

# New insights on the functional role of URG7 in the cellular response to ER stress

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**Background information.** Up-regulated Gene clone 7 (URG7) is an ER resident protein, whose expression is up-regulated in the presence of hepatitis B virus X antigen (HBxAg) during HBV infection. In virus-infected hepatocytes, URG7 shows an anti-apoptotic activity due to the PI3K/AKT signalling activation, does not seem to have tumorigenic properties, but it appears to promote the development and progression of fibrosis. However, the molecular mechanisms underlying URG7 activity remain largely unknown.

**Results.** To shed light on URG7 activity, we first analysed its interactome in HepG2 transfected cells: this analysis suggests that URG7 could have a role in affecting protein synthesis, folding and promoting proteins degradation. Moreover, keeping into account its subcellular localisation in the ER and that several viral infections give rise to ER stress, a panel of experiments was performed to evaluate a putative role of URG7 in ER stress. Our main results demonstrate that in ER-stressed cells URG7 is able to modulate the expression of Unfolded Protein Response (UPR) markers towards survival outcomes, up-regulating GRP78 protein and down-regulating the pro-apoptotic protein CHOP. Furthermore, URG7 reduces the ER stress by decreasing the amount of unfolded proteins, by increasing both the total protein ubiquitination and the AKT activation and reducing Caspase 3 activation.

**Conclusions.** All together these data suggest that URG7 plays a pivotal role as a reliever of ER stress-induced apoptosis.

**Significance.** This is the first characterisation of URG7 activity under ER stress conditions. The results presented here will help to hypothesise new strategies to counteract the antiapoptotic activity of URG7 in the context of the viral infection.



Additional supporting information may be found online in the Supporting Information section at the end of the article.

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**Key words:** Apoptosis, ER stress, PI3K/AKT, UPR, URG7.

**Abbreviations:** BiP, binding immunoglobulin protein/GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; ER, Endoplasmic Reticulum; HBV, Hepatitis B virus; HBxAg, Hepatitis B virus X antigen; MRP6, Multidrug-Resistance Protein 6; PARP-1, poly(ADP-ribose) polymerase; Tht, thioflavin T; TN, tunicamycin; UPR, Unfolded Protein Response; URG7, Up-regulated Gene clone 7; XBP1, X Box-Binding Protein 1/sXBP1, spliced X Box-Binding Protein 1.

## Introduction

Hepatitis B virus (HBV) infection is one of the most common chronic viral infection in the world: it is estimated that about 700,000 people die each year from complications of this form of hepatitis, the most important of which are both cirrhosis and liver cancer [Trépo *et al.*, 2014; Meireles *et al.*, 2015]. Despite many studies have been carried out, the exact

mechanisms underlying the HBV-mediated hepatocarcinogenesis are not fully understood [Xu *et al.*, 2014; Levrero and Zucman-Rossi, 2016].

The viral regulatory protein X (Hepatitis B x antigen, HBxAg/HBx) is a *trans*-activating protein involved in viral life cycles, viral–host interactions, cell cycle regulation, cell proliferation and apoptosis [Tang *et al.*, 2006; Feitelson *et al.*, 2014], affecting host gene expression [Hann *et al.*, 2004; Wang *et al.*, 2012]. Several differentially expressed mRNAs affected by HBx were identified by subtractive hybridisation [Lian *et al.*, 1999]; among these, the Up-Regulated Gene clone 7 (URG7) encodes a 99 amino acid polypeptide [Lian *et al.*, 2001; Ostuni *et al.*, 2014], sharing the first 74 residues with the N-terminal region of the multidrug-resistance protein 6 (MRP6) [Ostuni *et al.*, 2010, 2011; CuvIELlo *et al.*, 2015; Miglionico *et al.*, 2016]. Previous studies have demonstrated that URG7 contributes to the development and progression of fibrosis through the activation of the PI3K/AKT signalling pathway, which down-regulates the expression of the TGF $\beta$ 1 inhibitor,  $\alpha$ 2M [Feitelson *et al.*, 2012]. Furthermore, URG7 overexpression does not promote colony formation in soft agar nor tumour formation *in vivo* [Lian *et al.*, 2001]. Moreover, it seems that URG7 inhibits TNF $\alpha$ -mediated cell death by blocking caspases 3 and 8 and by stabilisation of  $\beta$ -catenin, suggesting its protective role in host cells during chronic HBV infection [Pan *et al.*, 2007]. Recently, we have demonstrated that URG7 is localised in the ER and its predicted transmembrane segment is integrated into the ER membrane with an N<sub>lumen</sub>-C<sub>cytosol</sub> orientation [Ostuni *et al.*, 2013; Lee *et al.*, 2014].

It is well known that during viral infection the large amount of viral/host proteins synthesised by the cell affects the endoplasmic reticulum (ER) homeostasis, resulting in the accumulation of unfolded and misfolded proteins in the ER-lumen. This stressful condition (ER stress) is sensed by an ER chaperone protein, the binding immunoglobulin protein (BiP)/glucose-regulated protein 78 (GRP78), allowing the cells to activate specific pro-survival signalling pathway known as Unfolded Proteins Response (UPR). This pathway increases protein folding ability, attenuates protein synthesis and degrades the excess of misfolded proteins. The key regulators of this adaptive mechanism are the transmembrane protein inositol-requiring protein-1

(IRE1 $\alpha$ ), the activating transcription factor-6 (ATF6) and the protein kinase RNA-like ER kinase (PERK), which are the three arms of the UPR [Malhi and Kaufman, 2011; Dufey *et al.*, 2014]. If the UPR fails to restore the ER homeostasis, it promotes cell death through the induction of the specific proapoptotic genes [Logue *et al.*, 2013].

It was suggested that HBV activates the UPR through the expression of HBx to relieve the ER stress, thus helping virus replication and cell survival, perhaps contributing to the pathogenesis of hepatic diseases [Li *et al.*, 2007].

In this context, the aim of this work is to elucidate the cellular mechanisms in which URG7 might be involved. We firstly analyzed its interactome in HepG2 transfected cells. Based on the results of the proteomic analysis and keeping into account the URG7 subcellular localisation in the ER, a panel of experiments was performed.

Our results demonstrated that URG7 is able to mitigate ER stress, promoting protein folding and ubiquitination and affecting the UPR response. These findings clarify many aspects of its anti-apoptotic activity and suggest that URG7 plays a crucial role as a reliever of ER stress-induced apoptosis.

## Results and discussion

### URG7 interactome investigation

In order to investigate the mechanism of action of URG7, a proteomic experiment was carried out, aimed at the isolation and identification of proteins able to interact with it. URG7 involving protein complexes were isolated by immunoprecipitation from a total protein lysate of FLAG-URG7 HepG2 transfected cells and individual protein components were separated by SDS-PAGE and identified by nanoLC-MS/MS. HepG2 cells transfected with mock were used as control. URG7 putative interactors occurring in both the sample and the control were discarded and only those proteins solely present in the sample were considered. The proteins identified as potential URG7 proteins partners are reported in Supplementary Table S1, where gene name, the UniProt code and other information are also reported.

The whole set of putative URG7 interacting proteins was analysed using the STRING functional protein interaction networks (<http://string-db.org/>).

**Figure 1 | The clustering analysis**

(A) The protein–protein interaction network was explored using STRING software. (B) According to “Kyoto Encyclopedia of Genes and Genomes (KEGG)” database, it is shown one of the most significant pathways (“Protein processing in endoplasmic reticulum”,  $P$  value:  $4.24E-6$ ). (C) According to Gene Ontology (GO), it is shown one of the most significant biological processes (“Protein localization to endoplasmic reticulum”,  $P$  value:  $2.09E-9$ ). In B and C, all proteins involved in the above-mentioned networks are shown in red. (D) URG7 associates with GRP78: proteins from HepG2 cell lysates were immunoprecipitated with a monoclonal anti FLAG-antibody in cells expressing 3XFLAG-URG7 (+) or the negative control expressing the empty vector. Immunocomplexes were fractionated by SDS-PAGE, blotted on nitrocellulose, and revealed by the anti-GRP78 antibody which revealed a protein band with an electrophoretic motility expected for GRP78.

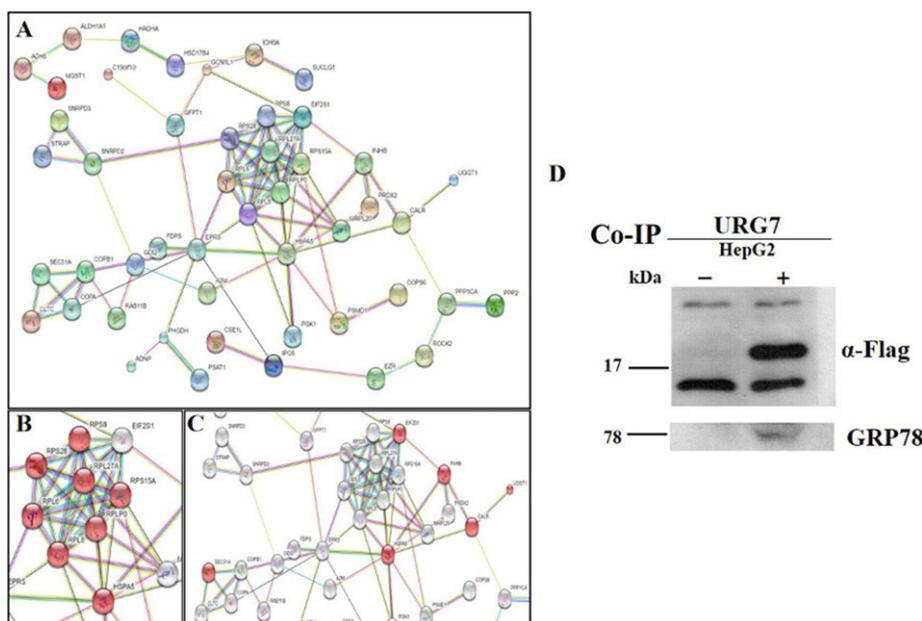


Figure 1A and Tables 1 and 2 show the statistically significant pathways and processes ( $P < 0.001$ ) predicted by Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology Biological Process (GO\_Biological Process), respectively.

In both KEGG pathway and GO Biological Process, a consistent number of putative URG7 interactors gathered with high scores in the functional classes named ‘Protein processing in endoplasmic reticulum’ ( $P$  value  $4.24E-6$ ) and ‘Protein localization to endoplasmic reticulum’ ( $P$  value  $2.09E-9$ ), respectively. The relevance of such processes is in agreement with the localization of URG7 in the endoplasmic reticulum membrane. ‘Protein processing in endoplasmic reticulum’ category involved the following proteins: UGGT1, EIF2S1, P4HB (PDIA1), HSPA5 (GRP78), CALR, SEC31A, while ‘Protein localization to endoplasmic reticulum’ included RPS28, RPL8, RPLP0, RPL27A, HSPA5 (GRP78), RPS8,

**Table 1 | KEGG Clustering Analysis**

Pathways	Number of Genes	$P$ Value
Ribosome	8	$1.54E-9$
Carbon metabolism	6	$2.82E-7$
Protein processing in endoplasmic reticulum	6	$4.24E-6$
Metabolic pathways	13	$7.42E-6$
Degradation of aromatic compounds	2	$2.05E-5$
Glycolysis / Gluconeogenesis	3	$5.35E-4$
Biosynthesis of amino acids	3	$8.4E-4$
Propanoate metabolism	2	$3.22E-3$

RPL6 and RPS15A. The graphical representation of the pathways and processes with proteins identified in this study (highlighted in red) were reported in Figures 1B and 1C.

**Table 2 | GO Biological Process Clustering Analysis**

Process	Number of Genes	P Value
Intracellular protein transport	14	1.55E-9
Protein localization to endoplasmic reticulum	8	2.09E-9
Translational initiation	9	5.94E-9
Cellular protein localization	16	6.03E-9
Catabolic process	19	1.1E-8
SRP-dependent cotranslational protein targeting to membrane	7	1.27E-8
Nuclear-transcribed mRNA catabolic process	8	1.52E-8
Viral transcription	7	1.75E-8
Response to endoplasmic reticulum stress	8	4.39E-8

Interestingly, among URG7 interacting proteins we found the binding immunoglobulin protein (BiP)/glucose-regulated protein 78 (GRP78), a chaperon primarily resident within the ER-lumen to facilitate the protein folding, also known to be the main cellular ER stress sensor [Zhang and Zhang, 2010]. Given the primary function of GRP78 to bind to many nascent polypeptides, this validated interaction (Figure 1D) could also explain the large number of proteins co-immunoprecipitated with URG7.

It is also meaningful the presence of other ER proteins among the identified interactors, such as: UDP-glucose glycoprotein glucosyltransferase (UGGT1) and calreticulin (CALR), both involved in the quality control for protein folding in the endoplasmic reticulum [Totani *et al.*, 2009; Gold *et al.*, 2010], and the prolyl 4-hydroxylase, subunit beta (P4HB or PDIA1), a multifunctional protein that catalyses the formation, breakage and rearrangement of disulphide bonds, also acting as a chaperone that inhibits aggregation of misfolded proteins [Kosuri *et al.*, 2012]. A large number of identified proteins has been classified by Gene Ontology as proteins involved in 'Intracellular protein transport' (14 genes,  $P = 1.55E-9$ ). In particular, the alpha (COPA) and beta1 (COPB1) subunits of the coatamer protein complex, as well as the SEC31A and the GDP dissociation inhibitor 2 (GDI2) are involved in cellular trafficking between ER and the Golgi apparatus [Barlowe and Miller, 2013]. The presence of these proteins and their

localisation at ER membrane may suggest a putative role for URG7 in the formation of vesicles travelling from ER to Golgi, which could be investigated in the future.

Among cytosolic proteins, particularly interesting are those involved in protein synthesis such as GCN1L1 and PSAT1 that control protein [Cambiaghi *et al.*, 2014] and amino acid synthesis [Basurko *et al.*, 1999], respectively. However, proteins involved in protein degradation are also present among URG7 interactors, such as the PSMD1 proteasome complex and the protein ubiquitin-conjugating enzyme UBE2N [Deng *et al.*, 2007; van Wijk *et al.*, 2009].

The occurrence of these putative interactions suggests that URG7 could have a role in the cellular response to ER stress either affecting protein synthesis, folding or promoting unfolded proteins degradation.

#### URG7 modulates UPR sensors expression in HepG2 cells treated with TN

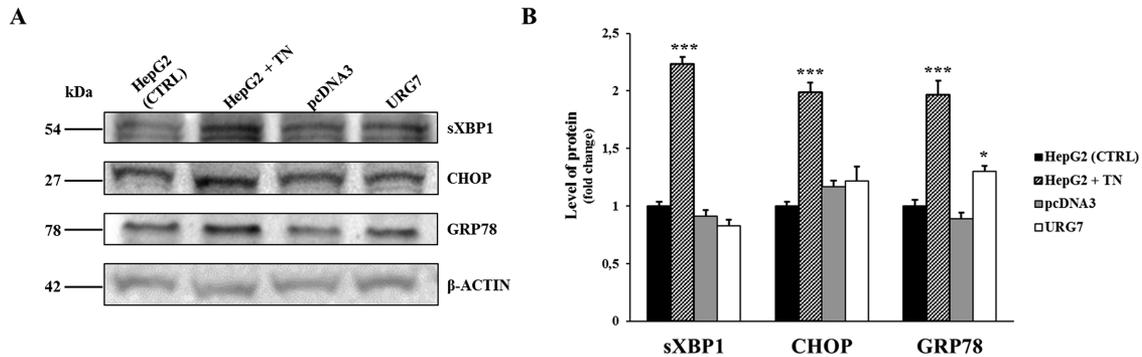
Based on the results of the proteomics analysis, a panel of experiments was performed. We first evaluated the putative role of URG7 in the specific pro-survival signalling pathway known as Unfolded Protein Response (UPR) in HepG2 transfected cells, monitoring the expression of specific ER stress markers: the GRP78 protein, previously found among URG7's interactors; the pro-apoptotic transcription factor C/EBP homologous protein (CHOP) [Oyadomari and Mori, 2004], and the spliced X Box-Binding Protein 1 (sXBP1), a transcription factor able to promote the expression of several genes, involved both in pro-survival or apoptotic pathways, depending on stress level [Lee *et al.*, 2003].

As shown in Figure 2 (A, Western blotting and B, densitometric analysis), in the HepG2 cells treated with the antibiotic TN for 15 h the expression levels of these markers were raised more than twofold compared with untreated cells, demonstrating that HepG2 cells are able to organize the UPR response when treated with an ER stressor.

To investigate whether URG7 expression was able to affect ER stress, the expression levels of the same ER stress markers, 48 h after URG7 transient transfection, were monitored and the results are reported in Figure 2. In URG7 HepG2 transfected cells, the protein expression levels of the ER stress markers sXBP1 and CHOP were almost unaltered

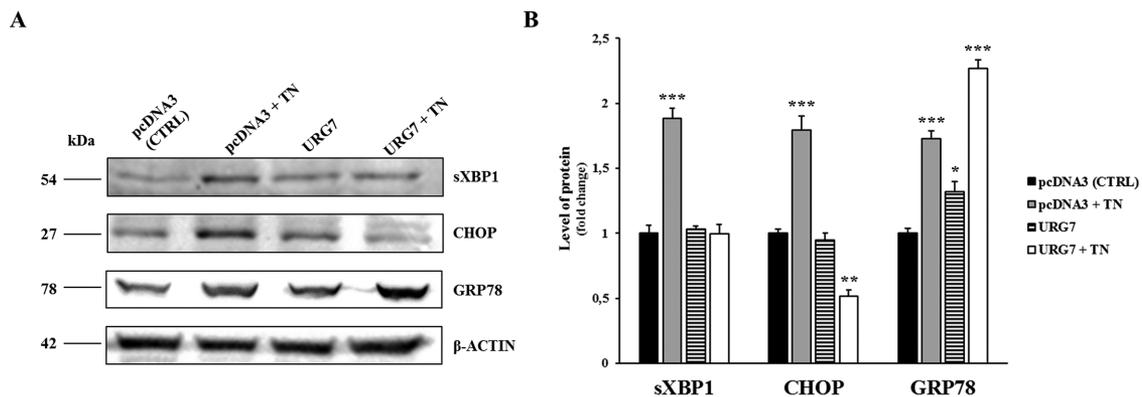
**Figure 2 | Effect of URG7 on ER stress markers expression levels**

(A) Representative Western blotting of ER-stress markers measured in mock and URG7 transfected cells. HepG2 cells treated with 3  $\mu\text{g/ml}$  of tunicamycin for 15 h are considered as the positive control of ER stress induction. In the densitometric analysis (B) (relative to three independent experiments) results are expressed as fold change compared with the control value (unstressed cells). Statistical analysis was carried out by a paired two-tailed Student's *t*-test ( $*P \leq 0.05$ ,  $***P \leq 0.001$ ,  $n = 3$ ). Data were represented as mean  $\pm$  S.E.M.



**Figure 3 | Effect of URG7 expression on tunicamycin-treated cells**

(A) Representative Western blotting followed by densitometric analysis (B) of ER-stress markers measured in mock and URG7 transfected cells treated with 3  $\mu\text{g/ml}$  of tunicamycin for 15 h. Results from three independent experiments are expressed as fold change compared with the control value (mock). Statistical analysis was carried out by a paired two-tailed Student's *t*-test ( $*P \leq 0.05$ ,  $**P \leq 0.01$ ,  $***P \leq 0.001$ ,  $n = 3$ ). Data were represented as mean  $\pm$  S.E.M.



compared with HepG2 control cells (CTRL), whereas the amount of GRP78 was slightly increased. This result suggests that the overexpression of URG7 does not affect *per se* the endoplasmic reticulum homeostasis and does not trigger significantly the UPR.

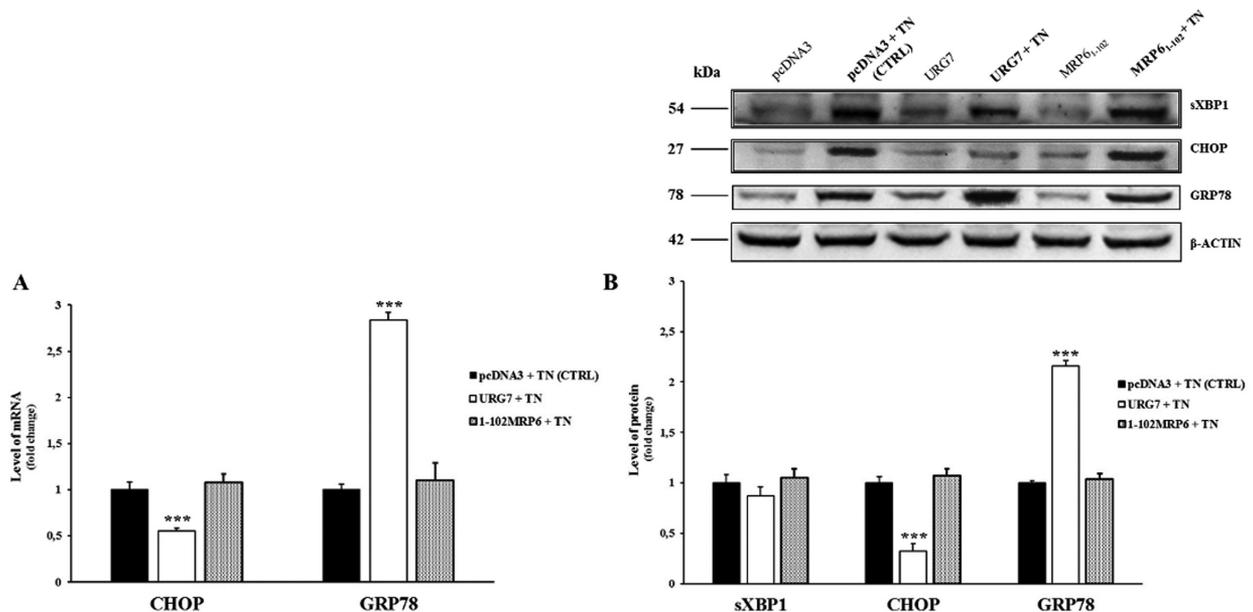
To investigate about a putative role of URG7 protein in response to ER stress, the expression of some UPR markers was monitored in URG7 transfected cells and then treated with TN. As shown in Fig-

ure 3, in mock cells treated for 15 h with TN, a clear up-regulation of about 70–80% of all three ER stress markers sXBP1, CHOP and GRP78 proteins was observed.

Conversely, the expression of URG7 protein led to a different effect in TN-treated cells: the expression of CHOP, a proapoptotic factor, was reduced by 50%, while the GRP78 expression was increased by about 100%, even more than the relative control

**Figure 4 | Specificity of the URG7 role in stressed transfected cells**

(A) Real time PCR and (B) representative Western blotting followed by densitometric analysis of ER-stress markers measured in pcDNA3, URG7 and MRP6<sub>1-102</sub> transfected cells, also treated with 3 µg/ml of tunicamycin for 15 h. Results from three independent experiments are expressed as fold change compared with the control value (mock + TN). Statistical analysis was carried out by a paired two-tailed Student's *t*-test ( $***P \leq 0.001$ ,  $n = 3$ ). Data were represented as mean  $\pm$  S.E.M.



(mock + TN). Finally, sXBP1 expression remained unchanged in both control and TN conditions, highlighting that the IRE1-XBP1 arm of UPR seems to be not activated at all.

These results clearly demonstrate that URG7 is able to differently modify the UPR under ER stress conditions, switching the expression pattern in favour of a pro survival response, especially knowing the well-documented role that the increase in GRP78 expression level has in cell survival [Ni *et al.*, 2011]. These data are in agreement with the observation that HBV is able to suppress apoptosis via inhibiting CHOP expression in hepatocarcinoma cells [Zhao *et al.*, 2017].

The specificity of URG7 in response to ER cellular stress was also investigated. For this purpose, HepG2 cells were transfected with a construct codifying a protein corresponding to the N-terminal portion (1–102) of the Multidrug Resistance Protein isoform 6 (MRP6), indicated as MRP6<sub>1-102</sub>. This polypeptide shares the first 74 amino acids with URG7, contains the first two predicted transmembrane  $\alpha$ -helices of MRP6 and it is inserted in the ER membrane with

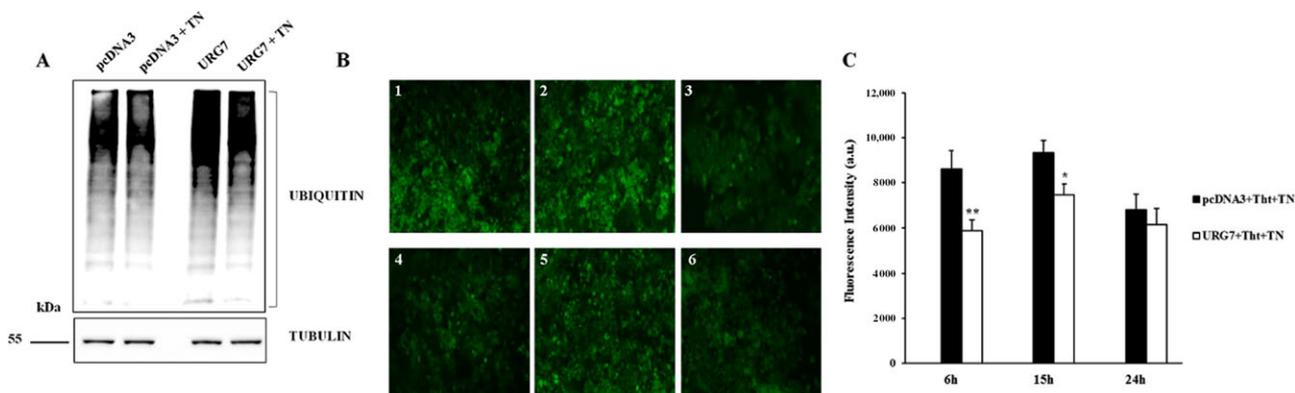
the N-terminus in the lumen [Ostuni *et al.*, 2013]. As shown in Figure 4, both the mRNA and protein levels of the sXBP1, CHOP and GRP78 markers were unchanged compared to TN-treated mock cells, revealing that the MRP6<sub>1-102</sub> overexpression in stressed cells does not affect the UPR. This suggests that the specific carboxy-terminal cytosolic tail of URG7 (75–99 aa) may be involved in the modulation of the UPR under ER stress conditions.

#### URG7 affects protein folding and ubiquitination

To further investigate about the putative role of URG7 as ER stress reliever, we evaluated the protein ubiquitination pattern in URG7 and mock transfected cells, after treatment with TN for 15 h. As shown in Figure 5A, URG7 expression caused a strong increase compared to the control, in the total amount of ubiquitinated proteins, under ER stress conditions induced by TN. These data perfectly agree with the previously reported demonstration that viral replication is favoured by inhibition of the proteasome [Zhang *et al.*, 2010] and could be eventually

**Figure 5 | URG7 affects misfolded protein elimination and protein ubiquitination**

(A) URG7 favours ubiquitination: representative western blotting of ubiquitinated proteins in mock and URG7 transfected cells, also treated with 3  $\mu\text{g/ml}$  of tunicamycin for 15 h. Statistical analysis was carried out by a paired two-tailed Student's *t*-test ( $*P \leq 0.05$ ,  $**P \leq 0.01$ ,  $n = 3$ ). Data were represented as mean  $\pm$  S.E.M. (B) Representative cell images and (C) 2.5  $\mu\text{M}$  thioflavin fluorescence intensity analysis, determined and quantified in HepG2 cells transfected with mock (1,2,3) and URG7 (4,5,6) plasmids and treated with 3  $\mu\text{g/ml}$  of tunicamycin for 6 h (a,d), 15 h (b,e) and 24 h (c,f). Images were analysed using Image J software program.



correlated with the presence of the ubiquitin ligase UB2EN among the URG7 interactors.

To test whether URG7 protein was also able to affect unfolded protein levels within ER under stressed conditions, the cells were stained with ThT, a fluorophore used to quantitate ER stress since it is able to bind protein aggregates into the endoplasmic reticulum [Beriault and Werstuck, 2013]. We found that the total amount of unfolded proteins was lower in the presence of URG7 compared with control (Figures 5B and 5C): this is also in agreement with a previous study showing that, during HBV infection, the ER associated degradation (ERAD) pathway is significantly activated [Lazar *et al.*, 2012], thus unburdening the endoplasmic reticulum. Our data suggest that URG7 allows a crucial mitigation of ER stress through an increased ubiquitination of proteins destined to subsequent degradation, and emphasize the relevance of the cellular protein folding and degradation machinery in the context of URG7 antiapoptotic activity.

**URG7 promotes cell survival and inhibits apoptosis in stressed conditions**

Considering that URG7 is an activator of the PI3K/AKT pathway [Pan *et al.*, 2007], the phospho-

rylation of AKT in URG7 and mock transfected cells, after treatment with TN for 15 h, was also evaluated. Figure 6 shows, in URG7 transfected cells, a higher level of AKT phosphorylation in stressed condition compared with the unstressed state.

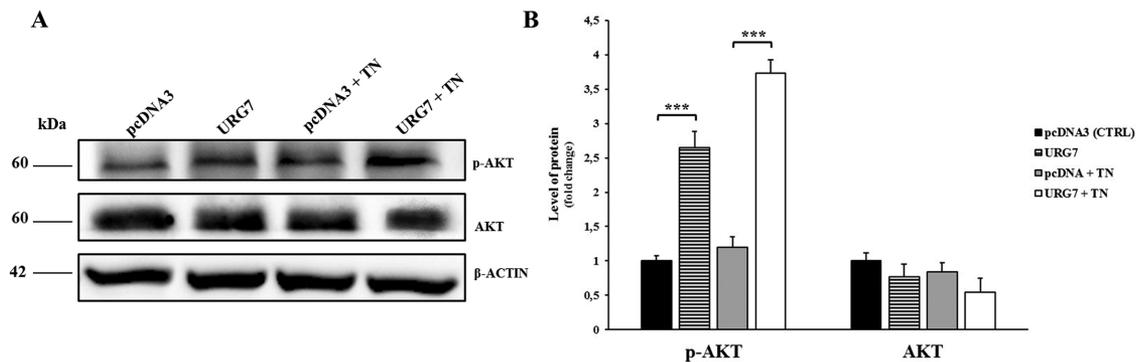
This result allows us to suggest that the abilities of URG7 both to modulate the ER stress as well as to trigger the AKT activation may be strictly linked, keeping also into account that the AKT activation is related to the expression levels of ER stress markers GRP78 [Dai *et al.*, 2010; Lin *et al.*, 2015; Song *et al.*, 2016] and CHOP [Yu *et al.*, 2016a, 2016b], or to the cellular protein ubiquitination [Yang *et al.*, 2010].

Finally, it was also evaluated, under ER stress condition, the expression level of the protein poly(ADP-ribose) polymerase (PARP-1), which is one of the main cleavage targets of caspase 3 *in vivo*. Western blotting analysis (Figure 7) shows a lower amount of the cleaved form (cPARP-1) in URG7 transfected cells, suggesting that the expression of URG7 counteracts the apoptosis activation.

Although the exact molecular mechanism through which URG7 is able to act as ER stress reliever is not yet fully clarified, our results let us to assert that the further activation of the survival PI3K/AKT pathway and the apoptosis inhibition are the consequence of URG7's ability to affect the ER stress.

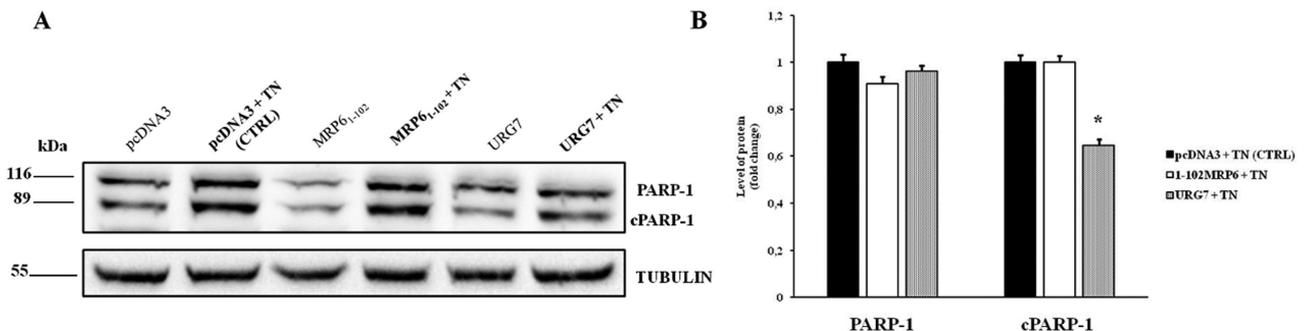
**Figure 6 | PI3K/AKT pathway markers analysis in HepG2 transfected and ER-stressed cells**

(A) Representative Western blotting followed by densitometric analysis (B) of AKT and p-AKT (Ser473) proteins measured in mock and URG7 transfected cells, also treated with 3  $\mu\text{g/ml}$  of tunicamycin for 15 h. Results from three independent experiments are expressed as fold change compared with the control value (mock + TN). Statistical analysis was carried out by a paired two-tailed Student's *t*-test ( $***P \leq 0.001$ ,  $n = 3$ ). Data were represented as mean  $\pm$  S.E.M.



**Figure 7 | Effects of URG7 on apoptosis-related protein expression in HepG2-stressed cells**

(A) Representative Western blotting followed by densitometric analysis (B) of PARP-1 and cleaved PARP-1 (cPARP-1) proteins measured in pcDNA3, URG7 and MRP6<sub>1-102</sub> transfected cells, also treated with 3  $\mu\text{g/ml}$  of tunicamycin for 15 h. Results from three independent experiments are expressed as fold change compared with the control value (mock + TN). Statistical analysis was carried out by a paired two-tailed Student's *t*-test ( $*P \leq 0.05$ ,  $n = 3$ ). Data were represented as mean  $\pm$  S.E.M.



## Conclusions

Although it is known that HBV infection causes ER stress, essentially due to the large amount of viral proteins produced, data reported in this study are the first characterization of the activity of URG7 protein under ER stress conditions. The protein-protein interactors network (many of which related to synthesis, folding and degradation of the cellular proteins) is consistent with the ability of URG7 to act as ER stress reliever. Moreover, the UPR switch towards an increased cell survival, in the presence of URG7, is probably related to the further activation of the PI3K/AKT pathway and we can speculate that

this activation may be actually mediated by the up-regulated UPR marker GRP78. This consideration is of importance because it suggests new opportunities to counteract the antiapoptotic activity of this small protein.

## Material and methods

### Reagents and antibodies

Dulbecco's Modified Eagle Medium (DMEM), tunicamycin (TN) and thioflavin (ThT) were purchased from Sigma-Aldrich. Trypsin-EDTA solution, foetal bovine serum (FBS), glutamine, penicillin-streptomycin and PBS solutions were purchased from Euroclone.

## URG7 and ER stress

Mouse anti-FLAG M5 was purchased from Sigma–Aldrich. Rabbit anti-CHOP and rabbit anti-XBP-1s were purchased from Biologend. Rabbit anti-GRP78 was purchased from Abcam. Rabbit anti-p-AKT (Ser473), anti-AKT and anti-PARP-1 were from Cell Signalling. Mouse anti-ubiquitin was purchased from Santa Cruz BioTechnology. Mouse anti- $\beta$ -actin was purchased from Abcam. Mouse anti-tubulin was purchased from Sigma. HRP-conjugated anti-mouse/rabbit IgG were purchased from Sigma.

GeneAmp RNA PCR core kit was from Applied Biosystem. Oligonucleotides were purchased from IDT.

## Plasmids

Cloning and construction of pcDNA3/URG7flag fusion plasmid (defined as URG7) used in this study were described previously [Ostuni *et al.*, 2013]. To clone into pcDNA3 (Invitrogen) vector, the sequence encoding the first 102 amino acids of the MRP6 protein, the forward primer 5'-AAAAGTTAACATGGACTACAAGGACGACGATGACAAGATGGCCGCGCCTGCTGAG-3' containing a flag tag (encoding DYKDDDDK at the N-terminus) and the reverse primer 5'-CGAGGTACC GAATTCTCATGGGCGCTCAGGCGT-3' were used. As template in PCR reaction, ABCC6/pcDNA.3.1D/V5-His-TOPO recombinant expression vector was used [Armentano *et al.*, 2008]. The 306 bp PCR product was cloned as an *HpaI*–*EcoRI* fragment. The resulting recombinant plasmid was defined as MRP6<sub>1–102</sub>. DNA sequencing (Eurofins MWG Operon) verified the construct.

## Cell culture and transfections

The human hepatocellular carcinoma cell line HepG2 was kindly gifted from Dr. V. Infantino (University of Basilicata-Italy). HepG2 cells were maintained in DMEM containing 4.5 mg/l glucose, 2 mM L-glutamine, 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C, 5% CO<sub>2</sub> in a humidified incubator. Cells grown to 80% confluence were transfected with URG7 or with MRP6<sub>1–102</sub> constructs, using TransIT-2020 Transfection Reagent (MIRUS), according to the manufacturer's instructions. Cells transfected with empty vector pcDNA3 (mock) were used as negative control. In order to induce ER-stress, 33 h after transient transfection, HepG2 cells were treated with 3  $\mu$ g/ml TN for 15 h at 37°C.

## URG7 immunoprecipitation

HepG2 cell lines transfected with URG7 or mock plasmids were lysated in 50 mM Tris–HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, complete protease inhibitors (Roche) and the complexes containing FLAG-URG7 were immunoprecipitated as reported [Licciardo *et al.*, 2003]. Briefly, the cell lysates were pre-cleared by 3 h incubation onto Sepharose Protein A beads (Sigma) and then incubated overnight at 4°C with the M2 anti-FLAG antibody-conjugated beads. Unbound proteins were discarded and the beads were extensively washed to eliminate non-specific bound proteins. The elution was carried out by competition incubating the mixture with FLAG peptide o/n at 37°C. The eluted proteins were precipitated in methanol/chloroform solution and then fractionated by 8–18% SDS-PAGE. The FLAG-URG7 and mock lanes were cut in 35 slices per lane. Each slice was crushed and treated for *in situ* hy-

drolysis [Caterino *et al.*, 2014; Spaziani *et al.*, 2014; Capobianco *et al.*, 2016].

## Mass spectrometry analysis

Peptide mixtures extracted from the gel were analysed by nano-chromatography tandem mass spectrometry, as reported [Pastore *et al.*, 2015; Caterino *et al.*, 2016]. Briefly, peptide mixtures analyses were performed by nLCMS/MS by using the LC/MSD Trap XCT Ultra (Agilent Technologies) equipped with a 1100 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture (0.5% TFA) was first concentrated and washed 4  $\mu$ l/min in a 40-nl enrichment column (Agilent Technologies chip) with 0.1% formic acid as the eluent. The sample was then fractionated on a C18 reverse-phase capillary column (75  $\mu$ m  $\times$  43 mm in the Agilent Technologies chip) at a flow rate of 300 nl/min with a linear gradient of eluent B (0.1% formic acid in acetonitrile) in A (0.1% formic acid) from 5 to 60% in 50 min. Elution was monitored on the mass spectrometers without any splitting device. Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 400 to 2000 *m/z*) followed by MS/MS scans of the three most abundant ions in each MS scan.

Raw data from LC–MS/MS analyses were converted into a Mascot format text and load on a MASCOT licensed software for protein identification in a non-redundant protein sequence database (NCBIInr) [Marucci *et al.*, 2013].

According to the probability-based Mowse score, the ion score is  $-10 \times \log(P)$ , where  $P$  is the probability that the observed match is a random event. Individual scores  $>38$  indicate identity or extensive homology ( $P < 0.05$ ). As reported [Caterino *et al.*, 2014; Di Pasquale *et al.*, 2016], MS/MS spectra with a Mascot score higher than 38 have a good ratio signal/noise. Single peptide identifications were not accepted.

## Clustering analysis

The whole group of identified proteins was analysed using the 'STRING: functional protein association networks' software 7.0 (<http://string-db.org/>) to evaluate the significant networks and canonical pathways associated with the differentially expressed proteins. STRING is a database of known and predicted protein interactions: the interactions include direct (physical) and indirect (functional) associations. The score of each process or pathway is equal to the negative logarithm of the  $P$  value and represents the likelihood that the assembly of the set of proteins identified in this study is part of significant canonical pathways or networks.

To validate URG7/GRP78 interaction, new preparations of FLAG-URG7 and mock immunoprecipitation were subjected to Western blot analysis, using anti-GRP78 antibody, as previously described [Iaconis *et al.*, 2017].

## Quantitative RT-PCR

HepG2 transfected cells were harvested and total RNA was extracted using the Quick-RNA<sup>TM</sup> MiniPrep kit (Zymo Research) according to the manufacturer's protocol. One microgram of RNA was used for cDNA preparation using High Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to the manufacturer's instructions. cDNA was amplified via Real-Time PCR using PowerSYBR Green PCR Master Mix

Table 3 | Primers Used in the Real-Time RT-PCR Assay

	Accession n°	Forward Primer	Reverse Primer
<b>URG7</b>	NM_001079528.3	5'-GCC ATC CCT GGG AGC CT-3'	5'-GAC TTC ACC AGG TTC CAG CC-3'
<b>GRP78</b>	NM_005347.4	5'-GAA TGC CCT GAC ACC TGA AGA-3'	5'-GTT TGC TGA TAA TTG GTT GAA CA-3'
<b>CHOP</b>	NM_001195053.1	5'-GTA CCT ATG TTT CAG CTC CTG-3'	5'-TCT CCT TCA TGC GCT GCT TTC-3'
<b><math>\beta</math>-Actin</b>	NM_001101.3	5'-CCT GGC ACC CAG CAC AAT -3'	5'-GCC GAT CCA CAC GGA GTA CT-3'

(Promega) on the 7500 Fast Real-Time PCR System (Applied Biosystems). Primers, designed with Allele ID program, span exon–exon junctions to eliminate any undesirable genomic DNA amplification. The cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, and at 72°C for 90 s and a final extension at 72°C for 10 min. To confirm PCR specificity, the PCR products were subjected to a melting-curve analysis. The comparative threshold cycle method ( $\Delta\Delta C_t$ ) was used to quantify relative amounts of product transcripts with  $\beta$ -actin as endogenous reference control. Primer sets are listed in Table 3.

#### Western blotting analysis

HepG2 transfected cells were harvested, washed with ice cold PBS and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% NP40, 0.5% NaDeoC, 0.1% SDS) supplemented with Protease Inhibitor Cocktail (Sigma). Protein concentration was determined by Bradford assay [Bradford, 1976] and equal amount of protein resolved on 10 or 15% SDS-PAGE. Proteins were electroblotted on nitrocellulose membrane at 360 mA for 90 min at 4°C. Following the transfer, the membranes were blocked with blocking solution (5% non-fat dried milk and 0.1% Tween-20 in PBS or 5% BSA in PBST) for 1 h at room temperature and incubated overnight with specific primary antibody (1:1000 anti-FLAG; 1:1000 anti-GRP78; 1:4000 anti- $\beta$ -actin; 1:1000 anti-CHOP and 1:500 anti-sXBP1; 1:1000 anti-Ubiquitin (P4D1); 1:750 anti-p-AKT(Ser473) and 1:1000 anti-AKT; 1:1000 anti-PARP-1). After incubation, membranes were washed three times with PBS-T (PBS containing 0.1% Tween 20) for 10 min and incubated with appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The membranes were washed three times with PBS-T and signals visualised by Chemiluminescent Peroxidase Substrate-1 (Sigma) or Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific), using Chemidoc XRS detection system equipped with Image Lab Software for image acquisition. The quantification of proteins bands was performed by determination of the relative optical density using ImageJ software (National Institute of Health).

#### Quantification of ER stress in HepG2 cells using the fluorescent compound ThT

HepG2 cells, cultured in complete DMEM, were seeded at a density of  $2 \times 10^5$ /well in 12-well plates and were allowed to adhere overnight to glass coverslips. Twenty-four hours after plasmids transfections, HepG2 cells were treated with 3  $\mu$ g/ml TN for 6, 15 and 24 h at 37°C to induce ER stress. Within the last 3 h of ER stress, cells were incubated with 2.5  $\mu$ M ThT, after

which they were carefully washed with cold PBS and observed with an inverted fluorescence microscopy (NIKON Eclipse 80i).

#### Author contribution

MFA and MaC conceived and performed the experiments and wrote the draft; RM and MCP performed the experiments; MM and FC supervised the proteomic analysis and gave technical support; AO, LM and MoC performed data analysis and interpretation; PP supervised the project; FB supervised the project, edited the manuscript and acted as corresponding author. All authors have read and approved the final manuscript.

#### Conflict of interest statement

The authors have declared no conflict of interest.

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