# Regulating the Expression of eEF1A to Decipher its Non Canonical Functions in Eukaryotic Cells

Kerry Ann Hallbrook

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Aston University

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**Abstract**

The canonical function of eEF1A is delivery of the aminoacylated tRNA to the A site of the ribosome during protein translation, however, it is also known to be an actin binding protein. As well as this actin binding function, eEF1A has been shown to be involved in other cellular processes such as cell proliferation and apoptosis. It has long been thought that the actin cytoskeleton and protein synthesis are linked and eEF1A has been suggested to be a candidate protein to form this link, though very little is understood about the relationship between its two functions. Overexpression of eEF1A has also been shown to be implicated in many different types of cancers, especially cancers that are metastatic, therefore it is important to further understand how eEF1A can affect both translation and the organisation of the actin cytoskeleton. To this end, we aimed to determine the effects of reduced expression of eEF1A on both translation and its non canonical functions in CHO cells. We have shown that reduced expression of eEF1A in this cell system results in no change in protein synthesis, however results in an increased number of actin stress fibres and other proteins associated with these fibres such as myosin IIA, paxillin and vinculin. Cell motility and attachment are also affected by this reduction in eEF1A protein expression. The organisational and motility phenotypes were found to be specific to eEF1A by transforming the cells with plasmids containing either human eEF1A1 or eEF1A2. Though the mechanisms by which these effects are regulated have not yet been established, this data provides evidence to show that the translation and actin binding functions of eEF1A are independent of each other as well as being suggestive of a role for eEF1A in cell motility as supported by the observation that overexpression of eEF1A protein tends to be associated with the cancer cells that are metastatic.

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them, two of the most inspirational women I have ever had the pleasure of spending time with and have had a big impact on making me the person that I am today.

**Table of Contents**

# List of Tables

# List of Figures

# List of Abbreviations

| | |
|---|---|
| **AA** | Amino acid |
| **aa-tRNA** | Aminoacyl transfer ribonucleic acid |
| **ABP** | Actin binding protein |
| **ABP-50** | Actin binding protein 50/EF1A |
| **ADF** | Actin depolymerising factor |
| **ADP** | Adenosine triphosphate |
| **ALS** | Amyotrophic lateral sclerosis |
| **Arp** | Actin related protein |
| **ATP** | Adenosine triphosphate |
| **$Ca^{2+}$** | Calcium |
| **CaM** | Calmodulin |
| **Cdc42** | Cell division control protein 42 |
| **CHO** | Chinese hamster ovary |
| **CHX** | Cycloheximide |
| **ECM** | Extracellular matrix |
| **eEF1A** | Eukaryotic elongation factor 1α |
| **eEF1β** | Elongation factor 1β |
| **eEF2** | Eukaryotic elongation factor 2 |
| **eIF** | Eukaryotic initiation factor |
| **ER** | Endoplasmic reticulum |
| **eRF** | Eukaryotic release factor |

| | |
|---|---|
| **F-actin** | Fibrous actin |
| **FAK** | Focal adhesion kinase |
| **G Protein** | Guanine nucleotide-binding protein |
| **G-actin** | Globular actin |
| **GAP** | GTPase accelerating protein |
| **GDP** | Guanosine diphosphate |
| **GEF** | Guanine nucleotide exchange factor |
| **Grb2** | Growth factor receptor-bound protein 2 |
| **GTP** | Guanosine-5'-triphosphate |
| **HBV** | Hepatitis B virus |
| **HIV** | Human immunodeficiency virus |
| **HPV** | Human papillomavirus |
| **IL-3** | Interleukin 3 |
| **Met** | Methionine |
| **MMP-2** | Matrix metalloproteinase 2 |
| **mRNA** | Messenger ribonucleic acid |
| **pAkt** | Phosphorylated Akt |
| **PI(4,5)$P_2$** | Phosphatidylinositol *4,5*-bisphosphate |
| **PI4kIIIβ** | Phosphatidylinositol 4-kinase IIIβ |
| **PKB** | Protein kinase B |
| **Prdx-1** | Peroxiredoxin-1 |
| **PTI-1** | Prostate tumour inducing gene 1 |
| **rasGAP** | Ras guanosine-5'-triphosphatase activating protein |

| | |
|---|---|
| **RF** | Release factor |
| **ROCK** | Rho Kinase |
| **SF** | Stress fibres |
| **SH2** | Src homology 2 |
| **SH3** | Src homology 3 |
| **siRNA** | Small interfering ribonucleic acid |
| **SMN** | Survival motor neuron protein |
| **tRNA** | Transfer ribonucleic acid |
| **UTR** | Untranslated region |
| **VASP** | Vasodilator stimulated phosphoprotein |
| **WASP** | Wiskott-Aldrich syndrome protein |
| **WAVE** | Wiskott-Aldrich syndrome protein (WASP) family verprolin homologous protein |
| **WNV** | West Nile virus |
| **ZNF9** | Zinc finger factor 9 |
| **ZPR1** | Zinc finger protein 1 |

# Chapter 1: Introduction

**Chapter 1 Table of Contents**

# 1.0 Introduction

## *1.1 Protein Translation and eEF1A*

Protein translation is an essential process to synthesise new proteins for growth and repair. It occurs in three stages that are carefully controlled by numerous proteins throughout each stage. Initiation is the first and is thought to be the most rate limiting step. It is during this phase that the correct positioning of the ribosome on the mRNA, at the initiation codon AUG is regulated to enable the accurate recognition of the open reading frame and therefore the correct synthesis of the amino acid sequence as shown in figure 1.1.1.

The ribosome is made up of the 40S and 60S subunits which come together to make up the complete 80S ribosome. In the first stages of initiation, the 40S subunit of the ribosome is bound by both eukaryotic initiation factor (eIF) eIF1A and eIF3 (Pain, 1995), and the 60S subunit possibly bound by eIF6 (Gingras et al., 1999). These steps are necessary to prevent the reassociation of the 40S and 60S subunits. In the early phases of the activation of the 40S ribosomal subunits, two different processes take place. First, the eIF2 in complex with guanine triphosphate (GTP) binds to initiator or methionine tRNA which results in the delivery of the met-tRNA-eIF2-GTP complex to the 40S subunit to form the preinitiation complex. This preinitiation complex then binds to the 5' cap of the mRNA through the activity of eIF4s, after which the complex migrates to the initiation AUG codon. The eIF4F complex is made up of eIF4E, which has the ability to bind the 5' cap of the mRNA, eIF4A, which is an RNA helicase, and eIF4G which is the backbone of the complex and can bind eIF4E, eIF4A and eIF3, which can bind to the 40S ribosomal subunit (Gingras et al., 1999). This means that eIF4F can interact with both the mRNA cap and the ribosomal subunit as well as providing a way to unravel the mRNA such that the ribosome can scan along the mRNA to find the initiation codon.

Once the preinitiation complex is positioned at the initiation codon, the GTPase accelerating protein activity (GAP) of eIF5 results in inactivation of eIF2 through the hydrolysis of the GTP to guanine diphosphate (GDP), which causes the release of the initiation factors from the complex, allowing the 60S subunit to associate to form the 80S ribosome ready for the elongation stage. The hydrolysed GDP complexed with eIF2 is then prevented from release by the guanine dissociation inhibition activity of eIF5 until the GDP is recycled back to GTP by the

guanine nucleotide exchange factor (GEF) eIF2B (Jennings et al., 2013). Most initiation events are reinitiation events, which occur on mRNA that are already associated with translating ribosomes resulting in polysomes associated with the mRNA.

Reunification of the two ribosomal subunits, along with the release of the different initiation factors and the recognition of the AUG codon ultimately lead to the completion of the initiation step. Following from this, the elongation phase will begin where the bulk of the amino acid incorporation takes place and where, in essence, the proteins are being synthesised, as shown in figure 1.1.2. In this phase, the peptide chain is elongated by delivery of the aminoacylated tRNA to the ribosome so that a codon anti codon match can be made and the correct amino acid added to the peptide chain. This step is performed by the eukaryotic elongation factor 1A (eEF1A).

The eukaryotic elongation factor 1A (eEF1A) is the guanine nucleotide binding protein responsible for transporting the aminoacylated tRNA to the A site of the ribosome. eEF1A binds to aminoacylated tRNA, and delivers them to the exposed A site of the ribosome. Matching of the mRNA codon with the proper tRNA triggers GTPase activity converting GTP back to GDP and causing eEF1A to dissociate from the ribosome (Riis et al., 1990). Binding of eEF1A to the γ subunit of the guanine nucleotide exchange factor (GEF) eEF1β allows GDP to be replaced by GTP, reactivating eEF1A in the process so that it can bind to another aminoacyl tRNA. eEF1A in its GDP bound form has also been shown to interact with deacylated tRNA, which can cause localisation of the eEF1A to aminoacyl tRNA synthetase (Petrushenko et al., 2002, Petrushenko et al., 1997).

Once the correct aminoacylated tRNA has been integrated in the A site of the ribosome, translocation occurs which transfers the deacylated tRNA to the E site of the ribosome and the peptidyl tRNA to the P site resulting in an empty A site for a new aminoacylated tRNA to enter (Merrick, 1992). When the next aminoacylated tRNA binds to the A site of the ribosome, the deacylated tRNA at the E site is released and the process continues until an in frame stop codon is reached at the end of the mRNA sequence.

Translocation of the ribosome on the mRNA is assisted by eEF2, a G protein which utilises the independence of the two different ribosomal subunits to move the ribosome one codon along the mRNA. When bound to GTP, eEF2 can bind to the 40S ribosomal subunit causing a rotation which moves the tRNAs from the A and P sites in the ribosome to the P and E sites. Hydrolysis of the GTP to GDP causes the release of eEF2 - GDP from the ribosome, with the

rotation of the ribosome returning, resulting in the repositioning of the tRNAs as well as the movement of the ribosome to the next codon, ready for the delivery of the next aminoacylated tRNA to the ribosome by eEF1A (Spahn et al., 2004).

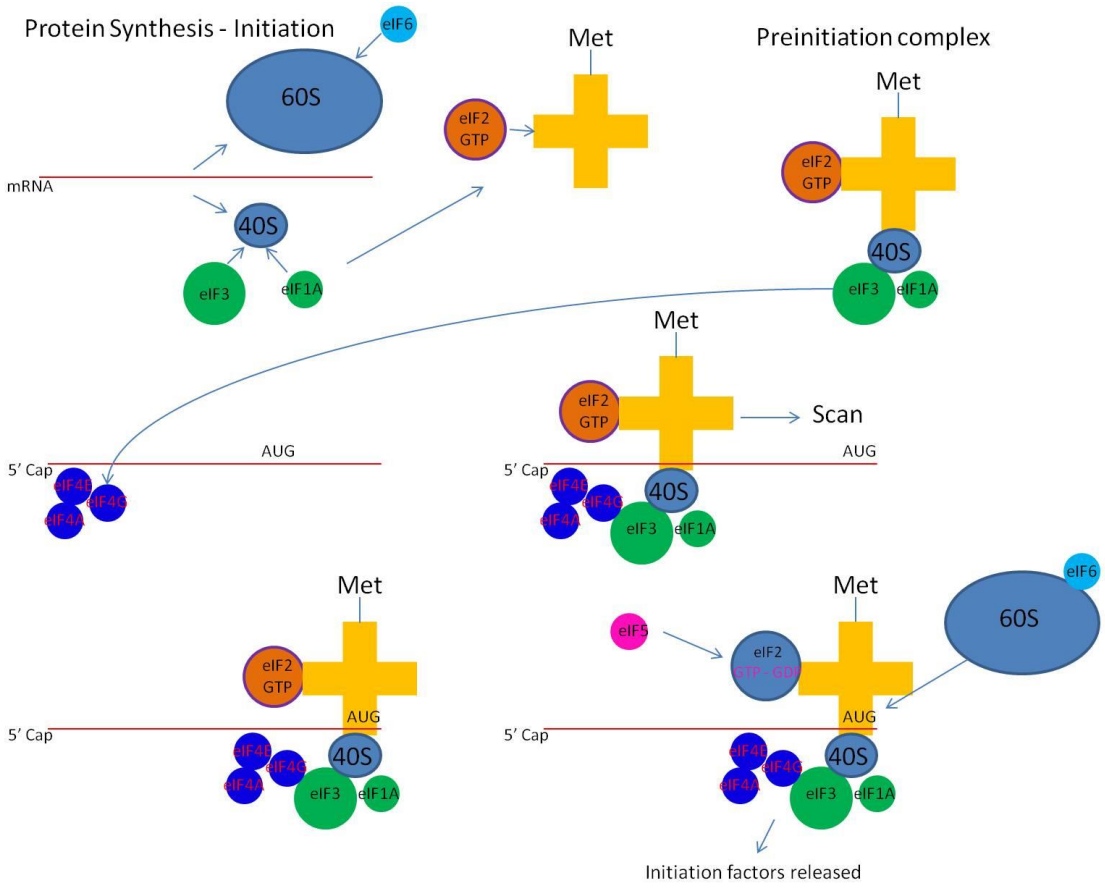



**Figure 1.1.1 –** Schematic representation of protein translation initiation. The 40 and 60S ribosomal subunits dissociate from the mRNA. The 40S subunit is bound by eIF3 and eIF1A, whilst the 60S subunit is bound by eIF6. eIF2 binds methionine tRNA allowing the delivery of this complex to the 40S complex to form the preinitiation complex. The preinitiation complex can then bind to the 5' cap of the mRNA through eIF4s, and the complex migrates along the mRNA to the start codon. eIF5 causes the inactivation of eIF2 by causing the hydrolysis of GTP-GDP which results in release of initiation factors allowing the 60S subunit to associate to form the assembled 80S ribosome on the mRNA ready for elongation.

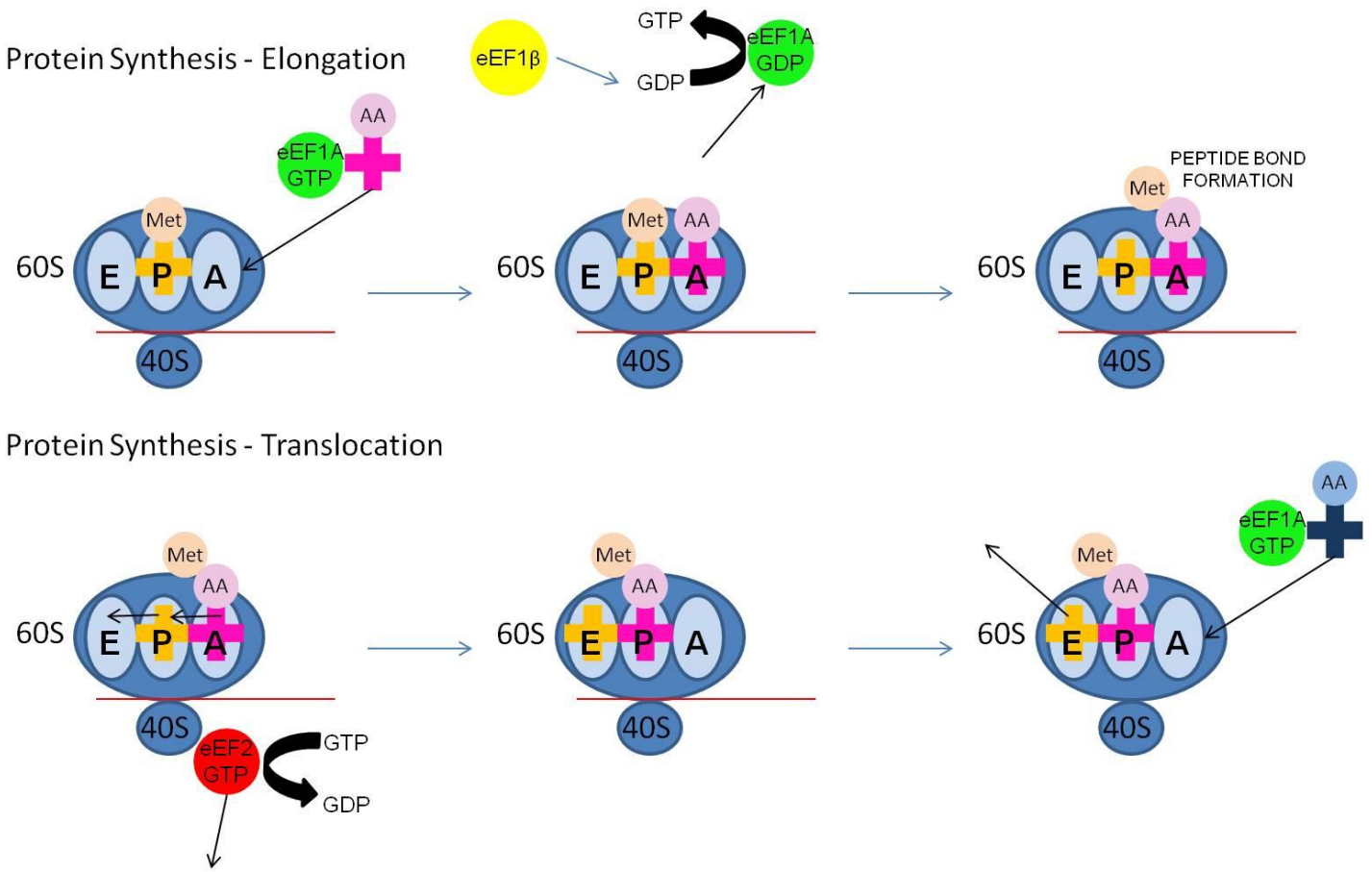



**Figure 1.1.2 –** Schematic representation of protein translation elongation. eEF1A-GTP delivers the aminoacylated tRNA to the A site of the ribosome. Upon the match of codon to anti-codon, the GTP is hydrolysed to GDP and eEF1A is released. A peptide bond is formed between the two amino acids, and when eEF2 binds to the 40S subunit, a rotation occurs which causes translocation which moves the tRNAs one space to the left, so that the two tRNAs are now occupying the E and P sites. The tRNA in the P site is then released and the next tRNA can enter the A site of the ribosome.

Protein synthesis is stopped by binding of a protein release factor (RF), which allows the fully translated protein to be released from the mRNA, and the ribosome to dissociate into its 40S and 60S subunits in the final stage of translation, termination, as described in figure 1.1.3. When one of the termination codons is reached, UAA, UGA or UAG, the polypeptide chain is released in a GTP dependent manner (Goldstein et al., 1970, Beaudet and Caskey, 1971). eRF1, which has a ribosome binding site, a termination codon recognition site, a peptidyl tRNA interaction site and an eRF3 binding site, recognises the termination codon, and binds to the ribosome (Kisselev et al., 2003). eRF3, a G protein, binds in its GDP bound state to the ribosome only when eRF1 is bound, after which the GDP dissociates leading to a complex of guanine free eRF3 with eRF1 bound to the ribosome. After hydrolysis of the ester bond in peptidyl tRNA, GTP can bind to eRF3 bound to the ribosome resulting in a conformational change which leads to the release of eRF1 from the complex. The hydrolysis of GTP to GDP results in a low affinity of eRF3 for the ribosome without eRF1 resulting in the dissociation of eRF3 from the ribosome ready for the next round of termination (Kisselev et al., 2003).

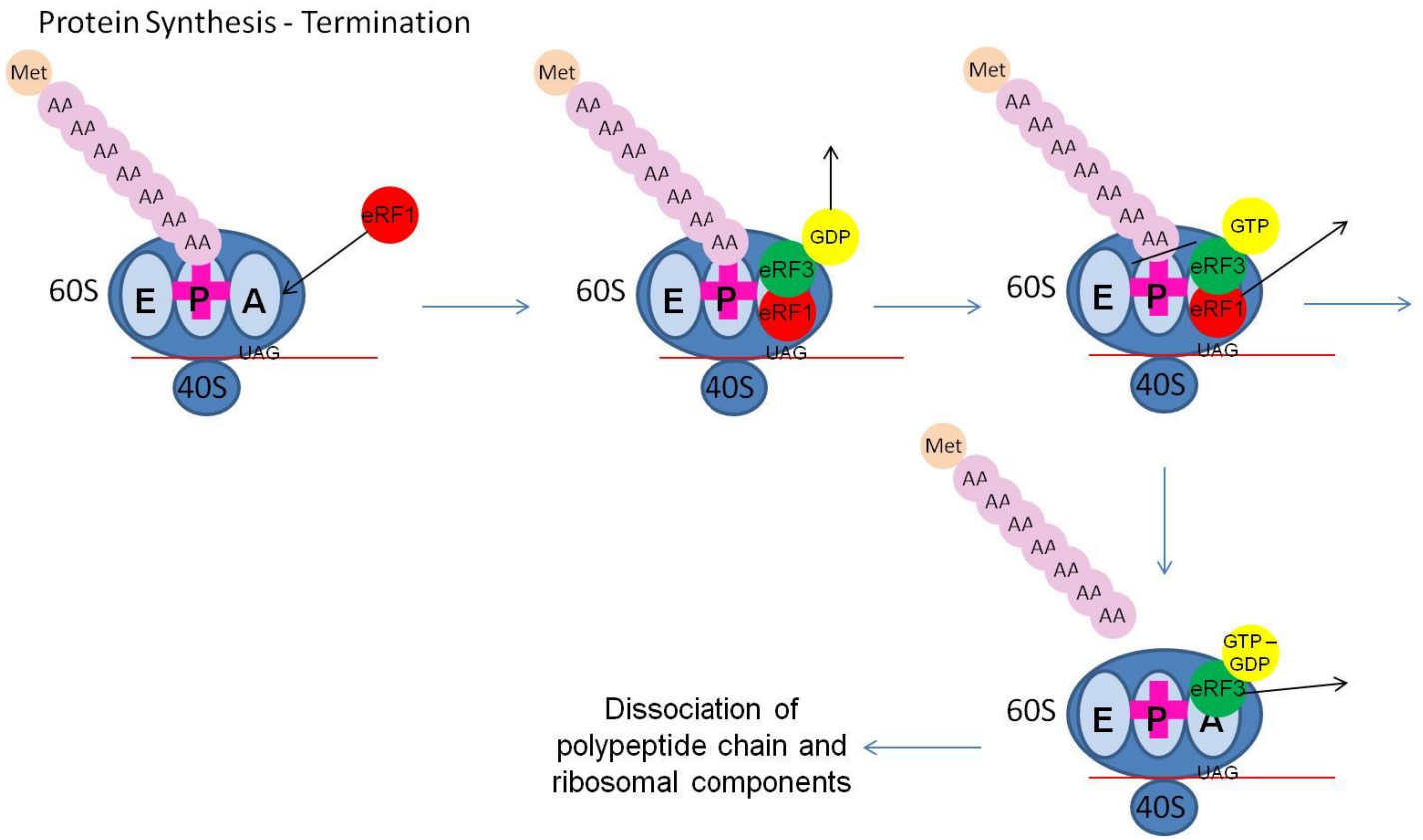



**Figure 1.1.3** – Schematic representation of protein translation termination. When the stop codon is reached, eRF1 can bind to the ribosome, and when eRF1 is bound, eRF3-GDP can also bind to the ribosome, after which the GDP dissociates. After hydrolysis of the ester bond between the amino acid and the tRNA, GTP can then bind to eRF3 causing a conformational change leading to release of eRF1. Hydrolysis of GTP to GDP then results in a lower affinity of eRF3, leading to its release and dissociation of the ribosomal complex from the mRNA.

## *1.2 Structure of eEF1A*

The eEF1A protein has been demonstrated to be composed of three structural domains. Domain I is responsible for GTP binding, domain II for tRNA binding and domain III for actin binding (Moore and Cyr, 2000). The guanine nucleotide exchange factor (GEF), eEF1β, interacts with both domain I and II of eEF1A (Andersen et al., 2001). The elucidation of the molecular structure of eEF1A in complex with eEF1β allowed further understanding of the way in which eEF1A works. eEF1β consists of two subunits, eEF1βα and eEF1βγ. The eEF1βα subunit is responsible for the catalysis of the GDP – GTP exchange. This subunit interacts with domain II of eEF1A at the same binding site as that of the aminoacylated tRNA, therefore competing for its binding (Andersen et al., 2001).

## *1.3 Isoforms of eEF1A*

In mammalian cells, there are two isoforms of eEF1A, eEF1A1 and eEF1A2 which have 75% similarity at the nucleotide level and 98% similarity at the protein level (Knudsen et al., 1993, Panasyuka et al., 2008) and demonstrate mutually exclusive expression. eEF1A1 is ubiquitously expressed in all tissues except in specific tissues such as in neurons, differentiated skeletal muscle, terminally differentiated heart and brain cells and human pancreatic islets, where eEF1A2 is expressed, (Knudsen et al., 1993, Kahns et al., 1998, Ahmed et al., 2005), comprising cells with specific architectures and generally of slow growth. Both isoforms have been shown to be present in brain and aorta tissue (Knudsen et al., 1993, Kahns et al., 1998, Kristensen et al., 1998, Ahmed et al., 2005). In rapidly proliferating cells, including tumours, the level of mRNA of eEF1A is greatly increased which suggests that eEF1A may have a role in cell proliferation (Condeelis, 1995).

It has further been suggested that eEF1A1 is the predominant form in these terminally differentiated tissues such as heart, brain and skeletal cells, until they are fully differentiated, during which time the level of eEF1A2 mRNA accumulates, and the level of eEF1A1 mRNA reduces until undetectable (Lee et al., 1995). This switch in eEF1A isoform expression has also been well characterised in mice in neuronal tissue with Pan et al, 2004, showing that eEF1A1 was the predominant form of eEF1A in neurons after 1 day, and then slowly declines until at

14 days, eEF1A1 expression was barely detectable and from 20 days onwards is undetectable. Conversely, eEF1A2 expression is only detectable after 14 days, slowly increasing until it is the dominant form after 20 days (Pan et al., 2004). This isoform switch has also been shown to be conserved in *Xenopus*, though the reduced level of eEF1A1 protein is controlled in a post transcriptional manner with eEF1A1 mRNA levels remaining at similar levels to eEF1A2 mRNA (Newbery et al., 2011). Interestingly, Khalyfa et al, 1999, showed that after skeletal tissue injury by Marcaine injection, the ratio of eEF1A1 to eEF1A2 switches back to that seen in the developmental stages, and after recovery from that injury the ratio reverts back to that of normal developed skeletal tissue. The reason for this is unknown but it has been suggested to be due to potential different secondary functions of eEF1A1 and eEF1A2 outside of its primary function in protein translation (Khalyfa et al., 1999). This is supported by the high level of conservation seen between the two isoforms across species, and the tightly regulated control of expression.

The translational activity of both isoforms has been shown to be very similar, though the affinity for GDP and GTP was found to be different, with eEF1A1 shown to bind GDP and GTP with similar affinity and eEF1A2 having a stronger relative affinity for GDP, most likely due to the difference in amino acid residue at position 197 which is very close to the known binding position of the guanine nucleotide (Kahns et al., 1998). This could be of interest as GTP binding has been shown to inhibit binding of eEF1A to G-actin (Dharmawardhane et al., 1991), therefore the higher affinity of eEF1A to GDP could potentially affect the likelihood of this isoform to be able to participate in cytoskeletal remodelling. The rate of GTP hydrolysis was found to be the same for both isoforms which was unsurprising as the amino acids proposed to be involved in GTP hydrolysis are conserved between the two isoforms (Kahns et al., 1998).

It has also been noted that eEF1A2 tends to be associated with tumour development in tissues that would normally only express eEF1A1 (Anand, 2002, Amiri et al., 2007) which supports the theory of the two different isoforms performing the same canonical function in protein translation, as shown by Kahns et al, 1998, but different non-canonical functions. Examples of these non canonical functions and the differing effects of the two isoforms will be discussed in detail in section 1.4.

Seven post translational modifications have been shown for eEF1A1 (Dever et al., 1989), at least 3 of which are conserved in eEF1A2, however the lysine residues at amino acids 55 and 165 were found to be trimethylated in eEF1A2 but only dimethylated in the eEF1A1 isoform (Kahns et al., 1998).

## *1.4 Non Canonical Functions of eEF1A*

The non-canonical roles of eEF1A are extensive and include roles in quality surveillance of newly synthesised proteins (Hotokezaka, 2002), ubiquitin dependent degradation (Chuang et al., 2005, Gonen et al., 1994), viral functions (Kinzy and Goldman, 2000), facilitating apoptosis (Duttaroy et al., 1998, Chen et al., 2000, Lamberti et al., 2004), interactions with the cytoskeleton from yeast through to mammals (Edmonds et al., 1996, Gross and Kinzy, 2005, Suda et al., 1999, Munshi et al., 2000), and microtubule binding, bundling and severing (Moore et al., 1998, Moore and Cyr, 2000, Ueno et al., 2003).

### 1.4.1 eEF1A and the Cytoskeleton

Translation has been shown to be dependent upon an intact cytoskeleton (Negrutskii and Deutscher, 1991, Gross and Kinzy, 2007), and mRNA has specifically been shown to localise to actin fibres (Bassell et al., 1994, Bassell and Singer, 1997), therefore eEF1A has been suggested to be a regulator of both translation and the actin cytoskeleton, mainly highlighted by the fact that eEF1A is the second most abundant protein after actin, making up between 1-10% of total cellular protein in normally growing cells (Liu et al., 1996b, Merrick, 1992), and studies have shown that there is a 17-35 fold molar excess of eEF1A when compared to ribosomes (Slobin, 1980). Based on this large excess in comparison to the other components of the translational machinery, it has been suggested that there may be 'pools' of eEF1A present for different functions where translational functions would be prioritised over others, including its regulatory properties on the actin remodelling.

### 1.4.1.1 Actin

The actin cytoskeleton has three main functions, to maintain spatial organisation of the cell contents, to allow the cell to connect to the extracellular matrix and to create coordinated forces to ensure maintenance of cell shape and motility (Fletcher and Mullins, 2010).

Actin is found in the cell as globular actin (G-actin) and fibrous actin (F-actin), usually observed as cortical actin which surrounds the cell or as stress fibres which are located throughout the cell. The polymerisation of monomeric G actin into filamentous F actin and vice versa is a tightly regulated process as maintenance of the actin cytoskeleton is essential for normal cellular functions. Actin filaments are polymerised by nucleation, where the addition of single G actin monomers occurs over time to form a structure containing 3-4 units. The addition of G actin monomers usually occurs at the fast growing/barbed end or the plus end of the fibre, with the removal of monomers from the pointed or minus end of the fibre (Dominguez, 2009). Polymerisation can be inhibited by the binding and sequestering of monomers by actin binding proteins such as thymosin. Another actin monomer binding protein is profilin, which completes with thymosin to bind the actin monomer resulting in prevention of addition of the monomer from the minus end, but allows addition to the plus end of the actin fibre (Remedios et al., 2002). Once the monomer-profilin complex is bound, a conformational change in the actin monomer occurs which reduces the affinity of profilin for actin, therefore releasing profilin and making the actin fibre one monomer longer (Remedios et al., 2002).

The main regulator of actin nucleation is the Arp2/3 complex, which when working together with the WASP/WAVE family of proteins, recruits actin and activates nucleation (Dominguez, 2009). The Rho GTPase Cdc42 has been shown to indirectly activate the Arp2/3 complex using the WASP family of proteins as adaptor proteins (Machesky and Insall, 1998), and it has been suggested that the Arp2/3 complex is likely to be the effector for Rac induced actin polymerisation using the SCAR/WAVE family of proteins as the adaptor (Welch, 1999). The WASP proteins have been shown to bind to actin monomers with the potential function of delivering the monomers to the plus end of the actin filament for polymerisation (Winder and Ayscough, 2005).

It has been suggested that, as in yeast, while the Arp2/3 complex is involved in the nucleation of short branched actin filaments, formins may be involved in the formation of longer unbranched actin filaments (Winder and Ayscough, 2005). Formins have been shown to bind

to barbed ends (Higgs, 2005) and interact with profilin, the actin monomer binding protein resulting in delivery of the monomer to the plus end of the actin filament (Evangelista, 2003). The FH2 domain of the formin Bni1p has been shown to bind to eEF1A, an actin binding/bundling protein (Umikawa et al., 1998), which could link the nucleation and polymerisation of actin with the organisation of the actin filaments.

### 1.4.1.2 Actin binding proteins

There are many different actin binding proteins (ABPs), at least 162 known, that regulate the binding and bundling of actin fibres into the cortical actin and stress fibres observed in the cell, as well as playing a role in regulating the ratio of G actin to F actin (Remedios et al., 2002). The different actin binding proteins can be categorised dependent on whether the protein binds to G actin or F actin, as well as where the protein binds to on the F actin fibre. These are summarised in figure 1.4.1.2.

Already discussed are the monomer binding proteins such as thymosin and profilin which compete to prevent the unregulated association of G actin into F actin filaments. Actin cap binding proteins also help to prevent both addition and removal of actin monomers to either the plus or minus end of the actin fibre by binding to the ends of the fibre. The Arp2/3 complex, as well as being involved in nucleation of the actin fibre, is also a capping protein. It binds to the minus end of the fibre with a high affinity, therefore capping the fibre so that monomer addition or removal cannot occur from that end of the fibre (Cooper and Schafer, 2000). CapZ is another capping protein but unlike Arp2/3, it binds to the plus end of the actin fibre (Cooper and Schafer, 2000). Both the blocking of the actin fibres by capping proteins such as these, as well as the regulation of the free pool of G actin monomer by the actin monomer binding proteins, ensures tight regulation of the polymerisation of the actin fibres such that when polymerisation is required, capping proteins can be removed and more G actin monomers can be made available.

Another group of actin binding proteins are the side filament binding proteins which include both tropomyosin and the ADF/cofilin family. Tropomyosin binds to the side of the actin filament, spanning 6-7 subunits giving strength and stability. The binding of tropomyosin protects the fibre from depolymerisation by DNase I and severing by the ADF/cofilin family, as

well as regulating the access of some other actin binding proteins (Cooper, 2002) and myosin Ib (Tang and Ostap, 2001). Tropomyosin has also been shown to inhibit the nucleation of actin fibres by Arp2/3 by preventing the binding of the complex, therefore tropomyosin can also affect polymerisation indirectly (Blanchoin et al., 2001).

The ADF/cofilin family are side binding proteins that shorten actin filaments, by both severing and increasing the rate of loss of monomers from filament ends (Cooper and Schafer, 2000). Cofilin can bind to the actin fibre in an ADP dependent manner, inducing the fibre to twist by reducing the number of contacts between the monomers and making the fibre more brittle and more susceptible to severing (Cooper and Schafer, 2000). This binding to actin filaments associated with ADP helps to differentiate between old and new actin filaments as newer filaments are associated with ATP, and older filaments with ADP.

The change in structure of the fibre induced by binding cofilin could also result in dissociation of other actin binding proteins such as the Arp2/3 complex from the ends of the filament resulting in increased actin depolymerisation (Cooper and Schafer, 2000). Binding of cofilin has also been shown to increase the rate of actin polymerisation by severing the filament providing more free filament ends for polymerisation to occur, possibly regulated by the nucleating activity of the Arp2/3 complex (Cooper and Schafer, 2000). This provides a view in which the ADF/cofilin family of proteins could potentially play a role in actin polymerisation and depolymerisation under differing circumstances. The binding of ADF/cofilin and tropomyosin has been shown to be mutually exclusive, therefore this competitive binding can also regulate the polymerisation and depolymerisation of actin filaments (Cooper, 2002).

In order for the actin cytoskeleton to exert any effect on the extracellular matrix, the cytoskeleton must connect to the ECM via membrane associated actin binding proteins. These can link the actin cytoskeleton to the plasma membrane, such as annexins or to the extracellular matrix, for example talin and vinculin (Winder and Ayscough, 2005). Both have the ability to bind to actin and link the cytoskeleton to integrins in the ECM therefore anchoring the cell to the matrix (Winder and Ayscough, 2005). These actin binding proteins will be further described in the section explaining focal adhesions (Section 1.4.1.7).

Lastly, the final group of actin binding proteins are the bundling or cross-linking proteins. These provide the structure to the actin filament by bundling into fibres and/or cross-linking into the required structure. Cross-linking proteins usually contain either multiple actin binding domains which are separated by a long flexible spacer domain α-actinin, or contain one actin

binding site, and therefore first need to dimerise to form a homodimer offering two actin binding sites separated by a spacer domain (Winder and Ayscough, 2005). An example of the latter class is filamin whose dimerisation results in the perpendicular formation of actin filaments in relation to each other with the filamin acting as a bracket for the filaments (Popowicz et al., 2006). The long spacer domain of filamin also acts as a scaffold for membrane receptor associated signalling proteins, suggesting further functions coinciding with its role as an actin cross-linker (Popowicz et al., 2006).

α – actinin also bundles actin filaments via the formation of homodimers. In this instance, the coupling of the two proteins is done in an antiparallel manner with a resultant helical spacer domain in between the two actin binding sites, causing an increased space between the two actin filaments and looser bundles, such as those seen in actin stress fibres (Winder and Ayscough, 2005). It has also been shown to cross-link actin into a disordered square lattice to create a rigid actin meshwork (Pelletier et al., 2003).

As well as having actin nucleating activity and being a capping protein, the Arp2/3 complex can also cross-link actin filaments by capping/binding to the end of one protein and simultaneously binding to the side of another filament forming a branched actin network (Welch, 1999).

Actin bundling proteins can be further sub grouped depending on whether they form loose or tightly associated actin filaments, as well as their orientations. Such properties, as explained earlier, can be determined by the number of actin binding sites, the size of the spacer domain and the angle at which they bind filaments. Fimbrin for example packs fibres into tight bundles, such as those found in microvilli, as its two actin binding sites are in close proximity to each other (Winder and Ayscough, 2005). The filaments within the actin bundles can also be organised into parallel or anti parallel arrangements depending on the bundling protein involved, for example, fascin only bundles filaments into a parallel orientation, a cross-linker such as α – actinin can bind actin fibres at many different angles (Courson and Rock, 2010), and eEF1A has been shown to be an anti parallel actin bundling protein (Owen et al., 1992).

The actin binding proteins all bind with differing affinity to actin. Binding affinity is measured by Kd, the dissociation constant, defined as the concentration at which 50% of the binding sites are occupied by the protein ligand. Actin capping proteins for example only require one molecule to cap one actin filament which contains many actin monomers, therefore small changes in the concentration of actin capping proteins can cause significant changes to actin

polymerisation and depolymerisation rates, resulting in a low Kd value, usually around 1nM. A side binding protein such as tropomyosin binds to the filament and spans only 6 to 7 actin monomers, therefore a higher concentration is required to have a significant protective effect on the actin filament, resulting in a higher Kd value usually between 0.1-1µM.

The consequence of the differences in affinity of the ABPs determines which ABP is more likely to bind, for example, if two proteins were present at the same concentration, the protein with the higher affinity or the lowest Kd would be more likely to occupy more binding sites than the protein with the lower affinity or the higher Kd. The monomer binding protein thymosin has a Kd of approximately 0.7µM, compared to the Kd of profilin, 0.1µM, which means that profilin can bind the actin more tightly than thymosin, hence why the binding of profilin to actin is preferential to that of thymosin to help regulate actin polymerisation (Pollard et al., 2007), however, not only does the binding depend on the affinity of the protein but also on many other factors such as concentration of the proteins involved and competitive binding.

The actin bundling protein eEF1A has been shown to bundle actin filaments in such as way as to exclude other actin binding proteins (Owen et al., 1992), and has a Kd of between 0.1-10µM, compared to other filament binding proteins such as filamin with a Kd of 0.5µM and α – actinin with a Kd of between 1-5µM (Pollard et al., 2007). This means that eEF1A, depending on the conditions, could have the ability to bind actin with a higher affinity than the other filament binding proteins, making it most likely to be bound, and providing further evidence to support the exclusion of other ABPs, and therefore potentially one of the main regulators of the organisation of the actin cytoskeleton.

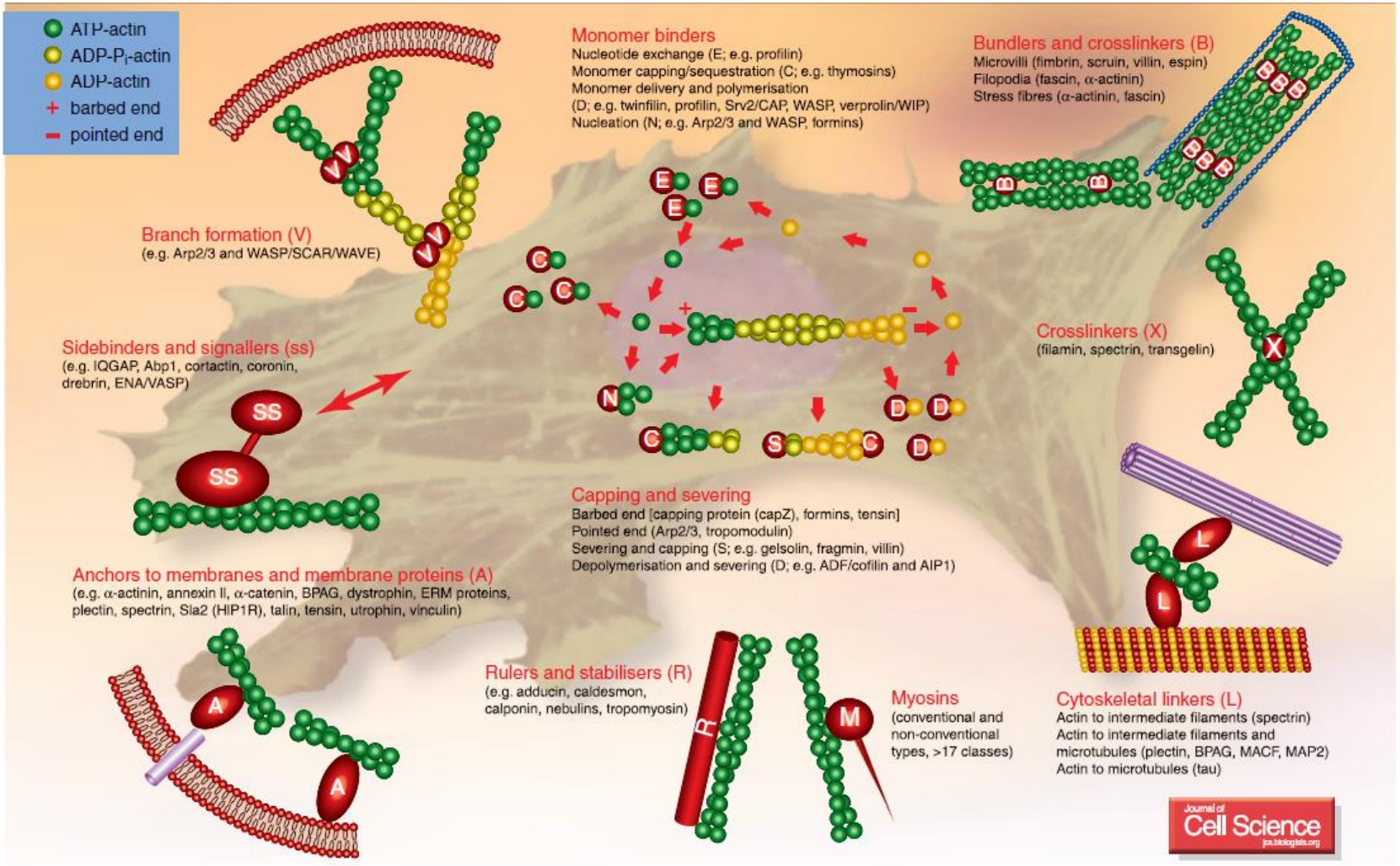



**Figure 1.4.1.2 –** A schematic representation of the different types of actin binding proteins, with examples, adapted from Winder and Ayscough, 2005.

### 1.4.1.3 eEF1A and Actin Binding/Bundling

eEF1A was first identified as an actin binding and bundling protein in 1990 from Dictyostelium discoideum, initially termed ABP-50 by Demma et al, 1990, through the isolation of a 50,000 dalton protein capable of actin filament bundling. ABP-50 was found to cross-link F-actin to form actin bundles in two different configurations, tightly packed, more ordered bundles and loosely packed less ordered bundles, as determined by electron microscopy (Demma et al., 1990). Further work showed that eEF1A can bundle actin in such a way that the actin filaments are rotated at $90^{o}$ relative to each other and are thought to have the ability to exclude other actin binding proteins (Owen et al., 1992). Immunofluorescence studies also showed that ABP-50/eEF1A was localised within the cell cortex, where actin bundles are known to reside. This ability of ABP-50 to bundle actin filaments was found to be unaffected by calcium ($Ca^{2+}$) and ATP. The same ABP-50 protein was subsequently identified as eEF1A by functional analysis and sequence comparison, resulting in the first direct connection between the actin cytoskeleton and the translational machinery (Yang et al., 1990).

#### *1.4.1.3.1 Regulation of eEF1A Binding to Actin by pH and Ionic Strength*

Changes in the cellular pH of Dictyostelium have been shown to affect the association of eEF1A with actin. At a low physiological pH, eEF1A was found to be mainly associated with actin, whereas with increasing pH, both the bundling and binding activities of eEF1A were diminished with different severity. Bundling loss was the first to occur followed by the loss of actin binding activity (Edmonds et al., 1995). This implies that eEF1A binding to actin filaments is less sensitive to changes in pH than actin bundling by eEF1A, and that actin binding and bundling by eEF1A are independent events, which was suggested to indicate multiple binding sites within the eEF1A protein. The interaction between eEF1A and actin was also found to be affected by the total ionic strength, suggesting that the interaction could be charge dependent (Edmonds et al., 1995), which is supported by the evidence that eEF1A is a basic protein, and actin is an acidic protein (Liu et al., 1996a).

It has been suggested that the change in pH could in effect mediate translational activity of eEF1A. Low pH conditions would favour the association of eEF1A with actin filaments,

reducing its interactions with other components of the translational machinery. As pH increases within the physiological range, eEF1A actin bundling activity is lost freeing the protein to associate with other components of the translational machinery (Edmonds et al., 1995). This was found to be likely as at a lower pH eEF1A binding to F-actin is favoured over eEF1A binding to aa-tRNA, whereas at higher pH, the opposite was true (Liu et al., 1996b). The binding affinity of eEF1A for aa-tRNA was found to be fairly constant over the range of pHs, whereas the binding affinity of eEF1A to actin was found to change (Liu et al., 1996b).

In the same study, both domains I and III of eEF1A showed actin binding activity, and although changes in pH affected both actin binding sites, the site in domain I was more sensitive than the site in domain III. This fits in with the finding that as pH is increased, actin bundling is decreased, as the binding site in domain I could be released leaving the single actin binding site in domain III to bind to actin filaments. As pH increases further, the actin binding site in domain III is also affected, and therefore both binding sites are released from the actin filament, leaving unbound eEF1A. The authors suggested that, as binding of eEF1A to actin or aa-tRNA seem to be independent, and binding of eEF1A to aa-tRNA blocks eEF1A actin binding, the binding sites for actin and aa-tRNA on the eEF1A protein are likely to be located on the same part of the protein, and that the sequences may even overlap.

### *1.4.1.3.2 Regulation of eEF1A Binding to Actin by Calcium and Calmodulin*

Previous studies have shown that eEF1A can bind to calmodulin (Kaur and Ruben, 1994) and that whilst actin bundling is inhibited by the presence of $Ca^{2+}$/calmodulin, actin binding was unaffected, though eEF1A has been shown to be insensitive to $Ca^{2+}$ in the absence of calmodulin (Kurasawa et al., 1996b).

This led the authors to hypothesise that two possibilities existed, either eEF1A has two separate actin binding sites, only one of which is affected by $Ca^{2+}$/calmodulin, or eEF1A has only one actin binding site and dimers are formed allowing cross-linking of the actin filaments and therefore actin bundle formation. The authors seem to favour the dimer formation theory, as the molar ratio of eEF1A to actin observed was 1:1, whereas if eEF1A contained two binding sites, the expected molar ratio would be 2:1 (Kurasawa et al., 1996b). There is currently evidence to support both of these theories.

Previous studies have shown that eEF1A can form dimers, and that whilst eEF1A monomers and dimers are both capable of binding actin, only eEF1A dimers have the ability to bundle actin in Tetrahymena, supporting the dimer theory of actin bundling (Bunai et al., 2006). Furthermore it was shown that dimer formation was regulated by $Ca^{2+}$/calmodulin, such that in the presence of $Ca^{2+}$/calmodulin, dimers are separated into monomers, and when $Ca^{2+}$ is chelated, dimers are formed, which supports the work of Kurasawa et al., 1996b. The authors suggested that in the absence of $Ca^{2+}$, calmodulin cannot bind to eEF1A, leaving it free to bind to another eEF1A molecule to form the dimer. In the presence of $Ca^{2+}$, the $Ca^{2+}$/calmodulin complex binds to eEF1A, preventing another eEF1A molecule from binding, inhibiting dimerisation. The binding of $Ca^{2+}$/calmodulin to eEF1A also reduces the affinity of eEF1A for F-actin leading to dissociation of the eEF1A/$Ca^{2+}$/calmodulin complex from the F-actin filament. This monomer would then be free to participate in translation, therefore $Ca^{2+}$/calmodulin could be a controlling link between the actin binding/bundling activity and the translational activity of eEF1A.

Continuing on with the work on eEF1A dimer formation it was found that domain III was responsible for dimer formation as well as F-actin binding, and that all three domains have the ability to bind calmodulin, though to varying degrees dependent on the concentration of calcium (Morita et al., 2008). At a $Ca^{2+}$ concentration of 1mM all three domains demonstrated the ability to bind calmodulin, however at a concentration of 1µM only domains I and III could bind to calmodulin, and at a concentration of 100nM only domain I showed calmodulin binding with domain III showing very slight calmodulin binding activity (Morita et al., 2008). In line with this, at a $Ca^{2+}$ concentration of 100nM F-actin bundles were unaffected, between 500nM - 1µM bundles were loosened, and at a concentration of between 100µM – 1mM the bundles were disassembled, suggesting a model in which $Ca^{2+}$ concentrations of below 100nM result in the binding of domain I to calmodulin, domain III to both another eEF1A molecule to form a dimer resulting in actin bundling, whereas when the concentration of $Ca^{2+}$ increases to 1µM, calmodulin binds to domain III of eEF1A causing the eEF1A dimers to split into monomers, therefore loosening the actin bundles and reducing the binding of eEF1A to actin filaments (Figure 1.4.1.3.1)

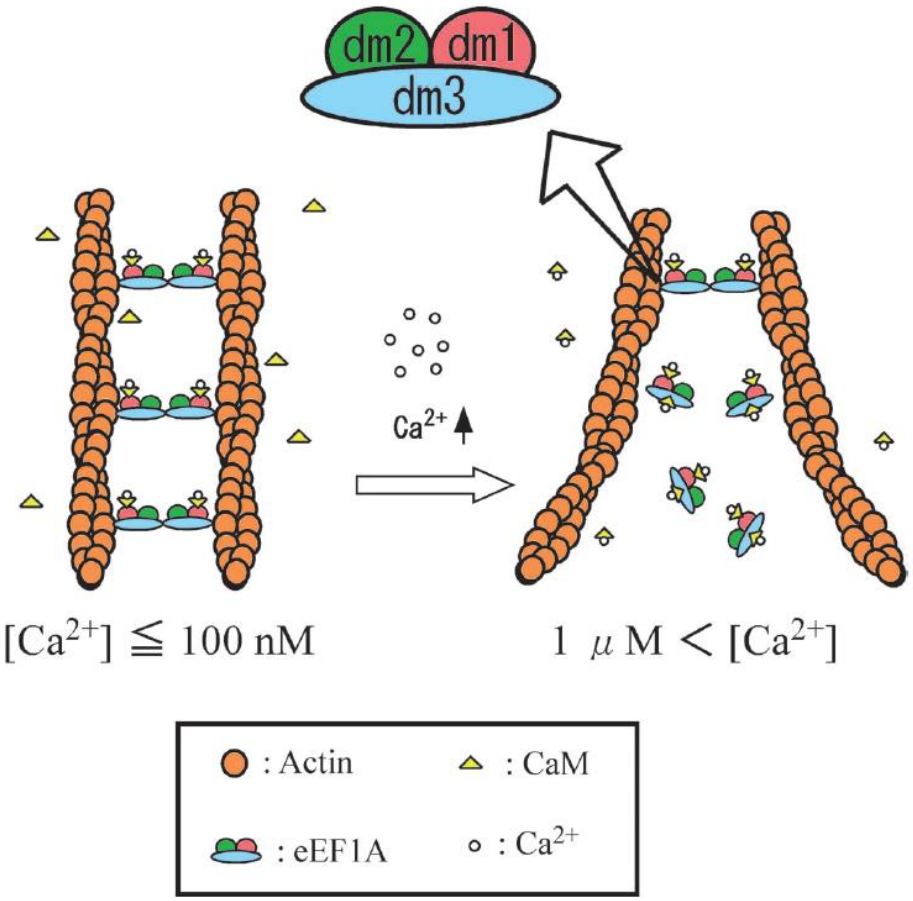



**Figure 1.4.1.3.2 –** The model of regulation of eEF1A actin binding by calcium and calmodulin based on eEF1A dimerisation adapted from Morita et al, 2008. In the presence of low concentrations of calcium, eEF1A can form dimers and therefore bundle actin, however at higher concentration of calcium, calcium can bind to domains I and III of eEF1A leading to the separation of dimers into monomers and the release of eEF1A from the actin fibre resulting in loosening and then loss of the actin bundles with an increasing calcium concentration.

### *1.4.1.3.3 Which eEF1A Domains are Important for Actin Binding/Bundling?*

As explained so far, the direct mechanisms to explain how eEF1A promotes its actin bundling properties have remained elusive as evidence for both dimerisation of single actin binding domain units or a single protein containing multiple binding sites have been reported. The regulation of actin binding and bundling by pH favoured the monomer actin binding theory, as both domains I and III of eEF1A were found to bind to actin (Liu et al., 1996b), however the regulation by $Ca^{2+}$/calmodulin favours the dimer theory as only domain III was shown to bind

to F actin, as well as binding to other eEF1A molecules (Kurasawa et al., 1996b), with Bunai et al, 2006, clearly demonstrating dimer formation.

In contrast to the dimer theory, a review by Liu et al., 1996a, favours the theory that eEF1A has two actin binding sites. The formation of the tightly packed bundles created by the bundling activity of eEF1A, would indicate that the cross-linking of actin filaments by eEF1A is restricted in space which supports the theory of the two different actin binding sites of eEF1A rather than dimer formation (Liu et al., 1996a).

Based on the strong evidence for both theories, it could be possible that eEF1A is capable of bundling actin by both monomer binding and dimerisation, as demonstrated in figure 1.4.1.3.3, but under different conditions.

The first study to experimentally try to identify specific regions within domain III of eEF1A that can bind to actin using mutagenesis and by creation of truncations, found that amino acids 414-427 of eEF1A, and mutations F422A, N305S and N329S are important, and that the mutants in which actin binding were affected, had no change in translational activity (Gross and Kinzy, 2005).

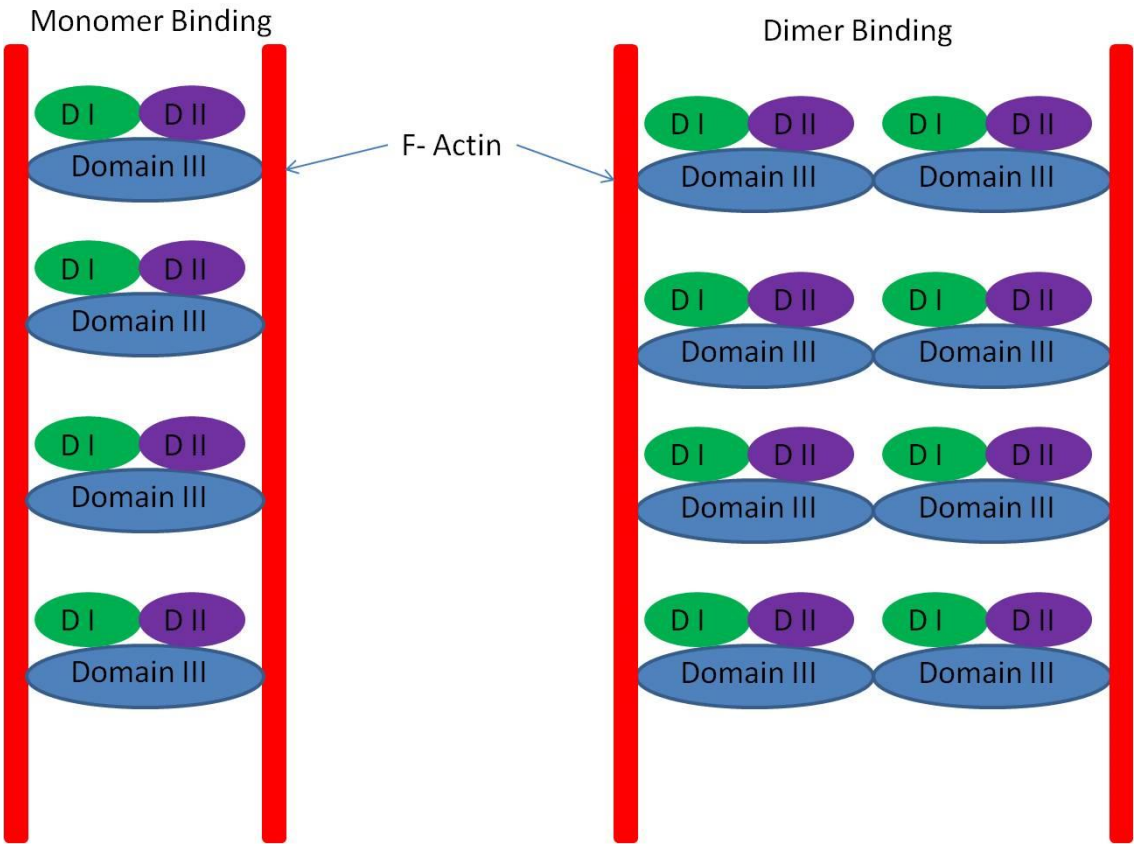



**Figure 1.4.1.3.3 –** Schematic representation of the two different actin binding theories for eEF1A. On the left, the monomer binding theory is shown. Domain III has been shown to have two actin binding sites, therefore it is possible that domain III is solely responsible for the bundling activity of eEF1A. On the right shows the dimer binding theory. Domain III has been shown to have the ability to bind to both itself and actin, therefore two eEF1A molecules could come together in order to bundle actin. Evidence has been shown for both theories.

### 1.4.1.4 eEF1A and Actin Polymerisation

Not only has eEF1A been shown to bind and bundle actin, it has also been found to affect the rate of polymerisation from both the barbed, fast growing end, as well as the pointed end of the actin filament, and this effect has been shown to be dependent on the actin bundling activity of eEF1A (Murray et al., 1996). It is thought that the bundling of actin by eEF1A causes the ends of the actin filaments to be unavailable for polymerisation to occur. eEF1A also

affects actin polymerisation in a concentration dependent manner so that at higher eEF1A levels, depolymerisation is inhibited.

### 1.4.1.5 eEF1A and G Actin

It was observed that eEF1A can bind to G-actin in the cytosol, and that GTP can inhibit this binding, though binding of F-actin by eEF1A is thought to be unaffected by both GDP and GTP (Dharmawardhane et al., 1991). It has also been shown that binding of eEF1A to G-actin can inhibit its translational activity (Bektaş et al., 2004).

### 1.4.1.6 Actin stress fibre subtypes

Actin stress fibres (SFs) are divided into four different types, the actin cap, transverse arcs, dorsal SFs and ventral SFs which can be differentiated based on their position within the cell, the proteins that localise to them and their connection to the extracellular matrix (ECM), as demonstrated in figure 1.4.1.6. The actin cap consists of stress fibres located above the nucleus, which provide it with structural support, especially during interphase (Tojkander et al., 2012). Myosin IIA is known to localise to the actin cap SFs. They attach to the extracellular matrix through focal adhesions at both ends of the fibre, and could therefore behave as mechanotransducers to allow force from the ECM to be relayed to the nucleus (Tojkander et al., 2012).

Transverse arcs are curved bundles of actin that can flow from the leading edge of the cell to the rear of the cell during migration (Pellegrin and Mellor, 2007, Tojkander et al., 2012). They alone do not associate with focal adhesion proteins, at either end of the fibre, however, they can connect with dorsal stress fibres (Tojkander et al., 2012). Dorsal stress fibres attach to the ECM via focal adhesion proteins at their distal end, with the other end usually connecting to the transverse arc. Unusually, myosin IIA does not localise to dorsal SFs, meaning that contractility from these fibres can only occur through their connection with transverse arcs.

Ventral stress fibres are the most common SF structures, with localisation of myosin IIA along the fibre, and focal adhesion proteins to both ends. This localisation of myosin IIA and the ability to attach to the ECM makes ventral stress fibres the main contractile machinery in some cells, and they have also been shown to affect directional cell migration by detaching from the rear of the cell and reattaching to adhesions at the front of the cell, with the new direction of motion aligning perpendicular to the direction of the newly attached fibre (Rid et al., 2005). Dorsal SFs and transverse arcs have been shown to merge to form ventral stress fibres (Hotulainen and Lappalainen, 2006).

The assembly and regulation of stress fibres is known to be promoted by both Rho A and Rac, small GTPases involved in the regulation of actin dynamics in the cell (Tojkander et al., 2012). mDia is an effector of Rho A and facilitates the polymerisation of actin filaments which are important for the formation of dorsal stress fibres, though it is likely that other proteins are also involved in the polymerisation of actin from the focal adhesion for this type of stress fibre, most likely another member of the formin family of proteins (Hotulainen and Lappalainen, 2006). The formation of transverse arcs was shown to be disrupted by siRNA knockdown targeted to the Arp2/3 complex, though this did not affect the formation of dorsal stress fibres, therefore it is suggested that the two stress fibres are formed by independent pathways with formins, specifically mDia involved in dorsal SF formation and the Arp2/3 complex involved in transverse arc formation (Hotulainen and Lappalainen, 2006).

Ventral stress fibres were found to be formed by the merging of two dorsal stress fibres with a transverse arc, and through contraction of the transverse arc, the fibres are aligned, and connect to the ECM through the focal adhesions formed at one end of the dorsal stress fibre (Hotulainen and Lappalainen, 2006).

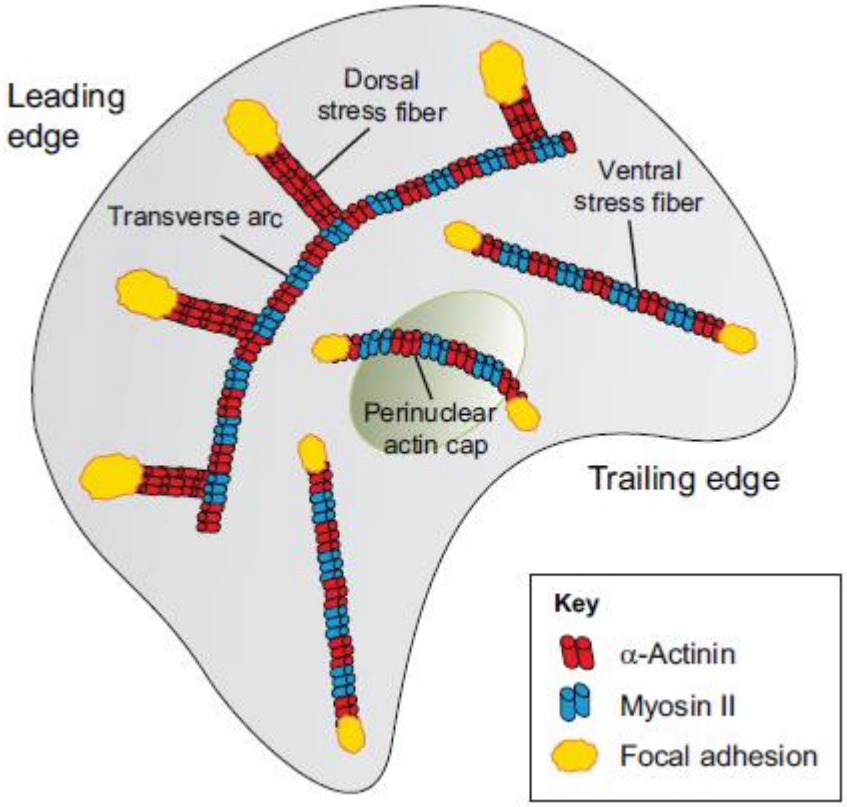



**Figure 1.4.1.6 –** A schematic representation of the different types of actin stress fibres adapted from Tojkander et al, 2012. Actin cap fibres which span the nucleus, ventral stress fibres which are associated with focal adhesions at either end, dorsal stress fibres which are only associated with focal adhesions at one end, and transverse arcs, which are not associated with focal adhesions however associate with dorsal stress fibres.

## 1.4.1.7 Focal adhesions

Focal adhesion complexes allow the actin cytoskeleton to attach to the ECM through interaction with integrins, as well as creating a point for signal transduction, with many different tyrosine phosphorylated proteins, tyrosine kinases, phosphatases and adapter proteins being recruited along with the focal adhesion proteins (Riveline et al., 2001). They are tightly regulated multi protein complexes that are formed when the cell attaches to the ECM. Newly formed focal complexes which mature into focal adhesions can be observed just behind the migrating front of the cell which has enabled the order of recruitment under

certain conditions to be established. The earliest focal adhesion proteins to be incorporated into hyaluronan mediated focal complexes were paxillin and talin, followed by vinculin, α – actinin, VASP and focal adhesion kinase (FAK) (Zaidel-Bar et al., 2004). Some focal complexes then disappear, however others mature into focal adhesions with this transition dependent on actomyosin contractility which requires the presence of an active form of RhoA to activate both Rho Kinase (ROCK) and mDia, both of which are involved in cytoskeletal dynamics (Zaidel-Bar et al., 2004). Some of the main components of focal adhesions are the adapter proteins paxillin and vinculin (Zaidel-Bar et al., 2004).

Paxillin has been shown to bind to vinculin, possibly to help to recruit vinculin to the focal adhesion complex (Turner et al., 1990). Myosin IIA activity has been shown to result in phosphorylation of paxillin by focal adhesion kinase (FAK), and that this phosphorylation can lead to the recruitment of vinculin to the focal adhesion to encourage focal adhesion maturation (Pasapera et al., 2010). Paxillin has also been shown to be a critical transducer of fibronectin signalling (Hagel et al., 2002). The phosphorylation of paxillin is essential for cell migration in some cell types (Huang et al., 2008), and it has been shown to be involved in transformation of cells as it is a common target for oncoproteins that contain tyrosine kinases (Sattler et al., 2000).

Whereas paxillin has an important role in the early stages of focal adhesion formation, vinculin appears later and has roles involved with cell spreading by coupling to integrins, and focal adhesion turnover (Ezzell et al., 1997, Saunders et al., 2006). Cells lacking vinculin were not only found to be deficient in cell spreading but were also not able to form actin stress fibres, suggestive of a role of vinculin in the promotion of stress fibre formation as well as reducing the amount of paxillin associated with the cytoskeleton (Ezzell et al., 1997). Complementing this link between vinculin and integrins, is the work that shows that vinculin stabilises focal adhesions, therefore inhibiting cell migration and that binding of the vinculin tail to inositol phospholipids has a role in focal adhesion turn over (Saunders et al., 2006).

In order for focal adhesions within the cell to make contact with the ECM, the adapter proteins such as talin connect to integrins which connect to the ECM. Integrins are made up of α and β subunits, of which there are many different types that can assemble to form different integrin receptors which can bind to different proteins of the ECM, for example, α5β1 integrins are most commonly associated with binding to fibronectin (Srichai and Zent, 2010). Binding of the ligand within the ECM to the extracellular domain of the integrin results in integrin clustering

and recruitment of proteins such as talin and paxillin (Zaidel-Bar et al., 2004), the first stage of focal complex formation.

### 1.4.1.8 Cell Migration

The process of overall cell motility is very complicated at the molecular level, there are however four main steps of cells migration that form a cycle; extension, attachment, contraction and release, after which the process restarts (Sheetz et al., 1998).

The first stage of the cycle, extension, involves the development of both filopodia and lamellipodium. Filopodia are spike like structures that are extensions of the cell membrane. Filopodia are often described as sensing structures which are extended in order to determine whether the surrounding area is suitable for cell adhesion and therefore determines which direction the cell migrates in. Lamellipodia have a different structure to filopodia and are often referred to as the leading edge of the cell. It is just behind the lamellipodium that actin polymerisation occurs allowing the cell to move in the appropriate direction and where initial focal complexes are formed leading to stable focal adhesions as the complexes mature (Schäfer et al., 2009). Jeganathan et al, 2008, found that increased levels of eEF1A2 can increase stimulation of filopodia formation by interacting with PI4kIIIβ, which in turn increases the amount of PI4P, which is the precursor for $PI(4,5)P_2$, which is essential for eEF1A2 mediated filopodia formation.

Once a favourable environment has been detected, focal adhesions and stress fibres are formed which attach the front of the cell to the ECM. Once the cell has extended using filopodia and lamellipodia, and made contact with the ECM through formation of focal adhesions, the cell must contract in order to migrate, hence the need for such complex control of actin organisation. Myosin II is thought to be involved in this contraction, by the double head of the myosin fibre attaching to two actin filaments and pulling the fibres. This is also thought to cause the focal adhesions at the rear of the cell to pull apart, therefore allowing the rear of the cell to move forward (Lauffenburger and Horwitz, 1996).

What becomes apparent is that the dynamic organisation of the actin cytoskeleton is an essential component of successful cell migration therefore any potential disruption of this

tightly controlled process would be highly likely to have a significant effect on overall cell migration rate. That being said, proteins such as eEF1A, which is known to have actin binding properties, are likely to be very closely monitored during this process and any change in level of such proteins could potentially lead to changes in migration phenotypes observed. This particular association between eEF1A, actin and cell migration is not completely understood therefore requires further investigation. The link between cell migration and cancer metastasis is well known, and the effect of eEF1A expression and its relation to increased cancer metastasis may suggest that by analysing the way in which eEF1A can affect cell migration at a molecular level, may result in potential targets for interruption of cancer metastasis in the future.

There are many different proteins that are involved in this migrational process, and Condeelis et al, 1992, argue that the process of metastasis is similar to the process of chemotaxis of motile cells along the cytokine gradient, therefore molecules identified as being involved in the process of chemotaxis, are also likely to be relevant during metastasis of the cancer cells. The migration of cells is highly dependent upon the rearrangement of the actin cytoskeleton upon cytokine stimulation. When cytokines bind to their receptors, the intracellular signalling cascade stimulates the production of polarised surface projections of newly synthesised actin (Devreotes and Zigmond, 1988) which are then stabilised by actin binding proteins.

### 1.4.1.9 Other Components of the Cytoskeleton

Microtubules are made up of α and β tubulin which form heterodimers that combine to form protofilaments. Thirteen of these protofilaments then bundle to form a hollow cylindrical fibre, a microtubule. Microtubules have a variety of functions, from maintaining the structure of the cell, transport of intracellular vesicles and organelles, and also their role in the formation of the mitotic spindle in cell mitosis. They have also been shown to be essential for directional cell migration in some cell types and have been shown to be stabilised by Rho GTPases and in turn microtubules can also affect the activities of the Rho GTPases (Watanabe et al., 2005).

The interaction between eEF1A and microtubules was first reported in higher plant cells by isolating and identifying proteins associated with microtubules (Durso and Cyr, 1994). This

association was found to stabilise the microtubule network in a calcium and calmodulin dependent manner (Durso and Cyr, 1994, Moore et al., 1998). Further studies demonstrated that the microtubule binding sites were located on domains I and III of eEF1A. Further analysis indicated that the binding site in domain III was found to bind to microtubules under normal cellular conditions, however, domain I was found to bind to the microtubule conditionally depending on the local pH, therefore suggestive of the latter domain being the regulating factor (Moore and Cyr, 2000). In contrast to this, eEF1A has also been shown to sever microtubules (Shiina et al., 1994), therefore it is possible that eEF1A has a role in regulating microtubules, with different activities based on different conditions.

Intermediate filaments function to maintain cell shape by enabling the cell to bear tension. They are generally made up of keratin or vimentin with lamin providing support for the nuclear envelope. There are six main types of intermediate filaments types I – VI. In epithelial cells, the most important types are I & II which consist of keratins, type III which consist of vimentin and type V which consist of nuclear lamins. The intermediate filaments form a complex meshwork of proteins which give the cell structural integrity especially when the cells are under stress, however unlike microtubules and actin filaments, they have no role in cell migration. No links between eEF1A and intermediate filaments have so far been established.

### 1.4.2 eEF1A and Cell Death

The first evidence to suggest a potential role of eEF1A in cell death was the finding that eEF1A localises to the nucleus in *Trypanosoma cruzi* during apoptosis, as shown by immunofluorescence studies (Billaut-Mulot et al., 1996). Further supporting evidence was provided when activation of p53, during the apoptosis process, was shown to upregulate the levels of eEF1A proteins (Kato et al., 1997). Induction of apoptosis through the use of sub lethal doses of hydrogen peroxide also resulted in transiently increased levels of eEF1A and that decreasing the level of eEF1A using antisense eEF1A protected the cells from apoptosis, and the level of protection, corresponds to the degree of repression of eEF1A expression (Chen et al., 2000), however there was no clear ideas whether such properties were due to translation of specific proteins involved in the apoptotic process, or other non-canonical functions.

Conversely to the results obtained by previous studies, a screen on IL-3 dependent cells identified eEF1A as a gene contributing to apoptotic resistance, as induced by both serum deprivation and by induction of endoplasmic reticulum (ER) stress (Talapatra et al., 2002). This protection from apoptosis was also found to occur in a dose dependent manner, with the highest level of eEF1A expression resulting in the greatest level of protection without correlating with total cellular protein level, cell size, or the rate of protein synthesis, indicating a translation independent role for eEF1A in apoptosis (Talapatra et al., 2002). No clear explanations were provided, however it was suggested that the protective effect of higher levels of expression of eEF1A seen in apoptosis could be due to better translational accuracy, resulting in fewer amino acid misincorporations, and therefore a reduction in protein misfolding leading to a decrease in accumulated misfolded proteins at the ER and reduced ER stress.

To add to the complexity and uncertainties regarding a role of eEF1A in cell viability, the two isoforms of eEF1A appear to have opposite roles in regulating apoptosis. Studies show that eEF1A1 seems to have a pro-apoptotic effect, with resistance to apoptosis occurring when eEF1A1 is disrupted, whereas eEF1A2 appears to have an anti-apoptotic effect, resulting in reduced cell death where expression of eEF1A2 is increased (Ruest, 2002, Chen et al., 2000, Borradaile et al., 2005). Duttaroy et al, 1998, suggested that eEF1A1 may be an essential regulator of apoptosis by showing that an increase in the expression of eEF1A resulted in an increased rate of apoptosis, whereas a decreased level of expression of eEF1A resulted in a decreased rate of apoptosis upon apoptotic stimuli. This potential role of both isoforms of eEF1A, in particular the anti-apoptotic potential of eEF1A2, may partially explain the oncogenic potential of the eEF1A2 isoform since eEF1A2 protected differentiated myotubes from caspase 3 mediated apoptosis, when the cells were deprived of serum (Ruest, 2002). This opposing effect of eEF1A1 and eEF1A2 in the process of apoptosis may allow eEF1A to have an important role in regulating apoptosis in cells, and as eEF1A2 is usually expressed in terminally differentiated cells, this finding that eEF1A2 could protect the cells from apoptotic cell death, could be physiologically important. The authors suggested that eEF1A2 may favour the translation of pro-survival genes or repress the translation of pro-apoptotic genes, with eEF1A1 working in the opposite way (Ruest, 2002).

Further studies into the effect of eEF1A on apoptosis found that only the eEF1A2 isoform interacts with peroxiredoxin-1 (Prdx-1), and that this interaction helps to protect cells against apoptotic cell death induced by hydrogen peroxide (Chang and Wang, 2007). Prdx-1 has been

shown to protect cells against oxidative stress by reducing reactive oxygen species (ROS), and transfection studies on Prdx-1 and eEF1A2 showed that alone, both genes had a protective effect against apoptosis, however, double transfections, with both genes, resulted in increased resistance, when compared to single transfections. The increase in eEF1A2 and Prdx-1 also correlates to an increase in the level of Akt, a survival factor, which previous studies have already shown to be affected by eEF1A2, as well as having a role in actin remodelling, and regulating migration, invasion and proliferation (Amiri et al., 2007, Pecorari et al., 2009). The findings from Li et al, 2010, that eEF1A2 knockdown in plasmacytomas increases apoptosis, also supports the idea that eEF1A2 has a protective effect against apoptotic cell death (Li et al., 2010).

eEF1A has also shown to be involved in anoikis, apoptosis caused by loss of cell anchorage (Itagaki et al., 2012). It has been suggested that, during serum starvation, NIH3T3 cells that adhere to fibronectin undergo anoikis caused by matrix metalloproteinase 2 (MMP-2) excretion which contributes to exposure of a cryptic anti-adhesive site within the fibronectin molecule. eEF1A then has the ability to mediate the anti-adhesive effect of the site, resulting in loss of cell anchorage and therefore death by anoikis. siRNA disruption of eEF1A expression resulted in resistance to anoikis, indicating the importance of eEF1A in this method of cell death.

A novel form of cell death was found to be induced by downregulation of eEF1A1 in tetraploids, cells in which the chromosomes did not segregate properly during mitosis, resulting in cells with four chromosomes rather than the usual two, which can facilitate tumour formation (Kobayashi and Yonehara, 2009). This cell death correlated with a decrease in eEF1A1 expression at both the mRNA and protein level, and transfection of the cells with an eEF1A1 plasmid resulted in reduction of cell death, suggesting that the downregulation of eEF1A1 induces the observed cell death. This increase in eEF1A1, and the corresponding decrease in this method of cell death resulted in an increased number of spontaneous binucleations, indicating that the novel form of cell death observed in this study contributes to the elimination of these naturally arising cells within a normal cell population (Kobayashi and Yonehara, 2009). The authors suggested that, as eEF1A2 was found to have a similar effect on this form of cell death when compared to eEF1A1, the tumorigenicity of eEF1A2 when overexpressed could be related to diminishing the cell death that would normally be induced by the reduction of expression of eEF1A1. It was found that the 5' untranslated region (UTR) from eEF1A1 is important to this downregulation of eEF1A1 expression.

Together all of this evidence suggests that overall expression of eEF1A1 may have a proapoptotic role, whereas eEF1A2 may have the reverse role, indicating an apparent difference in the non-canonical roles of the two isoforms. Suggestions were made by many authors regarding the mechanism by which eEF1A regulates apoptosis, with some indicating differences in translation of specific proteins essential for apoptotic cell death, with others suggesting the non-canonical function of eEF1A in actin binding and microtubule severing, and therefore overall cytoskeletal reorganisation as the mechanism for this regulation. So far there is little evidence to confirm or refute these theories.

### 1.4.3 eEF1A and Cell Cycle and Ageing

eEF1A has been thought to be associated with cell proliferation and ageing due to its involvement in different types of cancers when overexpressed and also due to the difference between expression of the different isoforms. The two isoforms are specifically expressed in cells with very different rates of cell proliferation, with eEF1A1 being expressed in rapidly dividing cells, and eEF1A2 in terminally differentiated cells, therefore providing the possibility that the isoform present could affect the rate of cell proliferation which may suggest that eEF1A could perform a regulatory role. In 1989, a study showed that an additional copy of the eEF1A gene lead to a longer life span in fruit flies, and Cavallius et al, 1989, showed that the activity of eEF1A decreased towards the end of the lifespan of human fibroblasts (Cavallius et al., 1986a). A more recent study identified eEF1A as an age related protein in human colonic epithelial cells, with the expression of eEF1A reduced in senescent cells (Yi et al., 2010). eEF1A expression was also found to be down regulated in different cancer cell lines that were undergoing induced cellular senescence in response to various stimuli, such as ionising radiation and hydrogen peroxide treatment, providing further evidence of the involvement of eEF1A, and potentially identifying eEF1A as a marker of cellular senescence and a potential therapeutic target for cancer treatment (Byun et al., 2009).

Reduced expression of eEF1A has been shown to reduce the proliferation rate of cells, and two independent studies on different cells have shown that reducing the expression of eEF1A results in a longer G1 phase resulting in the slower proliferation rate (Janse et al., 2003, Pecorari et al., 2009).

### 1.4.4 Other Secondary Functions

eEF1A has been implicated in the export of both tRNAs and proteins from the nucleus. Interruption by siRNA or mutation of the binding of eEF1A to the transcription dependent nuclear export motif of the protein resulted in defective nuclear export, with the suggestion that eEF1A stimulates nuclear export without entering the nucleus (Khacho et al., 2008).

When protein translation occurs, the synthesised protein undergoes quality surveillance to ensure there is no damage to the protein. How this is regulated is poorly understood, however eEF1A has been suggested to be involved by several studies, such as Hotokezaka et al, 2002, who showed that eEF1A associated with newly synthesised proteins that were not folded correctly, but not to proteins that were correctly folded (Hotokezaka, 2002), and Chuang et al, 2005, who found that the degradation of damaged proteins by the proteasome involved eEF1A by interaction with ubiquitinated proteins and the proteasome (Chuang et al., 2005). Further to this, eEF1A was also found to interact with the proteasome in Xenopus oocytes (Tokumoto et al., 2003). eEF1A has also been shown to bind to defective proteins which are released from the ribosomes which can then trigger aggresome formation (Meriin et al., 2012). eEF1A has also been shown to be required for the ubiquitin dependent degradation of certain proteins, including actin, though the mechanism by which this occurs is not understood (Gonen et al., 1994).

eEF1A has been shown to have a chaperone like function to allow renaturation of aminoacyl tRNA synthetases and other proteins which has been suggested to be important for maintaining enzyme activity and protecting the cells from stress (Lukash et al., 2004, Caldas et al., 1998).

All of the secondary functions mentioned show eEF1A to be a multi functional protein with many different roles, however, what is not certain is whether these functions are independent of its role in translation, for example if eEF1A protein expression is reduced, cell proliferation rate is decreased, but is this because protein translation is slower and therefore cell division is slowed down, or does eEF1A have an independent regulatory function in cell proliferation? The work by Gross and Kinzy, 2005, showed changes in actin binding while not affecting translation suggesting that the actin binding and translation functions of eEF1A are

independent. Further understanding is required to be able to identify if this is true for the other proposed secondary functions of eEF1A to determine which are true secondary functions and understand to how it is able to participate in so many different roles and have the ability to regulate such complex processes.

### 1.4.5 eEF1A Interactions

eEF1A has been shown to interact with a number of different molecules including phosphotidylinositol-4 kinase and calmodulin (Yang et al., 1993, Kaur and Ruben, 1994, Durso and Cyr, 1994, Shiina et al., 1994), phospholipase C-gamma 1 (Chang et al., 2002, Kim et al., 1999), non-receptor tyrosine kinase Txk of the Tec family (Maruyama et al., 2007), and Akt2 rho associated kinase (Lau et al., 2006). The molecules that eEF1A can interact with is also dependent on the isoform that is present as in some cases, eEF1A1 cannot bind to the same molecules as eEF1A2, for example, eEF1A2 has been shown to be able to interact with the SH2 domain of Shc and the SH3 domain of Crk, whereas eEF1A1 cannot (Panasyuka et al., 2008). In fact, by using the SH2 and SH3 domains of various different signalling molecules, Panasyuka et al, 2008, hypothesised that the difference in non-canonical functions between the two isoforms may be due to the difference in potential to participate in phosphotyrosine-mediated signalling. eEF1A2 appears to have greater potential when compared to eEF1A1 as eEF1A2 was demonstrated to have the ability to interact with more domains of signalling proteins, such as the SH2 domains of Grb2, rasGAP, and Shc, as well as the SH3 domains of Crk, Fgr and Fyn, whereas no such ability was found for eEF1A1. They also suggested that the increased oncogenicity of eEF1A2 compared to eEF1A1 may also be due to the difference in phosphotyrosine-mediated signalling.

## *1.5 eEF1A in Infection and Disease*

eEF1A has been implicated in many different infections and/or diseases including the 'wasted' mouse, autoimmune diseases, viral infections and many different cancers albeit for different reasons. The role of eEF1A in these different diseases is summarised below:

The ‘wasted’ mouse is a mouse model characterised by weight loss, progressive paralysis, tremors and ataxia after 21 days. By 28 days, the mice have died due to muscle wasting and neuronal degeneration. It was found that these mice have a homozygous mutation in which the promoter region and the first non coding exon of the eEF1A2 gene is deleted therefore there gene cannot be transcribed (Chambers et al., 1998). The time of development of this disease correlates to the isoform switch in mice which sees the expression of the eEF1A1 isoform replaced with the eEF1A2 isoform in skeletal muscle and neurons (Lee et al., 1993, Khalyfa, 2001), therefore in ‘wasted’ mice, as the eEF1A2 gene is not functional, when the level of eEF1A1 expression decreases, there is no isoform to replace this decrease, leading to cells deficient of eEF1A. This lack of eEF1A would mean that the cell could not synthesise new proteins, and would potentially affect the actin cytoskeleton leading to the wasting and degeneration of the tissue.

The ‘wasted’ mouse is used as a model for amyotrophic lateral sclerosis (ALS) (Newbery and Abbott, 2001), and a link between eEF1A and neuronal degeneration has been tentatively suggested due to the ability of eEF1A to form a complex with the zinc finger protein 1 (ZPR1) and alter the localisation of both proteins (Gangwani et al., 1998). A mutation of the survival motor neuron protein (SMN) has been linked to an early onset form of motor neuron disease (Monani, 2005), and ZPR1 has been shown to form a complex with SMN which has been shown to be essential for the correct localisation of SMN in neurons. Alongside this a protein of the same size as eEF1A co-purifies with the ZPR1/SMN complex suggesting that eEF1A may play a role in this complex, and potentially neuronal degeneration (Matera and Herbert, 2001). Interestingly the neuronal degeneration observed in the ‘wasted’ mouse was accompanied by the reduced expression of ZPR1 protein, which may add further credence to this theory (Murray et al., 2008). Reduced expression of eEF1A has also been noted to result in reduced protein translation in Myotonic Dystrophy 2, caused by a reduced expression of zinc finger factor 9 (ZNF9), which was shown to bind to eEF1A mRNA in myoblasts, suggesting that in normal myoblasts, ZNF9 may play some part in regulating the translation of eEF1A mRNA such that when it is reduced the level of eEF1A is also reduced leading to a decrease in total protein synthesis (Huichalaf et al., 2009).

eEF1A has been linked to Felty’s syndrome with Ditzel et al, 2000 finding that 66% of patients with this syndrome had anti-eEF1A1 antibodies in their serum, however the reason and significance for the presence of the autoantibodies in these patients is currently unknown (Ditzel et al., 2000).

Viruses require the host cell to provide the translational machinery in order to translate the viral genes needed for production of viral proteins and formation of further viral particles (Walsh and Mohr, 2011), it is therefore unsurprising that eEF1A, as a main component of the translational machinery, has been implicated in many different viral infections including the tombusvirus, HIV-1, human papillomavirus (HPV), hepatitis B and C, West Nile virus (WNV) and mosaic viruses (Li et al., 2009, Gamarnik et al., 2010, Warren et al., 2012, Yue et al., 2011, Lin et al., 2012, Kou et al., 2006, Davis et al., 2007, Yamaji et al., 2006, Thivierge et al., 2008, Yamaji et al., 2009). Unsurprisingly eEF1A can bind to viral RNA, as well as interact with viral polymerases and other proteins. It has also been shown to facilitate viral particle assembly (Li et al., 2013). Along with its translational function, the actin binding function of eEF1A has been shown to be utilised by viruses. The X protein from the hepatitis B virus (HBV) has been shown to bind to eEF1A and prevent its dimerisation and alter its actin bundling activity (Lin et al., 2012).

Viral infection can sometimes lead to the development of cancer such as with the HPV virus, which after persistent infection can lead to cervical cancer (Chen et al., 2011). Some of the HPV viruses have been shown to induce cellular immortalisation and transformation through expression of two oncoproteins, E6 and E7 (Ghittoni et al., 2009), the latter of which has been shown to associate with domain III of eEF1A, resulting in decreased actin binding by eEF1A and therefore disruption of the actin cytoskeleton (Yue et al., 2011).

## *1.6 eEF1A and Cancer*

Aberrant expression of eEF1A has been associated with many different types of cancer including breast, lung, ovarian and prostate cancer (Kulkarni et al., 2006, Li et al., 2006, Anand et al., 2002, Scaggiante et al., 2011), however, what is more interesting is that it has been shown to be associated with the metastatic process (Edmonds et al., 1996, Pencil et al., 1993). Based on the role of eEF1A in the organisation of the cytoskeleton, as well as its role in protein translation, it is perhaps unsurprising that changes in the level of eEF1A could lead to such phenotypes, though how this occurs is currently unknown. Determining the function of eEF1A in cell attachment and cell migration would therefore be of interest to help to understand this link. It has also been shown that the two different isoforms of eEF1A can both affect cancer progression and formation, particularly where expression of eEF1A2 is observed in tissues that would normally only express the eEF1A1 isoform (Anand et al., 2002, Amiri et al., 2007).

### 1.6.1 Prostate Cancer

Increased expression of both eEF1A1 and eEF1A2 has been observed in prostate cancer cells when compared to normal controls, in particular in metastatic tumour cell lines (Zhu et al., 2009, Scaggiante et al., 2011, Rehman et al., 2012). Furthermore reducing the eEF1A expression by siRNA resulted in decreased proliferation, invasion and migration, indicating that eEF1A could be a good therapeutic target for prostate cancer, as well as a good marker for tumour progression, however the specific isoform of eEF1A involved was not determined (Zhu et al., 2009). Further to this, an assay used to determine serum biomarkers for metastatic progression of human prostate cancer found that eEF1A1 protein expression was significantly increased in non progressing cancer compared to the normal control group, and a further significant increase in the progressing cancer, further confirming that the expression of eEF1A could be used as a biomarker for the progression of prostate cancer (Rehman et al., 2012). In contrast to these results, a study by Scaggiante et al, 2011, showed that eEF1A1 mRNA and protein levels were unchanged in the prostate cancer cell lines used, however, eEF1A2 levels were significantly increased in all the cancer cell lines used, with the highest expression observed in the least aggressive form of cancer (Scaggiante et al., 2011). Interestingly, prostate tumour inducing gene 1 (PTI-1), a truncated mutated form of eEF1A

(Mansilla et al., 2005), is also differentially expressed in metastatic prostate carcinomas in comparison to their normal and benign counterparts (Sun et al., 1997).

### 1.6.2 Ovarian Cancer

Amplification of the 20q13 locus is commonly observed in ovarian cancers (Sonoda et al., 1997). As this is the location of the eEF1A2 gene (Lund et al., 1996), it was an interesting target to determine any correlation with the incidence of ovarian cancer. eEF1A2 has been determined as an oncogene in ovarian cancer with eEF1A2 gene expression found to be overexpressed in 30% of ovarian cancers (Anand et al., 2002, Pinke et al., 2007), increased eEF1A2 protein expression in 75% of ovarian clear cell carcinomas (Tomlinson et al., 2007) and increased eEF1A2 gene expression coinciding with an increased likelihood of recurrence of epithelial ovarian cancer (Sharma et al., 2007). Similarly, a study by Sun et al, 2008, showed that an increase in eEF1A2 protein expression by lentiviral transfection resulted in apoptosis resistance, serum independence, and anchorage independence which could be reversed by siRNA interference (Sun et al., 2008). In contrast to these studies, serous tumours with a higher expression of eEF1A2 resulted in a better probability of survival than those with a lower expression suggesting that, as in the case of prostate cancer, an increased expression of eEF1A2 leads to a more favourable outcome (Pinke et al., 2007).

### 1.6.3 Breast Cancer

Amplification of chromosome 20 is commonly observed in breast cancer, in particular 20q12-13, the region in which eEF1A2 is found (Lund et al., 1996), which is amplified in 40% of breast cancer cell lines (Hodgson et al., 2003). eEF1A has been shown to be upregulated in breast cancer tissue (Chen, 2005), and has been suggested to be a potential diagnostic marker for breast cancer development (Hamrita et al., 2011). Both eEF1A and eEF1β were found to be upregulated in cancerous tissue when compared to normal breast tissue (Al-Maghrebi et al., 2005). Specifically eEF1A2 has been shown to be overexpressed in two thirds of breast tumours, and is suggested to be an oncogene in breast cancer, as well as a potential diagnostic marker and therapeutic target (Tomlinson et al., 2005). It was determined that patients with

tumours showing a high expression of eEF1A2 have an increased probability of 20 year survival when compared to those with tumours that show a low eEF1A2 expression (Kulkarni et al., 2006).

A study by Edmonds et al, 1996, showed that eEF1A protein expression is increased in metastatic rat mammary adenocarcinoma, and that its actin binding activity is reduced compared to non metastatic tumours resulting in potential reorganisation of the cytoskeleton and changes to the translation of specific mRNAs that are associated (Edmonds et al., 1996). It has been shown that as well as binding to actin, eEF1A can interact with pAkt-1, which is involved in regulating cell proliferation motility and angiogenesis (Pecorari et al., 2009). Downregulation of eEF1A by siRNA resulted in reduced expression of pAkt-1 leading to reduced proliferation, migration and invasion suggesting that eEF1A may regulate pAkt (Pecorari et al., 2009). Akt/PKB is a serine/threonine kinase that regulates many different cellular processes, in particular those involved in cancer such as proliferation, motility and survival (Liu et al., 2007), and activation of this can result in more aggressive tumour behaviour and decreased survival (Bellacosa et al., 2005). Pecorari et al, 2009, showed that decreased expression of eEF1A resulted in decreased pAkt, and inhibited cell proliferation, invasion and promoted apoptosis of HCC1937 cells, indicating that eEF1A is a regulator of pAkt activity, and agreeing with previous studies that show that eEF1A promotes tumourigenesis and indicate that eEF1A expression is required for many breast cancer cell properties via both pAkt dependent and Akt independent mechanisms. eEF1A2 has also been shown to stimulate cell migration in a PI3K and Akt dependent way in BT549 cells, suggesting that eEF1A2 regulates oncogenesis through these pathways by cytoskeletal remodelling (Amiri et al., 2007).

### 1.6.4 Lung Cancer

Two different studies have implicated the overexpression of eEF1A2 in lung cancer and have suggested it to be an oncogene, possibly acting as a biomarker for diagnosis and therapy (Li et al., 2006, Zhu et al., 2007). Indeed inhibition of eEF1A2 expression by siRNA was shown to reduce cell proliferation as well as increasing the rate of apoptosis in all four lung cancer cell lines that were tested (Li et al., 2006). Interestingly PRDX1, a protein which protects cells from oxidative stress by reducing reactive oxygen species, was also found to be increased in lung cancer cells and the cells adjacent to the cancer cells (Li et al., 2006). eEF1A, specifically

eEF1A2, has been shown to interact with PRDX1 to protect cells against apoptosis induced by oxidative cell stress (Chang and Wang, 2007), therefore the increased expression of both of these proteins in lung cancer cells could provide a mechanism for apoptotic resistance. Furthermore eEF1A2 has been shown to be increased in lung cancer cells, not by an increase in expression, but by a decrease in the degradation of eEF1A2 after phosphorylation by C-Raf, and that this increase appeared to have an anti-apoptotic effect on the cancer cells (Lamberti et al., 2007).

### 1.6.5 Other Cancers

As well as those cancers already mentioned, eEF1A has also been associated with other types of cancer which have not been studied as extensively. eEF1A2 has been shown to be a potential marker for patient outcome in gastric adenocarcinoma, with reduced expression of eEF1A2 being associated with poor survival (Xu et al., 2011). In cancer of the liver, eEF1A1 mRNA levels were found to be increased in both cancer cell lines analysed, and eEF1A2 in only one when compared to normal controls (Grassi et al., 2007). Wit et al, 2002, showed that eEF1A2 was downregulated in human melanoma cell lines (Wit et al., 2002). eEF1A2 has also been shown to promote cell growth and inhibit apoptosis in mouse plasmacytomas and some human multiple myelomas, potentially through regulation of the JAK/STAT or Akt signalling pathways (Li et al., 2010). A study on the expression of eEF1A2 in pancreatic cancer found that eEF1A2 was overexpressed in 83% of the pancreatic cancers studied, and that this overexpression resulted in increased cell growth, survival and invasion (Cao et al., 2009). Overexpression of eEF1A2 in NIH3T3 cells resulted in increased cell growth rate as well as making cells more tumourigenic by enhancing focus formation and allowing anchorage dependent growth (Anand et al., 2002).

### 1.6.6 Targeting of eEF1A in Cancer Therapy

eEF1A has been shown to be a candidate marker for the diagnosis and prognosis of different types of cancer, and its overexpression has been demonstrated to affect cell proliferation, migration and invasion as well as apoptosis, the main attributes for a cancer to become

metastatic. For this reason eEF1A, especially eEF1A2 has been suggested to be a good therapeutic target despite its essential function in translation, and could potentially explain why tumour cells that grow rapidly may be more sensitive to any potential interference (Lee, 2003).

Inhibition of PTI-1, the truncated mutated form of eEF1A with antisense PTI-1 resulted in reversal of the transformed cells into a more normal morphological phenotype suppressing anchorage independent growth (Su et al., 1998). Some success has also been achieved in increasing the survival of mice with metastatic melanomas by targeting eEF1A with narciclasine which reduces cancer cell proliferation with little effect on normal non cancerous cell lines (Van Goietsenoven et al., 2010). eEF1A2 has also been suggested to be a potential target of resveratrol which has been shown have the ability to reduce the proliferation rate of ovarian cancer cells (Lee et al., 2009).

eEF1A has been shown to be the binding target of cytotoxic GT oligomers that can have antiproliferative and cytotoxic effects on lymphoblasts (Dapas et al., 2003), haematopoietic human cancer cells (Scaggiante et al., 2006) and prostate cancer cells (Scaggiante et al., 2014). These are promising results using eEF1A as a target to combat cancers, and further support the idea that eEF1A is one of the main oncogenes, overexpression of which can significantly contribute to the cancerous phenotypes of increased cell proliferation, migration, invasion and decreased rates of apoptosis, making the mechanism by which eEF1A exerts this effect important to establish. eEF1A has also been shown to be upregulated in cells with acquired cisplatin resistance (Johnsson et al., 2000), therefore adding increased difficulty to treatments already used.

Early stage research has shown that targeting eEF1A in prostate cancer cells using an aptamer called GT75, a nucleic acid which has been shown to be able to bind to eEF1A, resulted in a decrease in cell proliferation and migration in cancer cells with little effect on the non tumourigenic control, as well as increasing the effect of conventional drugs (Scaggiante et al., 2014). This further confirms eEF1A as a causative agent of the increased growth and migration rates of progressive prostate cancers and highlights the importance of understanding the mechanism by which eEF1A causes this change to enable specific targeting of these processes with few side effects.

## *1.7 Aims and Objectives*

Most, if not all of the work so far, has focused on the aberrantly regulated effects of eEF1A expression, most notably eEF1A overexpression. Very little attention has been focused on trying to determine the physiological consequences of eEF1A expression in normal cells, due to direct implications related to the canonical function of these proteins, at least in mammalian cells. Through different strategies, cells constitutively expressing differential levels of eEF1A, including eEF1A1 and eEF1A2, were obtained. The aim of this work was to establish, both at the cellular and molecular levels, the implications of low expression of these proteins on protein synthesis and other reported non canonical functions of the factors. Using immunofluorescence and motility studies, changes in cytoskeletal organisation and cell migration were analysed to determine whether any differences could be observed when the level of eEF1A protein expression was reduced. Our main objectives were;

- To determine whether the reduced expression of eEF1A protein would affect its role in protein translation
- To determine whether the reduced expression of eEF1A proteins was sufficient to affect its role in actin binding and bundling
- To determine whether any of the above differences would affect the motility of the cells
- To prove that any changes observed were eEF1A dependent
- To observe whether there was any difference in rescue with eEF1A1 and eEF1A2

# Chapter 2: Materials & Methods

**Chapter 2 Table of Contents**

# 2.0 Materials and Methods

## *2.1 Materials for Mammalian Cell Work*

### 2.1.1 Antibodies Used

**Table 2.1.1.1 Antibodies Used for Immunofluorescence Staining**

| Name of Antibody | Secondary Antibody Used | Source | Dilution |
|---|---|---|---|
| **Immunofluorescence Staining – Primary Antibodies** | | | |
| Phalloidin (Actin) | Mouse | Invitrogen | 1 in 100 |
| eEF1A | Mouse | Millipore | 1 in 100 |
| Paxillin | Mouse | Invitrogen | 1 in 100 |
| Vinculin | Mouse | Sigma Aldrich | 1 in 200 |
| VASP | Rabbit | Abcam | 1 in 200 |
| Tubulin | Mouse | Sigma Aldrich | 1 in 100 |
| Vimentin | - | Sigma Aldrich | 1 in 200 |
| Myosin IIA | Rabbit | Covance | 1 in 2,000 |
| Arp3 | Mouse | Abcam | 1 in 500 |

| | | | |
|---|---|---|---|
| β-1 Integrin | Mouse | Millipore | 1 in 500 |
| **Immunofluorescence Staining – Secondary Antibodies** | | | |
| Anti- Mouse FITC | - | Dako | 1 in 100 |
| Anti- Rabbit FITC | - | Dako | 1 in 100 |

**Table 2.1.1.1 –** The primary and secondary antibody dilutions used for all immunofluorescence studies described in chapters 3 and 4, including the company purchased from, as well as the secondary antibody used where applicable.

**Table 2.1.1.2 Antibodies Used for Western Blotting**

| **Name of Antibody** | **Secondary Antibody Used** | **Source** | **Dilution** |
|---|---|---|---|
| **Western Blotting – Primary Antibodies** | | | |
| eEF1A1 | Rabbit | Gene Tex | 1 in 1,000 |
| eEF1A2 | Rabbit | Gene Tex | 1 in 1,000 |
| eEF1A | Mouse | Millipore | 1 in 5,000 |
| α-Adaptin | Mouse | Santa Cruz | 1 in 1,000 |
| Paxillin | Mouse | Invitrogen | 1 in 1,000 |

| | | | |
|---|---|---|---|
| Vinculin | Mouse | Sigma Aldrich | 1 in 2,000 |
| VASP | Rabbit | Abcam | 1 in 2,000 |
| Tubulin | Mouse | Sigma Aldrich | 1 in 5,000 |
| Vimentin | Mouse | Gene Tex | 1 in 1,000 |
| Myosin IIA | Rabbit | Covance | 1 in 20,000 |
| Arp3 | Mouse | Abcam | 1 in 1,000 |
| β-1 Integrin | Mouse | Millipore | 1 in 1,000 |
| Actin | Rabbit | Sigma Aldrich | 1 in 1,000 |
| Actin (From Kit) | Rabbit | Cytoskeleton | 1 in 500 |
| **Western Blotting – Secondary Antibodies** | | | |
| Anti- Mouse HRP | - | Sigma Aldrich | 1 in 5,000 |
| Anti- Rabbit HRP | - | Sigma Aldrich | 1 in 5,000 |

**Table 2.1.1.2 –** The primary and secondary antibody dilutions used for all Western blotting studies described in chapters 3 and 4, including the company purchased from, as well as the secondary antibody used where applicable.

**Table 2.1.1.3 Antibodies Used for ELISA**

| Name of Antibody | Secondary Antibody Used | Source | Dilution |
|---|---|---|---|
| **ELISA – Primary Antibodies** | | | |
| eEF1A | Mouse | Millipore | 1 in 1,000 |
| Tubulin | Mouse | Sigma Aldrich | 1 in 1,000 |
| **ELISA – Secondary Antibodies** | | | |
| Anti- Mouse HRP | - | Sigma Aldrich | 1 in 2,000 |

**Table 2.1.1.3 -** The primary and secondary antibody dilutions used for all ELISA studies described in chapters 3 and 4, including the company purchased from, as well as the secondary antibody used where applicable.

## 2.1.2 Primers Used for Mammalian Cell Work

| Primer Name | Primer Sequence 5'-3' | Digestion Enzyme |
|---|---|---|
| eEF1A1 Forward | CGGAATTCGCCACCATGGGAAAGGAAAAGACT | EcoRI |
| eEF1A1 Reverse | TGCTCTAGATCATTTAGCCTTCTGAGC | XbaI |
| eEF1A2 Forward | CGGAATTCGCCACCATGGGCAAGGAGAAGACC | EcoRI |
| eEF1A2 Reverse | TGCTCTAGATCACTTGCCCGCCTTCTG | XbaI |

**Table 2.1.2** – List of primers used for plasmid construction including sequences and digestion enzymes used.

### 2.1.3 Plasmids Used for Mammalian Cell Work

| Name as Referred to in Text | Description | Purpose | Sequence & Plasmid Map |
|---|---|---|---|
| pcDNA3.1 Zeo | pcDNA3.1 Zeocin, Ampicillin and Zeocin resistance | Backbone for all plasmids created | - |
| pstBLUE | Cloning vector, Ampicillin and Kanamycin resistance | To create plasmid with PCR product to allow more efficient digestion | - |
| SGB51 | eEF1A1 in pGEM-T easy vector, Ampicillin resistance | Plasmid used as PCR template for eEF1A1 insert | - |
| SGB42 | eEF1A2 in pcDNA3.0, Ampicillin and G418 resistance | Plasmid used as PCR template for eEF1A2 insert | - |
| eEF1A1 in Cloning Vector | eEF1A1 in pstBLUE-1 Cloning Vector, Ampicillin and Kanamycin resistance | Plasmid created with eEF1A1 PCR product to allow more efficient digestion | - |

| | | | |
|---|---|---|---|
| eEF1A2 in Cloning Vector | eEF1A2 in pstBLUE-1 Cloning Vector, Ampicillin and Kanamycin resistance | Plasmid created with eEF1A2 PCR product to allow more efficient digestion | - |
| eEF1A1 Zeo | eEF1A1 in pcDNA3.1 Zeo, Ampicillin and Zeocin resistance | Plasmid for transfection of eEF1A1 into 2E2 cells | Appendix 8.1 |
| eEF1A2 Zeo | eEF1A2 in pcDNA3.1 Zeo, Ampicillin and Zeocin resistance | Plasmid for transfection of eEF1A2 into 2E2 cells | Appendix 8.2 |

**Table 2.1.3 –** Plasmids used throughout chapter 4, including names, description and usage.

## *2.2 Methods Used for Mammalian Cell Work*

### 2.2.1 Cell Lines Used

Chinese hamster ovary cells (CHO cells) were used as a control cell line. CHO cells with a reduced expression of eEF1A were kindly donated by Borradaile et al, 2005, referred to from this point on as 2E2 cells. The 2E2 cells had been created by Borradaile in the process of studying resistance of CHO cells to lipotoxic cell death. They were generated using a vesicular stomatitis virus G protein (VSV-G) pseudotyped murine retrovirus, which encoded the ROSAβgeo retroviral promoter trap (Friedrich and Soriano, 1991). This virus was used to transduce the CHO-K1 cells such that there was only one integration per 10 genomes, to attempt to ensure that there was no more than one integration per cell. The mutated cells were then selected using 500μg/ml G418 in high glucose (4.5 mg/ml) DMEM and Ham's F-12 nutrient mixture (1:1), with 5% (v/v) fetal bovine serum (FBS), 2 mM l-glutamine, 50 U/ml penicillin G sodium, 50 U/ml streptomycin sulfate, and 1 mM sodium pyruvate for 8 days, after which 500μM palmitate was added to the media, sufficient to result in death of wild type CHO cells that had not acquired resistance.

Clones of cells that survived were then isolated, and 5' rapid amplification of cDNA ends (RACE) was used to identify the gene that had been disrupted by the integration, which was found to be eEF1A. Northern blotting, using a probe created from full length eEF1A1, and Western blotting using an eEF1A antibody that recognises both isoforms of eEF1A were performed and only a feint residual band was detected in the mutated cells when compared to the control CHO cells. PCR using primers specific to either eEF1A1, eEF1A2, or ROSAβgeo, showed that only the eEF1A1 gene was interrupted in the mutated cells, which led the authors to conclude that expression of eEF1A1 was completely knocked out, and that the residual band observed on the Northern and Western blots were due to the lack of specificity of the probes as the sequences of eEF1A1 and eEF1A2 are so similar at the amino acid level, resulting in a cell line that only expressed very small amounts of eEF1A2 and no eEF1A1. The selection used for the 2E2 cell line was the G418 antibiotic, and, in order to enable the control cell line to be grown in the same media, CHO cells were transfected with an empty plasmid containing the G418 resistance gene, neo, and selected using 500 μg/ml G418 containing medium. These CHO cells transfected with the empty G418 resistance plasmid are referred to as CHO G418 cells. 2E2 cells were also transfected with the pcDNA3.1 zeocin plasmid containing eEF1A1 or

eEF1A2 and both 2E2 and CHO G418 cells were transfected with the empty pcDNA3.1 zeocin plasmid as controls. These cells are referred to as 2E2 eEF1A1, 2E2 eEF1A2, 2E2 zeo and CHO G418 zeo respectively from this point forward.

## 2.2.2 Cell Culture

The control CHO cell line used was grown in DMEM. The 2E2 and CHO G418 cell lines were grown in DMEM media (Table 2.2.2) with 500 µg/ml G418. Transfected 2E2 cells and CHO G418 zeo cells were grown in DMEM with 500µg/ml G418 and 200µg/ml zeocin. All cell lines were incubated at 37$^{o}$C in a humidified atmosphere with 5% (v/v) $CO_2$ and 95% (v/v) air.

**Table 2.2.2 Composition of DMEM**

| **Name of Solution/Buffer** | **Composition of Buffer** |
|---|---|
| DMEM | DMEM<br>4500mg/L Glucose<br>< 1% (w/v) Sodium Pyruvate<br>< 0.37% (w/v) Sodium Bicarbonate<br>0.02% (w/v) L- Glutamine<br>10% (v/v) Foetal Bovine Serum (FBS)<br>0.01% (v/v) Non Essential Amino Acids<br>50U/ml Penicillin<br>50µg/ml Streptomycin |

**Table 2.2.2 –** Composition of basic DMEM used for cell culture.

### 2.2.3 Passaging of cells

In order to maintain a healthy supply of cells, the cell lines were grown in the above conditions until a confluency of around 70-80% was reached. The media was removed from the flask and the cells washed with an appropriate amount of serum free medium. 1x Trypsin with 2mM ethylenediaminetetraacetic Acid (EDTA) at pH 7.4, was then added to cover the bottom of the flask and the cells were incubated at 37$^{o}$C for a few minutes until the cells detached from the flask. Serum containing medium was then added to deactivate the trypsin, and the cells transferred into a 15ml centrifuge tube, before pelleting by centrifugation at 250g for 5 minutes at room temperature. The supernatant was then poured off leaving the pellet at the bottom, and the cells were resuspended with fresh complete medium ready for counting or replenishing growing stock. Cell counting was done by adding an appropriate amount of medium depending on the size of the pellet observed, then resuspending the cells and adding 10µl to both sides of a haemocytometer. A cell counter was then used to count the number of cells in the grid, allowing the number of cells to be seeded to be calculated.

### 2.2.4 Cell Freezing

The above method for passaging was followed until the cells were pelleted at the bottom of the tube. The cells were then resuspended in the freezing mixture made up of 90% (v/v) FBS and 10% (v/v) dimethyl sulphoxide (DMSO), using 1-1.5mls for each vial to be frozen. The mixture was then transferred to a cryovial and placed into the -20$^{o}$C freezer for 1 hour before being transferred into the -80$^{o}$C freezer. For longer term storage, cells were transferred to a liquid nitrogen tank.

### 2.2.5 Cell Thawing

Cells to be thawed were removed from the liquid nitrogen stock or -80$^{o}$C freezer and quickly thawed using a 37$^{o}$C water bath. The content of the vial was then gently transferred to a 25cm$^{2}$ flask and pre-warmed complete media was added very slowly to the flask whilst gently

mixing the solutions together. The cells were then incubated for 4-6 hours or until the cells attached, the media removed and fresh media was added. The cells were then placed back into the incubator.

## 2.2.6 Cell Homogenisation for SDS PAGE Analysis

500,000 cells or 1,000,000 cells were seeded into a 6cm or 10cm tissue culture dish, and left to grow for 2 days, or until confluent. If cells were treated with triton before harvesting, the media was removed, the cells washed with 1x PBS, then 1ml of 10% (v/v) triton added and incubated at room temperature for 2 minutes. The triton wash was then collected. Cells were washed with 1x PBS again, before scraping cells into 100µl homogenising buffer (Table 2.2.6) using a cell scraper sterilised using 75% (v/v) IMS and 25% (v/v) distilled water and transferred using a pipette into a 1.5ml eppendorf tube. The cells were then sonicated on ice at 6-8 Amps for 10 seconds, three times with a 30 second break in between. 20µl of sample was removed for the purposes of protein quantification and the remaining 80µl was kept on ice and 5x protein dye added ready to run on the gel for the Western blot. Cells that were not washed with triton were harvested by removing media, and washing cells with ice cold PBS 3 times, before adding 50µl or 100µl homogenising buffer, scraping the cells and then continued as described above.

**Table 2.2.6 Composition of Homogenising Buffer**

| Name of Solution/Buffer | Composition of Buffer |
|---|---|
| Homogenising Buffer | 0.25M Sucrose<br>5mM Tris-HCl pH 7.4<br>2mM EDTA<br>200mM Phenylmethylsulfonyl Fluoride (PMSF) |

**Table 2.2.6 –** Composition of homogenising buffer used to prepare cell lysate for Western blotting.

## 2.2.7 Protein Quantification

To determine the amount of total protein in the sample, 5µl was added to a 96 well plate in triplicate, 25µl Biorad protein assay reagent A added and 200µl Biorad protein assay reagent B added then pipetted up and down to mix whilst avoiding the production of bubbles. Alongside this, 5µl of standards containing different levels, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 10.0 mg/ml, of bovine serum albumin (BSA) were added to the 96 well plate to use as a standard curve. The absorbance at 750nm was then measured and recoded using a plate reader and a straight line graph created using the values obtained from the standards. The standard curve was then used to determine to total amount of protein in the sample.

## 2.2.8 SDS Gel Electrophoresis

**Table 2.2.8.1 Composition of Acrylamide Gels**

| Running Gel (8%) | Running Gel (10%) | Stacking Gel (3%) |
|---|---|---|
| 4.94 ml $dH_20$ | 4.4 ml $dH_20$ | 1.725 ml $dH_20$ |
| 1.7 ml 1.5M Tris pH8.8 | 1.7 ml 1.5M Tris pH8.8 | 1.7 ml 1.5M Tris pH8.8 |
| 1.1 ml 60% Sucrose | 1.1 ml 60% Sucrose | 33 µl 10% SDS |
| 67 µl 10% SDS | 67 µl 10% SDS | 0.375 ml acrylamide (40%) |
| 2.16 ml acrylamide (40%) | 2.7 ml acrylamide (40%) | 20 µl 10% APS |
| 50 µl 10% APS | 50 µl 10% APS | 3.3 µl TEMED |
| 3.3 µl TEMED | 3.3 µl TEMED | |

**Table 2.2.8.1 –** Both 10% (v/v) and 8% (v/v) running gels were used for Western blotting depending on the size of the protein that is being detected, prepared with the chemicals as shown. For proteins between 25-200 kDa, such as vinculin, the 8% (v/v) gel was used, though for smaller proteins, between 15-100 kDa, such as eEF1A and paxillin, the 10% (v/v) gel was used. The stacking gel stayed the same regardless of which running gel was used.

The running gel was made and mixed as shown in table 2.2.8.1 then pipetted into the gel cast leaving a gap of around 1-2cm at the top. The stacking gel was then made and mixed as shown by the table and pipetted into the gel cast until the cast was full. A 10 well comb was then placed into the gel cast using laboratory tissue to catch the overflow of extra gel. The gel was then left, either in the incubator at 37$^{o}$C or on the bench, to set. The gel was taken out of the setting cast and placed into the running cast and the tank filled with 1x running buffer (Table 2.2.8.2). An appropriate amount of sample, usually between 10-50µg protein, was then

loaded and the gel run at 60V to introduce the samples, and then increased to 120V until the sample reached the end of the gel.

**Table 2.2.8.2 Composition of All Buffers Used to Run Acrylamide Gels**

| **Name of Solution/Buffer** | **Composition of Buffer** |
|---|---|
| 5x Loading Dye | 250mM Tris pH 6.8<br>40% (v/v) Glycerol<br>4% (w/v) Sodium Dodecyl Sulphate (SDS)<br>4% (v/v) 2-Mercaptoethanol<br>Bromophenol Blue to add Colour |
| 10x Running Buffer | 30g Tris<br>144g Glycine<br>10g SDS<br>Made up to 1 Litre with Distilled Water |
| 1x Running Buffer | 100mls 10x running buffer<br>Made up to 1 Litre with Distilled Water |

**Table 2.2.8.2 –** Composition of all buffers used to run the acrylamide gels for separation of proteins ready for Western blotting

## 2.2.9 Western Blotting

The gel obtained from the gel electrophoresis was placed onto nitrocellulose and sandwiched between filter paper and foam then placed into a cassette. The cassette was placed into the

Western blot kit, along with an ice block, and the chamber filled with ice cold 1x electroblot (Table 2.2.9). The chamber was then surrounded by ice and the sample transferred onto the nitrocellulose at 200mA for 2 hours. The transfer was checked using ponceau stain (Table 2.2.9) and washed with TBS tween (Table 2.2.9), until all stain was removed. The membrane was then blocked for 30 minutes in TBS tween (Table 2.2.9) containing 5% (w/v) milk, before exchanging this for TBS tween with 5% (w/v) milk containing the primary antibody at the appropriate dilution as shown by table 2.1.2, and leaving overnight at 4$^{o}$C on the shaker. The primary antibody was then washed off using TBS tween and blocked for 30 minutes using TBS tween with 5% (w/v) milk. The secondary antibody was then added at the appropriate dilution as shown by table 2.1.2, and left on the shaker at room temperature for 2 hours. This was followed by washing again with TBS tween before adding ECL detection reagent (Table 2.2.9), and developing the Western blot using Kodak film and developer and fixer made as per manufacturer's instructions in the dark room. The bands observed were then quantified by densitometry using ImageJ.

**Table 2.2.9 Composition of Buffers Used for Western Blotting**

| **Name of Solution/Buffer** | **Composition of Buffer** |
|---|---|
| 10x Electroblot | 30.25g Tris<br>144g Glycine<br>Made up to 1 Litre with Distilled Water and pH to 8.3 with HCl |
| 1x Electroblot | 100mls 10x Electroblot<br>200mls Methanol<br>700mls Distilled Water |
| Ponceau Stain | 0.1% (w/v) Ponceau S<br>5% (v/v) Acetic Acid |

| | |
|---|---|
| 10x TBS | 0.1mM Tris<br>1.5mM Sodium Chloride<br>pH to 8.0 with HCl |
| TBS Tween | 100mls 10x TBS<br>0.5mls Tween 20<br>Made up to 1 Litre with Distilled Water |
| ECL | 1ml of 1M Tris pH 8.5<br>9ml distilled water<br>20µl 90mM Coumaric acid<br>50µl 250mM Luminol<br>3µl 37% $H_2O_2$ |

**Table 2.2.9 –** Composition of all buffers used for each different stage of Western blotting.

## 2.2.10 Cell Growth Determination

The MTT assay was performed by seeding 10,000 cells into each well on a 24 well plate in a total of 0.4mls complete medium. After a set period of time; 4 hours, 24 hours, 48 hours, 72 hours or 96 hours, 40µl of 5mg/ml (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate buffered saline (PBS) was added, and incubated at 37$^{o}$C for 30 minutes. The media was then removed and 0.4mls of DMSO was added and incubated at 37$^{o}$C for 10 minutes. 100µl of each sample was then transferred to a 96 well plate and the absorbance at 550nm was read using a plate reader and recorded. Any sample that had a reading of above 1.1 was diluted appropriately with DMSO in order to maintain accuracy.

The cell counting assay was carried out by seeding 10,000 cells into each well of a 24 well plate in a total of 0.4mls complete medium, and placed in the incubator at 37°C for a set period of time, either 4 hours, 24 hours, 48 hours, 72 hours or 96 hours. The cells were then washed with serum free medium to remove all traces of serum, and 100µl trypsin was used to remove the cells from the well. 10µl of the trypsin containing the cells was then mounted onto a haemocytometer and counted. If the cell density was too high, 1x PBS was added to dilute the cells in order to maintain accuracy whist counting, with this dilution taken into account when working out the total number of cells.

### 2.2.11 Scratch Assay

500,000 cells were seeded into each well of a 6 well plate with 5mls of complete medium, or 50,000 cells into each well of a 24 well plate with 0.4mls media. The cells were left to grow to confluency, usually overnight, and a scratch was made through the cells using a 100µl pipette tip. Media was then removed, the cells gently washed to remove any loose cells and fresh medium added. The cells were then observed using the Cell IQ machine, which consistently images the same point on each scratch of each well over a predetermined period of time. The software then allows the threshold of the scratch to be determined in order to quantify the width of the scratch as well as the area, from which the percentage closure over time can be determined. As with this method of creating the scratch, the size of the scratch is not always consistent, the percentage closure seemed to be the most reproducible measurement. Triplicate points on each scratch were selected and each well was repeated in triplicate, allowing for 9 repeated measurements, which were compiled into graphs showing the percentage closure curves of the individual points as well as an average percentage closure curve.

### 2.2.12 Immunofluorescence Staining

Round glass coverslips, measuring 13mm in diameter were placed into the wells of a 24 well plate. The lid of the 24 well plate containing the coverslips was removed and exposed to UV light for 10 minutes in order to sterilise. The coverslips were then coated with fibronectin, 2µg

per well dissolved in sterile 1x PBS, incubated for 30 minutes at 37°C, and washed twice with PBS. 10,000 cells per well were then seeded in 0.4mls medium and left in the incubator at 37°C to grow for 48 hours. The cells were washed with 1x cytoskeletal buffer (CB buffer) (Table 2.2.12) twice, and fixed in 3.7% (w/v) PFA in 1x CB buffer at 37°C for 20 minutes. 0.4mls of 30mM glycine in 1x CB buffer was added for 10 minutes at room temperature before the cells were permeabilised with either 5% or 10% (v/v) Triton X-100 in CB buffer for 2 or 10 minutes at room temperature. The cells were then washed with 1x CB buffer twice and blocked with 10% (v/v) goat serum in 1x CB buffer for 30 minutes at room temperature or left in the fridge overnight. The primary antibody in 1% (v/v) goat serum was added at the appropriate dilution as shown by table 2.1.2, and incubated at room temperature for 45 minutes. The coverslips were then washed three times in 1% (v/v) goat serum in 1x CB buffer before adding the correct secondary antibody at the appropriate dilution, as shown by table 2.1.2, and incubating again at room temperature for 45 minutes, before washing 3 times in 1% (v/v) goat serum in 1x CB buffer then twice in distilled water and mounting onto a glass microscope slide using vector shield mounting medium containing dapi staining. The slides were then viewed using the leica scanning confocal microscope using the 63x objective lens. When actin staining was included, 6.6μM phalloidin was added at a dilution of 1 in 100, at the same time as adding the secondary antibody, and the protocol continued as above. For cells transfected with a GFP plasmid, the cells were seeded and fixed as above before being mounted onto the glass slide.

**Table 2.2.12 Composition of Buffers Used for Immunofluorescence Staining**

| Name of Solution/Buffer | Composition of Buffer |
|---|---|
| 10x Cytoskeletal Buffer | 150mM Sodium Chloride<br>5mM Magnesium Chloride<br>5mM Ethylene Glycol Tetraacetic Acid (EGTA)<br>5mM Glucose<br>10mM 2-(n-morpholino)-ethansulphon Acid<br>pH to 6.1 |
| 1x Cytoskeletal Buffer | 100mls 10x Cytoskeletal Buffer<br>Made up to 1 Litre with Distilled Water |

**Table 2.2.12 –** Composition of cytoskeletal buffer used for immunofluorescence staining.

## 2.2.13 Plasmid Construction

### 2.2.13.1 Making and Running Agarose Gels

A 1% (w/v) agarose gel was made by adding 0.5g agarose to 50mls of 1x TAE buffer (Table 2.2.13.1) and heating in the microwave to dissolve the agarose, before adding 2.5µl ethidium bromide. The gel was then poured into the cast and left to set. Samples were loaded onto the gel by adding the appropriate amount of 5x loading dye, and run alongside 5µl of the 1kb DNA ladder from Fermentas to determine the correct size of plasmids or fragments. The gel was run at 90V, in 1x TAE, until the dye front was close to the end of the gel, and imaged using the gene snap imager.

**Table 2.2.13.1 Composition of Buffers for Agarose Gels**

| Name of Solution/Buffer | Composition of Buffer |
|---|---|
| 50x TAE Buffer | 242g Tris<br>51.7ml Glacial Acetic Acid<br>100ml 0.5M EDTA pH 8.0<br>Made up to 1L with Distilled Water |
| 1x TAE Buffer | 20mls 50x TAE Buffer<br>Made up to 1 Litre with Distilled Water |
| 5x Loading Dye | 2mls 0.5M EDTA pH 7.5<br>2mls 1% (w/v) Bromophenol Blue<br>6mls Glycerol |

**Table 2.2.13.1 –** Composition of the different components to make and run an agarose gel.

## 2.2.13.2 Isolation of plasmids

Plasmids were grown from a frozen -80$^{o}$C stock by inoculating 10mls 1x LB (Table 2.2.13.2) with 100µg/ml Ampicillin (LB Amp) or 40µg/ml Kanamycin (LB Kan) with the appropriate bacterial stock. Bacteria was pelleted by centrifugation at 3,000 rpm at room temperature for 5 minutes, resuspended in 250µl resuspension buffer (Promega kit) or Qiagen solution P1 (Table 2.2.13.2), before the cells were lysed by adding 250µl lysis buffer (Promega kit) or Qiagen solution P2 (Table 2.2.13.2), followed by neutralisation by adding 350µl neutralisation solution (Promega kit) or Qiagen solution P3 (Table 2.2.13.2), after which cells were transferred to an eppendorf tube. The tube was then centrifuged at 14,000 xg at room

temperature for 5 minutes, and transferred to a column from the Wizard® Plus SV Minipreps DNA Purification System kit from Promega, or into a fresh eppendorf tube for isopropanol precipitation.

If transferred to a column, the solution was incubated for 1 minute at room temperature, before centrifugation at 14,000 xg. Flow through was discarded and the column washed with 0.8 ml wash solution from the kit, by centrifuging for around 10 seconds, removing the flow through, then centrifuging again at 14,000 xg at room temperature for 5 minutes. The column was then transferred to an eppendorf tube, and 50µl distilled water pipetted onto the disc inside the column. This was incubated at 37°C for a couple of minutes before centrifugation at 14,000 xg at room temperature for 2 minutes.

If transferred to an eppendorf tube, an equal volume of isopropanol was added, the contents thoroughly mixed, before leaving in the -80°C freezer for 10 minutes followed by the -20°C freezer for 20 minutes. The tube was then centrifuged at 13,000 rpm at 4°C for 10 minutes, the supernatant removed, and the pellet washed with ice cold 70% (v/v) ethanol. The pellet was then air dried, and resuspended in 50µl distilled water. In both cases 5µl of the extracted DNA was run on a 1% (w/v) agarose gel to ensure purification was successful.

**Table 2.2.13.2 – Composition of Buffer Used for Plasmid Extraction**

| **Name of Solution/Buffer** | **Composition of Buffer** |
|---|---|
| 1x Luria Broth (LB) | 20g LB<br>Made up to 1 Litre with Distilled Water<br>Autoclave |
| Qiagen Solution P1 | 1ml 10mg/ml RNase A<br>5ml 1M Tris pH 8.0<br>2ml 0.5M EDTA<br>Made to 100ml with Distilled Water |

| | |
|---|---|
| Qiagen Solution P2 | 0.2M Sodium Hydroxide<br>1% SDS |
| Qiagen Solution P3 | 29.5g Potassium Acetate<br>Made to 100ml with Distilled Water<br>pH to 5.5 with Glacial Acetic Acid |

**Table 2.2.13.2 –** Composition of the different buffers used to grow and isolate plasmids from bacteria.

## 2.2.13.3 PCR of inserts

PCR was carried out by adding the following into a PCR tube;

2.5µl of 10x PCR buffer

2µl of 10mM dNTPs

5µl of 5uM forward primer

5µl of 5µM reverse primer

0.5µl DNA diluted 1 in 10

0.5µl Taq polymerase

9.5µl distilled water per reaction

If MgCl2 wasn't included in the PCR buffer, 1µl of 50mM MgCl2 was added and the amount of distilled water adjusted accordingly

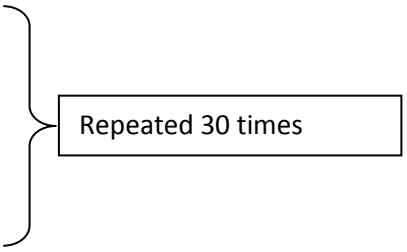
The tube was then placed into the PCR machine and run using the eEF1A programme, which was set up as follows;

Heat to 94°C and hold for 2 minutes

94°C for 30 seconds

50°C to 70°C for 30 seconds

72°C for 1 minute and 30 seconds




72°C for 5 minutes

4°C until removed from machine

The PCR product was then run on a 1% (w/v) agarose gel to ensure bands were observed.

## 2.2.13.4 Purification of DNA bands from 1% (w/v) agarose gels

The sample was run on a 1% (w/v) agarose gel, and the band of DNA to be purified identified, and the section of the gel containing the band cut out using a scalpel. The gel slice was then put into an eppendorf tube and purified using the Promega Wizard® SV Gel and PCR Clean-Up Kit in the following way. The gel slice was weighed and an equal volume of membrane binding solution added to the tube. The gel slice was then dissolved by heating at 60°C, with frequent mixing, after which the solution was transferred to a column from the kit, and incubated in the column at room temperature for 1 minute. The column was centrifuged at 16,000 xg for 1 minutes, the flow through discarded, and 0.8mls wash solution added to the column. Centrifugation for around 10 seconds at the same speed was followed by removal of the flow through, and further centrifugation at the same speed for 2 minutes. The columns were transferred to eppendorf tubes, and 50µl distilled water added to the disc inside the column, and incubated at 37°C for a couple of minutes, before centrifugation at the same speed for 2 minutes. 5µl sample was loaded onto a 1% (w/v) agarose gel to check whether the purification was successful.

## 2.2.13.5 Digestion of plasmids or PCR products

For each digestion the following was added into an eppendorf tube and incubated at 37°C for 2-3 hours or overnight;

20µl Plasmid/PCR product

2.5µl Digestion enzyme buffer

1µl Restriction enzyme (EcoR1 or EcoRV)

1.5µl distilled water

After digestion with a single restriction enzyme, to prevent religation of the plasmid without the insert, the digested plasmid was dephosphorylated by adding 2µl shrimp alkaline phosphatase to the eppendorf tube after digestion, and incubated at 37°C for 1-2 hours.

## 2.2.13.6 Insertion of PCR products into cloning vector pST-Blue

Once the purified PCR product was obtained, the overhangs had to be restored back onto the insert. This was done by adding the following into a PCR tube and leaving at 72°C for 10 minutes;

20µl Purified PCR product

2.5µl 10x PCR buffer

2µl 10 mM dNTPs

0.5µl Taq polymerase

The insert was then inserted into a cloning vector, pst blue, by adding the following to an eppendorf tube and incubating at 16°C for 2 hours;

0.5µl Acceptor vector

4µl Purified PCR product with overhangs restored

0.5µl Nuclease free water

5µl Cloneables 2x ligation premix from the pSTBlue-1 AccepTor™ Vector Kit from Novagen

## 2.2.13.7 Ligation of purified insert into backbone

The insert was ligated into the vector backbone by adding the following to an eppendorf tube and leaving for 3 hours at room temperature or overnight at 4°C;

3µl plasmid

15µl insert

2µl ligation buffer

1µl of ligase

## 2.2.13.8 Preparation of competent bacteria

Chemically competent cells were prepared by inoculating a colony of DH5α Escherichia *coli* (E.coli) cells in around 5mls 1x LB and growing overnight at 37°C shaking at 250rpm. The overnight culture was then diluted 1:1000 into 50mls of fresh 1x LB, and the cells grown in the same conditions until the optical density ($OD_{600}$) reached 0.3. The cells were placed on ice for 15 minutes, 50mls ice cold 2x TSS (Table 2.2.13.8) added, and the solution gently mixed, before leaving on ice for 20 minutes. The cells were then pelleted by centrifugation at 1000 xg at 4°C for 10 minutes, and the pellet resuspended in 5mls ice cold 1x TSS (Table 2.2.13.8), and the cells aliquoted into 100µl aliquots, and snap frozen by placing into liquid nitrogen, then storing in the -80°C freezer.

Electrocompetent bacteria were prepared by inoculating 500mls LB with a preculture of DH5α E.coli cells, and growing on a shaking incubator at 37°C at 300rpm to an optical density ($OD_{600}$) of around 0.5-0.7. Cells were then placed on ice for 20 minutes, centrifuged at 4000 xg for 15 minutes at 4°C. The supernatant was discarded, the pellet resuspended in 500mls ice cold 10% (v/v) glycerol, before centrifugation under the same conditions. The pellet was resuspended in 250mls 10% (v/v) ice cold glycerol, followed by centrifugation under the same conditions, then resuspended in 20mls 10% (v/v) ice cold glycerol, followed by centrifugation under the same conditions. The cells were then resuspended in 1-2ml 10% (v/v) ice cold

glycerol, and 100µl of the cell suspension placed into eppendorf tubes. The tubes were snap frozen by dropping the tubes into liquid nitrogen, then stored in the -80°C freezer until used.

**Table 2.2.13.8 Composition of Buffers Used to Make Competent Bacteria**

| **Name of Solution/Buffer** | **Composition of Buffer** |
|---|---|
| 2x TSS | 20% (w/v) Polyethylene Glycol (PEG)<br>100mM MgCl2<br>10% Dimethyl Sulphoxide (DMSO)<br>Dissolved in 1x LB<br>pH to 6.5 |
| 1x TSS | Dilute 2x TSS 1:1 with 1x LB |

**Table 2.2.13.8 –** Composition of the different buffers used to make competent bacteria.

## 2.2.13.9 Bacterial Transformation

Bacterial transformation was carried out in two different ways, by heat shocking the bacteria or by electroporation. When heat shocking, a tube of competent DH5α E.coli cells were removed from the -80°C freezer and placed on ice to thaw. 1µl of the ligated plasmid was added and the tube was gently agitated and replaced onto the ice for 30 minutes. The cells were then heat shocked at 42°C for 40 seconds, then placed back onto ice for 2 minutes. 800µl of 1x LB was added and the tube was placed into a shaking incubator set at 37°C and 250 rpm and left for 1 hour. The sample was then removed from the incubator, 100µl placed onto a 1x LB agar plate containing 100µg/ml ampicillin and a glass spreader sterilised with 75% (v/v) IMS in distilled water was used to gently spread the cells over the plate. The remaining

cells were then pelleted by centrifugation at 5,000rpm for 10 minutes at room temperature, the supernatant removed, and the cells resuspended in approximately 100µl LB and added to another plate and spread in the same way as before. The plates were then placed into a 37°C incubator overnight.

Electrotransformation was carried out by thawing the competent cells on ice, and adding 1µl of ligated plasmid to the cells before mixing and leaving on ice for 1 minute after which they were transferred to a 0.2cm electroporation cuvette. The cuvette was placed into the micropulser which was set to Ec2, and the pulse button pressed. 1ml 1x LB was added to the cells immediately, and the cells transferred to an eppendorf tube where they were placed in the shaking incubator at 37°C at 250rpm for 1 hour. The cells were then plated in the same way as the bacteria transformed by heat shock.

## 2.2.13.10 Screening of transformed colonies

30µl of screening lysis buffer (Table 2.2.13.10) per colony was added to an eppendorf tube. The same number of tubes was also set up containing 1ml 1x LB broth containing 100µg/ml ampicillin or 40µg/ml kanamycin. The colony was picked up using a yellow pipette tip, and then placed into the LB before being transferred into the screening lysis buffer, allowing some cells to be transferred into the LB and some cells to be transferred into the lysis buffer. The tubes with the lysis buffer were incubated at 37°C for 5 minutes, and then placed on ice for 5 minutes. They were then centrifuged at maximum speed for 1 minute, and the sample loaded onto a 1% (w/v) agarose gel. The size of the band was estimated by looking at migration of the band in the gel and comparing to a DNA ladder from Fermentas, and from this it was decided whether the plasmid was likely to contain the insert. The tubes containing the 1x LB with the cells were placed on the shaking incubator with the same settings as above and left for one hour after which the samples were removed from the incubator and the positive samples were kept. 100µl of this 1x LB stock with the potential positive plasmid was then used to inoculate 10mls 1x LB with 100µg/ml ampicillin, and left to grow overnight in the shaking incubator in the same conditions as used before. The plasmid was then extracted from the bacteria as described in section 2.2.13.2.

**Table 2.2.13.10 Composition of Buffer Used for Screening**

| Name of Solution/Buffer | Composition of Buffer |
|---|---|
| Screening Lysis Buffer | 10% (w/v) Sucrose<br>0.1M NaOH<br>0.6M KCl<br>0.5mM EDTA<br>0.05% SDS |

**Table 2.2.13.10 –** Composition of screening lysis buffer used for the screening of potentially positive plasmids.

## 2.2.13.11 Sequencing of newly created plasmids

Sequencing of the inserts ligated into the new plasmids was carried out at Birmingham University. The following was placed into a tube and the sequence determined using Sanger sequencing according to standard protocols;

200-500ng plasmid

3.2pMol Primer

Made to 10µl with distilled water

Sequence comparisons were carried out using ApE software.

## 2.2.14 Transfection

## 2.2.14.1 Transfection – Pool of transfected cells

Several conditions had to be trialled in order to optimise the best method for transfection of the cells. This was done using a lifeact plasmid which contains the green fluorescent protein (GFP) gene and allows visualisation of the actin cytoskeleton allowing the cells containing the plasmid to be identified. The manufacturer's protocol for JetPEI™ suggested the use of a ratio of 1.2µl of Jetpei™ to 1µg DNA for CHO cells, therefore this was used to determine any potential difference in transfection efficiency between CHO and 2E2 cells. 50,000 cells or 400,000 cells were seeded into a 24 well plate or a 6cm tissue culture dish and left to grow overnight. The media was removed, and 0.5, or 5mls fresh DMEM was added, and left for around 30 minutes. The concentration of plasmid DNA was quantified using the nanodrop, which allows the optical density at 260nm and 280nm to be determined to both quantify and determine the purity of the DNA sample, then 1µg DNA or 5µg DNA was added to 50µl or 250µl NaCl solution, and 1.2µl or 6µl jetPEI™ was added to 50µl or 250µl of NaCl solution provided, depending on the number of cells. The DNA and jetPEI™ solutions were vortexed, and the jetPEI™ added dropwise the DNA, after which the combined solution was vortexed and then incubated at room temperature for 30 minutes. The solution was then added dropwise to the cells, and incubated overnight. When quantifying, the number of transfected cells was counted along with the total number of cells, and a percentage calculated. When transfecting to create new cells lines, the media was changed to selective media, or the cells passaged into selective media, and left until cell death from selection was complete and a healthy flask of cells remained.

### 2.2.14.2 Transfection – Clonal cells

Transfection with jetPEI™ was carried out as described previously, though scaled up to a 10cm dish. The cells were then incubated for 24 hours, after which they were passaged into a 10cm dish with selective DMEM containing 500µg/ml G418 and 200µg/ml zeocin. The cells were then incubated until individual colonies started to form, and once these colonies were large enough, cloning cylinders were used to remove them. One side of a sterile cloning cylinder of appropriate size was dipped into sterile grease, and placed around the colony of interest. The cylinder was then gently pushed down to create a seal around the colony. 50µl trypsin was added and incubated at 37°C until cells were detached, after which 100µl of complete media was added, and the cells transferred to a well of a 24 well plate. Once the cells were confluent they were passaged into a 6 well plate, and then into a T25cm$^2$ flask, after which they were screened for expression of the plasmids by immunofluorescence and Western blotting.

## 2.2.15 Polysome Profiling

### 2.2.15.1 Mammalian Polysome Extraction With or Without CHX

500,000 cells were seeded into a 10cm tissue culture dish, and left to grow until around 90% confluence. If extracting in the presence of CHX, media was replaced with complete DMEM media containing 100µg/ml CHX, and incubated at 37°C for 15 minutes. Media was then removed, cells washed with cold PBS, and 0.25mls of polysome lysis buffer with or without 10mg CHX (Table 2.2.15.1) added. Cells were then scraped off the plate, transferred to an eppendorf tube and centrifuged at 4°C at 10,000 rpm for 15 minutes. The supernatant was then transferred to a fresh eppendorf tube and stored at -80°C until the sample was loaded onto a sucrose gradient.

**Table 2.2.15.1 Composition of Buffers Used for Polysome Extraction**

| Name of Solution/Buffer | Composition of Buffer |
|---|---|
| Polysome Lysis Buffer Without CHX | 20mM Tris pH 7.5<br>10mM Potassium Chloride (KCl)<br>10mM $MgCl_2$<br>1mM DTT<br>1% (v/v) NP-40<br>Protease Inhibitor Cocktail diluted to 1x<br>RNase Inhibitor diluted to 1x |

**Table 2.2.15.1 –** Composition of polysome lysis buffer without CHX used for polysome extractions.

## 2.2.15.2 Sucrose Gradients

In order to create polysome profiles, the extraction weas run on a sucrose gradient made up of layers containing 10, 20, 30, 40 and 50% (w/v) sucrose, with the highest concentration at the bottom. For each layer, the sucrose was weighed out, the 10x sucrose gradient buffer (Table 2.2.15.2) added so that a 1x solution is achieved, and made up to volume using distilled water. The sucrose was dissolved and 2mls of each sucrose layer was pipetted into a centrifuge tube with care to avoid the transfer of solution from the sides of the pipette to the tube. After each layer had been added, the gradients were frozen at -80$^{o}$C before the next layer added, to ensure no mixing of the layers occurred. The gradients were then stored in the -80$^{o}$C freezer until needed, when they were removed from the freezer and allowed to thaw, either overnight or on the day of use.

**Table 2.2.15.2 Composition of Buffer Used to Make Sucrose Gradients**

| Name of Solution/Buffer | Composition of Buffer |
|---|---|
| 10x Sucrose Gradient Buffer | 50mM Ammonium Chloride<br>50mM Tris Acetate pH7<br>12mM Magnesium Chloride<br>1mM Dithiothreitol |

**Table 2.2.15.2 –** Composition of the buffer used to make the sucrose gradients for separation of polysomes.

## 2.2.15.3 Quantification and Centrifugation of Polysome Extractions

The concentration of the polysome extract was determined by using the nanodrop. The concentration of each extraction was read three times, after which an average concentration was determined. 200µg of extract was then gently added onto the top of a thawed sucrose gradient, and centrifuged at 38,000rpm for 2 hours at 4$^{o}$C under a vacuum with slow deceleration. The polysome profiles were then obtained by running them on the Gradient Master connected to an EM1 detector, which allows UV detection of the polysomes at 254nm.

# Chapter 3:

# The effect of reduced eEF1A protein expression on biological processes in CHO cells

## Table of Contents

# 3.0 The effect of reduced eEF1A protein expression on biological processes in CHO cells

## *3.1 Introduction*

One protein that has been demonstrated to have essential functions in actin remodelling is eukaryotic elongation factor 1 alpha (eEF1A), which has the ability to both bind and bundle actin (Demma et al., 1990, Yang et al., 1990). In particular it has been suggested to bind actin filaments into anti parallel square packed bundles, which exclude other actin binding proteins (Owen et al., 1992), however the direct roles of eEF1A on actin remodelling are not clear in vivo. Overexpression of eEF1A has been linked to the metastatic properties of rat breast tumour cells (Edmonds et al., 1996). In such cells, a high correlation between the intracellular distribution of filamentous actin and eEF1A is thought to play a key role in regulating cellular motility. Further work in cells using the more amenable yeast organism, Saccharomyces cerevisiae, has also highlighted the importance of eEF1A as a regulator of actin organisation. Indeed, overexpression of eEF1A in yeast results in a significant disruption of the actin cytoskeleton (Munshi et al., 2000). Gross and Kinzy, 2005, demonstrated the importance of eEF1A in the overall physiological organisation of the actin cytoskeleton since mutant eEF1A proteins harbouring deficient actin bundling activities resulted in clear defects in overall actin remodelling (Gross and Kinzy, 2005, Gross and Kinzy, 2007). Interestingly, affecting actin organisation did not result in significant changes in protein translation elongation. eEF1A is present in large excess to the rest of the translational machinery, 17-35 fold molar excess compared to the ribosomes (Slobin, 1980), suggesting that eEF1A has other functions outside of translation. It has been hypothesised that eEF1A may be present in different pools within the cell allowing it to regulate different processes, for instance, it has been shown that eEF1B, the guanine nucleotide exchange factor (GEF) for eEF1A, is capable of distributing eEF1A to its different functions (Pittman et al., 2009).

eEF1A has also been shown to have other functions as well as its role in protein translation and actin binding/bundling including microtubule binding bundling and severing (Durso and Cyr, 1994, Shiina et al., 1994, Moore et al., 1998), influencing apoptosis (Billaut-Mulot et al., 1996, Kato, 1999, Duttaroy et al., 1998, Chen et al., 2000, Ruest, 2002, Talapatra et al., 2002, Borradaile et al., 2005), cell proliferation and cell cycle (Pecorari et al., 2009) where a

reduction of eEF1A protein expression resulted in an increase in the fraction of G1 cells and apoptotic cells and decrease in the fraction of cells in S phase (Pecorari et al., 2009).

Specifically determining which roles of eEF1A are due to a direct regulatory function of this protein on certain cell processes, or merely the consequent results of protein synthesis fluctuations has remained elusive. Furthermore, aberrantly regulating the levels of eEF1A expression has continuously been impeded by the abundance of the protein, as eEF1A is the second most abundant protein in the cell after actin, making up between 1-10% of total cellular protein in normally growing cells (Liu et al., 1996b, Merrick, 1992), therefore using the common method of siRNA to knock down the expression of eEF1A may not be sufficient to allow phenotypes to be observed and creating knockouts of eEF1A is not possible due to the essential nature of the gene. For this reason a different strategy was required. Whilst trying to identify genes involved in resistance to lipotoxic cell death, Borradaile et al, 2005, created a mutant CHO-K1 cell line which was the product of a single retroviral promoter trap integration. Interestingly, for one of the clones isolated, the integration was shown to have taken place in the promoter region of the eEF1A1 gene, deleting both the promoter and the first intron in the process resulting in the inactivation of that allele. Biochemical analysis further demonstrated a significant reduction in eEF1A protein expression without any direct effect on rates of protein synthesis (Borradaile et al., 2005).

Using this invaluable mammalian cell system, we sought to analyse how low levels of eEF1A would alter overall cell behaviours, including cellular motility/adhesion and changes in cytoskeletal architectures.

## *3.2 Materials and Methods*

### 3.2.1 Attachment Assay

1,000 cells were seeded in media either with or without serum into a 96 well tissue culture plate, of which some wells had previously been coated with fibronectin in PBS as described in section 2.2.12, but scaled down to a well of smaller size. The cells were left to attach for either 30 or 60 minutes before gentle washing with 1x PBS pH 7.4. The attached cells were then fixed and stained with 70% (v/v) ethanol with 0.5% (w/v) crystal violet, and incubated at room temperature for 15 minutes, after which the solution was removed, and the cells again washed with 1x PBS pH 7.4. The crystal violet was then solubilised with 100µl 30% (v/v) acetic acid and the absorbance read on a plate reader at 540nm.

### 3.2.2 Flow Cytometry for Cell Cycle

750,000 cells were seeded into a 10cm dish, and left to grow for 2 days. Cells were then trypsinised as before, and washed with 0.1% (w/v) BSA in PBS, before resuspending in 1ml 1x PBS. Fixation of cells was done by adding the 1ml of cells to 9mls ice cold 70% (v/v) ethanol, before vortexing to mix and storing in the -20$^{o}$C freezer overnight. The following day cells were pelleted at 250xg for 5 minutes, and washed in 0.1% (w/v) BSA in PBS, before resuspending in 0.5mls staining solution (Table 3.2.2) and incubating at room temperature in the dark for 30-60 minutes. The cells were then run on the flow cytometer using the cell cycle programme which measures the forward and side scatter, as well as the fluorescence intensity. 10,000 events were recorded for each cell line. Potential doublet cells were excluded from analysis.

**Table 3.2.2 Composition of Buffer for Flow Cytometry**

| Name of Solution/Buffer | Composition of Buffer |
|---|---|
| Staining Solution (Flow Cytometry) | 3.8mM Sodium Citrate pH 7.4<br>50µg/ml Propidium Iodide (PI)<br>0.5µg/ml RNase A |

**Table 3.2.2 –** Composition of the staining solution used during flow cytometry.

## 3.2.3 Ratio of G Actin to F actin Assay

The Cytoskeleton G-actin/F-actin In Vivo Assay Kit was used as per the manufacturer's protocol. 500,000 cells were seeded into a 6cm plate and left to grow for 2-3 days until confluent. The centrifuge was pre-warmed to 37$^{o}$C before starting the assay, and LAS2 buffer made up in the following way;

1ml LAS01 buffer (Lysis and F-actin stabilisation buffer)

10µl BSA04 (100mM ATP stock solution)

10µl PIC02 (100x protease inhibitor stock)

The LAS2 buffer was pre-warmed to 37$^{o}$C for 30 minutes before beginning the assay. Media was removed from cells, 250µl LAS2 buffer added, cells scraped off and transferred to an eppendorf tube. Cells were sonicated using a handheld sonicator set to around 8-10 amps and lysates incubated at 37$^{o}$C for 10 minutes. 100µl was then transferred to an eppendorf tube, centrifuged at 350xg for 5 minutes at room temperature, supernatant transferred into a centrifuge tube, and centrifuged at 100,000xg for 1 hour at 37$^{o}$C. Supernatants were transferred to a clearly labelled tube, and 100µl of F-actin depolymerisation buffer added to the pellet which was incubated on ice for 1 hour with pipetting up and down to help pellet resuspension every 15 minutes. 25µl of 5x SDS sample buffer was added to both the pellet and supernatant samples, and the samples stored at -20$^{o}$C until processed. Samples were

processed by running 10µl on a 10% (v/v) SDS gel as described in section 2.2.8 and the Western blot performed as described in section 2.2.9. The Western blot was probed with the actin antibody provided with the kit, at a dilution of 1 in 50 in 0.1% (w/v) TBS-T with milk, and incubated for 1 hour at room temperature.

### 3.2.4 Modified ELISA for detection of eEF1A

2,500, 5,000 and 10,000 cells were seeded into a 96 well plate and left to grow for 2-3days. Cells were then washed with 1x PBS and fixed with 3.7% (w/v) PFA in 1x PBS for 20 minutes at 37$^{o}$C. Cells were then washed with 1x PBS, and permeabilised with 0.1% (v/v) or 5% (v/v) triton in 1x PBS for 2-5 minutes before washing and blocking in 5% (w/v) milk in 1x PBS for 30 minutes at room temperature. Primary antibody was then added at the relevant dilution shown in table 2.1.2 and left at 37$^{o}$C for 1 hour. The primary antibody was then washed off with 5% (w/v) milk in 1x PBS, and the appropriate HRP conjugated secondary antibody added at the dilution stated in table 2.1.2 and left at room temperature for 2 hours. Cells were then washed with 5% (w/v) milk in 1x PBS, then again in 1x PBS, before 200µl of sigmafast OPD was added, made as per manufacturer's instructions. The plate was left in the dark at room temperature for 30 minutes for the colour to develop, before the plate was read at 490nm.

## *3.3 Results*

### 3.3.1 2E2 cells show a significant reduction in eEF1A expression when compared to control CHO cells

Following on from the promoter trapping experiment, and the isolation of a specific clone, Borradaile et al, 2005, reported the disruption of the eEF1A1 locus, resulting in the low expression of the eEF1A proteins in 2E2 cells as determined by Western and Northern blotting. They assumed that the resulting expression of eEF1A proteins present was caused by eEF1A2 expression (Borradaile et al., 2005). Such conclusions were based on the assumption that the remaining bands viewed on the Western blots showed eEF1A2 expression as a result of lack of specificity of the antibodies used, with the two isoforms indistinguishable. It was therefore important to determine the level of eEF1A reduction in the 2E2 cells as well as which of the isoforms were affected. It was also imperative to ensure that the decrease in eEF1A protein expression was uniform across the whole cell population, therefore Western blotting was used to check protein expression and immunofluorescence staining to ensure consistency throughout the cell population.

Western blotting was performed by homogenising cells, running protein on a 10% (v/v) acrylamide gel, transferring to nitrocellulose and probing for eEF1A, eEF1A1 eEF1A2 or a loading control protein, α – adaptin or tubulin. The blots were then developed and the intensity of the bands quantified using densitometry analysis on ImageJ.

Expression of eEF1A using the antibody that does not differentiate between the two isoforms was found to be reduced by around 40% in the 2E2 cells compared to the control CHO cells (Figure 3.3.1 A & B). When the blots were probed using the antibodies specific to human eEF1A1 or eEF1A2, no bands were observed for eEF1A1 (including in the positive control), suggesting that this antibody was not functioning properly, however the eEF1A2 antibody showed a reduced expression of around 67% (Figure 3.3.1 C & D). Hela cells were used as a positive control to ensure the antibodies, in particular the eEF1A2 antibody, were functioning as Hela cells have been shown to express both isoforms (Datu and Bag, 2013).

Cells for immunofluorescence staining were grown on coverslips and fixed and permeabilised before staining for eEF1A and viewing using fluorescence microscopy.

It was found that the level of eEF1A protein was uniform throughout the population in both CHO and 2E2 cells, and as already shown by Western blot, the 2E2 cells had a reduced level of eEF1A when compared to the control CHO cells (Figure 3.3.1 E).

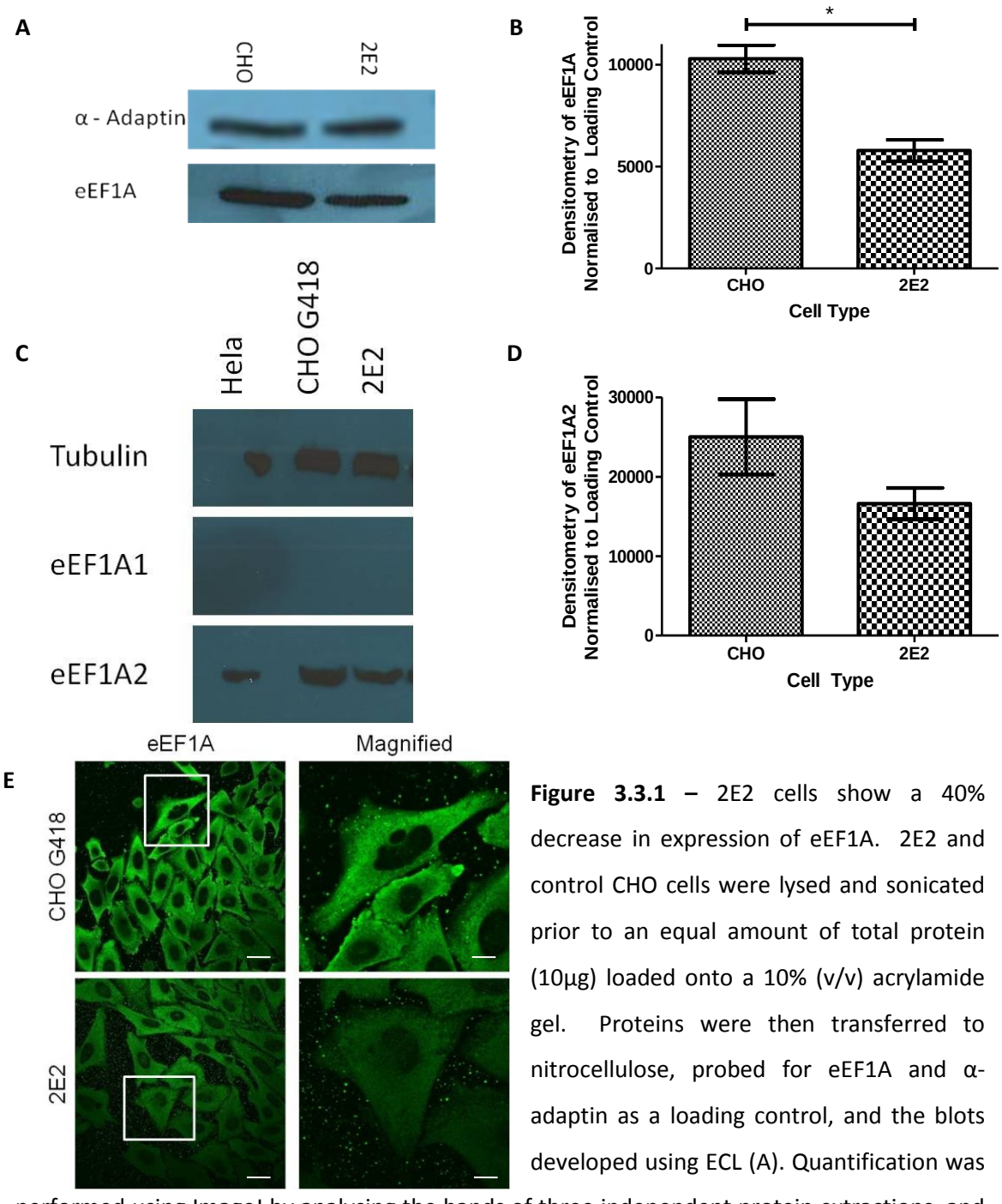



**Figure 3.3.1 –** 2E2 cells show a 40% decrease in expression of eEF1A. 2E2 and control CHO cells were lysed and sonicated prior to an equal amount of total protein (10µg) loaded onto a 10% (v/v) acrylamide gel. Proteins were then transferred to nitrocellulose, probed for eEF1A and α-adaptin as a loading control, and the blots developed using ECL (A). Quantification was performed using ImageJ by analysing the bands of three independent protein extractions, and plotting the average along with the standard error of the mean. Statistical significance was determined using an unpaired T test to a P value of <0.05 (B and D). Blots probed for eEF1A1 and eEF1A2 with tubulin as a loading control were prepared in the same way as above, though with 100µg total protein loaded onto the gel (C). Immunofluorescence was carried out by seeding cells onto a fibronectin coated coverslip, fixing, permeabilising and staining for eEF1A with a FITC conjugated secondary antibody before mounting onto a microscope slide and viewing using fluorescence microscopy (E). Scale bars are equal to 25µM.

### 3.3.2 2E2 cells show no elongation block when compared to control CHO cells

Borradaile et al, 2005, detected no change in rate of total protein translation reporting a rate of 1500-1700 cpm/μg protein for both cell lines using methionine incorporation (Borradaile et al., 2005), it is however possible that although no changes in total protein translation could be observed, some more subtle effects on the phase of elongation could be present. In order to assess such possibility, polysome profiling was undertaken where differences in initiation or elongation can be highlighted due to a change in the ratio of the polysome to monosome (80S) peaks with or without the antibiotic cycloheximide (CHX), though if elongation blocks are expected extraction is usually performed without CHX as CHX can cause artificial elongation blocks on polysome profiles (Rivest et al., 1982).

Polysomes were consequently extracted in the absence of CHX, separated on a sucrose gradient, and the gradient run on the gradient profiler. Interestingly, the polysome profiles from the two cell lines collected in the absence of CHX showed that there was no observable elongation block in the 2E2 cells, with the polysome/monosome (P/M) ratios of 1.25 for CHO and 1.34 for 2E2 cells showing no differences (Figure 3.3.2).

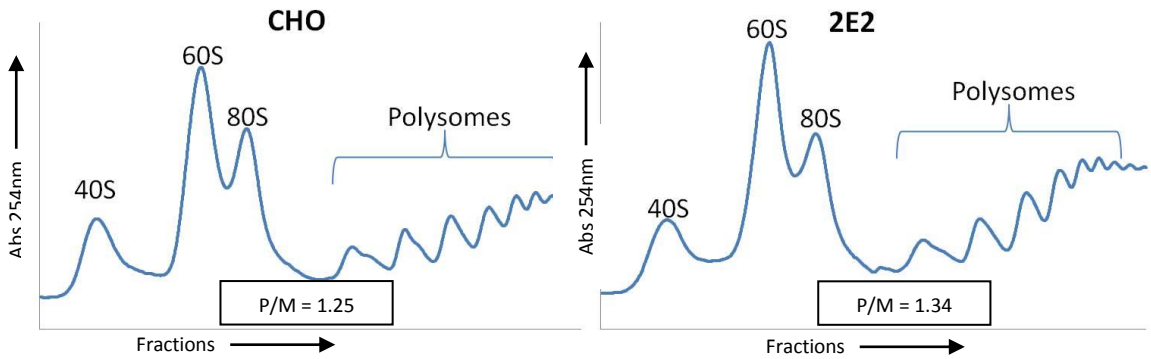


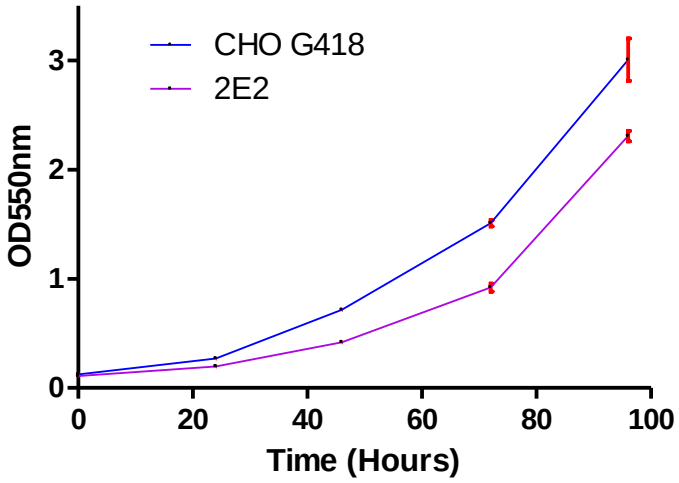

**Figure 3.3.2 –** The reduced expression of eEF1A observed in 2E2 cells causes no change in the polysome profile. 2E2 and control CHO cells were scraped into polysome lysis buffer with or without CHX, the supernatant collected and quantified and 200µg of the polysome extract loaded onto a sucrose gradient. The gradient was centrifuged at 38,000rpm for 2 hours and run on the gradient profiler. The polysome/monosome (P/M) ratio was worked out using ImageJ to calculate the area underneath the 80S peak (M), and the first four polysome peaks (P).

### 3.3.3 Reduced expression of eEF1A coincides with a slower growth phenotype

Reduced expression of eEF1A in breast cancer cells has been shown to be linked to a reduction in cell proliferation (Pecorari et al., 2009). To establish whether this observation could also be seen in our cell system, we sought to establish whether the decrease in eEF1A protein levels seen in the 2E2 cells would affect the growth rate of the cells. Growth curves were created using MTT and cell counting assays over a 96 hour period.

It was found that the 2E2 cells had a significantly lower growth rate when compared to the control CHO cells, with the difference observable as early as 24 hours and gradually increased until 72 hours when the CHO cells had an $OD_{550nm}$ of 3 compared to 2.3 for the 2E2 cells (Figure 3.3.3A). To determine whether the differences seen were due to changes in cell number, or possibly a reduction of metabolic activities, cell growth assays were also carried out. A similar reduction in cell number was seen in the 2E2 cells as observed in the MTT assay across the different time points. Whilst no changes could be seen at 24 hours, cell number decreased from 51000 in CHO cells to 43000 in 2E2 cells at 48 hours and much more significantly at the later time points, with more than 300000 cells per well for the control whilst 2E2 cells were around 150000 (Figure 3.3.3B).

A




B

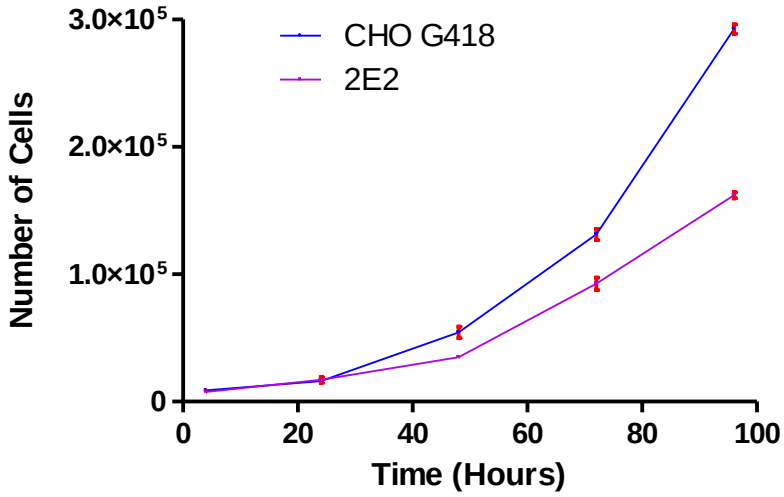



**Figure 3.3.3 –** Reduced expression of eEF1A in 2E2 cells coincides with a significant decrease in growth rate. For both assays, 10,000 cells were seeded, and left to grow for 4, 24, 48, 72 or 96 hours. Following on from this incubation, either MTT was added, solubilised with DMSO and the absorbance at 550nm read (A), or cells were trypsinised, and counted using a haemocytometer (B). Results are presented as average values with standard error and analysed for statistical significance by an unpaired T test * p = <0.01, ** p = <0.005.

### 3.3.4 Reduced expression of eEF1A coincides with a decrease of cells in S phase and an increase in apoptotic cells

Low levels of eEF1A have led to a decreased S phase fraction as well as an increase in the fraction of cells with subG1 DNA content which indicates apoptosis in breast cancer cells (Pecorari et al., 2009). A decrease in expression of eEF1A has also been linked to cellular senescence induced by many different factors (Byun et al., 2009), therefore given the changes we have seen in regards to their proliferation (Figure 3.3.3), we sought to determine if any differences in the stages of the cell cycle could be seen in our cell system.

Flow cytometry was used to analyse cell cycle after trypsination, fixation and staining with propidium iodide before 10,000 events were recorded. A decrease in the fraction of cells in S phase and G2/M phase, as well as an increase in the fraction of apoptotic cells could be observed in the 2E2 cells compared to the control cells (Figure 3.3.4).

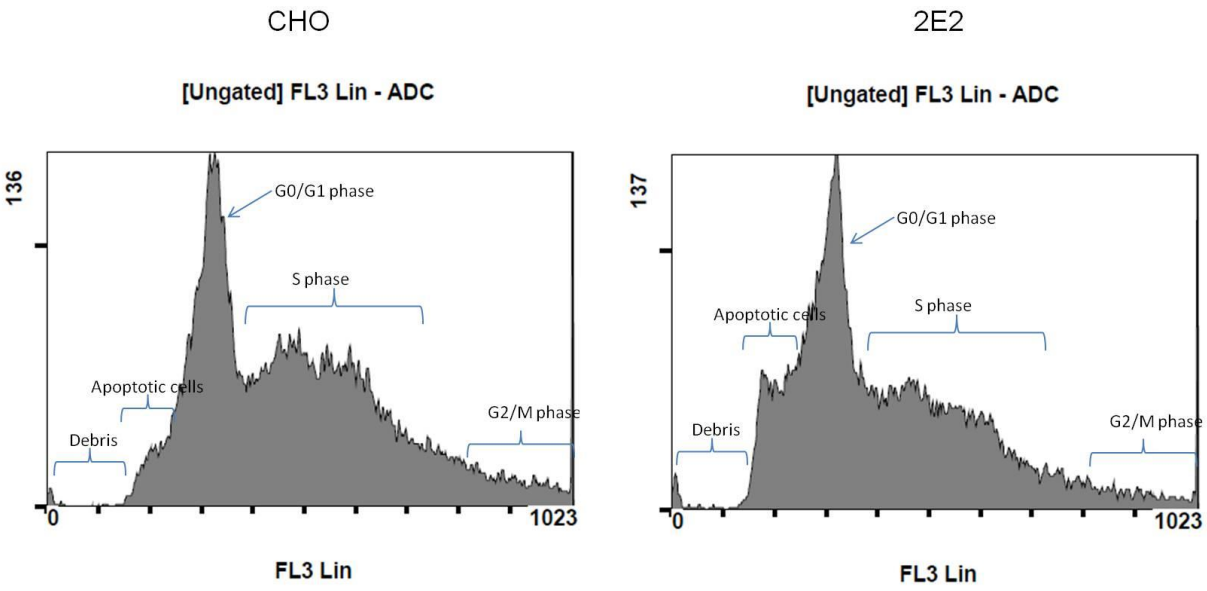



**Figure 3.3.4 –** Reduced expression of eEF1A coincides with a decrease in S phase cells and an increase in apoptotic cells in 2E2 cells compared to control CHO cells. Cells were trypsinised, washed and fixed in 70% (v/v) ethanol overnight at 4°C. The cells were then washed and resuspended in 50µg/ml propidium iodide for 60 minutes before running on the flow cytometer with 10,000 events recorded.

### 3.3.5 Reduced expression of eEF1A results in an increase in actin stress fibres

eEF1A was shown to be an actin binding and bundling protein as early as the 1990s (Demma et al., 1990, Yang et al., 1990), and since, this property has been shown to be conserved throughout many different species (Dharmawardhane et al., 1991, Collings et al., 1994, Gross and Kinzy, 2005, Liu, 2002). We decided to focus our attention on the actin architecture in our cell system to establish whether the low levels of eEF1A would affect their cytoskeletal organisation. In the first instance, we wanted to establish if the overall level of actin expression was affected by Western blotting as well as determine if the two cell lines presented any significance changes in their overall organisation using immunofluorescence staining.

Western blotting was performed by extracting the total protein, loading onto a gel, transferring and probing for actin (Arp3 as loading control). No difference in actin protein expression was detected between the CHO and 2E2 cells (Figure 3.3.5 A & B), indicating that the overall levels of actin protein are constant and that lowering eEF1A levels does not affect its overall expression.

Immunofluorescence staining for actin was carried out using rhodamine phalloidin (eEF1A co-stain) and viewed using confocal microscopy. The overall intensity for actin was comparable between the two cell lines in line with the data obtained after Western blotting, whilst a reduction in eEF1A protein in the 2E2 cells demonstrates a large presence of actin stress fibres throughout the cell body. Quantification of these fibres was carried out by assuming the striations across the cell as fibres, and counting the number of fibres that cross the cell. Stress fibre agglomeration at the cell periphery was not quantified as it is impossible to differentiate the different bundles.

Quantification of these stress fibres indicated a significant increase in actin stress fibres, with the 2E2 cells presenting an average of 14 +/- 7.1 arc stress fibres per cell whilst the control counterparts were found to have 1 +/- 1.3 (Figure 3.3.6 C & D). Large pools of actin punctate were however observed though not quantified in the control CHO cells, potentially likely to be short F-actin fibres which were absent in the 2E2 cells. The 2E2 cells also appear to show a thinner cortical actin ring around the cell when compared to CHO cells based on observation

of the images. In order to further characterise the observed stress fibres, other proteins known to be associated with actin stress fibres were analysed.

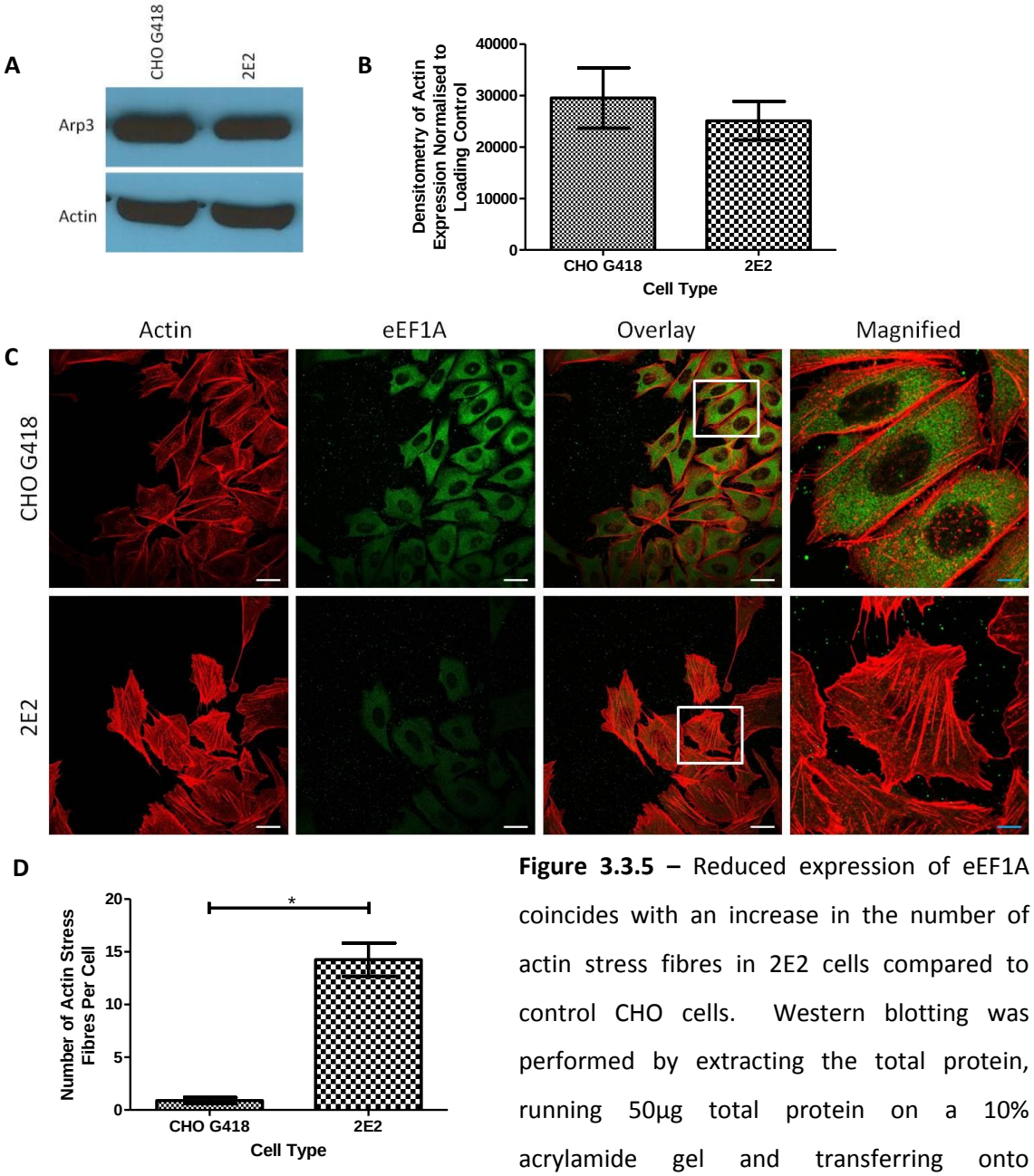



**Figure 3.3.5 –** Reduced expression of eEF1A coincides with an increase in the number of actin stress fibres in 2E2 cells compared to control CHO cells. Western blotting was performed by extracting the total protein, running 50µg total protein on a 10% acrylamide gel and transferring onto nitrocellulose, before probing for actin and Arp3, and detecting with ECL (A). ImageJ was used to quantify the bands from three independent protein extractions and plotting the average and standard error of the mean. Statistical analysis was carried out by unpaired T test to a P value of *<0.05 (B). Immunofluorescence staining was performed by seeding cells onto coated coverslips, before fixing, permeabilising, blocking and staining for actin (rhodamine phalloidin) and eEF1A (FITC conjugated secondary antibody). Coverslips were then mounted and viewed using confocal microscopy (C). Actin stress fibres were quantified by counting the number of fibres passing through the cells for each cell type with actin fibres surrounding the cell excluded from quantification as it was impossible to differentiate bundles of actin filaments (n=20). Statistical significance was determined by an unpaired T test to a P value of *<0.05 (D). White scale bars are equal to 25µM, blue scale bars are equal to approximately 7.5µM.

### 3.3.6 Reduced expression of eEF1A correlates with a change in localisation of myosin filaments

Actin stress fibres can be categorised into four different types, namely the perinuclear actin cap, transverse arcs, and dorsal and ventral stress fibres each with varying functions (Tojkander et al., 2012). The types of actin stress fibres can be differentiated using immunofluorescence staining by observing where the fibres are localised, as well as the proteins associated with the stress fibre, for example, it has been shown that dorsal stress fibres do not contain myosin II at the trunk of the fibre (Kovac et al., 2012), and only attach to focal adhesions at one end, with transverse arcs having no association with focal adhesions at all, therefore observation of myosin II and focal adhesion proteins would allow further characterisation of the stress fibres, allowing them to be classified, as well as giving some insight into their function. Myosin IIA is known to localise to actin filaments and stress fibres, giving the fibres the ability to contract (Weber and Groeschel-Stewart, 1974, Tojkander et al., 2011). Due to the increased number of actin stress fibres seen in the 2E2 cells compared to the control CHO cells (Figure 3.3.8), we sought to determine if myosin protein levels were affected and whether there were any differences in myosin IIA colocalisation with the observed fibres to further characterise the fibre structure.

Extraction of the total lysate from the 2E2 and control CHO cells was performed before samples were run on an 8% (v/v) acrylamide gel and transferred and probed for myosin IIA (tubulin as loading control) before developing. No difference in myosin IIA protein expression was observed between the two different cell lines (Figure 3.3.6 A & B), demonstrating that actual expression of the proteins is not affected in the cell system used.

Localisation of myosin IIA was observed by immunofluorescence staining after cell permeabilisation using confocal microscopy. Quantification of myosin IIA fibres was carried out in the same manner to those of actin (see in figure 3.3.5). Immunofluorescence staining showed that myosin IIA localised to the actin stress fibres in 2E2 cells with the average number of myosin IIA striations per cell being 1 +/- 0.7 in CHO cells and 14 in 2E2 +/- 4.6 cells, therefore a reduction in eEF1A protein expression correlates with a statistically significant increase in the number of myosin IIA fibres across the cell (Figure 3.3.6 C & D), with the number of striations correlating closely to the number observed for the actin stress fibres.

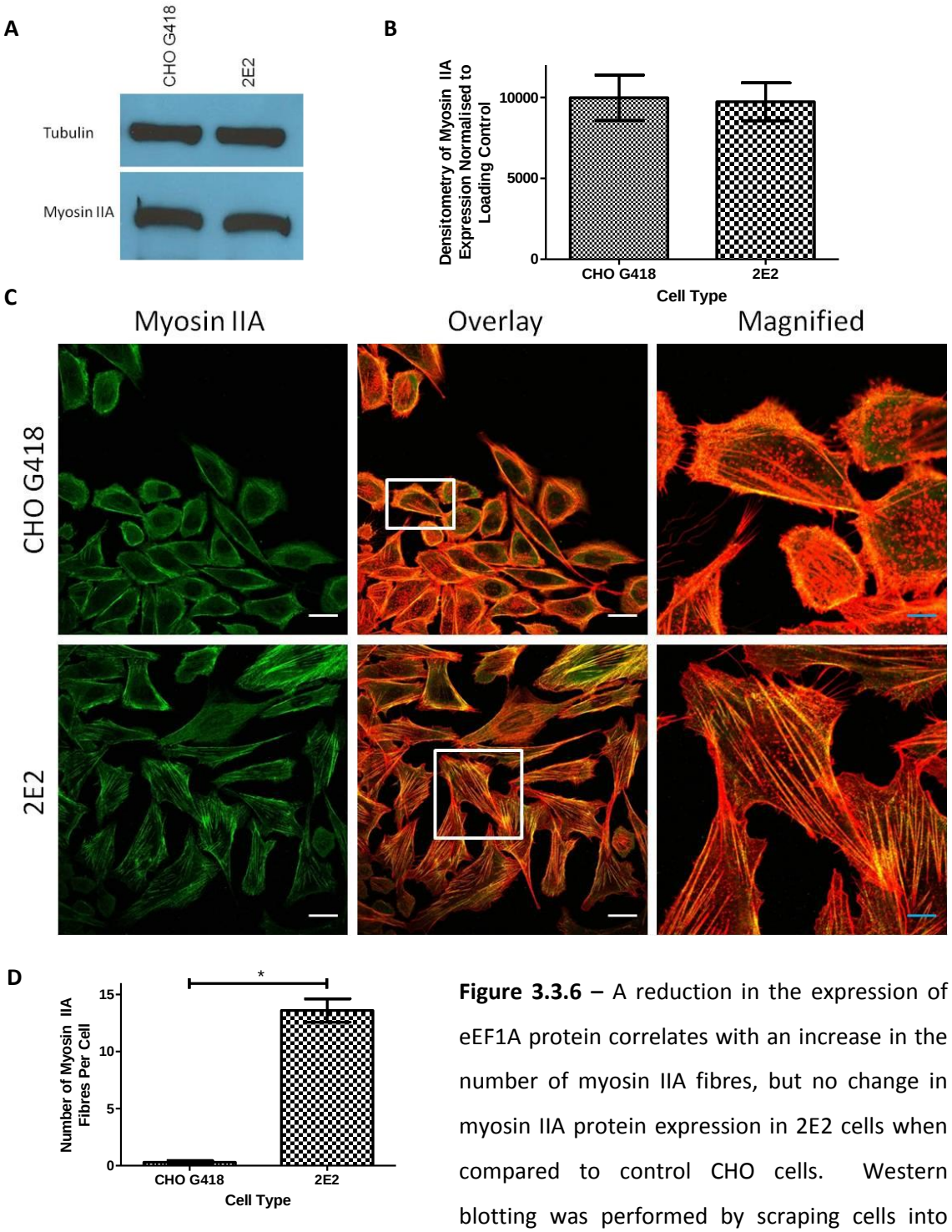



**Figure 3.3.6 –** A reduction in the expression of eEF1A protein correlates with an increase in the number of myosin IIA fibres, but no change in myosin IIA protein expression in 2E2 cells when compared to control CHO cells. Western blotting was performed by scraping cells into homogenising buffer, sonicating and quantifying the total protein. 25µg total protein was loaded onto an 8% (v/v) gel which was run, transferred, probed for myosin IIA (tubulin as a control) and developed using ECL (A). ImageJ was used to quantify the bands from three independent protein extractions, plotting the average and standard error of the mean (B). Immunofluorescence staining was carried out by fixing, permeabilising, blocking and staining cells for myosin IIA (FITC conjugated secondary antibody) and actin (rhodamine phalloidin). Coverslips were then mounted and viewed using confocal microscopy (C). Myosin IIA was

quantified by assuming the striations across the cells to be fibres similar to actin stress fibres and counting the number of fibres that passed through the cell in 20 cells for each cell line. The average fibre number was plotted with the standard error of the mean and statistical analysis was carried out by unpaired T test to a P value of *<0.05 (D). White scale bars are equal to 25µM, blue scale bars are equal to approximately 7.5µM.

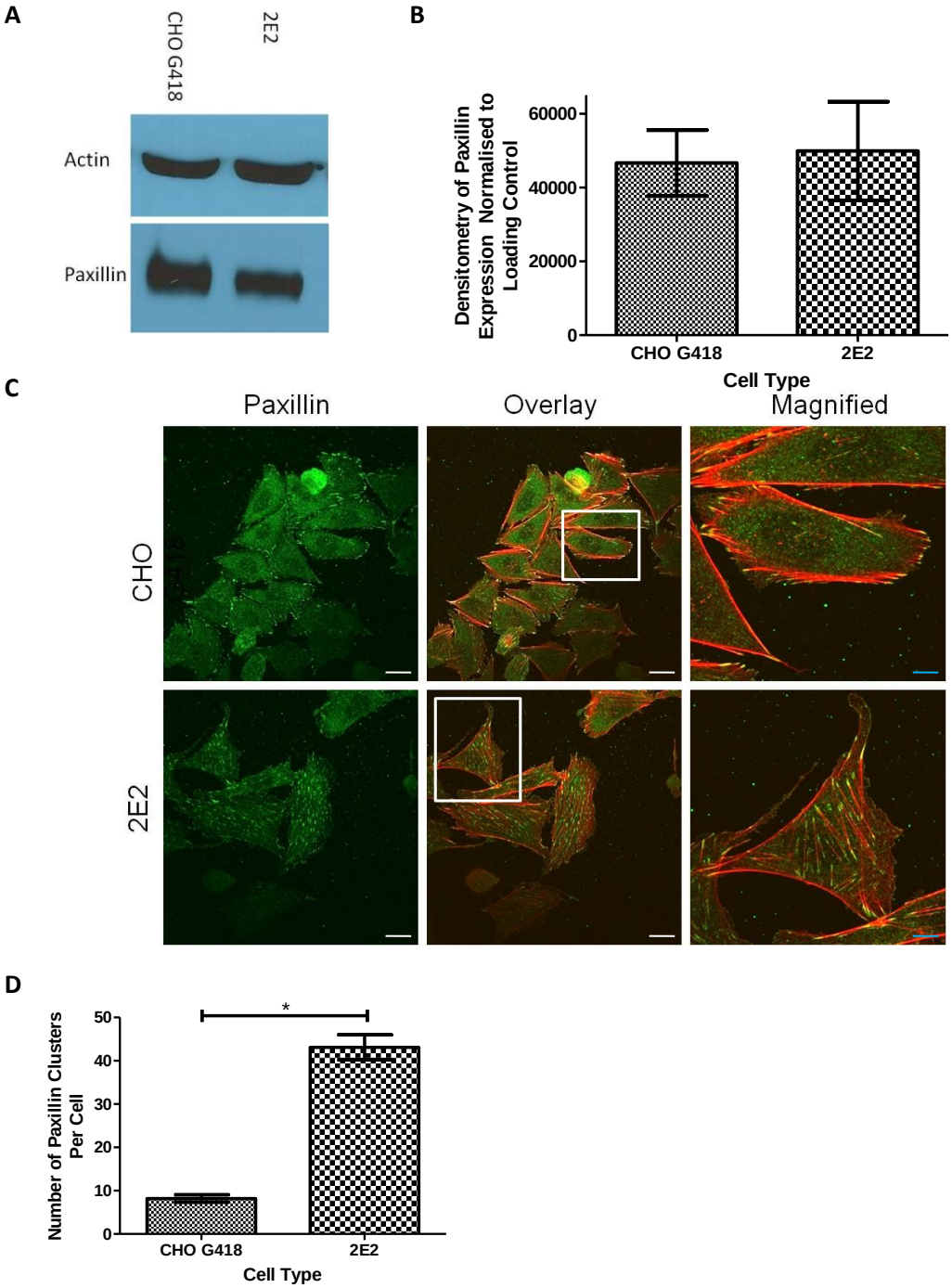
### 3.3.7 Reduced expression of eEF1A coincides with an increase in the number of paxillin and vinculin clusters

Based on the position of the actin stress fibres in relation to the cell (Figure 3.3.5), and the localisation of myosin IIA to the fibres (Figure 3.3.6), it is likely that the stress fibres present in the 2E2 cells are ventral stress fibres. Ventral stress fibres are the only type of stress fibre to attach the extracellular matrix by focal adhesions at both ends of the fibre (Pellegrin and Mellor, 2007), therefore to confirm the fibres as ventral stress fibres, and to provide further insight into the effect this increase in fibre number would have on the physical characteristics of the cell, focal adhesions were analysed. Paxillin is one of the earliest proteins present in the formation of focal complexes (Zaidel-Bar et al., 2004), and has been shown to localise to early stress fibre focal adhesions (Zimerman et al., 2004). Vinculin is another focal adhesion protein involved in the same complexes as paxillin, however it is recruited to focal complexes and the more mature focal adhesions later on in the formation process (Zaidel-Bar et al., 2004). Paxillin has also been shown to bind to vinculin potentially providing a mechanism for the recruitment of vinculin to the focal complex (Turner et al., 1990), therefore a change in paxillin localisation would potentially be mirrored by vinculin localisation.

Western blotting was carried out by quantifying the total protein, loading onto a gel, running and transferring onto nitrocellulose. The blots were then probed for paxillin (actin as loading control) or vinculin (tubulin as a loading control) and developed. No difference in protein expression was observed for paxillin or vinculin (Figure 3.3.7 A & B and E & F).

Visualisation of focal adhesion number was analysed using confocal microscopy by observing paxillin and vinculin clusters following immunofluorescence staining. Clusters were quantified by manually counting the number of clusters per cell in 20 cells of each type. 2E2 cells were found to have a statistically significant increase in the number of paxillin clusters when compared to control CHO cells, with 2E2 cells having an average of 43 +/- 12.9 clusters per cell and the CHO cells 8 +/- 3.8 clusters per cell (Figure 3.3.7 C & D). A similar phenotype was observed for vinculin. The number of vinculin clusters was significantly increased in the 2E2 cells compared to the control CHO cells, with the average number of vinculin clusters per cell in 2E2 cells being 50 +/- 11.5 and in CHO cells 13 +/- 5.2 (Figure 3.3.7 G & H). Both paxillin and vinculin clusters localised to both ends of the actin stress fibres, therefore strengthening the view that the stress fibres seen are ventral stress fibres, which allowed further characteristics

to be deduced regarding the nature and potential functions and consequences of this increase in ventral stress fibre number.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Actin
Paxillin
B
Densitometry of Paxillin Expression Normalised to Loading Control
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40000
20000
0
CHO G418
2E2
Cell Type
C
Paxillin
Overlay
Magnified
CHO
2E2
D
Number of Paxillin Clusters Per Cell
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40
30
20
10
0
*
CHO G418
2E2
Cell Type

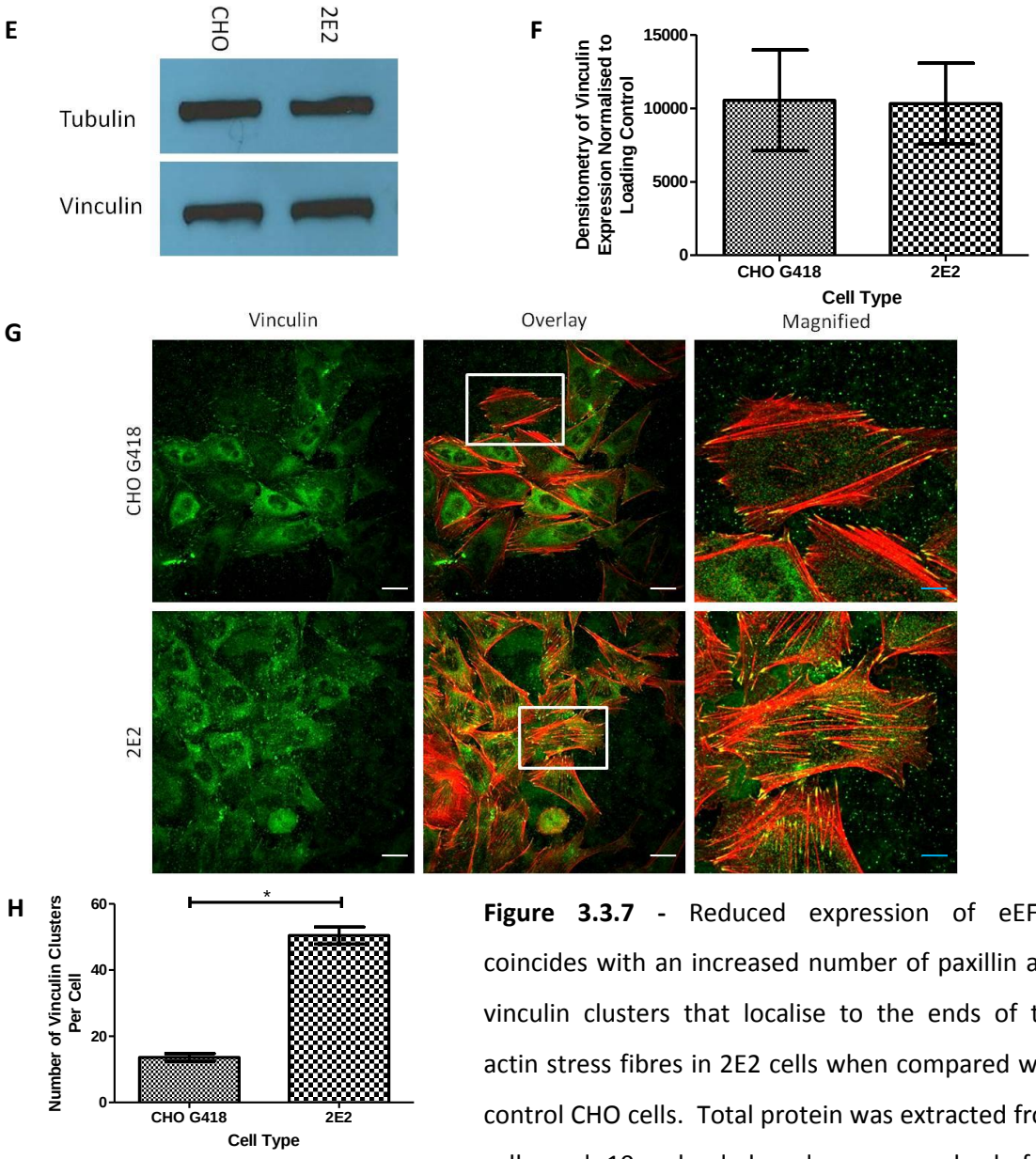



**Figure 3.3.7 -** Reduced expression of eEF1A coincides with an increased number of paxillin and vinculin clusters that localise to the ends of the actin stress fibres in 2E2 cells when compared with control CHO cells. Total protein was extracted from cells and 10µg loaded and run on gels, before transferring to nitrocellulose. The blots were then probed for paxillin (actin as control) or vinculin (tubulin as control) followed by HRP conjugated secondary antibodies. The blots were then developed using ECL (A & E). The intensity of bands from three independent protein extractions were analysed by densitometry analysis using ImageJ, and the average with standard error of the mean plotted (B & F). Immunofluorescence staining was carried out by growing cells on coated coverslips prior to fixing and staining for paxillin or vinculin (FITC conjugated secondary) and actin (rhodamine phalloidin). Cells were mounted and viewed using confocal microscopy. The white rectangles define the selected cell for the magnified image (C & G). Paxillin and vinculin clusters were quantified by counting the clusters in 20 random cells for each cell line, average and standard error of the mean plotted, and statistical

significance determined by unpaired T test to a P value of * < 0.05 (D & H). White scale bar equals 25µM, blue scale bar equals approximately 7.5µM.

### 3.3.8 Reduced expression of eEF1A results in no change in VASP localisation

Vasodilator-stimulated protein (VASP) has been shown to promote the assembly of actin filaments by recruiting profilin, a G-actin binding protein (Reinhard et al., 1995) and is known to be associated with focal adhesions and stress fibres (Reinhard et al., 1992, Price and Brindle, 2000), likely due to its ability to bind to vinculin (Brindle et al., 1996, Reinhard et al., 1996). This apparent recruitment to vinculin, along with the potential of VASP to elongate actin fibres through profilin recruitment make it an interesting to protein to analyse to determine whether the reduced level of eEF1A which coincides with the actin, myosin IIA, paxillin and vinculin phenotypes already observed, would affect the localisation of VASP.

Immunofluorescence staining was used to analyse any differences in localisation of VASP and was performed by seeding cells onto coverslips, fixing, permeabilising, blocking and staining for VASP, before mounting coverslips onto microscope slides and viewing by confocal microscopy. After analysis of the staining images, no difference in localisation of VASP was observed when eEF1A protein expression level was reduced (Figure 3.3.8).

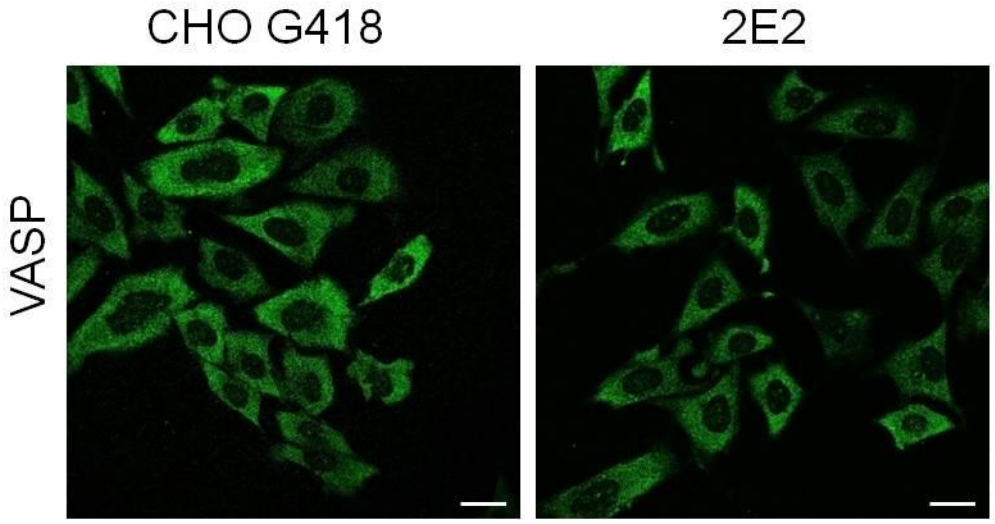



**Figure 3.3.8 -** Reduced expression of eEF1A does not lead to changes in VASP localisation. Cells were grown on fibronectin coated coverslips prior to fixing, permeabilising, blocking and staining for VASP (FITC conjugated secondary). Coverslips were mounted and viewed using confocal microscopy. Error bars are equal to 25µM.

### 3.3.9 Reduced expression of eEF1A does not coincide with a change in Arp3 protein expression or localisation

The increase in stress fibres observed in the 2E2 cells are thought to be ventral stress fibres, and although the protein expression of actin was unchanged in 2E2 cells compared to control CHO cells (Figure 3.3.5) what is unknown is where the F actin for the stress fibres is originating from, relocalisation of F actin fibres, polymerisation of new fibres or annealing of smaller fibres to create the longer fibres. The Arp2/3 complex been shown to be essential for the formation of transverse arcs, which have been suggested to combine with dorsal stress fibres to make ventral stress fibres (Hotulainen and Lappalainen, 2006), therefore observing the localisation of the Arp2/3 complex could help to answer the question. The Arp2/3 complex has also been shown to be associated with vinculin (DeMali et al., 2002), the localisation of which has already been shown to be affected by the decreased eEF1A expression observed in the 2E2 cells compared to the control CHO cells (Figure 3.3.7).

No difference in Arp3 protein expression, as demonstrated by densitometry analysis was detected between the two cell lines following separation of total proteins and Western blotting using an Arp3 antibody (Figure 3.3.9 A & B).

Immunofluorescence images were created by fixing, permeabilising, blocking and staining for Arp3 and actin, after which coverslips were mounted onto microscope slides, and viewed using confocal microscopy. No difference in Arp3 localisation was observed in the CHO and 2E2 cells, indicating that reduced levels of eEF1A have no effect on the overall distribution of Arp3 (Figure 3.3.9 C). It is however worth noting that the association of the Arp2/3 complex with vinculin observed by DeMali et al, 2002, was only observed in early focal complexes rather than mature focal adhesions, therefore association of Arp3 with vinculin may have been present, and as focal adhesion formation and disassembly is a dynamic, constantly changing process, it may be that Arp3 is associated with early focal complexes, but that these complexes mature too quickly to allow observation by this method.

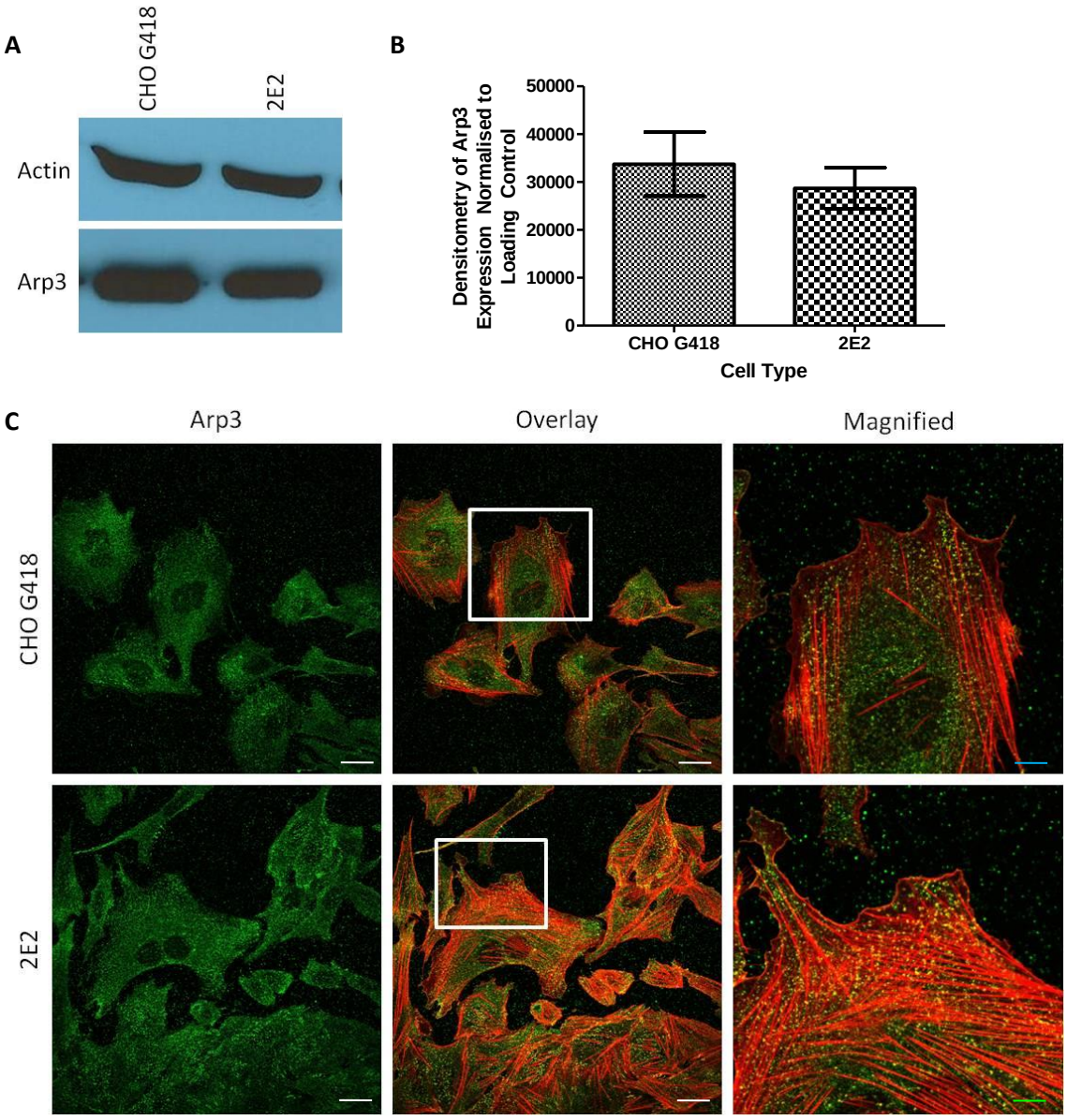



**Figure 3.3.9 –** Reduced expression of eEF1A results in no change in Arp3 protein expression or localisation in 2E2 cells compared to control CHO cells. Cells were lysed, protein quantified, and 50µg total protein loaded onto a 10% acrylamide gel which was run and transferred to nitrocellulose. The blot was probed for Arp3 and actin as a loading control, and developed using ECL (A). Bands from three independent protein extractions were quantified by densitometry using ImageJ, and the average and standard error of the mean plotted (B). Cells for staining were seeded onto fibronectin coated coverslips, and grown for 48 hours, after which they were fixed, permeabilised, blocked and stained for Arp3 with a FITC conjugated secondary antibody and co-stained for actin with rhodamine phalloidin. Coverslips were mounted onto microscope slides and viewed by confocal microscopy. The white rectangles define the selected cell for the magnified image (C). White scale bars equal 25µM, blue scale bar to 5µM and the green scale bar to 2.5µM.

### 3.3.10 Reduced expression of eEF1A does not affect beta-1 integrin localisation

All of the above observed phenotypes focus on the actin stress fibres and their association with focal adhesion proteins, where an increase in actin stress fibre numbers was equally mirrored by an increased number of focal adhesions (Figure 3.3.7). All focal adhesions connect to the extracellular matrix via integrins, therefore it was of interest to determine whether there was a similar change in the localisation of integrins. The problem faced when analysing this subset of proteins, is the range of different isoforms. However, many studies have established that CHO-K1 cells endogenously express β-1 integrin subunit (Jaspers et al., 1994, Balzac et al., 1994). Because of the importance of the β-1 integrin subunit and its role in fibronectin binding (Johansson et al., 1997), an antibody specific to β-1 integrin was chosen to determine any differences in localisation between the CHO and 2E2 cells.

Immunofluorescence staining was performed by fixing and staining cells for β-1 integrin and actin before viewing by confocal microscopy. No change in β-1 integrin localisation was observed in 2E2 cells when compared to control CHO cells, suggesting that a change in eEF1A protein expression does not have an effect (Figure 3.3.10). It is possible that other isoforms of the integrin family are affected by the change in eEF1A protein expression, however, the tools to observe all different isoforms were not readily available.

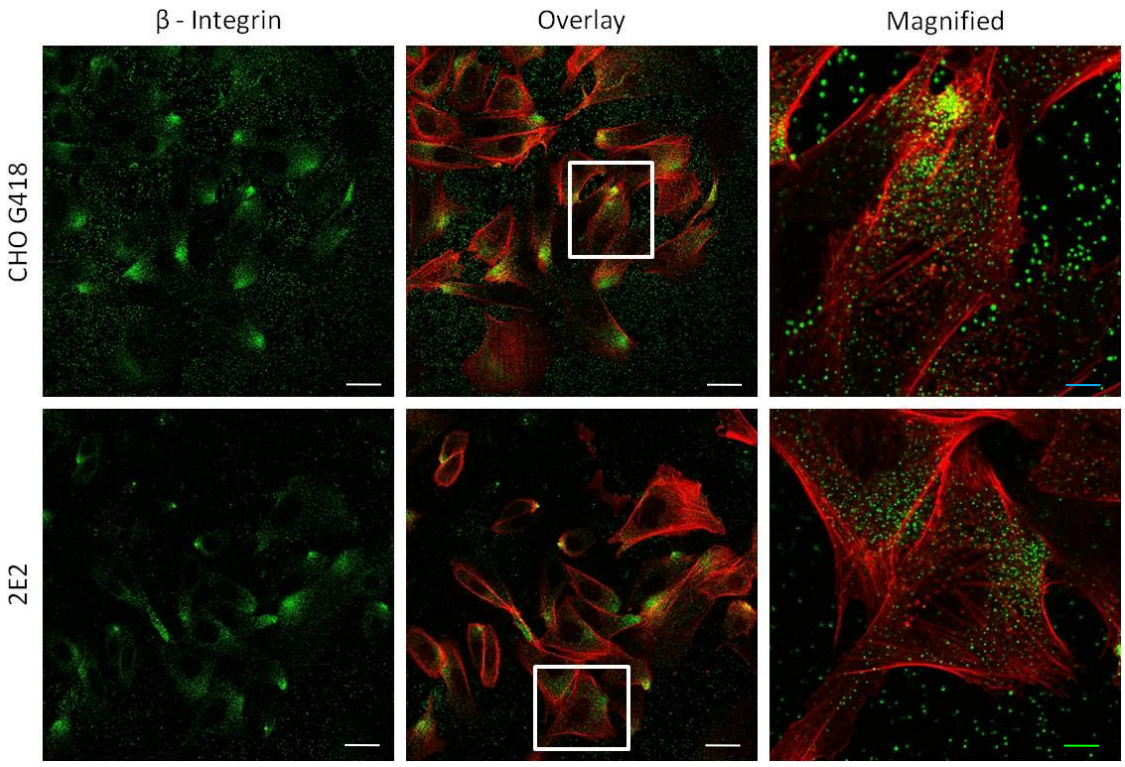
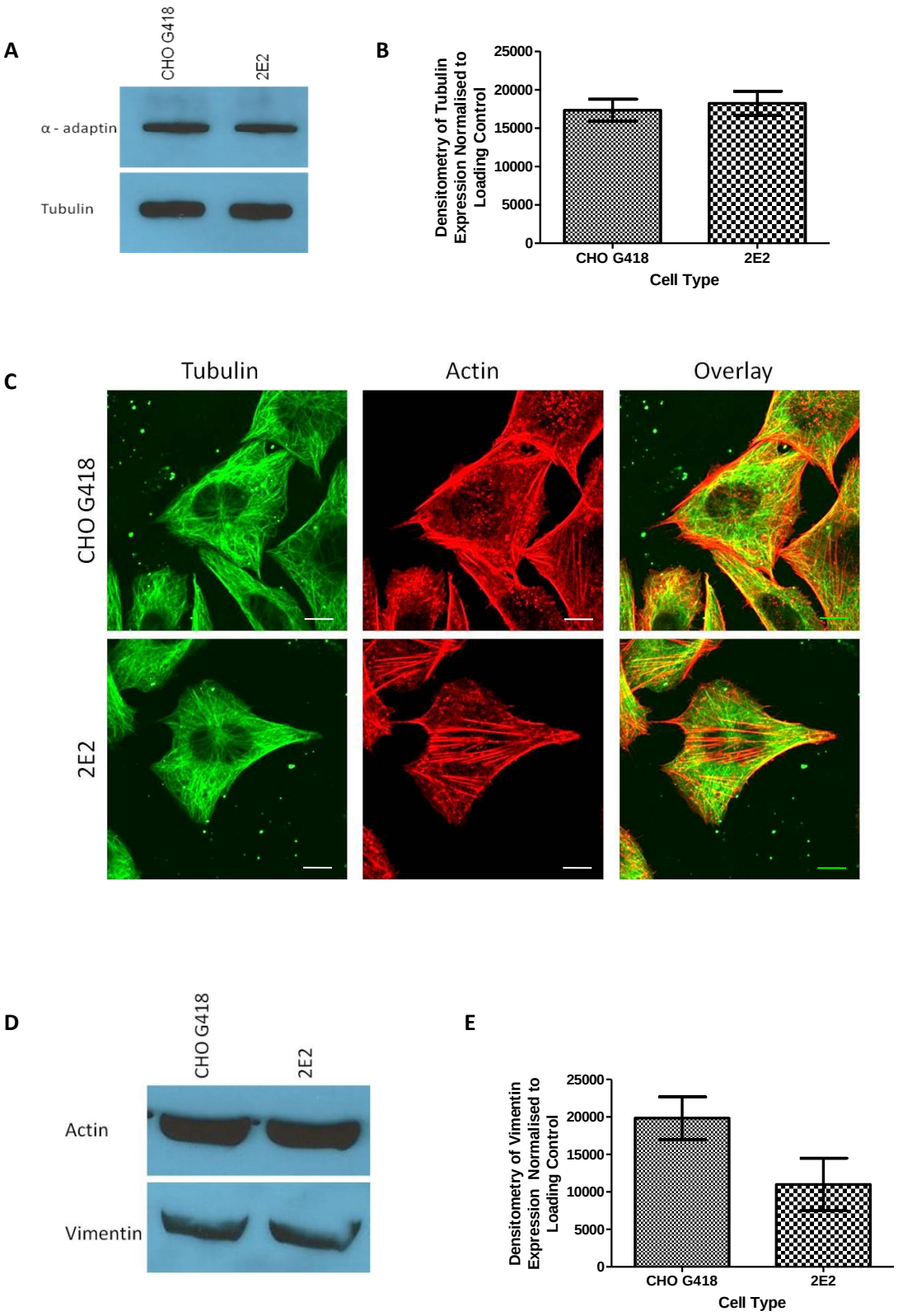



**Figure 3.3.10 –** Reduced expression of eEF1A does not affect the localisation of β-1 integrin in 2E2 cells compared to control CHO cells. Cells seeded onto fibronectin coated coverslips then fixed and stained for β-1 integrin with a FITC conjugated secondary antibody, and co-stained for actin with rhodamine phalloidin. Coverslips were then mounted onto microscope slides and viewed by confocal microscopy. The white rectangles define the selected cell for the magnified image. White scale bars are equal to 25µM, the blue scale bar 5µM and the green scale bar approximately 7.5µM.

### 3.3.11 Reduced expression of eEF1A has no observable effect on the expression level nor localisation of other cytoskeletal structures

Microfilaments have been shown to be affected by the reduced level of eEF1A in the 2E2 cells compared to the control CHO cells (Figures 3.3.5 & 3.3.6). Given that eEF1A is capable of binding/bundling, severing and stabilising other cytoskeletal structures, including microtubules (Durso and Cyr, 1994, Moore et al., 1998, Shiina et al., 1994), it was of interest to determine whether our observations so far were specific to the actomyosin architecture or were part of more global changes in cytoskeletal protein localisation.

Tubulin and vimentin protein expression were analysed by Western blotting by extracting the total protein, loading onto a gel and transferring to nitrocellulose prior to probing for tubulin or vimentin and developing. In parallel immunofluorescence staining for the same proteins was carried out prior to viewing using confocal microscopy. No significant difference in tubulin or vimentin protein expression was observed by Western blotting (Figure 3.11 A – B & D - E) and immunofluoresence staining showed no observable difference in tubulin or vimentin localisation in 2E2 cells compared to control CHO cells (Figure 3.11 C & F), confirming that the observed actin phenotype is not due to a global change in cytoskeletal protein, but is specific to actin and its associated proteins.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CHO G418
2E2
α - adaptin
Tubulin
B
Densitometry of Tubulin Expression Normalised to Loading Control
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20000
15000
10000
5000
0
CHO G418
2E2
Cell Type
C
Tubulin
Actin
Overlay
CHO G418
2E2
D
CHO G418
2E2
Actin
Vimentin
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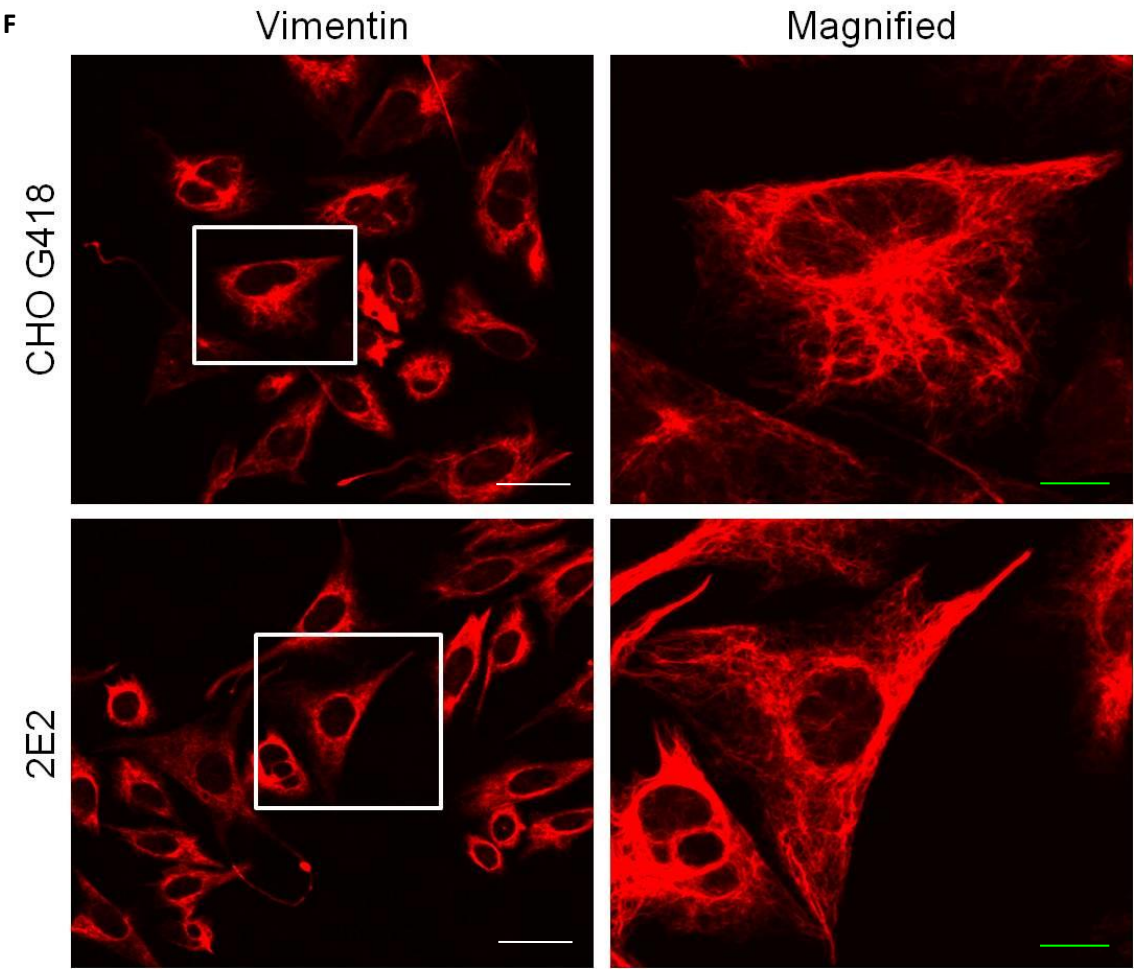



**Figure 3.3.11 –** Reduced expression of eEF1A does not affect protein expression or localisation of tubulin or vimentin. Western blotting was carried out by scraping cells into homogenising buffer, sonicating, quantifying and loading 10µg or 50µg of total protein onto a gel, which was run, then transferred onto nitrocellulose. The blots were then probed for tubulin (α – adaptin as control) or vimentin (actin as control), and developed using ECL (A & D). Bands from three independent protein extractions were quantified using densitometry analysis on ImageJ and the average and standard error of the mean plotted (B & E). Immunofluorescence staining was performed by seeding cells onto coated coverslips after which they were fixed, permeabilised, blocked and stained for tubulin (FITC conjugated secondary antibody) and actin (rhodamine phalloidin) or vimentin (cy3 conjugated primary antibody). Coverslips were then mounted and viewed using confocal microscopy by z-stack, with the presented image the merged view of the images from the z-stack. The white rectangles show the areas selected for the magnified photographs (C & F). White scale bars are equal to 25µM and the green scale bars approximately 7.5µM.

### 3.3.12 Reduced expression of eEF1A coincides with a significantly increased rate of cell attachment

Our results have so far shown that the number of actomyosin fibres is increased in 2E2 cells when compared to the control CHO cells (Figures 3.3.5 and 3.3.6), and that these fibres are more likely to be ventral stress fibres that are complexed to the focal adhesion proteins paxillin and vinculin (Figure 3.3.7). Focal adhesions allow the actin cytoskeleton to connect to the extracellular matrix through a link with integrins, therefore it was of interest to determine whether the differences reported so far would impact on the cells ability to attach and adhere to the substratum.

To detect any differences in cell attachment cells were seeded into a 96 well plate and left to attach for 30 or 60 minutes. Cells were then washed, and stained with crystal violet, before washing and solubilising prior to the optical density being measured. A robust and significant increase in the number of 2E2 cells attached compared to control CHO cells was seen at both time points with an approximate average absorbance of 0.6 +/- 0.06 in CHO cells after 30 minutes and 0.73 +/- 0.1 in 2E2 cells (Figure 3.3.12).

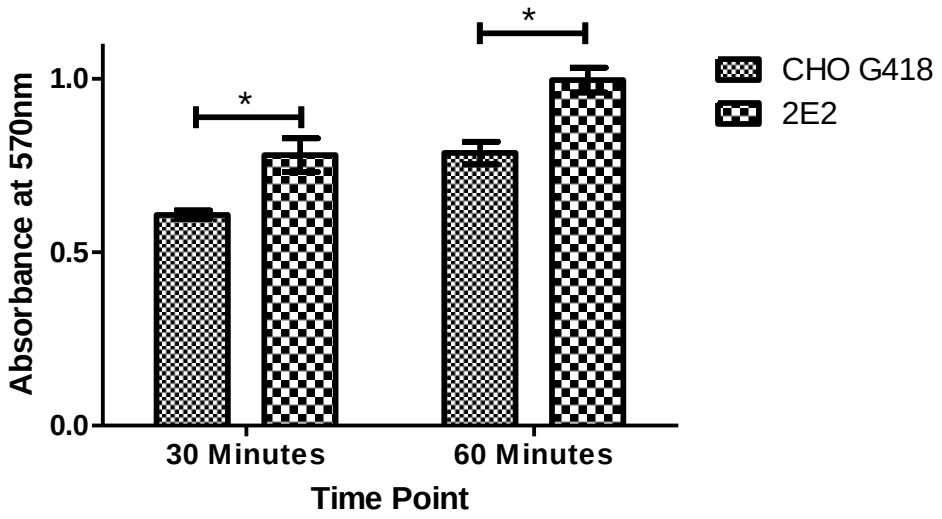



**Figure 3.3.12 –** 2E2 cells show a significant change in cell attachment when compared to control CHO cells. Cells were seeded into a well of a 96 well plate for 30 or 60 minutes after which the media was removed, the cells gently washed with PBS, 0.5% (w/v) crystal violet in 70% (v/v) ethanol added for 15 minutes at room temperature, before cells were washed again with PBS and the crystal violet solubilised with 30% (v/v) acetic acid and the absorbance at 570nm read and recorded. The differences observed were found to be statistically significant after analysis using a T test to compare CHO and 2E2 cells for each condition to a P value of *<0.05.

### 3.3.13 Reduced expression of eEF1A coincides with a reduction in cell migration

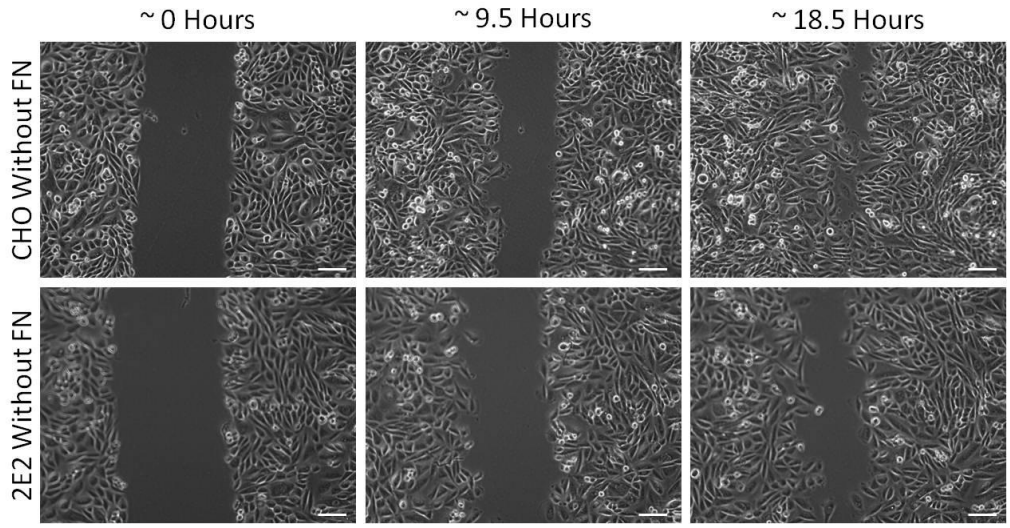
Cell migration relies on attachment of the cell to the ECM followed by contraction, and detachment of the adhesions at the rear of the cell (Lauffenburger and Horwitz, 1996). Attachment of the cell relies on the formation of focal complexes which mature to focal adhesions, which anchor the cell to the ECM. Contraction of the cell relies on the actomyosin fibres in the cell, whether cortical actin or stress fibres, with the contraction of the actomyosin fibres causing the detachment of the focal adhesions at the rear of the cell. A slight increase in cell attachment was observed in the 2E2 cells compared to the control CHO cells (Figure 3.3.12), therefore this slight increase in the attachment of the cell to the matrix could assist cell migration or hinder it. An increased number of actomyosin fibres was also observed in 2E2 cells compared to control CHO cells (Figures 3.3.5 and 3.3.6), which could affect the contractile force of the cells and therefore affect cell migration, therefore it was of interest to determine whether these changes would lead to a change in the motile properties of the different cell lines using the well defined would healing closure assay.

Cells were seeded with or without fibronectin and grown until confluent. The monolayer was scratched and cell motility analysed on the Cell IQ, with images being taken at specified intervals (Figure 3.3.13 A & B). Analysis of results allowed the percentage wound closure over time to be determined (Figure 3.3.13 C & D).

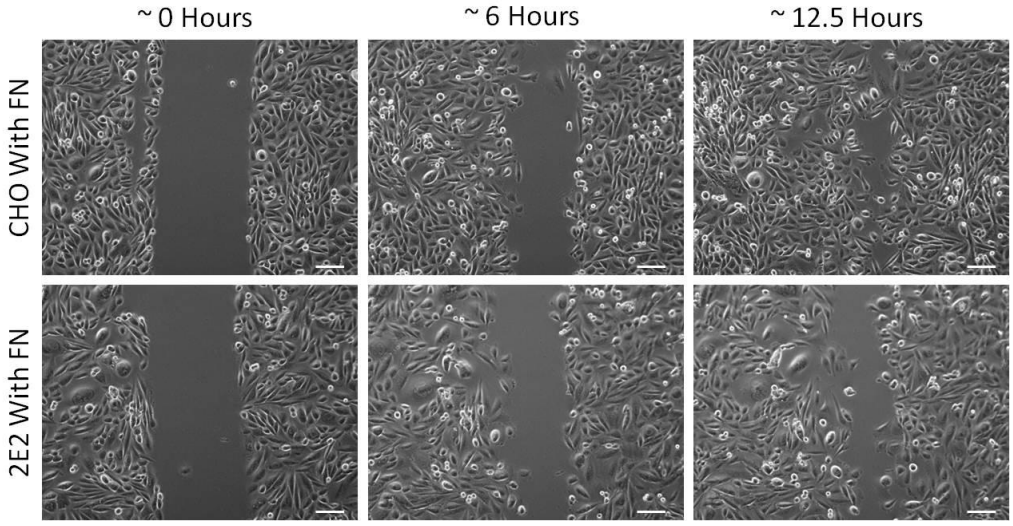
The reduced expression of eEF1A in the 2E2 cells results in a severe decrease in migration both on untreated plates or in the presence of fibronectin compared to the control CHO cells.

Fibronectin was seen to improve the overall motility of the CHO cells showing 95% closure after 10 +/- 2.8 hours in the presence of fibronectin whilst cells grown without coating were found to close the gap within 17 +/- 4.4 hours. Under the same conditions, cells expressing low levels of eEF1A were found to complete their migration in 18 +/- 3.0 hours with fibronectin and after 34 +/- 2.7 hours in uncoated conditions (Figure 3.3.13 E). Interestingly, video analysis suggested some further differences in the directionality or persistence of the cells, with the 2E2 cells appearing to migrate in a more random fashion than the consistent motility observed with the CHO cells.

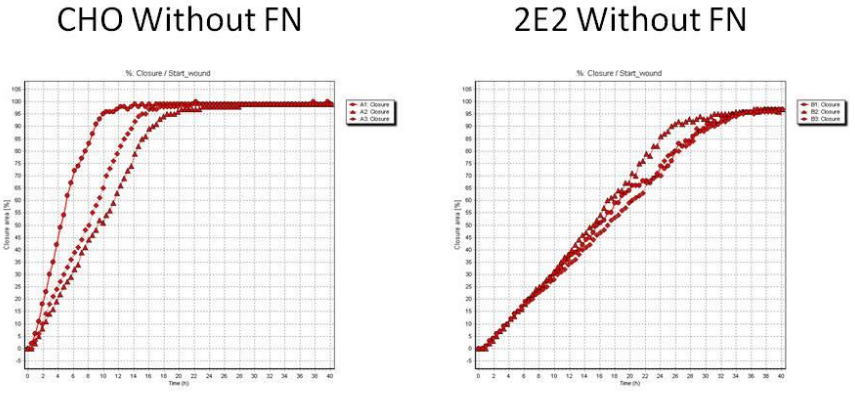
A


~ 0 Hours
~ 9.5 Hours
~ 18.5 Hours
CHO Without FN
2E2 Without FN

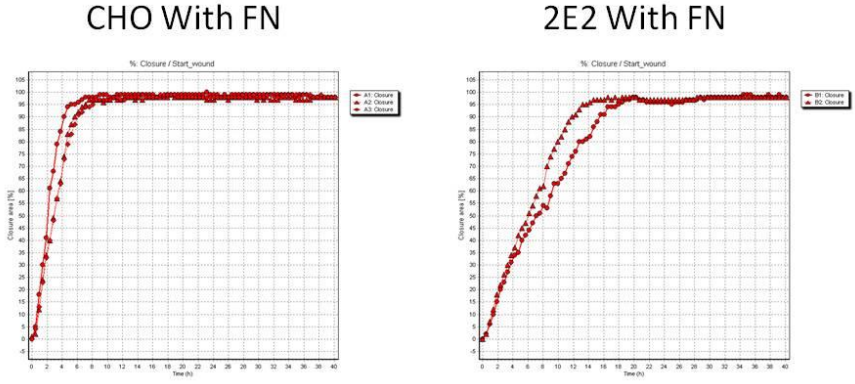
B


~ 0 Hours
~ 6 Hours
~ 12.5 Hours
CHO With FN
2E2 With FN

C


CHO Without FN
2E2 Without FN

D


CHO With FN
2E2 With FN

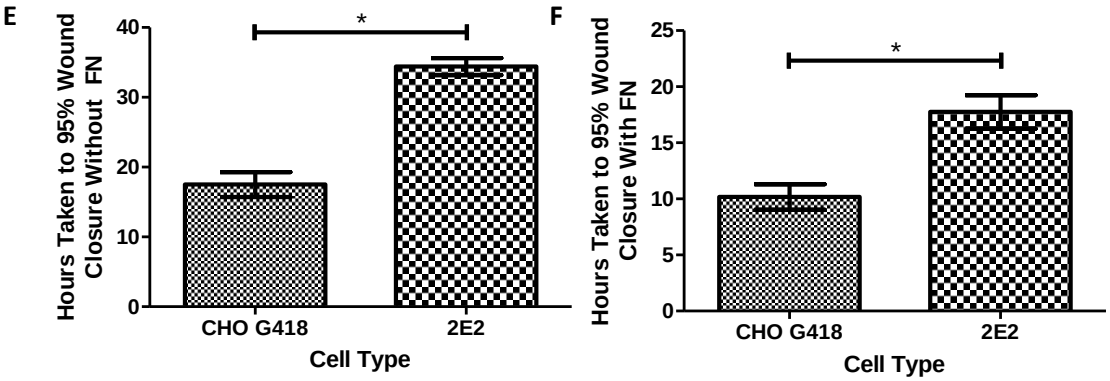



**Figure 3.3.13 –** Reduced expression of eEF1A in 2E2 cells results in a significant reduction in cell migration when compared to control CHO cells. Cells were grown until confluent on 6 well plates either untreated or coated with fibronectin and a scratch made through the confluent monolayer with a yellow pipette tip. Cells were washed, fresh media added, and cellular motility and wound closure measured over time using the Cell IQ setup. Images were taken approximately every 30 minutes. Images of three different time points for each condition are shown (A & B), with white scale bars equal to 50µM. Nine different points on each scratch were chosen on triplicate or duplicate scratches. The average wound closure of the 9 points was taken for each scratch resulting in two or three curves (C & D). The thresholds of the scratches were determined using the Cell IQ software, and the percentage wound closure of each individual scratch analysed. All of the closures from each individual scratch were combined and the percentage closure over time plotted (C & D). The average time taken for each scratch to reach 95% closure was then recorded and plotted, along with the standard error of the mean (E & F). The results were found to be statistically significant to a P value of *0.001 without fibronectin and *0.01 with fibronectin as determined by T test.

### 3.3.14 Migrating CHO cells show an increased number of actomyosin stress fibres

The reduced expression of eEF1A in the 2E2 cells compared to the control CHO cells resulted in a robust decrease in the closure of the wound and consequently a suggestion that these cells had a reduced speed of motility with a more random directionality of migration (Figure 3.3.13). Ventral actin stress fibres in migrating cells have been shown to orient perpendicular to the direction of motion, and have been suggested to have an effect on directionality of migration by detaching from the adhesions at the rear of the cell, and reattaching to adhesions at the front of the cell, with the new direction of movement perpendicular to that of the stress fibre that formed (Rid et al., 2005). Based on the increased number of actomyosin stress fibres in the 2E2 cells with the reduced level of eEF1A protein expression (Figures 3.3.5 and 3.3.6) we sought to determine if we could monitor changes in the number and/or orientation of actomyosin stress fibres in migrating cells. Observing localisation of myosin IIA to any stress fibres present would also enable the characteristics of the stress fibres to be determined, as myosin IIA creates the contractile force for the stress fibre to pull the cell along.

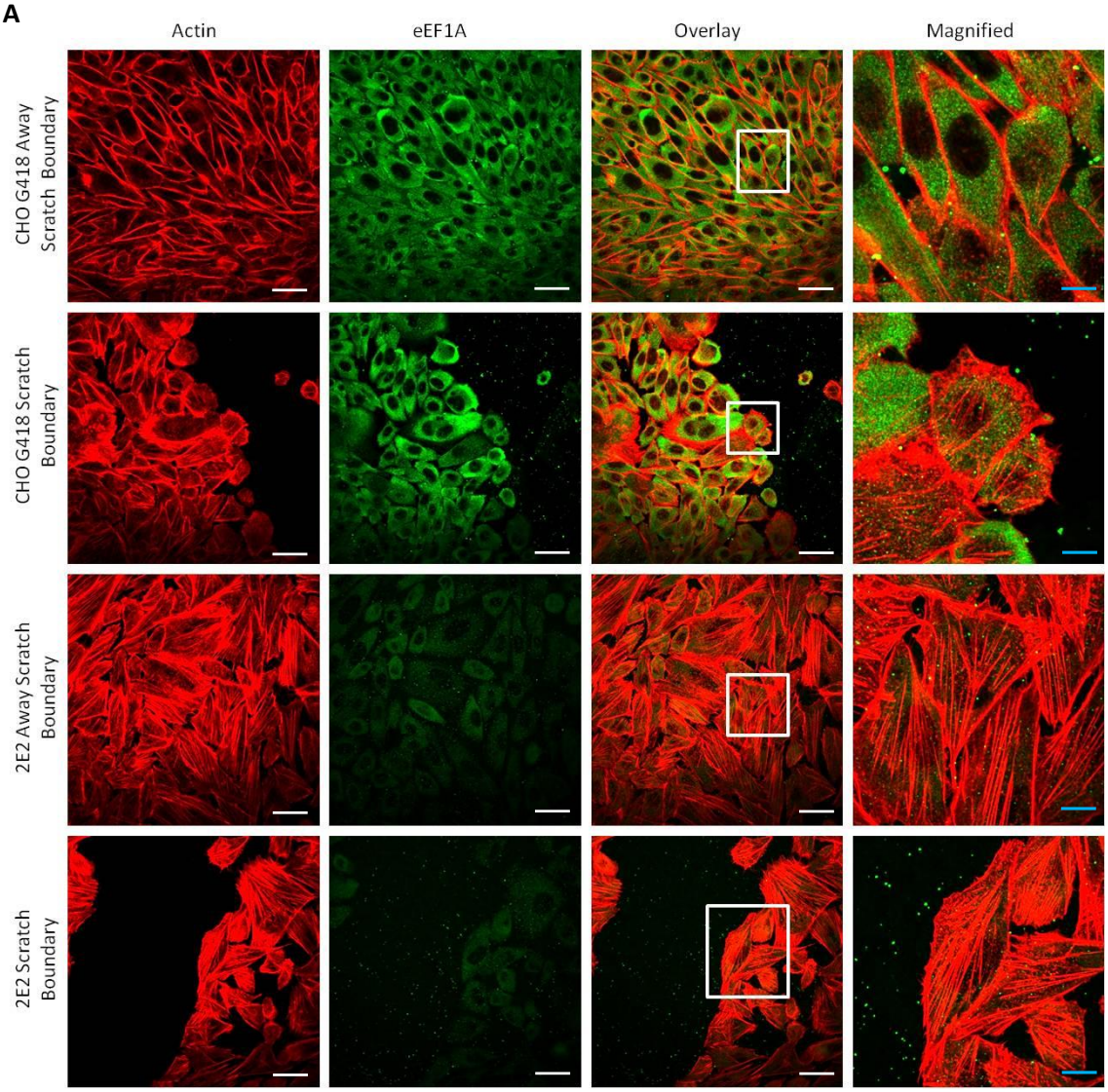
To assess this, cells were seeded onto fibronectin coated coverslips and grown to confluency prior to scratching through the monolayer. The cells were left to migrate for around 6 hours, prior to fixing, permeabilising and staining for actin and eEF1A or myosin IIA before viewing using confocal microscopy.

Stationary CHO cells showed a very limited number of actin stress fibres, presenting an average of only 1 +/- 1.1 per cell (as described previously (Figure 3.3.5), whereas migrating CHO cells, at the edge of the wound demonstrated a statistically significant increase in actin stress fibres in comparison, with an average of 5 +/- 2.6 fibres per cell. Interestingly, migrating CHO cells still demonstrated fewer actin stress fibres than both stationary 2E2 cells and migrating 2E2 cells which were both found to have an average of 12 +/- 3.0 fibres per cell. The difference in actin stress fibre number between the migrating and stationary CHO cells was statistically significant, as well as the difference between the stationary and migrating CHO cells and their 2E2 counterparts. No difference in actin stress fibre number was observed between migrating and stationary 2E2 cells. It was also observed that whereas stress fibres in the migrating CHO cells seemed to be perpendicular to the direction of the scratch, the stress

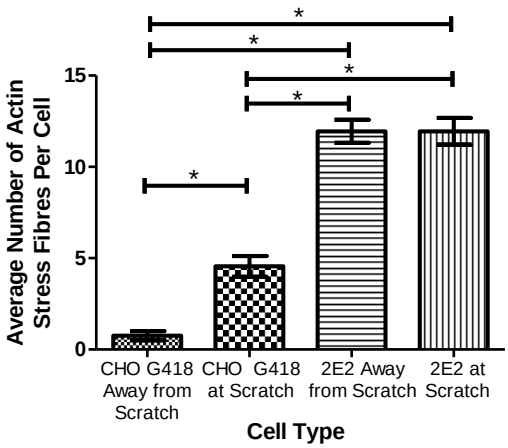
fibres in the 2E2 cells showed a less organised orientation in comparison, with a lot of fibres oriented parallel to the direction of the scratch (Figure 3.3.14 A & B).

Myosin IIA was shown to localise to the actin stress fibres observed in the 2E2 cells (Figure 3.3.6) therefore the localisation of myosin IIA to the newly formed stress fibres in the migrating CHO cells was unsurprising (Figure 3.3.14 C). Migrating CHO cells were found to have an average of 6 +/- 2.6 myosin IIA fibres per cell, and stationary CHO cells 1 +/- 1.1 with both stationary and migrating 2E2 cells having an average of 12 +/- 2.8 and 12 +/- 3.2 fibres per cell repectively (Figure 3.3.14 D). The orientation of the myosin IIA fibres appeared to be different between the CHO and 2E2 cells that were at the migratory front, with more of the myosin IIA fibres arranged so that they were perpendicular to the direction of motion compared to 2E2 cells, with the myosin IIA fibres in 2E2 cells appearing more randomly orientated.

A


Actin
eEF1A
Overlay
Magnified
CHO G418 Away Scratch Boundary
CHO G418 Scratch Boundary
2E2 Away Scratch Boundary
2E2 Scratch Boundary

B


Average Number of Actin Stress Fibres Per Cell
15
10
5
0
*
*
*
*
*
CHO G418 Away from Scratch
CHO G418 at Scratch
2E2 Away from Scratch
2E2 at Scratch
Cell Type

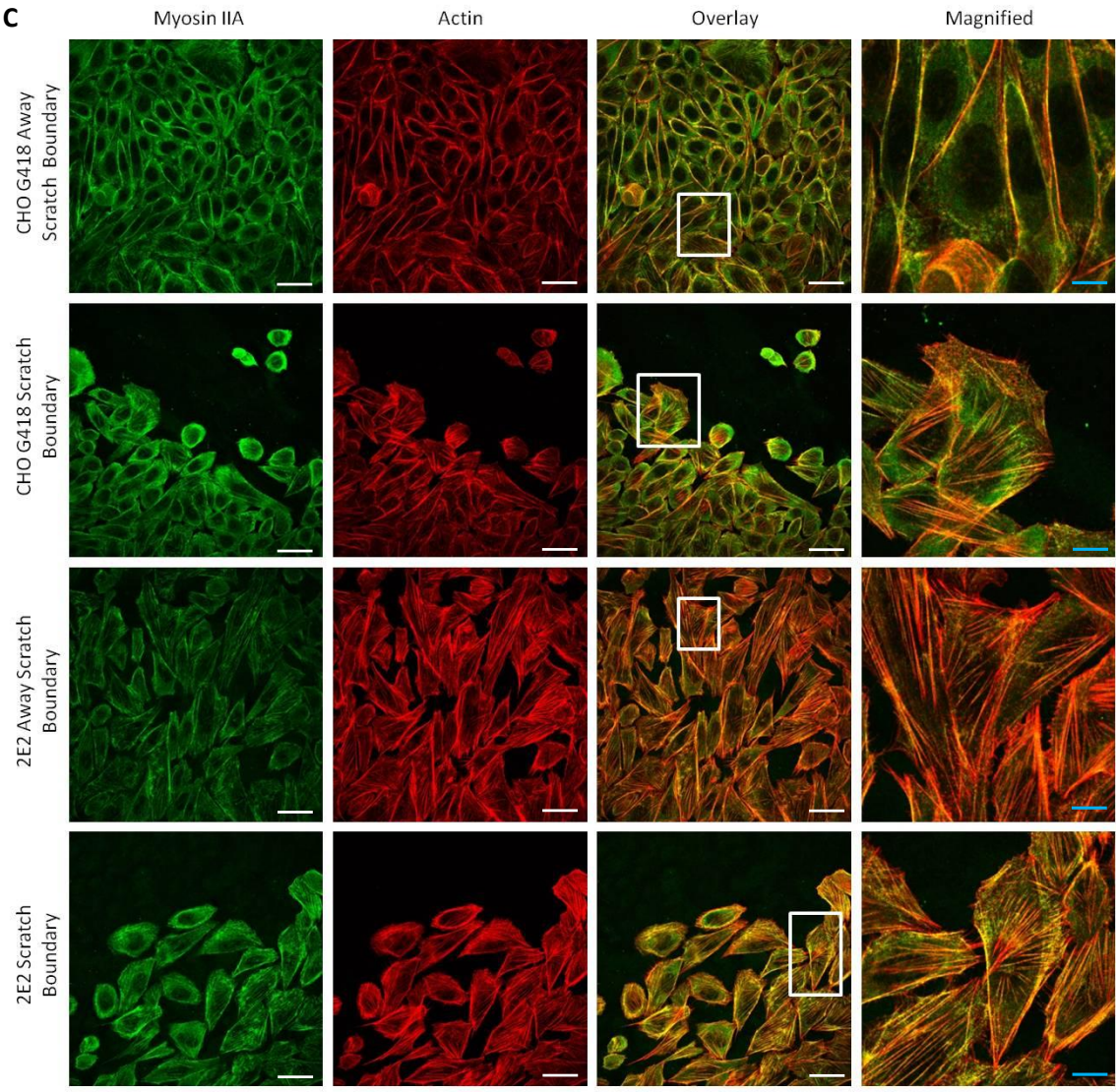


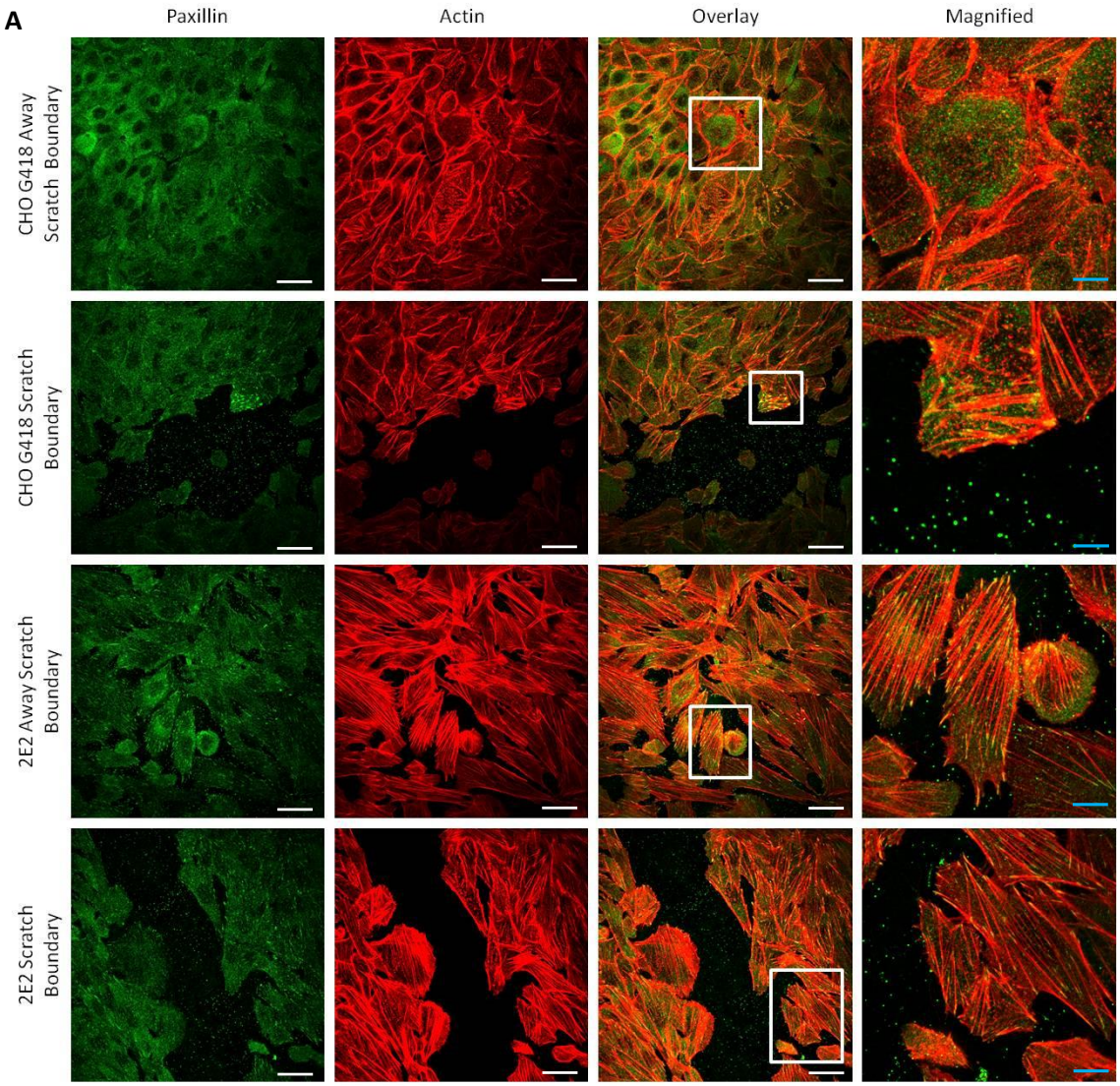

**Figure 3.3.14 –** Induced motility leads to a greater number of actomyosin stress fibres in CHO cells, but not in 2E2 cells. Cells were grown to confluence on a fibronectin coated coverslip, and a scratch made through the confluent monolayer. Once cells were migrating, they were fixed, permeabilised, blocked and stained for actin (rhodamine phalloidin) and eEF1A (FITC conjugated secondary antibody; A) or myosin IIA (FITC conjugated secondary antibody; C) and actin (rhodamine phalloidin). Coverslips were then mounted onto microscope slides and viewed using confocal microscopy (A & C). Actin and myosin IIA fibres were quantified by counting the number of fibres that crossed over the cell in 20 cells for each cell type, both at the migrational front and behind. The average number of fibres with the standard error of the mean was plotted and statistical analysis performed using GraphPad Prism, using the 1 way

ANOVA with Tukeys post test and results were significant to a P value of *<0.05 (B & D). White error bars are equal to 25µM. Blue error bars are equal to approximately 7.5µM.

### 3.3.15 Paxillin and vinculin localise to the ends of actin stress fibres in stationary and migrating cells

The increase in actomyosin stress fibres in migrating CHO cells compared to stationary CHO cells (Figure 3.3.14) indicated that this increase would provide the optimal condition for migration, however, in order for actin stress fibres to affect the motility of the cell, the fibres have to connect to the ECM. We sought to determine whether changes in the organisation and localisation of focal adhesion proteins would be observed following induced motility using the same type of experiments.

Observation of paxillin clusters during migration was carried out by growing the cells to confluence on a fibronectin coated coverslip, scratching and allowing them to migrate for approximately 6 hours into the newly created space prior to fixing, permeabilisation and staining for paxillin or vinculin and actin before viewing using confocal microscopy. Paxillin and vinculin clusters were observed again localised to the ends of the stress fibres in both CHO and 2E2 cells (Figure 3.3.15) as previously demonstrated (Figure 3.3.7). When cells were confluent, it could not be determined which cell the clusters at the periphery belonged too, therefore quantification of the clusters was not possible.


A
Paxillin
Actin
Overlay
Magnified
CHO G418 Away Scratch Boundary
CHO G418 Scratch Boundary
2E2 Away Scratch Boundary
2E2 Scratch Boundary

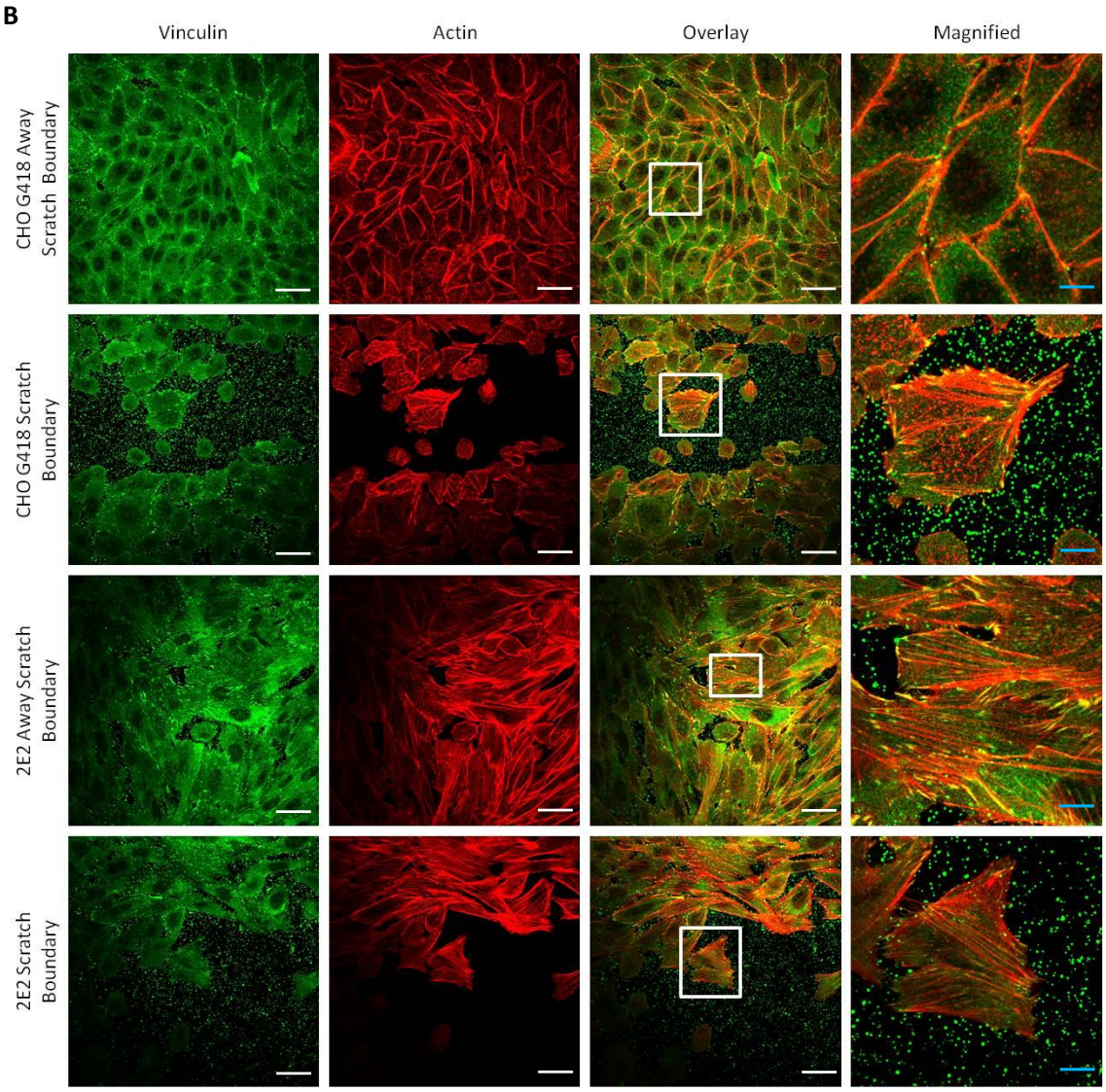



**Figure 3.3.15 -** Migrating CHO and 2E2 cells show paxillin and vinculin clusters localised to the ends of the observed actin stress fibres. Cells were grown to confluence on a fibronectin coated coverslip, and a scratch made through the confluent monolayer using a yellow pipette tip. Once cells were migrating, they were fixed, permeabilised, blocked and stained for paxillin or vinculin (FITC conjugated secondary antibody) and actin (rhodamine phalloidin). Coverslips were then mounted onto microscope slides and viewed using confocal microscopy (A & B). White error bars are equal to 25µM. Blue error bars are equal to approximately 7.5µM.

### 3.3.16 Arp3 localisation is not affected in cells with normal or reduced level of eEF1A protein during migration

No difference was detected in either protein expression or localisation of Arp3 when eEF1A protein level is reduced in 2E2 cells compared to CHO cells (Figure 3.3.9). An increase in the number of ventral actin stress fibres has been shown in 2E2 cells compared to CHO cells (Figure 3.3.5) as well as in migrating CHO cells compared to stationary CHO cells (Figure 3.3.14). Arp3 has been shown to be essential for formation of transverse arcs, which have been shown to connect with dorsal stress fibres to form ventral stress fibres (Hotulainen and Lappalainen, 2006), as well as being associated with focal adhesions, especially new focal adhesions (DeMali et al., 2002), therefore it was of interest to determine whether there was any localisation of Arp3 to the new stress fibres observed in the migrating CHO cells compared to the stationary CHO cells. The Arp2/3 complex has also been shown to be critical for the formation of lamellipodia, the sheet like protrusions that support cell migration and have been shown to be important for directional migration as well as being the site of formation of focal adhesions (Wu et al., 2012), therefore based on the change in cell motility of the 2E2 cells when compared to the control CHO cells, observing the localisation of Arp3 during cell migration was of interest.

Following confluent growth on a fibronectin coated coverslip, a scratch was made through the monolayer and the cells were left to migrate for approximately 6 hours before fixation, permeabilisation and staining for Arp3 and actin. The coverslips were then mounted onto microscope slides and viewed by confocal microscopy. No difference in Arp3 localisation was observed between migrating and stationary CHO cells, or between the CHO and 2E2 cells (Figure 3.3.16), suggesting that low levels of eEF1A do not affect overall Arp3 functions in these cells.

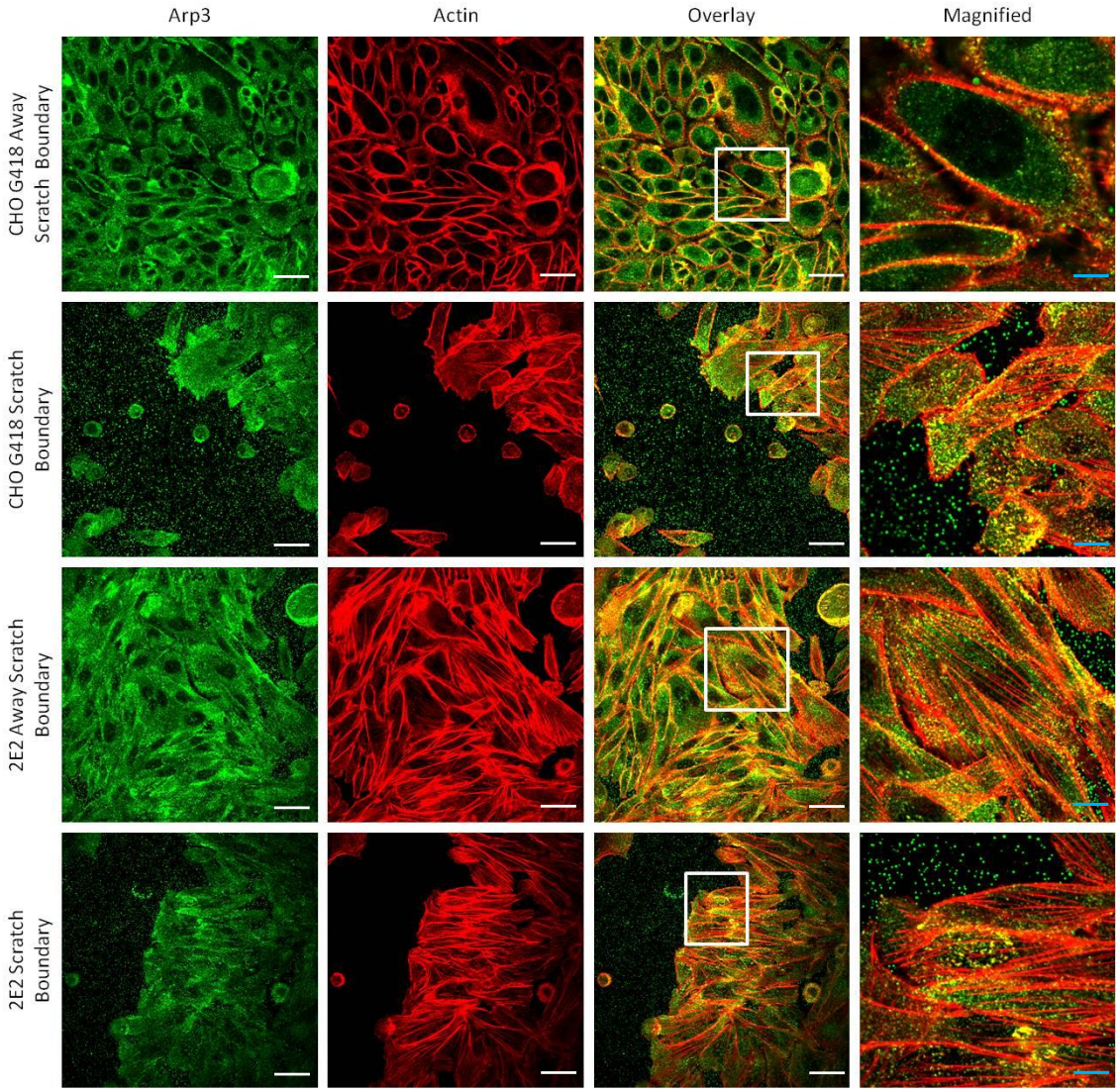



**Figure 3.3.16 –** Reduced expression of eEF1A results in no change in Arp3 localisation in migrating or stationary cells. Cells were seeded on to fibronectin coated coverslips, and grown to confluence. A yellow pipette tip was used to make a scratch through the confluent monolayer, and the cells left to start migrating. The cells were then fixed, permeabilised, blocked and stained for Arp3 with a FITC conjugated secondary antibody, and actin with rhodamine phalloidin. The coverslips were then mounted onto microscope slides, and viewed using confocal microscopy. The white rectangles show the areas selected for the magnified images. White error bars are equal to 25µM. Blue error bars are equal to approximately 7.5µM.

### 3.3.17 Beta-1 integrin localisation is not affected in cells with normal or reduced level of eEF1A protein during migration

The localisation of β-1 integrin was unaffected by the change in protein expression of eEF1A in the 2E2 cells compared to the control CHO cells (Figure 3.3.10). Integrins play an integral role in cell migration, and as it has been shown that there was an increase in actin stress fibres, and their associated focal adhesion proteins in migrating CHO cells compared to stationary CHO cells, it was of interest to determine whether there was any difference in localisation of β-1 integrin in migrating CHO cells compared to migrating 2E2 cells.

The cells were grown to confluence on a fibronectin coated coverslip, a scratch made through the monolayer, and the cells left for approximately 6 hours to start migrating, after which they were fixed and stained for β-1 integrin and actin. The coverslips were then mounted onto microscope slides and viewed by confocal microscopy. No difference in β-1 integrin was observed between CHO and 2E2 cells, or between migrating or stationary cells (Figure 3.3.17).

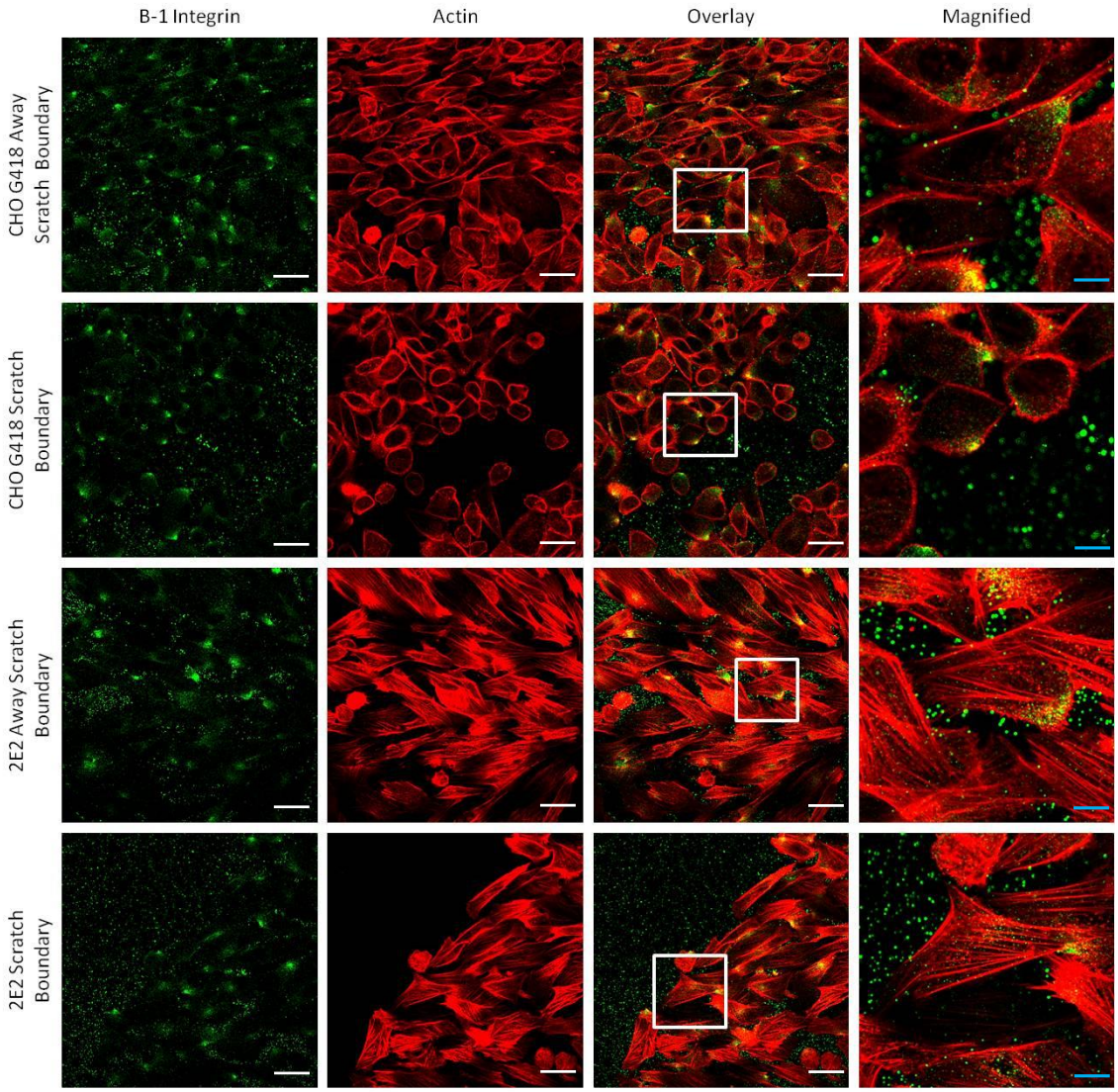



**Figure 3.3.17 –** Reduced expression of eEF1A does not affect β-integrin localisation in migrating or stationary cells. Cells were seeded onto fibronectin coated coverslips and grown to confluence. A yellow pipette tip was used to make a scratch through the confluent monolayer, and the cells left to migrate. Once migrating the cells were fixed and stained for β-1 integrin, with a FITC conjugated secondary antibody, and co-stained for actin with rhodamine phalloidin. The coverslips were then mounted onto microscope slides and viewed using confocal microscopy. The white rectangles show the areas selected for the magnified images. White error bars are equal to 25µM. Blue error bars are equal to approximately 7.5µM.

### 3.3.18 The ratio of G-actin to F-actin is increased in 2E2 cells compared to control CHO cells

The reduced expression of eEF1A results in an increase in the number of ventral stress fibres, without revealing significant changes in the overall levels of actin protein expression (Figure 3.3.5). Our data however suggests potential differences in the level of polymerised actin filaments between the CHO and 2E2 cells lines, an important criterion that cannot be assessed when performing Western blotting on whole cell lysates. It was of interest to determine whether the reduction in eEF1A level would correlate with differences in the levels of G- and F- actin and their overall ratio.

Cells were scraped and the lysate homogenised before centrifugation to separate the different pools of G and F-actin. Both the pellet and supernatant were collected, corresponding to the F- and G- actin respectively in cells at the time of isolation and the ratio of actin determined by Western blotting of the samples, followed by quantification using ImageJ.

In order to determine that the centrifugation was sufficient to separate the F actin and G actin, an inhibitor was used to increase the amount of F actin, therefore when the inhibitor was used, the amount of F actin should increase and G actin should decrease. It can be seen that when the inhibitor is used, the level of F actin becomes greater than the level of F actin, however when no inhibitor is used, the level of G actin is higher as would be expected in normal samples. Interestingly the ratio of F-actin to G-actin was found to be statistically increased in the 2E2 cells when compared to the control CHO cells from 1 : 0.47 +/- 0.09 to 1 : 0.67 +/- 0.04 indicating that there is an increase in the amount of F-actin in the 2E2 cells when compared to the CHO cells (Figure 3.3.18).

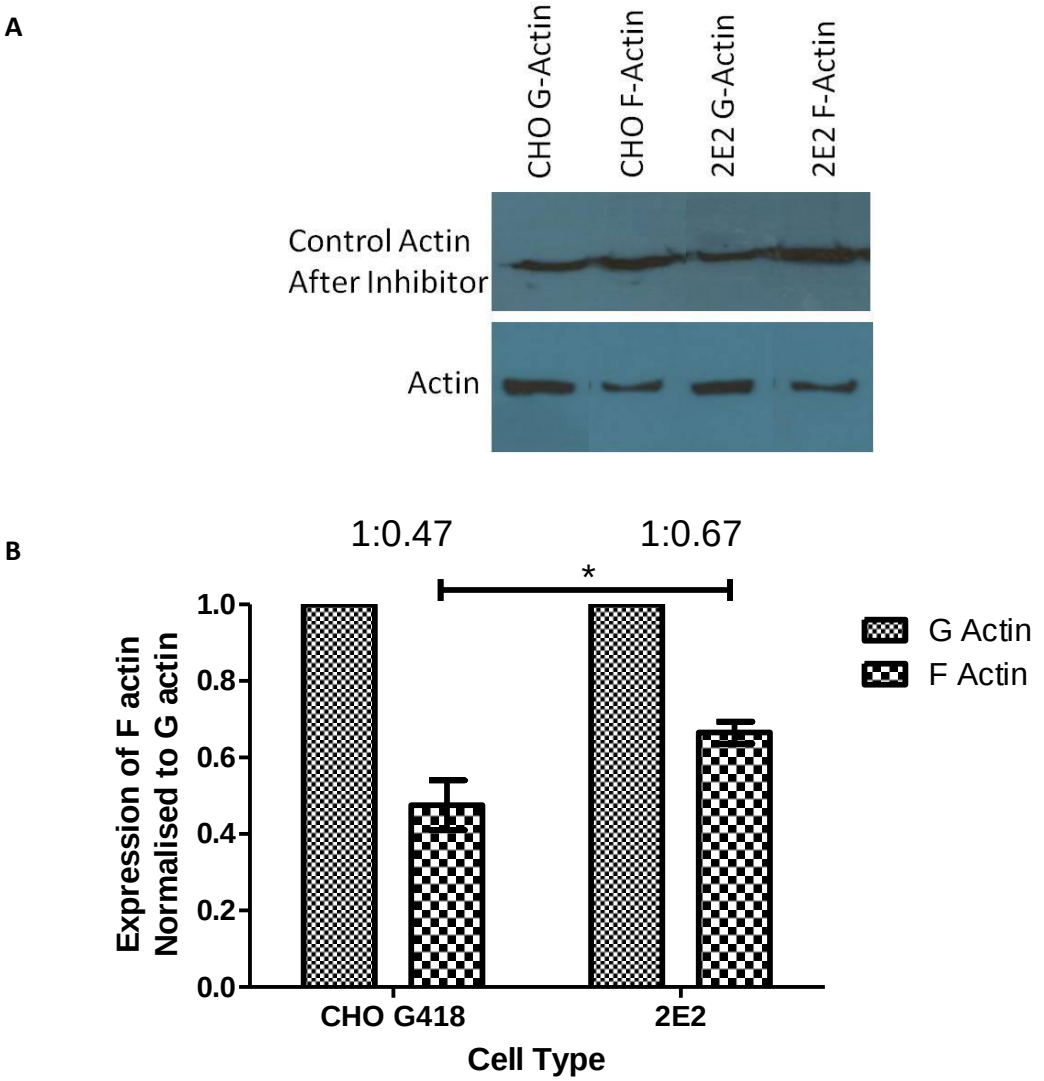



**Figure 3.3.18 –** 2E2 cells have a higher ratio of F-actin to G-actin when compared to control CHO cells. Cells were scraped into buffer, homogenised and centrifuged at 100,000 xg for 1 hour. The supernatants and pellets were collected and Western blotting performed by loading 10µl of each sample onto a 10% (v/v) acrylamide gel before running and transferring to nitrocellulose. The blots were probed for actin followed by detection with ECL (A). Duplicate bands were quantified using ImageJ, the average and standard error of the mean plotted, and ratios determined by normalising F-actin protein expression to G-actin. Ratios are included as numerical values on the graph for ease of viewing (B). Statistical analysis was done by T test and results were found to be significant to a P value of *<0.05.

### 3.3.19 2E2 cells show reduced localisation of eEF1A with the cytoskeleton when compared to control CHO cells

It has so far been demonstrated that the 2E2 cells show an increased number of ventral stress fibres compared to the control CHO cells (Figure 3.3.5 & 6), and that this change is due to an increase in the amount of F-actin compared to G-actin rather than relocalisation of the F-actin already present (Figure 3.3.18) suggesting that the decrease in eEF1A affects the polymerisation of G-actin to F-actin, whether directly or indirectly, and the newly formed F-actin is then organised into the observed stress fibres by an actin binding protein. eEF1A is a known actin binding protein (Demma et al., 1990, Yang et al., 1990), therefore suggesting that reducing its level would equally lead to a decrease in actin bundling, an observation that is not seen given the high numbers of stress fibres seen in the 2E2 cells (Figure 3.3.5). Importantly, eEF1A has been shown to bundle actin fibres into square packed bundles in a way that has been suggested to exclude other actin binding proteins (Owen et al., 1992). We hypothesise that, at physiological levels, when present at abundant levels, eEF1A prevents other actin binding proteins from forming actin stress fibres. By contrast, reducing the level of eEF1A protein expression creates the opportunity for other actin binding proteins to bind and bundle actin. For this reason we sought to analyse the localisation of eEF1A with the actin stress fibres, or the cortical actin surrounding the cell, using immunofluorescence staining, ELISA and Western blotting on triton washed cells, therefore removing the majority of cytosolic proteins, leaving only the triton insoluble cytoskeleton and proteins associated with the cytoskeleton behind (Kobayashia et al., 1982).

Immunofluorescence staining was carried out by seeding cells onto fibronectin coated coverslips before fixing, permeabilising with a high concentration of triton for two minutes before blocking and staining for eEF1A and viewing using confocal microscopy.

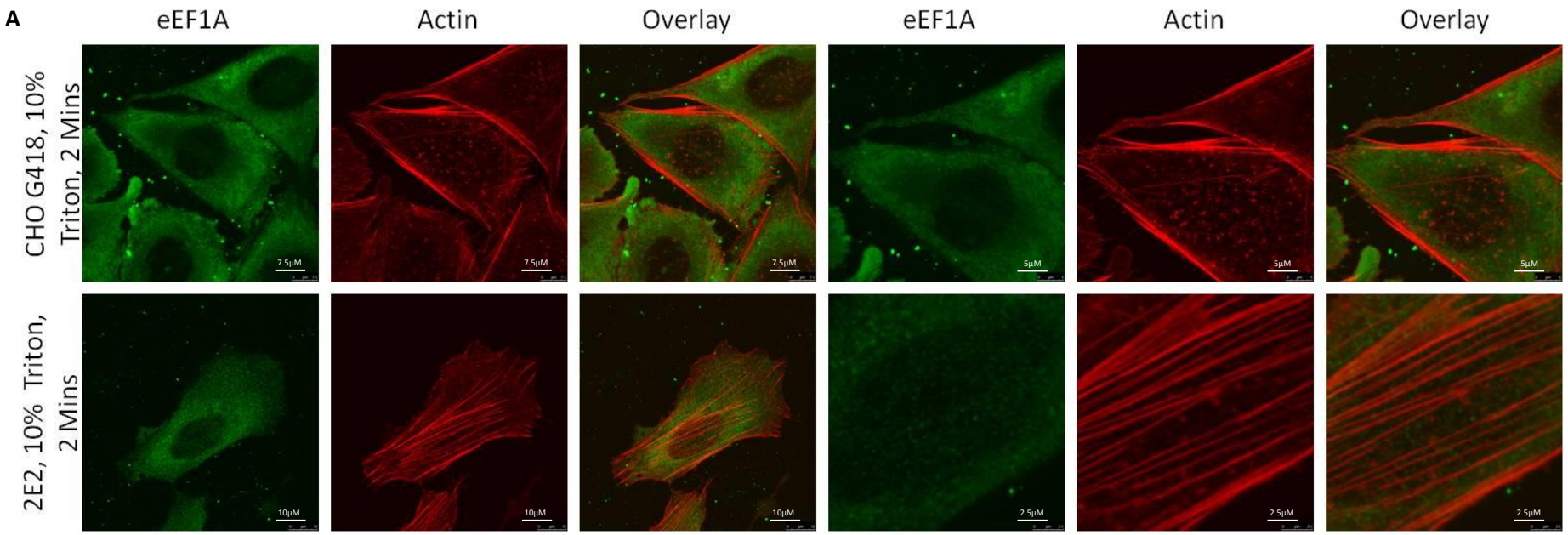
The ELISA was carried out by growing, fixing, and permeabilising cells with different concentrations of triton, and then blocking before probing for eEF1A, and developing using OPD as per manufacturer's instructions.

Western blotting with triton treatment was carried out by growing cells before washing, adding triton, washing, homogenising and loading total protein onto a gel. The gel was run, transferred to nitrocellulose, probed for eEF1A and actin and developed. Quantification of bands was performed by densitometry analysis using ImageJ.

Following immunofluorescence staining, actin fibres in both cell lines were magnified, to observe any localisation of eEF1A to the fibre, however no localisation was observed in either cell line to actin fibres around the outside of the cell or to the actin stress fibres across the cell (Figure 3.3.19 A).

ELISA quantification, following gentle cell permeabilisation, demonstrated a significant reduction in the level of eEF1A in 2E2 cells at 52% of the expression of CHO cells when normalised to tubulin expression (Figure 3.3.19 B), supporting the data collected during Western blotting, and further highlighting the suitability of this method for quantification of eEF1A levels. When a high concentration of triton was added to remove the majority of cytosolic eEF1A the 2E2 cells showed only 73% of the eEF1A protein levels when compared to CHO cells (Figure 3.3.19 B). If the level of eEF1A association with the cytoskeleton was the same in both cell lines, the percentage expression value for 2E2 cells should be 100%, therefore a value of 73% suggests that less eEF1A is associated with the cytoskeleton in 2E2 cells when compared to control CHO cells.

Protein was extracted from CHO and 2E2 cells without triton treatment, which showed a 40% decrease in eEF1A expression in 2E2 cells compared to CHO cells which correlates to the same observed difference in expression shown in figure 3.3.1, however, after treatment with triton, and after normalising eEF1A expression to actin, the treated 2E2 cells were shown to have a decreased proportion of eEF1A protein compared to the treated CHO cells, around 85% in comparison to 100% in control CHO cells, indicating that less eEF1A protein is associated with the cytoskeleton in 2E2 cells when compared to the control CHO cells (Figure 3.3.19 C & D).


A
eEF1A
Actin
Overlay
eEF1A
Actin
Overlay
CHO G418, 10% Triton, 2 Mins
2E2, 10% Triton, 2 Mins
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7.5μM
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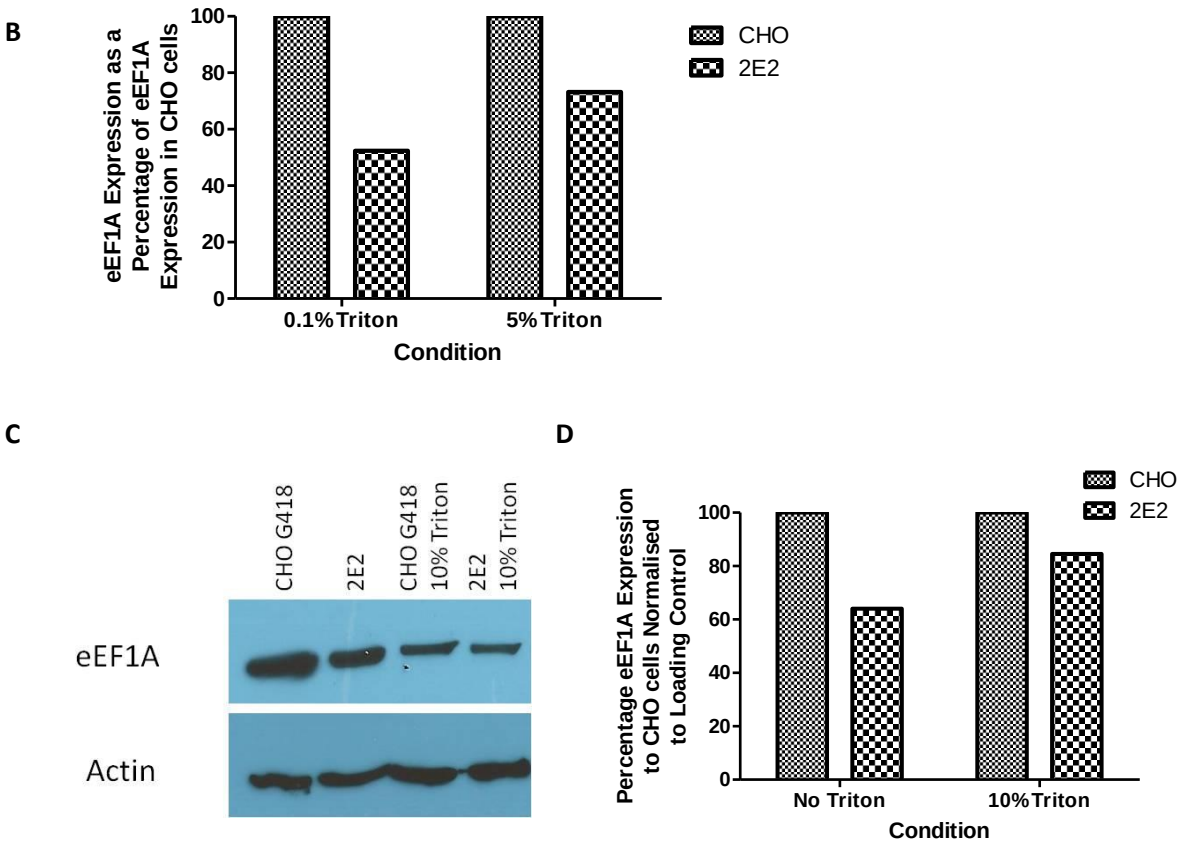



**Figure 3.3.19** – No localisation of eEF1A to actin fibres can be observed through immunofluorescence staining, however, less eEF1A is associated with the cytoskeleton in 2E2 cells when compared to control CHO cells as analysed by ELISA and Western blotting. Cells were fixed then permeabilised with 10% triton (v/v) for 2 minutes before blocking and staining for eEF1A (FITC conjugated secondary antibody) and actin (rhodamine phalloidin). Coverslips were mounted onto microscope slides and viewed using confocal microscopy. Actin fibres in individual cells were zoomed into to attempt to visualise localisation (A). For the ELISA, 2,500 or 5,000 cells were seeded into a 96 well plate and grown for two days before fixing, permeabilising with 0.1% (v/v) or 5% (v/v) triton, and blocking. Cells were then probed for eEF1A and tubulin with a HRP conjugated secondary antibody, and the ELISA developed using OPD as per the manufacturer's instructions. Results for 2,500 and 5,000 cells were combined to create an average reading, and percentage expression of eEF1A to control CHO cells presented after normalisation to the tubulin control (B). For Western blotting, cells were grown to confluence, before media was washed off, 10% (v/v) triton incubated for 1 minute at room temperature, after which cells were again washed, homogenised and total protein quantified. Protein was loaded onto a gel, the gel run and transferred onto nitrocellulose before being probed for eEF1A and actin (C). Blots were then developed and the bands quantified by densitometry analysis performed using ImageJ (D).

## *3.4 Discussion*

Previous studies on eEF1A have focused on determining how the cell is affected by overexpression of eEF1A (Munshi et al., 2000, Gross and Kinzy, 2005), as overexpression of eEF1A can lead to several different types of cancer. However, in order to establish the normal functions of eEF1A it is also important to determine the effect of reduced expression of eEF1A in cell systems. The canonical function of eEF1A is in the elongation stage of protein translation, however, another important function of eEF1A is actin binding and bundling (Dharmawardhane et al., 1991). Studies, at least in yeast, have so far suggested that these two functions are independent of each other, as work by Gross and Kinzy, 2005 & 2007, showed expression of mutated forms of eEF1A that affected actin binding/bundling without affecting protein translation, at least at the elongation step (Gross and Kinzy, 2005, Gross and Kinzy, 2007). Determining whether these functions are independent in mammalian cells is important to further understand the mechanism by which eEF1A can carry out its multiple functions.

CHO-K1 cells with a reduced expression of eEF1A were created by Borradaile et al, 2005, in their pursuit to determine factors affecting resistance to lipotoxic cell death through the random integration of a retroviral promoter trap in the eEF1A gene (Borradaile et al., 2005). The target of the random retroviral insertion was determined using 5' RACE (rapid amplification of cDNA ends), with BLAST comparison to which it was found to align with part of the 5' untranslated region of hamster eEF1A1. This was confirmed using PCR with primers specific to both eEF1A1 and eEF1A2, establishing that eEF1A1 was the only isoform affected, and that eEF1A1 expression was completely abolished. When protein levels were analysed, an eEF1A antibody was used that recognised both isoforms, therefore it was assumed that based on the previous data eEF1A1 expression was completely removed, and the remaining band was due to expression of eEF1A2. CHO-K1 cells are known to be functionally hemizygous at many different loci (Borradaile et al., 2005), therefore it is possible that by targeting one gene, the expression of that gene could be abolished and the likelihood of more than one retrovirus infecting the same cell and therefore affecting two different genes would be highly unlikely, as they used a low multiplicity of infection such that there was only one integration per every 10 genomes. Unfortunately no data could be found about whether CHO-K1 cells are hemizygous at the eEF1A1 or eEF1A2 locations.

Importantly, analysis of total protein translation by methionine incorporation in this system showed that there was no change in total protein synthesis between the two different cells (Borradaile et al., 2005). This therefore provided a tool in which the level of eEF1A had been greatly reduced with no global change in the level of protein translation, suggesting that the remaining eEF1A protein in these cells was sufficient to satisfactorily complete its translational role. We set about to determine if these cells presented differences in cellular behaviours and characteristics where eEF1A has been highlighted as a major regulator.

Initially it was important to clearly establish the level of reduction of eEF1A protein and also to decipher which isoforms of the factor were actually expressed. Western blotting was performed on the two cell lines with the same antibody used by Borradaile et al, 2005, confirming the low expression of the eEF1A protein. The reduction in eEF1A protein expression was however found to be less severe in our analysis, with a decrease of around 40% (Figure 3.3.1 A & B).

Antibodies specific to human eEF1A1 and eEF1A2 were purchased to determine the true changes in the protein expression of the two isoforms. A reduction in eEF1A2 expression could be seen whilst no detection was observed in either cell lines, including other controls when probing for eEF1A1 proteins (Figure 3.3.1 C & D).

These results alone would suggest that the expression of eEF1A2 has been affected by the retroviral insertion, however, the specificity of the antibody used was only determined in human cells, and the specific epitope recognised is unknown, therefore it is possible that the antibody is recognising both isoforms of eEF1A in the hamster cells used. Based on the different gene disruption it is also unlikely that induced mutation can affect more than one gene, and furthermore it is highly unlikely that both eEF1A1 and eEF1A2 would randomly be affected especially as the two isoforms are found on different chromosomes with eEF1A1 found on chromosome 6q14 and eEF1A2 found on chromosome 20q13.3 (Lund et al., 1996). It is at this stage difficult to explain how the different levels of eEF1A have been affected, since CHO-K1 cells are hemizygous at many loci (Borradaile et al., 2005), however it seems sensible to suggest that these cells may have two copies of the eEF1A1 gene. This would explain the 40% reduction in eEF1A protein expression as the retroviral insert would only affect one of the copies and therefore result in half of the normal expression. Throughout this work it was assumed based on the argument put forth before that eEF1A1 protein expression was reduced.

Although global protein synthesis had been shown not to be significantly affected by the reduction in eEF1A protein, it was unclear whether such a decrease was mirrored by more subtle changes in elongation, where eEF1A plays essential roles. To directly assess this, polysome profiling for the two cell lines was performed, allowing for a detailed and specific analysis of each stage of translation through changes in the polysome to monosome ratio. If an elongation block was to be observed, the polysome peaks would increase and the monosome peaks would decrease leading to an increased polysome ratio in the absence of cycloheximide. When the CHO and 2E2 cells were analysed in this context, the polysome profiles were found to be very similar with the polysome to monosome ratios of 1.25 and 1.34 respectively, suggesting that the reduced expression of eEF1A in the 2E2 cells does not affect the overall rate of protein synthesis elongation (Figure 3.3.2), confirming the results from Borradaile et al, 2005. Furthermore data collected throughout this chapter also clearly suggest that overall levels of protein expression is rarely affected, as a large panel of proteins, known as regular housekeeping genes (actin, tubulin and others) were always found at similar levels in the two cell lines (Figures 3.3.5-3.3.7, 3.3.9 & 3.3.11).

The concept that loss of such a high proportion of eEF1A does not lead to direct overall protein synthesis should not be surprising since the amount of eEF1A is in 17-35 fold molar excess to other components of the translational machinery (Slobin, 1980). This finding supports the theory that there is a large excess ofeEF1A protein in cells and suggests their use for other different but conserved cellular functions.

One of the best characterised secondary functions for eEF1A is its ability to bind and bundle actin in vitro (Demma et al., 1990, Yang et al., 1990), as well as its vital role in actin remodelling in yeast (Munshi et al., 2000). We were therefore interested to determine if any cytoskeletal changes in the mammalian CHO cell system could be observed given the large reduction in eEF1A protein expression.

Whilst low levels of eEF1A did not lead to significant changes in overall actin protein levels, they resulted in a significantly increased number of actin stress fibres as observed using confocal microscopy (Figure 3.3.5), therefore indicating that the difference observed relates to a regulatory process rather than more global changes in translation of actin. Myosin IIA was found to be localised to the actin stress fibres and its striations were significantly increased in the 2E2 cells compared to the control CHO cells, though no difference in myosin IIA protein expression was observed (Figure 3.3.6). Staining for both paxillin and vinculin (Figure 3.3.7), in CHO and 2E2 cells showed an increase in the number of clusters when eEF1A level was

decreased, correlating with the increase in the number of actin stress fibres. It was observed that the clusters were found at both ends of the actin stress fibres, which along with the localisation of myosin IIA within, further suggested that they were ventral stress fibres, as only this category of fibres attach to the extracellular matrix via focal adhesion proteins (Tojkander et al., 2012).

This increase in ventral actin stress fibres in the 2E2 cells poses the questions of 1) how are the fibres organised into stress fibres? and 2) where do the actin filaments come from? In fact, very little is known about the specific proteins that are involved in inducing stress fibre formation, however, Rho A is known to be involved in the signalling pathway, usually through the activation of ROCK which can increase the activity of myosin to potentially recruit actin fibres (Naumanen et al., 2007, Verkhovsky et al., 1995). Rho A has also been shown to activate formins, which can induce actin assembly, therefore increasing the number of fibres to be bundled.

Though eEF1A has been linked to actin binding and bundling, no studies have linked eEF1A with stress fibre formation, although a large proportion of eEF1A in cells have been shown to be associated with the actin cytoskeleton (Edmonds et al., 1995). Given that previous studies have shown, not only that can eEF1A bind and bundle actin fibres, but that this bundling results in the exclusion of other actin binding proteins, in particular orthogonal ABPs, (Owen et al., 1992), a reduced level of eEF1A could result in more eEF1A free actin filaments therefore giving the opportunity for other actin binding proteins to replace eEF1A, resulting in stress fibre formation.

α-actinin is an orthogonal cross-linking ABP that binds to the side of the actin filaments and has been shown to associate with transverse arcs, dorsal and ventral stress fibres. When eEF1A is abundantly present, it is possible to suggest that the binding of eEF1A to the side of the actin filament could be sufficient to compete with the binding of α-actinin (Owen et al., 1992) to the same filament, therefore preventing the bundling of filaments into stress fibres. α-actinin is known to be associated with stress fibre formation, in particular with its known alternating pattern with myosin IIA (Pellegrin and Mellor, 2007), it therefore presents a potential candidate which, when allowed to bind to the filaments in the absence of eEF1A in the 2E2 cells, could be involved in the formation of the ventral stress fibres observed. Similarly, tropomyosin is another side filament ABP which stabilises actin filaments which could be provided with the opportunity to bind when eEF1A is no longer present in abundance. eEF1A and α-actinin offer similar binding affinity, between 0.1-10μM (Pollard et

al., 2007) and 1-5μM (Pollard et al., 2007) respectively, and given the large difference in protein levels between the two, it is possible to hypothesise that the binding of eEF1A to actin would exclude the binding of α-actinin. This competitive binding of ABPs is not a new concept, it has been known for decades that actin binding proteins can compete for the same site of on the actin fibre, for example, α-actinin and tropomyosin are known to compete for the same binding site, and since this discovery many other actin binding protein have also been shown to compete, such as tropomyosin and cofilin (Drabikowski and Nowak, 1968, Remedios et al., 2002),

Since myosin IIA has also been shown to induce formation of actin bundles as well as influencing the alignment of actin and myosin filaments (Verkhovsky et al., 1995), it is possible that its presence in these stress fibres may not only help to characterise the fibres but may also influence their formation, as with reduced expression of eEF1A, more actin fibres would be free to be assembled into the observed stress fibres, potentially allowing the myosin IIA to induce formation of the actin bundles (Verkhovsky et al., 1995), possibly alongside the other actin binding proteins mentioned before.

To support our hypothesis that the reduced level of eEF1A leads to more availability for other actin binding proteins to bundle actin into the observed stress fibres, it was of interest to first determine if indeed eEF1A would be found to be less associated with the cytoskeleton in the 2E2 cells compared to the control CHO cells. Localisation of eEF1A with the actin cytoskeleton was first performed using immunofluorescence as performed by others (Edmonds et al., 1995). No localisation of eEF1A could be detected either with the cortical actin present mostly in the control CHO cells or with the actin stress fibres present in the 2E2 cells (Figure 3.3.19 A). This could be due to the diffuse nature of the eEF1A protein as well as its interactions with other cytoskeletal proteins such as microtubules. It is possible that any localisation of eEF1A to actin could be masked by this level of diffusion of the eEF1A protein, and therefore more specific, quantifiable methods were carried out.

The total amount of eEF1A associated with the cytoskeleton was also quantified by both a modified ELISA, as well as Western blotting. Cytosolic eEF1A was removed by treatment with triton, which has been shown to remove cytosolic proteins leaving behind the triton-insoluble material which is primarily made of cytoskeletal structures (Kobayashia et al., 1982). The amount of eEF1A was therefore quantified and validated to the amount present in both cell lines before removal of cytosolic eEF1A. It was found that less eEF1A was associated with the

actin cytoskeleton in the 2E2 cells when compared to the control CHO cells by both ELISA (Figure 3.3.19 B) and Western blotting after normalisation to the amount of actin present (Figure 3.3.19 C). This suggests that less eEF1A is indeed found in the triton insoluble material, possibly indicating that less may be bound to the actin fibres or actin bundles in the 2E2 cells when compared to the control CHO cells which provides further evidence to suggest the molecular mechanisms that may be put in place to promote the formation of the ventral actin stress fibres observed in the 2E2 cells.

As well as the question of which actin binding proteins are responsible for this increase in actin stress fibres, there is also a question as to where the stress fibres originate from. Current thinking suggests that they are formed due to 1) increased polymerisation of free G actin into F actin, 2) relocalisation of F actin stress fibres already formed from the cortical actin ring, 3) annealing of smaller F actin fibres into longer fibres or 4) a combination of relocalisation, polymerisation and/or annealing. Consequently, it is possible that in CHO cells, the fibres could be the result of increased polymerisation of F actin fibres from G actin. Though the level of actin protein expression was found to be unchanged between the two cell lines, the actin antibody used for Western blotting does not differentiate between the two forms of actin, therefore changes in the levels of G and F actin cannot be determined using this antibody.

An assay was therefore used in order to establish any differences in the actin pools in the different CHO cells. It was found that there was an increased ratio of G actin to F actin in 2E2 cells suggesting that there is an increase in polymerised actin molecules in the absence of eEF1A. This would rule out the appearance of the stress fibres as a relocalisation of current F actin fibres from the cortical actin ring, as well as the merging of smaller F actin fibres, suggesting that the stress fibres are formed by an increase in actin polymerisation of G actin to F actin. This is unsurprising as bundling of eEF1A has been shown to inhibit polymerisation of actin (Murray et al., 1996), therefore it is possible that the removal of eEF1A from actin would result in fewer eEF1A associated actin bundles and would therefore also remove this inhibitory effect of eEF1A allowing polymerisation of actin to occur. This was further supported by our work using Triton permeabilisation which equally demonstrated a reduction in eEF1A associated with the insoluble material (Figure 3.3.19). Such data further supports the hypothesis that the removal of the 'pool' of eEF1A for actin binding and bundling can provide the opportunity for the other actin binding protein mentioned previously to bind and bundle actin fibres that are now more abundant due to the lack of inhibition of actin polymerisation by eEF1A (Murray et al., 1996).

The Arp2/3 complex and VASP, are both ABPs that bind to the actin filament in different ways but can both affect actin polymerisation. The Arp2/3 complex is involved in both nucleation, as well as capping of actin filaments (Cooper and Schafer, 2000, Winder and Ayscough, 2005), therefore has an important role in regulating the polymerisation of actin filaments. It has also been shown to be involved in the formation of transverse arcs which can merge with two dorsal stress fibres to form a ventral stress fibre (Hotulainen and Lappalainen, 2006). It is therefore possible that Arp2/3 could be involved in the formation of the ventral stress fibres that are formed when eEF1A protein level is reduced. VASP has been shown to have a role as an anti capping protein by antagonising capping proteins at the barbed end of the actin filament (Krause, 2002), and has been shown to be involved in the formation of actin stress fibres in endothelial cells (Price and Brindle, 2000), therefore observing VASP localisation to the newly formed stress fibres was also of interest to determine whether VASP could play a role in the formation of the newly formed actin stress fibres. Both Arp2/3 and VASP have also been shown to bind to vinculin (Brindle et al., 1996, DeMali et al., 2002), which as vinculin has been shown to localise to both ends of the new actin stress fibres (Figure 3.3.7), provides further reason for these two proteins to potentially be affected in the new cell system.

Western blotting and immunofluorescence staining was carried out to determine if there was any difference in expression or localisation of VASP or Arp3 between the two cells lines, however no difference was detected for the former (Figures 3.3.8 and 3.3.9). The expression VASP was not determined, as the antibody used was apparently unsuitable for Western blotting, however the expression of Arp3 was unaffected by the reduced expression of eEF1A in the 2E2 cells compared to the control CHO cells. Based on the previous findings that actin, myosin IIA, paxillin and vinculin were found to have the same protein expression in both cell lines this result was unsurprising. The localisation of VASP was found to be rather diffuse throughout the cytosol, therefore it was impossible to determine any localisation to the fibres, for this reason it is difficult to say with any certainty whether VASP is involved in the formation of these stress fibres (Figure 3.3.8). The localisation of Arp3 showed no difference between the two cell lines, however, as with VASP no specific localisation could be observed within the cell (Figure 3.3.9). As the localisation of VASP or Arp3 to stress fibres could not be observed using this method, further studies on interactions between both VASP and Arp3 would be of interest to analyse any differences between the two cell lines in order to determine whether they play a part in the increased polymerisation of actin in the 2E2 cells, and whether eEF1A could play an inhibitory role in their activation.

Focal adhesions are connected to the ECM by integrin clusters, therefore for the increase in focal adhesions observed in the 2E2 cells compared to the control CHO cells to have an effect on cell adhesion or migration, a change in integrin localisation would be expected to enable the focal adhesions to contact the ECM in order to function. CHO cells are known to express β-1 integrin (Balzac et al., 1994, Jaspers et al., 1994), therefore β-1 integrin protein expression and localisation was observed. Localisation of β-1 integrin was found to be unaffected in either the 2E2 or counterpart control (Figure 3.3.10), however this does not mean that there is no change in other integrin types.

Whilst our data unequivocally demonstrated changes in the number of actomyosin fibres, perhaps coincidently, when eEF1A levels are regulated, it was unclear if remodelling of other cytoskeletal structures could be taking place. Indeed eEF1A has been shown to affect the binding and bundling of microtubules, as well as affecting their stabilization and severing (Moore and Cyr, 2000, Moore et al., 1998, Shiina et al., 1994). In our cell system, the reduced expression of eEF1A observed in the 2E2 cells resulted in no observable difference in either localisation or protein expression of tubulin or vimentin (Figure 3.3.11). These negative results further suggest that the difference in actin and myosin IIA localisation observed in the 2E2 cells are indeed specific to the loss of function of eEF1A and not due to a general collapse of cell integrity.

All of the cytoskeletal changes observed so far in the 2E2 cells show specific alteration of organisational morphology but do not provide any biological consequences of these changes. Ventral stress fibres are considered to be contractile structures under normal physiological conditions (Pellegrin and Mellor, 2007). The localisation of myosin IIA gives the fibres the ability to contract, therefore giving the cell the ability to move (Weber and Groeschel-Stewart, 1974, Tojkander et al., 2011). Ventral stress fibres have also been shown to be important in the retraction of the tail end of the cell, as well as potentially affecting the persistence of the cell by relocating to the front of the cell after detachment at the back (Rid et al., 2005). This increase in ventral stress fibres could therefore have a significant impact upon both cell adhesion and migration.

When cell attachment was monitored, the 2E2 cells were found to attach to the ECM significantly faster than the control CHO cells regardless of the presence of fibronectin coating (Figure 3.3.12). This suggests that the focal adhesions observed at the ends of the stress fibres are likely to be functional.

The presence of actin stress fibres in cells has been shown to impact cell migration both positively and negatively (Tojkander et al., 2012). There is a fine balance between cell attachment and motility, if the cells adhere too strongly then the adhesions at the rear of the cell will not be able to detach therefore impeding cell migration, however if the cells don't attach strongly enough there is not enough contact with the ECM for the cell to pull itself forward via myosin IIA contraction. For this reason it was of interest to determine whether there was any change in cell migration between the two cell lines.

It was found that the reduced expression of eEF1A coincided with a significantly reduced rate of migration in the 2E2 cells compared to the control CHO cells with and without fibronectin (Figure 3.3.13).

The increased speed of migration observed with fibronectin coating in both cell lines when compared to migration without fibronectin is unsurprising as, using fibronectin as the ECM would increase the ligand concentration, which has been shown to affect the migration rate of cells (Palecek et al., 1997). The rate of cell migration is dependent upon a fine balance between the contractility of the cell as well as the strength of cell adhesion to the extracellular matrix. It would be expected that as the concentration of fibronectin was increased past the optimal level the migration rate would start to reduce, and if the migration assay was carried out at varying concentrations of fibronectin a curve would be observed where the migration speed would be optimal at intermediate levels of fibronectin because, at lower levels the rate of migration would be slower due to the cells not adhering as well to the ECM, and at higher concentrations the cells would adhere that tightly that they would also take much longer to migrate (Palecek et al., 1997). In our cell system it would seem that the addition of fibronectin provides a more favourable environment for the cell, and potentially allows greater adherence to the ECM, therefore reducing the rate of cell migration. This can also explain the reduced speed of the 2E2 cells when compared to the control CHO cells, as the 2E2 cells have been shown to have more focal adhesions and therefore presumably more contacts with the ECM.

The 2E2 cells were shown to adhere to the ECM more strongly than the control CHO cells (both in the presence and absence of fibronectin), it is therefore logical to suggest that the increased adhesion of the 2E2 cells to the matrix in this system, may be in part responsible for the impediment in cell migration. The stress fibres observed in the 2E2 cells in much higher numbers than in the CHO cells were classified as ventral stress fibres by their location, the localisation of myosin IIA to the fibre and the localisation of both paxillin and vinculin to both ends of the fibre, therefore the increased strength of cell attachment is unsurprising as the

focal adhesions at the ends of the stress fibres would be highly likely to contribute towards an increased cell adhesion in the 2E2 cells compared to the control CHO cells.

Interestingly, the reduced expression of eEF1A in the 2E2 cells also appears to affect the directionality of migration of the cells compared to the control CHO cells. When observing individual cells migrate in the CHO cell population by observing single cells from the videos, the single cell starts to migrate in one direction, usually straight towards to wound gap with very little deviation, however when observing the individual cells in the 2E2 cell population, they appear to migrate in a less ordered fashion, with much greater deviation in the direction of motion. Though this is currently only an observation, it would be of interest to characterise or quantify this change in persistence or directional migration with migration studies using chemotaxis chambers and differing chemoattractants to enable better understanding of this phenomenon and by observing the orientation of the stress fibres while the cells were migrating.

Ventral stress fibres have been shown to affect rear cell retraction in migrating cells, as well as affecting the persistence by detaching from the rear of the cell and relocalising to attach at the front of the cell, the direction of the cell has then been shown to change according to the direction of the newly localised fibre (Rid et al., 2005). Both fibroblasts and epithelial cells have been shown to assemble stress fibres in response to mechanical tension during wound healing (Pellegrin and Mellor, 2007), and the rate of cell migration has been shown to be linked to the balance between cell attachment and cell contractility. Based on the change in cell migration observed between the CHO and 2E2 cells as well as the apparent change in cell persistence, stress fibres in migrating cells of both cell lines were observed by fixing the cells once they had started to migrate to determine any change in stress fibre number in either cell line and to visualise the orientation of the stress fibres in the 2E2 cells in particular.

It was found that whilst there was no change in the stress fibre number observed in the 2E2 cells during migration, the control CHO cells had a significantly increased number of stress fibres in migrating cells at the front of the scratch, when compared to the stationary cells imaged within the confluent monolayer behind the scratch (Figure 3.3.14 A). Myosin IIA was found to localise to these newly formed actin stress fibres, which is unsurprising as presumably it is the formation of the actin stress fibres that promotes the migration of the CHO cells at the scratch boundary (Figure 3.3.14 B). Both paxillin and vinculin were found to localise to these new actin stress fibres (Figures 3.3.15), allowing them to be characterised as ventral stress fibres, which are known to be contractile and can encourage cell movement as

they are anchored to the ECM at both ends of the fibre allowing the contractility of the fibre to be transferred to the ECM and therefore allow the cell to pull itself forward. This increase in the number of ventral stress fibres in migrating CHO cells would also be likely to help the rear tail retraction of the cell, as well as to help with persistence (Rid et al., 2005).

Though it was impossible to quantify or statistically analyse the orientation of the actin stress fibres, after visual comparison of the orientation of the stress fibres visible in the 2E2 cells with those visible in the control CHO cells, it appears that the fibres in both cell lines orient perpendicular to the direction of the cell movement, but there appear to be more cells along the migrational front with actin stress fibres perpendicular to the direction of the scratch in the control CHO cells when compared to the 2E2 cells, such that more 2E2 cells have fibres oriented with the direction of the scratch as opposed to perpendicular to it. This could provide some insight into why the cells move more randomly, as although the fibres align to the direction of cell migration, this direction does not appear to align perpendicular to the scratch to allow uniform scratch closure. Ventral stress fibres have been shown to affect persistence of cells through the mechanism of rear cell detachment, however, the proteins involved in ensuring that the ventral stress fibres are lined up perpendicular to the direction of motion have not yet been established. Palladin has been shown to affect the persistence of cells (Naumanen et al., 2007), and a wound healing assay on neural cells showed a reduced migration rate in null palladin cells (Luo et al., 2005), which would match nicely to the observed phenotype, including the presence of stress fibres, however this again is only one such protein that could impact this system and without further studies, it is impossible to say which proteins are involved in our cell system.

The Arp2/3 complex is involved in nucleation of new actin filaments, capping fibres and has been shown to be essential for the formation of transverse arcs, which can connect to dorsal stress fibres to form ventral stress fibres, therefore, though there was no difference in Arp3 localisation or protein expression in stationary cells (Figure 3.3.9), it was of interest to determine whether the newly formed ventral stress fibres observed in the migrating CHO cells would lead to a change in localisation of Arp3. This could give further indication as to the formation of the stress fibres in the 2E2 cells, as if localisation was observed with stress fibres in the migrating CHO cells, but no localisation is observed to the stress fibres in the 2E2 cells, it would point to a different regulatory mechanism of formation, however, no difference in Arp3 localisation was observed between either the migrating and stationary CHO or 2E2 cells (Figure 3.3.16).

Beta-1 integrin localisation was also observed through immunofluorescence staining in CHO and 2E2 cells, however no difference was detected (Figure 3.3.10), potentially as the stress fibres and their associated focal adhesion complexes attached to the ECM through a different isoform of integrin. As newly formed stress fibres were observed in migrating CHO cells when compared to stationary CHO cells, it was of interest to determine whether there was a difference in β-1 integrin localisation in migrating CHO and 2E2 cells when compared to stationary cells, which would give an indication as to which integrin isoform the migrating CHO stress fibres were interacting with and so whether they were behaving in the same way as the previously characterised stress fibres observed in the stationary and migrating 2E2 cells. No difference was detected in the migrating CHO or 2E2 cells when compared to either each other or their stationary counterparts, therefore it is likely that the stress fibres observed in the 2E2 cells compared to the control cells attach to the ECM through the same integrin isoform as the newly formed stress fibres in the migrating CHO cells (Figure 3.3.17), though without determining which isoform this is, it is not possible to say for sure that they complex with the same isoform, just that neither complex with β1 integrin.

To establish whether lower levels of eEF1A expression resulted in changes in proliferation rate, different viability assays were performed. Using both the MTT assay (Figure 3.3.3A) and a cell counting assay (Figure 3.3.3B), we found significant changes in growth rate between the two cell lines, with the control CHO cells presenting more rapid growth over 72 hours. Supporting differences in growth were also obtained from flow cytometry analysis where an increase in both apoptotic cells and G phase cells, and a decrease in S phase cells were obtained for the 2E2 cells (Figure 3.3.4). Aberrant eEF1A protein expression levels have been shown to affect the rate of proliferation in both yeast and mammalian cells (Gross and Kinzy, 2005, Pecorari et al., 2009, Anand et al., 2002). In human breast cancer cells, a reduction in eEF1A expression coincides with a decrease in cell proliferation, which was shown to correlate with an increase in the fraction of G1 phase and apoptotic cells and a decrease in the fraction of S phase cells as determined by flow cytometry.

Disruption of the actin cytoskeleton has been shown to induce apoptosis in both yeast and eukaryotic cells (Gourlay and Ayscough, 2005), therefore it is likely that the increase in the rate of apoptosis, as well as the reduced cell growth rate result from changes in the organisation of the actin cytoskeleton caused by the reduced level of eEF1A protein expression.

The actin cytoskeleton has been suggested to be an early modulator of commitment to apoptosis, as well as being a target of the apoptotic process (White et al., 2001). Many different actin binding proteins have also been shown to be biomarkers of the apoptotic process , therefore it seems sensible to suggest the reduced level of eEF1A expression, which coincides with the disruption of the organisation of the actin cytoskeleton and the suggested change in actin binding proteins that associate to the cytoskeleton in the absence of eEF1A, could lead to a change in apoptotic potential.

In order to provide an explanation for these observed phenotypes it must be proved that the change in expression of eEF1A is the sole difference between the two cell lines, and that the phenotypes are therefore eEF1A dependent. By up-regulating the level of eEF1A1 or eEF1A2 in the 2E2 cells, not only can we determine the dependence of the phenotypes on eEF1A but also potentially to determine whether there are any differences in non canonical functions between the two isoforms (Chapter 4).

# Chapter 4:
# Rescue of Phenotypes with Full Length eEF1A1 or eEF1A2

**Chapter 4 Table of Contents**

# 4.0 Rescue of Phenotypes with Full Length eEF1A1 or eEF1A2

## *4.1 Introduction*

The 2E2 cells used in the previous chapter were shown to have around a 40% reduction in eEF1A protein expression when compared to the control CHO cells (Figure 3.3.1). The cells were created by the random insertion of a retroviral promoter trap into the CHO-K1 cells which was found, by 5'RACE and confirmed by PCR, to have inserted into the eEF1A1 gene (Borradaile et al., 2005). Based on the low multiplicity of infection used when the cells were created it is highly unlikely that more than one gene was targeted, therefore eEF1A1 was assumed to be the only isoform affected, and the only gene affected in the 2E2 cells, however it remained to be determined whether all of the phenotypes observed in these cells, were solely due to the low expression of eEF1A. It was therefore essential to prove that increasing the level of eEF1A in the 2E2 cells back to that of physiological levels could restore the observed phenotypes.

It was also of interest to determine whether expression of either isoforms of the human eEF1A protein, namely eEF1A1 and eEF1A2 would equally be capable of restoring the different phenotypes. Human isoforms were chosen as they are almost identical to the hamster isoforms and were more readily available. eEF1A2 has been demonstrated to have the same canonical function in protein translation as eEF1A1 (Kahns et al., 1998), however, numerous studies have suggested that there may be differences in the non canonical functions of the two isoforms. In fact the eEF1A isoforms have in some instances been shown to have opposing effects, for example in apoptosis where eEF1A1 has been shown to have a pro-apoptotic effect whilst eEF1A2 supports a more anti-apoptotic role (Chen et al., 2000, Ruest, 2002). eEF1A2 has also been suggested to act as a possible oncogene, especially where aberrant eEF1A2 expression is observed in tissues that would normally only express eEF1A1 (Anand et al., 2002, Tomlinson et al., 2005, Lee and Surh, 2009), therefore understanding the functions of eEF1A may provide evidence about how eEF1A2 causes such problems.

Using the established CHO system, we aimed to generate both cellular pools and clones of cells expressing either eEF1A1 or eEF1A2. Generating such transgenes would allow to demonstrate the sole role of eEF1A in rescuing the phenotypes reported in the last chapter.

Furthermore the different lines would also provide a invaluable system to examine some of the non-canonical functions of eEF1A1 and eEF1A2 and determine how they could affect the restoration of different biological changes reported.

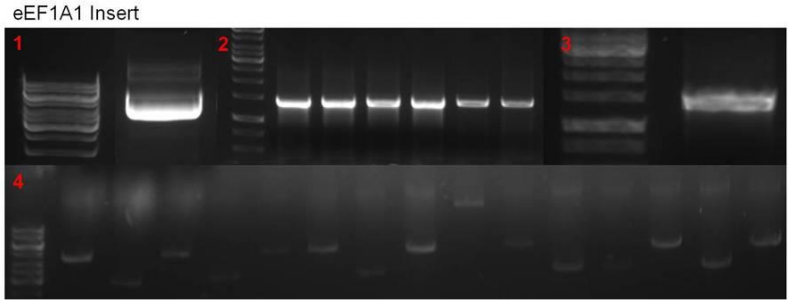
## *4.2 Results*

### 4.2.1 Creation of plasmids expressing human eEF1A1 or eEF1A2

In order to restore the level of eEF1A in the 2E2 cells to establish whether the phenotypes are eEF1A dependent, we first needed to engineer plasmids expressing the different isoforms. The vector backbone chosen for such purpose was the pcDNA3.1 zeo plasmid, given that selection others than G418 needed to be used, since the latter antibiotic had already been used for selection.

Appropriate cloning procedures were followed to first isolate and amplify the open reading frames of both eEF1A1 and eEF1A2. Purification of previously cloned eEF1A1 and eEF1A2 plasmids was first carried out by miniprep (Figure 4.2.1 A1), generating a profile and size that is expected for the vector of interest. Primers were designed for PCR amplification, allowing for addition of a kozak sequence and the restrictions sites XbaI and EcoRI. This produced a large amount of genetic material, consistent with the size expected, migrating at around 1.5kB (Figure 4.2.1 A2) and which was successfully purified further (Figure 4.2.1 A3). The purified PCR produce was then ligated into the cloning vector pST Blue-1, for ease of digestion, and the plasmids transformed into E.coli. The plasmids were then screened to determine which plasmids contained inserts (Figure 4.2.1 A4). The higher bands indicate potential positive plasmids. The pcDNA3.1 zeo backbone was prepared by extracting the plasmid by miniprep (Figure 4.2.1 B1), followed by digestion with EcoRI (Figure 4.2.1 B2), dephosphorylation to prevent 'self' ligation, and purified (Figure 4.2.1 B4). The eEF1A1 and eEF1A2 (not shown) inserts were digested out of the pST Blue-1 vector with EcoRI (Figure 4.2.1 B3), and purified (Figure 4.2.1 B4). The inserts were then ligated into the pcDNA3.1 zeo vector backbone and the plasmids screened for positives (Figure 4.2.1 B5). The potential positives were then extracted by miniprep (Figure 4.2.1 B6) and digested with EcoRI to ensure a fragment was released (Figure 4.2.1 B7). In order to ensure the orientation of the insert was correct, eEF1A1 plasmids were digested with EcoRV, where if a large insert was released, the plasmid should contain the insert with the correct orientation (Figure 4.2.1 B8), and eEF1A2 plasmids were digested with BamH1, where if a small insert was observed the plasmid should contain the insert in the correct orientation (not shown). The plasmids were then sent for sequencing at Birmingham University, and a sequence comparison carried out against the known sequences of eEF1A1 and eEF1A2 (Appendix 6.1 & 6.2).

A




B

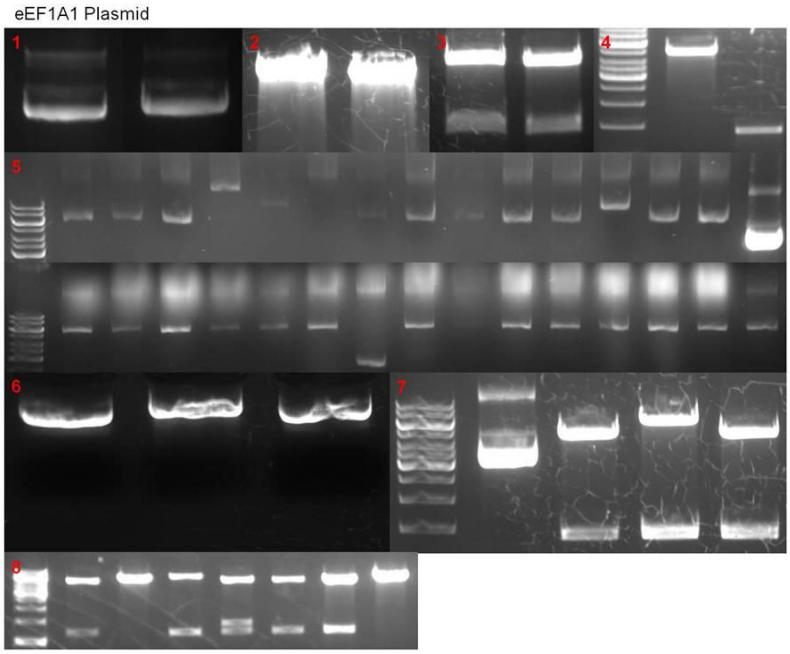



**Figure 4.2.1 –** Creation of eEF1A1 and eEF1A2 expressing plasmids in the pcDNA3.1 zeo backbone vector. **Inserts** - Plasmids containing either the eEF1A1 (SGB51 as described in Table 2.1.4) or eEF1A2 (SGB42 as described in Table 2.1.4) gene were isolated using a miniprep kit (A1), PCR was used to amplify the inserts from plasmids (A2), followed by gel purification from the agarose gel (A3). The inserts were then ligated into the sub-cloning vector pST Blue-1, the plasmids transformed into E.coli and the vectors screened for those containing the insert (A4). **Plasmids** - The pcDNA3.1 zeo plasmid backbone was isolated using a miniprep kit (B1), before digestion (B2) alongside the eEF1A1 or eEF1A2 pST Blue-1 cloning vector plasmid (B3) with EcoRI. The digested pcDNA3.1 zeo backbone was dephosphorylated, and the digested fragments purified using a gel purification kit (B4). The purified digested fragments were then ligated and transformed into E.coli, after which the colonies were screened for potential positives (B5). The positive colonies were grown, the plasmids isolated using a miniprep kit (B6), and digested with EcoRI to ensure that an insert was released (B7). The eEF1A1 plasmid was then digested with EcoRV to ensure correct orientation (B8).

### 4.2.2 Determination of the minimal zeocin concentration sufficient to cause death in both CHO G418 and 2E2 cells

The plasmid created for the expression of eEF1A1 and eEF1A2 in the 2E2 cells, along with the control plasmid to be transfected into the CHO G418 and 2E2 cells, contain the zeocin resistance gene, sh *ble*, the product of which can bind to zeocin to prevent it from cleaving the DNA, and therefore prevent cell death. To select for transfected cells, the concentration of zeocin that can kill wild type CHO and 2E2 cells needed to be established using an MTT assay after growing the cells in different concentrations of zeocin.

Cells were seeded in varying concentrations of zeocin between 0-500 µg/ml, and their effects determined over 96 hours by viability measurement using MTT solubilisation with DMSO, and the absorbance read on a plate reader.

Both CHO and 2E2 cells were found to die in a dose dependent manner, with concentrations above 200µg/ml reducing the absorbance to levels similar to the starting absorbance after 96 hours (Figure 4.2.2). One way ANOVA with Tukeys post test was used to analyse the significance of the values recorded after 96 hours, and significant differences were observed between the 0µg/ml concentration when compared with all others, and the 100µg/ml and 150µg/ml concentrations showed significantly different results compared to some of the higher concentrations, however no significant difference was observed between the 200µg/ml concentration when compared to the higher concentrations. For this reason, though it was found that both 100µg/ml and 150µg/ml did cause cytotoxicity in both CHO G418 and 2E2 cells, the 200µg/ml concentration was deemed to be the most efficient concentration for selection. All significance was measured to a P value of $<0.05$.

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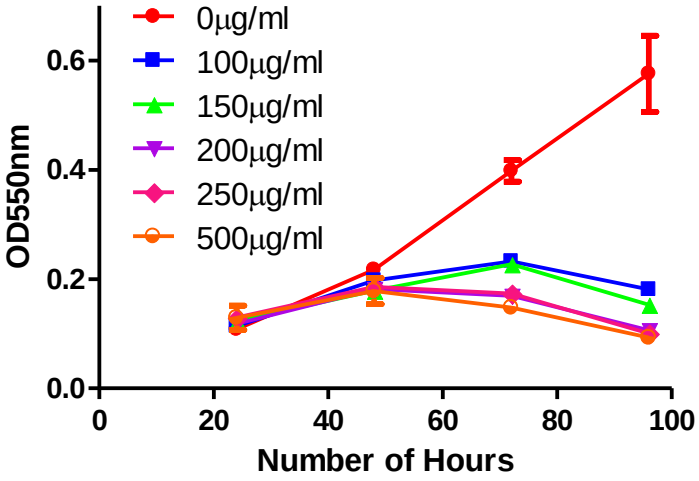


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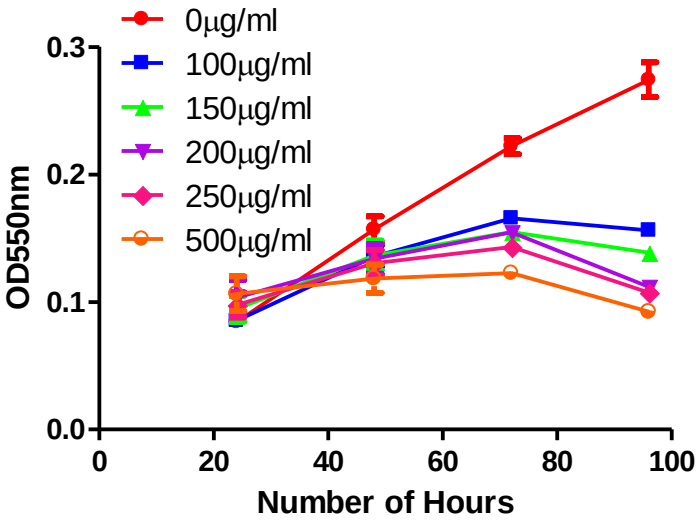


**Figure 4.2.2 –** Zeocin causes cytotoxicity in both CHO G418 and 2E2 cells in a dose dependent manner. 10,000 cells, either CHO (A) or 2E2 (B) were seeded into wells of a 24 well plate in DMEM with 500µg/ml G418 and 0, 100, 150, 200, 250 or 500µg/ml zeocin. The cells were then incubated for 24, 48, 72 or 96 hours, after which MTT was added, solubilised in DMSO, and the absorbance read at 550nm and recorded. One way ANOVA with Tukeys post test was used to determine which concentrations were significantly different, and 200µg/ml was found to be the lowest concentration that was significantly different to the lower concentrations to a P value of <0.05, whilst having no significant difference when compared to the higher concentrations.

### 4.2.3 Reduced expression of eEF1A causes no significant change in transfection efficiency

The reagent chosen for transfection was jetPEI™, which is provided with a manufacturers protocol with optimised conditions for CHO cells, however with the differences already observed in the 2E2 cells compared to the control CHO cells, as well as the control cells being CHO-K1 cells, it was important to first establish the optimal conditions for the two cell lines. A plasmid containing lifeact was used to allow visualisation of the transfected cells.

Cells were seeded onto a fibronectin coated coverslip, before transfection, using jetPEI™ and the lifeact plasmid. The mixture was added to the media, incubated for 24 hours then removed after which cells were fixed, coverslips mounted onto microscope slides, and viewed using fluorescence microscopy.

No significant difference in transfection efficiency was seen between the 2E2 cells and the control CHO cells, with CHO cells showing 23 +/- 9% efficiency, and 2E2 cells showing 32 +/- 12% efficiency (Figure 4.2.3), a yield which should be sufficient to generate stable transfected clones and a pool of cells. Statistical analysis was carried out using an unpaired T test to a P value of $<0.05$.

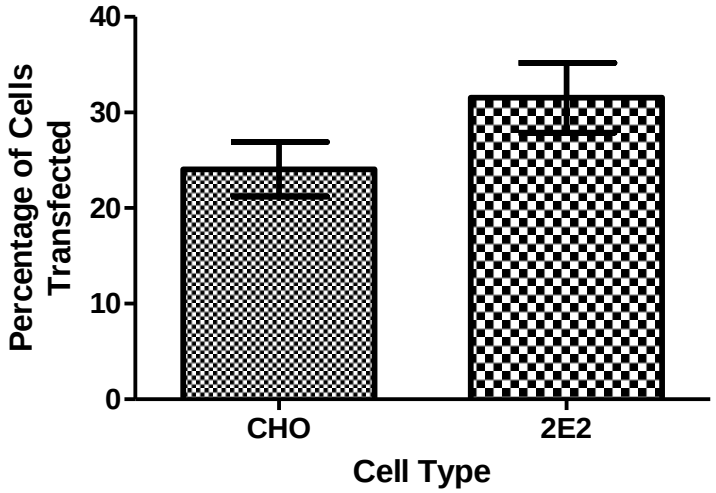


**Figure 4.2.3 –** No difference in transfection efficiency is observed between CHO and 2E2 cells. 50,000 cells were seeded onto coated coverslips and grown overnight. 1.2µg of jetPEI™ was added to 50µl NaCl, and 1µg lifeact plasmid DNA was added to 50µl NaCl. The jetPEI™ solution was added to the DNA solution, mixed and incubated at room temperature for 30 minutes. The solution was then added to the media on the cells and incubated for 24 hours. The cells were then fixed and the coverslips mounted with mounting media containing DAPI onto a microscope slide and viewed by fluorescence microscopy. The number of transfected cells was counted by observation of GFP fluorescent cells, and the percentage of cells transfected determined by comparing this number to the total number of cells counted by using DAPI staining. The average number of transfected cells was plotted with the standard error of the mean and statistical analysis by unpaired T test showed no statistical significance to a P value of $<0.05$.

### 4.2.4 Transfected 2E2 cells show a range of different levels of expression throughout the cell population

After optimisation of the transfection efficiency in 2E2 cells, and determining the optimal concentration for selection of transfected cells, the 2E2 cells were transfected with the eEF1A1 zeo and eEF1A2 zeo plasmids, and selected with 200µg/ml zeocin in order to generate a pool of stably transfected cells expressing different levels of the eEF1A proteins. Immunofluorescence staining for eEF1A was performed to observe the expression of eEF1A throughout the cell population.

Immunofluorescence staining was carried out by seeding cells onto coated coverslips, before fixing, permeabilising, and staining for the eEF1A proteins. The coverslips were then mounted onto microscope slides, and viewed using confocal microscopy.

It was found that, as would be expected from a pooled population of transfected cells, there was a range of different protein expression levels of eEF1A throughout the cell population, and the cells that were highly transfected could be easily identified using this method (Figure 4.2.4).

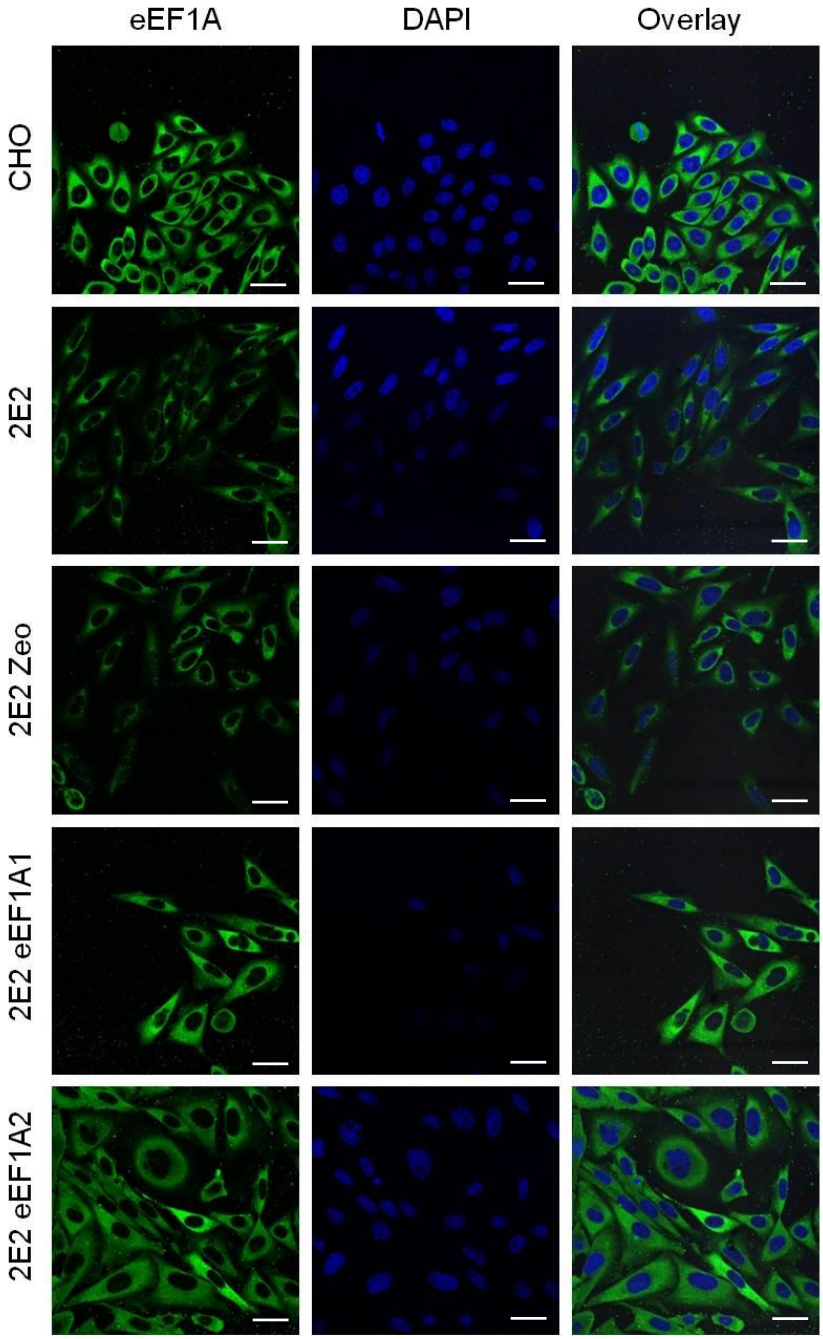


**Figure 4.2.4** – Transfection of 2E2 cells with eEF1A1 and eEF1A2 plasmids leads to a varied expression of eEF1A across the cell population, with both high and low expression of eEF1A being observed in cells. 10,000 cells were seeded, left to grow for 48 hours then fixed, permeabilised and stained for eEF1A with a FITC conjugated secondary antibody. Coverslips were then mounted with DAPI containing mounting media onto microscope slides and viewed using confocal microscopy. White scale bars are equal to 25µM.

### 4.2.5 High expression of either eEF1A1 and eEF1A2 in single 2E2 cells leads to fewer stress fibres

It was observed that the 2E2 cells had an increased number of actin stress fibres compared to control CHO cells, though no difference in actin protein expression was observed (Figure 3.3.5), therefore immunofluorescence staining was used to determine whether transfected cells with a high level of eEF1A1 or eEF1A2 expression showed a restoration in the number of actin stress fibres.

Cells were seeded onto coated coverslips, before fixing, permeabilising, blocking and staining for eEF1A and actin, the coverslips were then mounted onto a microscope slide and viewed using confocal microscopy. Cells with a high level of eEF1A, determined by the level of green fluorescence were observed, as well as cells with a low level of eEF1A, and actin stress fibres quantified by counting the number of actin fibres that passed across the cell in 20 different cells for both high and low expression of both plasmids, and control cells.

It was found that in cells that had a higher expression of eEF1A, as determined by visual comparison, the actin stress fibre number was low, 1 +/- 1.8 per cell for eEF1A1 transfected cells and 2 +/- 2.0 per cell for eEF1A2 transfected cells, similar to control CHO cells with 1 +/- 1.5 fibre per cell, however, in cells that were transfected but had only a low eEF1A protein expression, the number of actin stress fibres was similar to that of the control 2E2 and 2E2 zeo cell lines, with the low eEF1A1 transfected cells showing 10 +/- 4.4 fibres per cell, the low eEF1A2 transfected cells showing 11 +/- 2.5 fibres per cell and the 2E2 and 2E2 zeo cells showing 12 +/- 4.9 and 12 +/- 5 fibres per cell respectively (Figure 4.2.5). This difference between number of actin stress fibres in the highly transfected and low transfected 2E2 cells was found to be statistically significant to a P value of $<0.05$.

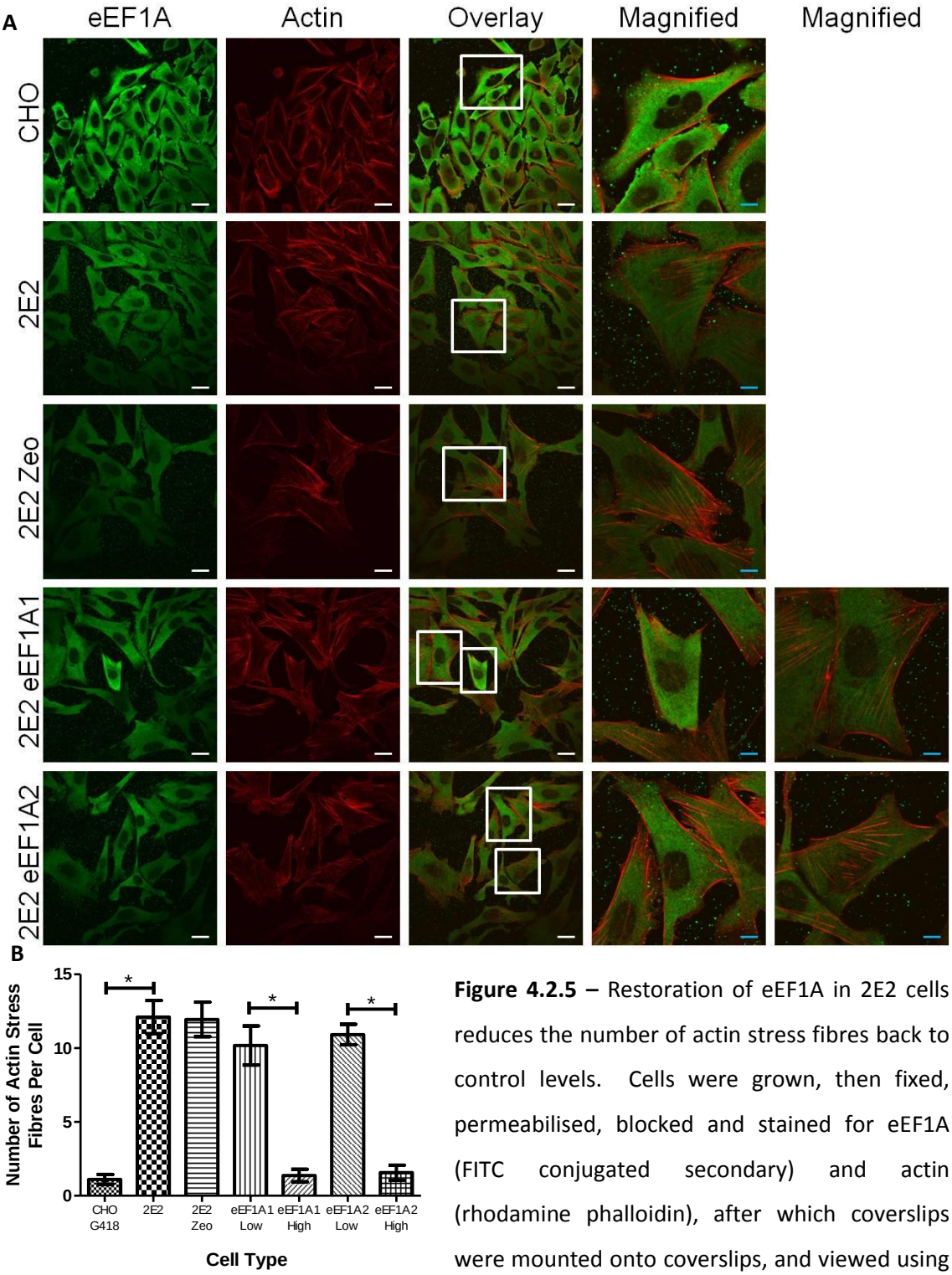


**Figure 4.2.5 –** Restoration of eEF1A in 2E2 cells reduces the number of actin stress fibres back to control levels. Cells were grown, then fixed, permeabilised, blocked and stained for eEF1A (FITC conjugated secondary) and actin (rhodamine phalloidin), after which coverslips were mounted onto coverslips, and viewed using confocal microscopy. Red rectangles show the areas selected for the magnified images (A). Actin stress fibres were quantified by counting the number of actin filaments that passed across the cell in 20 different cells for each cell type and plotting the average with the standard error of the mean (B). Differences were found to be statistically significant with a P value *<0.05 as determined by one way ANOVA with a Tukey post test. White scale bars are equal to 25µM, blue scale bars are equal to approximately 7.5µM.

### 4.2.6 Clonal transfected cell lines show a change in eEF1A protein expression level, with expression varying between different clones

The transfected cells used so far were created as a pool of transfected cells, and could therefore only be used to observe changes in phenotypes in single cells, but as a proof of principle, appeared to provide correlative evidences between high levels of eEF1A expression and number of actin stress fibres. In order to analyse how 2E2 cells with an increased eEF1A protein expression behave as a population, clonal cell lines expressing varying levels of the proteins needed to be established. Generating such cell systems would prove to be an invaluable tool for studying the possible reversions of all phenotypes analysed so far.

2E2 cells were therefore transfected using the JetPEI™ reagent and incubated in selective conditions until cell colonies started to form. A cloning cylinder was placed around the outside of a colony, and the cells within the cylinder detached with trypsin and expanded to create new clonal cell lines. The different cell lineages were grown until large amounts of cellular material could be isolated and characterise further.

Western blotting was used to determine eEF1A protein expression in each clone by lysing the cells and extracting protein. Protein was then loaded onto a gel, run, transferred to nitrocellulose and the blots probed for eEF1A and tubulin, before developing and quantifying using densitometry analysis on ImageJ.

All clones showed an increased expression of eEF1A when compared to the 2E2 cells, with the eEF1A2 clone 1 showing an intermediate level of eEF1A of approximately 80% eEF1A expression, and the other clones, such as eEF1A1 clone 3 showing an eEF1A expression level as high as the control CHO cells at 101%, and some clones showing a higher level of eEF1A expression than the control CHO cells, such as eEF1A1 clone 4, and eEF1A2 clones 3, 4 and 5, which show 116%, 121%, 130% and 157% expression respectively (Figure 4.2.6).

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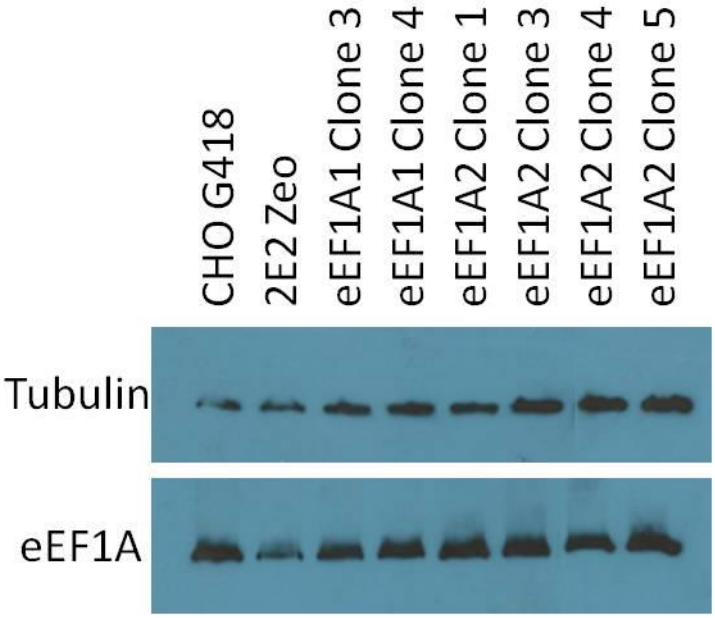


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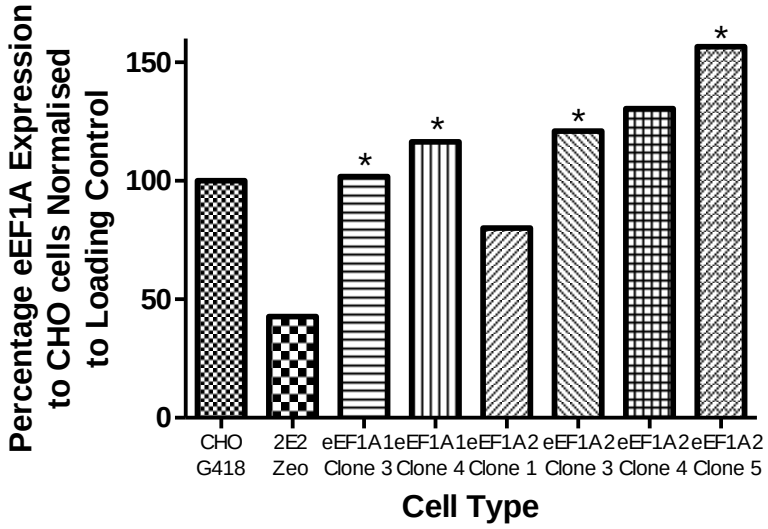


**Figure 4.2.6 –** Clones of transfected 2E2 cells show an increase in eEF1A protein expression at varying levels between clones. Cells were homogenised, protein extracted, quantified, 25µg total protein loaded, and run on a gel before transferring to nitrocellulose. The blot was then probed with eEF1A and α – adaptin or tubulin primary antibodies, and HRP conjugated secondary antibody. Blots were then developed (A) and the intensity of the bands quantified using densitometry analysis on ImageJ (B). The clones chosen for further experimentation are marked with an asterisk (*).

### 4.2.7 Increased expression of eEF1A in clonal cell lines can be seen as consistent throughout the cell population

After analysis of the level of eEF1A protein expression in the transfected clones which showed an increase in eEF1A protein expression in the clonal cell lines (Figure 4.2.6), it was essential to determine that the level of expression was consistent throughout the cell population, and that the clones truly were clones from one single cell. Immunofluorescence staining was used to analyse individual cells within the population.

Cells were seeded onto a coated coverslip, and the cells fixed, permeabilised, blocked and stained for eEF1A, after which the coverslips were mounted onto a microscope slide and viewed using confocal microscopy.

The staining for eEF1A was found to be similar in intensity throughout the whole cell population for all clones (Figure 4.2.7), and the expression level of eEF1A in most clones appeared higher than that of the 2E2 cells, which correlates to the eEF1A protein expression determined by the Western blotting (Figure 4.2.6). From the information gathered from Western blotting and staining it was determined that for further experiments, eEF1A1 clones 3 and 4, and eEF1A2 clones 3 and 5 would be used to analyse rescuing of the observed phenotypes.

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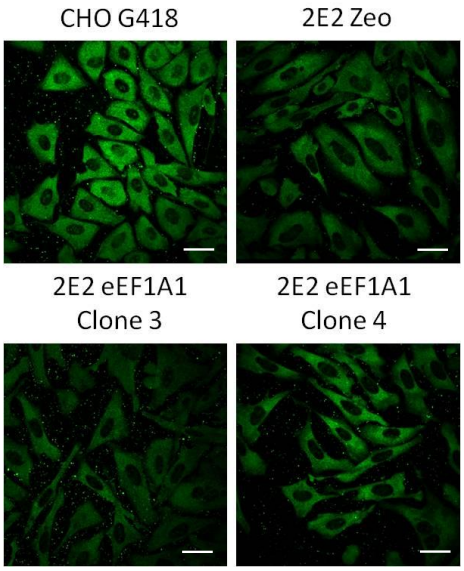


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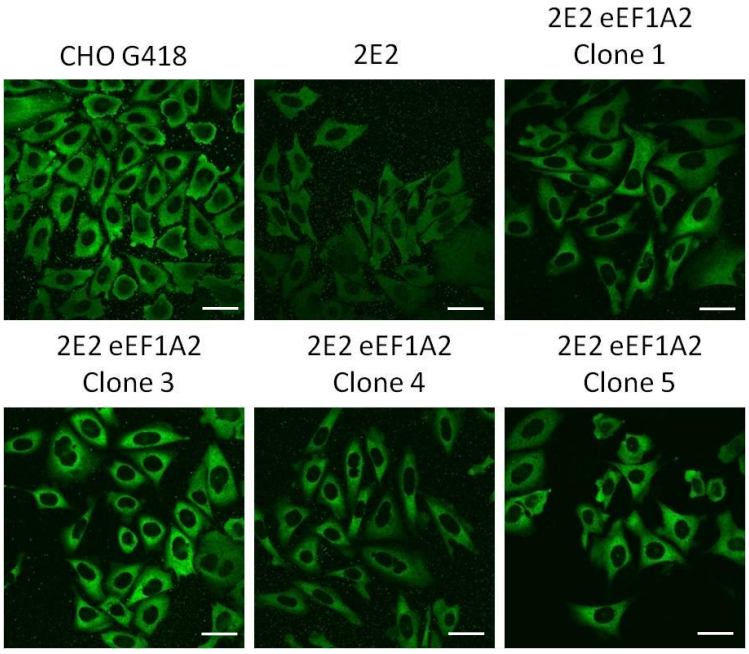


**Figure 4.2.7 –** All transfected cell lines show consistent eEF1A protein expression throughout the cell population. eEF1A1 clonal cells (A) and eEF1A2 clonal cells (B,) were fixed, permeabilised, blocked and stained for eEF1A (FITC conjugated secondary antibody), before mounting coverslips onto microscope slides and viewing using confocal microscopy. Scale bars are equal to 25µM.

### 4.2.8 Increased expression of neither eEF1A1 or eEF1A2 was sufficient to rescue the slow growth phenotype

It was previously shown that the decrease in eEF1A protein level in 2E2 cells compared to CHO cells resulted in a decrease in growth rate by both MTT and cell counting assays (Figure 3.3.3), which was consistent with previous findings by Pecorari et al, 2009. For this reason an MTT assay was used to determine whether restoring the level of eEF1A expression would be sufficient to rescue the previously observed phenotype.

Two clones for each eEF1A1 and eEF1A2 were chosen to determine restoration of the phenotypes. These clones, 3 and 4 for eEF1A1 and 3 and 5 for eEF1A2, were chosen as they gave either the same level of expression of eEF1A, or higher than was observed in the CHO cells. Cells were seeded and left to grow for the appropriate length of time, before MTT was added and solubilised with DMSO and the plate read at 550nm. The MTT assay was carried out in both the presence and absence of selection to ensure that any changes were not caused by changes in selective pressure between the cell lines (data not shown).

No difference in cell growth was determined with clones with increased eEF1A1 or eEF1A2 expression (Figure 4.2.8). The curves of the clones remained similar to that of the 2E2 cells whilst the rate of growth of the CHO cells can be seen to be faster. Statistical analysis by one way ANOVA with Tukeys post test showed a significant difference between the CHO cells and all 2E2 cells after 96 hours, however no statistical difference was detected between the other three cell lines for either eEF1A1 or eEF1A2 after 96 hours to a P value of $<0.05$.

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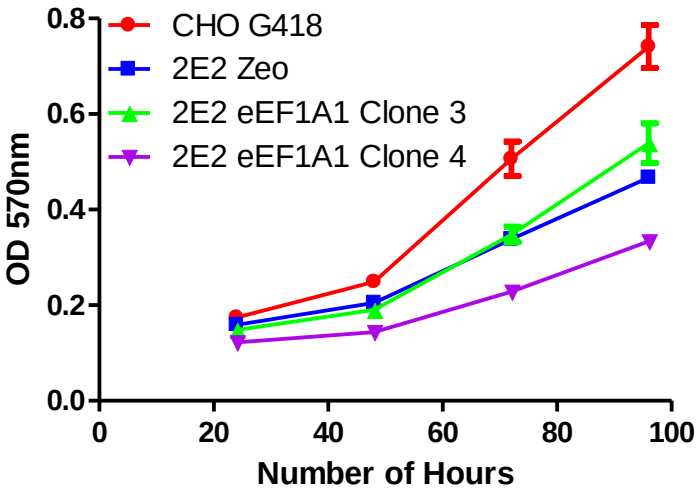


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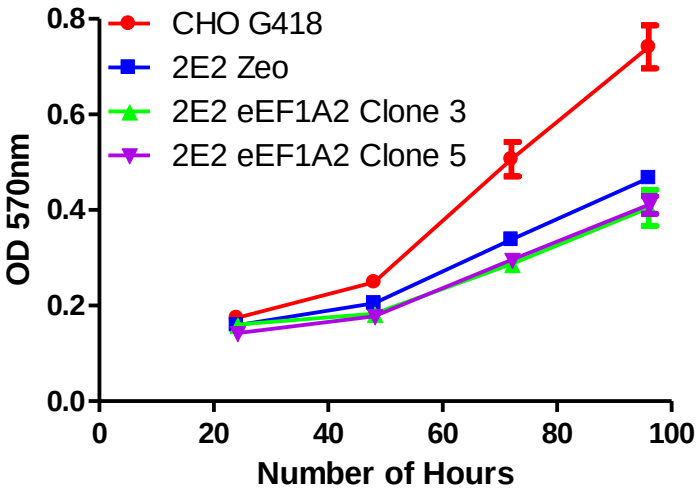


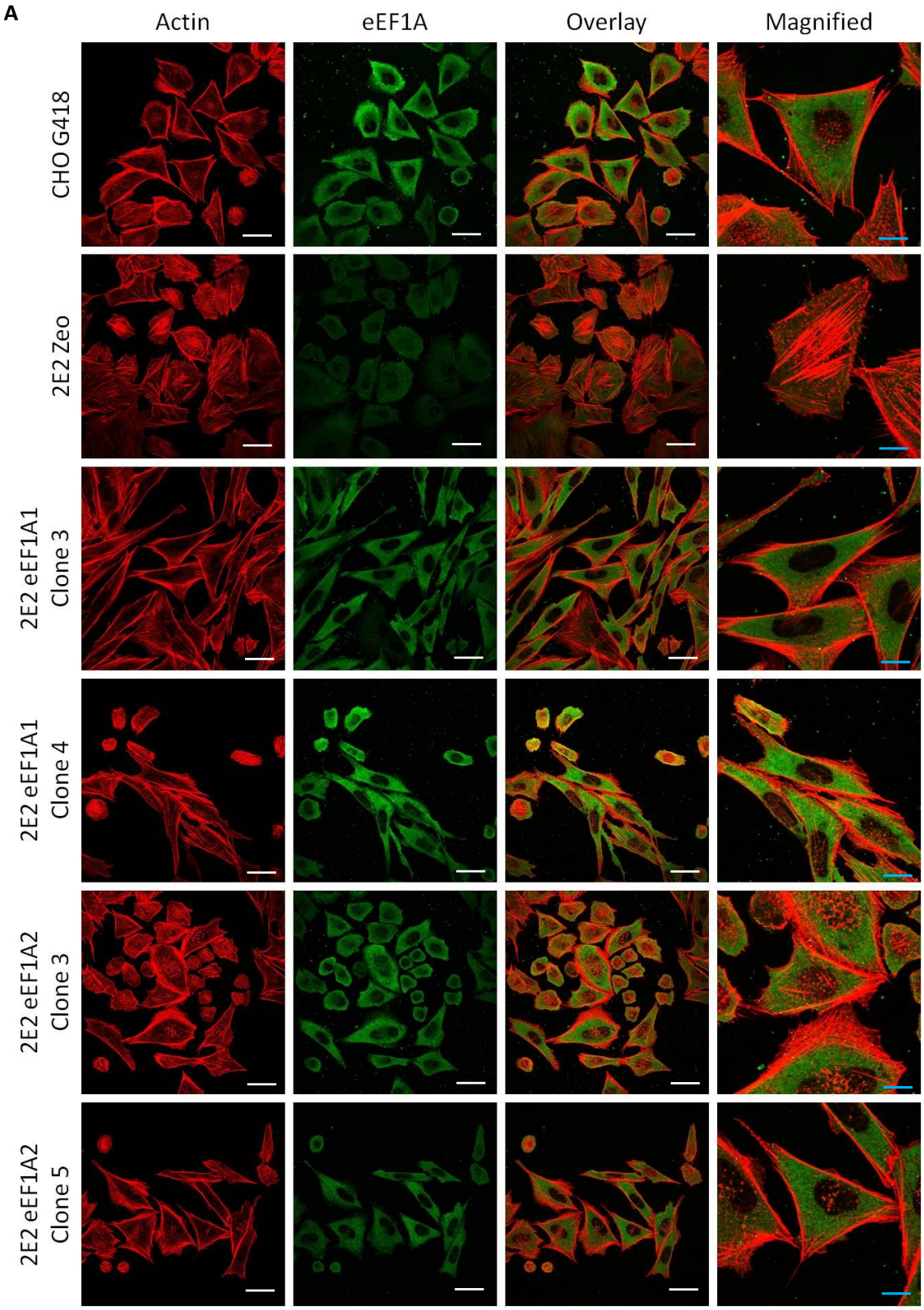

**Figure 4.2.8 –** Increased expression of eEF1A1 or eEF1A2 is not sufficient to restore the growth phenotype. 10,000 cells were seeded and left to grown for 24, 48, 72 or 96 hours, after which MTT added and solubilised, before the absorbance at 550nm was read and recorded. Results were normalised to the CHO cells at 24 hours so that curves could be directly compared (A & B). Statistical analysis was performed using one way ANOVA with Tukeys post test with the 96 hour time point to a P value of <0.05.

### 4.2.9 Restored level of eEF1A results in decreased numbers of actin stress fibres

When actin organisation was observed in 2E2 cells it was found that the decrease in eEF1A resulted in an increase in the number of actin stress fibres compared to the control CHO cells as determined by immunofluorescence staining, however, the protein expression of actin was unaffected (Figure 3.3.5). This change in the number of actin stress fibres was restored when actin localisation was observed in the pool of transfected cells expressing high levels of eEF1A (Figure 4.2.5). By analysing the number of actin stress fibres in the clonal cell lines, the rescue of the phenotype could be further confirmed.

Immunofluorescence staining was carried out by fixing, permeabilising, blocking and staining cells for eEF1A and actin before mounting coverslips and viewing using confocal microscopy. Fibres were quantified by counting the number of actin stress fibres that passed across the cell in 20 random cells from each cell line.

It was found that the transfected clones with increased expression of eEF1A protein had a decreased number of actin stress fibres compared to the control 2E2 zeo cells, with clones showing a similar number of actin stress fibres to that observed in control CHO cells (Figure 4.2.9). Control CHO cells were found to have an average of 3 +/- 2.5 actin fibres per cell, with the eEF1A1 clones having 4 +/- 2.6 and 4 +/- 2.5 and the eEF1A2 clones having 5 +/- 2.9 and 4 +/- 1.5, compared to the 2E2 cells which had an average number of 15 +/- 2.9. These results were found to be statistically significant to a P value of $< 0.05$.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Actin
eEF1A
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Magnified
CHO G418
2E2 Zeo
2E2 eEF1A1 Clone 3
2E2 eEF1A1 Clone 4
2E2 eEF1A2 Clone 3
2E2 eEF1A2 Clone 5

**B**

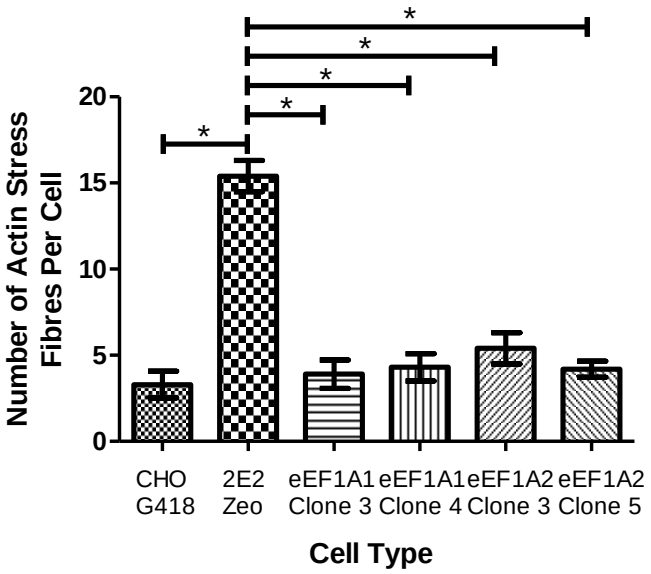



**Figure 4.2.9 –** Increased expression of eEF1A1 or eEF1A2 results in a reversion of the actin stress fibre phenotype. Cells were fixed, permeabilised, blocked and stained for eEF1A (FITC conjugated secondary antibody) and actin (rhodamine phalloidin), before coverslips were mounted onto microscope slides, and viewed by confocal microscopy (A). White scale bars are equal to 25µM, blue scale bars are equal to approximately 7.5µM. Fibres were quantified by counting the number of actin stress fibres that passed across the cell in 20 cells per cell line, with the average number of fibres and the standard error of the mean plotted (B). Statistical analysis was carried out by way of 1 way ANOVA followed by Tukey post test to a P value of *<0.05 as calculated using GraphPad Prism.

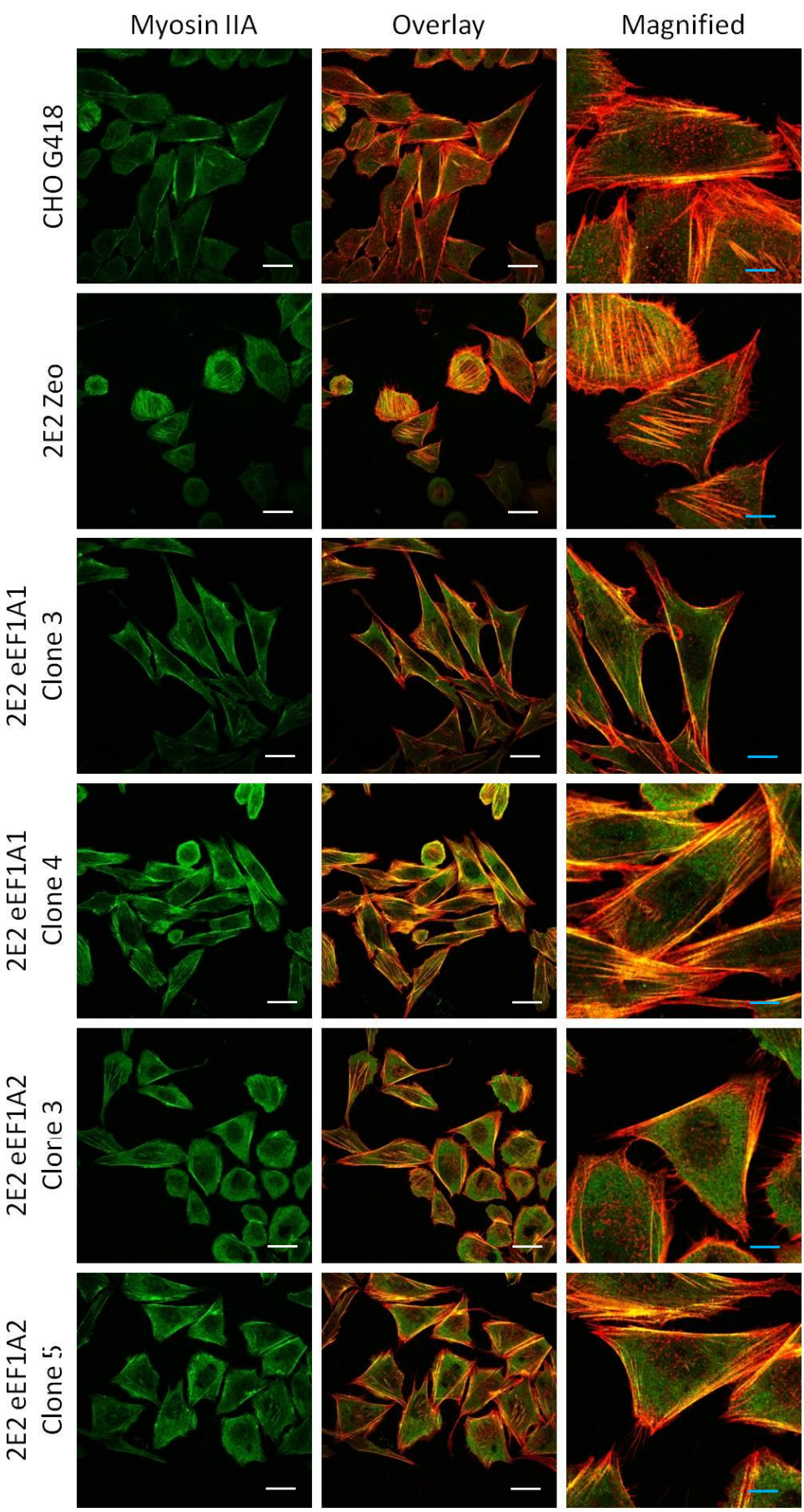
### 4.2.10 Increased level of eEF1A results in a decrease in myosin IIA fibres

Myosin IIA was shown to localise to the actin stress fibres observed in the 2E2 cells, with the number of myosin IIA fibres increased in 2E2 cells when compared to control CHO cells (Figure 3.3.6). Immunofluorescence staining of the clonal cells would determine whether the increase in eEF1A in the 2E2 cells would result in a rescue of the myosin IIA phenotype.

Cells were stained by fixing, permeabilising and blocking cells before staining for myosin IIA and actin. Coverslips were then mounted onto microscope slides and viewed using confocal microscopy, before myosin IIA was quantified by assuming the observed striations to be fibres, and counting the number of fibres that passed across the cell.

It was found that the increase in expression of both eEF1A1 and eEF1A2 in the clones was sufficient to restore the myosin IIA phenotype (Figure 4.2.10). The CHO cells were found to have an average number of 1 +/- 1 myosin fibres, with the eEF1A1 clones showing an average number of 2 +/- 2 and 3 +/- 3, and the eEF1A2 clones showing an average of 6 +/- 2 and 4 +/- 3, compared to the 2E2 cells which showed an average number of 14 +/- 4 myosin fibres. These results were found to be statistically significant to a P value of $<0.05$.

**A**


Myosin IIA
Overlay
Magnified
CHO G418
2E2 Zeo
2E2 eEF1A1 Clone 3
2E2 eEF1A1 Clone 4
2E2 eEF1A2 Clone 3
2E2 eEF1A2 Clone 5

**B**

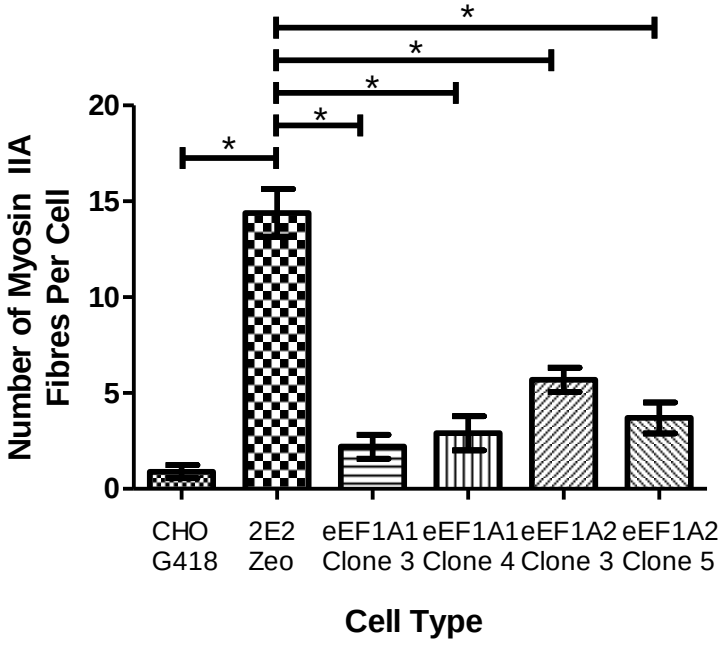


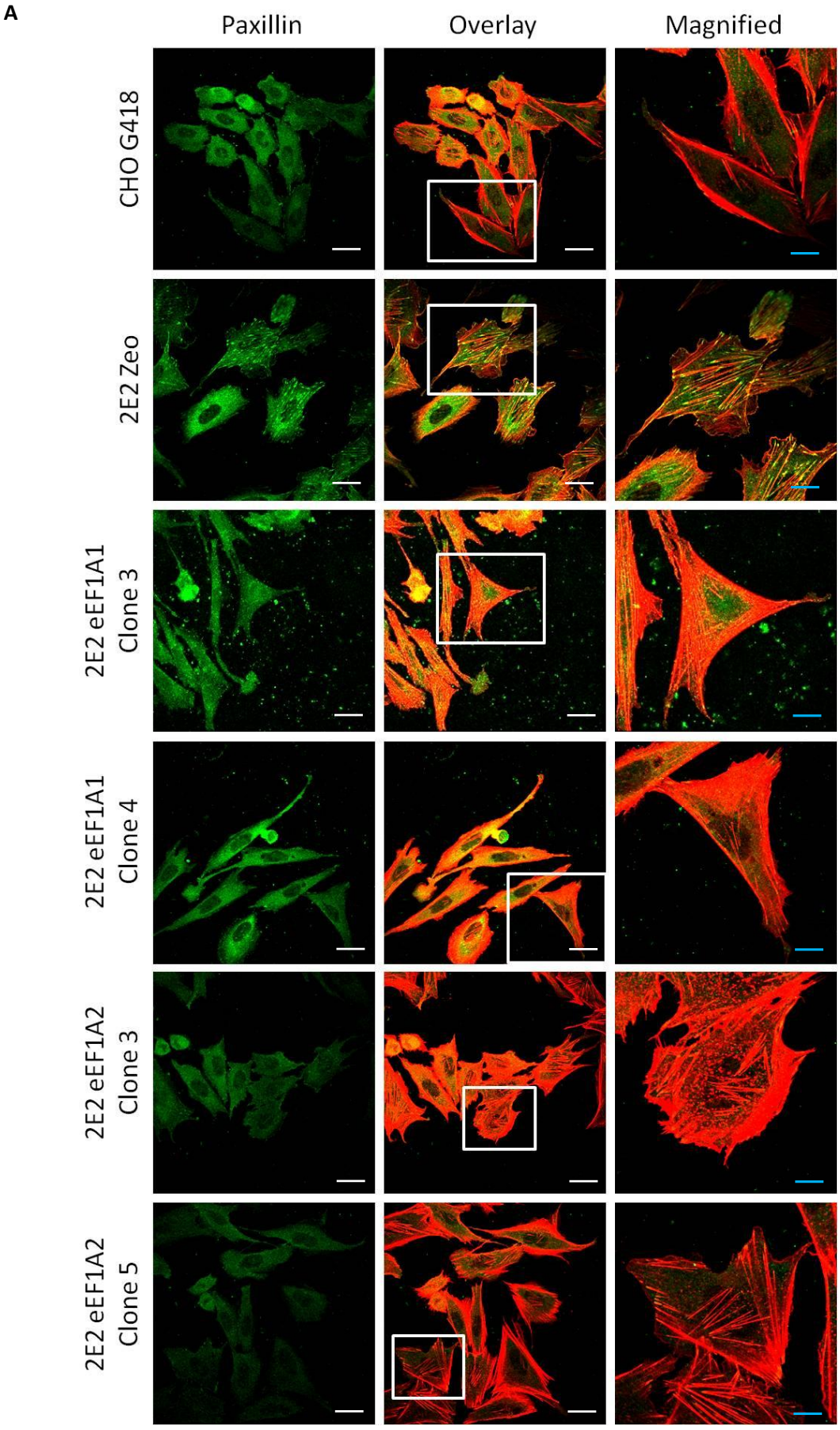

**Figure 4.2.10 –** Increased expression of eEF1A1 or eEF1A2 in clonal cell lines is sufficient to rescue the observed myosin IIA phenotype. Cells were fixed, permeabilised, blocked and stained for myosin IIA (FITC conjugated secondary) and actin (rhodamine phalloidin) before mounting coverslips onto microscope slides and viewing using confocal microscopy (A). White scale bars are equal to 25µM, blue scale bars are equal to approximately 7.5µM. Quantification was carried out by assuming the striations of myosin IIA observed to be fibres, and counting the number that pass across the cell in 20 cells of each cell type, with the average and standard error of the mean plotted (B). Statistical analysis was performed on GraphPad Prism by way of 1 way ANOVA followed by Tukey post test, and results deemed to be significant to a P value of *<0.05.

### 4.2.11 Increased level of protein expression of eEF1A is sufficient to rescue the number of paxillin and vinculin clusters

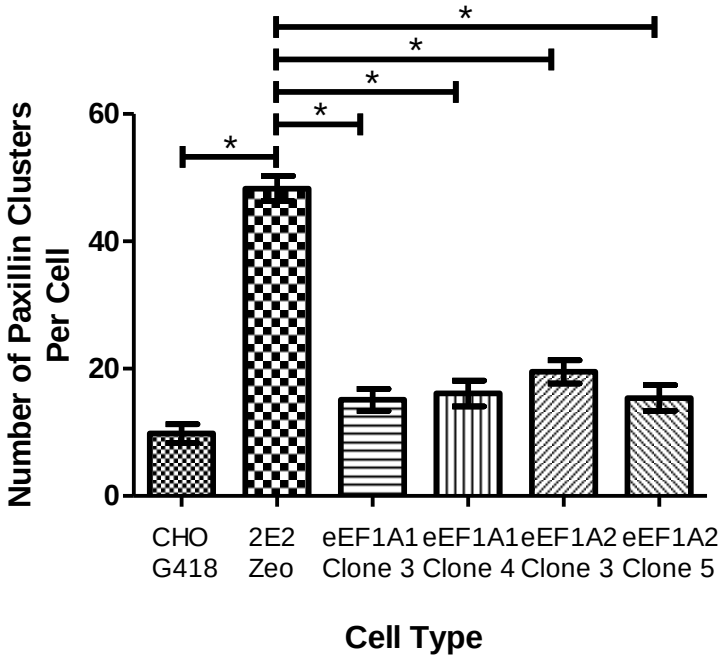
Immunofluorescence staining for paxillin and vinculin in CHO and 2E2 cells showed that when eEF1A protein expression level was decreased, the number of paxillin clusters increased, and localised to the ends of the actin stress fibres that passed through the cell (Figure 3.3.7). By using the clonal cell lines with a uniform expression of eEF1A throughout the cell population, it was possible to determine whether or not the increase in eEF1A1 or eEF1A2 was sufficient to restore the paxillin phenotype.

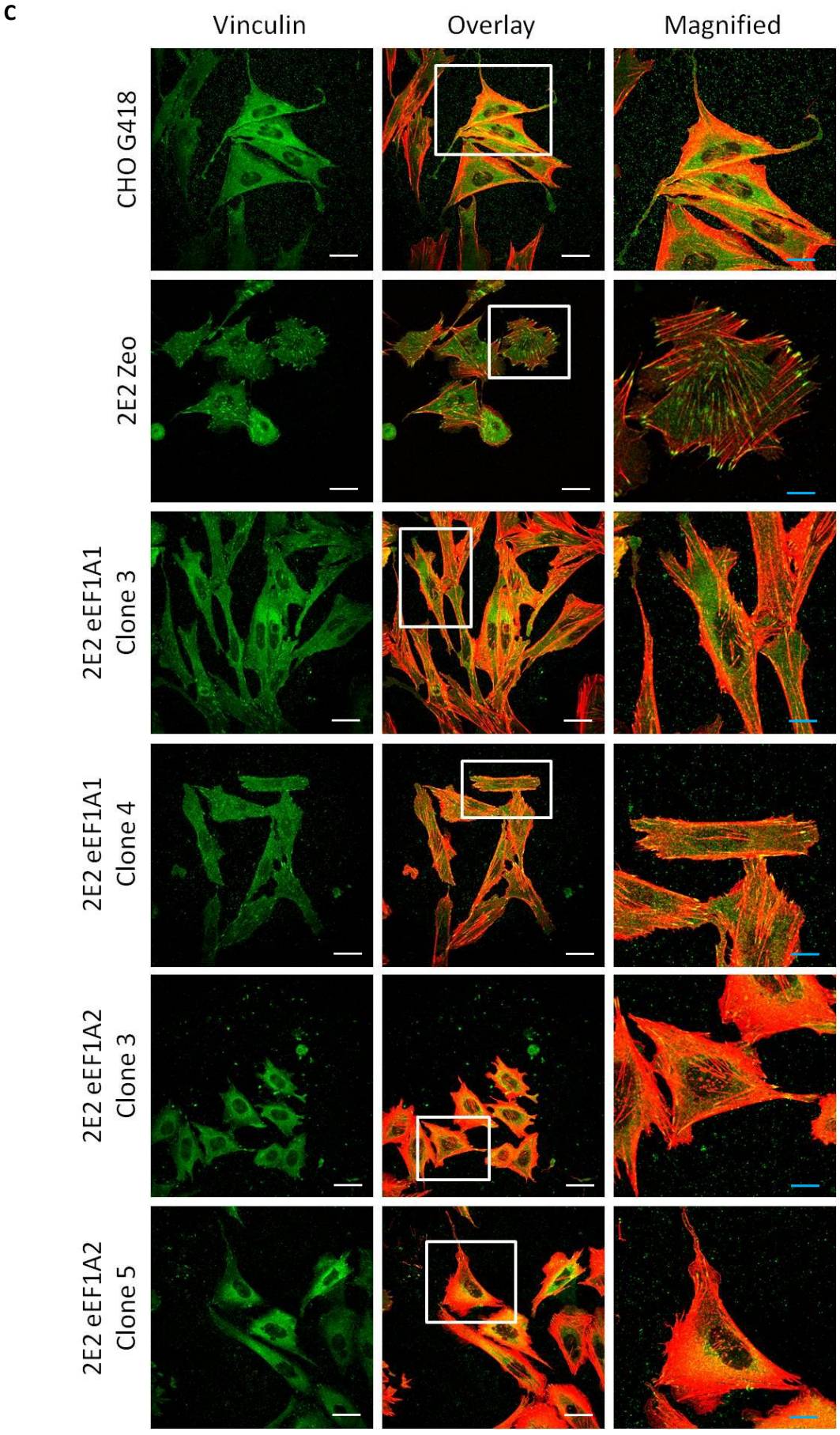
Cells were seeded onto coated coverslips, before fixing, permeabilising, blocking and staining for paxillin or vinculin and actin. The coverslips were then mounted onto a microscope slide and viewed using confocal microscopy. Clusters were quantified by counting the number of clusters per cell for 20 cells or each cell type.

Results showed that the expression of eEF1A1 or eEF1A2 in clonal cell lines was sufficient to rescue the paxillin phenotype, such that 2E2 and 2E2 cells showed an increased number of clusters compared to control CHO cells, but clonal transfected cells show a decreased number of clusters compared to the 2E2 cells, with a similar cluster number to the control CHO cells (Figure 4.2.11) with results shown to be statistically significant to a P value of $< 0.05$. The average number of paxillin clusters in the CHO cells was found to be 10 +/- 5, with the average number of paxillin cluster in the eEF1A1 clones being 15 +/- 5 and 16 +/- 6, and the eEF1A2 clones 20 +/- 6 and 15 +/- 6, compared to the 2E2 cells showing an average number of 48 +/- 6. The number of vinculin clusters was shown to be similar in clonal cells compared to control CHO cells, with the average number of vinculin clusters in CHO cells as 10 +/- 6, with the eEF1A1 clones having an average of 17 +/- 6 and 15 +/- 5, and the eEF1A2 clones having an average of 19 +/- 6 and 15 +/- 6. The 2E2 cells in contrast showed an average vinculin cluster number of 48 +/- 6.


A
Paxillin
Overlay
Magnified
CHO G418
2E2 Zeo
2E2 eEF1A1 Clone 3
2E2 eEF1A1 Clone 4
2E2 eEF1A2 Clone 3
2E2 eEF1A2 Clone 5

B


Number of Paxillin Clusters Per Cell
60
40
20
0
*
*
*
*
*
CHO G418
2E2 Zeo
eEF1A1 Clone 3
eEF1A1 Clone 4
eEF1A2 Clone 3
eEF1A2 Clone 5
Cell Type


c
Vinculin
Overlay
Magnified
CHO G418
2E2 Zeo
2E2 eEF1A1 Clone 3
2E2 eEF1A1 Clone 4
2E2 eEF1A2 Clone 3
2E2 eEF1A2 Clone 5

**D**

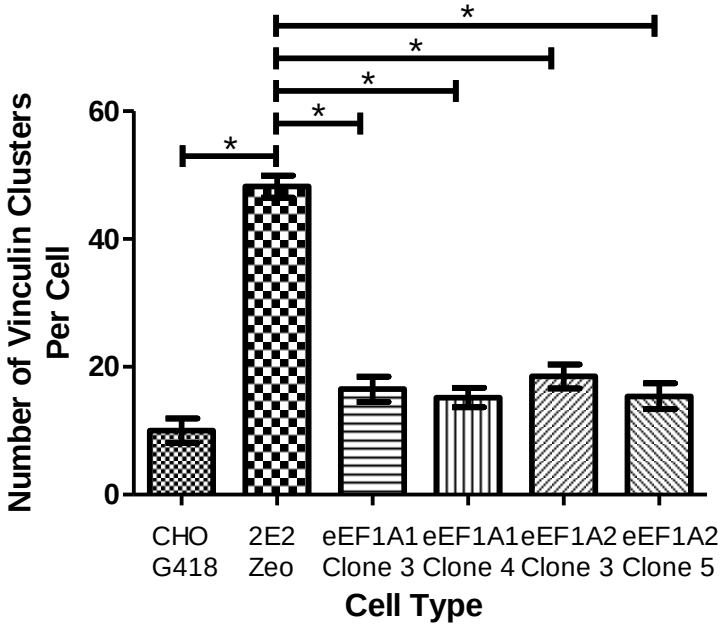


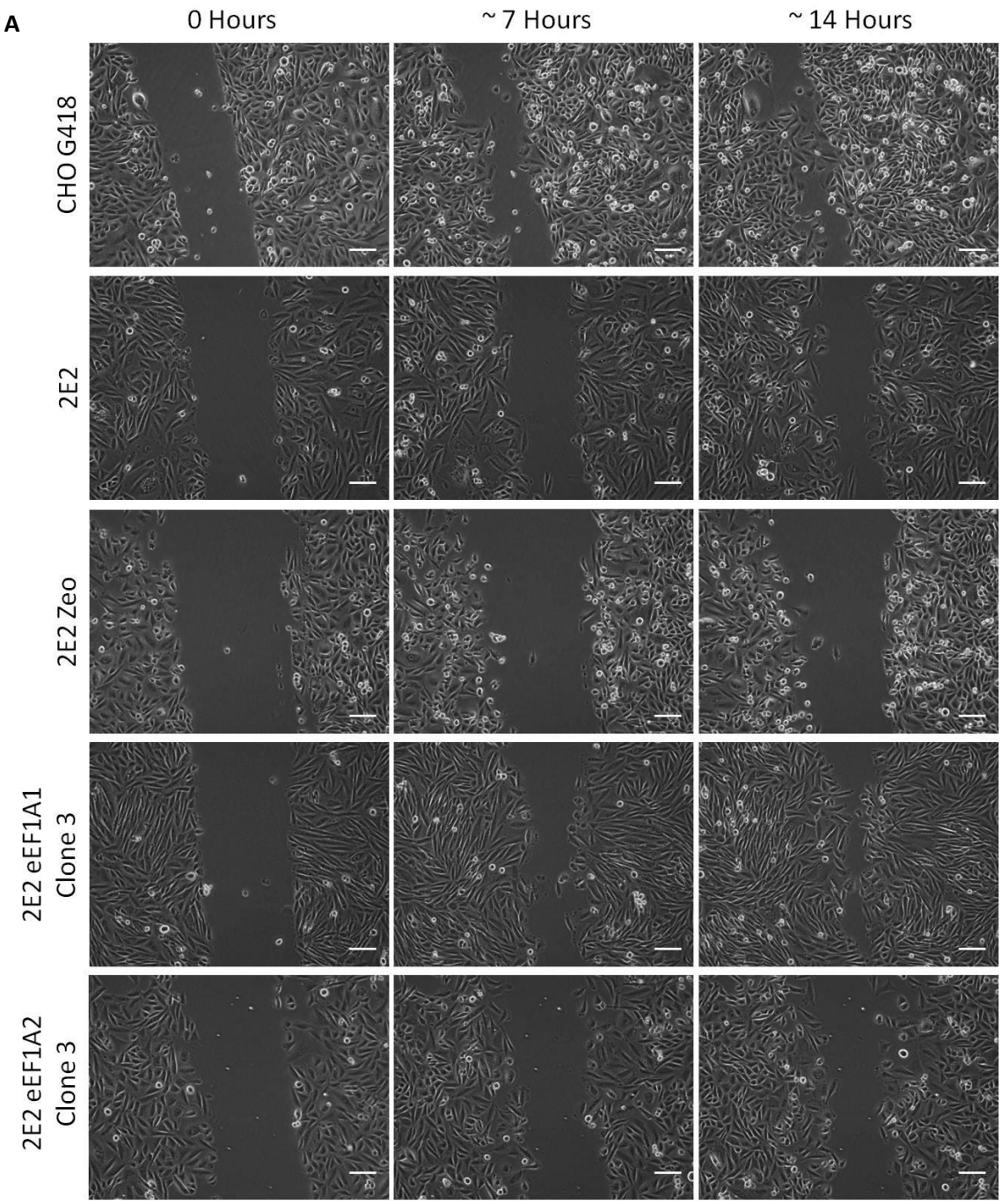

**Figure 4.2.11 –** Increased expression of eEF1A protein in clonal transfected cells results in a restoration of the paxillin and vinculin phenotypes. Cells were fixed, permeabilised, blocked and stained for paxillin (FITC conjugated secondary antibody) and actin (rhodamine phalloidin), before mounting coverslips onto microscope slides and viewing using confocal microscopy (A & C). White scale bars are equal to 25µM, blue scale bars are equal to approximately 7.5µM. Clusters were quantified by counting the total number of clusters per cell for 20 cells of each type, the average number and standard error of the mean plotted, and statistical significance determined using 1 way ANOVA on GraphPad Prism to a P value of *<0.05 (B & D).

### 4.2.12 Increased expression of eEF1A1, but not eEF1A2, resulted in restoration of the migration phenotype,

The reduced eEF1A protein expression in the 2E2 cells compared to the control CHO cells coincided with a reduced rate of cell migration as determined by a scratch assay analysed using the Cell IQ software (Figure 3.3.13), therefore to prove that this was dependent on eEF1A, the scratch assay was repeated on the clonal cell lines.

One clone for each eEF1A1 and eEF1A2 was chosen for the scratch assay. The clones chosen were those which showed the closest level of eEF1A expression to the control CHO cells. Cells were grown to confluence, before being scratched, washed and positions to be imaged selected. Images were taken and then wound closure analysed using the Cell IQ software.

Increased expression of eEF1A1 in clone 3 showed an increased rate of wound closure similar to that of the CHO cells, with the clonal cell line closing after approximately 15 hours compared to 18 hours for the CHO cells and 31 hours for the 2E2 cells, however no change in wound closure was seen in the eEF1A2 clone 3 when compared to 2E2 cells with a wound closure time of 28 hours (Figure 4.2.12).


A
0 Hours
~ 7 Hours
~ 14 Hours
CHO G418
2E2
2E2 Zeo
2E2 eEF1A1 Clone 3
2E2 eEF1A2 Clone 3

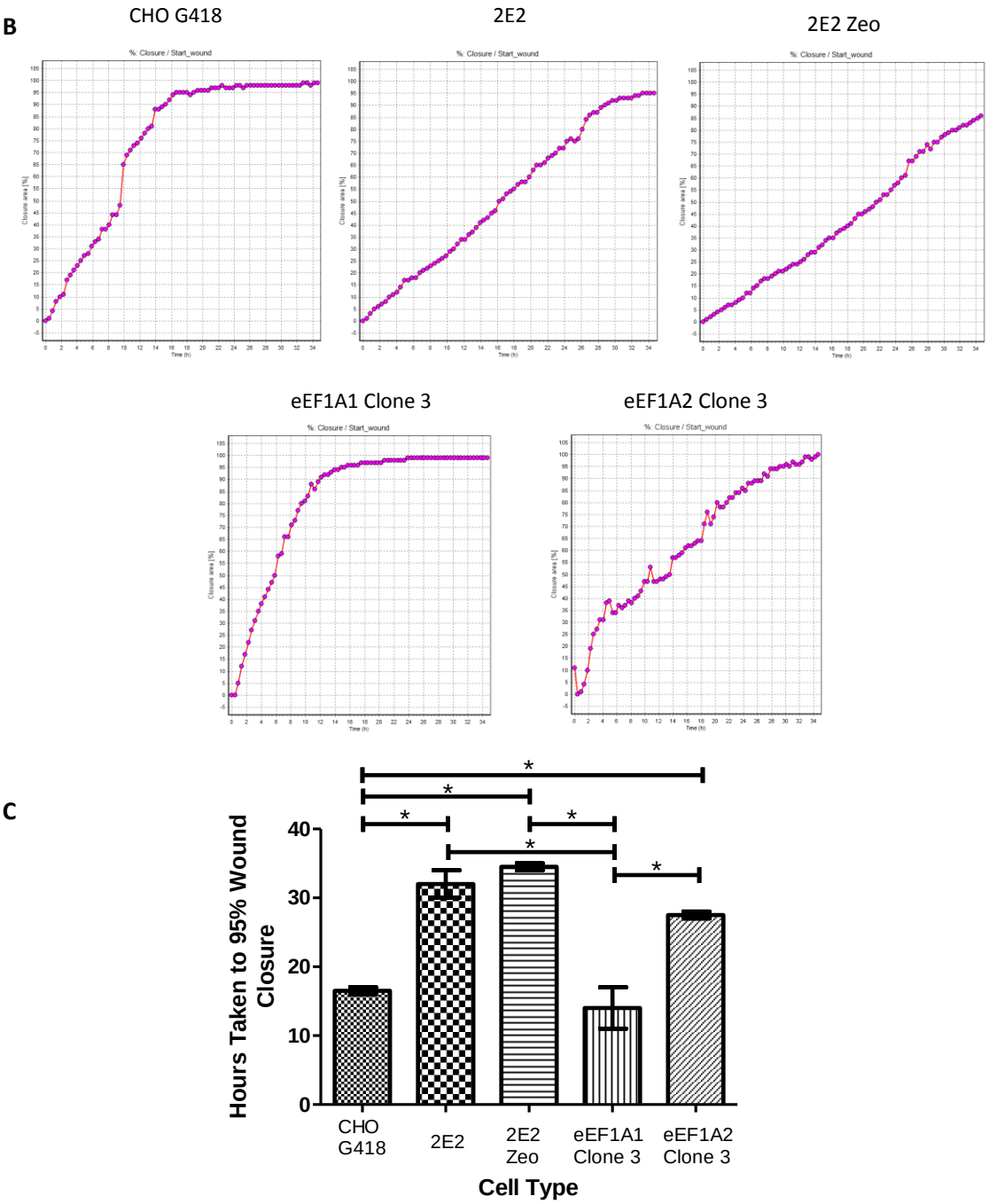



**Figure 4.2.12 –** Increased expression of eEF1A1 is capable of restoring the rate of cell migration, however increased expression of eEF1A2 is not sufficient. Cells were grown until confluent after which a scratch was made through the confluent monolayer with a yellow pipette tip. The cells were then washed and media replaced. The plate was loaded into the Cell IQ machine and three different points in each of the three replicate scratched were selected and images taken (A). Scale bars represent 50µM. The threshold of the scratch was then set, and the average percentage wound closure recorded over time (B). The average times at which each point reached 95% closure were recorded and plotted, along with standard error of the mean (C).

## *4.3 Discussion*

To restore the level of eEF1A in the 2E2 cells, a plasmid that would allow sufficient eEF1A expression and selection of transfected cells was created (Figure 4.2.1). The pcDNA3.1 zeocin plasmid was used as the 2E2 cells already had G418 resistance following a retroviral promoter trap insertion, therefore this plasmid should allow both sufficient expression and selection. To determine the concentration of zeocin required to achieve cell death, both CHO and 2E2 cells were grown in varying concentrations of zeocin and an MTT assay used to determine cell viability (Figure 4.2.2). All concentrations used, 100-500 µg/ml caused cell death, however the 200µg/ml zeocin concentration was the lowest concentration that was found to cause significant death after 24 hours, with the cell viability reduced to that of the starting $OD_{540nm}$ after 96 hours, therefore this concentration was deemed to be the least stressful concentration that would cause sufficient death to select cells that had been transfected.

Zeocin causes cell death by cleaving the DNA resulting in double strand breaks. The 200µg/ml concentration is in line with other studies using zeocin for selection of CHO-K1 cells which have used a selection concentration between 150-200µg/ml (Satoh et al., 2005, Vanblokland et al., 2007).

The transfection efficiency of the CHO and 2E2 cells was then determined using the standard protocol to ensure maximal transfection efficiency in each cell line (Figure 4.2.3). Both cell lines were found to have a similar efficiency, consistent with the conditions recommended for this cell type therefore the standard protocol for transfection was followed. This was important to establish as the function of eEF1A in integration of transfected genes is unknown therefore it is possible that reducing the level of eEF1A could affect transfection efficiency. Based on the similarity on transfection efficiency between the two cell lines it seems that eEF1A protein expression does not affect this integration.

After transfection and selection of the 2E2 cells, a pool of transfected cells was created which showed a varied eEF1A protein expression throughout the cell population (Figure 4.2.4). This varied pattern of expression of the transfected gene is common as the level of protein expression is dependent upon where the transfected gene integrates into the host cell genome. This varied protein expression allows for clones of differing expression levels to be achieved. The pool of transfected cells was a useful tool to observe cells expressing differing levels of eEF1A within the cell population by immunofluorescence which would allow a

correlation to be observed between eEF1A protein level and stress fibres (Figure 4.2.5), however, phenotypes such as migration and cell growth require cells expressing a homogenous protein level in order to observe any potential rescuing. For this reason, clonal cell lines were also created and showed a homogenous eEF1A protein expression throughout the cell population (Figure 4.2.7). Both the pool of transfected cells and the clones provided valuable tools to observe whether the phenotypes were restored with an increased level of eEF1A.

2E2 cells with a reduced level of eEF1A protein expression were found to have a significantly increased number of actin stress fibres (Figure 3.3.5), to which myosin IIA was found to localise (Figure 3.3.6). The focal adhesion proteins paxillin and vinculin were found to localise to both ends of the stress fibres (Figure 3.3.7), allowing them to be characterised as ventral stress fibres. With the pool of transfected cells allowing cells with both high and low eEF1A protein expression levels to be observed, co-staining of eEF1A and actin was carried out. It was found that the cells with increased eEF1A1 or eEF1A2 expression had a reduced number of actin stress fibres, with the number similar to the control CHO cells, and the cells with a low level of eEF1A1 or eEF1A2 were found to have a similar number of actin stress fibres to the 2E2 cells (Figure 4.2.5), indicating that the increased expression of either eEF1A1 or eEF1A2 is sufficient to restore the actin stress fibre number. This was further confirmed by immunofluorescence staining of the clonal cell lines which proved that the increased expression of both eEF1A1 and eEF1A2 was sufficient to restore the level of actin stress fibres (Figure 4.2.9).

Immunofluorescence of both eEF1A1 and eEF1A2 in clonal cell lines were found to result in the restoration of the myosin IIA phenotype with the number of myosin IIA fibres in the clonal cell lines being similar to that of the control cells (Figure 4.2.10). Using the clonal cells it was also determined that the both the paxillin and the vinculin phenotypes were also rescued, with the number of clusters in the 2E2 transfected clones being similar to that observed in the control CHO cells (Figure 4.2.11).

This rescue of the actin, myosin IIA, paxillin and vinculin phenotypes proved that the increased number of ventral stress fibres in the 2E2 cells was due to the reduced expression of eEF1A protein, and also that both eEF1A1 and eEF1A2 are capable of restoring these phenotypes. Though eEF1A1 and eEF1A2 have been suggested to have different non canonical functions, this result is unsurprising as both isoforms have been shown to have the ability to affect the actin cytoskeleton (Amiri et al., 2007, Doyle et al., 2011, Edmonds et al., 1995). It is therefore

possible that as suggested before, the reduced expression of eEF1A results in an insufficient amount of eEF1A protein that can bind to the actin fibre in order to prevent other actin binding proteins from causing rearrangement of the actin cytoskeleton. When eEF1A level is then boosted back up by transfection of the eEF1A1 or eEF1A2 plasmids, the eEF1A protein level is once again sufficient to bind actin in such a way that other actin binding protein are excluded (Owen et al., 1992).

In order to determine whether the migration phenotype observed could be rescued by eEF1A1 or eEF1A2, clonal cells had to be used. The pool of transfected cells was not suitable for this analysis as only a small percentage of cells were highly transfected, therefore any change in migration would have been undetectable from the overall population of cells. Once clonal cell lines had been isolated and expanded, the eEF1A expression level was detected by Western blotting (Figure 4.2.6), and uniform expression was determined by immunofluorescence staining (Figure 4.2.7). From these expanded clonal cell lines, two clones were chosen for both eEF1A1, clones 3 and 4, and eEF1A2, clones 3 and 5. These clones were chosen as they gave either the same level of expression of eEF1A, or higher than was observed in the CHO cells. For the migration assay, only one clone was chosen, which was decided based upon which of the two clones had the closest expression level to that of the control CHO cells.

It was found that eEF1A1 clone 3 was able to rescue the observed phenotype (Figure 4.2.12). This is unsurprising as the migration phenotype observed is likely to be due to the disruption of the actin cytoskeleton, therefore by restoring cytoskeletal organisation would result in restoration of the migration speed, however contradictory to this theory is that the eEF1A2 clone was not able to rescue the migration phenotype in spite of showing the ability to rescue the disruption to the actin cytoskeleton. This would therefore imply the possibility that eEF1A1 but not eEF1A2 could be capable of affecting cell migration independently of the observed change in the actin cytoskeleton. This supports the theory that the two isoforms carry out the same function in protein translation but have different non canonical functions, and could also explain the increased association of eEF1A with metastatic cancers (Pencil et al., 1993, Edmonds et al., 1996). The mechanism for how this may be regulated is unknown and would require further studies into the downstream effectors of the pathway.

eEF1A has been shown to be able to significantly increase the rate of actin polymerisation, therefore it could be possible that under normal conditions, the polymerisation of actin seen at the leading edge of the cell to form the filopodia and lamellipodia is controlled by eEF1A to regulate the speed and direction of migration. In our situation, where the level of eEF1A

protein expression has been reduced by such a significant amount, the level of eEF1A may not be sufficient to regulate this actin polymerisation in order to produce the structures required for cell migration at the same speed as when it is present in abundance. This could explain why the rate of cell migration is reduced in cells that have a reduced expression of eEF1A, but also why the migration phenotype is only rescued by eEF1A1 even though the actin stress fibres phenotype is rescued by both eEF1A1 and eEF1A2. It provides a mechanism by which eEF1A1 could regulate cell migration independently of its role in actin binding and bundling.

It was found that neither the eEF1A1 or eEF1A2 clones were sufficient to restore the cell growth defect observed (Figure 4.2.8). In chapter 3 this change in cell growth rate was suggested to be due to the disruption of the actin cytoskeleton which, as the actin cytoskeleton is essential for proper regulation of cell proliferation and cell cycle progression, would be a sensible suggestion, however the lack of restoration would seem to contradict this. A potential explanation for this would be that, although care was taken to ensure that only one gene was affected by the retroviral insertion when the cells were created, it is possible that more than one gene in the cells was disrupted, and this may have caused the change in cell proliferation observed, hence why it was not rescued by the increased level of eEF1A1 or eEF1A2 in the clones. The link between eEF1A expression and cell growth rate has been demonstrated in the past by various other studies, therefore it is also possible that the phenotype is eEF1A dependent, however the level of eEF1A in the clones is not sufficient to restore the phenotype as the species of eEF1A used to restore the level of eEF1A was not mouse but human, therefore overexpression of this isoform may be needed to provide the same effect. Although the level of eEF1A expression in the clones is similar to that of the control cells, it may be that, as the translational role of eEF1A along with the actin organisational role are likely to be the most highly conserved, it may take an increased level of expression in order to restore the proliferation phenotype. It would make sense for eEF1A to be a regulator of cell growth independently of actin organisation as, from the results achieved in this chapter, it would appear that eEF1A1 and eEF1A2 have similar actin binding activity, however, eEF1A1 is usually expressed in rapidly dividing cells, in comparison to eEF1A2 which is usually expressed in terminally differentiated cells which divide very slowly. This could implicate eEF1A directly in the regulation of cell growth by affecting the progression of the cell through the cell cycle, indeed, eEF1A has been shown to affect the cell cycle not only in this study (Figure 3.3.6), but also in other studies (Pecorari et al., 2009), as well as being implicated in cellular senescence (Byun et al., 2009, Cavallius et al., 1986b). Clones which overexpress eEF1A1 and eEF1A2 to levels higher than that observed in the control CHO cells would further

confirm whether the level observed is simply not high enough to provide the rescue of this phenotype, or whether the phenotype is not eEF1A dependent but caused by some other change in the cells used.

# Chapter 5:

# Reduction of eEF1A Protein Levels in Yeast and its effects on cellular processes

**Chapter 5 Table of Contents**

# 5.0 Reduction of eEF1A Protein Levels in Yeast and its effects on cellular processes

## *5.1 Introduction*

The primary function of eEF1A is in protein translation, however, eEF1A is present in a 17-35 fold molar excess compared to the other components of the translational machinery (Slobin, 1980). eEF1A has also been demonstrated to affect actin cytoskeletal organisation (Chapter 3 Figure 3.3.5) as well as many other cellular functions including microtubule binding, bundling and severing, apoptosis, and protein degradation (Shiina et al., 1994, Moore et al., 1998, Moore and Cyr, 2000, Duttaroy et al., 1998, Chen et al., 2000, Gonen et al., 1994, Chuang et al., 2005). Work has been done to show that overexpression of eEF1A can disrupt the actin organisation of the cell without affecting the rate of protein synthesis (Munshi et al., 2000, Gross and Kinzy, 2005) suggesting that these functions are independent.

The theory that there may be different 'pools' of eEF1A conserved for different functions is something that has been suggested, however not investigated. Indeed it has been shown that the intracellular pH can affect the affinity of eEF1A to actin, with the affinity decreasing as pH increases, which could potentially allow for an increased localised concentration of eEF1A that is freely available to interact with tRNA for protein synthesis as pH increases providing a potential mechanism for how eEF1A can carry out these two roles in an independent manner (Edmonds et al., 1995, Liu et al., 1996b). The role of the actin cytoskeleton in the regulation of protein translation has been hypothesised (Kim and Coulombe, 2010), and based on the regulatory function of eEF1A on the organisation of the actin cytoskeleton, as well as the apparent mutual exclusivity of binding of eEF1A to actin and tRNA which can be controlled by changes in the local pH, eEF1A is a very likely candidate for the link between the two cellular functions.

Our work so far has shown, in mammalian CHO-K1 cells, that a reduction of eEF1A level to around 50% of its normal content, leads to significant changes in cytoskeletal reorganisation and overall growth among other characteristics. We set about to further establish how growth, viability and other biological processes would be affected with varying reduced levels of eEF1A (from no expression to physiological levels) in the easy amenable yeast organism, Saccharomyces cerevisae. Using this model system we sought to determine whether

fluctuations in eEF1A protein level would lead to specific phenotypic changes in actin organisation and protein translation. Due to its very amenable genetics, different strategies for reducing the level of eEF1A in yeast are available including knock down using siRNA, gene knock out and transformation of plasmids with different copy numbers and/or transfection of plasmids with differing promoters into deletion strains, each with their own advantages and disadvantages. siRNA knock down should provide a range of different levels of eEF1A expression, however this method is not only expensive, but also time consuming, as a plasmid encoding the genes for Ago2, Dicer and TRBP has to be transformed into the cells before siRNA can be performed to allow it to be processed (Suk et al., 2011). Given that eEF1A is so abundant, to knock down the level of eEF1A to a sufficient level to affect translation by this method would be unlikely, therefore siRNA was deemed impractical to create the varying levels of eEF1A required for our purpose.

A very useful tool to enable manipulation of eEF1A expression levels in yeast was created by Sandbaken and Culbertson, 1988, by creating a strain in which both chromosomal copies, TEF1 and TEF2 were disrupted such that they were no longer functional, with the only copy of eEF1A found on an episomal plasmid (Sandbaken and Culbertson, 1988), providing us with an invaluable tool to use different plasmids to regulate the level of this protein.

The level of protein expression from a plasmid is dependent upon both the copy number and the promoter used. The copy number of a plasmid refers to the number of copies that are present in the host cell, therefore usually, the higher the copy number of the plasmid, the higher the level of protein expression controlled by the plasmid, however it has been found that there is an inverse link between plasmid copy number and plasmid stability, such that high copy number plasmids are less stable and therefore provide less stable phenotypes than those with a lower copy number (Futcher and Cox, 1984). For our purpose, a wide range of different protein expression levels were required, therefore as changing the copy number of the plasmid had limitations for both stability and the range of protein expression achievable, an alternative method was used.

Varying the promoter can create a big change in the level of expression of the protein of interest. Glycolytic promoters such as glyceraldhehyde-3-phosphate dehydrogenase (GAP) and alcohol dehydrogenase 1 (ADH1) are the strongest promoters in Saccharomyces *cerevisiae* and though initially thought to be constitutively active, were found to be induced by the addition of glucose (Romanos et al., 1992). The CYC1 promoter has been found to be a weak promoter, and the TEF promoter a strong promoter (Mumberg et al., 1995), therefore

transforming plasmids with different promoters such as the GAP, ADH1, TEF1 or CYC1 promoter, into a strain can result in differential expression of the protein of interest. The level of protein expression under the weaker promoter may also still be too high in order to achieve the desired end result. It has also been shown that constantly high ectopic expression of some proteins can be detrimental to the cell and therefore cause unwanted side effects (Weinhandl et al., 2014).

To achieve a wide range of expression levels, plasmids containing inducible or repressible promoters rather than constitutive promoters can be used, of which there are many different types available including the tetracycline, glucose/galactose and copper systems. The glucose/galactose system is the most commonly used, which causes an increase in expression of the protein of interest in the presence of galactose and a decrease in the expression in the presence of glucose which is regulated by the binding of the GAL4 and GAL80 proteins to the upstream activation sequences (UASs) (Romanos et al., 1992), however this system requires drastic changes to the growth media that could potentially affect the characteristics of the cell, and therefore lead to misleading phenotypes. The tetracycline system relies on addition of differing concentrations of tetracycline to the media to alter the expression of the protein of interest by regulating the binding of activator or repressor molecules to the tetO promoter (Bellí et al., 1998), however the limitation of this system is the presence of the antibiotic which could negatively affect cellular characteristics. Both of these regulatable promoter systems have been shown to allow tight regulation of the expression of the protein of interest. Further to these systems of regulatable promoters, the glucose repressible alcohol dehydrogenase 2 (ADH2) promoter has been shown to be able to affect the copy number of the plasmid when inserted in front of the CEN3 sequence, enabling the carbon source to affect expression of the protein of interest by affecting the copy number of the plasmid (Chlebowicz-Śledziewska and Śledziewski, 1985). Another regulatable system also available is the copper repressible system which was the system chosen for this study.

The copper repressible system is based on the CTR1 gene which encodes a membrane associated copper transport protein, the expression of which is repressed in the presence of copper, and increased during copper starvation (Labbe et al., 1997). The regulation of expression is dependent on two copper responsive cis acting elements, and controlled by a copper sensing transcription factor called Mac1, therefore the CTR1 promoter region was cloned into a plasmid with variable cloning sites to allow insertion of the gene of interest in order to study the differential expression of that gene under the control of the copper

repressible promoter (Labbe and Thiele, 1999). For our purposes the eEF1A coding sequence from the TEF1 gene was inserted downstream of the copper repressible promoter and the newly generated plasmid transformed into a yeast strain where both genomic copies of eEF1A, namely TEF1 and TEF2 had been removed providing us with a cellular organism where the amount of copper in the media should control the level of eEF1A transcription. This strategy was deemed most logical to allow a range of different eEF1A protein expression levels to be achieved consistently, with changes in expression occurring very rapidly and controlled by adding either bathocuproine disulphonate (BCS), a copper chelator, or copper sulphate ($CuSO_4$).

This chapter therefore focuses on carefully downregulating the expression of eEF1A in Saccharomyces *cerevisae*, with the aim to determine if the biological consequences of the regulated withdraw/reduction of the expression on different cellular process.

## *5.2 Materials and Methods*

### 5.2.1 Materials Used for Yeast Work

### 5.2.1.1 Yeast plasmids and Strains Used

**Table 5.2.1.1.1 Yeast Plasmids Used**

| Name | Description |
|---|---|
| **Plasmids** | |
| TKB561 | Cu414 expression vector CEN TRP1 $Cu^{2+}$ repressible |
| TKB579 | His tagged TEF1 into TKB561 digested with BamH1 |

**Table 5.2.1.1.1 –** Yeast plasmids used to create the yeast strain used.

**Table 5.2.1.1.2 Yeast Strains Used**

| Name | Description |
|---|---|
| **Strains** | |
| TKY102 | Strain with disrupted TEF1 and TEF2 genes with episomal TEF1 to maintain viability |
| TKY622 | An elongation defective yeast strain |
| SGY5 | TKY102 transformed with TKB579 after removal of TEF1 URA with FOA |
| TKY102 & TKB561 | TKY102 transformed with TKB561 |

**Table 5.2.1.1.2 –** Yeast strains used to create SGY5 strain and control strains.

## 5.2.1.2 Antibodies Used for Yeast Work

**Table 5.2.1.2 Antibodies Used for Western Blotting**

| Name of Antibody | Secondary Antibody Used | Source | Dilution |
|---|---|---|---|
| **Western Blotting – Primary Antibodies** | | | |
| eEF1A | Rabbit | | 1 in 10,000 |
| EF2 | Mouse | | 1 in 20,000 |
| **Western Blotting – Secondary Antibodies** | | | |
| Anti- Mouse | - | Sigma Aldrich | 1 in 5,000 |
| Anti- Rabbit | - | Sigma Aldrich | 1 in 5,000 |

**Table 5.2.1.2 –** Primary and secondary antibodies used for all Western blots for this chapter, including dilutions, and where they were purchased from.

## 5.2.2 Methods for Yeast Work

### 5.2.2.1 Creation of SGY5 strain

#### *5.2.2.1.1 Isopropanol Precipitation of Plasmid*

Bacteria containing the plasmid to be purified were grown from a -80°C stock in LB amp (Table 5.2.2.1.1) overnight in a shaking incubator at 37°C at 250 rpm. The bacteria were pelleted by centrifugation at 3,000 rpm for 5 minutes at room temperature, before the supernatant was removed, and the pellet resuspended in 250µl Qiagen solution P1 (Table 5.2.2.1.1). Cells were then lysed by adding 250µl Qiagen solution P2 (Table 5.2.2.1.1), and neutralised by adding 350µl Qiagen solution P3 (Table 5.2.2.1.1), before transferring to an eppendorf tube and centrifuging at 14,000 rpm for 5 minutes at room temperature. Supernatant was transferred to a new eppendorf tube, and an equal amount of isopropanol added. The tube was vigorously mixed, before transferring to the -80°C freezer for 10 minutes, followed by the -20°C freezer for 20 minutes, after which the tube was put in the centrifuge at 13,000 rpm for 20 minutes at 4°C. The supernatant was removed, and the pellet washed with ice cold 70% (v/v) ethanol, before drying the pellet and resuspending in 50µl of distilled water. Some of the purified plasmid was then mixed with 5x loading dye (Table 5.2.2.1.1), run on a 1% (w/v) agarose gel as described in section 2.2.13.1, and viewed using a UV box, to ensure that the plasmid was present, with the rest stored in the fridge until needed.

**Table 5.2.2.1.1 Composition of Buffers Used for Plasmid Extraction**

| Name of Solution/Buffer | Composition of Buffer |
|---|---|
| LB Amp | 20g LB powder<br>Made to 1L with distilled water<br>Autoclaved<br>Once cooled, 1ml 100mg/ml Ampicillin added |
| Qiagen Solution P1 | 1ml 10mg/ml RNase A<br>5ml 1M Tris pH 8.0<br>2ml 0.5M EDTA<br>Made to 100ml with Distilled Water |
| Qiagen Solution P2 | 10ml 2M Sodium Hydroxide<br>10ml 10% SDS<br>Made to 100ml with Distilled Water |
| Qiagen Solution P3 | 29.5g Potassium Acetate<br>Made to 100ml with Distilled Water<br>pH to 5.5 with Glacial Acetic Acid |
| 5x DNA Loading Dye | 250mM Tris pH 6.8<br>40% (v/v) Glycerol<br>4% (w/v) Sodium Dodecyl Sulphate (SDS)<br>4% (v/v) 2-Mercaptoethanol<br>Bromophenol Blue to Colour |

**Table 5.2.2.1.1 –** Composition of solutions used for growth of bacteria and extraction of plasmids

### *5.2.2.1.2 Transformation of Yeast Strain*

Yeast strain TKY102 was grown in 10mls YEPD (Table 5.2.2.1.2) overnight to an optical density at 600nm ($OD_{600}$) of between 0.3-1. Cells were centrifuged at 2,000 rpm for 5 minutes at room temperature, before the supernatant was discarded, the pellet resuspended in 5mls LiAc/TE (Table 5.2.2.1.2), and pelleted again by centrifugation under the same conditions, whilst the salmon sperm DNA (ssDNA) was boiled for 5 minutes, after which it was put onto ice. The supernatant from the cells was discarded, and the pellet resuspended in 1ml LiAc/TE, before the following was added to an eppendorf tube;

12µl ssDNA

10µl Plasmid DNA (Dependent on the concentration of the preparation)

100µl Yeast

1ml of 40% (w/v) PEG (Table 5.2.2.1.2) was then added and the tube inverted and tapped to mix, before placing in a 30°C incubator for 30 minutes. The cells were then placed at 42°C for 10 minutes to heat shock, before centrifugation at 3,000 rpm for 5 minutes. The supernatant was then removed, and the pellet resuspended in 100µl sterile distilled water, after which the cells were spread onto a c-trp plate (Table 5.2.2.1.2) and placed in the 30°C incubator until colonies appeared.

**Table 5.2.2.1.2 Composition of Buffers Used for Yeast Transformation**

| Name of Solution/Buffer | Composition of Buffer |
|---|---|
| YEPD | 20g Peptone<br>10g Yeast Extract<br>Made to 900mls with Distilled Water<br>Autoclaved<br>20g Glucose made up to 100mls with Distilled Water<br>Glucose Solution Sterile Filtered to make 1L |
| LiAc/TE | 5mls 1M Tris pH 7.5<br>5.1g Lithium Acetate<br>1ml 0.5M EDTA<br>Made up to 500mls with Distilled Water<br>Autoclave |
| 40% Polyethylene Glycol (PEG) | 5mls 1M Tris pH 7.5<br>5.1g Lithium Acetate<br>1ml 0.5M EDTA<br>200g PEG<br>Made up to 500mls with Distilled Water<br>Autoclave |

| | |
|---|---|
| c-trp Plates | 1.7g Yeast Nitrogen Base – Amino Acids<br>5g Ammonium Sulphate<br>20g Glucose<br>20g Agar<br>1.26g c-trp Drop Out Mix<br>Made to IL with Distilled Water<br>Autoclave<br>Pour into Petri Dishes |

**Table 5.2.2.1.2 –** Composition of solutions used to transform plasmids into yeast strains.

### *5.2.2.1.3 Removal of TEF2-Ura3 Plasmid from Transformed Strain*

Several colonies from the plate of transformed yeast were taken and transferred to a fresh c-trp plate, creating a square patch of cells for each colony, before plates were placed into the 30$^{o}$C incubator to grow. Once grown the cells were replica plated using sterile velvet fabric onto a c-trp and a 5-FOA plate (Table 5.2.2.1.3), and placed into the incubator at 30$^{o}$C to grow. The cells growing on the 5-FOA plate were then replica plated onto a fresh 5-FOA plate, and again placed into the 30$^{o}$C incubator, before finally being replica plated onto a c-trp plate and left to grow in the 30$^{o}$C incubator. Cells were then streaked onto a c-trp or YEPD plate to use as a stock plate. For long term storage the new SGY5 strain was frozen by growing the strain in 10mls c-trp, before taking 1ml of yeast and adding 0.5mls 60% (w/v) glycerol, and placing into the -80$^{o}$C freezer.

**Table 5.2.2.1.3 Composition of 5-FOA Plates**

| **Name of Solution/Buffer** | **Composition of Buffer** |
|---|---|
| 5-Fluoroorotic Acid (5-FOA) Plates | 1.7g Yeast Nitrogen Base – Amino Acids<br>5g Ammonium Sulphate<br>20g Glucose<br>20g Agar<br>1.3g Complete Amino Acid Mix<br>Make up to 700ml with Distilled Water<br>Autoclave<br>Dissolve 1g 5-FOA in 300mls Distilled Water<br>Filter Sterilise 5-FOA into Mix<br>Pour into Petri Dishes |

**Table 5.2.2.1.3 –** Composition of 5-FOA plates for removal of URA3 plasmid.

## 5.2.2.2 Liquid Growth Assay

A preculture of yeast was grown from the stock plate by inoculating a colony in c-trp (Table 5.2.2.2) or YEPD, and shaking at 250 rpm at 30$^{o}$C overnight. Cells were then diluted into c-trp or YEPD containing different concentrations of either bathocuproine disulfonate (BCS), or copper sulphate ($CuSO_4$), so that all bottles had the same $OD_{600}$ at the start time point. Cells were then left to grow for around 12-16 hours, after which 1ml of cells were transferred to a cuvette and the $OD_{600}$ read and recorded. If the $OD_{600}$ was higher than 1, the sample was diluted to maintain accuracy.

**Table 5.2.2.2 Composition of c-trp Media**

| **Name of Solution/Buffer** | **Composition of Buffer** |
|---|---|
| c-trp | 1.7g Yeast Nitrogen Base – Amino Acids<br>5g Ammonium Sulphate<br>20g Glucose<br>1.26g c-trp Drop Out Mix<br>Made to IL with Distilled Water<br>Autoclave |

**Table 5.2.2.2 –** Composition of c-trp media for growing yeast cells

## 5.2.2.3 Protein Extraction for Western Blotting

Cells were grown overnight in c-trp or YEPD to and $OD_{600}$ of between 0.3-0.8. Cells were centrifuged at 3,000 rpm for 5 minutes at room temperature, before discarding supernatant and resuspending the pellet in 5mls distilled water. Cells were then pelleted by centrifugation with the above conditions, after which the pellet was resuspended in 100µl cold lysis buffer (Table 5.2.2.3). The cells were transferred into an eppendorf tube containing around 50µl of glass beads and either vortexed for 30 seconds, followed by 30 seconds on ice repeated 7 times, or placed into the fast prep at 50 for 45 seconds. The eppendorf tubes were then centrifuged at 13,000 rpm for 5 minutes at 4$^{o}$C, and the supernatant collected in a fresh tube. Protein quantification was carried out as described in section 2.2.7. 50µl 5x loading dye was added to 50µl sample, and gel electrophoresis and Western blotting performed as described in sections 2.2.8 and 2.2.9 respectively.

**Table 5.2.2.3 Composition of Lysis Buffer**

| **Name of Solution/Buffer** | **Composition of Buffer** |
|---|---|
| Lysis Buffer | 0.5ml 1M Tris pH 8.0<br>1.65mls 60% (v/v) Glycerol<br>5µl 1M DTT<br>25µl 200mM PMSF<br>Made up to 5mls with Distilled Water |

**Table 5.2.2.3 –** Composition of lysis buffer used for protein extraction.

### 5.2.2.4 Solid Growth Assay

A 5ml preculture of cells was grown in c-trp overnight shaking at 250 rpm at 30°C. The $OD_{600}$ of the cultures was read, and diluted to equal the $OD_{600}$ of the lowest reading sample. 90µl of culture was the placed into the 1st well of a 96 well plate, and 90µl c-trp placed into the next 4 wells. 10µl of culture was taken from well 1, placed into well 2 and mixed, before 10µl of culture was taken from well 2 and transferred to well 3. This was continued until all 5 wells contained cells as a dilution across the wells. A spotter was then used to spot a drop of the culture from each well onto c-trp plates containing different concentrations of BCS or $CuSO_4$. One c-trp plate or each condition was inoculated by hand by pipetting 5µl of each culture onto the plate. The plates were then grown at 30°C until spots appeared, after which photographs were taken.

### 5.2.2.5 Actin Staining

Cells were grown in c-trp with varying concentrations of BCS or $CuSO_4$ overnight shaking at 250 rpm at 30°C. $OD_{600}$ was read, and the cells diluted into 5mls of YEPD such that the $OD_{600}$ would be around 0.2, and put back on the shaking incubator with the same conditions for 4 hours. The $OD_{600}$ was read again, and the cell pelleted by centrifugation at 2,000 rpm for 5 minutes at room temperature, before resuspending in 5mls fixation solution (Table 5.2.2.5), and incubating at room temperature on a rotary shaker for 90 minutes. Cells were then washed in 1 ml of 1x PBS, before resuspending again in 1 ml 1x PBS. Approximately 25 million cells were transferred to an eppendorf tube, and centrifuged under the same conditions as before, after which the pellet was resuspended in 100µl of 0.6µM rhodamine phalloidin in 1x PBS. Cells were then washed with 1x PBS three times before finally resuspending in 50µl mounting media (Table 5.2.2.5), after which cells were stored at -20°C until viewing. When viewed, 1µl of cells was added to a microscope slide, and a coverslips placed on top and firmly pressed down, and viewed by scanning confocal microscopy.

**Table 5.2.2.5 Composition of Buffers for Actin Staining**

| Name of Solution/Buffer | Composition of Buffer |
|---|---|
| Fixation Solution | 4.45mls 1x PBS<br>50µl $MgCl_2$<br>0.54ml Formaldehyde |
| Mounting Media | 2.4g Mowiol<br>6g Glycerol<br>6mls Distilled Water<br>12mls 0.2M Tris-Cl, pH 8.5<br>2.5% (w/v) 1,4-diazobicyclo-[2.2.2]-octane (DABCO) |

**Table 5.2.2.5 –** Composition of buffers used for actin staining of yeast cells.

## 5.2.2.6 Yeast Polysome Extraction With or Without CHX

10ml yeast cultures were grown overnight on the shaking incubator at 250 rpm at 30°C. The $OD_{600}$ of the precultures was read, and the appropriate amount added to 200mls c-trp or 100mls YEPD with or without BCS or $CuSO_4$, so that the cultures would have an $OD_{600}$ of around 0.8 by the time of extraction. If CHX was used, 0.1mg/ml CHX was added to the culture and incubated for a further 15 minutes. The cultures were transferred to 250ml centrifuge bottles, and filled with ice, before centrifugation at 7,000 rpm for 5 minutes at 4°C. The media was discarded, and the pellet resuspended in 10mls of freshly prepared polysome lysis buffer with or without CHX (Table 5.2.2.6), and the solution transferred to a 15 ml tube. The cells were pelleted at 3,000 rpm for 5 minutes at 4°C, the supernatant discarded and cells

resuspended in 0.25mls polysome lysis buffer. The solution was transferred to a breaking tube containing around half the volume of glass beads, and the cell lysed using the cell lyser at 50 for 3 minutes. The tubes were centrifuged at 13,000 rpm for 20 minutes at 4°C, before transferring the supernatant to a fresh tube. The extraction was quantified, centrifuged on sucrose gradients prepared as described in 2.2.15.2, and run on the gradient profiler as described in section 2.2.15.3.

**Table 5.2.2.6 Composition of Buffers Used for Polysome Extraction**

| **Name of Solution/Buffer** | **Composition of Buffer** |
|---|---|
| Polysome Lysis Buffer with CHX | 10mM Tris-HCl pH 7.5<br>0.1M NaCl<br>30mM $MgCl_2$<br>50µg/ml CHX<br>200µg/ml Heparin<br>0.2% (v/v) Diethyl phthalate (DEP) |
| Polysome Lysis Buffer without CHX | 10mM Tris-HCl pH 7.5<br>0.1M NaCl<br>30mM $MgCl_2$<br>200µg/ml Heparin<br>0.2% (v/v) DEP |

**Table 5.2.2.6 –** Composition of buffers used to extract polysomes in the presence and absence of CHX.

## *5.3 Results*

### 5.3.1 Creation of strain with copper regulatable levels of eEF1A

The yeast strain TKY102 was created by Sandbaken and Culbertson (1988), and the predominant characteristic of this strain is that both chromosomal copies of the genes encoding eEF1A, namely TEF1 and TEF2 have been disrupted, yielding no functional expression of the protein. To maintain viability, the TEF1 gene was inserted back into the TKY102 strain by way of an episomal plasmid with a URA3 marker (Cottrelle et al., 1985, Sandbaken and Culbertson, 1988). Plasmid shuffling can therefore be obtained through introduction of another form of TEF1/eEF1A followed by removal of the episomal URA3 plasmid using 5-fluoroorotic acid (5-FOA). For the purpose of this study an eEF1A regulatable expressing vector was chosen, TKB579. Plasmid shuffling following transformation of TKB579 and elimination of the episomal URA3 eEF1A plasmid created a strain in which the only form of eEF1A present was controlled by the copper repressible promoter, theoretically allowing varying levels of eEF1A to be achievable by increasing and or sequestering the concentration of copper in the media. This strategy is schematically represented in figure 5.3.1. The newly created yeast strain was named SGY5.

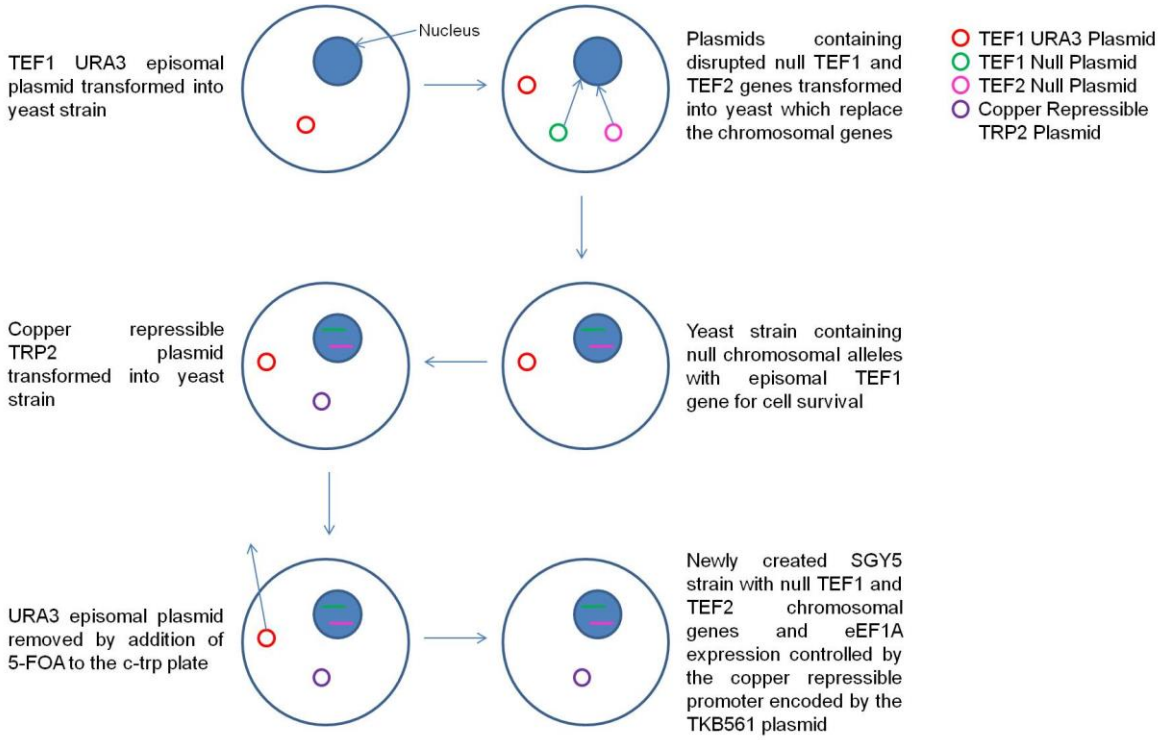



**Figure 5.3.1 –** Schematic representation of plasmid shuffling used to create the SGY5 strain. The plasmid containing eEF1A with the copper repressible promoter was transformed into the yeast strain, after which the episomal TEF1 plasmid was removed using 5-FOA to create the new SGY5 strain in which the only copy of eEF1A was controlled by the copper repressible promoter.

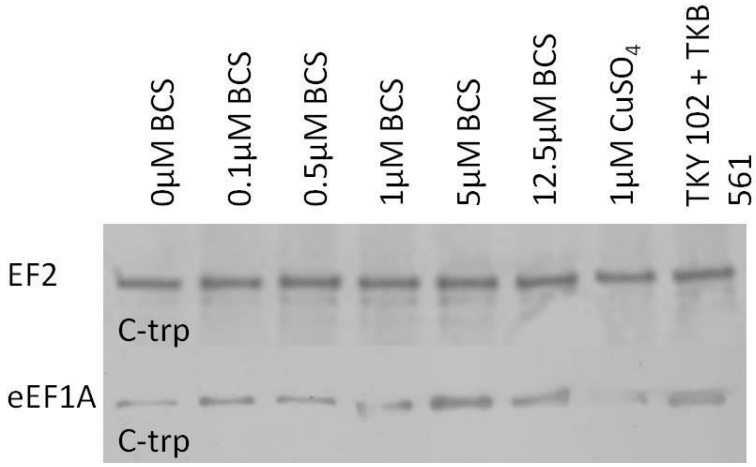
### 5.3.2 A range of different eEF1A expression levels were obtained from cells grown in media containing between 1µM $CuSO_4$ and 12.5µM BCS

After creation of the SGY5 strain containing the plasmid encoding expression of eEF1A controlled by the copper repressible promoter, we sought to establish a dose response effect of different concentrations of bathocuproine disulphonate (BCS) and copper sulphate ($CuSO_4$), in order to regulate the transcriptional activity of the promoter for eEF1A, to determine which concentrations would give the appropriate changes in eEF1A protein expression. A range of concentrations between 1µM $CuSO_4$ and 12.5µM BCS were selected after a simple growth experiment showed that this range would provide the greatest change in cell growth (data not shown).

To verify the level of expression of eEF1A, the yeast cells were grown for 24 hours in medium containing varying concentration of $CuSO_4$ and BCS, protein extracted, quantified and run on a gel, transferred to nitrocellulose, before being probed for eEF1A (EF2 as a loading control) and developed using standard Western blotting techniques. The presence of 12.5µM BCS resulted in a similar level of eEF1A protein expression as the control strain, suggesting that this concentration is capable of eliminating all traces of copper and inducing the full expression of eEF1A, replicating the level of expression seen in physiological conditions (Figure 5.3.2).

When the cells were grown without BCS, the level of eEF1A protein expression was reduced to approximately 40%, and the presence of 1µM $CuSO_4$ led to further reduction of the expression to a minimal 20% of the levels seen in the control strain, indicating that as the amount of copper in the media increases, the level of eEF1A protein expression decreases in a dose dependent fashion (Figure 5.3.2).

A




B

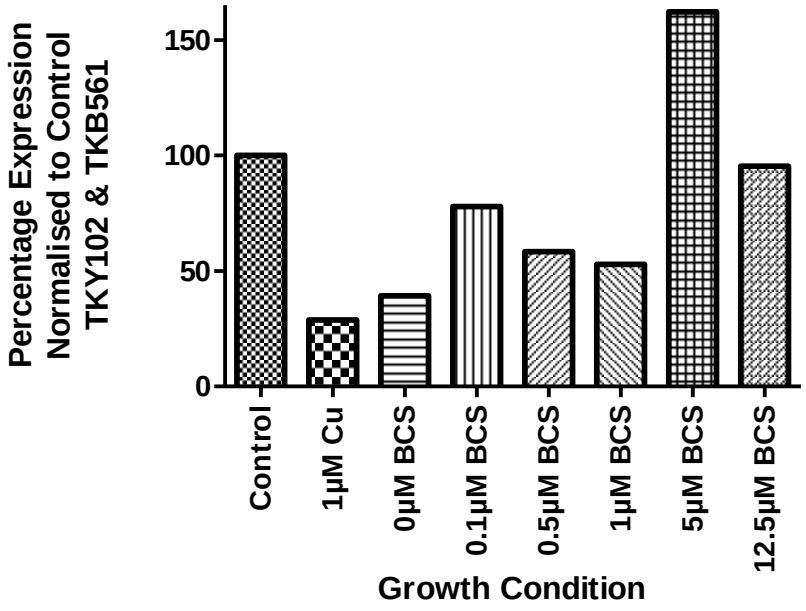



**Figure 5.3.2 –** Addition of varying levels of BCS or $CuSO_4$ to the synthetic growth media results in change in eEF1A protein expression. Cells were grown overnight in c-trp in exponential phase with varying concentrations of BCS or $CuSO_4$ to and $OD_{600}$ of approximately 0.8, before total protein was collected and quantified. 5ug of total protein was loaded onto a gel, run, transferred to nitrocellulose, before being probed for eEF1A, and EF2 as a loading control and developed using ECL (A). The intensity of the bands was quantified by densitometry analysis using ImageJ (B).

### 5.3.3 The same concentrations of BCS and $CuSO_4$ in YEPD show a less gradual change in eEF1A expression, resulting in a more all or nothing response

The previous analysis (Figure 5.3.2) clearly demonstrated the presence of copper in synthetic medium, indicating that this level, endogenously found in the different media preparation needs to be taken into consideration. It was therefore important to determine whether or not the same gradual change in eEF1A expression could be achieved by addition of BCS and $CuSO_4$ in YEPD medium. YEPD is a much more nutrient rich media, containing yeast extract and tryptone, where concentrations of copper are much less characterised. It is also a potentially more 'forgiving' medium.

In order to determine the regulatory effects of the copper inducible promoter on eEF1A expression under such conditions, cells were grown in YEPD with varying concentrations of BCS or $CuSO_4$ overnight, before total protein was extracted and quantified and a Western blot performed for eEF1A (EF2 as a loading control). Whereas in c-trp media, 12.5µM BCS gave an eEF1A protein expression level similar to that of the control strain (Figure 5.3.2), in YEPD, only around 40% expression was observed. The gradual reduction of eEF1A protein expression controlled by BCS/$CuSO_4$ seen in synthetic media was not observed in YEPD, as concentrations of BCS lower than 12.5µM BCS showed only minimal eEF1A protein expression, and addition of 1µM $CuSO_4$ resulted in almost undetectable levels of eEF1A protein expression (Figure 5.3.3), suggesting that in YEPD growth, the expression of eEF1A is much less regulatable and that synthetic medium should be the preferred media for future use.

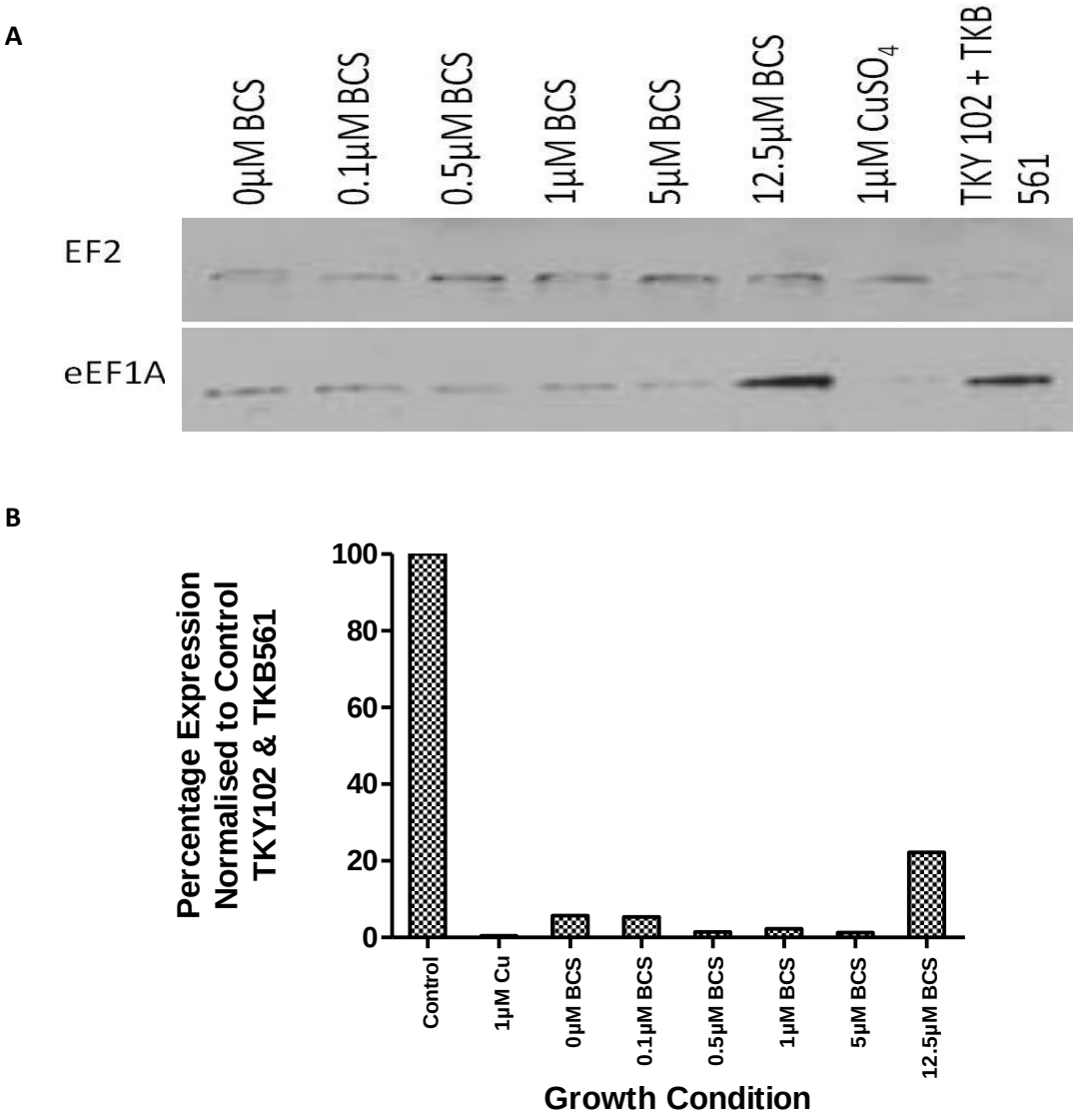



**Figure 5.3.3 –** Addition of BCS or $CuSO_4$ to the growth media results in change in eEF1A protein expression in YEPD. Cells were grown in YEPD overnight in exponential phase with the corresponding concentrations of BCS and $CuSO_4$ to an $OD_{600}$ of approximately 0.8, before total protein was extracted and quantified. 5μg total protein was then loaded onto a gel, run, transferred to nitrocellulose and probed for eEF1A and EF2 as a loading control, before developing using ECL (A). Intensity of the bands was quantified by densitometry analysis using ImageJ (B).

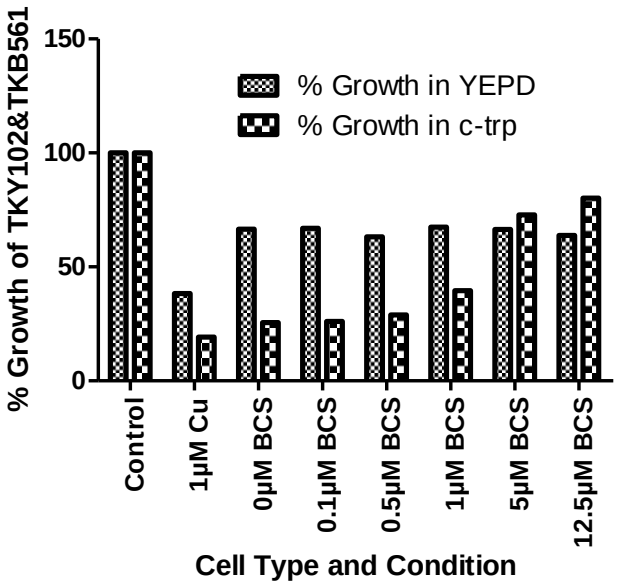
### 5.3.4 Level of expression of eEF1A as determined by addition of BCS and $CuSO_4$, correlates to growth rate in both C-trp and YEPD

Addition of varying concentrations of $CuSO_4$ and BCS lead to significant changes in the level of eEF1A expression in c-trp (Figure 5.3.2) but less so in YEPD (Figure 5.3.3). We now sought to determine how the downregulation of eEF1A protein level would affect yeast growth and viability on both liquid and solid cultures.

Growth assays were performed by monitoring either absorbance of overnight liquid growth or by colony size formation on plates in varying concentrations of BCS and CuSO4 concentrations as used to achieve the varying level of eEF1A protein expression (Figure 5.3.4). Cells grown in the presence of 12.5µM BCS in c-trp, which has an eEF1A protein expression level similar to that of the control strain, also demonstrated similar growth rates at around 90%. Cells grown in absence of BCS, which results in an eEF1A protein expression level of around 40% resulted in a growth rate of 40%, whilst cells incubated in 1µM $CuSO_4$, with an eEF1A protein expression level of 20% presented a growth rate of approximately 20%. This data indicates a high proportionally between eEF1A expression and growth. This difference in growth rate was confirmed using the solid growth assay, which showed a gradual decrease in growth with decreasing concentration of BCS, with the lowest growth rate observed in cells grown in the presence of 1µM $CuSO_4$ (Figure 5.3.4 B).

The correlation of growth to eEF1A protein expression was not as robust in YEPD conditions, since the different concentrations of BCS all led to a similar 80% growth rate, with only cells grown in 1µM $CuSO_4$ resulting in severely reduced eEF1A protein expression level showing around a 40% growth rate (Figure 5.3.4 A).

A




B

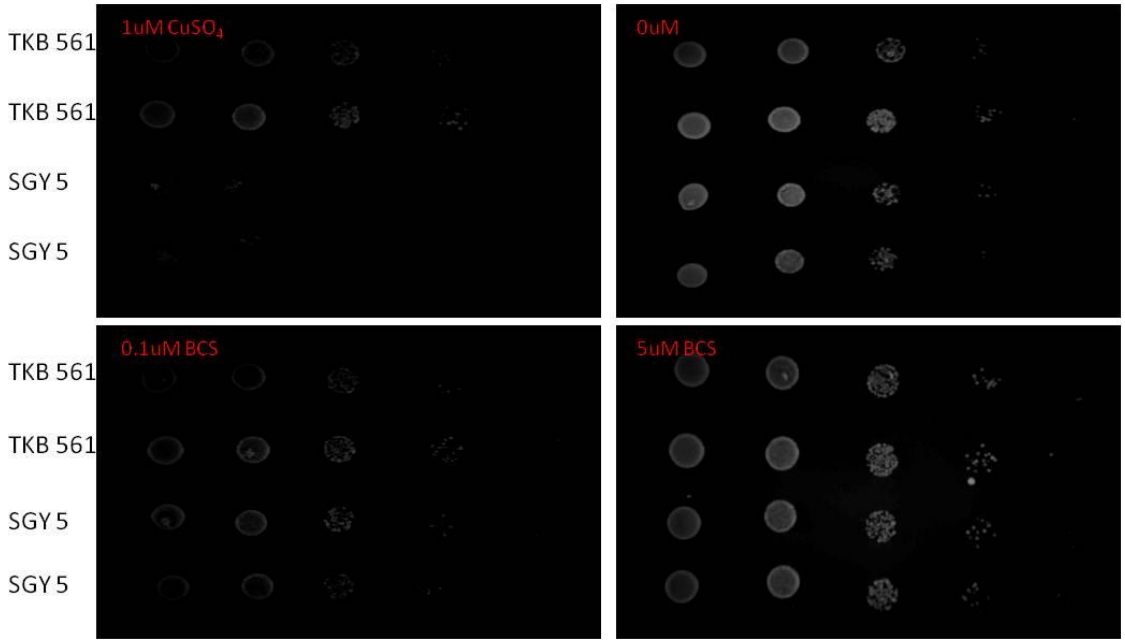



**Figure 5.3.4 –** The growth rate of yeast cells is reduced with a reduced level of eEF1A protein expression. Liquid growth assays were performed by using precultures to inoculate media at the same starting $OD_{600}$, grown overnight, and the $OD_{600}$ read and recorded the following day. The percentage expression compared to the control TKY102 & TKB561 strain was plotted onto a graph (A). Solid growth assays were performed by creating a serial dilution of cells by diluting a preculture of cells so that the starting concentrations were the same before adding 100µl to a 96 well plate. 10µl of this solution was taken and transferred to the next well along with 90µl of media, and this continued until 5 wells contained cells and spotting onto c-trp plates containing 0, 0.1 or 5µM BCS or 1µM $CuSO_4$. The plates were then incubated at 30oC for 2-3 days until colonies could be visualised, and the plates photographed (B).

### 5.3.5 Reduced expression of eEF1A results in changes in actin organisation

Overexpression of eEF1A in yeast has been shown to cause a slow growth phenotype that has been suggested to be due to the change in actin organisation rather than a change in protein translation (Munshi et al., 2000, Gross and Kinzy, 2005), however, the effect of reduced expression of eEF1A on actin organisation in yeast has not been investigated.

Using the SGY5 strain and the different concentrations of BCS and $CuSO_4$ to create a gradient of different eEF1A expression levels, the yeast were grown in c-trp, fixed and stained for actin using rhodamine phalloidin before viewing using confocal microscopy. As has been shown before, both the control strain, and SGY5 grown in 12.5µM BCS show an 'endogenous and physiological' level of eEF1A protein expression (Figure 5.3.2) and after observation were seen to present highly organised actin cytoskeletal architecture, made primarily of actin patches and fibres or cables, as indicated by the arrows on the photographs. Growing the same cells when the amount of BCS was reduced to 1 and 0.5µM, with around 50% eEF1A expression compared to the control strain, demonstrated far fewer fibres, though the actin patches still remain. Once $CuSO_4$ was added, to create the lowest level of eEF1A protein expression at around 20%, both actin buds and fibres were disrupted, and no clear actin organisation could be observed (Figure 5.3.5).

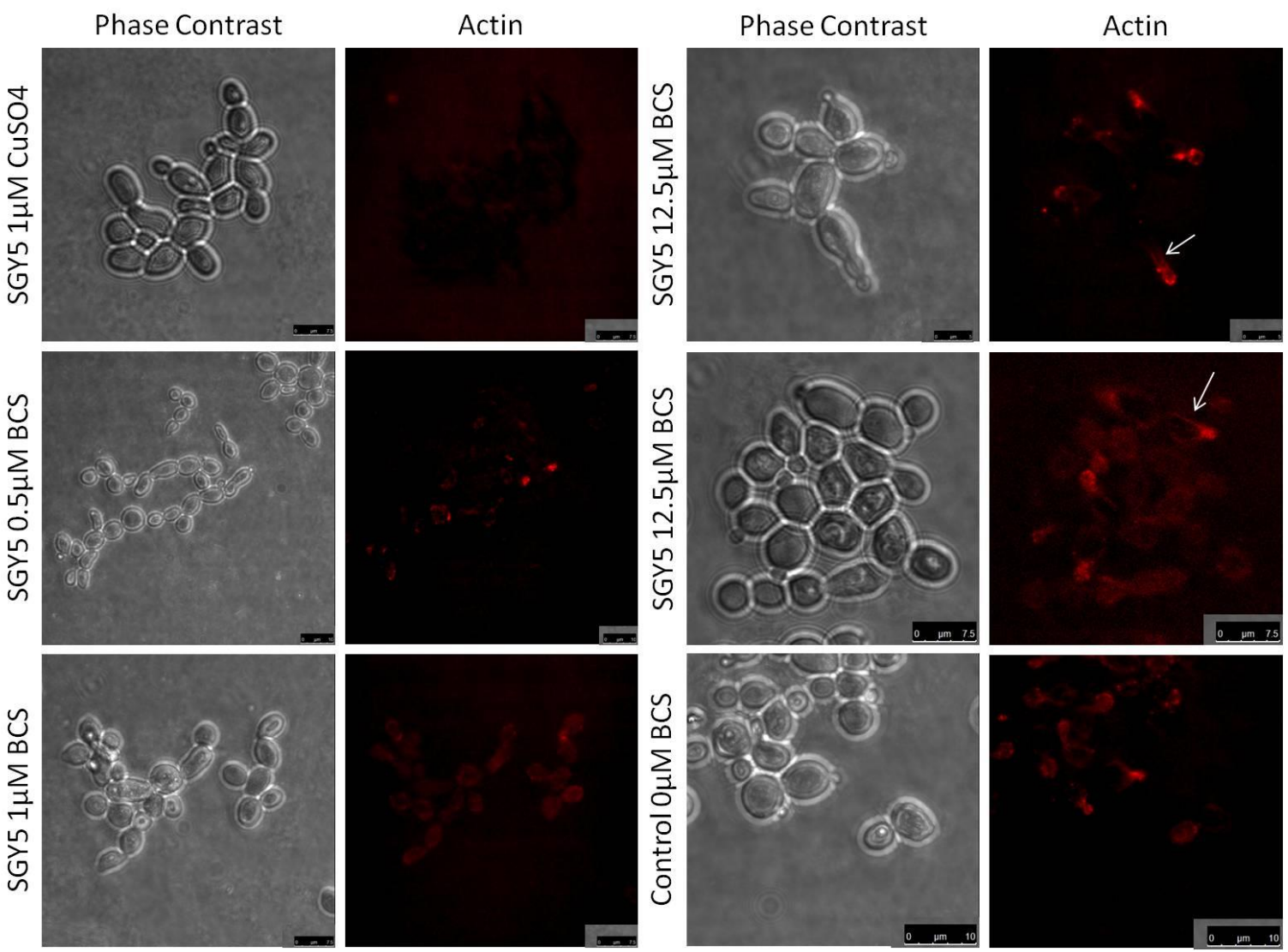



**Figure 5.3.5 –** Reduced expression of eEF1A results in a disruption of the actin organisation in yeast. Cells grown overnight in c-trp in the presence of 0, 0.5, 1 or 12.5µM BCS or 1µM $CuSO_4$ to exponential phase were transferred to YEPD for 4 hours before fixing and staining with rhodamine phalloidin, and mounted onto a microscope slide for viewing. Cells were observed by confocal microscopy with a 100x objective lens. White arrows point to actin fibres/cables.

### 5.3.6 Polysome profiles in c-trp and YEPD, with and without cycloheximide

The data presented so far demonstrates that a decrease in eEF1A expression results in growth defects and changes in actin organisation, both in a dose dependent manner, the most extreme phenotypes being observed in the presence of 1µM CuSO4 resulting in the lowest level of eEF1A protein expression. It is however unclear whether these reported differences are a direct result of reduction in protein synthesis or possibly due to the lowering of other sub fraction pools of eEF1A that affect other physiological processes, potentially whilst conserving the translational 'pool'.

To determine if these down-regulations of eEF1A lead to significant changes in protein synthesis, we set out to determine the effect of BCS and $CuSO_4$ addition to polysome profiles, however first the protocol needed to be established in the strain used in the media used. Polysome profiling is a common method of analysing translational activity of cells and an elongation block, which could potentially be caused by reduced levels of eEF1A, could be easily indentified due to an accumulation of ribosomes on the mRNA (polysome peaks) as long as the experiments are done in the absence of cycloheximide. As mentioned previously, due to the effect of CHX on elongation, it is not ideal to use as a stabilising agent when observing potential changes in elongation, especially as these changes have been shown to be observable on a polysome profile in some cell types (Rivest et al., 1982). This causes a problem with viewing an elongation block in the sample when extracted in the presence of cycloheximide, therefore the polysomes must be extracted without cycloheximide, requiring further optimisation. Most methods already established are also focused on extractions after growth in YEPD media, rather than selective media, therefore this provided another hurdle, as the 'gradient' of expression of eEF1A was only properly observed in c-trp, therefore only c-trp could be used. To ensure the protocol was working sufficiently, polysomes were first extracted after growth in YEPD with cycloheximide. Following on from that, polysomes were extracted in c-trp with cycloheximide, and then c-trp without cycloheximide. It was important to test whether polysome profiles could be obtained under each condition to ensure that any problems could be detected and solved quickly and easily. The best examples of these trial profiles are shown (Figure 5.3.6).

Polysomes were extracted with or without cycloheximide, after growth in YEPD or c-trp, separated by density on a sucrose gradient and analysed for absorbance at 254nms. The gradients were run on the gradient profiler to create the polysome profiles showing all characteristic features, proving that profiles can be obtained from cells grown in YEPD and in c-trp extracted both with and without cycloheximide (Figure 5.3.6). Characteristic features of a normal polysome profile include the 40S and 60S peaks, which represent the individual ribosomal subunits, the 80S peak, also known as the monosome peak, which represents the intact ribosome, and the polysome peaks, which represent the ribosome that are actively translating with the first peak corresponding to mRNA with one monosome translating and the second peak with two translating monosomes and so on. Changes in polysome profiles are usually quantified by the polysome/monosome (P/M) ratio, calculated using the areas underneath the respective peaks. Polysome profiles allow changes in the different stages of protein translation to be observed, with an initiation block resulting in an increase in the monosome peaks with a decrease in polysome peaks causing a decrease in the P/M ratio, and an elongation block observed by reduction of the monosome peak and an increase in the polysome peaks, resulting in an increase in the P/M ratio.

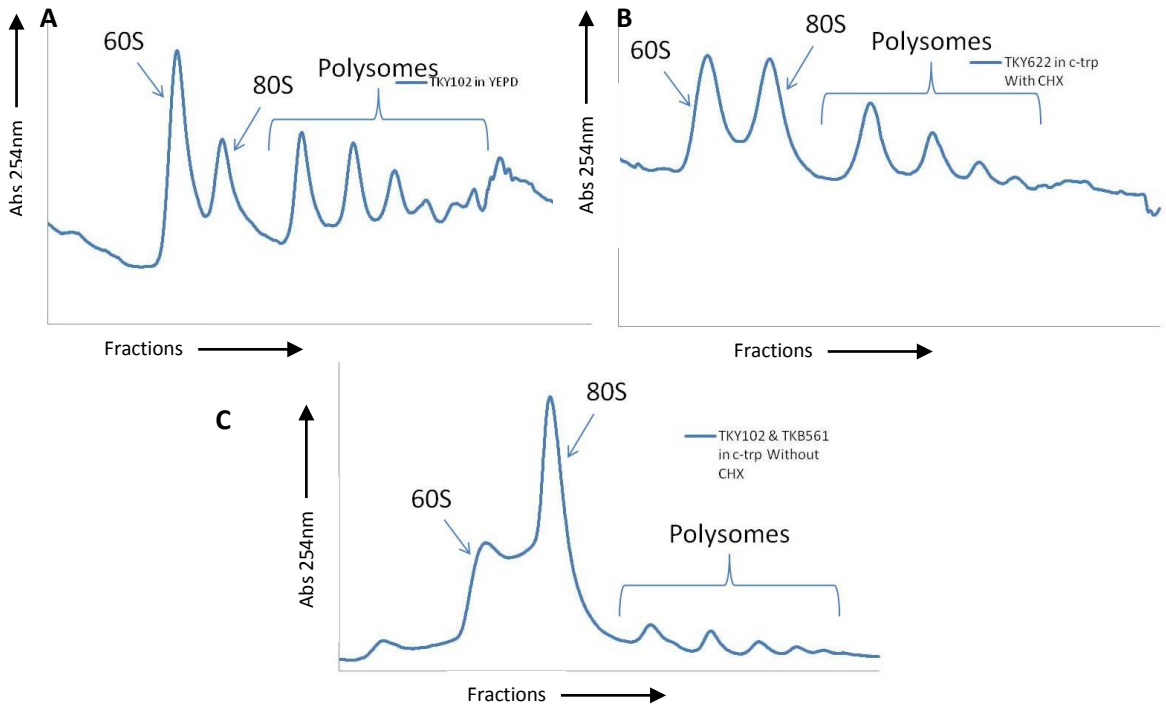



**Figure 5.3.6 –** Polysome profiles can be obtained in both YEPD and c-trp in the presence and absence of cycloheximide, with a clear elongation block observed in the strain with a translational defect. For polysomes extracted with CHX, cells were grown to exponential phase overnight in YEPD (A) or c-trp (B) before 0.1mg/ml cycloheximide was added for 15 minutes. Cells were then scraped into lysis buffer containing CHX, isolated, lysed and cellular content collected. 200µg of total nucleic acid was loaded onto a sucrose gradient, centrifuged at 38,000rpm for 2 hours, and analysed using the Biorad Gradient Profiler. Polysomes extracted in the absence of CHX were prepared in the same way without incubating with CHX, and by scraping into lysis buffer without CHX (C). The characteristic 60S, 80S and polysome peaks are identified in all three profiles.

### 5.3.7 SGY5 strain shows an initiation block in the presence of 1µM CuSO4 in YEPD medium, but an elongation block in c-trp medium, however no difference is seen with varying concentrations of BCS.

Having been able to establish appropriate polysome profiles that allow observation of changes in elongation ability both with and without the use of cycloheximide treatment, we sought to analyse whether the reduced expression of eEF1A caused by the variation in BCS or $CuSO_4$, would lead to changes in polysome profiling that could be detected with our setup. Cells were grown in either YEPD or c-trp with different concentrations of BCS and $CuSO_4$ before, polysomes extracted, loaded onto a sucrose gradient and run on the gradient profiler.

When cells were grown in YEPD and extracted with cycloheximide, the profile obtained for the SGY5 strain grown in the presence of 12.5µM BCS, which has similar or higher eEF1A protein expression level to the control strain, was comparable to that observed for the control strain, with the polysome/monosome (80S peak) ratio (P/M) very similar at 0.827 and 0.720 respectively. The profile obtained for the SGY5 strain grown in 1µM $CuSO_4$, however, which showed very minimal eEF1A protein expression, showed a reproducible and unexpected initiation block, as demonstrated by the P/M ratio changing from 0.827 with 12.5µM BCS to 0.168 with 1µM CuSO4, compared to 0.720 in the control strain (Figure 5.3.7A). Cells grown in c-trp, and extracted in the presence of cycloheximide with 12.5µM BCS presented a similar profile and P/M ratio similar to that of the control strain, 0.121 and 0.163 respectively. The presence of 1µM $CuSO_4$, in synthetic medium, which showed around 20% eEF1A protein expression compared to the control strain, resulted in a severe elongation block observed on the profile, with the P/M ratio changing from 0.121 with 12.5µM BCS to 0.865 with 1µCuSO4, compared to the control strain at 0.163 (Figure 5.3.7B). Though cycloheximide can cause an elongation block, the block caused by addition of the $CuSO_4$, was large and therefore observable in the presence of cycloheximide, however, smaller changes would not be observed, therefore to determine whether there were any changes in the profile of SGY5 grown in the presence of varying concentrations of BCS, the cells were extracted without cycloheximide. It was found that SGY5 cells grown in c-trp in the presence of either 0, 2.5 or 5µM BCS and extracted in the absence of cycloheximide, showed no difference with P/M ratios of 0.061, 0.067 and 0.077 respectively, compared to the control strain with a P/M ratio of 0.091 (Figure 5.3.7C), even though the level of eEF1A expression in cells grown in these conditions was around 40% compared to the control.

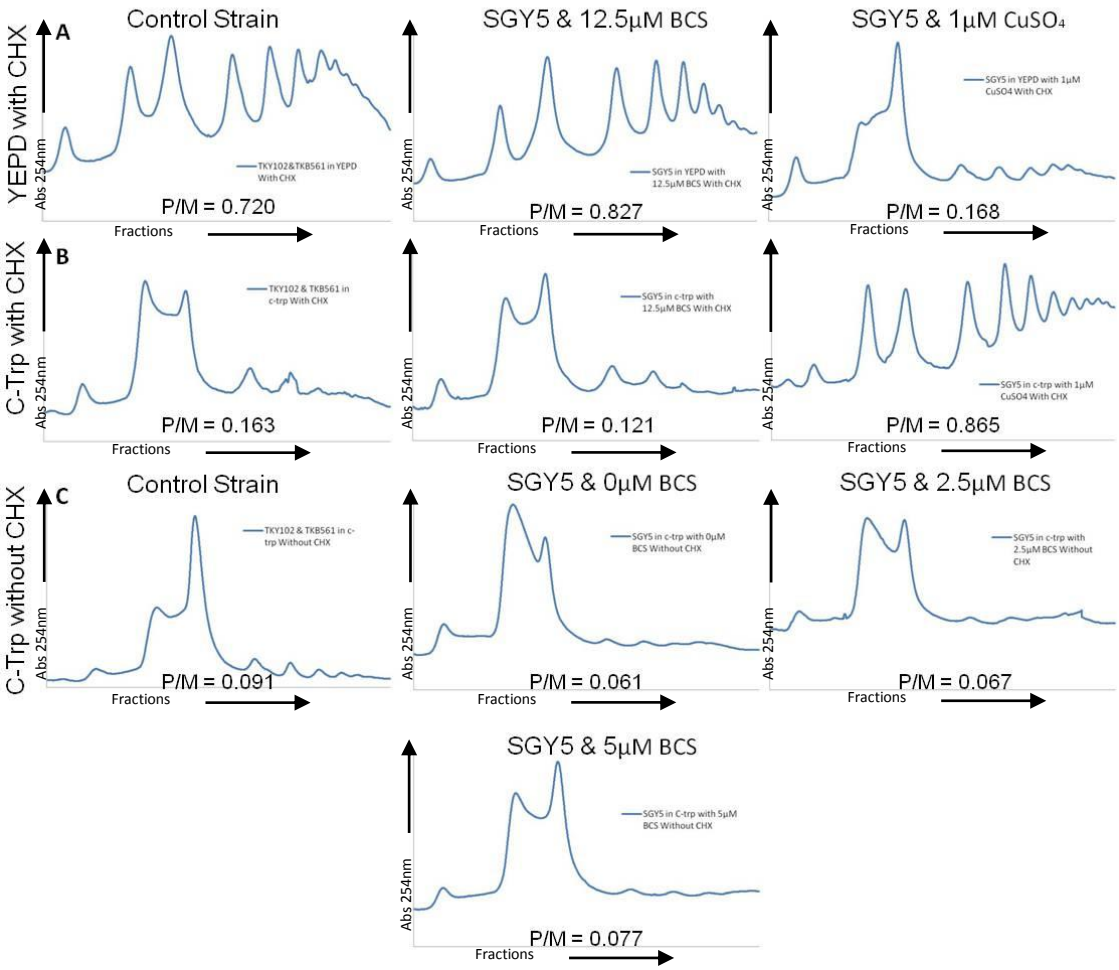



**Figure 5.3.7 –** Reduced expression of eEF1A only caused changes in protein translation at the maximum level of protein reduction in the presence of 1µM CuSO4, less drastic changes in eEF1A protein level resulted in no change in protein translation. Cells were grown overnight in YEPD with 0 or 12.5µM BCS or 1µM $CUSO_4$ (A) or c-trp with 0, 2.5, 5 or 12.5µM BCS or 1µM $CuSO_4$ (B & C) to an $OD_{600}$ of approximately 0.8. 0.1mg/ml of cycloheximide was added to those extracted with CHX for 15 minutes (A & B). Cells were then scraped into lysis buffer containing CHX (A & B), or lysis buffer without CHX (C), isolated, lysed and cellular content collected. 200µg of total nucleic acid was loaded onto a sucrose gradient, centrifuged at 38,000rpm for 2 hours, and analysed using the Biorad Gradient Profiler.

## *5.4 Discussion*

The focus of this chapter aimed to engineer a yeast strain in which the level of eEF1A protein expression could be tightly down-regulated in order to determine whether it was possible to extend our work to separate the function of eEF1A in protein translation and actin remodelling, this time in lower eukaryotes. This would lend credence to the theory of different 'pools' of eEF1A for different functions, with the 'pool' for translation conserved over others due the essential nature of the function to the survival of the cell.

To study this and in order to have controlled reduced levels of eEF1A, a yeast strain was engineered in which the only form of eEF1A expression was regulated through a copper repressible promoter (Figure 5.3.1). The change in eEF1A protein expression with varying concentrations of BCS and $CuSO_4$ in c-trp was confirmed by Western blotting (Figure 5.3.2), with the cells grown in the presence of 1µM $CuSO_4$ showing the greatest reduction in eEF1A protein expression whilst the addition of 12.5µM BCS to the media results in a similar eEF1A protein expression to the control strain. Interestingly, regulating the levels of BCS and $CuSO_4$ in YEPD did not result in a gradient of eEF1A protein expression, rather more of an 'all or nothing' response was observed, with a concentration of only 5µM BCS resulting in a large decrease in eEF1A protein expression (Figure 5.3.3). This is likely to be due to YEPD being a much more nutrient rich, less restrictive media therefore more extreme conditions are required in order to affect the level of protein expression in this media compared to synthetic medias such as c-trp.

Regulating the levels of BCS and $CuSO_4$ led to correlative evidence of eEF1A protein expression and cell growth rate although not always providing the best linearity (Figure 6.2.4). Much evidence has shown that cell growth is directly related to actin cytoskeletal organisation, with actin patches/buds and cables both having been shown to be involved in cell division in yeast (Moseley and Goode, 2006). Actin patches have been shown to localise to the region at which the daughter cell will bud off from the mother cell, and the actin cables have been shown to orient along the growth axis (Drubin, 1990). A change in expression of eEF1A has been shown to affect the organisation of the actin cytoskeleton without affecting the rate of total protein translation in previous studies (Gross and Kinzy, 2005) as well as in previous chapters (Figures 3.3.1-5), therefore it seems likely that the reduced growth rate observed in the presence of the different concentrations of BCS and CuSO4 in both c-trp and YEPD could be due to the disruption of the actin cytoskeleton.

In agreement with the suggested possibility that actin disruption could be the cause of the observed slow growth phenotype when eEF1A protein is reduced, the overall organisation of actin was found to be affected in those cells grown with BCS and CuSO4 (Figure 5.3.5). With intermediate eEF1A protein expression when cells were grown in the presence of 1 or 0.5μM BCS which correlates to approximately 50% eEF1A protein expression compared to the control strain, a reduction in actin fibres was observed, whilst when the level of eEF1A protein expression was reduced to its maximum to around 20% of the control when grown in the presence of 1μM CuSO4, both actin buds and actin fibres were disrupted. As eEF1A is a known actin binding protein (Yang et al., 1990, Demma et al., 1990), these results are not surprising and could suggest that since and insufficient amount of eEF1A is present in the cell, there is much less control of the actin cytoskeleton.

Though no studies have shown the effect of reduced expression of eEF1A on the actin cytoskeleton, overexpression studies have shown that when TEF1 or TEF2 are overexpressed in yeast, cell growth rate is reduced and actin cytoskeletal organisation is affected whilst no difference in protein translation was observed (Munshi et al., 2000). Gross and Kinzy, 2005, used this overexpression phenotype to observe the effect of eEF1A mutants that were shown to affect cytoskeletal organisation without affecting protein translation, and found that the mutants affecting the actin cytoskeleton were capable of restoring the slow growth phenotype, indicating that the observed phenotype was due to the effect of eEF1A on the cytoskeleton. Based on these studies, as well as the results reported in previous chapters which showed a reduced growth rate with a change in actin architecture and no change in protein translation caused by a decrease in eEF1A protein expression, it seems sensible to suggest that these changes in actin cytoskeletal organisation are likely to be the cause of the change in cell growth rate. Further supporting this is the finding that mutant forms of eEF1β can also result in similar effects on the actin cytoskeleton which also confers a slow growth phenotype (Pittman et al., 2009), therefore adding further credence to the implication that it is the disruption of the actin cytoskeleton that causes the altered growth rate. This suggests that eEF1A may be an important regulator of the actin cytoskeleton in yeast under these conditions, and, as the actin cytoskeleton can affect many other cellular functions, this reduced expression of eEF1A and its resulting effect on the cytoskeleton could make eEF1A a major regulator of many cellular roles.

The roles of eEF1A in actin binding and protein translation have been suggested to be independent based on studies showing changes in actin organisation without any effect on

translation by methionine incorporation or polysome profiling (Munshi et al., 2000, Gross and Kinzy, 2005), leading to the hypothesis of the different 'pools' of eEF1A for different functions. Based on the above findings it would be interesting to determine whether changes in translation were observed in our yeast cells under the different conditions.

Attaining polysome profiles of the yeast cells grown in c-trp in the absence of CHX, required many steps of optimisation to ensure that all characteristic features of the profile could be observed and therefore compared (Figure 5.3.6). Once the protocol was established, it was determined that when cells were grown in the presence of 1μM $CuSO_4$ in YEPD, an initiation block was observed (Figure 5.3.7A), however when the cells were grown in the presence of 1μM $CuSO_4$ in c-trp, an elongation block was observed (Figure 5.3.7B). The observed elongation block in c-trp was expected as, when 1μM $CuSO_4$ was added, the eEF1A protein level would have been reduced so drastically that there would not be sufficient protein available to allow protein translation to continue at a normal rate.

The initiation block observed when the cells were grown in 1μM CuSO4 in YEPD was unusual as eEF1A is an elongation factor therefore the elongation stage would be expected to be affected as observed in c-trp, however, the cells grown under these conditions have been shown to have a completely disrupted actin cytoskeleton, and disruption of the actin architecture has been shown to affect initiation both as a result expression of mutated eEF1A and independently of eEF1A (Gross and Kinzy, 2007). As several different initiation factors have been shown to associate with the cytoskeleton (Howe and Hershey, 1984, Gross and Kinzy, 2007), it is possible that the disruption of the cytoskeleton could render the initiation factors no longer functional therefore causing the observed initiation block. Why this is not observed in the c-trp samples under the same conditions is unknown, however it is possible that the level of cytoskeletal disruption in cells grown in YEPD is greater than that observed in c-trp resulting in an initiation block in YEPD and an elongation block in c-trp as there would not be sufficient eEF1A to continue the elongation stage.

Polysome profiles of the SGY5 strain grown in c-trp with 0, 2.5 or 5μM BCS however, showed no elongation block when compared to the control strain, showing that although the level of eEF1A protein expression is reduced at these concentrations (Figure 5.3.2), the translation function of eEF1A is unaffected (Figure 5.3.7C). This would suggest that under these conditions there was sufficient eEF1A protein available for the cell to utilise for protein translation, and given the observed changes in actin architecture under these same conditions,

supports the theory of there being different 'pools' of eEF1A for different functions, with the 'pool' for translation conserved over all other functions.

The results all taken together provide a picture that suggests that when eEF1A levels are reduced by the maximum amount, i.e. when grown in the presence of 1µM $CuSO_4$, actin organisation and translation are both affected, as well as having a profound impact on cell growth rate. The change in cell growth rate under these conditions is likely to be caused by the effect on both actin organisation and translation, as the integrity of the actin cytoskeleton is essential for cell growth, and translation of new proteins is essential to allow the cell to divide. However, when eEF1A levels are reduced to less dramatic levels, i.e. in the absence of BCS, the elongation stage of translation is unaffected whilst actin organisation is disrupted with fewer actin fibres observed. At these levels of eEF1A expression, cell growth rate is also found to be reduced. This provides further evidence to support previous studies that have suggested that the actin function of eEF1A is independent of its role in protein synthesis (Gross and Kinzy, 2005), as well as showing that this disruption of the actin cytoskeleton is capable of affecting cell growth rate proving a link between the two processes indicating that an intact, undisrupted actin cytoskeleton is essential for normal cell division. Furthermore, the evidence that intermediate levels of eEF1A protein expression allow for a normal translational function to the detriment of actin organisation suggests that the theory of different 'pools' of eEF1A for different functions could be the most likely explanation, and that the 'pool' for translation is conserved above all others due to its importance to the survival of the cell.

This potential role of eEF1A in regulating its different functions and affecting the integrity of the actin cytoskeleton would have great biological significance, as if there are different 'pools' of eEF1A conserved for different functions, the level of eEF1A protein expression in the cell could regulate the functions of the cell both spatially and chronologically. The actin cytoskeleton is a major regulator of many different functions within the cell and provides the structural basis by working as a scaffold for proteins to localise to, thereby allowing organisation and compartmentalisation of the cell, therefore by affecting the integrity of the actin cytoskeleton, the expression of eEF1A protein can cause changes in many different cellular functions. For example, the disruption of the actin cytoskeleton would affect other actin binding proteins. ABP-1 is an actin binding protein that has been shown to localise to actin patches but not to actin cables, and has been suggested to play a role in localised cell surface growth of yeast (Drubin, 1990), therefore any disruption of actin patches as observed in the 1µM $CuSO_4$ condition would also affect the localisation of ABP-1 and would therefore

affect this function of ABP-1. Similarly tropomyosin has been shown to localise to actin cables but not to actin patches, and disruption of the tropomyosin gene results in a slow growth phenotype (Liu and Bretscher, 1989), therefore the reduced expression of eEF1A protein observed in the intermediate conditions which disrupt actin cables while not affecting actin patches would disrupt this function of tropomyosin which could contribute to the observed phenotypes. These are just two examples of actin binding proteins that interact with actin in different ways but would not be able to exert their effect if the actin cytoskeleton was disrupted as is observed when the level of eEF1A protein is reduced to varying levels.

This chapter has focussed on the role of eEF1A in translation, actin organisation, and cell growth, however, eEF1A has been shown to have other non canonical functions, therefore to be able to observe these in this cell system or by similar methods in other cell systems would allow further understanding of the regulatory role of eEF1A in cellular functions.

# Chapter 6:
# General Discussion

**Chapter 6 Table of Contents**

# 6.0 General Discussion and Future Work

## *6.1 General Discussion*

Translation has been shown to be dependent upon a functional well organised actin cytoskeleton, and eEF1A provides the perfect link to ensure that the two functions are synchronised (Negrutskii and Deutscher, 1991, Gross and Kinzy, 2007). It is present in large excess the other components of the translational machinery (Slobin, 1980), therefore it is very easy to view it as a multifunctional protein that can perform several tasks simultaneously. It is a known actin binding protein, which can bundle actin into unique formations which have been suggested to be able to exclude other actin binding proteins (Owen et al., 1992), which would make it a likely candidate to be one of the main regulators of actin cytoskeletal organisation.

It is therefore our hypothesis that there are different 'pools' of eEF1A within the cell for its different functions in translation, actin binding, microtubule binding bundling and severing (Durso and Cyr, 1994, Shiina et al., 1994, Moore et al., 1998), influencing apoptosis (Billaut-Mulot et al., 1996, Kato, 1999, Duttaroy et al., 1998, Chen et al., 2000, Ruest, 2002, Talapatra et al., 2002, Borradaile et al., 2005) and cell proliferation and cell cycle (Pecorari et al., 2009), among others. This is supported by our findings that the 40% reduction in eEF1A in the 2E2 cells compared to the control CHO cells results in no change in protein translation, eEF1A's canonical function, however causes significant changes to the actin cytoskeleton, as well as the findings that as eEF1A is reduced in yeast cells, actin organisation is affected, whilst translation is unaffected, until a point at which eEF1A is reduced by such an extent that both the translational functions and the actin organisation are completely disrupted. eEF1A has been shown to bundle actin into square packed bundles which exclude other actin binding proteins (Owen et al., 1992). We suggest that this unique bundling of actin by eEF1A could explain the paradoxical observation that when you reduce the level of eEF1A an increase in actin bundling is observed as the reduced expression of eEF1A provides the opportunity for other actin binding proteins, such as those mentioned previously, to bundle actin into the observed actin stress fibres.

α-actinin is presented as a likely candidate for the bundling of actin into the observed stress fibres in the absence of abundance of eEF1A protein expression, though other ABPs could also be involved due to the presence of eEF1A as an side binding actin bundling protein.

All of these findings point towards eEF1A being a major regulator of the actin cytoskeleton, potentially by negatively regulating the activation of proteins or signalling pathways due to its abundance within the cell. Once this abundance is removed by reduced the protein expression of eEF1A, the inhibitory effect is removed or reduced therefore allowing processes that would normally be prevented to occur. The evidence supporting this seems reasonably strong based on the lack of change in protein synthesis and the drastic change observed in the actin cytoskeleton, along with other proteins associated with it.

It was found that the reduced expression of eEF1A resulted in reduced speed of cell migration and decreased cell proliferation, which were both initially thought to be a consequence of the disrupted actin cytoskeleton. In the study of yeast the cell growth rate was closely correlated to the level of eEF1A expression which was linked to the organisation of the cytoskeleton. As the level of eEF1A was reduced, the growth rate decreased, and the actin cytoskeleton became more disorganised which shows a link between the three areas, however, this link was questioned in mammalian cells, when the clonal cell lines failed to rescue the growth phenotype. This lack of rescue is thought to be due to other changes in the cells that have not yet been determined, or possibly due to the level of eEF1A expression not being high enough to support the rescue of this phenotype or due to the minor differences in hamster and human eEF1A. Further work to create overexpressing clones or clones expressing hamster eEF1A would determine which of these is correct, however as the link between eEF1A expression and cell growth is fairly well established it would be surprising if the phenotype was not eEF1A dependent.

Interestingly only eEF1A1 was capable of restoring the migration phenotype when both eEF1A1 and eEF1A2 were shown to be sufficient to rescue the disruption of the actin cytoskeleton, which also calls into question the dependence of the migrational defect on the disruption of the actin cytoskeleton. The rescue of the migration phenotype by eEF1A1 only could potentially be a difference in the non canonical functions of eEF1A1 and eEF1A2. It is possible that eEF1A1 could directly regulate cell migration, though by which pathway remains to be determined by further studies. It could be that under normal conditions, the level of

eEF1A is sufficient to support actin polymerisation to form the migrational structures, filopodia and lamellipodia, however when the protein expression level is reduced by such a large amount, this actin polymerisation support is removed and therefore migration speed is reduced.

## *6.2 Future Work*

Further work would aim to clarify suggestions made based on the results observed so far. Further characterisation of the phenotypes already established, for example analysing the motility defect in different ways using chemotaxis assays, and progressing to invasion assays, would be of great interest, as well as determining the pathways by which the regulation of migration by eEF1A occurs. Analysing the rate of actin polymerisation by eEF1A in the two different cell lines would enable us to determine whether it is this function of eEF1A that is responsible for the impaired migration phenotype.

Due to the abundance of secondary functions assigned to eEF1A, it would also be interesting to see if these other functions, for example apoptosis, which was appeared to be changed when analysing cell cycle, would be affected and which pathway is involved.

Based on the theory of pools of eEF1A, the CHO-K1 system used would be ideal to try to manipulate the level of eEF1A further by using siRNA to reduce the protein expression by even greater amounts to see the point at which translation would be affected, as well as observing the effects on the other functions, but also to try to restore differing levels of eEF1A1 and eEF1A2 to determine at what level of expression certain functions are returned to the cells. What would be essential to determine to further understand the observed phenotypes is the other actin binding proteins that are involved in this actin stress fibre formation, and whether there is any direct interaction of eEF1A with the other actin binding proteins or, as suggested by Owen et al, 1992, whether it is simply the way in which eEF1A bundles actin that excludes all other actin binding proteins (Owen et al., 1992).

# Chapter 7:

# References

# 7.0 References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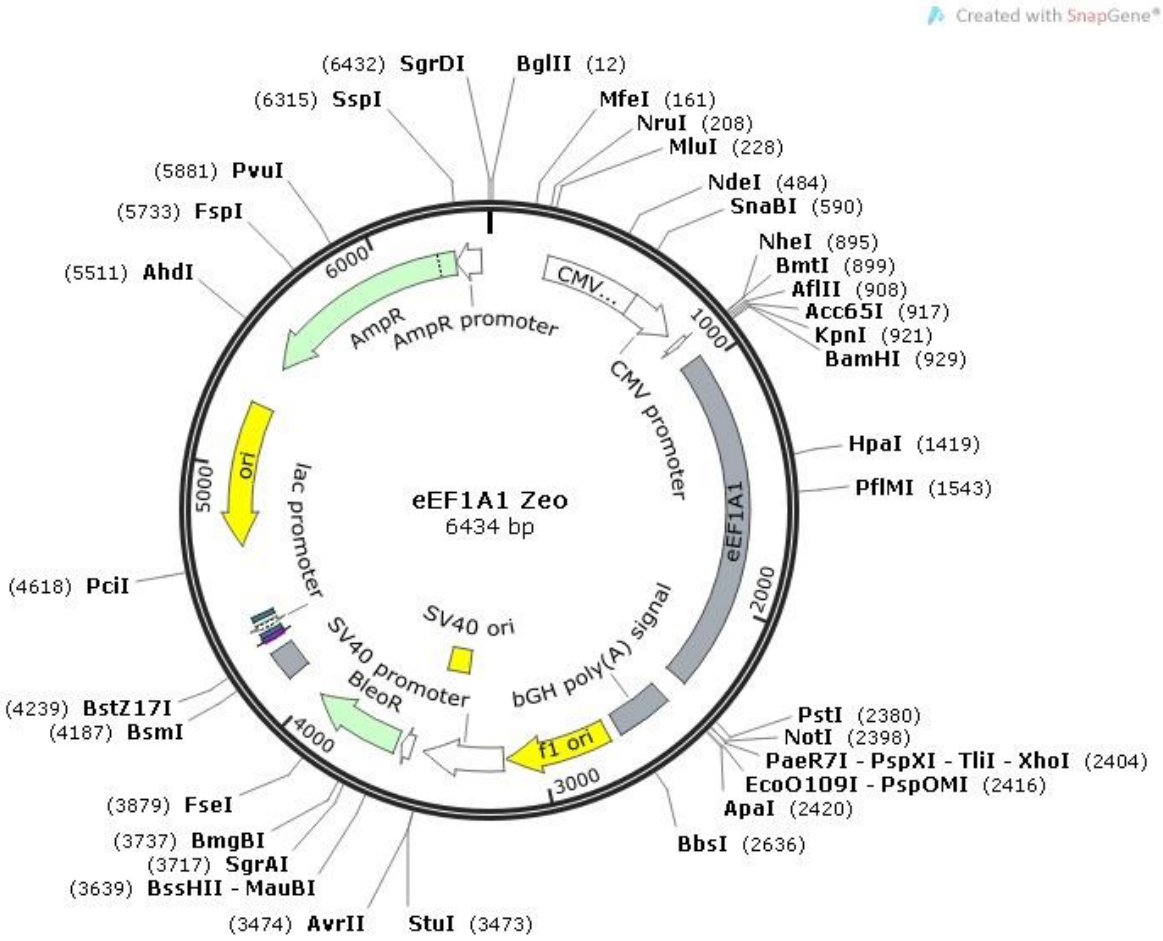
# Chapter 8:

# Appendix

# Table of Contents

# 8.0 Appendix

## *8.1 Plasmid Map and Sequence Comparison for eEF1A1 Zeo*



Fri Mar 11, 2011 11:21 -0000

New DNA from 1 to 1389

Alignment to

EF1A1 No1 in pcDNA3.1 zeo.ab1 basecalls from 1 to 934

Matches(|):845

Mismatches(#):11

Gaps( ):611

Unattempted(.):0

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                                                          *                                 *

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       |                       |  |   || |   |             ||    |                              ||||||||||#|
     1 ACMSSSRWWAGCWWRCGTTTAACTTAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCGCCACCATGGGAAARRAAAAGACTCACA 100
    23 tcaacattgtcgtcattggacacgtagattcgggcaagtccaccactactggccatctgatctataaatgcggtggcatcgacaaaagaaccattgaaaa 122
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   101 TCAACATTGTCGTCATTGGACACGTAGATTCGGGCAAGTCCACCACTACTGGCCATCTGATCTATAAATGCGGTGGCATCGACAAAAGAACCATTGAAAA 200
   123 atttgagaaggaggctgctgagatgggaaagggctccttcaagtatgcctgggtcttggataaactgaaagctgagcgtgaacgtggtatcaccattgat 222
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       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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   323 aggctgactgtgctgtcctgattgttgctgctggtgttggtgaatttgaagctggtatctccaagaatgggcagacccgagagcatgcccttctggctta 422
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   601 AGCACTTACATTAAGAAAATTGGCTACAACCCCGACACAGTAGCATTTGTGCCAATTTCTGGTTGGAATGGTGACAACATGCTGGAGCCAAGTGCTAACA 700
   623 tgccttggttcaagggatggaaagtcacccgtaaggatggcaatgccagtggaaccacgctgcttgaggctctggactgcatcctaccaccaactcgtcc 722
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   701 TGCCTTGGTTCAAGGGATGGAAAGTCACCCGTAAGGATGGCAATGCCAGTGGAACCACGCTGCTTGAGGCTCTGGACTGCATCCTACCACCAACTCGTCC 800
```

```
 723 aactgacaagcccttgcgcctgcctctccaggatgtctacaaaattggtggtattggtactgttcctgttggccgagtggagactggtgttctcaaaccc 822
     |||||||#|||||||||||||||||||#||||||||||||||||||||||||||||||||||||||||||||||||||||||||||#||||||||||||||||||
 801 AACTGACMAGCCCTTGCGCCTGCCTCTYCAGGATGTCTACAAAATTGGTGGTATTGGTACTGTTCCTGTTGGCCGAGTGGARACTGGTGTTCTCAAACCC 900

 823 ggtatggtggtcacctttgctccagtcaacgttacaacggaagtaaaatctgtcgaaatgcaccatgaagctttgagtgaagctcttcctggggacaatg 922
     ||||||||||#|##|#||||#|||||| |||
 901 GGTATGGTGGWCMMCWTTGCYCCAGTC-ACG---------------------------------------------------------------------- 930
 923 tgggcttcaatgtcaagaatgtgtctgtcaaggatgttcgtcgtggcaacgttgctggtgacagcaaaaatgacccaccaatggaagcagctggcttcac 1022

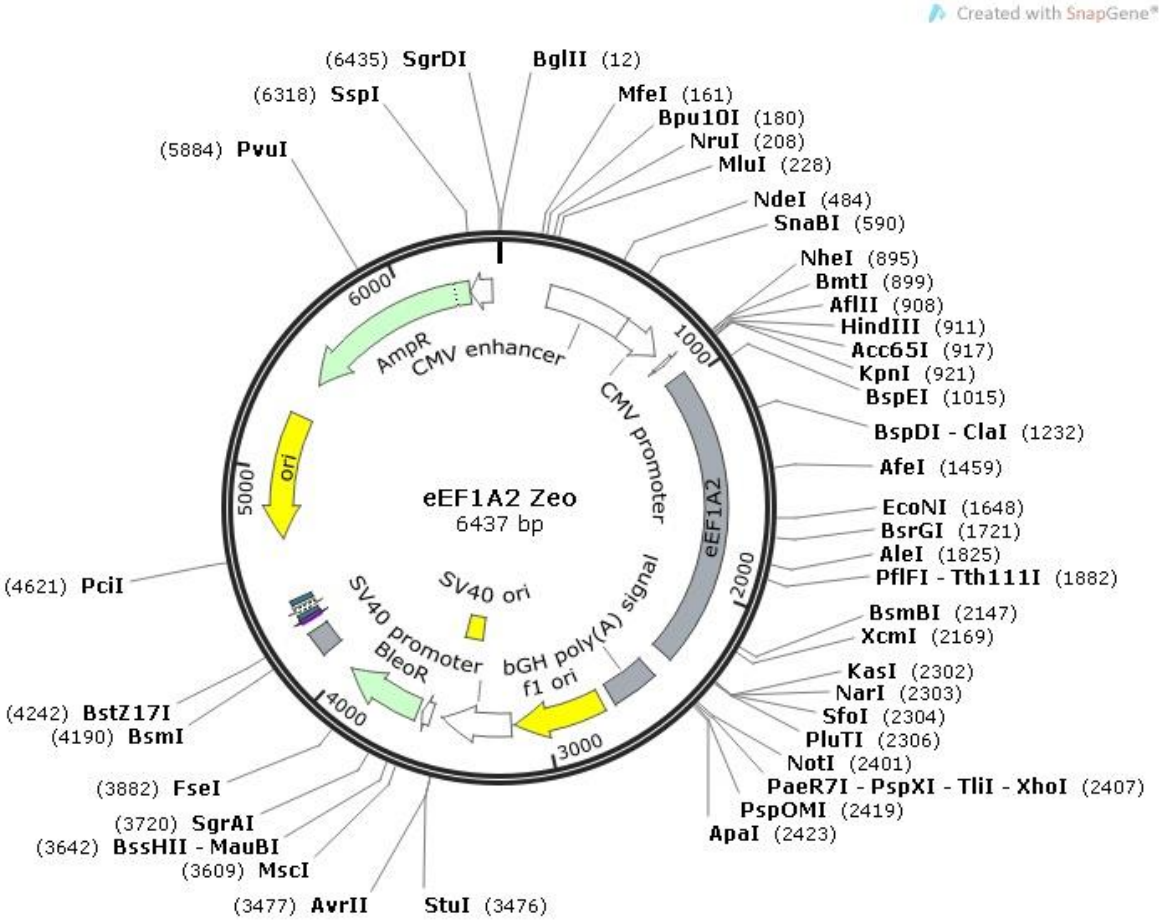
 931 ---------------------------------------------------------------------------------------------------- 931
1023 tgctcaggtgattatcctgaaccatccaggccaaataagcgccggctatgcccctgtattggattgccacacggctcacattgcatgcaagtttgctgag 1122

 931 ---------------------------------------------------------------------------------------------------- 931
1123 ctgaaggaaaagattgatcgccgttctggtaaaaagctggaagatggccctaaattcttgaagtctggtgatgctgccattgttgatatggttcctggca 1222

 931 ---------------------------------------------------------------------------------------------------- 931
1223 agcccatgtgtgttgagagcttctcagactatccacctttgggtcgctttgctgttcgtgatatgagacagacagttgcggtgggtgtcatcaaagcagt 1322

 931 ---------------------------------------------------------------------------------------------------- 931
1323 ggacaagaaggctgctggagctggcaaggtcaccaagtctgcccagaaagctcagaaggctaaatga 1389
                                                                    #|#|
 931 ------------------------------------------------------------KTWA 934
```

## *8.2 Plasmid Map and Sequence Comparison for eEF1A2 Zeo*



Fri Mar 11, 2011 11:24 -0000

New DNA from 1 to 1392

Alignment to

EF1A2 L in pcDNA3.1 zeo.ab1 basecalls from 1 to 1277

Matches(|):940

Mismatches(#):7

Gaps( ):78

Unattempted(.):444

```
                                                                                                  *        *
     1 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ATGGGCAAGGAGAAGACCCACATCA 25
                                                                                                  ||||||||||||||||||||||||||
     1 CMRMMCTWWGCWGCGTTTAACTTAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCGCCACCATGGGCAAGGAGAAGACCCACATCA 100
    26 ACATCGTGGTCATCGGCCACGTGGACTCCGGAAAGTCCACCACCACGGGCCACCTCATCTACAAATGCGGAGGTATTGACAAAAGGACCATTGAGAAGTT 125
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   101 ACATCGTGGTCATCGGCCACGTGGACTCCGGAAAGTCCACCACCACGGGCCACCTCATCTACAAATGCGGAGGTATTGACAAAAGGACCATTGAGAAGTT 200
   126 CGAGAAGGAGGCGGCTGAGATGGGGAAGGGATCCTTCAAGTATGCCTGGGTGCTGGACAAGCTGAAGGCGGAGCGTGAGCGCGGCATCACCATCGACATC 225
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   201 CGAGAAGGAGGCGGCTGAGATGGGGAAGGGATCCTTCAAGTATGCCTGGGTGCTGGACAAGCTGAAGGCGGAGCGTGAGCGCGGCATCACCATCGACATC 300
   226 TCCCTCTGGAAGTTCGAGACCACCAAGTACTACATCACCATCATCGATGCCCCCGGCCACCGCGACTTCATCAAGAACATGATCACGGGTACATCCCAGG 325
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   301 TCCCTCTGGAAGTTCGAGACCACCAAGTACTACATCACCATCATCGATGCCCCCGGCCACCGCGACTTCATCAAGAACATGATCACGGGTACATCCCAGG 400
   326 CGGACTGCGCAGTGCTGATCGTGGCGGCGGGCGTGGGCGAGTTCGAGGCGGGCATCTCCAAGAATGGGCAGACGCGGGAGCATGCCCTGCTGGCCTACAC 425
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   401 CGGACTGCGCAGTGCTGATCGTGGCGGCGGGCGTGGGCGAGTTCGAGGCGGGCATCTCCAAGAATGGGCAGACGCGGGAGCATGCCCTGCTGGCCTACAC 500
   426 GCTGGGTGTGAAGCAGCTCATCGTGGGCGTGAACAAAATGGACTCCACAGAGCCGGCCTACAGCGAGAAGCGCTACGACGAGATCGTCAAGGAAGTCAGC 525
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   501 GCTGGGTGTGAAGCAGCTCATCGTGGGCGTGAACAAAATGGACTCCACAGAGCCGGCCTACAGCGAGAAGCGCTACGACGAGATCGTCAAGGAAGTCAGC 600
   526 GCCTACATCAAGAAGATCGGCTACAACCCGGCCACCGTGCCCTTTGTGCCCATCTCCGGCTGGCACGGTGACAACATGCTGGAGCCCTCCCCCAACATGC 625
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   601 GCCTACATCAAGAAGATCGGCTACAACCCGGCCACCGTGCCCTTTGTGCCCATCTCCGGCTGGCACGGTGACAACATGCTGGAGCCCTCCCCCAACATGC 700
   626 CGTGGTTCAAGGGCTGGAAGGTGGAGCGTAAGGAGGGCAACGCAAGCGGCGTGTCCCTGCTGGAGGCCCTGGACACCATCCTGCCCCCCACGCGCCCCAC 725
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   701 CGTGGTTCAAGGGCTGGAAGGTGGAGCGTAAGGAGGGCAACGCAAGCGGCGTGTCCCTGCTGGAGGCCCTGGACACCATCCTGCCCCCCACGCGCCCCAC 800
```

```
 726 GGACAAGCCCCTGCGCCTGCCGCTGCAGGACGTGTACAAGATTGGCGGCATTGGCACGGTGCCCGTGGG-CCGGGTGGAGACCGGCATCCTGCGGCCGGG 824
     |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
 801 GGACAAGCCCCTGCGCCTGCCGCTGCAGGACGTGTACAAGATTGGCGGCATTGGCACGGTGCCCGTGGGGCCGGGTGGAGACCGGCATCCTGCGGCCGGG 900

 825 CATGGTGGTGACCTTTGCGCCAGTGAACATCACCACTGAGGTGAAGTCAGTGGAGATGCACCACGAGGCTCTGAGCGAAGCTCTGCCCGGCGACAACGTC 924
     #||||||||||||||||||#|#|||||||||||||||||||||||||||||||||#|||||||||#|||||||||||||||||||||||||#|||||| |||||
 901 SATGGTGGTGACCTTTGCSCMAGTGAACATCACCACTGAGGTGAAGTCAGTGGASATGCACCACSAGGCTCTGAGCGAAGCTCTGCCSGGCGAC-ACGTC 999
 925 GGC-TTCAATGTGAAGAACGTGTCGGTGAAGGACATCCGGCGGGGCAACGTGTGTGGGGACAGCAAGTCTGACCCGCCGCAGGAGGCTGCTCAGTTCACC 1023
     #|| ||||||||||||||||||||.....................................................................................
1000 SGCTTTCAATGTGAAGAACGTGTC---------------------------------------------------------------------------------- 1023
1024 TCCCAGGTCATCATCCTGAACCACCCGGGGCAGATTAGCGCCGGCTACTCCCCGGTCATCGACTGCCACACAGCCCACATCGCCTGCAAGTTTGCGGAGC 1123
     .....................................................................................................
1024 ---------------------------------------------------------------------------------------------------- 1024
1124 TGAAGGAGAAGATTGACCGGCGCTCTGGCAAGAAGCTGGAGGACAACCCCAAGTCCCTGAAGTCTGGAGACGCGGCCATCGTGGAGATGGTGCCGGGAAA 1223
     .....................................................................................................
1024 ----------------RGTGAGGACATCCGGCGGGGCAACKRGTGTGGGGAMCRSCAAGTCTGACCCGSCGCAGGAGGCWKGCTMRTTYMACCTCCCARGT 1108
1224 GCCCATGTGTGTGGAGAGCTTCTCCCAGTACCCGCCTCTCGGCCGCTTCGCCGTGCGCGACATGAGGCAGACGGTGGCCGTAGGCGTCATCAAGAACGTG 1323
     .....................................................................................................
1109 CRWCWWCCTGAACCACCCMGGGGYMGATKATCGCCGGCTACSWCCCGGGTMMAYSAMCTGGCACACMGRCCASAWYSGYGGGCAGGTKTGCGAGASYGAA 1208
1324 GAGAAGAAGAGCGGCGGCGCCGGCAAGGTCACCAAGTCGGCGCAGAAGGCGCAGAAGGCGGGCAAGTGA 1392
     ....................................................................|
1209 RGGAYACATTGACGGGCASTCTTGGCARAACYGRGRRGAMAACCCCYAGTTCCGTGAATTCKRKGARCA 1277
```