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Investigating the Restraints Upon Recombinant Protein Production in Mammalian Cells via the Manipulation of eEF2, eEF2K and mTOR

2014

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A thesis submitted to the University of Kent for the degree of Doctor of Philosophy

University of Kent

School of Biosciences

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent or any other University or Institute of learning

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Acknowledgments

I would like to thank Professor Mark Smales for his continuous support, guidance and advice throughout this work. I would also like to thank all of the members of the Smales lab for their support in and out of the lab, and their friendship, all of whom have made studying at Kent a great experience.

I would like to thank the BBSRC for their sponsorship of this project, enabling me to do this PhD.

Finally I would like to thank my family for their support and encouragement throughout this PhD.

Thanks to everyone.

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Abbreviations

4EBP1	4e binding protein 1
aa	Amnio acid
ADP	Adenosine diphosphate
Ala	Alanine
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
A-site	Acceptance site
ATP	Adenosine triphosphate
BNIP3	BCL2/Adenovirus E1B 19kD-Interacting Protein 3
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CHO	Chinese Hamster Ovary
Deptor	DEP-domain-containing mTOR-interacting protein
eEF	Eukaryotic elongation factor
eEF2	Elongation factor 2
eEF2K	Elongation factor 2 kinase
eIF	Eukaryotic initiation factor
ELISA	Enzyme-linked immunosorbant assay
E-site	Exit site
Fab	Fragment binding region
Fc	Fragment crystalisable
FLCN	Folliculn
FRET	Fluorescence Resonance Energy Transfer
FRT	Flp Recombinase Target
GAP	GTPase activating protein
GATOR	GAP activity towards Rags
GDP	Guanine diphosphate
Glu	Glutamic acid
GTP	Guanine triphosphate
HC	Heavy Chain
HEK	Human embryonic kidney

HELA	Henrietta Lack cells
HIF1α	Hypoxia-induced factor 1-alpha
HP	high producer
IBs	Inclusion bodies
Ig	Immuno globulin
IRES	Internal ribosome entry site
kDa	Kilo Daltons
LBK1	Liver Kinase B 1
LC	Light Chain
LCPS	Light counts per second
LP	Low producer
m^{7}G./m7GTP	7-methylguanylate cap
mAb	Monoclonal antibody
miR	Mirco RNA
mLST8/GβL	Mammalian lethal with Sec13 protein 8
MP	Mid producer
mSIN1	Mammalian stress-activated protein kinase interacting protein
mTOR	Mammalian target of rapamycin
mTORC 1/2	Mammalain target of rapamycin complex 1/2
PA	Phosphatidic acid
PABP	Ploly A binding protein
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
Pi	Phosphate group
PI3K	Phosphoinsitide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLD	Phospholipase D
PMA	Phorbol Myristate acetate
PML	Promyeloctic leukaemia tumour suppressor
PRAS40	Proline rich AKT substrate 40 kDa
Protor-1	Protein observed with Rictor-1
P-site	Poly-peptide site

PTM	Post translational modification
RAG	Ras-related GTPase
Raptor	Regulatory-associated protein of mTOR
rDNA	Recombinant DNA
REDD1	Regulated in development and DNA damage response 1
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
rPP	Recombinant protein production
rPs	Recombinant proteins
S6K1	Ribosomal protein S6 kinase beta 1
shRNA	Short hairpin RNA
SRP14	Signal recognition particle 14 kDa
tDNA	Transfer DNA
Thr	Threonine
TOR	Target of rapapmycin
TSC 1/2	Tuberous sclerosis complex

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Abstract

Since their first clinical application in the 1980's recombinant proteins have become an increasingly larger section of the drug market, growing into a multi-billion dollar global market. The pursuit of improving the design, production, and application of recombinant proteins for biotherapeutic uses is a key driver in industry and academia. The majority of the recombinant biotherapeutic proteins used in the clinic are produced in mammalian cell expression systems due to their capability to undertake human-like complex post translational modifications. The currently 'gold standard' mammalian cell expression system for the production of recombinant proteins is the Chinese hamster ovary (CHO) cell line. However, even with advances in mammalian cell expression technology, there is still a high cost and a long development period required for a recombinant protein therapeutic to go from design to market. As such, bottlenecks include the time taken for mammalian cells to grow and divide with slow doubling times compared to microbial systems and limited capacity to synthesise and secrete recombinant proteins. One of the cellular processes that underpins both cell growth and recombinant protein production is the translation of mRNA. Translation consists of three distinct steps: initiation, elongation and termination. One major cell signalling pathway that is considered a master regulator of both initiation and elongation of translation is mTOR, also involved in regulating ribosome biogenesis and cell proliferation. During the process of polypeptide elongation (mRNA translation), elongation factor 2 (eEF2) is a key control point that regulates protein synthesis via its de/phosphorylation. Phosphorylation of eEF2 results in its inactivation; slowing or halting elongation resulting in the attenuating of protein synthesis. This study set out to establish if manipulation of the mTOR signalling pathway and/or manipulation of phosphorylation of elongation factor 2 and the kinase that inactivates eEF2, eEF2K, in CHO cells impacts upon CHO cell growth and recombinant protein production yields. Transient expression of wild type and a Thr56Ala eEF2 mutant in CHOK1 cells affected the short term (24-48 hour) phosphorylation of eEF2 but did not appear to have an effect upon intracellular recombinant protein production and cellular growth in culture over 96 hours. Stable over expression of the wild type eEF2 construct in CHO cells resulted in a 2-fold increase in expression of eEF2 and a decrease in phosphorylation of eEF2 at the protein level; but the there was no change in the levels of total eEF2 mRNA expression. Stable expression of the Thr56Ala and Thr56Glu eEF2 mutants had a greater effect upon eEF2 expression resulting in a 3-5 fold increase in total eEF2 expression, however the phosphorylation of eEF2 was almost unchanged in an Ala56 eEF2 cell line, whereas it was reduced in the Glu56 eEF2 mutant cell

line. Growth of the CHO cells lines expressing the eEF2 mutants show that over expression of any of the eEF2 mutants resulted in a change in growth, but the Ala56 eEF2 mutant showed the largest change in cellular growth. Short term transient expression of recombinant firefly luciferase in the stable eEF2 cell lines revealed that the Thr56Ala mutant greatly increases the CHO cells total recombinant protein production. Further, mutation of eEF2 to Ala56 or Glu56 had little effect upon the mis-incorporation of amino acids during translation. The transient knockdown of eEF2K was achieved and this was shown to prevent eEF2 phosphorylation. However, CHO cells do not appear to tolerate the knockdown of eEF2K stably or at only very low levels. This suggests that a sustained, high level of eEF2K knockdown is lethal to the cell; which would result in the loss of eEF2 regulation for an extended period of time. Transient expression of the eEF2K shRNA into CHOK1D6 cells stably expressing firefly luciferase, a non-secreted protein, resulted in a 5-fold increase in luciferase expression showing that knockdown of eEF2K increased the short term productivity of these cells. Interestingly, the study of CHO cell lines with varying recombinant monoclonal antibody protein production capacities revealed that levels of total and phosphorylated eEF2 did not appear to change in correlation with the mAb titre. These data suggest that eEF2 activity is tightly regulated across cell lines and is not directly related to recombinant protein secretion in these industrially used CHO cell lines The addition of 2 µM of PMA, an activator of mTOR signalling, increased antibody production in a low producer cell line but had no effect upon the antibody production from a high producing cell line. Together the data presented here shows that manipulation of eEF2 and eEF2K activity can enhance cellular growth and recombinant protein production from CHO cells. As such, new engineering approaches that allow the manipulation of both elongation and polypeptide synthesis combined with the secretory capacity of the cell are likely to yield new CHO host cells with more predictable recombinant protein capacity and further advance our understanding of the role of mRNA translation in controlling cell proliferation, and both global and recombinant protein synthesis.

Chapter 1

Introduction

1.1 Biotherapeutic Recombinant Proteins

Recombinant proteins are proteins that have been produced by recombinant DNA (rDNA) technology using a specific expression system to generate the target protein of choice. These can be proteins made for example, for research purposes, for use in diagnostics or for biotherapeutic use in the clinic to treat a wide range of diseases (Holt et al. 2000; Amadori et al. 2002). Almost all recombinant proteins are made using cell expression systems, including the use of expression systems from both prokaryotes and eukaryotes such as plants and plant cells, insect cells, mammalian cells, yeasts, and bacteria. In the majority of cases the rDNA is delivered into the cell expression system on a plasmid to allow subsequent transcription and translation of the target gene of interest in live cells cultured under laboratory conditions. More details on particular expression systems are discussed below.

The use of recombinant proteins as medical therapeutics has become common-place since the first approved molecule in the 1980s. Naturally occurring proteins were the first recombinant proteins that were put into trials to be use as a medical therapeutic (Gulan et al. 1987). Through the study of protein sequencing and rational design the first recombinant therapeutic to be put into clinical use was Humulin®, recombinant human insulin, which was approved by the FDA in 1982 (Butler 2005). Since the commercialisation of insulin, other biotherapeutic proteins have become available through recombinant techniques that are able to treat other disorders and diseases.

Since the launch of the first recombinant biotherapeutic protein in the 1980's, biotherapeutic proteins have constituted a large and growing proportion of the total pharmaceutical market (which includes traditional small molecule drugs, e.g. antibiotics). As such, biologics based medicines as a whole now represent a major portion of total pharmaceutical sales, with a value of $150 billion (US$) in 2011, and are predicted to reach $252 billion in total sales by

2017. Of this $252 billion, monoclonal antibody (mAb) based products, are predicted to account for $89.9 billion of sales (Highsmith 2013). The majority of recombinant protein biopharmaceuticals are produced in cultured mammalian cells (Walsh 2010a), with the most commonly used industrial mammalian cell host being the Chinese hamster ovary (CHO) cell (Kim et al. 2012). As of 2010, the number of approved recombinant protein products in Europe and the US was 200 (Walsh 2010a), of which 27 had 'blockbuster' status (sales over $1 billion per year).

Biotherapeutic proteins therefore constitute an important section of health care medicines, but as these continue to grow in terms of the number of such biologic drugs and indications they can be used for they also contribute to increasing health costs globally (Sibbald 1999). In 2011, biopharmaceuticals accounted for more than half of all molecules in the drug development pipeline (Carter 2011). The costs to develop a biotherapeutic drug have been estimated as ranging from approximately $2000 to $20000 US$ per gram (Kelley 2009), associated with this high cost of development is the fact that the majority of drugs do not produce revenues that recover their research and development costs (Vernon et al. 2010). As a result there is a need to be able to develop and produce recombinant biotherapeutics more rapidly, efficiently, and cost-effectively, whilst maintaining or improving upon the product quality and authenticity of the final product molecule.

One of the ways to address the challenges of reducing time-lines for the development of such complex molecules, as well as improving efficiency and product quality whilst reducing costs would be to generate new host expression systems that are more amenable to production of a wider range of molecules, grow faster to higher biomass (thus reducing time lines) and produce higher yields of a high quality product. The work undertaken in this thesis was focused upon improvements to CHO host expression systems as outlined in more detail below to potentially improve product yield and quality therefore ultimately impacting upon the ability to produce the required quantities of these high value biotherapeutic molecules. Given the dominance of the CHO production platform (Kim et al. 2012) this was focused upon.

1.2 The Biotherapeutic Protein Landscape

Of the top ten pharmaceutical sales in the US in 2012, eight were protein-based biotechnology-produced products with only two being traditional small molecules synthesized by organic chemistry (Staton 2012). Of the eight protein biotherapeutic molecules, six were monodonal antibodies demonstrating the importance of this class of molecule in the market place (Modjtahedi et al. 2012; Parkinson et al. 2012; Staton 2012). These represent the top protein based drugs by sale. When biopharmaceuticals are looked at in terms of their use in different clinical areas, the top three applications in 2010/2011 were: haematopoietics, for the treatment of blood disorders; monodonal antibodies for treatment of a large number of different indications, and cytokines (Figure 1.1). Monoclonal antibodies therefore currently constitute the greatest number of biotherapeutic molecules in the R&D pipeline, the majority of top selling pharmaceuticals and a major portion of total amount of protein used in the clinical setting. Currently in development there are a large number of biopharmaceuticals, some of which are highlighted in table 1.1

Table 1.1 Biological therpetuics currently in development or recently released. The information was sources from the respective companies' public pipeline web pages. Information from Roche was gathered from their 2013 annual report.

Product name	Function/ disease treatment	Company	Year
Alemtuzumab	Multiple sclerosis	Genzyme	2014
Acid sphingomyelinase	Familial amyloid cardiomyopathy	Genzyme	2014
Neo-GAA	Pompe disease (2nd generation enzyme)	Genzyme	2014
Brodalumab	Psoriasis	Amgen	2014
Blinatumomab	Acute lumphoblastoc leukemia	Amgen	2014
Rilotumumab	Gastric cancer	Amgen	2014
Bococizumab (RN316) (PF-04950615)	Hyperlipidemia	Pfizer	2014
Inotuzumab ozogamicin	Actue Lymphoblastic Leukemia	Pfizer	2014
MabThera/Rituxan	Non-Hodgkins lymphoma, chronic lymphocytic leukemia and rheumatiod arhritis	Roche	2013
Avastin	Colorectal, breast, lung, kidney, ovraian cancer and glioblastomna	Roche	2013
Herceptin	HER2-positive breast and gastric cancer	Roche	2013
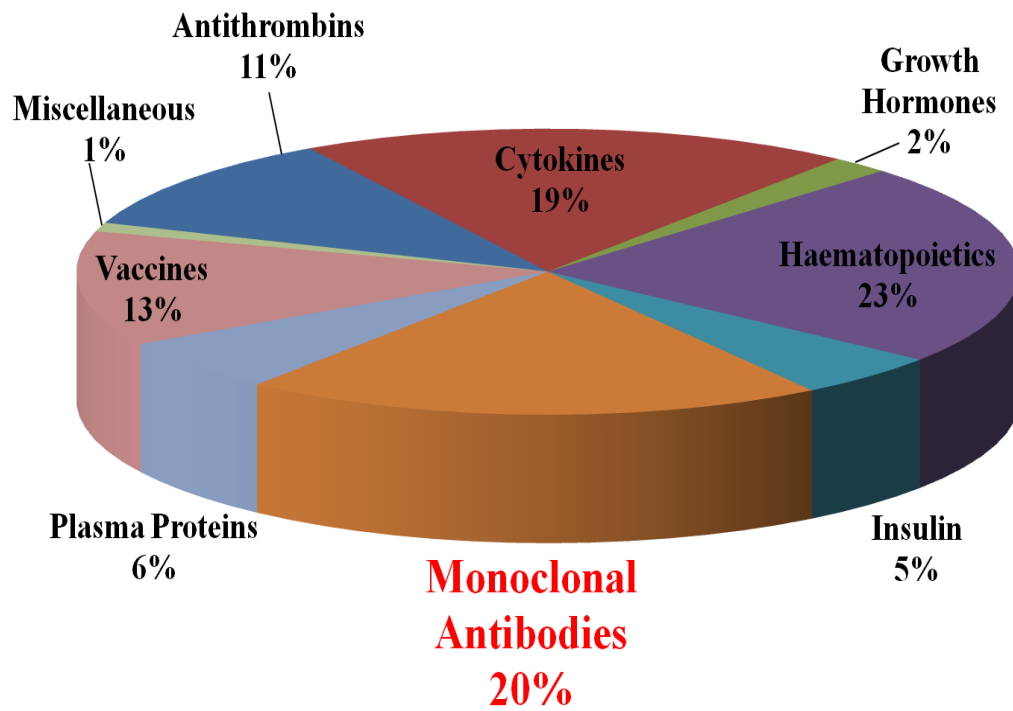
Figure 1.1 The distribution of the different classes of recombinant proteins in terms of how much each class of protein contributed to the market at 2010-2011 (Carter 2011)

1.3 Monoclonal Antibodies (mAbs)

Antibodies are naturally synthesised proteins produced by B-cells in complex organisms as a defence mechanism against foreign bodies or antigens (Reddy & Corley 1999). Antibodies are generated as part of the immune response whereby B-cell lymphocytes differentiate into Ig (antibody) secreting plasma cells when a foreign body is recognised (van Anken et al. 2003; Schmidlin et al. 2009). In order for the cell to be able to synthesis, assemble, post-translationally modify and secrete large amounts of antibody material, the plasma cell undergoes a wave of reprogramming events to support and enhance the required metabolic and secretory capacity of the cell to deliver the required Ig amounts (van Anken et al. 2003). Antibodies are made that are specific to a particular epitope of the foreign body, and via the immune response a pool of antibodies to different epitopes of the antigen are produced, thus generating a so called polyclonal pool of antibodies. For biotherapeutic purposes, monoclonal antibodies (mAbs) are usually used or desired where a specific antibody to a specific epitope is produced recombinantly.

The Ig monomer is traditionally depicted in the form a Y-shape structure as shown in Figure 1.2 and is approximately 150 kDa in size (Schroeder & Cavacini 2010). Each individual Ig molecule is assembled from two identical heavy chains (HC) and two identical light chains (LC) with each chain being made up of a number of different domains: the constant and variable domains. It is the variable domains that come together to form the antigen binding site with specificity for a particular epitope whilst the constant region of the heavy chain defines the class of Ig an antibody molecule belongs to. There are five Ig classes, IgA, IgD, IgE, IgG and IgM; in humans each isoform has a distinct function and localisation i.e. IgA is main found in saliva (Jefferis & Lefranc 2009).

As shown in Figure 1.2, the Ig structure is comprised of two Heavy chains (HCs, approximately 50 kDa each) and 2 light chains (LCs, approximately 25 kDa each). These chains form a Y shaped structure, with the two heavy chains forming the base of the structure and the light chains form the arms, with a hinge region at the intersect of the Y shape (Wang et al. 2007). The lower half of the heavy chain structure (CH2 and CH3) form the fragment crystallisable region (Fc), this being a sequence of amino acids that has a highly conserved sequence and structure for recognition within the immune system, which actives the compliment response

(Heyman 2000; Ochsenbein & Zinkernagel 2000; Sörman et al. 2014). Conversely, at the head of each arm of the antibody is the fragment antigen binding region (Fab); this region is produced on an antigen specific basis, making each antibody highly specific for its target (Arevalo et al. 1994). It is this Fab and Fc region functionality which makes antibodies a desirable therapeutic, the Fab can be engineered for a specific target where the Fc can be used to activate cell killing, to be inactive and use only the antigen binding region to neutralise a target or be modified to either carry a small molecule, or be probed using another antibody or molecule in an analytical test or experiment (Zola & Swart 2005).

Of the five isoforms, it is the IgG isoform that is currently and almost exclusively used in biotherapeutics applications. The IgG isoform has 4 sub classes IgG1-4, which are dependent upon the inter- and intra-chain bonding between the HCs and LCs and identified by these substructures and bonding patterns and their abundance in serum levels (Schroeder & Cavacini 2010). Mammalian cells are engineered to produce and secrete one of the four sub classes, IgG1 being the most commonly used sub class. It should also be noted that the heavy chain is N-glycosylated and the correct glycosylation is often important for the function of the molecule as a biotherapeutic molecule and can affect the *in vivo* half-life of the molecule (Zheng et al. 2011). Due to the complex nature of antibodies, their synthesis and assembly requires the correct folding, assembly and post-translational modification of two heavy chain (HC) and two light chain (LC) Ig polypeptides in the ER (Feige & Buchner 2014; Hendershot et al. 1987; Leitzgen et al. 1997) assisted by molecular chaperones, transport to the Golgi for further glycosylation processing (Werner et al. 2007) and then secretion out of the cell (Jefferis 2005; Holland et al. 2006). The manufacture of such complex molecules is therefore usually undertaken in cultured mammalian cells as these contain the correct machinery to correctly synthesise and assemble the desired product and provide human-like post-translational modifications that will not elicit immune responses in the patient (Jefferis 2005).
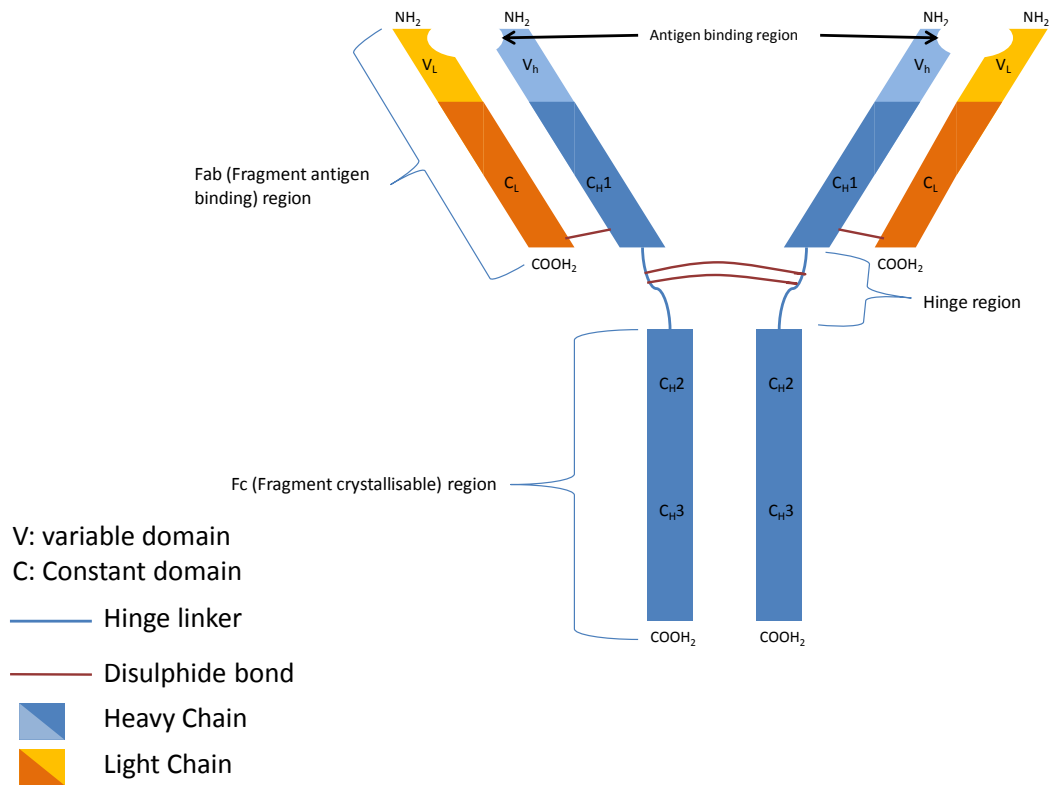
Figure 1.2 Schematic depicting the make up of an Ig antibody. The antigen binding regions are design specifically per antigen. The sub classes of the IgGs differ in disulphide bonds between the C_H2 regions of the F_C region and the C_H1 and C_l regions of the Fab regions.

In addition to classical antibody molecules, there has been a great deal of interest in generating novel types of antibody based molecule for biotherapeutic applications that are not naturally found or made. For example, monoclonal antibodies have been modified to carry small molecules and radioisotopes to specific targets, allowing for a greater specificity when treating disease such as cancer (Adams & Weiner 2005; Carter 2001). Novel molecules such as a mAb Fc region conjugated to a different small molecule or protein such as a cytokine, designed as an alternative to mAb therapy have also been investigated, often referred to as FC- fusion proteins. Such molecules are shown schematically in figure 1.3. These types of molecule have different properties compared to antibodies and allow for an increase in the half-life of the conjugated molecule and by preventing absorption of the Fc-fusion by the kidney or its depredation in an endosome (Carter 2011).

Fc fusion
proteins

R-protein/small
molecule

Radio
isotope

C_H2

C_H2

Fc (Fragment crystallisable) region

C_H3

C_H3

C: Constant domain

Hinge linker

Disulphide bond

Heavy Chain

$COOH_2$

$COOH_2$
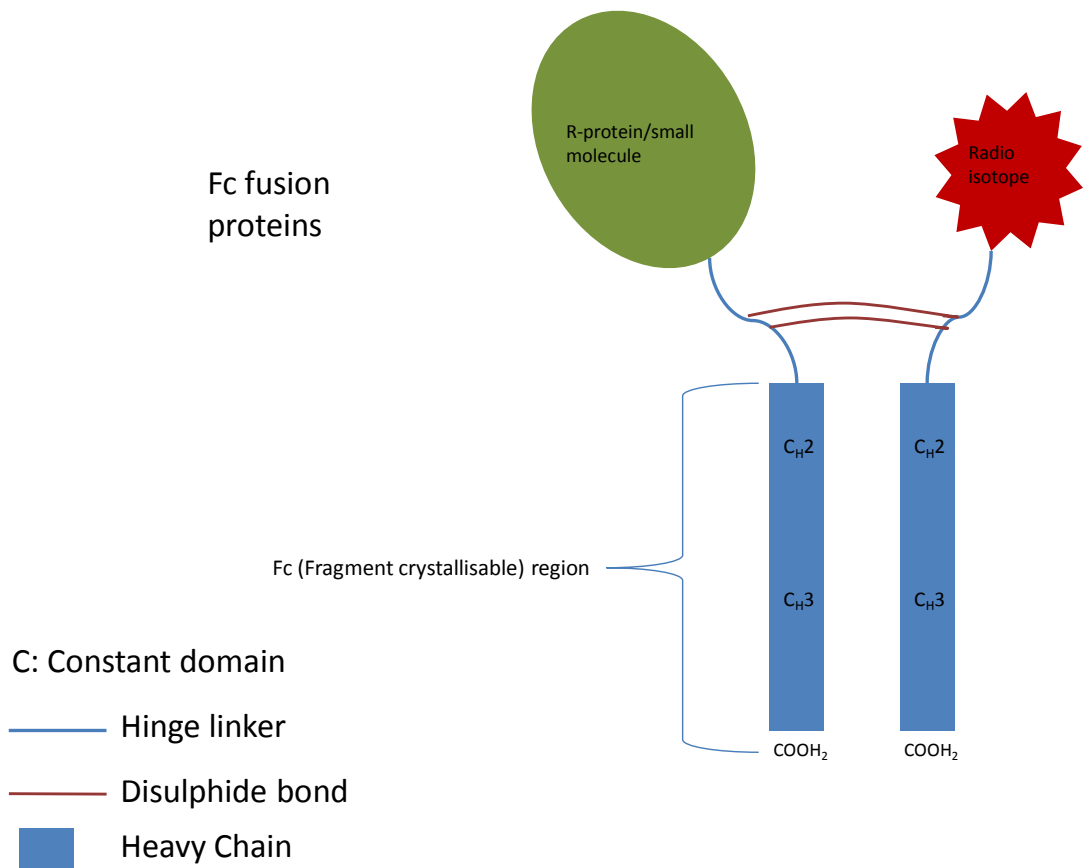
Figure 1.3 Fc fusion recombinant proteins; Fc regions of a monoclonal antibody, with a recombinant protein therapeutic or a small molecule replacing the Fab region.

1.4 Different Expression Systems for the Commercial Production of Biotherapeutic Proteins

As stated above, CHO cells are the predominant expression system used for the production of biotherapeutic proteins. However, a number of other expression systems are used and the capacity of these bacterial, insect, yeast and mammalian cells to produce high yields of recombinant biotherapeutic proteins in large-scale fermentations have been greatly enhanced through a combination of rational and empirical manipulations of host, vector and bioprocess engineering (Kotsopoulou et al. 2010; Le Fourn et al. 2014; Wurm 2004). Each system has advantages and disadvantages with respect to their application for the production of biotherapeutic recombinant proteins. Two expression systems are predominantly used for

production of biotherapeutic proteins in addition to CHO, *E. coli* and the yeast S. *cerevisiae,* together these three expression systems being responsible for >90% of all the biopharmaceuticals in clinical use, the majority of which are secretory proteins (Walsh 2010b).

E. coli are used to produce about a third of all biotherapeutic rPs approved to date (Walsh 2010b) , this includes the production of antibody fragments, insulin and various cytokines and growth factors. Biotherapeutic proteins expressed in *E.coli* can be expressed in the cytoplasm as a soluble protein, expressed as insoluble inclusion bodies (IBs) that require subsequent solubilisation and refolding; or can be exported into the periplasmic space (Balasundaram et al. 2009). The advantages of this expression system are that the bacteria grow rapidly, therefore fermentation times can be reduced, high yields can be achieved, transformation can be undertaken with small amounts of DNA and the physiology of *E. coli* is well characterised. However, there is a limited repertoire of folding and post-translational modification (PTM) machinery in these cells (e.g. lack ability to form disulphide bonds in the cytoplasm (Baneyx & Mujacic 2004) and hence the production of complex molecules that require human like PTMs is not possible.

On-the-other-hand, the use of the yeast (particularly *S. cerevisiae*) has advantages over the prokaryotic *E. coli* system as a eukaryotic expression host for biotherapeutic recombinant protein production because it can be easily genetically manipulated and has relatively simple downstream processing (Idiris et al. 2010)(Celik & Calık 2012). Unfortunately, although yeasts are able to undertake complex folding and PTMs, it is often limited in its ability to cope with the demands of high recombinant protein expression and secretion, and the glycosylation profiles are not human like. Such bottlenecks can potentially limit the yield and authenticity of the resulting recombinant target product (Idiris et al. 2010)(Celik & Calık 2012). In summary, yeast are able to grow fast, perform PTMs and secrete target proteins into the growth medium but have inappropriate human glycosylation, are inefficient as secretion and can produce proteases that can degrade the target product (Frenzel et al. 2013).

The mammalian CHO cell line is the current cell line of choice for the industrial production of complex biopharmaceuticals. This is demonstrated by the fact that of 58 new

biopharmaceutical products approved in the period 2006-2010, 32 were expressed in CHO cells (Walsh 2010a). CHO cells have a track record of approval by regulatory agencies for the production of biopharmaceuticals, and have complex cellular machinery required to complete the folding, assembly and post-translational modifications essential to the production of fully functional recombinant glycoproteins (Li et al. 2010)(Birch & Racher 2006). However, CHO cells are expensive to maintain due to the complex medium used to grow them, have a slow doubling rate meaning production cycles are lengthy as are the development processes and the high production cost. Further, whilst high yields of some products can be achieved, the yields of other products can be low. Therefore there is a need to further improve product quality (i.e. product heterogeneity, aggregation, glycosylation) to meet regulatory requirements and to further enhance yields and product activity, resulting in reduced costs and improve supply of biotherapeutic drugs.

1.5 The Chinese Hamster Ovary Cell Lines

As described above, the production of biotheraputic proteins, especially mass production in industry, relies upon the cells used to produce them. A number of different cell types from prokaryotes and eukaryotes, have been used as a production system over the years since the first implementation of recombinant proteins in medical therapies but the CHO cell has emerged as the expression system of choice in the majority of cases as described above. Other mammalian cells are also used in industry, such as human embryonic kidney-293 (HEK-293) cells and NS0 cells (a human carcinoma cell line) (Butler 2005)(Chu & Robinson 2001).

The original CHO cell line was developed in the 1950's (Robinson 1958) and has since emerged as a preferred host for the large scale production of biotherapeutic proteins. This is due to the fact they are robust and easy to handle and culture, they proliferate/divide approximately every 24 hours, tDNA can be transfected both transiently (Geisse & Henke 2005; Kim & Eberwine 2010; Daramola et al. 2013) and stably (Baldwin et al. 2003; Wurm 2004) into CHO cells which can then secrete the recombinant product into the culture medium, they are adaptable to different growth conditions, can undertake human like glycosylation and fold and assemble multi-domain/subunit proteins (e.g. antibodies). There are actually are number of different CHO cell hosts used industrially derived from the initial CHO cell line isolated by Puck. These include CHO-K1, a subclone from the parental CHO cell line in 1957 requiring proline for

growth (Robinson 1958). From the initial proline-dependent strains, a number of additional CHO cell lines have been subsequently generated (Urlaub & Chasin 1980; Urlaub et al. 1983; Hacker et al. 2009).

Major advances in the yields of recombinant proteins from CHO cells have been achieved since the 1980s. In the late 1980s yields of 10-20 pg/cell/day specific activity and 50-100 mg/L volumetric activity from a 7 day culture with a maximum cell density of 1-2 million cells/ml were being achieved (Hacker et al. 2009). More recently, up to 50-90 pg/cell/day specific activity and 1-5 g/L volumetric activity from a 21 day fed batch culture with a maximum cell density of 10-15 million cells/ml have been achieved (Li et al. 2010; Hacker et al. 2009). Further, the recent publication of the CHO genome in 2011 has made genetic engineering of CHO cells much easier. However, there have been a number of different approaches already taken to try and engineer CHO cells for improve culture lifetime, enhanced product yield and authenticity. These are discussed further below.

1.6 Approaches Taken to Date to Improve Biotherapeutic Recombinant Protein Expression from CHO Cells

There have been several advances in recombinant since their introduction into commercial use as bio-therapeutics. Research in every part of recombinant protein production has been conducted to improve recombinant protein yield from mammalian cells. Studies of cells capable of expressing recombinant proteins have revealed several limiting factors in the process of recombinant protein production. Several approaches have been taken to improve upon these limitations.

One such approach is to optimise the culture medium formulation to increase the recombinant protein produced from the mammalian cell lines used. Jordan et al. (2013) report that the 'reshuffling' of the cell culture media components resulted in an improvement of mAb titre from the CHO cells used in the study. Therefore provision of essential nutrients and components to the mAb expressing CHO cells can remove certain nutrient limiting factors regarding recombinant protein expression and provide data for further optimisation of the culture medium.

A review by Gustafsson et al. (2004) collated studies of gene/codon optimisation in various cell lines; stating that different cell lines show bias towards certain sequences from protein translation and that optimisation of the codon sequence improves the recombinant protein expression from the host cell line (Gustafsson et al. 2004). In 2010 Kotsopoulou et al. then show that optimisation of codons resulted in a lower copy number of mRNA, and subsequently stated that via improving the codon optimisation and increase the copy number results in an increase in recombinant protein production (Kotsopoulou et al. 2010).

Of course if the culture of cells producing recombinant protein longevity increases so would the total recombinant protein secretion. Sanchez et al. (2014) report that the depletion of miR-7 (mirco RNA 7), improves CHO cell culture longevity and recombinant protein yields. Although, it is possible that the depletion of miR-7 is responsible for the improved recombiant protein expression as well as the culture longevity. Le Forn et al. (2014) demonstrated that there are also limiting components within the secretory pathway e.g. SRP14, and suggests that engineering of this secretory pathway could improve upon the recombinant protein expression from CHO cells in the future (Le Fourn et al. 2014).

An area of particular relevance to this thesis where work has been undertaken with regard to identifying limitations in CHO cells with respect to recombinant protein production is the process of mRNA translation, are discussed in more detail is the following sections. Indeed, mRNA translation is important in controlling global and mRNA specific translation rates and hence protein synthesis. Before a cell can divide and produce more biomass, it must first double its protein biomass, an energy-expensive process which involves extensive protein synthesis. As described above, one of the rate limiting steps in the development of mammalian cell lines for the production of recombinant proteins is the doubling rate of the host cells used for bioproduction, which is typically around 24 h. As a result, much of the development time involves waiting for cells to reach sufficient biomass. Reducing the doubling time (increasing the proliferation rate) would allow faster cell line development times and hence reduce production costs and timelines, resulting in quicker delivery of material for clinical trial. Apart from cell growth, there are now a number of reports that suggest that the major cellular constraints upon therapeutic recombinant protein production throughout the gene expression pathway in mammalian cells are post-transcriptional (O'Callaghan et al. 2010; Fussenegger &

Betenbaugh 2002; Smales et al. 2004; Khoo & Al-Rubeai 2009; Mead et al. 2012). Among the key control points is mRNA translation: a number of reports having shown that translation is a key determinant of high producing cell lines (O'Callaghan et al. 2010; Khoo & Al-Rubeai 2009; Mead et al. 2012) and others that report that manipulation of ribosome biosynthesis can lead to the enhancement of recombinant protein yields (Santoro et al. 2009) Therefore, control of mRNA translation and ribosome biogenesis is important in determining the yield of recombinant proteins from mammalian cells. One master regulator of ribosome biogenesis, mRNA translation and cell growth is mTOR, discussed in more detail below. Protein synthesis is catalysed by ribosomes, and mTOR is a master regulator ribosome biogenesis as well as mRNA translation (i.e., protein synthesis) (Foster & Fingar 2010). A recent report has shown that the manipulation of mTOR simultaneously improved key processes underpinning recombinant protein production from CHO cells including cell growth, proliferation, viability and cell specific productivity and concluded that mTOR-based engineering of mammalian cell lines has a promising future for the improvement of the bioprocessing of biotherapeutic proteins (Dreesen & Fussenegger 2011b). A further study has also reported that in plasma cells (the cells that 'naturally' synthesize and secrete IgGs) protein synthesis is regulated by crosstalk between endoplasmic reticulum stress and mTOR signalling (Goldfinger et al. 2011). As such, there is a compelling argument for investigating the manipulation of mRNA translation and mTOR signalling in CHO cells as part of a strategy to develop new CHO cell lines with enhanced properties for biotherapeutic recombinant protein production.

1.7 mRNA Translation

mRNA translation is a key regulatory step in the control of gene expression in eukaryotes and a recent study has reported that the cellular abundance of proteins is predominantly controlled at the level of translation (Schwanhäusser et al. 2011). The perception of stress or a change in environmental conditions by eukaryotic cells leads to the global attenuation of cap-dependent mRNA translation via a range of mechanisms whilst the translational efficiency of specific mRNAs is actually increased (Pain 1996). The general mechanism for controlling the activity of the translational machinery is via the phosphorylation of translation initiation or elongation factors leading to their (in)activation (Pain 1996). mRNA translation is a three-stage process comprised of initiation, elongation and termination (Sonenberg & Hinnebusch 2009). Although it has been proposed that translational control is mostly exerted at the initiation phase (Sonenberg & Hinnebusch 2009), recent data suggest that elongation makes a major

contribution to the overall regulation of this process (Kenney et al. 2014). The different phases of translation in mammalian cells are discussed in more detail below.

1.7.1 Translation Initiation

Initiation of mRNA translation is the assembly of the ribosome at the AUG start codon of the mRNA and is centred around activities at the 5' prime cap of the mRNA. This occurs via two mechanisms: Cap-dependent and cap-independent translation. Only a small percentage of mRNAs are translated in a cap-independent manner in mammalian cells, around 5% (Merrick 2004). Cap independent translation occurs via insertion/formation of the ribosome into an internal ribosome entry site (IRES) coded for in the mRNA downstream of the 5'-cap but before the start AUG codon and is the method used by viruses to hijack the cellular machinery when viral infection results in shut-down of the cap-dependent mechanism (Spahn et al. 2001). Cap-dependant translation is a highly regulated process involving several eukaryotic initiation factors (eIFs) and is discussed in detail below.

1.7.1.1 Cap Dependant mRNA Translation Initiation

The 5' cap on an mRNA is added in the nucleus and aids export out of the nucleus as well as translation initiation and consists of a guanine nucleotide connected to the mRNA via a triphosphate linkage. The guanosine itself is methylated on the 7-position directly after capping *in vivo* by a specific methyl transferase and hence the cap is referred to as a 7-methylguanylate cap (abbreviated to m^7G.) (Banerjee 1980). The 'cap', as shown schematically in Figure 1.4, is attached to the 5 prime end of the mRNA and is the site where the eukaryotic initiation factor (eIF) eIF4E binds to recruit additional eIFs to form the eIF4F complex (Figure 1.4). Several of the eIFs involved in the formation of the ribosomal complex are regulated by mTOR as discussed further later in this chapter.

1.7.1.2 Formation of the 80S Ribosome Complex

Figure 1.4a depicts the formation of the eIF4F complex around the 5' prime mRNA cap. The cap is recognised by the cap binding protein eIF4E, which forms a complex with eIF4A/B (an ATP dependant RNA helicase) and eIF4G which binds both eIF4E and eIF4A to form the

complex eIF4F. eIF4G binds to the polyA binding protein PABP which binds to the 3' polyA tail of the mRNA resulting in circularisation of the mRNA (Figure 1.4a). The eIF4F complex therefore consists of eIF4E (cap-binding protein), eIF4G (scaffolding protein) and eIF4A (RNA helicase) to form the larger eIF4F complex as shown in figure 1.4a (Sachs et al. 1997; Gingras et al. 1999a; Komar & Hatzoglou 2005). The eIF4F complex is then able to recruit the 43S ribosomal subunit to the mRNA (Pain 1996; Spahn et al. 2001).

The 43S ribosomal subunit is formed from eIF3, eIF5, eIF1, eIF1A and a ternary complex (TC) comprised of eIF2, GTP and Met-tRNA ,and the 40S ribosomal subunit as depicted in Figure 1.4b (Merrick 2004). This complex if formed by recruitment of the TC to the 40S subunit by eIF3, eIF5, eIF1, eIF1A (Merrick 2004). Specifically, at the 40S subunit the initiator methionyl-tRNA (tRNA$_i^{MET}$) is recruited by eIF2·GTP (eIF2 complexed to GTP) and eIF5 (Shin et al. 2011). Along with these initiation factors, eIF3 and eIF1A (both prevent the 60S subunit binding at this stage) bind to the 40S subunit forming the 43S pre-initiation complex (Figure 1.4b) (Maag et al. 2005; Maag et al. 2006). The 43S subunit interacts with the 5' end of the mRNA through the interaction of eIF3 with eIF4F to form the 48S complex. The 48s complex then begins to scan the mRNA for the AUG start codon using eIF4G, eIF1 and eIF1A (Figure 1.5) (Pestova & Kolupaeva 2002; Merrick 2004). The first AUG is considered the initiating codon found in the consensus sequence of the mRNA during scanning (Gingras et al. 1999a). Factors eIF4A, eIF4B 'unwind/remove' any secondary structures in the mRNA using energy generated by ATP hydrolysis. Whist scanning, the GTP bound to the eIF2 unit is hydrolysed into GDP+Pi by eIF5, the GDP+pi+eIF2 complex is retained within the scanning complex unit, by eIF1, until a start codon is found (Merrick 2004; Sonenberg & Hinnebusch 2009). Once the initiator AUG is encountered, the initiation factors are released (Figure 1.5), these previously prohibiting the binding of the 60S subunit to the 40S. Once the eIFs are disassociated from the closed complex and the larger 60S ribosomal subunit is recruited by eIF5b via hydrolysis for GTP (Pestova et al. 2000) and translation elongation of the mRNA can begin (Figure 1.5b) (Sachs et al. 1997; Gingras et al. 1999a).
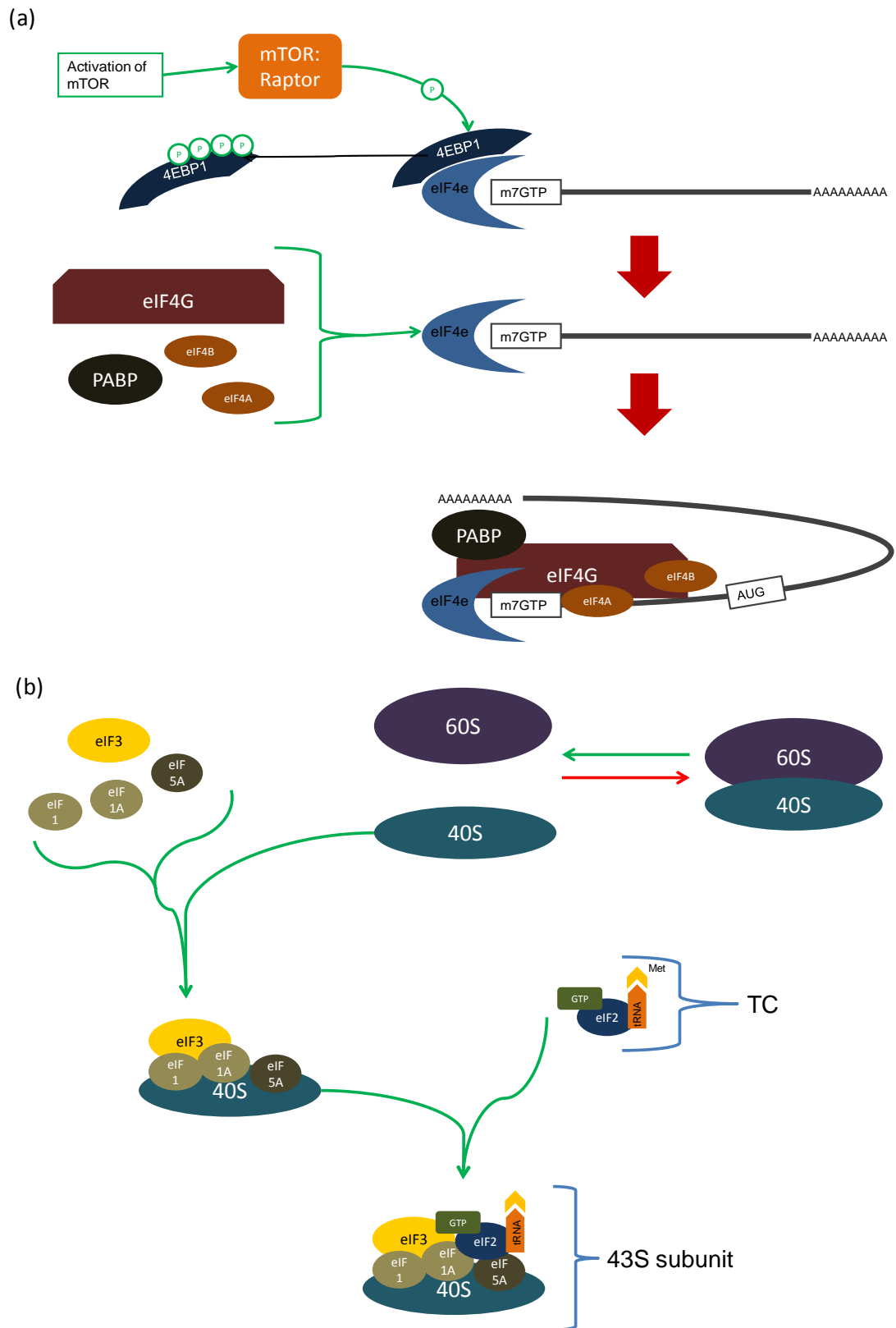
Figure 1.4 (a) Formation of the eIF4F cap complex around the m7GTP-mRNA. In a nutrient and energy rich environment mTOR:Raptor phosphorylates 4E binding protein 1 (4EBP1), reducing its affinity for eIF4E allowing it to recruit eIF4G, eIF4A, eIF4B and Poly A binding protein (PABP). **(b)** Formation of the 43S ribosomal subunit. When the 40S subunit is released from the 60S, eIF3 recruits eIF1A, eIF1 and eIF5A to the 40S subunit. Once bound to the 40S unit eIF3 recruits the ternary complex (TC) (eIF2:GTP:aa-tRNA) to form the 43S ribosomal subunit.
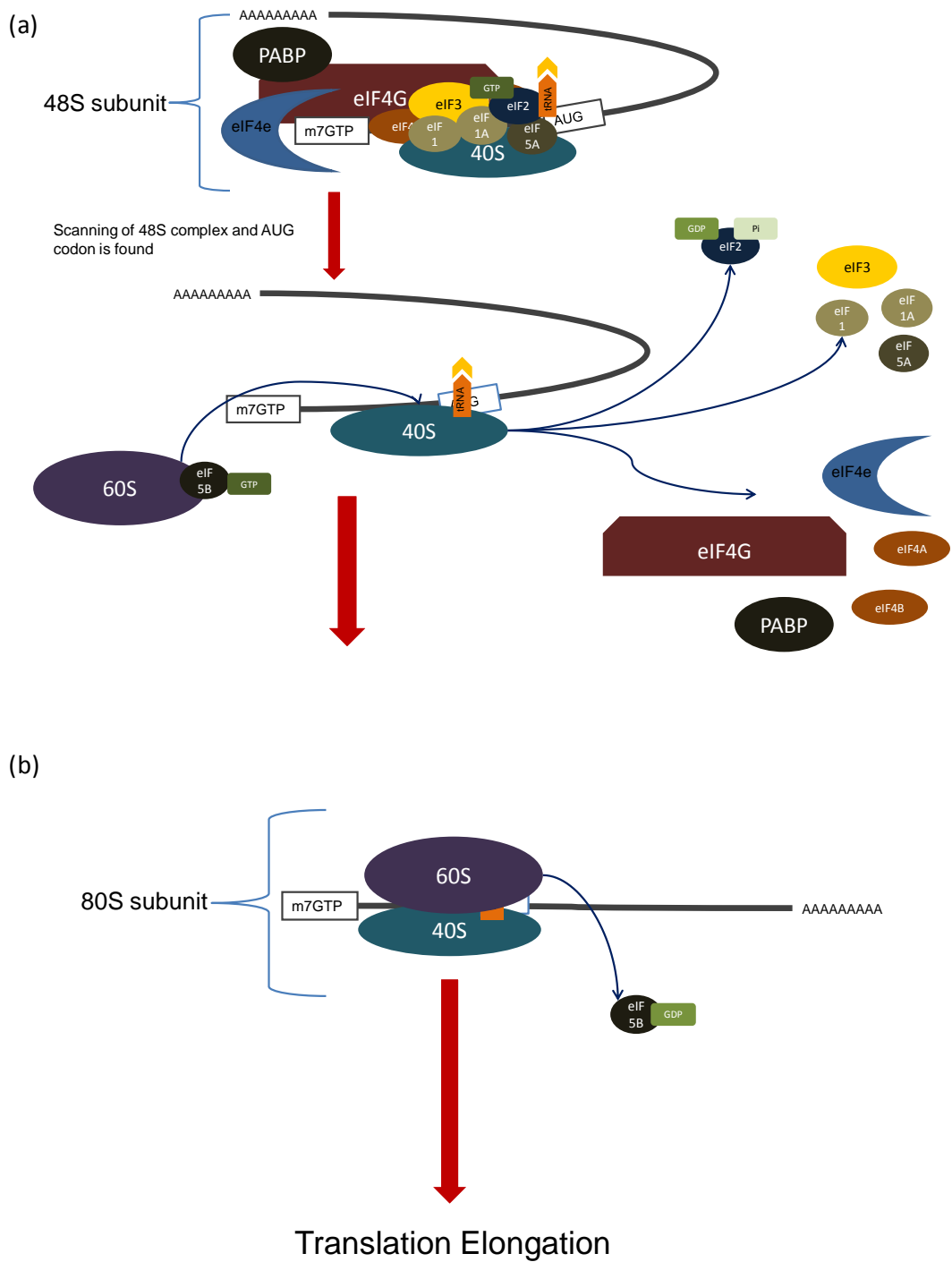
Figure 1.5 (a) via eIF3-eIF4G forming the 48S subunit; the mRNA is bound by the eIF5 to the 40S subunit. The subunit then begins to scan along the mRNA for a AUG start codon, once found the methionine-tRNA found in the TC complex is bound to the start codon in a GTP-dependent manor. The eIFs in the 48S complex disassociate from the complex as the 60S ribosomal subunit is recruited by eIF5B:GTP **(b)** The 60S unit is bound to the 40S subunit is catalysed by eIF5B in a GTP-dependent manner, this forms the 80S ribosome and elongation of the poly peptide can begin

1.7.1.3 mRNA Translation Elongation

Once the 80s ribosome is formed around the start AUG of the mRNA and the first Met amino acid has been placed in the P-site of the ribosome, elongation of the poly-peptide can proceed. There are two major factors involved in the elongation of a polypeptide, eukaryotic elongation factor 1A (eEF1A) and eEF2 (Figure 1.6). Elongation begins with eEF1A transporting an amino acid into the A-site of a ribosome in an eEF1A-GTP-aa-tRNA complex, by the tRNA having the complimentary anti-codon sequence to the 3 base pair codon sequence upon the mRNA within the A-site of the ribosome. Hydrolysis of the GTP moves the amino acid-tRNA complex into the A-site of the ribosome (Kaul et al. 2011; Browne & Proud 2002). Once in the A-site of the ribosome, eEF2 'pushes' the aa-tRNA complex over into the P-site of the ribosome, moving the ribosome along the mRNA strand, freeing the A-site for the next aa-tRNA complex to enter (Kapp & Lorsch 2004). As the aa-tRNA complexes move from the A-site into the P-site of the ribosome, the amino acid bound to the tRNA is removed from the tRNA and bound to another amino acid of the preceding tRNA by the peptidyl transferase centre of the ribosome, leaving the tRNA in the P-site (Kaul et al. 2011; Kapp & Lorsch 2004). This results in the polypeptide chain elongating. The tRNA then moves out of the P-site into the E-site of the ribosome and is released from the ribosome (Figure 1.6 and 1.7). Elongation of a polypeptide chain has high energy requirements and is a heavily regulated process. For the movement of one aa-tRNA through one site of the ribosome requires a total of 4 high energy bonds (Kaul et al. 2011). Control of elongation is mainly achieved via the phosphorylation status of eEF2 − when phosphorylated at Thr56 it is inactivated and hence protein synthesis is slowed (Rose et al. 2005; Kaul et al. 2011).
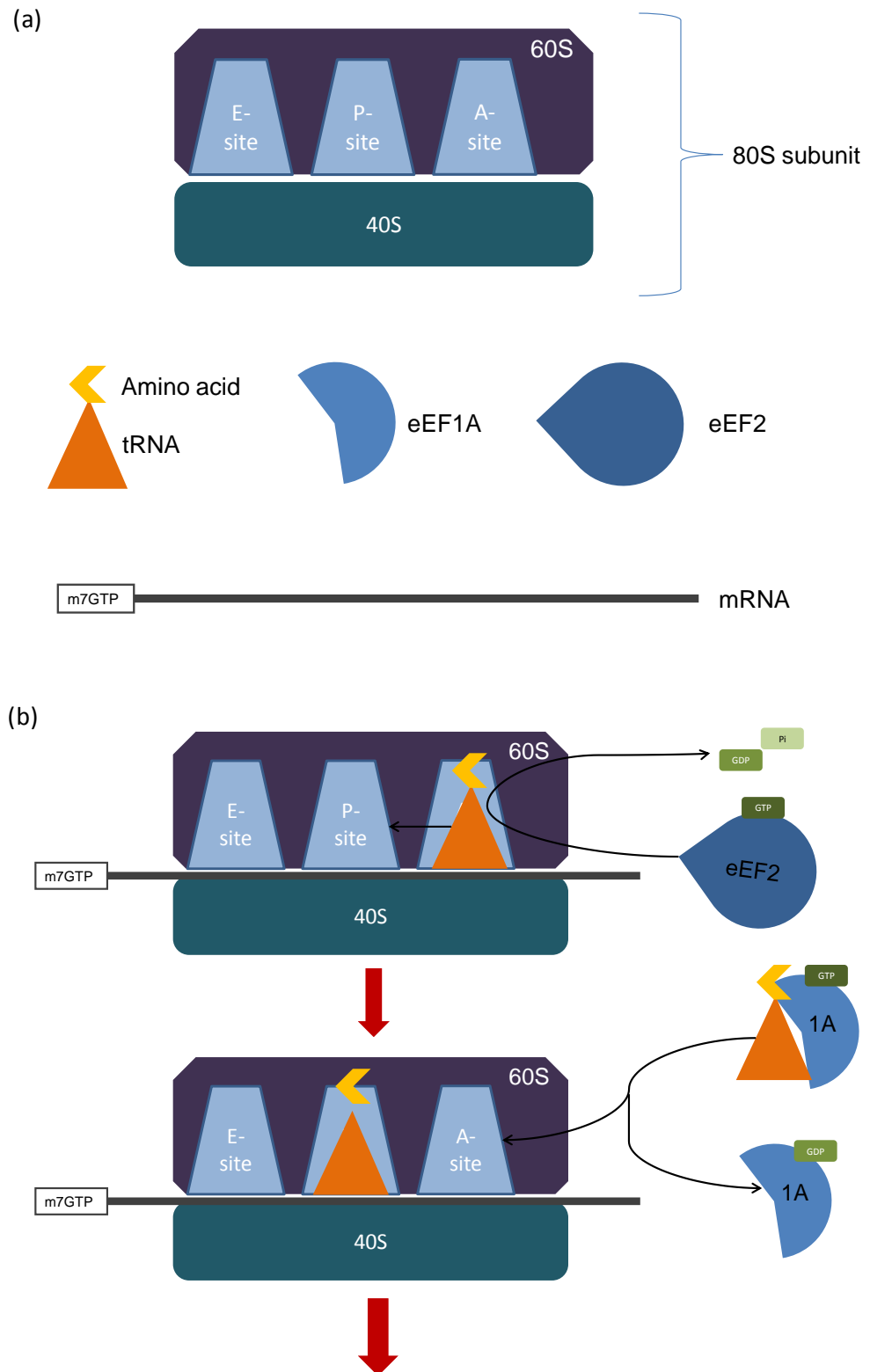
Figure 1.6 (a) Components of polypeptide elongation **(b)** As a result of completion of cap-dependent initiation the 80S subunit has a met:tRNA prepared in the A-site (Acceptance site) of the ribosome, translocation of the met:tRNA into the P-site (poly-peptide site) is catalysed by elongation factor 2 (eEF2) in a GTP dependent manor, where the Met disassociates from the tRNA. A new aa:tRNA is inserted into the A-site of the ribosome by elongation factor 1A (eEF1A) via GTP hydrolysis; dependent upon the complimentary codon-anti-codon pairing between the mRNA and aa:tRNA.
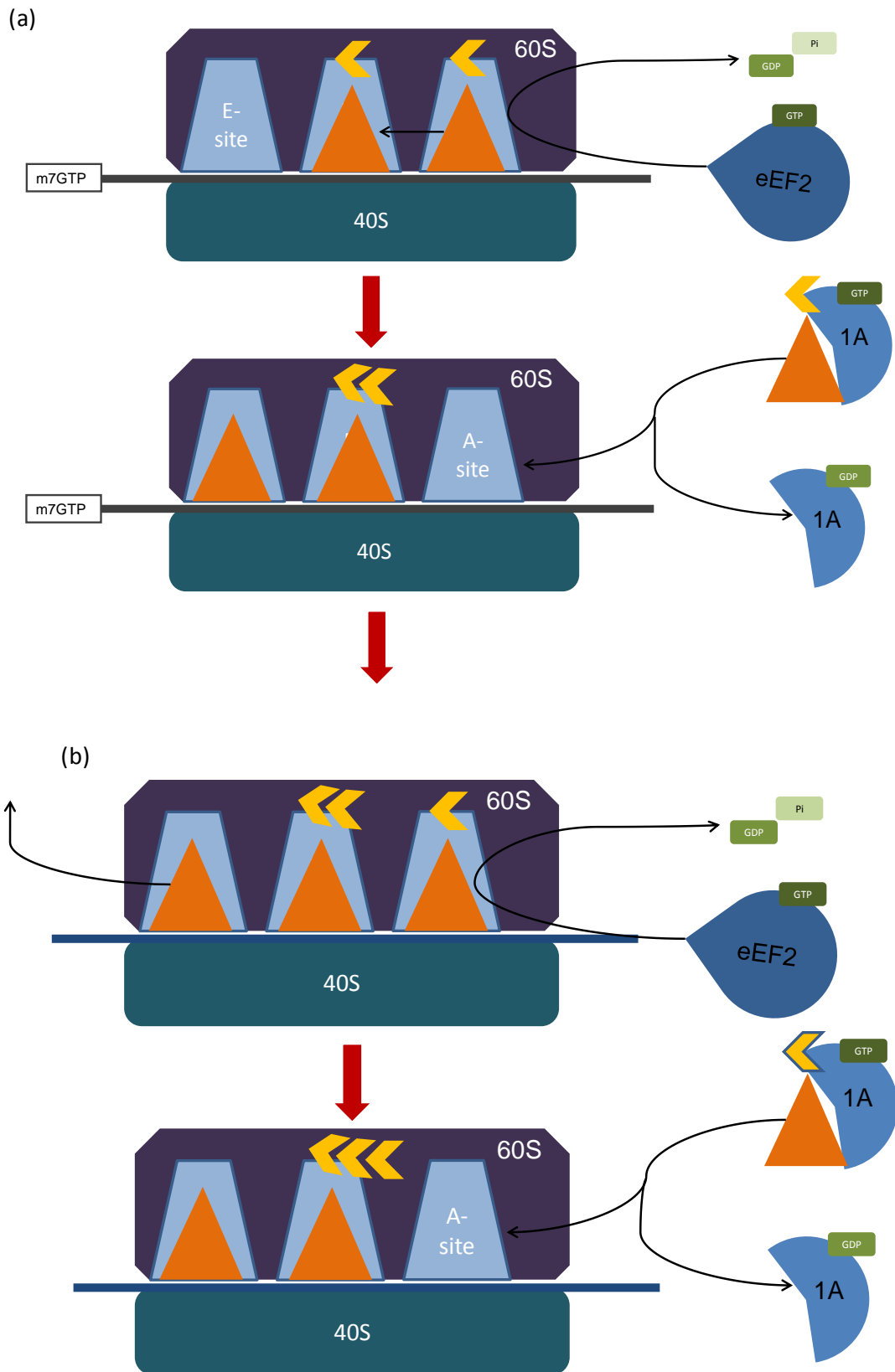
Figure 1.7 (a) Once a new aa:tRNA is inserted into the A-site of the ribosome eEF2 then translocates it into the P-site, moving the tRNA already occupying the P-site into the E-site (Exit-site), where it is ejected from the ribosome as translocation of the tRNAs through the ribosome proceeds. The amino acid bound to the tRNA disassociate in the P-site; peptide binding between the two amino acids within the P-site is the catalysed by the peptidyl transferase centre of the ribosome. **(b)** Elongation then continues in this cycle of translocation of aa:tRNA through the ribosome, with peptide binding occurring between the amino acids that enter the P-site in a sequential fashion, producing a specific polypeptide chain. Elongation continues, unless halted by regulators or a stop codon is reached in the mRNA sequence initiating termination of translation.

1.7.1.4 mRNA Translation Termination

Once elongation of the polypeptide is completed, when the ribosome encounters a stop codon in the mRNA, translation terminates. Termination results in the release of the mRNA and the newly formed polypeptide from the ribosome. Release of the mRNA is orchestrated by 2 distinct eukaryotic release factors (eRF): eRF1 and eRF3 (Figure 1.8). The release factors are split into two classes, class 1, eRF1, which recognises the stop codons: UAA, UAG, and UGA; eRF1 facilitates the release of the polypeptide chain via interaction with the peptidyl transferase centre. Class 2 release factors, eRF3, catalyse the activity of eRF1 release factors in a GTP dependent manor (Hauryliuk et al. 2006; Kapp & Lorsch 2004). Release factor 1 and 3 work in unison to terminate translation; eRF3 catalyses eRF1s activity within the A-site of the ribosome. Study of the release factors in yeast show that eRF3 GTP activity is the rate limiting step in the release of the poly peptide formation the mRNA (Salas-Marco & Bedwell 2004).
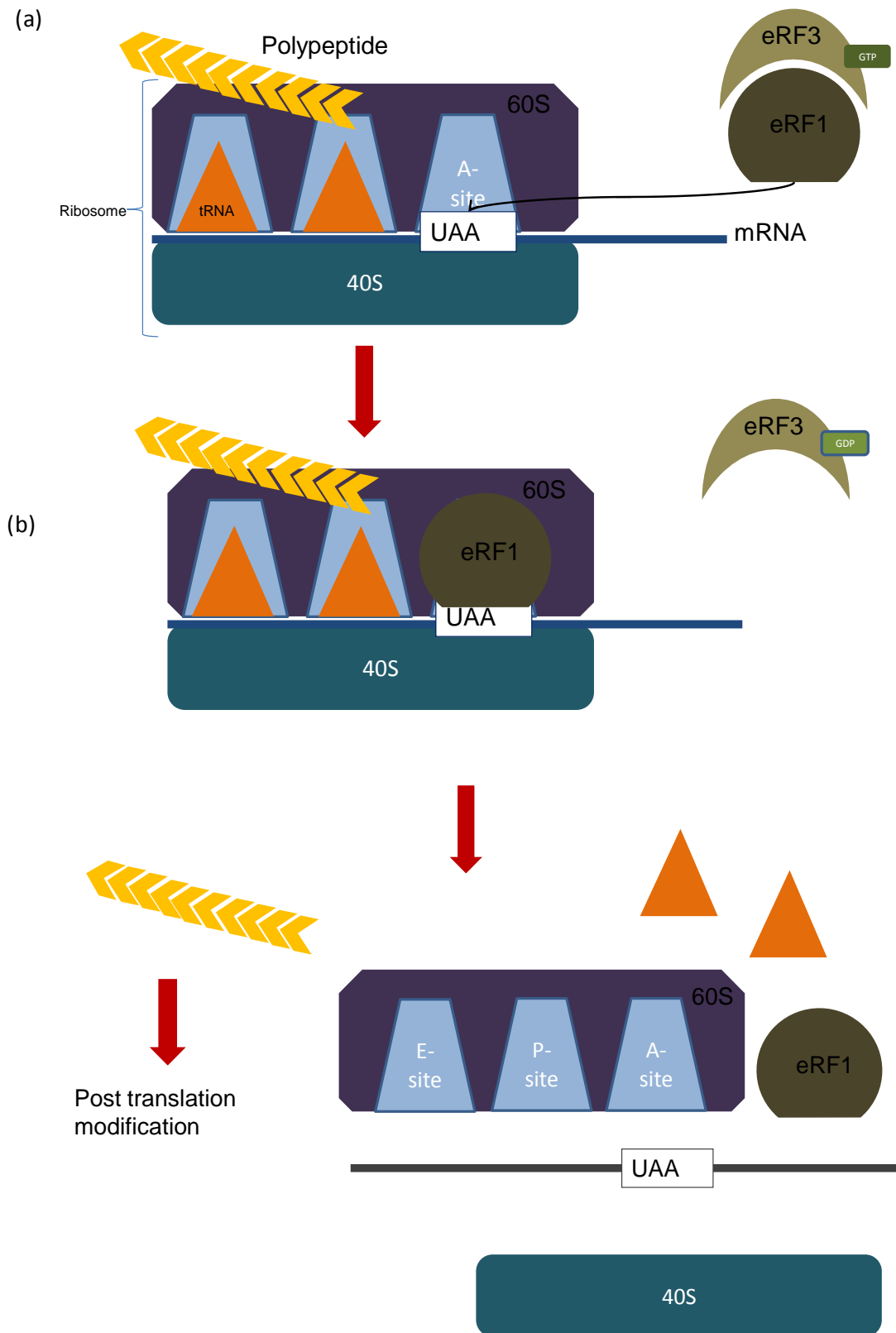
Figure 1.8 (a) Termination of polypeptide elongation is initiated when a stop codon, UAA, UAG or UGA enters the A-site of the ribosome, the stop codon is recognised by release factor 1 (eRF1). Entry of eRF1 in A-site of the ribosome is aided by eRF3 and hydrolysis of GTP. **(b)** Once in the A-site eRF1 catalyses the release of the mRNA from the 80S ribosome by promoting disassociation of the 60S and 40S subunit. The polypeptide is the transported to various locations within the cell.

1.8 Specific Translation Initiation Factors

1.8.1 Eukaryotic initiation factor 3 (eIF3)

Pertaining to this project and investigations into mTOR and a possible link to recombinant protein production, eIF3 is known to directly interact with the mTOR:Raptor and S6K1 regulators (Peterson & Sabatini 2005). eIF3 consists of 13 subunits, named eIF3a-eIF3m, and the total complex is the largest of the initiation factors at approximately 700 kDa (Dong & Zhang 2006). Some mammalian eIF3 subunits have a yeast homolog, for example subunits a, b, c, g and i, which are considered to form a core unit of the initiation factor (Hinnebusch 2006). There is less known about other subunits such as eIF3j, which also has a homolog in yeast, and has been found to change the interactions of the 43S subunit with the mRNA (Hinnebusch 2006). Deletion of eIF3j results in a change in cellular growth but doesn't affect the survival of cells (Fraser et al. 2007). Thus, the eIF3 core is still capable of executing its function, but eIF3j is not required for the core to function at full efficiency.

The primary function of eiF3 is to regulate the formation of the 43S PIC, prevent the 60S subunit prematurely binding to the 40S subunit and to act as a bridge to the eIF4F complex from the 43S complex (Hinnebusch 2006; Holz et al. 2005). It has also been shown that the eIF3 subunits influence other cellular functions. For example, eIF3a, eIF3e and eIF3k have been implemented in cell cycle regulation (Dong & Zhang 2006). Also, eIF3a over-expression has been linked to various cancers and is a possible target for cancer therapy (Dong & Zhang 2006). Changes in signalling and eIF3 expression has been shown to result in changes in cellular growth, therefore it is probable that it may influence recombination protein expression when signalling via mTOR is modified.

1.8.2 Eukaryotic initiation factor 4E (eIF4E)

As mention earlier, eIF4E is the mRNA cap binding protein that anchors the eIF4F complex to the mRNA cap. The regulation and binding of eIF4E is one of the rate limiting steps of translation inanition (Modrak-Wojcik et al. 2013). The structure of eIF4E resembles the shape of cupped hands, consisting of 8 anti-parallel beta sheets and 3 alpha helices (Rosettani et al. 2007). The mRNA cap binds in the mRNA-cap pocket of eIF4E, and 4E binding protein 1 (4EBP1)/eIF4G binds on the opposite side of the protein (Rosettani et al. 2007). 4EBP1 has

around a 10-fold higher affinity for eIF4E when it is hypophosphorylated at multiple potential phosphorylation sites (Abiko et al. 2007). During low nutrient/energy circumstances eIF4E is bound to a hypophosphorylated 4EBP1, blocking the binding of eIF4G and formation of the eIF4F cap complex. In conditions of high nutrients/energy, 4EBP1 is phosphorylated on multiple sites by an activated mTORC1, the level of phosphorylation being relative to the availability of the energy/nutrients (Fingar et al. 2002) causing 4EBP1s affinity for eIF4E to be reduce significantly and thus there is free eIF4E available to bind with eIF4G, allowing for translation to be initiated (Gingras et al. 1999a; Modrak-Wojcik et al. 2013). In recent years, the eIF4E, 4EBP1 and mTOR link to protein translation has garnered more attention as its regulation has been shown to be a bottle neck in protein synthesis (Gautsch et al. 1998) as well as being implicated in some cancers (Hsieh & Ruggero 2010).

1.8.3 Eukaryotic Elongation factor 2 (eEF2)

Elongation factor 2 is a 95.3 kDa protein, consisting of 857 amino acid monomeric chain, with a highly conserved amino acid sequence across mammalian species (Kaul et al. 2011). It has 3 structural blocks comprised of 5 domains: domains I-II, domain III, and domains IV-V (Taylor et al. 2007). Domain I has several conserved sequences that resemble other GTP binding pockets and which is located near the N-terminus of the protein (Taylor et al. 2007). It is not currently known how the complex eEF2-GTP forms, which is located in the interfacing region of the three structural blocks, which suggests that GTP binding and hydrolysis affect structural changes in eEF2. Elongation factor 2 is part of the G-protein super family, which undergo conformational changes when bound to GTP and upon hydrolysis of GTP. G-proteins have 3 regions that facilitate the binding and hydrolysis of GTP: a switch 1 region, also known as the effector loop, the switch 2 region which assists with the binding of Mg^{2+} and the P-loop, the phosphate-binding loop (Taylor et al. 2007; Spahn et al. 2004). Together these regions bind GTP in eEF2; hydrolysis of this GTP enables eEF2 to translocate the tRNAs within the ribosome.

Elongation factor 2 is responsible for the translocation of the aa-tRNA ribosome complex from the A-site to the P-site within the ribosome. Translocation of the tRNA within the ribosome via interaction with eEF2 is still not fully understood. It is known that translocation occurs when eEF2-GTP binds to the ribosome and eEF2 moves the aa-tRNA into the ribosome (Sengupta et al. 2008). When bound to the ribosome, eEF2 undergoes a conformational change, due to the

hydrolysis of GTP. The hydrolysis of GTP causes the domains within eEF2 to rotate, domains I-III rotate relative to domains VI-V (Kaul et al. 2011; Taylor et al. 2007). Domain IV is seen to move into the ribosome upon hydrolysis of GTP, causing the translocation of the aa-tRNA from the A-site to the P-site. The inactive eEF2-GDP complex then detaches from the ribosome, GDP is then released from eEF2, allowing for a new GTP molecule to take its place.

Elongation factor 2 has a unique histidine residue at position 715 in the amino acid sequence - dipthaminde, 2-(3Craboryamido-3-(trimetheylammonia) propyl) histidine) (Figure 1. 9) (Abdel-Fattah et al. 2013)**.** Several steps are required for this post translation modification to be completed and studies conclude that it is required for regular eEF2s function (Kimata & Kohno 1994). What is interesting about this residue is that it is a target of a bacterial exotoxin, exotoxin A, which is found in diphtheria toxin. The toxin inactivates eEF2 by transferring an ADP-ribosyl moiety of NAD+ onto the dipthamide imidazole (Kaul et al. 2011; Ortiz et al. 2006; Jørgensen et al. 2005)**.** Although the function of the dipthaminde residue is not known, it is believed to have a role in translation, the sequence surrounding the dipthaminde residue is highly conserved in mammalian sequences suggesting that is does have some unknown function in translation or elsewhere (Kaul et al. 2011). The activity of elongation factor 2 is regulated by a single kinase, elongation factor 2 kinase (eEF2K). Elongation factor 2 is regulated by phosphorylation of its threonine 56 residue by eEF2K. Phosphorylation of the Thr56 residue deactivates eEF2, blocking its interaction with the ribosome and prevents polypeptide elongation (Kenney et al. 2014; Ma & Blenis 2009). In this way elongation rates can be modulated by the cell in response to protein synthesis needs and cellular stress.
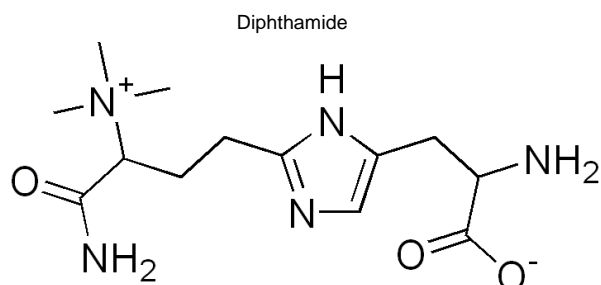
Figure 1.9 Chemical structure of the 715 Dipthamide residue of elongation factor 2

1.8.4 Eukaryotic Elongation factor 2 kinase (eEF2K)

Elongation factor 2 kinase is an α-kinase, monomeric protein around 100 kDa in size and is the sole regulator of elongation factor 2. There are two distinct structural domains, near the N-terminus there is a calmodulin (CaM) binding motif, for binding Ca^{2+}, immediately next to an α-kinase helix (Figure 1.10) (Kenney et al. 2014). Less is known about the C-terminus end of eEF2K, the predicted structure is that there are four α-helical regions of unknown role, possibly for protein-protein interactions with eEF2 (Pavur et al. 2000). Elongation factor 2 kinase activity is regulated by several mechanisms within the mammalian cell. There are four major regulatory pathways that regulate eEF2K activity. As mention above, eEF2K has a CaM binding site, meaning that eEF2K activity is Ca^{2+} dependent (Pigott et al. 2012). Other proteins phosphorylate eEF2K on various serine residues in its structure. For example, AMP-activated protein kinase (AMPK): Ser^{378}, cAMP (cyclic adenosine monophosphate)-dependent protein kinase via PKA (Protein kinase A): Ser^{499}, and S6K1 via mTORC1: $Ser^{78/359/366}$ (Kenney et al. 2014; Ma & Blenis 2009). In this way mTOR signalling can directly influence polypeptide elongation. The CaM binding site, AMPK and cAMP all activate eEF2K, to repress elongation. On-the-other-hand, mTOR controlled phosphorylation of S6K1 works to inactivate eEF2K and hence maintain or enhance elongation by maintaining eEF2 in a non-phosphorylated state.

Calmodulin activates eEF2K by an unknown mechanism related to calcium flux in the cell, particularly a release of Ca^{2+} ions into the cystol (Pigott et al. 2012). AMPK responds to the increase of the of intracellular ratio of ATP:AMP and ATP:ADP. As the levels of ADP, AMP rise, AMPK activates various catabolic processes to preserve levels of ATP as well as activating eEF2K (Leprivier et al. 2013a). cAMP is produced by signalling from G-proteins to adenylyl cyclase which circularises AMP into cAMP. cAMP activates protein PKA; PKA then actives eEF2K (Kenney et al. 2014; Hovland et al. 1999). AMPK and cAMP are both sensors of intra cellular energy and nutrients; therefore AMPK and cAMP activation of eEF2K is to preserve the intra cellular levels of nutrients and conserve energy. In this way elongation factor 2 kinase is therefore capable of protecting the cells from nutrient and energy deprivation. It has also been suggested that eEF2K is capable for protecting certain tissues from autophagy and cytotoxic stress (Leprivier et al. 2013a; Kenney et al. 2014).
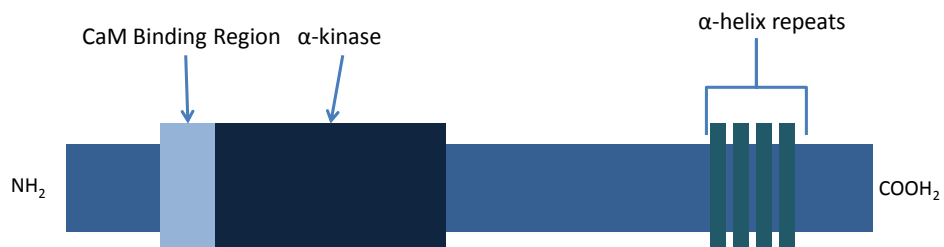
Figure 1.10 Schematic of elongation factor 2 kinase (eEF2K). The CaM binding region activates eEF2K upon Ca^{2+} and calmodulin binding. The α-kinase region directly phosphorylates elongation factor 2 (eEF2) on Thr56 when eEF2K is activate, deactivating eEF2. Four α-helix repeats near the C-terminus function is not fully known, but believed to be responsible for protein-protein interactions.

1.9 Mammalian Target of Rapamycin (mTOR) Signalling – A Master Regulator of Cell Proliferation and Protein Synthesis

Mammalian target of Rapamycin (mTOR) is considered a master regulator within mammalian cells, tying together extra cellular signals to intra cellular mechanisms. mTOR is a serine threonine kinase 289kDa in size and is part of the phosphoinsitide 3-kinase related family (PI3K) (Laplante & Sabatini 2009b). mTOR is considered a master regulator of protein synthesis, ribosome biogenesis and cell proliferation, and has recently been reported to influence cell growth and recombinant protein synthesis from mammalian cells. Protein synthesis and ribosome biogenesis are key determinants of cellular biomass and protein productivity. However, the roles that this signalling pathway plays in underpinning these phenotypes, and whether it can be manipulated to enhance them, have yet to be fully elucidated.

With respect to the growth of recombinant cell lines, mTOR regulates these processes via the coordination of signalling pathways in response to growth factors, nutrient availability (amino acids), energy status (ATP) and cell stress (Zoncu et al. 2011; Laplante & Sabatini 2009b), all factors that play key roles in regulating recombinant protein yields from mammalian cells.

mTOR is therefore likely to be a key global regulator of exactly those properties that are essential to achieving and maintaining high level recombinant protein production from mammalian cells including regulating cell growth and proliferation rates, cellular responses to nutrient and oxygen supplies, anabolic metabolism including ribosome biogenesis and mRNA translation/protein synthesis, and consequently cell-specific productivity.

Rapamycin was first derived from a bacterial strain called *Streptomyes hygroscopius* (Laplante & Sabatini 2012). After the discovery of rapamycin, an antifungal reagent, in the 1970's the target of rapamyicn (TOR) was subsequently discovered in the 1990's in budding yeast (Heitman et al. 1991). Shortly after the discovery of TOR, the mammalian homologue of TOR, mTOR was discovered (Sabatini et al. 1995). The TOR makeup is conserved throughout eukaryotic cells. Near the N-terminus there are several HEAT repeats which are involved with protein-protein binding as well as two FAT (FRAT-ATM-TRRAP) repeats either side of the FRB (FKBP12-rapamycin) binding site (Ma & Blenis 2009). In cells, rapamycin binds to the FKB12, then the rapamycin:FKB12 complex binds to mTORs active site, causing it to lose functionality (Foster & Fingar 2010).

1.9.1 mTOR Complexes

mTOR is the catalytic subunit of two functionally distinct complexes, mTORC1 and mTORC2. In the short term, the inhibitor rapamycin only effects mTORC1 signalling. mTORC1 consists of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8/GβL), proline rich AKT substrate 40 kDa (PRAS40) and DEP-domain-containing mTOR-interacting protein (Deptor) (Laplante & Sabatini 2009b). mTORC2 complex consists of rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1), protein observed with Rictor-1 (Protor-1), mLST8, and Deptor ((Laplante & Sabatini 2009b). Thus, each complex contains mTOR, mLST8/GβL (which binds the kinase domain) and Deptor, an inhibitor. However, the other partner proteins differ between the two complexes. mTORC1 contains raptor, which binds substrates for mTORC1, recruiting them for phosphorylation by mTOR, and PRAS40. mTORC2 contains the proteins rictor, mSin1 and PRR5 whereby rictor and mSin1 promote mTORC2 signalling (Foster & Fingar 2010; Laplante & Sabatini 2009b). The function of the constituent parts of mTORC1 and 2 are still not fully understood, but it is believed that PRAS40 and Deptor are inhibitors of mTORC1. It has been shown activity of mTOR is reduced when PRAS40 and Deptor are recruited into mTROC1 whereas, when mTORC1 activity is increased when they are phosphorylated and leave the complex (Peterson et al. 2010; Sancak et al. 2007). A schematic of mTOR and the complex is depicted in Figure 1. 11.
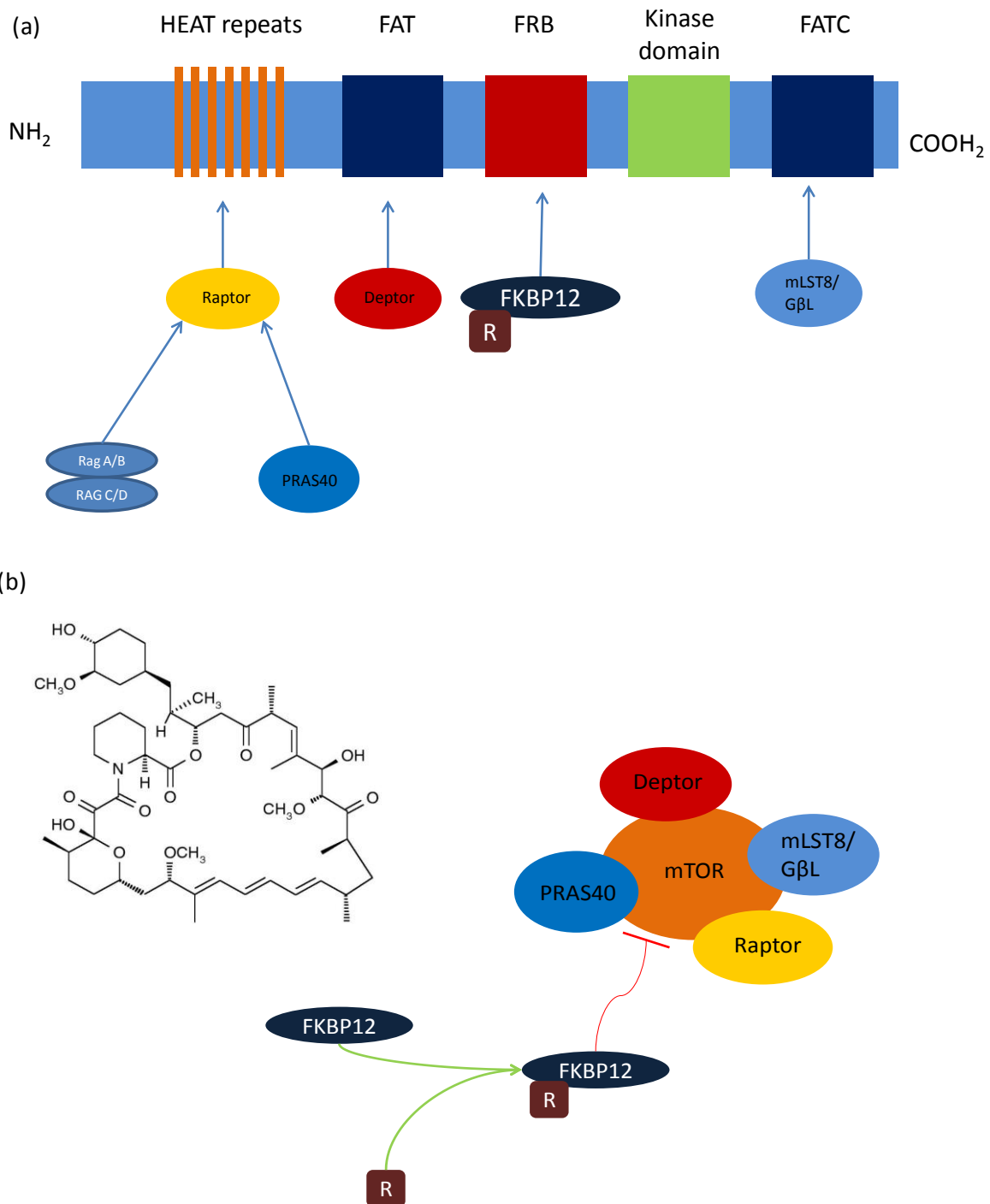
Figure 1.11 (a) Structure of mTOR, depicted with various interactions with mTOR related proteins. HEAT repeats that promote protein-protein interaction, FAT (FRAT-ATM-TRRAP) binds Deptor and FATC (C terminus FAT) binds mLST, for the formation of the mTORC1 complex, FRB domain binds the inhibitory complex of FKBP12 and rapamycin which reduces the activity of the adjacent kinase site. **(b)** Structure of rapamycin and its inhibition of mTORC1.

The signalling and role of mTORC1 in controlling cell growth, proliferation, ribosome biogenesis and protein synthesis are much better defined. mTORC1 senses extra- and intra-cellular signals whereby growth factors, nutrients and energy promote mTORC1-dependent cell growth/proliferation and protein synthesis. At the same time, mTORC1 promotes ribosome biogenesis by enhanced transcription of ribosomal RNAs and translation of mRNAs for ribosomal (r-)proteins to increase protein synthetic capacity. In response to reduced levels of growth factors, nutrients (specifically amino acids), energy (ATP) or cell stresses such as that encountered during rPP, mTORC1 action is attenuated to ensure that the biosynthetic processes are maintained at levels appropriate for such suboptimal conditions. mTORC1 also activates lipogenesis (Laplante & Sabatini 2009a) while reduced mTORC1 activity leads to activated macroautophagy, which mediates the breakdown of cellular components into building blocks (e.g. amino acids and other small molecules) to compensate for deficient nutrient supply (Foster & Fingar 2010). Finally, mTOR regulates mitochondrial oxidative function and is a key control point in regulating the balance between nutrient availability and energy metabolism and between mitochondrial vs. non-mitochondrial ATP generation (Schieke et al. 2006). There is less known about mTORC2, but it has been linked to cell survival, proliferation, and metabolism (Gibbons, Abraham, & Yu, 2009; Laplante & Sabatini, 2009)**.** Of the two complexes, mTORC1 is sensitive to the effects of rapamycin, whereas mTORC2 is not. This is shown by mTORC1s interaction with FKBP12, whereas mTORC2 does not. mTORC1 is of greater interest in this investigation, as it is responsible for the regulation of protein synthesis.

1.9.2 Regulation of mTORC1

There are several extra cellular and intra cellular signalling mechanisms that regulate the formation and activity of mTORC1. These mechanisms evolved to be responsive in a complex organism, therefore some of the signalling pathways would not have an effect upon cellular regulation when the cells are cultured in a simple culture i.e. mTORC1 responses to levels of insulin (Wang & Proud 2006; Wang et al. 2000); as there is no insulin, or changes to the concentration of insulin within culture, it is unlikely this will cause any signalling to mTOR. Therefore the relevant pathways that would regulate mTOR in culture are outline below (figure 1.12).Signalling to mTORC1 occurs directly through various mechanisms which all signal to mTORC1, or various single pathways converge on the TSC (tuberous sclerosis complex) complex which acts as a GTPase-activating protein, which deactivates Rheb (Ras homolog enriched in brain).(Ma & Blenis 2009; Zoncu et al. 2011)The inactivated Rheb is then incapable of   activating   mTORC1.
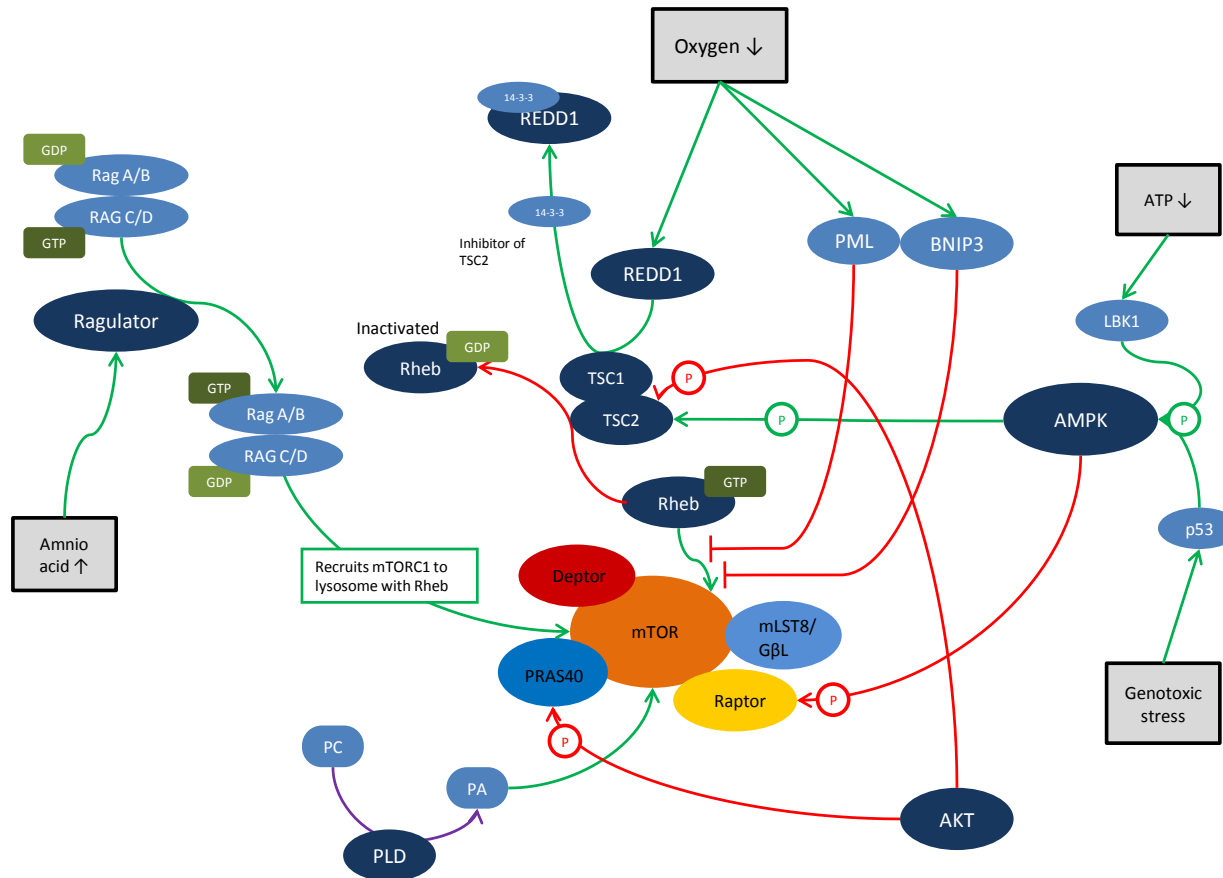
Figure 1.12 Various signalling pathways that regulate mTORC1 that are pertinent to cellular signalling within cellular culture. Activation and inhibition of mTORC1 occurs either via direct interaction with mOTRC1 or via a signalling cascade via Rheb (Ras homolog enriched in brain), an activator of mTOR. Rhebs regulator, the TSC (tuberous sclerosis complex) is a GAP (GTPase activating protein) ,when active, which inhibits Rhebs activity by reducing the GTP bound to Rheb to GDP, inactivating Rheb. In a state of hypoxia, low oxygen levels signal to REDD1 via hypoxia-induced factor 1-alpha (HIF1α), when activated REDD1 will recruit 14-3-3 (inhibitor of TSC2), removing it from TSC2, activating TSC and inhibit Rheb. HIF1α also activates PML (promyeloctic leukaemia tumour suppressor) and BNIP3 (BCL2/Adenovirus E1B 19kD-Interacting Protein 3) prevent Rheb binding to mTOR during a state of hypoxia preventing Rhebs activation of mTOR. Reduction of energy/ATP levels and genotixoc stress activate AMPK (AMP-activated protein kinase) via LBK1 and p53 respectively. AMPK then phosphorylates and subsequently activates the TSC complex resulting in reduced mTORC1 activity; AMPK has also been shown to interact with mTORC1 directly by phosphorylation and inhibition of Raptor, an activator of mTOR. In a nutrient/amino acid rich environment, RAG A/B and RAG C/D, are active by their regulator, Ragulator, which switches the positions of GTP and GDP between the monomers. The RAG dimer then recruits mTORC1 to the membrane of a lysosome where it is then activated by Rheb. Phosphatidic acid (PA) is produced by hydrolysis of phosphatidylcholine (PC) via PLD (phospholipase D). PC is derived from fatty acids found in several foods. PA stabilises mTORC1 which is essential for its activity. mTORC2 activates AKT, which then phosphorylates and inhibits PRAS40, a direct inhibitor of mTOR, and TSC2, ultimately activating mTOR.

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1.9.2.1 TSC1:TSC2 Complex Regulation of mTORC1

The TSC complex consists of 3 proteins, two hetero dimers TSC1 and TSC2, and TBC1D7 (Tre2-Bub2-Cdc16-1 domain family member 7) (Dibble et al. 2012). As a complex these proteins regulate mTOR via Rheb (Figure 1.12). TSC2 is a GAP (GTPase activating protein), whilst TSC1 acts as a stabilising scaffold for the other two proteins. AMPK, which responds to genotoxic stress and levels of ADP, phosphorylates and activates TSC2 (Figure 1.12). REDD1 also activates TSC2 by removal of a 14-3-3 inhibitor of TSC2 (Tan & Hagen 2013; Schneider et al. 2008; Ma & Blenis 2009). Activation of TSC2 causes it to deactivate Rheb preventing it from mTORC1 activation. AKT, insulin and various TNFs inhibit the TSC complex, usually in energy and nutrient rich environments, allowing for mTORC1 activation (Long et al. 2005; Ma & Blenis 2009). The mechanism by which the TSC complex regulates Rheb is still not fully understood.

1.9.2.2 Amino acids and mTORC1 Signalling

It has been shown that mTORC1 is capable of sensing and responding to the intra cellular levels of amino acids, specifically leucine (Lynch 2001; Gran & Cameron-Smith 2011; Proud 2004). Until recently the mechanisms for sensing the levels of amino acids had been unknown, but it has now been revealed that amino acid sensing and metabolism occurs on the membrane of the lysosome (Figure 1.13) (Bar-Peled & Sabatini 2014). There are several factors that recruit mTOR to the lysosome. Rag (Ras-related GTPase) A:C/B:D dimers, Ragulator v-ATPase, folliculn (FLCN) and GATOR (GAP activity towards Rags) 1 and 2 (Bar-Peled & Sabatini 2014). In an environment of low amino acids, v-ATPase and folliculn are inactive upon the membrane of the lysosome; the Rag A/C or Rag B/D have GDP bound to Rag A/B and GTP to Rag C/D and GATOR1 inhibits the Ragulators guanine nucleotide exchange factor activity which swaps the GDP/GTP binding on the Rag A/C: Rag B/D dimers. When there is an abundance of amino acids, GATOR1 becomes inhibited by GATOR2 and the TSC complex is inhibited; v-ATPase and Ragulator are activated, changing the position of the GTP from RagC/D to Rag A/B, which then recruits mTOR to the lysosome where it is subsequently activated by Rheb (Bar-Peled & Sabatini 2014; Abraham 2010; Sancak et al. 2008; Sancak et al. 2010). mTOR can then activate protein production via 4EBP1 and S6K1 signalling.
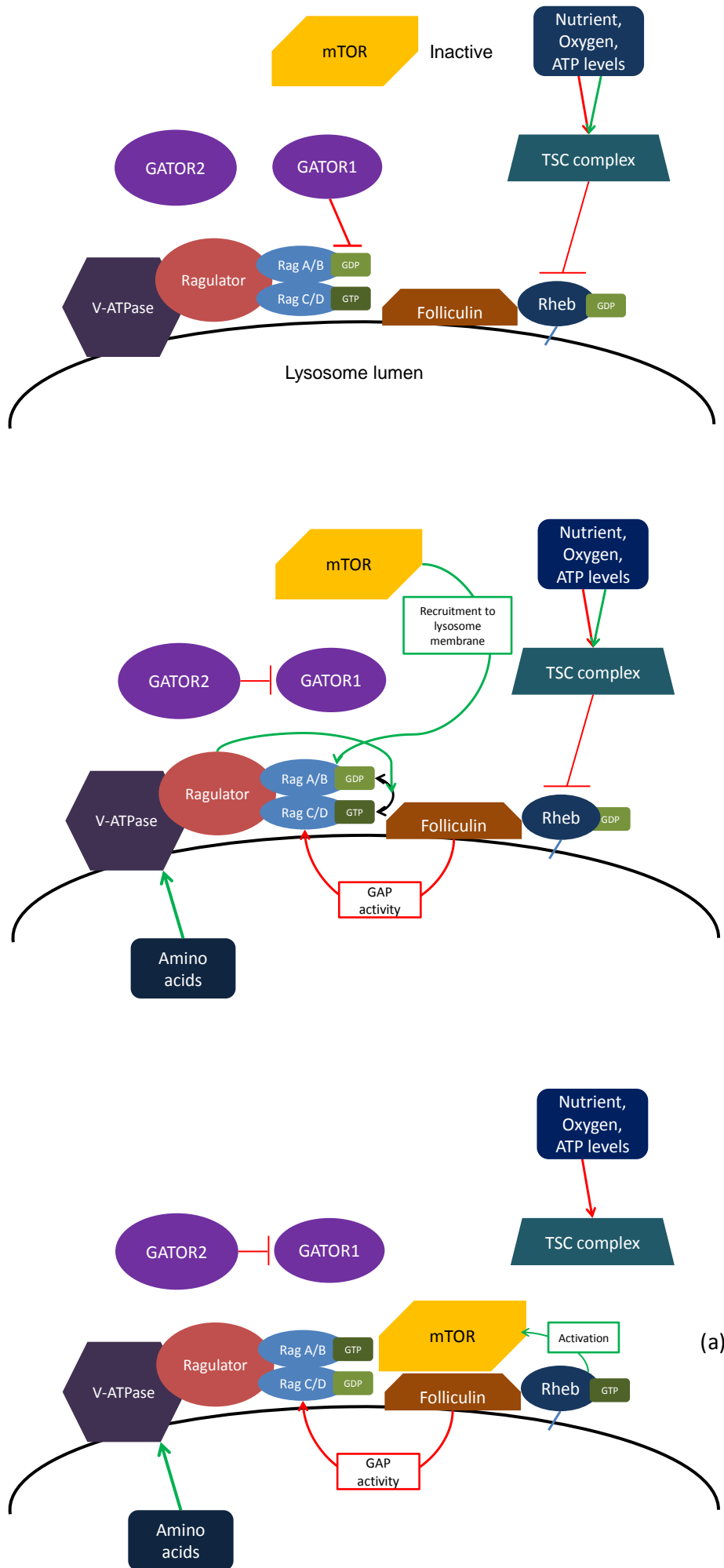
Figure 1.13 A more detailed view of mTOR recruitment and activation at the lysosome membrane in a nutrient/amino acid rich environment. Regulated by v-ATPase an amino acid sensor; GATOR (GAP activity towards Rags) 1 and 2, and Folliculin regulators of the Rag heterodimers; and the TSC complex regulator of Rheb, activator of mTOR. **(a)** In a staved state, mTOR remains inactive in the cystol; v-ATPase and folliculn are inactive upon the surface of the lysosome membrane. GATOR1 inhibits the exchange of GTP and GDP between the Rag monomers, and the TSC deactivates Rheb. **(b)** In the presence of amino acids V-ATPase is activated and GATOR1 is inhibited by GATOR2, this allows for Ragulator to transfer the GTP and GDP upon the Ragmonomers. Folliculin is also activated and prevents the GTP and GDP exchanging back to their inactive positions upon the Rag monomers, promoting Rag activity. The now active Rag A/B C/D dimer then recruits mTOR to the lysosome membrane **(c)** In a nutrient/amino acid rich environment the TSC complex is deactivated, activating Rheb. Once mTOR has translocated to the membrane it is activated by Rheb.

1.9.2.3 Oxygen and mTORC1 Signalling

There have been several studies into the effects of hypoxia and its effects upon mTOR signalling. Some of these studies have shown that the regulation of mTOR is linked to hypoxia-induced factor 1-alpha (HIF1α) (Connolly et al. 2006; Wouters & Koritzinsky 2008). During hypoxia, HIF1α becomes phosphorylated and down regulates mTORC1 activity through REDD1, a DNA damage response agent which increases the activity of the TSC1:2 complex (Figure 1.12) (Schneider et al. 2008). HIF1α also targets BNIP3 (BCL2/Adenovirus E1B 19kD-Interacting Protein 3), which inhibits Rheb activity, down regulating mTOR activity.

1.9.2.4 Glucose, ATP/ADP and mTORC1 Signalling

Intra cellular levels of key energy molecules are sensed by LKB1-AMPK pathway (Hardie et al. 2012). When the levels of ATP drops, AMPK then activates the TSC1:2 complex which down regulates mTOR activity (Figure 1.12) (Gleason et al. 2007; Chan et al. 2004). AMK has also been reported to interact with mTOC1 directly, by phosphorylation and deactivation of Raptor, preventing raptor from activating mTOR (Gwinn et al. 2008).

1.9.2.5 Phosphatidic acid (PA) and mTORC1 Signalling

PA is produced from intra cellular hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD). PA can also be produced from lysophosphatidic acid (LPA) and diacylglycerol, but the main sourse of PA is thought to be phosphatidylcholine (Foster 2009). PA directly interacts with mTOR, by binding to the same active site as rapamycin:FKBP12 on the mTOR molecule (Foster 2007; Toschi et al. 2009). PA has been shown to be essential to the stabilisation of the mTORC1 and mTORC2 complexes, but it still requires other signals for mTOR activation (Foster 2007; Foster 2009).

1.9.3 mTORC1 and Protein synthesis

mTORC1 is involved in a number of mechanisms of signalling and control over protein synthesis. It has been shown to regulate mRNA synthesis, mRNA translation initiation and elongation stages of protein synthesis as described below and depicted in Figure 1.14.
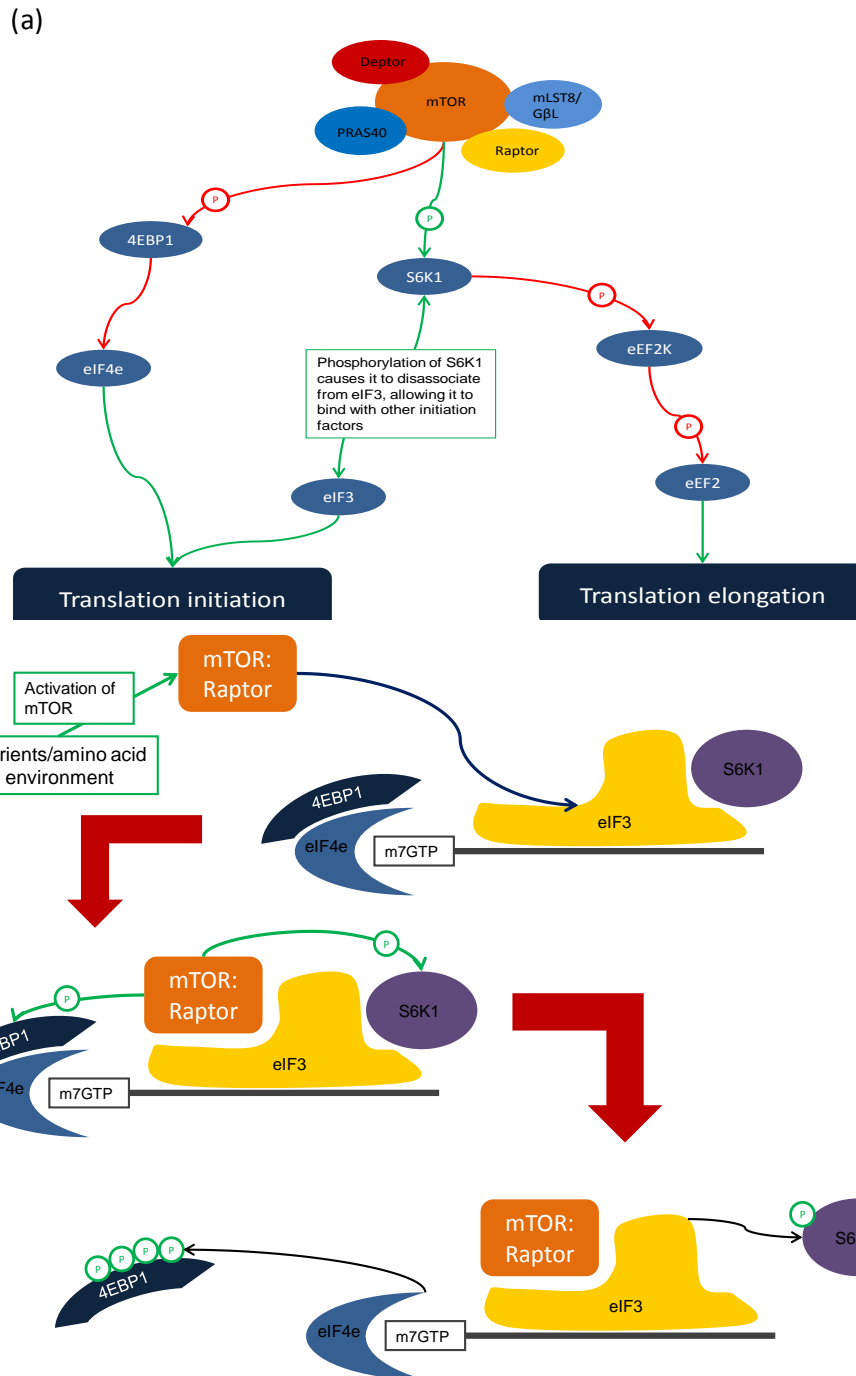
Figure 1.14 (a) An overview of mTORC1 signalling and control of protein synthesis within mammalian cells. (**b**) A detailed view of mTOR regulation of mRNA translation initiation. In a starved/low energy state eIF4E is bound to 4EBP1 (4e binding protein 1) which prevents formation of the the cap by blocking eIF4E binding with eIF4G; and an inactive S6K1 is bound to eIF3 preventing formation of the 43S complex. In a enriched nutrient/high energy state, mTOR is activated and recruited to eIF3 via raptor. When in proximity to eIF3 mTOR

then phosphorylates the S6K1 bound to eIF3, activating it, leading to is disassociation with eIF3. This allows eIF3 to being formation of the 43S pre-initiation complex. mTOR also phosphorylates 4EBP1 on multiple phosphorylation sites, causing it to loose affinity for eIF4E, releasing eIF4E to for the eIF4F cap complex for translation initiation.

1.9.3.1 mTORC1 and Translation Initiation

mTORC1 influences cap regulated translation through several regulatory mechanisms (Figure 1.14). The regulation of eukaryotic initiation factor 4E (eIF4E) through mTORC1 activation of 4E binding protein 1 (4EBP1) has been described previously (Dowling, Topisirovic, Fonseca, & Sonenberg, 2010; Fingar et al., 2002; Gingras, Raught, & Sonenberg, 1999). eIF4E forms a cap binding complex with eIF4F which binds to the 5'mRNA cap and recruits the 43S pre-initiation complex to the mRNA (Gingras et al. 1999b; Pestova & Kolupaeva 2002) and interacts with eIF4G to recruit the ribosome to the capped mRNA. 4EBP1 has a 10 fold higher affinity for eIF4E then the 5' mRNA cap does so when it is active. 4EBP1 binds eIF4E and prevents 4E/4F cap binding complex formation and translation beginning. 4EBP1 has several phosphorylation sites, which act as its regulatory sites for the protein. The more sites that are phosphorylated, the less active 4EBP1 becomes allowing eIF4E to become more active, reflecting the level of mTORC1 activity (Fingar et al. 2002; Foster & Fingar 2010).

As described previously, mTORC1 has also been shown to interact with eIF3, which also interacts with the eIF4F complex. eIF3 has 13 subunits, several that have implicated in various forms of cancer i.e. eIF3i, eIF3h. REF eIF3 is an integral part of the 43S pre-initiation complex, under certain conditions eIF3 is shown to bind to an inactive S6K1 and recruits the mTOR:Raptor complex to a 5' capped mRNA:eIF4e:4eBP1 complex; this allows the mTOR:raptor complex to phosphorylate and deactivate 4EBP1 (Peterson & Sabatini 2005). Full activation of mTOR:Raptor leads to phosphorylation of S6K1, which releases eIF3 resulting in the formation of the 43S pre-initiation complex, allowing for translation to be initiated.

1.9.3.2 mTORC1 and Translation Elongation

As well as initiation, mTOR is capable of regulating polypeptide elongation. When mTOR is active, it activates S6K1, which in turn deactivates eukaryotic elongation factor 2 kinase (eEF2K) by phosphorylation on the $Ser^{78/359/366}$ (Ma & Blenis 2009; Kenney et al. 2014). Deactivation of eEF2K prevents it from phosphorylating eukaryotic elongation factor 2, this allows for eEF2 to remain active and allows translation elongation to continue (Browne & Proud 2002).

1.10 Aims of this Project

Production of recombinant proteins and biopharmaceuticals/biotherapeutics is a multi-billion dollar industry, and their use in the place of small molecules has become more common place as medical treatment. As described above, mTORC1 is mediated through the binding of raptor to ribosomal S6 protein kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), which recruits these substrates to be phosphorylated by mTOR. When hypophosphorylated, 4E-BP1 acts as a translational repressor by binding to eIF4E, and preventing it interacting with eIF4G to promote ribosome recruitment to the mRNA. eIF4E also binds directly to the mRNA's 5'-cap structure. By directly phosphorylating 4E-BP1 at multiple sites ($Thr^{37/46}$, Thr^{70}, Ser^{65}), mTORC1 promotes its dissociation from eIF4E allowing the formation of the eIF4F complex and the initiation of cap-dependent translation. Recent work has shown that increased 4E-BP1 phosphorylation is correlated with enhanced recombinant interferon-γ production in CHO cells. The authors suggest this was due to the alleviation by mTORC1 of translation initiation repression (Chong et al. 2009). Others have also previously shown that, with respect to culturing Chinese hamster ovary (CHO) cells at reduced temperature ($<37^{o}C$), transient overexpression of eIF4E resulted in enhanced recombinant protein expression (Underhill et al. 2006). On the other hand, mTORC1-promoted phosphorylation of S6K1 may enhance translation via several possible mechanisms. Relevant substrates of S6K1 include the ribosomal protein S6, eIF4B and the elongation factor 2 kinase eEF2K which phosphorylates and inactivates elongation factor 2 (eEF2).

Several key environmental cues promote or attenuate mTORC1 signalling. They are all directly relevant to culturing mammalian cells for the production of rPs (e.g. growth factors, amino acids, osmolarity, the energy status of the cell, cell stress). With respect to recombinant production from mammalian cells, a recent report has shown that the manipulation of mTOR simultaneously improved key processes underpinning recombinant protein production from CHO cells including cell growth, proliferation, viability and cell specific productivity and concluded that mTOR-based engineering of mammalian cell lines has a promising future for the improvement of the bioprocessing of biotherapeutic proteins (Dreesen & Fussenegger 2011b). As described previously, a further study has also reported that in plasma cells (the cells that 'naturally' synthesize and secrete IgGs) protein synthesis is regulated by crosstalk between endoplasmic reticulum stress and mTOR signalling (Goldfinger et al. 2011) .

This project therefore set out to establish if manipulation of the mTOR signalling pathway and/or manipulation of phosphorylation of elongation factor 2 in CHO cells impacts upon CHO cell growth and recombinant protein production yields. The specific aims of the project were to:

- To investigate the effects of over expression of elongation factor 2 upon CHO cellular growth and recombinant protein production.

- Observe the effects of eEF2 mutation at the Thr56 residue, changing its regulation and the effects of this mutant eEF2 upon CHO cell growth and recombinant protein production.

- Investigate the effect of shRNA silencing of elongation factor 2 kinase upon CHO cellular growth metabolism and recombinant protein production.

- Investigate mTOR signalling within recombinant CHO cells that have different recombinant protein production capacity.

- Investigate the use of small molecule stimulation of mTOR and its effects upon recombinant protein production

The results of these investigations are presented and discussed in the following chapters.

Chapter 2

Materials and Methods

2.1 General Reagents

All reagents and materials used throughout the work described in this thesis were of analytical grade quality or better unless otherwise stated. All water used was of Milli-Q standard.

2.1 Mammalian cell culture

2.1.1 Cell culture maintenance

All adherent cell lines were maintained in flat T flasks (T25, T75 and T175; Starstedt) at 37°C in a 5% CO_2 atmosphere. To passage cells, spent medium was aspirated off and the cells washed with sterile phosphate buffered saline (PBS). The PBS was then aspirated off and trypsin (Invitrogen) added to detach the cells. Upon the addition of trypsin the cells were incubated for 2-5 min at 37°C, 5% CO_2. 10 ml of medium was then added to the flask to inhibit the trypsin and the desired number of cells/ volume for the seeding of a new flask was then added to a new flask which was then immediately placed back into the incubator. The adherent CHO lines were maintained in a number of different media as described below.

CHO Flp-In variant cell lines were routinely cultured in Ham's F12 medium containing 10% fetal bovine serum (FBS, Lonza). Every 4th passage, the Parental CHO Flp-In cell lines were cultured with 100 µg/ml zeocin (stock 100 mg/ml, Invitrogen), to maintain selection for CHO cells that contained the recombinase site within their genome. Stable cell lines that had been generated using the CHO Flp-In system, were cultured with 500 µg/ml hygromycin (Invitrogen) every 4th passage to maintain the selection for cells containing the gene of interest (GOI). All CHO Flp-In cells were cultured with their respective antibiotic selection before experimentation to ensure the cells within the culture were expressing their relevant GOI.

CHOK1 variant cell lines were routinely cultured in DEMEM containing 10% FBS with the addition of 15 ml of 200 mM L-glutamine, 5ml of L-glutamic acid + asparagine (6µg/ml of both L-glutamic acid and asparagine in ddH$_2$O), 10ml of nucleoside stock (175 mg each of adenosine, guanosine, cytidine, uridine and 60 mg of thymidine in 500 ml ddH$_2$O) and 5ml of non-essential amino acid solution (Sigma) in a total of 500 mL of medium. All CHO cell lines were maintained on a 3/4 day cycle of passage schedule, passaging at 1/50th of the cell concentration each passage.

Adherent HEK cells were maintained in DMEM containing 10% FBS and maintained on a once a week passage schedule, passaging at ~1/50 each passage. HEK Flp-In cells were maintained in the same manor with the addition of 150 µg/ml hygromycin (Invitrogen) every 3/4th passage to maintain the selection for cells containing the gene of interest (GOI).

Suspension CHO cell lines were maintained in 125 ml disposable Erlenmeyer flasks (Corning Inc), in CD-CHO medium (Invitrogen) containing 25 mM L-methionine sulfoximine (MSX). Cells were passaged once a week to a cell concentration of 0.3x10^6 viable cells/ml (cells were counted using a ViCell system (Beckman Coulter) in 10 ml of medium. Cells were then gassed with 5% CO$_2$ balanced air for 12 seconds each passage.

2.1.2 Recovery and cryopreservation of cells

Cryo stocks of all mammalian cells were made and preserved in liquid nitrogen. Cells were passaged for a minimum of 3 times in preparation for cyro preservation and cells for cryopreservation were taken from mid-exponential phase of growth. Prior to cryopreservation, cell cultures were assessed for cell numbers and viability using a commercially available ViCell instrument (Beckman Coulter). Cyropreservation of 1x10^7 viable cells/ml/vial in the appropriate medium (the medium the cells were maintained in) with 10% v/v DMSO were prepared and aliquot into 1.8 ml cryo-vials (Nunc). Vials were then stored in a Cryo 1°C freezing container (NALGENE, 5100-0001) and stored at -80°C overnight or at -20°C for 3-5 hours then -80°C overnight. Stocks were then stored in liquid nitrogen in a cryostat until required for further use.

When reviving cells from cryostorage, one vial of the desired cell line was thawed in a water bath at 37°C rapidly. The vial contents were then added to 10 ml of the appropriate media, the solution centrifuged at 1,000 rpm for 5 min and the supernatant removed. The cell pellet was then resuspended in 10 ml of fresh growth medium and placed in a T25 flask for 24-48 h at 37°C at which time the cells were then inspected under a microscope. Cells were then passaged 3 times before any experimentation was undertaken on them.

2.1.3 Counting of cells

Cells were either counted manually using a haemocytometer for determining cell viability and concentration by counting the number of cells in four large squares and then using the equation:

$$\text{Viable Cell Concentration/ml} = \text{Average no. of cells} \times \text{dilution factor} \times 10^4$$

$$\text{Viability} = \frac{\text{Total no. of living cells} \times 100}{\text{Total no. of cells}}$$

and using the trypan blue exclusion method. Alternatively, cell numbers were determined using a ViCell (Beckman Coulter) instrument.

2.1.4 xCELLegence monitoring of adherent cell growth

Prior to experimentation, mammalian cells were cultured in their appropriate medium with the required antibiotic selection. The xCELegence equipment programme for cell culture monitoring was prepared according the manufactures protocol. Electronic sweeps of the plates were set for every 30 minutes for 168 hours (7 days).

CHO/HEK cells were then taken from mid-log phase growth and seeded at 3000/5000 viable cells per well respectively in the appropriate 'E-plate' (Amgen) in the desired conditions, i.e. 32°C, 10nM rapamycin.

2.1. Generation of stable CHOK1 cell lines with the commercially available Invitrogen Flp-In system

CHOK1 cell lines expressing exogenous target genes of interest or shRNAs were generated using the commercially available CHOK1 Flp-In system essentially as described in the manufacturers manual (see Invitrogen Website). This system uses a Flp recombinase to insert the gene of interest into a specific *loci* in the genome. Briefly, CHOK1 Flp-In cells, maintained in Ham's F12 medium containing 10% v/v FBS and 100 µg/ml zeocin (stock 100 mg/ml, Invitrogen) were seeded 24 hours before transfection at one T25 flask per transfection, at a viable cell concentration of $5x10^5$ cells in a volume of 6.25 ml of medium without zeocin.

For transfection of the gene of interest into the CHOK1 cells, 9 µg of the pOG44 vector provided with the Flp-In system plus 1 µg of the FRT-cloning plasmid containing the gene of interest in the multiple cloning site were prepared in 625 µl of Optimum reagent (Invitrogen). In a separate tube, 30 µl of Lipofectamine 2000 transfection reagent (Invitrogen) was prepared in 625 µl Optimem**.** These preparations were then incubated for 5 minutes before being combined and mixed. The mixed solutions were then incubated for a further 20 minutes before adding all of the mixed preparation directly to the T25 flask of CHO Flp-In cells prepared 24 h previously as described above.

After the addition of the transfection reagent to the T25 flask, the cells were incubated for 5.5 hours at 37^oC, after which time the culture medium was removed, the cells washed with 10 ml PBS and fresh medium without zeocin added. This process was repeated again 24 hours post transfection. After a further 24 hours the cells were washed with 10 ml of PBS then incubated for 5 mins in 200 µl of trypsin-EDTA (Invitrogen), until the cells had detached from the surface of the T25 flask. The detached cells were then diluted into 20 ml of Flp-In medium containing 500 µg/ml hygromycin (Invitrogen). Into different wells of a 6 well tissue culture plate 2 ml per well of this cell suspension was then added and the plates put in an incubator at 37°C, 5% CO_2. The medium was then changed every 72 hours until colonies began to be observed by eye. Once cell colonies were visible by eye, the cell lines were either cloned or sub-cultured into T25 flasks and then T75 flasks so that cryopreservation of stocks could be performed. At this stage cells were considered ready for experimentation and validation of expression of target genes.

2.2 Construction, manipulation, transformation, mutation and transfection of DNA and RNA

2.2.1 Generation of cDNA from RNA

CHOK1 cells were cultured in T175 flasks for 72 hours before being lysed using a RLT lysis buffer provided by Qiagen. Total RNA was extracted from the lysate using a QIAGEN Shredder and RNease kit. The RNA was then used in a reverse transcriptase reaction consisting of 4 µg RNA + 4 µl oligo DT + 7 µl RNAse free water in a 1.5 ml Eppendorf tube, and then incubated for 5 minutes at 70°C before being transferred on to ice for 2 minutes. Once on ice, 10 µl of 5x MMLV buffer (Promega), 2.6 µl of 10 mM dNTPs, 1.4 µl RNase, 4 µl nuclease free water and 2 µl MMLV reverse transcriptase (Promega) was added to the reaction and left for 1 hour. The subsequently generated cDNA was then cleaned up using the wizard PCR clean-up kit (Promega) and stored at -20 or -80°C until required for use in experiments.

2.2.2 RNA extraction from mammalian cells for cloning or PCR amplification of target genes

Adherent CHO cell total RNA lysates were extracted from cells cultured in 6 well plates. Extraction of total RNA from mammalian cells was undertaken using the commercially available RNease kit and the protocol provided by the manufacturer (Qiagen). The total RNA was extracted from the lysate using a QIAGEN Shredder kit and the amount of RNA extracted determined by A260 nm measurement. Total RNA was stored at -80°C.

2.2.3 PCR methods

2.2.3.1 Amplification of target gene sequences

Primers designed for PCR generation of target genes of interest (GOI) are described in the relevant results chapters (see section 3.2.1.1). Genes were amplified from cDNA templates and hence are from mature mRNA transcripts and do not include any introns but the open reading frame only. UTRs were not amplified. For PCR amplification of target genes the Phusion high fidelity DNA polymerase (New England Bioloabs, NEB) was used to amplify specific sequences using the primers designed for amplification of the target GOI. Reaction

mixes for PCR of the GOI were prepared following the protocol provided with the polymerase and as detailed below.

PCR conditions:
- Initial denaturation step: 98°C, 30 sec.
- 30-35 cycles:
  - Denaturation: 98°C, 10 sec
  - Annealing: 60-64°C, 30 sec
  - Elongation: 72°C, 20 sec/1 kb of DNA
- 72°C hold, 10 mins
- final hold 20°C

For a 50 µl reaction PCR reaction:
- NF water (nudease free water) to 50 µl (variable)
- 10 µl 5x Phusion HF buffer (The default buffer provided for High Fidelity PCR reactions) or GC buffer (A buffer to be used with GC-rich templates, of those that have secondary structure)
- 1 µl 10 mM dNTPS
- 2.5 µl 10 µM forward primer
- 2.5 µl 10 µM reverse primer
- 1µg template cDNA
- 1.5 µl DMSO (optional)
- 0.5 µl Phusion DNA polymerase

2.2.3.2 PCR colony screening of competent DH5α E.coli cells transformed with a GOI+vector

GoTaq Polymerase (Promega) was used to screen colonies produced by transformation of a GOI PCR product after digestion with the appropriate restriction enzymes and ligation with the target acceptor plasmid vector followed by transformation of the ligation into DH5α *E. coli* competent cells. Colonies were picked from the LB agar plates (with the appropriate selection antibiotic) using 200 µl sterile pipettes. Part of the colony was suspended in 5 µl of NF water for the PCR reaction; the remainder was streaked onto another LB agar plate, as a preparation for if the screened colony was positive for the presence of the GOI insert.

The PCR parameters and conditions for colony screening for the presence of the correct insert/GOI are detailed in table 2.1. The resulting PCR products were analysed by agarose electrophoresis to determine if a band of the expected size was present. For PCR colony screening universal T7 (taatacgactcactataggg) and BGHR (tagaaggcacagtcgagg) cloning primers were used to amplify the desired target sequence. When agarose gel electrophoresis confirmed the presence of the desired target insert, the corresponding colony was grown in an overnight culture in a liquid LB broth as described in section 2.2.11. PCR cycles were set according to the protocol provided with the GoTaq kits used. The PCR conditions were as follows:

- Initial denaturation step: 94°C, 5 mins
- 30 cycles:
    - Denaturation: 94°C, 30 sec
    - Annealing: 50°C, 30 sec
    - Elongation: 72°C, 1 min/1 kb of DNA
- 72°C hold, 7 mins
- final hold 20°C

Table 2.1 Reaction constituents for PCR colony screens of DH5α *E. coli* cells for the presence of the required plasmid and GOI insert

GoTaq screening	GoTaq Hot start Green Master mix
25 µl reaction: • 5 µl of NF water + colony • 7.3 µl NF water • 4 µl GoTaq x5 Buffer • 1.6 µl 25 mM MgCl$_2$ • 0.4 µl 10 mM dNTPs • 0.8 µl Forward primer (T7) • 0.8 µl Reverse primer (BGHR) • 0.1 µl Gotaq polymerase	For a 25µl reaction: • 5 µl of NF water + colony • 12.5 µl GoTaq master mix x2 • 1 µl Forward primer 10 µM • 1 µl Reverse primer 10 µM • 5.5 µl NF water

2.2.4 DNA and RNA quantification

Quantification of DNA and RNA samples was carried out using a Nano-Drop Spectrometer ND-1000. For quantitation, 2 µl of the DNA/RNA solution was dotted into the Nano-Drops platform and measured at OD 260 nm. DNA/RNA was then quantified in ng/µl.

2.2.5 DNA and plasmid DNA analysis and separation via gel electrophoresis

DNA and plasmid DNA samples were analysed and separated using 1% (w/v) agarose/TAE gels and 3 µl of SyBR Green per 50 ml of agarose/TAE solution. Samples were suspended in 6x DNA sample buffer (Promega) and run at 100 V or 70 V for 30 mins or 60 mins. DNA bands were then visualised using a Transiluminator BioView UV lamp (Biostep) in a Gel logic 100 imaging system (Kodak). When required, DNA bands were cut from the agarose gel and the subjected to a gel dean-up using a commercially available kit to recover the DNA for further use.

2.2.6 DNA restriction enzyme digestion

All DNA digestions were undertaken using Promega or Fermentas restriction enzymes and the appropriate buffers as instructed by the manufacturers. Each restriction digest enzyme was used with the appropriate restriction site sequence as specified in the results section. The primer tables 3.1 and 3.2 show the specific DNA sequences on primers and hence the restriction enzymes used for the cloning of each GOI. Digests were undertaken following the protocols provided with the specified enzyme by each manufacturer.

2.2.7 DNA ligation reactions

Ligation of DNA inserts amplified by PCR was generally achieved using a commercially available T4 DNA ligase (Promega) following the protocol provided with the enzyme. The exception to this was the ligation of the wild type elongation factor 2 (eEF2) PCR into the FRT vector. This ligation was undertaken using the T4 ligase and a difference buffer. Concentrations of the various components of the buffer were:

- 132 mM Tris-HCl
- 20 mM $MgCl_2$
- 2 mM ATP
- 2 mM DTT
- 15% (v/v) PEG 6000
- Calibrate to pH 7.6 at 25°C

2.2.8 Generation of an shRNA for the knockdown of the kinase eEF2K

An shRNA for the knockdown/silencing of elongation factor 2 kinase (eEF2K) was constructed using the Gene clip U1 hairipin cloning system-Pyromycin (Promega, C8760), following the instructions provided with the kit. Further details on the primer design and exact protocol are described in section 4.2.1.

2.2.9 Generation of competent DH5a *E.coli* cells

To prepare competent DH5α *E.coli* cells, cells were initially streaked onto and cultured on an LB agar plate over night at 37°C. A single colony from the plate was then picked and incubated overnight in 2 ml of liquid LB broth in a 15 ml falcon tube at 37°C with orbital shaking at 200 rpm. 0.5 ml of this starter culture was then used to inoculate 40 ml of liquid LB broth and incubated at 37°C with shaking at 200 rpm, until an OD_{600} nm of between 0.3-0.5 absorbance was obtained. The DH5α cells were then pelleted (2500 g for 5 minutes) and then resuspended in 20 ml of 50 mM $CaCl_2$ and incubated on ice for 30 minutes. The cells were then pelleted again (2500 g for 5 minutes) and resuspended in 4 ml of ice-cold 50 mM $CaCl_2$ and incubated on ice for a further 30 minutes. 100 µl aliquots of the cell suspension were then placed into sterile 1.8 ml cryo-vials on dry ice. Aliquots of the competent cells were then stored at $-80^{o}C$ until required.

2.2.10 Transformation of competent E.coli cells with plasmid DNA

100 µl aliquots of competent DH5α cells were removed from storage at -80°C and thawed on ice. Either 1 µg of the required plasmid DNA construct or all of a recently ligated construct, was added to a single DH5α aliquot and incubated for 30 minutes on ice. The DH5α cells where then heat shocked in a water bath for 90 seconds at 42°C and then re-incubated on ice. The cells were then incubated for 1 hour at 37°C with shaking at 200 rpm with 900 µl LB medium added to each aliquot. 100 µl of the culture was then plated on LB ampicillin 50 µg/ml plates and incubated overnight at 37°C.

2.2.11 Plasmid DNA recovery from *E.coli* cells

DH5α *E.coli* cells transformed with DNA constructs were cultured in 6 ml or 250 ml liquid LB broth overnight with 50 µg/ml ampicillin antibiotic selection at 37°C with shaking at 200 rpm. DNA was extracted and recovered from transformed DH5α *E.coli* cells using the commercially available mini prep/high speed maxi prep kits from Qiagen following the protocols provided with each kit.

2.2.12 DNA sequencing

Aliquots of prepared DNA were sequenced using a sequencing service provided by Beckman-Coulter.

2.2.13 Site directed mutagenesis of DNA

Site directed mutagenesis of various DNA sequences described in the results sections of this thesis (see sections 3.2.1.1) were achieved using the commercially available QuikChange® Lightning Site-Directed Mutagenesis Kit (Aligent Technologies) and the protocol included within the kit. Primers for each of the relevant mutations generated are described in the relevant results sections (see sections 3.2.1.1 and 3.2.1.2). The presence of the correct mutation was confirmed by DNA sequencing.

2.2.14 Transfection of DNA into mammalian cells

Mammalian cells were seeded as follows for transfection. For experiments in 6 well tissue culture plates, 2×10^5 viable cells in 2.5 ml of media/well; for 24 well tissue culture plates, 6×10^4 viable cells in 600 µl of media /well. Cells were seeded into tissue culture plates 24 hours prior to transfection with the appropriate DNA. For transfection 1-2 µg (6 well plates) or 0.8 µg (24 well plates) of DNA was suspended in 250 µl or 50 µl of Optimen (invitrogen) respectively whilst in a separate tube 12 µl of the commercially available lipofectamine 2000 transfection reagent in 250 µl or 50 µl of Optimen was prepared per well. Both tubes were then incubated for 5 minutes at room temperature before the contents of each tube were mixed together and incubated for a further 20 minutes. 500 µl (6 well plates) or 100 µl (24 well plates) of the resulting solution was then added to each well and incubated at 37°C or 32°C, dependent upon the experiment, for the period of time required before cells or supernatants (or both) were harvested for analysis.

2.2.15 Quantitative PCR analysis (qRT-PCR) of target mRNA amounts

2.2.15.1 qRT-PCR analysis of eEF2 and eEF2K mRNA amounts

qRT-PCR analysis to determine the relative amounts of target mRNAs (relative to a house keeping mRNA, β-actin that was assumed not to change between samples) between samples was undertaken using the commercially available iScript™ One-Step RT-PCR Kit with SYBR® Green Kit (BioRad) and a Eppendorf Realplex master cycler instrument. RNA samples were diluted to a concentration of 25 ng/µl in sterile nuclease free water (sNF). Separate master mixes (25 µl per a reaction) for each experimental and housekeeping gene primer pairs were prepared on ice under sterile conditions following the dilutions described in table 2.2 below.

Table 2.2 Reaction mixture volumes for qRT-PCR analysis

Reagent	Volume per reaction (µl)
sNF	8.5
2x SYBR Green	12.5
Forward primer (10 µM)	0.75
Reverse primer (10 µM)	0.75
Reverse transcriptase	0.5
Template RNA	2

25 µl per a reaction of the master mixes were the pipetted into a Bio-Rad 96-well optical plate on ice. The plate was then sealed and placed in the Eppendorf Realplex master cycler (Thermocycler) and the following program used:

- 50°C for 10 minutes
- 95°C for 5 minutes
- 40 cycles of
  - 95°C for 10 seconds
  - 55°C for 30 seconds, SYBR emission reading
- 95°C for 15 seconds
- 55°C for 15 seconds
- Melting curve: 55°C to 95°C with 0.5°C
- 95°C for 15 seconds

The Eppendorf instrument excites the fluorescent dye used and the software calculates when the fluorescence reaches the critical threshold (C$_T$). The C$_T$ value is that when the fluorescence becomes detectable above the background. The C$_T$ value is inversely proportional to the logarithm of the initial copies of template DNA (Livak & Schmittgen 2001). Calculations using the C$_T$ values of the sample were normalised to that of the housekeeping gene (the endogenous control) β-actin. This comparative method follows the comparative C$_T$ method equation shown below:

$$\text{Relative Expression} = 2^{-\Delta\Delta C_T}$$

2.2.15.2 Qiagen RT2 Profiler PCR Array Mouse mTOR Signalling plates and analysis

cDNA from samples for analysis was prepared for the PCR array using a Qiagen RT2 First Strand Kit from CHO Flp-In RNA lysates. The cDNA and RT2 SYBR Green ROX qPCR Mastermix (Qiagen) was then added to the commercially available array plate (Catalogue number PAMM-098Z) in accordance with the protocol provided with the array. The Plates were then run in an Eppendorf Realplex master cyder (Thermocylcer) in accordance with the protocol provided with the kit. Analysis of the resulting data was undertaken using the Excel spread sheet provided by the manufacturer. The outputs of this are provided in Appendix 7.6.2.

2.3 Protein extraction and analysis protocols

2.3.1 Preparation of total protein cell lysates from cultured mammalian cells

2.3.1.1 Lysis buffer preparation

Mammalian cells were lysed in a buffer consisting of 200 mM HEPES-NaOH pH7.2, 100 mM NaCl, 1% v/v TX 100 and 10 mM Na-β-glycerophopate. Before use a complete mini, EDTA-free protease inhibitor tablet (Roche) was added to 10 ml of the lysis buffer as well as 50 mM NaF and 1 mM NaV (Na-Vanidate) (New England Biolabs).

2.3.1.2 Protein extraction

Protein from cells cultured in 6 well plates were extracted by washing the cells in 1 ml of PBS for each well and then the cells were incubated in 200 µl of cell lysis buffer per well on ice for 5 minutes. The wells were then scraped and the contents put into a sterile 1.5 ml Eppendorf tube. An additional 200 µl cell lysis buffer per well was used to wash each well and this was then collected and placed into the corresponding Eppendorf tube so that a combined 400 µl of cell lysate was collected per 6-well plate.

2.3.2 Bradford assay quantification of protein concentration in cell lysates

Protein quantification of cell lysates was determined using a Bradford assay with each lysate being measured in duplicate. Cell lysates were centrifuged for 1 minute at 13000 rpm and then kept on ice. 5 µl of lysate was then diluted in 45 µl with ddH$_2$O and then 1 ml of

Bradford reagent (BioRad) was added to the solution. The Bradford solution was then mixed and incubated for 10 minutes. The total protein was then quantified by reading the absorption of the solution at a wavelength of 600 nm using an Eppendorf BioPhotometer. The concentration of protein in each lysate was then calculated by comparison to a standard curve generated using known amounts of bovine serum albumin (BSA).

2.3.3 SDS-PAGE analysis of protein extracts

Tris-glycine SDS-polyacrylamide gels were routinely used for protein analysis. SDS page gels were prepared using: www.changbioscience.com/calculator/sdspc.htm for dilution calculations; 30% 29:1 polyacrylamide (BioRad), ammonium persulphate and tetramethylelthylenediaine (TEMED), all sourced from BioRad. SDS gels were prepared in Novex 1 mm gel cassettes (Invitrogen).

Protein lysate samples for analysis were diluted in NuPAGE LDS sample buffer x4 (Invitrogen) to the volume appropriate for the well size used or concentration of protein required (typically 5-20 μg/well) and number of runs per sample (well volume (μl) x number of runs = total volume of sample). Prior to loading, samples were boiled for 5 minutes at 85-95°C, then loaded into the SDS gel for analysis. Gels were assembled in a Novex mini cell tank (Invitrogen) and loaded with the prepared samples and electrophoresis undertaken at 125 V for 1 hour and 30 minutes or until the dye front of the samples had reached the bottom of the gel. Gels were extracted from the gel cassettes and either stained with Coomassie blue R-250 for 20-60 minutes and then destained until protein bands were visualised or prepared for transfer onto nitrocellulose for western blot analysis.

2.3.4 Western blot analysis of proteins

Proteins separated by SDS-PAGE were transferred to 0.45 μm nitrocellulose blotting membrane (Amersham Hybond ECL) via electrophoresis using a TE series transfer electrophoresis unit (Hoefer) for 1 hour at 4°C. After transfer, the blots were incubated in 20-30 ml of blocking buffer (5% powdered milk (Marvel) in TBST) for 30 minutes-1 hour. Blots were then washed in 0.1% TBST three times for 5 minutes per wash, then incubated overnight in a 10 ml dilution of the appropriate primary antibody prepared in 3% BSA-TBS.

After overnight incubation, the blots were washed 3 times with 0.1% TBST for 10 minutes, and then incubated in the appropriate secondary antibody dilution in blocking buffer for 1 hour. Blots were then washed again 3 times with 0.1% TBST for 10 minutes, and incubated for 5 minutes in ECL western blotting detection reagents (Amersham), following the instructions provided with the reagents by the manufacturer. The blots were then exposed to High performance chemiluminecsese film (Amersham Hyperfilm ECL), which was subsequently processed using a Compact X4 processor (Xograph imaging systems).

2.3.5 Luciferase assays

Mammalian cell cultures were prepared for firefly luciferase assays in 6 or 24 well plates seeded as follows; 6 well plates: 2×10^5 viable cells in 2.5 ml of media per well, 24 well plates: 6×10^4 viable cells in 600 µl of media per well. Cells were washed with warm (37°C) PBS then incubated for 5 minutes in 200 µl of luciferase lysis buffer (LLB) (500 µl PBS A [348 mM Na_2HPO_4, 70 mM NaH_2PO_4], 500 µl PBS B [18 mM $CaCl_2\cdot2H_2O$, 70 mM KCl, 18 mM $MgSO_4\cdot6H_2O$, 2740 mM NaCl] , 0.1% v/v Triton X, 8 ml ddH_2O, 1 mini-complete EDTA-free tablet (Roche)) for 5 minutes and lysates then scraped and collected into sterile 1.5 ml Eppendorf tubes. Samples could then be stored at -80°C. Lysate total protein concentration was then quantified using a Bradford assay. The luciferase assay was undertaken using 75 µl of lysate in a 96 well flat bottom white polystyrene plate (Optiplate) and 75µl of steady lite reagent provided with the kit (Prepared according to instructions provided with the kit, PerkinElmer). The plate was incubated for 10-15 minutes at room temperature, then light was measured as light counts per a second using a FLUOomega plate reader (BMG Labtech).

Luciferase assays were also undertaken using the steady lite kit directly on cells in a 24 well plate. For these assays, cells were washed with 1 ml of warm PBS (37°C), then 200 µl steady lite solution added directly onto cells and incubated for 15 minutes. Two aliquots of 100 µl of each lysate was added to a 96 well plate and light was measured as light counts per a second using a FLUOomega plate reader (BMG Labtech).

2.3.6 Florescence imaging of live cells expressing FRET

For live cell imaging, mammalian cells were seeded into glass bottom petri dishes, with cover slides that have a collagen coating (MatTek, part number P35GCol-10-14-C), at 2.5×10^5 viable cells in 2.5 ml of media per dish. Cells were left overnight to adhere, then transfected with the appropriate GOI using the same transfection protocol as described for 6 well plate transfections. The plates were then incubated for 24 hours then imaged using a temperature controlled LEICA DMIRE2 confocal microscope (TCSSP2 AOBS).

2.3.7 Immunoflorescence imaging of fixed CHO cells

For immunofluorescence imaging of cells, prior to seeding cells before transfection, cover slides were sterilised and placed 1 per well in 24 well plates. Cells were then seeded at 6×10^4 viable cells per well and incubated for 24 hours. 0.8 µg of the selected DNA plasmid was then added per well in triplicate wells using the 24 well plate transfection protocol described above and incubated for 24 hours. Cells were then prepared according to the following protocol:

Methanol (MeOH) fixing of cells

Cells were required to be fixed to the cover slips within each well:

- Siphon media from wells
- Wash the cells with 1 ml warm PBS (37°C)
- Siphon off PBS
- Add 1 ml MeOH (-20°C)
- Incubate the plate for 5 minutes on ice
- Siphon off MeOH and air dry (about 5 minutes)
- Keep plates at 4°C (for up to 1 week)

Antibody probing of the cover slides

Once the cells were fixed to the cover slides, the slides were probed using antibodies to visualise specific targets. This was achieved using the following protocol:

- MeOH fixed cells were rehydrated with 1 ml of PBS (room temperature, RT) for 5min
- PBS was then siphoned off
- The cover slips were then incubated with 250 µl blocking buffer (3% w/v BSA in PBS) per well for 15 minutes at RT

- 25 μl droplets of 1° antibody (diluted 1:500, anti-v5 (Sigma) or anti-eEF2) in 3% w/v BSA were placed on a sheet of parafilm in a damp box and the slides applied 'cell side' down. The slides were incubated overnight at 4°C in the damp box.
- 4 sets of 100 μl PBS with 0.1% Tween wash droplets were prepared on sheets of parafilm.
- Cover slips were then transferred from the damp box to the 1st wash droplet (being dipped in PBS and 0.1% v/v Tween between 1st antibody and 1st wash droplet). The cover slips were then transferred 'cell side down' through the wash droplets, allowing 5 minutes per wash.
- Whilst the cover slips were on the 1st droplet the 2° antibody was prepared: FITC and TRITC antibodies (Sigma) were diluted 1:100 in 3% w/v BSA dilutions prepared in light protected Eppendorf tubes and centrifuged (10 minutes, 13000 rpm) to remove aggregates; the 'supernatant' was then transferred to a fresh light protected tube.
- 25 μl droplets of 2° antibody were then aliquoted on to parafilm in a light protected damp box. Cover slips were transferred cells side down onto the 2° antibody droplets; blotting off any excess liquid from previous washes.
- The cover slips were then incubated in the dark for 2 hour at RT
- The cover slips were then washed again in 100 μl PBS with 0.1% Tween as they were after incubation with the 1° antibody.

Nuclear staining of DNA
- 25 μl droplets of DAPI (10 mg/ml) were prepared on parafilm in a light protected box.
- Cover slips were then placed cell side down on the DAPI droplets and incubated for 1 minute.
- Cover slips were then washed twice for 10 minutes in 100 μl PBS droplets, prepared on parafilm.

Anti-fading and preparation of slides for imaging
- During the washing process, either after incubation of 2° antibody or post staining with DAPI, 900 μl of Mowiol was mixed with with 100 μl of phendinediamine.
- 5 μl of the Mowiol/Phendinediamine mixture was then dripped on to a microscope slide and the cover slip applied on top of the 5 μl cell side down.
- Slides/cover slips were then left at 4°C overnight to set in a light protected box.

- The edges around cover slips were sealed with clear nail varnish and the top of the cover slip washed with ddH$_2$O and a cotton bud.

- Cells were then imaged on a LEICA LEITZ DMRD or a LEICA DMIRE2 confocal microscope (TCSSP2 AOBS).

- Wavelengths of excitation and emission of each colour visualised are shown in table 2.3.

Table 2.3 Wavelengths used for excitation and emission of colours visualised during immunoflorescence studies

Excitation wavelength (nm)	Emission wavelength (nm)
340-380 (Blue)	LP 425 (Blue)
450-490 (Blue)	LP 515 (Green)
515-560 (Green)	LP 590 (Red)

2.3.8 ELISA assays

ELISA Buffers

The following buffers were prepared and used for ELISA assays.

- **Coating buffer**: 0.8 g Na$_2$CO$_3$, 1.47 g NaHCO$_3$ in 500 ml water. Calibrate to pH 9.7

- **Block solution**: 1.59 g Na$_2$CO$_3$, 2.93 g NaHCO$_3$ in 1 litre water. Before use dissolve 1.0 g casein to 200 ml in block solution overnight.
Wash buffer: 5.84 g NaCl, 1.15 g Na$_2$HPO$_4$, 0.29 g NaH$_2$PO4.H$_2$O, 3.72 g EDTA, 200 ⍰l Tween-20, 10 ml L-butanol in 1 litre water. Calibrate to pH 7.2

- **Sample buffer**: 12.1 g Tris-HCl, 5.84 g NaCl, 200 µl Tween-20, in 1 litre water. Calibrate to pH 7.0

- **Substrate buffe:** 8.2 g Na acetate in 1 litre water. Calibrate to pH 5.0 (1 M citric acid)

ELISA IgG standards preparation

5 μl Human IgG (Human IgG reagent grade, 10 mg/ml, Sigma) in 5 ml sample buffer was prepared. Then 25 μl of the preparation was diluted in 975 μl of sample buffer. Seven serial dilutions were then prepared to provide a standard curve ranging from 250 ng/ml to 3.9 ng/ml of IgG.

ELISA Protocol

Prior to plating of samples, 96 well plates (Nunc) were coated with 100 μl/well AffiniPure F(ab')2 Fragment Goat Anti-Human IgG Fc fragment specific (minimal cross-reaction to Bovine, Horse, Mouse serum proteins) (Jackson Immune Research). The plates were stored in light protected conditions at 4°C overnight. Samples were then serial diluted using sample buffer and stored at 4°C. When required, the plates were washed twice with excess amounts of the wash buffer and the following steps were then taken to prepare the sample:

(Note, between each step the plates were washed twice with excess amounts of the wash buffer)

1. 200 μl per well of blocking solution was placed in each well for 1 hour with shaking at room temperature (RT).
2. 100 μl per well of sample/IgG standard was then added per well with shaking at RT for 1 hour.
3. 100 μl per well of secondary antibody solution (Anti-human kappa light chain (bound and free) HRP conjugate raised in goat (Sigma) diluted 1:10 000 in wash buffer) per well was then added for one hour with shaking and at room temperature (RT).
4. 100 μl per well of TMB (Sigma) substrate per well was then added.

The samples and standards would turn blue. Once the standards had developed to an appropriate depth of blue colour, 50 μl per well of 2.5 M H_2SO_4 was added, and the colour changed to yellow. The plate OD was then read at 450 nm using a jBio 96 well plate reader. The concentration of samples was then determined from the standard curve from the known concentrations of IgG.

Chapter 3

Manipulation of Translation Elongation Factor 2 (eEF2) and the Effect on Recombinant Protein Expression from Chinese Hamster Ovary (CHO) Cells

3.1 Introduction

Studies on elongation factor 2 (eEF2) have shown that it is a key regulator of translation and polypeptide synthesis, specifically of polypeptide elongation, and that increasing the activity and/or the level of eEF2 increases cellular protein production and growth, as well as the up regulation of eEF2 has been implicated in cancer (Nakamura et al. 2009), indicating that an increase in eEF2 activity results in an increase in cellular proliferation and global protein production.

Elongation factor 2 kinase (eEF2K) is the only known regulator of eEF2 protein, which phosphorylates eEF2 on the Thr56 residue of the protein (Kaul et al. 2011). Phosphorylation of eEF2 results in its inactivation; slowing or halting elongation and hence attenuating protein synthesis. Activation of eEF2K and the subsequent phosphorylation of eEF2 is regulated by a number of mechanisms, but particularly via the mTOR (mammalian target of rapamycin) signalling pathway. Of the two mTOR complexes mTORC1 is responsible for eEF2K regulation via S6K1.

This chapter describes investigations into the manipulation by over-expression and mutation of eEF2 in Chinese hamster ovary (CHO) cells and the effects this has upon cellular growth and recombinant protein production. Through the construction and profiling of stable (CHO) cell lines over expressing eEF2 and mutated variants: Thr56Ala and Thr56Glu it was possible to investigate the effect of eEF2/amount/activity and regulation upon cellular growth and recombinant protein production in this industrially important mammalian expression system.

3.2 Results

3.2.1 Transient Expression of eEF2 in CHO Cells

The initial investigations into the role or effect of translation elongation factor 2 (eEF2) in regulating recombinant protein production from cultured CHO cells was undertaken by transiently over-expressing either human of Chinese hamster eEF2 in CHO cells. In order to undertake such studies it was first necessary to generate mammalian expression vectors for the over-expression of CHO or human eEF2.

3.2.1.1 Construction of eEF2 Mammalian Expression Vectors

The initial step to clone the eEF2 gene was to identify the sequence using the NCBI database. The sequence for both human (NCBI accession number AY942181) and Chinese hamster (NCBI accession number M13708) eEF2 were retrieved and sequence alignment revealed that the sequences had 99.2% homology at the amino acid level and therefore are likely to be functionally interchangeable. At the nucleotide level the homology was much less at 87.3% (Appendix 7.1 & 7.2). At this stage it was anticipated that studies might be undertaken in both human (HEK/HELA) cells and CHO cells and hence both human and Chinese hamster eEF2 sequences were cloned. As these are 99% homologous at the amino acid level we might expect these to 'behave' in a similar manner when expressed in CHO cells. The human sequence was obtained by isolating total RNA from HELA cells, the Chinese hamster from total RNA extracted from CHO cells and then cDNA generated from this material by a reverse transcriptase reaction. The eEF2 sequence was then amplified from the resulting cDNA by PCR using the primers described in table 3.1. The primers were designed to allow cloning into the commercially available pcDNA3.1 expression vector. The PCR product was then ligated into the commercially available pcDNA3.1V5tag (Appendix 7.3) mammalian expression vector after digestion of the vector and the PCR insert with the appropriate restriction enzymes. Following transformation into competent *E.coli* cells the presence of the insert was confirmed by colony PCR screening (Figure 3.1). Finally, the correct sequence of the wild-type eEF2 (WT eEF2) was confirmed by DNA sequencing using a commercial provider.

Using the Chinese hamster wild-type eEF2 construct, a number of mutants were then constructed. Control of the activity of eEF2 has been shown to be switched on and off by phosphorylation of threonine residue 56 (Thr56) (Kaul et al. 2011) as outlined in the introduction to this thesis. The usual approach to mutate such threonine (or indeed serine) residues that may be phosphorylated to prevent this is to mutate these to alanine (Ala) residues (Browne & Proud 2004; Pavur et al. 2000). Such residues may also be mutated to a glutamic acid (Glu) in order to try and mimic a phosphorylated threonine residue. The Ala mutates eEF2 in such a way that it cannot be phosphorylated at Thr56 and hence is always in the 'on' state and the mimics of a permanent phosphorylated Thr56 residue (Glu) that eEF2 appears to the cell to always be in the 'off' state. This is because the glutamic acid residue has a negative side chain, therefore acts as if it is already phosphorylated. To generate these mutants here, the commercially available Quik Change kit (Qiagen) was used along with the primers descried in table 3.1 to mutate the Threonine 56 amino acid to an alanine (Ala56 eEF2) or glutamic acid (Glu56 eEF2) residue. The Thr56Ala mutant was thus generated in the Ppc3.1V5 plasmid backbone and the Thr56Glu in in the FRT plasmid backbone. The only difference between these was the single amino acid change compared to the wild type eEF2, this being confirmed by sequencing of the resulting construct. Using these two mutants and the wild type eEF2 it was then possible to begin investigating the effect(s) of transient over expression of eEF2 in CHO cells.

Table 3.1 Primers used to clone, sub-clone and mutate human and Chinese hamster elongation factor 2 (eEF2) sequences. eEF2 sequences for cloning into pc.1V5 were primed from human and Chinese hamster cDNA generated from HELA and Chinese hamster ovary cells (CHO) respectively. The Thr56Ala point mutation was achieved using the pc3.1V5 with an inserted CHO eEF2 wild type sequence, to generate an eEF2 Thr56Ala mutant inserted in the PC3.1V5 vector.

For insertion of eEF2 into FRT, the Chinese hamster eEF2 sequences were sub cloned from the pc3.1 + eEF2 vectors, with and without the V5 tag, using the primers described in the FRT section of the table. Point mutation of Thr56Glu was achieved using the FRT + eEF2 wild type vector once it was constructed.

PC3.1V5	**Forward Primers**	
	Hela BamH1 forward:	tatggatcccactatggtgaacttcacggtagacc
	CHO NotI:	tatgcggccgctgagaatccgtcgccaatg
	Reverse Primers	
	Hela XhoI reverse Stop:	atactcgagctacagtttgtccaggaagttgtcc
	Hela XhoI reverse V5:	atactcgagtacagtttgtccaggaagttgtcc
	CHO XbaI stop:	atatctagaacggatattctacagtttgtccaggaagttgtcc
	CHO XbaI V5:	atatctagaacggatatttacagtttgtccaggaagttgtcc
	THr56Ala mutation primers	
	CHO Ala56 sense	ggtgagacacgcttcgccgacacccgcaaggacgaacag
	CHO Ala56 anti-sense	ctgttcgtccttgcgggtgtcggcgaagcgtgtctcacc
FRT	**Forward Primers**	
	Stop Forward Kpn1	tatggtacctgagaatccgtcgccaatg
	V5 Forwards Pme1	tatgtttaaactgagaatccgtcgccaatggtg
	Reverse Primers	
	Stop Reverse Not1	atagcggccgcacggatattctacagtttgtccaggaagttgtcc
	V5 Reverse Pme1	ctgatcagcgggtttaaactcaatggtgatg
	Thr56Glu mutation	
	Forwards	ggtgagacacgcttcgaggacacccgcaaggacgaacag
	Reverse	ctgttcgtccttgcgggtgtcctcgaagcgtgtctcacc
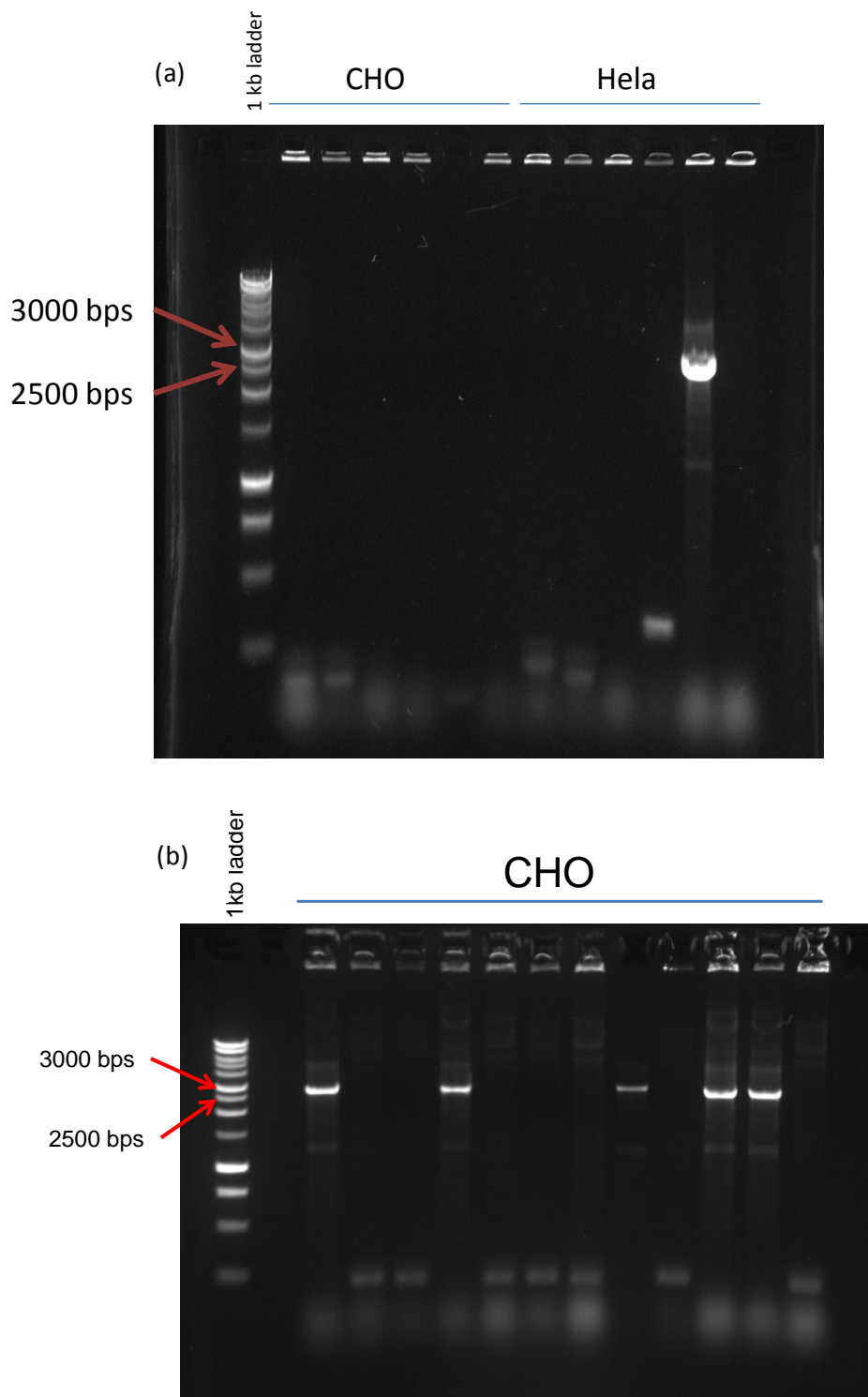
Figure 3.1 (a)(b) PCR colony screens of competent *E.coli* cells transformed with pc3.1V5 ligated with either **(a)** human or **(b)** Chinese hamster elongation factor 2, confirming insertion of eEF2 into the pc3.1V5 vector.

Initially, after the construction of the FRT eEF2 V5 vactor, the C-terminal V5 reporter tag was found to not be expressed I.e. not giving a signal on blots or in immunoflorescences experiments when probed for using antibodies specific for the V5 tag. Therefore the V5 tag was attached to the N- terminus of eEF2, to determine if the physical location of the V5 tag was effecting its expression. Elongation factor 2 was therefore cloned into an FRT vector with a N-terminal V5 tag using the primers shown in table 3.2.

Sequencing of the NV5 FRT eEF2 wild type vector was confirmed using commercial sequencing. Later it was found that both the NV5 and CV5 tagged eEF2 had an unexpected error within their sequencing. Within the CV5 tagged eEF2 the sequence between the deleted stop codon at the end of the eEF2 sequence   and the V5 tag had another stop codon inserted during cloning, preventing the expression of the V5 tag. In the NV5 FRT eEF2 vector, the sequence between the N-V5 tag and the start of eEF2 was out of frame by one nucleotide preventing the translation of eEF2 with an N-terminal V5 tag. To remedy these problems, the Quik Change lighting mutagenesis kit and primers (Table 3.2) was used on the CV5 stop codon mutated to a 'non-stop' codon; and a single nucleotide deletion within the sequence between the NV5 and the eEF2 sequence allowed for the expression of eEF2 with a N- or C- terminal V5 tag. These sequences were confirmed using commercial sequencing.

Table 3.2 Primers used for construction and mutation of FRT vectors for expression of eEF2 with either an N-terminal or a C-terminal tagged elongation factor 2.

eef2 insertion into nv5 vector		
Fwd(Age1)		tataccggttgagaatccgtcgccaatg
Rev(Age1)		ataaccggtacggatattctacagtttgtccag
v5 mutagenesis		
CV5	Fwd	ggacaacttcctggacaaactgtacatatccgttctagagggc
	Rev	gccctctagaacggatatgtacagtttgtccaggaagttgtcc
NV5	Fwd	cgattctacgcgtaccgtcactatggtgaacttcacggtagacc
	Rev	ggtctaccgtgaagttcaccatagtgacggtacgcgtagaatcg

3.2.1.2 Investigating the Effect of Transient eEF2 Over-Expression in CHO Cells

The constructs generated in section 3.2.1.1 above were used to transiently over-express eEF2 or the mutants in a model CHOK1 system. As described above, the constructs were generated in a vector with a read through the stop codon to a V5 tag on the C-terminal end. This allows detection of the recombinant material from the construct but not the endogenous material by using an anti-V5 antibody. Initially the human WT, eEF2 construct was transfected into the CHOK1 cells to check that expression of the recombinant eEF2 could be achieved; cell lysates were collected 24 and 48 h post-transfection. The resulting lysates were then analysed by SDS-PAGE and subsequent western blot analysis using an anti-V5 antibody undertaken and are shown in Figure 3.3 below.
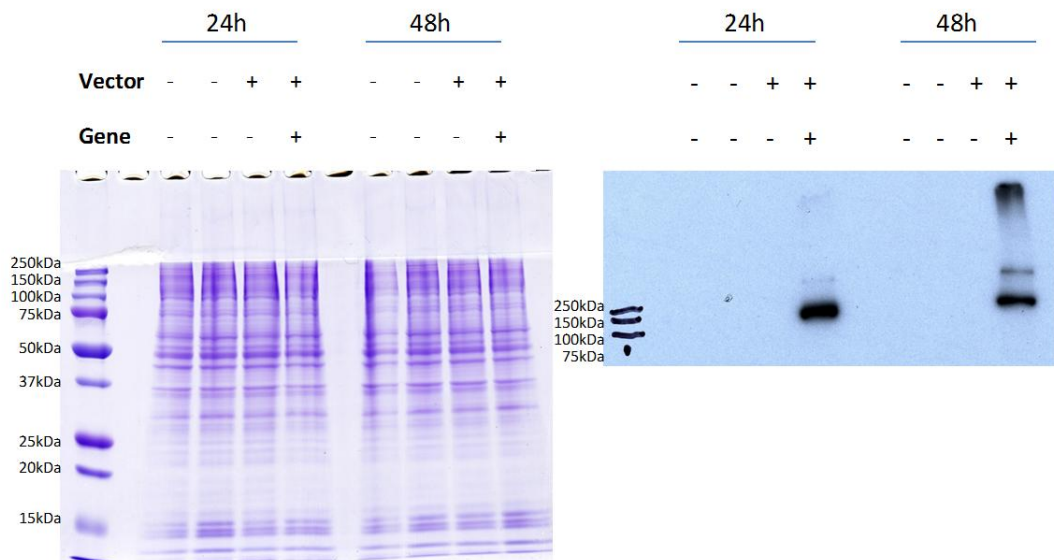
Figure 3.3 SDS-PAGE and western blot analysis of CHOK1 cells following transfection with wild-type eEF2 with a C-terminal V5-tag 24 and 48 h post-transfection. Vector = control empty vector, gene = eEF2 V5 tagged wild-type containing construct, (-) = not transfected, (+) = transfected. The left hand image shows a Coomassie blue stained image of all samples loaded, the right-hand image shows the western blot with the anti-V5 antibody.

The Coomassie stain of the cell lysates showed very similar banding patterns and protein content across the lanes (Figure 3.3) confirming that equal protein load was used from the different samples. The fact that there were no obvious differences in the banding pattern suggests that the transfection of the cells and over-expression of WT eEF did not

detrimentally affect the cells. The western blot analysis confirmed the recombinant expression of human wild-type eEF2 at 24 and 48 h respectively (Fig 3.3). A band of the expected size was observed at ~100 kDa at both the 24 and 48 h time points; however at the 48 h time point there was also a much higher band in the gel suggesting the presence of aggregate eEF2. In both lanes there was a band the size of a dimer that was more intense in the 48 h sample (Fig 3.3). These data confirm the over-expression of eEF2 but that as time progresses aggregate dimer and aggregate material appears to form.

Eventually the Chinese hamster sequence was inserted into the pc3.1V5 vector (Figure 3.1b), and the Thr56 residue mutated to Ala56 using the Quik change kit (Aligent) and primers described in table 3.1. The pc3.1V5 CHO eEF2 wild type and Ala56 mutant were then transfected into CHOK1 adherent cells, cell lysates were collected 24 and 48 hours post transfection and analysis via western blotting for total and phosphorylated eEF2 undertaken (Figure3.4). Consistent loading of the SDS-gels for the western blotting analysis was achieved using a Bradford assay to confirm equal amounts of protein were loaded. The analysis showed no change in total levels of eEF2 over 48 hours between the control cells and those transfected with the eEF2 WT and Ala56 mutant. The levels of phosphorylation after 24 hours of the Ala56 mutant transfected samples appeared to be slightly reduced compared to the plasmid control and the wild type samples, but after 48 hours the levels of phosphorylation appear to be uniform across all of the samples.

The initial decrease in phosphorylation of eEF2 after 24 hours post transfection could be due to stress imposed upon the cell by the transfection reagents; or as the phosphorylation levels are only decreased in the samples that were transfected with eEF2, it is possible that over expression of eEF2 causes a reduction in phosphorylation of both the endogenous and exogenous eEF2. However, this reduction in phosphorylation in the Ala56 samples suggests that the Ala56 mutant is effective at reducing the phosphorylation of eEF2; and that the CHO eEF2 is being expressed as a monomer at the correct size. The over expression of the pc3.1 V5 CHO eEF2 WT and Ala56 mutant appears to be short lived, only lasting around 24-48 hours.
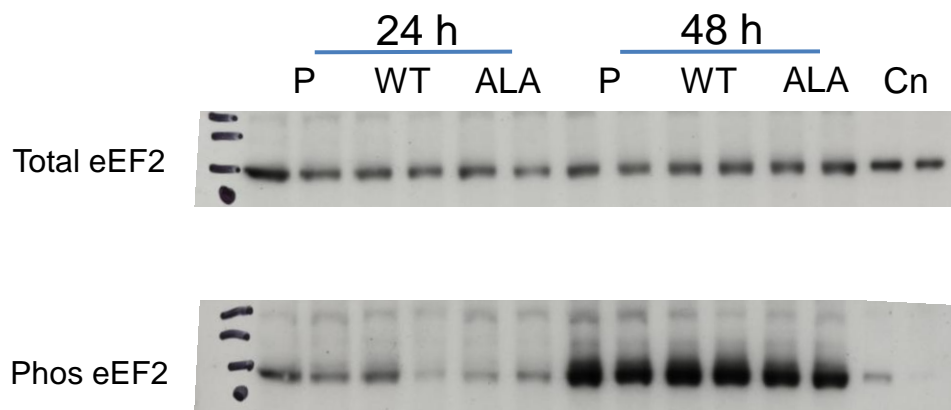
Figure 3.4 Western blot analysis of CHOK1 cells transfected with 2µg PC3.1V5 CHO eEF2 wild type or PC3.1V5 CHO eEF2 Ala56 mutant, 24 and 48 hours post transfection. Cn= control lysates, P= empty PC3.1V5 vector, WT= PC3.1V5 CHO eEF2 wild type, ALA= PC3.1V5 CHO eEF2 Ala56 mutant.

In addition to confirming expression of the human wild-type eEF2 by Western analysis, immunofluorescence was also undertaken using the anti-V5 antibody to detect the recombinant eEF2 and DAPI staining to visualise the nucleus (Figure 3.5). As expected, for the control and empty plasmid samples no recombinant eEF2 was detected using the anti-V5 antibody. However, where the pcDNA3.1V5 tagged wild-type eEF2 construct was transfected into CHOK1 cells eEF2 was detected (Figure 3.5). The immunofluorescence analysis showed the eEF2 to be present in the cytosol as expected and the transfection efficiency or number of cells expressing eEF2 24 hour post-transfection was approximately 20-30% (see Figure 3.5 for example). There was no direct evidence of aggregates in these images although it would be difficult to tell this from these images and the previous western blot analysis had shown there was little aggregate present at the 24 h time point.
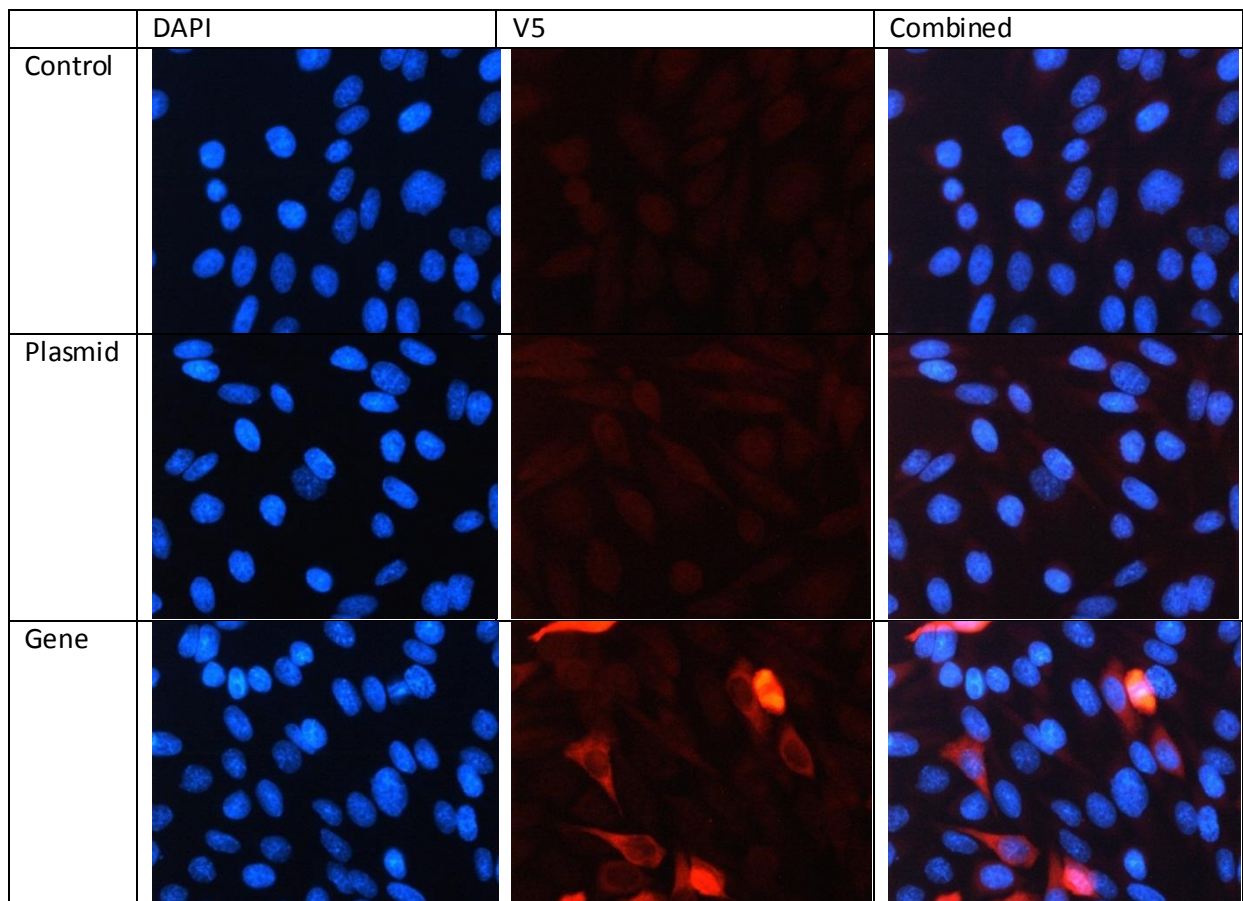
Figure 3.5 Immunofluorescence of CHOK1 cells 24 h post-translation with the human wild-type V5-tagged eEF2 construct. Blue = DAPI stain for nucleus, Red = V5 antibody stain. Control = transfection agents only transfected (no DNA), Plasmid = empty pcDNA3.1 plasmid with no gene sequence, Gene = eEF2 wild-type containing pcDNA3.1V5 construct. Combined shows an overlay of the V5 and DAPI stains.

Although the cellular protein composition did not appear to change upon transfection and expression of human wild-type eEF2 (see Figure 3.4 above), a longer time period was investigated post-transfection to determine if there was any effect on cellular growth in the transient transfections over a 96 hour period. These studies showed that there was no change in the cellular growth or viability profiles when CHOK1 cells were transfected with the wild-type human eEF2 construct (Figure 3.6). Both the control and eEF2 WT constructs when transfected gave comparable growth profiles suggesting that the expression of the exogenous V5-tagged eEF2 (in 20-30% of cells) was not detrimental to the cells, even if aggregate material was observed by western analysis 48 h post-transfection.
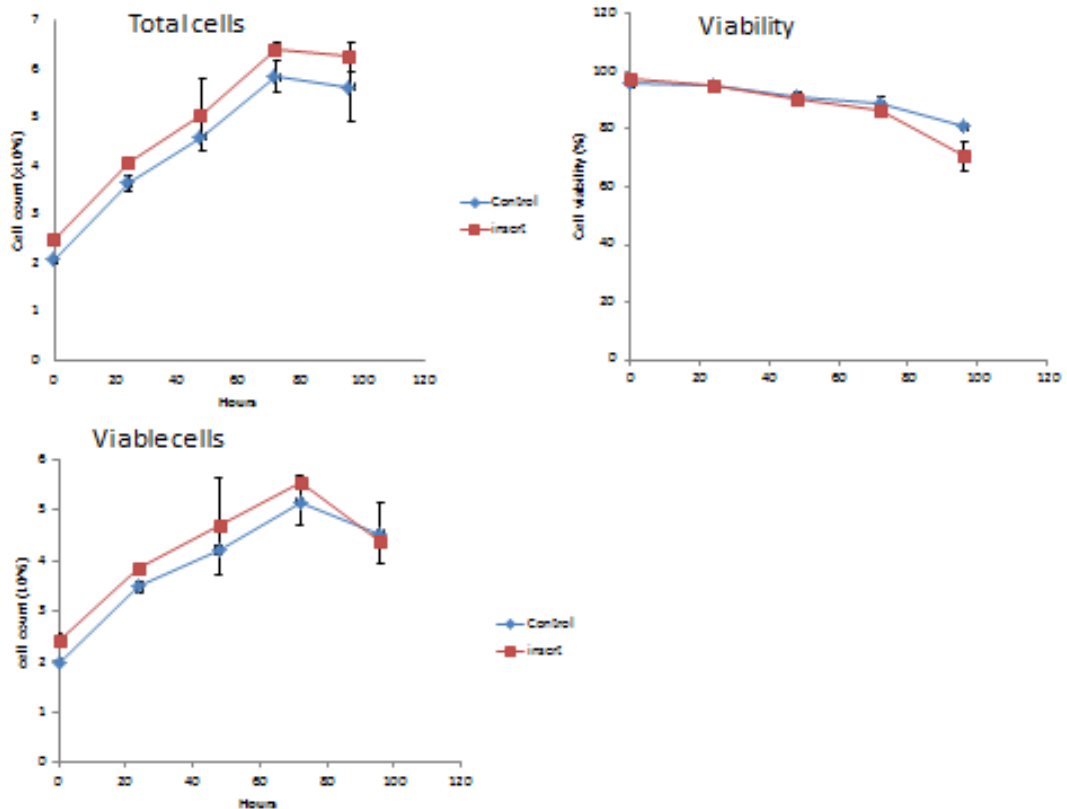
Figure 3.6 Growth profiling of CHOK1 cells transiently transfected with 2µg of pcDNA3.1V5 human eEF2 (insert) (red) or the empty pcDNA3.1V5 vector (control) (blue). n=2

The growth profile experiment was then also repeated with the CHO derived wild-type eEF2 and CHO eEF2 Ala56 mutant to determine if the inability to phosphorylate the exogenous eEF2 in transfected cells influenced the cell growth or viability. Again, the expression of the eEF2 Ala56 mutant did not appear to have any adverse effect upon the cellular growth or viability of the CHOK1 cells (Figure 3.7). This may be due to the cellular transfection rate being too low to have a significant impact, that the transfected cells compensate for the additional eEF2 by down regulating its activity by phosphorylation in the case of the wild type (but this would not be possible with the eEF2 Ala56 mutant), that the endogenous eEF2 is down regulated to compensate for the recombinant eEF2 being produced, or that the over expressed WT/Ala56 constructs ,or total eEF2 including the endogenous material, are being degraded and turned over to regulate and maintain homeostasis of the intracellular level of eEF2.
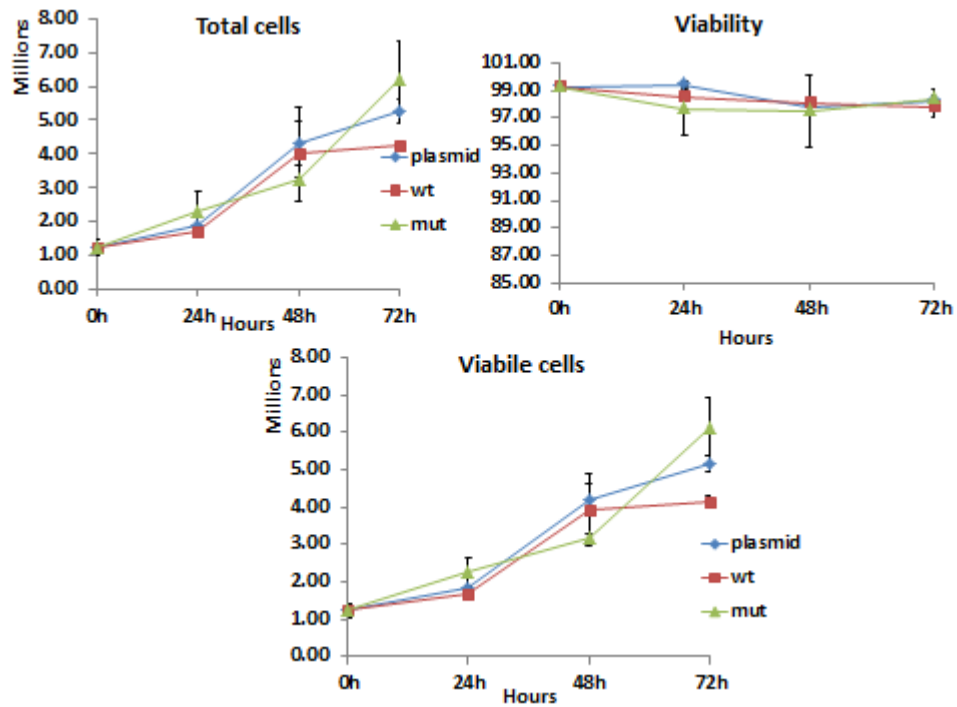
Figure 3.7 Growth profiling of CHOK1 cells transiently transfected with 2μg of empty pcDNA3.1V5 vector (plasmid), pcDNA3.1V5 CHO eEF2 (WT) construct, or the pcDNA3.1V5 CHO Ala56 mutant construct (mut). The data shows the total and viable cell numbers (x10^6 cells/mL) and cell viability. n=2

3.2.1.3 Investigating the Effect of Transient Over-Expression of Wild Type CHO eEF2 and Mutants of eEF2 on Recombinant Protein Expression using Firefly Luciferase as a Reporter Gene

To determine if the transient expression of eEF2 has any effect on recombinant protein expression from CHO cells a CHO cell line stably expressing the reporter gene firefly luciferase, CHOK1D6, was used. The amount of firefly luciferase being expressed at any one time can be rapidly assessed by measuring the amount of light produced when an activating substrate of firefly luciferase is present. In this way whether the over-expression of eEF2 or the mutants influences recombinant expression can be determined. It is noted that firefly luciferase is widely used as a reporter gene for such studies (Masterton et al. 2010) but is also a cytosolic protein whilst most biotherapeutic recombinant proteins, certainly those expressed from mammalian cells, are secreted proteins.

CHOK1D6 cells were therefore transiently transfected with either the empty pcDNA3.1 vector, a vector containing the eGFP gene, the pcDNA3.1 vector containing the wild-type eEF2 construct or the Ala56 mutated eEF2 construct. The resulting firefly luciferase activity 48 h post-transfection is reported in Figure 3.8. The transfection of the CHOK1D6 cells with the CHO eEF2 constructs resulted in no appreciable increase in luciferase expression 48 h post-transfection compared to the transfection reagent, pcDNA3.1 empty vector and eGFP control vectors (see Figure 3.8). This may reflect the low levels of over-expression achieved as demonstrated previously and discussed in the previous sections. This also may reflect the cells ability to tightly regulate the amounts of eEF2 in the cell or poor transient over-expression or a combination of both. In order to better assess the influence of eEF2 manipulation on CHO cell phenotypes, the production of stably over-expressing eEF2 cell lines was investigated.
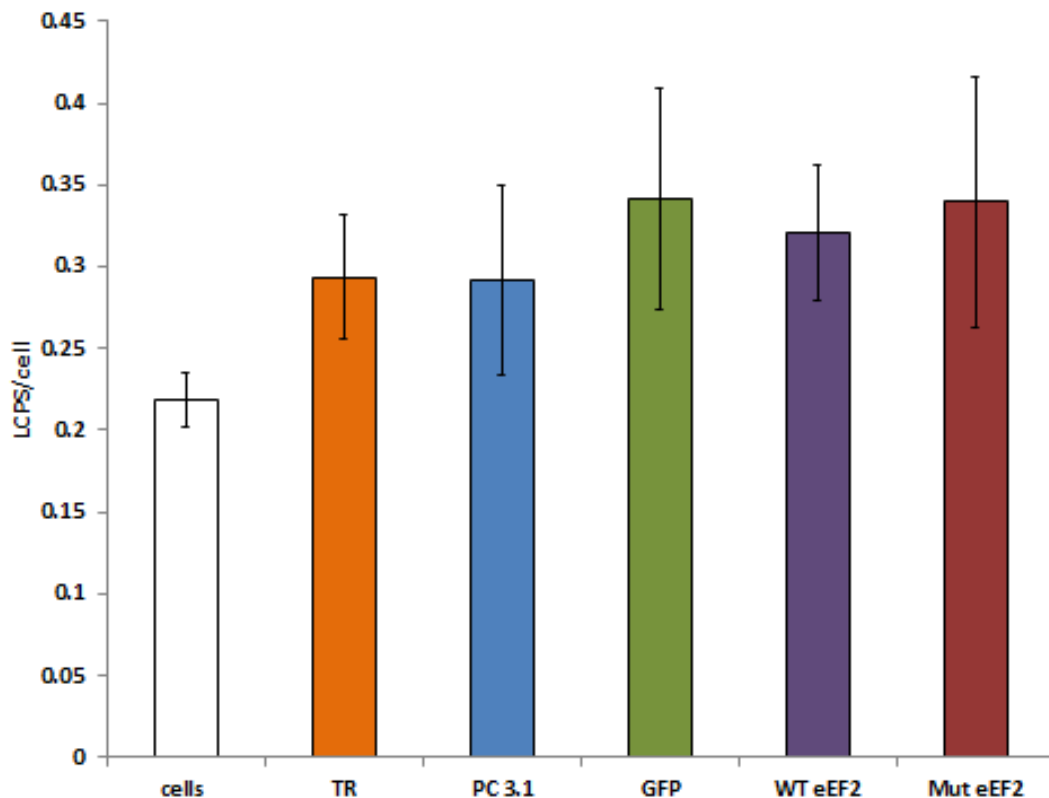
Figure 3.8 Firefly luciferase expression (shown as Light counts per second (LCPS)) per cell in CHOK1D6 cells 48 hours post transient transfection with 2 µg of CHO eEF2 constructs. TR, Cells treated with transfection regent only; PC 3.1, empty pcDNA3.1 V5 vector; GFP, eGFP vector; WT eEF2, wild type eEF2 in pcDNA3.1 V5 vector; Mut eEF2, Thr56Ala eEF2 mutant in pcDNA3.1 V5 vector. n=6, Wt eEF2 and Mut eEF2 p>0.05 compared to TR control

3.2.2 Generation and Investigation of Stably Over-Expressing Chinese Hamster eEF2 in CHO Cell Lines

Due to the low expression or transfection efficiency of the eEF2 constructs when transfected into CHO cells, the generation of cell lines stably expressing the various constructs was investigated, these being the CHO eEF2 WT, Thr56Ala, Thr56Glu and eEF2WT with a C/N terminus V5 tag. To generate stable cell lines the commercially available CHO Flp-In system (Invitrogen) was used. This system uses a recombinase site placed into the host CHO cell genome, in which the corresponding FRT cloning and expression vector can be stably integrated into the host cell rapidly and stable integration selected for using a hygromycin selection marker. This is shown schematically in Figure 3.9 below.

1. pFRT/lacZeo is stably transfected into the mammalian cells of interest to generate the Zeocin™-resistant Flp-In™ Host Cell Line(s)
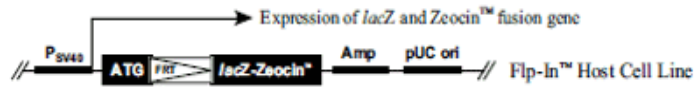
2. The pcDNA5/FRT expression vector containing your gene of interest (GOI) is cotransfected with pOG44 into the Flp-In™ Host Cell Line.
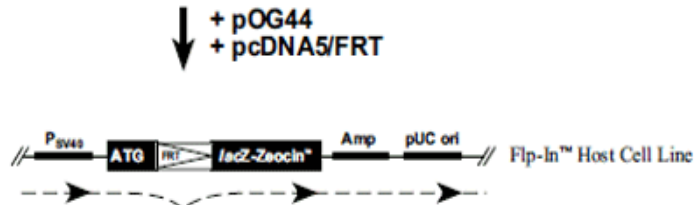
3. The Flp recombinase expressed from pOG44 catalyzes a homologous recombination event between the FRT sites in the host cells and the pcDNA5/FRT expression vector.
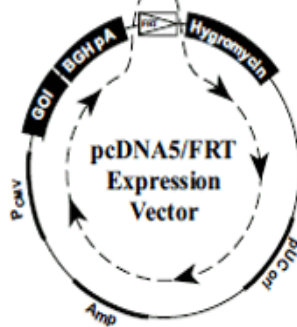
4. Integration of the expression construct allows transcription of the gene of interest (GOI) and confers hygromycin resistance and Zeocin™ sensitivity to the cells.
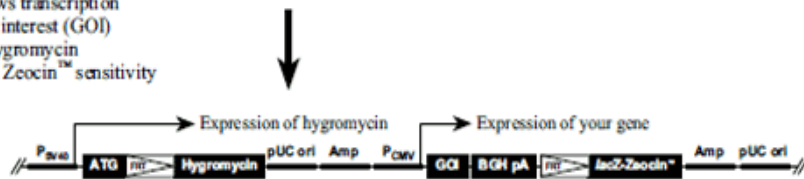
Figure 3.9 Schematic depicting the stable integration of the FRT construct of interest into the FRT site of the CHOK1 FRT host to generate stable expressing recombinant cell lines (taken from the Invitrogen manual, see www.lifetechnologies.com/uk/en/home/references/protocols/proteins-expression-isolation-and-analysis/protein-expression-protocol/flp-in-system-for-generating-constitutive-expression-cell-lines.html).

3.2.2.1 Subcloning of the eEF2 gene sequences from the pcDNA3.1 plasmid into the FRT cloning vector and generation of stably expressing cell lines

In order to sub-clone the various eEF2 constructs from the pcDNA3.1 vector into the FRT cloning vector, the primers listed in Table 3.1 were used to PCR amplify the different eEF2 sequences plus the V5 tag from the pcDNA3.1 vector. Bands of the expected size were obtained as confirmed by agarose gel electrophoresis, gel purified and then digested with the appropriate restriction enzymes and ligated into the FRT vector that had also been digested with the appropriate restriction enzymes. Following transformation, colonies containing the inserts of interest were confirmed by PCR screening with the primers used to amplify the insert of interest and a positive colony confirmed by commercially available sequencing for each construct (appendix 7.4).

In addition to generating the FRT constructs to generate these stable cell lines, CHO eEF2 WT FRT vectors were also generated to transiently express exogenous eEF2 with a V5 reporter at the C- and N- terminus, in case the presence of the tag at either end influences the activity of eEF2. Using the presence of the V5 tag the exogenous eEF2 can be detected by western blotting and immunofluorescence to confirm the expression and location within the cell of the exogenous eEF2 in the CHO Flp-In cell lines. In this way the exogenous eEF2 only is detected and not the endogenous and naturally expressed eEF2. Initially to confirm that these constructs were transient expressed in the CHO Flp-In host cells, transient over-expression of these constructs was undertaken.

Figure 3.10 Western blot analysis 24 and 48 hour post-transient transfection of 2 µg of either N-V5 or C-V5 V5 tagged eEF2 in FRT vector in CHO Flp-In cells. T.R, Transfection reagent control; C-V5, C-terminus V5 eEF2; N-V5, N-terminus V5 eEF2. eEF2 = total eEF2 in cell (both endogenous and exogenous), eEF2V5 = V5 tagged blot for V5 tagged eEF2. A tubulin loading control is shown.

The data shown in Figure 3.10 confirmed that the V5 tagged FRT eEF2 constructs were expressed in the CHO FRT host cell line both 24 and 48 h post-transfection. There were a number of interesting observations from these data. Firstly, and as expected, there was no signal in the control transfection reagent V5 blot. Further, the C-terminal tagged eEF2 gave a much higher signal than the N-terminal V5 tagged eEF2 suggesting that the presence of the V5 tag on the terminal can influence either the amount of protein produced or the stability of the recombinant eEF2. It is possible that the C-terminal V5 tag stabilises the eEF2 and hence could result in higher amounts of material being observed or that the N-terminal tag destabilises the eEF2 protein and hence this is turned over faster. Alternatively, the tag at either end could influence the protein synthesis rate. It was also interesting to note that although the C-terminal V5 tagged eEF2 protein was well expressed as determined by western blot analysis, there was little elevation in the overall (total) amounts of eEF2 observed (Figure 3.10). This suggests that the cell can 'sense' the amount of eEF2 present in the cell and regulates this to maintain a more-or-less constant amount. This regulation could only be achieved by reducing the amount of endogenously expressed eEF2 or by changing the turnover of eEF2 as the cell cannot directly control the synthesis rate of the exogenous material. These data all suggest careful control of eEF2 amounts by the cell. In addition to the western blot analysis of the transient expression of the C/N terminus V5 eEF2, immunofluorescence analysis was also undertaken (Figure 3.11). As expected endogenous eEF2 was observed in the cytosol of cells. In those transfected with the V5 eEF2 tagged constructs, the immunofluorescence data again showed more cells expressing higher amounts with the C-terminal tagged eEF2 than the N-terminal in agreement with the western blot analysis. There were also fewer cells in the eEF2 N-terminal tagged samples suggesting that this was not as well tolerated as the C-terminal exogenous material (Figure 3.11). These data combined suggest that the exogenous C-terminal V5 tagged eEF2 was better expressed in the CHO FRT cells than the N-terminal V5 tagged version.
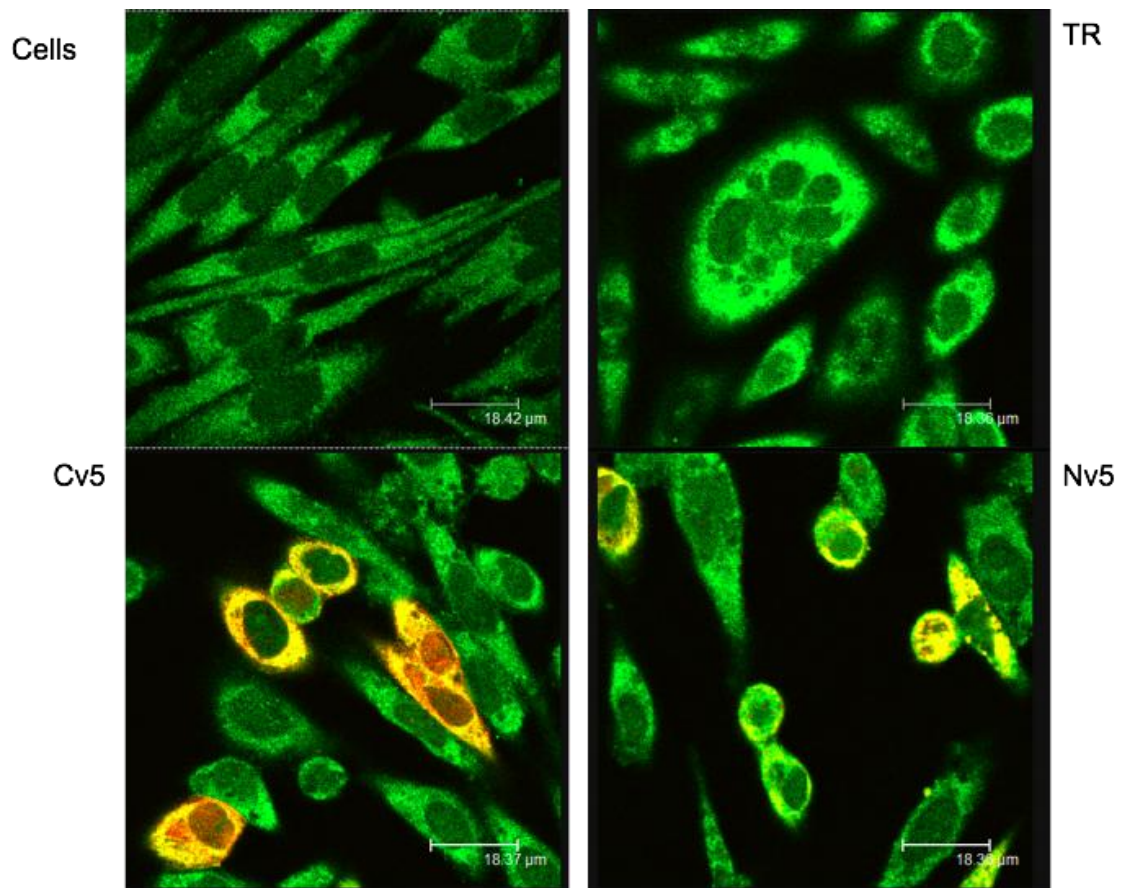
Figure 3.11 Combined immunofluorescent images of a 24 hour transient transfection of 0.8 µg of either N-V5 or C-V5 V5 tagged eEF2 in FRT vector in CHO Flp-In cells. Green, endogenous eEF2; Red, anti-V5. Each image shows an overlay of the green and red and hence yellow results from yellow and red overlay. Cells, untreated cells; TR, cells treated with transfection reagent only; Cv5, cells transfected with FRT eEF2 with a C-terminus V5 tag; Nv5, cells transfected with FRT eEF2 with a N-terminus V5 tag.

Once all the FRT vectors with the CHO eEF2 WT, Thr56Ala, and Thr56Glu were constructed and confirmed by sequencing (Appendix 7.4), these were used to generate stably expressing CHOK1 FRT cell lines following the instructions in the manual. Three polyclonal cell lines for experimentation were generated, one for each construct, these being the CHO eEF2WT, Thr56Ala, and Thr56Glu mutants. Alongside the generation of these eEF2 over-expressing cell lines, the parental CHO cell line (Host cell line) and a cell line containing an empty FRT vector (FRT, not containing a gene of interest) were also constructed as controls. The reason for generating stable pools and not clonal cell lines was that although the generation of cell lines in this way should allow for cells expressing the same amount of recombinant mRNA (transcriptional activity the same), there may be different translational limitations in different cells that mean varying amounts of recombinant eEF2 are expressed in different cells lines. As such, a polyclonal pool of cells was maintained to determine if this was the case and to ensure the 'average' eEF2 over-expression was being investigated.

3.2.2.2 Characterisation of the eEF2 Protein Levels in the Stable eEF2 Expressing CHO FRT Poly-clonal Cell Lines

Once the stably expressing eEF2 FRT CHO Flp-In polyclonal cell lines were established it was necessary to confirm expression of the exogenous eEF2. Initially this was achieved by western blotting of the CHO WT eEF2 stably expressing pools with either a C-terminal or N-terminal V5 tag. The resulting blot data is shown in Figure 3.12. Interestingly, V5 labelled exogenous eEF2 was observed in both the C-terminal and N-terminal V5 tagged eEF2 polyclonal cell lines, and possibly the amount of expression was marginally higher in the N-terminally tagged samples (Figure 3.12). This is in direct contrast to the transient results reported above where there was much less expression of the N-terminally V5 tagged eEF2 construct. This suggests that those cells that have survived the stable cell line generation and selection process are able to tolerate the N-terminally V5 tagged version of eEF2 as well as the C-terminally tagged.
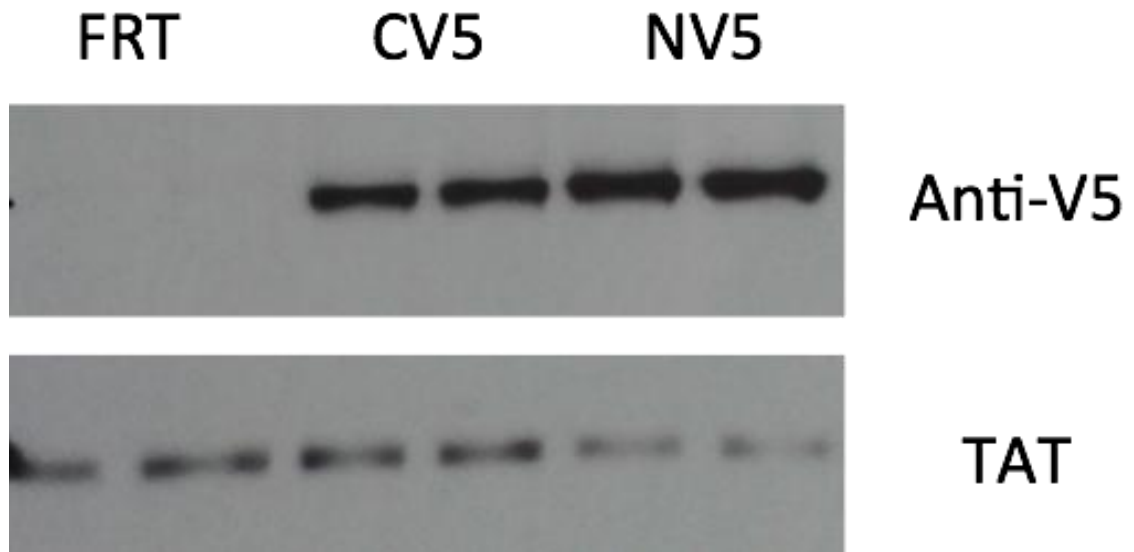
Figure 3.12 Western blot analysis of cell lysates collected from CHO Flp-In stable cell lines expressing either the empty FRT vector, human WT eEF2 with a C-terminus V5 tag or eEF2 with a N-terminus V5 tag. Alpha tubulin (TAT) is shown as a loading control. FRT, stable cell line expressing the empty FRT vector; CV5, stable cell line expressing eEF2 with C-terminus V5 tag; NV5, stable cell lines expressing eEF2 with N-terminus V5 tag.

In addition to the western analysis, immunofluorescence analysis was undertaken again for the expression of exogenous V5 tagged eEF2 (Figures 3.13 and 3.14 below). These data showed that all cells were expressing the recombinant exogenous V5 tagged eEF2 and that the N-terminal tagged eEF2 may express marginally more than the C-terminal in agreement with the western blot data again. When the C-terminal tagged V5 eEF2 polyclonal cell line was investigated in more detail, there was clearly V5 tagged exogenous eEF2 expressed in all cells and this overlaid with the total eEF2 in the cell (Figure 3.14). These stable cell lines therefore appear to tolerate the expression of endogenously V5 tagged eEF2 although the western blot analysis suggest that total eEF2 amounts are not increased, again suggesting that the amount of eEF2 must be tightly regulated by the cells.
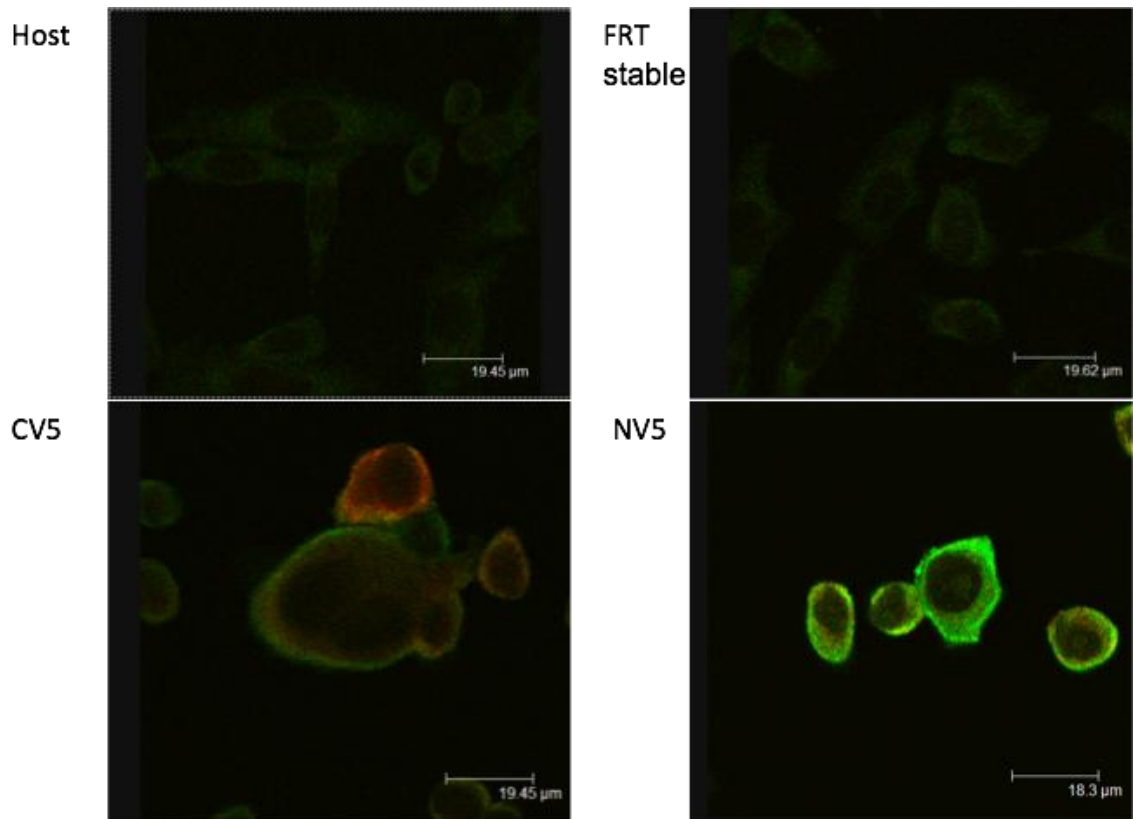
Figure 3.13 Combined immunofluorescent images of CHO Flp-in cells stably expressing eEF2 either N-V5 or C-V5 tagged 24 hours post seeding. Host = host FRT untransfected cell line; FRT stable = control FRT cell line containing empty FRT cloning vector; CV5 = C-terminal V5 tagged eEF2 stable cell line; NV5 = N-terminal V5 tagged eEF2 stable cell line. Red= V5, Green= eEF2

In addition to characterising the WT eEF2 V5 tagged polyclonal stable cell lines, western analyse was undertaken on cell lysates of the non-tagged stable cell line mutants and WT collected 24 h after cells were seeded into flasks when growth should be maximal. Western blot analysis was undertaken for the amount of total and phosphorylated eEF2 in each of the polyclonal cell lines generated, these being: the non-tagged version of the CHO wild-type eEF2 stably expressing cell line, the Ala56 and Glu56 eEF2 mutants and the control FRT cell line containing an empty FRT vector (no exogenous gene). The initial western blot analysis suggested that the amount of total eEF2 was elevated in the stably expressing WT eEF2, Ala56eEF2 and Glu56eEF2 polyclonal cell lines compared to the host and FRT control cell lines (Figure 3.15a).
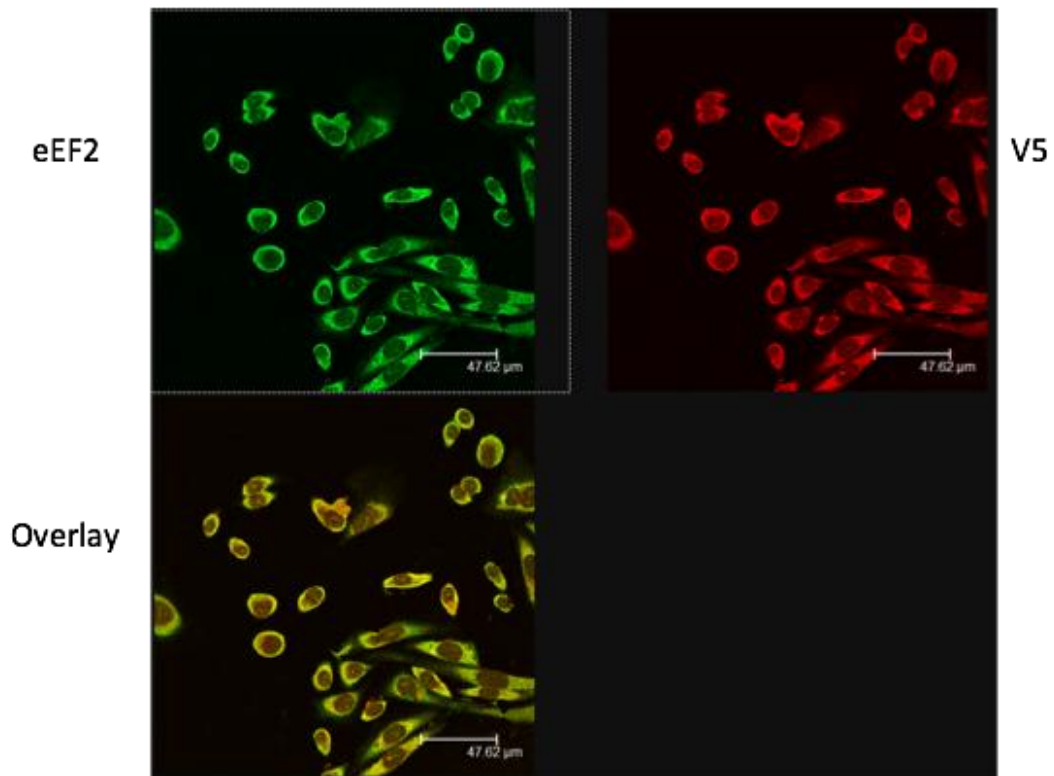
Figure 3.14 Immunofluorescent images of stable CHO Flp-in cell line expressing eEF2 with a C-terminus V5 tag 24 hours post seeding. Green= total eEF2; Red= anti-V5 tag; overlay, total and V5 overlay.

In order to determine if this elevation was real, the freeware ImageJ software was used to analysis the levels of total and phosphorylated eEF2 relative to the α-tubulin loading control for each sample, and then the levels in each cell line were expressed relative to the FRT control cells (Figure 3.15b). From this analysis the total level of eEF2 was reduced when the empty FRT vector was stably present in the CHO Flp-In cells. However, when the WT eEF2, Ala56 or Glu56 eEF2 mutant constructs were stably integrated into the CHO Flp-In cell line an increase in the amount of eEF2 present was confirmed (see Figure 3.15b).

The Ala56 eEF2 mutant cell line had the highest amount of total eEF2. The Glu56 eEF2 mutant cell line also had elevated amounts of total eEF2 compared to the controls; the WT eEF2 cell line also had increased amounts of eEF2 but this increase was less than that observed for either of the mutants (see Figure 3.15b). Although there was an increase in total eEF2 amounts in these untagged eEF2 cell lines, the amount of phosphorylated eEF2 was slightly reduced in the WT eEF2 cell line and reduced by almost 50% in the Glu56 eEF2 cell line. This reduction in phosphorylation in the Glu56 cell line suggests that the cells could be compensating for the expression of the inactive Glu56 eEF2 by activating endogenous eEF2. The amount of phosphorylated eEF2 in the Ala56 eEF2 mutant cell line was similar to that of the FRT control, suggesting that the cells may compensate for the expression of the presence of the active and non-phosphorylatable Ala56 eEF2 by phosphorylating a larger amount of the endogenous eEF2 within the cell.

These data suggest that the non-V5 tagged eEF2 WT and mutants were able to up-regulate eEF2 expression to a greater degree than the V5 tagged WT eEF2 and that eEF2 can be elevated in CHO Flp-In cells stably. Further, dependent upon the eEF2 mutant over-expressed, the cells adjust the amount of eEF2 phosphorylated to compensate for inactive/activate additional eEF2, at least when the cells are in rapid growth phase and are not nutrient deprived.

(a)

HOST   FRT   WT   ALA   GLU

eEF2 T

eEF2 P

α-tubulin

eEF2 T

eEF2 P
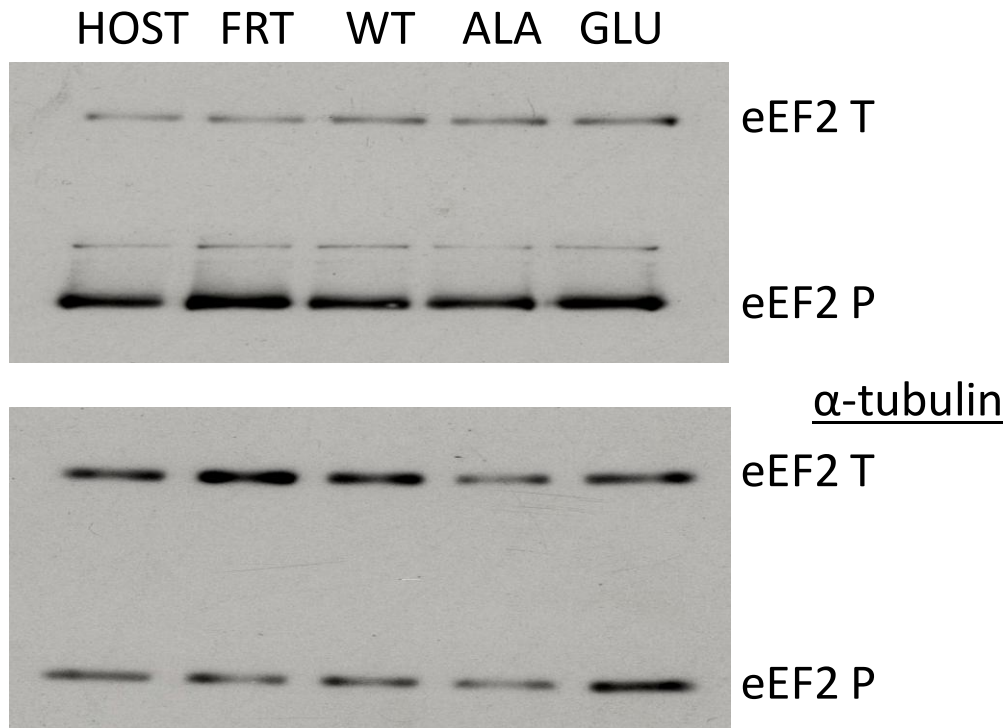
(b)
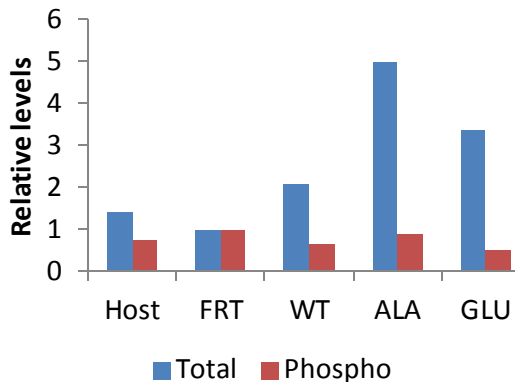
(c)
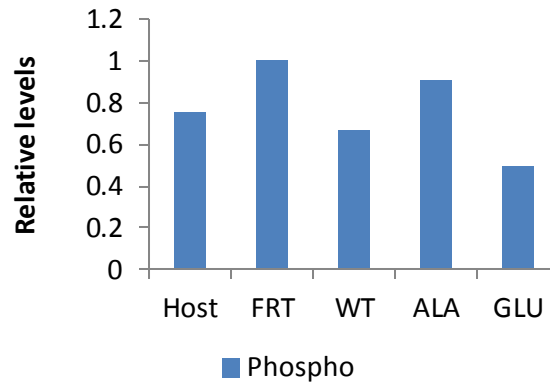
Figure 3.15 (a) Western blot analysis of stable CHO flp-in cell lines expressing the eEF2 variant lysates 24 hours after seeding cultures. **(b)&(c)** Densitometry analysis of total and phosphorylated eEF2 in each cell line relative to the loading control of alpha tubulin; the levels of each cell line were made relative to the FRT control cell line. HOST: CHO Flp-In parental cell line; FRT: CHO Flp-In cells stably transfected with an empty FRT vector; WT: CHO Flp-In cells stably expressing wild type CHO eEF2; ALA: CHO Flp-In cells stably expressing CHO Thr56Ala CHO eEF2; GLU: CHO Flp-In cells stably expressing CHO Thr56Glu CHO eEF2.

3.2.2.3 Characterisation of eEF2 mRNA Levels in the Stable non-V5 tagged eEF2 Expressing CHO FRT Polyclonal Cell Lines

As well as determining the over-expression of eEF2 at the protein level in the stable cell lines, the effect at the mRNA level was also determined. To do this, each cell line was cultured for 24 hours before extracting the total RNA and then using qRT-PCR the relative amount of total eEF2 mRNA was determined using the primers described in Table 3.3. mRNA levels of β-actin were also determined as a normaliser, assuming that these do not change. The resulting data is shown in Figure 3.16. All eEF2 mRNA amounts are shown relative to the FRT eEF2 mRNA levels. Despite the fact that the eEF2 protein amounts were elevated in the eEF2 WT, Ala56 and Glu56 mutant stable cell lines (see Figure 3.15), the eEF2 mRNA amounts were only raised to any extent in the Glu56 mutant cell line (see Figure 3.16). Therefore, only the eEF2 Glu56 mutant appears to have increased levels of mRNA, this may be because the cells are compensating for the production of 'deactivated' Thr56Glu eEF2 mutant by producing more endogenous eEF2 mRNA. . It also suggests that the cells modulate the total mRNA level to maintain this and this may be the mechanism by which protein amounts are maintained, even when over-expressing exogenous mRNA. When investigating the WT V5-tagged cell lines the V5 tagged protein is expressed although total amounts of protein were not elevated. These data suggest that endogenous mRNA amounts may be down-regulated in response to exogenous mRNA amounts to try and maintain the total amount of eEF2 mRNA and protein in the stable cell lines.

Table 3.3 Primers used for the mRNA analysis of the stale eEF2 CHO Flp-In cell lines. A universal forward primer was used, on specific primers were used for analysis of each cell line.

Primers	Sequences
Total eEF2 forward	agtgtcgacaggactcaagg
Total eEF2 reverse	acataccggcccatcatcag
β-actin forward	agctgagagggaaattgtgcg
β-actin reverse	gcaacggaaccgctcatt

Figure 3.16 qRT-PCR analysis of eEF2 mRNA amounts relative to the FRT control cell line in the various stably eEF2 expressing CHO FRT cell lines. Total mRNA was extracted from each cell 24 hours post seeding. HOST, parental CHO Flp-In cell line; FRT, CHO Flp-In cell line stably expressing an empty FRT vector; WT, CHO Flp-In cell line stably expressing wild type human eEF2; ALA, CHO Flp-In cell line stably expressing Thr56Ala eEF2 mutant; GLU, CHO Flp-In cell line stably expressing Thr56Glu eEF2 mutant. Error bars show one standard deviation of n=3 independent experiments. $p > 0.05$ when comparing the Host, WT and ALA values to the FRT control, whereas $p < 0.05$ when comparing the GLU and FRT control values.

3.2.3 Effect of Stable eEF2 and eEF2 Mutant Expression on CHO Cell Growth

3.2.3.1 Growth of Stable eEF2 Expressing CHO FRT Cell Lines

As described in the introduction to this thesis, elongation and eEF2 is a key control point of translation elongation and hence protein synthesis. For high recombinant protein producing cell lines it is ideal if a high cell concentration can be achieved, and/or the specific productivity (Qp) of each cell is increased– the more cells in the bioreactor producing a high amount of target protein results in a higher yield of recombinant protein. Therefore the ability to engineer cells to grow faster: to higher concentrations less time = less cost for a fermentation would be beneficial. Also with a greater Qp = higher recombinant protein titre per culture and therefore a reduction in the ratio of host cell protein to target protein, reducing the cost of downstream processing.

As a cell must double its protein mass before it can divide (Foster 2013; Conlon & Raff 2003) and elongation is a key control point of protein synthesis, the effects of whether the manipulation of eEF2 amounts or the expression of the mutant eEF2 molecules in the stably expressing CHO FRT cell lines influenced cell growth parameters was investigated. Further, temperature shift to a lower culture temperature is often used during bioprocessing (e.g. from 37 to 32°C) to prolong culture and increase recombinant protein yields, the effect at both these temperatures was investigated. At the lower temperature protein synthesis rates are usually decreased by approximately 50% compared to those observed at 37°C (Roobol et al. 2009).

The growth profiles of each of the non-V5 tagged stably over-expressing CHO eEF2 cell lines was established using the commercial Xcellegence system (Roche) (Host, FRT, WT, Ala56 mutant, Glu56 mutant cell lines) over a period of 7 days (Figure 3.17). This system uses impedance of an electronic current across the surface of the wells the cells are adhered to in order to measure the 'cell index' reflecting the number of cells present on the surface of the well. Triplicate cultures for each cell line were setup and an impedance reading was recorded every 30 minutes. At 37°C the growth profiles for the host cell line and FRT control were very similar to each other, however the three cell lines expressing additional exogenous CHO eEF2 all grew faster than the control cell lines and reached a higher cell

index more rapidly (Figure 3.17). All of these three cell lines grew much more rapidly during the first 60-70 h of culture even though they all started from a more-or-less equivalent cell index. The fastest growing cell line that also reached the highest cell index was that over-expressing the eEF2 Ala56 mutant that cannot be phosphorylated. This was the cell line that densitometry analysis of the western blots of total eEF2 amounts showed had the highest total eEF2 amounts (Figure 3.15). The eEF2 Glu56 and WT eEF2 cell lines grew with approximately the same profile although the Glu56 mutant declined earlier (Figure 3.17).

When the same cell lines were cultured at 32°C instead of 37°C, again the Ala56 mutant grew to the highest cell index. The host actually initially grew the fastest but reached the lowest cell index of any of the cell lines (Figure 3.17). The Glu56 mutant and FRT control both grew the slowest but reached a similar cell index to that of the eEF2 WT cell line. These data together suggest that the stable expression of a the Ala56 eEF2 mutant in CHO Flp-In cells gives an advantage at both 37 and 32°C in terms of the cell growth, maximum cell number (as indicated by cell index here) achievable and culture duration.
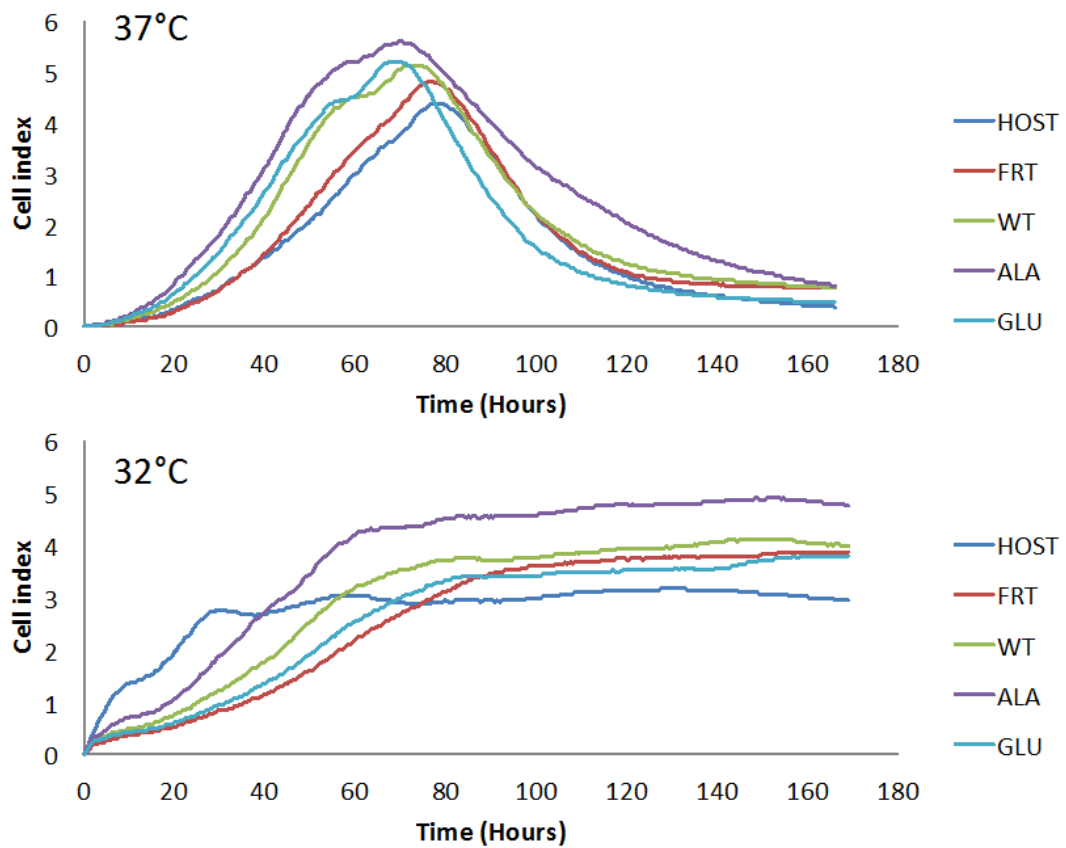
Figure 3.17 Growth profiles of the stably expressing eEF2 CHO FRT cell lines generated using the Xcellegence system (Roche). Each cell line profile was generated in triplicate with readings taken every 30 minutes over a 7 day period at 37 or 32°C. Host, parental CHO Flp-In cell line; FRT, CHO Flp-In cell line stably expressing the empty FRT vector; WT, CHO Flp-In cell line stably expressing wild type human eEF2; ALA, CHO Flp-In cell line stably expressing the Thr56Ala eEF2 mutant; GLU, CHO Flp-In cell lines stably expressing the Thr56Glu eEF2 mutant. Each profile represents the average of triplicate growth curves.

3.2.3.2 Effect of the Presence of Rapamycin on the Growth of the Stable eEF2 Expressing CHO FRT Cell Lines

Elongation factor 2 is indirectly regulated by mTOR (mammalian target of rapamycin), as described in the introduction chapter of this thesis. When mTOR is active eEF2 is not phosphorylated at the Thr56 residue because the kinase that phosphorylates eEF2 (eEF2K) is inactivated. However, when mTOR is inactivated, as in the presence of rapamycin, eEF2K is active and eEF2 becomes phosphorylated and inactivated. Therefore, the effect of the addition of 10 nM rapapmycin, enough to partially inhibit mTOR as shown in other studies (Raje et al. 2004; Lee & Lee 2012; Leontieva et al. 2012), on cell growth in the stably expressing CHO eEF2 cell lines was investigated.

In order to investigate the growth profiles of the different cell lines in the presence of rapamycin, once again the Xcellegence system was used to culture the cell lines: FRT, WT eEF2, Ala56 mutant and the Glu56 mutant in 10 nM rapamycin over 7 days. As the rapamycin is prepared and added in DMSO, the same volume of DMSO without rapamycin was used as the control conditions. The resulting growth profiles are shown in Figures 3.18 and 3.19. The growth profiles of each cell line were similar in that the presence of the rapamycin generally slowed the growth of the cell lines compared to the DMSO controls. The presence of the rapamycin therefore appears to have reduced the cells growth such that the maximum cell index is achieved ~48 hours later than in the presence of DMSO alone. Aside from the reduced growth rate, the FRT, WT and ALA cell lines exhibited similar growth patterns between the DMSO and rapamycin experiments.
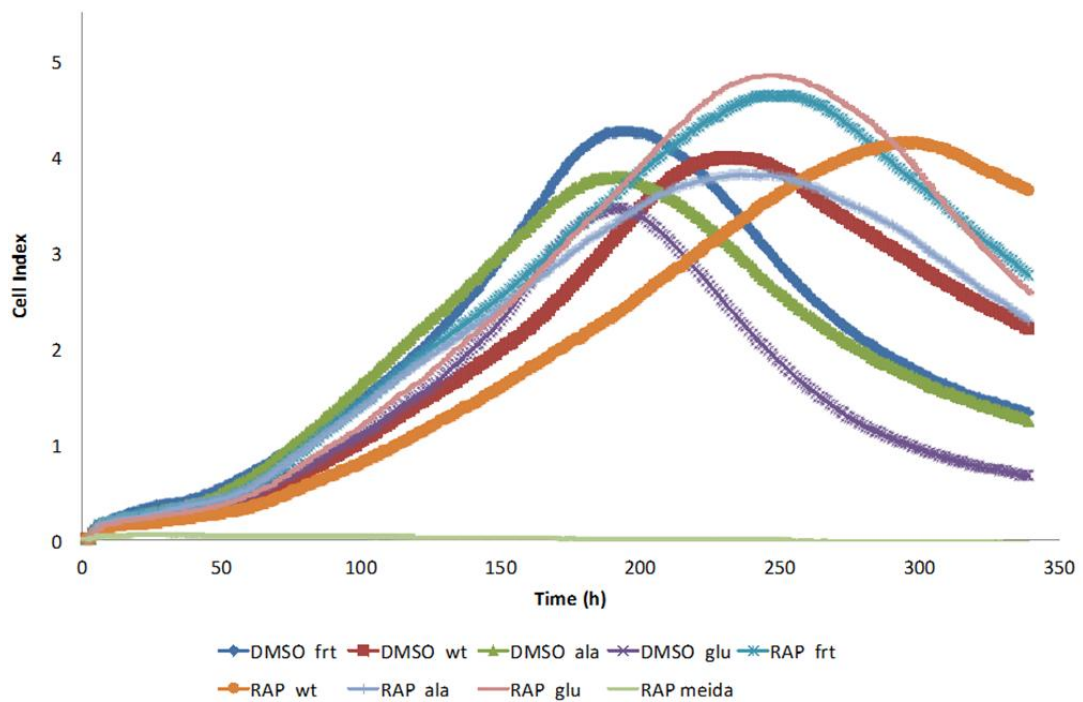
Figure 3.18 Growth profiles of CHO Flp-In cells stably expressing eEF2 constructs in triplicate using the Xcellegence system over 7 days. Cells were either grown in the presence of 10 nM rapapmycin in DMSO or in DMSO alone. DMSO, cells grown in the presence of DMSO only; RAP: cells grown in the presence of 10 nM rapamycin; FRT, CHO Flp-In cells stably expressing the empty FRT vector; WT, CHO Flp-In cells stably expressing wild type eEF2; ALA, CHO Flp-In cells stably expressing Thr56Ala eEF2; Glu, CHO Flp-In cells stably expressing Thr56Glu eEF2; RAP media: media with 10 nM rapamycin without cells present.
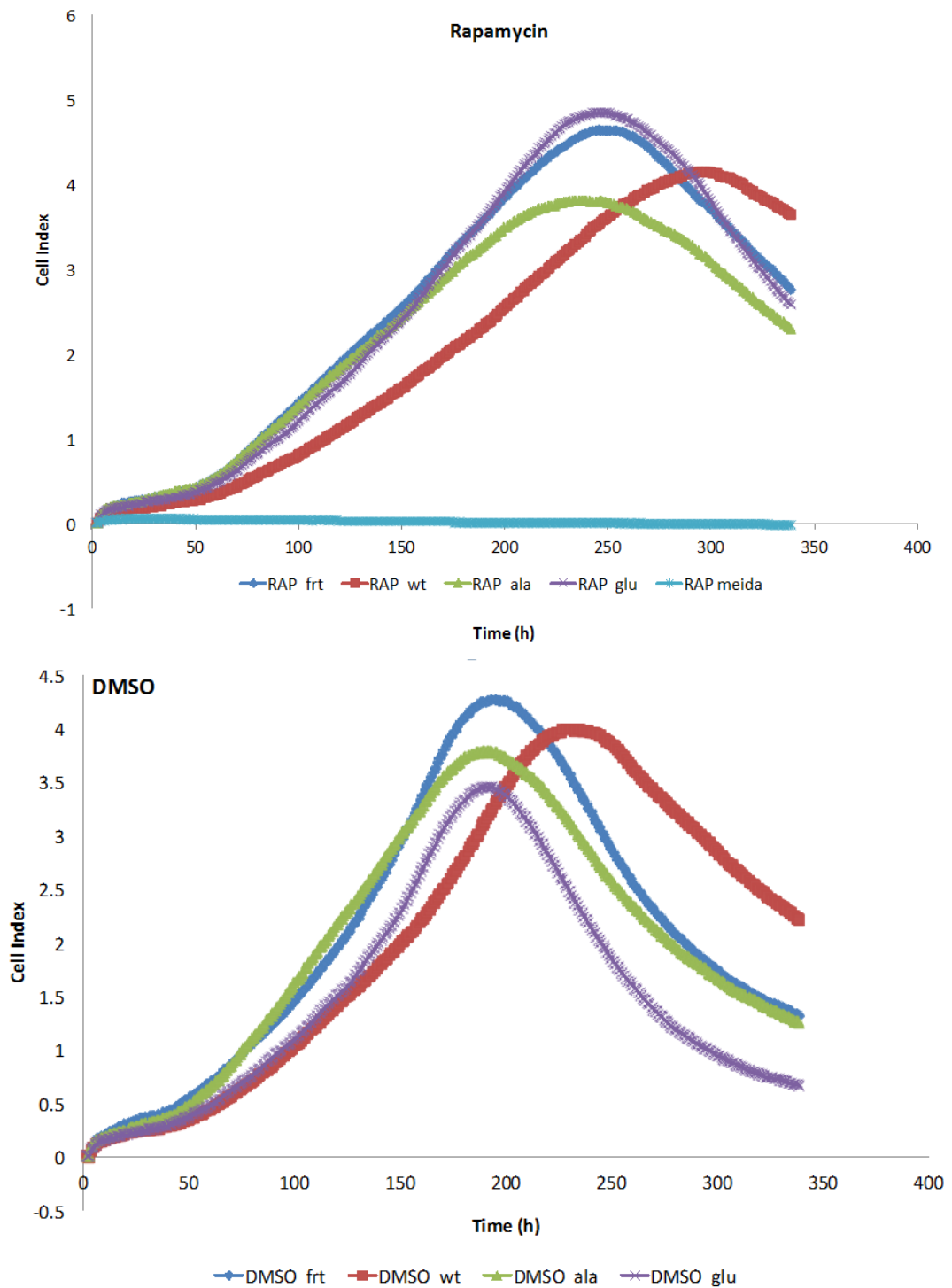
Figure 3.19 Growth profiles of the CHO Flp-In cells stably expressing eEF2 constructs in triplicate using the Xcellegence system over 7 days. In either DMSO alone (upper panel) of in DMSO + 10 nM rapamycin (lower panel). RAP: cells grown in the presence of 10 nM rapamycin; FRT, CHO Flp-In cells stably expressing the empty FRT vector; WT, CHO Flp-In cells stably expressing wild type eEF2; ALA, CHO Flp-In cells stably expressing Thr56Ala eEF2; Glu, CHO Flp-In cells stably expressing Thr56Glu eEF2; RAP media: media with 10 nM rapamycin without cells present. Each curve is the average of three replicate cultures.

3.2.4 Influence of Stable CHO WT eEF2 and eEF2 Mutant Expression on Recombinant Protein Production in CHO FRT Cell Lines

3.2.4.1 Effect of Stable Expression of Non-V5-Tagged eEF2 WT and Mutants in CHO Cells on Transient Firefly Luciferase Expression

In order to investigate any effect of expressing the non-tagged version of CHO WT eEF2 or the mutant Ala56 or Glu56 eEF2 on recombinant protein expression from the CHO Flp-In cell lines, transient transfection and expression of firefly luciferase from these cell lines was investigated. In the early experiments of this chapter, transient expression of eEF2 on stable firefly expression was investigated but the lack of eEF2 transfection and expression made it difficult to draw conclusions from these studies although it did appear that over-expression of eEF2 did enhance luciferase expression. In this experiment, the stable cells all express the eEF2 WT or mutants as appropriate and assuming that luciferase transfection is equal across the cell lines it should be more evident if there is an effect of eEF2 expression on luciferase expression. Therefore, the firefly luciferase construct pGL3 (commercially available from Promega) was transfected into each cell line and the luciferase expression determined 48 h post-transfection. Figure 3.20 reports on the total light counts per second or total luciferase expression in each sample 48 h post-transfection.

When the firefly luciferase pGL3 construct was transient transfected into the CHO FRT control or stably expressing WT eEF2 cell lines the total luciferase expression observed was more-or-less the same (Figure 3.20). A small but detectable increase in total luciferase expression was observed when the pGL3 construct was transfected into the stably expressing Glu56 eEF2 cell line above that of the FRT control. However, when the pGL3 construct was transfected into the stably expressing Ala56 eEF2 mutant an approximate 3-fold increase in the total amount of luciferase activity was observed (Figure 3.20). These data suggest that the total transient luciferase expression obtainable from the stably expressing Ala56 eEF2 cell line dramatically enhanced the luciferase expression compared to the control. In addition to the total luciferase expression detected, the luciferase expression was normalised for cell number to determine the luciferase activity per cell (Figure 3.21). Once again the CHO FRT and stably expressing WT eEF2 cell lines gave similar cell specific productivities (amount of luciferase produced per cell). However the amount of

luciferase produced per cell was approximately 50% higher in both the Ala56 and Glu56 eEF2 cell lines (Figure 3.21). This suggests that as the total amount of luciferase was the same in the Glu56 cell line compared to the control, there must be fewer Glu56 cells producing the same amount of total protein. The Ala56 mutant must have both of these: more cells each producing more luciferase, resulting in the approximate 3-fold increase in total luciferase observed. Together these data confirm that over expression of the eEF2 Ala56 mutant confers an advantage for the transient expression of firefly luciferase above that from control cells.
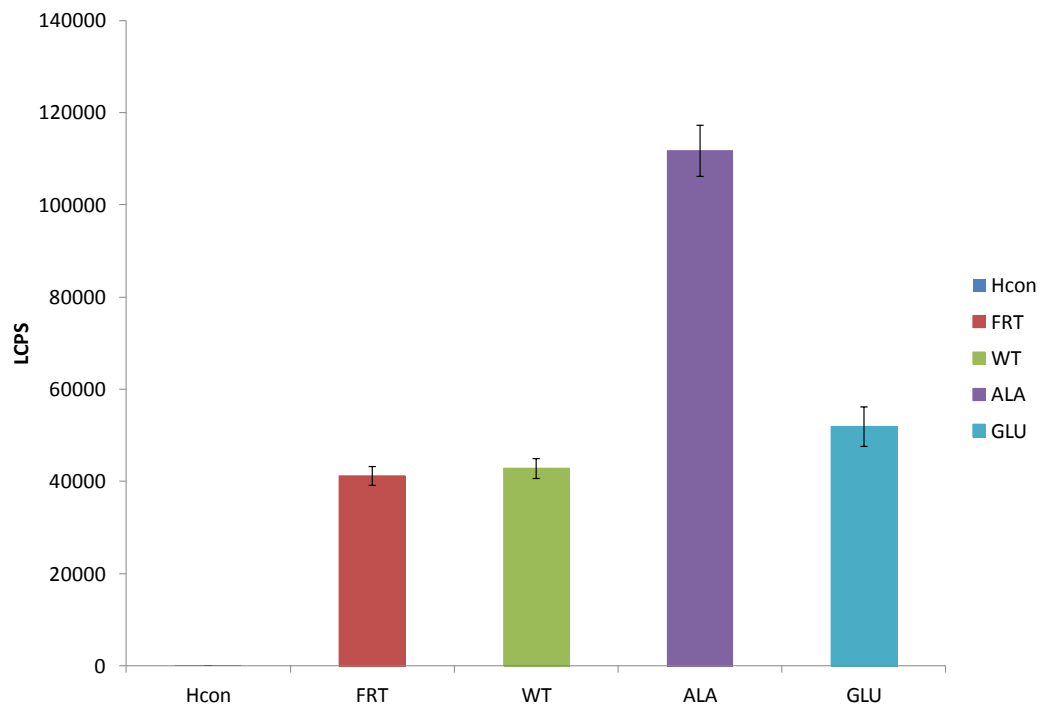
Figure 3.20 Total transient firefly luciferase expression from the FRT control and stably expressing eEF2 cell lines 48 h post-transfection. Hcon, FRT cell line transfected without luciferase DNA; FRT, CHO Flp-In cells stably expressing the empty FRT vector; WT, CHO Flp-In cells stably expressing wild type eEF2; ALA, CHO Flp-In cells stably expressing Thr56Ala eEF2; Glu, CHO Flp-In cells stably expressing Thr56Glu eEF2. Error bars represent standard deviation from n=4 independent experiments. $p < 0.05$ when comparing the FRT control values against the ALA values.
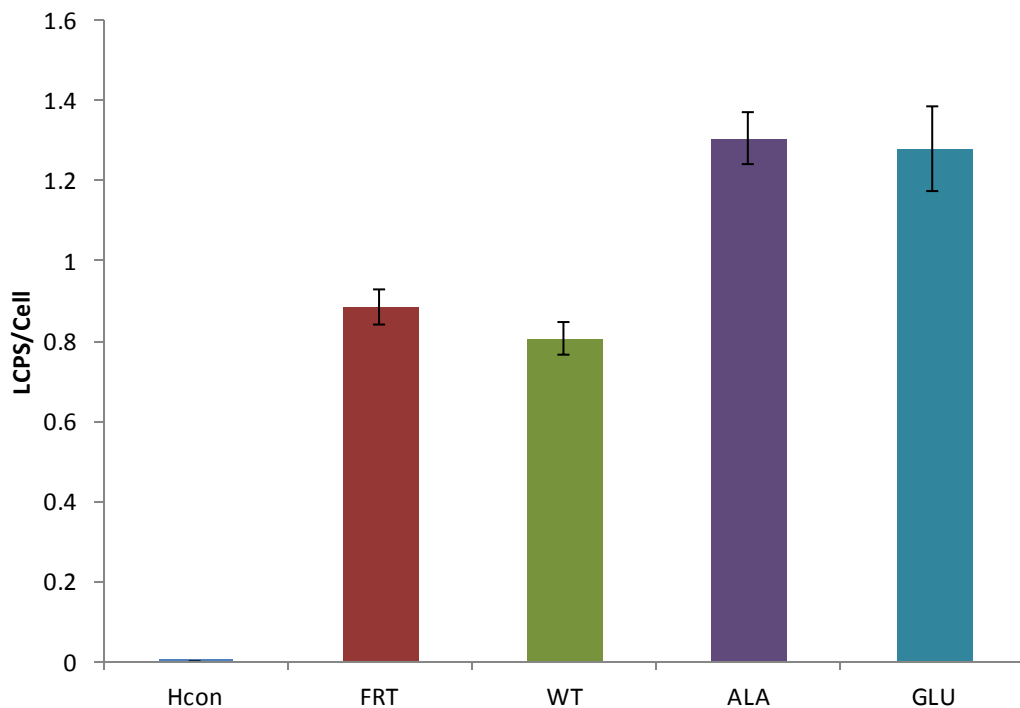
Figure 3.21 Transient firefly luciferase expression per cell from the FRT control and stably expressing eEF2 cell lines 48 h post-transfection. Hcon, FRT cell line transfected without luciferase DNA; FRT, CHO Flp-In cells stably expressing the empty FRT vector; WT, CHO Flp-In cells stably expressing wild type eEF2; ALA, CHO Flp-In cells stably expressing Thr56Ala eEF2; Glu, CHO Flp-In cells stably expressing Thr56Glu eEF2. Error bars represent standard deviation from n=4 independent experiments. p<0.05 when comparing the FRT control values to the ALA or GLU values.

3.2.4.2 Effect of Stable Expression of V5-Tagged eEF2 WT and Mutants in CHO Cells on Transient Firefly Luciferase Expression

In addition to investigating the effect of stable expression of the untagged eEF2 and mutants on firefly luciferase expression, any effect of stably expressing the V5-tagged eEF2 in CHO cells on recombinant firefly luciferase expression was also investigated. For this experiment, 1 µg of the firefly luciferase pGL3 was transfected into the CHO FRT control cell line and the stably expressing C-terminal and N-terminal V5-tagged eEF2 CHO cell lines; the luciferase activity determined 48 h post-transfection. In both the N- and C-terminal V5-tagged eEF2 cell lines there was a 2-3-fold increase in the total luciferase expression above that observed in the FRT control cell line (Figure 3.22). This increase was not previously observed when the WT untagged eEF2 cell line was transfected with the pGL3 and hence the data strongly suggests that the presence of the V5 tag influences the subsequent

luciferase expression. The reason for this is not clear from this data, or whether this is a direct effect of eEF2 and elongation or via some other unknown mechanism.
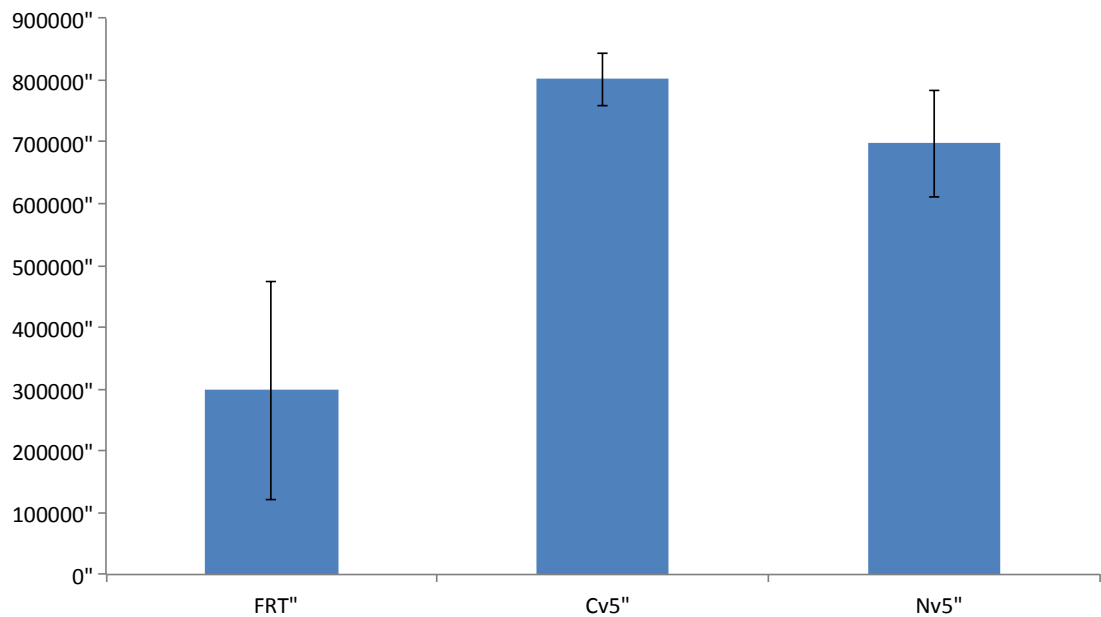
Figure 3.22 Total transient firefly luciferase expression from stable CHO Flp-In cell lines expressing eEF2 with either a V5 tag at the C-terminus (Cv5) or the N-terminus (Nv5), transiently transfected with a pGL3 firefly construct. Light counts per a second (LCPS) were measured 48 hours post transfection. FRT = control cell line. Error bars represent standard deviation from n=4 independent experiments. p<0.05 when comparing Cv5 or Nv5 values to the FRT control.

3.2.5 Effect of Stable eEF2 Expression in CHO FRT Cells on Protein Fidelity

The data shown in this chapter suggests that the stable expression of an eEF2 Ala56 mutant in CHO cells can result in increased firefly luciferase expression. However, increased expression and particularly polypeptide elongation could come with a cost of increased amino acid mis-incorporation compromising protein fidelity. To investigate this two mutant variants of the pGL3 firefly luciferase were kindly provided by Dr Anne Roobol; an R218S mutant which causes the luciferase molecule to lose its activity and an R218STOP mutant which results in a truncated luciferase protein with no activity (Conn & Qian 2014). Luciferase activity from these mutants is therefore only observed upon mis-translation or a loss of fidelity. An increase in luciferase activity is therefore indicative of a loss of protein fidelity.

When these constructs were transfected into the FRT CHO control cell line, or stably expressing WT eEF2 and mutant Ala56 and Glu56 eEF2 cell lines there was very low luciferase activity 48 h post-transfection indicative of very low infidelity. In the stably expressing WT eEF2 cell line there was actually a slight decrease in luciferase expression compared to the FRT control cell line suggesting an increase in fidelity in this cell line (Figure 3.23). There was little difference in the expression from the Glu56 mutant cell line compared to the FRT control. However, in the Ala56 mutant there was a small but noticeable increase in luciferase expression compared to the FRT control cell line for both constructs, indicating a very small loss in protein fidelity. However, this small increase is more than offset by the fact that this cell line gives the highest total luciferase expression by approximately 3-fold as shown in Figure 3.20. This may therefore not actually represent a true increase in infidelity but rather reflect the increased amount of luciferase expressed and as such could well be considered as an increase in fidelity (i.e. may expect a 3-fold increase in expression from these constructs in line with their total luciferase expression).
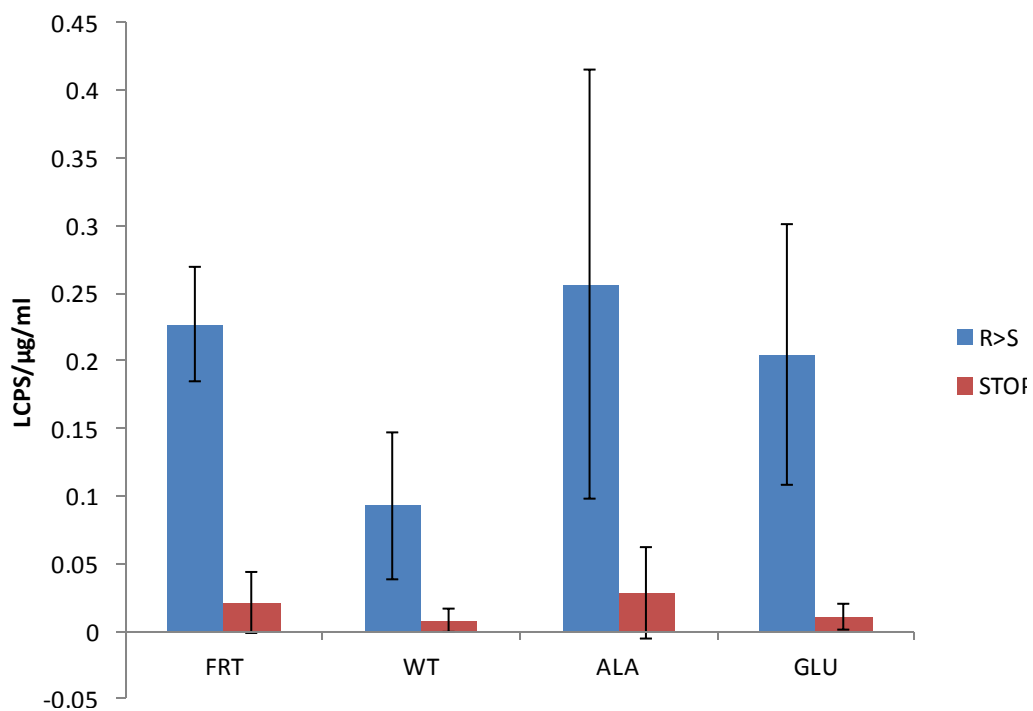
Figure 3.23 CHO Flp-In cell lines stably expressing eEF2 constructs, transiently transfected with mutant firefly luciferase constructs. R218S firefly luciferase mutant and R218STOP fire luciferase mutant. Lysates were taken 48 hours post transfection; light counts per a second (LCPS) were measured and normalised to total protein per a sample. FRT, CHO Flp-In cell line stably expressing the empty FRT vector; WT, CHO Flp-In cell line stably expressing wild eEF2; ALA, CHO Flp-In cell line stably expressing Thr56Ala eEF2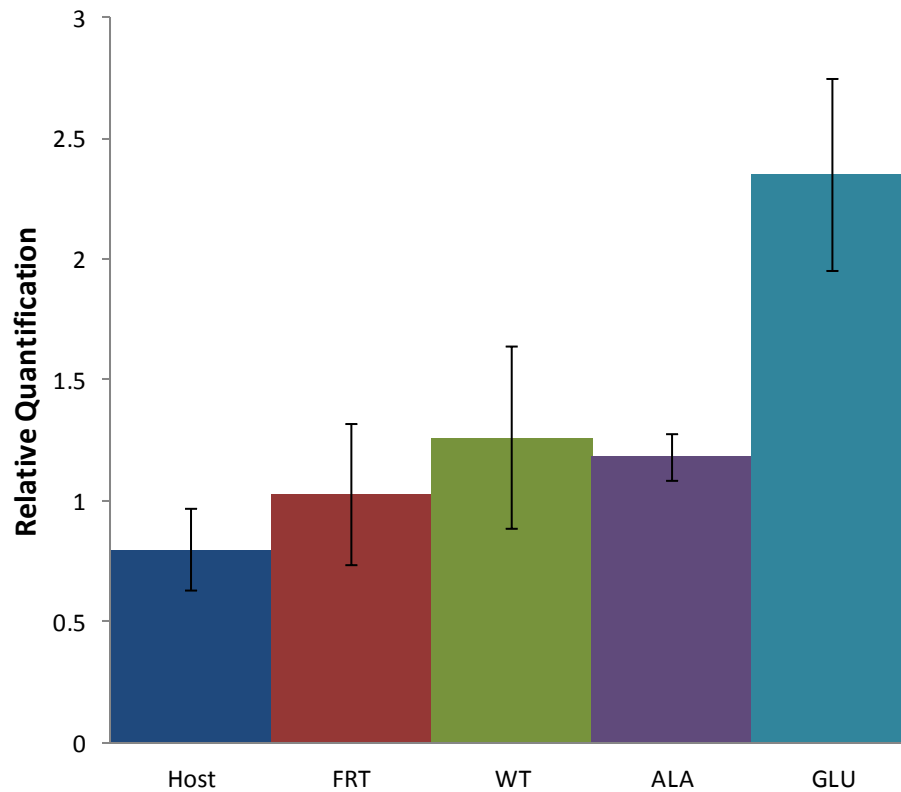; GLU, CHO Flp-In cell line stably expressing Thr56Glu eEF2. n=3, When comparing the R>S WT values to the R>S values, $p<0.05$.

3.3 Conclusions and Summary Statements from this Chapter

The main findings of this and subsequent chapters are discussed together in detail in the discussion chapter (chapter 6) of this thesis. Here the main findings are summarised.

To summarise, it was found that due to the conserved nature of the amino acid eEF2 sequence it was possible to use the human DNA sequence to express eEF2 (elongation factor 2) constructs in adherent CHOK1 cells, which was confirmed by western blot analysis, but transient expression of the human eEF2 in the CHOK1 cells did not affect their growth in culture. Transient expression of wild type and Thr56Ala eEF2 in CHOK1 cells appears to effect the short term (24-48 hours) phosphorylation of eEF2; but it does not appear to have an effect upon intra cellular recombinant protein production and cellular growth in culture over 96 hours. Immunofluorescence imaging of CHOK1 cells transfected with the eEF2 WT construct revealed that the translation efficiency of eEF2 in to the CHOK1 cells was only 20-30%. Therefore the majority of the cells within culture were not expressing the eEF2 constructs, skewing the results of the growth and western analysis of the transient experiments. Therefore to have all of the cells within the culture expressing the eEF2 construct for an extended period of time required stable expression of the construct.

Using the commercially available CHO Flp-In system (Invitrogen), CHO Flp-In cells stably expressing a variant of the CHO FRT eEF2 constructs (eeF2 WT or Thr56Ala or Thr56Glu) as well as a control cell line using an empty FRT vector were generated. Confirmation of the eEF2 constructs expression was confirmed via western blotting and immunofluorescence of an N- or C-terminal V5 reporter tag.

Initial stable over expression of the wild type eEF2 construct showed a 2-fold increase in expression of eEF2 and a decrease in phosphorylation of eEF2 at the protein level; but the there was no change in the levels of total eEF2 mRNA expression. These data suggest that there is some form of regulation of eEF2 expression between translation of the eEF2 mRNA and its expression as a functional protein.

Stable expression of the Thr56Ala and Thr56Glu mutants had a greater effect upon eEF2 expression resulting in a 3-5 fold increase in total eEF2 expression, however the phosphorylation of eEF2 was almost unchanged in the Ala56 cell line, whereas it was reduced in the Glu56 mutant cell line. This reflects the activity of the mutants, where the Thr56Ala mutant is considered to be continuously active; therefore phosphorylation of the endogenously expressed eEF2 is increased. The Thr56Glu cell line eEF2 mutant is considered to be continuously inactive; therefore the endogenous eEF2 must increase its activity to maintain protein expression, resulting in a reduction in eEF2 phosphorylation.

Growth of the CHO Flp-In cells lines expressing the eEF2 mutants show that over expression of any of the eEF2 mutants resulted in a change in growth, but the Ala56 eEF2 mutant showed the biggest change in cellular growth, increasing the cell index and prolonging the cellular culture compared to the FRT control. When cultured with rampamycin the opposite was observed, when under the stress of rapamycin the Ala56 cell line was the first to enter the decline phase of cellular culture and the maximum cell number was reduced. These data suggest that although additional exogenous eEF2 is capable of increasing cellular growth, this leaves the cell less resistant or able to adapt to additional stress on the mTOR signalling pathway.

Short term (48 hours) transient expression of recombinant firefly luciferase in the stable eEF2 cell lines revealed that the Thr56Ala mutant greatly increases the CHO cells total recombinant protein production. When normalised to cell number there was a change in luciferase levels the Ala56 and Glu56 cell lines that show a greater expression of luciferase compared to the Wt and FRT cell lines.

With the over expression of eEF2 read through or mis-incorporation of amino acids during translation of polypeptides is a potential concern. Using a mutant luciferase it was possible to investigate if the mutant of eEF2 would cause additional errors in mRNA translation. It was determined that mutation of eEF2 to Ala56 or Glu56 had little negative effect upon the mis-incorporation of amino acids during translation. However, over expression of the wild type eEF2 construct decreased the mis-incorporation of amino acids.

Finally, the V5 reporter tag itself appeared to have a positive effect upon recombinant protein production, both the N-terminal and C-terminal V5 tag resulted in an increase In recombinant firefly luciferase production as seen in Figure 3.22. The C-terminal V5 tag appears to be the more effective construct, as seen during transient immunofluorescence of the eEF2 WT V5 construct. The reason for the increase in eEF2 activity/ recombinant protein production due to the addition of a V5 reporter tag is still unknown, it is possible that it stabilises eEF2s structure or ha some similar effect as the Ala mutation. This is discussed in more detail in the discussion chapter of the thesis.

Chapter 4

Manipulation of the Kinase eEF2K and the Effect On Cell Growth and Recombinant Protein Expression in CHO Cells

4.1 Introduction

In the previous chapter the effect of (i) manipulating of elongation factor 2 (eEF2) amounts and (ii) the ability of exogenous eEF2 to be phosphorylated at Thr56 on CHO cell growth and reporter recombinant protein expression was investigated. These investigatinos were undertaken by the exogenous over-expression of either CHO WT eEF2, a non-phosphorylatable Thr56Ala eEF2 mutant or a mimic of phosphorylated eEF2: Thr-Glu56 eEF2. The results showed that the expression of the Ala56 mutant in particular offered advantages of better growth and higher recombinant protein expression as determined using a firefly luciferase reporter assay. One of the major disadvantages of this approach is that the endogenous CHO eEF2 is still present and thus the effect of manipulating the exogenous eEF2 is likely to be dampened by the endogenous material.

A more direct way of manipulating the endogenous eEF2 and its activity is to reduce or prevent its phosphorylation of the Thr56 residue by removing the kinase responsible, elongation factor 2 kinase (eEF2K). In this way it may be possible to prevent the slowing of elongation by phosphorylation of eEF2 although the cell may compensate for this by reducing the amount of total eEF2 produced. As such, the work described in this chapter focused upon investigating whether manipulation of eEF2K amounts in CHO cells could be used to reduce eEF2 Thr56 phosphorylation and if this subsequently influenced cell growth and recombinant protein expression from CHO cells.

As described in the introduction chapter to this thesis, eEF2K is the key regulator eEF2, through phosphorylation of the threonine 56 amino acid residue. When eEF2 is phosphorylated by eEF2K it is inactivated and hence polypeptide elongation during translation is slowed. In other words, the inactivation of eEF2 halts global protein synthesis,

which in turn would reduce recombinant protein production. In the previous chapter (Chapter 3) it was shown that eEF2 regulation is a key component in determining recombinant protein production and its amount and activity within CHO cells affected the volume and quality of the recombinant protein produced.

Elongation factor 2 kinase belongs to a super family of alpha kinases due to its active kinase site being an alpha helix instead of the more conventional beta sheet model ((Blakemore et al. 1998; Kenney et al. 2014); eEF2K has 2 structural domains: one at the N-terminus which has a calmodulin binding region and an alpha kinase region. At the C-terminus there are four alpha helical repeats, which have an unknown role but are believed to be involved with protein-protein interactions. Elongation factor 2 kinase is regulated by a number of other proteins including calmodulin, AMPK, mTORC1 and cAMPK (Kenney et al. 2014). Previous work has suggested and shown that manipulation of eEF2K and its activity has an effect on global protein expression (Kenney et al. 2014; Leprivier et al. 2013b; Heise et al. 2014). As eEF2K is the major regulator of eEF2, here the hypothesis that inactivation, knockdown/silencing of eEF2K results in increased/prolonged/enhanced CHO cell growth and recombinant protein production was investigated.

4.2 Results

4.2.1 Development of a shRNA for the Knockdown of CHO eEF2K

One method of modulating the amount of eEF2K in the cell and hence the phosphorylation of eEF2 is to reduce the amount of eEF2K in the cell by RNA silencing approaches. As such, the use of a short hairpin RNA (shRNA) approach for targeted silencing of eEF2K expression was utilised. In this way the amount of eEF2K in the cell can be reduced and subsequently the phosphorylation of eEF2 and eEF2s inactivation should be reduced. shRNAs work by generating a tight hairpin turn that supress mRNA translation of the target mRNA via RNA interference (RNAi) which reduces its expression, in this case expression of eEF2K. shRNAs are usually delivered via plasmid based systems which offers an advantage over the use of chemically synthesised siRNAs, that are directly transfected into cells, in that shRNA harbouring plasmids can be amplified in bacteria and also used to generate stably expressing shRNA cell lines where knockdown should be constitutive.

The shRNAs work by being transcribed in the nucleus after transfection and the resulting hairpin is a mimic of a pri-microRNA that is processed in the nucleus by Drosha. This 'pre-shRNA' is then transported into the cytosol by exportin-5 where it is further processed by Dicer and loaded into the RNA-induced silencing complex (RISC). The shRNA then silence's the expression of the mRNA either by repressing translation of the target to which it is complementary or the targeted mRNA is cleaved, again resulting in silencing.

A shRNA targeting eEF2K was therefore developed for use in this investigation. To do this the commercially available Gene Clip U1 Hairpin system (Promega) was used (Figure 4.1a) to investigate the effects of eEF2K knockdown upon recombinant protein production and cellular growth in CHO cells. In order to develop the eEF2K targeting shRNA, oligonudeotides for construction of the hairpin were designed based upon siRNA sequences for the knockdown of eEF2K. These oligonudeotides were

Table 4.1 Oligonucleotides used to construct the eEF2K shRNA hairpin using the Gene Clip U1 Hairpin system (Promega).

Oligonudeotide	Sequence
Oligo A	tctcctcatgcctgcaaccggatttctcgagaaatccggttgcaggcatgagct
Oligo B	ctgcagctcatgcctgcaaccggatttctcgagaaatccggttgcaggcatgag

The oligonudeotides were annealed together then ligated into the pGeneClip U1 plasmid before being transformed into competent *E.coli* cells. The presence of the hairpin insert in the resulting colonies was then confirmed by undertaking a digestion with the restriction enzyme PstI on minipreps of selected colonies (Figure 4.1b). When the hairpin is successfully inserted it causes a new restriction site to be constructed, therefore if the insert is present two bands are observed upon PstI digestion and only one when the insert is not present. As shown in figure 4.1b, in 4 of the colonies screened by this method two bands were observed at the expected sizes giving preliminary confirmation that the hairpin had been inserted correctly. In order to further confirm the correct insert and sequence was present, commercial sequencing of the hairpin constructs was undertaken which confirmed the presence of the hairpin sequence (see Appendix 7.5).
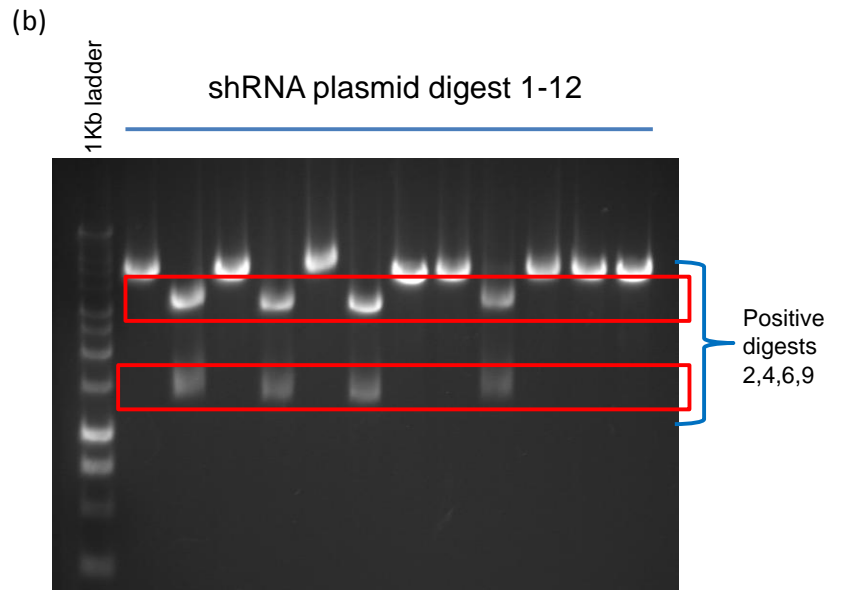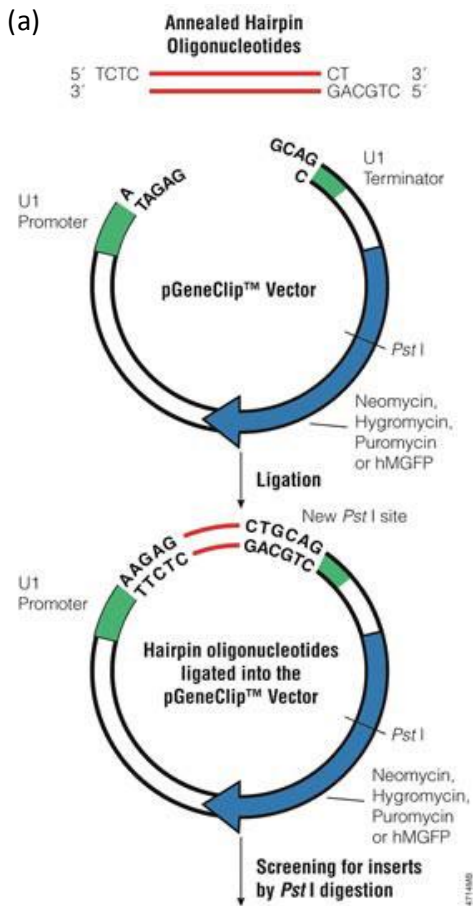
Figure 4.1 (a) Schematic depicting the pGeneClip U1 cloning system. Two oligonudeotides, designed as a short hairpin, that have been annealed together are inserted into the commercial pGeneClip™ vector. Once inserted, the newly constructed vector is screened using a Pst I digest restriction. If inserted correctly the annealed oligonudeotides result in a second Pst I digest site, allowing for quick screening of the ligated insert and vector.

(b) Screening of constructs for the presence of the eEF2K silencing hairpin by restriction digest with the PstI restriction enzyme. Agarose gel analysis shows four positive constructs had the short hair pin oligonudeutodies inserted into the pGeneClip™ vector by production of 2 bands at 3209 bp and 1402 bp. These construct where later confirmed with commercial sequencing.

4.2.2 The effect of transient transfection of the eEF2K shRNA into CHO Cells

4.2.2.1 Does Transient Transfection of the eEF2K shRNA into CHO Cells Reduce eEF2K Expression and Subsequently Reduce eEF2 Phosphorylation?

In order to determine if the eEF2K shRNA was expressed and resulted in knockdown of eEF2K at the protein level in CHO cells, transient transfection was undertaken into the CHOK1 Flp-In host cell line. To achieve this both 0.5 and 1 μg of the shRNA plasmid was transfected into 6 well plates containing the CHOK1 Flp-In host cell line as described in the methods chapter and cell lysates collected 24 and 48 h post-transfection. A control containing just the transfection reagents was also undertaken and lysates collected at the same time points.

The lysates were then examined for eEF2K levels by western blot analysis probing with an anti-eEF2K antibody. A loading control blot was also undertaken. The results of these blots are shown in Figure 4.2. The western analysis showed that there was good knockdown of eEF2K compared to the control when using 1 μg of the shRNA plasmid 24 h post-transfection (Figure 4.2). The effect was still present but not to the same extent at 48 h as there appeared to be less eEF2K even in the control sample. The knockdown was not as obvious in the 0.5 μg sample at 24 h post-transfection but still present. The short-term knockdown may partially reflect the regulation and rapid turnover of eEF2K, which has been reported to have a half-life of around 6 hours (Arora et al. 2005; Wiseman et al. 2013). Regardless, the 24 h 1 μg data confirmed that a high level of eEF2K knockdown could be achieved at least transiently in CHO cells using the eEF2K shRNA.

Once it had been established that the eEF2K shRNA could give a high amount of eEF2K protein knockdown, the same cell lysates were probed by western blotting for total eEF2 and phosphorylated eEF2 levels. The blot for these targets showed that the levels of total eEF2 did not change in response to the knockdown of eEF2K (Figure 4.2). However, there was a very distinct and large reduction in the amounts of phosphorylated eEF2 in the 1 μg knockdown sample 24 h post-transfection (Figure 4.2). These data confirm that the knockdown of eEF2K was sufficient to reduce the ability of eEF2K to phosphorylated eEF2 and hence inactive eEF2. There was still an effect upon eEF2 phosphorylation levels 48 hours post transfection compared to the control samples. In the 0.5 μg transfected samples

there was still a reduction in phosphorylated eEF2 24 h post-transfection but this was not as dramatic as transfecting 1 μg of the shRNA. Once again there did appear to be an effect on eEF2 phosphorylation 48 h post-transfection in the 0.5 μg samples but reduced eEF2 phosphorylation compared to the 1 μg sample (Figure 4.2). Together these data show that the shRNA was able to silence eEF2K expression at the protein level and that this subsequently reduced the observed phosphorylation of eEF2 in the CHO Flp-In host cell lines for at least 48 h post-transfection.
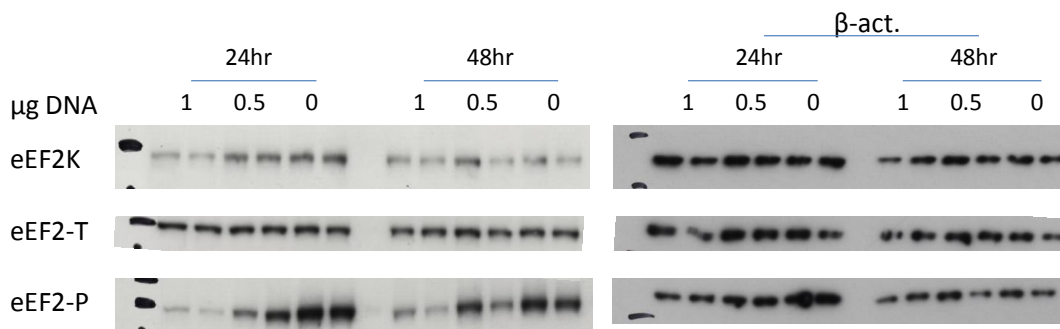
Figure 4.2 Western blot analysis of the knockdown of eEF2K using the shRNA plasmid generated in this study and the subsequent effect on total and phosphorylated eEF2 levels in CHO Flp-In parental cells. These blots confirm the knockdown of eEF2K 24 hours post transfection of 1ug of the eEF2K shRNA; this is corroborated by the reduction of eEF2 phosporylation in the same sample and that levels of total eEF2 are unchanged. eEF2K: elongation factor 2 kinase, eEF2-T: total elongation factor 2, eEF2-P: Phosphorylated elongation factor 2.

4.2.3 The Effect of Transient eEF2K Knockdown On Recombinant Protein Expression

4.2.3.1 Effect of Transient eEF2K Knockdown upon Stable Firefly Luciferase Expression from CHO Cells

Once it had been established that the shRNA eEF2K targeting plasmid could successfully knockdown endogenous CHO eEF2K it was used to assess whether transient knockdown of eEF2K had any effect on CHO cells capability of achieving recombinant protein production. This was initially investigated using the cytosolic firefly luciferase reporter gene. To achieve this, the CHOK1D6 cell line that stably expresses firefly luciferase were transfected with 1 µg of the eEF2K shRNA in 24 well plates as described in the methods section. Control experiments with transfection reagent only and the cells only with no additions were also undertaken. 24 hours post-transfection the firefly luciferase expression was determined in the different samples as light count per second (LCPS). The resulting data is presented in Figure 4.4. This data shows that the cells transfected with the eEF2K shRNA produced a much higher amount of luciferase per cell than in the controls, the amount of expression being approximately 5-fold higher (Figure 4.4).

As the transient knockdown of the eEF2K had such a pronounced effect on the stable firefly luciferase expression, the effect of a co-transfection of the eEF2 constructs used in Chapter 3 and the eEF2K shRNA on firefly luciferase expression was investigated. In doing this it is noted that the major effect of over-expressing eEF2 reported in chapter 3 was in the stably expressing Ala56 cell line and that the transient over-expression had less to no effect. To investigate this transient co-expression of the eEF2 and shRNA eEF2K knockdown plasmid, the CHOK1D6 cells were initially transfected cells with the different eEF2 constructs described in Chapter 3 (eEF2 WT, eEF2 Thr56Ala, eEF2 Thr56Glu) and 24 hours post-transfection the eEF2 WT transfected cells were transfected again with the eEF2K shRNA targeting plasmid to silence eEF2K activity. Then 24 hours post the 2nd transfection the amount of luciferase produced from each sample was determined. The results from this experiment are shown in Figure 4.5. From these data it appears that in the stably expressing firefly luciferase cell line that the knockdown of eEF2K has a greater effect upon recombinant firefly luciferase protein production than mutation of eEF2 that cannot be phosphorylated. This would be expected as the transient eEF2 manipulation reported in

chapter 3 showed little effect on luciferase expression (as opposed to stable expression of the Ala56 mutant).

Further, when the mutant Thr56Ala eEF2 is transfected into the cells, endogenous eEF2 is still expressed and can be phosphorylated by the cell to control elongation as described in the introduction to this cell. In the samples where the WT eEF2 was over-expressed and eEF2K was knocked down there appeared to be a decrease in luciferase expression (Figure 4.5) compared to the knockdown. Co-transfections are likely to result in additionally stress upon the cell as opposed to a single transfection and this may also explain why less luciferase expression was observed as compared to the knockdown alone (Figure 4.5). These data confirm that the transient knockdown of eEF2K enhances firefly luciferase expression but the transient expression of eEF2 appears to have little effect on luciferase expression.
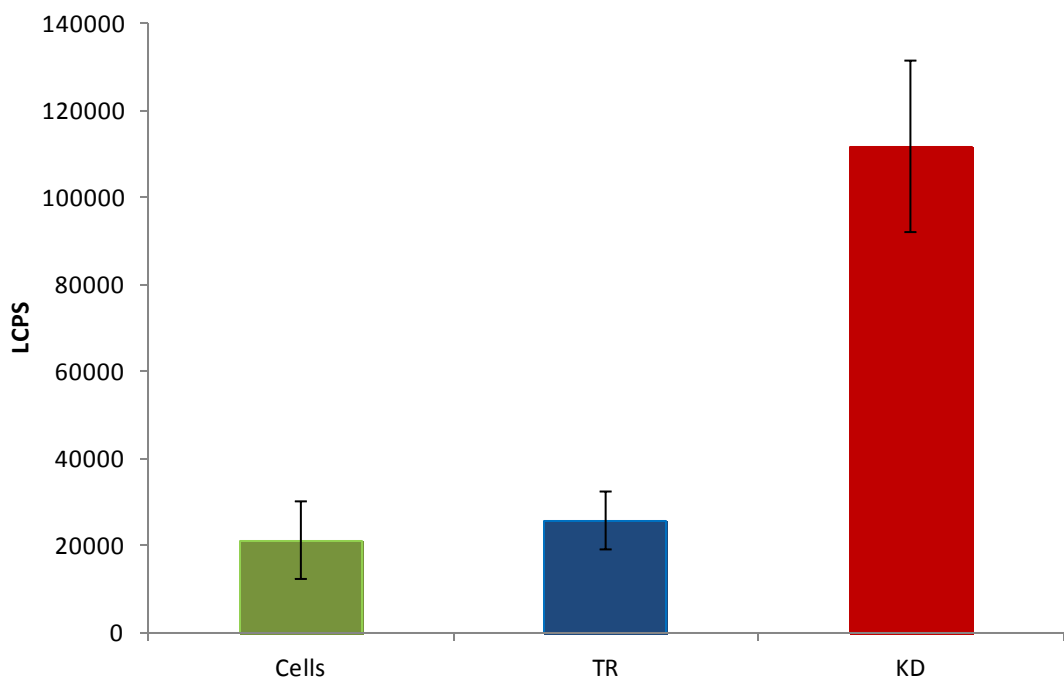
Figure 4.4 Expression of firefly luciferase measured as LCPS (Light counts per a second) from CHOK1D6 cells 48 hours post transfection of 1µg eEF2K shRNA (KD). Control samples of CHOK1D6 cells were treated with transfection reagent (TR) and others incubated without any treatment. Knockdown of eEF2K resulted in a 5-fold increase of LCPS expression and therefore luciferase production. Readings were taken in n=4 and averages and standard deviations calculated. n=8, $p<0.05$ when comparing the KD to the TR control and Cells control values.
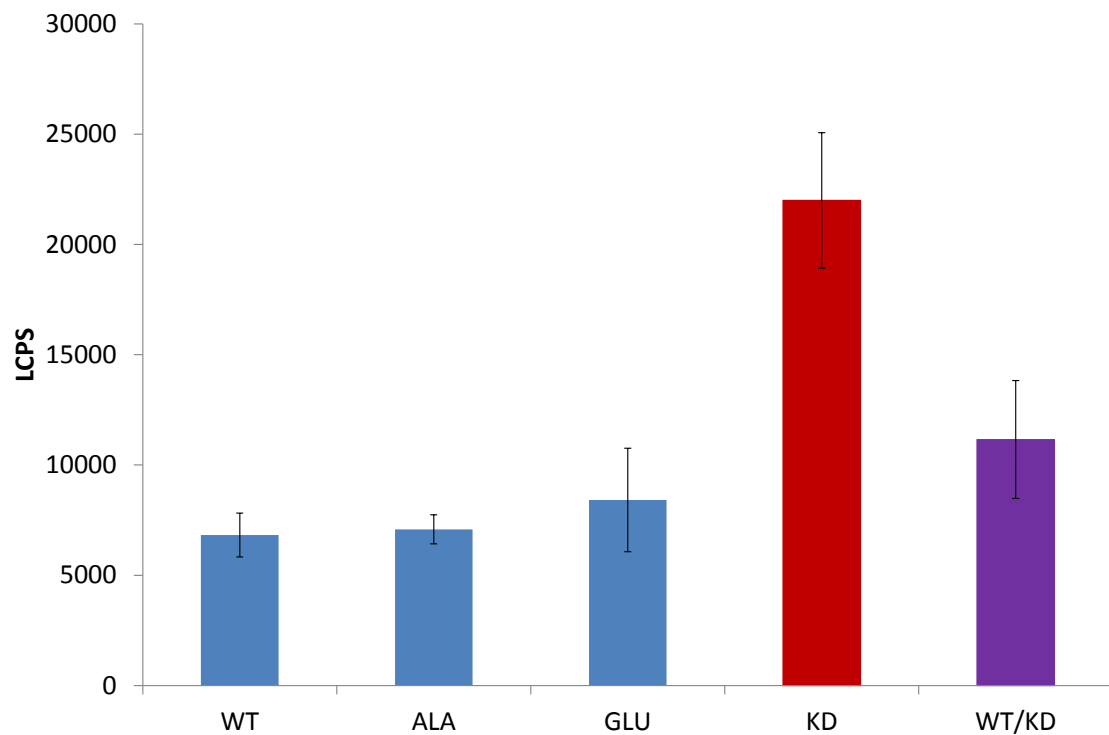
Figure 4.5 Determination of firefly luciferase activity via measurement of LCPS (Light counts per a second) in CHOK1D6 cells stably expressing firefly luciferase 48 hours post transfection of wild type eEF2 (WT), Thr56Ala eEF2 (ALA), Thr56Glu eEF2 (GLU)or eEF2K shRNA (KD). 24 hours post transfection of the wild type eEF2, a sample of CHOK1D6 cells were transfected again with 1μg eEF2K shRNA and then incubated for a further 24 hours (WT/KD). n=6, $p<0.05$ when comparing the GLU, KD and WT/KD values against the WT values.

4.2.3.2 Effect of Transient eEF2K Knockdown upon Stable Gaussia Luciferase Expression from CHO Cells

The transient knockdown of eEF2K was shown to be able to enhance cytosolic firefly luciferase expression; however biotherapeutic proteins expressed from mammalian cells are secreted proteins therefore the effect of the eEF2K knockdown upon the production of secreted proteins are of more interest industrially. As a model secreted protein gaussia luciferase was chosen as this can be rapidly assayed like firefly luciferase and stably expressing CHO cells were available for investigation. Further, previously studies have used this to determine the effect of manipulation of gene expression in CHO cells on secreted recombinant protein productivity (Jossé et al. 2010). One disadvantage of using gaussia luciferase is that the molecule itself appears to be less stable than firefly luciferase and this must be considered when interpreting expression data. The expression of gaussia luciferase was therefore determined by western blotting instead of an activity assay as used in the case of the firefly luciferase protein.

Two cell lines stably expressing gaussia luciferase available in the laboratory at Kent (cell line 12 and cell line 14) were transiently transfected with 1 μg of the eEF2K targeting shRNA and left for 24 hours before both supernatant and cell lysates were collected from control and knockdown samples. The resulting supernatant and cell lysates were then analysed by western blot analysis for total and phosphorylated eEF2, eEF2K and gaussia luciferase. This experiment was repeated twice. As can be seen in western blot data shown in figure 4.6a and 4.6c, gaussia luciferase was detected in the supernatant of all samples. For intracellular analysis of eEF2 and eEF2K the α-tubulin loading suggested that less protein had been loaded in the knockdown cell lysate samples (Figure 4.6a and 4.6c). As all cell lysates were collected with a set volume of lysis buffer, this suggest that there were less cells in the knockdown samples which may be due to the knockdown or could be due to cell seeding differences. As such, the gaussia luciferase expression was therefore normalised to the α-tubulin bands as an estimate to normalise to total protein or total cell amount. This is discussed more below (Figure 4.6). There was little or no gaussia luciferase expression detected in the cell lysates (Figure 4.6).

As described above, the western blot data shown in figures 4.6 showed less tubulin in the knockdown samples indicative of a lower protein load, presumably due to fewer cells. Therefore, it was necessary to consider this when determining the level of knockdown in

the cells and hence densitometry analysis was undertaken (Figure 4.6b and 4.6d). The blots suggested a knockdown of eEF2K even with a reduced tubulin band and the densitometry analysis confirmed this (Figure 4.6b and 4.6d). There was also a decrease in the amount of eEF2 phosphorylation. The total amount of guassia luciferase present in the supernatant was reduced in eEF2K knockdown samples as the same amount of supernatant was loaded from each sample (Figure 4.6). However, when the amount of luciferase was normalised to the tubulin loading control for the amount of cells, cells transfected with the eEF2K shRNA generally produced more gaussia luciferase compared to the control cells (untreated or treated with transfection reagent only). This was particularly true for cell line 12 but less pronounced for cell line 14 (see figure 4.6).

These data together suggest that knockdown of eEF2K influenced the total cellular protein amount (e.g. cell biomass generated over the time course) but that the amount of gaussia luciferase generated was increased on a total protein biomass basis upon eEF2K knockdown. Further, there was a cell line specific effect suggesting that the knockdown of eEF2K may have a different level of effect on different cells, probably due to a different balance in the amount of eEF2K, eEF2 and other translation factors. This has important implications for potential cell line engineering strategies by manipulation of eEF2K. Firstly, if the knockdown of eEF2K does reduce biomass then this is not desirable, as it will potentially limit target recombinant protein and cell biomass amounts. Secondly, from the results in this chapter so far, transient manipulation of eEF2K does appear to enhance recombinant protein expression but that this effect in stably expressing cell lines may be cell line specific and therefore not predictable.

It is noted that knockdown of eEF2K was attempted in the stably over-expressing eEF2 Ala56 mutant as the work in chapter 3 had shown this to have the most effect on cell growth and recombinant protein production. However, when knockdown was attempted in this stable cell line all cells died and the knockdown was not tolerated. This suggests that in the cell line the knockdown is lethal to the cell, possibly because it results not only in a reduced ability to phosphorylate endogenous eEF2 but the exogenous, non-phosphorylatable eEF2 is also present. This in all likelihood results in elevated levels of eEF2 that is not phosphorylated and the work in the previous chapter suggested that eEF2 levels are controlled by the cell to be maintained at a buffered level. The effect of the knockdown in this cell line may result in uncontrolled elongation and cell death.
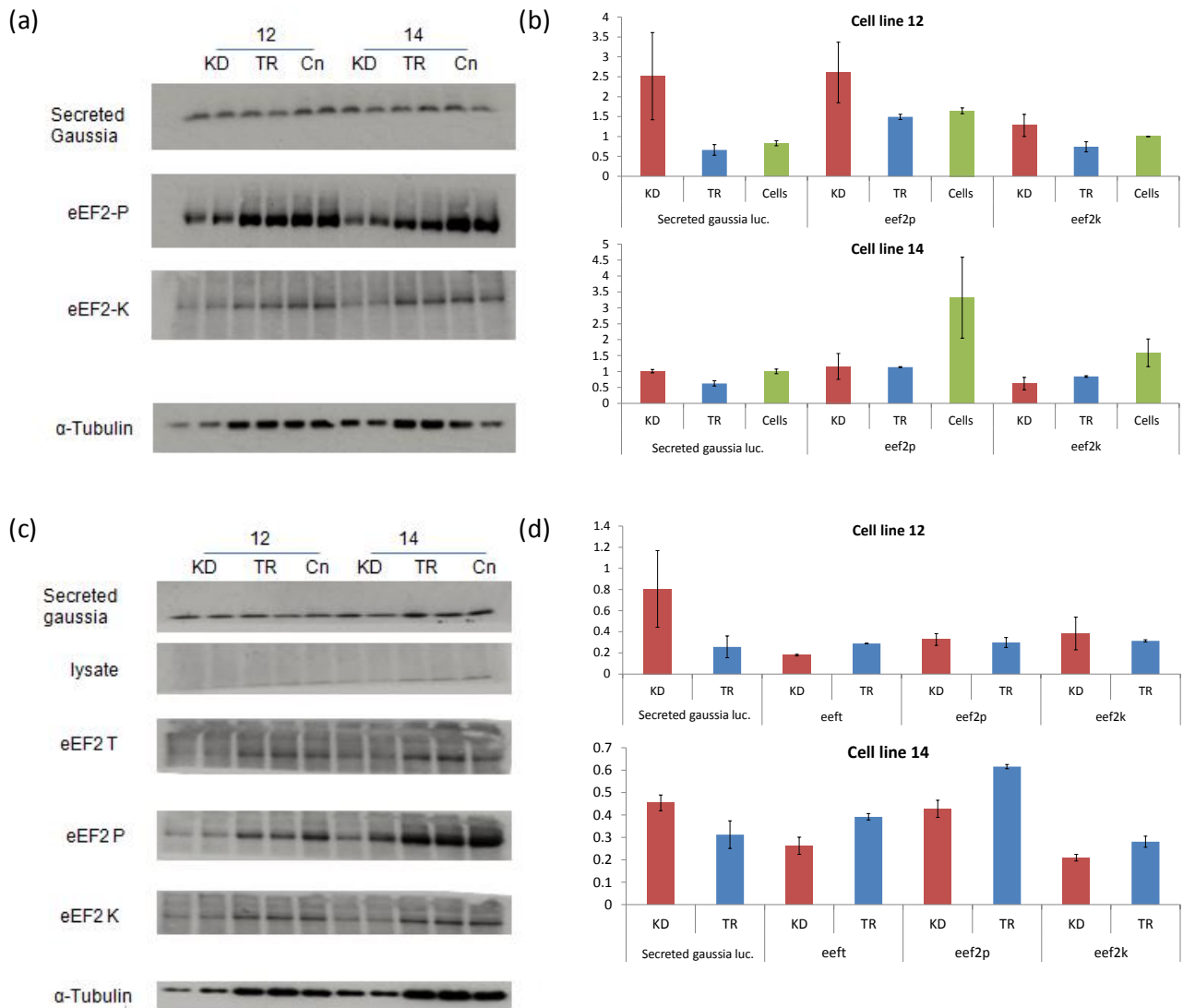
Figure 4.6 (a)(c) Western blot analysis of two CHO Flp-In cell lines stably expressing gaussia luciferase (12 and 14) 24 hours post transfection of 1 µg eEF2K shRNA (KD) or treatment with transfection reagents (TR). Duplicate supernatant samples were probed for secreted gaussia luciferase and lysates of the corresponding CHO Flp-In cell lines were probed for gaussia luciferase (lysate), levels of total (eEFF2 T) and phosphorylated (eEF2 P) elongation factor, elongation factor 2 kinase (eEF2 K) ,and alpha tubulin (α-Tubulin) as a loading control. Due to the unequal loading of the samples densitometry analysis was undertaken; relative levels of gaussia luciferase, eEF2 and eEF2K levels were calculated (relative to alpha tubulin) **(b)(d).** Blots **(a)** and anaylsis **(c)** have duplicate samples of the control sample 'cells': untreated CHO Flp-In cells expressing gaussia luciferase.

4.2.4 Transient Knockdown of eEF2K in CHO Cells upon Leucine Depletion in the Culture Media

As described in the introduction chapter of this thesis, eEF2K activity is controlled via mTOR signalling which keeps eEF2K activity low when mTOR is active. However, upon nutrient depravation, particularly leucine depravation, mTOR signalling is impaired (Lynch 2001; Gran & Cameron-Smith 2011; Proud 2004) and eEF2K activity is increased to slow translation elongation. The effect of leucine depletion upon eEF2K knockdown in CHO cells was therefore investigated using the CHO gaussia luciferase 14 expressing cell line.

To undertake this, eEF2K was knocked down in cell line 14 for 24 hours and then the cells placed in leucine depleted media (LD media) for 24 hours and cell lysates and supernatant collected. Control samples where eEF2K was not knocked down were also generated. Western blot analysis of the cell lysates for eEF2K, total eEF2, phosphorylated eEF2 and gaussia luciferase are shown in Figure 4.7a. A blot for α-tubulin was also undertaken as a loading control. As described above, the tubulin loading was less in the knockdown cells; therefore densitometry analysis was undertaken on the bands and is shown in Figure 4.7b.

The densitometry data suggested that there had been little knockdown of the eEF2K in the samples (see Figure 4.7b) although the phosphorylation of eEF2 was decreased. The cells with transfection reagent only also had decreased phosphorylation of eEF2 although not to the same extent as the samples transfected with the eEF2K shRNA knockdown plasmid. There was no difference in the total amount of eEF2 between the different samples. It is difficult to determine how eEF2 phosphorylation levels were down when eEF2K levels were not in the knockdown samples. It may be that the amounts of eEF2K were difficult to normalise with the tubulin and an over-representation of the amount of eEF2K present resulted. Alternatively, it is possible that due to the leucine depletion even though eEF2K was being knocked down, the cell responded to this to increase the eEF2K amounts in response under the leucine deficient conditions so that the eEF2K amounts were more-or-less the same as the control samples. However, this would not account for a reduction in phosphorylation of eEF2. Finally, although the normalised amount of gaussia luciferase in the supernatant appeared increased over the controls, the total amount detected was reduced (Figure 4.7).

(a)
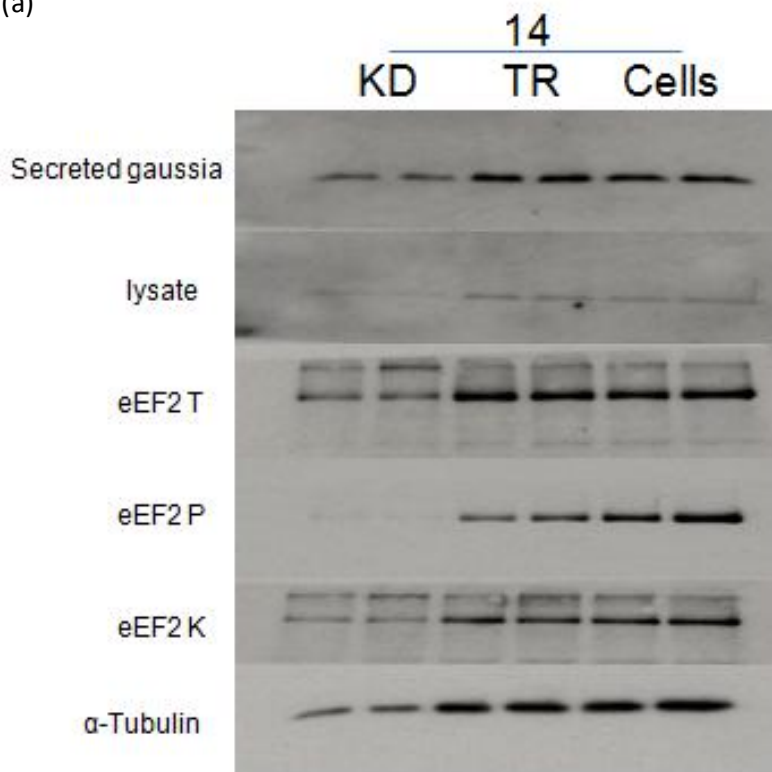
(b)
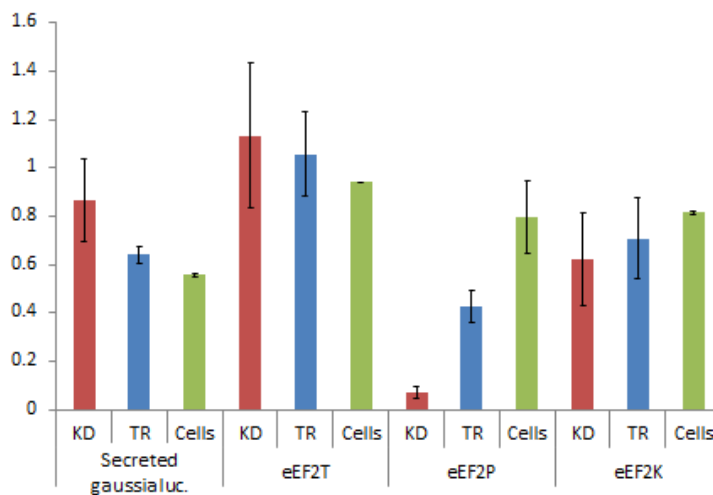
Figure 4.7 (a) Western blot analysis of CHO Flp-In cells stably expressing gaussia luciferase (cell line 14) transfected with 1 µg eEF2K shRNA and incubate for 24 hours, then incubated in leucine depleted media for a further 24 hours, after which supernatant and lysate were collected. Supernatant were probed for secreted gaussia luciferase and lysates of the corresponding CHO Flp-In cell lines were probed for gaussia luciferase (lysate), levels of total (eEFF2 T) and phosphorylated (eEF2 P) elongation factor, elongation factor 2 kinase (eEF2 K), and alpha tubulin (α-Tubulin) as a loading control. KD = cells transfected with 1 µg eEF2K shRNA, TR = cells treated with transfection reagent, Cell s= untransfected cells. **(b)** Densitometry analysis of the relative levels of gaussia luciferase, total and phosphorylated eEF2 and eEF2K levels were calculated relative to the α-Tubulin loading control.

4.2.5 Calcium Flux in the ER and eEF2K Knockdown

As described in the introduction chapter of this thesis, eEF2K has several regulators, one of these being calmodulin. The activity of calmodulin is responsive to calcium ions, therefore the control of eEF2K activity may respond to calcium flux levels. Although the tools to investigate a direct link were not available, changes in calcium amounts in the ER could be monitored using a calcium fluorescence based probe in stable cells available in the laboratory. Specifically, for this experiment, HEK Flp-In cells stably expressing a FRET genetically encoded calcium sensor that reports as a fluorescent indicator of calcium ion concentration in the ER was used (Palmer & Tsien 2006).

The FRET molecule consists of two arms with a CFP donor which has an excitation at 440 nm and emission at 480 nm, as well as a YFP acceptor arm which has an emission of 535 nm, with a calcium binding pocket between the arms. When excited by a laser at 440 nm and calcium is not bound, only the CFP donor emits a signal. However, when calcium binds in the calcium binding pocket this results in a conformation change that means the CFP donor and YFP acceptor arms become close to each other, causing the excitation energy to transfer to the YFP acceptor resulting in emission at 535 nm. Previous work in the laboratory at Kent has shown that during mild cold-shock of these HEK cells, calcium ions are released from the ER into the cytosol and hence there is a loss of the YFP signal and increase in CFP. This translocation of calcium ions from the ER to the cytosol could activate eEF2K via calmodulin activation and subsequently increase phosphorylation of eEF2. Therefore, whether knockdown of eEF2K had any effect on the calcium ion redistribution upon mild cold-shock was investigated. It was not expected that this would be the case, but as mild cold-shock is often used for the production of recombinant proteins from mammalian cells (Thaisuchat et al. 2011; Roobol et al. 2009) it was important to confirm this if engineering of eEF2K knockdown was to be considered as an approach for generating host cells for recombinant protein production.

HEK D1ER cells stably expressing the FRET molecule localised to the ER were therefore transfected with the eEF2K shRNA plasmid and then 24 hours post transfection cells were incubated at 37°C for a further 1 hour 20 minutes or at 32°C for 1 hour 20 minutes. The FRET in the cells was then observed using a confocal microscope to determine any differences in calcium ion concentrations in the ER under the different conditions. The

results are shown in Figure 4.15. For samples maintained at 37°C, the initial most striking feature was a change in the morphology of the cells (see figure 4.8). Whilst the control cells had a 'typical' HEK cell morphology with an extensive ER network and cytosol, the cells that had been transfected with the eEF2K targeting shRNA plasmid were more rounded and had a less extensive cytosol and ER network as determined from the fluorescence imaging.

Further, there appeared to be less YFP expression and more CFP in the knockdown cells suggesting that the knockdown experiment had in some way interfered with calcium homeostasis in the ER (see figure 4.8), which was unexpected. The difference in the extent of the cytosol could explain the lower total protein amounts in the lysates of the gaussia expressing cells discussed in the previous section. The potential change in calcium distribution from the ER could only occur if the cell responds to the knockdown of eEF2K by releasing calcium from the ER to try and activate more eEF2K in response to the knockdown, such that control and slowing of protein synthesis occurs, or if the transfection and expression procedure somehow compromises the ER membrane integrity or calcium transporters across the ER so that calcium is released into the cytosol, reducing that in the ER and hence reducing the YFP signal. It is not obvious from these data, which, if either, of these mechanisms is responsible for the observed effect.

In the cold-shocked control samples there appeared to be very little difference in the fluorescence signal between the 37°C and 32°C samples (Figure 4.8). However, once again there was a loss of the YFP signal in the eEF2K knocked down cells, suggesting a change in ER calcium amounts upon the knockdown procedure as in the 37°C knockdown samples. The general trend was for the loss of YFP signal to be greater at 32°C in the knockdown cells than that in the 37°C cells suggesting that temperature increased the effect observed. A hypothesis to explain these somewhat unexpected results is that the cell may try to adjust for reduced eEF2K levels, and hence a lack of ability to phosphorylate and inactivate eEF2, by modulating calcium amounts to try and activate eEF2K to provide an ability to continue controlling protein synthesis rates at the elongation stage via eEF2 phosphorylation. Further work would need to be undertaken to confirm if this was indeed the case.
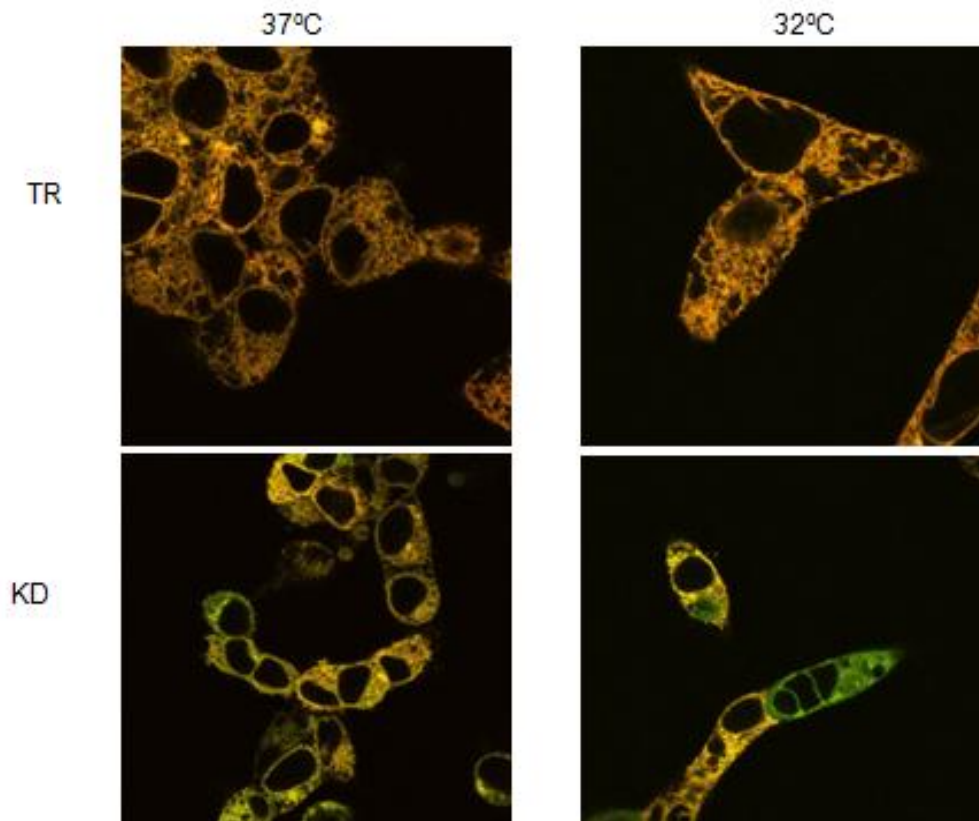
Figure 4.8 Visualisation of live HEK (Human embryonic kidney cells) stably expressing FRET genetically encoded calcium sensor localized to the ER 25 h 20 minutes post a 24 hour transfection of 1 µg eEF2K shRNA (KD) or treated with transfection reagent (TR) and a 1 h 20 minute cold shock (32°C), HEK cells that were not subjected to cold shocked were incubated at 37°C for the same length of time. The FRET molecule has two arms: a CFP donor with excitation at 440 nm and emission at 480 nm and an YFP acceptor with emission of 535 nm, with a calcium binding pocket between the arms. Excitation by a laser at 440 nm, without calcium binding, the CFP donor emits a signal at 480 nm. With calcium binding a conformation change occurs that closes CFP donor and YFP acceptor together, causing the excitation energy to transfer to the YFP acceptor resulting in emission at 535 nm. False colour was assigned to both the CFP (Green) and YFP (Red). Compositions of the red and green images were made using the freeware tool ImageJ.

4.2.6 Generation of CHO Cells with Stable Knockdown of eEF2K and the Effect on Protein Synthesis and Cell Growth

The work described in section 4.2.2 showed that eEF2K could be transiently knocked down in CHO cells resulting in reduced eEF2 phosphorylation and increased firefly luciferase expression. However, if such an approach was to be utilised in a biotechnological setting it would be necessary to generate stably knockdown eEF2K cell lines that exhibited the same effect. Whether stably knocked down eEF2K cell lines could be established, and the effect of this on cell growth and recombinant protein productivity, was therefore investigated.

4.2.6.1 The Generation of CHO Cell Lines Stably expressing the eEF2K targeting shRNA Plasmid

For construction of CHO cells stably expressing the eEF2K shRNA, the plasmid construct generated in the gene clip system could be used as this contains antibiotic selection for Puromycin. By application of the puromycin antibiotic, selection after transfection for the presence of the eEF2K shRNA would be possible to create stable cell lines expressing the eEF2 targeting shRNA. This was therefore achieved following the protocols provided with the Gene Clip kit to generate stable cell lines with puromycin resistance. The host cell line used was the CHO Flp-In parental cell lines. It is noted here that the recombinase targeting is not utilised in this approach as the shRNA was not on the appropriate vector nor was the plasmid with the recombinase co-transfected at the same time. This approach will lead to selection of random integration in the genome as is usually undertaken when generating recombinant cell lines and as such the resulting cells that survive would be expected to show different expression levels of the shRNA.

To generate the stably eEF2K shRNA targeting expressing cells, the CHO Flp-In parental were cells were transfected with 1 µg of the eEF2K shRNA as used in the transient expression studies previously described. 24 hours post-transfection the media was changed for selection media containing 10 µg/mL of the pyromycin antibiotic. The cells were then cultured in this selection media for two weeks before use in experimentation. Initially upon addition of puromycin the majority of cells died as expected, but then colonies of cells that survived the selection began to emerge and a pool of cells was developed.

In order to determine if the eEF2K targeting shRNA selected cells had reduced eEF2K and phosphorylated eEF2-levels, western blotting for these targets was undertaken. Initially the pool of stable cells was cultured over a 96 h period with cell samples taken every 24 h for western blot analysis. In order to ensure equal amounts of protein were loaded for each cell lysate, the protein concentration in each sample was determined by Bradford analysis and then equal amounts of protein loaded into each well. The resulting blot is shown in Figure 4.9. The blot showed low amounts of the eEF2K present early in culture but that this increased as the culture progress and this was mirrored by an increase in phosphorylation of eEF2.

In order to ascertain if the amounts of eEF2K were reduced compared to the host a second experiment was run where the amounts in the host of each of these proteins was directly compared to the amounts in the eEF2K shRNA stable pool of cells (Figure 4.9). These data showed that there was a small reduction in the amount of eEF2K present in the knock down cell line and an associated delay in eEF2-phosphorylation but the effect was far less obvious than that observed in the transient studies described earlier. This suggests that cells with a more pronounced knockdown of eEF2K did not survive the selection process providing further evidence that knockdown of this kinase and the loss of control over translation elongation is not tolerated by CHO cells. Further, the fact that eEF2K expression is observed later in culture in the knockdown cells also suggest that these cells are able to up-regulate eEF2K expression to overcome the shRNA effect or that the shRNA is not highly/well expressed in these cells. However, as there was a small knockdown of eEF2K the stable pool was investigated further.
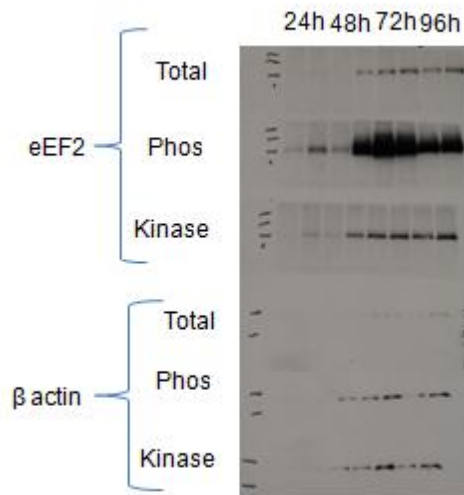
Figure 4.9 (a) Western blot analysis of CHO cells selected for during construction of eEF2K knockdown stable cell lines. Stably expressing CHO cells were cultured for 96 hours with biological duplicate lysates taken every 24 hours. The samples were then probed for levels of total and phosphorylated eEF2, eEF2K and β-actin as a loading control.

(b)
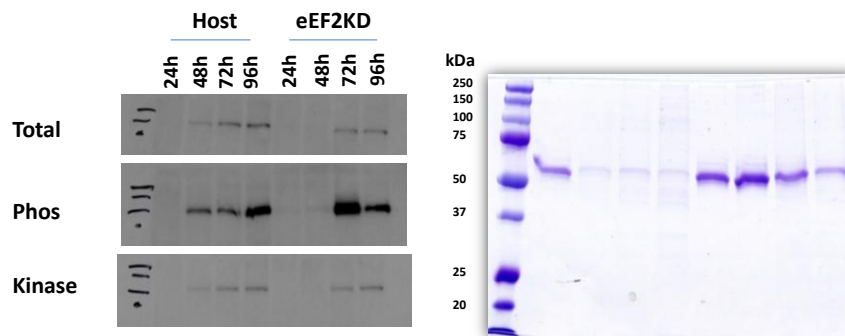
Figure 4.9 (b) Repeat western blot analysis of the eEF2K knockdown stable cells lines (eEF2KD) and the CHO Flp-In parental cell line (Host) used in the construction of the eEF2KD cell line. Both cell lines were cultured for a period of 96 hours with cell lysates collected every 24 hours. The samples were then probed for levels of total and phosphorylated eEF2, eEF2K. Samples were also separated on an SDS-PAGE gel and stained with Coomassie brilliant blue as a loading control.

4.2.6.2 Growth of the stable eEF2K shRNA cell lines

To assess if there was any effect on the growth of the CHO cells stably expressing the eEF2K shRNA construct, the cells were seeded into T25 flasks whilst control cells were seeded at the same time. The growth profile cultures were set up in biological duplicate and the viable cell concentration determined in each flask over a four day period. The resulting growth profiles are shown in Figure 4.10. As can be seen in Figure 4.10, there was no appreciable difference in cell concentration or viability of the cells expressing the eEF2K shRNA when compared to the control cell line (CHO Flp-In parental cells) across the four-days of culture. This is likely to reflect the fact that there was only a small knockdown in eEF2K amounts as determined by western blotting. It is also possible that a greater effect may have been observed at longer culture duration when nutrients become limiting and translation is slowed but this was not determined here. It is most likely that the small knockdown of eEF2K is not sufficient to influence cell growth characteristics.

(a)

(b)
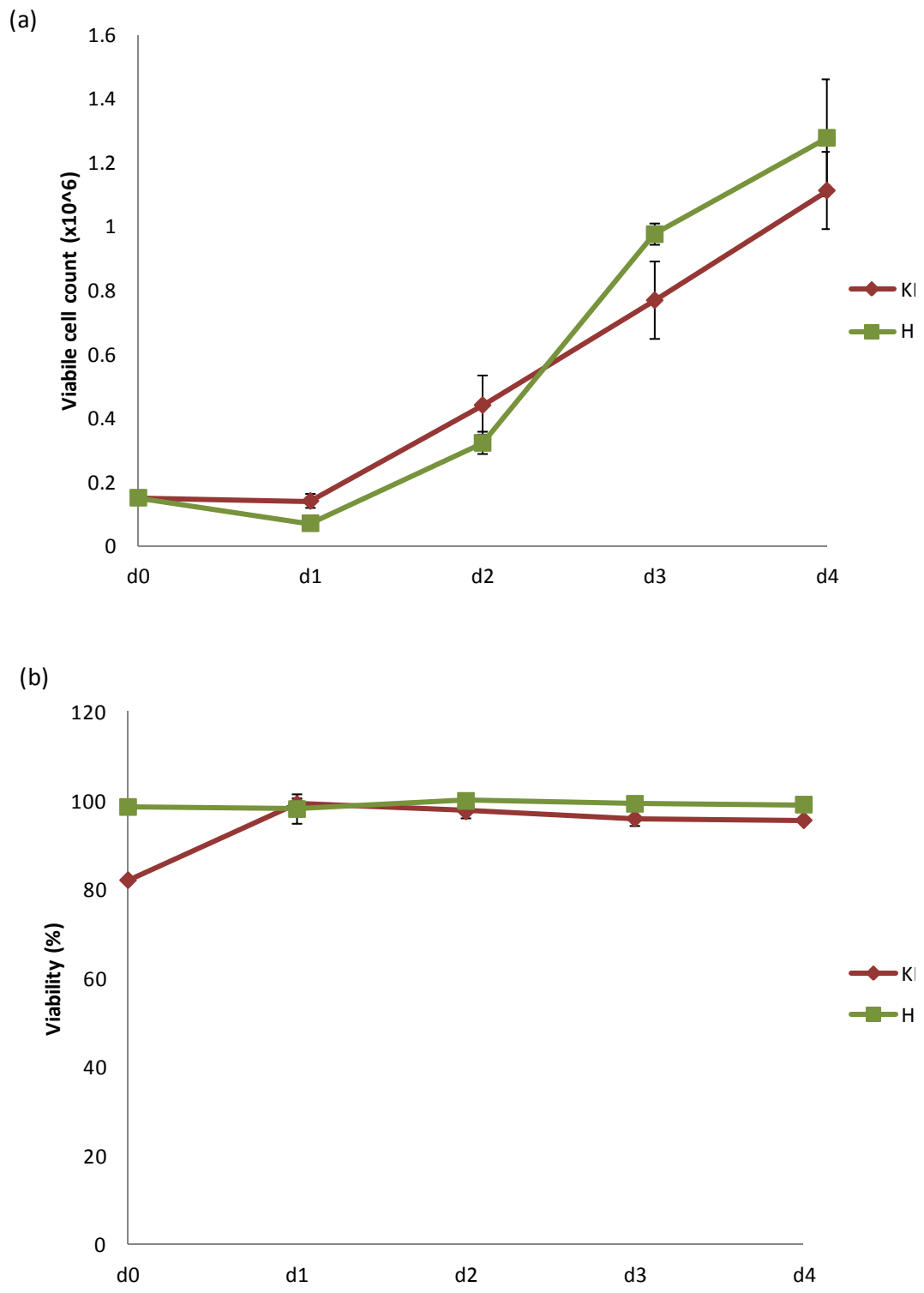
Figure 4.10 The average viable cell number **(a)** and average cell viability **(b)** of CHO Flp-In parental cells (H) and the eEF2K shRNA stable cell lines (KD) over a period of 4 days/96 hours. Cells were counted in biological duplicate, n=2 which were counted every 24 hours in triplicate.

4.2.6.3 Use of ^{35}S Metabolic Labelling of Nascent Polypeptides to Determine if Stable Over-Expression of the eEF2K Targeting shRNA Effects Global Protein Synthesis

The western blot data on the stably generated eEF2K targeting shRNA suggested that those cells generated using the process did not exhibit a significant knockdown of eEF2K and hence there was not a major effect upon eEF2 phosphorylation as determined by western blot. Further, the growth curve data again suggested there was little difference between the host control and stably expressing eEF2K shRNA knockdown plasmid. To further investigate if there was any effect on protein synthesis in the eEF2K shRNA harbouring cells, ^{35}S Met metabolic labelling studies were undertaken on the control host and knockdown cells.

For radiolabelling, the routine maintenance media was supplemented for 1770 kBq/ml Pro-Mix L-[^{35}S] cell labelling mix (GE Healthcare) then incubated for 1 h before cell lysates were collected to determine ^{35}S incorporation over the 1 h period which would be indicative of protein synthesis. This process was used rather than changing to cysteine- and methionine-deficient media as this in itself can result in stress to the cell and changes in protein synthesis.

From the growth data of the eEF2K targeted shRNA stable CHO Flp-In cells, two time points were determined for the addition of the ^{35}S isotope, 68 hours post-seeding of the cells when these were considered to be in the rapid growth phase of growth, and 120 hours post-seeding of the cells where the cells would be in the stationary phase of growth (see Figures 4.10). It was important to investigate both these potential time points as during rapid cell growth when there is also no nutrient limitations upon the cells, it would be expected that knockdown of eEF2K would have little effect on protein synthesis rates as protein synthesis would be at a maximum. However, when cell growth slows and nutrients become limiting protein synthesis might be expected to decrease and elongation slowed.

The cell lysates were collect and separated by SDS-PAGE, the gels dried and then exposed to X-ray film for 5 hours. The resulting autoradiograph is shown in Figure 4.11. The autoradiographs showed the same banding patterns between the host and eEF2K shRNA

harbouring cells at both the 68 and 120 h time points. Whilst the banding intensity was more-or-less equivalent at the 120 h time point, indicating a similar level of protein synthesis between the two samples, there was a reduced banding intensity in the eEF2K knockdown samples (Figure 4.11). It was expected that the knockdown cell line would if anything show enhanced protein synthesis compared to the host cell line. However, it appears that the knockdown either has no effect or has a negative effect upon global protein synthesis. The growth curve data also showed a lower number of cells around 3 days into culture in the eEF2K cells than the host, suggesting that protein synthesis and cell growth may be marginally compromised in these cells at this stage of culture. However, the lack of an obvious knockdown probably means that there is insufficient difference in eEF2K amounts between the control and shRNA harbouring cells to detect a difference in the protein synthesis. When cell lysate samples from these cell lines were probed by western blotting for eEF2K and eEF2 at 68 and 120 h of culture time points there was no obvious difference in the amounts of eEF2K or eEF2 phosphorylation (see Figure 4.12). Densitometry analysis of the western data suggested that eEF2 phosphorylation may have been elevated in the shRNA eEF2K harbouring cells compared to that observed in the host cell line (Figure 4.12a).
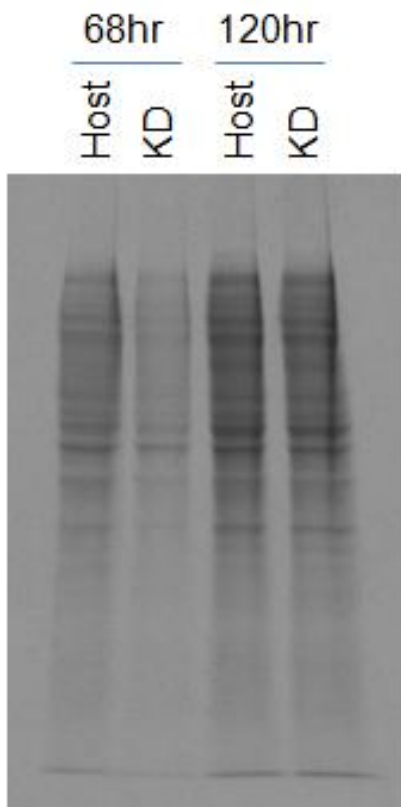
Fig 4.11 Lysates of CHO Flp-In parental cells (H) and the eEF2K shRNA stable cell lines (KD) cultured for 67 and 119 hours and then incubated with a ^{35}S Met metabolic label in routine maintenance media for 1 hour. Lysates were separated by SDS-page electrophoresis; the subsequent gel was then dried and exposed to x-ray film for 5 hours. Gel loading was normalised using a Bradford assay to quantify total protein per a lysate.
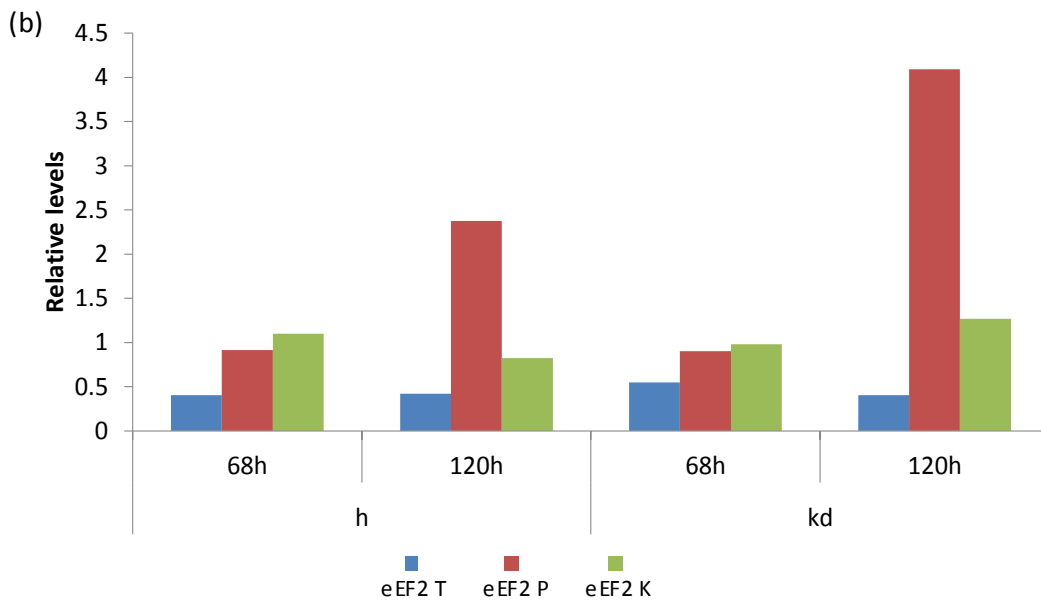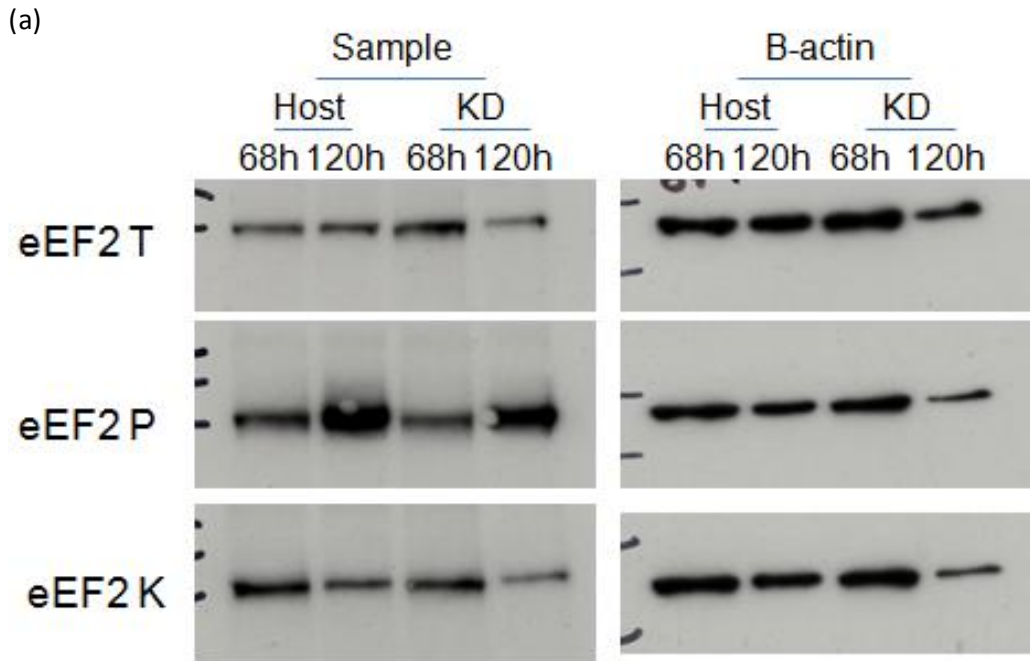
Figure 4.12 (a) Lysates of CHO Flp-In parental cells (h) and the eEF2K shRNA stable cell lines (kd) cultured for 68 and 120 hours analysed via western blotting, lysates were collected in conjunction with the ^{35}S Met radiolabelling experiment. Cells were cultured without the addition of the ^{35}S Met label. Lysates were probed for levels of total and phosphorylated eEF2, eEF2K and β-actin as a loading control. **(b)** Densitometry analysis of the band intensities were normalised to their corresponding β-actin and relative levels of total and phosphorylated eEF2 and eEF2K were determined, using the freeware tool ImageJ.

4.2.6.4 Effect of Stable eEFK shRNA Expression upon Recombinant Protein Production

The work described earlier in this chapter where eEF2K was knocked down transiently showed this could result in enhanced firefly luciferase expression in CHO cells (see section 4.2.3.1). Although the stable expression of the eEF2K shRNA appeared to result in the selection of cells whereby knockdown was limited compared to the control cells, it may be possible that the effect of the shRNAs knockdown was sufficient to alter the cellular protein production of the firefly luciferase reporter gene. To investigate this, the eEF2K shRNA cell lines were transiently transfected with the pGL3 firefly luciferase construct. 48 hours post transfection, light counts per second were measured as well as viable cell counts and from this the amount of luciferase (as determined by luciferase activity) per cell was calculated. The resulting firefly luciferase expression observed is reported in Figure 4.13. There was no change in the firefly expression per cell produced from the stable eEF2K knockdown cells when compared to the CHO Flp-In parental cells used as a control (Figure 4.13).

These data collectively confirm that the knockdown of eEF2K by stably expressed eEF2K shRNA is not as potent as transient expression of the shRNA. It is likely that CHO cells are not capable of maintaining a higher level of stable shRNA expression, such as that observed in the transient experiments. If this is the case, it is likely that during the stable cell construction, those cells with higher expression of the shRNA eEF2K targeting hairpin are not able to survive the antibiotic selective pressure as well as the knockdown of eEF2K. Meanwhile, those cells that had a lower level of shRNA expression survived the antibiotic pressure as well as being able to maintain eEF2K levels and control of eEF2 over longer term culture.
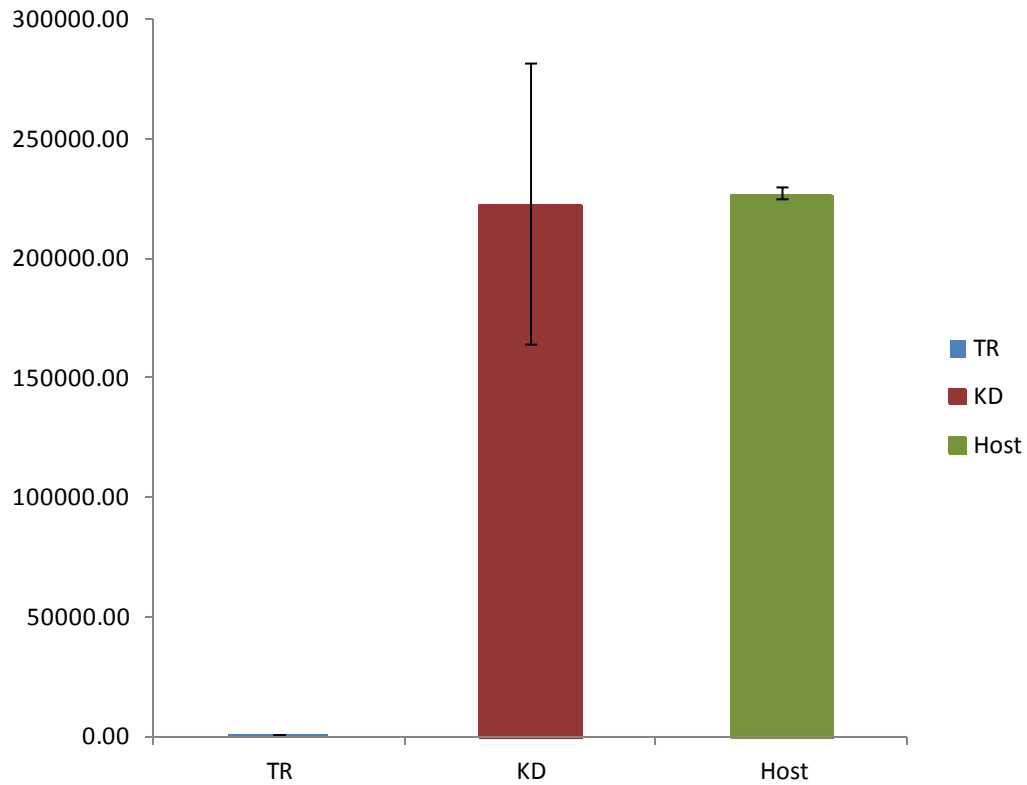
Fig 4.13 Determination of firefly luciferase activity via measurement of LCPS (Light counts per a second) 48 hours post transfection of 2µg pGL3 firefly luciferase into CHO Flp-In parental cells (Host) and the eEF2K shRNA stable cell lines (KD); Cells were also treated with transfection reagent only (TR) as a control. Cell counts were taken in correspondence of the LCPS activity to determine LCPS/cell. n=3 p>0.05.

4.3 Conclusions and Summary Statements from this Chapter

The data presented in this chapter shows that the transient expression of an shRNA plasmid targeted to eEF2K can be used to successfully knockdown eEF2K transiently and to prevent eEF2 phosphorylation. However, CHO cells do not appear to tolerate the knockdown of eEF2K stably or at only very low levels. This suggests that a sustained, high level of eEF2K knockdown is lethal to the cell; which would result in the loss of eEF2 regulation for extended period of time. It has been suggest by Ashour et al. (2014) in tumour cells eEF2K down regulation results in in cellular death. This supports the data shown in this chapter that it is only possible to stably express a low level of eEF2K shRNA. Therefore if eEF2K is not present within the cell, the cell is more susceptible to pro-apoptotic signalling.

The data in this chapter therefore suggests that any cells expressing high levels of the eEF2K shRNA would succumb to apoptosis/cell death during the antibiotic selection process of stable cell line production; as well as the cells that had not integrated the shRNA plasmid. This would mean only a small pool of CHO cells that have integrated the eEF2K shRNA plasmid into their genome, allowing the cells to survive the antibiotic selection process, with a low eEF2K shRNA expression, would survive. A possible method to circumvent this would be to use an inducible expression system whereby expression of the shRNA could be in the 'off' mode the majority of the time but switched to 'on' when it might be most useful later in culture

Any effect of the stable expression of the eEF2K shRNA appears to be until only around 48 hours after seeding of a culture, approximately the same length as the transient expression. Although the levels of eEF2K within the CHO Flp-In parental cell line are similar to that of the eEF2K knockdown cell line post 24 hours seeding, the rate at which eEF2K is expressed is reduced within the eEF2K knockdown cell line around 24 hours (Figure 4.9b). As the growth profile of the stable CHO Flp-In eEF2K knockdowns is similar to that of the original parental cell line (Figure 4.10), the delay of eEF2K expression is most likely due to the knockdown effects of the shRNA. It is likely that after the effects of the antibiotic selection within the culture media has diminished, so does the expression of the eeF2K shRNA.

Transient knockdown of eEF2K was shown to be a much more effective approach to knocking down eEF2K protein expression. The effect of the shRNA knockdown is only a short period of 24-48 hours, but with the majority of eEF2K protein expression suppressed over this period (Figure 4.2). This could in part be seen in the stable eEF2K knockdown cell lines as the eEF2K expression increase 72 hours post seeding (Figure 4.9), suggesting either the reduced effect of the antibiotic selection or an up regulation of the eEF2K mRNA transcription and translation.

Signalling to eEF2 in response to the transient transfection of the eEF2K shRNA appears to be at least partially mediated via the Ca^{2+}/calmodulin/eEF2K axis. Live cellular imagining of HEK cells stabling expressing an ER localised FRET molecule that responds to Ca^{2+} (Figure 4.8) showed an influx of Ca^{2+} out of the ER into the cystol 24 hours post transfection of eEF2K shRNA. Due to the reduced eEF2K expression, the cells appear to release calcium into the cystol in an attempt to activate calmodulin and in turn active eEF2K. These releases of calcium in to the cystol would also result in the activation/modulation of other proteins regulated by calcium such as the RAS GTPases (Aspenström 2004) and calcium dependant transcription factors (Fodor et al. 2013). Interestingly, visualisation of HEK cells revealed a change in cellular morphology upon eEF22K knockdown. Cells that had been transfected with the eEF2K shRNA were more rounded and the ER capacity reduced compared to those cells only treated with transfection reagent.

Transient expression of the eEF2K shRNA into CHOK1D6 cells stably expressing firefly luciferase, a non-secreted protein, resulted in a 5-fold increase in luciferase expression (Figure 4.4) showing that a knockdown of eEF2K did increase the short term productivity of these cells. However, in the biopharmaceutical industry the therapeutic proteins are secreted from the cell therefore a similar transfection in two CHO Flp-In cell lines stably expression gaussia luciferase, a secreted protein was undertaken (figure 4.6).

The effect of the shRNA appears to depend upon the activity and capacity of each cell line, as knockdown of eEF2K in cell line 14 only marginally increased the level of gaussia luciferase produced where as in cell line 12 there was little to no knockdown of eEF2K, but the levels of gaussia luciferase showed a greater increase in expression. The reason for this is not clear but collectively the results provide further evidence to support the idea that the up-regulation of elongation can increase recombinant protein production.

When cell line 14 was put under nutrient stress by being cultured in leucine depleted media for 24 hours post transfection of eEF2K shRNA, the only change was the levels of eEF2 phosphorylation (see figure 4.7) but levels of eEF2K remained similar to those of the control samples. It is possible that eEF2 activity has been increased due to the stress induced by the deprivation of leucine. Further, investigations into the levels of eEF2 and eEF2K mRNA as well as an extension of the time in culture before samples were collected could lead to a clearer picture of what is occurring under these conditions.

When compared to the short term (48 hour) transient effects of over expression of wild type eEF2 and the Thr56Ala and Thr56Glu eEF2 mutants in CHOK1D6 cells that stably express firefly luciferase transient knockdown of eEF2K had a greater effect upon intra cellular protein production as shown in figure 4.5. When co-expressed with the wild type eEF2 the knockdown effect appears to be dismissed. It is possible that is due to the stress caused by a second transfection. When the eEF2K shRNA plasmid was transiently transfected into the stable eEF2 CHO Flp-In stable cell lines the cells did not survive. It would appear that the effects of the eEF2K shRNA and consistent over expression of eEF2 were not tolerated by the cell.

Chapter 5

Investigations into the Link between Mammalian Target of Rapamycin (mTOR) Signalling, eEF2K, eEF2 Phosphorylation and CHO Cell Growth and Recombinant Protein Productivity

5.1 Introduction

The mammalian target of rapamycin (mTOR) is considered a master regulator of intracellular processes, such as protein and lipid synthesis, autophagy and cellular metabolism (Laplante & Sabatini 2009b). There are two known complexes of mTOR: mTORC1 and mTORC2 (mTOR complex 1/2). In this chapter the investigation was focused on the manipulation of mTORC1 via signalling pathways and the effects of this manipulation upon protein synthesis.

As outlined in the introduction to this thesis, the development of mammalian cell lines for high level expression of recombinant proteins has progressed such that cell line construction projects now routinely produce cell lines that are capable of delivering gram per litre yields of product in fed-batch cultures. In previous decades, such yields were unheard of whereby titres of 50-100 mg/L were the norm. The improvement of protein yield has largely been the result of improved expression vectors, development of medium and feeding optimisation, and advanced screening techniques that have dramatically improved the viable cell concentrations obtained in the bioreactor and reduced the time taken to produce stable production cell lines (Hacker et al. 2009; Wurm 2004). Despite such improvements, key areas remain that require further development in an industrial sense to reduce the timelines/cost of generating cell lines and improve the cell specific and volumetric productivities. One approach to addressing these issues is via engineering of cellular systems utilising the ever-improving understanding of the biology of cell growth and recombinant protein synthesis, folding, assembly and secretion. With regard to this, the manipulation of the mTOR pathway, a master regulator of protein synthesis, ribosome biogenesis and cell proliferation, has recently been reported to influence cell growth and recombinant protein synthesis from mammalian cells (Dreesen & Fussenegger 2011b).

Protein synthesis and ribosome biogenesis are key determinants of cellular biomass and protein productivity. However, the roles that these signalling pathway plays in these phenotypes, and whether it can be manipulated to enhance them, have yet to be fully elucidated. The studies described here investigated the role of mTOR signalling in determining the cellular phenotype of recombinant mammalian cell lines and whether this signalling system, and the environmental cues to which it responds, may be manipulated to enhance such phenotypes.
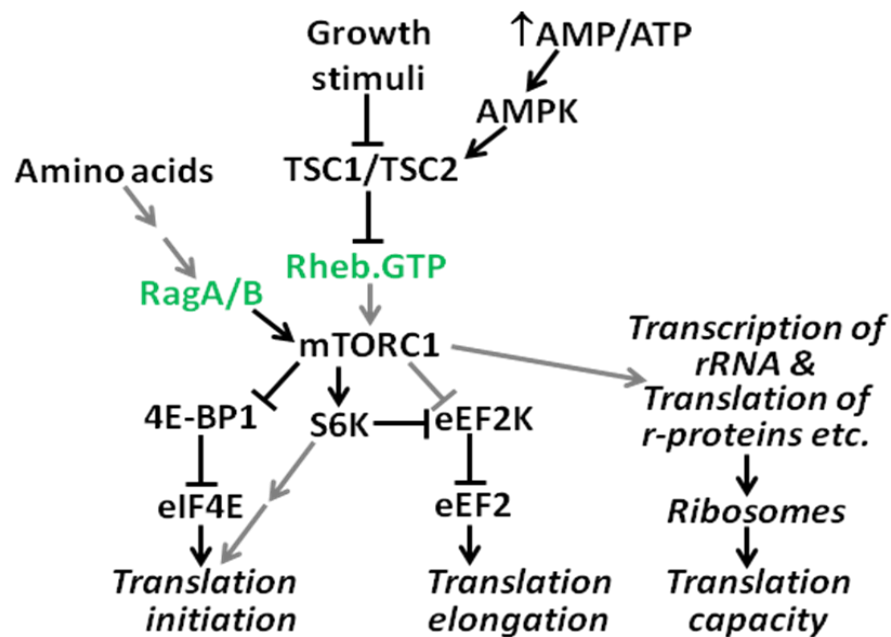
Figure 5.1 Simple schematic of mTORC1 signalling in mammalian cells. Grey lines denote processes that are not fully understood.

In the previous chapters of this thesis, the investigation was focused on elongation factor 2 (eEF2) and its regulator, elongation factor 2 kinase (eEF2K). mTORC1 regulates protein synthesis via S6K kinase, which regulates eEF2K (see Figure 5.1), in this way eEF2K is controlled by mTORC1. When mTORC1 signalling is attenuated, such as under conditions of amino acid starvation, eEF2K is activated as mTORC1 inactivation of eEF2K, via S6k kinase, is removed and hence eEF2K can phosphorylate eEF2, inactivating it and slowing elongation (see Figure 5.1). mTORC1 also regulates initiation of translation via eukaryotic initiation factor 4E (eIF4E). mTORC1 mediation of translation is achieved through the binding of raptor to ribosomal S6 protein kinase 1 (S6K1) and eukaryotic initiation factor 4E binding

protein 1 (4E-BP1), which recruits these substrates to be phosphorylated by mTOR. When hypophosphorylated, 4E-BP1 acts as a translational repressor by binding to eIF4E (Figure 5.1), preventing it interacting with eIF4G to promote ribosome recruitment to the mRNA; eIF4E also binds directly to the mRNA's 5'-cap structure. By directly phosphorylating 4E-BP1 at multiple sites (Thr$^{37/46}$, Thr^{70}, Ser^{65}), mTORC1 promotes its dissociation from eIF4E allowing the formation of the eIF4F complex and the initiation of cap-dependent translation (Fingar et al. 2002).

In this manner mTORC1 signalling directly controls protein synthesis and manipulation of the mTOR pathways has been shown to increase cellular proliferation, size and protein synthesis and reduce cell death (Dreesen & Fussenegger 2011b; Fingar et al. 2002; Schneider et al. 2008). There are now a number of studies that describe (i) how improved media composition and feeding can increase cell biomass of recombinant cell lines (Wurm 2004), (ii) that amino acid deprivation, osmolarity and general culture stress influence recombinant protein production from mammalian cells (Han et al. 2010), (iii) that mRNA translation can limit recombinant protein synthesis from mammalian cells (O'Callaghan et al. 2010; Khoo & Al-Rubeai 2009) (iv) that ribosome biogenesis can be manipulated to improve recombinant protein expression (Santoro et al. 2009)(vi) that chaperones play a key role in antibody biogenesis (Feige et al. 2009; Vanhove et al. 2001), and (vii) that mTOR related processes correlated with recombinant protein production (Chong et al. 2009; Dreesen & Fussenegger 2011b). Despite these studies, the precise role that mTOR signalling plays in determining doubling time, proliferation and recombinant protein synthesis has not been defined. Therefore, here it was investigated whether an increase or manipulation of mTOR activity in CHO cells producing recombinant protein cells results in an increase in protein synthesis, cell growth and specifically recombinant protein synthesis.

5.2 Results

5.2.1 Investigation of the Relationship between mTOR Signalling and Recombinant Protein Production in Recombinant CHO Cell Lines

In order to investigate any relationship between mTOR signalling and recombinant protein production during batch culture of CHO cells, two CHO suspension cell lines with different monoclonal antibody (mAb) productivities CHO High producer (CHOHP): regarded as high productivity - 560 mg/L in batch shake flasks, CHO Low producer (CHOLP): regarded as low productivity – 170 mg/L in batch shake flasks) were initially cultured in CD-CHO media and cell pellets harvested during culture for analysis of protein target amounts by western blot. The growth data from these cultures is shown in Figure 5.2. The growth curves for the CHOHP and CHOLP cell lines (Figure 5.2) show that the cell viability begins to decline after seven days of growth. Therefore lysates of both cell lines between days 4-7 were prepared and probed for total and phosphorylated eEF2 and total eEF2K amounts. The subsequent blot analysis suggested that there was no obvious change in the amounts of these factors present, or the phosphorylation status of eEF2, across this section of the time course or between the cell lines (Figure 5.3). In order to allow comparisons between the cell lines and time points, the total amount of protein loaded was normalised by a Bradford assay and the same total number of cells were lysed. These data suggest that at least across the time course investigated for these two cell lines, there was little difference in the control of protein synthesis at the level of polypeptide elongation between the two cell lines.
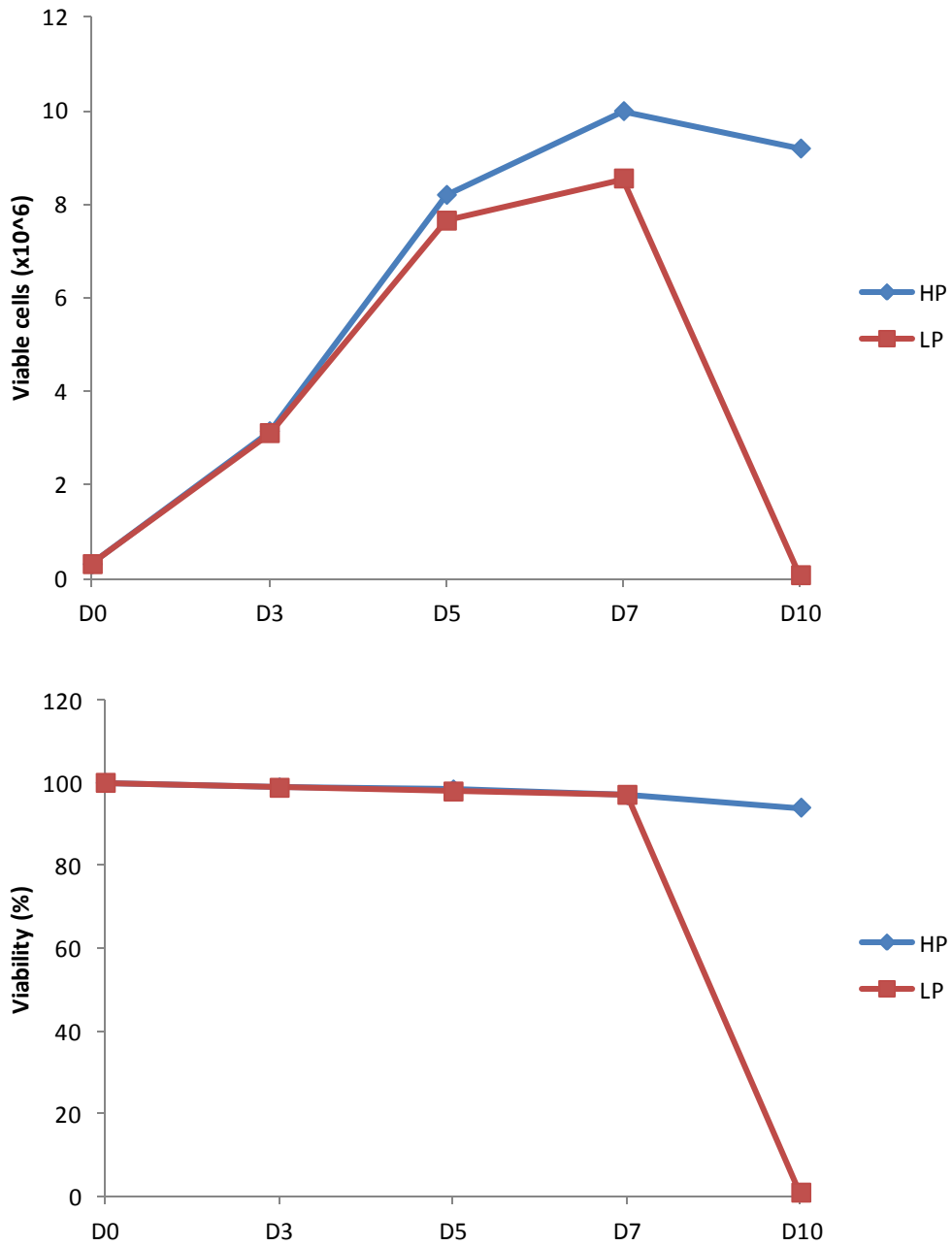
Figure 5.2 Growth curve data showing viable cell concentration and cell viability of the recombinant CHO high producer and CHO low producer cell lines expressing a model monoclonal antibody. Samples were collected across days 4-7 for western blotting.
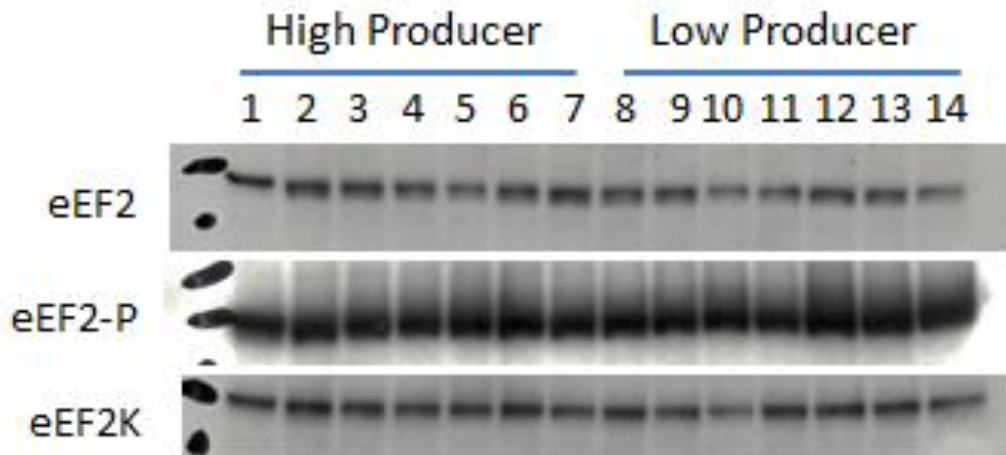
Figure 5.3 Western blot analysis of eEF2, phosphorylated eEF2 (eEF2-P) and eEF2K on days 4, 5 ,6 and 7 of batch culture for the CHO High producer and CHO low producer recombinant cell lines expressing a model monoclonal antibody. Lane 1 and 2 = day 4 samples, lane 3 and 4 = day 5 samples, lane 5 = day 6 sample, lane 6= day 7 sample, lane 7 and 8= day 4 samples, lane 9 and 10= day 5 samples, lane 11 and 12= day 6 samples, lane 13 and 14= day 7 samples.

In order to further investigate any potential relationship between the recombinant CHO cell lines ability to produce recombinant monoclonal antibody (mAb) and translation elongation status determine by eEF2 and eEF2K levels, a wider panel of mAb 'producer' CHO cell lines with a range of mAb titres over a batch culture were therefore investigated. The range of antibody yields from these cell lines in a batch culture were from 240 to 690 mg/ml. The CHO cell lines investigated were labelled as H1, H2, M1, M2, L1 and L2 (High prouder 1 & 2, Mid producer 1 & 2 and Low producer 1 & 2) which had antibody productivities of 691, 541, 415, 372, 272 and 244 mg/L each in a batch culture when harvested on the same day (10 days of batch culture). Cell lysates were collected on days 6 and 8 of culture when the cells were in early stationary and late stationary phase, where the majority of antibody production occurred. These samples were then probed for total eEF2 and phosphorylated eEF2 and eEF2K, the resulting blots are shown in Figure 5.4. From the western analysis alone there was no obvious trend in the change of either of these proteins or the phosphorylation status of eEF2 in relation to mAb productivity (see Figure 5.4).
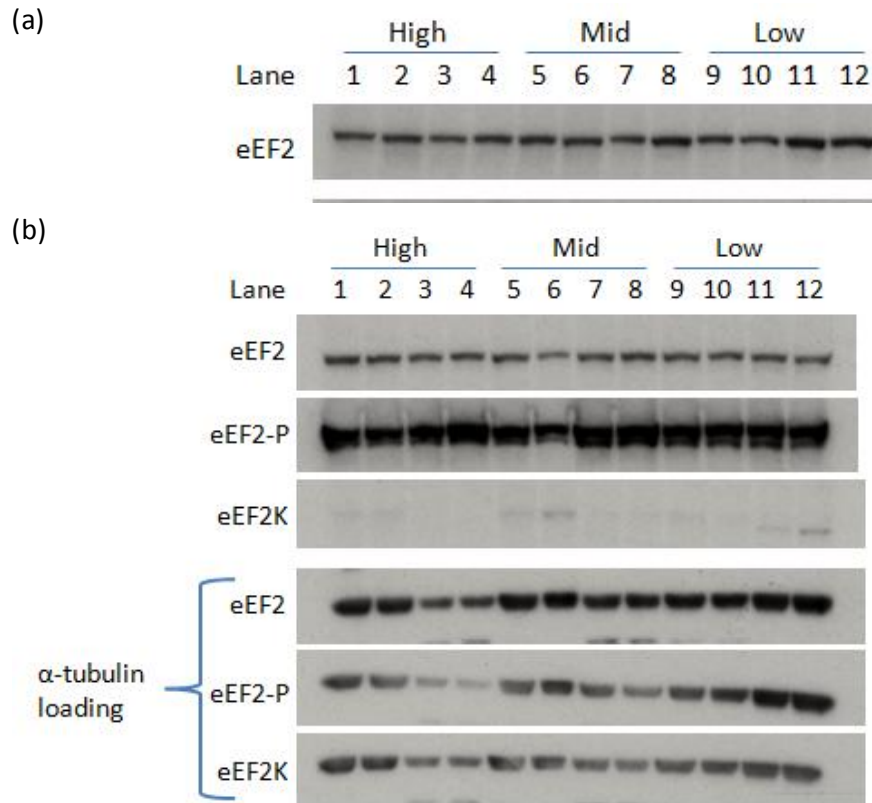
Figure 5.4 Western blot analysis of eEF2, phosphorylated eEF2 (eEF2-P) and eEF2K on days 6 (A) and 8 (B) during batch culture of a panel of recombinant CHO cell lines expressing different amounts of a model monoclonal antibody. Lane 1 and 2 = High level producing cell line 1, Lane 3 and 4= High level producing cell line 2, Lane 5 and 6= Mid level producing cell line 1, Lane 7 and 8= Mid level producing cell line 2, Lane 9 and 10= Low level producing cell line 1, Lane 11 and 12= Low level producing cell line 2.

Although an equal amount of protein was loaded for analysis from each sample as determined using a Bradford assay, the α-tubulin control blot suggested different protein loading and therefore densitometry analysis was undertaken on the images and the amount of each band normalised to the α-tubulin loading control to determine if this revealed any relationship between these band intensities and culture productivity. The densitometry analysis confirmed the results of the initial study looking at the CHOHP and CHOLP cell lines; that the levels of total and phosphorylated eEF2 did not relate to a recombinant cell lines capacity for mAb production (Figure 5.5). Between day 6 and day 8 levels of total eEF2 appeared to reduce slightly across most of the cell lines, with cell lines H2 and L2 being the exception with a larger decrease in eEF2 levels. Phosphorylation of eEF2 generally increased from day 6 to day 8, which was expected, as eEF2 phosphorylation

increases over time in cell culture due to nutrient deprivation and increases in culture stress. Levels of eEF2K were difficult to detect but remained relatively constant throughout culture (Figure 5.5). These data suggest that the amounts of eEF2 and eEF2K are tightly controlled by the cell but do not underpin the recombinant capability of a CHO cell for producing monoclonal antibody. This agrees with the data in the previous chapters whereby the over-expression of eEF2 or knockdown of eEF2K was difficult in stable cell lines, once again suggesting that if the regulation of these crucial elongation control points is lost the cell does not survive.
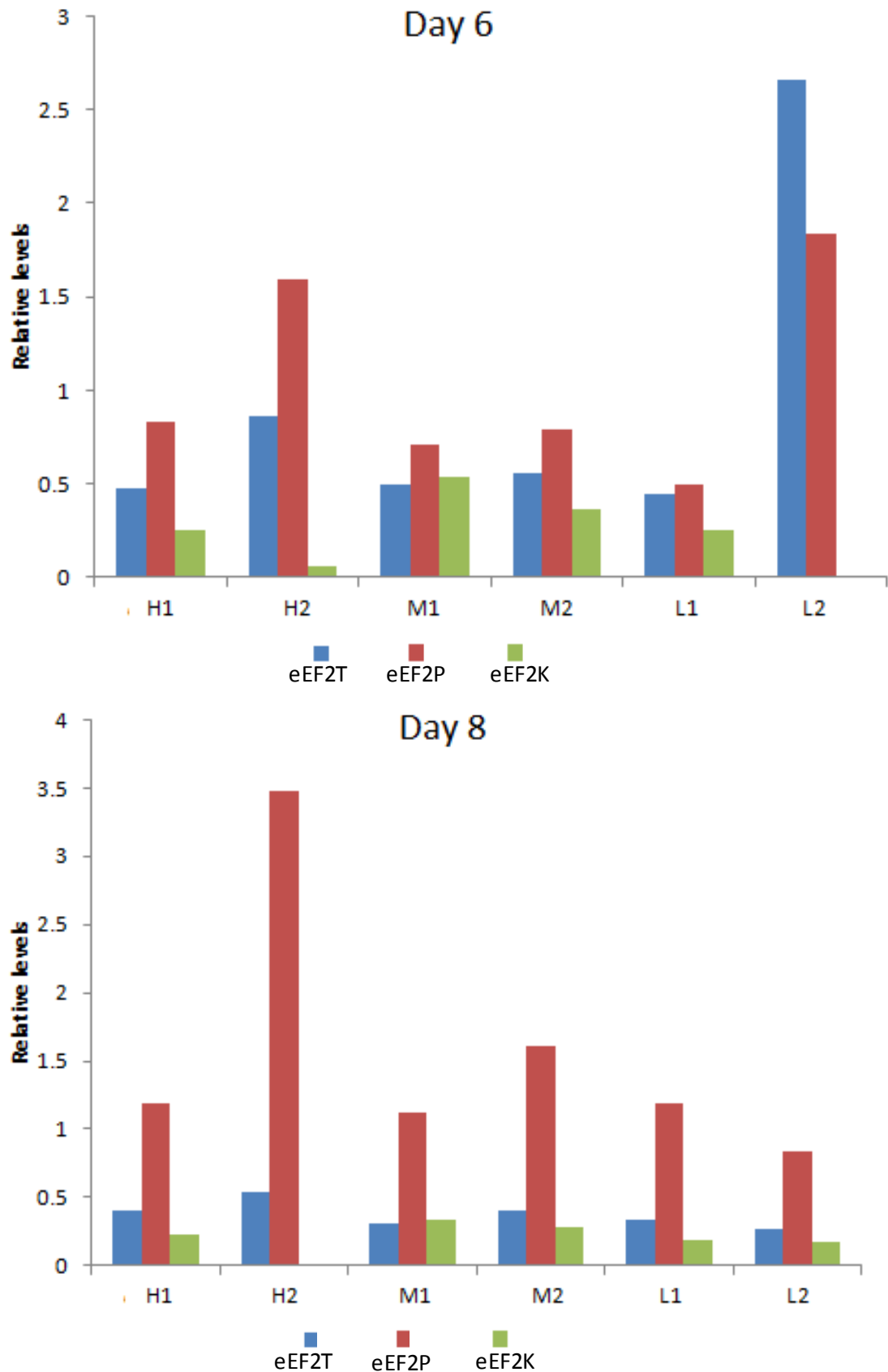
Figure 5.5 Densitometry analysis of the western blots images shown in Figure 5.4. Analysis was undertaken using the ImageJ freeware. The density of each band was determined and then normalised to the tubulin loading control to compare expression of each target on the different days of culture in the different CHO antibody producing cell lines. H1 and H2= high producing cell lines 1 and 2, M1 and M2= Mid producing cell lines 1 and 2, L1 and L2= Low producing cell lines 1 and 2.

5.2.2 Investigating changes in mTOR signalling throughout culture in a host CHO cell line using mRNA profiling

As described in the introduction to this thesis and this chapter, eEF2K and hence phosphorylation of eEF2 is controlled by mTOR signalling, particularly in response to nutrient depravation. Further, several reports have now suggested that mTOR signalling may play a role in the control of CHO cell growth and properties that underpin the ability to generate/produce recombinant protein from these cells. A wider study to investigate how mTOR signalling changes throughout batch culture was therefore undertaken using the commercially available mTOR Signaling RT[2] Profiler PCR Array from QIAGEN. The array used was a panel of primers designed to allow the profiling of the expression of 84 genes in the mTOR signalling pathway. This includes profiling of mTORC1 and mTORC2 complexes, positive and negative regulators of each of these complexes, mTOR downstream effectors, and genes involved in other cellular processes related to mTOR signalling. Details of all genes in the array are provided in Appendix 7.6. As there are many inputs/outputs into and out of the mTOR signalling pathway, it was envisaged that using this screening approach additional targets may be identified for the manipulation and control of mTOR signalling.

The monetary cost of these arrays allowed the ability to only undertake repeat experiments on two samples. The CHO Flp In parental cell line was chosen as the cell line to profile as it had been used extensively in Chapter 3 and 4. CHO cell lysates were therefore collected 68 h and 120 h post-seeding of a culture when the cells would be in exponential growth or stationary phase. The total RNA was then extracted from these cell lysates and used for the qRT-PCR array screening. The screens were run according to the manufacturer's instructions and then the data analysed following the manual instructions provided by the manufacturer and the Excel spreadsheet provided by Qiagen. Using these data and the house keeping gene normalises provided on the array plate, a fold-change in expression was calculated for each of the genes at the mRNA level between the 120 and 68 h time points. The resulting calculations from this (the Excel Spreadsheet) are shown in Appendix 7.6.2. The fold-change reported here (see Table 5.1) is calculated using the crossing point threshold normalised to the control expression and the equation:

Fold-Change = (2^(- Delta Delta Ct)) in the normalized gene expression (2^(- Delta Ct)) in the Test Sample divided the normalized gene expression (2^(- Delta Ct)) in the Control Sample.

The fold-regulation or change represents fold-change results in a biologically meaningful way; where the fold-change values greater than 1 indicate a positive or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than 1 indicate a negative or down-regulation. In addition to the fold-change, the software also comments on the reliability of the data. Where 'okay' is placed in the comments box, meaning that the data is considered reliable and robust; comments A-C (see Appendix 7.6.2) generally mean that a higher number of replicate results should be run to be confident in the data. For the purposes of this investigation only genes where the data confidence was okay and a fold-change greater than 2 was observed were considered. The genes that were found to meet these criteria are listed in Table 5.1 below.

The data from the comparison of the mTOR screen at 68 and 120 h of growth showed that all genes, that meet the criteria except for one (Hsp90ab1) were up-regulated at 120 h compared to 68 h. This suggests that at 120 h the cells were responding to changes in growth conditions but were not in a 'critical' state such that global gene expression was being down-regulated. None of the genes involved in autophagy or energy stress were differentially regulated to the extent of those above >2-fold; again suggesting that at the 120 h time point the cell was not sufficiently stressed compared to the 68 h time point to result in the up-regulation of genes involved in these responses/processes.

Interestingly, there were both positive (e.g. Akt1/2 and Rag A/C) and negative up-stream regulators (e.g. Pten and Lkbkb) of both mTORC1 and mTORC2 up-regulated at the 120 h time point relative to that observed at the 68 h time point. This suggests that at 120 h of culture, control of mTOR signalling by the cell was important. Further, the majority of these changes were up-regulation of positive regulators suggesting that the first responses to any stress later in culture is to try and maintain mTOR signalling (and thus protein synthesis) in order for the cell to continue growing and synthesising protein. The maintenance of mTOR signalling allows the downstream effectors to be maintained as well. This balance is obviously a crucial point for cell survival and the ability to continue producing recombinant protein and suggests that the engineering of upstream regulators of mTOR may allow the extended performance of cells. This of course would need to be further investigated by manipulating the amounts of those targets identified here to see if this did indeed give the cell an advantage in terms of survival and recombinant protein synthesis.

Table 5.1 mTOR related genes shown to be differentially regulated up- or down by >2-fold at the mRNA level between 120 and 68 h of batch culture of the CHO host FRT cell line using the commercially available mTOR Signaling RT^2 Profiler PCR Array from QIAGEN. A –ve fold-change means a decrease in mRNA expression at 120 h compared to 68 h, a +ve fold-change means and increase in mRNA expression between the 120h and 68 h time point.

Gene	Fold Change	Function Grouping				
		mTOR Complexes	mTOR Upstream Regulators	mTOR Downstream Effectors	Cellular Processes	Others
Akt1	2.82		mTORC1/C2 +ve regulator			
Akt2	2.86		mTORC1/C2 +ve regulator			
Cab39l	3.62		mTORC1/C2 -ve regulator			
Gsk3b	2.78			mTORC2 +ve regulator		
Hras1	2.98		mTORC1 +ve regulator			
Lkbkb	2.84		mTORC1/C2 -ve regulator			
mTOR**	2.92	mTORC1 mTORC2				
Pdpk1	3.13		mTORC1/C2 +ve regulator		Insulin signalling	
Ppp2ca	2.90			mTORC1 -ve regulator	Translation	
Prkca	2.18			mTORC2 +ve regulator	Cytoskeleton organisation	
Pten	2.24		mTORC1 -ve regulator			
Rhoa	2.18			mTORC2 +ve regulator	Cytoskeleton organisation	
Rps6ka5	2.58		mTORC1/C2 +ve regulator		Growth factor response Translation	
Rps6kb1	2.78			mTORC1/C2 +ve regulator	Translation	
Rptor	3.15	mTORC1				
Rraga	2.28		mTORC +ve regulator		Amino acid response	
RRagc	3.11		mTORC +ve regulator		Amino acid response	
Sgk1	3.57			mTORC2 +ve regulator		
Vegfa	2.37			mTORC1 +ve regulator	Angiogenesis	
Vegfb	2.67			mTORC1 +ve regulator	Angiogenesis	
Actb	2.11				Cytoskeleton	
Hsp90ab1	-4.39					Heat shock protein

**mTOR did not meet the requirement of 'okay' on the comment as described in the text, but is included for comparisons sake.

5.2.3 The Effects of adding Phosphatidic acid (PA) or Phorbol Myristate acetate (PMA) to CHO Cells, eEF2 Phosphorylation and Recombinant Protein Production

Another direct way to try and influence mTOR signalling is to add molecules to the cell culture supernatant that can act upon the mTOR pathway. A number of such molecules have now been identified, although many of these can also in/activate other pathways as well and therefore it can be difficult to determine if any effects observed are related to mTOR signalling or due to other, off-target, effects. Despite this, the addition of such molecules to cell cultures and observation of the ensuring response(s) can be useful, particularly if investigating the effect on specific targets such as eEF2 phosphorylation and recombinant protein synthesis.

A number of molecules are known to activate the mTOR pathway and enhance cell growth or protein production (Xu et al. 2004; Hagland et al. 2013; Hayashi & Proud 2007; Avila-Flores et al. 2005; Roux et al. 2004; Dreesen & Fussenegger 2011b). A study into the literature around the mTOR pathways and the mTOR RNA panel revealed that there were several small molecules that can directly/indirectly activate mTOR. Two of these molecules were selected for further experimental study, based upon their availability and activity within the mTOR pathway. These molecules were phosphatidic acid (PA) and phorbol myristate acetate (PMA). Figure 5.6 below shows where and how these molecules elicit a response on the mTOR pathway.

PA directly interacts with/activates mTOR within the cytosol of the cell; therefore addition of PA could provide artificial activation of mTOR. PA can directly activate mTORC1 after being produced from a number of sources, such as phosphatidylcholine. On-the-other-hand, PMA is known to promote cell growth through the mTOR and MAP kinase pathways, via activation of protein kinase C (Roux et al. 2004). Both of these were therefore investigated in CHO cell cultures.
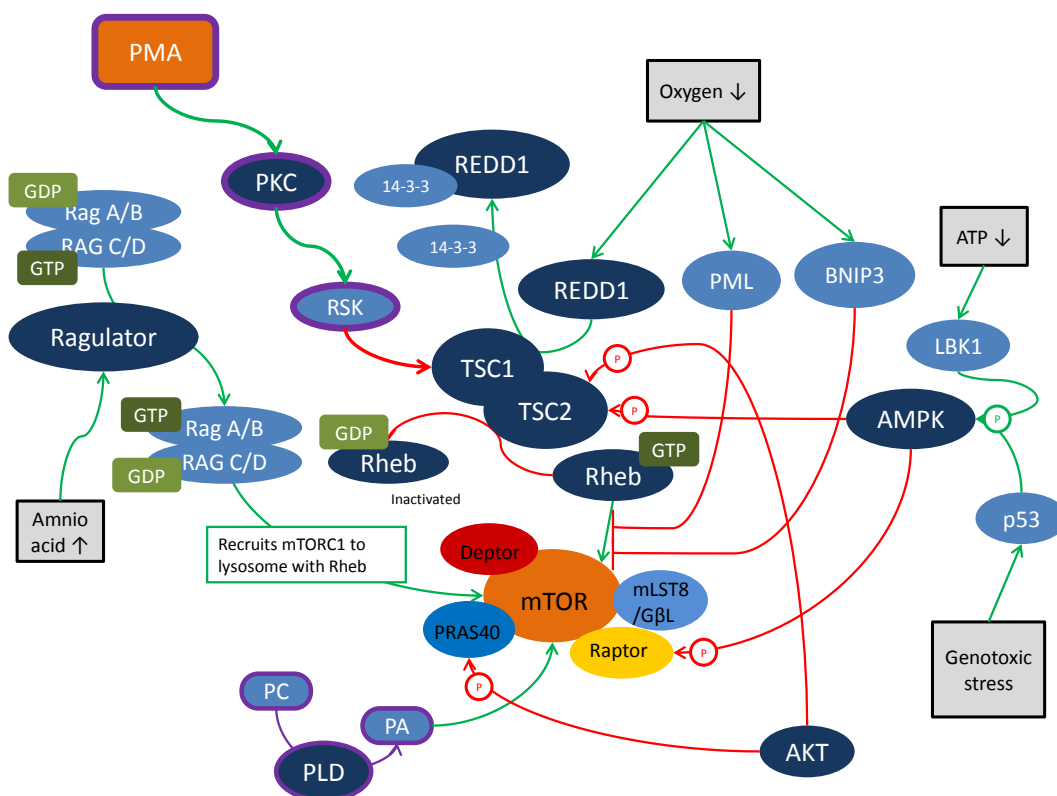
Figure 5.6 Schematic of mTOR signalling showing where and how Phosphatidic acid (PA) or Phorbol Myristate acetate (PMA) act and potentially elicit a cellular response. Highlighted with purple outlines.

5.2.3.1 The Effects of Adding Phosphatidic acid (PA) to CHO Cells, eEF2 Phosphorylation and Recombinant Protein Production

The initial investigations were to add PA to a recombinant CHO cell lines expressing a model monoclonal antibody. For this, the cell line CHOHP described and used in section 5.2.1 was utilised. The PA molecule used in these experiments was derived from egg yolk lectin, it is therefore safe to handle and easy to acquire. As PA activates mTOR within the cystol, it is necessary for it to be taken up through the plasma membrane. The PA was added on day 4 of a culture of CHOHP cells and then samples taken 24 and 48 h post-addition for analysis of mTOR, eEF2, eEF2-phospho and eEF2K amounts in the cell lysates. A total of 100µM of PA (in a buffer of 150mM NaCl, 10mM Tris-HCL pH 8) was added to each culture and controls without PA for comparison. This initial data suggested that addition of 100µM of PA (Toschi

et al. 2009; Lim et al. 2003) to the CHO suspension cells on did not result in any major changes to the levels of mTOR, eEF2K and eEF2 observed (Figure 5.7). This is most likely due to PA needing to cross the cellular membrane, either PA was not able to cross the membrane due to the hydrophobicity of the PA molecule or the concentration was not high enough to cause an effect.
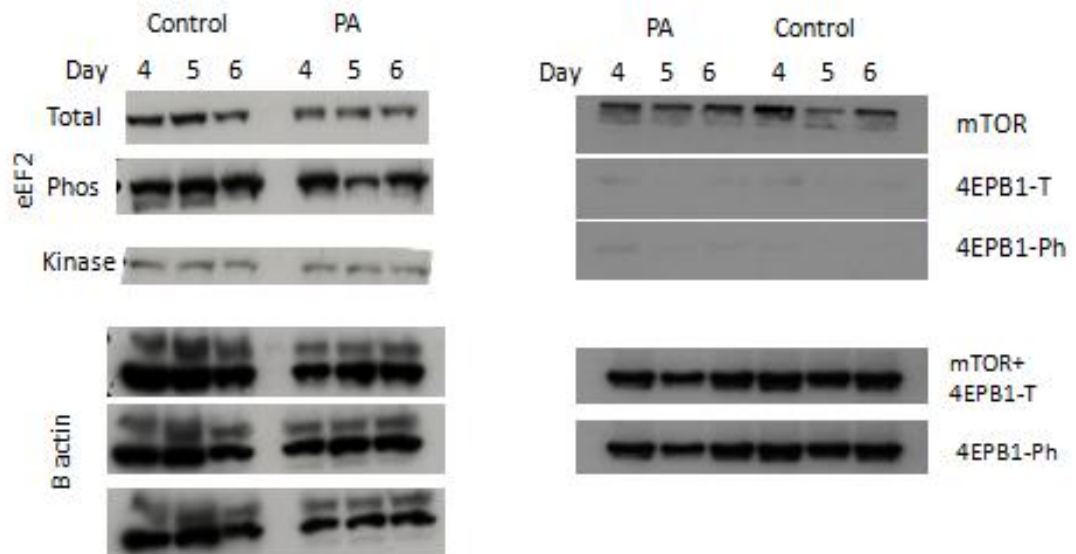
Figure 5.7 Western blot analysis of eEF2, phosphorylated eEF2, eEF2K, mTOR, 4EBP-1 and phosphorylated 4EBP-1 in the CHO high producer samples taken at 0, 24, and 48 h after addition of 100μM PA on day 4 of culture. β-actin is shown as a loading control.

As PA is most likely difficult to deliver through the cellular membrane, this is likely to pose a significant barrier for the treatment to be effective. Addition of a precursor of PA could be more effective in mTOR activation or genetic manipulation of Phospholipase (PLD)/ diacylglycerol kinase (DAGK)/ Lysophosphatidic acid acyltransferase-beta (LPAAT). Unfortunately, due to time restrictions and availability of DAG these experiments were not possible to complete or further investigation of PA on recombinant protein synthesis.

5.2.3.2 The Effects of Adding Phorbol Myristate Acetate (PMA) to CHO Cells and Subsequent Recombinant Protein Production

In addition to investigating the effect of PA on CHO cells, PMA (phorbol myristate acetate) was also investigated. PMA is a class 2 drug, known for its tumorigenic properties (Roux et al. 2004). It is known to promote cell growth through the mTOR and MAP kinase pathways, via activation of protein kinase C (see Figure 5.6). As PMA is capable of passing through the cellular membrane it was likely to provide a more efficient method of experimentation on these signalling pathways and any effect on recombinant protein expression. PMA indirectly activates mTOR via activation of PKC (protein kinase C), which actives RS6K (Ribosomal S6 Kinase), which inhibits the Tsc1/Tsc2 (tuberous sclerosis) complex and therefore allows for Rheb (Ras homolog enriched in brain) to become active and activate mTOR (see Figure 5.6).

Previous research into PMA activity has shown that a 100 nM concentration is enough to elicit an effect in cells (Roux et al. 2004; Aeder et al. 2004). Therefore based upon this, CHOK1D6 cells stably expressing firefly luciferase were treated with 0.5-5 µM PMA and 24 hours post PMA treatment the luciferase expression was determined. As the PMA is delivered in DMSO, cells where DMSO alone had been added were used as a control for each concentration of the drug investigated. The resulting luciferase expression for each sample is shown in Figure 5.8. This data showed that as the amount of DMSO added was increased, an increase in firefly luciferase expression was also observed, suggesting that the addition of the DMSO resulted in an increase in recombinant protein expression independent of the PMA. However, the PMA also appeared to further enhance the increase in recombinant firefly luciferase expression observed (Figure 5.8). In order to determine the effect of the PMA alone, the luciferase expression in the PMA samples was expressed as a percentage of the control DMSO samples and is shown in Figure 5.8. This showed that even at a concentration of 0.5 µM there was an approximately 150% increase in firefly luciferase expression. The 'greatest' response from the PMA relative to the DMSO control was observed at a concentration of 2 µM PMA (approximately 325% of the DMSO control) and at 5 µM this was reduced to approximately 200% of the control. The actual highest amount of firefly luciferase was observed in the 5 µM sample but the amount of DMSO here elevated the amount more than that observed in all other samples (Figure 5.8).
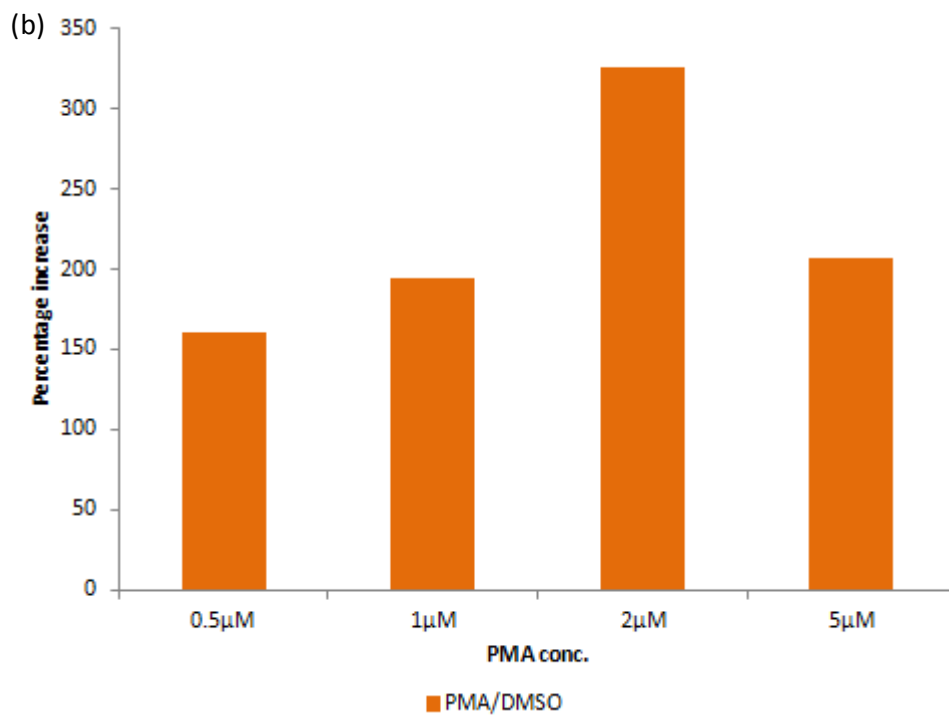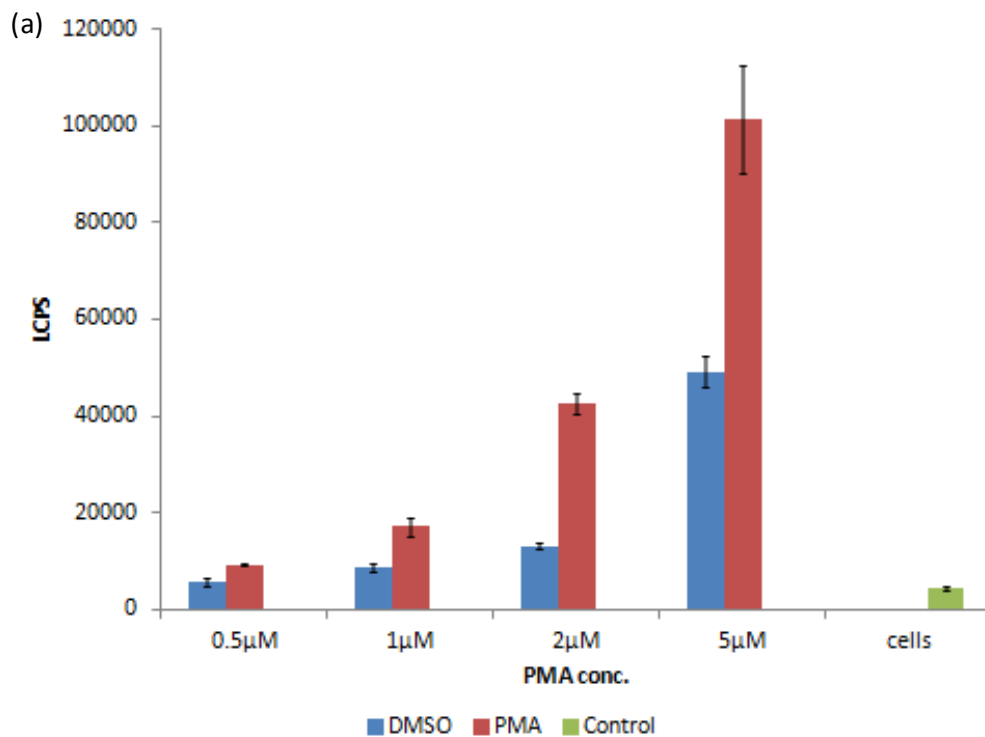
Figure 5.8 (a) Expression of firefly luciferase measured in light counts per a second (LCPS) from CHOK1D6 cells 24 hour post addition of 0.5-5 µM PMA or the equivalent volume of DMSO, n=4, p<0.05 when comparing the DMSO values against the PMA values at 2 µM and 5 µM PMA **(b)** The percentage increase of LCPS between the CHOK1D6 cells incubated with 0.5-5µM PMA and DMSO.

The addition of DMSO and PMA therefore appeared to have an effect on recombinant firefly luciferase expression, with 2 µM PMA giving the greatest response due to addition of PMA. A second experiment was therefore undertaken whereby 2 µM PMA in DMSO was added to the CHO recombinant monoclonal antibody producing cell lines CHOHP and CHOLP described in section 5.2.1 and the effect on recombinant antibody production, eEF2 phosphorylation and mTOR determined. The 2 µM PMA was added on day 6 of culture and samples taken 24 and 48 h post addition for both western blot analysis and analysis of secreted antibody in the supernatant. The western blot analysis from these samples is shown in figure 5.9.
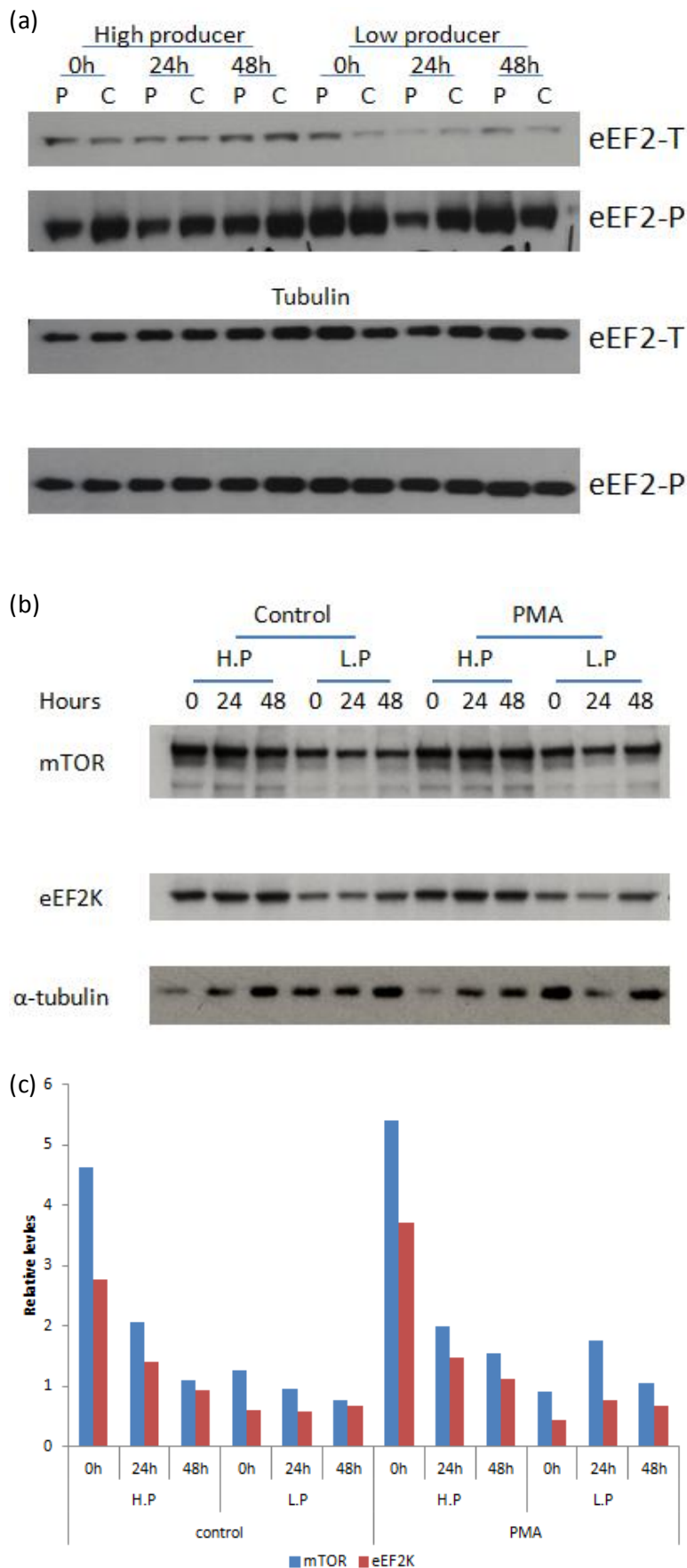
Figure 5.9 Western blot analysis of **(a)** total eEF2 and phosphorylated eEF2 in the high producing CHO cell and low producing CHO cell samples taken at 0, 24, and 48 h after addition of PMA on day 4 of culture. P= Cells treated with 2 µMPMA, C= Cells treated with DMSO, eEF2-T= total eEF2, eEF2-P= Phosphorylated eEF2 **(b)** Western blot analysis of mTOR and eEF2K in the same samples. H.P= High producer, L.P= Low producer. **(c)** Densitometry analysis of the banding pattern of mTOR and eEF2K in the high and low producer cell lines, normalising the band intensities to the α-tubulin band.

The western blot data of the CHO recombinant cell lines expressing a monoclonal antibody showed that PMA did reduce the amount of phosphorylated eEF2 observed in samples compared to the control samples (eEF2-P levels) but not the total levels of eEF2 (Figure 5.9). This shows that PMA can influence eEF2 phosphorylation by addition to the supernatant of cells, but that the reduction in eEF2 phosphorylation was much less than that observed upon eEF2K knockdown reported in Chapter 4. Western blot data for the eEF2K and mTOR samples were normalised to the levels of α-tubulin, the loading control, via densitometry analysis due to the fluctuation of tubulin between samples. The analysis showed that eEF2K and mTOR levels decrease in the high producer cell line over 48 hours. The low producer cell lines show a 'spike' of mTOR and eEF2K levels 24 hours post PMA treatment with eEF2K levels slightly elevated and the mTOR levels increased by approximately 50%. This increase of mTOR activity coincides with the decrease of eEF2 phosphorylation, therefore it suggested that the addition of PMA has influenced mTOR activity, which in turn reduces eEF2K activity, in spite of the slight increase of eEF2K levels which result in a decrease of eEF2 phosphorylation.

In order to determine whether this change in eEF2 phosphorylation and mTOR levels across the 48 h time course when PMA was added influenced antibody amounts in the supernatant from the recombinant CHO cells, the amount of antibody in the supernatant over the 48 hour time course was analysed by ELISA. The resulting data is shown in Figure 5.10 where the amounts are relative to that observed at the time when the PMA was added (0 hours, 0 h). In the lower producing CHO cell line there was an almost 4.5-fold increase in the amount of antibody in the supernatant 48 h post-addition of the PMA compared to the 0 h time point that was not reflected in the DMSO control (Figure 5.10). Such an increase was not observed in the higher producing cell line. These data suggest that in the low producing cell line the PMA is able to elicit a response that overcomes a potential limiting block(s) in this cell line that is not present in the high producing cell line. There was little difference in the total eEF2 and phosphorylated eEF2 amounts between these cell lines when initially investigated, suggesting that although phosphorylation is reduced this is not the key response that results in the observed increase in antibody production from the low producing cell line. It is interesting that this response is only observed in the low producing cell line. Thus, whatever the mechanism by which PMA results in increased antibody production from the low producing cell line might not be limiting in the high producing cell lines. Further investigations into identifying this might provide biomarkers for the

identification of high and low producing cell lines. Unfortunately there was not time to undertake such studies during this study.
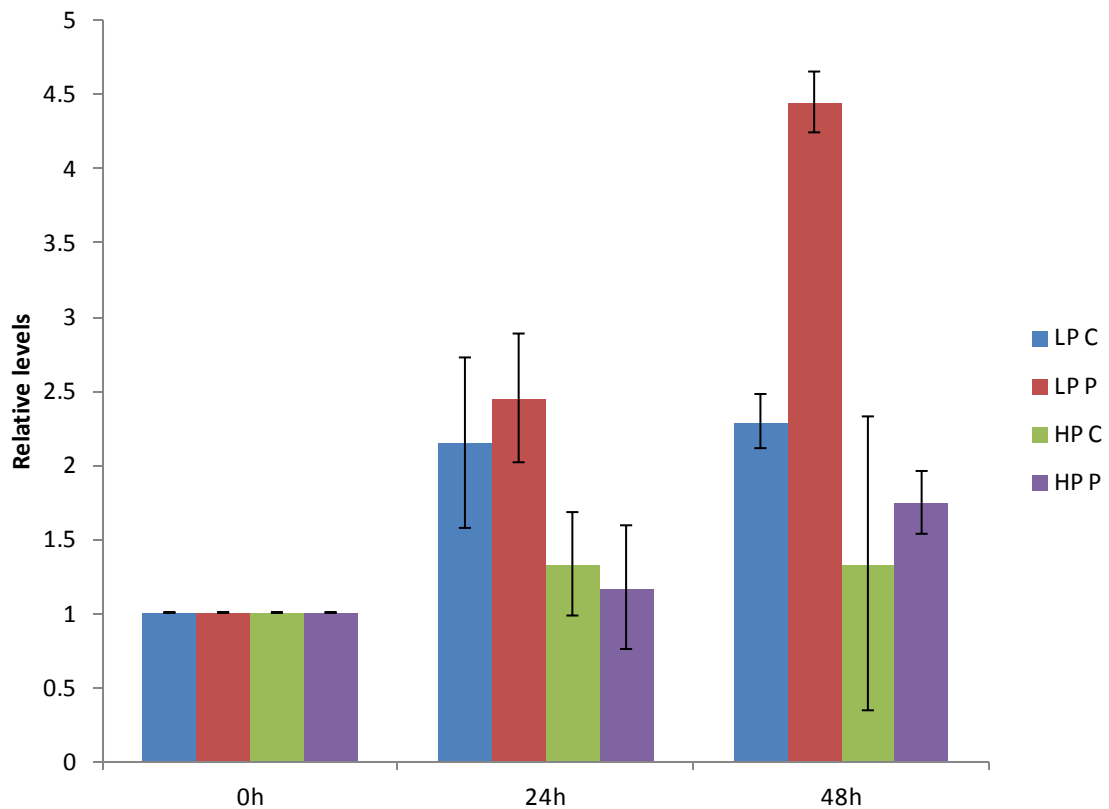
Figure 5.10 Antibody concentrations in the supernatant of CHOHP and CHOLP cells 24 and 48 h after the addition of PMA to the cultures on day 6 of culture. Concentrations are shown relative to those on day 6 when the PMA was added. HP= High producer, LP= Low producer P = PMA addition, C = control DMSO addition. n=3, student t-test shows that at 48 hours in the LP difference between the P and C samples are $p<0.05$. Base levels (ng/ul/cell) at 0h were LP C: 43103.51, LP P: 52607.84, HP C: 68173.91, HP P: 76047.06

In addition to the analysis of eEF2 phosphorylation, mTOR and antibody production over the 48 h period post-PMA addition to the CHOHP and CHOLP cell lines, the viable cell concentration and viable cell number was followed. The resulting growth data is shown in Figure 5.11 below. Overall the PMA did not have any major effect upon the CHOHP cell viability or number and therefore the lack of an increase in antibody concentration was not due to cell death. The CHOLP cells did show a small decrease in viable cell numbers on days 7 and 8 relative to that when the PMA was added on day 6. This might suggest these cells were more 'stressed' by the addition of the PMA, possibly resulting in induction of stress responses that might account for the increase in antibody concentration observed. Once again, further studies would need to be undertaken to investigate this hypothesis.
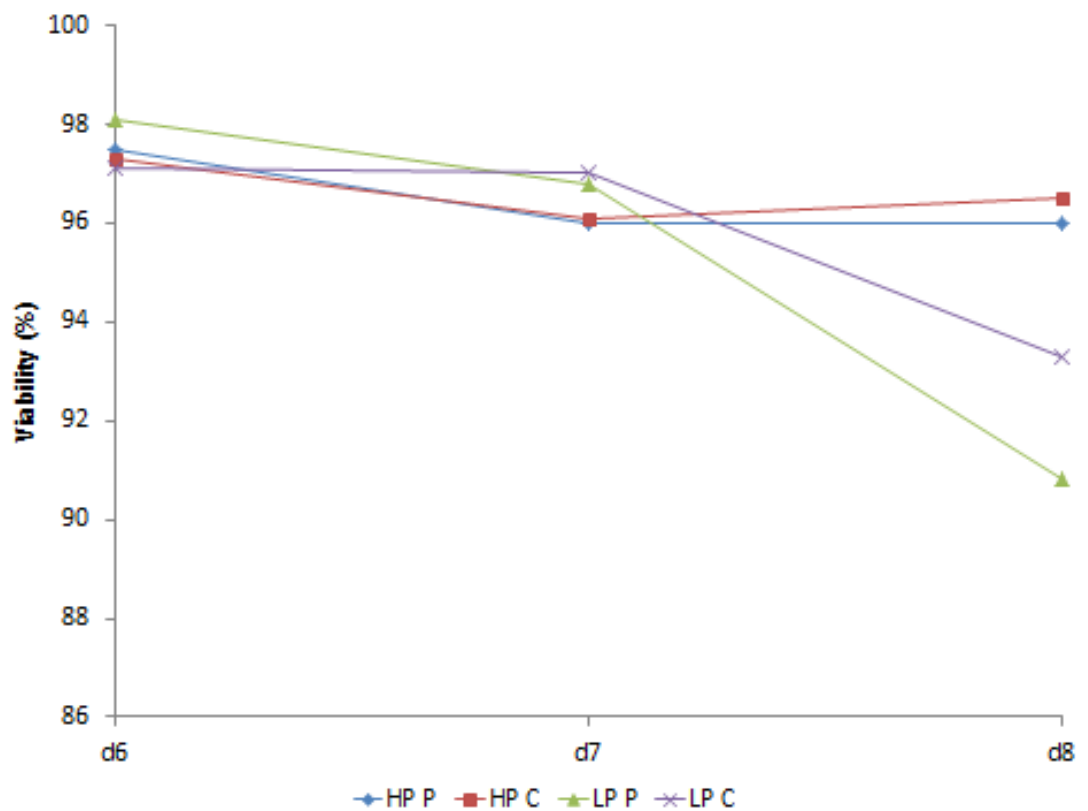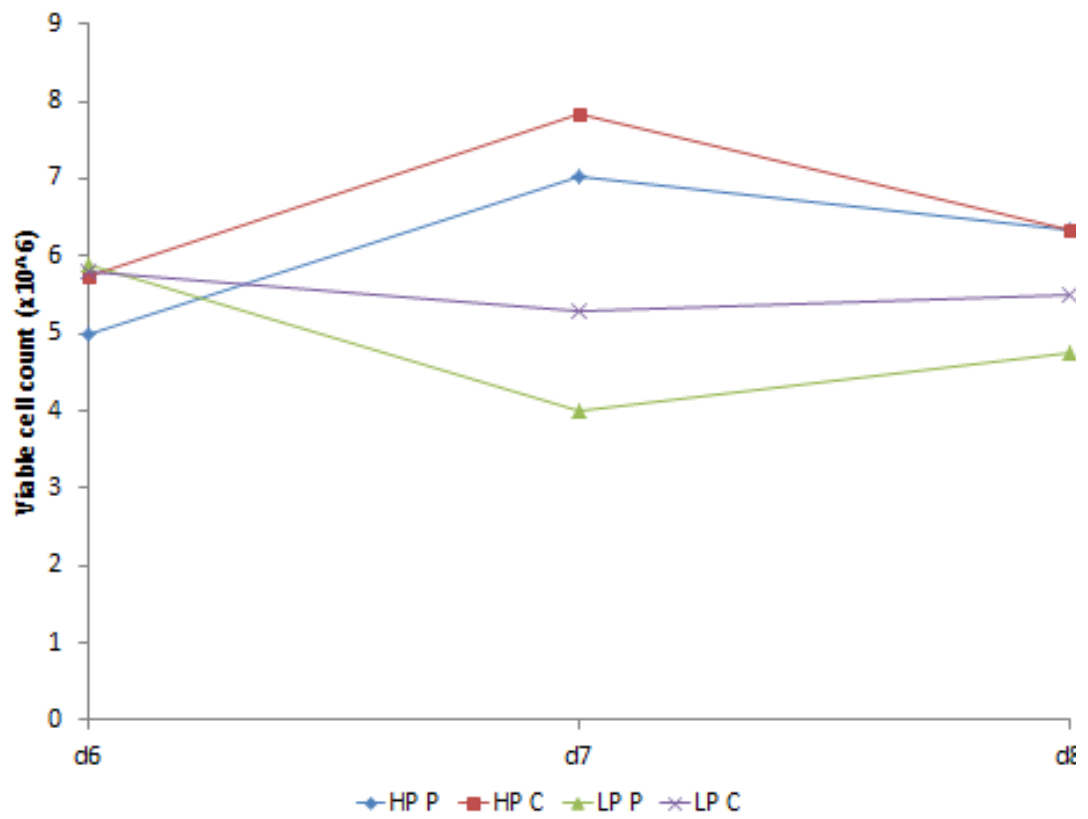
Figure 5.11 Total viable cell numbers (viable cells/ml) and culture viabilities in the CHOHP (HP) and CHOLP (LP) cultures at the time of, 24 hours and 48 hours post addition of 2 μM PMA to cultures. P= addition of 2 μM PMA, C= Control cell, incubated with the equivalent volume of DMSO.

5.2.3.3 The Effects of Adding Phorbol Myristate Acetate (PMA) to CHO Cell Growth

As PMA was shown to be effective at increasing CHOK1D6 cell firefly luciferase recombinant protein production and a lower producing antibody producing cell line's productivity, further investigation into how PMA addition might influence the growth and proliferation of CHO cells across a longer time period was undertaken. Further, the addition of PMA and the mTOR inhibitor rapamycin were added separately and together to CHO cells. Using the Xcellegence system (Roche) described in earlier chapters and the host CHO Flp In cell line. The cells were cultured in the presence of either 2 μM PMA, 10 nM rapamycin or both. The cells were cultured at 37°C for 136 hours and electrical impedance measured every 30 minutes to follow cell growth. The resulting growth profiles are shown in Figure 5.12 below.

The addition of 10 nM rapamycin resulted in the CHO cells entering into decline phase after 100 hours of culture whilst the cells grown in the presence of 2 μM PMA maintained lag phase for another ~24 hours. These cells also grew to a much higher cell index suggesting higher cell numbers in the presence of PMA. Cells that were grown in the presence of both rapamycin and PMA showed slower growth initially but reached the higher cell index (or electrical impedance) observed in the PMA alone samples and were maintained in the stationary phase of growth. Surprisingly, cells grown in the presence of a double dose of a singular molecule showed different profiles. A 4 μM dose of PMA appeared to result in almost complete cell death and 20 nM rapamycin resulted in an unexpected increase in the initial cellular proliferation and the cell index reached the same level as the cells grown in the presence of PMA but entered into decline phase of growth around 100 hours post-seeding (Figure 5.12).

One of the objectives of the simultaneous treatment of the CHO cells using rapamycin, a known mTOR inhibitor, and PMA was to try and determine if the PMA effect was via mTOR or another pathway. The result from the 20 nM rapamycin grown cells was not as expected and suggests this initiates some other response that results in enhanced cell proliferation. The PMA alone did result in a higher cell index being obtained, suggesting increased cell proliferation or a longer growth period before nutrient depletion was sensed, possibly through an mTOR mechanism. However, from these data alone it is not possible to determine if the PMA effect on cell growth is mediated via an mTOR or mTOR independent mechanism.
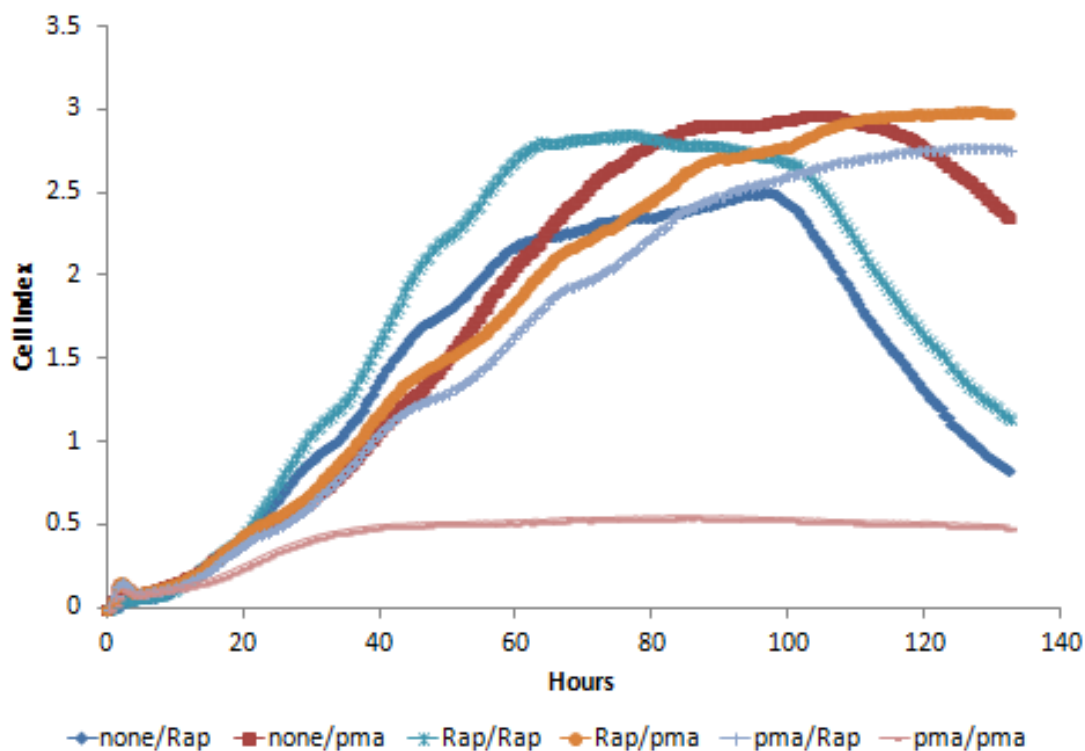
Figure 5.12 Growth profiles of the CHO Flp-in host cell line in the presence or absence of PMA, rapamycin or both determined using the commercially available Xcellegence culturing system (Roche). n=2

5.2.4 Investigating mTOR Signalling in CHO Cells Stably Expressing eEF2 Mutants in Leucine Depleted Media

As described earlier in this thesis, it is well established that mTOR signalling is sensitive to intracellular nutrient concentrations and levels of amino acids; one of the responses to nutrient depletion is mTOR regulated attenuation of protein synthesis. It has been demonstrated that mTOR is particularly sensitive to leucine concentrations or availability (Gran & Cameron-Smith 2011; Proud 2002). In order to investigate mTOR signalling further, and how this might influence recombinant protein synthesis, the CHO Flp-In cells over expressing eEF2 mutants (WT: wild type; ALA: Thr56Ala mutation; GLU: Thr56Glu mutation) detailed in Chapter 3 were cultured in leucine depleted media over a short period of time (24 h) and targets of mTOR signalling investigated. The Ala mutant should always be in the 'on' position as it cannot be phosphorylated and therefore inactivated, whilst the Glu mutant is a phosphorylation mimic and hence should be inactive.

In order to investigate mTOR and recombinant protein synthesis in the CHO eEF2 over-expressing cell lines, two experiments were undertaken. In the first, the cell lines were grown in either leucine efficient (leucine present in the media) or leucine depleted media for 24 h and then cell pellets collected and lysates prepared for the analysis of total and phosphorylated eEF2, eEF2K and mTOR. In a second experiment, the CHO Flp-In eEF2 mutants were transiently transfected with a firefly luciferase plasmid, 6 hours post transfection the media was changed and the cells were then incubated in leucine depleted media for 24 hours before the firefly luciferase expression from each cell line was determined and compared.

Surprisingly, there was no significant difference in the levels of total and phosphorylated eEF2 and eEF2K between the cell lines in the presence or absence of leucine (Figure 5.13). It would have been expected that depleted leucine would result in increased eEF2 phosphorylation after 24 h but this was not observed. However, the cells incubated in the leucine depleted media showed a decline in mTOR expression compared to those cultured in the regular media (Figure 5.13). There was once again no observable difference between the control and eEF2 over-expressing cell lines suggesting that this did not influence the response to leucine depravation.

When firefly luciferase was transiently expressed in the different cell lines in the leucine depleted media, there was actually a reduction in the firefly luciferase expression in the Ala56 and Glu56 eEF2 mutant cell lines compared to the control and WT over-expressing cell lines (Figure 5.14). This is in direct contrast to the results reported in chapter 3 where the Ala stably expressing cell line was able to produce more luciferase in the presence of leucine. In the eEF2 over-expressing WT cell line there appeared to be a similar amount of luciferase expression as in the FRT control cell lines. The reasons for this are not clear as the blot data in the absence of leucine and firefly shown in Figure 5.13 show similar amounts of eEF2 and phosphorylated eEF2. The removal of leucine must therefore result in a greater impact upon firefly luciferase expression in the Ala56 and Glu56 eEF2 cell lines than that in the other two cell lines, presumably due to the presence of the exogenous eEF2. It is possible that in the CHO Ala56 eEF2 mutant stable cell line, as the eEF2 Thr56Ala mutant is considered to be constantly active, the cell takes other measures to slow protein production such as the prevention of translation initiation via eIF2α, eiF3 and 4EBP-1 (initiation factors show to have a link to mTOR).
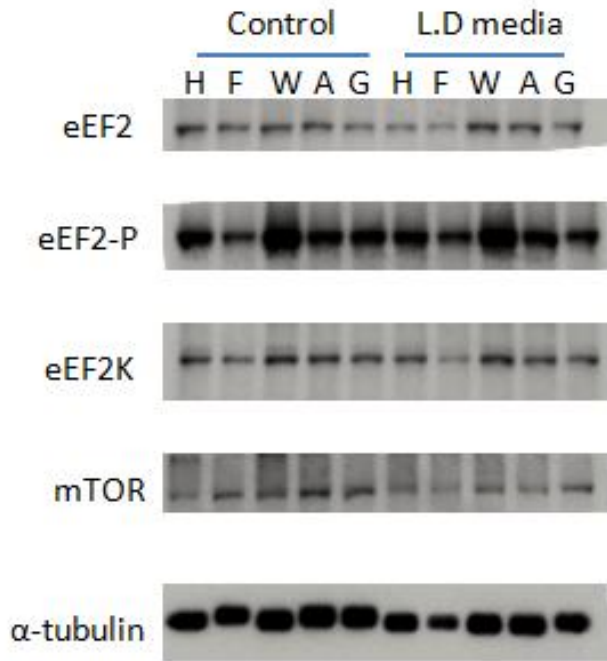
Figure 5.13 Western blot analysis of cell lysates from the stably expressing eEF2WT, eEF2Thr56Ala and eEF2 Thr56Glu mutants after growing for 24 h in the presence or absence of the amino acid leucine. The blots shows total and phosphorylated eEF2, eEF2K and mTOR levels in the different cell lines in + or − leucine media. H= CHO Flp-In parental cells, F= CHO Flp-In cells stably trasfected with an empty FRT vector, W= CHO Flp-In cells stably expressing wild type eEF2, A= CHO Flp-In cells stably expressing Thr56Ala eEF2 mutant, G= CHO Flp-In cells stably expressing Thr56Glu eEF2mutant.
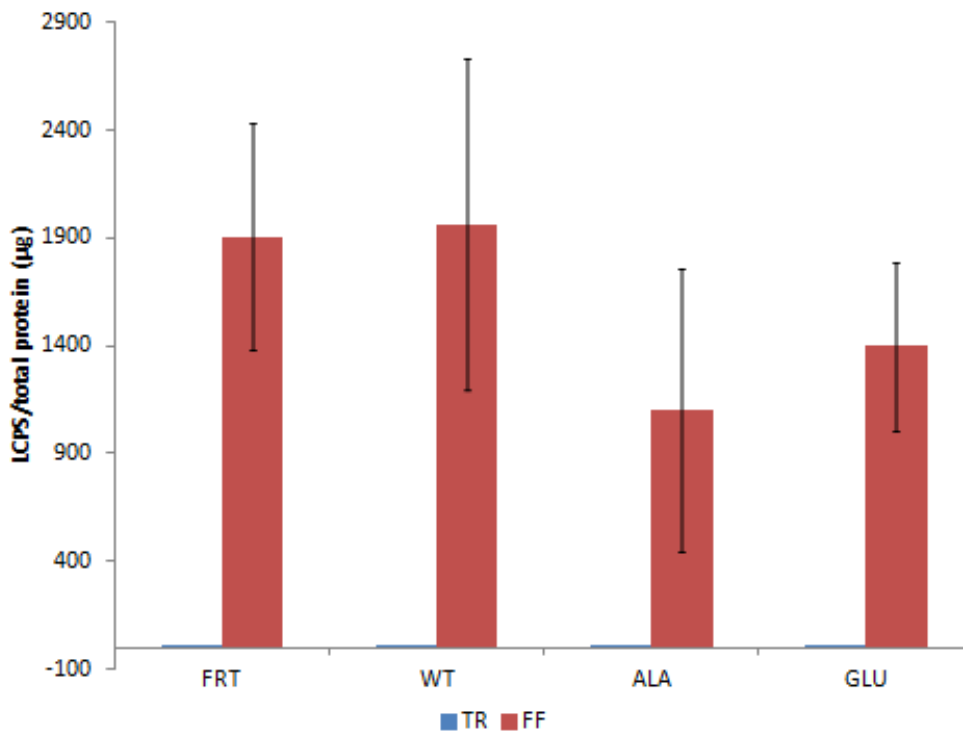
Figure 5.14 Transient firefly luciferase expression measured as light counts per a second (LCPS) normalised to total protein from CHO Flp-In cells in the stably expressing empty FRT vector (FRT), wild type eEF2 (WT), Thr56Ala eEF2 (ALA) and Thr56Glu eEF2 (GLU) mutants for 24h post transfection of the pGL3 firefly luciferase construct (FF) or incubation with the equivalent volumes of transfection reagent (TR), incubated in leucine depleted media. n=3, error bars show one standard deviation.

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5.2.5 Investigation of mTOR and Translation Initiation Factors in HEK Cells

It has been established in this thesis that manipulation of elongation via over-expression of eEF2 or knockdown of eEF2K can result in enhanced recombinant protein synthesis and enhanced growth rate in CHO cells. Other studies have shown that by manipulating mTOR signalling translation elongation is controlled and hence protein synthesis rates. However, translation itself is made up of initiation, elongation, termination and a ribosome recycling step. Therefore before elongation can occur, mRNA translation initiation has to take place first. Reports have shown that mTOR regulates translation initiation as well as elongation, largely through the phosphorylation of 4E-binding protein, 4E-BP1 (Fingar et al. 2002; Gibbons et al. 2009). However, a more recent study has suggested that the phosphorylation of eIF3i might occur in an mTOR dependent manner and this could also influence translation initiation (Roobol et al. 2014). In order to investigate if cells over-expressing eIF3i are mTOR sensitive, HEK cell lines stably over-expressing eIF3i or eIF3h were investigated and the amounts of eEF2, eEF2K and cellular growth investigated to determine if there was a link between the over-expression of these initiation factors and elongation control. The eIf3h cell line was included as this is not thought to be mTOR sensitive.

HEK (Human Embryonic Kidney) Flp-In cell lines (Invitrogen) each separately stably expressing either eIF3iV5, eIF3hV5 (both have a c-terminus V5 reporter tag) or eIF3h, were used to investigate the effect of stable initiation factor over expression. These cell lines were kindly provided by Dr Anne Roobol, University of Kent. This system is the same system used to create the stable eEF2 CHO mutants presented in chapter 3 except HEK cells were used as the mammalian host cell. For initial characterisation of these cell lines (FRT HEK control, eIF3i-V5 tagged, eIF3h-V5 tagged, eIF3h (no tag)) they were grown and after 24 and 48 h of growth in static adherent culture, cell lysates were generated for western blotting. The lysates were analysed via western bottling for total and phosphorylated eEF2, eEF2K and the presence of the V5 tag (Figure 5.15). Subsequently, densitometry analysis was undertaken on blots and the results are shown in Figure 5.16.

The V5 probe blot confirmed that the HEK cells were over-expressing the eIF3iV5 and eIF3hV5 tagged initiation factors (Figure 5.15). From this we assume that the eFI3h HEK cell line is also stably over-expressing this subunit although without an antibody for eIF3h it was not possible to confirm this. The western blot and densitometry data showed that 24 hours after seeding the cells, the levels of eEF2 across the cell lines did not appear to differ to a

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great extent. However, the level of eEF2 phosphorylation and eEF2K did appear to be elevated compared to the FRT control. On the other hand, after 48 hours the levels of eEF2 were dropped slightly in the eIF3 over-expressing cell lines relative to the control, and whilst the phosphorylation of eEF2 in the eIF3iV5 and eIF3h cell lines was similar, the eIF3hV5 cell line appeared to have a slightly elevated level of eEF2 phosphorylation. This could suggest that the presence of the V5 tag on the eIF3h subunit could be causing an artificial rise in eEF2 phosphorylation. Interestingly, the levels of eEF2K were also drastically decreased at the 48 h time point compared to that of the control cell line in each of the eIF3 expressing cell lines in contrast to that observed at the 24 h time point (see Figure 5.16). This may account for the decrease in eEF2 phosphorylation observed. The eEF2K levels may be reduced in response to the cells requirements: the HEK cells will have been entering into the log phase of growth, during this time eEF2 is very active and therefore deactivation by phosphorylation is not required. However, the question remains why this would be more pronounced in the eIF3 expressing cell lines than the control cell line. One explanation would be if there was enhanced protein synthesis and growth in the eIF3 over-expressing cell lines compared to the control cell line. This was therefore further investigated.
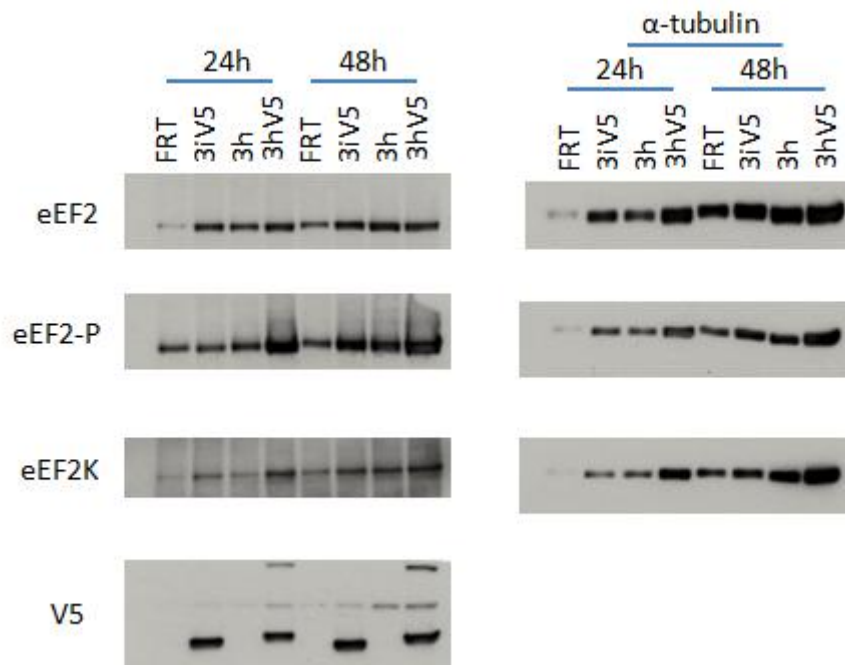
Figure 5.15 Western blot analysis of cell lysates from HEK Flp In cells stably expressing eIF3iV5, eIF3hV5, eIF3h and FRT control cell lines 24 and 48 h after seeding of cells. Cell lysates were probed for the presence of a V5 tag, eEF2, phosphorylated eEF2 and eEF2K amounts.

(a)

(b) Relative to FRT
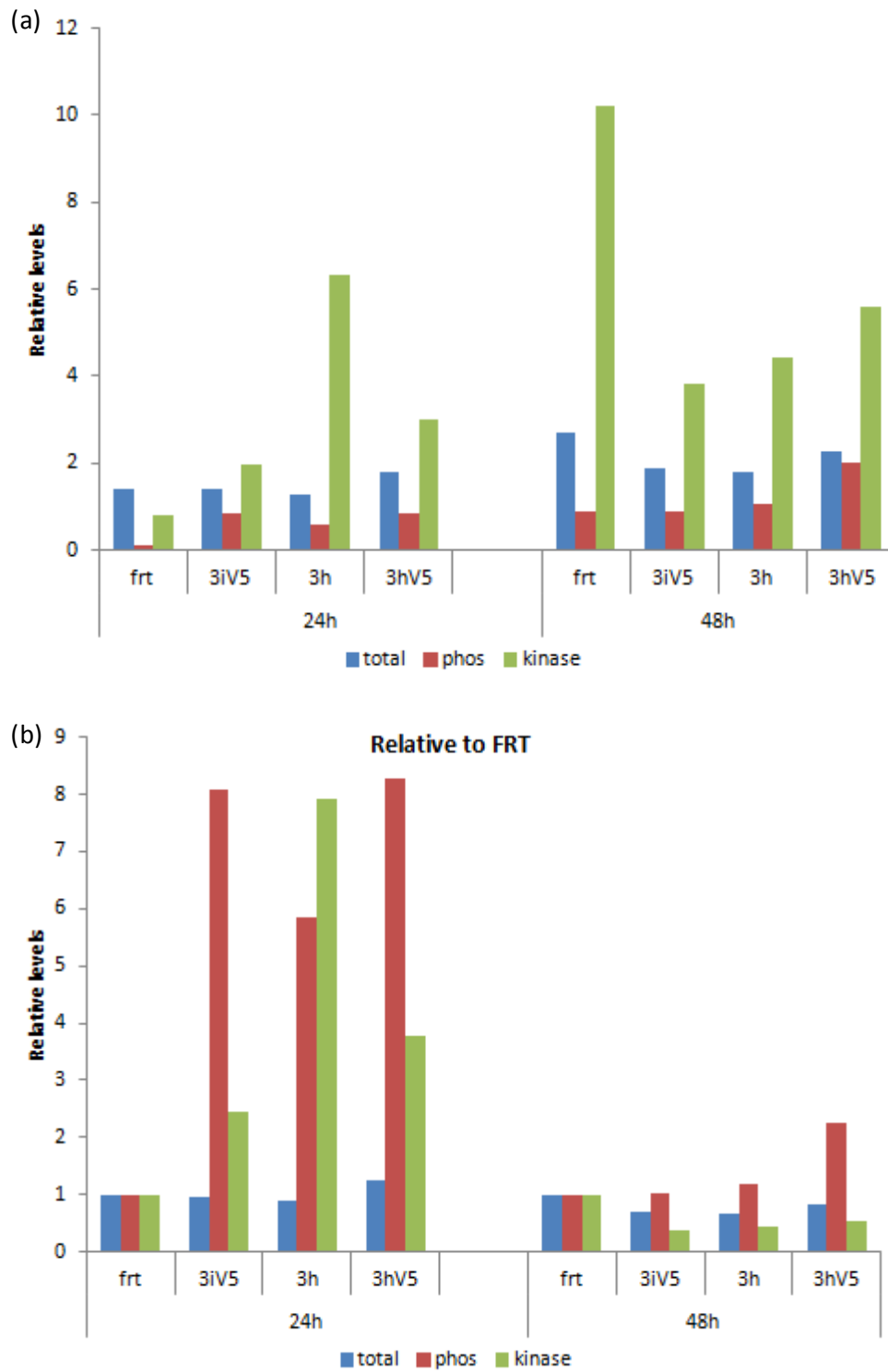
Figure 5.16 (a) Densitometry analysis of the western blots present in figure 5.15. Levels of total eEF2 (total), phosphorylated eEF2 (Phos) and eEF2K (kinase) were normalise to α-tubulin. Samples were collected from HEK Flp-In cells stably expressing eIF3iV5, eIF3hV5, eIF3h and a FRT control cell lines, 24 and 48 h post seeding. **(b)** Levels of total eEF2, phosphorylated eEF2 and eEF2K relative to the FRT control cell line.

To investigate whether the over-expression of the eIF3 subunits offered a growth advantage over the control cell line and if there was a possible link that mTOR signalling was in any way related to the effects that the eIF3 over expression had upon cellular growth of the HEK Flp-In cells, HEK cells stably expressing the eIF3 mutants as well as HEK cells expressing an empty FRT vector were cultured in 10 nM rapamycin or the equivalent volume of DMSO over 136 hours.

The growth curves were generated using the Xcelligence system (Roche) previously described and the resulting data is shown in Figure 5.17. These data show that the HEK cells are more sensitive to the presence of DMSO and rapamycin than the CHO cells previously investigated. The eIF3iV5 and the eIF3h cells cultured in DMSO showed a greater sensitivity to DMSO than the FRT and eIF3hV5 cell lines. In the FRT and eIF3hV5 cell lines the lag phase of growth was dramatically extended. When the cell lines were grown in the presence of rapamycin all of the cells showed little growth and long extended lag times. After 120 h of culture there was only growth from the eIF3hV5 and FRT host cell line. These data suggest that the over-expression of eIF3hV5 confers some sort of protective effect against rapamycin in the HEK cells but over expression of eIF3h and eIF3iV5 does not. Why this is not observed in the untagged eIF3h cell line is not clear although over-expression of eIF3h in this cell line could not be confirmed. Due to the sensitivity of the HEK cells to DMSO and rapamycin it was not possible to determine from these data if any mTOR related effects are observed upon over-expression of the eIF3 subunits and further work would be required.
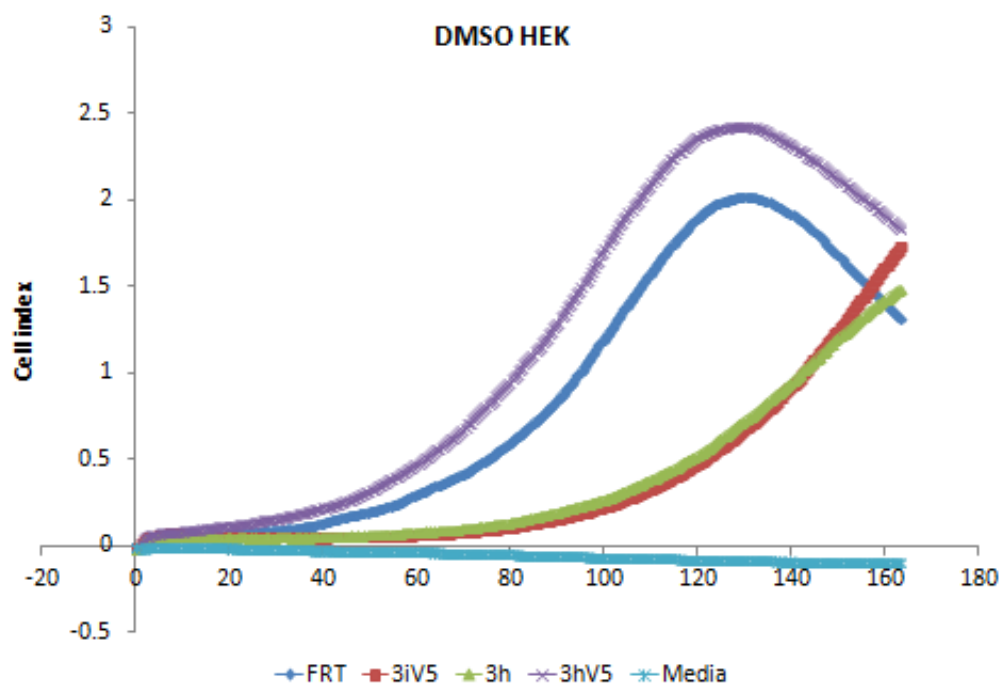
Figure 5.17 Growth profiles of the HEK293 FRT control host cell line, and HEK FRT cell lines stably over-expressing eIF3iV5, eIF3hV5 and eIF3h in the presence of DMSO over seven days.
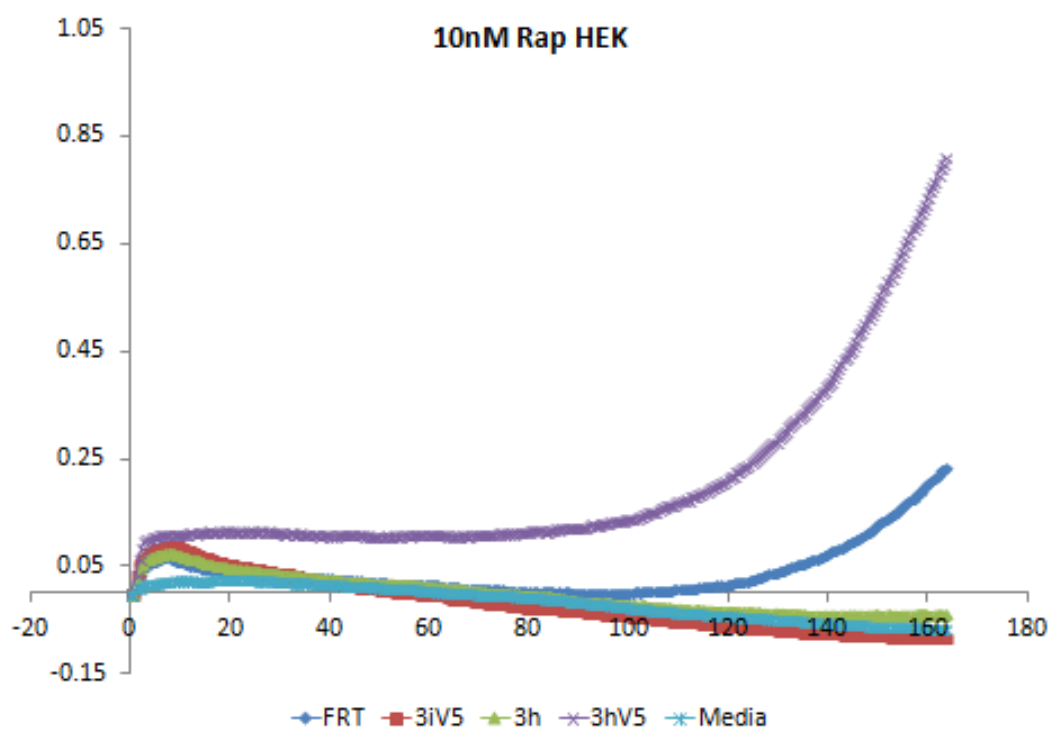
Figure 5.18 Growth profiles of the HEK293 FRT control host cell line, and HEK FRT cell lines stably over-expressing eIF3iV5, eIF3hV5 and eIF3h in the presence of 10 nM rapamycin over seven days.

5.3 Conclusions and Summary Statements from this Chapter

The findings of this chapter are discussed in detail in Chapter 6 but the main findings of this chapter are summarised here. The study of the CHO cell lines with varying recombinant monoclonal antibody protein production capacities revealed that levels of total and phosphorylated eEF2 do not appear to change in correlation with the mAb titre. This data suggest that eEF2 activity is tightly regulated across cell lines and is not directly related to recombinant protein secretion in these industrially used CHO cell lines

When qRT-PCR analysis was used to investigated changes in mTOR signalling over time during culture of a model host CHO cell line, the majority of the changes detected in the mTOR qRT-PCR panel were up-regulation of +ve mTOR regulators. These data suggest that the first response to stress later in culture is to try and maintain mTOR signalling (and thus protein synthesis) in order for the cell to continue growing and synthesising protein. The maintenance of mTOR signalling allows the downstream effectors to be maintained as well. This balance is obviously a crucial point for cell survival and the ability to continue producing recombinant protein. However, the addition of PA to the media during culture, to enhance mTOR activity, did not appear to have an effect upon CHO cells. This is probably either due to the hydrophobicity of the PA molecule or the concentration was not enough to elicit a response from the cells/ mTOR pathways. On-the-other-hand, addition of 0.5-5µM PMA to adherent CHO cells increased the production of recombinant firefly luciferase and 2 µM PMA appeared to elicit the largest response. Western blot analysis revealed that addition of PMA, at least in part, signals to eEF2K via mTOR. It is very likely that PMA signals to other pathways.

The addition of 2 µM of PMA also increased mAb production in a low producer cell line but had no effect upon the mAb production from a high producing cell line. This data suggests that a limiting factor(s) in the terms of mAb production in the lower producing cell line was over come upon PMA addition, but why and what these limitations could be is unknown and would require further investigation. Addition of PMA to the CHO producer cells does not appear to effect the growth of the cells, with only a few percent between the viability of the cells treated with PMA and the control cells. Finally with regards to PMA, CHO Flp-In parental cells cultured with 2 µM PMA and 10nM rapamycin, showed that PMA recovers

the cells from the effects of rapamycin, strongly suggesting that PMA is, in part, influencing mTOR signalling.

Cell lines stably expressing the eEF2 mutants constructed in chapter 3 (eEF2 wild type, eEF2 Thr56Ala and eEF2 Thr56Glu) transiently transfected with firefly luciferase and subsequently cultured in leucine depleted media exhibited behaviour the opposite of what was observed in Chapter 3. When the stable eEF2 cell lines were transiently expressing firefly luciferase the Ala56 eEF2 mutant cell line expressed the highest levels of luciferase. Conversely, when cultured in the leucine deplete media, the Ala56 eEF2 mutant cell line expressed the least amount of firefly luciferase. The data could be explained by the fact that the Ala56 eEF2 mutants is continuously activity and when this cell line is put under nutrient stress and the cells are still expending resources the cell takes other actions to dampen/suppress protein expression; such as the slowing of translation initiation. Further investigation into this behaviour would be required to determine this if it was the case.

Experimentation with HEK cells over expressing eIF3 subunits i and h with a V5 tag, and h without a V5 tag were also undertaken. This was to determine if any links between over expression of these subunits and mTOR regulated initiation and elongation were present. 48 hours post seeding the cells over expressing the eI3iV5 and 3h subunits showed a small increase in eEF2 phosphorylation, but the cells over expressing the eI3hV5 subunit showed a larger increase in eEF2 phosphorylation. The reason why the presence of the V5 tag results in an increase in eEF2 phosphorylation is unknown. Over expression of the eIF3 subunits appears to sensitise the HEK cells to the effects of rapamycin and DMSO, although the V5 tagged eIF3h subunit exhibits improved growth over its non-tagged counterpart, again suggesting that the V5 tag has some protective or enhancing effect upon the cells mechanisms.

Chapter 6

General Discussion

The data presented in the preceding chapters of this thesis describe a number of investigations into the role of polypeptide elongation and its control in Chinese hamster ovary cells and how this may impact upon recombinant protein production. Here an overall discussion of the results arising from these studies is provided.

6.1 Polypeptide Elongation during mRNA Translation and Elongation Factor 2 (eEF2)

Polypeptide elongation during protein synthesis on ribosomes is a key control point of gene expression and mRNA translation as described in the introduction chapter. Indeed, mRNA translation can limit recombinant protein synthesis from mammalian cells (Gustafsson et al. 2004; Wang & Proud 2006; Schwanhäusser et al. 2011; Modrak-Wojcik et al. 2013) and ribosome biogenesis can be manipulated to improve recombinant protein expression (Santoro et al. 2009), demonstrating the importance of translational control in recombinant protein expression from mammalian expression systems. The elongation of polypeptides is regulated via elongation factor 2 (eEF2) and elongation factor 1A (eEF1A). Phosphorylation of eEF2 at Thr^{56} inhibits its activity by inhibiting its binding to ribosomes. This phosphorylation is catalysed by eEF2K (Kaul et al. 2011; Browne & Proud 2002). Over expression of eEF2 and its subsequent mutation in mammalian cells has been shown to result in an increase in cellular growth and protein synthesis capacity (Nakamura et al. 2009), therefore it was hypothesised that the manipulation of eEF2 in CHO cells could improve the cells' capacity for recombinant protein production as well as extend the length of culture, both of which are of biological and industrial relevance.

6.1.1 Transient Expression of Human and Hamster eEF2 in CHO Cells

The initial construction of expression vectors for the expression of human and Chinese hamster eEF2 was undertaken to allow the investigation into the effects of eEF2 in human or Chinese hamster ovary cell (CHO) expression systems, as both are used in the industrial setting. Further, although the sequences are highly homologous (see Chapter 3), it is possible that the human eEF2 may 'behave' differently in the CHO cell than the endogenous CHO sequence. For example, stability and turnover, or the ability for the human eEF2 to play its role in elongation may be compromised or enhanced in the CHO system relative to the endogenous Chinese hamster eEF2.

Section 3.2.1.2 reports, that transient expression of the human eEF2 was possible in CHO cells when tagged at the C-terminal with a V5 reporter tag. Further, the expression of the human eEF2 form did not appear to affect the growth of the CHO cells or the regulation of the endogenous eEF2. As there did not appear to be any difference between the CHO and human exogenous eEF2 at the amino acid level; this suggests that the human eEF2 is either inactive or is not detrimental to the CHO cells homeostasis. As a result of these early experiments with human and hamster eEF2 sequences, it was decided that the majority of the work to be undertaken would focus upon investigations using the CHO cell expression system and the hamster eEF2 sequence, as CHO cells are considered the current gold standard mammalian expression system in the industrial setting (Kotsopoulou et al. 2010; Becerra et al. 2012).

Mutants of eEF2 in which Thr^{56} had been converted to a non-phosphorylatable residue (Ala) were therefore generated and transiently express in the model systems to determine the influence on recombinant protein synthesis. Complementary to this, and as described further below, eEF2K was knocked down by shRNAi to determine the effect on general and recombinant protein synthesis. Transient over-expression of wild type Chinese hamster and the Thr56Ala eEF2 mutant in CHOK1 cells affected the short-term (24-48 hour) amount of phosphorylated eEF2 present but had no appreciable effect upon intra-cellular recombinant protein production or cell growth in culture over a 96 hour period. Immunofluorescence imaging of CHOK1 cells transfected with the eEF2 hamster WT construct revealed that the translation efficiency/exogenous expression of eEF2 in the CHOK1 cells was approximately

20-30%. Therefore, the majority of the cells within the culture were not expressing the exogenous eEF2 constructs and as such interpreting the results from the transient expression studies is difficult. Transient expression also subjects the cells to the stress of the transfection protocol itself that can result in short-term cell responses and each cell can be expressing different amounts of the exogenous gene due to being transfected with differing copies of the plasmid. As such, these approaches are not idea for determining the effect of eEF2 manipulation on cell growth and recombinant protein productivity. Therefore, in order to ensure all of the cells within the culture were expressing the exogenous eEF2 constructs and at approximately the same amount for an extended period of time, CHO cell lines stably expressing the eEF2 constructs were generated.

6.1.2 Stable Over-expression of Wild Type, Thr56Ala and Thr56Glu eEF2 Mutants in CHO Cells

Using the commercially available CHO Flp-In system (Invitrogen), CHO Flp-In cell lines stably expressing either the wild-type or mutants of the CHO eEF2 constructs (eEF2 WT or Thr56Ala or Thr56Glu) as well as a control cell line using an empty FRT vector were constructed. Whilst the Ala mutant cannot be phosphorylated and hence should always be in the 'on' or active state, the Glu mutant is a mimic of phosphorylated Thr56 and should therefore always be in the 'off' state in terms of elongation activity. Further, stable expression of eEF2 and the eEF2 mutant variants in CHO cells removed the stress of transfection reagents upon the cells used for the transfection of the eEF2 constructs, and allows for transient transfection of another construct, such as the reporter firefly luciferase pGL3 construct. This also allowed for a more representative assessment of the effects of eEF2 over-expression and subsequent mutations on the CHO cell population. A limitation of this system however is that while it makes it possible to stably express the eEF2 constructs in all CHO cells, it is not possible to eliminate or suppress the expression of the endogenous or naturally expressed eEF2 within the CHO Flp-In cells. As the constructs at most had a 2 base pair mutation (from Thr to Ala or Glu), it was not possible to suppress the endogenous eEF2 via RNA silencing as it would also supress the exogenous eEF2 constructs that were under investigation.

To begin addressing this it could be possible to mutate the dipthamide H715 residue on the eEF2 constructs to render them immune to the effects of diphtheria toxin (Argüelles et al. 2014; Abdel-Fattah et al. 2013) thereby culturing the CHO Flp-In cells with diphtheria toxin would render the native eEF2 inactive and the 'flipped in' eEF2 would be the only active eEF2 within the cells. This was not put into action because it has been previously observed that the dipthamide H715 residue is linked to the functionality of eEF2 (Argüelles et al. 2014; Meeting et al. 2006; Kaul et al. 2011) and therefore by mutating it unknown effects and introduction of a second variable into the investigation would occur in addition to the mutation being directly investigated (e.g. Thr56 mutations). Further, using diphtheria toxin as a selective pressure would have an unknown effect upon the CHO Flp-In cells themselves. A further possible way to address this would be to silence the endogenous eEF2 by genome editing knockout technology in the stable cell lines to prevent endogenous eEF2 expression. This could be targeted to the chromosome location of the cell lines but is a long and time-consuming process. The investigation into the expression of exogenous eEF2 and the Ala/Glu56 mutants was therefore undertaken with the knowledge that as well as expression of the exogenous eEF2 constructs there would as expression of the endogenous/native eEF2 that the cell might modulate in response to the exogenous material.

6.1.2.1 Stable Over-Expression of Wild Type eEF2 and Thr56Ala and Thr56Glu eEF2 Mutants Changes the Intra-Cellular eEF2 Amounts in CHO Flp-In Cells

Western blot analysis and the subsequent densitometry analysis of these blots showed that stable over expression of the eEF2 constructs in the CHO Flip-In cells increased the amount of eEF2 present at the protein level (Figure 3.10). Conversely, the mRNA levels did not show any increase aside from the CHO Flp-In cell line stably expressing Thr56Glu eEF2 (Figure 3.16). Over-expression of the hamster wild type eEF2 construct resulted in a 2-fold increase in expression of eEF2 and a small decrease in phosphorylation of eEF2 at the protein level; but there was no change in the levels of total eEF2 mRNA expression. The over-expression of the exogenous eEF2 construct provides another transcript for the translation of eEF2, this transcript being transcribed from the CMV promoter in the expression plasmid whose control is not naturally linked to eEF2 expression in mammalian cells. This suggests that there is some form of regulation of eEF2 mRNA expression between translation of the eEF2 mRNA and its expression as a functional protein. It may be possible

that the cell can 'sense' the amount of available eEF2 protein and/or mRNA and hence reduce the promoter activity transcribing eEF2 to maintain the mRNA amounts at an acceptable amount to the cells. It is noted that no very high expressing eEF2 stable cell lines were obtained, suggesting these either do not survive or that the cell regulates eEF2 amounts. The exogenous amount could be controlled via increased mRNA or protein turnover although this was not investigated in this study.

Expression of the Thr56Ala eEF2 mutant resulted in a 5-fold increase in total eEF2 levels as determined from western analysis and densitometry, but as for the wild type construct, there was little change in the mRNA levels. The mutation of Thr56 to Ala56 on eEF2 results in a 'continuously activate' eEF2, therefore to maintain homeostasis of eEF2 amounts within the cell it would require reduction of the levels of eEF2/increased protein turnover or regulation of transcription or translation initiation of the endogenous gene within the cells to compensate for the inability to control the Ala56 eEF2 mutant. What was observed from the densitometry analysis was unexpected in that the levels of total eEF2 increase but the levels of eEF2 phosphorylation were similar to that of the FRT control (it might be expected that phosphorylation of endogenous material would be increased to inactive this and compensate for the 56Ala expression).

The relatively larger increase in the Ala56 eEF2 cell line eEF2 amounts can therefore, at least partly, be attributed to the continuous constitutive expression of the exogenous eEF2. Further, as the Ala56 eEF2 mutant is always 'active', this could result in more polypeptides being translated/elongated faster, including the Ala56 eEF2 itself. This may be particularly so if the exogenous eEF2 Ala56 transcript is highly abundant although the data suggests this is not the case. The increased elongation would only be possible in a nutrient rich environment as the elongation of polypeptides is an 'energy heavy' dependent process (See section 1.7.1.3). However, once stressed by nutrient depravation it is likely that the cells would need to drastically reduce their eEF2 activity or become starved of nutrients or die. This could be further investigated in future studies.

Overexpression of the Thr56Glu eEF2 mutant resulted in an increase in both eEF2 protein and mRNA expression and the increase of total eEF2 protein expression in the Glu56 cell line relates to the levels of eEF2 mRNA. This increase may be more tolerated by the cell as the Glu56 eEF2 mutant is an 'inactive' mutant and may be seen as little more than a harmless recombinant protein. However, the eEF2 mutant may also compete for interaction with the ribosome with wild type eEF2 and hence one might argue that an increase in endogenous eEF2 might occur to counter balance the inactive exogenous material. Alternatively, the levels of phosphorylation of wild-type eEF2 may be decreased in an effort to compensate for the expression of the inactive Glu56 eEF2. The data generated from this study was not able to confirm whether either of these scenario's occurred and once again this could be further investigated in the future.

6.1.2.2 Stable Over-Expression of Wild Type eEF2 or the Thr56Ala and Thr56Glu eEF2 Mutants Affects the Growth of the CHO Flp-In Cell Lines Expressing Them

The cellular growth in culture of the CHO Flp-In cell lines stably expressing the different exogenous eEF2 constructs were investigated using an Xcellegence system developed by Roche. The system is advantageous for the measurement of growth of adherent cell lines. The system uses 12 well plates and measures electrical impedance of a current passed across the bottom each well. The current was passed through the wells every 30 minutes; this allowed for a measurement for each cell line (5 in total) in triplicate without disturbing the cells during the culture period. The disadvantage of this system is that the passing of the current across the wells cannot account for the mass of the cells adhered to the surface; the system cannot distinguish single cells via the electrical impedance cause by the cells.

At 37°C the growth of all of the CHO Flp-In cell lines expressing any of the eEF2 constructs was increased as determined using this instrument and hence it is assumed the proliferation of the cells, in early stages of log phase growth in culture. The cell line expressing the Ala56 eEF2 mutant showed the largest increase in growth/proliferation. This is the 'active' mutant, however perhaps surprisingly, the Glu56 eEF2 cell line containing mutant grew more rapidly than the other cells lines except the Ala56 mutant. This growth pattern coincides with the levels of eEF2 in the different cell lines, with the Ala56 eEF2 cell

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line having the largest amount of eEF2, then the Glu56 eEF2 cell line, and lastly the wild type eEF2 cell line. These data suggest that increasing eEF2 amounts impacts upon cell growth/proliferation, presumably this can occur faster when eEF2 is up-regulated as protein can be synthesised faster. This could come with compromised protein quality, something that was investigated later in the project.

Interestingly, the Glu56 and wild type eEF2 cell lines each achieved a similar maximum cell number in culture, although the Glu56 cell line reached this a little earlier than the wild type cell line. The Ala56 cell line attained the highest maximum cell number and appeared to 'hold' this peak cell number for the longest time. Late in culture, it was the Glu56 eEF2 cell line that began to decline in viability/cell number first, the wild type cell line follows the same decline as the FRT and parental (HOST) cell line; but the Ala56 eEF2 cell line showed a more sustained cell index. These data collectively suggest that at 37°C in nutrient rich conditions over-expression of any of the eEF2 constructs confers a benefit to cellular proliferation. The Glu56 eEF2 mutation does not appear to have any negative effects until later in culture, were the cell culture appears to decline only a few hours before the wild type eEF2. Under mild cold-shock growth conditions (32°C) the Ala56 eEF2 mutation within the cell line appears to promote cellular proliferation and increase the maximum cell number attained whereas the over expression of the wild type eEF2 and Glu56 mutant eEF2 only increase CHO cells proliferation, but the maximum cell number attained remained the same as the FRT control cell line. Interestingly, mild cold shock has been reported to slow protein synthesis and mRNA translation by approximately 50% (Roobol et al. 2009). The fact that the over-expression of the Ala56 eEF2 mutant, that cannot be inactivated by phosphorylation, appears to increase the growth of CHO cells at 32°C, suggests that it is polypeptide elongation that is one of the major limiting steps or control points limiting protein synthesis at such temperatures. This may therefore be a possible route to increase cell biomass at 32°C for the enhanced production of recombinant proteins at such sub-physiological temperatures.

From the data presented in this thesis with the stably expressing CHO cell lines it could therefore be suggested that when the cells are put under stress from lack of nutrients or reduced temperature, polypeptide elongation is slowed by reducing polypeptide elongation rates to preserve nutrients and energy. However, this can be negated to some extent by expressing more of a more active eEF2 in the form of the Ala56 eEF2 mutant. Although it is not possible to discern the levels of the over expressed exogenous eEF2 constructs from the native eEF2 within the cells from these experiments, it is clear that the over expression of eEF2 affects the growth of CHO cells and can be used to enhance CHO cell biomass or the speed at which this is achieved.

6.1.2.3 Culturing of the Wild Type eEF2 or Thr56Ala and Thr56Glu eEF2 Mutants in CHO Flp-In Cells with 10 nM Rapamycin Changes the CHO Cells Response and Growth

Rapamycin inhibits mTOR activity by binding to FKBP12 and this complex binds to mTOR reducing its activity (see section 1.9). mTOR regulates eEF2 via S6K1 that phosphorylates elongation factor 2 kinase (eEF2K) (Kenney et al. 2014). Therefore, the CHO Flp-In cells stably expressing the eEF2 variants were cultured in the presence of rapamycin to investigate any link between mTOR and whether over expression of eEF2 or the Thr56 eEF2 mutant affects the growth of the cells. The CHO Flp-In cell lines were cultured over 7 days with 10 nM rapamycin using the Xcellegence system. The rampamycin used was dissolved in DMSO as a solvent, therefore the control cultures were cultured with the equivalent volume of DMSO. When cultured with DMSO/rapamycin the CHO Flp-In cells exhibit a different behaviour to that when they are cultured in nutrient rich conditions or under mild cold shock. DMSO exhibits an toxic effect upon the cells (Stevens et al. 2014) and the eEF2 stable cell lines appear to have a different reaction to that of cold shock. Rapamycin resulted in the proliferation rate of all of the cell lines to decrease in line with this inactivating mTOR, prolonging the log phase of growth by approximately 50 hours. Interestingly, all of the recombinant eEF2 cell lines attained the same maximum cell number as the DMSO control except the Glu56 eEF2 cell line which showed an increase in cell index from 3.5 to 5. One plausible explanation for this is that the rapamycin slows the proliferation of each of the cell lines, the Glu56 having some protection against this already with the inactive eEF2 present, giving it a competitive advantage.

6.1.2.4 Stable Over-Expression of Wild Type eEF2 or Thr56Ala and Thr56Glu eEF2 Mutants can Increase the Levels of Transient Recombinant Firefly Luciferase Expression

Firefly luciferase is an intra-cellular expressed recombinant protein and is often used as a reporter to give an estimate of a cells capacity for recombinant protein expression (Masterton et al. 2010). The advantage of this system is that the protein is not secreted as for most therapeutic recombinant proteins and therefore the direct result of manipulation of translation can be investigated. For secretory proteins limitations in secretion could mask any effects on translation when elongation is manipulated. Therefore, transient expression of firefly luciferase from the CHO Flp-In cell lines stably expressing the eEF2 variants was investigated 48 hours post-transfection of the firefly luciferase (Figure 3.20). The over-expression the Ala56 eEF2 mutant increased the CHO cells ability to generate recombinant protein production as determined from these luciferase assays. This suggests that the Ala56 eEF2 cell line overcomes a limitation in elongation that restricts firefly luciferase expression from CHO cells.

The Glu56 eEF2 cell line produced a similar total amount of luciferase compared to the FRT control and wild type eEF2 cell lines. The Glu56 cell line was shown to have around a 3-fold increase in eEF2 (see figure 3.15) and was the only cell line to show an increase in eEF2 mRNA levels. However, the Glu56 cell line produced a higher amount of luciferase than the control on a per-cell basis and more-or-less the same amount on a per cell basis as the Ala56 mutant. This suggests that the fact luciferase levels were not increased in terms of total amounts in the Glu56 cell line was down to a biomass issue, reduced compared to that in the Ala56 cell line over the same time period.

One potential issue mentioned above is that increased elongation and translation could result in more errors during translation. An experiment using mutant luciferase was therefore undertaken to determine levels of read through and amino acid mis-incorporation into the poly peptide during translation elongation (see figure 3.23). It was observed that the wild type eEF2 expressing cell line had a reduced level of read through and mis-incorporation during recombinant luciferase protein production compared to the control cells. It appears from these data that the wild type eEF2 engineered cell lines results in more accurate translation elongation. It has been observed that during an increase in

protein synthesis there usually is a loss of translation fidelity (Conn & Qian 2014). On-the-other-hand, the read though appeared to be increased in the Ala56 eEF2 mutant expressing cell line when the total amount of read through is considered. However, when the amount of read through is considered as a proportion of the total amount of luciferase made, the amount of read through is actually reduced. Thus, the data suggests that increasing eEF2 amounts can both increase the fidelity of recombinant protein produced and the yield.

6.1.2.5 The Addition of the V5 Tag to Stably Expressed Wild Type eEF2 at the C- and N-Terminus Increases the Expression of Recombinant Firefly Luciferase

The use of the V5 reporter tag was essential in this project as confirmation of the 'expression' of the exogenous eEF2 in the CHO Flp-In cells. This allows the distinction between the native eEF2 within the CHO cells and the exogenous eEF2 constructs although as different antibodies are used this cannot tell us about the relative amounts of endogenous and exogenous eEF2. Western blot analysis and immunofluorescent imaging confirmed expression of a C- and N- terminus V5 tagged wild type eEF2 in the CHO Flp-In cells. Due to difficulty in constructing/expressing the V5 tagged eEF2 (see section 3.2.2.1) only the wild type eEF2 cell lines were constructed with the C/N-terminal V5 tag. Interestingly, transient transfection of firefly luciferase into the C-terminus V5 (Cv5) eEF2 cell line and the N-terminus (Nv5) eEF2 cell line stable CHO Flp-In cell lines showed a 2- to 3-fold increase in luciferase when compared to the FRT control cell line (Figure 3.22). Previously the transient expression of luciferase in the wild type eEF2 stable cell line showed a similar luciferase expression to the FRT cell line (it was the Ala56 mutant where an increase was observed). Therefore, the presence of the V5 tag appeared to influence the amount of recombinant material produced. The reason why the addition of a V5 tag to a terminus of the eEF2 would influence recombinant luciferase expression is unclear. Possible explanations are that the V5 tag provides the eEF2 molecule with some sort of activity advantage in its interactions regarding polypeptide elongation. The V5 tag may change the conformation of eEF2s structure so that the domains of eEF2 are shifted to a more 'active' position. It is also possible that the V5 tag enhances the interaction between eEF2 and the ribosome complex. An alternative may be that the V5 tag prevents eEF2K interacting with eEF2 and therefore preventing its phosphorylation and subsequent deactivation, although there was little evidence of changes in eEF2 phosphorylation in these samples.

6.2 The Effect of Silencing Elongation Factor 2 Kinase (eEF2K) upon eEF2 Activity and Recombinant Protein Production

After investigation into the effects of eEF2 over-expression and mutation upon recombinant protein production from CHO cells, the regulator of eEF2, eEF2K was investigated. As eEF2K is the only known regulator of eEF2, manipulation of eEF2K to inactive this and prevent phosphorylation of the Thr56 residue and thus promote eEF2 activity was investigated as a means of modulating CHO cell growth and recombinant protein production. As described in section 1.8.4, eEF2K phosphorylates eEF2 at the Thr56 residue resulting in the inactivity of eEF2 and therefore the silencing of eEF2K via siRNA would prevent the phosphorylation of eEF2 and maintain eEF2 in an 'active' state. Further, the amount of phosphorylated eEF2 can therefore be used as a direct readout of eEF2K activity.

6.2.1 Transient Knockdown of eEF2K via Transfection of an shRNA (short hairpin RNA) Plasmid Construct

Construction of the eEF2K shRNA was achieved using the commercial kit Gene Clip U1 Hairpin system (Promega). The oligo nucleotides designed for the hairpin were based upon a commercial, verified sequence provided by Sigma. As both the plasmid vector and the hairpin sequences were commercial available and the sequencing of the construct was verified it provided confidence that the hairpin would be effective at eEF2K silencing.

6.2.1.1 Short-Term (48 hour) Transient Transfection of the eEF2K shRNA Reduces the Amounts and Activity of eEFK

The results in Section 4.2.2 show that transient transfection of the eEF2K shRNA effectively knocked down eEF2K, as shown by western blot analysis. There was also an associated drastic decrease in phosphorylation of eEF2 while total eEF2 levels remained constant over the 48 hour time period (Figure 4.2). The shRNA was therefore successful in silencing eEF2K expression at least over the short term and was therefore used to investigate the effect of this on recombinant protein production.

6.2.2 The Transient Knockdown of eEF2K Effects Recombinant Protein Production in CHO Cells

The effect of transient knockdown of eEF2K on recombinant protein production was investigated using CHO cell lines stably expressing the reporters firefly or gauusa luciferase. Using both firefly and gaussia luciferase would give data on how the knockdown of eEF2K effects intra cellular protein production (Firefly luciferase is a intra cellular protein) and how it may effect production of a protein that is secreted from the cell (gaussia luciferase in secreted from the cells), which mirrors the majority of recombinant proteins produced in the industrial setting, certainly those expressed in mammalian cells.

6.2.2.1 Transient Knockdown of eEF2K Increases Firefly Luciferase Expression and is more Effective than Transient Expression of eEF2 and Thr56 Mutant eEF2

The transient knockdown of eEF2K, in CHOK1D6 cells stably expressing firefly luciferase resulted in a 5-fold increase in firefly luciferase production. Comparison against the transient transfection of the wild-type eEF2 shows that the knockdown of eEF2K is more effective at increasing firefly protein production. When cells were co transfected with wild type eEF2 and the eEF2K shRNA, the knockdown of eEF2K appeared to enhance the production of firefly luciferase the most.   This could reflect the low transfection rate/expression of eEF2 described in chapter 3. The enhanced effect of eEF2K knockdown over eEF2 expression probably reflects the fact that the knockdown of eEF2K targets all of the eEF2K within the cell, removing its ability to phosphorylated eEF2, resulting in a larger population of 'active' eEF2. Expression of the eEF2 and mutants does not remove the effect of endogenous eEF2 as described previously. Transfection of the eEF2K shRNA into the CHO Flp-In cells stably over expressing the eEF2 mutants was also investigated, however 24 hours post transfection the CHO cells did not survive this. These data suggest that the loss of native eEF2K and the over expression of mutant eEF2 was a lethal combination to the cells. Therefore, the cells must maintain some measure of eEF2 and hence polypeptide elongation control. These findings agree with other studies of various cancer cells that targeting eEF2K results in cell death (Arora et al. 2003; Ashour et al. 2014).

6.2.3 Transient Knockdown of eEF2K in CHO Cells Stably Expressing Gassiua Luciferase is Cell Line Dependant

Two CHO Flp-In cell lines expressing gaussia luciferase (cell lines 12 and 14), each at a different level, were used to investigate the effect of eEF2K knockdown upon secreted recombinant protein expression. Western blotting of the gaussia luciferase produced was the preferred method of analysis for the gaussia protein instead of measurement via luminescence due to the instability of the gaussia protein activity. Both gaussia cell lines appeared to secrete similar quantities of gauissa luciferase, but when transfected with the eEF2K shRNA, cell line 12 showed an increase in gausssia secretion but an actual increase in eEF2K expression. The response from cell line 14, on average, was the opposite; with a decrease of eEF2K levels but only a marginal if any increase in gaussia expression. These data were observed across two independent experiments, showing that the effects of the eEF2K knockdown appear to be cell line specific.

It is possible the transfection efficiencies between the two cell lines are different, therefore the level of eEF2K knockdown achieved is different. From the data presented it would appear that there is a positive correlation between the amounts of eEF2K and gaussia luciferase secretion. It is possible that through some unknown mechanism eEF2K influences secretion of guassia recombinant protein. One scenario by which this might occur is that if large amounts of luciferase are already made in these cells (e.g. cell line 12), there may be a limitation upon secretion or folding in the ER in the secretory pathway. Upregulation of eEF2K may actually result in a slowing of translation and entry of gaussia luciferase into the ER and secretory pathway which may result in more correctly folded and thus secreted material being produced. Further experimentation would need to be undertaken to confirm if this hypothesis is correct.

The depletion of leucine during knockdown of eEF2K in the gaussia cell line 14 resulted in a decrease of phosphorylation of eEF2 as well as a small knockdown of eEF2K and increase in secreted gaussia luciferase. The depletion of leucine inactivates mTOR signalling which results in the activation of eEF2K and in turn the phosphorylation of eEF2, although culturing in leucine deplete media resulted in a decrease in eEF2 phosphorylation. As the depletion of leucine inactivates mTOR, it is also possible that other pathways are

in/activated that could potentially result in the activation of eEF2K as eEF2K activity is controlled via other pathways (Lynch 2001; Suryawan et al. 2012).

6.2.4 Transient Knockdown of eEF2K is Associated with a Calcium Ion Flux Across the ER Membrane

Another regulator of eEF2K that is linked both directly and indirectly to mTOR is calmodulin (Browne & Proud 2004; Gulati et al. 2008). Calcium flux can activate eEF2K and, it was possible that by knocking down eEF2K the cell may change the intra cellular calcium flux to try and compensate for reduced eEF2K activity. Live cell imaging of HEK cells stably expressing a FRET reporter molecule of calcium in the ER was therefore used to visualise any change in intracellular calcium ion distribution upon transient knockdown of eEF2K by shRNA. These studies showed that a flux of calcium ions out of the ER into the cytoplasm of HEK cells transfected with the eEF2K shRNA occurred along with changes to the cellular morphology.

The knockdown of eEF2K increased intracellular firefly recombinant protein production, as described in section 4.2.3.1 In response to eEF2K knockdown, the imaging undertaken here in HEK cells suggests that calcium is transported from the ER into the cytosol. This may activate calmodulin and any eEF2K. With an increase of calcium ions within the cytosol, activation of other pathways and the transcription of various genes may also be de/activated. In eukaryotic cells, the release of calcium ions into the cytosol is often undertaken in response to stress upon the cells (Egnatchik et al. 2014). Therefore, it is likely that the stress/burden of increased recombinant protein production and depletion of eEF2K and the loss of control of elongation contributes to a cellular response involving the flux of calcium from the ER into cytosol in order to try and compensate for this. Further evidence that the shRNA eEF2K transfected cells are under stress is provided by the fact that the cells show a rounder morphology and a smaller ER. The control cells had a larger ER and more of a 'splayed' morphology suggesting that the cells were under less stress.

6.2.5 Stable Expression of an eEF2K Targeting shRNA in Adherent CHO Cells

The shRNA vector provided with the kit for construction of the eEF2K shRNA contains a selection marker for pyromycin antibiotic resistance allowing for a stable selection process in CHO cells for those expressing the eEF2K shRNA vector. Stable expression of the shRNA was appealing as it would allow for longer term experiments (4-7 days) and remove the inconsistences and stress of transient transfection. The stable expression system relies upon random integration of the shRNA plasmid into the cells genome instead of the use of a recombinase site like the CHO Flp-In system.

CHO cells stably expressing the shRNA plasmid did not show any change in their grow profile compared to control cells and when analysed by western blot the silencing of eEF2K was not as apparent as in the transient experiments. After 72 hours the level of eEF2 phosphorylation was increased in the shRNA eEF2K cells compared to the control, suggesting that the knockdown of the eEF2K was only apparent during the first 48-72 h of growth of the stably expressing cell lines. Further, the fact that no cells with complete knockdown of eEF2K were isolated suggests that these were not able to survive, again providing further evidence that permanent eEF2K knockdown is toxic to cells. The initial transient experiments also suggested that the CHO cells could only sustain a minimal level of eEF2K expression over a short time period. When investigating protein synthesis in these cells by radiolabelling of nascent polypeptides, the resulting ^{35}S autoradiograph also showed a decrease in protein in the stable eEF2K shRNA CHO cells. This again suggests a reduction in protein synthetic capacity in these cells. This is an undesirable effect where recombinant protein production is concerned. However, an alternative to this might be to use an inducible knockdown system whereby a cell line could be generated where eEF2K expression could be silenced at a particular time in culture by inducing expression of an shRNA eEF2K knockdown sequence. In this way cells could normally be cultured without the expression of the eEF2K targeting hairpin being turned 'on', but when it was beneficial to switch on knockdown this could be induced. This may give a phenotype similar to that observed with the transient knockdown but further experiments would have to be undertaken to confirm this.

6.3 Investigating Links between mTOR, eEF2 and Recombinant Protein Production

Mammalian target of rapapmycin (mTOR) is known to be a regulator of global protein synthesis and its manipulation, via SGK1 regulation of eEF2K, has been shown to increase protein production as well as other process within the cells (Laplante & Sabatini 2012; Dreesen & Fussenegger 2011a). Therefore the potential links between mTOR and polypeptide elongation with respect to recombinant protein production were investigated.

6.3.1 There is no Correlation between mAb Titre in CHO Suspension Cell Lines and Levels of eEF2 and eEF2K

Over expression of mTOR and its dis-regulation have been shown to increase intracellular protein production within mammalian cells (Dreesen & Fussenegger 2011a). In the industrial setting there is a desire to find mammalian cells capable of producing high levels of recombinant proteins of appropriate quality (e.g. correctly folded, no mis-incorporation of amino acids), therefore whether there is a link between industrially constructed cell lines capability of monoclonal (mAb) secretion and their expression of eEF2 and its regulation was investigated.

Initially two cell lines, a high mAb producer and a low producer, were used to investigate eEF2 and eEF2K amounts in these cell lines between days 4-7 of growth in batch culture, the period when the mAb production begins. The levels of eEF2 and eEF2K were more-or-less consistent between the cell lines and across the days of culture sampled. Therefore, the investigation was opened up into a wider panel of cell lines consisting of 6 cell lines of varying mAb titre. For this wider mAb producer panel samples were taken at day 6 and day 8 of batch culture. The samples were screened for eEF2 and eEF2K amounts using western blotting techniques and the resulting data analysed using densitometry software. The results of the day 6 and day 8 producer panel (Figure 5.4 and 5.5) revealed that there was no discernible correlation between eEF2 and eEF2K activity and the cell lines mAb titre levels. The levels of total eEF2 appear to remain relativity consistent between all of the cell lines while the levels of eEF2K varied only slightly. The levels of eEF2 phosphorylation appeared to relate to the levels of total eEF2 in the cell lines i.e. the more eEF2 the more phosphorylated eEF2 was detected.

These data show and agree with the data collected in the other experiments within this study as well as other studies (Kaul et al. 2011; Nakamura et al. 2009) in that eEF2 is tightly regulated within the cell and the analysis of the producer panel revels that eEF2 and eEF2K do not play a key role in the production of the secreted recombinant protein investigated here. This does not mean that changes to eEF2 and eEF2K cannot influence recombinant protein activity as the previous sections have demonstrated that this can be achieved, but confirms that the cell controls the amounts of these and elongation closely. Thus any strategy to manipulate this must be carefully constructed in order that the cell can survive such intervention.

6.3.2 Changes in the Environment During Batch Culture are Reflected in Changes in mTOR Signalling and Reveals Possible Targets for Cell Engineering

The investigation into mTOR signalling and possibly extrapolating a link between mTOR, eEF2 and recombinant protein production would require experimentation and investigation into several pathways at the protein and mRNA level, resulting in a lengthy and extensive work load. Therefore, the use of a commercially available mTOR mRNA array was used as a starting point to identify potential targets that could be manipulated to up-regulate mTOR signalling and/or eEF2 activity via mTOR.

In order to undertake such studies, the RNA from two phases of adherent CHO cell growth were collected, during log growth phase and stationary phase, to discern which genes become up or down regulated once nutrients start to become limiting. These genes could then become a potential target for manipulation and subsequent enhancement of mTOR activity and recombinant protein production. When comparing the changes in the gene array between the log phase (68 hour) and stationary phase (120 hour) samples several positive and negative regulators, up and down stream of mTOR, were seen to be up regulated over 2-fold after 120 hours of growth compared to that at 68 hours of growth. The initial assumption to such a result is that mTOR activity is modulated within the cell with time and environmental conditions (e.g. nutrient availability) in order to maintain cell 'health'. This view is corroborated in several reviews of the mTOR literature stating the complexity involved in the regulation of mTOR and the numerous input and outputs from the mTOR complexes (Laplante & Sabatini 2012; Laplante & Sabatini 2009b). The function

of each gene identified as being differentially regulated between the two time points is described in appendix 7.6 which was provided with the data analysis spreadsheet provided with the array plates. Ideally this experiment would be repeated at least once more, and undertaken with other sample time points.

Several of the genes identified in the array panel are regulators of, or involved in, several pathways including AKT, which regulates several responses involving mTORC1 and MTOR2 and response from insulin receptors (Zoncu et al. 2011). Therefore, mTORC1 manipulation itself may be a the most likely route to deliver global change and regulation or effectors of the global pathway. For this reason two small molecules that are activators of mTOR: phosphatidic acid (PA), which interacts with mTOR directly, stabilising the formation of the mTORC1 complex (Foster 2009; Roux et al. 2004) and phorbol myristate acetate (PMA), a tumor promoting small molecule which activates protein kinase C and subsequently mTOR (see schematic 5.6), were selected for further study.

6.3.2.1 Addition of Phosphatidic Acid (PA) to Cell Culture does not Effect mTOR Signalling in CHO Cells

PA is known to interact with mTORC1/C2, stabilising the complex (Foster 2009; Foster 2007) therefore it was investigated whether the addition of PA to the cell culture media would increase mTOR activity. Although PA is essential to mTOR activity, it appears to require other signalling for mTOR activation(Foster 2009) but it was unknown if addition of further PA would increase mTOR signalling. PA was therefore added to a culture of a high producing mAb CHO cell line on day 4 (mid log phase of growth) of culture and samples collected 48 hours post addition of PA and mTOR, eEF2 and eEF2K activity analysed via western blotting. The analysis of the samples revealed no changes in the levels of eEF2, eEF2K and mTOR 48 hours post incubation with PA. This lack of response to PA maybe because the PA molecule was not able to cross the lipid membrane of the cell. PA is derived from several intra cellular pre-cursors (see section 1.9.2.5). Further investigation of the manipulation mTOR via PA would require either the addition of the pre cursor elements of PA or genetic manipulation of the kinases such as phospholipase D (PLD) involved in its *in vivo* synthesis. This could be a viable route for the investigation as PA interacts with mTOR directly and therefore could demonstrate that intracellular PA directly stimulates mTOR, whereas other methods inadvertently activate other regulatory pathways due to the complex mature of the mTOR pathway.

6.3.2.2 Addition of Phorbol Myristate Acetate (PMA) to Cell Culture Changes mTOR Signalling and Increases Recombinant Protein Production in CHO in a Cell Line Dependant Manner

PMA was also chosen to use in the project due to its known turmogenic properties coupled with studies that show mTOR is implicated in some cancers and activated by PMA. Further, unlike PA, PMA has been shown in other studies to induce a cellular response at a low concentration of 10 µM and is known to be able to cross the plasma membrane into the cell (Roux et al. 2004). The initial investigation with PMA was to investigate if it could increase cellular recombinant protein production over a 48-hour period, as well as determining a suitable concentration that was not toxic but elicited a response for use in future experiments. Using CHOK1D6 cells stably expressing firefly luciferase revealed that concentrations as little as 0.5 µM of PMA can elicit an increase in firefly luciferase

production (Figure 5.8) and 2 µM was the optimum concentration of PMA of those investigated. With this confirmation of PMAs ability to elicit an increase in protein production from CHO cells, the investigation was moved to mAb producing CHO cells. Using the same high and low producer CHO cell lines from previous experiments (section 5.2.1), the effect of PMA upon mAb production within these cells as well as PMAs effects upon mTOR, eEF2K and eEF2 signalling was investigated. The experiment was conducted over days 6-8 of batch culture, where the majority of mAb production occurs and therefore would have the most impact upon the cells protein production mechanisms. PMA was added to the culture on day 6 and samples taken 24 and 48 hours later.

PMA activates protein kinase C (PKC), which in turn goes on to activate a number of different pathways within the cell including the Nox enzymes which produce reactive oxygen species (Kizub et al. 2014; Cosentino-Gomes et al. 2012) and additional pathways involved in cellular responses and regulation. PKC activates mTOR via activation of RS6K (Ribosomal S6 Kinase), which inhibits the Tsc1/Tsc2 (tuberous sclerosis) complex, which in turn allows for Ras homolog enriched in brain (Rheb) to activate mTOR. Analysis of cellular lysates (western blotting) and supernatant (ELISA assay) revealed that the effect of PMA was cell line specific. The ELISA data revealed that only the low producing cell line increased its mAb output post PMA treatment. This suggests that the lower producing cell line was limited by one of the mechanisms/pathways that PMA activates. It is not possible to know what limiting factors were 'overcome' without an in depth study of the pathways regulated by PKC. However, through the western blot analysis it is possible to determine if mTOR signalling was increased in the low producing cell line. This did indeed appear to be the case as evidenced by a reduction of eEF2 phosphorylation upon PMA addition as well as an increase in mTOR levels. These data suggest that in the high producing cell lines investigated, mTOR signalling was sufficient and not limiting. This further suggests that the cell line selection and development process either selects for cell lines with appropriate mTOR signalling or that high producing cell lines must not have limitations in mTOR signalling or else the cell lines by definition cannot be high producing.

6.3.2.3 Culturing of Cells with PMA and Rapamycin Recovers Cell Growth Over that Seen When adding Rapamycin Alone

As described above, rapamycin is known to inhibit mTORC1 activity and reduce cellular growth (Fingar et al. 2002; Dowling et al. 2010). To determine if PMA could recover cellular growth during culture via promoting mTOR activity in the presence of rapamycin, CHO Flp-In cells were incubated with rapamycin and/or PMA, over a 7 day period using the Xcellegence system (Roche). Surprisingly, cells that were incubated with 20 nM rapamycin showed enhanced cellular growth; it is not know why this occurred. It is possible that the concentration of rapamycin passed a sensory threshold that triggered a stress response within the cells that was not reached in the cells incubated with 10 nM rapamycin. A concentration of 4 μM PMA appeared to be lethal to the CHO cells, suggesting over activation of mTOR results in cellular death. As expected, cells cultured with 10 nM rapamycin declined in cell viability early in the cell culture at 100 hours, were as when cultured in the presence of 2 μM PMA the cells recovered their growth and the culture appeared to have an extended stationary phase. This data further suggests that PMA, at least in part, elicits its responses in a mTOR-dependant manner. Further investigation of the cellular signalling occurring under these conditions by investigating the response of the different components of the signalling systems involved would allow the unravelling as to whether PMA signalling occurs largely in an mTOR dependant manner or via a number of different mechanisms.

6.3.2.4 Depletion of Leucine Adversely Effects the Expression of Recombinant Proteins in CHO Flp-In Cells Stably Over-Expressing Wild Type or Mutant Thr56Ala or Thr56Glu eEF2

Leucine has been shown to be a key signal of intra cellular amino acid levels to mTORC1 and modulate mTORC1 activity (see section 1.9.2.2). The CHO cell lines stably over expressing either wild type eEF2 or the Thr56Ala or Thr56Glu mutants were shown to increase the intra cellular recombinant protein levels of firefly luciferase. Cells were therefore incubated in leucine depleted media for 24 hours and cell lysates were collected to determine if this influenced mTOR signalling in these cell lines. Cells were also incubated for 48 hours in leucine depleted media for 48 hours post transient transfection of the pGL3 firefly luciferase construct. These experiments were conducted to determine how and if the

leucine depletion would affect mTOR signalling in the CHO cells, and if this signalling effects polypeptide elongation in the CHO cells over expressing the eEF2 variants.

Western blot analysis revealed that there was little change in the amount of eEF2 phosphorylation and eEF2K across the cell lines compared to the control cells, although there was a decrease in mTOR levels in the cells incubated in the leucine depleted media. Although expression of eEF2 and eEF2K were unchanged 24 hours post incubation in leucine depleted media, the firefly luciferase assay revealed that compared to previous assays (see figure 3.20) there was a reduction of firefly luciferase expression, with the Ala56 eEF2 cell line showing the largest decrease compared to that of the control cell line. This contrasted the results under nutrient rich conditions where the Ala56 eEF2 cell line showed a 5-fold increase in total luciferase expression and 2-fold increase in the amount of luciferase made per cell. This suggests that the presence of the exogenous Ala56 eEF2 that cannot be phosphorylated and inactivated compromises the ability of the cells to respond to the severe conditions of total depletion of the amino acid leucine. This inability to slow elongation must therefore be over-compensated for by mechanisms to slow protein synthesis such as attenuation of translation elongation. Further studies could therefore investigate whether translation elongation is attenuated in these cells to a greater extent under these conditions than the control cells.

6.4 Investigating if Over-Expression of eIF3 Subunits 3i and 3h in HEK Flp-In Cells Leads to Increased Cell Growth and Productivity.

Before translation elongation can begin, mRNA translation must be initiated. Therefore there are direct links and controls between translation initiation and elongation, including mTOR signalling which regulates translation initiation as well as translation elongation (Wang & Proud 2006; Holz et al. 2005). mTOR regulates initiation via 2 key points, 4E binding protein 1 (4EBP1) and regulation of eIF3, both of which are essential to initiation of cap-dependant translation. Therefore, a possible link between 'mTOR' translation initiation and elongation was investigated in HEK Flp-In cells stably over expressing eIF3 subunits 3h or 3h/3i with a C-terminus V5 tag. Cell lysate samples were analysed via western blotting for levels of eEF2 and eEF2K as an initial indication if over expression of these initiation subunits would influence elongation in these cells. The hypothesis was that if translation

initiation is enhanced then elongation may be as well to prevent ribosome traffic jams on the mRNA and keep elongation rates in line with translation initiation.

After 24 hours of culture there was an observable difference between the three cell lines in terms of eEF2 amounts, but 48 hours into culture the expression of eEF2 was more-or-less the same in the cell lines and the only discernible difference was an increase in eEF2 phosphorylation in the eIF3hV5 cell line. However, after 48 hours the levels of eEF2K were approximately half that of the FRT control cell line, this suggesting that the expression of the eIF3 subunits has a negative effect upon eEF2 activity and therefore the required eEF2K to regulate eEF2 is reduced.

The HEK cells were cultured in 10 nM rapamycin over 7 days to observe, if any, effects the inhibition of mTOR has upon the over expression of the eIF3 subunits. Compared to the CHO cells, the HEK cells appear to be more sensitive to the effects of DMSO and rapamycin. Further, the over expression of eIF3iV5 and 3h appears to have a detrimental effect upon the cells survival, showing complete culture death when cultured with rapamycin and a severely delayed growth when cultured with DMSO. In previous experiments, where eEF2 was tagged with a V5 reporter, cellular recombinant protein output was increased. Once again the data suggests that the V5 sequence influences cellular responses. The reason for this is unclear and would require extensive investigation, although the manipulation of the V5 tag alone could prove to be a potential method of enhancing cellular process at minimal intervention/intrusion into the cells regular processes.

6.5 General Conclusions

This project set out to investigate the effects of over expression of eEF2 and the subsequent mutation of eEF2 Thr56 residue upon CHO cellular growth and recombinant protein production. Further, silencing of eEF2K using shRNA to reduce eEF2 phosphorylation was undertaken and an investigation of the signalling of mTOR between CHO cells that have a different capacity for recombinant protein production as well as an investigation into the effects of small molecule activators of mTOR signalling and their effects upon the recombinant protein capacity of the cells.

This project has shown that:

1. Stable over expression of a Thr56Ala mutant eEF2 in CHO cells increases cellular growth and recombinant protein production although it alters the cells ability to respond to various environmental stresses including reduced temperature shift, osmotic stress and rapamycin. This would suggest that eEF2, both the non-phosphorylated and phosphorylated form, are integral to the survival of the cell.

2. Stable knockout of eEF2K is not tolerated by CHO cells and some eEF2K must remain or cellular death results. eEF2K is the sole regulator of eEF2, and knockdown results in the loss of eEF2 control. A partial transient knockdown of eEF2K greatly improves the cells capacity for intra cellular recombinant protein production, although secretion of recombinant proteins is dependent on the cells capacity for secretion, which is not improved by the silencing of eEF2K. This shows that although polypeptides can be synthesised faster by knock down of eEF2K, there are still other limiting factors down stream of poly peptide elongation that limit a cells capacity for secreted recombinant protein production, such as post-translation modification (Feige & Buchner 2014) and transport of the recombinant protein from the ER to and through the golgi apparatus (Le Fourn et al. 2014; Idiris et al. 2010) and out of the cell.

3. Stable over expression of eEF2 and the transient knockdown of eEF2K is not tolerated by CHO cells, showing that the eEF2-eEF2K regulatory pathway is strictly controlled.

4. The 'master regulator' mTOR, which is responsible for several cellular process ranging from gene regulation (Cunningham et al. 2007), lipid synthesis (Laplante & Sabatini 2009a) and protein synthesis (Hayashi & Proud 2007), has been studied extensively since its discovery and it is still not fully understood as a regulator within the cell. However, small chemical additives may enhance mTOR signalling and be used to increase recombinant protein expression from CHO cells and be useful experimental tools.

6.6 Concluding Remarks

To conclude, this project has shown that manipulation of eEF2 and eEF2K activity within certain limitations can enhance cellular growth and recombinant protein production from CHO cells. This suggests further efforts to understand and manipulate this process should be taken to apply such knowledge in an industrial sense. However, limitations in secretory productivity of a cell may further limit potential advances from manipulation of elongation alone and hence engineering strategies that combine enhancing the cells secretory capacity with enhanced elongation may yield the largest increases in recombinant protein production. Inducible systems that allow the control of elongation may be required to over-come the cells lack of ability to survive in the absence of eEF2K and elongation control. Such manipulations would shed further light on elongation control more widely in addition to its importance in recombinant protein synthesis from CHO cells. It may also be possible to manipulate the mTOR pathway to improve recombinant protein production via addition of small molecules on a cell-by-cell basis, but the effect of this manipulation will effect several pathways connected to mTOR and may be unpredictable. As such, new engineering approaches that allow the manipulation of both elongation and polypeptide synthesis combined with the secretory capacity of the cell are likely to yield new host cells with more predictable recombinant protein capacity than the use of such small molecules.

Appendices

7.1 Alignment of human and hamster elongation factor 2 DNA sequences using a web based alignment tool.

```
Comparison of:
(A) ./wwwtmp/.17214.1.seq human 2582 bp
- 2582 nt
(B) ./wwwtmp/.17214.2.seq hamster 2577 bp
- 2577 nt
 using matrix file: DNA (5/-4), gap-open/ext: -14/-4 E(limit)   0.05

  87.3% identity in 2577 nt overlap (1-2577:1-2577); score: 9933
E(10000):      0


                10        20        30        40        50        60
human   ATGGTGAACTTCACGGTAGACCAGATCCGCGCCATCATGGACAAGAAGGCCAACATCCGC
        ::::::::::::::::::::::::::::::: ::::: ::::::::::: ::::::::::::
hamste  ATGGTGAACTTCACGGTAGACCAGATCCGTGCCATTATGGACAAGAAAGCCAACATCCGG
                10        20        30        40        50        60

                70        80        90       100       110       120
human   AACATGTCTGTCATCGCCCACGTGGACCATGGCAAGTCCACGCTGACAGACTCCCTGGTG
        :::::::: :::::::: ::::::::::: ::::::::::: ::::: ::::::::::::::
hamste  AACATGTCAGTCATCGCTCACGTGGACCACGGCAAGTCCACACTGACGGACTCCCTGGTG
                70        80        90       100       110       120

               130       140       150       160       170       180
human   TGCAAGGCGGGCATCATCGCCTCGGCCCGGGCCGGGGAGACACGCTTCACTGATACCCGG
        :::::::::: :::::::::: ::   : ::::: ::::::::::::::: :: :::::
hamste  TGCAAGGCGGGTATCATCGCCTCTGCAAGAGCCGGTGAGACACGCTTCACAGACACCCGC
               130       140       150       160       170       180

               190       200       210       220       230       240
human   AAGGACGAGCAGGAGCGTTGCATCACCATCAAGTCAACTGCCATCTCCCTCTTCTACGAG
        :::::::: :::::::: ::::::: :::::::: :: :::::::::::::::: :::
hamste  AAGGACGAACAGGAGCGCTGCATCACTATCAAGTCCACGGCCATCTCCCTCTTCTATGAG
               190       200       210       220       230       240

               250       260       270       280       290       300
human   CTCTCGGAGAATGACTTGAACTTCATCAAGCAGAGCAAGGACGGTGCCGGCTTCCTCATC
        ::::: :::::::::: :::::::::::::::::::::::::: ::   : ::::: ::::::
hamste  CTCTCTGAGAATGACCTGAACTTCATCAAGCAGAGCAAGGATGGATCTGGCTTTCTCATC
               250       260       270       280       290       300

               310       320       330       340       350       360
human   AACCTCATTGACTCCCCCGGGCATGTCGACTTCTCCTCGGAGGTGACTGCTGCCCTCCGA
        :::::::: ::::: :: :: ::::: :: :::::::: :::::::: ::::: :: ::
hamste  AACCTCATCGACTCTCCAGGCCATGTGGATTTCTCCTCAGAGGTGACAGCTGCACTTCGT
               310       320       330       340       350       360
```

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             370        380        390        400        410        420
human  GTCACCGATGGCGCATTGGTGGTGGTGGACTGCGTGTCAGGCGTGTGCGTGCAGACGGAG
       ::::::::::: ::   : :::::::::::::: ::::: :: ::::::::::::: :::
hamste GTCACCGATGGAGCTCTTGTGGTGGTGGACTGTGTGTCTGGTGTGTGCGTGCAGACTGAG
             370        380        390        400        410        420




             430        440        450        460        470        480
human  ACAGTGCTGCGGCAGGCCATTGCCGAGCGCATCAAGCCTGTGCTGATGATGAACAAGATG
       :: :::::::::::::::::::::::::::::::::::::: ::::::::: ::::::
hamste ACCGTGCTGCGGCAGGCCATTGCCGAGCGCATCAAGCCTGTCCTGATGATGAATAAGATG
             430        440        450        460        470        480

             490        500        510        520        530        540
human  GACCGCGCCCTGCTGGAGCTGCAGCTGGAGCCCGAGGAGCTCTACCAGACTTTCCAGCGC
       ::::: :::::::: :::::::::::::::::: ::::: :: ::::::::: :::::::::
hamste GACCGTGCCCTGCTTGAGCTGCAGCTGGAGCCTGAGGAACTATACCAGACCTTCCAGCGC
             490        500        510        520        530        540

             550        560        570        580        590        600
human  ATCGTGGAGAACGTGAACGTCATCATCTCCACCTACGGCGAGGGCGAGAGCGGCCCCATG
       :: :::::::: :: :::::::::: ::::::: ::::::: :::::::::::: :::::::
hamste ATTGTGGAGAATGTCAACGTCATCATTTCCACCTATGGCGAGGGCGAGAGTGGACCCATG
             550        560        570        580        590        600

             610        620        630        640        650        660
human  GGCAACATCATGATCGATCCTGTCCTCGGTACCGTGGGCTTTGGGTCTGGCCTCCACGGG
       :: :: :: ::::: :: :: ::::: :: :: :::::::::: ::::::::: :: ::
hamste GGAAATATTATGATTGACCCCGTCCTGGGCACTGTGGGCTTTGGCTCTGGCCTGCATGGC
             610        620        630        640        650        660

             670        680        690        700        710        720
human  TGGGCCTTCACCCTGAAGCAGTTTGCCGAGATGTATGTGGCCAAGTTCGCCGCCAAGGGG
       :::::::::: ::::::::::::::::::: :::::::::::: ::::: :: :: :::::
hamste TGGGCCTTCACTCTGAAGCAGTTTGCGGAGATGTATGTGGCTAAGTTTGCAGCAAAGGGT
             670        680        690        700        710        720

             730        740        750        760        770        780
human  GAGGGCCAGTTGGGGCCTGCCGAGCGGGCCAAGAAAGTAGAGGACATGATGAAGAAGCTG
       :::::::::: :::::::::: ::::::::::::::::::: ::::::::::::::::: ::
hamste GAGGGCCAGCTGGGGCCTGCTGAGCGGGCCAAGAAAGTGGAGGACATGATGAAGAAGTTG
             730        740        750        760        770        780

             790        800        810        820        830        840
human  TGGGGTGACAGGTACTTTGACCCAGCCAACGGCAAGTTCAGCAAGTCAGCCACCAGCCCC
       ::::: ::   :::: ::::: :: ::::: ::::: ::::::::::: :: : ::::::
hamste TGGGGAGATCGGTATTTTGATCCCGCCAATGGCAAATTCAGCAAGTCCGCTAACAGCCCT
             790        800        810        820        830        840

             850        860        870        880        890        900
human  GAAGGGAAGAAGCTGCCCACGCACCTTCTGCCAGCTGATCCTGGACCCCATCTTCAAGGTG
       :: ::::::::: :::::::::::::::: :::::::: ::::::::::::::::::::::::
hamste GATGGGAAGAAACTGCCCACGCACCTTTTGCCAGCTCATCCTGGACCCCATCTTCAAGGTG
             850        860        870        880        890        900
```

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              910        920        930        940        950        960
human   TTTGATGCGATCATGAATTTCAAGAAAGAGGAGACAGCAAAACTGATAGAGAAACTGGAC
        ::::: :: :::::::: :::::  :: :::::::: :: :: ::::: ::::: :::::
hamste  TTTGACGCCATCATGAACTTCAGAAAGGAGGAGACGGCCAAGCTGATTGAGAAGCTGGAT
              910        920        930        940        950        960


              970        980        990       1000       1010       1020
human   ATCAAACTGGACAGCGAGGACAAGGACAAAGAAGGCAAACCCCTGCTGAAGGCTGTGATG
        ::::: ::::::::::::: :::::: :: ::::: :::::::::::::::::::::::::
hamste  ATCAAGCTGGACAGCGAGGACAAAGACAAGGAGGGCAAGCCCCTGCTGAAGGCTGTGATG
              970        980        990       1000       1010       1020



             1030       1040       1050       1060       1070       1080
human   CGCCGCTGGCTGCCTGCCGGAGACGCCTTGTTGCAGATGATCACCATCCACCTGCCCTCC
        ::::: :::::::: :: :: ::::::: :: ::::::::::: :::::::::::: :: :::
hamste  CGCCGGTGGCTGCCCGCAGGGGACGCCCTGCTGCAGATGATAACCATCCACCTCCCTTCC
             1030       1040       1050       1060       1070       1080


             1090       1100       1110       1120       1130       1140
human   CCTGTGACGGCCCAGAAGTACCGCTGCGAGCTCCTGTACGAGGGGCCCCCGGACGACGAG
        :: :: :: :::::::: ::::::::::: :: ::::::::::: :: :: :: :::
hamste  CCCGTCACCGCCCAGAAATACCGCTGCGAGCTGCTCTACGAGGGGCCACCTGATGATGAG
             1090       1100       1110       1120       1130       1140


             1150       1160       1170       1180       1190       1200
human   GCTGCCATGGGCATTAAAAGCTGTGACCCCAAAGGCCCTCTTATGATGTATATTTCCAAA
        :: :::::::::::::::::::::::: ::::: :: :: ::::::::::: :::::::
hamste  GCGGCCATGGGCATTAAAAGCTGTGATCCCAAGGGTCCCCTTATGATGTACATTTCCAAG
             1150       1160       1170       1180       1190       1200


             1210       1220       1230       1240       1250       1260
human   ATGGTGCCAACCTCCGACAAAGGTCGGTTCTACGCCTTTGGACGAGTCTTCTCGGGGCTG
        ::::::::::::::::::::::::: :: ::::::::::::: :::: ::::: ::: :
hamste  ATGGTGCCAACCTCCGACAAAGGCCGCTTCTACGCCTTTGGTAGAGTGTTCTCCGGGGTA
             1210       1220       1230       1240       1250       1260


             1270       1280       1290       1300       1310       1320
human   GTCTCCACTGGCCTGAAGGTCAGGATCATGGGGCCCAACTATACCCCTGGGAAGAAGGAG
        :: :: :: :: :: :::::: : :::::::: :::::::: :: :::::::::::::::
hamste  GTGTCGACAGGACTCAAGGTCCGCATCATGGGCCCCAACTACACGCCTGGGAAGAAGGAG
             1270       1280       1290       1300       1310       1320


             1330       1340       1350       1360       1370       1380
human   GACCTCTACCTGAAGCCAATCCAGAGAACAATCTTGATGATGGGCCGCTACGTGGAGCCC
        :: :: :::::::::: :::::::::: ::: ::::::::::: :: :::::::
hamste  GAACTGTACCTGAAGCCCATCCAGAGAACCATCCTGATGATGGGCCGGTATGTGGAGCCA
             1330       1340       1350       1360       1370       1380


             1390       1400       1410       1420       1430       1440
human   ATCGAGGATGTGCCTTGTGGGAACATTGTGGGCCTCGTGGGCGTGGACCAGTTCCTGGTG
        :: ::::: ::::: :::::::::::::::: :: :: :: :: :: :::::::: ::::::
hamste  ATTGAGGACGTGCCCTGTGGGAACATTGTCGGGCTGGTTGGTGTAGACCAGTTTCTGGTG
             1390       1400       1410       1420       1430       1440


             1450       1460       1470       1480       1490       1500
human   AAGACGGGCACCATCACCACCTTCGAGCACGCGCACAACATGCGGGTGATGAAGTTCAGC
        ::::: :: :: :::::::::: :: ::::: :: :::::::: ::::::::::::::::
hamste  AAGACAGGGACTATCACCACCTTTGAACACGCACATAACATGCGTGTGATGAAGTTCAGC
             1450       1460       1470       1480       1490       1500
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209

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              1510      1520      1530      1540      1550      1560
human   GTCAGCCCTGTTGTCAGAGTGGCCGTGGAGGCCAAGAACCCGGCTGACCTGCCCAAGCTG
        ::::::::::: ::::::::::: :::::::::::::::::: :::::::::::::: :::
hamste  GTCAGCCCTGTCGTCAGAGTGGCTGTGGAGGCCAAGAACCCAGCTGACCTGCCCAAACTG
              1510      1520      1530      1540      1550      1560


              1570      1580      1590      1600      1610      1620
human   GTGGAGGGGCTGAAGCGGCTGGCCAAGTCCGACCCCATGGTGCAGTGCATCATCGAGGAG
        :::::::: ::::::::::::: :: :: ::::: :::::::::::::::: ::::::
hamste  GTGGAGGGCCTGAAGCGGCTGGCAAAATCTGACCCTATGGTGCAGTGCATCATTGAGGAG
              1570      1580      1590      1600      1610      1620


              1630      1640      1650      1660      1670      1680
human   TCGGGAGAGCATATCATCGCGGGCGCCGGCGAGCTGCACCTGGAGATCTGCCTGAAGGAC
        :: :: ::::: ::::: :: :: :: :: :::::::::::::: :::::::: ::::::
hamste  TCTGGGGAGCACATCATTGCTGGAGCAGGTGAGCTGCACCTGGAAATCTGCCTCAAGGAC
              1630      1640      1650      1660      1670      1680


              1690      1700      1710      1720      1730      1740
human   CTGGAGGAGGACCACGCCTGCATCCCCATCAAGAAATCTGACCCGGTCGTCTCGTACCGC
        :::::::::::::::::::::::::::::::::::::::::::::: :: ::::: :::::
hamste  CTGGAGGAGGACCACGCCTGCATCCCCATCAAGAAATCTGACCCTGTTGTCTCATACCGG
              1690      1700      1710      1720      1730      1740


              1750      1760      1770      1780      1790      1800
human   GAGACGGTCAGTGAAGAGTCGAACGTGCTCTGCCTCTCCAAGTCCCCCAACAAGCACAAC
        ::::: :: :: :: ::::: :: ::::: ::::: :::::::: ::::::::::::::::
hamste  GAGACAGTGAGCGAGGAGTCAAATGTGCTGTGCCTGTCCAAGTCGCCCAACAAGCACAAC
              1750      1760      1770      1780      1790      1800


              1810      1820      1830      1840      1850      1860
human   CGGCTGTACATGAAGGCGCGGCCCCTTCCCCGACGGCCTGGCCGAGGACATCGATAAAGGC
        ::    ::::: ::::::::: :::::::::: :: :::::::::::::::::: :: ::
hamste  CGATTGTATATGAAGGCCCGGCCCCTTCCCTGATGGCCTGGCCGAGGACATCGACAAGGGT
              1810      1820      1830      1840      1850      1860


              1870      1880      1890      1900      1910      1920
human   GAGGTGTCCGCCCGTCAGGAGCTCAAGCAGCGGGCGCGCTACCTGGCCGAGAAGTACGAG
        :: ::::: :: :: ::::::::::::    :: :: ::::: :: :: :: ::::::::
hamste  GAAGTGTCTGCTCGCCAGGAGCTCAAGGCACGCGCACGCTATCTAGCTGAAAAGTACGAA
              1870      1880      1890      1900      1910      1920


              1930      1940      1950      1960      1970      1980
human   TGGGACGTGGCTGAGGCCCGCAAGATCTGGTGCTTTGGGCCCGACGGCACCGGCCCCAAC
        ::::: :: ::::: :::::::::::::::::::::::::::::: :: ::::: :::::
hamste  TGGGATGTTGCTGAAGCCCGCAAGATCTGGTGCTTTGGGCCTGATGGCACTGGCCCCAAC
              1930      1940      1950      1960      1970      1980


              1990      2000      2010      2020      2030      2040
human   ATCCTCACCGACATCACCAAGGGTGTGCAGTACCTCAACGAGATCAAGGACAGTGTGGTG
        :: :: ::::: ::::::::::::::::::::::::: :: :: ::::::::::::::::
hamste  ATTCTTACCGATATCACCAAGGGTGTGCAGTACCTGAATGAAATCAAGGACAGTGTGGTG
              1990      2000      2010      2020      2030      2040


              2050      2060      2070      2080      2090      2100
human   GCCGGCTTCCAGTGGGCCACCAAGGAGGGCGCACTGTGTGAGGAGAACATGCGGGGTGTGT
        :: :::::::::::::::::: ::::::::::: :: :::::::::::::::::: ::::::
hamste  GCTGGCTTCCAGTGGGCCACTAAGGAGGGCGCTCTCTGTGAGGAGAACATGCGTGGTGTGT
              2050      2060      2070      2080      2090      2100
```

```
              2110      2120      2130      2140      2150      2160
human   CGCTTCGACGTCCACGACGTCACCCTGCACGCCGATGCCATCCACCGCGGAGGGGGCCAG
        :::::::::  :::::  ::  ::  :::::  ::  ::  ::::::::::::::  :::::  ::  :::
hamste  CGCTTCGATGTCCATGATGTGACCCTACATGCTGATGCCATCCACCGAGGAGGTGGTCAG
              2110      2120      2130      2140      2150      2160


              2170      2180      2190      2200      2210      2220
human   ATCATCCCCACAGCACGGCGCTGCCTCTATGCCAGTGTGCTGACCGCCCAGCCACGCCTC
        :::::  :::::::::  ::  ::  ::  ::  ::::::::::::  ::::  ::  :::::  ::::::
hamste  ATCATTCCCACAGCTCGTCGTTGTCTGTATGCCAGTGTGTTGACTGCACAGCCCCGCCTC
              2170      2180      2190      2200      2210      2220


              2230      2240      2250      2260      2270      2280
human   ATGGAGCCCATCTACCTTGTGGAGATCCAGTGTCCAGAGCAGGTGGTCGGTGGCATCTAC
        :::::::::  :::::::::  :::::::::  :::::::::  ::  :::::  ::  ::::::::::::
hamste  ATGGAGCCTATCTACCTGGTGGAGATTCAGTGTCCTGAACAGGTCGTGGGTGGCATCTAC
              2230      2240      2250      2260      2270      2280


              2290      2300      2310      2320      2330      2340
human   GGGGTTTTGAACAGGAAGCGGGGCCACGTGTTCGAGGAGTCCCAGGTGGCCGGCACCCCC
        ::  ::   ::::::::::::  :::::  :::::  ::  ::::::::::::::  :::::::::
hamste  GGTGTCCTGAACAGGAAGCGTGGCCATGTGTTTGAAGAGTCCCAGGTGGCTGGCACCCCC
              2290      2300      2310      2320      2330      2340


              2350      2360      2370      2380      2390      2400
human   ATGTTTGTGGTCAAGGCCTATCTGCCCGTCAACGAGTCCTTTGGCTTCACCGCTGACCTG
        :::::::::  :::::::::::::::  ::  :::::::::::::::::  ::  ::  :::::::::
hamste  ATGTTTGTAGTCAAGGCCTATCTTCCAGTCAACGAGTCCTTTGGTTTTACAGCTGACCTG
              2350      2360      2370      2380      2390      2400


              2410      2420      2430      2440      2450      2460
human   AGGTCCAACACGGGCGGCCAGGCGTTCCCCCAGTGTGTGTTTGACCACTGGCAGATCCTG
        :  :::::::::  ::  :::::  ::  :::::::::::::::::  :::::::::::::::  :::
hamste  CGCTCCAACACTGGTGGCCAAGCTTTCCCCCAGTGTGTGTTCGACCACTGGCAGATTCTG
              2410      2420      2430      2440      2450      2460


              2470      2480      2490      2500      2510      2520
human   CCCGGAGACCCCTTCGACAACAGCAGCCGCCCCAGCCAGGTGGTGGCGGAGACCCGCAAG
        :::::  :::::  :::::::::::::::::::  :::::::::  :::::::::  :::::::::::
hamste  CCCGGGGACCCGTTCGACAACAGCAGCCGTCCCAGCCAAGTGGTGGCTGAGACCCGCAAG
              2470      2480      2490      2500      2510      2520


              2530      2540      2550      2560      2570
human   CGCAAGGGCCTGAAAGAAGGCATCCCTGCCCTGGACAACTTCCTGGACAAATTGTAG
        :::::  ::  ::  ::  ::  :::::::::  ::  :::::::::::::::::::  :::::
hamste  CGCAAAGGTCTAAAGGAGGGCATCCCAGCACTGGACAACTTCCTGGACAAACTGTAG
              2530      2540      2550      2560      2570
```

7.2 Alignment of human and hamster elongation factor 2 amino acid sequences using a web based alignment tool.

```
Comparison of:
(A) ./wwwtmp/.26055.1.seq human 858 bp
- 858 aa
(B) ./wwwtmp/.26055.2.seq hamster 858 bp
- 858 aa
 using matrix file: BL50 (15/-5), gap-open/ext: -14/-4 E(limit)
0.05

  99.2% identity in 858 aa overlap (1-858:1-858); score: 5677
E(10000):      0


              10        20        30        40        50        60
human   MVNFTVDQIRAIMDKKANIRNMSVIAHVDHGKSTLTDSLVCKAGIIASARAGETRFTDTR
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
hamste  MVNFTVDQIRAIMDKKANIRNMSVIAHVDHGKSTLTDSLVCKAGIIASARAGETRFTDTR
              10        20        30        40        50        60


              70        80        90       100       110       120
human   KDEQERCITIKSTAISLFYELSENDLNFIKQSKDGAGFLINLIDSPGHVDFSSEVTAALR
        :::::::::::::::::::::::::::::::::::::.:::::::::::::::::::::::::
hamste  KDEQERCITIKSTAISLFYELSENDLNFIKQSKDGSGFLINLIDSPGHVDFSSEVTAALR
              70        80        90       100       110       120


             130       140       150       160       170       180
human   VTDGALVVVDCVSGVCVQTETVLRQAIAERIKPVLMMNKMDRALLELQLEPEELYQTFQR
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
hamste  VTDGALVVVDCVSGVCVQTETVLRQAIAERIKPVLMMNKMDRALLELQLEPEELYQTFQR
             130       140       150       160       170       180


             190       200       210       220       230       240
human   IVENVNVIISTYGEGESGPMGNIMIDPVLGTVGFGSGLHGWAFTLKQFAEMYVAKFAAKG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
hamste  IVENVNVIISTYGEGESGPMGNIMIDPVLGTVGFGSGLHGWAFTLKQFAEMYVAKFAAKG
             190       200       210       220       230       240


             250       260       270       280       290       300
human   EGQLGPAERAKKVEDMMKKLWGDRYFDPANGKFSKSATSPEGKKLPRTFCQLILDPIFKV
        ::::::::::::::::::::::::::::::::::::::::::.::.::::::::::::::::
hamste  EGQLGPAERAKKVEDMMKKLWGDRYFDPANGKFSKSANSPDGKKLPRTFCQLILDPIFKV
             250       260       270       280       290       300


             310       320       330       340       350       360
human   FDAIMNFKKEETAKLIEKLDIKLDSEDKDKEGKPLLKAVMRRWLPAGDALLQMITIHLPS
        ::::::::.:::::::::::::::::::::::::::::::::::::::::::::::::::::
hamste  FDAIMNFRKEETAKLIEKLDIKLDSEDKDKEGKPLLKAVMRRWLPAGDALLQMITIHLPS
             310       320       330       340       350       360


             370       380       390       400       410       420
human   PVTAQKYRCELLYEGPPDDEAAMGIKSCDPKGPLMMYISKMVPTSDKGRFYAFGRVFSGL
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::.
hamste  PVTAQKYRCELLYEGPPDDEAAMGIKSCDPKGPLMMYISKMVPTSDKGRFYAFGRVFSGV
             370       380       390       400       410       420
```

```
                430       440       450       460       470       480
human   VSTGLKVRIMGPNYTPGKKEDLYLKPIQRTILMMGRYVEPIEDVPCGNIVGLVGVDQFLV
        ::::::::::::::::::::::.::::::::::::::::::::::::::::::::::::::::
hamste  VSTGLKVRIMGPNYTPGKKEELYLKPIQRTILMMGRYVEPIEDVPCGNIVGLVGVDQFLV
                430       440       450       460       470       480


                490       500       510       520       530       540
human   KTGTITTFEHAHNMRVMKFSVSPVVRVAVEAKNPADLPKLVEGLKRLAKSDPMVQCIIEE
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
hamste  KTGTITTFEHAHNMRVMKFSVSPVVRVAVEAKNPADLPKLVEGLKRLAKSDPMVQCIIEE
                490       500       510       520       530       540


                550       560       570       580       590       600
human   SGEHIIAGAGELHLEICLKDLEEDHACIPIKKSDPVVSYRETVSEESNVLCLSKSPNKHN
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
hamste  SGEHIIAGAGELHLEICLKDLEEDHACIPIKKSDPVVSYRETVSEESNVLCLSKSPNKHN
                550       560       570       580       590       600


                610       620       630       640       650       660
human   RLYMKARPFPDGLAEDIDKGEVSARQELKQRARYLAEKYEWDVAEARKIWCFGPDGTGPN
        :::::::::::::::::::::::::::::::: :::::::::::::::::::::::::::::
hamste  RLYMKARPFPDGLAEDIDKGEVSARQELKARARYLAEKYEWDVAEARKIWCFGPDGTGPN
                610       620       630       640       650       660


                670       680       690       700       710       720
human   ILTDITKGVQYLNEIKDSVVAGFQWATKEGALCEENMRGVRFDVHDVTLHADAIHRGGGQ
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
hamste  ILTDITKGVQYLNEIKDSVVAGFQWATKEGALCEENMRGVRFDVHDVTLHADAIHRGGGQ
                670       680       690       700       710       720


                730       740       750       760       770       780
human   IIPTARRCLYASVLTAQPRLMEPIYLVEIQCPEQVVGGIYGVLNRKRGHVFEESQVAGTP
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
hamste  IIPTARRCLYASVLTAQPRLMEPIYLVEIQCPEQVVGGIYGVLNRKRGHVFEESQVAGTP
                730       740       750       760       770       780


                790       800       810       820       830       840
human   MFVVKAYLPVNESFGFTADLRSNTGGQAFPQCVFDHWQILPGDPFDNSSRPSQVVAETRK
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
hamste  MFVVKAYLPVNESFGFTADLRSNTGGQAFPQCVFDHWQILPGDPFDNSSRPSQVVAETRK
                790       800       810       820       830       840


                850
human   RKGLKEGIPALDNFLDKL
        ::::::::::::::::::
hamste  RKGLKEGIPALDNFLDKL
                850
```

7.3 Schematics of commercial vectors used in the cloning and expression of elongation factor 2

(a)
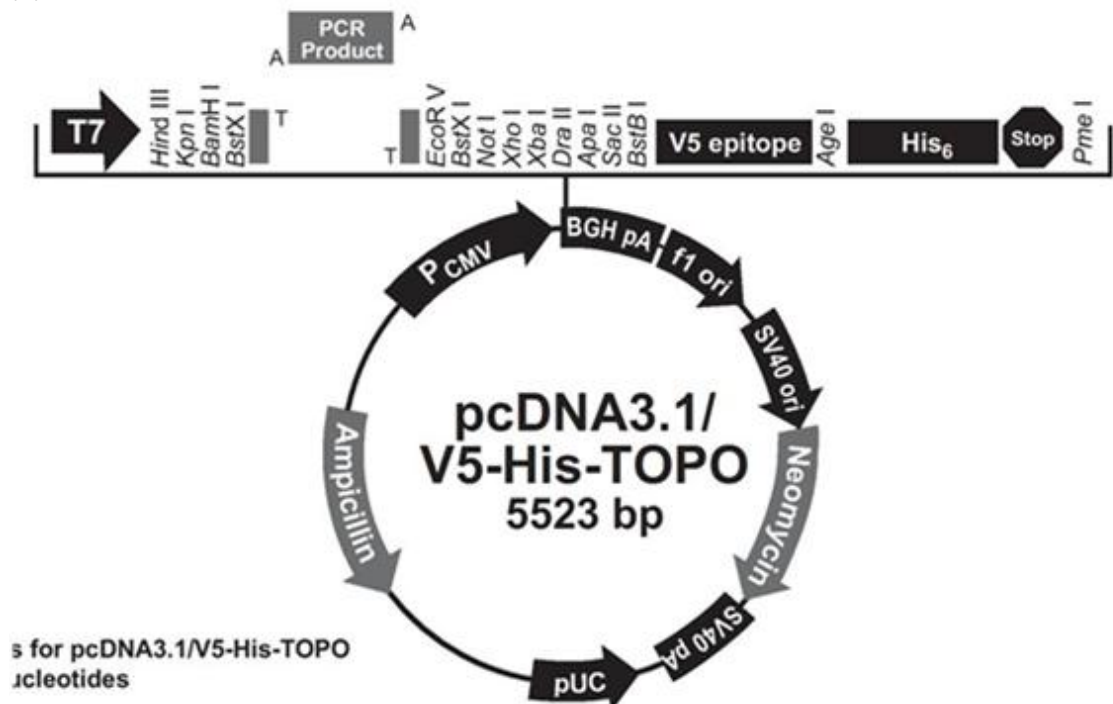
(b)
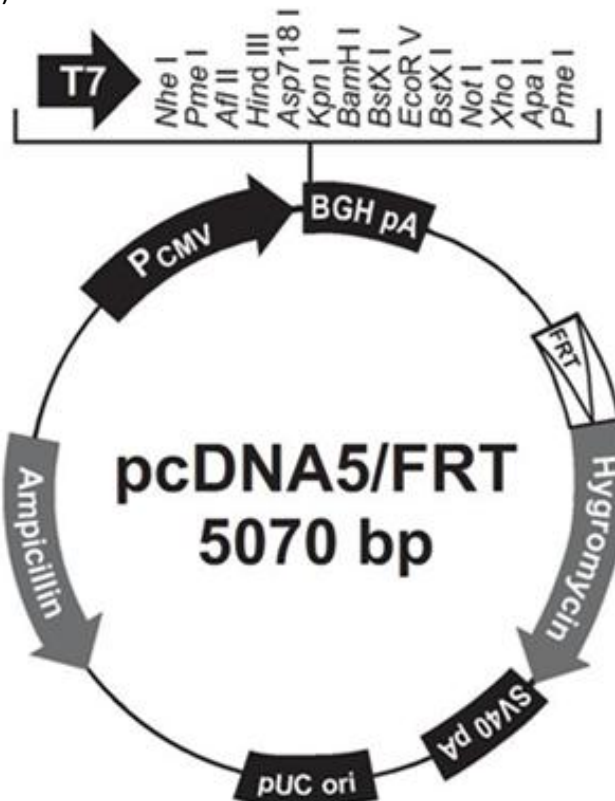
Schematic 7.3.1 Vectors used in the mutation, transient and stable expression of wild type eEF2 and the eEF2 mutants Ala56 and Glu56 **(a)** Vector map of the pcDNA3.1 plasmid used in the transient expression of eEF2 **(b)** Vector map of FRT (pcDNA5/FRT) plasmid used in the stable expression of eEF2

7.4 Alignment of Hamster wild type (WT)/ Thr56Ala (Ala56) / Thr56Glu (Glu) Elongation factor 2

Sequence comparrision of the wild type WT/ Thr56Ala Ala56 / Thr56Glu elongation factor 2 constructs. Mutation of Thr56 is highlighted below

Glu 56= CHO eEF2 Thr56Glu mutant sequence

Ala56= CHO eEF2 Thr56Glu mutant sequence

eEF2 = hamster eEF2 sequence (NCBI accession number M13708)

WT= Wild type eEF2 sequence

```
Glu56    ------NGGGNNNGTTACTTAGCTTGGTACTGAGATCCGTCGCCATCCGCCACTATGGT
Ala56    NCCNNNNNNNNNN------------NNGTACTGANATCCGTCGCCATCCGCCACTATGGT
eEF2     ----------------------------------------------------atggt
WT       --NNNNNNNNNNNNGTTACTTAGCTNNGNNCTGANANCCGTCGCCATCCGCCACTATGGT
                                                             *****

Glu56    GAACTTCACGGTAGACCAGATCCGTGCCATTATGGACAAGAAAGCCAACATCCGGAACAT
Ala56    GAACTTCACGGTAGACCAGATCCGTGCCATTATGGACAAGAAAGCCAACATCCGGAACAT
eEF2     gaacttcacggtagaccagatccgtgccattatggacaagaaagccaacatccggaacat
WT       GAACTTCACGGTAGACCAGATCCGTGCCATTATGGACAAGAAAGCCAACATCCGGAACAT
         ************************************************************

Glu56    GTCAGTCATCGCTCACGTGGACCACGGCAAGTCCACACTGACGGACTCCCTGGTGTGCAA
Ala56    GTCAGTCATCGCTCACGTGGACCACGGCAAGTCCACACTGACGGACTCCCTGGTGTGCAA
eEF2     gtcagtcatcgctcacgtggaccacggcaagtccacactgacggactccctggtgtgcaa
WT       GTCAGTCATCGCTCACGTGGACCACGGCAAGTCCACACTGACGGACTCCCTGGTGTGCAA
         ************************************************************

Glu56    GGCGGGTATCATCGCCTCTGCAAGAGCCGGTGAGACACGCTTCGAGGACACCCGCAAGGA
Ala56    GGCGGGTATCATCGCCTCTGCAAGAGCCGGTGAGACACGCTTCGCCGACACCCGCAAGGA
eEF2     ggcgggtatcatcgcctctgcaagagccggtgagacacgcttcacagacacccgcaagga
WT       GGCGGGTATCATCGCCTCTGCAAGAGCCGGTGAGACACGCTTCACAGACACCCGCAAGGA
         *********************************************.. *************

Glu56    CGAACAGGAGCGCTGCATCACTATCAAGTCCACGGCCATCTCCCTCTTCTATGAGCTCTC
Ala56    CGAACAGGAGCGCTGCATCACTATCAAGTCCACGGCCATCTCCCTCTTCTATGAGCTCTC
eEF2     cgaacaggagcgctgcatcactatcaagtccacggccatctccctcttctatgagctctc
WT       CGAACAGGAGCGCTGCATCACTATCAAGTCCACGGCCATCTCCCTCTTCTATGAGCTCTC
         ************************************************************

Glu56    TGAGAATGACCTGAACTTCATCAAGCAGAGCAAGGATGGATCTGGCTTTCTCATCAACCT
Ala56    TGAGAATGACCTGAACTTCATCAAGCAGAGCAAGGATGGATCTGGCTTTCTCATCAACCT
eEF2     tgagaatgacctgaacttcatcaagcagagcaaggatggatctggctttctcatcaacct
WT       TGAGAATGACCTGAACTTCATCAAGCAGAGCAAGGATGGATCTGGCTTTCTCATCAACCT
         ************************************************************
```

```
Glu56    CATCGACTCTCCAGGCCATGTGGATTTCTCCTCAGAGGTGACAGCTGCACTTCGTGTCAC
Ala56    CATCGACTCTCCAGGCCATGTGGATTTCTCCTCAGAGGTGACAGCTGCACTTCGTGTCAC
eEF2     catcgactctccaggccatgtggatttctcctcagaggtgacagctgcacttcgtgtcac
WT       CATCGACTCTCCAGGCCATGTGGATTTCTCCTCAGAGGTGACAGCTGCACTTCGTGTCAC
         ************************************************************

Glu56    CGATGGAGCTCTTGTGGTGGTGGACTGTGTGTCTGGTGTGTGCGTGCAGACTGAGACCGT
Ala56    CGATGGAGCTCTTGTGGTGGTGGACTGTGTGTCTGGTGTGTGCGTGCAGACTGAGACCGT
eEF2     cgatggagctcttgtggtggtggactgtgtgtctggtgtgtgcgtgcagactgagaccgt
WT       CGATGGAGCTCTTGTGGTGGTGGACTGTGTGTCTGGTGTGTGCGTGCAGACTGAGACCGT
         ************************************************************

Glu56    GCTGCGGCAGGCCATTGCCGAGCGCATCAAGCCTGTCCTGATGATGAATAAGATGGACCG
Ala56    GCTGCGGCAGGCCATTGCCGAGCGCATCAAGCCTGTCCTGATGATGAATAAGATGGACCG
eEF2     gctgcggcaggccattgccgagcgcatcaagcctgtcctgatgatgaataagatggaccg
WT       GCTGCGGCAGGCCATTGCCGAGCGCATCAAGCCTGTCCTGATGATGAATAAGATGGACCG
         ************************************************************

Glu56    TGCCCTGCTTGAGCTGCAGCTGGAGCCTGAGGAACTATACCAGACCTTCCAGCGCATTGT
Ala56    TGCCCTGCTTGAGCTGCAGCTGGAGCCTGAGGAACTATACCAGACCTTCCAGCGCATTGT
eEF2     tgccctgcttgagctgcagctggagcctgaggaactataccagaccttccagcgcattgt
WT       TGCCCTGCTTGAGCTGCAGCTGGAGCCTGAGGAACTATACCAGACCTTCCAGCGCATTGT
         ************************************************************

Glu56    GGAGAATGTCAACGTCATCATTTCCACCTATGGCGAGGGCGAGAGTGGACCCATGGGAAA
Ala56    GGAGAATGTCAACGTCATCATTTCCACCTATGGCGAGGGCGAGAGTGGACCCATGGGAAA
eEF2     ggagaatgtcaacgtcatcatttccacctatggcgagggcgagagtggacccatgggaaa
WT       GGAGAATGTCAACGTCATCATTTCCACCTATGGCGAGGGCGAGAGTGGACCCATGGGAAA
         ************************************************************

Glu56    TATTATGATTGACCCCGTCCTGGGCACTGTGGGCTTTGGCTCTGGCCTGCATGGCTGGGC
Ala56    TATTATGATTGACCCCGTCCTGGGCACTGTGGGCTTTGGCTCTGGCCTGCATGGCTGGGC
eEF2     tattatgattgaccccgtcctgggcactgtgggctttggctctggcctgcatggctgggc
WT       TATTATGATTGACCCCGTCCTGGGCACTGTGGGCTTTGGCTCTGGCCTGCATGGCTGGGC
         ************************************************************

Glu56    CTTCACTCTGAAGCAGTTTGCGGAGATGTATGTGGCTAAGTTTGCAGCAAAGGGTGAGGG
Ala56    CTTCACTCTGAAGCAGTTTGCGGAGATGTATGTGGCTAAGTTTGCAGCAAAGGGTGAGGG
eEF2     cttcactctgaagcagtttgcggagatgtatgtggctaagtttgcagcaaagggtgaggg
WT       CTTCACTCTGAAGCAGTTTGCGGAGATGTATGTGGCTAAGTTTGCAGCAAAGGGTGAGGG
         ************************************************************

Glu56    CCAGCTGGGGGCCTGCTGAGCGGGCCAAGAAAGTGGAGGACATGATGAAGAAGTTGTGGGG
Ala56    CCAGCTGGGGGCCTGCTGAGCGGGCCAAGAAAGTGGAGGACATGATGAAGAAGTTGTGGGG
eEF2     ccagctgggggcctgctgagcgggccaagaaagtggaggacatgatgaagaagttgtgggg
WT       CCAGCTGGGGGCCTGCTGAGCGGGCCAAGAAAGTGGAGGACATGATGAAGAAGTTGTGGGG
         ************************************************************
```

```
Glu56    AGATCGGTATTTTGATCCCGCCAATGGCAAATTCAGCAAGTCCGCTAACAGCCCTGATGG
Ala56    AGATCGGTATTTTGATCCCGCCAATGGCAAATTCAGCAAGTCCGCTAACAGCCCTGATGG
eEF2     agatcggtattttgatcccgccaatggcaaattcagcaagtccgctaacagccctgatgg
WT       AGATCGGTATTTTGATCCCGCCAATGGCAAATTCAGCAAGTCCGCTAACAGCCCTGATGG
         ************************************************************

Glu56    GAAGAAACTGCCACGCACCTTTTGCCAGCTCATCCTGGACCCCATCTTCAAGGTGTTTGA
Ala56    GAAGAAACTGCCACGCACCTTTTGCCAGCTCATCCTGGACCCCATCTTCAAGGTGTTTGA
eEF2     gaagaaactgccacgcaccttttgccagctcatcctggaccccatcttcaaggtgtttga
WT       GAAGAAACTGCCACGCACCTTTTGCCAGCTCATCCTGGACCCCATCTTCAAGGTGTTTGA
         ************************************************************

Glu56    CGCCATCATGAACTTCAGAAAGGAGGAGACGGCCAAGCTGATTGAGAAGCTGGATATCAA
Ala56    CGCCATCATGAACTTCAGAAAGGAGGAGACGGCCAAGCTGATTGAGAAGCTGGATATCAA
eEF2     cgccatcatgaacttcagaaaggaggagacggccaagctgattgagaagctggatatcaa
WT       CGCCATCATGAACTTCAGAAAGGAGGAGACGGCCAAGCTGATTGAGAAGCTGGATATCAA
         ************************************************************

Glu56    GCTGGACAGCGAGGACAAAGACAAGGAGGGCAAGCCCCTGCTGAAGGCTGTGATGCGCCG
Ala56    GCTGGACAGCGAGGACAAAGACAAGGAGGGCAAGCCCCTGCTGAAGGCTGTGATGCGCCG
eEF2     gctggacagcgaggacaaagacaaggagggcaagcccctgctgaaggctgtgatgcgccg
WT       GCTGGACAGCGAGGACAAAGACAAGGAGGGCAAGCCCCTGCTGAAGGCTGTGATGCGCCG
         ************************************************************

Glu56    GTGGCTGCCCGCAGGGGACGCCCTGCTGCAGATGATAACCATCCACCTCCCTTCCCCCGT
Ala56    GTGGCTGCCCGCAGGGGACGCCCTGCTGCAGATGATAACCATCCACCTCCCTTCCCCCGT
eEF2     gtggctgcccgcaggggacgccctgctgcagatgataaccatccacctcccttccccccgt
WT       GTGGCTGCCCGCAGGGGACGCCCTGCTGCAGATGATAACCATCCACCTCCCTTCCCCCGT
         ************************************************************

Glu56    CACCGCCCAGAAATACCGCTGCGAGCTGCTCTACGAGGGGGCCACCTGATGATGAGGCGGC
Ala56    CACCGCCCAGAAATACCGCTGCGAGCTGCTCTACGAGGGGGCCACCTGATGATGAGGCGGC
eEF2     caccgcccagaaataccgctgcgagctgctctacgaggggccacctgatgatgaggcggc
WT       CACCGCCCAGAAATACCGCTGCGAGCTGCTCTACGAGGGGGCCACCTGATGATGAGGCGGC
         ************************************************************

Glu56    CATGGGCATTAAAAGCTGTGATCCCAAGGGTCCCCTTATGATGTACATTTCCAAGATGGT
Ala56    CATGGGCATTAAAAGCTGTGATCCCAAGGGTCCCCTTATGATGTACATTTCCAAGATGGT
eEF2     catgggcattaaaagctgtgatcccaagggtcccctttatgatgtacatttccaagatggt
WT       CATGGGCATTAAAAGCTGTGATCCCAAGGGTCCCCTTATGATGTACATTTCCAAGATGGT
         ************************************************************

Glu56    GCCAACCTCCGACAAAGGCCGCTTCTACGCCTTTGGTAGAGTGTTCTCCGGGGGTAGTGTC
Ala56    GCCAACCTCCGACAAAGGCCGCTTCTACGCCTTTGGTAGAGTGTTCTCCGGGGGTAGTGTC
eEF2     gccaacctccgacaaaggccgcttctacgcctttggtagagtgttctccggggggtagtgtc
WT       GCCAACCTCCGACAAAGGCCGCTTCTACGCCTTTGGTAGAGTGTTCTCCGGGGGTAGTGTC
         ************************************************************
```

```
Glu56    GACAGGACTCAAGGTCCGCATCATGGGCCCCAACTACACGCCTGGGAAGAAGGAGGACCT
Ala56    GACAGGACTCAAGGTCCGCATCATGGGCCCCAACTACACGCCTGGGAAGAAGGAGGACCT
eEF2     gacaggactcaaggtccgcatcatgggcccccaactacacgcctgggaagaaggaggaact
WT       GACAGGACTCAAGGTCCGCATCATGGGCCCCAACTACACGCCTGGGAAGAAGGAGGACCT
         ************************************************************. **

Glu56    GTACCTGAAGCCCATCCAGAGAACCATCCTGATGATGGGCCGGTATGTGGAGCCAATTGA
Ala56    GTACCTGAAGCCCATCCAGAGAACCATCCTGATGATGGGCCGGTATGTGGAGCCAATTGA
eEF2     gtacctgaagcccatccagagaaccatcctgatgatgggccggtatgtggagccaattga
WT       GTACCTGAAGCCCATCCAGAGAACCATCCTGATGATGGGCCGGTATGTGGAGCCAATTGA
         ************************************************************

Glu56    GGACGTGCCCTGTGGGAACATTGTCGGGCTGGTTGGTGTAGACCAGTTTCTGGTGAAGAC
Ala56    GGACGTGCCCTGTGGGAACATTGTCGGGCTGGTTGGTGTAGACCAGTTTCTGGTGAAGAC
eEF2     ggacgtgccctgtgggaacattgtcgggctggttggtgtagaccagtttctggtgaagac
WT       GGACGTGCCCTGTGGGAACATTGTCGGGCTGGTTGGTGTAGACCAGTTTCTGGTGAAGAC
         ************************************************************

Glu56    AGGGACTATCACCACCTTTGAACACGCACATAACATGCGTGTGATGAAGTTCAGCGTCAG
Ala56    AGGGACTATCACCACCTTTGAACACGCACATAACATGCGTGTGATGAAGTTCAGCGTCAG
eEF2     agggactatcaccacctttgaacacgcacataacatgcgtgtgatgaagttcagcgtcag
WT       AGGGACTATCACCACCTTTGAACACGCACATAACATGCGTGTGATGAAGTTCAGCGTCAG
         ************************************************************

Glu56    CCCTGTCGTCAGAGTGGCTGTGGAGGCCAAGAACCCAGCTGACCTGCCCAAACTGGTGGA
Ala56    CCCTGTCGTCAGAGTGGCTGTGGAGGCCAAGAACCCAGCTGACCTGCCCAAACTGGTGGA
eEF2     ccctgtcgtcagagtggctgtggaggccaagaacccagctgacctgcccaaactggtgga
WT       CCCTGTCGTCAGAGTGGCTGTGGAGGCCAAGAACCCAGCTGACCTGCCCAAACTGGTGGA
         ************************************************************

Glu56    GGGCCTGAAGCGGCTGGCAAAATCTGACCCTATGGTGCAGTGCATCATTGAGGAGTCTGG
Ala56    GGGCCTGAAGCGGCTGGCAAAATCTGACCCTATGGTGCAGTGCATCATTGAGGAGTCTGG
eEF2     gggcctgaagcggctggcaaaatctgaccctatggtgcagtgcatcattgaggagtctgg
WT       GGGCCTGAAGCGGCTGGCAAAATCTGACCCTATGGTGCAGTGCATCATTGAGGAGTCTGG
         ************************************************************

Glu56    GGAGCACATCATTGCTGGAGCAGGTGAGCTGCACCTGGAAATCTGCCTCAAGGACCTGGA
Ala56    GGAGCACATCATTGCTGGAGCAGGTGAGCTGCACCTGGAAATCTGCCTCAAGGACCTGGA
eEF2     ggagcacatcattgctggagcaggtgagctgcacctggaaatctgcctcaaggacctgga
WT       GGAGCACATCATTGCTGGAGCAGGTGAGCTGCACCTGGAAATCTGCCTCAAGGACCTGGA
         ************************************************************

Glu56    GGAGGACCACGCCTGCATCCCCATCAAGAAATCTGACCCTGTTGTCTCATACCGGGAGAC
Ala56    GGAGGACCACGCCTGCATCCCCATCAAGAAATCTGACCCTGTTGTCTCATACCGGGAGAC
eEF2     ggaggaccacgcctgcatccccatcaagaaatctgaccctgttgtctcataccgggagac
WT       GGAGGACCACGCCTGCATCCCCATCAAGAAATCTGACCCTGTTGTCTCATACCGGGAGAC
         ************************************************************
```

```
Glu56    AGTGAGCGAGGAGTCAAATGTGCTGTGCCTGTCCAAGTCGCCCAACAAGCACAACCGATT
Ala56    AGTGAGCGAGGAGTCAAATGTGCTGTGCCTGTCCAAGTCGCCCAACAAGCACAACCGATT
eEF2     agtgagcgaggagtcaaatgtgctgtgcctgtccaagtcgcccaacaagcacaaccgatt
WT       AGTGAGCGAGGAGTCAAATGTGCTGTGCCTGTCCAAGTCGCCCAACAAGCACAACCGATT
         ************************************************************

Glu56    GTATATGAAGGCCCGGCCCTTCCCTGATGGCCTGGCCGAGGACATCGACAAGGGTGAAGT
Ala56    GTATATGAAGGCCCGGCCCTTCCCTGATGGCCTGGCCGAGGACATCGACAAGGGTGAAGT
eEF2     gtatatgaaggcccggcccttccctgatggcctggccgaggacatcgacaagggtgaagt
WT       GTATATGAAGGCCCGGCCCTTCCCTGATGGCCTGGCCGAGGACATCGACAAGGGTGAAGT
         ************************************************************

Glu56    GTCTGCTCGCCAGGAGCTCAAGGCACGCGCACGCTATCTAGCTGAAAAGTACGAATGGGA
Ala56    GTCTGCTCGCCAGGAGCTCAAGGCACGCGCACGCTATCTAGCTGAAAAGTACGAATGGGA
eEF2     gtctgctcgccaggagctcaaggcacgcgcacgctatctagctgaaaagtacgaatggga
WT       GTCTGCTCGCCAGGAGCTCAAGGCACGCGCACGCTATCTAGCTGAAAAGTACGAATGGGA
         ************************************************************

Glu56    TGTTGCTGAAGCCCGCAAGATCTGGTGCTTTGGGCCTGATGGCACTGGCCCCAACATTCT
Ala56    TGTTGCTGAAGCCCGCAAGATCTGGTGCTTTGGGCCTGATGGCACTGGCCCCAACATTCT
eEF2     tgttgctgaagcccgcaagatctggtgctttgggcctgatggcactggccccaacattct
WT       TGTTGCTGAAGCCCGCAAGATCTGGTGCTTTGGGCCTGATGGCACTGGCCCCAACATTCT
         ************************************************************

Glu56    TACCGATATCACCAAGGGTGTGCAGTACCTGAATGAAATCAAGGACAGTGTGGTGGCTGG
Ala56    TACCGATATCACCAAGGGTGTGCAGTACCTGAATGAAATCAAGGACAGTGTGGTGGCTGG
eEF2     taccgatatcaccaagggtgtgcagtacctgaatgaaatcaaggacagtgtggtggctgg
WT       TACCGATATCACCAAGGGTGTGCAGTACCTGAATGAAATCAAGGACAGTGTGGTGGCTGG
         ************************************************************

Glu56    CTTCCAGTGGGCCACTAAGGAGGGCGCTCTCTGTGAGGAGAACATGCGTGGTGTGCGCTT
Ala56    CTTCCAGTGGGCCACTAAGGAGGGCGCTCTCTGTGAGGAGAACATGCGTGGTGTGCGCTT
eEF2     cttccagtgggccactaaggagggcgctctctgtgaggagaacatgcgtggtgtgcgctt
WT       CTTCCAGTGGGCCACTAAGGAGGGCGCTCTCTGTGAGGAGAACATGCGTGGTGTGCGCTT
         ************************************************************

Glu56    CGATGTCCATGATGTGACCCTACATGCTGATGCCATCCACCGAGGAGGTGGTCAGATCAT
Ala56    CGATGTCCATGATGTGACCCTACATGCTGATGCCATCCACCGAGGAGGTGGTCAGATCAT
eEF2     cgatgtccatgatgtgaccctacatgctgatgccatccaccgaggaggtggtcagatcat
WT       CGATGTCCATGATGTGACCCTACATGCTGATGCCATCCACCGAGGAGGTGGTCAGATCAT
         ************************************************************

Glu56    TCCCACAGCTCGTCGTTGTCTGTATGCCAGTGTGTTGACTGCACAGCCCCGCCTCATGGA
Ala56    TCCCACAGCTCGTCGTTGTCTGTATGCCAGTGTGTTGACTGCACAGCCCCGCCTCATGGA
eEF2     tcccacagctcgtcgttgtctgtatgccagtgtgttgactgcacagccccgcctcatgga
WT       TCCCACAGCTCGTCGTTGTCTGTATGCCAGTGTGTTGACTGCACAGCCCCGCCTCATGGA
         ************************************************************
```

```
Glu56    GCCTATCTACCTGGTGGAGATTCAGTGTCCTGAACAGGTCGTGGGTGGCATCTACGGTGT
Ala56    GCCTATCTACCTGGTGGAGATTCAGTGTCCTGAACAGGTCGTGGGTGGCATCTACGGTGT
eEF2     gcctatctacctggtggagattcagtgtcctgaacaggtcgtgggtggcatctacggtgt
WT       GCCTATCTACCTGGTGGAGATTCAGTGTCCTGAACAGGTCGTGGGTGGCATCTACGGTGT
         ************************************************************

Glu56    CCTGAACAGGAAGCGTGGCCATGTGTTTGAAGAGTCCCAGGTGGCTGGCACCCCCATGTT
Ala56    CCTGAACAGGAAGCGTGGCCATGTGTTTGAAGAGTCCCAGGTGGCTGGCACCCCCATGTT
eEF2     cctgaacaggaagcgtggccatgtgtttgaagagtcccaggtggctggcacccccatgtt
WT       CCTGAACAGGAAGCGTGGCCATGTGTTTGAAGAGTCCCAGGTGGCTGGCACCCCCATGTT
         ************************************************************

Glu56    TGTAGTCAAGGCCTATCTTCCAGTCAACGAGTCCTTTGGTTTTACAGCTGACCTGCGCTC
Ala56    TGTAGTCAAGGCCTATCTTCCAGTCAACGAGTCCTTTGGTTTTACAGCTGACCTGCGCTC
eEF2     tgtagtcaaggcctatcttccagtcaacgagtcctttggttttacagctgacctgcgctc
WT       TGTAGTCAAGGCCTATCTTCCAGTCAACGAGTCCTTTGGTTTTACAGCTGACCTGCGCTC
         ************************************************************

Glu56    CAACACTGGTGGCCAAGCTTTCCCCCAGTGTGTGTTCGACCACTGGCAGATTCTGCCCGG
Ala56    CAACACTGGTGGCCAAGCTTTCCCCCAGTGTGTGTTCGACCACTGGCAGATTCTGCCCGG
eEF2     caacactggtggccaagctttcccccagtgtgtgttcgaccactggcagattctgcccgg
WT       CAACACTGGTGGCCAAGCTTTCCCCCAGTGTGTGTTCGACCACTGGCAGATTCTGCCCGG
         ************************************************************

Glu56    GGACCCGTTCGACAACAGCAGCCGTCCCAGCCAAGTGGTGGCTGAGACCCGCAAGCGCAA
Ala56    GGACCCGTTCGACAACAGCAGCCGTCCCAGCCAAGTGGTGGCTGAGACCCGCAAGCGCAA
eEF2     ggacccgttcgacaacagcagccgtcccagccaagtggtggctgagacccgcaagcgcaa
WT       GGACCCGTTCGACAACAGCAGCCGTCCCAGCCAAGTGGTGGCTGAGACCCGCAAGCGCAA
         ************************************************************

Glu56    AGGTCTAAAGGAGGGCATCCCAGCACTGGACAACTTCCTGGACAAACTGTAGAATATCCG
Ala56    AGGTCTAAAGGAGGGCATCCCAGCACTGGACAACTTCCTGGACAAACTGTAAATAT-CCG
eEF2     aggtctaaaggagggcatcccagcactggacaacttcctggacaaactgtag-------
WT       AGGTCTAAAGGAGGGCATCCCAGCACTGGACAACTTCCTGGACAAACTGTAGAATATCCG
         ***********************************************.
```

7.5 Elongation factor 2 kinase short hairpin RNA sequencing

Design and sequencing of eEF2K shRNA plasmid.

<u>Oligo A sequence design for elongation factor 2 kinase shirt hairpin</u>

TCTCCTCATGCCTGCAACCGGATTTCTCGAGAAATCCGGTTGCAGGCATGAGCT

<u>T7 forwards seqeuencing of the eEF2K shRNA plasmid</u>

```
NNNNNNNNGGGCGANNNNNNNGANATTGGATCCTAAGACAGCTTCTTTGGGAGAGAACAGACGCAG
GGGCGGGAGGGAAAAAGGGAGAGGCAGACGTCACTTCCCCTTGGCGGCTCTGGCAGCAGATTGGT
CGGTTGAGTGGCAGAAAGGCAGACGGGGACTGGGCAAGGCACTGTCGGTGACATCACGGACAGGG
CGACTTCTATGTAGATGAGGCAGCGCAGAGGCTGCTGCTTCGCCACTTGCTGCTTCACCACGAAG
GAGTTCCCGTGCCCTGGGAGCGGGTTCAGGACCGCTGATCGGAAGTGAGAATCCCAGCTGTGTGT
CAGGGCTGGAAAGGGCTCGGGAGTGCGCGGGGCAAGTGACCGTGTGTGTAAAGAGTGAGGCGTAT
GAGGCTGTGTCGGGGCAGAGGCCCAAGATCTCCTCATGCCTGCAACCGGATTTCTCGAGAAATCC
GGTTGCAGGCATGAGCTGCAGTCTGGAGTTTCAAAAGTAGACTGGGCGGCCGCATCGATGTTAAC
CTCGAGGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTT
GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATT
GCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGG
CCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTGATCTGCGCAGCACCATGG
CCTGAAATAACCTCTGAAAGAGGAACTTGGTTAGGTACCTTCTGAGGCGGAAAGAACCAGCTGTG
GAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCA
TGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATG
CAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCT
AACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAAAGG
CCGAGGCCGCCTCGGCCTCTGAACTATTCCAGAAGTAATNGAGGAGNNNTTTTTGGAGGCCTAGN
NTTTTGCAAANNNNTTGANTTNNNNGAACCTAGCNNNCATGACCGANNACAGNCCCNGGGGGGNNN
NNNCCCCCNGGNNAAATTCCCCGGGNCGTAAGCCCCNNNNNNNNNGGTTNNCGGNN
```

<u>Alignment</u>

```
100.0% identity in 54 nt overlap (1-54:419-472); score:  270
E(10000): 1.6e-15


              10        20        30        40        50
Oligo  TCTCCTCATGCCTGCAACCGGATTTCTCGAGAAATCCGGTTGCAGGCATGAGCT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query  TCTCCTCATGCCTGCAACCGGATTTCTCGAGAAATCCGGTTGCAGGCATGAGCT
      420       430       440       450       460       470
```

7.6 Qiagen RT2 Profiler PCR Array Mouse mTOR Signaling data

7.6.1 mTOR signalling array gene description

Details of all genes in the array: mTOR Signaling RT^2 Profiler PCR Array from QIAGEN

PCR Array Catalog #:		PAMM-098Z			
Position	UniGene	RefSeq	Symbol	Description	Gene Name
A01	Mm.6645	NM_009652	Akt1	Thymoma viral proto-oncogene 1	Akt, PKB, PKB, Akt, PKBalpha, Rac
A02	Mm.148007	NM_026270	Akt1s1	AKT1 substrate 1 (proline-rich)	1110012J22Rik, AI227026, Lobe, Lobel, PRAS40
A03	Mm.177194	NM_007434	Akt2	Thymoma viral proto-oncogene 2	2410016A19Rik, AW554154, MGC14031, PKB, PKBbeta
A04	Mm.235194	NM_011785	Akt3	Thymoma viral proto-oncogene 3	AI851531, D930002M15Rik, Nmf350
A05	Mm.26135	NM_133781	Cab39	Calcium binding protein 39	AA408805, AA960512, C78372, MO25, MO25alpha
A06	Mm.179091	NM_026908	Cab39l	Calcium binding protein 39-like	1500031K13Rik, 2810425O13Rik, 4930520C08Rik, AA589432, MO2L
A07	Mm.1022	NM_009861	Cdc42	Cell division cycle 42 homolog (S. cerevisiae)	AI747189, AU018915
A08	Mm.3996	NM_007700	Chuk	Conserved helix-loop-helix ubiquitous kinase	AI256658, Chuk1, Fbx24, Fbxo24, IKBKA, IKK1, Ikka, MGC25325, NFKBIKA
A09	Mm.21697	NM_029083	Ddit4	DNA-damage-inducible transcript 4	5830413E08Rik, AA415483, REDD1, Rtp801, dig2
A10	Mm.250841	NM_030143	Ddit4l	DNA-damage-inducible transcript 4-like	1700037B15Rik, 1700108M02Rik, REDD2, RTP801L, Smhs1
A11	Mm.295397	NM_145470	Deptor	DEP domain containing MTOR-interacting protein	4731402B04Rik, 9130412E02Rik, D15Ertd336e,

					D15Ertd597e, Depdc6, Depdc6-001, Depdc6-002, KIAA4200, R75183, mKIAA4200
A12	Mm.290022	NM_145625	Eif4b	Eukaryotic translation initiation factor 4B	2310046H11Rik, AL024095, C85189, Eif4a2
B01	Mm.3941	NM_007917	Eif4e	Eukaryotic translation initiation factor 4E	EG668879, Eif4e-ps, If4e, MGC103177, eIF-4E
B02	Mm.6700	NM_007918	Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1	4e-bp1, AA959816, PHAS-I
B03	Mm.259516	NM_010124	Eif4ebp2	Eukaryotic translation initiation factor 4E binding protein 2	2810011I19Rik, 4E-BP2, AA792569, BC010348, MGC141219, PHAS-II
B04	Mm.278458	NM_008019	Fkbp1a	FK506 binding protein 1a	12kDa, FKBP12, FKBP12-T1, FKBP12-T2, Fkbp, Fkbp1, mFKBP1, mFKBP12
B05	Mm.141864	NM_010223	Fkbp8	FK506 binding protein 8	38kDa, FKBP-38, FKBP-8, Fkbp38
B06	Mm.394930	NM_019827	Gsk3b	Glycogen synthase kinase 3 beta	7330414F15Rik, 8430431H08Rik, C86142, GSK-3, GSK-3beta, GSK3
B07	Mm.3879	NM_010431	Hif1a	Hypoxia inducible factor 1, alpha subunit	AA959795, HIF1alpha, MOP1, bHLHe78
B08	Mm.334313	NM_008284	Hras1	Harvey rat sarcoma virus oncogene 1	H-ras, Ha-ras, Harvey-ras, Hras-1, Kras2, c-H-ras, c-Ha-ras, c-rasHa, ras
B09	Mm.239865	NM_008300	Hspa4	Heat shock protein 4	70kDa, AI317151, APG-2, Hsp110, Hsp70RY, KIAA4025, mKIAA4025
B10	Mm.268521	NM_010512	Igf1	Insulin-like growth factor 1	C730016P09Rik, Igf-1, Igf-I
B11	Mm.29254	NM_008343	Igfbp3	Insulin-like growth factor	AI649005,

				binding protein 3	IGFBP-3, IGgfbp3
B12	Mm.277886	NM_010546	Ikbkb	Inhibitor of kappaB kinase beta	AI132552, IKK-2, IKK-beta, IKK2, IKK[b], IKKbeta
C01	Mm.274846	NM_010562	Ilk	Integrin linked kinase	AA511515, ESTM24
C02	Mm.4946	NM_008387	Ins2	Insulin II	AA986540, Ins-2, InsII, Mody, Mody4, proinsulin
C03	Mm.268003	NM_010568	Insr	Insulin receptor	4932439J01Rik, CD220, D630014A15Rik, IR, IR-A, IR-B
C04	Mm.4952	NM_010570	Irs1	Insulin receptor substrate 1	G972R, IRS-1
C05	Mm.196581	NM_011949	Mapk1	Mitogen-activated protein kinase 1	9030612K14Rik, AA407128, AU018647, C78273, ERK, Erk2, MAPK2, PRKM2, Prkm1, p41mapk, p42mapk
C06	Mm.8385	NM_011952	Mapk3	Mitogen-activated protein kinase 3	Erk-1, Erk1, Ert2, Esrk1, Mnk1, Mtap2k, Prkm3, p44, p44erk1, p44mapk
C07	Mm.270866	NM_177345	Mapkap1	Mitogen-activated protein kinase associated protein 1	AI591529, D230039K05Rik, Sin1
C08	Mm.289516	NM_019988	Mlst8	MTOR associated protein, LST8 homolog (S. cerevisiae)	0610033N12Rik, AA409454, AI505104, AI851821, Gbl
C09	Mm.21158	NM_020009	Mtor	Mechanistic target of rapamycin (serine/threonine kinase)	2610315D21Rik, AI327068, FRAP, FRAP2, Frap1, MGC118056, RAFT1, RAPT1, flat
C10	Mm.234502	NM_008659	Myo1c	Myosin IC	C80397, MMIb, MYO1E, NMI, mm1beta, myr2
C11	Mm.10504	NM_011062	Pdpk1	3-phosphoinositide dependent protein kinase 1	Pdk1
C12	Mm.194127	NM_181414	Pik3c3	Phosphoinositide-3-kinase, class 3	5330434F23Rik, Vps34
D01	Mm.260521	NM_008839	Pik3ca	Phosphatidylinositol 3-	6330412C24Rik,

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				kinase, catalytic, alpha polypeptide	MGC161268, caPI3K, p110, p110alpha
D02	Mm.213128	NM_029094	Pik3cb	Phosphatidylinositol 3-kinase, catalytic, beta polypeptide	1110001J02Rik, AI447572, MGC150132, p110beta
D03	Mm.229108	NM_008840	Pik3cd	Phosphatidylinositol 3-kinase catalytic delta polypeptide	2410099E07Rik, 2610208K16Rik, AW545373, p110delta
D04	Mm.101369	NM_020272	Pik3cg	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	5830428L06Rik, PI3Kgamma, p110gamma
D05	Mm.212039	NM_008875	Pld1	Phospholipase D1	AA536939, C85393, Pld1a, Pld1b
D06	Mm.260177	NM_008876	Pld2	Phospholipase D2	-
D07	Mm.260288	NM_019411	Ppp2ca	Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	PP2A, R75353
D08	Mm.26134	NM_028392	Ppp2r2b	Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform	2900026H06Rik, 6330404L05Rik, E130009M08Rik, MGC113781, PP2A-PR55B, PR55-BETA, SCA12
D09	Mm.275393	NM_138748	Ppp2r4	Protein phosphatase 2A, regulatory subunit B (PR 53)	2610042B21Rik, C77440, MGC60706, MGC7129, N28142, PR53
D10	Mm.207004	NM_0010133 67	Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	AI194361, AI450832, AL024255, AMPKalpha1, C130083N04Rik
D11	Mm.48638	NM_178143	Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit	2310008I11Rik, A830082D05, AMPKalpha2
D12	Mm.458152	NM_031869	Prkab1	Protein kinase, AMP-activated, beta 1 non-catalytic subunit	1300015D22Rik, AU021155, E430008F22
E01	Mm.31175	NM_182997	Prkab2	Protein kinase, AMP-activated, beta 2 non-catalytic subunit	5730553K21Rik, AW049591, BB124140
E02	Mm.6670	NM_016781	Prkag1	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	AA571379, BB036179, Prkaac
E03	Mm.33649	NM_145401	Prkag2	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	2410051C13Rik, AAKG, AAKG2, AI854673, H91620p,

					WPWS
E04	Mm.166501	NM_153744	Prkag3	Protein kinase, AMP-activated, gamma 3 non-catatlytic subunit	AMPKg3L, AMPKg3S, Amkg3, Ampkg3
E05	Mm.222178	NM_011101	Prkca	Protein kinase C, alpha	AI875142, Pkca
E06	Mm.207496	NM_008855	Prkcb	Protein kinase C, beta	A130082F03Rik, PKC-Beta, Pkcb, Prkcb1, Prkcb2
E07	Mm.7980	NM_011102	Prkcc	Protein kinase C, gamma	MGC130440, PKCgamma, Pkcc, Prkcg
E08	Mm.24614	NM_011104	Prkce	Protein kinase C, epsilon	5830406C15Rik, PKC[e], PKCepsilon, Pkce, R75156
E09	Mm.245395	NM_008960	Pten	Phosphatase and tensin homolog	2310035O07Rik, A130070J02Rik, AI463227, B430203M17Rik, MGC183880, MMAC1, TEP1
E10	Mm.319175	NM_053075	Rheb	Ras homolog enriched in brain	-
E11	Mm.757	NM_016802	Rhoa	Ras homolog gene family, member A	Arha, Arha1, Arha2
E12	Mm.275811	NM_030168	Rictor	RPTOR independent companion of MTOR, complex 2	4921505C17Rik, 6030405M08Rik, AVO3, AW492497, D530039E11Rik
F01	Mm.379007	NM_009096	Rps6	Ribosomal protein S6	MGC102571, MGC103209
F02	Mm.301827	NM_009097	Rps6ka1	Ribosomal protein S6 kinase polypeptide 1	Rsk1, p90rsk, rsk
F03	Mm.268383	NM_011299	Rps6ka2	Ribosomal protein S6 kinase, polypeptide 2	90kDa, D17Wsu134e, Rps6ka-rs1, Rsk3, p90rsk, pp90rsk
F04	Mm.220417	NM_153587	Rps6ka5	Ribosomal protein S6 kinase, polypeptide 5	3110005L17Rik, 6330404E13Rik, AI854034, MGC28385, MSK1, MSPK1, RLPK
F05	Mm.394280	NM_028259	Rps6kb1	Ribosomal protein S6 kinase, polypeptide 1	2610318I15Rik, 4732464A07Rik, 70kDa, AA959758, AI256796, AI314060, S6K1, p70, 85s6k, p70s6k

F06	Mm.271937	NM_021485	Rps6kb2	Ribosomal protein S6 kinase, polypeptide 2	70kDa, S6K2
F07	Mm.209933	NM_028898	Rptor	Regulatory associated protein of MTOR, complex 1	4932417H02Rik, Rap, Raptor, mKIAA1303
F08	Mm.31178	NM_178376	Rraga	Ras-related GTP binding A	1300010C19Rik, AI255374, FIP-1, RAGA
F09	Mm.190922	NM_001004154	Rragb	Ras-related GTP binding B	MGC69750, MGC95567
F10	Mm.220922	NM_017475	Rragc	Ras-related GTP binding C	AU041672, Gtr2, MGC47404, RAGC, TIB929, YGR163W
F11	Mm.300814	NM_027491	Rragd	Ras-related GTP binding D	5730543C08Rik, AI467523, C030003H22Rik, D4Ertd174e
F12	Mm.28405	NM_011361	Sgk1	Serum/glucocorticoid regulated kinase 1	Sgk
G01	Mm.44231	NM_011492	Stk11	Serine/threonine kinase 11	AA408040, Lkb1, Par-4, R75140
G02	Mm.286006	NM_172656	Stradb	STE20-related kinase adaptor beta	AA792893, Als2cr2, B830008M19, D1Ucla2, ILPIP, ILPIPA, PRO1038, Papk, Syradb
G03	Mm.288702	NM_027880	Telo2	TEL2, telomere maintenance 2, homolog (S. cerevisiae)	1200003M09Rik, AI415602, Tel2, mKIAA0683
G04	Mm.222	NM_011640	Trp53	Transformation related protein 53	Tp53, bbl, bfy, bhy, p44, p53
G05	Mm.224354	NM_022887	Tsc1	Tuberous sclerosis 1	hamartin, mKIAA0243
G06	Mm.30435	NM_011647	Tsc2	Tuberous sclerosis 2	Tcs2
G07	Mm.271898	NM_009469	Ulk1	Unc-51 like kinase 1 (C. elegans)	AU041434, Unc51.1, mKIAA0722
G08	Mm.162025	NM_013881	Ulk2	Unc-51 like kinase 2 (C. elegans)	A830085I22Rik, AU015340, Unc51.2, mKIAA0623
G09	Mm.282184	NM_009505	Vegfa	Vascular endothelial growth factor A	Vegf, Vegf120, Vegf164, Vegf188, Vpf
G10	Mm.15607	NM_011697	Vegfb	Vascular endothelial growth factor B	VEGF-B, Vrf
G11	Mm.1402	NM_009506	Vegfc	Vascular endothelial growth factor C	AW228853, VEGF-C

G12	Mm.289630	NM_011739	Ywhaq	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	2700028P07Rik, AA409740, AU021156, MGC118161, R74690
H01	Mm.328431	NM_007393	Actb	Actin, beta	Actx, E430023M04Rik, beta-actin
H02	Mm.163	NM_009735	B2m	Beta-2 microglobulin	Ly-m11, beta2-m, beta2m
H03	Mm.343110	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Gapd, MGC102544, MGC102546, MGC103190, MGC103191, MGC105239
H04	Mm.3317	NM_010368	Gusb	Glucuronidase, beta	AI747421, Gur, Gus, Gus-r, Gus-s, Gus-t, Gus-u, Gut, asd, g
H05	Mm.2180	NM_008302	Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1	90kDa, AL022974, C81438, Hsp84, Hsp84-1, Hsp90, Hspcb, MGC115780
H06	N/A	SA_00106	MGDC	Mouse Genomic DNA Contamination	MIGX1B
H07	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H08	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H09	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H10	N/A	SA_00103	PPC	Positive PCR Control	PPC
H11	N/A	SA_00103	PPC	Positive PCR Control	PPC
H12	N/A	SA_00103	PPC	Positive PCR Control	PPC

7.6.2 Raw data collected from the mTOR Signaling RT^2 Profiler PCR Array from QIAGEN using CHO Flp-Inparental cell RNA samples taken 68 hours and 120 hours from culture.

Symbol	Well	AVG ΔC$_t$ (Ct(GOI) - Ave Ct (HKG))		2^-ΔC$_t$		Fold Change	T-TEST	Fold Up- or Down-Regulation	Comments
		120Hr	68Hr	120Hr	68Hr	120Hr/68Hr	p value	120Hr/68Hr	
Akt1	A01	-3.12	-1.63	8.7E+00	3.1E+00	2.82	N/A	2.82	OKAY
Akt1s1	A02	5.05	5.89	3.0E-02	1.7E-02	1.80	N/A	1.80	B
Akt2	A03	-1.98	-0.47	4.0E+00	1.4E+00	2.86	N/A	2.86	OKAY
Akt3	A04	0.92	1.63	5.3E-01	3.2E-01	1.64	N/A	1.64	OKAY
Cab39	A05	6.60	7.65	1.0E-02	5.0E-03	2.08	N/A	2.08	C
Cab39l	A06	-2.29	-0.44	4.9E+00	1.4E+00	**3.62**	N/A	**3.62**	OKAY
Cdc42	A07	3.61	4.43	8.2E-02	4.6E-02	1.77	N/A	1.77	B
Chuk	A08	N/A	N/A	N/A	N/A	**N/A**	N/A	**N/A**	C
Ddit4	A09	3.71	3.37	7.7E-02	9.6E-02	0.79	N/A	-1.26	B
Ddit4l	A10	N/A	N/A	N/A	N/A	**N/A**	N/A	**N/A**	C
Deptor	A11	-1.07	-0.42	2.1E+00	1.3E+00	1.58	N/A	1.58	OKAY
Eif4b	A12	N/A	7.65	N/A	5.0E-03	**N/A**	N/A	**N/A**	C
Eif4e	B01	1.92	2.28	2.6E-01	2.1E-01	1.29	N/A	1.29	A
Eif4ebp1	B02	N/A	N/A	N/A	N/A	**N/A**	N/A	**N/A**	C
Eif4ebp2	B03	N/A	7.65	N/A	5.0E-03	**N/A**	N/A	**N/A**	C
Fkbp1a	B04	6.60	7.65	1.0E-02	5.0E-03	2.08	N/A	2.08	C
Fkbp8	B05	0.88	0.87	5.4E-01	5.5E-01	1.00	N/A	-1.00	OKAY
Gsk3b	B06	-2.10	-0.63	4.3E+00	1.5E+00	2.78	N/A	2.78	OKAY
Hif1a	B07	N/A	7.65	N/A	5.0E-03	**N/A**	N/A	**N/A**	C
Hras1	B08	-0.97	0.60	2.0E+00	6.6E-01	2.98	N/A	2.98	OKAY
Hspa4	B09	4.82	2.95	3.5E-02	1.3E-01	**0.27**	N/A	**-3.64**	B
Igf1	B10	6.60	7.62	1.0E-02	5.1E-03	2.04	N/A	2.04	B
Igfbp3	B11	6.60	6.60	1.0E-02	1.0E-02	1.00	N/A	1.00	B
Ikbkb	B12	0.25	1.75	8.4E-01	3.0E-01	2.84	N/A	2.84	OKAY
Ilk	C01	-0.73	-0.10	1.7E+00	1.1E+00	1.55	N/A	1.55	OKAY
Ins2	C02	6.60	6.96	1.0E-02	8.0E-03	1.29	N/A	1.29	B
Insr	C03	6.60	6.92	1.0E-02	8.2E-03	1.25	N/A	1.25	B
Irs1	C04	2.33	3.78	2.0E-01	7.3E-02	2.74	N/A	2.74	B
Mapk1	C05	3.19	1.92	1.1E-01	2.6E-01	0.42	N/A	-2.40	A
Mapk3	C06	6.60	7.65	1.0E-02	5.0E-03	2.08	N/A	2.08	C
Mapkap1	C07	N/A	7.65	N/A	5.0E-03	**N/A**	N/A	**N/A**	C
Mlst8	C08	1.79	3.26	2.9E-01	1.0E-01	2.78	N/A	2.78	B
Mtor	C09	2.23	3.77	2.1E-01	7.3E-02	2.92	N/A	2.92	B
Myo1c	C10	6.57	5.43	1.1E-02	2.3E-02	0.46	N/A	-2.19	B
Pdpk1	C11	-1.01	0.63	2.0E+00	6.4E-01	**3.13**	N/A	**3.13**	OKAY
Pik3c3	C12	4.43	4.48	4.6E-02	4.5E-02	1.04	N/A	1.04	B
Pik3ca	D01	N/A	N/A	N/A	N/A	**N/A**	N/A	**N/A**	C
Pik3cb	D02	2.61	3.08	1.6E-01	1.2E-01	1.39	N/A	1.39	B
Pik3cd	D03	6.60	6.70	1.0E-02	9.6E-03	1.08	N/A	1.08	B
Pik3cg	D04	6.60	6.15	1.0E-02	1.4E-02	0.74	N/A	-1.36	B
Pld1	D05	3.12	4.38	1.2E-01	4.8E-02	2.41	N/A	2.41	B

Pld2	D06	N/A	N/A	N/A	N/A	**N/A**	N/A	**N/A**	C
Ppp2ca	D07	-4.55	-3.02	2.3E+01	8.1E+00	2.90	N/A	2.90	OKAY
Ppp2r2b	D08	N/A	6.83	N/A	8.8E-03	**N/A**	N/A	**N/A**	B
Ppp2r4	D09	N/A	7.65	N/A	5.0E-03	**N/A**	N/A	**N/A**	C
Prkaa1	D10	3.36	3.16	9.8E-02	1.1E-01	0.87	N/A	-1.14	B
Prkaa2	D11	6.34	3.85	1.2E-02	6.9E-02	**0.18**	N/A	**-5.59**	B
Prkab1	D12	6.60	N/A	1.0E-02	N/A	**N/A**	N/A	**N/A**	C
Prkab2	E01	1.94	3.64	2.6E-01	8.0E-02	**3.26**	N/A	**3.26**	B
Prkag1	E02	6.25	6.43	1.3E-02	1.2E-02	1.14	N/A	1.14	B
Prkag2	E03	-0.27	0.71	1.2E+00	6.1E-01	1.98	N/A	1.98	OKAY
Prkag3	E04	N/A	7.65	N/A	5.0E-03	**N/A**	N/A	**N/A**	C
Prkca	E05	-0.24	0.88	1.2E+00	5.4E-01	2.18	N/A	2.18	OKAY
Prkcb	E06	N/A	7.65	N/A	5.0E-03	**N/A**	N/A	**N/A**	C
Prkcc	E07	N/A	N/A	N/A	N/A	**N/A**	N/A	**N/A**	C
Prkce	E08	5.87	7.19	1.7E-02	6.8E-03	2.51	N/A	2.51	B
Pten	E09	-3.08	-1.92	8.5E+00	3.8E+00	2.24	N/A	2.24	OKAY
Rheb	E10	2.88	2.15	1.4E-01	2.2E-01	0.61	N/A	-1.65	A
Rhoa	E11	-4.62	-3.50	2.5E+01	1.1E+01	2.18	N/A	2.18	OKAY
Rictor	E12	N/A	N/A	N/A	N/A	**N/A**	N/A	**N/A**	C
Rps6	F01	4.53	3.24	4.3E-02	1.1E-01	0.41	N/A	-2.43	B
Rps6ka1	F02	2.51	3.61	1.8E-01	8.2E-02	2.15	N/A	2.15	B
Rps6ka2	F03	6.60	7.05	1.0E-02	7.5E-03	1.37	N/A	1.37	B
Rps6ka5	F04	0.79	2.15	5.8E-01	2.2E-01	2.58	N/A	2.58	OKAY
Rps6kb1	F05	-3.06	-1.59	8.4E+00	3.0E+00	2.78	N/A	2.78	OKAY
Rps6kb2	F06	4.92	6.26	3.3E-02	1.3E-02	2.54	N/A	2.54	B
Rptor	F07	-0.78	0.87	1.7E+00	5.5E-01	**3.15**	N/A	**3.15**	OKAY
Rraga	F08	-2.20	-1.02	4.6E+00	2.0E+00	2.28	N/A	2.28	OKAY
Rragb	F09	0.90	1.76	5.4E-01	2.9E-01	1.82	N/A	1.82	OKAY
Rragc	F10	-2.96	-1.33	7.8E+00	2.5E+00	**3.11**	N/A	**3.11**	OKAY
Rragd	F11	N/A	7.20	N/A	6.8E-03	**N/A**	N/A	**N/A**	B
Sgk1	F12	-0.07	1.76	1.1E+00	2.9E-01	**3.57**	N/A	**3.57**	OKAY
Stk11	G01	3.95	3.89	6.5E-02	6.7E-02	0.96	N/A	-1.04	B
Stradb	G02	N/A	N/A	N/A	N/A	**N/A**	N/A	**N/A**	C
Telo2	G03	6.60	7.47	1.0E-02	5.6E-03	1.84	N/A	1.84	B
Trp53	G04	6.60	7.65	1.0E-02	5.0E-03	2.08	N/A	2.08	C
Tsc1	G05	6.60	7.65	1.0E-02	5.0E-03	2.08	N/A	2.08	C
Tsc2	G06	5.35	4.76	2.5E-02	3.7E-02	0.67	N/A	-1.50	B
Ulk1	G07	6.60	N/A	1.0E-02	N/A	**N/A**	N/A	**N/A**	C
Ulk2	G08	6.30	6.41	1.3E-02	1.2E-02	1.08	N/A	1.08	B
Vegfa	G09	-1.98	-0.74	4.0E+00	1.7E+00	2.37	N/A	2.37	OKAY
Vegfb	G10	-0.31	1.10	1.2E+00	4.7E-01	2.67	N/A	2.67	OKAY
Vegfc	G11	6.60	7.65	1.0E-02	5.0E-03	2.08	N/A	2.08	C
Ywhaq	G12	N/A	N/A	N/A	N/A	**N/A**	N/A	**N/A**	C
Actb	H01	-7.26	-6.19	1.5E+02	7.3E+01	2.11	N/A	2.11	OKAY
B2m	H02	6.60	7.65	1.0E-02	5.0E-03	2.08	N/A	2.08	C
Gapdh	H03	N/A	N/A	N/A	N/A	**N/A**	N/A	**N/A**	C
Gusb	H04	N/A	N/A	N/A	N/A	**N/A**	N/A	**N/A**	C
Hsp90ab1	H05	0.67	-1.47	6.3E-01	2.8E+00	**0.23**	N/A	**-4.39**	OKAY

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Legend (taken from PCR array data analysis Excel spreadsheet tool:

Fold-Change (2^(- Delta Delta Ct)) is the normalized gene expression (2^(- Delta Ct)) in the Test Sample divided the normalized gene expression (2^(- Delta Ct)) in the Control Sample.

Fold-Regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change

Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change.

Fold-change and fold-regulation values greater than 2 are indicated in red; fold-change values less than 0.5 and fold-regulation values less than -2 are indicated in blue.

p-values: The p values are calculated based on a Student's t-test of the replicate 2^(- Delta Ct) values for each gene in the control group and treatment groups, and p values less than 0.05 are indicated in red.

Comments:

A: This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30).

These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result.

This fold-change result may also have greater variations if p value > 0.05; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.

B: This gene's average threshold cycle is relatively high (> 30), meaning that its relative expression level is low, in both control and test samples, and the p-value for the fold-change is either unavailable or relatively high (p > 0.05).

This fold-change result may also have greater variations; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.

C: This gene's average threshold cycle is either not determined or greater than the defined cut-off (deault 35), in both samples meaning that its expression was undetected, making this fold-change result erroneous and un-interpretable.

8 References

Abdel-Fattah, W. et al., 2013. Insights into diphthamide, key diphtheria toxin effector. *Toxins*, 5(5), pp.958–68.

Abiko, F. et al., 2007. Binding preference of eIF4E for 4E-binding protein isoform and function of eIF4E N-terminal flexible region for interaction, studied by SPR analysis. *Biochemical and biophysical research communications*, 355(3), pp.667–72.

Abraham, R.T., 2010. Lysosomal Rag-ulation of mTOR complex 1 activity. *Cell metabolism*, 11(5), pp.341–2.

Adams, G.P. & Weiner, L.M., 2005. Monoclonal antibody therapy of cancer. *Nature biotechnology*, 23(9), pp.1147–57.

Aeder, S.E. et al., 2004. PKC-eta mediates glioblastoma cell proliferation through the Akt and mTOR signaling pathways. *Oncogene*, 23(56), pp.9062–9.

Amadori, M. et al., 2002. Use of recombinant proteins in antibody tests for bovine tuberculosis. *Veterinary microbiology*, 85(4), pp.379–89.

Van Anken, E. et al., 2003. Sequential waves of functionally related proteins are expressed when B cells prepare for antibody secretion. *Immunity*, 18(2), pp.243–53.

Arevalo, J.H. et al., 1994. Structural analysis of antibody specificity. Detailed comparison of five Fab'-steroid complexes. *Journal of molecular biology*, 241(5), pp.663–90.

Argüelles, S. et al., 2014. Elongation factor 2 diphthamide is critical for translation of two IRES-dependent protein targets, XIAP and FGF2, under oxidative stress conditions. *Free radical biology & medicine*, 67, pp.131–8.

Arora, S. et al., 2003. Identification and Characterization of an Inhibitor of Eukaryotic Elongation Factor 2 Kinase against Human Cancer Cell Lines Identification and Characterization of an Inhibitor of Eukaryotic Elongation Factor 2 Kinase against Human Cancer Cell lines. *Cancer Research* , 63, pp.6894–6899.

Arora, S., Yang, J. & Hait, W.N., 2005. Identification of the Ubiquitin-Proteasome Pathway in the Regulation of the Stability of Eukaryotic Elongation Factor-2 Kinase. *Cancer Research*,65 pp.3806–3810.

Ashour, A. a et al., 2014. Targeting elongation factor-2 kinase (eEF-2K) induces apoptosis in human pancreatic cancer cells. *Apoptosis : an international journal on programmed cell death*, 19(1), pp.241–58.

Aspenström, P., 2004. Integration of signalling pathways regulated by small GTPases and calcium. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1742(1-3), pp.51–58.

Avila-Flores, A. et al., 2005. Modulation of the mammalian target of rapamycin pathway by diacylglycerol kinase-produced phosphatidic acid. *The Journal of biological chemistry*, 280(11), pp.10091–10099.

Balasundaram, B., Harrison, S. & Bracewell, D.G., 2009. Advances in product release strategies and impact on bioprocess design. *Trends in biotechnology*, 27(8), pp.477–85.

Baldwin, S. et al., 2003. Transient and stable transfection of Chinese hamster ovary cells with the recombinant feline erythropoietin gene and expression, purification, and biological activity of feline erythropoietin protein. *American Journal of Veterinary Research*, 64(12), pp.1465–1471.

Banerjee, a K., 1980. 5'-Terminal Cap Structure in Eucaryotic Messenger Ribonucleic Acids. *Microbiological reviews*, 44(2), pp.175–205.

Baneyx, F. & Mujacic, M., 2004. Recombinant protein folding and misfolding in Escherichia coli. *Nature biotechnology*, 22(11), pp.1399–408.

Bar-Peled, L. & Sabatini, D.M., 2014. Regulation of mTORC1 by amino acids. *Trends in cell biology*, 24(7), pp.400–406.

Becerra, S. et al., 2012. Exploring the effect of mild hypothermia on CHO cell productivity. *Biochemical Engineering Journal*, 60, pp.1–8.

Birch, J.R. & Racher, A.J., 2006. Antibody production. *Advanced drug delivery reviews*, 58(5-6), pp.671–85.

Blakemore, C. et al., 1998. Take four neuroscientists Alpha-kinases : a new class of protein kinases with a novel catalytic domain. , pp.43–45.

Browne, G.J. & Proud, C.G., 2004.(Apr) A Novel mTOR-Regulated Phosphorylation Site in Elongation Factor 2 Kinase Modulates the Activity of the Kinase and Its Binding to Calmodulin. *Molecular and Cellular Biology*, pp.2986-2997

Browne, G.J. & Proud, C.G., 2002. Regulation of peptide-chain elongation in mammalian cells. *European Journal of Biochemistry*, 269(22), pp.5360–5368.

Butler, M., 2005. Animal cell cultures : recent achievements and perspectives in the production of biopharmaceuticals. *Applied Microbiology Biotechnology* , pp.283–291.

Carter, P., 2001(November). Improving the Efficacy of Antibody-based Cancer Therapies. *Nature reviews*. 1 , pp.118-129

Carter, P.J., 2011. Introduction to current and future protein therapeutics: a protein engineering perspective. *Experimental cell research*, 317(9), pp.1261–9.

Celik, E. & Calık, P., 2012. Production of recombinant proteins by yeast cells. *Biotechnology advances*, 30(5), pp.1108–18.

Chan, A.Y.M. et al., 2004. Activation of AMP-activated protein kinase inhibits protein synthesis associated with hypertrophy in the cardiac myocyte. *The Journal of biological chemistry*, 279(31), pp.32771–32779.

Chong, W.P.K. et al., 2009. Enhanced IFNγ production in adenosine-treated CHOCells: A mechanistic study. *Biotechnology progress*, 25(3), pp.866–873.

Chu, L. & Robinson, D.K., 2001. Industrial choices for protein production by large-scale cell culture. *Biochemical Engineering*, pp.180–187.

Conlon, I. & Raff, M., 2003. Differences in the way a mammalian cell and yeast cells coordinate cell growth and cell-cycle progression. *Journal of Biology*, pp.1–10.

Conn, C.S. & Qian, S.-B., 2014. Nutrient Signaling in Protein Homeostasis: An Increase in Quantity at the Expense of Quality. , *Sci Signal* 6(271), pp.1–22.

Connolly, E. et al., 2006. Hypoxia Inhibits Protein Synthesis through a 4E-BP1 and Elongation Factor 2 Kinase Pathway Controlled by mTOR and Uncoupled in Breast Cancer Cells †.*Molecular and Cellular Biology* , 26(10), pp.3955–3965.

Cosentino-Gomes, D., Rocco-Machado, N. & Meyer-Fernandes, J.R., 2012. Cell Signaling through Protein Kinase C Oxidation and Activation. *International journal of molecular sciences*, 13(9), pp.10697–721.

Cunningham, J.T. et al., 2007. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature*, 450(7170), pp.736–740.

Daramola, O. et al., 2013. A high-yielding CHO transient system: coexpression of genes encoding EBNA-1 and GS enhances transient protein expression. *Biotechnology progress*, 30(1), pp.132–41.

Dibble, C.C. et al., 2012. TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1. *Molecular cell*, 47(4), pp.535–46.

Dong, Z. & Zhang, J.-T., 2006. Initiation factor eIF3 and regulation of mRNA translation, cell growth, and cancer. *Critical reviews in oncology/hematology*, 59(3), pp.169–80.

Dowling, R.J.O. et al., 2010. Dissecting the role of mTOR: Lessons from mTOR inhibitors. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1804(3), pp.433–439.

Dreesen, I. J. & Fussenegger, M., 2011a. Ectopic expression of human mTOR increases viability, robustness, cell size, proliferation, and antibody production of chinese hamster ovary cells. *Biotechnology and Bioengineering*, 108(4), pp.853–866.

Dreesen, I. J. & Fussenegger, M., 2011b. Ectopic expression of human mTOR increases viability, robustness, cell size, proliferation, and antibody production of chinese hamster ovary cells. *Biotechnology and bioengineering*, 108(4), pp.853–66.

Egnatchik, R. et al., 2014. ER calcium release promotes mitochondrial dysfunction and hepatic cell lipotoxicity in response to palmitate overload. *Molecular metabolism*, 3(5), pp.544–53.

Feige, M.J. et al., 2009. An unfolded CH1 domain controls the assembly and secretion of IgG antibodies. *Molecular cell*, 34(5), pp.569–79.

Feige, M.J. & Buchner, J., 2014. Principles and engineering of antibody folding and assembly. *Biochimica et biophysica acta*, pp.12–14.

Fingar, D.C. et al., 2002. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes & development*, 16(12), pp.1472–87.

Fodor, J. et al., 2013. Store-operated calcium entry and calcium influx via voltage-operated calcium channels regulate intracellular calcium oscillations in chondrogenic cells. *Cell Calcium*, 54(1), pp.1–16.

Foster, D. , 2013. Phosphatidic acid and lipid-sensing by mTOR. *Trends in endocrinology and metabolism: TEM*, 24(6), pp.272–8.

Foster, D. , 2009. Phosphatidic acid signaling to mTOR: Signals for the survival of human cancer cells. *Biochim Biophys Acta*, 1791(9), pp.949–955.

Foster, D. , 2007. Regulation of mTOR by phosphatidic acid? *Cancer research*, 67(1), pp.1–4.

Foster, K.G. & Fingar, D.C., 2010. Mammalian target of rapamycin (mTOR): conducting the cellular signaling symphony. *The Journal of biological chemistry*, 285(19), pp.14071–7.

Le Fourn, V. et al., 2014. CHO cell engineering to prevent polypeptide aggregation and improve therapeutic protein secretion. *Metabolic engineering*, 21, pp.91–102.

Fraser, C.S. et al., 2007. eIF3j is located in the decoding center of the human 40S ribosomal subunit. *Molecular cell*, 26(6), pp.811–9.

Frenzel, A., Hust, M. & Schirrmann, T., 2013. Expression of recombinant antibodies. *Frontiers in immunology*, 4(July), p.217.

Fussenegger, M. & Betenbaugh, M.J., 2002. Metabolic engineering II. Eukaryotic systems. *Biotechnology and bioengineering*, 79(5), pp.509–31.

Gautsch, T. et al., 1998. Availability of eIF4E regulates skeletal muscle protein synthesis during recovery from exercise. *The American journal of physiology*, 274(2 Pt 1), pp.C406–14.

Geisse, S. & Henke, M., 2005. Large-scale transient transfection of mammalian cells: a newly emerging attractive option for recombinant protein production. *Journal of structural and functional genomics*, 6(2-3), pp.165–70.

Gibbons, J.J., Abraham, R.T. & Yu, K., 2009. Mammalian target of rapamycin: discovery of rapamycin reveals a signaling pathway important for normal and cancer cell growth. *Seminars in oncology*, 36 Suppl 3(6), pp.S3–S17.

Gingras, a C., Raught, B. & Sonenberg, N., 1999a. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annual review of biochemistry*, 68, pp.913–63.

Gingras, a C., Raught, B. & Sonenberg, N., 1999b. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annual review of biochemistry*, 68, pp.913–63.

Gleason, C.E. et al., 2007. The role of AMPK and mTOR in nutrient sensing in pancreatic beta-cells. *The Journal of biological chemistry*, 282(14), pp.10341–51.

Goldfinger, M. et al., 2011. Protein synthesis in plasma cells is regulated by crosstalk between endoplasmic reticulum stress and mTOR signaling. *European journal of immunology*, 41(2), pp.491–502.

Gran, P. & Cameron-Smith, D., 2011. The actions of exogenous leucine on mTOR signalling and amino acid transporters in human myotubes. *BMC physiology*, 11(1), p.10.

Gulan, M., Gottesman, I.S. & Zinman, B., 1987. Biosynthetic human insulin improves postprandial glucose excursions in type I diabetics. *Annals of internal medicine*, 107(4), pp.506–9.

Gulati, P. et al., 2008. Amino acids activate mTOR complex 1 via Ca2+/CaM signaling to hVps34. *Cell metabolism*, 7(5), pp.456–65.

Gustafsson, C., Govindarajan, S. & Minshull, J., 2004. Codon bias and heterologous protein expression. *Trends in biotechnology*, 22(7), pp.346–53.

Gwinn, D.M. et al., 2008. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Molecular cell*, 30(2), pp.214–26.

Hacker, D.L., De Jesus, M. & Wurm, F.M., 2009. 25 Years of Recombinant Proteins From Reactor-Grown Cells - Where Do We Go From Here? *Biotechnology advances*, 27(6), pp.1023–7.

Hagland, H.R. et al., 2013. Induction of mitochondrial biogenesis and respiration is associated with mTOR regulation in hepatocytes of rats treated with the pan-PPAR activator tetradecylthioacetic acid (TTA). *Biochemical and Biophysical Research Communications*, 430(2), pp.573–578.

Han, Y.K. et al., 2010. Hyperosmotic stress induces autophagy and apoptosis in recombinant Chinese hamster ovary cell culture. *Biotechnology and bioengineering*, 105(6), pp.1187–92.

Hardie, D.G., Ross, F. a & Hawley, S. a, 2012. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nature reviews. Molecular cell biology*, 13(4), pp.251–62.

Hauryliuk, V. et al., 2006. Class-1 release factor eRF1 promotes GTP binding by class-2 release factor eRF3. *Biochimie*, 88(7), pp.747–57.

Hayashi, A. a & Proud, C.G., 2007. The rapid activation of protein synthesis by growth hormone requires signaling through mTOR. *American journal of physiology. Endocrinology and metabolism*, 292(6), pp.E1647–55.

Heise, C. et al., 2014. Elongation factor-2 phosphorylation in dendrites and the regulation of dendritic mRNA translation in neurons. *Frontiers in cellular neuroscience*, 8(February), p.35.

Heitman, J., Movva, N.R. & Hall, M.N., 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science*, 253(5022), pp.905–9.

Hendershot, L. et al., 1987. Assembly and secretion of heavy chains that do not associate posttranslationally with immunoglobulin heavy chain-binding protein. *The Journal of cell biology*, 104(3), pp.761–7.

Heyman, B., 2000. Regulation of Antibody Repsonses via Antibodies, Comlement, And Fc Receptors.*Annu. Rev. Immunol*, (5), pp.709–737.

Highsmith, J., 2013. *Biologic Therapeutic Drugs: Technologies and Global Markets. BCC Research report BIO079B*,

Hinnebusch, A.G., 2006. eIF3: a versatile scaffold for translation initiation complexes. *Trends in biochemical sciences*, 31(10), pp.553–62.

Holland, M. et al., 2006. Differential glycosylation of polyclonal IgG, IgG-Fc and IgG-Fab isolated from the sera of patients with ANCA-associated systemic vasculitis. *Biochimica et biophysica acta*, 1760(4), pp.669–77.

Holt, L.J. et al., 2000. The use of recombinant antibodies in proteomics. *Current opinion in biotechnology*, 11(5), pp.445–9.

Holz, M.K. et al., 2005. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell*, 123(4), pp.569–80.

Hovland, R. et al., 1999. cAMP inhibits translation by inducing Ca2+/calmodulin-independent elongation factor 2 kinase activity in IPC-81 cells. *FEBS letters*, 444(1), pp.97–101.

Hsieh, A.C. & Ruggero, D., 2010. Targeting eukaryotic translation initiation factor 4E (eIF4E) in cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 16(20), pp.4914–20.

Idiris, A. et al., 2010. Engineering of protein secretion in yeast: strategies and impact on protein production. *Applied microbiology and biotechnology*, 86(2), pp.403–17.

Jefferis, R., 2005. Glycosylation of Recombinant Antibody Therapeutics. *Biotechnol. Prog.* 21, pp.11–16.

Jefferis, R. & Lefranc, M., 2009. Human immunoglobulin allotypes. ,*mAbs,* 1(4), pp.1–7.

Jordan, M. et al., 2013. Cell culture medium improvement by rigorous shuffling of components using media blending. *Cytotechnology*, 65(1), pp.31–40.

Jørgensen, R. et al., 2005. Exotoxin A-eEF2 complex structure indicates ADP ribosylation by ribosome mimicry. *Nature*, 436(7053), pp.979–84.

Jossé, L., Smales, C.M. & Tuite, M.F., 2010. Transient expression of human TorsinA enhances secretion of two functionally distinct proteins in cultured Chinese hamster ovary (CHO) cells. *Biotechnology and bioengineering*, 105(3), pp.556–66.

Kapp, L.D. & Lorsch, J.R., 2004. The molecular mechanics of eukaryotic translation. *Annual review of biochemistry*, 73, pp.657–704.

Kaul, G., Pattan, G. & Rafeequi, T., 2011. Eukaryotic elongation factor-2 (eEF2): Its regulation and peptide chain elongation. *Cell Biochemistry and Function*, 29(3), pp.227–234.

Kelley, B., 2009. Industrialization of mAb production technology: the bioprocessing industry at a crossroads. *mAbs*, 1(5), pp.443–52.

Kenney, J.W. et al., 2014. Eukaryotic elongation factor 2 kinase, an unusual enzyme with multiple roles. *Advances in biological regulation*, 55, pp.15–27.

Khoo, S.H.G. & Al-Rubeai, M., 2009. Detailed understanding of enhanced specific antibody productivity in NS0 myeloma cells. *Biotechnology and bioengineering*, 102(1), pp.188–99.

Kim, J.Y., Kim, Y.-G. & Lee, G.M., 2012. CHO cells in biotechnology for production of recombinant proteins: current state and further potential. *Applied microbiology and biotechnology*, 93(3), pp.917–30.

Kim, T.K. & Eberwine, J.H., 2010. Mammalian cell transfection: the present and the future. *Analytical and bioanalytical chemistry*, 397(8), pp.3173–8.

Kimata, Y. & Kohno, K., 1994. Elongation factor 2 mutants deficient in diphthamide formation show temperature-sensitive cell growth. *The Journal of biological chemistry*, 269(18), pp.13497–501.

Kizub, I. V, Klymenko, K.I. & Soloviev, A.I., 2014. Protein kinase C in enhanced vascular tone in diabetes mellitus. *International journal of cardiology*, 174(2), pp.230–42.

Komar, A. a & Hatzoglou, M., 2005. Internal ribosome entry sites in cellular mRNAs: mystery of their existence. *The Journal of biological chemistry*, 280(25), pp.23425–8.

Kotsopoulou, E. et al., 2010. Optimised mammalian expression through the coupling of codon adaptation with gene amplification: maximum yields with minimum effort. *Journal of biotechnology*, 146(4), pp.186–93.

Laplante, M. & Sabatini, D.M., 2009a. An emerging role of mTOR in lipid biosynthesis. *Current biology : CB*, 19(22), pp.R1046–52.

Laplante, M. & Sabatini, D.M., 2009b. mTOR signaling at a glance. *Journal of cell science*, 122(Pt 20), pp.3589–94.

Laplante, M. & Sabatini, D.M., 2012. mTOR Signaling. *Cold Spring Harbor perspectives in biology*, 4(2), pp.2012–2015.

Lee, J.S. & Lee, G.M., 2012. Rapamycin treatment inhibits CHO cell death in a serum-free suspension culture by autophagy induction. *Biotechnology and bioengineering*, 109(12), pp.3093–102.

Leitzgen, K., Knittler, M.R. & Haas, I.G., 1997. Assembly of Immunoglobulin Light Chains as a Prerequisite for Secretion: A model for Oliomerization-dependent Subunit Folding. *Journal of Biological Chemistry*, 272(5), pp.3117–3123.

Leontieva, O. V et al., 2012. Hypoxia suppresses conversion from proliferative arrest to cellular senescence. *Proceedings of the National Academy of Sciences of the United States of America*, 109(33), pp.13314–8.

Leprivier, G. et al., 2013a. The eEF2 kinase confers resistance to nutrient deprivation by blocking translation elongation. *Cell*, 153(5), pp.1064–79.

Leprivier, G. et al., 2013b. The eEF2 kinase confers resistance to nutrient deprivation by blocking translation elongation. *Cell*, 153(5), pp.1064–79.

Li, F. et al., 2010. Cell culture processes for monoclonal antibody production. *mAbs*, 2(5), pp.466–479.

Lim, H.-K. et al., 2003. Phosphatidic acid regulates systemic inflammatory responses by modulating the Akt-mammalian target of rapamycin-p70 S6 kinase 1 pathway. *The Journal of biological chemistry*, 278(46), pp.45117–45127.

Livak, K.J. & Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)*, 25(4), pp.402–8.

Long, X. et al., 2005. Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. *The Journal of biological chemistry*, 280(25), pp.23433–6.

Lynch, C.J., 2001. Symposium : Leucine as a Nutritional Signal Role of Leucine in the Regulation of mTOR by Amino Acids : Revelations from Structure – Activity Studies 1 , 2. , pp.861–865.

Ma, X.M. & Blenis, J., 2009. Molecular mechanisms of mTOR-mediated translational control. *Nature reviews. Molecular cell biology*, 10(5), pp.307–18.

Maag, D. et al., 2005. A conformational change in the eukaryotic translation preinitiation complex and release of eIF1 signal recognition of the start codon. *Molecular cell*, 17(2), pp.265–75.

Maag, D., Algire, M. & Lorsch, J.R., 2006. Communication between eukaryotic translation initiation factors 5 and 1A within the ribosomal pre-initiation complex plays a role in start site selection. *Journal of molecular biology*, 356(3), pp.724–37.

Masterton, R.J. et al., 2010. Post-translational events of a model reporter protein proceed with higher fidelity and accuracy upon mild hypothermic culturing of Chinese hamster ovary cells. *Biotechnology and bioengineering*, 105(1), pp.215–20.

Mead, E.J. et al., 2012. Experimental and in silico modelling analyses of the gene expression pathway for recombinant antibody and by-product production in NS0 cell lines. *PloS one*, 7(10), p.e47422.

Meeting, F. et al., 2006. Translation UK. , (July 2005), pp.21–23.

Merrick, W.C., 2004. Cap-dependent and cap-independent translation in eukaryotic systems. *Gene*, 332, pp.1–11.

Modjtahedi, H., Ali, S. & Essapen, S., 2012. Therapeutic application of monoclonal antibodies in cancer: advances and challenges. *British medical bulletin*, 104, pp.41–59.

Modrak-Wojcik, A. et al., 2013. Eukaryotic translation initiation is controlled by cooperativity effects within ternary complexes of 4E-BP1, eIF4E, and the mRNA 5' cap. *FEBS letters*, 587(24), pp.3928–34.

Nakamura, J. et al., 2009. Overexpression of eukaryotic elongation factor eEF2 in gastrointestinal cancers and its involvement in G2/M progression in the cell cycle. *International journal of oncology*, 34(5), pp.1181–9.

O'Callaghan, P.M. et al., 2010. Cell line-specific control of recombinant monoclonal antibody production by CHO cells. *Biotechnology and bioengineering*, 106(6), pp.938–51.

Ochsenbein, A.F. & Zinkernagel, R.M., 2000. Natural Antibodies and Complement Link Innate and acquired immunity. *Immunology Today*, 5699(12), pp.624–630.

Ortiz, P. a et al., 2006. Translation elongation factor 2 anticodon mimicry domain mutants affect fidelity and diphtheria toxin resistance. *The Journal of biological chemistry*, 281(43), pp.32639–48.

Pain, V.M., 1996. Initiation of protein synthesis in eukaryotic cells. *European journal of biochemistry,* 236(3), pp.747–71.

Palmer, A.E. & Tsien, R.Y., 2006. Measuring calcium signaling using genetically targetable fluorescent indicators. *Nature protocols*, 1(3), pp.1057–65.

Parkinson, D.R., Johnson, B.E. & Sledge, G.W., 2012. Making personalized cancer medicine a reality: challenges and opportunities in the development of biomarkers and companion diagnostics. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 18(3), pp.619–24.

Pavur, K.S., Petrov, a N. & Ryazanov, a G., 2000. Mapping the functional domains of elongation factor-2 kinase. *Biochemistry*, 39(40), pp.12216–24.

Pestova, T. V et al., 2000. The joining of ribosomal subunits in eukaryotes requires eIF5B. *Nature*, 403(6767), pp.332–5.

Pestova, T. V & Kolupaeva, V.G., 2002. The roles of individual eukaryotic translation initiation factors in ribosomal scanning and initiation codon selection. *Genes & development*, 16(22), pp.2906–22.

Peterson, T.R. et al., 2010. NIH Public Access. , 137(5), pp.873–886.

Peterson, T.R. & Sabatini, D.M., 2005. eIF3: a connecTOR of S6K1 to the translation preinitiation complex. *Molecular cell*, 20(5), pp.655–7.

Pigott, C.R. et al., 2012. Insights into the regulation of eukaryotic elongation factor 2 kinase and the interplay between its domains. *The Biochemical journal*, 442(1), pp.105–18.

Proud, C.G., 2004. mTOR-mediated regulation of translation factors by amino acids. *Biochemical and Biophysical Research Communications*, 313(2), pp.429–436.

Proud, C.G., 2002. Regulation of mammalian translation factors by nutrients. *European Journal of Biochemistry*, 269(22), pp.5338–5349.

Raje, N. et al., 2004. Combination of the mTOR inhibitor rapamycin and CC-5013 has synergistic activity in multiple myeloma. *Blood*, 104(13), pp.4188–4193.

Reddy, P.S. & Corley, R.B., 1999. The contribution of ER quality control to the biologic functions of secretory IgM. *Immunology today*, 20(12), pp.582–8.

Robinson, A.R.T.H.U.R., 1958. Genetics of Somatic Mammalain cells. *The Journal of Experimental Medicine* , 108, pp.945-955.

Roobol, A. et al., 2009. Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming. *FEBS Journal*, 276(1), pp.286–302.

Roobol, A. et al., 2014. The chaperonin CCT interacts with and mediates the correct folding and activity of three subunits of translation initiation factor eIF3: b, i and h. *The Biochemical journal*, 458(2), pp.213–24.

Rose, A.J. et al., 2005. Exercise rapidly increases eukaryotic elongation factor 2 phosphorylation in skeletal muscle of men. *The Journal of physiology*, 569(Pt 1), pp.223–228.

Rosettani, P. et al., 2007. Structures of the human eIF4E homologous protein, h4EHP, in its m7GTP-bound and unliganded forms. *Journal of Molecular Biology*, 368(3), pp.691–705.

Roux, P.P. et al., 2004. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. , 101(37), pp.13489–13494.

Sabatini, D.M. et al., 1995. The Rapamycin and FKBP12 Target (RAFT) Displays Phosphatidylinositol 4-Kinase Activity. *Journal of Biological Chemistry*, 270(36), pp.20875–20878.

Sachs, B., Sarnow, P. & Hentze, M.W., 1997. Starting at the beginning, middle, and end: translation initiation in eukaryotes. *Cell*, 89(6), pp.831–8.

Salas-marco, J. & Bedwell, D.M., 2004. GTP Hydrolysis by eRF3 Facilitates Stop Codon Decoding during Eukaryotic Translation Termination. *Molecular and Cellular Biology*, 24(17).

Sancak, Y. et al., 2007. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Molecular cell*, 25(6), pp.903–15.

Sancak, Y. et al., 2010. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell*, 141(2), pp.290–303.

Sancak, Y. et al., 2008. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science*, 320(5882), pp.1496–501.

Sanchez, N. et al., 2014. CHO cell culture longevity and recombinant protein yield are enhanced by depletion of miR-7 activity via sponge decoy vectors. *Biotechnology Journal*, 9(3), pp.396–404.

Santoro, R., Lienemann, P. & Fussenegger, M., 2009. Epigenetic engineering of ribosomal RNA genes enhances protein production. *PloS one*, 4(8), p.e6653.

Schieke, S.M. et al., 2006. The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *The Journal of biological chemistry*, 281(37), pp.27643–52.

Schmidlin, H., Diehl, S. a & Blom, B., 2009. New insights into the regulation of human B-cell differentiation. *Trends in immunology*, 30(6), pp.277–85.

Schneider, A., Younis, R.H. & Gutkind, J.S., 2008. Hypoxia-induced energy stress inhibits the mTOR pathway by activating an AMPK/REDD1 signaling axis in head and neck squamous cell carcinoma. *Neoplasia*, 10(11), pp.1295–302.

Schroeder, H.W. & Cavacini, L., 2010. Structure and function of immunoglobulins. *The Journal of allergy and clinical immunology*, 125(2 Suppl 2), pp.S41–52.

Schwanhäusser, B. et al., 2011. Global quantification of mammalian gene expression control. *Nature*, 473(7347), pp.337–42.

Sengupta, J. et al., 2008. Visualization of the eEF2-80S ribosome transition-state complex by cryo-electron microscopy. *Journal of molecular biology*, 382(1), pp.179–87.

Shin, B.-S. et al., 2011. Initiation factor eIF2γ promotes eIF2-GTP-Met-tRNAi(Met) ternary complex binding to the 40S ribosome. *Nature structural & molecular biology*, 18(11), pp.1227–34.

Sibbald, B., 1999. Making a case for a $2700-a-month drug. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne*, 161(9), p.1173.

Smales, C.M. et al., 2004. Comparative proteomic analysis of GS-NS0 murine myeloma cell lines with varying recombinant monoclonal antibody production rate. *Biotechnology and bioengineering*, 88(4), pp.474–88.

Sonenberg, N. & Hinnebusch, A.G., 2009. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell*, 136(4), pp.731–45.

Sörman, A. et al., 2014. How antibodies use complement to regulate antibody responses. *Molecular immunology*, 61(2), pp.79–88.

Spahn, C.M. et al., 2001. Hepatitis C virus IRES RNA-induced changes in the conformation of the 40s ribosomal subunit. *Science (New York, N.Y.)*, 291(5510), pp.1959–62.

Spahn, C.M.T. et al., 2004. Domain movements of elongation factor eEF2 and the eukaryotic 80S ribosome facilitate tRNA translocation. *The EMBO journal*, 23(5), pp.1008–19.

Staton, T., 2012. No Title. *Top 10 Best-selling Cancer Drugs*. Available at: http://www.fiercepharma.com/special-reports/top-10-best-selling-cancer-drugs/top-10-best-selling-cancer-drugs.

Stevens, A.-S. et al., 2014. Toxicity profiles and solvent-toxicant interference in the planarian Schmidtea mediterranea after dimethylsulfoxide (DMSO) exposure. *Journal of applied toxicology : JAT*, (February).

Suryawan, A. et al., 2012. Differential regulation of protein synthesis in skeletal muscle and liver of neonatal pigs by leucine through an mTORC1-dependent pathway. *Journal of Animal Science and Biotechnology*, 3(1), p.3.

Tan, C.Y. & Hagen, T., 2013. Post-translational regulation of mTOR complex 1 in hypoxia and reoxygenation. *Cellular signalling*, 25(5), pp.1235–44.

Taylor, D.J. et al., 2007. Structures of modified eEF2 80S ribosome complexes reveal the role of GTP hydrolysis in translocation. *The EMBO journal*, 26(9), pp.2421–31.

Thaisuchat, H. et al., 2011. Identification of a novel temperature sensitive promoter in cho cells. *BMC biotechnology*, 11(1), p.51.

Toschi, A. et al., 2009. Regulation of mTORC1 and mTORC2 complex assembly by phosphatidic acid: competition with rapamycin. *Molecular and cellular biology*, 29(6), pp.1411–1420.

Underhill, M.F. et al., 2006. On the Effect of Transient Expression of Mutated eIF2 α and eIF4E Eukaryotic Translation Initiation Factors on Reporter Gene Expression in Mammalian Cells Upon Cold-Shock. , 34.

Urlaub, G. et al., 1983. No TitleDeletion of the diploid dihydrofolate reductase locus from cultured mammalian cells. *Cell Press*, 2(33), pp.405–12.

Urlaub, G. & Chasin, L. a, 1980. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proceedings of the National Academy of Sciences of the United States of America*, 77(7), pp.4216–20.

Vanhove, M., Usherwood, Y.K. & Hendershot, L.M., 2001. Unassembled Ig heavy chains do not cycle from BiP in vivo but require light chains to trigger their release. *Immunity*, 15(1), pp.105–14.

Vernon, J.A., Golec, J.H. & Dimasi, J.A., 2010. Drug Development Costs when Financial Risk is Measured Using the Fama-French Three-Factor Model.*Health Economics Letters*, 1005(August 2009), pp.1002–1005.

Walsh, G., 2010a. Biopharmaceutical benchmarks 2010. *Nature biotechnology*, 28(9), pp.917–24.

Walsh, G., 2010b. Post-translational modifications of protein biopharmaceuticals. *Drug discovery today*, 15(17-18), pp.773–80.

Wang, L., Wang, X. & Proud, C.G., 2000. Activation of mRNA translation in rat cardiac myocytes by insulin involves multiple rapamycin-sensitive steps. *American journal of physiology. Heart and circulatory physiology*, 278(4), pp.H1056–H1068.

Wang, W. et al., 2007. Antibody structure, instability, and formulation. *Journal of pharmaceutical sciences*, 96(1), pp.1–26.

Wang, X. & Proud, C., 2006. The mTOR pathway in the control of protein synthesis. *Physiology*, 21, pp.362–369.

Werner, R.G., Kopp, K. & Schlueter, M., 2007. Glycosylation of therapeutic proteins in different production systems. *Acta paediatrica (Oslo, Norway : 1992). Supplement*, 96(455), pp.17–22.

Wiseman, S.L. et al., 2013. Proteasomal degradation of eukaryotic elongation factor-2 kinase (EF2K) is regulated by cAMP-PKA signaling and the SCFβTRCP ubiquitin E3 ligase. *The Journal of biological chemistry*, 288(24), pp.17803–11.

Wouters, B.G. & Koritzinsky, M., 2008. Hypoxia signalling through mTOR and the unfolded protein response in cancer. *Nature reviews. Cancer*, 8(11), pp.851–864.

Wurm, F.M., 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nature Biotechnology*, 22(11), pp.1393–8.

Xu, Y. et al., 2004. Activation of mTOR signaling by novel fluoromethylene phosphonate analogues of phosphatidic acid. *Bioorganic and Medicinal Chemistry Letters*, 14(6), pp.1461–1464.

Zheng, K., Bantog, C. & Bayer, R., 2011. The impact of glycosylation on monoclonal antibody conformation and stability. *mAbs*, 3(6), pp.568–76.

Zola, H. & Swart, B., 2005. The human leucocyte differentiation antigens (HLDA) workshops: the evolving role of antibodies in research, diagnosis and therapy. *Cell Research*, 15(9), pp.691–4.

Zoncu, R., Efeyan, A. & Sabatini, D.M., 2011. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature reviews. Molecular Cell Biology*, 12(1), pp.21–35.