DNA Damage and Cellular Stress Responses

Association of the von Hippel–Lindau Protein with AUF1 and Posttranscriptional Regulation of VEGFA mRNA

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Abstract

The von Hippel–Lindau (VHL) tumor suppressor gene product is the recognition component of an E3 ubiquitin ligase and is inactivated in patients with VHL disease and in most sporadic clear-cell renal cell carcinomas (RCC). pVHL controls oxygen-responsive gene expression at the transcriptional and posttranscriptional levels. The VEGFA mRNA contains AU-rich elements (ARE) in the 3'-untranslated region, and mRNA stability or decay is determined through ARE-associated RNA-binding factors. We show here that levels of the ARE-binding factor, AUF1, are regulated by pVHL and by hypoxia. pVHL and AUF1 stably associate with each other in cells and AUF1 is a ubiquitylation target of pVHL. AUF1 and another RNA-binding protein, HuR, bind to VEGF ARE RNA. Ribonucleoprotein (RNP) immunoprecipitations showed that pVHL associates indirectly with VEGF mRNA through AUF1 and/or HuR, and this complex is associated with VEGF mRNA decay under normoxic conditions. Under hypoxic conditions pVHL is downregulated, whereas AUF1 and HuR binding to VEGF mRNA is maintained, and this complex is associated with stabilized mRNA. These studies suggest that AUF1 and HuR bind to VEGF ARE RNA under both normoxic and hypoxic conditions, and that a pVHL–RNP complex determines VEGF mRNA decay. These studies further implicate the ubiquitin–proteasome system in ARE-mediated RNA degradation. Mol Cancer Res; 10(1); 108–20. ©2011 AACR.

Introduction

Germline inactivation of the von Hippel–Lindau (VHL) tumor suppressor gene is linked with development of VHL disease, an autosomal dominantly inherited cancer syndrome. VHL patients are predisposed to develop various vascular tumors, including hemangioblastomas of the retina and central nervous system, clear-cell renal cell carcinomas (RCC), pancreatic cysts and adenocarcinomas, and adrenal pheochromocytomas (1). The VHL gene is also inactivated in the majority of patients with sporadic clear-cell RCC (1).

The VHL gene encodes proteins (pVHL) of 25 and 19 kDa through use of alternative translation initiation codons, and both isoforms seem to possess tumor suppressor activity (2). pVHL has α and β structural domains that are critical to its function as the substrate recognition component of a cullinRING E3 ubiquitin ligase (CRL; ref. 3–5). The N-terminal β domain is associated with target protein recognition, whereas the α domain contains a SOCS box that interacts with elongin C and links pVHL to the ubiquitin–ligase complex containing elongin B and C and Cul2. Perhaps the best characterized pVHL CRL targets are the hypoxia-inducible factor (HIF; ref. 6). Hydroxylation of conserved prolines in HIFα subunits under normoxic (21% O₂) conditions provides a substrate recognition motif for pVHL polyubiquitylation and proteasomal degradation. In hypoxia (1% O₂) prolyl hydroxylase activity is inhibited, unmodified HIFα subunits are stabilized, and the hypoxia response is initiated. We recently showed that pVHL levels are suppressed in hypoxia (7), providing an additional mechanism for HIFα upregulation in hypoxia. HIF activation stimulates transcription of several hundred genes whose products have varied functions, including nutrient uptake, glycolytic metabolism, and neovascularization (8, 9). However, many of the hypoxia-inducible genes also exhibit control at the level of mRNA stability. Such mRNAs are labile under normal conditions and become stabilized in hypoxia. This regulation is mediated through adenine/uridine-rich elements (ARE) that are typically found in the 3'-untranslated regions (UTR) of these mRNAs, and interactions with ARE-binding proteins
determines the stability or decay of the mRNA [reviewed in von Roretz and colleagues (10)]. Two well characterized are AUFI and HuR (10). AUFI (or hnRNP D) represents a family of proteins of 37, 40, 42, and 45 kDa that are produced through alternative splicing of a single pre-mRNA (11). All AUFI proteins contain 2 nonidentical RNA recognition motifs (RRM), a consensus RNA-binding domain, and may bind to ARE RNA as heterologous oligomers (12). Sequences flanking the RRMs seem to contribute to ARE binding, including an alanine-rich region near the N-terminus and a short glutamine-rich region near the C terminus. Although AUFI is frequently associated with mRNA destabilizing activity, several studies have indicated that AUFI stabilizes some mRNAs [reviewed in Zucconi and colleagues (13)]. The independence and/or interdependence of the various AUFI isoforms is not yet fully understood. HuR is a 36 kDa protein that is ubiquitously expressed and is a member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins. HuR has 3 RRMs and is generally considered to be involved in the stabilization of ARE-containing mRNAs (10). Overexpression of HuR has been noted in malignancies, including RCC (14).

Overexpression of a key angiogenic factor, VEGF, has long been appreciated in RCC and VHL-associated tumors. The importance of VEGFA activity in RCC is underscored by the fact that anti-VEGF therapies are now the first-line standard of care for patients with metastatic RCC [reviewed in Rini and colleagues (15)]. VEGFA is a classical hypoxia-inducible gene product. In normoxia, basal levels of VEGFA transcription may be controlled by the SP1 general tran-

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After a shift to hypoxia an 8- to 30-fold increase in steady-state VEGF mRNA levels in various cell lines has been observed [reviewed in Levy and colleagues (22)]. VEGF induction in hypoxia occurs through increased transcription initiation mediated through HIF binding to VEGFA promoter enhancer elements (23), as well as through an increased mRNA half-life of up to 4 hours (21). The involvement of several RNA-binding proteins in the regulation of VEGFA mRNA levels has been described, including HuR (24), hnRNP L (25, 26), and Tis11b (27).

In this article, we examined further the role of pVHL in regulating VEGFA mRNA stability. RCC cell lines with inactivated pVHL exhibit a condition that has been termed pseudohypoxia, high expression levels of classically defined hypoxia-responsive gene products in the presence of normal oxygen levels. Restoration of pVHL expression in RCC cell lines restores the normal hypoxic response. We show here that the ARE RNA-binding protein, AUFI, is regulated by pVHL and by hypoxia, that pVHL and AUFI directly associate with each other, and pVHL seems to target p45AUFI and p42AUFI for ubiquitylation. AUFI and HuR bind to VEGFA and TNFα ARE RNA sequences under both normoxic and hypoxic conditions. In normoxia pVHL is also found in these complexes, and this binding is associated with rapid decay of the ARE-containing RNA. In hypoxia, when pVHL levels are decreased, the continued binding of AUFI and HuR is associated with stability of the ARE-containing RNA. These results directly link pVHL, and perhaps its ubiquitin–ligase activity, with enhanced degradation of ARE-containing RNA.

Materials and Methods

Antibodies and reagents

Monoclonal antibodies (mAb) directed against hemagglutinin (HA, HA.11), tubulin, and ubiquitin (P4G7) were from Covance; M2 anti-FLAG mAb was from Sigma; HuR and Cul2 mAb, rabbit polyclonal pVHL antibody (FL181), normal rabbit IgG, and normal mouse IgG were from Santa Cruz Biotechnology; pVHL mAb (lg32) was from Pharmingen. Rabbit affinity purified AUFI antibody was previously described (28) or from Upstate Biotechnology or Phoenix Biochemicals. Proteasome inhibitors were from Calbiochem and desferrioxamine was from Sigma.

Cell culture

Cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS in a humidified, 5% CO2 incubator at 37 °C. A complete description of the cell lines used in these studies is provided in Supplementary Table S1. UOK121 RCC cells are from the Urologic Oncology Branch cell line repository and were provided by Dr. W. Marston Linehan, National Cancer Institute. Human embryonic kidney 293T cells and 786-O RCC cells were obtained from the American Type Culture Collection. 786-O G7F cells and 786-O 157A express wild-type Flag-tagged VHL or truncated and inactive pVHL (amino acids 1–157) cDNAs, respectively (7). HA-VHL-expressing 786-O RCC cells (WT7) and control cells (ARZ2 and pRC9) were provided by Dr. William G. Kaelin, DanaFarber/Harvard Cancer Center. All of the cell lines used during the course of these studies were maintained in continuous culture for 4 months or less.

Hypoxia experiments were carried out in a chamber monitored by a BioSpherix C21 dual O2/CO2 controller that was calibrated to 1% O2 levels according to the manufacturer’s specifications. In a typical experiment, cells were seeded in 100-mm tissue culture dishes and cultured in 5% CO2 in normoxia (room air) for 18 to 24 hours before transfer to the hypoxia chamber.

Immunoprecipitation

Cells were harvested at less than 75% confluence in all experiments by scraping in ice-cold PBS and centrifugation at 500 × g. Cells were lysed in either Igelal lysis buffer [100 mmol/L NaCl, 0.5% Igepal CA630 (Sigma-Aldrich), 20 mmol/L Tris-HCl, pH 7.0, 5 mmol/L MgCl2, 1 mmol/L sodium orthovanadate, 5 mmol/L levanisole, aprotinin (1 μg/mL), 0.5 mmol/L 4-(2-aminoethyl) benzene sulphonyl fluoride; all from Sigma], and complete protease inhibitor cocktail (Roche), or radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 20 mmol/L Tris-HCl, pH 7.0,
5 mmol/L MgCl2, and the same inhibitors as above. For immunoprecipitations, 500 μg cell extract was precleared overnight at 4°C with protein G Sepharose (50% v/v; Sigma) that had been preblocked with PBS containing 20 mg/mL bovine serum albumin (BSA), 5 mg/mL heparin, and 0.1 mg/mL yeast tRNA. Immunoprecipitating antibody (1–2 μg) was added to the precleared lysates and incubated at 4°C for 2 hours to overnight, followed by 2 hours incubation with preblocked protein G Sepharose (50% v/v) on a rotator at 4°C. Beads were washed 5 times in lysis buffer, samples were resolved in SDS-polyacrylamide gels, and electrotransferred onto polyvinylidene difluoride membranes. Western blots were developed using chemiluminescence (ECL, Amersham).

In vitro protein interactions and ubiquitylation assays

Full-length or truncated GST-VHL and GST-AUF1 clones in pGEX-2T were prepared by PCR amplification of cDNA clones. cDNAs encoding p45AUF1 or p37AUF1 were used to generate GST-AUF1 clones that either contained both or lacked both exon 2- and exon 7-encoded sequences, respectively. Fusion protein expression in Escherichia coli BL21 with IPTG induction was conducted. Glutathione S-transferase (GST) pull-down assays were done by prebinding GST fusion proteins to glutathione-Sepharose (50% v/v; Sigma) in PBS containing 1% NP-40, washing extensively, and then adding radiolabeled in vitro translated protein in PBS+1% NP-40, 1 mg/mL heparin, 5 mg/mL L-levamisole, 1 mg/mL sodium orthovanadate and Complete protease inhibitors. Incubations were for 1 hour at 4°C, and the beads were then washed 4 times with PBS containing 500 mmol/L NaCl, 1% NP-40, and 0.5 μg/mL BSA. Samples were resolved by 12% SDS-PAGE, and gels were dried and autoradiographed.

In vitro ubiquitylation assays were done with p25VHL and AUF1 proteins that were translated in vitro using the TNT coupled reticulocyte lysate system (Promega). Glutathione S-transferase (GST) pull-down assays were done by prebinding GST fusion proteins to glutathione-Sepharose (50% v/v; Sigma) in PBS containing 1% NP-40, washing extensively, and then adding radiolabeled in vitro translated protein in PBS+1% NP-40, 1 mg/mL heparin, 5 mg/mL L-levamisole, 1 mg/mL sodium orthovanadate and Complete protease inhibitors. Incubations were for 1 hour at 4°C, and the beads were then washed 4 times with PBS containing 500 mmol/L NaCl, 1% NP-40, and 0.5 μg/mL BSA. Samples were resolved by 12% SDS-PAGE, and gels were dried and autoradiographed.

RNA–protein interaction assays

VEGFA ARE regions defined by Levy and colleagues (ref. 29; see Supplementary Fig. S3A) were amplified by PCR using a VEGFA 3′-UTR cDNA clone as template. Each amplicon had a T7 RNA polymerase site at the 5′ end, and T7 in vitro transcription reactions were done in the presence of α-32P-UTP or biotin-11–GTP (Perkin Elmer). Complementary double-strand DNA oligonucleotides were synthesized corresponding to the TNFα ARE (5′-AATTATTATTATTATTATTATTATTATTTTAA-3′) or a mutated ARE sequence (5′-AATGATGATCACTACTTGTTCTATGGTCCCTTGGAA-3′; ref. 30), were cloned into pGEM4, and run-off transcripts were made with T7 RNA polymerase using linearized plasmid as template. Radiolabeled transcripts (2 × 10⁵ cpm/reaction) were incubated with 30 μg cytosolic extracts in binding buffer (10 mmol/L HEPES, pH 7.5, 10% glycerol, 5 mmol/L MgCl2, 50 mmol/L KCl, 0.5 mmol/L EGTA, 0.5 mmol/L DTT, 100 μg/mL yeast tRNA, and 5 mg/mL heparin), and incubated at 30°C for 20 minutes followed by RNase treatment (40 units RNase T1 and 1 μg RNase A) for 15 minutes at room temperature. Samples were run in 8% nondenaturing polyacrylamide gels in 0.5X TBE buffer, gels were dried, and subjected to autoradiography.

RNP immunoprecipitation and RT-PCR

For ribonucleoprotein (RNP) immunoprecipitation, cultures at 75% confluence were washed with cold PBS and extracts were prepared using 0.5 ml/100-mm dish RNase-free lysis buffer (50 mmol/L HEPES, pH 7.5, 10 mmol/L sodium pyrophosphate, 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.5% Igepal CA630, 10% glycerol, 100 mmol/L sodium fluoride, 0.2 mmol/L sodium orthovanadate, 1 mmol/L EGTA, and Complete protease inhibitor cocktail). Insoluble material was removed by centrifugation for 30 minutes at 20,000 × g. Antibodies were added to 1 μg/mL and incubated for 2 hours at 4°C on a rotator. Immune complexes were collected with the addition of preblocked protein G Sepharose (50% v/v) for an additional 1 hour at 4°C on a rotator. Samples were washed 5 times in RNase-free lysis buffer and then were extracted with 50 μL TRIzol (Invitrogen). The aqueous phase was isolated and ethanol precipitated at −80°C overnight with 1 μg glycogen added as carrier. Ethanol precipitates were centrifuged at 20,000 × g for 1 hour at 4°C, washed with 70% ethanol, and the pellets were solubilized in 8 μL water for 15 minutes at 65°C. The entire contents of each tube were used for first-strand cDNA synthesis (Invitrogen) according to the manufacturer’s protocol. PCR was done using primers specific for VEGFA or actin as previously described (31), except that 35 amplification cycles were carried out. In control reverse transcriptase PCR (RT-PCR) reactions first-strand cDNA that was prepared from total cellular RNA was used, and 25 amplification cycles were carried out. PCR products were resolved in 2% agarose-1000 (Invitrogen) gels.

Reporter plasmid constructs and transfection

Fragments from the VEGFA 3′-UTR were generated by PCR and subcloned into the XhoI restriction site downstream of the firefly luciferase gene translation termination codon in the pGL3-Promoter vector (Promega). A 193 bp (base pair) fragment, corresponding to VEGFA 3′-UTR nucleotides 306 to 498 (relative to the translation termination codon; GenBank submission number Y08736), contains an ARE that was shown previously to confer stability under hypoxic conditions (25, 26, 32). A control, non-ARE

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containing 193 bp fragment corresponding to VEGFA nucleotides 113 to 305 was also cloned into the pGL3-Promoter vector. The orientation and sequence of the inserts were verified by DNA sequencing. Cells were transfected in 60-mm tissue culture dishes (Lipofectamine 2000; Invitrogen). After 24 hours, the cultures were washed, trypsinized, and divided onto two 60-mm tissue culture dishes, which were then cultured for an additional 6 hours and then either transferred to the hypoxia incubator (1% oxygen) or maintained in room air. After 12 hours, the cells were extracted with RNP-ip lysis buffer or with TRIzol and analyzed by RNP-ip or RT-PCR as described above. Luciferase-specific PCR primers were: forward 5'-AGATGCACATATC-GAGGTGGACAT-3' and reverse 5'-ATCGTATTTGT-CAATCAGGTCGCT-3', which amplifies a 786 bp fragment. Aliquots of the cell extracts were also used for luciferase assays, and transfection efficiencies were determined by cotransfection of a plasmid expressing CMV-β-galactosidase.

Gene silencing
pSilencer-AUF1 and control plasmids (33) were obtained from Dr. M Gorospe, National Institute of Aging, NIH. Verified, pooled HuR siRNA were obtained from Dharmacon. 293T cells were transfected using Lipofectamine 2000, and after 48 hours total RNA or RIPA extracts were prepared for quantitative RT-PCR or Western blotting analyses, respectively. Primers for VEGFA, AUF1, HuR, 18S rRNA, and TBP were obtained from Qiagen, and quantitative PCR analyses were done with the ABI StepOnePlus Real-Time PCR system with SYBR green detection. Target mRNA expression levels were measured in triplicate and relative quantitation (RQ) values were determined relative to TBP mRNA levels, and data are presented as fold change relative to normoxia. All experiments were repeated at least three times.

Results
AUF1 protein levels are regulated by pVHL and hypoxia
To understand better regulation of ARE-mediated mRNA stability and decay in normoxia and hypoxia, we examined AUF1 expression in RCC and 293T cells. AUF1 protein levels were lower in pVHL-expressing 786-O G7F RCC cells as compared with 786-O 157A RCC cells that were cultured (A) under normoxic (room air) or hypoxic (1% O2) conditions for 18 hours or (B) in the presence of the hypoxia mimetic, desferrioxamine (DFO; 100 μmol/L), for 2 to 18 hours. U, untreated cells. C, 293T cells were cultured under normoxic or hypoxic conditions for 18 hours. D, 786-O G7F RCC cells were cultured in the absence or the presence of the proteasome inhibitor, MG132 (20 μmol/L), for 2 hours. For the experiments in panels A-D, protein extracts were prepared with RIPA buffer, and 40 μg cellular protein was resolved by 12% SDS-PAGE followed by Western blotting using the indicated antibodies. Tubulin or Cul2 detection were used as protein loading controls. E, quantitative real-time RT-PCR analysis of VEGF and AUF1 mRNAs in 786-O RCC cells that were cultured in normoxia or hypoxia for 18 hours. The primers used for PCR are specific for VEGF exons 1 and 3 and AUF1 exons 3 and 5, which are contained in all alternatively spliced forms of these mRNAs. Expression levels of the target mRNAs were determined in triplicate and averages are expressed relative to 18S rRNA levels. F, relative AUF1 mRNA levels in pVHL-positive, WT7, and pVHL-negative, PR9, RCC cells were determined by quantitative real-time RT-PCR. AUF1 mRNA expression levels were determined relative to TBP mRNA levels, and data are expressed as relative RQ values with WT7 = 1.

was equally expressed in pVHL-positive or pVHL-negative RCC cells and that HuR expression was unaffected by hypoxia (Fig. 1A). AUF1 levels also increased within 4 hours of addition of the hypoxia mimetic, desferrioxamine, in 786-O G7F RCC cells, whereas desferrioxamine treatment had no effect on AUF1 levels in 786-O 157A RCC cells (Fig. 1B). AUF1 shuttles between the nucleus and the cytosol. Immunofluorescence staining suggested that although desferrioxamine treatment increased total AUF1 levels, AUF1

Figure 1. VHL-, oxygen-, and proteasome-dependent regulation of AUF1 expression in RCC and 293T cells. AUF1 levels in pVHL-positive (786-O G7F) and pVHL-negative (786-O 157A) RCC cells that were cultured (A) under normoxic (room air) or hypoxic (1% O2) conditions for 18 hours or (B) in the presence of the hypoxia mimetic, desferrioxamine (DFO; 100 μmol/L), for 2 to 18 hours. U, untreated cells. C, 293T cells were cultured under normoxic or hypoxic conditions for 18 hours. D, 786-O G7F RCC cells were cultured in the absence or the presence of the proteasome inhibitor, MG132 (20 μmol/L), for 2 hours. For the experiments in panels A-D, protein extracts were prepared with RIPA buffer, and 40 μg cellular protein was resolved by 12% SDS-PAGE followed by Western blotting using the indicated antibodies. Tubulin or Cul2 detection were used as protein loading controls. E, quantitative real-time RT-PCR analysis of VEGF and AUF1 mRNAs in 293T cells that were cultured in normoxia or hypoxia for 18 hours. The primers used for PCR are specific for VEGF exons 1 and 3 and AUF1 exons 3 and 5, which are contained in all alternatively spliced forms of these mRNAs. Expression levels of the target mRNAs were determined in triplicate and averages are expressed relative to 18S rRNA levels. F, relative AUF1 mRNA levels in pVHL-positive, WT7, and pVHL-negative, PR9, RCC cells were determined by quantitative real-time RT-PCR. AUF1 mRNA expression levels were determined relative to TBP mRNA levels, and data are expressed as relative RQ values with WT7 = 1.
subcellular localization was not changed (Supplementary Fig. S1A). AUFI expression levels also increased after culture of 293T cells in hypoxia (Fig. 1C). As we previously showed (7), p25VHL expression and, to a lesser extent, p19VHL is suppressed by culture in hypoxia (Supplementary Fig. S1B). These results suggest that the hypoxia-induced increase in AUFI protein levels in 786-O G7F RCC cells or 293T cells may be linked to decreased pVHL levels.

We treated 786-O G7F RCC cells with the proteasome inhibitor, MG132, and found that AUFI levels increased (Fig. 1D), suggesting that the decreased AUFI protein levels detected in these cells was proteasome dependent. Similar results were seen after treatment with proteasome inhibitors, MG115 and lactacystin, as well as the calpain inhibitors ALLN and ALLM, which also inhibit proteasome activity (ref. 34; Supplementary Fig. S1C). On the other hand, treatment of 786-O 157A RCC cells with these same inhibitors had no effect on AUFI protein levels (Supplementary Fig. S1C). Our results suggest that AUFI protein is downregulated in pVHL-expressing RCC cells as compared with isogenic pVHL-negative RCC cells, and this down-regulation is dependent on proteasomal activity.

The data presented in Fig. 1A–C suggest that AUFI is a hypoxia-responsive gene product. However, quantitative RT-PCR analyses showed that total AUFI mRNA levels were decreased in 293T cells after 6 and 16-hour culture in hypoxia to 65% and 49%, respectively, of the levels of normoxic 293T cells (Fig. 1E). We also found that total AUFI mRNA levels were similar in the pVHL-positive and pVHL-negative cell lines that were tested (Fig. 1F). Therefore, the increased AUFI protein levels detected in hypoxia or in pVHL-positive and pVHL-negative cells was not likely to be due to differing AUFI mRNA levels, suggesting that a posttranscriptional mechanism accounts for the pVHL- and oxygen-dependent regulation of AUFI protein levels.

pVHL and AUFI associate in cells

We next found that AUFI and pVHL associate in cells. Either anti-pVHL or anti-AUFI antibodies coimmunoprecipitated a pVHL–AUFI complex from cells extracts from 786-O G7F RCC cells (Fig. 2A). 786-O G7F cells express both p25 and p19 VHL with a carboxy terminal Flag epitope tag, and both isoforms were coimmunoprecipitated with AUFI antisera. In reciprocal experiments, anti-Flag antibodies coimmunoprecipitated AUFI isoforms (Fig. 2A). Similar results were obtained with HA-VHL-expressing WT7 cells (Fig. 2B), providing independent confirmation of the pVHL–AUFI interaction. We also determined association of endogenous AUFI and endogenous pVHL in 293T cells. We previously showed that 293T cells express only p19 VHL (7), and p19 VHL–AUFI1 complexes were detected in reciprocal coimmunoprecipitations (Fig. 2C). Cul2 was present in both pVHL and AUFI immunoprecipitations (Fig. 2B and C), suggesting that the pVHL CRL complex associates with AUFI. Taken together, our results indicate that stable pVHL–AUFI complexes are isolated from pVHL-expressing RCC and 293T cell lines.

pVHL targets p45AUFI and p42AUFI for ubiquitylation

Because the pVHL CRL complex associates with AUFI and AUFI protein levels exhibited pVHL and oxygen dependence, we asked whether pVHL targets AUFI for ubiquitylation. UOK121 RCC cells, which do not express VHL mRNA or protein (35), were cotransfected with plasmids expressing Myc-tagged pVHL and HA-tagged ubiquitin and cultured in the presence of proteasome inhibitor MG132. pVHL coimmunoprecipitated with high molecular weight, polyubiquitylated AUFI species (Fig. 3A, lane 2), and the levels of polyubiquitylated AUFI species were enhanced when cells also overexpressed HA-tagged ubiquitin (Fig. 3A, lanes 1 and 6). We additionally carried out in vitro ubiquitylation assays and found that p45AUFI was targeted for polyubiquitylation when incubated with p25VHL (Fig. 3B, compare lanes 2 and 4 with lane 6). p45AUFI polyubiquitylation was reduced in the absence of added E2 (Fig. 3B, compare lanes 2 and 4), which may be due to low-level E2 activity contained within the reticulocyte extracts that were used to synthesize these proteins. Under our experimental conditions pVHL seemed to target p42AUFI for polyubiquitylation less efficiently than p45AUFI because the high molecular weight species were decreased relative to p45AUFI (Fig. 3B, compare lanes 2 and 3). In addition, anti-ubiquitin Western blotting showed that the major p42AUFI ubiquitylated species migrated with relative...
molecular weights of approximately 50 to 100 kDa (Fig. 3B, lane 3), suggesting multibiquitylation rather than polyubiquitylation. Ubiquitylation of p42\textsc{AUF1} was dependent on addition of both p25\textsc{VHL} and E2 ubiquitin-conjugating enzyme (Fig. 3B, lanes 1 and 5 versus lane 3). In similar experiments, we failed to detect polyubiquitylation of p37\textsc{AUF1} or p40\textsc{AUF1} (Fig. 3C). It is possible that pVHL may not target these isoforms for ubiquitylation, or that our experimental conditions (e.g., the use of a single E2 enzyme, UbcH5b) were not optimal for these in vitro reactions.

Mapping pVHL-AUF1–binding domains in vitro

To characterize pVHL–AUF1 interactions, we carried out GST fusion protein pull-down studies. We found that all 4 AUF1 isoforms bound to GST–VHVL\textsubscript{1-213} (i.e., full-length p25\textsc{VHL}) but not to GST alone (Fig. 4A). Because GST-pVHL associated with each AUF1 isoform in vitro, we further determined binding of the largest AUF1 isoform, p45, to a series of progressive GST-pVHL C-terminal deletions (Fig. 4B). We found that both GST-pVHL\textsubscript{1-173} and GST-pVHL\textsubscript{1-114} were able to bind p45\textsc{AUF1}, indicating that deletion of the Elongin C–binding pVHLα domain did not affect AUF1 binding (Fig. 4B). We also tested GST-pVHL\textsc{β} domain mutations that correspond to truncation at each of the first 4 \textbeta sheets as indicated in Fig. 4B (3, 4). Deletion of \textbeta sheet 4 (construct 1–101) and \textbeta sheet 3 (construct 1–89) did not disrupt p45 AUF1 binding, whereas p45 AUF1 binding to the GST-pVHL\textsubscript{1-78} construct was diminished (Fig. 4B), suggesting that AUF1 requires either \beta sheet 2, the loop between \beta sheet 1 and 2, or both for binding to pVHL. Therefore, pVHL seems to bind AUF1 through its target protein recognition domain.

To determine the region of AUF1 that binds to pVHL, we tested a series of GST-AUF1 deletion constructs (Fig. 4C). pVHL bound most strongly to GST-AUF1 constructs containing C-terminal sequences and, in particular, those GST-AUF1 constructs that contained amino acids 285 to 334, which are encoded by the AUF1 alternatively spliced exon 7 and the C-terminus (compare pVHL binding with GST-AUF1 constructs 3 or 5 with weak binding GST-AUF1 construct 2; Fig. 4C). Our data suggest that the RNA recognition motifs (RRM1 and RRM2) or the glutamine-rich domain (Q) are not necessary for pVHL binding to AUF1 (compare pVHL binding with GST-AUF1 constructs 3, 4, and 5; Fig. 4C). However, weak pVHL binding was also detected with AUF1 amino acids 1 to 112 (Fig. 4C), and sequences encoded by the AUF1 alternatively spliced exon 2 (amino acids 78–98) seemed to be required for this interaction (compare pVHL binding with GST-AUF1 constructs 6 and 7; Fig. 4C). It is likely that pVHL binding to GST-AUF1 construct 1 is also mediated through AUF1 amino acids 78 to 98 (Fig. 4C). Our in vitro interaction studies suggest that pVHL may associate with 2 separate AUF1 domains, which are focused in or around amino acids encoded by the alternatively spliced AUF1 exons 2 and 7 (see Fig. 4E). Although there does not seem to be significant

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We also tested the ability of internal pVHL deletions to bind to GST-AUF1 construct # 3, containing AUF1 amino acids 174 to 355. Full-length p25 VHL (1–213) and p19 VHL (54–213) both bound GST-AUF1<sup>174-355</sup> equally well (Fig. 4D), indicating that the pVHL acidic N-terminus is not required for AUF1 binding. Deletion of pVHL amino acids 115 to 155 (encoded by alternatively spliced exon 2 and corresponding to β sheets 5, 6, and 7 of the pVHL β domain) also did not affect VHL binding to GST-AUF1<sup>174-355</sup> (Fig. 4D). However, deletion of pVHL amino acids 60 to 114 (deleting β sheets 1–4) abrogated VHL binding to GST-AUF1<sup>174-355</sup> (Fig. 4D). Taken together, these results further suggest that the N-terminal portion of the pVHL β domain is required for AUF1 binding.

**AUF1 and HuR bind to VEGFA ARE RNA**

The relative roles of AUF1 and HuR in regulating VEGFA mRNA expression were determined through expression silencing experiments. Suppression of AUF1 expression in 293T cells resulted in a 1.7-fold increase in VEGFA mRNA levels (Fig. 5A), suggesting that AUF1 expression is associated with decreased VEGFA mRNA levels. In contrast, suppression of HuR expression resulted in a 30% decrease in VEGFA mRNA levels (Fig. 5A), which suggests that HuR binding to VEGFA mRNA is associated with mRNA stability and is in accord with earlier studies (24, 36). Suppression of AUF1 or HuR expression was verified by RT-PCR analyses (Fig. 5A) as well as by Western blotting (Supplementary Fig. S2). These experiments were done using 293T cells that were cultured under normoxic conditions. When similar silencing experiments were done with cells that were cultured in hypoxia, we did not detect efficient suppression of AUF1 or HuR mRNA or protein levels (data not shown) relative to control cells. The reason for this is unclear but suggests that the silencing apparatus may be sensitive to cellular oxygen levels.

The results presented above suggest that AUF1 may play a role in VEGF mRNA instability in normoxia. Each of the AUF1 isoforms in purified, recombinant form bound to VEGFA ARE1-RNA (see Supplementary Fig. S3A) as determined by RNA electrophoretic mobility shift assay (EMSA) or UV cross-linking (Fig. 5B). In addition, AUF1 antibody supershift assays resulted in retarded migration of protein–ARE complexes when 786-O RCC cytosolic RNA probes that were tested (Supplementary Fig. S3B and S3C),
suggested further that AUF1 may play a role in VEGFA regulation.

We next determined the functional effects of pVHL–AUF1 interactions by examining the role of AUF1 in regulating VEGFA mRNA stability. VEGFA mRNA levels were low in 786-O G7F RCC cells that were cultured in normoxia but were upregulated nearly 15-fold when these cells were cultured under hypoxic conditions (Fig. 6A). pVHL-negative 786-O 157Δ RCC cells expressed similar VEGFA mRNA levels when cultured either in normoxia or hypoxia (Fig. 6A). Because AUF1 and HuR regulate ARE-containing mRNA stability and seem to have opposing effects on VEGFA mRNA levels (Fig. 5A), we asked whether these proteins associate with VEGFA mRNA in RCC cells. Both AUF1 and HuR were found to be associated with VEGFA mRNA in 786-O 157Δ RCC cell extracts (Fig. 6B). Actin mRNA was not immunoprecipitated by AUF1 or HuR antibodies from these cells under our experimental conditions, and VEGFA mRNA was not amplified from irrelevant IgG nor from anti-pVHL immunoprecipitations (Fig. 6B). These results suggest that the RNP immunoprecipitations did not copurify mRNAs nonspecifically, and that AUF1 and HuR may play a role in controlling VEGFA mRNA levels in RCC cells.

Figure 5. AUF1 binds to VEGFA ARE RNA. A, 293T cells were transfected with a vector expressing AUF1 shRNA (32), an HuR siRNA pool, or appropriate controls. Total RNA was extracted 48 hours posttransfection and quantitative real-time RT-PCR analyses were done using VEGF-, AUF1-, or HuR-specific PCR primers. Expression levels of the target mRNAs were determined in triplicate and averages are expressed relative to TBPl mRNA levels, which were unaffected in these experiments. B, recombinant 6xHis-tagged AUF1 isoforms were expressed in bacteria and purified by metal affinity chromatography. For EMSA 100 ng recombinant protein was incubated with radiolabeled VEGF 3′-UTR ARE1 probe, treated with RNase A, and resolved on nondenaturing 8% polyacrylamide gels followed by autoradiography. For UV cross-linking (UV XL) complexes were exposed to UV light (300 mjoules), treated with RNase A, and resolved by 12% SDS-PAGE, and autoradiographed. C, radiolabeled VEGF 3′-UTR ARE1 probe was incubated in the presence of 100 μg protein cytosolic extracts that were prepared from 786-O RCC cells. Protein–RNA complexes were incubated with anti-AUF1, irrelevant rabbit anti-serum, or 1 mg/mL BSA. Samples were resolved on nondenaturing 8% polyacrylamide gels followed by autoradiography. AUF1-specific supershift complexes are indicated.

Figure 6. AUF1 and HuR associate with VEGFA mRNA in vivo and regulate VEGFA mRNA levels. A, pVHL-positive RCC cells (786-O G7F) and pVHL-negative RCC cells (786-O 157Δ) were cultured either under normoxic conditions (room air) or hypoxic conditions (1% O2) for 18 hours. Total cellular RNA was prepared and quantitative real-time RT-PCR reactions were done with VEGFA-specific PCR primers. VEGFA expression levels were determined in triplicate and averages are expressed relative to 18S rRNA levels. B, RNP immunoprecipitations were done from pVHL-negative 786-O 157Δ RCC cells with antibodies directed against AUF1, HuR, or pVHL, or an irrelevant rabbit IgG (rIgG). Immunoprecipitates were washed in lysis buffer, RNA was extracted, and RT-PCR was done using VEGF-specific or actin-specific PCR primers. Products of PCR reactions were resolved on 2% low molecular weight agarose gels containing ethidium bromide and photographed under UV light. Reverse photographic images are shown. The VEGFA PCR primers amplify products of 567, 495, 363, and 267 bp due to expression of alternatively spliced forms of the VEGF mRNA, and the β-actin primers amplify a 600-bp product (30).
pVHL associates with RNP–ARE RNA complexes under normoxic but not hypoxic conditions

As discussed above, VEGFA mRNA levels are regulated both transcriptionally and posttranscriptionally. To focus on posttranscriptional regulation, a heterologous luciferase reporter construct was tested in which VEGFA ARE1 was subcloned downstream of the luciferase open reading frame (Fig. 7A). This region contains a consensus AUUUA ARE site as well as (UA)x repeats, which form stem loop structures (26). Luciferase activity was detected in all of the cells that were transiently transfected with the luciferase–ARE1 construct, and when cells that express wild-type pVHL (293T cells and WT7 RCC cells) were cultured in hypoxia, luciferase activity was increased, 1.5-fold and 4-fold, respectively (Fig. 7B). Luciferase activity was comparable in the pVHL-negative ARZ2 cells when cultured under normoxic or hypoxic conditions (Fig. 7B). Luciferase activity of a construct containing a control fragment from the VEGFA 3′-UTR lacking ARE sequences (Fig. 7A) did not exhibit regulation by pVHL nor by hypoxia (Fig. 7B). Luciferase

Figure 7. Oxygen-dependent and pVHL-dependent expression of luciferase-VEGFA ARE1 constructs. A, a schematic diagram of the proximal portion of the VEGF 3′-UTR showing the regions that were independently subcloned into the 3′-UTR of the luciferase reporter. Nucleotide numbering is relative to the VEGFA translation termination codon. B, cell lines 293T, WT7 (pVHL positive), and ARZ2 (pVHL negative) were transiently transfected with plasmids containing either VEGFA ARE1 (Luc-ARE1) or control sequences (Luc control). After 24 hours transfected cultures were trypsinized and divided evenly into 2 dishes, and after an additional 6 hours of culture, one dish was transfected to a hypoxia (1% O2) incubator, and one dish was maintained in an incubator in room air (normoxia). Cell lysates were harvested after 12 hours and luciferase assays were done. CMV–β-gal plasmid was cotransfected and β-galactosidase activity was measured and used to normalize for transfection efficiency. **; *P = 0.00071; **; *P = 0.0093 (Student’s 2-tailed t test). C, 293T cells were transiently transfected with pGL3-Promoter luciferase reporter plasmid containing either VEGFA ARE1 (Luc-ARE1) or VEGF control sequences (Luc control), and cells were cultured under normoxic (room air) or hypoxic (1% O2) conditions as described above. Total RNA was extracted and RT-PCR using luciferase-specific PCR primers was done. D, 293T cells were transfected and cultured under normoxic (room air) or hypoxic (1% O2) conditions as described above. Actinomycin D (10 μg/mL) was added and total RNA was isolated at the indicated time points. RT-PCR analyses for luciferase mRNA expression were done and resolved on 2% low molecular weight agarose gels containing ethidium bromide. Band intensities were quantitated using the Quantity One software package (Bio-Rad), and luciferase mRNA levels were plotted relative to the untreated “0” time point. Nonlinear regression analyses showed that the luciferase-VEGF ARE1 mRNA had a half-life of 0.8 hours in normoxic cells and 3.8 hours in hypoxic cells. E, 293T cells were transfected as described above, and RNP-immunoprecipitations were done with antibodies directed against pVHL, AUF1, HuR, or irrelevant rabbit IgG (rIgG). Immunoprecipitates were washed in RNP immunoprecipitation buffer, RNA was extracted, and RT-PCR was done using luciferase-specific PCR primers. Products of PCR reactions were resolved on 2% low molecular weight agarose gels containing ethidium bromide and photographed under UV light. Reverse photographic images are shown.
mRNA levels were also determined by RT-PCR. In WT7 RCC cell transfectants luciferase–ARE1 mRNA levels were induced 3- to 5-fold in hypoxia, whereas luciferase control mRNA levels or actin mRNA levels were unaffected by hypoxia in those cells (Fig. 7C). Therefore, hypoxic induction of luciferase enzymatic activity reflected differences in luciferase mRNA levels.

Luciferase–ARE1 activity was consistently lower in pVHL-positive RCC cell transfectants as compared with pVHL-negative RCC cells. The SP1 transcription factor binds to GC boxes contained within the SV40 promoter of this vector and stimulates constitutive luciferase transcription. Because pVHL binds to SP1 and suppresses its activity (16, 37), it is possible that we are observing both transcriptional and posttranscriptional effects in these studies. To determine whether pVHL played a role in regulating luciferase–ARE1 mRNA stability in RCC cells, WT7 RCC cells were transfected with the luciferase–ARE1 construct and then cultured in normoxia or hypoxia in the presence of the transcription inhibitor, actinomycin D. RT-PCR and nonlinear regression analyses of the data determined that the half-life of luciferase–ARE1 mRNA was 0.8 hours in normoxic WT7 RCC cells and 3.8 hours in hypoxic WT7 RCC cells (Fig. 7D). Our results suggest that the VEGFA ARE1 sequence functions as a destabilizing element when expressed in the luciferase mRNA 3′-UTR, and that this mRNA is stabilized in hypoxic pVHL-expressing RCC cells.

Because the luciferase–ARE1 mRNA exhibited both pVHL- and hypoxia-dependent posttranscriptional regulation, we determined whether AU1 and HuR associate with the luciferase–ARE1 mRNA in transiently transfected 293T cells. Both AU1 and HuR antibodies were able to coimmunoprecipitate luciferase mRNA from luciferase–ARE1 transfectants that had been cultured either in normoxia or in hypoxia (Fig. 7E). Neither AU1 nor HuR antibodies immunoprecipitated the luciferase control mRNA (Fig. 7F). We also carried out parallel RNP immunoprecipitations with pVHL antibody and found that pVHL coimmunoprecipitated with luciferase–ARE1 mRNA in 293T cell transfectants that were cultured in normoxia but not when cultured in hypoxia when pVHL levels are suppressed (Fig. 7E). Therefore, pVHL is found in a complex with ARE mRNA, but we cannot determine from these studies whether pVHL binds ARE RNA directly or indirectly.

Association of AU1 or HuR complexes with an independent ARE from the TNFα ARE was also determined. Both AU1 and HuR exhibited specific binding to the TNFα ARE RNA but little to no binding to the ARE-mutated RNA that was tested (Fig. 7F). We next incubated extracts from normoxic and hypoxic 293T cells with radiolabeled TNFα ARE RNA, subjected the protein–RNA complexes to UV cross-linking, and carried out immunoprecipitations. We found that AU1, HuR, and pVHL antibodies each coimmunoprecipitated TNFα ARE RNA from normoxic 293T cell extracts (Fig. 7G). In hypoxia, when pVHL levels are suppressed, anti-pVHL did not immunoprecipitate TNFα ARE RNA (Fig. 7G). The RNP complexes that were identified in anti-pVHL immunoprecipitations migrated in SDS-polyacrylamide gels between 30 to 50 kDa and were similar to the RNP complexes seen in AU1 or HuR immunoprecipitations. These results suggest that pVHL did not bind directly to the TNFα ARE RNA because we did not detect a signal migrating at approximately 19 kDa in the anti-pVHL immunoprecipitations. Because pVHL associates with AU1 (this study) and with HuR (38), our results suggest an indirect association of pVHL with ARE RNA through AU1 and/or HuR complexes.

**Discussion**

Our focus in this article was on interactions between pVHL and the ARE RNA-binding protein, AU1. AU1
protein levels were elevated in the pVHL-negative RCC cells as compared with the isogenic pVHL-positive RCC cells that were tested. In addition, in pVHL-positive cells, AUF1 protein levels were increased in hypoxia or with culture in the presence of proteasome inhibitors. pVHL binds to AUF1 through its target protein recognition domain and targeted p45<sub>AUF1</sub> and p42<sub>AUF1</sub> for ubiquitylation in vitro. In hypoxia, when pVHL expression is suppressed, AUF1 protein levels were elevated. Taken together, our results are consistent with a model in which pVHL controls at least some AUF1 isoforms through ubiquitin-mediated proteasomal degradation and that AUF1 proteins may be commonly overexpressed in clear-cell RCC with inactivated pVHL.

We also found that AUF1 bound to ARE RNA under both normoxic and hypoxic culture conditions. The association of AUF1 with VEGFA ARE RNA in normoxia is consistent with a destabilizing activity of AUF1. However, because pVHL levels are suppressed in hypoxia (7) and AUF1 levels are increased in hypoxia (Fig. 1), our results also indicate that AUF1 binding to ARE RNA may be associated with VEGFA mRNA stability in hypoxia. These results suggest that the presence or absence of pVHL in the ARE-binding complex constitutes at least one determining factor for VEGFA ARE-mediated decay or stability, respectively (Fig. 7H). Whether pVHL ubiquitin-ligase activity is required for the targeting of ARE RNA for degradation remains to be determined. This is a difficult question to address experimentally because uncoupling pVHL from the CRL complex renders pVHL unstable (39).

The relationship between proteasome activity and turnover of ARE-containing mRNA has been explored previously. Proteasome inhibition resulted in suppressed ARE-mediated RNA decay, suggesting the importance of the ubiquitin–proteasome system for mRNA degradation (40). p37<sub>AUF1</sub> and p40<sub>AUF1</sub> were shown to be targeted for ubiquitylation in response to heat shock (41), and p37<sub>AUF1</sub> and p40<sub>AUF1</sub>, but not p42<sub>AUF1</sub> or p45<sub>AUF1</sub>, were found to be ubiquitylated in vitro in studies that used HeLa cell extracts as a source of E1, E2, and E3 enzymes (42). Although we did not detect pVHL-dependent ubiquitylation of p37<sub>AUF1</sub> and p40<sub>AUF1</sub> under our experimental conditions (Fig. 3), it is clear that the ubiquitin–proteasome system plays a role in ARE-mediated RNA decay.

AUF1 was found to bind ARE RNA in both normoxia and hypoxia. Suppression of AUF1 expression resulted in elevated VEGFA mRNA levels in normoxia, suggesting that AUF1 destabilizes VEGFA mRNA (Fig. 5). However, we could not determine the effects of AUF1 suppression on VEGFA mRNA in hypoxia. Because VEGFA mRNA is stabilized in hypoxia (Figs. 6 and 7), this would suggest that AUF1 may stabilize this mRNA in hypoxia, or alternatively, AUF1 may play some other regulatory role. For example, the association of AUF1 with VEGFA ARE RNA in hypoxia could provide a mechanism to regulate mRNA translation. AUF1 complexes with translation initiation factor 4G (eIF4G) and poly(A)-binding protein (PABP; ref. 41), suggesting that AUF1 may play a role in translation initiation. AUF1 may stimulate VEGFA translation in a manner similar to its enhancing effect on c-Myc translation by competing for ARE binding with a translation inhibitor, TIAR (43). Because AUF1 protein levels were found to increase in hypoxia (Fig. 1), this would provide an advantage for AUF1 binding in competition with other ARE-binding factors. On the other hand, several reports showed that ARE-mediated RNA degradation was dependent on translation (44–46). Association of AUF1 with VEGFA mRNA in hypoxia may limit the amount of VEGFA protein produced or provide a mechanism to rapidly degrade VEGFA mRNA when hypoxic stress is alleviated.

We characterized here pVHL interactions with AUF1. Earlier studies showed that pVHL binds to other ARE-binding proteins: hnRNP A2 (47), HuR (38), and Tis11b (27). The interaction between hnRNP A2 and pVHL required formation of the pVHL CRL complex, and hnRNP A2 protein levels were downregulated by pVHL in RCC cells. These results are similar to those shown in this article for AUF1. Datta and colleagues (38) found that pVHL modulated binding of HuR to ARE RNA. They concluded that pVHL expression leads to VEGFA mRNA decay by blocking the ARE RNA stabilizing activity of HuR. We did not detect modulation of HuR protein levels in the cell lines tested in our studies (Fig. 1), and we detected HuR association with ARE sequences in both pVHL-positive and pVHL-negative RCC cells. However, our RNP immunoprecipitation studies were not quantitative assays, and, therefore, we cannot determine whether total levels of HuR or AUF1 that bound to the ARE RNAs tested were modulated by pVHL or hypoxia.

AUF1 and HuR have been shown to bind independently to some mRNAs and concurrently to other mRNAs (33), which was not addressed in our studies. Because AUF1 binds ARE RNA as hexamers (48) and active HuR binds as a homodimer (49), it is possible that higher order complexes containing pVHL, AUF1, and HuR exist. Other proteins may also be involved. For example, AUF1 associates with eIF4G and PABP (discussed above), as well as with Hsc70-Hsp70 and Hsp27. This complex has been referred to as an AUF1- and signal transduction-regulated complex, which recruits RNases to degrade ARE-containing RNA (50). Thus, the stoichiometry of protein–protein interactions and protein–ARE RNA interactions may ultimately determine mRNA stability or decay. Stoichiometry will be affected by the relative expression levels of the various proteins, and we predict that events such as pVHL loss in RCC or hypoxic induction of AUF1 would lead to stabilization of ARE-containing mRNAs. In addition, various posttranslational modifications, including phosphorylation, ubiquitylation, SUMOylation, and/or methylation, of pVHL, AUF1, HuR, or other interacting partners are likely to play a role in determining ARE-mediated RNA stability or decay.

The relationship between ARE-mediated mRNA stability or decay and miRNAs is not yet clear. Protein–ARE RNA interactions may recruit miRNA-containing RNA-induced silencing complex or they may directly block miRNA interactions with target sequences. Protein binding may also influence RNA secondary structure as was recently shown in the VEGFA 3′-UTR (26), which could in turn affect miRNA interactions. Although our data suggest that
VEGFA mRNA stability was mediated through protein–ARE RNA interactions, the relationship between AUFI and/or HuR association with ARE RNA and miRNAs remains to be explored.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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