



Published in final edited form as:

Biochem J. 2008 January 15; 409(2): 389–398. doi:10.1042/BJ20070860.

Polyamines modulate the subcellular localization of RNA-binding protein HuR through AMP-activated protein kinase-regulated phosphorylation and acetylation of importin $\alpha 1$

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Abstract

Polyamines are required for maintenance of intestinal epithelial integrity, and a decrease in cellular polyamines increases the cytoplasmic levels of RNA-binding protein HuR stabilizing p53 and nucleophosmin mRNAs, thus inhibiting IEC (intestinal epithelial cell) proliferation. The AMPK (AMP-activated protein kinase), an enzyme involved in responding to metabolic stress, was recently found to be implicated in regulating the nuclear import of HuR. Here, we provide evidence showing that polyamines modulate subcellular localization of HuR through AMPK-regulated phosphorylation and acetylation of Imp $\alpha 1$ (importin $\alpha 1$) in IECs. Decreased levels of cellular polyamines as a result of inhibiting ODC (ornithine decarboxylase) with DFMO (D,L- α -difluoromethylornithine) repressed AMPK activity and reduced Imp $\alpha 1$ levels, whereas increased levels of polyamines as a result of ODC overexpression induced both AMPK and Imp $\alpha 1$ levels. AMPK activation by overexpression of the *AMPK* gene increased Imp $\alpha 1$ but reduced the cytoplasmic levels of HuR in control and polyamine-deficient cells. IECs overexpressing wild-type Imp $\alpha 1$ exhibited a decrease in cytoplasmic HuR abundance, while cells overexpressing Imp $\alpha 1$ proteins bearing K22R (lacking acetylation site), S105A (lacking phosphorylation site) or K22R/S105A (lacking both sites) mutations displayed increased levels of cytoplasmic HuR. Ectopic expression of these Imp $\alpha 1$ mutants also prevented the increased levels of cytoplasmic HuR following polyamine depletion. These results indicate that polyamine-mediated AMPK activation triggers HuR nuclear import through phosphorylation and acetylation of Imp $\alpha 1$ in IECs and

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that polyamine depletion increases cytoplasmic levels of HuR as a result of inactivation of the AMPK-driven Imp α 1 pathway.

Keywords

AMP-activated protein kinase (AMPK); growth arrest; HuR; importin α 1; intestinal epithelium; ornithine decarboxylase

INTRODUCTION

The mammalian intestinal mucosa has the most rapid turnover rate of any tissue in the body and undergoes a continual renewal process, characterized by active proliferation of stem cells localized near the base of the crypts, progression of these cells up the crypt–villus axis with cessation of proliferation and subsequent differentiation and apoptosis [1,2]. This dynamic and constant state of intestinal epithelial renewal is highly regulated and critically controlled by numerous factors, including cellular polyamines [3,4]. The natural polyamines spermidine and spermine and their precursor putrescine are organic cations found in all eukaryotic cells [5], and the regulation of cellular polyamines has been recognized for many years as a central convergence point for the multiple signalling pathways driving different IEC (intestinal epithelial cell) functions [3,6]. It has been shown that IEC proliferation in the intestinal mucosa depends on the supply of polyamines to the dividing cells in the crypts [3,4] and that decreasing cellular polyamines inhibits cell renewal *in vivo* [3,4,9] as well as *in vitro* [7–11]. Although few specific functions of polyamines at the molecular level have been identified to date, an increasing body of evidence indicates that polyamines regulate IEC proliferation by virtue of their ability to modulate expression of various growth-related genes [3,5,12–15]. Polyamines positively regulate the transcription of growth-promoting genes such as *c-fos*, *c-jun* and *c-myc* [7,12,13], but negatively modulate expression of growth-inhibiting genes, including *p53* [9], *p21* [14,15], the gene coding for NPM (nucleophosmin) [11], *junD* [8,43] and the genes coding for TGF β (transforming growth factor β) receptors [6,16] through modifications at the post-transcriptional level.

Although stress stimuli trigger alterations in gene expression patterns via transcriptional and post-transcriptional mechanisms, the important contribution of post-transcriptional events, such as mRNA processing, transport, turnover and translation, has been becoming increasingly recognized recently [17,18]. Among these post-transcriptional processes, mRNA stability and translation are tightly regulated by the association of the mRNAs with specific RBPs (RNA-binding proteins) and are emerging as highly effective means of controlling the profiles of expressed gene products [17,19,20]. The best-characterized *cis*-acting elements regulating mRNA stability and translation are U-rich elements and AREs (AU-rich elements) that are usually located in the 3′-UTRs (3′-untranslated regions) of many labile mRNAs [17]. Likewise, different RBPs selectively recognize and bind to AREs of target mRNAs, leading to either an increase or decrease in the transcript half-life and translation rate [21,22]. HuR is a ubiquitously expressed member of the Hu/ELAV (embryonic lethal abnormal vision in *Drosophila melanogaster*) family of RBPs, which also comprises the neuron-specific proteins HuC, HuD and Hel-N1 [20]. HuR is predominantly

nuclear in unstimulated cells, but it rapidly translocates to the cytoplasm in response to various stress stimuli and binds with great affinity and specificity to AREs in a variety of mRNAs that typically present one or several hits of a recently identified RNA motif [23–25]. Although the precise processes regulating HuR function remain to be fully understood, it is clear that HuR subcellular localization is intimately linked to its effects on target transcripts. We [3,26] have recently demonstrated that polyamines modulate the HuR shuttle between the nucleus and the cytoplasm in IECs and that depletion of cellular polyamines increases cytoplasmic levels of HuR-stabilizing p53 and NPM mRNAs, thus contributing to the inhibition of IEC proliferation. However, the exact mechanism by which polyamines regulate HuR subcellular distribution remains elusive.

The AMPK (AMP-activated protein kinase) is a cellular sensor of metabolic stress, by which cells in both mammals and lower organisms sense and respond to changes in energy status [27]. Mammalian AMPK is a heterotrimer of three subunits: one catalytic (α) and two regulatory (β and γ) subunits [28,29], and its activity is ubiquitous, although different isoforms of AMPK exhibit tissue-specific distribution and preferential subcellular localization [30,31]. Recently, several studies have revealed that AMPK is implicated in regulating the nuclear import of HuR and that decreased AMPK activity results in an elevation of cytoplasmic HuR levels; conversely, AMPK activation causes a reduction in cytoplasmic HuR [32–35]. Previous studies also have shown that *Imp α 1* (importin α 1), an adaptor protein, is a critical downstream target of AMPK and functions as a key mediator of AMPK-mediated HuR nuclear import [33–35]. Given that polyamines are absolutely required for IEC proliferation and their cellular levels are tightly regulated by the status of cell growth and stress stimulation [3] and given that AMPK-triggered reduction in cytoplasmic HuR levels profoundly affects the stability of target mRNAs [32], we sought to determine whether polyamines modulate subcellular localization of HuR by altering AMPK-regulated *Imp α 1* activity in IECs. The results presented in the current study demonstrate that decreased levels of cellular polyamines repressed AMPK expression, whereas increased polyamines induced AMPK levels. The present study further shows that activated AMPK following increased polyamines triggered HuR nuclear import through phosphorylation and acetylation of *Imp α 1* and that polyamine depletion increased cytoplasmic HuR levels as a result of inactivation of the AMPK-driven *Imp α 1* pathway.

MATERIALS AND METHODS

Chemicals and supplies

Disposable culture ware was purchased from Corning Glass Works (Corning, NY, U.S.A.). Tissue culture medium and dFBS [dialysed FBS (fetal bovine serum)] were purchased from Invitrogen (Carlsbad, CA, U.S.A.), and biochemicals were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Antibodies against HuR, β -tubulin and lamin B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and antibodies against pAMPK α (phosphorylated AMPK α), AMPK α and phospho-acetyl-CoA-carboxylase (Ser⁷⁹) were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). The monoclonal antibody against *Imp α 1* or ODC (ornithine decarboxylase) was from Sigma. DFMO (D,L- α -difluoromethylornithine) was purchased from Genzyme (Cambridge, MA, U.S.A.).

Cell culture

The IEC-6 cell line was purchased from the A.T.C.C. at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. [54]. IEC-6 cells originated from intestinal crypt cells as judged by morphological and immunological criteria. They are non-tumorigenic and retain the undifferentiated characteristics of intestinal crypt cells. Passages 15–20 were used in experiments, which exhibit a stable phenotype [8–10]. ODC-IEC cells (ODC-overexpressing IEC-6 cells) cells were developed as described in our previous publications [26,55] and expressed a more stable ODC variant with full enzyme activity [36]. Stock cells were maintained in T-150 flasks in DMEM (Dulbecco's modified Eagle's medium) supplemented with 5% (v/v) heat-inactivated FBS, 10 μg of insulin/ml and 50 μg /ml gentamicin. Flasks were incubated at 37°C in a humidified atmosphere of 90% air/10% CO₂.

Recombinant viral construction and infection

Adenoviral vectors were constructed by using the Adeno-X Expression System (Clontech, Mountain View, CA, U.S.A.) according to the protocol provided by the manufacturer. Briefly, the full-length cDNA of human AMPK α was cloned into the pShuttle by digesting with BamHI/HindIII and ligating the resultant fragments into the XbaI site of the pShuttle vector. AdAMPK α (pAdeno-AMPK α) was constructed by digesting the pShuttle construct with PI-SceI/I-CeuI and ligating the resultant fragment into the PI-SceI/I-CeuI sites of the pAdeno-X adenoviral vector. Recombinant adenoviral plasmids were packaged into infectious adenoviral particles by transfecting HEK-293 cells (human embryonic kidney cells) using Lipofectamine™ Plus reagent (Gibco-Bethesda Research Laboratory, Gaithersburg, MD, U.S.A.). The adenoviral particles were propagated in HEK-293 cells and purified by caesium chloride ultracentrifugation [14000 rev./min for 1 h in a type SA-600 rotor (Sorvall)]. Titres of the adenoviral stock were determined by standard plaque assay. Recombinant adenoviruses were screened for the expression of the introduced gene by Western-blot analysis using anti-AMPK α antibody. pAdeno-X, which was the recombinant replication-incompetent adenovirus carrying no AMPK α cDNA insert (Adnull), was grown and purified as described above and served as a control adenovirus. IEC-6 cells were infected with AdAMPK α or Adnull, and expression of AMPK α was assayed at 24 or 48 h after the infection.

Generation of stable Imp α 1-transfected IECs

The WT (wild-type)-Imp α 1 and the Imp α 1 proteins bearing either a lysine-to-arginine mutation on residue 22 [Imp α 1(K22R)] or a serine-to-alanine mutation on residue 105 [Imp α 1(S105A)] or both mutations [Imp α 1(S105A/K22R)] were generated using the QuikChange® site-directed mutagenesis kit (Stratagene) and were expressed in the plasmid pcDNA3.1/V5-His-TOPO (Invitrogen). All these constructs were verified by sequencing before being used for transfection. IEC-6 cells were transfected with either the WT-Imp α 1, various point-mutated Imp α 1 constructs or the control vector lacking Imp α 1 cDNA by using Lipofectamine™ 2000, and the transfected cells were selected by incubation with the selection medium containing G418 (600 μg /ml). Clones resistant to the selection medium

were isolated, cultured and screened for Imp α 1 expression by Western-blot analysis using a specific anti-His-tag antibody.

Preparation of cytoplasmic and nuclear proteins and Western-blot analysis

Cytoplasmic and nuclear proteins were prepared via the procedure described previously [11,26], and the protein contents in different preparations were measured using the Bradford [37] method. Cell samples, placed in SDS sample buffer [250 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol and 5% mercaptoethanol], were sonicated and then centrifuged (10000 g) at 4°C for 15 min. The supernatant from cell samples was boiled for 5 min and then subjected to SDS/PAGE (7.5% polyacrylamide) by the method of Laemmli [38]. After the transfer of protein on to nitrocellulose filters, the filters were incubated for 1 h in 5% (w/v) non-fat dry milk in PBS-T [1×PBS/Tween 20: 15 mM NaH₂PO₄, 80 mM Na₂HPO₄, 1.5 M NaCl, pH 7.5, and 0.5% (v/v) Tween 20]. Immunological evaluation was then performed for 1 h in 1% BSA/PBS-T buffer containing 1 μ g/ml of the specific antibody against HuR, AMPK α or Imp α 1 proteins. The filters were subsequently washed with 1×PBS-T and incubated for 1 h with the secondary antibody conjugated with peroxidase by protein cross-linking with 0.2% glutaraldehyde. After extensive washing with 1×PBS-T, the immunocomplexes on the filters were developed by the ECL® method according to the manufacturer's instructions (Amersham Biosciences, Arlington Heights, IL, U.S.A.).

Immunofluorescence staining

The immunofluorescence staining procedure was carried out according to the methods described previously [39,40]. After the monolayers of control and polyamine-deficient cells were fixed in 3.7% (v/v) formaldehyde in PBS and rehydrated, they were incubated with the primary antibody against AMPK α or Imp α 1 in the block buffer [5% (w/v) non-fat dried milk in 1% TBS-T (25 mM Tris base, 137 mM NaCl, 3 mM KCl and 0.1% (v/v) Tween 20), pH 7.5]] at a 1:300 dilution at 4°C overnight and then incubated with secondary antibody conjugated with Alexa Fluor® 594 (Molecular Probes, Eugene, OR, U.S.A.) for 2 h at room temperature (22°C). After rinsing three times, the slides were incubated with TO-PRO3 (Molecular Probes) at a concentration of 1 μ M for 10 min to stain the cell nuclei. Finally, the slides were washed, mounted and viewed through a Zeiss confocal microscope (model LSM410). Images were processed using Photoshop software (Adobe, San Jose, CA, U.S.A.).

Polyamine analysis

The cellular polyamines content was analysed by HPLC analysis as previously described [8,35]. Briefly, after 0.5 M HClO₄ was added, the cells were frozen at -80°C until ready for extraction, dansylation and HPLC analysis. The standard curve encompassed 0.31–10 μ M. Values that fell >25% below the curve were considered undetectable. The results are expressed as nanomoles of polyamines per milligram of protein.

Statistics

All data are expressed as means \pm S.E.M. for three or six dishes. Immunoblotting results and immunofluorescence staining were repeated three times. The significance of the difference

between means was determined by ANOVA. The level of significance was determined using Duncan's multiple-range test [41].

RESULTS

Reduced levels of AMPK and *Impa1* after polyamine depletion

Polyamine depletion increases the levels of cytoplasmic HuR, which in turn stabilizes p53 and NPM mRNAs in IECs [3,26], and increasing AMPK activity lowers cytoplasmic HuR levels by promoting *Impa1*-mediated nuclear import of HuR. Therefore we hypothesized that decreasing cellular polyamines might induce HuR cytoplasmic translocation by altering AMPK/*Impa1* signalling. As reported in our previous studies [8,15,42], exposure of IEC-6 cells to 5 mM DFMO for 4 and 6 days completely inhibited ODC enzyme activity and almost totally depleted cellular polyamines. The levels of putrescine and spermidine were undetectable on days 4 and 6 after treatment with DFMO, and spermine was decreased by ~60% (results not shown). The results presented in Figure 1(A) show that polyamine depletion by DFMO decreased the levels of total (by%), cytoplasmic (by ~60%) and nuclear (by ~50%) AMPK α and lowered its activity as indicated by decreased levels (by ~65% on day 4 and by ~95% on day 6 respectively) of pAMPK α at Thr¹⁷² (pT172AMPK α). The reduced AMPK α activity in polyamine-deficient cells was associated with a significant decrease in the levels of total (by ~60%), cytoplasmic (by ~70%), and nuclear (by ~25%) *Impa1*. Putrescine (10 μ M) given together with DFMO prevented the decreases in levels of AMPK α and *Impa1*. Addition of spermidine (5 μ M) had an effect equal to putrescine on AMPK α and *Impa1* when it was added to cultures containing DFMO (results not shown). To monitor the quality and abundance of the cytoplasmic and nuclear fractions, the levels of β -tubulin (a cytoplasmic protein) and lamin B (a nuclear protein) were also examined and served as loading controls. Assessment of these markers revealed that there was no contamination between cytoplasmic and nuclear fractions.

These changes in levels of AMPK α and *Impa1* after polyamine depletion were further studied by immunofluorescence staining. In control IEC-6 cells (Figure 1B*a*), AMPK α and *Impa1* immunoreactive signals were localized in both the cytoplasm and nucleus. Consistent with the Western blotting results, the levels of cytoplasmic and nuclear AMPK α and *Impa1* immunostainings decreased significantly in polyamine-deficient cells (Figure 1B*b*). Combined treatment with DFMO and putrescine prevented the decreased AMPK α and *Impa1* signals, rendering the subcellular staining patterns similar to those of untreated cells (Figure 1B*c*). The results presented in Figure 1(C) further show the time course of pT172AMPK α and *Impa1* levels after polyamine depletion. Significant decreases in levels of pT172AMPK α and *Impa1* were observed on day 3, while the maximal reduction of both AMPK α and *Impa1* occurred on day 4 after treatment with DFMO. In contrast, there were no significant changes in the levels of pT172AMPK α or *Impa1* between days 1 and 4 after plating in control cells (without DFMO treatment). These results indicate that decreasing cellular polyamines repressed AMPK α expression and decreased *Impa1* levels.

Increasing cellular polyamines by ODC overexpression increases the cytoplasmic levels of AMPK and Imp α 1

Given our previous findings that increasing cellular polyamines decreased the cytoplasmic levels of HuR in IEC-6 cells [26], we sought to examine if there were changes in the levels of AMPK α and Imp α 1 in stable ODC-IEC cells that were recently developed in our laboratory [15,55]. These ODC-IEC cells expressed high levels of ODC protein (Figure 2, top), exhibited greater than 50-fold higher ODC enzyme activity and had elevated cellular polyamines putrescine, spermidine and spermine (by 12-fold, 2-fold and 25% compared with control populations respectively). Increased levels of cellular polyamines by ODC overexpression induced the levels of total and cytoplasmic AMPK α , although there were no significant changes in the levels of nuclear AMPK α in ODC-IEC cells (Figure 2). On the other hand, ODC overexpression increased the cytoplasmic levels of Imp α 1 (Figure 2, middle) and correspondingly decreased its nuclear abundance (Figure 2, bottom); no net changes in whole-cell Imp α 1 levels (Figure 2, top) were seen in ODC-IEC cells compared with control cells (vector alone). The effect of ODC overexpression on the levels and subcellular distribution of AMPK α and Imp α 1 were not simply due to clonal variation, since two stable clones, ODC-IEC-C1 and ODC-IEC-C2, showed identical responses. These results indicate that increasing cellular polyamines not only increases AMPK α expression but also promotes the cytoplasmic accumulation of AMPK α and Imp α 1 in IECs.

AMPK activation increases Imp α 1 and decreases cytoplasmic HuR levels

To define the exact role of polyamine-modulated AMPK α in regulating HuR subcellular trafficking, the following two studies were carried out. First, we examined the effects of increasing AMPK α by treatment with its chemical activator antimycin A [27] on the cytoplasmic levels of HuR in IEC-6 cells. The results presented in Figure 3(A) show that exposure of control and polyamine-deficient cells (pretreatment with DFMO for 6 days) to 5 μ M antimycin A not only increased the levels of total and pAMPK α protein but it also stimulated its kinase activity as indicated by increased levels of pACC (phospho-acetyl-CoA carboxylase), which is a specific AMPK substrate, and its phosphorylation at Ser⁷⁹ is exclusively performed by active AMPK. Induced AMPK phosphorylation and its activity by antimycin A were associated with an increase in Imp α 1. Significant inductions of AMPK activation and Imp α 1 occurred by 4–6 h after administration of antimycin A. Treatment with antimycin A also reduced the cytoplasmic levels of HuR in control (Figure 3A, left) and polyamine-deficient cells (Figure 3A, right). The levels of cytoplasmic HuR in control cells decreased by ~60% at 4 h and by ~80% at 6 h after treatment with antimycin A, while the cytoplasmic levels of HuR in polyamine-deficient cells decreased by ~83% at 4 h and 95% at 6 h respectively.

Secondly, we examined changes in the levels of cytoplasmic HuR after overexpression of the AMPK α gene. An adenoviral vector containing the corresponding full-length human AMPK α cDNA under the control of the cytomegalovirus immediate early gene promoter (AdAMPK α) was constructed as described previously [34]. This delivery vector was chosen over other delivery methods because adenoviruses have been shown to infect a variety of cultured rat and human IECs with nearly 100% efficiency [10,15]. More than 95% of IEC-6 cells were positive by 24 h following infection with a control adenoviral vector

encoding GFP (green fluorescent protein; results not shown). As shown in Figure 3(B), AMPK α protein levels increased in cells infected with progressively higher viral loads. When AdAMPK α was used to infect at 10, 50 and 100 pfu (plaque-forming units)/cell, the levels of AMPK α protein increased by ~2-fold (controls) or 3-fold (polyamine-deficient cells) at 10 pfu, ~10-fold at 50 pfu and 15-fold at 100 pfu respectively. The increased levels of AMPK α protein after AdAMPK α infection were paralleled by significant increases in levels of pACC, indicating that this overexpressed AMPK α has sufficient kinase activity. An adenovirus that lacked an insert (Adnull) was used as a negative control and did not induce AMPK α levels (results not shown). Overexpression of the AMPK α gene increased Imp α 1 and decreased the cytoplasmic levels of HuR. When control cells were infected with different concentrations of AdAMPK α (Figure 3B, left), the whole-cell levels of Imp α 1 increased by ~2-fold at 10 pfu, 8-fold at 50 pfu and 11-fold at 100 pfu respectively. Conversely, the levels of cytoplasmic HuR decreased by ~60% at 50 pfu and 93% at 100 pfu after the infection. In polyamine-deficient cells (Figure 3B, right), infection with AdAMPK α decreased cytoplasmic HuR levels by ~47% at 10 pfu, ~75% at 50 pfu and ~91% at 100 pfu respectively. These findings indicate that increased AMPK α activity elevates Imp α 1 abundance and decreases the levels of cytoplasmic HuR in the presence or absence of cellular polyamines.

Polyamine depletion increases cytoplasmic HuR levels by inhibiting AMPK-regulated phosphorylation and acetylation of Imp α 1

To determine whether increased levels of cytoplasmic HuR following polyamine depletion result from inactivation of AMPK-regulated phosphorylation and acetylation of Imp α 1, stable WT and point-mutant Imp α 1-transfected IECs (mutated Imp α 1-IECs) were developed and characterized. His-tagged Imp α 1 constructs were prepared from pcDNA3.1/V5-His-TOPO to express either WT-Imp α 1 or Imp α 1 carrying specific amino acid mutations: Imp α 1 mutants lacking Lys²² [Imp α 1(K22R)], Ser¹⁰⁵ [Imp α 1(S105A)] alone or in combination [Imp α 1(K22R/S105A)] as described in our previous publication [34]. Previous studies showed that Ser¹⁰⁵ and Lys²² in Imp α 1 are necessary for its phosphorylation and acetylation respectively and that point mutations in these modification sites impair the functional activity of Imp α 1 [32,34]. As shown in Figure 4, four clones resistant to the selection medium containing 0.6 mg/ml of G418 expressed high levels of either WT- or mutated-Imp α 1 as indicated by an increase in levels of their His tag. Overexpression of WT-Imp α 1 did not change total HuR levels, but it significantly reduced cytoplasmic HuR levels and increased HuR nuclear abundance. In contrast, IECs overexpressing Imp α 1 bearing mutations at K22R (which abrogated acetylation at Lys²²), S105A (which abrogated phosphorylation at Ser¹⁰⁵) or K22R/S105A (which prevented both modifications) displayed a significant increase in the levels of cytoplasmic HuR, although there were no changes in whole-cell HuR levels.

Overexpression of Imp α 1 mutants, including Imp α 1(K22R), Imp α 1(S105A) and Imp α 1(K22R/S105A), prevented the AMPK α -induced nuclear import of HuR, as indicated by a decrease in cytoplasmic HuR levels in IECs. Ectopic expression of the AMPK α by infection with AdAMPK α decreased cytoplasmic HuR levels in both control cells (Figure 5, left) and cells expressing WT-Imp α 1 (Figure 5, middle), and had no effect on total HuR

levels. However, neither cytoplasmic HuR levels nor its whole-cell levels were changed after overexpression of AMPK α in various stable IEC clones ectopically expressing *Imp α 1* point mutants (Figure 5, right). There were no significant differences in the levels of cytoplasmic and total HuR between mutated *Imp α 1*-IECs infected with Adnull (lacking AMPK α cDNA) and those infected with AdAMPK α .

The results presented in Figure 6 further show that inhibition of phosphorylation and acetylation of *Imp α 1* by overexpression of its various mutants totally blocked the polyamine depletion-induced cytoplasmic translocation of HuR. In control IECs, depletion of cellular polyamines by treatment with DFMO for 6 days not only decreased AMPK α but also increased the cytoplasmic levels of HuR, which was completely prevented by putrescine given together with DFMO (Figure 6, left). Consistently, polyamine depletion also decreased the levels of AMPK α and increased cytoplasmic HuR levels in cells expressing WT-*Imp α 1* (Figure 6, middle). On the other hand, in IEC clones expressing mutant *Imp α 1* proteins, polyamine depletion by DFMO did not alter cytoplasmic HuR levels, although it significantly decreased the levels of total and cytoplasmic AMPK α (Figure 6, right). The levels of cytoplasmic HuR in the *Imp α 1* mutant IEC clones exposed to DFMO alone or DFMO plus putrescine were indistinguishable from those observed in control cells. The finding that overexpression of all *Imp α 1* mutants (K22R, S105A or both mutations) have similar inhibitory effects on the cytoplasmic translocation of HuR following polyamine depletion suggests that both phosphorylation and acetylation of *Imp α 1* through AMPK α contribute to the polyamine-modulated subcellular trafficking of HuR in IECs.

Based on the results presented here and our previous studies [26,34], we propose a model delineating the role of polyamines in the regulation of the subcellular localization of HuR in IECs (Figure 7). In this model, increased levels of cellular polyamines stimulate AMPK activity, in turn triggering HuR nuclear import through the activation of both phosphorylation (Ser¹⁰⁵) and acetylation (Lys²²) of *Imp α 1*, whereas polyamine depletion increases the cytoplasmic levels of HuR by inactivating the AMPK-mediated dual modifications of *Imp α 1*.

DISCUSSION

Increases in the levels of cellular polyamines enhance IEC proliferation, at least partially, by suppressing the expression of growth-inhibiting genes such as *p53* [9], *NPM* [11] and *junD* [8,43,44], whereas decreases in the levels of cellular polyamines activate the expression of these growth-inhibiting genes. Our previous studies [3,8,9] and others [5,14,45] further indicate that polyamines negatively regulate expression of these growth-inhibiting genes at the post-transcriptional level and that depletion of cellular polyamines stabilizes these mRNAs without affecting their transcription rates [6,8,9]. Induced levels of nuclear p53, NPM and JunD in polyamine-deficient cells are shown to interact directly with their downstream target genes [e.g. they increase p21 but repress CDK4 (cyclin-dependent kinase 4)] [8,11,44], leading to increased arrest in the G₁-phase of the cell cycle. Our recent studies [26] have further demonstrated that polyamine depletion increases HuR abundance in the cytoplasm, where it binds to the 3'-UTR of p53 and NPM, in turn controlling the stability of these mRNAs and influencing their protein levels. The silencing of HuR prevented the

stabilization of p53 and NPM mRNAs and hence lowered p53 and NPM protein expression in polyamine-deficient cells. In the present study, we provide new evidence showing that polyamines modulate the subcellular trafficking of HuR in IECs through AMPK-regulated phosphorylation and acetylation of *Imp α 1*, thereby advancing our understanding of the molecular functions of cellular polyamines.

The results reported here show that cellular polyamines critically influence AMPK expression and activity. Depletion of cellular polyamines by inhibiting ODC with DFMO decreased AMPK protein and repressed its activity, as indicated by a reduction in pT172AMPK α levels (Figure 1). Because decreases in AMPK expression and its activity in DFMO-treated cells were completely prevented by the addition of exogenous putrescine, these observed changes in AMPK are most likely related to polyamine depletion rather than to the non-specific effect of DFMO. Furthermore, increases in the levels of cellular polyamines by ectopic *ODC* overexpression induced AMPK levels, which was predominantly located in the cytoplasm (Figure 2). Although AMPK was discovered almost three decades ago, details of its regulation have only recently begun to be elucidated. In mammalian cells, AMPK lies at the foot of an activating signal transduction cascade, in which the AMPK α subunit is phosphorylated on Thr¹⁷² by an upstream kinase [AMPKK (AMPK kinase)] [46]. Although not yet defined in molecular terms, an AMPKK activity has been purified from liver extracts [47], and is co-immunoprecipitated by antibodies against the β -subunit of AMPK, suggesting that two kinases, AMPKK and AMPK, are normally associated in a complex. In addition to covalent modification, AMPK activity is also activated directly by elevation of AMP and repressed by high concentrations of ATP [27,46]. Although how polyamines modulate AMPK expression and activity remains an open question, it is plausible that these compounds influence multiple distinct signalling pathways leading to alterations of gene transcription [12,13], post-transcriptional events [3,8], protein phosphorylation [48], and protein trafficking [49,50], at least in part through AMPK-elicited processes. Given the pleiotropic effects of AMPK on the cell's metabolism, further efforts to elucidate the role of AMPK in the processes mediated by polyamines within the intestinal epithelium are warranted.

The results from the present study also show that polyamine-mediated AMPK modulates subcellular trafficking of HuR through a process involving *Imp α 1* in IECs. It has been reported that *Imp α 1* functions as an adaptor that associates with *Imp β* , which transports bound cargoes through the nuclear pore complex [51]. Recently, *Imp α 1* was identified as a key cytoplasmic HuR ligand mediating nuclear import of HuR [33–35]. In the present study, inhibition of AMPK expression levels and function by polyamine depletion decreased *Imp α 1* abundance (Figure 1), which was associated with an increase in the cytoplasmic accumulation of HuR but did not alter whole-cell HuR levels [3,26]. In contrast, increased AMPK by ODC overexpression induced *Imp α 1* expression and decreased the levels of cytoplasmic HuR, with a corresponding increase in nuclear HuR abundance [26]. Activation of AMPK by either treatment with antimycin A or ectopic overexpression of a constitutively active isoform of AMPK not only increased *Imp α 1* but also decreased cytoplasmic HuR levels regardless of the presence (controls) or absence (DFMO treatment for 6 days) of cellular polyamines (Figure 3), suggesting that decreased levels of *Imp α 1* and subsequently increased levels of cytoplasmic HuR result primarily from AMPK inactivation

following polyamine depletion. These findings are consistent with previous observations [33–35] that show that decreased AMPK activity led to an elevation in cytoplasmic HuR levels and that AMPK activation through interventions such as treatment with AICAR (5-amino-4-imidazolecarboxamide riboside) and ectopic expression of the *AMPK* gene results in a reduction of cytoplasmic HuR. The results presented in Figure 3(A) also show that treatment of IEC-6 cells with antimycin A significantly increased levels of total AMPK α protein regardless of the presence or absence of cellular polyamines. To our knowledge, this is the first report showing changes in total AMPK α protein after exposure to antimycin A. Consistent with the findings reported by others [27], treatment of IEC-6 cells with antimycin A also increased AMPK α phosphorylation. Although the mechanisms underlying the induction of AMPK α by antimycin A remain unknown, our results suggest that increased AMPK activation by antimycin A in IECs results from inductions of both total AMPK α protein levels and its phosphorylation. Because the decrease in cytoplasmic HuR in polyamine-deficient cells by transfection with its specific siRNA (small interfering RNA) is accompanied by a reduction in complexes of HuR bound to p53 and NPM mRNAs, a decrease in the stability of such mRNAs, a lessening of the expression of the corresponding protein products and an ensuing inhibition of cell growth [3,11,26], the subcellular distribution of HuR via an AMPK-regulated *Imp α 1* pathway following changes in the levels of cellular polyamines is of great biological significance in maintaining the intestinal epithelial integrity.

The findings presented here further indicate that phosphorylation and acetylation of *Imp α 1* are crucial for regulating the relative subcellular localization of HuR by polyamine-mediated AMPK activation. *Imp α 1* has an N-terminal *Imp β* -binding domain that also includes an autoinhibitory region, followed by a nuclear localization sequence-binding domain and a C-terminal domain that is necessary for binding its export factor CAS (cellular apoptosis susceptibility protein) [52,53]. AMPK was found to phosphorylate *Imp α 1* at Ser¹⁰⁵ and also indirectly acetylate *Imp α 1*, through phosphorylation of p300, which in turn acetylates *Imp α 1* at Lys²² [34]. Phosphorylation and/or acetylation of *Imp α 1* increase its affinity for *Imp β* and also increase its affinity for nuclear import cargoes such as HuR [32,34]. Consistent with these observations, the results presented in Figure 4 show that overexpression of WT-*Imp α 1*, but not point mutants of *Imp α 1* lacking the phosphorylation and acetylation sites, enhanced the nuclear import of HuR in IECs. In contrast, overexpression of *Imp α 1* bearing mutations K22R (to disrupt its acetylation), S105A (to disrupt its phosphorylation) or K22R/S105A (to disrupt both modifications) displayed a significant increase in the levels of cytoplasmic HuR. Importantly, ectopic overexpression of different point mutants of *Imp α 1*, including K22R, S105A and K22R/S105A, also prevented the increased levels of cytoplasmic HuR following polyamine depletion in IEC-6 cells (Figure 6), suggesting that both AMPK-regulated phosphorylation and acetylation of *Imp α 1* are required for the stimulation of HuR nuclear import following polyamine-mediated AMPK activation.

In summary, these results indicate that polyamines modulate the subcellular localization of HuR through AMPK-driven modification of *Imp α 1* function in IECs. Depletion of cellular polyamines repressed AMPK expression and activity, whereas increased polyamines by *ODC* overexpression increased AMPK levels. In the absence of cellular polyamines,

decreased levels of *Imp α 1* were associated with an increase in the levels of cytoplasmic HuR, which was completely overcome by AMPK activation through ectopic expression of the WT *AMPK* gene. The results obtained in the present study further show that overexpression of *Imp α 1* mutants, lacking either phosphorylation site, acetylation site, or both, suppressed the nuclear accumulation of HuR and also prevented the increased levels of cytoplasmic HuR in polyamine-deficient cells. Since cellular polyamines are highly regulated by the status of epithelial cell growth and stress stimuli, these findings suggest that polyamine-modulated HuR subcellular distribution through AMPK-regulated phosphorylation and acetylation of *Imp α 1* plays an important role in regulating IEC proliferation and epithelial integrity under physiological and pathological conditions.

Acknowledgments

This work was supported by a Merit Review Grant (to J.-Y.W.) from the Department of Veterans Affairs and by NIH grants DK-57819, DK-61972, DK-68491 (to J.-Y.W.) and AI-68432 and AT-4148 (to H.Z.). J.-Y.W. is a Research Career Scientist at the Medical Research Service, the U.S. Department of Veterans Affairs. M.G. is supported by the NIA-IRP, NIH.

Abbreviations used:

AdAMPKα	pAdeno-AMPK α
AMPK	AMP-activated protein kinase
AMPKK	AMPK kinase
ARE	AU-rich element
DFMO	D,L- α -difluoromethylornithine
FBS	fetal bovine serum
HEK-293 cell	human embryonic kidney cell
IEC	intestinal epithelial cell
Impα1	importin α 1
MOI	multiplicity of infection
NPM	nucleophosmin
ODC	ornithine decarboxylase
ODC-IEC cells	ODC-overexpressing IEC-6 cells
pACC	phospho-acetyl-CoA carboxylase
pAMPKα	phosphorylated AMPK α
PBS-T	1 \times PBS/Tween 20
pfu	plaque-forming units

RBP	RNA-binding protein
WT	wild-type
UTR	untranslated region

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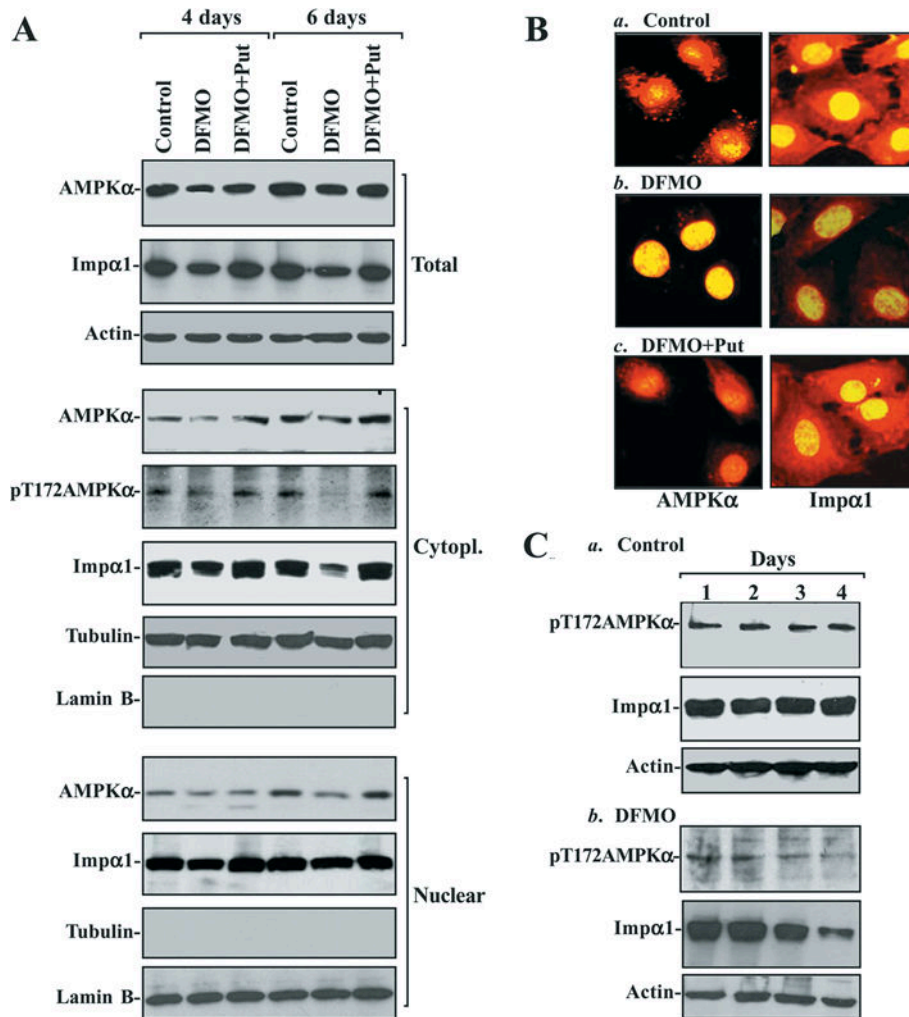


Figure 1. Changes in levels and cellular distribution of AMPK and Imp α 1 in control IEC-6 cells and cells treated with either DFMO (5 mM) alone or DFMO plus putrescine (Put; 10 μ M) for 4 and 6 days

Whole-cell lysates and cytoplasmic and nuclear fractions were prepared for Western blotting. (A) Representative Western blots in total (top), cytoplasmic (middle) and nuclear (bottom) proteins. Total, cytoplasmic and nuclear proteins (30 μ g of each) were subjected to SDS/PAGE (10% polyacrylamide), and levels of AMPK α and Imp α 1 were measured by using specific antibody against AMPK α or Imp α 1. The pAMPK α was identified by using the antibody that specifically recognizes pAMPK α at Thr¹⁷² (pT172AMPK α). After detecting AMPK α and Imp α 1, blots were re-probed to detect β -actin in whole-cell lysates, β -tubulin in cytoplasmic proteins or lamin B in nuclear proteins to control for the quality of the fractionation procedure and the even loading of samples. Three experiments were performed and they showed similar results. (B) Cellular distribution of AMPK α (left) and Imp α 1 (right) in cells described in (A). (a) Control; (b) DFMO treatment for 6 days; (c) DFMO plus putrescine treatment for 6 days. AMPK α and Imp α 1 were first detected using specific antibodies recognizing either AMPK α or Imp α 1 and then with anti-IgG conjugated with Alexa Fluor[®] 594 (red fluorescence), while nuclei were stained with TO-PRO3 (green fluorescence). The images shown result from the merging of these two individual signals.

Yellow signals reveal the areas where red has merged with green in the nuclear area; and brighter yellow colour indicates a decrease in AMPK α or Imp α 1 in the area. Original magnification, \times 1000. (C) Time course of levels of pT172AMPK α in controls (*a*) and cells exposed to DFMO (*b*). Whole-cell lysates were harvested at different time points after treatment with 5 mM DFMO, and levels of pT172AMPK α were measured by Western-blot analysis.

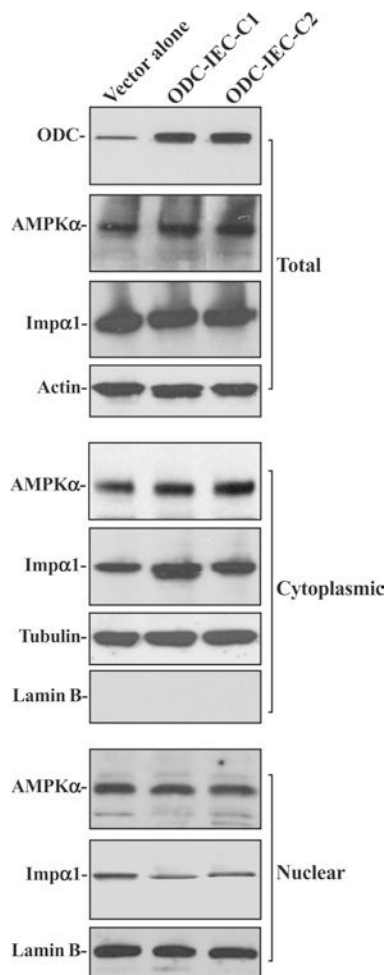


Figure 2. Changes in levels of AMPK α and Imp α 1 proteins in stable ODC-IEC cells
Whole-cell lysates were harvested from clonal (C) populations of ODC-IEC cells and in control IEC-6 cells (vector alone), and cytoplasmic and nuclear fractions were prepared for Western-blot analysis. β -Actin signals served to assess the equality of protein loading in whole-cell lysates; β -tubulin served as loading of cytoplasmic proteins, while lamin B immunoblotting served as loading of nuclear preparations. Three experiments were performed and they showed similar results.

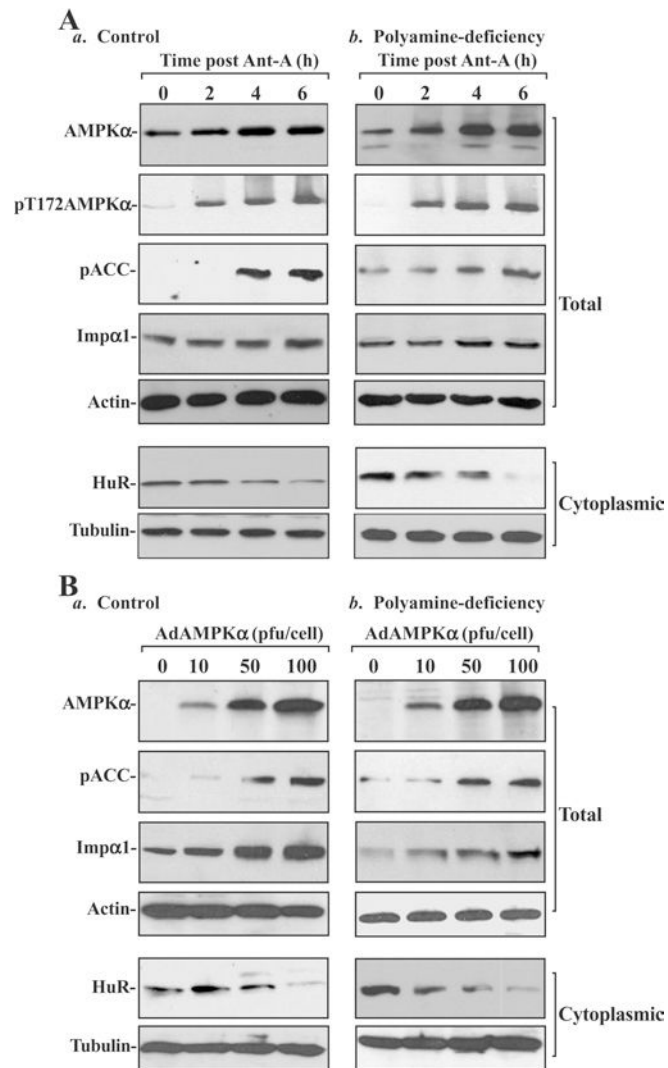


Figure 3. Effect of stimulation of AMPK α by treatment with its chemical activator antimycin A (Ant-A) and ectopic expression of WT AMPK α gene on cytoplasmic levels of HuR in control and polyamine-deficient cells

(A) Changes in levels of total and pAMPK α , pACC, Imp α 1 and HuR after treatment with Ant-A. (a) Control; (b) polyamine-deficient cells. IEC-6 cells were cultured in the control medium and a medium containing DFMO for 6 days and then exposed to Ant-A (5 μ M). Whole-cell lysates were harvested at different times after administration of Ant-A, and total and cytoplasmic fractions were prepared for Western-blot analysis. Levels of AMPK α , pAMPK α , pACC, Imp α 1 and HuR were identified by using the specific antibodies, and equal loading was monitored by immunoblotting of β -actin in total proteins and β -tubulin in cytoplasmic proteins. Three experiments were performed and they showed similar results. (B) Changes in levels of AMPK α , pACC, Imp α 1 and HuR after overexpression of the AMPK α gene. (a) Control; (b) polyamine-deficient cells. After cells were grown in control cultures and cultures containing 5 mM DFMO for 4 days, they were infected with the adenoviral expression vector encoding the complete open reading frame of the human AMPK α cDNA (AdAMPK α) or adenoviral vector lacking AMPK α cDNA (Adnull) at an

MOI (multiplicity of infection) of 10–100 pfu per cell. Whole-cell lysates were harvested 48 h after the infection in the presence or absence of DFMO, and levels of AMPK α , pACC, Imp α 1 and HuR were measured by Western-blot analysis. Three experiments were performed and they showed similar results.

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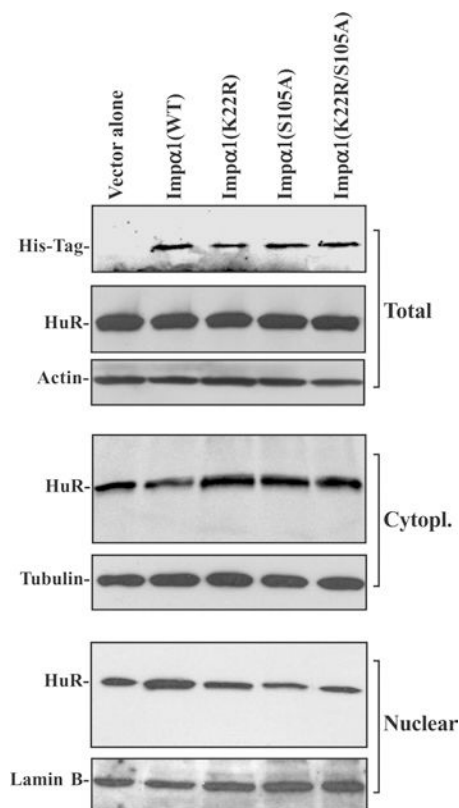


Figure 4. Effect of overexpression of WT and point-mutated Imp α 1 on subcellular distribution of HuR in IEC-6 cells

His-tagged Imp α 1 constructs were generated from the vector pcDNA3.1/V5-His-TOPO to express either WT-Imp α 1 or Imp α 1 bearing point mutations. IEC-6 cells were transfected with the constructs indicated by using Lipofectamine™, and clones resistant to the selection medium were isolated and screened for Imp α 1 expression by Western-blot analysis using a specific anti-His-tag antibody. Total, cytoplasmic and nuclear proteins were prepared, and levels of HuR were measured by using a specific antibody. Equal loading in total, cytoplasmic and nuclear proteins was monitored by β -actin, β -tubulin and lamin B respectively. Three experiments were performed and they showed similar results.

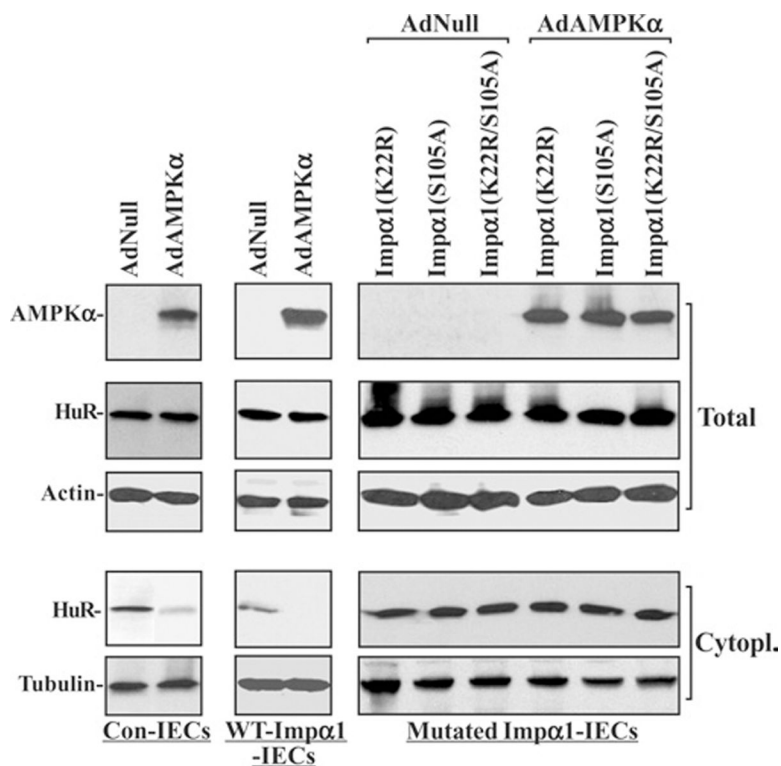


Figure 5. Effect of ectopic expression of AMPK α gene on the levels of cytoplasmic HuR in stable WT and point-mutated Imp α 1-transfected IEC-6 cells

Control (Con) IEC-6 cells transfected with the empty vector (left), stable WT-Imp α 1-transfected cells (middle) and stable point-mutated Imp α 1-transfected cells (right) were infected with the AdAMPK α or Adnull at an MOI of 100 pfu per cell. Whole cell lysates were harvested 48 h after the infection, and levels of total and cytoplasmic HuR were measured by Western-blot analysis. Equal loading of samples was monitored by β -actin in total cell lysates and β -tubulin in cytoplasmic lysates. Three experiments were performed and they showed similar results.

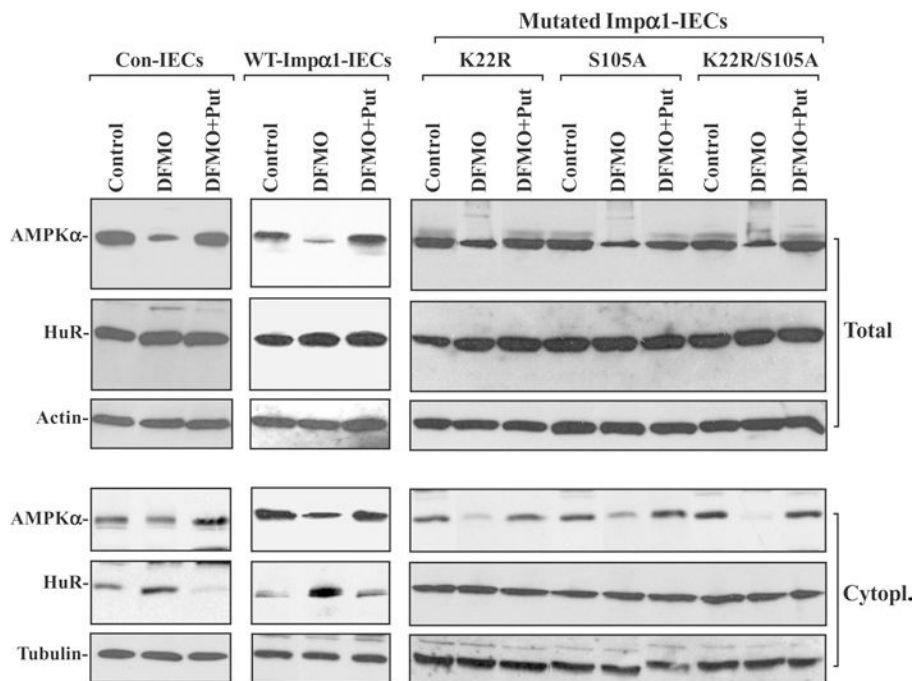


Figure 6. Effect of polyamine depletion on the levels of cytoplasmic HuR in stable WT and point-mutated Imp α 1-transfected IEC-6 cells

Whole cell lysates were harvested after control (Con) IEC-6 cells (left), stable WT-Imp α 1-transfected cells (middle) and stable point-mutated Imp α 1-transfected cells (right) were grown in the control cultures and cultures containing DFMO (5 mM) alone or DFMO plus putrescine (10 μ M) for 6 days. Total and cytoplasmic proteins were prepared and levels of AMPK α and HuR were measured by Western-blot analysis. Immunoblottings of β -actin in total cell lysates and β -tubulin in cytoplasmic lysates were examined to serve as equal loading controls. Three experiments were performed and they showed similar results.

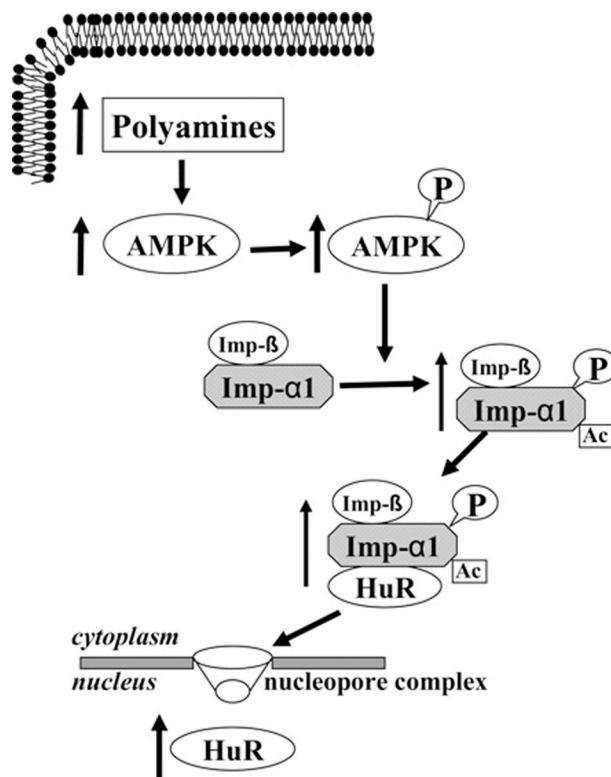


Figure 7. Proposed model depicting the role of cellular polyamines in modulating the subcellular localization of HuR through AMPK-regulated phosphorylation (P) and acetylation (Ac) of Imp- α 1

Increased polyamines result in AMPK activation that enhances the nuclear accumulation of HuR through a mechanism involving Imp α 1. The activation of AMPK by polyamines promotes Imp α 1-mediated nuclear import of HuR via modification of Imp α 1 on two residues. In addition, Imp β is also proposed to participate in the formation of HuR–Imp α 1 complexes for HuR nuclear import.