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HuR Contributes to TRAIL Resistance by Restricting Death Receptor 4 Expression in Pancreatic Cancer Cells

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Abstract

Pancreatic ductal adenocarcinoma (PDA) is one of the most lethal cancers, in part, due to resistance to both conventional and targeted therapeutics. Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) directly induces apoptosis through engagement of cell surface Death Receptors (DR4 and DR5), and has been explored as a molecular target for cancer treatment. Clinical trials with recombinant TRAIL and DR-targeting agents, however, have failed to show overall positive outcomes. Herein, we identify a novel TRAIL resistance mechanism governed by Hu antigen R (HuR, ELAV1), a stress-response protein abundant and functional in PDA cells. Exogenous HuR overexpression in TRAIL-sensitive PDA cell lines increases TRAIL resistance whereas silencing HuR in TRAIL-resistant PDA cells, by siRNA oligo-transfection, decreases TRAIL resistance. PDA cell exposure to soluble TRAIL induces HuR translocation from the nucleus to the cytoplasm. Furthermore, it is demonstrated that HuR interacts with the 3'-untranslated region (UTR) of DR4 mRNA. Pre-treatment of PDA cells with MS-444 (Novartis), an established small molecule inhibitor of HuR, substantially increased DR4 and DR5 cell surface levels and enhanced TRAIL sensitivity, further validating HuR's role in affecting TRAIL apoptotic-resistance. NanoStringTM analyses on the transcriptome of TRAIL-exposed PDA cells identified global HuR-mediated increases in anti-apoptotic processes. Taken together, these data extend HuR's role as a key regulator of TRAIL-induced apoptosis.

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Implications—Discovery of an important new HuR-mediated TRAIL resistance mechanism suggests that tumor-targeted HuR inhibition increases sensitivity to TRAIL-based therapeutics and supports their re-evaluation as an effective treatment for PDA patients.

Keywords

Apoptosis; DR4; TRAIL; HuR and PDA

Introduction

Pancreatic ductal adenocarcinoma (PDA) is predicted to become the 2nd leading cause of cancer-related deaths in the United States by the end of this decade (1). A diagnosis of advanced stage PDA typically translates into less than a year of survival (1), attributable to late diagnosis, aggressive biology, and an inherent resistance to conventional treatments (2). Newer combinatorial chemotherapeutic regimens such as FOLFIRINOX and gemcitabine-abraxane (3), only marginally extend survival outcomes (4). Thus, the clinical and research community are in desperate pursuit of enhancing or developing effective targeted therapies.

One rationally dismissed alternative, potent therapeutic strategy is the use of a recombinant soluble form of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL; CD253), a type-II transmembrane protein that is a key player in the cancer immunosurveillance system (5). TRAIL potently and selectively triggers caspase-dependent apoptosis through activation of two TNF-related death domain (DD)-containing receptors, death receptor (DR)4 [TRAIL-receptor (R)1, CD261] and DR5 (TRAIL-R2, CD262). Functional TRAIL receptors are widely expressed by many cancers, including PDA cells (6, 7) and by triggering them, one can selectively kill pancreatic and other tumor cells, while sparing normal cells (5, 8). Unfortunately, multiple TRAIL-based pre-clinical and clinical trials have not led to positive therapeutic outcomes because most primary tumor cells acquire innate resistance to DR apoptotic signaling (7).

A number of mechanisms have been attributed to TRAIL resistance in PDA and other solid tumors, such as the aberrant overexpression of caspase inhibitors or pro-survival/anti-apoptotic proteins (9). Despite isolated reports of epigenetic silencing or gene deletion/mutation to explain TRAIL receptor loss in some cancers (10–13), these infrequent events cannot account for TRAIL receptor down regulation and resistance observed in solid tumors. Another well-documented mechanism frequently associated with TRAIL resistance in cancer is down-regulation of functional TRAIL receptor (DR4 and DR5) expression demonstrated when TRAIL sensitivity was restored by re-expression of DR4 or DR5 (14–16). To date, the mechanisms regulating DR4 expression have not been completely solved.

One possible mechanism is post-transcriptional gene regulation, which is mediated by microRNAs (miRNA) and RNA-binding proteins (RBP) that associate primarily with the 3'-untranslated region (UTR) of target mRNAs. A key regulator of post-transcriptional gene regulation is Hu antigen R (HuR, or *ELAVL1*), a member of the embryonic lethal, abnormal vision in *Drosophila*-like (ELAVL) family (17). Structurally, HuR contains three RNA recognition motifs (RRM) that selectively bind U- and AU-rich elements (AREs) within the 5'- and 3'-UTRs of select target mRNAs. HuR, a predominantly nuclear protein,

translocates to the cytoplasm upon exposure to specific stressors such as those present in the tumor microenvironment (e.g., hypoxia, nutrient deprivation) or associated with cancer treatment (e.g., chemotherapy) (6, 18–20). As with other RBPs, HuR can rapidly modulate aspects of the transcriptome by altering the mRNA half-life or rate of translation of target mRNAs, profoundly impacting protein expression, and thus, signaling pathways (21–23). In PDA and other cancers, an abnormally high level of HuR imparts a survival advantage to cancer cells under tumor-associated stress by supporting an anti-apoptotic and pro-survival gene expression network (19–21, 24). Consequently, elevated HuR levels have been linked to both PDA cell survival and poor clinicopathologic features (24–27).

Recent studies have established HuR as a promoter of several pro-tumorigenic processes, but they have not addressed HuR's role in mediating TRAIL resistance in PDA and other cancers (24). Previously, we provided the first evidence establishing HuR's role in regulating the TRAIL receptor apoptotic pathway (6). We demonstrated that HuR binds to DR5 mRNA and represses DR5 protein expression, causing a decrease in apoptosis induced by agonist anti-DR5 mAb. We also documented an inverse correlation between HuR levels and DR5 expression *in vitro* and in PDA patient samples (6). However, since DR4, and not DR5, was shown to be a much more potent trigger for TRAIL-induced apoptosis of PDA cells (8), we have herein expanded upon our previous work. We now show elevated HuR levels inversely regulate DR4 protein expression levels and thus increase TRAIL resistance in PDA.

Material and Methods

Cell culture

Human PDA-derived cell lines were cultured in Dulbecco's modified Eagle's (MiaPaCa2, CaPan1 and Panc-1) or RPMI (Su.86.86, Hs766T and BxPC3) medium (Life Technologies, Grand Island, NY, USA) (ATCC, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Gemini, West Sacramento, CA), 1% L-glutamine (Gemini) and 1% penicillin-streptomycin (Gemini). Cultures were grown at 37°C in a humidified atmosphere containing 5% carbon dioxide.

DNA constructs

HuR overexpression was achieved by transfecting MiaPaCa-2 cells with a green fluorescent protein (GFP)-tagged construct expressing full-length HuR (28), as previously described (19). Empty vector (GFP-only) was used as a control. For non-GFP constructs, the coding region of full-length human DR4 or HuR was subcloned in to the Kpn I and Not I sites of the pcDNA3.0 vector (Life Technologies) and transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Plasmids used for determining HuR binding were constructed by generating PCR fragments corresponding to either the 5' UTR (forward: 5'-CGTACCTCGAGCACTCCGAATGCGAAGTTCTG -3'; reverse: 5'-GTCTAGCGGCCGACCTGCCAGGTCAATCCAAGAAGCAG -3') or 3' UTR (forward: 5'-CGTACCTCGAGAGACTCTTTTACCAGAGGTTTCCT -3'; reverse: 5'-GTCTAGCGGCCGCAACCAAATCTTTGCATAGGTACCAA -3') of DR4 and subcloning them into the Xho I and Not I sites of psiCHECK2 (Promega, Madison, WI, USA). Cloning of the Pim-1 3' -UTR into psiCHECK2 was described (29).

siRNA transfections

Short-term knockdown of HuR, DR4 and DR5 was performed by transfecting 2×10^6 cells with 1 μ M of scrambled control, HuR-specific siRNA, DR4- or DR5-specific siRNA (Life Technologies) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. For double-knockdown siRNA transfections, we used 0.5 μ M of each siRNA. Cells are treated or analyzed, as described, 48 h after siRNA transfections.

Immunoblot analysis

A Nu-Per kit (Thermo Fisher, Scientific, Grand Island, NY) was used to perform subcellular fractionation of cell pellets. Lysates were then quantitated by Pierce BCA Protein Assay Kit (Thermo Fisher) and loaded equally onto SDS-PAGE gels. Proteins were electrotransferred onto Immobilon-P membranes (EMD Millipore, Billerica, MA) and incubated with mouse anti-HuR (Santa Cruz, Dallas, TX, USA), rabbit anti-Lamin A/C (Cell Signaling, Danvers, MA, USA), anti-DR4 (Thermo Fisher) or mouse anti- α -tubulin (Life Technologies) primary antibodies for 12 h in Odyssey blocking buffer (Licor, Lincoln, NE). The corresponding secondary antibodies were used at 1:10,000 dilutions (Santa Cruz). Immunoblots were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, model #9120). Densitometric quantification was performed using Odyssey Infrared Imaging System software to verify the amount of protein on each immunoblot. The numbers under sample bands indicate the densitometric quantification values, first normalized to their respective loading control and expressed as fold-change compared to their respective control sample.

Immunofluorescence microscopy

MiaPaCa-2 cells were plated on 4-well chamber slides at 1,000 cells per chamber and immunostained with anti-HuR Ab (Santa Cruz) for 12 hours, as previously described (18). Nuclei were visualized by staining with Hoechst 33342 nucleic acid stain for 5 min. Chamber slides were fixed, mounted and analyzed with a Zeiss LSM-510 Confocal Laser Microscope.

Cell viability assay

Cells from the experimental and control groups were seeded in triplicate at 1×10^3 cells per well in 96-well plates and treated as described. Following 5 days of incubation, cell viability was measured with a PicoGreen dsDNA assay kit (ThermoFisher Scientific), as previously described (6). Nonlinear regression plots were generated using Prism (GraphPad, La Jolla, CA, USA).

Statistical methods

Quantitative data are reported as the mean $\pm$ SD of at least three replicate samples. Group comparisons were analyzed using the Student's *t* test calculated from Prism (GraphPad). For calculation of the *P* values, parameters of a two-tailed, 95% confidence interval were used for all analyses. A *P* value of < 0.05 was considered significant.

Reverse-Transcription Real-Time Polymerase Chain Reaction analysis (RT-qPCR)

Total RNA was isolated from cells using an RNeasy Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was transcribed using MultiScribe Reverse Transcriptase (Life Technologies). Gene expression levels of HuR, DR4, DR5, and Parp-1 were determined by real-time PCR using gene-specific probe sets (ThermoFisher Scientific) and measured with TaqMan Universal PCR Master Mix (ThermoFisher Scientific) according to the manufacturer's instructions. *Renilla* luciferase and *18s* rRNA was measured using SYBR Green real-time PCR master mix (ThermoFisher Scientific) following the manufacturer instructions. Primer sets for *Renilla* luciferase (forward: 5'-TGAGGAG TTCGCTGCCTACC-3'; reverse: 5'-TGCGGACAATCTGGACGACG-3') and *18s* rRNA (forward: 5'-GTAACCCGTTGAACCCCAT-3'; reverse: 5'-CCATCCAATCGGTAGTAGCG-3') were synthesized by IDT (Coralville, IA, USA). All PCR reactions were run on an ABI 7500 machine (ThermoFisher) and data calculated using the 2deltaCt method and normalized to *18S* rRNA. Corrected data were normalized to values from untreated control siRNA-transfected samples.

Ribonucleoprotein immunoprecipitation (RNP-IP) assays

Panc-1 cells were plated and grown to ~65% confluency in T-150 flasks. For some samples, recombinant human soluble (s)TRAIL (ENZO) was added at 60 ng/mL for 1h prior to RNP-IP. RNA transcripts were immunoprecipitated from cell lysates with Protein A sepharose-bound anti-HuR Ab or non-specific IgG (MBL International, Woburn, MA), as previously described (30). The quantity of bound RNA was determined by RT-qPCR or measured using NanoString's (Seattle, WA) nCounter technology.

Flow cytometry

PDA cell lines were immunostained with either phycoerythrin (PE)- or allophycocyanin (APC)- conjugated mAb specific for human DR5 (clone DJR2-4) and DR4 (BioLegend, San Diego, CA), respectively, or with corresponding PE- or APC-conjugated isotype control Ab for 30 min on ice, washed twice and analyzed using a FACSCalibur flow cytometer (BD Biosciences, East Rutherford, NJ). Histograms and calculated relative mean fluorescence intensity (MFI) was produced using FlowJo software (Tree Star, Inc., Ashland, OR). Apoptosis was measured by incubating the cells for 30 min with CellEvent Caspase-3/7 detection reagent (ThermoFisher Scientific), according to the manufacturer's instructions, and then analyzed by flow cytometry.

mRNA expression data analysis

We followed the methods we have previously utilized for NanoString™ data analysis (31). NanoString™ data set has endogenous controls and RNA spike-ins, i.e., additional negative controls from other species purposefully added to the assay to assure that reads were not from non-specific binding. Total count normalization was performed per the manufacturer's protocol for normalizing NanoString™ data.

The normalized expression level for i^{th} mRNA in j^{th} sample was calculated as:

$$exp_{ij} = \frac{Count_{ij}}{TotalCount_j} * 100000$$

Where $Count_{ij}$ is the raw count, $TotalCount_j$ is the sum of counts for all mRNAs, positive and negative controls in j^{th} sample, and 100000 is a normalization factor. In essence, this calculation accounts for different amounts of total number of counted molecules across samples. Data were log normalized (base 2), and all mRNAs with maximum normalized expression levels < maximum expression values of all the negative controls and spike-ins per sample were removed from subsequent analysis. For each transcript, the normalized count from the IgG group was subtracted from each sample bound to HuR. The IgG subtracted data was assessed for statistical significance via a one-way ANOVA that considered treatment with sTRAIL as the independent factor, taking in to account three levels, Untreated, Low Dose sTRAIL (30 ng/mL), High Dose sTRAIL (60 ng/mL). Results were then evaluated by a *post hoc* Tukey Honest Significant Difference approach using *aov* and *TukeyHSD* functions in the core *stats* package of the R platform (32).

Results

Relative levels of HuR inversely correlate with DR4 expression and predict TRAIL efficacy

Previous studies indicate TRAIL sensitivity directly correlates with its receptors' levels in various cancer types (15, 16). We surveyed several PDA cell lines for TRAIL receptor surface expression, determined by flow cytometry (Supplementary Fig. S1A). To determine whether DR4 or DR5 expression can predict PDA cell sensitivity to soluble (s)TRAIL treatment, we compared the half maximal inhibitory concentration (IC_{50}), determined for each cell line from its sTRAIL killing curve (Supplementary Fig. S1B), to its corresponding DR4 or DR5 cell surface expression, expressed as mean fluorescence intensity (MFI). Plotting TRAIL receptor expression levels versus TRAIL IC_{50} values using a linear regression analysis revealed a correlation ($R^2 = 0.490$) between DR4 surface expression and TRAIL sensitivity (Fig. 1A). A much weaker correlation was observed for TRAIL IC_{50} and cell surface DR5 expression ($R^2 = 0.298$, Supplementary Fig. S1C), consistent with previously published studies (8, 15, 16).

Given HuR's established role in regulating anti-apoptotic and survival genes, we determined if there was a correlation between DR4 expression and HuR levels in the same PDA cell lines. A regression analysis comparing cytoplasmic HuR protein levels relative to α -tubulin, determined by Western blot (Supplementary Fig. S2), to cell surface DR4 expression revealed a small ($R^2 = 0.354$) negative correlation (Fig. 1B). For example, Hs766T cells had the highest level of HuR and the least amount of DR4 whereas Su.86.86 cells had low cytoplasmic HuR and high DR4 levels. To ascertain whether this negative correlation between cytoplasmic HuR and DR4 expression is meaningful in regards to TRAIL sensitivity, we next compared HuR levels to TRAIL IC_{50} values (Fig. 1C) for each cell line. This regression analysis revealed a highly correlative positive relationship ($R^2 = 0.925$) between relative cytoplasmic HuR levels and TRAIL resistance. Collectively, these data

indicate cytoplasmic HuR levels in these PDA cells may be a useful predictor of TRAIL efficacy and suggest HuR plays a role in mediating TRAIL resistance.

TRAIL treatment induces HuR cytoplasmic translocation

HuR executes its post-transcriptional gene regulation in the cytoplasm following stress-induced nuclear-to-cytoplasmic translocation and accumulation (19). Various cancer-associated stressors can induce this HuR cytoplasmic translocation including hypoxia (29), glucose deprivation (18), DNA damaging agents (33), anti-DR5 mAb (6) and gemcitabine (34). To determine whether TRAIL treatment of PDA cells can induce HuR translocation from the nucleus to the cytoplasm, we performed immunoblot analysis of cytoplasmic extracts from PDA cells treated with increasing doses of sTRAIL and discovered a more than 2-fold increase in cytoplasmic HuR levels in BxPc-3 cells treated for 3 h at 40 ng/ml of sTRAIL, compared to untreated samples (Fig. 2A). There was no significant change in nuclear HuR levels with TRAIL treatment or in the amount of total HuR in the cells (Fig. 2B). Cytoplasmic HuR accumulation was also visualized using immunofluorescence (IF) staining and confocal microscopy. HuR cytoplasmic translocation was induced in BxPc-3 cells with sTRAIL treatment (120 ng/ml) for 2 h (Fig. 2C) and the level of HuR cytoplasmic translocation was comparable to a 3 h dose of 1 μ M gemcitabine, a widely used chemotherapeutic drug for PDA (34) (Fig. 2C). These results establish that TRAIL, as with other cancer-associated stressors, can induce HuR translocation from the nucleus to the cytoplasm.

HuR post-transcriptionally binds DR4 mRNA via the 3'-UTR

The correlation between baseline HuR levels and DR4 expression in PDA cells suggested a role for HuR in the direct regulation of DR4 expression. We used a ribonucleoprotein immunoprecipitation (RNP-IP) assay to probe whether DR4 mRNA is a target of HuR in PDA cell lines. To confirm the specificity of the anti-HuR Ab used in the IP samples, an HuR immunoblot of samples taken before (pre-clear) and after (post-IP) the IP step demonstrated that only HuR was successfully immunoprecipitated in the HuR Ab post-IP lysates, whereas the control IgG had no detectable bands for HuR or α -tubulin (Fig. 3A). RT-qPCR of RNA isolated from anti-HuR antibody RNP-IP samples prepared from Panc-1 cells contained 11-fold more DR4 mRNA than RNA extracted from control IgG RNP-IP samples, demonstrating anti-HuR specificity and an association of HuR with DR4 mRNA (Fig. 3B). Panc-1 cells treated for 1 h with sTRAIL resulted in a 19-fold increase in HuR-specific binding to DR4 mRNA versus control, suggesting TRAIL-induced cytoplasmic translocation of HuR increased this HuR-DR4 mRNA association. As a positive control, we probed for DR5 mRNA, a validated HuR target (6), and showed 14-fold more DR5 mRNA bound to HuR than control IgG, which increased further with sTRAIL treatment. As a negative control, HuR did not bind to PARP-1 mRNA, a target mRNA transcript which lacks HuR target sequences (Fig. 3B) (unpublished, Chand, S.N. et. al.).

Inspection of the DR4 mRNA sequence indicates the presence of multiple AU-rich elements containing HuR motifs within the 3'-UTR with potential for HuR binding (35). RBP association determined from RIP-Chip analysis by the ENCODE/SUNY project and hosted in the UCSC genome browser and database (<http://genome.ucsc.edu/>) also predicted HuR

binding sites within the DR4 mRNA. To authenticate a direct association between DR4 mRNA and HuR, and to localize the site of interaction, constructs containing the 5' - or 3' - UTR of DR4 subcloned immediately upstream or downstream, respectively, of the *Renilla* reporter gene of psiCHECK2 vector. These reporter constructs were then transfected into Panc-1 cells, as previously described (6, 29, 33). We determined by RT-qPCR that the total input RNA for the RNP-IP contained equivalent DR4 and PIM1 sequences (Supplemental Fig. S3) and the validation of the RNP-IP samples for HuR was confirmed by immunoblot analyses (Fig. 3C). RT-qPCR of RNA extracted from anti-HuR RNP-IP samples from Panc-1 transfectants revealed an association between HuR and the DR4 3' -UTR sequence relative to IgG IP samples or empty vector controls (Fig. 3D). HuR binding to the DR4 3' -UTR was 9-fold greater than a similarly constructed vector containing the HuR binding sequences of the PIM1 3' -UTR (29).

HuR repression of DR4 protein expression causes TRAIL resistance in PDA cells

Previously we have demonstrated HuR inhibits DR5 protein expression, therefore, silencing HuR also enhances anti-DR5 antibody-mediated cell death (6). Herein we show that HuR levels inversely correlate with DR4 protein expression in PDA cell lines (see Fig. 1B), suggesting a role for HuR in DR4 protein regulation. To assess directly the effect of HuR levels on DR4 protein expression, HuR was silenced in PDA cell lines (MiaPaCa-2 and Panc-1) using HuR-specific siRNA (siHuR) and silencing of expression was confirmed using RT-qPCR to assess the level of HuR mRNA relative to control siRNA (siCtrl) in treated cells (Supplemental Fig. 4A). HuR silencing substantially increased the cell surface expression of DR4 protein levels, measured 48 h after siRNA knockdown by flow cytometry (Fig. 4A). The gain in DR4 mean fluorescence intensity (MFI) was comparable to the rise in DR5 cell surface expression for the PDA cell lines, suggesting HuR acts more broadly to repress TRAIL death receptor expression. In addition, HuR knockdown increased total cellular DR4 protein expression (Fig. 4B), indicating HuR is not merely functioning to block DR4 and DR5 trafficking to the cell surface. HuR association with target mRNA transcripts commonly results in changes in mRNA stability, but HuR has also been shown to regulate the rate of translation of some of its target mRNAs (22, 23, 36). In the MiaPaCa-2 PDA cell line, there was no difference in the steady-state DR4 mRNA levels between control and HuR siRNA treated cells after 48 h (Fig. 4C). However, in the presence of sTRAIL, HuR knockdown resulted in a substantial increase in DR4 mRNA levels by 60 minutes that did not occur in control siRNA treated cells (Fig. 4D), suggesting HuR plays a role in the downregulation of TRAIL-induced DR4 mRNA expression. Overall, these results suggest HuR functions to restrict the abundance of DR4 on the cell surface.

Having demonstrated that HuR inversely regulates DR4 protein expression and a direct correlation exists between high cytoplasmic HuR expression and TRAIL resistance (see Fig. 1C), we next addressed whether HuR could influence TRAIL sensitivity in PDA cells. HuR levels were inhibited by transfection with HuR-specific siRNA oligos 24h prior to treatment with increasing concentrations of sTRAIL. HuR silencing markedly increased TRAIL sensitivity in multiple PDA cell lines, as measured by their IC₅₀ values, when compared to control siRNA-treated cells (Fig. 4E, Supplemental Fig. 4B–D and Supplemental Table 1 and 2). For Panc-1 and Hs766T cells (Fig. 4E), HuR knockdown reduce their TRAIL IC₅₀

values by 58% and 48.6% respectively. For MiaPaCa-2 and BxPc-3 cells treated with sTRAIL (Supplemental Fig. 4A–C and Supplemental Table 2), HuR knockdown induced approximately 3-fold more cell death with than control siRNA-treated cells. To determine the relative contribution of DR4 and DR5 in HuR-mediated TRAIL sensitization, we performed siRNA double knockdown of HuR and either DR4 or DR5. TRAIL resistance increased significantly with knockdown of DR4 in both Panc-1 and Hs766T cells, and when combined with HuR silencing, DR4 knockdown completely reversed the increase in TRAIL sensitivity observed with HuR knockdown alone (Fig. 4E). In contrast, DR5 silencing caused only a minor increase in TRAIL resistance in just one of the cell lines (Panc-1) and could be completely ablated when combined with HuR knockdown. Together, these data indicate that TRAIL resistance in PDA cell lines is due in large part to HuR-regulated downregulation of DR4 expression, with very little resistance attributable to DR5 inhibition.

Elevated cytoplasmic HuR is common in pancreatic cancer patient samples relative to normal pancreatic ductal and acinar cells (19, 20). To demonstrate that HuR overexpression induces TRAIL resistance, we created stable transfectants expressing a green fluorescence protein-tagged version of full-length HuR (GFP-HuR) in a low-HuR cell line (MiaPaCa-2) (25). Transfectants expressing GFP only (GFP) were used as a control and expression of GFP-tagged and endogenous HuR was validated by GFP (Supplemental Fig. 5A) and HuR immunoblots (Supplemental Fig. 5B). HuR overexpression resulted in a significant increase in TRAIL resistance when compared to GFP only, increasing the IC₅₀ approximately 3-fold (Supplemental Fig. 5C and Supplemental Table 3). To determine whether HuR-mediated inhibition of TRAIL-induced apoptosis is primarily due to inhibition of DR4 expression, and not a result of other apoptotic pathway components, we performed DR4 rescue experiments as we previously performed with PIM1 (29). Panc-1 cells were transfected with empty vector (pcDNA3.0) or expression plasmids for HuR, the DR4 coding region, or co-transfection with both plasmids, and overexpression was confirmed with HuR and DR4 immunoblots (Fig. 4F). HuR overexpression resulted in an increase in TRAIL resistance compared to empty vector (Fig. 4G and Supplemental Table S4), whereas DR4 overexpression, whether alone or in combination with HuR overexpression, caused a significant increase in TRAIL sensitivity, suggesting DR4 overexpression supersedes HuR-dependent inhibition of apoptosis.

Inhibition of HuR stimulates TRAIL-induced apoptosis in PDA cells

To further determine the specificity of HuR's regulation of apoptosis, whether through the death receptor (extrinsic) or mitochondrial (intrinsic) pathways, we examined HuR regulation of caspase-3 cleavage, one of the key downstream effector caspases (37). In Panc-1 cells pretreated with control or HuR siRNA, apoptosis was triggered with sTRAIL or staurosporine, an established inducer of intrinsic apoptosis and HuR engagement (25, 38). HuR knockdown caused a significant increase in TRAIL-induced caspase-3 cleavage, but not in staurosporine-treated cells (Fig. 5A). Furthermore, lysates from Panc-1 cells pretreated with control or HuR siRNA and then sTRAIL were analyzed on immunoblots probed for caspase-3 or caspase-8, the pivotal DR4 initiator caspase that binds to the death-inducing signaling complex (DISC). Combining HuR knockdown with sTRAIL treatment resulted in an increase in both caspase-3 and -8 cleavage, in each case, substantially more

than was observed with sTRAIL alone (Fig. 5B). This data establishes HuR as an important regulator of signaling within the death receptor apoptotic pathway in PDA cells, and in particular, DR4.

The small molecule HuR inhibitor, MS-444, enhances TRAIL-induced apoptosis

High cytoplasmic HuR levels are common in PDA patient samples and correlate with low DR5 protein expression (6). Thus, strategies aimed at decreasing cytoplasmic HuR in PDA patients may increase the efficacy of certain treatment modalities such as sTRAIL. To test this hypothesis, we used a low molecular weight drug, MS-444 (Novartis), previously shown to block HuR dimerization within the nucleus and thus prevent its cytoplasmic trafficking (26, 39, 40), as a therapeutic approach to block cytoplasmic HuR. First, we determined the optimal biological dose (OBD) of MS-444 for each PDA cell line by treating the cells with increasing concentrations of MS-444 for 6 d and examining them for viability to determine the maximal concentration exhibiting minimal toxicity. To demonstrate its effectiveness, Panc-1 (Fig 6A), and BxPc3 (Supplemental Fig. 6) cells pre-treated with MS-444 were treated with sTRAIL or gemcitabine to induce nuclear-to-cytoplasmic translocation and examined by confocal immunofluorescence microscopy. Compared to cells treated with TRAIL or gemcitabine alone, MS-444 pre-treatment effectively blocked cytoplasmic HuR accumulation. To quantitate the potency of MS-444 blockade of TRAIL-induced HuR translocation from the nucleus to the cytoplasm, cell lysates from BxPC-3 cells were fractionated into cytoplasmic, nuclear and total lysates and probed for HuR. MS-444 pre-treatment prevented a near 2-fold increase in sTRAIL-induced cytoplasmic HuR (Fig. 6B), whereas there was no significant change in HuR levels in the nuclear (Fig. 6B) and total fractions (Fig. 6C). In addition, MS-444 treatment also reduced the baseline cytoplasmic HuR levels in these cells (Fig. 6B), indicating its potential usefulness in studies of HuR inhibition in PDA cells.

To determine the effect of MS-444 on HuR-mediated repression of DR4 protein expression, PDA cell lines (MiaPaCa-2, Panc-1, Su.86.86 and BxPc-3) were treated with MS-444 for 24 h to inhibit cytoplasmic HuR accumulation. Cells were then fluorescently-labeled with DR4 and DR5 mAb and analyzed by flow cytometry (Fig. 6D). MS-444 treatment substantially increased DR4, and to a lesser degree DR5, cell surface expression and the gain in DR4 mean fluorescence intensity (MFI) was comparable to the increase in DR4 observed with HuR siRNA knockdown (see Fig. 4A). As a consequence of increasing DR4 expression, MS-444 treatment would be expected to improve TRAIL sensitivity. Pre-treatment of BxPC-3 and Su.86.86 cells with the OBD of MS-444 for 24 h significantly increased sTRAIL-induced cell death compared to DMSO-only controls (Fig. 6E), lowering the TRAIL IC₅₀ for each cell line tested by approximately three-fold (Supplemental Table 5).

Caspase-3 cleavage, required for apoptosis in response to both intrinsic and extrinsic stimuli, is often used as a more definitive measure of apoptosis than cell death (41). Pre-treatment with 12 μ M MS-444 induced a majority (59.0 %) of Panc-1 cells treated with 60 ng/ml sTRAIL to undergo caspase-3 cleavage, compared to only 26.4% of the cells receiving sTRAIL alone, as determined by flow cytometry (Fig. 6F). Treatment of Panc-1 cells with MS-444 alone for 24 h also induced a low level of caspase-3 cleavage (18.8 %), most likely

a result of HuR's role in supporting the expression of many pro-survival genes (24). These data support the notion of an alternative anti-cancer therapeutic approach whereby HuR inhibition could be used as a potent sensitizer for TRAIL-based therapies.

Abundance of HuR-targeted transcripts is altered with sTRAIL treatment

To more broadly determine the influence that TRAIL-induced HuR activation has on apoptosis and other cancer-related pathways, we looked for changes in the amount of mRNA bound to HuR among a panel of 236 cancer-associated genes following low- and high-dose TRAIL treatment. HuR-bound mRNA was analyzed in MiaPaCa-2 cells, from 13 individual anti-HuR Ab RNP-IP samples (1 non-specific IgG, 4 untreated, 4 low dose and 4 high dose sTRAIL), using the NanoString nCounter® assay. The results revealed 83 transcripts among the 236 assayed transcripts that displayed expression levels above the background threshold (non-specific IgG). Among those, 70 transcripts (Fig. 7A, Set 2) showed greater than four-fold enrichment over background across all treatment groups (i.e., untreated, low sTRAIL and high sTRAIL). Moreover, these HuR-targeted transcripts exhibited differential enrichment along a gradient, from lowest enrichment in the untreated samples to the greatest enrichment in the high sTRAIL samples. The remaining thirteen HuR-specific transcripts (Fig. 7A, Set1) that did not exhibit at least four-fold enrichment above background did not show a significant change with TRAIL treatment. Such a monotonic enrichment of HuR-targeted mRNAs with increasing amounts of TRAIL treatment suggests that the level of HuR activation correlates with and is sensitive to the amount of TRAIL treatment.

We also analyzed the data sets for HuR targets that are differentially enriched between the treatment groups. This analysis (Fig. 7B) revealed eight transcripts that were differentially expressed in both the low- and high dose sTRAIL-treated samples when compared to the untreated samples (*Junb*, *Jun*, *Fos*, *IL-8*, *Mcl-1*, *Pcna*, *Serpine1*, and *Gas1*). Another set of six targets were significantly enriched with only high sTRAIL exposure compared to the untreated samples (*E2f3*, *Cav1*, *Fosl2*, *Pik3ca*, *Cdk6*, and *S100a4*). These results suggest that increased TRAIL stress leads to HuR-mediated stabilization of transcripts corresponding to cell proliferation and anti-apoptotic processes (21, 42). For example, *Cav1* is known to negatively regulate TRAIL-mediated apoptosis in hepatocellular carcinoma cells (43). Our results suggest that HuR targeting of *Cav1* transcript at least partly contributes to the *Cav1*-mediated counteraction of TRAIL-induced apoptosis. Two other HuR targets (*NRAS* and *Cdk4*) were differentially enriched only with low sTRAIL treatment compared to untreated samples (Fig. 7B). Focusing on apoptosis-related transcripts, four transcripts were at least four-fold enriched (Fig. 7A –Set 2) with sTRAIL compared to non-specific IgG background (*Mcl1*, *Birc5*, *Tp53* and *Casp2*) and another two (*Akt2* and *Bcl2*) were measurably enriched, but by less than four-fold (Fig. 7A –Set 1).

Discussion

The development and clinical application of TRAIL-based anti-cancer therapeutics targeting DR4 (e.g., mapatumumab), DR5 (e.g., lexatumumab), or a recombinant form of TRAIL targeting both receptors (i.e., dulanerin) have recently undergone widespread clinical investigation for PDA and other solid tumors (44). Despite their considerable promise,

TRAIL receptor-targeting agents have generally exhibited disappointing results in early phase clinical trials, with only partial responses reported when combined with other anti-cancer compounds. These disheartening results are believed to be the consequence of *de novo* apoptosis resistance mechanisms acquired during oncogenesis (45). Multiple molecular mechanisms have been linked to TRAIL-induced apoptosis resistance, but the loss of membrane DR4, and to a lesser extent DR5, expression in PDA surgical specimens is a key culprit of TRAIL-resistance in pancreatic cancer (14). In this report, we show that HuR, a pro-survival RBP overexpressed in PDA cells (20) functions to down-regulate DR4 and DR5 expression (6) enabling PDA cells to escape apoptosis induced by TRAIL.

Mechanistically, cytoplasmic HuR typically binds to ARE's within the 3'-UTR of target mRNA's and enhances gene expression by increasing mRNA stability. Conversely, several studies have shown HuR association with either the 5'-(*p27*) or 3'-UTR (*Wnt5a*, *Fas*) of target mRNA's inhibits translation, repressing gene expression (22, 23, 36). For DR4 mRNA, HuR bound to the 3'-UTR (Fig. 3), resulting in reduced DR4 protein expression (Fig. 4). These results established an association between HuR and DR4 mRNA, yet attempts to show HuR-mediated regulation of luciferase gene expression using the DR4 3'-UTR-containing psiCHECK-2 vector yielded inconclusive results (data not shown), suggesting a complex mechanism of regulation that may involve other proteins and HuR ligands (46). *c-myc* is one HuR target mRNA where the mechanism of HuR-mediated repression of translation has been further elucidated (47). In the case of *c-myc*, translational repression required HuR-mediated recruitment of the miRNA let-7 to the 3'-UTR binding site. We observed no change in the steady-state levels of DR4 mRNA following HuR knockdown, but found a rapid increase in TRAIL-induced DR4 mRNA in the absence of HuR (Fig. 4C and D). These data support the notion that HuR may restrict DR4 transcription and/or translation to inhibit gene expression.

Compared with normal cells, HuR is expressed at higher levels in tumorigenic cells (48) and accordingly by definition, higher HuR expression should be associated with a more abundant nuclear to cytoplasmic translocation. Others and us have recently demonstrated that tumor microenvironment-associated stressors, specifically glucose deprivation (18) and hypoxia (29) promote a HuR-dependent growth and survival response in PDA cells. In this report, we show that TRAIL is a rapid and potent driver of HuR translocation from the nucleus to the cytoplasm (Fig. 2). Although the precise mechanism of TRAIL-induced HuR translocation from the nucleus to the cytoplasm is unknown, TRAIL binding to its receptors recruits receptor interacting protein (RIP) kinases to the DISC, activating NF- κ B. NF- κ B then transcriptionally promotes the expression of several anti-apoptotic proteins (49, 50). Importantly, HuR was shown to be a downstream transcriptional target of NF- κ B signaling, promoting tumorigenesis in gastric cancer cells (49). Other mechanisms of TRAIL-induced HuR translocation may also be involved, such as the phosphorylation of specific serine residues embedded in the HuR protein sequence by kinases that promote cytoplasmic and/or nuclear accumulation of HuR (51).

Our discovery of HuR-regulated post-transcriptional regulation of DR4 presents a potential means to restore TRAIL sensitivity in HuR abundant resistant cells and prompted us to explore avenues of therapeutically targeting HuR in PDA and other cancers. Among the

handful of known HuR inhibitors, MS-444, a small molecule drug that interferes with the formation HuR dimers required for nuclear-to-cytoplasmic trafficking, has been found to be a potent inhibitor of cytoplasmic HuR accumulation (26). Pre-treatment of PDA cells with micro-molar amounts of MS-444 mimicked the effects we observed with siRNA knockdown of HuR. MS-444 caused blockade of TRAIL-induced HuR cytoplasmic activation (Fig. 6A – C), restored DR4 and DR5 protein expression (Fig. 6D), increased TRAIL sensitivity (Fig. 6E) and increased TRAIL-induced apoptosis (Fig. 6F). MS-444 has been used previously in animal models and has advantageous *in vivo* pharmacokinetic properties (26, 40) and thus now emerges as a useful therapeutic molecule for modulating TRAIL resistance *in vivo* via HuR inhibition. Moreover, HuR has been shown to regulate the global expression of many of the critical regulators of apoptosis and survival pathways involved in the signaling and execution of apoptosis (Fig. 7) (6, 28). Thus, therapeutic administration of a molecular-targeted inhibitor of HuR in the setting of PDA could potentially have a major clinical benefit when combined with TRAIL or DR agonists, or in combination with most other anti-cancer (e.g., chemotherapeutic) agents (6, 29, 33) whose activity relies on activation of intrinsic or extrinsic apoptotic pathways (52). Ongoing studies are exploring alternative approaches of inhibiting HuR that could easily be incorporated in a regimen that includes TRAIL (53, 54).

This study supports the notion that HuR is a key pro-survival molecule in PDA cells, or even a hub, for regulating TRAIL-induced apoptosis and other pro-tumorigenic processes (24). Our discovery of HuR-regulated repression of the TRAIL receptors' (DR4/DR5) expression levels for the first time solidifies HuR to TRAIL resistance in cancer at a critical point in the signaling pathway (i.e., receptor signaling). Clinically, tumor HuR status could be used as an indicator in real time to predict the therapeutic potential of TRAIL or other related therapies (19, 20). Specifically, TRAIL is a potent activator of HuR (Fig. 2), suggesting that pre-screening TRAIL based therapies for their effects on cytoplasmic HuR translocation may help predict outcomes. These findings may help to support the resurrection of a once-promising TRAIL-targeted strategy for PDA and others cancers (5, 14, 15).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Comparative regression analyses of surface DR4 expression, TRAIL responsiveness, and cytoplasmic HuR protein in PDA cell lines. A, regression analysis comparing flow cytometric examination of DR4 surface protein levels, expressed as MFI, to TRAIL responsiveness, expressed as IC₅₀ values, for each PDA cell line. TRAIL IC₅₀ values were determined for six PDA cell line incubated with sTRAIL for 6 d and measured for survival by PicoGreen staining. The coefficient of determination (R^2) is 0.490. B, regression analyses of cytoplasmic HuR expression for each cell line, from a representative anti-HuR immunoblot, compared to DR4 surface protein levels expressed as MFI. $R^2 = 0.354$. C, regression analysis of HuR expression compared to TRAIL IC₅₀ values for each PDA cell line. $R^2 = 0.925$ and $P = 0.001$. Corresponding TRAIL survival curves, HuR immunoblot and death receptor surface protein histograms are shown in Supplemental Fig. 1.

Figure 2.

TRAIL-mediated stress rapidly induces HuR cytoplasmic translocations. A, representative immunoblots of cell extracts from BxPc-3 cells treated with increasing amounts of sTRAIL for 3 h, separated into cytoplasmic and nuclear fractions, and probed with Ab against HuR, nuclear membrane-associated lamin A/C (used to determine fractionation purity) and α -tubulin, used as a loading control. The number under each band is the ratio of the protein above and the loading control. B, immunoblot of total cellular extracts of BxPC-3 cells treated and probed as in Fig. 2A. C, confocal immunofluorescence photomicrographs (40x) of BxPc-3 cells treated for 2 h with sTRAIL (120 ng/mL) or for 4 h with Gemcitabine (1mM) and stained with anti-HuR Ab (green) and Hoechst 33342 nucleic acid stain (blue).

Figure 3. HuR binds the 3' UTR of DR4 mRNA. A, anti-HuR immunoblot of Panc-1 cell lysates used for RNP-IP analyses (B), separated into pre- and post-IP samples. Anti- α -tubulin was used to control for the specificity of the anti-HuR Ab. B, ribonucleoprotein immunoprecipitation (RNP-IP) assay of HuR-associated mRNA from Panc-1 cells treated where indicated with sTRAIL. The amount of DR4 mRNA was determined by RT-qPCR, represented as fold-change enrichment relative to IgG isotype controls. Parp-1 was used as a negative control and DR5 was used as a positive control for HuR binding. C, anti-HuR immunoblot of Panc-1 cell lysates used for RNP-IP analyses (D), separated into pre- (PC) and post-IP samples. Staining with Ab against α -tubulin shows the specificity of the anti-HuR Ab for HuR in the IP samples. D, RNP-IP analysis of HuR-associated mRNA from Panc-1 cells individually transfected with four expression constructs, empty vector (EV; psiCHECK-2 only) or psiCHECK-2 containing the 5' -UTR region of DR4 mRNA (DR4 5' -UTR), the 3' -UTR region of DR4 mRNA (DR4 3' -UTR) or the 3' -UTR region of PIM1 mRNA (PIM1 3' -UTR), used as a positive control. EV was used as a control for non-specific binding, and the amount of bound *Renilla* luciferase mRNA was determined by qRT-PCR as in (A).

Figure 4.

HuR regulation of DR4 mRNA and protein levels alters TRAIL sensitivity in PDA cell lines. A, histogram overlays comparing DR4 or DR5 surface protein expression in PDA cell lines transfected with control (thin black line) or HuR siRNA (thick black line) 48 h prior to immunostaining. The percent change in MFI between control and HuR siRNA treated samples is indicated in the upper right corner of each histogram. An isotype matched control Ab (grey line) depicts non-specific binding. B, whole cell lysates from MiaPaCa-2 and BxPc3 cells pre-treated with control or HuR siRNA were immunoblotted and probed with Ab against HuR and DR4, or α -tubulin as a loading control. C, RT-qPCR of HuR and DR4 mRNA isolated from MiaPaCa-2 cells pre-treated with control or HuR siRNA. D, RT-qPCR of DR4 mRNA isolated from MiaPaCa-2 cells pre-treated with control or HuR siRNA and incubated with sTRAIL (75 ng/ml) for 30 or 60 min. E, TRAIL responsiveness of Panc-1 (top panel) and Hs766T (bottom panel) PDA cell lines pre-treated with indicated combinations of either control siRNA (siCtrl), HuR siRNA (siHuR), DR4 siRNA (siDR4) or DR5 siRNA (siDR5) for 24h, measuring cell viability on day 7. For Hs766T: siCtrl/siCtrl (C/C) vs. siCtrl/siHuR (C/H), $P = 0.0025$, C/C vs. siCtrl/siDR4 (C/D4), $P = 0.0071$. C/C vs. siCtrl/siDR5 (C/D5), $P = \text{n.s.}$ C/H vs. H/D4, $P = 0.0027$, C/H vs. C/D5, $P = 0.0031$. C/D4 vs. H/D4, $P = \text{n.s.}$ and C/D5 vs. H/D5, $P = 0.025$. Corresponding TRAIL IC_{50} values in

Supplemental Table S1. F, immunoblots of Panc-1 cells transfected with expression constructs expressing pcDNA3.0 only (–), full-length HuR in pcDNA3.0 (pcDNA3.0/HuR), and/or full-length DR4 in pcDNA 3.0 (pcDNA3.0/DR4), as indicated, and probed with Ab against HuR, DR4 or α -tubulin, used as a loading control. The number under each band is the normalized ratio of DR4 or HuR protein. G, TRAIL sensitivity of Panc-1 stable transfectants overexpressing full-length HuR (pcDNA3.0/HuR), DR4 (pcDNA3.0/DR4), or double transfectants expressing HuR and DR4, and compared to empty vector (EV; pcDNA3.0 only) as control. DR4 vs. DR4 and HuR, $P = \text{n.s.}$, HuR vs. HuR and DR4, $P = 0.0062$, EV vs. HuR, $P = 0.0157$, EV vs. HuR and DR4, $P < 0.05$. Corresponding TRAIL IC_{50} values in Supplemental Table S4.

Figure 5. HuR regulation of DR4 impacts TRAIL-induced apoptosis in PDA cell lines. A, representative histograms of Panc-1 cells pre-treated with control or HuR siRNA and either left untreated or incubated for 3 h with sTRAIL (60 ng/ml) or staurosporine (1nM) and then measured for cleaved caspase-3 by flow cytometry. The numbers indicate the percentage of cleaved caspase-3 positive cells. B, immunoblots of Panc-1 cells pre-treated with control or HuR siRNA and incubated with sTRAIL (60 ng/ml) for 3 h and probed with Ab against HuR and both pro- and cleaved caspase-8 or caspase-3.

Figure 6.

The drug MS-444 inhibits cytoplasmic HuR accumulation, increasing DR4 expression and TRAIL-induced apoptosis in PDA cell lines. A, confocal immunofluorescence photomicrographs (40x) of Panc-1 cells pre-treated with MS-444 (15 mM) or vehicle only (DMSO), then treated for 2 h with sTRAIL (75 ng/mL) or for 4 h with Gemcitabine (1mM) and stained with anti-HuR Ab (green) and Hoechst 33342 nucleic acid stain (blue). White arrows indicate cytoplasmic HuR. B, representative immunoblots of BxPc3 cells pre-treated with MS-444 or DMSO and incubated for 2 h with sTRAIL (120 ng/mL), separated into cytoplasmic and nuclear fractions, and probed with Ab against HuR, lamin A/C and α -tubulin. C, immunoblot of total cellular extracts of BxPc3 cells treated and probed as in Fig. 6B. D, histogram overlays comparing DR4 or DR5 surface protein expression in PDA cell lines treated with DMSO (thin black line) or MS-444 (thick black line) 24 h prior to immunostaining. The MS-444 concentration used for the MiaPaCa-2, Panc-1, Su.86.86 and BxPc-3 cells was 12, 12, 10 and 15 μ M, respectively. The percent change in MFI between DMSO and HuR siRNA treated samples is indicated in the upper right corner of each histogram. An isotype matched control Ab (grey line) depicts non-specific binding. E, TRAIL responsiveness of Su.86.86 and BxPc-3 cells pre-treated for 24 h with DMSO or MS-444, measuring cell viability on day 7. Su.86.86, $P < 0.05$ and BxPc-3, $P = 0.0083$ ($n = 3$). Corresponding TRAIL IC_{50} values in Supplemental Table S5. F, representative histograms of Panc-1 cells pre-treated with DMSO or MS-444 and then either left untreated

or incubated for 3 h with sTRAIL (60 ng/ml) and examined for cleaved caspase-3 by flow cytometry. The numbers indicate the percentage of cleaved caspase-3 positive cells.

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Figure 7.

Global analysis of cancer-associated transcripts that are potential HuR targets shows differential enrichment upon TRAIL-induced stress. A, Heatmap showing relative intensities of the differentially expressed genes in MiaPaCa-2 cells treated with low- or high-dose sTRAIL and compared to untreated cells. HuR-associated mRNAs from RNP-IP samples were identified by NanoString™ analysis. HuR-associated mRNAs above a 4-fold threshold relative to IgG RNP-IP samples revealed 70 HuR targets (Set 2) among 236 potential cancer-associated genes that were differentially expressed across all treatment groups. These are in contrast to 13 HuR-target mRNAs that did not meet the 4-fold threshold criteria and were not differentially expressed across all groups (Set 1). # indicates the HuR target transcripts associated with the apoptosis pathway. B, representation of 16 HuR target transcripts differentially expressed between treatment groups, represented as fold change relative to IgG. The top 8 graphs correspond to the transcripts enriched in both low- and high-dose TRAIL groups compared to the untreated cells at the same *p*-value, and the bottom graphs correspond to the transcripts differentially enriched between the untreated and TRAIL-treated groups. No significant enrichment was found between low and high TRAIL stress. *****P*<0.001, ****P*<0.01, ***P*<0.05, **P*<0.10 For statistical analysis, a one way ANOVA followed by a Tukey's Honest Significance Differences (HSD) test was used.