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Proteins Enhancing IRES-Mediated Translation

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Proteins Enhancing IRES-Mediated Translation

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Abstract

Certain RNAs are proposed to have an internal ribosome entry site (IRES) in their 5' untranslated region (UTR). RNAs with an IRES include the encephalomyocarditis virus (EMCV), poliovirus (PV), hepatitis C virus (HCV), cricket paralysis virus (CrPV) and the cellular myc mRNA. Using a traditional dual luciferase construct, we can determine the translation efficiency of IRES-containing RNAs in cells in the presence and absence of cellular proteins.

To identify cellular proteins important for IRES-mediated translation, such as ribosomal proteins and translation factors, we have established shRNA knockdowns in HeLa cells. We specifically depleted ribosomal proteins eS25 and RACK1, and translation initiation factors eIF4E, DAP5, and eIF3D to test their involvement in IRES-mediated translation. Based on the literature, we hypothesized that loss of RACK1, DAP5, eS25 and eIF3D may decrease IRES-mediated translation of specific IRESs, while eIF4E should not be required for IRES-mediated translation. First, we validated successful knockdown of these proteins in HeLa cells compared to a control by immunoblotting.

We next determined the translation efficiency of the EMCV, PV, HCV, and CrPV IRESs using dual luciferase constructs. As expected, the CrPV IRES only required eS25, while the other IRESs required eS25 and RACK1. We further found that the HCV IRES additionally required eIF3D, while only the EMCV IRES required all four proteins, but not eIF4E. Overall, my experiments established a system to dissect the protein requirements for cellular IRESs in the future.

Keywords: *MYC, IRES, Protein factors, Translation, Transcription factors*

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Introduction

Cap-independent translation initiation mechanisms are mainly used when cap-dependent translation is compromised. One cap-independent mechanism uses **i**nternal **r**ibosome **e**ntry **s**ites (IRESs). IRESs are RNA secondary structures that are able to mediate translation. These structures are found on both viral and cellular mRNA transcripts. It has been established that certain physiological, pathophysiological and stress conditions contribute to the inhibition of cap-dependent translation and increase the use of IRES-mediated translation in a eukaryotic cell. Cellular mRNAs proposed to have an internal ribosome entry site (IRES) in their 5' untranslated region (UTR) are the c-myc and L-myc mRNAs as well as, c-jun, and DAP5 mRNAs (Komar & Hatzoglou, 2011).

With our main interest in the mechanism of IRES-mediated translation, the goal of this project was to identify cellular proteins, such as ribosomal proteins and translation factors, important for IRES-mediated translation. By depleting mRNAs and proteins using shRNA knockdowns in HeLa cells, we aimed at depleting ribosomal proteins RACK1 and eS25 and translation factors eIF4E, DAP5, and eIF3D. We next determined the translation efficiency of different viral IRESs in these cells using previously validated dual luciferase constructs (Jan & Sarnow, 2002; Johannes & Sarnow, 1998; Thompson, 2012).

After establishing that the knockdown cell lines possessed the expected characteristics, our next step will be to investigate which cellular proteins are needed for IRES-mediated translation in certain cellular mRNAs such as c-myc, c-Jun, and DAP5. C-myc is a 62 kDa protein transcription factor involved in cell differentiation, proliferation, and apoptosis (Sammak et al., 2019). C-jun is a protooncogene that is also involved in cell proliferation, cell death and

malignant transformation as well as inducing genes for cell cycle progression and repressing tumor suppressors like p53 (Blau et al., 2012). Lastly, DAP5 is a member of the eIF4G family known to participate in programmed cell death via caspase cleavage (Marash & Kimchi, 2005).

Mechanism of translation

The translation of the mRNA sequence into a protein sequence is performed by molecular machines called ribosomes, which are composed of several RNA molecules and numerous proteins. In prokaryotes, ribosomes are composed of 30S and 50S subunits and in eukaryotes of 40S and 60S subunits. Translation can be divided into three stages, initiation, elongation, and termination. During eukaryotic initiation, Met-tRNA$_i$, eIF2 and GTP form a ternary complex, and assemble with the 40S ribosomal subunit together with initiation factors such as eIF1, eIF1A, eIF3 and eIF5, consequently forming a pre-initiation complex (PIC).

The pre-initiation complex is recruited to the 5' end of an mRNA, which contains a 7-methyl guanosine cap (m^7G cap). The mRNA cap is also bound by other proteins, specifically the eIF4F complex, composed of the cap-binding protein eIF4E, the scaffolding protein eIF4G and the helicase eIF4A. Translation factors eIF3 and the poly(A)-binding protein (PABP) bind to eIF4G and mediate mRNA circularization. Next, the PIC scans the 5' UTR in an open conformation in search for a start (AUG) codon; upon start codon recognition, GTP hydrolysis and P_i release cause a conformational change in eIF2. The scanning mechanism is then arrested due to the formation of a closed conformation of the pre-initiation complex that stabilizes the mRNA and Met-tRNA$_i$ interaction. The GDP-bound eIF2 and eIF5 then dissociate, activating another GTPase (eIF5B) which allows for 60S subunit joining and formation of the 80S elongation competent ribosome (Aitken & Lorsch, 2012).

The process of elongation which constitutes of peptide bond formation begins when the peptidyl-tRNA enters the P site of the ribosome. The peptide from the tRNA in the P-site (peptidyl-tRNA) is transferred to the amino group of the A-site aminoacyl-tRNA (aatRNA), which lengthens the peptide by another amino acid (Figure 1). During this process, tRNAs adjust themselves in three hybrid states through subunit rotation. In the first hybrid state (unrotated complex), the new peptidyl-tRNA in found in the A-site while the deacylated tRNA is found in the P-site. In the second hybrid state (rotated-1 complexes), deacylated tRNA transforms into a hybrid P/E state with a mRNA paired to an anti-codon at the P-site, the acceptor arm of the tRNA in the E-site and the peptidyl-tRNA still at the A-site. In the third hybrid state (rotated-2-state), deacylated tRNA positions itself in a P/E hybrid state whiles peptidyl-tRNA reassembles itself in A/P hybrid state. The mRNA at this point is still paired with an anticodon in the A site and the growing peptide attaches its acceptor arm in the P site. Eukaryotic elongation factor 2 (eEF2), induces translocation of tRNAs to the E and P sites. Specifically, eEF2 binds to the A site and decodes helix h44 nucleotides in the presence of the codon-anticodon duplex. Deacylated t-RNA then fully moves to the E site and peptidyl-tRNA assembles in the P site (Dever et al., 2018).

Lastly, the process of termination is facilitated when a UAA, UAG or UAG stop codon enters the A site. These stop codons are recognized by eukaryotic release factors 1 or 2 (eRF1, eRF2). Following stop codon recognition by eRF1 or eRF2, a conformational change triggers hydrolysis of the peptidyl-tRNA chain and peptide release. Binding of eukaryotic translation termination factor (eRF3) and GTP facilitates dissociation of eRF1/2, while GTP hydrolysis and P_i release enable dissociation of eRF3. Lastly, ribosome release factor RRF in conjunction with

EFG and GTP hydrolysis releases mRNA, peptidyltRNA and the ribosomal subunits (Mitkevich et al., 2006)

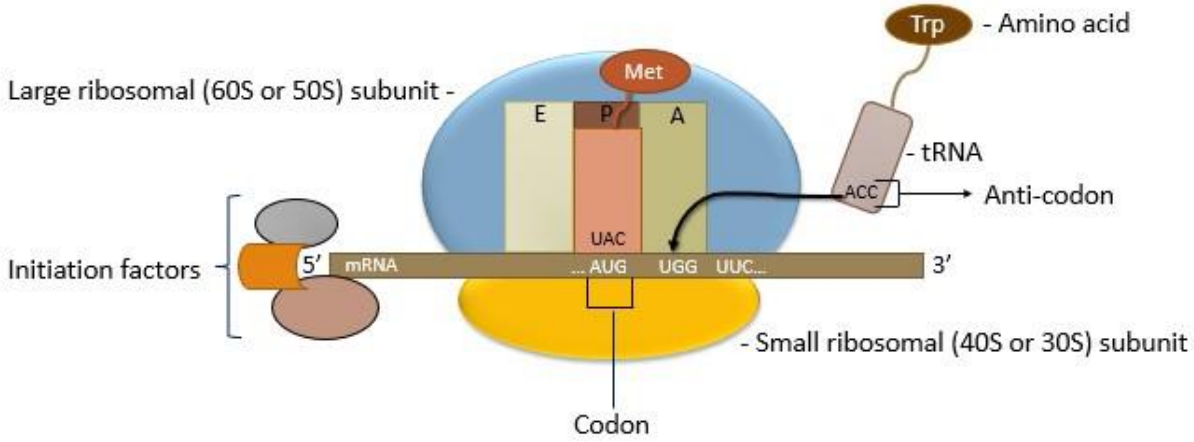


Figure 1

Schematic of translation in the ribosome

Note. First, the large and small ribosomal subunits assemble on the start codon and recruit an amino acid using a tRNA into the A site. The codon-anticodon base pairing with the mRNA leads to peptide formation in a step called elongation. When a stop codon enters the A site, mRNA, peptide, and ribosomal subunits are released.

Cap- dependent and Cap-independent translation in eukaryotes

Unlike cap-dependent translation, cap-independent translation forsakes the need for m^7Gcap as the terminal nucleotide on the 5′ UTR of a mRNA transcript. Cap-independent translation initiates by using RNA secondary structures to recruit the small (40S) subunit and the large (60S) subunit of a ribosome with minimal initiation factors (Figure 2). Such mRNA secondary structures that can recruit ribosomal subunits cap-independently are known as an IRES (Komar & Hatzoglou, 2011).

IRESs are found on both viral and cellular mRNA transcripts. Cellular IRESs are thought to be mainly used when cap-dependent translation is compromised. It has been established that

certain physiological, pathophysiological and stress conditions contribute to the inhibition of cap-dependent translation and increase IRES-mediated translation in a eukaryotic cell (Komar & Hatzoglou, 2011). Hence, cellular IRES-containing mRNAs are preferentially translated during conditions of stress. Examples of stressful conditions that utilize the mechanism of cellular IRES-mediated translation include endoplasmic reticulum (ER) stress, hypoxia, nutrient limitation, mitosis, and cell differentiation. After much investigation, it is known that mRNAs with cellular IRESs either protect cells from these stressful conditions or facilitate automatic programmed cell death (apoptosis). Therefore, cellular IRESs play an important role in cell-fate decision (Komar & Hatzoglou, 2011).

Certain IRESs can directly recruit the 40S ribosomal subunit to the start codon. Such IRESs are the viral IRESs found in Hepatitis C Virus (HCV) and Cricket Paralysis Virus (CrPV). Other IRESs, including select cellular IRES-containing mRNAs may also engage in the "land" (in the vicinity of initiation codon) and "scan" mechanism. IRESs found in myc are proposed to use this mechanism of internal initiation (Komar & Hatzoglou, 2011). The "land" and "scan" mechanism entail ribosomes and corresponding initiation factors binding near the 5' end of the mRNA, a process facilitated by the presence of a cap structure. The ribosome then scans the mRNA sequence until a pertinent initiation codon is found, enabling the process of cap-independent translation of cellular IRES-containing mRNAs (Pelletier & Sonenberg, 1988).

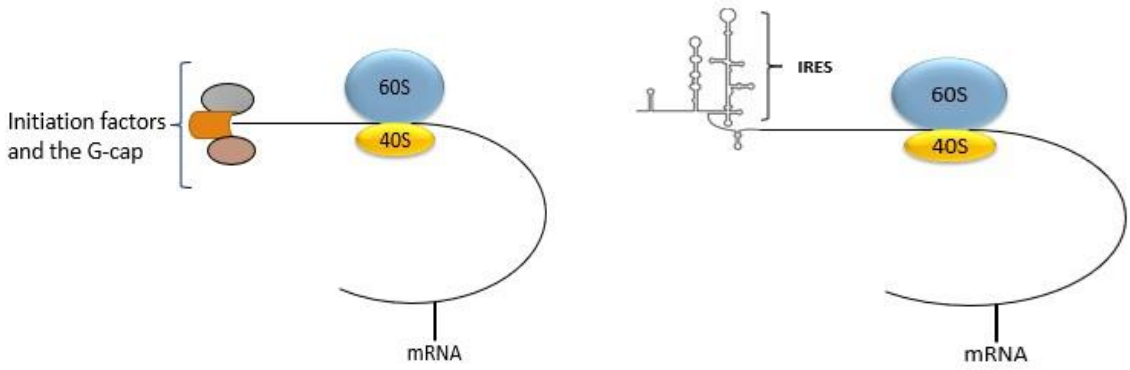


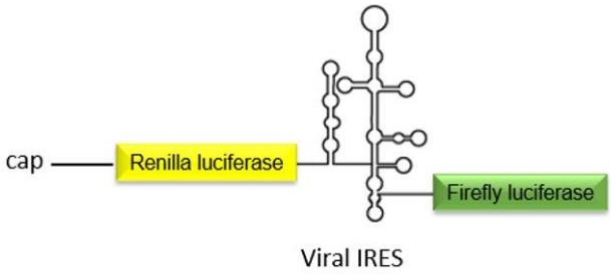
Figure 2

*Cap-dependent (left) and cap-independent (right) translation***.**

Note. In cap dependent translation, translation factors bind to the 7-methyl guanosine residue that caps the 5' end of the mRNA. For cap-independent translation, an IRES is utilized to initiate translation.

Cellular RNAs to measure IRES-mediated translation

Although cellular IRESs have been suggested two decades ago, their existence has remained elusive. Specifically, the presence of the m^7G cap on all cellular mRNAs made it challenging to prove their existence. To test for IRES activity, a dual reporter assay is



$$\text{IRES activity} = \frac{\text{Firefly value}}{\text{Renilla value}}$$

Figure 3

Schematic of reporter proteins Renilla and Firefly luciferase on the same messenger RNA.

Note. Renilla luciferase translates the mRNA without an IRES whiles Firefly luciferase translation is mediated by an IRES. This dual reporter assay ratio is a proxy for IRES activity in a viral IRES.

frequently used. These constructs encode two reporter proteins, often *Renilla* and Firefly luciferase, on the same mRNA. In the construct shown in Figure 3, the *Renilla* luciferase is translated cap-dependently, while translation of the Firefly luciferase is mediated by an IRES.

However, this assay has been shown to also be associated with false-positive results, as cryptic promoters within the IRES can give rise to a capped and well-translated monocistronic construct, which exaggerates IRES activity (Figure 4) (Thompson, 2012). Similarly, cryptic splice sites and ribosome readthrough can also elevate Firefly protein levels in absence of an IRES.

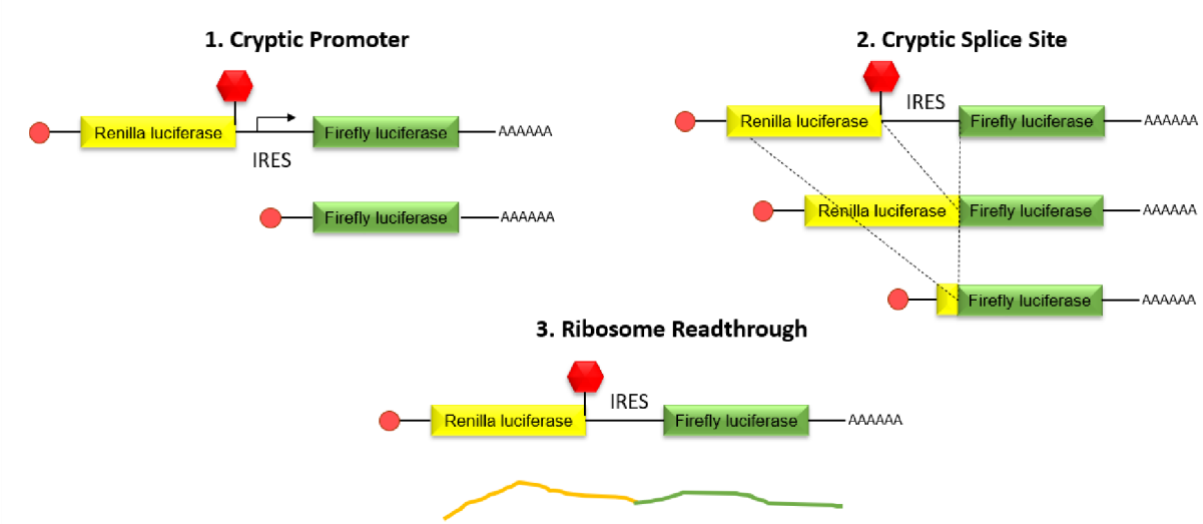


Figure 4

Effect of cryptic promoter, cryptic splice site and ribosome readthrough on IRES activity.

Note. Cryptic promoters enhance the translation of monocistronic construct, interfering with IRES activity in a mRNA. In addition, cryptic splice site and ribosome readthrough elevates Firefly protein levels in the absence of an IRES.

Cellular RNAs proposed to be involved IRES-mediated translation are myc, c-jun and DAP5. MYC also known as c-Myc, is a 62 kDa protein and part of the basic helix-loophelix zipper (bHLHZip) class of transcription factors. Additionally, it has a N-terminus that contains a transcription activation domain (TAD) and two MYC boxes (MBI and MBII) that participate in transcription regulation and protein stabilization (Sammak et al., 2019). Over the years, c-Myc has gained popularity given its active involvement in many cancers since it controls cell proliferation, differentiation, and apoptosis (Thoma et al., 2004). Furthermore, its poly(A) tail enables translation via an IRES during cell stress, summoning the assembly of the 48S complex and initiating translation cap-independently.

C-jun, a protooncogene, is also involved in cell proliferation, cell death and malignant transformation. C-jun primarily initiates cell progression through two mechanisms, namely the (a) induction of genes supporting cell cycle progression like cyclin D1 and (b) repression of tumor suppressor genes like p53. When c-jun is exposed to extracellular stimuli like growth factors, cytokines, cellular stress or UV radiation, its expression is elevated. The accumulation of c-jun transcripts is shown to be meditated by an IRES and forsakes the need to activate the MAPK family of serine/threonine kinases, to initiate translation (Blau et al., 2012). However, literature support for the c-jun IRES is much weaker as only a single reference is referenced in the IRESite database (Mokrejs et al., 2006).

DAP5, Death Associated Protein 5, is a part of the eIF4G family with no binding site for elF4E. Like c-Myc and c-jun, it is also involved in programmed cell death when activated by caspase cleavage, which results in a C-terminal-truncated protein of 86 kDa. The p86 form induces translation by an IRES of many mRNAs like Apaf-1, c-Myc. XIAP,

HIAP2 and DAP5 itself as it also harbors an IRES element in its own 5'UTR. The DAP5 IRES enables translation of the DAP5 protein during cell stress using a positive feedback loop, during cap-independent translation (Marash & Kimchi, 2005).

Viral IRESs

Viral IRES-mediated translation by encephalomyocarditis virus (EMCV), polio virus (PV), hepatitis C virus (HCV) and cricket paralysis virus (CrPV) (Figure 5) does not utilize a m^7G cap to initiate translation and only requires fewer cellular proteins to together with the 40S ribosomal subunit. This alternative pathway enables the 40S ribosomal subunit to have strong affinity to the 5' Untranslated Region (UTR) of the viral mRNA (Lukavsky, 2009).

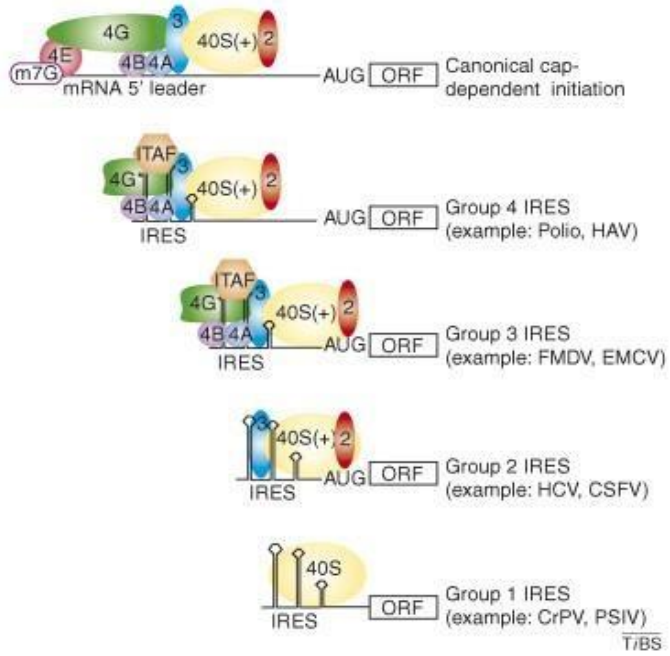


Figure 5

Schematic of IRES-mediated translation in PV, EMCV, HCV and CrPV.

Note. Majority of the viral IRESs use a few ribosomal proteins and translation initiation factors to translate mRNAs via the IRES (Kieft, 2008).

The CrPV IRES is a 190 nucleotide (nt) secondary mRNA structure located upstream of the first alanine codon in the second CrPV cistron. It consists of three structural regions that have a pseudoknot (PK) required for viral IRES translation activity (Johnson et al., 2017). The HCV IRES is 340 nt in length and located in the 5' UTR of the HCV gene. It has multiple folded domains that bind the 40S ribosomal subunit and the eIF3 complex and contributes to translation initiation activities (Johnson et al., 2017). Similarly, both PV and EMCV IRESs are also found in the 5' UTR and utilize cap-independent translation for their mRNAs (Schmid & Wimmer, 1994).

Proteins involved in IRES mediated translation

Multiple cellular proteins have been shown to support viral IRES-mediated translation, particularly the ribosomal proteins Receptor for activated C Kinase 1 (RACK1) (LaFontaine et al., 2020; Majzoub et al., 2014; Olivares et al., 2014) and eukaryotic ribosomal protein 25(Hertz et al., 2013; Landry et al., 2009). Cellular translation factors Death-associate protein 5 (DAP5) and eukaryotic initiation factor 3D (eIF3D) have been shown to mediate translation of c-Myc (de la Parra et al., 2018). In contrast, the cap-binding protein eukaryotic initiation factor 4E (eIF4E) is not required for IRES-mediated translation, although a recent report showed that it can enhance IRES-mediated translation independent of its cap-binding properties (Avanzino et al., 2017).

RACK1, a eukaryotic ribosomal protein of the small ribosomal subunit (Figure 6), has been found to be heavily involved in cellular regulation. This adapter protein interacts with multiple signaling molecules and is located on the 40S ribosomal subunit of the ribosome. Additionally, it plays a major role in 80S ribosome formation by activating a

signaling cascade which allows initiation factor eIF6 to bind with the 60S subunit of the ribosome and is phosphorylated by the RACK1 protein, releasing it from the 60S subunit (Ceci et al., 2003). RACK1 involvement in IRES-mediated translation has been shown in Drosophila C virus (DCV), HCV and PV translation, which proceeds cap-independently using an IRES (LaFontaine et al., 2020; Majzoub et al., 2014).

RPS5 or eS25, like RACK1, is also a eukaryotic ribosomal protein, which is located near the head of the 40S ribosomal protein (RP) directly involved in the mechanism of protein synthesis. Like other ribosomal proteins, eS25 partakes in the production of proteins through specific molecular interactions with ribosome-bound RPs and mRNA transcripts by manipulating its levels to allow the ribosome to generate certain mRNA transcripts (Johnson et al., 2020)

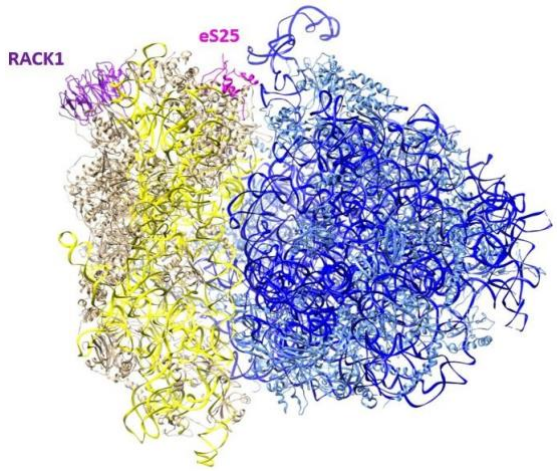


Figure 6

Structure of the eukaryotic ribosome with the 40S ribosomal subunit in yellow and the 60S subunit in blue.

Note. Ribosomal proteins RACK1 in purple and eS25 in magenta are highlighted (PDB 5T2C, RCSB PDB-5T2C: CryoEM Structure of the Human Ribosome at 3.6 Angstrom Resolution, n.d.; Zhang et al., 2016).

DAP5, also known as p97 or NAT1, participates in programmed cell death or apoptosis. It is a member of the eIF4G family that does not contain an elF4E binding site. Its involvement in apoptosis is triggered by a caspase cleavage, that results in a deformed C terminal of protein 86 KDa or p86. The structure of p86 promotes cap-independent or IRES-mediated translation in multiple mRNAs, which includes DAP5 itself. The DAP5 IRES promotes a positive feedback loop that prolongs translation of the DAP5 protein in conditions that favors cap-independent translation (Marash & Kimchi, 2005). In addition, DAP5 has been shown to mediate the initial round of viral translation of coxsackivirus (Dave et al., 2019).

eIF3D is a translation initiation factor that can be located behind the head of the 40S ribosomal subunit adjacent to ribosomal protein RACK1 (Lee et al., 2016). Like the proteins above, it is needed for cap-independent translation (Lee et al., 2016). Additionally, when eIF3D directly binds to DAP5, cap-dependent translation is accelerated in a messenger RNA (de la Parra et al., 2018).

eIF4E, eukaryotic translation initiation factor 4E, binds to the m^7G-cap and helps to recruit the 40S ribosomal subunit of the ribosome to the 5' end of the mRNA for cap-dependent translation. It is heavily controlled by two mechanisms: the binding of eIF4E to translational repressor 4E-binding proteins (4EBPs) which prevent binding to eIF4G and formation of the eIF4F complex. Furthermore, elF4E combines signals from the mTOR and ERK signaling pathways, which are triggered by growth hormones to facilitate translation of certain mRNAs. These particular mRNAs are involved in cell growth, proliferation, and neuroplasticity (Uttam et al., 2018).

Materials and Methods

Cloning of shRNAs

The pLKO plasmid (a generous gift by Dr. Cara Pager) was cut with AgeI and EcoRI overnight at 37°C, dephosphorylated and purified via agarose gel electrophoresis. The cut plasmid was gel extracted using the QIAGEN Gel Extraction kit following the manufacturer's protocol. The following shRNA primer sequences were ordered from IDT:

Table 1 shRNA Primer Sequences

shRNA oligo name	shRNA oligo sequence
RACK1 shRNA 1 Fwd	**CCGGT**CAAGCTGAAGACCAACCACAT**CTCGA**GATGTGGTTGGTCTTCAGCTTG**TTTTTG**
RACK1 shRNA 1 Rev	AATTC AAAAACAAGCTGAAGACCAACCACATCTCGAGATGTGGTTGGTCTTCAGCTTG A
RACK1 shRNA 2 Fwd	**CCGG**TACCCTGGGTGTGTGCAAATA**CTCGA**GTATTTGCACACACCCAGGGTA**TTTTTG**
RACK1 shRNA 2 Rev	AATTC AAAAATACCCTGGGTGTGTGCAAATACTCGAGTATTTGCACACACCCAGGGTA A
RACK1 shRNA3 Fwd	**CCGGT**GATGTGGTTATCTCCTCAGAT**CTCGAG**ATCTGAGGAGATAACCACATC**TTTTTG**
RACK1 shRNA3 Rev	**AATTC**AAAAAGATGTGGTTATCTCCTCAGATCTCGAGATCTGAGGAGATAACCACATC**A**
RACK1 shRNA4 Fwd	**CCGGT**CGAGATAAGACCATCATCAT**CTCGAG**ATGATGATGGTCTTATCTCGA**TTTTTG**
RACK1 shRNA4 Rev	**AATTC**AAAAATCGAGATAAGACCATCATCATCTCGAGATGATGATGGTCTTATCTCG**A**
RPS25 shRNA 1 Fwd	**CCGGT**CGGGACAAGCTCAATAACTTA**CTCGA**GTAAGTTATTGAGCTTGTCCCG**TTTTTG**
RPS25 shRNA 1 Rev	AATTC AAAAACGGGACAAGCTCAATAACTTACTCGAGTAAGTTATTGAGCTTGTCCCG A
RPS25 shRNA 2 Fwd	**CCGGT**GCTCAATAACTTAGTCTTGTT**CTCGA**GAACAAGACTAAGTTATTGAGC**TTTTTG**
RPS25 shRNA 2 Rev	AATTC AAAAAGCTCAATAACTTAGTCTTGTTCTCGAGAACAAGACTAAGTTATTGAGC A
eIF3D shRNA 1 Fwd	**CCGGT**GCGTCATTGACATCTGCATGA**CTCGA**GTCATGCAGATGTCAATGACGC**TTTTTTG**
eIF3D shRNA 1 Rev	AATTC AAAAAAGCGTCATTGACATCTGCATGACTCGAGTCATGCAGATGTCAATGACGC A
eIF3D shRNA 2 Fwd	**CCGGT**GACGACATGGATAAGAATGAA**CTCGA**GTTCATTCTTATCCATGTCGTC**TTTTTTG**
eIF3D shRNA 2 Rev	AATTC AAAAAAGACGACATGGATAAGAATGAACTCGAGTTCATTCTTATCCATGTCGTC A
DAP5 shRNA1 Fwd	**CCGG**GTGCTCCTTGATGTTAAGTAA**CTCGAG**TTACTTAACATCAAGGAGCAC**TTTTTTG**
DAP5 shRNA1 Rev	AATTCAAAAAAGTGCTCCTTGATGTTAAGTAACTCGAGTTACTTAACATCAAGGAGCAC
DAP5 shRNA2 Fwd	**CCGG**CCCTTTGGTGAAATCCTATTT**CTCGAG**AAATAGGATTTCACCAAAGGG**TTTTTTG**
DAP5 shRNA2 Rev	AATTCAAAAAACCCTTTGGTGAAATCCTATTTCTCGAGAAATAGGATTTCACCAAAGGG
eIF4E shRNA1 Fwd	**CCGG**CGGCTGATCTCCAAGTTTGAT**CTCGAG**ATCAAACTTGGAGATCAGCCG**TTTTTG**
eIF4E shRNA1 Rev	AATTCAAAAACGGCTGATCTCCAAGTTTGATCTCGAGATCAAACTTGGAGATCAGCCG
eIF4E shRNA2 Fwd	**CCGG**CCGACTACAGAAGAGGAGAAA**CTCGAG**TTTCTCCTCTTCTGTAGTCGG**TTTTTG**
eIF4E shRNA2 Rev	AATTCAAAAACCGACTACAGAAGAGGAGAAACTCGAGTTTCTCCTCTTCTGTAGTCGG

Primers were resuspended to a concentration of 100 µM. 1 µl of corresponding forward and reverse primers were phosphorylated at 37°C for 30 min in T4 DNA Ligase

buffer and annealed by heating to 95°C for 5 minutes and slow cooling to room temperature in a thermocycler.

Annealed primers were diluted 1:250 in water, and 1 µl of the diluted annealed primers were used for a ligation reaction with 50 ng of the digested pLKO plasmid. Following ligation for 15 min at room temperature, 3 µl of the ligation reactions were transformed into DH5alpha competent bacteria cells. Following a 30-minute incubation on ice, 30 s heat shock at 42°C and 2 minutes on ice, 50 µl of SOC media was added, bacteria were allowed to recover for 30 min at 37°C in a shaker and were plated on LB Carb plates and grown overnight.

Two colonies for each shRNA construct were picked and grown overnight in LB Amp media. The miniprep plasmid extraction was performed following the Fuchs lab protocol using buffers P1, P2 and P3 from the Machery-Nagel Midiprep kit, followed by isopropanol precipitation and 75% ethanol wash. Pellets were resuspended in 50 µl water. The presence of the shRNA sequence was confirmed by Sanger sequencing (Figure 7).

Transfection of HEK293FT cells for lentivirus packaging, lentivirus transduction of HeLa cells, and subsequent selection with puromycin was performed by Dr. Fuchs.

Validating potential shRNAs knockdowns by immunoblotting

To validate the potential shRNA knockdowns, an SDS-PAGE followed by immunoblotting was performed. First, the cells were washed with PBS, scraped, transferred into microcentrifuge tubes and gently pelleted at 1000 rpm. PBS was removed, and cells were vortexed in 50 µl Radio Immuno Precipitation Assay (RIPA) lysis buffer (PBS with 0.1% SDS, 0.5% sodium deoxycholate, 1% TritonX-100, protease inhibitor

tablet (Roche)) 8% and 12% SDS PAGE gels were prepared. Next, 6 µl of protein sample (RACK1, RPS25, DAP5, eIF3D and eIF4E shRNA expressing HeLa cell lysate, respectively) were mixed with 4 µl of 2X loading dye with DTT, heated to 95°C for 5 minutes and loaded on the gels. RACK1 and DAP5 were loaded on the 8% gel, the others were loaded on the 12% gel. The gels were run at a 100 Volts until the bromophenol loading dye was no longer visible at the bottom of the gel.

The gels were transferred to a nitrocellulose membrane at 100 V for 60 min in Towbin transfer buffer with SDS (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol).

Following transfer, the membrane was blocked in 5% milk- phosphate buffered saline with 0.1% Tween-20 (PBS/T) for 1 hour at room temperature. Residual milk residues were removed by washing the membrane three times at room temperature for 10 minutes each with PBS/T. The membranes were incubated in primary antibody solutions made up in 5% bovine serum albumin (BSA)-PBS/T overnight at 4°C. The antibodies used in this study were: RACK1 antibody (1:1000, Cell Signaling D59D5), DAP5 (1:2000, 17728-1-AP, ProteintechGroup), eS25 (1:1000, Abcam ab102940), eIF3D (1:1000, ProteintechGroup, 66024-1-Ig).

The next day, the membrane was washed three times at room temperature for 10 minutes each with PBS/T, then incubated with secondary antibody solution (1:10,000 anti-rabbit IgG or 1:10,000 anti-mouse IgG (Jackson) in 5% milk-PBS/T) for 1 hour at room temperature. Following additional three washes in PBS/T at room temperature, the

membranes were visualized with ECL reagent (ThermoFisher) using the BIORAD ChemiDoc XRS imager.

Seeding and Harvesting potential shRNA Knockdown cell lines in preparation for transfection assay

The growth media was aspirated, cells were washed with PBS and PBS was aspirated. Cells were trypsinized at 37°C for approximately 5 min. The cells were resuspended in

Dulbecco's modified Eagle media (DMEM). 10 µl of the cell suspension was counted in a BIORAD cell counter.

Cells were diluted to a concentration of 20,000 cells/100 µl, and 100 µl of cell suspension was added to each well of a 96-well plate. Cells were grown overnight at 37°C, 5% CO_2.

Transfection

For each reaction, we transfected 100 ng of dual luciferase constructs containing either the

EMCV, PV, HCV or CrPV IRES. Each reaction was designed to contain 1 µl of both the plasmid.

For 1 reaction:

Mastermix: 5 µl OptiMEM + 0.15 µl Lipofectamine 3000

DNA dilution: 5 µl OptiMEM + 1 µl DNA + 0.2 µl P300

Add Mastermix to DNA dilution and incubate for 15 mins at room temperature. These reactions were done in triplicate for each cell line.

Harvesting cells

24 hours post transfection, the media from the 96-well plate was discarded, and cells were lysted in 50 µl 1X passive buffer. Following incubation at room temperature for 15 minutes, 20 ul of the cell suspension was pipetted into an opaque white 96-well plate. 40 µl of luciferase assay reagent (LAR) as well as 40 µl of Stop 'N Glo *Renilla* luciferase reagent was injected, and luminescence was measured using a Promega GloMax luminometer.

The Firefly/Renilla ratios were calculated in Excel, and the results were plotted using GraphPad Prism 9 software. Error bars represent the 95% confidence interval. The statistical analysis (multiple analysis, one-way ANOVA, Dunnett's test) was also performed in GraphPad Prism 9. Statistical significance is as follows: $p < 0.05$ = *, $0.05 > p > 0.001$ = **.

Results

Following ligation of the annealed and phosphorylated shRNA primer sequences into the pLKO plasmid, successful shRNA sequence insertion into the pLKO plasmid was confirmed via Sanger sequencing. The chromatogram for eIF3D shRNA 1 clone 2 is shown in figure 7.

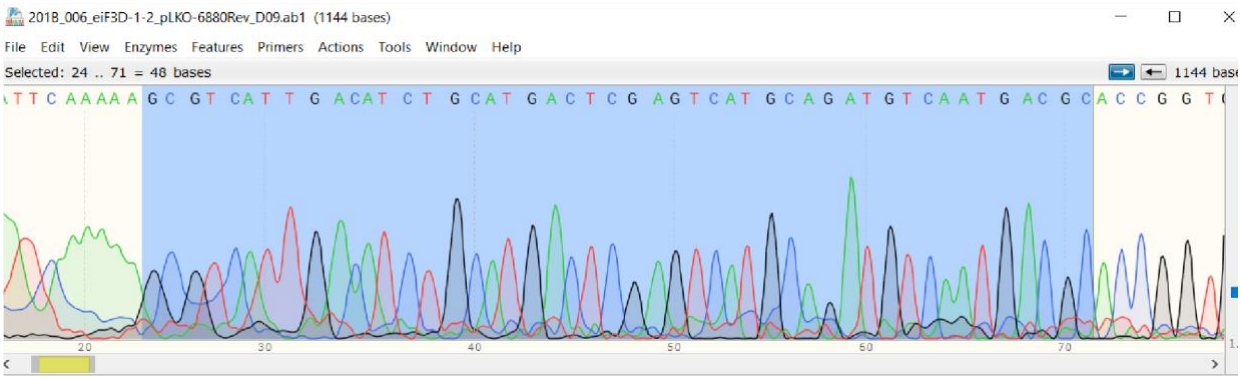


Figure 7

Sanger sequencing chromatogram for eIF3D shRNA 1 clone 2

Note. Sanger sequencing chromatogram confirmed successful insertion of the eIF3D shRNA sequence highlighted in blue.

To determine successful shRNA knockdowns for this study, samples from shRNA samples targeting RACK1, DAP5, eS25, eIF3D and eIF4E had been prepared using RIPA buffer. My first goal was to confirm depletion of these cellular proteins by immunoblotting or western blotting. Following separation of the total protein lysate by SDS-PAGE, we performed a western blot for all of our samples and quantified it using the volume tools in ImageLab to measure the intensity of the bands as compared to actin which in this case was the loading control for all the cellular proteins listed above. Per our expectations, we observed efficient protein depletion for eS25, eIF4E and DAP5. RACK1

was depleted by approximately 50%, while eIF3D depletion was with 30% depletion the least efficient.

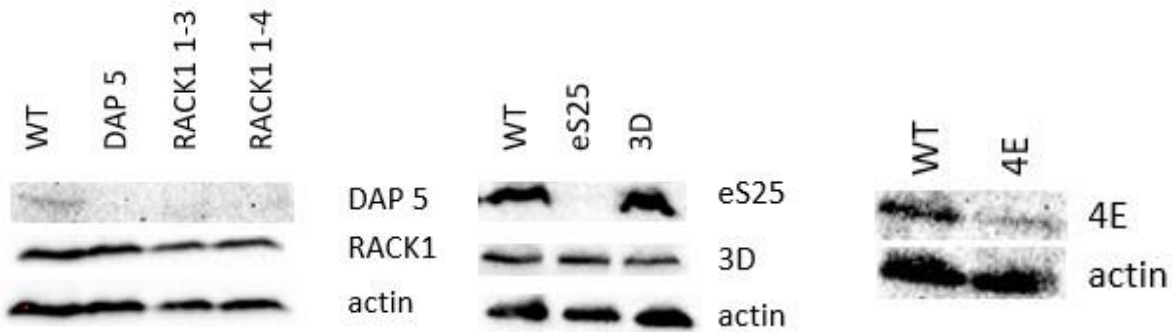


Figure 8

Knockdowns identified in RACK1, DAP5 and eIF4E.

Note. Western blotting illustrates DAP5 and RACK 1 on the left, eS25 and eIF3D in the middle and eIF4E on the right. Protein levels are decreased in the Hela cell lines as compared to the actin band. There is an efficient depletion in DAP5, eS25 and eIF4E. Additionally, there is only a 50% depletion in both RACK1 and eIF3D.

After validating the shRNA knockdowns, we proceeded with testing the translation efficiency of EMCV, PV, HCV and CrPV viral IRESs in RACK1, eS25, eIF3D, DAP5 and eIF4E-depleted cell lines.

Based on literature, cellular proteins RACK1, and eS25 should be required for translation from several viral IRESs, and hence served as positive controls. In contrast, eIF4E is not expected to facilitate IRES-mediated translation and served as a negative control. Although DAP5 and eIF3D have been shown to facilitate c-Myc mRNA translation, it is unclear if these proteins facilitate translation from the (a) c-Myc IRES or (b) viral IRESs.

I seeded, transfected, and harvested wildtype HeLa or HeLa cells expressing different shRNAs over the span of 48 hours and performed a dual luciferase assay to measure firefly and *Renilla* luciferase produced in these cell lines. The firefly/*Renilla* ratio was then plotted for comparisons. Figure 9 shows that the translation efficiency from the EMCV IRES is strongly decreased in all cell lines with the eIF4E being the only exception.

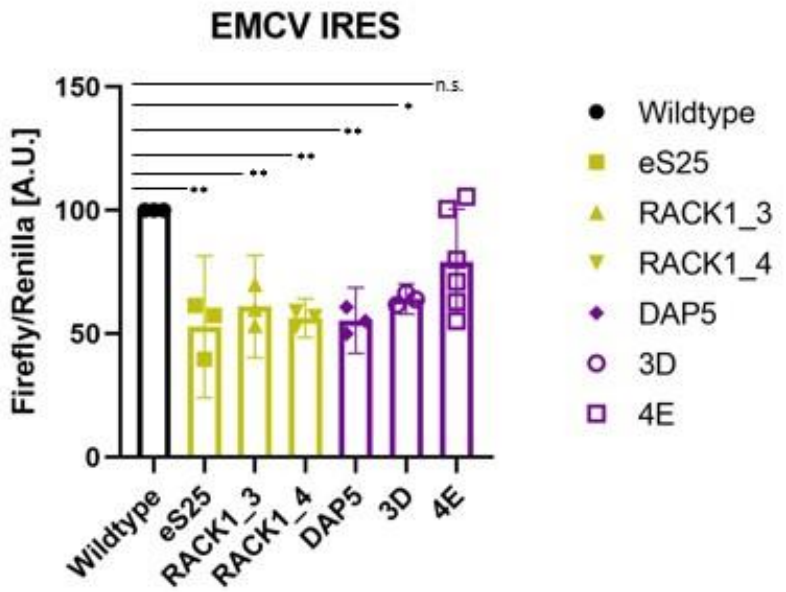


Figure 9

Depletion of eS25, RACK1, DAP5, and eIF3D proteins decreased translation efficiency from the EMCV IRES.

Note. The quantification of EMCV IRES translation efficiency is decreased in cells depleted of eS25, RACK1, DAP5, and eIF3D. The depletion of the eIF4E protein increased translation in the EMCV IRES because the cap binding protein eIF4E is known to participate in cap-dependent translation.

We next tested the translation efficiency from the PV IRES in cells depleted of these ribosomal proteins and translation factors. We expected that the depletion of eS25

and RACK1 proteins would decrease the translation of the PV IRES as these findings have been published earlier (Hertz et al., 2013; LaFontaine et al., 2020). As shown in Figure 10, we can see eS25 is required for viral IRES-mediated translation in PV. However, the depletion of RACK1 only had a small effect on PV IRES-mediated translation, possibly because the knockdown was not as efficient. Interestingly, depletion of eIF3D and eIF4E increased PV IRES translation, indicating that these proteins are not required. Rather, these results suggest that their depletion decreased cap-dependent translation of the *Renilla* luciferase, which in turn increased the Firefly/*Renilla* ratio.

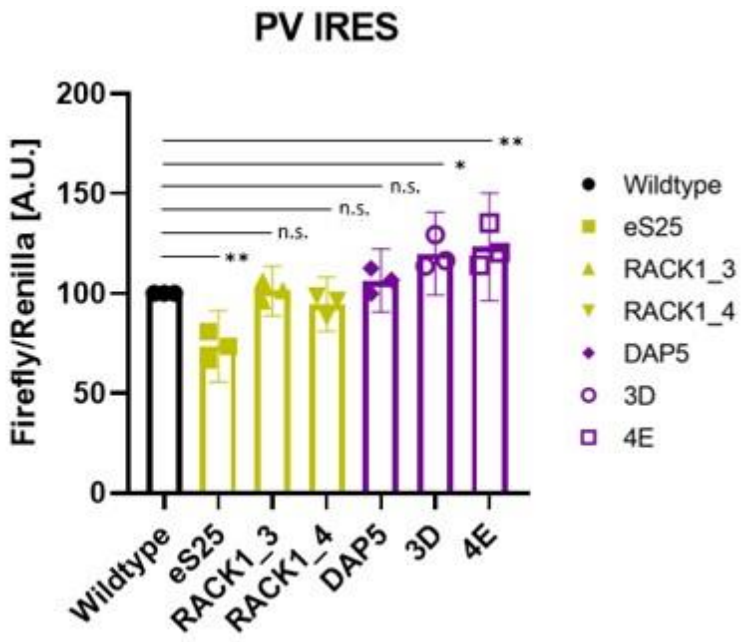


Figure 10

Depletion of ribosomal protein eS25 decreased translation of the PV IRES.

Note. As illustrated above, translation efficiency from the PV IRES is greatly decreased when eS25 depleted. The depletion of RACK1 is not statistically significant, possibly due to its inefficient knockdown.

We next tested the translation efficiency of the HCV IRES using the same approach. We expected that upon depletion of eS25 and RACK1 proteins, HCV IRES-mediated translation would decrease. As shown in Figure 11, loss of eS25 and RACK1 indeed decrease HCV IRES-mediated translation. We also observed that the depletion of the eIF3D protein decreased HCV IRES-mediated translation, signifying that it is necessary for its translation. In contrast, depletion of eIF4E and DAP5 does not affect translation from the HCV IRES, indicating that these proteins are not necessary for HCV IRES mediated translation.

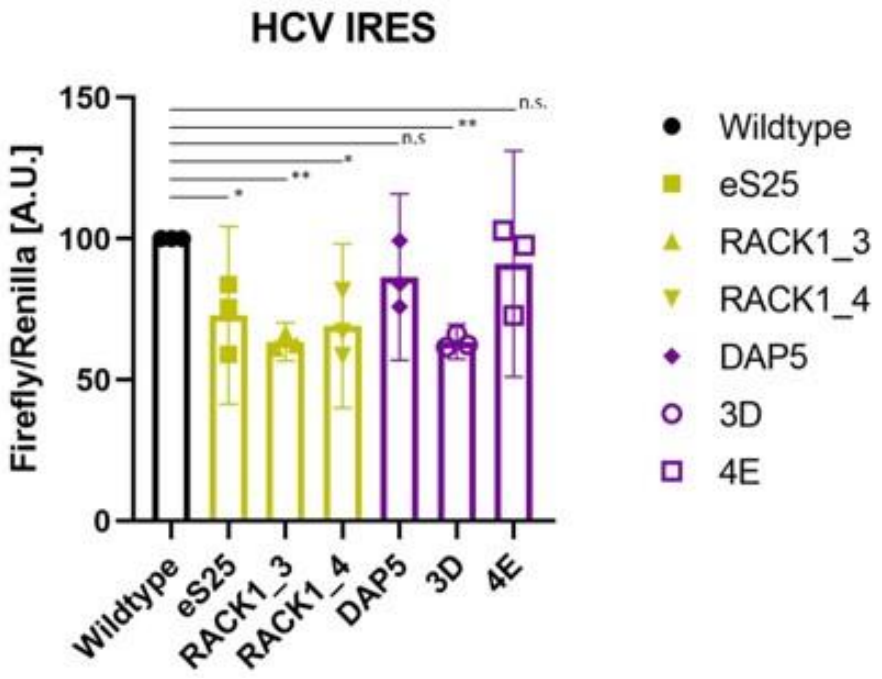


Figure 11

Depletion of eS25, RACK1 and eIF3D protein decreased HCV IRES mediated translation.

Note. As illustrated above, translation efficiency from the HCV IRES is decreased when eS25, RACK1 and eIF3D are depleted. The depletion of eIF4E and DAP5 does not affect HCV IRES mediated translation.

Finally, we tested translation efficiency the CrPV IRES upon mRNA and protein depletion. We expected that upon depletion of eS25, CrPV IRES-mediated translation would decrease as previously reported (Landry et al., 2009). As shown in Figure 12, depletion of eS25 indeed reduces CrPV IRES-mediated translation. However, due to the rather large errors bars this effect is not statistically significant. In contrast, we did observe that the depletion of DAP5, eIf3D and eIF4E increased translation from the CrPV IRES, although the increase is only statistically significant for DAP5. These results suggest that the depletion of these proteins enhances CrPV IRES translation.

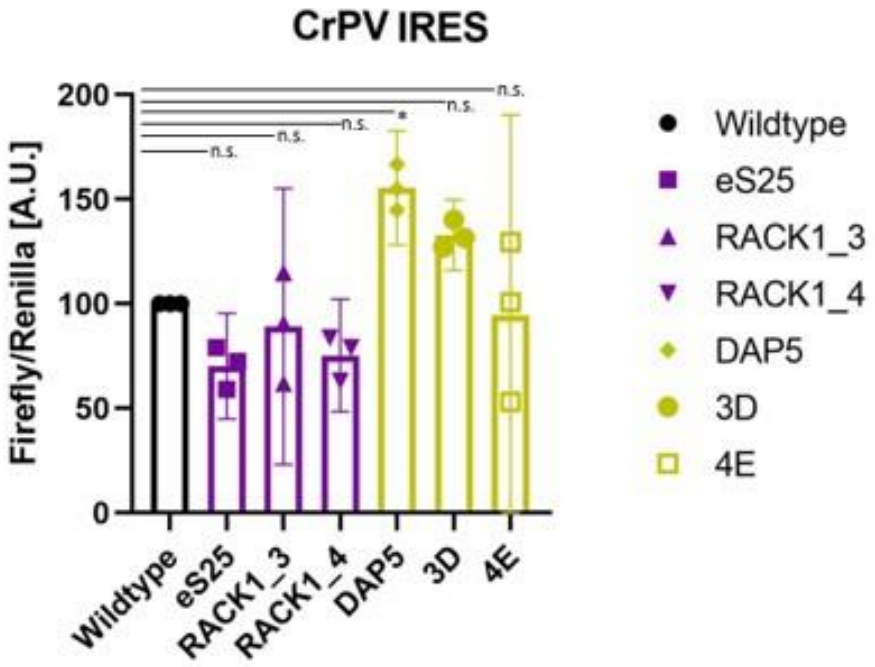


Figure 12

Depletion of eS25 decreased translation of the HCV IRES.

Note. As illustrated above, the level of translation efficiency in the HCV IRES is decreased when eS25 is depleted. In contrast, depletion of eIF4E, eIF3D and DAP5 increased CrPV IRES mediated translation.

Discussion

The primary goal of this research was to test if the cellular proteins DAP5 and eIF3D are required for viral IRES-mediated translation. Based on previously published reports, RACK1 and eS25 were used as positive controls whiles eIF4E was used as a negative control.

We first created shRNA knockdown cell lines and validated the knockdown by immunoblotting. While knockdown of eS25, DAP5 and eIF4E was very efficient, RACK1 and eIF3D were only depleted by 50% and 30%, respectively.

As expected, depletion of eIF4E had no effect on translation efficiency of all our viral IRESs. This result was expected because eIF4E is known to mainly function in cap dependent translation initiation. Hence, we can then conclude that eIF4E is indeed not required for viral IRES-mediated translation with any of our IRESs.

Due to previous reports of RACK1 functioning in IRES-mediated translation (LaFontaine et al., 2020; Majzoub et al., 2014), depletion of RACK1 was expected to impact EMCV, PV and HCV IRES-mediated translation, while eS25 is expected to reduce translation of all four viral IRESs. Although our observations overall agree with these previous reports, due to partial depletion of these proteins and thresholds of depletion required to have an effect, the observed effects were not always statistically significant. We also observed that depletion of eIF3D reduced translation from EMCV, PV and HCV IRESs, but not the CrPV IRES. Instead, eIF3D depletion increased CrPV IRES-mediated translation, although this effect was not statistically significant. Since the CrPV IRES does not require translation initiation factors, it is likely that the observed effect is due to the

measurement of the Firefly/*Renilla* ratio. A decrease in cap-dependent translation would increase the Firefly/*Renilla* ratio, which yielded the observed effect. So rather than depletion of eIF3D increasing CrPV IRES-mediated translation, the depletion likely was caused by a decrease in cap-dependent *Renilla* translation. Similarly, DAP5 depletion appeared to increase

CrPV IRES-mediated translation, while the effect is more likely caused by a decrease in *Renilla* translation. Interesting, DAP5 depletion reduced EMCV IRES-mediated translation but not translation from HCV and PV IRESs. Together, our results validated the use of these cell lines stably expressing shRNAs as a tool to investigate IRES-mediated translation. In the future, we are planning to clone different cellular IRESs and measure translation efficiency in the here established cell lines. Using the requirements for different ribosomal proteins and translation factors, we can use the viral IRESs as a guide to classify the cellular IRESs based on their requirement for translation factors, which overall will help to further identify and characterize cellular IRESs.

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