Biochemical Analysis of Translational Recoding Driven by 2A Peptide

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Thesis submitted to Newcastle University in candidature for the degree of Doctor of Philosophy

May 2018

Declaration

I hereby declare that I alone have composed the thesis and that, except where stated, the work presented herein is my own.

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ABSTRACT

2A/2A-like peptides are short sequences (20-30 amino acids) encoded predominantly within open reading frames (ORFs) of RNA viruses. They drive a non-canonical translation, in which the nascent chain is released from the ribosome at a sense (proline) codon, followed by continued translation to generate a separate downstream protein, initiated from the same proline codon. The aim of this study is to investigate the role of ribosomal factors in the 2A reaction in *Saccharomyces cerevisiae* cells. Results obtained showed that reduced activity of eRF1/3 inhibits the 2A reaction. This inhibition did not strongly correlate with the effect that mutations have on termination at stop codons. In particular, several mutations within the NIKS motif, which is essential for stop codon recognition, had minimal effect on the 2A reaction. To confirm these results, we developed a new reporter to investigate the 2A activity, where the green fluorescent protein (GFP) sequence was separated with a 2A sequence, between residues 157 and 158. This reporter was utilised to confirm the effects of eRF1 mutations, previously assessed by immunoprecipitation, and results, observed by flow cytometry, revealed consistency in terms of the role of eRFs in the 2A reaction. In summary, these observations provide evidences supporting recruitment of eRFs to the ribosome to drive the non-canonical termination event that releases the first part of the 2A reaction.

Acknowledgements

Firstly, I must thank my sponsor, The Ministry of Higher Education and the University of Mosul/IRAQ, for providing me the opportunity to achieve my career towards obtaining my PhD and also my supervisor, **Dr. Jeremy Brown** (Newcastle University), for his help, support and his willingness to impart his knowledge to his students during my four years of study.

I would also like to thank **Dr. Peter Banks** (Professor Lydall's lab, Newcastle University) for providing yeast strains for my study and **Dr. Tobias von der Haar** (University of Kent) for providing plasmids used in some of my experiments. I extend my thanks to **Dr. Andrew Filby** and all the staff in the Flow Cytometry Core Facility for their help. Finally, I would like to thank all my friends and colleagues who helped me in some points of my research during the past four years and to **Soulaf** and **Shiny** who gave me much support and help with my work, which I greatly appreciated.

I am deeply indebted to **my mother and brother, Faris**, whose blessing and support continued during a period of bitter experience in our country while I was privileged to study in a peaceful environment. I am especially grateful to my lovely long lost cousin, **Fatin** for her care and support. Being reunited with her after so many years was a source of comfort by restoring a sense of family.

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List of Abbreviations

ABCE1	ATP-binding cassette 1
ADP	adenosine 5'-diphosphate
Amp	ampicillin
APS	ammonium persulphate
ATP	adenosine 5'-triphosphate
bp	base pair
ddH_2O	double distilled water
dH_2O	distilled water
DNA	deoxynucleic acid
dNTPS	dideoxynucleoside triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eEFs	Eukaryotic Elongation Factors
eIFs	Eukaryotic Initiation Factors
eRFs	Eukaryotic Release Factor
g	gram(s)
GFP	green fluorescent protein
GTP	guanine 5'-triphosphate
H_2O_2	hydrogen peroxide
IRES	Internal Ribosomal Entry Sites
kb	kilo base
kDa	kilodalton
L	litre(s)
LB	Lysogenic Broth
M	Mole
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
MOPS	Morpholinopropanesulfonic acid
mRNA	messenger ribonucleic acid
nt	nucleotides

OD	optical density
ORF	open reading frame
PAC	puromycin N-acetyltranferase
PCI	phenol:chloroform:isoamylalcohol
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIC	Pre-initiation complex
PMSF	phenylmethylsulphonyl fluoride
PTC	peptidyl-transferase centre
Rli1	Ribonuclease inhibitor 1
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolution per min
RRF	Ribosomal Recycling Factor
SDS	sodium dodecyl sulphate
sec	seconds
Ski7	Super killer 7
TBS	tris buffered saline
TC	Ternary complex
TCA	tricholoroacetic acid
TEMED	tetramethylethylenediamine
Tris	tris hydroxymethylaminomethane
tRNA	transfer ribonucleic acid
µg	micro gram(s)
µl	micro litre(s)
Ub	ubiquitin
UTR	Untranslated region
V	volt(s)
v/v	volume per unit volume
w/v	weight per unit volume
YPD	yeast peptone dextrose

Amino Acid Nomenclature

Amino acids	Symbols		Codons
Alanine	Ala	A	GCA, GCC, GCG, GCU
Cysteine	Cys	C	UGC, UGU
Aspartic acid	Asp	D	GAC, GAU
Glutamic acid	Glu	E	GAA, GAG
Phenylalanine	Phe	F	UUC, UUU
Glycine	Gly	G	GGA, GGC, GGG, GGU
Histidine	His	H	CAC, CAU
Isoleucine	Ile	I	AUA, AUC, AUU
Lysine	Lys	K	AAA, AAG
Leucine	Leu	L	UUA, UUG, CUA, CUC, CUG, CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC, AAU
Proline	Pro	P	CCA, CCC, CCG, CCU
Glutamine	Gln	Q	CAA, CAG
Arginine	Arg	R	AGA, AGG, CGA, CGC, CGG, CGU
Serine	Ser	S	AGC, AGU, UCA, UCC, UCG, UCU
Threonine	Thr	T	ACA, ACC, ACG, ACU
Valine	Val	V	GUA, GUC, GUG, GUU
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAC UAU

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1 INTRODUCTION

1.1 Protein synthesis

Translation is the mechanism by which proteins are made in the cell, using the information stored in a molecule of mRNA. The mRNA sequence is decoded by the ribosome, with the help of the translation factors. Regulation of transcription and the consequent translation, in addition to the mRNA turnover, define the amount and activity of the synthesized protein (Rodnina, 2016). Proteins are also subjected to post-translational modification after translation is completed, this ensures the full activity of the protein with proper folding and functional groups on the protein structure, and post-translational modification frequently plays a key role in regulating protein activity. Eukaryotic canonical translation includes four steps: initiation, elongation, termination and ribosomal recycling. Initiation and elongation are the steps at which most translation is regulated and which also provide checkpoints for the accuracy of translation. A variety of signals on the mRNA sequence may control initiation through the selection of the open reading frame (ORF) (Rodnina, 2016). This thesis focusses on translation in eukaryotes, and thus most of the information below is specific to eukaryotic protein synthesis.

1.2 Messenger Ribonucleic Acid (mRNA)

mRNA is a single stranded nucleic acid, carrying genetic codes for proteins to the ribosome. mRNA comprises ~5% of the total RNA in the cell. In eukaryotes, mRNA is transcribed by RNA polymerase II, and following transcription, the initial precursor mRNA is modified by splicing to remove introns, as well as addition of a cap and poly(A) tail. The cap is an inverted guanosine, which is methylated on the position 7-nitrogen of the guanosine ring (m^7G). The cap structure stabilises the mRNA from exonucleolytic digestion and also directs binding of the ribosome. The cap is normally followed by a non-coding untranslated region (the 5’ UTR). The 5’ UTR provides the space for the 40S subunit to bind and initiate translation. Eukaryotic mRNAs typically contain one open reading frame (ORF), starting with a start codon (AUG for methionine) and ending with a stop codon. The ORF is decoded in the 5’ to 3’ direction. Each codon within the ORF consists of three bases, representing a specific amino acid. Following the ORF, there is another UTR (3’ UTR). The mRNA ends in a poly(A) tail (50-200 nucleotides) (figure 1.1). This tail is added by poly(A) polymerase and, like the cap structure, provides stability to the mRNA.

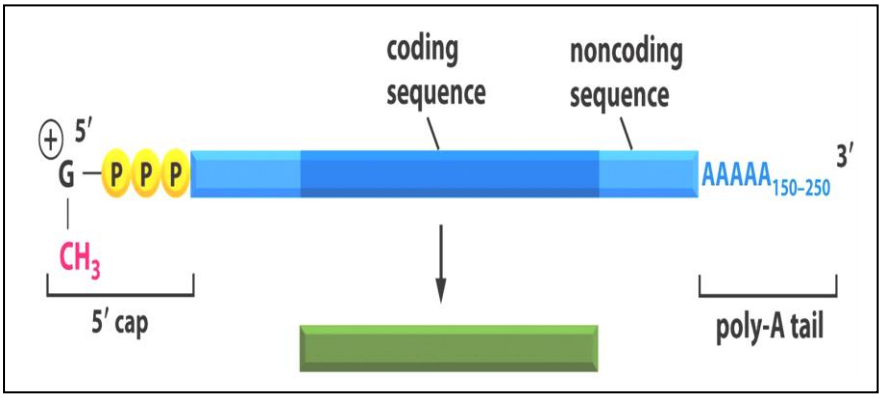

Figure 1.1: Structure of eukaryotic mRNA, see text for details. Adapted from: http://molbiol4masters.masters.grkraj.org/html/Ribose_Nucleic_Acid8-Stability_of_mRNAs_and_its_Regulation.htm

1.3 The ribosome

Ribosomes, in both eukaryotes and prokaryotes, are responsible for all protein synthesis. Ribosomes comprise two subunits, termed small and large, which differ slightly in size depending on their source. Thus the small subunit is 30S in bacteria, 40S in eukaryotes, and the large subunit 50S in bacteria, 60S in eukaryotes. Both subunits are composed of ribosomal RNA (rRNA) and protein, with the eukaryotic ribosome containing more rRNA and ribosomal proteins (r-proteins) than their bacterial counterparts.

About 7000 nucleotides of rRNA are contained in eukaryotic ribosomes. The 40S subunit consists of 18S rRNA with 33 proteins, while the 60S subunit consists of 5S, 5.8S, and 25S rRNA and 46 proteins. The assembly of the 40S and 60S subunits yields the 80S ribosome, which contains three binding sites for transfer RNA (tRNA); A (aminoacyl) site, where each codon is recognized and the corresponding aminoacyl tRNA binds, P (peptidyl) site, where the peptide-tRNA chain is hosted and E (exit) site where the de-acylated tRNA leaves the ribosome (figure 1.2). The two key activities of the ribosome, codon recognition and catalysis of peptide bond formation, are found in the 40S and 60S subunits respectively (Yusupova and Yusupov, 2017).

The junction between A and P sites within the 60S subunit is called the peptidyl transferase centre (PTC), this is the location for the peptide bond formation between the aminoacyl-tRNA in the A site and the peptidyl-tRNA in the P site. An aqueous ‘exit’ tunnel, with 15 Å average diameter, is extended from the PTC through the P site (figure 1.2) (Schneider-Poetsch *et al.*, 2010) and the whole 60S subunit. This tunnel hosts the growing nascent peptide chain. The main constituent of the exit tunnel inner walls is rRNA with only 3 r-proteins contributing to

the tunnel wall (rpL4, L22, L39), these r-proteins assume the co-translation processing for the nascent peptide chain (Marino *et al.*, 2016).

Cryo-EM examination of the yeast ribosome has revealed that the rRNA is incorporated in the core of each subunit, whereas r-proteins are largely located on the surface. The 18S rRNA sequences form four secondary domains, which tend to fold into tertiary structures, these tertiary structures with r-proteins form the main skeleton for the 40S subunit. The 5.8S and 25S rRNAs in the 60S subunit also tend to fold, but into six domains (I–VI), which ensures proper base pairing between 5.8S and 25S rRNAs (Woolford *et al.*, 2013).

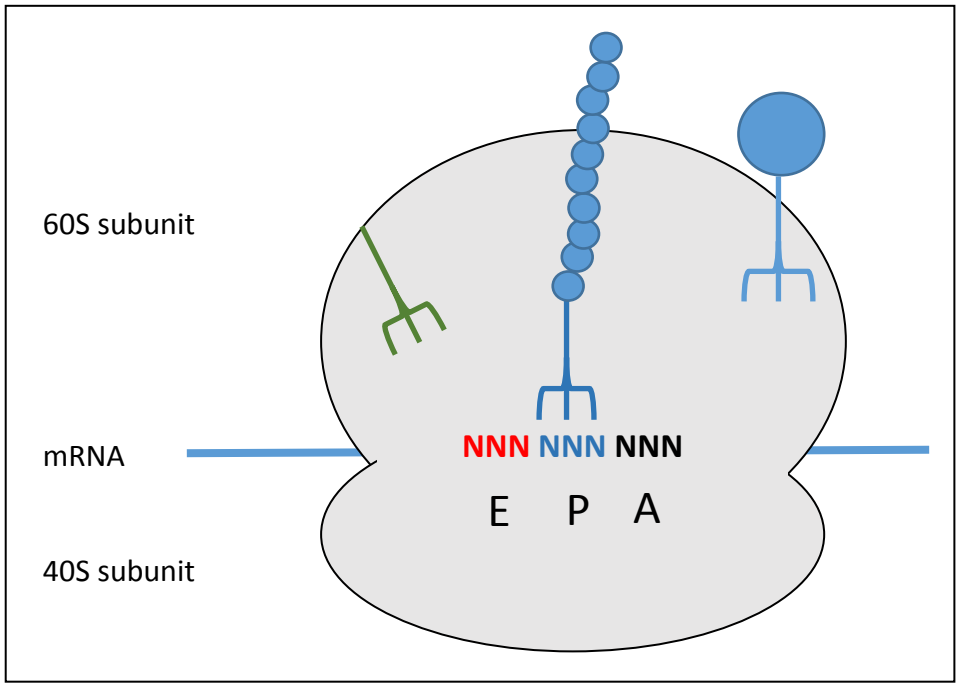

Figure1.2: Structural outlines of the eukaryotic ribosome.

A schematic structure of the eukaryotic ribosome shows the 40S and 60S ribosomal counterparts with mRNA at the interface. A codon of an amino acid is contained in the ribosomal A site, and an appropriate aminoacyl-tRNA, carried by elongation factor 1A (eEF1A), binds the codon in the A site. The peptidyl-tRNA is attached to the ribosomal P site, whereas the de-acylated tRNA leaves the ribosome from the ribosomal E site. The scheme is an outlined example for the canonical elongation.

1.4 The steps of translation

1.4.1 Initiation

Translation initiation is the first step of translation which undertakes the proper binding of the mRNA to the ribosome with the aid of ~20 initiation factors (eIFs) to find the proper start codon on the mRNA sequence. The involvement of many eIFs in the initiation rates this step as the most complicated step among the translation steps (Schmidt *et al.*, 2016). Under various

stressors, canonical initiation is impeded, which reduces the overall protein synthesis (Piccirillo *et al.*, 2014).

1.4.1.1 Cap-dependent initiation

In general, initiation is the rate-limiting step of translation (Piccirillo *et al.*, 2014), and in eukaryotes, translation on most mRNAs occurs through the 'cap-dependent mechanism'. This requires the 5' m^7G cap structure of the mRNA to be recognized by the 40S subunit and eIFs (Hinnebusch *et al.*, 2016). A key step in initiation is the formation of the ternary complex, this consists of eIF2 bound to $tRNA^{met}$ (met-tRNA) and GTP, a process which is controlled by the guanine nucleotide exchange factor eIF2B (Gomez and Faurobert, 2002). Ternary complex binds to the 40S subunit, along with eIF1, eIF1A, eIF3 and eIF5, generating the 43S pre-initiation complex (PIC), which is competent for binding to the mRNA. At this stage, eIF3 may change the conformational status of the 40S subunit to permit access for the Met-tRNA, attached to the eIF2 (des Georges *et al.*, 2015). eIF5 serves as an adaptor between 40S subunit-eIF3 and the ternary complex (des Georges *et al.*, 2015) (figure 1.3).

In order for a mRNA to bind to the PIC, the 5′ m^7G cap must be bound by eIF4F, a complex comprising eIF4E, eIF4G and eIF4A (Dhote *et al.*, 2012). Within the eIF4F complex, eIF4E binds to the cap structure (a rate-limiting step for initiation). eIF4E also stimulates the activity of DEAD-box helicase, eIF4A, the ATPase activity of this factor is utilised for the 5′ UTR duplex unwinding (Pause and Sonenberg, 1992). eIF4G acts as a scaffold and has binding sites for both eIF4E and eIF4A, as well as eIF3 via eIF3c, eIF3d and eIF3e subunits, to help the recruitment of the PIC (Wagner *et al.*, 2014). A further factor, eIF4B also stimulates eIF4A helicase activity. It has been recently demonstrated that there is an eIF4F-independent role for eIF4B in this context, which is added to its known function as eIF4A cofactor (Pestova and Kolupaeva, 2002; Mancera-Martinez *et al.*, 2017). After the PIC properly assembles on the mRNA, a process termed scanning starts. If the 5′ UTR is unstructured, a minimum set of factors, 40S plus eIF1, eIF2-Met-tRNA and eIF3, is required to scan the 5′ UTR. This indicates that scanning is an intrinsic property of the 40S subunit. However, in most cases, the 5′ UTR is structured, and it requires further helicase activity for scanning. Specifically, the DEAD-box helicase DHX29 has been identified as a factor that promotes scanning through structured 5' UTRs (Pisarev *et al.*, 2008). In addition, ATP hydrolysis promotes the function of some DEAD-box family members either through the helicase activity or to stimulate the ribosome translocation in a 5′ to 3′ direction (Pisareva and Pisarev, 2016). Once the complex finds the appropriate AUG, which is characterized by a favourable context (purine in position 3 with G

in +4), scanning stops. The PIC components eIF1 and eIF1A both function to improve scanning and are displaced from the ribosomal P site to ensure base-pairing between Met-tRNA and the AUG (Lomakin and Steitz, 2013). Within the PIC a basic loop of eIF1 is located in the P site, which stabilises the association of the AUG with the tRNA anticodon. This interaction, along with GTP hydrolysis, leads to the displacement of the eIF1 basic loop, and this plus other structural reorganisation of the complex results in formation of the 48S complex (Lomakin and Steitz, 2013). eIF2-GDP is then released from the complex and eIF5B acts to stabilize Met-tRNA (Pisareva and Pisarev, 2014). At this point, the 60S subunit joins the complex, a reaction promoted by the GTPase eIF5B, forming the 80S ribosome which is ready now to decode the mRNA and generate peptide chains (basic steps of cap-deprndent initiation are indicated in figure 1.3).

1.4.1.2 Cap-independent initiation of viral ORFs

While the majority of translation initiation in eukaryotes is driven by the canonical pathway outlined above, some mRNAs are efficiently translated while the recognition of the m^7G cap is impaired. To address this mechanism, studies have focused on this point and an alternative cap-independent initiation pathway has been described which requires Internal Ribosomal Binding Sites (IRES) (Jackson, 2013). IRES were first discovered in 1988 when Pelletier and Sonenberg (1988) detected an efficient internal initiation of translation on a structured 5′ UTR of Polioviruses. This mechanism bypasses the requirement for eIF4E to bind the cap structure. The key feature of this mechanism is direct recruitment of 40S subunits to internal portions of a mRNA. Subsequent analysis has revealed that IRES-driven translation can occur via several different routes, with some using scanning downstream of the initial 40S binding-site in search of the AUG, while in other cases the 40S subunit is directly loaded to the vicinity of the AUG. IRES also have different requirements for canonical initiation factors. In some, such as the Dicistrovirus intergenic IRES, they are entirely independent of any eIFs, while others require a subset of initiation factors, such as eIF4G and eIF3 (Lozano and Martinez-Salas, 2015; Abaeva *et al.*, 2016). Many viruses have been shown to use IRES sequences to direct the translation of the viral protein, typically coupled with the ability to de-activate host cap-dependent translation initiation through cleaving eIFs, and increasing the abundance of the viral mRNA over the eukaryotic mRNA (Pelletier and Sonenberg, 1988).
The secondary and tertiary structures of IRES are likely to account for an effective, direct or indirect, interaction with the 40S ribosomal subunit. Indirect interaction needs assistance of some eIFs and IRES trans-acting factors (ITAFs), which helps the binding of the 40S subunit to the viral mRNA through their ability to stabilize specific conformations of IRES elements

(Komar and Hatzoglou, 2015). An example of this is binding of ITAF45 to the foot-and-mouth disease (FMDV) IRES which causes changes in the structure of the IRES that in turn enhances binding of both eIF4A and eIF4G (Song *et al.*, 2005). In general, the requirement for eIFs and ITAFs is reduced in the more structured IRES. Figure 1.4 illustrates the outlines of cap-independent initiation.

While IRES were first characterised as being important in viral gene expression, cellular mRNAs also contain IRES. These cellular IRES are different from each other, in terms of both sequence and secondary structure (Baird *et al.*, 2006). Mammalian species having this IRES are rodents and ruminants, but it is also found in other vertebrates such as birds and frogs (Sherrill and Lloyd, 2008). Putative cellular IRES are considered, on the base of eIFs requirements, closest to Picorna virus IRES (Jackson, 2013).

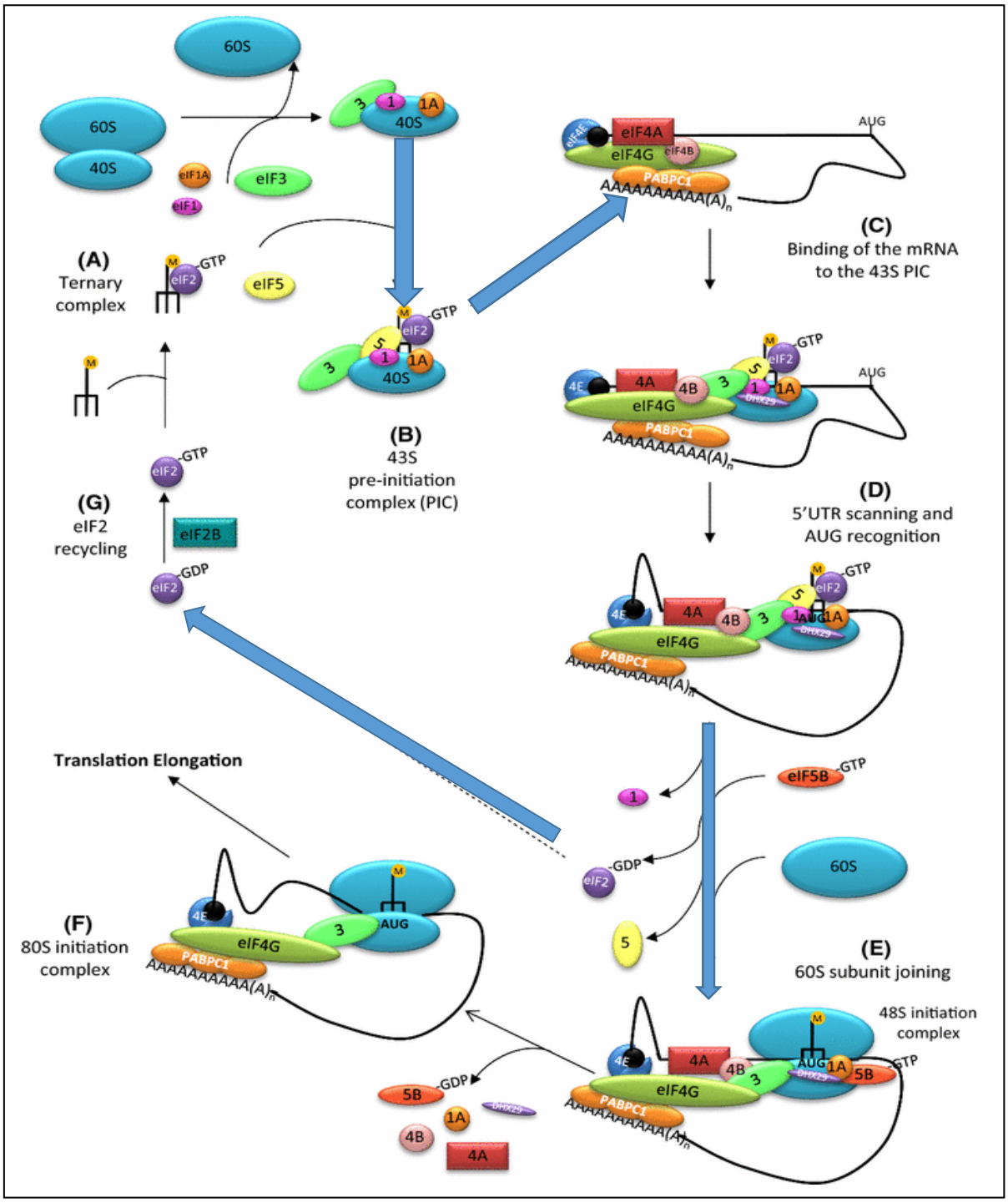

Figure 1.3: Canonical initiation of translation in eukaryotes. Adapted from: Lacerda *et al* (2016).

Schematic model for eukaryotic canonical initiation. (A) The formation of ternary complexes, eIF2 associated with Met-tRNA and GTP. (B) The ternary complex binds to the 40S subunit with the help of eIF1, eIF1A, eIF3 and eIF5, forming the 43S pre-initiation complex. (C) The 43S binds to the cap structure at mRNA 5′ end. (D) The scanning ends when the 43S complex encounters the AUG to bind and form the 48S initiation complex. (E) The 60S subunit joins to form the 80S ribosome. eIF5B hydrolyses the eIF2–GTP. (F) Releasing the initiation factors, eIF5B–GTP then binds to the 40S subunit and accelerates the rate of 60S subunit joining. (G) The ribosome enters the elongation phase, eIF2 is recycled to enable a next initiation step.

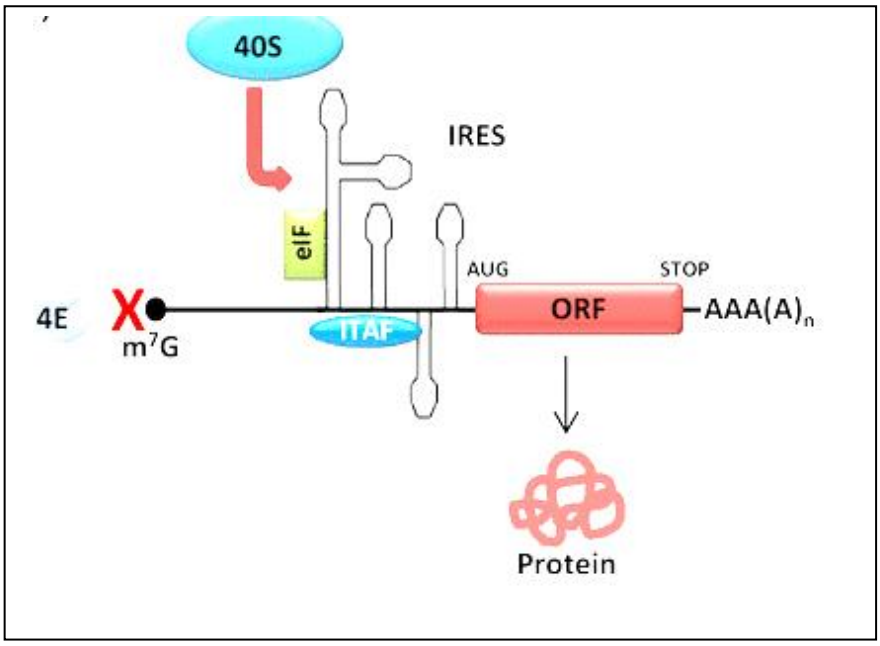

Figure 1.4: Cap-independent initiation. Adapted from: Lacerda *et al* (2016).

Schematic model of IRES-dependent initiation. mRNA secondary structures recruit the 40S subunit to the AUG of the ORF, skipping, or not, the scanning process. eIFs and/or IRES (ITAFs) may or may not assist in this context. See text for details.

Diverse mechanisms have been employed by viruses to yield efficient translation of their mRNA, and to out-compete cellular mRNAs (Lopez-Lastra *et al.*, 2010). The dependence of host translation on the m^7G cap structure is exploited by viruses. Normally, the cap structure at the 5' end of the mRNA is recognized by eIF4F complex, then, the 40S subunit is recruited to form the 43S PIC, which scans the mRNA for the appropriate AUG. Plant and animal viral mRNAs are uncapped, i.e. they lack the m^7G cap structure at the 5' of the mRNA, thus eIF4F is not recruited. Many mechanisms are used by viruses to inhibit the cap-dependent initiation, these mechanisms include de-activation of eIFs (Lloyd, 2006). A common cap-independent translation, carried out by viruses, is IRES-mediated initiation. IRES recruit the required eIFs and ribosomal subunits to a vicinity of the appropriate AUG on the viral mRNA. This form of initiation was first identified in poliovirus, and has since been observed in a variety of viral and cellular mRNAs including: Picornaviruses, Hepatitis C virus and Pestvirus etc (Griffiths and Coen, 2005). About 84 nts of unstructured sequence has been identified just upstream to the *Turnip crinkle virus* (TCV) ORF of the coat protein. This promotes the expression of the coat protein from genomic RNA. The coat protein IRES is universally conserved in viruses of the same genus. IRES is eIF4G-dependent, but not eIF4E, in terms of the activity. The translation of the coat protein from the viral genomic RNA seems to be essential to stimulate the host immune system. It has been suggested that both cellular and viral IRES have same strategies

for translation (Bugaud *et al.*, 2017). Infection with RNA viruses produces cell stress, and one of the cellular responses is activation of the eIF2α kinase PKR, which recognises double stranded RNA. PKR and other eIF2 kinases phosphorylate the α subunit of eIF2 (Garcia *et al.*, 2007), resulting in repression of translation thereby depleting the ternary complex. Most viral initiation steps require a ternary complex. However, viral RNA encodes proteins that counter translation of cellular mRNA. An example of this is ICP34.5 (Herpes Simplex Viral Protein), that requires phosphatase PI which dephosphorylates eIF2α and reactivates translation, after being initially inhibited (Li *et al.*, 2011). As noted above (section 1.4.1.2), the *Dicistroviridae* inter-genic IRES does not require any eIFs. It resembles tRNA, inserts into the ribosomal P site and triggers initiation at an Ala codon, which circumvents the requirements for Met-tRNA. A number of animal viruses have developed a strategy to cope with the deactivation of eIF2α, by using alternative proteins to deliver the initiator tRNA including, eIF2A (Merrick and Anderson, 1975), and ligatin (known as eIF2D) (Dmitriev *et al.*, 2010) and eIF5B (Terenin *et al.*, 2008).

1.4.2 Elongation

Elongation is the second step of translation during which the ORF is decoded, and the amino acid corresponding to each codon is added to the growing chain. In fungi including S*accharomyces cerevisiae,* elongating the peptide chain requires three elongation factors, eEF1 complex in addition to eEF2 and eEF3 (Bourne *et al.*, 1991).

The eEF1 consists of three subunits: eEF1A (50 kDa), eEF1Bα (23 kDa) and eEF1Bγ (48 kDa). It delivers the aminoacylated tRNA to the ribosomal A site. The structure of eEF1A is conserved between eukaryotes and prokaryotes (where it is termed EF-Tu). It is composed of three domains, termed I, II and III. Domain I binds guanine nucleotides, while domains II and III contain the binding site for aminoacyl tRNA (aa-tRNA). A hydrophobic pocket between domains I and II is a binding site for eEF1Bα (Andersen *et al.*, 2003). eEF1A binds aminoacylated tRNA in a ternary complex with GTP, and this form binds to the elongating ribosome in a codon-anticodon dependent manner in the ribosomal A site.

A general model for translation elongation starts by binding of an eEF1A-GTP-aa-tRNA ternary complex (TC) to the A site, GTP hydrolysis is then triggered by codon recognition, allowing the accommodation of the specific aa-tRNA in the ribosomal A site. Once this is achieved, the ribosome catalyses peptide bond formation. Nucleophilic attack of the aa-tRNA in the ribosomal A site occurs on the ester carbon of the peptidyl-tRNA in the P site. It has been proposed that a transient intermediate product is formed to be then broken down into deacylated tRNA and elongated peptidyl-tRNA. A76 of the peptidyl-tRNA may play a key role by serving

as a proton shuttle which extracts a proton from the amino group to be donated to the de-acylated 3′ hydroxyl (Kuhlenkoetter *et al.*, 2011). Following peptide bond formation, eEF1 is released from the ribosome, the de-acylated P site tRNA and A site peptidyl-tRNA are moved into hybrid P/E and A/P positions respectively, with the ends of tRNA in both E and P sites. eEF2-GTP binds the ribosome in this stage, the tip of domain IV of this factor is inserted in the A site. A structural model suggested that domains I and II move relative to domains III, IV and V of this factor during ribosomal translocation, which triggers the translocation of the ribosome, by sliding three nucleotides over the mRNA, which promotes the positioning of the de-acylated tRNA in the E site to be then released from the ribosome. When eEF2-GTP binds to the ribosome, a strong conformation of the 40S subunit is then induced, Domains I and II (the head of eEF2) rotate ~18º to the E site, resulting in structural changes, and allowing the movement of the amino acyl tRNA to the post state (Zhou *et al.*, 2013). Steps of elongation are outlined in figure 1.5.

eEF3 is a translation factor unique to fungi, which binds the ribosome close to the E site and promotes the release of the de-acylated tRNA from the E site (Pittman *et al.*, 2006). This in turn facilitates the delivery of the next aa-tRNA to the A site by eEF1A (Triana-Alonso *et al.*, 1995). eEF3 is essential in fungi, and has ATPase activity. The function of the eEF3 is still entirely unclear, however its function is likely to be linked to the eEF1A function (Petropoulos and Green, 2012).

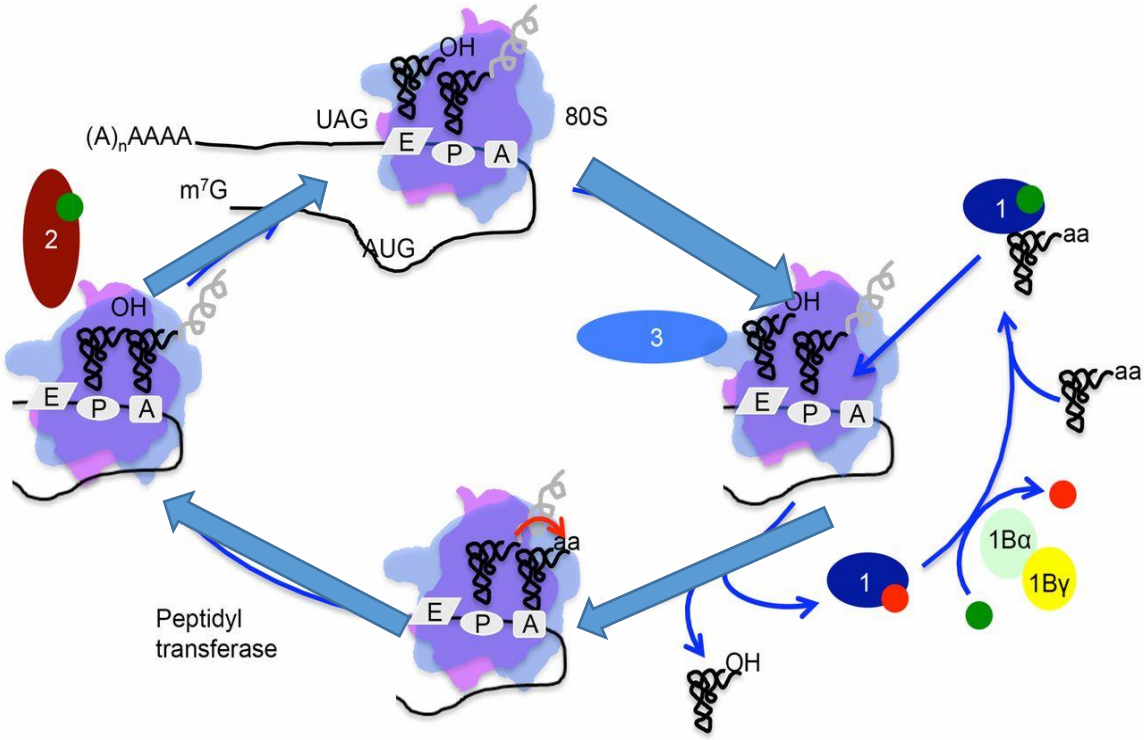

Figure 1.5: Steps of translational elongation. Adapted from: Dever *et al* (2016).

Eukaryotic translational elongation starts at the top of the figure. The ribosome in this stage contains a peptidyl-tRNA and a de-acylated tRNA in P and E sites respectively. (1) eEF1A bound to GTP, shown in green, binds aminoacyl-tRNA and delivers it to the cognate codon, contained in the ribosomal A site. The binding of appropriate aminoacyl-tRNA to the codon triggers a conformational change in the eEF1A, leading to GTP hydrolysis and consequent release of eEF1A-GDP, shown in red. Peptide bond formation is catalysed, followed by ribosomal translocation, catalysed by eEF2 (2) Binding to GTP, which stimulates the peptidyl-tRNA from A to P sites, and the de-acylated tRNA from P to E sites. The fungal essential factor, eEF3 (3), interacts with the eEF1A and possibly helps the release of tRNA from the E site.

1.4.3 Termination

Open Reading Frames (ORFs) usually end with a stop codon (UAA, UAG or UGA). When a stop codon occupies the ribosomal A site, this triggers the termination step of translation. Release of the nascent peptide chain from the ribosome, which is then followed by recycling of ribosomal subunits (Dever and Green, 2012). In eukaryotes, two termination (release) factors (eRF1 and eRF3) catalyse termination, with the help of ABCEI.

1.4.3.1 Class I release factor

Class I release factor, eRF1 (or Sup45 in yeast), is a part of the ternary complex which is required for translation termination, along with a class II factor eRF3 (Sup35) and GTP. Eukaryotic RF1 (eRF1) was first characterized in the 1990s, following studies on prokaryotic RFs. X-ray crystal analysis has revealed that eRF1 is composed of three domains, termed N, M and C, and has similar dimensions to a tRNA (Song et *al.*, 2000). Domain N, in *S. cerevisiae*,

contains residues 1-138, while domain M comprises residues 139-271 and domain C residues 272-431. Domain N is primarily responsible for recognition and binding of the three stop codons, mainly via the conserved TASNIKS motif (residues 55-61 yeast numbering), but with the help of the GTS (residues 28-30 yeast numbering) and YxCxxxF motifs (residues 125-131 yeast numbering) (Ito *et al.*, 2002; Kryuchkova *et al.*, 2013; Preis *et al.*, 2014). A further conserved surface residue on eRF1 is E55, which has also been suggested to interact with mRNA (Kolosov *et al.*, 2005). Unlike prokayotic RFs, eRF1 is omnipotent, able to decipher all the three stop codons. Mutations in residues of the GTS and YxCxxxF motifs implied that these motifs are essential for stop codon recognition (Cheng *et al.*, 2009). It has recently been shown that the GTS motif/loop performs a specific conformational perturbation during termination (Pillay *et al.*, 2016).

A universally conserved motif (GGQ) is contained in the M domain, and is positioned at the tip of the protein, in a position corresponding to the CCA-end of a tRNA (Mora *et al.*, 2003). The GGQ motif is critical for nascent peptide release and it activates hydrolytic activity at the PTC. Mutating the central glycine in GGQ motif to alanine (G181A) results in a loss of the GGQ motif function, whereas mutations to Q182 have different effects rated from minor to complete loss of activity (Hauryliuk *et al.*, 2006). The M domain carries out a specific interaction with the ribosomal 60S subunit whereas no interaction has been detected with the 40S subunit (Ivanova *et al.*, 2008). The C domain of eRF1 provides an interface with eRF3. Residues Y410 and T295 are located on the protein surface thus, it is supposed that they mediate the interaction with eRF3 (Merkulova *et al.*, 1999a; Akhmaloka *et al.*, 2008).

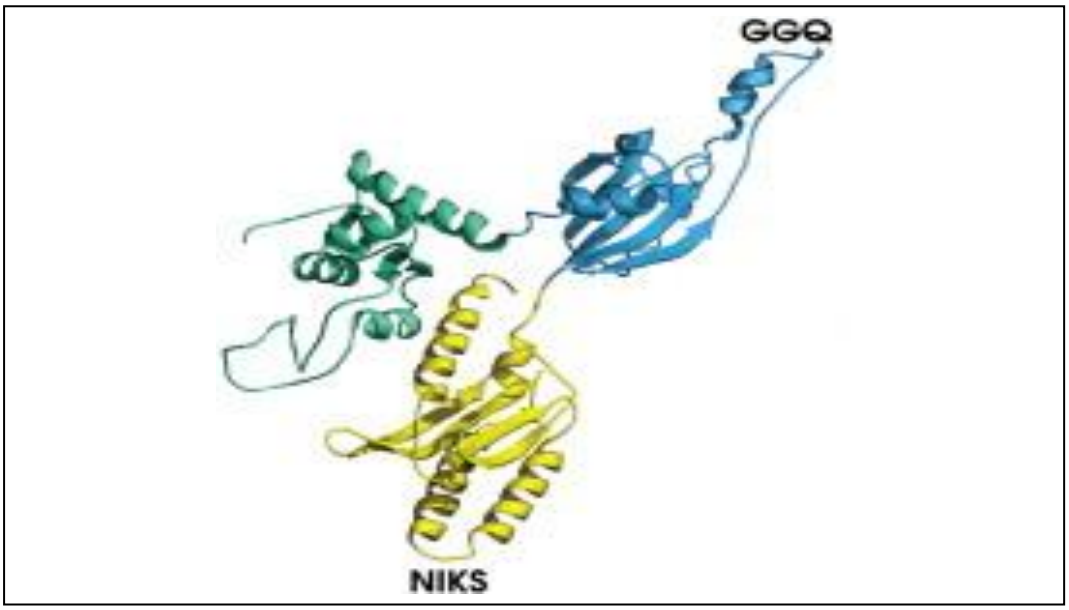

Figure 1.6: The structure of eRF1. Protein Data Bank Code 1EVV Kisselev (2002).

The structure of eukaryotic RF1 (Sup45 in yeast) shows the functional regions in each domain. The NIKS motif is shown on the epitope of the N domain, the function of this motif is decoding the stop codon in the ribosomal A site. The GGQ motif is highlighted in the M domain, this motif functions to catalyse the release of the nascent chain from the ribosome as the final action of termination.

The fidelity of translation termination is affected by point mutations in yeast eRF1 (Sup45). The effect on termination displays some pleiotropic phenotypes, including osmotic stress sensitivity (Singh, 1977), chromosome instability (Borchsenius *et al.*, 2000), respiratory deficiency (Ter-Avanesyan *et al.*, 1982) and cytoskeletal and cell cycle defects (Valouev *et al.*, 2002). Furthermore, the defects in termination, induced by *sup45* alleles, are likely to be strain specific (Merritt *et al.*, 2010), which means some alleles show significant effects on termination in particular strains backgrounds rather than others. Moreover, the effect of some *sup45* alleles on termination fidelity varies between *in vitro* and *in vivo* investigations. Some alleles produce effects in an *in vitro* analysis that mirror the relevant outcomes observed from *in vivo* analysis. These alleles include, N58A and S61A (Merritt *et al.*, 2010). Wheras others such as E52A and R62A significantly influence termination in an *in vitro* analysis but no *in vivo* effect was observed (Merritt *et al.*, 2010).

The efficiency of termination is usually assessed by the read-through assay, using a dual-luciferase reporter. This provides an idea about the ratio of ribosomes reading-through a particular stop codon, inserting a cognate aminoacyl-tRNA to decode the stop codon. It was reported that one of the most conserved peptides among a variety of organisms in the sequence of eRF1 is 54-GTASNIKSR-62 (*S. cerevisiae* numbering) (Inagaki *et al.*, 2002). However, yeast can tolerate amino acid substitution more than other organisms in this particular region. Mutations to G54 or K60 yield inviable strains whereas N58A mutant is viable, although it

causes severe defects in terms of termination efficiency. In contrast, T55A, S61A and R62A mutants cause mild or undetectable termination defects (Merritt *et al.*, 2010). It has been found that S30P, I32F and P38L mutations cause suppression of the three stop codons, increasing the read-through, whereas mutations to residues in/close to the GGQ motif may result in a minority of ribosomes stalling at the stop codon (Bertram *et al.*, 2000).

1.4.3.2 Class II release factor

Class II release factor, eRF3 (Sup35 in yeast) is the second component of the ternary complex which stimulates the translational termination. The N terminal region (residues 1-253) of eRF3 is non-conserved and not essential for the activity of the protein (Kononenko *et al.*, 2008). The remainder of the protein is composed of 3 domains: domain 1 (residues 254-488), domain 2 (residues 489-576) and domain 3 (residues 577-685). eRF3 is a GTPase and domain 1 corresponds to the G domain. Domains 2 and 3 of eRF3 are β-barrels, providing an overall structure similar to elongation factors, eEF2 and eEF1A (Kononenko *et al.*, 2010). eRF3 interacts with eRF1 via domains M and C. The functional role of eRF3 is not conserved between eukaryotes and prokaryotes. Prokaryotic RF3 is essential for recycling of class I factors (RF1 and RF2) after termination, whereas no role in this context has been found in eukaryotic RF3 (eRF3). eRF3 enters the ribosome in complex with eRF1, and hydrolysis of GTP on eRF3 is linked to a necessary conformational change within the pre-termination complex (Alkalaeva *et al.*, 2006; Wada and Ito, 2014). Each of eRF1 and eRF3 possess two regions critical for mutual binding to each other. In eRF1, residues 281-305 and 411-415, whereas in eRF3, residues 478-530 and 628-637 are supposed to be the key regions for eRF1-eRF3 binding (Merkulova *et al.*, 1999a). The availability of eRF1 affects the function of eRF3; there is ~5 times increase in the eRF3 capability to stimulate the peptide release, at saturated pool of eRF1 (Salas-Marco and Bedwell, 2004; Wada and Ito, 2014).

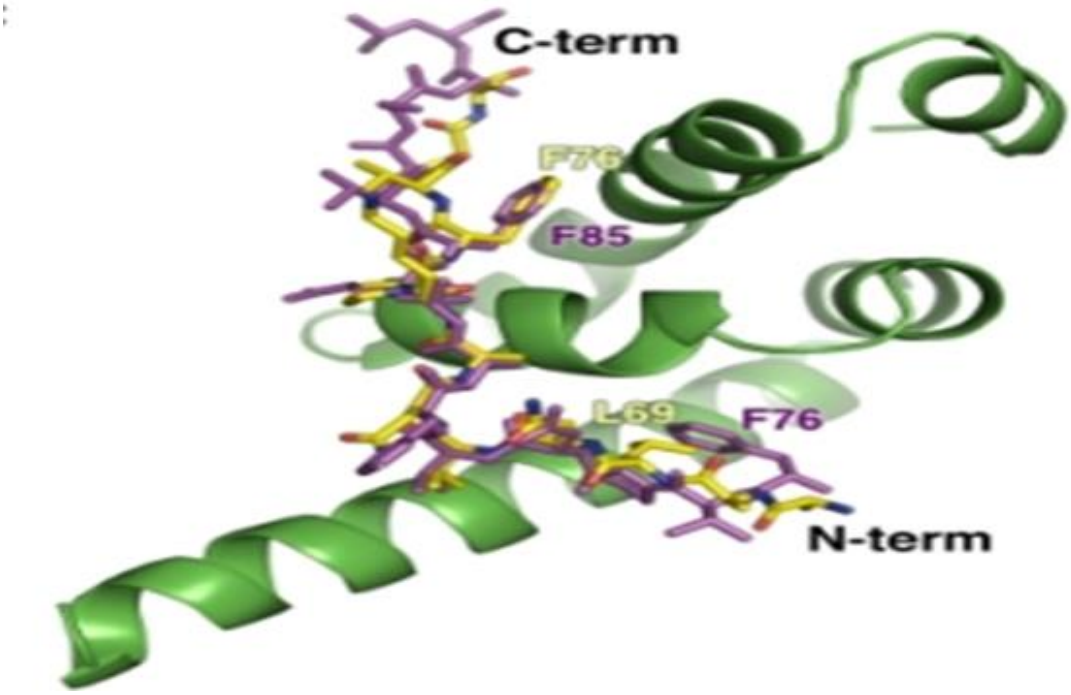

Figure 1.7: Structure of eRF3. Adapted from: Kozlov and Gehring (2010).

During the course of this study, we analysed several mutations in eRF3 (*sup35*) for their effects on the 2A reaction. In addition, we used a strain, limited in eRF3 activity, termed [PSI$^+$], along with the relevent wild type, [*psi*$^-$], for the same purpose. Translation termination is epigenetically modified by [PSI$^+$] in *S. cerevisiae* (Tuite and Cox, 2006). An intrinsic disordered prion-determining region is carried on Sup35 at the N terminus, when this region turns to the aggregating conformation, also termed prion conformation. There is a limited availability of Sup35 for termination, which increases the read-through across stop codons (Bidou *et al.*, 2000). It was reported that different phenotypes can result from [PSI$^+$] in different genetic backgrounds, the majority of the effects induced by these phenotypes are recapitulated by different degrees of loss of Sup35 function (True *et al.*, 2004). It has been suggested that some phenotypic effects shown by [PSI$^+$] is a result of ribosomal frame-shifting (Namy *et al.*, 2008). In addition, it has also been shown that the variation in read-through among different yeast strains might be a result of ribosomal bypass and reading into the 3' UTR (Shorter and Lindquist, 2005).

1.4.3.3 ATP-Binding Cassette E1 (ABCEI)

The third key factor for termination is ABCEI (also known as RNAase L inhibitor/Rli1 in yeast). This protein is found in all eukaryotes and archaea. It belongs to the ATP-binding cassette transporter protein family, which carries out a variety of functions such as translocating

substrates across membranes, DNA repair, DNA replication, histone biosynthesis, chromosome maintenance as well as translation (Toompuu *et al.*, 2016). Typically, ABC proteins possess double nucleotide-binding domains (NBDs), and these couple ATP hydrolysis to conformational changes in their substrate-binding sites. ABCEI is a 68 kDa protein which contains the typical two NBDs, arranged in a head-to-tail direction (Karcher *et al.*, 2008). Among the members of the ABC protein family, ABCE1 contains an N-terminal domain comprising two iron-sulphur (FeS) clusters (Barthelme *et al.*, 2007). Each NBD contains a bi-lobed fold of ATPase domains, as well as ATP-binding sites. The specific arrangement of the two NBDs forms two binding sites. Among ABC proteins family, the cysteine-rich N-terminus is conserved among species and unique among ABC proteins. The FeS cluster is composed of non-heme iron molecule with organic sulphide; the function of this kind of cluster is mostly the mediation of electron transport in redox reactions, however, they can stabilize protein folding, substrate binding and activation (Lill *et al.*, 2006). The FeS clusters of the *S. cerevisiae* ABCE1 homologue Rli1 are essential, mutation of most of the conserved Cys that co-ordinates iron in the FeS cluster lead to a loss of activity (Barthelme *et al.*, 2007). Thus the framework for this domain, in addition to its functional interactions with the NBD domains, are the keys for ABCEI function.

Loss or reduced activity of ABCEI is lethal, or causes severe defects (Winzeler *et al.*, 1999). In addition to its function in translational termination and recycling, ABCEI is involved in ribosomal biogenesis via the FeS clusters on its N domain (Kispal *et al.*, 2005). The involvement of ABCEI in several steps of translation has recently been established. It associates with the 40S subunit as well as initiation factors eIF2, eIF3 and eIF5 suggesting a distinct role in the formation of pre-initiation complex (Andersen and Leevers, 2007). ABCE1 has also been identified as a quality control factor to dissociate stalled ribosomes (Stec *et al.*, 2015). ABCEI directly associates with Hcr1 (eIF3j), which is a subunit of eIF3 involved in several steps of translation and in rRNA processing (Beznoskova *et al.*, 2013). Beyond translation, ABCE1 has roles in other processes including HIV capsid assembly (Karblane *et al.*, 2015).

1.4.3.4 Mechanism of translational termination

When the elongating ribosome encounters one of the three stop codons in the A site, the termination ternary complex (TTC) which consists of eRF1:eRF3-GTP recognizes the stop codon. Motifs of eRF1 which function in stop codon recognition are TASNIKS, GTS and YxCxxxF. Then, eRF3 triggers GTP hydrolysis, which allows the eRF1 to fully accommodate in the A site, positioning the M domain in the PTC, and orienting the GGQ motif at the ester bond between the tRNA and the nascent peptide chain. The GTS motif is located close to the

pentose of the +3 base of the stop codon (Wong *et al.*, 2012) therefore, when UAG stop codon is contained in the ribosomal A site, the threonine (T29 of *S. cerevisiae* numbering) in the GTS motif faces the +3 base of the stop codon sequence, forming a hydrogen bond with the nitrogen atom of the guanosine of the stop codon (Cheng *et al.*, 2009). This mechanism of stop codon recognition is structurally and functionally different from that in bacteria. eRF1 N and M domains contact the tRNA in the P site and a structural helix (α2 helix), located in the N domain, interacts with the anticodon loop of the tRNA in the P site (Brown *et al.*, 2015). The TASNIKS motif, located at the end of α2 helix, interacts directly with the uridine in the first position of the stop codon with hydrogen bonds between side chains of both asparagine and lysine in the TASNIKS motif and the uracil carbonyl groups. This lysine is hydroxylated, and in the absence of this modification termination efficiency is reduced (Feng *et al.*, 2014). Therefore, uridine is universal as the first nucleotide in stop codons, due to its ability to form hydrogen bonds with eRF1, and other nucleotides are not able to make productive interactions with the TASNIKS motif, explaining the universality of uridine in the first position of the stop codon.

The discrimination of stop codons from sense codons is carried out via interaction of the YxCxxxF motif with the +2 and +3 purines, through forming hydrogen bonding between purines in the stop codon and residues in the YxCxxxF motif (Brown *et al.*, 2015).

The pre-termination complex binds the ribosome, via the eRF1 N domain, which binds the 40S subunit. The recognition of the first two nucleotides in the stop codon is followed by a conformation of the N domain of eRF1, allowing decoding of the stop codon (Kryuchkova *et al.*, 2013). Helices α8 and α11 of eRF1 contact the C domain of eRF3 whereas the M domain of eRF1 anchors it to the 40S subunit. The C domain of eRF1 is then packed against the three domains of eRF3, forming a large interface which extends to the switch regions of the G domain of eRF3 (Preis *et al.*, 2014). In this stage, GTP hydrolysis is triggered, which acts as a key point to induce an appropriate conformation of eRF1. It has been found that eRF1 enhances the GTPase activity of eRF3, via acting as a guanine nucleotide exchange factor (Frolova *et al.*, 1996). It has been proposed that eRF3 may have a role here, by simulating the eRF1, through maintaining recycling of eRF1 (Zavialov *et al.*, 2001). However, others suggested that the eRF3's GTPase activity might be a result of stop codon recognition, maintained by eRF1 (Salas-Marco and Bedwell, 2004).

As a result of these events, a locked conformation sandwiches the GGQ motif between both 40S subunit and the G domain of eRF3, but with an incompatible position to trigger the peptide release in this stage, a dramatic conformation is then undergone for proper positioning of the GGQ loop in the PTC (Preis *et al.*, 2014).

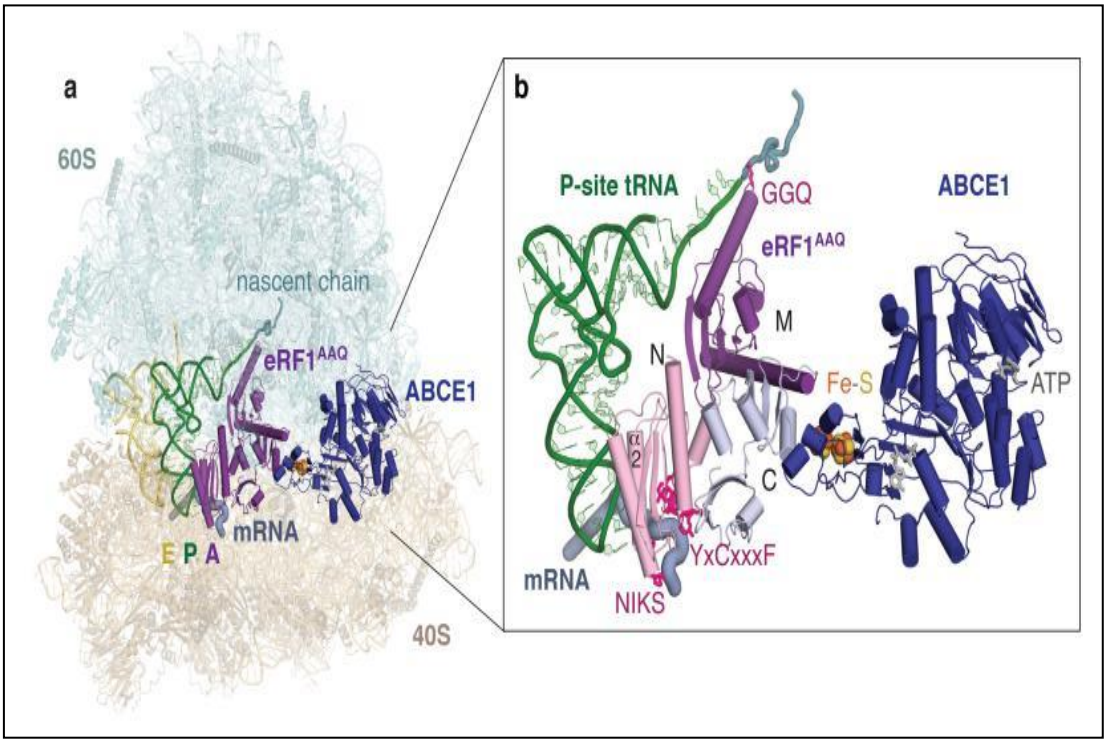

Figure 1.8: Structure of eukaryotic translation termination complex. Adapted from: Brown *et al* (2015).

(a) Structure of an eRF1-eukaryotic ribosome-nascent chain complex with UAG, E-(yellow) and P-site (green) tRNAs, eRF1 (purple) in the A site, and ABCE1 (blue) in the GTPase centre. (b) Zoom of eRF1 coloured by domain (N, M, C) with the GGQ, NIKS and YxCxxxF motifs highlighted (pink). The P-site tRNA (green), nascent polypeptide (teal), the mRNA containing the UAG stop codon (slate), and ABCE1 (blue) with its FeS (orange/yellow) and nucleotide binding sites (grey).

eRF3 with GDP are then released from the ribosome, to allow recruitment of the ABCEI which has an overlapping binding site on the ribosome to eRF3. ABCEI stimulates an additional conformational change of eRF1 to trigger the release of the nascent peptide chain from the ribosome (Dever and Green, 2012). Indeed, peptide release is accelerated in a GTP-hydrolysis-dependant manner (Eyler *et al.*, 2013). The FeS domain of ABCE1 contacts the C domain of eRF1, and causes a drastic conformation in the M domain of eRF1 resulting rearrangements in helix 95 (H95) of the 60S subunit, these events lead to multiple contacts of ABCE1 to rRNA, which results in an extension of the eRF1 M domain toward the P site-tRNA. All together, these events locate the GGQ motif at the PTC in close vicinity to the CCA of the peptidyl-tRNA, in this active conformation; ABCE1 exerts stabilization of eRF1 via the active contact of eRF1. This confirms the role of ATPase in eRF1-dependent peptide release (Shoemaker and Green, 2011).

1.4.4 Ribosome recycling

Once the nascent peptide chain is released from the ribosome, recycling of the ribosomal subunits takes place so that they are available for subsequent cycles of translation. In some cases, only partial dissociation of the complex takes place to allow re-initiation, when the ribosome translates more than one ORF however, most ribosomes undergo full dissociation after releasing the nascent peptide. In bacteria, a specific ribosome recycling factor (RRF) dissociates ribosomal subunits from mRNA, this factor interacts with the post-termination complex following the dissociation of the RF1/RF2. Eukaryotes have no homologues of RRF, and current evidence indicates that eRF1 remains associated with the ribosome following termination (Pisarev *et al.*, 2007). Thus, eRF1-bound post-termination complex, in addition to the deacylated tRNA, must be targeted by the recycling process in eukaryotes. Initially, it was considered that eIF3 plays a role in recycling of eukaryotic ribosomes (Pisarev *et al.*, 2007), stabilizing separated subunits through its capacity to bind 40S in a region overlapping with the subunit interface. However, the current model is that the multifunctional ABCE1 is a key factor for eukaryotic ribosomal recycling factor (Pisarev *et al.*, 2010; Barthelme *et al.*, 2011; Shoemaker and Green, 2011). Since eRF1 remains associated with the ribosome after termination (Pisareva *et al.*, 2011), eRFs may slowly trigger subunit dissociation (Pisarev *et al.*, 2007). However, ABCE1 stimulates subunit dissociation in an ATP-dependent manner (Pisarev 2011; Shoemaker and Green 2011). In this context, energy from ATP hydrolysis is proposed to be converted to a mechanical motion, used to separate ribosomal subunits: ATP hydrolysis is essential to promote fully closed ABCE1 conformational state which is required for efficient dissociation of the ribosomal subunits (Becker *et al.*, 2012).

Two further factors are involved in ribosomal recycling in eukaryotes. These include Dom34 and Hbs1 which function in cases of stalled ribosomes on aberrant mRNAs. Dom34 and Hbs1 are related to eRF1 and eRF3 respectively, they bind to the ribosomal A site and promote subunit dissociation in codon-independent manner (Pisareva *et al.*, 2011).

1.5 Translational Recoding

While translation mostly follows the standard rules of decoding, ribosomes, in particular cases do not follow the mRNA-protein co-linearity and participate in alternative decoding of the codons (Baranov *et al.*, 2006). This is termed recoding, a translational event when the outcome of translation is not what would be expected by standard reading of the mRNA, and is driven by signals in the mRNA sequence or occasionally the nascent peptide chain. Furthermore, in some mRNAs in competition with standard decoding, stop codons are occasionally redefined to amino acids such as glutamine, tryptophan or the 21st amino acid

selenocysteine (Gesteland and Atkins, 1996). Recoding is particularly prevalent in RNA viruses, providing them with control over gene expression that might be governed by transcription in DNA viruses/cellular genes. Examples of translational recoding are outlined below.

1.5.1 mRNA-mediated recoding

1.5.1.1 Frameshifting

Frameshifting is a common recoding event which occurs in either + or – direction on the mRNA. The ribosome shifts by one or two nucleotides, thereby changing the reading frame (Caliskan *et al.*, 2015; Dunkle and Dunham, 2015). Typically, only a small percentage of ribosomes undergo frame-shifting, and this is therefore a means by which a protein needed in relatively small amounts can be generated at an appropriate level. The vast majority of frameshifting sequences have been identified in viruses, and it is typically used for expression of replicase proteins (Liao *et al.*, 2011). For example, in HIV, frameshifting is essential to produce the DNA polymerase, in addition, it defines the ratio of Gag to Pol proteins, as it should not exceed a certain limit in order to maintain assembly of this virus, as well as genome maturation (Brakier-Gingras *et al.*, 2012). It has been recently suggested that frameshifting regulates mRNA and DNA stability in addition to its established role in controlling protein production. Thus frameshifting signals in four mRNAs encoding proteins essential for yeast telomerase maintenance limit the amount of protein made from these mRNAs and contribute towards the proteins being made in the correct stoichiometry (Advani *et al.*, 2013).

Several elements, embedded in the mRNA, promote -1 frame shifting. An essential element is the so-called slippery sequence. This is a heptametrical sequence with a X XXYYYZ or ZYYYXX X pattern which ensures proper base pairing between codons in the mRNA and the tRNA anticodons during the slippage. 0 frame and +/-1 can be decoded by the same tRNA according to the wobble interaction at the third position (Caliskan *et al.*, 2015). In addition to the slippery sequence, many frame-shift signals contain secondary structures such as pseudoknots and stem-loops downstream to the slippery sequence in the mRNA, which stimulate frame-shifting (Howard *et al.*, 2004).

Mechanistically, the structures in the mRNA are considered to pause the ribosome with tRNAs at the slippery site. This sequence aids to re-pair the non-wobble bases of the amino acid-tRNA and the peptidyl-tRNA, together with the -1 frame codons (Jacks and Varmus, 1985). Other mRNA structures are also capable of filling the role of the pseudoknots, by providing an energetic barrier to elongating ribosomes, as well as positioning them right over the slippery sequence (Yu *et al.*, 2011).

In contrast to the -1 frameshifting, in +1 frameshifting, the elongating ribosome bypasses a nucleotide while moving in the 3' direction. The signals for +1 frameshifting seem to be more specific with different mechanisms. In eukaryotes, a strong secondary mRNA structure located 3' of the slippery site is implicated in this context (Ivanov *et al.*, 2004). Many +1 frame-shift sites contain the stop codon UGA in the A site (in the 0 frame), which stimulates the frame-shift, they also depend on the tRNA:mRNA interaction in the P site. The yeast Ty retrotransposable elements are good examples of +1 frameshifting, which controls the synthesis of gag-pol protein (Farabaugh, 1995). Here, the ribosome slips on the sequence CUU AGG C. Frameshifting is primarily driven when the P site tRNA slips from CUU to UUA. The Ty frame-shift site does not contain the UGA stop codon, rather, AGG in the A site. This is decoded by a low abundance tRNA (Arg-tRNA^{CCU}). Overexpression of this tRNA results in a 50-fold decrease in frameshifting, whereas deletion of the gene encoding it increases the efficiency (Kawakami *et al.*, 1993).

1.5.1.2 Ribosome shunting

Ribosome shunting occurs when the 40S subunit bypasses an RNA structure, after translating a short ORF, to reinitiate translation of the downstream main ORF (Ogawa, 2013). Ribosomal shunting is well-studied in *Caulimoviridae*. Some members of this family have 3' ORFs on their mRNAs. A long 5' UTR has been identified in these mRNAs, comprising a short ORF downstream of the cap structure, which is then followed by a long stem loop of ~480 nucleotides (Pooggin *et al.*, 2012). The ability of the ribosome to carry out the shunt depends on retaining initiation factors, particularly eIF2, while translating the short ORF. The position and probably the length of the short ORF are essential for shunting, but not the sequence, ie. more efficient shunting is expected with shorter ORFs (Pooggin *et al.*, 2012).

1.5.1.3 Stop codon read-through

Although translational termination is an efficient process, the nature of the stop codon and/or the sequence context may significantly influence termination. Noticeably, some stop codons contexts are leaky, allowing the ribosome to read-through the stop codon at a frequency of 0.3-5% (Bertram *et al.*, 2001). In this case, a near-cognate tRNA decodes the stop codon to carry on translation toward the next stop codon, extending the protein. In the case of viruses, as with frame-shifting, this frequently allows expression of specific functions such as polymerases (Nam and Rousset, 2010). In Luteovirus, while the mRNA is translated, the ribosome reads-through at the end of the coat protein gene, generating a protein necessary for aphid

transmission (Brault *et al.*, 1995). Elements located either on the 5' or 3' of the suppressed stop codon can affect the efficiency of read-through. The presence of A in the two positions preceding the stop codon can increase the read-through efficiency in yeast, and this feature is also commonly seen in viruses such as tobamoviruses, poleroviruses and luteoviruses (Tork *et al.*, 2004). Two kinds of downstream stimuli have been recognized: the nucleotides directly adjacent to the suppressed stop codon and more distal elements which form secondary structures. The 3' sequences are thought to involve the identification of the stop codon (Bonetti *et al.*, 1995) thereby a competition between eRFs and potential near cognate tRNA to bind to the stop codon is expected. A mechanism has suggested that stop codon read-through might be a result of either mRNA-protein or mRNA-rRNA interactions. This interaction may modulate ribosome activity, and also alter eRFs function, although some stimulatory structures on mRNA do not significantly pause the ribosome (Napthine *et al.*, 2012).

1.5.1.4 Translational coupling

Many mRNAs contain short, cap-proximal ORFs (<~30 amino acids). Following translation and termination on these ORFs, the 40S ribosomal subunit is frequently able to resume scanning and re-initiate at a downstream AUG (Jackson *et al.*, 2012). For this to occur, certain eIFs must remain associated with the 40S subunit, and during scanning towards the downstream AUG, the complex must also reacquire ternary complex (eIF2-Met-tRNA-GTP). In a number of cases, cap-proximal ORFs have been shown to play key roles in regulating expression of the main ORF (Kojima *et al.*, 2005). In contrast to re-initiation following a short upstream ORF, re-initiation is extremely rare after translation of a long coding ORF. However, this has been shown to occur in a few cases, termed translational coupling, and produces key proteins in a number of viruses (Jackson *et al.*, 2012). An example of translational coupling is seen in Calicivirus. Caliciviral RNA contains three protein-coding ORFs: ORF1 encodes the non-structural proteins, whereas ORF2 and ORF3 encode the major capsid protein and a protein which is a component of the virion respectively (Herbert *et al.*, 1996). In some genera, ORF2 is continuous with ORF1 thus, some capsid proteins are generated as fusion proteins with the non-structural counterparts. The initiation codon of ORF3 is located close to the termination codon of ORF2 (either AUGnnUGA or overlapping as UAAUG or AUGA), and ORF3 is usually translated via translational coupling (Napthine *et al.*, 2009). Re-initiation of translation in this case, depends on RNA motifs, ranging from 40 to 90 nt, located upstream to the ORF2 stop codon. Using yeast cells as an experimental system in which to examine the coupling mechanism. An interaction between this motif and the 18S rRNA was determined to be required to increase the efficiency of re-initiation (Luttermann and Meyers, 2009). It has also been

suggested that eIF3 binds to a proportion of 40S subunits of ribosomes terminating the translation of the ORF2, this seems to be followed by recruitment of the ternary complex to the ribosome. eIF3-bound subunits then undergo subsequent initiation at ORF3 start codon (Poyry *et al.*, 2007). When mutations are induced at the normal ORF3 start site, initiation codons downstream to it can be utilised, but less efficiently and, in addition, initiation may occur on non-AUG initiation, though it is less preferred (Poyry *et al.*, 2007; Luttermann and Meyers, 2009; Powell *et al.*, 2011). A second example of translational coupling is seen in *Caulimovirus* and *Soymovirus*. In contrast to coupling in calicivirus, where only a fraction of ribosomes undergo re-initiation, these events are highly efficient and are driven by a specific viral protein termed transactivator viroplasmin (Schepetilnikov *et al.*, 2011).

1.5.2 Nascent peptide-mediated recoding

1.5.2.1 Stop-carry on/Stop Go

This recoding event is mediated by amino acid motifs rather than mRNA sequence. This is driven by the 2A peptide, the focus of this thesis, and will be described in more detail in section 1.7. Briefly, 2A peptides, encoded within the body of an ORF, interact with the ribosomal PTC and exit tunnel to prevent the formation of a peptide bond at the end of the 2A sequence, specifically to a proline. This results in release of the nascent chain in a stop-codon independent manner. Translation is then resumed with proline as the first amino acid of a separate downstream product. The 2A reaction is a primary processing step in the resolution of polyproteins encoded by a number of viruses, and 2A peptides are found in a number of RNA viruses including aphthoviruses, cardiviruses, boviruses and teschoviruses (Luke *et al.*, 2008).

1.5.2.2 Secretion monitor (SecM)

In particular cases, ribosomes are affected by some peptides, while being translated, but with no recoding observed. A few examples of this are mentioned below.

SecM is a regulatory sequence located upstream to SecA, which encodes an ATPase required for protein secretion in bacteria. After translation, the SecM peptide chain pauses the ribosome. This pause is released if the signal sequence of SecM is engaged in the protein conducting channel. However, if translocation is inefficient the pause is prolonged. Stalled ribosomes on SecM disrupt secondary structures in mRNA which otherwise impair recognition of the Shine-Dalgarno sequence of SecA. Prolonged ribosomal pausing on SecM thus increases the synthesis of SecA. SecM carries out the stalling effect on the ribosome via a conserved motif (amino

acids 150-166) (Nakatogawa and Ito, 2001). Although prolyl-tRNA binds to the ribosomal A site, proline is not included in the nascent chain (Garza-Sanchez *et al.*, 2006).

1.5.2.3 Crb^{cmlA} peptide

The N-terminus of Crb^{cmlA} nascent chain starts with eight amino acids (MSTSKNAD), encoded by an uORF to the gene Tn1696 which is responsible for the development of chloramphenicol resistance, thereby, inhibiting the peptidyl transferase activity. Tn1696 was initially isolated with a plasmid from *Pseudomonas aeruginosa* (Partridge *et al.*, 2001). The regulatory mechanism is pausing the ribosome via *Crb*^{cmlA} in response to chloramphenicol, the inhibitory upstream nascent chain regulates the activity of the PTC through altering the conformation of certain domains of 23S rRNA. Therefore, the paused ribosome modulates the translation of the downstream gene (Harrod and Lovett, 1995).

1.5.2.4 TnaC

TnaC is a peptide composed of 24 residues, encoded by a short ORF upstream to the *E. coli* tryptophanase (*tnaA*) gene. Similar to the mechanisms described above, the interaction of nascent chain with the ribosome structure regulates the expression of the tryptophanase operon in *E. coli*. At high concentration of tryptophan ribosomes pause at the end of TnaC, and termination is impeded. The particular conformation of the stalled ribosome occludes binding sites for the *rho* terminator, allowing transcription of the downstream *tnaA* and *tnaB* within the operon. The peptide chain of TnaC interacts with the exit tunnel, resulting in stalling of the ribosome at the Pro24 of TnaC thus termination is impaired. It has been found that both Pro24 and Trp12 in addition to rRNA nucleotides U2609, A749-753 and L22 protein are the key elements for ribosomal pausing on TnaC (Konan and Yanofsky, 2000).

1.5.2.5 Arginine attenuator peptide

The yeast carbamoylphosphate synthase (Cpa1) is encoded by the *CPA1* gene. Cpa1 is a key enzyme in the pathway of arginine biosynthesis. The concentration of arginine in the cell modulates translation of *CPA1* mRNA. *CPA1* mRNA contains a leader sequence composed of 250 nucleotides which contains a 25 codon uORF. The sequence of amino acids in this uORF, but not the mRNA sequence, is the essential feature for the repression of the *CPA1* by arginine level (Hood *et al.*, 2007).

1.6 Translational quality control

Translation is a highly accurate process which ensures that the codons in mRNA are faithfully interpreted. Despite the high accuracy level, up to one in ten of synthetized proteins have been found to contain at least one miscoded amino acid (Wolff *et al.*, 2014). Moreover, failure to maintain proteostasis are potential consequences of mRNA processing and modification (Simms *et al.*, 2014; Simms *et al.*, 2017). A number of quality control mechanisms have evolved to detect errors and subject mRNA/protein to degradation (van Hoof and Wagner, 2011).

1.6.1 Non-stop decay (NSD)

The lack of a stop codon (non-stop mRNA) leads the ribosome to stall at the 3' end of the RNA due to the inability of the subunits to dissemble through recycling. Non-stop mRNA is rapidly degraded by actions of both cytoplasmic endonucleases and exonucleases (Barthelme *et al.*, 2011). NSD was first discovered in yeast, in which constructs lacking stop codons turn over rapidly. The GTPase Ski7 is essential for initial recognition of the defective mRNA (van Hoof *et al.*, 2002). Ski7 is an eRF3-related protein, and a component of the yeast exosome. Also, Ski7 is the paralog of NGD protein, Hbs1, they both arise from the same origin (van Hoof, 2005). In other organisms, Hbs1 tends to simulate the function of Ski7, interestingly, deletion of Ski7 is complemented by overexpression of Hbs1 however, the molecular mechanism of Ski7/Hbs1 role in the recognition of defective mRNA is not fully understood. Most non-stop cellular mRNAs originate from premature polyadenylation, in contrast to truncated mRNA, and the ribosome stalls while decoding the polyA tail into Lys (Kervestin and Jacobson, 2012). Here, the endonucleolytic cleavage of mRNA is driven by Dom34:Hbs1 complex (homologs of eRF1 and eRF3 respectively). This process is performed through the Dom34:Hbs1 activity to dissociate stalled ribosomes at the 3' end of non-stop mRNA, and then stimulates the degradation by exosomes (Kobayashi *et al.*, 2010). However, an endonucleolytic cleavage of mRNA can be observed in the absence of Dom34:Hbs1 complex (Kuroha *et al.*, 2010). It has recently been revealed that the Dom34:Hbs1 complex also promotes peptidyl-tRNA drop-off in addition to direct the elongation complex into the subunits dissociation and release the mRNA with the help of ABCE1/Rli1 (Pisareva *et al.*, 2011). During elongation, the Dom34:Hbs1 is supposed to compete with the eEF1α for binding the ribosomal A site, and therefore the number of nucleotides downstream of the P site should be critical for the Dom34:Hbs1 to recognise the ribosome (Pisareva *et al.*, 2011). The binding of Dom34 to the ribosomal A site destabilizes the mRNA, and probably disrupts codon-anticodon binding in the P site (Shoemaker and Green, 2011). The degradation of non-stop mRNA is carried out by the

exosome, along with the Ski2-Ski3-Ski8 helicase complex (van Hoof *et al.*, 2000; Araki *et al.*, 2001). Ski7 interacts with Ski2-Ski3-Ski8 complex via its N terminus, this kind of interaction is essential for the 3' to 5' direction decay pathway of normal mRNAs. Whereas the C-terminus of the Ski7 simulates the GTP-binding domain of eEF1α, the C-terminus has a role in the non-stop mRNA decay in a poly A-dependent manner (Tsuboi *et al.*, 2012). The N domain of Dom34 contains loops which are essential for the recognition of the stalled ribosome in the A site (Kobayashi *et al.*, 2010). Although Ski7 might not be required for the dissociation of stalled ribosomes at the 3' end of non-stop mRNA, the C terminus of Ski7 may play a role in recognizing stalled ribosomes at the 3' end of a polyA tail, independently of Dom34:Hbs1 (Tsuboi *et al.*, 2012).

1.6.2 No-Go-Decay (NGD)

NGD is triggered by structures that block ribosome progress on the mRNA template. Dom34:Hbs1 is involved in this pathway (Doma and Parker, 2006). The Dom34:Hbs1-GTP ternary complex splits the 80S ribosome into 40S and 60S subunits, a process which is carried out with an independency of the codon in the A site (Shoemaker *et al.*, 2010). Rli1 has a significant role in this process by increasing the Dom34-mediated ribosome dissociation, presumably functioning analogously to how it functions in recycling of ribosomal subunits post-termination (Shoemaker and Green, 2011). The recognition of the defective ribosome is-in part-driven by the interaction between Hbs1 and the 40S subunit (Becker *et al.*, 2011). The N domain of Hbs1 binds the mRNA entry tunnel, while ribosomes are in elongation and the entry tunnel is dwelt by the mRNA, thus, Hbs1 is not effective (Shoemaker and Green, 2011). The defective mRNA is then cleaved by the endonucleolytic activity in the vicinity of the stall site (Tsuboi *et al.*, 2012). The cleavage yields an uncapped mRNA, which is degraded by the 5-3' exonuclease termed, Xrn1.

1.6.3 Nonsense-Mediated Decay (NMD)

NMD is the mechanism through which cells degrade mRNAs that contain a premature stop codon. Premature stop codons may arise from several causes such as mutations and inaccurate transcription. NMD was first discovered in yeast, in which some mutations in *ura3* were noted to reduce the transcript level with no effect on the rate of synthesis (Losson and Lacroute, 1979). However, after about four decades of studying the mechanism of NMD, it is still not fully understood. Genetic studies in yeast and nematodes identified a group of conserved factors to be involved in the NMD, acting as bridges to connect the mRNA sequence with eRFs (Chakrabarti *et al.*, 2011). The binding between mRNA and eRFs induces post-

translational modification to Upf1 which results in a stimulatory signal for NMD via the recruitment of mRNA-degrading factors (Okada-Katsuhata *et al.*, 2012). Also, this connection seems to inhibit interactions between poly A-binding protein (PABP) and eRF3 which might be a key step for normal termination (Kashima *et al.*, 2006). Two models have been put forward to uncover the mechanism of target recognition during NMD, the efficiency of both is related to the length of the UTR. The first model suggests that the proximity between both the premature stop codon and the poly A-tail is a proposed reason for the inhibition of interaction between PABP and eRF3 (Singh *et al.*, 2008). The Upf proteins are then recruited to bind the mRNA, marking it as an NMD target. The second model has suggested that Upf1 binds and coats the 3' UTR (Hogg and Goff, 2010). For this reason, longer UTRs recruit a higher concentration of Upf1.

1.7 The 2A peptide

It has been briefly described earlier that 2A peptides are short peptides that act to co-translationally separate the nascent chain into two molecules, driving a skip in the formation of a specific peptide bond, between Gly18 and Pro19, the last two amino acids in the 2A sequence (Ryan *et al.*, 1991; de Felipe *et al.*, 1999; Donnelly *et al.*, 2001b; Doronina *et al.*, 2008). 2A is found in many RNA viruses and, in addition, it has been identified in non LTR-retrotransposons of *Trypanosoma* spp. as well as cellular genes in sea urchin and a number of other species. The first identified and most studied 2A peptide is encoded within the foot-and-mouth disease Virus (FMDV) RNA (figure 1.9).

A key feature of 2A is that it can be transposed into other contexts (polyprotein systems/ reporters) and retains its activity (Ryan and Flint, 1997). Thus 2A is autonomous and there are no other requirements for other parts of the virus. This trait enabled the usage of 2A as a tool for co-translational processing of proteins (Ryan *et al.*, 1991; Ryan and Drew, 1994; de Felipe *et al.*, 2006). The activity of 2A is driven by the amino acid sequence rather than the mRNA sequence. Thus, synonymous substitution of codons in the sequence does not change activity, whereas changing the reading frame over the 2A sequence or introducing non-synonymous changes affects activity (Ryan and Flint, 1997; Donnelly *et al.*, 2001a; Donnelly *et al.*, 2001b; Sharma *et al.*, 2012).

A conserved motif has been identified in all 2A and 2A-like sequences, D(V/I)EXNPG-P, which lies at the C-terminus and this is preceded by a non-conserved N-terminus (Donnelly *et al.*, 2001a). 2A peptides have been subjected to extensive mutational analysis. This has revealed that most of the C-terminal conserved amino acids, including G_{18}-P_{19}, are critical for activity of

the peptide. In addition, mutations to the non-conserved sequences at the N-terminus can also affect the 2A activity. Further, efficiency of the 2A reaction is enhanced by addition of residues (5-14) from the C-terminus of the upstream FMDV 1D protein. On the other hand, addition of viral (2B) sequences downstream of 2A has no effect on activity (Donnelly *et al.*, 2001a; Odon *et al.*, 2013). Table 1.1 shows 2A sequences from different viruses.

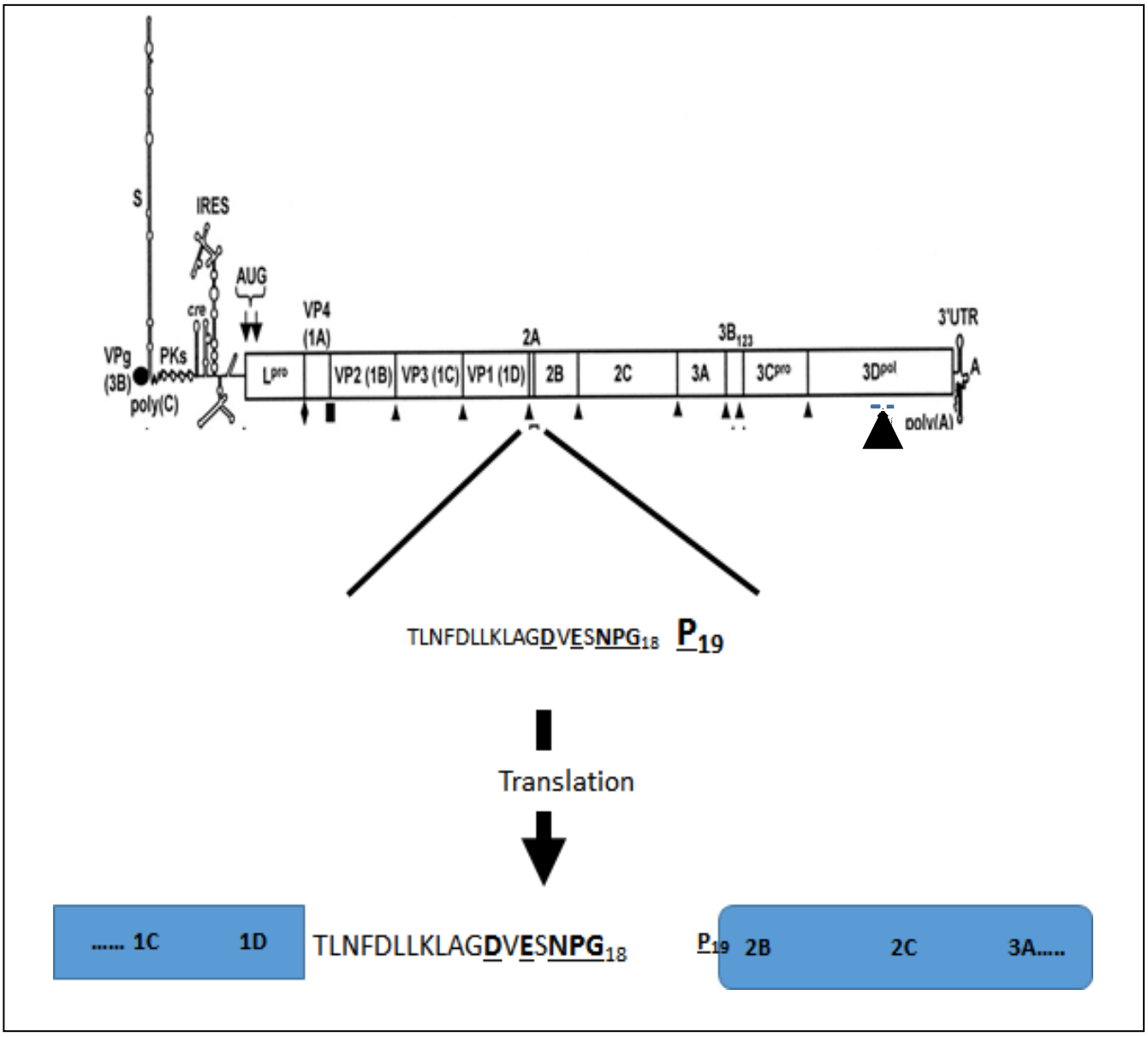

Figure 1.9: Structure of FMDV ORF and 2A sequence.

The ORF of FMDV is shown on the top with sites of primary and secondary cleavage for different segments to produce mature viral proteins. The amino acid sequence is shown with the conserved C-terminus motif highlighted. In the bottom, the outcomes of translation of 2A-containing ORF are shown, which separate the products into an upstream, containing 2A and a downstream, starting with P19.

Family	Type	Amino acid sequence
Cypoviruses	BmCPV 1	RTAFDFQQDVFRSNYDLLKLCGDIESNPGP
	LdCPV 1	MTAFDFQQAVFRSNYDLLKLCGDVESNPGP
	DoCPV 1	MTAFDFQQAVFRSNYDLLKLCGDVESNPGP
	OpbuCPV 18	IHANDYQMAVFKSNYDLLKLCGDVESNPGP
Totiviridae	IMNV 2A1	WDPTYIEISDCMLPPPDLTSCGDVESNPGP
	IMNV 2A2	RDVRYIEKPFDKEEHTDILLSGDVESNPGP
Rotaviruses	PoRV C	GNGNPLIVANAKFQIDKILISGDVELNPGP
	BoRV C	GIGNPLIVANSKFQIDRILISGDIELNPGP
	HuRV C	GAGYPLIVANSKFQIDKILISGDIELNPGP
	ADRV N	FFDSVWVYHLANSSWVRDLTRECIESNPGP
Dicistroviridae	CrPV	LVSSNDECRAFLRKRTQLLMSGDVESNPGP
	DCV	QGIGKKNPKQEAARQMLLLLSGDVETNPGP
	ABPV	TGFLNKLYHCGSWTDILLLLSGDVETNPGP
	KPV	IGFLNKLYKCGTWESVLNLLAGDIELNPGP
	IAPV	IGFLNKLYRCGDWDSILLLLSGDIEENPGP
Tetraviridae	TaV	RGPRPQNLGVRAEGRGSLLTCGDVEENPGP
	EeV	RRLPESAQLPQGAGRGSLVTCGDVEENPGP
	PrV 2A1	LEMKESNSGYVVGGRGSLLTCGDVESNPGP
	PrV 2A2	NSDDEEPEYPRGDPIEDLTDDGDIEKNPGP
	PrV 2A3	TLMGNIMTLAGSGGRGSLLTAGDVEKNPGP
Iflaviruses	IFV	PSIGNVARTLTRAEIEDELIRAGIESNPGP
	EoPV 2A1	GQRTTEQIVTAQGWAPDLTQDGDVESNPGP
	PnPV 2A1	GQRTTEQIVTAQGWVPDLTVDGDVESNPGP
	EoPV 2A2	TRGGLQRQNIIGGGQRDLTQDGDIESNPGP
	PnPV 2A2	TRGGLRRQNIIGGGQKDLTQDGDIESNPGP
Picornaviridae	EMCV	VFGLYRIFNAHYAGYFADLLIHDIETNPGP
	TMEV	FREFFKAVRGYHADYYKQRLIHDVEMNPGP
	T-LV	FSDFFKHVREYHAAYYKQRLMHDVETNPGP
	SAF-V	FTDFFKAVRDYHASYYKQRLQHDVETNPGP
	FMDV	HKQKIVAPVKQTLNFDLLKLAGDVESNPGP
	ERAV	RHKFPTNINKQCTNYSLLKLAGDVESNPGP
	ERBV1	EATLSTILSEGATNFSLLKLAGDVELNPGP

	PTV	AMTVMAFQGPGATNFSLLKQAGDVEENPGP
	BRV2	LRLTGEIVKQGATNFELLQQAGDVETNPGP
	LV	YFNIMHSDEMDFAGGKFLNQCGDVETNPGP
	SVV	RAWCPSMLPFRSYKQKMLMQSGDIETNPGP
	DHV1	AFELNLEIESDQIRNKKDLTTEGVEPNPGP

Table 1.1: Sequence analysis of various 2A peptides (Luke *et al.*, 2008).

1.7.1 The 2A reaction

The 2A reaction is not proteolytic, but is instead a co-translational event. The use of artificial polyprotein reporters such as CAT-2A-GUS, GUS-2A-GFP, GFP-2A-GUS revealed that the ratios resulting from the reaction are not consistent with the 1:1 ratio expected from a proteolytic event (Donnelly *et al.*, 1997; Donnelly *et al.*, 2001a; Donnelly *et al.*, 2001b): three different translation products are observed from such reporters, the full length protein and separated upstream and downstream proteins, frequently with a molar excess of the upstream product over the downstream. Furthermore, prolonged incubation of the reaction does not alter the outcomes (Donnelly *et al.*, 2001a; de Felipe *et al.*, 2010).

Mutational analysis targeting nucleophilic residues in the conserved motif did not affect the 2A activity, however, mutagenesis targeting several conserved positions abolished the reaction, confirming that they are essential for the normal activity of the 2A. These residues are P_{17}, G_{18} and P_{19}. Proline and glycine have the least nucleophilic properties, thus, the 2A reaction is not expected to be a self-proteolytic reaction (Donnelly *et al.*, 2001a) which suggests that 2A reaction is unique in terms of the mode of action (de Felipe *et al.*, 2003). To further investigate, an inactive version of 2A (P17A), termed 2A*, was developed and constructed into a reporter consisting of α Factor-2A/2A*-GFP. Through testing the localization of the translation products, it was concluded that the 2A reaction occurs before the emergence of the nascent chain from the ribosome (de Felipe *et al.*, 2003). The 2A reporter, CAT-2A-GUS was expressed in prokaryotic cell model, using *E. coli*. As a result of translation, only a full-length product was obtained, indicating that the 2A peptide is non-functional in prokaryotes (Donnelly *et al.*, 1997).

1.7.2 Proposed 2A mechanism

It has been demonstrated by ribosomal toe-printing that the ribosome at 2A peptide pauses with codons encoding Gly18 and Pro19 in P and A sites respectively (Doronina *et al.*, 2008). Given the dependence of the reaction on the peptide sequence, this suggests that the

nascent chain makes specific interactions with the ribosomal exit tunnel to facilitate ribosomal pausing and to drive the 2A reaction. It has been suggested that the majority of the 2A peptide may take alpha helical conformation in the exit tunnel, and the conserved C-terminus forms a tight loop (de Felipe *et al.*, 1999; Ryan *et al.*, 1999; Donnelly *et al.*, 2001). The specific interaction between both conserved and non-conserved regions of 2A on one side and, the ribosome on the other side, prevents the formation of the peptide bond between glycine and proline in the PTC however, Doronina and her collaborators suggested a substitution in this context; they further investigated the C terminus of the upstream 2A product. Using *N. crassa* extracts, they confirmed that translating ribosomes pause at the 3' end of the 2A-coding sequence. Furthermore, the upstream fragment from peptidyl 2A-tRNA is rapidly released from the ribosome. They also suggested that termination factors might be involved in the 2A reaction, through detecting reduction in the 2A reaction in *S. cerevisiae* strains with defective eRFs (Doronina *et al.*, 2008). Further, high level expression of mRNA encoding 2A peptides in such strains led to growth inhibition and enhanced stop codon read-through, suggesting that the mRNA encoding 2A was able to exert a titration effect on the available eRFs (Doronina *et al.*, 2008). On the other hand, more recently, Machida *et al* (2014) aimed to define translation factors involved in the 2A reaction, and recruited to the ribosome. They reconstituted an HCV IRES-dependent translation system, with eEF1, eEF2, eRF1, eRF3, aminoacyl tRNA synthetases and tRNA, being added to a human-derived ribosome. Using this they found that while translating 2A with this system, 2A and subsequent 2B processing does not recruit eRFs, but instead, they suggested that the process is entirely an elongation event (Machida *et al.*, 2014). The current study provides further evidence that the process is a termination-elongation event.

1.7.3 Biological activity of the 2A reaction

As described above in section 1.7.2, 2A activity can be found in several organisms. As the main example of 2A peptide used in this study was derived from the FMDV, it would be logical to briefly discuss the previous knowledge and related work on this version of 2A peptide. FMDV is a member of *Picornaviridae* which also includes Cardiovirus, Enterovirus, Hepatovirus, Parechovirus and Rhinovirus. More than 200 serotypes of *Picornaviridae* have been isolated, with the most ancient type (1400 BC) from Egypt. A variety of organisms, including humans are potentially the hosts of Picorna viruses. For example in humans these viruses cause Polio, Hepatitis A and common colds. FMDV was first discovered in 1898 in cattle (Rott and Siddell, 1998). It causes Foot and Mouth Disease (FMD) which is infective among the species (Barrionuevo *et al.*, 2018). Although FMD mainly infects animals, a few

cases of infection in human have been diagnosed world wide. Outbreaks in animals usually result in huge economical loss. Although FMD normally is not fatal, especially for adult animals, the economic loss comes from the acute reduction of animal production such as milk production and weight gain in livestock. Unfortunately no successful treatment has been introduced to control FMD. FMDV exists in seven serotypes but also with sub-serotypes thus vaccines have limited ability to control the disease due to their high specificity against a particular serotype (Lyons *et al.*, 2016).

The genetic information of all *Picornaviridae* is encoded on sense single stranded RNA, which includes a single ORF, translated by the host ribosome. Despite the presence of only one ORF, the translation end result is co-and post-translationally processed into polyprotein, carried out by viral proteases. The 5' UTR of *Picornaviridae* varies in length (624-1199 bp), and contains an IRES structure, whereas the 3' UTR is relatively short (47-125 bp) and it is followed by the poly A tail (Curry *et al.*, 2007; King *et al.*, 2016).

During the translation of the FMDV ORF, it is not expected to obtain the full length protein. This is due to the 2A cleavage activity, which functions to separate the product into two parts. The first part consists of peptides, 1A, 1B, 1C and 1D, termed VPs which represents the capsid protein. The other counterpart of the translation (2B, 2C, 3A, 3B and 3D) is the viral replicative protein. Additional processing of the products results in up to 12 different products. The 2A, and similar activity shown by 3C, are for polyprotein processing (Donnelly *et al.*, 1997).

1.7.4 Employment of 2A peptides in biotechnology

Classically, selection of traits in different microorganisms was achieved by selection of parents carrying the desired trait to be then identified in offspring however, the generation time in some cases places major obstacles on the process. Many studies have made a significant progress in terms of gene therapy although limits in technologies still exist, one of the major limits is the non-sufficient coding capacity of viral vectors (Tahara and Lotze, 1995; Walther and Stein, 2000). Only small inserts can be accommodated in commonly used delivery systems, which limits the size of the target protein to be expressed, in addition to difficulties accompanying the co-expression of two or more proteins. A revolution in protein co-expression was made after introducing 2A to biotechnology (Qian *et al.*, 2001). The small size of 2A and 2A-like sequences, in addition to their ability for efficient co-expression, when placed between genes, made 2A as the best tool to be utilised in this field, which overcomes disadvantages observed when IRES were used for the same purpose (Donnelly *et al.*, 2001a; Donnelly *et al.*, 2001b). 2A peptide was first reported to be used in biotechnology by Percy and colleagues who generated a neuraminidase and chloramphenicol acetyl transferase genes linked together by 2A

sequence (Furler *et al.*, 2001). This success led to a revolution in terms of genes co-expression, such as the expression of IL-12 subunits (Kessels *et al.*, 2001). Many examples have been published on how 2A and 2A-like sequences are successfully employed in different fields of biology including, genetic engineering, plant biotechnology, recombinant protein production, metabolism engineering, gene therapy and many other applications. Some of these utilisations are briefly discussed below:

1.7.4.1 Generation of transgenic organisms

2A activity has been invested to generate a wide variety of transgenic mice (Szymczak *et al.*, 2004; Alli *et al.*, 2008; Chaparro *et al.*, 2008; O'Connell-Rodwell *et al.*, 2008; Trichas *et al.*, 2008; Rothwell *et al.*, 2010; Bettini *et al.*, 2012; Rawlins and Perl, 2012; Tittel *et al.*, 2012; van der Velden *et al.*, 2016), fish (Provost *et al.*, 2007; Dempsey *et al.*, 2012), rats (Herold *et al.*, 2008), pigs (Yang *et al.*, 2010; Deng *et al.*, 2011), birds (Bower *et al.*, 2011), amphibians (Lin *et al.*, 2012), insects (Diao and White, 2012) and sheep (Tian *et al.*, 2013).

1.7.4.2 Co-expression of multiple genes

Multiple gene co-expression at a desired amount is an attractive aspect in biomedicine. The expression includes cellular reprogramming (Takahashi and Yamanaka, 2006; Vierbuchen *et al.*, 2010). Reprogramming of multiple subunits of complex multimeric proteins is one application of gene therapy (Zitvogel *et al.*, 1994; Szymczak *et al.*, 2004). Tagging of a particular protein for isolation or estimation (Huh *et al.*, 2003; Park *et al.*, 2014). The strategy which was used for multiple gene co-expression includes utilisation of multiple vectors or multiple promoters within a single vector. However, introducing the self-cleaving 2A peptides into these strategies represented a huge step to improve genes co-expression (Liu *et al.*, 2017). 2A peptide was adopted to *Trichoderma reesei* fungus in order to obtain an easily screenable marker protein which can be co-translated with a target protein (Subramanian *et al.*, 2017). In plants, such as tobacco cells, 2A has been utilised for coordinated expression of multiple proteins. Experiments were undertaken using an Ssp DnaE mini-intein variant, engineered for N-terminal auto-cleavage of intein. Ssp DnaE mini-intein is covalently linked to a 2A peptide, which yields an Int-2A self-cleaving fusion protein domain, that acts, in *cis*, to effectively release the flanking protein (POIs), which is the protein of interest. The efficiency of polyprotein processing, mediated by Int-2A domain, has also been employed in lettuce and *Nicotina benthamina* (Zhang *et al.*, 2017). Another study described a novel strategy for co-expression of adeno-viral vascular endothelial growth factor (VEGF) and fibroblast growth

factor 2 (FGF2), connected together through 2A peptide in umbilical cord monocytes. After injection into transgenic mice, co-expression of both factors was observed in spinal cord after 1 month (Garanina *et al.*, 2016). 2A cleavage properties have also been used to produce multi-transgenic pigs. 2A sequence was constructed in a double promoter expression vector, by which the expression of four fluorescent proteins is mediated. This vector was transfected into porcine fetal fibroblasts, and the four fluorescent proteins were found to be evenly expressed in the reconstructured embryos (Deng *et al.*, 2011). 2A has been used to co-express the a and b chains of T lymphocyte cell receptors, which can be successfully assembled into the active form. This made a substantial advance in the field of cancer therapy (Szymczak *et al.*, 2004). This strategy has shown success in treatment of various types of cancers such as, metastatic melanoma, synovial cell sarcomas, renal cell carcinomas, colorectal cancer and renal cell carcinomas (Chhabra *et al.*, 2008; Johnson *et al.*, 2009; Parkhurst *et al.*, 2009; Govers *et al.*, 2010; Hudecek *et al.*, 2010; Leisegang *et al.*, 2010; Robbins *et al.*, 2011; Wang *et al.*, 2011).

1.7.4.3 Stable co-expression of transgenes

The co-expression of multiple genes opens the door for therapeutic attempts to treat multi-gene deficiency. However, problems, such as the inability to ensure efficient co-expression, halted these attempts. The usage of IRES to achieve this met success to some extent, thereby upto 4 genes were able to be co-expressed from a polycistronic vector (Fussenegger, 2001). However, two major problems were identified in this context, the imbalance and the large size of IRES which interferes with the accurate detection of the downstream products (Mizuguchi *et al.*, 2000).

Vectors containing 2A sequences have been used to drive equimolar expression of multiple transgenes from one promoter. Co-expression of antibody fragment and enhanced yellow fluorescent protein was established from lentiviral shuttle plasmids through inserting 2A sequence between the two cDNAs. The order of the transgenes relative to the 2A influences the level of expression i.e. reduced expression of transgenes located downstream of 2A compared to the upstream is expected (Appleby *et al.*, 2013).

1.7.4.4 Enhancement of productivity in plants

In plants, the early findings, which proved that 2A is fully active in wheat germ extracts, made 2A as a strong candidate to be used in co-expression of genes. Consequently, 2A was successfully used to co-express multiple proteins in transgenic tobacco cells in addition to its efficiency in terms of plant virus genome manipulation. In this context, recombinant plant

viruses encoding 2A have been used to co-express high quality proteins. Accordingly, 2A has been used to produce a broad range of novel traits in plants such as drought resistance, high nutritional value crops, breeds with high productivity in addition to crops with modified metabolic pathways (Halpin *et al.*, 1999; Francois *et al.*, 2002; Abbadi *et al.*, 2004; Yasuda *et al.*, 2005; Obro *et al.*, 2009; Ha *et al.*, 2010; Petrie *et al.*, 2012; Ruiz-Lopez *et al.*, 2015). Furthermore, employment of 2A came with a solution for resistance against the herbicide, glyphosate and *Bacillus thuringiensis* toxins (Sun *et al.*, 2012).

2A-based polyprotein constructs have been introduced in plants to generate novel products. An example is potato strains which show resistance against drought, generated by co-expression of both trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase genes from the yeast, *Zygosaccharomyces rouxii* (Yeo *et al.*, 2000).

1.7.4.5 Recombinant virus vectors

Using strong promoters is a critical step in recombinant expression. Cytomegalovirus (CMV) promoter is commonly used for monoclonal antibody expression in Chinese Hamster Ovary cells. This is performed by using four bicistronic vectors encoding 2A sequences, which is an efficient tool to produce viable antibodies, used for therapeutic and diagnostic aspects (Ebadat *et al.*, 2017). Moreover, the non-oxygen-dependent fluorescent protein (iLOV) is a promising alternative to the green fluorescent protein (GFP). The sequence of iLOV was fused to the C terminus of Tobacco mosaic virus coat protein, which carries a 2A sequence. A systemic transfection with this recombinant vector was achieved in the plant, *Nicotiana benthamiana*, and by testing the presence of iLOV, detected by electron microscopy, it was indicated that this vector is efficient for production of proteins when combined with 2A sequence, which allows the production of proteins in different cells (Roder *et al.*, 2017). Another study combined 2A with recombinant adeno associated virus to perform long term overexpression of nerve growth factor (*VGF*) gene together with a fluorescent marker gene to track it in the transfected neurons. The relatively short sequence of the 2A allowed successful overexpression on *VGF* gene combined with the ability to follow the protein (Lewis *et al.*, 2015).

1.7.4.6 Gene therapy

Adenovirus-associated vectors encoding 2A sequence have been used to generate therapeutic proteins. Examples for these proteins are human-synclein which is expressed in Parkinson disease (Furler *et al.*, 2001). Also, this system was successfully used in homeobox

protein of target genes (Klump *et al.*, 2001). Furthermore, 2A sequence was successfully constructed in a vector bearing three genes and 2A between them to separate human iduronidase gene as well as the firefly luciferase and DsRed2. This allowed an expression of active forms of the three individual proteins which enabled a supratherapuetic iduronidase in addition to efficient protein tracking (Osborn *et al.*, 2005).

1.7.4.7 Cancer diagnosis and treatment or monoclonal antibody production

The challenge of cancer treatment and diagnosis has benefited from 2A properties. The utilisation of 2A enhanced the employment of monoclonal antibodies in this approach by overcoming the difficulties of antibodies limited production in addition to reduce the high cost. Due to the ability to co-express multiple proteins, stable production of a full-length antibodies was established by using 2A properties to express both heavy and light chains from a single ORF (Fang et al., 2005).

1.7.4.8 Stem cells applications

One application of 2A in biomedicine is that related to the treatment with stem cells. Utilisation of 2A overcame problems associated with isolation of stem cells for therapeutic purposes. Four genes were defined in this context, *Oct-3/4, SOX2, c-Myc* and *Klf4*, which when co-expressed together result in production of induced stem cells. These genes were linked to each other using 2A sequences (Okita *et al.*, 2008), which ensures the co-expression of the desired proteins in the same cells.

1.8 Aims of the study

Despite the fact that 2A reaction has been subjected to investigations and/or employment for around two decades, many aspects of the reaction, including the molecular mechanism, are still vague. As discussed above, the presence of 2A-encoding sequence in eukaryotic cellular system dictates an efficient production of separated proteins during translation. There is, however, a contradiction on the mechanism of the 2A reaction. In 2008, Doronina and co-workers suggested that eRFs are involved, at least partially, in the reaction (Doronina *et al.*, 2008), whereas in 2014, Machida and his team argued against this idea, and through *in vitro* experiments, suggested that eRFs are not required, and that 2A is an elongation rather than a termination event (Machida *et al.*, 2014).

In the work carried out in this thesis, I have biochemically tested the involvement of cellular factors in the 2A reaction, using *Saccharomyces cerevisiae* strains lacking/limited in these factors. In addition, I examined a wide variety of mutations in eRF1/eRF3, to further investigate the involvement of these factors in the reaction and to identify features of the protein that are important to drive a normal 2A reaction.

A further part of this study was to develop new reporters to study 2A activity. Reporters that have been used thus far for this purpose are either based on polyprotein systems (de Felipe *et al.*, 2006) or rely on metabolic pathways such as adenine biosynthesis to report on 2A activity (Sharma *et al.*, 2012). For *in vivo* investigations, metabolic conditions may interfere with cell growth and the consequent result. Thus, the aim here was to develop a novel reporter which works efficiently and independently of cellular pathways, and which could be used to estimate the activity of the 2A reaction under different conditions.

2 MATERIALS AND METHODS

2.1 Materials

All chemicals/reagents and enzymes, except where indicated, were purchased from Sigma-Aldrich Chemical Co, Fisher, New England Biolabs or Promega. Ingredients for media were from Melford or Formedium.

2.2 Strains and growth conditions

S. cerevisiae

Strain	Genotype	Source
JDY2	*MATa, trp1-289, lys2-801, his3-352, ura3-52, ade2-101*	Derived from TR1 (Parker *et al.*, 1988)
JDY4	*MATa, trp1-Δ99, his3-Δ200, ura3-Δ99, leu2-Δ1, ade2-101, cir^{o}*	(Sharma *et al.*, 2012)
JDY778 (MT557/1d)	*MATa, sup45-2, ade2-1, leu2-3,-112 ura3-1*	(Doronina *et al.*, 2008)
JDY795 (IS37/7b)	*MATa, sup45::hisG, leu2-3,-112, ura3-52, met8-1, SUQ5, ade2-1, [pGB1-702]*	(Bertram *et al.*, 2000)
JDY808	*MATa, sup45::kanMX4-tetO$_7$-SUP45, ura3-52, leu2-Δ1, his3-Δ200, GAL2, CMVp(tetR'-SSN6)::LEU2, trp1::tTA*	(Doronina *et al.*, 2008)
JDY809	*MATa, sup35::kanMX4-tetO$_7$-SUP35, ura3-52, leu2-1, his3-200, GAL2, CMVp(tetR'-SSN6)::LEU2, trp1::tTA*	Laboratory strain
JDY832	*MATα, ade1-14, his3, leu2, trp1, ura3, [PSI^{+}]STRONG*	(Doronina *et al.*, 2008)
JDY833	*MATα, ade1-14, his3, leu2, trp1, ura3, [PSI^{+}]WEAK*	(Doronina *et al.*, 2008)
JDY834	*MATα, ade1-14, his3, leu2, trp1, ura3, [psi^{-}]*	(Doronina *et al.*, 2008)
JDY837	*MATa, leu2-3,-112, his3-11,-15, trp1-1, ura3-1, ade1-14, sup35Δ::HIS3, pCEN-TRP1-SUP35, [psi^{-}]*	Laboratory strain derived from YDB498 (Salas-Marco and Bedwell, 2004) by plasmid shuffling

JDY838	*MATa, leu2-3,-112, his3-11,-15, trp1-1, ura3-1, ade1-14, sup35Δ::HIS3, pCEN-TRP1-sup35R419G, [psi$^-$]*	Laboratory strain derived from YDB498 (Salas-Marco and Bedwell, 2004) by plasmid shuffling
JDY839	*MATa, leu2-3,-112, his3-11,-15, trp1-1, ura3-1, ade1-14, sup35Δ::HIS3, pCEN-TRP1-sup35H348Q, [psi$^-$]*	Laboratory strain derived from YDB498 (Salas-Marco and Bedwell, 2004) by plasmid shuffling
JDY876 (W303)	*MATa, ade2-1, his3-11,-15, leu2-3,-112, ura3-2, trp1-1*	(Thomas and Rothstein, 1989)
JDY879	*MATa, ura3-52, leu2- 1, his3-Δ200, GAL2, CMVp(tetR'-SSN6)::LEU2, trp1::tTA, sup35::kanMX6, [CEN-TRP1 tet-SUP35]*	Laboratory strain
JDY895 (BY4741)	*MATa, ura3-Δ0, leu2-Δ0, met15-Δ0, his3-Δ1*	(Brachmann *et al.*, 1998)
JDY1253	*MATa, ura3-Δ0, leu2-Δ0, his3-Δ0, met15-Δ0, dom34Δ::kanMX4*	(Giaever *et al.*, 2002)
JDY1254	*MATa, ura3-Δ0, leu2-Δ0, his3- Δ0, met15-Δ0, hbs1Δ::kanMX4*	(Giaever *et al.*, 2002)
JDY1255	*MATa, ura3-Δ0, leu2-Δ0, his3- Δ0, met15-Δ0, hcr1Δ::kanMX4*	(Giaever *et al.*, 2002)
JDY1256	*MATa, ura3-Δ0, leu2-Δ0, his3-Δ0, met15-Δ0, ski7Δ::kanMX4*	(Giaever *et al.*, 2002)
JDY1257	*MATa, ura3-Δ0, leu2-Δ0, his3-Δ0, met15-Δ0, CYH2+, DAmP-RLI1*	(Breslow *et al.*, 2008)
JDY1296	*MATa, ura3-Δ0, leu2-Δ0, his3-Δ0, met15-Δ0, CYH2+, DAmP-SUP45*	(Breslow *et al.*, 2008)
JDY1297	*MATa, ura3-Δ0, leu2-Δ0, his3-Δ0, met15-Δ0, tef1Δ::kanMX4*	(Giaever *et al.*, 2002)
JDY1298	*MATa, ura3-Δ0, leu2-Δ0, his3-Δ0, met15-Δ0, CYH2+, DAmP-TEF4*	(Breslow *et al.*, 2008)
JDY1299	*MATa, ura3-Δ0, leu2-Δ0, his3-Δ0, met15-Δ0, SOD1-DHFR [F1,2]::natMX*	Laboratory strain

JDY1300	*MATa, ura3-Δ0, leu2-Δ0, his3-Δ0, lys2-Δ0, SOD1-DHFR [F3]::hohMX*	Laboratory strain
YTH89	*MATα, ade1-14, trp1-2889, his3-Δ200, ura3-52, leu2-3,-112, sup45Δ::kanMX4*	Tobias von der Haar lab
YTH91	*MATa/α, his3-Δ1/his3-Δ1, leu2-Δ0/leu2-Δ0, LYS2/lys2-Δ0, met15-Δ0/MET15, ura3-Δ0/ura3-Δ0, sup45Δ::kanMX4*	Tobias von der Haar lab

Table 2.1: Strains of *S. cerevisiae* used in the study with the genotype.

Table 2.1 shows strains of *S. cerevisiae*, used in this study. Yeast were grown in non-selective rich medium, YPD (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, with 2% w/v agar for plates), or selective media (0.67% w/v yeast nitrogen base, 2% w/v glucose, with 2% w/v agar for plates, supplemented with required amino acids and/or nucleobases, according to growth requirements. Selective media containing 0.1% w/v 5-fluoroorotic acid (5FOA) was used for selection against URA^+ cells (Boeke *et al.*, 1984). Expression of 2A reporters from the galactose-dependent *GAL1* promoter was performed for immunoprecipitation experiments, by growing cells in selective media with 2% w/v raffinose, rather than glucose, then galactose was added to concentration of 2% w/v for ~5 hours. Yeast cells were incubated at 30 ºC, unless they were thermosensitive.

E. coli

E. coli strain, DH10b *BF- endA1 recA1 galK6 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC)λ-* (Grant *et al.*, 1990) was used in this study to produce plasmid

DNA and to transform ligation reactions. Cells were grown in lysogeny broth (LB) media (2% w/v tryptone, 1% w/v yeast extract, 1% w/v NaCl, with 2% w/v agar for plates), with 100 µg/ml ampicillin added when necessary for plasmid selection, and incubated at 37 ºC.

2.2.1 Plasmids

Plasmids, and their details, which were used in this study are stated in table 2.2.

Name	Vector	Insert	Source/Reference
pJNY11	pMW20 *GAL* (CEN, *URA3*)	-	(de Felipe *et al.*, 1999)
pJNY59	pMW20	αFactor-2A-GFP	(de Felipe *et al.*, 1999)
pJNY161	YCplac22	Wild type *SUP35*	(Salas-Marco and Bedwell, 2004)
pJNY162	YCplac23	H438Q *SUP35*	
pJNY163	YCplac24	G419A *SUP35*	
pJNY175	pMW20	αF-2A TAV-GFP	Lab collection
pJNY176	pMW21	αF-2A* TAV-GFP	
pJNY250	pDB0688	read-through cassette CAAA s	(Du *et al.*, 2009) Gifted by Bedwell
pJNY251	pDB0688	Renilla-read-through cassette UAAA-Fire fly	
pJNY253	pDB0688	Renilla-read-through cassette UAGC-Fire fly	
pJNY252	pDB0688	Renilla-read-through cassette CGAC-Fire fly	
pJNY254	pDB0688	Renilla-read-through cassette UAGu-Fire fly	
pJNY278	pJNY11	3HA-αF-2A*-GFP	Lab collection
pJNY279	pJNY12	3HA-αF-2A-GFP	Lab collection
pET17b	_	Protein induction plasmid	

pJN132	pET17b	*SUP45*-STREP tagged	Lab collection
pJN134	pET17b	*SUP35*	Lab collection
pJN141	pET17b	αF-2A-PPL SP6	Lab collection
pJN142	pET17b	αF-2A*-PPL SP6	Lab collection
pJN177	pSP65	aF-2A-pL poly A	Lab collection
pJN178	pSP66	aF-2A*-pL poly A	Lab collection
pJN197	pET3-a	Wild type *SUP35*	Lab collection
pJN198	pET3-a	H438Q *sup35*	Lab collection
pJN199	pET3-a	G419A *sup35*	Lab collection
pJN239	pDB1083	HIS-*SUP45*	Gifted by David Bedwell
pTH353	pRS315	WT *SUP45*	(Merritt *et al.*, 2010)
pTH399	pRS316	S30P *SUP45*	
pTH313	pRS317	I32F *SUP45*	
pTH314	pRS318	P38L *SUP45*	
pTH438	pRS319	L49A *SUP45*	
pTH439	pRS320	E52A *SUP45*	
pTH355	pRS321	T55A *SUP45*	
pTH371	pRS322	S61A *SUP45*	
pTH373	pRS323	R62A *SUP45*	
pTH374	pRS324	V68A *SUP45*	
pTH316	pRS325	V107D *SUP45*	
pTH443	pRS326	P174Q *SUP45*	
pTH444	pRS327	G180A *SUP45*	
pTH376	pRS328	G181A *SUP45*	
pTH397	pRS329	Q182E *SUP45*	
pTH445	pRS330	I222S *SUP45*	
pTH357	pRS331	T295A *SUP45*	
pTH363	pRS332	T388A *SUP45*	
pTH366	pRS333	F401Y *SUP45*	
pTH365	pRS334	Y410F *SUP45*	
pTH769	pRS313	WT *SUP45*	

pTH770	pRS314	R320I *SUP45*	
pTH773	pRS315	G357D *SUP45*	
pTH774	pRS316	S416F *SUP45*	
pDHFR	pRS317	WT DHFR	This study
pDH	pRS318	F3 fragment of DHFR	This study
pFR	pRS319	F1,2 fragment of DHFR	This study
pDHFR/A	pRS320	FMDV 2A in the middle of DHFR	This study
pDHFR/*	pRS321	2A in the middle of DHFR	This study
pGFP	pRS316	WT GFP	This study
pTAV 19	pRS317	Short TAV 2A in GFP	This study
pTAV* 19	pRS318	Short TAV 2A* in GFP	This study
pTAV 31	pRS319	Long TAV 2A in GFP	This study
pTAV* 31	pRS320	Long 2A* in GFP	This study
pFM	pRS321	FMDV 2A in GFP	This study

Table 2.2: Plasmids used in this study.

A collection of plasmids was constructed during the course of this study, which encodes the GFP-2A reporter (see Chapter 5). We used different sequences of 2A peptides and cloned them, into a pRS316 vector, which carries a *URA3* marker. 2A sequence was cloned between bases encoding the amino acid residues 157 and 158 of the GFP sequence, with few extra sequences on both sides to allow folding flexibility. The whole construct was cloned in the plasmid poly-linker which is flanked by T7 initiator and T3 terminator. The initial strategy of cloning included amplification of PCR DNA encoding the required 2A sequence using the forward specific oligonucleotide and T3 universal oligonucleotide as a reverse. This produces a DNA

fragment with a 2A sequence at the 5', starts with BglII site, and ends with T3 terminator at the 3' end, flanked with EcoRI site. BglII and EcoRI sites, which are unique in the vector backbone, were successfully used to clone PCR fragments encoding different 2A/2A* sequences-extra amino acid codons to allow folding flexibility-GFP sequence starts with sequence encoding residue 158 -T3 terminator sequence, into a pRS316 vector.

2.2.2 Oligonucleotides

Oligonucleotides used in this study were supplied by Sigma Aldrich. Sequences of oligonucleotides are stated in table 2.3.

Name	**Sequence 5'-3'**	**Usage**
2A Fwd	CCGTCTAGAGCATGCGCACCG	19 amino acids 2A inserted in DHFR
2A Rvs	CCTCCATGGCATGACTGCCCGGGGCCC	
DHFR Fwd	AGGGGATCCACGGTTCGACCATTGAACTG	DHFR F1,2
DHFR Intrvs	GGCCCATGGGAATCTAGAGCCACTGCCCAATTCCGGT TGTTCAATAAGTC	
DHFR intfwd	CGGACCGGTCCATGGGGGAGCGGCGCAAGTAAGTAG ACATGGTTTGG	DHFR F3
DHFR intrvs	ACGCTGCAGTTAGTCTTTCTTCTCGTAGAC	
RLI1FED	CCGGAGCTCCCGCCGACGTGTCATTTTCG	*RLI1* amplification
RLI1RVS	CCGTTACCCGGGAATACCGGTGTTATCCAAGAAAAAG	
eif3jfwd xba1	GCATCTAGAATTGGTCGCG CACTAAATTTG	*HCR1* (eIF3j) amplification
eif3jrvsBamHI	GCTGGATCCAACGGCAATATTGGCTACATTC	
Sup45fwd	GCCCATATGGATAACGAGGTTGAAAAAAATATTG	*SUP45* amplification
Sup45rvs	GCCTCTAGATTAATGATGATGATGATGATGATGATG AATGAAATCATAGTCGGATCC	

IFV 2A	CCGAGATCTCCCTCAATTGGTAATGTCGCGCGGACTCT GACGAGGGCGGAGATTGAGGATGAATTGATTCGTGCA GGAATTGAATCAAATCCAGGTCCATCCATCCGGA	Various sequences of 2A peptides from different viruses
PrV2A	CCGAGATCTTTGGAGATGAAGGAGTCTAATAGTGGTT ACGTAGTCGGTGGTCGGGGGTCTCTTCTCACTTGTGGG GACGTTGAATCCAATCCAGGTCCATCCGGA	
DCV 2A	CCGAGATCTCAAGGCATCGGTAAGAAGAATCCGAAAC AGGAAGCTGCACGTCAGATGTTGCTCTTGTTATCAGG AGATGTTGAGACTAATCCGGTCCATCCGGA	
PTV	CCGAGATCTGCTATGACTGTGATGACATTCCAGGGAG GAGGTGCAACAAACTTCTCCCTCTTGAAACAAGCAGG AGATGTTGAGGAAAATCCAGGTCCATCCGGA	
IMNV	CCGAGGTCTTGGGACCCAACCTACATTGAAATTTCTG ATTGTATGCTGCCACCTCCAGACCTTACATCGTGCGGG ACGTTGAGAGTAATCCAGGTCCATCCGGA	
IFV 2A	CCGAGATCTCCCTCAATTGGTAATGTCACGCGGACTCT GACGAGGGCGGAGATTGAGGATGAATTGATTCGTGCA GGAATTGAATCAAATCCAGGTCCATCCGGA	
PrV2A1	CCGAGATCTTTGGAGATGAAGGAGTCTAATAGTGGTT ACGTAGTCGGTGGTCGGGGGTCTCTTCTCACTTGTGGG GACGTTGAATCCAATCCAGGTCCATCCGGA	
DCV 2A	CCGAGATCTCAAGGCATCGGTAAGAAGAATCCGAAAC AGGAAGCTGCACGTCAGATGTTGCTCTTGTTATCAGG AGATGTTGAGACTAATCCAGGTCCATCCAGGA	
PTV	CCGAGATCTGCTATGACTGTGATGACTTCCAAGGGAC CAGGTGCAACAAACTTCTCCCTCTTGAAACAAGCAGG AGATGTTGAGGAAAATCCAGGTCCATCCGGA	
IMNV	CCGAGATCTTGGGACCCAACCTACATTGAAATTTCTG ATTGTATGCTGCCACCTCCAGACCTTACATCGTGCGGG GACGTTGAGAGTAATCCAGGTCCATCCGGA	
IFV 2A	CCGAGATCTCCCTCAATTGGTAATGTCGCGCGGACTCT GACGAGGGCGGAGATTGAGGATGAATTGATTCGTGCA GGAATTGAATCAAATCCAGGTCCATCCGGA	

TaV 2A	GGTAGATCTAGGGGCCCCAGACCGCAGAACCTCGGAG TCAGAGCTGAAGGTAGAGGTTC	
T3	AATTAACCCTCACTAAAGGG	For amplification of 2A using pRS316 template
OPT*	CCAAGATCTAGACATAAACAAAAAATTGTTGCTCCAG TTAAACAAACTTTGAATTTTGATTTGTTGAAATTGGCT GGTGATGTTGAATCTAATGCTGGTCCATCCGGAGG	Optimised 2A sequence
S15I	CCAAGATCTAGACATAAACAAAAAATTGTTGCTCCAG TTAAACAAACTTTGAATTTTGATTTGTTGAAATTGGCT GGTGATGTTGAAATTAATCCAGGTCCATCCGGAGG	De-optimised 2A sequence
N16H	CCAAGATCTAGACATAAACAAAAAATTGTTGCTCCAG TTAAACAAACTTTGAATTTTGATTTGTTGAAATTGGCT GGTGATGTTGAATCTCATCCAGGTCCATCCGGAGG	Mutations in the FMDV2A sequence
E14Q	CCAAGATCTAGACATAAACAAAAAATTGTTGCTCCAG TTAAACAAACTTTGAATTTTGATTTGTTGAAATTGGCT GGTGATGTTCAATCTAATCCAGGTCCATCCGGAGG	
TaV2A	GGTAGATCTAGGGGCCCCAGACCGCAGAACCTCGGAG TCAGAGCTGAAGGTAGAGGTTC	19 amino acids Tav2A sequence
FMDV 2A*	CCAAGATCTAGACATAAACAAAAAATTGTTGCTCCAG TTAAACAAACTTTGAATTTTGATTTGTTGAAATTGGCT GGTGATGTTGAATCTAATGCTGGTCCATCCGGAGG	Inactive FMDV2A

Table 2.3: Oligonucleotides used in this study.

2.3 Molecular biology techniques

Standard techniques including plasmid preparation, PCR amplification, DNA ligation, digestion were achieved according to Sambrook and Gething (1989), or following manufacturer's instructions when kits were used. Sequencing was carried out by Source Bioscience.

2.3.1 Separation and visualization of DNA fragments

Separation of DNA was performed using gel electrophoresis, with 1% w/v agarose in 1X TAE (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA) with 1 µg/ml ethidium bromide. Gels were run at 140 V until suitable resolution of fragments was achieved. DNA was visualised under UV light, and images captured using gel documentation system (BIO RAD Gel Doc™ XR).

2.3.2 Separation of proteins

Proteins were resolved by polyacrylamide gel electrophoresis (SDS-PAGE) (Table 2.4), using tris-glycine gels (Zhang *et al.*, 2016). Protein samples were run on a vertical electrophoresis system, using 1X protein running buffer (25 mM Tris-HCl, 192 mM Glycine, 0.1% w/v SDS). Gels were run at 150 V until the dye-front reached the bottom of the gel. When necessary, visualization of protein fractions was carried out using Coomassie blue staining (0.25% w/v Coomassie brilliant blue, 50% v/v methanol, 7% v/v acetic acid) for 15 minutes, then de-stained for ~2 hours, using destain solutions (45% v/v methanol, 7% v/v acetic acid).

Ingredient (ml)	**Gel (%)**			
	10	12	15	Stacking
Separating buffer	2.5	2.5	2.5	1.25
40% Acrylamide	2.5	3	3.75	1.25
2% Bis acrylamide	1.5	1.6	2	0.66
10% SDS	0.1	0.1	0.1	0.1
H2O	3.23	2.6	1.4	6.4
TEMED	0.004	0.004	0.004	0.005
10% APS	0.167	0.167	0.167	120

Table 2.4: Ingredients used in different concentrations of SDS-PAGE.

2.3.3 Polymerase chain reaction (PCR)

PCR was performed using Velocity DNA polymerase (BIOLINE), or homemade polymerase for general diagnostic procedures. A standard PCR reaction (total volume 100 µl) consists of 10 µl of 10X polymerase buffer (provided by manufacturer or 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.01% w/v gelatin diluted from a 10X stock), 1 mM dNTPs, 1-2 units of DNA polymerase, 1 pM of each oligonucleotide and 1 ng/µl of DNA template. A

standard 3 step programme was used and reactions were run in PTC-100 (MJ Research), as follows (table 2.5):

Step	Temperature (ºC)	Time
Initial denaturation	96	5 min.
Denaturation	96	30 sec.
Annealing*	55	30 sec.
Extension	72	1 min.
Extension in total	72	10 min.

Table 2.5: The three step program used to amplify PCR fragments.

*Annealing temperature was optimized according to the oligonucleotides used in the reaction. 25-35 cycles for the denaturation, annealing and extension were used to obtain considerable concentration of PCR products, while a final extension step was set on 10 minutes.

2.3.4 Preparation of competent *E. coli* cells

Competent *E. coli* cells of DH10b strain were prepared according to Sambrook and Gething (1989), using the $CaCl_2$ method. A culture was grown in LB at 37 ºC overnight, the culture was then diluted 200 times into 100 ml of LB medium and incubated again until it reached OD_{600} ~ 0.5. Cells were incubated on ice for 10 minutes, then centrifuged at 5000 rpm for 5 minutes using Avanti J20-XP (Beckman Coulter). Cell pellets were re-suspended in 40 ml TFBI (30 mM KOAc, 100 mM $RuCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% v/v glycerol, pH 5.8 adjusted with acetic acid) at 4 ºC. Cells were then incubated on ice for 5 minutes, pelleted at 3000 rpm for 10 minutes and suspended in 4 ml TFBII (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RuCl_2$, 15% v/v glycerol, pH 6.5 adjusted with KOH). Cells were incubated on ice for 15 minutes, then dispensed as 200 µl aliquots into Eppendorf tubes and stored at -80 ºC.

2.3.5 Transformation of *E. coli*

Transformation of competent *E. coli* cells was carried out as follows. Appropriate number of competent cell aliquots (60 µl per sample) prepared as in section 2.3.4 were thawed on ice, 1-100 ng of plasmid DNA or 10 µl of a ligation reaction were added, mixed and the tube incubated on ice for 15-30 minutes. Cells were exposed to heat shock at 42 ºC for two minutes and then placed back on ice for another minute. 1 ml of LB media was added to each reaction, and cells were then incubated at 37 ºC for 45 minutes. Cells were harvested; 8000 rpm for 5 minutes, and most of the liquid was discarded. Pellets were re-suspended in the remaining

liquid, and either plated onto LB plates or inoculated into LB liquid supplemented with 100 µg/ml ampicillin as appropriate. Plates/flasks were then incubated at 37 ºC overnight.

2.3.6 Plasmid preparation from *E. coli*

Plasmid preparation from *E. coli* was performed in small and large scales namely mini and midi preparations. Both rely on alkaline lysis of cells (Birnboim and Doly, 1979).

2.3.6.1 Midi preparation

Cells were harvested from 50 ml of overnight culture by centrifugation at 3000 rpm for 15 minutes in a 50 ml Falcon tube using a Beckman J6 centrifuge. The culture media was discarded and the pellet re-suspended in 2 ml of TEG buffer (1% w/v glucose, 2 mM EDTA, 2.5 mM Tris-HCl pH 8.0). 4 ml of SDS/NaOH reagent were added (0.2 M NaOH, 1% w/v SDS) and the tubes mixed by inversion. Then, 2.5 ml of 3 M sodium acetate (pH 5.2) were added and the tubes, mixed by vortexing. Samples were centrifuged as above, supernatants transferred to fresh Falcon tubes, mixed with an equal volume of isopropanol, and centrifuged again as above. Pellets were washed with 70% v/v ethanol, allowed to air dry and re-suspended in 0.5 ml Tris EDTA (TE) (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Solutions were transferred to Eppendorf tubes and 5 µl of 10 mg/ml RNAase A were added. Tubes were then incubated at 37 ºC for 30 minutes. PCI (25:24:1 phenol:chloroform:isoamylalcohol) extraction was applied, by adding an equal volume to each tube, vortexing, and centrifuging at full speed for 5 minutes in a microcentrifuge. The top aqueous layers were separated into fresh tubes, and PCI extraction was repeated 3-5 times until the interface between the layers was clear. DNA was then precipitated by addition of an equal volume of isopropanol and 1/10 volume of 3 M sodium acetate (pH 5.2), and centrifuged at full speed for 15 minutes in a microcentrifuge. DNA pellets were washed in 70% v/v ethanol and allowed to air dry. Finally, DNA was dissolved into 250 µl TE and frozen at -20 ºC.

2.3.6.2 Mini preparation

3 ml of overnight culture of *E. coli* were harvested in Eppendorf tubes (by filling the same tube twice) in a microcentrifuge at full speed for 3 minutes. The pellet was re-suspended in 100 µl TEG buffer and 200 µl of SDS-NaOH were added and mixed by inversion, and 150 µl of 3 M sodium acetate were also added and mixed. 10 µl RNAase A were added, and samples were incubated at 37 ºC for 30 minutes. Samples were centrifuged, and supernatants were transferred to fresh tubes. One PCI extraction cycle was applied, and upper layers were

transferred to fresh tubes. DNA was precipitated by addition of an equal volume of isopropanol, and centrifugation at full speed in a microcentrifuge for 15 minutes. Supernatants were removed and pellets were washed with 70% v/v ethanol, allowed to air dry, and dissolved in 25 µl TE.

2.3.7 Restriction enzyme digestion of DNA

Standard DNA digestion reactions were performed as follows in table 2.6:

Componenets	Volume (µl)
DNA	2-5 (1 µg)
Restriction enzyme	1
Optimum manufacturer's buffer (10X)	2
dH_2O	To 20

Table 2.6: Basic components of DNA digestion reaction.

Reactions were incubated at recommended temperatures for maximum enzyme activity for 1 hour and then run on a 1% w/v agarose gel (section 2.2.1).

2.3.8 DNA ligation

Ligation reactions were assembled as follows in table 2.7:

Components	Volume (µl)
Vector DNA	50 -200 ng
Insert	Equal molar ratio (3-5 times of the vector)
T4 DNA ligase	1
10X T4 ligase buffer	2
dH_2O	To 20

Table 2.7: Basic components of ligation reaction.

Reactions were incubated at room temperature for 1-3 hours and then transformed into *E. coli* (section 2.3.5).

2.3.9 DNA transformation into yeast

2.3.9.1 *S. cerevisiae* transformation

DNA including plasmids and fragments were introduced into *S. cerevisiae* cells using the lithium acetate method (Ito *et al.*, 1983). Overnight cultures of yeast cells were grown at 30 ºC, diluted 10-times the next morning and incubated again to an OD_{600} of ~0.5. Cells were harvested at 3000 rpm for 5 minutes in 50 ml Falcon tubes in a Beckman J6 centrifuge and pellets washed with lithium acetate mix (0.1 M LiOAc pH 7.3) and re-suspended in 1 ml of the same reagent. 0.1-1 µg of transforming DNA was added to 100 µl of cell suspension, 10 µl of carrier DNA (10 mg/ml) (freshly denatured and chilled salmon sperm DNA) and 700 µl polyethylene glycole (PEG) mix (46% w/v polyethylene glycol-2000, 0.1 M LiOAc pH 7.3, 1X TE) were added and mixed gently with the cell suspension, and then samples were incubated at room temperature (RT) for 30 minutes. Heat shock was applied at 42 ºC for 20 minutes, and cells were then pelleted at 5000 rpm for 1 minute in a microcentrifuge. Most of the liquid was discarded; cells were re-suspended in the remaining volume and plated on appropriate selective plates.

2.3.10 Plasmid recovery from *S. cerevisiae*

To isolate plasmid DNA from *S. cerevisiae*, 2 ml cultures were grown in selective media overnight. Cells were harvested in a 15 ml Falcon tube at 3000 rpm for 5 minutes. Pellets were washed with TE, and then re-suspended in 100 µl of STET buffer (8% w/v sucrose, 50 mM Tris-HCl pH 8.0, 50 mM EDTA and 5% v/v Triton X-100). Suspensions were transferred to Eppendorf tubes and Zirconium beads (Biospec Products) were added to just below the liquid surface. Cells were lysed using a bead-beater (Mini-Beadbeater-16, Biospec products) twice for 20 seconds, with 2-3 minutes incubation on ice in between to cool down the lysate. A further volume (100 µl) of STET were added, and samples were boiled for 10 minutes and centrifuged for 10 minutes at 14,000 rpm, in a cooled microcentrifuged. 100 µl of liquid were taken to a fresh Eppendorf tube containing 50 µl of 7.5 M ammonium acetate. Tubes were then incubated at -20 ºC for 2 hours, and then centrifuged at 14,000 rpm for 10 minutes in a cooled microcentrifuge. 100 µl of the resulting supernatant were transferred to a fresh tube with 200 µl ethanol. Samples were incubated on ice for 15 minutes before repeating the centrifugation step to pellet DNA. The resulting pellets were washed with 70% v/v ethanol, allowed to air dry and then re-suspended in 10-20 µl of TE. 10 µl of the final volume was used for transformation into *E. coli*.

2.3.11 Extraction of genomic DNA from *S. cerevisiae*

To isolate the genomic DNA from *S. cerevisiae*, cultures (5 ml) of relevant strains were grown overnight in YPD at 30 ºC. Cells were harvested by centrifugation at 3000 rpm for 5 minutes, using a bench-top centrifuge, washed twice with sterile water, and re-suspended in 200 µl of lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% w/v SDS, 2% v/v Triton X-100). Cell suspensions were transferred to Eppendorf tubes, 200 µl of phenol: chloroform: isoamylalcohol (25:24:1) were added, and zirconium beads were then added to just below the liquid surface. Cell lysis was achieved using a bead-beater, twice for 20 seconds, with 2-3 minutes incubation on ice in between. Samples were then centrifuged, and 200 µl of supernatants transferred to fresh tubes. To precipitate the DNA, 20 µl of 3 M sodium acetate (pH 5.2) in addition to 400 µl of ethanol were added, and samples were centrifuged at 14,000 rpm for 15 minutes in a microcentrifuge. Pellets were washed in 70% v/v ethanol, allowed to air dry, and then re-suspended in 100 µl of dH_2O.

2.4 Biochemical techniques

2.4.1 Protein expression

50 ml of *S. cerevisiae* culture were incubated overnight at 30 ºC in appropriate selective media. For induction from a *GAL1* promoter, overnight cultures contained 2% w/v raffinose (instead of glucose) to remove repression on the promoter. Cultures were diluted on the next day to OD_{600} 0.2 and re-incubated with addition of 2% w/v galactose. Cells were harvested when cultures reached OD_{600} ~0.5 by centrifugation at 3000 for 5 minutes, and washed twice with dH_2O at 4 ºC. Cell suspensions were transferred to Eppendorf tubes and centrifuged at 5000 rpm for 1 minute and processed as required for downstream experiments.

2.4.2 Western blotting

2.4.2.1 Cell lysis and protein extraction for western blotting

Pellets of *S. cerevisiae* cells were re-suspended in 0.5 ml of native buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA pH 8, 5 mM DTT, 1 mM PMSF), Zirconium beads were added and cells were lysed using the bead-beater, twice for 20 seconds with 2-3 minutes incubation on ice in between. The lysate was removed from the beads to fresh tubes and centrifuged at 13000 for 20 minutes at 4 ºC in a microcentrifuge. Clarified lysates were then transferred to fresh tubes. Protein concentration was measured using Bradford assay (Biorad), and a standard curve generated using Bovine Serum Albumin (BSA).

2.4.2.2 Blotting technique

Samples were adjusted for equal concentration of protein, mixed with equal volume of SDS-PAGE sample loading buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 10% v/v glycerol, 2% w/v SDS, 0.1% w/v bromophenol blue), heated at 65 ºC for 5 minutes and a volume corresponding to 10-20 µg lysate run on an SDS-PAGE gel (see section 2.2.2). Proteins were transferred from the gel onto nitrocellulose membrane (Schleicher & Schuell) using a semi-dry blotter (Bio-Rad Trans-Blot) according to manufacturer's instructions. Transferred proteins were visualised by applying Ponceau stain for 1 minute and then rinsing the membrane in water. Blocking of the membrane was performed through incubation in block solution (5 % w/v skimmed milk, 0.5% v/v Tween, 1X TBS, for 1X TBS, 50mM Tris-HCl, 120 mM NaCl pH 7.6) for 30-60 minutes. The membrane was then exposed to the primary antibody (1:1000 dilution in block solution) and incubated 1 hour at room temperature or overnight at 4 ºC. The membrane was then washed 3 times for 5 minutes with block solution, and then exposed to the secondary antibody for 1 hour at room temperature (1:2000 in block solution) and washed as previously. A final wash was carried out with 1X TBS. The membrane was then incubated with developing solution (1.25 mM luminal, 0.2 mM p-coumaric acid in 1M Tris-HCl pH 8.5 to which 0.03% v/v H_2O_2 was added just before use). The signal was detected on photographic film (Kodak BIOMAX light).

2.4.3 Pulse labelling and immunoprecipitation

2.4.3.1 Protein expression

Culture growth and protein induction was performed as stated in section 2.4.1. Cells were harvested from a volume of culture corresponding to 2-5 OD_{600}, by centrifugation at 3000 rpm for 5 minutes in a bench-top centrifuge. Cells were washed once in dH_2O, transferred to screw top Eppendorf tubes and centrifuged at 5000 rpm for 1 minute. Cells were then washed once in -Met -Cys selective media, containing 2% w/v each of raffinose and galactose, re-suspended in 1 ml of the same media and incubated at 30 ºC for 15 minutes. 50 µCi [^{35}S]-labelled Met/ Cys (EXPRES protein labelling mix, Perkin Elmer, 11 mCi/ml) were added per OD, and incubation continued for a further 10 minutes. Cells were harvested at 5000 rpm for 1 minute in a microcentrifuge, media removed and tubes placed into liquid nitrogen.

2.4.3.2 Cell lysis and protein extraction for immunoprecipitation

Pellets of yeast were re-suspended in 0.5 ml TCA buffer (20 mM Tris-HCl pH 8.0, 50 mM ammonium acetate, 2 mM EDTA), and 0.5 ml 30% w/v TCA. ~0.5 ml Zirconium beads

was added and cells were lysed in a mini bead-beater (Biospec products) twice for 30 seconds, with a 2-3 minute incubation on ice in between. Lysate was transferred to fresh tubes, and beads were washed with 400 µl of 1:1 (TCA buffer:30% w/v TCA), and this liquid was added to the initial lysates. Precipitated proteins were collected by centrifugation at 14000 rpm for 10 minutes in a microcentrifuge, supernatants were discarded, and pellets re-suspended in 40 µl/ OD of resuspension buffer (3% w/v SDS, 100 mM Tris-HCl pH 10.8, 3 mM DTT). The samples were heated at 65 ºC for 5 minutes and at 100 ºC for another 5 minutes prior to centrifugation at 14000 rpm for 5 minutes in a microcentrifuge. Protein solutions were transferred to fresh tubes in 40 µl aliquots and frozen in liquid nitrogen.

2.4.3.3 Immunoprecipitation

Aliquots of labelled proteins were thawed and the following were added to each: 560 µl of IPSII (13.3 mM Tris-HCl pH 7.5, 50 mM NaCl, 1% v/v Triton X100, 0.02% w/v NaN_3), 6 µl of 100 mM PMSF and 30 µl of protein G resin (Amintra), pre-equilibrated in IPSI (0.2% w/v SDS, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% v/v Triton X, 0.02% w/v NaN_3). Samples were incubated at 4 ºC for 1 hour on a rotating wheel, and centrifuged at 2000 rpm for 1 minute in a microcentrifuge. The liquid was transferred to fresh tubes and 1-2 µl of antibody were added to each sample and incubated for 1 hour on the rotating wheel. 30 µl of protein G resin in IPSI were added and incubation continued for 1 more hour. Samples were centrifuged, supernatants discarded and resin washed 5X with TBS. Resin was transferred to fresh tubes before the last washing steps. After removal of the final wash, 30 µl of protein loading dye were added to samples, which were then heated at 65 ºC for 5 minutes and loaded onto SDS-PAGE gels. After electrophoresis, gels were stained with Coomassie brilliant blue for 15 minutes and de-stained for 2 hours. Gels were dried on a vacuum drier and [^{35}S] Met/Cys detected using phosphor storage screens and a Typhoon Trio Scanner (GE Healthcare). Quantification of bands was carried out using ImageQuant. Relative amounts of protein were normalized according to the number of methionine and cysteine residues contained in the detected different proteins.

2.4.4 Dual reporter assay

Dual luciferase assay was carried out in the presence of 2A over-expression. The activity of luciferases generated from Renilla and Firefly were determined using home-made reagents and values read using a fluorimeter (POLARstar Omega plate reader).

2.4.4.1 Protein expression

Cultures of *S. cerevisiae* expressing dual luciferase reporters were grown at 30 ºC overnight in selective media, diluted the next day and re-grown to OD_{600} ~0.5. Cells were harvested in 50 ml Falcon tubes at 3000 rpm for 5 minutes in a bench-top centrifuge. Cell pellets were re-suspended in 1 ml dH_2O, transferred to Eppendorf tubes and cells re-pelleted by centrifugation at 5000 rpm for 1 minute in a microcentrifuge. The resulting pellets were re-suspended in 500 µl of passive lysis buffer (Promega). Zirconium beads were added to just below the top of the liquid and cells lysed using the mini bead beater, 2X for 20 seconds, with 2-3 minutes incubation on ice in between. Lysates were transferred to fresh tubes and centrifuged at 13000 rpm for 20 minutes at 4 ºC in a microcentrifuge. Supernatants were taken to fresh tubes, and protein concentrations determined as in section 2.4.2.1.

2.4.4.2 Measurement of luciferase activity

Lysate corresponding to 10-20 µg protein was used to measure Renilla and Firefly luciferase activity. Two reagents were used; reagent A (200 mM Tris-HCl pH 7.4, 15 mM $MgSO_4$, 0.1 mM EDTA, 25 mM DTT, 1 mM ATP (Calbiochem), 0.2 mM Co-enzyme A (Calbiochem), 200 µM D-luciferin substrate (Sigma Aldrich)), and reagent B (25 mM NaPPi, 10 mM NaAc, 500 mM Na_2SO_4, 500 mM NaCl, 50 µM Luciferase Inhibitor (Calbiochem), 4 µM colentrazine-h (Melford)). Reagent A measures Firefly luciferase activity, whereas reagent B inhibits Firefly luciferase and allows the measurement of Renilla luciferase activity. Measurement was performed in 96-well microplates, with lysate being added to each well, then the plate inserted into the fluorometer. Reagents A and B were prepared separately in 15 ml Falcon tubes, and connected to injectors on the fluorimeter. The reagents were then added and Renilla (first ORF) and Firefly (second ORF) activities measured automatically and background (i.e. measurements from lysates not containing any luciferase) subtracted from the measured values. Average values for each enzyme were calculated from 26 interval measurements and the ratio of the Firefly (downstream)/Renilla (upstream) was then established.

2.5 *In vitro* translation

2.5.1 Yeast translation competent extracts

Lysates were made essentially as described (Ng *et al.*, 1994). Cultures of *S. cerevisiae* strains were grown in 2.8 L flasks in YPD at 30 ºC overnight, diluted on the next day using the same media, and re-grown again to OD_{600} 0.5. Cells from 10-12 L culture were harvested at 3000 rpm for 5 minutes at 4 ºC in 1 L centrifuge bottles in a Beckman Coulter J-20 XP

centrifuge. Pellets were collected, snap frozen by squirting from a syringe into liquid nitrogen, and stored at -80 °C. Cells were lysed while frozen by homogenization in a Waring blender equipped with a stainless steel vessel in the presence of a small volume of liquid nitrogen. Blending was for 15 seconds and the resulting frozen lysate was thawed into 3 ml of buffer A (0.1 M KOAc pH 7.5, 2 mM MgOAc, 4 mM HEPES KOH pH 7.4, 0.5 mM PMSF, 3 mM DTT). Lysates were centrifuged at 6000 rpm for 15 minutes at 4 ºC in JA20 Beckman tubes. The clear supernatant was collected and centrifuged again at 29000 rpm for 35 minutes at 4 ºC in a Beckman Ti80 ultracentrifuge rotor. Following this step, the clear middle layer of the resulting supernatant was recovered from the tubes and loaded into a 100 ml sephadex G25 column equilibrated in 1X buffer A containing 14 % v/v glycerol. Fractions were collected every 2 minutes, and samples with highest OD_{260} were pooled, aliquoted and snap frozen at -80 °C. Alternatively, the clear fraction from the ultracentrifugation step was loaded onto Zeba spin desalting columns (Thermofisher Scientific), according to the manufacturer's instructions. Protein fractions were collected and frozen as above.

2.5.2 *In vitro* transcription

Capped mRNA templates used for *in vitro* translation were generated from DNA templates, using a commercial kit (RiboMAX™ Large Scale SP6, Promega). Plasmid DNA, encoding the desired 2A reporter, was linearized using an appropriate restriction enzyme to be used as a template, purified by PCI, precipitated with ethanol and re-suspended in an appropriate volume of RNAase-free water. Transcription reactions were assembled according to the manufacturer recommendations as follows in table 2.8:

Component	**Qty (µl)**
5X SP6 transcription buffer	4
rNTPs (25 mM ATP, CTP, UTP and 3 mM GTP)	4
DNA template	2 (5-10 µg)
40 mM Ribo cap analog (Promega)	1.5
enzyme mix	2
RNAase-free water	To final volume 20

Table 2.8: The components of RNA transcription reactions.

Reactions were incubated at 37 ºC for 4-5 hours. RNA was then extracted with PCI, precipitated with ethanol and re-suspended in RNAase-free water. RNA concentration was estimated using a Nanodrop spectrophotometer (ThermoFisher), and integrity checked by mixing 2-4 µg with RNA-loading dye (90% v/v formamide, 0.5% w/v EDTA, 0.1% v/v xylene cyanol, 0.1% w/v bromphenol blue), heating at 65 ºC for 5 minutes and resolving on pre-warmed 20X20 cm 6% w/v acrylamide 8 M urea 1X TBE gels. Gels were run at 20 W for 2-3 hours. RNA was visualized by staining with ethidium bromide (1 µg/ml).

2.5.3 Translation reactions

Yeast translation competent extracts and *in vitro* transcripts (sections 2.5.1 & 2.5.2) were used for *in vitro* translation. Endogenous mRNAs in yeast extracts were degraded by treatment with micrococcal nuclease: 4.2 µl micrococcal nuclease (20000 U/ml), 5.6 µl of 0.1 M $CaCl_2$ were added to 1 ml of yeast extract which was then incubated at room temperature for 20 minutes. 14 µl of 0.1 M EGTA were then added and the extract mixed and aliquoted (50 µl/tube), and the aliquots snap frozen and kept at -80 ºC. A 6X buffer was used for *in vitro* translation prepared as follows in table 2.9:

Component	**Qty. (µl)**
1M HEPES. KOH pH 7.4	66
1M MgOAc	6
800 mM Creatine Phosphate	93.8
100 mM ATP	2.25
100 mM GTP	3
1 mM amino acid mixture without Met	120
1M DTT	5.1
dH_2O	183.6

Table 2.9: The ingredients used to prepare 6X *in vitro* translation buffer.

7 µl [^{35}S] labelled Met EasyTag, Perkin Elmer, 10.25 mCi/ml) and 3 µl CPK (10 mg/ml in 50% v/v glycerol, Sigma Aldrich) were added to 25 µl of 6X buffer and the translation reaction was assembled as follows in table 2.10:

Component	**Qty. (µl)**
1M KOAc	1.2
yeast extract	3.3
mRNA	3.2 (1 µg)
6X buffer plus CPK and ^{35}S labelled Met	2.3

Table 2.10: The reaction components of an *in vitro* translation reaction.

Reactions were incubated for 30 minutes at room temperature. 10 µl of protein loading dye (80 mM Tris-HCl, 10% v/v glycerol, 0.0006% w/v bromophenol blue, 0.1 M DTT) were added to the samples which were heated at 65 ºC for 5 minutes and loaded onto SDS-PAGE gels. Following electrophoresis, gels were fixed and stained with Coomassie brilliant blue (0.1% w/v Coomassie brilliant blue R-250, 50% v/v methanol, 10% v/v glacial acetic acid) for 15 minutes and de-stained for 2 hours using de-staining solution (50% v/v methanol, 10% v/v glacial acetic acid). Gels were dried using a vacuum drier, exposed to phosphor storage screens, which were scanned in a phosphorimager (Typhoon Trio, GE Healthcare).

2.6 Flow cytometry

Yeast cells were exposed to cell sorting and flow cytometry to measure the GFP fluorescence in different constructs. Strains expressing GFP reporters were grown overnight in selective media, diluted in the morning, and allowed to grow again to OD_{600} ~0.5. A volume corresponding to 5X OD_{600} was harvested at 3000 rpm for 5 minutes in a Beckman J-6 centrifuge. Pellets were washed in sterile PBS, and re-suspended in 1 ml PBS. Suspensions of cells were sonicated (MESONIX SONICATOR 3000), on 0.5 mA for 10 seconds per sample. Cells then were exposed to the cell sorting using a Fortessa X20 cell analyzer. The equipment was set up according to the GFP standard parameters. This was performed with the excitation set at 488 nm and the emission at 520 nm to detect GFP fluorescence. Fluorescein channel was used for sorting target cells in the total number of cells (40,000 events per second) and GFP gating to calculate the GFP-positive cells numbers, keeping the sample flow constant.

3 CHAPTER 3: INVESTIGATING THE ROLES OF RIBOSOME-ASSOCIATED FACTORS IN 2A REACTION.

3.1 Introduction

As discussed (introduction section 1.9), It had suggested that eRF1 and 3 might be required for the 2A reaction (Doronina *et al.*, 2008), whereas a more recent paper (Machida *et al.*, 2014) found that eRFs are not required for the 2A reaction, but instead suggested that the 2A reaction is an elongation event. The premise of work carried out in this thesis was that eRF1 and 3 are essential for the 2A reaction. Thus, as a first step in this project, the effect of reducing levels of eRFs on the 2A reaction was investigated. Furthermore, experiments were carried out to investigate prospective roles of eEFs. Alongside translation factors, other proteins have been shown to influence termination efficiency. These include ABCE1/Rli1 and eIF3j/Hcr1. These are also examined in this chapter for involvement in the 2A reaction. eIF3j is loosely associated with the core initiation factor eIF3, facilitating the formation of the 43S pre-initiation complex and hence scanning for the appropriate AUG. However, *hcr1Δ* yeast strains have increased stop codon read-through, and this can be suppressed by over-expression of Rli1, which concomitantly suppresses the cold-sensitive phenotype of these cells. This led to the conclusion that a key role of this protein is in promoting dissociation of eRF3 from the termination complex thereby, promoting binding of ABCE1 (Beznoskova *et al.*, 2013).

Further factors examined in this chapter are Dom34, Hbs1 and Ski7, all of which are required in mechanisms that rescue ribosomes during abnormal translation such as NSD and NGD. Since ribosomes pause at 2A, we reasoned that factors which could influence the 2A reaction are those that resolve stalled ribosomes. Dom34 and Hbs1 are key factors in this context. These are eRF1- and eRF3-related proteins that are important for resolving stalled ribosome-mRNA complexes in the NSD and NGD pathways. As mentioned earlier in the literature review, section 1.7, Dom34 and Hbs1 share structural similarities with eRF1 and eRF3, respectively, though Dom34 lacks the motifs specific for stop codon recognition and nascent peptide chain release (Shoemaker and Green, 2011). The Dom34:Hbs1 complex can recognise stalled ribosomes and, concomitantly, it recruits endonucleases to degrade the aberrant mRNA (Passos *et al.*, 2009). It has been shown that the Dom34:Hbs1 complex triggers the dissociation of stalled ribosomes in cases of NGD (Shoemaker and Green, 2011). Rli1 is also involved in these pathways, particularly in dissociation of ribosomal subunits along with the Dom34-Hbs1 complex (Kashima *et al.*, 2014). Ski7 (Super killer protein 7) is, like Hbs1, similar to eRF3. It interacts via its N-terminus with the exosome on one side and the SKI complex on the other side, and is required for the general 3'-5' RNA decay pathway (Jamar *et al.*, 2017). In addition, it has a role in NSD, mutations to the C-terminal GTP binding domain of the protein specifically

affect its ability to function in this pathway where it is proposed to act competitively to Dom34/Hsb1 (Horikawa *et al.*, 2016). Furthermore, Ski7 mutant cells are sensitive to hygromycin B, suggesting a role in translation (Kowalinski *et al.*, 2015).

3.2 Reporter of the 2A reaction

Experiments reported in this chapter used an artificial polyprotein reporter of the 2A reaction. This reporter derived from a previous reporter, αF-2A-GFP (de Felipe *et al.*, 2003), has the signal sequence of the alpha factor replaced by three copies of the haemagglutinin (HA) epitope-tag (YPYDVPDYA). Expression of the fusion protein is driven by the inducible *GAL1* promoter in the plasmid pMW20 (Zieler *et al.*, 1995). A control containing the non-functional P17A (2A*) mutation was included in all experiments to confirm the size of the full-length translation product. When 2A is active, the expression of this reporter in *S. cerevisiae* generates 3HAαF-2A and GFP separately. In contrast, the absence of 2A activity yields the intact fusion protein. 2A activity is assessed through pulse-labelling and immunoprecipitation of proteins from cell extracts with either anti-HA or anti-GFP antibodies, followed by quantification of the amount of full-length translation product and separated upstream and downstream fragments by phosphorimaging. The pMW20 vector has a *URA3* selectable marker, and to provide further flexibility, a second set of reporters were generated in which *HIS3* was inserted into the middle of the *URA3* gene on the pMW20 vector. A schematic figure of the reporters and the expected protein bands outcomes are presented in figure 3.1.

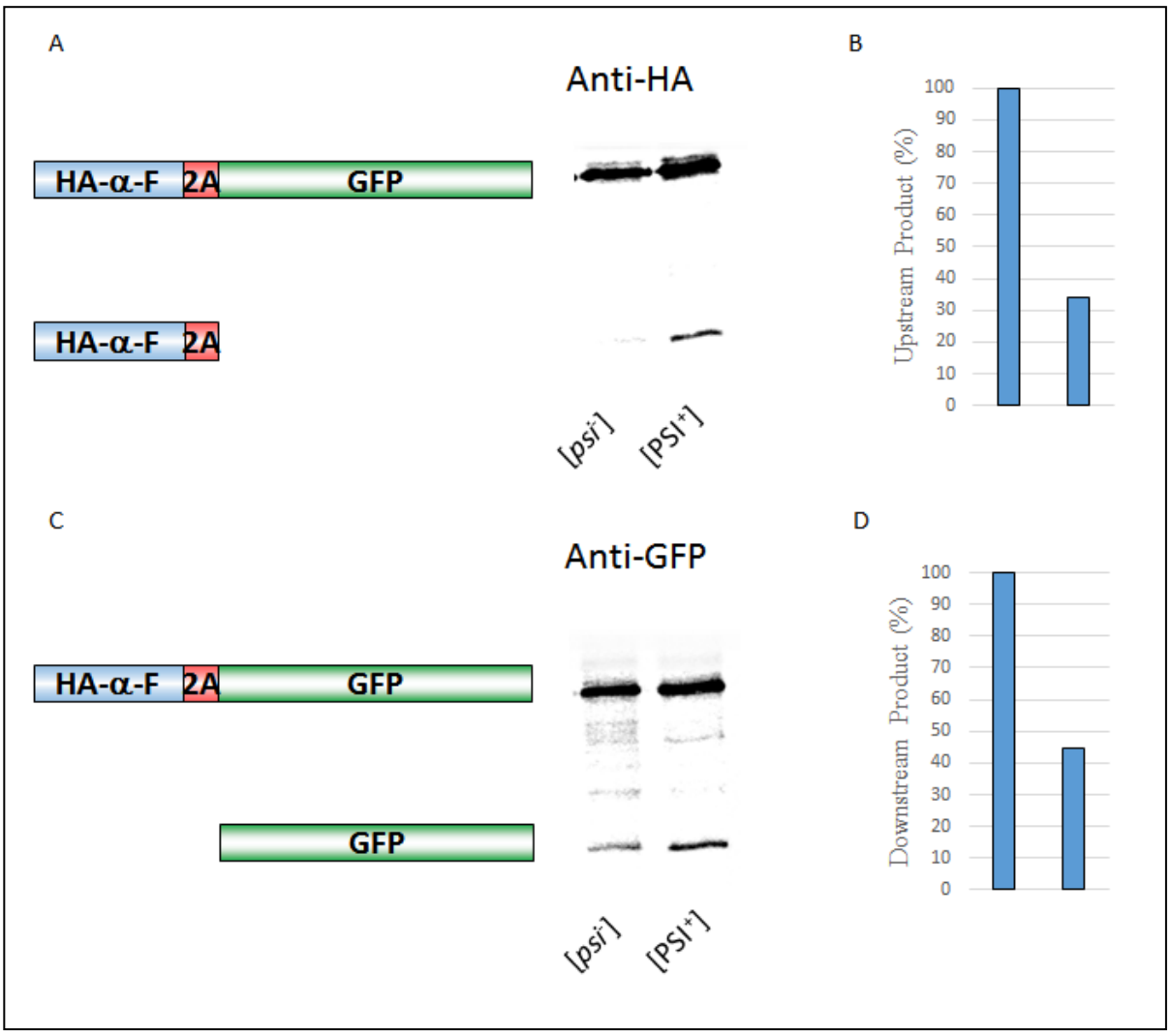

Figure 3.1: 2A reporters used in the immunoprecipitation experiments.

The 2A-containing reporter and the expected translation products are expressed from a *GAL1* promoter. [PSI$^+$] and [*psi*$^-$] strains expressing the 3HAαF-2A-GFP reporter were pulse-labelled with [^{35}S] methionine and cysteine, and translation products retrieved from cell lysates by immunoprecipitation with either anti-HA (A and B) or anti-GFP (C and D) antibodies. Images of gels (A and C) and quantification of them (B and D) are presented. Quantified data are expressed as the ratio of upstream (B) or downstream (D) to total translation events. The ratio for wild type [*psi*$^-$] (control) cells was set to 100%, and the ratio for [PSI$^+$] cells expressed relative to this.

3.3 Decreased eRF1 and eRF3 levels inhibit the 2A reaction

3.3.1 Limited availability of eRF1 increases the read-through over the 2A peptide

Three strains of *S. cerevisiae* were tested to address whether reducing Sup45 activity affects the 2A reaction. Two contained point mutations to the gene, the *sup45-42* and *sup45-2* mutations (Stansfield *et al.*, 1997), while the third contained a DAmP (Decreased Abundance by mRNA Perturbation) allele of *sup45* (Breslow *et al.*, 2008). The *sup45-2* allele is a missense mutation, which imposes thermosensitivity due to loss of activity of the protein at higher temperatures, whereas *sup45-42* is a nonsense mutation leading to expression of a C-terminally truncated protein lacking 27 amino acids (Stansfield *et al.*, 1996). The truncated protein is non-

functional and the allele is lethal in an otherwise wild type yeast strain. The strain used here contains a suppressor tRNA that leads to sufficient full-length Sup45 being produced to maintain viability.

Initially, the Sup45 availability was tested in the *DAmP-sup45* strain, through western blotting. This was to confirm that there is a reduction in the Sup45p expressed in this strain. *DAmP-sup45* strain, along with wild type were grown at 30 ºC and diluted on the next day for protein extraction (see section 2.3.2.1) and western blotting was undertaken as described in Materials and Methods, section 2.3.2.2. Results of protein blottings showed that there is a considerable reduction in Sup45 in the *DAmP-sup45* strain, compared to the isogenic wild type which expresses the normal level of Sup45p (figure 3.2). This confirms that the *DAmP-sup45* strain is suitable to be used to examine the effect of eRF1 on 2A reaction, due to the significant lower *SUP45* expression.

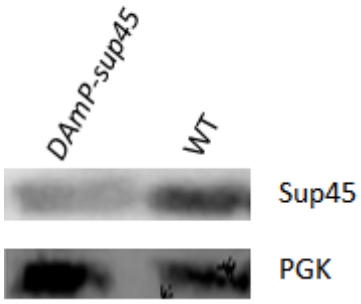

Figure 3.2: *DAmP-sup45* strain produces less Rli1 protein compared to the wild type.

Western blotting analysis was performed to examine the level of Sup45 in the *DAmP-sup45* strain, against the wild type (JDY895). Cultures were incubated at 30 ºC and diluted on the next day, and allowed to grow again to OD_{600} ~0.5. Cells were harvested in 50 ml Falcon tubes at 3000 rpm for 5 minutes, using bench-top centrifuge, washed and lysed in native buffer using zirconium beads in a bead beater (2X, for 30 seconds each). Proteins were observed from lysates by centrifugation (12.000 rpm for 20 minutes at 4 ºC), and clear lysates were collected into fresh tubes. Protein concentration was estimated (Bradford assay), and 10-15 µg of protein was denatured by heating with protein loading dye at 65 ºC for 5 minutes. Protein samples were resolved onto 10% SDS-PAGE, and then blotted on nitrocellulose membrane. The membrane was blocked using blocking solution and exposed to the primary antibody (1:1000 of mouse monoclonal, Abcam), washed (3X) with TBS and exposed to the secondary antibody (1:2000 of mouse polyclonal, Abcam). The membrane was then washed (3X) with TBS and the protein signal was detected on light films through exposing the membrane to ECL. Developing the films was performed using auto-developer. Phosphoglycerate kinase (PGK) was used as a reference protein to ensure equal sample loading. Primary mouse monoclonal anti PGK antibody (1:1000) and secondary mouse polyclonal anti PGK antibody (1:2000) were used to detect the signal for the reference protein.

The three strains and isogenic wild types were transformed with plasmids from which the 3HA-αF-2A-GFP polyprotein reporter is expressed, pulse-labelled and proteins containing 2A peptides then immunoprecipitated from cell extracts using either anti-HA antibody to detect the upstream product, or anti-GFP to detect the downstream product. Immunoprecipitated proteins were quantified and data presented as ratios of up/downstream fragments to the whole

translation products (figures 3.3 & 3.4). In all cases, at least 3 independent experiments were carried out and the results are expressed as an average of these repeats. All assays were carried out with cells grown at 30 ºC. This is a permissive temperature for *sup45-42*.

Initial results revealed that, excluding the *sup45-42* strain which shows a reduction only in the upstream products, the expression of 2A-containing polyproteins in the other two strains revealed a highly significant reduction ($P \leq 0.01$) in the ratios of both upstream and downstream products of the 2A reaction to full-length extended proteins (figure 3.4).

Reduction in the ratio of upstream fragment in the three strains tested varied, with the *sup45-42* mutation having the least effect, reducing the amount of upstream fragment to ~50%, while the *sup45-2* and *DAmP-sup45* strains reducing it to ~30% of that seen in the equivalent wild type strains. This effect is most readily attributed to the reduced activity of Sup45 in these strains, with more ribosomes then reading through the 2A peptide and elongating into the downstream sequences.

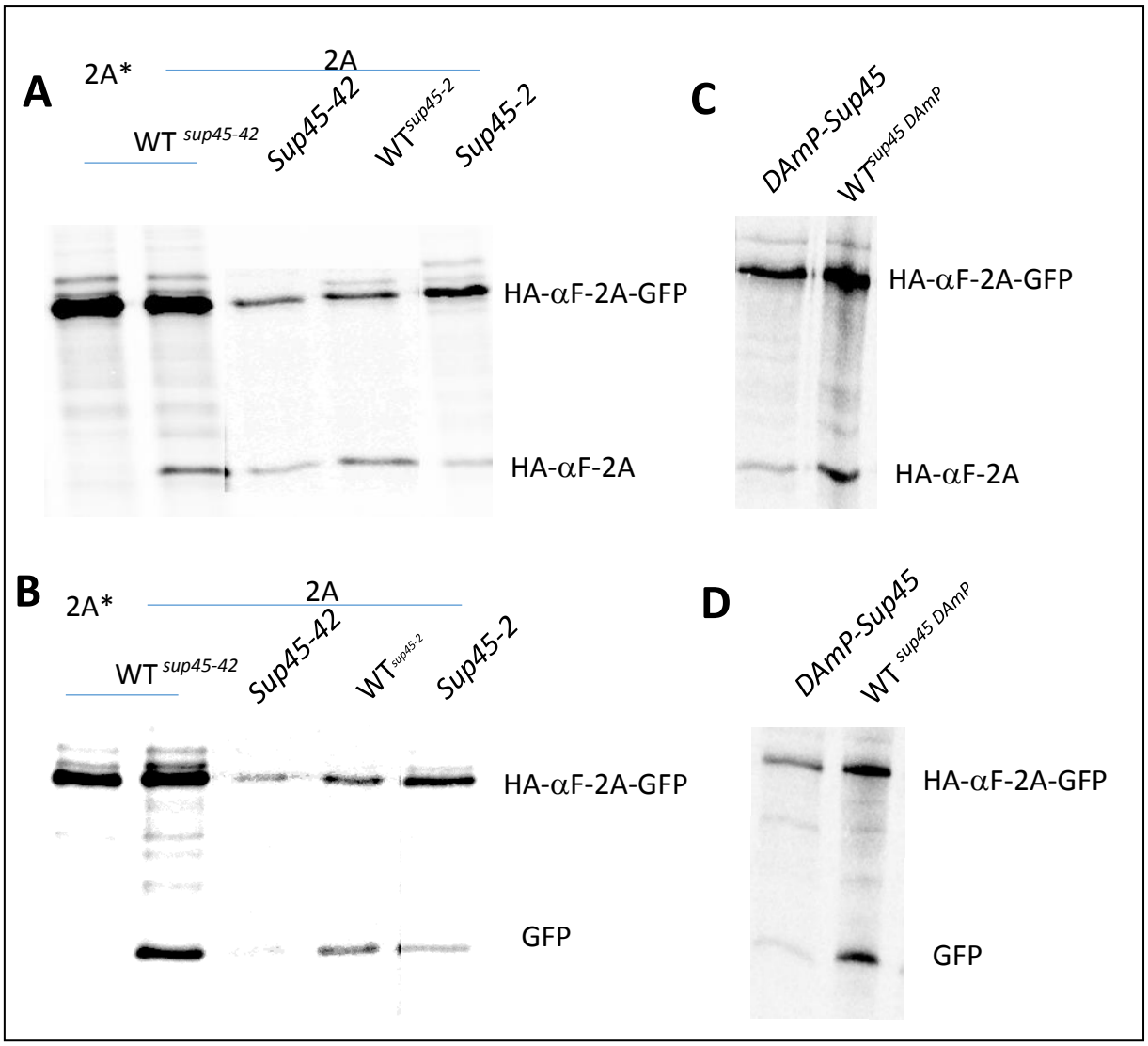

Figure 3.3: Immunoprecipitation of proteins from strains with defective eRF1.

Plasmids carrying 2A signal were transformed into the indicated strains and grown in selective media with 2% w/v galactose before pulse labelling and immunoprecipitation. Negative controls include the empty vector in wild-type strain. In addition, inactive 2A (P17A), namely 2A*, was used. Pulse labelling and immunoprecipitation were carried out using anti-HA and anti-GFP antibodies. Samples were loaded onto SDS-PAGE, gels fixed, dried and exposed to phosphor exchange screen, and scanned using Typhoon scanner. (A) proteins from *sup45-42* and *sup45-2* strains with isogenic wild types, detected by anti-HA antibody. (B) proteins from *sup45-42* and *sup45-2* strains with isogenic wild types, detected by anti-GFP antibody. (C) proteins from the *DAmP-sup45* strain, detected by anti-HA antibody. (D) proteins from the *DAmP-sup45* strain, detected by anti-GFP antibody.

A direct consequence of ribosomes failing to undergo the first step in the 2A reaction, i.e. terminate translation at the Pro 19 of the 2A sequence, would be that less of the downstream fraction should be generated. This was the case in all three strains. For both *sup45-2* and *DAmP-sup45* strains, the reduction of downstream product was similar to the reduction of upstream fragment. In the case of the *sup45-42* strain, the upstream product was decreased significantly more than the downstream (figure 3.4). Putting these results together with those above regarding the upstream product, it can be concluded that when ribosomes are translating 2A

peptides, lowered eRF1 activity leads to reduce upstream and downstream proteins, suggesting that an increased number of ribosomes are reading-through the 2A peptide.

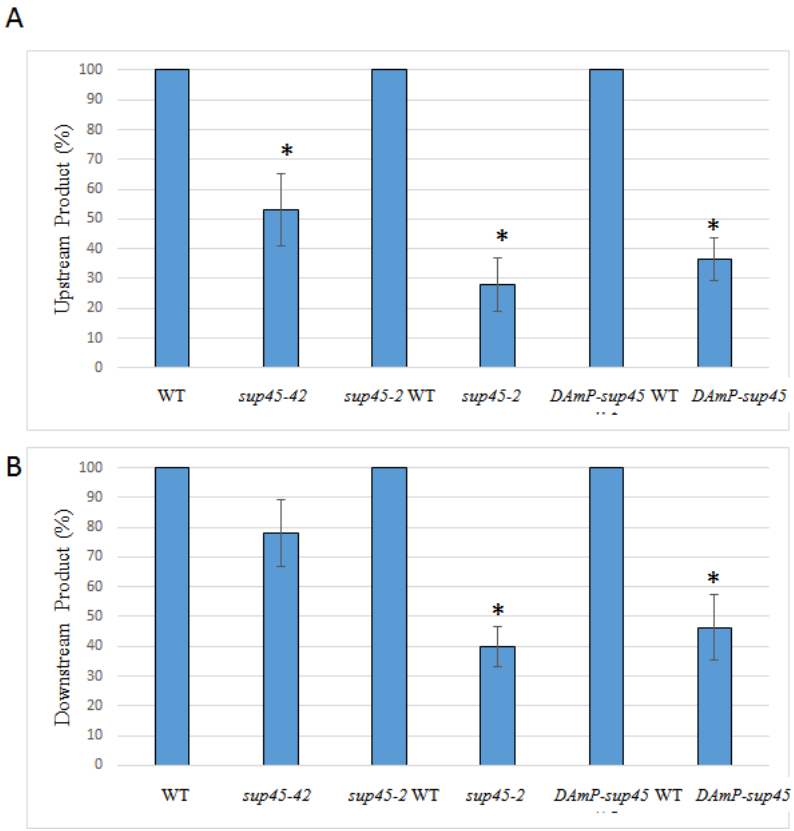

Figure 3.4: Defects in eRF1 inhibit both steps of the 2A reaction.

Gels from 3 experiments for each of the strains (as shown in figure 3.3) were quantified, values normalized according to the number of methionine and cysteine contained in each product, and the fractions of either upstream (A) or downstream (B) translation product out of the total translation events were calculated and expressed as a percentage. The average for each mutant strain is shown relative to the wild type, which is set to 100%. * refers to significance ($p \leq 0.01$).

3.3.2 eRF3 is also required for the 2A reaction

As described in the introduction (section 1.4.3.2), termination requires a complex of both eRF1 and eRF3. To this point, data have confirmed that eRF1 is important for the 2A reaction. Here, the involvement of the second component of this complex, eRF3, was investigated. Levels of eRF3 in yeast are conveniently affected by the [PSI^+] state. In [PSI^+] strains, eRF3/Sup35 is present in an aggregating prion-like conformation (Derkatch *et al.*, 1996). This leads to reduced availability and hence activity of this essential protein. Here, a [PSI^+] and control [*psi*$^-$] strain with the same genetic background were used. In addition to the [PSI^+]/[*psi*$^-$] cells, strains expressing mutations in the GTPase domain of eRF3 previously used to examine the importance of eRF3 for the 2A reaction were also examined (Doronina *et al.*, 2008). As described above, strains were transformed with 2A reporters, pulse labelled, proteins immunoprecipitated and the amount of upstream, downstream and elongation product quantified.

3.3.2.1 Effects of limiting eRF3 on the 2A reaction

As with strains with reduced eRF1 activity, lower abundance of eRF3 ([PSI^+] strain) negatively affected the generation of both the upstream and downstream fragments of the 2A polyprotein reporter, compared to the isogenic wild type ([*psi*$^-$] strain) (figure 3.5). In the [PSI^+] strain, both the upstream and downstream products of the reaction were reduced to ~40% of that seen in the [*psi*$^-$] cells. This is similar to what was found with the eRF1 experiments, which confirms the role of both eRF1 and eRF3 in 2A reaction.

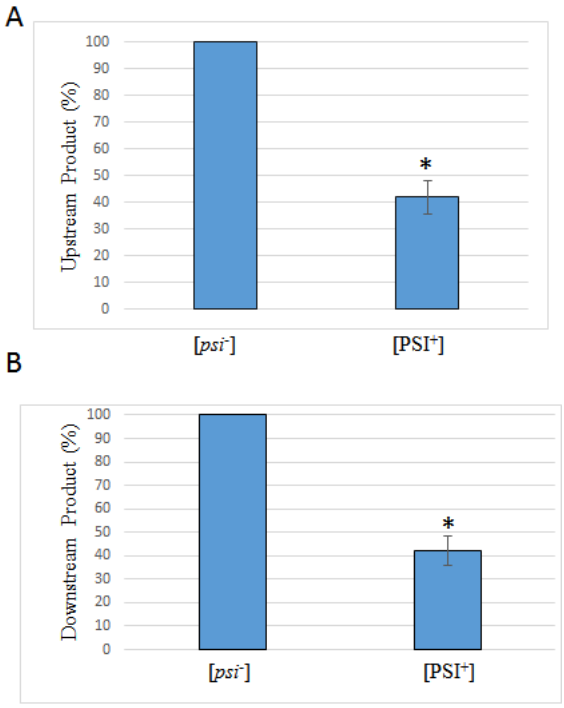

Figure 3.5: 2A reaction is inhibited by limited activity of eRF3.

Scans of gels were quantified, values normalized according to the number of methionine and cysteine contained in each product, and the fraction of free upstream (A) or downstream (B) translation product out of the total translation events expressed as a percentage. Values are obtained from 3 independent experiments. *refers to significance ($p \leq 0.01$).

3.3.2.2 Mutations within the GTPase domain of eRF3 have no effect on 2A reaction

To further extend analysis of the involvement of eRF3, the effect of mutations in the GTPase domain of this protein was tested. eRF3 functions as a part of the ternary complex, which includes eRF1, eRF3 and GTP. This complex binds the ribosomal A site, and GTP is then hydrolysed by eRF3, and promotes a specific conformation of the eRF1 to contact the PTC and trigger the peptide release from the exit tunnel. Two specific mutations, H348Q and R419G, which reduce the activity of the GTPase domain of eRF3 were used here (Salas-Marco and Bedwell, 2004). These were generated by plasmid shuffling (materials and methods section 2.1.1.1), transformed with the 2A reporter and analysed as described above.

In previous analysis (Doronina *et al.*, 2008), cells expressing these mutant eRF3 as the only version of the protein revealed an increase in the level of upstream product compared to the wild type. However, here, analysis of the strains expressing H348Q and R419G did not show significant effect on the production of either the upstream or downstream product. Indeed, values of the two *sup35* alleles tested were in the same range as the isogenic wild type (figure 3.6). This suggests that GTP hydrolysis accompanying termination at 2A peptide is catalysed

by eRF3, but in a slightly different scenario than that seen in the canonical termination, due to the special conformation undergoen mainly by eRF1 at a sense (Pro) codon.

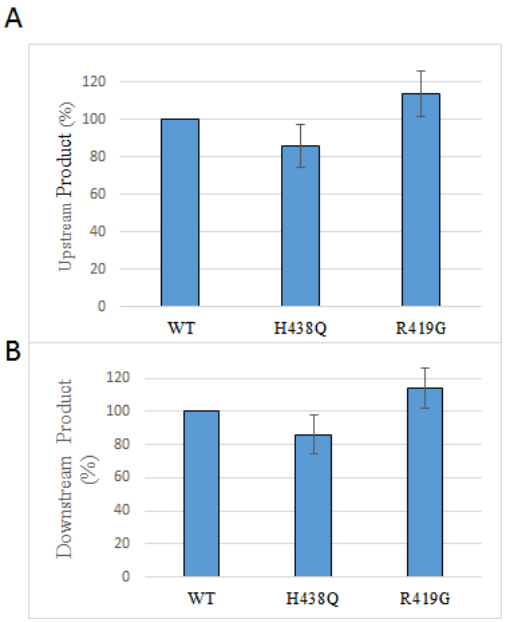

Figure 3.6: Mutations in the GTPase motif of eRF3 do not affect the 2A reaction.

Gels of relevant protein samples were quantified, values normalized according to the number of methionine and cysteine contained in each product, and the fraction of free upstream (A) or downstream (B) translation product out of the total translation events was calculated. Values are obtained from 3 independent experiments and expressed as means±SE.

To this point, we have confirmed that both eRF1 and eRF3 are required for the 2A reaction, although these results may suggest different features for proteins, particularly eRF3, from that involved in the canonical translation, in the presence of a stop codon in the ribosomal A site. Thus, how do release factors mediate the 2A reaction? Further investigations will be performed to address the roles of translational termination factors in the 2A reaction.

3.4 Investigating the involvement of further termination factors and ribosome-rescue factors in the 2A reaction

Beyond eRF proteins, several other factors that influence termination (ABCE1/Rli1, eIF3j/Hcr1) are required for the rescue of stalled ribosomes (Dom34, Hbs1, and Ski7) (see introduction sections 1.7). None of these had previously been tested to investigate whether they are required for or influence the efficiency of the 2A reaction. For this analysis, strains lacking these factors, through deletion of the respective gene, were used. An exception was Rli1, which is an essential factor, and a strain from the DAmP strain collection (Breslow *et al.*, 2008; Finkel *et al.*, 2011) expressing reduced levels of the protein was used. Rli1 level was investigated in

the *DAmP-rli1* strain using western blotting, and results revealed that this strain produces less amount of Rli1 in comparison with the wild type strain, which expresses the normal level of protein, that is essential to undergo the biological functions in the ribosome (figure 3.7).

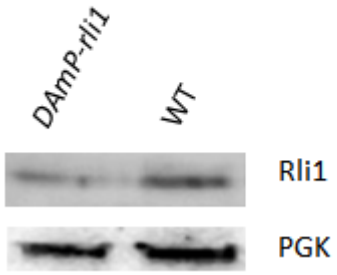

Figure 3.7: *DAmP-rli1* strain produces less Rli1 protein compared to the wild type strain.

Western blotting analysis was performed to examine the level of Rli1 in the *DAmP-rli1* strain , against the wild type strain (JDY895). Cultures were incubated at 30 ºC and diluted on the next day, and allowed to grow again to OD_{600} ~0.5. Cells were harvested in 50 ml Falcon tubes at 3000 rpm for 5 minutes, using bench-top centrifuge, washed and lysed in native buffer using zirconium beads in bead beater (2X, for 30 seconds each). Proteins were observed from lysates by centrifugation (12.000 rpm for 20 minutes at 4 ºC), and clear lysates were collected into fresh tubes. Protein concentration was estimated (Bradford assay), and 10-15 µg of protein was denatured by heating with protein loading dye at 65 ºC for 5 minutes. Protein samples were resolved onto 10% SDS-PAGE, and then blotted on nitrocellulose membrane. The membrane was blocked using blocking solution and exposed to the primary antibody (1:1000 of mouse monoclonal, Abcam), washed (3X) with TBS and exposed to the secondary antibody (1:2000 mouse polyclonal. Abcam). The membrane then was washed (3X) with TBS and the protein signal was detected on light films through exposing the membrane to ECL, and developing the films was performed using auto-developer. Phosphoglycerate kinase (PGK) was used as a reference protein to ensure equal loading. Primary mouse monoclonal anti PGK antibody (1:1000) and secondary mouse polyclonal anti PGK antibody (1:2000) were used to detect the signal for the reference protein.

To investigate whether these factors play a role in the 2A reaction, mutant strains and isogenic wild types were transformed with a plasmid from which the HA-αF-2A-GFP reporter was expressed, and analysed similarly to the strains limited for eRF activity. Example images of SDS-PAGE gels on which immunoprecipitated proteins were run and bar graphs representing ratios of upstream/downstream to the full-length averaged for 3 independent experiments are shown in figures 3.8 & 3.9.

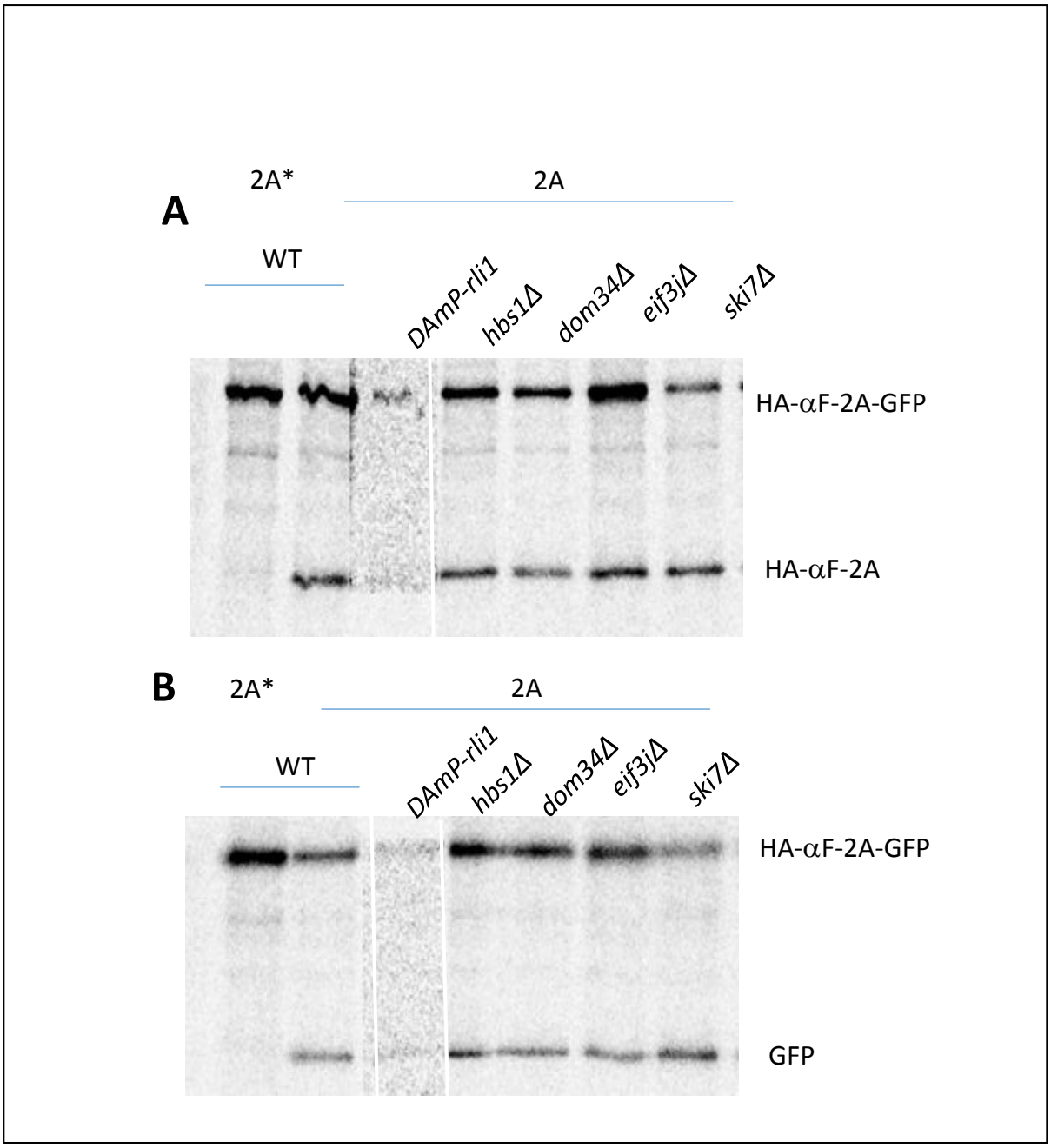

Figure 3.8: Immunoprecipitation of proteins from cells lacking/ limited in termination and ribosomal rescue factors.

Plasmids expressing HA-αF-2A-GFP or the non-active 2A* were transformed into the indicated strains which were grown in selective media with 2% w/v galactose to induce the *GAL1* promoter before pulse labelling and immunoprecipitation with either anti-HA (A) or anti-GFP (B) antibodies. Samples were loaded onto SDS-PAGE, gels fixed, dried and exposed to phosphor exchange screen, and scanned using Typhoon scanner. Note that the contrast of the DAmP-*rli1* strain immunoprecipitations was altered compared to the other lanes as the signal was relatively weak in this experiment.

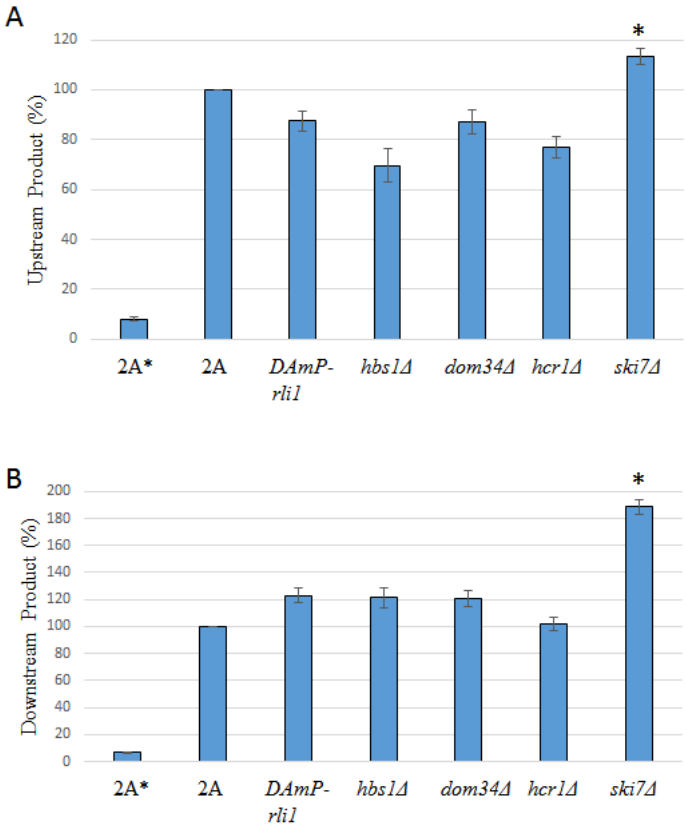

Figure 3.9: Quantification of protein bands observed from strains lacking/ limited in termination and ribosomal rescue factors.

Gels from 3 experiments for each of the strains (as shown in figure 3.8) were quantified, values normalized according to the number of methionine and cysteine contained in each product, and the fractions of either upstream (A) or downstream (B) translation product out of the total translation events were expressed as a percentage. The average for each mutant strain is shown relative to the wild type, which is set to 100%. * refers to significance ($p \leq 0.01$).

From the data presented in figure 3.9, no evidence for the majority of factors analysed being involved in the 2A reaction was found. The fraction of ribosomes undergoing the 2A reaction is statistically similar in all strains analysed, which has been observed with the anti-HA, and/ or anti-GFP antibodies. Strains with *DAmP-rli1*, or lacking *dom34*, *hcr1* or *hbs1* have revealed an insignificant increase in the downstream product compared to the wild type strain. An exception has been observed here, which is the *ski7Δ* strain. Specifically, in this strain, the relative amount of downstream product is doubled. In general, it is unlikely that the ribosome generates more downstream fragments than the upstream. However, if there is less downstream product, it would indicate ribosomal drop off, then an increase of outcomes of the second part of the 2A reaction could be explained as a result of re-initiation, which may become more efficient in the absence of Ski7. This would then imply that normally Ski7 interferes with the completion of the 2A reaction.

To briefly sum up the above results, deletion of ribosomal factors including Hbs1, Dom34 and Hcr1 in yeast does not alter 2A activity. Moreover, limiting the availability of Rli1 also had no significant effect on the 2A reaction, suggesting that these factors are not required for the translational recoding event driven by the 2A peptide. On the other hand, deletion of Ski7 increased both upstream and downstream products ratios, which may reflect a certain effect on the ribosome, possibly the absence of this factor during translation of 2A promotes the re-initiation of ribosomes stalled at the C terminus of the 2A peptide. These effects will be followed with more investigations for deeper insight.

3.5 Investigating growth rates of strains overexpressing 2A-encoding mRNA

It has been shown that overexpression of a 2A-containing polyprotein (from a *GAL1* promoter) inhibits the growth of strains with limited release factor activity (Doronina *et al.*, 2008). The reason for this is not clearly understood, though it has been suggested that the presence of multiple copies of the 2A-containing mRNA may somehow titrate the remaining available release factors. This is perhaps through affecting the conformation of release factors when they are released from the ribosome such that they take longer to be recycled. To complement the labelling and immunoprecipitation experiments, growth profiles of the same strains used in immunoprecipitations when the same 2A-containing reporter was expressed were generated and compared with the growth of the same strain in the presence of the 2A*-containing control reporter.

3.5.1 The overexpression of 2A-containing proteins inhibits the growth of cells with defective eRF1

Strains containing the *sup45-42*, *sup45-2* and *DAmP-sup45* alleles, used for immunoprecipitation experiments in section 3.3.1, were used here. Strains were transformed with plasmids from which the HA-αF-2A/A*-GFP reporters is expressed from the inducible *GAL1* promoter. Growth curves were generated from the strains on expression of the reporters and this revealed that while the overexpression of reporters did not perturb growth of the wild type strain, the 2A-, but not 2A*-containing reporter resulted in a reduction in growth of the *sup45-42*, *sup45-2* and *DAmP-sup45* strains (table 3.1). This is charactarised by a significant increase in doubling time in strains expressing 2A peptide, compared to the same strain expressing 2A* (inactive 2A). Interestingly, the *DAmP-sup45* strain grew significantly slower than other strains (doubling time~300% of the wild type). Doubling time was also increased significantly in the *sup45-2* strain expressing 2A compared to the wild type, whereas the

elevation observed in the *sup45-42* strain was not significant, probably due to the high significance level used in our study ($p \leq 0.01$).

A

Strain Expressing 2A	**Growth Doubling Time (%) (mean±SE)**
WT	107.287± 4.90
DAmP-sup45	295.034± 13.48*
sup45-42	143.914± 6.57
sup45-2	175.403± 8.01*

B

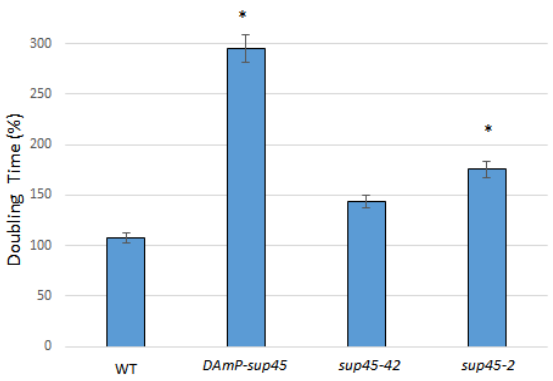

Figure 3.10: Growth doubling time in strains with defective eRF1 while expressing 2A peptide

Strains with defects in eRF1 (Sup45) which includes, *DAmP-sup45*, *sup45-42* and *sup45-2*, were transformed with 2A and 2A*-containing reporters (pJNY278 and 279), and grown overnight at 30 ºC in selective media. Cultures were diluted on the next day with the same media plus 2% w/v galactose to induce the *GAL1* promoter of the 2A reporter. OD_{600} was recorded every one hour for eight hours, and data were processed to find the doubling time for each strain expressing active 2A, and normalised to the doubling time of the strain expressing 2A*. Averages of 3-4 independent experiments are presented above ± standard error. (A) Table of doubling time values. (B) Chart of values corresponding to A. * refers to significance $p \leq 0.01$.

3.5.2 Growth rates of strains lacking/limited in ribosome-rescue factors

Strains investigated for their prospective effect on 2A reaction, by means of immunoprecipitation, were subjected here to measurement of growth rate when the HA-αF-2A/A*-GFP mRNAs were expressed in them. Comparison of each strain was achieved through comparing data of each active and inactive 2A of the same strain.

A

Strain Expressing 2A	Growth Doubling Time (%) (mean±SE)
WT	111.239± 5.08
dom34Δ	112.514± 5.14
DAmP-rli1	143.322± 6.54
hbs1Δ	132.509± 6.05
eif3jΔ	113.075± 5.16
ski7Δ	111.860± 5.11

B

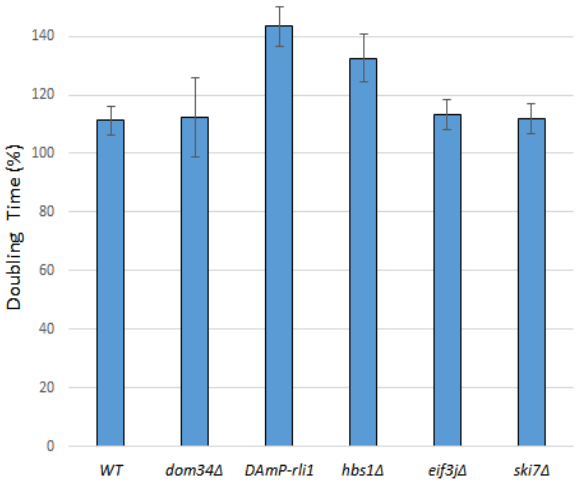

Figure 3.11: Growth doubling time of strains with defects in some ribosomal factors while expressing 2A peptide.

Strains with defects in several ribosomal factors (JDY1253-1257) which includes, *DAmP-rli1*, *dom34Δ, hbs1Δ, eif3jΔ and ski7Δ*, were transformed with 2A and 2A*-containing reporters and grown overnight at 30 ºC in selective media. Cultures were diluted on the next day with the same media plus 2% w/v galactose to induce the *GAL1* promoter of the 2A reporter. OD_{600} was recorded every one hour for eight hours, and data were processed to find the doubling time for each strain expressing active 2A, and normalised to the doubling time of the strain expressing 2A*. Averages of 3-4 independent experiments are presented above ± standard error. (A) Table of doubling time values. (B) Chart of values corresponding to A.

Growth profiles of the strains reveal no effect of the 2A peptide overexpression on doubling time: each strain grew at a similar rate with the active and inactive 2A peptides (table 3.2, figure 3.11). Thus the presence of mRNA encoding 2A in strains lacking Dom34, Hbs1, eIF3j/Hcr1, Ski7 or limited for ABCE1/Rli1 does not compromise growth in the way seen with cells limited for eRFs. From these and the immunoprecipitation data, it has been confirmed that the ribosome can maintain normal 2A reaction in *S. cerevisiae*, even when cells lack one of these factors. Thus, Dom34, Hbs1, Rli1, Hcr1 or Ski7 are not required to promote a normal 2A reaction in yeast cells.

3.5.3 Limited activity, but not GTPase mutations, of eRF3 inhibits the growth of cells overexpressing 2A peptide

Growth profiles of both [PSI^+] and [*psi*$^-$] strains, along with strains with mutations in the GTPase domain of eRF3, were measured while expressing HA-αF-2A/A*-GFP (table 3.3. figure 3.12). The [PSI^+] strain grew slower when HA -αF-2A-GFP was expressed. On the other hand, the overexpression of active 2A peptide in strains carrying H438Q or R419G mutations in *sup35* did not alter the growth profile of cells compared to the strain expressing the inactive 2A peptide (2A*).

A

Strain Expressing 2A	Growth Doubling Time (%) (mean±SE)
R419G	118.537± 5.41
H438Q	105.340± 4.81
[PSI$^+$]	193.367± 14.51*
[*psi*$^-$]	112.248± 5.12

B

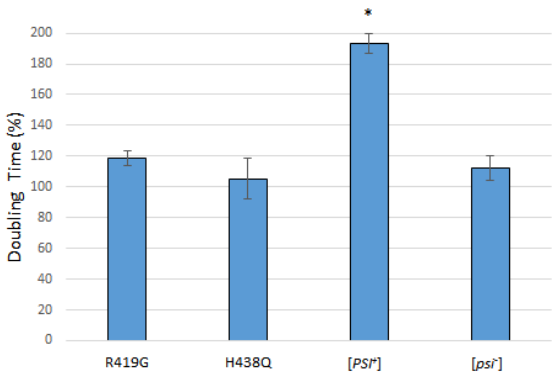

Figure 3.12: Growth doubling time of strains with defective eRF3 (Sup35) while expressing 2A peptide. .

Strains with defects in eRF3 (Sup35) namely, [PSI$^+$] (JDY832) in addition to two mutations in GTPase domain of *sup35* which are, H438Q and R419G (JDY838 and 839), along with the wild type, [*psi*$^-$] (JDY832) were transformed with 2A and 2A*-containing reporters (pJNY278 and 279) and grown overnight at 30 ºC in selective media. Cultures were diluted on the next day with the same media plus 2% w/v galactose to induce the *GAL1* promoter of the 2A reporter. OD_{600} was recorded every one hour for eight hours, and data were processed to find the doubling time for each strain expressing active 2A, and normalised to the doubling time of the strain expressing 2A*. Averages of 3-4 independent experiments are presented above ± standard error. (A) Table of doubling time values. (B) Chart of values corresponding to A. * refers to significance ($p \leq 0.01$).

Data shown in table 3.3 and figure 3.12 are consistent with the outcomes of immunoprecipitation experiments using the same strains. The overexpression of a functional 2A peptide in the [PSI$^+$] strain, within which much of the eRF3 in the cell is inactive, strongly inhibited the growth of this strain, compared to the expression of a non-functional 2A peptide (2A*), which is detected by the significant increase in the doubling time of [PSI$^+$] strain expressing 2A. On the other hand, no influence of 2A peptide expression, in terms of growth, was detected in strains expressing mutations in the eRF3 GTPase domain. To this point, it has been confirmed that the limited availability of eRF3, which has been examined in the strong [PSI$^+$] strain, inhibits the 2A reaction, and one consequence of this effect is an inhibition of the growth rate of this strain, compared to the equivalent [*psi*$^-$] strain.

3.6 Elongation factors are also involved in the 2A reaction

In this section, the prospective roles of eEF1α and eEF1β in the 2A reaction were analysed. The second step of the 2A reaction requires the ribosome to proceed, with the Pro 19 codon encoding the first amino acid of the downstream protein. Following the termination reaction that releases the upstream product, this is in the A site, and presumably prolyl-tRNA^{Pro} must bind to it and then be translocated to the P site. Prolyl-tRNA^{Pro} will associate with the ribosome in complex with eEF1-GTP and translocation requires eEF2 (see introduction section 1.4.2). In previous work (J Brown, F Yan and E Leung unpublished), a number of mutations in

eEF2 were found not to affect the 2A reaction. Therefore here the focus was on eEF1. Strains of *S. cerevisiae* with reduced levels of eEF1α (TEF1) or eEF1β (eEF1Bγ) (the G-nucleotide exchange factor for eEF1α) were used to investigate the effects of these factors on the 2A reaction. In the case of eEF1β, a DAmP allele was used, whereas for eEF1α, a strain lacking one copy (*tef1*) of this duplicated gene was used. These two strains, along with the isogenic wild types, were transformed with plasmids from which the HA-αF-2A/A*-GFP reporters were expressed from the *GAL1* promoter. Cells were grown and pulse labelled, proteins were extracted and immunoprecipitated with anti-HA and anti-GFP antibodies (figure 3.13).

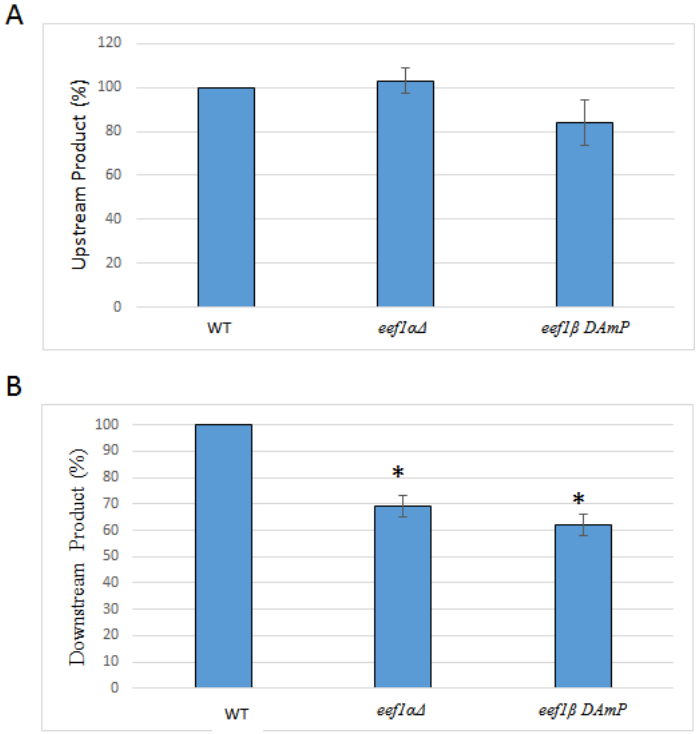

Figure 3.13: Reduction of eEF1 activity inhibits the second step of the 2A reaction.

Gels from 3-5 independent experiments for each of the strains indicated were quantified and compared to the WT strain (JDY895). Values normalised according to the number of methionine and cysteine contained in each product, and the fractions of either upstream (A) or downstream (B) translation product out of the total translation events were expressed as a percentage ± standard error. The average for each mutant strain is shown relative to the wild type strain, which is set to 100%. * refers to significance ($p \leq 0.01$).

The reduction in levels of either eEF1α or its exchange factor eEF1β had no effect on the generation of the upstream fragments of the 2A reporter during the first step of the reaction, but in both strains the amount of downstream product produced was significantly reduced (figure 3.13). These data are consistent with the first part of the reaction being a termination step (and hence not requiring elongation factors) but that the restart to translation after release of the upstream product is particularly sensitive to abundance of the ternary complex prolyl-tRNA^{Pro}-

eEF1-GTP. Limiting the availability of this complex through either of the routes used reduced the amount of downstream fragment by ~30%, leaving the ribosome stalled at the C-terminus of the 2A peptide.

3.7 Overexpression of *SUP45* and *RLI1* enhances the 2A reaction in *S. cerevisiae*

It has been found that the overexpression of Rli1 improves the efficiency of canonical termination, even when cells are mutants or deficient in one or more factor (Khoshnevis *et al.*, 2010). Here, the effect of overexpressing Rli1 or eRF1 on the 2A reaction has been investigated using a centromeric plasmid expressing Rli1 or Sup45 from *GAL1* promoter (kind gift of Prof. D. Lydall, Newcastle). The *SUP45*/*RLI1* genes in the plasmids mentioned above are His-tagged, which enabled testing the efficiency of the overexpression of such gene through western blotting. Both strains (*DAmP-sup45* and *DAmP-rli1*) were transformed with plasmids encoding *SUP45/RLI1* genes, and proteins were extracted to undergo western blotting. *RLI1* was overexpressed in both *DAmP-sup45* and *DAmP-rli1* strains, whereas *sup45* was only overexpressed in the *DAmP-sup45* strain, as the previous examination of the *DAmP-rli1* strain, undergone by immunoprecipitation, did not show effects on the 2A reaction (figure 3.9).

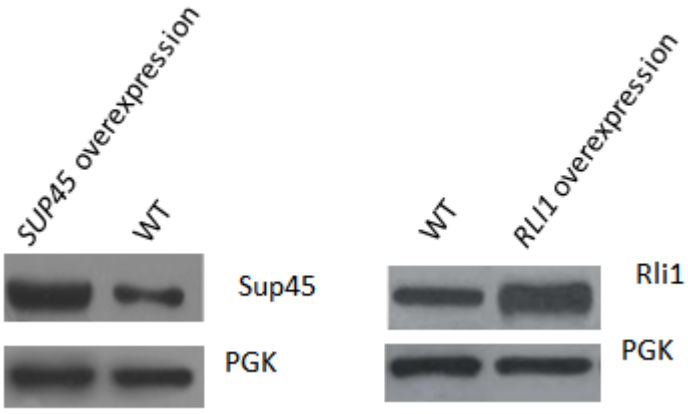

Figure 3.14: Western blotting of *SUP45* and *RLI1* overexpression.

Plasmids encoding *SUP45* and *RLI1* genes (Professor D. Lydal lab), under a *GAL1* promoter and with a His tag, were transformed into strains limited in these genes (JDY1296 and 1257). Cultures of these strains, along with the appropriate wild type (JDY895) were incubated in selective media with 2% w/v raffinose as a source of carbon at 30 ºC, and diluted on the next day using the same media but with 2% w/v galactose, and allowed to grow again to OD_{600} ~0.5. Cells were harvested in 50 ml Falcon tubes at 3000 rpm for 5 minutes, using bench-top centrifuge, washed and lysed in native buffer using zirconium beads in bead beater (2X, for 30 seconds each). Proteins were observed from lysates by centrifugation (12.000 rpm for 20 minutes at 4 ºC), and clear lysates were collected into fresh tubes. Protein concentration was estimated (Bradford assay), and 10-15 µg of protein was denatured by heating with protein loading dye at 65 ºC for 5 minutes. Protein samples were resolved onto 10% SDS-PAGE, and then blotted on nitrocellulose membrane. The membrane was blocked using blocking solution and exposed to the primary antibody (1:1000 of mouse monoclonal anti His antibody, Abcam), washed (3X) with TBS and exposed to the secondary antibody (1:2000 of mouse polyclonal anti His antibody, Abcam). The membrane then was washed (3X) with TBS and the protein signal was detected on light films through exposing the membrane to ECL, and developing the films using auto-developer. Phosphoglycerate kinase (PGK) was used as a reference protein to ensure equal loading. Primary mouse monoclonal anti PGK antibody (1:1000) and secondary mouse polyclonal anti PGK antibody (1:2000) were used to detect the signal for the reference protein.

As seen in figure 3.14, the overexpression of both *SUP45* and *RLI1* genes in strains limited in these genes, induced by DAmP feature, increased the amount of protein detected by western blotting compared to the appropriate wild type strain, which expresses the normal level of the protein with no overexpression. Obviously, the expression of these genes from an inducible (*GAL1*) promoter has an efficient effect to increase the protein abundance over that seen in the wild type, which can overcome the effects observed by limiting the studied factors in the DAmP strains.

Now, to examine the effects of gene overexpression in DAmP strains stated above, the same strains used in western blotting (figure 3.14) were then transformed with the 2A reporters with *HIS3* as a selective marker. Pulse labelling and immunoprecipitation were performed as described in earlier sections.

3.7.1 Overexpression of *SUP45* in *S. cerevisiae*

The overexpression of eRF1 from an inducible promoter restored the efficiency the 2A reaction caused by reduced expression in a strain containing the *DAmP-erf1* allele to a similar

amount to that seen in a wild type strain (figure 3.15). A significant increase in both upstream and downstream products was observed, compared to the control strain, where no eRF1 overexpression was induced. In contrast, overexpressing eRF1 did not increase the efficiency of the reaction in a wild type strain. A previous study has shown that overexpression of eRF1 does not reduce stop codon read-through (Salas-Marco and Bedwell, 2004), and this suggests that for both canonical termination and the 2A reaction eRF1 is not limiting. In yeast, eRF1 overexpression did not alter the 2A reaction in strains lacking *eef1α*, or *ski7* or limited in *eef1β* (figure 3.15). This may indicate an independence of these factors to drive a 2A reaction far from termination factors, and for eEF1 with this factor affecting a different step (restart to translation).

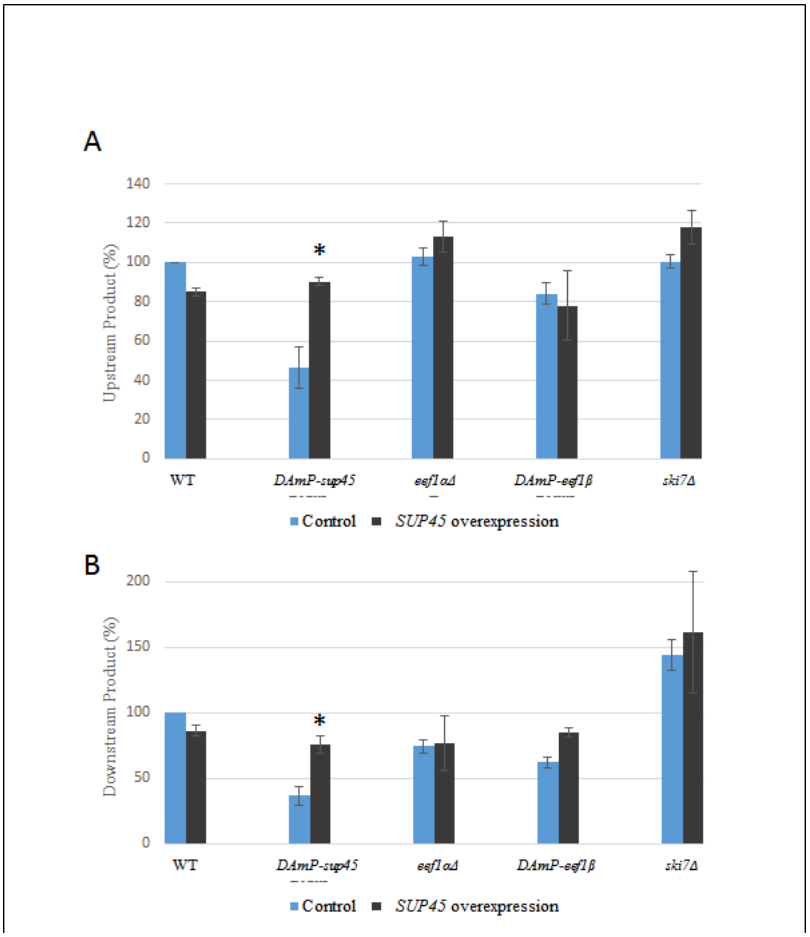

Figure 3.15: Overexpression of eRF1(*SUP45*) enhances the 2A reaction in strains with limited Sup45p.

A plasmid encoding the *SUP45* gene, expressed from the *GAL1* promoter, was transformed in the above strains, which are limited/lacking one factor, and expressing 2A reporter from a plasmid. Cultures were grown in selective media with 2% w/v galactose. Pulse labelling, immunoprecipitation and quantification of protein bands were performed as described above. (A) Quantification of the upstream product. (B) Quantification of the downstream product. * refers to significance ($p \leq 0.01$).

3.7.2 Overexpression of *RLI1* in *S. cerevisiae*

Here, the overexpression of Rli1 was tested in several strains including, *DAmP-sup45, rli1 DAmP*, *ski7Δ, eef1αΔ*, *DAmP-eef1β* as well as isogenic wild type cells. Reduction of Rli1 levels does not reduce the efficiency of the 2A reaction (figure 3.9) and this was also seen here. Overexpression of *RLI1* from the *GAL1* promoter in wild type cells and the *DAmP-rli1* strain did not alter the efficiency of the 2A reaction. Thus Rli1 is not limiting for the efficiency of the reaction. However, overexpression of *RLI1* (ABCE1) led to an increase in 2A activity in cells with reduced eRF1 (*DAmP-sup45*) activity. This increased activity was detected by increased upstream and downstream products generated from the ribosome. In cells with reduced eEF1 activity, in which generation of the downstream fragment of the 2A reaction is reduced, overexpression of *RLI1* compensated for this defect, especially in cells with reduced levels of eEF1. This suggests that while Rli1 may not be a limiting factor for the first step of the 2A reaction, when the ternary prolyl-tRNA^{Pro}-eEF1-GTP complex is limiting, efficient completion of the first step of the 2A reaction/release of termination factors from the ribosome by the action of Rli1 promotes completion of the whole reaction (figure 3.16). Finally, the overexpression of *RLI1* had no effect on cells lacking Ski7, which has already been found to increase the ratios of the smaller fragments of the 2A reporter. The above results confirm that Rli1 plays an essential role in ribosomes translating ORFs with 2A peptides although limiting the abundance of Rli1 in cells overexpressing 2A reporter did not affect the 2A activity.

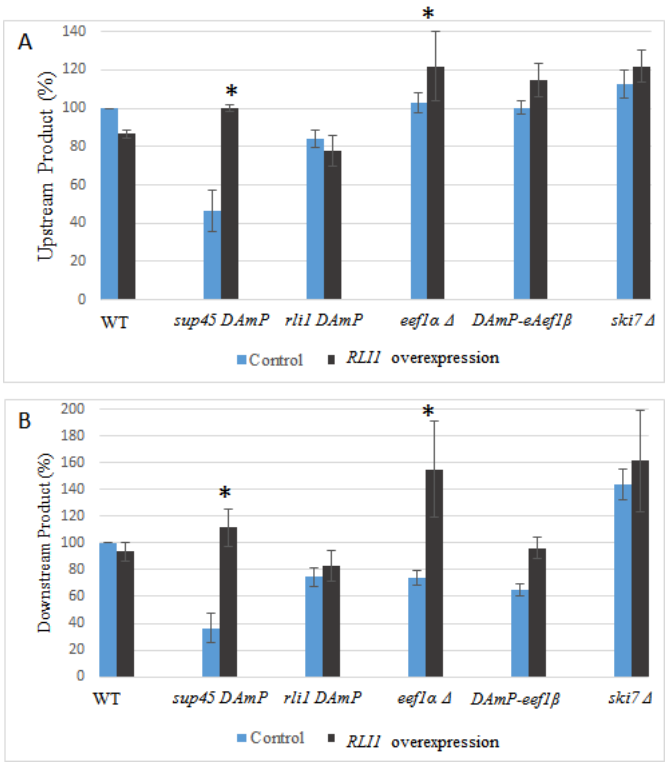

Figure 3.16: Overexpression of *RLI1* enhances the 2A reaction in strains limited in Sup45p and eEF1.

A plasmid encoding *RLI1* gene, expressed from the *GAL1* promoter, was transformed in the above strains, which are limited/lacking one factor, and expressing 2A reporter from a plasmid. Cultures were grown in selective media with 2% w/v galactose. Pulse labelling, immunoprecipitation and quantification of protein bands were performed as described above. (A) Quantification of the upstream product. (B) Quantification of the downstream product. * refers to significance ($p \leq 0.01$).

3.7.3 The overexpression of *SUP45* and *RLI1* improves the growth of the strain with defective eRF1

In this section, the growth rate of the strain limited in *erf1* (DAmP strain) is measured against the overexpression of eRF1 and *RLI1* in this strain. It was shown that the 2A activity is significantly diminished in *S. cerevisiae* cells limited in the availability of eRF1. Furthermore, in sections 3.7.1 and 3.7.2, data confirm the role of *RLI1* and *SUP45* overexpression in enhancing the activity of 2A peptide in those cells, achieved by immunoprecipitation. To perform this part, the same strains used in experiments stated in sections 3.7.1 and 3.7.2 have been subjected to the growth rate measurement, as described earlier.

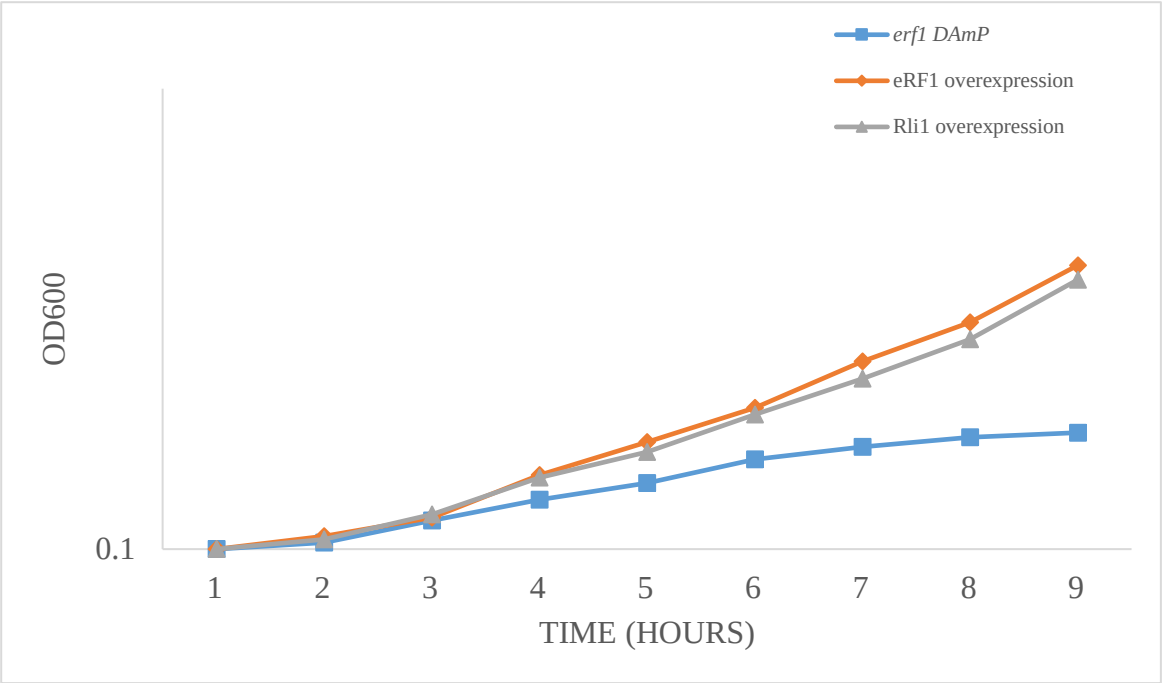

Figure 3.17: The overexpression of *SUP45* and *RLI1* improves the growth of cells limited for eRF1 cells while expressing 2A peptide.

The indicated strains were grown in selective media, containing 2% w/v raffinose and diluted in the morning using the same media in addition to 2% w/v galactose to induce the *GAL1* promoter. Growth rate was measured in the *DAmP-erf1* strain, with and without overexpression of *SUP45* and *RLI1*, through measuring the OD_{600} every 1 hour. Data are plotted on a semi logarithmic chart.

As shown in figure 3.17, overexpression of eRF1 improved the slow growth rate of the *DAmP-sup45* strain. This is consistent with the *GAL1-SUP45* allele restoring levels of the eRF1 protein. Intriguingly, overexpression of *RLI1* in these cells had a similar effect which confirms the recruitment of Rli1 to the ribosome along with eRF1 and 3 to maintain a normal 2A reaction. Although the limited availability of this factor did not influence the reaction, it seems that a significant ratio of ribosomes fail to undergo termination at Pro19 unless Rli1 is recruited to the ribosome. This suggests that over-expression of eRF1 suppressed the 'titration' effect of the 2A-encoding mRNA on eRF1 availability in these cells.

3.8 Discussion

3.8.1 Ribosome rescue factors, Rli1 and eIF3j, are not required for the 2A reaction

One aspect of this study was to investigate whether several factors associated with termination and ribosome rescue are involved in the 2A reaction. The factors tested were Dom34, Hbs1, Rli1, eIF3j (Hcr1) and Ski7. Experiments carried out were both pulse-labelling and immunoprecipitation to examine the 2A reaction itself and growth curves to assess whether

it is affected by the expression of a 2A-containing mRNA. Data presented in section 3.4 regarding immunoprecipitation of 2A-containing polyprotein reporters and growth curves respectively, showed that no effects on the 2A reaction have been found in cells lacking Dom34, Hbs1, Hcr1 or limited in Rli1 abundance. Specifically, the 2A reaction occurred with similar efficiency and the growth rate of these cells was unaffected by the expression of a 2A-containing mRNA, when compared to the wild type control. One exception has been detected here, which is Ski7. The absence of this factor led to an increase in both steps of the 2A reaction, which may suggest a role of this factor in promoting ribosomes to read through the 2A site, removing ribosomes, and/or degrading the mRNA when they are stalled at the C-terminus of the 2A peptide. Given the known role of Ski7 in NGD and its importance in coupling recognition of the defective mRNA to degradation, it is perhaps more likely to be involved in suppressing the 2A reaction by removal of ribosomes/mRNA before the first step of the reaction or between the first and second steps. Ski7 is proposed to bind an empty A site on the ribosome, which may occur more frequently than during normal translation when ribosomes arrive at the 2A site since prolyl-tRNA^{Pro} does not have a productive interaction with the ribosome and the interaction of eRF1 with the ribosome is non-canonical. Thus, the absence of Ski7 may allow ribosomes to be more likely to undergo both steps of the reaction. Furthermore, the growth rate for these strains was not altered during 2A overexpression.

Depletion of Rli1 increases the read-through along stop codons (Khoshnevis *et al.*, 2010). Structural models of Rli1 have shown that it binds to the same site as eRF3, interacting with the C-terminus of eRF1, and that it promotes the placement of the GGQ motif of eRF1 in the PTC (Becker *et al.*, 2012). As Rli1 is crucial for yeast survival, a *DAmP-rli1* strain was used. Since Rli1 is required for normal translation termination, it would be reasonable to expect that a decreased availability of this protein (as in the DAmP allele) would also affect the 2A reaction. However, given the unusual nature of the 2A reaction and the likely negative effect of ribosomal dissociation, also promoted by Rli1, it may be that this factor is not involved or indeed could promote ribosomes dissociating at 2A rather than continuing to translate the downstream product. A further experiment that sheds light on this was overexpression of *RLI1* and testing the outcome of translation through 2A. This revealed a significant improvement of the 2A reaction in the *DAmP-sup45* strain, which underlines this factor as a contributor in the 2A reaction, although the limited availability of this factor did not affect such step of the 2A reaction.

Dom34 and Hbs1 promote ribosomal subunit dissociation during NGD and/or NSD, and this activity is directed by Rli1. Given that ribosomes pause temporarily at 2A (Doronina *et al.*,

2008), it is possible that these factors could be recruited to and either interfere with the normal reaction or be part of the mechanism of the 2A reaction. In the current model of the 2A reaction, where there is no need for the subunits to dissociate from each other, after upstream peptide release, the intact ribosome continues to translate the remaining open reading frame. However, the reaction could proceed via other pathways. The main function of Ski7 is to target mRNAs in the NSD pathway for decay via interactions between the Ski complex and the exosome, However, its similarity to eRF3 and Hbs1 (Kowalinski *et al.*, 2015), and ability to bind stalled ribosomes, suggest it as a candidate protein that could be recruited to the 2A reaction. Hcr1 (eIF3j) has been proposed to promote eRF3 release and binding of Rli1 to the termination complex (Beznoskova *et al.*, 2013). Despite the role of this protein in termination, the protein appears not to be involved in the 2A reaction.

3.8.2 eRFs are required to drive the 2A reaction

Initial experiments using strains with reduced levels of eRF1 and 3 activity confirmed the indicated involvement of translational termination factors in the 2A reaction. Although our initial observations about eRF1 confirm that they participate in the 2A reaction, it remains unclear how these factors are recruited to drive the 2A reaction and trigger the release of the nascent peptide chain from the ribosome, in the absence of a stop codon in the ribosomal A site. A wider analysis of the role of eRF1 in 2A reaction is described in the next chapter, in order to address this point and to generate information on features of eRFs required for their productive interaction with the ribosome in this case.

As we have shown in sections 3.4 and 3.5, reduced availability/activity of eRF1 and eRF3 reduces the 2A activity. Three strains of eRF1 have been used to investigate this aspect: BSC483-*sal4-42* which expresses reduced eRF1 activity due to a mutation in the *SUP45* gene, *sup45-2* which encodes an isoleucine residue in place of serine, and finally *DAmP-sup45*, as the *SUP45* gene is essential for yeast survival. Whereas strains that were used to analyse the effects of eRF3 on 2A reaction were [PSI^+], H438Q and R419G, with relevant wild types as controls. It has previously been found that reduced availability of eRF1 in eukaryotic ribosomes leads the ribosome to read-through the stop codon, generating different products from that encoded in the ORF on the mRNA, whereas mutations or reduced activity of eRF3 impose pauses on the ribosome (Merritt *et al.*, 2010; Eyler *et al.*, 2013). These outcomes might be different with ribosomes translating active 2A peptides. The observations of pulse labelling and immunoprecipitation of 2A reporters in the current study have revealed that deficient strains, in such release factors, promote the ribosome to read-through the 2A peptide, generating a higher

ratio of the full-length protein. This was observed through quantifying both upstream and downstream products and calculating their ratios to the relevant whole products. The decrease in the ratio of both upstream and downstream proteins suggests that the ribosome skips the release of the nascent chain at the Pro 19 codon, including 2A fragment, and carries on translation to the end of the reporter ORF, as a result of the limited availability of eRF1/3, which are specified to terminate the canonical translation on a stop codon, contained in the ribosomal A site. Unexpectedly, in contrary to other strains tested in this context, the *sup45-42* strain has shown a decrease in the upstream product of the reaction, but not the downstream. This may raise a question: does the truncated *sup45-42* protein retain any activity/ability to bind ribosomes? Given that this is the case, it could bind, but does not activate termination however, the ribosome could not be able to proceed which may lead the ribosome to 'dropoff' and thus a greater effect on downstream than upstream.

The 2A reaction was investigated in two strains expressing Sup35 with reduced GTPase activity. These strains were previously found to display increased release of the upstream product of the 2A reaction (Doronina *et al.*, 2008). In the current study, these strains have shown no effects on the 2A reaction, and the growth rate of these strains was unaffected by overexpression of the 2A-encoding mRNA. However, limited availability of eRF3, in [PSI^+] cells, led to the same effect on 2A reaction that has been detected with *sup45* alleles. This confirms our results, as both eRF1 and eRF3 trigger termination as a counterpart of the PTC, in addition to GTP. The eRF3 is essential for eRF1 (Dever and Green, 2012; Eyler *et al.*, 2013). eRF3 is important for the proper positioning of eRF1 in the ribosome and triggering GTP hydrolysis therefore, both release factors are recruited to the ribosome in the 2A reaction however, the way to hydrolyse GTP might be different.

Normally, the eRF1 within the PTC decodes the stop codon in the ribosomal A site, but in the case of 2A, no stop codon in the mRNA is encountered by the ribosome. This suggests that eRF1 binds the ribosome with different conformational changes, and contacts the PTC to trigger the peptide release from the exit tunnel. However, more investigations, in terms of the eRFs residues and biochemical analysis, are required to propose a model for the 2A mechanism, according to the current outcomes.

Consistent with the results of pulse labelling and immunoprecipitation, strains with limited availability of eRF1/3 have shown inhibition in the growth profiles, while expressing functional 2A peptide from a strong promoter. The reason behind the growth inhibition is not clear, however, it is consistent with the results of Doronina *et al.*, (2008). The inhibition of growth rate detected in limited pool of eRFs suggests a toxic effect exerted by the overexpression of

2A peptide, which might be attributed to depletion of eRFs from the ribosome through arresting them, and impairing the recycling process, which maintains the normal level of release factors. This will also be further investigated in the next chapter.

4 CHAPTER 4: DISSECTING REQUIREMENTS FOR eRF1 AND eRF3 IN THE 2A REACTION THROUGH ANALYSIS OF POINT MUTANTS.

4.1 Introduction

Work presented in Chapter 3 established that both eRF1 and eRF3 are essential factors to maintain the normal 2A reaction and the data obtained support the conclusion that they drive the reaction by triggering peptide release from the ribosome, which then resumes translation of the remaining ORF. As outlined in Chapter 1, eRF1 and 3 bind the ribosome as a complex together with GTP. Under normal circumstances, a key initial step in binding of eRFs to the ribosome is stop codon recognition, mediated by specific motifs in the eRF1. The presence of a Pro codon in the A site during the 2A reaction makes it unclear at this point if eRF1 binds the ribosome using the same motifs used to bind stop codons. At the least, it could be expected that there is a slight alteration of the N domain conformation to properly accommodate into the ribosomal A site, and then enable the M domain to contact the peptidyl transferase centre (PTC). In this chapter a collection of mutations, mainly in eRF1, but also eRF3, were analysed to identify similarities and differences in the interaction of/requirements for eRFs with the ribosome at 2A peptide. The same strategies, including pulse labelling and immunoprecipitation, of 2A-containing proteins as used in Chapter 3 were used to carry out these experiments.

4.2 Biochemical analysis of eRF1 mutants for effects on the 2A reaction.

4.2.1 Laboratory materials

A strain of *S. cerevisiae* was used in the experiments presented in this chapter; YTH91 (see table 2.1). The *SUP45* gene is deleted in this strain, and viability maintained by a centromeric plasmid (pTH400 CEN/*URA3* (Merritt *et al.*, 2010), expressing wild type *SUP45* from a *GAL1* promoter. Alongside this strain, a collection of *sup45* alleles (Merritt *et al.*, 2010) was used. This collection was assembled in the laboratory of Dr. Tobias von der Haar and includes alleles described in previous studies and all alleles are placed into the same context, the CEN/*LEU2* plasmid pRS315 (Sikorski and Hieter, 1989), under control of the *SUP45* promoter. Importantly, this collection includes alterations to amino acids in and close to key conserved motifs of eRF1, and all have been characterised for their effects on growth and defects in termination (table 4.1).

Protein	Mutation	Domain	Protein abundance (%) (Merritt *et al.*, 2010)	Stop codon read-through (Merritt *et al.*, 2010)
eRF1	S30P	N	101	↑
	I32F	N	30	↑↑
	P38L	N	96	↑↑
	L49A	N	Nd	↑
	E52A	N	Nd	↑
	T55A	N	86	↑
	N58A	N	140	↑↑↑
	S61A	N	Nd	↑
	R62A	N	87	↑
	V68A	N	85	↑↑
	V107D	N	29	↑↑
	P174Q	M	82	↑
	I222S	M	85	↑
	T295A	C	Nd	↑
	T388A	C	Nd	↑
	F401Y	C	Nd	↑
	Y410F	C	74	↑
eRF3	R320I	G		
	G357D	G		
	S416F	GTPase		

Table 4.1: The mutations in eRF1 and eRF3 used in the study and their locations in the protein.

4.2.2 The effect of mutations to residues in the N domain of eRF1 on the 2A reaction

To analyse different *sup45* alleles, strains were transformed with plasmids containing the desired *sup45* variant and the *LEU2* selectable marker. Transformed cells were plated on media lacking leucine and then onto 5-FOA-containing plates, to select for cells that had lost the *URA3*-marked plasmid containing the wild type gene (Materials and Methods). These cells were then maintained on glucose plates/media. Both strains and plasmids (Merritt *et al.*, 2010), used in this section, were obtained from Dr. Tobias von der Haar, University of Kent. Strains expressing *sup45* mutations were transformed with plasmids from which the HA-αF-2A/2A*-GFP polyprotein reporters were expressed, and cells cultured, pulse labelled, proteins extracted, and immunoprecipitation and quantification performed as described in Chapter 3. An example of immunoprecipitations from a variety of the strains analysed here is presented in figure 4.1. Each strain was analysed at least 3 times and graphed data in figures 4.2-4.6 is averages +/- the standard error.

The TASNIKS motif, and specifically the NIKS sequence, imposes a strict requirement for U at the first position of the stop codon (Bulygin *et al.*, 2010). Yeast cells expressing the HA-αF-2A-GFP reporter protein and Sup45 containing mutations mapping to residues upstream, within and just downstream of this motif (L49A, N58A, T55A, S61A, R62A, V68A) were pulse-labelled and the effect of the Sup45 mutations on the 2A reaction examined by immunoprecipitation and quantification of 2A reaction products. When compared to the reaction in cells expressing wild type Sup45, cells expressing two of the mutations, L49A and R62A, revealed a significant ($p<0.01$) decrease in the production of both the upstream and downstream products of the 2A reaction (figure 4.2). This indicates that in the presence of these mutations to Sup45, more ribosomes read through 2A and into the downstream sequence. Neither of these mutations lead to increase in stop codon read-through (Merritt *et al.*, 2010). In contrast, the T55A mutant led only to a significant decrease in downstream product but a similar amount of upstream product to the wild type cells. This is consistent with a similar fraction of ribosomes undergoing the first (i.e. termination) part of the 2A reaction, but fewer proceeding to re-start translation to generate the downstream product. The remaining 3 mutations to this region of Sup45 did not affect the 2A reaction significantly. Of these 2, N58A and S61A, are within the core of the TASNIKS motif. The N58A mutation leads to strongly increased read-through of all 3 stop codons, consistent with the role of the TASNIKS motif in stop codon recognition.

Overall, these results are consistent with the interactions of the TASNIKS motif necessary for the 2A reaction, where no stop codon is present in the ribosomal A site, being different to those required for efficient termination at stop codons.

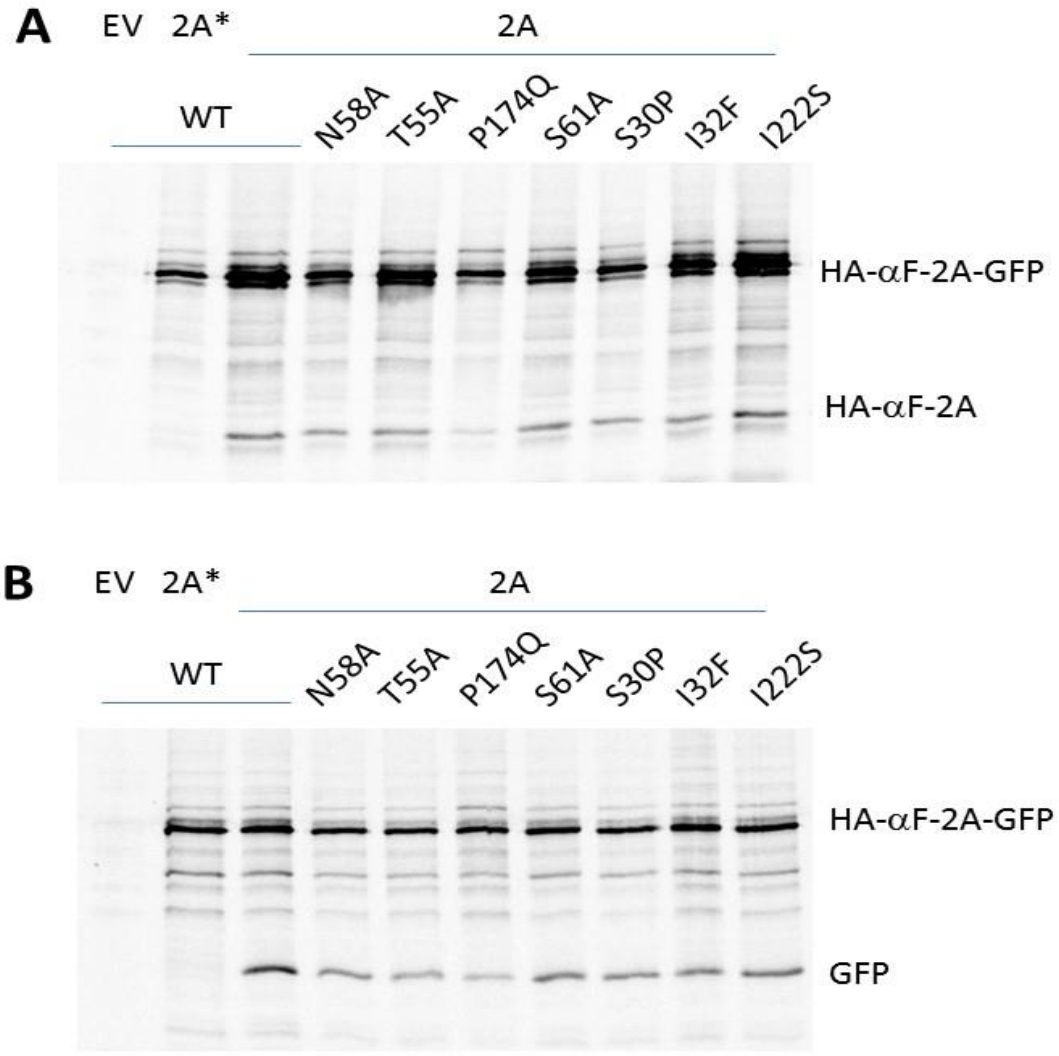

Figure 4.1 Immunoprecipitation of proteins derived from strains expressing mutations in various regions of eRF1.

Strains expressing the variant of Sup45 indicated were transformed with empty vector (EV) or a plasmid expressing the 2A reporter or non-functional (2A*) variant, pulse labelled with [^{35}S] methionine/cysteine and translation products were detected by imunoprecipitation with anti-HA (A) or -GFP (B) antibodies. Immunoprecipitated proteins were resolved on an SDS-PAGE gel which was subsequently fixed and dried and exposed to a phosphorimager screen. Screens were scanned using Typhoon phosphoimager.

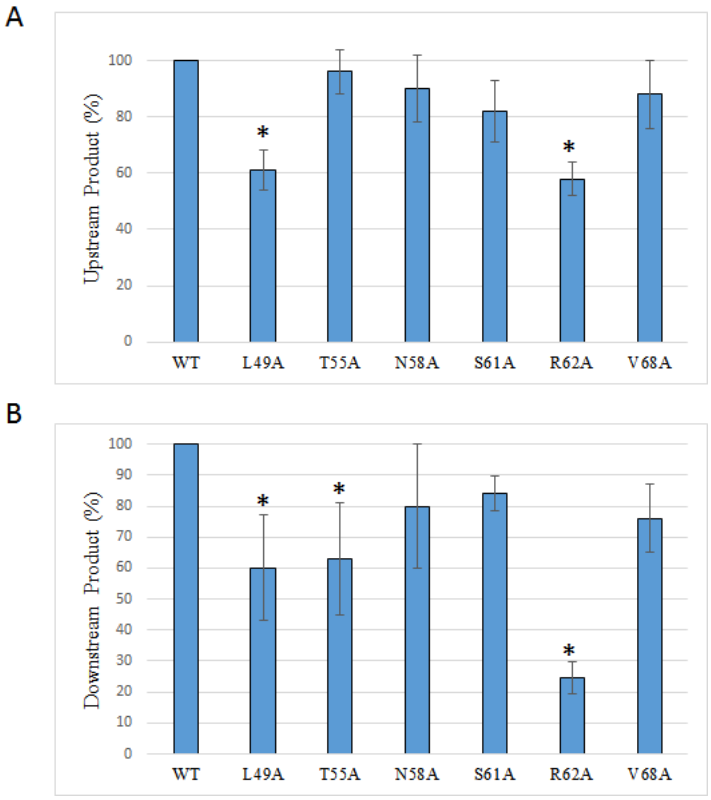

Figure 4.2: Mutations in TASNIKS but not the NIKS core motif inhibit the 2A reaction.

Pulse-labelled proteins extracted from strains containing the indicated Sup45 variants as the only version of the protein, and expressing the HA-αF-2A/2A*-GFP reporter, were isolated by immunoprecipitation with anti-HA (A) or anti-GFP (B) and the immunoprecipitated proteins resolved on SDS-PAGE gels. Gels were fixed and dried, and then exposed to phosphor screens. Screens were scanned using Typhoon phosphoimager, and bands quantified using ImageQuant to find the ratios of smaller products to the full-length. Values presented are from 3-5 independent experiments. * refers to significance $p \leq 0.01$.

4.2.3 Mutations affecting recognition of the second and third bases of the stop codon

The YxCxxxF motif and E52 (E55 in mammalian eRF1) interact with the purines at positions 2 and 3 of the stop codon (Seit-Nebi *et al.*, 2002) and along with the GTS motif to provide the key region of eRF1 that allows specificity of recognition of the bases at these positions (Blanchet *et al.*, 2015). No mutations were available directly affecting the YxCxxxF motif, but the nearby amino acid V107 is supposed to perform a function related to it. Yeast strains expressing Sup45, and containing the V107D mutation, changes within and close to the GTS (S30P, I32F, P38L) or the E52A as the only version of the protein were tested for effects on the 2A reaction.

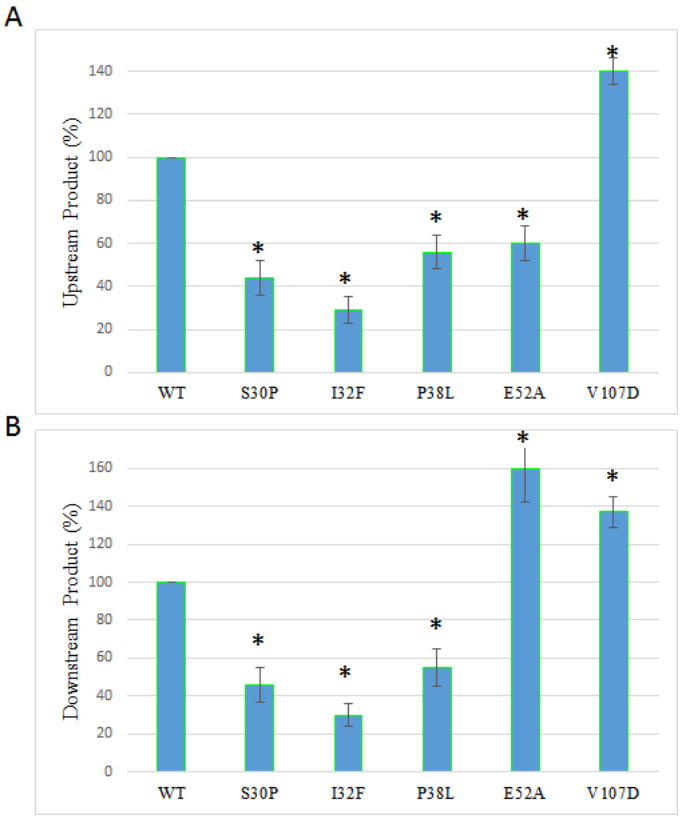

Figure 4.3: Mutations to residues specified for recognition of the second and third bases of the stop codon affect the 2A reaction.

Pulse-labelled proteins extracted from strains containing the indicated Sup45 variants as the only version of the protein, and expressing the HA-αF-2A/2A*-GFP reporter, were isolated by immunoprecipitation with anti-HA (A) or anti-GFP (B) and the immunoprecipitated proteins resolved on SDS-PAGE gels. Gels were fixed and dried, and then exposed to phosphor screens. Screens were scanned using Typhoon phosphoimager, and bands quantified using ImageQuant to find the ratios of smaller products to the full-length. Values presented are from 3-5 independent experiments. * refers to significance $p \leq 0.01$.

As seen in figure 4.3, all mutations that have been examined here affect the 2A reaction either by elevating or inhibiting the products of the reaction. Expression of HA-αF-2A-GFP in strains expressing the S30P mutation to the core GTS motif, in addition to mutations beyond this motif (I32F and P38L), led to reduced levels of both upstream and downstream products of the reaction when compared to the wild type strain, indicating that a greater fraction of ribosomes read through the 2A sequence. In contrast, the E52A mutation significantly reduced the upstream product and increased the downstream. In this case, the ribosome seems to pause at the Pro 19 codon, releasing the nascent chain then, a high ratio of these ribosomes may re-start again to generate the downstream product. The V107 residue is located close to the YxCxxxF motif, and assumes a function in stop codon decoding. Here, the V107D increased both products of the 2A reporter, indicating an increase in the efficiency of both steps of the 2A reaction over the level of the strain expressing wild type eRF1.

4.2.4 Mutations affecting the M domain inhibit the 2A reaction

The M domain contains the key GGQ motif that extends to the PTC and drives release of the nascent chain. Mutations within the GGQ motif itself are inviable and/or unstable in *S. cerevisiae* (Merritt *et al.*, 2010) and thus 2 other mutations were tested here, P174Q (Pillay *et al.*, 2016) and I222S, which is the analog of the *sup45-2* strain (see Chapter 3 for details). These are important in the peptide chain release, together with the GGQ motif (residues 180-182). The P174 residue is located 6 residues prior to the GGQ motif, which contacts the PTC and triggers the peptide release from the ribosome.

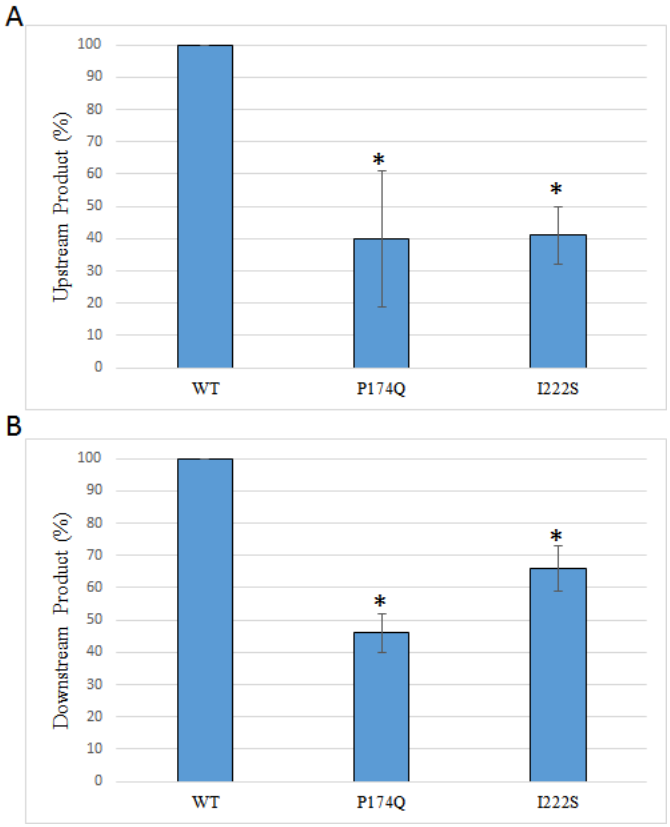

Figure 4.4: Mutations in the M domain of eRF1 inhibit the 2A reaction.

Pulse-labelled proteins extracted from strains containing the indicated Sup45 variants as the only version of the protein, and expressing the HA-αF-2A/2A*-GFP reporter, were isolated by immunoprecipitation with anti-HA (A) or anti-GFP (B) and the immunoprecipitated proteins resolved on SDS-PAGE gels. Gels were fixed and dried, and then exposed to phosphor screens. Screens were scanned using Typhoon phosphoimager, and bands quantified using ImageQuant to find the ratios of smaller products to the full-length. Values presented are from 3-5 independent experiments. * refers to significance $p \leq 0.01$.

Expression of the 2A reporter in strains expressing the P174Q or I222S mutation in Sup45 as the only version of the protein reduced the ratio of upstream and downstream products of the 2A peptide-containing polyprotein (figure 4.4). A 40% reduction in the first step of the reaction was detected for both P174Q and I222S mutations (figure 4.4: A), whereas the second step was reduced about 45% in the strain expressing P174Q, and about 66% in the strain expressing I222S, when compared to the wild type strain (figure 4.4: B).

4.2.5 Effect of mutating residues in the C domain of eRF1 on the 2A reaction

eRF1 interacts with eRF3 via their C domains. Thus, mutating residues in eRF1 which are responsible for interaction with eRF3 may affect the 2A reaction. In this section, yeast cells expressing the HA-αF-2A-GFP 2A reporter protein and Sup45 with mutations in the eRF1 interface with eRF3, as the only copy of the protein, were tested for effects on the 2A reaction. Four mutations were tested, T295A, T388A, F401Y and Y410F. As before, cells were pulse-labelled, proteins generated from the reporter immunoprecipitated with anti-GFP or anti-HA antibodies and quantified.

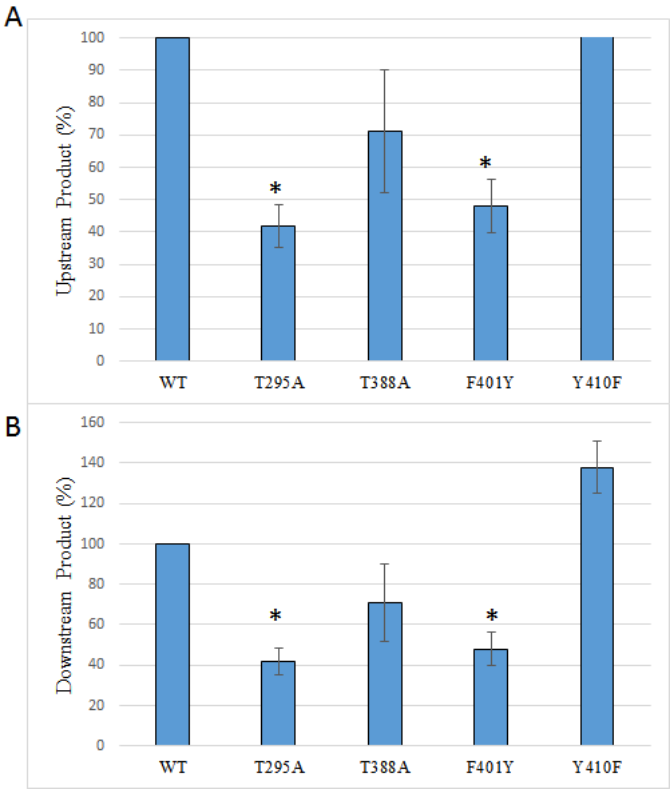

Figure 4.5: Effect of mutations in the C domain of eRF1 on the efficiency of the 2A reaction.

Pulse-labelled proteins extracted from strains containing the indicated Sup45 variants as the only version of the protein, and expressing the HA-αF-2A/2A*-GFP reporter, were isolated by immunoprecipitation with anti-HA (A) or anti-GFP (B) and the immunoprecipitated proteins resolved on SDS-PAGE gels. Gels were fixed and dried, and then exposed to phosphor screens. Screens were scanned using Typhoon phosphoimager, and bands quantified using ImageQuant to find the ratios of smaller products to the full-length. Values presented are from 3-5 independent experiments. * refers to significance $p \leq 0.01$.

Immunoprecipitation data revealed that T295A and F401Y reduce the overall efficiency of the 2A reaction. Both steps of the reaction were significantly ($p \leq 0.01$) reduced, by ~ 40 and 50% in strains expressing the T295A and F401Y mutations respectively (figure 4.5). The T388A mutation also reduced the efficiency of the reaction, though not to the same level of significance. Although Y410 is stated as a key residue for eRF1 binding to eRF3 (Song *et al.*, 2006), the Y410F mutation in the current study has shown no significant effect on both upstream and downstream products. It increased the production of the downstream fragment, but below the significance level used in our study.

4.3 Biochemical analysis of eRF3 mutants for effects on the 2A reaction.

The data in the preceeding section significantly add to the understanding of the contribution of several different regions of eRF1 to the 2A reaction. The effect of mutations within the protein on the 2A reaction differs from the effect on canonical termination in many instances. The determinant here is the absence of a stop codon in the ribosomal A site.

We found in Chapter 3 of this study that cells limited in eRF3 activity ([PSI^+]) significantly inhibit the 2A activity, in terms of reducing ratios of both upstream and downstream products. On the other hand, mutating residues in the GTPase domain of eRF3 did not affect the reaction activity (see Chapter 3). Based on the structure of eRF1-eRF3 interaction in the ribosome, we identified mutations in eRF1's C domain, in residues which serve as binding points between eRF1 and eRF3, that inhibit the 2A reaction by reducing both upstream and downstream ratios. To further address the effect of eRF3 on 2A reaction, we have investigated, in this section, the effect of some mutations in eRF3 C domain.

4.3.1 Laboratory materials

A strain of *S. cerevisiae* (JDY879) was used to investigate the effects of eRF3 on the 2A peptide. In this strain *SUP35* transcription is driven by the repressible *kanMX4-tet07* promoter. The strain was transformed with plasmids containing *sup35* alleles (provided by Dr. Tobias von der Haar) or the wild type under control of the *SUP35* promoter maintained on media containing doxycycline 15 µg/ ml to repress the genomic *SUP35*, leaving cells reliant on expression from the plasmid-borne copy of the gene.

4.3.2 The S416F mutation in Sup35/eRF3 inhibits the 2A reaction

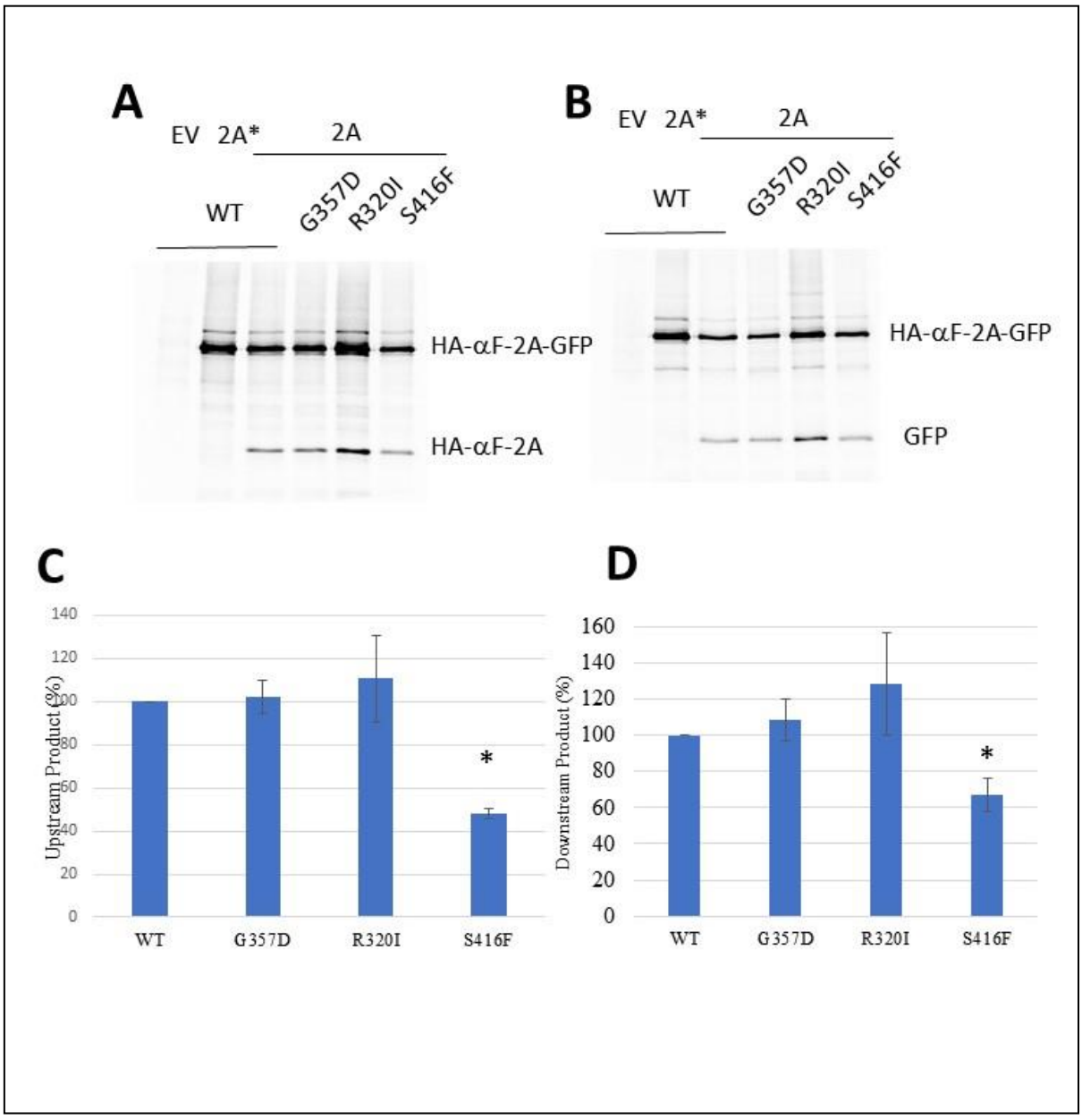

Figure 4.6: Analysis of *sup35* mutations for effects on the 2A reaction.

Strains expressing the variant of Sup35 indicated were transformed with empty vector (EV) or a plasmid expressing the 2A reporter or non-functional (2A*) variant, pulse labelled with [^{35}S] methionine/cysteine and translation products detected by imunoprecipitation with anti-HA (A) or -GFP (B) antibodies. Immunoprecipitated proteins were resolved on an SDS-PAGE gel which was subsequently fixed and dried and exposed to a phosphorimager screen. (C) Quantification of the upstream products. (D) Quantification of the downstream products. Data presented are the average of 3-4 independent experiments. * refers to significance $p \leq 0.01$.

Three mutations to Sup35 were tested: G357D, R320I and S416F, all located in domain 1 of the C terminus of eRF3. This is the G-nucleotide binding domain of the protein, which assumes its function in GTP hydrolysis during canonical termination (Fabret *et al.*, 2008). Only the S416F mutation revealed a significant inhibition of the 2A reaction (figure 4.6), diminishing the amount of both upstream and downstream products by 50 and 70% respectively. Whereas the G357D and R320I mutations had no effect on either step of the reaction. These outcomes confirm the role of eRF3 in the 2A reaction, together with eRF1, the process which needs GTP hydrolysis to ensure the efficiency of the reaction.

4.4 Measurement of growth rates of cells with mutant release factors, expressing a 2A-containing reporter

To add to data from immunoprecipitation experiments, we evaluated the effect of over-expressing the 2A reporter in cells expressing mutant forms of eRF1 and eRF3. The experimental approach was similar to that adopted in chapter 3 (section 3.5) and was accomplished by measuring optical densities of cultures at 600 nm. Strains expressing the same variants of Sup45 (or Sup35) as the only form of the protein as used in chapter 3 were transformed with plasmids from which either HA-αF-2A-GFP or the inactive HA-αF-2A*-GFP were expressed from the *GAL1* promoter. Growth of cultures of cells containing these plasmids were compared. Data presented here is in the same order used in the immunoprecipitation section.

4.4.1 Mutations in the TASNIKS motif

Analysis of mutations in or close to the TASNIKS motif indicated that only L49A and R62A affected the overall 2A reaction while the T55A mutation led to a specific reduction in generation of the downstream product indicating a defect in the second (re-initiation) step in the 2A reaction (section 4.2.2.1). L49A and R62A mutations do not affect the ability of Sup45 to terminate translation at stop codons, while T55A has only a very minor defect, specifically at UAA stop codons (Merritt *et al.*, 2010). The same set of mutations that was examined for effects on the 2A reaction (L49A, T55A, N58A, S61A, R62A and V68A) were assessed in the same manner explained above for growth rate when the 2A- or 2A*-containing reporter was expressed.

As seen in figure 4.7, the presence of 2A-coding sequence peptide in cells expressing any of the TASNIKS-motif associated mutations does not alter the growth rate/doubling time. They grew equally well in the presence of 2A or 2A*. The measurement and comparison of growth

in strains expressing active 2A peptides has shown no effect compared to strains with inactive 2A (2A*).

A

Strain Expressing 2A	Growth Doubling Time (%) (mean±SE)
WT	103.505± 4.73
L49A	123.981± 5.66
T55A	112.067± 5.12
N58A	112.798± 5.15
S61A	120.610± 5.51
R62A	132.509± 6.06
V68A	111.626± 5.10

B

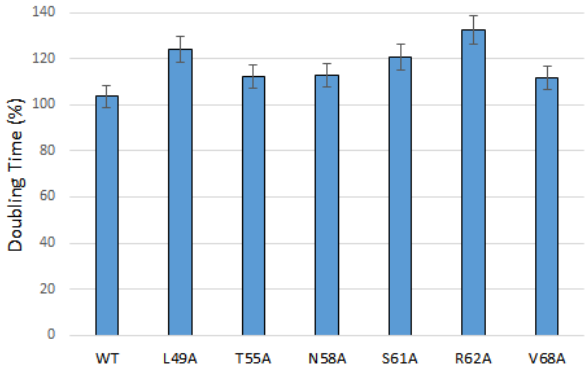

Figure 4.7: Growth doubling time is not affected in strains with mutations in the TASNIKS motif while expressing 2A peptide.

Strains with mutations in TASNIKS motif of Sup45 (YTH91 transformed with pTH438, 355, 371, 373 and 374), along with the wild type (YTH91 transformed with pTH353), were transformed with 2A and 2A*-containing reporters (pJNY279 and 278) and grown overnight at 30 ºC in selective media. Cultures were diluted on the next day with the same media plus 2% w/v galactose to induce the *GAL1* promoter of the 2A reporter. OD_{600} was recorded every one hour for eight hours, and data were processed to find the doubling time for each strain expressing active 2A, and normalised to the doubling time of the strain expressing 2A*. (A) Table of doubling time values. (B) Chart of values corresponding to A.

4.4.2 Growth doubling time with mutations affecting the recognition of the second and third bases in the stop codon sequence

The same strains tested in section 4.2.3 for their tendency to inhibit the 2A reaction are tested here to address the inhibitory effect of 2A overexpression on the growth of these cells. These strains include the core GTS mutation, S30P as well as two other mutations located close to this motif, I32F and P38L. Furthermore, strains expressing E52A and V107D have been included to this range of mutations.

A

Strain Expressing 2A	Growth Doubling Time (%) (mean±SE)
WT	103.505± 4.73
S30P	230.211± 10.52*
I32F	130.814± 5.97
P38L	112.513± 5.14
E52A	100.889± 9.54
V107D	111.922± 5.11

B

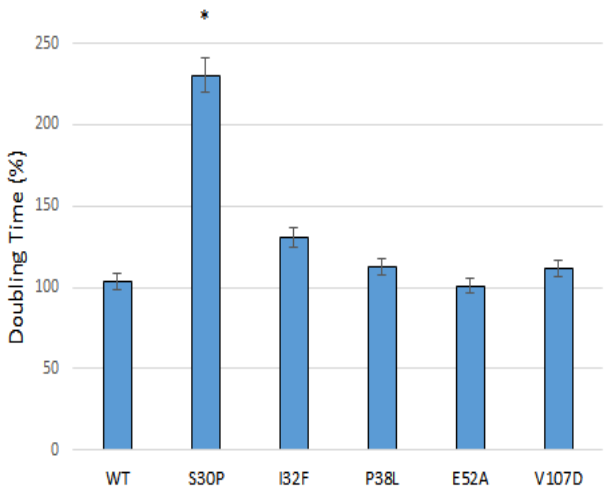

Figure 4.8: Growth doubling time of strains with mutations to residues which recognise the 2nd and 3d bases of the stop codon.

Strains expressing S30P, I32F, P38L, E52A and V107D in Sup45 (YTH91 transformed with pTH399, 313, 314, 439 and 316), along with the wild type (YTH91 transformed with pTH353), were transformed with 2A and 2A*-containing reporters (pJNY278 and 279) and grown overnight at 30 ºC in selective media. Cultures were diluted on the next day with the same media plus 2% w/v galactose to induce the *GAL1* promoter of the 2A reporter. OD_{600} was recorded every one hour for eight hours, and data were processed to find the doubling time for each strain expressing active 2A, and normalised to the doubling time of the strain expressing 2A*. Averages of 3-4 independent experiments are presented above± standard error. (A) Table of doubling time values. (B) Chart of values corresponding to A. * refers to significance $p \leq 0.01$.

Mutations tested in the GTS motif (S30P) significantly increased the doubling time of cells expressing 2A compared to the wild type cells, whereas the increase in the doubling time detected in the I32F mutation, which is located beyond the GTS motif, was insignificant at p level 0.01. However, the P38L did not affect the growth pattern of cells expressing 2A peptide. (figure 4.8) It has already been detected in this study that all the three mutations affected the 2A reaction, by reducing the production of both upstream and downstream reaction products in relation to read-through product (section 4.2.3). In contrast, cells expressing the Sup45-P38L, E52A or V107D mutation grew equally well with no detectable difference in doubling time compared to the wild type (figure 4.8).

4.4.3 Mutations affecting the M domain inhibit the growth in strains expressing 2A peptide

As presented above in regard to other functional parts of the eRF1, mutations before and beyond the universally conserved GGQ motif were examined here, whether they affect the growth of yeast cells, while expressing 2A peptide. Two mutations were tested in this context, P174Q and I222S.

A

Strain Expressing 2A	Growth Doubling Time (%) (mean±SE)
WT	103.505± 4.73
P174Q	124.074± 5.67
I222S	215.813± 9.86*

B

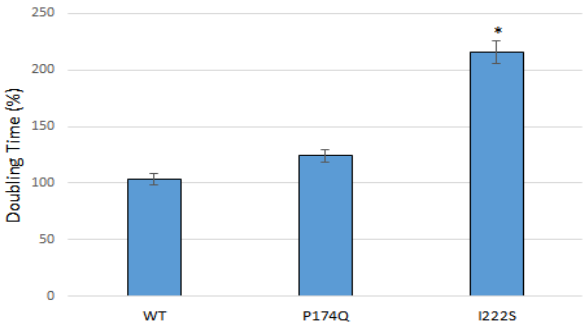

Figure 4.9: Growth doubling time in strains expressing mutations to residues close to the GGQ motif.

Strains expressing P174Q and I222S in Sup45 (YTH91 transformed with pTH443 and 445), along with the wild type (YTH91 transformed with pTH353), were transformed with 2A and 2A*-containing reporters (pJNY278 and 279) and grown overnight at 30 ºC in selective media. Cultures were diluted on the next day with the same media plus 2% w/v galactose to induce the *GAL1* promoter of the 2A reporter. OD_{600} was recorded every one hour for eight hours, and data were processed to find the doubling time for each strain expressing active 2A, and normalised to the doubling time of the strain expressing 2A*. Averages of 3-4 independent experiments are presented above ± standard error. (A) Table of doubling time values. (B) Chart of values corresponding to A. * refers to significance $p \leq 0.01$.

Of the 2 Sup45 variants tested, only I222S, which is the same change to the protein as that in the *sup45-2* allele (see Chapter 3 for details), has revealed an inhibition in the growth, detected by increased doubling time, while expressing active 2A, compared to the same strain expressing inactive 2A peptide. The doubling time in this strain was approximately double of that observed in the wild type. The strain expressing Sup45-P174Q revealed no effect in this context as a result of expressing the construct encoding active 2A (figure 4.9).

4.4.4 Growth of cells with mutations in the C domain of eRF1

A

Strain Expressing 2A	Growth Doubling Time (%) (mean±SE)
WT	103.505± 4.73
T295A	178.932± 8.17*
T388A	119.679± 5.64
F401Y	109.313± 4.99
Y410F	290.667± 13.28*

B

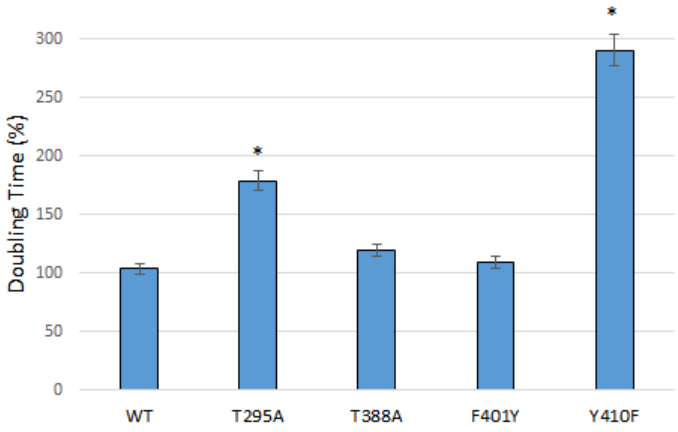

Figure 4.10: Growth doubling time is increased in some strains expressing mutations in the C domain of Sup45.

Strains expressing mutations in Sup45 C domain which includes, T295A, T388A, F401Y and Y410F (YTH91 transformed with pTH363, 365, 366 and 357), along with the wild type (YTH91 transformed with pTH353), were transformed with 2A and 2A*-containing reporters (pJNY278 and 279) and grown overnight at 30 ºC in selective media. Cultures were diluted on the next day with the same media plus 2% w/v galactose to induce the *GAL1* promoter of the 2A reporter. OD_{600} was recorded every one hour for eight hours, and data were processed to find the doubling time for each strain expressing active 2A, and normalised to the doubling time of the strain expressing 2A*. Averages of 3-4 independent experiments are presented above ± standard error. (A) Table of doubling time values. (B) Chart of values corresponding to A. * refers to significance $p \leq 0.01$.

As with mutations in other regions of Sup45, the 4 variants in the C domain of the protein yielded different growth patterns and thereby different doubling time, when the 2A-containing reporter was expressed. Thus the growth of cells expressing the T295A and Y410F variants was inhibited and showed a significant increase in the doubling time compared to the wild type strain, whereas those expressing T388A and F401Y were growing normally. The strain with F401Y-*sup45* has revealed a significant inhibition of the 2A reaction, when biochemically assessed by immunoprecipitation (section 4.2.5). Surprisingly, the growth of this strain here is normal while expressing 2A peptide, compared to the control expressing 2A* (figure 4.10). Conversely, the presence of active 2A in the Y410F strain has inhibited the growth, relative to the overexpression of 2A* in the same strain background, although no effect was detected with this strain in terms of immunoprecipitation (figure 4.5). Worth noting however, 4-5 repeats of growth measurement have been performed to confirm these results.

4.5 Measurement of growth of strains with mutant eRF3

The same selection of mutations within the GTP binding domain of Sup35 were tested as in the immunoprecipitation experiments (section 4.2).

A

Strain Expressing 2A	Growth Doubling Time (%) (mean±SE)
WT	115.178± 5.26
R320I	205.047± 9.37*
G357D	105.916± 4.84
S416F	109.145± 4.98

B

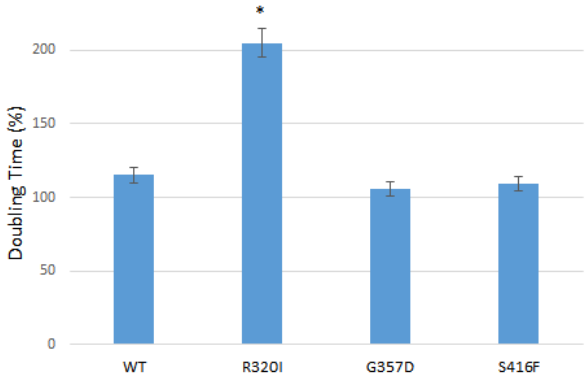

Figure 4.11: Growth doubling time of strains with mutations in the GTP-binding domain of Sup35 while expressing 2A peptide.

Strains expressing mutations in Sup35 GTP-binding domain which includes, R320I, G357D and S416F (JDY879 transformed with pTH770, 773 and 774), along with the wild type (895 transformed with pTH769), were transformed with 2A and 2A*-containing reporters (pJNY278 and 279) and grown overnight at 30 ºC in selective media. Cultures were diluted on the next day with the same media plus 2% w/v galactose to induce the *GAL1* promoter of the 2A reporter. OD_{600} was recorded every one hour for eight hours, and data were processed to find the doubling time for each strain expressing active 2A, and normalised to the doubling time of the strain expressing 2A*. Averages of 3-4 independent experiments are presented above ± standard error. (A) Table of doubling time values. (B) Chart of values corresponding to A. * refers to significance $p \leq 0.01$.

The R320I strain grew slower when the active 2A-coding construct was expressed, but other strains were not affected. S416F is not different in terms of doubling time compared to the wild type, while expressing 2A, although it showed a significant reduction in the 2A reaction outcomes, when assessed using immunoprecipitation (figure 4.6). This strain revealed a significant high doubling time, referring to the slow growth induced by expression of 2A peptide relative to the strain expressing 2A*, and compared to the wild type. Worth noting, the strain expressing the G357D mutation did not reveal changes in the growth compared to the wild type (figure 4.11).

4.6 Overexpression of 2A-containing protein inhibits termination

Overexpression of mRNA encoding 2A may lead to increase in stop codon read-through in *trans*, indicating a titration effect on eRF activity. The experiments carried out by Doronina *et al* (2008) used a β-galactosidase-STOP-luciferase construct, to examine stop codon read-through. Extending from that work, here a similar dual luciferase reporter system (Grentzmann *et al.*, 1998) was used to test whether expression of the 2A-encoding mRNA leads to increased

read-through of stop codons in cells expressing the range of Sup45 and Sup35 variants used in the experiments above.

4.6.1 The dual luciferase reporters

Dual luciferase reporters for analysing translational recoding were first developed by Atkins and colleagues (Grentzmann *et al.*, 1998), and subsequently adapted for use in yeast (Keeling *et al.*, 2004; Salas-Marco and Bedwell, 2004). These comprise sequences encoding Renilla luciferase followed by the stop codon, and then Firefly luciferase and are available with each stop codon with every one of the 4 possible nucleotides after them. Here, two stop codons (UAG and UAA), with different 3' nucleotides, have been investigated for the possibility of being suppressed by expression of 2A-coding mRNA (see section 2.4.3 for details). An empty vector was used to provide a background measurement for the luciferase assays, and a version of the dual luciferase reporter with a sense codon provided a positive (100% 'read-through') control.

4.6.2 Strains

The same collection of strains used in the immunoprecipitation and growth experiments above were used here. This collection expresses a variety of mutations in eRF1. Controls are used with each sample (mutation) including vectors (no luciferase reporter), 2A* (inactive 2A peptide), in addition to the active 2A, each tested with both sense and stop codon-containing luciferase reporters. Strains expressing different variants of Sup45 were transformed with either 2A or 2A*-encoding plasmids and then each of these was transformed with a plasmid containing the dual luciferase reporter, the positive (sense) control plasmid or the empty vector negative control. Cells were then grown under conditions that induce expression of the 2A/2A* encoding mRNA, and luciferase measured in extracts of the cells. Readings were processed, and normalised to values obtained with the construct containing the sense codon (100%), and empty vector (0%). For each strain, data are presented as a comparison of read-through in strains expressing active 2A-and the inactive 2A*-containing mRNA and an empty vector. Statistical analysis was performed by comparing the value of stop codon read-through of strains to the value of isogenic wild type eRF1. Example of data is shown below in figure 4.12. Of note, with these strains, the one expressing the wild type eRF1 has shown an increased stop codon read-through in all experiments undergone with the dual-luciferase reporter.

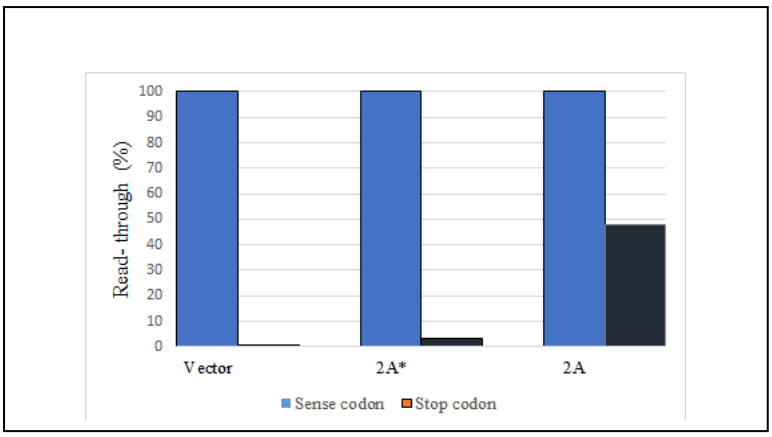

Figure 4.12: Stop codon read-through in wild type *S. cerevisiae* with sense and stop codon-containing dual luciferase plasmids.

Yeast strains transformed with the HA-αF-2A/2A*-GFP plasmids and also containing either the sense or stop codon UAG/UAA/UGA (pJNY250-255), and containing a version of the dual luciferase plasmid were grown, lysates made and luciferase activities measured as described in section 2.4.4 (Materials and methods). The ratio of firefly values to the Renilla was calculated from the average of each sample readings, setting the value obtained with the sense codon version of the dual luciferase plasmid to 100%.

4.6.3 Mutations in the TASNIKS motif increase stop codons read-through in cells expressing 2A peptide

Dual-luciferase activity was measured in yeast cells expressing mutations to the TASNIKS motif and adjacent amino acids as used in previous experiments (L49A, N58A, T55A, S61A, R62A, V68A) and HA-αF-2A/2A*-GFP reporters. A comparison of read-through of UAA and UAG stop codons is presented in figure 4.13. Two different contexts of the UAG stop codon (UAGA and UAGC) were used, as the 4th position has a significant effect on the recognition of the stop codon, with C being the 3' base that yields the highest basal levels of read-through (Salas-Marco and Bedwell, 2004). As seen in figure 4.13, compared to the effect on wild type cells, expression of HA-αF-2A-GFP led to significant increase in read-through in strains expressing L49A, T55A and R62A variants of Sup45 with all 3 tested stop codons/contexts. The V68A mutation caused a significant increase in read-through with the UAGC context sequence, compared to the wild type, whereas the elevation in this strain was insignificant with other tested stop codons (figure 4.13).

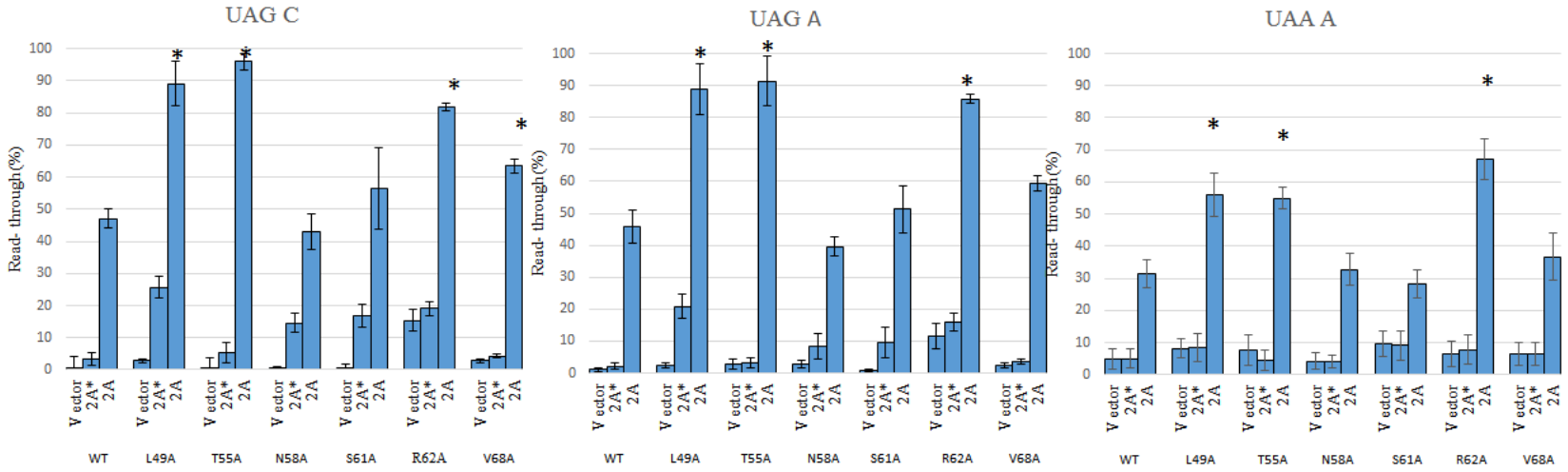

Figure 4.13: Expression of 2A leads to increased stop codon suppression in *trans* in strains expressing mutations close to the TASNIKS motif.

Cultures of the indicated strains, expressing dual luciferase reporter with sense, UAG or UAA stop codons followed by cytosine or adenine as a context sequence with plasmids encoding empty vector, 2A* or 2A, were grown overnight in selective media. Proteins were extracted and 10-20 µg were used for luciferase measurement of each sample. Data obtained were processed to find averages, values of sense codons were normalised to 100%, and samples were calculated accordingly. Values of the active 2A in each strain with a stop codon were statistically compared to the relevant value of the isogenic wild type. * refers to significance $p \leq 0.01$.

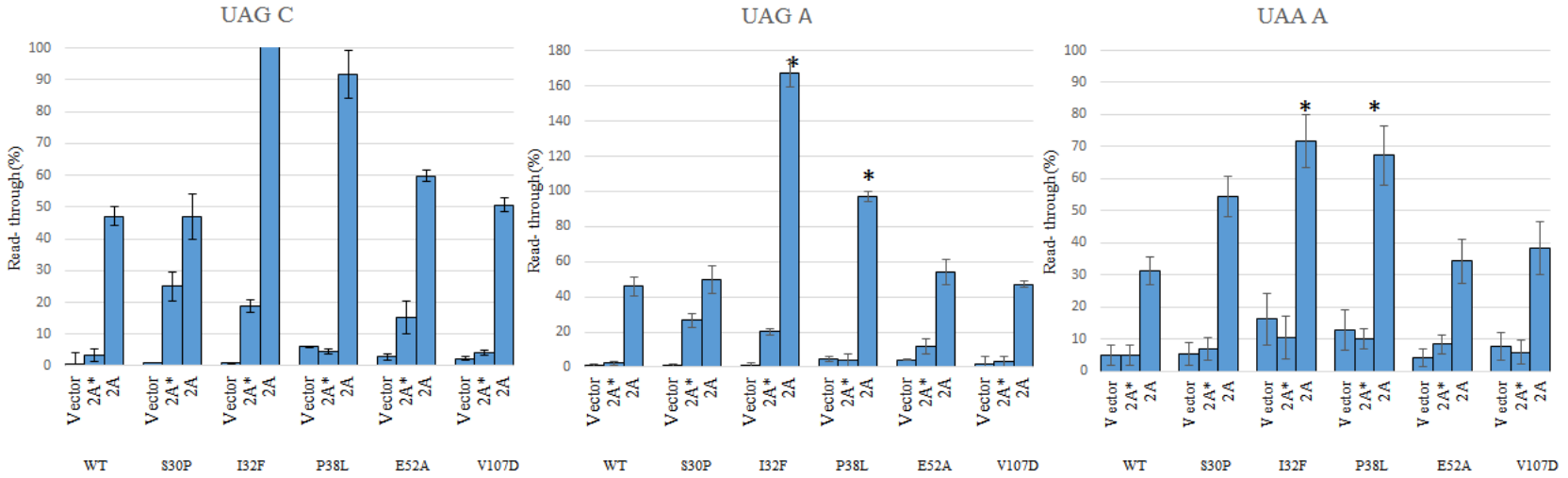

Figure 4.14: Stop codon read-through in *trans* in strains expressing 2A peptide, and maintaining termination with eRF1 expressing mutations to residues in necessary for stop codon recognition.

Cultures of the indicated strains, expressing dual luciferase reporter with sense, UAG or UAA stop codons followed by cytosine or adenine as a context sequence with plasmids encoding empty vector, 2A* or 2A, were grown overnight in selective media. Proteins were extracted and 10-20 µg was used for luciferase measurement of each sample. Data obtained were processed to find averages, values of sense codons were normalised to 100%, and samples were calculated accordingly. Values of the active 2A in each strain with a stop codon were statistically compared to the relevant value of the isogenic wild type. * refers to significance $p \leq 0.01$.

4.6.4 Mutations in the stop codon recognition residues increase stop codon read-through when cells express 2A-encoding mRNA

As seen in figure 4.14, yeast strains expressing eRF1 with mutations beyond the GTS motif, I32F and P38L, have increased the read-through level over the wild type strain. The level of read-through approaches 100% with the UAG C reporter for both strains, whereas with the UAG A, read-through increase was apparently greater than that seen with the sense-codon containing construct (160%) in the strain expressing I32F and 100% in the strain expressing P38L. Strains expressing either of these eRF1 variants have reduced 2A activity. A third eRF1 variant that reduces 2A activity, S30P (see section 4.2.3 for details), did not significantly affect stop codon read-through in *trans*.

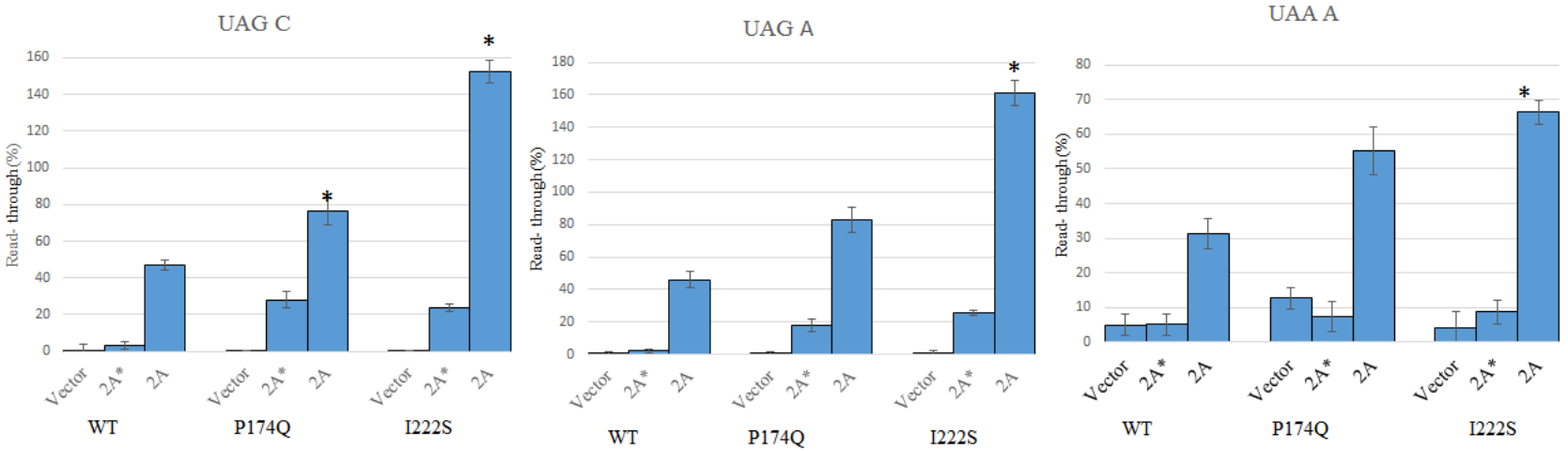

Figure 4.15: Stop codon read-through in *trans* in strains with mutations to residues close to the GGQ motif of eRF1 while expressing 2A peptide.

Cultures of the indicated strains, expressing dual luciferase reporter with sense, UAG or UAA stop codons followed by cytosine or adenine as a context sequence with plasmids encoding empty vector, 2A* or 2A, were grown overnight in selective media. Proteins were extracted and 10-20 µg was used for luciferase measurement of each sample. Data obtained were processed to find averages, values of sense codons were normalised to 100%, and samples were calculated accordingly. Values of the active 2A in each strain with a stop codon were statistically compared to the relevant value of the isogenic wild type. * refers to significance $p \leq 0.01$.

4.6.5 Mutations in the M domain of eRF1 show elevated read-through of stop codons when 2A-containing mRNA is expressed

The two eRF1 variants in the M domain that affect the 2A reaction (P174Q and I222S) were tested using the dual-luciferase reporters. It was observed (figure 4.15) that expression of 2A-containing mRNA in a strain expressing the I222S variant of eRF1 led to a significant increase in stop codon read-through with all codons tested here. This result is in accordance with our previous observation with immunoprecipitation, where we found that this mutation caused a significant inhibition in the 2A reaction. On the other hand, the P174Q mutation, which is located 6 amino acids before the universally conserved GGQ motif (180-182 *S. cerevisiae* numbering), caused a significant elevation of read-through only of the UAGC codon. However, these results confirm the role of eRF1 in the 2A reaction, which depletes/deactivates eRF1, leaving the ribosome undergoing defective termination.

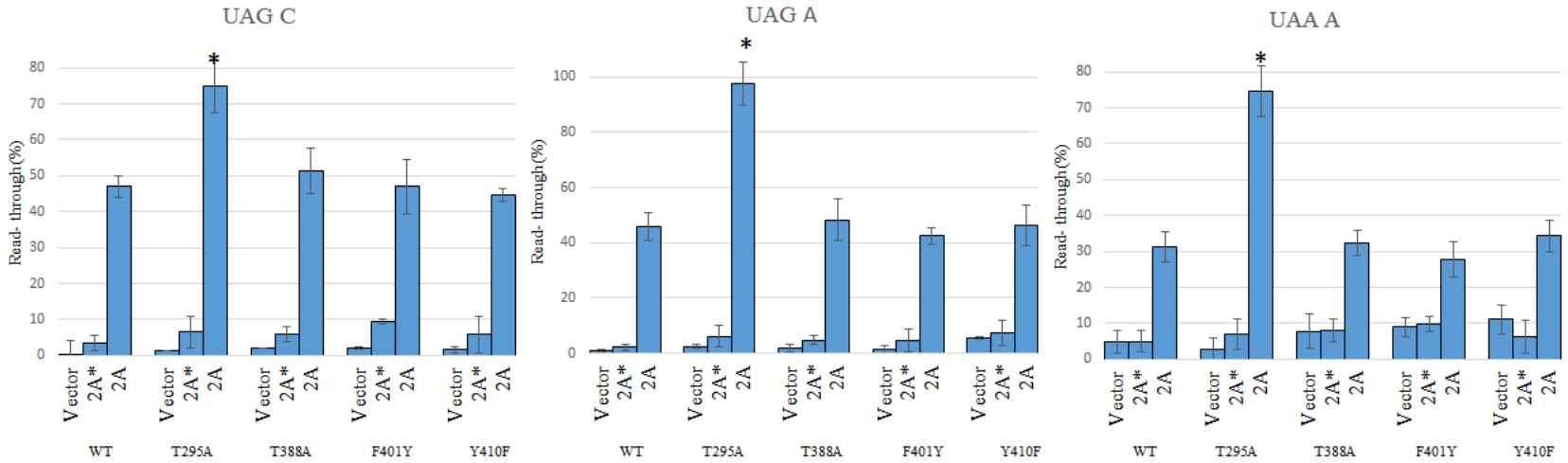

Figure 4.16: Stop codon read-through in strains expressing 2A peptide, with mutant eRF1 in the C domain as the only version of the protein.

Cultures of the indicated strains, expressing dual luciferase reporter with sense, UAG or UAA stop codons followed by cytosine or adenine as a context sequence with plasmids encoding empty vector, 2A* or 2A, were grown overnight in selective media. Proteins were extracted and 10-20 µg was used for luciferase measurement of each sample. Data obtained were processed to find averages, values of sense codons were normalised to 100%, and samples were calculated accordingly. Values of the active 2A in each strain with a stop codon were statistically compared to the relevant value of the isogenic wild type. * refers to significance $p \leq 0.01$.

4.6.6 The effect of 2A overexpression on stop codon read-through in strains expressing eRF1 with mutations in the C domain

Here, the same mutations examined for the effect on the 2A reaction were tested for their effect on stop codon inhibition. The overexpression of 2A-encoding mRNA in these strains revealed an increased read-through in cells expressing the T295A mutation only (figure 4.16), whereas the other mutations analysed in this domain have no effect on read-through. Given that the T295 is one of the key residues in eRF1 for its interaction with eRF3 in the ternary complex. The variation in the importance of eRF1 residues between canonical termination and 2A confirms that the conformation of eRF1 in the ribosome translating 2A sequence is different from that known in canonical translation/termination.

4.6.7 Expression of 2A-encoding mRNA leads to increased stop codon read-through in strains with reduced eRF3 activity

Strains which were analysed for the effect of alterations to Sup35 activity on the 2A reaction (section 4.3.2) were examined to determine whether the expression of the 2A-containing mRNA affected read-through of stop codons in *trans*. As with strains containing alterations to Sup45, dual-luciferase reporters were used with the same stop codons/contexts.

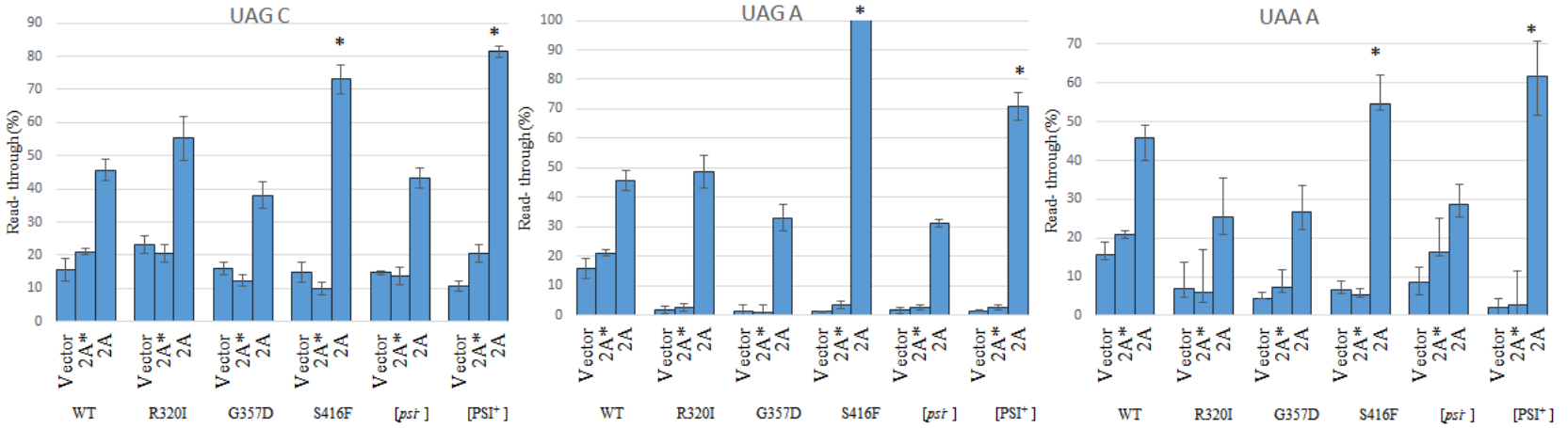

Figure 4.17: Stop codon read-through in *trans* in strains with mutant eRF3 while expressing 2A peptide.

Cultures of the indicated strains, expressing dual luciferase reporter with sense, UAG or UAA stop codons followed by cytosine or adenine as a context sequence with plasmids encoding empty vector, 2A* or 2A, were grown overnight in selective media. Proteins were extracted and 10-20 µg was used for luciferase measurement of each sample. Data obtained were processed to find averages, values of sense codons were normalised to 100%, and samples were calculated accordingly. Values of the active 2A in each strain with a stop codon were statistically compared to the relevant value of the isogenic wild type. * refers to significance $p \leq 0.01$.

As seen in figure 4.17, a range of mutations in eRF3, particularly in the GTP-binding domain, along with the [PSI$^+$] and [*psi*$^-$], were tested here for the tendency to read-through stop codons, while overexpressing 2A-encoding mRNA. The results of the dual luciferase assay are in accordance with what we have found in regards to the effect of these mutations of eRF3 on the 2A reaction (see section 4.3.2). In all stop codons tested, the strain expressing S416F mutation in eRF3 showed a significant increase in read-through, over that seen in the wild type strain, whereas, values of other mutations were statistically similar to the wild type strain. The [PSI$^+$] strain, in which the available eRF3 is significantly reduced through formation of aggregates of the protein, also revealed a significant increase in read-through of stop codons, compared to the equivalent [*psi*$^-$] strain (figure 4.17).

4.7 *In vitro* analysis of effects of some mutations in eRF1 on 2A reaction

To complement the *in vivo* biochemical analysis of the 2A reaction, another biochemical analysis was applied through an *in vitro* translation experiments, which were carried out using translation-competent extract from yeast. Strains chosen were ones that had revealed defects in the 2A reaction *in vivo* (I32F, P38L, L49A, T55A, R62A, P174Q and [PSI^+]). These were assembled into *in vitro* translation reactions (Materials and Methods) with [^{35}S]-labelled methionine and capped mRNA transcripts encoding αF, followed by 2A/2A*, and GFP.

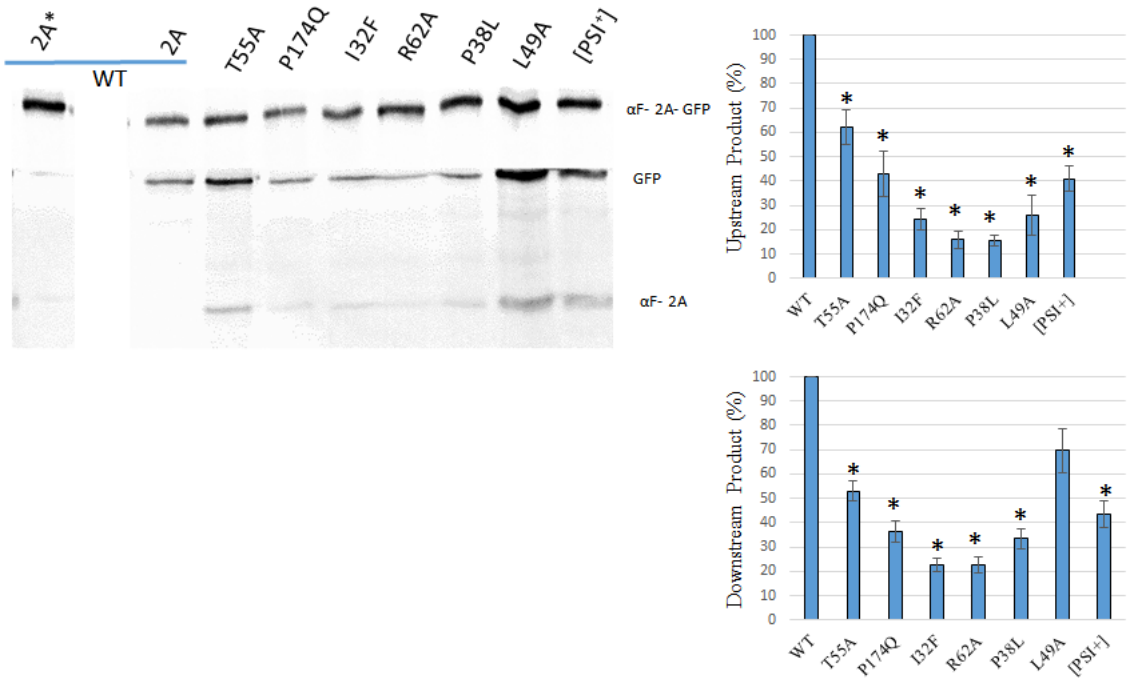

Figure 4.18: *In vitro* analysis of the 2A reaction.

In vitro translation reactions were assembled using extracts from wild type yeast cells expressing the indicated Sup45 variant or [PSI$^+$] cells with mRNA transcripts encoding 2A/2A* reporters. Reactions were resolved onto SDS-PAGE, gels were fixed and dried, and exposed to exchange screen. Gels from 3 independent experiments were quantified, values normalised according to the number of methionine residues in the proteins and the amount of upstream and downstream fragments of the 2A reaction was calculated and expressed as a fraction of the amount in the reaction assembled with the wild type extract set to 100%. * Significance at 0.01.

Data obtained from the *in vitro* translation experiments confirmed those obtained from *in vivo* analysis. Thus, *in vitro* translation of the 2A-containing reporter revealed a significant decrease in upstream/downstream products in extracts from all the mutants tested here. I32F, P38L, (located beyond the GTS motif) and R62A (just downstream to the NIKS motif), had the most impact on the 2A reaction representing 25, 18 and 15% respectively of the WT value of the upstream product, and 22, 23 and 34% respectively in the downstream products. Two observations are inconsistent here with the *in vivo* experiments undergone by immunoprecipitation; first, the T55A mutation caused a significant decrease here in the upstream product whereas it did not change the ratio of this product in the immunoprecipitation experiments (see figure 4.2). In addition, the L49A mutation caused insignificant decrease in the downstream product here, whereas it was significant in the immunoprecipitation. It is unclear as to why these differences were seen, though the difference in conditions between the *in vivo* and *in vitro* experiments may impact on the outcome of the reaction. The experiments do though confirm that the mutations in eRF1 and the [PSI$^+$] state impact on the 2A reaction.

4.8 Discussion

Much research has been carried out using 2A peptides and the 2A reaction. However, relatively few studies have investigated the 2A reaction mechanism. The main focus of this chapter is to investigate the involvement of a broad range of residues in the eRF proteins in the 2A reaction. In Chapter 3, it was demonstrated that limited activity of eRF1 and eRF3 in cells inhibits the 2A activity below the wild type strains. This led to the conclusion that the initial step in the 2A reaction is a non-canonical termination. According to data presented in this chapter, a variety of residues, in different motifs/domains, of eRF1 are essential for cells of *S. cerevisiae* to maintain a normal 2A reaction. Further, alteration to S416 in eRF3 also impaired the 2A reaction. Data obtained in this chapter are summarised in Table 4.2.

Mutation	Location	**Biochemical Effect**					
		2A Reaction Products		Stop Codon Read-through in *trans*			Growth Doubling Time
		Upstream	Downstream	UAGC	UAGA	UAAA	
S30P	N (GTS)	↓	↓	NS	NS	NS	↓
I32F	N	↓	↓	↑	↑	↑	NS
P38L	N	↓	↓	↑	↑	↑	NS
L49A	N	↓	↓	↑	↑	↑	NS
E52A	N	↓	↑	NS	NS	NS	NS
T55A	N (TASNIKS)	NS	↓	↑	↑	↑	NS
N58A	N (TASNIKS)	NS	NS	NS	NS	NS	NS
S61A	N (TASNIKS)	NS	NS	NS	NS	NS	NS
R62A	N	↓	↓	↑	↑	↑	NS
V68A	N	NS	NS	↑	NS	NS	NS
V107D	N	↑	↑	NS	NS	NS	NS

P174Q	N	↓	↓	↑	NS	NS	NS
I222S	M	↓	↓	↑	↑	↑	↓
T295A	C	↓	↓	↑	↑	↑	↓
T388A	C	NS	NS	NS	NS	NS	NS
F401Y	C	↓	↓	NS	NS	NS	NS
Y410F	C	NS	NS	NS	NS	NS	↓
R320I	G	NS	NS	NS	NS	NS	↓
G357D	G	NS	NS	NS	NS	NS	NS
S416F	G	↓	↓	↑	↑	↑	NS
[PSI^+]		↓	↓	↑	↑	↑	↓
[*psi*$^-$]		NS	NS	NS	NS	NS	NS

Table 4.2: Mutations in Sup45 and Sup35 used in this study and their effects on the 2A reaction.

The point mutation analysis undertaken in this study is summarised in the table above. The table shows mutations in each Sup45 and Sup35 with their locations in the protein structure in addition to the functional motif/loop. The different effects of such mutation are presented either by ↑, stands for elevation, ↓, stands for reduction or NS, stands for No Significance. It is also presented in the table the observed effects in terms of stop codon inhibition in addition, the different effects of such mutation studied on the level of growth of strains expressing such *sup45/sup35* variants.

eRF1 triggers peptidyl-tRNA hydrolysis, thereby releasing the nascent peptide chain from the ribosome. A wealth of biochemical and genetic studies have led to recognition of the importance of three highly conserved motifs in the N domain, GTS, NIKS and YxCxxxF, for stop codon recognition. A universally conserved tripeptide, GGQ is located in the M domain of eRF1, and is critical for peptidyl-tRNA hydrolysis in the PTC (Zhuravleva *et al.*, 2007). It has been suggested that in termination complexes, uridine in the first position of the stop codon contacts the NIKS motif (Chavatte *et al.*, 2002). On the other hand, purines in the second and third positions contact the GTS and YxCxxxF motifs (Bulygin *et al.*, 2011). Studies also referred to different conformational changes adopted by the N domain to recognize signals in the ribosomal A site (Wong *et al.*, 2012). Recent cryo-electron microscopy studies have confirmed the direct role of the three implicated motifs in the N domain in deciphering the stop codon (Muhs *et al.*, 2015). Structurally, differences in positioning of the GGQ motif have been found relative to the PTC, GGQ in the pre-termination complex is divergent from the PTC,

which indicates inactivity of eRF1 in this stage (Matheisl *et al.*, 2015). Conversely, complexes in the post-termination stage impose shifting of the M domain, positioning the GGQ tripeptide directly in the PTC, with vicinity to the 3'-terminus of the P site tRNA (Preis *et al.*, 2014).

4.8.1 eRF1 is recruited to the 2A reaction

The analysis of a wide range of eRF1 mutants described in this chapter confirms the role of this protein in the 2A reaction, and provides information about residues involved in maintaining the normal 2A reaction in *S. cerevisiae* cells. Specific residues located around both GTS and TASNIKS motifs of eRF1 are important in this context. The S30P mutation, which is located in the core of the GTS motif, as well as the I32F and P38L mutations all adversely affect the 2A reaction, as assessed using the HA-αF-2A-GFP reporter. Experiments revealed a significant reduction of the 2A activity, characterized by reducing amounts of both upstream and downstream products, indicating that in cells expressing these variants of Sup45, a higher ratio of ribosomes read-through the 2A peptide to generate the full-length protein of the 2A reporter. GTS motif/loop (28-30 of *S. cerevisiae* numbering) has an essential role in stop codon recognition, in cases of canonical translation, through decoding purines in the second and third positions, and probably the first context base just after the stop codon (Conard *et al.*, 2012). This is carried out through a specific conformation of eRF1 in the ribosomal A site, where the GTS motif aids the YxCxxxF motif, in addition to the E55 residue (Brown *et al.*, 2015). Despite the different situation in the case of the 2A reaction, where no stop codon is occupying the ribosomal A site, it seems that the same residues specified to decode stop codons are involved in maintaining the 2A reaction. Using NMR techniques, studies have revealed different conformations undergone by the GTS loop, which impacts specificity to bind stop codons (Wong *et al.*, 2012). Noticeably, GTS loop in termination lies in vicinity of guanine residues in the second or third position of the stop codon, moreover, mutagenesis of human eRF1 identified some essential residues for termination (Kryuchkova *et al.*, 2013). This study highlighted S33 (equivalent to S30 in yeast) as the main residue involved in guanine decoding. It has been reported through NMR analysis of several mutations in the N domain of eRF1 that mutations of the S30 residue may change the local conformation in the eRF1 N domain, which confirms the importance of the shape of a serine residue at this position. Mutagenesis of the S30 residue causes shifting in other residues across different motifs, one of the affected residues is I32, which is located in the secondary sequence of the eRF1 protein (Blanchet *et al.*, 2015). Thus, this effect may also include the P38 residue, which inhibited the 2A reaction as a result of mutating it to leucine.

The next residues which have been examined locate in the TASNIKS motif, these include L49, T55, N58, S61, R62 and V68. Only N58 and S61 lie in the core NIKS motif, and notably do not influence the 2A activity. As described by Ito and his collaborators, the NIKS loop normally functions to interact with the first uracil of the stop codon through the anti-codon mimicry (Ito *et al.*, 2002). On the other hand, our data reveal that residues just before or after the NIKS loop have shown a significant inhibition of the 2A reaction, these include T55 and R62, which may indicate a slightly different conformation of the protein, undergone in the ribosome in the absence of a stop codon in the ribosomal A site. R62 is located at the edge of the pocket 1 cavity in the N domain of the eRF1 tertiary structure, and it is presumed to perform a role in terms of the protein structure and activity. Furthermore, arginine is a positively charged amino acid, thus there is a possibility to interact mainly with mRNA via the negatively charged phosphate group, but probably interaction occurs with the rRNA as well (Blanchet *et al.*, 2015). It has been demonstrated that threonine 55, together with alanine 56 and serine 57, which imply to the first three residues in TASNIKS motif, may function as an anticodon peptide. Furthermore, TAS, along with the NIKS region, forms a flexible sequence, which can adopt a tight or loose conformation to decode the stop codon, specifically, the second nucleotide of the UGA stop codon, whereas the asparagine 58 has been found to assume the recognition of the third nucleotide (Nakamura *et al.*, 2000; Muramatsu *et al.*, 2001). Finally, a study found that TASNIKS loop undergoes a function to promote the right conformation of eRF3 in the TC, in addition, this loop may also trigger the GTP hydrolysis (Fan-Minogue *et al.*, 2008). It has been found that the valine in position 68 of *S. cerevisiae* eRF1 functions in the recognition of the adenine residues in stop codons structure (Fan-Minogue *et al.*, 2008; Blanchet *et al.*, 2015).

Furthermore, structural studies have revealed that the distance between the Y-C-F and the GGQ motif is ~80 Å, which is similar to the distance between the anticodon and the CCA end of the tRNA, thus it can easily be located in vicinity with the stop codon when the GGQ is placed at the PTC close to the CCA end of the peptidyl-tRNA (Inagaki *et al.*, 2002). Mutations in the Y-C-F motif abolish the recognition of the three known stop codons (Seit-Nebi *et al.*, 2002). However, we were not able to carry out assessment for such mutation in this motif during the course of this study, as none could maintain cell viability (Merritt *et al.*, 2010).

The eRF1 structure resembles the letter ‘Y’, which in part mimics the ‘L’- shaped of the tRNA moiety, where the M domain of eRF1 simulates the tRNA acceptor stem (Nissen *et al.*, 2000). Thus, it is suggested that the GGQ function is equivalent to the 3’-CCA end of tRNA (Ivanova *et al.*, 2007). The amino acid sequence between leucine 173 and alanine 207 in *S. cerevisiae* eRF1 is strictly conserved and contains the invariant GGQ motif. Apart from this motif, some nearby residues are also conserved among species, these residues include proline 174 and serine

183, which are both located in the loop region. The conserved residues in the M domain may suggest a functional role through forming a surface for the eRF1 to bind mRNA (Ivanova *et al.*, 2007). The particular geometry of residues 177-184 region corresponds to a loop that connects secondary structures (Song *et al.*, 2000). The molecular mechanism of the peptide release in the PTC has not been fully uncovered. However, it has been found that there would be a process of water coordination to the GGQ. The glutamine 182 side chain acts as a coordinator for the water molecule, which performs nucleophilic attack on the peptidyl-tRNA bond. The neighbouring residues, including the conserved glycines 180 and 181, enhance the contact with the phosphate group of both rRNA and the acceptor stem of the ribosomal P site tRNA (Song *et al.*, 2000). Moreover, it has been reported that mutating isoleucine 222 of the eRF1 to serine yields a significant inactivation of the functional protein. This particular mutation namely, *sup45-2* in *S. cerevisiae*, reduces the proteins ability to bind the ribosome as well as the ability to trigger the peptide chain release (Stansfield *et al.*, 1997).

As stated above, eRF1 binds to eRF3 to form a complex which binds the ribosome to trigger termination. The binding of both eRFs is carried out through their C domains thus changing residues in eRF1's C domain may alter the termination efficiency, and concomitantly the 2A reaction activity. In this context, we tested several mutations in the eRF1's C domain: these include: T295A, T388A, F401Y and Y410F, using immunoprecipitation of both upstream and downstream products of the 2A reporter. We found that both T295A and F401Y have reduced the smaller products (upstream or downstream), indicating more ribosomes undertaking read-through across the C terminus of the 2A peptide. Studies have demonstrated that deletion of 6-19 amino acids from the eRF1's C domain in *S. cerevisiae*, and 17 amino acids of *S. pombe*, results in a complete loss of binding capacity to eRF3 (Ito *et al.*, 1998; Eurwilaichitr *et al.*, 1999). It has been identified that two regions in each eRF1 and 3 are the key regions for mutual binding, these include residues 281-305 and 411-415 (GILRY) in eRF1, whereas residues 478-530 and 628-637 represent the binding interface in eRF3 (Merkulova *et al.*, 1999b). A computer modelling study suggested that two residues threonine 295 and tyrosine 410 are exposed to be the surface of the Sup45 protein molecule. Furthermore, among amino acids in this region, tyrosine residue can interact with residues on the Sup35 surface since it contains hydroxyl group (Akhmaloka *et al.*, 2008). A motif has been distinguished, termed AMLRY, which lies at positions 406-410 of yeast eRF1. In general, mutations at the C terminus of eRF1 do not significantly affect the overall function as these residues are stabilized by water solvent. However, substitution of Y410 alters the secondary structure of residues 343-349, despite the relative long distance between these two regions. This might be attributed to the capability of the proton in the tyrosine molecule to be readily ionized, in addition to the functional group and electrons

contained in the tyrosine molecule. An exception, substitution of tyrosine to phenylalanine does not disturb the amino acids structure in the stated region (Akhmaloka *et al.*, 2008).

4.8.2 Effect of eRF3 on the 2A reaction

The effect of three mutations of the eRF3 protein, R320I, G357D and S416F, on the 2A reaction was tested. All lie in the functional G domain region of eRF3, however only S416F revealed a significant inhibition of the 2A reaction, reducing the amount of both upstream and downstream fragments generated. S416 is located in the hydrophobic pocket (residues 385-429), which holds the guanine base of G-nucleotides (Andersen *et al.*, 2001). Thus, mutating serine 416 to phenylalanine clearly impairs the GTPase function of this domain, and hence the termination efficiency while translating 2A peptide. However, mutation of glycine 357 and arginine 320, which assume a role in the GTPase activity of eRF3 (Salas-Marco and Bedwell, 2004), did not significantly alter the reaction outcomes. The change in the eRF1 conformation in the ribosome, caused by the presence of the Pro 19 codon rather than a stop codon in the ribosomal A site, may result in a change in the normal conformation of eRF3, seen in the canonical termination, for better binding to eRF1, thus, change in specific residues for eRF3 binding are expected in the case of 2A. This conclusion is based on the reduction of the 2A activity caused by mutating threonine 295 (section 4.3.2), one of the key residues in the eRF1 binding site to the eRF3. eRF3 contributes in peptide release by increasing the rate ~5 times over the basal rate (Eyler *et al.*, 2013). This effect is possibly due to the role of eRF3-GTP in mediating the proper conformation of eRF1 in the TC. This confirms that eRF3 is also recruited to the ribosome in the presence of 2A peptide.

4.8.3 Overexpression of 2A inhibits the growth of cells expressing some eRF1/3 variants

The growth doubling time of strains with some mutations in eRF1/3 was inhibited by expression of a 2A (but not 2A*) -encoding mRNA. These data are in accordance with what was obtained from the same strains when we exposed them to the pulse labelling. These data are also consistent with the results of Doronina *et al.*, (2008). However, the former work was carried out only on strains with reduced eRF1/3 activity (*sup45-42*), or [PSI^+] respectively, but they confirmed that 2A peptides from other viruses such as *Thosea asigna* virus and Theiler's murine encephalitis virus are also able to inhibit the growth. Strains expressing some Sup45 variants showed a significant reduction of the 2A reaction, detected by immunoprecipitation, but no effect on the growth, for example, P38L, P174Q and L49A strains. On the other hand, the strain expressing the Y410F mutation of eRF1 has shown a significant increase in the

doubling time while expressing both active and inactive 2A peptides, which might be attributed to a toxicity in the strain background, although this curve was repeated several times to confirm the current result. This is inconsistent with Doronina *et al* (2008), who suggested that the growth inhibition is not "*sup45* allele specific". On the other hand, the same study found that the expression of 2A-containing polyprotein from a "moderate strength promoter" such as *PHO5* does not alter the growth of strains with reduced eRF activity. Those findings, and data from the current study, suggest that the expression of 2A is toxic for cells, especially in presence of certain mutations in the ribosomal termination factors.

4.8.4 Stop codon read-through in cells expressing 2A and undergoing termination with eRF1/3 variants

To investigate the effect of 2A expression on termination efficiency in *S. cerevisiae* cells with mutant eRF1, plasmids encoding dual-luciferase reporters with different stop codons and/ or context sequences were used. These effects have been investigated on UAA and UAG stop codons, with adenine as a context sequence for the former and adenine or cytosine for the last stop codon. It has been reported that there is an increase in stop codon read-through in cells expressing 2A (Doronina *et al.*, 2008), however the former study did not consider mutations in the eRFs. In the current study, we observed a highly significant stop codon suppression, expressed by the ratio of ribosomes undergoing read-through over different stop codons. Excluding the S30P-*sup45*, our results are quite consistent with what we observed from the immunoprecipitation experiments; all strains have shown different levels of read-through compared to the strain expressing isogenic wild type *SUP45*. It has already been demonstrated that mutations in eRF1 induce stop codon read-through. To confirm that the stop codon read-through results from the 2A peptide expressed in yeast cells, we used different controls for each sample, which is the 2A*, this an inactive version of 2A carries P17A mutation, which inactivates the peptide. Therefore, we can attribute the increase in the read-through ratio to the effect of 2A expression in cells. Our analysis has revealed a high base line read-through being promoted by UAG C, which is consistent with other studies (Rakwalska and Rospert, 2004). Thus, this stop codon is an efficient and sensitive indicator of termination defects, with almost no effect of the context sequence in terms of measuring the read-through ratio in the strain expressing isogenic wild type eRF1. The stop codon read-through was lower with the UAA A stop codon, compared to the other stop codons. This was revealed by the wild type basal read-through however, it is still above the normal value, such an effect might be attributed to the presence of a large pool of 2A peptides. The same results, in terms of increased stop codon read-through, have been detected in strains expressing *sup35* alleles, with the most significant

elevation undergone by the [PSI$^+$] strain. The increased basal level of stop codon inhibition is unlikely to be eRF-specific, but instead, 2A-induced effect, given that the wild type eRFs are also increasing the stop codon inhibition while expressing active 2A peptide compared to the inactive 2A. The increased stop codon read-through, in *trans*, in the presence of multiple copies of 2A, suggests that 2A may deplete termination factors in the cytosol, and these factors may remain bound to the ribosome at 2A, which reduces the availability of these factors for canonical termination. We have confirmed that 2A reaction is mainly a termination event, driven by eRF1/3, which are recruited, somehow, to the ribosome. Although mutations in eRF1/3 produce a significant ratio of full-length product of the 2A reporter, Ribosome could be stalled at the 3' end of mRNA, and ribosomal subunits might stay bound to each other, with no proper release of eRFs from the ribosome. Our findings, when reduced Rli1 abundance (*DAmP-rli1*) did not affect the reaction, support this theory. Thus, a high ratio of eRF1 will not be recycled and hence it is functionally inactive to drive successful termination and decode stop codons. It has been found that 2A peptide has no effect on cellular eRFs levels (Doronina *et al.*, 2008). Furthermore, it has been found that the reduction in Sup45 level in *S. cerevisiae* is the main reason for increased stop codon read-through (Salas-Marco and Bedwell, 2004).

SUMMARY

Data presented in this chapter confirm results presented in Chapter 3, that both eRF1 and eRF3 are essential to drive the 2A reaction. The analysis of a broad range of mutations in eRF1, which covers the most key regions for the protein activity, revealed a clear involvement of some residues of the protein in the 2A reaction. However, this involvement is likely to be different from that undergone in canonical termination, in the presence of a stop codon in the ribosomal A site. A modification/shifting in the normal conformation of eRF1 is proposed here for proper accommodation of eRF1 in the ribosome, and thereby successful releasing of the nascent peptide chain from the PTC. Given that eRF3 assumes an essential role for eRF1 function, in terms of driving translation, eRF3 seems to be an important counterpart of the 2A reaction, through its GTPase activity. These main results have been confirmed by 1) measuring growth doubling time of strains expressing 2A and driving termination using s*up45* alleles, and 2) assessing the propensity of the former strains to suppress stop codons, and thereby promoting read-through. Although the outcomes of growth rates are not fully consistent with the results of the main biochemical experiments, the observations regarding to the read-through assay are found to be in accordance with the biochemical analysis, undergone by cell pulse labelling and immunoprecipitation. To sum up, changing the availability, activity, or even replacing particular key residues of eRF1/3 results in a significant inhibition of the 2A reaction,

promoting the ribosome to read-through the 2A peptide coding sequence, and pauses at the 3’ end of the ORF.

5 CHAPTER 5: DEVELOPING NOVEL REPORTERS FOR STUDYING 2A ACTIVITY

5.1 Introduction

Polyprotein reporters that have been used thus far to study the activity of the 2A peptide lie in two categories: 1) reporters such as αF-2A-GFP, which has previously been described in Chapters 2, 3 and 4. Analysis of translation products of this reporter requires at a minimum western blotting and for accurate determination of activity pulse-labelling, immunoprecipitation and quantification are required. 2) Reporters such as Ub-R-2A-ADE2 (Sharma *et al.*, 2012). In this reporter, the 2A reaction releases Ade2 protein from degradation imposed by an N-terminal arginine (exposed after proteolytic cleavage of the translation product). Activity is then scored on the basis of yeast colony pigmentation: in a genetically *ade2* background, which yields red cells on media containing limited adenine, this produces cells of decreasing colouration as 2A activity increases. Although the adenine reporter is easy and quick to implement, it relies on several cellular metabolic pathways and enzymes, both to generate the red pigment and to process the Ub-R-2A-Ade2 protein. Indeed, attempts to use this reporter in screens for cellular mutations that affect the 2A reaction have thus far only identified false-positives (Sharma *et al.*, 2012). In this chapter, alternative strategies were considered, aiming to generate reporters which are less reliant on cellular pathways for function. Two reporters are described, based on the use of DHFR or GFP.

5.2 Dihydrofolate reductase (DHFR) reporter

A dihydrofolate reductase (DHFR)-based reporter of the 2A reaction was generated by placing sequences coding for 2A within DHFR at the position used to split the protein into the 2 fragments (termed F1,2 and F3), used in the protein fragment complementation assay (PCA) (Tarassov *et al.*, 2008). With the knowledge that the DHFR protein split at this position is unable to form a functional enzyme, and that fusion of sequences to the protein at this point does not leave the fragments unable to form a functional enzyme when they are brought together, made this a strong candidate for a suitable position to place 2A. Activity of 2A should separate the enzyme into 2 inactive fragments (F1,2 and F3), whereas any full-length protein produced, e.g. through the effect of either a mutation within 2A that inactivates it or a trans-acting mutation that leads to the 2A reaction being inefficient, would hopefully be active.

DHFR activity can be scored using Methotrexate (Mtx). Mtx is a competitive inhibitor of DHFR, a key enzyme in folate metabolism that catalyses synthesis of tetrahydrofolate, which is required for *de novo* synthesis of thymidine (Miran *et al.*, 2017). The mouse DHFR used to

generate the new reporter exhibits increased resistance to Mtx (10,000 times less sensitive to Mtx than the endogenous yeast enzyme), due to specific mutations within its sequence. At the levels of methotrexate used to test constructs containing this DHFR sequence, the yeast enzyme is completely inhibited.

The F1,2 and F3 fragments of the DHFR gene were amplified by PCR from yeast strains containing plasmids which included sequences encoding these (kind gift of Dr P. Banks, Newcastle University). These were cloned with the two parts of DHFR separated by appropriate restriction sites to allow subsequent cloning of the 2A-coding sequences (figure 5.1). Two induced mutations in the DHFR coding sequence were obtained; L23Y and F32S, these were confirmed via sequencing the constructs which have been cloned.

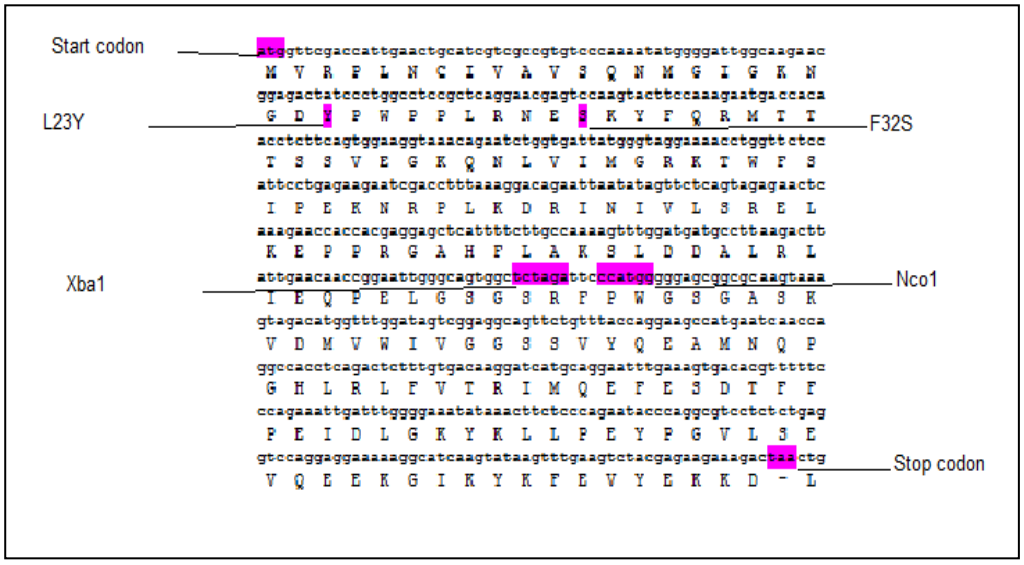

Figure 5.1: The sequence of the DHFR used to develop the 2A reporter.

The sequence of pDHFR obtained after cloning. The mutations that confer increased resistance to methotrexate (L23Y and F32S) and restriction sites into which 2A/2A* were subsequently cloned are indicated.

Three plasmids were generated, one containing the full-length DHFR coding sequence (termed pDHFR), and two with FMDV 2A sequences inserted between the F1,2 and F3 fragments. Of these, one contained the P17A mutation that inactivates 2A, termed 2A*. Wild type yeast strains (JDY2, 4 and 895) were transformed with plasmids pDHFR, pDHFR-2A, and pDHFR-2A* and then screened for resistance to Mtx by spotting serial dilutions onto plates containing different concentrations, 50, 100 and 200 µg/ml of Mtx (data not shown). It was found that the highest concentration (200 µg/ml) is the optimal concentration for testing growth of yeast in the PCA using Mtx selection (Tarassov *et al.*, 2008).

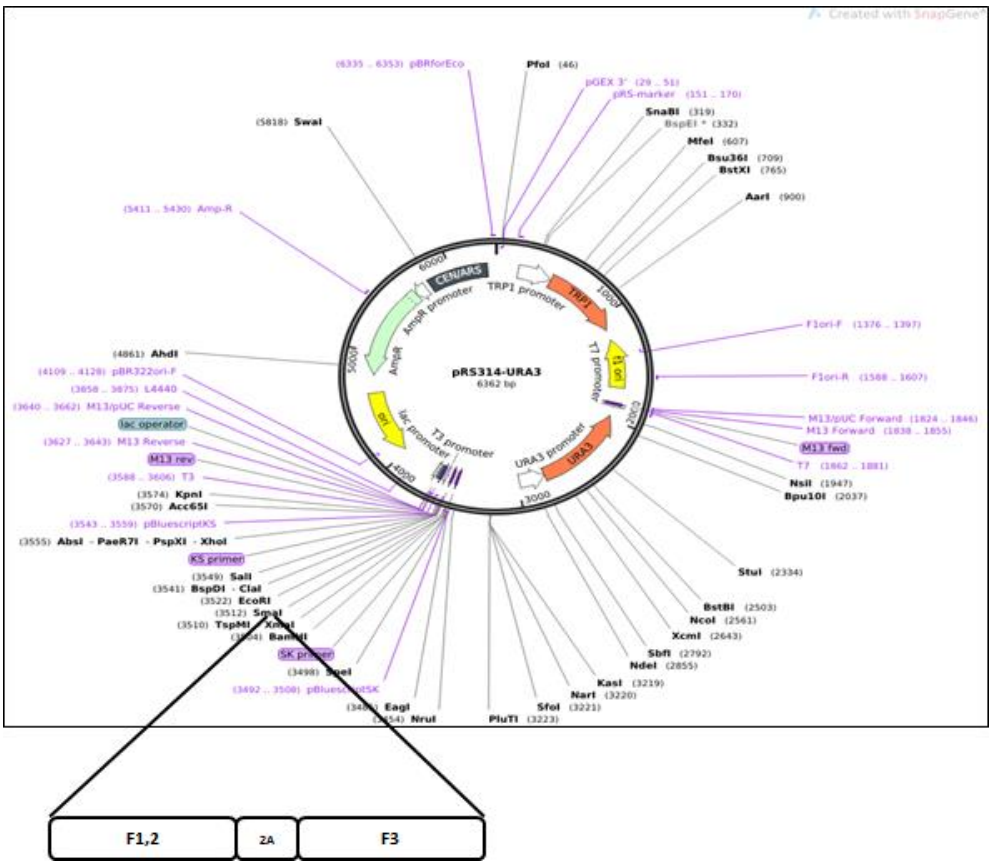

Figure 5.2: Scheme of the DHFR reporter cloning strategies.

The PCR-5' and 3' fragments of the DHFR open reading frame (coding for the F1,2 and F3 fragments of the protein) were cloned into a pRS314 vector using the restriction enzymes indicated. The whole construct was basically cloned using single digestion strategy into a SmaI site. This generated pDHFR, containing a linker of NcoI, AgeI and XbaI restriction sites. Subsequently, 2A or 2A* fragments were cloned between the NcoI and AgeI sites.

Testing of the constructs in wild type strains of *S. cerevisiae* revealed no significant differences in the growth rates of cells containing the four plasmids (2A and 2A* cloned between F1,2 and F3 of DHFR) on Mtx plates compared to the controls (empty vector and wild type DHFR) in wild type strains used (Data not shown). Additionally, growth of cells transformed with these plasmids in liquid media was monitored and it was found that they all grew similarly in the presence of Mtx, with no growth inhibition observed due to the presence of active 2A peptide in the middle of DHFR (data not shown).

The most concerning aspect to this experiment is that the negative control yeast strains, which does not express mouse DHFR, grew similarly to those expressing DHFR on Mtx plates. Several tests were carried out to determine the reason for this. It was possible that there may be an interaction between Mtx and one or more components in the media, thus preventing it from being available for uptake into the yeast cells. Several experiments were undertaken to try to determine if media components were to blame, and specifically the agar used since it was

highlighted by Professor D. Lydall (Newcastle University) that some agars may inhibit the activity of Mtx, whereas noble agar does not. However, cell growth on plates containing noble agar was similar to that on plates containing the agar that was typically used (Formedium) (data not shown). A second possibility was that the Mtx used had low activity/was inactive. To investigate the activity of the Mtx, and also to see whether the strain background used in the experiments above could be more resistant to Mtx than the strain used by the Lydall laboratory, control yeast strains were obtained (Lydall laboratory), expressing the F1,2 (Nourseothricin, NAT resistant strain) and F3 (Hygromycine B, HYG resistant strain) fragments of DHFR fused to Sod1, which has a strong interaction due to the dimerization of Sod1 (Shibasaki *et al.*, 2008). These were mated and the resulting diploid tested alongside the haploid strains on Mtx plates. The two strains, expressing either F1,2 or F3 fragments, were used as controls along with a diploid one, all were tested on plates containing appropriate antibiotics (100 µg/ml w/v NAT and 200 µg/ml w/v HYG). The haploid strains did not grow on Mtx screens; in contrast, the diploid grew due to the expression of intact DHFR (figure 5.3).

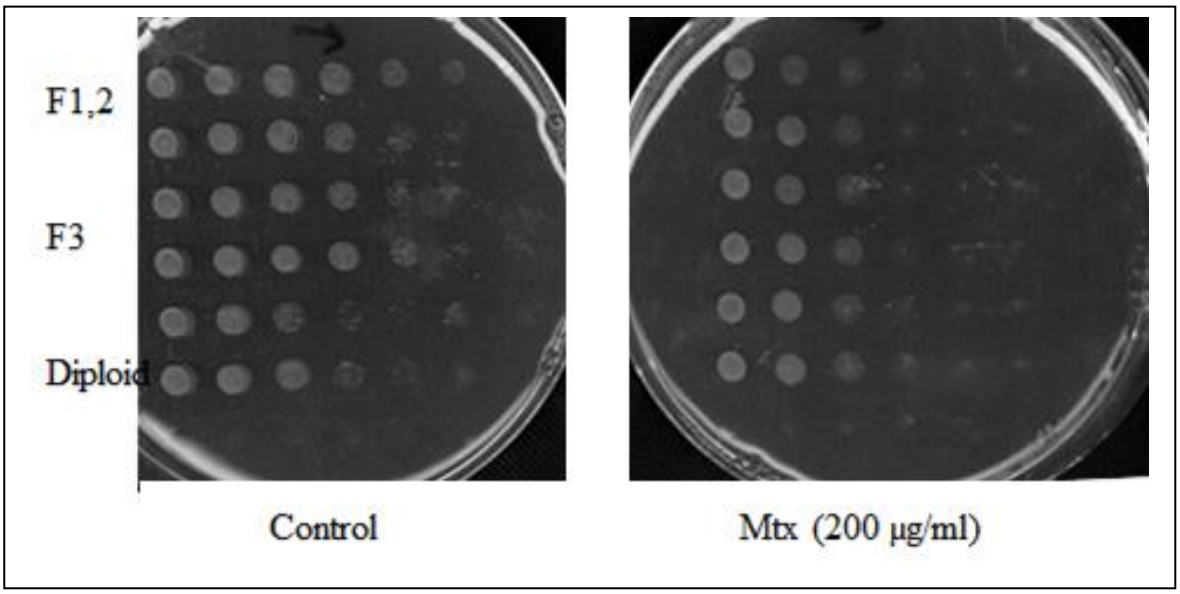

Figure 5.3: Testing the activity of Mtx using control strains.

Haploid strains expressing F1,2 fragment (NAT resistant) and F3 fragment (HYG resistant) of DHFR from centromeric plasmids (JDY1299 and 1300 respectively) were mated to generate a diploid strain (NAT and HYG resistant), which expresses the intact DHFR gene. Serial dilutions from each strain were prepared and spotted on plates containing Mtx (200 µg/ml) against control plates (-Mtx), and incubated at 30 ºC. Rows are duplicated for each strain.

The growth rate of the diploid strain was not very fast on Mtx-containing plates, but the same growth rate was observed on the control plate for the same strain, whereas the other two strains, which do not express an intact DHFR showed an inhibition in terms of their growth on Mtx.

This suggests that replacing the Mtx with another one, supplied by Sigma, was successful to resolve the problem.

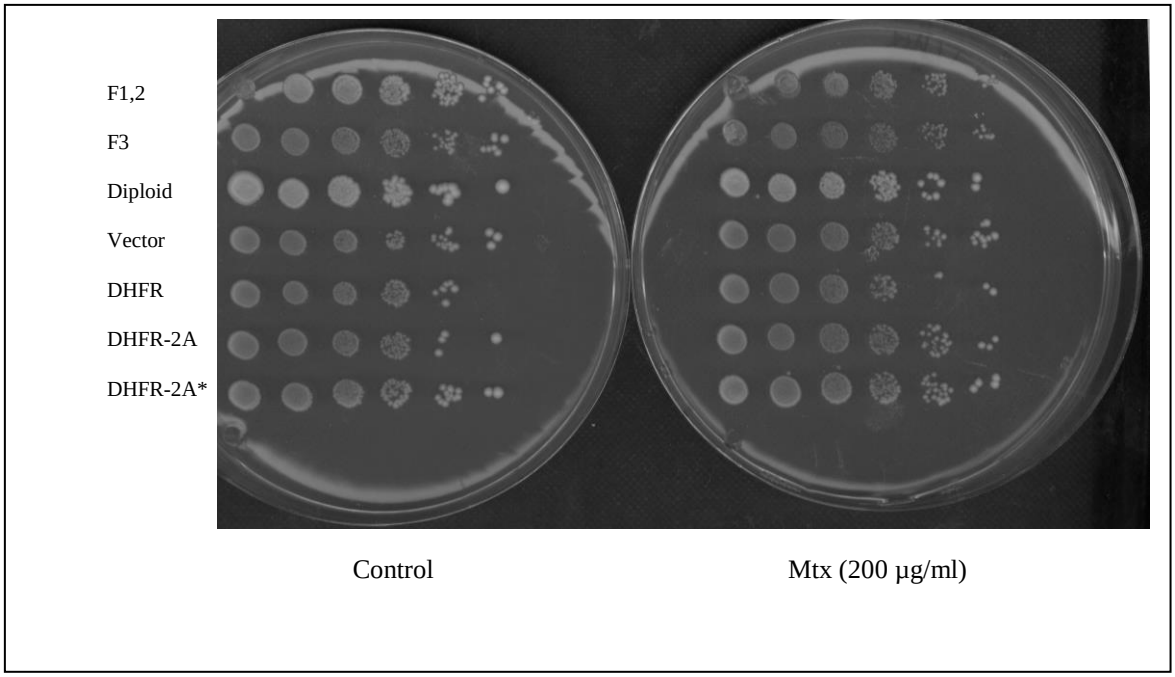

Figure 5.4: Testing the efficiency of the DHFR reporter on Mtx screen.

Plasmids (pRS314) encoding wild type DHFR, 2A or 2A* inserted between F1,2 and F3 fragments of DHFR were transformed in a wild type yeast strain (JDY895) (rows 4,5,6 and 7) to obtain strains expressing DHFR gene, interrupted by 2A sequence, as well as a control, which expresses a wild type DHFR (not interrupted by 2A sequence). These strains, along with the once which carry F1,2, F3 and the diploid mentioned above (rows 1.2 and 3) (see legend to figure 5.3 for details), were grown, and serial dilutions were prepared from each and spotted on Mtx plates. Plates were incubated at 30 ºC.

Although the issue with Mtx is likely resolved, we did not find what is expected from expressing active 2A peptide within the DHFR sequence. Theoretically, it is supposed that the strain which is transformed with a plasmid encoding 2A sequence between F1,2 and F3 fragments of DHFR gene, to reduce the growth on Mtx plates, compared to strains transformed with a plasmid encoding 2A* between F1,2 and F3 fragments or a plasmid encoding the intact DHFR gene. The reason for this, is the expression of DHFR, which enables the strain to grow on Mtx plates. Here, we found that the growth of the tested strains is not affected by the DHFR expression i.e. the control strains lacking the intact DHFR grew similarly on media containing or lacking Mtx. Furthermore, no difference on the growth is detected between the strain expressing the active or inactive version of the FMDV 2A peptide (figure 5.4). Given that the two haploid and the diploid strains respond to the Mtx-containing plates, we can confirm that there is no issue with the activity of the drug. These outcomes may indicate another interference/s with the 2A

activity, and suggest that the DHFR reporter does not compromise the 2A reaction, and cannot be implemented at moment as a tool to investigate the 2A activity.

5.3 Green fluorescent protein (GFP) reporter

The second reporter that was developed used GFP, and the same principle was followed as with DHFR i.e. separation of the protein into 2 fragments by the 2A reaction, the fragments then not being able to re-associate. It has been reported that insertion of sequences between residues 157 and 158 of GFP, located in an extended loop in its structure, does not influence expression, folding, and fluorescence of the protein. On the other hand, expressing GFP as 2 fragments separated between residues 157 and 158 yields no reassembled fluorescent protein (Gosh *et al.,* 2000) (figure 5.5).

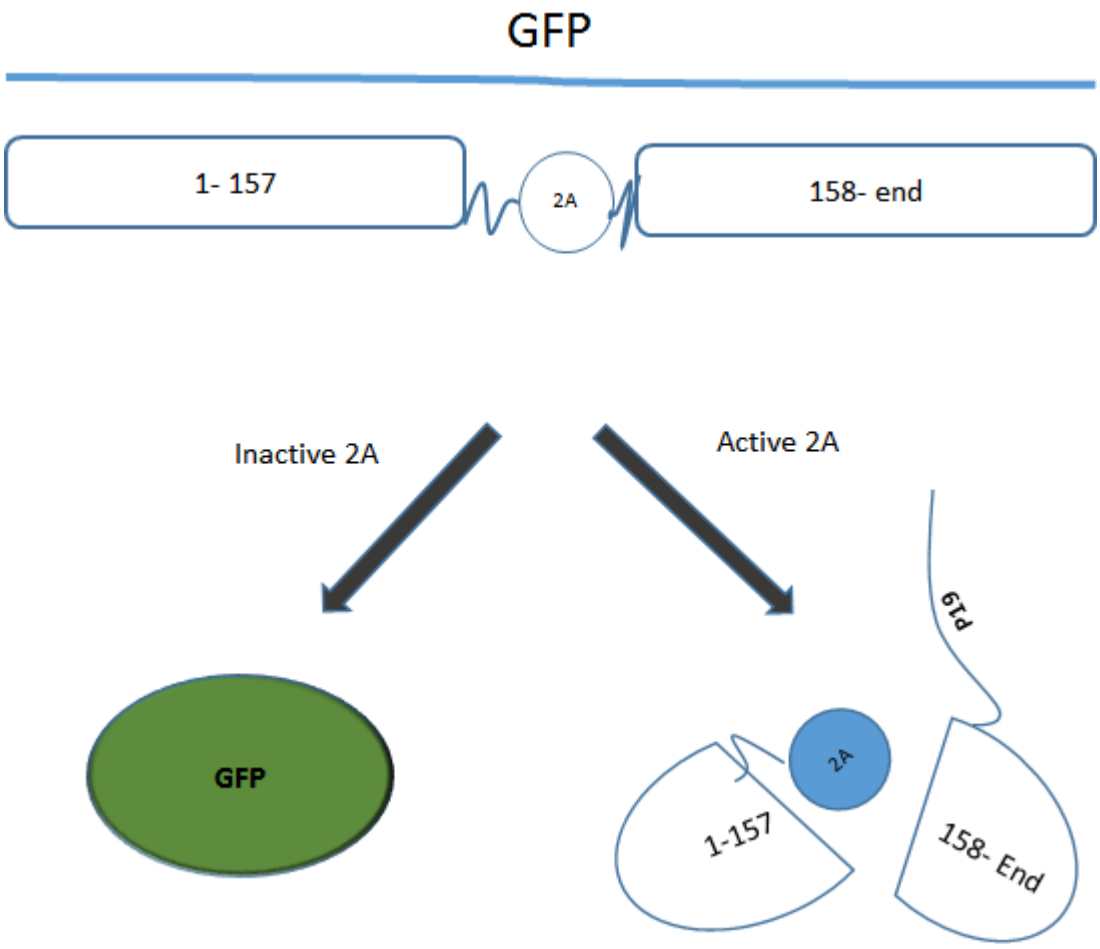

Figure 5.5: Scheme of the GFP reporter.

GFP fragments (sequence encoding residues 1-157 and 158-end) were generated by PCR using appropriate oligonucleotides. Also, sequences of 2A were generated using the specific forward oligonucleotide encoding the 2A sequence and a T3 oligonucleotide. This generates fragments of DNA encoding 2A sequence at the 5' in addition to the second fragment of GFP (sequence of residues 158-end) in addition to the T3 terminator. Initial cloning for GFP sequence was performed using SmaI restriction site to clone the basic construct (GFP 1-157-2A-GFP 158-end). Different 2A sequences were then cloned to the vector by replacing the first sequence of 2A using BglII site at the 5' and EcoRI at the 3' of T3 terminator. Cloning was done into pRS316, *URA3* yeast vector. Plasmids encoding GFP reporters were transformed into a wild type strain (JDY895) of *S. cerevisiae*, and assessed using different strategies. In general, when 2A is inactive, it is expected the GFP to show normal fluorescent activity. Whereas inserting active 2A in GFP decreases the fluorescence depending on the 2A activity.

2A coding sequences were cloned between codons 157 and 158, flanked with linkers to give structural flexibility and hopefully promote proper folding of the protein. Cloning was performed into the *URA3*-marked low copy (centromeric) pRS316 plasmid (Sikorski and Hieter, 1989), and the gene initially expressed from *SRP14* promoter. A variety of 2A sequences were used including, FMDV, IFV, DCV, TaV, PTV, PrV, amplified by PCR using T3 as a reverse primer, and such cloning was achieved using BglII and EcoRI sites which flank 2A-second part of GFP (158-end)-T3. Controls were provided by the 2A* and an uninterrupted wild type GFP.

Preliminary experiments were carried out using fluorescence microscopy and western blotting (data not shown) to examine the efficiency of the reporter. These experiments revealed that cells expressing active 2A within the GFP structure showed decreased fluorescence compared with cells expressing the inactive 2A*.

5.3.1 Fluorescent microscopy of the GFP reporter

The initial constructs of the GFP reporters included cloning of 2A/A* sequences from *Thosea asigna* virus (TaV) 2A encoding for 19 amino acids. The efficiency of these constructs has been initially examined under the fluorescent microscope (figure 5.6).

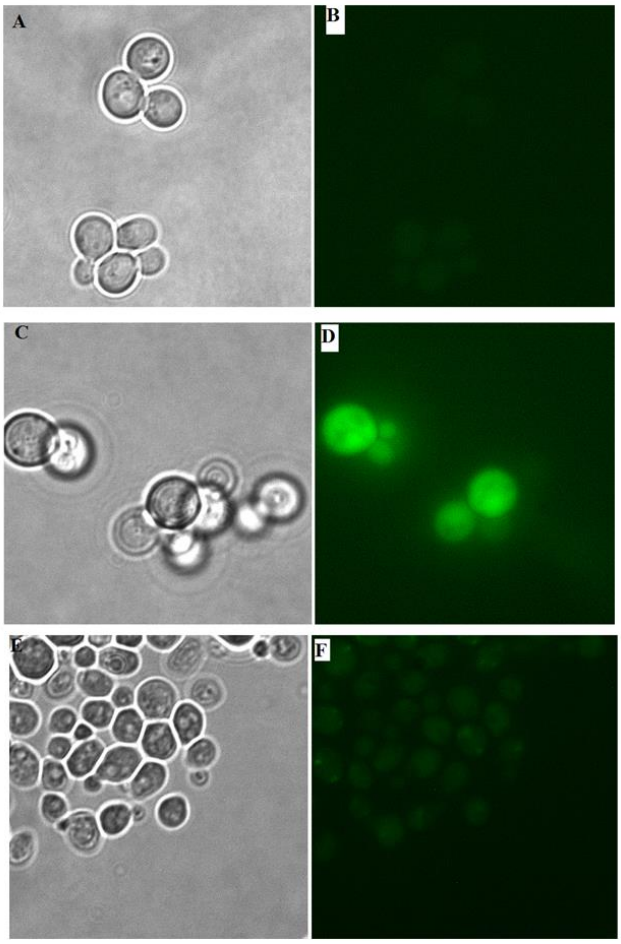

Figure 5.6: Fluorescence activity of yeast cells expressing TaV GFP reporter**.**

Reporters were constructed with active 2A and inactive (P17A, 2A*) and cloned into the coding sequence for GFP between the codons for amino acids 157 and 158 of the protein. Versions of 2A used in this experiment were TaV-2A (19 amino acids), TaV-2A* (19 amino acids), and an empty vector. These constructs were transformed into a wild type yeast strain, BY4741 (Cubillos *et al.*, 2009) and grown overnight at 30 ºC. Cultures were diluted in the morning and following regrowth for 2-4 hours, cells fixed in low melting point agarose on slides. Cells were visualised and images captured using an Axiovert 200 microscope (Zeiss) with a 100X lens. (A & B) Phase contrast and GFP fluorescence of the empty vector. (C & D) Phase contrast and fluorescence of the TaV-2A*/ respectively. (E & F) Phase contrast and fluorescence of TaV 2A respectively. All fluorescence and all phase contrast images were captured with the same settings using Zeiss Axiovision software, exported as TIF files, and processed in the same way using Photoshop (Adobe).

Microscopic test revealed strong fluorescence from some, but not all, cells containing TaV 2A* (figure 5.6 C and D). In contrast, cells expressing GFP with active TaV 2A showed less fluorescent activity which was expected in the presence of active 2A (figure 5.6 E & F). Of note, all constructs expressing GFP showed higher fluorescent activity compared to the empty vector which does not express GFP (figure 5.6 , A & B), suggesting that the TaV 2A peptide was not fully active, leading to some intact GFP being expressed. However, these data cannot be confirmed unless compared to a wild type GFP.

5.3.2 Measurement of GFP fluorescence in yeast using flow cytometry

To this point, the GFP reporter provides a qualitative assessment for 2A activity, where we can roughly estimate the reaction efficiency. GFP fluorescence can be quantified on a single cell level using flow cytometry, allowing the expression profile across a population of cells to be examined. This was of particular interest since the initial fluorescence microscopy experiments (e.g. figure 5.6 D) had revealed differences in fluorescence in cells and some that appeared not to fluoresce. Cultures of each strain were incubated overnight in selective media

at 30 ºC, diluted on the next day, and allowed to grow 2-3 hours. The OD_{600} was measured and five ODs were harvested by centrifugation, washed with sterile dH_2O and then PBS, and re-suspended in 1 ml PBS. Suspensions were sonicated (0.5 mA) for 10 seconds. Fluorescence was measured using FORTESSA X20 in the Medical School Flow Cytometry Core Facility (FCCF), using appropriate settings for GFP fluorescence (figure 5.7).

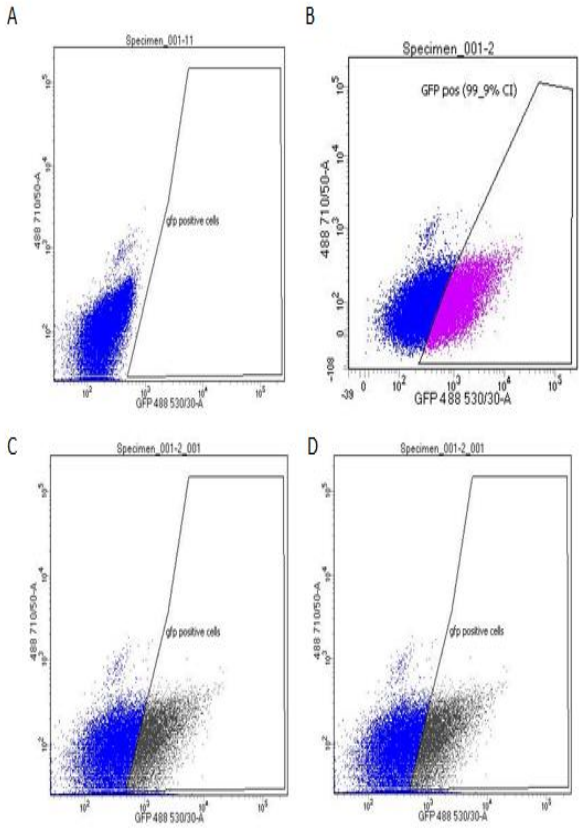

Figure 5.7: Measurement of GFP fluorescence using flow cytometry.
Cultures of the yeast strain JDY895 already transformed with plasmids encoding GFP reporter including: wild type GFP, where no 2A was inserted, 2A*, 2A and empty vector to subtract the background, were incubated at 30 ºC in the afternoon. Cultures were diluted in the morning and allowed to re-grow again for 2-3 hours. 5 OD_{600} were harvested from each strain, washed twice with water and PBS, and re-suspended in 1 ml PBS each. Sonication at low power was performed for 10 seconds to reduce cells aggregation. Fluorescence measurement was achieved on FORTESSA X20 using specific GFP settings (488 710/50-A against 488 530/30-A. A propagate was set around the population of the empty vector sample (A). This is to estimate the noise/background in the strain expressing the empty vector, where no GFP is expressed, and to clarify the GFP-positive events in other tested strains, which are shown outside the gate in B, C and D in such measurement. (A) Empty vector. (B) Wild type GFP. (C) 2A* and (D) 2A

5.3.3 The length of the 2A peptide is directly related to the activity

Next, we compared the activity of short amino acid sequences (19 residues) with longer sequences (31 residues) of the 2A peptide (figure 5.8). Results of fluorescent microscopy (data not shown) and/or western blotting revealed that longer 2A peptides derived from TaV have higher activity, compared to the shorter peptides. The protein expression in yeast, observed by western blotting is higher with the 19 amino acids length of the TaV 2A peptide. This is confirmed by measuring the GFP fluorescence using flow cytometry, which reflects lower 2A activity, thus the ribosome is promoted to assume continuous translation through the proline 19, with no release of the upstream fragment. Consequently, the higher fraction of intact protein produced in cases of lower 2A activity shows higher GFP fluorescence in cells under the microscope.

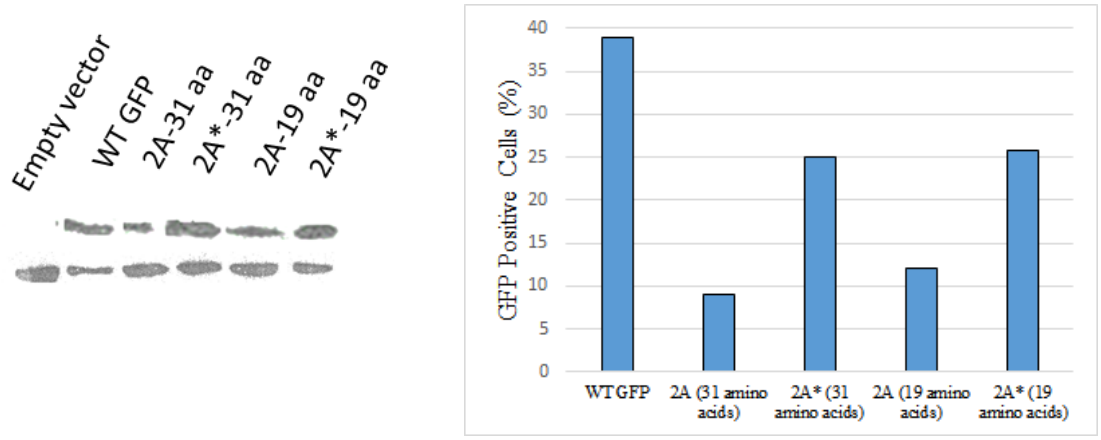

Figure 5.8: Comparative activity of different lengths of 2A peptides derived from TaV 2A peptide.

The effect of peptide length has been investigated, and results are presented regarding to both western blotting (left) and flow cytometry (right). Western blotting for proteins extracted from cells expressing 19 and 31 amino acids was undergone according to the standard method described in the Materials and Methods, and proteins on the membrane were treated with primary anti-GFP antibody (1:1000 mouse monoclonal antibody supplied by Abcam). The same laboratory procedures presented in the above figures have also been followed here. Data analysis of the flow cytometry measurement. Values presented represent the ratio of GFP positive cells in such strain. The ratios of GFP-positive cells were obtained from the oparating software (BD FACSDiva™ *software*) which is used with the FORTESSA X20 operation. This is performed by relating the GFP-positive cells to the parent cells.

From data presented in figure 5.8, longer TaV 2A peptide showed higher activity, observed by fluorescent microscopy, western blotting and flow cytometry. The longer peptide sequence of 2A shows more activity, obtained by decreased GFP fluorescence, it represents less than 25% of the activity shown by the wild type GFP, whereas it is ~30% in the shorter peptide sequence of 2A (19 amino acids). Both shorter and longer inactive 2A peptides have shown similar fluorescence activity, which is less than that in the strain expressing wild type GFP, where no 2A is inserted. This suggests a direct relationship between the length of the 2A peptide and the activity. Of note, it has clearly been observed that the insertion of 2A peptide reduces the ability of GFP to show a normal fluorescence activity. Noticeably, data from both longer and shorter 2A* indicate that although 2A is inactive, the presence of this sequence in the middle of GFP protein may influence the florescence, which is found to be in these strains ~65% of that seen in the strain expressing wild type GFP. Furthermore, data observed from both fluorescent microscopy and/or flow cytometry show that not all cells express the GFP in the same manner, in other words, GFP reporter is being expressed in a fraction of cells rather than the total cell in the culture (figures 5.6 & 5.7). This may reduce the efficiency of the reporter and interfere with the accuracy. Efforts for further optimisation of the GFP reporter will be carried out for optimum results.

5.3.4 Investigating the effect of codon usage bias in yeast on the 2A activity using the GFP reporter

Codon usage bias (CUB) is species-specific differences in the prevalence of synonymous codons, reflecting the abundance of the tRNAs that bind to them (Pop *et al.*, 2014). It has been proposed that CUB helps to maintain fast translation with high degree of accuracy. Given that yeast cells are not the natural host of viruses encoding 2A peptide, 2A reaction might not be optimised in yeast, at least partly due to differences in codon usage. Depending on CUB, we proposed that changing particular nucleotides in the 2A-coding sequence with others preferred by *S. cerevisiae* translation machinery may have positive or negative effects on 2A activity. Here, we used 31 amino acid FMDV sequences encoding 2A and 12 upstream amino acids to undergo the current expriments. Two versions were constructed, 'optimised' and 'de-optimised' with respect to synonymous codon usage in yeast, specifically across the conserved GDVESN motif just prior to the end of the 2A peptide sequence (figure 5.9). In the optimised sequence, we used the most preferred codons in *S. cerevisiae*, whereas a standard 2A sequence of the FMDV was used as a de-optimised sequence. The expected outcome of these differences in codons would be that ribosomes translating the de-optimised sequence would be slower as they approached the critical glycine-proline codons.

Optimised: 5'-
CCAAGATCTAGACATAAACAAAAAATTGTTGCTCCAGTTAAACAAACTTTGAATTTTGAT
P R S R H K Q K I V A P V K Q T L N F D
TTGTTGAAATTGGCTGG**T**GA**T**GT**T**GA**A**TC**T**AA**T**CCAGGTCCATCCGGA-3'
L L K L A G D V E S N P G P S G

De-optimised: 5'-
CCAAGATCTAGACATAAACAAAAAATTGTTGCTCCAGTTAAACAAACTTTGAATTTTGAT
P R S R H K Q K I V A P V K Q T L N F D
TTGTTGAAATTGGCTGG**G**GA**C**GT**G**GA**G**TC**G**AA**C**CCAGGTCCATCCGGA-3'
L L K L A G D V E S N P G P S G

Figure 5.9: Comparative sequence of optimised and de-optimised 2A peptides derived from FMDV.

Nucleotide and consequent amino acid sequences of optimised *vs* de-optimised (a wild type sequence) 2A peptides of the FMDV are presented for comparison. Different nucleotides between the two sequences are underlined. The standards of *S. cerevisiae* codon usage bias were taken into account when primers were designed.

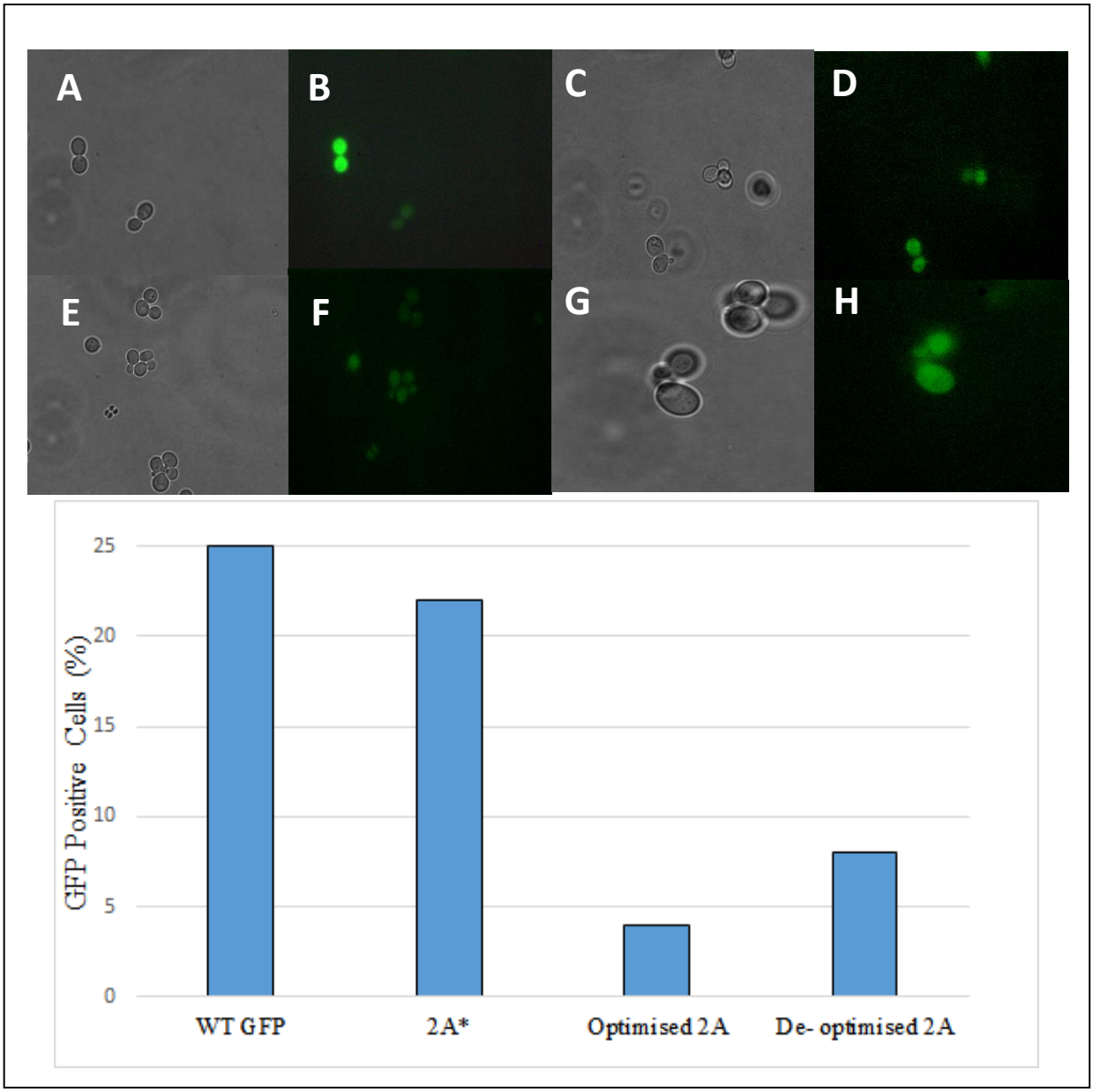

Figure 5.10: Comparative data of optimised versus de-optimised 2A peptides from FMDV.

Fluorescence microscopy of a wild type yeast strain (JDY895) transformed with plasmids encoding GFP reporters with optimised/de-optimised FMDV 2A peptide. (A& B) Phase contrast and GFP fluorescence of the wild type GFP. (C& D) Phase contrast and GFP fluorescence of the 2A*. (E& F) Phase contrast and GFP fluorescence of the optimised FMDV 2A peptide. (G& H) Phase contrast and GFP fluorescence of the de-optimised 2A. The chart underneath represents the flow cytometry data observed from same strains when exposed to cell sorting. Cells for both techniques were treated as described earlier in the Materials and Methods. The ratios of GFP-positive cells were obtained from the operating software (BD FACSDiva™ *software*) which is used with the FORTESSA X20 operation. This is performed by relating the GFP-positive cells to the parent cells.

The optimisation of the third nucleotide in codons of the C-terminus conserved motif of the 2A peptide increased the activity of the reaction, observed by decreased GFP fluorescent activity shown by both fluorescent microscopy and flow cytometry. The 40% decreased 2A activity observed in the strain expressing the optimised 2A, compared to the de-optimised 2A, and the 75% decrease compared to the inactive 2A peptide, confirms the concept of more efficient

translation when considering the optimum codons depending on the codon usage bias. On the other hand, de-optimising the DVESN conserved motif showed more GFP activity than the optimised one, but still less than the inactive 2A with about 40% (figure 5.10).

5.3.5 Testing the efficiency of the GFP reporter with mutations in the conserved motif of FMDV 2A peptide

In this section, we investigate the effects of three mutations in the conserved motif, located at the C-terminus of the FMDV 2A peptide. These mutations include: E14Q, S15I and N16H. These mutations were created in a longer sequence of FMDV 2A peptide in order to observe higher activity, given that we already approved above that longer sequences of 2A peptides are more active than the standard sequence (19 amino acids) (figure 5.8).

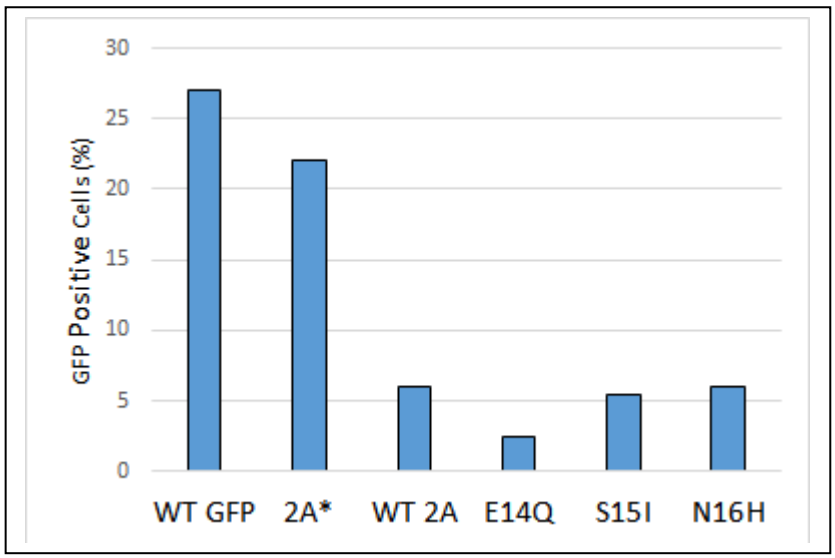

Figure 5.11: Flow cytometry analysis of GFP fluorescence with mutations in the conserved motif of FMDV 2A peptide.

Three point mutations in the C-terminus conserved motif of 2A peptide including, E14Q, S15I and N16H, were induced using PCR. Appropriate oligonucleotides encoding a single mutation in each were used to amplify PCR DNA fragments. DNA fragments were cloned into pRS316, *URA3* vector, and plasmids were transformed into a wild type strain of *S. cerevisiae*. Transformed strains were grown in selective media at 30 °C, and overnight cultures were diluted on the next day using the same media, and allowed to grow again until reaching OD_{600}~0.5. Five ODs were harvested in Falcon tubes using bench-top centrifuge at 3000 rpm for 5 minutes and washed with sterile PBS. Pellets were re-suspended in 1 ml PBS and sonicated for 30 seconds. Samples were exposed to flow cytometry as described in Materials and Methods.

Mutating S15 or N16 residues does not affect the GFP fluorescence detected by flow cytometry whereas, the E14Q mutation caused a sharp reduction (~50%) in the fluorescence compared with the wild type FMDV 2A activity (figure 5.11). This suggests an improvement in the 2A activity with this mutation over the value of the wild type FMDV 2A peptide, where the normal codons for the whole sequence is not changed. However, the value of the wild type FMDV 2A represents ~30% of that of the 2A*.

5.3.6 Comparative analysis of 2A activity from different viruses

To this point, we successfully developed an efficient tool to study the 2A activity. From experiments carried out and presented above, the GFP reporter responds properly and reflects accurate results where we can assess the 2A activity during different conditions. Further work has been undertaken here to optimise the reporter efficiency. In this section, we have utilised the GFP reporter to investigate the activity of 2A peptides from different viruses, and to find out if the activity is different when 2A sequence is expressed in *S. cerevisiae* cells. Thus far, longer sequences of both FMDV and TaV 2A peptides were used in the previous experiments. Here, it seems logical to investigate activities of different 2A peptides in *S. cerevisiae* cells as a comparative analysis. DNA sequences of 2A peptides presented in table 5.1 were cloned into the same vector encoding the GFP, the resulting plasmids were transformed into a wild type yeast strain (JDY895). Experiments were carried out as described in above sections.

Infectious flacherie virus (IFV) (AB000906)	P S I G N V A R T L T R A E I E D E L I R A G I E S N P G P
Perina nuda picorna-like virus (PrV2A1) (AF548354)	L E M K E S N S G Y V V G D R G S L L T C G D V E S N P G P
Drosophila C virus (DCV)	Q G I G K K N P K Q E A A R Q M L L L L S G D V E T N P G P
Porcine tescho virus-1 (PTV)	A M T V M T F Q G P G A T N F S L L K Q A G D V E E N P G P
Infectious myonecrosis virus (IMNV)	W D P T Y I E I S D C M L P P P D L T S C G D V E S N P G P
Thosea asigna virus (TaV)	G R S R G P R P Q N G S G E G R G S L L T C G D V E N P G P
Foot and Mouth Disease Virus (FMDV)	P R S R H K G S G V K Q T L N F D L L K L A G D V E S N P G P

Table 5.1: Different 2A peptides used for comparison with their amino acid sequences.

The comparative activity of these different sequences was assessed via western blotting and flow cytometry. Different amino acid sequences showed different activities when expressed in *S. cerevisiae* cells. It has been shown in figure 5.12 that different 2A peptides have different fluorescence, thereby different reaction activity. Here, we have chosen the most active 2A peptides amongst the collection which has been examined.

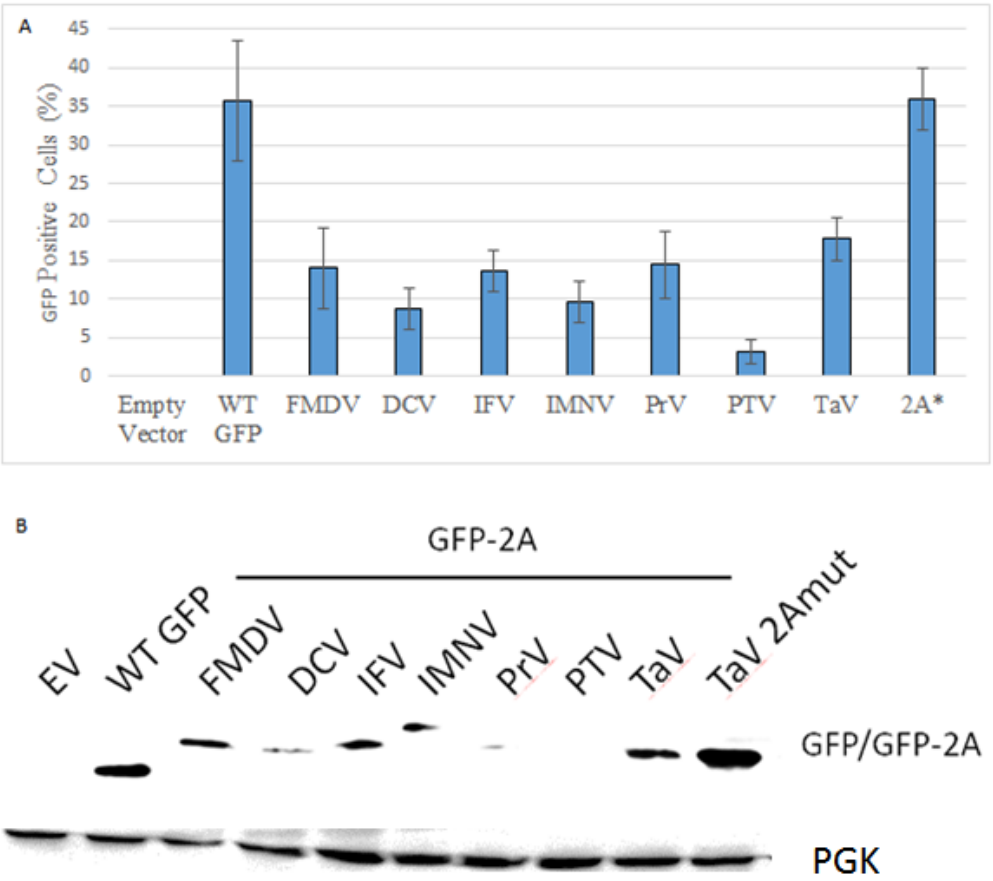

Figure 5.12: Different 2A peptides from several viruses illustrate different activities in *S. cerevisiae* cells.

Different sequences of 2A peptides encoded in several viruses are compared here in terms of their activities. Different DNA sequences of 2A peptides encoded in several viruses were amplified using PCR, and resulting fragments were cloned into pRS316, *URA3* vectors. Plasmids were transformed into a wild type *S. cerevisiae* strain, and grown in selective media at 30 ºC. Cultures were diluted on the next day and allowed to grow again to the OD_{600}~0.5. Five ODs were harvested from each culture and treated as decribed previously for flow cytometry. Proteins were extracted from the same cultures for western blotting (see Materials and Methods for details). 20 µg of each protein were resolved into SDS-PAGE and trans blotted onto hemicellulose membrane. Protein bands were detected on the transblotted membranes using anti GFP monoclonal antibody. Panel (A) Flow cytometry analysis of strains expressing a variety of 2A-coding sequences, placed in the middle of GFP as a reporter, compared to the wild type GFP and/or 2A*. Panel (B) Western blotting of proteins extracted from yeast strains expressing the same GFP reporters with different 2A peptides, presented in panel A. Protein bands observed in this panel demonstrates slight differences in term of their sizes, due to differences in their amino acid sequences, and hence, molecular mass.

From data presented above (figure 5.12), different amino acid sequences of 2A peptides have shown different activities, assessed by both western blots and flow cytometry, and confirmed by fluorescent microscopy (data not shown). Noticeably, the amino acid sequence in different 2A peptides is implicated in its activity when expressed in *S. cerevisiae* cells. The most active 2A peptide amongst the range that has been tested is the peptide taken from PTV 2A. In this peptide, the fluorescence of GFP is at its minimum level, representing ~10% of the controls, which include both wild type GFP and 2A*. The high efficiency of PTV permitted no intact GFP to be produced by the ribosome, concomitantly, no fluorescence has been detected. Furthermore, DCV and IMNV have also shown higher activity (30% of the controls) obtained by flow cytometry and/or western blotting outcomes. However, it is still below the level that has been shown by the PTV 2A peptide. The PrV 2A results are slightly contradictory, the fluorescent activity of this peptide, observed by flow cytometry, is ~50% of that in the wild type GFP, whereas, it reflects a high activity by western blot. Furthermore, these results show that the least active 2A peptide among the selection examined was the TaV 2A peptide, this is demonstrated in the flow cytometry outcomes, and confirmed by western blotting. The control used in this experiment consists of the wild type GFP, where the sequence of the protein was not interrupted by the 2A peptide, as well as the TaV 2A*mut* (2A*). Both controls approached the same levels of fluorescence although the western blotting screen demonstrates a higher intensity of the protein band corresponding to the 2A* (figure 5.12 A& B).

5.3.7 Optimisation of the GFP reporter efficiency

As we have previously mentioned, the GFP reporter is currently efficient in measuring the 2A activity in different conditions, and indicating increased/decreased activity when reaction circumstances are changed accordingly. However, the cells in fluorescent microscopy and/or flow cytometry do not show equality in their fluorescence, in other words, yeast cells in the same culture have different levels of fluorescence although they express the same reporter-encoding sequence from the same promoter. To address the reason for the variable fluorescence among cells in culture, we proposed that the plasmid copy number during cells replication may be the reason for this unequal fluorescence, accordingly a few steps were taken to improve the reporter efficiency.

5.3.7.1 Integration of the GFP reporter into S. cerevisiae genome

To investigate whether the plasmid copy number of pRS316, the basic vector of the GFP reporter, is the issue in regard to the different fluorescent activity among cells, integration of the same GFP construct, with 2A peptide inserted at the 157 residue has been carried out into the cells genome. If the plasmid copy number was to blame, the integration ensures higher expression of the construct encoding the GFP reporter, regardless of the plasmid copy number in cytoplasm.

To achieve this integration, we made use of the *his3-Δ1* mutation in the wild type strain (JDY895) that we have used. This mutation includes an internal deletion of 187 base pairs from an area flanked with two HindIII sites (nt 305-492). Thus, we excised the GFP reporter from its basic vector (pRS316), and cloned it into a *HIS3* vector (pRS303). GFP reporter/s were amplified using specific oligonucleotides flanked with SmaI restriction sites on both 5' and 3' ends using PCR. The resulting DNA was cloned into a pRS303 vector using SmaI sites, and the cloned plasmids were linearized by HindIII restriction enzyme and integrated into the JDY895 strain, using standard yeast transformation protocol described in the Materials and Methods chapter.

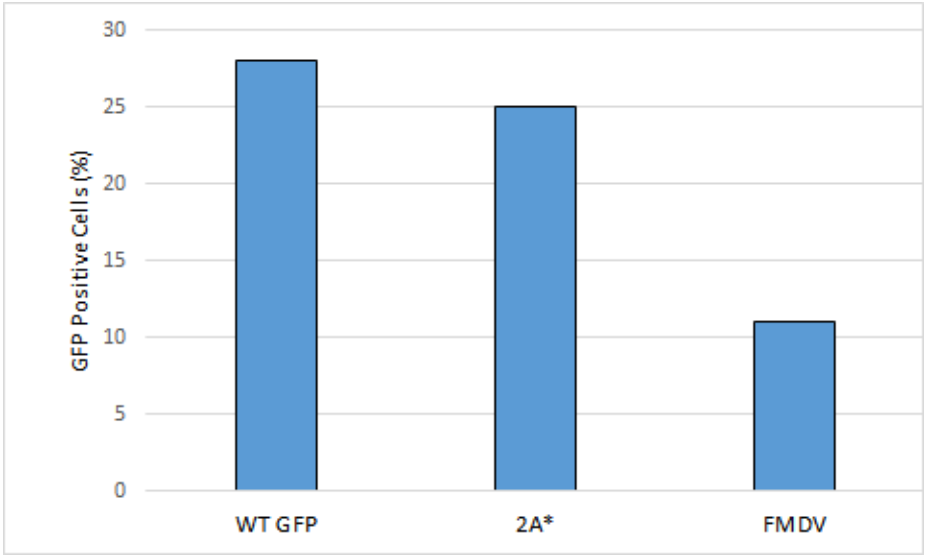

Figure 5.13: GFP fluorescence of TaV 2A peptide sequence integrated into the cell genome.

Plasmids (pRS303) encoding WT GFP, 2A* and active FMDV 2A peptides were linearized, and extracted from the digests. 10 µg of each linearized plasmid was integrated into a WT *S. cerevisiae* strain genome (JDY895) using standard yeast transformation protocol (see Materials and Methods). Transformed cells were plated on plates lacking histidine thus, integrated cells were able to grow, as histidine gene is expressed from the integrated plasmid. Growth conditions and flow cytometry procedures are similar to previous experiments shown above.

Increasing the expression of the GFP reporter via integration into the genome did not increase the ratio of cells which are positive to GFP. The number of yeast cells properly expressing the protein in the strain integrated with the wild type GFP is ~30% of the parent cells sorted by the flow cytometer. This number is about the same in the strain integrated with the inactive 2A construct however, the number of cells expressing GFP is decreased in the strain integrated with the active 2A construct (figure 5.13). Given that only 30% of cells express the entire GFP in controls, where no 2A activity is supposed to be detected, the integration did not resolve the problem, and another solution is going to be suggested below.

5.3.7.2 Overexpression of the GFP reporter

Here, we presumed using an inducible promoter would increase the expression of the reporter in all cells, leading to an increase in the efficiency of the reporter in terms of assessing 2A activity. The *GAL1* promoter has been used for this purpose, and sequences encoding wild type GFP, GFP-2A*, GFP-TaV-2A and GFP-PTV-2A were cloned into a vector, already cloned with a *GAL1* promoter. The *GAL1* sequence from pJNY11 vector (the basic vector of the 2A reporter used in the immunoprecipitation) was used here by excising the *GAL1* gene sequence and clone it upstream to the GFP-2A sequence into the pRS316 vector. Then, to change the selection marker from *URA3* to *HIS3*, the *HIS3* gene was taken from pRS313 vector and cloned into appropriate sites within the *URA3* gene sequence of the newly developed vector, to end up with a plasmid expressing the GFP reporter from a *GAL1* promoter, and carrying a *HIS3* selection marker. This time, the GFP reporter was utilised to examine the efficiency of the 2A peptide in the presence of different mutations of eRF1/3. The newly cloned GFP reporter into the *GAL1* vector was transformed into the strains, which maintain termination via mutant eRF1/3 (same strains used in experiments presented in Chapter 4). As previously mentioned in this thesis, strains of *S. cerevisiae* with attenuated *SUP45* gene with *kanmix* (YTH91, table 2.1) were used. Therefore, they undertake termination by a plasmid-born (centromeric plasmid *URA3*) copy of wild type *SUP45*. These strains were transformed with plasmids with *LEU2* selection marker. These plasmids encode point mutations in *SUP45* (pTH313-774, table 2.2). Cells were selected on 5-FOA plates, to select colonies with only the *LEU2* plasmid copy of *sup45* being expressed. Cultures of these strains were incubated overnight in selective media at 30 ºC with 2% w/v raffinose, diluted on the next day with the same media plus 2% w/v galactose, and allowed to grow for ~4 hours. Cells were harvested and diluted as described above for the flow cytometry measurements. The highest efficiency detected amongst the tested selection was with the PTV 2A peptide (section 5.3.6), thus we started assessing the effect of Sup45 mutations using the GFP reporter containing PTV coding sequence. Despite the

overexpression of the GFP reporter used in this experiment, the high activity of the PTV 2A peptide overrides the prospective effects of mutant eRF1, when the high efficiency of the reaction diminished to a great extent, the fraction of ribosomes undergoing read-through over the proline 19 of the 2A peptide (figure 5.14).

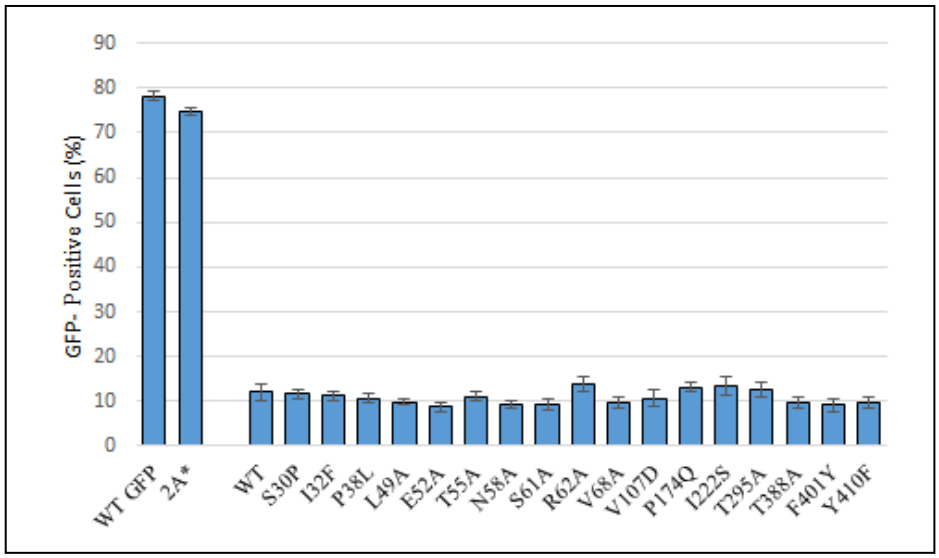

Figure 5.14: GFP fluorescence in cells expressing mutant eRF1.

GFP reporter with PTV 2A-coding sequence was used to examine the effects of mutations in different domains of eRF1. The GFP reporter was under a *GAL1* promoter, and plasmids encoding wild type GFP, PTV-2A* and PTV-2A were transformed in yeast strains expressing *sup45* as the only copy of eRF1 for termination (YTH91 strain transformed with plasmids, pTH313-774, table 2.2 see text in section 5.3.7.2 for details). Strains were inoculated into selective media with 2% w/v raffinose and incubated at 30 ºC overnight, diluted in the morning with the same media plus 2% w/v galactose. Further incubation was applied for 4-5 hours, and 5 OD_{s600} were harvested, washed twice with dH_2O and PBS respectively, and re-suspended in 1 ml PBS. Suspensions were sonicated for 10 seconds each, and exposed to the flow cytometry analyser. Data were automaticslly processed, by the analyser operating system (BD FACSDiva™ *software*) to obtain the ratio of cells expressing GFP (GFP-positive cells) from each strain, detected by FORTESSA X20 analyser. Data presented as means±SE. Values are from 4 independent experiments.

To avoid the interfering effect of the high activity of 2A peptide, expressed by the PTV-2A, we replaced the PTV with a less active 2A peptide from TaV (figure 5.15). This was performed using the same cloning strategy mentioned for the GFP cloning which includes, the amplification of the TaV 2A sequence with the second part of GFP (sequence of residues 158-end) using the specific forward oligonucleotides for TaV and T3 oligonucleotide as the reverse primer. The PCR fragment was cloned into the modified pRS316 vector (with a *HIS3* selection marker and *GAL1* promoter, see text in 5.3.7.2 for details) using BglII site which flanks the 5' end of the fragment and EcoRI at the 3'. This plasmid was transformed into strains maintaining termination through a range of Sup45 mutations (YTH91 transformed with pTH313-774). The

former experiment was repeated to investigate whether the usage of the GFP reporter gives the same results observed by the immunoprecipitation experiments, presented in Chapters 3 & 4.

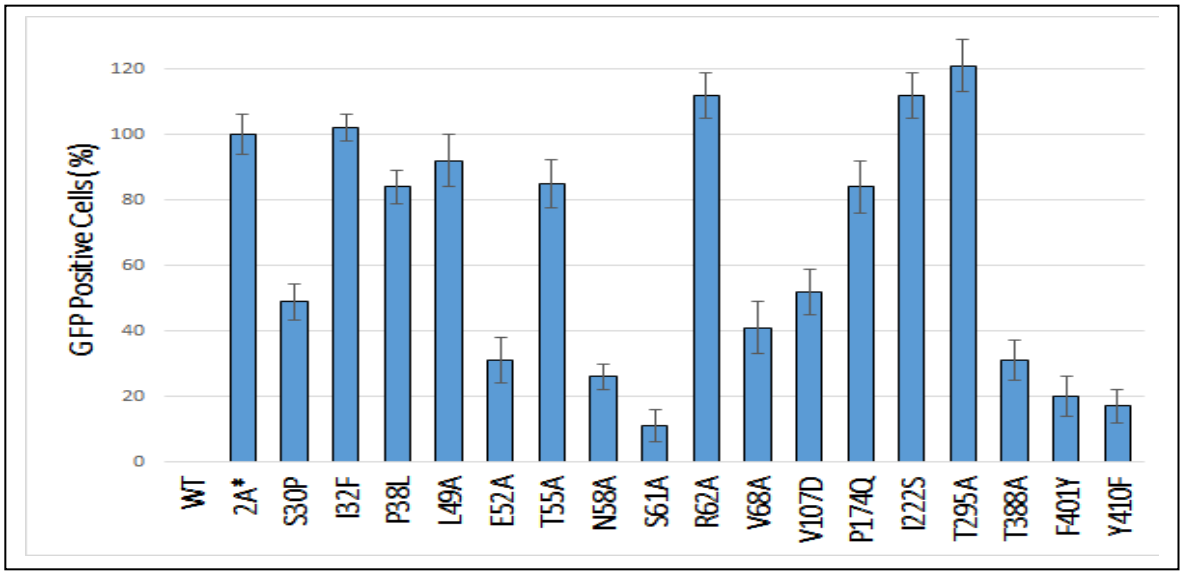

Figure 5.15: GFP reporter is sensitive to changes in the 2A activity produced by mutations in eRF1.

GFP fluorescence of strains with a variety of *sup45* alleles, expressed by flow cytometry is presented above. Plasmids encoding a GFP reporter with TaV 2A were transformed in yeast strains expressing *sup45* alleles as the only copy of eRF1 for termination. The GFP reporter encodes TaV 2A peptide, and expressed from *GAL1* promoter in presence of 2% w/v galactose in the growth media. All procedures are similar to the experiments explained above. Values of GFP fluorescence here were normalised according to the value of the strain expressing the reporter with 2A* where the 2A is inactive (shows the maximum read-through/fluorescence). The fluorescence value of this strain was set to 100. Then, other samples were normalised using the formula: Z=Y*100/X, where Z: the value of the sample/mutation presented in the chart. Y: the original value of the sample observed by the flow cytometer. X: the original value of the strain expressing 2A* observed by the flow cytometer (the value before being set to 100). Data were plotted to present differences in the read-through triggered by each mutation/strain, compared to the wild type. Values were taken from three independent experiments and expressed as mean±SE.

The utilisation of TaV-2A peptide within the GFP reporter resolved, to a great extent, the former problem obtained when the PTV-2A peptide was used. It has been clearly shown in figure 5.15 that the expression of the GFP reporter from a *GAL1* promoter results in about 7 fold increase in the ratio of GFP-positive cells in such sample, retaining the variation in the GFP fluorescence among strains expressing different mutations of eRF1. The analysis presented in figure 5.15 reveals massive differences in 2A activity, in strains maintaining termination with mutant eRF1, observed by GFP fluorescence. The differences in the GFP fluorescence imply changes in ratios of ribosomes translating the reporter, which is caused by the inhibitory effect of eRF1 mutations on the 2A activity. The majority of mutations tested here reveal consistent results with those observed from immunoprecipitation. Most mutations such as, I32F, L49A, T55A, R62A, P174Q, I222S and T295A, show an increased GFP fluorescence, which approaches the value observed in the inactive 2A, suggesting more fractions of ribosomes undergoing translation of the entire GFP. These data are in accordance with the pulse labelling and immunoprecipitation

data. The test of most mutations of eRF1 revealed a status of read-through across proline 19 of 2A peptide in the polyprotein reporter, observed by reducing the ratio of each upstream and downstream product (table 4.2). Of note, data regarding the strains expressing S30P and F401Y are not consistent with that obtained by immunoprecipitation. However, this experiment proves that the GFP reporter is sufficiently accurate to be used to investigate the 2A activity

5.4 Discussion

The structure of GFP, shown in figure 5.16, illustrates a barrel-like formation with a diameter of about 24 Å and a height of about 42 Å. The chromophore is located in the centre of the barrel, and mutational analysis revealed that almost the entire structure is required for chromophore formation. Residues 2-232 or 7-229 are essential for the GFP fluorescence. GFP folds into an 11-strand barrel, which is likely to be essential for the chromophore formation. Intramolecular cyclization of Ser65-Tyr66-Gly67 is responsible for the chromophore formation, which is an autocatalytic event (Heim *et al.*, 1994). Therefore, the amino acid sequence and the consequent three dimensional structure of the protein is responsible for the intramolecular cyclization of residues 65-67. The barrel structure of the protein provides protection for the chromophores and, in addition, it aids to stabilize the GFP structure (figure 5.12) (Ward and Bokman, 1982). Although the GFP protein folds quickly, the chromophore formation, and the consequent oxidation are likely to be a slower processes (Reid and Flynn, 1997).

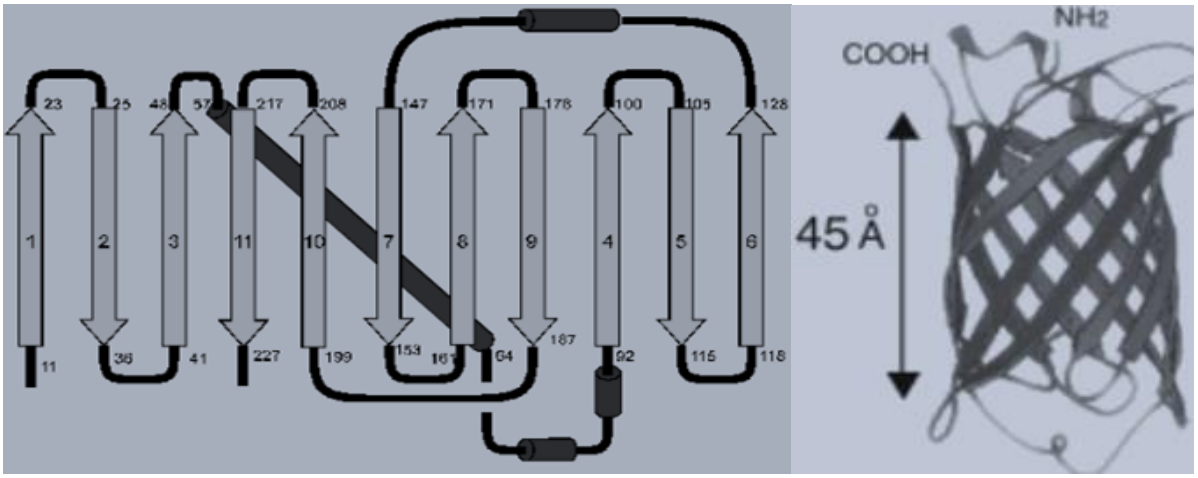

Figure 5.16: The structure of GFP barrel and folding direction. Adapted from http://www.cryst.bbk.ac.uk/PPS2/projects/jonda/structur.htm

The diagram shows the topology and pattern of GFP folding. On the right, ribbon structure of the GFP β-barrel with the loop, on the top, corresponding to the site of possible separation (residues 157-158). The diagram on the left represents the pattern and the direction of protein folding to produce the normal fluorescent activity.

It has been previously shown that the dissection of GFP at the loop located between residues 157 and 158 can accommodate an insertion of 20 amino acids residues (Abedi *et al.*, 1998). The ultimately chromophore-forming residues, Ser65, Tyr66 and Gly67, will be contained in the N terminus of the protein, and will configure properly in terms of fluorescence (Tsien, 1998). Two sites in the GFP protein structure have been defined for insertion of short peptides; one of these sites is within a smaller loop (Ala154-Gly160), this site possesses more mobility than other loops, as it is not integral to the structure of the protein. Although several sites in GFP have been defined to be the best accommodation locations for peptide insertion, the protein does not produce fluorescence levels as high as the wild type GFP (Abedi *et al.*, 1998).

In this chapter, we used the properties of GFP to accept insertions at particular locations to develop a novel reporter to study the 2A reaction. This has been tested according to the GFP fluorescence and/or protein expression, therefore cells with less 2A activity would be sorted as having higher fluorescence, whereas, cells with higher 2A activity give less/undetectable fluorescence. Initial experiments indicated promising results, assessed by fluorescent microscopy, where strains expressing functional 2A peptide have shown low level of fluorescence *vs* strains expressing 2A*.

Using the newly-developed GFP reporter, we found that the longer sequence of 2A peptides, the more efficient. We used TaV 2A peptide to achieve this part. Two versions of TaV 2A encoding sequences were used, 19 and 31 amino acids. The differences between the two lengths of 2A peptides, on the level of protein expression, are not clear, in addition, the images of fluorescent microscopy show a massive difference between wild type GFP, 2A* on one side, and active 2A on the other side, but it is unclear between the 19 and 31 amino acids (data not shown). Cell sorting techniques provided a good analysis of strains expressing different lengths of 2A peptides, when fluorescence of such is measured. Values obtained from flow cytometry suggest that the longer sequence of 2A peptide is the stronger in activity, given that the fluorescence of the strain expressing the 31 amino acid length 2A peptide is less than that of the strain expressing the 19 amino acid 2A (figure 5.8).

Despite the efficiency of the 31 amino acids TaV 2A peptide over the 19 amino acids 2A, TaV-derived 2A seems to be the less efficient 2A amongst the range of 2A peptides that have been tested. PTV 2A shows the maximum activity detected by the lowest fluorescence measurement by flow cytometry (~10% of the wild type), which is in accordance with the results of (Kim *et al.*, 2011). Furthermore, both DCV and PrV 2A peptides show higher activity than others, fluorescence: 25% and 46% respectively of the wild type. However, the western blotting shows lower protein expression in the lane corresponding to the PrV 2A (figure 5.12 A&B).

Donnelly and colleagues demonstrated that the TaV 2A peptide shows a high activity (~100%), followed by both PTV and FMDV 2A peptides (Donnelly *et al.*, 2001a). In contrast to these results, Szymczak and colleagues reported that the highest activity of 2A peptides has been observed in both FMDV and TaV 2A peptides (Szymczak *et al.*, 2004). The results of this study seem more consistent with Donnelly's group results. Since previous studies were based on an *in vitro* translation of transcripts encoding these 2A sequences, a question remains unclear about how 2A reaction may act in *in vivo* experiments. It has been found that the PTV 2A peptide is the highest, in terms of activity, among a range of 2A sequences that have been tested in human cell lines, zebrafish embryos and mice liver (Szymczak *et al.*, 2004). The mechanism by which PTV 2A peptide exhibits a high activity is still unclear, however it has been proposed that 2A activity can be promoted (de Felipe *et al.*, 2006) by inclusion of a GSG linker at the N-terminus of a 2A sequence (Szymczak *et al.*, 2004; Holst *et al.*, 2006). In addition, insertion of C-terminus residues of the 1D fragment in the N-terminus of the 2A peptide can also elevate the efficiency of the reaction, this means that the addition of the C-terminus five amino acids, APVKQ, to the N-terminus of the 2A increases the activity in the FMDV up to 13% in an *in vitro* study (Donnelly *et al.*, 1997). Furthermore, it has been demonstrated that the insertion of C-terminal seven residues, APVKQLL, of the FMDV 1D peptide to the N-terminus of its 2A raises the activity up to 15% in baby hamster kidney and porcine alveolar macrophages cells (Groot Bramel-Verheije *et al.*, 2000). These data are in agreement with our findings, in terms of the high 2A efficiency associated with the increased length of the 2A peptide. Although previous studies provided knowledge about activities of some 2A peptides, they did not account for the differences in the activity among 2A peptides from different viruses, as they assessed the activity of 2A peptide derived from the same virus, but in presence or absence of extra residues. Noteworthy, it was found that the 19 amino acids length of 2A is more efficient than the 22 amino acids in human cell lines. However, the three extra amino acids may not induce a detectable effect (Szymczak *et al.*, 2004) which is inconsistent with our data. Given that the current study is carried out on yeast cells, variations, in terms of 2A activity, are expected due to the ability of cells to replicate plasmids as well as the nature of vectors/promoters encoding the 2A sequence. There might be other factors governing the differences in cleavage efficiency among different 2A peptides.

2A peptides have been confirmed to function in eukaryotic cells such as, yeast (de Felipe *et al.*, 2003), plants (Park *et al.*, 2009), mammalian cells (Hu *et al.*, 2009) and transgenic animals (Trichas *et al.*, 2008). Thus, the expression of 2A-containing ORFs occurs independently of cell type, since the 2A reaction is only dependent on the presence of eukaryotic translating machinery, which is universally conserved in terms of structure.

The 2A/2B of FMDV 2A reaction is a translational modification applied in the ribosome by the 2A peptide, allowing the release of upstream protein, associated with 2A from the translation machinery, while the downstream protein synthesis is permitted (Donnelly *et al.*, 2001a). Meanwhile, the efficiency of the 2A cleavage is not 100% i.e the translation through an ORF with 2A coding sequence may also yield a proportion of ribosomes generating a full-length product (Wang *et al.*, 2007). The specific site of cleavage of the 2A reaction, G18/P19, participates a main role in the recoding event (Sharma *et al.*, 2012). Therefore, mutations at this site abrogate or at least reduce the 2A activity (Doronina *et al.*, 2008; Sharma *et al.*, 2012). Mutational analysis of the 2A peptide mainly concentrates on the conserved C-terminus motif (Minskaia *et al.*, 2013), however some studies also performed single amino acid mutational analysis for the N-terminus extension. It has been found that the 2A activity is completely abrogated when G18 is mutated (Sharma *et al.*, 2012).

Synonymous yeast codon usage bias plays an important role in this context (Kramer *et al.*, 2009; Tuller *et al.*, 2010). Gene expression can be affected with even a single nucleotide mutation (Kimchi-Sarfaty *et al.*, 2007). Additional information might be contained in the mRNA, which can fine-adjust protein folding, therefore ribosomal pausing at a rare codon may also impact protein folding (Komar, 2009). 2A-induced cleavage is an instant event following the synthesis of the polypeptide chain in the PTC, when the ester bond is hydrolysed and the nascent peptide chain is concomitantly released from the tRNA. In this point, the G18 seems to be the key amino acid to maintain the 2A activity, along with the conserved motif, due to its location right upstream to the 2A peptide cleavage site. The importance of G18 comes from its specific location which contributes to the assumption that mutating this particular residue may influence the 2A activity (Gao *et al.*, 2014).

In general, it has been found that the amino acid sequence in GFP structure plays a role in the protein function/and expression. Codon usage is a major determinant that may alter dynamics and/or speed of translation (Novoa *et al.*, 2012; Novoa and Ribas de Pouplana, 2012). It is well defined that ribosomes synthesise proteins on a linear substrate, and no uniform speed has been recognized for this process. Therefore, some co-translational effects can be a result of transient pausing of ribosomes, these effects may include protein folding (Li *et al.*, 2012). Sharma and colleagues (Sharma *et al.*, 2012) confirmed that there is an interaction between the nascent peptide chain, in case of 2A reaction, and the exit tunnel, which may dictate a status of co-translational recoding (stop carry on). It has been found, through a comprehensive analysis of the mRNA sequence and its role in the protein synthesis, the corresponding nucleotide sequence may influence the structure of newly formed protein, even when the protein retains its amino acid sequence (Kimchi-Sarfaty *et al.*, 2007). Modelling of the 2A function has proposed that

the G18-P19 may take a shape of tight-turn straight away beyond α-helix (Donnelly *et al.*, 2001a). Gao and colleagues investigated the 2A sequence to test the codon usage bias, particularly G18. They replaced the GGT codon of G18 with GGG and found that the GGG is the most frequent codon used by CHO cells (Gao *et al.*, 2014). However this codon needs more energy than cognate codons to break the codon-anti codon bond between guanine and cytosine, compared to the required energy used by adenine and thymine. Codons with high pairing energy possess a strong tendency to bind the anti-codon on the tRNA while the ribosome is translating 2A peptide. This may reduce the translation efficiency (Cannarozzi *et al.*, 2010). It has been suggested, based on the above findings, codon usage bias could slightly alter the speed of translation, resulting in differences in the 2A activity. In the context of our results, the finding stated above lie in accordance with our current results regarding to the codon usage bias. The optimised 2A peptide showed ~20% fluorescence compared to the strain expressing the wild type GFP, whereas the GFP fluorescence (read-through level) of the strain expressing the de-optimised 2A has slightly elevated (~30% of the wild type GFP) (figure 5.10). However, we did not subject the G18 to the codon analysis, but instead concentrated on the conserved C-terminus motif of the 2A peptide.

The newly developed GFP reporter has been utilised to confirm the results mainly presented in Chapter 4, regarding to the mutational analysis undertaken on eRF1. The differences in the GFP fluorescence, detected by flow cytometry, are consistent to what was observed by immunoprecipitation. However, two mutations which were found to promote the read-through propensity in Chapter 4 showed no effect when assessed by the GFP reporter. The S30P mutation has been found to reduce the 2A activity through quantifying the outcomes of immunoprecipitation, and also caused a reduction in the strain growth rate, however its effect on the stop codon read-through was mild. In this case, the expression of the GFP reporter might be blamed. The degree of expression could be an important factor to obtain clear results, we found that the differences in fluorescence among the range of mutations in eRF1 were not significant when the construct was expressed from *SRP1* promoter (data not shown), although they depict a clear feature about the effect of such mutation on the 2A activity. Whereas the differences in the fluorescence were clearer when the GFP reporter was expressed from an inducible (*GAL1*) promoter (figure 5.15). However the fluorescence of the strain expressing S30P mutation was not altered.

Regarding to the DHFR reporter, it seems that the growth inhibition due to methotrexate treatment is not related to the change in DHFR expression induced by the 2A peptide. However, control strains expressing F1,2 and F3 showed what is expected, in terms of growth, on

methotrexate screen. Changing strain background also did not resolve the problem, which suggests that the expression/folding of DHFR is defective when 2A is inserted in between.

SUMMARY

The GFP reporter is a sensitive tool, which can be successfully used to examine the 2A activity. This reporter is independent of metabolic pathways in yeast, and fast to generate data. The results presented in this chapter suggest that using this reporter to be expressed from a plasmid in yeast is sufficient to obtain satisfactory results with consequent significance.

6 CHAPTER 6. SUMMARY AND DISCUSSION

6.1 Background

The 2A reaction comprises 2 unusual translational events; an 'early' nascent peptide release from the ribosome at the end of 2A sequence, with no requirement for a stop codon to occupy the ribosomal A site, which is instead occupied by the Pro 19 codon, and a re-start to translation using the same Pro 19 codon (Ryan and Flint, 1997). The mechanism of re-starting translation is still unknown, though it is coupled to the first step of the reaction. It has been proposed that amino acids of the 2A peptide have specific interactions with one or more components of the ribosome, modifying the activity of the PTC such that it cannot carry out its normal function (Donnelly *et al.*, 2001a; de Felipe *et al.*, 2010). Thus, its capability to generate a peptide bond between the Gly 18 and Pro 19 is hindered. Despite the N-terminal region of the 2A amino acid sequence being highly variable, it has been found that the whole amino acid sequence of 2A peptides are essential for activity: many changes to the N-terminal of the peptide reduce its activity (Sharma *et al.*, 2012). Unlike other cases of recoding, 2A activity is derived from peptide, rather than RNA sequence (Doronina *et al.*, 2008; Sharma *et al.*, 2012). Many 2A sequences, including the FMDV peptide, have a high alpha helical propensity. This has been suggested to be important for the peptide's activity (Donnelly *et al.*, 2001b). However, recent data indicate that 2A peptides from a variety of viruses, including FMDV, are extended in the ribosomal exit tunnel. Thus, specific contacts, and possibly the conformation, of a few key amino acids of 2A are likely to drive the changes to PTC activity.

6.2 Methodology

Limited set of mutations to eRF1 and 3, plus strains in which the proteins were depleted, were used in this study. A strain, *DAmP-sup45*, was used to investigate the effects of depleting eRF1. This was complemented with an extensive set of strains expressing a variety of mutations, located in different domains of eRF1 and also eRF3, and which have different effects on canonical termination. The approach taken was to use methods that had previously been informative in analysing the 2A reaction (pulse-labelling and immunoprecipitation, measurement of growth and stop codon read-through in cells expressing a 2A-containing mRNA to high levels, and *in vitro* translation), alongside a newly developed GFP-based reporter of the 2A reaction.

The novel GFP reporter, developed in this study, allows efficient assessment of the 2A activity via measurement of the GFP fluorescence, using flow cytometry. This reporter has several

advantages including positive selection of the GFP positive cells, and no interference with cellular metabolic pathways. In addition, it provides a quick and accurate tool for studying the 2A activity. Data observed in this study through immunoprecipitation were confirmed using the GFP reporter, and results from the 2 methods were almost entirely consistent.

6.3 Key findings

The work in this thesis proved that both eRF1 and eRF3 are involved in the 2A reaction, and extend this to reveal that alteration to some residues affect the reaction more than others. Of particular note, some residues which are highly important for efficient canonical termination are not essential in the case of 2A, whereas changes to other amino acids that do not strongly affect canonical termination have much larger effects on the 2A reaction.

Further to this, the first inroads into understanding key features of the second step in the 2A reaction emerged from the work. Specifically, reduction in levels of eEF1 activity reduces the generation of the downstream product. This strongly supports the suggestion that the generation of the first peptide bond of the downstream peptide is less favourable than other peptide bond formation events in the ribosome.

6.4 Expanding discussion of the findings

Sup45: Data presented in Chapter 3 clearly show that decreased abundance of eRF1 (*SUP45*) results in significant inhibition of the 2A reaction. This was detected in the *DAmP-sup45* strain (figures 3.2 & 3.3). The inhibition of the 2A reaction was demonstrated as a reduction of both upstream and downstream products of the 2A reaction, revealing that a larger fraction of ribosomes in this case translated Pro 19 as per canonical decoding, to generate full-length translation product. Other experiments confirmed the data obtained with strains carrying the *sup45-2* and *sup45-42* alleles: these both reduce 2A activity. Expression of the 2A peptide in the *sup45-42* strain, results in a significant reduction (at $p \leq 0.01$) only in the upstream product. This is perhaps attributable to variation among experiment repeats, and the relatively high standard error (about 12): here, only the high significant difference ($p \leq 0.01$) was considered.

A key confirmatory experiment demonstrating the involvement of eRF1 in the 2A reaction was to overexpress *SUP45* in the strains with defective eRF1 (see section 3.7.1). The overexpression of *SUP45* in the *DAmP-sup45* strain restored the fractions of both upstream and downstream products of the 2A reaction (figure 3.12). Intriguingly, overexpression of *RLI1*, in the *DAmP-sup45* strain, also brought the products ratios to the level seen in the wild type. This suggests

that the increased abundance of Rli1 could enhance the efficiency of eRF1 in catalysing the 2A reaction, which is consistent with the established roles of Rli1 in termination (Muhs *et al.*, 2015; Mancera-Martinez *et al.*, 2017).

As presented in Chapter 4, a broad range of mutations located in different domains of eRF1 was analysed for effects on 2A reaction. A number of these revealed reduction of the 2A reaction, with both products of the reaction being reduced. The conclusion obtained from this work is that these mutations result in more ribosome passing through the 2A site without undergoing the reaction. Exceptions to this are E52A, which showed a decrease in the ratio of the upstream, whereas the downstream was significantly increased, compared to the wild type eRF1 (figure 4.3). By reviewing the absolute values of both upstream and downstream products, obtained after quantification and correction according to Met/Cys in each product, it is clear that this particular mutation (E52A) exhibits this effect. Absolute ratios of 2A reaction products (before normalising the values), corresponding to this mutation revealed 34% *vs* 36% in the wild type for the upstream product, and 32% *vs* 14% for the downstream. This confirms the accuracy of this part of the results, in which the downstream product is almost doubled. An additional mutation V107D, located in the N domain close to the Y-C-F motif, revealed a significant increase in both products of the 2A reaction (figure 4.3). In the case of V107D mutation, it seems that this alteration to eRF1 promotes increased efficiency of the 2A reaction over the strain expressing the wild type eRF1. Presumably this specific amino acid change alters the conformation/interaction of eRF1 such that it improves its ability to interact with the ribosome-nascent 2A peptide complex. Interestingly, this effect is diametrically opposite to the effect of this mutation on termination at stop codons, which is reduced for all three (Merritt *et al.*, 2010). Differences between the effects of a mutation in eRF1 on canonical termination and the 2A reaction were also seen with a number of other eRF1 variants. The point mutations analysed in this study in yeast eRF1 revealed specific eRF1-associated phenotypes in the case of the 2A reaction. This indicates that there may be specific eRF1 functional epitopes that are key for the 2A reaction.

Aberrant termination at a sense codon is rare in eukaryotes (~10^{-4}) (Arava *et al.*, 2005). Given that eRFs are the main factors to drive the 2A reaction, how eRFs are prompted to efficiently bind the ribosome in the absence of a stop codon is unclear. Depending on the fact that ribosomes undergo different conformational changes during the cycle of peptide bond formation and termination, Doronina *et al.* (2008) suggested that 2A may overcome the necessity for a stop codon through imposing a similar conformation on the ribosome to that undergone in the case of stop codons. This, to some extent, is inconsistent with our results. Data

in the current study suggest that eRFs, particularly eRF1, undergo a modified conformation in the functional domains to structurally, and functionally accommodate in the A site and trigger an early termination. Moreover, 2A imposes a different conformation on the ribosome/interactions of the ribosome with factors, this is supported by the observation that if the Pro 19 codon is replaced by a stop codon then ribosomes are unable to pass 2A and instead stall (Sharma *et al.*, 2012). The conformation is sufficiently unusual that a stop codon cannot be recognised and termination is effected normally. However, it is not very far from the normal conformation of the ribosome during translation as replacement of Pro 19 with other sense codons leads to efficient: it read-through is only disturbed enough to alter the ability of prolyl-$tRNA^{Pro}$ and eRF1 to interact differently to normal.

The ribosome translating a 2A peptide may pause, for a longer time than that seen in the canonical termination, at the 3' of the 2A-coding sequence, where eRF1 is supposed to be associated, yielding low concentration of effective eRF1 being available for next rounds of the reaction, this may influence/delay the recycling efficiency of eRF1 to ensure sufficient pool of eRF1 for successful termination.

Our results confirm the role of eRF1 and 3 in the 2A reaction, as key factors catalysing termination as the first step in the reaction. Machida (Machida *et al.*, 2014) argued against this scenario, suggesting that termination factors are "likely" not required for the reaction. This team proposed an alternative mechanism including interaction of the C-terminus of 2A with the exit tunnel, this possibly affects the PTC activity, leading to hydrolysis of the 2A-peptidyl-tRNA bond, rather than the reaction being catalysed by eRF1. This model is based on the understanding that peptide bonding by proline is slower than other amino acids residues (Skabkin *et al.*, 2010). This study also suggested a possible contact between the 2A C-terminus and the ribosomal protein L10e to activate the PTC, however, this study provided no evidence for this. A second possibility this study raised is that some ribosome-associated proteins could not be removed from the translation system they purified, and might be involved. A potentially confounding aspect to the Machida study is that the *in vitro* translation experiments were carried out for several hours and no time courses are presented to show how they proceed during that time. It is possible that the 2A reaction may proceed at slower (uncatalysed) rate in the absence of eRFs, and that this is what was being detected in the experiments in this paper. Furthermore, the fact that proline is relatively slower than other amino acids, in terms of peptide bond formation, is in support of our proposal, in which a competition may occur between prolyl-tRNA and eRF1 to occupy the A site of the ribosome. Our study provides clear evidence, mainly *in vivo*, but also *in vitro*, that eRFs are the key elements that are required to maintain the 2A reaction, based on biochemical analysis of mutation effects in both eRF1 and eRF3.

A three dimentional structure of eRF1 with residues examined in this study highlighted in functional domains/motifs of the protein is presented below in figure 6.1.

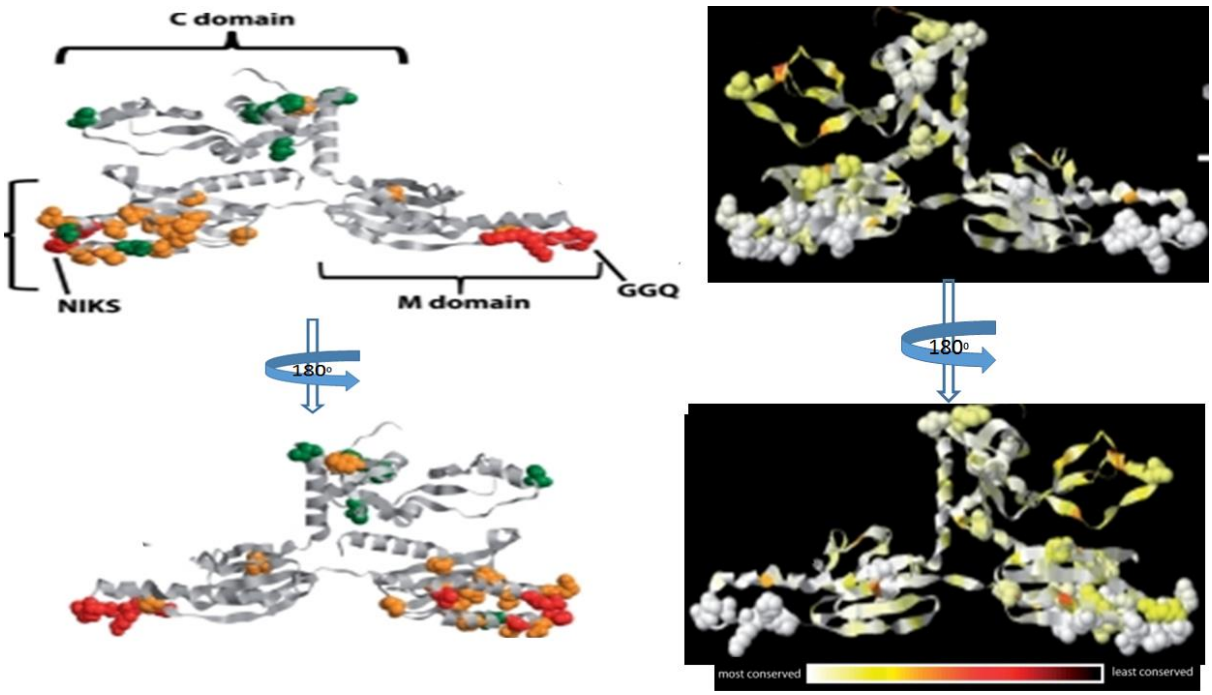

Figure 6.1: The structure of eRF1 and point mutations analysed in this study. Figures adapted from Merritt *et al* (2010).

(Top left) mutations to residues in eRF1 are presented in spacefill in a ribbon model of Sup45. Mutations are shown in orange/green. (Bottom left) same of the top with 180° rotation of the protein and mutations to residues highlighted. (Top and bottom right) the conservation of amino acids in different domains/motifs are indicated in spacefill.

6.5 Rli1 takes part in the 2A reaction

Depletion of Rli1 leads to defects in termination and ribosome recycling (Shoemaker and Green, 2011). The *DAmP-rli1* strain had no detected defect in the 2A reaction. Thus Rli1 is not a limiting factor for eRFs activity in promoting termination at 2A. However, Rli1 can enhance the reaction through overexpressing of this factor in cells with defective eRF1, to approach the value seen in cells expressing a wild type eRF1. This is in accordance with the function of Rli1 in canonical termination.

Ski7 perhaps competes with Rli1 (Rli1 promoting recycling of eRF1 from the 2A complex). Examination of strains deficient in factors that promote a variety of ribosome rescue/mRNA degradation pathways (Chapter 3) revealed an increase in the efficiency of the 2A reaction in a *ski7Δ* strain (figure 3.7). When a polylysine segment interacts with the ribosomal exit tunnel, a halt of translation is expected. This increases the probability for the mRNA to be exposed to endonucleases (Tsuboi *et al.*, 2012). This endonucleolytic activity might be provided by the exosome, where a nuclease is recruited to the ribosome by the Dom34:Hsb1 complex, however, some evidence refers to the effect of the ribosome itself to recruit this unknown nuclease (Hoshino, 2012; Inada, 2017). In contrast, no nuclease recruitment, with a continuous

translation of the mRNA, was detected in yeast strains with *ski7Δ* (Hoshino, 2012). It has been confirmed that Ski7 plays an essential role in release of polypeptides encoded by stop codon-less mRNA (Chiabudini *et al.*, 2014). Given these findings, it is possible that Ski7 is recruited to some ribosomes paused at the 2A site, leading to degradation of the mRNA. Thus Ski7 could be considered as an inhibitor of the reaction. Further experiments should then test the abundance and degradation (half-life) of 2A-encoding mRNA in strains containing or lacking Ski7.

Protein	**Mutation**	**Domain**	**Effect on 2A**		**Effect on termination**	**Protein abundance**
			Upstream	**Downstream**		
eRF1	S30P	N	↓↓↓	↓↓↓	↓	=
	I32F	N	↓↓↓	↓↓↓	↓↓	↓↓↓
	P38L	N	↓↓	↓↓	↓↓	=
	L49A	N	↓↓	↓↓	↓	Nd
	E52A	N	↓↓	↑↑	↓	Nd
	T55A	N	=	↓↓	↓	↓
	N58A	N	=	=	↓↓↓	↓↓
	S61A	N	=	=	↓	↓
	R62A	N	↓↓	↓↓↓	↓	↓
	V68A	N	=	=	↓↓	↓
	V107D	N	↑↑	↑↑	↓↓	↓↓↓
	P174Q	N	↓↓	↓↓	↓	↓
	I222S	M	↓↓	↓↓	↓	↓
	T295A	C	↓↓	↓↓	↓	Nd
	T388A	C	=	=	↓	Nd
	F401Y	C	↓↓	↓↓	↓	Nd
	Y410F	C	=	=	↓	↓

Table 6.1: Comparative effects of eRF1 mutations between stop codon and 2A reaction.

Mutations of eRF1 tested in this study, along with locations in the protein structure are summarized in this table. Comparison is made between effects of mutations on the 2A reaction and canonical termination at a stop codon. In addition, protein abundance of such mutation is shown (Merritt *et al.*, 2010). Nd: not determined, =: no effect, ↑: increase, ↓: decrease.

As seen in table 6.1, although most mutations tested here have a reducing effect on the level of protein abundance, their inhibitory effects, when found, on the 2A reaction is at least two times of that seen on protein abundance. For example, the S30P mutation inhibited the 2A reaction by ~65%, whereas it shows no effect on the protein abundance. Also, P38L inhibited the 2A reaction by ~40% with no effect on the protein abundance. On the other hand, both N58A and V68A have shown no effect on the 2A reaction but the protein abundance in these mutations is reduced (Merritt *et al.*, 2010). The evidence confirms that the inhibitory effect of such eRF1 mutations on the 2A reaction and the consequent effects on growth rates and stop codon read-through are not affected by protein availability of such mutations, but instead by the key role of each residue to maintain a normal 2A reaction. Data in support of this, the N58A and S61A mutations, which did not alter the 2A reaction, show a sharp decrease in protein abundance. Furthermore, the V107D increased both upstream and downstream products, but also has a low protein abundance. This confirms that effects seen here on 2A reaction are not results of reduced protein abundance.

The alleles tested in this study for their effects on the 2A reaction are corresponding to human eRF1 i.e. they affect canonical termination in human ribosomes. They were previously examined for their effects on the canonical termination by both *in vivo* and *in vitro* analysis, it is not surprising that both N58A and S61A, located in the core functional NIKS loop of the TASNIKS motif, have inhibitory effect on canonical termination (cause increased read-through), detected by both *in vivo* and *in vitro* assays (Frolova *et al.*, 2002; Merritt *et al.*, 2010). In our study, we chose a few mutations for an *in vitro* analysis, and found that the results mirror the corresponding *in vivo* biochemical analysis of such allele examined by pulse labelling and immunoprecipitation. The highest inhibitory effect in both upstream and downstream products was seen in S30P and I32F. Whereas R62A revealed the same effect on the downstream product but less inhibition on the upstream. P38L, L49A, P174Q, I222S, T295A and F401Y revealed less inhibition on both translation products than that observed in both S30P and I32F.

Models for stop codon decoding involve linear positioning upon physical binding between TASNIKS motif and the stop codon (Muramatsu *et al.*, 2001; Chavatte *et al.*, 2002), in addition to a cavity model, which propose that several cavities contained in the N domain of eRF1 may bind the three nucleotides of the stop codon (Fan-Minogue *et al.*, 2008; Hatin *et al.*, 2009). It has been reported that stop codons are physically linked to individual amino acids. This was assessed by an *in vitro* assay used s4U-label cross links the first base of the stop codon to either R62, S61 or K60 (yeast numbering) (Chavatte *et al.*, 2002). This concept seems to be acceptable

by our outcomes, given that R62A is amongst the mutations which show the highest inhibitory effect of the 2A reaction, determined by immunoprecipitation, growth rate measurement and consequent doubling time, stop codon read-through and confirmed by the GFP reporter. Unfortunately, we were unable to examine the K60 due to its effect on cell viability, but given that the S61A mutation has shown no effect on the 2A reaction, we presumed that the R62 residue assumed a unique role to bind a base in the Pro 19 codon in the A site. The specific conformation undergone by eRF1 includes the involvement of the S30, located in the core GTS motif, as well as I32 and P38, which are incorporated with in the YxCxxxF motif. Unexpectedly, when mutating valine 107, which is also located adjacent to the Y-C-F motif, to aspartic acid, it was revealed an increase in the 2A reaction efficiency over the value of the wild type, suggesting that the aspartic acid in this conformation plays an essential role to improve the translation. However, more investigations are required to utilise this point mutation as a tool to improve the 2A reaction.

Consistent with our observations, regarding the functional role of TASNIKS in stop codon recognition, during the canonical termination, the ribosome adopts a particular conformation, similar to that seen in stalled ribosomes in the NMD, and the pre-termination complex in mammals, containing eRF1 and 3 (Becker *et al.*, 2011; des Georges *et al.*, 2014). eRF1 contacts the ribosomal 40S subunit via its N domain, directing the TASNIKS loop in vicinity of the stop codon in the ribosomal A site. The core NIKS motif is then localised opposite to the stop codon, simulating the model suggested in bacteria (Korostelev *et al.*, 2008). A two-step model has been proposed to recognize the first two bases of the stop codon, which is followed by a conformation of the N domain of eRF1, facilitating the decoding of the second and third bases (Kryuchkova *et al.*, 2013). eRF1 contacts domain III of eRF3 via the C domain. A mini domain has been recognized in the eRF1's C domain (Mantsyzov *et al.*, 2010), which anchors eRF1 in this point, to the "beak" of the 40S subunit, while the M domain is packed against the eRF3's domains, providing an interaction surface, where the switch regions of the eRF3's G domain contact eRF1. Both M and C domains of eRF1, together with eRF3, bind to each other in such a position that the functionally essential GGQ loop is positioned between the 40S subunit and the G domain of eRF3, in a tight conformation that prevents peptide release activity at this stage. To activate the release activity, a dramatic conformational change is required to localize the GGQ loop in the PTC (Preis *et al.*, 2014).

We also found a role for eEFs in the 2A reaction, particularly in catalysing the generation of the downstream product of the reporter. This confirms that the second step of the 2A reaction

is indeed an elongational event. As the main function of eEF1α is to deliver the aminoacyl-tRNA to the ribosome, the role of eEF1β is complementary, by triggering guanine exchange. Both eEF1α and β altered the second step of the 2A reaction, by reducing the ratio of the downstream product.

To sum up our findings, it seems plausible that NIKS motif is not involved in the 2A reaction. Given that the main function of NIKS is to bind the first uridine of the stop codon (Chavatte *et al.*, 2002), which is replaced, in the case of 2A with Pro 19 codon (CCA), therefore it is not expected that NIKS loop interacts with the cytidine. Moreover, purines in the +1 position of the stop codon are likely to be incompatible, in terms of propensity to form hydrogen bond. On the other hand, GTS loop, which normally binds purines in the second and third positions (Bulygin *et al.*, 2011), may be involved in the 2A reaction by binding the adenine in the third position of Pro codon. This is perhaps carried out by S30, which, when mutated, significantly inhibited the reaction, probably caused by shifting of particular residues across different motifs (Blanchet *et al.*, 2015).

R62 is supposed to play an important role in the 2A reaction. Literatures in support of our results demonstrate that arginine in this position interacts with rRNA in the ribosome via its negative charge, allowing proper conformation for eRF1 (Blanchet *et al.*, 2015).

Finally, we have suggested a model for the 2A reaction, according to data obtained from this study, which includes the involvement of both termination and elongation factors in the reaction (figure 6.2).

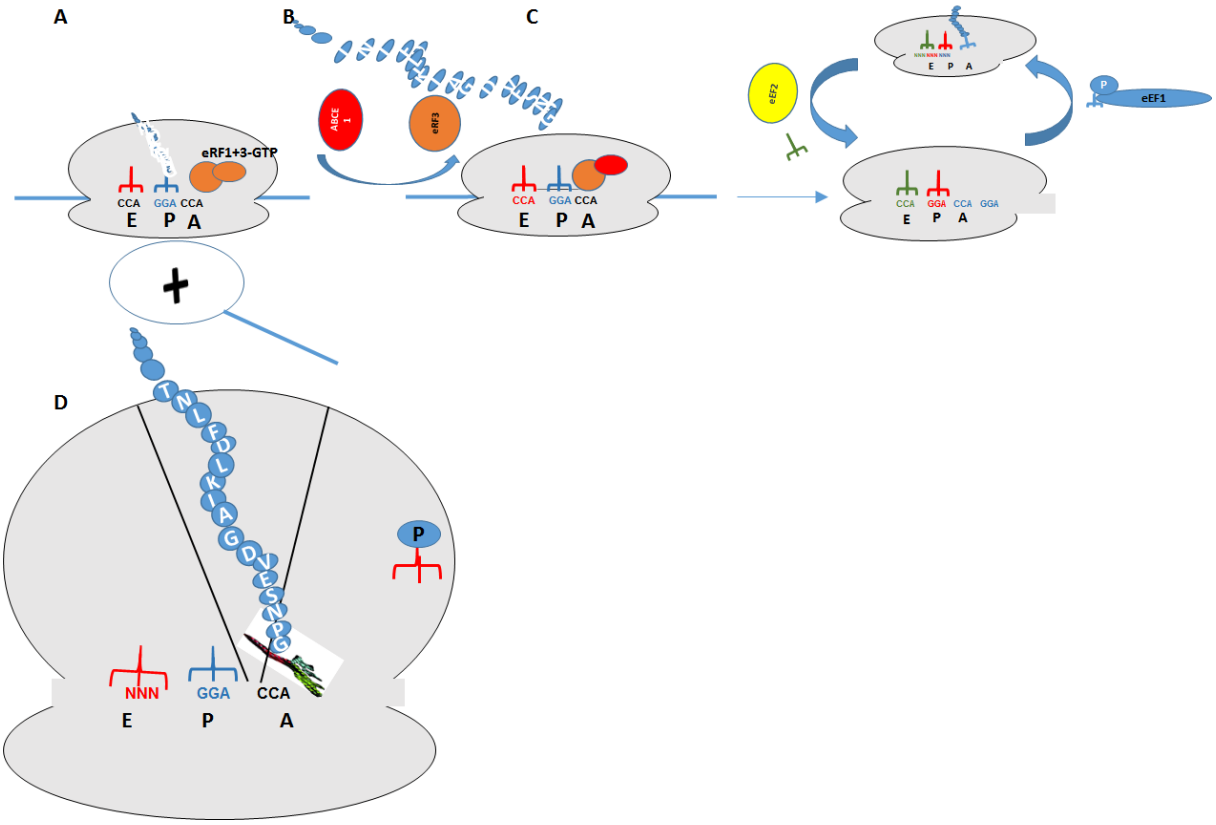

Figure 6.2: A schematic model of 2A reaction.

A model of the 2A reaction is presented according to the data of the current study. (A) The ribosome reaches the end of the 2A peptide sequence, where a Pro 19 codon (CCA) is in the A site. A recruitment of the pre-termination complex is triggered, then eRF1 binds the Pro codon, with different conformation than that with a stop codon. (B) Release of eRF3 and binding of ABCE1 to promote the peptide release. (C) After peptide release, the ribosome resumes the translation, with the help of the eEF1 to deliver the prolyl-tRNA to the A site to restart the translation of the downstream peptide. (D) Magnification view of A, showing the peptide chain of 2A inside the exit tunnel before being released from the ribosome by the action of eRF1, which is associated here to the ribosomal A site with the M domain/GGQ motif in the PTC to catalyse the peptide release.

Conclusions

2A reaction is mainly a termination event for the first part of the reaction, which includes release of nascent peptide chain from the ribosome.

eRF1 can be recruited to the ribosome to trigger termination on a sense codon rather than a stop codon.

The conformation of eRF1 undergone on 2A peptide (Pro codon) is different from that seen in canonical termination, which includes involvement of residues that are not important for the termination at a stop codon, and also, exclude the role of some residues that are essential in canonical termination.

eRF3 and ABCE1 are also involved in 2A reaction to complement the function of eRF1 in the ternary complex.

Elongation factors are also recruited to the ribosome to drive the second step of the 2A reaction, which includes the translation of the downstream mRNA sequence.

GFP reporter is a novel tool for investigating the 2A activity. It provides an independent assessment of the reaction with accurate and quick outcomes.

7 References

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