Published online 8 December 2009 *Nucleic Acids Research, 2010, Vol. 38, No. 5* ***1547–1558***
doi:10.1093/nar/gkp1114

Reduced nuclear export of HuR mRNA by HuR is linked to the loss of HuR in replicative senescence

Jie Yi[1], Na Chang[1], Xinwen Liu[1], Gaier Guo[2], Lixiang Xue[1], Tanjun Tong[1], Myriam Gorospe[3] and Wengong Wang[1,*]

[1]Research Center on Aging, Department of Biochemistry and Molecular Biology, Peking University Health Science Center, 38 Xueyuan Road, Beijing 100083, [2]Department of Biochemistry, Changzhi Medical College, Changzhi, Shanxi 046000, P. R. China and [3]Laboratory of Cellular and Molecular Biology, National Institute on Aging, National Institutes of Health, 251 Bayview Blvd., Baltimore, MD 21224, USA

Received July 12, 2009; Revised October 27, 2009; Accepted November 12, 2009

ABSTRACT

The RNA-binding protein, HuR, associates with the HuR mRNA, but the consequences of this interaction are unknown. Here, we use human diploid fibroblasts (HDFs) and cervical carcinoma cells to study this regulatory paradigm. Ectopic overexpression of HuR potently enhanced the translation and cytoplasmic levels of endogenous HuR, but did not affect HuR mRNA levels. Inhibition of CRM1 function by Lemptomycin B or by knockdown of CRM1 greatly diminished the cytoplasmic levels of endogenous HuR mRNA and hence blocked the induction of endogenous HuR by exogenous HuR. Further studies showed that HuR interacted with the 3′-untranslated region (UTR) of HuR and that overexpression of HuR increased the cytoplasmic levels of a chimeric luciferase-HuR 3′-UTR reporter transcript, as well as luciferase activity; conversely, HuR knockdown reduced both parameters. Moreover, the loss of HuR in senescent, late-passage HDFs was accompanied by a reduced cytoplasmic presence of endogenous HuR mRNA, ectopic Luc-HuR-3′UTR reporter transcript, and luciferase activity relative to what was observed in young, early-passage cells. Our results reveal a positive feedback mechanism for the regulation of HuR, which may play an important role in the regulation of HuR during replicative senescence.

INTRODUCTION

In mammalian cells, gene expression is strongly regulated at the post-transcriptional level through altered mRNA export, turnover, and translation. RNA-binding proteins (RBPs) are key regulators of these processes. The ubiquitous member of the Hu family of RBPs, HuR, recognizes specific RNA signature sequence that are typically U- or AU-rich, usually found in the 3′-untranslated region (UTR) of short-lived messenger RNAs (mRNAs), such as those encoding VEGF, $p21^{CIP1}$, cyclin A, cyclin B1, c-fos, SIRT1, COX-2, p53, β-actin, myoD and myogenin (1–7). HuR has been shown to stabilize many target mRNAs, but it also can enhance the translation of certain mRNAs (e.g. MKP-1, p53, prothymosin α, HIF-1α) and repress the translation of other mRNAs (e.g. p27, Wnt5a, IGF-IR) (8–12).

Although the precise mechanisms by which HuR stabilizes and regulates the translation of target mRNAs are largely unknown, HuR's cytoplasmic presence, post-translational modification (phosphorylation, methylation and ubiquitination) and interaction with nuclear ligands have been shown to influence HuR's ability to regulate mRNA turnover or translation (1,12,13–15). Among them, the cytoplasmic presence of HuR strongly influences the fate of HuR target mRNAs and has been studied most extensively. We previously showed that exposure to stresses, such as ultraviolet light irradiation, hydrogen peroxide, prostaglandins and alkylating agents, enhanced the stability of the mRNA encoding cyclin-dependent kinase (CDK) inhibitor $p21^{CIP1}$ by increasing HuR's association with the p21 mRNA in the cytoplasm of the stressed cells (6). Cytoplasmic HuR levels fluctuated during the cell division cycle, being highest during S and G_2, the period of greatest stability of HuR target mRNAs encoding cyclin A and B1 (7). The elevation of cytoplasmic HuR in various cancers, such as breast cancer, ovarian carcinoma, colon carcinoma and gastric cancer (16–18), was linked to the stabilization of mRNAs encoding cancer-related genes such as COX-2, VEGF, β-catenin, etc. (2,4,14,19) and correlated with the tumor grade in human breast and colon cancers as well as with

*To whom correspondence should be addressed. Tel: +86 10 8280 2949; Fax: +86 10 8280 2949; Email: wwg@bjmu.edu.cn

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

poor outcome in human ovarian carcinoma (17,20). Moreover, the AMP-activated protein kinase (AMPK) and cyclin-dependent kinase 1 (Cdk1) have been shown to regulate cytoplasmic HuR levels by different mechanism, which in turn influence HuR function to stabilize the mRNAs encoding cyclin A, cyclin B1 and c-fos, and other proteins implicated in cell division and replicative senescence (1,21,22).

Besides the aforementioned factors, the presence of HuR in the cytoplasm is affected by changes in total HuR levels. For example, the decrease of cytoplasmic levels resulting from the loss of HuR during replicative senescence was linked to the reduced expression of proliferative genes, such as cyclin A, cyclin B1 and c-fos, and the stagnant growth of senescent cells (23). Likewise, the elevation of HuR in human cancers leads to higher cytoplasmic levels, which in turn increases COX-2 expression through stabilizing the COX-2 mRNA (17,20,24,25). A recent study described that miR-519 acts as a negative regulator of HuR translation in human colon cancer (26).

Given that HuR is predominantly localized in the nucleus, there has been much interest in identifying HuR functions other than those of mRNA stabilization and translation in the cytoplasm. In this regard, HuR has been proposed to act as an important regulator of the nuclear export of CD83, COX-2 and c-fos mRNAs (8,13,14,27,28,29).Here, we present studies that suggest a positive feedback regulatory mechanism for HuR. HuR was found to associate with the 3′UTR of the HuR mRNA and upregulated HuR translation by promoting the nuclear export of HuR mRNA. We propose that this mechanism of regulation may be responsible for the loss of HuR during replicative senescence.

MATERIALS AND METHODS

Cell culture, transfection and treatment

Human IDH4 fibroblasts were generously provided by J. W. Shay and described previously (30). Early-passage [Young, ~28 population doublings (pdl)], middle-passage (45 pdl) and late-passage (Senescent, ~60 pdl) human diploid 2BS fibroblasts (National Institute of Biological Products, Beijing, China), and HeLa cells 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. Unless otherwise indicated, IDH4 cells were further supplemented with dexamethasone (Dex) for constitutive expression of SV40 large T antigen to suppress senescence and stimulate proliferation. To induce senescence of IDH4 cells, Dex was removed from the medium, and regular serum was replaced with charcoal-stripped serum, whereupon cells were cultured for five additional days for further experiments in which senescent cells were required. To inhibit CRM1-dependent mRNA export, cells were treated with 10 nM Lemptomycin B (LMB, Roche) overnight (12 h) and collected for further experiments. All plasmids were transfected using lipofectamine 2000 (Invitrogen) and small interfering RNA (siRNA) targeting HuR (positions 649–669, AAGAGGCAAUUACC AGUUUCA) were transfected using oligofectamine (Invitrogen) following the manufacturer's instructions. Cells were collected 48–72 h after transfection for further analysis.

RNA–protein-binding assays

Complementary DNA (cDNA) was used as a template for polymerase chain reaction (PCR) amplification of different HuR 3′UTR and coding regions (CRs). All 5′ primers contained the T7 promoter sequence CCAAGCTTCTAA TACGACTCACTATAG GGAGA-3′ (T_7). To prepare the HuR CRs (positions 192–1145) transcript, primers (T_7)ATGGCCGAAGACTGCAGGGGTGAC and TTT GTGGGACTTGTTGGTTTTGA were used. To prepare fragment A spanning both CR and 3′ UTR (positions 720–1650), CCAACCCCAACCAGAACAAAAA and ACGGGACCTGCCTGGAAAAGGA were used. To prepare the HuR 3′-UTR fragments B (positions 1645–2550), C (positions 1645–1814), D (positions 1814–1983) or E (positions 1983–2550), we used these primer pairs. CCCGTTGCCACCTCCTGCTCAC and CCTTC CTCCGGGCTCCTGGTTTA for B, CCCGTTGCCAC CTCCTGCTCAC and AACTCTTTGGTCCATTCCCT for C, GGGAATGGACCAAAGAGTT and GCTCTA GACGGGAGAAATTATCGTGAA for D and TTCAC GATAATTTCTCCCG and CCTTCCTCCGGGCTCCT GGTTTA for E. For biotin pull-down assays, PCR-amplified DNA was used as template to transcribe biotinylated RNA by using T7 RNA polymerase in the presence of biotin-UTP, as described (7). Six micrograms of purified biotinylated transcripts were incubated with 100 μg of cytoplasmic extracts or 20 μg of nuclear extracts for 30 min at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dynal, Oslo), and the pull-down material was analyzed by western blotting.

RNA isolation and PCR analysis

Total cellular RNA was prepared using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. To isolate cytoplasmic and nuclear RNA, 2×10^5 cells were trypsinized, rinsed with phosphate-buffered saline, incubated in 100 μl of hypotonic buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$] supplemented with 2 μl RNasin (Promega) on ice for 3 min, lysed by addition of 12.5 μl of buffer A containing 2.5% NP-40 plus RNasin and centrifuged at 3500 r.p.m. for 4 min at 4°C. Cytoplasmic or nuclear RNA was prepared from the supernatant (cytoplasmic RNA) or the pellet (nuclear RNA) using RNeasy Mini Kit (Qiagen) as described above. For reverse transcription (RT)-PCR analysis to detect HuR transcripts, primers A TGAAAGACAACGCCCAATC and CCAACCCCAAC CAGAACA were used for HuR pre-mRNA, and TTCAC ATCCGATTCAGCC and TCTACTGCCATCATTACA CG for HuR mRNA. To analyze the total, cytoplasmic, and nuclear levels of Luciferase, Renilla or U6 mRNA by RT-PCR, primers GATTACCAGGGATTTCAGT and GACACCTTTAGGCAGACC, GAAACGGATGATA

ACTGG and TCGCCATAAATAAGAAGAG as well as GCCTATTTCCCATGATTC and ACGGTGTTTCGTC CTTTC were used, respectively.

Constructs and reporter gene assays

For the construction of vectors expressing flag-HuR, HA-HuR and myc-HuR, full-length HuR was amplified by PCR using flag-tagged, HA-tagged or myc-tagged primers and inserted between EcoRI and Xbal sites in pcDNA3.1 vector (Clontech), respectively. The pRc/CMV-Hsp72 plasmid expressing flag-Hsp72 was generously provided by Knowlton A and the pIRES-CSIG vector expressing flag-CSIG was described previously (31). For the construction of a vector expressing CRM1 siRNA, oligonucleotides corresponding to the siRNA targeting the CRM1 CR (UGUGGUGAA UUGCUUAUAC) and a control siRNA (AAGTGTAGT AGATCACCAGGC) were inserted into the BamHI and HindIII sites in pSilencer 2.1-U6 neo vector (Ambion) to generate vectors expressing CRM1 and control siRNAs following the manufacturer's instructions. For reporter gene assays, pGL3-CR, pGL3-A, pGL3-B, pGL3-C, pGL3-D or pGL3-E was constructed by inserting the HuR mRNA fragments described in 'RNA–protein binding assays' section into the XbaI site of pGL3 vector (Promega) and confirmed by sequence analysis. Transient transfection of HeLa cultures with the reporters was carried out by Lipofectamine 2000 (Invitrogen). Co-transfection of pRL-CMV served as an internal control. Firefly and Renilla luciferase activities were measured with a double luciferase assay system (Promega, Madison, WI, USA) following the manufacturers' instructions. All firefly luciferase measurements were normalized to Renilla luciferase measurements from the same sample.

Northern blot, subcellular fractionation, western blot and protein stability analysis

For northern blot analysis, total RNA was isolated using RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. For detection of HuR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs, cDNA inserts excised from pcDNA 3.1-HuR were labeled by random primer extension with α-^{32}P-dCTP using Prime-a-Gene® Labeling System (Promega). Northern blot analysis was performed as previously described (6). Northern signals were visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). For western blot analysis, whole-cell, nuclear or cytoplasmic lysates were prepared as previously described (6). Lysates were size-fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto poly-vinylidene difluoride (PVDF) membranes. Monoclonal antibodies recognizing HuR, CRM1, p53, HDAC1 or GAPDH were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The monoclonal antibody recognizing Hsp72 was from Stressgene. To detect CSIG, polyclonal antiserum recognizing CSIG was obtained after immunizing rabbits as described previously (31). After secondary antibody incubation, signals were detected by SuperSignal WestPico Chemiluminescent Substrate (Pierce) following the manufacturer's instructions and quantified by densitometric analysis with ImageMaster VDS software. For HuR protein stability assays, HeLa cells were transiently transfected with either pcDNA-flag-HuR or the empty vector; 48 h later, cells were treated with cycloheximide (50 μg/ml), collected at the times indicated and subjected to western blot analysis.

Analysis of nascent protein

One million cells were incubated with 1 mCi (1 Ci = 37 GBq) L-[^{35}S]methionine and L-[^{35}S]cysteine (Easy Tag EXPRESS, NEN/Perkin–Elmer) per 60-mm plate for 20 min, whereupon cells were lysed by using TSD lysis buffer (50 mM Tris, pH 7.5/1% SDS/5 mM DTT), and lysates were immunoprecipitated by using either monoclonal anti-HuR antibody (Santa Cruz Biotechnology), anti-GAPDH antibody (Bios. Biotechnology) or Immunoglobulin G (IgG) for 1 h at 4°C. After extensive washes in TNN buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA and 0.5% NP-40), the immunoprecipitated material was resolved by 12% SDS-PAGE, transferred onto PVDF membranes and visualized by using a PhosphorImager (Molecular Dynamics).

RESULTS

Induction of endogenous HuR by the expression of exogenous HuR

The current study was initiated by our finding that endogenous HuR was markedly induced by transiently transfecting vectors that expressed ectopic, tagged HuR in HeLa cells. As analyzed by western blot in Figure 1A (upper panels) and B, expression of flag-HuR, HA-HuR,or myc-HuR induced endogenous HuR ~4.5-, 3.8- or 4.2-fold, respectively. As negative controls, expression of flag-tagged CSIG or Hsp72 did not influence the abundance of endogenous HuR, or CSIG or Hsp72 (Figure 1A, bottom panels). Similar results were obtained in HEK-293 cells (data not shown). These findings support the notion that a positive feedback mechanism may be involved in the regulation of HuR expression.

Exogenous HuR increases HuR mRNA cytoplasmic abundance and HuR translation

We next investigated the mechanisms underlying the induction of endogenous HuR by the expression of flag-HuR. We transfected plasmid flag-HuR or an empty vector control in HeLa cells, and collected cells 48 h later. Total RNA was then prepared for RT-PCR analysis to assess the levels of endogenous HuR mRNA and pre-mRNA. As shown in Figure 2A, expression of flag-HuR did not influence the levels of endogenous HuR pre-mRNA or mRNA. To address the possible involvement of protein turnover in the feedback regulation of HuR, HeLa cells were transfected to express

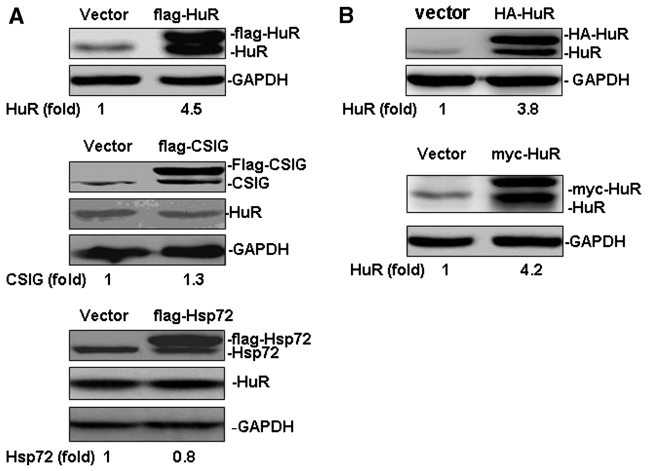

Figure 1. Induction of endogenous HuR by the expression of exogenous HuR. (**A**) HeLa cells were transfected with vectors expressing flag-HuR (upper panels), flag-CSIG (middle panels), flag-Hsp72 (bottom panels) or the empty vectors. Forty-eight hours later, whole-cell lysates were prepared and subjected to western blot analysis using antibodies that recognized HuR, CSIG or Hsp72. GAPDH served as a loading control. The relative abundance of HuR was measured by densitometry and expressed as fold increase relative to the levels in empty vector-transfected cells. (**B**) HeLa cells were transfected with vectors expressing HA-HuR, Myc-HuR or the corresponding empty vectors. Forty-eight hours later, whole-cell lysates were prepared and subjected to western blot analysis to detect ectopic and endogenous HuR. GAPDH was included as a loading control.

flag-HuR or empty vector; 48 h later, cells were treated with cycloheximide (50 μg/ml) and collected at times indicated (Figure 2B) for western blot analysis. As shown in Figure 2B, HuR protein levels remained unchanged even after exposure of cells to cycloheximide for 24 h. Expression of flag-HuR had no significant effect on the stability of endogenous HuR. No cell death was observed throughout the cycloheximide treatment period. To confirm the effective inhibition of translation by cycloheximide, the rate of p53 clearance in flag-HuR- and empty vector-transfected cells was assessed by western blot analysis. As anticipated, p53 protein in both transfection groups was undetectable by 4 h after treatment with cycloheximide; the expression of flag-HuR led to a significant increase in p53 expression, in keeping with the previous finding that HuR promotes p53 translation (11). These results indicate that the feedback regulation of HuR does not involve regulation at the levels of transcription, mRNA turnover or protein turnover.

Instead, our results suggest that the expression of endogenous HuR was induced by exogenous HuR at the level of translation. To test this possibility directly, HeLa cells transiently expressing flag-HuR were incubated with L-[^{35}S] methionine and L-[^{35}S] cysteine for 20 min, whereupon cell lysates were prepared and the 'nascent'-labeled HuR protein was analyzed by immunoprecipitation. As shown in Figure 2C, *de novo* synthesized endogenous HuR protein was ~4.0-fold more abundant in flag-HuR expressing cells than in empty vector-transfected cells. As a control, expression of flag-HuR did not influence the levels of nascent GAPDH protein. Therefore, exogenous HuR induces endogenous HuR by enhancing its translation.

HuR has been reported to regulate target gene expression at the translational level by processes that also enhance the cytoplasmic presence of the target mRNA (27,14,8,28). To address if the nuclear export of HuR mRNA is involved in this feedback regulatory process, HeLa cells expressing flag-HuR were exposed to Lemptomycin B (LMB), a CRM1 inhibitor which has been proposed to affect HuR translocation through the nuclear envelope. Expression of endogenous HuR was assessed by western blot analysis. Consistent with earlier results (Figure 1A), HeLa cells expressing flag-HuR exhibited 3.8-fold-higher levels of endogenous HuR (Figure 3A, left, lane 2) relative to what was observed in control transfected cells (left, lane 1). However, treatment of flag-HuR-expressing cells with LMB nearly abolished the effect of the expression of flag-HuR in inducing endogenous HuR (left, lanes 2 and 4). Notably, treatment of empty vector-transfected cells with LMB had a weak inhibitory effect (~0.6-fold, left, lane 3) on the expression of endogenous HuR. LMB is also an inhibitor of the export of HuR protein. To verify the effective inhibition of CRM1 by LMB, HeLa cells were either treated with LMB or left untreated, and whole-cell, nuclear and cytoplasmic lysates were subjected to western blot analysis. As shown in Figure 3A (right), exposure of cells to LMB reduced cytoplasmic HuR levels by ~3.3-fold. Moreover, LMB inhibited HuR expression (Figure 3A, left, lanes 1 and 3; Figure 3A, right, lanes 1, 2, 5 and 6) in whole cell and nucleus by ~2.0- and ~1.7-fold, respectively; these reductions likely reflected the lower rates of HuR export, which in turn led to overall lower HuR translation and steady-state levels. As controls for loading and purity of subcellular fractionation, HDAC1 and GAPDH were measured. HDAC1 was not detectable in the cytoplasmic extracts and remained unchanged in both whole-cell and nuclear lysates, and GAPDH was unchanged in both whole-cell and cytoplasmic lysates but only showed weak signals in the nuclear extracts. To further confirm the role of CRM1 on the feedback regulation of HuR, expression of HuR was assessed in CRM1-silenced cells. As shown in Figure 3B, transfection of HeLa cells with a vector expressing CRM1 siRNA inhibited CRM1 levels by ~85% and reduced HuR expression by ~2.5-fold, compared with what was observed in control siRNA-transfected cells, while the knockdown of CRM1 did not alter the levels of GAPDH.

These observations prompted us to test if regulation at the level of mRNA nuclear export is involved in flag-HuR-induced expression of endogenous HuR. To this end, whole-cell RNA, cytoplasmic RNA and nuclear RNA were isolated from control and flag-HuR-expressing cells and endogenous HuR was detected by RT-PCR analysis. As shown in Figure 3C and D, neither expression of flag-HuR nor exposure to LMB or knockdown of CRM1 significantly affected the total cellular HuR mRNA levels. However, expression of flag-HuR increased the cytoplasmic level (~3.9-fold) and decreased the

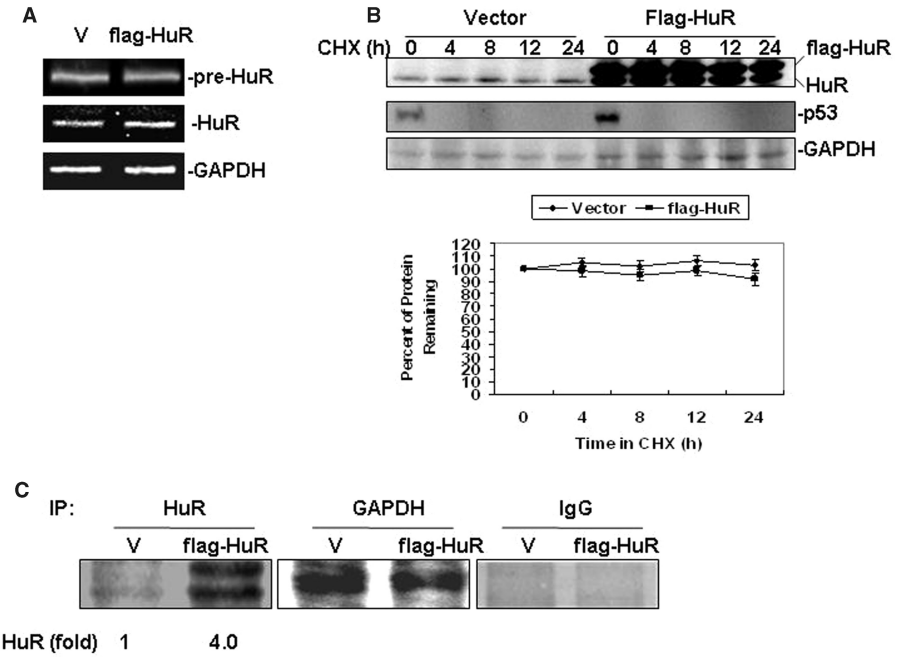

Figure 2. Exogenous expression of HuR induces the translation of endogenous HuR by increasing the cytoplasmic presence of endogenous HuR mRNA. (**A**) HeLa cells were transfected with vector expressing flag-HuR or the empty vector. Forty-eight hours later, total RNA were prepared and subjected to RT-PCR analysis to determine the levels of the mRNA and pre-mRNA of HuR. The HuR pre-mRNA was evaluated by RT-PCR using primers specifically recognizing sequence spanning the second and third exons of HuR ('Materials and Methods' section). RT-PCR analysis of GAPDH mRNA served to monitor differences in loading among samples. (**B**) Forty-eight hours later after transfection of HeLa cells with a vector expressing flag-HuR or the empty vector control were treated with cycloheximide (50 μg/ml) for the time periods indicated, whereupon whole-cell lysates were prepared and subjected to Western blot analysis for HuR and p53. Values represent mean ± SEM from three independent experiments. GAPDH served as a loading control. (**C**) Analysis of HuR translation in flag-HuR-expressing cells. Newly translated HuR was measured by incubating cells with L-[^{35}S]methionine and L-[^{35}S]cysteine for 20 min, followed by immunoprecipitation using either anti-HuR antibody, anti-GAPDH antibody or IgG, resolving immunoprecipitated samples by SDS-PAGE, and transferring for visualization of signals by using a PhosphorImager.

nuclear level (~5.0-fold) of endogenous HuR mRNA (Figure 3C, right). Treatment of cells expressing flag-HuR with LMB dramatically reduced the effect of flag-HuR in inducing the cytoplasmic (1- versus 1.3-fold) and reducing the nuclear (1- versus 0.9-fold) presence of endogenous HuR mRNA (Figure 3C, left). Likewise, although flag-HuR expression decreased endogenous HuR mRNA in the nucleus by ~3.3-fold and increased it level in the cytoplasm by ~4.0-fold in control siRNA-expressing cells (Figure 3D, right), the effect of flag-HuR was nearly abolished in CRM1-silenced cells. As shown in Figure 3D (left), expression of flag-HuR failed to increase the cytoplasmic presence (1- versus 1.1-fold) and decrease the nuclear presence (1- versus 0.9-fold) of endogenous HuR mRNA. Verification that nuclear mRNA did not leak into the cytoplasm during the isolation process was obtained through subsequent RT-PCR analysis of the levels of U6, a small RNA that localizes exclusively in the nucleus. As a loading control, neither the expression nor the subcellular distribution of GAPDH mRNA was influenced by LMB treatment or CRM1 knockdown (Figure 3C and D). Together, these results suggest that expression of flag-HuR induces endogenous HuR by specifically promoting the nuclear export of endogenous HuR mRNA in a CRM1-dependent manner.

Analysis of HuR 3′UTR heterologous reporter

To ascertain the ability of HuR to interact with HuR mRNA, biotinylated transcripts spanning segments of HuR mRNA (Figure 4A), as well as nuclear and cytoplasmic HeLa cell extracts were prepared and used for pull-down analysis as previously described (7). As shown by western blotting of HuR in the pull-down materials (Figure 4B), fragments B and E were found to interact with HuR in both the nucleus and the cytoplasm, but fragments CR, A, C or D were not. In control pull-down analyses, the biotinylated RNA fragments described in Figure 4A could not interact with GAPDH present in cytoplasmic lysates or with HDAC1 in nuclear lysates. These results showed that HuR was capable of interacting with the HuR 3′UTR *in vitro*.

To further test whether the association of HuR protein with the HuR 3′UTR was important for the feedback regulation of HuR, we constructed a series of pGL3-derived reporter constructs containing HuR fragments CR, A, B, C, D or E (Figure 4 schematic). These constructs were individually co-transfected either with a vector expressing flag-HuR or with the empty vector. Forty-eight hours later, the activity of the reporter genes was analyzed by luciferase assays. As shown in Figure 4C, expression of flag-HuR greatly increased the luciferase activity of pGL3-B (~6.7-fold) and pGL3-E (~6.1-fold), both

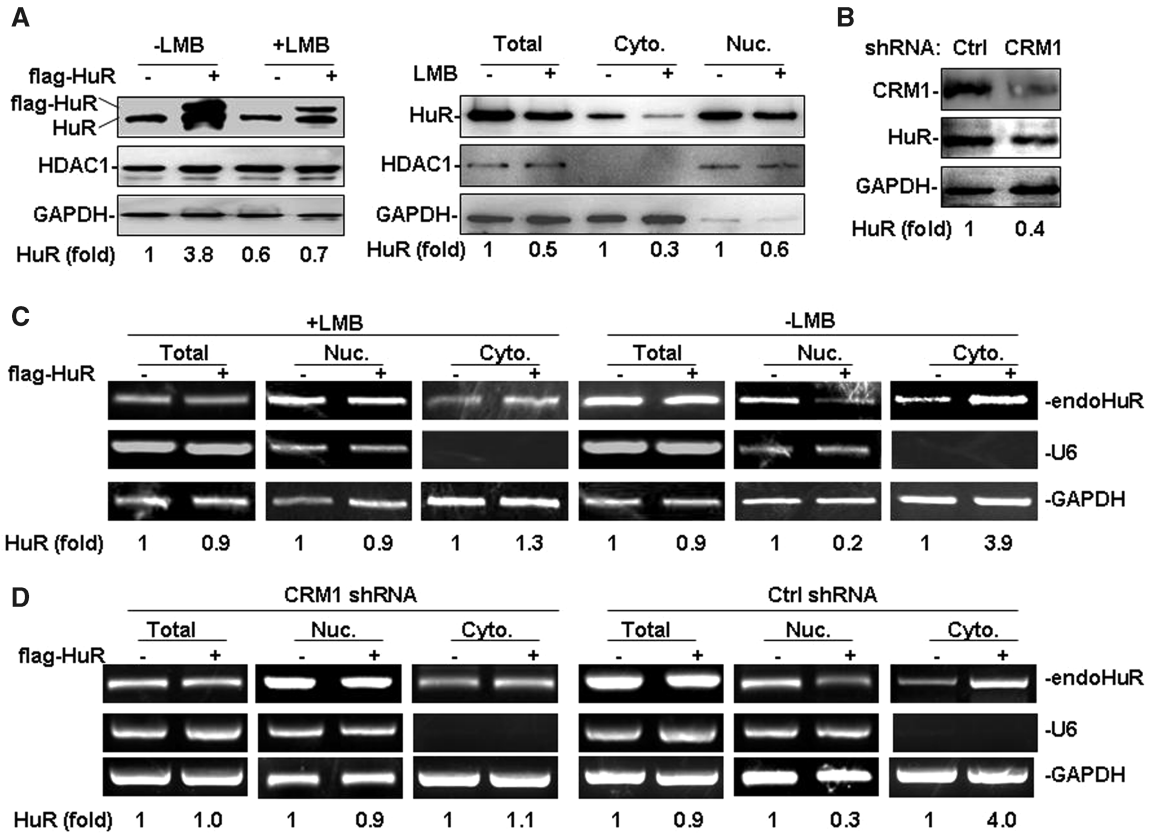

Figure 3. HuR regulates the nuclear export of HuR mRNA in a CRM1-dependent manner. (**A**) (Left) HeLa cells transiently transfected with a vector expressing flag-HuR or the empty vector were either treated with 10 nM Lemptomycin B (LMB) for 12 h or left untreated and whole-cell lysates were prepared for western blot analysis to evaluate the levels of flag-HuR and endogenous HuR. GAPDH and HDAC1 served as loading controls. (Right) HeLa cells were either treated with Lemptomycin B (LMB) for 12 h or left untreated, whole-cell, nuclear and cytoplasmic lysates were prepared and subjected to western blot analysis of HuR, HDAC1 and GAPDH abundance. (**B**) HeLa cells were transfected with vectors that expressed either CRM1 shRNA (pSilencer-CRM1) or control shRNA for 48 h, whole-cell lysates were prepared and subjected to western blot analysis for CRM1, HuR and GAPDH (**C** and **D**). Whole-cell mRNA, cytoplasmic RNA and nuclear RNA were prepared from cells that were transfected as described in Figure 3A and B and subjected to RT-PCR analysis to evaluate the expression and nuclear export of endogenous HuR mRNA. RT-PCR analysis of U6 and GAPDH served to assess the quality of the cell fractionation procedure and to monitor differences in loading and transfer among samples.

reporters bear the fragments of HuR 3′UTR which were shown to interact with HuR (Figure 4B). In contrast, the luciferase activity of pGL3, pGL3-CR, pGL3-A, pGL3-C or pGL3-D was not apparently affected by the expression of flag-HuR. To test whether knockdown of HuR could have an effect opposite to that of flag-HuR, HeLa cells were transfected with a siRNA directed against the HuR CR (targeting positions 648–668), but not to fragment A (931-bp, spanning CR and 3′UTR, positions 720–1650). Twenty-four hours later, cells were then transfected with pGL3-A, pGL3-B or pGL3 and cultured for an additional 24 h, whereupon luciferase activity was analyzed as described in Figure 4C. As shown in Figure 4D, transient transfection of HuR siRNA reduced HuR abundance by ~90% (bottom panel), compared with control siRNA-transfected cells. As a result, knockdown of HuR reduced ~76% of the activity of pGL3-B, relative to what was seen in the control transfection group, while the activity of pGL3 and pGL3-A reporters remained nearly unchanged (upper panel). These results indicate that the association of HuR with the 3′UTR of the HuR mRNA is a necessary step for the feedback regulation of HuR expression.

We next sought to determine if the observed effect of HuR, as a regulator of pGL3-B activity, was linked to changes in the whole-cell or cytoplasmic levels of the pGL3-B chimeric transcript. To this end, whole-cell RNA, cytoplasmic RNA and nuclear RNA were isolated from the cells described in Figure 4C and D and measured by RT-PCR. As shown in Figure 5, transient overexpression of HuR by transfection of flag-HuR greatly induced the presence of pGL-3B chimeric transcript in the cytoplasm(~9.7-fold) (Figure 5A, right, upper panels), while knockdown of HuR markedly reduced the cytoplasmic level of pGL-3B chimeric transcript (~3.3-fold) (Figure 5B, right, upper panels), compared with the results obtained from the control cells. Conversely, HuR overexpression decreased the levels of pGL3-B chimeric transcript in the nucleus (~10-fold) (Figure 5A, right, middle panels), while knockdown of HuR increased the nuclear presence of pGL3-B chimeric transcript (~11-fold) (Figure 5B, right, middle

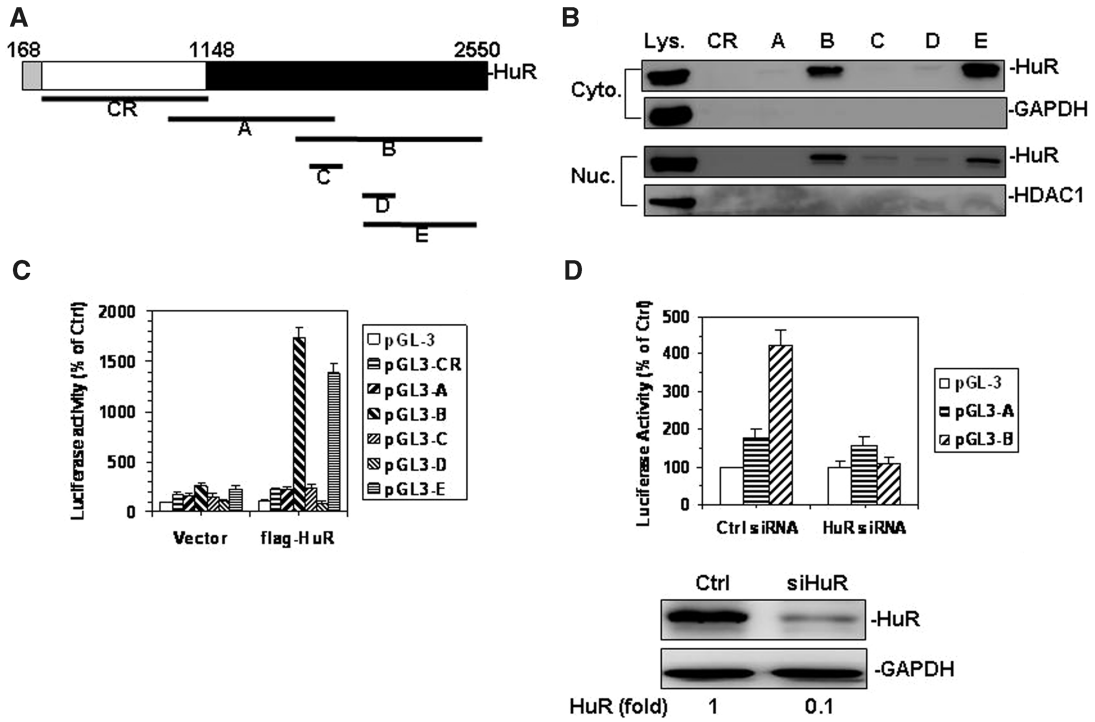

Figure 4. The HuR 3′UTR is recognized by HuR and confers HuR responsiveness to a reporter construct containing Luciferase-HuR-3′UTR. (**A**) Schematic presentation of the HuR mRNA and various transcripts derived from the coding region (CR) and 3′ UTR used in this study. (**B**) Cytoplasmic and nuclear extracts (100 μg) were prepared from HeLa cells. RNA pull-down assays were performed using biotinylated fragments to detect bound HuR by western blotting. A 10-μg aliquot of whole-cell lysates (Lys.) and binding of GAPDH (negative control) to HuR mRNA were also tested. (**C**) HeLa cells were co-transfected with vector expressing flag-HuR or the empty vectors plus pGL3, pGL3-CR, pGL3-A, pGL3-B, pGL3-C, pGL3-D or pGL3-E reporter vector along with pRL-CMV control reporter; 48 h later, firefly luciferase activity was determined and normalized to Renilla luciferase activity. Values represent the means ± SEM from five independent experiments. (**D**) (Upper panel) HeLa cells were transfected with HuR-directed siRNA; 24 h later, cells were co-transfected with pGL3, pGL3-A or pGL3-B reporter vectors along with the pRL-CMV control reporter and an additional 24 h later, luciferase activity was analyzed as described in (C). Western blot analysis was performed to evaluate the effect of HuR knockdown (bottom panel).

panels). In keeping with the findings shown in Figures 3C, 4C and D, neither overexpression nor knockdown of HuR significantly affected the total levels of pGL3-B chimeric transcript. In addition, as anticipated, neither overexpression nor knockdown of HuR altered the total level or nuclear export of pGL3 or pGL3-A transcripts (Figure 5A and B, left and middle panels). As controls for monitoring loading and quality of mRNA isolation process, the total cellular Renilla mRNA levels and their distribution in the cytoplasm and nucleus, and the total cellular as well as nuclear levels of U6 were unaffected by either overexpression or knockdown of HuR; U6 was not detectable in the cytoplasm (Figure 5A and B). Taking together, our results show that the HuR 3′UTR fragment containing an HuR-binding site is capable of eliciting feedback regulation of HuR through a mechanism linked to HuR mRNA nuclear export.

The feedback regulation of HuR plays a critical role in the loss of HuR during replicative senescence in IDH4 and 2BS cells

Thus far, our results revealed a positive feedback mechanism for HuR regulation. We previously reported that loss of HuR was responsible for the reduced expression of proliferative genes during replicative senescence (23). However, the mechanisms underlying the reduction of HuR during cellular senescence have not been studied. Based on the above results, we hypothesized that a positive feedback regulatory mechanism of HuR might be important for the loss of HuR during senescence. To study this possibility, the expression of HuR in early-passage [Young, ~28 population doublings (pdl)] and late-passage (senescent, ~60 pdl) 2BS cells was examined by western blot and northern blot analyses. As shown in Figure 6A, compared with early-passage 2BS cells (Young, Y), HuR levels in middle-passage (M) or late-passage 2BS cells (Senescent, S) were reduced by ~3.3-fold or ~10-fold, respectively. Northern blot analysis showed that HuR mRNA levels remained unchanged in senescent 2BS cells. As shown in Figure 6B, two HuR mRNA variants (2.7 kb and 1.5 kb) were detected, and their abundance was comparable in young, middle-aged and senescent 2BS cells. A recent study observed three variants of HuR mRNA (of sizes 6.0 kb, 2.7 kb and 1.5 kb) in HeLa cells, due to alternative polyadenylation (32). However, the 6.0-kb variant was not detectable by northern blot analysis, due to its low abundance in 2BS cells. The reduction of HuR in replicative senescence was also studied in IDH4 cells, another model cell line for studies of cellular senescence (30). In IDH4

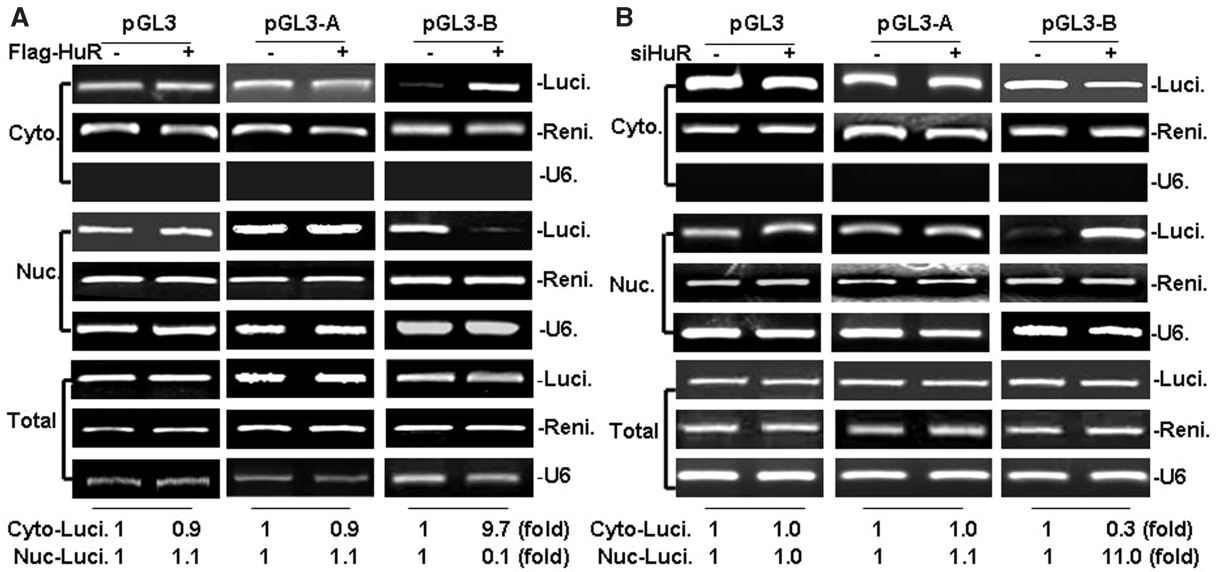

Figure 5. HuR regulates the nuclear export of luciferase-HuR 3′UTR chimeric transcripts. (**A**) HeLa cells were co-transfected with a vector expressing flag-HuR or the empty vector and pGL3, pGL3-A or pGL3-B reporter vector along with pRL-CMV control reporter; 48 h later, total, cytoplasmic and nuclear RNAs were isolated and subjected to RT-PCR analysis to determine the expression and nuclear export of the chimeric transcripts. RT-PCR analysis of U6 RNA and Renilla mRNA served to assess the quality of the cell fractionation procedure and to monitor differences in loading and transfer among samples. (**B**) HeLa cells were transfected with HuR-directed siRNA; 24 h later, cells were transfected with pGL3, pGL3-A or pGL3-B reporter vector along with pRL-CMV control reporter, and an additional for 24 h later, the expression and nuclear export of the chimeric transcripts were assessed as described in (A).

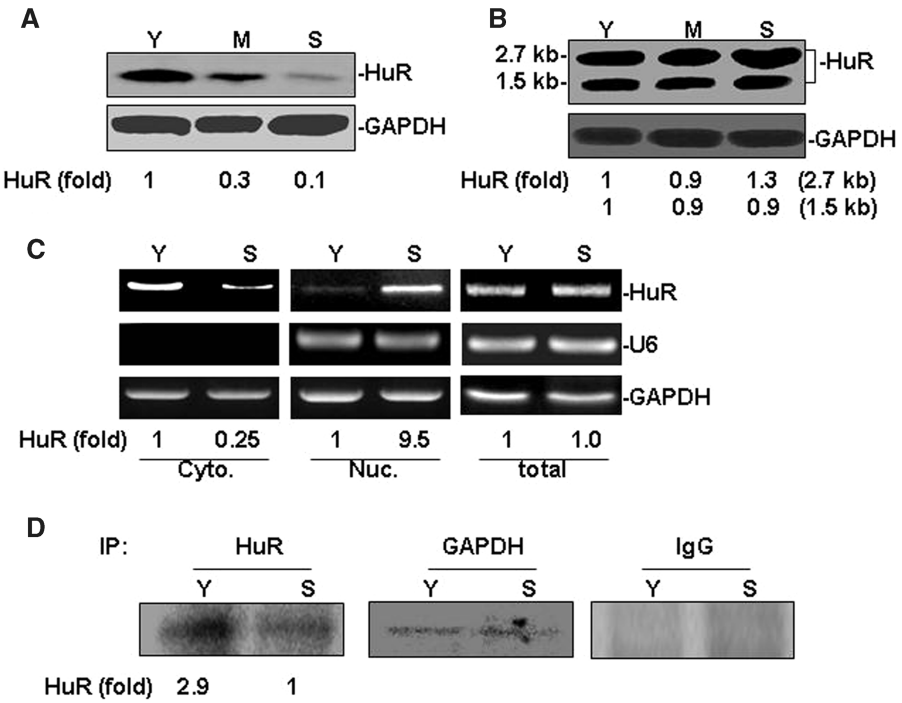

Figure 6. Reduction of translation and cytoplasmic mRNA levels of HuR in senescent 2BS cells. (**A**) Western blot analysis of HuR levels in early-passage (Young, ~27 pdl, Y), middle-passage, (~45 pdl, M) and late-passage (Senescent, ~60 pdl, S) 2BS cells; GAPDH served as loading control. Whole-cell RNA, cytoplasmic RNA and nuclear RNA were prepared from young (S) and senescent 2BS cells. The expression of HuR mRNA was assessed either by northern blot analysis using cDNA probes of HuR and GAPDH (**B**) or by RT-PCR (**C**, right). The cytoplasmic and nuclear mRNA levels of HuR were analyzed by RT-PCR (C, middle and left panels). RT-PCR analysis of U6 RNA and GAPDH mRNA served to assess the quality of the cell fractionation procedure and to monitor differences in loading and transfer among samples. (**D**) The levels of nascent HuR protein in early- (Y, ~27 pdl) and late-passage (S, ~60 pdl) 2BS cells were analyzed as described in Figure 2C.

cells, we also observed a ~10-fold difference in HuR abundance between young and senescent cells, but no change in cellular HuR mRNA levels (Figure 7A and B). To evaluate the importance of mRNA export for the regulation of HuR during cellular senescence, we analyzed the subcellular distribution of HuR mRNA. As shown, the HuR mRNA levels were decreased in the cytoplasm (~14-fold) and increased in the nucleus (~11-fold) of senescent 2BS (Figure 6C) and IDH4 cells (Figure 7C), while the whole-cell HuR mRNA remained unchanged, while whole-cell levels of GAPDH mRNA and U6 RNA, the nuclear levels of U6, as well as the distribution of GAPDH mRNA in the nucleus and cytoplasm was not altered in senescent 2BS or IDH4 cells. As verification for the purity of cytoplasmic RNA, the small nuclear RNA U6 was not detectable in the cytoplasm of either 2BS or IDH4 cells (Figures 6C and 7C). The loss of HuR in senescent cells resulted from reduced translation, as determined by assessing the levels of nascent HuR. As shown in Figure 6D, *de novo* synthesized HuR in young (Y) 2BS cells was ~2.9-fold higher than in senescent cells (S). These results suggest that the feedback regulation of HuR is an important mechanism for the loss of HuR with senescence.

To further address the role of HuR in the nuclear export of HuR mRNA in replicative senescence, pGL3-A, pGL3-B and pGL3 vectors were individually transfected into IDH4 cells. After transfection, dexamethasone was removed from the medium for 5 days to induce cell senescence. This cell system ensured equal transfection efficiency between young and senescent cells, unlike 2BS cells, which were not amenable to such experiments due to the uneven transfection rates between young and senescent cells. Reporter gene activity and the distribution of the reporter transcripts in the cytoplasm and nucleus were analyzed as described in Figures 4 and 5. As shown in Figure 7D, pGL3-B luciferase activity in young IDH4 cells, which has higher levels of HuR than senescent cells do (Figure 7A), was ~4.3-fold higher than in senescent cells. As controls, the reporter gene activity of pGL3-A

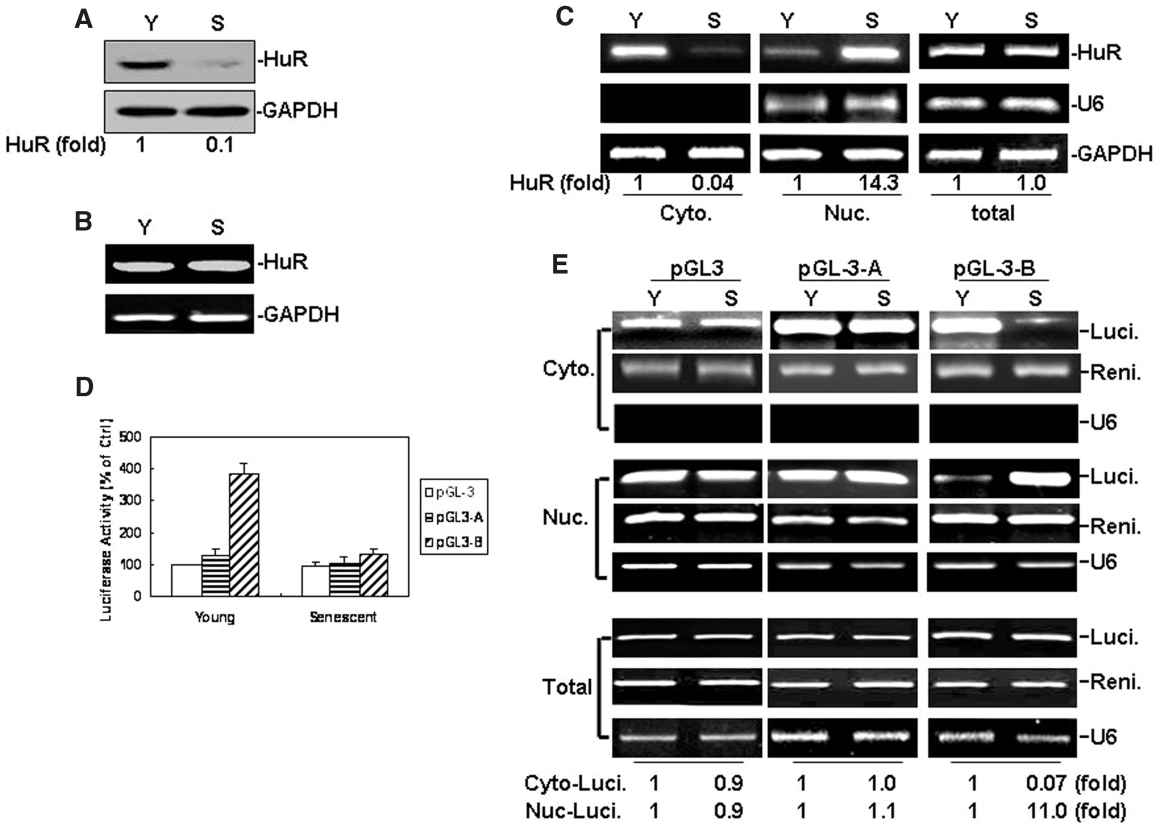

Figure 7. Loss of HuR is linked to the reduction of pGL-3B reporter gene activity and the nuclear export of the chimeric transcript in senescent IDH4 cells. (**A, B**) IDH4 cells were either cultured in the presence (Young, Y) or absence (Senescent, S) of Dexamethasone (Dex) for 5 days, whereupon total protein and mRNA were prepared for western blot and RT-PCR analyses to assess the expression of HuR protein (A) and HuR mRNA levels (B). GAPDH was included as a loading control. (**C**) Total, cytoplasmic and nuclear mRNA were prepared from Young (+Dex) and Senescent (–Dex for 5 days) IDH4 cells. The levels of HuR mRNA and its presence in the cytoplasm and nucleus were analyzed by RT-PCR. RT-PCR analysis of U6 RNA and GAPDH mRNA served to assess the quality of the cell fractionation procedure and to monitor differences in loading and transfer among samples. (**D**) IDH4 cells (+Dex) were co-transfected with pGL3, pGL3-A or pGL3-B plus pRL-CMV control reporter; 48 h later, firefly luciferase activity was determined and normalized against Renilla luciferase activity. Values represent the means ± SEM from five independent experiments. (**E**) IDH4 cells were co-transfected with pGL3, pGL3-A or pGL3-B plus pRL-CMV control reporter and further cultured in the presence (Y) or absence (S) of Dex for 5 days, whereupon total, cytoplasmic and nuclear mRNAs were prepared and subjected to RT-PCR analysis to assess the expression and presence of the reporter gene mRNA in the cytoplasm and nucleus. RT-PCR analysis of U6 RNA and Renilla mRNA served to assess the quality of the cell fractionation procedure and to monitor differences in loading and transfer among samples.

and pGL3 between young and senescent cells was comparable. Consistent with these observations, the levels of pGL3-B chimeric transcript increased in the cytoplasm and decreased in the nucleus, while the distribution of pGL3 and pGL3-A transcripts in the cytoplasm and nucleus remained unchanged during IDH4 cell senescence. The data presented above strongly suggests that the feedback regulation of HuR acts as a critical mechanism for the regulation of HuR during replicative senescence.

DISCUSSION

In this study, we set out to explore the mechanism underlying the regulation of HuR in replicative senescence. We first obtained data showing that expression of exogenous HuR (flag-HuR) elevates endogenous HuR (Figure 1A and B), suggesting that a positive feedback regulatory mechanism controlled the expression of HuR. Further study showed that expression of flag-HuR had no effect on either the mature or the pre-mRNA levels of endogenous HuR (Figure 2A) and found that the turnover of HuR was not influenced by the expression of flag-HuR (Figure 2B). Therefore, mechanisms other than transcription, mRNA turnover, mRNA maturation and HuR protein turnover were involved in the feedback regulation of HuR. Our results show that ectopic overexpression of HuR by transfecting vectors that express flag-HuR increased the cytoplasmic levels and the translation of endogenous HuR mRNA (Figures 2C and 3C). Investigation into the mechanism showed that HuR associated with the HuR 3′UTR (Figure 4B) in both the nucleus and the cytoplasm. This *cis*-acting element is functional, as its presence in a reporter construct rendered it responsive to HuR overexpression and silencing (Figures 4C and D, and 5). Based on these findings, we propose that HuR regulates its own expression by promoting the nuclear export of HuR mRNA, and consequently increasing the efficiency of HuR translation.

While the analysis of HuR mRNA export poses major technical limitations, we believe that this process is controlled by HuR based on several observations. First, the effect of exogenous HuR in inducing endogenous HuR production was greatly diminished by treatment of cells with LMB (Figure 3A), an inhibitor of the export factor CRM1 [which was also shown to block the nuclear export for certain mRNAs (13,33–35)]. Second, knockdown of CRM1 had similar consequences (Figure 3B). HuR has also been proposed to regulate the nuclear export of c-fos, COX-2 and CD83 mRNA (8,14,28); our evidence strongly suggests that HuR can also promote the export of the HuR mRNA. A recent study described an autoregulation of HuR by increased mRNA stability (32). However, in the present study, induction of endogenous HuR by the expression of flag-HuR may not result from increased mRNA stability because the cellular HuR mRNA levels remained unchanged (Figures 2A, 3C and D). One possible reason is that the endogenous HuR (e.g. in HeLa and HEK293 cells) is sufficient to render HuR mRNA stable. As a result, expression of flag-HuR may have limited influence on the stability of HuR mRNA Evidence support for this point is obtained from the observations that knockdown of HuR is always more effective than overexpression of HuR in interfering the expression of target genes (data not shown). In addition, mRNA turnover may not be an important mechanism for the regulation of HuR during cellular senescence because the mRNA levels of HuR are not reduced in senescent 2BS and IDH4 cells (Figures 5B and 6B). However, regulation of HuR at the level of mRNA turnover could be possible in human breast cancer because a moderate elevation of HuR mRNA was observed when flag-HuR was expressed (unpublished data). In addition, the function of HuR in stabilizing target transcripts may depend upon other factors. A typical example is the stabilization of $p21^{CIP1}$ mRNA by HuR. Exposure of cells to UVC increases the cytoplasmic presence of HuR and stabilized p21 mRNA (6). However, HuR did not function in the stabilization of p21 mRNA in senescent IDH4 cells, since HuR was greatly reduced (23). As with other HuR target mRNAs, it is likely that HuR could sequentially participate in mRNA export, mRNA stabilization and translational regulation. Such was the case, for example, for c-fos and COX-2 mRNAs, whose nuclear export and turnover were influenced by HuR (5,14,23,28). It is challenging to distinguish conclusively between altered mRNA export and altered mRNA transcription or stability. However, the evidence obtained here suggests that HuR is likely responsible for promoting the transport of HuR mRNA, and likely that of other target mRNAs, including CD83, c-fos and COX-2 mRNAs.

It is well accepted that the cytoplasmic presence of HuR is of critical importance for its actions on target mRNA turnover and translation. For example, under stress conditions, the increased cytoplasmic HuR levels were linked to the stabilization of $p21^{CIP1}$ mRNA by HuR (6). Similarly, during the cell division cycle, HuR stabilized mRNAs encoding cell cycle regulatory proteins in accordance with its fluctuating presence in the cytoplasm (7). However, it appears that not all stimuli that increase HuR in the cytoplasm can promote HuR mRNA export and increased translation, since HuR levels often remained unchanged after various treatments (6,7,21–23). It is important to note that, while increases in cytoplasmic HuR should be expected to decrease nuclear HuR levels, even strong increases in cytoplasmic HuR typically do not lead to measurable changes in the nuclear HuR pool, since so much more HuR is nuclear at all times (6,21).

Without a doubt, investigation into the mechanisms underlying the regulation of HuR will be helpful to explain its altered function in cancer and aging/senescence, where increases or reductions in HuR expression were accompanied with its alteration in cytoplasmic abundance (19,23). In the present report, we employ two model cell lines for replicative senescence studies, IDH4 and 2BS cells, to explore if the reduced HuR abundance in turn influences the loss of HuR in cellular senescence. The IDH4 human fibroblast model of reversible senescence was developed by Wright and colleagues (10,30). In this model of cell senescence, constitutive, dexamethasone-driven SV40 large-T antigen expression allows cells to suppress senescence and continue to

proliferate as young, early-passage cells. We have previously described that loss of HuR in senescent IDH4 and WI-38 cells reduced the expression of proliferative genes including c-fos, cyclin A and cyclin B1 (23). In keeping with these observations, reduction of HuR in protein level observed in senescent 2BS cells (Figure 6A) is accompanied by reduced translation of HuR (Figure 6D). Strikingly, analysis of HuR mRNA levels by RT-PCR or northern blot indicated no changes among these populations of IDH4 or 2BS cells (Figures 6B and 7B), while the cytoplasmic levels of HuR mRNA were markedly reduced (Figures 6C and 7B). These findings suggest that the mRNA nuclear export mechanism is at least partly involved in the regulation of HuR in replicative senescence. Further supporting evidence was obtained from the reporter gene assays. The reporter activity and the cytoplasmic mRNA level of pGL3-B reporter were reduced in senescent IDH4 cells (Figure 7D and E), which exhibit less HuR protein than do young cells (Figure 7A). Together, these results strongly indicate that the positive feedback mechanism is of great importance for the reduction of HuR during cellular senescence. Although the role of the feedback regulation of HuR in human cancer has not been studied, the expression of HuR in human colon cancer cells is likely to be regulated at the translational level because HuR mRNA levels remain unchanged (26). Likewise, we recently observed that HuR levels in human breast cancer are highly elevated, while the HuR mRNA levels only exhibited a moderate elevation (unpublished data). Therefore, it is possible that the feedback regulation of HuR could be a critical mechanism for the elevation of HuR in human cancer.

After maturation, mRNA export from the nucleus to the cytoplasm is required for the expression of cellular proteins. CRM1-dependent and ARE-dependent nuclear export are two important mechanisms for the export of mRNAs, including those that encode COX2, IFN-α, c-fos, cyclin D1 and CD83, from the nucleus to the cytoplasm (33–35,36). The data presented in our study suggest that the nuclear export of HuR is ARE-dependent because the binding of HuR to the ARE localized in the 3′UTR of HuR mRNA is critical for its function to regulate the nuclear export of Luc-HuR 3′-UTR (pGL3-B) chimeric transcripts (Figure 5). In addition, LMB inhibits the expression of both flag-HuR (without 3′UTR; 'Materials and Methods' section) and endogenous HuR, and inhibits the nuclear export of the endogenous HuR transcript (Figure 3A and C). Therefore, the nuclear export of HuR mRNA is a complex process that involves both CRM1- and ARE-dependent mechanisms. These findings not only emphasize the function of HuR as a regulator of the nuclear export of HuR mRNA, but also reveal a positive feedback regulatory mechanism for HuR. Perhaps other RNA-binding proteins, many of which are themselves shuttling proteins, participate in similar regulation of mRNA export.

In conclusion, the present study has revealed a positive feedback mechanism for HuR expression. However, key questions remain about how the reduction of HuR during cellular senescence (or the elevation of HuR in cancer) is initiated. It is reasonable to postulate that feedback regulation makes HuR progressively less expressed during replicative senescence, and progressively more abundant during cancer. A complex set of regulatory mechanisms may participate in the control of HuR expression during replicative senescence or human cancer. The U- and AU-rich elements in the 3′-UTR of HuR may be recognized by other RNA-binding proteins such as AUF1, CUGBP2, TIA-1, TTP, etc. Studies to address if these RNA-binding proteins or other microRNAs are involved in the regulation of HuR translation in cancer or aging are underway in our laboratory. We anticipate our findings will broaden the spectrum of mechanisms by which HuR regulates gene expression in both cancer and aging.

ACKNOWLEDGEMENTS

We are grateful to J. A. Shay for providing the IDH4 cells and to A. A. Knowlton for providing the pRc/CMV-flag-Hsp72 plasmid.

FUNDING

Funding for open access charges: The Major State Basic Research Development Program of China (Grant 2007CB507400); the National Science Foundation of China (Grants 30672202 and 30621002); the Ministry of Education of People's Republic of China (Grant B07001, 111 project); and the National Institute on Aging-IRP, National Institutes of Health (to M.G.).

Conflict of interest statement. None declared.

REFERENCES

1. Abdelmohsen,K., Pullmann,R. Jr, Lal,A., Kim,H.H., Galban,S., Yang,X., Blethrow,J.D., Walker,M., Shubert,J., Gillespie,D.A. *et al.* (2007) Phosphorylation of HuR by Chk2 regulates SIRT1 expression. *Mol. Cell*, **25**, 543–557.
2. Dormoy-Raclet,V., Ménard,I., Clair,E., Kurban,G., Mazroui,R., Di Marco,S., von Roretz,C., Pause,A. and Gallouzi,I.E. (2007) The RNA-binding protein HuR promotes cell migration and cell invasion by stabilizing the beta-actin mRNA in a U-rich-element-dependent manner. *Mol. Cell. Biol.*, **27**, 5365–5380.
3. Figueroa,A., Cuadrado,A., Fan,J., Atasoy,U., Muscat,G.E., Muñoz-Canoves,P., Gorospe,M. and Muñoz,A. (2003) Role of HuR in skeletal myogenesis through coordinate regulation of muscle differentiation genes. *Mol. Cell. Biol.*, **23**, 4991–5004.
4. Levy,N.S., Chung,S., Furneaux,H., Levy,A.P. and Levy,N.S. (1998) Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. *J. Biol. Chem.*, **273**, 6417–6423.
5. Sengupta,S., Jang,B.C., Wu,M.T., Paik,J.H., Furneaux,H. and Hla,T. (2003) The RNA-binding protein HuR regulates the expression of cyclooxygenase-2. *J. Biol. Chem.*, **278**, 25227–25233.
6. Wang,W., Furneaux,H., Cheng,H., Caldwell,M.C., Hutter,D., Liu,Y., Holbrook,N. and Gorospe,M. (2000) HuR regulates p21 mRNA stabilization by UV light. *Mol. Cell. Biol.*, **20**, 760–769.
7. Wang,W., Caldwell,M.C., Lin,S., Furneaux,H. and Gorospe,M. (2000) HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation. *EMBO J.*, **19**, 2340–23450.
8. Fries,B., Heukeshoven,J., Hauber,I., Grüttner,C., Stocking,C., Kehlenbach,R.H., Hauber,J. and Chemnitz,J. (2007) Analysis of nucleocytoplasmic trafficking of the HuR ligand APRIL and its influence on CD83 expression. *J. Biol. Chem.*, **282**, 4504–4515.

9. Kullmann,M., Göpfert,U., Siewe,B. and Hengst,L. (2002) ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5′UTR. *Genes Dev.*, **16**, 3087–3099.
10. Ma,W., Cheng,S., Campbell,C., Wright,A. and Furneaux,H.M. (1996) Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. *J. Biol. Chem.*, **271**, 8144–8151.
11. Mazan-Mamczarz,K., Galbán,S., López de Silanes,I., Martindale,J.L., Atasoy,U., Keene,J.D. and Gorospe,M. (2003) RNA-binding protein HuR enhances p53 translation in response to ultraviolet light irradiation. *Proc. Natl Acad. Sci. USA*, **100**, 8354–8359.
12. Peng,S.S., Chen,C.Y., Xu,N. and Shyu,A.B. (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *EMBO J.*, **17**, 3461–3470.
13. Brennan,C.M., Gallouzi,I.E. and Steitz,J.A. (2000) Protein ligands to HuR modulate its interaction with target mRNAs in vivo. *J. Cell Biol.*, **151**, 1–14.
14. Doller,A., Akool,el-S., Huwiler,A., Müller,R., Radeke,H.H., Pfeilschifter,J. and Eberhardt,W. (2008) Posttranslational modification of the AU-rich element binding protein HuR by protein kinase C delta elicits angiotensin II-induced stabilization and nuclear export of cyclooxygenase 2 mRNA. *Mol. Cell. Biol.*, **28**, 2608–2625.
15. Li,H., Park,S., Kilburn,B., Jelinek,M.A., Henschen-Edman,A., Aswad,D.W., Stallcup,M.R. and Laird-Offringa,I.A. (2002) Lipopolysaccharide-induced methylation of HuR, an mRNA-stabilizing protein, by CARM1. Coactivator-associated arginineмethyltransferase. *J. Biol. Chem.*, **277**, 44623–44630.
16. Denkert,C., Weichert,W., Winzer,K.J., Müller,B.M., Noske,A., Niesporek,S., Kristiansen,G., Guski,H., Dietel,M. and Hauptmann,S. (2004) Expression of the ELAV-like protein HuR is associated with higher tumor grade and increased cyclooxygenase-2 expression in human breast carcinoma. *Clin. Cancer Res.*, **10**, 5580–5586.
17. Erkinheimo,T.L., Lassus,H., Sivula,A., Sengupta,S., Furneaux,H., Hla,T., Haglund,C., Butzow,R. and Ristimäki,A. (2003) Cytoplasmic HuR expression correlates with poor outcome and with cyclooxygenase 2 expression in serous ovarian carcinoma. *Cancer Res.*, **63**, 7591–7594.
18. Heinonen,M., Bono,P., Narko,K., Chang,S.H., Lundin,J., Joensuu,H., Furneaux,H., Hla,T., Haglund,C. and Ristimäki,A. (2005) Cytoplasmic HuR expression is a prognostic factor in invasive ductal breast carcinoma. *Cancer Res.*, **65**, 2157–2161.
19. Dixon,D.A., Tolley,N.D., King,P.H., Nabors,L.B., McIntyre,T.M., Zimmerman,G.A. and Prescott,S.M. (2001) Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells. *J. Clin. Invest.*, **108**, 1657–1665.
20. Denkert,C., Weichert,W., Pest,S., Koch,I., Licht,D., Köbel,M., Reles,A., Sehouli,J., Dietel,M. and Hauptmann,S. (2004) Overexpression of the embryonic-lethal abnormal vision-like protein HuR in ovarian carcinoma is a prognostic factor and is associated with increased cyclooxygenase 2 expression. *Cancer Res.*, **64**, 189–195.
21. Wang,W., Fan,J., Yang,X., Fürer-Galban,S., Lopez de Silanes,I., von Kobbe,C., Guo,J., Georas,S.N., Foufelle,F., Hardie,D.G. *et al.* (2002) AMP-activated kinase regulates cytoplasmic HuR. *Mol. Cell. Biol.*, **22**, 3425–3436.
22. Wang,W., Yang,X., Kawai,T., López de Silanes,I., Mazan-Mamczarz,K., Chen,P., Chook,Y.M., Quensel,C., Köhler,M. and Gorospe,M. (2004) AMP-activated protein kinase-regulated phosphorylation and acetylation of importin alpha1: involvement in the nuclear import of RNA-binding protein HuR. *J. Biol. Chem.*, **279**, 48376–48388.
23. Wang,W., Yang,X., Cristofalo,V.J., Holbrook,N.J. and Gorospe,M. (2001) Loss of HuR is linked to reduced expression of proliferative genes during replicative senescence. *Mol. Cell. Biol.*, **21**, 5889–5898.
24. Nabors,L.B., Gillespie,G.Y., Harkins,L. and King,P.H. (2001) HuR, a RNA stability factor, is expressed in malignant brain tumors and binds to adenine- and uridine-rich elements within the 3′ untranslated regions of cytokine and angiogenic factor mRNAs. *Cancer Res.*, **61**, 2154–2161.
25. Young,L.E., Sanduja,S., Bemis-Standoli,K., Pena,E.A., Price,R.L. and Dixon,D.A. (2009) The mRNA binding proteins HuR and tristetraprolin regulate cyclooxygenase 2 expression during colon carcinogenesis. *Gastroenterology*, **136**, 1669–1679.
26. Abdelmohsen,K., Srikantan,S., Kuwano,Y. and Gorospe,M. (2008) miR-519 reduces cell proliferation by lowering RNA-binding protein HuR levels. *Proc. Natl Acad. Sci. USA*, **105**, 20297–20302.
27. Chemnitz,J., Pieper,D., Grüttner,C. and Hauber,J. (2009) Phosphorylation of the HuR ligand APRIL by casein kinase 2 regulates CD83 expression. *Eur. J. Immunol.*, **39**, 267–279.
28. Higashino,F., Aoyagi,M., Takahashi,A., Ishino,M., Taoka,M., Isobe,T., Kobayashi,M., Totsuka,Y., Kohgo,T. and Shindoh,M. (2005) Adenovirus E4orf6 targets pp32/LANP to control the fate of ARE-containing mRNAs by perturbing the CRM1-dependent mechanism. *J. Cell Biol.*, **170**, 15–20.
29. Prechtel,A.T., Chemnitz,J., Schirmer,S., Ehlers,C., Langbein-Detsch,I., Stülke,J., Dabauvalle,M.C., Kehlenbach,R.H. and Hauber,J. (2006) Expression of CD83 is regulated by HuR via a novel cis-active coding region RNA element. *J. Biol. Chem.*, **281**, 10912–10925.
30. Wright,W.E., Pereira-Smith,O.M. and Shay,J.W. (1989) Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. *Mol. Cell. Biol.*, **9**, 3088–3092.
31. Ma,L., Chang,N., Guo,S., Li,Q., Zhang,Z., Wang,W. and Tong,T. (2008) CSIG inhibits PTEN translation in replicative senescence. *Mol. Cell. Biol.*, **28**, 6290–6301.
32. Wijdan,A., Al-Ghamdi,M., Al-Haj,L., Al-Saif,M. and Khabar,K.S.A. (2009) Alternative polyadenylation variants of the RNA binding protein, HuR: abundance, role of AU-rich elements and auto-Regulation. *Nucleic Acids Res.*, **37**, 3612–3624.
33. Hutten,S. and Kehlenbach,R.H. (2007) CRM1-mediated nuclear export: to the pore and beyond. *Trends Cell Biol.*, **17**, 193–201.
34. Jang,B.C., Muñoz-Najar,U., Paik,J.H., Claffey,K., Yoshida,M. and Hla,T. (2003) Leptomycin B, an inhibitor of the nuclear export receptor CRM1, inhibits COX-2 expression. *J. Biol. Chem.*, **278**, 2773–2776.
35. Kimura,T., Hashimoto,I., Nagase,T. and Fujisawa,J. (2004) CRM1-dependent, but not ARE-mediated, nuclear export of IFN-alpha1 mRNA. *J. Cell Sci.*, **117(Pt 11)**, 2259–2270.
36. Culjkovic,B., Topisirovic,I., Skrabanek,L., Ruiz-Gutierrez,M. and Borden,K.L. (2006) eIF4E is a central node of an RNA regulon that governs cellular proliferation. *J. Cell Biol.*, **175**, 415–426.