Krüppel –Like Factor 8 is a Stress-Responsive Transcription Factor that Regulates Expression of HuR

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HuR • KLF8 • Cellular stress • Kidney • Transcriptional regulation • Alternative promoters

Abstract
Background/Aims: HuR is an RNA-binding protein that regulates the post-transcriptional life of thousands of cellular mRNAs and promotes cell survival. HuR is expressed as two mRNA transcripts that are differentially regulated by cell stress. The goal of this study is to define factors that promote transcription of the longer alternate form. Methods: Effects of transcription factors on HuR expression were determined by inhibition or overexpression of these factors followed by competitive RT-PCR, gel mobility shift, and chromatin immunoprecipitation. Transcription factor expression patterns were identified through competitive RT-PCR and Western analysis. Stress responses were assayed in thapsigargin-treated proximal tubule cells and in ischemic rat kidney. Results: A previously described NF-κB site and a newly identified Sp/KLF factor binding site were shown to be important for transcription of the long HuR mRNA. KLF8, but not Sp1, was shown to bind this site and increase HuR mRNA levels. Cellular stress in cultured or native proximal tubule cells resulted in a rapid decrease of KLF8 levels that paralleled those of the long HuR mRNA variant. Conclusions: These results demonstrate that KLF8 can participate in regulating expression of alternate forms of HuR mRNA along with NF-κB and other factors, depending on cellular contexts.

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Introduction

HuR, a ubiquitously expressed RNA-binding protein of the embryonic lethal, altered vision (ELAV) family, exerts powerful effects on cell growth and survival through its diverse effects on thousands of eukaryotic mRNAs. HuR is best-studied for its promotion of mRNA stability, effected through binding of adenine- and uridine-rich regions in the 3’ untranslated and intronic regions of mRNAs [1, 2]. HuR has also been demonstrated to alter translation of selected transcripts [3-6], and more recently, to inhibit microRNA-mediated suppression or activation of certain target mRNAs [7, 8]. HuR binding to intronic sequences has been suggested to alter pre-mRNA splicing, although its role in this process is not well established [8, 9]. HuR’s target mRNAs include many involved in cell proliferation and survival [10], and as such, its expression is key in protecting cells from lethal damage during stress. However, when hyperactivated, these same pathways can lead to oncogenic transformation. Indeed, elevated expression of HuR has been identified in many types of tumors, and higher HuR levels have been associated with worse prognosis in multiple cancers, including breast, ovarian, lung, and colon [11].

Because HuR function must be finely tuned to create a balance between cell proliferation and survival on one hand, and oncogenic transformation on the other, its expression is tightly controlled at multiple levels [12]. At a post-transcriptional level, HuR mRNA levels are controlled by multiple regulators of mRNA stability [13, 14], including HuR itself, since its expression may be autoregulated [15, 16]. More than a half dozen microRNAs (miRNAs) have been identified as repressors of HuR translation [17-22], and ubiquitin- and caspase-mediated pathways control its degradation [23-26]. In this study, we continue to examine the regulation of HuR at a transcriptional level. Our previous studies demonstrated that HuR mRNA is expressed as two alternate transcripts that are expressed at similar levels but vary in their 5’ untranslated (5’ UTR) regions [27, 28]. The longer 5’ UTR is about 150 bases, depending on the host species, and is highly G+C-rich (~75%). Meanwhile, the shorter 5’ UTR is about 20 bases, and has very little G+C content (~25%). Given the predicted differences in secondary structure between these forms, it is not surprising that the translatabilities of these alternate mRNAs also differ. In vitro translation of these mRNAs showed the HuR transcript with the short 5’ UTR to produce far more protein than the alternate transcript in standard cellular extracts [27]. In addition, we found that cellular stress induced greater expression of the short, more translatable form, while decreasing expression of the longer, less translatable form [28]. From these findings, we hypothesized that the alternate mRNAs provide a way to maintain constant levels of HuR protein under conditions where the normal translation process might be compromised.

Our additional studies demonstrated that the shorter, readily translatable HuR mRNA is under control of Smad 1/5/8 transcription factors that bind just upstream of the transcriptional start [28]. About the same time, other researchers identified a functional NF-κB binding site an additional ~150 bases upstream, in a region positioned properly to promote transcription of the long HuR mRNA transcript [29]. However, while their study and ours [30] demonstrated the importance of NF-κB to overall HuR mRNA levels, the contribution of this transcription factor to regulation of the alternate HuR transcripts was not determined. In this study, we specifically identify transcription factors that regulate the long HuR mRNA with an emphasis on regulation of its expression during cell stress.

Materials and Methods

Cell culture & reagents

LLC-PK1 cells from the American Type Culture Collection (ATCC), Manassas, VA, were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing penicillin/streptomycin and 10% fetal bovine serum in a humidified incubator at 5% CO₂. The cells were harvested and RNA was extracted using Trizol (Invitrogen) according to the manufacturer’s protocol. Transfections were performed using an Amaxa
nucleofector with kit L (Lonza, MD). For these experiments, 2 × 10⁶ transfected cells were plated in each well of a six-well plate and media was replaced 12 hours after nucleofection. Cells were harvested 48 hours after transfection for assays. To induce endoplasmic reticulum stress, thapsigargin (Sigma) was added to normal growth medium at 1 μM concentration for the indicated durations. Some cells were allowed to recover from thapsigargin treatment by washing with phosphate buffered saline and replacing the normal DMEM medium. The NF-κB inhibitor BAY11-7082 (Sigma) was added to cells at 2 μM, while the Sp1 inhibitor mithramycin was added to cells at 0-200 nM. Expression plasmids for p65 and Sp1 were kind gifts of Drs. Denis Guttridge and Arthur Strauch (The Ohio State University), respectively. Expression plasmids for KLF8 and Smad1 were obtained from the American Type Culture Collection.

Reverse transcription/polymerase chain reaction (RT-PCR)

Total RNA was extracted from LLC-PK₁ cells with Trizol. mRNA levels were assessed by competitive RT-PCR, a quantitative mRNA assay with superior accuracy in determining small expression differences [31]. An internal standard for competitive RT-PCR of the long porcine HuR mRNA was synthesized using a previously described procedure [28]. Ten picograms of the internal standard RNA and 2.5 μg of total LLC-PK₁ RNA were mixed and reverse transcribed using the SuperScript II First Strand System (Invitrogen). The cDNA was then subjected to PCR amplification using PrimeStar HS DNA polymerase and GC-rich buffer (Takara Bio-Clontech laboratories, CA) using the following primers and conditions:

- 5′-CGCGCTGAGGAGGAGCC-3′ (forward), and 5′-CCTGGGTCATGTTCTGAGGGAG-3′ (reverse); 3 min at 94°C followed by 40 cycles of 94°C for 10 sec, 64°C for 15 sec, then 72°C for 30 sec. The resulting DNA was electrophoresed in a 2% agarose gel and visualized and quantified using a Chemi-doc image analyzer (Bio-Rad) with Quantity One software system. KLF family member transcripts were reverse transcribed using the SuperScript II First Strand System, then amplified in either standard PCR buffer (KLFs 7, 8, 10) or in Takara GC-rich buffer and 10% DMSO, using PrimeStar HS DNA polymerase (KLFs 13-16). Cycling conditions were 2 min at 94°C followed by 35 cycles of 94°C for 10 sec, 58°C for 30 sec, then 68°C for 1 min. The primers used were as follows and were derived from previously published porcine sequences [32] (Table 1).

Western analysis

SDS-PAGE resolved proteins were transferred to Hybond P membrane (GE healthcare) and probed with polyclonal rabbit antibodies (Sigma) at 1:1000 for anti-KLF8 and anti-NFκB p65. An anti-Sp1 antibody, also from Sigma, was used at 1:750. Antibodies used for loading controls (anti-β-actin at 1:1000 and anti-GAPDH at 1:7500) were obtained from Cell Signaling. Primary antibodies were detected using horse radish peroxidase conjugated secondary antibody and a clarity ECL western substrate system (Bio-Rad). For LLC-PK₁ experiments, protein lysates were harvested using M-PER (Pierce). Protein lysates from rat kidney cortex had been prepared as previously published [27].

Gel Mobility Shift Assay

Nuclear extracts were prepared from LLC-PK₁ cells as previously described. [28]. Double-stranded oligonucleotide probes were end-labeled with [γ-³²P] ATP and T4 polynucleotide kinase using a Gel Shift Assay System from Promega (Madison, WI). Eight micrograms of nuclear extracts were incubated with radiolabeled probe and binding buffer from the kit at room temperature for twenty minutes prior to separation in a non-denaturing 4% polyacrylamide gel. The gel was then fixed in 10% acetic acid +10% methanol for 20 min at room temperature and exposed to autoradiography film at -80°C for 30-60 min. Oligonucleotide competition reactions were set up by pre-incubating the nuclear extracts with 100-fold excess unlabeled oligonucleotide for 15 min at room temperature prior to addition of the corresponding labeled probes.
**Chromatin Immunoprecipitation (ChIP)**

Assays were performed with the Magna ChiP A kit (EMD-Millipore, CA). LLC-PK₁ cells (10 x 10⁶) were fixed using 1% formaldehyde at room temperature, and unreacted formaldehyde was quenched using 0.125M glycine. DNA was sheared using a Virsonic 100 sonicator at 5W and 5 x 15 sec. DNA bound to p65-NFκB or KLF8 was detected by co-immunoprecipitation using 10 μg of the appropriate antibody (Rockland Immunochemicals Inc, PA and Sigma Aldrich, MO). To amplify the p65-NFκB or KLF8 binding region, the precipitated DNA was subjected to PCR using PrimeSTAR hi-fidelity DNA polymerase and GC Rich buffer (Takara Bio-Clontech laboratories, CA) under the following conditions: 5′-GCCGACCTTTCTCAAGC -3′ (sense), and 5′-TAGCGGTGGCGCAGCG-3′ (antisense); 3 min at 94 °C followed by 40 cycles of 98 °C for 10s, 64 °C for 15s, then 72 °C for 30s. Specificity of the transcription factor binding to the HuR promoter was controlled using primers designed to amplify regions of HuR exon 6 and intron 5. The primers for exon 6 were 5′-ATTGCTTTCCCAAGCAGGTCC-3′ (sense) and 5′-TTGGTCCATTCCCATTGGG-3′ (antisense), while the primers for intron 5 were 5′-TCTGGCATTGCTGTGAGCTG-3′ (sense) and 5′-AGGAGCTGCACCATTGTGGT-3′ (antisense). Competitive N-ChIP [33] was performed similarly except that DNA-protein complexes were not cross-linked with formaldehyde prior to sonication, and 0 to 900 nM of competitor oligonucleotide was added simultaneously with the immunoprecipitating antibody.

**Statistical Analysis**

All statistical analyses were performed using Student’s t-test for paired samples. Statistical significance is indicated when \( P < 0.05 \).

**Results**

Proximal tubule cells of the kidney are particularly sensitive to cell stress due to an extreme need for ATP that supports their high levels of ongoing solute reabsorption and secretion. We have previously employed two proximal tubule cell lines, the porcine LLC-PK₁ and the human HK-2, in well-established models of cell stress to study HuR expression [27, 28, 30, 34, 35]. We demonstrated that in LLC-PK₁ cells, the long and short HuR mRNA forms are expressed at approximately equal levels, but that cellular stress can increase expression of the short form while decreasing expression of the long form [28]. Here we use the LLC-PK₁ model to understand regulation of the long HuR mRNA.

As described above, previous studies identified a functional NF-κB binding site in an appropriate position to promote transcription of the HuR long form mRNA. To determine whether NF-κB regulates expression of this variant, LLC-PK₁ cells were incubated with the NF-κB inhibitor BAY11-7082 and the mRNA was detected by competitive RT-PCR. As shown in Figure 1A, 60 minutes in the inhibitor was sufficient to almost completely abolish the HuR transcript. In addition, overexpression of the NF-κB subunit p65 increased HuR long form expression in a dose-dependent manner (Fig. 1B). These experiments demonstrate that NF-κB is a positive regulator of the long HuR transcript. We previously showed that a Smad 1/5/8 binding site, approximately 100 bp downstream of the NF-κB site, was a positive regulator of the short HuR mRNA. We also found that transcriptional upregulation of the short form was accompanied by decreased long form expression. To determine whether interference between the NF-κB and Smad 1/5/8 sites might account for this outcome, the effect of Smad1 on p65 activation was tested. As shown in Figure 1C, Smad1 co-transfection inhibited induction of the long form by p65, likely contributing at least in part to the reciprocal relationship in expression between the long and short HuR mRNAs.

We next examined how NF-κB and HuR levels may change during cellular stress. LLC-PK₁ cells were incubated with 1 μM thapsigargin, an inducer of endoplasmic reticulum (ER) stress that was previously shown to decrease levels of the long HuR transcript [28]. Western analysis demonstrated that total p65 levels increased during this treatment (Fig. 2A), a finding consistent with NF-κB’s known activation by ER stress [36]. In contrast, competitive RT-PCR confirmed that thapsigargin treatment over a 6 hour period markedly decreased levels of the long HuR transcript (Fig. 2B). These results suggest that other transcription
factors may predominate in regulating expression of this mRNA during stress. Analysis of the HuR 5’ flanking region indicated potential additional transcription factor binding sites near the NF-κB binding sequence. One region, positioned just upstream of the long form transcriptional start, contained two putative core Sp/KLF sites that might bind the related Sp or KLF families of transcription factors (GC boxes, Fig. 3A, B). The first set of experiments was performed to determine whether the widely-expressed Sp1-like family of proteins binds to the HuR promoter. Gel mobility shift analysis was performed on nuclear extracts from LLC-PK₁ cells, using probes and competitive oligonucleotides shown in Figure 3B. As demonstrated in Figure 3C, a probe containing the full Sp/KLF region but not the NF-κB or Smad 1/5/8 sites (probe S/K), was capable of creating a band shift. In contrast, probes containing either the individual (probes Δ1 and Δ2) or mutated (probes Δ2mut and mut) GC boxes did not. These results indicate that transcription factor binding requires both Sp/
KLF core sequences. To better determine the identity of the protein binding this region, gel mobility shift analysis using additional probes and cold competitor oligonucleotides was performed. Figure 3D shows that unlabeled probes containing the individual GC boxes (Δ1 and Δ2, lanes 11 and 4) were unable to compete for binding of the protein, confirming that both sites are necessary for factor binding. Other reactions were prepared to determine whether the protein that bound the S/K probe belonged to the Sp1-like family of proteins. Addition of an unlabeled Sp1-binding oligonucleotide was unable to inhibit protein binding to the Sp/KLF region (lane 5). In addition, a commercial, labeled Sp1 binding sequence did

Fig. 3. A putative Sp/KLF binding region just upstream of the transcriptional start site binds a factor that is not Sp1. (A) Schematic of the proximal promoter region of HuR. (B) Sequences of the sense strand of probes used in protein-DNA binding assays. Underlined sequences indicate core GC boxes, while bolded letters indicate introduced mutations. (C) Gel mobility shifts assays demonstrate that a probe spanning the full Sp/KLF binding region strongly shifted nuclear protein, but individual or mutated GC boxes did not. (D) Gel mobility shift assays performed with unlabeled competitive oligonucleotides demonstrate that the shifted band produced by the Sp/KLF probe is not Sp1. (E) Addition of the Sp1 inhibitor mithramycin at varying concentrations for 24 hours did not inhibit expression of the long HuR mRNA. (F) Overexpression of an Sp1 expression plasmid did not increase levels of the long HuR mRNA as indicated by competitive RT-PCR. At the top right, a Western blot demonstrates increased expression of Sp1 in this experiment. A Western blot of GAPDH is shown as a loading control.
not produce shifted bands when combined with the LLC-PK₁ extracts (lane 7). We were also unable to supershift the protein complex using anti-Sp1 antibodies (not shown). These results are consistent with findings demonstrating that Sp1 is expressed at very low levels in mature proximal tubule cells [37], and together suggest that Sp1 does not bind this region of the HuR promoter. To confirm this hypothesis, LLC-PK₁ cells were alternatively treated for 24 hours with varying concentrations of mithramycin, an inhibitor of Sp1 (Fig. 3E), or were transfected with a plasmid expressing Sp1 (Fig. 3F). Neither treatment resulted in changed levels of either the long HuR transcript, as shown, or total HuR mRNA levels (not shown).
shown). Figure 3F also shows Western analysis of Sp1 levels in untransfected cells and in cells transfected with an Sp1 expression plasmid. This blot demonstrates that Sp1 levels in normal LLC-PK\(_1\) cells are very low or absent, and that even when Sp1 is present, it does not stimulate expression of HuR mRNA. These results together reveal that Sp1 and related proteins do not significantly contribute to regulation of the HuR transcript.

We next turned our attention to determining what KLF family member(s) might be involved in HuR regulation. The KLF family is large, with 17 members, and previous studies had identified at least eight of these in the kidney [38]. To determine which KLF factors were present in LLC-PK\(_1\) cells, primers were designed against KLFs 7-16. RT-PCR resulted in detection of mRNA for KLF 7, 8, 10, and 13-16, though signals for KLF15 and KLF16 were very weak (Fig. 4A). Others had previously demonstrated the presence of KLF 4 and KLF6 in proximal tubule cells [39, 40]. A review of the literature indicated that KLF8 in particular shared many functional attributes with HuR, including promotion of tumor development, growth, and metastasis, and a commonality of target genes [41]. We therefore focused our attention on this transcription factor. Chromatin immunoprecipitation (ChIP) analysis of the region containing the NF-κB and KLF binding sites was able to detect binding to this region of both p65 and KLF8 (Fig. 4B). The specificity of this reaction is demonstrated by the failure of either magnetic beads alone (lane 1) or irrelevant antibody (lane 2) to precipitate a product. Further, only primers against the HuR promoter were able to amplify DNA of the correct size, while primers designed against irrelevant regions of chromatin (HuR exon 6 and intron 5) did not detect co-precipitated DNA. In addition, the S/K double-stranded oligonucleotide was capable of competitively inhibiting a native ChIP assay (N-ChIP), while the mutant S/K oligonucleotide was not (Fig. 4C). These reactions definitively demonstrate the ability of KLF8 to specifically bind the HuR promoter. Further, overexpression of KLF8 in LLC-PK\(_1\) cells caused an increase in the HuR long form transcript (Fig. 4D). KLF8 levels were also found to decrease during thapsigargin treatment, with kinetics similar to that of the HuR transcript.
Although thapsigargin causes acute suppression of new protein synthesis, KLF8 levels remained at baseline for at least 2 hours of treatment before rapidly diminishing. This may be due to the intrinsic stability of KLF8 since we found that KLF8 mRNA levels begin to drop within 1 hour of thapsigargin treatment (data not shown). Interestingly, HuR mRNA levels showed the same lag time prior to a precipitous drop. These results are consistent with KLF8 acting as a transcriptional activator of HuR and potentially playing a dominant role in expression of the HuR long form mRNA during cellular stress.

To determine what factors may be involved during immediate recovery from cellular stress, LLC-PK$_1$ cells were incubated for 2 hours with thapsigargin, but were allowed to recover in growth medium for several hours more. Levels of the long HuR mRNA transcript transiently increased 2 to 3 hours after treatment but returned to their baseline levels (Figure 5A). Similarly, total p65 levels transiently increased within 2-3 hours after thapsigargin treatment, although with somewhat slower kinetics. Although the finding that p65 levels increase after HuR may initially appear counter-intuitive, this may be explained by our previous discovery that HuR promotes NF-κB activity in a positive feedback loop [30]. In contrast, KLF8 levels markedly decreased in the hours after thapsigargin treatment, demonstrating the sensitivity of this transcription factor to stress. These findings suggest that KLF8 is unlikely to regulate expression of HuR during recovery from cellular stress.

We previously showed that in native rat kidneys transiently subjected to ischemic stress for 30 minutes, expression of the long HuR mRNA (and HuR protein) in the renal cortex approximately doubled by 14 days of reperfusion, when damaged proximal tubules have regenerated and are proliferating [27]. To understand the expression of the NF-κB and KLF8 transcription factors in native kidneys, protein lysates from the cortex of rat kidneys that had been subjected to temporary ischemic injury were analyzed by Western blot [27]. As shown in Figure 6, p65 expression remained relatively constant immediately following ischemia but increased during reperfusion. However, KLF8 expression was more sensitive to ischemic injury and diminished markedly but began to recover within 1 hour. After 14 days, expression of KLF8 also appeared to have increased over baseline but this increase did not reach statistical significance due to some scatter in the data. Nonetheless, these findings are consistent with our in vitro studies that revealed KLF8’s sensitivity to cellular stress. Further, both p65 and KLF8 levels appear to correlate with that of the HuR long mRNA before and after renal injury, suggesting that these factors are likely to play important roles in stress responses in native kidney.
Discussion

The results described here demonstrate a role for KLF8 in binding to the promoter region of HuR and stimulating its expression. KLF8 is one of a family of zinc-finger transcription factors that are highly conserved across species. Seventeen Krüppel-like factors have been identified that share homologous C-terminal zinc-finger domains, and each possesses a distinct amino-terminal region that determines its specificity for binding partners [38]. Sp1 also shares homology with the KLFs in its zinc-finger domains, resulting in their binding to similar GC-rich sequences. However, some distinctions do exist in binding preferences, with the KLFs having a preference for 5’-CACCC-3’ (or 5’-GGGTG-3’) sequences [42]. This core sequence is not present in the region of the HuR promoter tested, though chromatin immunoprecipitation studies readily demonstrated the binding of KLF8 in these studies.

Although kidney cells express mRNAs for many members of the KLF family, we focused on KLF8 for a number of reasons. First, though KLF8 is expressed at low levels in many tissues, it is at its highest levels in kidney [43]. Further, the influence of KLF8 on cellular activity is similar to the influences of HuR. For example, overexpression of KLF8 has been associated with oncogenic transformation in a number of cell types, including renal cells [44], as has HuR [45]. Additionally, many of KLF8’s transcriptional target genes are also regulated by HuR’s post-transcriptional effects. These include cyclin D1 [46-48], Bcl-2 family members [10, 46, 49], and MMP9 [46, 50], which regulate cell proliferation, survival, and motility/invasion.

Further, KLF8 expression is regulated by activation of PI3K/Akt signaling [51], as is HuR expression [29, 30]. KLF8 expression was recently shown to be sensitive to oxygen tension in placenta, indicating its role as a stress-responsive regulator of cellular function [52]. Interestingly, knockout of HuR in mice showed HuR to be critical to appropriate placental morphogenesis [53]. Given the findings of our current study, it is tempting to speculate that KLF8’s effects on cell growth may be mediated by its transcriptional activation of HuR expression. However, definitive proof of this hypothesis will require specific manipulation of KLF8 and HuR levels and subsequent examination of cellular function.

Large-scale characterization of full-length cDNAs has estimated that about half of human genes are regulated by alternate promoters [54]. Transcription from alternate promoters has been demonstrated in multiple cellular contexts, including differentiation, growth, and stress. The existence of alternate HuR transcripts with very different 5’ untranslated regions and consequently distinct translatabilities offers cells a means to keep HuR protein levels stable under a variety of conditions. A moderate level of HuR expression is required for cell survival, since its absence results in cell death [55]. Yet, its overexpression is well-established to play a role in oncogenic transformation and tumor invasiveness [11, 12, 22, 29, 45, 56-64]. Therefore, even under conditions of cell stress, when normal translational regulation may be impaired, it is critical that HuR expression is maintained at appropriate levels. Our studies indicate that the longer form of HuR mRNA is translated under normal growth conditions, but during a stress event, translation of the shorter form predominates (manuscript in preparation). This shorter form has a ~20 base, AT-rich, unstructured 5’ untranslated region, which permits rapid translation in in vitro assays. In contrast, the longer HuR mRNA is much more poorly translated under the same conditions [28]. In this respect, the longer form of HuR mRNA is similar to class I mRNAs, which have been defined as those encoding transcription factors, growth factors and their receptors, proto-oncogenes and similar transcripts that are not well translated under normal conditions. This class of mRNAs is notable in that >90% of their 5’ UTRs are longer than 100 bases and have extensive secondary structure [65]. In contrast, the shorter HuR transcript is similar to mRNAs of class III, which consists of highly expressed genes whose levels of expression are controlled primarily at the transcriptional level and are thought to be efficiently translated. These mRNAs were found to have short 5’ UTRs that were free from secondary structure [65]. A stress-induced increase in the shorter transcript would permit ready translation of HuR under poor cellular conditions. Indeed, we previously showed that HuR translation actually increased in renal proximal tubule (LLC-PK₁) cells that were undergoing an almost total depletion of ATP [35]. However, upon return...
of the cells to normal growth medium, HuR levels returned to baseline. The differential expression of HuR mRNAs with alternate 5' UTRs and translatabilities provides a mechanism for this stress-responsive regulation of HuR expression.

Although we have not yet explored potential interactions between KLF8 and NF-κB, it is clear that both factors are individually involved in promoting transcription of the long HuR mRNA. It is of interest that expression of KLF8 was previously demonstrated to be upregulated by PI3K/Akt signaling in human ovarian cells [51]. HuR expression was also demonstrated to be stimulated by PI3K/Akt signaling in both renal proximal tubule cells [30] and gastric tumor cells [29]. In fact, we found that HuR participates in a positive feedback loop for Akt signaling in proximal tubule cells [30]. We do not know how KLF8 might integrate into this signaling pathway in our model system. However, it seems unlikely to be part of this feed-forward mechanism, given that its expression decreases in the context of cellular stress, when Akt activation occurs. More studies are required to delineate its responses to PI3K activity in the kidney.

We did not observe a role for Sp1-like transcription factors in regulation of HuR, and this was consistent with findings that Sp1 is expressed at very low levels in mature proximal tubule cells [37]. Even overexpression of Sp1 in LLC-PK₁ cells did not promote expression of the long HuR transcript, indicating a role for KLF family members in this core promoter region of the HuR gene. Numerous KLF family members have been identified in kidney, but more work is needed to definitively identify the expression patterns of all these factors in specific cell types in development and disease. However, some KLF factors are beginning to be understood in this organ system. For example, KLF15 (formerly KKLF, kidney-enriched Krüppel-like factor), is enriched in endothelial and mesangial cells of the kidney and a few nephron segments, excluding proximal tubules [66]. KLF15 has been shown to inhibit mesangial cell proliferation, promote podocyte differentiation, repress transcription of the kidney-specific chloride channel CLC-K1, and may inhibit renal interstitial fibrosis [66-69]. Another Krüppel-like factor not expressed in proximal tubule cells is KLF12, which is restricted to collecting ducts and promotes expression of the urea transporter UT-A1 [70]. Few KLF proteins have been characterized in proximal tubules. One example is KLF4, which functions as a tumor suppressor in a number of tissues, and was recently shown to be expressed in proximal tubule cells and downregulated in renal cell carcinoma tissues [40]. Additionally, in a screen for mRNAs that are upregulated in the earliest stages of renal ischemia/reperfusion injury, KLF6 (formerly Zf9) was detected. This factor was expressed at barely detectable levels in control kidneys, but demonstrated a rapid (within 1-3 hours) induction after ischemic injury. It also was rapidly induced in cultured proximal tubule cells subjected to ATP depletion, and its expression paralleled that of TGF-β1, a previously identified target [39]. Both KLF4 and KLF6 have temporal expression patterns that contrast sharply with KLF8, which is upregulated in renal cell carcinoma, and which rapidly decreases upon ischemic injury or thapsigargin treatment. It is clear that individual KLF family members adopt distinct spatio-temporal patterns in kidney cells, and comprehending how these factors are expressed will give us a clearer understanding of the regulation of their target genes.

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