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HuB and HuD repress telomerase activity by dissociating HuR from *TERC*

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ABSTRACT

The ubiquitous RNA-binding protein HuR (ELAVL1) promotes telomerase activity by associating with the telomerase noncoding RNA *TERC*. However, the role of the neural-specific members HuB, HuC, and HuD (ELAVL2–4) in telomerase activity is unknown. Here, we report that HuB and HuD, but not HuC, repress telomerase activity in human neuroblastoma cells. By associating with AU-rich sequences in *TERC*, HuB and HuD repressed the assembly of the TERT–*TERC* core complex. Furthermore, HuB and HuD competed with HuR for binding to *TERC* and antagonized the function of HuR that was previously shown to enhance telomerase activity to promote cell growth. Our findings reveal a novel mechanism controlling telomerase activity in human neuroblastoma cells that involves a competition between HuR and the related, neural-specific proteins HuB and HuD.

INTRODUCTION

Telomeres include single-stranded DNA repeats at the 3′ ends of linear chromosomes and serve to maintain genomic stability (1,2). Telomeres become progressively shorter after each cell division, and when they reach a critically short size, they elicit a DNA damage response that triggers cellular phenotypes such as senescence (3). The maintenance of telomeric DNA length requires telomerase, a holoenzyme comprising the non-coding RNA *TERC* and the catalytic subunit TERT (4). *TERC* functions as a template for telomerase to catalyze the addition of single-stranded telomere DNA repeats onto the 3′ ends of linear chromosomes (5,6). Increasing evidence suggests that factors targeting *TERC* modulate the levels or function of *TERC*, and thereby telomerase activity (7).

Proteins such as DKC1 (dyskerin), TCAB1, GAR1, NHP2, NOP10, Pontin/Reptin, DAXX, the core protein components of box H/ACA small nucleolar ribonucleoprotein particles (snoRNPs), and telomeric proteins TIN2 and TPP1, are found to regulate telomerase activity through associating with *TERC* (8–17). HuR, 'human antigen R', also known as HuA and ELAVL1 (embryonic lethal abnormal vision-like 1), is the ubiquitous member of the Hu/elav RNA-binding protein family protein. It recognizes AU-rich or U-rich elements of almost all species of RNAs, in turn regulating processes such as cell proliferation, differentiation, senescence, and apoptosis (18,19). Interestingly,

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HuR is able to regulate telomerase activity by associating with *TERC* and promoting *TERC* modification at m^5C (20). Neuronal cells express all of the members of the Hu RNA-binding protein family (HuR, HuB, HuC and HuD) even though their RNA-binding abilities are largely similar (21,22). Although the involvement of neural Hu proteins in neuronal differentiation, behavior, development, and maturation has been studied in detail (23–26), it is not known if they regulate telomerase activity and cellular senescence.

In the present study, we describe evidence that HuB and HuD associate with *TERC* in human neuroblastoma cells. The binding of HuB and HuD to *TERC* prevents HuR binding to *TERC*, thereby antagonizing the function of HuR in promoting telomerase activity and in delaying cellular senescence. Our findings reveal a concomitant regulation of telomerase activity by Hu RNA-binding proteins in human neuroblastoma cells.

MATERIALS AND METHODS

Cell culture, transfections, and SA-β-galactosidase activity

Human osteosarcoma U2OS cells and human neuroblastoma cells (SH-SY5Y) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37°C in 5% CO_2. All plasmid transfections were performed using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. SA-β-gal activity was assessed by using a senescence β-Galactosidase Staining Kit (GENMED Scientifics Inc., Wilmington, USA).

EdU-incorporation and cell counting

EdU (5-ethynyl-2′-deoxyuridine) incorporation assays were performed by using a kit (RIBOBIO, Guangzhou, China) following the instructions from the manufacturer. Briefly, cells were digested and transferred to 96-well plates at a density of 4×10^3–1×10^5 cells per well, then incubated with 50 mM EdU for 3 h and washed twice with PBS. After paraformaldehyde fixation, cells were neutralized with 2 mg/mL glycine solution, permeabilized with osmotic agent (0.5% PBS of Triton X-100), and stained with 1 × Apollo® dye solution at room temperature for 30 min. After washing twice with osmotic agent, cells were stained again 1 × Hoechst 33342 reaction solution for 30 min. Cells then were washed with PBS and imaged by fluorescence microscopy.

For cell counting, a cell counting kit-8 (CCK8 kit**,** Abmole Bioscience Inc., Houston, USA) was used for cell counting following the manufacturer's instructions. Cells were digested and transferred to 96-well plates at a density of 1000–4000/well. Each sample had two duplicates. Cells were incubated with CCK8 agent for 2 h and the signal (OD) was detected at 450 nm by spectrophotometry.

Silencing of HuR, HuB, and HuD

To silence HuR, HuB, or HuD, siRNAs targeting HuR (AAGAGGCAAUUACCAGUUUCA), HuB (GCUAAUAACCCAAGCCAAA) or HuD (GAAUAUGACCCAAGAAGAA) were used; siRNA (UUGUUCGAACGUGUCACGUUU) was used as a control. Transfections were performed by using Oligofectamine (Invitrogen) following the manufacturer's instructions. Unless otherwise indicated, cells were collected for analysis 48 h after transfection.

To stably silence HuR, HuB, or HuD, SH-SY5Y cells were infected with lentiviruses bearing vector expressing shHuR, shHuD or shHuB. Forty-eight hours later, cells were cultured in medium containing puromycin (1.5 μg/ml) and cultured for an additional 7 days. Cells then were cultured in medium containing puromycin (1.0 μg/ml) until cells were collected for experiments.

Immunofluorescence analysis

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then permeabilized in 0.1% Triton X-100. To detect HuB, HuD, and Coilin, cells were blocked with 5% BSA for 30 min and incubated with antibodies recognizing HuB (1:200),HuD (1:100),HuC(1:100),HuR(1:200) or Coilin (1:200) overnight at 4°C. After washing with PBS, cells were incubated with FITC-conjugated or TRIFC-conjugated secondary antibodies (1:200) at room temperature for 1 h. Cells were then stained with DAPI and mounted. Fluorescence was visualized using a Leica microscope.

Western blot analysis and reverse transcription (RT) followed by real-time quantitative (q)PCR analysis

Western blot analysis was performed following standard procedures. Monoclonal antibodies recognizing GAPDH, α-tubulin (TUBA), or HuR were from Santa Cruz and a monoclonal anti-flag antibody was from Sigma. Polyclonal antibodies recognizing HuB and HuC were from Proteintech. Monoclonal anti-HuD antribody was from ABcam. For reverse transcription (RT) followed by real-time quantitative (q)PCR (RT-qPCR) analysis to detect human *TERC* and human *TERT* mRNA, we used the following primer pairs: TCTAACCCTAACTGAGAAGGGCGTAG and GTTTGCTCTAGAATGAACGGTGGAAG for *TERC*, and TCCACTCCCCACATAGGAATAGTC and TCCTTCTCAGGGTCTCCACCT for *TERT* (forward and reverse, respectively). *GAPDH* mRNA was measured to normalize RT-qPCR results and was amplified using primer pairs CTGGGCTACACTGAGCACC and AAGTGGTCGTTGAGGGCAATG.

RNA pulldown, UV-crosslinking RNP IP and UV-crosslinking rEMSA

For RNA pulldown assays, PCR-amplified DNA was used as template to transcribe biotinylated RNA by using T7 RNA polymerase in the presence of biotin-UTP. One microgram of purified biotinylated transcripts was incubated with 100 μg of cytoplasmic extracts for 30 min at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dynal, Oslo), and the pulldown material was assessed by western blot analysis.

For UV-crosslinking RNP IP assays, lysates from cells exposing to UVC (400 mJ/cm^2) were used for immunoprecipitation by using anti-HuR, anti-HuB, anti-HuD, or anti-flag

antibody. The IP materials were washed twice with a stringent buffer (100 mM Tris–HCl, pH 7.4, 500 mM LiCl, 0.1% Triton X-100, 1 mM DTT, protease Inhibitor Cocktail) and twice with the IP buffer. The RNA in RNP IP was assessed by RT-qPCR analysis.

For UV-crosslinking RNA electrophoretic mobility shift assay (UV-crosslinking rEMSA), the RNA-protein interaction mixtures (0.02 ml) contained 50 mM Tris (pH 7.0), 150 mM NaCl, 0.25 mg/ml tRNA, 0.025 mg/ml bovine serum albumin, 500 nM of purified his-HuB or his-HuD and 500 nM of *in vitro*-transcribed *TERC*. Reactions were incubated at 25 °C for 30 min, and digested with RNase T1 (100 U/reaction) for 15 min at 37°C. After crosslinking of complexes with UV light (1800 J/m^2), reactions were used for western blot analysis.

Constructs

For construction of the pcDNA3.1 vector (QIAGEN) expressing HuB, the coding region of HuB was amplified by using primer pairs GCGGATCCGCGATG GAAACACAACTGTCT and GCGAATTCGCTTAG GCTTTGTGCGTTTTGT and inserted between the BamH I and EcoR I sites of the vector. For construction of the pcDNA3.1 vector expressing HuD, the coding region of HuD was amplified by using primer pairs CGAAGCTTGCGATGGTTATGATAATTAGCAC and GCGGATCCCGTCAGGACTTGTGGGCTTTG and inserted between the Hind III and BamH I sites of the vector. To construct vectors expressing his-HuB or his-HuD in E. coli, the coding regions of HuB and HuD were amplified using primer pairs CAGGATCCATGGAAACACAACTGTCTAA and CGCTCGAGTTAGGCTTTGTGCGTTTTG and primer pairs GCGGATCCATGGTTATGATAATTAGCACCA TGG and ATCTCGAGTCAGGACTTGTGGGCTTTG, respectively, and inserted between BamH I and XhoI sites of pet28a-His vector (QIAGEN). To construct vector expressing shHuB, oligo pairs GATCCGGCTAATAACCCAAGCCAAATTCAA GAGATTTGGCTTGGGTTATTAGCTTTTTTC and AATTGAAAAAAGCTAATAACCCAAGCCAAA TCTCTTGAATTTGGCTTGGGTTATTAG CCG were annealed and inserted between BamH I and EcoR I sites of PHBLV-zsGreen-U6 plasmid. To construct the vector expressing shHuD, oligomer pairs GATCCGGAATATGACCCAAGAAGAATTCAA GAGATTCTTCTTGGGTCATATTCTTTTTTC and AATTGAAAAAAGAATATGACCCAAGAAGAATC TCTTGAATTCTTCTTGGGTCATATT CCG were annealed and inserted between BamH I and EcoR I sites of PHBLV-zsGreen-U6 plasmid. To construct the vector expressing shHuR, oligo pairs GATCCG AAGAGGCAATTACCAGTTTCATTCAAGAGA TGAAACTGGTAATTGCCTCTTCTTTTTTC and AATTGAAAAGAAGAAGAGGCAATTACCAGTTT CATCTCTTGAATGAAA CTGGTAATTGCCTCTTCG were annealed and inserted between BamH I and EcoR I sites of plasmid PHBLV-zsGreen-U6. The pcDNA 3.0 (+) vectors (Invitrogen) expressing *TERC*, flag-tagged TERT (human TERT), HuR, as well as the p3 × Flag-CMV 10 vector expressing full-length human flag-TERT) were described previously (20,27).

Preparation of transcripts

cDNA was used as a template to amplify the different fragments of *TERC*. All 5′ primers contained the T7 promoter sequence CCAAGCTTCTAATACGACTCACTA TAGGGAGA-3′ (T7). To amplify templates for synthesizing RNAs *TERC*, *TERC*-A, and *TERC*-B, we used following primer pairs; (T7) GGGTTGCGGAGGGTGGGC CT and GCATGTGTGAGCCGAGTCCTGG for *TERC*, (T7) GGGTTGCGGAGGGTGGGCCT and AGGCCG AGGCTTTTCCGCC for *TERC-A*, and (T7) GCCGCC TTCCACCGTTCATT and GCATGTGTGAGCCGAG TCCTGG for *TERC-B*. The p27 5′UTR and CR fragments were described previously (28). The templates for variants of *TERC* (U40A, U100A and U40A+U100A) were amplified from the pcDNA 3.0 (+) vector expressing these variants, as described previously (20). PCR-amplified DNA was used as the template to transcribe biotinylated RNA by using T7 RNA polymerase in the presence of biotin-UTP.

Telomere repeat amplification protocol (TRAP) and telomere length measurement

For TRAP assays, cells were collected and used for the analysis of telomerase activity by using polyacrylamide gels (12%), and visualized by staining with SYBR Safe (Invitrogen), as described previously (29). Telomere length was determined by using a kit from Roche (Cat. No. 12 209 136 001) following the manufacturer's instructions. Genomic DNA was digested with Hinf I and Rsa I, and resolved on 0.8% agarose gels. After denaturation, gels were hybridized with a Digoxin-labeled, telomere-specific hybridization probe (TAGGG), and then exposed to X-ray film. The mean terminal restriction fragment (TRF) length was calculated according to the formula: mean TRF = $\sum(OD_i)/\sum(OD_i/L_i)$ (where OD_i and L_i are the signal intensity and the molecular weight at the position i, respectively).

Statistical analysis

Two-tailed Student's t test was used to analyze the significance of the data. Significance was indicated only when P value <0.05.

RESULTS

HuB and HuD associate with *TERC*

In a previous study, we found that the HuR, the ubiquitous member of Hu/elav RNA-binding protein family, regulates telomerase activity by associating with *TERC* (20). To test if the neuronal Hu/elav proteins (HuB, HuC and HuD) might play similar role as HuR, RNA pulldown assays were performed by using human neuroblastoma SH-SY5Y cell lysate and *in vitro*-transcribed biotinylated *TERC*. As shown in Figure 1A by western blot analysis, HuB, HuD,

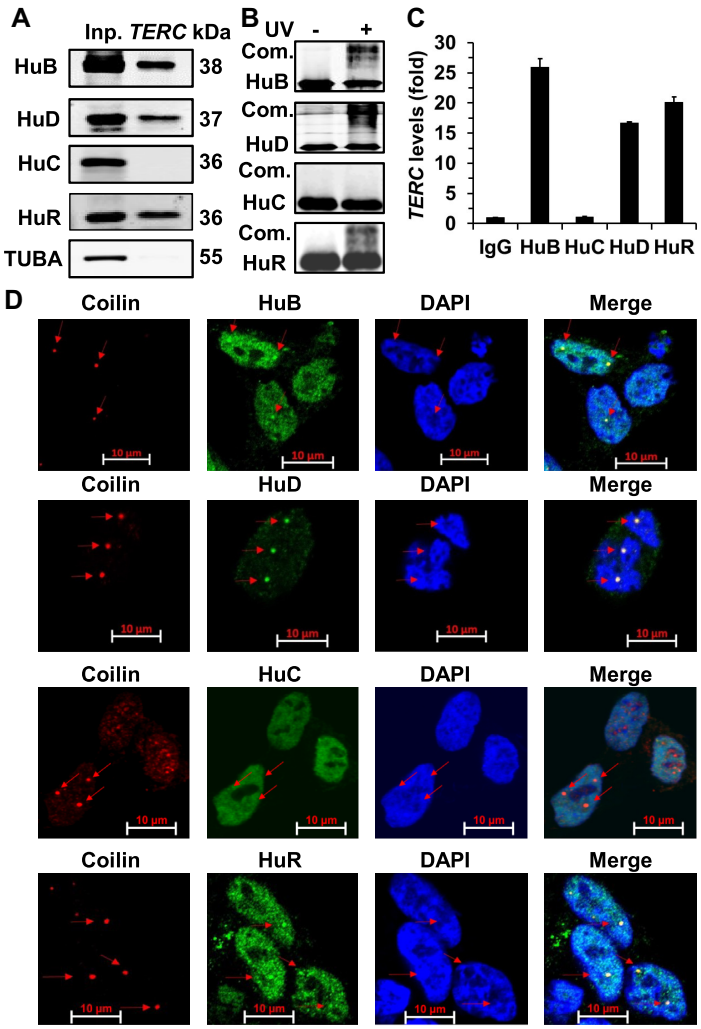

Figure 1. HuB and HuD associate with *TERC*. (**A**) RNA pulldown assays were performed by using SH-SY5Y cell lysates and *in vitro*-transcribed *TERC*. The presence of HuB,HuD,HuC or HuR in the pulldown materials was assessed by western blot analysis. (**B**) UV crosslinking rEMSA assays were performed by using purified his-HuB,his-HuD,his-HuC and his-HuR as well as *in vitro*-transcribed *TERC*. The covalently bound HuB or HuD (complex, 'Com.') was detected by western blot analysis. (**C**) The association of HuB, HuC and HuD with *TERC* was assessed by using RNP IP assays. The presence of HuB, HuC and HuD in the IP materials was assessed by RT-qPCR analysis. IP using an anti-IgG antibody and an anti-HuR antibody served as positive and negative controls, respectively. Data are the means ± SD from three independent experiments. (**D**) Immunofluorescence assays were performed to test the co-localization of Coilin with HuB,HuD,HuC orHuR. DAPI was used to visualize nuclei. Data are representatives from three independent experiments.

and HuR, but not HuC, were present in the pulldown materials, suggesting that HuB and HuD may associate with *TERC*. This interaction was validated by UV-crosslinking complexes that contained purified his-tagged HuB, purified his-tagged HuD or purified his-tagged HuR and *TERC in vitro*; as shown by western blot analysis (Figure 1B), HuB, HuD, and HuR, but not HuC, bound to *TERC* migrated as larger complexes. By RNP IP assays (Figure 1C), *TERC* was found enriched in the ribonucleoprotein immunoprecipitation (RIP) materials recovered by using an anti-HuB antibody, an anti-HuD antibody, or an anti-HuR antibody, but not after RIP with an anti-HuC antibody. In addition, HuB, HuD and HuR, but not HuC, were found to co-localize with coilin in Cajal bodies, where *TERC* undergoes maturation (Figure 1D).

HuR associates with *TERC* at the 5′end (positions 1–139) (20). To further determine if HuB and HuD associated with *TERC* at the same region, we tested if HuB and HuD bound the full-length *TERC*, *TERC* fragment A (positions 1–139) and/or *TERC* fragment B (positions 140–451) (Figure 2A, schematic) by biotin–RNA pulldown assays. As shown in Figure 2B, HuB and HuD associate with the full-length *TERC* and fragment A, but not with fragment B. Fragment A contains two AU-rich elements (ARE1 and ARE2, locating at positions 38–43 and positions 98–103, respectively) that are recognized by HuR (20). To test if HuB and HuD were capable of recognizing these AREs, *in vitro*-transcribed *TERC* and the variants bearing mutations at ARE1 (U40A), ARE2 (U100A) or both (U40A + U100A) (Figure 2C, schematic) were used for pulldown assays. Mutating U40, but not U100, greatly reduced the association of both HuB and HuD with *TERC* ($P < 0.01$), while mutating both (U40A + U100A) exhibited similar effect as the mutation of the ARE1 in reducing this association (P

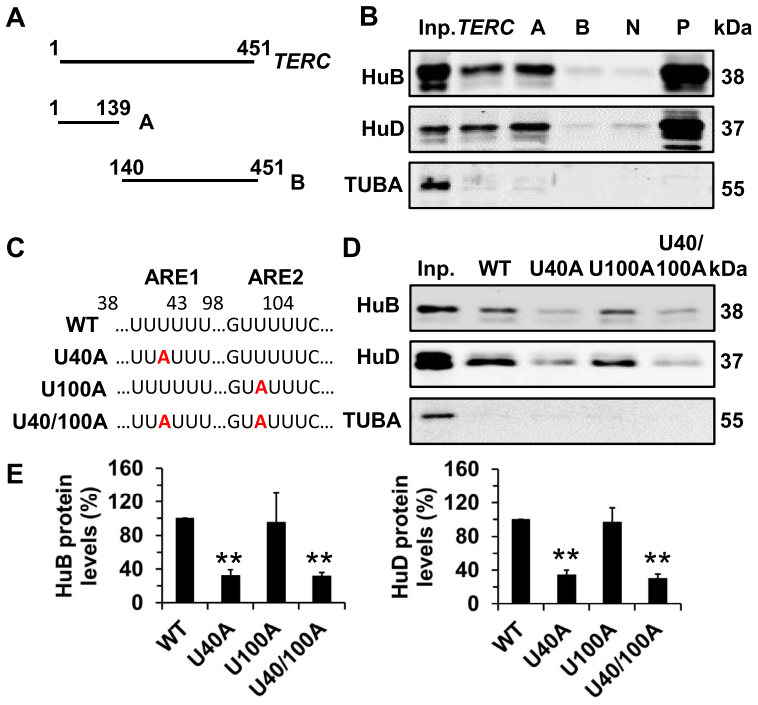

Figure 2. HuB and HuD associate with *TERC* in the same motif. (**A**) Schematic representation depicts the fragments of *TERC* used for RNA pulldown assays. (**B**) RNA pulldown assays were performed using SH-SY5Y cell lysates and *in vitro*-transcribed *TERC* fragments depicted in (A). The CR (coding region) and 3′UTR fragments of *p27* mRNA served as negative (N) and positive (P) controls, respectively. A 5-μg aliquot input (Inp.) and binding to GAPDH were also assessed. (**C**) Schematic representation depicts full length *TERC* (WT) and its variants used for pulldown assays. (**D**) The association of HuB and HuD with *TERC* variants bearing mutations U40A, U100A, or U40A + U100A (U40/100A) (Figure 2C, Schematic) was determined by using RNA pulldown assays. (**E**) Quantification of the bands detected by western blot analysis in (D); data are the means ± SD of the signals from three independent experiments and significance is analyzed by two-tailed Student's *t* test (**$P < 0.01$).

< 0.01) (Figure 2D and E). Therefore, HuB and HuD associate with *TERC* at the ARE1.

HuB and HuD antagonize the association of HuR with *TERC*

Next, we asked if HuB and HuD influenced the association of HuR with *TERC* by silencing HuB or HuD in SH-SY5Y cells. As shown, knockdown of HuB or HuD markedly increased the association of HuR with *TERC*, while knockdown of HuR greatly increased the association of HuB or HuD with *TERC* ($P < 0.05$ or $P < 0.01$) (Figure 3A-B and Supplementary Figure S1A and B). On the other hand, overexpression of HuB or HuD repressed the association of HuR with *TERC*, while overexpression of HuR repressed the binding of HuB or HuD to *TERC* ($P < 0.05$ or $P < 0.01$) (Figure 3C, D and Supplementary Figure S1C and D). HuR was previously shown to have no effect on the expression levels of TERT and *TERC* (20). Similarly, knockdown of HuB or HuD did not influence the levels of *TERT* mRNA and protein nor the levels of *TERC* (Supplementary Figure S2). Furthermore, knockdown of HuB or HuD did not alter the levels of HuR; knockdown of HuR had no effect on the levels of HuB and HuD (Supplementary Figure S3A and B). Moreover, knockdown of HuB did not alter HuD protein levels, and HuD knockdown did not alter HuB protein levels (Supplementary Figure S4). Together, these results indicate that HuB and HuD antagonize the association of HuR with *TERC*, and *vice versa*.

HuB and HuD repress telomerase activity by inhibiting telomerase assembly

To evaluate the effect of HuB and HuD in regulating telomerase activity, cell lysates from SH-SY5Y cells with silenced HuB, HuD, or both HuB and HuD were used for TRAP assays. As shown in Figure 4A, knockdown of HuB or HuD increased telomerase activity significantly (by ∼1.65-fold and ∼1.67-fold, respectively; $P < 0.01$); knockdown of both HuB and HuD was much more effective than knockdown of HuB or HuD alone in increasing telomerase activity (by ∼3.24-fold; $P < 0.01$). On the other hand, overexpression of HuB or HuD reduced telomerase activity by ∼54.7% and ∼69.3%, respectively ($P < 0.01$); overexpression of both HuB and HuD was much more effective than overexpression of HuB or HuD alone in reducing telomerase activity (∼84.2%, $P < 0.01$) (Figure 4B). These results suggest that HuB and HuD cooperatively repress telomerase activity.

To further assess the role of HuB and HuD in repressing telomerase activity, SH-SY5Y cells with stably silenced HuB, HuD, or both HuB and HuD were used for measuring telomere length. As shown in Figure 5A and B, although knockdown of HuB or HuD alone increased telomerase activity (Figure 4), neither transient (3 days) nor stable (60 days) knockdown of HuB or HuD exhibited significant effect in altering telomere length. However, stable simultaneous knockdown of HuB and HuD (60 days), but not transient simultaneous knockdown of HuB and HuD (3 days),

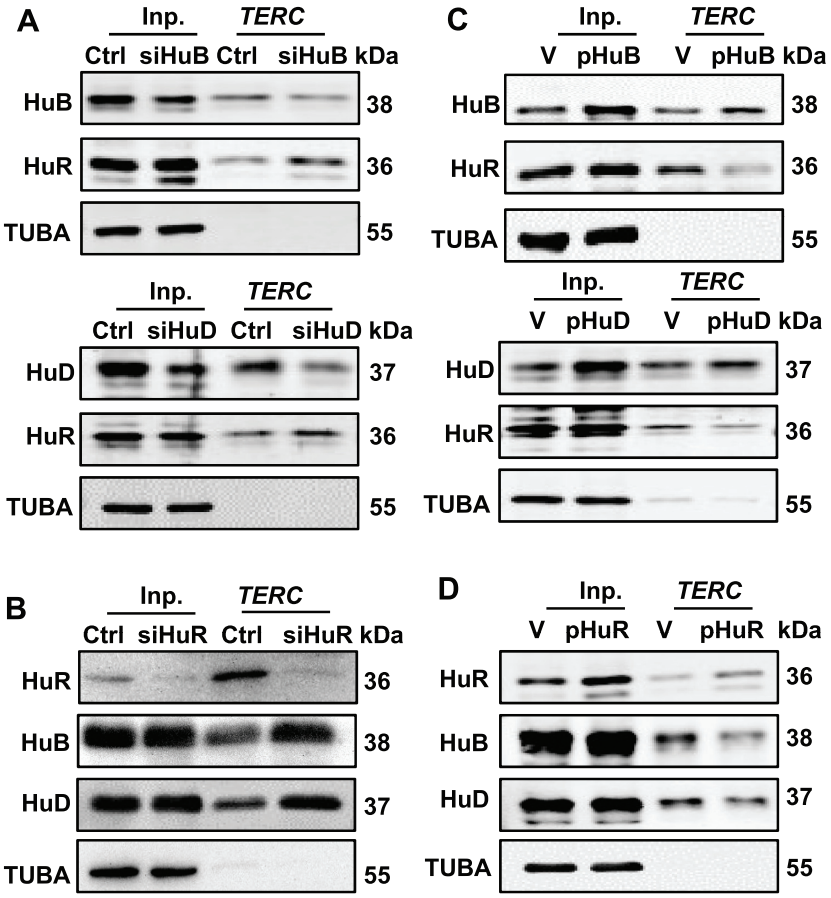

Figure 3. HuB and HuD compete with HuR to associate with *TERC*. (**A**, **B**) SH-SY5Y cells were transfected with a siRNA targeting HuB or HuD (A), or HuR (B) for 48 h. RNA pulldown assays were performed to evaluate the association of HuR, HuB or HuD with *TERC*. (**C**, **D**) SH-SY5Y cells were co-transfected with a vector expressing HuB together with a vector expressing HuD (C) or transfected with a vector expressing HuR (D). Forty-eight hours later, RNA pulldown assays were performed by using *in vitro*-transcribed *TERC*. The levels of HuR, HuB, HuD and TUBA were monitored by western blot analysis (Input, Inp.). Data in all panels are representative of three independent experiments.

significantly extended telomere length, confirming the co-operative regulation of telomere activity by HuB and HuD ($P < 0.01$) (Figure 5C).

The association of HuR with *TERC* promotes the assembly of the telomerase complex, in turn enhancing telomerase activity (20). Specifically, we asked if HuB and HuD influenced the assembly of TERT-*TERC* complex through associating with *TERC*. As shown in Figure 6A and B by RNP IP assays, knockdown of HuB or HuD in SH-SY5Y cells increased the association of flag-TERT with *TERC* ($P < 0.01$), while overexpression of HuB or HuD, exhibited opposite effect ($P < 0.01$). Human osteosarcoma U2OS cells, which do not express endogenous human TERT or *TERC* (30), were used for evaluating the effect of ectopic expression of HuB and HuD in TERT-*TERC* assembly. This cell line was transfected with a vector expressing flag-tagged-TERT (human TERT) together with a vector expressing human *TERC*. The effect of the ectopically expressed HuB and HuD on telomerase assembly was then evaluated. By RNA pulldown assays using *in vitro* transcribed *TERC*, ectopic co-expression of HuB and HuD in U2OS cells reduced the presence of flag-TERT in the pulldown materials ($P < 0.01$) (Figure 6C). Further study showed that co-expression of HuB and HuD decreased the telomerase activity in U2OS cells expressing ectopic flag-TERT and *TERC* (Figure 6D) ($P < 0.01$). Therefore, HuB and HuD are able to repress telomerase activity by inhibiting the assembly of TERT–*TERC* complex.

HuB and HuD antagonize the effect of HuR in regulating telomerase activity

Based on the findings that HuB and HuD antagonized the association of HuR with *TERC* and both HuB and HuD repressed telomerase activity (Figures 3–5), we proposed that HuB and HuD might antagonize the effect of HuR in regulating telomerase activity. To test this possibility, HuR alone (shHuR), HuB and HuD (shHuB/D) or HuR, HuB and HuD (shHuB/D/R) were silenced stably in SH-SY5Y cells. TRAP assays and Southern blot analysis were used to determine telomerase activity and telomere length, respectively. In agreement with previous findings (20), knockdown of HuR reduced telomerase activity and shortened telomere length (Figure 7A–C, lanes 2). As anticipated, the combined knockdown of HuB and HuD increased telomerase activity and extended telomere length (Figure 7A–C, lanes 3). Importantly, however, simultaneous knockdown of HuR, HuB and HuD did not significantly alter telomerase activity or

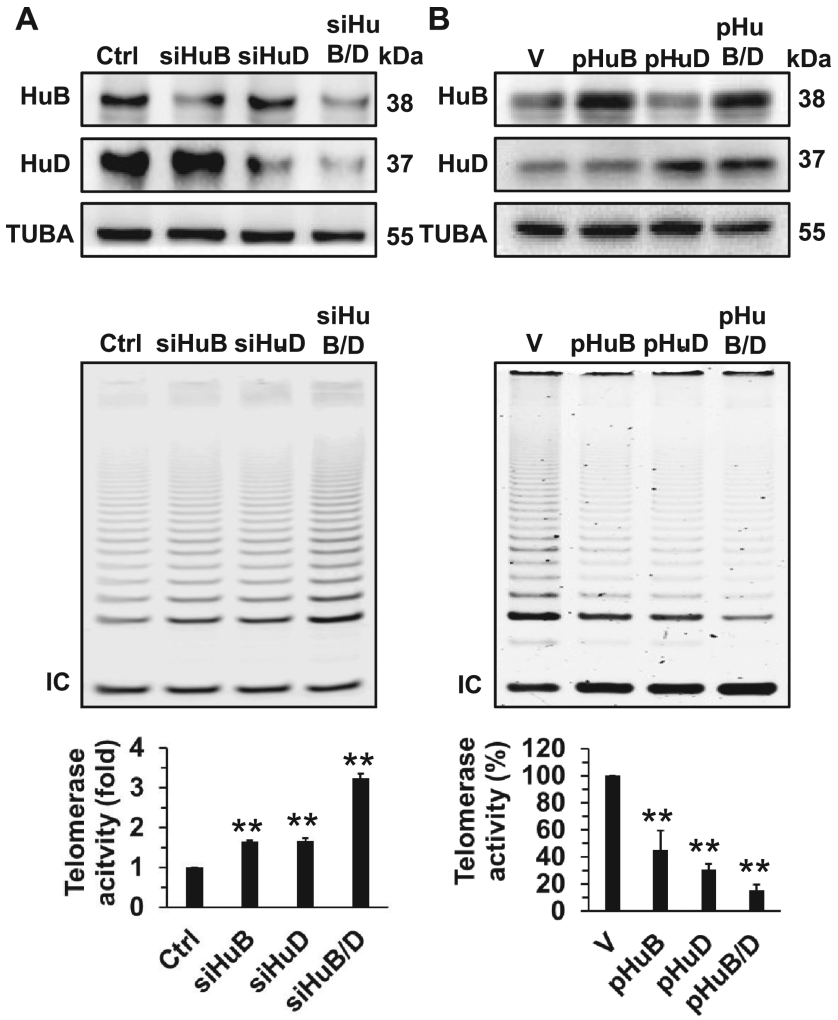

Figure 4. HuB and HuD repress telomerase activity. (**A**) *Top*, SH-SY5Y cells were transfected with a siRNA targeting HuB or HuD, or co-transfected with both siRNAs (siHuB/D). Forty-eight hours later, the levels of HuB, HuD, and TUBA were monitored by western blot analysis. *Middle*, cells described in top panels were used for TRAP assays. *Bottom*, the density of the signals in the middle panels was scanned and plotted as the means ± SD from three independent experiments; significance is analyzed by two-tailed Student's *t* test (**$P < 0.01$). (**B**) *Top*, vectors expressing HuB (pHuB) or HuD (pHuD), or co-transfected with both vectors (pHuB/D). Forty-eight hours later, the levels of HuB, HuD and TUBA were monitored by western blot analysis. *Middle*, cells described in top panels were used for TRAP assays. *Bottom*, the density of the signals in the middle panels is scanned and plotted as the means ± SD from three independent experiments; significance is analyzed by two-tailed Student's *t* test (**$P < 0.01$).

telomere length (Figure 7A–C, lanes 4). In sum, HuB and HuD antagonized the effect of HuR as promoter of telomerase activity, and *vice versa.*

HuB/D-telomerase regulatory process impacts on cellular senescence

Telomerase and telomere are critically important for cell growth and senescence (1–3). To further address if HuB and HuD influenced cell senescence by preventing telomerase activity, SH-SY5Y cells described in Figure 7 were further subjected to EdU incorporation assay (Figure 8A and B), cell counting assay (by using CCK8 kit) (Figure 8C) and SA-β-gal staining assays (Figure 8D and E). As anticipated, knockdown of HuR in SH-SY5Y cells decreased EdU incorporation and inhibited cell growth, thereby accelerating cell senescence. Conversely, knockdown of HuB and HuD together increased EdU incorporation, induced cell growth and delayed cellular senescence. In addition, knockdown of HuB, HuD and HuR together failed to influence the DNA replication, cell growth and cellular senescence, as observed from the results of EdU incorporation, CCK8 and SA-β-gal staining assays. Together, the competition between HuR and HuB/D modulates telomerase activity and cellular senescence.

DISCUSSION

The ubiquitous RNA-binding protein HuR associates with *TERC* and promotes telomerase activity (20). The present study supports the notion that the neural-specific ELAVL family proteins HuB and HuD are also able to associate with *TERC* (Figure 1), in turn reducing the ability of HuR to associate with *TERC* and reducing telomerase activity (Figures 3–5). However, our results suggest that the association of HuB and HuD with *TERC* represses telom-

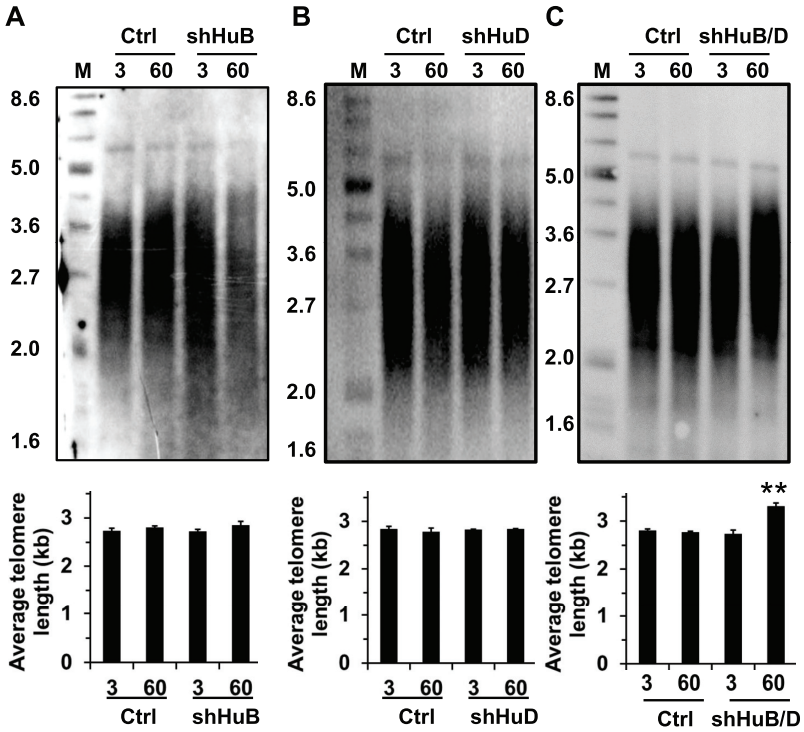

Figure 5. HuB and HuD repress telomerase activity. (**A–C**) *Top*, SH-SY5Y cells were transfected with a vector expressing shHuB (A), shHuD (B) or co-transfected with a vector expressing shHuB together with a vector expressing shHuD (C). After times indicated (3, 3 days; 60, 60 days), DNA was isolated and subjected to Southern blot analysis to assess the length of the telomeres. *Bottom*, the density of the signals shown in the upper panels of (A–C) is presented as the means ± SD from three independent experiments; significance is analyzed by two-tailed Student's *t* test (**$P < 0.01$).

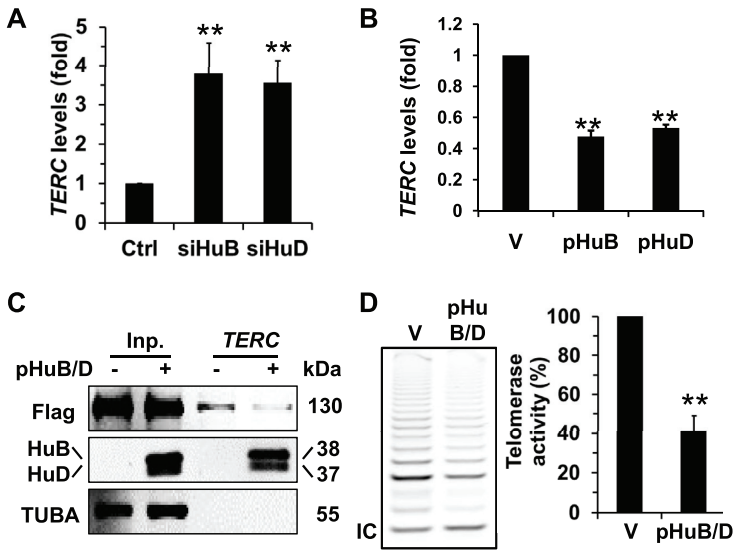

Figure 6. HuB and HuD repress the assembly of TERT-*TERC* complex. (**A**, **B**) SH-SY5Y cells were transfected with a vector expressing flag-tagged human TERT (flag-TERT). Twenty-four hours later, cells were further transfected with a siRNA targeting HuB or HuD (A), or transfected with a vector expressing HuB (pHuB) or HuD (pHuD) (B) and cultured for additional 48 h. RNP IP assays were performed by using an anti-flag antibody to test the levels of *TERC* in the IP materials. Data are the means ± SD from three independent experiments; significance was analyzed by two-tailed Student's *t* test (**$P < 0.01$). (**C**) U2OS cells were co-transfected with vectors expressing flag-TERT and *TERC*. Twenty-four hours later, cells were further co-transfected with vectors expressing HuB and HuD. After an additional 48 h, RNA pulldown assays were performed by using biotinylated *TERC* to test the presence of flag-TERT (flag) and HuB/D (using both HuB and HuD antibodies). The levels of flag-TERT, HuB/D and TUBA were monitored (Input, Inp.) by western blot analysis. Data are the means ± SD from three independent experiments; significance is analyzed by two-tailed Student's *t* test (**$P < 0.01$). (**D**) *Left*, U2OS cells processed as described in (C) were used for TRAP assays. *Right*, the density of the signals of TRAP analysis from the left panels is presented as the means ± SD from three independent experiments; significance is analyzed by two-tailed Student's *t* test (**$P < 0.01$).

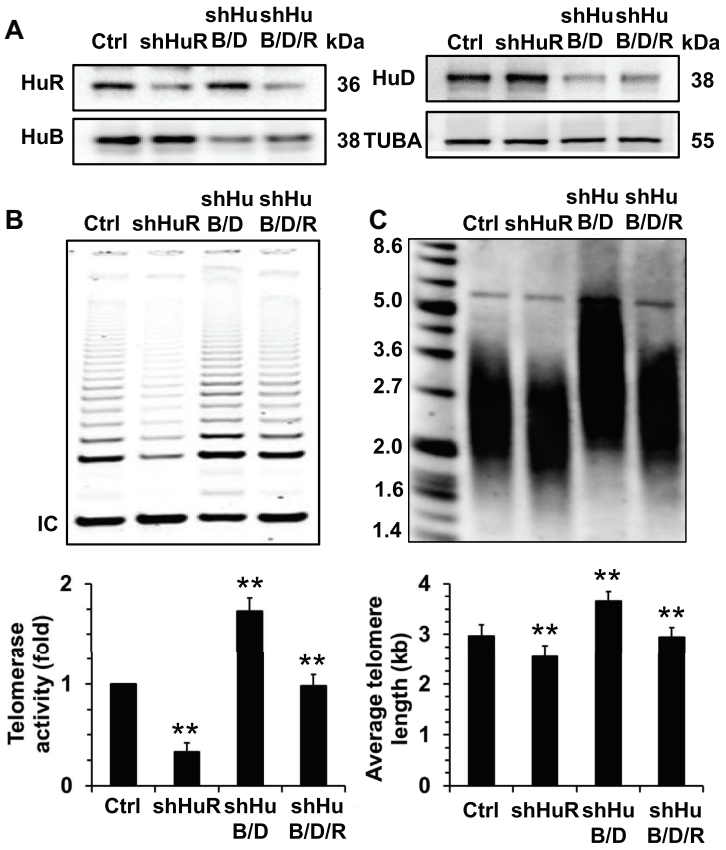

Figure 7. HuB and HuD compete with HuR to regulate telomerase activity. (**A**) *Top*, SH-SY5Y cells were transfected with a vector expressing shHuR, co-transfected with vectors expressing shHuB and shHuD (shHuB/D), or co-transfected with vectors expressing shHuB, shHuD and shHuR (shHuB/D/R). Sixty days later, western blot analysis was used to monitor the levels of HuR, HuB, HuD and TUBA. Data are representative from three independent experiments. (**B**) *Top*, cells described in (A) were used for TRAP assays. *Bottom*, the density of the signals shown in the upper panels of (A) was presented as the means ± SD from three independent experiments; significance is analyzed by two-tailed Student's *t* test (**$P < 0.01$). (**C**) *Top*, cells processed as described in (A) were subjected to Southern blot analysis to assess telomere length. The density of the signals shown in the top panels of (C) is presented as the means ± SD from five independent experiments (*bottom*); significance is analyzed by two-tailed Student's *t* test (**$P < 0.01$).

erase activity (Figures 6 and 7); this is opposite to what we found earlier for HuR, which promoted the telomerase activity (20). Importantly, the regulation of telomerase activity by HuB and HuD impacted upon cell proliferation and senescence (Figure 8). These findings support the view that Hu/elav RNA-binding proteins in the nerve system and other tissues expressing HuD or HuB (e.g. testis, ovary, small lung cancer, pancreatic islets, etc.) (22,31,32) may represent a way to fine-tune or balance telomerase activity, by eliciting both positive and negative regulation. Given that telomere homeostasis is also important for cell differentiation, apoptosis, DNA damage response and mitochondrial metabolism (33–35), the concurrent regulation of telomerase by the Hu/elav proteins may also influence these processes.

Although the joint silencing of HuB and HuD effectively extended telomere length, individual knockdown of HuB or HuD alone did not significantly alter telomere length (Figure 5), even though HuB and HuD each associated with *TERC* and repressed telomere activity (Figures 1, 4 and 5). Therefore, it is plausible that the two proteins, HuB and HuD, work together in regulating telomerase activity. Whether they form a dimer or only one RNA-binding protein physically associates with *TERC* is not known at present. In addition, while HuR, HuB and HuD appear quite abundant in human neuroblastoma cells, their relative abundance in neurons in the body remains to be further studied. HuR is found to maintain telomerase activity through promoting *TERC* C106 methylation (m5C methylation) (20). Interestingly, knockdown of HuB and HuD increased the levels of C106 methylation of *TERC* ($P < 0.01$, Supplementary Figure S5), while simultaneous knockdown of HuR diminished the effect of HuB and HuD knockdown in increasing the levels of C106 methylation. Therefore, HuB and HuD may also regulate telomerase activity through inhibiting *TERC* methylation.

The mechanisms underlying the roles of HuR, HuD and HuB in cellular senescence in these cells likely depends on their influence on other mRNAs, as mRNAs encoding other factors associated with proliferation and senescence (p27, p16, p21, c-fos, cyclin A, cyclin B and numerous cytokines) (18,19) are similarly recognized by all three proteins. In fact, the members of the Hu/elav proteins share affinities for many of the same RNAs and are sometimes viewed as having redundant actions on gene expression. However, as in the neuronal paradigm illustrated here, they may also have

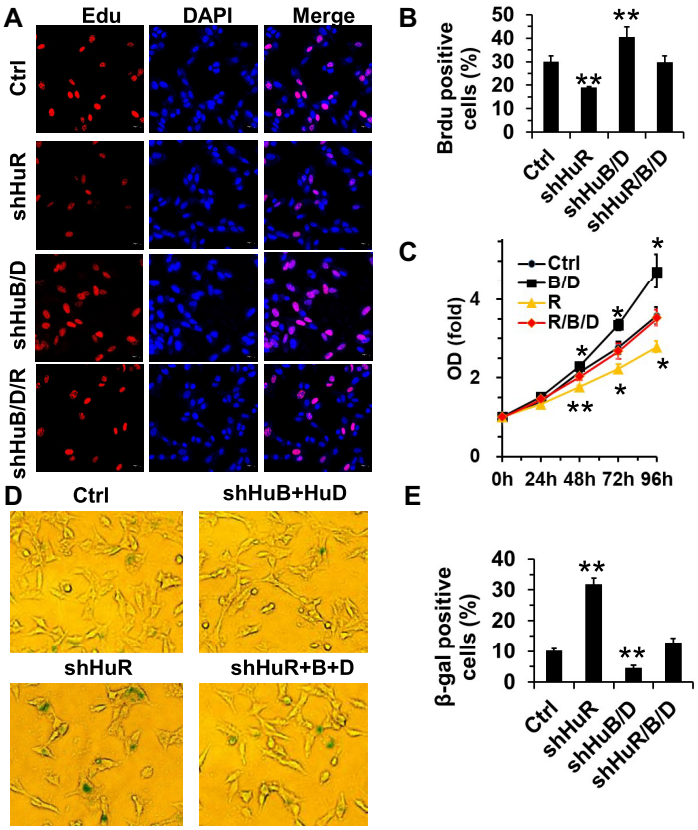

Figure 8. HuB and HuD compete with HuR in regulating cellular senescence. (**A**, **B**) SH-SY5Y cells were stably transfected with a vector expressing shHuR, co-transfected with vectors expressing shHuB and shHuD (shHuB/D), or co-transfected with vectors expressing shHuB, shHuD and shHuR (shHuB/D/R). Cells then were subjected to EdU incorporation assays. Representative images (A) and the means ± SD from three independent experiments (B) are shown. (**C**) Cells processed as described in (A) were cultured for the times indicated and used for cell counting assays to evaluate the cell growth. Data are the means ± SD from three independent experiments. (**D**, **E**) Cells described in (A) were cultured for 72 h and subjected to SA-β-gal staining. Representative images (D) and the means ± SD from three independent experiments (E) are shown. Significance in (B), (C) and (E) is analyzed by two-tailed Student's *t* test (*$P < 0.05$; **$P < 0.01$).

opposite effects on target RNAs and hence in cellular processes (like telomerase activity and telomere length) and thus contribute in this manner to homeostatic regulation of neuronal tissues.

DATA AVAILABILITY

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files. The raw data for dot graphs and uncropped versions of any gels or blots or micrographs presented in the figures and supplementary figures are included in the Data Source File. The vectors used in this study will be available from the corresponding author upon reasonable request.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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