A Novel Factor-dependent Internal Ribosome Entry Site and Programmed Frameshifting Signal in the Bemisia-associated Dicistrovirus 2

by

Yihang Chen

B.Sc., University of British Columbia, 2020

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

December 2022

© Yihang Chen, 2022

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

A Novel Factor-dependent Internal Ribosome Entry Site and Programmed Frameshifting Signal in the Bemisia-associated Dicistrovirus 2

Submitted by	Yihang Chen	in partial fulfilment of the requirements for
the degree of	Master of Science	
in	Biochemistry and Mole	cular Biology

Examining Committee:

Dr. Eric Jan, Professor, Biochemistry and Molecular Biology, UBC

Supervisor

Dr. Honglin Luo, Professor, Pathology and Laboratory Medicine, UBC

Supervisory Committee Member

Dr. Ethan Greenblatt, Assistant Professor, Biochemistry and Molecular Biology, UBC Supervisory Committee Member

Dr. LeAnn Howe, Professor, Biochemistry and Molecular Biology, UBC Additional Examiner

Abstract

Internal ribosome entry sites (IRES) are structured RNA elements that recruit ribosomes using a subset of translation factors in a 5' cap-independent manner. The dicistrovirus IRES uses the most streamlined mechanism: the IRES recruits ribosomes directly without the use of initiation factors and directs translation from a non-AUG codon. The IRES adopts three pseudoknots, stem-loop and unpaired regions that interact with specific domains of the ribosomal 40S and 60S subunits. Analysis of dicistrovirus-like genomes identified via metagenomic studies predicts potentially novel atypical IRES RNA structures. Specifically, the Bemisia-associated dicistrovirus 2 (BaDV-2) IRES contains an unusual RNA structure that lacks conserved elements and altered structures that do not conform to the typical dicistrovirus IRES mechanism. Moreover, the BaDV-2 genome contains a predicted -1 frameshifting signal (FSS) with an RNA stem-loop structure that would direct translation of the RNA-dependent RNA polymerase (RdRp) motif. In this study, using a bicistronic reporter, we demonstrate that BaDV-2 IGR supports internal ribosome entry activity in in vitro insect Sf-21 and rabbit reticulocyte translation extracts. Using deletion and mutagenesis analyses, we showed that the BaDV-2 IRES activity is within a minimal 140 nucleotide element containing a predicted stem-loop. Moreover, the IRES is sensitive to eIF2 and eIF4A inhibitors, NSC1198983 and hippuristanol, respectively, indicating that the BaDV-2 IRES is factor-dependent, unlike typical factor-less dicistrovirus IRESs. Preliminary data shows that a chimeric dicistrovirus infectious clone does not support virus infection in Drosophila cells. Finally, we show that the predicted stem-loop structure -1 frameshifting signal can direct programmed frameshifting in vitro using a luciferase-based bicistronic reporter. In summary, we have provided evidence of the first -1 frameshifting signal

and a novel IRES mechanism in this viral family, thus highlighting the diversity of viral strategies to direct viral protein synthesis.

Lay Summary

Protein synthesis is an essential process in the viral life cycle. RNA viruses generally hijack the host translational machinery to direct viral protein synthesis. One prominent viral mechanism is by using structured RNA elements called internal ribosome entry site (IRES). This thesis focuses on an atypical IRES found in an insect virus that infects the sweet potato whitefly *Bemisia tabaci*, which is an agriculture pest. Using biochemical and molecular approaches, we provide insights into the factor requirements and structure of this IRES. This thesis reveals a novel IRES mechanism within this viral family which may be exploited for mRNA therapeutics, or biological control strategies to combat agriculture pests.

Preface

The work presented here was conducted by Yihang Chen, under the supervision of Dr. Eric Jan at the University of British Columbia. Except for the parts listed below:

Validation for mutagenesis analysis for IRES (**Figure 2.2**) was partially performed by Jodi Chien, who was an undergraduate student in the lab.

SEC-MALS were performed by Yihang Chen under the supervision of Dr. Higor S. Pereira and Dr. Trushar Patel in the Patel lab at the University of Lethbridge (Figure 2.7 A, B). SAXS data was analyzed by Dr. Higor S. Pereira (Figure 2.7 C).

Table of Contents

Abstract	iii
Lay Summa	ryv
Preface	vi
Table of Con	itentsvii
List of Figur	resx
List of Symb	olsxii
List of Abbr	eviationsxiii
Acknowledg	mentsxix
Chapter 1: Iı	ntroduction1
1.1 Eukar	yotic translation1
1.1.1	Canonical cap-dependent translation initiation in eukaryotes1
1.1.2	Alternative cap-independent translation mechanisms in viruses
1.1	1.2.1 Internal ribosomal entry sites: four types and mechanism10
1.2 Recod	ling mechanisms in viruses: Programmed ribosomal frameshifting19
1.2.1	-1 Programmed ribosomal frameshifting (-1 PRF)19
1.2.2	+1 Programmed ribosomal frameshifting (+1 PRF)21
1.2.3	+2 Programmed ribosomal frameshifting (+2 PRF)23
1.3 Dicist	rovirus23
1.3.1	Classification

1.3.2	Genome organization
1.3.3	Viral strategies of translational control
1.3.4	Viral discovery by metagenomic approaches35
1.4 Thesis	Investigation

Chapter 2: A novel factor-dependent internal ribosome entry site in the Bemisia-associated dicistrovirus 2		
2.1 Introducti	on	
2.2 Material a	and methods	
2.3 Results		
2.3.1 Ba	aDV-2 IGR supports internal ribosome entry42	
2.3.2 M	inimal BaDV-2 IGR that directs IRES activity44	
2.3.3 Ba	DV-2 IGR IRES initiates translation from an AUG start codon48	
2.3.4 Ba	aDV-2 IGR IRES does not bind to purified 80S ribosome50	
2.3.5 Ba	aDV-2 IGR IRES requires eIF4A and eIF2 to recruit ribosomes51	
2.3.6 Ba	aDV-2 minimal IRES contains predicted stem loops54	
2.3.7 Ba	aDV-2 IGR does not support IRES activity in S2 cells56	
2.4 Discussion	n60	

Chapter 3: A novel programmed -1 frameshifting signal in the Bemisia-associated

dicistrovirus 2		
3.1 Introduction	63	
3.2 Material and methods	64	
3.3 Results	65	

3	3.3.1	BaDV-2 IGR supports a -1 PRF	65
3	3.3.2	Characterization of the BaDV-2 -1FSS	69
3.4 D	Discuss	sion	73
Chapter 4:	: Sum	mary and future directions	75
Bibliograp	ohy		79

List of Figures

Figure 1.1.	Overview of the general eukaryotic translation initiation pathway4
Figure 1.2.	Model of the eukaryotic translation elongation pathway7
Figure 1.3.	Model of the eukaryotic translation termination pathway8
Figure 1.4.	Examples of the diversity of mechanisms of viral IRES RNAs10
Figure 1.5.	Structure of CrPV IGR IRES in the Ribosome13
Figure 1.6.	The secondary structure of Type IVa and IVb dicistrovirus IGR IRESs17
Figure 1.7.	The predicted model of dicistrovirus IRES18
Figure 1.8.	The genome organization of a standard picornavirus and a standard
dicistrovirus.	
Figure 1.9.	The RNA genome and polyprotein processing of dicistroviruses
Figure 1.10.	Recycling of eIF2 during translation initiation
Figure 2.1.	BaDV-2 IGR and its IRES activity43
Figure 2.2.	Predicted secondary model of BaDV-2 IGR IRES45
Figure 2.3.	Deletion analysis of BaDV-2 IGR IRES47
Figure 2.4.	Stop codon mutations to confirm the BaDV-2 IGR IRES initiation site49
Figure 2.5.	Affinity of 80S-BaDV-2 IGR IRES complexes51
Figure 2.6.	Inhibitor treatment to test the requirement of initiation factors

Figure 2.7.	Structural analysis of BaDV-2 IGR IRES55	5
Figure 2.8.	BaDV-2 IGR IRES activity in infectious clone and in cells	3
Figure 3.1.	The predicted secondary structure of BaDV-2 -1 FSS60	5
Figure 3.2.	In vitro translation assays in Sf-21 cell extracts and RRL68	3
Figure 3.3.	Deletion analysis of BaDV-2 -1 FSS70)
Figure 3.4.	Mutation analysis of BaDV-2 -1FSS72	2

List of Symbols

- α Alpha
- β Beta
- γ Gamma
- μ Micron
- Δ Delta
- k Kilo
- ° Degree
- % Percent

List of Abbreviations

aa-tRNA	aminoacyl-tRNA
ABCE1	ATP-binding cassette protein
Ad	Adenovirus
A-site	aminoacyl-site
ASL	anticodon stem-loop
ATP	adenosine triphosphate
BaDV-2	Bemisia-associated dicistrovirus 2
BUNV	Bunyamwera virus
cDNA	complementary deoxyribonucleic acid
CHX	cycloheximide
CrPV	Cricket paralysis virus
cryo-EM	cryo-electron microscopy
CSFV	classic swine fever virus
DCV	Drosophila C virus
DENR	density- regulated protein
DMS	dimethyl sulfate
DTT	dithiothreitol

4E-BP	eukaryotic initiation factor 4E-binding protein
eEF	eukaryotic elongation factor
EF	elongation factor
eIF	eukaryotic initiation factor
eIF2D	ligatin
EMCV	encephalomyocarditis virus
E-site	exit-site
ER	endoplasmic reticulum
FLuc	firefly luciferase
FMDV	foot-and-mouth disease virus
FSS	frameshifting signal
GCN2	general control non-derepressible-2
GDI	GDP-dissociation inhibitor
GEF	guanine nucleotide exchagne factor
GTP	guanosine triphosphate
HAV	hepatitis A virus
HCV	hepatitis C virus
HIV-1	human immunodeficiency virus type I

h p.i.	hours post-infection
h p.t.	hours post-transfection
HRI	heme-regulated inhibitor
HSV-1	herpes simplex virus-1
HTLV-II	human T-cell leukemia virus type II
IAPV	Israeli acute paralysis virus
IFN	interferon
IGR	intergenic region
IRES	internal ribosome entry site
ITAF	IRES trans-acting factor
KD	dissociation constant
KOAc	potassium acetate
L	Loop
m6A	N 6 -methyladenosine
m ⁷ G cap	7-methylaguanosine cap
MCT1	multiple copies in T-cell lymphoma-1
Met-tRNA _i	Methionine-transfer Ribonucleic acidi
MgOAc	magnesium acetate

miRNA	microRNA
MLOGD	maximum likelihood overlapping gene detector
mRNA	messenger ribonucleic acid
MT	Mutant
NMR	nuclear magnetic resonance
PABP	poly(A) binding protein
P-bodies	processing-bodies
PCBP	poly(C)-binding protein
PERK	PKR-like endoplasmic reticulum kinase
Pi	inorganic phosphate
PIC	preinitiation complex
PIPO	Pretty Interesting Potyviridae ORF
РК	pseudoknot
PKR	interferon-inducible RNA-dependent kinase
PRF	programmed ribosomal frameshift
P-site	peptidyl site
РТВ	pyrimidine track binding protein
PTC	peptidyl transferfase center

PTV-1	porcine teschovirus 1
PV	poliovirus
RBP	RNA-binding protein
RdRp	RNA-dependent RNA polymerase
RF	release factor
RLuc	Renilla luciferase
RRL	rabbit reticulocyte lysate
rRNA	ribosomal ribonucleic acid
S2	Drosophila Schneider 2 cell line
SARS	severeve acute respiratory virus
SAXS	small angle X-ray scattering
SD	Shine-Delgarno
SEC-MALS	size exclusion chromatography with multi-angle light scattering
Sf-21	Spodoptera frugiperda
SG	stress granule
SHAPE	selective 2' hydroxyl acylation analyzed by primer extension
SL	stem-loop
smFRET	single-molecule fluorescence resonance energy transfer

SV	Sindbis virus
TC	ternary complex
TLS	tRNA-like structure
TMEV	Theiler's encephalomyelitis virus
tmRNA	transfer-messenger ribonucleic acid
tRNA	transfer ribonucleic acid
TSV	Taura syndrome virus
TuMV	Turnip mosaic virus
5'-UTR	5'-untranslated region
VLR	variable loop region
VPg	viral protein genome-linked
VSV	Vesicular stomatitis virus
WT	wild-type

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Eric Jan. Eric, thank you for "adopting" me as a graduate student from a totally different undergraduate background. You are such an enthusiastic scientist, and I am inspired by your passion for pure science and tons of crazy ideas every day. It was a privilege to learn how to conduct solid scientific research from you. Also, thank you for always being so kind and supportive. That meant a lot to me.

I also want to thank my supervisory committee members, Dr. Ethan Greenblatt and Dr. Honglin Luo for your expertise, insight, and constructive feedback throughout my MSc.

To the Jan lab members and alumnus, it has been a delightful journey to work with all of you. Jibin, thank you for being such a supportive mentor and friend, for sharing joy and sorrows, in early mornings and late nights. And thank you for being such a good role model of devoting yourself to teach and help others. Reid, thank you for leading me the whole biochemistry and virology field. It was your endless patience and support as the BIOC410 TA that gave me the courage to continue even when my biochemistry knowledge was so weak. Dan, thank you for always willing to share your expertise and insights in experiment techniques, speech improvement, and of course, cute kittens. Nicolas, thank you for always sharing your extraordinary bioinformatics knowledge so patiently. Jodi, you are so supportive, patient, and thoughtful, the best colleague and friend who I could ever imagine having. I want to thank everyone in Jan lab and whom I have ever encountered during my MSc. for accepting me, teaching me to be brave to ask questions, and for exploring the unknown in science together.

Finally, to my family and friends, thank you for always being there for me.

Chapter 1: Introduction

1.1 Eukaryotic translation

1.1.1 Canonical cap-dependent translation initiation in eukaryotes

Protein synthesis is an essential process in gene expression. Regulation of translation is vital for maintaining homeostasis in cells, development, cell fate determination, cellular stress and virus infection (Hershey, Sonenberg, & Mathews, 2019). Eukaryotic protein synthesis involves four main processes: initiation, elongation, termination and ribosome recycling (C. U. T. Hellen, 2018; Jackson, Hellen, & Pestova, 2010; Merrick & Pavitt, 2018). The ways that ribosomes are recruited to the messenger RNA (mRNA) and recognize initiation sites differ in prokaryotes and eukaryotes (Kozak, 1999). Importantly, translation initiation is considered as the rate-limiting step and is highly regulated in eukaryotes (Mathews & Sonenberg, 2000). As this thesis focuses on non-canonical translation in eukaryotes, I will provide a brief overview of each process in the following sections.

Translation initiation is the process of assembling the elongation-component 80S ribosomes on an mRNA. There are two main steps: the formation of the 48S initiation complex and the joining of 60S subunits. To start with, the initiator methionyl-transfer Ribonucleic acid_i (Met-tRNA_i) is recruited to the ribosome with eIF2-GTP. eIF2 is a GDP/GTP binding G protein complex consisting of three subunits, alpha (α), beta (β), and gamma (γ). GDP has a 400-fold higher affinity for eIF2 than GTP, and eIF2-GDP is in a relatively stable form (Kapp & Lorsch, 2004; Panniers, Rowlands, & Henshaw, 1988). GDP exchange to GTP on eIF2 is mediated by the guanine nucleotide exchange factor (GEF) eIF2B (G D Pavitt, Ramaiah, Kimball, & Hinnebusch, 1998; Rowlands, Panniers, & Henshaw, 1988). In the GTP bound state, eIF2-GTP has a high affinity for Met-tRNAi (Kapp & Lorsch, 2004). The formation of eIF2•GTP•MettRNA_i complex as ternary complex (TC) is considered as the first step of translation initiation (Merrick & Pavitt, 2018). Then eIF5, eIF3, eIF1 and eIF1A recruit the TC to the 40S ribosomal subunit to form the 43S preinitiation complex (PIC) (Merrick & Pavitt, 2018; Pisarev, Hellen, & Pestova, 2007). eIF1 is a small protein that binds close to the 40 ribosomal peptidyl-site (P-site) and mRNA entry channel (Rabl, Leibundgut, Ataide, Haag, & Ban, 2011). eIF1A binds 40S directly adjacent to eIF1 (Carter et al., 2001), located in the aminoacyl-site (A-site) of 40S ribosomal subunit (Weisser, Voigts-Hoffmann, Rabl, Leibundgut, & Ban, 2013), and binds to the head of the 40S subunit through its helical subdomain (Yu et al., 2009). eIF1 and eIF1A together induce 40S conformational changes that opens up a cleft between the 40S head and body to facilitate TC binding (Maag, Fekete, Gryczynski, & Lorsch, 2005; Passmore et al., 2007; Sokabe & Fraser, 2014; Weisser et al., 2013). In a mammalian system, the eIF3 octamer core binds around the 40S mRNA exit channel. eIF3 is critical for bringing the TC to the 40S subunit, which then stabilizes mRNA at both the entry and exit channels (Aitken et al., 2016), and also facilitates subsequent steps in initiation, ribosomal recycling and reinitiation (Beznosková, Wagner, Jansen, von der Haar, & Valášek, 2015; C. U. T. Hellen, 2018; Mohammad, Munzarová Pondelícková, Zeman, Gunišová, & Valášek, 2017; Pisarev et al., 2007; Valášek et al., 2017).

The next important step is mRNA recruitment, which requires eIF4A, eIF4B, eIF4E and eIF4G (Figure 1.1). eIF4E is the major 7-methylguanosine (m⁷G) cap recognition factor (J. D. Gross et al., 2003). eIF4G is a scaffold protein that contains the binding site for eIF4E, poly(A) binding protein (PABP) and RNA to form a circular mRNA/RNA-binding protein complex. eIF4G together with eIF4E and eIF4A forms eIF4F complex, and eIF4G enhances eIF4E's affinity for the m⁷G cap (J. D. Gross et al., 2003; O'Leary, Petrov, Chen, & Puglisi, 2013;

Yanagiya et al., 2009). eIF4A is an RNA helicase that unwinds mRNA secondary structures (Kumar, Hellen, & Pestova, 2016) or disrupts RNA-binding proteins from RNA (Jankowsky, Gross, Shuman, & Pyle, 2001) to facilitate loading of the single-strand mRNA into the mRNA channel on the 43S PIC (Merrick & Pavitt, 2018) and the subsequent scanning (Pestova & Kolupaeva, 2002; Sonenberg, 1988; Svitkin et al., 2001). The helicase activity requires ATP and is greatly enhanced by eIF4B (Abramson et al., 1987; Harms, Andreou, Gubaev, & Klostermeier, 2014; Rozen et al., 1990).

After 43S PIC is formed and loaded onto mRNA, it scans along mRNA from the m⁷G cap for the initiation codon. GTP binds to the γ subunit of eIF-2 whereas eIF-5 binds to the β subunit of eIF2. eIF5 is recruited once eIF2 ternary complex reaches the AUG start codon and causes a conformational change on eIF2 which releases inorganic phosphate (Pi) through GTP hydrolysis (Algire, Maag, & Lorsch, 2005; Majumdar & Maitra, 2005; Sizova, Kolupaeva, Pestova, Shatsky, & Hellen, 1998). The conformational change on eIF2 lowers its affinity for Met-tRNA_i (Algire et al., 2005). The dissociation of eIF2-GDP and eIF5 from the 40S ribosomal subunit allows the 60S ribosomal subunit recruitment via eIF5B (Merrick & Pavitt, 2018; Yamamoto et al., 2014). eIF5B and eIF1A reorient the Met-tRNA_i to facilitate 60S ribosomal subunit to join (Lapointe et al., 2022). Then eIF5B-GDP and eIF1A dissociate and the 80S ribosome is formed with Met-tRNA_i in the P-site, ready for the elongation (Merrick & Pavitt, 2018).



Figure 1.1. Overview of the general eukaryotic translation initiation pathway. Blue text shows a series of major steps that are important for recruiting tRNA to mRNA AUG codon in the context of an 80S ribosome. Black text labels individual eukaryotic initiation factors and nucleotide hydrolysis and inorganic phosphate release are shown by blue arrow.

Adapted with permission from (Merrick & Pavitt, 2018).

Translation elongation is conserved between eukaryotes and bacteria (Dever, Dinman, & Green, 2018). As shown in **Figure 1.2**, starting with the end of translation initiation, where MettRNAⁱ^{Met} is bound to P-site of the ribosome and the next codon of the ORF sits in the A-site. For context, there are three tRNA-binding sites on the 80S ribosome: aminoacyl-site (A-site), the peptidyl-site (P-site) and the exit-site (E-site). The eukaryotic translation elongation factor eEF1A is activated by binding to guanosine triphosphate (GTP) and forms a ternary complex with a cognate aminoacyl-tRNA (aa-tRNA) (Dever et al., 2018). The ternary complex delivers the aa-tRNA to the ribosomal A-site. Cognate base-pairing between the codon of mRNA at Asite and the anticodon of the aa-tRNA triggers eIF1A-GTP hydrolysis, leading to the release of eEF1A-GDP complex and the accommodation of the aa-tRNA into the A-site (Dever et al., 2018). eEF1A-GDP is recycled to eEF1A-GTP by guanine nucleotide exchange factor (GEF) eEF1B (Dever et al., 2018). eIF5A with hypusine modification then binds in the ribosomal E-site and interacts with the acceptor arm of the peptidyl-tRNA to position it in a favorable spot for peptide bond formation (Dever, Gutierrez, & Shin, 2014; Melnikov et al., 2016; Schmidt et al., 2016; Shin et al., 2017). During peptide bond formation, the nascent peptide is transferred from the peptidyl-tRNA in P-site to the amino group of the aa-tRNA in the A-site. In the meantime,

tRNAs are positioned into a hybrid state: the deacylated tRNA adopts a hybrid P/E state where its anticodon pair with the mRNA codon in the P-site and its acceptor arm is in the E-site; the newly-formed peptidyl-tRNA adopts a hybrid A/P state where its anticodon pair with the mRNA codon in the A-site and its acceptor arm with peptide is in the E-site (Behrmann et al., 2015; Budkevich et al., 2011; Dever et al., 2018). eEF2-GTP then binds to the A-site and promotes the translocation of the deacylated tRNA from the P-site to the E-site and the peptidyl-tRNA from the A-site to the P-site through GTP hydrolysis (Dever et al., 2018). eEF2-GDP is then released from ribosomes. The deacylated tRNA now occupies the E-site, and its release is coupled with the delivery of the next eEF1A-GTP-aa-tRNA ternary complex in the A-site (Anand, Chakraburtty, Marton, Hinnebusch, & Kinzy, 2003; Triana-Alonso, Chakraburtty, & Nierhaus, 1995), ready for another elongation cycle (Dever et al., 2018).



Figure 1.2 Model of the eukaryotic translation elongation pathway. Starting with delivery of an eEF1A-GTP-aa-tRNA ternary complex to the A-site of an 80S ribosome, GTP is hydrolysis and eEF1A-GDP is released, leading to accommodation of the aa-tRNA in the A-site. eEF1A-GDP is recycled to eEF1A-GDP by eEF1B. Substrate positioning for peptide bond formation is catalyzed by the binding of eIF5A with its hypusine modification (green) in the E-site. After peptide bond formation, eEF2-GTP with diphthamide modification (magenta) binds in the A-site and promotes tRNAs translocation to the P- and E-sites, respectively.

Adapted with permission from (Dever et al., 2018).

Translation termination occurs when ribosomes encounter a stop codon (UAA, UAG, UGA), and is mediated by the release factors eRF1 and eRF3 that form a ternary complex eRF1eRF3-GTP (**Figure 1.3**) (Alkalaeva, Pisarev, Frolova, Kisselev, & Pestova, 2006; Dever & Green, 2012; C. U. T. Hellen, 2018; Jackson, Hellen, & Pestova, 2012). eRF1 contains an aminoterminal domain (N) that recognizes the stop codon in the A-site (Bertram, Bell, Ritchie, Fullerton, & Stansfield, 2000; A. Brown, Shao, Murray, Hegde, & Ramakrishnan, 2015; Matheisl, Berninghausen, Becker, & Beckmann, 2015; Shao et al., 2016), a middle domain (M) that induces the release of the polypeptide from the peptidyl-tRNA on the ribosomal P-site (Frolova et al., 1999), a carboxy-terminal domain(C) that binds to eRF3 with a flexible minidomain close to N-terminal domain and is responsible for stop codon specificity (C. U. T. Hellen, 2018; Mantsyzov et al., 2010). The eRF1 recognizes the stop codon and the ternary complex binds to the pre-termination complex (pre-TC) (**Figure 1.3**) (C. U. T. Hellen, 2018). eRF3 undergoes GTP hydrolysis and eRF1 accommodates in the peptidyl-transferase center (PTC), resulting in peptide release (C. U. T. Hellen, 2018).



Figure 1.3 Model of the eukaryotic translation termination pathway. The pretermination complex (pre-TC) has a peptidyl-tRNA in the P-site and a stop codon in the A-site. The eRF1/eRF3-GTP recognizes the stop codon and binds to the A-site. GTP hydrolysis causes eRF1

conformational changes and accommodates it in the peptidyl-transferase center (PTC), inducing peptide release, leaving the post-termination complex (post-TC).

Adapted with permission from (C. U. T. Hellen, 2018).

The 80s ribosomes are then split by the ATP-binding cassette protein (ABCE1), releasing the 60S subunit (C. U. T. Hellen, 2018). The following dissociation of deacetylated tRNA and mRNA from the 40S subunit is mediated by factors, ligatin (eIF2D), or density-regulated protein (DENR) and multiple copies in T-cell lymphoma-1 (MCT1) (C. U. T. Hellen, 2018; Pisarev et al., 2007, 2010; Skabkin et al., 2010).

1.1.2 Alternative cap-independent translation mechanisms in viruses

Under some cellular stress including viral infections, cap-dependent translation is inhibited, which leads to global shutoff of protein synthesis (Stern-Ginossar, Thompson, Mathews, & Mohr, 2019; Wek, 2018). Several translation factors are targeted via PTM and cleavage. As such, viruses have developed their own mechanisms to hijack the host translational machinery, one of which is via internal ribosome entry sites (IRESs). IRESs are RNA elements that can recruit the ribosome in a 5'end independent manner, thus IRES can recruit the ribosome internally within the mRNA (Terenin, Smirnova, Andreev, Dmitriev, & Shatsky, 2017). The first IRES was discovered in 1988 from polioviruses (PV) (Pelletier & Sonenberg, 1988; Prats et al., 2020). As my thesis focuses on viral cap independent translation mechanisms, several types of viral IRESs are described in more detail below.

1.1.2.1 Internal ribosomal entry sites: four types and mechanisms

According to the IRES database, 774 eukaryotic IRESs and 554 viral IRESs have been identified from 11 eukaryotic organisms and 198 viruses, respectively, up until 2020 (Zhao et al., 2020). Viral IRESs are categorized into four main types (Figure 1.4), based on the secondary structure, requirement for initiation factors and the initiation mechanism (Yang & Wang, 2019).



Figure 1.4 Examples of the diversity of mechanisms of viral IRES RNAs. There are four main types of viral IRESs. Type I and II IRESs, exemplified by the PV and FMDV, respectively, have similar initiation factor requirements, except that ribosomes recruited by type I IRES scans for AUG start codon. Type III IRES, exemplified by HCV, only require eIF2 and eIF3 to recruit

the 40S. Type IV IRES, observed in dicistrovirus IGR, does not require any initiation factor to recruit the 40S and can initiate translation at a non-AUG start codon.

Adapted with permission from (Plank & Kieft, 2012).

Type I IRESs require many factors to facilitate the recruitment of the 40S ribosomal subunit to mRNA (Pelletier & Sonenberg, 1988). Specifically, Type I IRESs require initiation factors: eIF2, eIF3, eIF4A, the central domain of eIF4G called eIF4Gm, as well as IRES transacting factor (ITAF): pyrimidine tract binding protein(PTB), poly(C)-binding protein 1(PCBP1) and PCBP2 (Sweeney, Abaeva, Pestova, & Hellen, 2014). ITAFs are cellular RNA-binding proteins that typically are not the core translation factors and that can facilitate IRES translation by modulating an active RNA structural conformation (Yu, Abaeva, Marintchev, Pestova, & Hellen, 2011). 40S subunits that are recruited to Type I IRESs typically scan downstream for the AUG initiation codon. Type I IRESs are found in poliovirus, EV71, BEV and Coxsackievirus (Sweeney et al., 2014).

Type II IRESs are in general similar to Type I IRES in terms of factor dependency except that 40S subunits recruited to the IRES do not scan and instead recruits the 40S near or at the AUG start codon. Type II IRESs require the ITAFs PTB and ITAF45 and initiation factors: eIF2, eIF3, eIF4A, eIF4B and eIF4G. Type II IRESs include IRESs from viruses such as encephalomyocarditis virus (EMCV), foot-and-mouth disease virus (FMDV) and Theiler's encephalomyelitis virus (TMEV) (Yang & Wang, 2019).

Type III was first identified in hepatitis A virus(HAV) of the family *Picornaviridae* (E. A. Brown, Day, Jansen, & Lemon, 1991). Type III IRESs were then identified in Hepatitis C

virus (HCV), classic swine fever virus (CSFV) and porcine teschovirus 1 (PTV-1) (Yang & Wang, 2019). The 5' UTR IRES of HAV contains six domains, and domains IV and V form the key IRES core that is located adjacent to the initiation codon AUG (E. A. Brown, Zajac, & Lemon, 1994). Type III IRESs have the ability to directly bind to 40S ribosomal subunit and bring the 40S near the AUG codon and then subsequently, requiring only eIF2, eIF3 and Met-tRNA_i to properly position the 40S at the AUG codon (Pisarev et al., 2004; Rijnbrand, van der Straaten, van Rijn, Spaan, & Bredenbeek, 1997; Tsukiyama-Kohara, Iizuka, Kohara, & Nomoto, 1992). Interestingly, HCV IRES can direct factorless translation under high salt conditions (MgCl₂ = 5 mM) (Lancaster, Jan, & Sarnow, 2006).

Type IV IRESs are the most streamlined translation mechanism to date: these IRESs require no initiation factors nor ITAFs for ribosome recruitment. They are found in the singlestranded positive sense RNA viruses that belong to the family *Dicistroviridae*. Type IV IRES contains three main pseudoknots (PKI, PKII, and PKIII) that directs ribosome binding and directs translation (Jan & Sarnow, 2002; Kanamori & Nakashima, 2001; Nishiyama et al., 2003; Spahn et al., 2004). Structural studies have shown that the IRES binds within the inter-subunit space between the 40S and 60S subunits, spanning over all three ribosomal sites: PKI forms one domain that occupies the A-site and mimics a tRNA anticodon-codon interaction (Koh, Brilot, Grigorieff, & Korostelev, 2014; Warsaba, Sadasivan, & Jan, 2020), and PKII and PKIII forms the ribosome binding domain that occupies the E and P-sites (Figure 1.5) (Jan & Sarnow, 2002; Nishiyama et al., 2003; Pfingsten, Costantino, & Kieft, 2006, 2007; Wilson, Pestova, Hellen, & Sarnow, 2000). Remarkably, the PKI domain can be functionally replaced by an authentic tRNA anticodon stem-loop (H. H. T. Au & Jan, 2012). These two domains can function independently: PKI can fold independently, PKI disruption does not impact ribosome assembly, and PKII/PKIII domain can also fold independently and bind to ribosomes (D. Costantino & Kieft, 2005; Fernández, Bai, Murshudov, Scheres, & Ramakrishnan, 2014; C. J. Jang & Jan, 2010; Jan & Sarnow, 2002; Nishiyama et al., 2003).



Figure 1.5 Structure of CrPV IGR IRES in the Ribosome. A) The secondary structure of the CrPV IGR IRES with three PKs and stem-loops. B) Overview of the CrPV IGR IRES in the isolated yeast ribosomes. Inside the black box shows the CrPV IGR IRES spans over the E, P, A-sites of the 80S ribosome.

Adapted from (Fernández et al., 2014). Permission is not required for non-commercial use.

Structural analysis showed that distinct IRES domains interact with specific regions of the ribosome. Loop 1.1 (L1.1) region directly interacts with the L1 stalk of the 60S ribosomal subunit (Fernández et al., 2014). The L1 stalk of the 60S subunit normally functions as stabilizing a hybrid P/E tRNA state through an inward movement during translocation

(Tourigny, Fernández, Kelley, & Ramakrishnan, 2013). Mutations in L1.1 disrupts 60S subunit joining and IRES translation (C. J. Jang, Lo, & Jan, 2009). Stem loop IV (SLIV) and SLV interact with the ribosomal rpS5 and rpS25 to promote 40S binding (D. A. Costantino, Pfingsten, Rambo, & Kieft, 2008; C. J. Jang et al., 2009; Kieft, 2008; Landry, Hertz, & Thompson, 2009; Nishiyama, Yamamoto, Uchiumi, & Nakashima, 2007; Pfingsten, Castile, & Kieft, 2010; Pfingsten et al., 2006; Schmidt et al., 2016; Spahn et al., 2004). Mutations in these two loop regions abolish the interaction with 40S ribosomal unit and translation initiation (D. Costantino & Kieft, 2005; Jan & Sarnow, 2002; Nishiyama et al., 2003). In the initial assembled 40S:IRES complex, the CrPV PKI domain adopts a conformation that resembles a deacylated tRNA in a P/E hybrid state on the ribosome, thus indicating that the ribosome: IRES is primed for translocation (D. A. Costantino et al., 2008; Yamamoto, Nakashima, Ikeda, & Uchiumi, 2007). Translocation of the PKI domain from the A to P-sites is mediated by eEF2. eEF2-GTP binds to the IRES/80S complex when IRES is in a rotated state, interacting with IRES PKI and ribosomal A-site, and thus promotes the PKI domain to translocate from ribosomal A-site to P-site (Abeyrathne, Koh, Grant, Grigorieff, & Korostelev, 2016; Fernández et al., 2014; Muhs et al., 2015; Murray et al., 2016; Yamamoto et al., 2007).

IRES translocation proceeds with a swiveling movement of the head of 40S in an orthogonal direction with respect to the back rotation of the 40S to recover a canonical configuration (Pisareva, Pisarev, & Fernández, 2018; Ratje et al., 2010). After eEF2 leaves the ribosome, the head of 40S returns to its non-swiveled configuration (Noller, Lancaster, Zhou, & Mohan, 2017; Pisareva et al., 2018). Since translocation of the PKI domain to the P-site is not stabilized in reactions with just eEF2, the PKI domain can back translocate (Warsaba et al., 2020). Stable translocation of the PKI domain to the P-site requires delivery of the first aa-tRNA to the A-site by eEF1A (Muhs et al., 2015; Warsaba et al., 2020). The entire process is known as pseudotranslocation as no peptide bond is formed (Ruehle et al., 2015; Warsaba et al., 2020). Type IV IRESs requires in total two translocation events to place the first aa-tRNA in the P-site, then proceeding with canonical elongation (Abeyrathne et al., 2016; Murray et al., 2016; Pisareva et al., 2018). The mechanism of the second translocation still largely remains unknown. However, a recent Cryo-EM study revealed that the IRES PKI domain resembles an acceptor tRNA and flips from the decoding E-site in the 40S to the 60S subunit, and the head swiveling of 40S plays an important role in the second translocation of IRESs (Pisareva et al., 2018).

To date, there are two sub-classes of the Type IV IRES based on different features: Type IVa and Type IVb, characterized by cricket paralysis virus (CrPV) intergenic region (IGR) IRES and Taura syndrome virus (TSV) IGR IRES as examples, respectively (Figure 1.6) (Jan, 2006; Nakashima & Uchiumi, 2009). Type IVb IRESs contains a longer L1.1 region and an extra stem-loop, SLIII, as indicated in **blue box in Figure 1.6**, compared with Type IVa IRESs. Studies have shown that mutations in SLIII can still assemble 80S ribosomes in RRL but deletion of SLIII abolishes both 80S positioning and IRES-driven translation activity (Hatakeyama, Shibuya, Nishiyama, & Nakashima, 2004; C. J. Jang & Jan, 2010; Pfingsten et al., 2007). Interestingly, Type IVa and IVb IGR IRESs are thought to function in a similar mechanism (Warsaba et al., 2020). PKI domains of Type IVa and IVb IGR IRESs are functionally interchangeable (Hertz & Thompson, 2011; C. J. Jang & Jan, 2010).

A subset of type IV IRESs from dicistroviruses, including IAPV, Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV) and Solenopsis invicta virus 1 (SINV), contains a overlapping +1 ORF (ORFx) within the viral structural protein coding region (H. H. Au et al., 2015; Firth, Wang, Jan, & Atkins, 2009; Sabath, Price, & Graur, 2009). For example, the IAPV

IRES directs both 0 and +1 frame translation (Ren et al., 2012). Specific mutations within the SLIII of the IAPV IRES uncouples 0 and +1 frame translation (H. H. Au et al., 2015). Furthermore, the integrity of the SLIII domain and two unpaired adenosines at the core junction of three helices in PKI are important for IRES-mediated reading frame selection (H. H. Au et al., 2015). The CrPV IRES also can drive 0 and +1 frame translation, however, the mechanism mediated the CrPV IRES to drive +1 translation is distinct from that of the IAPV IRES (Kerr et al., 2018). In summary, the type IV IRESs have provided excellent models to study fundamental ribosome functions.



Figure 1.6 The secondary structure of Type IVa and IVb dicistrovirus IGR IRESs. A) Type IVa CrPV IGR IRES. B) Type IVb TSV IGR IRES. In green box shows the extra stem-loop, SLIII.

Adapted with permission from (C. J. Jang & Jan, 2010)

Recently, metagenomics studies have revealed many novel dicistrovirus-like genomes (Boros, Pankovics, Simmonds, & Reuter, 2011; Culley, Lang, & Suttle, 2007; Shi et al., 2016). For example, the IGR from Halastavi árva virus (HalV), isolated from the intestinal contents of freshwater carp (*Cyprinus carpio*) (Boros et al., 2011), was identified to contain novel IRES features (Abaeva et al., 2020). HaIV IGR IRES is predicted to form a large hairpin at the 3' end of ORF1 and consist of two domains: domain 1 and domain 3 (Figure 1.7A) (Abaeva et al.,
2020). Domain 1 contains PKII with helical elements P1.1, P1.2, and P1.3, and internal loops, including L1.1. Domain 3 contains PKI (Figure 1.7A) (Abaeva et al., 2020). Interestingly, even though HaIV IGR IRES lacks PKIII and SLIV and SLV that a typical Type IV IRES has (Figure 1.7B) and is not able to bind to individual 40S subunit, it is still able to bind performed 80S ribosomes (Abaeva et al., 2020). The discovery of HaIV IGR IRES leads to the possibility of new IRES-dependent mechanisms.



Figure 1.7 The predicted model of dicistrovirus IRES. A) The predicted model of HaIV IGR IRES. B) The secondary structure model of CrPV IGR IRES, which is a typical Type IV IRES.

Adapted from (Abaeva et al., 2020). Permission is not required for non-commercial use.

1.2 Recoding mechanisms in viruses: Programmed ribosomal frameshifting

IRESs are not the only viral RNA structures that regulate translation. Some viruses utilize translation recoding, which is a strategy to regulate and expand the coding capacity in some cellular mRNA and RNA viruses (W. P. Tate et al., 1995). As I described above, a subset of Type IV IRESs can initiate translation in 0 and +1 frames in order to increase coding capacity. Another well-studied viral recoding strategy is programmed ribosome frameshifting (PRF), typically classified into -1, +1 and +2 types. I will review these frameshift mechanisms in brief with more focus on the -1PRF as this is one of the main aims of this thesis.

1.2.1 -1 Programmed ribosomal frameshifting (-1 PRF)

A -1 PRF generates a shift of the reading frame by one nucleotide towards the 5' direction. Specifically, elongating ribosomes that interact with the -1 frameshifting signal (FSS) will shift the reading frame and continue translate in the -1 frame. -1 PRF is commonly used by viruses such as coronaviruses and retroviruses (Giedroc, Theimer, & Nixon, 2000; Hatfield & Oroszlan, 1990). In some viruses such as human immunodeficiency virus type I (HIV-1), the frameshifting(FS) frequency is finely tuned to ensure an optimal ratio of structural and nonstructural protein expression for virus infection (J D Dinman, Ruiz-Echevarria, & Peltz, 1998). Inhibition or enhancement of -1 FS efficiency can inhibit HIV infection, thus -1 FS is an effective target for antiviral strategies (Hilimire et al., 2017).

The precise location of -1 FS depends on a slippery sequence, typically an AU rich element, that allows the realignment of tRNA anticodons over this sequence (Choi, O'Loughlin,

Atkins, & Puglisi, 2020). The slippery site in eukaryotes is featured by a sequence of N NNW WWH, where N is any base, W is A or U, and H is A, C or U, with some exceptions (Brierley, Jenner, & Inglis, 1992). Ribosomal frameshifting over a slippery sequence alone is in general very weak (~2%) (Sharma et al., 2014; Weiss, Dunn, Shuh, Atkins, & Gesteland, 1989). The other element that promotes -1 FS is a downstream stimulatory element, which is generally a structured RNA such as a pseudoknot, stem-loop or long-range kissing loop interactions (Baril, Dulude, Steinberg, & Brakier-Gingras, 2003; Barry & Miller, 2002; Giedroc et al., 2000; Herold & Siddell, 1993; Liston & Briedis, 1995; Plant & Dinman, 2005). The length of the spacer sequence is also important in determining the frameshifting efficiency (Qiao et al., 2017). The common length of the spacer sequence is 5-6 nucleotides for bacterial ribosomes and 5-9 for mammalian ribosomes. A longer or shorter spacer length can reduce the -1 frameshifting frequency significantly (Brierley et al., 1992; Kontos, Napthine, & Brierley, 2001; Larsen, Gesteland, & Atkins, 1997; Qiao et al., 2017), possibly because the optimal pausing is required for the intrinsic helicase in ribosomes to restore the contact with mRNA and unwind it for decoding (Kontos et al., 2001). In summary, the key elements for a -1 PRF are a heptameric "slippery site", a 3' stimulatory element and the "spacer" sequence between the slippery sequence and the stimulatory element (Brierley, Digard, & Inglis, 1989; ten Dam, Pleij, & Bosch, 1990).

Recent model suggests that the ribosome is intrinsically susceptible to frameshift before translocating, and this transient state is promoted by the downstream stimulatory element (Choi et al., 2020). In *Escherichia coli*, single-molecule fluorescence resonance energy transfer (smFRET) studies on a frameshift model showed that during elongation factor - G (EF-G) - catalyzed translocation, a portion of ribosomes spontaneously switches from rapid, accurate

20

translation to a slow, frameshifting-prone translocation mode, where the movements of peptidyland deacylated tRNA become uncoupled (Poulis, Patel, Rodnina, & Adio, 2022). Peptidyl-tRNA fluctuates between chimeric and post-translocation states while deacylated tRNA translocates from the ribosomal P to E-sites (Poulis et al., 2022). After the release of deacylated tRNA, peptidyl-tRNA gains access to the -1 frame codon, leading to the -1 FS slippage and the recruitment of corresponding aa-tRNA at the A-site (Poulis et al., 2022). Interestingly, a transacting factor, viral protein 2A, has recently been shown to bind to an RNA stem-loop, beginning 14 nt downstream of the frameshifting slippery sequence, to form an RNA: protein complex, inducing higher PRF efficiency in EMCV (Napthine et al., 2017). Thus, -1 PRF can be stimulated by a downstream stimulatory RNA element or by a protein, highlighting the diverse mechanisms that could affect translation recoding.

1.2.2 +1 Programmed ribosomal frameshifting (+1PRF)

+1 FSS directs elongating ribosomes to shift 1 nucleotide towards the 3' direction of the mRNA. +1 PRF is not as commonly reported as -1 FSS in eukaryotes. Exceptionally, one study analyzed the genome of *Euplotes octocarinatus* and identified 3700 +1 PRF events from 32,353 transcripts (Klobutcher & Farabaugh, 2002; Lobanov et al., 2017; R. Wang, Xiong, Wang, Miao, & Liang, 2016). Interestingly, the mechanisms underlying +1 PRF are distinct (Jonathan D Dinman, 2012). In E.coli *prfB* mRNA, which encodes for the termination factor release factor 2 (RF2), ribosomes translate and encounter the UGA stop codon in the slippery sequence U CUU UGA. Typically, when a stop codon is encountered by the ribosome, RF2 recognizes the UGA termination codon leading to termination (Craigen, Cook, Tate, & Caskey, 1985). However, low

levels of RF2, will lead to inefficient recognition of the UGA stop codon, causing the ribosome to pause. A Shine Delgarno-like (SD-like) sequence at the 5' of the slippery site will then interact with the anti-SD sequence on the 16S rRNA to relocate the ribosomes in the +1 frame. As a result, the +1 FSS leads to translation of the full-length RF2 and increasing expression of RF2. Thus, the +1 PRF in the RF2 mRNA undergoes an autoregulation to control levels of RF2 (Adamski, Donly, & Tate, 1993). Mathematical modeling showed the +1 PRF efficiency is related to destabilization of deacylated tRNA in the E-site, rearrangement of peptidyl-tRNA in the P-site, and the availability of cognate aa-tRNA corresponding to the A-site (Liao, Gupta, Petrov, Dinman, & Lee, 2008).

In eukaryotic cells, +1 PRF is driven by other mechanisms. The mammalian ornithine decarboxylase antizyme such as OAZ uses a strong secondary structure at the 3' of the slippery site as the main kinetic trap for +1 PRF. Almost all vertebrate OAZ +1 PRFs have pseudoknots (Ivanov, Anderson, Gesteland, & Atkins, 2004). However, similar to E.coli *prfB*, the +1 PRF in OAZ is activated by a 0-frame UGA codon at A-site, and also is negatively related to the tRNA-mRNA interactions in the ribosomal P-site (Ivanov et al., 2004). Interestingly, OAZ +1 PRF is regulated by polyamines (Rom & Kahana, 1994). Polyamines are small polycationic molecules such as putrescine, spermidine and spermine that are important for many basic cellular functions (Cohen & -, 1998; Tabor & Tabor, 1984). Although mechanisms remain unknown, a high concentration of polyamine is found to be able to induce promiscuous +1 frameshifting (Oguro et al., 2020).

1.2.3 +2 Programmed ribosomal frameshifting (+2 PRF)

+2 FSS directs the elongating ribosome to shift two nucleotides towards 3' direction of the mRNA. +2 PRF was observed typically in the *Potyviridae* family, which consists of about 30% of the known plant viruses (Riechmann, Laín, & García, 1992). These viruses are positive sense RNA viruses that contain one single long ORF around 10 kb in length. The ORF is translated into a large polyprotein, which is later cleaved into about 10 mature proteins. +2 PRF allows for the translation of a short overlapping ORF, Pretty Interesting Potyviridae ORF (PIPO), embedded within the P3 cistron of the polyprotein (Chung, Miller, Atkins, & Firth, 2008). PIPO was first identified in an alignment of Turnip mosaic virus (TuMV) sequences using bioinformatics tool Maximum Likelihood Overlapping Gene Detector (MLOGD) (Chung et al., 2008; Firth & Brown, 2006; J. A. Walsh & Jenner, 2002). +2 PRF in *Potyviridae* begins at the G1-2A6-7 motif, which is generally (G) GAA AAA A(A) (Chung et al., 2008). The resulting polyprotein P3N-PIPO fusion protein is vital for cell-to-cell movement during viral infection (Chung et al., 2008). The exact RNA structure or mechanism to stimulate the frameshifting is still under investigation, but evidence suggests that a polymerase slippage may be involved (Chung et al., 2008; Rodamilans et al., 2015).

1.3 Dicistrovirus

Dicistrovirus are single-stranded, positive-sense RNA viruses, which mainly infect arthropods. Well-studied examples include CrPV and *Drosophila* C virus (DCV). CrPV was originally isolated from two species of field crickets, *Teleogryllus oceanicus and Teleogryllus commodus*, in 1970 (Reinganum, O'Loughlin, & Hogan, 1970). DCV infects *Drosophila* *melanogaster* and was firstly reported as an unclassified member of *Picornaviridae* in 1995 (Murphy et al., 1995). CrPV has a wide host range (over 40 species) and can replicate in most *D. melanogaster* cell lines, thus providing a cell culture infection model. In cultured *D. melanogaster* cell lines, CrPV infection causes cytopathic effect which eventually leads to cell lysis. DCV can also cause cytopathic effect in *D. melanogaster* cells, which can lead to cell lysis through mainly cell clumping and cell detachment.

1.3.1 Classification

CrPV and DCV are RNA viruses with picornavirus-like characteristics ((Johnson & Christian, 1998). Debates about whether CrPV and DCV belong to the family *Picornaviridae* existed for a long time (Norman F. Moore, Reavy, Pullin, & Plus, 1981; N F Moore, Kearns, & Pullin, 1980). CrPV and DCV and many other small RNA viruses have been previously classified in *Picornaviridae* based on their physicochemical properties, and the presence of three major structural proteins (Jousset, Bergoin, & Revet, 1977; N F Moore et al., 1980; Plus, Croizier, Reinganum, & Scotti, 1978). Picornaviruses has a single strand of positive sense RNA, which encodes one open reading frame, flanked by a 5' untranslated region (5'UTR), a 3' UTR and a poly(A) tail. The open reading frame of a picornavirus genome is translated into a single polyproteins, which is then cleaved into individual proteins in three segments: P1, P2 and P3, as shown in Figure 1.4 (Jacobson & Baltimore, 1968; J. Tate et al., 1999). P1 segment encodes for the structural proteins, including precursor protein VP0, which contains VP4 and VP2, VP3 and VP1 (Figure 1.8A). Segment P2 and P3 encodes the non-structural proteins. P2 contains the 2A protease and 2C. P3 contains protease 3C and RNA-dependent RNA polymerase 3D (J. Tate et al.

al., 1999).



Figure 1.8 The genome organization of a standard picornavirus and a standard dicistrovirus. A) The organization of a standard picornavirus genome, with the structural proteins (encoded in the P1 segment) at the 5' end. B) The genome organization of CrPV. P1 segment is at the 3' end. Two open reading frames were flanked by a proposed IRES.

Adapted with permission (J. Tate et al., 1999).

Sequencing of the viral genomes revealed that DCV and CrPV genome organizations are different compared to picornavirus genomes (Johnson & Christian, 1998; J. Tate et al., 1999). Instead of a single polyprotein ORF observed in picornaviruses, dicistroviruses encode two main ORFs with the non-structural proteins encoded in ORF1 and the structural proteins encoded in ORF2, thus the non-structural proteins are encoded upstream of structural proteins. These viral genomes were subsequently classified into a distinct family, *Dicistroviridae*. *Dicistroviridae* is a rapidly growing family of RNA viruses that mainly infect arthropods (**Figure 1.8B**) (Bonning, 2009; Warsaba et al., 2020). The detailed genome organization is introduced in the following section.

1.3.2 Genome organization

As mentioned above, the dicistrovirus genome encodes two main ORFs, ORF1 and ORF2 (Figure 1.9). The translation of each ORF is directed by the 5' untranslated region internal ribosome entry site (5'UTR IRES) and the intergenic region internal ribosome entry site (IGR IRES), respectively. The upstream ORF1 encodes the non-structural proteins including the viral silencing suppressor 1A protein, the 2B protein, the 2C RNA helicase, the 3A protein, the viral protein genome-linked (VPg), the 3C protease and the RNA-dependent RNA polymerase (RdRP) 3D proteins (Warsaba et al., 2020). The downstream ORF2 encodes the structural proteins VP2, VP4, VP3, VP1 and an ORFx in the +1 reading frame (Ren et al., 2012; J. Tate et al., 1999). The ORFs are translated into polyproteins which are then processed into individual proteins by virally encoded 3C protease, a 2A "stop-go" translation mechanism and other yet to be identified mechanisms (Warsaba et al., 2020).

The functions of specific dicistrovirus proteins are still not fully understood, however some viral proteins are characterized. CrPV 1A inhibits the insect antiviral RNA interference pathway and stress granule (SG) assembly (Khong & Jan, 2011; Khong et al., 2017; Nayak et al., 2010; Sadasivan et al., 2022). 2B has similarity to poliovirus 2B and is predicted to function in remodeling membranes (Aldabe, Barco, & Carrasco, 1996). 2C is a RNA helicase and is also predicted to mediate membrane permeabilization (Gorbalenya, Koonin, & Wolf, 1990; Mirzayan & Wimmer, 1994; Rodríguez & Carrasco, 1993). 3A is predicted to be responsible for viral replication complex formation (Fujita et al., 2007). VPg is covalently linked to the 5' end of viral genomic RNA and is important for priming viral replication (Hwang et al., 2015; Warsaba et al., 2022). CrPV has four nonidentical copies VPg and deletion analysis showed a threshold number of VPgs is important for productive infection (Hwang et al., 2015; Warsaba et al., 2022). 3C is a protease that processes the viral polyprotein into individual viral proteins (Nakashima & Nakamura, 2008). 3D encodes the RNA-dependent RNA polymerase which is responsible for viral RNA synthesis and viral replication (Bonning & Miller, 2010). Dicistroviruses are nonenveloped viruses that are roughly spherical consisting of three major capsid proteins VP1, 2 and 3 (J. Tate et al., 1999). VP3 and VP4 are firstly translated as precursor protein VP0 before cleavage. VP1 subunits form a pentamer, whereas VP2 and VP3 form heterohexamers. VP4 is attached to the inner surface of the viral asymmetric unit, and is important to membrane permeabilization during viral entry (Sánchez-Eugenia, Goikolea, Gil-Cartón, Sánchez-Magraner, & Guérin, 2015; Warsaba et al., 2020).

27





Adapted from (Warsaba et al., 2020). Permission is not required for non-commercial use.

1.3.3 Viral strategies of translational control

After entering host cells, some viruses have evolved strategies to modulate the host translation machinery in order to redirect ribosomes to viral RNAs for viral protein synthesis as well as to dampen antiviral responses (H. H. T. Au & Jan, 2014). I will describe a few notable examples.

Internal Ribosome Entry Site hijack host translational machinery:

Type IV IRESs for example, as mentioned previously in chapter 1.2.2.1., initiate translation internally by functionally mimicking a tRNA to span over ribosomal A, P, E-sites, and direct a non-AUG codon to the ribosomal A-site. Thus, they bypass the requirements for initiation factors or Met-tRNA_i, giving the advantage for translation initiation when initiation factors are disrupted by other viral strategies.

Inactivation of Cap-Binding Complexes:

Translation initiation is the rate-limiting step for protein synthesis, therefore it is tightly regulated by initiation factors, the eIF4F cap-binding complex, which consists of eIF4G, eIF4E and eIF4A, is one of the major targets for translational control (H. H. T. Au & Jan, 2014). For example, poliovirus (genus Enterovirus, family Picornaviridae) (PV) encodes two viral protease: 2A protease and 3C protease, that are responsible for the cleavage of the viral polyprotein and specific host proteins (Glaser & Skern, 2000; Kräusslich, Nicklin, Lee, & Wimmer, 1988; Kundu, Raychaudhuri, Tsai, & Dasgupta, 2005). 2A protease targets the scaffold proteins eIF4GI and eIF4GII, resulting in two fragments: an amino-terminal fragment that contains eIF4E binding site (Lamphear, Kirchweger, Skern, & Rhoads, 1995; Mader, Lee, Pause, & Sonenberg, 1995), and a carboxyl-terminal fragment that contains the eIF3- and eIF-4 binding sites (Imataka & Sonenberg, 1997; Lamphear et al., 1995). PV requires only the carboxyl-terminal fragment for its translation, which is mediated by the Type I IRES (Ohlmann, Rau, Pain, & Morley, 1996). Moreover, PV can also cleave PABP by viral 2A and 3C proteases, the same as coxsackievirus 2A protease (Joachims, Van Breugel, & Lloyd, 1999). As a result, this cleavage impairs overall cap-dependent translation but not poliovirus translation (Gradi, Svitkin, Imataka, & Sonenberg,

1998).

eIF4E is another target during virus infections. CrPV infection in *Drosophila* S2 results in the dissociation of eIF4G and eIF4E early in infection, however the mechanism underlying this s inhibition remains elusive (Garrey, Lee, Au, Bushell, & Jan, 2010). Enterovirus 71 infection induces microRNA (miRNA)-144 upregulation in host cells, which subsequently targets eIF4E mRNA for degradation (Ho et al., 2011). Transfection of antagomiR-141, which is the antisense miRNA-144 showed a delay in inhibition of eIF4E and attenuated virus production (Ho et al., 2011). eIF4E is also regulated by binding to its repressor 4E-BP (Garrey et al., 2010; Haghighat, Mader, Pause, & Sonenberg, 1995; Lin et al., 1994). 4E-BP and eIF4G compete for binding to eIF4E (Mader et al., 1995). Under Vesicular stomatitis virus (VSV), PV and EMCV infection, eIF4E is dephosphorylated at serine 209 and binds to activated 4E-BP, leading to inhibition of host protein synthesis (Connor & Lyles, 2002; Gingras, Svitkin, Belsham, Pause, & Sonenberg, 1996). Adenovirus (Ad) infection prevents host translation by displacing eIF4E kinase Mnk1 from eIF4F to block eIF4E phosphorylation with Ad 100k protein (Cuesta, Xi, & Schneider, 2000).

Activation of Cap-Binding Complexes:

Some cap-binding complexes have the effect of inhibiting translation initiation and could be activated by viral infection. For example, EMCV and PV infection results in the dephosphorylation of 4E-BP1, which activates it to bind to eIF4E, shutting off the host protein synthesis (Gingras & Sonenberg, 1997; Gingras et al., 1996).

Some viruses that do not use IRES elements to recruit ribosomes, can actively assemble eIF4F complexes to mediate their own cap-dependent viral protein synthesis, even when host protein synthesis is inhibited. For example, during herpes simplex virus -1 (HSV-1, which is a DNA virus) infection, the viral protein ICP6 binds to eIF4G and promotes eIF4F assembly (D. Walsh & Mohr, 2006).

Inactivation of eIF2 and Alternate Initiation Factors:

One central player in the innate immune response is eIF2. The formation of TC, which is composed of eIF2, GTP and an initiator tRNA (commonly, Met-tRNA_i), is critical to initiation and initiation codon (commonly, AUG) selection, and it is highly regulated through the innate immune response (Alan G Hinnebusch, 2011). The inactive eIF2-GDP is required to be recycled by eIF2B to exchange the bound GDP for GTP, preparing the active eIF2-GTP for initiation cycles. However, the α subunit of eIF2 can be phosphorylated by kinases under stress conditions, such as viral infection. eIF2αP within the eIF2-GDP state has a high affinity to eIF2B, thereby reducing the overall GTP exchange for GDP on eIF2, leading to global translational repression (Figure 1.10). Eukaryotes have four eIF 2α kinases: PKR-like endoplasmic reticulum (ER) kinase (PERK), general control non-derepressible-2 (GCN2), heme-regulated inhibitor (HRI) and interferon-inducible RNA-dependent kinase (PKR), activated by different stresses (Dever, 2002). Among those, PKR, GCN2 and PERK have antiviral effects (Berlanga et al., 2006; Sharp, Xiao, Jeffrey, Gewert, & Clemens, 1993; Won et al., 2012). PKR is activated by double-strand RNA (dsRNA), a pathogen-associated molecular pattern (PAMP) and the host protein PACT (A G Hinnebusch, 2000; Mohr & Sonenberg, 2012; Sharp et al., 1993; Tavantzis, 2001). GCN2 is activated by amino acid degradation and UV irradiation (Deng et al., 2002; A G Hinnebusch, 2000). GCN2 is also activated when its histidyl-tRNA synthetase-related domain binds to the Sindbis virus (SV) genomic RNA (Berlanga et al., 2006). PERK can be activated by ER stress induced by viral infection (Graham D Pavitt & Ron, 2012; Ron & Walter, 2007; Shrestha et al.,

2012). Interestingly, several viruses have evolved strategies to antagonize PKR via virus-coded dsRNA decoy molecules, dsRNA-binding proteins, or PKR-binding proteins, to counteract PKR activation and eIF2α phosphorylation (Mohr & Sonenberg, 2012). For example, Influenza A virus contains the non-structural 1A (NS1A) protein that can not only bind to dsRNA to prevent it from activating PKR but also bind to PKR linker region to prevent the conformational change required for PKR autophosphorylation (Kim, Jeong, & Jang, 2021; S. Li, Min, Krug, & Sen, 2006; Mohr & Sonenberg, 2012). HCV contains the viral nonstructural 5A (NS5A) protein that can also bind to PKR and suppress the dsRNA-dependent PKR activation and IFN regulatory factor-1 (IRF-1) activation during HCV infection (Mohr & Sonenberg, 2012; Pflugheber et al., 2002).



Figure 1.10 Recycling of eIF2 during translation initiation. During translation initiation, the eIF2-Met-tRNA_i-GTP ternary complex is recruited to the 43S PIC for initiation codon recognition. After GTP hydrolysis, the inactive eIF2-GDP is required to be recycled by eIF2B to exchange the bound GDP for GTP. However, under cellular stress, eIF2 can be phosphorylated at the α unit (eIF2 α P) by several protein kinases (PERK, HRI, PKR, GCN2). Phosphorylated eIF2 acts as an inhibitor of eIF2B GEF, thus inhibiting overall protein synthesis initiation as a regulatory mechanism.

Adapted with permission from (H. H. T. Au & Jan, 2014).

Change the Distribution of Translation Initiation Factors:

Another host process that viral infection changes is the subcellular distribution of translation factors that are required by host cap-dependent translation. For example, poliovirus infection leads to relocalization of eIF4E to the nucleus (Sukarieh, Sonenberg, & Pelletier, 2010). EMCV 2A is released during infection, which has a nuclear localization signal and a eIF4E binding site, that is able to relocalize eIF4E to the nucleus upon interaction (Groppo, Brown, & Palmenberg, 2011). In Bunyamwera virus (BUNV), rotavirus, and HSV-1 infections, PABP is retained in the nucleus (Blakqori, van Knippenberg, & Elliott, 2009; Dobrikova, Shveygert, Walters, & Gromeier, 2010; Harb et al., 2008).

Manipulation of host RNA:

Lastly, translation can be modified by manipulating host RNA. Influenza viral polymerase contains three subunits: PA, PB1 and PB2 (Dias et al., 2009; Garcin et al., 1995; Pfingsten et al., 2006; Plotch, Bouloy, Ulmanen, & Krug, 1981). The PB2 subunits can bind to host pre-mRNAs (Fechter et al., 2003; Guilligay et al., 2008; M. L. Li, Rao, & Krug, 2001), use the endonuclease active site in PA to cleave 10-13 nucleotides after the 5' cap of host pre-mRNAs (Dias et al., 2009), and use the capped RNA fragment to prime viral transcription (Engelhardt, Smith, & Fodor, 2005; Whelan & Pelchat, 2022). Furthermore, the severe acute respiratory virus (SARS) coronavirus use protein Nsp1 to associate with the 40S ribosomal subunits and selectively cleaves host mRNA to suppress host translation (Huang et al., 2011; Kamitani, Huang, Narayanan, Lokugamage, & Makino, 2009; Kamitani et al., 2006).VSV infection suppresses the export of cellular mRNA from the nucleus to inhibit the translation of host defense-related proteins (Faria et al., 2005).

1.3.4 Viral discovery by metagenomic approaches

Viruses are considered as the most abundant entities in the global biosphere (Koonin, Krupovic, & Dolja, 2022). The ongoing metagenomic-metatranscriptomic revolution showed that there is a vast scale of diversity in virus genomes (Dolja & Koonin, 2018; Dutilh, 2014). Next generation sequencing and accelerated computing power have transformed and accelerated the discovery of novel viral genomes. Currently, many independent studies exploring thousands of metatranscriptomes from diverse environments greatly increased the number of known RNA viruses (Edgar et al., 2022; Kuhn et al., 2021; Neri et al., 2022; Shi et al., 2016; Zayed et al., 2022). For example, Shi et al. performed deep transcriptome sequencing on more than 220 invertebrate species, and through Illumina HiSeq sequencing, they identified at least 1,445 phylogenetically distinct virus genomes or genome segments that has RdRp domain, and thus largely expanded the viromes characteristics of diverse invertebrates (Shi et al., 2016). Serratus, which is a cloud computing infrastructure that enables ultra-high-throughput sequencing alignment at the petabase scale, has been utilized to search 5.7 million biologically diverse samples for RdRp domains and identified over 10⁵ novel RNA viruses (Edgar et al., 2022). Scaleable, network-based, iterative clustering approach has been developed to analyse around 28 terabases of Global Ocean RNA sequences to extend Earth's RNA virus catalogs, taxonomy and evolution origins (Zayed et al., 2022).

With all the new RNA viruses being identified, outstanding questions remain including identifying the host for virus infection, improving genome completeness, and capturing RNA virus particles from environmental samples to assess their diversity in a more targeted manner (Zayed et al., 2022), leading to new exciting research in the future.

1.4 Thesis Investigation

Recently, using high-throughput sequencing of total RNA extracted from whiteflies, Inoue-Nagata et al. identified a novel dicistrovirus member, Bemisia-associated dicistrovirus 2 (BaDV-2), that showed distinct features from other well-studied dicistroviruses (Hedil et al., 2020). The predicted IGR region contains an atypical RNA structure that lacks conserved elements and altered structures that do not conform to the typical dicistrovirus IGR IRES structure. BaDV-2 genome also contains a predicted -1 FS RNA stem-loop structure within ORF1 that would direct the translation of the RNA-dependent RNA polymerase (Hedil et al., 2020). However, whether these BaDV-2 RNA elements direct IRES and/or -1 FSS has not been investigated.

In Chapter 2, I hypothesize that BaDV-2 IGR supports IRES activity and BaDV-2 IGR IRES functionally and structurally resemble a typical dicistrovirus IRES. I use molecular, biochemical, and structural assays to validate the BaDV-2 IGR IRES and investigate the functions and structures of BaDV-2 IGR IRES. In Chapter 3, I hypothesize that BaDV-2 utilizes -1 FSS to direct the translation for RdRp motif. I use molecular assays to validate the BaDV-2 -1 FSS and investigate its secondary structure. In summary, I am hoping my work helps to expand our knowledge of the factorless translation mechanisms in diverse dicistroviruses, and thus providing a framework for future studies into novel translation mechanisms and into the evolution of IGR IRES translation in dicistrovirus field, and hopefully reveal some new translation initiation and recoding mechanisms.

Chapter 2: A novel factor-dependent internal ribosome entry site in the Bemisia-associated dicistrovirus 2

2.1 Introduction

IRESs are typically structured RNAs that recruit ribosomes using a subset of translation factors in a 5' cap-independent manner (S. K. Jang, Pestova, Hellen, Witherell, & Wimmer, 1990). The dicistrovirus IGR IRES uses the most streamlined mechanism: the IRES recruits ribosomes directly without the use of initiation factors and directs translation from a non-AUG codon (C. U. T. Hellen, 2007). The IRES adopts three overlapping pseudoknots containing key stem-loops and unpaired regions that interact with specific domains of the ribosomal 40S and 60S subunits (Fernández et al., 2014; Jan & Sarnow, 2002; Kanamori & Nakashima, 2001; Nishiyama et al., 2003; Spahn et al., 2004).

Analysis of dicistrovirus-like genomes identified via metagenomic studies predict potentially novel atypical IRES RNA structures (Abaeva et al., 2020; Boros et al., 2011; Culley et al., 2007; Shi et al., 2016). Specifically, the Bemisia-associated dicistrovirus 2 (BaDV-2) genome contains a predicted IGR IRES containing an unusual RNA structure that lack conserved elements and altered structures that do not conform to the typical dicistrovirus IRES mechanism. For instance, the L1.1 and SLIV sequence, which are generally conserved across dicistrovirus IGR IRESs, are distinct in the predicted BaDV-2 IGR secondary structure (Hedil et al., 2020). The sequence identities in L1.1 and SLIV are important because they are responsible for the interaction with ribosomes (Fernández et al., 2014). Therefore, distinct sequences might lead to an atypical translation initiation mechanism by the predicted BaDV-2 IGR IRES. However, the IRES activity of BaVD-2 IGR needs to be validated and the molecular mechanism needs to be uncovered. Therefore, I hypothesize that BaDV-2 IGR contains IRES activity, and it structurally and functionally resembles a typical dicistrovirus IGR IRES. In this chapter, I demonstrate that the BaDV-2 IGR supports IRES activity using a bicistronic reporter in *in vitro* insect *Spodoptera frugiperda* (Sf) 21 and rabbit reticulocyte (RRL) translation extracts. Using deletion and mutagenesis analyses, I demonstrate that the BaDV-2 IRES activity is within a minimal 140 nucleotide element containing a predicted stem-loop. Moreover, the IRES is sensitive to eIF2 and eIF4A inhibitors, NSC1198983 and hippuristanol, respectively, indicating that this IRES is factor-dependent which is unlike typical factorless dicistrovirus IRES mechanisms. In summary, we have provided evidence of a novel IRES mechanism in this viral family, thus highlighting the diversity of viral strategies to direct viral protein synthesis.

2.2 Material and methods

Cell Culture

Drosophila Schneider 2 cell line (S2) cells (Invitrogen) were maintained in Shields and Sang M3 insect medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and at 25 °C.

Plasmids

BaDV-2 IGR IRES gene fragment (nucleotides 5325 to nucleotides 5694, Accession No. MN231041) (Twist Biosciences) was cloned into bicistronic and monocistronic reporter plasmids using Gibson assembly. Deletion mutations and specific nucleotide mutations were generated by PCR-based methods. BaDV-2 IGR IRES with a downstream P2A peptide

38

sequence (Twist Biosciences) was cloned into the CrPV infectious clone (Kerr et al., 2015), replacing the CrPV IGR IRES.

In vitro Transcription

Monocistronic, bicistronic and infectious clones were linearized by NarI, BamHI and Eco53KI (NEB) restriction enzymes, respectively. RNA was transcribed using T7 RNA polymerase and subsequently purified with a RNeasy kit (Qiagen). 5'capping and polyadenylation were performed post-transcriptionally (CellScript). The RNA integrity and purity was confirmed by denaturing agarose gel analysis and concentration was measured by a spectrophotometer (Nanodrop).

Transfection

3 ug of bicistronic or infectious clone RNA was transfected into S2 cells (3×10^6 cells) using Lipofectamine 2000 reagent (Thermo Fisher Scientific). For monitoring reporter RNA translation under CrPV infection, S2 cells were transfected with bicistronic RNA for 1 hour, followed by infection with CrPV (MOI = 20) for 4.5 hours (Kerr et al., 2015). For infectious clone transfection experiments, S2 cells were transfected and then harvested at the indicated times and lysed in 1× passive lysis buffer (Promega). Lysates were cleared and protein concentration was measured by Bradford assay (Bio-Rad). Western blotting using an anti-VP2 antibody was performed as previously described (Kerr et al., 2015).

In vitro Translation Reactions

In vitro transcribed bicistronic (1 ug) and infectious clone RNA (2 ug) were incubated in Sf-21 cell extract (Promega) or RRL (Promega) for 2 hours or 45 minutes, respectively, in the presence of [³⁵S]-methionine/cysteine. Inhibitors (hippuristanol or NSC119889) at desired concentrations were added to the reaction 5 minutes prior to the addition of RNA. Reactions were either loaded and resolved on 15% SDS-PAGE gels and analyzed by phosphorimager analysis (Amersham Typhoon) or analyzed for enzymatic luciferase activity (Promega) using a luminometer (Turner Designs TD-20/20).

Ribosome Filter Binding Assay

40S and 60S ribosomal subunits were purified as previously described (Jan & Sarnow, 2002; X. Wang, Vlok, Flibotte, & Jan, 2021). RNA was dephosphorylated by FastAP thermosensitive alkaline phosphatase (ThermoFisher) then labelled with T4 polynucleotide kinase (NEB) and [γ^{32} P]-ATP. [γ^{32} P]-RNA (final concentration: 0.5 nM) was heated at 65°C for 3 min prior to the addition of 1× buffer E (final concentration: 20 mM Tris pH7.5, 100 mM KCl, 2.5mM MgOAc, 0.25 mM Spermidine and 2 mM DTT) and gently cooled to room temperature for 20 min, allowing for proper folding. The RNA was then incubated with purified 40S ribosomal subunits from 0.1 nM to 100 nM for 5 minutes, followed by 60S ribosomal subunits from 0.15 nM to 150 nM for 15 minutes, with 50 ng/ul *in vitro* transcribed noncompetitor RNA prepared from pcDNA3 (linearized with EcoRV) to prevent non-specific binding (Jan & Sarnow, 2002). Reactions were loaded to the Bio-Dot filtration apparatus (Bio-Rad) with nitrocellulose and nylon membranes (Zeta-Probe, Bio-Rad). The membranes were then washed three times with 1× buffer E, then dried and imaged by phosphoimager analysis (Amershan Typhoon, Image Quant). The fraction bound and the dissociation constant (K_D) were calculated as previously described (X. Wang et al., 2021).

RNA purification and Size Exclusion Chromatography with Multi-angle Light Scattering (SEC-MALS)

In vitro transcribed BaDV-2 IGR IRES RNA was purified by size exclusion chromatography(SEC) with an ÄKTA pure fast protein liquid chromatography (FPLC) (Global Life Science Solutions USA LLC) as previously described (D'Souza et al., 2022). RNA containing fractions were then pooled and purified by ethanol precipitation with resuspension in HEPES folding buffer (final concentration: 50 mM HEPES, 150 mM NaCl, 15 mM MgCl2, 3% Glycerol, and at pH 7.4) for SEC-MALS analysis and small-angle X-ray scattering (SAXS) submission. SEC-MALS analysis was performed as previously described with Optilab Refractive Index System (Wyatt Technology) (D'Souza et al., 2022).

Small-angle X-Ray Scattering (SAXS)

SAXS was performed as previously described in (D'Souza et al., 2022). Briefly, SAXS data for BaDV-2 minimal IRES RNA were collected at 2mg/ml. Samples were run at Diamond Light Source Ltd. synchrotron (Didcot, Oxfordshire, UK) on the B21 SAXS beamline, with a high-performance liquid chromatography (HPLC) system attached upstream to ensure the monodispersity (Cowieson et al., 2020). A specialized flow cell was employed in conjunction with an inline Agilent 1200 HPLC system (Agilent Technologies, Stockport, UK). RNA samples were injected onto a Shodex KW403-4F (Showa Denko America Inc., New York, NY, USA) size exclusion column, with a flow rate maintained at 0.160mL/minute. The eluted samples are

exposed to X-rays with 3 second exposure time and 600 frames. Analysis of scattering data was performed using the ATSAS suite and as previously described (Badmalia, Sette Pereira, Siddiqui, & Patel, 2022; D'Souza et al., 2022; Manalastas-Cantos et al., 2021). RNA models were generated using DAMMIN (Badmalia et al., 2022; D'Souza et al., 2022; Svergun, 1999).

2.3 Results

2.3.1. BaDV-2 IGR supports internal ribosome entry

We investigated whether the BaDV-2 IGR supports IRES activity. Our initial analysis predicted an atypical RNA structure that had features (i.e. PK) like known dicistrovirus IRES structures (Hedil et al., 2020). We cloned the IGR of BaDV-2 (nucleotide 5325 to 5694) into the IGR of a RLuc-FLuc bicistronic reporter construct (**Figure 2.1A**). The predicted start site based on the proposed IRES structure starts at a GCG codon (nucleotide 5668 to 5670). We incubated *in vitro* transcribed RNAs into Sf-21 extracts and monitored scanning-dependent RLuc translation and IRES-mediated FLuc translation. As shown previously, the CrPV IGR IRES directed FLuc expression and activity, whereas a mutant CrPV IGR IRES that disrupts PKI base pairing (CC₆₂₁₄₋₅ to GG mutant) abolished IRES activity (**Figure 2.1A**). We calculated IRES activity as a ratio of FLuc/RLuc, where RLuc serves as a normalization control across experiments. The BaDV-2 IGR IRES (**Figure 2.1A**).

Furthermore, we added a hairpin loop right before RLuc to abolish RLuc translation and investigated if FLuc translation was coupled to RLuc translation (**Figure 2.1B**). Without the hairpin loop, RLuc and FLuc were translated (**Figure 2.1B**, **black bars**), whereas with the hairpin loop, there was only FLuc translation but not RLuc translation (**Figure 2.1B**, **red bars**).

This suggested that FLuc translation was not coupled to RLuc translation therefore further validating that BaDV-2 IGR has IRES activity.



Figure 2.1. BaDV-2 IGR and its IRES activity. A) Bicistronic reporter RNAs containing either the CrPV IGR or the BaDV-2 IGR (top schematic) were incubated in Sf-21 extracts. The FLuc and RLuc activities were measured and the FLuc/RLuc ratio was calculated and normalized to the wild-type CrPV bicistronic RNA as shown in the bar graph. An ANOVA statistical test was used to determine the p value and thus the significance levels. B) A bicistronic RNA containing an upstream hairpin to block scanning dependent translation was incubated in Sf-21 extracts. Translation of FLuc and RLuc was measured by either (left) radioisotope [³⁵S]- methionine/cysteine incorporation followed by SDS-PAGE and phosphorimager analysis or (right) luciferase assays. A paired t-test was used to determine the p value and thus the significance levels. * represents p<0.05, ** represents p<0.01. Shown are a representative SDS PAGE gel and the averages from at least three independent experiments ± standard deviation.

2.3.2 Minimal BaDV-2 IGR that directs IRES activity

The predicted BaDV-2 IRES structure adopts several stem-loop and pseudoknot structures (Hedil et al., 2020). A series of mutations were generated that disrupt predicted base pairing (Figure 2.2A). In summary, the majority of mutants did not affect BaDV-2 IRES activity (Figure 2.2 B), suggesting there is an alternative RNA structure and possibly mechanism for translation initiation.



Figure 2.2 Predicted secondary model of BaDV-2 IGR IRES. A) Specific nucleotides were mutated to validate the predicted secondary structure of BaDV-2 IGR IRES. B) The luciferase activities were measured through luciferase assays for each mutation via bicistronic reporter RNA assays in Sf-21 extracts. An ANOVA statistical test was used to determine the p value and thus the significance levels. Groups were compared to WT BaDV. * represents p<0.05, ** represents p<0.01. Shown are the averages from at least three independent experiments \pm standard deviation.

We next generated a series of 5' and 3' deletions mutations within the IGR region to pinpoint the minimal sequence for BaDV-2 IRES translation. As a reminder, nucleotides 5325 to 5694 of BaDV-2 was initially cloned and showed IRES activity (**Figure 2.1**). Compared to the wild-type BaDV-2 IRES, 5' deletions from nucleotide (nt) 5325-5366 (5'Del1) still displayed IRES activity, however, deleting nt 5325-5421 (5'Del2) inhibited IRES activity (**Figure 2.3A**), thus setting the boundaries from the 5'end of the BaDV-2 IRES. Using a similar approach from the 3'end, the majority of 3' deletions from 5506-5694 (3'Del1-5) resulted in IRES activity as similar to or higher as wild-type IRES activity (**Figure 2.3B**). The only exception was 3'DelΔ3, which showed 60% of wild-type IRES activity (**Figure 2.3B**). To further pinpoint the minimal IRES, we generated combinations of 5' and 3' deletions. Combining 5'Del 1 and 3'Del 4 or 3'Del 5 still resulted in IRES activity, whereas combining 5'Del 2 and 3'Del 4 inhibited IRES activity to ~40% of wild-type IRES activity (**Figure 2.3B**). Thus, our deletion analysis showed that the minimal BaDV-2 IRES is contained within a 140 nucleotide region from nt 5366-5506.



Figure 2.3 Deletion analysis of BaDV-2 IGR IRES. A) 5' and 3' deletions were generated within the BaDV-2 IGR. " \blacktriangle " = del = deletion B) Bicistronic RNAs containing the corresponding deletion mutants were incubated in Sf-21 extracts and RLuc and FLuc activities measured and normalized to the wild-type BaDV-2 IRES. An ANOVA statistical test was used to determine the p value and thus the significance levels. Groups were compared to WT BaDV. * represents p<0.05, ** represents p<0.01. Shown are the averages from at least three independent experiments \pm standard deviation.

2.3.3 BaDV-2 IGR IRES initiates translation from an AUG start codon

We next determined the start site of the BaDV-2 IGR IRES. In-frame stop codons (SC) were inserted across the full-length BaDV-2 IGR. Insertion of stop codons at nt 5503, 5608 and 5668 (SC1-3) all decreased IRES activity to ~5-25% of wild-type IRES activity (Figure 2.4), thus suggesting that the start site is upstream of nt 5503. Moreover, these experiments are in line with the deletion analysis that the minimal IRES is contained between nt 5366-5506. A stop codon insertion at nt 5444 (SC4) did not affect IRES activity, thus indicating that the start codon is between nts 5444 and 5503 (Figure 2.4). We hypothesized that an AUG (nt 5491-3) codon may be the initiation codon of the IRES. We mutated the AUG (nt 5491-3) codon to UGA (M1) or GCG (M2) or deleted the AUG (Del). Deletion of the AUG codon or mutating to UGA (M1) dramatically inhibited IRES translation by ~75-80% compared to wild-type IRES activity (Figure 5B). We reasoned that the IRES may recruit ribosomes and then scan downstream to initiate translation from the AUG codon of FLuc. To address this, we deleted the AUG codon of FLuc in combination with the deletion of and mutant AUG (nt 5491-3). Deleting the AUG codon of FLuc within the wild-type BaDV-2 IGR IRES inhibited IRES activity by ~10-15% as compared to the wild-type IRES with the AUG codon of FLuc. By contrast, combining the deleted AUG codon of FLuc with deletion of AUG (nt 5491-3) or M1 or M2 mutants abolished IRES-mediated FLuc activity completely. These results strongly demonstrated that BaDV-2 IRES initiates from the AUG (nt 5491-3) codon and that a fraction of 40S subunits of ribosomes recruited to the IRES can scan to initiate translation from a downstream AUG codon.



Figure 2.4 Stop codon mutations to confirm the BaDV-2 IGR IRES initiation site. A) Schematic of stop codon mutations along the BaDV-2 IGR IRES (upper). *In vitro* translation assays were performed in Sf-21 cell lysate with the indicated mutations using bicistronic reporter RNAs. RLuc and FLuc activities were measured and normalized to the wild-type BaDV-2 bicistronic RNA (bottom). An ANOVA statistical test was used to determine the p value and thus the significance levels. Groups were compared to WT BaDV. * represents p<0.05, ** represents p<0.01. Shown are the averages from at least three independent experiments \pm standard deviation.

2.3.4 BaDV-2 IGR IRES does not bind to purified 80S ribosome

A unique feature of dicistrovirus IGR IRESs is its ability to recruit ribosomes directly without the aid of initiation factors. The CrPV IGR IRES can assemble ribosomes by first recruiting 40S subunits followed by 60S subunit joining (Jan, Kinzy, & Sarnow, 2003), however, at ~8% of the time, the CrPV IRES can also bind to pre-formed 80S ribosomes (Petrov, Grosely, Chen, O'Leary, & Puglisi, 2016). We accessed the ribosomal binding using an established filter binding assay (H. H. T. Au, Elspass, & Jan, 2018). Incubating increasing concentration of purified salt-washed ribosomes with wild-type CrPV IRES resulted in increasing fraction of CrPV IRES: ribosome complexes with a K_D of 1.4 ± 0.3 nM (Figure 2.5A), which is similar to previous reports (H. H. T. Au et al., 2018; X. Wang et al., 2021). By contrast, a mutant CrPV IRES containing disruptions that disrupt all three pseudoknot structures did not show appreciable 80S binding (Figure 2.5A, K_D = 321.0 ± 523.0 nM) (H. H. T. Au et al., 2018). Similarly, the BaDV-2 IRES did not bind to purified ribosomes (Figure 2.5A, K_D = 940.3 ± 4377 nM), thus indicating that this IRES is likely not using a factorless mechanism for translation.



Figure 2.5 Affinity of 80S-BaDV-2 IGR IRES complexes.

(A) Filter binding assays. [32 P]-BaDV-2 IGR IRES, [32 P]-Cricket Paralysis Virus (CrPV) IGR IRES or mutant (Δ PKI/II/III) CrPV IGR IRES were incubated with increasing amounts of purified salt-washed 80S. The fractions bound were quantified by filter binding assay followed by phosphorimager-analysis. CrPV WT has a K_D value of 1.4 nM, CrPV MT has a K_D value of 321.0 nM and WT BaDV-2 has a K_D value of 940.3 nM. Shown are the averages from at least three independent experiments \pm standard deviation.

2.3.5 BaDV-2 IGR IRES requires eIF4A and eIF2 to recruit ribosomes

Our results so far point to an alternative IRES mechanism that drives BaDV-2 IGR IRES activity. To determine whether the BaDV-2 IRES uses translation factors, we used inhibitors that target specific translation initiation factors (**Figure 2.6 A**). Specifically, hippuristanol inhibits eIF4A helicase activity and NSC119889 blocks eIF2 from binding to initiator Met-tRNAⁱ (Cencic & Pelletier, 2016; Novac, Guenier, & Pelletier, 2004; Robert et al., 2006). We pre-incubated these inhibitors in RRL or Sf-21 followed by addition of IRES-containing reporter

RNAs. Incubating with hippuristanol (10 uM and 15 uM) or NSC119889 (12.5 uM) significantly decreased scanning dependent RLuc translation, as expected (**Figure 2.6 B, C**). By contrast, CrPV IRES-mediated FLuc translation was relatively resistant to hippuristanol or NSC119889, in line that the CrPV IRES drives factorless translation (**Figure 2.6 B, C**) (C. J. Jang & Jan, 2010). Interestingly, BaDV-2 IRES translation was sensitive to both drug treatments, thus demonstrating that eIF4A and eIF2 ternary complexes are required for BaDV-2 IRES translation.



Figure 2.6 Inhibitor treatment to test the requirement of initiation factors. A) Schematic illustration of bicistronic reporter RNAs with CrPV or BaDV-2 IGR IRESs. *In vitro* transcribed reporter RNAs were incubated in RRL with B) hippuristanol or C) in Sf21 extracts with NSC119889 at the indicated concentrations. RLuc and FLuc activities were measured and normalized to DMSO-treated lysates with wild-type CrPV or BaDV-2 IGR IRES bicistronic RNAs. An ANOVA statistical test was used to determine the p value and thus the significance
levels. * represents p<0.05, ** represents p<0.01. Shown are the averages from at least three independent experiments \pm standard deviation.

2.3.6 BaDV-2 minimal IRES contains predicted stem loops

Small angle X-Ray scattering (SAXS) is a method to resolve the size and shape of monodispersed macromolecules (Stetefeld, McKenna, & Patel, 2016). A prerequisite for SAXS is a pure, monodisperse sample that displays similar hydrodynamic properties (Dzananovic et al., 2014). We firstly purified BaDV-2 minimal IRES RNA made from T7 promoter by FPLC Superdex 200 (S200). The elution profile of the RNAs is as shown in **Figure 2.7A** for pure monomer BaDV-2 minimal IRES RNA collection. Combined RNA went straight to SEC-MALS for further purity and molecular weight analysis. SEC-MALS confirmed BaDV-2 IGR IRES had a molecular weight of 47.53 (± 1.675%) kDa (**Figure 2.7B**), consistent with the predicted molecular weight (49.22kDa). Taken together, the results suggest BaDV-2 minimal IRES RNA sample is appropriate for sending for SAXS analysis. After obtaining SAXS data, DAMMIN was performed to obtain low-resolution structures for BaDV-2 minimal IRES RNA (D'Souza et al., 2022). Here, we showed a representative model in **Figure 2.7C** and its corresponding secondary structure model in **Figure 2.7D**. Mutations were made at the stem loop areas and results showed mutations at the UA-rich region was important for IRES activity (**Figure 2.7D**, **M4**).



Figure 2.7 Structural analysis of BaDV-2 IGR IRES. A) FPLC profiles associated with BaDV-2 IGR minimal IRES IVT RNA purification. B) SEC-MALS traces of the peak from the BaDV-2 minimal IRES RNA run and the absolute molecular weight across them. C) Predicted

BaDV-2 minimal IRES RNA structure model from SAXS data. D) The secondary structural model of BaDV-2 minimal IRES corresponding to the predicted model as shown in C). Mutation analysis of BaDV-2 minimal IRES with bicistronic reporter Sf-21 translation assays were performed and the results (M1-7) were measured by luciferase assays and normalized to wild-type (WT) BaDV-2 IGR IRES minimal IRES (bottom). Mutations marked in red (M8-9) are interesting mutants that can be tested in the future. Groups were compared to WT BaDV. An ANOVA statistical test was used to determine the p value and thus the significance levels. * represents p<0.05, ** represents p<0.01. Shown are the averages from at least three independent experiments \pm standard deviation.

2.3.7 BaDV-2 IGR does not support IRES activity in S2 cells

Next, we wanted to test how well the BaDV-2 IGR IRES functions in mock- and CrPVinfected S2 cells. We tested in both mock and infected cells as it has been shown that IRES translation is stimulated under virus infection (Kerr, Ma, Jang, Thompson, & Jan, 2016). Firstly, we transfected *in vitro* transcribed RNA (WT and MT CrPV IRES, WT, 3 del 5 and minimal BaDV-2 IRES) into S2 cells, infect with CrPV virus at MOI = 20 after 1 hour for the CrPVinfected groups, and measured RLuc and FLuc activity at 6 hours after transfection. Translation of transfected reporter RNAs in S2 cells are active at this time after transfection. The results showed that only wild-type CrPV but not BaDV-2 IGR (including WT, 3 del 5 and minimal IRES) was able to support FLuc translation in mock and CrPV-infected cell conditions (**Figure 2.8A**).

Next, we determined whether the BaDV-2 IRES could support viral translation in an infectious clone model. We previously showed that the dicistrovirus IAPV IRES and an ancient Northwest Territories Cripavirus IRES can support virus translation and in infection using a heterologous chimeric CrPV infectious clone (H. H. T. Au et al., 2018; X. Wang et al., 2021). Briefly, we replaced the CrPV IGR IRES with the BaDV-2 IGR IRES (full length, nt 5325 to 5694) in the CrPV infectious clone that was previously made in our lab (Figure 2.8B) (Kerr et al., 2015; Warsaba et al., 2020). We also generated a clone with an inserted P2A "stop-go" peptide site between BaDV-2 IGR IRES and ORF2 to ensure proper expression of CrPV ORF2 (Q. S. Wang, Au, & Jan, 2013). We first determined whether the BaDV-2 IRES could support translation of the infectious clone in Sf-21 extracts. Our results showed that the CrPV but not BaDV-2 IGR IRES supported translation of ORF2 proteins (Figure 2.8C). To determine whether the CrPV-BaDV-2 chimeric clone is infectious, we transfected in vitro transcribed infectious clone RNAs into S2 cells and then followed VP2 expression by immunoblotting at 144 hours after transfection. We previously showed that detection of VP2 after transfection of CrPV infectious clone RNA reflects productive CrPV infection in S2 cells (Kerr et al., 2015; Warsaba et al., 2020). VP2 was detected in S2 cells transfected with wild-type CrPV clone but not a mutant CrPV clone containing a stop codon in ORF1 or with the CrPV-BaDV-2 IGR IRES chimeric clone (both with and without P2A peptide site) (Figure 2.8D). Moreover, cytopathic effects were observed in S2 cells transfected with the wild-type CrPV clone but not with the other clones (data not shown). In summary, the BaDV-2 IRES is not active in the infectious clone and does not support CrPV infection using a chimeric infectious clone.



Figure 2.8 BaDV-2 IGR IRES activity in infectious clone and in cells. A) Bicistronic reporters were transfected into S2 cells followed by mock infection or CrPV infection (MOI = 20). Cells were collected 6 hour post transfection. Bicistronic RNAs tested contain the wild-type CrPV, mutant CrPV (CC₆₂₁₄₋₅ to GG at PKI, previously shown abolished CrPV IGR IRES activities, (X. Wang et al., 2021)), wild-type BaDV-2 full-length IGR IRES (WT BaDV-2), BaDV-2 3' del 5 (as shown in **figure 2.3**) and BaDV minimal IRES (double deletion at both 3' and 5' end, as shown in **figure 2.3**). Luciferase activities were measured and normalized to

wild-type CrPV with mock infection. A paired t-test was used to determine the p value and thus the significance levels. * represents p<0.05, ** represents p<0.01. Shown are the averages from at least three independent experiments \pm standard deviation. B) Schematic of infectious clone CrPV (pCrPV, CrPV_IGR_IRES) and chimeric clones with CrPV IGR IRES replaced with BaDV-2 IGR IRES (BaDV-2 IGR IRES) and with P2A-site added after BaDV-2 IGR IRES (BaDV-2 IGR IRES+P2A). C) Infectious clone RNAs were incubated in Sf-21 extracts containing [³⁵S]-methionine/cysteine and then monitored by SDS-PAGE analysis. Mock = mock transfection; OS = ORF1Stop; CrPV = pCrPV; BaDV= BaDV-2 IGR IRES; P2A= BaDV-2 IGR IRES+ P2A. Shown is a representative gel from at least three independent experiments. D) *In vitro* transcribed infectious clone RNAs were transfected into S2 cells for 144 hours. VP2 expression was detected by immunoblotting. Shown are a representative SDS PAGE gel and the averages from at least three independent experiments \pm standard deviation.

2.4 Discussion

Dicistroviruses IGR IRESs utilizes the most streamlined eukaryotic translation mechanism to date. It is presumed that translation mechanisms within a viral family would have conserved viral translation strategies. In this chapter, I demonstrated that the IGR of the dicistrovirus BaDV-2 contains IRES activity. Moreover, unlike typical dicistrovirus IRESs, the BaDV-2 IRES cannot bind to ribosomes directly and requires translation factors, eIF2 and eIF4A, for IRES activity. Finally, the BaDV-2 minimal IRES within a 140 nucleotides region is sufficient to direct translation from an AUG codon. Therefore, I propose that the BaDV-2 IRES represents a novel IRES mechanism within the *Dicistroviridae* family.

Although we did not observe BaDV-2 IRES activity in S2 cells, possibly because there is a cell type specificity that allows for it to function, further experiments are needed to draw definite conclusions. Infectious clones are a great tool to investigate the viral life cycle and pathogenesis (Kerr et al., 2015). Transfection S2 cells with infectious clone RNA helps to uncover the fundamental host-viral interactions in insect cells (Kerr et al., 2015). Unfortunately, an infectious clone for BaDV-2 is not available. Instead, we used bicistronic reporters and chimeric clones to investigate BaDV-2 IGR IRES mechanisms, which are also considered as handy and powerful tools for IRES studies (Q. S. Wang et al., 2013). Chimeric infectious clones should enable CrPV ORF2 translation when transfecting S2 cells. However, we were unable to detect VP2 expression in both chimeric clones, CrPV-BaDV-2 IGR IRES and CrPV-BaDV-2 IGR IRES +P2A. A possible explanation is that BaDV-2 IGR IRES-dependent translation requires eIF4A and eIF2 ternary complexes. Under viral infection, $eIF2\alpha$ kinases are activated and phosphorylate eIF2 α , which prevents eIF2-GDP from exchanging to GTP (H. H. T. Au & Jan, 2014; Garrey et al., 2010; A G Hinnebusch, 2000). The lack of activated eIF2 ternary complex will disable BaDV-2 IGR IRES from recruiting ribosomes and initiating ORF2 translation, leading to lack of VP2 expression.

In summary, in chapter 2 I concluded that the BaDV-2 IRES contains a novel IRES mechanism that is distinct from a typical dicistrovirus IGR IRES. Knowing that BaDV-2 IGR IRES recruit ribosomes in an eIF4A- and eIF2- dependent manner to initiate translation at an AUG codon, we are interested to investigate further about the details in its translation mechanism. Other molecular assays could be further performed to uncover this mechanism in the future. For example, toeprinting can validate the BaDV-2 ORF2 initiation site (Gu, Mao, Jia, Dong, & Qian, 2021). Mutations at the specific regions in minimal IRES could be performed to validate the predicted secondary structure of BaDV-2 minimal IRES. Chemical modification combined with sequencing is a great tool to validate the BaDV-2 IGR IRES and minimal IRES structures, assisting with our SAXS data. For example, selective 2' hydroxyl acylation analyzed by primer extension (SHAPE) utilizes hydroxyl-selective electrophiles such as NMIA to react with the 2'hydroxyl group in flexible nucleotides to form a stable 2'-O-ester adduct, which is later used to identify exposed nucleotides through primer extension (Chahal et al., 2019; Wilkinson, Merino, & Weeks, 2006). Dimethyl sulfate (DMS) mutational profiling with sequencing (DMS-MaSeq) uses DMS modification within the Watson-Crick face to investigate RNA structures in vivo (Zubradt et al., 2017). Furthermore, reconstitution assays that add back different initiation factors together can be used to systematically validate eIF2 and eIF4A and other initiation factor requirements for BaDV-2 IRES to function (Lancaster et al., 2006).

In conclusion, our work in BaDV-2 IGR IRES provides a systematic workflow to investigate viral IRESs and expands the current knowledge on the mechanism that dicistrovirus

IGR IRESs use to recruit ribosomes and drive translation initiation. It will be of great interest to investigate this novel IRES further, both functionally and structurally.

Chapter 3: A novel programmed -1 frameshifting signal in the Bemisiaassociated dicistrovirus 2

3.1 Introduction

As described previously in chapter 1, -1 PRF is a common strategy for viral recording to expand the viral genome coding capacity. -1 PRF directs elongating ribosome to shift one nucleotide towards the 5' direction of the mRNA and is a critical process in coronavirus and retrovirus life cycles. Generally , a -1 PRF consists of a heptameric "slippery site", a 3' stimulatory element and the "spacer" sequence between the slippery sequence and the stimulatory element (Brierley et al., 1989; ten Dam et al., 1990). The stimulatory element has been proposed to hinder elongating ribosomes such that ribosomes decoding over the slippery sequence shifts in the -1 frame, as previously described in chapter 1.2.1.

BaDV-2 was isolated from the sweet potato whitefly *Bemisia tabaci* (Hedil et al., 2020). The 8012 nt genomic sequence of BaDV-2 contains (GeneBank accession No. MN231041) three ORFs (ORF1a, ORF1b, and ORF2) with a 5'UTR, an intergenic region (IGR), a 3'UTR and poly(A) tail (Hedil et al., 2020). ORF1a contains an RNA helicase (nt 1780-2121) and a 3C cysteine protease motif (nt 3541-3654) (Hedil et al., 2020). ORF1b is in -1 frame and contains an RdRp motif (nt 4047-5261) (Hedil et al., 2020). ORF2 contains two picornavirus capsid proteinlike Rhv motifs (nt 5893 to 6177 and 6730 - 7002, respectively), a calicivirus coat-protein motif (nt 7021 to 7209) and a CrPV-capsid protein-like motif (nt 7336 to 7977) (Hedil et al., 2020). A putative -1 frameshifting signal (-1 FSS) was proposed to drive the translation of the full-length ORF1 polyprotein, consisting of a slippery sequence (GUCUUUU, nt 3780-6) and a putative pseudoknot (Hedil et al., 2020). The -1 FSS is required to direct translation of the RdRP replicase motif. In this chapter, I hypothesize that BaDV-2 utilizes a -1 FS strategy to express RdRP domain of the ORF1 polyprotein. Overall, I validated that the putative BaDV-2 -1 FSS is a bona fide FSS. Mutational and deletion analyses revealed that a stem-loop structure and slippery sequence are essential for FS activity. This is the first report of a bona fide -1 FSS in the *Dicistroviridae* family.

3.2 Material and methods

Plasmids

The BaDV-2 predicted frameshifting signal fragment (nucleotides 3742 to 3884, Accession No. MN231041) (Twist Biosciences) was cloned into bicistronic reporter plasmids using Gibson assembly. Deletion mutations and specific nucleotide mutations were generated by PCR-based mutagenesis methods.

In vitro Transcription

Bicistronic plasmids were linearized using BamHI (NEB) restriction enzyme. RNA was transcribed using T7 RNA polymerase and subsequently purified with a RNeasy kit (Qiagen). The RNA integrity and purity was confirmed by denaturing agarose gel analysis and concentration was measured by a spectrophotometer (Nanodrop).

In vitro Translation Reactions

In vitro transcribed bicistronic (1 ug) was incubated in *Sf-21* cell extract (Promega) or rabbit reticulocyte lysate (Promega) for 2 hours or 45 minutes, respectively, in the presence of [³⁵S]-methionine/cysteine. Reactions were either loaded and resolved on 15% SDS-PAGE gels and analyzed by phosphorimager (Amersham Typhoon) or analyzed for enzymatic luciferase activity (Promega) using a luminometer (Turner Designs TD-20/20).

3.3 Results

3.3.1 BaDV-2 IGR supports a -1 PRF

A predicted pseudoknot structure with an upstream of a putative slippery sequence as characteristics of a -1 programmed frameshift signal within the BaDV-2 genome was proposed (Brierley et al., 1989; Hedil et al., 2020) (Figure 3.1A). To determine whether this element can support FS, we cloned the putative -1 FS element (nucleotides 3742 to 3884, Accession No. MN231041) into a bicistronic reporter between the Renilla (RLuc, 0 frame) and Firefly luciferase (FLuc, -1 frame) open reading frames (Figure 3.1B). We cloned sequences upstream and downstream of the predicted -1 FSS to ensure the entire element is present. We also generated an in-frame control reporter whereby the RLuc and FLuc ORFs are in the same frame and a mutation (UUU₃₇₈₃₋₈₅ to CCC) (SS MUT) that disrupts the putative slippery sequence (Figure 3.1B).



Figure 3.1. The predicted secondary structure of BaDV-2 -1 FSS. A) Simplified schematic of BaDV-2 genome. B) The predicted secondary structure of BaDV-2 -1 frameshifting signal, and schematic of the bicistronic reporter with RLuc and FLuc.

We incubated *in vitro* transcribed bicistronic reporter RNAs in Sf-21 insect cell lysate (Sf-21) and rabbit reticulocyte lysate (RRL) and monitored luciferase activities and expression by incorporation of [³⁵S]-methionine/cysteine (**Figure 3.2A, 3.2B**). FLuc activity was detected suggesting that -1 frameshifting was occurring. Therefore, a fusion RLuc-FLuc protein product (~100 kDa) was detected supporting that -1 frameshifting occurred (**Figure 3.2A, 3.2B**, **lane 2**). An RLuc protein was also detected indicating that a fraction of translation ribosomes do not

undergo frameshifting (Figure 3.2A, 3.2B, lane 2). As expected, the in-frame control reporter RNA resulted in robust expression of the 100 kDa RLuc-FLuc fusion protein and loss of RLuc expression (Figure 3.2A, 3.2B, lane 3). Based on the relative luciferase activities, the -1 FSS activity occurred at 4 and 8% efficiency in Sf-21 and RRL, respectively. Mutant bicistronic RNAs containing either a premature stop codon at the end of RLuc (Figure 3.2A, 3.2B, lane 1) or the slippery sequence mutant (SS Mut, UUU₃₇₈₃₋₅CCC) (Figure 3.2A, 3.2B, lane 4) abolished FLuc translation and activity, thus supporting the presence of a -1 FSS. These results demonstrated that the BaDV-2 genome contains a bona fide -1 FSS and the fact that it can be detected in both Sf-21 and RRL suggests this activity is species-independent.



Figure 3.2 *In vitro* **translation assays in Sf-21 cell extracts and RRL.** Bicistronic reporter RNAs containing wildtype or mutant BaDV-2 -1 FSS, and in-frame controls were incubated in A) Sf-21 or B) RRL extracts. "RLuc Stop" contians a premature stop codon at the end of RLuc; "RLuc_FS_-1FLuc" contains wild-type -1 FSS with FLuc in -1 frame; "SS Mut" contains a mutant slippery sequence (UUU₃₇₈₃₋₅CCC); "0FLuc" is the in-frame control where RLuc and FLuc are both in the 0-reading frame. (left) Reactions were resolved on SDS-PAGE gel and monitored by phosphor-imager analysis. Shown are a representative SDS PAGE gel. (right) Luciferase activities were measured and calculated as a ratio of FLuc/RLuc and normalized to the in-frame control (0FLuc). Groups were compared to 0FLuc. An ANOVA statistical test was

used to determine the p value and thus the significance levels. * represents p<0.05, ** represents p<0.01. Shown are averages from at least three independent experiments \pm standard deviation.

3.3.2 Characterization of the BaDV-2 -1FSS

The proposed -1 FSS of BaDV-2 contains a putative pseudoknot structure seven nucleotides downstream of the slippery sequence. To determine whether other elements are important, systematic 3' end deletions were created (Figure 3.3A). Progressive 3' deletions significantly inhibited -1 FSS activity. Of note, the largest deletion (3'del 3), which includes the putative pseudoknot base-pairing inhibited -1 FSS activity the most (Figure 3.3B). These results suggested that the elements downstream of the putative -1 FSS element may promote frameshifting.



Figure 3.3. Deletion analysis of BaDV-2 -1 FSS. A) Schematic of deletion mutants surrounding the putative BaDV-2 -1 FSS. B) *In vitro* transcribed RNAs were incubated in RRL and luciferase activities were measured and normalized to wild type BaDV-2 -1 FSS (BaDV-2 FS WT). The red dot line represents the background characterized by a mutation in the slippery site (SS MUT, UUU₃₇₈₃₋₅CCC) that abolishes -1 FS activity. Groups were compared to BaDV-2 FS WT. An ANOVA statistical test was used to determine the p value and thus the significance levels. * represents p<0.05, ** represents p<0.01. Shown are averages from at least three independent experiments ± standard deviation.

To determine whether the predicted stem-loop and pseudoknot are important for -1 FSS, we generated mutations that would disrupt base pairing and tested-1 FSS activity in RRL (Figure 3.4A). Mutations that disrupt the bottom base paired domain of the stem-loop (M1, M2) abolished -1 FSS activity to the same extent as the SS MUT, however, compensatory mutations (M1+M2) that restored base pairing did not rescue activity, suggesting that the sequence identities may be important (Figure 3.4B). Mutations that disrupt the upper base-paired region (M4) inhibited -1 FSS activity, however, the corresponding disrupted base-paired region (M5) had only a minor effect (~84% of the wild type) (Figure 3.4B). Compensatory mutations (M4+M5) rescued -1 FSS activity to wild-type levels, suggesting that nucleotide identity of ACC (nt 3804 to 3806) may be important (Figure 3.4B). Note, a potential pseudoknot was proposed, however, mutant (M5), which would disrupt the pseudoknot base pairing, had only a minor effect on -1 FSS activity, thus indicating that the putative pseudoknot may not be critical for frameshifting (Figure 3.4B). Mutations that disrupt the apical loop (M3) did not affect -1 FSS activity (Figure 3.4B). In summary, we have demonstrated that the BaDV-2 genome contains a bona fide -1 FSS signal and identified several key nucleotides and a stem-loop important for activity.



Figure 3.4 Mutation analysis of BaDV-2 -1FSS. A) Schematic of BaDV-2 -1 FSS mutations. The mutation marked in red (M6) represent an interesting mutation can be tested in the future. B) *In vitro* transcribed RNAs were incubated in RRL, and luciferase activities were measured and normalized to wild type BaDV-2 -1 FSS (BaDV-2 WT). The red dot line represents the background characterized by a mutation in the slippery site (SS MUT, UUU₃₇₈₃₋₅CCC) that abolishes -1 FSS activity. Groups were compared to BaDV-2 WT. An ANOVA statistical test was used to determine the p value and thus the significance levels. * represents p<0.01. Shown are averages from at least three independent experiments \pm standard deviation.

3.4 Discussion

A -1 FSS has not been identified or biochemically validated in dicistrovirus (Hedil et al., 2020). In this chapter, I validated for the first time that the BaDV-2 -1 FSS can support -1 frameshifting *in vitro*. Using bicistronic reporters, I found that the frameshifting efficiency of BaDV-2 -1FSS is 4% and 8% in Sf-21 and RRL, respectively. I delineated that the -1 FSS is within nucleotides 3742 - 3884. Deletion analysis demonstrated the region downstream of the stem-loop may promote frameshifting. Moreover, mutagenesis analysis demonstrated that the slippery sequence and some sequence identities in the stem-loop are important for -1 FSS, however, the predicted pseudoknot may not be important.

The current approach that we used in this study has some limitations. Firstly, as mentioned previously in chapter 2, we lack the optimal vector, BaDV-2 infectious clone, to test the BaDV-2 -1 FSS. An infectious clone can allow us to investigate the significance of this frameshifting mechanism under BaDV-2 infection and may provide a framework to identify frameshifting elements key to BaDV-2 infection. Moreover, BaDV-2 -1 FSS may be more active in a more physiologically relevant cell line, such as *Bemisia*, however, there are not available cell lines to date. Thirdly, the real structure of the BaDV-2 -1 FSS requires further investigation. Deletion and mutagenesis analyses identified that the slippery sequence, the structure of the stem-loop, some sequence identities in the stem-loop and a downstream region (nt 3827-3842) are key to BaDV-2 FSS. However, it is still possible that an alternative secondary structure is responsible for this -1 FSS, considering that viral frameshifting such as the SARS-CoV-2 -1 frameshifting can undergo distinct conformations (Huston et al., 2021; Lan et al., 2022). RNA secondary structure prediction, such as using mFold Web Server (Zuker, 2003), combined with

molecular assays, such as mutagenesis analysis, can be used to further explore the possible secondary structures.

The investigation on -1 PRF has potential in developing antiviral drugs as -1 FSS is essential for the viral life cycle and thus is an effective target for antiviral strategies (Hilimire et al., 2017). For example, studies showed that triazole-containing compounds are able to bind the HIV FSS with high affinity and selectivity and can strongly inhibit HIV replication (Hilimire et al., 2017). The explosive expansion of viral genome discovery has resulted in opportunities to search for novel viral mechanisms such as IRESs and PRFs. Using bioinformatics analysis, our lab has recently identified more possible -1 FSS and other novel features in several dicistroviruses (data not shown). The validation of BaDV-2 -1 FSS provides a solid foundation for other potential -1 FSS in dicistroviruses to be investigated.

Chapter 4: Summary and future directions

Insects like whitefly Bemisia tabaci causes significant agricultural and economical loss in crops worldwide, including tomato, pepper and soybean (Hedil et al., 2020; Navas-Castillo, Fiallo-Olivé, & Sánchez-Campos, 2011). Chemical control is challenging because whiteflies can rapidly develop resistance to insecticides (Palumbo, Horowitz, & Prabhaker, 2001). Biological control includes the use of predators, fungi, and parasitoids, however, none of them are quite effective so far (Horowitz, Antignus, & Gerling, 2011). Therefore, entomopathogenic viruses are considered to be the new potential to control these insects (ACHOR, 2001; Nakasu et al., 2017, 2019; Hedil et al., 2020). Previously known viruses transmitted by whiteflies include begomoviruses, criniviruses, ipomoviruses (Navas-Castillo et al., 2011). Recently, BaDV-2 was identified from whitefly Bemisia tabaci isolates and classified as dicistrovirus (Hedil et al., 2020). Dicistroviruses are single-stranded positive sense RNA viruses that mainly infect arthropods, and are great models to study virus-host interactions, such as viral translational control mechanisms and insect innate immune pathways (Warsaba et al., 2020). As the name implies, dicistrovirus genome contains two ORFs: ORF1 and ORF2, where translation of each is directed by 5' UTR IRES and IGR IRES, respectively. It was assumed that the translational mechanism utilized by viruses within a viral family would be conserved and similar. However, this may not be true given that the 5' UTR IRESs of dicistroviruses have distinct mechanisms, at least the ones that have been studied (L. Gross et al., 2017; Roberts & Groppelli, 2009). For the IGR IRES of dicistroviruses, the IRESs studied so far are structurally similar, having a ribosome binding domain through PKII/PKIII and a tRNA anticodon-codon mimicry domain through PKI. However, a recent report showed that the Halastavi IGR IRES contains an atypical structure that

binds to preformed 80S, suggesting that IGR IRESs within the dicistrovirus family may be more diverse than initially thought (Abaeva et al., 2020).

Focusing on the translation initiation mechanism of BaDV-2, in chapter 2, I demonstrated that BaDV-2 IGR supports IRES activity using a bicistronic reporter in insect Sf-21 and RRL extracts. I also showed that the IRES activity is within a 140-nucleotide region via deletion and mutagenesis analyses. Moreover, unlike a typical dicistrovirus IGR IRES, BaDV-2 IGR IRES is factor-dependent and requires eIF2 and eIF4A to initiate translation at an AUG start codon. One main limitation is that we were not able to generate an infectious clone for BaDV-2 to fully access other potentially factors encoded in the BaDV-2 genome that may be key to BaDV-2 IGR IRES activity. Instead, we used bicistronic reporters and chimeric clones, which are also powerful and handy tools for IRES studies. Interestingly, we were able to detect BaDV-2 IRES activity in a bicistronic reporter but not a chimeric clone. This might be because some initiation factors, such as eIF2, are cleaved or inhibited through phosphorylation under CrPV infection and can no longer support BaDV-2 IRES activity. Furthermore, although we lack solid data to support the predicted secondary structure model of BaDV-2 IGR IRES, future investigation combing SAXS-predicted models with molecular assays such as mutagenesis and chemical modification combined with sequencing, such as DMS probing and SHAPE, can be used to uncover the BaDV-2 IGR IRES structure and thus its translation initiation mechanism.

In Chapter 3, I showed that BaDV-2 genome contains a -1 FS strategy to direct the translation of a RdRp motif. Using a bicistronic reporter with luciferase assays and [³⁵S]- methionine/cysteine labeling, I validated the putative BaDV-2 -1 FSS with an efficiency of 4% and 8% in Sf21 and RRL, respectively. Mutagenesis and deletion analyses revealed that a stem-loop structure, sequence identities in the stem-loop and the slippery sequence are key to the

frameshifting activity. Main limitations include that we did not have an BaDV-2 infectious clone and were not able to validate the predicted -1 FSS secondary structure model. However, we managed to identify some key nucleotide identities and showed evidence that the predicted pseudoknot was not essential for -1 FSS. Structural probing analysis such as SHAPE or DMS analysis should provide further insights into its structure. Given the expansion of the RNA virome, it will also be interesting to determine whether -1 FSS is utilized in other dicistrovirus genomes.

Overall, using BaDV-2 as a model, we provided strong evidence of a novel IRES and -1 frameshifting mechanisms in dicistrovirus, and thus highlighting the diversity of viral strategies to direct viral protein synthesis. Importantly, the BaDV-2 IRES and -1 frameshifting viral mechanisms can be targeted for antivirals. There is precedent for targeting RNA structures by small molecules or antisense strategies. For example, small molecules such as triazole-containing compounds are able to bind the FSS with high affinity and selectivity and thus strongly inhibit HIV replication (Hilimire et al., 2017). Another small molecule based on benzimidazole can cause conformational change in FMDV IRES, leading to reduced IRES activity (Lozano et al., 2015). Therefore, exploration of these screening strategies has significant antiviral potentials, especially for some of these viruses impacting agriculture.

Furthermore, the recent advances in RNA therapeutics have drawn tremendous attention to new RNA designs including the use of IRESs. For example, IRESs are used in stable circular RNAs to direct translation (Chen et al., 2021). The knowledge of the BaDV-2 IRES could be potentially used in such RNA technologies including in circRNAs or linear RNAs. Although the BaDV-2 IRES is not relatively strong, it may be possible to select for stronger IRES activities via directed evolution (ex. SELEX) or to try duplicated IRES elements that may possibly drive translation more.

The history of nucleic acid-encoded drugs has been developing since the first direct injection of *in vitro* transcribed mRNA into the skeletal muscle of mice, and was found to be able to express the encoded protein in the injected musical in 1990 (Wolff et al., 1990). Since then, the potential of using mRNA as a therapeutic has slowly gained attention. It was only recently through the COVID-19 pandemic that we saw the true potential of mRNA vaccines and therapeutics. IRES containing mRNAs to drive therapeutic proteins can be important in specific cellular conditions like diseased cells, such as in hypoxic tumours or pancreatic beta-cells that undergo ER-stress induced apoptosis, that have impaired cap-dependent translation. Thus, inclusion of an IRES, which require fewer factors, could be used in therapeutic mRNAs to drive translation under diseased cells. Indeed, IRESs such as the CrPV IGR IRES are translated well under ER stress despite a shutdown of global cap-dependent translation (C. U. Hellen & Sarnow, 2001). Therefore, there is a lot of future potential for type IV IRES as they can hijack host translational machinery in a factor-less manner and initiate translation even under stress. Working with dicistrovirus, it is of great interest to use them as a model to study viral translation initiation and to screen for the IRES could boost the translation efficiency mostly to be used in mRNA therapeutics.

In summary, my thesis provides a deeper understanding of the IRES-dependent translation mechanism in BaDV-2 and the -1 FSS. Our findings extended the current understanding of the dicistrovirus IGR IRES-mediated mechanism and frameshifting in this field and hopefully brought new potentials in utilizing these in antiviral drugs and mRNA therapeutics by boosting mRNA translation.

78

Bibliography

- Abaeva, I. S., Vicens, Q., Bochler, A., Soufari, H., Simonetti, A., Pestova, T. V., Hashem, Y., et al. (2020). The Halastavi árva Virus Intergenic Region IRES Promotes Translation by the Simplest Possible Initiation Mechanism. *Cell reports*, *33*(10), 108476.
- Abeyrathne, P. D., Koh, C. S., Grant, T., Grigorieff, N., & Korostelev, A. A. (2016). Ensemble cryo-EM uncovers inchworm-like translocation of a viral IRES through the ribosome. *eLife*, *5*.
- Abramson, R. D., Dever, T. E., Lawson, T. G., Ray, B. K., Thach, R. E., & Merrick, W. C. (1987). The ATP-dependent interaction of eukaryotic initiation factors with mRNA. *The Journal of Biological Chemistry*, 262(8), 3826–3832.
- ACHOR, D. S. (2001). Replication of Insect iridescent virus 6 in a whitefly cell line. *Journal of invertebrate pathology (Print)*, 77(2), 144–146.
- Adamski, F. M., Donly, B. C., & Tate, W. P. (1993). Competition between frameshifting, termination and suppression at the frameshift site in the Escherichia coli release factor-2 mRNA. *Nucleic Acids Research*, 21(22), 5074–5078.
- Aitken, C. E., Beznosková, P., Vlčkova, V., Chiu, W.-L., Zhou, F., Valášek, L. S., Hinnebusch,
 A. G., et al. (2016). Eukaryotic translation initiation factor 3 plays distinct roles at the
 mRNA entry and exit channels of the ribosomal preinitiation complex. *eLife*, 5.
- Aldabe, R., Barco, A., & Carrasco, L. (1996). Membrane permeabilization by poliovirus proteins 2B and 2BC. *The Journal of Biological Chemistry*, *271*(38), 23134–23137.
- Algire, M. A., Maag, D., & Lorsch, J. R. (2005). Pi release from eIF2, not GTP hydrolysis, is the step controlled by start-site selection during eukaryotic translation initiation. *Molecular Cell*, 20(2), 251–262.

- Alkalaeva, E. Z., Pisarev, A. V., Frolova, L. Y., Kisselev, L. L., & Pestova, T. V. (2006). In vitro reconstitution of eukaryotic translation reveals cooperativity between release factors eRF1 and eRF3. *Cell*, *125*(6), 1125–1136.
- Anand, M., Chakraburtty, K., Marton, M. J., Hinnebusch, A. G., & Kinzy, T. G. (2003).
 Functional interactions between yeast translation eukaryotic elongation factor (eEF) 1A and eEF3. *The Journal of Biological Chemistry*, 278(9), 6985–6991.
- Au, H. H., Cornilescu, G., Mouzakis, K. D., Ren, Q., Burke, J. E., Lee, S., Butcher, S. E., et al. (2015). Global shape mimicry of tRNA within a viral internal ribosome entry site mediates translational reading frame selection. *Proceedings of the National Academy of Sciences of the United States of America*, 112(47), E6446-55.
- Au, H. H. T., Elspass, V. M., & Jan, E. (2018). Functional Insights into the Adjacent Stem-Loop in Honey Bee Dicistroviruses That Promotes Internal Ribosome Entry Site-Mediated Translation and Viral Infection. *Journal of Virology*, 92(2).
- Au, H. H. T., & Jan, E. (2012). Insights into factorless translational initiation by the tRNA-like pseudoknot domain of a viral IRES. *Plos One*, *7*(12), e51477.
- Au, H. H. T., & Jan, E. (2014). Novel viral translation strategies. *Wiley interdisciplinary reviews*. *RNA*, *5*(6), 779–801.
- Badmalia, M. D., Sette Pereira, H., Siddiqui, M. Q., & Patel, T. R. (2022). A comprehensive review of methods to study lncRNA-protein interactions in solution. *Biochemical Society Transactions*, 50(5), 1415–1426.
- Baril, M., Dulude, D., Steinberg, S. V., & Brakier-Gingras, L. (2003). The frameshift stimulatory signal of human immunodeficiency virus type 1 group O is a pseudoknot. *Journal of Molecular Biology*, 331(3), 571–583.

- Barry, J. K., & Miller, W. A. (2002). A -1 ribosomal frameshift element that requires base pairing across four kilobases suggests a mechanism of regulating ribosome and replicase traffic on a viral RNA. *Proceedings of the National Academy of Sciences of the United States of America*, 99(17), 11133–11138.
- Behrmann, E., Loerke, J., Budkevich, T. V., Yamamoto, K., Schmidt, A., Penczek, P. A., Vos,
 M. R., et al. (2015). Structural snapshots of actively translating human ribosomes. *Cell*, *161*(4), 845–857.
- Berlanga, J. J., Ventoso, I., Harding, H. P., Deng, J., Ron, D., Sonenberg, N., Carrasco, L., et al. (2006). Antiviral effect of the mammalian translation initiation factor 2alpha kinase GCN2 against RNA viruses. *The EMBO Journal*, 25(8), 1730–1740.
- Bertram, G., Bell, H. A., Ritchie, D. W., Fullerton, G., & Stansfield, I. (2000). Terminating eukaryote translation: domain 1 of release factor eRF1 functions in stop codon recognition. *RNA (New York)*, 6(9), 1236–1247.
- Beznosková, P., Wagner, S., Jansen, M. E., von der Haar, T., & Valášek, L. S. (2015).
 Translation initiation factor eIF3 promotes programmed stop codon readthrough. *Nucleic Acids Research*, 43(10), 5099–5111.
- Blakqori, G., van Knippenberg, I., & Elliott, R. M. (2009). Bunyamwera orthobunyavirus Ssegment untranslated regions mediate poly(A) tail-independent translation. *Journal of Virology*, 83(8), 3637–3646.
- Bonning, B. C., & Miller, W. A. (2010). Dicistroviruses. *Annual Review of Entomology*, 55, 129–150.
- Bonning, B. C. (2009). The Dicistroviridae: An emerging family of invertebrate viruses. *Virologica Sinica*, *24*(5), 415–427.

- Boros, Á., Pankovics, P., Simmonds, P., & Reuter, G. (2011). Novel positive-sense, singlestranded RNA (+ssRNA) virus with di-cistronic genome from intestinal content of freshwater carp (Cyprinus carpio). *Plos One*, *6*(12), e29145.
- Brierley, I., Digard, P., & Inglis, S. C. (1989). Characterization of an efficient coronavirus ribosomal frameshifting signal: requirement for an RNA pseudoknot. *Cell*, *57*(4), 537–547.
- Brierley, I., Jenner, A. J., & Inglis, S. C. (1992). Mutational analysis of the "slippery-sequence" component of a coronavirus ribosomal frameshifting signal. *Journal of Molecular Biology*, 227(2), 463–479.
- Brown, A., Shao, S., Murray, J., Hegde, R. S., & Ramakrishnan, V. (2015). Structural basis for stop codon recognition in eukaryotes. *Nature*, 524(7566), 493–496.
- Brown, E. A., Day, S. P., Jansen, R. W., & Lemon, S. M. (1991). The 5' nontranslated region of hepatitis A virus RNA: secondary structure and elements required for translation in vitro. *Journal of Virology*, 65(11), 5828–5838.
- Brown, E. A., Zajac, A. J., & Lemon, S. M. (1994). In vitro characterization of an internal ribosomal entry site (IRES) present within the 5' nontranslated region of hepatitis A virus RNA: comparison with the IRES of encephalomyocarditis virus. *Journal of Virology*, 68(2), 1066–1074.
- Budkevich, T., Giesebrecht, J., Altman, R. B., Munro, J. B., Mielke, T., Nierhaus, K. H., Blanchard, S. C., et al. (2011). Structure and dynamics of the mammalian ribosomal pretranslocation complex. *Molecular Cell*, 44(2), 214–224.
- Carter, A. P., Clemons, W. M., Brodersen, D. E., Morgan-Warren, R. J., Hartsch, T., Wimberly,
 B. T., & Ramakrishnan, V. (2001). Crystal structure of an initiation factor bound to the 30S ribosomal subunit. *Science*, 291(5503), 498–501.

- Cencic, R., & Pelletier, J. (2016). Hippuristanol A potent steroid inhibitor of eukaryotic initiation factor 4A. *Translation (Austin, Tex.)*, *4*(1), e1137381.
- Chahal, J., Gebert, L. F. R., Gan, H. H., Camacho, E., Gunsalus, K. C., MacRae, I. J., & Sagan,
 S. M. (2019). miR-122 and Ago interactions with the HCV genome alter the structure of the viral 5' terminus. *Nucleic Acids Research*, 47(10), 5307–5324.
- Chen, C.K., Cheng, R., et al. (2021). Structured elements drive extensive circular RNA translation. *Mol Cell*, *81*(20), 4300-4318.
- Choi, J., O'Loughlin, S., Atkins, J. F., & Puglisi, J. D. (2020). The energy landscape of -1 ribosomal frameshifting. *Science Advances*, *6*(1), eaax6969.
- Chung, B. Y.-W., Miller, W. A., Atkins, J. F., & Firth, A. E. (2008). An overlapping essential gene in the Potyviridae. *Proceedings of the National Academy of Sciences of the United States of America*, 105(15), 5897–5902.

Cohen, S. S., & -. (1998). guide to the polyamines. Oxford University Press.

- Connor, J. H., & Lyles, D. S. (2002). Vesicular stomatitis virus infection alters the eIF4F translation initiation complex and causes dephosphorylation of the eIF4E binding protein 4E-BP1. *Journal of Virology*, 76(20), 10177–10187.
- Costantino, D., & Kieft, J. S. (2005). A preformed compact ribosome-binding domain in the cricket paralysis-like virus IRES RNAs. *RNA (New York)*, *11*(3), 332–343.
- Costantino, D. A., Pfingsten, J. S., Rambo, R. P., & Kieft, J. S. (2008). tRNA-mRNA mimicry drives translation initiation from a viral IRES. *Nature Structural & Molecular Biology*, *15*(1), 57–64.
- Cowieson, N. P., Edwards-Gayle, C. J. C., Inoue, K., Khunti, N. S., Doutch, J., Williams, E., Daniels, S., et al. (2020). Beamline B21: high-throughput small-angle X-ray scattering at

Diamond Light Source. Journal of Synchrotron Radiation, 27(Pt 5), 1438–1446.

- Craigen, W. J., Cook, R. G., Tate, W. P., & Caskey, C. T. (1985). Bacterial peptide chain release factors: conserved primary structure and possible frameshift regulation of release factor 2. *Proceedings of the National Academy of Sciences of the United States of America*, 82(11), 3616–3620.
- Cuesta, R., Xi, Q., & Schneider, R. J. (2000). Adenovirus-specific translation by displacement of kinase Mnk1 from cap-initiation complex eIF4F. *The EMBO Journal*, *19*(13), 3465–3474.
- Culley, A. I., Lang, A. S., & Suttle, C. A. (2007). The complete genomes of three viruses assembled from shotgun libraries of marine RNA virus communities. *Virology Journal*, *4*, 69.
- D'Souza, M. H., Mrozowich, T., Badmalia, M. D., Geeraert, M., Frederickson, A., Henrickson, A., Demeler, B., et al. (2022). Biophysical characterisation of human LincRNA-p21 sense and antisense Alu inverted repeats. *Nucleic Acids Research*, 50(10), 5881–5898.
- ten Dam, E. B., Pleij, C. W., & Bosch, L. (1990). RNA pseudoknots: translational frameshifting and readthrough on viral RNAs. *Virus Genes*, *4*(2), 121–136.
- Deng, J., Harding, H. P., Raught, B., Gingras, A.-C., Berlanga, J. J., Scheuner, D., Kaufman, R.
 J., et al. (2002). Activation of GCN2 in UV-irradiated cells inhibits translation. *Current Biology*, *12*(15), 1279–1286.
- Dever, T. E., Dinman, J. D., & Green, R. (2018). Translation elongation and recoding in eukaryotes. *Cold Spring Harbor Perspectives in Biology*, 10(8).
- Dever, T. E., & Green, R. (2012). The elongation, termination, and recycling phases of translation in eukaryotes. *Cold Spring Harbor Perspectives in Biology*, *4*(7), a013706.

Dever, T. E., Gutierrez, E., & Shin, B.-S. (2014). The hypusine-containing translation factor

eIF5A. Critical Reviews in Biochemistry and Molecular Biology, 49(5), 413–425.

- Dever, T. E. (2002). Gene-specific regulation by general translation factors. *Cell*, *108*(4), 545–556.
- Dias, A., Bouvier, D., Crépin, T., McCarthy, A. A., Hart, D. J., Baudin, F., Cusack, S., et al. (2009). The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. *Nature*, 458(7240), 914–918.
- Dinman, Jonathan D. (2012). Mechanisms and implications of programmed translational frameshifting. *Wiley interdisciplinary reviews. RNA*, *3*(5), 661–673.
- Dinman, J D, Ruiz-Echevarria, M. J., & Peltz, S. W. (1998). Translating old drugs into new treatments: ribosomal frameshifting as a target for antiviral agents. *Trends in Biotechnology*, 16(4), 190–196.
- Dobrikova, E., Shveygert, M., Walters, R., & Gromeier, M. (2010). Herpes simplex virus proteins ICP27 and UL47 associate with polyadenylate-binding protein and control its subcellular distribution. *Journal of Virology*, *84*(1), 270–279.
- Dolja, V. V., & Koonin, E. V. (2018). Metagenomics reshapes the concepts of RNA virus evolution by revealing extensive horizontal virus transfer. *Virus Research*, *244*, 36–52.
- Dougherty, J. D., White, J. P., & Lloyd, R. E. (2011). Poliovirus-mediated disruption of cytoplasmic processing bodies. *Journal of Virology*, *85*(1), 64–75.
- Dutilh, B. E. (2014). Metagenomic ventures into outer sequence space. *Bacteriophage*, 4(4), e979664.
- Dzananovic, E., Patel, T. R., Chojnowski, G., Boniecki, M. J., Deo, S., McEleney, K., Harding,
 S. E., et al. (2014). Solution conformation of adenovirus virus associated RNA-I and its interaction with PKR. *Journal of Structural Biology*, *185*(1), 48–57.

- Edgar, R. C., Taylor, J., Lin, V., Altman, T., Barbera, P., Meleshko, D., Lohr, D., et al. (2022). Petabase-scale sequence alignment catalyses viral discovery. *Nature*, *602*(7895), 142–147.
- Engelhardt, O. G., Smith, M., & Fodor, E. (2005). Association of the influenza A virus RNAdependent RNA polymerase with cellular RNA polymerase II. *Journal of Virology*, *79*(9), 5812–5818.
- Faria, P. A., Chakraborty, P., Levay, A., Barber, G. N., Ezelle, H. J., Enninga, J., Arana, C., et al. (2005). VSV disrupts the Rae1/mrnp41 mRNA nuclear export pathway. *Molecular Cell*, *17*(1), 93–102.
- Fechter, P., Mingay, L., Sharps, J., Chambers, A., Fodor, E., & Brownlee, G. G. (2003). Two aromatic residues in the PB2 subunit of influenza A RNA polymerase are crucial for cap binding. *The Journal of Biological Chemistry*, 278(22), 20381–20388.
- Fernández, I. S., Bai, X.-C., Murshudov, G., Scheres, S. H. W., & Ramakrishnan, V. (2014). Initiation of translation by cricket paralysis virus IRES requires its translocation in the ribosome. *Cell*, 157(4), 823–831.
- Firth, A. E., & Brown, C. M. (2006). Detecting overlapping coding sequences in virus genomes. BMC Bioinformatics, 7, 75.
- Firth, A. E., Wang, Q. S., Jan, E., & Atkins, J. F. (2009). Bioinformatic evidence for a stem-loop structure 5'-adjacent to the IGR-IRES and for an overlapping gene in the bee paralysis dicistroviruses. *Virology Journal*, 6, 193.
- Frolova, L. Y., Tsivkovskii, R. Y., Sivolobova, G. F., Oparina, N. Y., Serpinsky, O. I., Blinov,
 V. M., Tatkov, S. I., et al. (1999). Mutations in the highly conserved GGQ motif of class 1
 polypeptide release factors abolish ability of human eRF1 to trigger peptidyl-tRNA
 hydrolysis. *RNA (New York)*, 5(8), 1014–1020.

- Fujita, K., Krishnakumar, S. S., Franco, D., Paul, A. V., London, E., & Wimmer, E. (2007).
 Membrane topography of the hydrophobic anchor sequence of poliovirus 3A and 3AB proteins and the functional effect of 3A/3AB membrane association upon RNA replication. *Biochemistry*, 46(17), 5185–5199.
- Garcin, D., Lezzi, M., Dobbs, M., Elliott, R. M., Schmaljohn, C., Kang, C. Y., & Kolakofsky, D. (1995). The 5' ends of Hantaan virus (Bunyaviridae) RNAs suggest a prime-and-realign mechanism for the initiation of RNA synthesis. *Journal of Virology*, 69(9), 5754–5762.
- Garrey, J. L., Lee, Y.-Y., Au, H. H. T., Bushell, M., & Jan, E. (2010). Host and viral translational mechanisms during cricket paralysis virus infection. *Journal of Virology*, 84(2), 1124–1138.
- Giedroc, D. P., Theimer, C. A., & Nixon, P. L. (2000). Structure, stability and function of RNA pseudoknots involved in stimulating ribosomal frameshifting. *Journal of Molecular Biology*, 298(2), 167–185.
- Gingras, A. C., & Sonenberg, N. (1997). Adenovirus infection inactivates the translational inhibitors 4E-BP1 and 4E-BP2. *Virology*, *237*(1), 182–186.
- Gingras, A. C., Svitkin, Y., Belsham, G. J., Pause, A., & Sonenberg, N. (1996). Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus. *Proceedings of the National Academy of Sciences of the United States of America*, 93(11), 5578–5583.
- Glaser, W., & Skern, T. (2000). Extremely efficient cleavage of eIF4G by picornaviral proteinases L and 2A in vitro. *FEBS Letters*, *480*(2–3), 151–155.
- Gorbalenya, A. E., Koonin, E. V., & Wolf, Y. I. (1990). A new superfamily of putative NTPbinding domains encoded by genomes of small DNA and RNA viruses. *FEBS Letters*,

262(1), 145–148.

- Gradi, A., Svitkin, Y. V., Imataka, H., & Sonenberg, N. (1998). Proteolysis of human eukaryotic translation initiation factor eIF4GII, but not eIF4GI, coincides with the shutoff of host protein synthesis after poliovirus infection. *Proceedings of the National Academy of Sciences of the United States of America*, 95(19), 11089–11094.
- Groppo, R., Brown, B. A., & Palmenberg, A. C. (2011). Mutational analysis of the EMCV 2A protein identifies a nuclear localization signal and an eIF4E binding site. *Virology*, 410(1), 257–267.
- Gross, J. D., Moerke, N. J., von der Haar, T., Lugovskoy, A. A., Sachs, A. B., McCarthy, J. E.G., & Wagner, G. (2003). Ribosome loading onto the mRNA cap is driven byconformational coupling between eIF4G and eIF4E. *Cell*, *115*(6), 739–750.
- Gross, L., Vicens, Q., Einhorn, E., Noireterre, A., Schaeffer, L., Kuhn, L., Imler, J.-L., et al. (2017). The IRES5'UTR of the dicistrovirus cricket paralysis virus is a type III IRES containing an essential pseudoknot structure. *Nucleic Acids Research*, *45*(15), 8993–9004.
- Guilligay, D., Tarendeau, F., Resa-Infante, P., Coloma, R., Crepin, T., Sehr, P., Lewis, J., et al. (2008). The structural basis for cap binding by influenza virus polymerase subunit PB2. *Nature Structural & Molecular Biology*, 15(5), 500–506.
- Gu, Y., Mao, Y., Jia, L., Dong, L., & Qian, S.-B. (2021). Bi-directional ribosome scanning controls the stringency of start codon selection. *Nature Communications*, 12(1), 6604.
- Haghighat, A., Mader, S., Pause, A., & Sonenberg, N. (1995). Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. *The EMBO Journal*, 14(22), 5701–5709.
- Harb, M., Becker, M. M., Vitour, D., Baron, C. H., Vende, P., Brown, S. C., Bolte, S., et al.

(2008). Nuclear localization of cytoplasmic poly(A)-binding protein upon rotavirus infection involves the interaction of NSP3 with eIF4G and RoXaN. *Journal of Virology*, *82*(22), 11283–11293.

- Harms, U., Andreou, A. Z., Gubaev, A., & Klostermeier, D. (2014). eIF4B, eIF4G and RNA regulate eIF4A activity in translation initiation by modulating the eIF4A conformational cycle. *Nucleic Acids Research*, 42(12), 7911–7922.
- Hatakeyama, Y., Shibuya, N., Nishiyama, T., & Nakashima, N. (2004). Structural variant of the intergenic internal ribosome entry site elements in dicistroviruses and computational search for their counterparts. *RNA (New York)*, *10*(5), 779–786.
- Hatfield, D., & Oroszlan, S. (1990). The where, what and how of ribosomal frameshifting in retroviral protein synthesis. *Trends in Biochemical Sciences*, *15*(5), 186–190.
- Hedil, M., Nakasu, E. Y. T., Nagata, T., Wen, J., Jan, E., Michereff-Filho, M., & Inoue-Nagata,A. K. (2020). New features on the genomic organization of a novel dicistrovirus identified from the sweet potato whitefly Bemisia tabaci. *Virus Research*, 288, 198112.
- Hellen, C. U., & Sarnow, P. (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes & Development*, 15(13), 1593–1612.

Hellen, C. U. T. (2007). Bypassing translation initiation. Structure, 15(1), 4–6.

- Hellen, C. U. T. (2018). Translation termination and ribosome recycling in eukaryotes. *Cold Spring Harbor Perspectives in Biology*, *10*(10).
- Herold, J., & Siddell, S. G. (1993). An "elaborated" pseudoknot is required for high frequency frameshifting during translation of HCV 229E polymerase mRNA. *Nucleic Acids Research*, 21(25), 5838–5842.

Hershey, J. W. B., Sonenberg, N., & Mathews, M. B. (2019). Principles of translational control.
Cold Spring Harbor Perspectives in Biology, 11(9).

- Hertz, M. I., & Thompson, S. R. (2011). In vivo functional analysis of the Dicistroviridae intergenic region internal ribosome entry sites. *Nucleic Acids Research*, *39*(16), 7276–7288.
- Hilimire, T. A., Chamberlain, J. M., Anokhina, V., Bennett, R. P., Swart, O., Myers, J. R.,
 Ashton, J. M., et al. (2017). HIV-1 Frameshift RNA-Targeted Triazoles Inhibit Propagation of Replication-Competent and Multi-Drug-Resistant HIV in Human Cells. *ACS Chemical Biology*, *12*(6), 1674–1682.
- Hinnebusch, Alan G. (2011). Molecular mechanism of scanning and start codon selection in eukaryotes. *Microbiology and Molecular Biology Reviews*, 75(3), 434–67, first page of table of contents.
- Hinnebusch, A G. (2000). Mechanism and regulation of initiator methionyl-tRNA binding to ribosomes. *COLD SPRING HARBOR MONOGRAPH*
- Horowitz, A. R., Antignus, Y., & Gerling, D. (2011). Management of Bemisia tabaci Whiteflies.
 In W. M. O. Thompson (Ed.), *The Whitefly, Bemisia tabaci (Homoptera: Aleyrodidae) Interaction with Geminivirus-Infected Host Plants* (pp. 293–322). Dordrecht: Springer
 Netherlands.
- Ho, B.-C., Yu, S.-L., Chen, J. J. W., Chang, S.-Y., Yan, B.-S., Hong, Q.-S., Singh, S., et al.
 (2011). Enterovirus-induced miR-141 contributes to shutoff of host protein translation by targeting the translation initiation factor eIF4E. *Cell Host & Microbe*, 9(1), 58–69.
- Huang, C., Lokugamage, K. G., Rozovics, J. M., Narayanan, K., Semler, B. L., & Makino, S. (2011). SARS coronavirus nsp1 protein induces template-dependent endonucleolytic cleavage of mRNAs: viral mRNAs are resistant to nsp1-induced RNA cleavage. *PLoS Pathogens*, 7(12), e1002433.

- Huston, N. C., Wan, H., Strine, M. S., de Cesaris Araujo Tavares, R., Wilen, C. B., & Pyle, A.
 M. (2021). Comprehensive in vivo secondary structure of the SARS-CoV-2 genome reveals novel regulatory motifs and mechanisms. *Molecular Cell*, 81(3), 584-598.e5.
- Hwang, H.-J., Min, H. J., Yun, H., Pelton, J. G., Wemmer, D. E., Cho, K.-O., Kim, J.-S., et al. (2015). Solution structure of the porcine sapovirus VPg core reveals a stable three-helical bundle with a conserved surface patch. *Biochemical and Biophysical Research Communications*, 459(4), 610–616.
- Imataka, H., & Sonenberg, N. (1997). Human eukaryotic translation initiation factor 4G (eIF4G) possesses two separate and independent binding sites for eIF4A. *Molecular and Cellular Biology*, 17(12), 6940–6947.
- Ivanov, I. P., Anderson, C. B., Gesteland, R. F., & Atkins, J. F. (2004). Identification of a new antizyme mRNA +1 frameshifting stimulatory pseudoknot in a subset of diverse invertebrates and its apparent absence in intermediate species. *Journal of Molecular Biology*, 339(3), 495–504.
- Jackson, R. J., Hellen, C. U. T., & Pestova, T. V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. *Nature Reviews. Molecular Cell Biology*, 11(2), 113–127.
- Jackson, R. J., Hellen, C. U. T., & Pestova, T. V. (2012). Termination and post-termination events in eukaryotic translation. *Advances in protein chemistry and structural biology*, 86, 45–93.
- Jacobson, M. F., & Baltimore, D. (1968). Polypeptide cleavages in the formation of poliovirus proteins. Proceedings of the National Academy of Sciences of the United States of America, 61(1), 77–84.

- Jang, C. J., & Jan, E. (2010). Modular domains of the Dicistroviridae intergenic internal ribosome entry site. *RNA (New York)*, *16*(6), 1182–1195.
- Jang, C. J., Lo, M. C. Y., & Jan, E. (2009). Conserved element of the dicistrovirus IGR IRES that mimics an E-site tRNA/ribosome interaction mediates multiple functions. *Journal of Molecular Biology*, 387(1), 42–58.
- Jang, S. K., Pestova, T. V., Hellen, C. U., Witherell, G. W., & Wimmer, E. (1990). Capindependent translation of picornavirus RNAs: structure and function of the internal ribosomal entry site. *Enzyme*, 44(1–4), 292–309.
- Jankowsky, E., Gross, C. H., Shuman, S., & Pyle, A. M. (2001). Active disruption of an RNAprotein interaction by a DExH/D RNA helicase. *Science*, *291*(5501), 121–125.
- Jan, E., Kinzy, T. G., & Sarnow, P. (2003). Divergent tRNA-like element supports initiation, elongation, and termination of protein biosynthesis. *Proceedings of the National Academy* of Sciences of the United States of America, 100(26), 15410–15415.
- Jan, E., & Sarnow, P. (2002). Factorless ribosome assembly on the internal ribosome entry site of cricket paralysis virus. *Journal of Molecular Biology*, *324*(5), 889–902.
- Jan, E. (2006). Divergent IRES elements in invertebrates. Virus Research, 119(1), 16–28.
- Joachims, M., Van Breugel, P. C., & Lloyd, R. E. (1999). Cleavage of poly(A)-binding protein by enterovirus proteases concurrent with inhibition of translation in vitro. *Journal of Virology*, 73(1), 718–727.
- Johnson, K. N., & Christian, P. D. (1998). The novel genome organization of the insect picornalike virus Drosophila C virus suggests this virus belongs to a previously undescribed virus family. *The Journal of General Virology*, 79 (Pt 1), 191–203.

Jousset, F. X., Bergoin, M., & Revet, B. (1977). Characterization of the Drosophila C virus. The

Journal of General Virology, 34(2), 269–283.

- Kamitani, W., Huang, C., Narayanan, K., Lokugamage, K. G., & Makino, S. (2009). A twopronged strategy to suppress host protein synthesis by SARS coronavirus Nsp1 protein. *Nature Structural & Molecular Biology*, 16(11), 1134–1140.
- Kamitani, W., Narayanan, K., Huang, C., Lokugamage, K., Ikegami, T., Ito, N., Kubo, H., et al. (2006). Severe acute respiratory syndrome coronavirus nsp1 protein suppresses host gene expression by promoting host mRNA degradation. *Proceedings of the National Academy of Sciences of the United States of America*, 103(34), 12885–12890.
- Kanamori, Y., & Nakashima, N. (2001). A tertiary structure model of the internal ribosome entry site (IRES) for methionine-independent initiation of translation. *RNA (New York)*, 7(2), 266–274.
- Kapp, L. D., & Lorsch, J. R. (2004). GTP-dependent recognition of the methionine moiety on initiator tRNA by translation factor eIF2. *Journal of Molecular Biology*, 335(4), 923–936.
- Kerr, C. H., Ma, Z. W., Jang, C. J., Thompson, S. R., & Jan, E. (2016). Molecular analysis of the factorless internal ribosome entry site in Cricket Paralysis virus infection. *Scientific Reports*, 6, 37319.
- Kerr, C. H., Wang, Q. S., Keatings, K., Khong, A., Allan, D., Yip, C. K., Foster, L. J., et al. (2015). The 5' untranslated region of a novel infectious molecular clone of the dicistrovirus cricket paralysis virus modulates infection. *Journal of Virology*, 89(11), 5919–5934.
- Kerr, C. H., Wang, Q. S., Moon, K.-M., Keatings, K., Allan, D. W., Foster, L. J., & Jan, E.
 (2018). IRES-dependent ribosome repositioning directs translation of a +1 overlapping ORF that enhances viral infection. *Nucleic Acids Research*, *46*(22), 11952–11967.

Khong, A., & Jan, E. (2011). Modulation of stress granules and P bodies during dicistrovirus

infection. Journal of Virology, 85(4), 1439–1451.

- Khong, A., Kerr, C. H., Yeung, C. H. L., Keatings, K., Nayak, A., Allan, D. W., & Jan, E.
 (2017). Disruption of stress granule formation by the multifunctional cricket paralysis virus 1A protein. *Journal of Virology*, *91*(5).
- Kieft, J. S. (2008). Viral IRES RNA structures and ribosome interactions. *Trends in Biochemical Sciences*, *33*(6), 274–283.
- Kim, H. J., Jeong, M. S., & Jang, S. B. (2021). Structure and Activities of the NS1 Influenza Protein and Progress in the Development of Small-Molecule Drugs. *International Journal* of Molecular Sciences, 22(8).
- Klobutcher, L. A., & Farabaugh, P. J. (2002). Shifty ciliates: frequent programmed translational frameshifting in euplotids. *Cell*, *111*(6), 763–766.
- Koh, C. S., Brilot, A. F., Grigorieff, N., & Korostelev, A. A. (2014). Taura syndrome virus IRES initiates translation by binding its tRNA-mRNA-like structural element in the ribosomal decoding center. *Proceedings of the National Academy of Sciences of the United States of America*, 111(25), 9139–9144.
- Kontos, H., Napthine, S., & Brierley, I. (2001). Ribosomal pausing at a frameshifter RNA pseudoknot is sensitive to reading phase but shows little correlation with frameshift efficiency. *Molecular and Cellular Biology*, *21*(24), 8657–8670.
- Koonin, E. V., Krupovic, M., & Dolja, V. V. (2022). The global virome: How much diversity and how many independent origins? *Environmental Microbiology*.
- Kozak, M. (1999). Initiation of translation in prokaryotes and eukaryotes. *Gene*, 234(2), 187–208.

Kräusslich, H. G., Nicklin, M. J., Lee, C. K., & Wimmer, E. (1988). Polyprotein processing in

picornavirus replication. Biochimie, 70(1), 119–130.

- Kuhn, J. H., Adkins, S., Agwanda, B. R., Al Kubrusli, R., Alkhovsky, S. V., Amarasinghe, G. K., Avšič-Županc, T., et al. (2021). 2021 Taxonomic update of phylum Negarnaviricota (Riboviria: Orthornavirae), including the large orders Bunyavirales and Mononegavirales. *Archives of Virology*, *166*(12), 3513–3566.
- Kumar, P., Hellen, C. U. T., & Pestova, T. V. (2016). Toward the mechanism of eIF4F-mediated ribosomal attachment to mammalian capped mRNAs. *Genes & Development*, 30(13), 1573– 1588.
- Kundu, P., Raychaudhuri, S., Tsai, W., & Dasgupta, A. (2005). Shutoff of RNA polymerase II transcription by poliovirus involves 3C protease-mediated cleavage of the TATA-binding protein at an alternative site: incomplete shutoff of transcription interferes with efficient viral replication. *Journal of Virology*, 79(15), 9702–9713.
- Lamphear, B. J., Kirchweger, R., Skern, T., & Rhoads, R. E. (1995). Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. *The Journal of Biological Chemistry*, 270(37), 21975–21983.
- Lancaster, A. M., Jan, E., & Sarnow, P. (2006). Initiation factor-independent translation mediated by the hepatitis C virus internal ribosome entry site. *RNA (New York)*, 12(5), 894– 902.
- Landry, D. M., Hertz, M. I., & Thompson, S. R. (2009). RPS25 is essential for translation initiation by the Dicistroviridae and hepatitis C viral IRESs. *Genes & Development*, 23(23), 2753–2764.
- Lan, T. C. T., Allan, M. F., Malsick, L. E., Woo, J. Z., Zhu, C., Zhang, F., Khandwala, S., et al.

(2022). Secondary structural ensembles of the SARS-CoV-2 RNA genome in infected cells. *Nature Communications*, *13*(1), 1128.

- Lapointe, C. P., Grosely, R., Sokabe, M., Alvarado, C., Wang, J., Montabana, E., Villa, N., et al. (2022). eIF5B and eIF1A reorient initiator tRNA to allow ribosomal subunit joining. *Nature*, 607(7917), 185–190.
- Larsen, B., Gesteland, R. F., & Atkins, J. F. (1997). Structural probing and mutagenic analysis of the stem-loop required for Escherichia coli dnaX ribosomal frameshifting: programmed efficiency of 50%. *Journal of Molecular Biology*, 271(1), 47–60.
- Liao, P.-Y., Gupta, P., Petrov, A. N., Dinman, J. D., & Lee, K. H. (2008). A new kinetic model reveals the synergistic effect of E-, P- and A-sites on +1 ribosomal frameshifting. *Nucleic Acids Research*, 36(8), 2619–2629.
- Lin, T. A., Kong, X., Haystead, T. A., Pause, A., Belsham, G., Sonenberg, N., & Lawrence, J. C. (1994). PHAS-I as a link between mitogen-activated protein kinase and translation initiation. *Science*, *266*(5185), 653–656.
- Liston, P., & Briedis, D. J. (1995). Ribosomal frameshifting during translation of measles virus P protein mRNA is capable of directing synthesis of a unique protein. *Journal of Virology*, 69(11), 6742–6750.
- Li, M. L., Rao, P., & Krug, R. M. (2001). The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits. *The EMBO Journal*, *20*(8), 2078–2086.
- Li, S., Min, J.-Y., Krug, R. M., & Sen, G. C. (2006). Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology*, *349*(1), 13–21.

Lobanov, A. V., Heaphy, S. M., Turanov, A. A., Gerashchenko, M. V., Pucciarelli, S., Devaraj,

R. R., Xie, F., et al. (2017). Position-dependent termination and widespread obligatory frameshifting in Euplotes translation. *Nature Structural & Molecular Biology*, *24*(1), 61–68.

- Lozano, G., Trapote, A., Ramajo, J., Elduque, X., Grandas, A., Robles, J., Pedroso, E., et al.
 (2015). Local RNA flexibility perturbation of the IRES element induced by a novel ligand inhibits viral RNA translation. *RNA Biology*, *12*(5), 555–568.
- Maag, D., Fekete, C. A., Gryczynski, Z., & Lorsch, J. R. (2005). A conformational change in the eukaryotic translation preinitiation complex and release of eIF1 signal recognition of the start codon. *Molecular Cell*, *17*(2), 265–275.
- Mader, S., Lee, H., Pause, A., & Sonenberg, N. (1995). The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. *Molecular and Cellular Biology*, 15(9), 4990–4997.
- Majumdar, R., & Maitra, U. (2005). Regulation of GTP hydrolysis prior to ribosomal AUG selection during eukaryotic translation initiation. *The EMBO Journal*, *24*(21), 3737–3746.
- Manalastas-Cantos, K., Konarev, P. V., Hajizadeh, N. R., Kikhney, A. G., Petoukhov, M. V., Molodenskiy, D. S., Panjkovich, A., et al. (2021). ATSAS 3.0: expanded functionality and new tools for small-angle scattering data analysis. *Journal of Applied Crystallography*, 54(Pt 1), 343–355.
- Mantsyzov, A. B., Ivanova, E. V., Birdsall, B., Alkalaeva, E. Z., Kryuchkova, P. N., Kelly, G., Frolova, L. Y., et al. (2010). NMR solution structure and function of the C-terminal domain of eukaryotic class 1 polypeptide chain release factor. *The FEBS Journal*, 277(12), 2611– 2627.
- Matheisl, S., Berninghausen, O., Becker, T., & Beckmann, R. (2015). Structure of a human translation termination complex. *Nucleic Acids Research*, *43*(18), 8615–8626.

- Mathews, M. B., & Sonenberg, N. (2000). Origins and principles of translational control. *COLD* SPRING
- Melnikov, S., Mailliot, J., Rigger, L., Neuner, S., Shin, B.-S., Yusupova, G., Dever, T. E., et al. (2016). Molecular insights into protein synthesis with proline residues. *EMBO Reports*, *17*(12), 1776–1784.
- Merrick, W. C., & Pavitt, G. D. (2018). Protein synthesis initiation in eukaryotic cells. *Cold Spring Harbor Perspectives in Biology*, *10*(12).
- Mirzayan, C., & Wimmer, E. (1994). Biochemical studies on poliovirus polypeptide 2C: evidence for ATPase activity. *Virology*, *199*(1), 176–187.
- Mohammad, M. P., Munzarová Pondelícková, V., Zeman, J., Gunišová, S., & Valášek, L. S. (2017). In vivo evidence that eIF3 stays bound to ribosomes elongating and terminating on short upstream ORFs to promote reinitiation. *Nucleic Acids Research*, 45(5), 2658–2674.
- Mohr, I., & Sonenberg, N. (2012). Host translation at the nexus of infection and immunity. *Cell Host & Microbe*, *12*(4), 470–483.
- Moore, Norman F., Reavy, B., Pullin, J. S. K., & Plus, N. (1981). The polypeptides induced in Drosophila cells by Drosophila C virus (strain Ouarzazate). *Virology*, *112*(2), 411–416.
- Moore, N F, Kearns, A., & Pullin, J. S. (1980). Characterization of cricket paralysis virusinduced polypeptides in Drosophila cells. *Journal of Virology*, *33*(1), 1–9.

Muhs, M., Hilal, T., Mielke, T., Skabkin, M. A., Sanbonmatsu, K. Y., Pestova, T. V., & Spahn,
C. M. T. (2015). Cryo-EM of ribosomal 80S complexes with termination factors reveals the translocated cricket paralysis virus IRES. *Molecular Cell*, *57*(3), 422–432.
Murphy F. A., Fauquet C. M., Bishop D. H. L., Ghabrial S. A., Jarvis A. W., Martelli G. P., Mayo M. A., Summers M. D. (1995). Sixth Report of the International Committee on

Taxonomy of Viruses. Virus Taxonomy.

- Murray, J., Savva, C. G., Shin, B.-S., Dever, T. E., Ramakrishnan, V., & Fernández, I. S. (2016).
 Structural characterization of ribosome recruitment and translocation by type IV IRES.
 eLife, 5.
- Nakashima, N., & Nakamura, Y. (2008). Cleavage sites of the "P3 region" in the nonstructural polyprotein precursor of a dicistrovirus. *Archives of Virology*, *153*(10), 1955–1960.
- Nakashima, N., & Uchiumi, T. (2009). Functional analysis of structural motifs in dicistroviruses. *Virus Research*, *139*(2), 137–147.
- Nakasu, E. Y. T., Hedil, M., Nagata, T., Michereff-Filho, M., Lucena, V. S., & Inoue-Nagata, A.
 K. (2019). Complete genome sequence and phylogenetic analysis of a novel dicistrovirus associated with the whitefly Bemisia tabaci. *Virus Research*, *260*, 49–52.
- Nakasu, E. Y. T., Melo, F. L., Michereff-Filho, M., Nagata, T., Ribeiro, B. M., Ribeiro, S. G., Lacorte, C., et al. (2017). Discovery of two small circular ssDNA viruses associated with the whitefly Bemisia tabaci. *Archives of Virology*, 162(9), 2835–2838.
- Napthine, S., Ling, R., Finch, L. K., Jones, J. D., Bell, S., Brierley, I., & Firth, A. E. (2017). Protein-directed ribosomal frameshifting temporally regulates gene expression. *Nature Communications*, *8*, 15582.
- Navas-Castillo, J., Fiallo-Olivé, E., & Sánchez-Campos, S. (2011). Emerging virus diseases transmitted by whiteflies. *Annual Review of Phytopathology*, *49*, 219–248.
- Nayak, A., Berry, B., Tassetto, M., Kunitomi, M., Acevedo, A., Deng, C., Krutchinsky, A., et al. (2010). Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in Drosophila. *Nature Structural & Molecular Biology*, 17(5), 547–554.

Neri, U., Wolf, Y. I., Roux, S., Camargo, A. P., Lee, B., Kazlauskas, D., Chen, I. M., et al.

(2022). A five-fold expansion of the global RNA virome reveals multiple new clades of RNA bacteriophages. *BioRxiv*.

- Nishiyama, T., Yamamoto, H., Shibuya, N., Hatakeyama, Y., Hachimori, A., Uchiumi, T., & Nakashima, N. (2003). Structural elements in the internal ribosome entry site of Plautia stali intestine virus responsible for binding with ribosomes. *Nucleic Acids Research*, *31*(9), 2434–2442.
- Nishiyama, T., Yamamoto, H., Uchiumi, T., & Nakashima, N. (2007). Eukaryotic ribosomal protein RPS25 interacts with the conserved loop region in a dicistroviral intergenic internal ribosome entry site. *Nucleic Acids Research*, *35*(5), 1514–1521.
- Noller, H. F., Lancaster, L., Zhou, J., & Mohan, S. (2017). The ribosome moves: RNA mechanics and translocation. *Nature Structural & Molecular Biology*, *24*(12), 1021–1027.
- Novac, O., Guenier, A.-S., & Pelletier, J. (2004). Inhibitors of protein synthesis identified by a high throughput multiplexed translation screen. *Nucleic Acids Research*, *32*(3), 902–915.
- O'Leary, S. E., Petrov, A., Chen, J., & Puglisi, J. D. (2013). Dynamic recognition of the mRNA cap by Saccharomyces cerevisiae eIF4E. *Structure*, *21*(12), 2197–2207.
- Oguro, A., Shigeta, T., Machida, K., Suzuki, T., Iwamoto, T., Matsufuji, S., & Imataka, H. (2020). Translation efficiency affects the sequence-independent +1 ribosomal frameshifting by polyamines. *Journal of Biochemistry*, *168*(2), 139–149.
- Ohlmann, T., Rau, M., Pain, V. M., & Morley, S. J. (1996). The C-terminal domain of eukaryotic protein synthesis initiation factor (eIF) 4G is sufficient to support cap-independent translation in the absence of eIF4E. *The EMBO Journal*, 15(6), 1371–1382.
- Palumbo, J. C., Horowitz, A. R., & Prabhaker, N. (2001). Insecticidal control and resistance management for Bemisia tabaci. *Crop Protection*, 20(9), 739–765.

- Panniers, R., Rowlands, A. G., & Henshaw, E. C. (1988). The effect of Mg2+ and guanine nucleotide exchange factor on the binding of guanine nucleotides to eukaryotic initiation factor 2. *The Journal of Biological Chemistry*, 263(12), 5519–5525.
- Passmore, L. A., Schmeing, T. M., Maag, D., Applefield, D. J., Acker, M. G., Algire, M. A., Lorsch, J. R., et al. (2007). The eukaryotic translation initiation factors eIF1 and eIF1A induce an open conformation of the 40S ribosome. *Molecular Cell*, 26(1), 41–50.
- Pavitt, Graham D, & Ron, D. (2012). New insights into translational regulation in the endoplasmic reticulum unfolded protein response. *Cold Spring Harbor Perspectives in Biology*, 4(6).
- Pavitt, G D, Ramaiah, K. V., Kimball, S. R., & Hinnebusch, A. G. (1998). eIF2 independently binds two distinct eIF2B subcomplexes that catalyze and regulate guanine-nucleotide exchange. *Genes & Development*, 12(4), 514–526.
- Pelletier, J., & Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature*, *334*(6180), 320–325.
- Pestova, T. V., & Kolupaeva, V. G. (2002). The roles of individual eukaryotic translation initiation factors in ribosomal scanning and initiation codon selection. *Genes & Development*, 16(22), 2906–2922.
- Petrov, A., Grosely, R., Chen, J., O'Leary, S. E., & Puglisi, J. D. (2016). Multiple parallel pathways of translation initiation on the crpv IRES. *Molecular Cell*, *62*(1), 92–103.
- Pfingsten, J. S., Castile, A. E., & Kieft, J. S. (2010). Mechanistic role of structurally dynamic regions in Dicistroviridae IGR IRESs. *Journal of Molecular Biology*, *395*(1), 205–217.
- Pfingsten, J. S., Costantino, D. A., & Kieft, J. S. (2006). Structural basis for ribosome recruitment and manipulation by a viral IRES RNA. *Science*, *314*(5804), 1450–1454.

- Pfingsten, J. S., Costantino, D. A., & Kieft, J. S. (2007). Conservation and diversity among the three-dimensional folds of the Dicistroviridae intergenic region IRESes. *Journal of Molecular Biology*, 370(5), 856–869.
- Pflugheber, J., Fredericksen, B., Sumpter, R., Wang, C., Ware, F., Sodora, D. L., & Gale, M. (2002). Regulation of PKR and IRF-1 during hepatitis C virus RNA replication. *Proceedings of the National Academy of Sciences of the United States of America*, 99(7), 4650–4655.
- Pisarev, A. V., Chard, L. S., Kaku, Y., Johns, H. L., Shatsky, I. N., & Belsham, G. J. (2004). Functional and structural similarities between the internal ribosome entry sites of hepatitis C virus and porcine teschovirus, a picornavirus. *Journal of Virology*, 78(9), 4487–4497.
- Pisarev, A. V., Hellen, C. U. T., & Pestova, T. V. (2007). Recycling of eukaryotic posttermination ribosomal complexes. *Cell*, 131(2), 286–299.
- Pisarev, A. V., Skabkin, M. A., Pisareva, V. P., Skabkina, O. V., Rakotondrafara, A. M., Hentze, M. W., Hellen, C. U. T., et al. (2010). The role of ABCE1 in eukaryotic posttermination ribosomal recycling. *Molecular Cell*, 37(2), 196–210.
- Pisareva, V. P., Pisarev, A. V., & Fernández, I. S. (2018). Dual tRNA mimicry in the Cricket Paralysis Virus IRES uncovers an unexpected similarity with the Hepatitis C Virus IRES. *eLife*, 7.
- Plank, T.-D. M., & Kieft, J. S. (2012). The structures of nonprotein-coding RNAs that drive internal ribosome entry site function. *Wiley interdisciplinary reviews*. *RNA*, *3*(2), 195–212.
- Plant, E. P., & Dinman, J. D. (2005). Torsional restraint: a new twist on frameshifting pseudoknots. *Nucleic Acids Research*, 33(6), 1825–1833.
- Plotch, S. J., Bouloy, M., Ulmanen, I., & Krug, R. M. (1981). A unique cap(m7GpppXm)-

dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell*, *23*(3), 847–858.

- Plus, N., Croizier, G., Reinganum, C., & Scotti, P. D. (1978). Cricket paralysis virus and Drosophila C virus: Serological analysis and comparison of capsid polypeptides and host range. *Journal of invertebrate pathology*, *31*(3), 296–302.
- Poulis, P., Patel, A., Rodnina, M. V., & Adio, S. (2022). Altered tRNA dynamics during translocation on slippery mRNA as determinant of spontaneous ribosome frameshifting. *Nature Communications*, 13(1), 4231.
- Prats, A.-C., David, F., Diallo, L. H., Roussel, E., Tatin, F., Garmy-Susini, B., & Lacazette, E. (2020). Circular RNA, the key for translation. *International Journal of Molecular Sciences*, 21(22).
- Qiao, Q., Yan, Y., Guo, J., Du, S., Zhang, J., Jia, R., Ren, H., et al. (2017). A review on architecture of the gag-pol ribosomal frameshifting RNA in human immunodeficiency virus: a variability survey of virus genotypes. *Journal of Biomolecular Structure & Dynamics*, 35(8), 1629–1653.
- Rabl, J., Leibundgut, M., Ataide, S. F., Haag, A., & Ban, N. (2011). Crystal structure of the eukaryotic 40S ribosomal subunit in complex with initiation factor 1. *Science*, *331*(6018), 730–736.
- Ratje, A. H., Loerke, J., Mikolajka, A., Brünner, M., Hildebrand, P. W., Starosta, A. L.,
 Dönhöfer, A., et al. (2010). Head swivel on the ribosome facilitates translocation by means of intra-subunit tRNA hybrid sites. *Nature*, *468*(7324), 713–716.
- Reinganum, C., O'Loughlin, G. T., & Hogan, T. W. (1970). A nonoccluded virus of the field crickets Teleogryllus oceanicus and T. commodus (Orthoptera: Gryllidae). *Journal of*

invertebrate pathology, 16(2), 214–220.

- Ren, Q., Wang, Q. S., Firth, A. E., Chan, M. M. Y., Gouw, J. W., Guarna, M. M., Foster, L. J., et al. (2012). Alternative reading frame selection mediated by a tRNA-like domain of an internal ribosome entry site. *Proceedings of the National Academy of Sciences of the United States of America*, 109(11), E630-9.
- Riechmann, J. L., Laín, S., & García, J. A. (1992). Highlights and prospects of potyvirus molecular biology. *The Journal of General Virology*, 73 (Pt 1), 1–16.
- Rijnbrand, R., van der Straaten, T., van Rijn, P. A., Spaan, W. J., & Bredenbeek, P. J. (1997). Internal entry of ribosomes is directed by the 5' noncoding region of classical swine fever virus and is dependent on the presence of an RNA pseudoknot upstream of the initiation codon. *Journal of Virology*, 71(1), 451–457.
- Roberts, L. O., & Groppelli, E. (2009). An atypical IRES within the 5' UTR of a dicistrovirus genome. *Virus Research*, *139*(2), 157–165.
- Robert, F., Kapp, L. D., Khan, S. N., Acker, M. G., Kolitz, S., Kazemi, S., Kaufman, R. J., et al. (2006). Initiation of protein synthesis by hepatitis C virus is refractory to reduced eIF2.GTP.Met-tRNA^{iMet} ternary complex availability. *Molecular Biology of the Cell*, *17*(11), 4632–4644.
- Rodamilans, B., Valli, A., Mingot, A., San León, D., Baulcombe, D., López-Moya, J. J., & García, J. A. (2015). RNA polymerase slippage as a mechanism for the production of frameshift gene products in plant viruses of the potyviridae family. *Journal of Virology*, *89*(13), 6965–6967.
- Rodríguez, P. L., & Carrasco, L. (1993). Poliovirus protein 2C has ATPase and GTPase activities. *The Journal of Biological Chemistry*, 268(11), 8105–8110.

- Rom, E., & Kahana, C. (1994). Polyamines regulate the expression of ornithine decarboxylase antizyme in vitro by inducing ribosomal frame-shifting. *Proceedings of the National Academy of Sciences of the United States of America*, 91(9), 3959–3963.
- Ron, D., & Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews. Molecular Cell Biology*, 8(7), 519–529.
- Rowlands, A. G., Panniers, R., & Henshaw, E. C. (1988). The catalytic mechanism of guanine nucleotide exchange factor action and competitive inhibition by phosphorylated eukaryotic initiation factor 2. *The Journal of Biological Chemistry*, 263(12), 5526–5533.
- Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W. C., & Sonenberg, N. (1990).
 Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F.
 Molecular and Cellular Biology, *10*(3), 1134–1144.
- Ruehle, M. D., Zhang, H., Sheridan, R. M., Mitra, S., Chen, Y., Gonzalez, R. L., Cooperman, B.S., et al. (2015). A dynamic RNA loop in an IRES affects multiple steps of elongation factor-mediated translation initiation. *eLife*, *4*.
- Sabath, N., Price, N., & Graur, D. (2009). A potentially novel overlapping gene in the genomes of Israeli acute paralysis virus and its relatives. *Virology Journal*, *6*, 144.
- Sadasivan, J., Vlok, M., Wang, X., Nayak, A., Andino, R., & Jan, E. (2022). Targeting Nup358/RanBP2 by a viral protein disrupts stress granule formation. *PLOS Pathogens*, 18(12), e1010598.
- Sánchez-Eugenia, R., Goikolea, J., Gil-Cartón, D., Sánchez-Magraner, L., & Guérin, D. M. A. (2015). Triatoma virus recombinant VP4 protein induces membrane permeability through dynamic pores. *Journal of Virology*, 89(8), 4645–4654.

Schmidt, C., Becker, T., Heuer, A., Braunger, K., Shanmuganathan, V., Pech, M.,

Berninghausen, O., et al. (2016). Structure of the hypusinylated eukaryotic translation factor eIF-5A bound to the ribosome. *Nucleic Acids Research*, *44*(4), 1944–1951.

- Shao, S., Murray, J., Brown, A., Taunton, J., Ramakrishnan, V., & Hegde, R. S. (2016). Decoding Mammalian Ribosome-mRNA States by Translational GTPase Complexes. *Cell*, 167(5), 1229-1240.e15.
- Sharma, V., Prère, M.-F., Canal, I., Firth, A. E., Atkins, J. F., Baranov, P. V., & Fayet, O. (2014). Analysis of tetra- and hepta-nucleotides motifs promoting -1 ribosomal frameshifting in Escherichia coli. *Nucleic Acids Research*, 42(11), 7210–7225.
- Sharp, T. V., Xiao, Q., Jeffrey, I., Gewert, D. R., & Clemens, M. J. (1993). Reversal of the double-stranded-RNA-induced inhibition of protein synthesis by a catalytically inactive mutant of the protein kinase PKR. *European Journal of Biochemistry / FEBS*, 214(3), 945– 948.
- Shin, B.-S., Katoh, T., Gutierrez, E., Kim, J.-R., Suga, H., & Dever, T. E. (2017). Amino acid substrates impose polyamine, eIF5A, or hypusine requirement for peptide synthesis. *Nucleic Acids Research*, 45(14), 8392–8402.
- Shi, M., Lin, X.-D., Tian, J.-H., Chen, L.-J., Chen, X., Li, C.-X., Qin, X.-C., et al. (2016). Redefining the invertebrate RNA virosphere. *Nature*, *540*(7634), 539–543.
- Shrestha, N., Bahnan, W., Wiley, D. J., Barber, G., Fields, K. A., & Schesser, K. (2012). Eukaryotic initiation factor 2 (eIF2) signaling regulates proinflammatory cytokine expression and bacterial invasion. *The Journal of Biological Chemistry*, 287(34), 28738– 28744.
- Sizova, D. V., Kolupaeva, V. G., Pestova, T. V., Shatsky, I. N., & Hellen, C. U. (1998). Specific interaction of eukaryotic translation initiation factor 3 with the 5' nontranslated regions of

hepatitis C virus and classical swine fever virus RNAs. *Journal of Virology*, 72(6), 4775–4782.

- Skabkin, M. A., Skabkina, O. V., Dhote, V., Komar, A. A., Hellen, C. U. T., & Pestova, T. V. (2010). Activities of Ligatin and MCT-1/DENR in eukaryotic translation initiation and ribosomal recycling. *Genes & Development*, 24(16), 1787–1801.
- Sokabe, M., & Fraser, C. S. (2014). Human eukaryotic initiation factor 2 (eIF2)-GTP-MettRNAi ternary complex and eIF3 stabilize the 43 S preinitiation complex. *The Journal of Biological Chemistry*, 289(46), 31827–31836.
- Sonenberg, N. (1988). Cap-binding proteins of eukaryotic messenger RNA: functions in initiation and control of translation. *Progress in Nucleic Acid Research and Molecular Biology*, 35, 173–207.
- Spahn, C. M. T., Jan, E., Mulder, A., Grassucci, R. A., Sarnow, P., & Frank, J. (2004). Cryo-EM visualization of a viral internal ribosome entry site bound to human ribosomes: the IRES functions as an RNA-based translation factor. *Cell*, *118*(4), 465–475.
- Stern-Ginossar, N., Thompson, S. R., Mathews, M. B., & Mohr, I. (2019). Translational Control in Virus-Infected Cells. *Cold Spring Harbor Perspectives in Biology*, 11(3).
- Stetefeld, J., McKenna, S. A., & Patel, T. R. (2016). Dynamic light scattering: a practical guide and applications in biomedical sciences. *Biophysical reviews*, 8(4), 409–427.
- Sukarieh, R., Sonenberg, N., & Pelletier, J. (2010). Nuclear assortment of eIF4E coincides with shut-off of host protein synthesis upon poliovirus infection. *The Journal of General Virology*, 91(Pt 5), 1224–1228.
- Svergun, D. I. (1999). Restoring low resolution structure of biological macromolecules from solution scattering using simulated annealing. *Biophysical Journal*, 76(6), 2879–2886.

- Svitkin, Y. V., Pause, A., Haghighat, A., Pyronnet, S., Witherell, G., Belsham, G. J., & Sonenberg, N. (2001). The requirement for eukaryotic initiation factor 4A (elF4A) in translation is in direct proportion to the degree of mRNA 5' secondary structure. *RNA (New York)*, 7(3), 382–394.
- Sweeney, T. R., Abaeva, I. S., Pestova, T. V., & Hellen, C. U. T. (2014). The mechanism of translation initiation on Type 1 picornavirus IRESs. *The EMBO Journal*, 33(1), 76–92.

Tabor, C. W., & Tabor, H. (1984). Polyamines. Annual Review of Biochemistry, 53, 749-790.

- Tate, J., Liljas, L., Scotti, P., Christian, P., Lin, T., & Johnson, J. E. (1999). The crystal structure of cricket paralysis virus: the first view of a new virus family. *Nature Structural Biology*, 6(8), 765–774.
- Tate, W. P., Poole, E. S., Horsfield, J. A., Mannering, S. A., Brown, C. M., Moffat, J. G.,
 Dalphin, M. E., et al. (1995). Translational termination efficiency in both bacteria and
 mammals is regulated by the base following the stop codon. *Biochemistry and Cell Biology* (*Biochimie et Biologie Cellulaire*), 73(11–12), 1095–1103.
- Tavantzis, S. M. (Ed.). (2001). *dsRNA Genetic Elements: Concepts and Applications in Agriculture, Forestry, and Medicine* (illustrated ed.). CRC Press.
- Terenin, I. M., Smirnova, V. V., Andreev, D. E., Dmitriev, S. E., & Shatsky, I. N. (2017). A researcher's guide to the galaxy of IRESs. *Cellular and Molecular Life Sciences*, 74(8), 1431–1455.
- Tourigny, D. S., Fernández, I. S., Kelley, A. C., & Ramakrishnan, V. (2013). Elongation factor G bound to the ribosome in an intermediate state of translocation. *Science*, 340(6140), 1235490.

Triana-Alonso, F. J., Chakraburtty, K., & Nierhaus, K. H. (1995). The elongation factor 3 unique

in higher fungi and essential for protein biosynthesis is an E-site factor. *The Journal of Biological Chemistry*, *270*(35), 20473–20478.

- Tsukiyama-Kohara, K., Iizuka, N., Kohara, M., & Nomoto, A. (1992). Internal ribosome entry site within hepatitis C virus RNA. *Journal of Virology*, *66*(3), 1476–1483.
- Valášek, L. S., Zeman, J., Wagner, S., Beznosková, P., Pavlíková, Z., Mohammad, M. P.,
 Hronová, V., et al. (2017). Embraced by eIF3: structural and functional insights into the
 roles of eIF3 across the translation cycle. *Nucleic Acids Research*, 45(19), 10948–10968.
- Walsh, D., & Mohr, I. (2006). Assembly of an active translation initiation factor complex by a viral protein. *Genes & Development*, 20(4), 461–472.
- Walsh, J. A., & Jenner, C. E. (2002). Turnip mosaic virus and the quest for durable resistance. *Molecular Plant Pathology*, 3(5), 289–300.
- Wang, Q. S., Au, H. H. T., & Jan, E. (2013). Methods for studying IRES-mediated translation of positive-strand RNA viruses. *Methods*, 59(2), 167–179.
- Wang, R., Xiong, J., Wang, W., Miao, W., & Liang, A. (2016). High frequency of +1 programmed ribosomal frameshifting in Euplotes octocarinatus. *Scientific Reports*, 6, 21139.
- Wang, X., Vlok, M., Flibotte, S., & Jan, E. (2021). Resurrection of a Viral Internal Ribosome Entry Site from a 700 Year Old Ancient Northwest Territories Cripavirus. *Viruses*, *13*(3).
- Warsaba, R., Sadasivan, J., & Jan, E. (2020). Dicistrovirus-Host Molecular Interactions. *Current issues in molecular biology*, *34*, 83–112.
- Warsaba, R., Stoynov, N., Moon, K.-M., Flibotte, S., Foster, L., & Jan, E. (2022). Multiple Viral Protein Genome-Linked Proteins Compensate for Viral Translation in a Positive-Sense Single-Stranded RNA Virus Infection. *Journal of Virology*, 96(17), e0069922.

- Weisser, M., Voigts-Hoffmann, F., Rabl, J., Leibundgut, M., & Ban, N. (2013). The crystal structure of the eukaryotic 40S ribosomal subunit in complex with eIF1 and eIF1A. *Nature Structural & Molecular Biology*, 20(8), 1015–1017.
- Weiss, R. B., Dunn, D. M., Shuh, M., Atkins, J. F., & Gesteland, R. F. (1989). E. coli ribosomes re-phase on retroviral frameshift signals at rates ranging from 2 to 50 percent. *The New biologist*, 1(2), 159–169.
- Wek, R. C. (2018). Role of eIF2α Kinases in Translational Control and Adaptation to Cellular Stress. *Cold Spring Harbor Perspectives in Biology*, 10(7).
- Whelan, M., & Pelchat, M. (2022). Role of RNA Polymerase II Promoter-Proximal Pausing in Viral Transcription. *Viruses*, 14(9).
- White, J. P., Cardenas, A. M., Marissen, W. E., & Lloyd, R. E. (2007). Inhibition of cytoplasmic mRNA stress granule formation by a viral proteinase. *Cell Host & Microbe*, *2*(5), 295–305.
- Wilkinson, K. A., Merino, E. J., & Weeks, K. M. (2006). Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution. *Nature Protocols*, 1(3), 1610–1616.
- Wilson, J. E., Pestova, T. V., Hellen, C. U., & Sarnow, P. (2000). Initiation of protein synthesis from the A-site of the ribosome. *Cell*, 102(4), 511–520.
- Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., & Felgner, P. L.
 (1990). Direct gene transfer into mouse muscle in vivo. *Science*, 247(4949 Pt 1), 1465–1468.
- Won, S., Eidenschenk, C., Arnold, C. N., Siggs, O. M., Sun, L., Brandl, K., Mullen, T.-M., et al. (2012). Increased susceptibility to DNA virus infection in mice with a GCN2 mutation. *Journal of Virology*, *86*(3), 1802–1808.

- Yamamoto, H., Nakashima, N., Ikeda, Y., & Uchiumi, T. (2007). Binding mode of the first aminoacyl-tRNA in translation initiation mediated by Plautia stali intestine virus internal ribosome entry site. *The Journal of Biological Chemistry*, 282(11), 7770–7776.
- Yamamoto, H., Unbehaun, A., Loerke, J., Behrmann, E., Collier, M., Bürger, J., Mielke, T., et al. (2014). Structure of the mammalian 80S initiation complex with initiation factor 5B on HCV-IRES RNA. *Nature Structural & Molecular Biology*, 21(8), 721–727.
- Yanagiya, A., Svitkin, Y. V., Shibata, S., Mikami, S., Imataka, H., & Sonenberg, N. (2009).
 Requirement of RNA binding of mammalian eukaryotic translation initiation factor 4GI (eIF4GI) for efficient interaction of eIF4E with the mRNA cap. *Molecular and Cellular Biology*, 29(6), 1661–1669.
- Yang, Y., & Wang, Z. (2019). IRES-mediated cap-independent translation, a path leading to hidden proteome. *Journal of Molecular Cell Biology*, 11(10), 911–919.
- Yu, Y., Abaeva, I. S., Marintchev, A., Pestova, T. V., & Hellen, C. U. T. (2011). Common conformational changes induced in type 2 picornavirus IRESs by cognate trans-acting factors. *Nucleic Acids Research*, 39(11), 4851–4865.
- Yu, Y., Marintchev, A., Kolupaeva, V. G., Unbehaun, A., Veryasova, T., Lai, S.-C., Hong, P., et al. (2009). Position of eukaryotic translation initiation factor eIF1A on the 40S ribosomal subunit mapped by directed hydroxyl radical probing. *Nucleic Acids Research*, *37*(15), 5167–5182.
- Zayed, A. A., Wainaina, J. M., Dominguez-Huerta, G., Pelletier, E., Guo, J., Mohssen, M., Tian,
 F., et al. (2022). Cryptic and abundant marine viruses at the evolutionary origins of Earth's
 RNA virome. *Science*, *376*(6589), 156–162.

Zhao, J., Li, Y., Wang, C., Zhang, H., Zhang, H., Jiang, B., Guo, X., et al. (2020). Iresbase: A

comprehensive database of experimentally validated internal ribosome entry sites. Genomics, proteomics & bioinformatics / Beijing Genomics Institute, 18(2), 129–139.

- Zhou, W.-Y., Cai, Z.-R., Liu, J., Wang, D.-S., Ju, H.-Q., & Xu, R.-H. (2020). Circular RNA: metabolism, functions and interactions with proteins. *Molecular Cancer*, *19*(1), 172.
- Zubradt, M., Gupta, P., Persad, S., Lambowitz, A. M., Weissman, J. S., & Rouskin, S. (2017).
 DMS-MaPseq for genome-wide or targeted RNA structure probing in vivo. *Nature Methods*, 14(1), 75–82.
- Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research*, *31*(13), 3406–3415.