating HuR expression. A, schematic presentation of RNPC1a, Δ RNP2, and Δ RNP1. B, REMSA was performed by mixing 32 P-labeled RNA probe A with recombinant GST, GST-HA-RNPC1a, GST-HA- Δ RNP1, and GST-HA- Δ RNP2 proteins, respectively. C, Δ RNP1 and Δ RNP2 mutants are unable to increase HuR expression. MCF7 cells (1×10^6) transiently transfected with 1 μ g of empty vector or vectors expressing HA-RNPC1a, Δ RNP1, or Δ RNP2 for 48 h. Cell lysates were collected, and the level of RNPC1, Δ RNP1 and Δ RNP2, HuR, and actin was determined by Western blot analysis. The basal level of HuR was arbitrarily set at 1.0, and relative -fold change of each protein level is shown below each lane. The data are representative of three independent experiments.

known to contain a RNPC1-binding site, was used as a control (25, 28). We showed that recombinant GST-fused HA-RNPC1, but not GST protein, formed a complex with probe A or p21 probe, but not probes CR and B (Fig. 3C, compare lanes 1, 3, 5, and 7 with lanes 2, 4, 6, and 8, respectively). Moreover, the formation of RNA-protein complex was gradually inhibited by adding various amounts of cold probe A (Fig. 3D) or p21 probe (Fig. 3E), but not by cold probe CR (Fig. 3F). Furthermore, the specificity of this RNA-protein complex was confirmed by supershift analysis with anti-HA, which recognizes HA-tagged RNPC1 (Fig. 3G, lane 3).

The RNA-binding Domain in RNPC1 Is Required for Regulating HuR Expression—The RNA binding domain in RNPC1 is composed of two RNA-binding submotifs: RNP1 and RNP2 (25). Thus, we determined whether the RNA-binding activity of RNPC1 is required for regulating HuR expression. To address this, two RNPC1 mutants, lacking either RNP1 or RNP2 (Fig. 4A), were used for the REMSA assay. We found that these two deletion mutants were unable to bind HuR transcript *in vitro* (Fig. 4B, compare lane 2 with lanes 3 and 4). Consistent with this, the deletion mutants failed to increase HuR expression (Fig. 4C, compare lane 2 with lanes 3 and 4). Together, these data suggest that the RNA binding domain in RNPC1 is required for binding to the HuR transcript and enhancing HuR expression.

Knockdown of HuR Attenuates RNPC1-mediated Growth Suppression—We have previously reported that RNPC1 can induce growth suppression at least in part by stabilizing the p21 transcript (25). Because HuR is also a critical regulator of the cell survival and death pathways, we sought to determine whether the increased expression of HuR by RNPC1 plays a role

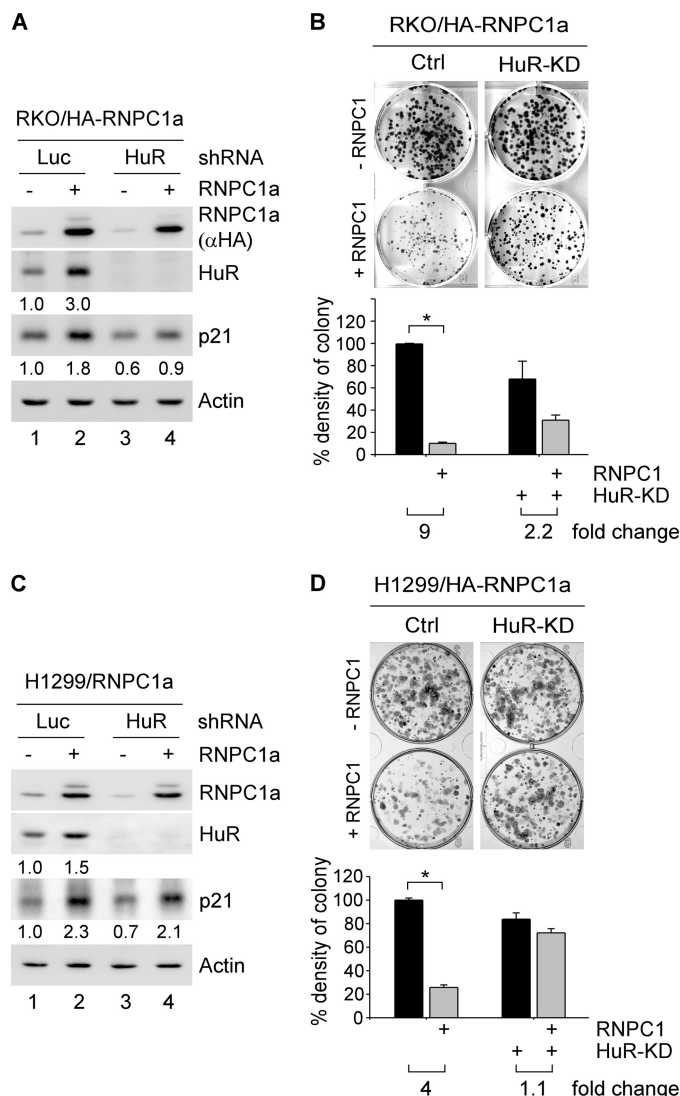


FIGURE 5. HuR knockdown attenuates RNPC1-mediated growth suppression. A, RKO cells were transduced with a lentivirus expressing control luciferase shRNA or HuR shRNA, selected by puromycin for 3 days, and then uninduced or induced to express RNPC1a for 48 h. Cell lysates were collected, and the levels of RNPC1, HuR, p21, and actin were measured by Western blot analysis. The basal level of HuR or p21 was arbitrarily set at 1.0, and relative -fold change of each protein level is shown below each lane. The data are representative of three independent experiments. B, HuR knockdown attenuates RNPC1-induced growth suppression in RKO cells. Upper panel, colony formation assay was performed using the cells treated as in A. Lower panel, colony numbers from the wells in the upper panel were quantified, and the rate of colony formation at the control condition was set as 100%. The -fold change is the ratio of colonies formed in the absence of RNPC1 versus that in the presence of RNPC1. *, $p < 0.05$ by two-tailed Student's *t*-test. C, the experiment was performed as in A, except that H1299 cells were used. D, HuR knockdown attenuates RNPC1-induced growth suppression in H1299 cells. Upper panel, colony formation assay was performed as using the cells treated as in C. Lower panel, quantitative analysis was performed as in B, lower panel.

in RNPC1-mediated growth suppression. To address this, a lentivirus expressing a control or HuR shRNA was transduced into RKO cells that can inducibly express RNPC1, followed by a colony formation assay. As shown in Fig. 5A, the level of RNPC1 was detectable upon induction (Fig. 5A, lanes 2 and 4). However, the increased HuR expression by RNPC1 was completely abolished by HuR shRNA (Fig. 5A, compare lanes 1 and 2 with lanes 3 and 4). Interestingly, the level of p21 was still increased

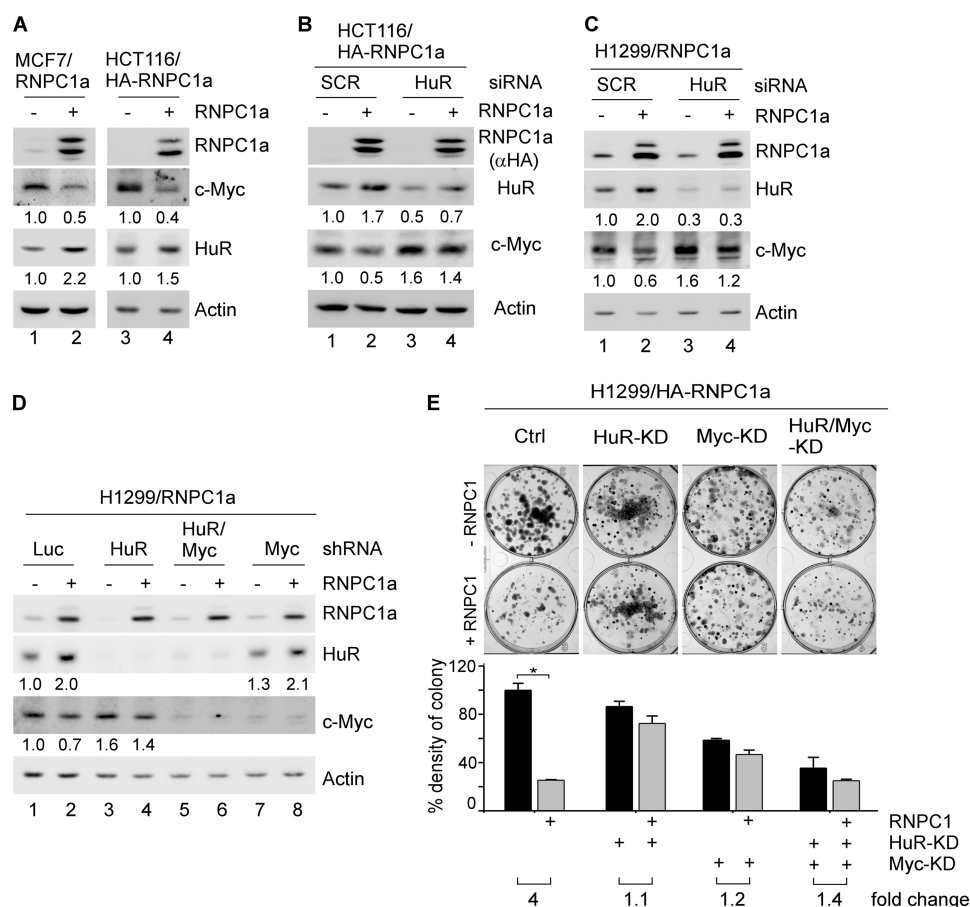


FIGURE 6. HuR-mediated c-Myc repression is critical for the growth suppression by RNPC1. *A*, the level of c-Myc is repressed by RNPC1. MCF7 and HCT116 cells were un-induced or induced to express RNPC1 for 48 h, and the level of RNPC1a, HuR, c-Myc, and actin was measured by Western blot analysis. The basal level of c-Myc or HuR was arbitrarily set at 1.0, and relative -fold change of each protein level is shown *below* each lane. The data are representative of three independent experiments. *B* and *C*, RNPC1 repression of c-Myc is HuR-dependent. HCT116 (*B*) and H1299 (*C*) cells were transiently transfected with scrambled or RNPC1a siRNA for 24 h, and then un-induced or induced to express RNPC1 for 48 h. The level of RNPC1, HuR, c-Myc, and actin was measured by Western blot analysis. The basal level of HuR or c-Myc was arbitrarily set at 1.0, and relative -fold change of each protein level is shown *below* each lane. The data are representative of three independent experiments. *D*, H1299 cells were transduced with a lentivirus expressing control luciferase shRNA or shRNAs against HuR and/or c-Myc, selected by puromycin for 3 days and then un-induced or induced to express RNPC1a for 48 h. Cell lysates were collected, and the levels of RNPC1, HuR, c-Myc, and actin were measured by Western blot analysis. The basal level of HuR or c-Myc was arbitrarily set at 1.0, and -fold change is shown *below* each lane. The data are representative of three independent experiments. *E*, HuR and/or c-Myc knockdown attenuates RNPC1-induced growth suppression. *Upper panel*, colony formation assay was performed with H1299 cells treated as in *D*. *Lower panel*, colony numbers from the wells in the *upper panel* were quantified and the rate of colony formation at the control condition was set as 100%. The -fold change is the ratio of colonies formed in the absence of RNPC1 *versus* that in the presence of RNPC1. *, $p < 0.05$ by two-tailed Student's *t*-test.

by RNPC1, although to a less extent upon HuR knockdown (Fig. 5*A*, compare *lanes 1* and *3* with *lanes 2* and *4*, respectively). Significantly, the colony formation assay indicated that HuR knockdown greatly diminished the growth suppression mediated by RNPC1 (Fig. 5*B*, *upper panel*). Quantitative analysis indicated that, upon knockdown of HuR, the ability of RKO cells to form colonies was reduced by RNPC1 from 9- to 2.2-fold (Fig. 5*B*, *lower panel*). Nevertheless, knockdown of HuR alone led to a 32.1% of reduction of colony formation in the absence of RNPC1 expression, consistent with a previous report (11). Furthermore, we showed that HuR knockdown greatly attenuated RNPC1-mediated growth suppression in H1299 cells from 4- to 1.1-fold (Fig. 5, *C* and *D*). Together, these data suggest that HuR mediates RNPC1-dependent growth suppression.

HuR-mediated Repression of c-Myc Is Required for RNPC1-dependent Growth Suppression—Because p21 can be increased by RNPC1 independent of HuR (Fig. 5, *A* and *C*), we postulated

that the increased expression of HuR by RNPC1 may affect other HuR targets that control cell proliferation. One candidate is the proto-oncogene c-Myc, a master regulator of cell proliferation, which has been shown recently to be repressed by HuR (8, 34). In this regard, the level of c-Myc was measured in MCF7 and HCT116 cells that can inducibly express RNPC1. We found that c-Myc was repressed by RNPC1, concomitantly with increased expression of HuR (Fig. 6*A*, compare *lanes 1* and *3* with *lanes 2* and *4*, respectively). Next, to determine whether HuR plays a role in RNPC1-mediated repression of c-Myc, scrambled siRNA or siRNA against HuR was transiently transfected into HCT116 cells in the presence or absence of RNPC1. We found that the overall level of c-Myc was increased upon HuR knockdown as expected (Fig. 6*B*, compare *lanes 1* and *3* with *lanes 2* and *4*, respectively). However, RNPC1-mediated repression of c-Myc was nearly abolished by HuR knockdown (Fig. 6*B*, compare *lanes 1* and *3* with *lanes 2* and *4*, respectively). Similarly, knockdown of HuR abrogated RNPC1 to suppress c-Myc expression in H1299 cells

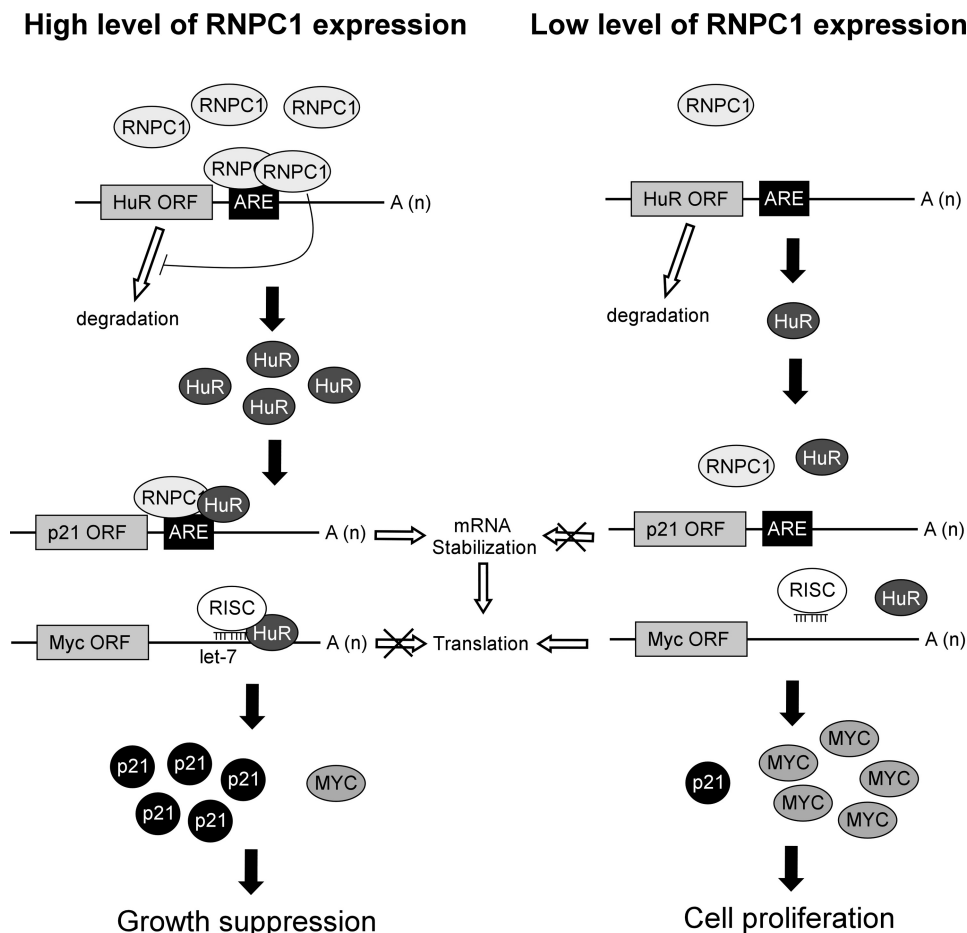


FIGURE 7. A model for the role of RNPC1 and HuR in regulating cell proliferation.

(Fig. 6C, compare lanes 1 and 3 with lanes 2 and 4, respectively). Furthermore, to examine whether the repression of c-Myc via HuR plays a role in RNPC1-dependent growth suppression, H1299 cells were transduced with a lentivirus expressing a control shRNA or shRNAs against HuR and/or c-Myc in the presence or absence of RNPC1 expression for colony formation assay. We showed that RNPC1 was expressed upon induction, whereas HuR and/or c-Myc were knocked down upon transduction of shRNA-expressing lentivirus (Fig. 6D). Importantly, we found that, upon knockdown of HuR and/or c-Myc, the ability of RNPC1 to suppress colony formation was abrogated (Fig. 6E). Nevertheless, we would like to note that knockdown of HuR and/or c-Myc was able to suppress colony formation in the absence of RNPC1 (Fig. 6E, upper panel). Quantitative analysis indicated that, upon knockdown of HuR and/or c-Myc, the ability of H1299 cells to form colonies was reduced by RNPC1 from 4- to 1.4-fold or less (Fig. 6E, lower panel). Together, these data indicate that HuR, by repressing c-Myc expression, facilitates RNPC1-mediated growth suppression.

DISCUSSION

HuR plays a critical role in regulating cell cycle progression, differentiation, and immune response. In the current study, we have uncovered a novel regulation of HuR by RNA-binding protein RNPC1 via mRNA stability. Specifically, we showed that overexpression of RNPC1 increases, whereas knockdown

or knock-out of RNPC1 decreases, the level of HuR transcript and protein. In addition, we showed that RNPC1 increases the half-life of HuR transcript. Moreover, we showed that RNPC1 directly binds to the 3'-UTR of HuR transcripts. Finally, we showed that HuR, by repressing c-Myc expression, facilitates RNPC1-mediated growth suppression. Based on these findings, we propose a model for RNPC1-regulated HuR expression and its role in regulating cell proliferation (Fig. 7).

The Regulation of HuR by RNPC1—Our results indicate that RNPC1 can stabilize HuR transcript via binding to the AU-rich element in HuR 3'-UTR (Figs. 2 and 3). In addition, we showed that the RNA-binding domain in RNPC1 is required for stabilizing the HuR transcript (Fig. 4). To our knowledge, this is the first study indicating that HuR is subjected to post-transcriptional regulation via mRNA stability. However, several questions still remain. For example, a precise RNPC1-binding site in HuR 3'-UTR needs to be mapped. In addition, we showed that RNPC1b, the small isoform of RNPC1 with an intact RNA binding domain, and mutant RNPC1 deficient in RNA-binding activity, were unable to regulate HuR expression. This suggests that the RNA-binding activity of RNPC1 is not sufficient for regulating HuR expression. It is likely that RNPC1 recruits other factors to cooperatively regulate HuR mRNA stability. Therefore, it will be interesting to identify other components that interact with RNPC1 and/or jointly bind HuR transcript

and, consequently, regulate HuR mRNA decay. Finally, it is not clear whether RNPC1 is involved in suppressing the exosome, a pivotal component of the RNA degradation machinery, or competing with miRNAs known to regulate HuR (24, 35), to stabilize HuR mRNA. Therefore, further studies to address these questions would help us better understand how HuR is regulated.

The Role of HuR in RNPC1-mediated Growth Suppression—In this study, we found that, in the absence of RNPC1 expression, knockdown of HuR led to a decreased colony formation (Fig. 5, *B* and *D*), consistent with the role of HuR in promoting cell proliferation (11). By contrast, in the presence of RNPC1 expression, knockdown of HuR attenuates growth suppression by RNPC1 (Fig. 5, *B* and *D*), suggesting that HuR is a mediator of RNPC1 in growth suppression. We speculate that, owing to potent growth suppression by RNPC1, HuR is unable to activate pro-survival pathways to facilitate cell recovery, but instead to promote cell death. Consistent with this idea, HuR was reported to promote cell death in cells treated with a lethal dose of staurosporine (20). However, upon mild stress, HuR was able to prevent cell death by promoting translation of prothymosin α , an anti-apoptotic factor (36). Thus, depending on the nature of stress signals, HuR functions as a stress sensor and regulates the stability and/or translation of transcripts encoding pro- and anti-apoptotic proteins. Nevertheless, future study is warranted to examine how RNPC1 affects the ability of HuR to associate with its targets and thus, alter cell fate.

To uncover the underlying mechanism by which HuR facilitates RNPC1-mediated growth suppression, we showed that RNPC1 is able to inhibit *c-Myc* expression in a HuR-dependent manner (Fig. 6, *A–C*). Furthermore, we showed that knockdown of *c-Myc* alone or together with HuR nearly abolishes RNPC1 to suppress cell growth (Fig. 6*E*). Together, these data suggest that increased expression of HuR by RNPC1 leads to decreased expression of *c-Myc* and, subsequently, promotes RNPC1-mediated growth suppression. Interestingly, although both HuR and RNPC1 can stabilize the p21 transcript (2, 25, 28), p21 can be increased by RNPC1 independent of HuR (Fig. 5, *A* and *C*). This probably explains why HuR knockdown is unable to completely abolish RNPC1-mediated growth suppression (Fig. 5, *B* and *D*). Future studies are needed to address whether other factors regulated by HuR participate in this process.

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