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Ornithine Decarboxylase mRNA is Stabilized in an mTORC1-dependent Manner in Ras-transformed Cells

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Ornithine decarboxylase mRNA is stabilized in an mTORC1- dependent manner in Ras-transformed cells

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Abstract:

Upon *ras* activation, ornithine decarboxylase (ODC) is markedly induced, and numerous studies suggest that ODC expression is controlled by Ras effector pathways. ODC is therefore a potential target in the treatment and prevention of Ras-driven tumors. We compared ODC mRNA translation profiles and stability in normal and Ras12V-transformed rat intestinal epithelial (RIE-1) cells. While translation initiation of ODC increased modestly in Ras12V cells, ODC RNA was stabilized 8-fold. Treatment with the specific mTORC1 inhibitor rapamycin or siRNA knockdown of mTOR destabilized the ODC message, but rapamycin had only a minor effect on ODC translation initiation. Inhibition of mTORC1 also reduced the association of the mRNA binding protein HuR with the ODC transcript. We have shown previously that HuR binding to the ODC 3'UTR results in significant stabilization of the ODC mRNA, which contains several AU- rich regions within its 3'UTR that may act as regulatory sequences. Analysis of ODC 3'UTR deletion constructs suggests that *cis*-acting elements between bases 1969 and 2141 of the ODC mRNA act to stabilize the ODC transcript. These experiments thus define a novel mechanism of ODC synthesis control. Regulation of ODC mRNA decay could be an important means of limiting polyamine accumulation and subsequent tumor development.

Keywords: ornithine decarboxylase, mRNA stability, translation initiation, mTOR, rapamycin, HuR

INTRODUCTION

Increased activity of ornithine decarboxylase (ODC), the first biosynthetic enzyme of the polyamine pathway, has been demonstrated in a variety of human malignancies, including prostate cancer, squamous cell carcinoma of the skin and colon cancer (reviewed in [1]). Intracellular levels of ODC are tightly regulated, and multiple means of ODC dysregulation can occur in response to oncogenic stimuli. We have used *in vitro* and *in vivo* models of Ras activation to establish that ODC activity is regulated by and necessary for Ras-dependent cellular transformation, as well as transformation brought about by the Ras effectors MEK and eIF4E [2–5]. Activation of ODC transcription and protein synthesis is dependent on pathways downstream of Raf/MEK/ERK and PI3K/mTOR in both fibroblast and epithelial models [3, 6]. The cooperation of pathways controlled by Raf and PI3K/mTOR is necessary for complete Ras transformation of several types of epithelial cells (reviewed in [7]). Since most solid

tumors are epithelial in origin, understanding how ODC synthesis is controlled by these pathways is crucial in defining the role of ODC in maintaining a transformed phenotype.

Cap-dependent translational regulation of ODC through its 5'-untranslated region (5'UTR) is well-established, and ODC activity and translation are induced in eIF4E-overexpressing fibroblasts (4E-P2 cells) [2, 8]. However, our studies in rat intestinal epithelial cells (RIE-1 cells) described here suggest an alternate post-transcriptional regulatory mechanism for ODC protein synthesis. In this system, ODC synthesis is regulated primarily by changes in the levels of ODC RNA associated with polysomes, rather than changes in translation initiation. The mechanism of this regulation is a marked stabilization of the ODC mRNA in Ras12V-transformed RIE-1 cells (Ras12V cells) compared to their nontransformed parental controls, which appears to be regulated at least in part by pathways downstream of mTOR Complex 1 (mTORC1). Although the primary function of mTORC1 is in controlling the availability of eIF4E for translation initiation (reviewed in [9]), several studies show that TOR inhibition results in RNA stabilization. In *S. Cerevisiae* inhibition of TORC1 using the specific inhibitor rapamycin induced destabilization of multiple mRNAs, suggesting that TORC1 functions also involve regulation of mRNA turnover [10, 11]. In mammalian systems, rapamycin treatment of mouse embryo fibroblasts increased the degradation of mRNAs corresponding to Cyclin D1 and c-Myc in an Akt-dependent manner [12], while treatment of breast cancer MDA-MB-231 cells with rapamycin resulted in destabilization of IL-8 mRNA [13].

Regulation of mRNA stability is recognized to play a pivotal role in controlling gene expression. Sequences defined as adenylate- and uridylate-rich elements (AREs), which are classified based on the number and context of the sequence 5'-AUUUA-3', are present within the 3'UTRs of many proto-oncogene, transcription factor and cytokine mRNAs (reviewed in [14, 15]), and can act as determinants of mRNA stability. The mouse, rat and human ODC 3'UTR sequences, each of which is between 600–700 bases in length, have several potential AREs within approximately 300 bases the stop codon. A number of regulatory proteins are known to interact with ARE sequences. These proteins not only control transcript decay, but can also influence translational efficiency, or cause the bound RNA transcript to move to

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a processing body (P-body) for storage [16]. We have shown recently that the ubiquitous member of the ELAV protein family HuR associates with ODC mRNA in transformed cells and causes the ODC transcript to be stabilized [17]. Our results described here suggest that changes in ODC mRNA stability are mediated by *cis*-acting elements within the ODC 3'UTR and pathways downstream of mTORC1 play a role in this regulation at least in part by controlling the association of HuR with the ODC transcript.

EXPERIMENTAL

Cell culture and inhibitors

RIE-1 cells (a kind gift of Dr. Kenneth Brown, University of Cambridge, UK) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 μg/ml penicillin and streptomycin. Ras12V cells were generated from RIE-1 cells as described previously [6]. Sources for inhibitors were as follows: PD98059 was purchased from EMB4Biosceinces (Rockland, MA). CGP57380 was purchased from Tocris Bioscience (Ellisville, MO). Rapamycin was obtained from the Developmental Therapeutics Program of the National Cancer Institute.

Western blot analysis

Cells were plated and treated with inhibitors in DMSO as described in the figure legends, then extracted in RIPA buffer (EMB4Bioscience). Total cellular protein (20μg) was used for analysis. Membranes were probed using a purified rabbit polyclonal antibody raised against a 6Xhis-Tagged ODC protein as described previously [3], or a mouse monoclonal antibody against HuR (Santa Cruz Biotech, Santa Cruz, CA). Other antibodies used were as follows: GAPDH, 4EBP1, S6K1 and ERK1/2 polyclonal antibodies (Cell Signaling Technology, Danvers, MA); Actin and Lamin B polyclonal antibodies (Santa Cruz Biotech). To prepare cytoplasmic and nuclear extracts, cells were fractionated using the Pierce Fractionation Kit as per the manufacturer's instructions (Pierce, Rockford, IL).

Generation of pGL3-ODC3'UTR constructs

The full-length construct was made by PCR amplification of the 735 base ODC 3'UTR from Ras12V cells and ligation into the pGL3-

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control expression vector (Promega, Madison, WI). Orientation of the 3'UTR was confirmed by sequencing. Deletion mutants of the ODC 3'UTR were constructed by PCR using the same template and antisense primers designed to produce deletions from the distal end of the 3'UTR.

Immunoprecipitation analysis of translation initiation factors and Methyl-7-GTP sepharose chromatography

Ras12V cells (7×10^5 cells/plate) and RIE-1 cells (9×10^5 cells/plate) were plated on 10cm plates, allowed to adhere overnight and extracted 24h later. For inhibitor treatments Ras12V cells were treated for 2 h with either DMSO or 100 nM rapamycin after 24 h of growth. Cell extracts were prepared as described previously [18].

Polysome analysis

Polysome analysis was carried out as described previously [19] using 9×10^5 RIE-1 or 7×10^5 Ras12V cells per 10 cm plate. Analysis was performed after 24 h of growth. Sucrose gradients (20–47%) were centrifuged at 34000 rpm for 5 h using a SW41 rotor (Beckman Coulter, Brea, CA). Absorbance was followed at 254 nm. A total of 11 fractions were collected every 30 sec. For each fraction equal volumes of RNA were analyzed by Northern blot hybridization. Integrity of RNA was ensured by staining formaldehyde agarose gels with Ethidium bromide.

Synthesis of radiolabeled cDNA probes

cDNA probes were prepared by radiolabeling 100ng DNA with [32-P]-dCTP using the Rad prime DNA kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A full-length ODC probe was prepared from pGEM-ODC [3]. To prepare probes complementary to eEF1A and Cyclophilin, RNA from Ras12V cells was reverse transcribed and amplified by PCR.

RNA stability assay

Cells were cultured on 10 cm plates similar to polysome analysis. After 24 h of growth cells were treated with 5μg/ml Actinomycin D (Sigma-Aldrich, St. Louis, MO) and RNA was isolated at defined time points using the RNAqueous kit (Ambion, Austin, TX).

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Northern analysis was carried out using 20 μg total RNA and radiolabeled cDNA probes synthesized for ODC and Cyclophilin as described above. Half-life of ODC RNA was determined by curve fit analysis. For Ras12V cells an approximate half-life was determined by extrapolation. Stability of pGL3-ODC3'UTR and mutant transcripts were compared by Northern blot analysis using a [32-P]-labelled firefly luciferase probe following transfection into RIE-1 and Ras12V cells.

siRNA experiments

Previously validated rat mTOR siRNA and scrambled control oligonucleotides [20] were synthesized, annealed *in vitro* and transfected using oligofectamine (Invitrogen) at 80 nM final concentration into Ras12V cells. At 48 h after transfection, Actinomycin D was added to the cells and stability of the ODC RNA was measured as described above. Extent of mTORC1 knockdown was assessed by measuring levels of hyperphosphorylated 4EBP1 by Western blot.

Biotin-labeled RNA protein-binding assays

A synthetic ODC transcript was generated by isolating total RNA from Ras12V cells, then using reverse transcriptase to produce cDNA. The cDNA was used as a template for PCR amplification of the full length 3'UTR of ODC. The 5' primer sequence began with the T7 RNA polymerase promoter sequence (T7): CCAAGCTTCTAATACGACTCACTATAGGGAGA to allow further amplification. The ODC 3'UTR was amplified and PCR products were purified and used as templates to transcribe biotinylated RNA using T7 RNA polymerase in the presence of biotinylated CTP [21]. The GAPDH 3'UTR was amplified in the same manner for use as a negative control. The resulting biotinylated products were used in RNA pull-down assays essentially as described [22]. Western blot analysis was used to examine the association of HuR with the pull-down materials. For analysis of HuR binding to the DEL02 truncation mutant, biotinylated probes were synthesized and analyzed as described previously [17]. Mutation of the AUUUA sequence to GGGUA was by site-directed mutagenesis using the Quikchange Mutagenesis Kit as per the manufacturer's instructions (Stratagene, La Jolla, CA).

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Ribonucleoprotein immunoprecipitation (RNP IP)

For immunoprecipitation of endogenous RNA-protein binding complexes, 2000 μg of cytoplasmic lysate was incubated for 2 h at room temperature with 50% (v/v) suspension of Protein A-Sepharose beads (Sigma-Aldrich) that had been pre-coated with 30 μg of either mouse IgG (Invitrogen) or HuR antibodies as described [23]. The immunoprecipitated material was analyzed by washing the beads with NT-2 buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, and 0.05% Nonidet P-40), and further incubated with 0.5 mg/ ml Proteinase K, NT-2 buffer, and 0.1% SDS for 15 min at 55°C. RNA was precipitated using phenol-chloroform extraction in the presence of glycoblue (Qiagen, Valencia, CA). RNA from the immumoprecipitation was then reverse transcribed to detect the presence of ODC mRNA.

RESULTS

Stabilization of ODC mRNA upon Ras transformation of RIE-1 cells

RIE-1 cells and their Ras-transformed counterparts are a valuable model in which to study the regulation of cellular polyamines [22, 24, 25], The studies described here use RIE-1 cells stably expressing a constitutively active Ras12V mutant (Ras12V cells), which results in about a 25-fold induction in ODC activity, and empty vector parental controls [6]. To determine whether the increase in ODC activity observed could be explained by changes in ODC translation efficiency in response to Ras activation, polysome profiles were performed (Figure 1A). In addition to a slight shift in ODC RNA from lighter to heavier polysome fractions in Ras12V cells, which one would expect with increased translation initiation, we observed an increase in the amount of total polysome-associated RNA in the Ras12V cells in comparison to the control RIE-1 cells (Figure 1A). By normalizing the amount of ODC RNA in each fraction to the relatively constant levels of Cyclophilin RNA associated with polysomes in both Ras12V and RIE-1 cells, we observed a 5-fold increase in the amount of ODC RNA associated with the polysomal fractions in the Ras12V cells (Figure 1A).

The increase in ODC RNA associated with polysomes may result from increased interaction with stabilizing RNA binding proteins (RBPs)

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or changes in its interaction with microRNAs (miRNAs). We have recently shown that the ODC transcript is stabilized by the RBP HuR in cells derived from skin carcinomas [17]. To determine whether changes in rate of mRNA decay could contribute to the dramatic ODC activity induction seen in Ras12V cells, we examined ODC RNA stability in both cell lines after exposure to Actinomycin D. The t1/2 for RIE-1 ODC RNA was 3.0 h (Figure 1B, C), while the t1/2 in Ras12V cells was calculated to be 23.7 h, indicating an 8-fold increase in stability (Figure 1B, 1C). Cyclophilin served as a control.

Inhibition of mTORC1 results in modest changes in ODC translation efficiency and a marked increase in ODC mRNA decay

We have shown that post-transcriptional regulation of ODC is dependent on mTORC1- controlled pathways in several experimental systems [3, 6]. Therefore, we were interested to determine whether treatment with rapamycin affected cap-dependent translation initiation of ODC in Ras12V cells. Treatment of Ras12V cells with rapamycin reduced ODC protein in a time-dependent manner to about 15% of protein levels found in untreated cells by 24 h (Figure 2A). This is in agreement with previous results showing inhibition of ODC enzyme activity by rapamycin in the same time frame [6].

Treatment with rapamycin for 2 h had only a modest effect on ODC translation efficiency (Figure 2B, 2C). On the other hand, the same treatment time inhibited translation of eEF1α by causing a shift in EF1α RNA from polysomal fractions to non-polysomal fractions (Figure 2B, 2C), confirming that rapamycin inhibited translation initiation of known targets in this time frame. Rapamycin treatment also increased the association of 4EBP1 with eIF4E as assayed by both IP analysis of eIF4E and by m7GTP sepahrose chromatography (Supplemental Figure 1). IP analysis revealed a decrease in the association of eIF4G with eIF4E as well (Supplemental Figure 1). These results together suggest that mTORC1 controls cap complex formation in Ras12V cells. Exposure of cells to rapamycin for 24 h also produced little change in ODC translation efficiency (Figure 2B, 2C), although this prolonged treatment caused both a dramatic decrease in ODC protein levels (Figure 2A) and an overall decrease in the levels of 40S, 60S, and 80S ribosomal subunits (Figure 2B, 2C).

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To determine whether mTOR-dependent pathways may be involved in ODC mRNA stability control, we examined the effect of rapamycin on ODC mRNA decay in Ras12V cells. Since treatment with rapamycin for either 2 h or 24 h had similar effects on ODC polysome profiles (Figure 2), we decided to mimic the constitutive down-regulation of mTOR-dependent signaling previously observed in RIE-1 cells [6] by treating the Ras12V cells with rapamycin for 24 h. In vehicle-treated cells, the ODC RNA was very stable, with a calculated half-life of over 15 h. In cells pre-treated with rapamycin (100 nM) the $t_{1/2}$ was calculated to be about 6 h (Figure 3A, 3B). Rapamycin treatment does not change the half- life of ODC mRNA in RIE-1 cells (data not shown), consistent with the already low mTOR activity in these cells [6].

To investigate the contribution of other Ras-effector pathways to control of ODC mRNA decay, we used inhibitors of MEK (PD98059, 50 μM) or the eIF4E kinase Mnk1 (CGP57380, 20 μM). We have shown previously that both MEK and Mnk1 contribute to ODC synthesis in Ras12V cells by enhancing internal ribosome entry site (IRES)-mediated translation [6], and MEK-dependent pathways control ODC transcription [3]. However, the results shown in Figure 3A and 3B suggest that neither pathway appears to play a major role in controlling ODC mRNA stability. To confirm the specificity of each inhibitor for its target, we measured activation of both mTORC1 and MEK signaling. Treatment with rapamycin completely inhibited phosphorylation of S6K1 and reduced hyperphosphorylation of 4EBP1, indicating inhibition of mTORC1-dependent pathways (Figure 3C). Activation of ERK is also seen in rapamycin-treated cells, consistent with previous results showing up-regulation of the MEK-ERK signaling cascade upon S6K1 inhibition [26]. On the other hand, inhibition of MEK with PD98059, while abolishing ERK phosphorylation, had no effect on mTORC1 activity (Figure 3C). Although ERK can phosphorylate and inhibit TSC2, leading to activation of mTOR, we have shown previously that the levels of activated Akt in Ras12V cells are very high [6]. These results suggest that TSC2 is phosphorylated primarily by Akt in these cells. CGP57380, which we previously showed to be a very effective inhibitor of Mnk1 in Ras12V cells [6], had no effect on either mTORC1 or ERK signaling cascades (Figure 3C).

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mTOR knockdown mimics the effect of rapamycin treatment on ODC mRNA decay

To confirm the effect of rapamycin on the destabilization of ODC mRNA, we knocked down mTOR using an siRNA approach. Reduction in mTOR by siRNA results in inhibition of both mTORC1- and mTORC2-dependent pathways, while mTORC2 is not sensitive to rapamycin in most (though not all) models of tumorigenesis [27, 28]. We estimated the extent of mTOR knockdown 48h after transfection by measuring the levels of both total and phosphorylated 4EBP1. When compared to 4EBP1 levels in parental RIE-1 cells, both total 4EBP1 and its hyperphosphorylated gamma form were clearly increased in Ras12V cells transfected with a scrambled siRNA, consistent with increased mTOR activity and protein synthesis in Ras12V cells compared to RIE-1 cells [6]. In contrast, hyperphosphorylated 4EBP1 was largely eliminated in Ras12V cells transfected with the mTOR siRNA (Figure 4A). This result suggests that mTORC1 activity is effectively inhibited in the mTOR siRNA-transfected cells.

To determine ODC message stability in the presence of mTOR knockdown, Actinomycin D was added and Northern analysis was performed using Ras12V cells transfected with either scrambled siRNA or mTOR siRNA. These results show that knockdown of mTOR destabilizes the ODC transcript compared to a scrambled siRNA that does not knock down mTOR (Figure 4B). Quantitation of the mRNA showed about a 15 h half-life in cells treated with a scrambled siRNA, and a 7 h half-life in cells exposed to an siRNA directed to mTOR (Figure 4C). These results are very similar to those obtained with rapamycin treatment (Figure 3) and confirm that pathways dependent on mTORC1 regulate the decay of the ODC mRNA in Ras12V cells.

Endogenous HuR associates with the ODC 3'UTR in Ras12V cells in a rapamycin- dependent manner

We have shown previously using keratinocyte-derived cell lines that the RBP HuR associates with the ODC 3'UTR and stabilizes the ODC message in carcinoma cells but not in non-transformed cells [17]. To confirm that those results are not limited to the skin, we first performed an experiment designed to determine the intracellular localization of HuR in unstimulated parental and Ras12V-transformed

RIE-1 cells (Figure 5A). While HuR was predominantly nuclear in both cell lines, there was significant accumulation of HuR in the cytoplasm as well. Quantitation of bands revealed about a 2-fold increase in cytoplasmic HuR in Ras12V cells compared to their non-transformed counterparts (Figure 5A, 5B).

These results differ from those reported previously in keratinocyte-derived cells, where HuR was exclusively nuclear in normal keratinocytes, but present in both the nucleus and cytoplasm of carcinoma cells [17].

We assessed the intracellular association of ODC mRNA with HuR in parental RIE-1 cells and their Ras-transformed counterparts using RNP IP assays, which allow the association between HuR and its target mRNAs to be maintained. Interestingly, almost no ODC mRNA was bound to HuR in lysates from RIE-1 cells, in spite of the presence of HuR in the cytoplasm. On the other hand, a strong association between HuR and ODC was observed in Ras12V cells (Figure 5B), similar to our results in skin carcinoma cells [17]. The lack of association in RIE-1 cells was not due to the absence of ODC mRNA from RIE-1 lysates. Indeed, ODC message is clearly present in both cell lines (Figure 5B).

Ras12V cells were next treated with 100 nM rapamycin for 1 h, 8 h, or 24 h, then harvested and analyzed as described above. This concentration of rapamycin blocks phosphorylation of both of the mTORC1 targets 4EBP1 and S6-kinase in Ras12V cells ([6] and Figure 3). We observed a time-dependent decrease in the amount of ODC message present in the HuR immunoprecipitate in the presence of rapamycin (Figure 5C), suggesting that HuR interaction with the ODC mRNA is mediated in an mTORC1-dependent manner. Further, the time course of reduction in HuR association with the ODC message corresponds to the change in ODC activity in rapamycin-treated cells shown in Figure 1. There was no measurable change in either total HuR or the ratio of nuclear to cytoplasmic HuR in Ras12V cells, even after 24 h exposure to rapamycin (Figure 5D). This is consistent with the observation that RIE-1 cells also contain HuR in both the nucleus and cytoplasm (Figure 5A), in spite of very low levels of mTOR activity.

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Identification of cis-acting stability elements in the ODC 3'UTR

The regulation of mRNA decay involves interaction of *trans*-acting factors with *cis*-acting sequences that confer stability to a transcript. Many *cis*-acting sequences contain AREs within them, and the ODC 3'UTRs from mouse, rat and human contain several sequences that would be identified as AREs within the first 300 bases after the termination codon (Supplemental Figure 2). Our previous results suggest that elements within this ODC 3'UTR proximal end are responsible for stabilizing the ODC message by interacting with HuR [17].

In order to identify *cis*-acting elements within the ODC 3'UTR, we constructed several expression vectors in which the firefly luciferase open reading frame is linked to the full length mouse ODC 3'UTR sequence (pGL3-ODC3'UTR), or deletion mutants corresponding to those used previously for HuR binding studies [17]. Stability of pGL3- ODC3'UTR and deletion transcripts was compared following transfection. The half-life of RNA derived from a luciferase reporter construct containing the full-length ODC 3'UTR (725 bases) was approximately 22h in Ras12V cells but only 7.5h in RIE-1 cells (Figure 6A). This is in good agreement with the half-life of the endogenous ODC transcript (Figure 1B). Deletions in the 3'UTR from the distal end showed that deleting the last 381 bases to create a 344 base long 3'UTR (DEL01) had no effect on mRNA decay in Ras12V cells (Figure 6A). However, when an additional 172 bases were deleted (DEL02, 172 bases long), the ODC mRNA t1/2 decreased about 3-fold, and was not changed substantially by further deletion to create a 53 base long 3'UTR (DEL03). Steady state mRNA levels of transcripts derived from all transfected vectors were not significantly different (data not shown), suggesting that transfection efficiencies for the four vectors were similar.

Mouse, rat and human ODC 3'UTR sequences all contain one canonical 5'-AUUUA-3' pentamer between bases 1857 and 1861 of the ODC transcript (Supplemental Figure 2, [17]). To determine if the presence of this pentamer influences HuR binding, we used biotinylated synthetic transcripts corresponding to our DEL02 construct containing either the wild-type AUUUA sequence, or a mutation of the

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putative HuR binding site to GGGUA. The results show not only that HuR binds to the GGGUA-containing transcript, but it appears to associate more strongly when compared to the wild-type transcript (Figure 6B).

DISCUSSION

Previous experiments from our lab and others have demonstrated cap-dependent translational regulation of ODC in a fibroblast model. Our data reported here suggest that in response to Ras transformation of RIE-1 cells, rates of ODC translation initiation increase only slightly. However, ODC mRNA is markedly stabilized, and is available to be recruited onto polysomes, resulting in increased ODC synthesis. The clear increase in ODC mRNA stability observed is a potentially important component of the mechanism responsible for increasing ODC protein and activity in a variety of tumorigenic models. There are a number of reports in the literature demonstrating that both global mRNA stability and the stability of specific transcripts are functionally linked to translation, particularly during stress responses [29–32]. While the ODC transcript is much more stable in Ras12V cells compared to RIE-1 cells, the polysome distributions of endogenous ODC mRNA suggest that it is quite poorly translated in both cell lines, with most of the ODC mRNA present in early fractions corresponding to polysomes containing a small number of ribosomes. This supports the idea that increased mRNA stability in Ras12V cells plays an important role in the accumulation of ODC protein in these cells. Indeed, the 2–3 fold increase in transcription shown previously in Ras12V cells [6], coupled with the calculated 8-fold increase in RNA stability shown here, would potentially account for most of the 20–25-fold increase in ODC enzyme activity that we have observed in RIE-1 cells transformed by Ras [6].

Although the mechanisms controlling mRNA decay are only beginning to be understood, treatment with rapamycin results in destabilization of several target RNAs in mouse embryo fibroblasts and breast cancer cell lines [12, 13], suggesting an alternative method of post- transcriptional control by mTORC1-dependent pathways. In agreement with this, our results show that inhibition of mTORC1 in Ras12V cells destabilizes ODC mRNA while only modestly changing translation initiation of the ODC transcript. Further, treatment of Ras12V cells with rapamycin reduces association of the stabilizing RBP

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HuR with the ODC transcript. Taken together with our previous results, which show a functional link between HuR binding and ODC mRNA stability [17], these data suggest that activation of mTORC1 may help regulate ODC transcript decay by directing the association of this stabilizing factor with *cis*-acting elements within the ODC sequence.

Our data suggest that elements between base 1969 and base 2141 of the ODC sequence are necessary to stabilize the ODC transcript. This includes the sequence that we identified previously as containing multiple HuR binding sites [17]. In this context, it is interesting to note that in the *in vitro* assay used for these experiments HuR is capable of binding to the DEL02 deletion mutant, yet this transcript is significantly less stable than the full length ODC 3'UTR when transfected into Ras12V cells, suggesting that additional binding proteins are acting in concert or competition with HuR to control the decay of the ODC mRNA. This interpretation is supported by our results showing that endogenous HuR present in the RIE-1 cytoplasmic lysate does not interact with the ODC 3'UTR. Although previous results suggest that the sub-cellular localization of HuR responds to changes in activity of AMP-activated protein kinase, which is upstream of mTOR [33, 34], we see no change in HuR localization in rapamycin-treated Ras12V cells even though association of HuR with the ODC transcript is decreased. This further supports the idea of other *trans* factors competing with HuR for binding to the ODC message.

HuR binding to the ODC 3'UTR is not abolished by mutation of the consensus ARE sequence 5'-AUUUA-3' to GGGUA, suggesting this is not an important site of interaction. While AUUUA is classically defined as a canonical ARE, other more heterogeneous AU- and U-rich sequences have also been shown to bind RBPs, including HuR [15], and consensus sequences controlling mRNA stability have not yet been strictly defined. It is also well known that the ODC 5'UTR has a profound affect on ODC translation [35–39], and a contribution of the 5'UTR to transcript stability cannot be ruled out from these experiments. However, the RNA half-life of our 3'UTR full-length reporter plasmid is quite similar to the half-life of the endogenous ODC transcript in both cell lines.

Neither the increased stability of ODC RNA and its recruitment into polysomes in Ras12V cells, nor the destabilization of the ODC

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message in the presence of rapamycin, are fully reflected in the total RNA levels. One hypothesis to explain these observations is that in RIE-1 cells much of the ODC RNA is stored in cytoplasmic P-bodies, aggregates of translationally repressed mRNA that are associated with decapping and deadenylation machinery. Upon activation of Ras-dependent pathways, ODC RNA is released from these bodies to be recruited into polysomes in an mTOR-dependent manner. This notion is plausible, as it has been shown that P-bodies are dynamic foci that are able to assemble and disassemble based upon changes in cellular conditions [40]. Other studies have suggested that HuR may interact with miRNAs through an interdependent mechanism that causes release of specific transcripts from P-bodies and their entry onto polysomes in response to various types of cellular stresses [41]. Although application of a genome-wide target prediction algorithm [42] predicts that several miRNAs may interact with the ODC 3'UTR with favorable energies, a role for miRNAs in ODC post-transcriptional regulation has not been defined.

In conclusion, we have demonstrated that, analogous to other growth regulatory proteins such as Cyclin D1, VEGF and c-Myc, the synthesis of ODC can be regulated at the levels of both mRNA translation and mRNA stability [23, 43–45]. Changes in ODC mRNA stability are at least partly dependent on the activity of mTORC1, which we show for the first time can not only regulate initiation of ODC translation but can also control ODC mRNA decay. Binding of the stabilizing RBP HuR to the ODC transcript is reduced when mTORC1 is inhibited, suggesting that mTOR-dependent pathways regulate this association, and possibly that of additional RBPs. Thus, these experiments extend our previous findings [17] to further define the mechanism by which ODC synthesis is controlled, and identify additional targets that influence intracellular ODC activity.

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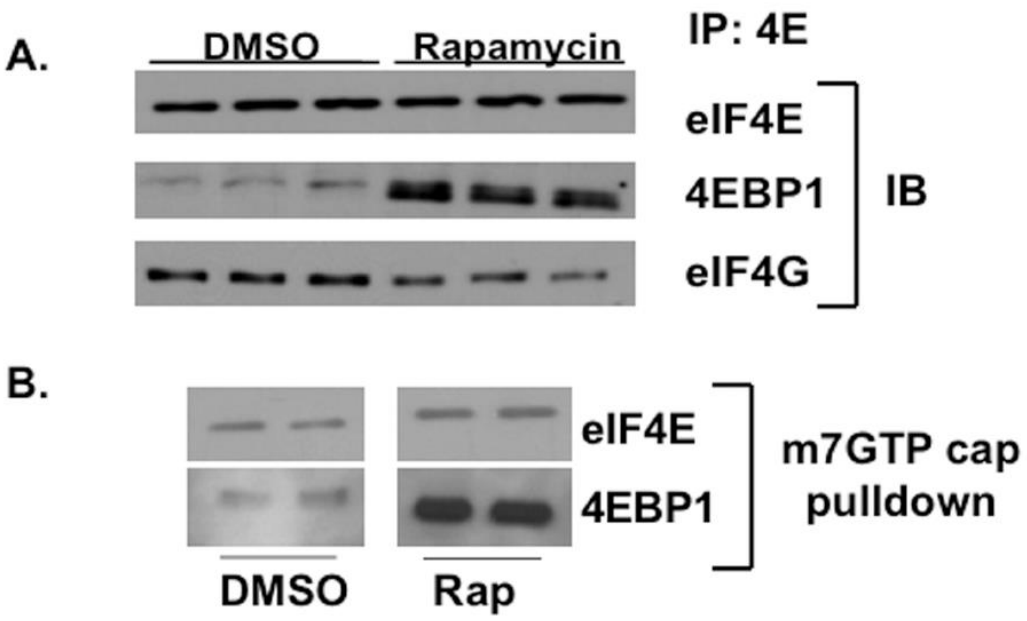
Supplementary Material

Supplemental figure 1



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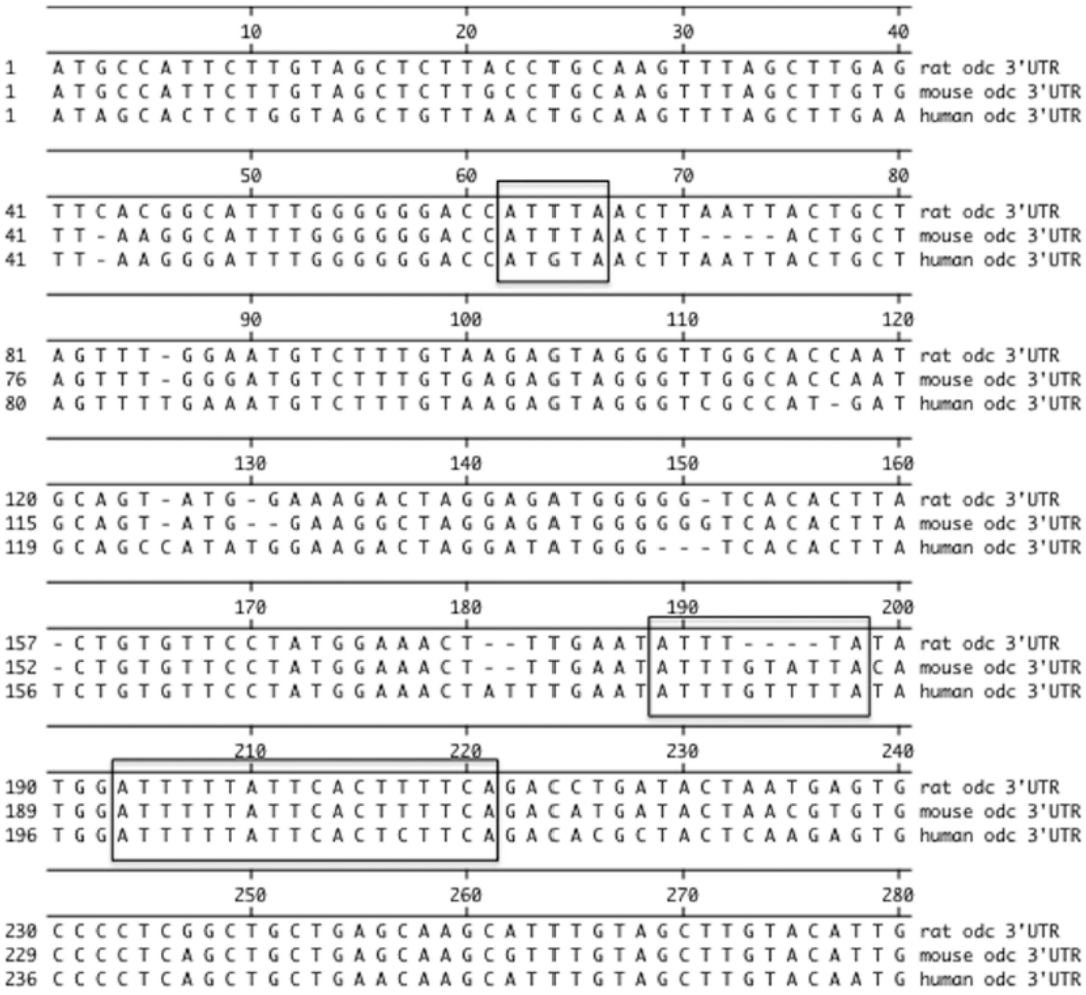
Supplemental figure 2



Supplemental figure legend

Supplemental Figure 1.

Rapamycin treatment inhibits cap complex formation in Ras12V cells. Plates grown in parallel with those shown in Figure 2A were harvested and subjected to either IP analysis or m7GTP sepharose chromatography. (**A**) Using IP of total eIF4E, the amount of 4EBP1 or eIF4G associated with eIF4E was determined by Western blot analysis. IP analysis using mouse IgG served as a negative control. (**B**) m7GTP sepharose chromatography was performed and the amount of eIF4E or 4EBP1 associated with the m7GTP cap was analyzed by immunoprecipitation.

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Supplemental Figure 2.

Alignment of the proximal mouse, rat and human ODC 3'UTRs. Potential AREs, either 5'-AUUUA-3' pentamers or extended AU-rich regions, are shown in boxes.

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Abbreviations used

ARE	adenylate- and uridylate-rich element
eEF1A	eukaryotic elongation factor 1A
eIF4E	eukaryotic initiation factor 4E
ERK	extracellular signal-regulated kinase
MEK	mitogen-activated protein kinase kinase
miRNA	microRNA
RNP IP	ribonucleoprotein immunoprecipitation
mTOR	mammalian target of Rapamycin
ODC	ornithine decarboxylase
P-body	processing body
PI3K	phosphoinositide 3-kinase
RBP	RNA binding protein
siRNA	small interfering RNA
UTR	untranslated region

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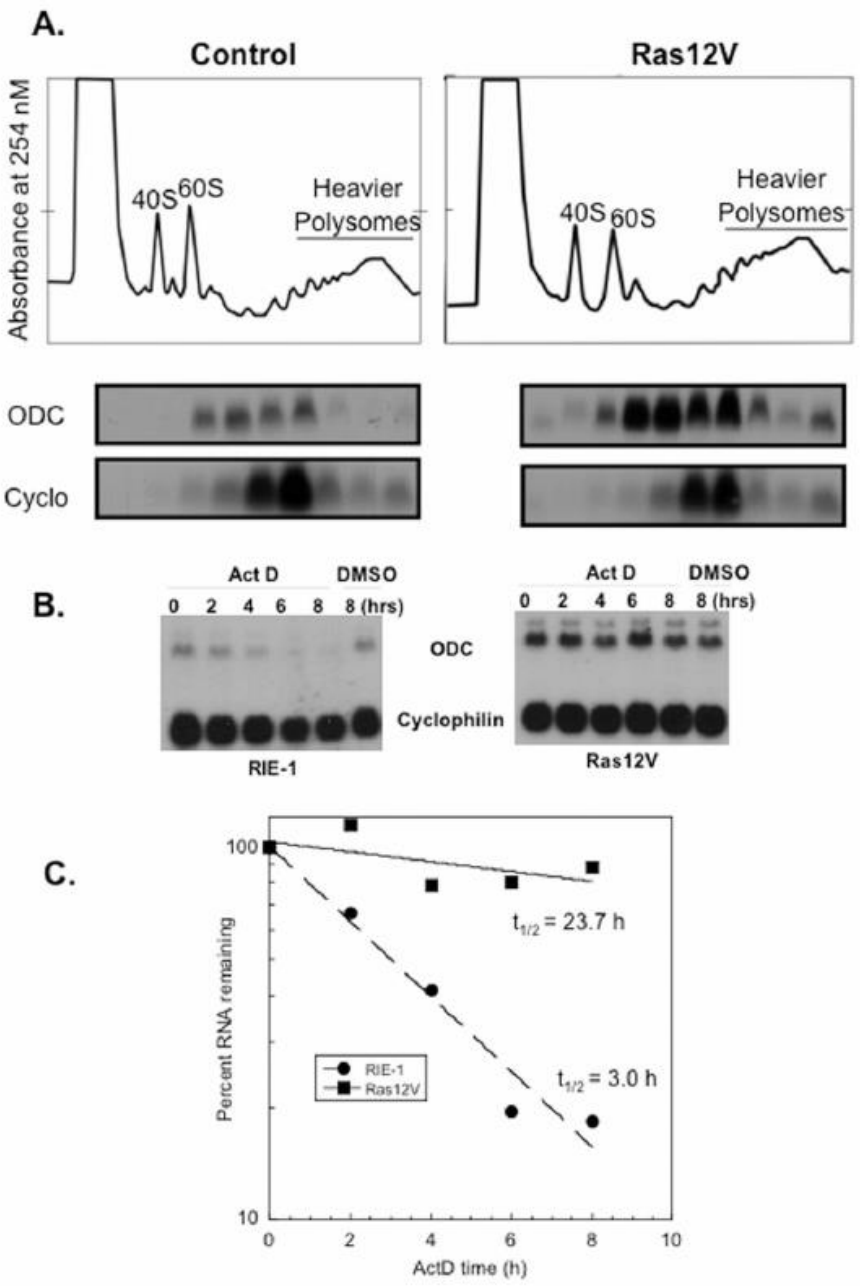

Figure 1. Analysis of ODC RNA translation and stability

(A) Polysomal analysis of ODC RNA was carried out in both RIE-1 and Ras12V cells as described in the Experimental section. Equal volumes of RNA were loaded per lane and Northern blots were probed with radiolabeled cDNA complementary to full length ODC, then stripped and reprobed with cDNA complementary to cyclophilin (Cyclo). The experiment was performed in duplicate with reproducible results. **(B)** To measure RNA stability, cells were treated with either 5 μg/ml Actinomycin D (Act D) or DMSO vehicle. RNA was isolated immediately after addition of Act D at 0 h and thereafter at 2, 4, 6 and 8 h and at 8 h for DMSO-treated cells. For Northern analysis, 20μg of total RNA was loaded per lane and probed for ODC and cyclophilin simultaneously. The experiment was performed in duplicate with reproducible results. **(C)** Quantitation of the results presented in (B). The absolute values for absorbance corresponding to time=0 were 5.21 for Ras12V cells and 2.10 for RIE-1 cells (arbitrary units).

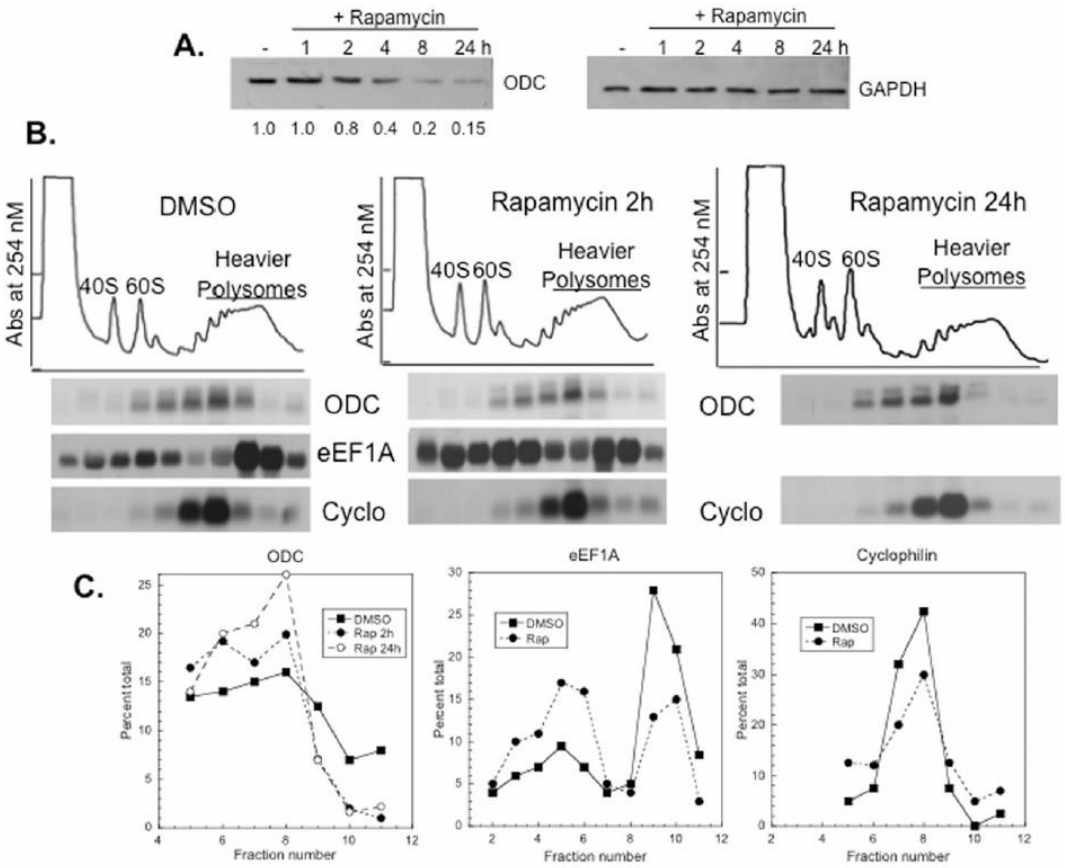

Figure 2. Effect of mTOR inhibition on ODC translation initiation and protein levels in Ras12V cells

(A) Cells were treated with either DMSO or 100 nM rapamycin (Rap) for the times indicated and harvested for Western blots of ODC protein as described in the Experimental section. Quantitation of bands was performed by densitometry and expressed as a fraction of the band corresponding to untreated cells (−). GAPDH

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served as a control. **(B)** Cells were treated with either DMSO or 100 nM rapamycin for the times indicated and polysome analysis was carried out as described in the Experimental section. Northern blots were simultaneously probed for ODC and Cyclophilin, then were stripped and reprobed for eEF1A. The experiment was performed in duplicate with reproducible results. **(C)** Quantitation of the polysome profiles shown in (B).

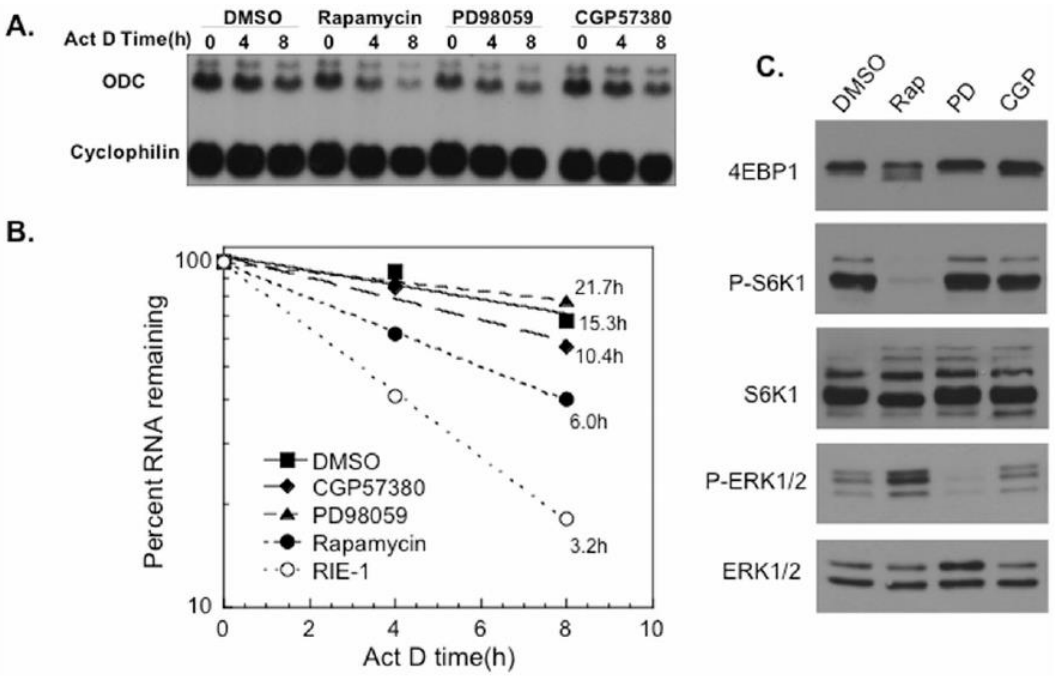

Figure 3. Effect of Ras downstream signaling inhibition on ODC mRNA decay

(A) Stability of ODC RNA was analyzed as described in Figure 1, except 100 nM rapamycin, 50 μM PD98059, 20 μM CG57380 or vehicle DMSO were added 24 h before the addition of Actinomycin D (ActD). RNA was collected immediately after addition of ActD at 0 h, and 4 h and 8 h later. **(B)** Quantitation of the results in (A). The absolute values for absorbance corresponding to time=0 were as follows: DMSO=2.11; Rapamycin=1.84; PD98059=1.49; CGP57380=2.00 (arbitrary units). **(C)** Cells were treated with inhibitors at the concentrations described in (A), harvested after 24 h in RIPA buffer, and analyzed for 4EBP1 and P-S6K1 as a measure of mTORC1 activity and P-ERK1/2 as a measure of MEK activity. P-S6K1 and P-ERK1/2 blots were then stripped and re-probed for total protein. All experiments were performed in duplicate or triplicate with reproducible results.

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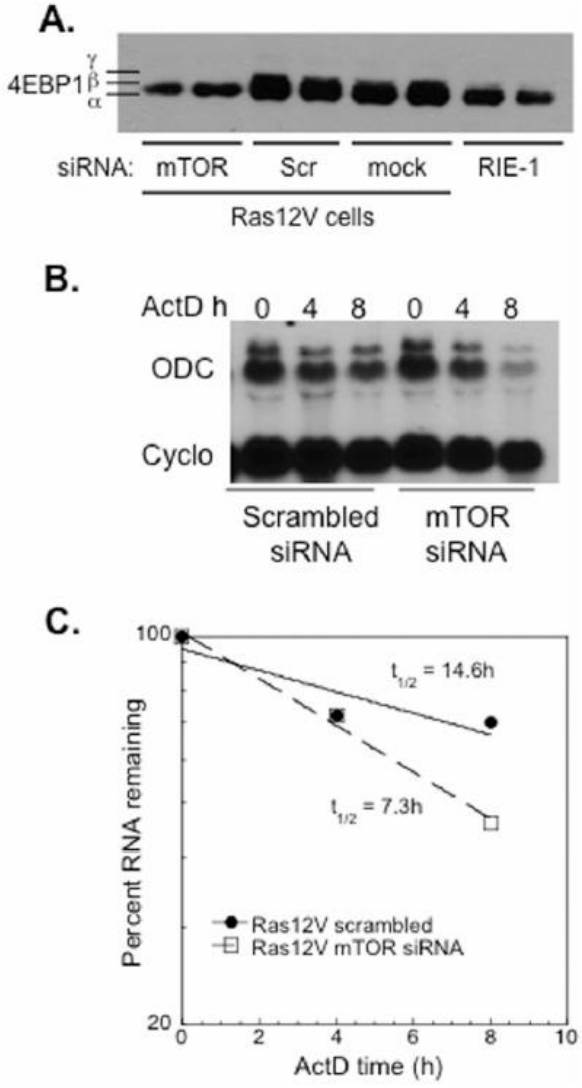

Figure 4. Regulation of ODC RNA stability by mTOR-dependent pathways

(A) Knockdown of mTOR with siRNA was performed as described in the Experimental section. Extent of mTORC1 knockdown was assessed by measuring the levels of hyperphosphorylated 4EBP1 using Western blot analysis and comparing to parental RIE-1 cells. **(B)** ODC mRNA stability in the presence of siRNA directed against mTOR or a scrambled control siRNA was measured by Northern blot analysis.
(C) Quantitation of the results in (B). All experiments were performed in duplicate with reproducible results.

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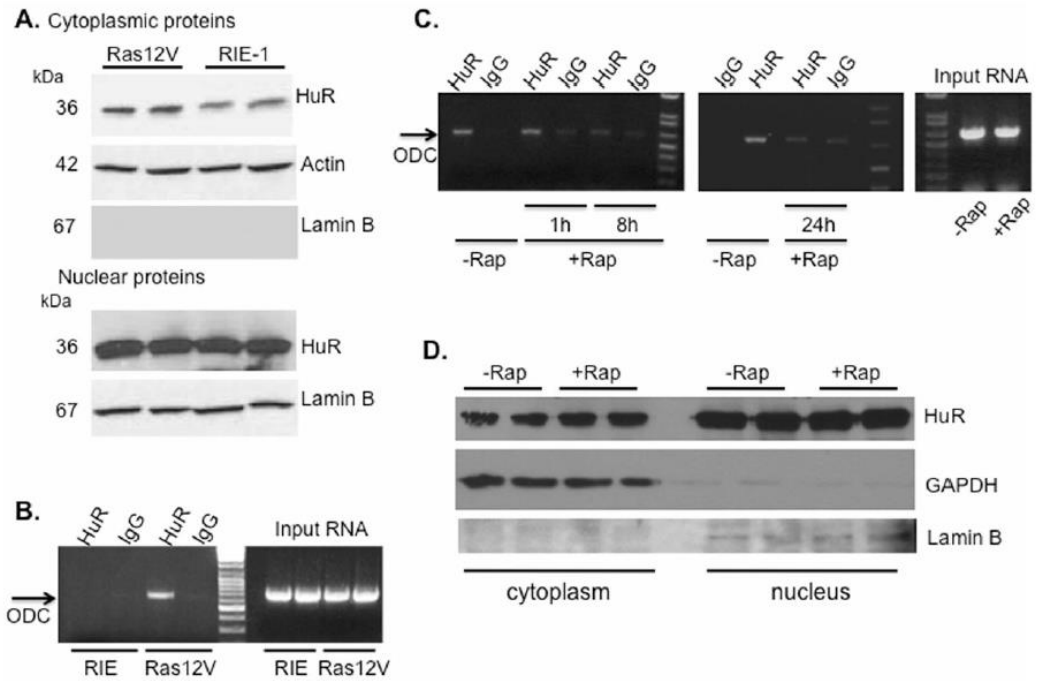

Figure 5. The mRNA binding protein HuR associates with the ODC transcript in Ras12V cells in a rapamycin-dependent manner

(A) RIE-1 and Ras12V cells were fractionated into cytoplasmic and nuclear extracts and HuR localization was detected by Western blot analysis. Actin was used as a cytoplasmic marker and Lamin B was used as a nuclear marker. Band quantitation estimated a 2-fold increase in cytoplasmic HuR in Ras12V cells. **(B)** Cytoplasmic lysates from Ras12V and RIE-1 cells were used for RNP IP analysis with anti-HuR or nonspecific mouse IgG. RNA in the IP material was extracted with phenol-chloroform, used in a PCR reaction, and visualized on a 2% agarose gel. The levels of ODC mRNA were assessed by PCR using primers specific for the coding region. The same primers were used to estimate the amount of input RNA using cDNA derived from each cell line as a template. The experiment was conducted in triplicate with reproducible results. **(C)** The RNP IP experiment described in was repeated using Ras12V cells that had been pre-treated for 1 h, 8 h, or 24 h with 100 nM rapamycin or with DMSO vehicle for 24 h. Input RNA was assayed in cells treated with rapamycin or DMSO for 24 h. The experiment was conducted in triplicate with reproducible results. **(D)** Nuclear/cytoplasmic fractionation was performed as described in (A) using Ras12V cells treated with rapamycin or DMSO vehicle for 24 h. GAPDH served as cytoplasmic marker and Lamin B served as nuclear marker.

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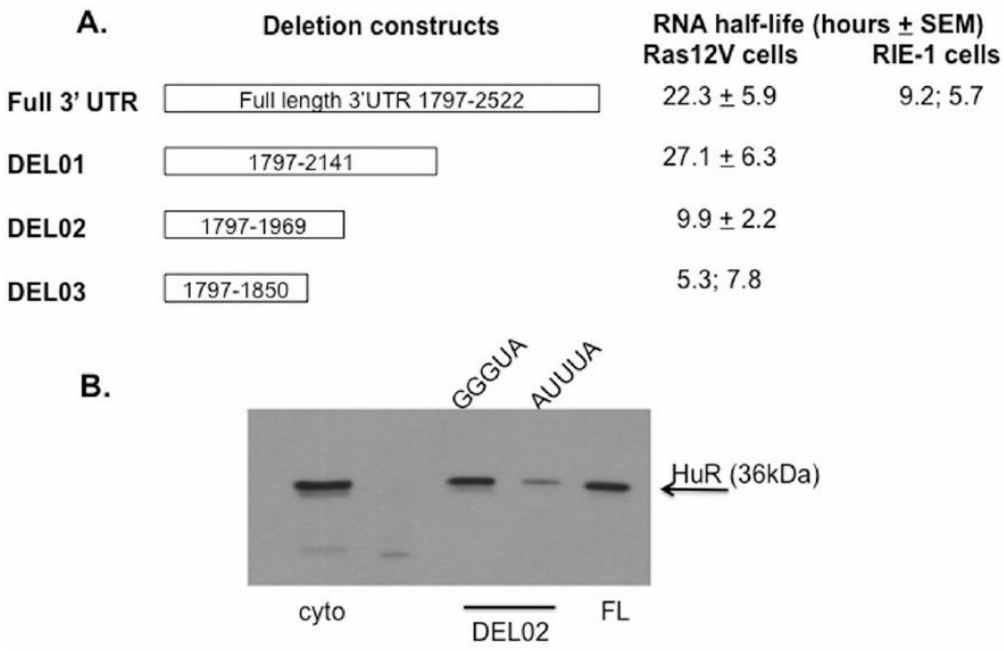

Figure 6. The ODC 3′UTR contains a *cis*-acting RNA stability element

(A) Cells were grown for 48 h then transfected with luciferase expression plasmids containing either the full length ODC 3′UTR or one of three 3′UTR deletion constructs, as shown in the schematic representation. RNA half-life was measured 48 h later as described in Figure 1. Results are from 2–5 independent experiments. Where two experiments were performed, results from both experiments are shown, others are the means ± SEM. **(B)** Ras12V cytoplasmic lysate (120 μg) was incubated with 15 μl of biotin-labeled synthetic probes corresponding to the DEL02 sequence containing either the wild-type (AUUUA) or mutated (GGGUA) ARE and Western blot for HuR was performed. The full length 3′UTR (FL) was used as a positive control. Cytoplasmic lysate (cyto) is also shown. This experiment was done in duplicate with similar results.

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