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HuR controls mitochondrial morphology through the regulation of Bcl_{xL} translation

Danielle Durie,[1] Maria Hatzoglou,[2] Pranesh Chakraborty[3,4] and Martin Holcik[1,3,*]

[1]Apoptosis Research Centre, Children's Hospital of Eastern Ontario Research Institute; Ottawa, ON Canada; [2]Department of Nutrition; Case Western Reserve University; School of Medicine; Cleveland, OH USA; [3]Department of Pediatrics; University of Ottawa; Ottawa, ON Canada; [4]Newborn Screening Ontario; Children's Hospital of Eastern Ontario; Ottawa, ON Canada

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Bcl_{xL} is a key prosurvival factor that in addition to controlling mitochondrial membrane permeability regulates mitochondrial network dynamics. The expression of Bcl_{xL} is regulated at the level of transcription, splicing and selective translation. In this study, we show that the RNA-binding protein HuR, which is known to orchestrate an anti-apoptotic cellular program, functions as a translational repressor of Bcl_{xL}. We show that HuR binds directly to the 5'UTR of Bcl_{xL}, and represses Bcl_{xL} translation through the inhibition of its internal ribosome entry site (IRES). Reduction of HuR levels leads to the derepression of Bcl_{xL} translation and subsequent rearrangement of the mitochondrial network. Our results place Bcl_{xL} into the HuR-regulated operon and provide further insight into the regulation of cellular stress response by HuR.

Introduction

Mitochondrial dynamics has recently emerged as an important regulatory process in cellular bioenergetics, apoptosis and disease.[1] Mitochondria exist as dynamic networks alternating between fusion and fission states that allow for the exchange and movement of cellular components such as mtDNA, proteins, ATP and lipids. This process is necessary for the proper maintenance of mitochondrial homeostasis and its misregulation is directly linked to a variety of human disease states.

One of the best studied regulatory mechanisms that is intimately linked to mitochondrial morphology is the cellular apoptotic machinery.[2] Mitochondria serve as cellular sentinels that detect, integrate and propagate death signals. The key factors participating in this process are members of the Bcl-2 family of proteins which regulate membrane permeabilization and the release of pro-apoptotic factors such as cytochrome c and Smac/DIABLO, thus triggering cell death.[3] Recently, studies have suggested an uncoupling between Bcl-2 family-mediated cell survival and mitochondrial dynamics.[4] For example, different forms of Mcl-1 were shown to reside in distinct mitochondrial locations where they perform separate functions.[5]

Bcl_{xL} is a key pro-survival member of the Bcl-2 family whose sole overexpression is sufficient to inhibit cell death.[6] The role of Bcl_{xL} in mitochondrial dynamics is not clear; overexpression of Bcl_{xL} increased mitochondrial fission in ~50% of cells while the remaining cells exhibited increased mitochondrial fusion.[7,8] Similarly, re-expression of Bcl_{xL} in Bcl_{xL}-null mice promoted both fusion and fission rates and mitochondrial mass in healthy neurons.[9] The expression of Bcl_{xL} is known to be regulated at the level of transcription and alternative splicing.[10,11] In particular, Bcl-x pre-mRNA can be spliced into a long, anti-apoptotic isoform (xL) or a short, pro-apoptotic isoform (xS). Various stimuli, along with different splicing factors regulate the switch between these isoforms. For instance, mitogenic pathways promote the expression of the xL isoform whereas the sphingolipid ceramide stimulates expression of the xS isoform. The relative levels of these factors determine whether cells will be resistant to death triggers or engage in apoptosis.[11] Additional point of Bcl_{xL} regulation was identified recently. Yoon and colleagues have shown that translation of Bcl_{xL} mRNA is specifically impaired in cells harbouring mutation of *dyskerin*, the gene mutated in patients suffering from Dyskeratosis congenita.[12] They further demonstrated that the 5' UTR of Bcl_{xL} harbours an IRES element which drives Bcl_{xL} translation under stress. Additional recent reports confirmed the 5' UTR of Bcl_{xL} as a key regulatory *cis*-element controlling expression of Bcl_{xL}. Cytoplasmic accumulation of hnRNP A1 in cells undergoing osmotic shock was shown to inhibit Bcl_{xL} mRNA translation by binding to the 5' UTR and tipping the survival balance in favor of apoptosis.[13] Similarly, binding of PDCD4 to the 5' UTR of Bcl_{xL} specifically repressed Bcl_{xL} translation, and this repression was relieved in cells treated in FGF-2, resulting in the degradation of PDCD4 and de-repression of Bcl_{xL} translation, thus contributing to enhanced chemoresistance.[14]

In the present study we demonstrate a link between the translational control of Bcl_{xL} expression and mitochondrial morphology. We show that an RNA binding protein HuR specifically and directly binds to the 5' UTR of Bcl_{xL} and functions as a repressor

*Correspondence to: Martin Holcik; Email: martin@arc.cheo.ca
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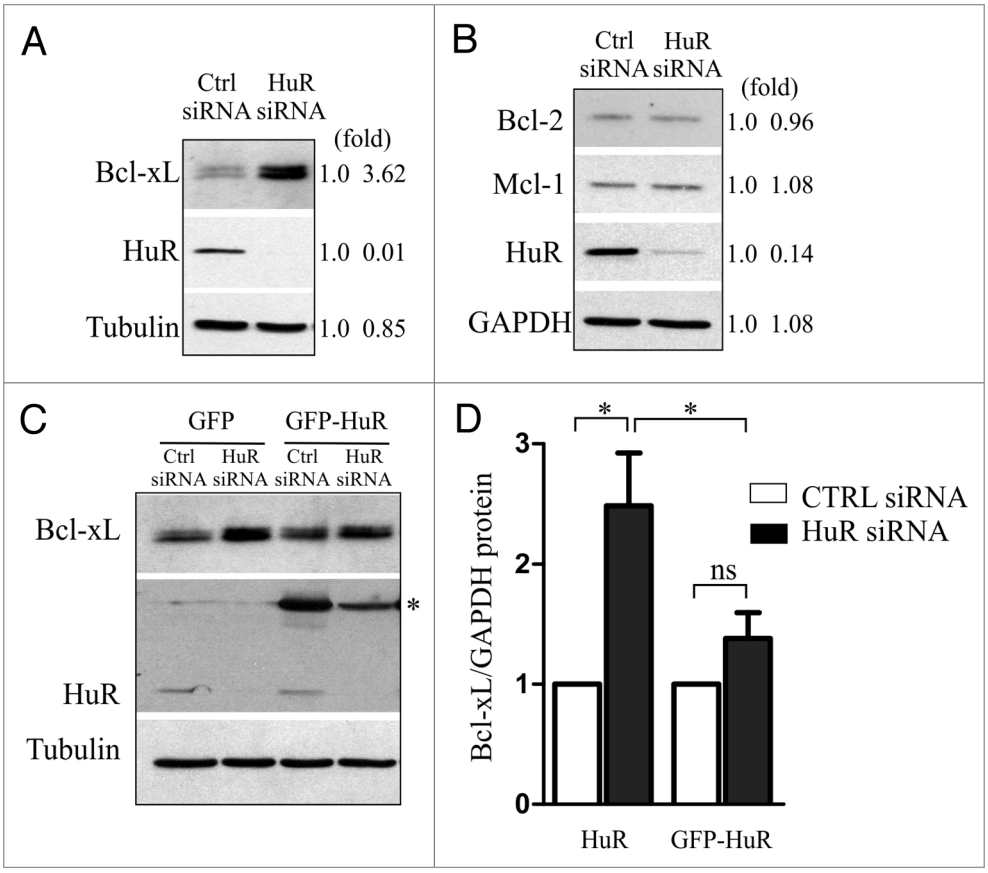

Figure 1. Cellular levels of HuR control the expression of Bcl_{xL}. U2OS cells were transiently transfected with HuR-targeting or non-silencing (Ctrl) siRNA and the levels of Bcl_{xL} (**A**), or Bcl-2 and Mcl-1 (**B**) were determined 72 h after transfection by western blot analysis. Signal intensities were quantified by densitometry and are shown on the right (*fold*). (**C**) U2OS cells were transiently transfected with HuR-targeting or non-silencing (Ctrl) siRNA, and 48 h later transfected with GFP- or GFP-HuR expressing plasmid as indicated. The expression levels of Bcl_{xL} were determined 24 h following the plasmid transfection by western blot analysis (*denotes GFP-HuR). Signal intensities were quantified by densitometry and are shown in (**D**). (*$p < 0.05$; $n = 3$).

of Bcl_{xL} translation in cells. Reduction in HuR levels by siRNA results in marked increase in Bcl_{xL} expression and subsequent fragmentation of the mitochondrial network. Our results suggest that HuR contributes to the maintenance of the mitochondrial network by controlling Bcl_{xL} expression.

Results

HuR is a translational repressor of Bcl_{xL}. We have shown recently that HuR regulates translation of XIAP through the interaction with the XIAP 5' UTR.[15] Since XIAP and Bcl_{xL} translation were shown to be coordinately regulated[12-14] we hypothesized that HuR might also control translation of Bcl_{xL}. We therefore examined whether modulating levels of HuR, either by knockdown with small interfering RNA (siRNA) or by overexpression, have any effect on the translation of endogenous Bcl_{xL} mRNA. U2OS cells were transiently transfected with either HuR-targeting or non-silencing control siRNA and the levels of Bcl_{xL} protein were determined 72 h later by western blot analysis. We found that reducing the levels of HuR by siRNA resulted in ~3.5 fold increase in Bcl_{xL} protein levels compared with a non-silencing control (**Fig. 1A**). This increase in expression was specific to Bcl_{xL}, since reducing the levels of HuR had no effect on the expression of closely related members of the Bcl-2 family of proteins, Bcl-2 and Mcl-1 (**Fig. 1B**). Importantly, the increase in Bcl_{xL} protein levels caused by reduction in HuR could be significantly blunted by restoring the levels of HuR (**Fig. 1C and D**). These observations suggest that HuR is a negative regulator of Bcl_{xL} expression.

To further demonstrate that HuR controls the translation and not the steady-state levels of endogenous Bcl_{xL} mRNA we examined the mRNA levels of Bcl_{xL} by quantitative RT-PCR in cells in which the expression of HuR was either reduced by siRNA, or increased by ectopic expression. Bcl_{xL} is one of the two alternatively spliced variants of the Bcl_x gene;[16] we therefore determined the steady-state levels of both Bcl_{xL} and Bcl_{xS} splice isoforms using a common primer set (Bcl_x; **Fig. 2A**) and specifically just that of Bcl_{xL} (**Fig. 2B**). We found that modulating the levels of HuR had no effect on the abundance of Bcl_{xL} mRNA. Furthermore, to eliminate the possibility that the increase in Bcl_{xL} is due to the change in the splicing ratio between Bcl_{xL} and Bcl_{xS} we examined the abundance of each isoform by isoform specific RT-PCR. Again, we didn't observe any differences in the ratio of the two isoforms in cells with reduced expression of HuR (**Fig. 2C**). To demonstrate that HuR indeed controls the translation of

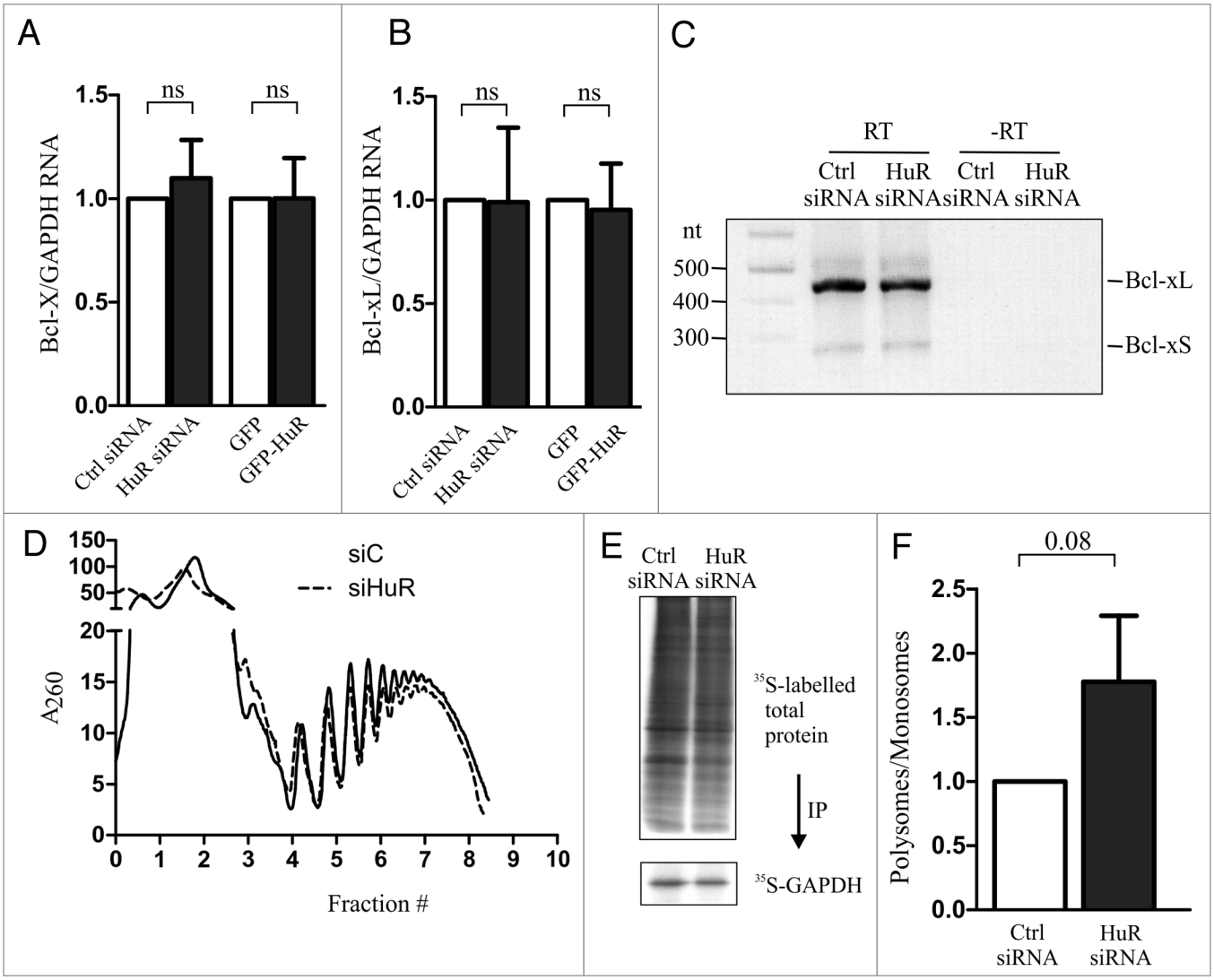

Figure 2. HuR regulates translation of Bcl_{xL}. U2OS cells were transiently transfected with HuR-targeting or non-silencing (Ctrl) siRNA, or with GFP- or GFP-HuR expressing plasmid and the steady-state levels of Bcl_x (**A**) and Bcl_{xL} (**B**) mRNAs were determined by quantitative RT-PCR. (**C**) Splice-variant specific RT-PCR was performed on RNA extracted from U2OS cells transiently transfected with HuR-targeting or non-silencing (Ctrl) siRNA. The position of Bcl_{xL} and Bcl_{xS} is indicated on the right. (**D**) Representative polysome profile trace of HE K293 cells transiently transfected with HuR-targeting or non-silencing (Ctrl) siRNA. Fractions (0 - top; 10 - bottom) are indicated below the graph. (**E**) Analysis of de novo proteins synthesis by L-[^{35}S]methionine labeling of HE K293 cells after transfection with either HuR-targeting or non-silencing (Ctrl) siRNA. GAPDH was immunoprecipitated using anti-GAPDH antibodies. (**F**) The relative polysome abundance of Bcl_{xL} mRNA calculated from polysome profiling (monosomes - fractions 1–4; polysomes- fractions 5–9).

endogenous Bcl_{xL} mRNA, we used polysome profiling to examine the association of Bcl_{xL} mRNA with translating ribosomes in cells with reduced levels of HuR. Because we were unable to obtain sufficient amount of ribosomes-associated RNA from U2OS cells, we used HEK293 cells which recapitulate the HuR-mediated changes in Bcl_{xL} expression seen in U2OS cells (data not shown). We observed that reducing HuR levels by siRNA had no measurable effect on the overall polysome profile (**Fig. 2D**), indicating that reduced levels of HuR do not affect global rate of protein synthesis. This was further corroborated by measuring ^{35}S-Met incorporation which indicated no measurable difference in global de novo protein synthesis, nor a difference in de novo synthesis of GAPDH, a representative house-keeping gene, in cells with reduced levels of HuR (**Fig. 2E**). We could not test de novo synthesis of Bcl_{xL}, as the anti-Bcl_{xL} antibody did not precipitate sufficient amount of labeled Bcl_{xL} for analysis. However, reverse transcriptase-PCR amplification of Bcl_{xL} mRNA from individual polysome fractions showed increased polysome association in cells with reduced HuR levels (**Fig. 2F**), in keeping with the notion that HuR functions as a translation repressor of Bcl_{xL}.

HuR represses Bcl_{xL} translation through interaction with its 5' UTR. We wished to elucidate the molecular basis for the translational repression of Bcl_{xL} by HuR. In previously published reports HuR was frequently found to interact with the 5' UTR regions of mRNAs whose translation was regulated by HuR.[15,17-19] We therefore questioned whether HuR interacts with the 5'

UTR of Bcl_{xL} as well. To determine whether HuR binds directly to the Bcl_{xL} 5' UTR we performed a UV-crosslinking experiment using a radiolabelled Bcl_{xL} 5' UTR RNA probe and purified recombinant GST-HuR. Increasing amounts of GST-HuR were incubated with [^{32}P]-labeled full length Bcl_{xL} 5' UTR RNA (**Fig. 3A**; probe A), followed by crosslinking and separation by SDS-PAGE. We found that Bcl_{xL} 5' UTR RNA is crosslinked to GST-HuR in a dose-dependent manner (**Fig. 3B**), indicating that HuR does indeed bind directly to Bcl_{xL} 5' UTR.

We further assessed the interaction between HuR and the Bcl_{xL} 5' UTR by measuring the apparent equilibrium dissociation constant (K_d) of the recombinant GST-HuR and Bcl_{xL} 5' UTR RNA. To determine the K_d of this interaction we performed a nitrocellulose filter binding assay, in which varying amounts of GST-HuR are incubated with a constant amount of RNA. We found the K_d for the interaction between GST-HuR and Bcl_{xL} 5' UTR RNA is 105 ± 61 nM (**Fig. 3C**). In order to determine the region of HuR interaction with the Bcl_{xL} 5' UTR we generated a series of deletions (**Fig. 3A**) which were subsequently subjected to the filter binding assay with GST-HuR as described above. We found that GST-HuR does not effectively bind probes B, C, and E (**Fig. 3C**; we were unable to determine the actual K_d value because we were unable to saturate binding). In contrast, probe D of the Bcl_{xL} 5' UTR showed strong binding with the K_d similar to that of the full length RNA (132 ± 40 nM). Importantly, deletion of segment D from the full length 5' UTR failed to bind GST-HuR (**Fig. 3C**; probe F). Therefore, HuR specifically associates with Bcl_{xL} 5' UTR RNA.

We have shown that cellular levels of HuR control translation of Bcl_{xL} and that HuR binds to the 5' UTR region of Bcl_{xL} mRNA. The 5' UTR of Bcl_{xL} harbours an IRES element that controls translation of the Bcl_{xL} mRNA under various physiological and pathophysiological conditions.[12-14] We therefore examined whether modulating levels of HuR has any effect on the activity of Bcl_{xL} IRES. To assess Bcl_{xL} IRES activity, we used a previously characterized bicistronic reporter plasmid containing the Bcl_{xL} IRES (pßgal/BclxL/CAT[13,14]). In this construct, expression of the first cistron (β-galactosidase, βgal) is cap dependent, whereas expression of the second cistron (chloramphenicol acetyl transferase, CAT) is driven by Bcl_{xL} 5' UTR. HEK293 cells were transiently co-transfected with pßgal/BclxL/CAT reporter plasmid and with either GFP- or a GFP-HuR expressing plasmid. We found that overexpression of GFP-HuR caused ~60% reduction in CAT expression compared with that of a GFP control (**Fig. 3D**). In contrast, overexpression of GFP-HuR had no effect on the expression of βgal. This observation was somewhat surprising since we have reported previously that overexpression of HuR caused a decrease in overall translation;[15] however, the decrease in global translation was observed by polysome profile analysis whereas in the current study we only followed expression of a single, ectopically expressed transgene. Overexpression of GFP-HuR had no effect on the expression of either reporter protein from the control plasmid pßgal/CAT, suggesting that HuR specifically regulates translation controlled by the Bcl_{xL} 5' UTR. We wished to test the activity of Bcl_{xL} 5' UTR deleted of the HuR binding site (probe F). However, this deletion causes the loss of basal IRES activity[12] (and data not shown) and we were therefore unable to test this construct in cells. Similarly, we were unable to perform the converse experiment, since downregulation of HuR by siRNA caused rapid degradation of the reporter RNA irrespective of which reporter plasmid was used in the assay.

HuR regulates mitochondrial morphology through Bcl_{xL}. Members of the Bcl-2 family of proteins, including Bcl_{xL} regulate the morphology of the mitochondrial network.[4,20] We therefore wished to investigate if reducing the levels of HuR, and the consequent increase in Bcl_{xL} expression, would impact mitochondrial morphology. U2OS cells were transiently transfected with siRNA targeting HuR or a non-silencing control, and the mitochondrial network was visualized 72 h later by staining the fixed cells for the outer mitochondrial marker Tom20. We observed that there was a 2.2-fold increase in fragmented mitochondria in HuR siRNA treated cells when compared with cells treated with non-silencing siRNA (**Fig. 4A and B**). Importantly, siRNA-mediated knockdown of Bcl_{xL} significantly reduced the portion of fragmented mitochondria (**Fig. 4A and B**). These results indicate that the induction of Bcl_{xL} expression through HuR downregulation contributes to the rearrangement of the mitochondrial network.

Discussion

Bcl_{xL} is a key component of the cellular anti-apoptotic machinery. In addition to regulating the release of pro-apoptotic mitochondrial factors such as cytochrome c and Smac/DIABLO, recent data suggests that Bcl_{xL} also actively participates in the control of mitochondrial dynamics that is independent of the regulation of apoptosis.[4] We and others have shown that in addition to the regulation of Bcl_{xL} splicing and transcription, control of Bcl_{xL} translation is the key regulatory step, in particular in response to cellular stress.[12-14] The 5' UTR of Bcl_{xL} was shown to harbour an IRES element that allows the Bcl_{xL} mRNA to be translated in times of cellular stress when global translation is attenuated. Translation of the majority of cellular mRNAs under normal growth conditions occurs by a cap-dependent mechanism that requires interaction of specific initiation factors with the 5' cap of the mRNA, followed by recruitment of ribosomal subunits, recognition of the AUG start codon and commencement of polypeptide chain elongation.[21] In contrast, IRES-mediated translation initiation was identified as key mechanism which supports cap-independent translation under conditions of cellular stress such as nutrient deprivation, hypoxia or gamma irradiation.[22] Although the precise mechanism of cellular IRES translation remains unclear, it has been postulated that cellular IRES require some of the canonical initiation factors as well as auxiliary proteins (termed IRES trans-acting factors, ITAFs) for their proper function.[23,24] Two such factors were identified previously for the Bcl_{xL} IRES. hnRNP A1, which accumulates in the cytoplasm in response to various forms of cellular stress, was shown to bind to the 5' UTR of Bcl_{xL} and to attenuate its translation during osmotic stress.[13] Although the precise mechanism of this inhibition was not elucidated, it was suggested that hnRNP A1 specifically sequesters Bcl_{xL} in stress granules in response to

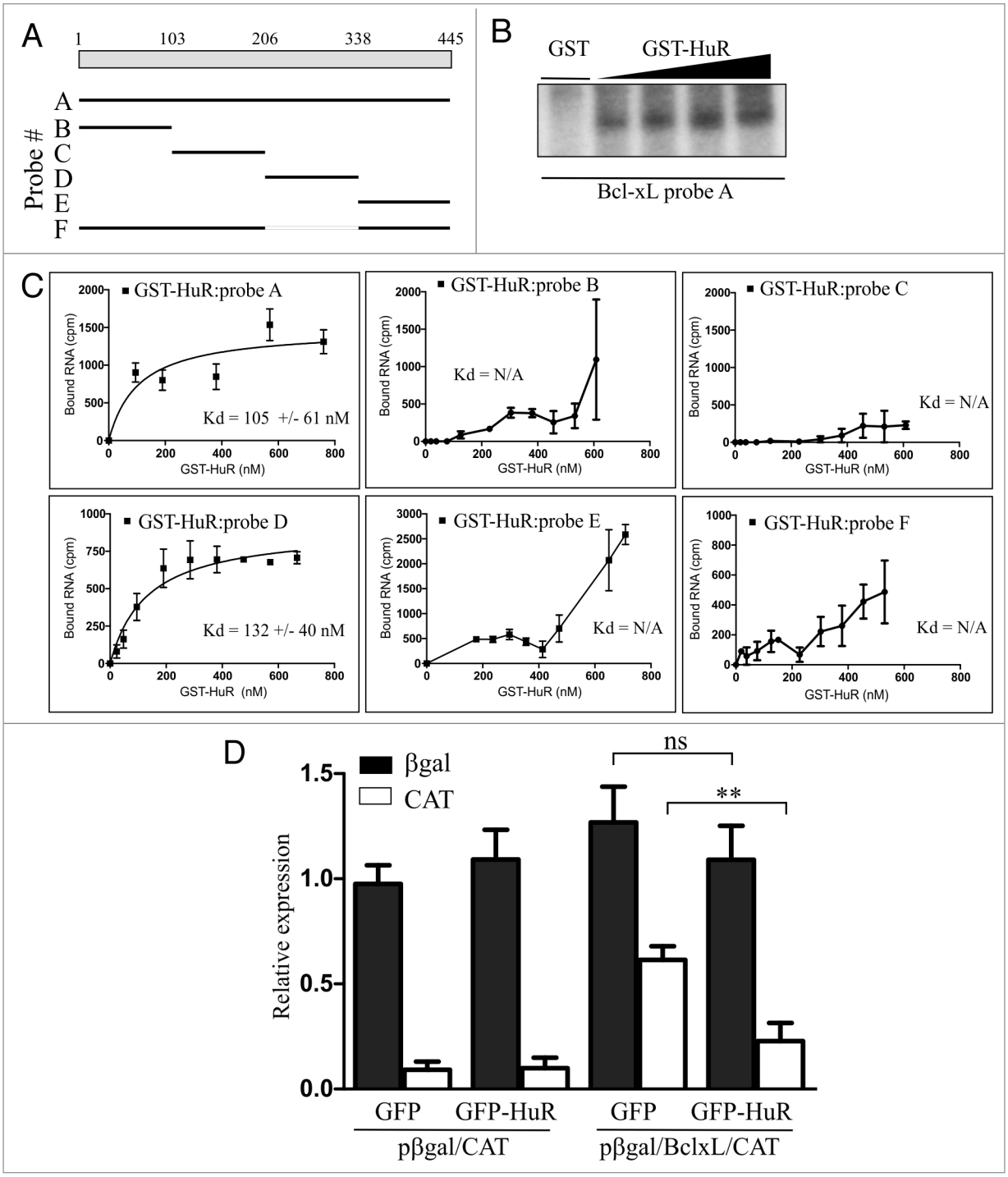

Figure 3. HuR binds directly to the 5' UTR of Bcl_{xL} and regulates its translation. (**A**) Schematic diagram of Bcl_{xL} 5' UTR and the deletion fragments used in the binding assays. (**B**) Recombinant GST-HuR was incubated in the presence of ^{32}P-labeled, in vitro transcribed RNA (probe A) and subjected to UV cross-linking. RNA-protein complexes were separated by SDS-PAGE and analyzed by autoradiography. GST was used as a negative control. (**C**) Increasing concentrations of GST-HuR were incubated with indicated, ^{32}P-labeled, in vitro transcribed RNAs and nitrocellulose filter binding assays were performed as described in Material and Methods. Levels of filter-bound RNA are plotted as a function of protein concentration. Apparent dissociation constants (K_d) are shown for each probe (mean +/- S.E.M., n = 3). (**D**) Bicistronic DNA construct containing Bcl_{xL} 5' UTR (pßgal/BclxL/CAT), or a parental vector (pßgal/CAT) were co-transfected into HEK293 cells along with GFP- or GFP-HuR expressing plasmids and the expression levels of each reporter gene were determined 24 h after transfection (**p < 0.01; n = 3).

hypertonic stress, thus inhibiting its translation. The second protein that similarly inhibits Bcl_{xL} translation is PDCD4.[14] PDCD4 is a tumor suppressor which was shown to act as an inhibitor of eIF4A, an RNA helicase component of the eIF4F complex

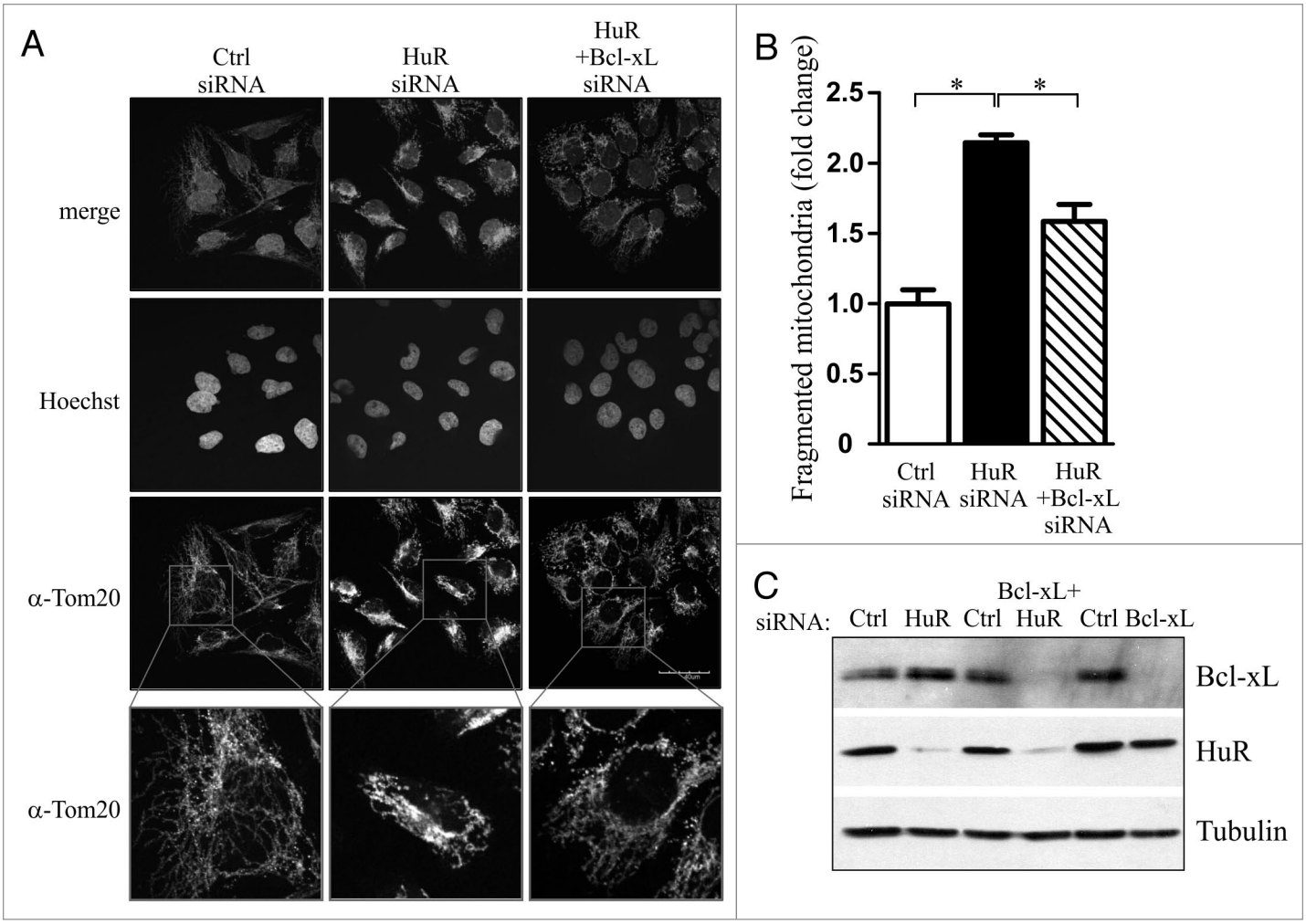

Figure 4. HuR regulates mitochondrial morphology through Bcl_{xL}. (**A**) U2OS were transiently transfected with HuR-targeting, non-silencing (Ctrl), or a combination of HuR- and Bcl_{xL} -targeting siRNA and the cells were prepared for immunofluorescent microscopy 72 h later. Mitochondrial morphology was visualized by staining with anti-Tom20 antibody. (**B**) The fraction of cells with fragmented mitochondria from (A) were determined by two blind observers and is plotted for each treatment (*$p < 0.05$; n = 83 cells for each treatment). (**C**) Expression levels of Bcl_{xL} and HuR in lysates from cells treated as in (A) were determined by western blot analysis.

which is required for the recruitment of capped mRNA to the ribosome.[25] Although PDCD4 is generally viewed as a general translation repressor, recent evidence suggests that PDCD4 preferentially represses mRNA with highly structured 5' UTRs[26,27] and those harbouring an IRES.[14] We have shown previously that PDCD4 specifically interacts with the 5' UTRs of at least two anti-apoptotic factors, XIAP and Bcl_{xL}, and mediates repression of their translation by interfering with the assembly of the 48S initiation complex.[14] In response to FGF-2 treatment of cells, PDCD4 is phosphorylated by S6K2 and targeted for proteosomal degradation, thus de-repressing translation of Bcl_{xL} and resulting in enhanced chemoresistance.[14,28] In the present study we have identified a third repressor of Bcl_{xL} translation. We show that HuR binds directly and specifically to the 5' UTR of Bcl_{xL}. Importantly, we showed that HuR translationaly regulates Bcl_{xL} through the IRES element located within the 5' UTR. Using the 5' UTR reporter construct we demonstrated that the IRES activity of Bcl_{xL} decreases upon overexpression of HuR, while the loss of HuR results in enhanced recruitment of Bcl_{xL} mRNA into polyribosomes and increase in Bcl_{xL} protein levels. Interestingly, the same three Bcl_{xL} ITAFs were also shown to regulate expression of another anti-apoptotic protein, XIAP[14,15,29] suggesting an existence of a common regulatory network(s) that may control selective translation of mRNAs involved in the regulation of cell survival in response to stress. However, in contrast to hnRNP A1 and PDCD4, HuR functions as an enhancer of XIAP but a repressor of Bcl_{xL} translation. Such dichotomy has been shown for HuR previously since it inhibits translation of some (e.g., p27;[30]) while enhancing activity of other (e.g., HCV;[31]) IRES. Although the basis for this dichotomy is not known, it is possible that conformational changes elicited by the binding of HuR to some IRES will render these elements non-functional, while binding to other IRES will induce conformation that is amenable to ribosome recruitment. In addition, the cohort of other ITAFs and possibly canonical initiation factors, which seems to be more or less distinct for various IRES elements, will play a significant

role in rendering HuR a repressor or activator of the given IRES. Of note, translation of Bcl_{xL} mRNA was shown previously to be significantly enhanced by overexpression of the cap-binding protein eIF4E[32] and this increase led to the inhibition of cytochrome c release from the mitochondria. Interestingly, the cohorts of HuR and eIF4E targets are at least partially overlapping[33] illustrating the interdependent regulation of critical genes involved in the regulation of cell growth, proliferation and apoptosis.

HuR is a multifunctional protein, a member of the ELAV family of RNA-binding proteins that has been implicated in a variety of RNA metabolism processes including mRNA stability and translational control.[34] Interestingly, HuR appears to play a dual role in the regulation of cell death and survival. It has been shown to orchestrate both the pro- and anti-apoptotic cascades depending on the upstream pathways that are triggered. It is believed that activation of distinct signaling pathways triggers HuR-dependent control of specific groups of mRNAs (HuR regulons), thereby altering the cellular state.[35] HuR prevents cell death by upregulating expression of inhibitors of apoptosis such as XIAP, ProT-α, p21, p53 and Survivin.[15,36-38] Conversely, HuR suppresses pro-apoptotic factors such as Fas1.[39] This HuR-dependent pro-survival regulation correlates well with the observations that HuR levels are elevated in numerous cancers and suggests that HuR could attenuate cell death from occurring in malignant cells and tumors, thereby promoting resistance to radiation and chemotherapies.[40] However, other data suggests that when cellular stress is prolonged or lethal, a threshold is reached where HuR engages the apoptotic cascade. For example, upon staurosporine treatment, HuR is translocated to the cytoplasm in association with apoptosome activator pp32/PHAP-1 and is subsequently cleaved by caspases into a fragment (HuR-CP1) which was found to amplify the apoptotic response.[41] Interestingly, it was observed that in muscle cells the HuR-CP1 fragment caused cytoplasmic accumulation of full length HuR and subsequent enhancement of myogenesis.[42] Our data places Bcl_{xL} within the HuR-regulated operon and shows that HuR, in addition to directly orchestrating cellular antiapoptotic program also contributes to the control of mitochondrial morphology.

Material and Methods

Cell culture, expression constructs and transfection. U2OS and HEK293 cells were maintained at 37°C, 5% CO_2 in complete Gibco®'s DMEM (1% FBS, 1% glutamine, 100,000 U/L penicillin and 100 μg/L streptomycin). The bicistronic reporter plasmid pßgal/BclxL/CAT has been described previously[14] and contains the 5' UTR of Bcl_{xL}.[12] The GST-HuR and GFP-HuR expressing plasmids were described previously.[41] For siRNA knockdown experiments, $1.5x10^5$ cells were reverse transfected in a 6-well plate with 10 nM HuR siNRA, 30 nM Bcl_{xL} siRNA, or corresponding non-silencing controls (Dharmacon) following the manufacturer's protocol (Lipofectamine™ RNAiMAX, Invitrogen). Cells were harvested 72 h later. For overexpression experiments, $1.5x10^5$ cells were seeded in a 6-well plate for 24 h, then 2 μg of GFP or GFP-HuR plasmid DNA was transfected following the manufacturer's protocol (JetPrime™, Polyplus transfection). Cells were harvested 24 h later for RNA extraction or Western Blot analysis. For rescue experiments, $1.5x10^5$ cells were first reverse transfected with 10 nM HuR siRNA or non-silencing control for 48 h, then 2 μg of GFP or GFP-HuR plasmid DNA was forward transfected for an additional 24 h and cells were harvested for further analysis.

UV-crosslinking and nitrocellulose filter binding assays of RNA-protein complexes. Radioactive Bcl_{xL} 5' UTR probes for UV-crosslinking and nitrocellulose filter binding assays were prepared by an in vitro transcription using Maxiscript® kit (Ambion) and gel purified. RNA-protein UV-crosslinking experiments and the nitrocellulose binding assay were conducted as previously described.[14,15]

β-galactosidase and CAT analysis. Transiently transfected cells were washed in 1 ml of phosphate-buffered saline and harvested in 300 μl CAT ELISA kit lysis buffer according to the protocol provided by the manufacturer (Roche Molecular Biochemicals). β-galactosidase (β-gal) enzymatic activity was determined by spectrophotometric assay using o-nitrophenyl-β-D-galactopyranoside as previously described.[43] CAT levels were determined using the CAT ELISA kit according to the protocol provided by the manufacturer (Roche Molecular Biochemicals).

Western blot analysis. Cells were washed in 1 ml phosphate-buffered saline and lysed in 150 μl RIPA buffer (50 mM Tris base pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% SDS, 0.25 g deoxycholate in 50 mL water; 1 mM PMSF and 10 μg/mL leupeptin were added fresh) for 30 min at 4°C, followed by centrifugation at 12,000 g for 10 min to pellet debris. Protein concentration was determined by the BCA Protein Assay Kit (Pierce Biotechnology) and equal amounts of protein extract were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane by wet transfer. Samples were analyzed by western blotting using rabbit anti-Bcl_{xL} (Cell Signaling Technologies), mouse monoclonal anti-HuR (Santa Cruz Biotechnology), mouse anti-GAPDH (Advanced Immunochemical Inc.), mouse anti-Tubulin (Abcam), rabbit anti-Mcl-1 (Santa Cruz Biotechnology), or rabbit anti-Bcl-2 (Cell Signaling Technologies) followed by secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG; Cell Signaling Technologies). Antibody complexes were detected using the ECL or ECL Plus systems (GE Healthcare) and were quantified using Odyssey densitometry software (Li-Cor, Lincoln).

RNA extraction, RT-PCR and quantitative RT-PCR analysis. Total RNA was isolated from transfected cells using RNazol according to the manufacturer's instructions (Molecular Research Center). cDNA was generated using an oligo dT18 primer and the qScript™ cDNA Supermix according to the protocol provided by the manufacturer (Quanta Biosciences). The synthesized cDNA was used as the template for quantitative PCR using the QuantiTect SYBR Green PCR kit (Qiagen) along with gene specific primers for Bcl_x, Bcl_{xL} (QuantiTect Primer Assay, Qiagen), and GAPDH,[15] and analyzed on a Mastercycler realplex (Eppendorf) real-time thermocycler using the associated realplex software. Relative expression levels were determined using the standard curve method. Controls lacking RT demonstrated no significant genomic DNA amplification (> 10 cycle difference).

For splice-specific variant RT-PCR, the cDNA was used as a template in standard PCR with KOD polymerase and primers Bcl-X2 and Bcl-X3 as described.[44]

Polysome profiling. HEK293 cells were reverse transfected with 10 nM HuR siRNA or non-silencing control siRNA for 72 h in 6-well plates (2 plates per condition) and polysome isolation was done as described.[14] Gradient fractions were collected from the top using the ISCO Teledyne programmable gradient fractionation system, and RNA was monitored at 254 nm. RNA was isolated from individual fractions (1 ml) by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Equal quantities of RNA from each fraction were used to generate cDNA as described above. Quantitative RT-PCR was used to determine the abundance of Bcl_{xL} and GAPDH mRNA in each fraction as described above.

Immunofluorescent microscopy. U2OS cells were seeded at a density of 7.5 x 10^4 cells on coverslips in 6-well plates for 24 h before transfection with HuR siRNA, Bcl_{xL} siRNA, or a non-silencing control siRNA for 72 h. Cells were rinsed 3 times in phosphate-buffered saline, fixed with 3.7% paraformaldehyde for 15 min and then permeabilized with 0.2% Triton-X100 for 15 min before blocking in 1% FBS for 15 min. Coverslips were incubated with α-Tom20 anti-rabbit antibody (Santa Cruz; 1:2000 diluted in 0.2% Triton-X100/ 0.004% BSA) for 1 h, washed 3x for 5 min in Triton-X100/0.004% BSA buffer before incubation with secondary antibody for 1 h (Alex Fluor A594 goat anti-rabbit, Invitrogen, diluted in 0.2% Triton-X100/ 0.004% BSA). Hoechst dye was added (1 μg/mL) for 10 min to stain the nuclei and cells were washed 4x for 5 min in phosphate-buffered saline prior to mounting. Coverslips were mounted on slides using Dako Fluorescent Mouting Medium. Confocal microscopy was performed using the 60X objective with water (Olympus Fluoview FV1000, Richmond Hill, Ontario Canada).

Statistical analysis. All data are expressed as means ± standard error of the mean (SE), with a minimum of three independent experimental replicates unless otherwise noted. For reporter assays and for quantitative RT-PCR, independent replicates consisted of three biological triplicate experiments. A student t-test was performed to determine data significance using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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