DISSERTATION

ROLE OF HUR, AUF1 AND ZETA-CRYSTALLIN IN MEDIATING pH-RESPONSIVE INCREASE IN RENAL PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) mRNA ABUNDANCE IN KIDNEY CELLS

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ABSTRACT

ROLE OF HUR, AUF1 AND ZETA-CRYSTALLIN IN MEDIATING pH-RESPONSIVE INCREASE IN RENAL PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) mRNA ABUNDANCE IN KIDNEY CELLS

The maintenance of blood acid-base balance is essential for survival. However, metabolic acidosis is a common clinical condition that is characterized by a significant decrease in plasma pH and bicarbonate concentration. This alteration is caused by genetic or acquired defects in metabolism, in renal handling of bicarbonate, and in the excretion of titratable acid. In addition, metabolic acidosis could pose a secondary complication in patients with cachexia, trauma, uremia, end stage renal disease, osteomalacia, HIV infection and in patients with degenerative diseases. Increased renal ammoniagenesis and gluconeogenesis from plasma glutamine constitute an essential physiological response to metabolic acidosis that partially restores acid-base balance. A portion of this adaptive response is the rapid and pronounced increase in the cytosolic isoform of phosphoenolpyruvate carboxykinase (PEPCK) that occurs within the renal proximal convoluted tubule. Previous *in vitro* biochemical studies have mapped the binding of AUF1, HuR and ζ-crystallin (ζ-Cryst) to various AU-rich sequences within the 3'UTR of PEPCK mRNA. This response is reproduced in LLC-PK_1-F^+9C cells that are treated with acidic (pH 6.9) medium. It is mediated, in part, by stabilization of PEPCK mRNA.

Here I have used a combination of approaches to characterize the dynamic interaction of *trans*-acting factors with the *cis*-acting elements in mediating the pH-responsive stabilization of PEPCK mRNA. In chapter III I show that binding of HuR and

AUF1 have opposite effects on basal expression, but their co-ordinate interaction is required to mediate the pH-responsive adaptation. Consistent with this, while the individual recruitment of a chimeric protein containing the MS2 coat protein and either HuR or p40AUF1 failed to produce a pH-responsive stabilization, the concurrent expression of both chimeric proteins was sufficient to produce a pH-responsive increase in the half-life of the reporter mRNA. This study also demonstrated that HuR and AUF1 underwent profound altered post-translational modifications when LLC-PK_1-F^+9C cells were switched from basal to acid-pH medium conditions. In Chapter IV I went on to demonstrate that HuR makes direct interaction with PEPCK mRNA and that HuR/ AUF1 form hetero-oligomeric complex in an RNA-dependent manner. Finally in Chapter V I investigated the functional significance of the ζ-Cryst binding to the PEPCK-3'UTR. These experiments suggested that ζ-Cryst may serve as a key co-factor along with HuR and AUF1 to restrict the basal expression and that only HuR and AUF1 are required for the pH-responsive increase of PEPCK mRNA.

Based upon the findings of the current study, I proposed a model depicting the co-ordinate role of HuR, AUF1 and ζ-Cryst in post-transcriptional regulation of PEPCK mRNA turnover and, more importantly in mediating the sustained pH-responsive increase of PEPCK mRNA. Under normal acid-base conditions, phosphorylated HuR, covalently modified AUF1 and ζ-crystallin are co-recruited to the 3'-UTR of PEPCK mRNA and may form a complex through direct protein-protein interactions. The binding of the three RNA-binding proteins leads to recruitment of a deadenylase that removes the poly-A tail and leads to the subsequent decapping and 5'→ 3' decay of the deadenylated PEPCK mRNA. However onset of metabolic acidosis leads to alterations in post-

translational modifications including decreased phosphorylation of HuR and an increased phosphorylation of AUF1. These changes may promote the dissociation of ζ-crystallin from the RNA-binding complex. This remodeling of the ribonucleoprotein-complex blocks the association of deadenylases and maintains a poly-A tail that is well protected by the poly-A binding protein (PABP). Therefore, this remodeling of the protein/mRNA complex mediates the enhanced stabilization and translation of PEPCK mRNA.

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CHAPTER 1

INTRODUCTION

1.1 Metabolic Acidosis

The human kidney consists of approximately one million specific structural units called nephrons. Each nephron starts with a renal glomerulus, which is the site of primary filtration of blood. The filtrate enters the proximal convoluted tubule where approximately 80% of the water and solutes are reabsorbed. The remaining segments, the loop of Henle, the distal tubule and collecting ducts are responsible for reabsorption of the appropriate amount of water and solutes to maintain fluid and electrolyte balance. The kidneys also participate in maintenance of acid-base balance (pH 7.40 ± 0.04) of the extracellular environment. The relative concentrations of the HCO_3^- and CO_2 are the primary determinants of plasma pH and are regulated by the kidneys and the lungs, respectively. However, blood pH values outside the normal range define an acidosis (pH < 7.36) or an alkalosis (pH > 7.44).

The term metabolic acidosis (MA) refers to a systemic disorder that is characterized by a decrease in plasma [HCO_3^-] and extracellular pH. It can result from either increased acid production or impairment in renal acid excretion. Major causes of metabolic acidosis are:

I. Increased acid production that occur during

a) Lactic acidosis, ketoacidosis (uncontrolled diabetes mellitus).

b) A high protein diet or conditions that cause the rapid utilization of endogenous protein also produce an acidosis.

c) Loss of bicarbonate either due to gastrointestinal disorders (diarrhea) or a defect in the renal excretion of H^+ ions or in the reabsorption of the HCO_3^- ions from the body (renal tubular acidosis)

II. Decreased acid excretion either due to

a) Decreased NH_4^+ excretion (renal failure) or

b) Decrease in glomerular filtration rate (Type 1 and Type 4 renal tubular acidosis)

1.2 Glutamine

Glutamine serves as a primary carrier of nitrogen between organs. Glutamine is synthesized by the glutamine synthetase, which catalyzes the ATP-dependent ligation of an ammonium ion and glutamate to form glutamine (Eq 1).

Eq 1: $\text{L-Glutamate}^- + NH_4^+ + ATP \longrightarrow \text{L-Glutamine} + ADP + P_i$

Glutamine synthetase is a cytosolic enzyme that is expressed most abundantly in muscle, lungs, brain, liver and adipose tissues. The catabolism of glutamine is initiated by a mitochondrial glutaminase that catalyzes the hydrolytic cleavage of glutamine to form glutamate and an ammonium ion (Eq 2).

Eq 2: $\text{L-Glutamine} + H_2O \longrightarrow \text{L-Glutamate}^- + NH_4^+$

The body expresses two isoforms of the mitochondrial glutaminase (Krebs 1935), liver-type glutaminase (LGA) and kidney-type glutaminase (KGA). The two isoenzymes not only differ in their structural and kinetic properties, which leads to their unique function and short-term regulation, but the two GA isoforms also vary in their pH optima and their

inhibition by the end product of the reaction, glutamate (Meister 1975). Glutamine plays a crucial role in metabolic pathways; in the liver, glutamine is an important substrate for ureagenesis and gluconeogenesis (Watford et al. 2002) , while in kidney it plays a key role in gluconeogenesis and ammoniagenesis (Curthoys and Gstraunthaler 2001). In addition, glutamine is also consumed as a primary metabolic fuel for rapidly dividing cells, including intestinal epithelial cells (Mithieux 2001), lymphocytes (Newsholme 2001), and various transformed cells (Medina 2001).

1.2.1 Renal Glutamine metabolism during normal physiology

During normal acid-base balance, the kidney extracts and metabolizes very little of the plasma glutamine (Squires et al. 1976). The measured rat renal arterial-venous difference is less than 3 percent of the arterial concentration of glutamine. Although approximately 20% of the plasma glutamine enters the filtrate and is extracted from the lumen of the nephron, but very little is catabolized by the kidney (Squires et al. 1976). In fact most of the filtered glutamine is reabsorbed within the proximal convoluted tubule (Silbernagl 1980) and then transported across the basolateral membrane via the LAT2, a Na^+- independent neutral amino acid transporter (Seow et al. 2004). Utilization of a small amount of glutamine that is extracted by the proximal tubule cells requires its transport into the mitochondria, which is facilitated by a mersalyl-sensitive electroneutral uniporter (Sastrasinh and Sastrasinh 1989). In the mitochondrial matrix, glutamine is deamidated by a phosphate-activated glutaminase (GA) and then glutamate dehydrogenase (GDH) oxidatively deaminates the glutamate to form α-ketoglutarate, which enters the citric acid cycle. Thus during normal acid-base balance, two-thirds of the ammonium ions produced

by GA and GDH are trapped in the tubular lumen and excreted into the urine, which is slightly acidified (pH 6.8); the remainder is added to the renal venous blood (Fig.1.1).

1.2.2 Renal ammoniagenesis following acute metabolic acidosis

Acute onset of a metabolic acidosis produces a rapid increase in the renal catabolism of glutamine, resulting in increased renal ammoniagenesis and gluconeogenesis (Sleeper et al. 1978). Within 1-3 hrs, there is a two-fold increase (Hughey et al. 1980) in the arterial plasma glutamine pool that is facilitated by release of glutamine from the muscle tissues (Schrock et al. 1980). Significant renal extraction of glutamine becomes evident as the arterial plasma concentration is increased. The net extraction reaches 30 percent of the plasma glutamine, and therefore exceeds the percent filtered by the glomeruli. Thus, the direction of the basolateral glutamine transporter (LAT2) must be reversed in order for the proximal convoluted tubule to extract glutamine from both the glomerular filtrate and the venous blood. Furthermore, the transport of glutamine into the mitochondria is also acutely activated (Sastrasinh and Sastrasinh 1990). Additionally, the apical Na^+/H^+ exchanger (NHE3) is activated to produce a prompt acidification of the urine (Peng et al. 2001), which facilitates the rapid excretion of cellular ammonium ions in the urine (Tannen and Ross 1979). Finally, a pH-induced activation of α-ketoglutarate dehydrogenase reduces the intracellular concentrations of glutamate and α-ketoglutarate (Lowry and Ross 1980). Thus, acute acidosis leads to an increase in the catabolism of glutamine, which initially results from a rapid activation of the key transport processes, increased glutamine availability, and a decreased

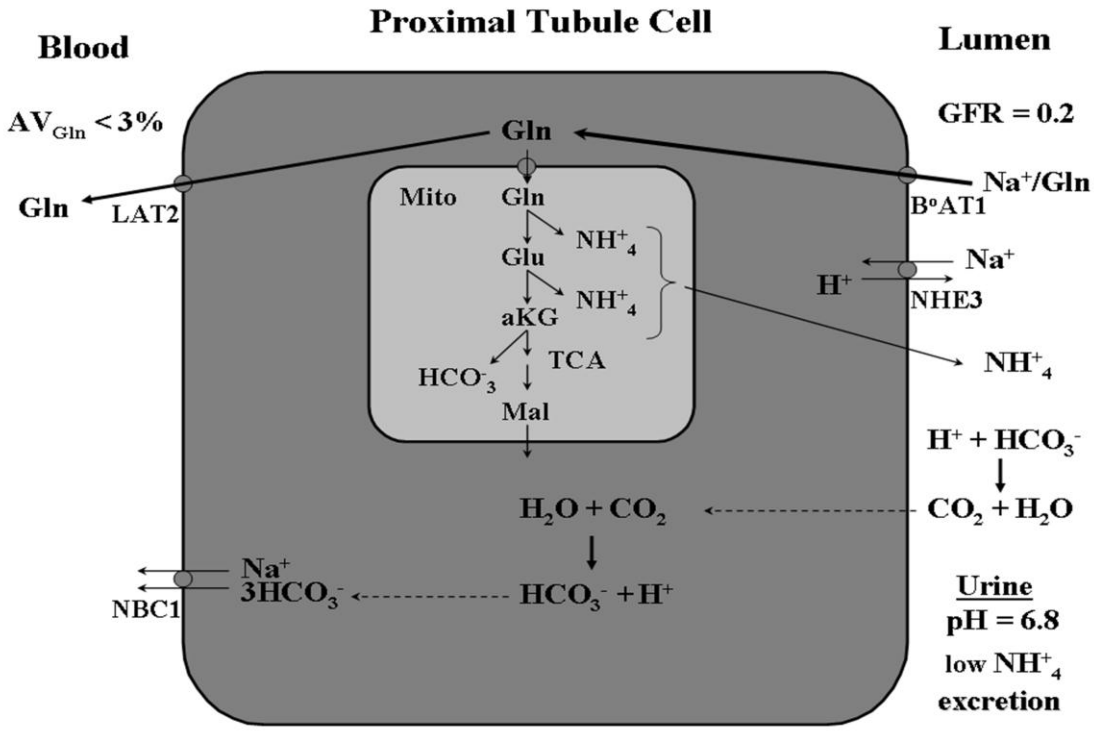

Fig.1.1: Pathways of renal glutamine catabolism and bicarbonate reabsorption during normal acid-base balance (adapted from (Curthoys 2011) with permission). The glomerular filtrate is slightly acidified by the apical Na^+/H^+ exchanger, NHE3. The translocated H^+ ions titrate most of the filtered HCO_3^- ions producing carbonic acid which is converted to CO_2 and H_2O by the apical carbonic anhydrase. The CO_2 diffuses into the proximal tubule and is hydrated by the cellular carbonic anhydrase to reform bicarbonate ions that are translocated across the basolateral membrane by NBC1. The glutamine filtered by the glomeruli is transported across the basolateral membrane by LAT2. A small portion of the recovered glutamine is transported into the mitochondria where it is catabolized to generate the basal level of ammonium ions that are excreted in the slightly acidified urine.

concentration of the products of GA and GDH reactions. The net effect is rapid and enhanced excretion of ammonium ions and titratable acid in the urine (Sleeper et al. 1978).

1.2.3. Chronic adaptations to renal metabolic acidosis

During chronic metabolic acidosis, most of the acute adaptations are partially compensated. However, the kidney continues to extract more than one-third of the total plasma glutamine (Squires et al. 1976). The increased expression of the genes that encode the mitochondrial GA, mitochondrial GDH, and the cytosolic phospho*enol*pyruvate carboxykinase (PEPCK) facilitate the renal catabolism of the absorbed glutamine. The activities of these three key enzymes are increased only in the proximal tubule cells, which is also the primary site of renal ammoniagenesis (Burch et al. 1978). The adaptive increases in gene expression may be initiated by a decrease in the intracellular pH (pH_i). The adaptations in GA and PEPCK levels result from increased rates of synthesis of the proteins (Iynedjian et al. 1975; Tong et al. 1986) that correlate with concomitant increases in the levels of their respective mRNAs (Cimbala et al. 1982; Hwang et al. 1991). However, the initial increase in the PEPCK mRNA levels results from enhanced transcription of the *PEPCK* gene (Hanson and Reshef 1997), whereas mRNA stabilization produces the increase in the GA mRNA levels (Laterza et al. 1997) and contributes to the sustained increase in PEPCK mRNA (Mufti et al. 2011). The levels of the mitochondrial glutamine transporter (Sastrasinh and Sastrasinh 1990), the apical Na^+/H^+ exchanger, NHE3 (Preisig and Alpern 1988), the basolateral SN1 glutamine transporter (Karinch et al. 2002), and the apical Na^+-dicarboxylate cotransporter, NaDC-

1(Aruga et al. 2000), the basolateral Na^+ /$3HCO_3^-$ co-transporter, NBC1 (Preisig and Alpern 1988) are also increased during chronic acidosis. The increased renal ammoniagenesis provides an expendable cation that facilitates the excretion of titratable acids while conserving sodium and potassium ions. The increased apical Na^+ /H^+ exchanger activity sustains the acidification of the fluid in the tubular lumen and promotes the tubular reabsorption of HCO_3^- ions. In addition, the generated α-ketoglutarate is converted to glucose, generating 2 HCO_3^- ions per α-ketoglutarate. The increase in basolateral Na^+ /$3HCO_3^-$ co-transporter, NBC-1, facilitates the translocation of the reabsorbed and the *de novo* synthesized HCO_3^- ions into the renal venous blood. Thus, the combined adaptation creates a net release of HCO_3^- ions that contribute to the ability of the kidney to compensate the systemic acidosis (Fig.1.2).

1.3 Life span of an mRNA and its regulation

Eukaryotic gene expression can be regulated at multiple stages, including mRNA transcription, splicing, export, stability, localization and translation. Depending on the physiological stimuli and the type of mRNA species, the mRNA may be destined for regulated turnover at the end of its life cycle. It is also subjected to strict surveillance throughout its biogenesis, processing and translation to ensure the quality of the resulting protein products. The complex and coordinated interplay between the *trans*-acting factors-primarily RNA binding proteins (RBPs) (Keene and Tenenbaum 2002; Keene and Lager 2005) and the multiple *cis*- regulatory elements within the untranslated regions (UTR) of mRNA contribute to the regulated degradation of mRNAs. (Chen and Shyu 1995; Vlasova and Bohjanen 2008; Clark et al. 2009; Lee et al. 2010).

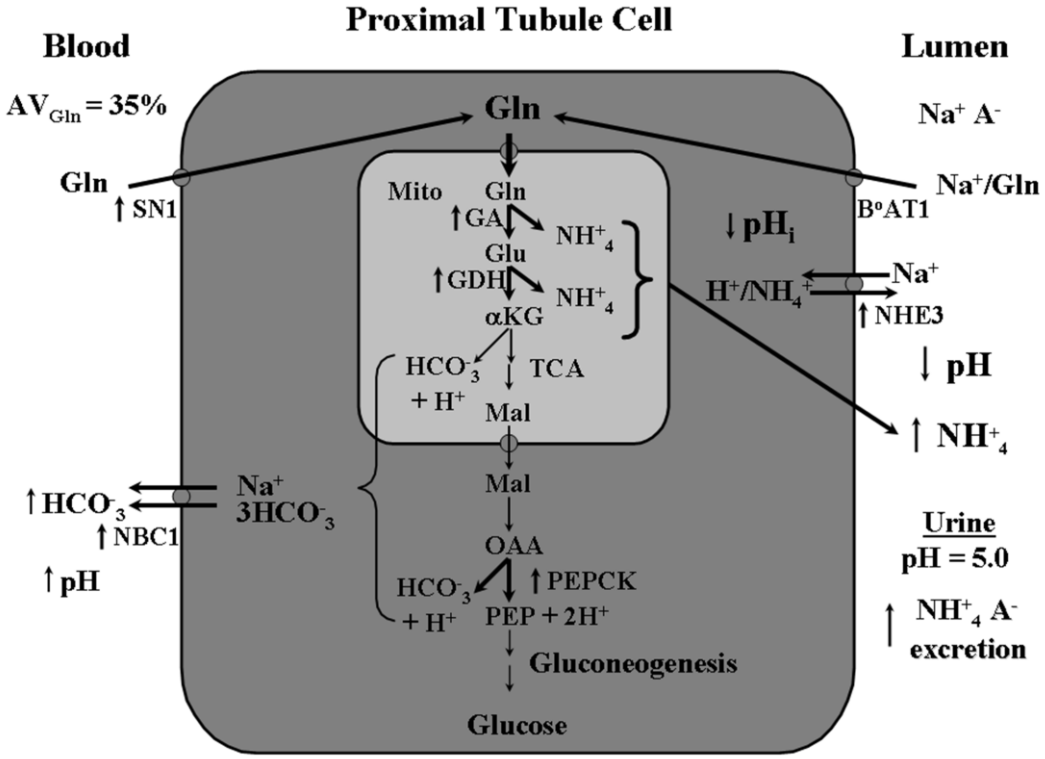

Fig.1.2: **Pathway of renal proximal tubular glutamine catabolism during chronic acidosis (adapted from (Curthoys 2011) with permission).** Increased renal catabolism of glutamine is sustained during chronic acidosis by increased expression of the genes that encode glutaminase (GA), glutamate dehydrogenase (GDH), phospho*enol*pyruvate carboxykinase (PEPCK), the mitochondrial glutamine transporter, the apical Na^+/H^+ exchanger (NHE3), the basolateral glutamine transporter (SN1), and the basolateral $Na^+/3HCO_3^-$ co-transporter (NBC1). The increased proteins are indicated by the thicker arrows. Some of the ammonium ions are transported across the apical membrane by NHE3. The combined increases in renal ammonium ion excretion and gluconeogenesis result in a net synthesis of HCO_3^- ions.

1.4 ARE (AU-rich element) mediated RNA decay

AREs or AU-rich elements were one of the first and best-characterized *cis*-encoded regulatory sequences within the 3'-UTR of several cytokines and oncogenes (Caput et al. 1986). It has been estimated that AREs are present in 8% of mammalian transcripts (Bakheet et al. 2006). AREs can range in size from 50-150 nucleotides in length (Bevilacqua et al. 2003) and have been classified into three classes based on the number and distribution of the AUUUA pentamers (Chen and Shyu 1995). Class I AREs contain one to three scattered copies of pentamers embedded in U-rich regions of 3'-UTRs of c-fos and c-myc mRNAs. TNF-α and GM-CSF mRNAs contain the class II AREs, with the characteristic feature of two or more overlapping copies of UUAUUUAUU nanomeric sequence. The Class III AREs lack the consensus ARE-AUUUA pentamer, but require a U-rich and possibly other unknown features that render the mRNA unstable, as in case of c-jun mRNA (Xu et al. 2001). Binding of multiple RBPs to these AU-rich regions can either stabilize or destabilize the mRNA (Chen and Shyu 1995) and regulate translation of the mRNA (Ross 1995).

1.5 Trans-acting factors and their role in modulating mRNA stability

A plethora of RBPs are recruited to the primary sequence elements of the mRNA, often displaying tissue or cell-type specific expression pattern. Another important aspect of RBP regulation is the coordinated binding of a given RBP to multiple mRNA species (Morris et al. 2010), and *vice-versa*, depending on the physiological stimuli and cellular localization of the RBP(s) and associated mRNA(s). Among the number of ARE-binding proteins known to regulate the post-transcriptional fate of the associated mRNA, HuR

(ELAV- family) and AUF1 (AU-binding factor 1/ hnRNP D) are well-studied RBPs. However, there is far more to be explored in terms of their mechanism of regulating mRNA decay.

HuR- HuR belongs to the Hu-family of RBPs that are involved in diverse molecular roles affecting the post-transcriptional fate of the bound mRNA. HuR is an ubiquitously expressed family member with key roles in the apoptotic-response of certain genes involved in DNA damage and other types of stresses (Lal et al. 2005; Mazroui et al. 2008). HuR regulates the post-transcriptional fate of mRNAs involved in oncogenesis, muscle differentiation, immune response and other cellular-lethal agents. In most cases HuR contributes to the stabilization of target mRNAs (Hinman and Lou 2008). The protein structure of HuR (Fig.1.3) reveals a typical RBP like structure with three RNA recognition motifs (RRM), where RRM1 and RRM2 were shown to bind to the AU-rich region of the mRNA (Wang and Tanaka Hall 2001). RRM3 is thought to bind the poly-A tail to aid in stabilizing the RNA-protein complex or mediate protein-protein interactions (Beckel-Mitchener et al. 2002; Kasashima et al. 2002). Unlike the RRMs, the less conserved hinge region between RRM2 and 3 contains the HuR nucleocytoplasmic shuttling sequence (HNS). This segment includes both a nuclear localization signal and a nuclear export signal, allowing HuR to shuttle between nucleus and cytoplasm (Fan and Steitz 1998). While predominantly nuclear, the HNS domain in association with the adaptor proteins, pp32/PHAP-1, APRIL and transportin-1 and 2, mediate the nucleocytoplasmic export of HuR (Brennan et al. 2000; Gallouzi and Steitz 2001; Fan et al. 2003; Rebane et al. 2004).

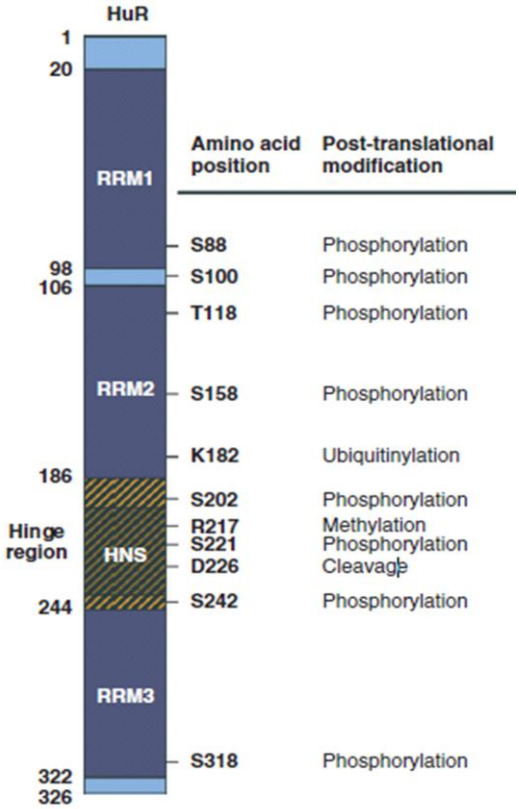

Fig.1.3: Schematic representation of HuR, showing three RNA-Recognition Motifs (RRMs) (adapted from (Abdelmohsen and Gorospe 2010). Hinge region (186-244) containing HuR nucleocytoplasmic shuttling (HNS) domain is spanned between RRM2 and RRM3. Covalent modifications on different residues of HuR are depicted, which were identified either by mass-spectrometry or mimetic-mutants.

Although there is an enormous literature on HuR and its ability to stabilize mRNA ligands, the actual molecular mechanism by which HuR acts as a stabilizing RNA-binding factor is still elusive. Nevertheless, there are few hypotheses that are popular in the field. The foremost observation is that HuR is largely translocated to the cytoplasm at the onset of cellular stresses, where it promotes mRNA stability and increases the rate of translation of target mRNAs. In addition, post-translational phosphorylation of HuR by protein kinase C (PKC), the mitogen activated protein kinase p38, and cyclin-dependent kinase 2(Chk2) alters its association with mRNA, and thus its stabilizing effect (Masuda et al. 2011). HuR methylation of R217 in the hinge region, via coactivator- associated arginine methyltransferase 1(CARM1), enhanced mRNA stabilization (Li et al. 2002). Another potential mechanism is that HuR may contribute to mRNA stabilization by packaging oligomeric protein complexes on mRNA substrates through cooperative binding, as observed with the TNF-α ARE (Fialcowitz-White et al. 2007). Furthermore, FRET analysis has established that HuR can undergo homomultimerization (David et al. 2007). Therefore, HuR oligomerization could block the association of destabilizing proteins. The RRM3 was shown to be a key component in mediating this co-operative binding of HuR (Fan and Steitz 1998). Finally, HuR may function as an mRNA stabilizing factor by recruiting bound mRNAs to the translation machinery, as opposed to destabilizing RNA binding proteins which associate with decay machinery (Hinman and Lou 2008).

AUF1-ARE/ poly(U)-binding/ degradation factor1 (AUF1) also known as hnRNPD is one of the best characterized ARE-binding proteins. It binds not only bind to poly(A) segments but also to poly(U) regions (Sagliocco et al. 2006). The AUF1 gene gives rise (Pinol-Roma et al. 1988) to four isoforms that are designated by their molecular weights as p37, p40, p42, and p45 (Fig.1.4). The four isoforms result from alternative splicing of a single pre-mRNA (Wagner et al. 1998). The four isoforms differ in the absence or presence of exon 2 and exon 7. All four AUF1 isoforms share a common N-terminal alanine-rich stretch of 28 amino acids potentially required for protein oligomerization, and a C-terminal glutamine-rich domain required for AUF1-RNA interaction. Of the four isoforms, p37 is the smallest in length. It lacks both the 19-amino acids encoded by exon 2 and 49 amino acids from exon 7. While p40 and p42 isoforms contain the sequence from exon 2 and exon 7, respectively, the largest isoform, p45 has both inserts (DeMaria et al. 1997). While AUF1 is predominantly nuclear, all four isoforms shuttle between the nucleus and cytoplasm depending on the physiological stimulus (Sarkar et al. 2003). The C-terminal domains of p37AUF1 and p40AUF1 contain a nuclear import signal (NIS) and thus are largely retained in the nucleus. However, the inclusion of exon 7 in p42AUF1 and p45AUF1 causes them to be localized primarily in the cytoplasm (Sarkar et al. 2003).

Among the various post-translational modifications identified thus far in AUF1 proteins, phosphorylation and ubiquitination are thought to be the most important in terms of regulating their function. Original i*n vitro* experiments and prediction from an online the phosphodatabase search engine PhosphoBase suggest two serines, Ser83 and Ser87 located within exon 2 as possible kinase sites (Tolnay et al. 2000). The

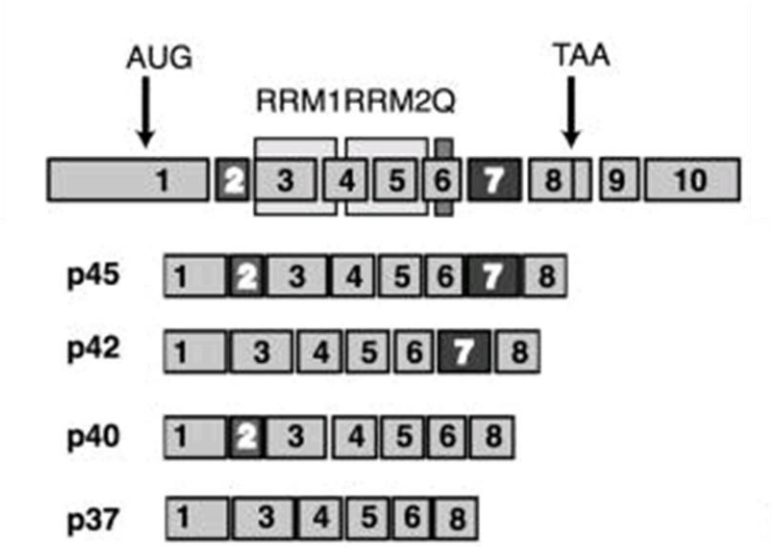

Fig.1.4: Diagrammatic representation of mRNAs encoding four alternately spliced isoforms (p37, p40, p42 and p45) of AUF1 gene (adapted from (Raineri et al. 2004) with permission). The two larger isoforms (p45 and p42) are unique in the presence of exon 7 which blocks their nuclear export. In contrast, the two smaller isoforms (p40 and p37) which differ only by the presence of exon 2 in p40, shuttle between the nucleus and the cytosol.

implications of p40AUF1phosphorylation state have been extensively studied. For example, both the *TNFα* and *IL1β* transcripts have been shown to be stabilized following the simultaneous dephosphorylation of p40AUF1, perhaps by altering AUF1-RNA affinity (Wilson et al. 2003).Ubiquitination and protein degradation is another important mode of AUF1 regulation. AUF1 pull-down experiments with lysates from heat-shocked cells established a close link between mRNA decay, ubiquitin-proteasome degradation pathway (UPP), and AUF1 degradation (Laroia et al. 1999). *In vitro* assays have confirmed that only p40AUF1 and p37AUF1 are predisposed to ubiquitination, consistent with their potential role in mRNA degradation. On the other hand, the insertion of the amino acids encoded by exon 7 in p42AUF1 and p45AUF1 is apparently sufficient to block their ubiquitin-mediated decay (Laroia and Schneider 2002). In conclusion, the spliced isoforms demonstrate functional diversity contingent upon the tissue/ cell line in which they are expressed, the specific mRNA with which they are interacting, the environmental cues that cause the post-translational modifications, and most importantly, the relative levels of individual AUF1 isoforms, instead of the absolute levels of all four isoforms.

ζ-Crystallin- ζ-Crystallin (ζ-Cryst) is an enzyme that exhibits multiple functions (Porte et al. 2009). ζ-Cryst was first identified as a structural protein in the ocular lens of some vertebrate species. It is also found in plants and yeast (Lapucci et al. 2010). Interestingly, ζ-Cryst gene was also shown to have an enzymatic function as an oxidoreductase. In liver and kidney (Rao et al. 1992), it catalyzes NADPH-dependent reduction of various quinones (Fernandez et al. 2007). More recently, ζ-Cryst was also identified as an RNA-binding protein that binds to AU-rich sequences within glutaminase

(Tang and Curthoys 2001) and glutamate dehydrogenase (Schroeder et al. 2003) mRNAs that function as pH-response elements (pHREs). It also contributes to the pH-responsive stabilization of the $Na^+K^+2Cl^-$ -cotransporter in the thick ascending limb of the nephron (Szutkowska et al. 2009). In another recent study (Lapucci et al. 2010), ζ-Cryst was identified as a bcl-2 mRNA binding protein, involved in *bcl-2* overexpression in T-cell acute lymphocytic leukemia.

1.6 mRNA decay pathways

mRNAs are co-transcriptionally decorated with a 5'-methylguanosine cap and a 3'-poly (A) tail as a mechanism to protect them from a variety of eukaryotic ribonucleases. Consequently, in the cytoplasm, the mature transcript is bound by poly (A)-binding protein (PABPC1) and the cap-binding protein eIF4E, preventing its degradation and promoting its association with mRNA translation initiation factors (Coller and Parker 2004; Garneau et al. 2007). The majority of cellular mRNAs are degraded following deadenylation, a process that shortens or removes the poly (A)-tail (Coller and Parker 2004) (Fig.1.5). However there are deadenylation-independent pathways of mRNA decay.

1.6.1 Deadenylation-dependent decay

Initial shortening of the poly (A) tail is reversible. Thus, an mRNA transcript that is apparently destined for decay, can be returned to polysomes to resume translation (Curtis et al. 1995). However, if the mRNA undergoes decay, deadenylation is followed by mRNA degradation, primarily by two major pathways that involve 3'→5' or 5'→3'

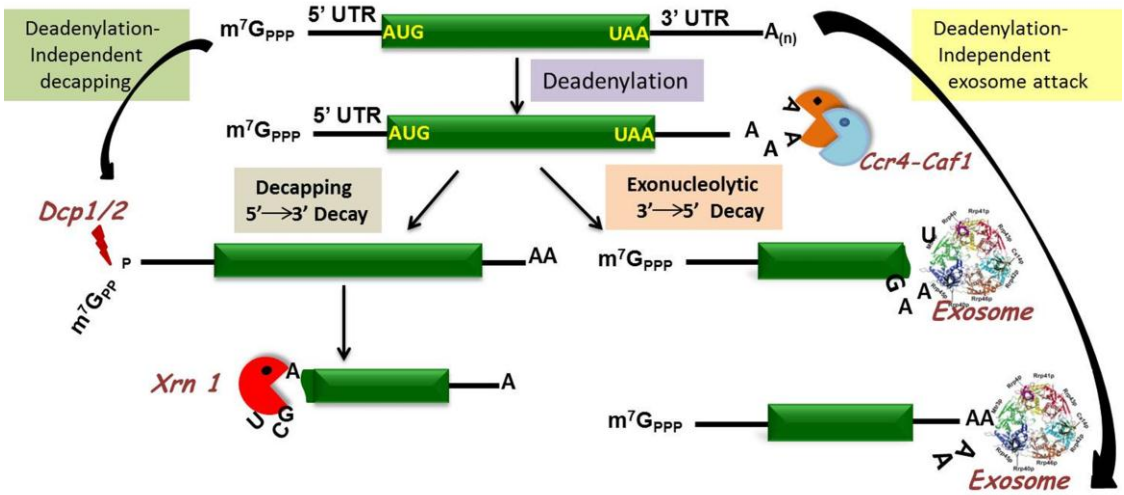

Fig. 1.5: Schematic diagram showing eukaryotic mRNA turnover pathways in the cytoplasm. Degradation of majority of mRNAs begins with deadenylation, followed by either a 5'→ 3' or 3'→5' exoribonucleolytic decay. Alternatively, deadenylation-independent pathways also target the mRNA for exoribonucleolytic decay or endoribonucleolytic cleavage.

decay. The 3'→5' decay pathway is mediated by the exosome, a 9-subunit complex of 3'-5' exoribonucleases, which targets the unprotected 3'end of the transcript. Alternatively, 5'→3'decay, is mediated by association of the LSM1-7 complex with the 3'end of the mRNA. This leads to decapping via Dcp1/Dcp2 complex. Following removal of the 5'cap, the transcript is predisposed to degradation by the 5'-3' exonuclease , Xrn1(Coller and Parker 2004).

Thus far, three different deadenylase complexes have been identified. In yeast, the Ccr4/Pop2/Not complex is the predominant deadenylase complex. While Ccr4p belongs to the ExoIII family of nuclease, Pop2p belongs to the RNase D family of nuclease. Although both of these proteins exhibit deadenylase activity (Daugeron et al. 2001; Tucker et al. 2002), they require various accessory factors such as Not1-Not5p, Caf4, Caf16, Caf40 and Caf130p to efficiently deadenylate the RNA (Tucker et al. 2001; Denis and Chen 2003). The second enzyme complex consists of the Poly (A) Nuclease, Pan2/Pan3, which is dependent on PABPC1 being associated with the poly (A)-tail (Sachs and Deardorff 1992). The third enzyme is the poly (A) ribonuclease or PARN protein. This enzyme is a cap-dependent deadenylase, meaning the presence of cap stimulates PARN activity (Gao et al. 2000). However, the presence of PABPC1on the poly (A)-tail inhibits both PARN and Ccr4/Pop2/Not complex (Sachs and Deardorff 1992).

1.6.2 Sites of mRNA decay

Stress granules (SGs) and processing bodies (PBs) are discrete dynamic cytoplasmic RNA-protein granules that are spatially, compositionally and functionally

connected. SGs are the sites of mRNA triage that appear when the cells are exposed to various environmental stresses such as heat shock, UV exposure or oxidative stress (Anderson and Kedersha 2009). Stalled translation preinitiation complexes are temporarily stored in the SGs that are elicited by the phosphorylation of the alpha subunit of eukaryotic translation initiation factor-2 (eIF2α) (Dever 2002). Nevertheless, SG assembly is reversed if the cells are relieved from stress and the mRNA can be sent back to normal pool of mRNAs for reinitiating translation or targeted to PBs. PBs are also the cytoplasmic sites of mRNA degradation that may also be triggered by environmental stress. PBs execute the decay of its harbored mRNAs via deadenylases and decapping enzymes (Dcp1, Xrn1, and Lsm proteins) (Spector 2006). Several proteins involved in translation repression such as the helicase, Dhh1/Rck, (Coller and Parker 2005) and the components (Argonautes) of small interfering RNA (siRNA) mediated translational silencing are clustered in PBs (Pillai et al. 2007). Although the fate of harbored mRNAs is yet to be deciphered, there is evidence to suggest a dynamic exchange of mRNA substrates and the associated proteins and, in fact also an *in vivo* overlap between the two cytosolic dynamic entities of SGs and PBs (Stoecklin and Anderson 2007).

1.6.3 Miscellaneous methods of mRNA decay

Other methods of mRNA decay are initiated by defects in the mRNA, and are a part of mRNA surveillance. mRNAs with nonsense mutations are recognized and degraded by a quality control process termed nonsense-mediated mRNA decay (NMD) (McGlincy and Smith 2008; Rebbapragada and Lykke-Andersen 2009). Similarly, mRNAs which lack translation termination codons could make abnormal proteins with C-

terminal extensions. These mRNAs are recognized and rapidly targeted for decay in a translation-dependent process termed non-stop mRNA decay pathway (NSD) (van Hoof et al. 2002) (Frischmeyer et al. 2002). No-go decay (NGD) clears the stalled ribosomes that are halted by the presence of the secondary structures on the mRNA, and degrades the transcript through endonucleolytic cleavage followed by exonucleolytic decay (Doma and Parker 2006).

1.6.4 MicroRNA mediated-mRNA decay

MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) when incorporated into RNA-induced silencing complex (RISC) can result in post-transcriptional gene silencing. miRNAs belong to a family of short ~21 base non-coding RNAs, estimated to regulate the activity of as much as 60% of all protein-coding genes (Friedman et al. 2009). miRNAs bind to 3'UTR of target mRNAs with perfect complementarity in a 7-8 nucleotide seed region and inhibit protein synthesis either by repressing translation or by endoribonucleolytic cleavage of the bound mRNAs via the miRISC complex. However, as there are exceptions to every rule in nature, some miRNAs were also reported to upregulate mRNA translation (Vasudevan et al. 2007). Core components of miRISCs include the AGO2 protein which directly secures onto the associated miRNA, and the effector protein, GW182, which acts downstream of AGO2. GW182 initiates miRNA-mediated repression through recruiting the deadenylation machinery CCR4/CAF1 and PAN2/PAN3 (Chen et al. 2009). Subsequently, the miRNA-repressed mRNAs are targeted to the cytoplasmic foci of mRNA degradation i.e P-bodies (Parker and Sheth 2007) (Eulalio et al. 2007) or to stress granules (Leung et al. 2006). Since miRNAs are

implicated as key players in pathogenesis of some diseases, misregulation of miRNAs can impact post-transcriptional gene-expression (Chang and Mendell 2007). Nevertheless, keeping in view the length and breadth of literature on RBPs and the emerging miRNA era, it would be prudent to acknowledge the cross-talk between the two players in modulating the associated mRNA-stability via RNA 3'end. Ironically, in most cases miRNA-mediated repression and overexpression of the well-studied RNA regulatory protein are linked to different types of cancers. HuR is reported to antagonize the repressive effect of miR-122 on the CAT-1 mRNA that encodes the high-affinity cationic amino acid transporter. While it is unclear as to the role of HuR in the relief of miRNA-mediated downregulation of CAT-1 mRNA, it is hypothesized that perhaps HuR oligomerization displaces the miRNP complex or inhibits its repressive effect on the translation of CAT-1 mRNA. By contrast, HuR complements the let-7 miRNA mediated repression of proto-oncogene c-Myc mRNA and protein levels (Meisner and Filipowicz 2010). In conclusion, it is plausible that either the distance of RNA binding protein from the miRNP binding site on the mRNA or the post-translational modifications on these proteins could determine their downstream effect on the mRNA stability.

1.7 Phosphoenol pyruvate carboxykinase (PEPCK)

Phospho*enol* pyruvate carboxykinase catalyzes the committed step in hepatic and renal gluconeogenesis (Rognstad 1979). It utilizes GTP as a phosphate donor to form phosphoenolpyruvate (PEP) (Mukhopadhyay et al. 2001).

$$\text{Oxaloacetate} + \text{GTP} \longleftrightarrow \text{PEP} + CO_2 + \text{GDP}$$

In all eukaryotes, the PEPCK protein is expressed as two isoforms, a mitochondrial form (PEPCK-M) and a cytosolic form (PEPCK-C). The PEPCK isoforms have similar kinetic

properties and approximately the same molecular weight but are encoded by separate nuclear genes (Hanson and Patel 1994). The human PEPCK-C gene is on chromosome-20, while the human PEPCK-M is located on chromosome-14 (Okamura et al. 2007).The two isoforms of PEPCK are expressed predominantly in liver, kidney, and adipose tissues. In kidney, the PEPCK gene is expressed solely within the convoluted and straight segments of the proximal tubule (Burch et al. 1978). PEPCK is a well-studied enzyme and known to be a key player in multiple metabolic pathways (Yang et al. 2009). Most importantly, it is involved in gluconeogenesis and in oxidation of the carbon skeletons of amino acids such as glutamine and serine. PEPCK is appropriately described as a cataplerotic enzyme in that its primary function is to remove citric acid cycle generated anions and prevent their accumulation. In the process, PEPCK and pyruvate kinase play a central role in catabolism of the carbon backbones of amino acids. The process involves the conversion of the carbon skeletons of amino acids, such as glutamine and glutamate to malate, which exits the mitochondria. The malate is then oxidized to oxalacetate and, subsequently decarboxylated to phospho*enol*pyruvate via PEPCK-C. The phospho*enol*pyruvate could either enter the gluconeogenic pathway or be converted to pyruvate via pyruvate kinase and then oxidized to carbon dioxide in the citric acid cycle (Fig.1.6). The level of renal PEPCK is also increased in response to glucocorticoids, parathyroid hormone or angiotensin II (Hanson and Patel 1994). The latter hormones increase the cAMP levels within the proximal tubule. Since renal gluconeogenesis is primarily coupled to renal ammoniagenesis and the maintenance of acid-base balance,

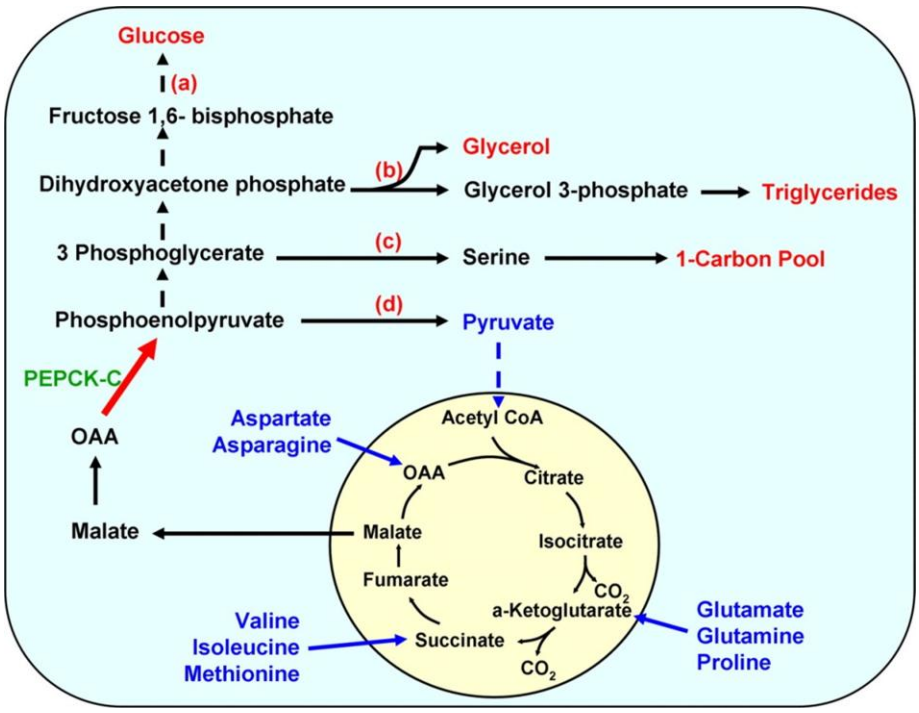

Fig.1.6: **PEPCK is a key player in multiple metabolic pathways (adapted from (Hanson and Patel 1994) with permission).** PEPCK is one of the several cataplerotic enzymes involved in scavenging the TCA cycle intermediates and feeding them into biosynthetic pathways to be converted into either glucose (gluconeogenesis), fatty acids (lipogenesis), or glycerol (glycerogenesis).

renal PEPCK gene expression is also induced by acidosis (Curthoys 2011).

The *PCK-1* gene, that encodes PEPCK-C, is composed of 10 exons and 9 introns, spanning 6-kb in length (Beale et al. 1985). The *PCK-1* genes from rat, mouse and human share more than 90 percent nucleotide sequence identity within their coding regions. The rat gene encodes a 2.6-kb mRNA that includes 143 and 615 nucleotides of 5'-and 3'-untranslated (UTR) sequences, respectively (Fig 1.7). The protein consists of 621 amino acids and has a molecular mass of 69,300 Daltons. The - 490 and + 73 bp region of the *PCK-1*gene contains at least 13 distinct *cis*-regulatory elements (Hanson and Reshef 1997). The level of PEPCK mRNA in rat kidney is increased rapidly following acute onset of acidosis (Hwang et al. 1991). The increase is initiated within 1h and reaches a maximum within 7 h at a level that is 6-fold greater than normal. The 6-fold induced level of PEPCK mRNA is sustained in rats that are made chronically acidotic for 7-d. Transcription run off experiments were conducted using isolated rat renal nuclei (Hwang and Curthoys 1991). The relative rate of transcription of the PEPCK gene increased 3-fold within 2 h after acute onset of acidosis, reached a maximum of 4-fold induction by 6 h, and then decreased slightly after 20 h. The observed changes in PEPCK mRNA levels closely correlated with earlier data that measured changes in the relative rates of PEPCK protein synthesis in normal and acidotic rats (Iynedjian et al. 1975). Interestingly, the 6-fold induced level of PEPCK mRNA is sustained in rats that are made chronically acidotic even though the relative rate of transcription gradually decreases and plateaus at a level that is only 2-fold greater than observed in normal rats (Hwang et al. 1991).

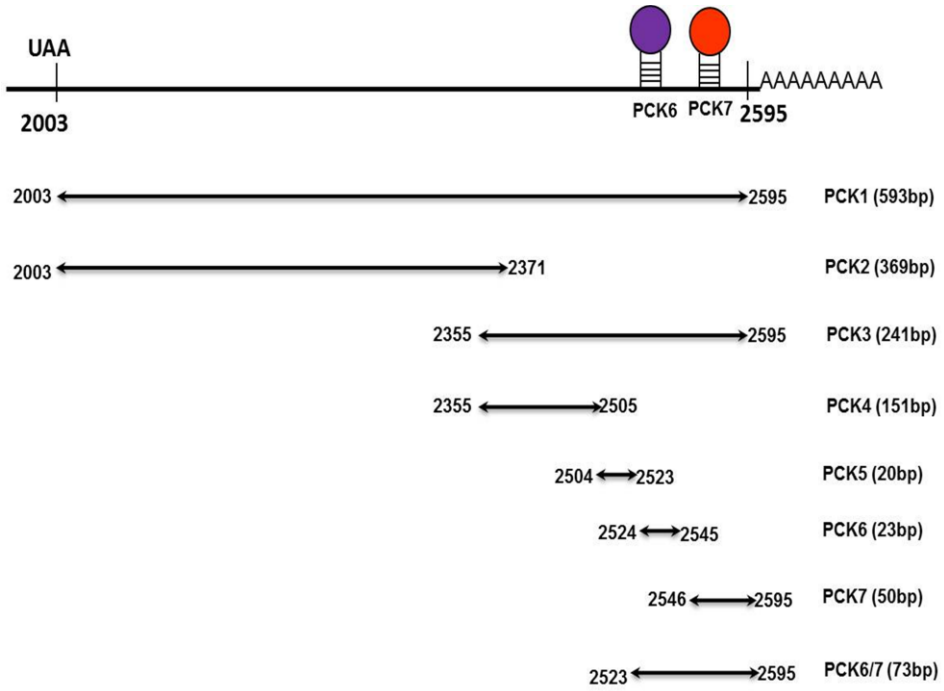

Fig.1.7: Deletion constructs of 3'-UTR of PEPCK mRNA . Diagrammatic sketch showing full-length 3'-UTR and deletion constructs of PEPCK-3'UTR containing conserved AU- and CU-rich sequences, were cloned into β-globin reporter plasmid. The numbers across each construct indicate the corresponding nucleotide base pair within the PEPCK cDNA. The lengths of the PEPCK segments are indicated in nucleotides (nt) adjacent to the construct name.

Several studies were performed to determine the role of the *PCK-1* promoter in the tissue-specific expression and during altered acid-base balance. The bovine growth hormone (bGH) gene was fused to a 2.3-kb segment of the *PCK-1*promoter to produce a construct that was expressed at high levels in the liver and adipose tissue, but was barely expressed in kidney. In contrast transgene that contains only 362 bp of the promoter but all of the downstream exons and introns of the rat *PCK-1*gene was expressed at normal levels in the kidney (Eisenberger et al. 1992). This transgene differed from the endogenous *PCK-1* gene only by the substitution of a segment of the chicken *PCK-1*gene into the portion of the final exon that encodes the 3'-UTR of the PEPCK mRNA. In addition, the CRC-362 construct showed significant renal-specific induction when the transgenic mice were made acidotic (Cassuto et al. 2003).

Previous studies established that the half-life of the PEPCK mRNA was increased in response to cAMP (Hod and Hanson 1988) and glucocorticoids (Petersen et al. 1988). Thus, it was proposed that perhaps the increased stability might also contribute to the sustained induction of renal PEPCK mRNA during chronic acidosis (Hwang et al. 1991). Porcine kidney tubule LLC-PK_1 cells lack endogenous fructose-1,6 bisphosphatase 1 (FBP1) and the ability to produce glucose. Selection of cells by glucose starvation produced a line of cells that express FBP1 and are gluconeogenic (LLC-PK_1-F^+) (Gstraunthaler et al. 1985; Gstraunthaler and Handler 1987). Cell culture studies indicated that LLC-PK_1-F^+ exhibit a pH-responsive induction of PEPCK mRNA (Gstraunthaler et al. 2000). A tetracycline-regulated promoter system was used to map the instability elements in various chimeric β-globin-PEPCK mRNA constructs

containing different segments of the 3'-UTR of the PEPCK mRNA (TβG-PCK), expressed in LLC-PK_1-F^+ cells (Hajarnis et al. 2005) (Fig.1.7). Half-life analysis showed that TβG-PCK1 mRNA containing the full-length 3'UTR is degraded with a half-life ($t_{1/2}$) of 1.2 h. RNase H treatment indicated that rapid deadenylation occurred concomitant with degradation of the TβG-PCK1 mRNA. However, TβG-PCK7, that contained only a 50-nt segment from the 3'-end of the PEPCK 3'-UTR, had half-life of 17 h. With inclusion of adjacent PCK-6 (23-bp) AU-rich sequence, the half-life (TβG-PCK6/7) decreased to 3.6 h. The TβG-PCK3-mRNA that contains the 3'-half of 3'-UTR was degraded with a similar half-life. Interestingly, TβG-PCK2-mRNA which contains 5'-half of the 3'UTR was also degraded rapidly ($t_{1/2}$ =5.4 h). RNA gel-shift analyses established that AUF1 binds to the PCK-7, PCK-6 and PCK-2 segments with high affinity and specificity. Mutational analysis indicates that AUF1 binds to a UUAUUUUAU sequence within PCK-6 and a stem-loop structure and adjacent CU-region of PCK-7. Thus, AUF1 binds to multiple destabilizing elements within the 3'-UTR that participate in the rapid turnover of the PEPCK mRNA.

HuR is an mRNA binding protein that contributes to the stabilization of mRNAs that contain AU-rich elements. Therefore, electrophoretic mobility shift assays were performed to determine if recombinant HuR binds to specific sites within the 3'UTR of PEPCK mRNA. When initially tested, only the 3'-fragment (PCK3), but not the 5'-half (PCK2) of the 3'UTR of PEPCK mRNA formed a shifted RNA: HuR complex. This lead to the gel-shift analysis of multiple deletion constructs (PCK-4 and PCK-5) that comprise the 5'end of the PCK-3 RNA. While the PCK-4 RNA did not bind to HuR, PCK-5 formed two shifted bands with HuR which suggested that multiple RNA: protein

complexes were formed. The PCK-5 RNA is a short GU-rich segment. However, a higher concentration of HuR (360-1800 nM) was required to form a shift with the PCK-5 RNA than with the PCK-3 RNA (56-392 nM). This observation suggests that HuR may bind with higher affinity to additional elements that are contained within the 3'-end of the PCK-3 RNA. Therefore, HuR binding to the combined PCK-6/7 RNA was analyzed. PCK6/7 RNA contains the 73 nucleotides that comprise the 3'-end of the PEPCK mRNA. HuR formed at least three separate complexes with the PCK-6/7. A competition assay using the [^{32}P]-labeled and excess unlabeled PCK-6/7 RNA established the specificity of HuR binding to the PCK-6/7 RNA. Next, the individual PCK-6 and PCK-7 RNAs were tested and found at least one binding site in PCK6 and two sites in PCK-7 that can synergistically interact to enhance the binding with HuR.

1.8. Significance of cross-linking in cells

Studies to date have clearly demonstrated that mRNA binding proteins are integrally involved in post-transcriptional regulation, at the levels of pre-mRNA processing and mRNA stability (Tenenbaum et al. 2002) and that ribonucleoprotein (RNP) complexes form the fundamental unit of post-transcriptional regulation (Beach and Keene 2008). Traditionally, researchers have used several *in vitro* approaches to study RNA-protein interactions, including UV-crosslinking, nitrocellulose filter binding, and RNA electrophoretic mobility shift assays (Cilley and Williamson 1999). Although these biochemical methods have enhanced our understanding in the structure/function relationship of RNP complexes, they are limited in their ability to reflect *in vivo* interactions (Niranjanakumari et al. 2002). This eventually led to the development of *in*

vivo assays, of which, the yeast three-hybrid system was developed to identify binding partners when one of the components was known (SenGupta et al. 1996). Unfortunately, this technique has been limited due to procedural drawbacks (Keene et al. 2006). As the era of mRNA target discovery advanced, new techniques were developed for purifying endogenously formed RBP-mRNA complexes. RNA-binding protein immunoprecipitation-microarray (RIP-chip) assay was widely adapted, either with or without chemical cross-linking. In the recent past, RIP-chip assays were applied to mammalian cells and have proven useful in providing insights into the infrastructure of coordinated eukaryotic post-transcriptional gene expression (Keene et al. 2006). Despite the usefulness of this procedure, it is possible that the efficacy of the technique is compromised when using cell lysates containing either non-crosslinked complexes or post cell lysis-crosslinked complexes. As a result, this can introduce potential artifacts due to redistribution of cellular compartments upon cell disruption (Niranjanakumari et al. 2002). Thus, while studying macromolecular interactions, inclusion of an initial step of "fixing" or "immobilization" of inherent RNA-protein interactions before further processing of cell lysates may be essential. Although physical (ultraviolet-UV light) and chemical (formaldehyde) crosslinking agents have been explored in a wide range of *in vivo* experiments, cross-linking or fixing cells with an optimal dose of formaldehyde allows one to safely reverse the crosslinks and thus perform quantitative analysis of RNA from the recovered complexes. Additionally, formaldehyde reagent has been used for a variety of purposes including 'fixing' the cells, so it could help protect the integrity of cell organelles while stabilizing the RNA-Protein interactions (Moller et al. 1977). In contrast, *in vivo* UV-crosslinking forms irreversible bonds between the complexes and

requires longer exposure hours which could lead to cell damage (Niranjanakumari et al. 2002). Cross-linking and immunoprecipitation (CLIP) (Licatalosi et al. 2008) and photoactivatable ribonucleoside-enhanced CLIP (PAR-CLIP) (Hafner et al. 2010) are two popular methods employed for high-throughput screening of RNA-binding protein binding sites, including HuR binding sites. Quantitative analysis of CLIP versus PAR-CLIP methods essentially produced comparable hits for HuR binding sites, even though PAR-CLIP employs cross-linking at slightly higher wavelength (365nm) and milder nuclease-digestion conditions (Kishore et al. 2011).

PAR-CLIP (photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation) (Hafner et al. 2010) is a novel method that was developed to characterize the genome wide *in vivo* interaction of a specific RNA binding protein. The first step in this approach requires incorporation of photoreactive ribonucleoside analogs by growing cells in the presence of 4-thiouridine. A brief exposure to UV-light irradiation (365 nm) allows covalent crosslinking between the nascent RNA and RNA-binding proteins. Immunoprecipitation of Ribonucleoprotein complex, followed by reverse transcription of the bound nascent RNA marks the site of cross-linking as a U → C transition in the cDNA. The presence of mutations allows the detection of precise point of cross-linking between the RNA and the associated protein.

1.9 Modified Halo-Chip System for studying RNA: protein interaction

The mRNP complexes in the cell extracts can be immunoprecipitated with an antibody specific for the RNA binding protein of interest. However, constraints such as the lack of availability of specific antibodies for a particular protein or inaccessibility of

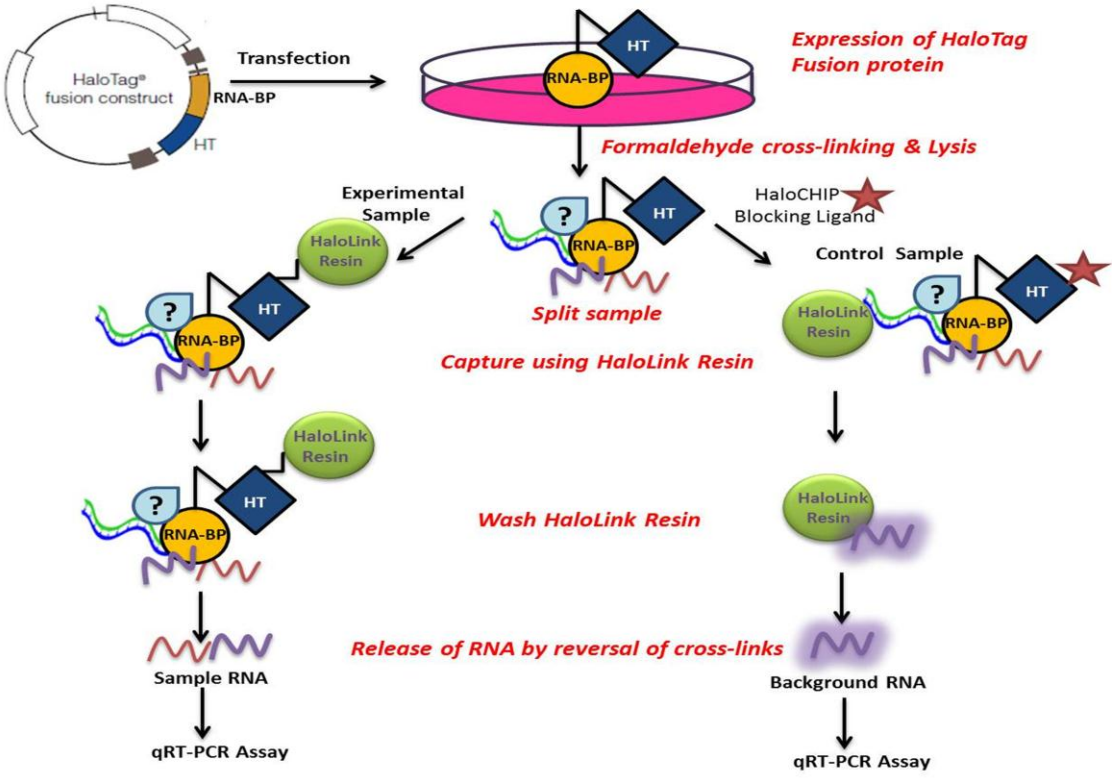

Fig.1.8: Schematic diagram showing HaloRNA-pull down Assay (modified from Promega). RNA-binding proteins (RNA-BPs) of interest are cloned into pFC14A HaloTag CMV Flexi vector to add a c-terminal Halo Tag (HT). The LLC-PK_1-F^+ 9C cells are transfected with halo-chimeric construct to produce either transient or stable expression. The transformed cells were cross-linked with formaldehyde (0.3%) to stabilize the *in-vivo* RNA-protein and protein-protein interactions. The cross-linking reaction is quenched and the cells were lysed. The cell lysate is split into two halves prior to incubation with the haloLink Resin. While the cross-linked complexes from the lysate of the experimental sample are directly captured via covalent interaction between the HT-epitope and the HaloLink Resin, the lysate designated for the control sample is pre-incubated with halo-blocking peptide. Stringent washing allows for the removal of non-specifically bound RNA, DNA and proteins. Subsequent heat reversal of the cross-links releases RNA bound to the HT-fusion protein. The eluted RNA from both control and experimental sample is analyzed in qRT-PCR assay.

components of the mRNP-complex have lead researchers to take the alternative approach of epitope-tagging of RBPs. The Halo-ChIP system (Promega) is an innovative technique that covalently captures intracellular protein: DNA complexes without using antibodies. HaloChIP was modified to perform a HaloRNA-pull down assay (Fig.1.8), where RNA binding proteins of interest were expressed as HaloTag fusion proteins, cross-linked to RNA with formaldehyde, and then captured on HaloLink Resin. The HaloLink Resin is a synthetic ligand comprising of a chloroalkane linker attached to a solid surface. The HaloTag sequence is a mutated haloalkane dehalogenase (hydrolase) that forms an irreversible covalent linkage to its substrate that is highly specific and forms under physiological conditions. Hence, the resin can be stringently washed to effectively remove non-specific RNAs and proteins. The cross-links are reversed by heating and the released RNA fragments can be analyzed by quantitative real time PCR (qRT-PCR). Therefore, the HaloRNA-pull down assay will be used to quantify the *in vivo* association of the individual AUF1isoforms and HuR with the 3'-UTR of PEPCK mRNA, without using large amounts of different antibodies.

1.10 Recruitment of proteins to RNAs by bacteriophage proteins

The use of reporter genes is one of the classic approaches to study the functional role of an RNA binding protein under *in vivo* conditions (Keryer-Bibens et al. 2008). A recruitment assay using the bacteriophage MS2-coat protein (MS2cp) to bind specific proteins to an RNA is one such approach that has been widely explored. The MS2cp is derived from an RNA bacteriophage, where it acts as a translational repressor of the RNA replicase by binding to its stem-loop structure (Bernardi and Spahr 1972). Adaptation of

the tethering approach not only enabled analysis of the post-transcriptional activities of a number of different RNA binding proteins, but also the molecular mechanisms of translational silencing and mRNA degradation by miRNAs. The MS2 reporter assay was also extensively utilized to decipher the role of RNA binding protein in mRNA decay (Lykke-Andersen and Wagner 2005). Furthermore, application of this reporter assay was extended to track the localization of mRNAs in living cells (Bertrand et al. 1998) and to study the mechanism of CRM1-Rev mediated export of RNAs (Yi et al. 2002). Although there are many variations to this technique, the tethered assay in principle employs two recombinant molecules: a recombinant reporter mRNA that contains the stem-loop elements that bind the MS2-coat protein and a chimera of the MS2cp and the RNA binding protein of interest. Theoretically, when both of the recombinant constructs are co-expressed in a cell, the fusion protein is tethered to the reporter mRNA via the exogenous RNA-binding domain and thereby triggers the molecular consequences of its association with the mRNA (Keryer-Bibens et al. 2008). Essentially this technique allows us to investigate the ability of an RNA binding protein to mediate a specific response. However, the simultaneous recruitment of two MS2coat protein fusion proteins to a single reporter RNA containing multiple MS2-RNA hairpins can also be used to explore the potential interaction of two RNA binding proteins. Alternatively, the combined use of the two distinct stem-loop sequences (MS2 and PP7) in a single pre-mRNA molecule was employed to study the post-transcriptional events of exon splicing (Gesnel et al. 2009). Kathleen Collin's lab pioneered the use of PP7 coat protein derived from *Pseudomonas aeruginosa* for affinity purification of ribonucleoproteins (Hogg and Collins 2007). More recently, Singer and his colleagues used the truncated PP7 coat protein (13.6 kDa) for

live cell imaging of single mRNA molecule (Chao et al. 2008). Chao made the truncated version of original PP7 coat protein. This protein retained the ability to dimerize and bind the RNA, but was compromised in its capsid assembly. The structure of the PP7 protein was solved as a combined effort between Singer and Almo's lab (Chao et al. 2008). The two RNA elements for the MS2cp and the PP7 coat protein differ in the sequence of the loop and the position of a bulged adenosine residues (Lim and Peabody 2002) (Fig.1.9). Consequently, this allows the two coat proteins to discriminate in the recognition of their cognate RNA binding site, but still allow one to study the effect of binding of two chimeric proteins in a single reporter mRNA.

1.11 Hypotheses and Statement of problem

1.11.1 Co-regulatory effects of HuR and AUF1on PEPCK mRNA turnover

Previous electrophoretic mobility shift studies established that HuR and AUF1 bind *in-vitro* to overlapping sites within the instability elements in the 3'UTR of PEPCK mRNA with high affinity and specificity (Hajarnis et al. 2005; Mufti et al. 2011). Nevertheless, a moderate reduction in AUF1 via siRNA silencing did not significantly alter either the basal level or the pH-responsive increase in PEPCK expression. By contrast, a 90% knockdown of HuR led to significant reduction in the basal levels, and a modest decrease in PEPCK mRNA and protein levels (Mufti et al. 2011). There is also evidence in the literature suggesting competitive or concurrent binding of HuR and AUF1 in regulating the stability of AU-rich mRNA targets (Lal et al. 2004).

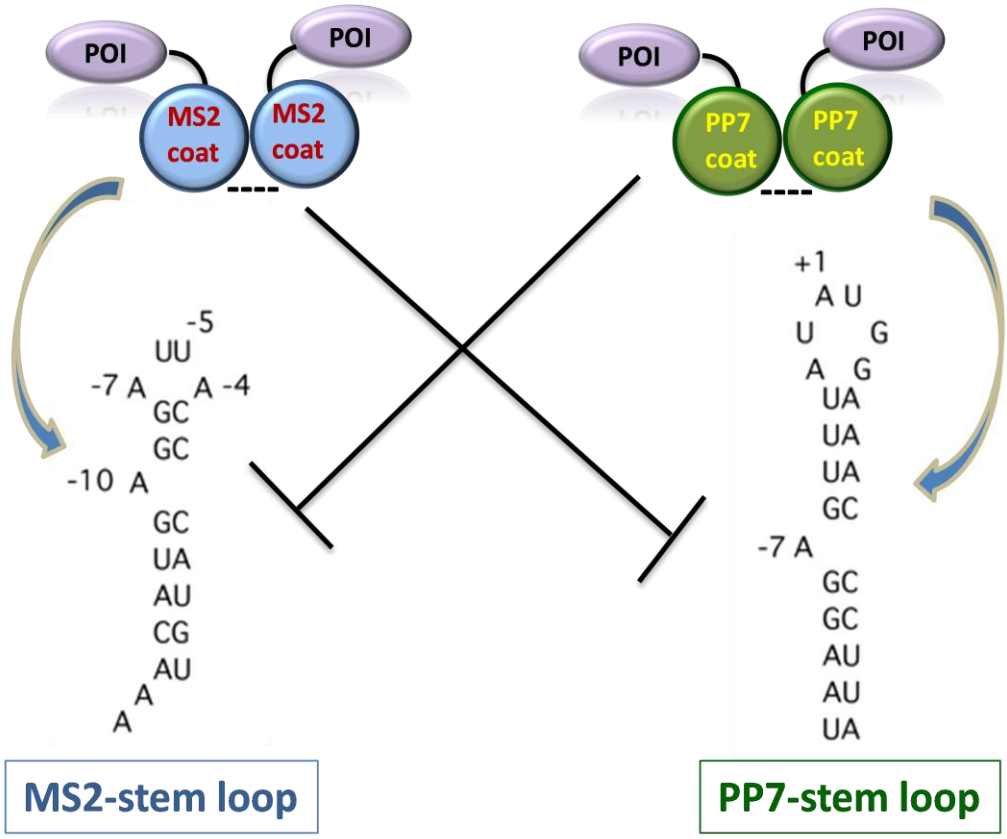

Fig.1.9: Bacteriophage coat fusion proteins and their cognate RNA-stem loop binding sequence (Modified from (Lim and Peabody 2002) with permission). Homodimers of MS2 or PP7 coat fusion proteins have binding specificities to their respective RNA-stem loop sequences and show very little affinity to the hairpin sequence of the opposite coat protein.

Recent studies confirmed that the pH-responsive increase in PEPCK expression in the LLC-PK_1-F^+-9C kidney cells is mediated at least in part, by a 2-fold stabilization of the PEPCK mRNA. This pH-responsive stabilization was recapitulated in a Tet-responsive expression of chimeric β-globin mRNA that contains the entire 3'-UTR of PEPCK mRNA (Mufti et al. 2011). *In addition, since p40AUF1 and HuR have overlapping binding sites within the 3'UTR (Hajarnis et al. 2005; Mufti et al. 2011), we hypothesized that either the competitive or simultaneous binding between the two RNA binding proteins is necessary to mediate the rapid turnover and the pH-responsive stabilization of PEPCK mRNA.*

1.11.2 Covalent modifications on HuR and AUF1 in response to metabolic acidosis.

Recent studies also showed that neither the levels nor the subcellular-localization of HuR and AUF1 are altered when cells were switched from basal to acidic medium (Mufti et al. 2011). *Hence, we hypothesized that perhaps post-translational modifications are required to cause remodeling of the protein/ protein and consequently protein/ RNA complex to produce the pH-responsive increase in renal PEPCK expression.*

1.11.3 Determine the *in vivo* interaction of HuR and AUF1 with PEPCK mRNA.

To characterize *in vivo* association of HuR and AUF1 to the 3'-UTR of PEPCK mRNA and determine the protein-protein interactions between RNA binding proteins, HuR, AUF1 and ζ-Cryst. *Since the in-vitro binding of HuR and AUF1was earlier established with the pH-response element, here we wanted to test the same in the in-vivo*

condition using the Halo-Tag pull down assay. This experimental approach will also be used to further assess co-purifying proteins in the eluted mRNA/protein complex.

1.11.4 Functional significance of ζ-Cryst binding in PEPCK mRNA regulation.

Previous experiments using RNA affinity purification, mass spectrometry and western blot analysis confirmed the identity of ζ-Cryst as another RNA binding protein, that binds with high affinity to the 3'-end (PCK6/7) of the 3'UTR of PEPCK mRNA. Intriguingly, p40AUF1 and HuR also have overlapping binding sites within this segment. Nevertheless, neither transient nor stable knockdown of ζ-Cryst alone significantly altered the basal or pH-responsive increase in PEPCK protein. *Thus it was clear that ζ-Cryst was not the sole mediator of this response. However, we postulated that a combinatorial interaction of HuR and/or AUF1 with ζ-Cryst may mediate either the rapid degradation or the pH-responsive stabilization of the PEPCK mRNA. We proposed to further characterize the potential effect of ζ-Cryst on the stability of PEPCK mRNA using LLC-PK$_1$-F$^+$-9C cells that either overexpress the mouse ζ-Cryst or an shRNA to knockdown the porcine ζ-Cryst.* In summary, the central focus of this thesis is to investigate the functional role of the trans-acting factors (RNA-binding proteins) in modulating the PEPCK mRNA turnover.

CHAPTER II

MATERIALS AND METHODS

2.1 Cell culture and siRNA transfections

LLC-PK_1-F^+9C cells (Mufti et al. 2011) were cultured in DMEM-Base medium (Sigma) supplemented with penicillin/streptomycin (Sigma), 10% fetal bovine serum (FBS), 5mM glucose, 26mM $NaHCO_3$, 17mM NaCl, 2mM glutamine, 5mM pyruvate, 5µM phenol red, and 10mM HEPES, pH 7.4 at 37^{o}C in a 5% CO_2 atmosphere. A physiologic mimic of metabolic acidosis was recapitulated using an acidic medium (pH 6.9 medium) which was prepared as above, except 9mM $NaHCO_3$ and 34mM NaCl were added to reduce pH while maintaining equivalent osmolarity (Gstraunthaler et al. 2000). Pre-annealed double stranded stealth siRNA (Invitrogen) targeting porcine HuR coding region (HuR97forward strand sequence- CAGGAGGAGUUACGAAGUCUGUUCA), and a control siRNA that is not encoded in the human, rat or mouse genome (ctrl378 forward sequence- UGUAGGUAGAAGCUAUCAUUACGUG) were used at a final concentration of 30 nM and 50 nM, respectively. To silence pig AUF1, two separate siRNA oligos that target exon 3, which is common to all four isoforms of AUF1, (378AUF forward sequence-CACUCUGAAGUUAGAUCCUAUCACA; 429AUF forward sequence- UUUAGGAUCAAUCACCUUCCCAUUC) were used in a combination of 50 nM each. The siRNAs were transfected into 70-80% confluent cells grown in 12-well plates using Lipofectamine RNAiMAX (Invitrogen) as described previously (Mufti et al. 2011). Forty eight hours following transfection, cells were treated

with either pH 7.4 or pH 6.9 medium for 24 h prior to harvesting in 100 µl lysis buffer for western blots (O'Hayre et al. 2006) or in 250 µl TRIzol® Reagent for RNA isolation.

2.2 RNA extraction and Real-time quantitative PCR (qRT-PCR)

Total RNA was isolated using TRIzol® (Invitrogen) as per the manufacturer's protocol. An Oligo-dT_{18} primer (IDT) and the avian myeloblastosis virus-reverse transcriptase (Promega) were used to reverse transcribe 1µg of RNA. Gene-specific primers and Taqman probes for the detection of endogenous porcine PEPCK mRNA and the control GAPDH mRNA were used for the qRT-PCR analysis as previously reported (Mufti et al. 2011).

2.3 Chimeric MS2-expression plasmids

A chimeric reporter construct that expresses a β-globin-PCK2 mRNA from a tetracycline-responsive promoter was created and maintained as reported earlier (Hajarnis et al. 2005). This construct contains a tetracycline (Tet)-responsive promoter, the coding region of the rabbit β-globin (βG) gene, the 5'fragment (381bp) of 3'-UTR of the rat PEPCK cDNA, and ployadenylation site of bovine growth hormone (bGH) cDNA. The pPC-β6bs plasmid (Lykke-Andersen et al. 2000), which is a derivative of pcDNA3.0 (Invitrogen) containing six copies of MS2 coat protein-binding sites, was obtained as a kind gift from the Wilusz lab (Dept. of Microbiology, Immunology and Pathology, CSU). Each of these MS2 stem-loops differ from wild type by a A→C high affinity mutation in the loop sequence, as indicated in Fig.2.1A. *Not I*/ *Xba I* digestion of pPC-β6bs released a 314 bp fragment containing six-repeats of MS2 coat protein-binding sites. This fragment

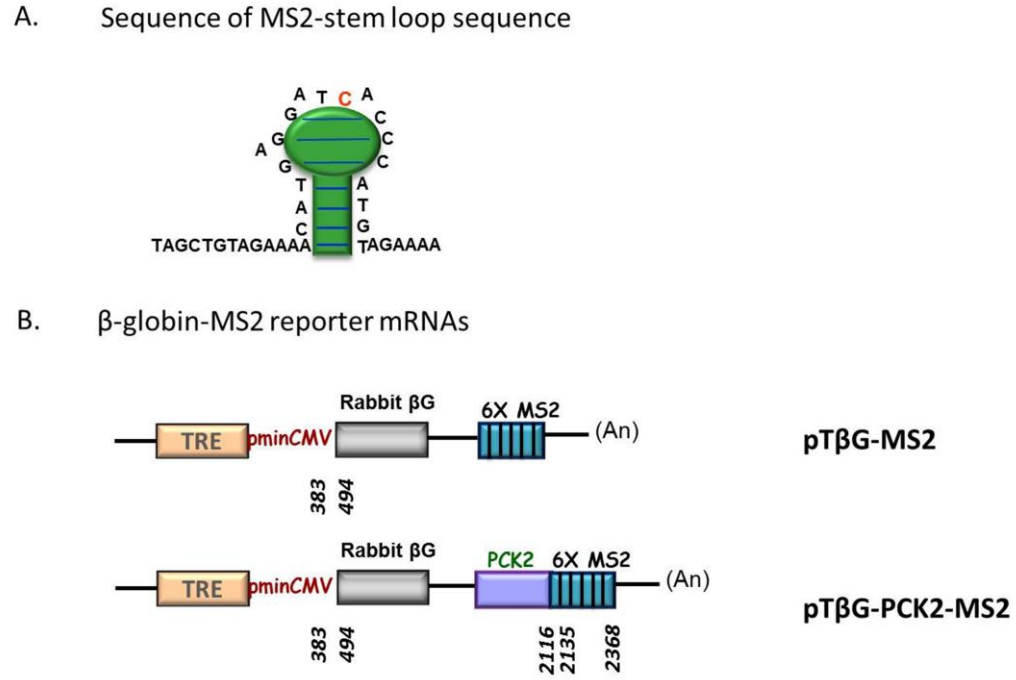

Fig.2.1. Schematic of the β-globin reporter mRNAs with MS2 coat protein binding sequence. The following constructs were used to determine the effect of HuR and AUF1 recruitment on the half-life of the reporter mRNA.
A) Nucleotide sequence and secondary structure of an MS2 stem-loop.
B) The pTβG-MS2 plasmid contains a tetracycline responsive elements (TRE) fused to a minimal CMV promoter. The complete coding region for rabbit β-globin, and a 3'UTR that includes six copies of the MS2 coat protein binding sites (6x MS2). The pTβG-PCK2-MS2 plasmid also incorporates the PCK-2 segment that contains the 5'-half of the 3'UTR of rat PEPCK mRNA. Test reporter βG with downstream in-frame PCK2 (5'-end of PEPCK 3'UTR) followed by six copies of the MS2cp-binding sites (6 x MS2), under the control of Tet-responsive elements.

was inserted into TβG-PCK2 reporter between *Not I* and *Xba I* sites to create the MS2-tether reporter, TβG-PCK2-MS2, that retains a single AUF-1 binding site (Fig.2.1B). A control non-ARE reporter TβG-MS2 (Fig.2.1B) was also created using the same strategy as above, except that the starting plasmid lacked the PCK-2 sequence.

2.4 Creation of cell lines that stably express MS2 reporter mRNAs.

Cell lines that stably express the TβG-MS2 or TβG-PCK2-MS2 constructs were made by lipofectamine-2000 (Invitrogen) transfection of the corresponding constructs and pcDNA3.1/Hygro (Invitrogen) into LLC-PK_1-F^+9C tTA cells, a line of LLC-PK_1-F^+9C cells that over-express the tTA protein (Mufti et al. 2011). At 6 h post-transfection, the medium was removed, the cells were washed twice with phosphate buffered saline, and then fresh medium containing 0.2 mg/ml G-418 was added. At 24 h post-transfection and every two days thereafter, fresh medium containing 0.2 mg/ml G-418 and 0.8 mg/ml Hygromycin B was added. After 15-20 days, individual colonies were isolated and lifted with trypsin. When the clonal transformants were eventually seeded on10-cm plates, the Hygromycin B concentration was reduced to 0.2 mg/ml. The cell lines were tested for pH and Dox-responsiveness by maintaining the cells for 24 h in medium in the presence or absence of 1 μg/ml Dox or by treating with acidic medium minus Dox. Total RNA was isolated from the cells and analyzed by qRT-PCR assay.

2.5 MS2-fusion proteins

The pcNMS2-Flag (Lykke-Andersen et al. 2000) plasmid was also obtained as a kind gift from Wilusz lab. pcNMS2-Flag encodes an N-terminal flag peptide sequence in-frame with MS2-coat protein (MS2cp). The inserted MS2cp sequence encodes a mutated form of the bacteriophage MS2cp that contains two point mutations (V75E; A81G). The two mutations prevent protein multimerization but retain high binding affinity to the MS2-stem loop binding sites (LeCuyer et al. 1995). The open reading frame of mouse HuR was PCR amplified with 5'flanking *Bgl II* (GACTAGATCTAGCGCCATGTCTAATGG) and 3'*XbaI* (CGATTTCTAGATTAAACTTTGTGGGACTTG) sequences from pGEMTeasy-mHuR and cloned in frame to the C-terminus of the MS2cp sequence. Similarly, the p40AUF1coding sequence was PCR amplified (forward- 5'AATTGGATCCAAAGCCATGTCGGAGG 3' and (reverse- 5' GTCCGATGCTAGCTTAAACGTATGGTTTGTAGC 3') from pGEMTeasy-p40AUF1 and inserted in-frame at *BamHI* and *NheI* sites that are downstream of the MS2-sequence (Fig.2.2).

2.6 mRNA half-life analysis

To assess mRNA half-lives, the β-globin reporter RNA plasmids were stably co-transfected with pcDNA3.1/Hygro (Invitrogen) into 9C cells that stably expressed the tTA protein (Schroeder et al. 2006)**.** The stable transformants were grown in 6-well plates in normal medium containing minimal Dox (100 ng/ ml) until 70% confluet. The cells were then transiently transformed with Flag-MS2cp-HuR (2µg) or Flag-MS2cp-p40AUF1 (2µg) plasmid and maintained in normal (pH 7.4) medium minus Dox for two days, before treating with acidic medium (pH 6.9) for 24 h. RNA was isolated at various times after complete

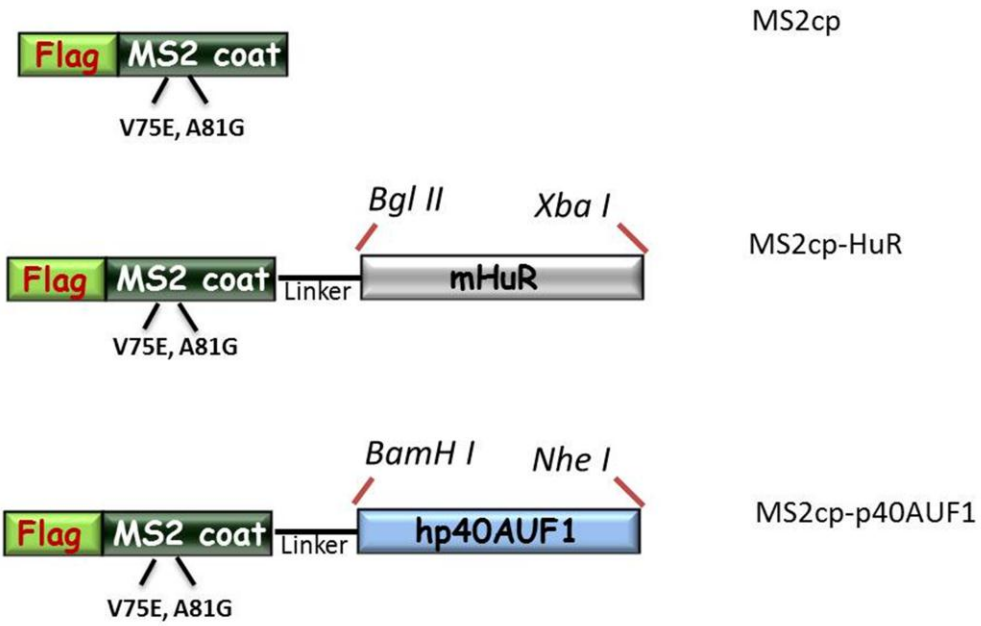

Fig.2.2. Schematic of the MS2 fusion proteins.
The MS2cp fusion contains an N-terminal FLAG epitope that is in frame with MS2coat protein sequence. The remaining fusion proteins contain this sequence followed by a hydrophilic linker sequence followed by HuR or AUF1.

arrest of transcription with addition of 1µg/ ml Dox. The relative levels of β-globin and GAPDH mRNAs were quantified by RT-qPCR and normalized to GAPDH mRNA. Since, the kidney cells do not express β-globin, the RT-PCR assay detects only the reporter RNA and serves as a common read-out for all the β-globin based reporters. The RT-PCR assay for βG-cDNAs used forward (5'TCAGTGAGGGTCTGAATCACC 3') and reverse (5'CTGCACCTGAGGAGTGAATTC 3') primers and a Taqman probe-(5'FAM-CACCTTTGCTAAGCTGAGTGAACTGCAC-BHQ1 3').

2.7 Subcellular fractionation of LLC-PK$_1$-F$^+$9C cells

Nuclei were collected from the cells by adapting the protocol from (Pan et al. 2005). Briefly, cells were washed twice with PBS and then lysed by incubating cells with ice-cold buffer containing 85 mM KCl, 5 mM PIPES, pH 8.0 and 0.5% IGEPAL supplemented with Halt phosphatase inhibitor (Thermo scientific) and Protease Inhibitor Cocktail (Sigma). The lysate was centrifuged at 3000 rpm for 5 min and the supernatant (cytosolic fraction) was collected and the resulting nuclear pellet was resuspended in the same lysis buffer (Mufti et al. 2011).

2.8 Western blot analysis

Bradford assays (Bradford 1976) were performed to determine the concentration of the protein lysates. Samples containing 15 µg of protein were resolved by 10 % SDS-PAGE and transferred to Immobilon-F membranes (Millipore). The blots were probed with mouse monoclonal antibodies to HuR (Santa Cruz Biotechnology) and β-tubulin (Sigma) and rabbit polyclonal antibodies to AUF1(Millipore) and PEPCK (Abgent). Subsequently, the blots

were developed with secondary antibodies, goat anti-rabbit 800 and goat anti-mouse 680 (LiCor) and signals were detected using an Odyssey Infra-red Imager.

2.9 HaloLink Resin Pull-Down Assay

Halo RNA-pull down assay (Fig.1.8) was employed to "visualize" the relative changes in binding interactions between multiple RNA-binding proteins and PEPCK mRNA during normal and acidotic conditions. Our goal was to characterize the binding of HuR and p40AUF1 to the PEPCK mRNA in intact cells by attaching a HaloTag to the individual RNA binding proteins. The HaloTag is a unique peptide sequence (mutated hydrolase) that forms a covalent linkage to the Halo resin. An alternative goal in using the HaloTag technology was to identify *in vivo* protein-protein interactions between HuR, AUF1 and ζ-Cryst and to determine if those interactions were RNA-mediated.

2.10 Cloning of RNA binding proteins into the Halo tag vector

For this application, the protein of interest was cloned into pFC14A (HaloTag) CMV Flexi vector (Fig.2.3), which allows expression of C-terminal tagged proteins (Danette. D.H. 2007). The coding regions of HuR and p40 AUF1 were PCR amplified using Flexi primers to append on *SgfI* site just upstream of the start codon of the protein-coding region and a *PmeI* site after the carboxy - terminus. The *PmeI* site contains a stop codon. The amplicons were then cloned into an acceptor vector (pFC14A) that contains a *SgfI* site and a blunt-ended *EcoICRI* site. Upon successful ligation of the insert, the blunt

PmeI and *EcoICRI* ends are joined to destroy the stop codon, allowing read through into the C-terminal Halo peptide sequence (Fig.2.4). Mouse HuR and human p40 AUF1

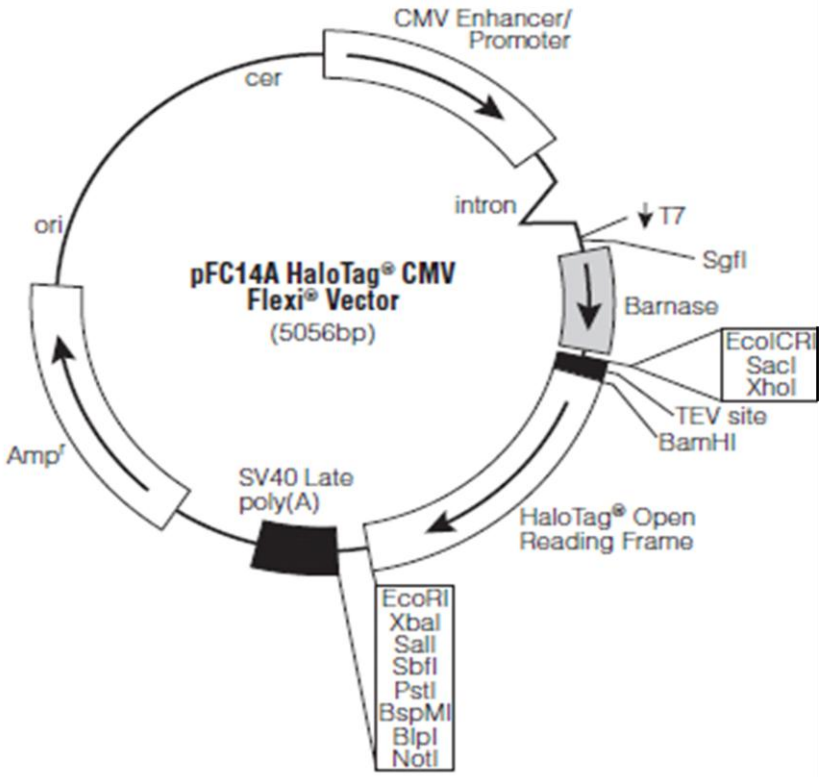

Fig.2.3. pFC14A HaloTag CMV Flexi Vector circle map (adapted from Promega website). This vector is useful for appending a C-terminal Halo Tag (33 k Da) to the fusion protein, and is driven by the human cytomegalovirus (CMV) promoter which allows constitutive expression in mammalian cells. The presence of lethal gene (barnase) allows for positive selection for the insert. The unique TEV protease site downstream of the protein of interest can be used to cleave off the Halo Tag from the fusion protein. The *SgfI* and *EcoICRI* offer unique restriction sites to allow easy insertion of the protein coding sequence of the insert.

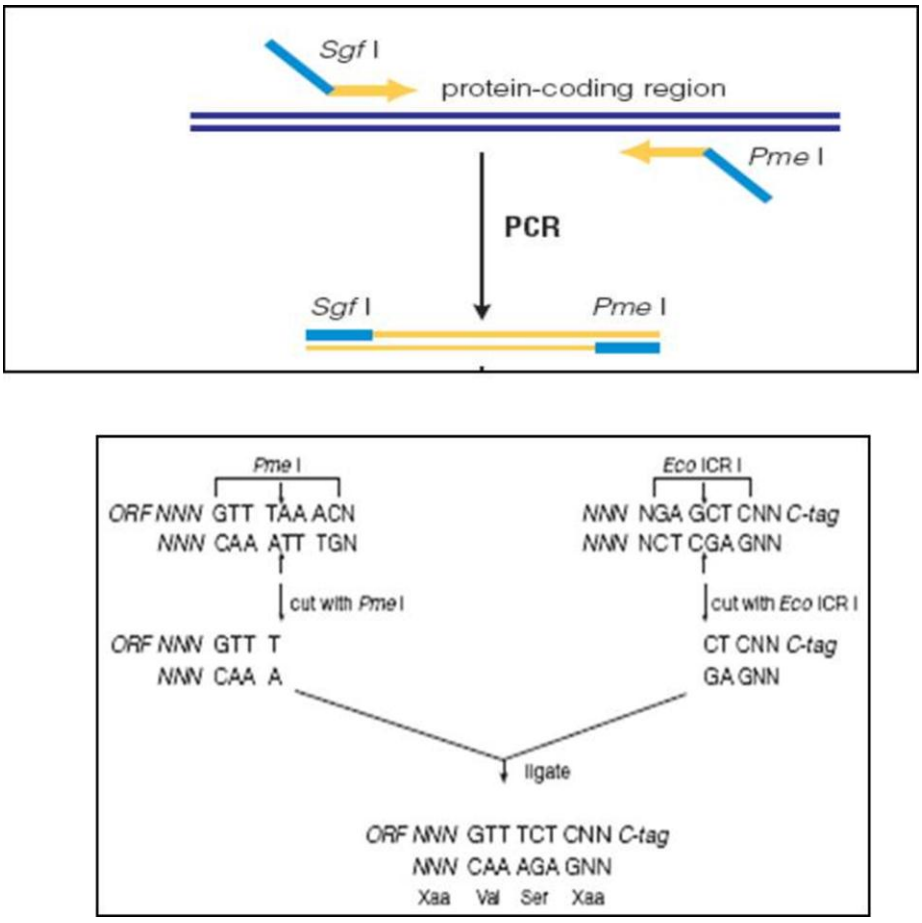

Fig.2.4 Cloning strategy for inserting ORF of protein of interest into pFC14A HaloTag CMV Flexi Vector (adapted from Promega website). PCR amplify sequence of the coding region with flanking *SgfI* and *PmeI* restriction sites. Joining the blunt ends of *PmeI* and *EcoICRI* creates an in-frame Ser codon that appends the downstream sequence-terminal protein-coding region contained on the Flexi Vector backbone).

were PCR amplified with 5'*SgfI* and 3'*PmeI* (primer sequences listed in Table 2.1 from previously available plasmids in the lab, using Flexi Vector Primer Design Tool (Promega).

2.11 Expression of halo-fusion constructs

Individual chimeric halo-fusion constructs were co-transfected with pcDNA 3.1 hygro into LLC-PK_1-F^+-9C cells. The transformed cells were grown in selection medium containing 1µg/ ml hygromycin. Stable cell lines were obtained for only the p40AUF1 fusion-constructs. Transient transformation was used to express halo-chimeric HuR. Halo-HuR construct (3 µg) was transiently transfected using Lipofectamine-2000 (Invitrogen) into LLC-PK_1-F^+9C cells. After 6 h of transfection the medium was removed, the cells were washed twice with phosphate buffered saline, and then fresh medium containing Penicillin/Streptomycin was added.

2.12 HaloLink Resin Pull-down assay with Halo-fusion construct

LLC-PK_1-F^+-9C cells that either transiently express Halo-HuR or stably express Halo-p40AUF1 were either maintained in normal (pH 7.4) medium or transferred to acidic (pH 6.9) medium for 24 h. The cells were then cross-linked with 0.3 % formaldehyde for 10 min and harvested with nuclei swelling buffer (5 mM PIPES, pH 8.0; 85 mM KCl; 0.5% Nonidet P-40), supplemented with 150 mM sodium chloride, Halt Phosphatase inhibitor (1%), protease inhibitor cocktail (10%) and RNasin Plus RNase Inhibitor (1u/µl) . The total cell extract was spun at 3000 rpm (237 xg) for 5 min at 4^oC and the nuclei pellet was discarded. The supernatant containing the cytoplasmic extract

Table 2.1: List of Halo-Flexi primer sequences. Halo Flexi primers appending *Sgf I* site on the forward primer and *PmeI* to the reverse primer. These primer sequences were made using Flexi Vector Primer Design Tool (Promega).

Primers for generation of the halo mHuR and halo p40AUF1		
Starting Plasmid	Primer Name	Oligonucleotide Sequence 5'➔ 3'
pET-21b his-HuR	mHuR-Flexi-ShortF	AGGAGCGATCGCCATGGAAGACTGCAGGGATGACATT
	mHuR-Flexi-longF	GTCGGCGATCGCCATGTCTAATGGTTATGAAGACCACA TGGCGGAAGACTGCAGGGATGACATTGGGAGAACG
	mHuR-Flexi-shortR	CTTAGTTTAAACTTTGTGGGACTTGTTGGTTTTG
hp40AUF1	hAUF-FlexiF	GGGGGCGATCGCCATGTCGGAGGAGCAGTTCGG
	hAUF-FlexiR	ATGCGTTTAAACGTATGGTTTGTAGCTATTTTGATGACCAC
Primers for sequencing final halo-clones	pFC14A Flex.Vec.978 Forward	TTCTCTCCACAGGTGTCC
	pFC14A Flex.Vec.1545 ReversePr	CAGGACTTCCACATAATGG

was used for RNA-pull down assays. Triplicates of pull-down samples were collected for each treatment, while controls were harvested in duplicates. Control samples were pre-incubated with Halo-blocking peptide that binds to the HaloTag protein and prevents interaction with the HaloLink Resin. Multiple binding conditions were tested and 350 µg of cytosolic protein extract and 125 µl of resin slurry incubated for 4-5 h with rocking at 4^{o}C produced optimal binding with low background. Following incubation, the resin-protein was pelleted by centrifugation and the supernatant containing unbound halo-chimeric HuR, along with the input sample were analyzed on a western blot. Stringent washing of the resin was achieved by washing with high-salt wash buffers and nuclease-free water (Promega.; 2011) to ensure removal of protein or RNA that bind through non-specific interactions. The HaloTag protein:RNA complexes on the resin were subsequently heated to 70^{o}C for 45 min in presence of 100 mM $MgCl_2$ and RNAsin plus RNAse inhibitor to reverse the cross-links between the RNA and the fusion protein and release the captured RNA. RNA from either the input samples or the precipitated complex were purified using TRIzol reagent and quantified using a qRT-PCR assay using specific primers and taqman probes for the AU-rich region of the 3'UTR PEPCK mRNA (PCK67; Hex), and the coding region of porcine glyceraldehyde 3-phosphate dehydrogenase (p-GAPDH; Texas Red).

At this point, the immobilized haloTag protein is still covalently bound to the haloLink Resin. Treatment of the precipitated complex with boiling SDS sample buffer releases all the co-purifying proteins that were interacting with HaloTag-fusion protein via ionic interactions (Fig. 2.5)

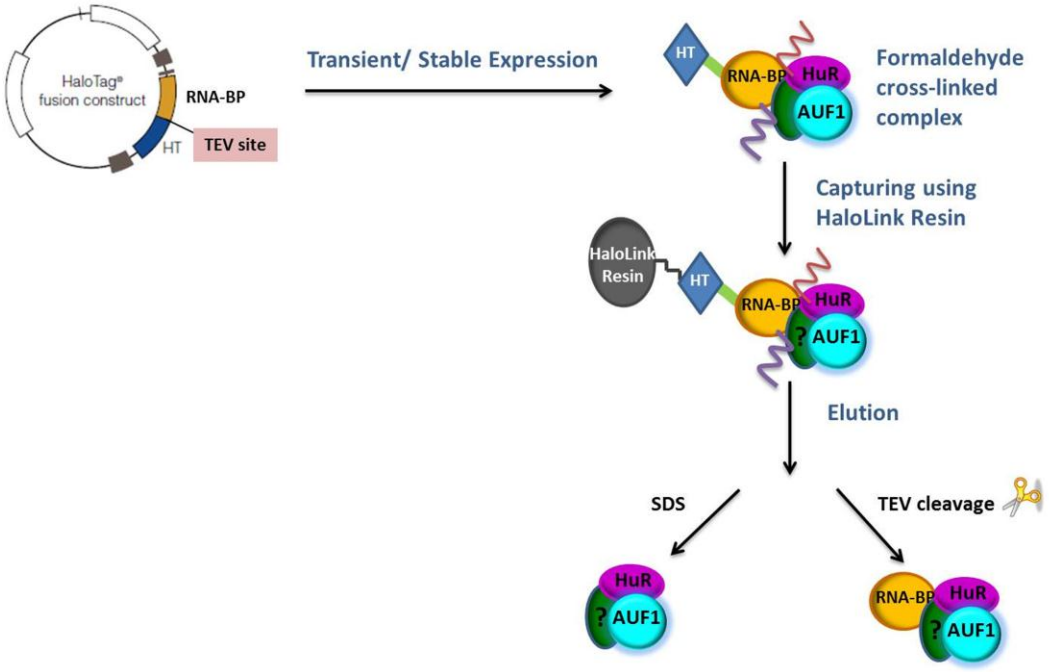

Fig.2.5. Schematic showing *in vivo* HaloTag-pull down protocol for identifying protein-protein interactions (modified from Promega website).
Expression of HaloTag (HT) fusion protein and formation of stable complexes with interacting protein partners. The stable complexes are captured on HaloLink Resin followed by stringent washing. The bound protein complexes can be eluted off via two means- SDS elution releases associated protein partners of HT-fusion protein; the whole complex including the protein of interest are recovered with TEV protease digestion.

2.13 Selection of stable cell line over-expressing Neo-ζ-Cryst

pcDNA 3.1/Neo-ζ-Cryst plasmid was constructed and maintained as referenced in Hend Ibrahim's thesis. This plasmid was used to select stable mixed cell line of LLC-PK_1-F^+9C that over-express ζ-Cryst. This was accomplished by transfection of subconfluent cells with Lipofectamine-2000 reagent (Invitrogen). Fresh DMEM medium minus Fetal Bovine serum and with no antibiotics was added 1 h prior to the addition of 3 μg or 5 μg of plasmid DNA. After 6 h, the transfection medium was removed, and the cells were washed two times with phosphate-buffered saline before fresh pH 7.4 medium containing 0.8 mg/ml neomycin was added. The medium was changed every two days. After 10-15 days, neomycin resistant colonies were treated with trypsin and the cells were grown on larger plates. Following the initial split, the cells were grown in pH 7.4 medium containing 0.2 mg/ml neomycin and checked for the level of overexpression of mouse ζ-Cryst via western analysis.

2.14 ShRNA knockdown of ζ-Cryst

The pSilencer ζ-Cryst shRNA expression vector was created and transformed into XL1 Blue cells (unpublished data of Hajarnis, et.al). The resulting plasmid was purified, sequenced and stably transformed into LLC-PK_1-F^+9C along with pcDNA3.1 neo, which allowed selection with 0.8 mg/ ml G418. Clonal lines of the stably transfected cells were tested for knockdown of ζ-Cryst. Clone 9 was used for the reported experiments.

CHAPTER III

CONCURRENT BINDING AND POST-TRANSLATIONAL MODIFICATION OF AUF1 AND HUR MEDIATE THE PH-RESPONSIVE STABILIZATION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE MRNA IN KIDNEY CELLS[†]

3.1 Introduction

The maintenance of blood acid-base balance is essential for survival. However, metabolic acidosis is a common clinical condition that is characterized by a significant decrease in plasma pH and bicarbonate concentration (Stern 2004; Wagner 2007). This disturbance is caused by genetic or acquired defects in metabolism, in renal handling of bicarbonate, and in the excretion of titratable acid. In addition, patients with cachexia, trauma, uremia, end stage renal disease or HIV infection frequently develop acidosis as a secondary complication that adversely affects their outcome. Chronic acidosis causes mental retardation in children and osteomalacia, nephrocalcinosis and urolithiasis in adults. Increased renal ammoniagenesis and gluconeogenesis from plasma glutamine constitute an essential physiological response to metabolic acidosis that partially restores acid-base balance (Tannen and Sahai 1990; Halperin 1993; Curthoys 2007). A portion of this adaptive response is the rapid and pronounced increase in the cytosolic isoform of phosphoenolpyruvate carboxykinase (PEPCK) that occurs within the renal proximal convoluted tubule (Curthoys and Gstraunthaler 2001).

[†] (This chapter was recently submitted to The *Journal of Biological Chemistry* for publication) (Lakshmi Gummadi, Lynn Taylor and Norman P. Curthoys)

Previous transcription run-off experiments established that the rapid increase in PEPCK mRNA during acute onset of metabolic acidosis correlates with an increased transcription of the *PCK1* gene (Hwang and Curthoys 1991; Hwang et al. 1991). However, the resulting data also suggested that the sustained increase in PEPCK expression during chronic acidosis is due, at least in part, to stabilization of the PEPCK mRNA. The pH-responsive increases in PEPCK mRNA and protein are reproduced when LLC-PK_1-F^+-9C cells (Mufti et al. 2011), a clonal line of porcine proximal tubule-like kidney cells, are treated with an acidic medium (pH 6.9, 9 mM HCO_3^-) for 24 h. The resulting 4-fold increase in PEPCK protein is derived from increased transcription and a 2-fold stabilization of the PEPCK mRNA. Furthermore, the pH-responsive stabilization was recapitulated using a chimeric reporter mRNA, containing the full-length 3'-UTR of PEPCK, which was stably expressed in the LLC-PK_1-F^+-9C cells from a Tet-regulated promoter. Previous studies also identified the combined PCK6 and PCK7 segments within the 3'UTR of PEPCK mRNA as the elements that contribute to its rapid turnover (Hajarnis et al. 2005) and mediate the pH-responsive stabilization (Mufti et al. 2011). The same segments contain highly conserved AU-rich elements (ARE) that bind two well-characterized RNA binding proteins, the 40-kDa isoform of AU binding-factor-1 (p40AUF1) and Human Antigen R (HuR), with high affinity and specificity.

HuR is a 36-kDa protein that normally is localized predominantly in the nucleus (Keene 1999). It contains three conserved RNA recognition motifs (Burd and Dreyfuss 1994) and a 33-amino acid hinge region that functions as a nucleocytoplasmic shuttling sequence (Fan and Steitz 1998). In response to various stress conditions, HuR affects the translation and enhances the stability of many mRNAs that contain an AU-rich element

(ARE) (Fan and Steitz 1998). However, the mechanism by which HuR mediates the stabilization of mRNAs is poorly understood (Keene 1999; Brennan and Steitz 2001). The shuttling of HuR to the cytoplasm in response to stress conditions, such as heat shock (Gallouzi et al. 2000), UV irradiation (Wang et al. 2000), amino acid starvation (Yaman et al. 2002), chronic ethanol exposure (McMullen 2003), hypoxia (Levy et al. 1998), and ATP depletion (Jeyaraj et al. 2006) is primarily linked to its ability to stabilize mRNAs (Wang et al. 2000; Lopez de Silanes et al. 2004). Alternatively, post-translational modifications of HuR may affect its RNA binding affinity or its ability to associate with additional RNA stabilizing factors (Abdelmohsen et al. 2007; Doller et al. 2007; Lafarga et al. 2009; Lopez de Silanes et al. 2009).

The AUF1 or hnRNP D family of proteins contains four isoforms (p37, p40, p42 and p45) that are produced by alternative splicing of a single pre-mRNA (Zhang et al. 1993; Ehrenman et al. 1994; DeMaria and Brewer 1996; Wagner et al. 1998). Previous studies have established that AUF1 can either stabilize or destabilize various mRNAs by binding to specific ARE- containing segments. The specific effect of AUF1 binding has been postulated to be cell specific, due to changes in the relative abundance of the AUF1 isoforms, or may result from various post-translational modifications (Laroia and Schneider 2002; Wilson et al. 2003; Raineri et al. 2004). HuR and AUF1 share a number of common ARE binding sites and multiple studies have demonstrated that the two RNA binding proteins can interact and form a physical association (Lal et al. 2004; Palanisamy et al. 2008; Masuda et al. 2011). Thus, the dynamic interaction between HuR and AUF1 may determine whether the complex mediates the rapid turnover or facilitates the stabilization of a specific mRNA (Barker et al. 2012).

Previous studies have established that HuR and AUF1 bind to multiple overlapping sites within 3'-UTR of PEPCK mRNA (Hajarnis et al. 2005; Mufti et al. 2011). siRNA knockdown of HuR in LLC-PK_1-F^+-9C cells had a significant effect on basal expression, but only a slight effect on the pH-responsive stabilization of PEPCK mRNA. By contrast, the partial knockdown of AUF1 failed to affect either the basal or the pH-responsive expression of PEPCK mRNA (Mufti et al. 2011). Therefore the focus of the current study was to assess the potential combinatorial role of HuR and AUF1 in mediating the pH-responsive stabilization of PEPCK mRNA in LLC-PK_1-F^+-9C cells. Interestingly, the resulting data established that the concomitant reduction of HuR and AUF1 is required to completely abolish the pH-responsive increases in PEPCK mRNA and protein. This conclusion was corroborated by demonstrating that the co-recruitment of chimeric constructs of the MS2 coat protein (MS2cp) and of HuR and of p40AUF1 is necessary to impart a pH-responsive stabilization to a β-globin-PCK2 (βG-PCK2) reporter mRNA that contains six MS2 stem-loop elements. Furthermore, two-dimensional western blot analyses indicated that the extent of phosphorylation of HuR and possibly AUF1 are altered in response to treatment of LLC-PK_1-F^+-9C cells with an acidic medium. Therefore, a remodeling of the HuR/AUF1 complex associated with the 3'UTR may mediate the stabilization of PEPCK mRNA when kidney cells are challenged with an acidotic stress.

3.2 Materials and Methods

3.2.1 Cell culture and siRNA transfections

LLC-PK_1-F^+-9C cells (Mufti et al. 2011) were cultured in DMEM-Base medium (Sigma) supplemented with penicillin/streptomycin (Sigma), 10% fetal bovine serum, 5 mM glucose, 26 mM $NaHCO_3$, 17 mM NaCl, 2 mM glutamine, 5 mM pyruvate, 5 µM phenol red, and 10 mM HEPES, pH 7.4 at 37^{o}C in a 5% CO_2 atmosphere. A physiologic mimic of metabolic acidosis was recapitulated using an acidic (pH 6.9) medium, which was prepared as above, except that 9 mM $NaHCO_3$ and 34 mM NaCl were added to reduce the pH while maintaining equivalent osmolarity (Gstraunthaler et al. 2000). A pre-annealed double stranded stealth siRNA (Invitrogen) targeting the coding region of porcine HuR (HuR97 forward strand - CAGGAGGAGUUACGAAGUCUGUUCA) and a control siRNA that is not encoded in the human, rat or mouse genome (ctrl378 forward strand - UGUAGGUAGAAGCUAUCAUUACGUG) were used at a final concentration of 30 nM and 50 nM, respectively. To silence pig AUF1, two separate siRNA oligos that target exon 3, which is common to all four isoforms of AUF1 (378AUF forward strand - CACUCUGAAGUUAGAUCCUAUC ACA and 429AUF forward strand - UUUAGGAUCAAUCACCUUCCCAUUC) were used in a combination of 50 nM each. The siRNAs were transfected into 70-80% confluent cells in 12-well plates using Lipofectamine RNAiMAX (Invitrogen) as described previously (Mufti et al. 2011). After 48 h, cells were treated with either pH 7.4 or pH 6.9 medium for 24 h prior to harvesting in 100 µl lysis buffer for western blots (O'Hayre et al. 2006) or in 250 µl TRIzol® Reagent for RNA isolation.

3.2.2 RNA extraction and Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated using TRIzol® (Invitrogen) as per the manufacturer's protocol. An Oligo-dT_{18} primer (IDT) and the avian myeloblastosis virus-reverse transcriptase (Promega) were used to reverse transcribe 1 µg of RNA. Gene-specific primers and Taqman probes for the detection of endogenous porcine PEPCK mRNA and the control GAPDH mRNA were used for the RT-qPCR analysis as previously reported (Mufti et al. 2011).

3.2.3 Chimeric MS2-expression plasmids

A chimeric reporter construct (pβG-PCK2) that expresses a β-globin-PCK2 mRNA from a tetracycline-responsive promoter was created and maintained as reported earlier (Hajarnis et al. 2005). This construct contains a tetracycline-responsive promoter, the coding region of the rabbit β-globin (βG) gene, the 5'fragment (381bp) of the 3'-UTR of rat PEPCK cDNA (PCK2), and the polyadenylation site of bovine growth hormone (bGH) cDNA. The pPC-β6bs plasmid (Lykke-Andersen et al. 2000), which is a derivative of pcDNA3.0 (Invitrogen) that encodes six copies of MS2 coat protein-binding sites, was obtained as a kind gift from the Wilusz lab (Department of Microbiology, Immunology and Pathology, Colorado State University). Each of the MS2 stem-loops differs from the wild type by an A→C high affinity mutation in the loop sequence as indicated in Fig. 3.1A. A *Not I*/*Xba I* digestion of pPC-β6bs released a 314 bp fragment

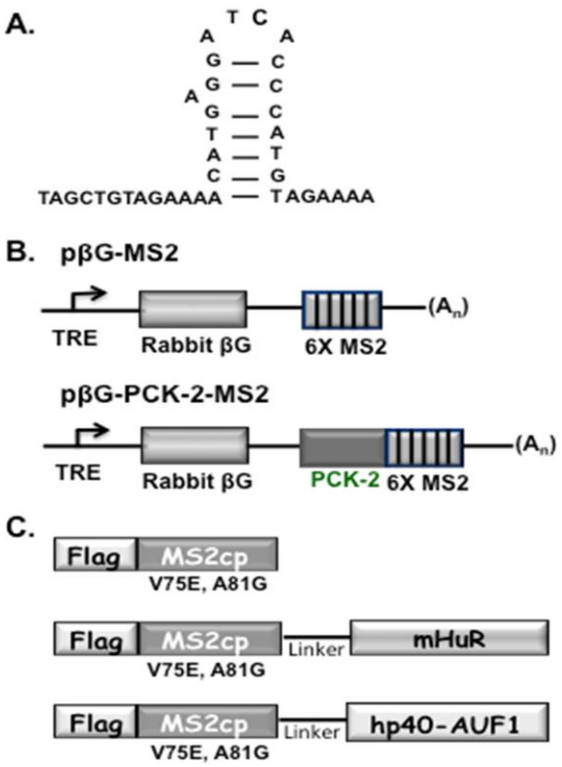

Fig.3.1. Reporter mRNAs and MS2 fusion proteins used in the recruitment assay. A. Nucleotide sequence and secondary structure of an MS2 stem-loop. B. The pβG-MS2 plasmid contains a tetracycline-responsive promoter element (TRE), the complete coding region for rabbit β-globin (βG), and a 3'UTR that contains six copies of the MS2 stem-loop sequence (6x MS2). The pβG-PCK2-MS2 plasmid also encodes the PCK-2 sequence that constitutes the 5'-half of the 3'UTR of rat PEPCK mRNA. C. The MS2cp fusion contains an N-terminal FLAG epitope that is in frame with the MS2 coat protein sequence. The remaining fusion proteins also contain a hydrophilic linker sequence followed by the mouse HuR or human p40-AUF1 sequence.

containing six-repeats of the MS2 coat protein-binding sites. This fragment was inserted into the *NotI* and *XbaI* sites of pβG-PCK2 to create the MS2 reporter construct, pβG-PCK2-MS2. This construct retains a single AUF-1 binding site within the PCK2 segment (Fig.3.1B). A control reporter construct that lacks the AUF1 binding site, pβG-MS2 was also created using the same strategy, except that the starting plasmid lacked the PCK-2 sequence.

3.2.4 MS2-fusion proteins

The pcNMS2-Flag (Lykke-Andersen et al. 2000) plasmid was also obtained from the Wilusz lab. pcNMS2-Flag encodes an N-terminal flag peptide sequence that is in-frame with MS2-coat protein (MS2cp). The inserted MS2cp sequence encodes a mutated form of the MS2 bacteriophage coat protein that contains two point mutations (V75E; A81G). The two mutations prevent protein multimerization but retain high binding affinity to the MS2-stem loop binding sites (LeCuyer et al. 1995). The open reading frame of mouse HuR was PCR amplified from pGEMTeasy-mHuR (Mufti et al. 2011) with primers containing 5'-*BglII* ($^{5'}$GACTAGATCTAGCG CCATGTCTAATGG$^{3'}$) and 3'-*XbaI* ($^{5'}$CGA TTTCTAGATTAAACTTTGTGGGACTTG$^{3'}$) sequences and cloned in frame with the C-terminus of the MS2cp sequence (Fig. 3.1C). Similarly, the p40AUF1coding sequence was PCR amplified from pGEMTeasy-p40AUF1 (Hajarnis et al. 2005) with forward ($^{5'}$AATTGGATCCAAAGCCATGTCGG AGG$^{3'}$) and reverse ($^{5'}$GTCCGATGCTAGC TTAAACGTATGGTTTGTAGC$^{3'}$) primers and inserted in-frame at the *BamHI* and *NheI* sites that are downstream of the MS2cp-sequence.

3.2.5 mRNA half-life analysis

To assess mRNA half-lives, the β-globin reporter RNA plasmids were stably co-transfected with pcDNA3.1/Hygro (Invitrogen) into LLC-PK_1-F^+-9C cells that stably express the tTA protein (Schroeder et al. 2006)**.** The stable transformants were grown in 6-well plates in normal medium containing minimal Dox (100 ng/ml) until 70% confluent. The cells were then transiently transformed with pFlag-MS2cp-HuR (2 μg) or pFlag-MS2cp-p40AUF1 (2 μg) plasmid and maintained in normal (pH 7.4) medium minus Dox for two days, before treating with acidic medium (pH 6.9) for 24 h. RNA was isolated at various times after addition of 1 μg/ml Dox to completely arrest transcription. The relative levels of β-globin and GAPDH mRNAs were quantified by RT-qPCR and normalized to GAPDH mRNA. Since the kidney cells do not express β-globin, the RT-PCR assay detects only the reporter RNA and serves as a common read-out for all the β-globin based reporters. The RT-PCR assay for βG-cDNAs used forward ($^{5'}$TCAGTGAGGGTCTGA ATCACC$^{3'}$) and reverse ($^{5'}$CTGCACCTGAGG AGTGAATTC$^{3'}$) primers and a Taqman probe ($^{5'}$FAM-CACCTTTGCTAAGCTGAGTGAACTG CAC-BHQ1$^{3'}$).

3.2.6 Western blot analysis

Bradford assays (Bradford 1976) were performed to determine the concentration of the protein lysates. Samples containing 15 μg of protein were resolved by 10% SDS-PAGE and transferred to Immobilon-F membranes (Millipore). The blots were probed with mouse monoclonal antibodies to HuR (Santa Cruz Biotechnology) and β-tubulin (Sigma) and rabbit polyclonal antibodies to AUF1 (Millipore) and PEPCK (Abgent).

Subsequently, the blots were developed with goat anti-rabbit 800 and goat anti-mouse 680 (LiCor) secondary antibodies and the resulting fluorescence was quantified using an Odyssey Infra-red Imager.

3.2.7 Two Dimensional Western Blot analyses

Total cell extracts were prepared from 10-cm plates of confluent LLC-PK_1-F^+-9C cells that were either maintained in pH 7.4 medium or treated with pH 6.9 medium for 24 h prior to lysis. The freshly prepared lysis buffer contained 7 M urea, 2 M thiourea, 4% w/v CHAPS and 30 mM Tris, pH 8.8 (Curthoys et al. 2007) supplemented with 0.1 mg/ml PMSF, 10% Protease Inhibitor Cocktail (Sigma) and 3x Halt phosphatase inhibitor (Roche). The cell extracts were incubated on ice for 10 min followed by centrifugation for 5 min at 4,000 x g at 4 ^oC. A sample containing 30 µg of the supernatant of the total cell lysate was diluted to 450 µl with rehydration buffer and applied to a 7-cm precast IPG Immobiline DryStrip (pH 6-11) purchased from GE Healthcare. Strips were covered with mineral oil and actively rehydrated for 16 hours on IPGphor apparatus (GE Healthcare) at 50 µA/strip at 20 ^oC on the IPGphor apparatus followed by first dimension focusing using 500V for 1 h, 1000V for 1 h, and 8000V for 6 h. After focusing, the strips were rinsed, reduced with 1% DTT in equilibration buffer (7M Urea, 2M Thiourea, 4% CHAPS, 1% Bromophenol blue) and then treated with 2.5% iodoacetamide. The proteins were resolved in a second dimension on a 10% SDS-polyacrylamide gel, blotted to an Immobilon-F membrane at 180V for 55 min, and then probed with the appropriate antibodies.

3.3 Results

3.3.1 Treatment with acidic medium does not significantly change the subcellular localization of HuR and AUF1

While HuR is normally located predominantly in the nucleus, shuttling to the cytoplasm occurs in response to various stress conditions such as heat shock (Gallouzi et al. 2000), UV irradiation (Wang et al. 2000), amino acid starvation (Yaman et al. 2002), chronic ethanol exposure (McMullen et al. 2003), hypoxia (Levy et al. 1998), and ATP depletion (Jeyaraj et al. 2006). Therefore, nuclei and post-nuclear fractions were isolated to determine whether treatment of LLC-PK_1-F^+-9C cells with pH 6.9 medium causes the redistribution of HuR and/or AUF1. Treatment of cells with acidic medium for 18 h caused a pronounced increase in PEPCK protein that is recovered solely in the post-nuclear fraction. However, western blot analysis (Fig.3.2) showed no significant changes in the cytoplasmic levels of HuR. Only 12% of the HuR protein was recovered in the cytosolic fraction of cells grown in normal medium. Furthermore, the proportion of HuR recovered in this fraction was unchanged when the cells were treated with acidic medium. Similarly, only 11% of the total p45 AUF was recovered in the post-nuclear fraction. By contrast, nearly 60% of the combined p40/p42 AUF1 was cytosolic. However, treatment of cells for 18 h with acidic medium again had no effect on the subcellular distribution of AUF1.

Therefore acidic pH treatment did not cause a change in abundance of HuR or AUF1 isoforms or a significant alteration in the subcellular localization of the proteins in LLC-PK_1-F^+-9C cells. Thus, a remodeling of the binding of HuR and AUF1 to the

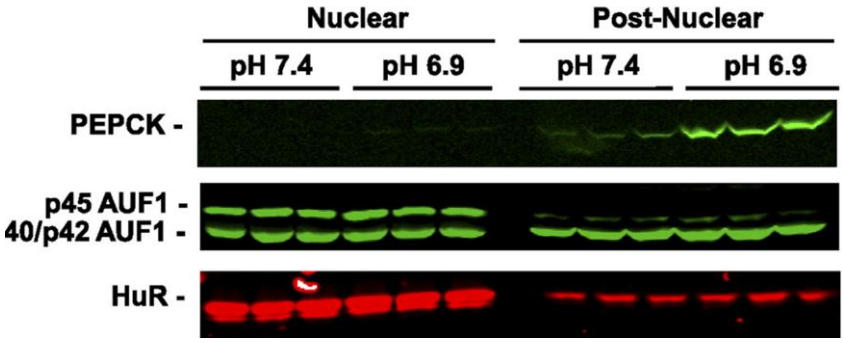

Fig.3.2. Treatment with acidic medium does not change the sub-cellular distribution of HuR or AUF1 (This figure generated by Lakshmi Gummadi and was published in (Mufti et al. 2011). Western blot analysis of the nuclear and post-nuclear fractions derived from LLC-PK_1-F^+-9C cells that were either maintained in normal medium or were treated with acidic medium for 18 h. The cells were lysed and immunoblotted to quantify PEPCK, HuR and AUF1 proteins.

3′-UTR of PEPCK mRNA during acidosis is not driven by changes in the relative cytosolic concentrations of the two RNA binding proteins (Mufti et al. 2011).

3.3.2 HuR is necessary for the pH-responsive stabilization of PEPCK mRNA

Previous studies (Mufti et al. 2011) established that the half-life of the endogenous PEPCK mRNA is increased from 3.2 h to 6.1 h when LLC-PK_1-F^+-9C cells are transferred from normal to acidic medium. The 2-fold stabilization was reproduced using a chimeric reporter mRNA (βG-PCK1), which contains the full-length 3'UTR of PEPCK mRNA. However, this response was lost when a binding site for HuR within the highly conserved AU-sequence of the PCK6 segment was mutated. To further investigate the possible role of HuR in the stabilization of PEPCK mRNA, a small interfering (si)RNA was used to knockdown the level of HuR in LLC-PK_1-F^+-9C cells. Western blot analysis demonstrated that this treatment decreased HuR expression by 90% (Fig. 3.3A). As reported previously (Mufti et al. 2011), the decreased expression of HuR caused a pronounced decrease in the basal level of PEPCK protein and reduced the pH-responsive increase in expression (Fig. 3.3B). With the decreased expression of HuR, the measured half-lives of the PEPCK mRNA in normal ($t_{1/2}$ = 4.0 h± 0.03) or acidic medium ($t_{1/2}$ = 4.5 h± 0.02) were not significantly different (Fig. 3.3C). Therefore, a decrease in HuR expression also prevents the pH-responsive stabilization of PEPCK mRNA. Thus, HuR is a necessary component of the mechanism that mediates this response.

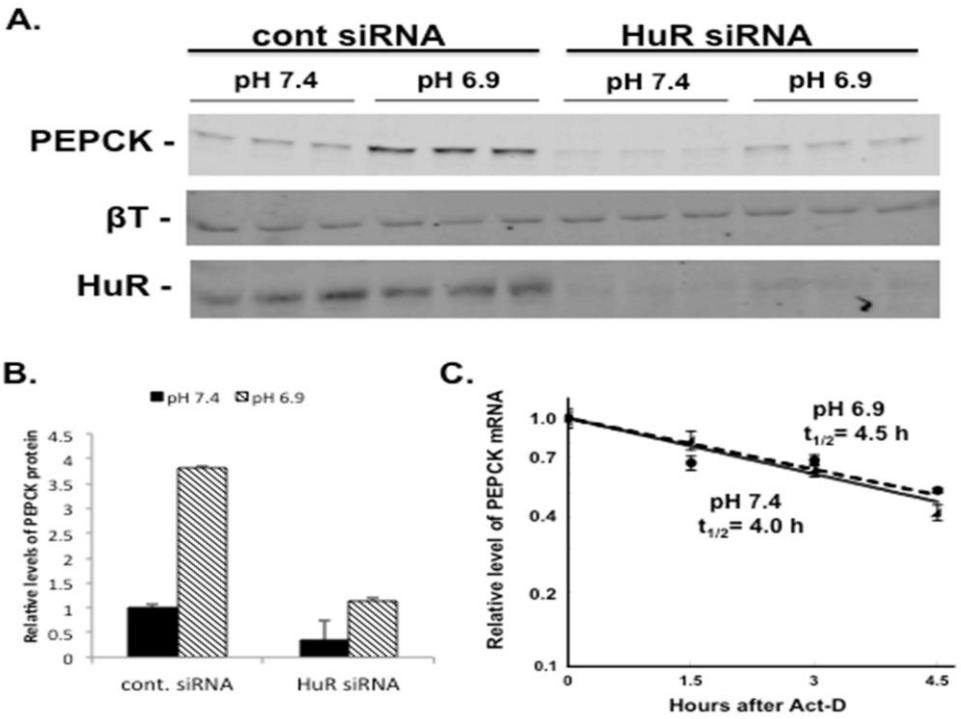

Fig.3.3. siRNA knockdown of HuR prevents the pH-responsive stabilization of PEPCK mRNA. A. LLC-PK_1-F^+-9C cells were transfected with 50 nM control siRNA or 30 nM HuR siRNA and treated with either normal (pH 7.4) or acidic (pH 6.9) medium for 24 h before harvesting. Western blot of the cell lysates were probed for PEPCK, β-tubulin (βT) and HuR. B. siRNA knockdown of HuR led to a significant reduction in the basal level and the pH-responsive increase of PEPCK protein. C. Knockdown of HuR prevents the pH-responsive stabilization of PEPCK mRNA. LLC-PK_1-F^+-9C cells were cultured in 6-well plates and transfected with 30 nM HuR siRNA. The cells were treated for 24 h in either normal (pH 7.4) or acidic (pH 6.9) medium and then exposed to 8 μg/ml Actinomycin D. RNA was isolated at the indicated times and the levels of PEPCK and GAPDH mRNAs were quantified by RT-qPCR. The log of the relative level of PEPCK mRNA was plotted versus time after addition of Actinomycin D to assess the half-life. The reported data are the mean of +/- the S.E. of triplicate samples for each time point.

3.3.3 Co-knockdown of HuR and AUF1 abolishes the pH-responsive increase in PEPCK mRNA and protein

Previous studies (Hajarnis et al. 2005) established that AUF1 binds to many of the same segments within the 3'UTR of PEPCK mRNA that bind HuR (Mufti et al. 2011). LLC-PK_1-F^+-9C cells express high levels of the p45-, p42-, and p40-isoforms of AUF1. Previous attempts to knockdown AUF1 expression using a single siRNA resulted in only a modest decrease in the 3 proteins (Mufti et al. 2011). However, by using a combination of two new siRNAs that are complementary to sequences contained in all four AUF1 mRNAs, an 80% reduction in total AUF1 protein expression was achieved (Fig. 3.4A). This level of knockdown produced a slight increase in basal levels of PEPCK mRNA (Fig.3.4B) and protein (Fig.3.4C) and decreased the pH-responsive increase in PEPCK. Thus, AUF1 may contribute to the rapid degradation of PEPCK mRNA under normal conditions but it may also participate in the pH-responsive stabilization. Most importantly, the silencing of both HuR and AUF1 had little effect on expression in normal medium, but completely blocked the pH-responsive increase of PEPCK mRNA (Fig.3.4B) and protein (Fig.3.4C). These results support the view that HuR may enhance expression of PEPCK in normal medium, while AUF1 may have an inhibitory effect. However, a cooperative interaction between the two RNA binding proteins is required to mediate the pH-responsive stabilization of PEPCK mRNA.

3.3.4 Concurrent binding of HuR and AUF1 to an MS2-based reporter mRNA

A recruitment assay was developed to further test the hypothesis that the pH-responsive increase in PEPCK mRNA requires a coordinate interaction between AUF1

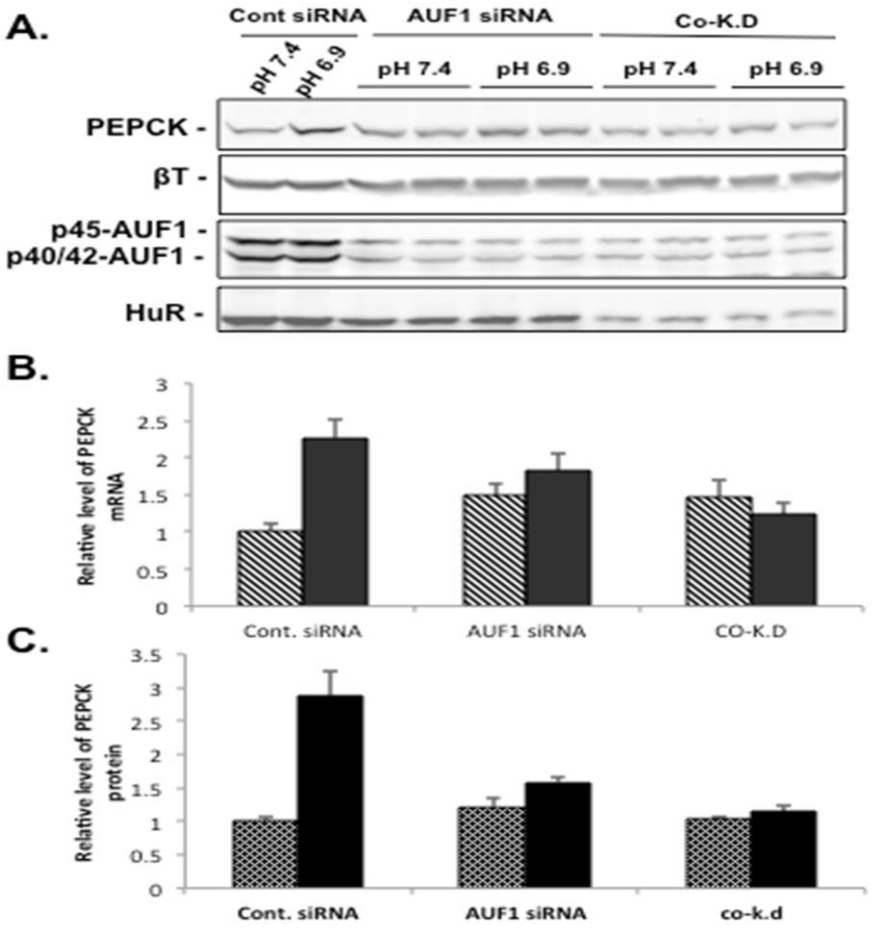

Fig.3.4. Co-knockdown of HuR and AUF1 abolishes the pH-responsive increase in PEPCK mRNA and protein. LLC-PK_1-F^+-9C cells were transfected with 50 nM control siRNA, 30 nM HuR siRNA and/or 100 nM AUF1 siRNAs. After 2 d, the cells were maintained in either normal (pH 7.4) or acidic medium (pH 6.9) for 24 h and then harvested with lysis buffer or TRIzol. A. Western blot analysis was performed to monitor the expression of PEPCK, HuR, AUF1 and β-tubulin (βT) proteins. B. Levels of PEPCK and GAPDH mRNAs were determined by RT-qPCR. C. The levels of PEPCK and β-tubulin proteins were quantified from the western blots. The relative levels of PEPCK mRNA (Panel B) and protein (Panel C) in the normal (hatched bars) and acidic samples (solid bars) are the mean +/- SE of triplicate samples.

and HuR. This assay makes use of the high affinity interaction between the MS2 coat protein (MS2cp) and a unique RNA binding site within the MS2 phage that forms a steam-loop structure.

Initially, a chimeric βG-MS2 mRNA was stably expressed in LLC-PK_1-F^+-9C cells from a tetracycline-responsive promoter. However, preliminary experiments indicated that this mRNA has a half-life of 17 h in cells grown in normal medium (data not shown). Therefore, an alternate reporter mRNA (βG-PCK2-MS2) was developed. The latter mRNA contains the standard rabbit β-globin coding sequence followed by the PCK2 segment of the 3'UTR of PEPCK mRNA and six MS2 stem-loops. The PCK2 segment contains an instability element that binds AUF1, but it does not contribute to the pH-responsive stabilization of PEPCK mRNA (Hajarnis et al. 2005). Inclusion of the PCK2 segment produced a reporter mRNA that decays with a half-life ($t_{½}$ = 2.9 h) that can be more accurately quantified and that facilitates the identification of stabilizing interactions. Most importantly, the half-life of the βG-PCK2-MS2 reporter mRNA is not affected by treating the cells with an acidic medium (data not shown). To determine the effect of HuR or AUF1 recruitment, cells that stably express the βG-PCK2-MS2 mRNA were transiently transfected with a plasmid that expresses a chimeric MS2cp-HuR or MS2cp-p40AUF1 protein. Western blot analyses confirmed that the chimeric MS2cp-HuR and MS2cp-p40AUF1 were expressed at levels similar to their respective endogenous proteins (Fig. 3.5A). Expression of the chimeric MS2cp-HuR binding protein increased the half-life of the βG-PCK2-MS2 mRNA approximately 1.5 fold ($t_{1/2}$ = 4.7 h) in cells grown in normal medium (Fig. 3.5B). However, the reporter mRNA did not exhibit a significant change in stability when cells were grown in acidic medium

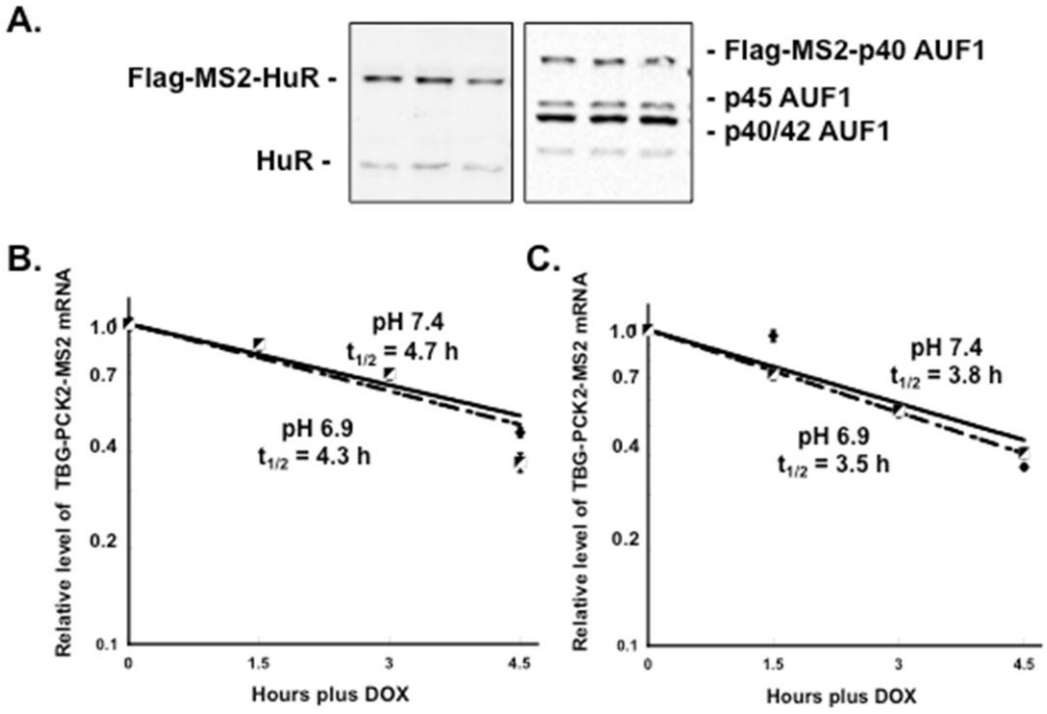

Fig.3.5. Recruitment of MS2cp-HuR or MS2cp-p40AUF1 is not sufficient to impart a pH-responsive stabilization of βG-PCK2-MS2 mRNA. A. LLC-PK$_1$-F$^+$-9C cells that stably express the βG-PCK2-MS2 reporter RNA were transiently transfected with 2 µg of pFlag-MS2cp-hHuR or 2 µg of pFlag-MS2cp-p40AUF1 and then maintained in normal (pH 7.4) medium for 48 h before harvesting with lysis buffer. Western blot analysis was performed to assess the expression of the endogenous and chimeric forms of HuR and AUF1. Half-life analysis of the βG-PCK2-MS2 reporter RNA in LLC-PK$_1$-F$^+$-9C cells that transiently express MS2cp-HuR (Panel B) or MS2cp-p40AUF1 (Panel C). Cells were maintained in minimal Dox (100 ng/ml) until 70% confluent. Following transiently transfection with 2 µg of either expression plasmid, the cells were maintained in normal (pH 7.4) medium minus Dox for 48 h. The cells were then treated with normal or acidic medium for 24 h. RNA was isolated at various times after addition of 1µg/ml Dox to arrest transcription. The relative levels of β-globin and GAPDH mRNAs were quantified by RT-qPCR. The log of normalized data was then plotted against the time after Dox addition. The reported data are the mean ± S.E. of triplicate samples.

($t_{1/2}$ = 4.3 h). The transient expression of the chimeric MS2-p40AUF1 binding protein also had a modest stabilizing effect (~ 1.3-fold) on the half-life of the βG-PCK2-MS2 mRNA ($t_{1/2}$ = 3.8 h) in cells grown in normal medium (Fig. 3.5C). The recruitment of MS2-p40AUF1 also failed to produce a significant change in half-life of the reporter mRNA when the cells were treated with an acidic medium ($t_{1/2}$ = 3.5 h). By contrast, the concomitant binding of MS2cp-HuR and MS2cp-p40AUF1 proteins produced a significant stabilization of the βG-PCK2-MS2 mRNA when cells were switched from normal ($t_{1/2}$ = 4.1 h± 0.01) to acidic medium ($t_{1/2}$ = 6.1 h± 0.01) (Fig. 3.6). This finding strongly supports the hypothesis that the pH-responsive stabilization requires the concurrent recruitment of HuR and p40AUF1 to the terminal segment of the 3-UTR of the PEPCK mRNA. This finding also implies that HuR and AUF1 are simultaneously associated with adjacent binding sites in the PEPCK mRNA.

3.3.5 Two-dimensional gel analyses of potential post-translational modifications of HuR and AUF1

Both the co-knockdown experiments and the MS2-recruitment studies suggest that the pH-responsive stabilization of PEPCK mRNA requires a remodeling of the concurrent binding of HuR and AUF1 to the PCK6/7 segment of the 3'UTR. However, neither HuR nor AUF1 exhibit a significant increase in expression or cytoplasmic localization when LLC-PK_1-F^+-9C cells were treated with an acidic medium (Mufti et al. 2011). Therefore, 2-dimensional gel electrophoresis and western blotting were used to assess whether changes in post-translational modifications may contribute to the stabilizing effect of the bound HuR/AUF1 complex. HuR has a molecular mass of

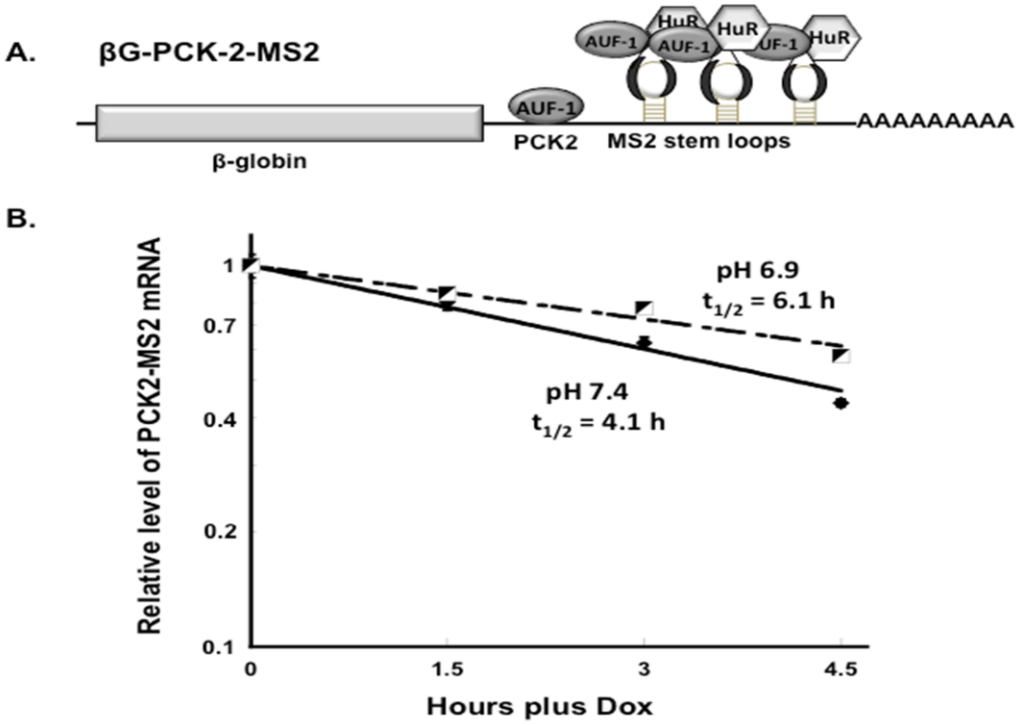

Fig.3.6. Recruitment of both MS2cp-HuR and MS2cp-p40AUF1 imparts a pH-responsive stabilization of βG-PCK2-MS2 mRNA. A. Schematic diagram of the co-recruitment of MS2cp-HuR and MS2cp-p40AUF1 to the βG-PCK2-MS2 mRNA. B. Half-life analysis of the βG-PCK2-MS2 reporter RNA in LLC-PK_1-F^+-9C cells that transiently express MS2cp-HuR and MS2cp-p40AUF1. Cells were maintained in minimal Dox (100 ng/ml) until 70% confluent. Following transiently transfection with 2 µg of both expression plasmids, the cells were maintained in normal (pH 7.4) medium minus Dox for 48 h. The cells were then treated with normal or acidic medium for 24 h. RNA was isolated at various times after addition of 1µg/ml Dox to arrest transcription. The relative levels of β-globin and GAPDH mRNAs were quantified by RT-qPCR. The log of normalized data was then plotted against the time after Dox addition. The reported data are the mean ± S.E. of triplicate samples.

36 kDa and a predicted pI of 9.9. In extracts of LLC-PK_1-F^+-9C cells that were maintained in normal medium, HuR migrates with the expected molecular mass, but contains 3 distinct species that differ slightly in isoelectric points (Fig. 3.7A). The predominant species in normal lysates is the form with the intermediate isoelectric point. When the cell lysate was pretreated with Lambda protein phosphatase, the three species are predominately shifted to a new form that has a more basic pI, consistent with the removal of phosphate groups. When the lysate was prepared from LLC-PK_1-F^+-9C cells that were treated with acidic medium, the same 3 species were observed (Fig. 3.7B). However, the pattern is shifted compared to normal extracts in that the most basic isoform was now the predominant species. Treatment of this lysate with the protein phosphatase again shifted the 3 charged isoforms to the single more basic form as observed following phosphatase treatment of normal lysates. Therefore, HuR may be phosphorylated at multiple sites in LLC-PK_1-F^+-9C cells and the treatment of cells with acidic medium may reduce the overall level of phosphorylation of HuR.

LLC-PK_1-F^+-9C cells express high levels of the p45-, p42- and p40-isoforms, but not the p37-isoform of AUF1. However, the p42- and p40-isoforms co-migrate on an SDS-gel. Thus, only two apparent molecular weight species were identified when the 2-dimensional gels were probed with AUF1 antibodies (Fig. 3.8). However, each isoform produced multiple variants that differ in apparent pI and that may reflect differences in covalent modifications. Pretreatment of the normal lysate with Lambda protein phosphatase produced only a slight increase in the more basic species of the multiple AUF1 variants. Thus, the complex pattern of AUF1 variants may result primarily from covalent modifications other than phosphorylation. By contrast, a lysate prepared from

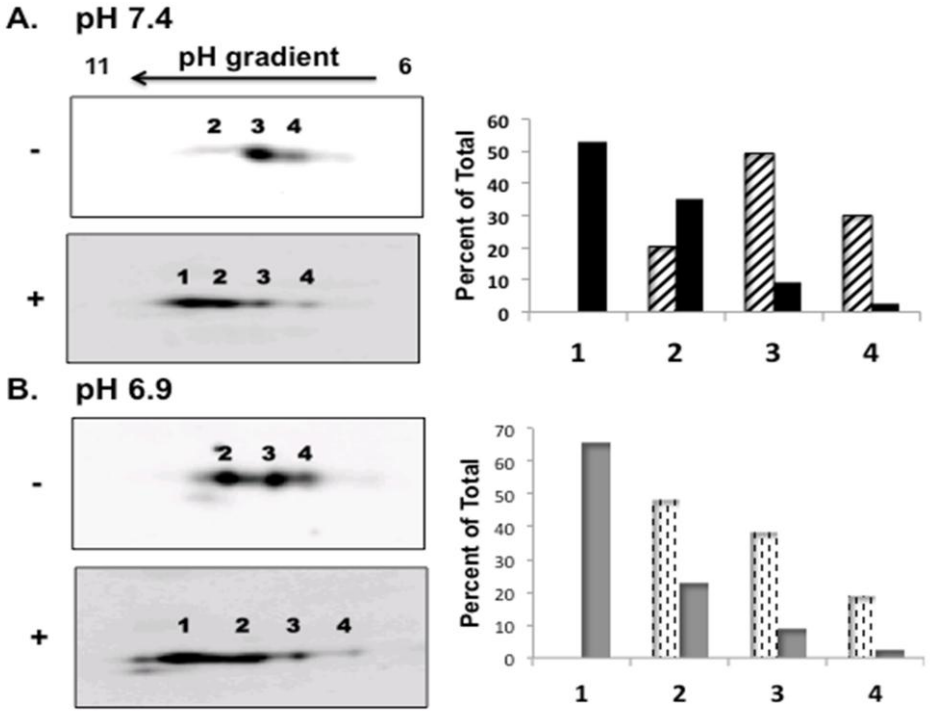

Fig.3.7 Two-dimensional gel analysis of the effect of pH on phosphorylation of HuR. Confluent cultures of LLC-PK_1-F^+-9C cells were treated with normal or acidic medium for 24 h and then lysed with urea and thiourea. The extracts were resolved by two-dimensional gel electrophoresis using isoelectric focusing strips that ranged from pH 6 to 11. The resulting blots were probed with anti-HuR antibody. Resolution of HuR isoforms in cells treated with normal (Panel A) or acidic (Panel B) medium before (-) or after (+) treatment with protein phosphatase. The intensities of the individual spots in the samples before (hatched bars) or after (solid bars) treatment with protein phosphatase were quantified using an Odyssey Infra-red Imager and graphed as a percent of the total intensity.

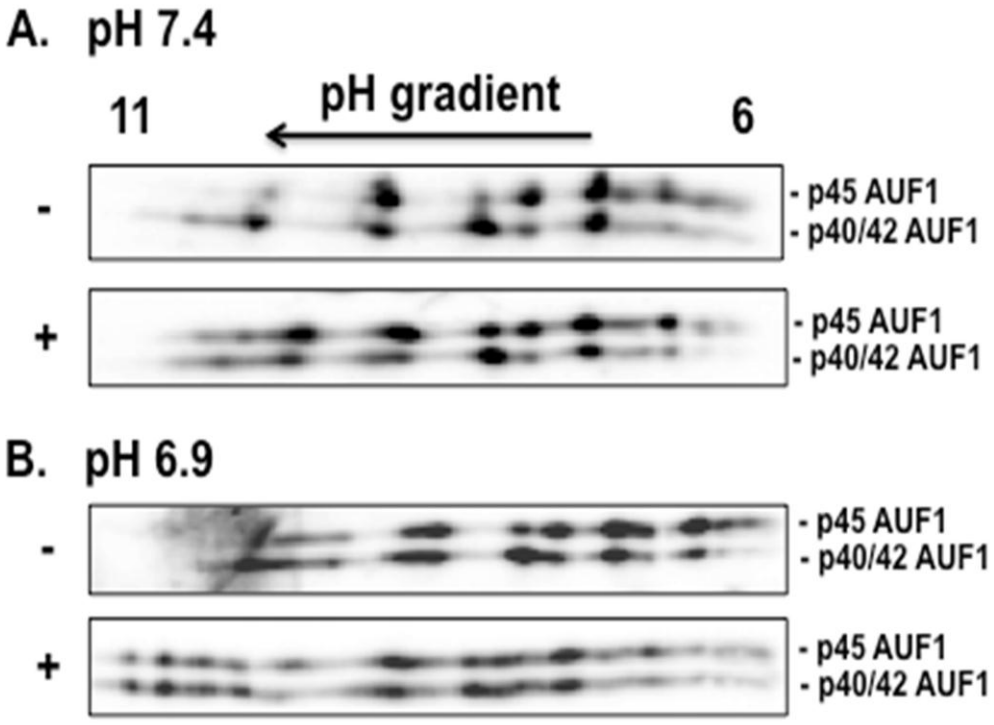

Fig.3.8. Two-dimensional gel analysis of the effect of pH on phosphorylation of AUF1. Confluent cultures of LLC-PK_1-F^+-9C cells were treated with normal or acidic medium for 24 h and then lysed with urea and thiourea. The extracts were resolved by two-dimensional gel electrophoresis using isoelectric focusing strips that ranged from pH 6 to 11. The resulting blots were probed with anti-AUF1 antibody. Resolution of AUF1 isoforms in cells treated with normal (Panel A) or acidic (Panel B) medium before (-) or after (+) treatment with protein phosphatase.

cells treated with acidic medium exhibits a pattern in which the more acidic variants of both the p45- and p42/p40-AUF1 isoforms are more abundant. When this lysate is pretreated with protein phosphatase, the pattern becomes more similar to that observed for the non-phosphatase- and phosphatase-treated normal lysates. However, additional variants, which migrate with a more basic pI, are also formed (Fig. 3.8). These data indicate that acidic treatment of LLC-PK_1-F^+-9C cells may lead to an increase in phosphorylation of the various isoforms of AUF1.

3.4 Discussion

Previous experiments demonstrated that siRNA silencing of the RNA-binding protein, HuR, decreased both the basal and the pH-stimulated levels of PEPCK mRNA and protein in LLC-PK_1-F^+-9C cells (Mufti et al. 2011). In the current study, the effect of siRNA knockdown of HuR on the half-life of the endogenous PEPCK mRNA was assessed directly. Transfer of LLC-PK_1-F^+-9C cells from normal to acidic medium produces a 2-fold increase in the half-life of PEPCK mRNA (Mufti et al. 2011). However, following the knockdown of HuR, the endogenous PEPCK mRNA decayed with same half-life ($t_{1/2}$ = 4.0 h and 4.5 h) in cells treated with normal or acidic medium, respectively (Fig. 3.3). This finding indicates that HuR is at least one of the *trans*-acting factors that are required for the pH-responsive stabilization of PEPCK mRNA. The loss of a pH-responsive stabilization caused only a slight decrease in the fold increase in PEPCK mRNA and protein that occur when cells are transferred from normal to acidic medium. The residual response is probably due to an increased transcription of the *PCK1* gene (Feifel et al. 2002; O'Hayre et al. 2006). The combined analyses also indicated that

the observed decrease in basal expression of PEPCK mRNA and protein was not due to a more rapid decay of the PEPCK mRNA. Thus, the binding of HuR may also enhance the processing, nuclear export, and/or translation of the PEPCK mRNA. These observations are consistent with the accepted roles of HuR in enhancing the translation and stability of its cognate mRNAs (Brennan and Steitz 2001; Lopez de Silanes et al. 2009).

By contrast, siRNA-mediated silencing of AUF1 caused a slight increase in the basal levels of PEPCK mRNA and protein, but partially inhibited the pH-responsive increases that normally occur when the cells are transferred to an acidic medium (Fig. 3.4). Not surprisingly, the co-knockdown of HuR and AUF1 has offsetting effects on basal expression of PEPCK mRNA and protein. However, the concurrent reduction of both RNA-binding proteins completely blocked the pH-responsive increase of PEPCK mRNA and protein. Therefore, HuR and AUF1 may impart opposing effects on the stability of PEPCK mRNA, but a co-operative interaction between the two RNA-binding proteins may be required to mediate the pH-responsive increase in renal PEPCK.

An MS2-recruitment assay was developed to further assess the prerequisite for co-ordinate binding of HuR and AUF1. Previous studies have used reporter mRNAs containing multiple MS2 binding elements to study mRNA processing, trafficking, localization and decay (Coller and Wickens 2002; Baron-Benhamou et al. 2004). These events often require the interaction of several RNA binding proteins. To study mRNA stabilization, it was necessary to design a reporter mRNA that decayed with a half-life that was easily quantified and could still be used to detect a significant stabilization. This required the inclusion of the PCK-2 segment that constitutes the 5'-end of the 3'UTR of the PEPCK mRNA and contains an instability element (Hajarnis et al. 2005). The

resulting reporter mRNA, βG-PCK-2-MS2, was identical to the βG-PCK-1 reporter mRNA except that the portion of the 3'UTR of PEPCK mRNA that contains the pH-responsive elements was replaced with six copies of the MS2 binding element. The two reporter mRNAs decayed with similar half-lives when the cells were grown in normal medium. However, in contrast to the βG-PCK-1 mRNA, the βG-PCK-2-MS2 mRNA was not stabilized when cells were transferred to acidic medium. In addition, the individual recruitment of MS2cp-HuR or MS2cp-p40AUF1 failed to produce a pH-responsive stabilization of the βG-PCK-2-MS2 mRNA. However, the co-expression of the two fusion proteins produced a 1.5-fold increase in the half-life of the reporter mRNA when cells were transferred to acidic medium. This response is slightly less than the 2-fold stabilization observed with the endogenous PEPCK mRNA or the βG-PCK-1 reporter mRNA. There are numerous possibilities as to why the recruitment of the tethered RNA binding proteins may be less than optimal compared to the direct binding of HuR and AUF1 to the normal pH-response element. However, the observed stabilization was both reproducible and significant. Thus, the observed pH-responsive stabilization of the βG-PCK-2-MS2 mRNA strongly supports the hypothesis that the concurrent association and probable interaction of HuR and p40AUF1 within the terminal segment of the 3'-UTR is necessary to mediate the pH-responsive stabilization of PEPCK mRNA in LLC-PK_1-F^+-9C cells.

The levels and nucleocytoplasmic shuttling of HuR and AUF1 are unaltered when LLC-PK_1-F^+9C cells are transferred from normal to acidic medium (Mufti et al. 2011). Therefore, 2-dimensional gel electrophoresis was used to determine if HuR or AUF1 underwent pH-responsive changes in covalent modification. Isoelectric focusing of HuR

identified 3 variants, each of which has a pI that is more acidic than the unmodified protein. Treatment of the cell lysates with a protein phosphatase produced a pronounced shift to a new variant that had a more basic pI, consistent with the removal of multiple phosphates. Comparison of the relative abundance of the 3 variants indicated that treatment of cells with acidic medium caused a decrease in phosphorylation of HuR. Previous studies have identified multiple post-translational modifications of HuR that occur in response to various stimuli, which activate different signaling pathways (Doller et al. 2008; Abdelmohsen and Gorospe 2010). At least eight potential sites of phosphorylation have been identified within the three RNA recognition motifs and the hinge region of HuR. The various modifications have been reported to affect the interaction of HuR with various mRNAs, its nucleocytoplasmic distribution, and its ability to stabilize specific mRNAs. For example, oxidative stress promotes the dissociation of HuR from the SIRT1 mRNA causing its more rapid degradation (Abdelmohsen et al. 2007). Analysis of the effects of expressing various non-phosphorylatable mimetics of HuR demonstrated that phosphorylation on T118 and S88 promoted HuR binding, while phosphorylation of S100 inhibited its interaction with the SIRT1 mRNA. Thus, a change in phosphorylation of HuR may also effect its interaction with the PEPCK mRNA.

Previous studies have used 2-D gel electrophoresis to assess changes in covalent modifications of AUF1 in RAW 264.7 cells by lipopolysaccharide stimulation (Cok et al. 2004) and in parathyroid cells upon changes in serum Ca^{+2} and phosphate (Bell et al. 2005). The previous analyses also concluded that each isoform of AUF1, produced by alternative splicing, undergoes extensive and variable covalent modifications to produce

multiple variants. Thus, the complex pattern observed with lysates of LLC-PK_1-F^+-9C cells is consistent with the previous reports. However, the lysates from the cells treated with acidic medium exhibit a significant increase in the variants that have a more acidic pI. This shift was reversed by treatment with protein phosphatase, consistent with the removal of phosphate groups. By contrast, protein phosphatase treatment of the normal lysates had little effect on the observed pattern of variants. Thus, multiple variants of AUF1 may be produced by covalent modification other than phosphorylation. However, treatment with an acidic medium may result in an increase in phosphorylation of AUF1. The combined analyses suggest that opposite changes in phosphorylation of HuR and AUF1 may promote the remodeling of their interaction that leads to stabilization of the PEPCK mRNA in response to conditions that model a metabolic acidosis.

CHAPTER IV

DEVELOPMENT OF HALO-TAG/RNA PULL-DOWN ASSAYS TO CHARACTERIZE THE BINDING OF HUR AND AUF1 TO PHOSPHOENOLPYRUVATE CARBOXYKINASE MRNA IN LLC-PK$_1$-F$^+$-9C† CELLS

4.1 Introduction

Metabolic acidosis is a common clinical condition that is characterized by a decrease in blood pH and bicarbonate concentration (Wagner 2007). To partially restore acid-base balance, the kidneys initiate multiple responses that include increased ammoniagenesis and gluconeogenesis from glutamine to facilitate excretion of acid and generate bicarbonate ions, respectively (Tannen and Sahai 1990; Halperin 1993; Curthoys et al. 2007). This adaptation results, in part, from a rapid and sustained increase in expression of phospho*enol*pyruvate carboxykinase (PEPCK). Previous nuclear run-on experiments established that the rapid increase in PEPCK expression is initiated primarily by increased transcription of the *PCK1* gene, but the adaptive increase is sustained in large part by stabilization of the PEPCK mRNA (Hwang and Curthoys 1991; Hwang et al. 1991). Recent studies have established that the latter response is effectively modeled in LLC-PK$_1$-F$^+$-9C cells, a clonal line of porcine renal proximal tubule like cells (Mufti et al. 2011).

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†(This chapter will be submitted as a manuscript to The *RNA* journal for publication)

(Lakshmi Gummadi and Norman P. Curthoys)

UV-crosslinking experiments, RNA gel-shift assays, and mutational analyses mapped the binding of HuR, AUF1 (Hajarnis et al. 2005; Mufti et al. 2011) and ζ-crystallin (unpublished data of S. Hajarnis and N.P. Curthoys) to various instability elements within the 3'-UTR of PEPCK mRNA While AUF1 binds to multiple segments of the 3'UTR (PCK-2, PCK-6, and PCK-7), HuR binds only to stem loop structures in PCK-6 and PCK-7, and ζ-crystallin binds only to the PCK-7 segment. However, it is unknown whether the three RNA binding proteins actually compete for the same sites or bind simultaneously to adjacent sites within the identified segments of the 3'-UTR of PEPCK mRNA. Thus, it is important to determine which of these interactions also occur in intact cells under normal conditions and under conditions that model a metabolic acidosis.

RNA-protein immunoprecipitation (Peritz et al. 2006) is frequently used to detect RNA binding proteins that are bound to specific transcripts. However, there are no commercially available antibodies that are specific for the individual isoforms of AUF1. Therefore, an alternate approach was developed by modifying the Halo-ChIP system (Promega) and adding a Halo-Tag to various RNA binding proteins. This approach was used to pull-down RNA: protein complexes that were crosslinked with formaldehyde in intact cells. Unlike other conventional protein tags, such as FLAG, His and Myc, a Halo-Tag protein forms a covalent linkage with a haloalkane substrate that is attached to the HaloLink Resin (Los et al. 2008). The formation of an irreversible linkage allows for more stringent washing to remove contaminants that bind through non-specific interactions.

4.2 Materials and Methods

4.2.1 Cell culture and Western blot analysis

LLC-PK_1-F^+-9C cells (Mufti et al. 2011) were cultured in DMEM-Base medium (Sigma) supplemented with penicillin/streptomycin (Sigma), 10% fetal bovine serum, 5 mM glucose, 26 mM $NaHCO_3$, 17 mM NaCl, 2 mM glutamine, 5 mM pyruvate, 5 µM phenol red, and 10 mM HEPES, pH 7.4 at 37^{o}C in a 5% CO_2 atmosphere. An acidic medium (pH 6.9), which was used to mimic metabolic acidosis, was prepared as above except that 9 mM $NaHCO_3$ and 34 mM NaCl were added to reduce the pH while maintaining equivalent osmolarity (Gstraunthaler et al. 2000).

A dye binding assay (Bradford 1976) was used to determine the concentration of protein in the cell lysates. Samples containing 15 µg of protein were resolved by 10 % SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-F membranes (Millipore). The blots were probed with mouse monoclonal antibodies to HuR (Santa Cruz Biotechnology) and β-tubulin (Sigma) and rabbit polyclonal antibodies to AUF1 (Millipore) and ζ-crystallin (provided by J. S. Zigler, Jr., National Eye Institute). Subsequently, the blots were developed with goat anti-rabbit 800 and goat anti-mouse 680 secondary antibodies (LiCOR) and quantified using an Odyssey Infra-red Imager.

4.2.2 Cloning and expression of the chimeric Halo-RNA binding proteins

The coding regions of mouse HuR and human p40AUF1 were PCR amplified using Flexi primers to append an *SgfI* site just upstream of the start codon and a *PmeI* site that introduces a stop codon after the carboxy-terminus. The primers were designed using the Flexi Vector Primer Design Tool (Promega). The amplicons were then cloned into the

pFC14A (HaloTag) CMV Flexi vector (Promega) that contains a *SgfI* site and a *EcoICRI* site. Upon ligation of the blunt *PmeI* and *EcoICRI* ends, the stop codon is eliminated, allowing read through into the C-terminal HaloTag peptide sequence.

The Halo-p40AUF1 construct was co-transfected with pcDNA3.1-hygro into LLC-PK_1-F^+-9C cells and stable transformants were selected with medium containing 1 µg/ml hygromycin. The selected cells were maintained in normal medium containing 0.2 µg/ml of hygromycin. Higher expression of the Halo-HuR protein was obtained by transient transfection of LLC-PK_1-F^+-9C cells using Lipofectamine-2000 (Invitrogen) to introduce 3 µg of the Halo-HuR plasmid. After 6 h, the transfection medium was removed and the cells were washed twice with phosphate buffered saline before adding fresh medium containing Penicillin/Streptomycin.

4.2.3 HaloLink Resin pull-down assay

LLC-PK_1-F^+-9C cells that either transiently express Halo-HuR or stably express Halo-p40AUF1 were either maintained in normal medium (pH 7.4) or transferred to acidic medium (pH 6.9) for 24 h. The protocol for formaldehyde crosslinking was adapted from the study of Niranjanakumari (Niranjanakumari et al. 2002). Briefly, the cells were washed twice with phosphate-buffered saline (PBS) and then incubated with 0.3% formaldehyde in PBS for 10 min at room temperature with slow rocking. The crosslinking reaction was arrested by the addition of 0.25 M glycine in PBS, pH 7.0, and incubating for 5 min at room temperature. The cells were subsequently harvested with lysis buffer containing 85 mM KCl, 150 mM sodium chloride, 0.5% Nonidet P-40 and 5 mM PIPES, pH 8.0, supplemented with 1% Halt Phosphatase Inhibitor (Pierce), 10%

Protease Inhibitor Cocktail (Sigma) and 1 unit/µl RNasin Plus RNase Inhibitor (Promega). The cell lysate was centrifuged at 3000 rpm (237xg) for 5 min at 4^{o}C and the pellet was discarded. The supernatant was used for the pull down assays. Triplicate pull-down samples were collected for each treatment, while controls were harvested in duplicate. Control samples were pre-incubated with Halo-blocking peptide that is a substrate for the HaloTag protein. Covalent attachment of the blocking peptide prevents the binding of the HaloTag protein to the HaloLink Resin. The HaloLink Resin was preequilibrated by washing twice with Tris-buffered saline supplemented with 0.6% IGEPAL CA-630. Optimal binding with a low background was obtained by incubating 350 µg of protein extract and 125 µl of preequilibrated HaloLink Resin at 4^{o}C for 4 h with rocking. The low temperature was required to maintain integrity of the RNA. Following incubation, the samples were centrifuged at 800xg for 2 min at 4^{o}C and the supernatants, along with an untreated input sample, were analyzed by western blotting to assess the level of unbound Halo-protein. The resin was subsequently washed with 1 ml of lysis buffer containing 1% Triton X-100 and 0.1% sodium deoxycholate followed by washing with nuclease-free water. The beads were then washed with a high salt wash-buffer containing 50 mM Tris-HCl, pH 7.5, 700 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, and 5 mM EDTA followed by washing with nuclease-free water. The resin samples containing the HaloTag:RNA:protein complexes were subsequently heated to 70^{o}C for 45 min in presence of 100 mM $MgCl_2$ and 1 unit/µl of RNAsin plus RNAse inhibitor to reverse the crosslinks and release the captured RNA. RNA from untreated input samples and the precipitated complexes were purified using TRIzol reagent and quantified by qRT-PCR assay using specific primers and Taqman probes for

the AU-rich region of the 3'-UTR of PEPCK mRNA and the coding region of porcine glyceraldehyde 3-phosphate dehydrogenase (Mufti et al. 2011). Alternatively, the precipitated complexes were treated with boiling SDS sample buffer to release the proteins that co-purify with the chimeric HaloTag protein.

4.3 Results

4.3.1 Halo-Tag/RNA pull-down assay

The Halo-ChIP system (Promega) is an innovative technique that covalently captures intracellular protein:DNA complexes without using antibodies. The Halo-ChIP assay was modified to perform a Halo-RNA pull down assay (Fig.4.1). The RNA binding proteins of interest were expressed as HaloTag fusion proteins, cross-linked to RNA with formaldehyde, and then captured on the HaloLink Resin. The HaloTag protein is a mutated haloalkane dehalogenase that under physiological conditions forms an irreversible ester linkage to its substrate (Los et al. 2008). A synthetic chloroalkane substrate for the HaloTag protein is covalently linked to the HaloLink Resin. Hence, after incubation to form a covalent linkage, the resin can be stringently washed to effectively remove RNAs and proteins that are non-specifically associated. Subsequent heating reverses the formaldehyde cross-links and the released RNA and proteins can be analyzed by quantitative real time PCR (qRT-PCR) and western blotting, respectively. Therefore, the Halo-RNA pull down assay was used to quantify the intracellular association of HuR with the 3'-UTR of PEPCK mRNA and the p40 isoform of AUF1.

The recombinant Halo-p40AUF1 construct was stably expressed in LLC-PK_1-F^+-9C cells. Western blot analysis (Fig. 4.2 A) demonstrated that the Halo-p40AUF1 protein

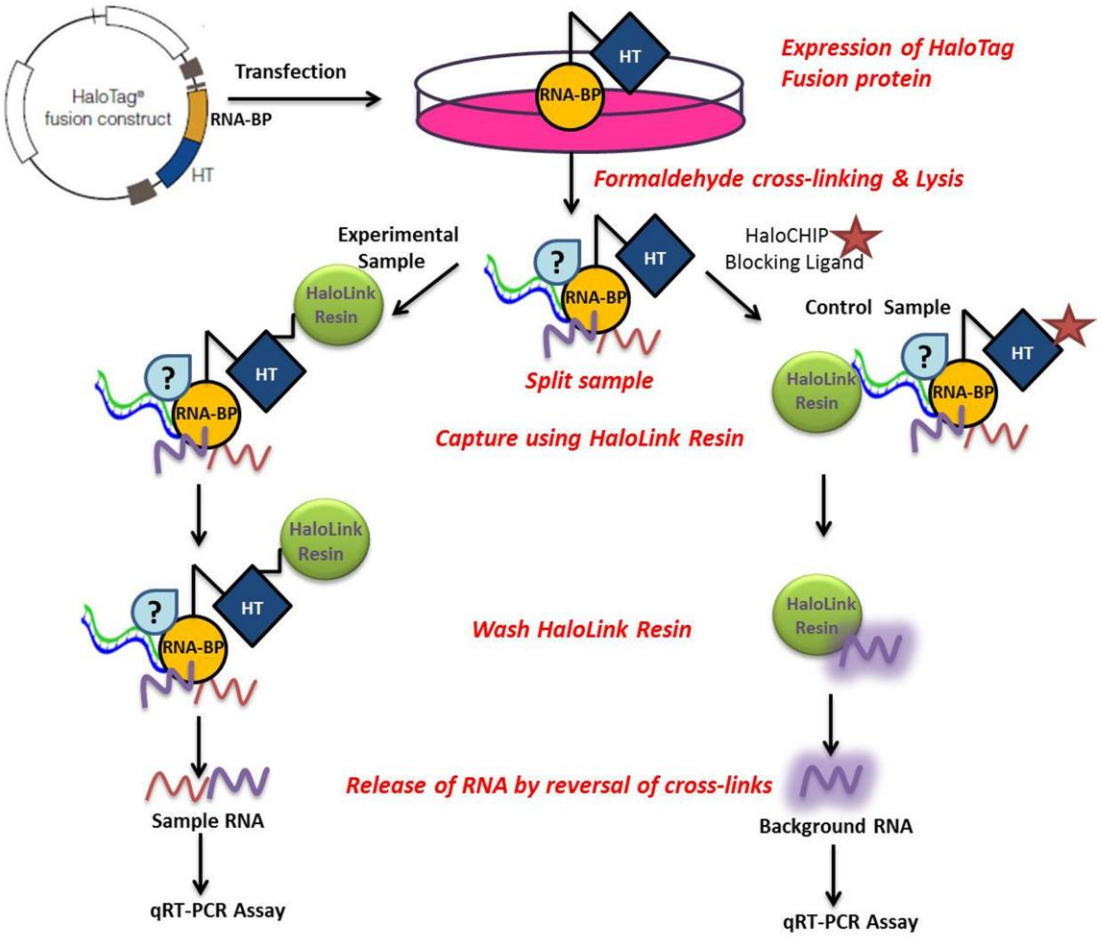


Fig.4.1. Schematic diagram of the Halo-RNA pull down assay. RNA-binding proteins (RNA-BPs) of interest were cloned into the pFC14A HaloTag CMV Flexi vector to add a C-terminal HaloTag (HT). LLC-PK_1-F^{+}-9C cells were either stably or transiently transfected with the chimeric Halo-proteins. The transformed cells were treated with 0.3% formaldehyde to *in-vivo* crosslink the RNA-protein and associated protein-protein interactions. The experimental cell lysates were incubated directly with the HaloLink Resin to form a covalent linkage to the HaloTag domain. By contrast, the control sample was pre-incubated with Halo-blocking peptide, which forms a covalent linkage to the HaloTag domain and prevents its subsequent binding to the Halo-Link Resin. Stringent washing removes the non-specifically bound RNA, DNA and proteins. Subsequent heat reversal of the cross-links releases the RNAs and proteins that were cross-linked to the chimeric HaloTag protein. The RNAs eluted from the control and experimental sample were analyzed in qRT-PCR assay. Proteins associated with the Halo-Tag/RNA complexes were recovered from separate samples and analyzed by western blotting.

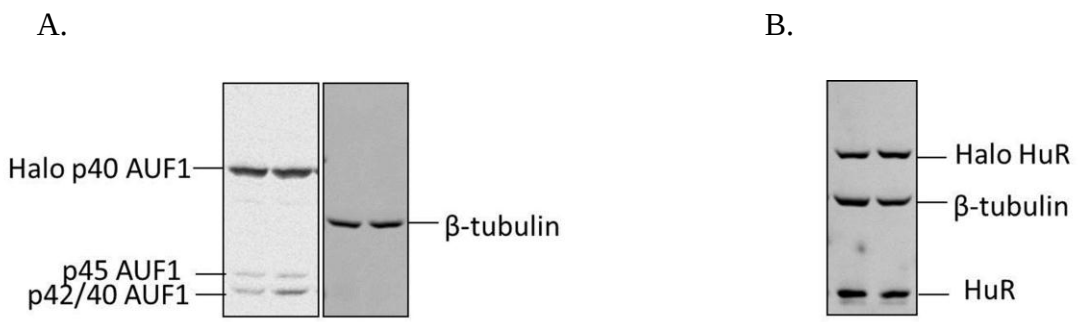


Fig.4.2. Level of Halo-fusion protein expression. Stable expression of Halo-p40AUF1 (Panel A) and, transient expression of Halo-HuR (Panel B) in LLC-PK_1-F^+-9C cells. Cell lysates were separated by 10% SDS-PAGE and probed with antibodies to AUF1 (green), β-tublin (red) and HuR (red).

was expressed at a level slightly greater than the sum of the endogenous AUF1 isoforms. By contrast, stable transformants of the Halo-HuR expressed relatively low levels of the chimeric protein. However, transient expression consistently produced a level of the Halo-HuR protein that is similar to the level of the endogenous HuR (Fig. 4.2 B).

4.3.2 Halo-RNA pull down assay using transiently expressed Halo-HuR

Initial experiments were performed to optimize the Halo-RNA pull down assay in LLC-PK_1-F^+-9C cells that transiently express Halo-HuR. Cell lysates were prepared from cells grown in normal medium (Fig.4.3). A control sample (BL) was pre-incubated with the Halo-blocking peptide to prevent interaction with the HaloLink Resin. Duplicate samples (S1 and S2) were incubated with the HaloLink Resin for 4 h at 4^oC. Following this incubation, the bound proteins were removed by centrifugation and the resultant supernatants were characterized by western blotting (Fig. 4.3). As expected, Halo-HuR was depleted in the supernatant fraction of the actual pull-down samples, but the binding to the HaloLink Resin was significantly inhibited in the blocking ligand control. However, the levels of β-tubulin were similar in the input sample and the supernatant fractions of both the experimental samples and the BL control sample. Surprisingly, the levels of endogenous HuR in the different samples showed a pattern similar to the Halo-HuR protein. SDS elution of an experimental sample (Eluate) caused the release of significant levels of Halo-HuR and endogenous HuR, but not β-tubulin. Further treatment with TEV protease failed to release any detectable levels of Halo-HuR or endogenous HuR. Thus, the formaldehyde cross-linked complex contains both the Halo-HuR and significant amounts of endogenous HuR, but not β-tubulin.

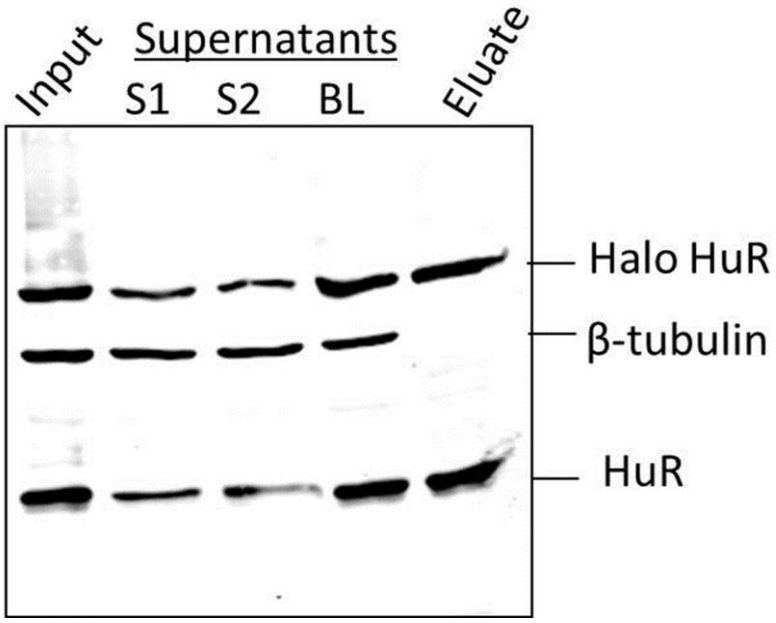


Fig.4.3 Halo-HuR and endogenous HuR are recovered in a Halo-HuR pull down assay. LLC-PK_1-F^+-9C cells were transfected with the Halo-HuR construct, cross-linked with 0.3 % formaldehyde and lysed to obtain total cell extracts. A sample of starting material (Input) was removed before incubation with the HaloLink Resin. Following the incubation, the supernatant from the experimental samples (S1, S2) was collected to assess the unbound fraction of Halo-HuR. The control sample (BL) represents the supernatant from a sample that was pretreated with blocking ligand peptide prior to incubating with the resin. SDS elution of an experimental sample (Eluate) releases the captured Halo-HuR and endogenous HuR, but not β-tubulin, indicating the specificity of the bound protein complex.

The Halo-HuR pull down assay was used to determine if HuR is bound to the 3'-UTR of PEPCK mRNA in LLC-PK_1-F^+-9C cells that were crosslinked with formaldehyde. Total RNA samples (Input) were derived from cells that were crosslinked with formaldehyde and heat-treated to reverse the formaldehyde crosslinks. The total RNA samples recovered from cells grown in acidic medium exhibit a 2-fold increase in PEPCK mRNA, while the levels of GAPDH mRNA were unchanged. Control RNA samples were derived from cell lysates that were preincubated with the blocking peptide before incubating with the HaloLink Resin. The control RNA samples contained non-detectable levels of PEPCK and GAPDH mRNAs. This finding is consistent with the western blot data (Fig.4.3), which indicated that pre-treatment with blocking peptide significantly reduced the binding of Halo-HuR to the HaloLink Resin. By contrast, the RNA recovered from the experimental lysates of cells grown in normal medium contained significant levels of PEPCK mRNA ($4.5 \pm 0.4 \times 10^3$ copies), but only trace amounts of the GAPDH mRNA (< 5 copies). Experimental samples isolated from cells treated with acidic medium (pH 6.9) exhibit a 3-fold increase in recovery of PEPCK mRNA ($12.5 \pm 3.6 \times 10^3$ copies) but no change in GAPDH mRNA (< 5 copies). When expressed as a percent of the input mRNA, a 1.5-fold increase in binding of Halo-HuR to PEPCK mRNA was observed in the samples obtained from the pH 6.9-treated cells (Fig.4.4). The combined data establish the specificity of the pull-down assay and confirm the hypothesis that HuR is bound to the PEPCK mRNA in intact LLC-PK_1-F^+-9C cells. Furthermore, it suggests that HuR binding may be increased when cells are treated with acidic medium.

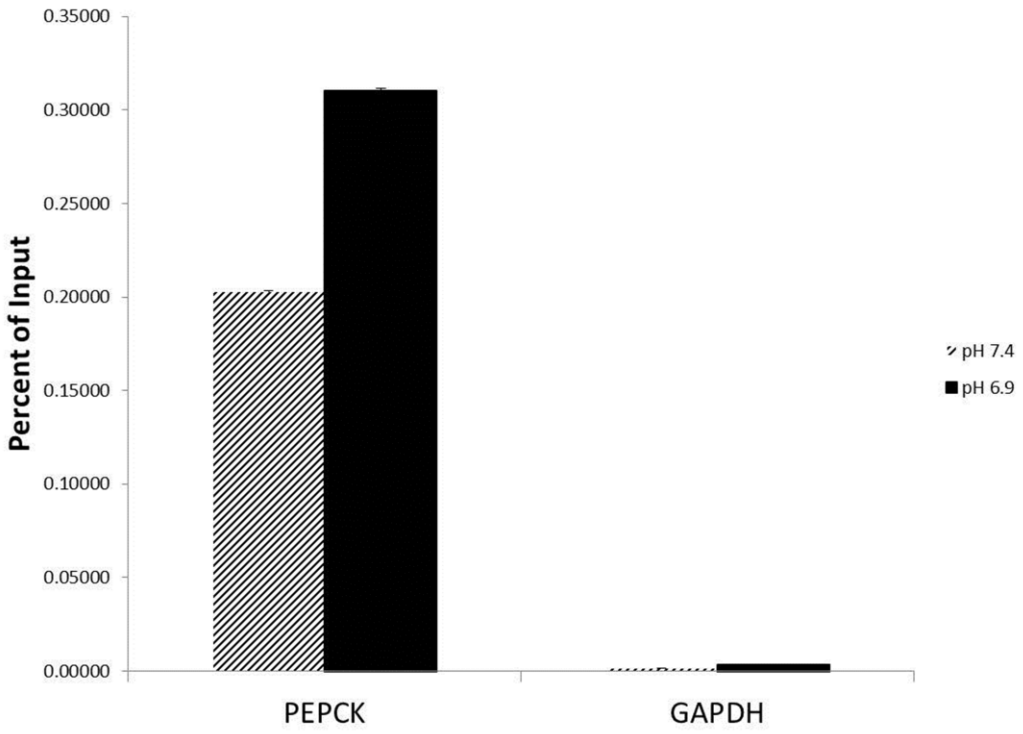


Fig.4.4. Treatment with acidic medium increases the *in vivo* binding of Halo-HuR to the pH-response element in PEPCK mRNA. The percent recoveries of PEPCK and GAPDH mRNAs were calculated by comparing the experimental samples and the control sample treated with blocking peptide (BL) versus the mRNA levels in the input sample. The data are the mean +/- S.E. of triplicate samples.

The samples from a Halo-HuR pull down experiment were also analyzed for AUF1 (Fig.4.5). Both Halo-HuR and endogenous HuR were depleted in the supernatant fractions of the experimental samples (S1, S2) and recovered in the eluted fractions. By contrast, neither halo-HuR nor endogenous HuR were depleted in control samples treated with the blocking ligand or recovered in the eluates of the control samples. Surprisingly, p45AUF1 and possibly p40AUF1 were also depleted in the supernatant fractions of experimental samples, and recovered in the eluted fractions of the corresponding samples. Again, this interaction was not evident in the samples pretreated with blocking ligand.

4.3.3 Isolation of protein-complex from Halo-p40 AUF1 pull-down samples

Halo-p40AUF1 pull-down assays were performed using either non-crosslinked or formaldehyde-treated cellular lysates of LLC-PK_1-F^+-9C cells that stably express Halo-p40AUF1. The initial cell lysate (input) and the final protein-complex recovered following capture of Halo-p40AUF1 by the HaloLink Resin (S1 and S2) were analyzed by western blotting (Fig. 4.6A). A significant proportion of the Halo-p40AUF1 was recovered from the pull-down fraction of the lysates of cells that were fixed with formaldehyde. However, the recovery of Halo-p40AUF1 from the extracts of non-cross-linked cells was minimal. In addition, a large fraction of the endogenous HuR was recovered in Halo-p40AUF1/RNA/protein-complex (Fig. 4.6B). This observation mirrors the results observed with the Halo-HuR pull down assay. In both cases, β-tubulin was absent from the complex. In addition, ζ-crystallin failed to co-purify with the isolated

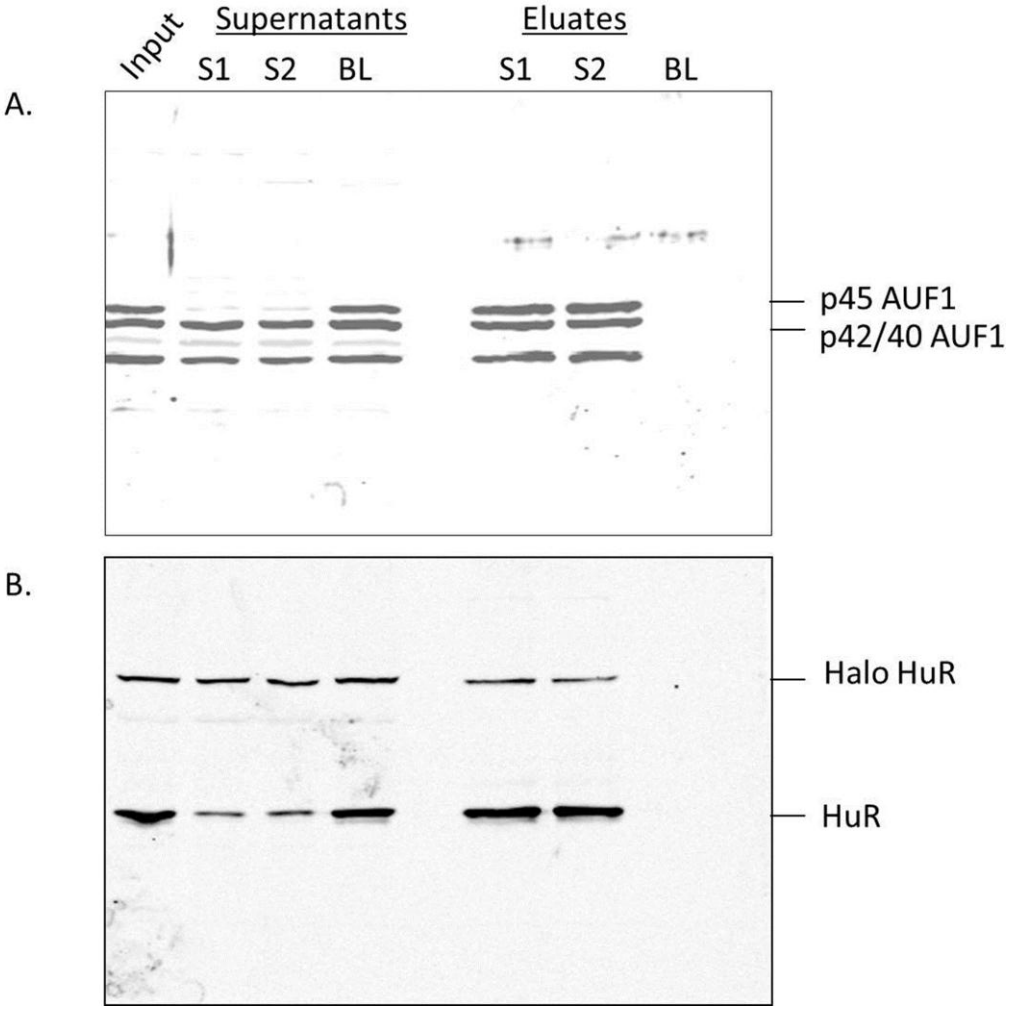
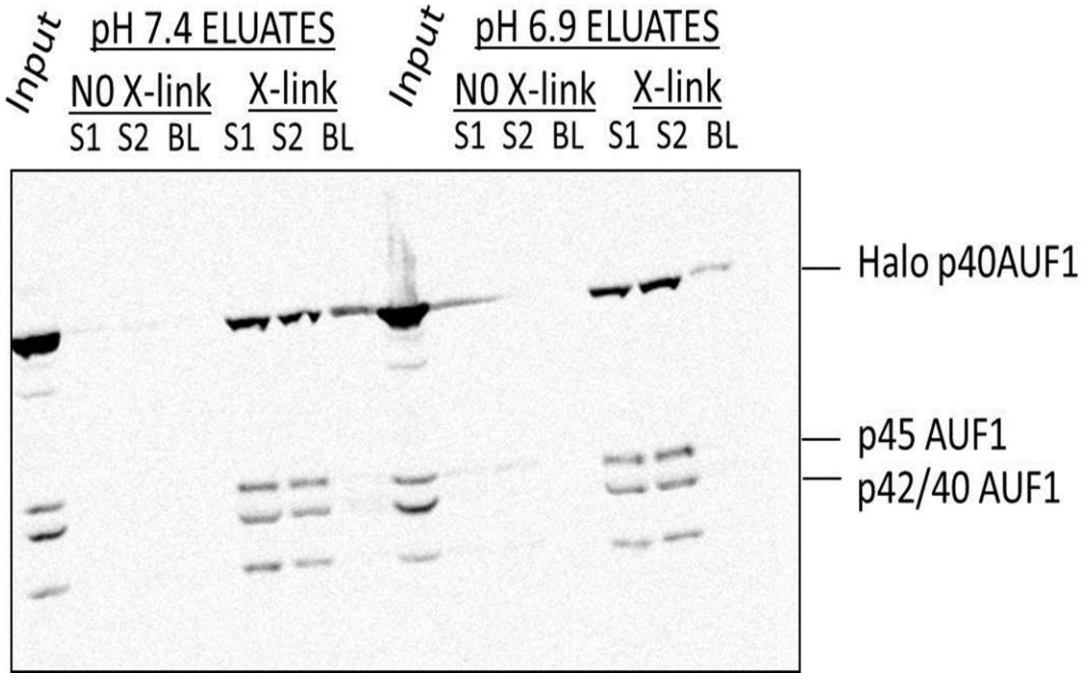
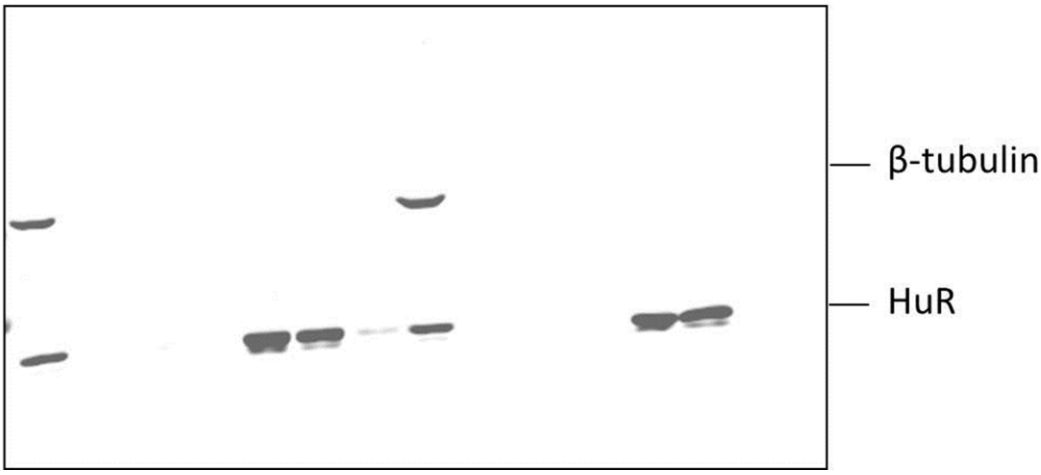


Fig. 4.5. Recovery of endogenous p45AUF1 in a Halo-HuR pull down assay. LLC-PK_1-F^+-9C cells were transiently transfected with Halo-HuR plasmid, cross-linked with 0.3 % formaldehyde, and lysed to obtain cell extracts. A sample of starting material (Input) was removed before incubation with HaloLink Resin. Following the incubation, the supernatants from the experimental samples (S1, S2) were collected to assess the fraction of unbound Halo-HuR. The supernatant was also recovered from a control sample that was pretreated with blocking ligand peptide (BL) prior to incubating with the resin. After extensive washing, the resins were treated with hot SDS sample buffer to obtain the final eluates. SDS elution of the experimental sample released the captured Halo-HuR and endogenous HuR (Panel B) and AUF1 (Panel A).

A.


Input
pH 7.4 ELUATES
NO X-link
X-link
S1 S2 BL S1 S2 BL
Input
pH 6.9 ELUATES
NO X-link
X-link
S1 S2 BL S1 S2 BL
Halo p40AUF1
p45 AUF1
p42/40 AUF1

B.


β-tubulin
HuR

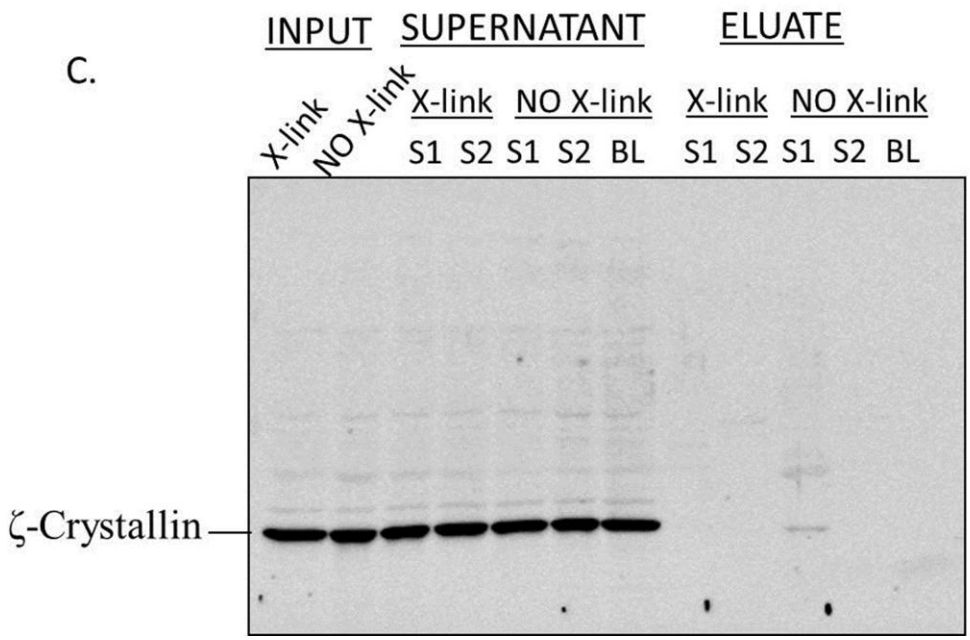


Fig.4.6. Recovery of endogenous HuR in a Halo-p40-AUF1 pull-down assay. LLC-PK$_1$-F$^+$-9C cells that stably express Halo-p40AUF1 were grown in normal medium (pH 7.4) or treated with acidic medium (pH 6.9) for 24 h. The cells were either not crosslinked (no x-linked) or crosslinked (x-linked) with 0.3 % formaldehyde and then lysed. A sample of starting material (Input) was removed before incubation with the HaloLink Resin. Following the incubation, the resin was pelleted, washed extensively, and the bound proteins were eluted with hot SDS sample buffer. Panel A & B. The eluates from the experimental samples (S1, S2) and the blocking peptide control sample (BL) from both sets of cell lysates were analyzed on a western blot by probing with primary antibodies for HuR, AUF1 and β-tubulin. Panel C. Inputs, supernatant and eluate fractions from the cells maintained in normal medium were probed with ζ-crystallin antibody.

Halo-p40AUF1 protein complex (Fig. 4.6C) in either the fixed or non-crosslinked cell lysates. ζ-Crystallin was also not recovered in the eluates from the Halo-HuR pull down experiments (data not shown). This finding further confirms the specificity of the RNA-binding protein complex.

4.3.4 Halo-HuR pull downs of ribonuclease treated extracts

Halo-HuR pull downs were performed using cell extracts that were pretreated either with (+) or without (-) ribonucleaseONE (Fig. 4.7). The Halo-HuR was depleted in the supernatant fractions of the experimental samples irrespective of the RNase treatment, but not in the control pretreated with blocking ligand (BL). However, significant depletion of endogenous HuR and p45AUF1 occurred in the untreated extracts, but not in the extracts treated with ribonucleaseONE. In addition, only the protein-complexes recovered in HaloHuR-Pull down from untreated extracts were enriched in both endogenous HuR and AUF1. Therefore, treatment of the protein complex with ribonucleaseONE prevented the association of endogenous HuR and p45AUF1 with the formaldehyde crosslinked complex. Thus, the co-association of the endogenous RNA binding proteins required the presence of intact RNA.

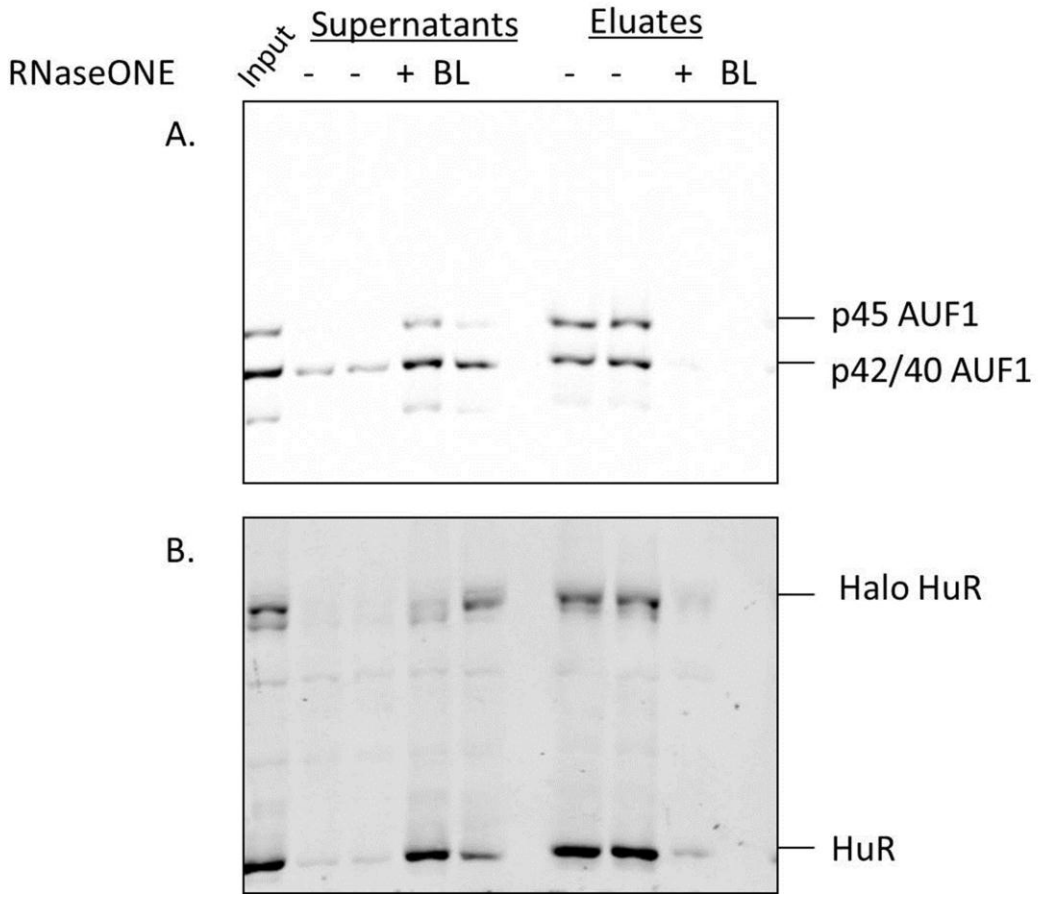


Fig.4.7 Pretreatment with ribonuclease prevents the recovery of endogenous HuR and AUF1 in a Halo-HuR pull-down assay. Lysates of LLC-PK$_1$-F$^+$-9C cells that transient express Halo-HuR were either untreated or treated with RibonucleaseOne prior to incubation with HaloLink Resin. A sample of starting material (Input) was removed before incubation with the HaloLink resin. Following the incubation, the supernatants of the untreated (-) and treated (+) samples were collected to assess the unbound proteins. The control sample (BL) was pretreated with blocking ligand peptide prior to incubating with the resin. The resin was pelleted, washed extensively, and the bound proteins were eluted with hot SDS sample buffer. The eluates from both the RibonucleaseOne untreated (-) and treated (+) lysates were analyzed on a western blot that was probed with primary antibodies for HuR (Panel B), AUF1 (Panel A).

4.4 Discussion

Stable expression of Halo-p40AUF1 in LLC-PK_1-F^+-9C cells produced a level of the chimeric protein that was suitable for the pull-down experiments. However, only a low level of Halo-HuR expression occurred in stably transformed cells. Thus, LLC-PK_1-F^+-9C cells were transiently transformed to achieve a level of Halo-HuR expression similar to that of the endogenous HuR. Initially, the Halo-HuR RNA-pull down assay was optimized to achieve efficient binding of Halo-HuR to the HaloLink Resin in lysates of cells that were grown in normal medium. The final conditions produced 80-90% removal of Halo-HuR from the supernatant fraction. RT-PCR analysis of the RNA isolated from the Halo-HuR pull-down samples indicated an enrichment of PEPCK mRNA relative to the recovery of GAPDH mRNA. In addition, the samples obtained from cells treated with acidic medium exhibit a 3-fold increase in recovery of PEPCK mRNA compared to the normal samples. This increase was slightly greater than the 2-fold increase in total PEPCK mRNA that was observed in the input samples. These data establish that HuR is bound to PEPCK mRNA in intact LLC-PK_1-F^+-9C cells and indicate that the amount of HuR bound to the 3'-UTR of PEPCK mRNA may increase slightly when cells are treated with an acidic medium.

Western blot analysis established that the pull-down of Halo-HuR consistently resulted in a corresponding depletion of endogenous HuR from the supernatant fractions. However, the removal of both the Halo-HuR and endogenous HuR did not occur in the control samples that were treated with blocking ligand. In addition, the abundant cytosolic protein, β-tubulin was always excluded from the protein-complex recovered in the Halo-HuR pull-downs. The specificity of the eluted protein-complex was further

confirmed by the observation that another cytosolic RNA-binding protein, ζ-crystallin, was not recovered in either the Halo-HuR or Halo-p40AUF1 pull-down assays. These data establish the specificity of the Halo-Tag pull-downs. However, SDS elution released a large proportion of the Halo-HuR and the endogenous HuR contained in the Halo-HuR/RNA/protein complex. Only non-covalent interactions should be dissociated following treatment of the Halo-Link Resin with the hot SDS-sample buffer. Thus, this observation indicates that Halo-HuR may also form oligomers with Halo-HuR and the endogenous HuR. By comparison, subsequent TEV cleavage, which should hydrolyze the linkage between the Halo-HuR and the resin, released very little protein. This may result from incomplete reversal of the formaldehyde crosslinks and the retention of a large complex that blocks access of the TEV protease.

The formation of a large multiprotein/RNA complex is also consistent with reports in the literature, which characterized the ability of HuR to oligomerize in a cooperative manner in the presence of AU-rich sequences (Fialcowitz-White et al. 2007). The observation, that recombinant Halo-HuR associates with the endogenous HuR and the halo-HuR to form multimers, indicates that Halo-HuR functions similarly to the endogenous HuR. Also intriguing is the finding that p45AUF1, and possibly p40AUF1, are significantly reduced in the supernatant fractions and recovered in the SDS eluted fractions. A HuR/AUF1 complex was also recovered when Halo-p40AUF1 was bound to the HaloLink Resin. These data support the conclusion that in LLC-PK_1-F^+-9C cells, HuR also forms a strong association with some isoforms of AUF1. Fluorescence resonance energy transfer (FRET) experiments previously detected protein-protein interactions between HuR and AUF1 in both nucleus and the cytoplasm of cells (David et al. 2007).

However, HuR and p45AUF1 are predominantly localized within the nucleus of LLC-PK_1-F^+-9C cells (Mufti et al. 2011). Therefore, a large proportion of the Halo-Tag/RNA/protein complex must be formed in the nucleus. Furthermore, experiments using ribonucleaseOne treated cytosolic extracts failed to co-precipitate HuR and AUF1, indicating that the formation of HuR/AUF1 heteromultimers is RNA-mediated. Thus, the association and/or complimentary binding of HuR and AUF1 may be a key component of pH-responsive stabilization of PEPCK mRNA. Finally, while the HuR/AUF1 complexes were enriched in the SDS eluates of both Halo-HuR and Halo-p40AUF1, the recovery of the complex was minimal in the non-crosslinked extracts. This finding is consistent with the previous studies that recommend minimal formaldehyde cross-linking to "seal" the *in-vivo* RNA-protein interactions, but still allow reversal of RNA while keeping the specificity of protein complex in the eluate (Niranjanakumari et al. 2002).

Ribonucleoprotein precipitation (RIP) (Keene et al. 2006), Crosslinking and Immunoprecipitation (CLIP) (Licatalosi et al. 2008) and Photoactivatable Ribonucleoside-Crosslinking and Immunoprecipiation (PAR-CLIP) (Hafner et al. 2010) are alternative techniques that can be used to investigate RNA/protein interactions. Each technique also offers unique advantages to detect the array of transcripts bound to the target protein. However, they share a common feature in that the three approaches require the use of antibodies to initially precipitate the RNA-protein complex. This is a significant limitation for some RNA-binding proteins due to the lack of commercial antibodies that have a high specificity or sufficient titer. The experiments reported in this study establish that in such cases, Halo-Tag-RNA pull-down assays offer an effective alternative. With this approach, the recovered RNAs can be PCR amplified to quantify

changes in RNA/protein interaction. In addition, the isolated RNA could be used for microarray analysis to identify the potential target genes for a specific RNA binding protein.

CHAPTER V

ROLE OF ZETA-CRYSTALLIN IN ASSOCIATION WITH AUF1 AND HUR IN REGULATING PEPCK MRNA TURNOVER IN KIDNEY CELLS

5.1 Introduction

Previous studies (Ibrahim et al. 2008) established that selective mRNA stabilization contributes to the increased expression of the mitochondrial glutaminase (GA) (Hansen et al. 1996) (Schroeder et al. 2006) glutamate dehydrogenase (GDH) and the cytosolic PEPCK (Hajarnis et al. 2005) (Hod and Hanson 1988) during chronic metabolic acidosis. Stabilization of rat GA mRNA is mediated by a direct repeat of 8-nt AU-rich sequences that function as a pH-response element (pHRE) (Laterza et al. 1997). Affinity chromatography using a biotinylated RNA identified ζ-crystallin (ζ-Cryst), an NADPH: quinone reductase, as the primary pHRE binding protein in rat renal cortical extracts (Tang and Curthoys 2001). This protein also binds to two 8-base pHREs within the 3'UTR of rat GDH mRNA, that are 88 % identical to one of the AU-sequences in the GA mRNA (Schroeder et al. 2003). However, ζ-Cryst lacks a recognizable RNA binding motif and was not previously known to function as an RNA binding protein. However, ζ-Cryst was previously shown to bind to single-stranded DNA and this interaction was effectively competed by NADPH (Gagna et al. 1998). Thus, the NADPH binding site of ζ-Cryst may constitute a portion of the nucleic acid binding site. More recent studies have established that yeast and human ζ-Cryst also bind to AU-rich elements in RNA with high affinity and specificity (Fernandez et al. 2007). Therefore, it was initially hypothesized that ζ-Cryst may contribute to mRNA

stabilization by preventing the binding of proteins that participate in mRNA turnover (Laterza and Curthoys 2000).

Previous studies established that expression of the $Na^+K^+2Cl^-$ - cotransporter (NKCC2) in the rat medullary thick ascending limb (TAL) is also increased during metabolic acidosis through the process of selective mRNA stabilization (Karim et al. 2006). More recent studies (Szutkowska et al. 2009) indicate that mouse TAL cells express ζ-Cryst and that the level of this protein is increased 2-fold when the cells are treated with an acidic medium (pH 7.1). Overexpression of mouse ζ-Cryst also resulted in an increased level of NKCC2 mRNA, but had no effect on expression of a construct lacking a specific AU-rich element. In addition, cells that overexpress ζ-Cryst failed to exhibit a further increase in NKCC2 mRNA when treated with acidic medium. Finally, siRNA knockdown of ζ-Cryst by 60 % reduced the basal level and completely abolished the pH-responsive increase in NKCC2 mRNA. Thus, ζ-Cryst may play an essential role in the pH-responsive stabilization of NKCC2 mRNA in TAL cells.

The identification and characterization of ζ-Cryst as an RNA-binding factor that affects expression of the human antiapoptotic *bcl-2* mRNA was also recently reported by Capaccioli's group (Lapucci et al. 2010). Previous studies established that human t (14:18) B-cell leukemias/ lymphomas exhibit increased Bcl-2 protein production due primarily to increased transcriptional activation of the *bcl-2*/ *IgH* fusion gene (Graninger et al. 1987). Later studies also showed that overproduction of Bcl-2 was also due in part to stabilization of the *bcl-2* mRNA (Capaccioli et al. 1996; Morelli et al. 1997). Multiple RNA-binding proteins have been characterized to bind to AU-rich elements within 3'-UTR of *bcl-2* mRNA (Lapucci et al. 2002; Bevilacqua et al. 2003; Donnini et al. 2004; Sengupta et al.

2004; Ishimaru et al. 2009). In a recent study, ζ-Cryst was reported as an additional protein that binds to *bcl-2* mRNA and overexpression of ζ-Cryst was shown to proportionally enhance the stability of *bcl-2* mRNA (Lapucci et al. 2010).

A novel RNA affinity purification protocol was used to isolate a protein from rat renal cortex that forms a complex with the PCK-6/7 RNA. Mass spectrometry and western blot analysis identified the primary PCK-6/7 RNA binding protein as ζ-Cryst. Direct binding assays and competition analyses established that purified ζ-Cryst binds to the PCK-6/7 RNA with high affinity and specificity. Further RNA gel shift analyses using truncated and mutated RNAs revealed that ζ-Cryst binds to a single site within the 3'-UTR of the PEPCK mRNA. This interaction requires specific sequences within both the stem and the AU-rich loop portions of a secondary structure within the PCK-7 segment (Unpublished data of Hajarnis.et al,). As shown in Fig.5.1, AUF1 binds to the PCK-2 RNA and the AU-rich sequences in PCK-6 and PCK-7 RNAs (Hajarnis et al. 2005) while HuR binds only to PCK-6 and PCK-7 (Mufti et al. 2011). The 11-nt AU-rich sequence that constitutes the loop in PCK-7 is identical in 11 of the 27 fully sequenced mammalian *PCK1* genes, including pig, rat, mouse, and human. In the remainder of these genomes, this sequence is highly conserved. In addition, this sequence contains an 8-nt stretch in which 7 of the 8 nucleotides are identical to one of the pHREs in the glutaminase mRNA (Laterza et al. 1997). Therefore, the sequence that binds ζ-Cryst may function as the pHRE within the PEPCK mRNA.

Earlier preliminary experiments to test the function of ζ-Cryst in LLC-PK_1-F^+ cells used transient expression of an siRNA to knockdown ζ-Cryst. This approach produced a 75% decrease in ζ-Cryst expression, but had no effect on the normal level or the pH-responsive increase in the PEPCK protein. To achieve a greater knockdown, four

clonal lines of LLC-PK_1-F^+ cells that stably express the shRNA were isolated. These cells exhibit between 85-95 % knockdown of ζ-Cryst with no effect on the basal level of PEPCK. However, ζ-Cryst knockdown did produce a slight, but not significant, enhancement of the pH-responsive increase in PEPCK protein (Unpublished data of Hajarnis. et al.,). Thus, ζ-Cryst binding is not likely to be the sole factor that accounts for the rapid degradation or the pH-responsive stabilization of PEPCK mRNA in the proximal convoluted tubule. In the current study, the clonal LLC-PK_1-F^+-9C cells were used to characterize the effects of overexpressing mouse ζ-Cryst or reducing the levels of endogenous ζ-Cryst by stable expression of an shRNA. These cells were used to analyze the effects of HuR and/or AUF1 knockdown.

5.2 Results

5.2.1 Effect of ζ-Cryst expression on the abundance of PEPCK protein

To initially assess the functional significance of the ζ-Cryst binding to the 3'-UTR of PEPCK mRNA(Fig.5.1), stable transformants of LLC-PK_1-F^+ 9C cells expressing either ζ-Cryst shRNA (9C -ZC) or mouse ζ-Cryst (9C +ZC) were compared versus control LLC-PK_1-F^+ 9C cells. There is a 3-fold decrease in ζ-Cryst in the 9C- ZC cells and about 5-fold increase in total ζ-Cryst in the 9C+ZC cells compared to the control LLC-PK_1-F^+ 9C cells (Fig 5.2A and 5.2B). Western blot analysis (Fig.5.2C) indicated that the effective knockdown of ζ-Cryst in stable 9C –ZC cells produced a modest increase (1.4-fold) (Fig.5.2D) in both the basal and acid pH-induced PEPCK protein levels when compared to their respective levels in control LLC-PK_1-F^+ 9C cells. By contrast, the constitutive overexpression of ζ-Cryst caused an average ~0.7-fold decrease

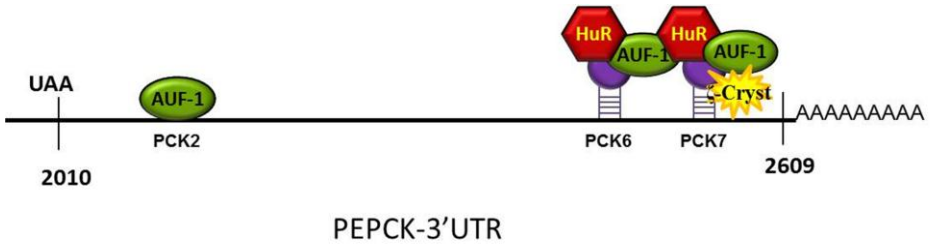


Fig. 5.1 The 3'-UTR of PEPCK mRNA contains multiple sites which bind various RNA binding proteins. *In vitro* studies have mapped the binding of HuR, p40AUF1 and ζ-crystallin to various elements within the PEPCK-3'UTR. While AUF1 has multiple binding sites (PCK-2, PCK-6 and PCK-7), HuR binds only to the PCK-6 and PCK-7 stem loops and ζ-Cryst binds to only one site within PCK-7.

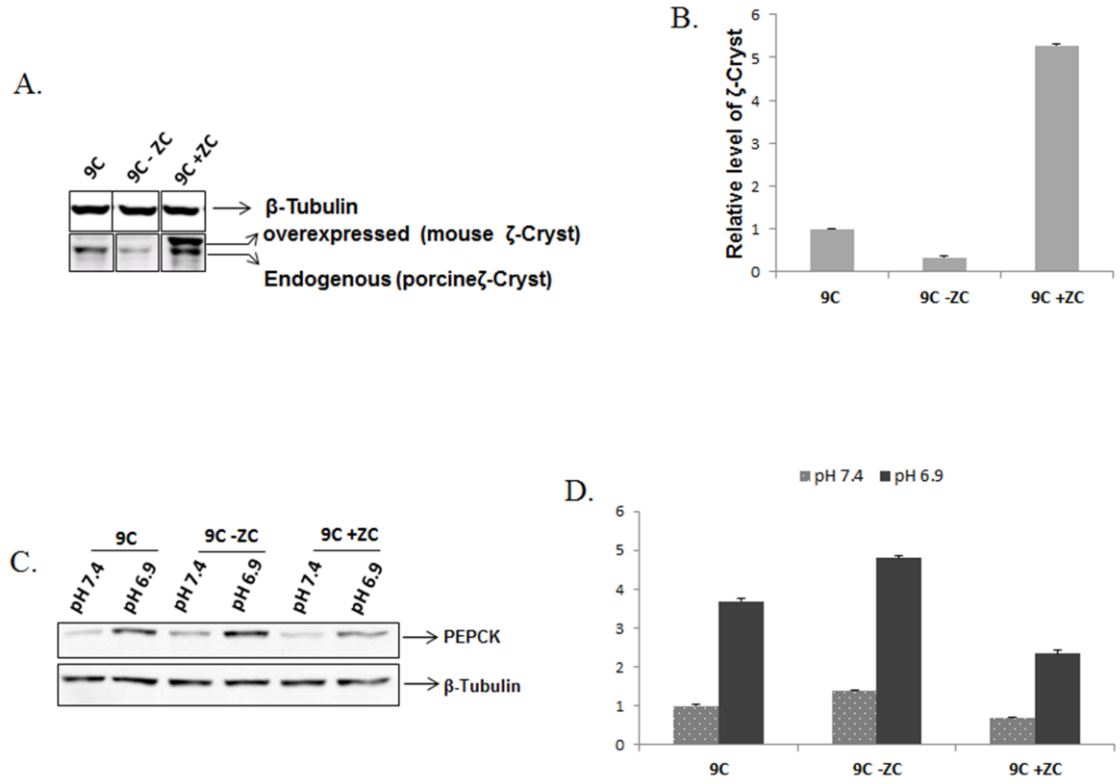


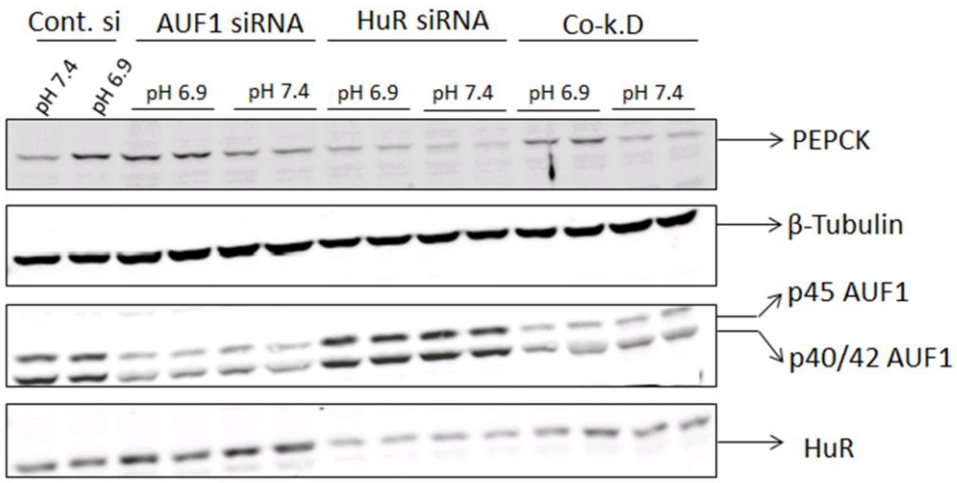
Fig.5.2 Effect of shRNA knockdown or overexpression of ζ-Cryst on the expression of PEPCK protein. LLC-PK_1-F^+-9C cells were stably transformed with either a ζ-Cryst shRNA (9C-ZC) or ζ-Cryst expression vector (9C+ZC) and maintained in normal medium (pH 7.4, 27 mM HCO_3^-) or treated with acidic medium (pH 6.9, 9 mM HCO_3^-) for 24 h . Extracts were prepared in RIPA buffer and used for western blot analysis. The blots were probed using antibodies that are specific for ζ-Cryst, PEPCK, and β-tubulin. The latter protein was used as a loading control. The blots were imaged using an Odyssey Infra-red Imager and the bands were normalized relative to the intensities of the β-tubulin bands. Panel A. Immunoblot showing level of ζ-Cryst expression in control LLC-PK_1-F^+-9C cells versus 9C-ZC and 9C+ZC cells and the corresponding quantification for relative level of ζ-Cryst represented in Panel B. Panel C. Immunoblot showing level of PEPCK expression in control LLC-PK_1-F^+-9C cells versus 9C –ZC and 9C +ZC cells. The corresponding quantification for relative level of PEPCK protein is shown in Panel D.

in PEPCK protein in 9C+ZC cells compared to the control LLC-PK_1-F^+ 9C cells, maintained in either normal or acidic medium. This suggested that ζ-Cryst may contribute to the rapid degradation of PEPCK mRNA under normal and acid-pH conditions. Nevertheless, the increased or decreased abundance of cytoplasmic protein ζ-Cryst failed to significantly alter the pH-responsive increase in PEPCK protein, which was approximately 3.6-fold in both 9C -ZC cells and the 9C +ZC cells, on par with pH-induced PEPCK protein response in the untransformed LLC-PK_1-F^+ 9C cells (Fig 5.2C and 5.2D). Therefore, it appears that ζ-Cryst alone may not be the sole regulator of basal and pH-responsive increase in PEPCK expression.

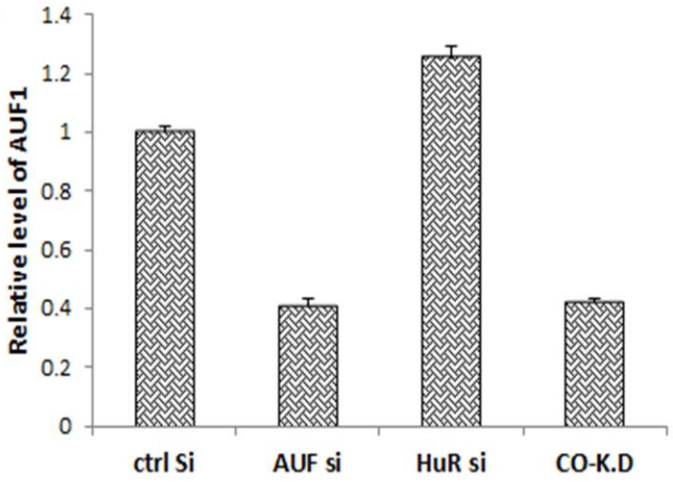
5.2.2 Effect of co-knockdown of HuR and AUF1 on the PEPCK mRNA and protein in 9C +ZC cells

The studies reported in Chapter III established that co-silencing of HuR and AUF1 is required to completely inhibit the pH-responsive increase of PEPCK mRNA and protein. Therefore, the effect of siRNA knockdown of HuR and/or AUF1 on the basal and pH-responsive expression of PEPCK protein was determined in cells that overexpress ζ-Cryst (Fig. 5.3A). The parent LLC-PK_1-F^+ 9C cells express high levels of the p45-, p42-, and p40-isoforms of AUF1. A combination of two siRNA oligos targeting different regions of exon 3, common to all four isoforms, gave a modest 60 % decrease in the three AUF1 isoforms (Fig.5.3B). Similarly, the transient transfection of an siRNA that is complimentary to HuR mRNA produced ~ a 60% reduction in HuR protein expression (Fig.5.3C). The 9C +ZC cells treated with a control siRNA retain a normal

A.


Cont. si
AUF1 siRNA
HuR siRNA
Co-k.D
pH 7.4
pH 6.9
pH 6.9
pH 7.4
pH 6.9
pH 7.4
pH 6.9
pH 7.4
PEPCK
β-Tubulin
p45 AUF1
p40/42 AUF1
HuR

B.


Relative level of AUF1
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1.2
1
0.8
0.6
0.4
0.2
0
ctrl Si
AUF si
HuR si
CO-K.D

C.

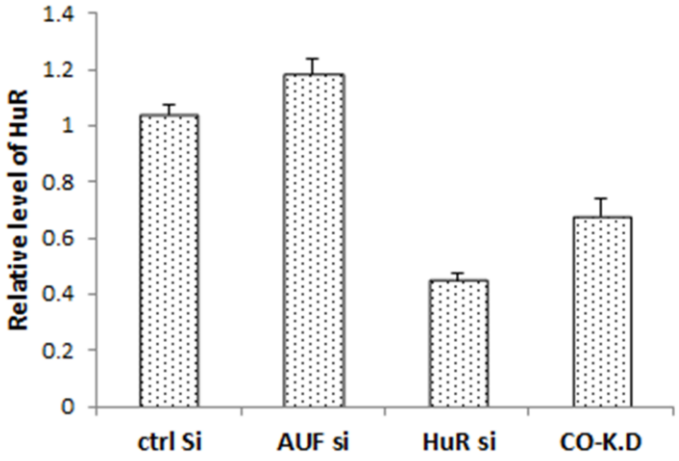

Relative level of HuR
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ctrl Si
AUF si
HuR si
CO-K.D

Fig. 5.3 Level of HuR and AUF1knockdown in the 9C +ZC cells.
Two days after transfection with 50nM control siRNA, 30nM HuR siRNA or 100nM AUF1 siRNA, 9C +ZC cells were maintained either in normal medium (pH 7.4) or treated with acidic medium (pH 6.9) for 24 h before harvesting with lysis buffer (protein).(A). Western blot analysis to monitor expression of PEPCK, HuR, AUF1 and β-tubulin loading control proteins.(B). Quantification of western blot data showing levels of AUF1 relative to β-tubulin at pH 7.4 and at pH 6.9. Data are the mean +/- SE of triplicate samples.(C). Quantification of western blot data showing levels of HuR relative to β-tubulin at pH 7.4 and at pH 6.9. Data are the mean +/- SE of triplicate samples.

pH-responsive increase in the PEPCK mRNA (2-fold) and protein (3-fold). The knockdown of HuR or AUF1 had little effect on the basal levels of PEPCK mRNA (Fig.5.4A) or the protein (Fig.5.4B) in 9C +ZC cells when compared to the cells treated with the control siRNA (Fig.5.4A). By contrast, the co-reduction of HuR and AUF1 produced nearly a 2-fold decrease in basal expression of PEPCK mRNA (Fig.5.4A) and protein (Fig.5.4B). However, the knockdown of HuR and/ or AUF1 caused a significant decrease in the pH-responsive increase in both PEPCK mRNA and protein levels in the 9C +ZC cells. Therefore, ζ-Cryst might play a dominant protective role of PEPCK mRNA expression in normal medium because the presence or absence of HuR or AUF1 failed to significantly influence the PEPCK mRNA or protein levels under these conditions. However, following treatment with acidic medium, the overexpression of ζ-Cryst fails to overcome the effects of HuR or AUF1 knockdown on the pH-responsive increase of PEPCK mRNA and protein.

5.2.3 Effect of co-knockdown of HuR and AUF1 on the PEPCK mRNA and protein levels in 9C -ZC cells.

Western blot analysis (Fig.5.5A) established that transient transfection of the 9C -ZC cells with siRNAs specific for either HuR or AUF1 proteins again achieved approximately 60% decrease in HuR (Fig.5.5B) and AUF1 (Fig.5.5C). Treatment of the 9C -ZC cells transfected with the control siRNA, with acidic medium for 24 h again produced a 2.2-fold (Fig.5.6A) and a 3.3-fold (Fig.5.6B) increase in PEPCK mRNA and protein, respectively. The knockdown of AUF1produced a slight increase in the basal

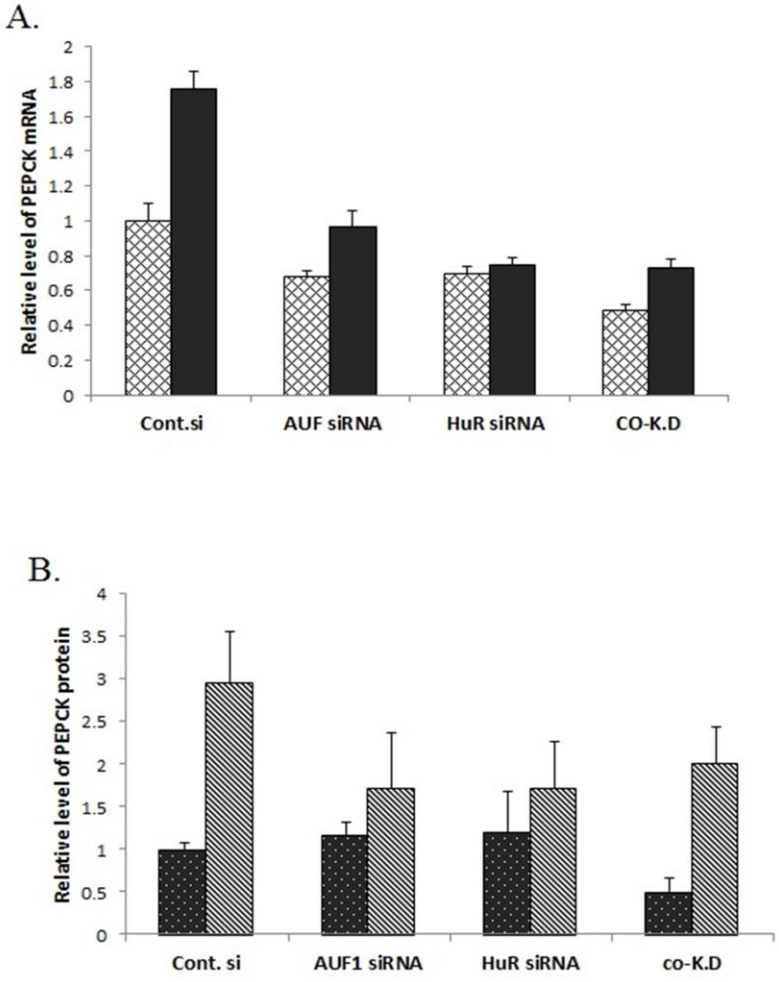
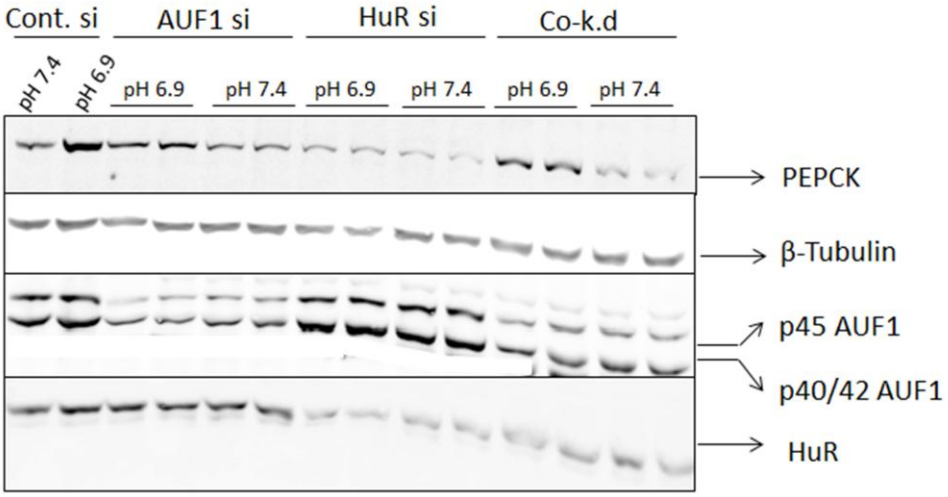
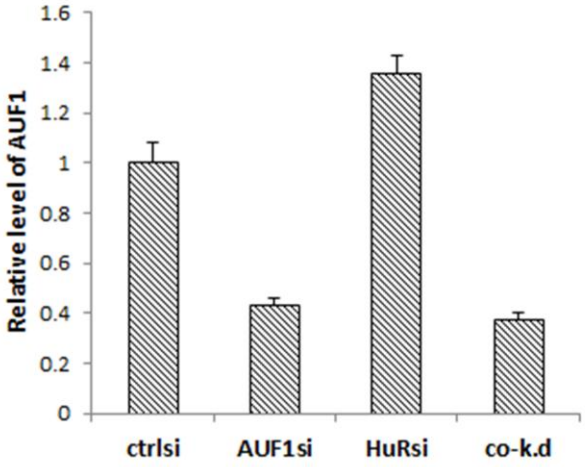


Fig. 5.4 Effect of Co-silencing of HuR and AUF1 in the 9C +ZC cells. Two days after transfection with 50nM control siRNA, 30nM HuR siRNA or 100nM AUF1 siRNA, 9C +ZC cells were maintained either in normal medium (pH 7.4) or treated with acidic medium (pH 6.9) for 24 h before harvesting with TRIzol (RNA) or lysis buffer (protein). (A). Levels of PEPCK mRNA relative to GAPDH mRNA at pH 7.4 (hatched bars) and at pH 6.9 (solid bars) were determined by RT-qPCR. Data are the mean +/- SE of triplicate samples. (B). Quantification of western blot data showing levels of PEPCK protein relative to β-tubulin at pH 7.4 (black patterned) and at pH 6.9 (grey hatched). Data are the mean +/- SE of triplicate samples.

A.


Cont. si
AUF1 si
HuR si
Co-k.d
pH 7.4
pH 6.9
pH 6.9
pH 7.4
pH 6.9
pH 7.4
pH 6.9
pH 7.4
PEPCK
β-Tubulin
p45 AUF1
p40/42 AUF1
HuR

B.


Relative level of AUF1
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ctrlsi
AUF1si
HuRsi
co-k.d

C.

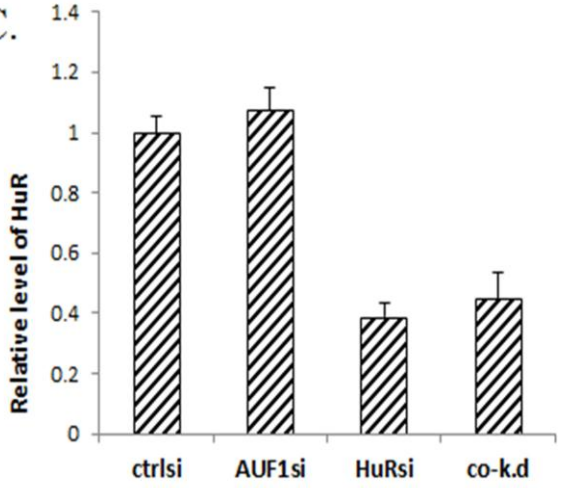

Relative level of HuR
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ctrlsi
AUF1si
HuRsi
co-k.d

Fig. 5.5 Level of HuR and AUF1 knockdown in the 9C -ZC cells.
Two days after transfection with 50nM control siRNA, 30nM HuR siRNA or 100nM AUF1 siRNA, 9C -ZC cells were maintained either in normal medium (pH 7.4) or treated with acidic medium (pH 6.9) for 24 h before harvesting with lysis buffer (protein). (A). Western blot analysis to monitor expression of PEPCK, HuR, AUF1 and β-tubulin loading control proteins. (B).Quantification of western blot data showing levels of AUF1 relative to β-tubulin at pH 7.4 and at pH 6.9. Data are the mean +/- SE of triplicate samples. (C). Quantification of western blot data showing levels of HuR relative to β-tubulin at pH 7.4 and at pH 6.9. Data are the mean +/- SE of triplicate samples.

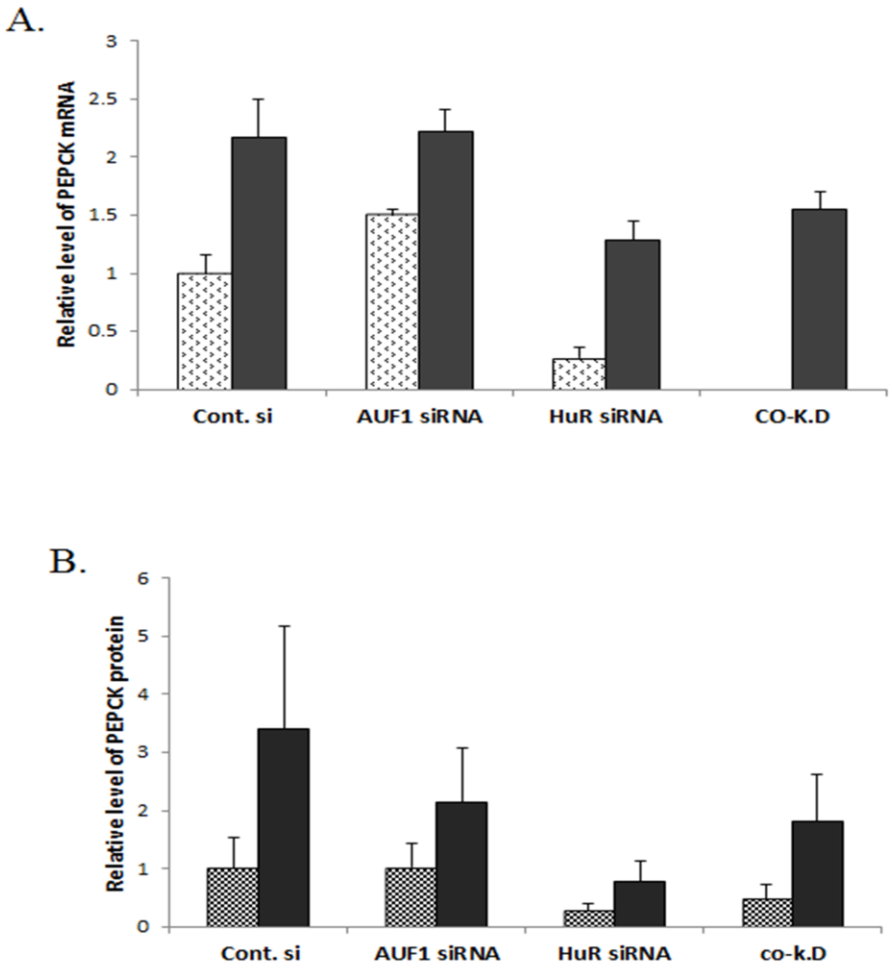


Fig. 5.6. Effect of Co-silencing of HuR and AUF1 in the 9C -ZC cells.
Two days after transfection with 50nM control siRNA, 30nM HuR siRNA or 100nM AUF1 siRNA, 9C -ZC cells were maintained either in normal medium (pH 7.4) or treated with acidic medium (pH 6.9) for 24 h before harvesting with TRIzol (RNA) or lysis buffer (protein). (A). Levels of PEPCK mRNA relative to GAPDH mRNA at pH 7.4 (hatched bars) and pH 6.9 (solid bars) were determined by RT-qPCR. Data are the mean +/- SE of triplicate samples. (B). Quantification of western blot data showing levels of PEPCK protein relative to β-tubulin at pH 7.4 (grey bars) and at pH 6.9 (black solid bars). Data are the mean +/- SE of triplicate samples.

level but no change in pH induced level of the PEPCK mRNA. By contrast, siRNA knockdown of AUF1 had no effect on the PEPCK protein level, but decreased pH-induced level when compared to the control siRNA treated cells. Decreased expression of HuR decreased both the basal and the pH-induced levels of the PEPCK mRNA (Fig.5.6A) and protein (Fig.5.6B). Lastly, transient knockdown of both HuR and AUF1 in the LLC-PK_1-F^+ 9C cells caused substantial decrease in basal expression of PEPCK mRNA and pronounced decrease in the PEPCK protein. However, the reduced levels of above three RNA-binding proteins had little effect on the induced levels of PEPCK mRNA and protein (Fig.5.6A and 5.6B). Consistent with ζ-Cryst over expression, knockdown of ζ-Cryst also indicates that ζ-Cryst is an important co-factor, along with HuR and AUF1, that contributes to the basal expression of PEPCK mRNA in LLC-PK_1-F^+ 9C cells. However, it apparently plays a minor role in the pH-responsive stabilization of PEPCK mRNA.

5.2.4 Comparing the effect of HuR and/ or AUF1knockdown on PEPCK mRNA levels in LLC-PK_1-F^+-9C cells, 9C +ZC cells and, 9C -ZC cells

To confirm the possible roles of HuR, AUF1 and ζ-Cryst on PEPCK mRNA expression, RNA samples were isolated from the three cell lines that were treated with the various siRNAs and quantified in a single RT-qPCR assay (Fig.5.7). The first striking observation is that in cells treated with the control siRNA, overexpression of ζ-Cryst significantly decreased the level of PEPCK mRNA, but the stable knockdown of ζ-Cryst had little effect compared with the control LLC-PK_1-F^+-9C cells. Another noteworthy observation from this data (Fig.5.7) is that the overexpression or knockdown of ζ-Cryst

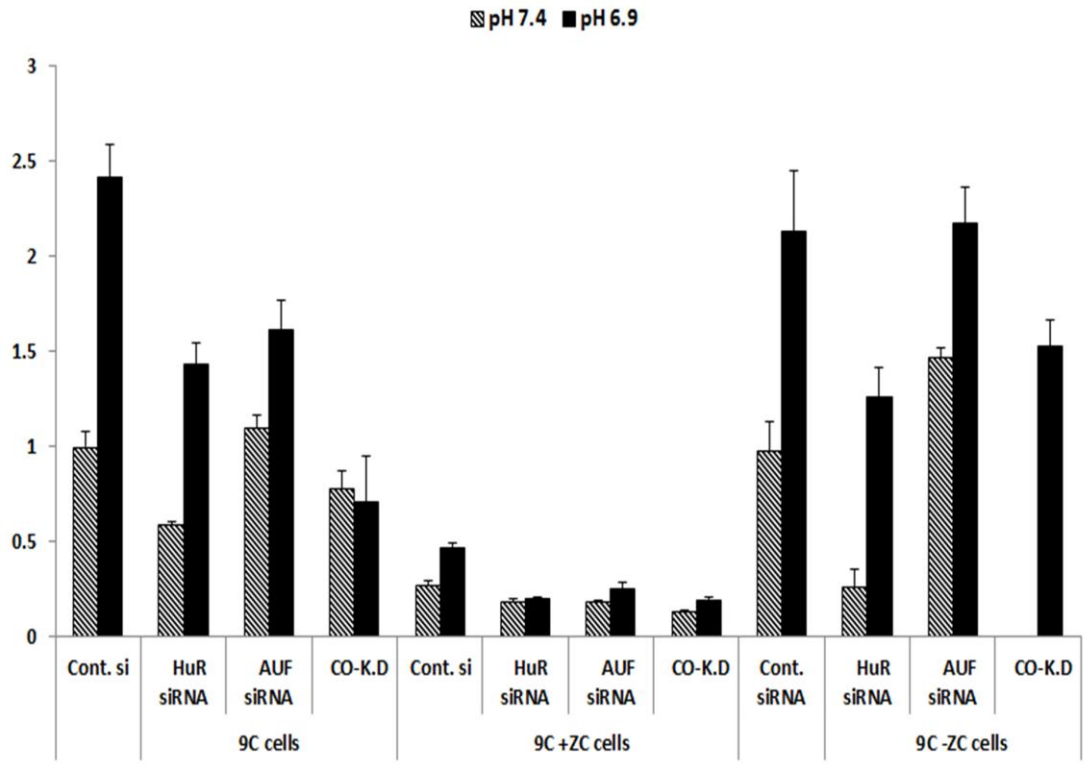


Fig. 5.7. Effect of Co-silencing of HuR and AUF1 in the LLC-PK$_1$-F$^+$-9C cells, 9C +ZC cells and 9C –ZC cells. Two days after transfection with 50 nM control siRNA, 30 nM HuR siRNA or 100 nM AUF1 siRNA, cells were maintained either in normal (hatched bars-pH 7.4) or treated with acidic medium (solid bars-pH 6.9) for 24 h before harvesting with TRIzol (RNA). Levels of PEPCK mRNA relative to GAPDH mRNA were determined by RT-qPCR. Data are the mean +/- SE of triplicate samples.

has little effect on the pH-responsive increase in PEPCK mRNA, suggesting that ζ-Cryst may not function as pH-responsive *trans*-acting factor. The knockdown of HuR in LLC-PK_1-F^+-9C cells again reduced normal and pH-induced levels of PEPCK mRNA. However, when the similar experiment was performed in 9C +ZC cells, HuR reduction totally abolished the pH-responsive increase in PEPCK mRNA expression. This data indicates that ζ-Cryst and HuR may play opposing roles when the cells are treated with acid-pH medium. This finding was further corroborated in 9C -ZC cells, wherein, with lowered endogenous ζ-Cryst levels, HuR siRNA treated cells exhibit a greater fold medium. By contrast, AUF1 knockdown again caused increase in basal level of PEPCK mRNA in LLC-PK_1-F^+-9C cells. This effect was even more evident when the AUF1 knockdown was performed in 9C -ZC cells that have reduced ζ-Cryst levels. However, with the overexpression of ζ-Cryst in the 9C +ZC cells, the AUF1 silencing failed to significantly enhance the PEPCK mRNA levels in comparison with the respective control siRNA treatment. Presumably, these findings demonstrate a complimentary role between AUF1 and ζ-Cryst and, that they are primarily involved in PEPCK mRNA turnover at both normal and pH-induced conditions. More interestingly, with co-knockdown of HuR and AUF1, the basal levels of PEPCK mRNA were maintained in LLC-PK_1-F^+-9C cells and 9C +ZC cells, but a drastic reduction of basal PEPCK mRNA occurred in the 9C -ZC cells. However, this effect is reversed when the 9C -ZC cells are treated with acidic medium. This is a clear evidence to support the hypothesis that ζ-Cryst is an important *trans*-acting factor, which along with HuR and AUF1 is required for the basal expression of PEPCK mRNA, but not the pH-responsive increase.

5.3 Discussion

In light of earlier RNA-gel shift and mapping experiments, PCK-7 was mapped as a common binding site for AUF1(Hajarnis et al. 2005), HuR (Mufti et al. 2011) and ζ-Cryst (Hajarnis and Curthoys 2012). Here we report the comparative analyses of ζ-Cryst overexpression and ζ-Cryst silencing experiments in parallel with HuR/ AUF1 knockdown to gain insight into the individual and joint role of these proteins in regulating the post-transcriptional fate of PEPCK mRNA under basal and pH-induced conditions. The foremost observation from these studies was that overexpression of ζ-Cryst alone decreased both normal and acidic pH-induced levels of PEPCK mRNA (Fig.5.7) and protein (Fig.5.2). Nevertheless, overexpression of ζ-Cryst restored the pH-responsiveness of PEPCK mRNA (Fig.5.7) and protein (Fig.5.4) in LLC-PK_1-F^+-9C cells in which AUF1 and HuR expression was decreased. By contrast, the knockdown of ζ-Cryst alone had little effect on the basal or pH-responsive increase in PEPCK expression. However, the knockdown of all three RNA binding proteins greatly inhibited basal expression, but not the pH-induced expression of PEPCK mRNA and protein. Thus, ζ-Cryst binding may contribute the rapid turnover of PEPCK mRNA in cells grown in normal medium and interact with HuR and or AUF1 to effect the pH-responsive stabilization and/ or translation of PEPCK mRNA.

Interestingly, transient transfection of siRNA oligos complimentary to HuR in LLC-PK_1-F^+-9C cells achieved 90 % reduction in HuR levels (Chapter 3).However, with the similar conditions of transfection in 9C +ZC and/ or 9C –ZC cells, HuR levels were decreased by only 60 % when compared to the control siRNA treated cells (Fig.5.3 & Fig.5.5). Similarly, while we successfully reduced AUF1 levels by 80% with siRNA mediated silencing in LLC-PK_1-F^+-9C cells (Chapter 3), the same treatment yielded a

maximum of 60% reduced AUF1 levels (Fig.5.3 & Fig.5.5) in 9C +ZC and 9C –ZC cells. More intriguingly, it was also evident that in the cell lines with either overexpression of ζ-Cryst levels (9C +ZC) or stable knockdown of ζ-Cryst (9C –ZC cells), the knockdown of HuR positively impacted the levels of AUF1 and vice-versa (Fig.5.3 & Fig.5.5). However, a similar pattern was not observed when HuR or AUF1 levels were knocked down in parent LLC-PK_1-F^+-9C cells. These observations indicate that expression of ζ-Cryst may affect expression of HuR and AUF1 and their response to environmental stimuli.

In summary, the resulting data indicates that ζ-Cryst acts as a co-factor but requires either HuR or AUF1 to contribute the basal turnover of PEPCK mRNA and, perhaps the dual role of HuR and AUF1 is sufficient to sustain the pH-responsive stabilization of PEPCK mRNA. These observations are consistent with ζ-Cryst overexpression studies in 9C +ZC cells.

While we showed that ζ-Cryst functions as a key co-factor *in-vivo* in maintaining normal levels of PEPCK mRNA, ζ-Cryst failed to co-purify with neither Halo-HuR nor Halo-p40AUF1 pull-downs (Chapter 4). This finding suggested that either ζ-Cryst does not directly bind to the 3'-UTR of PEPCK mRNA or that it is excluded from the ribonucleoprotein complex which HuR and AUF1 form on 3'-UTR of PEPCK mRNA.

CHAPTER VI

CONCLUSIONS AND PERSPECTIVES

6.0 Conclusions and future perspectives

Onset of metabolic acidosis leads to a pronounced increase in renal expression of phospho*enol*pyruvate carboxykinase (PEPCK). This response, which is mediated, in part, by stabilization of PEPCK mRNA, is reproduced in LLC-PK_1-F^+9C cells that are treated with an acidic medium (pH 6.9). Previous *in vitro* biochemical studies mapped the binding of AUF1, HuR and ζ-crystallin to two AU-rich sequences within the 3'-UTR of PEPCK mRNA. Based upon this finding, it was hypothesized that stabilization of PEPCK mRNA is regulated by the dynamic interplay between the three RNA binding proteins. The current study has significantly increased our understanding of the individual and combinatorial roles of the RNA binding proteins, HuR, AUF1 and ζ-crystallin, in modulating the turnover of the PEPCK mRNA in LLC-PK_1-F^+-9C cells under normal conditions and conditions that model a metabolic acidosis. This study has also found evidence for post-translational modifications of HuR and AUF1 in response to treatment of LLC-PK_1-F^+-9C cells with an acidic medium. The results of this study are summarized below.

6.1 The joint role of HuR and AUF1 in mediating the pH-responsive increase in PEPCK mRNA and protein

The resulting data from this section established that binding of HuR and AUF1 have opposite effects on basal expression, but their co-ordinate interaction is required to mediate the pH-responsive adaptation. This conclusion was reinforced by the results of a recruitment assay that used a β-globin-PCK reporter mRNA in which the pH-response elements of the 3'-UTR of PEPCK mRNA were replaced with six MS2 stem-loop sequences. The individual recruitment of a chimeric protein containing the MS2 coat protein and either HuR or p40AUF1 to the reporter failed to produce a pH-responsive stabilization. However, the co-recruitment of both chimeric constructs did produce a pH-responsive stabilization of the reporter mRNA. Two-dimensional western blot analysis indicated that AUF1 and HuR exhibit different levels of covalent modifications in LLC-PK_1-F^+-9C cells that were either maintained in basal medium or treated with acidic medium. Strikingly, HuR was present as multiple pI variants in cells treated with either condition. In both cases, treatment of the cell lysates with a protein phosphatatse caused the multiple variants to collapse into a single variant with a lower pI, suggesting that HuR is phosphorylated. Comparison of the pI profiles suggests that stimulation of cells with acidic medium caused a pronounced decrease in phosphorylation of HuR. AUF1 generated multiple covalent variants with a wide range of pIs. However, predominant phosphorylation, as indicated by a shift in mobility following treatment with protein phosphatase, was apparent only in cell lysates from cells treated with acidic medium. Thus, we conclude that the pH-responsive stabilization of PEPCK mRNA requires the

concurrent binding of HuR and AUF1 and may be mediated by changes in their extent of covalent modification.

In order to validate the requirement of co-recruitment of HuR and AUF1, one could use a reporter with two alternate RNA hairpin-sequences a (MS2 and PP7 stem-loop sequences). This construct will again utilize a Tet-Off promoter and will be stably expressed in the clonal LLC-PK_1-F^+-9C cells. Initially, the half-life of the reporter mRNA will be determined in cells that are grown in normal or acidic medium. Subsequently, the effects of transient expression of either MS2cp-HuR or PP7coat-AUF1, a chimeric protein composed of the PP7 binding protein and p40AUF1, on the half-life of the reporter mRNA in both normal and acidic medium. Finally, test the effect of co-expression of both chimeric RNA binding properties on the double tether reporter RNA. It is anticipated that this approach will both confirm our preliminary results and produce a greater pH-responsive stabilization when the two chimeric proteins are co-expressed and recruited to separate but adjacent sites.

The results of the reported experiments indicate changes in phosphorylation of HuR may contribute the pH-responsive stabilization of PEPCK mRNA. Therefore, the effects of various kinase and phosphatase inhibitors on the basal half-life and the pH-responsive stabilization could be tested to identify the signaling pathway that mediates the changes in phosphorylation of HuR. A single threonine and seven serine residues in HuR are phosphorylated in HuR in response to various conditions or stimuli (Abdelmohsen and Gorospe 2010). Thus, it would be feasible to test effects of phosphomimetic mutations of these residues on the expression and stability of PEPCK mRNA. A similar strategy will be adapted to identify phospho-modifications on AUF1

isoforms in LLC-PK_1-F^+-9C cells maintained in normal medium or stimulated with pH 6.9 medium.

6.2 The in-vivo binding of HuR to the PEPCK mRNA and the RNA-dependent HuR/ AUF1 heteromultimerization

A HaloTag-RNA pull-down assay using HaloTag-HuR was developed and successfully optimized. This analysis demonstrated that HuR is bound to the pH-response element of PEPCK mRNA in intact LLC-PK_1-F^+ cells and that this interaction may increase slightly in cells treated with acidic medium. Furthermore, protein complexes eluted with Halo-HuR pull-down assay also contained endogenous HuR, p45AUF1 and possibly p40AUF1. The same protein-complex was recovered when the reciprocal pull-down assays were performed with HaloTag-p40AUF1. However, with either Halo-HuR or Halo-p40AUF1 pull-downs, the abundant cytosolic protein β-tubulin and ζ-crystallin did not associate with the ribonucleoprotein complex. Now that the HaloTag-assay conditions are optimized for RNA-pull down, the *in-vivo* binding of Halo-p40AUF1 and Halo-ζ-crystallin with PEPCK mRNA will be tested.

6.3 Role of ζ-crystallin in association with HuR and AUF1 in regulating the PEPCK mRNA turnover

The complimentary studies of ζ-crystallin overexpression and ζ-crystallin knockdown suggested that ζ-crystallin may contribute to the basal expression of PEPCK mRNA, but unlike HuR and AUF1, it is does not contribute to expression of PEPCK mRNA in cells treated with acidic medium. To more directly assess the effect of ζ-

crystallin on PEPCK mRNA stability, half-life studies can be performed on 9C –ZC and 9C +ZC cell lines, by using Actinomycin-D to arrest of transcription. This will establish if ζ-crystallin contributes to the basal turnover and/or the pH-responsive stabilization of PEPCK mRNA in LLC-PK_1-F^+-9C cells.

Based upon the findings of the current study, the following model is proposed (Fig.6) depicting the dynamic interplay of *trans*-acting factors of HuR, AUF1 and ζ-crystallin in regulating the PEPCK mRNA turnover in LLC-PK_1-F^+-9C cells. Under normal acid-base conditions, phosphorylated HuR, covalently modified AUF1 and ζ-crystallin are co-recruited to the 3'-UTR of PEPCK mRNA and may form a complex through direct protein-protein interactions. The binding of the three RNA-binding proteins leads to recruitment of a deadenylase that removes the poly-A tail and leads to the subsequent decapping and 5'→ 3' decay of the deadenylated PEPCK mRNA. However onset of metabolic acidosis leads to alterations in post-translational modifications including decreased phosphorylation of HuR and an increased phosphorylation of AUF1. These changes may promote the dissociation of ζ-crystallin from the RNA-binding complex. This remodeling of the ribonucleoprotein-complex blocks the association of deadenylases and maintains a poly-A tail that is well protected by the poly-A binding protein (PABP). Therefore, this remodeling of the protein/mRNA complex mediates the enhanced stabilization and translation of PEPCK mRNA.

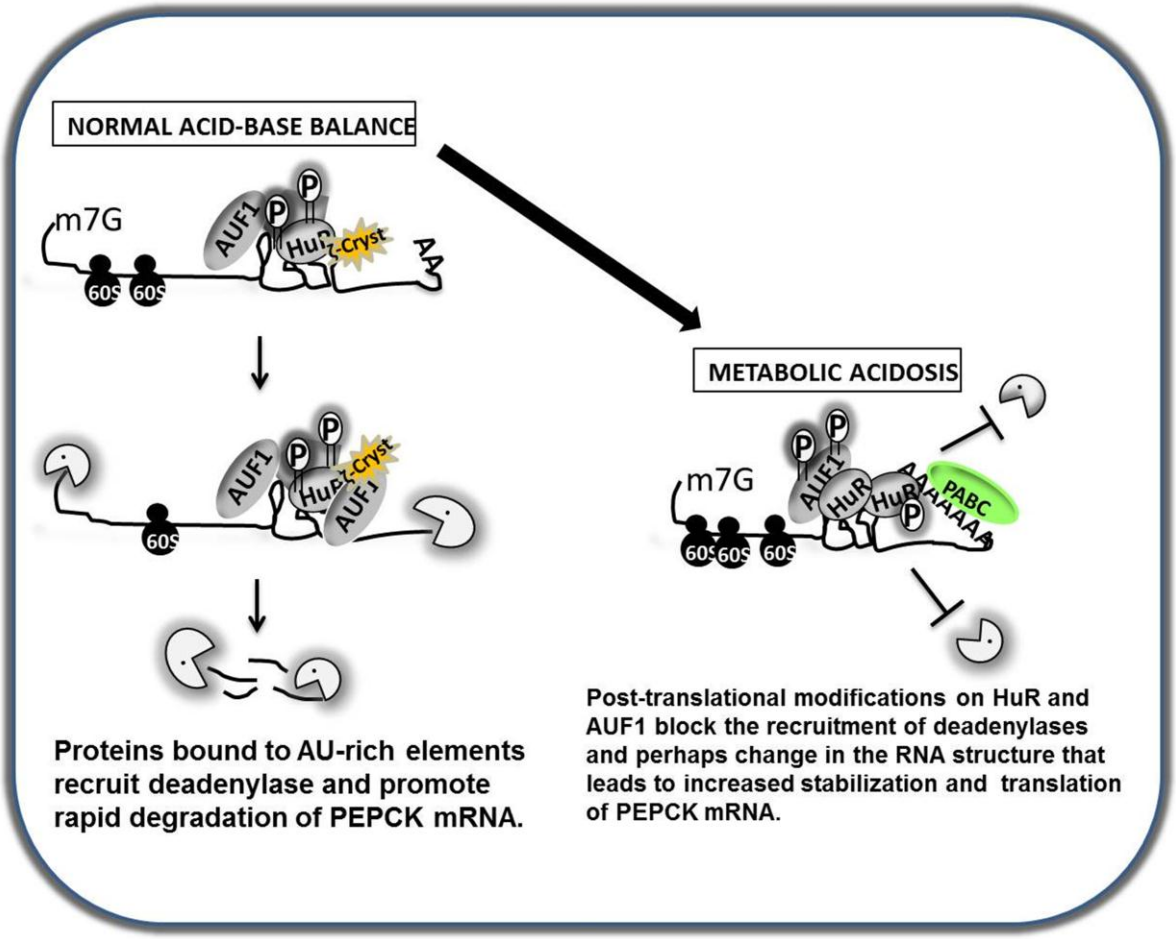


Fig.6.1 Model: Under basal pH-conditions AU-rich elements are bound by RNA-binding proteins of ζ- Cryst, phosphorylated HuR and unphosphorylated AUF1, which could recruit deadenylases. The deadenylated PEPCK mRNA is further targeted by other exoribonucleases for further 5' → 3' decay. However, at the onset of metabolic acidosis, increased phosphorylation of AUF1 and decreased phosphorylation of HuR and possible dissociation of ζ-Cryst presumably leads to remodeling of bound RNA-binding proteins and thus the RNA-structure, thereby keeping away the deadenylases. Therefore, this remodeling of the protein/ mRNA complex contributes to enhanced stabilization and translation of PEPCK mRNA.

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