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Internal ribosome entry site-mediated translational regulation of ATF4 splice variant in mammalian unfolded protein response

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ABSTRACT

Activating transcription factor 4 (ATF4) is a master regulator of genes involved in unfolded protein response (UPR) and its translation is regulated through reinitiation at upstream open reading frames. Here, we demonstrate internal ribosome entry site (IRES)-mediated translation of an alternatively spliced variant of human ATF4. This variant that contains four upstream open reading frames in the 5′ leader region was expressed in leukocytes and other tissues. mRNA and protein expression of this variant was activated in the UPR. Its translation was neither inhibited by steric hindrance nor affected by eIF4G1 inactivation, indicating a cap-independent and IRES-dependent mechanism not mediated by ribosome scanning-reinitiation. The IRES activity mapped to a highly structured region that partially overlaps with the third and fourth open reading frames was unlikely attributed to cryptic promoter or splicing, but was activated by PERK-induced eIF2α phosphorylation. Taken together, our findings reveal a new mechanism for translational regulation of ATF4 in mammalian UPR.

1. Introduction

Accumulation of unfolded protein in the endoplasmic reticulum (ER) causes stress and elicits the unfolded protein response (UPR) comprised of multiple intracellular signaling pathways [1]. The UPR modulates gene expression at both transcriptional and translational levels to adjust biosynthetic capacity and maintain homeostasis. Activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and protein kinase R-like ER kinase (PERK) are three major UPR sensors that reside in the ER membrane and they transduce the activation signal to three downstream pathways of the UPR [2]. Particularly, PERK phosphorylates translation initiation factor eIF2α leading to attenuation of global protein synthesis and translational activation of ATF4, which controls the expression of ER chaperones and other proteins required for stress amelioration, protein synthesis and folding [3]. In addition to PERK, there exist three mammalian eIF2α kinases that can also impact ATF4 translation. These stress-sensing kinases named GCN2, PKR and HRI are activated by nutrient limitation, viral infection and heme deprivation, respectively [4]. In this sense, ATF4 is pivotally important in cellular response to various stress conditions [5].

Translational regulation of human ATF4 transcript is mediated through two upstream open reading frames (uORFs) in the 5′ leader region [6]. Mechanistically, scanning ribosomes initiate translation at uORF1 and reinitiate at uORF2 efficiently in unstressed cells in which eIF2α is hypophosphorylated and eIF2-GTP is abundant. The translation of ATF4 is therefore repressed. In contrast, phosphorylation of eIF2α in stressed cells leads to a reduction in eIF2-GTP level and a failure in reinitiation at uORF2. As a result, scanning ribosomes bypass the inhibitory uORF2 and translate ATF4 [7,8]. This model for regulation of mammalian ATF4 is very similar to the mechanism that controls the translation of yeast GCN4 [9,10]. Interestingly, uORF-regulated expression of ATF4 is activated in human cells with a defect in translation termination [11].

We are interested in cellular proteins that interact with retroviral oncoprotein Tax and regulate transcription from the long terminal repeats of human T-cell leukemia virus type 1 [12,13]. ATF4 is one of these proteins implicated in retroviral pathogenesis [14]. When ATF4 was initially identified as a protein that binds to the viral CRE-like element in the long terminal repeats of human T-cell leukemia virus type 1, an alternatively spliced ATF4 variant was cloned [15]. This variant designated variant 1 (V1) in GenBank is generated by intron retention. In other words, it harbors in its first exon an additional segment, which is skipped in the more extensively studied V2 [7,8]. Interestingly, V1 contains four uORFs in its 5′ leader region, resembling yeast GCN4 [9]. This raises the possibility that translation of V1 might be regulated through a similar mechanism based on ribosome scanning and reinitiation.

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In this work, we carried out a comparative study on translational control of V1 and V2 of human ATF4. To our surprise, translation of V1 was not mediated by reinitiation. An internal ribosome entry site (IRES) was found in the 5′ leader region of V1 and its activity was induced by the UPR. Our findings reveal another level of complexity in the regulation of ATF4 expression in mammalian UPR.

2. Materials and methods

2.1. Plasmid constructs

Expression vectors for rhinovirus 2A protease wild-type and its C106A defective mutant were generously provided by Dr. Joachim Seipelt [16]. Expression plasmids for eIF2α mutants S51D and S51A were gifts from Dr. David Ron [17]. Human PERK and its K621M mutant were kindly provided by Dr. Ronald Wek [18]. Human GADD34 was a gift from Dr. Nai Sum Wong.

Expression of β-galactosidase was driven by a cytomegalovirus (CMV) promoter and the vector pCMV-βGal was from Invitrogen. Monocistronic firefly luciferase reporter constructs were derived from pGL3-control (Promega), whereas bicistronic reporter plasmids were constructed on the backbone of pSP-FL + NF and pRL-CMV (Promega). In all reporter constructs that contain 5′ leader sequence of human ATF4, the first 32 codons of human ATF4 gene were fused in frame with coding sequence of firefly luciferase. In the monocistronic reporter constructs, expression of a firefly luciferase gene is driven by the SV40 promoter and enhancer. The expression cassette in the bicistronic reporter constructs contains a CMV promoter, a *Renilla* luciferase gene, followed by a firefly luciferase gene. For construction of the bicistronic reporter plasmids, V1 and V2 fragments of human ATF4 were first cloned into pSP-FL + NF fusion vector (Promega). The resulting ATF4-firefly luciferase fusion genes (i.e. V1-F and V2-F) were further subcloned into the downstream of *Renilla* luciferase gene in the pRL-CMV plasmid. Promoterless bicistronic vectors were generated by removing the region that contains the entire CMV enhancer and immediate early promoter. The predicted transcription start sites in V1-F, V2-F, R-V1-F and R-V2-F transcripts produced from the monocistronic and bicistronic vectors were verified by 5′ rapid amplification of cDNA ends. The cDNA of EMCV IRES (nucleotides 260–842) was PCR amplified from pIRESneo (Clontech) using primers 5′-GAAGATCTCA AGCTTCGAAT TCTGCAGTCG AC-3′ and 5′-GAAGATCTGG TTGTGGCCAT ATTATCATCG TG-3′. All V1 and V2 fragments of human ATF4 were amplified by PCR. Site-directed mutagenesis was carried out to introduce a G → A mutation that disrupts a consensus 5′ splice site in V1. Primers used in site-directed mutagenesis were 5′-CCAGCGGCTT AAGCCATGGC ATGAGTAC-3′ and 5′-GTACTCATGC CATGGCTTAA GCCGCTGG-3′.

Primers for PCR amplification and subcloning of V1 sequence were 5′-GCGGTACCGC GTGTGCGTTT TCCCTCCTC-3′ and 5′-GCGGAT CCCC TAGGCTTTCT TCAGCCCCC-3′. Forward primers for PCR amplification of V1 truncated mutants were 5′-CTAGCTAGCC TTTGCAGCGG CGGCAGCA-3′ (F1: nucleotides 124–886), 5′-CTAGCTAGCG GCGCGG GTTT TGGATTGGTG-3′ (F2: nucleotides 230–886), 5′-CTAGCTAGCG TAGTCGGGTG CCCGGACT-3′ (F3: nucleotides 368–886), 5′-CTAGC TAGCG GCTGTTGCCC CACGAAACGT G-3′ (F4: nucleotides 450–886), 5′-CTAGCTAGCA CGGAGCGCTT TCCTCTTGGC G-3′ (F5: nucleotides 572–886), 5′-CTAGCTAGCG CAAATACAAC TGCCCTGTTC CCG-3′ (F6: 663–886) and 5′-CTAGCTAGCC TGATTCTCAT TCAGGCTTCT CACGG-3′ (F7: 777–886). The common reverse primer for all V1 mutants was 5′-GGACTAGTTT ACACGGCGAT CTTTCCGCCC-3′.

To generate V1M in which a G-to-A mutation was introduced to the 5′ splice site (+189) in the 5′ UTR of V1, site-directed mutagenesis was performed by PCR with primers 5′-CCAGCGGCTT AAGCCAT GGC ATGAGTAC-3′ and 5′-GTACTCATGC CATGGCTTAA GCCGCTGG-3′.

Stem-loop structures inserted into different locations of the 5′ UTR are as follows. SL1Luc inserted into the NcoI site of pGL3-control: 5′-CCATGGTGGTGGAGC TTCCACCACCATGG-3′ (ΔG = −24 kcal/mol). V1SL1 inserted into the Bsu36I site in the upstream of uORF1 in V1: 5′-CCTCAGGTGGTGGAGCTTCCACCACCTCAGG-3′ (ΔG = −21 kcal/mol). V1SL2 inserted into the MluI site between uORF2 and uORF3 in V1: 5′-ACGCGTTGGTGGAGCTTCCACCAACGCGT-3′ (ΔG = −23 kcal/mol). V1SL3 inserted between uORF3 and uORF4 of V1 by PCR: 5′-ACGCGTTGGTGGAGC TTCCACCAACGCGT-3′ (ΔG = −23 kcal/mol). V2SL1 inserted into the PstI site between uORF1 and uORF2 in V2: 5′-CTGCAGTGGTGGAGCTTCCACCACTGCAG-3′ (ΔG = −24 kcal/mol). Nucleotides contributing to the stem were underlined. ΔG was calculated by using a web-based Vienna RNA secondary structure prediction program (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

2.2. RNA analysis

HeLa and THP-1 cells were cultured and RNA analysis was performed as described [19,20]. Northern blotting was carried out using the MTN blot (Clontech). RNA on this blot was isolated from different human tissues pooled from multiple healthy donors. Particularly, leukocyte RNA was isolated from pooled human peripheral blood. The leukocytes contain granulocytes, lymphocytes, monocytes and macrophages. Probes were labeled with [α-^{32}P] dCTP (Amersham) using random primers. Unincorporated nucleotides were removed using ProbeQuant™ G-50 micro-columns (Amersham). For quantitative real-time RT-PCR analysis [21,22], total RNA was extracted from cells using Trizol reagent (Invitrogen). RNA samples were digested with DNase I and cDNA was synthesized with oligo (dT) and Thermoscript reagents (Invitrogen).

Real-time PCR was performed using SYBR Green PCR master mix in StepOnePlus System (Applied Biosystems). Relative amounts of V1, V2 and GRP78 mRNAs were normalized to the levels of β-globin or GAPDH transcript by the comparative threshold cycle (CT) method where fold difference $= 2^{-(\Delta CT \text{ of gene of interest } - \Delta CT \text{ of } \beta-\text{globin})} = 2^{-\Delta\Delta CT}$. Primer pairs used were 5′-CCAAGGGGGA AGCGATTTAA CGAGCG-3′ and 5′-TGAGAATCAG AAGCCAACTC CCATTAGTGG-3′ (V1), 5′-CTTAAGCCAT GGCGCTTCTC AC-3′ and 5′-GGAGAAGGCA TCCTCCTTGC TG-3′ (V2), 5′-CCAACGCCAA GCAACCAAAG-3′ and 5′-AGTCGAGCCA CCAACAAGAA C-3′ (GRP78); 5′-AGCGTACTCC AAAGATTCAG GTT-3′ and 5′-TACAT GTCTC GATCCCACTT AACTAT-3′ (β-globin), and 5′-GCAGGGGGGA GCCAAAAGGG-3′ and 5′-TGCCAGCCCC AGCGTCAAAG-3′ (GAPDH).

2.3. Polysome analysis

Polysome analysis was carried out essentially as previously described [23,24]. In brief, HeLa cells cultured to 75–80% confluency were treated with DMSO, 300 nM Tg or 2.5 μg/ml Tu for 16 h. Cells were treated with cycloheximide (100 μg/ml) for 15 min at 37 °C before harvest. Washed cells were then centrifuged for 10 min at full speed. Pellet was resuspended in hypotonic lysis buffer containing 5 mM Tris–HCl, pH 7.5; 2.5 mM $MgCl_2$ and 1.5 mM KCl. Cells were incubated on ice with 100 μg/ml cycloheximide, 2 mM DTT, 200 μM phenyl methyl sulfonyl fluoride (PMSF), EDTA-free protease inhibitor cocktail (Roche) and 2 μl RNAsin inhibitor (40 U/μl) for 15 min. Cells were incubated on ice for another 10 min in the presence of 0.5% Triton X-100 and 0.5% sodium deoxycholate. Cell extracts were centrifuged for 15 min at 14,000 rpm. Supernatants were collected and loaded onto a pre-chilled 10–50% sucrose gradient. Gradients were placed in a Hitachi P40ST rotor and centrifuged at 35,000 rpm for 2 h at 4 °C. Sucrose gradients were fractionated, and monitored for absorbance at 254 nm. Fractions were collected, and RNA was extracted with Trizol reagent.

2.4. RNA transfection

RNA was prepared from linearized DNA template by in vitro transcription with T7 RNA polymerase as described [25–27]. DNA template

was removed by DNase I. Capped RNA transcripts were transfected into cells by using DMRIE-C reagent (Invitrogen).

2.5. Western blot analysis

Western blotting was carried out as described [13,28]. Cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) supplemented with 2 mM PMSF and EDTA-free protease inhibitor cocktail (Roche). Proteins on blots were visualized by enhanced chemiluminescence (Amersham). Rabbit polyclonal anti-eIF4G1 (ab2609) was purchased from Abcam. Mouse monoclonal anti-β-actin was from Sigma. Rabbit monoclonal anti-phospho-eIF2α (S51) (119A11) was from Cell Signaling. Rabbit polyclonal anti-eIF2α (sc-11386) and anti-ATF4 (sc-200) were from Santa Cruz.

2.6. Luciferase reporter assay

Dual luciferase reporter assay was performed as described using a reagent kit from Promega [29,30]. Luminescence was measured with an LB9570 luminometer (EG&G). Relative luciferase activity derived from mono- or bicistronic reporter constructs was determined by normalizing readouts of firefly luciferase to those of *Renilla* luciferase or β-galactosidase reporter, respectively. β-Galactosidase activity was assayed by using a luminescent detection kit from Clontech.

2.7. RNA secondary structure prediction

RNA secondary structure was predicted by the Mfold program, which calculates the overall minimum free energy of RNA molecule [31].

3. Results and discussion

3.1. An alternatively spliced variant of human ATF4

The V1 and V2 variants of human ATF4 transcript differ only in the 5′ leader region and they encode the same protein (Fig. 1A). The resemblance of V1 to yeast GCN4 in having four uORFs prompted us to compare the expression pattern of V1 and V2 in human tissues. Northern blot analysis indicated that V1 was widely expressed in many tissues including heart and peripheral blood leukocytes (Fig. 1B, lanes 2 and 12). In addition, in most tissues the abundance of V1 was significantly lower than that of V2 (Fig. 1B, middle panel).

When we searched the current databases for matches of the V1-specific sequence, 121 expressed sequence tags (ESTs) that are characteristic of the V1 transcript were identified. Among them, 28

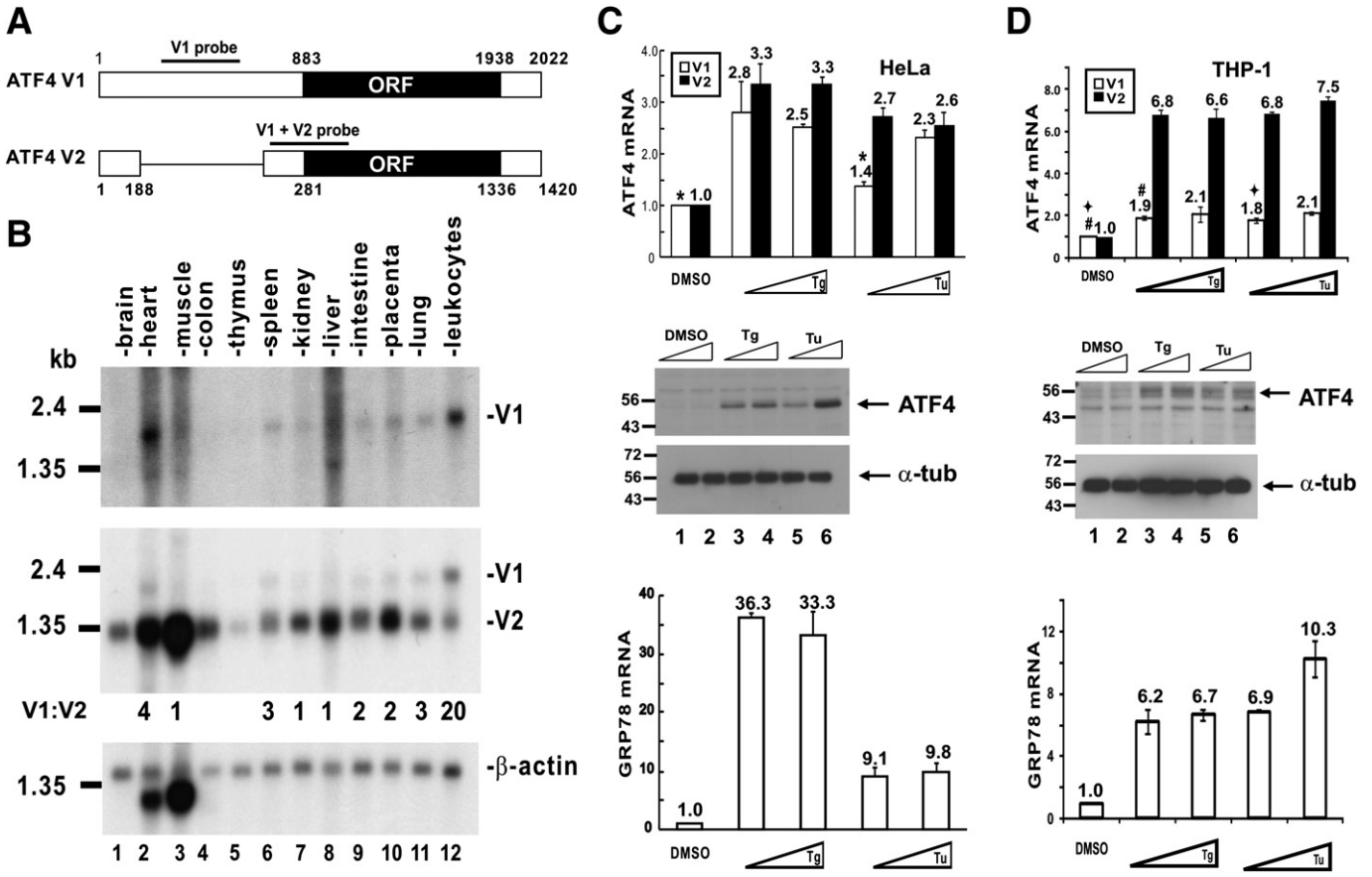


Fig. 1. Alternatively spliced variants of human ATF4. (A) Diagram of V1 and V2. Positions of probes specific for V1 and V1 + V2 are indicated. V1 and V2 sequences are identical except for the region missing in V2, which is indicated by a line and not a box. (B) Expression of V1 and V2 in different tissues. Northern blot analysis was carried out on MTN blots (Clontech) with probes specific for V1, V1 + V2, and β-actin. Ratios of V1 to V2 determined by densitometry were indicated below the middle panel. To facilitate comparison between lanes, level of V1 relative to V2 in the liver was arbitrarily set as 1. (C, D) UPR induction of V1 and V2 expression. HeLa (C) and THP-1 (D) cells were treated with DMSO, Tg (150 or 300 nM) or Tu (2.5 or 5 μg/ml) for 16 h. mRNA was then analyzed by quantitative real-time RT-PCR with primers specific for V1, V2, GRP78, β-globin or GAPDH. ATF4 protein was detected by Western blotting. α-Tubulin (α-tub) served as a control for equal loading. Expression levels of V1, V2 and GRP78 transcripts were normalized to those of β-globin or GAPDH mRNA, which did not fluctuate between treatment groups (Fig. S1). Results are representative of three independent experiments and error bars indicate *s.d.* Numbers at the top of the error bars indicate fold activation. *, # and ✦: The differences between the two groups are statistically significant by Student *t* test ($P = 0.0112$, $P = 0.0212$ and $P = 0.0138$, respectively).

were derived from B cells, leukocytes, leukemia, lymphoma and myeloma cells; 25 were from testis, colon, rectum, intestine, stomach, heart, spleen, bladder and cartilage; and 7 were from embryonic stem cells. V1-specifc ESTs were also found in various types of cancer. The most 5′ end of the V1 and V2 transcript as indicated in Fig. 1A was verified by 5′ rapid amplification of cDNA ends (RACE) and was also supported by the V1 and V2 EST clones in the databases. No further sequence could be identified either by 5′ RACE or from the EST clones. It is noteworthy that V1 retains only the first of the two introns located between the first two exons in human *ATF4* gene. Specific removal of the second intron in the V1 band seen in Fig. 1B suggested that it unlikely represents ATF4 hnRNA.

We next investigated whether the transcription of V1 was induced in the UPR. HeLa cells were treated with thapsigargin (Tg) and tunicamycin (Tu), two well-known stimuli of UPR [1]. The RNA levels were analyzed by quantitative real-time RT-PCR. In this setting, the expression of ATF4 protein was strongly induced by Tg or Tu (Fig. 1C, middle panel). As a result, the mRNA level of ER chaperone GRP78 was also elevated (Fig. 1C, lower panel). We then compared the induction of V1 and V2 transcripts. We found that V1 and V2 were equally inducible by Tg and Tu. For example, Tg at 150 nM stimulated the transcription of V1 and V2 and by 2.8 and 3.3 fold, respectively (Fig. 1C, top panel). Because V1 is more abundantly expressed in leukocytes and leukemia cells (Fig. 1B), we also analyzed the induction of V1 and V2 mRNAs by UPR stimuli in human monocytic leukemia THP-1 cells. Expression of both ATF4 protein and GRP78 mRNA in THP-1 cells was induced by Tg or Tu (Fig. 1D, middle and bottom panels). Induction of V1 and V2 transcripts was also observed, but the elevation of V1 transcript induced by Tg or Tu was less robust than that of V2 mRNA in THP-1 cells (Fig. 1D, top panel). Since the basal level of V1 transcript was already high in peripheral blood leukocytes (Fig. 1B) and THP-1 cells, one of the trans-acting factors specifically required for translation of V2 transcript, but not V1 transcript, might become limiting when ATF4 protein synthesis was further induced by the ER stressors. This implied that the translation of ATF4 protein from V1 and V2 transcripts could be regulated differentially. Nevertheless, both V1 and V2 transcripts were inducible in the UPR and contributed to the elevation of ATF4 protein expression in stressed cells.

The expression of ATF4 in the UPR is also regulated at translational level through the 5′ leader region [6]. Retention of 189–790 nucleotides in the first exon renders the 5′ leader region of V1 considerably longer than that of V2 (Fig. 1A). It is therefore of interest to see whether and how the long 5′ leader sequence of V1 might affect ATF4 translation. To determine whether V1 and V2 transcripts are actively translated, we performed polysome profile analysis using

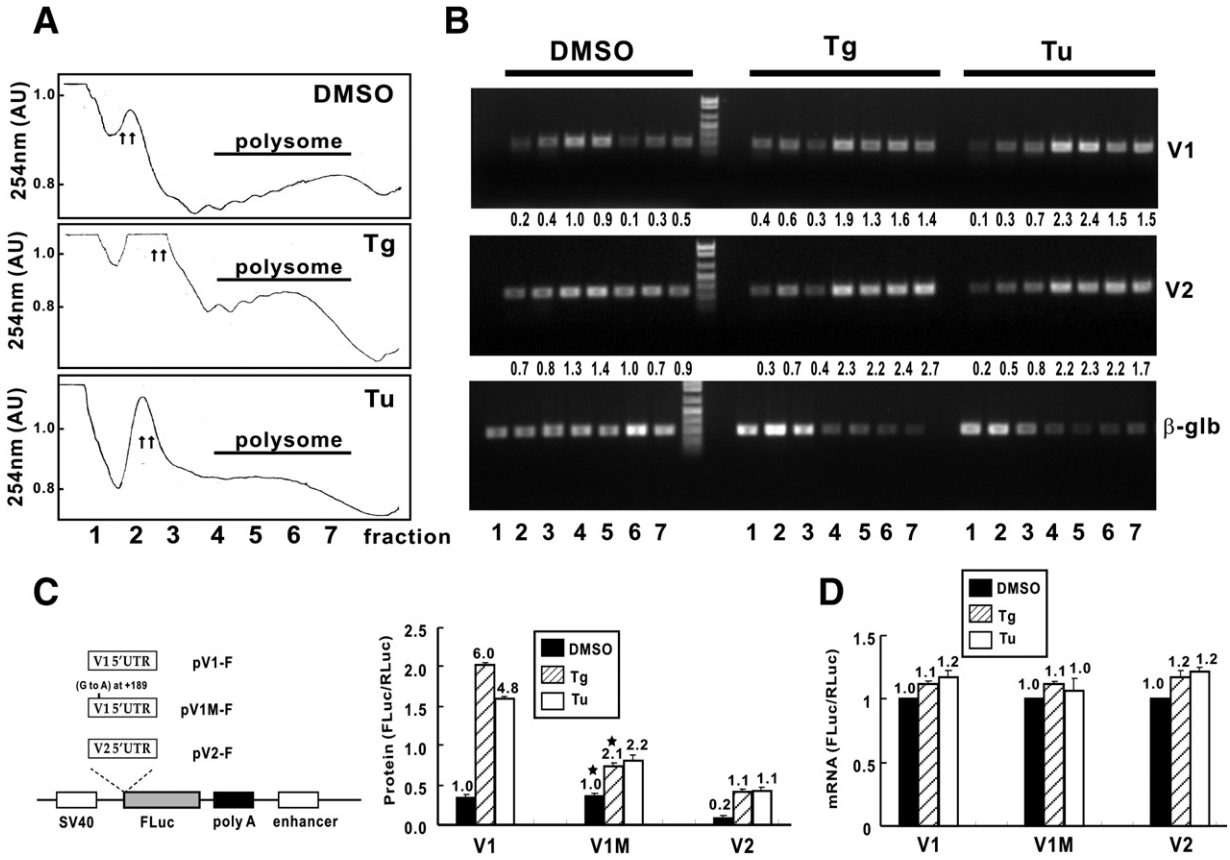


Fig. 2. Translation of V1 transcript is induced by ER stress. (A) Polysome analysis. HeLa cells were treated with DMSO, 300 nM Tg and 2.5 μg/ml Tu for 16 h. Cell extracts were subjected to sucrose gradient centrifugation and fractions were monitored by absorbance units (AU) at 254 nm. Polysome fractions are highlighted. 40S and 80S ribosomal fractions are indicated by arrows. The ratios of monosomes to polysomes were found to be increased 2- to 3-fold in cells treated with Tg or Tu. (B) Polysome association of V1 mRNA. Levels of V1, V2 and β-globin (β-glb) transcripts in different fractions were analyzed by RT-PCR. Primers that specifically recognize the spliced transcript of V1 or V2 were used. Shown below the panels are relative levels of V1 or V2 transcript normalized to β-globin mRNA determined by densitometry. (C) UPR induction of V1 and V2 translation. HeLa cells transfected with luciferase reporter plasmids driven by the 5′ leader sequence of V1, V1M and V2 (pGL3-CMV-V1/V1M/V2-Luc) were treated with DMSO, Tg (300 nM) and Tu (5 μg/ml) for 16 h. V1M is a mutant of V1, in which a G-to-A mutation was introduced into nucleotide 189 of the 5′ UTR of V1M to disrupt the 5′ splice site. Shown on the left is a schematic representation of V1, V1M and V2 reporter constructs. Dual luciferase assay was performed and relative luciferase activity (FLuc/RLuc) in arbitrary units was calculated by normalizing the readouts of firefly luciferase to those of *Renilla* luciferase expressed from a co-transfected plasmid. Normalization was not a major contributor to the difference observed. Results represent three independent experiments and error bars indicate *s.d.* Relative fold activation is indicated at the top of the error bars. ★: The difference between the two groups is statistically significant by Student *t* test ($P = 0.0165$). (D) V1 and V2 mRNAs introduced were unaffected by UPR. Steady-state amounts of V1, V1M and V2 transcripts expressed from the transfected plasmids were measured by quantitative real-time RT-PCR.

sucrose gradient centrifugation. In this assay mRNA association with large polysome fractions indicates robust translation, whereas transcripts present in the monosome and disome fractions are weakly translated [23,24]. Levels of monosomes and free ribosomes were indeed increased in Tg- or Tu-treated HeLa cells (Fig. 2A). Notably, V1 transcript, which was weakly associated with polysomes in the absence of Tg or Tu, was found to be more prevalent in the polysome fractions in Tg- and Tu-treated cells (Fig. 2B, fractions 4–7). A similar distribution pattern of V2 mRNA in the earlier and polysome fractions was also observed in cells treated with Tg or Tu. In contrast to V1 and V2 transcripts, β-globin mRNA was actively translated all the time in the absence of ER stressors and its association with polysomes was diminished in the presence of Tg or Tu. That is to say, translation of β-globin was repressed in response to ER stress.

We went on to compare the translational activity of V1 and V2 using reporter constructs in which the expression of luciferase is regulated by the 5′ leader sequences of V1 and V2. Similar constructs (Fig. 2C) have previously been used to assess the translational activity of the 5′ leader region of V2 successfully [7,8]. Interestingly, the relative luciferase activity derived from the reporter construct driven by the 5′ leader region of V1 was consistently 5- to 10-fold higher than that generated by the counterpart V2 plasmid (Fig. 2C). Moreover, Tg or Tu further stimulated the translational activity of V1 and V2 to similar magnitudes in this experimental setting (Fig. 2C). To prevent

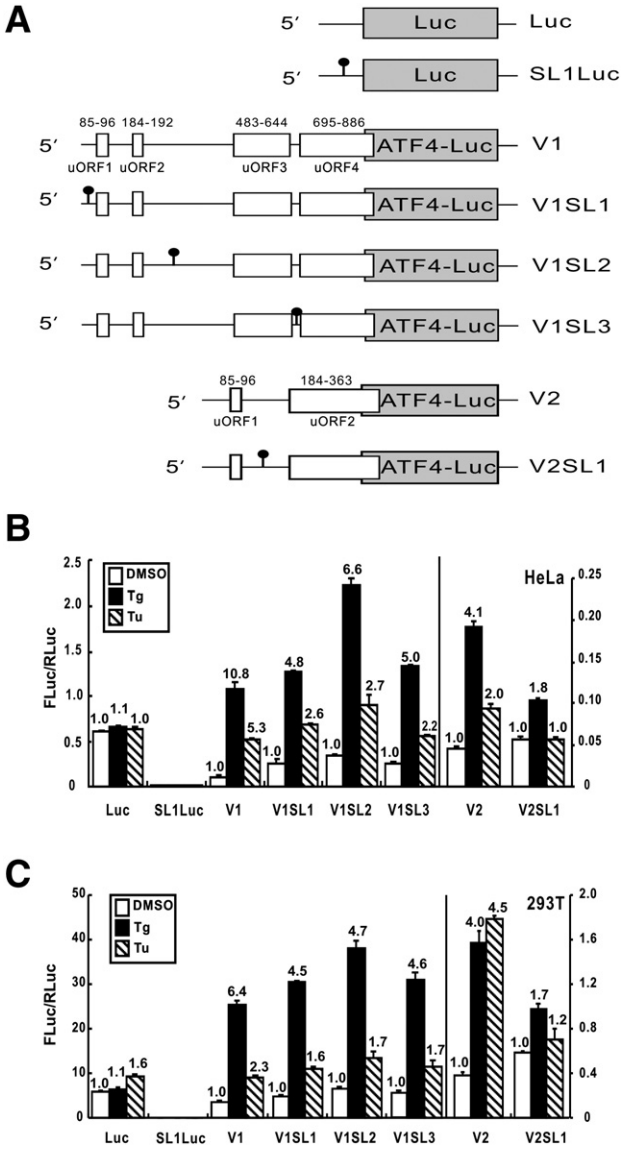


Fig. 3. Translation of V1 transcript is not mediated by ribosome scanning. (A) Schematic representation of V1 and V2 reporter constructs. A stem-loop was inserted into different locations of the 5′ leader region of V1 and V2 as depicted. (B, C) Effect of stem-loop insertion on translational activity of V1 and V2. HeLa and HEK293T cells transfected with the indicated reporter constructs were treated with DMSO, Tg (300 nM) or Tu (5 μg/ml) for 16 h. Dual luciferase assay was performed and results were presented as in Fig. 2. Numbers at the top of the error bars indicate relative fold activation over the DMSO control.

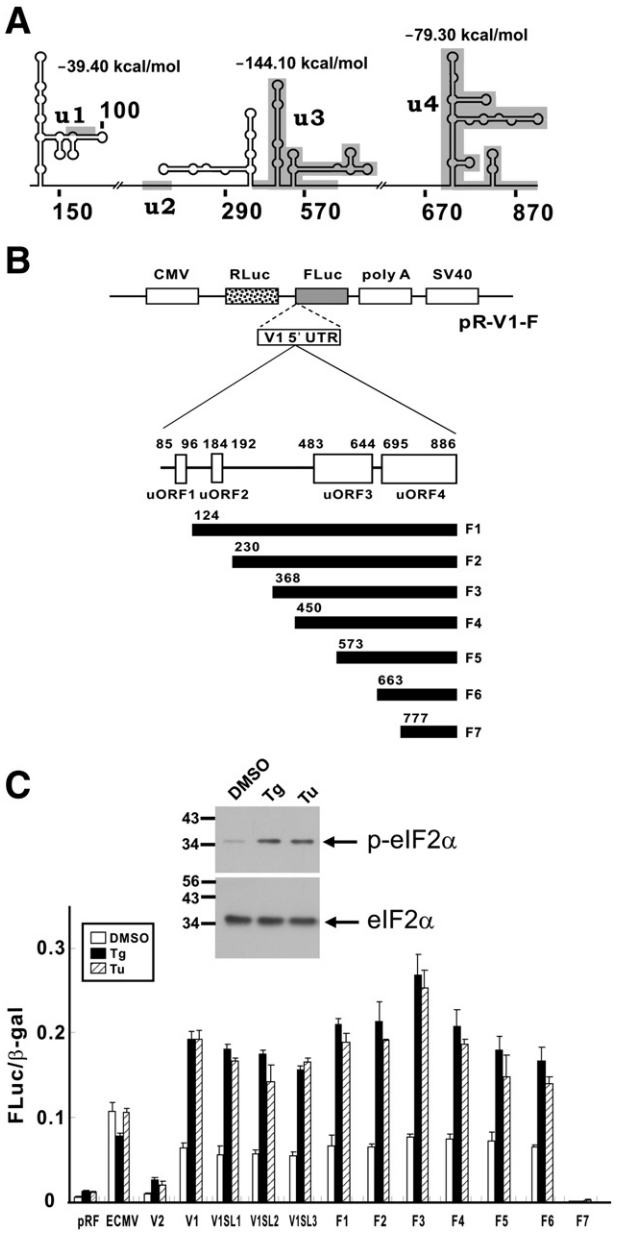
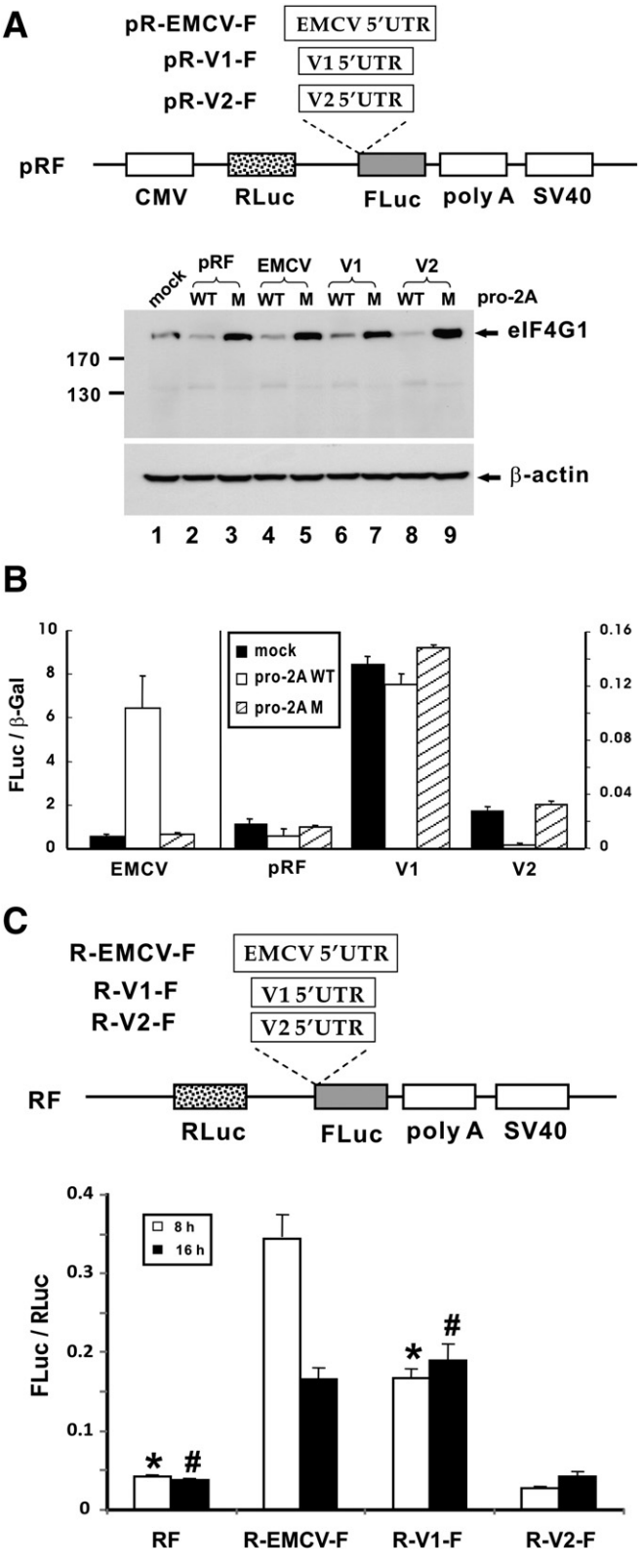


Fig. 4. IRES-dependent translation of V1 transcript. (A) Predicted secondary structure of the 5′ leader region (1–883) of V1. ΔG values of the three structures (29–150, 293–577 and 670–875 of GenBank NM_001675) are indicated. The four uORFs (u1–u4) are highlighted in gray. (B) Schematic diagram of bicistronic V1 reporter constructs. (C) Translational activity of stem-loop-inserted and truncated mutants of V1. HEK293T cells were co-transfected with the indicated reporter constructs plus an expression vector for β-galactosidase. Cells were treated with DMSO, Tg (300 nM) or Tu (5 μg/ml) for 16 h, and then assayed for firefly luciferase activity normalized to β-galactosidase activity, which was unaffected by either IRES or ER stress. Readouts of β-galactosidase activity increased linearly with mRNA concentrations. Western blot analysis of phospho-eIF2α in Tg- and Tu-treated cells was presented in the inset. *Renilla* luciferase activity was not used in the normalization since the translation of *Renilla* luciferase fluctuated slightly in the presence of a downstream IRES from either EMCV or V1 for unknown reason (see Fig. S2 for reference). However, normalization was not a major contributor to the difference observed. Results represent the mean ± *s.d.* of three independent experiments.

V1 from splicing into V2, we also analyzed a splice site mutant of V1 named V1M (Fig. 2C). Although it was less robust compared to the effect on V1, the induction of V1M translation by Tg or Tu was still significant (Fig. 2C). Importantly, Tg or Tu did not affect the mRNA levels of V1, V1M and V2 expressed from the transfected plasmids (Fig. 2D), indicating that the enhancement of V1 expression observed in Fig. 2C occurred at the level of protein translation. These results suggested that although the abundance of V1 was relatively low, significant amount of ATF4 protein could still be produced from this transcript in response to ER stress. Their association with polysomes in stressed cells (Fig. 2B) further suggested that both V1 and V2 would be physiologically important variants of ATF4 in the UPR. Consistent with this notion, an increase of V1 transcript was previously found to occur in stressed heart tissue [32].



3.2. Translation of V1 and V2 transcripts is mediated through different mechanisms

V1 has an extended 5′ leader region harboring 4 uORFs (Fig. 3A), resembling yeast GCN4. This raises the possibility that V1 is a prototypic GCN4-like human transcript regulated by ribosome scanning and reinitiation [10], whereas V2 represents a simplified version of this model of translational control. Alternatively, uORFs in mammalian genes are also known to regulate translation through other mechanisms such as ribosome shunting and IRES [33–36].

To understand whether reinitiation mediated translation of V1, we modified the reporter constructs used in Fig. 2C by inserting a stem-loop structure into different locations of the 5′ leader region of V1 and V2 (Fig. 3A). If ribosome scanning and reinitiation occurred, the stem-loop structure would constitute a physical barrier to the progression of the ribosome along the mRNA. Indeed, a similar stem-loop was previously shown to block reinitiation at uORF2 of V2 effectively when inserted between uORF1 and uORF2 [8]. Likewise, placing a stem-loop in front of uORF1 impeded translation of a V2-GFP reporter [7]. Consistent with previous findings, we observed that insertion of this stem-loop between uORF1 and uORF2 of V2 in the V2SL1 construct dampened the activation of V2 translation by Tg or Tu in HeLa cells (Fig. 3B, V2SL1 compared to V2). Notably, basal translation from a luciferase transcript without uORFs was completely abrogated when the stem-loop was inserted to the upstream of the start codon (Fig. 3B, SL1Luc compared to Luc). In other words, the steric hindrance created by the stem-loop is sufficient for blocking ribosome scanning both in the presence and in the absence of uORFs. In contrast, V1 translation was stimulated by Tg or Tu modestly when a similar stem-loop was inserted in front of uORF1, between uORF2 and uORF3, or between uORF3 and uORF4 (Fig. 3B, V1SL1, V1SL2 and V1SL3 compared to V1). In addition, similar activity profiles of the V1 and V2 constructs were also observed in HEK293T cells (Fig. 3C), attesting that the results were not cell type-specific. These results suggested that translation of V1 and V2 might be mediated through different mechanisms. In other words, the strong translational activity of V1 might not be ascribed to reinitiation. Because ribosome shunting is cap-dependent and starts from scanning [37], it should also be impeded by stem-loop insertion. Hence, translation of V1 uninhibited by a stem-loop was unlikely mediated by ribosome shunting.

Fig. 5. Cap-independent translation of V1 transcript. (A) Inactivation of eIF4G1 by rhinovirus 2A protease. HEK293T cells were co-transfected with the indicated bicistronic reporter constructs, an expression vector for β-galactosidase, and an expression vector for wild-type (WT) or mutant (M) form of 2A protease (pro-2A). Cells in lane 1 received no 2A protease. Shown above is a schematic representation of V1, V2 and EMCV reporter constructs. Steady-state amounts of eIF4G1 were analyzed by Western blotting. β-Actin served as a control for equal loading. (B) Translation of V1 does not require eIF4G1. HEK293T cells were transfected as in A and firefly luciferase activity normalized to β-galactosidase activity was measured. *Renilla* luciferase activity was not used in the normalization since the translation of *Renilla* luciferase was slightly perturbed by the IRES from either EMCV or V2. Results of firefly luciferase activity normalized to readouts of *Renilla* luciferase were presented in Fig. S2. Normalization was not a major contributor to the difference observed. (C) Translational activity of V1 transcript transfected into cells. HEK293T cells were transfected with in vitro transcribed bicistronic RNAs with the indicated 5′ leader sequence inserted between the two cistrons. Shown above is a schematic representation of V1 and V2 reporter transcripts used in RNA transfection. Dual luciferase activity was measured at 8 h and 16 h after transfection. Data represent the mean of three independent experiments and error bars indicate *s.d.* * and #: The differences between the two groups are statistically significant by Student *t* test ($P = 0.0128$ and $P = 0.0114$, respectively).

3.3. Translation of V1 transcript is driven by IRES

One alternative mechanism of eukaryotic translation initiation is through IRES [36–38]. Although consensus motifs for IRES have not been identified, IRESs are more frequently found in long 5′ untranslated regions with upstream AUG codons, high GC content and extensive secondary structure [39]. The 5′ leader regions of V1 and V2 comprise 882 and 280 nucleotides, while their GC contents are 63.2% and 58.9%, respectively. In addition, the longer and GC-richer 5′ leader region of V1 was predicted to be highly structured (Fig. 4A). This prompted us to test whether it might have IRES activity.

Despite limitations, the bicistronic reporter assay is still a common and useful test for IRES activity [36]. In this assay, the sequence of interest was placed between the *Renilla* and firefly luciferase genes (RLuc and FLuc) in the pRF reporter construct (Fig. 4B). The prototypic and well-characterized IRES element from encephalomyocarditis virus (EMCV) was included as a positive control [40]. This viral IRES has previously been shown to require eIF2α for function, but it responds differentially to the activation of eIF2α kinases [41,42]. Treatment of HEK293T cells with Tg or Tu induced phosphorylation of eIF2α as anticipated (Fig. 4C inset). Compared to EMCV, V1 exhibited modest IRES activity which was further potentiated by Tg or Tu (Fig. 4C). In addition, insertion of a stem-loop structure into different locations of the 5′ leader region of V1 transcript (see Fig. 3A for reference) did not significantly affect the IRES activity. In contrast, the activity of V2 in this assay was weak and only minimally higher than that of the empty pRF vector. Progressively truncated mutants of V1 designated F1-F7 were constructed (Fig. 4B) and their activity profiles mapped the ER stress-inducible IRES element to a region between F6 and F7 (nucleotides 663–776), which is downstream of uORF3 and overlaps with 5′ end of uORF4 (Fig. 4C). Whereas it was apparently intact in F6, the IRES was lost in F7 which contains no highly structured region (Fig. 4A). The structural element in F6 might interact with cellular trans-acting factors to mediate the activity of an ER stress-regulated IRES. Exactly what factors might be bound with this element requires further investigation.

It is not understood exactly why the point mutation at nucleotide 189 in V1M substantially affected the responsibility of IREB-dependent translation to the UPR (Fig. 2C). Both V1 and V2 transcripts were associated with polysomes in the presence of Tg or Tu (Fig. 2B). Their translation was also highly inducible by the UPR (Fig. 2C). This inducibility was largely intact even when the most 5′ part of V1 transcript (up to nucleotide 663) was removed (Fig. 4C). Whether the point mutation in V1M might disrupt secondary structure and long-range interaction with the IRES remains to be elucidated.

Major criticisms on the bicistronic reporter assay concerned the presence of cryptic promoters or splice sites and the low efficiency of internal initiation [43–45]. In response to these criticisms, more stringent RNA test procedures were recommended for detection of IRES [46,47]. With this in mind, we used multiple tests to rule in or rule out the possibility that translation of V1 was driven by IRES. First, we assessed whether eIF4G1 was necessary for translation of V1. The V1, V2 and EMCV constructs (Fig. 5A) were transfected into HEK293T cells. eIF4G1 is a scaffolding protein in the cap-binding complex required for cap-dependent translation mediated through ribosome scanning [36]. Rhinovirus encodes 2A protease that cleaves eIF4G1 to inhibit cap-dependent translation [16]. Indeed, the amounts of eIF4G1 were substantially reduced in cells expressing wild type 2A protease compared to cells expressing its C106A defective mutant (Fig. 5A, lanes 2, 4, 6 and 8 compared to lanes 3, 5, 7 and 9). Consistent with previous findings [48], the translation from the IRES of EMCV was not repressed but further enhanced when eIF4G1 was inactivated by 2A protease (Figs. 5B and S2). Whereas the translation driven by 5′ leader region of V2 was blocked almost completely in cells with compromised eIF4G1, the IRES activity of V1 was largely unaffected in these cells (Figs. 5B and S2). These results suggested that the 5′ leader region of V1 was able to drive translation in an eIF4G1-independent manner. Differential effect of eIF4G1 cleavage on viral and cellular IRESs has previously been reported [48]. It remains to be understood why IRESs of EMCV and V1 might respond differentially to eIF4G1 cleavage. Plausibly, these IRESs

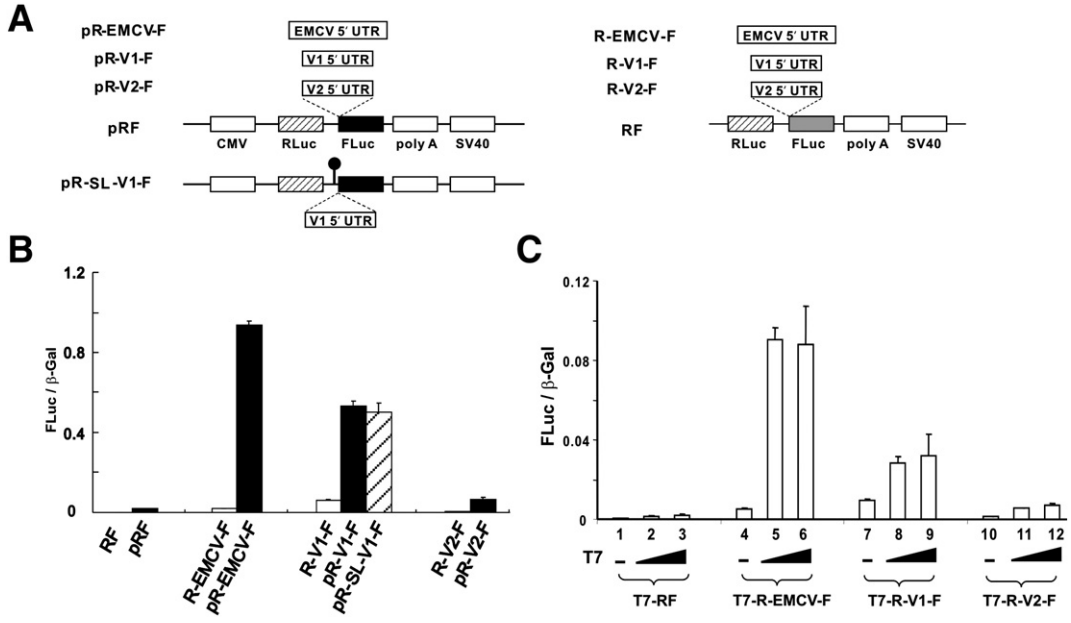


Fig. 6. Protein expression from V1 transcript is not mediated by cryptic promoter. (A, B) Schematic representation and activity of CMV promoter-driven and promoterless bicistronic reporter constructs. HEK293T cells were transiently transfected with the indicated reporters and assayed for relative firefly luciferase activity. (C) Protein expression from V1 DNA requires T7 RNA polymerase-driven transcription. HEK293T cells were co-transfected with PCR fragments containing T7 promoter and increasing amounts of an expression vector for T7 RNA polymerase. Relative firefly luciferase activity was then measured. Results represent three independent experiments and error bars indicate *s.d.*

could recruit different trans-acting factors to drive translation. Because residual amounts of eIF4G1 might exist in the presence of 2A protease and the cleaved eIF4G1 could also support cap-independent translation [49], the possibility that residual or cleaved eIF4G1 might still be involved in the translation of V1 transcript cannot be excluded.

Next, we conducted RNA transfection assay to assess the IRES activity of V1. In vitro transcribed bicistronic RNAs were directly transfected into HEK293T cells and the relative luciferase activity was measured (Fig. 5C). Both EMCV and V1 showed pronounced translational activity over the empty bicistronic RNA termed RF. In contrast, V2 had no IRES activity in this assay (Fig. 5C). Because RNAs directly transfected into cells unlikely underwent further splicing [44,45], these data lent further support to the functionality of IRES in V1.

To formally address whether a cryptic promoter might account for the observed IRES activity of V1, we constructed a series of promoterless bicistronic reporter constructs and compared them with the counterpart CMV-driven plasmids (Fig. 6A). While both EMCV and V1 displayed weak promoter activity over the empty RF vector, their IRES activity derived from the RNA transcripts was more pronounced. In the same setting, no promoter activity was detected for V2 (Fig. 6B). In addition, insertion of a stem-loop in front of the 5′ UTR of V1 did not affect its IRES activity (Fig. 6B, pR-SL-V1-F compared to pR-V1-F). Considered together with the vibrant expression of luciferase reporter driven by V1 (Fig. 2C), the weak promoter activity could unlikely account for its translational activity observed. Consistent with this, when we transfected bicistronic constructs harboring a T7 polymerase-dependent promoter into cells, the EMCV- and V1-driven expression of luciferase reporter was dependent on the co-expression of T7 RNA polymerase (Fig. 6C, columns 5 and 6 compared to column 4, and columns 8 and 9 compared to column 7). Collectively, our results suggested that cryptic promoter might not be a significant concern in the study of V1 translation.

Splicing is thought to mediate the activity of at least some previously-reported cellular IRESs [50]. Because V1 is an alternatively spliced variant of V2 generated by intron retention (Fig. 1A), it was not surprising that V1 might be spliced further into V2. Indeed, V2-specific transcript was detected in cells transfected with pR-V1-F plasmid, although the relative abundance was lower than that of V1 mRNA (Fig. 7A, lane 3). To rule out the possibility that splicing might contribute to the observed translational activity of V1, we made a non-spliceable point mutant of V1 (V1M) in which a 5′ splice site was disrupted (Fig. 7A). We confirmed by RT-PCR that this mutant was not spliced into V2 (Fig. 7A, lane 5 compared to lane 3). No other alternatively spliced transcripts were detected by RT-PCR or Northern blotting (data not shown). When we compared the translational activity of EMCV, V1, V2 and V1M, we noted that V1M exhibited the same pattern as V1 (Fig. 7B), whereas the activity of V2 was much lower. Thus, splicing might not explain the vibrant translational activity of V1 in cultured cells. This was consistent with two of our observations presented earlier. First, results from our stem-loop insertion experiment (Fig. 3) could not be explained by splicing of V1 into V2. Second, data from our RNA transfection experiment did not support a role for splicing (Fig. 5C). Taken together, neither cryptic promoter nor splicing could mediate the potent translational activity of V1.

3.4. IRES activity of V1 is further stimulated by eIF2α phosphorylation

The translation of V2 was known to be stimulated potently by eIF2α phosphorylation [7,8]. In addition, the IRES activity in several cellular and viral transcripts including cationic amino acid transporter 1, cricket paralysis virus, c-Myc and interferon regulatory factor 2 was also activated by eIF2α phosphorylation [51–53]. On the other hand, translation of XIAP mRNA, which was thought to be mediated by IRES, was eIF2α-independent [54]. In light of this, we asked whether

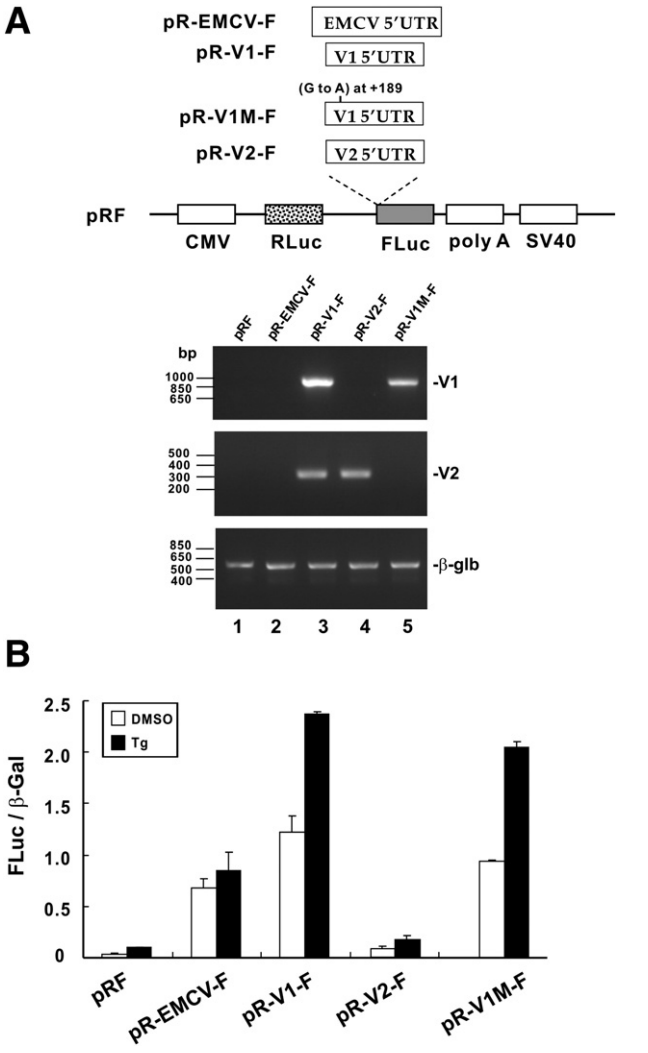


Fig. 7. Protein expression from V1 transcript is not mediated by splicing. (A) Abrogation of alternative splicing in V1 mutant. HEK293T cells were transfected with the indicated bicistronic plasmids. Cells were then analyzed by RT-PCR with primers specific for V1 and V2 transcripts, as well as β-globin (glb). Shown above is a schematic representation of V1, V1M and V2 reporter constructs. A G-to-A mutation at nucleotide 189 was introduced into 5′ UTR of V1M to disrupt the 5′ splice site. Forward primer for amplification of V1 was 5′-CTTAAGCCAT GGCGTGAGTA CC-3′ (GenBank NM_001675, positions 176–197). Forward primer for amplification of V2 was 5′-CTTAAGCCAT GGCGCTTCTC A-3′ (GenBank NM_182810, positions 176–197). (B) Translational activity of V1 mutant. HEK293T cells were transfected with the indicated bicistronic plasmids and assayed for relative firefly luciferase activity. Results are representative of three independent experiments and error bars indicate *s.d.*

the IRES activity of V1 might be affected by eIF2α phosphorylation. Dominant active and inactive mutants of eIF2α were expressed in cells and their impact on V1 and V2 translation was assessed. In contrast to the S51D mutant that mimics constitutively phosphorylated eIF2α, S51A is a non-phosphorylatable mutant of eIF2α [17]. The dominant active eIF2α mutant S51D stimulated translation of V2 from the monocistronic reporter plasmid pV2-F (see Fig. 6A for diagram) 1.7-fold, whereas dominant inactive eIF2α mutant S51A repressed V2 translation by 30% (Fig. S3). This experiment indicated that both mutants behaved as anticipated. Translation of V1 from the bicistronic construct pR-V1-F was stimulated by S51D 2.2- and 6.1-fold in HeLa and HEK293T cells, respectively (Fig. 8, A and B). In contrast, S51A did not activate IRES activity of V1 in either cell line (Fig. 8, A and B). As a control, the eIF2α mutants did not substantially affect the activity of pRF or pR-V2-F reporter plasmid in this assay. Similar experiments were also performed on V1, V2 and truncated

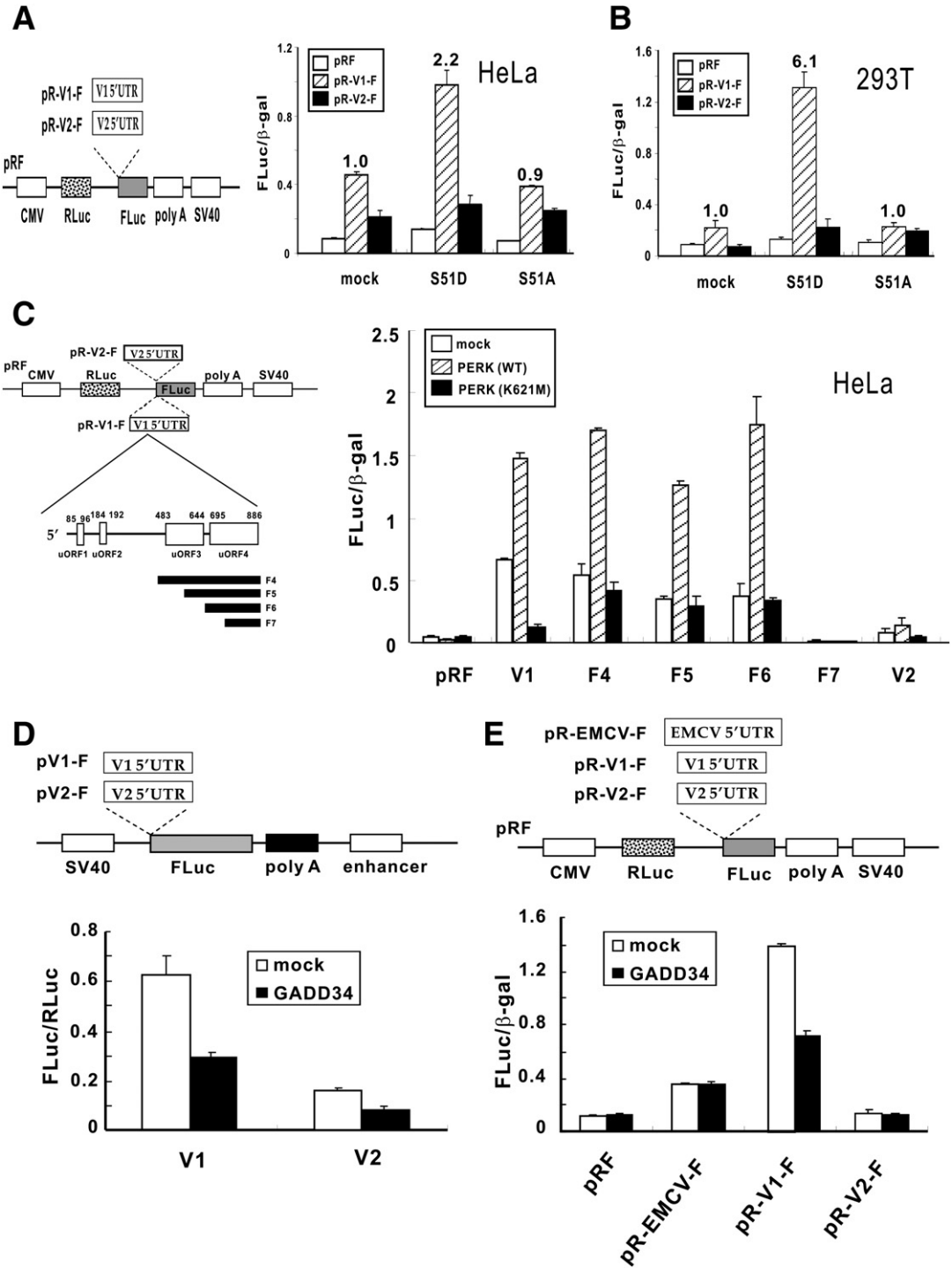


Fig. 8. Translation of V1 transcript is stimulated by eIF2α phosphorylation. HeLa and HEK293T cells were transiently transfected with the indicated combination of bicistronic (A, B, C and E) or monocistronic (D) reporter plasmids and expression vectors. Cells were harvested for measurement of relative luciferase activity. Data represent the mean ± *s.d.* of three independent experiments. Schematic representations of reporter constructs used are shown. Numbers at the top of the error bars indicate relative fold activation.

V1 mutants (F4-F7; Fig. 8C) with PERK wild type and dominant inactive mutant K621M [18]. Whereas PERK augmented the IRES activity of V1 and its mutants F4, F5 and F6, the K621M mutant of PERK showed the opposite effect. In comparison, PERK had no or minimal influence on the activity of pRF, pR-F7-F or pR-V2-F (Fig. 8C). Finally, expression of GADD34, the phosphatase that inactivates eIF2α [17], mitigated protein translation from a V1 or V2 monocistronic expression cassette (Fig. 8D). This is consistent with previous findings that the reinitiated translation of V2 is instigated by eIF2α phosphorylation [7,8]. In contrast, in a bicistronic reporter assay only the IRES activity of V1 was suppressed by GADD34, but the activity of EMCV IRES or V2 was unaffected (Fig. 8E). Collectively, our results indicated that phosphorylation of eIF2α activates IRES-dependent translation of V1.

It is generally accepted that IRES-dependent translation is selectively activated in response to cellular stress [38,39,55]. Recent evidence suggested that this activation might be mediated through facilitators of IRES-mediated translation such as PCBP2, DAP5/p97

and hnRNP A1 [56–58]. Particularly, DAP5/p97 and hnRNP A1 were activated during the UPR [57,58]. Further investigations are required to elucidate whether these IRES activators might be involved in the activation of V1 translation in the UPR and how they relate to eIF2α phosphorylation.

In this study, we demonstrated IRES-dependent translation of an ATF4 splice variant in mammalian UPR using stringent test procedures. The IRES activity was verified by multiple experiments including RNA transfection (Fig. 5C), eIF4G1 inhibition (Fig. 5B) and stem-loop insertion (Fig. 3). Additional analyses were also carried out to exclude the contribution of cryptic promoter or splicing to the potent translational activity of V1 (Figs. 6 and 7). Interestingly, this IRES activity in V1 was induced in the UPR (Fig. 2C) and stimulated by eIF2α phosphorylation (Fig. 8).

ATF4 is a master regulator of UPR genes and it is implicated in various human diseases including cancer, diabetes and skeletal dysplasia [3,59,60]. ATF4 expression is regulated at both transcriptional and translational levels [61,62]. Several lines of evidence support that the V1 transcript characterized in this study is a naturally occurring and physiologically relevant variant of ATF4. First, our Northern blot analysis indicated the expression of V1 in different tissues, particularly peripheral blood leukocytes (Fig. 1B). Second, as shown in GenBank, the V1 transcript was cloned from various human tissues and cells including lymphocytes, gastrointestinal tract, testis and embryonic stem cells, as well as tumor samples. A V1-specific transcript was also found to be elevated in stressed heart [32]. Third, association of the V1 transcript with polysomes in stressed cells suggested that it is efficiently translated (Fig. 2B). Finally, both transcription and translation of V1 were inducible by ER stress (Figs. 1 and 2).

It will be of great interest to elucidate the exact roles of V1 in the UPR. However, because both V1 and V2 encode the same ATF4 protein, it is difficult to determine the relative contribution of V1 versus V2 to induced expression of endogenous ATF4 protein in the UPR. In this regard, rescue experiments in ATF4-null cells and variant-specific siRNAs might prove useful in future attempts to distinguish the roles of V1 and V2 transcripts. However, the V1-specific siRNAs we made were also found to be able to affect the production of V2 transcript plausibly through an effect on splicing (data not shown). Nevertheless, our current findings suggest an unrecognized role of IRES in translational control of ATF4 in mammalian UPR.

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Author contribution

C.-P. Chan, K.-H. Kok, H.-M. V. Tang, C.-M. Wong and D.-Y. Jin designed experiments. C.-P. Chan performed experiments. C.-P. Chan, K.-H. Kok, H.-M. V. Tang, C.-M. Wong and D.-Y. Jin analyzed the data. C.-P. Chan, C.-M. Wong and D.-Y. Jin wrote the paper.

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References

[1] M. Schröder, R.J. Kaufman, The mammalian unfolded protein response, Annu. Rev. Biochem. 74 (2005) 739–789.
[2] P. Walter, D. Ron, The unfolded protein response: from stress pathway to homeostatic regulation, Science 334 (2011) 1081–1086.
[3] D.R. Fels, C. Koumenis, The PERK/eIF2α/ATF4 module of the UPR in hypoxia resistance and tumor growth, Cancer Biol. Ther. 5 (2006) 723–728.
[4] R.C. Wek, D.R. Cavener, Translational control and the unfolded protein response, Antioxid. Redox Signal. 9 (2007) 2357–2371.
[5] K. Ameri, A.L. Harris, Activating transcription factor 4, Int. J. Biochem. Cell Biol. 40 (2008) 14–21.
[6] H.P. Harding, I. Novoa, Y. Zhang, H. Zeng, R. Wek, M. Schapira, D. Ron, Regulated translation initiation controls stress-induced gene expression in mammalian cells, Mol. Cell 6 (2000) 1099–1108.
[7] P.D. Lu, H.P. Harding, D. Ron, Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response, J. Cell Biol. 167 (2004) 27–33.
[8] K.M. Vattem, R.C. Wek, Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 11269–11274.
[9] P.P. Mueller, A.G. Hinnebusch, Multiple upstream AUG codons mediate translational control of GCN4, Cell 145 (1986) 201–207.
[10] N. Sonenberg, A.G. Hinnebusch, Regulation of translation initiation in eukaryotes: mechanisms and biological targets, Cell 136 (2009) 731–745.
[11] H. Ait Ghezala, B. Jolles, S. Salhi, K. Castrillo, W. Carpentier, N. Cagnard, A. Bruhat, Y.P. Fafournoux, O. Jean-Jean, Translation termination efficiency modulates ATF4 response by regulating ATF4 mRNA translation at 5′ short ORFs, Nucleic Acids Res. 40 (2012) 9557–9570.
[12] Ching, A.C.S. Chun, K.T. Chin, Z.Q. Zhang, K.T. Jeang, D.Y. Jin, Specific TATAA and bZIP requirements suggest that HTLV-I Tax has transcriptional activity subsequent to the assembly of an initiation complex, Retrovirology 1 (2004) 18.
[13] Y.T. Siu, K.T. Chin, K.L. Siu, E.Y.W. Choy, K.T. Jeang, D.Y. Jin, TORC1 and TORC2 coactivators are required for Tax activation of the human T-cell leukemia virus type 1 long terminal repeats, J. Virol. 80 (2006) 7052–7059.
[14] T.R. Reddy, H. Tang, X. Li, F. Wong-Staal, Functional interaction of the HTLV-1 transactivator Tax with activating transcription factor-4 (ATF4), Oncogene 14 (1997) 2785–2792.
[15] A. Tsujimoto, H. Nyunoya, T. Morita, T. Sato, K. Shimotohno, Isolation of cDNAs for DNA-binding proteins which specifically bind to a Tax-responsive enhancer element in the long terminal repeat of human T-cell leukemia virus type I, J. Virol. 65 (1991) 1420–1426.
[16] M. Petz, D. Kozina, H. Huber, T. Siwiec, J. Seipelt, W. Sommergruber, W. Mikulits, The leader region of laminin B1 mRNA confers cap-independent translation, Nucleic Acids Res. 35 (2007) 2473–2482.
[17] I. Novoa, H. Zeng, H. Harding, D. Ron, Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α, J. Cell Biol. 153 (2001) 1011–1022.
[18] K. Ma, K.M. Vattem, R.C. Wek, Dimerization and release of molecular chaperone inhibition facilitate activation of eukaryotic initiation factor-2 kinase in response to endoplasmic reticulum stress, J. Biol. Chem. 277 (2002) 18728–18735.
[19] A.C.-S. Chun, K.-H. Kok, D.-Y. Jin, REV7 is required for anaphase promoting complex-dependent ubiquitination and degradation of translesion DNA polymerase REV1, Cell Cycle 12 (2013) 365–378.
[20] K.T. Chin, H.T. Xu, Y.P. Ching, D.Y. Jin, Differential subcellular localization and activity of kelch repeat proteins KLHDC1 and KLHDC2, Mol. Cell. Biochem. 296 (2007) 109–119.
[21] Y.T. Siu, Y.P. Ching, D.Y. Jin, Activation of TORC1 transcriptional coactivator through MEKK1-induced phosphorylation, Mol. Biol. Cell 19 (2008) 4750–4761.
[22] M.H.J. Ng, T.H. Ho, K.H. Kok, K.L. Siu, J. Li, D.Y. Jin, MIP-T3 is a negative regulator of innate type I interferon response, J. Immunol. 187 (2011) 6473–6482.
[23] D. Zhou, L.R. Palam, L. Jiang, J. Narasimhan, K.A. Staschke, R.C. Wek, Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions, J. Biol. Chem. 283 (2008) 7064–7073.
[24] L.R. Palam, T.D. Baird, R.C. Wek, Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance CHOP translation, J. Biol. Chem. 286 (2011) 10939–10949.
[25] K.H. Kok, M.H.J. Ng, Y.P. Ching, D.Y. Jin, Human TRBP and PACT interact with each other and associate with Dicer to facilitate the production of small interfering RNA, J. Biol. Chem. 282 (2007) 17649–17657.
[26] K.H. Kok, P.Y. Lui, M.H.J. Ng, K.L. Siu, S.W.N. Au, D.Y. Jin, The double-stranded RNA-binding protein PACT functions as a cellular activator of RIG-I to facilitate innate antiviral response, Cell Host Microbe 9 (2011) 299–309.
[27] P.C. Woo, S.K. Lau, G.K. Choi, Y. Huang, J.L. Teng, H.W. Tsoi, H. Tse, M.L. Yeung, K.H. Chan, D.Y. Jin, K.Y. Yuen, Natural occurrence and characterization of two internal ribosome entry site elements in a novel virus, canine picodicistrovirus, in the picornavirus-like superfamily, J. Virol. 86 (2012) 2797–2808.
[28] C.P. Chan, K.L. Siu, K.T. Chin, K.Y. Yuen, B. Zheng, D.Y. Jin, Modulation of the unfolded protein response by severe acute respiratory syndrome coronavirus spike protein, J. Virol. 80 (2006) 9279–9287.
[29] C.P. Chan, T.Y. Mak, K.T. Chin, I.O.L. Ng, D.Y. Jin, N-linked glycosylation is required for optimal proteolytic activation of membrane-bound transcription factor CREB-H, J. Cell Sci. 123 (2010) 1438–1448.
[30] C.P. Chan, Y.T. Siu, K.H. Kok, Y.P. Ching, H.M.V. Tang, D.Y. Jin, Group I p21-activated kinases facilitate Tax-mediated transcriptional activation of the human T-cell leukemia virus type 1 long terminal repeats, Retrovirology 10 (2013) 47.
[31] M. Zuker, Mfold web server for nucleic acid folding and hybridization prediction, Nucleic Acids Res. 31 (2003) 3406–3415.
[32] Z. Gao, H. Xu, D. DiSilvestre, V.L. Halperin, R. Tunin, Y. Tian, W. Yu, R.L. Winslow, G.F. Tomaselli, Transcriptomic profiling of the canine tachycardia-induced heart failure model: global comparison to human and murine heart failure, J. Mol. Cell. Cardiol. 40 (2006) 76–86.
[33] D.R. Morris, A.P. Geballe, Upstream open reading frames as regulators of mRNA translation, Mol. Cell. Biol. 20 (2000) 8635–8642.
[34] H.A. Meijer, A.A.M. Thomas, Control of eukaryotic protein synthesis by upstream open reading frames in the 5′-untranslated region of an mRNA, Biochem. J. 367 (2002) 1–11.

[35] S.J. Morley, M.J. Coldwell, A cunning stunt: an alternative mechanism of eukaryotic translation initiation, Sci. Signal. 1 (2008) pe32.
[36] B.L. Semler, M.L. Waterman, IRES-mediated pathways to polysomes: nuclear versus cytoplasmic routes, Trends Microbiol. 16 (2008) 1–5.
[37] C.U.T. Hellen, P. Sarnow, Internal ribosome entry sites in eukaryotic mRNA molecules, Genes Dev. 15 (2001) 1593–1612.
[38] A.A. Komar, M. Hatzoglou, Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states, Cell Cycle 10 (2011) 229–240.
[39] M. Holcik, N. Sonenberg, Translational control in stress and apoptosis, Nat. Rev. Mol. Cell Biol. 6 (2005) 318–327.
[40] C.Y. Chen, P. Sarnow, Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs, Science 268 (1995) 415–417.
[41] E. Welnowska, M.A. Sanz, N. Redondo, L. Carrasco, Translation of viral mRNA without active eIF2: the case of picornaviruses, PLoS One 6 (2011) e22230.
[42] M. Licursi, Y. Komatsu, T. Pongnopparat, K. Hirasawa, Promotion of viral internal ribosomal entry site-mediated translation under amino acid starvation, J. Gen. Virol. 93 (2012) 951–962.
[43] M. Kozak, A second look at cellular mRNA sequences said to function as internal ribosome entry sites, Nucleic Acids Res. 33 (2005) 6593–6602.
[44] E.S. Mardanova, L.A. Zamchuk, N.V. Ravin, Contribution of internal initiation to translation of cellular mRNAs containing IRESs, Biochem. Soc. Trans. 36 (2008) 694–697.
[45] W.V. Gilbert, Alternative ways to think about cellular internal ribosome entry, J. Biol. Chem. 285 (2010) 29033–29038.
[46] M.E. Van Eden, M.P. Byrd, K.W. Sherrill, R.E. Lloyd, Demonstrating internal ribosome entry sites in eukaryotic mRNAs using stringent RNA test procedure, RNA 10 (2004) 720–730.
[47] A.A. Komar, B. Mazumder, W.C. Merrick, A new framework for understanding IRES-mediated translation, Gene 502 (2012) 75–86.
[48] A. Castelló, E. Alvarez, L. Carrasco, Differential cleavage of eIF4GI and eIF4GII in mammalian cells: effects on translation, J. Biol. Chem. 281 (2006) 33206–33216.
[49] T. Ohlmann, M. Rau, V.M. Pain, S.J. Morley, The C-terminal domain of eukaryotic protein synthesis initiation factor (eIF) 4G is sufficient to support cap-independent translation in the absence of eIF4E, EMBO J. 15 (1996) 1371–1382.
[50] B.T. Baranick, N.A. Lemp, J. Nagashima, K. Hiraoka, N. Kasahara, C.R. Logg, Splicing mediates the activity of four putative cellular internal ribosome entry sites, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 4733–4738.
[51] J. Fernandez, I. Yaman, P. Sarnow, M.D. Snider, M. Hatzoglou, Regulation of internal ribosomal entry site-mediated translation by phosphorylation of the translation initiation factor eIF2α, J. Biol. Chem. 277 (2002) 19198–19205.
[52] G. Gerlitz, R. Jagus, O. Elroy-Stein, Phosphorylation of initiation factor-2α is required for activation of internal translation initiation during cell differentiation, Eur. J. Biochem. 269 (2002) 2810–2819.
[53] D. Dhar, S. Roy, S. Das, Translational control of the interferon regulatory factor 2 mRNA by IRES element, Nucleic Acids Res. 35 (2007) 5409–5421.
[54] N. Thakor, M. Holcik, IRES-mediated translation of cellular messenger RNA operates in eIF2α-independent manner during stress, Nucleic Acids Res. 40 (2012) 541–552.
[55] K.A. Spriggs, M. Bushell, A.E. Willis, Translational regulation of gene expression during conditions of cell stress, Mol. Cell 40 (2010) 228–237.
[56] K. Fujimura, F. Kano, M. Murata, Identification of PCBP2, a facilitator of IRES-mediated translation, as a novel constituent of stress granules and processing bodies, RNA 14 (2008) 425–431.
[57] S.M. Lewis, S. Cerquozzi, T.E. Graber, N.H. Ungureanu, M. Andrews, M. Holcik, The eIF4G homolog DAP5/p97 supports the translation of select mRNAs during endoplasmic reticulum stress, Nucleic Acids Res. 36 (2008) 168–178.
[58] F. Damiano, A. Rochira, R. Tocci, S. Alemanno, A. Gnoni, L. Siculella, hnRNP A1 mediates the activation of the IRES-dependent SREBP-1a mRNA translation in response to endoplasmic reticulum stress, Biochem. J. 449 (2013) 543–553.
[59] K.Y. Tsang, D. Chan, J.F. Bateman, K.S.E. Cheah, In vivo cellular adaptation to ER stress: survival strategies with double-edged consequences, J. Cell Sci. 123 (2010) 2145–2154.
[60] J. Ye, M. Kumanova, L.S. Hart, K. Sloane, H. Zhang, D.N. De Panis, E. Bobrovnikova-Marjon, J.A. Diehl, D. Ron, C. Koumenis, The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation, EMBO J. 29 (2010) 2082–2096.
[61] J.D. Blais, V. Filipenko, M. Bi, H.P. Harding, D. Ron, C. Koumenis, B.G. Wouters, J.C. Bell, Activating transcription factor 4 is translationally regulated by hypoxic stress, Mol. Cell. Biol. 24 (2004) 7469–7482.
[62] S. Dey, T.D. Baird, D. Zhou, L.R. Palam, D.F. Spandau, R.C. Wek, Both transcriptional regulation and translational control of ATF4 are central to the integrated stress response, J. Biol. Chem. 285 (2010) 33165–33174.