Post-transcriptional regulation of Nrf2-mRNA by the mRNA-binding proteins HuR and AUF1

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Abstract

The Nrf2-driven antioxidant response (AR) is a target of covalent drugs and bioactive native electrophiles. However, much of our understanding of AR has centered on protein-level regulation of Nrf2. Here we report a post-transcriptional modality to directly regulate Nrf2-mRNA. Our initial studies focused on the effects of the key mRNA-binding protein (mRBP) HuR on global transcriptomic changes incurred upon oxidant or electrophile stimulation. These data led us to discover a novel role of HuR in regulating Nrf2/AR, and in the process we further identified the related mRBP AUF1 as an additional novel Nrf2/AR regulator. Both mRBPs regulate AR by direct interaction with the Nrf2 transcript. Our data showed that HuR enhances Nrf2-mRNA maturation and promotes its nuclear export; whereas AUF1 stabilizes Nrf2-mRNA. Both mRBPs target the 3'–UTR of Nrf2-mRNA. Using an AR-reporter zebrafish strain, we document that this post-transcriptional control of AR is conserved at the whole-vertebrate level.

Introduction

The antioxidant response (AR) pathway is the cell's linchpin stress defense (Ma, 2013). The central player in AR is Nrf2, a transcription factor that controls the expression of numerous genes for cytoprotection and detoxification (Hayes and Dinkova-Kostova, 2014). AR is intrinsically linked to reactive electrophilic and oxidative species (RES/ROS), which are increasingly appreciated as bona fide cell signaling cues (Parvez et al., 2018). Upon stimulation with either endogenous or environmental RES/ROS, the cell mounts a stress defense through Nrf2/AR upregulation. Nrf2/AR is tightly regulated by an extensive network of proteins (Hayes and Dinkova-Kostova, 2014). Among the most notable players are Keap1 and β-TrCP, which independently maintain low steady-state levels of Nrf2 protein in non-stimulated cells through Nrf2-proteasomal targeting (Chowdhry et al., 2013; Taguchi et al., 2011). Deregulation of protein-level control of Nrf2/AR is common in various cancers (Nioi and Nguyen, 2007). Recent efforts to modulate Nrf2 function by targeting protein-based Nrf2 regulators like Keap1 have underscored the pharmaceutical relevance of AR (Copple, 2012). In addition to protein-level regulation of Nrf2/AR, some microRNAs (miRNAs) target the AR pathway either by direct Nrf2-targeting, or targeting of other regulators such as Keap1 (Cheng et al., 2013). However, expression of these miRNAs tends to be highly tissue/context-specific. A *general* regulatory mechanism (e.g., through interaction with a ubiquitously-expressed protein) of Nrf2/AR at the mRNA level has not been reported.

Regulation of mRNA is a complex process that can be mediated through structural (Strobel et al., 2016), sequence (Clery et al., 2008), or epitranscriptomic (Roundtree et al., 2017) elements within a transcript. The ubiquitously-expressed mRNA-binding protein (mRBP) HuR (ELAVL1) is a postulated stress-relevant protein that binds to AU-rich sites within regulatory mRNA targets and regulates their expression via post-transcriptional mechanisms (von Roretz et al., 2011). These binding sites typically reside within 3'–untranslated regions (UTRs) of target transcripts. However, binding within introns, coding regions, and 5'–UTRs has also been observed. Regulation of mRNA targets by HuR can occur via direct binding, or indirectly by miRNA-dependent mechanisms (Chang and Hla, 2011; Simone and Keene, 2013).

State-of-the-art sequencing techniques such as PAR-CLIP (Spitzer et al., 2014; Wang et al., 2015a) have revealed thousands of functionally-diverse targets of HuR (Lebedeva et al., 2011; Mukherjee et al., 2011). Although Nrf2 mRNA has been detected by PAR-CLIP analysis of HuR, no functional validations nor interaction studies have been made, likely because the Nrf2 transcript appears as a low-frequency hit [with only 46 T-to-C/A-to-G conversions for HuR (marking crosslinks to HuR), compared to hundreds to thousands of conversions for the most highly-ranked transcripts such as AKT3 and POLA1]. However,

PAR-CLIP rankings generally correlate poorly with HuR target affinity (Table S1); PAR-CLIP conversion number may be affected by varying expression levels of different targets or artifacts of the PAR-CLIP procedure. Accordingly, there remains a need to mechanistically investigate how HuR regulates important disease-relevant targets such as Nrf2-mRNA.

In addition to the difficulty in ranking importance of hits from high-throughput data sets, the multimodal regulatory activities of HuR render predicting functional consequences of a HuR-mRNA binding event difficult. HuR—distributed between the nucleus and cytosol in 10:1 ratio in HeLa cells (Lal et al., 2004)—largely modulates its target transcripts through alterations in mRNA-stability, typically by stabilizing bound transcripts (Brennan and Steitz, 2001). Additional regulatory mechanisms employed by HuR on its target transcripts have also been reported: e.g., nuclear export of the cyclooxygenase COX-2 mRNA (Doller et al., 2008; Fan and Steitz, 1998) and splicing regulation of the death receptor FAS and the translational regulator eIF4Enif1 (Chang et al., 2014; Izquierdo, 2008). However, the generality of these nuanced regulatory roles remains largely unclear, and their importance in Nrf2 regulation is completely unknown.

HuR is strongly linked to disease as a key player in inflammation and cancers among other disorders (Srikantan and Gorospe, 2012). The growing appreciation of HuR as a major player in disease is underscored by recent efforts to screen for inhibitors of the HuR–RNA interaction (Meisner et al., 2007; Wang et al., 2015b; Wu et al., 2015). The non-covalent inhibitors identified from such screens disrupt target-transcript binding or HuR oligomerization (key to HuR function). Some HuR inhibitors have shown promise in arresting cell cycle and inducing apoptosis in cultured lung cancer cells (Muralidharan et al., 2017). The mRNA-stabilizing ability of HuR is also affected by small-molecule stress-inducers such as the electrophilic prostaglandin A2 (PGA2) and lipopolysaccharide (LPS), which triggers oxidative stress (Cok et al., 2004; Lin et al., 2000; Wang et al., 2000; Yang et al., 2004). However, no study has directly compared the role of HuR regulation of Nrf2/AR under conditions of oxidative or electrophilic stress.

Understanding HuR regulatory circuits is further complicated by the presence of multiple mRBPs that bind to target mRNAs at the same/similar sites as HuR, but generally elicit antagonistic outputs to HuR binding. One such mRBP is AUF1 (HNRNPD), which shares significant target overlap with HuR based on PAR-CLIP analysis (Yoon et al., 2014). Mechanistically, in contrast to the canonical role of HuR in promoting mRNA stability, AUF1 typically promotes degradation of target mRNAs (Gratacos and Brewer, 2010). However, other regulatory mechanisms employed by AUF1 have recently come into focus: AUF1 can promote translation of myocyte enhancer factor MEF2C, but suppresses translation of profilin 1

(Panda et al., 2014; Yoon et al., 2014). Crosstalk between HuR and AUF1 in regulating common mRNA targets has also been investigated for the cyclin-dependent kinase inhibitor p21 and cyclin D1 (Lal et al., 2004). Stability of both of these targets was regulated positively by HuR and negatively by AUF1. However, general understanding of these co-regulatory events by these two mRBPs remains limited. Whether HuR and AUF1 bind to different sites or compete for the same sites within a specific co-regulated transcript also remains unclear (Lal et al., 2004). Nevertheless, AUF1 generally functions as the antipode of HuR in terms of target transcript regulation.

Herein, we describe novel post-transcriptional regulatory modes of the Nrf2/AR pathway, controlled by HuR and AUF1. Our initial RNA-sequencing analysis of HuR-dependent global transcriptomic changes in response to cell stimulation by H_2O_2 and 4-hydroxynonenal (HNE)—the prototypical oxidant (ROS) and electrophile (RES)—revealed that only the electrophile HNE causes significant induction of Nrf2-driven AR-genes. Surprisingly, we found that HuR depletion generally diminished Nrf2/AR activity in non-stimulated cells. Yet, upon electrophile stimulation, AR was more strongly upregulated in HuR-depleted cells. Our RNA-seq data implicate the involvement of different subsets of Nrf2-driven genes underlying these two effects. Our further investigations into HuR's regulation of Nrf2/AR in non-stimulated cells led us to discover a previously-unrecognized regulatory loop controlling AR, which our data show is conserved from cultured cells to zebrafish. This newly-identified post-transcriptional regulatory mode of AR potentially offers a novel alternative intervention to modulate Nrf2/AR in disease.

Results

HuR depletion perturbs the global transcriptomic status in electrophile-stimulated and non-stimulated cells

HuR has been implicated as a stress-responsive protein: cytoplasmic translocation of HuR is promoted by several small-molecule stressors, e.g., H_2O_2, arsenite, and the cyclopentenone prostaglandin A2 (PGA2), a Michael acceptor RES (Wang et al., 2000; Yang et al., 2004). Treatment with PGA2 also increases the affinity of HuR for p21 mRNA (Wang et al., 2000). Intrigued by reports of the stress-relevance of HuR, we launched gene-expression profiling studies in HuR-knockdown cells, in the presence or absence of small-molecule stress signals. HNE—a native RES with a reactive core chemically similar to that of PGA2—was selected as a representative bioactive RES (Jacobs and Marnett, 2009; Schopfer et al., 2011) and H_2O_2 was selected as a representative ROS. HuR-knockdown HEK293T cells were first generated (Table S2). Relative to a non-targeted shControl, HuR was knocked down by 70% in

these cells (Figure S1A). We then treated these shControl and shHuR cells with HNE (25 µM, 18 h) or H_2O_2 (225 µM, 18 h) and subsequently sequenced their RNA, post ribosomal RNA (rRNA) depletion. The chosen concentrations of HNE/H_2O_2 correspond to the approximate EC_{75} for growth inhibition, measured over 48 h (Figure S2). Significant differential expression was evaluated with CuffDiff (Trapnell et al., 2013), wherein gene-level pairwise comparisons having q-value (p-value corrected for multiple tests) < 0.05 are considered significantly differentially expressed (SDE).

Compared to non-treated control, HNE-stimulated shControl cells showed considerable upregulation of 11 genes out of 14 total genes SDE (79%). The data skewed in the positive direction overall, indicating a general stimulation of gene transcription [Figures 1A (data in blue) and S3; Tables S3 and S4]. In contrast, HNE-treated shHuR cells (compared to non-stimulated shHuR cells) showed upregulation of only 58% of the 12 genes SDE, and the dataset skewed in the negative direction overall [Figures 1A (data in red) and S3; Tables S3 and S4]. Thus, depletion of HuR compromises the ability of cells to mount positive transcriptional responses following electrophile stimulation. By contrast, neither shHuR nor shControl cells mounted as robust a response to H_2O_2 as to HNE. Compared to the number of genes SDE in HNE-stimulated shControl and shHuR cells (14 and 12, respectively), only 2 genes were SDE in H_2O_2-treated shControl cells, and no genes were SDE in H_2O_2-treated shHuR cells (Figure 1B; Table S3). Both control and HuR-knockdown datasets with H_2O_2-treatment skewed slightly in the negative direction (Figure S3; Tables S3 and S4).

HuR is a context-specific regulator of antioxidant response

Beyond HuR-dependent effects on global transcriptional status, we compared the expression of Nrf2-driven AR-genes in shHuR and shControl cells following HNE stimulation. We identified 4 AR-genes (ME-1, TXNRD1, FTL, and HMOX1) that were upregulated in both shHuR and shControl cells treated with HNE (Figures 1A and C). Intriguingly, 3 of these genes were upregulated to a greater extent in shHuR cells than in shControl cells: ME-1, TXNRD1, and FTL, showed 1.05–1.2-fold higher for fold-change $(FC)_{(treated/untreated)}$ in shHuR cells versus that in shControl cells (Figures 1A and C). This trend contrasts with the overall downregulation of global transcriptional activity following HNE-stimulation in shHuR- compared to in shControl-lines (Figures 1A and S3). We did not observe significant differential expression of these genes between *non-stimulated* shHuR and shControl cells (Figure 1D and Table S3). However, we identified two other Nrf2-driven AR-genes (SLC2A3 and MGST1), each downregulated by about 55% in shHuR cells relative to shControl cells under non-treated conditions (Figure 1D and Table

S3). These data give good initial evidence that HuR is a context-dependent regulator of Nrf2/AR, modulating different subsets of AR genes in electrophile-stimulated vs. non-stimulated conditions.

HuR-depleted cells manifest altered HNE-promoted antioxidant responsivity

These interesting findings led us to examine HuR-dependent regulation of Nrf2/AR in greater detail. Specifically, we examined the extent to which cellular AR-responsivity to HNE is HuR-dependent. AR was measured using a luciferase reporter under the transcriptional control of a Nrf2-activatable promoter, normalized to a constitutively expressed *Renilla* luciferase control (Figure 1E). Interestingly, shHuR cells under non-RES-stimulated conditions consistently featured a significant (two-fold) suppression of AR compared to shControl cells (Figure 1F), consistent with suppression of some AR-genes revealed by our RNA-sequencing analysis. Upon ectopic expression of HuR (optimized to give only ~2.5-fold above endogenous HuR levels, Figure S4) in these shHuR cells, normal basal AR levels were restored (Figure 1G).

Whole-cell stimulation with HNE (25 µM; 18 h) resulted in 2-fold higher levels of AR in HuR-depleted cells compared to shControl cells (Figure 1H). Under the same treatment conditions, ectopic expression of HuR in shHuR cells restored the extent of HNE-stimulated AR-upregulation to that observed in shControl cells (Figure 1I). Thus, the suppression of AR in non-stimulated shHuR cells, as well as their greater HNE-induced AR upregulation observed in both our reporter assay (Figures 1F and H) and our RNA-seq data (Figures 1A and D) are HuR-specific effects.

We validated these findings in mouse embryonic endothelial cells. When these cells were depleted of HuR (Figure S5A), they featured suppressed AR in the non-stimulated state (Figure S5B), and greater AR upregulation following HNE-stimulation [25µM; 18 h (Figure S5C)]. Consistent with our RNA-seq data, no significant difference was observed between shHuR and shControl HEK293T cells following H_2O_2 stimulation (Figure 1J).

HuR modulation of antioxidant responsivity is Nrf2-dependent

To confirm that the HuR-associated HNE-responsive effects observed above are Nrf2-dependent, we used siRNA (Figure S1B and Table S5) to simultaneously knockdown HuR and Nrf2. Multiple Nrf2 siRNAs led to ~40% suppression of AR on average (Figure S6A), validating the ability of our reporter assay to measure changes in Nrf2-driven AR activity. This result is also consistent with the generally narrow

dynamic range of AR assays as we explain further below. Knockdown of either HuR or Nrf2 in non-stimulated cells suppressed AR levels by about 50% compared to control cells; simultaneous knockdown of HuR and Nrf2 did not lead to any further suppression (Figure S6B). Upon HNE treatment, Nrf2 depletion completely arrested the ability of cells to mount an AR, in the presence or absence of simultaneous HuR-knockdown (Figure S6C). Thus, the effects of HuR knockdown on AR measured above are Nrf2-dependent.

Nrf2-dependence of HuR-regulation of AR is an important finding, especially given the emerging success and promise in therapeutic targeting of Nrf2-dependent pathways (Cuadrado et al., 2019). However, the multifaceted regulatory modalities of Nrf2 remain poorly understood, and Nrf2-regulation at the post-transcriptional level remains an untapped arena. Thus, we chose to delve deeper—through the following series of experiments—to understand this novel post-transcriptional mechanism of Nrf2-mRNA regulation by HuR in non-stimulated cells.

HuR and AUF1 co-regulate AR in non-stimulated cells

Considering that AUF1 is generally considered to be the antipode of HuR in regulating target transcripts, and that AUF1 binds to similar AU-rich elements within target transcripts as HuR, we probed the effects of both HuR and AUF1 on AR. We generated multiple HEK293T shAUF1 lines [each expressing a single shRNA-sequence targeting AUF1 mRNA (Table S2)] to deplete AUF1 levels. Because there are four isoforms of human AUF1 ($AUF1^{p37}$, $AUF1^{p40}$, $AUF1^{p42}$, and $AUF1^{p45}$), shRNAs targeting sequences conserved across all the isoforms were used (Table S2). Relative to a non-targeted shControl, total AUF1 was knocked down by 50–75% (Figure S1C). Because AUF1 typically destabilizes target transcripts, we expected that AUF1-knockdown would promote AR (Brennan and Steitz, 2001; Gratacos and Brewer, 2010). Contrary to this prediction, knockdown of AUF1 suppressed AR by 50-60% (Figure 2A).

We investigated the effects of simultaneous knockdown of HuR, using the siRNAs from above, and AUF1, achieved by shRNA. Cells transfected with siHuR alone featured 40–50% suppression in AR (Figure 2B) relative to cells transfected with siControl. Fold AR suppression upon HuR knockdown in shAUF1 (1) cells (30% on average) was not significantly different from fold suppression of AR upon HuR knockdown in shControl cells (Figures 2C and S7A). However, increased suppression of AR [relative to that observed in shAUF1 (1) and shControl cells] occurred in shAUF1 (2) (Figures 2C and S7A). These observations likely indicate that HuR- and AUF1-regulation of Nrf2/AR function through independent mechanisms.

However, because Nrf2/AR has multiple upstream effectors in addition to HuR/AUF1, we further address the issue of functional (in)dependence more directly below.

Modulation of AR by HuR and AUF1 is relevant at the organismal level

To extend the relevance of our findings, we turned to zebrafish (*Danio rerio*). HuR and AUF1 are both conserved in zebrafish (zebrafish gene names *elavl1a* and *hnrnpd*, respectively; we refer to these genes below as zHur and zAuf1, respectively, for clarity). The key regulators of AR are also conserved in zebrafish (Ma, 2013). Using an established AR-reporter strain, *Tg(gstp1:GFP)* (Tsujita et al., 2011), we used antisense morpholino oligonucleotides (MOs) to deplete zHur and zAuf1 in developing embryos (Table S6). An MO blocking translation initiation of zHur (Li et al., 2014) successfully depleted zHur levels at 24 hours post-fertilization (hpf) by ~25% (Figure S1D). This is likely an underestimate of knockdown efficiency given that the commercially-available HuR antibody detects other Hu-family proteins *not* targeted by this MO. zAuf1 has only two isoforms. We designed two MOs to deplete zAuf1: one to block translation initiation (ATG-MO), and one to block a splice site (SPL-MO). Interestingly, each MO depleted only one isoform of zAuf1 (Figure S1E).

Larvae heterozygotic for the AR reporter were injected with MOs at the single-cell stage. 24-hours following MO-injection, we used immunofluorescent (IF) staining to assess GFP protein levels. Knockdown of either zHur or zAuf1 (by either MO for zAuf1) led to a significant 10–15% decrease in reporter protein fluorescence (Figure 2D and E). Although the magnitude of this suppression is modest, the dynamic range of AR responsivity in these fish (like many other systems) is narrow: knockdown of *nfe2l2a* [the zebrafish Nrf2 homolog which drives AR (Kobayashi et al., 2009)] led to only a 20%-suppression of GFP levels in these fish (Figure S8). Therefore, these data are consistent our initial cell-based data (Figures 2A and B) and confirm that AR suppression promoted by depleted HuR or AUF1 is functionally relevant in a whole vertebrate animal.

HuR and AUF1 bind directly to Nrf2-mRNA in cells

We next confirmed that HuR/AUF1 and Nrf2-mRNA interact directly. RIP experiments were performed using HEK293T cells ectopically expressing either Flag-HuR or Flag-AUF1 isoforms. qRT-PCR analysis of eluted mRNA revealed that Nrf2 mRNA co-eluted with both HuR and all AUF1 isoforms (Figure 3A and B), confirming that both HuR and AUF1 bind directly to Nrf2-mRNA in cells.

HuR and AUF1 bind common sites in the Nrf2 3'–UTR with nanomolar affinities

The interaction of HuR and AUF1 with Nrf2-mRNA was further characterized *in vitro* by electrophoretic mobility gel shift assay (EMSA). Since HuR and AUF1 most commonly bind to sites within UTRs of target transcripts, three candidate binding sites within the 3'–UTR of Nrf2-mRNA were selected (Figure S9A and Table S7) based on reported consensus binding motifs of HuR and AUF1 (Barker et al., 2012; Yoon et al., 2014). Both HuR and AUF1^{p37} bound most of these sites with nanomolar affinity (Figure S9B–C; Table 1). With the exception of Nrf2 site-2, which showed negligible binding to HuR. These affinities are on par with those previously reported for HuR (Sengupta et al., 2003) and AUF1 (DeMaria et al., 1997) to their target mRNA-binding sites, implying that the interactions with Nrf2-transcript we characterized are physiologically relevant (Table S1).

Regulation depends on the 3'–UTR of Nrf2-mRNA

At this juncture, we hypothesized that suppression of AR in HuR-deficient cultured cells and larval fish stems at least in part from loss of regulation at the 3'–UTR of Nrf2-mRNA. To test this hypothesis, we utilized a luciferase reporter in which the 3'–UTR of Nrf2 is fused to a firefly luciferase transcript (Yang et al., 2011). A constitutively expressed *Renilla* luciferase is used as an internal normalization control (Figure 3C).

Cells deficient of HuR showed significantly reduced luciferase activity (30–60%) compared to controls (Figures 3D). We found a similar degree (40–50%) of reporter suppression in shAUF1 cells (Figure 3E). The magnitude of these effects on the 3'–UTR reporter (~50%) is similar to the extent of AR suppression we observed in both HuR- (~50%) and AUF1-knockdown cells (~60%; Figures 2A and B). Thus, regulation of the Nrf2 3'–UTR makes a significant contribution to the mechanism through which knockdown of AUF1 and HuR affects AR. Importantly, these findings further substantiate that the effects of HuR/AUF1-knockdown on Nrf2/AR occur specifically at the Nrf2-mRNA-level, ruling out effects of HuR/AUF1 on protein-level regulation of Nrf2.

Simultaneous knockdown of AUF1 and HuR resulted in a further decrease in luciferase activity relative to AUF1-knockdown alone (Figure 3F). Specifically, HuR knockdown suppressed 3'–UTR reporter levels by ~45% regardless of the presence or absence of AUF1 (Figure S7B). Because this assay is a more direct readout of Nrf2-mRNA regulation by HuR, than our AR-reporter assay above (Figure 2C and S7A), we conclude that HuR and AUF1 act independently on Nrf2-mRNA.

Nrf2-mRNA stability is reduced by AUF1 knockdown, but not HuR knockdown

Having established that HuR and AUF1 bind directly to Nrf2-mRNA and modulate AR, we sought to understand the specific mechanisms underlying this novel regulatory event. A common mode of HuR/AUF1 regulation involves stabilization or destabilization of the target upon binding (Brennan and Steitz, 2001).

We began by considering that HuR positively regulates Nrf2 transcript stability. Levels of endogenous, mature Nrf2-mRNA were reduced in shHuR cells (20–25% relative to shControl cells Figure S10A). mRNA levels of the 3'–UTR reporter transcripts were also significantly suppressed by 55% in siHuR (2)-treated cells (Figure S10B), which featured a stronger suppression in the luciferase reporter assay than siHuR (1) (Figure 3D). We expected that the half-life of Nrf2-mRNA in these cells would be similarly reduced. However, endogenous mature Nrf2 mRNA showed a non-significant (20% with 25% error) difference in half-life between shHuR and shControl cells [$t_{1/2}$ = 98 (k = 0.007 ± 0.002 min^{-1}), and 112 min h (k = 0.006 ± 0.0005 min^{-1}), respectively (Figure S10C), which, regardless of statistical significance, is not sufficient in magnitude to explain the two-fold AR suppression we consistently observed above.

Recent transcriptome-wide analysis of the stability of AUF1-targeted transcripts revealed the ability of AUF1 to positively regulate about 25% of target transcripts. Because our data showed that AR is suppressed in shAUF1 cells, we hypothesized that the stability of Nrf2-mRNA would be reduced in these cells. Consistent with this hypothesis, the half-life of Nrf2-mRNA was reduced by 20–60% upon knockdown of AUF1 (Figure S10D). These data also substantiate that our half-life measurements are sufficiently robust to reliably measure 2-fold changes in the stability of Nrf2-mRNA. The average suppression in half-life (50%) in shAUF1 cells is sufficient to explain the majority of the effect of AUF1-knockdown on AR. Thus, AUF1 *positively* regulates Nrf2/AR by stabilizing Nrf2-mRNA. Collectively, the mechanistic differences between the effect of AUF1 and HuR (which we elaborate on below) further point to independent effects of HuR and AUF1 on AR.

HuR enhances Nrf2-transcript splicing

Having ruled out significant effects on Nrf2-mRNA stability upon HuR knockdown, we investigated other potential means by which HuR regulates Nrf2/AR. In addition to its classical role in stabilizing target mRNAs, HuR also regulates maturation of some of its targets (Izquierdo, 2008). We reasoned that

modulations in Nrf2-mRNA processing (e.g. splicing, translocation) could lead to decreased levels of mature Nrf2-mRNA and contribute to the suppression in AR we observed. Importantly, premature Nrf2-mRNA does not contribute to the pool of functional Nrf2-mRNA (competent for translation into Nrf2-protein), because it is not exported from the nucleus and cannot be translated to give a functional protein (Figure S11A).

We first turned to our RNA-seq data for evidence of mis-regulation of maturation. Because we prepared our RNA-sequencing library using rRNA depletion and deep-sequenced the resulting library, we were able to examine the levels of intronic RNA present in the Nrf2 transcript. Nrf2-mRNA is composed of 5 exons with 4 intervening introns. The first intron of Nrf2 is extremely long at ~30,000 bp (Figure S11B), putting it in the longest 10% of human introns (Sakharkar et al., 2004). When we examined the levels of intronic RNA in the Nrf2-transcript in shHuR and shControl cells, we found that Nrf2 transcripts in shHuR cells contained higher levels of intronic content (Figure 4A). Thus, knockdown of HuR *suppresses* the maturation of Nrf2-mRNA, leading to an accumulation of unspliced, premature Nrf2-mRNA.

To probe this effect further, we utilized a firefly luciferase reporter with either an exon–intron–exon ("intron reporter") sequence or an exon–exon ("intron-less reporter") sequence from Nrf2-mRNA fused upstream of the luciferase reporter. The Nrf2 3'–UTR was also retained downstream of the luciferase reporter. Reporters were constructed containing 3 different Nrf2 exon pairs, with or without the intervening introns (Figure 4B). Regulation of these mRNA reporters can be assessed by measuring firefly luciferase enzyme activity in lysates of cells expressing these reporters (normalized to a constitutively expressed *Renilla* luciferase control). Because of the length of the 1st intron of Nrf2 (Figure S11B), this intron was not investigated in this assay.

Expression of the intron-less reporters in shHuR cells led to a 50% decrease in reporter activity relative to shControl cells (Figure 4C), consistent with our 3'–UTR luciferase reporter data above (Figure 3D). Because this effect (~50% reduction in shHuR cells) was similar across all three reporters containing different exons, it is likely attributable to the 3'–UTR (the common factor between the three reporters). However, two of the intron-containing reporters showed a further suppression upon HuR knockdown relative to their intron-less counterparts (Figure 4C, inset at right), consistent with our RNA seq data (Figure 4A). The greatest suppression effects were observed for introns 2 and 3, whereas intron 4 was not strongly suppressed in shHuR cells in either the RNA-seq or the reporter data.

Overall, these data agree with PAR-CLIP results indicating nucleotide conversions (i.e., HuR binding) in introns, as well as the presence of multiple putative binding sites in the introns based on consensus HuR-binding sequences (Lebedeva et al., 2011). These data collectively indicate that HuR enhances the splicing and maturation of Nrf2-mRNA. The magnitude of this effect is on par to the suppression of endogenous Nrf2-mRNA levels we observed above.

HuR regulates nuclear export of Nrf2-mRNA

Nuclear export of mature mRNA is intrinsically linked to the splicing/maturation process because maturation is a prerequisite for export. Coupling of these processes is an appreciated mechanism to enhance gene expression (Valencia et al., 2008). mRNA that is not exported due to improper or incomplete splicing is rapidly degraded in the nucleus (Moore, 2002). Given the ability of HuR to shuttle between the nucleus and cytosol (Fan and Steitz, 1998), we hypothesized that HuR governs Nrf2-mRNA export from the nucleus. Indeed, for some mRNA targets such as COX-2, HuR plays a key role in regulating export from the nucleus (Doller et al., 2008). To test whether a similar regulatory program is at play for Nrf2-mRNA, we employed nuclear/cytosolic fractionation in shHuR/shControl cells coupled with qPCR detection of endogenous, mature Nrf2-mRNA. These experiments revealed that the cytosolic/nuclear ratio of endogenous Nrf2-mRNA decreased by 35% on average in HuR-deficient cells (Figure 4D), indicating a *decrease in Nrf2-mRNA nuclear export* upon depletion of HuR. Faster turnover of nuclear-mRNA may contribute to the reduction in overall levels of Nrf2-mRNA upon HuR-knockdown (Figure S10A) and to the slight reduction in Nrf2-mRNA half-life we observed (Figure S10C). In sum, we conclude that the principal mechanisms by which HuR regulates Nrf2-mRNA are control of Nrf2-mRNA splicing/maturation and nuclear export of the mature transcript. Importantly, these results collectively point to a novel regulatory program that functions specifically at the Nrf2-mRNA level to modulate AR (Figure 4E).

Discussion

We began by exploring HuR-dependent global transcriptomic changes in cells stimulated with RES/ROS, using H_2O_2 and HNE as representative native ROS/RES. Contrary to previous reports of the sensitivity of HuR to oxidative stress(Mehta et al., 2016), we found no transcriptional responses specifically attributable to HuR following H_2O_2 stimulation (Figure 1B). Differences in cell type may explain this observed discrepancy, as we observed a muted transcriptional response to H_2O_2 stimulation overall in

our HEK293T cell lines. Conversely, HNE-stimulation of HuR-knockdown cells elicited a general downregulation of global transcriptional activity compared to control cells (Figure 1A and S3). Given the important roles that pleiotropic covalent drugs and native RES play on AR (Hayes and Dinkova-Kostova, 2014; Parvez et al., 2018) we homed in on differentially-expressed Nrf2-driven AR genes. This analysis revealed an interesting bifurcation between HuR-dependent effects on AR in non-stimulated shHuR cells versus HNE-stimulated cells. AR genes that were more strongly upregulated in HNE-stimulated shHuR cells (ME-1, TXNRD1, and FTL) were not significantly differentially expressed in non-stimulated shHuR cells relative to shControl cells (Figures 1A and C). However, a separate subset of AR-genes (SLC2A3 and MGST1) was suppressed in non-stimulated shHuR cells (Figure 1D). Together with our AR reporter data (Figures 1F and H) which was consistent with our RNA seq analysis, these findings highlight context-specific complexities inherent in the regulation of AR by HuR. Because of these intricacies, we chose to focus our further investigations on non-stimulated cells.

For both HuR and AUF1, which we also identified as a novel regulator of Nrf2/AR, we found 2–3-fold suppression in AR in both HuR- and AUF1-knockdown cells relative to control cells (Figures 1F, 2A, 2B, and S5). These changes are indicative of significant regulatory events because we and others have previously documented the narrow dynamic range (2–4-fold) of Nrf2/AR modulation in various readouts, including luciferase AR-reporter assays, flow cytometry-based analyses, as well as qRT-PCR and western blot analyses which assay endogenous Nrf2-driven AR-genes(Huang et al., 2012; Levonen et al., 2004; Long et al., 2017; Parvez et al., 2015). Two to three-fold changes are also typical magnitudes of response in other pathways regulated by electrophiles. For instance, knockdown of the electrophile sensor Pin1 elicits only about 30% decrease in viability upon treatment with HNE relative to knockdown control (Aluise et al., 2012). Our findings that HuR knockdown-induced AR suppression extends to whole zebrafish depleted of HuR or AUF1 (Figures 2D and E), as well as to mouse endothelial cells MEECs (Figure S5B) demonstrate a broader generality that is consistent with the fact that HuR and AUF1 are ubiquitously expressed. These novel general regulatory roles of HuR/AUF1 on Nrf2 mRNA stand in contrast to previously reported Nrf2-mRNA regulatory events by miRNAs that are highly cell/context specific (Cheng et al., 2013). Thus, in terms of generality and magnitude, our study goes a long way in demonstrating that Nrf2-mRNA regulation is an important modulator of AR.

HuR and AUF1 can function in concert to destabilize mRNAs such as $p16^{INK4}$ (Chang et al., 2010), and enhance the translation of mRNAs such as TOP2A (Yoon et al., 2014), among other examples.

However, we found that HuR and AUF1 act independently on Nrf2-mRNA, despite similar binding affinities to common sites we identified within the 3'–UTR (Table 1). The distinct, orthogonal mechanisms by which HuR and AUF1 regulate Nrf2-mRNA—control of Nrf2-mRNA splicing and export; and stabilization of Nrf2-mRNA, respectively—agree with our AR data indicating independent effects of HuR and AUF1 on Nrf2/AR-axis. These effects could not have been predicted based on the previously-reported PAR-CLIP binding data alone. The complexities we uncovered in this system collectively speak to the need for careful mechanistic evaluation of "on-target" effects of HuR/AUF1-knockdown, particularly when common binding sites are involved.

As is appreciated in the mRBP field (Gratacos and Brewer, 2010; Lebedeva et al., 2011), both HuR and AUF1 regulate their targets through multiple mechanisms. Although the magnitude of the UTR-reporter suppression (Figure 3D) and nuclear-export suppression (Figure 4E) we observed in HuR-knockdown cells at first glance seem sufficient to explain the magnitude of AR-suppression, our data testify that additional regulatory modes beyond UTR-regulation are at play. For instance, using multiple approaches, we identified Nrf2-mRNA splicing as a subtle but significant component of HuR regulation of Nrf2-mRNA (Figure 4A–C). These alternate/complementary mechanisms may explain why we unexpectedly observed a reduction in the global pool of Nrf2-mRNA without a similar fold-change in the half-life of Nrf2-mRNA upon HuR-knockdown, for instance. Our mechanistic interrogations ultimately established that reduced splicing and reduced nuclear export of Nrf2-mRNA are the principal mechanisms by which AR is suppressed upon HuR-knockdown. These mechanisms are likely linked as splicing is generally a prerequisite for nuclear export (Valencia et al., 2008), although the details of this mechanistic coupling for Nrf2-mRNA remain to be explored. Nevertheless, downregulation of both splicing/maturation and nuclear export of Nrf2-mRNA upon HuR-knockdown likely explains why we observed a suppression in the pool of mature Nrf2-mRNA (Figure S10A). Overall, the mechanisms we identified strongly and clearly point to on-target, Nrf2-mRNA-specific events that support HuR/AUF1 regulation of AR.

In sum, these data highlight an important intersection between proven disease-relevant players: HuR, AUF1, and Nrf2 are all upregulated in cancers (Abdelmohsen and Gorospe, 2010; Menegon et al., 2016); therapeutic targeting of Nrf2/AR axis through exploitation of protein regulators such as Keap1 continues to be a promising small-molecule intervention (Copple, 2012; Hur and Gray, 2011). The ubiquitous expression of HuR and AUF1, along with the conservation of their regulation of Nrf2/AR

across multiple cell types and whole organisms underscore the importance of this newly identified regulatory program. Because this regulation of Nrf2/AR occurs specifically at the mRNA-level, it potentially offers an orthogonal therapeutic strategy to modulate this conserved pathway of validated pharmacological significance.

Figure Legends

Figure 1. RNA-seq expression profiling indicates context-specific HuR-regulation in global and AR-specific transcriptional activities. (**A** and **B**) Differential expression from RNA-seq analyses in shHuR or shControl HEK293T cells following HNE (25 uM, 18 h) or H_2O_2 (225 uM, 18 h) stimulation relative to respective non-stimulated cells. Genes significantly differentially expressed (SDE) are denoted with dark, opaque points; Nrf2-driven AR-genes SDE in at least one comparison are labeled. Dashed lines to gene names indicate that the gene was not SDE in that comparison. See also Figures S1A, S2, S3, and Tables S2 and S3. (**C**) Fold changes (FCs) for selected Nrf2/AR-genes of interest upon HNE stimulation relative to untreated. Q-values were calculated with CuffDiff. See also Tables S2 and S3. (**D**) Differential expression from RNA-seq analysis in non-stimulated shHuR cells relative to shControl cells. Genes SDE are denoted with blue points. Two Nrf2-driven AR genes found to be SDE are labeled. See also Tables S2 and S3. (**E**) AR reporter system in which firefly luciferase is driven by Nrf2 and a constitutive *Renilla* luciferase is co-expressed as an internal normalization control. (**F**) AR activity of non-HNE-stimulated shHuR and shControl cells (mean±SEM, n=24). (**G**) AR activity was measured as in **F**, but ectopic HuR was expressed in non-HNE-stimulated shHuR cells (mean±SEM, n=7 for shHuR+HuR and n=8 for other conditions). See also Figure S4. (**H**) AR activity in HNE (25 µM, 18 h)-stimulated shHuR and shControl (mean±SEM, n=16)]. (**I**) AR activity was measured as in **H**, but ectopic HuR was expressed in HNE(25 µM, 18 h)-stimulated shHuR cells (mean±SEM, n=8). See also Figure S4. (**J**) AR activity in H_2O_2(225 µM, 18 h)-stimulated shHuR and shControl cells (mean±SEM, n=7 for shHuR and 8 for shControl)]. All p-values were calculated with Student's T-test. For **A** and **B**, data were derived from n=2 independent biological replicates per treatment condition. Skewness was calculated with Prism.

Figure 2. Depletion of HuR or AUF1 in cells and larval zebrafish suppresses AR activity. (**A** and **B**) Basal AR activity in HEK293T cells depleted of HuR and AUF1, respectively [mean±SEM of n=8 (HuR) and n=12 (AUF1) independent replicates per condition]. See also Figures S1B and C. (**C**) AR activity upon simultaneous knockdown of HuR and AUF1 (mean±SEM of n=4 independent replicates). See also Figure S7A. (**D and E**) AR activity in *Tg(gstp1:GFP)* zebrafish upon knockdown of zHur and zAuf1. Larvae are

stained with a red fluorescent antibody because green background fluorescence at this developmental stage prevents accurate quantitation of the GFP reporter signal. *Inset:* quantitation (mean±SEM) of mean fluorescence intensity measured using the Measure tool of ImageJ [sample sizes analyzed: E: n=26 (Control MO), 44 (ATG-MO); F: n=38 (Control MO), 12 (ATG-MO), 32 (SPL-MO)]. Each point represents a single fish. p-values were calculated with Student's T-test. Scale bars, 500 µm. See also Figures S1D and E, and S8.

Figure 3. HuR and AUF1 bind directly to the 3'–UTR of Nrf2-mRNA in cells. (**A**) RNA-binding protein immunoprecipitation (RIP) was carried out by expressing flag-tagged HuR or AUF1 in HEK293T cells and subjecting lysates to Flag IP. (**B**) Nrf2-mRNA co-eluting with enriched proteins was detected with qRT-PCR (mean±SEM of n=4 independent replicates). (**C**) 3'–UTR reporter system consisting of Nrf2-mRNA fused to a firefly luciferase transcript and normalized to *Renilla* luciferase. (**D** and **E**) Knockdown of HuR and AUF1, respectively, reduces the 3'–UTR reporter activity in HEK293T cells (mean±SEM of n=8 independent replicates for each bar). (**F**) Knockdown of both proteins leads to a further suppression of the 3'–UTR reporter activity (mean±SEM of n=4 independent replicates). See also Figure S7B. All p-values were calculated with Student's t-test.

Figure 4. HuR-knockdown suppresses Nrf2-mRNA splicing and nuclear export of Nrf2-mRNA. (**A**) Integrative Genomics Viewer (Robinson et al., 2011) view of splicing tracks for Nrf2 (NFE2L2) from RNA-sequencing analysis of shHuR and shControl HEK293T cells. The area of the blue tracks corresponds to the levels of intronic RNA detected. Shown is one representative replicate per cell line. See also Figure S11. (**B**) Reporters to readout the effect of introns were constructed by fusing a portion of Nrf2-mRNA (with or without the intron) upstream of a firefly luciferase reporter. As with the 3'–UTR reporter (Figure 3C), effects on this construct can be assayed by measuring luciferase activity in the lysates of cells expressing these reporters. (**C**) Reporter levels upon HuR knockdown in non-stimulated HEK293T cells for both the intron-less reporter (left) and the intron reporter (right) (mean±SEM of n≥7 per set). *Inset at right*: Comparison of reporter activity in shHuR cells (i.e., blue bars from the main plot in **C** upon

introduction of introns. (**D**) qRT-PCR was used to measure the ratio of endogenous Nrf2-mRNA in nuclear and cytosolic extracts of HEK293T cells depleted of HuR (mean±SEM of n=8 for siHuR (1 and 2) and n=7 for siControl). (**E**) Model of posttranscriptional regulation of Nrf2-mRNA by HuR and AUF1. HuR regulates Nrf2-mRNA maturation and nuclear export, and AUF1 stabilizes Nrf2-mRNA. Shown in blue text/boxes are the experimental evidence supporting each facet of this regulatory program. All p-values were calculated with Student's t-test.

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Note. RNA-Sequencing data have been submitted to the Gene Expression Omnibus (GEO; accession number pending).

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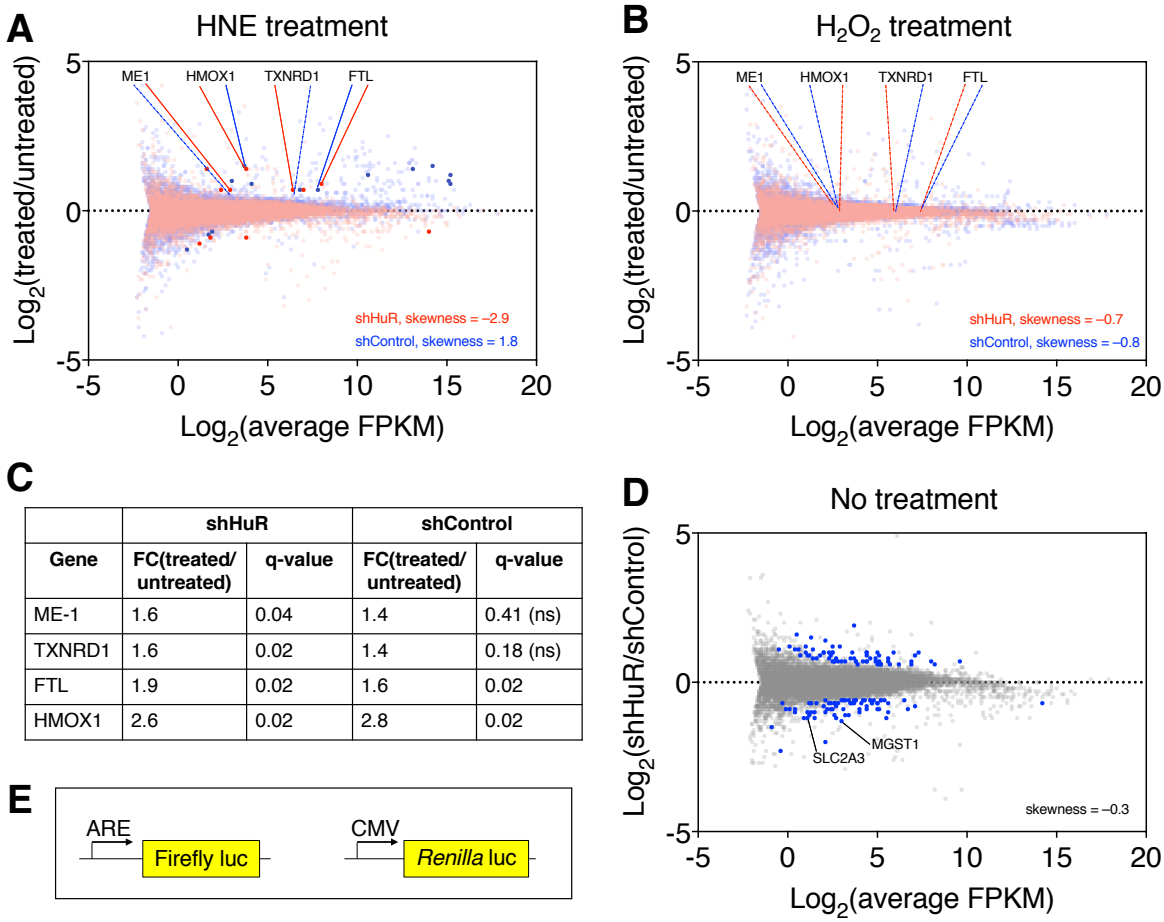
Figure 1



C

	shHuR		shControl	
Gene	FC(treated/ untreated)	q-value	FC(treated/ untreated)	q-value
ME-1	1.6	0.04	1.4	0.41 (ns)
TXNRD1	1.6	0.02	1.4	0.18 (ns)
FTL	1.9	0.02	1.6	0.02
HMOX1	2.6	0.02	2.8	0.02

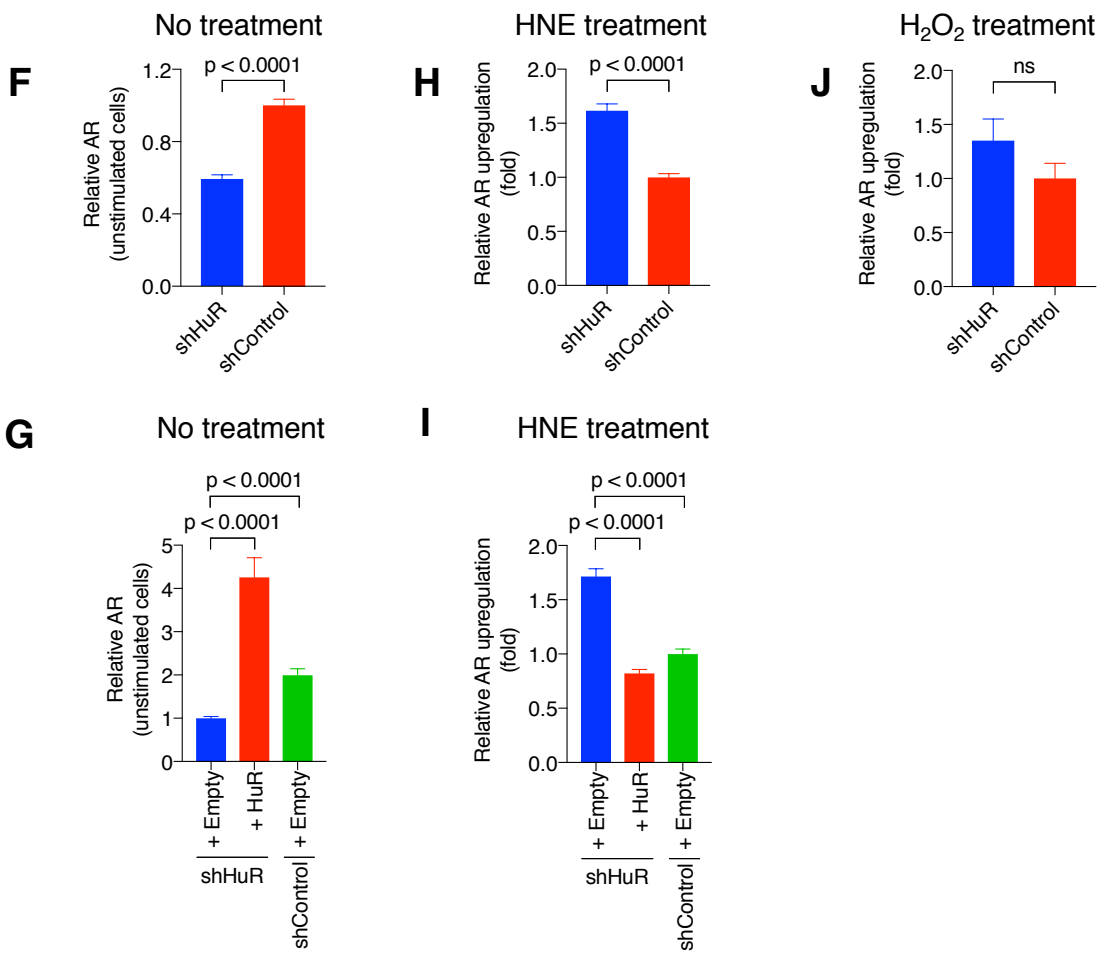


Figure 2

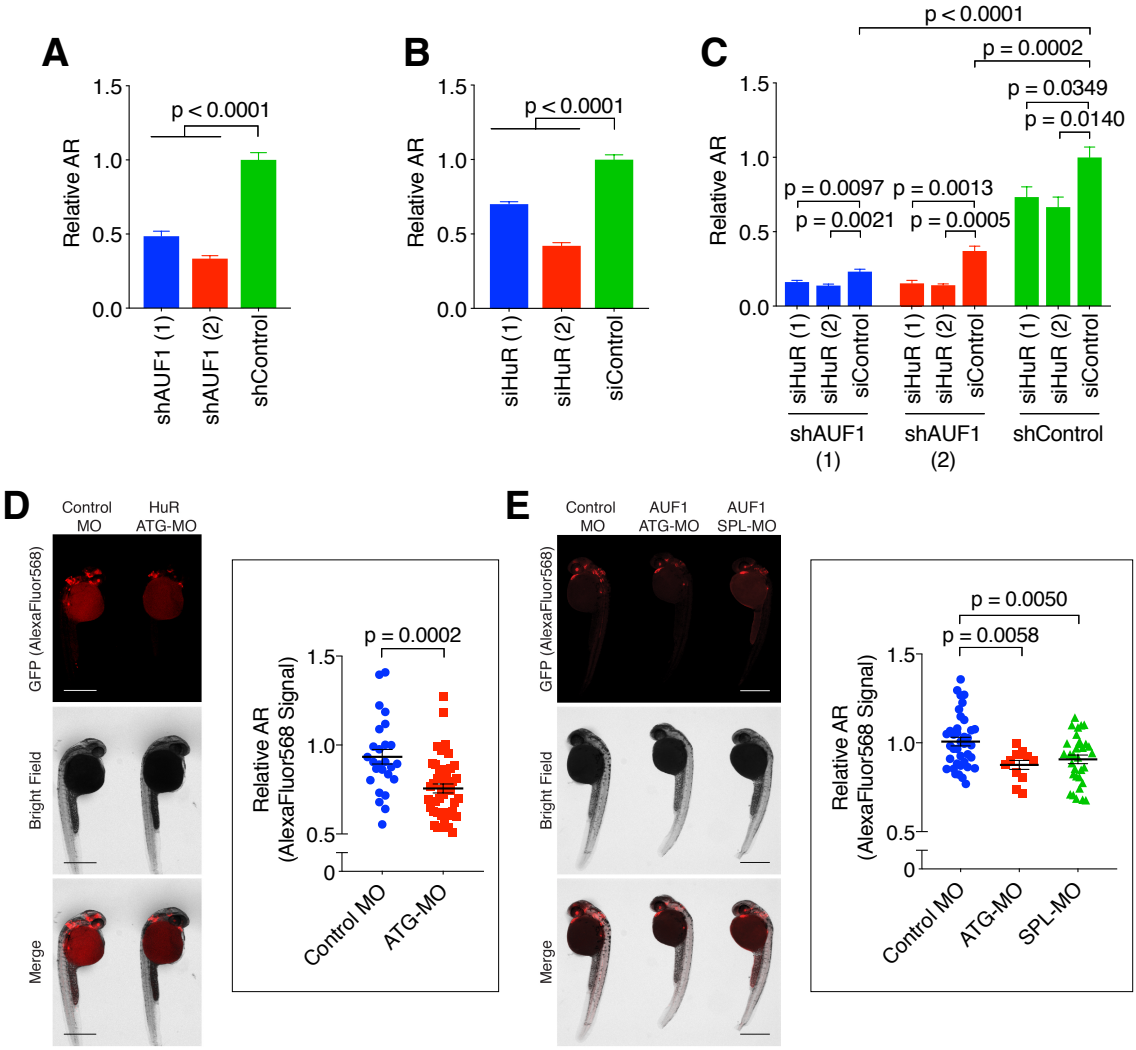

A
Relative AR
1.5
1.0
0.5
0.0
p < 0.0001
shAUF1 (1)
shAUF1 (2)
shControl
B
Relative AR
p < 0.0001
siHuR (1)
siHuR (2)
siControl
C
Relative AR
p < 0.0001
p = 0.0002
p = 0.0349
p = 0.0140
p = 0.0097
p = 0.0021
p = 0.0013
p = 0.0005
siHuR (1)
siHuR (2)
siControl
shAUF1 (1)
shAUF1 (2)
shControl
D
Control MO
HuR ATG-MO
GFP (AlexaFluor568)
Bright Field
Merge
Relative AR (AlexaFluor568 Signal)
p = 0.0002
Control MO
ATG-MO
E
Control MO
AUF1 ATG-MO
AUF1 SPL-MO
GFP (AlexaFluor568)
Bright Field
Merge
Relative AR (AlexaFluor568 Signal)
p = 0.0050
p = 0.0058
Control MO
ATG-MO
SPL-MO

Figure 3

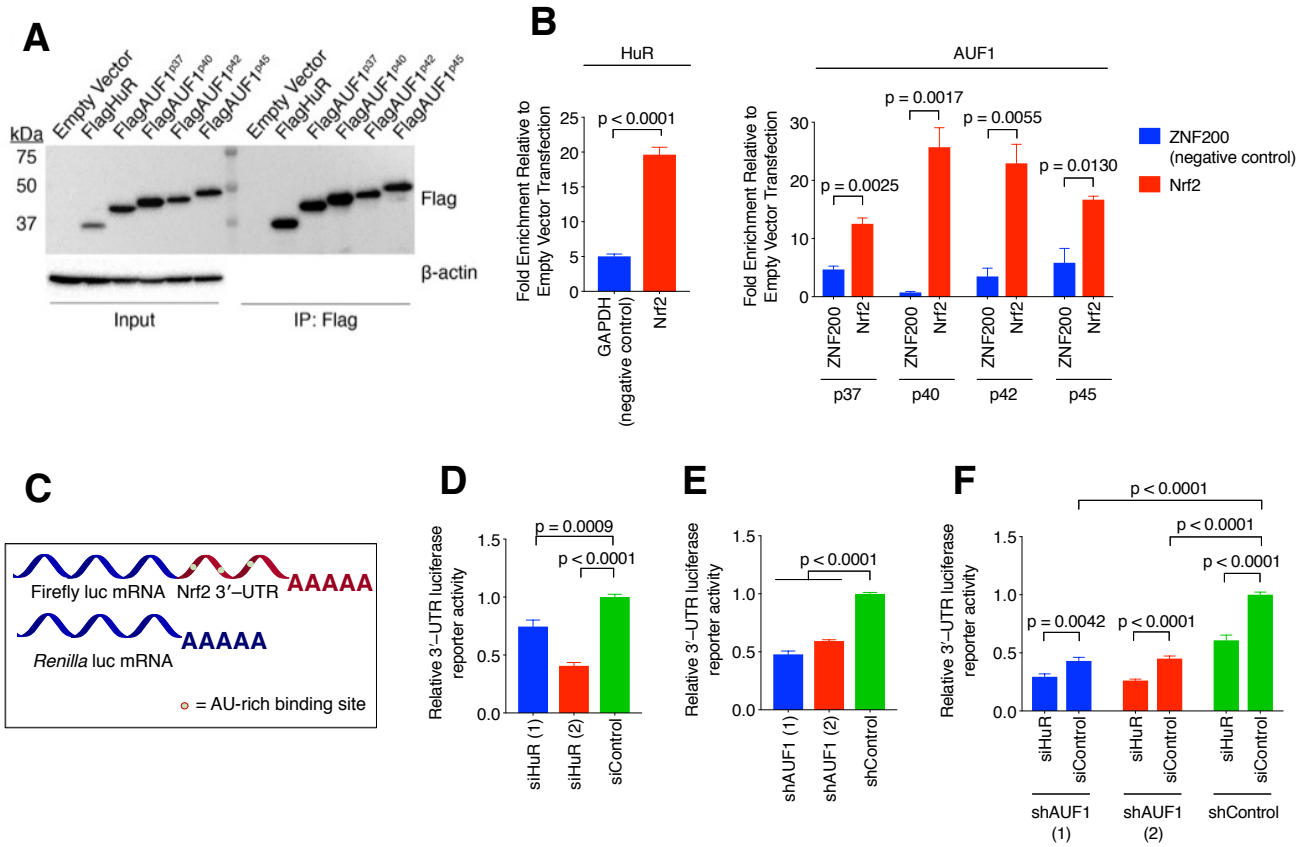

A
Empty Vector
FlagHuR
FlagAUF1p37
FlagAUF1p40
FlagAUF1p42
FlagAUF1p45
kDa
75
50
37
Flag
β-actin
Input
IP: Flag
B
HuR
AUF1
Fold Enrichment Relative to Empty Vector Transfection
p < 0.0001
GAPDH (negative control)
Nrf2
p = 0.0025
p = 0.0017
p = 0.0055
p = 0.0130
ZNF200
p37
p40
p42
p45
ZNF200 (negative control)
Nrf2
C
Firefly luc mRNA
Nrf2 3′–UTR
AAAAA
Renilla luc mRNA
= AU-rich binding site
D
Relative 3′–UTR luciferase reporter activity
p = 0.0009
p < 0.0001
siHuR (1)
siHuR (2)
siControl
E
shAUF1 (1)
shAUF1 (2)
shControl
F
p < 0.0001
p = 0.0042
siHuR
siControl
shAUF1 (1)
shAUF1 (2)
shControl

Figure 4

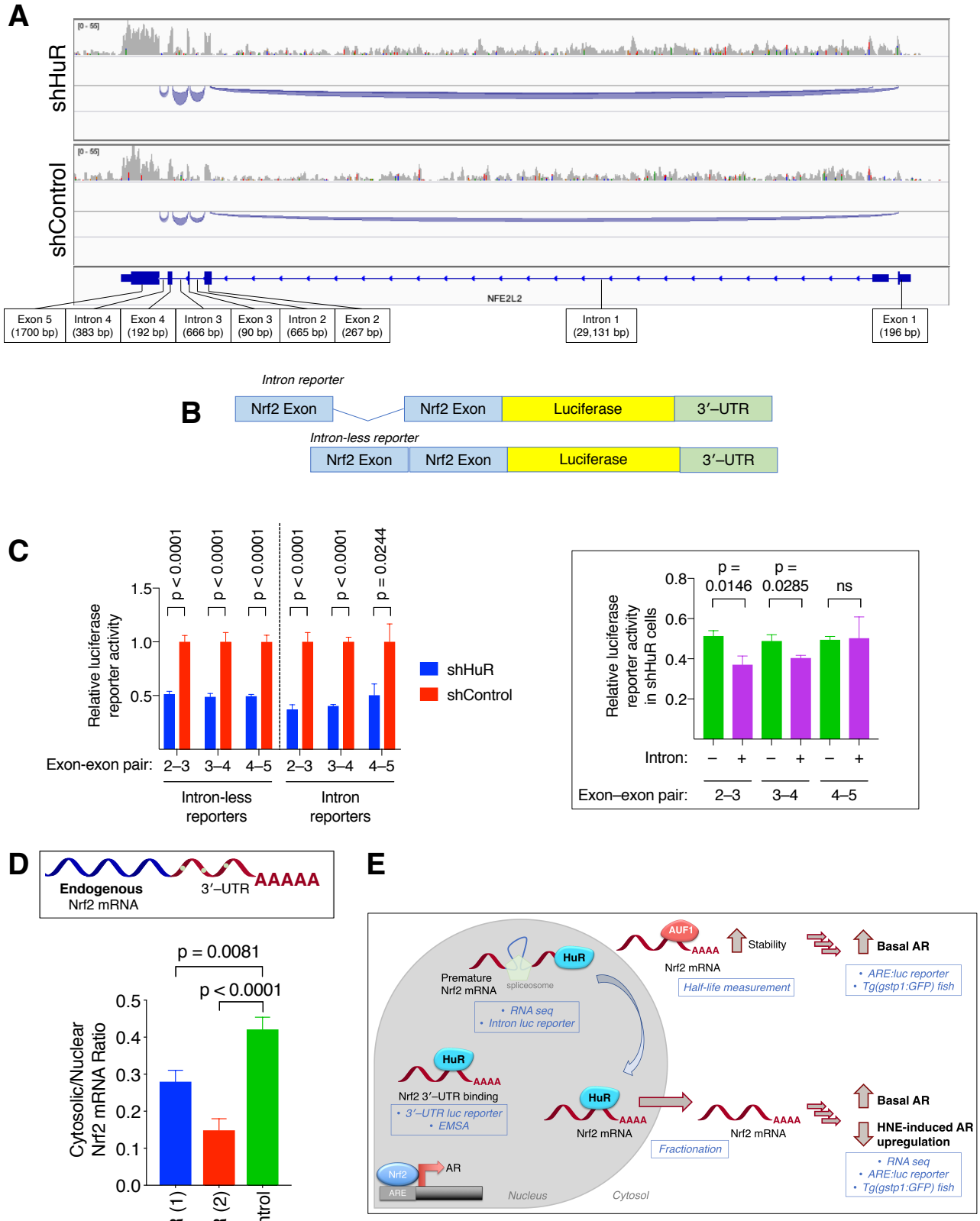

A
shHuR
shControl
NFE2L2
Exon 5 (1700 bp)
Intron 4 (383 bp)
Exon 4 (192 bp)
Intron 3 (666 bp)
Exon 3 (90 bp)
Intron 2 (665 bp)
Exon 2 (267 bp)
Intron 1 (29,131 bp)
Exon 1 (196 bp)
B
Intron reporter
Nrf2 Exon
Nrf2 Exon
Luciferase
3′–UTR
Intron-less reporter
Nrf2 Exon
Nrf2 Exon
Luciferase
3′–UTR
C
Relative luciferase reporter activity
p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001
p = 0.0244
shHuR
shControl
Exon-exon pair: 2–3 3–4 4–5 2–3 3–4 4–5
Intron-less reporters
Intron reporters
Relative luciferase reporter activity in shHuR cells
p = 0.0146
p = 0.0285
ns
Intron: – + – + – +
Exon–exon pair: 2–3 3–4 4–5
D
Endogenous Nrf2 mRNA
3′–UTR
AAAAA
p = 0.0081
p < 0.0001
Cytosolic/Nuclear Nrf2 mRNA Ratio
siHuR (1)
siHuR (2)
siControl
E
Premature Nrf2 mRNA
spliceosome
HuR
• RNA seq
• Intron luc reporter
AUF1
Nrf2 mRNA
Stability
Half-life measurement
Basal AR
• ARE:luc reporter
• Tg(gstp1:GFP) fish
Nrf2 3′–UTR binding
• 3′–UTR luc reporter
• EMSA
Nrf2 mRNA
Fractionation
Nrf2 mRNA
Basal AR
HNE-induced AR upregulation
• RNA seq
• ARE:luc reporter
• Tg(gstp1:GFP) fish
Nrf2
AR
ARE
Nucleus
Cytosol

Table 1. Dissociation constants measured for HuR and AUF1^{p37} binding to Nrf2 3′–UTR sites.

	HuR K_d (nM)	**AUF1^{p37} K_d (nM)**
Nrf2 site 1	20 ± 5	6 ± 2
Nrf2 site 2	–	25 ± 4
Nrf2 site 3	75 ± 20	670 ± 82