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Posttranscriptional regulation of cyclin D1 by ARE-binding proteins AUF1 and HuR in cycling myoblasts

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RNA binding proteins (RBPs) can regulate the stability and/or translatability of messengerRNAs (mRNAs) through interactions with their 3′-untranslated regions. However, individual mRNAs may be regulated simultaneously or successively by more than one RBP, as well as by Argonaute (AGO)-bound miRNAs; the coordination of these various influences on an individual mRNA is therefore complex and not well studied. In this report we examine the roles of two RBPs that bind to AU-rich elements (ARE) – AUF1 and HuR – in the stability and translation of cyclin D1 (Ccnd1) mRNA in rat myoblasts transiting the G phase of the cell cycle, and their interactions with miRNAs. Knockdown (KD) of AUF1 resulted in (1) transient upregulation of the mRNA level as well as an advancement of translation onset time (TOT) from 6 to 5 h post-serum addition, (2) loss of miRNA loading on AGO1 and AGO2 and (3) reduction in the level of AGO-1 and AGO-2 bound mRNA. In contrast, KD of HuR had no effect on the mRNA level, or on the AGO–mRNA complexes, but delayed TOT by 1 h independent of miRNA let-7. Thus the dynamics of RBP–mRNA binding and –RBP–AGO–miRNA interactions are coordinated to fine tune the expression of Ccnd1 in the G1 phase.

Keywords. Argonaute; AUF1; cyclin D1; HuR; myoblast; translation

1. Introduction

Many eukaryotic microRNAs (mRNAs) are subject to complex regulation at the level of translation initiation or mRNA turnover by RNA binding proteins (RBPs) and miRNAs (reviewed in Jackson *et al*. 2010). The 3′ untranslated regions (UTRs) of such mRNAs contain binding sites for multiple RBPs and Argonaute (AGO)-loaded miRNAs that individually may have different effects on mRNA translation or turnover, but how these effects are coordinated to result in expression of the protein at an appropriate time and at an appropriate rate under a given physiological condition, is not understood.

It is axiomatic that the cellular gene regulatory network undergoes specific temporal alterations finely tuned to the environment. As proliferating cells traverse various phases of the cell cycle, the molecular network ebbs and flows like an orchestra. It has been demonstrated that the concentration/activity of cell cycle regulators change at specific times. Recent studies have revealed the genome-wide occurrence of translational control of such mRNAs during the progression of the cell cycle (Stumf *et al*. 2013). Among the critical cell cycle regulators are the cyclins, including cyclin D1 (Ccnd1), which, in association with cyclin-dependent kinases (Cdks), promotes passage of cells through the G1 phase of the cell cycle. The 3′-UTR of Ccnd1 mRNA contains known or putative binding sites for a number of miRNAs and RBPs. In various asynchronous cell cultures, Ccnd1 expression has been shown to be post-transcriptionally regulated by the miRNAs let-7 (Bakre *et al*. 2012; Guo *et al*. 2013) and miR-1 (Zhang *et al*. 2012). In synchronized rat myoblasts transiting the G1 phase, miR-1 and let-7 were shown to regulate Ccnd1 mRNA levels, and the onset of Ccnd1 translation, respectively (Ghosh and Adhya 2016).

In addition to miRNAs, the binding of AU-rich element (ARE) RBPs AUF1 and HuR to human Ccnd1 mRNA 3′-UTR has been reported; knockdown (KD) of AUF1 results in upregulation of the mRNA and protein, while KD of HuR has opposite effects (Lal *et al*. 2004). Recent evidence suggests that at least some effects of 3′-UTR binding RBPs involve direct or indirect interactions with the miRNA silencing machinery. While AUF1 promotes miRNA-mediated gene silencing (Yoon *et al*. 2015), HuR may antagonize (Kundu *et al*. 2012) or facilitate (Kim *et al*. 2009) miRNA activity. AUF1 binds directly to miRNAs such as let-7 and assists in miRNA loading onto AGO2 (Yoon *et al*. 2015). How these various effects of AUF1 and HuR are integrated

Table 1. Antibodies

Antigen	Source	Vendor	Catalogue no.	Dilutions	
				WB	CLIP
CCND1	Rabbit	Cell Signalling	2978	1/1000	1/100
AGO 1	Rabbit	Abcam	ab155104	1/1000	1/100
AGO 2	Rabbit	Abcam	ab156870	1/1000	1/100
GAPDH	Rabbit	Abcam	ab9485	1/500	N.D.
HuR	Rabbit	Millipore	07-1735	1/1000	1/100
AUF1	Rabbit	Millipore	07-260	1/1000	1/100
Anti-Mouse IgG-HRP	Goat	Merck	62114068001A	1/4000	N.D.
Anti-Rabbit IgG-HRP	Goat	Merck	62114068001A	1/4000	N.D.

N.D.: not done.

in real time to promote Ccnd1 expression is an unresolved question.

In this report we used synchronized rat myoblasts to examine the roles of AUF1 and HuR in switching on Ccnd1 expression in the G1 phase of the cell cycle. We observe that these two RBPs have opposite effects on translation onset and differ in their interactions with AGO–miRNA complexes. The results indicate that derepression and activation of this mRNA is a precisely timed, stepwise process.

2. Materials and methods

2.1 *Cell culture*

L6 rat myoblasts were grown as monolayers in DMEM plus 10% fetal bovine serum, starved for 24 h in serum-free medium and then re-exposed to serum for the indicated times.

2.2 *RNA-crosslinking immunoprecipitation (R-CLIP)*

Cellular ribonucleoproteins were formaldehyde-crosslinked and immunoprecipitated with antibody-coated protein A-Sepharose beads, de-crosslinked and analysed for Ccnd1 mRNA by RT-PCR, or western blot, as described previously (Ghosh and Adhya 2016).

2.3 *Western and Northern blots*

Western blots were probed with the primary antibody (table 1) followed by chemiluminescence-based detection. Northern blots were probed with 5′-^{32}P-labelled antisense oligonucleotides (table 2) and autoradiographed (Ghosh and Adhya 2016).

2.4 *Knockdown*

Target-specific or scrambled sequence (scr) small interfering (si) RNA pools (table 3) were delivered by transfection to L6 cells for 48 h before serum starvation and stimulation as described previously (Ghosh and Adhya 2016). Cells were transfected with locked nucleic acid (LNA) antagomirs (table 4) during serum starvation, and then serum-stimulated as above.

Table 2. Primers and probes

Oligo no.	Sequences (5′–3′)	Gene	Position	Species	Orientation
O-320	CTACCGACTGAGCTAGCCCGGGC	tRNA^{lys}	+5	Rat	AS
O-390	**GGCTCGAG**GGGCCACCGGGCAGGCG	Ccnd1	3′-UTR +889	Rat	S
O-401	**GGCTCGAG**GTGTTGTTACCAGAAGG	Ccnd1	3′-UTR +982	Rat	S
O-402	GCCAAAGCTGTGCCTTTTCAGC	Ccnd1	3′UTR +1384	Rat	AS
O-405	CTCCCTATACTCAGGGTGATGC	CCND1	3′UTR +1119	Rat	AS
O-413	GGGTACATAAAGAAGTATGTGC	rno-miR-1a	+13	Rat	AS
O-414	AACTATACAACCTACTACCTCA	rno-let-7a	+8	Rat	AS
OUB-1	AGTATGACTCTACCCACGGC	GAPDH	+137	Rat	S
OUB-2	TGAAGACGCCAGTAGACTCC	GAPDH	+282	Rat	AS

AS: antisense; S: sense.

Table 3. siRNAs (SMARTpool, Dharmacon)

Product description	Sequences (5′–3′)
siGENOME Rat Ago1 (313594) siRNA – SMARTpool	GGGCAGCGGUGCAUUAAGA, GCAGAAGCAUACCUAUUUG, AGAUUGACGUCUACCAUUA, UACCGCGUGUGUAAUGUUA
siGENOME Rat Ago2 (59117) siRNA – SMARTpool	CCAGUGAUCGAGUUUGUUU, GCAAAUUGAUGCGAAGUGC, AAUUAAAGGUCUAAAGGUG, UAAAGAACAUGACAGCGCU
siGENOME Rat Elavl1 (HuR) (363854) siRNA – SMARTpool	CAACAAGUCCCACAAAUAA, GAUCAGACCACAGGUUUGU, GGGUGUAGAUCACAUGAGU, GAACUAUGUUACUGCAAAA
siGENOME Rat Hnrnpd (AUF1) (79256) siRNA – SMARTpool	GGAAAGUCAUUGAUCCUAA, GAUCGACGCCAGUAAGAAU, CUAUUUAAAGAGUCGGAGA, CCGGAAUGGAAGUAUGACGU
siGENOME nontargeting siRNA pool #1	UAGCGACUAAACACAUCAA, UAAGGCUAUGAAGAGAUAC, AUGUAUUGGCCUGUAUUAG, AUGAACGUGAAUUGCUCAA

2.5 *RNA quantification*

miRNAs were quantified by Northern blots probed with ^{32}P-labelled antisense oligonucleotides and measurement of mean luminosity of the scanned autoradiographic bands. The Ccnd1 mRNA level in cellular extracts or immunoprecipitates (IPs) was quantified by SYBR-Green-based real-time RT-PCR (Ghosh and Adhya 2016). From the Ct values of the target and GAPDH (loading control) of each sample, the fold change of mRNA between control and KD cells was computed as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct=\Delta Ct(\text{treated})-\Delta Ct(\text{control})$, and $\Delta Ct=Ct(\text{target})-Ct(\text{GAPDH})$. For IPs, the percent control of bound mRNA was calculated as $100\times2^{\Delta Ct}$, where $\Delta Ct=Ct(\text{control})-Ct(\text{KD})$.

2.6 *Sequence analysis*

miRNA targets in the Ccnd1 3′-UTR were computed using the miRNA-Target Gene Prediction database at EMBL (*http://targetscan.org*). AU-rich binding motifs identified for AUF (White *et al*. 2016) and HuR (Ray *et al*. 2009) were located between the miR-1 and let-7 target sites in the 3′ UTR (figure 1).

Table 4. Anti-miRNA LNAs (Exiqon)

Product description	Sequences (5′–3′)
rno-let-7a-5p	ACTATACAACCTACTACCTC
Scrambled (negative control A)	TAACACGTCTATACGCCCA

3. Results

3.1 *Transient downregulation of mRNA level and translation by AUF1*

The rat Ccnd1 3′-UTR includes an AU-rich region, containing putative binding motifs for AUF1 and HuR, between the miR-1 and let-7 target sites (figure 1A). We examined the kinetics of AUF1 expression in serum stimulated rat myoblasts. Between 3 and 8 h post-serum addition, there was a steady decrease in AUF1 levels until 6–7 h (figure 1B and D). The binding of AUF1 to Ccnd1 mRNA over the same time period, assessed by R-CLIP, revealed a parallel decline in RNA binding over time (figure 1C and D).

To determine the role of AUF1, rat L6 myoblasts were transfected with anti-AUF1 siRNA; in transfected cells the level of AUF1 was specifically reduced to 27% of normal (figure 1E). Serum stimulation of the AUF1-depleted cells showed that at 4 h the level of Ccnd1 mRNA was significantly increased (figure 1F); this effect was transient, as no significant difference was observed at 8 h (figure 1F).

In normal cells (Ghosh and Adhya 2016), or in cells transfected with scr siRNA (figure 1G), the translation onset time (TOT), i.e. the time at which the Ccnd1 protein was first detectable, was 6 h. In AUF1-KD cells, the TOT of the Ccnd1 protein was reduced from 6 to 5 h (figure 1G). Thus, AUF1 affects both the abundance and translation of Ccnd1 mRNA, and both these effects are transient and are manifested at different times after serum stimulation (4 h *vs* 6 h).

Both AGO1 and AGO2 are bound to Ccnd1 mRNA at early times in serum-stimulated myoblasts (Ghosh and Adhya 2016). To determine the relation between the binding of AUF1 and AGO1/2, crosslinked ribonucleoproteins were immunoprecipitated with the anti-AUF1 or -AGO1/2 antibody and the amount of Ccnd1 mRNA pulled down was determined by RT-PCR (R-CLIP assay). R-CLIP using the anti-AUF1 antibody was performed with cells transfected with scrambled, anti-AGO1 or anti-AGO2 siRNA. It was observed that the amount of AUF1–mRNA complex was comparable in these three cells at 4 h of serum stimulation (figure 2A), indicating that AUF1 binding is independent of AGO1/2. In the reverse experiment, R-CLIP using the anti-AGO1 or anti-AGO2 antibody was carried out in cells transfected with anti-AUF1 siRNA. Quantification of Ccnd1 mRNA bound showed that in AUF1 KD cells the binding of AGO1 or AGO2 was reduced by about 50% or 40%, respectively (figure 2B). Thus AUF1 facilitates AGO1 as well as AGO2 binding to mRNA.

IPs of both AGO1 and AGO2 contained AUF1, as shown by western blotting (figure 3A), indicating that AUF1 associates directly or indirectly with AGO proteins. Analysis of immunoprecipitates of AGO1 or AGO2 from cells transfected with scrambled or anti-AUF1 siRNA by Northern blot showed that (1) KD of AUF1 had no significant effect on the

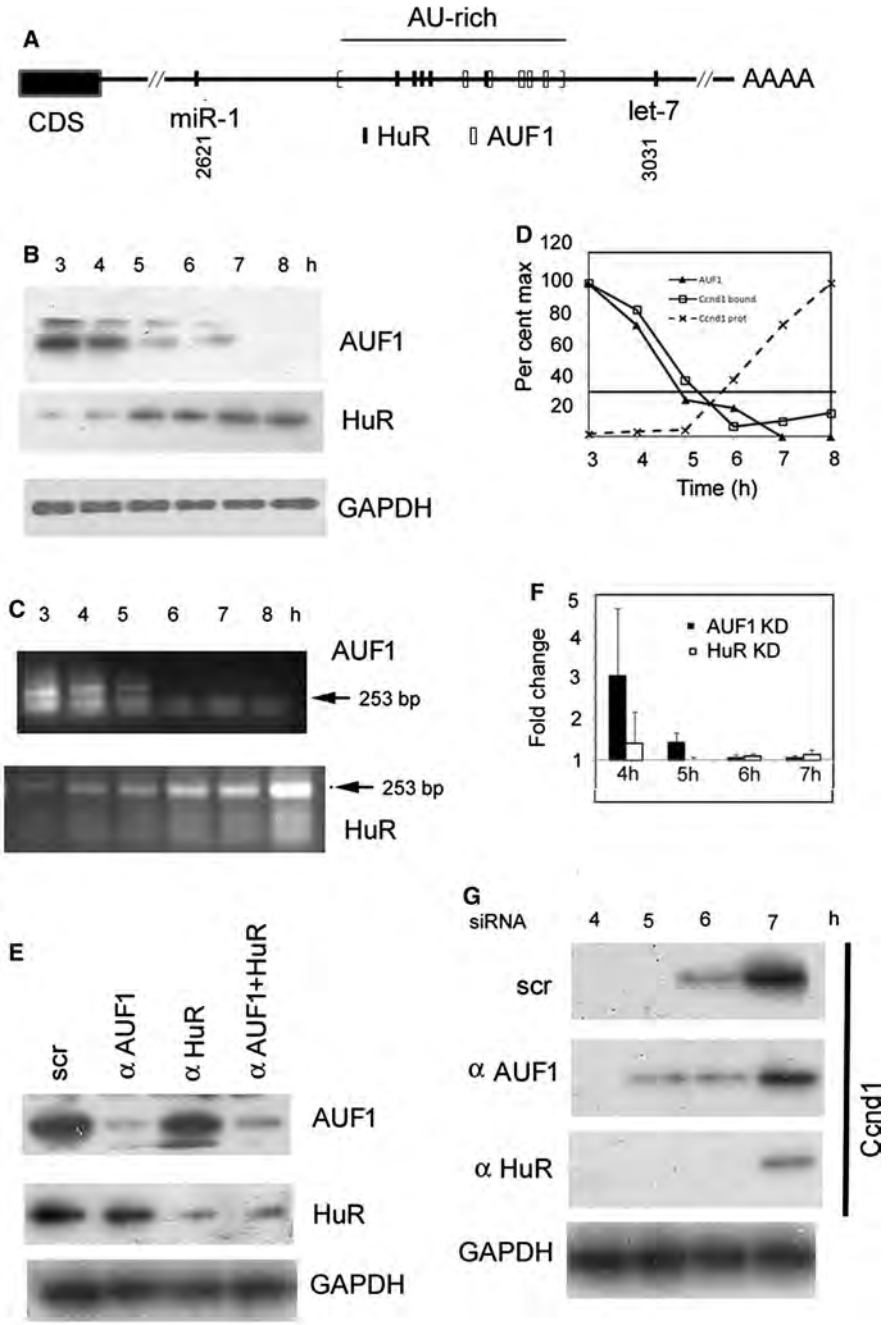

Figure 1. Effects of AUF1 and HuR on translation and turnover of Ccnd1 mRNA. (**A**) Map of part of the 3′-UTR of rat Ccnd1 mRNA showing the relevant miRNA target sites flanking RBP binding motifs in the AU-rich region. (**B**) Time profiles (Western blots) of total AUF1 (top), HuR (middle) or GAPDH (bottom) during serum stimulation of myoblasts. The multiple bands in the top panel represent AUF1 isoforms (see figure 3). (**C**) Time profiles of Ccnd1 mRNA-bound AUF1 (top) or HuR (bottom) during serum stimulation of myoblasts. Ccnd1 mRNA was detected by R-CLIP as a 253-bp RT-PCR product. The lower band present in all lanes probably represents primer dimers. (**D**) Quantification of total AUF1 protein (panel B) and Ccnd1 mRNA bound to AUF1 (panel C). The dashed line shows expression of the Ccnd1 protein in normal or scrambled sequence transfected cells (Ghosh and Adhya 2016; see also figure 1G, top panel, and figure 4, top panel). (**E**) siRNA-mediated KD of AUF1 or HuR. Western blots of total protein from cells transfected with scr, anti-AUF1 or anti-HuR siRNAs or a combination of both, probed with the anti-AUF1 (top) or anti-HuR (bottom) antibody. (**F**) Quantification of Ccnd1 mRNA in AUF1 KD (filled bars) or HuR KD cells (open bars) at indicated times of serum stimulation by real-time RT-PCR. Fold change at a specific time point is the level of mRNA in KD cells compared with that in the scr control at the same point. (**G**) Onset of Ccnd1 translation in control, AUF1 KD or HuR KD cells.

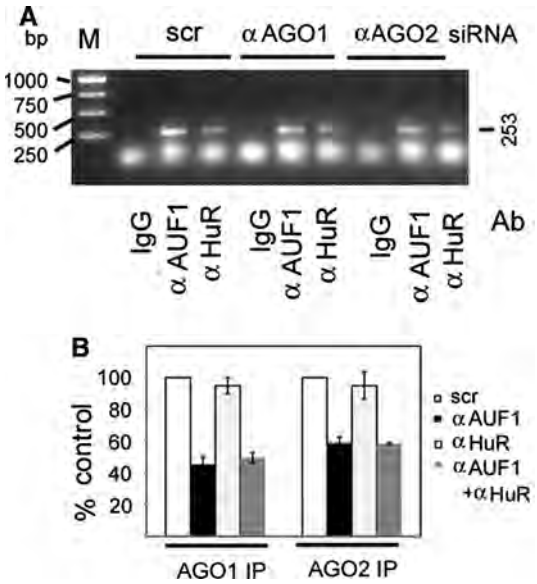

Figure 2. Relationship between AGO and RBP binding to Ccnd1 mRNA. (**A**) Cells were transfected with scr, anti-AGO1 or anti-AGO2 siRNAs, immunoprecipitated with control IgG, anti-AUF1 or anti-HuR antibody, and the RBPs at 4 h of serum stimulation were analysed by RT-PCR. The 253-bp Ccnd1 band is indicated. (**B**) Cellular RBPs from normal, AUF1 KD, HuR KD or AUF1–HuR double KD cells at 4 h of serum stimulation were crosslinked and immunoprecipitated with the anti-AGO1 or anti-AGO2 antibody, and the bound Ccnd1 mRNA was quantified by real-time PCR. Percent control is the amount of bound Ccnd1 mRNA in KD cells relative to that in control cells.

total level of let-7 or miR-1 (figure 3B, compare input lanes); (2) a significant fraction of the total miR-1 or let-7 was bound to either AGO1 or AGO2 in control cells and (3) binding of either miRNA was reduced to undetectable levels in AUF1-depleted cells (figure 3B). Therefore, AUF1 promotes loading of let-7 or miR-1 on to AGO1 as well as AGO2. The ∼50% of the mRNA–AGO1/2 complex in AUF1-KD cells (figure 2B) is therefore unlikely to contain miRNA, and may represent unloaded AGO bound to mRNA at nontarget sites.

3.2 *HuR positively regulates translation independent of miRNA*

Within the AU-rich region of the Ccnd1 mRNA 3′-UTR there are several high-scoring HuR binding motifs (figure 1A). Serum stimulation of L6 myoblasts resulted in a steady increase in the intracellular HuR concentration (figure 1B). In parallel, the level of the HuR–Ccnd1 mRNA complex increased (figure 1C). KD of HuR in myoblasts resulted in reduction of the endogenous HuR level by 66% (figure 1E). Upon serum stimulation of the HuR KD cells, there was no significant alteration in the Ccnd1 mRNA level compared with controls (figure 1F). However, there was a delay in TOT from 6 to 7 h (figure 1G). Thus, HuR positively regulates translation of Ccnd1 mRNA.

The level of the HuR–mRNA complex was not altered in AGO1- or AGO-2 KD cells (figure 2A), indicating that, as in the case of AUF1, the binding of HuR occurred independent of AGO. Additionally, in HuR-KD cells there was no significant change in the amount of Ccnd1 mRNA bound to either AGO1 or AGO2 compared with the control (figure 2B). In cells doubly deficient for HuR and AUF1, the amount of AGO1- or AGO2-bound Ccnd1 mRNA was identical to that in AUF1-deficient cells alone (figure 2B), indicating that HuR does not functionally compensate for the lack of AUF1 by, e.g. binding to the AUF1 sites on mRNA, as postulated elsewhere (Lal *et al*. 2004). Thus, the binding of HuR and AGO1/2 to Ccnd1 mRNA appear to be independent of each other.

KD of HuR resulted in a delay of Ccnd1 translation onset from 6 to 7 h (figures 1G and 4). In normal cells, the level of let-7 declines between 5 and 7 h, with the TOT at 6 h (Ghosh and Adhya 2016). Antagomir-mediated KD of let-7, on the other hand, caused an advancement of TOT from 6 to 5 h (figure 4), as shown earlier (Ghosh and Adhya 2016). One possible explanation for the effect of HuR is that it downregulates let-7, so that KD of HuR leads to overexpression of let-7 and translation delay. However, the TOT of 7 h in HuR-depleted cells was unaffected further by KD of let-7 (figure 4), indicating that in this system HuR acts independently of let-7 to expedite translation onset.

4. Discussion

Our results show that AUF1 and HuR have distinct effects on the level and translatability of Ccnd1 mRNA. While AUF1 downregulates both of these properties, HuR has no effect on the mRNA level but upregulates translation. Importantly, our time course studies with synchronized cells show that these effects are transient in nature, and result in subtle changes in TOT that would not be detectable in asynchronous cultures. Thus, both RBPs, as well as miRNAs such as let-7, effectively act as translation timers in cycling myoblasts.

KD of AUF1 affected the level of Ccnd1 mRNA at early but not at late times of serum stimulation (figure 1F). We have shown the effect of siRNA treatment for a total of 28 h (24 h of serum starvation followed by 4 h of serum stimulation) on AUF1 and HuR (figure 1E). The low AUF1 and HuR levels are maintained in the KD cells during the time frame of the experiment (4–7 h; data not shown). It is theoretically possible that the lack of stimulation of the Ccnd1 mRNA level in AUF1 KD cells at late times is due to restoration of AUF1 caused by degradation

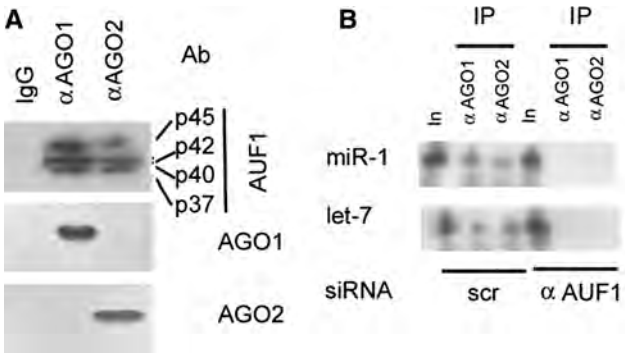

Figure 3. Role of AUF1 in miRNA loading. (**A**) IPs of AGO1 or AGO2 from 4 h lysates was western blotted and probed with the indicated antibodies. The four cross-reacting AUF1 isoforms p37, p40, p42 and p45 are indicated. (**B**) RNA in IPs of AGO1 or AGO2 from cells transfected with scr or anti-AUF1 siRNA at 4 h of serum stimulation was Northern blotted and probed with anti-let-7 or anti-miR-1 oligonucleotide.

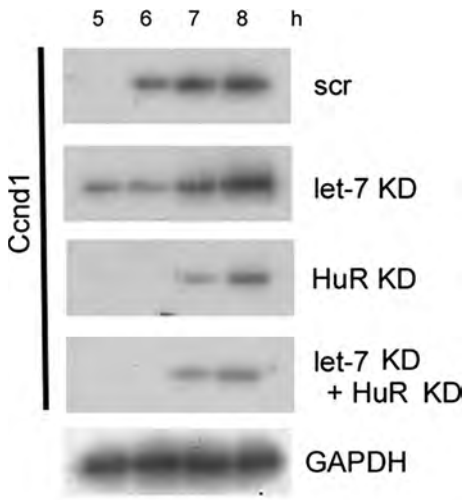

Figure 4. HuR positively regulates Ccnd1 translation independent of let-7. Ccnd1 protein expression at different times of serum stimulation in cells transfected with scr, anti-let-7 LNA, anti-HuR siRNA or a combination of both.

of the siRNA between 5 and 7 h, but this would not make any difference to the result, because AUF1 acts through the miR-1–AGO1 complex to destabilize the mRNA, and miR-1 decays by 5 h, as we have shown previously (Ghosh and Adhya 2016). Thus the transient effect of AUF1 on Ccnd1 mRNA is actually a reflection of the transient nature of the functional AUF1–AGO1–miR-1 ternary complex on the mRNA.

During the passage of myoblasts through the G1 phase, the intracellular concentrations of the negative regulators AUF1 (figure 1) and several miRNAs (Ghosh and Adhya 2016) decline at characteristic rates, while the level of the positive regulator HuR increases (figure 1). These levels correlate with the concentration of the corresponding complexes with mRNA (figure 1), but not with the extent or timing of protein expression. Rather, the onset of translation occurs when the concentration of the negative regulator (let-7 or AUF1), or of the corresponding RBP–mRNA complex, falls below ∼30% of the initial concentration, in this particular case, at 6 h of serum stimulation (figure 1D; Ghosh and Adhya 2016). This suggests the presence of a threshold concentration below which the mRNA is activated through titration of the regulator by nontarget sites, as observed elsewhere for miRNA-mediated silencing (Mukherji *et al*. 2011). In the case of AUF1 or let-7 KD cells, which have been exposed to siRNA for 24 h prior to serum addition, the concentration of the regulator is maintained at a constant level below 30% from the beginning to the end of the observation window (3–8 h; data not shown), but expression is advanced by only an hour in each case (figures 1 and 4). This can be attributed to a second rate limiting step in translation activation, in this case, the derepression of the 5′-cap through phosphorylation of 4EBP1, which starts at 5 h (Ghosh and Adhya 2016).

An emerging question arises with regard to the relationship, if any, between RBPs and miRNAs targeting the same mRNA. It has been recently observed that AUF1 associates indirectly (through RNA) with AGO2, interacts directly with AU-rich miRNAs such as let-7 and promotes miRNA loading on AGO2 (Yoon *et al*. 2015). Our results confirm and extend these observations: thus, AUF1 can interact with and load miRNAs on both AGO isoforms 1 and 2 (figure 3). However, a significant amount of mRNA (∼50%) is bound to AGO1/2 in AUF1-deficient cells, in which miRNA loading is abolished (figure 2). This suggests the presence of nontarget, non-miRNA loaded AGO bound to mRNA. Random binding of AGO to mRNA could represent an early step in target search by unloaded AGO, followed by miRNA loading and stabilization at the cognate site through miRNA–target annealing.

We observed that AUF1 interacts with AGO2 (figure 3), and promotes let-7 loading (figure 3). Moreover, KD of AUF1 (figure 1), or of AGO2 (Ghosh and Adhya 2016), or of let-7 (figure 4; Ghosh and Adhya 2016) has the same effect on TOT of Ccnd1, i.e. advancement of TOT from 6 to 5 h. Thus, it can be reasonably concluded that the Ccnd1 translation switch is composed of AUF1, let-7 and AGO2. A functional AUF1–AGO2–let-7 complex has been observed on mRNAs encoding pyruvate dehydrogenase phosphatase subunit 2 (PDP2) and RNA polymerase II subunit D (POLR2D) in human embryonic kidney cells (Yoon *et al*. 2015). Our studies additionally show that a second complex, AUF1–AGO1–miR-1, which regulates mRNA turnover. The fact that the AUF1 interaction region in the Ccnd1 3′-UTR is flanked by miR-1 and let-7 target sites (figure 1) enables the operation of both these switches by the same RBP.

HuR is generally regarded as an mRNA stabilizing protein, and there is evidence in other systems that it either

antagonizes (Kundu *et al*. 2012) or facilitates (Kim *et al*. 2009) miRNA-mediated silencing. We find, however, that HuR acts independently of miRNA and has no significant effect on Ccnd1 mRNA levels, or on the level of the AGO–mRNA complex, but specifically affects translation (figures 1 and 2). The effect of HuR depletion on TOT of Ccnd1 (postponement) was in the opposite direction to that of AUF1 depletion (advancement) (figure 1). Further, depletion of the AUF1–AGO2–let-7 translation switch through KD of let-7 failed to advance TOT in HuR-depleted cells (figure 4), indicating that the HuR switch acts independently of the AUF1 switch. A specific effect of HuR on p53 translation has been observed (Mazan-Mamczarz *et al*. 2003). Thus, the effect of HuR, as well as of AUF1, is context-specific, and may be related to facilitation or competition between the different regulators on the same mRNA. The mechanism of translational facilitation by HuR remains unknown, but could be due to displacement of a 3′-UTR bound repressor by HuR or recruitment of translation initiation factors at the 5′-end of the circularized translation template. The apparent complexity of HuR action awaits resolution.

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