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Involvement of HuR in the serum starvation induced autophagy through regulation of Beclin1 in breast cancer cell-line, MCF-7

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ABSTRACT

Starvation is a cellular stress that induces autophagy, a conserved cellular self-digestion mechanism that allows cells to degrade and recycle damaged proteins and organelles. The present study illustrated that during serum deprivation, Beclin1, a crucial gene that is essential for autophagosome formation in autophagy, gets controlled post-transcriptionally in breast cancer cell-line MCF-7. RNA affinity chromatography and co-immunoprecipitation confirmed the association of HuR with 3'-UTR of *beclin1* mRNA. After cytosolic translocation, HuR enhances beclin1 protein synthesis in response to serum starvation by enhancing the association of *beclin1* mRNA to the polysomes. Partial silencing of HuR resulted in reduction of beclin1 expression both at mRNA and protein levels, which in turn decreased starvation-induced autophagic flux. Thus, in conclusion, fine-tuning of *beclin1* gene expression at post-transcriptional level by HuR is one of the key regulatory mechanisms of starvation induced autophagy in breast cancer cell-line, MCF-7.

1. Introduction

Autophagy is an evolutionarily conserved mechanism responsible for cellular homeostasis. The key role of autophagy is to protect the cells under metabolic and environmental stress; however, autophagy can also result in cellular death. Thus, autophagy could be considered as a crucial player in both cell survival and death [1]. Autophagy initiates with the formation of autophagosome, a double-membrane vesicle that engulfs cytoplasmic proteins, damaged organelles and other materials, eventually fusing with the lysosomes form autolysosomes where the cytoplasmic cargos are degraded by lysosomal hydrolases [2]. During starvation, amino acids and fatty acids are produced by degradation of cytoplasmic materials that can be used to synthesize new proteins and ATP for cell survival [3]. Other than stress management, autophagy is involved in development, immunity and in many human patho-physiologies, like cancer, neurodegeneration, gastrointestinal, heart and liver diseases *etc.* [4–6]. Dysfunction of the genes regulating autophagy perturbs homeostasis and potentially leads the cells to different pathological conditions.

Serum or nutrient deprivation activated autophagy is a well-known phenomenon for both normal and cancer cells [7]. It has been found that in the cases of normal like breast epithelial cell-line MCF-10A and NIH 3 T3 (mouse embryonic fibroblast cell-line) nutrient starvation leads to autophagy [7,8] whereas in human embryonic kidney cells, HEK293 and NRK cell-line (rat kidney fibroblast) amino acid starvation leads to autophagy [9,10]. Serum deprivation-induced autophagy were reported in the cases of cancer cells like MDA-MB-231 (triple negative breast cancer), LNCaP (prostate cancer) [11,12], where in DLD-1 (colorectal cancer derived cell-line) autophagy was activated under nutrient deprived condition [13].

Beclin1 (Becn1) acts as a key gene that participates in the early stage of autophagosome nucleation [14]. It is also essential for induction of autophagy in plants, slime-molds, nematodes, fruit flies, mice as well as in human cells [15]. The structural analysis of Becn1 is revealed the presence of Bcl-2 homology domain (BH3), a coiled-coil domain (CCD) and an evolutionarily conserved domain (ECD), important for multiple protein interactions [16–18]. Through its ECD, Becn1interacts with the class III phosphatidyl inositol 3-kinase (PtdIns3KC3) or Vps34 (involved in autophagic vesicle nucleation) and subsequent recruitment of additional Atg proteins, like ATG14L and UVRAG to orchestrate autophagosome and autolysosome formation respectively [19,20]. ATG14L recruits Becn1 from the Trans-Golgi Network (TGN) to autophagosomes and UVRAG-containing Becn1/Vps34 complex facilitates autophagosome maturation, particularly the fusion of autophagosome with lysosomes [21]. Thus, adequate expression and interaction of Becn1 is necessary for performing autophagy [14]. Other than its role in

Abbreviations: becn1, Beclin; mRNA, Messenger RNA; UTR, Untranslated region; ARE, AU-rich element; RNABP, RNA binding protein; HuR, Human Antigen R; LC3, Microtubule associated protein1 light chain3; GFP, Green fluorescent protein; RFP, Red fluorescent protein; siRNA, Small interfering RNA

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autophagy, it also serves important functions in endocytic trafficking, phagocytosis, control of cytokinesis *etc.* [22,23]. Till date, the mechanism of regulation of expression of this important gene remained largely unexplored, thus demands thorough investigation.

Regulation of gene expression at post-transcriptional level is one of the most important events where complex interplay between RNA Binding Proteins (RBPs) with the target mRNA decides its fate. Specific association of several RBPs, including AUF1, HuR, nucleolin, PCBP1, PCBP2, TTP, KSRP *etc.* with the specific sequences (*cis*-elements) present in the 3'-UTR significantly play roles in mRNA stability, translation and turn-over of the target mRNAs [24–28]. One such stabilizing protein, Human Antigen R or HuR, is a ubiquitously expressed member of ELAV/Hu family proteins that predominantly localized in the nucleus of the cell and under stressed conditions shuttles from nucleus to cytoplasm. Generally, the cytosolic HuR is found to be involved in stabilization and polysomes association of the target mRNAs [29,30].

The mounting evidences indicate that modulation of the post-transcriptional regulatory network of autophagy related genes could exert significant effect on autophagy under various physiological conditions as well as in diverse types of diseases related stressed conditions, thus, appropriate study on the mechanism of RBPs-mediated regulation of autophagy requires immense attention [31–33]. The present article is aimed to identify the mechanism of post-transcriptional regulation of *becn1* during serum starvation in breast cancer cell-line, MCF-7.

2. Materials and methods

2.1. Cell culture and treatment with Actinomycin-D

Human epithelial breast carcinoma cell-line, MCF-7, MDA-MB-231, PC-3 and HaCaT was obtained from National Centre for Cell Science, Pune, India and maintained in DMEM media containing high glucose, 10% Fetal Bovine Serum, Penicilin (100 IU/ml), Amphotericin-B and Streptomycin (100 μg/ml). Serum starved condition was obtained according to [7]. The half-life of *becn1* mRNA was measured according to [34].

2.2. Cloning

Portion of 3'-UTR of *becn1 mRNA* [nucleotides# 13,534–13,834, (NM_003766.3)] was cloned in pTZ57R/T (Thermo Scientific, USA)by RT-PCR using primer pair (5'-CTT-TTT-TCC-TTA-GGG-GGA-G-3′/5′-CAA-CTC-AGT-TAA-AAA-AAA-GAA-AAG-C-3′) to produce pT-bec-A. The clone was confirmed by sequencing in Gene Analyzer 3130 (Thermo Scientific, USA). This region was further digested with restriction enzymes and subcloned into the mammalian expression vector pEGFPC1 downstream *gfp* reporter gene to produce pE-bec-A. The Full length coding region of *becn1* mRNA with and without 3′-UTR was cloned in pC-DNA vector downstream HA reporter to produce pC-HA-bec-FL and pC-HA-bec-Δ3′UTR using set of primers (5′-TTA-CGC-TTC-ACT-CGA-CGG-CGG-CTA-CCG-G-3′ and 5′-GAC-ACT-ATA-GAA-TAG-CAG-TTT-TCA-GAC-TGC-AGC-AAA-TCT-TTT-ATT-ACA-AAT-AAT-TAA-ATC-3′/ 5′-GAC-ACT-ATA-GAA-TAG-TCA-TTT-GTT-ATA-AAA-TTG-TGA-GGA-CAC-CCA-AGC-AAG-3′) suitable for in-fusion cloning strategy. The clone was confirmed by sequencing.

2.3. Transfection of plasmids and siRNAs

MCF-7 cells (2×10^5) were transfected with plasmids (pE-becA, pC-HA-becFL and pC-HA-bec-Δ3′UTR) or siRNA [scramble or HuR (sense 5′-CCA-GUU-UCA-AUG-GUC-AUA-A55–3′ and anti-sense 5′-UUA-UGA-CCA-UUG-AAA-CUG-G55–3′) duplex RNA (Eurogentec, Belgium)] using Turbofect reagent (Thermo Fisher Scientific, Waltham, MA, USA) or jet PRIME plasmid/siRNA transfection reagent (Polyplus-transfections, Illkirch, France) according to manufacturer's instructions. The cells were harvested after 48 h for extraction of RNAs or proteins.

2.4. RNA extraction and qRT-PCR

Semi-quantitative and quantitative PCR were performed with the cDNAs prepared from total cellular RNAs (2 μg) of MCF-7 cells according to [35] and primer pair (5′-GAAATTTCAGAGATACCGACTTG TTC-3′/ 5′- CCTTTCTCAACCTCTTCTTTGAAC -3′) specific for becn1 mRNA using KAPA SYBR FAST qPCR kit in Step One Plus System (Applied Biosystems). *Neomycin* and *β-actin* levels were used as transfection control and internal control respectively. For the reporter assays, *gfp* and HA RNA levels were quantified by qRT-PCR and the relative expressions were analyzed by ΔΔCt method. Primer sequences for neomycin, *β-actin* and *gfp* are mentioned in [36]. Specific primer pair was used for HA qRT-PCR (5′-ATGGCCTACCCATATGATG-3′/ 5′-GAA GCGTAATCTGGAACATC-3′).

2.5. RNA affinity column chromatography

In vitro-transcribed polyadenylated *bec-A* RNA (using a poly A kit according to the manufacturer's protocol) was incubated with the oligo (dT)-agarose beads (100 μg in200 μl) for 2 h at 4 °C, were incubated with the samples (pre-cleared cytosolic extract of serum-starved MCF-7 cells) for 2 h at 4 °C with gentle shaking followed by centrifugation at 10,000 *r.c.f.* for 1 min. The proteins were eluted from the matrix with a 0.2 M step gradient of NaCl (0.2 to 1.0 M) in buffer B. Eluted fractions were run on 10% SDS-PAGE.

2.6. RNA-protein co-immunoprecipitation assay

Immunoprecipitation of RNA-protein complexes was performed as described in [37]. Briefly, serum starved MCF-7 cells (5×10^7) in 10 ml of PBS were cross-linked with formaldehyde [final concentration of 0.1% (v/v)], then quenched with glycine (pH 7.0, 0.25 M) were re-suspended in 1 ml of RIPA buffer containing protease inhibitors. The pre-cleared cell extracts were incubated for 2 h with shaking at 4 °C with 20 μl protein A/G sepharose beads which were pre-incubated for 1 h with 4 μg of monoclonal anti-HuR (Santa Cruz; 0.2 μg/μl) or normal mouse IgG (0.2 μg/μl) antibodies. The A/G sepharose beads were washed, reverse cross-linked at 70 °C for 45 mins. Finally, RNA was extracted from the immunoprecipitated samples using TRIzol, treated with DNase I, reverse transcribed and amplified by semi-quantitative PCR for observing mRNA levels of *becn1*and *β-actin*. Results were also quantified by qRT-PCR.

2.7. Western blot

Whole cell and cytoplasmic extracts of untreated and starved MCF-7 cells were prepared according to [38] and western blots were performed using anti- Beclin1, anti-LC3, anti-HuR, anti-β-Actin, anti-GAPDH (Santa Cruz) and anti-HA (Sigma) antibodies.

2.8. Polysome analysis

Serum starved or transfected MCF-7 cells (20×10^6) were homogenized in polysome lysis buffer containing cycloheximide (0.1 mg/ml) as cited in [39]. Briefly, 10–50% (*w/v*) sucrose gradient was prepared in Biocomp Gradient Station (Biocomp, Canada) and after its stabilization the cytoplasmic extract was loaded on top of the sucrose gradient and ultra-centrifuged at 30,000 rpm for 4 h in SW41·Ti rotor (Beckman Coulter, USA). Fractions were collected using a programmable gradient fractionator (Biocomp, Canada) and absorbance at 254 nm (BioRad, USA) was measured. RNA was isolated from the fractions by phenol-chloroform extraction and ethanol precipitation while protein was obtained by precipitation with 30% tri-chloro acetic acid (TCA) followed by acetone wash and re-suspension in 1 × protein loading buffer.

2.9. Detection of autophagic vacuoles by confocal microscope

MCF-7 cells were stably transfected with ptfLC3 vectors (Addgene, MA, USA) and single colony (expressing both *gfp* and *rfp*) was isolated by limiting dilution in 96-well plates and were maintained in individual flasks in presence of G418 50 μg/ml [38].

MCF-ptfLC3 cells transfected with both si-HuR and si-Scramble were fixed with 4% p-formaldehyde under Serum starved and non-starved conditions (control). The cells were permeabilized with 0.1% Triton X 100 for10 mins and were visualized by Olympus confocal laser scanning microscope (IX81) at 40× magnification after staining with DAPI (5 ng/ml) and mounting with n-propyl galate.

2.10. Statistical analysis

All graphs were generated in Microsoft Office Excel 2010 (Microsoft Corporation, Washington, USA). Error bars indicate mean ± SEM of atleast three independent experiments. Parametric paired *t*-test was used for analysis of statistical significance with KyPlot version 2.0 (KyensLab Incorporated, Tokyo, Japan). *P*-values < 0.05 were considered to be statistically significant while $P > 0.05$ were considered non-significant (NS).

3. Results

3.1. Induction of Beclin1 in MCF-7 cells during serum starvation

The autophagic activity of MCF-7 cells during serum starvation (in presence of 0.5% FBS) was monitored (up to 18 h) by immune-blot of LC3B-II/ LC3B-I, where, LC3B-II was found to be upregulated with gradual declination of LC3B-I *i.e.* LC3B-I to LC3B-II conversion was increased with increasing time of serum starvation (Fig. 1A) indicating induction of autophagy in a canonical manner [40]. Under the same condition, levels of *becn1*mRNA [quantitatively (Fig. 1B; upper panel) and semi-quantitatively (Fig. 1B; lower panel)] and protein (Fig. 1C) were measured. Both the mRNA and protein levels of becn1were found to be up-regulated progressively with time of serum starvation. To check whether this phenomenon is exclusive for MCF-7 or not, we have measured the level of becn1 mRNA in triple negative breast cancer cell-line, MDA-MB-231, Prostate cancer cell-line, PC-3 and normal skin keratinocyte cell-line, HaCaT. The results showed upregulation of becn1 mRNA on serum starvation in all three cases (fig. S1). The half-lives ($t_{1/2}$) of *becn1* mRNA under untreated or starved (18 h) were compared after inhibiting transcription with actinomycin D. Fig. 1D showing the semi-log plot of relative expression (fold change after normalization with respective *β-actin* mRNA levels) of *becn1* mRNA in untreated control or serum starved MCF-7 cells. It was observed that the half-life of *becn1* mRNA was increased from 3.4 h (control) to $> > 6$ h during serum starvation indicating contribution of mRNA stability in the observed increase of the level of *becn1* mRNA. As it is well known that the 3′-UTR of any gene is the major contributor towards its stability, thus, we have checked the role of 3′-UTR on the stability of *becn1* gene. For this purpose *becn1* gene [coding region with entire 3′-UTR (pC-HA-bec-FL) and Δ3′-UTR (pC-HA-bec-Δ3UTR)] were cloned at the 3′ end of HA reporter. MCF-7 cells were transfected with the said vectors and expression of HA (both RNA and protein levels) was monitored before and 18 h after deprivation of serum. Fig. 1E showing that the cells transfected with pC-HA-bec-FL exhibited more expression (3.5 fold) of HA (at RNA level measured by qPCR) under serum starved condition as compared to the control (unstarved condition), whereas by removal of 3′-UTR decreased expression of HA for both the cases [starved (~ 50%) and unstarved (~ 90%)]. Similar phenomenon was observed for the expression of HA protein under same conditions (Fig. 1F). The results confirmed the role of the entire 3′-UTR of *becn1* gene in the expression of beclin 1 mRNA and protein (as measured by HA tagged beclin1 in the presence and absence of its 3′-UTR).

3.2. Binding of HuR to beclin1 3′-UTR participates in its enhanced translation

In silico study indicated that the proximal region of *becn1*–3′-UTR (300 bp) named as *bec-A* contains putative *cis*-elements for binding of RNA binding proteins (RNA-BPs). To identify the contribution of *bec-A* in stability to the *becn1* mRNA, *bec-A* region of the 3′-UTR of *becn1* mRNA was introduced at the 3′-end of a fairly stable reporter mRNA (*gfp*) and the half-life of the reporter mRNA was measured by actinomycin-D chase experiment. As shown in Fig. 1G, the half-life of *gfp* mRNA was 3.3 h and due to serum starvation that was increased to $> > 4$ h, suggesting the role of *bec-A* region of *becn1* mRNA in its destabilizing and serum starvation-induced stabilization.

For identification of the interacting proteins that are involved in *becn1*mRNA stability, affinity chromatography (oligo-dT) was performed using *in vitro* transcribed poly-adenylated *bec-A* transcript to pull-out the cytoplasmic proteins from the extract of serum starved MCF-7 cells. The proteins were eluted from the affinity column with increasing concentrations of NaCl (200 mM – 1 M) and eluted fractions were separated by SDS-PAGE. Proteins having molecular weights of 70, 62, 48, 36 kDa were visible in the commassie stained gel (Fig. 2A-i). Presence of HuR (36 kDa) in the eluted fractions was revealed by western blot, however, the specificity of this interaction was confirmed by absence of β-actin in the same sample (Fig. 2A-ii).

A time-dependent nuclear to cytoplasmic translocation of HuR that starts even as early as 3 h during serum starvation was evident from the western blot (Fig. 2B); absence of any band in the western blot using anti-histone antibody (Fig. 2B) demonstrated insignificant nuclear protein contaminations in the cytosolic extract.

In vivo association of HuR with *becn1* mRNA was apparent from co-immunoprecipitation (co-IP) of RNA-HuR complex pulled-down using anti-HuR antibody from the lysate of serum-starved MCF-7 cells. Both semi-quantitative RT-PCR (Fig. 2C; upper panel) and qPCR (Fig. 2D) confirmed the presence of *becn1* mRNA in the RNA-HuR complex. Absence of *becn1* mRNA in the similar co-IP under untreated condition and absence of *β-actin* RNA in all the immunoprecipitates is indicative of specific and exclusive *in vivo* interaction of HuR with *becn1* mRNA during serum starvation. Mouse-IgG antibody was used as negative control. Western blots of untreated and serum starved samples (Fig. 2C; lower panel) were suggestive of proper immunoprecipitation.

To check the effect of HuR on *becn1* translation in the case of starvation induced autophagy, polysome profiling was performed, where fractions were obtained after sucrose density gradient centrifugation of untreated or serum starved MCF-7 cytosolic extracts. The samples (fractionation profile shown in Fig. 2E; upper panel)from each fractions were subjected to semi-quantitative RT-PCR and western blots to measure the mRNA level of *becn1* and *β-actin* (Fig. 2E; middle panel) and the protein levels of HuR or β-actin (Fig. 2E; lower panel) respectively. The results revealed that, during serum starvation *becn1* mRNA and HuR protein were associated more with polysome fractions as compared to control (untreated), signifying enhanced translation of *becn1* mRNA. As it is evident from the previous results (Fig. 2A, C and E) that *becn1* mRNA remains associated with HuR, thus, during serum starvation, HuR might be helping the association of *becn1*mRNA to polysomes.

3.3. Partial silencing of HuR regulates beclin1 expression and starvation induced autophagy

To ascertain the direct role of HuR on translational regulation of-*becn1* mRNA, partial knock down of HuR was done in MCF-7 cells by introducing siRNA against HuR. It is evident that the cells that were transfected with scramble si-RNA (si-Scr) showed increase in intrinsic level of *becn1* mRNA (Fig. 3A) and protein (Fig. 3C) during nutrient limited condition as compared to normal, whereas, its level remained almost the same (no induction of *becn1* mRNA) when si-HuR transfected

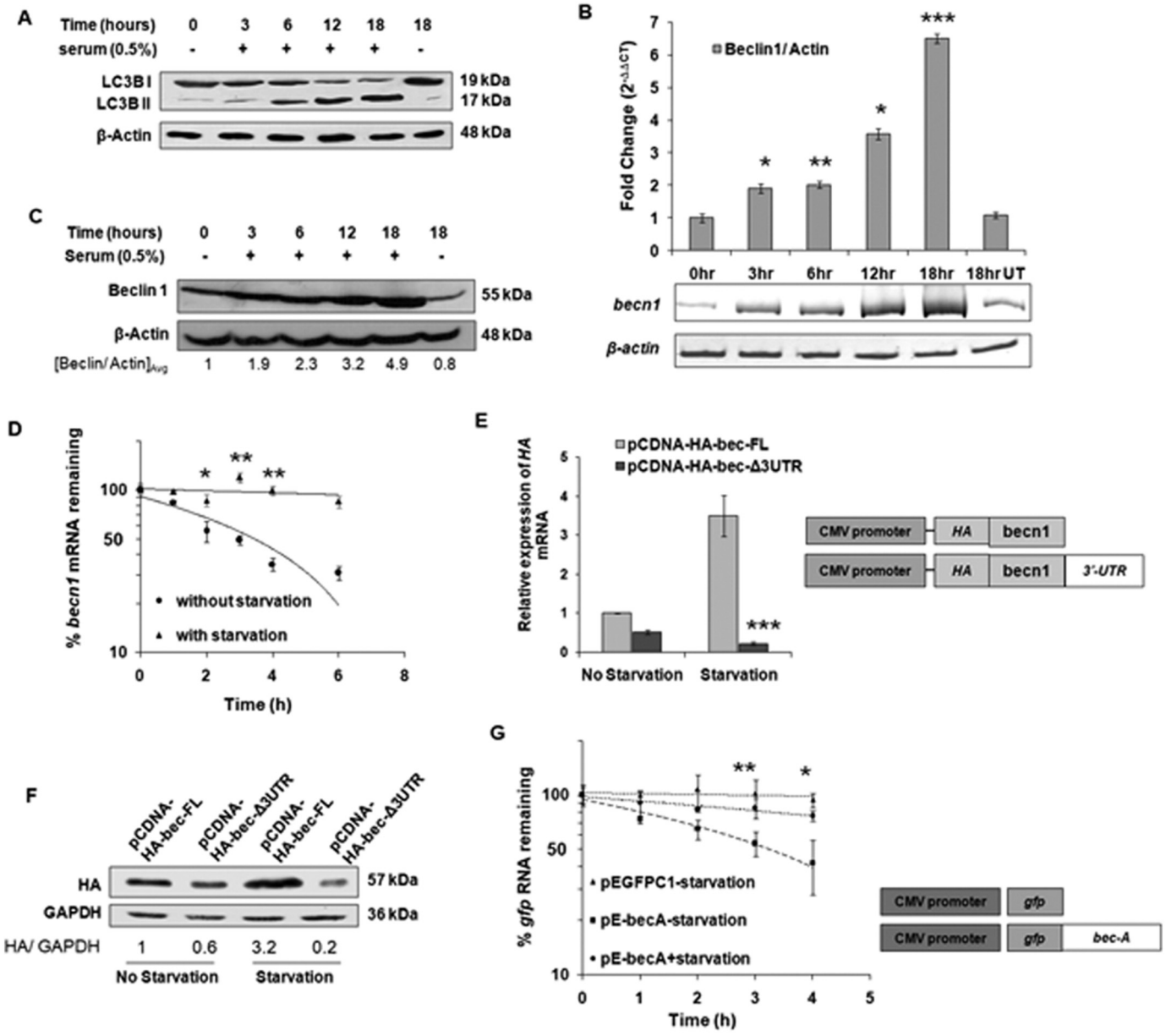

Fig. 1. Post-transcriptional regulation of *beclin1* mRNA during serum starvation induced autophagy. (A) Western blot using total cellular proteins (80 μg each) from serum starved (0.5% FBS) MCF-7 cells, resolved in 14% SDS-PAGE and probed with LC3B specific antibody and anti β-Actin antibody (loading control). (B) Bar diagram showing relative expression (RNA level) of *beclin1* mRNA level during starvation-induced autophagy at indicated time points measured by qRT-PCR using $\Delta\Delta C_T$ method and normalized with *β-actin* mRNA (upper panel). Ethidium bromide stained PCR products separated on 6% native PAGE/TBE gel (lower panel). (C) Representative western blot showing Beclin1 protein level during starvation-induced autophagy where cellular proteins (30 μg) were resolved in 12% SDS-PAGE and blotted with Beclin1 specific primary antibody and re-probed with anti β-Actin antibody (loading control). The bands were quantified using Image-J software and the average becn1/actin ratio is shown at the bottom of the corresponding bands. (D) Control or starved cells were grown for 14 h followed by treatment with actinomycin D (5 μg/ml) for 0–6 h. Semi-log plot showing decay of *becn1* mRNA where the levels of mRNAs were measured by qRT-PCR (using $\Delta\Delta C_T$ method normalized with β-actin mRNA). (E) Relative expression of reporter gene *HA* was measured (by $\Delta\Delta C_T$ method and normalized with *β-actin* mRNA) with RNA prepared from MCF-7 cells transfected with pC-HA-bec-FL or pC-HA-bec-Δ3′UTR plasmid followed by starvation for 18 h. Data plotted in B, D, E and G are means of three independent experiments and presented as mean ± SEM where NS indicates ($P > .05$), * is ($P \leq .05$), ** is ($P \leq .01$) and *** is ($P \leq .001$). (F) Western blot of anti-HA and anti-GAPDH antibody using cellular extracts (40 μg) of MCF-7 cells transfected with plasmids (pC-HA-bec or pC-HA-bec- Δ3′UTR) followed by starvation for 18 h. The bands were quantified using Image-J software and the average HA/GAPdH ratio (of three independent experiments) are shown at the bottom of the corresponding bands. (G) pEGFPC1 or pE-bec-A transfected MCF-7 cells harvested at 0, 1, 2, 3 and 4 h after treatment with actinomycin D (5 μg/ml). The relative expression of reporter (*gfp)* was quantified by q-RT PCR (using $\Delta\Delta C_T$ method after normalization with *neomycin* RNA). Statistical significance of was calculated at each point compared to the expression of pE-becA with or without starvation.

cells were serum starved. Additionally, during nutrient limited condition, Becn1 protein level was decreased significantly in the cells that were transfected with si-HuR as compared to si-Scr (Fig. 3C). Similar experiments were performed in triple negative breast cancer cell-line, MDA-MB-231, Prostate cancer cell-line, PC-3 and normal skin keratinocyte cell-line, HaCaT for validation of the result in other cancer cell-lines as well as normal. The results showed upregulation of becn1 mRNA on serum starvation in all three cases, but when si-HuR transfected cells were serum starved no significant induction of *becn1* mRNA were observed (fig. S2). These results confirmed that downregulation of

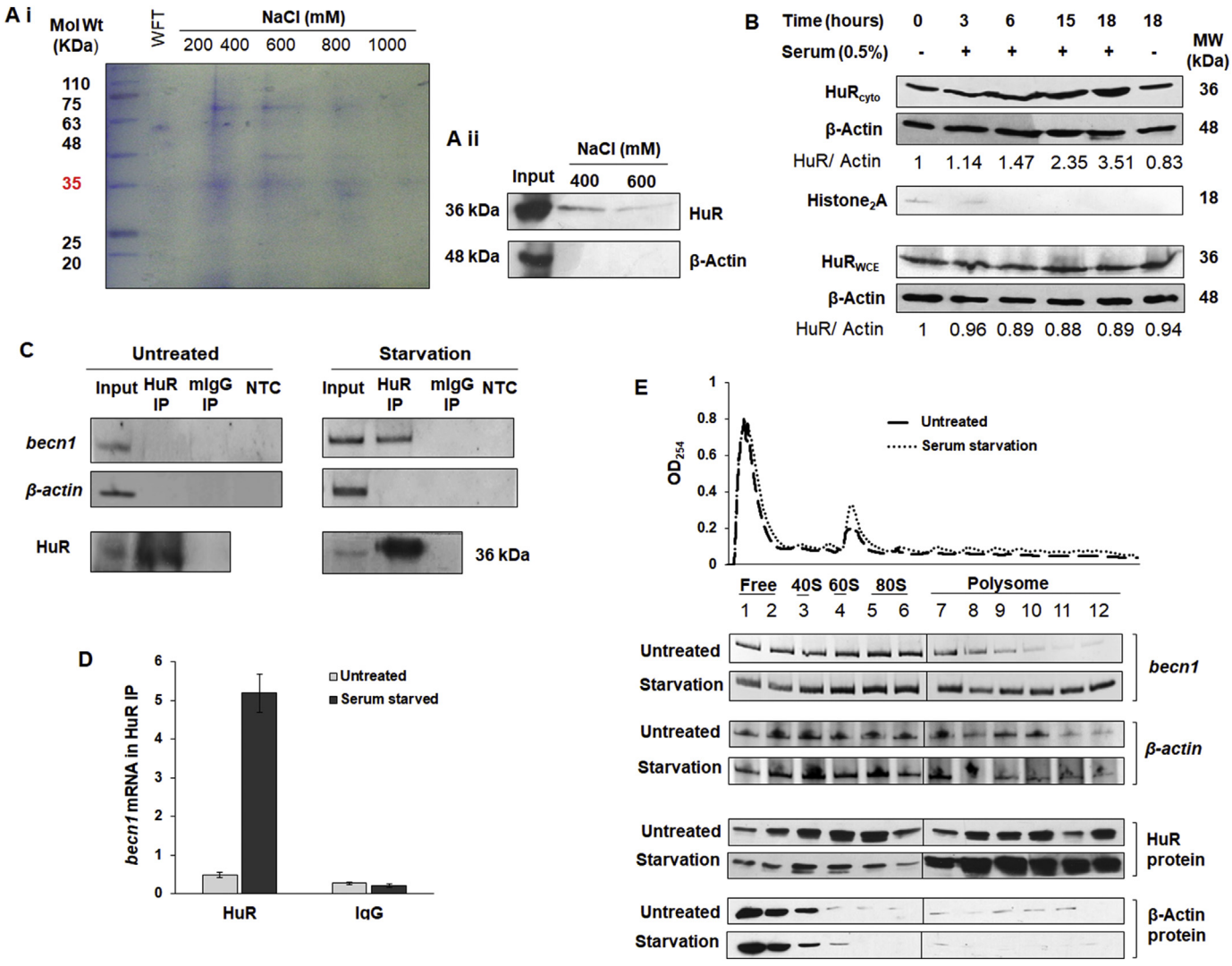

Fig. 2. Identification and involvement of HuR in *beclin1* post-trancriptional regulation (A i) Coomassie Blue stained SDS-PAGE (10%) image showing elution profile of the proteins in serum starved MCF-7 cytoplasmic extracts bound to the RNA affinity (polyadenylated-*bec-A* bound oligo-dT beads) column eluted with NaCl. (A ii) Western blot of eluted column fractions probed with anti-HuR and anti-β-Actin antibodies. (B) Western blots of with whole cell and cytoplasmic extracts (30 μg each) of untreated or starved MCF-7 cells resolved in 12% SDS-PAGE, probed with anti-HuR and anti-β-Actin (loading control) antibodies, where absence of Histone-2A showing no nuclear contamination in cytosolic extracts. The bands were quantified using Image-J software and the average HuR/actin ratio is shown at the bottom of the corresponding bands. (C) Elucidation of interactions of *bec-A* mRNA with HuR *in-vivo*. Upper panel: Ethidium bromide stained gel picture of semi-quantitative RT-PCR products of *bec-A* and *β-actin* RNA of chemically crosslinked RNA-protein complexes from untreated and serum starved MCF-7 cells immunoprecipitated with anti-HuR or IgG antibodies. Lower Panel: Western blot with anti-HuR of immunoprecipitated samples (as indicated). (D) Quantitative analysis of *becn1* mRNA immune-precipitation with HuR or IgG antibodies of untreated or serum starved MCF-7 cell lysates. The relative expression was calculated by $\Delta\Delta C_T$ method and normalized with *β-actin* mRNA. Plotted data are means of three independent experiments and presented as mean ± SEM. (E) Polysome profiles of untreated and serum starved MCF-7 cells (top). Semi-quantitative RT-PCR of *becn1* mRNA and *β-actin* from polysome fractions of untreated and serum starved MCF-7 cells (middle). Western Blots of HuR and β-Actin from polysome fractions of untreated and serum starved MCF-7 cells (bottom). Polysome profiling was repeated twice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
[Abbreviations: MW, molecular weight markers; WFT, wash flow through; cyto, cytoplasmic extracts; WCE, whole cell extracts; IP, immunoprecipitate; NTC, No template control].

HuR protein leads to decreased expression of becn1 in cancer as well as normal cell-lines.

Further to check the role of the specific interaction of *bec-A* region of 3′-UTR of *becn1* mRNA with HuR, a construct containing the 3′-UTR of *becn1* gene (*bec-A*) cloned at the 3′-end of *gfp* reporter was introduced in MCF-7 cells by transfection. The expression of *gfp* reporter was monitored after silencing HuR specifically by introduction of si-HuR. Silencing of HuR hindered the upregulation of *gfp* mRNA (~3.5 fold decrease as compared to cells transfected with scramble si-RNA) during serum starvation (Fig. 3B), *i.e.* suppression of HuR has no impact on untreated MCF-7 cells, but, could downregulate the expression of *becn1* both at mRNA and protein levels during starvation. Thus, it was clear that the induction of *becn1* mRNA by serum starvation require specific interaction of HuR with the *bec-A* region of *becn1* mRNA.

As HuR was found to be the key regulator of Becn1 expression, it was intriguing to know its functional activities on cellular autophagic induction. To serve this purpose, autophagic flux was evaluated during serum starvation by measuring the conversion of LC3B-I to LC3B-II in MCF-7 cells whose HuR was partially knocked down by si-HuR. As expected, an increased autophagic flux was observed with serum starvation in the cells transfected with scramble si-RNA, whereas, starvation induced autophagy was found to be abrogated (LC3B-II/ LC3B-I was decreased) in si-HuR transfected cells (Fig. 3D), implying the importance of HuR in starvation mediated canonical autophagy.

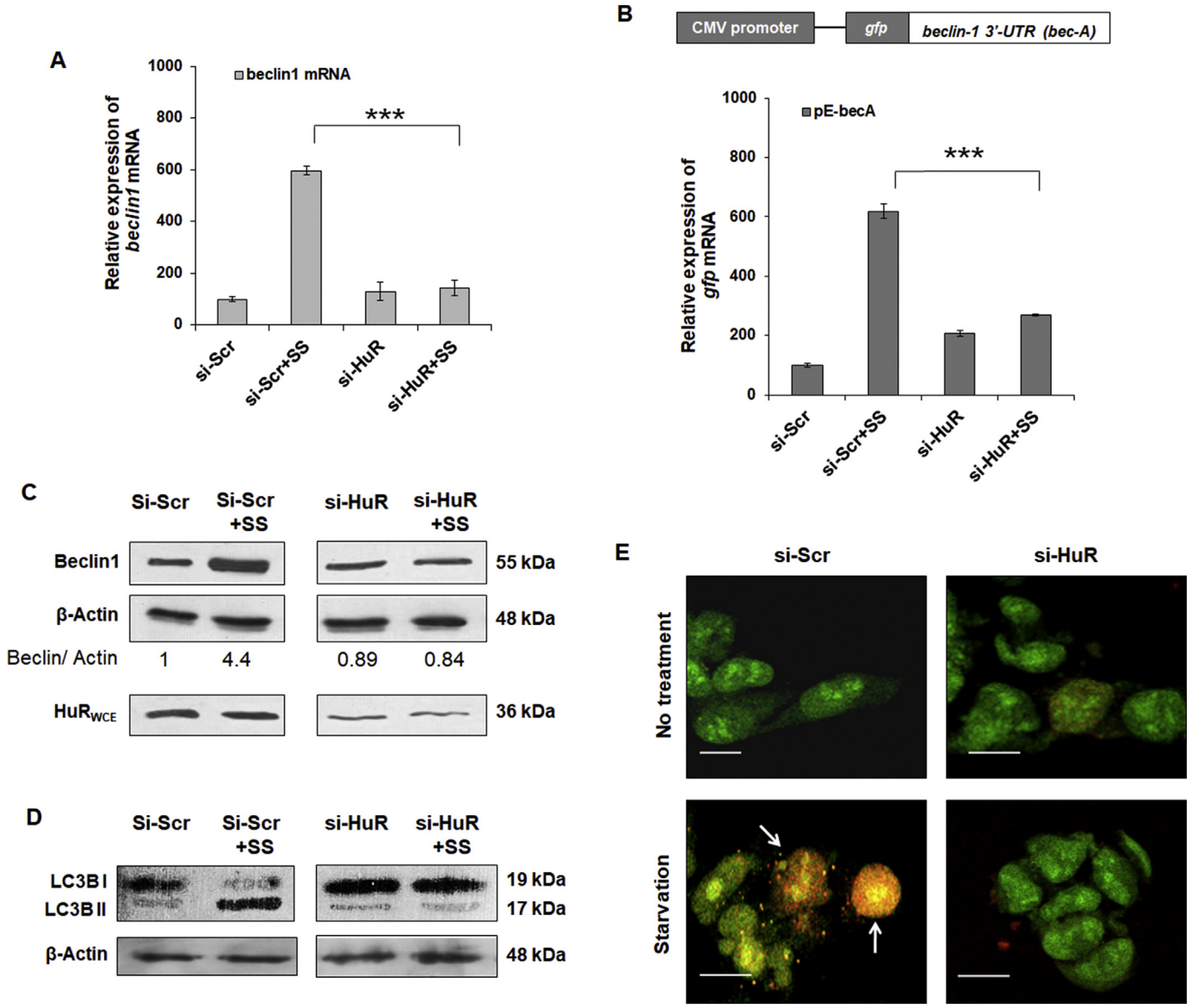

Fig. 3. Regulation of beclin1 expression and serum starvation induced autophagy by HuR. (A) Relative expression of intrinsic *becn1* mRNA (normalized to *β-actin* mRNA) measured by q-RT-PCR using $\Delta\Delta C_T$ method using RNA prepared from MCF-7 cells transfected 50 nM siRNA (scramble or HuR specific) for 48 h followed by starvation for 18 h. (B) Relative expression of reporter gene *gfp* was measured (by $\Delta\Delta C_T$ method and normalized with *β-actin* mRNA) with RNA prepared from MCF-7 cells co-transfected with 10 μg pE-becA plasmid and 50 nM siRNA (scramble or HuR) followed by starvation for 18 h. Data plotted in A and B are means of three independent experiments and presented as mean ± SEM where NS indicates ($P > 0.05$), * is ($P \leq 0.05$), ** is ($P \leq 0.01$) and *** is ($P \leq 0.001$). (C) Representative western blot of Becn1, HuR and β-Actin (three independent experiments) with cellular extracts from MCF-7 cells transfected with siRNA (scramble or HuR) followed by starvation for 18 h. The bands were quantified using Image-J software and the average becn1/β-actin ratio is shown at the bottom of the corresponding bands. (D) Detection of autophagic flux by LC3B immunoblot in MCF-7 cells, transfected with 50 nM siRNA (scramble or HuR) for 24 h followed by starvation for another 18 h. (E) Monitor of cellular autophagic activity in MCF-7ptfLC3 cells by confocal microscope. Cells were transfected with 50 nM siRNA (scramble or HuR) for 24 h followed by starvation for another 18 h. [Abbreviations: si-scr, scramble si-RNA; si-HuR, HuR si-RNA; SS, serum starvation]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Expression of RFP-GFP-LC3 fusion proteins at neutral pH (in autophagosomes) gives yellow signal due to expression of both RFP and GFP, where in autolysosomes, it fluoresces red due to quenching ofGFP fluorescence in acidic pH of the autolysosomal compartments [7,38]. Diffused green fluorescence (GFP-stain) in the cytoplasm was observed in MCF-7-ptfLC3 cells transfected with scrambled si-RNA (Fig. 3E, upper left panel),which upon starvation showed mostly red with few yellow puncta representing structures of autolysosomes and autophagosomal bodies (Fig. 2E; lower left panel) indicating increased autophagic flux in nutrient limited condition. In contrary, partial silencing of HuR by si-HuR caused diffused green fluorescence upon serum starvation (no change in fluorescence pattern). Altogether, this observations supported that declined expression of HuR could abrogate starvation induced autophagy.

4. Discussion

This study uncovered the involvement of a ubiquitously expressed RNA binding protein, HuR in the control of autophagy induced by serum starvation. Several groups had already reported that serum starvation induces autophagy in normal cell-lines like MCF-10A [8], HEK 293 [9], NIH 3 T3 [7]. On the other hand, in several cancer cell-lines like, MDA-MB-231 [11], MCF-7 cells [41], LNCaP [12] deprivation of serum caused autophagy, whereas, in DLD-1 autophagy was

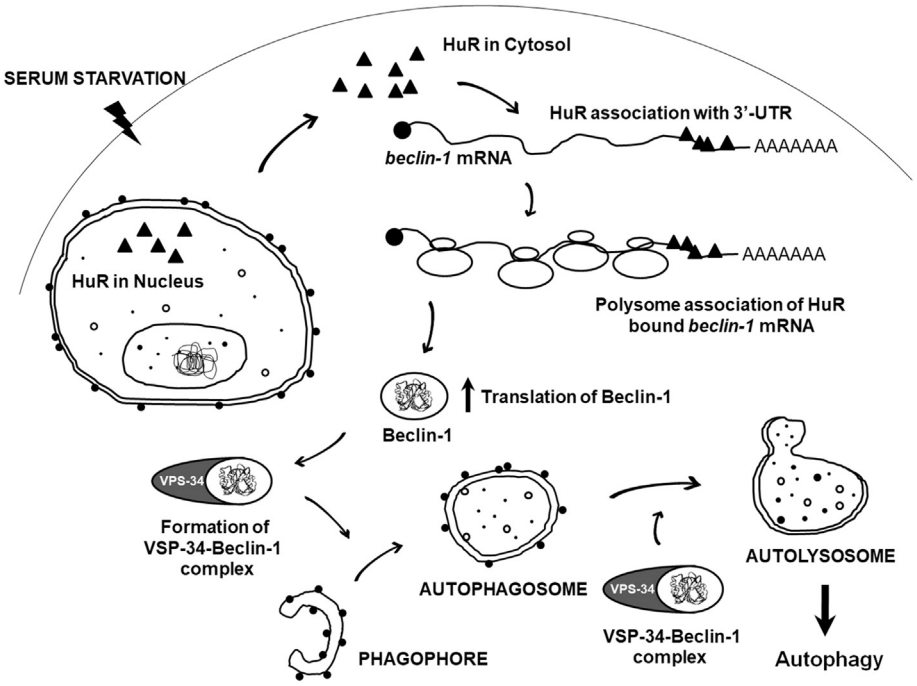

Fig. 4. Proposed model of HuR mediated post-transcriptional regulation of *beclin1* during serum starvation induced autophagy. Upon serum starvation, HuR translocated to the cytosol gets associated more with 3′-UTR of *beclin1* mRNA and led it to the translational machinery. Actively synthesized Beclin1 is complexed with Vps-34 and participated in autophagosome formation and autolysosome processing.

activated under nutrient deprived condition [13]. In this article, induction of autophagy caused by serum starvation was measured by increasing autophagic flux concomitant with up-regulation of both mRNA and protein levels of *becn1*. The calculated translational yield of becn1 during starvation was not changed significantly (1.0 to 0.8). Moreover, actinomycin-D chase experiments revealed that due to starvation in MCF-7 cells *becn1* mRNA was stabilized indicating moderate mRNA stabilization with pre-dominant protein synthesis. *In-silico* characterization of the proximal region of *becn1*3′-UTR (300 bp) or *bec-A* revealed putative binding sites for RNA-BPs as it contains Class I and Class III AREs, thus, claiming the importance of this region of *becn1* mRNA in its post-transcriptional regulation.

HuR functions primarily as a positive regulator for mRNA stability and it also regulates translation of the target mRNAs by associating it with polysomes [30]. Our results indicated that during serum starvation, HuR translocated from nucleus to cytosol and associated with the 3′-UTR of *becn1* mRNA, specifically to *bec-A* region. When HuR in MCF-7 cells was partial knocked down by siRNA, the cells showed no significant difference *w.r.t.* the control (si-scramble) in respect to either induction or suppression of autophagy, whereas, under serum starved condition, the MCF-7 cells showed significant suppression of *becn1* mRNA and protein along with changes in autophagic flux, which is pointing towards the fact that HuR is one of the crucial component in serum starvation mediated activation of autophagy. An early report stated that HuR inhibited autophagy activation by accumulating SQSTM1/P62 in human retinal pigment epithelial cells [42]. Interestingly, in our system, more polysome association of *becn1* mRNA and HuR protein was detected during starvation as compared to normal condition, indicating possible role of HuR in localization of *becn1* mRNA to translationally active polysomes during serum starvation. Similar to our observation, another group showed that over-expression of PCBP1, another ARE-BP, down-regulated serum starvation induced autophagy by targeting LC3B expression through decreasing its mRNA stability [43].

Though the conclusion is based on the experiments conducted in breast cancer cell-line MCF7, but was validated in triple negative breast cancer cell-line, MDA-MB-231, prostate cancer cell-line, PC-3 and normal skin keratinocyte cell-line, HaCaT. The conclusion is drawn from the experiments done on few cancerous and non-cancerous cell-lines, thus, it should not represent a general mode of autophagy regulation by serum starvation. But from the results it is clear that in all of these cells the mechanism of serum starvation induced autophagy involves the regulation of beclin1 through its interaction with HuR protein as it is found for MCF-7 cells.

Thus, all aforementioned observations that graphically represented (Fig. 4) resolved that during serum starvation, a ubiquitously expressed mRNA stabilizing protein, HuR upon cytosolic translocation binds to the 3′-UTR of *beclin1* mRNA, in turn stabilizes the mRNA and associates it to the translational machinery thus causing translational upregulation of the protein. Hence, actively synthesized Beclin1, subunit of Vps34-PI3K complex that is responsible for vesicle nucleation (autophagosome formation) and participate in autolysosome (fusion of autophagosome with lysosome) formation, finally leads to induction of the canonical autophagy.

Declaration of conflict of interest

No conflict of interest.

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Availability of data and material

All the raw data and material used in the published article should be available.

Authors' contributions

SD performed most of the experiments, statistical analysis and wrote the manuscript.

SS conceived the idea, wrote and corrected the manuscript.

S Das performed some of the experiments and corrected the manuscript.

Competing interests

Authors do not have any competing interest.

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Appendix A. Supplementary data

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