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HuR Regulates Alternative Splicing of the *TRA2*β Gene in Human Colon Cancer Cells under Oxidative Stress

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Hu antigen R (HuR) regulates stress responses through stabilizing and/or facilitating the translation of target mRNAs. The human *TRA2*β gene encodes splicing factor transformer 2β (Tra2β) and generates 5 mRNA isoforms (*TRA2*β*1* to *-5*) through alternative splicing. Exposure of HCT116 colon cancer cells to sodium arsenite stimulated checkpoint kinase 2 (Chk2)- and mitogen-activated protein kinase p38 ($p38^{MAPK}$)-mediated phosphorylation of HuR at positions S88 and T118. This induced an association between HuR and the 39-nucleotide (nt) proximal region of *TRA2*β exon 2, generating a *TRA2*β*4* mRNA that includes exon 2, which has multiple premature stop codons. HuR knockdown or Chk2/$p38^{MAPK}$ double knockdown inhibited the arsenite-stimulated production of *TRA2*β*4* and increased Tra2β protein, facilitating Tra2β-dependent inclusion of exons in target pre-mRNAs. The effects of HuR knockdown or Chk2/$p38^{MAPK}$ double knockdown were also confirmed using a *TRA2*β minigene spanning exons 1 to 4, and the effects disappeared when the 39-nt region was deleted from the minigene. In endogenous HuR knockdown cells, the overexpression of a HuR mutant that could not be phosphorylated (with changes of serine to alanine at position 88 [S88A], S100A, and T118A) blocked the associated *TRA2*β*4* interaction and *TRA2*β*4* generation, while the overexpression of a phosphomimetic HuR (with mutations S88D, S100D, and T118D) restored the *TRA2*β*4*-related activities. Our findings revealed the potential role of nuclear HuR in the regulation of alternative splicing programs under oxidative stress.

The Hu/embryonic lethal abnormal vision (ELAV) protein family comprises 3 primarily neuronal proteins (HuB, HuC, and HuD) and one ubiquitously expressed protein, HuR (Hu antigen R; also known as HuA). Hu family proteins contain 3 RNA recognition motifs (RRMs) that mediate the specific interaction of Hu proteins with RNA (1). RRMs specifically bind to short, single-stranded stretches of uridines separated by adenosines or, less commonly, other bases (1, 2), which are also known as RNA recognition elements (RREs) or AU-rich elements (AREs), in the 3′ untranslated region (UTR) of target mRNAs. HuR plays a crucial role in the regulation of gene expression in cells exposed to mitogenic, differentiation, immune, and stress-inducing agents (1, 3) through stabilizing and/or facilitating the translation of ARE-containing mRNAs for various proteins, including tumor suppressors (p53 and von Hippel-Lindau tumor suppressor), cyclins (A, B1, and D1), proto-oncogene products (c-Fos and c-Myc), growth factors (vascular endothelial growth factor), cytokines (transforming growth factor β and tumor necrosis factor alpha), cyclin-dependent kinase (Cdk) inhibitors (p21 and p27), antiapoptotic factors (prothymosin α [ProTα], B-cell CLL/lymphoma 2 [Bcl-2], and myeloid cell leukemia sequence 1 [Mcl-1]), and signaling molecules, such as mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1) (4–15). Recently, several key aspects of HuR signaling have emerged; for example, the set of RNAs (including pre-mRNAs and noncoding RNAs) controlled by HuR, the function of HuR in the nucleus, and the influence of microRNAs/RNA-induced silencing complex (RISC) on the posttranscriptional fate of HuR targets have been elucidated. In the nucleus, HuR is thought to participate in splicing and polyadenylation (16–19). However, the exact functions of HuR in the nucleus are not fully understood.

Alternative splicing of pre-mRNAs is a major process contributing to transcriptome diversity in various physiological and pathological situations. Over 90% of human genes generate several mRNAs with distinct exon contents through alternative splicing. The precise splicing events depend on the presence of consensus sequences at 5′ and 3′ exon splice sites and additional intronic and exonic regulatory elements (20). These regulatory elements are known as splicing enhancers or silencers and function to regulate splice site selection for normal or aberrant splicing (21, 22). Unlike classical splice sites, enhancers and silencers are highly degenerated sequences. Splice sites in higher eukaryotes are actually quite degenerate as well (23, 24). RNA-binding proteins are key splicing regulators that interact with intronic and/or exonic sequences. In general, each splicing factor has a positive or a negative effect on splicing. For example, serine/arginine-rich (SR) proteins act as splicing enhancers, whereas hnRNPA1/A2 proteins act as splicing silencers. However, their final effects on splicing are dependent on their binding sites (21), which ultimately facilitate specific interactions with spliceosomes on the nascent transcripts.

Transformer 2β (Tra2β) is a prototypical SR-like protein splicing factor that is ubiquitously expressed in metazoan genomes (25). Tra2β regulates the splice site selection of several genes, including those encoding calcitonin/calcitonin gene-related peptide (*CGRP*), survival motor neuron 1 and 2 (*SMN1* and *SMN2*), mi-

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crotubule-associated protein tau (*TAU*), and receptor-interacting serine-threonine kinase 2 (*RIPK2*) (26–29). The human *TRA2*β gene consists of 10 exons and 9 introns and generates 5 mRNA isoforms (*TRA2*β*1* to *-5*) through alternative splicing (30). Because of the existence of multiple premature termination codons (PTCs) in exon 2, *TRA2*β*1* mRNA, which encodes a functional, full-length Tra2β protein, does not include exon 2. On the other hand, the *TRA2*β*4* mRNA isoform, which is composed of 10 exons, is not translated and is normally actively degraded through nonsense-mediated mRNA decay (NMD), a surveillance mechanism that degrades PTC-containing mRNAs. Recent transcriptome analyses have revealed that oxidants regulate a large number of alternative splicing events in normal tissues and cancer cell lines (31–34). In addition, oxidative stress can modify the abundance of splicing factors or their activities (35, 36). We also reported that oxidative stress can modify the alternative splicing of the splicing regulator gene *TRA2*β and facilitate the production of the *TRA2*β*4* isoform in rat gastric mucosa and AGS human gastric cancer cells (37). However, little is known about how a specific signal is transduced and which molecules control pre-mRNA processing. Because of the broad role of HuR in oxidative stress responses, it is possible that HuR directly or indirectly controls the oxidant-stimulated generation of the *TRA2*β*4* isoform.

In the work discussed here, we showed that cell cycle checkpoint kinase 2 (Chk2)- and MAP kinase p38 ($p38^{MAPK}$)-mediated phosphorylation of HuR initiated the association between HuR and exon 2 of *TRA2*β pre-mRNA, facilitating the inclusion of exon 2 in colon cancer cells (HCT116 cells). HuR reduced Tra2β protein when exposed to sodium arsenite, at least in part by directly regulating alternative splicing of *TRA2*β pre-mRNA and probably by inhibiting the translation of Tra2β protein, resulting in the prevention of Tra2β-dependent aberrant alternative splicing of target pre-mRNAs. Our findings revealed the potential role of HuR in the regulation of alternative splicing under oxidative stress.

MATERIALS AND METHODS

Cell culture and transfection experiments. HCT116 cells were maintained in Dulbecco's modified essential medium supplemented with 10% (vol/vol) fetal bovine serum and penicillin G-streptomycin. Chk2 and $p38^{MAPK}$ small interfering RNAs (siRNAs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An siRNA targeting the coding region (AAGAGGCAATTACCAGTTTCA) or 3′ UTR (AACGACTCAAT TGTCCCGATA) of *ELAVL1* (*HUR*) mRNA and a control siRNA (AATT CTCCGAACGTGTCACGT) were obtained from Qiagen (Hilden, Germany). These siRNAs were transfected into HCT116 cells at a final concentration of 10 nM using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA). A control plasmid encoding a tandem affinity purification (TAP) tag (pTAP) or a plasmid encoding TAP-tagged wild-type (WT) HuR [pHuR (WT)-TAP] was transfected into HCT116 cells using Lipofectamine-2000 (Invitrogen). Plasmids encoding a TAP-tagged HuR mutant that could not be phosphorylated [with mutations at 3 phosphorylation sites, resulting in changes of serine to alanine at position 88 (S88A), S100A, and T118A; termed pHuR (3A)-TAP] and a TAP-tagged, phosphomimetic HuR [with mutations of S88D, S100D, and T118D; termed pHuR (3D)-TAP] were generated by site-directed mutagenesis and transfected into HCT116 cells using Lipofectamine-2000.

RIP analysis. Immunoprecipitation (IP) of ribonucleoprotein complexes (RIP) was performed as previously described (38). Briefly, cytoplasmic or nuclear lysates (1 mg protein) prepared from either untreated or arsenite-treated cells were incubated for 1 h at 4°C with 80 μl of a 50% (vol/vol) suspension of protein A-Sepharose beads precoated with 20 μg of mouse IgG1 (Santa Cruz Biotechnology) or a mouse anti-HuR antibody (Santa Cruz Biotechnology). The beads were washed 5 times with NT2 buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM $MgCl_2$, and 0.05% Nonidet P-40). For RNA analysis, the beads were incubated with 100 μl NT2 buffer containing 20 U of RNase-free DNase I for 15 min at 37°C, washed twice with 1 ml NT2 buffer, and further incubated for 30 min at 55°C in 100 μl NT2 buffer supplemented with 0.1% sodium dodecyl sulfate (SDS) and 0.5 μg/μl proteinase K. RNA was extracted using a phenol-chloroform mixture and precipitated in the presence of Dr. GenTLE precipitation carrier (Takara Bio, Otsu, Japan). Nuclear lysates were also prepared from cells transfected with pTAP, pHuR (WT)-TAP, pHuR (3A)-TAP, or pHuR (3D)-TAP and were subjected to IP using rabbit IgG-agarose (Sigma-Aldrich, St. Louis, MO) as described above. RNA in the IP materials was subjected to reverse transcription (RT) using a PrimeScript RT reagent kit (Takara Bio). Amplification and quantification of the PCR products were performed using the Applied Biosystems 7900 system (Applied Biosystems, Foster City, CA) and Power SYBR green PCR master mix (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was used as an endogenous quantity control. For RNase protection assays, lysates prepared from either untreated or arsenite-treated cells were incubated for 15 min at room temperature in 100 μl NT2 buffer supplemented with 1 U/μl RNase T1 and subjected to IP using a mouse anti-HuR antibody. RNA in the IP materials was subjected to RT as described above. The following primer sets were used to amplify 3 different fragments of *TRA2*β exon 2: GTTAATGTTGAAGAAGGAAAATGC and ATAAAA CTTGTCAAATGACGAC for the region from nucleotide (nt) 312 to 362, ACAAGTTTTATAAATGAGTATTTG and TTCAGCTTCACTTA TTCCTGAG for the region from nt 351 to 400, and GGAATAAGTGA AGCTGAAATTTG and TATTCTACAAGTGGGACTTCTG for the region from nt 382 to 487. PCR products were then separated on agarose gels.

Protein analysis. Whole-cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Rockford, IL, USA) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The extracted proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). After blocking for 1 h at room temperature with 5% nonfat milk (Cell Signaling Technology, Danvers, MA), membranes were incubated overnight at 4°C with anti-HuR (Santa Cruz Biotechnology), anti-Chk2 (Santa Cruz Biotechnology), anti-phosphorylated Chk2 (T68, phos-Chk2; Santa Cruz Biotechnology), anti-$p38^{MAPK}$ (Cell Signaling Technology), anti-phosphorylated $p38^{MAPK}$ (phos-$p38^{MAPK}$; Cell Signaling Technology), anti-Tra2β (Abcam, Cambridge, MA), anti-α-tubulin (Abcam), anti-hnRNPC1/C2 (Santa Cruz Biotechnology), or anti-GAPDH (Santa Cruz Biotechnology) antibody. Following incubation with an appropriate secondary antibody for 1 h at room temperature, bound antibodies were detected with an ECL (enhanced chemiluminescence) Western blotting detection system (GE Healthcare, Piscataway, NJ). For detection of the phosphorylated form of HuR, 150 μM acrylamide-pendant Phos-tag ligand (Wako Pure Chemical Industries, Osaka, Japan) and 0.1 mM $MnCl_2$ were added to the separating gel before polymerization (39). Phosphorylation of HuR was detected as shifted bands using an anti-HuR antibody.

Generation of *TRA2*β minigene and analysis of alternative splicing by RT-PCR. The region of the human *TRA2*β gene from exon 1 to 4 (*Tra* minigene [MGTra]-WT) was amplified from a genomic clone (NG_029862.1) using the primers MGTra-F-Bam (GGGGATCCGACCG GCGCGTCGTGCGGGGCT and MGTra-R-Xho GGGCTCGAGTACCC GATTCCCAACATGACG). The minigene was excised using BamHI and XhoI restriction sites that were introduced using these primers and was cloned into pCR3.1 (Invitrogen). The MGTra-Δ39 minigene, in which the 39-nt proximal region of exon 2 was deleted, was generated using a KOD mutagenesis kit (Toyobo, Osaka, Japan) with the primers MGTra-Δ39-S

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TABLE 1 List of primer sets used in qPCR

Gene name(s)[a]	Sequence	Position
TRA2β1 + *TRA2β1*	5′-GGAAGTGCTCACGGATCGG-3′	Exon 3 forward
	5′-GACATGGGAGAATGGCTGTGGC-3′	Exon 4 reverse
TRA2β1	5′-CGGCGAGCGGGAATCCCG-3′	Exon 1 forward
	5′-GACATGGGAGAATGGCTGTGGC-3′	Exon 1-3 junction reverse
TRA2β4	5′-AAGTCCCACTTGTAGAATATTGAGC-3′	Exon 2 forward
	5′-TCCGTTTCAAATTCTGACTTCTT-3′	Exon 2 reverse
GAPDH	5′-AGCCACATCGCTCAGACAC-3′	Forward
	5′-GCCCAATACGACCAAATCC-3′	Reverse
18S	5′-CGATTGGATGGTTTAGTGAGG-3′	Forward
	5′-AGTTCGACCGTCTTCTCAGC-3′	Reverse
SMN1 (total)	5′-GGGTTTGCTATGGCGATG-3′	Exon 4 forward
	5′-TCATCCCAAATGTCAGAATCAT-3′	Exon 5 reverse
SMN1 (including)	5′-GAAGGAAGGTGCTCACATTCCT-3′	Exon 7 forward
	5′-GAGTTACCCATTCCACTTCCTTTT-3′	Exon 8 reverse
SMN1 (excluding)	5′-ATATGGAAATGCTGGCATAGAGC-3′	Exon 6-8 junction forward
	5′-GAGTTACCCATTCCACTTCCTTTT-3′	Exon 8 reverse
SMN2 (total)	5′-CTTTCCCCAATCTGTGAAGTAGC-3′	Exon 4 forward
	5′-ATGGAGCAGATTTGGGCTTG-3′	Exon 5 reverse
SMN2 (including)	5′-TGATGATGCTGATGCTTTGGG-3′	Exon 6 forward
	5′-GGAATGTGAGCACCTTCCTTC-3′	Exon 7 reverse
SMN2 (excluding)	5′-GCTATTATATGGAAATGCTGGCATA-3′	Exon 6-8 junction forward
	5′-CGCTTCACATTCCAGATCTGTC-3′	Exon 8 reverse
TAU (total)	5′-AACCTCCAAAATCAGGGGATCG-3′	Exon 9 forward
	5′-ACCACTGCCACCTTCTTGG-3′	Exon 9 reverse
TAU (including)	5′-AGTCCAAGTGTGGCTCAAAGGATAA-3′	Exon 10 forward
	5′-CCCAATCTTCGACTGGACTCTG-3′	Exon 11 reverse
TAU (excluding)	5′-GGCGGGAAGGTGCAAATAGTCT-3′	Exon 9-11 junction forward
	5′-TGGCCACCTCCTGGTTTATG-3′	Exon 11 reverse
RIPK2 (total)	5′-TCAAAGTGGCGCATGATGTC-3′	Exon 3 forward
	5′-CTGGCCCTTGATTTTTGTCCAG-3′	Exon 3 reverse
RIPK2 (including)	5′-TGAAGCACCTGCACATCCAC-3′	Exon 1 forward
	5′-TCAGGCTCATTGCAAATTCCC-3′	Exon 2 reverse
RIPK2 (excluding)	5′-CACAAACTCGCCGACCTG-3′	Exon 1 forward
	5′-TCAGGATATTCAGTTTTCTGTCG-3′	Exon 1-3 junction reverse

[a] total, total transcript; including, isoform that includes Tra2β-targeted exon; excluding, isoform that excludes Tra2β-targeted exon.

(ACAAGTTTTATAAATGAGTATTTGAAGCTC) and MGTra-Δ39-AS (CTTAATAGAAAAAGAACAGGATGAAGAATA). For RT, a vector-specific primer, pCR-RT-reverse (GCCCTCTAGACTCGAGCTCGA), was used to avoid amplification of endogenous transcripts. MGTra-F-Bam and MGTra-R-Xho were used to amplify transfected cDNA. PCR products were then separated on agarose gels.

Quantitative real-time RT-PCR (qPCR). Total RNAs were extracted from HCT116 cells using TRIzol reagent (Invitrogen). One microgram of isolated RNA was reverse transcribed using a PrimeScript RT reagent kit (Takara Bio). The amounts of *TRA2β1*, *TRA2β4*, or *TRA2β1*-plus-*TRA2β4* mRNAs were measured using the specific primer sets listed in Table 1 and SYBR green master mix (Applied Biosystems) as previously described (37). *GAPDH* mRNA and 18S levels were also measured as internal controls for normalization (38, 40). Transcript levels for isoforms that included *TRA2*β-targeted exons, those that excluded *TRA2*β-targeted exons, and total transcripts of *SMN1*, *SMN2*, *TAU*, and *RIPK2* were measured using the specific primer sets listed in Table 1.

Biotin pulldown analysis. cDNA from HCT116 cells was used as a template for *in vitro* synthesis of biotinylated transcripts by PCR. The T7 RNA polymerase promoter sequence (CCAAGCTTCTAATACGACTCA CTATAGGGAGA [T7]) was added to the 5′ end of all fragments. The

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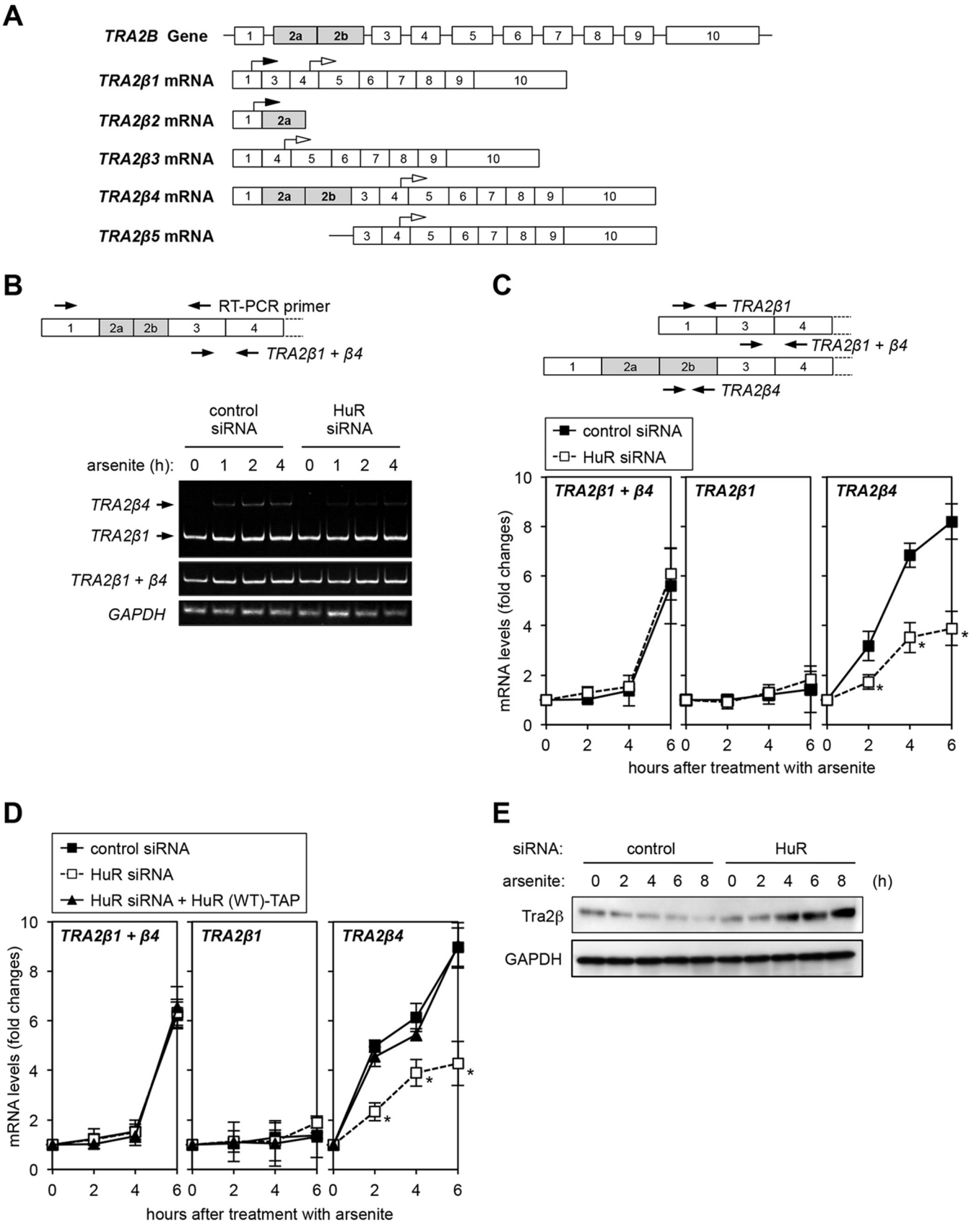

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biotinylated *GAPDH* 3′ UTR was prepared as described previously (7). The primers used for the preparation of biotinylated transcripts spanning exons 1, 2, 3, and 10 of *TRA2*β were as follows: T7-GTGCGGGACGCGC TGCAGCTGGA and GACTCCTGGCTGCTGTCGCCGGT for *TRA2*β exon 1, T7-GTTAATGTTGAAGAAGGAAAATGC and TTAGCGTAGT GCTTTCTGATTC for exon 2, T7-GAATCCCGTTCTGCTTCCAG AAGT and ATGGCTGTGGCTGTGCCGTCTA for exon 3, and T7-AGC ATGAAGACTTTCTGAAACCT and GAATACCCTGGATTCAGTA GAAA for exon 10. The following primer sets were used to prepare 6 different fragments of *TRA2*β exon 2: T7-GTTAATGTTGAAGAAGGAA AATGC and TATTCTACAAGTGGGACTTCTG for the fragment from nt 312 to 463 (F1), T7-GAATGCATGCTAATTATCAGAC and TTAGCG TAGTGCTTTCTGATTC for the fragment from nt 420 to 587 (F2), T7-GTTAATGTTGAAGAAGGAAAATGC and TTCAGCTTCACTTATTCC TGAG for the fragment from nt 312 to 400 (F3), T7-GGAATAAGTGAA GCTGAAATTTG and TATTCTACAAGTGGGACTTCTG for the fragment from nt 382 to 463 (F4), T7-ACAAGTTTTATAAATGAGTA TTTG and TTAGCGTAGTGCTTTCTGATTC for the fragment from nt 351 to 587 (ex2 Δ39), and T7-GGAATAAGTGAAGCTGAAATTTG and TTAGCGTAGTGCTTTCTGATTC for the fragment from nt 382 to 587 (ex2 Δ70). Biotinylated RNAs were synthesized using a MaxiScript T7 kit (Ambion, Austin, TX). Whole-cell lysates (40 μg for each sample) were incubated with one of the purified biotinylated fragments (4 μg) for 1 h at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated beads (Dynabeads M280 streptavidin; Invitrogen), and bound proteins in the pulldown materials were assayed by Western blotting using an anti-HuR antibody as described above.

RESULTS

Involvement of HuR in the arsenite-induced production of the *TRA2*β*4* mRNA isoform. The human *TRA2*β gene generates 5 mRNA isoforms (*TRA2*β*1* to -*5*) through alternative splicing (Fig. 1A). Gastric cancer cells (AGS cells) and colon cancer cells (HCT116 cells) constitutively express *TRA2*β*1* and a small amount of the *TRA2*β*4* isoform (around 10% that of *TRA2*β*1*) (37, 40). These cells preferentially produce the *TRA2*β*4* splice variant without changing *TRA2*β*1* mRNA expression during the initial 4 h after exposure to arsenite or oxidants (37, 40), after which HCT116 cells upregulate the transcription of the *TRA2*β gene in an Ets- and HSF1-dependent manner (40). Therefore, we first reexamined time-dependent changes in *TRA2*β*1*, *TRA2*β*4*, and *TRA2*β*1*-plus-*TRA2*β*4* mRNA levels by RT-PCR and qPCR using the primer sets indicated in Fig. 1B and C. There was no increase in *TRA2*β*1* mRNA during the initial 4 h after exposure to arsenite. In contrast, arsenite rapidly upregulated *TRA2*β*4* mRNA production during the initial 4 h (Fig. 1B and C). We also confirmed that HCT116 cells did not express detectable amounts of other *TRA2*β mRNA isoforms before or after exposure to arsenite (data not shown).

We were particularly interested in the mechanism and physiological relevance of the oxidant-induced production of the PTC-containing transcript *TRA2*β*4*. Notably, HuR knockdown significantly inhibited the arsenite-induced generation of the *TRA2*β*4* transcript during the initial 4 h of treatment (Fig. 1C). To examine the involvement of HuR in *TRA2*β*4* mRNA production, endogenous HuR was silenced with an siRNA targeting the 3′ UTR of *ELAVL1* (*HUR*) mRNA, and a HuR (WT) vector containing the coding region of *ELAVL1* was reintroduced into HuR-silenced cells (data not shown). The reintroduction of this HuR (WT) vector cancelled the inhibitory effects of HuR siRNA (Fig. 1D). At the same time, exposure to arsenite gradually reduced the Tra2β protein levels, while transfection with HuR siRNA promoted the arsenite-dependent induction of Tra2β protein after 4 h of treatment (Fig. 1E). These results suggested that the production of the HuR-dependent splice variant may regulate Tra2β protein levels following exposure to oxidants.

Association between HuR and *TRA2*β*4* mRNA. Next, we investigated the mechanisms through which HuR regulated the production of *TRA2*β*4* mRNA. Previous studies have shown that HuR binds to its target mRNAs and regulates the stability and/or translation of target transcripts. RIP using an anti-HuR antibody was employed to detect associations between HuR and *TRA2*β*1* or *TRA2*β*4* mRNAs. Compared to RIP with IgG, RIP with an anti-HuR antibody did not enrich *TRA2*β*1* or *TRA2*β*4* mRNAs in either cytoplasmic or nuclear lysates of untreated control cells (Fig. 2A). Interestingly, HuR associated specifically with *TRA2*β*4* and not with *TRA2*β*1* mRNA only in nuclear lysates from arsenite-treated cells (Fig. 2A), suggesting that arsenite might initiate the interaction of HuR with *TRA2*β*4* mRNA in the nucleus, thereby stabilizing *TRA2*β*4* mRNA. As shown by the results in Fig. 2B, exposure to sodium arsenite actually increased the stability of *TRA2*β*4* but not that of *TRA2*β*1* in control siRNA-transfected cells. Unexpectedly, however, HuR knockdown did not affect the stability of *TRA2*β*4* in HCT116 cells, regardless of arsenite treatment. Thus, our data suggested that HuR was likely to interact with *TRA2*β*4* mRNA when exposed to arsenite and that this interaction may stimulate the production of *TRA2*β*4* mRNA rather than stabilizing it.

We next examined whether HuR facilitated the inclusion of *TRA2*β exon 2 under oxidative stress. To this end, we cloned the MGTra minigene spanning from exon 1 to exon 4 of the *TRA2*β gene (MGTra-WT), as previously reported (41), and transfected this minigene into HCT116 cells. HuR knockdown did not activate the transcription of MGTra-WT and did not change the stability of its transcripts before or after exposure to arsenite (Fig. 2C,

FIG 1 Involvement of HuR in arsenite-stimulated expression of the *TRA2*β*4* mRNA isoform. (A) Diagram of *TRA2*β mRNA isoforms. The inclusion of each exon is indicated by Arabic numbers. The translation start sites in exons 1 and 4 are indicated by filled arrows and open arrows, respectively. (B) HCT116 cells were treated with 10 nM control siRNA or HuR siRNA for 48 h and then exposed to 100 μM sodium arsenite for the indicated times. RT-PCR was performed with primers specific for exons 1 and 4 or exons 3 and 4. Primer sets are indicated in the diagram. (C) Primer sets designed to measure *TRA2*β*1*, *TRA2*β*4*, or transcripts containing exons 3 and 4 (*TRA2*β*1* + β*4*) are indicated in the diagram. HCT116 cells were treated with 10 nM control siRNA or HuR siRNA for 48 h and then exposed to 100 μM sodium arsenite for the indicated times. Amounts of *TRA2*β*1*, *TRA2*β*4*, and *TRA2*β*1* plus *TRA2*β*4* were measured by qPCR, using *GAPDH* mRNA as an endogenous control. Values are expressed as fold changes (means ± standard deviations [SDs], $n = 5$) compared with the respective values in untreated control cells (0 h). *, significantly different from the control value by Student's *t* test ($P < 0.05$). (D) Control plasmids encoding the TAP tag or plasmids encoding HuR (WT)-TAP were transfected into HCT116 cells in which endogenous HuR was silenced using siRNA targeting the 3′ UTR of *HUR* mRNA. Cells were left untreated or were treated with 100 μM sodium arsenite for the indicated times. Levels of *TRA2*β*1*, *TRA2*β*4*, and *TRA2*β*1* plus *TRA2*β*4* were measured by qPCR, using *GAPDH* mRNA as an endogenous control. Values are expressed as fold changes (means ± SDs, $n = 5$) compared with the respective values in untreated control cells (0 h). *, significantly different from the control value by one-way analysis of variance followed by Tukey's multiple-comparison test ($P < 0.05$). (E) HCT116 cells were treated with 10 nM control siRNA or HuR siRNA for 48 h and exposed to 100 μM sodium arsenite for the indicated times. Tra2β levels were measured by Western blotting, using GAPDH as a loading control.

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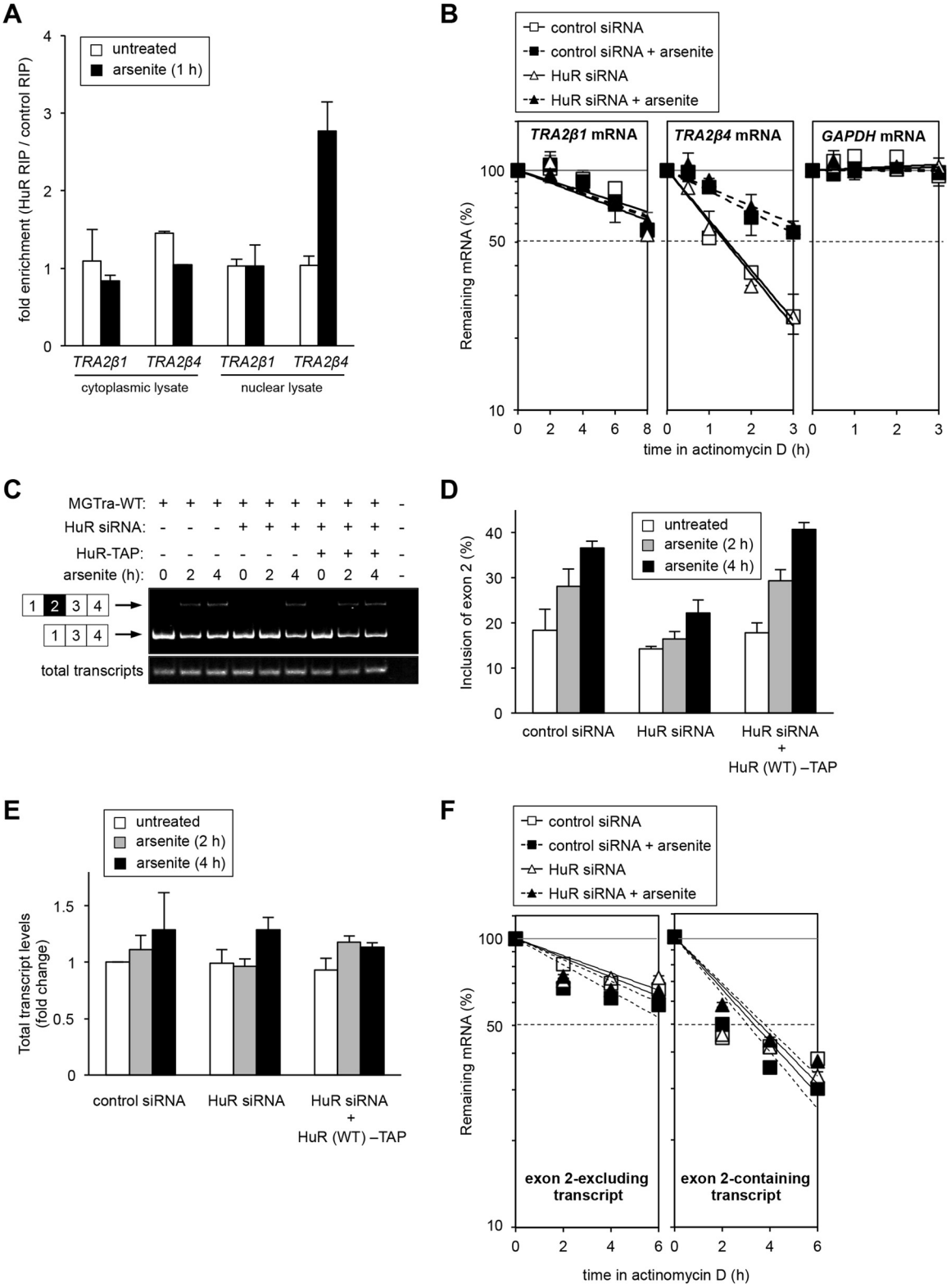

FIG 2 Association of HuR with *TRA2β4* mRNA. (A) Possible association between HuR and *TRA2β4* mRNA. Cytoplasmic and nuclear lysates were prepared from HCT116 cells before (untreated) or 1 h after treatment with 100 μM sodium arsenite. HuR-associated *TRA2β1* and *TRA2β4* mRNAs in these lysates were isolated by RIP, and their levels were measured by qPCR and normalized to *GAPDH* mRNA levels. Data are expressed as fold enrichment of *TRA2β1* or *TRA2β4* mRNA levels in HuR IP relative to those in IgG IP. Values are means ± SDs ($n = 4$). (B) Effects of HuR knockdown on the stability of *TRA2β1* and *TRA2β4* mRNA. After exposure of control siRNA- or HuR siRNA-transfected HCT116 cells to 100 μM sodium arsenite in the presence of 2 μg/ml transcription inhibitor, actinomycin D, amounts of *TRA2β1*, *TRA2β4*, and *GAPDH* mRNAs were measured by qPCR and normalized to 18S rRNA levels. Data (means ± SDs, $n = 3$) are expressed as percentages of *TRA2β1*, *TRA2β4*, or *GAPDH* mRNA levels before exposure to arsenite (time zero). (C) Effects of control siRNA or HuR siRNA

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E, and F). Treatment with arsenite for 2 or 4 h stimulated the production of a transcript containing exon 2 in MGTra-WT-transfected cells. HuR knockdown suppressed the inclusion of exon 2, and this effect was cancelled by exogenously introduced HuR (WT)-TAP, which was resistant to the HuR siRNA targeting the 3′ UTR (Fig. 2C and D), suggesting that HuR may regulate the alternative splicing of *TRA2*β pre-mRNA in the nucleus under oxidative stress.

Interaction between HuR and *TRA2*β exon 2. The RIP assay and minigene experiments suggested that *TRA2*β exon 2, which is an ARE and has a predicted HuR motif identified by photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) (Fig. 3A) (18), may be a potential target for nuclear HuR following exposure to sodium arsenite. To detect the association between HuR and *TRA2*β exon 2 by pulldown assays, we prepared biotinylated transcripts encoding exon 1, 2, 3, or 10 (full-length 3′ UTR) of *TRA2*β mRNA (Fig. 3A). Exon 2 was further subdivided into 2 overlapping fragments, F1 (nt 312 to 463) and F2 (nt 420 to 587). Nuclear lysates were prepared from HCT116 cells before and after treatment with 100 μM sodium arsenite for 2 h, and lysates were then incubated with one of the biotinylated RNA fragments. After RNA-protein complexes were pulled down using streptavidin-coated beads, HuR recovered in the precipitates was examined by Western blotting. As shown by the results in Fig. 3B, HuR associated with exon 2 in addition to the 3′ UTR but did not associate with exon 1, exon 3, or the *GAPDH* 3′ UTR. Putative sites for interaction with HuR were confirmed to be present in F1 of exon 2 (Fig. 3B). F1 was then subdivided into 2 overlapping fragments (F3 and F4) (Fig. 3C), and we found that exon 2a possessed an arsenite-responsive HuR-interacting site(s) (Fig. 3D). To further define the minimal region in exon 2 for interacting with HuR, we constructed serially truncated fragments of exon 2 (Fig. 3E). As shown by the results in Fig. 3F, deletion of the 39-nt region from positions 312 to 350 resulted in loss of the HuR-binding ability of exon 2. Limited RNase T1 digestion followed by RIP analysis with an anti-HuR antibody also demonstrated the interaction between the 39-nt region of exon 2 and endogenous HuR in nuclear lysates from arsenite-treated cells (Fig. 3G and H).

We next examined whether this region affected the arsenite-induced changes in alternative splicing of *TRA2*β pre-mRNA. To this end, we generated an MGTra minigene lacking the 39-nt region (MGTra-Δ39). The introduction of this deletion construct completely blocked the arsenite-induced production of an exon 2-containing transcript (Fig. 4A and B). HuR knockdown did not modify the transcription of the MGTra-Δ39 minigene and did not change the stability of its transcripts before or after exposure to arsenite (Fig. 4A, C, and D). Taken together, our data suggested that exposure to arsenite stimulated the interaction of HuR with the 39-nt proximal region of *TRA2*β*4* exon 2. This interaction may facilitate the inclusion of exon 2 when exposed to oxidative stress.

Involvement of Chk2 and p38^{MAPK} in HuR-mediated inclusion of *TRA2*β exon 2. HuR primarily resides in the nucleus. Moreover, phosphorylated HuR translocates from the nucleus to the cytoplasm in response to oxidants or UV irradiation and enhances binding to its target mRNAs in the cytoplasm (15). To evaluate the *in vivo* phosphorylation status of HuR, we employed Mn^{2+}–Phos-tag SDS-PAGE (39) for separating phosphorylated forms of the protein, followed by Western blotting using an anti-HuR antibody. Although a small amount of HuR was translocated into the cytoplasm after arsenite treatment, the majority of HuR was found in the nucleus, and arsenite initiated the phosphorylation of nuclear HuR within 2 h (Fig. 5A). The association of HuR with its target transcripts is modulated by Chk2- (4, 38), protein kinase C (PKC)- (42–44), and p38^{MAPK}-dependent phosphorylation (45). Consistent with an earlier report (4), exposure to arsenite resulted in phosphorylation of HuR within 1 h in association with Chk2 and p38^{MAPK} phosphorylation (Fig. 5B). As shown by the results in Fig. 5C, knockdown of either Chk2 or p38^{MAPK} did not block the arsenite-stimulated phosphorylation of HuR; however, phosphorylation was inhibited when both Chk2 and p38^{MAPK} were silenced (Fig. 5C and D). The association of HuR with *TRA2*β*4* mRNA was also blocked only after double knockdown of Chk2 and p38^{MAPK} (Fig. 5E). Consequently, arsenite-stimulated *TRA2*β*4* mRNA production was significantly inhibited only when both Chk2 and p38^{MAPK} were silenced (Fig. 5F and G). In contrast, knockdown of Chk2 and/or p38^{MAPK} did not affect *TRA2*β*1* mRNA expression (Fig. 5F and G). In accordance with the results in HuR knockdown cells, knockdown of endogenous Chk2 and p38^{MAPK} sequentially increased the amount of Tra2β protein after exposure to arsenite (Fig. 5H). Minigene experiments also demonstrated that Chk2/p38^{MAPK} double knockdown inhibited the inclusion of exon 2 (Fig. 6A and B) without changing the transactivation of the MGTra minigene (Fig. 6A and C). These results suggested that both Chk2- and p38^{MAPK}-dependent phosphorylation of HuR may promote the inclusion of exon 2, possibly through stimulating its interaction with exon 2.

Roles of HuR phosphorylation sites in the association of HuR with *TRA2*β*4* mRNA. To further analyze the effects of HuR phosphorylation on its interaction with *TRA2*β*4* mRNA, we constructed HuR mutants with modified S88, S100, and T118 residues, as indicated in Fig. 7A. All 3 residues that could be phosphorylated by Chk2 or p38^{MAPK} (S88, S100, and T118) were replaced with alanine in the HuR (3A)-TAP mutant. Endogenous HuR in HCT116 cells was silenced using siRNA targeting the 3′ UTR of *HUR* mRNA (data not shown), and the cells were transfected with plasmids encoding HuR (WT), HuR (3A), HuR (S88A), HuR (S100A), or HuR (T118A) for 48 h (data not shown). In RIP assays, HuR (3A), HuR (S88A), and

on alternative splicing of the MGTra minigene. Control plasmids expressing the TAP tag or plasmids expressing HuR (WT)-TAP were transfected into HCT116 cells in which endogenous HuR was silenced using siRNA targeting the 3′ UTR of *HUR* mRNA. Cells were transfected with MGTra-WT minigene (1 μg) for 24 h and left untreated or treated with 100 μM sodium arsenite for the indicated times. The inclusion of exon 2 and levels of transcripts containing exons 3 and 4 were analyzed by RT-PCR. (D) After treatment of HCT116 cells as explained for panel C, the percentage of exon 2 inclusion in transcripts from the MGTra-WT minigene was measured by qPCR using transcripts containing exons 3 and 4 as a quantity control. Values are means ± SDs ($n = 5$). (E) After treatment of HCT116 cells as explained for panel C, the amounts of transcripts derived from the MGTra minigene were analyzed by qPCR, using GAPDH mRNA as an endogenous control. Values are expressed as fold changes (means ± SDs, $n = 5$) compared with the respective values in untreated control cells. (F) MGTra-WT was cotransfected with control siRNA or HuR siRNA for 48 h. Cells were then treated with 100 μM sodium arsenite in the presence of 2 μg/ml transcription inhibitor, actinomycin D. The amounts of exon 2-excluding transcripts and exon 2-containing transcripts were analyzed as described for panel B.

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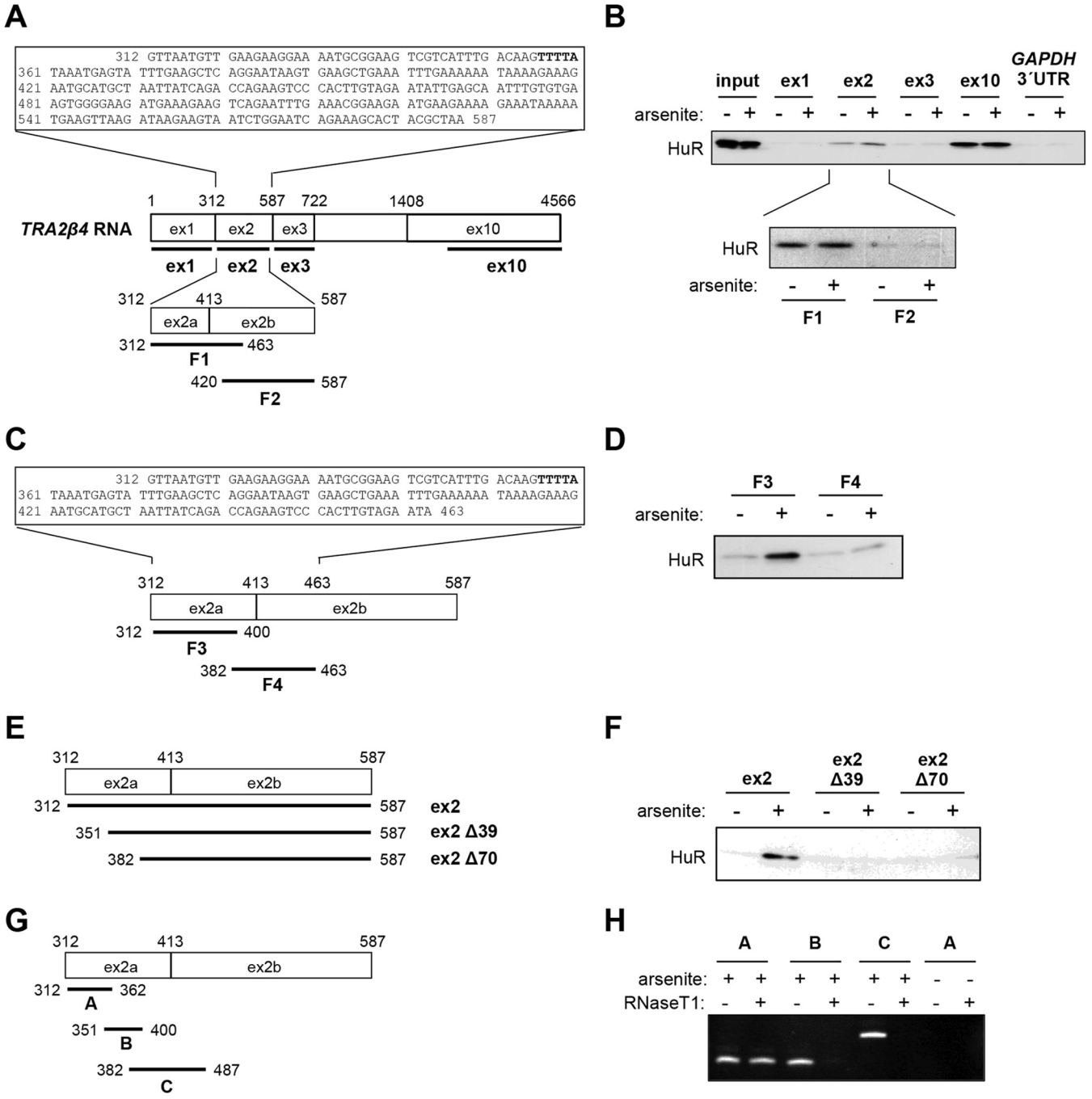

FIG 3 Detection of *TRA2*β mRNA-associated HuR. (A) Nucleotide sequence of the *TRA2*β exon 2 and schematic diagram of the biotinylated transcripts of *TRA2*β exon 1 (ex1), exon 2 (ex2), exon 3 (ex3), exon 10 (ex10), and exon 2 fragments (F1 and F2) used for biotin pulldown assays. A predicted hit of a previously identified HuR motif is depicted in boldface. (B) Biotinylated *TRA2*β fragments (ex1, ex2, ex3, ex10, ex2 F1, and ex2 F2) and a biotinylated fragment of the *GAPDH* 3′ UTR (negative control) were prepared. Associations between HuR and each fragment were tested by biotin pulldown assay using cell lysates from HCT116 cells left untreated or treated with 100 μM sodium arsenite for 2 h. Amounts of HuR in pulldown samples were measured by Western blotting using a specific anti-HuR antibody. Similar results were obtained in 3 independent experiments. (C) Schematic diagram of *TRA2*β exon 2 fragments 3 (F3) and 4 (F4). (D) Association of biotinylated F4 or F3 with HuR was examined by biotin pulldown assay, and F3- or F4-associated HuR was detected by Western blotting. The results are representative of 3 independent experiments. (E) Schematic diagram of *TRA2*β exon 2 (ex2) and exon 2 fragments (ex2 Δ39 and ex2 Δ70). (F) Associations between HuR and biotinylated exon 2, ex2 Δ39, and ex2 Δ70 were analyzed as described above. The results are representative of 3 independent experiments. (G) Schematic diagram of the *TRA2*β exon 2 fragments (A, B, and C). (H) Nuclear lysates were prepared from HCT116 cells before (−) or after (+) a 1-h treatment with 100 μM sodium arsenite. HuR-associated *TRA2β4* mRNAs in nuclear lysates were isolated by HuR IP performed after RNase T1 digestion and were analyzed by RT-PCR. The results are representative of 3 independent experiments.

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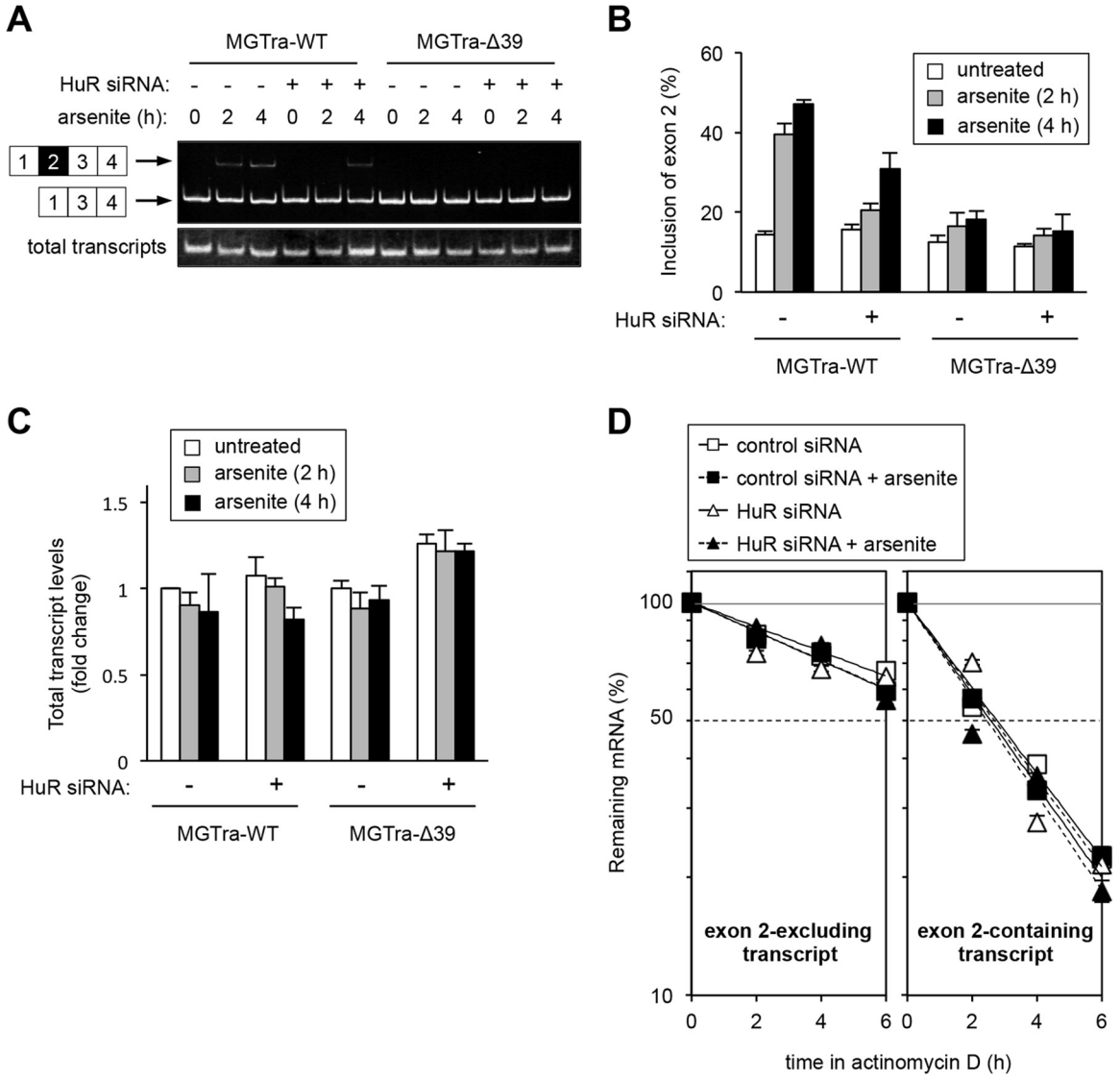

FIG 4 Involvement of the HuR-binding region in the arsenite-induced inclusion of *TRA2*β exon 2. (A) Endogenous HuR was silenced using siRNA targeting the 3′ UTR of *HUR* mRNA, and the MGTra-WT or MGTra-Δ39 minigene was transfected into HuR knockdown cells. Cells were then left untreated or were treated with 100 μM sodium arsenite for the indicated times. The inclusion of exon 2 and levels of transcripts containing exons 3 and 4 were analyzed by RT-PCR. (B) After treatment of HCT116 cells as explained for panel A, the percentages of exon 2 inclusion in transcripts from each MGTra minigene were measured by qPCR using transcripts containing exons 3 and 4 as a quantity control. (C) After treatment of HCT116 cells as explained for panel A, the amounts of transcripts derived from the indicated plasmids were analyzed as described in the legend to Fig. 2E. (D) MGTra-Δ39 was cotransfected with control siRNA or HuR siRNA for 48 h. Cells were then treated with 100 μM sodium arsenite in the presence of 2 μg/ml transcription inhibitor, actinomycin D. The amounts of transcripts derived from MGTra-Δ39 were analyzed as described in the legend to Fig. 2F.

HuR (T118A) did not show any association with *TRA2*β*4* mRNA after treatment with arsenite (Fig. 7B). Moreover, the transfection of plasmids encoding HuR (3A), HuR (S88A), and HuR (T118A) into HuR-silenced cells failed to increase *TRA2*β*4* isoform expression in response to arsenite (Fig. 7C). In contrast, the amount of *TRA2*β*1* mRNA was not significantly affected by the introduction of each HuR mutant before or after exposure to arsenite (Fig. 7D). These results indicated that phosphorylation at S88 and/or T118 was important for the generation of *TRA2*β*4* mRNA by regulating the association between HuR and *TRA2*β*4* in response to oxidative stress.

To further confirm that both Chk2- and $p38^{MAPK}$-dependent phosphorylation of HuR at S88 and T118 were crucial for arsenite-stimulated generation of the *TRA2*β*4* isoform, we prepared a plasmid vector expressing a constitutively active form of HuR with the 3 target residues replaced by aspartic acid; this construct was termed HuR (3D)-TAP (Fig. 7A). RIP assays showed that HuR (3D) constitutively associated with *TRA2*β*4* mRNA before and after treatment with arsenite (Fig. 7B). After silencing endogenous HuR, Chk2, and $p38^{MAPK}$ using appropriate siRNAs, HuR (WT), HuR (3A), or HuR (3D) was reintroduced into HCT116 cells. HuR (3D) significantly increased the levels of *TRA2*β*4* mRNA in both untreated and arsenite-treated cells (Fig. 7E), although the increase in untreated cells was not significant as measured by RT-PCR (data not shown). Collectively, these results suggested that Chk2- and $p38^{MAPK}$-dependent phosphorylation

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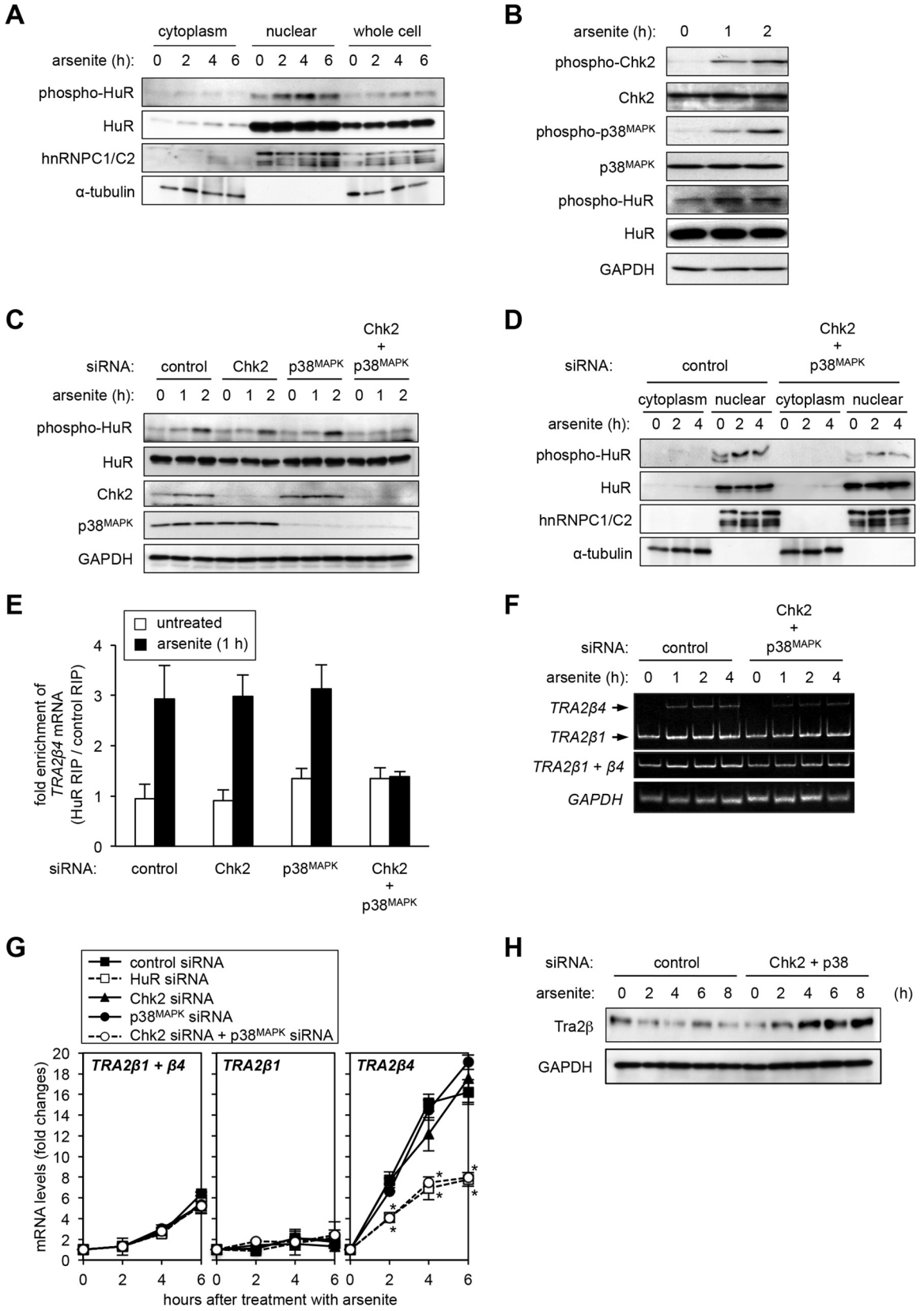

FIG 5 Involvement of Chk2 and p38^{MAPK} in arsenite-stimulated phosphorylation of HuR and alternative splicing of *TRA2*β pre-mRNA. (A) Subcellular distribution and phosphorylation states of HuR. Whole-cell, cytoplasmic, and nuclear fractions were prepared from HCT116 cells before (0) and 2, 4, or 6 h after exposure to 100 μM sodium arsenite. Amounts of HuR, α-tubulin (cytoplasmic marker), and hnRNPC1/C2 (nuclear marker) were measured by Western

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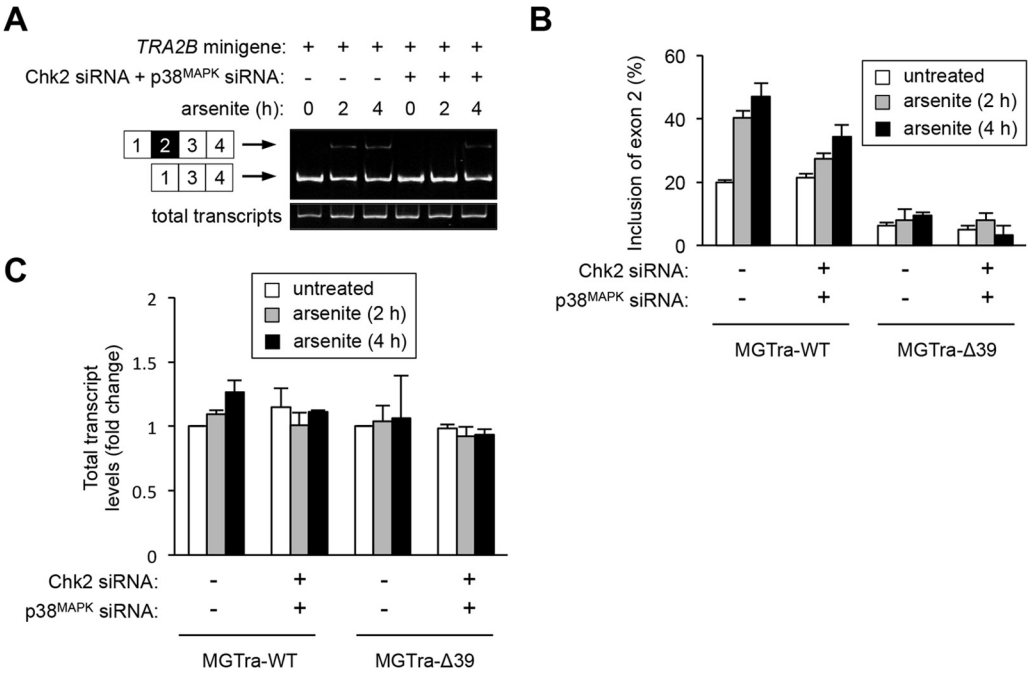

FIG 6 Involvement of Chk2 and p38^{MAPK} in the arsenite-induced inclusion of *TRA2*β exon 2. (A) The MGTra-WT minigene was cotransfected with control siRNA or Chk2 and p38^{MAPK} siRNAs for 48 h, and cells were treated with 100 μM sodium arsenite for the indicated times. Inclusion of exon 2 was analyzed by RT-PCR. (B) After treatment of HCT116 cells as described for panel A, the percentage of exon 2 inclusion in transcripts from each MGTra minigene was measured by qPCR using transcripts containing exons 3 and 4 as a quantity control. Values are means ± SDs ($n = 5$). (C) After treatment of HCT116 cells as described for panel A, the amounts of transcripts derived from the MGTra minigene were analyzed by qPCR, using *GAPDH* mRNA as an endogenous quantity control. Values are expressed as fold changes (means ± SDs, $n = 5$) compared with the respective values in untreated control cells.

of HuR may facilitate exon 2 inclusion in *TRA2*β pre-mRNA through the interaction of HuR with *TRA2*β exon 2 under oxidative stress.

Involvement of HuR in the regulation of Tra2β-dependent alternative splicing under oxidative stress. We next examined whether the arsenite-stimulated increase in Tra2β protein in HuR or Chk2 and p38^{MAPK} knockdown cells affected Tra2β-dependent alternative splicing. To this end, we analyzed calcitonin/calcitonin gene-related peptide (*CGRP*), *SMN1*, *SMN2*, *TAU*, and *RIPK2* genes, which have been reported to exhibit altered splicing patterns in a Tra2β-dependent manner (26–29). Of these targets, HCT116 cells did not express any detectable amounts of calcitonin or *CGRP* mRNAs before or after exposure to arsenite (data not shown). Moreover, exposure to arsenite for 6 h did not change the transactivation of *SMN1* (Fig. 8B and C), *SMN2* (Fig. 8B and D), or *TAU* (Fig. 8B and F), but it increased *RIPK2* transcription (Fig. 8B and E). Tra2β is expected to be able to bind to *SMN1* exon 7, *SMN2* exon 7, *RIPK2* exon 2, and *TAU* exon 10 and stimulate their inclusion in mRNAs (26–29). Therefore, we used qPCR to measure the levels of different isoforms containing or excluding these putative Tra2β target exons. In untreated cells, HuR knockdown or Chk2/p38^{MAPK} double knockdown did not affect transcripts containing or excluding these exons for all mRNAs tested. Knockdown of HuR or Chk2/p38^{MAPK} double knockdown significantly increased the arsenite-induced generation of transcripts containing these exons for *SMN1*, *SMN2*, and *RIPK2* mRNAs without affecting the levels of the corresponding transcripts excluding these exons (Fig. 8B to E). However, HuR knockdown or

blotting. Levels of phosphorylated HuR were analyzed by Phos-tag SDS-PAGE, followed by Western blot analysis using an anti-HuR antibody. (B) Before (0) and 1 or 2 h after treatment of HCT116 cells with 100 μM sodium arsenite, the levels of Chk2, phosphorylated Chk2 at Thr68, p38^{MAPK}, phosphorylated p38^{MAPK} at Thr180/Tyr182, and HuR were measured by Western blotting, using GAPDH as a loading control. Levels of phosphorylated HuR were analyzed as described for panel A. (C) After transfection with 10 nM control siRNA, Chk2 siRNA, p38^{MAPK} siRNA, or both Chk2 and p38^{MAPK} siRNAs for 48 h, HCT116 cells were left untreated (0 h) or were treated (1 or 2 h) with 100 μM sodium arsenite. Levels of Chk2, p38^{MAPK}, and HuR were measured by Western blotting, using GAPDH as a loading control. Levels of phosphorylated HuR were analyzed as described for panel A. (D) After transfection with 10 nM control siRNA or both Chk2 and p38^{MAPK} siRNAs for 48 h, HCT116 cells were left untreated (0 h) or were treated (2 or 4 h) with 100 μM sodium arsenite, and cytoplasmic and nuclear fractions were prepared. HuR, α-tubulin, hnRNPC1/C2, and phosphorylated HuR levels were analyzed as described for panel A. (E) After treatment of HCT116 cells as described for panel C, the association between HuR and *TRA2*β*4* mRNA was examined by RIP analysis and qPCR, using *GAPDH* mRNA as a quantity control. *TRA2*β*4* mRNA levels enriched in HuR IP compared with those in IgG IP are shown as fold changes. Values are means ± SDs ($n = 4$). (F) HCT116 cells were transfected with 10 nM control or Chk2-plus-p38^{MAPK} siRNA for 48 h and exposed to 100 μM sodium arsenite. RT-PCR was performed with primers specific for exons 1 and 4 or exons 3 and 4 as described in the legend to Fig. 1B. (G) HCT116 cells were transfected with 10 nM control, HuR, Chk2, p38^{MAPK}, or Chk2-plus-p38^{MAPK} siRNA for 48 h and exposed to 100 μM sodium arsenite. The levels of *TRA2*β*1*, *TRA2*β*4*, and *TRA2*β*1*-plus-*TRA2*β*4* in these cells were measured by qPCR, using *GAPDH* mRNA as an endogenous control. Values are means ± SDs ($n = 4$). *, significantly different by analysis of variance and Tukey's multiple-comparison test ($P < 0.05$). (H) HCT116 cells were transfected with 10 nM control siRNA or both Chk2 and p38^{MAPK} siRNAs for 48 h and exposed to 100 μM sodium arsenite for the indicated times. The levels of Tra2β were measured by Western blotting, using GAPDH as a loading control.

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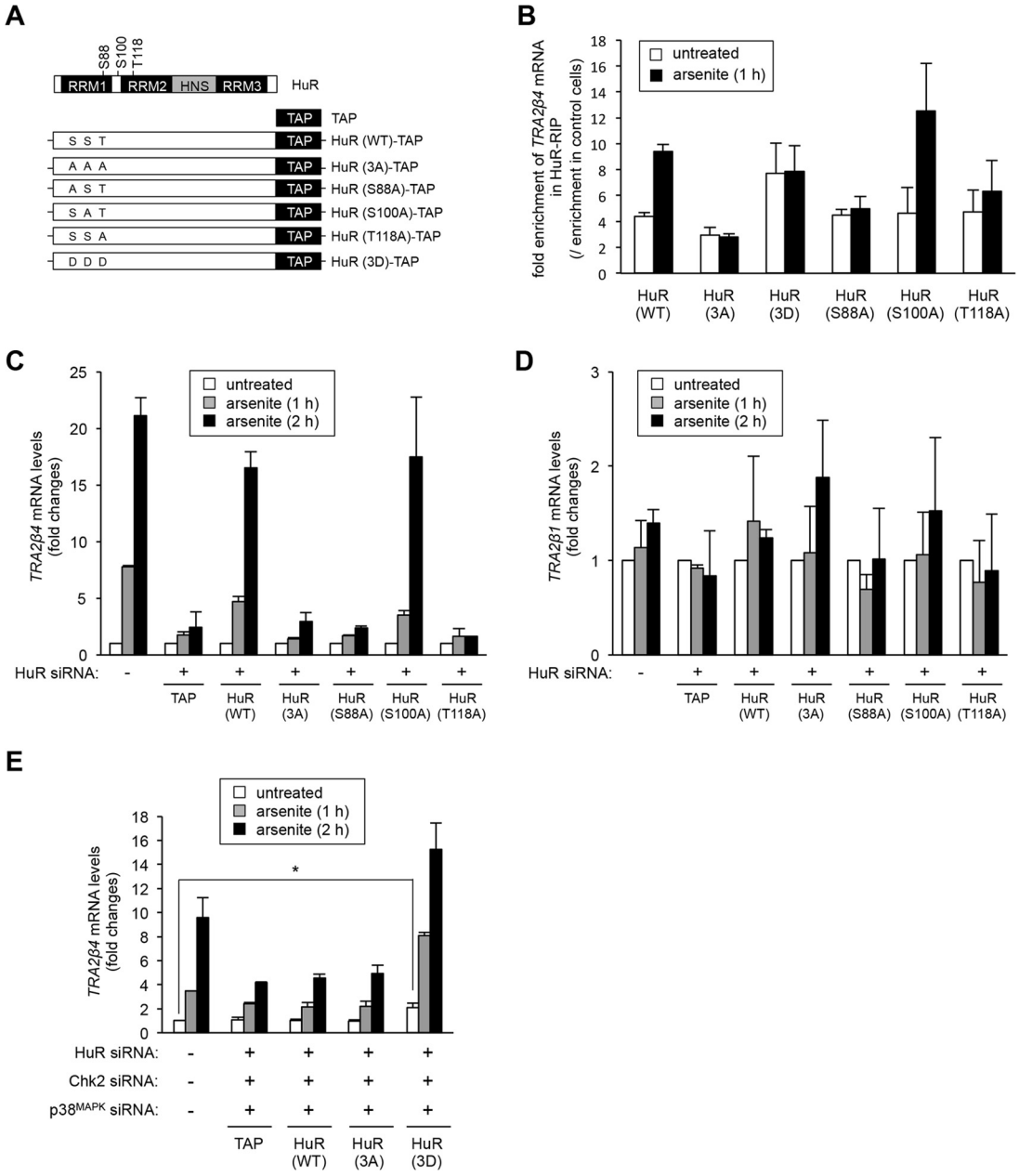

FIG 7 Effects of phosphorylation of HuR at S88, S100, or T118 on arsenite-stimulated binding to *TRA2β4* mRNA and the expression of *TRA2β4* mRNA. (A) Schematic diagram of plasmids encoding TAP, wild-type HuR (WT)-TAP, nonphosphorylatable mutant HuR (3A)-TAP (S88A, S100A, and T118A), HuR (S88A)-TAP, HuR (S100A)-TAP, HuR (T118A)-TAP, and phosphomimetic mutant HuR (3D)-TAP (S88D, S100D, and T118D). HNS, HuR nucleocytoplasmic shuttling sequence. (B) Endogenous HuR was silenced using siRNA targeting the *HUR* 3′ UTR, and TAP tag, HuR (WT)-TAP, HuR (3A)-TAP, HuR (3D)-TAP, HuR (S88A)-TAP, HuR (S100A)-TAP, or HuR (T118A)-TAP was overexpressed in these cells. Cells were then left untreated or treated with 100 μM sodium arsenite for 1 h. Data are expressed as the relative enrichment of *TRA2β4* in chimeric HuR-expressing cells compared with the levels in TAP tag-transfected and *HUR* 3′ UTR siRNA-transfected cells. (C) Chimeric HuR (WT)-TAP, HuR (3A)-TAP, HuR (S88A)-TAP, HuR (S100A)-TAP, and HuR (T118A)-TAP proteins were overexpressed by transfection of the corresponding plasmids into endogenous-HuR-knockdown HCT116 cells. Before (untreated) and 1 or 2 h after treatment with 100 μM sodium arsenite, *TRA2β4* mRNA levels were measured by qPCR, using *GAPDH* mRNA as an endogenous quantity control. Values are means ± SDs ($n = 4$). (D) After treatment of HCT116 cells as described for panel C, *TRA2β1* mRNA levels were measured by qPCR, using *GAPDH* mRNA as an endogenous quantity control. Values are means ± SDs ($n = 3$). (E) Plasmids encoding TAP tag, HuR (WT)-TAP, HuR (3A)-TAP, or HuR (3D)-TAP were transfected into HCT116 cells in which endogenous HuR, Chk2, and $p38^{MAPK}$ were silenced. *TRA2β4* mRNA levels in these cells were measured by qPCR before (untreated) and 1 or 2 h after treatment with 100 μM sodium arsenite. Values are expressed as fold changes (means ± SDs, $n = 5$) compared with the respective values in untreated control cells (0 h). *, significantly different from control value by Student's t test ($P < 0.05$).

Chk2/$p38^{MAPK}$ double knockdown did not alter the amount of *TAU* mRNA containing these exons (Fig. 8B and F). To further confirm the effects of Tra2β, endogenous Tra2β was silenced in HuR knockdown cells (Fig. 8A). As shown by the results in Fig. 8B to E, knockdown of endogenous Tra2β almost completely cancelled the stimulatory effects of HuR siRNA on the production of exon-containing variants. These results suggested that HuR may function to maintain Tra2β levels by regulating alternative splic-

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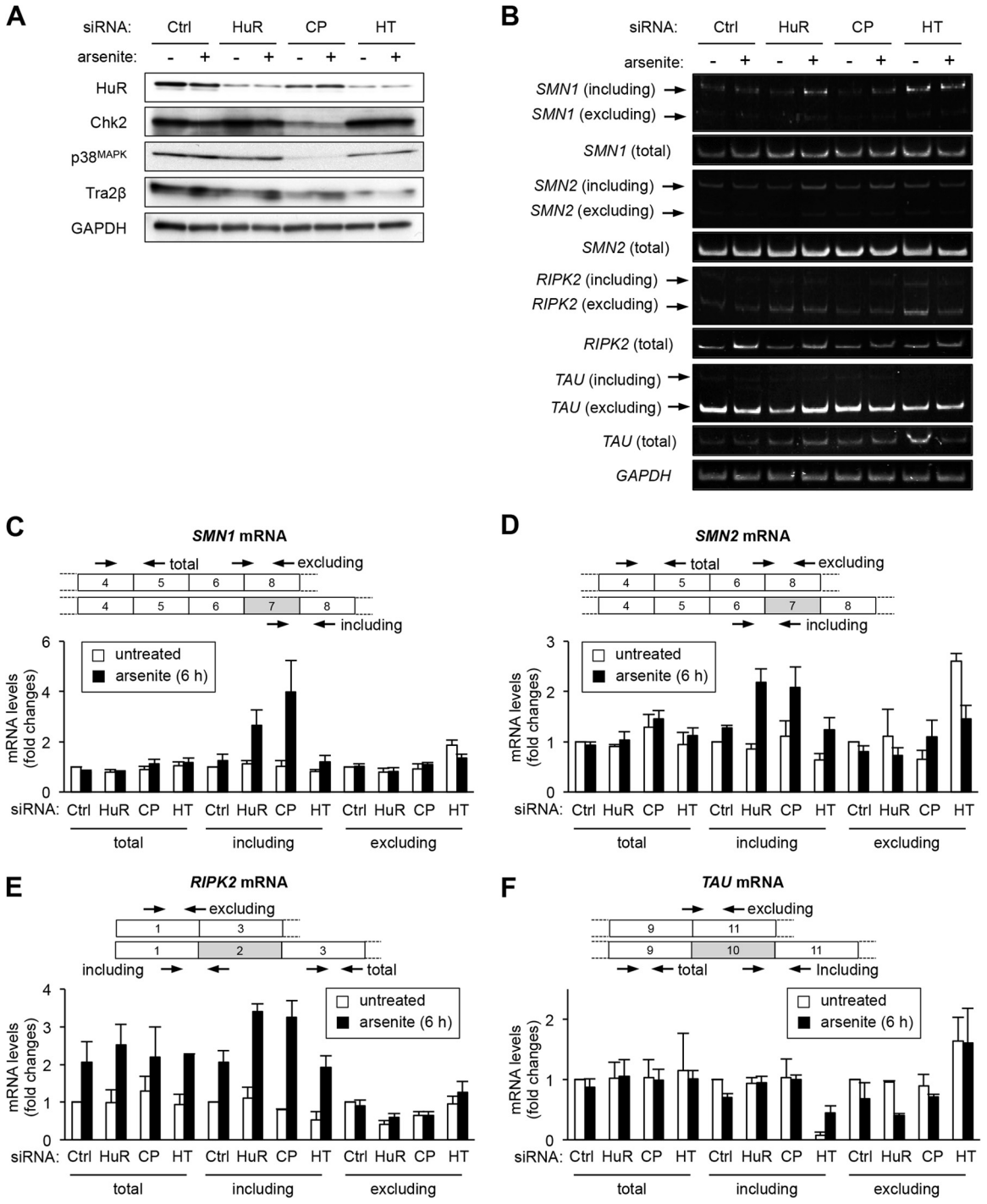

FIG 8 Involvement of HuR, Chk2, and p38^{MAPK} in alternative splicing of Tra2β-target mRNA. (A) HCT116 cells were transfected with 10 nM control siRNA (Ctrl), HuR siRNA (HuR), both Chk2 and p38^{MAPK} siRNA (CP), or both HuR and TRA2β siRNAs (HT) for 48 h, and whole-cell lysates were prepared from these cells before (−) and 6 h after exposure to 100 μM sodium arsenite. The levels of HuR, Chk2, p38^{MAPK}, and Tra2β were measured by Western blotting, using GAPDH as a loading control. (B) After treatment of HCT116 cells as described for panel A, transcript levels for isoforms that include *TRA2*β-targeted exons [indicated by (including)], those that exclude *TRA2*β-targeted exons [indicated by (excluding)], and total transcripts [indicated by (total)] of *SMN1*, *SMN2*, *RIPK2*, and *TAU* mRNAs were analyzed by RT-PCR. (C to F) After treatment of HCT116 cells as described for panel A, transcript levels for isoforms that include *TRA2*β-targeted exons (including), those that exclude *TRA2*β-targeted exons (excluding), and total transcripts (total) of *SMN1* (C), *SMN2* (D), *RIPK2* (E), and *TAU* (F) mRNAs were measured by qPCR, using *GAPDH* mRNA as an endogenous quantity control. Primer sets are indicated in the diagrams.

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ing of *TRA2*β pre-mRNA, thereby preventing aberrant alternative splicing.

DISCUSSION

In this study, we showed that nuclear HuR associated with exon 2a of *TRA2*β pre-mRNA and facilitated the generation of *TRA2*β*4* mRNA containing exon 2 in colon cancer cells only when exposed to oxidants, such as sodium arsenite. Importantly, Chk2- and $p38^{MAPK}$-mediated phosphorylation of HuR at S88 and T118 was a key step in promoting exon 2 inclusion. Transfection of a phosphomimetic HuR [HuR (3D)] stimulated arsenite-induced expression of *TRA2*β*4* mRNA in Chk2/$p38^{MAPK}$ double knockdown cells, while mutation of HuR at S88 and T118 blocked the inclusion of exon 2. Furthermore, HuR knockdown induced Tra2β protein expression after exposure to arsenite, facilitating Tra2β-dependent alternative splicing of target pre-mRNAs, including *SMN1*, *SMN2*, and *RIPK2*. Our results suggested that nuclear HuR may function as a regulator for oxidative stress-induced changes in alternative splicing of distinct genes.

Recently, studies have demonstrated that neuronal members of the Hu family (HuB, HuC, and HuD) regulate alternative splicing of the calcitonin/calcitonin gene-related peptide (46), neurofibromatosis type 1 (*NF1*) (47), and Ikaros (48) pre-mRNAs in neuronlike cells. In contrast, the nuclear localization of HuR has been shown to represent a means of storing nuclear mRNAs not yet ready for export, perhaps to avoid their premature degradation or translation. On the other hand, a previous report showed that HuR participated in the splicing of *FAS* pre-mRNA (16). Recent transcriptome analyses, such as PAR-CLIP (photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation), RIP-chip (RIP followed by microarray analysis), and RNA-seq (transcriptome sequencing), have revealed the presence of dozens of HuR targets in the nucleus (17, 18). In addition to evidence demonstrating that HuR can interact with introns, these previous investigations make a compelling case for a potential role of HuR in splicing, as illustrated for several randomly chosen mRNAs (*ZNF207*, *GANAB*, *PTBP2*, and *DST*) (17). Thus, several lines of evidence suggest that HuR has a role as a splicing regulator.

Mukherjee and coworkers showed that HuR knockdown preferentially downregulated the expression of splicing factors, such as FOX2, NOVA, PTB, and hnRNPC, all of which are targets for HuR (18). HuR may indirectly change alternative splicing through regulating the expression of these splicing regulators. At the same time, however, HuR knockdown was also shown to alter the inclusion of some exons containing adjacent HuR-binding sites, suggesting a direct role of HuR in the regulation of their alternative splicing (18). *TRA2*β exon 2 contains an ARE, and biotin pulldown assays suggested the presence of adjacent binding sites for HuR in exon 2a. Moreover, deletion of the 39-nt proximal region of exon 2a completely blocked the arsenite-induced generation of *TRA2*β*4* mRNA. RNA protection assays demonstrated the interaction of endogenous HuR with this region in arsenite-treated cells. Although this region does not contain any known HuR-binding motifs, our results suggest the involvement of HuR in splicing regulation of *TRA2*β pre-mRNA under oxidative stress through interaction with *TRA2*β exon 2.

Hu proteins exert their functions mainly by counteracting the actions of other proteins that regulate the same target mRNAs through direct competition for binding to AU-rich sequences. This mode of action is also seen in Hu-mediated regulation of pre-mRNA splicing. Neuron-specific Hu proteins (HuB, HuC, and HuD) can directly block the activity of T-cell intracellular antigen 1 on neuron-specific alternative RNA processing of its target pre-mRNAs (46). Hu proteins have also been reported to regulate alternative splicing by the induction of local histone hyperacetylation around alternative exons when they associate with their target sequences on *NF1* and *FAS* pre-mRNA (49). In this mode of action, Hu proteins directly interact with histone deacetylase 2 and inhibit its deacetylation activity. In contrast, HuR was reported to promote skipping of *FAS* exon 6 by binding to an exonic splicing silencer (16), although this is the only known example demonstrating a direct role of HuR in pre-mRNA splicing. In the case of *TRA2*β, HuR was likely to interact with *TRA2*β exon 2a and facilitate exon 2 inclusion. In response to arsenite, HCT116 cells rapidly generated *TRA2*β*4* mRNA through changing alternative splicing, followed by a gradual increase in *TRA2*β*1* mRNA levels, with a peak around 6 h after exposure to arsenite (40). An Ets1-binding site present at bp −64 to −55 of the *TRA2*β promoter is crucial for basal transcription, and 3 heat shock elements (HSEs) located at bp −145 to −99 mediate the oxidant-induced transactivation of *TRA2*β (40). Tra2β regulates alternative splice site selection in a concentration-dependent manner; therefore, Tra2β must be maintained at a proper level. After arsenite treatment, Tra2β protein levels were gradually reduced, resulting in the prevention of inadequate splicing of its target pre-mRNAs. Human *TRA2*β*1* pre-mRNA utilizes its own exon 2 for a negative feedback loop. Excess amounts of Tra2β bind to 4 enhancers present in exon 2 and stimulate the inclusion of exon 2, resulting in the generation of a *TRA2*β*4* variant that cannot be translated. As a consequence, Tra2β synthesis is effectively switched off (41). The binding sites for Tra2β are encoded in exon 2b (41); however, HuR bound to exon 2a. Moreover, we could not detect the association between HuR and Tra2β protein (data not shown). Thus, HuR may also control Tra2β expression independently of the autoregulatory mechanism. In this study, we found that HuR knockdown led to an increase in Tra2β protein after arsenite treatment without significant changes in *TRA2*β*1* isoform levels. HuR did not bind to *TRA2*β*1* isoform (Fig. 2A). However, it is possible that HuR also indirectly blocks the translation of Tra2β protein, although the precise mechanism remains to be elucidated.

The arsenite-induced association between HuR and *TRA2*β*4* is consistent with previous findings that treatment of colorectal carcinoma cells with H_2O_2 promotes the binding of HuR to a *CDKN1A* 3′ UTR transcript (15) and the formation of HuR-*ProT*α mRNA complexes (4). With a few exceptions (50), the stimulus-induced activation of HuR depends on 2 regulatory steps: the subcellular localization of HuR and the interaction of HuR with target mRNAs. Accumulating evidence indicates that phosphorylation of HuR by several different kinases, including PKCα, PKCδ, Cdk1, $p38^{MAPK}$, and Chk2, can change the subcellular localization of HuR and its binding to target mRNAs in response to various stimuli (4, 6, 44, 45). A recent study showed that poliovirus protease 2A induces the translocation of nuclear TIA-1 and TIAR to the cytoplasm without changing HuR localization, and this asymmetric distribution of HuR and TIA-1/TIAR modulates the splicing of the human *FAS* exon 6 (51). Under our experimental conditions, a small amount of nuclear HuR was translocated into the cytoplasm in response to arsenite, while the majority of HuR remained in the nucleus. Distinct phosphoryla-

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tion sites were found to specifically modulate the binding of HuR to target mRNA (1, 52). However, each HuR phosphorylation site is likely to exert an opposite effect on HuR binding, and the final outcome also depends on the target mRNA (52). Previous studies have revealed that H_2O_2 activates Chk2, which in turn phosphorylates HuR. Phosphorylation at each of the 3 HuR residues S88, S100, and T118 influences the association of HuR with *SIRT1* mRNA distinctly, with S88 having little effect, S100 reducing binding, and T118 promoting binding (4, 53). $p38^{MAPK}$ has also been reported to phosphorylate HuR following exposure to DNA-damaging agents (45); for example, following ionizing radiation, $p38^{MAPK}$ phosphorylates HuR at T118, leading to the cytoplasmic accumulation of HuR and the increased binding of HuR to *CDKN1A* mRNA. Our results revealed that arsenite-activated Chk2 and $p38^{MAPK}$ phosphorylated nuclear HuR, facilitating the association between HuR and *TRA2*β*4* exon 2a and the inclusion of exon 2. The triple nonphosphorylatable mutant [HuR (3A)-TAP] could not bind to *TRA2*β*4* mRNA in response to arsenite, an effect that recapitulated the results seen with the nonphosphorylatable HuR (S88A) or HuR (T118A) mutant. We also confirmed that phosphorylation of HuR at Ser100 was not involved in regulating the arsenite-induced binding of HuR to the *TRA2*β*4* isoform using a HuR (S100A) mutant. Finally, we showed that the triple phosphomimetic mutant [HuR (3D)-TAP] could bind to *TRA2*β*4* in untreated cells, inducing the expression of the *TRA2*β*4* isoform in Chk2/$p38^{MAPK}$ double knockdown cells. These results suggested that HuR phosphorylation by both Chk2 and $p38^{MAPK}$ may participate in the regulation of alternative splicing programs under oxidative stress.

In most cases, HuR is thought to bind to AREs in the 3′ UTR of target mRNAs (54, 55). Recent studies have shown that one-third of HuR-RNA associations occur at introns and the remaining two-thirds occur at 3′ UTRs (17, 18). Based on an early classification, AREs can be divided into 3 classes based on sequence similarities (56). Class I AREs contain multiple copies of the pentameric AUUUA motif within U-rich regions, class II AREs contain 2 or more overlapping nonamers containing the AUUUA motif, and class III AREs are U-rich regions without AUUUA pentamers. HuR has been shown to bind to all 3 ARE classes (57–59). Recent transcriptome analyses, such as individual-nucleotide-resolution cross-linking and immunoprecipitation (iCLIP) and PAR-CLIP, have shown that HuR binds to variably sized U-rich-hairpin loops and/or motifs containing a stretch of 3 or 4 uracils separated by an A or C (18, 60). *TRA2*β exon 2 contains several U-rich regions (Fig. 3A). Our biotin pulldown and RNase protection assays suggested that the proximal region of exon 2a from nt 312 to 350 may contain arsenite-responsive binding sites for HuR (Fig. 3F and H). However, previous studies did not predict the presence of any HuR motifs in this region (2, 18, 60). Therefore, elucidation of the precise molecular interaction between HuR and *TRA2*β exon 2 requires further investigation.

HuR is frequently overexpressed in several different types of tumors, including colorectal cancer, and the overexpression of HuR is one of the central regulators of cancer-related gene expression (13, 61–64). Our results indicated the possible involvement of HuR in adaptive or aberrant alternative splicing in cancer cells following exposure to oxidative stress. This function of HuR may be one of the crucial determinants of cancer cell fate.

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REFERENCES

1. **Hinman MN, Lou H.** 2008. Diverse molecular functions of Hu proteins. Cell. Mol. Life Sci. **65:**3168–3181. http://dx.doi.org/10.1007/s00018-008-8252-6.
2. **Lopez de Silanes I, Zhan M, Lal A, Yang X, Gorospe M.** 2004. Identification of a target RNA motif for RNA-binding protein HuR. Proc. Natl. Acad. Sci. U. S. A. **101:**2987–2992. http://dx.doi.org/10.1073/pnas.0306453101.
3. **Abdelmohsen K, Kuwano Y, Kim HH, Gorospe M.** 2008. Posttranscriptional gene regulation by RNA-binding proteins during oxidative stress: implications for cellular senescence. Biol. Chem. **389:**243–255. http://dx.doi.org/10.1515/BC.2008.022.
4. **Abdelmohsen K, Pullmann R, Jr, Lal A, Kim HH, Galban S, Yang X, Blethrow JD, Walker M, Shubert J, Gillespie DA, Furneaux H, Gorospe M.** 2007. Phosphorylation of HuR by Chk2 regulates SIRT1 expression. Mol. Cell **25:**543–557. http://dx.doi.org/10.1016/j.molcel.2007.01.011.
5. **Abdelmohsen K, Lal A, Kim HH, Gorospe M.** 2007. Posttranscriptional orchestration of an anti-apoptotic program by HuR. Cell Cycle **6:**1288–1292. http://dx.doi.org/10.4161/cc.6.11.4299.
6. **Kim HH, Abdelmohsen K, Lal A, Pullmann R, Jr, Yang X, Galban S, Srikantan S, Martindale JL, Blethrow J, Shokat KM, Gorospe M.** 2008. Nuclear HuR accumulation through phosphorylation by Cdk1. Genes Dev. **22:**1804–1815. http://dx.doi.org/10.1101/gad.1645808.
7. **Kim HH, Kuwano Y, Srikantan S, Lee EK, Martindale JL, Gorospe M.** 2009. HuR recruits let-7/RISC to repress c-Myc expression. Genes Dev. **23:**1743–1748. http://dx.doi.org/10.1101/gad.1812509.
8. **Kullmann M, Gopfert U, Siewe B, Hengst L.** 2002. ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5′ UTR. Genes Dev. **16:**3087–3099. http://dx.doi.org/10.1101/gad.248902.
9. **Lal A, Kawai T, Yang X, Mazan-Mamczarz K, Gorospe M.** 2005. Antiapoptotic function of RNA-binding protein HuR effected through prothymosin alpha. EMBO J. **24:**1852–1862. http://dx.doi.org/10.1038/sj.emboj.7600661.
10. **Lal A, Mazan-Mamczarz K, Kawai T, Yang X, Martindale JL, Gorospe M.** 2004. Concurrent versus individual binding of HuR and AUF1 to common labile target mRNAs. EMBO J. **23:**3092–3102. http://dx.doi.org/10.1038/sj.emboj.7600305.
11. **Levy NS, Chung S, Furneaux H, Levy AP.** 1998. Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J. Biol. Chem. **273:**6417–6423. http://dx.doi.org/10.1074/jbc.273.11.6417.
12. **Mazan-Mamczarz K, Galban S, Lopez de Silanes I, Martindale JL, Atasoy U, Keene JD, Gorospe M.** 2003. RNA-binding protein HuR enhances p53 translation in response to ultraviolet light irradiation. Proc. Natl. Acad. Sci. U. S. A. **100:**8354–8359. http://dx.doi.org/10.1073/pnas.1432104100.
13. **Dixon DA, Tolley ND, King PH, Nabors LB, McIntyre TM, Zimmerman GA, Prescott SM.** 2001. Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells. J. Clin. Invest. **108:**1657–1665. http://dx.doi.org/10.1172/JCI200112973.
14. **Wang W, Caldwell MC, Lin S, Furneaux H, Gorospe M.** 2000. HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation. EMBO J. **19:**2340–2350. http://dx.doi.org/10.1093/emboj/19.10.2340.
15. **Wang W, Furneaux H, Cheng H, Caldwell MC, Hutter D, Liu Y, Holbrook N, Gorospe M.** 2000. HuR regulates p21 mRNA stabilization by UV light. Mol. Cell. Biol. **20:**760–769. http://dx.doi.org/10.1128/MCB.20.3.760-769.2000.
16. **Izquierdo JM.** 2008. Hu antigen R (HuR) functions as an alternative pre-mRNA splicing regulator of Fas apoptosis-promoting receptor on exon definition. J. Biol. Chem. **283:**19077–19084. http://dx.doi.org/10.1074/jbc.M800017200.
17. **Lebedeva S, Jens M, Theil K, Schwanhausser B, Selbach M, Landthaler M, Rajewsky N.** 2011. Transcriptome-wide analysis of regulatory interactions of the RNA-binding protein HuR. Mol. Cell **43:**340–352. http://dx.doi.org/10.1016/j.molcel.2011.06.008.
18. **Mukherjee N, Corcoran DL, Nusbaum JD, Reid DW, Georgiev S, Hafner M, Ascano M, Jr, Tuschl T, Ohler U, Keene JD.** 2011. Integrative

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regulatory mapping indicates that the RNA-binding protein HuR couples pre-mRNA processing and mRNA stability. Mol. Cell **43:**327–339. http://dx.doi.org/10.1016/j.molcel.2011.06.007.
19. **Zhu H, Zhou HL, Hasman RA, Lou H.** 2007. Hu proteins regulate polyadenylation by blocking sites containing U-rich sequences. J. Biol. Chem. **282:**2203–2210. http://dx.doi.org/10.1074/jbc.M609349200.
20. **Kornblihtt AR, Schor IE, Allo M, Dujardin G, Petrillo E, Munoz MJ.** 2013. Alternative splicing: a pivotal step between eukaryotic transcription and translation. Nat. Rev. Mol. Cell Biol. **14:**153–165. http://dx.doi.org/10.1038/nrm3525.
21. **Matlin AJ, Clark F, Smith CW.** 2005. Understanding alternative splicing: towards a cellular code. Nat. Rev. Mol. Cell Biol. **6:**386–398. http://dx.doi.org/10.1038/nrm1645.
22. **Wang Z, Burge CB.** 2008. Splicing regulation: from a parts list of regulatory elements to an integrated splicing code. RNA **14:**802–813. http://dx.doi.org/10.1261/rna.876308.
23. **Schwartz SH, Silva J, Burstein D, Pupko T, Eyras E, Ast G.** 2008. Large-scale comparative analysis of splicing signals and their corresponding splicing factors in eukaryotes. Genome Res. **18:**88–103. http://dx.doi.org/10.1101/gr.6818908.
24. **Barbosa-Morais NL, Carmo-Fonseca M, Aparicio S.** 2006. Systematic genome-wide annotation of spliceosomal proteins reveals differential gene family expansion. Genome Res. **16:**66–77. http://dx.doi.org/10.1101/gr.3936206.
25. **Novoyatleva T, Heinrich B, Tang Y, Benderska N, Butchbach ME, Lorson CL, Lorson MA, Ben-Dov C, Fehlbaum P, Bracco L, Burghes AH, Bollen M, Stamm S.** 2008. Protein phosphatase 1 binds to the RNA recognition motif of several splicing factors and regulates alternative pre-mRNA processing. Hum. Mol. Genet. **17:**52–70. http://dx.doi.org/10.1093/hmg/ddm284.
26. **Kondo S, Yamamoto N, Murakami T, Okumura M, Mayeda A, Imaizumi K.** 2004. Tra2 beta, SF2/ASF and SRp30c modulate the function of an exonic splicing enhancer in exon 10 of tau pre-mRNA. Genes Cells **9:**121–130. http://dx.doi.org/10.1111/j.1356-9597.2004.00709.x.
27. **Singh NN, Androphy EJ, Singh RN.** 2004. In vivo selection reveals combinatorial controls that define a critical exon in the spinal muscular atrophy genes. RNA **10:**1291–1305. http://dx.doi.org/10.1261/rna.7580704.
28. **Tran Q, Coleman TP, Roesser JR.** 2003. Human transformer 2beta and SRp55 interact with a calcitonin-specific splice enhancer. Biochim. Biophys. Acta **1625:**141–152. http://dx.doi.org/10.1016/S0167-4781(02)00600-0.
29. **Anderson ES, Lin CH, Xiao X, Stoilov P, Burge CB, Black DL.** 2012. The cardiotonic steroid digitoxin regulates alternative splicing through depletion of the splicing factors SRSF3 and TRA2B. RNA **18:**1041–1049. http://dx.doi.org/10.1261/rna.032912.112.
30. **Nayler O, Cap C, Stamm S.** 1998. Human transformer-2-beta gene (SFRS10): complete nucleotide sequence, chromosomal localization, and generation of a tissue-specific isoform. Genomics **53:**191–202. http://dx.doi.org/10.1006/geno.1998.5471.
31. **Ashibe B, Hirai T, Higashi K, Sekimizu K, Motojima K.** 2007. Dual subcellular localization in the endoplasmic reticulum and peroxisomes and a vital role in protecting against oxidative stress of fatty aldehyde dehydrogenase are achieved by alternative splicing. J. Biol. Chem. **282:**20763–20773. http://dx.doi.org/10.1074/jbc.M611853200.
32. **Hang X, Li P, Li Z, Qu W, Yu Y, Li H, Shen Z, Zheng H, Gao Y, Wu Y, Deng M, Sun Z, Zhang C.** 2009. Transcription and splicing regulation in human umbilical vein endothelial cells under hypoxic stress conditions by exon array. BMC Genomics **10:**126. http://dx.doi.org/10.1186/1471-2164-10-126.
33. **Hirschfeld M, zur Hausen A, Bettendorf H, Jager M, Stickeler E.** 2009. Alternative splicing of Cyr61 is regulated by hypoxia and significantly changed in breast cancer. Cancer Res. **69:**2082–2090. http://dx.doi.org/10.1158/0008-5472.CAN-08-1997.
34. **Matsuo N, Ogawa S, Imai Y, Takagi T, Tohyama M, Stern D, Wanaka A.** 1995. Cloning of a novel RNA binding polypeptide (RA301) induced by hypoxia/reoxygenation. J. Biol. Chem. **270:**28216–28222. http://dx.doi.org/10.1074/jbc.270.47.28216.
35. **Kattapuram T, Yang S, Maki JL, Stone JR.** 2005. Protein kinase CK1alpha regulates mRNA binding by heterogeneous nuclear ribonucleoprotein C in response to physiologic levels of hydrogen peroxide. J. Biol. Chem. **280:**15340–15347. http://dx.doi.org/10.1074/jbc.M500214200.
36. **Panchenko MP, Silva N, Stone JR.** 2009. Up-regulation of a hydrogen peroxide-responsive pre-mRNA binding protein in atherosclerosis and intimal hyperplasia. Cardiovasc. Pathol. **18:**167–172. http://dx.doi.org/10.1016/j.carpath.2008.03.008.
37. **Takeo K, Kawai T, Nishida K, Masuda K, Teshima-Kondo S, Tanahashi T, Rokutan K.** 2009. Oxidative stress-induced alternative splicing of transformer 2beta (SFRS10) and CD44 pre-mRNAs in gastric epithelial cells. Am. J. Physiol. Cell Physiol. **297:**C330–C338. http://dx.doi.org/10.1152/ajpcell.00009.2009.
38. **Masuda K, Abdelmohsen K, Kim MM, Srikantan S, Lee EK, Tominaga K, Selimyan R, Martindale JL, Yang X, Lehrmann E, Zhang Y, Becker KG, Wang JY, Kim HH, Gorospe M.** 2011. Global dissociation of HuR-mRNA complexes promotes cell survival after ionizing radiation. EMBO J. **30:**1040–1053. http://dx.doi.org/10.1038/emboj.2011.24.
39. **Kinoshita E, Kinoshita-Kikuta E, Takiyama K, Koike T.** 2006. Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol. Cell. Proteomics **5:**749–757. http://dx.doi.org/10.1074/mcp.T500024-MCP200.
40. **Kajita K, Kuwano Y, Kitamura N, Satake Y, Nishida K, Kurokawa K, Akaike Y, Honda M, Masuda K, Rokutan K.** 2013. Ets1 and heat shock factor 1 regulate transcription of the Transformer 2beta gene in human colon cancer cells. J. Gastroenterol. **48:**1222–1233. http://dx.doi.org/10.1007/s00535-012-0745-2.
41. **Stoilov P, Daoud R, Nayler O, Stamm S.** 2004. Human tra2-beta1 autoregulates its protein concentration by influencing alternative splicing of its pre-mRNA. Hum. Mol. Genet. **13:**509–524. http://dx.doi.org/10.1093/hmg/ddh051.
42. **Amadio M, Bucolo C, Leggio GM, Drago F, Govoni S, Pascale A.** 2010. The PKCbeta/HuR/VEGF pathway in diabetic retinopathy. Biochem. Pharmacol. **80:**1230–1237. http://dx.doi.org/10.1016/j.bcp.2010.06.033.
43. **Doller A, Akool el, Huwiler SA, Muller R, Radeke HH, Pfeilschifter J, Eberhardt W.** 2008. Posttranslational modification of the AU-rich element binding protein HuR by protein kinase Cdelta elicits angiotensin II-induced stabilization and nuclear export of cyclooxygenase 2 mRNA. Mol. Cell. Biol. **28:**2608–2625. http://dx.doi.org/10.1128/MCB.01530-07.
44. **Doller A, Huwiler A, Muller R, Radeke HH, Pfeilschifter J, Eberhardt W.** 2007. Protein kinase C alpha-dependent phosphorylation of the mRNA-stabilizing factor HuR: implications for posttranscriptional regulation of cyclooxygenase-2. Mol. Biol. Cell **18:**2137–2148. http://dx.doi.org/10.1091/mbc.E06-09-0850.
45. **Lafarga V, Cuadrado A, Lopez de Silanes I, Bengoechea R, Fernandez-Capetillo O, Nebreda AR.** 2009. p38 mitogen-activated protein kinase- and HuR-dependent stabilization of p21(Cip1) mRNA mediates the G_1/S checkpoint. Mol. Cell. Biol. **29:**4341–4351. http://dx.doi.org/10.1128/MCB.00210-09.
46. **Zhu H, Hasman RA, Barron VA, Luo G, Lou H.** 2006. A nuclear function of Hu proteins as neuron-specific alternative RNA processing regulators. Mol. Biol. Cell **17:**5105–5114. http://dx.doi.org/10.1091/mbc.E06-02-0099.
47. **Zhu H, Hinman MN, Hasman RA, Mehta P, Lou H.** 2008. Regulation of neuron-specific alternative splicing of neurofibromatosis type 1 pre-mRNA. Mol. Cell. Biol. **28:**1240–1251. http://dx.doi.org/10.1128/MCB.01509-07.
48. **Bellavia D, Mecarozzi M, Campese AF, Grazioli P, Talora C, Frati L, Gulino A, Screpanti I.** 2007. Notch3 and the Notch3-upregulated RNA-binding protein HuD regulate Ikaros alternative splicing. EMBO J. **26:**1670–1680. http://dx.doi.org/10.1038/sj.emboj.7601626.
49. **Zhou HL, Hinman MN, Barron VA, Geng C, Zhou G, Luo G, Siegel RE, Lou H.** 2011. Hu proteins regulate alternative splicing by inducing localized histone hyperacetylation in an RNA-dependent manner. Proc. Natl. Acad. Sci. U. S. A. **108:**E627–E635. http://dx.doi.org/10.1073/pnas.1103344108.
50. **Abdelmohsen K, Srikantan S, Yang X, Lal A, Kim HH, Kuwano Y, Galban S, Becker KG, Kamara D, de Cabo R, Gorospe M.** 2009. Ubiquitin-mediated proteolysis of HuR by heat shock. EMBO J. **28:**1271–1282. http://dx.doi.org/10.1038/emboj.2009.67.
51. **Alvarez E, Castello A, Carrasco L, Izquierdo JM.** 2013. Poliovirus 2A protease triggers a selective nucleo-cytoplasmic redistribution of splicing factors to regulate alternative pre-mRNA splicing. PLoS One **8:**e73723. http://dx.doi.org/10.1371/journal.pone.0073723.
52. **Eberhardt W, Doller A, Pfeilschifter J.** 2012. Regulation of the mRNA-binding protein HuR by posttranslational modification: spotlight on phosphorylation. Curr. Protein Pept. Sci. **13:**380–390. http://dx.doi.org/10.2174/138920312801619439.
53. **Wilusz CJ, Wilusz J.** 2007. HuR-SIRT: the hairy world of posttranscrip-

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tional control. Mol. Cell **25:**485–487. http://dx.doi.org/10.1016/j.molcel.2007.02.001.

54. **Bakheet T, Frevel M, Williams BR, Greer W, Khabar KS.** 2001. ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. Nucleic Acids Res. **29:**246–254. http://dx.doi.org/10.1093/nar/29.1.246.
55. **Bakheet T, Williams BR, Khabar KS.** 2003. ARED 2.0: an update of AU-rich element mRNA database. Nucleic Acids Res. **31:**421–423. http://dx.doi.org/10.1093/nar/gkg023.
56. **Chen CY, Shyu AB.** 1995. AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem. Sci. **20:**465–470. http://dx.doi.org/10.1016/S0968-0004(00)89102-1.
57. **Dean JL, Wait R, Mahtani KR, Sully G, Clark AR, Saklatvala J.** 2001. The 3′ untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR. Mol. Cell. Biol. **21:**721–730. http://dx.doi.org/10.1128/MCB.21.3.721-730.2001.
58. **Jain RG, Andrews LG, McGowan KM, Pekala PH, Keene JD.** 1997. Ectopic expression of Hel-N1, an RNA-binding protein, increases glucose transporter (GLUT1) expression in 3T3-L1 adipocytes. Mol. Cell. Biol. **17:**954–962.
59. **Levine TD, Gao F, King PH, Andrews LG, Keene JD.** 1993. Hel-N1: an autoimmune RNA-binding protein with specificity for 3′ uridylate-rich untranslated regions of growth factor mRNAs. Mol. Cell. Biol. **13:**3494–3504.
60. **Uren PJ, Burns SC, Ruan J, Singh KK, Smith AD, Penalva LO.** 2011. Genomic analyses of the RNA-binding protein Hu antigen R (HuR) identify a complex network of target genes and novel characteristics of its binding sites. J. Biol. Chem. **286:**37063–37066. http://dx.doi.org/10.1074/jbc.C111.266882.
61. **Abdelmohsen K, Gorospe M.** 2010. Posttranscriptional regulation of cancer traits by HuR. Wiley Interdiscip. Rev. RNA **1:**214–229. http://dx.doi.org/10.1002/wrna.4.
62. **Denkert C, Koch I, von Keyserlingk N, Noske A, Niesporek S, Dietel M, Weichert W.** 2006. Expression of the ELAV-like protein HuR in human colon cancer: association with tumor stage and cyclooxygenase-2. Mod. Pathol. **19:**1261–1269. http://dx.doi.org/10.1038/modpathol.3800645.
63. **Lim SJ, Lee SH, Joo SH, Song JY, Choi SI.** 2009. Cytoplasmic expression of HuR is related to cyclooxygenase-2 expression in colon cancer. Cancer Res. Treat. **41:**87–92. http://dx.doi.org/10.4143/crt.2009.41.2.87.
64. **Lopez de Silanes I, Fan J, Yang X, Zonderman AB, Potapova O, Pizer ES, Gorospe M.** 2003. Role of the RNA-binding protein HuR in colon carcinogenesis. Oncogene **22:**7146–7154. http://dx.doi.org/10.1038/sj.onc.1206862.

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