HuR Uses AUF1 as a Cofactor To Promote p16^{INK4} mRNA Decay[▽]

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In this study, we show that HuR destabilizes p16^{INK4} mRNA. Although the knockdown of HuR or AUF1 increased p16 expression, concomitant AUF1 and HuR knockdown had a much weaker effect. The knockdown of Ago2, a component of the RNA-induced silencing complex (RISC), stabilized p16 mRNA. The knockdown of HuR diminished the association of the p16 3' untranslated region (3'UTR) with AUF1 and vice versa. While the knockdown of HuR or AUF1 reduced the association of Ago2 with the p16 3'UTR, Ago2 knockdown had no influence on HuR or AUF1 binding to the p16 3'UTR. The use of EGFP-p16 chimeric reporter transcripts revealed that p16 mRNA decay depended on a stem-loop structure present in the p16 3'UTR, as HuR and AUF1 destabilized EGFP-derived chimeric transcripts bearing wild-type sequences but not transcripts with mutations in the stem-loop structure. In senescent and HuR-silenced IDH4 human diploid fibroblasts, the EGFP-p16 3'UTR transcript was more stable. Our results suggest that HuR destabilizes p16 mRNA by recruiting the RISC, an effect that depends on the secondary structure of the p16 3'UTR and requires AUF1 as a cofactor.

HuR, the ubiquitously expressed member of the Hu RNA-binding protein family, has been broadly shown to stabilize various mRNAs, including those that encode cell cycle regulators like p21^{CIP1} and cyclins A, B1, D1, and E (10, 18, 35, 36), proliferation-associated proteins such as c-fos (2), and factors controlling tumor growth, such as vascular endothelial growth factor (VEGF), COX-2, β -actin, tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and IL-8 (6, 7, 21, 33). Besides stabilizing target mRNAs, HuR also acts as an important regulator of the translation of several target mRNAs (e.g., those encoding MKP-1, p53, prothymosin α , HIF-1 α , p27, Wnt5a, and IGF-IR [9, 16, 17, 19, 20, 25, 26]) or their nuclear export (e.g., those encoding CD83, COX-2, and c-fos [4, 11, 13]).

The molecular distinction between HuR's influence as a stabilizing factor for some target mRNAs and a factor that modulates the translation of other target mRNAs is not understood in detail. It is well accepted that HuR binds to mRNAs bearing U-rich or AU-rich elements (AREs), which typically are present in their 3' untranslated regions (3'UTRs) (3, 23, 27, 31). The secondary structure also is important for the interaction of HuR with target mRNAs. For example, in almost all mRNAs reported to be HuR targets, a 17- to 20-base-long RNA motif rich in uracils is present in the 3'UTR. This HuR motif forms a specific secondary structure and is conserved in >50% of human and mouse homologous genes (22). Besides the sequence of the target mRNA, the function

of HuR could be influenced by other regulatory factors involved in posttranscriptional regulation. For example, AUF1 appears to antagonize HuR function under certain conditions, as both proteins bind to and compete for the binding of mRNAs such as those that encode cyclin D1 and ATF3 (18, 29). Recently, HuR has been described to recruit the microRNA (miRNA) let-7 to the 3'UTR of c-Myc mRNA and repress c-Myc translation (15). Interestingly, the effect of HuR and let-7 on c-Myc translation is interdependent, as HuR requires let-7 to repress c-Myc translation and vice versa.

The contribution of mRNA turnover in replicative senescence is becoming increasingly apparent. Our previous studies showed that HuR stabilized mRNAs encoding cyclin A, cyclin B1, c-fos, and SIRT1 during replicative senescence (1, 36). In addition, HuR levels were markedly reduced in human fibroblasts undergoing replicative senescence and were modestly reduced in cultured skin fibroblasts from elderly individuals. As a result, the half-lives of the aforementioned HuR target mRNAs were lower in senescent cells. The overexpression of HuR delayed the process of replicative senescence, whereas the knockdown of HuR accelerated senescence. In another study, we identified a region within the 3'UTR of the p16 mRNA that confers transcript instability in early-passage human diploid fibroblasts (37). AUF1 was discovered to bind this instability region and promoted p16 mRNA decay, thereby influencing cell senescence.

In the present study, we have identified a common binding site for HuR and AUF1 in the p16 3'UTR. Unexpectedly, the interaction of HuR with p16 mRNA reduced p16 mRNA stability. Instead of binding to targets competitively and having opposite functions, HuR and AUF1 showed an interdependent interaction, and each destabilized the p16 mRNA. A stem-loop structure localized within the p16 3'UTR was required for

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HuR and AUF1 function. Our results indicate that the fate of p16 mRNA (stabilization or destabilization) depends on the secondary structure in its 3'UTR and on the interaction between HuR and AUF1.

MATERIALS AND METHODS

Cell culture, transfection, and computational analysis. Human IDH4 fibroblasts (generously provided by J. W. Shay) (38) and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO₂. Unless otherwise indicated, IDH4 cells were further supplemented with dexamethasone (Dex) for the constitutive expression of simian virus 40 (SV40) large T antigen to suppress senescence and stimulate proliferation. To induce the senescence of IDH4 cells, Dex was removed from the medium and regular serum was replaced with charcoal-stripped serum, whereupon cells were cultured for 3 additional days for further experiments. All plasmid transfections were performed using Lipofectamine 2000 (Invitrogen) by following the manufacturer's instructions. Unless otherwise indicated, cells were collected for analysis 48 h after transfection.

The computational analyses of RNA motifs, their binding sites, and their secondary structures were conducted according to the methods described by López de Silanes et al. (22) and using the software RNASTRUCTURE (available at the Turner laboratory homepage: <http://rna.chem.rochester.edu>) (24).

Subcellular fractionation and Western blot analysis. Whole-cell and cytoplasmic extracts were prepared as described previously (35). Western blot analysis was performed using standard procedures and the following antibodies: monoclonal anti-HuR, monoclonal anti-p16, and polyclonal anti-AUF1 from Santa Cruz Biotechnologies, and monoclonal anti-Ago2 and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) from Abcam.

RNA isolation, quantitative reverse transcription-PCR (RT-qPCR), and real-time qPCR. Total cellular RNA was prepared using the RNeasy mini kit (Qiagen) by following the manufacturer's protocol. Primers CAACGCACC GAATAGTTACG and GGTCTTTCAATCGGGGAT were used to detect p16, and CGAGTCAACGGATTGGTGGTAT and AGCCTTCTCCATGG TGAAGAC were used to detect GAPDH. Primers TGGAGGCGGGGGCG CTGCCCA and TCGTGCACGGGTGCGGTGAGA were used to detect p16 mRNA, TACAACCTACAACAGCCACAACG and ATCCTGCTCTCCAC CTCC to detect enhanced green fluorescent protein (EGFP)-derived reporter transcripts, and GATTACCAGGGATTTCAGT and GACACCTTTA GGCAGACC to detect pGL3-derived transcripts (Luciferase mRNA).

RNA-protein binding assays, immunoprecipitation (IP), and UV cross-link RNP IP assays. cDNA was used as a template for the PCR amplification of the different fragments of p16 mRNA. All 5' primers contained the T7 promoter sequence CCAAGCTTCTAATACGACTCACTATAGGGAGA-3' (T₇). To prepare templates for the 5'UTR (positions 88 to 305) and CR (coding region; positions 290 to 728), as well as the 3'UTR fragments FL (full length; positions 736 to 1186), A (positions 734 to 925), B (positions 858 to 1053), C (positions 998 to 1202), D (positions 734 to 845), E (positions 734 to 895), F (positions 878 to 928), G (positions 912 to 1003), H (positions 734 to 869), I (positions 870 to 928), J (positions 878 to 959), and K (positions 960 to 1186), we used the following primer pairs: (T7)CTTGCTGCTTTTAAACG and GACATTTACGGTAGTGGG for F, (T7)CCCAGTGAAGAACACAGAG and GTTCT GCCATTGCTAGCAG for FL, (T7)CCCGATTGAAAGAACACAGAG and TTTACGGTAGTGGGGGAAGG for A, (T7)AGAAAATAGAGCTTTTAAA AATGTCCTG and CCACATGAATGTGCGCTTAG for B, (T7)TGCTTTT CACTGTGTTGGA and TTTTATTTGAGCTTTGGTTCTGC for C, (T7)CCC GATTGAAAGAACACAGAG and CGAAGCGGGGGGGTTG for D, (T7)C CCGATTGAAAGAACACAGAG and CGTTAAAAGGCAGGACAT for E, (T7)ATGTCTGCTGCTTTTAAACG and GACATTTACGGTAGTGGG for F, (T7)CCCAGTGAAGAACACAGAG and GCTCTATTTTCTAAATGAAA for G, (T7)TTTAAAATGTCTGCTT and GACATTTACGGTAG TGGG for I, (T7)ATGTCTGCTGCTTTTAAACG and TATAAGAATATATAA AAAAT for J, and (T7)AAAATGTAAAAGAAAAAC and GTTCTGC CATTTGCTAGCAG for K. To generate p16 fragment B mutants B1, B2, B3, B4, and B3Δ, the HuR-binding motif (positions 929 to 970 inside the B fragment) CCAUUUAUAUCAUUUUUAUAUAUUCUUAUAAAAUGUAA was mutated to CCUAAUAUAUCAUUUUUAUAUAUUCUUAUAAAAU GUAA (B1), CCAUUUAUAUCAUUUUUAUAUAUUCUUAUAAAAU GUAA (B2), CCAUUUAUAUCAUUUUUAUAAAAUUCUUAUAAAAU GUAA (B3), CCAUUUAUAUCAUUUUUAUAAAAUUCUUAUAAAAU GUAA (B4), and CCAUUUAUAUCAUUUUUAUAAAAUUCUUAUAAAAU AAUGUAA (B3Δ) by overlapping RT-PCR. To generate fragments B5 (positions 857 to 952) and B6 (positions 953 to 1053), primer pairs (T7)AGAA AATAGAGCTTTTAAAAATGTCCTG and AATATATAAAAAATGATA TAAA and (T7)CTTATAAAAAATGTAAAAA and CCACATGAATGTGCGC TTAG were used. The full-length 3'UTR of p21 was generated as described previously (35). For biotin pull-down assays, PCR-amplified DNA was used as the template to transcribe biotinylated RNA by using T7 RNA polymerase in the presence of biotin-UTP, as described previously (37). 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 (Dyna, Oslo), and the pull-down material was analyzed by Western blotting.

For UV cross-link RNP IP assays, cells were exposed to UVC (400 mJ/cm²), and cytoplasmic extracts were prepared for immunoprecipitation using monoclonal anti-HuR, anti-Ago2, and polyclonal anti-AUF1 antibodies, as described by López de Silanes et al. (22). The IP materials were washed twice with stringent buffer (100 mM Tris-HCl, pH 7.4, 500 mM LiCl, 0.1% Triton X-100, 1 mM dithiothreitol [DTT], 2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM phenylmethyl-sulfonyl fluoride) and twice with the IP buffer (22). The mRNA in RNP was analyzed by real-time qPCR. IP assays were performed using the cytoplasmic extracts from HeLa cells, as described previously (15).

Knockdown of HuR, AUF1, Ago2, Dicer, and Drosha. To transiently silence HuR or AUF1, cells were transfected with vectors expressing shHuR (pSuper.retro-HuR) or shAUF1 (pSilencer-AUF1) by Lipofectamine 2000 (Invitrogen) by following the manufacturer's instructions. To transiently silence Ago2, Dicer, and Drosha, small interfering RNAs (siRNAs) targeting Ago2 (GCACGGAAGUCCAUUGAA TT), Dicer (AAGGCTTACCTTCTCCAGGCT), Drosha (AACGAGTAGGCTT CGTGACTT), or a control siRNA (UUGUUCGACGUGUCACGUTT) were transfected by oligofectamine (Invitrogen) by following the manufacturer's instructions. Cells were collected 24 to 48 h after transfection for further analysis. All knockdowns exhibited less than 1% cell death (by fluorescence-activated cell sorting [FACS] analyses [data not shown]).

Constructs and mRNA half-life measurement. For the construction of vectors expressing Flag-HuR, the full-length coding region of HuR was amplified by PCR using Flag-tagged primer GGAATTCATGGACTACAAGGACGACGAT GACAAGTCTAATGGTTATGAA and primer GCTCTAGATTATTTGTGG GACTTGTGG and inserted between EcoRI and XbaI sites of the pcDNA 3.1 vector (Clontech). For the construction of vectors expressing HuR or control short hairpin RNAs (shRNAs), oligonucleotides corresponding to shRNA targeting HuR (AAGAGGCAAUUACCAGUUUCA) or a control shRNA (AAG TGTAGTAGATCACCAGGC) were inserted between the HindIII and BglII sites in the pSuper.retro (Oligoengine) vector by following the manufacturer's instructions. The plasmid expressing AUF1 shRNA (pSilencer-AUF1) was described previously (37). For the construction of pTRE-d2EGFP-derived reporter plasmids, the p16 fragments depicted in Fig. 2B and 4B were amplified by PCR using the primers without the T7 promoter sequence, inserted into the pTRE-d2EGFP vector (Clontech) or pGL3 vector (for pGL3-B and pGL3-B4; Clontech), and confirmed by sequence analysis. To measure the half-life of endogenous p16 mRNA, the expression of p16 mRNA was shut off by adding actinomycin D (2 µg/ml) into the cell culture medium, and total RNA was prepared at the times indicated and subjected to RT-qPCR analysis using p16-specific primers. To test the half-lives of the EGFP-p16 chimeric transcripts, HeLa cells stably transfected with the pTet-Off plasmid were further transfected with each of the EGFP-p16 fragment constructs. Twenty-four h later, the expression of the EGFP-p16 chimeric transcripts was shut off by the addition of doxycycline (Dox; 1 µg/ml), whereupon total RNA was prepared at the times indicated and the transcript half-lives were evaluated by real-time qPCR using EGFP-specific primers. Data were plotted as the means ± standard deviations (SD) from five independent experiments, and the half-lives were calculated as previously described (37).

RESULTS

HuR represses p16^{INK4} expression by destabilizing p16 mRNA. The present study was prompted by the discovery that the modulation of HuR expression altered p16 levels. As shown in Fig. 1A and B by Western blotting, the expression of flag-HuR in HeLa cells reduced the level of p16 protein by ~76% on average (Fig. 1A, top and bottom), whereas the

FIG. 1. HuR represses p16 by destabilizing the p16 mRNA. (A) Forty-eight hours after the transfection of HeLa cells with a vector expressing Flag-HuR (left) or the HuR shRNA (right), lysates were prepared to assess the levels of HuR, p16, and loading control GAPDH by Western blot analysis. (B) RNA isolated from cells described for panel A was subjected to RT-qPCR to assess the mRNA levels of p16 and loading control GAPDH. The Western blotting and RT-qPCR data are representatives of three or more experiments. Where indicated (bottom panels), the signals were quantified by densitometry and represented as means $\pm$ SD from three independent experiments. (C) Cells described for panel A were exposed to actinomycin D (2 μ g/ml), whereupon the cellular RNA was isolated at the times indicated and subjected to real-time PCR to assess the half-life of p16 mRNA.

knockdown of HuR increased p16 protein by ~ 7.3 -fold on average (Fig. 1B, top and bottom). These alterations were specific for p16, as neither the overexpression nor the knockdown of HuR influenced the levels of GAPDH. HuR has been described as a critical regulator of the turnover and translation of target mRNAs. To further address the mechanism underlying the repression of p16 by HuR, the levels of p16 mRNA in cells treated as described in Fig. 1A and B were assessed by RT-qPCR analysis. As shown in Fig. 1A (middle and bottom), the overexpression of HuR reduced p16 mRNA levels by $\sim 82\%$ on average, whereas the knockdown of HuR increased p16 mRNA by ~ 6.7 -fold on average (Fig. 1B, middle and bottom). As a control, neither the overexpression nor the knockdown of HuR altered the levels of GAPDH mRNA. These results suggested that HuR decreases the stability of p16 mRNA. To test this possibility, cells were exposed to actinomycin D (2 μ g/ml), and total cellular RNA then was prepared at the times indicated and subjected to real-time, quantitative PCR to assess the half-life of p16 mRNA. As shown in Fig. 1C, the half-life of p16 mRNA in flag-HuR-expressing cells was markedly shorter (~ 2.1 h) than that observed in control vector-transfected cells (~ 4.1 h). In contrast, the half-life of p16 mRNA in HuR-silenced cells was much longer (~ 3.4 h) than that observed in control shRNA-expressing cells (~ 1.9 h). These results supported the view that HuR destabilized the p16 mRNA.

The p16 3'UTR interacts with HuR and confers HuR-dependent expression to a heterologous reporter. To test the ability of HuR to interact with p16 mRNA, biotinylated fragments of p16 mRNA [5'UTR and the coding region (CR), as well as the 3'UTR fragments A, B, and C (Fig. 2A, upper), and cytoplasmic extracts of HeLa cells were prepared and used for pull-down analysis as previously described (37). As shown, fragments A and B interacted with cytoplasmic HuR, but fragments 5'UTR, CR, and C did not (Fig. 2A, bottom). In control pull-down analyses, none of the biotinylated RNA fragments interacted with GAPDH present in the cytoplasmic lysates. These results indicated that HuR was capable of associating with the p16 3'UTR.

To further test if the association of HuR with the p16 3'UTR affected the turnover of p16 mRNA, we constructed a series of EGFP-derived reporter constructs bearing the p16 fragments CR, FL (full-length 3'UTR), A, B, and C (Fig. 2B, upper, schematic). The half-lives of the encoded chimeric RNAs were tested using a transcriptional pulse strategy based on the Tet-regulatory system as described previously (37). HeLa cells stably transfected with the pTet-off plasmid were individually cotransfected with each of the EGFP-p16 vectors plus a vector expressing either HuR shRNA or control shRNA. Forty-eight h later, total RNA was prepared at the times indicated and subjected to real time-qPCR to assess the half-lives of the reporter transcripts. Parallel RT-qPCR analysis confirmed that

FIG. 2. p16 3'UTR interacts with HuR and confers the responsiveness of EGFP-p16 3'UTR reporters to the ectopic modulation of HuR expression. (A) Upper panels, schematic representation of the p16 mRNA fragments prepared for biotin pull-down assays. Bottom panels, biotin pull-down assays using biotinylated fragments to detect bound HuR by Western blotting. A 5- μ g aliquot of whole-cell lysate (Lys.) and bound GAPDH were included. (B) Upper panels, schematic representation of the EGFP-p16 fragment reporters. HeLa cells stably transfected with the plasmid pTet-Off were cotransfected with each of the EGFP reporters (as depicted in the upper panels) plus a vector expressing either control or HuR shRNA. Forty-eight hours later, RNA was prepared and subjected to RT-qPCR to monitor the levels of the EGFP-p16 chimeric transcripts, using GAPDH mRNA as a loading control (bottom panels). (C) The transfections in Fig. 2B (bottom panels) were exposed to doxycycline (Dox, 2 μ g/ml) to shut off the expression of the EGFP-p16 chimeric transcripts, whereupon the RNA was prepared at the times indicated and subjected to real-time qPCR to assess the half-lives of the EGFP-p16 chimeric transcripts.

the knockdown of HuR induced the levels of EGFP-FL (~9.5-fold) and EGFP-B (~6.3-fold) chimeric mRNAs but not the levels of EGFP, EGFP-CR, EGFP-A, or EGFP-C mRNAs (Fig. 2B, bottom). Importantly, as shown in Fig. 2C, the knockdown of HuR significantly extended the half-lives of EGFP-FL (~3.6 h) and EGFP-B (~3.2 h) compared to those observed in control shRNA-expressing cells (~1.8 h for both EGFP-FL and EGFP-B). However, the half-lives of EGFP, EGFP-CR, EGFP-A, and EGFP-C chimeric transcripts were not influenced by HuR knockdown. Therefore, HuR is capable of interacting with the 3'UTR of p16 and destabilizing the EGFP-p16 3'UTR chimeric transcripts.

The secondary structure within the HuR interaction motif determines the function of HuR to destabilize p16 mRNA. Although HuR thus far is known as a stabilizer of target mRNAs, our data suggested that HuR had the opposite effect on p16 mRNA. To further address the mechanisms underlying this pathway of regulation, a series of small fragments of the p16 3'UTR,

depicted in Fig. 3A, were transcribed in the presence of biotinylated UTP. RNA pull-down assays were performed by using these biotinylated transcripts and the cytoplasmic extracts of HeLa cells. The interaction of the full-length p16 3'UTR with HuR and AUF1 served as a positive control. As shown in Fig. 3B by Western blotting, HuR and AUF1 proteins were detected in the pull-down materials of fragments FL, E, G, H, and J but not those of fragments D and K. These results identified two HuR and AUF1 common interaction regions in the p16 3'UTR: one between positions 845 and 869 and the other between positions 929 and 960. Interestingly, AUF1 also was present in the pull-down materials of fragments F and I, which were not recognized by HuR, indicating that their affinities for different segments of the p16 3'UTR were not identical. Because the EGFP-A reporters did not respond to the knockdown of HuR and AUF1 (Fig. 2C; also see Fig. 6B), we conclude that the HuR- and AUF1-responsive decay element of the p16 3'UTR was localized within positions 929 and 960.

FIG. 3. Identification of the HuR interaction motif in the p16 3'UTR. (A) Schematic representation of the p16 mRNA, depicting fragments used for biotin pulldown assays. (B) Biotin pulldown assays using biotinylated fragments to detect bound cytoplasmic HuR and AUF1 by Western blotting. A 5- μ g aliquot of whole-cell lysate (Lys.) and bound GAPDH were included as controls.

We next asked why HuR, instead of stabilizing p16 mRNA, promoted its decay. First, the analysis of the secondary structure of the HuR response element in the p16 3'UTR showed two possible stem-loop structures, SL1 and SL2, whose composition was partly shared (Fig. 4A). SL1 is the predicted structure for HuR interaction (22), but positions 931 and 935 spanned an AUUUA pentamer, a sequence frequently found in labile mRNAs (3, 23, 31). To study if the AUUUA motif and SL1 or SL2 were important for HuR interaction and function, several mutants of fragment B (Fig. 4B, schematic) were generated by overlapping PCR, as described in Materials and Methods. These fragments (B to B6 and B3Δ) were prepared as biotinylated transcripts and used for RNA pulldown assays. As shown in Fig. 4C, the B1 mutant (wherein AUUUA was changed to UAAUA) completely lost the ability to interact with both HuR and AUF1. However, mutants B3 and B4, in which SL1 and SL2 structures were impaired, remained associated with HuR and AUF1. Likewise, the B6 fragment (positions 953 to 1053) lacking the AUUUA pentamer and the moiety in the 5' end of SL1 and SL2 structures did not interact with either HuR or AUF1. The fragment B5 (between positions 857 and 952), which deleted the moiety in the 3' end of SL1 or SL2, associated with HuR and AUF1. In contrast, the mutant B3Δ, bearing complementary mutations to completely repair the SL2 structure and partly repair the SL1 structure of B3, associated with HuR and AUF1. The B2 mutant specifically mutating on SL2 but not on the SL1 structure still interacted with HuR and AUF1. Interestingly, Ago2, an important RISC (RNA miRNA-induced silencing complex) component, exhibited binding similar to those of HuR and AUF1. These results suggest that the AUUUA region, but not the stem-loop structures (SL1 or SL2), is critically important for the interaction of p16 mRNA with HuR, AUF1, and Ago2.

FIG. 4. Analysis of the secondary structure of the HuR interaction motif in the p16 3'UTR. (A) Schematic representation of the secondary structure (SL1 and SL2), analyzed as described in Materials and Methods. (B) Schematic representation depicting the wild-type fragment B and its mutants (B1 to B6 and B3Δ). The mutated sites are marked by capital letters. (C) Biotin pulldown assays were performed using biotinylated fragments (B to B6 and B3Δ) to evaluate the influence of the AUUUA pentamer and the SL1 or SL2 structure in the association of HuR, AUF1, and Ago2 with the p16 3'UTR. A 5-μg aliquot of whole-cell lysate (Lys.) and bound GAPDH were included as controls.

We next studied the influence of the AUUUA motif as well as the SL1 and SL2 structure on the stability of EGFP-B chimeric transcripts. To this end, EGFP-derived reporters bearing the mutated or deleted fragment B (B1 to B6 and B3Δ) were constructed (Fig. 5A, schematic). Each of these vectors was cotransfected with a vector expressing HuR shRNA or the AUF1 shRNA in pTet-off plasmid stably transfected HeLa cells. Forty-eight h later, the half-lives of these chimeric transcripts were assessed by real-time qPCR analysis. As shown in Fig. 5B (upper), consistently with the result obtained in Fig. 2B and C, the knockdown of HuR increased the level of EGFP-B transcript. The half-life of EGFP-B chimeric transcript in HuR-silenced cells was markedly longer (~3.4 h) than that observed in control shRNA-expressing cells (~1.9 h) (Fig. 5B, bottom). Likewise, the knockdown of AUF1 increased the half-life of EGFP-B but not that of EGFP-A and EGFP-C transcripts (Fig. 6B). As anticipated, because B1 and B6 were unable to interact with HuR and AUF1 (Fig. 4C), both the levels and the half-lives of EGFP-B1 and EGFP-B6 chimeric transcripts were unaltered by HuR or AUF1 knockdown (Fig. 5B and 6B). However, the knockdown of HuR (Fig. 5B) or AUF1 (Fig. 6B) had no influence on the half-lives of EGFP-B2, EGFP-B3, EGFP-B4, and EGFP-B5 mRNAs, even though B2, B3, B4, and B5 were shown to interact with HuR and AUF1 (Fig. 4C). It appears that SL2 is more important than SL1 for the destabilization of p16 mRNA by HuR or AUF1, since the fragments B3, B4, and B5 impaired both SL1 and SL2 structures but the B2 fragment specifically mutated the SL2 structure. Notably, the knockdown of HuR (Fig. 5B) or AUF1 (Fig. 6B) increased the half-life of EGFP-B3Δ transcript, indicating that the complementary mutation repaired the SL

FIG. 5. Influence of the SL1 or SL2 structure in HuR-mediated destabilization of EGFP-B chimeric transcript. HeLa cells stably transfected with pTet-Off plasmid were cotransfected with each of the EGFP-derived reporters bearing fragments (B to B6 and B3Δ) and a vector expressing HuR or the control shRNA. (B) Forty-eight hours later, the levels and the half-lives of the chimeric transcripts were assessed by RT-qPCR (upper panels) and real-time qPCR (bottom panels), respectively, as described in Materials and Methods.

structures of B3 and could rescue the destabilizing influence of HuR and AUF1 upon the EGFP-B3 chimeric transcript. Taken together, these results suggest that the function of HuR and AUF1 to destabilize p16 mRNA not only requires the association of HuR and AUF1 to p16 3'UTR but also depends upon the formation of the local hairpins.

HuR and AUF1 interact with the p16 3'UTR, recruit the RISC, and destabilize p16 mRNA in a cooperative manner. AUF1 was found to destabilize p16 mRNA (37). The analysis of the half-lives of the EGFP-p16 3'UTR chimeric transcripts indicated that the AUF1-responsive element localizes within fragment B (Fig. 6B), as does the HuR response element. Because AUF1 was reported to antagonize HuR in binding and regulating cyclin D1 and p21 mRNAs (18), we next asked whether HuR and AUF1 influenced each other's ability to affect the turnover of p16 mRNA. To answer this question, HeLa cells were transfected with vectors expressing HuR shRNA or AUF1 shRNA or were cotransfected with both vectors. Forty-eight hours later, Western blot and RT-PCR analyses were performed to assess the levels of p16 protein and mRNA, respectively. As shown in Fig. 7A, the knockdown of

HuR (lane 2) or AUF1 (lane 3) increased p16 protein levels by ~5.9- or ~7.8-fold (on average), respectively. However, the concomitant knockdown of HuR and AUF1 (lane 4) only moderately induced p16 protein levels (~1.8-fold, on average). Likewise, the knockdown of HuR or AUF1 individually elevated the levels of p16 mRNA on average by ~8.7- (lane 2) or ~8.4-fold (lane 3), and the joint knockdown of HuR and AUF1 only modestly induced the abundance of p16 mRNA (lane 4; ~2.2-fold, on average) (Fig. 7A). The RISC was implicated in the translation and turnover of mRNAs targeted by RNA binding proteins (RBPs) and microRNAs (14, 15). Because Ago2 was found to associate with fragment B (Fig. 4C), we asked whether the RISC participated in the regulation of p16 mRNA turnover. Interestingly, the knockdown of Ago2 increased p16 protein and mRNA levels by ~3.4- and ~4.5-fold (on average), respectively (Fig. 7B). The half-life of p16 mRNA was extended in cells with silenced Ago2 (~3.6 h) compared to that of the control siRNA-transfected cells (~2.9 h) (Fig. 7C). Because of the competitive nature of the binding of HuR and AUF1 to p21 and cyclin D1 mRNAs (18), we studied whether HuR, AUF1, and Ago2 influenced each

FIG. 6. SL1 or SL2 structure within the B fragment is required for AUF1 to destabilize the EGFP-B chimeric transcript. HeLa cells stably transfected with the pTet-Off plasmid were further transfected with a vector expressing AUF1 or control shRNA along with each of the EGFP-derived reporter vectors (Fig. 2B and 5A). Twenty-four hours later, the levels of AUF1 were monitored by Western blot analysis (A) and the half-lives of the chimeric transcripts were assessed as described in Materials and Methods (B).

other's interaction with the p16 3'UTR. As shown by biotin pulldown analysis (Fig. 7D), the knockdown of HuR not only reduced the association of HuR with fragments B and B4 but also reduced the binding of AUF1 and Ago2 with fragments B and B4 (left). Likewise, in AUF1-silenced cells, the association of HuR and Ago2 with fragments B and B4 was reduced (Fig. 7D, right). In contrast to the individual knockdown of HuR or AUF1, the joint knockdown of HuR and AUF1 more strongly reduced the association of HuR, AUF1, and Ago2 with fragment B (Fig. 7E). Additional evidence was obtained from the analysis of RNP; as shown in Fig. 7F, the knockdown of HuR reduced the levels of AUF1- and Ago2-bound pGL3-B chimeric transcript in RNP IP materials (left two graphs), and the knockdown of AUF1 reduced the HuR- and Ago2-bound pGL3-B transcript (right two graphs). On the other hand, although the knockdown of HuR or AUF1 reduced the association of Ago2 with pGL3-B transcript (Fig. 7D, E, and F), the knockdown of Ago2 did not affect the association of HuR and AUF1 with fragment B or B4, as shown by biotin pulldown (Fig. 8A) and RNP IP (Fig. 8B) analyses. In contrast, in keeping with previous studies (18), the knockdown of HuR increased the association of p21 3'UTR with AUF1, while the knockdown of AUF1 increased that with HuR (Fig. 9A and B). In coimmunoprecipitation assays using an antibody recognizing HuR, AUF1, or Ago2, we observed that HuR, AUF1, and Ago2 associated with each other in an RNA-dependent manner, as digestion by RNase A disrupted the interaction of HuR, AUF1, and Ago2 (Fig. 10A and B). To address if the interac-

tion between Ago2/RISC and HuR (or AUF1) was dependent on microRNAs, HeLa cells were transfected with siRNA targeting Dicer or Drosha, and the expression of Ago2, HuR, AUF1, and p16 was monitored by Western blot analysis. As shown, the knockdown of either Dicer or Drosha failed to alter the levels of Ago2, HuR, AUF1, or p16 (Fig. 11A and B, left). In addition, the knockdown of Dicer or Drosha did not significantly influence the association of p16 fragment B with Ago2, AUF1, or HuR, as shown by biotin pulldown (Fig. 11A and B, right) and RNP IP analyses (Fig. 11C and D). Taken together, these results indicate that HuR and AUF1 interact with the p16 3'UTR, recruit the RISC in an RNA-dependent but miRNA-independent manner, and cooperatively destabilize p16 mRNA. The functional consequences on p16 mRNA decay, but not the association of HuR and AUF1 with the p16 3'UTR, requires the presence of structured hairpins.

Impact of HuR-mediated p16 mRNA destabilization on the elevation of p16 in replicative senescence. We next tested if HuR affected p16 mRNA levels in replicative senescence. For this purpose, the human senescence model cell line IDH4 (36–38) was cotransfected with pTet-off and EGFP-B or EGFP-CR plasmid. After transfection, dexamethasone was removed from the medium for 3 days to induce cell senescence. This cell system ensured equal transfection efficiency between young and senescent cells, unlike other model cell lines for cell senescence (e.g., WI-38 and 2BS), which were not amenable to such experiments due to the uneven transfection rates between young and senescent cells. The rate of clearance (turnover) of

FIG. 7. HuR and AUF1 interact with the p16 3'UTR, recruit the RISC, and destabilize p16 mRNA in a cooperative manner. HeLa cells were individually transfected with a vector expressing HuR (lane 2) or AUF1 (lane 3) shRNA or cotransfected with both vectors (lane 4). (A) Forty-eight h after transfection, whole-cell lysate and RNA were prepared and subjected to Western blotting and RT-qPCR analysis to assess the protein and mRNA levels of p16. GAPDH served as a loading control. (B) HeLa cells were transfected with siRNA targeting Ago2 (siAgo2, +) or the control siRNA (siAgo2, -). Twenty-four hours later, whole-cell lysate and RNA were prepared and subjected to Western blotting and RT-qPCR to assess the protein and mRNA levels of p16. GAPDH served as a loading control. Where indicated, the Western blot and RT-qPCR signals were quantified by densitometry and are represented as means $\pm$ SD from three independent experiments. (C) The half-life of p16 mRNA was assessed from the transfections in Fig. 7B, as described in Materials and Methods. (D) HeLa cells were transfected with a vector expressing HuR (left panels) or AUF1 (right panels) shRNA. Forty-eight hours later, cytoplasmic extracts were prepared for biotin pull-down assays using biotinylated p16 fragments B and B4. The bound HuR, AUF1, and Ago2 in the pull-down materials were detected by Western blotting. A 5- μ g aliquot of whole-cell lysate (Lys.), pull-down using CR fragment (Neg.), and bound GAPDH were included as controls. (E) Cytoplasmic extracts from cells described for panel A were subjected to biotin pull-down analysis using biotinylated p16 fragment B. The bound HuR, AUF1, and Ago2 in the pull-down materials were detected by Western blotting, as described for panel D. (F) HeLa cells were transfected with a vector expressing HuR (left panels) or AUF1 shRNA (right panels) along with pGL3-B reporter vector. Forty-eight hours later, UV cross-link RNP IP assays were performed using cytoplasmic extracts, as described in Materials and Methods. The AUF1- and Ago2-associated pGL3-B chimeric transcripts in HuR-silenced cells (left two panels) as well as the HuR- and Ago2-bound pGL3-B chimeric transcripts in AUF1-silenced cells (right two panels) were quantified by real-time PCR. Values represent means $\pm$ SD from three independent experiments.

the chimeric transcripts then was tested by the addition of doxycycline (Dox). As shown in Fig. 12A by Western blotting, the levels of HuR (upper) and Ago2 (lower) were reduced by $\sim$ 82 and $\sim$ 92% in senescent cells (S) compared to that of young cells (Y). Consequently, the half-life of EGFP-B chimeric transcript was longer in senescent cells ($\sim$ 5.6 h) than in young cells ($\sim$ 2.7 h) (Fig. 12B, right). In contrast, the half-life of EGFP-CR chimeric transcript was comparable between young and senescent IDH4 cells (Fig. 12B, left). To further confirm the role of HuR and Ago2/RISC in the turnover of the p16 mRNA-derived chimeric transcript, the half-lives of

EGFP-B and EGFP-CR chimeric transcripts were evaluated in HuR- and Ago2-silenced IDH4 cells. As shown in Fig. 12C, the knockdown of HuR reduced the protein level of HuR by $\sim$ 90%. As a result, the half-life of EGFP-B transcript (Fig. 12D, right), but not that of EGFP-CR transcript (Fig. 12D, left), was extended in HuR-silenced cells ($\sim$ 4.8 h) compared to that observed in control shRNA-expressing cells ($\sim$ 2.5 h). Likewise, the knockdown of Ago2 reduced Ago2 by $\sim$ 85% (Fig. 12E) and extended the mRNA half-life of EGFP-B ($\sim$ 4.5 h) (Fig. 12F, right), but not that of EGFP-CR (Fig. 12F, left), compared to that observed in control siRNA-transfected cells

FIG. 8. Knockdown of Ago2 has no influence on the association of the p16 3'UTR with HuR or AUF1. (A) HeLa cells were transfected with the siRNA targeting Ago2 or the control siRNA. Twenty-four hours later, cytoplasmic extracts were prepared and subjected to biotin pulldown assays using biotinylated fragments B and B4. The bound Ago2, HuR, and AUF1 were detected by Western blotting as described in the legend of Fig. 7D. (B) HeLa cells were transfected with pGL3-B vector; 24 h later, cells were further transfected with the siRNA targeting Ago2 or the control siRNA and cultured for an additional 24 h. UV cross-link RNP IP assays were performed using cytoplasmic extracts, as described in Materials and Methods. The HuR- or AUF1-bound pGL3-B chimeric transcripts in cells with silenced Ago2 were tested by real-time qPCR. Values represent means $\pm$ SD from three independent experiments.

(3.0 h) (Fig. 12F, right). Therefore, the HuR-p16 regulatory pathway is of critical importance for the elevation of p16 in replicative senescence.

DISCUSSION

Over the past decade, much effort has been directed toward studying the function and mechanisms underlying RBP-regulated mRNA turnover. The binding motifs of several decay-regulatory RBPs are characterized by the presence of U- or AU-rich elements (AREs). Interaction with the AREs enables RBPs to either stabilize or destabilize the mRNAs targeted. For example, tristetraprolin (TTP) destabilizes COX-2, IL-8, and gamma interferon (IFN- γ) mRNAs (28, 34, 39); AUF1 destabilizes cyclin D1, p21, and p16 mRNAs (18, 37) but stabilizes the TNF- α and parathyroid hormone (PTH) mRNAs (8, 32). In contrast, HuR and other members of the Hu RNA-binding protein family, such as HuD and Hel-N1 (30), have been found to function exclusively on stabilizing target mRNAs. The present study describes an exceptional role for HuR as a protein that destabilizes the p16 mRNA (Fig. 1). This function of HuR was further confirmed by using EGFP-derived reporter constructs (Fig. 2B and C, 5B, and 12D).

HuR interacts with collections of target mRNAs and regulates their turnover, translation, and nuclear export. Among the numerous HuR target transcripts predicted by bioinformatics analysis (22), only a small portion of them have been shown to be regulated by HuR. Given that different RNA-binding proteins

may bind to the same target, the fate of certain target mRNAs (turnover, translation, etc.) may be determined by the interaction between these RBPs. For example, such was the case for AUF1 and HuR, which were shown to bind to the 3'UTR of p21 and cyclin D1 mRNAs on both distinct, nonoverlapping sites and on common sites in a competitive fashion. Consequently, HuR stabilized p21 and cyclin D1 mRNAs, but AUF1 destabilized both mRNAs (18). However, instead of competitively interacting with target mRNAs such as p21 mRNA (Fig. 9), HuR and AUF1 show an interdependent association with the p16 3'UTR (Fig. 7D, E, and F). The different interaction modalities with target mRNAs ultimately determines whether HuR and AUF1 compete (e.g., p21 and cyclin D1) or cooperate (e.g., p16) (Fig. 2, 5, and 6) in regulating mRNA turnover.

RBPs (e.g., TTP and HuR) have been found to influence the translation or turnover of mRNAs by targeting miR-16- or let-7-loaded Ago2/RISC onto certain mRNAs (14, 15). The present study showed that Ago2/RISC also was involved in the regulation of p16, because the knockdown of Ago2 increased the expression and mRNA stability of p16 mRNA (Fig. 7B and C). Although the underlying mechanism is unclear, HuR and AUF1 were found to recruit Ago2/RISC to the p16 3'UTR, as the knockdown of HuR or AUF1 reduced the association of Ago2 with the p16 3'UTR, whereas the knockdown of Ago2 had no influence on the association of HuR or AUF1 with the p16 3'UTR (Fig. 7D, 7E, 7F, and 8). In light of the recent discovery that Ago2 can associate directly with many target mRNAs without microRNAs (5) and *in vitro* evidence that Ago2 binds the p16 3'UTR in an miRNA-independent manner

FIG. 9. HuR and AUF1 competitively associated with the p21 3'UTR. The cytoplasmic extracts described in the legend to Fig. 7D were used for biotin pulldown assays using biotinylated p21 3'UTR. Bound HuR and AUF1 in HuR (A)- or AUF1 (B)-silenced cells were assessed by Western blotting. A 5- μ g aliquot of whole-cell lysate (Lys.) and bound GAPDH were included as controls.

FIG. 10. HuR, AUF1, and Ago2 interact in the cytoplasm in an RNA-dependent manner. The cytoplasmic extracts prepared from HeLa cells were incubated with RNase A or left untreated. IP assays were performed using HuR or AUF1 (A) or Ago2 (B) antibody. The IP materials then were subjected to Western blotting to detect the presence of HuR, AUF1, and Ago2. IP with IgG served as a negative control.

FIG. 11. Knockdown of Dicer or Drosha has no influence on the association of the p16 3'UTR with HuR, AUF1, or Ago2. Twenty-four hours after the transfection of HeLa cells with pGL3-B, cells were further transfected with siRNAs targeting Dicer (A), Drosha (B), or control siRNA and cultured for an additional 24 h. (A) On the left, the levels of Dicer, Ago2, HuR, AUF1, and p16 were assessed by Western blot analysis of whole-cell lysates. GAPDH was used as a loading control. On the right, cytoplasmic extracts were prepared and subjected to biotin pulldown assays using biotinylated fragment B; bound Ago2, HuR, and AUF1 were detected by Western blotting, as described in the legend to Fig. 7D. (B) On the left, the levels of Drosha, Ago2, HuR, AUF1, and p16 were assessed by Western blot analysis using whole-cell lysates. GAPDH was used as a loading control. On the right, cytoplasmic extracts were prepared and subjected to biotin pulldown assays using biotinylated fragment B. The bound Ago2, HuR, and AUF1 were detected by Western blotting, as described in the legend to Fig. 7D. (C and D) HeLa cells were transfected with pGL3-B vector. Twenty-four hours later, cells were further transfected with siRNAs targeting Dicer (C) or Drosha (D) and cultured for an additional 24 h. UV cross-link RNP IP assays were performed using cytoplasmic extracts, as described in Materials and Methods. The HuR-, AUF1-, and Ago2-bound pGL3-B chimeric transcripts in cells silenced for Dicer (C) or Drosha (D) were tested by real-time qPCR. Values represent means $\pm$ SD from three independent experiments.

(Fig. 11), it is likely that Ago2 directly associates with p16 mRNA in the cell. Furthermore, HuR, AUF1, and Ago2/RISC interact with each other in an RNA-dependent manner, since they failed to show association in the cytoplasmic extracts pre-incubated with RNase A (Fig. 10).

Besides the U-rich or AU-rich elements, the secondary structure of mRNAs is of critical importance for the interaction of RBPs with target mRNAs. The HuR-bound mRNAs have been characterized by the presence of specific stem-loop structures within their binding motifs (22). The secondary structure of p21 and TNF- α mRNAs has been reported to determine the mRNA destabilization by AUF1 (p37) (8). Iakova et al. (12) reported that a stem-loop structure localized at the 5'UTR of p21 determined the competitive interaction of CRT and CUGBP1 with the p21 5'UTR and thereby affected the fate of p21 translation. The SL1 and SL2 stem-loop structures predicted by bioinformatic analysis in the p16 3'UTR (Fig. 4A) are not necessary for binding by AUF1 and HuR, since disrupting these structures did not influence the association of HuR or AUF1 with fragment B (Fig. 4C). Because HuR and AUF1 could interdependently interact with either

wild-type fragment B or its mutant (B4) (Fig. 7D, E, and F), it is clear that the SL1 or SL2 structure is not a determinant for the interdependent interaction between HuR and AUF1. However, the SL1 or SL2 structure is indispensable for the interdependent function of HuR and AUF1 on destabilizing p16 mRNA, since the knockdown of HuR or AUF1 increases the half-life of EGFP-derived chimeric transcripts bearing SL1 or SL2 (EGFP-B) or similar (EGFP-B3 Δ) structures but did not influence the turnover of transcripts in which the structures were disrupted (B2 to B6) (Fig. 5 and 6). Apart from fragment B, there exists another common interaction motif for HuR and AUF1 in fragment A (within positions 845 and 869) (Fig. 3B). Unlike fragment B, fragment A neither bears SL1- or SL2-like structures nor affects the turnover of p16 mRNA by HuR or AUF1 (Fig. 2 and 6). Therefore, the destabilization of p16 mRNA by HuR depends on the SL1 or SL2 structure in the p16 3'UTR and requires AUF1 as a cofactor.

In the process of replicative senescence, where numerous genes are elevated or reduced by altered mRNA turnover or translation, the role of HuR and other RBPs is becoming increasingly apparent. HuR has been described as a critical

FIG. 12. Fragment B of the p16 3'UTR is a HuR response element in replicative senescence. (A and B) IDH4 cells were transfected with EGFP-CR or EGFP-B reporter vector, whereupon the cells were cultured either in regular medium plus dexamethasone (1 μ g/ml; Y) or in medium containing charcoal-stripped serum in the absence of dexamethasone (to induce cell senescence; S) for 3 days. (A) Whole-cell lysates then were prepared to assess the level of HuR and Ago2 as well as the loading control GAPDH by Western blotting. (B) The half-lives of the EGFP-CR or EGFP-B transcripts were determined as described in Materials and Methods. Values represent means $\pm$ SD from five independent experiments. (C and D) IDH4 cells were transfected with pTet-Off plasmid. Twenty-four hours later, cells were further cotransfected with a vector expressing HuR or the control shRNA as well as EGFP-CR or EGFP-B reporter vector and cultured for an additional 24 h. Whole-cell lysate was prepared and subjected to Western blotting to monitor the level of HuR and loading control GAPDH (C). The half-lives of EGFP-CR and EGFP-B transcripts were tested as described in Materials and Methods (D). (E and F) IDH4 cells were transfected with pTet-Off plasmid along with EGFP-CR or EGFP-B reporter vector. Twenty-four hours later, cells were further transfected with siRNA targeting Ago2 or the control siRNA and cultured for an additional 24 h. Ago2 and GAPDH protein levels (E) as well as the half-lives of EGFP-CR and EGFP-B transcripts (F) were tested as described for panels C and D. Values represent means $\pm$ SD from five independent experiments. Where indicated, the Western blotting signals were quantified by densitometry.

stabilizer of mRNAs encoding cell proliferative factors (cyclin A, cyclin B1, and c-fos) in cell senescence (36). The present study provides evidence that HuR also functions in the destabilization of p16 mRNA, which inhibits cell growth and accelerates replicative senescence (Fig. 12). Notably, HuR represses the translation of p27, an important cell growth-inhibitory gene (16). In sum, our findings lend further support to

HuR's role in controlling the process of cell growth and senescence.

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