

LncRNA *OIP5-AS1/cyrano* sponges RNA-binding protein HuR

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ABSTRACT

The function of the vast majority of mammalian long noncoding (lnc) RNAs remains unknown. Here, analysis of a highly abundant mammalian lncRNA, *OIP5-AS1*, known as *cyrano* in zebrafish, revealed that *OIP5-AS1* reduces cell proliferation. In human cervical carcinoma HeLa cells, the RNA-binding protein HuR, which enhances cell proliferation, associated with *OIP5-AS1* and stabilized it. Tagging *OIP5-AS1* with MS2 hairpins to identify associated microRNAs revealed that miR-424 interacted with *OIP5-AS1* and competed with HuR for binding to *OIP5-AS1*. We further identified a ‘sponge’ function for *OIP5-AS1*, as high levels of *OIP5-AS1* increased HuR-*OIP5-AS1* complexes and prevented HuR interaction with target mRNAs, including those that encoded proliferative proteins, while conversely, lowering *OIP5-AS1* increased the abundance of HuR complexes with target mRNAs. We propose that *OIP5-AS1* serves as a sponge or a competing endogenous (ce)RNA for HuR, restricting its availability to HuR target mRNAs and thereby repressing HuR-elicited proliferative phenotypes.

INTRODUCTION

Post-transcriptional regulatory processes critically influence eukaryotic gene expression programs. Pre-mRNA splicing and maturation, as well as mRNA transport, stability, storage, editing and translation are all subject to post-transcriptional gene regulation through the actions of non-coding RNAs (ncRNAs), including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), and RNA-binding proteins (RBPs) (1–5).

Despite the fact that ncRNAs were discovered decades ago (6–8), only with the recent advent of techniques for deep genomic analysis (e.g. tiling arrays, RNA sequencing) have we begun to appreciate that tens of thousands of ncRNAs are pervasively transcribed from >90% of genomes of eu-

karyotic species from yeast to human. In their mature form, some ncRNAs are small (e.g. miRNAs, small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs)) (9). Among these, miRNAs robustly regulate gene expression programs and have been studied in detail. They are transcribed as primary (pri-)miRNA transcripts and processed into miRNA precursors (pre-miRNAs) by the microprocessor complex, which includes the RNase Drosha. Following export to the cytoplasm, pre-miRNAs are cleaved by Dicer to form mature miRNAs, which are then loaded into the RNA-inducible silencing complex (RISC). MicroRNA-RISC complexes, which include the protein Argonaute 2 (AGO2), can target specific mRNAs, mainly through partial complementarity with the 3'-untranslated region (UTR) of the mRNA. In turn, this interaction leads to lower stability and/or translation of the target mRNA (10).

LncRNAs (>200 nt in length) also modulate gene expression programs through transcriptional and post-transcriptional mechanisms (5,11,12). Through their impact on gene expression patterns, lncRNAs are emerging as key regulators of cellular processes (proliferation, apoptosis, stress response, differentiation, senescence) as well as physiologic and pathologic processes (immune adaptation, cancer, neurodegeneration, cardiovascular disease and aging) (13–19). Akin to mRNAs, lncRNAs are transcribed as precursor transcripts and are subject to splicing and maturation in the nucleus, as well as cytoplasmic export, editing, transport and decay. In both the nucleus and the cytoplasm, lncRNAs are believed to control gene expression by interacting with chromatin regulators, transcriptional activators and repressors, chromosomal DNA, microRNAs, RBPs and mRNAs (5,15,20). However, the full spectrum of functions for the vast class of lncRNAs is poorly understood.

Ulitsky *et al.* (21) found evolutionarily conserved lncRNAs with biological significance in vertebrates. Transcriptional analyses from zebrafish to human identified *OIP5-AS1* (*OIP5* antisense transcript 1) as the mammalian homolog of the zebrafish transcript *cyrano*. *OIP5-AS1* is highly expressed in the nervous system and is important for controlling neurogenesis during development (21). While

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Table 1. siRNA duplexes

siRNA (Provider)	Sequence
Ctrl siRNA (Qiagen)	AATTCTCCGAACGTGTCACGT
HuR siRNA (Qiagen)	AATCTTAAGTTTCGTAAGTTA TTCCTTAAGATATATATATA CGCAGAGATTCAGGTTCTCC
<i>OIP5-AS1</i> (IDT)	Sense1 rGrGrCrUrGrArGrUrUrCrArUrUrGrArArArCrArGrGTG Antisense1 rCrArCrCrUrGrUrUrCrArArArUrGrArArArCrUrCrArGrCrUrU Sense2 rCrArUrGrCrArGrUrGrCrArUrCrUrGrArCrUrUrArUGG Antisense2 rCrCrArUrArArGrUrCrArGrArUrGrCrArCrUrGrCrArUrGrArG Sense3 rCrArCrCrArArArCrArGrGrCrUrUrGrUrGrUrCrCrUTA Antisense3 rUrArArGrGrArArCrArCrArArArGrCrCrUrGrUrUrGrGrUrGrGrU CAGCAGCAAUUC AUGUUUUGAA (Cat. No. MSY0001341)
miR-424 mimic (Qiagen)	

the sequence conservation for *OIP5-AS1* is limited among the genomes from vertebrates examined, its gene structure and localization between the *CHP1* and *OIP5* genes (*OIP5-AS1* is transcriptionally divergent from gene *OIP5*) are highly conserved (21,22).

To investigate in molecular detail the function of *OIP5-AS1*, we examined its interaction partners in HeLa (human cervical carcinoma) cells. Along with triggering suppression of HeLa cell proliferation, *OIP5-AS1* associated with HuR, an interaction that rendered *OIP5-AS1* stable. HuR is the ubiquitous member of the Hu/ELAV (human/embryonic lethal abnormal vision) RBP family and is predominantly nuclear, but its export to the cytoplasm is linked to the stabilization and/or translation of many target mRNAs, which typically bear U-rich 3' UTRs (23–25). HuR is highly abundant in cancer and numerous HuR target mRNAs encode proteins that promote different aspects of tumorigenesis, such as cell proliferation, angiogenesis, cell survival, invasion and metastasis (26–29). Importantly, some of HuR actions depend on its interaction with miRNAs (30). For example, HuR can compete with miRNA-RISC for binding and regulation of targets (e.g. *CAT1* and *TOP2A* mRNAs (31,32)), but it can also recruit miRNAs to certain target transcripts, as shown for *MYC* mRNA and for *LINC-RAP21* (33,34). Our findings reveal that the interaction of *OIP5-AS1* with HuR was competed by miRNA miR-424, thereby shifting HuR availability from *OIP5-AS1* to target mRNAs. The interaction between *OIP5-AS1* and HuR was found to sequester HuR away from target mRNAs, leading us to propose that *OIP5-AS1* was a 'sponge' for HuR. Accordingly, lowering HuR enhanced miR-424 binding to *OIP5-AS1*, while overexpression of miR-424 reduced HuR binding to *OIP5-AS1*, in turn, freeing up HuR for binding to target mRNAs encoding proliferative proteins. We propose that two *trans*-acting regulators, HuR and miR-424, compete for binding lncRNA *OIP5-AS1*, and the balance of this interaction directly influences cell phenotypes controlled by HuR.

MATERIALS AND METHODS

Cell culture, transfection, analysis and fractionation

Human cervical carcinoma HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics. siRNA duplexes in Table 1 were transfected at 50 nM final concentration using Lipofectamine 2000 (Invitrogen). Cells were counted using an automatic cell counter (Bio-Rad) after staining with 0.4% Trypan Blue (Sigma). [³H]-thymidine

(Amersham) incorporation and fluorescence-activated cell sorting (FACS) analyses were performed using standard procedures. Cellular fractionation was performed using the NE-PER nuclear and cytoplasmic extraction reagent (Thermo Fisher Scientific), following the manufacturer's protocol.

RNA analysis

Total RNA was prepared using Trizol (Invitrogen) and analyzed by conventional reverse transcription (RT) using random hexamers and Maxima reverse transcriptase (Thermo Fisher Scientific) followed by real-time, quantitative PCR (qPCR) using target-specific primers (Table 2) and SYBR green master mix (Kapa Biosystems) in an Applied Biosystems 7300 instrument. For quantification of miRNAs (Table 3), RT was performed with Mir-X™ First-Strand synthesis kit (Clontech) and qPCR amplification was carried out using an Applied Biosystems 7900HT instrument and normalized to *U6* RNA levels.

Protein analysis

Total protein lysates were prepared in RIPA buffer containing protease inhibitor and 1 mM dithiothreitol (DTT). Proteins were size-separated by SDS-PAGE and transferred onto nitrocellulose membrane (Invitrogen). For Western blot analysis, primary antibodies were used that recognized HuR (1:1000), CCNA2 (1:1000), SIRT1 (1:1000), α -tubulin (TUBA, 1:2000), HSP90 (1:20 000) (all from Santa Cruz Biotechnology) and CCND1 (1:2000; from Cell Signaling). After incubation with appropriate secondary antibodies, protein signals were developed using chemiluminescence.

GST pulldown and ribonucleoprotein immunoprecipitation (RIP) analyses

For GST pulldown analysis, glutathione magnetic beads (Pierce) were incubated with cell lysate for 1 h at 4°C. For RIP analysis, agarose beads coated with protein A were pre-incubated with 2 μ g each of antibody and isotype IgG for 4 h at 4°C. Cell lysates prepared in NT2 buffer containing RNase inhibitor, protease inhibitor and 1 mM DTT were incubated with pre-incubated beads at 4°C. After incubation for 1 h and washes in NT2 buffer, protein and RNA were extracted from the beads using NuPAGE sample buffer (Invitrogen) with 10% β -mercaptoethanol and Trizol (Invitrogen), respectively.

Table 2. mRNA qPCR primers

mRNA	Sequence
<i>CCNA2</i> -F	AACTTCAGCTTGTGGGCACT
<i>CCNA2</i> -R	AAACTCTGCTACTTCTGGGGG
<i>CCNB1</i> -F	CAAGCCCAATGGAAACATCT
<i>CCNB1</i> -R	GGATCAGCTCCATCTTCTGC
<i>CCNB3</i> -F	CCCATCTGAAAAGACGGGGG
<i>CCNB3</i> -R	GGGCTGGACAGGTTGACATT
<i>CCND1</i> -F	TGTTTGCAAGCAGGACTTTG
<i>CCND1</i> -R	TCATCCTGGCAATGTGAGAA
<i>CCNE1</i> -F	CCGAGTATCCCCAGCAAAT
<i>CCNE1</i> -R	AAGGCCGAAGCAGCAAGTAT
<i>CDK1</i> -F	CGTAGCTGGGCTCTGATTGG
<i>CDK1</i> -R	TGGTAGATCCGCGCTAAAGG
<i>CDK2</i> -F	CCTGAAATCCTCCTGGGCTG
<i>CDK2</i> -R	CCCAGAGTCCGAAAGATCCG
<i>HuR</i> -F	CGCCAACCTGTACATCAGCG
<i>HuR</i> -R	TAAACGCAACCCCTCTGGAC
<i>VHL</i> -F	GACTGGACATCGTCAGTCTG
<i>VHL</i> -R	ATCCGTTGATGTGCAATGCG
<i>SIRT1</i> -F	TTGCAACAGCATCTTGCCCTG
<i>SIRT1</i> -R	GTTTCATCAGCTGGGCACCTA
<i>PTMA</i> -F	GAACCAAAAACCTCCAAGGCC
<i>PTMA</i> -R	GCTGGTTTGGTCATCCGAGA
<i>TP53</i> -F	AGGCCTTGGAACTCAAGGAT
<i>TP53</i> -R	TGAGTCAGGCCCTTCTGTCT
<i>CDC25A</i> -F	GAACAGCGAAGACAGCGTGA
<i>CDC25A</i> -R	AATCCAAACAACAGTGGCGG
<i>WEE1</i> -F	CGAGTGCGGCACCGATAA
<i>WEE1</i> -R	AAAGGCATCCTATGGCTCGG
<i>OIP5-AS1</i> -F	TGCGAAGATGGCGGAGTAAG
<i>OIP5-AS1</i> -R	TAGTTCCCTCCTCTGGCCG
Mid- <i>OIP5-AS1</i> -F	TCCAGTTTCAGCCACTACCA
Mid- <i>OIP5-AS1</i> -R	TCACAGGATGAGCCAGGATT
<i>7SL</i> -F	CAAAACCTCCCGTGCATCA
<i>7SL</i> -R	GGCTGGAGTGCAGTGGCTAT
<i>18S</i> -F	CGAACGCTGCCCCTATCAACT
<i>18S</i> -R	ACCCGTGGTCACCATGGTA

Table 3. miRNA qPCR primers

miRNA	Sequence
hsa-let-7b	TGAGGTAGTAGGTTGTGTGGTT
hsa-let-7c	TGAGGTAGTAGGTTGTATGGTT
hsa-let-7d	AGAGGTAGTAGGTTGCATAGTT
hsa-let-7e	TGAGGTAGGAGGTTGTATAGTT
hsa-let-7g	TGAGGTAGTAGTTTGTACAGTT
hsa-miR-16-1	TAGCAGCACGTAAATATTGGCG
hsa-miR-16-2	TAGCAGCACGTAAATATTGGCG
hsa-miR-17	CAAAGTGCTTACAGTGCAGGTAG
hsa-miR-17*	ACTGCAGTGAAGGCACTGTAG
hsa-miR-18a	TAAGGTGCATCTAGTGCAGATAG
hsa-miR-126	TCGTACCGTGAGTAATAATGCG
hsa-miR-140-5p	CAGTGGTTTTACCCTATGGTAG
hsa-miR-183	TATGGCACTGGTAGAATTCCT
hsa-miR-20a	TAAAGTGCTTATAGTGCAGGTAG
hsa-miR-21*	CAACACCAGTTCGATGGGCTGT
hsa-miR-25	CATTGCACTTGTCTCGGTCTGA
hsa-miR-26b	TTCAAGTAATTCAGGATAGGT
hsa-miR-27b	TTACAGTGGCTAAGTTCTGC
hsa-miR-29a	TAGCACCATCTGAAATCGGTTA
hsa-miR-30b	TGTAAACATCCTACACTCAGCT
hsa-miR-301a	CAGTGCAATAGTATTGTCAAAGC
hsa-miR-424	CAGCAGCAATTCATGTTTTGAA
hsa-miR-425	AATGACACGATCACTCCCGTTGA
hsa-miR-452	AACTGTTTGCAGAGGAAACTGA
hsa-miR-497	CAGCAGCACACTGTGGTTTGT
hsa-miR-671	AGGAAGCCCTGGAGGGGCTGGAG
hsa-miR-96	TTTGGCACTAGCACATTTTGTCT
hsa-miR-98	TGAGGTAGTAAGTTGTATTGTT
<i>U6</i>	CACCACGTTTATACGCCGGT

Statistical analysis

For statistical analysis of signals on Western blots, the intensities of bands from three independent experiments were quantified using ImageJ, the means \pm S.E.M. were calculated, and *P*-values were determined using Student's *t*-test in each comparison. For statistical analysis of RNA levels, data from three independent qPCR results were calculated

by the $2^{-\Delta\Delta C_t}$ method and represented as the means \pm S.D. for steady-state RNA levels, or the means \pm S.E.M. for the levels of RBP-bound RNAs. *P*-values were determined using Student's *t*-test in each comparison. Only *P*-values lower than 0.05 were considered to be significant.

RESULTS

lncRNA *OIP5-AS1/cyrano* reduces proliferation of human cervical cancer cells

To begin examining the function of lncRNA *OIP5-AS1*, we silenced it in HeLa cells by transfecting a pool of siRNAs directed at *OIP5-AS1* and studied the ensuing changes in cell numbers. As shown in Figure 1A, silencing *OIP5-AS1* promoted cell proliferation, as assessed by counting cell numbers for up to 5 days; at the times examined, *OIP5-AS1* levels were efficiently reduced, as determined by RT followed by qPCR analysis (Figure 1B). The individual siRNAs had a similar effect (Supplementary Figure S1). Analysis of [³H]-thymidine incorporation into replicating DNA confirmed that silencing *OIP5-AS1* increased cell proliferation (Figure 1C), and FACS showed that silencing *OIP5-AS1* increased the relative sizes of the S and G2/M cell compartments (Figure 1D). Taken together, these results indicate that lowering *OIP5-AS1* enhances progression through the cell division cycle and suggest that *OIP5-AS1* inhibits cell proliferation.

RNA-binding protein HuR binds to and stabilizes *OIP5-AS1*

Among the vast collection of lncRNAs annotated for human sequences (9837 lncRNAs, Ensembl v72) (35,36), *OIP5-AS1* was identified as the human homolog of *cyrano*, a lncRNA that plays a role in zebrafish development (21), and showed significant conservation in gene structure (22). *OIP5-AS1* (15.6-kb long) comprises three short exons and a long fourth exon after the longest intron; the mature transcript (1.9-kb long) is shown in Figure 2A. A search for *OIP5-AS1* interaction partners revealed that the RBP HuR associated with *OIP5-AS1* at four different sites (24, GSM738185) (Figure 2A, green).

We validated the interaction between HuR and *OIP5-AS1* by ribonucleoprotein immunoprecipitation (RIP) analysis using anti-HuR and control IgG antibodies; after extracting RNA from the IP samples, RT-qPCR analysis was used to measure *OIP5-AS1* levels, and normalized to *GAPDH* mRNA levels in each IP sample. *OIP5-AS1* was found to be highly enriched (>15-fold) in HuR IP samples relative to IgG IP samples (Figure 2B), revealing that HuR selectively associates with *OIP5-AS1*. To investigate the biological significance of HuR binding to *OIP5-AS1*, we first examined if HuR regulates *OIP5-AS1* stability. By 48 h after silencing HuR in HeLa cells, RT-qPCR analysis from total RNA revealed that *OIP5-AS1* steady-state levels were reduced 3-fold (Figure 2C). To study if this reduction was due to increased *OIP5-AS1* decay, HeLa cells were incubated in the presence of actinomycin D to block *de novo* transcription, and the half-life of *OIP5-AS1* was measured as the time required to reach 50% of the RNA levels present at time 0 h (before adding actinomycin D); a very stable lncRNA, *7SL* (37), was assessed side by side. Compared with control cells, where *OIP5-AS1* RNA was quite stable, silencing HuR lowered *OIP5-AS1* half-life to 9.5 h (Figure 2D), indicating that HuR contributed to stabilizing *OIP5-AS1*.

Identification of miRNAs with enhanced binding to *OIP5-AS1* when HuR is silenced

To explore the mechanisms whereby HuR stabilizes *OIP5-AS1*, we investigated if other factors might be implicated in regulating *OIP5-AS1* half-life. *In silico* analyses employing ‘RegRNA 2.0’ and ‘IntaRNA’ identified 30 miRNAs possibly interacting with *OIP5-AS1*. RIP analysis of Argonaute 2 (AGO2), an essential component of the miRNA-associated RISC, indicated that *OIP5-AS1* was enriched in AGO2 complexes, as further evidence that *OIP5-AS1* associated with miRNAs (Supplementary Figure S2A).

In order to study if *OIP5-AS1* directly bound to these miRNAs in the cell, we prepared a vector that expressed *OIP5-AS1* tagged with MS2 hairpins and coexpressed the chimeric RNA (*OIP5-AS1-MS2*) in HeLa cells along with a fusion protein that contained a glutathione-S-transferase domain fused to a domain that recognizes MS2 hairpins (MS2-GST). After *OIP5-AS1-MS2* formed a complex with MS2-GST, miRNAs associated with the chimeric RNA were pulled down by using glutathione (GSH)-coated beads (Figure 3A). Following extraction of RNA from the beads, we employed RT-qPCR analysis to screen miRNAs predicted to have sites of interaction with *OIP5-AS1*, and further tested if silencing HuR (as in Figure 2C) changed the extent of interaction. Among these, nine miRNAs showed enhanced binding to *OIP5-AS1-MS2* when HuR was silenced (Supplementary Figure S2B, red arrowheads). MiRNAs miR-424 and miR-671 were preferentially enriched in the *OIP5-AS1-MS2* pulldown material over control transfected cells and over MS2 pulldown alone, although miR-671 was substantially less enriched in the MS2 pulldown than was miR-424 (Figure 3B, left). miR-7, a miRNA known to bind *OIP5-AS1* (21) was included as a positive control and found to bind to *OIP5-AS1-MS2* in pulldown experiments (data not shown). Collectively, these findings suggest that silencing HuR enhanced the access of certain miRNAs to *OIP5-AS1*, prompting us to hypothesize that miR-424 and HuR may compete for binding to *OIP5-AS1*.

miR-424 competes with HuR for binding *OIP5-AS1*

The miR-424 site on *OIP5-AS1* is not located immediately adjacent to any of the HuR sites (Figure 4A). However, even though the nearest HuR site is >200 nucleotides away, folding of *OIP5-AS1* could bring one or several HuR sites in proximity to the miR-424 site, such that the secondary structure of the RNA could lead to functional interactions among distal sites (31,33). To examine if miR-424 and HuR competed for binding to lncRNA *OIP5-AS1*, we mutagenized the miR-424 binding site on *OIP5-AS1* at nucleotide positions 27–34 (Figure 4A). HeLa cells expressing the wild type (wt) *OIP5-AS1-MS2* and mutated *OIP5-AS1 (mut)-MS2* were subjected to MS2 pulldown as described above (Figure 3A); miR-424 showed robust enrichment in wt *OIP5-AS1* pulldown, but showed much less binding to *OIP5-AS1 (mut)* (Figure 4B). Overexpression of miR-424 (Pre-miR-424) did not change *OIP5-AS1* levels (Figure 4C), nor did it significantly change either *OIP5-AS1* transcription rate, *OIP5-AS1* interaction with AGO2 or *OIP5-AS1* stability (Supplementary Figure S3).

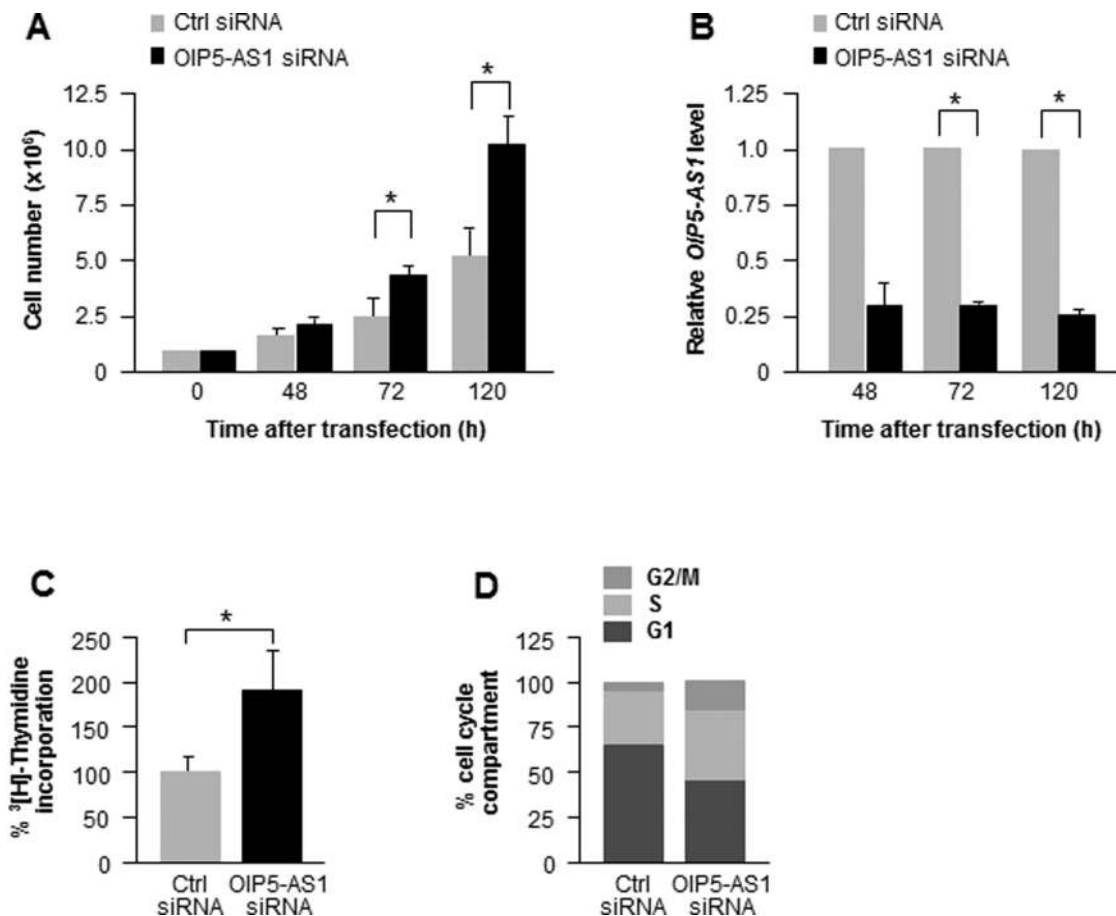


Figure 1. Silencing *OIP5-AS1* promotes cell proliferation. (A) HeLa cells were counted at the times indicated following transfection of siRNAs to silence *OIP5-AS1*. (B) Silencing efficiencies at each time point following transfection were examined by RT-qPCR analysis using total RNA; data were normalized to the levels of *ACTN* mRNA. (C and D) Seventy-two hours after transfection of HeLa cells with Ctrl or *OIP5-AS1* siRNAs, [³H]-thymidine incorporation was measured during a 16-h period (C) and the relative percentages of HeLa cells in each cell cycle compartment were assessed by FACS analysis (D). **P* < 0.05.

Conversely, analysis of the pulldown samples by Western blotting revealed that HuR was more abundant in *OIP5-AS1* (*mut*) pulldown samples (Figure 4D), suggesting that reduced miR-424 binding through mutation of the binding site on *OIP5-AS1* permitted enhanced HuR binding to *OIP5-AS1*. To test this idea further, we overexpressed miR-424 by transfecting HeLa cells with Pre-miR-424, and measured HuR binding to *OIP5-AS1* by RIP analysis. As shown, the 10-fold enrichment in HuR binding to *OIP5-AS1* in control cells declined significantly to a 6-fold enrichment when miR-424 was overexpressed (Figure 4E). In summary, mutations which interfered with the access of miR-424 to *OIP5-AS1* allowed enhanced HuR binding to *OIP5-AS1*, and conversely, when miR-424 was overexpressed, HuR binding to *OIP5-AS1* was markedly reduced. These findings led us to conclude that miR-424 and HuR compete with each other for binding to *OIP5-AS1*.

Competitive binding of HuR and miR-424 to *OIP5-AS1* affects HuR binding to target mRNAs, influences target mRNA fate

Next, we examined the consequences of HuR and miR-424 competing for binding to *OIP5-AS1*. Given that HuR binds

to many mRNAs and that *OIP5-AS1* is highly expressed in HeLa cells, we hypothesized that the interaction between *OIP5-AS1* and HuR may influence the ability of HuR to bind other target mRNAs. To test this possibility, we silenced *OIP5-AS1* in HeLa cells and performed RIP analysis of HuR interaction with target *CCNA2* and *CCND1* mRNAs (encoding cyclins A2 and D1, respectively), *SIRT1* mRNA (encoding sirtuin 1), *VHL* and *TP53* mRNAs (encoding the tumor suppressors VHL and p53), and *WEE1* mRNA (encoding the cell cycle kinase WEE1). As shown in Figure 5, silencing *OIP5-AS1* and overexpressing miR-424 each enhanced the interaction of HuR with target mRNAs relative to those seen in the corresponding control (Ctrl siRNA) transfections. The increased enrichments in HuR-mRNA complexes suggested that lowering *OIP5-AS1* or increasing the abundance of competitor miR-424 ‘freed up’ HuR for binding to target mRNAs.

To investigate if the increased binding of HuR to target mRNAs due to miR-424 overexpression or *OIP5-AS1* silencing influenced the levels of endogenous target mRNAs and proteins, we selected *CCNA2*, *CCND1* and *SIRT1* for further analysis. RT-qPCR and Western blot analyses after silencing *OIP5-AS1* and after miR-424 overexpression

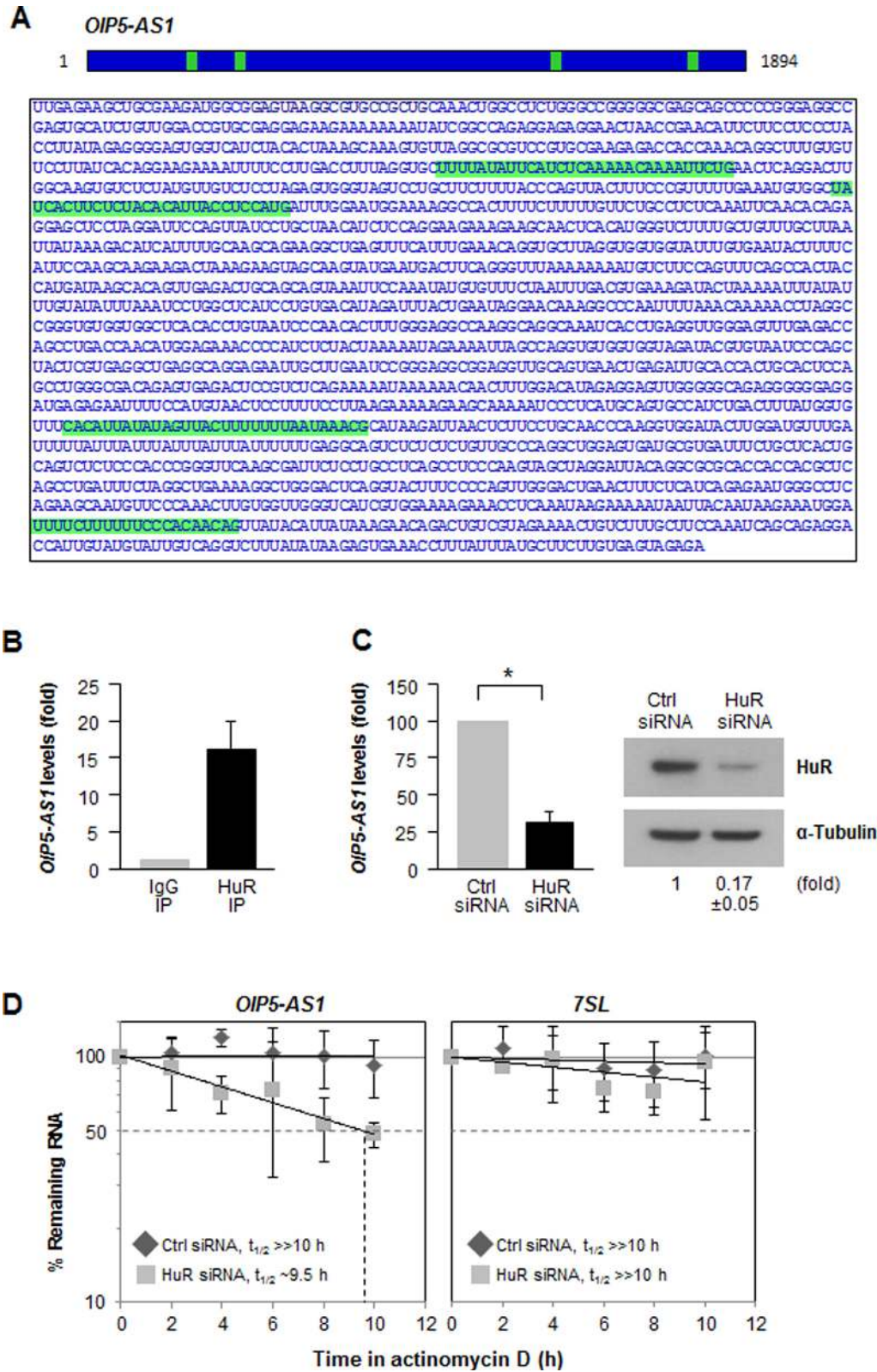


Figure 2. HuR binds to *lncRNA OIP5-AS1* and increases its stability. (A) HuR interaction sites (green) on *OIP5-AS1*, as identified using GSM738185.bed.gz. (B) RIP analysis of HuR interaction with *OIP5-AS1* in HeLa cells. Following HuR immunoprecipitation (IP) or control using IgG IP, *OIP5-AS1* levels were measured by RT-qPCR analysis and normalized to *7SL* levels. (C) Seventy-two hours after transfecting Ctrl or HuR siRNAs, the levels of *OIP5-AS1* (left) and HuR (right) were assessed by RT-qPCR and Western blot analyses, respectively. Data shown are the means and SEM from three independent experiments. (D) Cells transfected as in (C) were treated with actinomycin D to block *de novo* transcription and the levels of *OIP5-AS1* and *7SL* (a stable *lncRNA*) were assessed by RT-qPCR analysis and normalized to *18S* rRNA levels, also quantified by RT-qPCR analysis; the half-life ($t_{1/2}$) of *OIP5-AS1* was measured as the time required for the levels of *OIP5-AS1* to decline to 50% of their initial levels at 0 h.

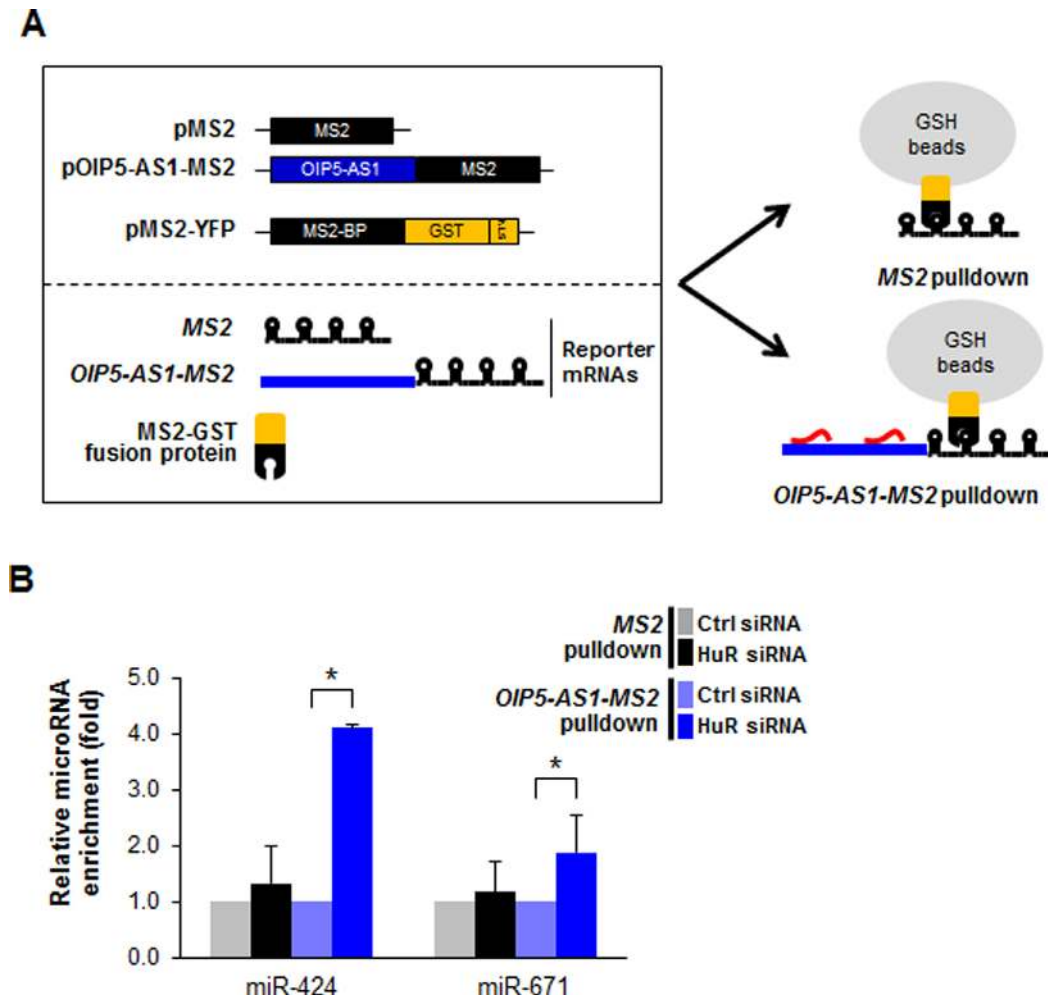


Figure 3. MiRNAs interacting with lncRNA *OIP5-AS1/cyano* in the presence and the absence of HuR. **(A)** Schematic of constructs used for MS2 pull-down, including plasmids pMS2 (a control vector expressing MS2 RNA), pOIP5-AS1-MS2 (a vector expressing the chimeric RNA *OIP5-AS1-MS2*) and pMS2-GST, expressing a fusion protein (MS2-GST) that recognizes MS2 RNA tags and can be pulled down using glutathione (GSH) beads. **(B)** Forty-eight hours after transfection with either Ctrl or HuR siRNAs, cells were co-transfected with the plasmids in (A) and 24 h after that, cells were lysed and the lysates were analyzed by pull-down using GSH-conjugated beads. The relative interaction of miR-424 and miR-671 with *OIP5-AS1-MS2* (selected among nine miRNAs that were chosen for further screening because they had predicted sites on *OIP5-AS1* (Supplementary Figure S2B)) was examined in pull-down material and the impact of silencing HuR on the magnitude of the interactions was assessed. Fold changes were calculated over Ctrl siRNA group in each MS2 pull-down and normalized to *U6* RNA levels. Error bars indicate SEM from three independent experiments in each pull-down.

(Figure 6A–C) revealed that the levels of *CCNA2* mRNA and *SIRT1* mRNA increased significantly, with a concomitant rise in CCNA2 and SIRT1 protein levels, while *CCND1* mRNA levels did not change significantly, but CCND1 protein levels rose moderately. Conversely, overexpression of *OIP5-AS1* as a fusion transcript (*OIP5-AS1-MS2*) reduced *CCNA2* and *SIRT1* mRNA levels and protein abundance, and also lowered CCND1 protein abundance but did not affect *CCND1* mRNA levels (Figure 6D–F). Considering that HeLa cells have ~1300 copies of HuR in the cytoplasm (17 200 total in the cell) (Supplementary Figure S4) and some 70 cytoplasmic copies of *OIP5-AS1* (~75 total in the cell) (38–40), and that each individual *OIP5-AS1* transcript has 4–6 binding sites for HuR (Supplementary Figure S5C), *OIP5-AS1* could sponge 30% of cytoplasmic HuR. If one further considers that each site can accommodate multiple copies of HuR, as HuR can multimerize (41,42), and that some HuR is unable to bind RNA (for example, HuR

phosphorylated at certain residues (43,44)), then *OIP5-AS1* could well sequester the entire pool of cytoplasmic HuR. In this regard, the levels of HuR-*OIP5-AS1* complexes in HeLa cytoplasm were far greater than those in the nucleus (Supplementary Figure S4E). In sum, these data support the notion that *OIP5-AS1* can control the availability of HuR to bind and modulate the post-transcriptional fate of target mRNAs. Accordingly, we propose that *OIP5-AS1* functions as a sponge for HuR.

OIP5-AS1 HuR sites linked to regulation of HuR availability for binding mRNA

To solidify evidence for the notion that *OIP5-AS1* can sponge HuR away from mRNAs, we sought to delete HuR binding sequences from *OIP5-AS1*. With knowledge of HuR binding sequences obtained from PAR-CLIP analysis (Figure 2A), we designed constructs in which the HuR

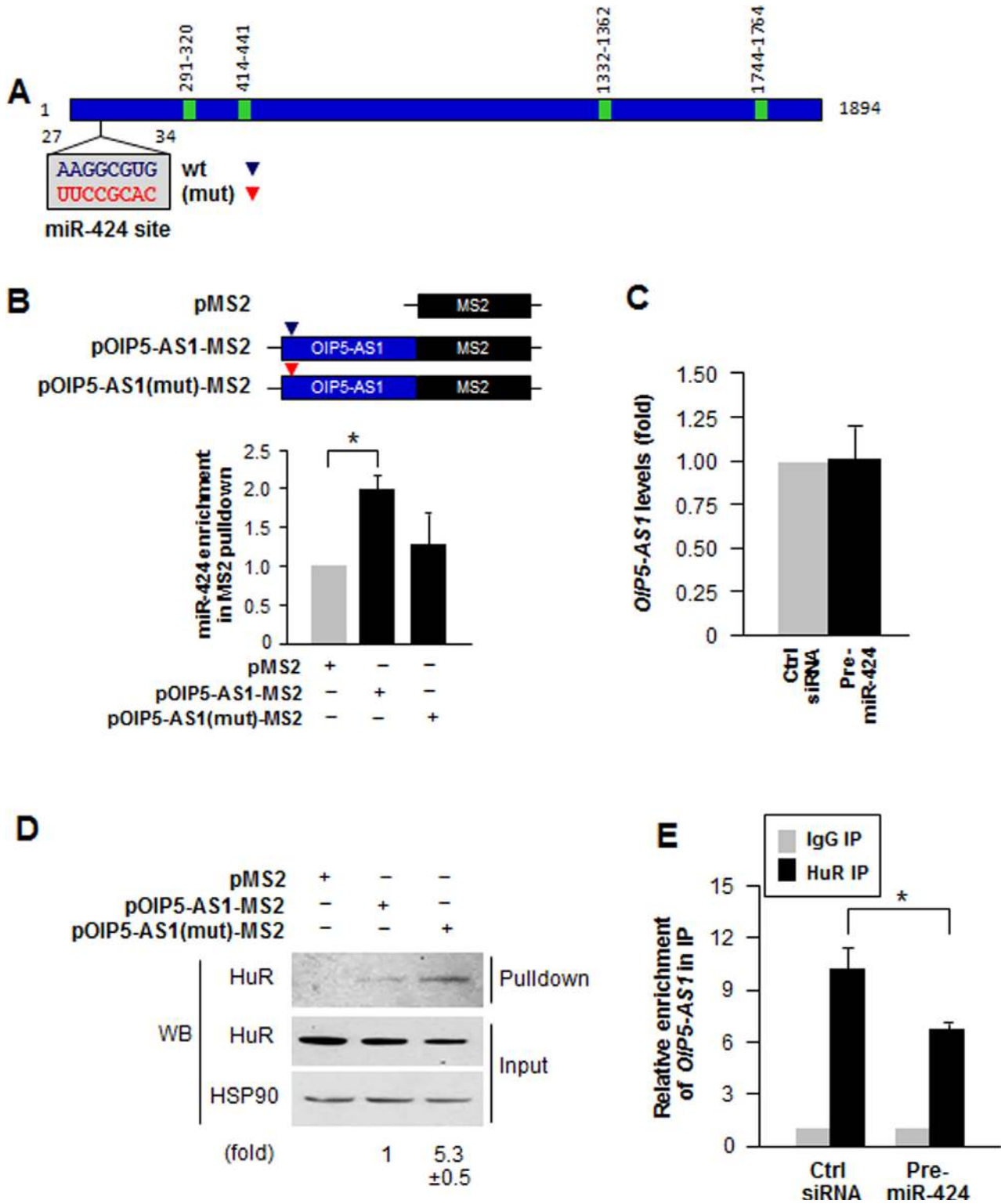


Figure 4. Mutation of miR-424 binding site enhances HuR binding to *OIP5-AS1*. (A) Schematic of the mutation of miR-424 binding site on *OIP5-AS1*. (B) HeLa cells transfected with plasmids pMS2, pOIP5-AS1-MS2 (wt) or pOIP5-AS1(mut)-MS2 were used for pull-down analysis using GSH beads to test the impact of mutating the *OIP5-AS1* miR-424 site on miR-424 binding to the tagged *OIP5-AS1*. The level of miR-424 associated with each MS2-tagged RNA was measured by RT-qPCR analysis. (C) Steady-state levels of *OIP5-AS1* in HeLa cells transfected with Pre-miR-424. Fold changes were relative to Ctrl siRNA transfection. (D) Cells transfected as in (B) were subjected to MS2 pull-down followed by detection of HuR levels associated with each MS2-tagged RNA by Western blot analysis; HuR and loading control HSP90 in the input material was also measured. (E) Twenty-four hours after transfecting HeLa cells with Ctrl siRNA or Pre-miR-424, the association of HuR with *OIP5-AS1* was assessed by RIP analysis. The relative interaction levels were calculated by normalization with IgG IP. In B–E, errors indicate standard deviation (SD) from three independent experiments. * $P < 0.05$.

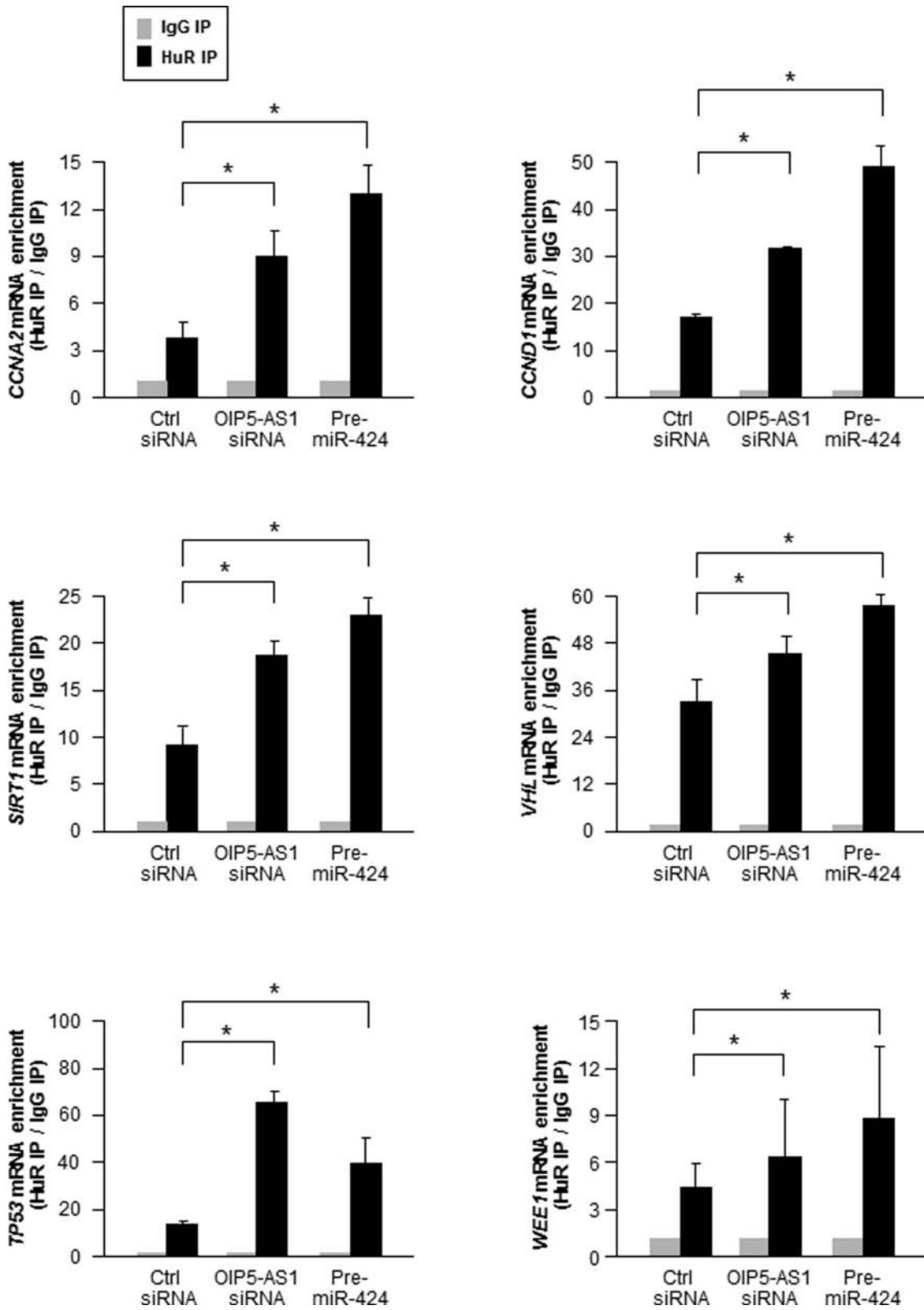


Figure 5. Effect of silencing *OIP5-AS1* or overexpression miR-424 on HuR binding to target mRNAs. By 72 h after transfecting Ctrl siRNA, OIP5-AS1 siRNA or Pre-miR-424, the relative binding of *CCNA2*, *CCND1*, *SIRT1*, *VHL*, *TP53* and *WEE1* mRNAs to HuR was calculated as 'fold enrichment' over IgG IP for each sample. Data are shown as the means and SEM from three independent experiments. * $P < 0.05$.

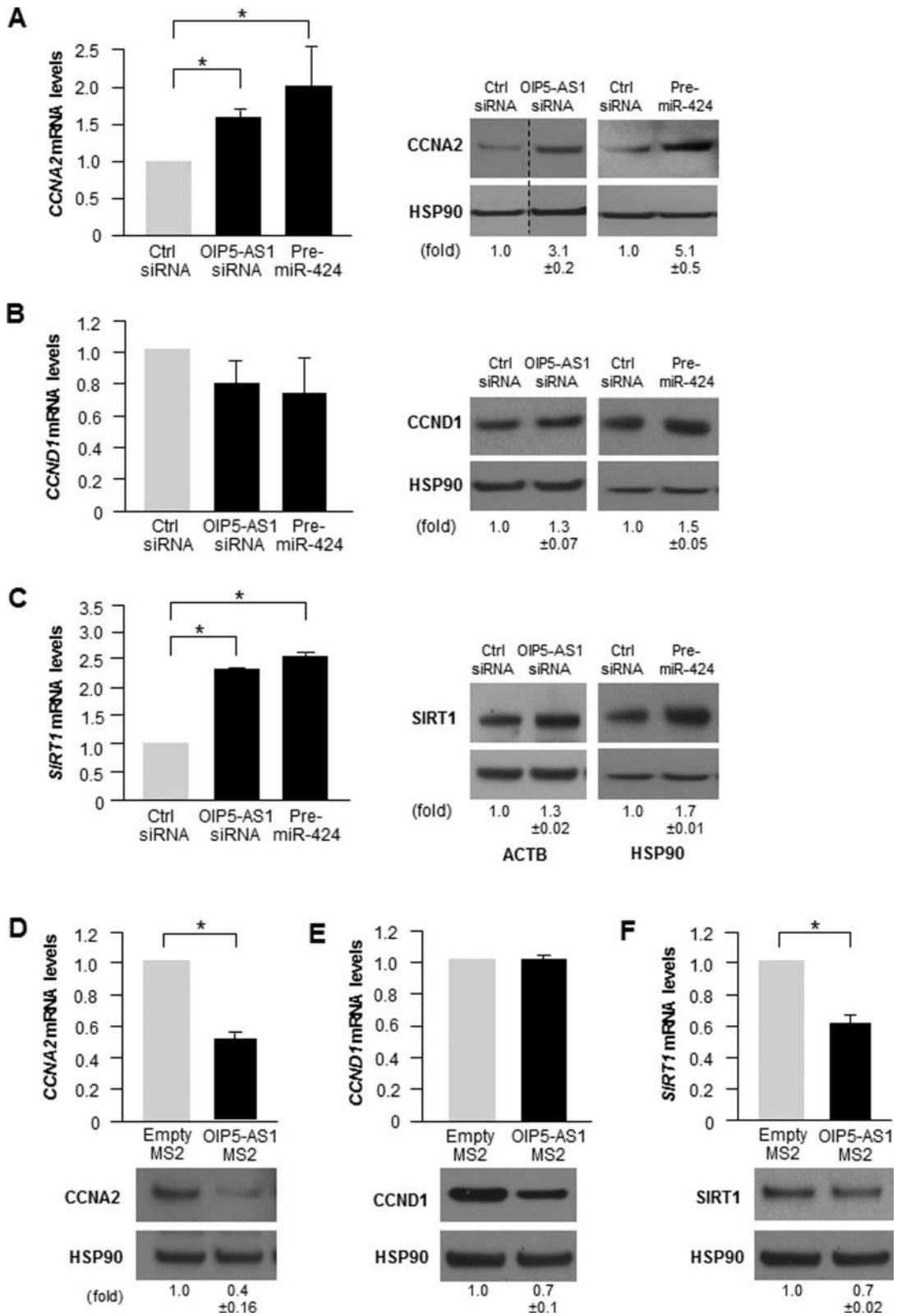


Figure 6. Impact of silencing *OIP5-AS1* or overexpressing miR-424 on the expression of HuR target mRNAs. (A–C) Steady-state levels of *CCNA2* mRNA (A), *CCND1* mRNA (B) and *SIRT1* mRNA (C), as measured by RT-qPCR analysis, as well as the encoded proteins, as measured by Western blot analysis, in cells that had been transfected 72 h earlier with Ctrl siRNA, *OIP5-AS1* siRNA or Pre-miR-424. *18S* rRNA was used to normalize mRNA levels (left), and HSP90 or ACTB was used to normalize protein input (right). In A, discontinuous lines flank one lane in order to exclude irrelevant lanes from that blot. (D) Steady-state levels of *CCNA2*, *CCND1* and *SIRT1* mRNAs and corresponding proteins in HeLa cells 72 h after transfection with empty pMS2 and pOIP5-AS1-MS2. Data are the means and SD from three independent experiments. Protein levels were quantified using ImageJ. **P* < 0.05.

sites were deleted at the 3' and the 5' ends (pOIP5-AS1-MS2- Δ 5'3' [p Δ 5'3']) (Figure 7A, left). This construct was used to evaluate the function of *OIP5-AS1* as a sponge for HuR, compared to full-length (FL) *OIP5-AS1* (pOIP5-AS1-MS2-FL [pFL]). First, we examined the efficiency of HuR binding to MS2-tagged RNAs (Figure 7A, right). As anticipated, FL RNA expressed from pOIP5-AS1-MS2 showed robust binding to HuR, but RNA expressed from p Δ 5'3' (pOIP5-AS1-MS2- Δ 5'3'), pulled down substantially less HuR, suggesting that the deletion of the HuR binding sites strongly reduced the physical interaction between HuR and *OIP5-AS1*. Analysis of the residual binding of HuR to the *OIP5-AS1-MS2- Δ 5'3'* RNA using a biotinylated partial RNA confirmed that HuR was capable of binding modestly to this region. Further examination of this segment revealed that Kishore *et al.* (45) had identified two additional putative HuR sites within this region (Supplementary Figure S5C).

Using this set of expression constructs, we tested whether HuR binding to target mRNAs was differentially influenced by *OIP5-AS1* transcripts containing or lacking HuR binding sites. HuR IP was performed 72 h after overexpressing each construct in HeLa cells; after extracting RNA, RT-qPCR was used to quantify the levels of target *CCNA2*, *CCND1* and *SIRT1* mRNAs and represented as fold enrichment in HuR IP relative to control IgG IP unless. When we overexpressed FL *OIP5-AS1*, binding of HuR to target mRNAs was significantly suppressed compared to cells expressing empty pMS2 (a plasmid that does not contain *OIP5-AS1* segments); however, this suppression disappeared when *OIP5-AS1-MS2- Δ 5'3'* was expressed instead, restoring the levels of HuR binding to target mRNAs to the levels seen when empty pMS2 was expressed (Figure 7B–D). The pattern of expression of proteins *CCNA2*, *CCND1* and *SIRT1* generally followed the pattern of binding, with proteins showing rescue after expressing the deletion mutant *OIP5-AS1-MS2- Δ 5'3'* compared to *OIP5-AS1-MS2-FL*. Overexpression of *OIP5-AS1-MS2- Δ 5'3'* caused HuR to bind more to *CCNA2* and *CCND1* mRNAs than other groups (Figure 7B and C, left) and led to higher production of *CCND1* (Figure 7C, right), suggesting that the deletion mutant may selectively influence regulatory factors that affect adversely these mRNAs.

In sum, HuR binds *OIP5-AS1* in competition with miR-424; given that HuR binding to *OIP5-AS1* reduced HuR availability to target mRNAs, *OIP5-AS1* mutants with impaired ability to bind HuR were unable to sponge HuR, permitting HuR to bind target mRNAs and enhance their expression. Taken together, our findings support a model whereby the accumulation of *OIP5-AS1* can lead to sponging of HuR, sequestering it away from target mRNAs. Conversely, when *OIP5-AS1* abundance declines or it becomes unavailable due to binding by miR-424, HuR availability increases, permitting HuR to elicit its post-transcriptional function on target mRNAs (Figure 8).

DISCUSSION

We have identified a novel transcript interacting with HuR, the lncRNA *OIP5-AS1*, and have characterized the consequences of this interaction. Given that *OIP5-AS1* reduced

cell division (Figure 1), we examined the possibility that the interaction or *OIP5-AS1* with HuR might impact the pro-proliferative phenotype attributed to HuR. After establishing that HuR binding *OIP5-AS1* stabilized this RNA and increased its abundance, and that HuR competed with miR-424 for binding *OIP5-AS1*, we found that the availability of HuR for binding target mRNAs (e.g. those encoding *CCNA2*, *CCND1*, *SIRT1*, *VHL*, *TP53*, *WEE1*), increased when HuR interaction with *OIP5-AS1* declined, whether it was by increasing miR-424 levels or by silencing *OIP5-AS1*. We propose that HuR stabilizes lncRNA *OIP5-AS1*, and in turn, *OIP5-AS1* associates with and sponges HuR. This positive feedback loop is interrupted when miR-424 competes with HuR for binding to *OIP5-AS1*; loss of interaction with *OIP5-AS1* releases HuR for binding to target mRNAs relevant for other cellular functions including proliferation. In this manner, *OIP5-AS1* and miR-424 jointly work to fine-tune HuR binding to mRNAs (Figure 8).

Regulation of HuR function

As mentioned above, HuR binds to hundreds of cellular mRNAs and is best known for stabilizing and/or regulating their translation, although it has also been implicated in splicing and in nuclear export of mRNAs. HuR can achieve these actions in different ways, typically by interfering with or by recruiting different RNA-binding factors to target RNAs. For example, by modulating the associations of miRNAs and RBPs with subsets of HuR target RNAs, HuR can influence their splicing, transport, turnover and translation (30,32,46). The function of HuR has been found to be regulated in three main ways: by controlling its abundance, its export to the cytoplasm, and its interaction with mRNAs (29,47). HuR export to the cytosol requires a specialized HuR nucleocytoplasmic shuttling domain (HNS) and several proteins, including transportins 1 and 2, the chromosome region maintenance 1 (CRM1), and importin-1 α ; this process is controlled by cyclin-dependent kinase 1 (Cdk1), AMP-activated protein kinase (AMPK), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) p38, which phosphorylate HuR and HuR transport proteins (48–52). HuR abundance is regulated transcriptionally by NF- κ B and by SMADs (53,54), and post-transcriptionally via miRNAs (including miR-125 and miR-519), and also through HuR ubiquitination and cleavage via caspases (55,56). HuR binding to mRNAs is likewise regulated via phosphorylation. Phosphorylation by the checkpoint kinase Chk2 at HuR residues S88, S100 and T118 (located between and within the HuR RNA-recognition motifs RRM1 and RRM2) modulates HuR binding to several target mRNAs (43,44,47). Phosphorylation by PKC at HuR S158, S221 and S318 promoted HuR binding to mRNAs. HuR methylation at N271 by CARM1 (coactivator-associated arginine methyltransferase 1) in response to lipopolysaccharide stimulation promoted HuR binding to and stabilization of a target mRNA (*TNF* mRNA) (57).

The regulation of HuR function reported here has some similarities with the function previously proposed in a study by Barnhart *et al.* (58), which showed that mRNAs encoded by the Sindbis virus contain 3' UTRs that bind HuR ro-

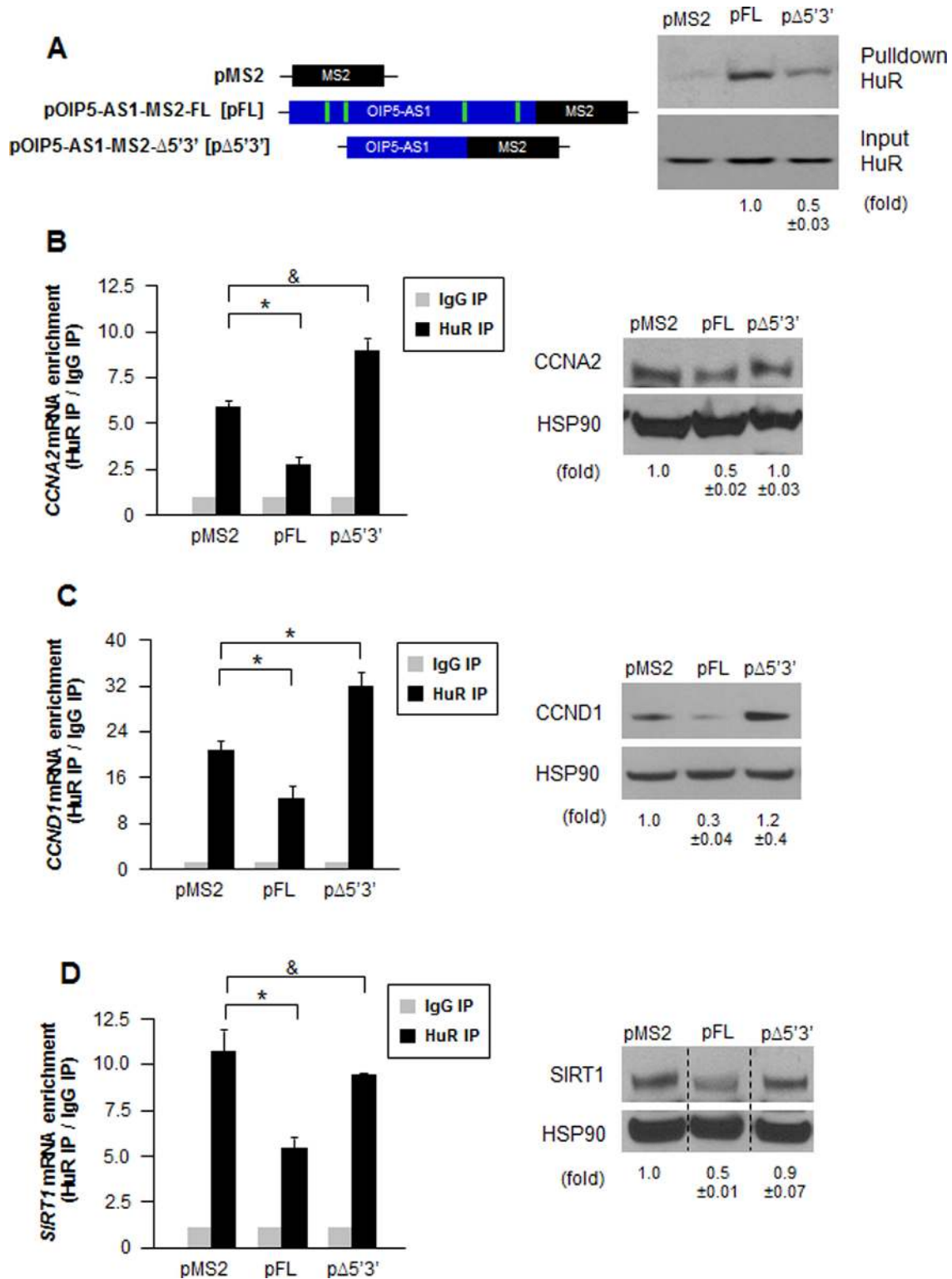


Figure 7. *OIP5-AS1* having deletions of HuR binding sites promotes HuR to bind with target mRNAs. (A) *Left*, plasmids pMS2 (parental vector), pOIP5-AS1-MS2-FL (pFL, expressing wild type, full-length *OIP5-AS1*) and pOIP5-AS1-MS2-Δ5'3' (pΔ5'3', expressing deletions on the HuR binding sites at the 5' and 3' ends) that were transfected into HeLa cells. The extent of HuR interaction with the RNAs expressed from each vector was assessed 72 h later by MS2 pull-down followed by Western blot analysis (*right*); HuR signals were quantified using ImageJ and indicated below. (B–D) HuR RIP analysis to quantify the interaction of HuR with target *CCNA2* mRNA (B), *CCND1* mRNA (C) and *SIRT1* mRNA (D), each detected by RT-qPCR analysis (*left*), was performed 72 h after transfection of the different constructs shown in (A) into HeLa cells. After each transfection, Western blot analysis was used to determine the level of target protein expressed (*right*). In B–D, *18S* rRNA was used for normalization of RNA and HSP90 for normalization of protein. In D, discontinuous lines flank one lane from a blot that was moved to exclude irrelevant lanes from that blot. Data are shown as the means and SEM from three independent experiments; **P* < 0.05 and &, not significant. Images are a representative from three independent experiments.

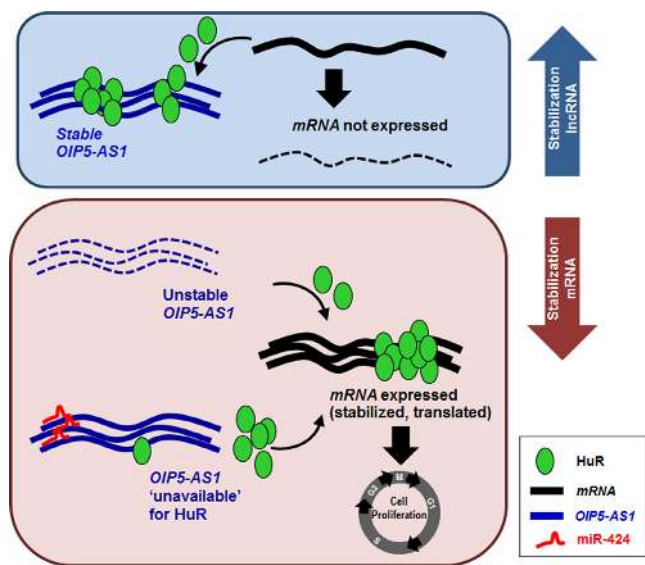


Figure 8. *OIP5-AS1* sponges HuR, preventing its interaction with target mRNAs, miR-424 binds *OIP5-AS1* and releases HuR to coding mRNAs. Model proposed to explain our findings. When HuR binds *OIP5-AS1*, it does not bind target coding mRNAs and they are not expressed (HuR may stabilize them and/or enhance their translation). When HuR dissociates from *OIP5-AS1* (including via displacement by miR-424), HuR becomes available to target mRNAs and promotes their expression, an effect linked to cell proliferation. HuR, *OIP5-AS1*, miR-424 and mRNA are represented in the box. Solid line, stable RNA; dashed line, unstable RNA (or untranslated mRNA).

bustly. This interaction excluded HuR from target mRNAs and caused alternative polyadenylation, splicing and degradation of cellular HuR target mRNAs. In the report by Barnhart, HuR sequestration by viral RNA helped elicit a gene expression program that favored viral infection. In our study, HuR is bound instead to a noncoding transcript, *OIP5-AS1*, and sequestration by *OIP5-AS1*, appeared to contribute to the growth inhibitory phenotype attributed to *OIP5-AS1*.

lncRNAs can sponge RBPs, not only miRNAs

Some lncRNAs have been reported to function as competing endogenous (ce)RNAs by serving as sponges that bind and sequester away miRNAs (59–66). For instance, this function was shown for lncRNA *linc-MD1*, which sponged miRNAs miR-133 and miR-135 (67), for lncRNA *HOTAIR* and miR-331–3p (68), and for lncRNA *H19* and miRNAs miR-138 and miR-200a (69).

Based on our findings, we posit that lncRNAs may also function as endogenous competing RNAs for RBPs, sponging their activity away from target mRNAs. In the present report, the abundant lncRNA *OIP5-AS1* sponged HuR away from HuR target mRNAs, generally lowering expression of these target transcripts. However, similar functions may be discovered for other highly expressed lncRNAs that interact with other RBPs. Like *OIP5-AS1* and HuR, such sponge lncRNAs are predicted to be abundant and to have long half-lives and multiple sites of interaction with RBPs.

miR-424

It is possible that other lncRNAs that sponge RBPs also involve the action of miRNAs, as shown here for miR-424. Besides a physical competition, miR-424 appears to compete with HuR functionally, as miR-424 has a tumor suppressive function in many cancer types (70–74). In our study, miR-424 competed with HuR for binding *OIP5-AS1* and hence counteracted the stabilizing effects of HuR on target mRNAs, which included proliferative and cancer-associated proteins. In this regard, it will be interesting to investigate in the future if miR-424 counteracts the growth suppressive phenotype of *OIP5-AS1*. HuR function is also closely linked to the stress response. While the impact of *OIP5-AS1* was not studied in the context of cell damage, it is interesting to note that some stresses (e.g. ischemia/reperfusion (I/R) injury) elevated miR-424 expression in the brain. If this rise in miR-424 after stresses occurs broadly, miR-424 could mobilize HuR away from *OIP5-AS1*, enabling HuR actions in the stress response. Unexpectedly, miR-424 did not promote the decay of *OIP5-AS1* and thus perhaps miR-424 serves primarily as an endogenous competitor, not a destabilizer of *OIP5-AS1*. In support of this notion was evidence that AGO2 interaction with *OIP5-AS1* rose minimally after overexpressing miR-424 (Supplementary Figure S3A), and that while miR-424 overexpression displaced HuR, two-thirds of the cellular HuR-*OIP5-AS1* complex was still detectable (Figure 4E), suggesting that much HuR is still bound and hence protecting *OIP5-AS1*. Further studies should test if *OIP5-AS1* or miR-424 modulates the tumorigenic influence of HuR *in vivo*.

Concluding remarks

In closing, our findings indicate that HuR and miR-424 bind to *OIP5-AS1* competitively and that the net impact of these interactions determine the level of HuR bound to *OIP5-AS1*, and hence the concentration of HuR available for other target mRNAs. The cytoplasmic-to-nuclear *OIP5-AS1* ratio is close to 3:1 (Supplementary Figure S2C). The sponging action of *OIP5-AS1* is expected to be particularly effective in the cytoplasm, since HuR abundance in this compartment is relatively low (Supplementary Figure S4B and D). Since PAR-CLIP analysis indicates that there are other abundant HuR-interacting lncRNAs (e.g. *SNHG1*, *SNHG15* and *MIR17HG*), it is likely that there are additional RNA sponges for HuR in the cell. Our findings pave the way for future studies of other RBPs controlled via lncRNA sponges, as we endeavor to elucidate how RBPs and noncoding RNAs coordinately regulate gene expression programs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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